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INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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CHROMATOGRAPHY AND ELECTROPHORESIS IN FORENSIC ANALYSIS

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JOURNAL OF CHROMATOGRAPHY A

Preface

Chemists are often called upon to testify as expert witnesses in court. There they find a distinct difference between the type of explanation and defense of chemical data required by the legal profession and that accepted by their peers. To satisfy both professions strict adherence to the highest standards of both is called for. This is particularly evident for DNA typing laboratories, which in the USA are now subject to mandatory proficiency testing.

Although it may be difficult to explain the principles of chromatographic analysis to lay persons, lawyers have been quick to grasp the significance of the information it produces. Chromatography and electrophoresis can be exquisitely sensitive and specific, but without controls and standards they are apt to lead to fatal mistakes.

This special issue on *Chromatography and Electrophoresis in Forensic Analysis* is our first attempt to bring the techniques and problems characteristic of this branch of chemical analysis into focus. It is our hope that comparison of different approaches to the identification and quantitation of physical evidence will ultimately lead to improvement in the testimony of the expert witness.

Orinda, CA (USA) Erich Heftmann

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JOURNAL OF CHROMATOGRAPHY A

Potential and pitfalls of chromatographic techniques and detection modes in substance identification for systematic toxicological analysis

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Abstract

The potential and the constraints of thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC) towards substance identification, together with their detection modes, are considered. The latter include colour reactions on the plate, molecular masses through chemical ionization mass spectrometry (MS) and diode-array UV spectrophotometry. Evaluations are carried out by the mean list length approach. Not surprisingly, GC–MS and HPLC–diode array detection qualify as the two most powerful combinations. However, one does not necessarily need to have access to these sophisticated detection modes: the identification power of TLC and colour reactions plus GC or HPLC retention indices is high and even a suitable combination of TLC and colour reactions remains a valuable tool. After analysis, the findings for the unknown substance(s) must be matched against databases containing the behaviour of reference substances. The search process for the computerized retrieval of potential candidates must allow the handling of all possible combinations of identification techniques applied.

1. Introduction

Systematic toxicological analysis (STA) can be defined as the logical chemical–analytical search for an unknown substance of toxicological relevance. As such, it represents a most important aspect of all toxicological analyses, such as clinical, forensic, environmental, occupational, workplace and traffic toxicology, and also in drugs of abuse testing and doping. First, it has to be established whether suspicious substances can be detected (screening); then the identity of the detected compounds must be established beyond reasonable doubt (identification or confirmation).

With regard to the actual analysis, toxicologists may choose from a series of analytical techniques and systems, such as thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC), and an array of immunoassay tests. The chromatographic techniques may be combined with appropriate detection modes, such as colour reactions, element-specific detections, UV and diode-array detection (DAD) and mass spectrometry (MS) which can provide additional identification parameters.

However simple as STA may appear at first sight, it poses many difficulties. As the ultimate

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aim of STA is to exclude all relevant substances except one, the major issues are not to miss any substance in the screening phase (false negatives) and to make sure that the substances found are properly identified (false positives). In order to achieve this, the following prerequisites are to be considered: (a) the utility of the various analytical systems towards STA needs to be established and the best choices can then be designated as recommended systems; and (b) reference substances are to be run in these systems so that databases can be compiled of their appropriate parameters (R_F values, retention indices, ultraviolet and mass spectra, etc.). Identifications can then be performed by comparing the behaviour of an unknown substance in the recommended systems with that of the reference substances in the databases. Of course, these databases should be up to date and as comprehensive as possible with respect to hazardous substances and their metabolites, but they should also contain data on therapeutic medicines, endogenous compounds, omnipresent contaminants (*e.g.*, plasticizers), etc. This explains why it is impossible for individual laboratories to develop and maintain their own databases. Instead, they must have access to large databases that have been developed for interlaboratory use.

In this paper we discuss the potential of TLC, GC and HPLC towards STA, taking into account their retention parameters and their detection modes. The latter include colour reactions on the plate for TLC, molecular masses through chemical ionization MS for GC and diode-array detection for HPLC. In addition, the possibilities for identification by means of computerized database searches are considered.

2. The mean list length approach (MLL)

In order to evaluate the suitability of a given analytical technique or system for STA, we developed the MLL concept so that its identification power can be expressed in a concise and objective way [1]. In this statistical approach, it is established for a given analytical parameter in a given system [*e.g.*, a retention index (RI) in a GC system] how many substances from a large population would qualify for identification. The number of substances that qualify is called the list length. If this exercise is repeated for all RI values in that GC system and the individual list lengths are averaged, the so-called mean list length (MLL) for that system is obtained. The shorter the MLL, the better is that system for STA. This can be done for individual systems, but also for any combination of systems, for example, a TLC system (R_F) plus a GC system (RI), two TLC systems (two R_Fs), a TLC system plus a GC system plus an HPLC system (RI), a TLC system (R_F) plus a colour reaction on the plate, a GC system (RI) plus a molecular mass via GC–MS or a TLC system plus an HPLC system plus a diode-array spectrum. The ultimate is reached when an MLL value of 1.00 is found, which means that each individual substance in the data set can be unequivocally identified against the background of all other substances in that set. It will be clear that an MLL of 1.00 can never be obtained by a single system and also that the number of systems required will increase with increasing number of substances in the data set.

Application of the MLL concept has shown that in order for chromatographic techniques to be suitable for STA, the following criteria should be met [1,2]: (1) the relevant substances should show proper migration and be evenly spread over the entire chromatographic range; (2) the retention parameters should be standardized in such a way that good reproducibility is obtained on an interlaboratory scale; and (3) when more than one chromatographic system is used, there must be a low correlation between these systems.

3. Evaluation of TLC, GC and HPLC systems

In recent years, extensive evaluations of TLC and GC systems have been carried out on an interlaboratory basis, which also resulted in extensive databases. Because it is customary to perform a sample work-up on the basis of a pH-dependent extraction, TLC systems were selected that handle either acidic and neutral

drugs (A/N systems) or basic and neutral drugs (B/N systems). This resulted in the recommendation of four TLC systems for A/N drugs and seven for B/N drugs [3]. Table 1 gives an overview of these systems, with their corresponding interlaboratory reproducibilities. The correlations between these systems are given in Table 2. To compensate for variations in the experimental conditions, the R_F values observed are to be corrected by means of reference substances that are run on the same plate as the unknown. In this way, interlaboratory variations can be decreased considerably. The reference substances for each recommended system are also listed in Table 1. The databases for the above TLC systems contain about 1800 substances [3].

For GC, a dimethylsilicone column, either packed or capillary, appears to be the best for screening in STA. Retention is expressed as Kováts retention indices [4], but to cover all substances in one run and to obtain a good spread over the entire chromatogram, temperature-programmed runs are recommended. Under the latter circumstances, there is an almost linear relationship between the carbon number of the *n*-alkanes and the net retention time [not log(retention time) as is the case in the isothermal mode]. Here too, variations in interlaboratory conditions can be compensated for by using a mixture of reference substances of toxicologically relevant compounds. Details can be found in ref. 5. The database in ref. 5 contains data on *ca.* 6000 substances. However, even though GC lends itself better than TLC to screening because of its high separation efficiency (particularly in capillary GC) and good reproducibility, the dimethylsilicone system is the only recommended system. This is due to the fact that other GC systems are all highly correlated with the dimethylsilicone system, so that relatively little additional information is gained by applying a second one [5,6]. Hence it would be a waste of time and effort to undertake the enormous task of setting up a database for a second system.

As for HPLC, its good separation power and general applicability, including thermolabile and non-volatile compounds, seem to make it an attractive technique for STA. Moreover, HPLC offers a wide variety of separation modes and possibilities to vary the stationary and mobile phases. However, difficulties in producing stationary phase materials with sufficient batch-to-batch reproducibility (in addition to brand-to-brand reproducibility) has long been a serious drawback. However, the situation has improved recently and a first HPLC screening system has been recommended [7,8]. It is based on reversed-phase (RP) C_{18} or C_8 columns, using a gradient of 25 m*M* triethylammonium phosphate buffer (pH 3.0) and acetonitrile as organic modifier. Retention is expressed as retention indices based on linear interpolation between consecutive homologues of nitroalkanes (nitromethane to 1-nitrooctane, RI values between 100 and 800). Again, mixtures of reference drugs are used to correct for variations in experimental conditions [9]. A database is under development. As in TLC, it will be no problem to find a second or third HPLC system with low correlations with the RP system described above. It remains to be seen, however, to what extent the former can comply with the other prerequisites of providing a good spread of the substances of interest over the entire run and providing good interlaboratory reproducibility.

4. Evaluation of combinations of systems

When one wishes to evaluate chromatographic systems and to express the utility of the retention parameter in an MLL value, the statistical calculation program is relatively simple [1,10], even when more than one system is being used or when different chromatographic techniques are applied (*e.g.*, a TLC system and an HPLC system). However, evaluating the identification power of detection modes, either as stand-alone technique or in combination with chromatographic systems, is much more complicated. Fortunately, recent developments in our laboratory have now provided calculation programs capable of handling such diverse parameters as

Table 1
Recommended TLC systems

Solvent[a]		Adsorbent	Reference compounds[b]	hR_F[c]	Error window[d]
(1)	Chloroform–acetone (80:20)	Silica	Paracetamol	15	7
			Clonazepam	35	
			Secobarbital	55	
			Methylphenobarbital	70	
(2)	Ethyl acetate	Silica	Sulfathiazole	20	8
			Phenacetin	38	
			Salicylamide	55	
			Secobarbital	68	
(3)	Chloroform–methanol (90:10)	Silica	Hydrochlorothiazide	11	8
			Sulfafurazole	33	
			Phenacetin	52	
			Prazepam	72	
(4a)	Ethyl acetate–methanol–conc. ammonia[e] (85:10:5)	Silica	Sulfadimidine	13	11
			Hydrochlorothiazide	34	
			Temazepam	63	
			Prazepam	81	
(4b)	Ethyl acetate–methanol–conc. ammonia[e] (85:10:5)	Silica	Morphine	20	10
			Codeine	35	
			Hydroxyzine	53	
			Trimipramine	80	
(5)	Methanol	Silica	Codeine	20	8
			Trimipramine	36	
			Hydroxyzine	56	
			Diazepam	82	
(6)	Methanol–*n*–butanol (60:40); 0.1 mol/l NaBr	Silica	Codeine	22	9
			Diphenhydramine	48	
			Quinine	65	
			Diazepam	85	
(7)	Methanol–conc. ammonia[e] (100:1.5)	Silica impregnated with 0.1 mol/l KOH and dried	Atropine	18	9
			Codeine	33	
			Chlorprothixene	56	
			Diazepam	75	
(8)	Cyclohexane–toluene–diethylamine (75:15:10)	Silica impregnated with 0.1 mol/l KOH and dried	Codeine	6	8
			Desipramine	20	
			Prazepam	36	
			Trimipramine	62	
(9)	Chloroform–methanol (90:10)	Silica impregnated with 0.1 mol/l KOH and dried	Desipramine	11	11
			Physostigmine	36	
			Trimipramine	54	
			Lidocaine	71	
(10)	Acetone	Silica impregnated with 0.1 mol/l KOH and dried	Amitriptyline	15	9
			Procaine	30	
			Papaverine	47	
			Cinnarizine	65	

[a] Eluent composition in v/v. Saturated systems are used except for systems 5 and 6, which are used with unsaturated solvent tanks. System 4 is split: 4a for acidic and neutral substances and 4b for basic and neutral substances.
[b] Solutions of the four reference compounds at a concentration of approximately 2 mg/ml of each substance.
[c] Database R_F values times one hundred from ref. 3.
[d] The error window for each system is based on multiplying by three the interlaboratory standard deviation of measurement of hR_F values.
[e] Conc. ammonia contains 25% NH_3.

Table 2
Correlation coefficients for pairs of recommended TLC systems 1–10

	1	2	3	4	5	6	7	8	9	10
1	–									
2	0.820									
3	0.890	–								
4	0.530	0.748	–	–						
5		0.464	0.593	0.460	–					
6				0.436	0.614	–				
7				0.700	0.745	0.552	–			
8				0.593	−0.128	−0.045	0.228	–		
9				0.723	0.748	0.472	0.728	0.342	–	
10				0.710	0.750	0.655	0.771	0.206	0.820	–

colour reactions on the plate, molecular masses and full-scan diode-array spectra [11]. They were applied as follows.

4.1. Test set

Evaluations were made with a test set of 99 basic and neutral drugs, which were selected to represent various classes/structures of toxicologically relevant drugs. The substances are listed in Table 3, together with their respective chromatographic and detection parameters. UV spectra are not given for technical reasons.

4.2. TLC systems

Three systems, recommended for B/N drugs [3], were used: TLC 1, ethyl acetate–methanol–25% ammonia (85:10:5) on silica gel GF_{254}, standard deviation (S.D.) of measurement 3.8; TLC 2, methanol on silica gel GF_{254}, S.D. 2.8; and TLC 3, cyclohexane–toluene–diethylamine (75:15:10) on silica gel GF_{254}, S.D. 3.0.

Systems 1 and 3 were run in paper-lined tanks, presaturated with solvent vapour for 30 min; system 2 was run in unsaturated tanks. Plates for system 3 were impregnated with KOH prior to development by dipping in 0.1 *M* KOH in methanol and letting the methanol evaporate for at least 24 h. Plates were 20 × 10 cm and were developed over the shortest distance of the plate to 7 cm over the starting points. Developments were carried out at ambient temperatures (20–24°C) and relative humidities of 30–70%. No heat activation of the plates was applied. They were stored in cabinets at ambient temperature and relative humidity.

After development, the plates were dried with a cold hair blower until the smell of the solvents had disappeared. Then a total of four colour reactions were carried out on the same plate in sequence and after each step the colour was noted and encoded by means of a colour chart [12]. The colour reactions (CR) were taken from the Toxi-Lab Drug Compendium [13] and consisted of the following steps: CR 1, expose to formaldehyde vapour, then dip in concentrated sulphuric acid containing 0.1% ammonium vanadate (Marquis–Mandelin reaction) and observe the colour; CR 2, dip in water (exothermic reaction) and observe the colour; CR 3, observe fluorescence under UV light of 366 nm and observe the colour; and CR 4, dip in modified Dragendorff reagent and observe the colour.

Observed R_F values on the plate were corrected by means of reference mixtures run on the same plate, as described in ref. 3. Observed colours were encoded numerically as described in ref. 12, using a colour wheel with eight reference colours.

4.3. GC system

The GC system consisted of an HP-1 fused-silica dimethylsilicone column (12.5 m × 0.53 mm I.D., film thickness 0.88 μm). The tempera-

Table 3
Test set of basic and neutral drugs in the MLL evaluation of combined systems, with their respective chromatographic and detection parameters

Substance	TLC 1		TLC 2		TLC 3		GLC RI	M_r	HPLC RI
	hR_F	Colour codes	hR_F	Colour codes	hR_F	Colour codes			
Acebutolol	33	0 0 0 3	13	0 0 0 3	0	0 0 0 3	2811	336	311
Aminophenazone	62	5 0 0 3	70	0 0 0 3	21	5 5 0 3	1895	231	243
Amitriptyline	69	3 4 1 3	27	4 3 1 3	50	3 4 1 3	2194	277	440
Amitriptyline M/nortriptyline	46	3 3 7 3	87	3 3 3 3	28	3 3 2 3	2215	263	400
Amphetamine	43	1 1 7 3	12	1 1 7 3	26	1 1 7 3	1125	135	238
Atenolol	22	5 5 0 3	14	5 5 0 3	0	5 5 0 3	2385	266	224
Atropine	24	0 0 0 3	5	0 0 0 3	5	0 0 0 3	2190	289	287
Benperidol	60	5 5 0 3	62	5 5 0 3	3	5 5 0 3	3433	381	371
Bromazepam	63	1 1 3 3	73	1 1 3 3	6	1 1 3 3	2665	316	378
Caffeine	52	0 0 0 6	59	0 0 0 6	3	0 0 5 6	1800	194	265
Carbamazepine	56	2 1 7 3	79	2 2 7 3	2	2 2 7 3	2285	236	380
Chlordiazepoxide	52	0 0 2 3	76	0 0 2 3	2	0 0 2 3	2797	300	357
Chloroquine	46	0 0 0 4	4	0 0 0 3	14	0 0 0 3	2605	320	265
Chlorphenamine	46	0 0 0 3	12	0 0 0 3	35	0 0 0 3	1996	275	348
Chlorpromazine	70	3 3 0 3	25	3 3 0 3	45	3 3 0 3	2495	319	452
Clobazam	75	0 0 0 3	84	0 0 0 3	8	0 0 0 3	2558	301	484
Clomipramine	72	0 7 8 3	26	0 7 8 3	53	0 7 8 3	2415	315	470
Clonazepam	67	0 0 7 3	85	0 0 7 3	0	0 0 7 3	2823	316	451
Clopenthixol	44	4 4 2 3	45	4 4 2 3	7	4 4 2 3	3400	401	456
Clorazepic acid	68	0 0 7 3	83	0 0 1 0	3	0 0 1 0	2457	315	464
Cocaine	77	0 0 0 3	35	0 0 0 3	45	0 0 0 3	2187	303	345
Cocaine M/benzoylecgonine	2	0 0 0 3	22	0 0 0 3	0	0 0 0 3	2570	289	295
Codeine	35	7 7 0 3	21	7 7 0 3	6	7 7 0 3	2375	299	250
Demoxepam	41	0 0 1 3	81	0 0 2 3	0	0 0 1 3	2529	287	388
Diamorphine	49	5 5 0 3	26	5 5 0 3	15	5 5 0 3	2615	369	327
Diazepam	76	0 0 1 3	82	0 0 1 3	27	0 0 1 3	2428	285	520
Diazepam M/nordazepam	69	0 0 1 3	82	0 0 1 3	3	0 0 1 3	2490	271	464
Diphenhydramine	65	1 1 5 0	27	1 1 5 0	44	1 1 5 3	1870	255	390
Dipyridamole	44	4 4 0 0	82	4 4 0 0	0	4 4 0 0	1640	505	387
Disopyramide	60	0 0 0 3	9	0 0 0 3	7	0 0 0 3	2505	339	327
Doxepin	63	4 4 0 0	24	5 5 0 0	48	5 5 0 0	2220	279	730
Droperidol	58	5 5 0 3	71	5 5 0 3	2	5 5 0 3	3430	379	369
Ephedrine	25	1 0 0 3	10	1 0 0 3	5	0 0 0 3	1365	165	218
Ethosuximide	66	0 0 6 0	84	0 0 6 0	5	0 0 6 0	1205	141	284
Flecainide	49	1 1 5 3	28	1 1 5 3	6	1 1 5 3	2250	414	410
Flumazenil	61	0 0 0 3	76	0 0 0 3	3	0 0 0 5	2660	303	362
Flunarizine	88	4 4 7 3	83	4 4 5 3	45	4 4 7 3	3035	404	571
Flunitrazepam	74	0 0 7 3	80	0 0 7 3	10	0 0 7 3	2600	313	459
Flupenthixol	46	2 2 1 3	50	2 2 1 3	6	2 2 1 3	3058	435	487
Flurazepam	71	0 0 1 3	52	0 0 1 3	30	0 0 1 3	2780	388	392
Glibenclamide	11	0 0 5 3	90	0 0 5 3	0	0 0 5 3	9999	494	623
Gliclazide	9	0 0 0 3	84	0 0 0 3	0	0 0 0 3	1456	494	538
Glutethimide	80	0 0 0 3	86	0 0 0 3	31	0 0 0 3	1830	217	430
Haloperidol	76	0 9 0 3	51	0 9 0 3	11	0 9 0 3	2930	376	409
Hydroxyzine	54	0 0 0 3	57	0 0 0 3	10	0 0 0 3	2849	375	435
Imipramine	67	8 8 1 3	21	8 8 1 3	48	0 8 1 3	2230	280	437
Imipramine M/desipramine	40	8 7 8 3	7	8 7 8 3	19	8 7 8 3	2235	266	423
Ketamine	79	0 0 0 3	68	0 0 0 3	37	0 0 0 3	1840	238	294
Ketazolam	74	0 0 0 3	83	0 0 0 3	14	0 0 0 3	2444	369	583

Table 3 (*continued*)

Substance	TLC 1		TLC 2		TLC 3		GLC RI	M_r	HPLC RI
	hR_F	Colour codes	hR_F	Colour codes	hR_F	Colour codes			
Labetalol	29	3 3 5 3	32	3 3 5 3	0	3 3 5 3	1230	328	350
Levomepromazine	76	3 4 0 3	32	3 4 0 3	47	3 3 0 3	2525	162	440
Lidocaine	80	4 2 2 3	72	3 3 2 3	35	3 3 2 3	1870	234	278
Loprazolam	40	0 0 0 3	26	0 0 0 3	1	0 0 8 3	3258	465	379
Lorazepam	43	0 0 1 0	82	0 0 1 0	1	0 0 1 3	2410	321	422
Maprotyline	36	6 6 7 3	6	3 3 2 3	18	3 3 2 3	2356	277	440
Medazepam	78	0 5 7 3	79	0 5 7 3	41	5 5 7 3	2235	271	395
Metamizole	2	0 0 5 3	85	0 0 5 3	0	0 0 0 3	1990	351	289
Methadone	77	7 7 8 3	16	7 7 8 3	59	7 7 8 3	2145	310	441
Methamphetamine	42	1 1 0 3	9	1 1 0 3	28	1 1 0 3	1175	149	246
Methaqualone	78	0 0 0 3	79	0 0 0 3	36	0 0 0 3	2135	250	450
Metoclopramide	51	3 4 0 3	17	3 3 5 3	1	5 5 0 3	2620	300	308
Metoprolol	44	5 5 7 3	20	5 5 7 3	10	5 5 7 3	2035	267	317
Mianserin	68	7 5 7 3	48	5 5 7 3	39	5 5 7 3	2210	264	390
Midazolam	60	0 0 7 3	69	0 0 7 3	6	0 0 7 3	2575	326	386
Morphine	20	5 5 0 3	18	5 5 0 3	0	5 5 0 3	2445	285	200
Nifedipine	71	5 5 0 3	79	6 6 8 3	1	4 3 8 3	2170	346	503
Nitrazepam	64	0 0 7 3	84	0 0 7 3	0	0 0 7 3	2740	281	430
Opipramol	38	1 1 8 3	35	2 2 8 3	6	2 2 8 3	3050	363	387
Orphenadrine	68	2 2 7 3	25	1 1 7 0	48	1 1 7 3	1935	269	416
Oxazepam	45	0 0 1 3	82	0 0 1 3	0	0 0 1 3	2325	287	441
Paracetamol	45	5 5 2 3	77	0 0 2 3	0	0 0 2 3	1665	151	234
Pentazocine	70	5 5 2 3	34	5 5 5 3	16	5 5 2 3	2280	285	357
Periciazine	51	4 4 7 3	46	2 2 0 3	4	2 4 7 3	3260	366	405
Perphenazine	42	4 4 5 3	40	4 4 0 3	7	4 4 5 3	2207	404	438
Pethidine	60	0 0 0 3	34	0 0 0 3	37	0 0 0 3	1754	247	334
Phenazone	45	0 0 0 3	66	0 0 2 3	4	0 0 0 3	1850	188	303
Pindolol	43	7 7 0 3	18	7 7 0 3	2	6 6 0 3	2245	248	277
Pipamperone	43	0 0 0 3	33	0 0 0 3	1	0 0 0 3	3040	375	286
Prazepam	81	0 0 7 3	84	0 0 7 3	36	0 0 7 3	2648	325	648
Procainamide	39	0 0 0 3	17	0 0 0 3	1	0 0 0 3	2200	235	202
Prochlorperazine	55	4 4 0 3	26	2 4 0 3	34	4 4 0 3	2955	374	462
Promazine	62	4 4 0 3	18	1 1 5 3	38	1 1 0 3	2315	284	418
Promethazine	65	4 4 0 3	30	1 1 5 3	36	1 1 0 3	2267	284	411
Propoxyphene	82	5 5 8 3	50	5 5 8 3	58	5 5 8 3	2190	339	438
Propranolol	49	8 8 1 3	21	8 8 2 3	6	8 8 2 3	2147	259	370
Propyphenazone	74	0 5 0 3	81	5 5 0 3	32	5 5 0 3	1920	230	422
Quinidine	49	0 0 7 3	30	0 0 7 3	4	0 0 7 3	2790	324	316
Quinine	45	0 0 7 3	26	0 0 7 3	2	0 0 7 3	2800	324	398
Strychnine	32	5 2 0 3	8	5 2 0 3	8	5 4 0 3	3116	344	292
Sulpiride	34	0 0 0 3	17	0 0 0 3	0	0 0 0 3	3102	341	240
Temazepam	62	0 0 1 3	82	0 0 1 3	8	0 0 1 3	2595	301	466
Terfenadin	74	3 3 8 3	45	3 3 8 3	13	3 3 8 3	3436	472	567
Thioridazine	67	7 7 0 3	20	7 7 0 3	42	7 7 0 3	3115	371	504
Tocainide	44	1 0 0 3	42	1 1 0 3	2	1 1 0 0	1714	193	251
Trazodone	66	7 5 5 3	64	7 5 5 3	10	7 5 5 3	3330	372	358
Triazolam	44	0 0 0 3	68	0 0 0 3	1	0 0 0 3	3080	343	452
Trimethoprim	45	2 2 2 3	45	2 2 2 3	0	2 2 2 3	2558	290	345
Verapamil	73	5 5 0 3	43	5 5 0 3	23	5 5 0 3	3150	455	454
Zopiclone	47	0 0 0 3	42	0 0 0 3	4	4 0 0 3	3062	389	314

hR_F values from ref. 3. Colour codes (CC) (according to ref. 12): 1 = yellow, 2 = orange, 3 = brown, 4 = red, 5 = purple, 6 = black, 7 = blue, 8 = green, 0 = no spot observed. GLC RI values from ref. 5. HPLC RI values (according to ref. 5): determined jointly in the laboratory of the authors and at the Department of Forensic Medicine, Cracow, Poland (Dr. M. Klys). UV spectra: not shown, but measured in conjunction with the HPLC RI values.

ture programme was 120°C for 2 min, increased at 10°C/min to 215°C and then at 8°C/min to 300°C, with 5 min at the final temperature. Flame ionization or nitrogen–phosphorus detection was used. Retention times were converted into retention indices as described in ref. 5, using reference mixtures of drugs as calibrators. The S.D. was 25.

4.4. HPLC system

The HPLC system consisted of an RP-Select B C_8 column (12.5 cm × 4.6 mm I.D.), run in a gradient mode from 100% B to 70% A–30% B in 30 min, where solvent A was acetonitrile and solvent B was 0.025 *M* triethylammonium phosphate buffer (pH 3.0). A Hitachi–Merck L-3000 diode-array detector was used. Retention times were converted into retention indices based on nitroalkanes [9], using reference mixtures of drugs as calibrators [7]. The S.D. was 7. Spectral comparisons were made over the range 200–360 nm (65 diodes).

4.5. MLL calculations

MLL calculations were performed for single systems and detection modes as described in ref. 1. For the evaluation of combinations of systems and their corresponding detection modes we used the recently extended calculation models [11].

Table 4 gives the MLL values for single systems and detection modes. As expected, the three TLC systems have low identification powers (IP), owing to their limited separation efficiencies and low reproducibilities. However, the importance of a good spread of the substances over the chromatographic run can be clearly seen from Fig. 1: in TLC 2 the R_F values are evenly spread across the entire run, whereas the R_F distributions in TLC 1 and TLC 3 are skewed. TLC 2 also has the best reproducibility with an S.D. of 2.8, compared with 3.8 and 3.0 for the other two systems. This results in an MLL of 14.09 for TLC 2. Of the chromatographic techniques, GC offers the best IP with an MLL of 7.20, but HPLC is close behind at 9.08. This indicates an effective gradient and a good separation efficiency for the RP-HPLC system on a column only 12.5 cm long. With regard to the detection modes, it is obvious that the molecular mass (M_r) is highly discriminative. However, as the present test set of 99 substances contains a few entries with the same M_r, the MLL does not reach the ideal value of 1.00. On the other hand, it is interesting that the combination of four colour reactions results in an MLL of about 15 and that the MLL of the DAD spectra is of the order of 10. Hence these detection modes provide about the same IP as the corresponding separation techniques.

By using the recently extended mathematical models [11], we could also assess the IPs of combinations of systems and/or detection modes. Table 5 gives the results for combina-

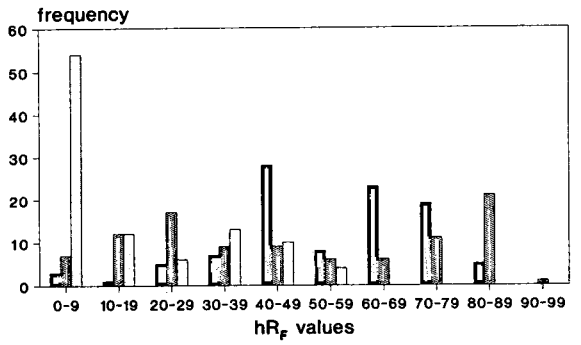

Fig. 1. Frequency distributions of hR_F values in TLC systems 1, 2 and 3. Black bars, TLC 1, ethyl acetate–methanol–ammonia (85:10:5); hatched bars, TLC 2, methanol; screened bars, TLC 3, cyclohexane–toluene–diethylamine (75:15:10).

Table 4
MLL values for single systems and single detection modes

System	MLL	Detection	MLL
TLC 1	19.60	CC 1	14.99
TLC 2	14.09	CC 2	16.36
TLC 3	22.12	CC 3	15.60
GC	7.20	M_r	1.40
HPLC	9.80	DAD	10.13

Table 5
MLL values for combinations of single systems with their appropriate detection modes

System and detection	MLL	System and detection	MLL
TLC 1–CC 1	9.46	GC–M_r	1.10
TLC 2–CC 2	7.61	HPLC–DAD	3.52
TLC 3–CC 3	9.88		

tions of single systems and their corresponding detection modes. Interestingly, the combination of TLC R_F values with four colour codes now results in MLL values between 7 and 10, indicating that a simple, rapid TLC test plus a few colour reactions provides the same IP as a GC or HPLC retention index. The highest IP is obviously provided by GC–M_r, but it should be noted that even for this limited test set GC–M_r is unable to provide unequivocal identification for all 99 substances.

A substantial gain in IP is obtained when two separation systems are applied, together with their appropriate detection modes, as demonstrated in Table 6. When the information on R_F values in two TLC systems and the subsequent colour codes is utilized, MLL values of the order of 2 are obtained, whilst the inclusion of GC–M_r or HPLC–DAD, or both, results in MLLs close to 1. Still, even the combination of GC–M_r and HPLC–DAD does not result in an MLL of 1.00.

The MLL values for combinations of three separation systems and detection modes are given in Table 7. The use of all three TLC systems and the color codes now results in an MLL value of 1.35. Combinations including GC–M_r or HPLC–DAD, or both, are even better but there is only one combination that provides the ideal MLL value of 1.00, namely TLC 2–CC plus GC–MS plus HPLC–DAD.

Hence these MLL evaluations provide an objective assessment of the potential of the various systems and detection modes for the identification of unknown substances, leading to some valuable and interesting conclusions. Obviously, HPLC–DAD and GC–M_r are very powerful techniques, but even for this limited test set additional information from a suitable TLC–CC system is necessary to yield unambiguous identification. This contrasts with the widely held belief in analytical toxicology that identification can be achieved by applying two techniques based on different physico-chemical principles. Clearly, the latter is a serious oversimplification, even when MS is included. Although we utilized molecular masses derived from MS, we have good reasons to believe that the same is true if MS information is used in the form of electron impact (EI) mass spectra. More detailed calculations on EI-MS are in progress.

On the other hand, for those workers who do not have access to the more powerful coupled detection modes, such as DAD and MS, it is good to see that identifications can be approached very well on the basis of TLC–CC plus GC and HPLC retention indices alone. This is demonstrated in Table 7, in which the MLL values in parentheses were obtained by excluding M_r and DAD parameters: the best combination

Table 6
MLL values for combinations of two systems and their appropriate detection modes

System and detection	System and detection			
	TLC 2–CC	TLC 3–CC	GC–M_r	HPLC–DAD
TLC 1–CC	2.17	2.65	1.04	1.24
TLC 2–CC	–	2.20	1.04	1.19
TLC 3–CC	–	–	1.06	1.18
GC–M_r	–	–	–	1.02

Table 7
MLL values for combinations of three systems and their appropriate detection modes

System and detection	System and detection		
	TLC 3–CC 3	GC–M_r[a]	HPLC–DAD[a]
TLC 1–CC 1 plus			
TLC 2–CC 2	1.35	1.02 (1.06)	1.08 (1.11)
TLC 3–CC 3	–	1.04 (1.19)	1.12 (1.16)
GC–M_r[a]	–	–	1.02 (1.06)
TLC 2–CC 2 plus			
TLC 3–CC 3	–	1.02 (1.08)	1.08 (1.10)
GC–M_r[a]	–	–	1.00 (1.04)
TLC 3–CC 3 plus			
GC–M_r[a]	–	–	1.02 (1.08)

[a] Values in brackets have been obtained by omitting M_r and DAD parameters.

of TLC 2–CC plus GC and HPLC now gives an MLL value of 1.04, which is only marginally higher than 1.00 when DAD and M_r are included. The values for combinations of two TLC–CC systems with either GC or HPLC RIs are not much higher, between 1.06 and 1.19, respectively. Moreover, even those laboratories which can only afford TLC (*e.g.*, in developing countries) should not despair: the combination of the present three TLC systems and the colour codes already has a very good identification power with an MLL of 1.35.

5. Computerized identification

After having selected the most suitable systems and detection modes for STA on the basis of their MLL values, databases need to be established on an interlaboratory scale. As explained, these bases are to contain data on a great many substances of toxicological relevance. When unknown substances are encountered, their parameters are then to be compared with those in the database to find possible matches. It will be clear that this is to be done with the aid of a bench-top computer that is directly accessible in the laboratory. We are currently developing such a system that is capable of handling TLC–CC, GC–MS (allowing both EI and CI mass spectra) and HPLC–DAD. This system is called MTSS and will be commercially available through Merck (Darmstadt, Germany) from mid-1994. It will contain systems for acidic and neutral substances and also for basic and neutral substances. Users may add their own data to the built-in databases and may create databases on their own.

The system works as follows. After having run the unknown sample in any combination of analytical systems and developing mode one chooses, the parameters found are entered into the computer, together with the data for the reference mixtures used to calibrate the chromatographic systems. The latter allow the computer to correct the R_F values and/or to calculate the RI values. After pressing the "Search" key, the computer then compares the values for the unknown(s) with the values in the databases to find possible matches within the allowed error windows. It can do this for up to five unknown spots or peaks per sample and it automatically checks all possible configurations [14] for combinations of spots and peaks. It is also able to deal with situations where the number of spots and peaks do not match (*e.g.*, two TLC spots and three HPLC peaks). Finally, the computer prints out a list of substances that give acceptable matches for all the analytical parameters entered, in decreasing order of possi-

bility. Also, a similarity index is given for each listed substance, indicating the difference between the measured data and those in the database. Obviously, when data for only one analytical system are entered, the list of candidates will be very long, but the length of the list is drastically decreased when data for a multitude of systems are entered (as already seen under the MLL evaluations). Of course, the ultimate is reached when the final list contains only one substance with an acceptable similarity index. If the list continues to contain more than one candidate, it means that additional systems and detection modes must be applied. The best choice is strongly dependent on the substances involved.

Future versions of the MTSS may include additional HPLC systems and immunoassay systems. Work on the evaluation of the latter is in progress.

The use of a computerized search is indispensable in cases in which no prior information is available, such as the so-called general unknowns. However, it should be stressed that also in cases that appear to be routine and where abundant prior information is available, the MTSS may be very valuable: it may hint at a substance that is not seen very often but whose data are similar to those for a better known substance. Also, even when the identity of at least one toxicant is already known, the computer will check the likelihood of the presence of all other substances in the database, so that no substance is overlooked. This satisfies the ultimate aim of STA, to exclude all substances except the one(s) present.

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JOURNAL OF CHROMATOGRAPHY A

Review

Gas chromatography with surface ionization detection in forensic analysis

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Abstract

Surface ionization detection (SID) for gas chromatography (GC) is a recent technique that is very sensitive and specific to tertiary amino compounds. The instrumentation for this detector and the application of this method to analyses of drugs, such as tricyclic antidepressants, phenothiazines, butyrophenones, local anaesthetics, narcotic analgesics and diphenylmethane antihistaminics, are reviewed. Specific structures that affect the SID response are discussed and SID is compared with flame ionization, nitrogen–phosphorus and electron-capture detection. Many drugs of medico-legal interest can be determined by GC–SID with extremely high sensitivity and specificity. The high sensitivity may allow trace determintions of drugs present in small samples, such as blood stains and hair, extending its applicability in forensic toxicology.

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1. Introduction

In 1923, Kingdom and Langmuir [1] first observed the formation of positive caesium ions on an incandescent tungsten surface. When atoms or molecules impinge on an incandescent metal surface, they may evaporate partly as neutral particles and partly as positive or negative ions, as shown in Fig. 1. In 1985, Fujii and Arimoto [2] developed a surface ionization detection (SID) method for gas chromatography (GC), which detected positive ions formed on a hot platinum surface, and showed that it provided extremely high and specific responses to compounds containing tertiary amino groups.

This review demonstrates that many medicolegally important drugs can be determined by GC–SID with extremely high sensitivity and specificity. Recently, Fujii and Arimoto [3] have also published a review mainly on basic aspects of SID, such as its principles, theory and instrumentation.

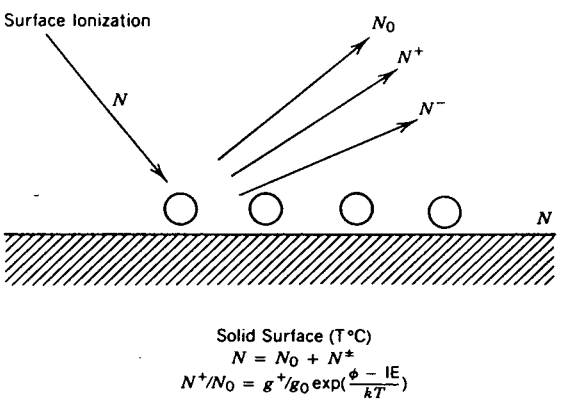

Fig. 1. Formation of neutral atoms and positive and negative ions on a hot metal surface. The surface with a layer of adsorbed particles of concentration N emits fluxes N_0 of neutral particles, N^+ of positive ions and N^- of negative ions. From ref. 3 (© 1992 Wiley).

2. Instrumentation for surface ionization detection

SID is a structural modification of standard nitrogen–phosphorus detection (NPD) for GC [2]; it consists of almost the same components as in NPD except that the alkali-salt bead emitter in NPD is replaced with a platinum emitter and the applied potential is reversed. A conventional flame ionization detection (FID) system might also be modified to a SID system if the platinum emitter is mounted in the gas flow path through the hole of detector envelope that is used for igniting the flame.

Fig. 2 shows the SID system develop by Fujii and Arimoto [2]. The platinum emitter is positioned between the quartz nozzle and the collector electrode. The ring electrode around the quartz nozzle is held at a positive potential of +200 V; the emitter and the ion collector are always at a negative potential of −200 V *versus* the ring electrode. Positive ion current directed to the collector is measured with an electrome-

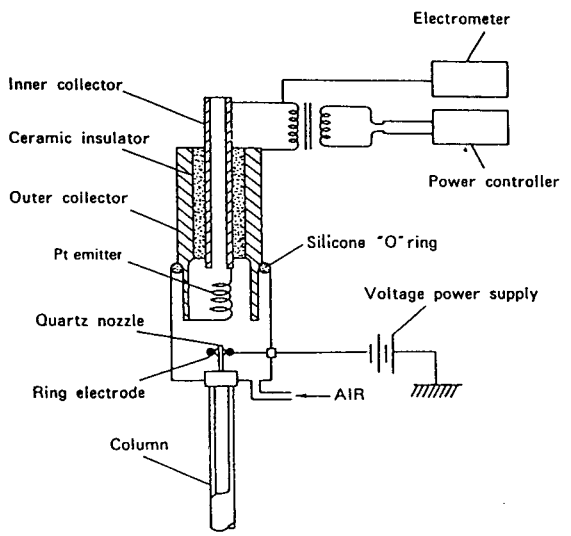

Fig. 2. SID system with a platinum emitter. From ref. 2 (© 1985 American Chemical Society).

ter. Electronics for variable heating of the emitter filament are required. The emitter is ten-turn coiled platinum (99.9%, 0.25 mm diameter), which is capable of withstanding temperatures around 1200°C for much longer than 1 month in the environment of a fast flow of a helium–air mixture. Platinum was chosen as an emitter material primarily because it has a higher work function (5.65 eV) than other typical refractory metals such as tungsten and rhenium. This property easily allows positive thermionic emission from the surface. The SID system is now commercially available from Shimadzu (Kyoto, Japan).

The SID conditions for capillary GC routinely used in our laboratories are as follows: heating current through the platinum emitter, 2.2 A; emitter temperature, *ca.* 600°C; and ring electrode bias voltage, +200 V *versus* the collector electrode.

3. Application to drug analyses

3.1. Tricyclic antidepressants

As a first study of the application of GC–SID, we tested the tricyclic antidepressants imipramine, amitriptyrine, trimipramine, chlorimipramine, desipramine, carpipramine, clocapramine and lofepramine [4]. Of these eight compounds, lofepramine, carpipramine and clocapramine, which had cyclic or aromatic side-chain structures, were found to give multiple peaks owing to heat decomposition in their underivatized form under medium-bore capillary GC conditions. Desipramine, with a secondary amino side-chain, showed much lower sensitivity. Therefore, the main experiments were performed on imipramine, amitriptyrine, trimipramine and chlorimipramine, which have tertiary amino groups with straight aliphatic side-chain structures, because they were relatively stable to heat.

Imipramine, amitriptyline, trimipramine and chlorimipramine (5 ng of each) were added to 1 ml of urine, plasma and whole blood, and extracted with Sep-Pak C_{18} cartridges. The four drugs were separated well from biological impurities on the gas chromatograms, but imipramine and trimipramine appeared overlapped with the use of an SPB-1 fused-silica capillary column (30 m × 0.32 mm I.D., film thickness 0.25 μm) (Fig. 3). The recovery of these drugs added to each body fluid was more than 60%.

The backgrounds obtained from plasma and whole blood were fairly clean, but that for urine showed many impurity peaks, which may be due to the excretion of many methylated metabolites of endogenous and exogenous amines in the urine; however, the drug peaks did not overlap any impurity peak in the urine extract. The baselines remained steady as the column temperature was increased (Fig. 3).

The four drugs showed satisfactory linearity in the range 10–80 pg in the injected volume. The detection limit (signal-to-noise ratio = 3) was 5–10 pg on-column (0.5–1.0 ng/ml in a sample).

Fujii *et al.* [5] also mentioned briefly that imipramine and chlorimipuramine can be determined with high sensitivity by GC–SID.

3.2. Phenothiazines

Phenothiazines are used widely as antipsychotics (major tranquillizers), anti-parkinsonism drugs and antihistaminics. They are frequently encountered in forensic chemistry and clinical toxicology because of their relatively small safety dose ranges.

We tested fourteen phenothiazines as shown in Table 1 [6], which gives their relative peak-area intensities measured by GC–SID when 2 pmol of each were injected into the GC port. The sensitivity was highest for trimeprazine, levomepromazine and promazine and lowest for thioproperazine and thiethylperazine.

Calibration graphs of peak area *versus* drug amount were drawn for chlorpromazine, trimeprazine and promazine. The three drugs showed excellent linearity with r values of 0.9984–0.9999 in the range of 0.25–3 pmol on-column. The detection limits of these phenothiazines were *ca.* 5–10 pg on-column, which is equivalent to 250–500 pg/ml in a sample.

Addition tests were made with a non-polar

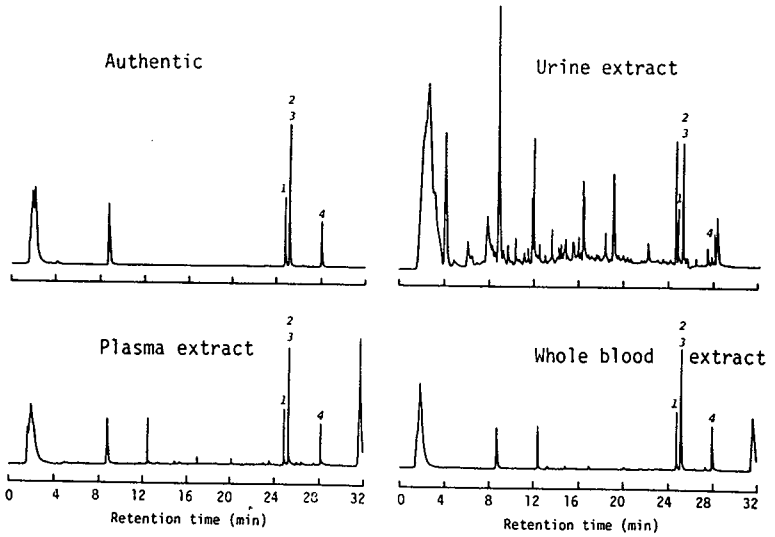

Fig. 3. Capillary GC–SID for tricyclic antidepressants extracted from urine, plasma and whole blood with the use of Sep-Pak C_{18} cartridges. Peaks 1 = amitriptyline; 2 = imipramine; 3 = trimipramine; 4 = chlorimipramine. GC was carried out with an SPB-1 fused-silica capillary column (30 m × 0.32 mm I.D., film thickness 0.25 μm). GC conditions: column temperature, increased from 100 to 280°C at 6°C/min; injection temperature, 200°C; and helium flow-rate, 22 cm/s. The samples were injected in the splitless mode at a column temperature of 100°C and the splitter was opened after 2 min. A mixture of the four tricyclic antidepressants (5 ng of each) was added to 1 ml of urine, plasma or whole blood.

Table 1
Retention times and relative peak-area intensities of phenothiazines measured by GC–SID

Compound[a]	Retention time (min)	Relative peak-area intensity[b]
Trifluprom azine	19.3	0.67
Isothipendyl	19.8	0.80
Promethazine	19.9	1.36
Trimeprazine	20.3	2.30
Promazine	20.6	1.95
Ethopropazine	21.4	1.33
Chlorpromazine	23.0	1.00
Levomepromazine	23.6	2.01
Trifluoperazine	25.6	0.41
Perazine	27.1	0.64
Prochlorperazine	28.7	0.20
Thioridazine	31.1	0.34
Thiethylperazine	32.7	0.09
Thioproperazine	46.9	0.04

[a] Aliquots of 2 pmol of each drug were injected into the GC port.
[b] Chlorpromazine = 1.00.

capillary column for whole blood and urine, with and without the addition of 100 pmol each of ten phenothiazines to 1-ml whole blood and urine samples. Prochlorperazine, thiethylperazine, thioproperazine and thioridazine were omitted because of their low responses or long retention times. All drug peaks were well separated from impurity peaks except that of promethazine, which partially overlapped an impurity peak for the whole blood extract. The recoveries of the ten phenothiazines were more than 79% for both whole blood and urine samples. The baselines remained steady during the increase in column temperature.

3.3. Butyrophenones

Butyrophenones are classified as major tranquillizers and are widely used for the treatment of schizophrenia. They are also frequently encountered in forensic science practice. We found

that some drugs of this group showed a high response to SID [7]. The drugs tested were haloperidol, moperone, bromperidol and pipamperone. They were added (50 pmol of each) to 1 ml of whole blood or urine and extracted with Sep-Pak C_{18} cartridges. The four drugs were well separated from each other and from impurities with a DB-1 fused-silica capillary column (15 m × 0.32 mm I.D., film thickness 0.25 μm).

Excellent linearity was observed in the range of 0.2–4 pmol on-column for the four compounds. The detection limit of the drugs was about 40 pg (0.1 pmol) on-column.

Haloperidol was determined with this method in whole blood and urine samples obtained from a 76-year-old male schizophenic patient who had received 3 mg of haloperidol daily; the levels found were 7.18 and 43.2 pmol/ml for blood and urine, respectively.

3.4. Local anaesthetics

Local anaesthetics are occasionally encountered in forensic science practice, especially in medical accidents. Fujii *et al.* [5] reported that lidocaine, a typical local anaesthetic, could be determined with high sensitivity by GC–SID. We also tested lidocaine, procaine, dibucaine, ethyl *p*-aminobenzoate (benzocaine), tetracaine, mepivacaine, bupivacaine, propitocaine, oxybuprocaine and *p*-(butylamino)benzoic acid 2-(diethylamino)ethyl ester by this method [8,9]. All drugs were relatively stable to heat and did not decompose during capillary GC analysis. They can be separated from each other and from impurities with use of either a non-polar or intermediate-polarity capillary column [10].

The sensitivity of the local anaesthetics to SID varied considerably. The highest sensitivity was apparent with lidocaine, mepivacaine and bupivacaine. These three drugs showed excellent linearity of the calibration graphs in the range 10–100 pg on-column. The detection limits of the drugs were 5–10 pg on-column (0.5–1.0 ng/ml in a sample). The three drugs (5 ng of each) added to 1 ml of whole blood and cerebrospinal fluid (CSF) could be measured with excellent recoveries.

The sensitivity for procaine, dibucaine, tetracaine, oxybuprocaine and *p*-(butylamino)benzoic acid 2-(diethylamino)ethyl ester was *ca.* 20 times lower and that for ethyl *p*-aminobenzoate and propitocaine was *ca.* 100 times lower.

3.5. Narcotic analgesics and their analogues

Meperidine, a narcotic analgesic drug with a piperidine ring structure, has been reported to be determined with high sensitivity by GC–SID [11]. Meperidine, together with the internal standard (diphenylpyraline), was added to whole blood and urine and extracted with a Sep-Pak C_{18} cartridge. The compounds were determined by GC–SID was a DB-17 fused-silica capillary column (15 m × 0.32 mm I.D., film thickness 0.25 μm). The recovery of meperidine was close to 100% and the detection limit was 10 pg on-column.

Pentazocine has also been determined by GC–SID [12], although its sensitivity was lower than that of meperidine. Pentazocine, which had been added to whole blood and urine samples and extracted with a Sep-Pak C_{18} cartridge, could be detected with a good recovery of more than 90%. The detection limit of this drug was about 50 pg on-column. Extracts of whole blood and urine samples obtained 3 h after intramuscular injection of 15 mg of pentazocine showed intense peaks of the drug.

Dextromethorphan and dimemorphan, morphine analogue antitussives, could be determined with relatively high sensitivity by this method [13]. The detection limit of these drugs was about 20 pg on-column.

Suzuki *et al.* [14] found that fentanyl and its derivatives could be determined with high sensitivity by GC–SID, although their experiments were carried out only with authentic standards.

3.6. Diphenylmethane antihistaminics and their analogues

Diphenylmethane antihistaminics and their analogues are one of the most commonly used drug groups for the treatment of colds, asthma and other allergic diseases, and are often abused;

fatal cases involving their ingestion are frequently encountered.

Most of the drugs contain tertiary amino groups and are very suitable for determination by GC–SID. We tested eleven drugs of this group [15] and obtained extremely high sensitivities for some of them. Table 2 gives the retention times and relative peak-height intensities of eleven diphenylmethane antihistaminics and their analogues (2 pmol of each injected). The response was highest with doxylamine, diphenhydramine, orphenadrine, chlorpheniramine, carbinoxamine and diphenylpyraline; their detection limits were 2–5 pg (*ca.* 6–20 fmol) on-column. The other drugs in Table 2 had sensitivities about ten times lower than those for the above six drugs.

The gas chromatograms for the extracts of whole blood and urine, with and without addition of 50 pmol each of the six drugs to 1-ml samples, with the use of a non-polar DB-1 fused-silica capillary column, showed satisfactory separation of the test peaks from each other and from impurities. The recovery of the drugs added to whole blood and urine was more than 60% with extraction with Sep-Pak C_{18} cartridges.

Table 2
Retention times and relative peak-height intensities of diphenylmethane antihistaminics and their analogues measured by GC–SID

Compound[a]	Retention time (min)[b]	Relative intensity[c]
Diphenhydramine	13.7	1.00
Doxylamine	14.3	1.19
Orphenadrine	14.7	0.84
Chlorpheniramine	15.5	0.66
Carbinoxamine	16.4	0.64
Diphenylpyraline	16.7	0.54
Terodiline	14.9	0.04
Benactyzine	18.7	0.21
Homochlorcyclizine	19.7	0.19
Clemastine	20.5	0.09
Pipethanate	21.0	0.13

[a] A 2-pmol amount of each drug was injected into the GC port.
[b] GC conditions as in Fig. 4.
[c] Diphenydramine = 1.00.

In all the chromatograms, baselines were also steady and did not rise with increase in column temperature.

3.7. Miscellaneous

Kawano *et al.* [16] reported the sensitive determination of aprindine, an antiarrhythmic drug, in serum; its detection limit was 16 pg.

We have recently obtained good results by GC–SID for cocaine, benzoylecgonine, atropine, antitussive drugs such as clobutinol, carbetapentane and oxeladin and paraquat after reduction.

4. Specific structures that affect the response of surface ionization detection

4.1. Tertiary amino groups

SID gives a high sensitivity to compounds with tertiary amino groups [2]. It seems that tertiary amino groups with straight side-chain structures give a slightly higher SID response than cyclic tertiary amino groups when the sensitivities for various types of phenothiazines (Table 1) and for diphenylmethane antihistaminics are compared (Table 2). It should be mentioned that compounds with N-alkylpiperidine rings, such as mepivacaine, bupivacaine [8], meperidine [11], butyrophenones [7] and fentanyl [14], give relatively high sensitivities.

Secondary amino compounds, such as terodiline [15] and propitocaine [8], gave sensitivities 25–100 times lower than tertiary amino compounds.

4.2. Amide groups

A nitrogen atom directly bound to a carbonyl group does not give a high SID response; we failed to detect barbiturates, benzodiazepines, pyrazolones and hydantoins, all of which contain amide groups, by GC–SID with high sensitivity (unpublished observations). However, a carbonyl group present in the β-position to the nitrogen of a tertiary amino group showed no negative effects for SID; this conclusion is based on the

very high sensitivity observed for lidocaine, bupivacaine and mepivacaine, which contain amide structures in their side-chains together with tertiary amino groups [8].

4.3. Halogen groups

Chlorpromazine and trifluprom azine, which contain halogen groups on the phenothiazine nucleus, showed lower responses than promazine (Table 1). The same is true when the data for prochlorperazine and trifluoroperazine are compared with those for perazine. Chlorpheniramine and carbinoxamine gave lower responses than diphenhydramine and doxylamine (Table 2). Hence, the presence of a halogen group may lower the SID response. This is not surprising because of the electrophilic properties of halogen groups.

4.4. Sulphur groups

Thiethylperazine and thioproperazine, which contain sulphur groups on the phenothiazine nucleus, showed the lowest sensitivity, suggesting negative effects of sulphur groups for SID (Table 1).

5. Comparison with other detectors for gas chromatography

5.1. Flame ionization detection

Compounds with tertiary amino groups could be determined by GC–SID with a sensitivity 100–1000 times higher than that of conventional FID for tricyclic antidepressants [4], local anaesthetics [8], dextromethorphan, dimemorphan [13] and diphenylmethane antihistaminics [15].

5.2. Nitrogen–phosphorus detection

In each study of the GC–SID of drugs with tertiary amino groups [4,6–9,11–13,15], the sensitivity of SID was carefully compared with that of NPD either on the basis of our own experiments or literature data. In view of the signal-to-

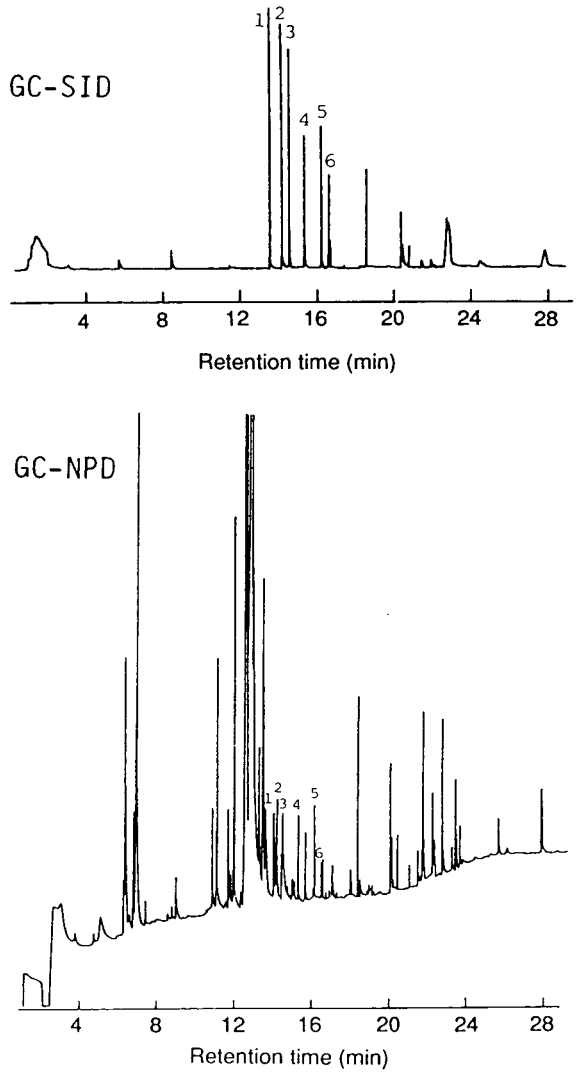

Fig. 4. Comparison of GC–SID with GC–NPD for a human whole blood extract in the presence of six diphenylmethane antihistaminics (50 pmol of each for 1-ml samples) with the use of Sep-Pak C_{18} cartridges for extraction. Peaks: 1 = diphenhydramine; 2 = doxylamine; 3 = orphenadrine; 4 = chlorpheniramine; 5 = carbinoxamine; 6 = diphenylpyraline. GC was carried out with a DB-1 fused-silica capillary column (30 m × 0.32 mm I.D., film thickness 0.25 μm). GC conditions: column temperature, increased from 100 to 280°C at 8°C/min; injection temperature, 280°C; detector temperature, 280°C; and helium flow-rate, 22 cm/s. The samples were injected in the splitless mode at a column temperature of 100°C and the splitter was opened after 1 min. A drug mixture of 50 pmol of each was added to 1 ml of whole blood. Both chromatograms were obtained with samples from the same extract vial.

Table 3
Summary of determinations of drugs by GC–SID without derivatization

Drug	Sample[a]	Extraction	Elution solvent	GC column[b]	Detection limit (on-column) (pg)	Ref.
Tricyclic antidepressants						
Amitriptyline, chlorimipramine, imipramine, trimipramine	WB, P, U	Sep-Pak C_{18}	Chloroform–2-propanol (9:1)	SPB-1 (100–280°C)	5–10	4
Phenothiazines						
Chlorpromazine, ethopropazine, isothipendyl, levomepromazine, promazine, promethazine, triflupromazine, trimepromazine	WB, U	Sep-Pak C_{18}	Chloroform–acetonitrile (8:2)	DB-1 (120–280°C)	5–10	6
Perazine, trifluoperazine	WB, U	Sep-Pak C_{18}	Chloroform–acetonitrile (8:2)	DB-1 (120–280°C)	25–50	6
Butyrophenones						
Bromperidol, haloperidol, moperone, pipamperone	WB, U	Sep-Pak C_{18}	Chloroform–ethanol (9:1)	DB-1 (180–300°C)	40	7
Local anaesthetics						
Bupivacaine, lidocaine, mepivacaine	WB, CSF	Sep-Pak C_{18}	Chloroform–methanol (9:1)	Ulbon HR-1 (100–280°C)	5–10	8
Benoxinate, dibucaine, procaine	WB, CSF	Sep-Pak C_{18}	Chloroform–ethanol (4:1)	DB-17 (120–180°C)	100–200	9
Narcotic analgesics and analogues						
Meperidine	WB, U	Sep-Pak C_{18}	Chloroform–ethanol (9:1)	DB-17 (100–220°C)	10	11
Pentazocine	WB, U	Sep-Pak C_{18}	Chloroform–ethanol (9:1)	DB-17 (150–280°C)	50	12
Dextromethorphan, dimemorphan	WB, U	Sep-Pak C_{18}	Chloroform–ethanol (9:1)	DB-17 (100–280°C)	20	13
Fentanyl and derivatives	Authentic standard	–	–	CBP-1 (100–320°C)	5	14
Diphenylmethane antihistaminics and analogues						
Carbinoxamine, chlorpheniramine, diphenhydramine, diphenylpyraline, doxylamine, orphenadrine	WB, U	Sep-Pak C_{18}	Chloroform–methanol (8:2)	DB-1 (100–280°C)	2–5	15
Benactyzine, clemastine, homochlorcyclizine, pipethanate, terodiline	WB, U	Sep-Pak C_{18}	Chloroform–methanol (8:2)	DB-1 (100–280°C)	20–50	15
Other						
Aprindine	Serum	Ethyl acetate–hexane (9:1)	–	DB-17 DB-1 (240°C)	16	16

[a] WB = Whole blood; P = plasma; U = urine.
[b] Fused-silica capillaries.

noise ratio and baselines, the sensitivity of GC–SID is 10–100 times higher than that of GC–NPD. A typical comparison of SID with NPD for diphenylmethane antihistaminics is shown in Fig. 4; the two traces were obtained with the same sample. In the SID chromatogram, the baseline was steady and did not rise with increase in column temperature, whereas in the NPD chromatogram many large impurity peaks appeared at various stages of the GC measurements and the baseline fluctuated and rose slightly with increase in column temperature.

Compounds with secondary or primary amino groups can probably be determined with higher sensitivity by GC–NPD than by GC–SID.

5.3. Electron-capture detection (ECD)

GC–ECD often gives a very high sensitivity comparable to that of GC–mass spectrometry with selected-ion monitoring for compounds with halogen or nitro groups. GC–SID gave a sensitivity comparable to or even higher than that given by GC–ECD for haloperidol [7] and chlorpheniramine [15].

6. Conclusions

GC–SID is a recent method developed in 1985 [2] and its application to drug analyses has begun. A brief summary of the applications is given in Table 3, which covers many drugs important in forensic science practice. GC–SID is especially recommendable for the determination of compounds with tertiary amino groups without halogen or nitro groups. The extremely high sensitivity of this method may also allow trace determinations of drugs present in small samples, such as blood stains and hair, extending its applicability in forensic toxicology.

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Review

Determination of volatile substances in biological samples by headspace gas chromatography

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Abstract

This review surveys headspace gas chromatographic (HS-GC) methods as used in the determination of volatile substances in body fluids and tissues from the viewpoints of the design of HS-GC instrumentation, partition coefficients and matrix effects of biological samples, additives to the liquid phase and stabilities of volatile substances in biological samples. It includes extensive tables that detail published static HS-GC methods that have been applied in forensic, clinical and environmental analyses.

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1. Introduction

Volatile substances are defined as those which vaporize easily when warmed. The detection and determination of volatile toxic substances is very important in forensic science [1], *e.g.*, in deducing causes of death and in the investigation of driving offences involving alcohol. Within clinical practice, they include the routine monitoring of industrial workers for exposure to hazardous compounds and the detection of organic endogenous metabolites in certain clinical diagnoses. In anaesthesiology, the solubility of inhaled anaesthetics in blood and tissues has been determined. In the environmental sciences, certain volatile compounds in biological samples have been measured as a means of monitoring environmental pollution. The headspace gas chromatographic (HS-GC) method was originally developed for use in anaesthesiology [2], toxicology [3,4] and food science [5] research. This method is now a powerful tool for analyses for volatile substances in a wide range of research and practical applications; including forensic science, clinical chemistry, anaesthesiology, environmental science, food science, polymer science and basic physico-chemistry (*i.e.*, in the determination of physical constants).

There are several monographs [6–8] and reviews [9–14] covering the HS-GC method. However, there has never been an extensive review of the HS-GC method as applied to biological analyses. This paper, therefore, centres on the static HS-GC method and its use in the determination of volatile substances in body fluids and tissues in relation to forensic science, and in addition briefly discusses its applications in clinical chemistry and environmental science. Obviously, many types of body fluids and tissues from humans and animals are the objects of analysis and the complexity of these samples alone presents a unique challenge to the researcher. Representative liquid samples include blood, urine, vitreous humor and milk. Solid biological samples include tissues such as the liver, lung, brain, kidney, muscle and various types of fatty tissue. In addition, clinical samples requiring analysis may originate from patients with a wide range of conditions including healthy, diseased, burned or deceased (*i.e.*, forensic examination). The analysis of breath is excluded from this review as it is considered a gaseous sample. In addition, the analysis of other biological samples, along with foods and beverages, is also excluded as these subjects have been reviewed elsewhere [15].

The aim of this paper is to provide a critical review of the HS-GC method as applied to the determination of volatile substances in body fluids and tissues. In addition, it provides reference methods and special precautions necessary for toxicologists engaged in the establishment of a practical HS-GC method. An extensive review of the literature concerning the fundamentals of HS-GC methodology is presented in terms of application to biological sampled. This literature review is presented in tables that are organized according to the chemical class of the analyte.

2. Gas chromatographic method for the determination of volatile substances

The determination of the level of volatile substances in samples has been accomplished through the use of titrimetric, spectrophotometric or chromatographic methods. The titrimetric and spectrophotometric techniques lack specificity and usually do not provide sufficient sensitivity. In contrast, the detection of volatile substances using gas chromatography (GC) is both qualitative (with respect to retention time) and quantitative (with respect to peak signal intensity). As a result, GC analysis is generally used for these types of determinations [16]. The three methods used for the preparation of samples for

GC analysis are as follows: (1) solvent extraction (SE), (2) direct aqueous injection (DAI) and (3) direct injection of the headspace volume (HS). The SE method involves the extraction of the volatile analyte(s) of interest from the sample using an organic solvent. An aliquot of this extract is then introduced into the GC column. This method is used extensively for the pretreatment of non-volatile analytes, allows for the concentration of analytes, thereby increasing sensitivity, and is also effective for volatile substances with high boiling points. However, the SE method has several disadvantages, including a time-consuming and laborious extraction procedure. In addition, precise determination of the analyte(s) cannot be expected, especially for substances with low boiling points. The solvent peak itself can interfere with the elution of analytes and many non-volatile components can be co-extracted, thus interfering with peak detection. This method requires ultra-high-purity organic solvents because the sensitivity is limited by background signals which may occur due to any trace contaminants.

The second technique, DAI, is a GC injection method in which minute amounts of the sample are injected directly into the GC column. The advantages of this procedure include a rapid and simple preparation of the sample. However, contamination of the GC injector and GC column with sample matrix is a serious disadvantage and necessitates frequent maintenance of the injector port. Also, attaining a stable baseline is often difficult and this method does not provide high sensitivity owing to the limited sample volume. In addition, water vapour can also disturb the chromatographic separation. For these reasons, the DAI method is now used almost exclusively for the analysis of aqueous samples only.

The HS technique was developed as a viable alternative to the above two methods. This method involves the equilibration of volatile analytes between a lower liquid (or solid) phase and an upper gaseous phase, with only the gaseous phase sampled. This method has significant advantages over the previous two. For example, gaseous sampling avoids any contamination of non-volatile components which may be found in the sample matrix or in an organic solvent.

The HS-GC technique can be divided into the two following categories: static (equilibrium) HS and dynamic (non-equilibrium) HS, also referred to as the "purge and trap" method. The static HS method involves the equilibration of volatile analyte within the sample with the vapour phase at a defined temperature. The vapour phase containing the analyte is then injected into the GC column in a closed system. This method is simple, minimizes the number of artifacts during analysis, can provide precise quantification and can effectively measure volatile substances with relatively low water solubility. A drawback, however, is that it is considerably less sensitive when analysing volatile substances with high water solubility. The second technique, dynamic HS, involves passing a carrier gas over the sample for a specified period of time and trapping the analyte in a cryogenic or adsorbent trap. The concentrated analyte is then introduced using pulsed heating. Several variations of this technique have been developed apart from the simple dynamic HS method (the inert gas is purged over a liquid sample), including through-solution gas stripping (the inert gas is bubbled into the liquid phase) and thermal desorption (the sample is retarded and heated on a solid support while the gas is purged). In general, the dynamic HS method is effective for the measurement of volatile substances of moderate to high water solubility. In addition, this method offers increased sensitivity when compared with static HS, DAI and SE methods owing to the concentration after trapping of the volatile analyte. The dynamic HS technique, however, is difficult and costly owing to the complex instrumentation necessary. Precise quantification of analytes suffers from incomplete recovery after the purging, trapping and desorption steps. The dynamic HS method may also result in artificats due to impurities present in the purging gas. There are many commercially available automated dynamic HS machines.

3. General

3.1. Theory concerning static HS-GC method

According to Raoult's law, in a closed system where a volatile component is in thermodynamic equilibrium between the liquid phase and the gaseous phase, the following equation holds:

$$P_i = P_i^0 x_i \gamma_i \quad (1)$$

where P_i = partial vapour pressure of the volatile component i, P_i^0 = vapour pressure of the pure component i, x_i = mole fraction of component i in solution and γ_i = activity coefficient of component i in solution. If the components do not interact, $\gamma_i = 1$. In contrast, non-ideal solutions can show positive or negative deviations from Raoult's law. The extent of deviation depends on the types of volatile substances analysed and on the solvent [17]. The value of γ_i is constant when the concentration of volatile substance in the liquid phase is below 1% [18], and Raoult's law can be simplified to Henry's law. The concentration of analyte in the gaseous phase is then proportional to that in the liquid phase:

$$C_L / C_G = k \quad (2)$$

Considering the mass balance of volatile analyte between two phases, the following equation is valid, as illustrated in Fig. 1:

$$C_L^0 V_L = C_L V_L + C_G V_G \quad (3)$$

where C_L^0 represents the analyte concentration in the liquid phase prior to HS equilibrium, C_L and C_G represent the concentrations in the liquid and gaseous phases, respectively, after equilibrium and V_L and V_G represent the volumes of the liquid and gaseous phases. The partition coefficient (k) and phase ratio (β) are defined as C_L/C_G and V_G/V_L, respectively. Eq. 3 can be transformed as follows:

$$C_G = C_L^0 / (k + \beta) \quad (4)$$

Eq. 4 demonstrates that the GC sensitivity is related to C_G and is dependent on k, β and C_L^0. The k value is constant with a dilute analyte [18], constant temperature and identical liquid matrix. The k value decreases with increase in temperature and changes according to the character of the matrix. The principles and applications of HS-GC methods have been reviewed elsewhere [10,18].

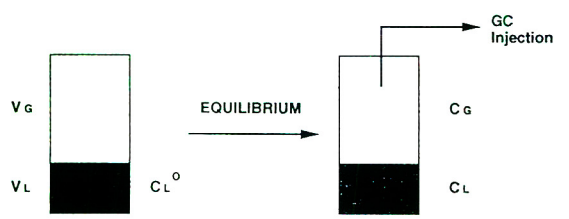

Fig. 1. Schematic diagram of static headspace gas chromatography. For symbols, see text.

3.2. System and instrumentation

Originally the HS-GC technique was applied to the measurement of the solubility of anaesthetics [2,19–21] and to the analysis of gases [3,4,22–24], alcohols [25,26] and solvents [27,28] in biological samples. The instrumentation and procedures were simple, but there were many drawbacks, a major one being the lack of precise quantification. Through improved instrumentation and further theoretical developments, the HS-GC method has been refined to overcome previous drawbacks and can now provide accurate quantitative analyses.

The HS-GC system consists of an HS element (pretreatment) and a GC element (measurement). The HS element can either be manual or automated and consists of a vaporization container where equilibrium is obtained, a heating device which keeps the HS container at a constant temperature and an injection device which transfers the vapour phase from the HS container into the GC column. Initially, the HS element consisted of a glass vial sealed with a rubber septum with transfer through a gas-tight syringe [2,4,19,20,25–27,29]. In general, a hard glass vial is recommended as the container. The container is sealed by either a screw-cap or a crimped cap. A septum is necessary for sealing the container, maintaining the physical integrity of the sample vial with increase in internal pressure, and allowing for sample withdrawal via

a syringe. Originally butyl rubber or silicone rubber septa were used but were found to introduce serious errors due to adsorption of the analyte on these materials, resulting in a time-dependent decrease in vapour concentration [30]. Currently, septa are coated with either polytetrafluoroethylene (PTFE; Teflon) or aluminium foil to prevent adsorption. All components of the HS container and injection equipment which contact the sample must be composed of chemically inert materials [31].

Even stir bars made of PTFE have been found to adsorb benzene and toluene [32]. Because of the limitations on sample volume and vapour volumes which can be injected into the GC system, HS containers with capacities between 5 and 25 ml have frequently been used. Manual injection with gas-tight syringes is the transfer method of choice. Unless special pressure corrections are employed [33,34], the use of pressure-lock-type syringes is recommended to prevent the loss of sample vapour. The syringe should be prewarmed to a temperature higher than the HS temperature in order to prevent analyte or water condensation. Contamination of the syringes is a major concern as it can lead to non-quantitative results [35]. It is possible to minimize contamination by cleaning the syringe with solvent and drying under vacuum at high temperature between analyses. Septumless injection equipment has also been devised to prevent cross-contamination [36–40]. A further disadvantage of the manual injection method is its low reproducibility due to sampling inaccuracies, and therefore automated injection instruments have been developed and are now commercially available. At present, there are two types of automated HS samplers: the sampling loop type [41] and the pneumatic control type [42–44]. The sampling loop type is a system where the HS vial is pressurized by a GC carrier gas for a constant time. At a given point, the vapour phase is released to atmospheric pressure, fills the sample loop and is subsequently introduced into the GC column. The pneumatic control type is a system whereby the HS container is pressurized by a GC carrier gas for a constant time and the vapour phase is injected into the GC column under pneumatic control at a given time. A stainless-steel transfer line of the HS sampler has been found to introduce ghosting problems, and it has been overcome by lining the transfer tube with deactivated fused silica [45].

Other HS containers that have been used are glass syringes, facilitating easy introduction of the HS vapour phase [2,3,21,28,46–50]. In addition, specially designed devices have also been used where carrier gas is employed either to sweep vapour over the HS phase or to by-pass it by a switching valve [40,51–54].

The ratio of the gaseous phase to liquid phase (β) and the injection volume will both vary depending on the HS-GC analyses. In general, phase ratios for most common analytes are 5–20. Injection volumes that offer appropriate sensitivity and peak resolutions should be low enough that they do not disturb the HS equilibrium. Usually, approximately 10% of the gaseous phase (0.05–2 ml) is introduced into the GC system.

Because the GC aspect is beyond the scope of this review, only the most important GC advances developed for increasing the sensitivity and resolution and shortening the analysis time of HS-GC are considered, namely the use of capillary columns [55–60] and cryofocusing systems [44,56,57,61–64].

3.3. Partition coefficients

Static HS-GC determination of volatile analyte requires the determination of a partition coefficient. Because matrix effects are unpredictable, special consideration must be given to the effects that the matrix may have on the determination of partition coefficients.

3.3.1. Data on partition coefficients

Published data on partition coefficients are widely available. In the physico-chemical meaning, the partition coefficient is equal to the Ostwald solubility coefficient, and so may be calculated from available physical solubility data (as in the case of priority pollutants) [65]. In anaesthesiology, the partition coefficients of

anaesthetics in various liquid phases such as water, oil and biological fluids are needed in order to obtain pharmacokinetic information such as the anaesthetic uptake and body distribution. These values are published in comprehensive tables [66–69]. Partition coefficients are also necessary to develop physiologically based pharmacokinetic models used to access uptake, distribution, metabolism and elimination of volatile pollutants [70–75]. Harger *et al.* [76] reported partition coefficients for ethanol in biological fluids by using spectrophotometric measurement of the vapor phase. Aqueous phase partition coefficients are available for components of food [77], organophosphorus compounds [78] and solvents [79] as determined by static HS-GC methods.

Partition coefficients are based on many parameters, *e.g.*, polarity, volatility and molecular mass. In general, partition coefficients of volatile substances in water increase with increasing water solubility and therefore the following order among chemical classes is observed: aromatics > cycloalkanes > alkenes > alkanes. Within each chemical class a decrease in partition coefficient occurs as the molecular mass increases [77].

Partition coefficients also differ according to the properties of the liquid phase [80,81]. Partition coefficients in biological samples are significantly influenced by the matrix components. Anaesthesiological and toxicological studies have indicated that partition coefficients of untested volatile substances within complex biological samples can be predicted based on a knowledge of the oil and saline partition coefficients and a knowledge of the relative lipophilicity and hydrophilicity of various biological fluids and tissues [72]. The relative ranking of increasing lipid character and decreasing aqueous character based on these coefficients is water < urine (body fluids) < blood < muscle < liver < fat tissue < oil. The partition coefficients for aromatic hydrocarbons in blood are closely correlated with the product of the partition coefficients for these compounds in both water and oil, but coefficients for ketones are more dependent on the coefficients determined in water [72]. For congeners of alkylbenzenes and chlorinated aliphatic hydrocarbons, those with a short alkyl chain are more soluble in water but less soluble in blood and olive oil than those with a long chain [72,73]. Although the partition coefficient can be regarded mainly as a simple function of lipid content, it can also be affected by other components within biological samples, such as water, salts, proteins and carbohydrates. Diethyl ether represents the only known exception to the rule that compounds show greater solubility in protein solutions than in water [67]. Partition coefficients are not identical for all types of protein or lipids within a liquid matrix and any variation in the composition of the matrix can alter the partition coefficient [67].

3.3.2. Effect of salt addition

The solubility of many non-electrolytes decreases in the presence of salts. This phenomenon, known as the "salting-out effect", produces an increased activity coefficient (γ) with increasing salt concentration. In contrast, a decrease in salt concentration produces a "salting-in effect". The degree of change in k values is correlated with the concentration and type of salt and volatile substance. For example, the chemical properties of both the salt ion and the volatile analyte, presumably charge density, size and resultant effects on bulk water structure, affect this behaviour. Theoretical predictions of the magnitude of this effect may be made and are based mainly on electrostatic theory [82]. The addition of salt causes a decrease in hydrogen bonding between analyte and water as free water is sequestered by the tight hydration shell surrounding the salt ion. For example, partition coefficients of ketones, aldehydes, alcohols [83] and ethyl acetate [84] are all decreased by the addition of sodium sulphate. A salting-out effect is also observed on addition of sugar compounds [85].

3.3.3. Matrix effects of biological samples

Biological samples consist of complicated components such as water, proteins, lipids, saccharides and salts, all in varying concentrations. Biological samples also differ in chemical compo-

sition, specimen type, specimen origin (species and individual) and clinical conditions. For example, blood samples can differ in haematocrit value, haemoglobin concentration and lipid content. Frequently encountered complications include alterations in the condition of the sample through post-mortem changes such as putrefaction, or the contamination of blood with other body fluids. These considerations make the preparation of a matrix identical with that in the unknown samples, for use in quantification and calibration, very difficult. Forensic and clinical toxicologists should therefore be aware of the limitations of quantitative HS-GC in the analysis of biological samples. Dilution of the biological samples with water can reduce the influence of complex matrix effects.

An association phenomenon between volatile analyte and matrix components may contribute to decreased partitioning [34]. For example, the slopes of the calibration graphs for tetrachloromethane [86] and ketones [87] were shown to differ among different specimens. In addition, lipid components of blood were found to retain halothane to varying extents and thereby resulted in poor correlation across samples, differences in partition coefficients and difficulty in obtaining accurate calibration graphs [88]. Fatty components in biological samples were also found to affect the vaporization of organic solvents [89] and ethanol [90]. The variable fat content of liver, as a consequence of the state of alcohol consumption, might affect the relative partition coefficient of ethanol, other alcohols or an internal standard [91]. Blood samples containing higher than normal cholesterol and triglyceride levels resulted in larger k values for halothane [88]. No clear correlation can be drawn between partition coefficients of ethanol and the following blood constituents; haemoglobin, albumin, total protein, urea, electrolyte and glucose levels [91]. Different matrix components within biological samples had differing effects on determinations of partition coefficients for analyte and internal standard pairs in the determination of ethanol [92] and cyanide [33], again demonstrating difficulties inherent in the internal standard method.

Despite unpredictable matrix effects in unknown samples, the partition coefficients of many volatile substances in urine and the other body fluids are similar to those in water. Differences in urine density resulted in negligible matrix effects on the partition coefficients for trichloroethanol (TCE) and the methyl ester of trichloroacetic acid (TCA) [93,94]. In addition, matrix effects have been examined for methanethiol in urine [95].

The distribution of ethanol between plasma and whole blood should be roughly the same as that of water [96]. Ethanol, a highly soluble volatile substance, equilibrates freely between the water component of biological samples and the overlying gaseous phase. Ethanol does not significantly bind to any endogeneous constituents of plasma and whole blood. The partition coefficient of ethanol was found to be correlated to the water content of the liquid phase in biological samples, and water content value (moisture ratio) is useful in the prediction of partition coefficients [97] or in the correction of results [98] related to ethanol content. The concentration of acetone in plasma was higher than that in an equal volume of whole blood, which probably reflects the greater proportion of water in plasma as compared with whole blood [91].

3.3.4. *Effect of temperature*

To facilitate the determination of trace amounts of a volatile substance, it is possible to alter the ratio of volatile substances in an HS container by altering the equilibration temperature. In general, raising the HS temperature decreases the partition coefficients [39,80], with a linear relationship between ln k and the reciprocal of temperature [68,78,99]. The slope of this plot differs with the type of volatile substance. The more soluble the volatile substance, the greater is the change in solubility with a given temperature change [67,68]. Raising the HS temperature is a popular technique for increasing vapour pressure and facilitating the sensitive detection of volatile analytes. However, raising the temperature can increase artifacts due to the enhanced chemical reaction in the bio-

logical samples. High temperatures are necessary for the HS-GC determination of certain volatile substances that have slow HS equilibrium values due to slow diffusion within certain biological materials.

3.4. Effect of various additives

To facilitate the HS-GC determination of volatile analytes, specific compounds can be added to the samples prior to analysis. Some additives are introduced immediately after sample retrieval, *e.g.*, EDTA to prevent blood coagulation and sodium fluoride to prevent the growth of microorganisms. As discussed above, salts can also be added to produce a salting-out effect. The addition of salt has historically been used in the determination of ethanol [5,100], and extensive improvements have been made in this area. The particular salts to be added should be selected based on their specific salting-out effect and the accuracy and precision desired. For example, ammonium sulphate has been used for both salting-out and to prevent the oxidation of ethanol [101]. Many types of salts have been investigated for the optimized salting-out of ethyl acetate [102]. In addition, acid has been used to convert cyanide into protonated, volatile hydrogen cyanide. Alkaline salts have been added to convert amines into their volatile free bases [103–106]. Sulphuric acid has been employed to prevent the degradation of ethyl acetate by hydrolytic enzymes in tissue [107]. Anhydrous copper(II) sulphate has been used for the deproteination and dehydration (salting-out) of samples in the determination of ethanol [53]. Sodium fluoride was also found to be effective for the storage and salting-out of ethanol containing samples [108]. Formic acid has been used to overcome ghosting problems in the determination of short-chain fatty acids [45]. Simultaneous addition of sodium nitrite and sodium fluoride prevented the oxidation of ethanol and bacterial growth and resulted in a salting-out effect [109]. Sodium chloride has been used to denature hydrolytic enzymes and to produce a salting-out effect in the determination of methyl methacrylate levels in blood [110]. A combined treatment with potassium hydroxide, olive oil and Triton X-100 has been used to solubilize tissue samples in the determination of toluene [89]. In general, the addition of olive oil has been found to reduce the matrix effect of tissue samples owing to its ability to minimize differences between the fat content of standard and unknown samples [89]. After conversion to volatile ester derivatives, non-volatile oxidized metabolites of alcohols have been detected by HS-GC [111–117]. Sodium nitrite and sulphuric acid were used for the conversion of ethanol into nitrite ester [118]. Likewise, non-volatile cationic acetylcholine has been detected by bacterial conversion to volatile trimethylamine [103]. Trichloroethanol and phenol conjugates have been converted into their respective free forms by β-glucuronidase [119,120] and sulphatase [121] treatment. Hydrolytic enzymes and derivatizing reagents were also used to convert conjugates of TCE and TCA into the volatile TCE and ester derivatives, respectively [120]. Bromide ion levels have been measured following conversion of bromoform by citric acid, potassium permanganate, manganese dioxide and sulphuric acid [122]. Sulphuric acid and ammonium sulphate have been used to convert TCA into its volatile ester and to produce a salting-out effect in the determinations of TCA and trichloroethanol [93,94,123,124]. For esters such as ethyl and methyl acetates, the addition of sodium fluoride has been recommended in order to minimize esterase activity [59].

A further reason for adding a concentrated salt solution is its ability to minimize the matrix effects of unknown samples [1]. This consequence was first reported in the determination of ethanol levels in various biological fluids [100]. Previous differences found in the partition coefficients between specimens can be alleviated by the addition of a salting-out reagent [125]. Recently, however, detailed studies that directly investigated the ability of salt saturation to minimize matrix effects have been published [92,126]. These studies demonstrated that discrepancies in the partition coefficients for ethanol in water and blood were magnified in the presence of saturating salt [92]. The matrix effect

was abolished only after substantial dilution of blood specimens [97]. Even with the introduction of a salting-out agent, the type of biological specimen still influences the partitioning of alcohols between liquid and HS vapour phases [126]. For example, the use of saturated sodium chloride did not result in equal activity coefficients across specimens, nor was the magnitude of the effect the same for ethanol and the common internal standard used in its determination [126].

3.5. Mode of quantification

One can determine the initial liquid phase concentration of a volatile analyte through the measurement of the equilibrated vapour phase concentration given a knowledge of the partition coefficients, or by using standard calibration methods. Because of its ease and simplicity, absolute calibration and internal standard methods have been the most commonly used for HS-GC analysis. Unless an automated sampling device is used, the accuracy and reproducibility of absolute calibration method may be low owing to sampling inaccuracy. The internal standard (I.S.) method does not suffer from inaccuracies due to sampling error. Experimental errors due to evaporation during storage and tissue homogenization will be minimized if the I.S. is added to the sample immediately after removal from the body. These calibration methods have additional limitations if the properties of the sample matrix are unknown, thereby prohibiting precise quantification. These limitations are based on differing lipid concentrations and other components within biological samples which influence the vapour phase concentration and can result in unreliable calibration graphs [88–90]. Watts and McDonald [92] have reported suitable internal standards for ethanol determination that take into account matrix differences. Drozd *et al.* [127] have shown theoretically that the I.S. method will produce systematic errors. Ideal internal standards are stable isotope-labelled compounds when mass spectrometric detection is possible [125,128–130].

Recently, to cancel the matrix effect of unknown samples, three different methods have been introduced into HS-GC analyses: standard addition method, multiple HS extraction method and full evaporation technique [131]. The standard addition method involves the preparation of standard addition (SA) samples spiked with known amounts of the analyte substance of interest followed by HS-GC analysis of the unknown and SA samples. The original concentration of analyte in the sample is calculated by comparison of the GC peak intensities with and without the standard addition. The added amount of standard analyte does not change the values of V_G and V_L and also does not change the thermodynamic properties of the solution. Therefore, the partition coefficient of the analyte does not change even in a complex matrix. The main constraint of this method is that the SA samples and unknown samples must be analysed under identical conditions. Theoretical investigations of this method in aqueous solutions have been reported [61,132–134]. Koupil *et al.* [88] reported the usefulness of this method in the determination of halothane in biological samples and did not observe matrix effects, such as from differing cholesterol concentrations. This method was also found to be valid for the determination of carbon monoxide in tissues [135]. However, in heterogeneous biological samples, there may be differences in evaporation properties between the volatile analyte in the original sample and the added substance due to differing amounts of diffusion of the volatile substance through the biological matrix.

The multiple headspace extraction (MHE) method is based on a stepwise extraction of the volatile substances into a gas, with subsequent HS-GC analysis of the extract. Following each sampling step, equilibrium is rapidly regained and a volume of inert gas equal to that removed is introduced into the HS container. The vapour concentration in each step of the analysis is reduced exponentially, and it is possible to obtain the original concentration and partition coefficient by extrapolation to zero time using a plot of the logarithm of vapour phase concentration *versus* the extraction step number, irrespective of the sample conditions. The total

amount of each volatile substance can be calculated after only a few extraction steps. This method is useful for samples that are insoluble, such as certain polymers or residual solvents in printed foils, as these cannot be analysed using standard calibration methods. However, the MHE method requires special instrumentation and is a complex procedure. Suzuki *et al.* [136] first described this method as applied to the determination of organic solvents within adhesive tape. McAullife [137] described the theoretical basis for this method. Practical theory, instrumentation and many applications have also been reviewed [138–145]. Several examples of this procedure as applied to the analysis of solid samples have been published [146–148], and it has proved to be more quantitative than other methods [140].

3.6. Tissue homogenization

Owing to their liquid nature, body fluids, such as blood, urine and cavity fluids, are reliable HS samples as they attain vapour equilibrium easily and completely. Tissues such as liver, kidney and brain are also suitable for the determination of volatile substance levels when blood or urine samples cannot be obtained owing to delays in post-mortem sampling. The analysis of these tissues involves homogenization of the tissue to facilitate the vaporization of any volatile substances. The homogenization is usually done with a blender at low temperatures. Often homogenization is done in an open vessel, which results in a significant loss of volatile analytes prior to HS-GC analysis [89]. Improved recovery from tissue has been obtained by homogenization of the sample in the presence of organic solvents [149]. The recovery of ethanol levels was also optimized by homogenization in the presence of thiourea and perchloric acid [150].

Obviously, the homogenization of tissue samples should be done in a closed HS container to prevent loss of the volatile analyte. Miyaura and Isono [89] described a treatment involving the presence of KOH, olive oil and Triton X-100 in order to solubilize tissues within closed HS containers. Homogenization has also been done through the use of ultrasonic irradiation. This method of disruption has been useful in the determination of ethanol [151] and carbon monoxide levels in coagulated blood [152] and tissues [153,154]. In addition, enzymatic homogenization of tissues using collagenase [155] and subtilisin A [59,156] has also been described. A combination of physical (ultrasonic irradiation), chemical (detergent) and biochemical (collagenase) treatments which liquify samples has been effective in the analysis of chloroform levels in various kinds of tissues [155].

3.7. Stability of volatile substances within biological samples

One purpose of toxicological analyses is to obtain a quantitative measure of volatile analyte levels within the body. Realistically, the measured results often do not reflect the true physiological levels. According to Chace *et al.* [157], the following two post-mortem alterations in volatile substances should be considered: phase I, a change in volatile substance which occurs between the time of death and the time of sampling; and phase II, the change which occurs between the time of sampling and the time of HS-GC analysis, *i.e.*, the time for storage and transfer. In addition, this author would like to add consideration of a further change, phase III: the change that can occur during analysis. These changes in analyte emphasize that HS-GC results in forensic and toxicological studies of absolute levels of volatile analytes within biological samples need to be evaluated critically.

Phase I changes have been investigated for a variety of substances. The post-mortem phase I change of inhaled ethyl acetate and toluene has been reported [158–162]. The change and distribution of toluene in eggs (used as a biological sample) after burning were investigated, in relation to an actual case of a fire [128]. Liquified petroleum gas has been found to be metabolized to more polar volatile substances [163]. The changes in the carboxyhaemoglobin percentage in blood during heating and putrefaction have been investigated [164–167]. Post-mortem formation of carbon monoxide in tissue has also

been reported [168,169]. Post-mortem ethanol formation has been considered in relation to the formation of *n*-propanol [98].

To limit the amount of phase II change there are several important storage characteristics that should be considered: type of container, temperature, time and β value (the ratio of air volume to sample volume). The stability of carbon monoxide during storage has been examined [52,157,164,165,170,171]. The stability of ethanol in blood and tissues has been extensively investigated and stabilizing procedures have been found [29,90,172–174]. Three processes could result in the loss of ethanol from stored blood samples: loss due to the growth of contaminating microorganisms (prevented by the addition of sodium fluoride), loss by evaporation due to improper capping of the storage container and decomposition that occurs in samples stored over long periods (accelerated at elevated temperatures). Storage conditions have also been investigated for toluene, mainly with respect to the use of appropriate containers [158,175–178]. The stability of carbon tetrachloride in blood and urine samples has also been examined [179], in addition to changes in cyanide concentration in blood [33,180]. An additional phase II change could involve the contamination of samples during storage. For example, chlorinated solvents commonly used in the laboratory should be considered, given that capped vials are rarely airtight. The integrity of the septum decreases with storage at low temperature and could allow gaseous contamination to reach the samples [181]. Most volatile substances are relatively stable in blood if certain simple precautions are taken [59]. It is generally recommended that samples be stored at low temperatures in the presence of preservatives, and in glass containers sealed tightly with an inert septum. Further, the containers should have minimal air space above the sample and be opened at low temperature and only when required for analysis [59].

There are several examples of phase III changes in the literature. Raising the temperature can introduce a change in analyte level and therefore requires special precautions. Ethanol concentration has been found to decline progressively during HS equilibrium [182]. Ethanol can be oxidized to acetaldehyde at elevated temperature. This reaction is catalysed by oxyhaemoglobin and is limited only by the amount of oxygen in the sealed vial [182,183]. Acetaldehyde shows rapid disappearance during HS incubation [29]. Acetaldehyde can also be formed from ethanol during deproteinization procedures prior to HS-GC analysis [184–189]. Other phase III changes include the conversion of thiocyanate into cyanide in the presence of haemoglobin and acid in the analysis of blood for cyanide [33] and the degradation of ethyl acetate to acetic acid and ethanol during the analysis of liver samples [107].

4. Detailed conditions of HS-GC methodology

Tables 1–10 present detailed HS-GC conditions for the determination of several groups of volatile substances in a variety of biological matrices. For each chemical group the following variations of HS-GC methodology are considered: HS conditions (equilibration times and temperatures, the use and types of additives, the use and types of IS, HS containers, HS sampling system), GC conditions (columns, temperatures, carrier gas, detectors) and overall HS-GC sensitivity (detection and quantification limits). Within each table references are cited in chronological order.

4.1. Definition of terms used in tables

In the "Substances" and "Additive" columns, the following abbreviations are used: AcH = acetaldehyde; ACA = acetoacetic acid; AcOEt = ethyl acetate; AcMe = acetone; MeCN = acetonitrile; $AmSO_4$ = ammonium sulphate; Bz = benzene; nBuOH = *n*-butanol; tBuOH = *tert.*-butanol; sBuOH = *sec.*-butanol; CH = chloral hydrate; ClBz = chlorobenzene; AcOH = acetic acid; BCE = 1-bromo-2-chloroethane; IBA = isobutyraldehyde; Bu_2NH = dibutylamine; 1,2-DCE = 1,2-dichloroethane; 1,1-DCE = 1,1-dichloroethane; cisDCE = *cis*-1,2-dichloroethylene; DCPOH = 1,3-dichloro-2-pro-

panol; Et_2S = diethyl sulphide; Me_2SO_4 = dimethyl sulphate; Me_2S = dimethyl sulphide; EtOH = ethanol; EtBz = ethylbenzene; EDTA = ethylenediaminetetraacetate; EtSH = ethanethiol; EtHgCl = ethylmercury(II) chloride; EtONO = ethyl nitrite; FBz = fluorobenzene; βG = β-galactosidase; BHB = β-hydroxybutyric acid; IAA = iodoacetic acid; BKB = β-ketobutyric acid; MeOH = methanol; AcEt = methyl ethyl ketone (2-butanone); AciBu = methyl isobutyl ketone; MeHgCl = methylmercury(II) chloride; MeHgI = methylmercury(II) iodide; AcPr = 2-pentanone; PCA = perchloric acid; PheHgCl = phenylmercury(II) chloride; nPrOH = 1-propanol; iPrOH = 2-propanol; SDS = sodium dodecyl sulphate; SSA = sulphosalicylic acid; THF = tetrahydrofuran; THP = tetrahydropyran; MeBz = toluene; TSA = toluenesulphonic acid; TCA = trichloroacetic acid; $C_2H_3Cl_3$ = 1,1,1-trichloroethane; $iC_2H_3Cl_3$ = 1,1,2-trichloroethane; TCE = trichloroethanol; C_2HCl_3 = trichloroethylene; TMB = 1,2,4-trimethylbenzene; TSA = toluenesulphonic acid; TX = Triton X-100; Xyl = xylene; oXyl = *o*-xylene; mXyl = *m*-xylene; pXyl = *p*-xylene.

In the "Specimen" column the following abbreviations are used: AP = adipose tissue; AQ = water; BL = blood; BR = brain; CF = cavity fluid; FT = fat tissue; FB = fermentation broth; GC = gastric content; HE = heart; KD = kidney; LV = liver; LN = lung; ML = milk; PL = plasma; MS = muscle; SR = serum; SL = saliva; SP = spleen; ST = stomach content; UR = urine; VH = vitreous humor.

In the "HS temperature" column the ambient temperature (room temperature) is abbreviated as RT.

In the "HS container" column the following abbreviations are used: VS = Van Slyke volumetric apparatus; WN = Warburg nanometer.

In the "HS injection" column, abbreviations of method are as follows: LP = automated injection method by which a constant volume of vapour phase sample in loop is introduced; MA = manual injection using syringe; PN = automated injection method by which pressurization and injection are controlled pneumatically; SW = injection method by which carrier gas is used to sweep vapour phase; VE = injection method by which vapour phase is vacuum extracted using Van Slyke apparatus.

In the "Column" column, liquid phase (percentage concentrations and types), support particle size (mesh/mesh, mesh number × particle diameter (mm) = 14.9) and column dimensions (length × I.D.) are cited for packed column analysis. For capillary column analysis, column (liquid phase type and film thickness), dimensions (length × I.D.) and splitting ratio are cited. The following abbreviations are used: for liquid-phase material, AG = Apiezon grease; CW = Carbowax; DIP = diisodecyl phthalate; HC = Hallcomid; PEG = polyethylene glycol; for column support material, AW = acid washed; CP = Carbopack; CS = Chromosorb; DMCS = dimethyldichlorosilane; HP = Haloport; HMDS = deactivated with hexamethyldisilazane; MS = molecular sieve; NAW = non-acid-washed; PP = Porapak; SL = Shimalite; SCOT = support-coated open-tubular capillary column, UP = Uniport; UB = Unibeads.

In the "Oven temperature" column, the temperature values are cited for isothermal analysis. For temperature-programmed analysis, the initial temperature (°C) and its hold time (min) in parentheses, temperature ramp rate (°C/min) (number/m) and the final temperature (°C) are cited.

In the "Carrier gas" column, type of gas and flow-rate are cited except for special cases which describe controlled-pressure values.

In the "Detection" column, the following abbreviations are used: βID = β-ionization detection; ECD = electron-capture detection; FID = flame ionization detection; FTD = flame thermionic detection; HECD = Hall electrolytic conductivity detection; MS = mass spectrometry; MIPT = microwave-induced plasma emission detection; NPD = nitrogen–phosphorus detection; PID = photoionization detection; FPD = flame photometric detection; TCD = thermal conductivity detection.

4.2. Carbon monoxide

The determination of carbon monoxide (CO) levels in blood is important in cases such as CO

inhalation due to fires and in industrial hygiene [190]. CO binds strongly to the reduced form of haemoglobin (Hb), and therefore the carboxyhaemoglobin percentage in blood (% CO-Hb) is a critical parameter that predicts CO levels. A spectrophotometric method has been used to measure the % CO-Hb, but this method is not reliable in certain instances, *e.g.*, in forensic analysis such as fire accident victims or putrefied bodies. Denatured haemoglobin molecules give false results in this method owing to the altered absorbance spectrum. Alternatively, the HS-GC method has been used for blood CO determinations. The HS-GC procedure involves the initial liberation of CO gas from blood Hb by the addition of hexacyanoferrate(III) [to oxidize Hb to methaemoglobin (met-Hb)] or strong acid (to denature Hb). The released CO gas is then measured by GC using TCD or FID (after CO has been converted into methane by postcolumn reduction with hydrogen on a nickel catalyst). Molecular sieves (5 Å) are used as the column matrix, and provide a good separation without interference. The following two injection methods are used in this analysis; static HS injection and a method in which CO gas is liberated in a special reaction vessel and introduced into the GC system by a carrier gas swept over the HS phase.

The partition coefficient of CO is very low (0.014–0.036), and therefore all liberated CO in blood samples is available for GC analysis. In order to facilitate the liberation of CO gas from samples, saponin or detergent is added as a haemolysation reagent. The HS temperature does not need to exceed room temperature owing to its small k value. The HS-GC method has been shown to be reliable as compared with other methods [54,191–197].

To obtain a % CO-Hb value in blood, a reference of 100% CO-Hb needs to be measured using one of the following two methods. The first is measurement of the CO binding capacity, obtained by CO saturation of the blood sample and subsequent analysis. The second is to measure the total Hb concentration by the cyanomethaemoglobin spectrophotometric method or by analysing for total iron content by atomic absorption spectrometry. The first method is easily performed using the specially designed sweeping apparatus, but is unreliable owing to the inconsistent CO binding capacity found for special samples [51,198,199]. For instance, Met-Hb (oxidized Hb molecule having no CO binding capacity) is found in subjects who have died due to fire, does not have a complete CO binding capacity and can therefore result in incorrect % CO-Hb measurements. Detailed HS-GC conditions are presented in Table 1.

4.3. Alcohols and their metabolites

Blood ethanol levels are tested in many countries as a means of addressing and deterring citizens from driving under the influence of alcohol. Compared with other methods, such as spectrophotometry, the GC technique is advantageous because it provides both qualitative (retention time) and quantitative (peak signal) results. The direct injection technique has been used in the past, but contamination of samples in the GC injector port and column is a serious disadvantage. Pyrolysis GC has recently been developed for blood ethanol determination [206]. In European countries, HS-GC has been made a legal method for ethanol determination. Ethanol determination in post-mortem samples is important in order to determine blood alcohol levels after traffic accidents.

Numerous reviews have been published concerning ethanol determination [207–211]. Extensive improvements in HS-GC have been made such that it is now possible to determine results for numerous samples quickly and accurately [212]. The selection of the column, internal standard, additives for sample preservation and induction of salting-out effect, automated systems and data systems have been extensively discussed [212]. The critical determinates in the selection of an I.S. for ethanol determinations are that it is not an endogenous compound and that it has similar chemical properties and GC retention time, and for these reasons *n*-propanol and *tert.*-butanol are used extensively. Sodium chloride is the most commonly used salting-out reagent. Various HS temperatures have been used that either optimize sensitivity (high temperature) or accuracy (room temperature)

Table 1
Carbon monoxide

Specimen	Haemolysis reagent	CO release reagent	HS container	HS injection[a]	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit	Reference[b]	Ref.
BL tissue	Saponin	$K_3Fe(CN)_6$ AcOH	Syringe	MA RT	MS 5A (160) 2 m	75	He 135	TCD	20 ppm (v/v)	CO Sat	3
BL		$K_3Fe(CN)_6$	Vessel	SW 5 min	MS 5A 3 m	100	He 30	TCD		CO Sat	51
BL	Saponin	$K_3Fe(CN)_6$ Lactic acid	VS	SW 3 min	MS 5A 2.4 m	70	He 15 p.s.i.	TCD	10 ppm (v/v)		200
BL	Saponin	$K_3Fe(CN)_6$ Lactic acid	VS	SW 2 min	MS 5A (40/60) 2.7 m	100	He 80	TCD	50 nl/ml		201
BL	TX	$K_3Fe(CN)_6$	Syringe	SW 10 min	Silica 2 m × 6.4 mm	30	He 60	TCD		CO Sat	52
BL		HCl, lactic acid	VS	SW 2 min	MS 5A (50) 1.8 m × 6.4 mm	105	He 135	TCD		Hi-CN	202
BL	Sterox	$K_3Fe(CN)_6$	Vessel	SW 5 min	MS 5A (30/80) 1.8 m × 6.4 mm	100	He 50	FID	20 nl/ml		54
BL	Saponin TX	$K_3Fe(CN)_6$	Syringe	MA 10 min RT	MS 5A (60/80) 1.5–1.8 m × 6.4 mm	100 ~ 120	He 45	TCD		Hb Fe (AA)	90
BL	Saponin	$K_3Fe(CN)_6$	VS	SW 0.5 min	MS 5A (30/60) 2 m × 3 mm	50	He 50	TCD		CO Sat	170
BL	Saponin	$K_3Fe(CN)_6$ Lactic acid	Vial	SW 3 min	PP Q (80/100) 0.91 m × 6 mm MS 13X (40/60) 6 mm × 3.6 mm	70	He 100	TCD	50 nl/ml		191
BL	TX	$K_3Fe(CN)_6$	Vessel	SW 5 min	MS 5A (40/60) 3 m × 3 mm	100	He 30	TCD		CO Sat	203
BL	TX	$K_3Fe(CN)_6$	Vessel	SW 5 min	MS 5A (40/60) 3 m × 3 mm	100	He 30	TCD		Hi-CN	198
BL	Saponin	$K_3Fe(CN)_6$	Vessel	SW 5 min	MS 5A (30/60) 2 m × 3 mm	80	He 35	TCD		CO Sat	199
BL		H_2SO_4	Vial	MA 10 min RT	MS 5A (60/80) 1.65 m × 3.2 mm	110	H_2 20	FID		CO-Oxi Hi-CN	192
BL		$K_3Fe(CN)_6$	Vessel	MA 5 min RT	MS 5A 2 m × 3 mm	80	He 35	TCD			204
BL		$K_3Fe(CN)_6$ Lactic acid	Tube	MA 30 min RT	MS 5A (100/120) 1.8 m × 3.2 mm	140	He 15	FID		CO Sat	205
BL		$K_3Fe(CN)_6$	Chamber	LP 75 s	MS 5A 2 m × 3 mm	80	He 35	TCD		CO Sat	152
BL	Saponin	$K_3Fe(CN)_6$	Syringe	MA 4 min RT	MS 5A (80/100) 1 m × 3 mm	90	He 40	TCD		CO Sat	49
BL		H_2SO_4	Syringe	MA 10 min	MS 5A 1.8 m × 6.4 mm	70	He 30 p.s.i.	TCD		Hi-CN	48
BL		$K_3Fe(CN)_6$	Vial	PN 1 h 90°C	MS 3A (60/80) 1 m × 3.2 mm	135	He 36 p.s.i.	FID	0.1% CO-Hb	Hi-CN	193
BL,CF	TX	$K_3Fe(CN)_6$	Syringe	MA 5 min RT	MS 5A (60/80) 2.1 m × 3 mm	100	He 24	TCD		Hi-CN	50 169
BL	Saponin TX	$K_3Fe(CN)_6$	Bottle	MA 10 min RT	MS 5A (60/80) 1.8 m × 3.2 mm	70	He 20	TCD		CO Sat Hi-CN Fe(AA)	194
BL	Saponin	$K_3Fe(CN)_6$	Vial	LP 10 min	MS 5A (30/60) 2 m × 3 mm	70	N_2 0.6 kg/cm^2	TCD FID		CO Sat	153
LV		$K_3Fe(CN)_6$	Vial	MA 5 min RT	MS 5A	80	N_2 35	FID		Fe (AA)	154
BL		HC, lactic acid	Chamber	SW 5 min	MS 5A (60/80) 1.8 m × 3.2 mm	50 ~ 70	He	TCD		Hi-CN	196
BL		$K_3Fe(CN)_6$	Vessel	SW 2.5 min	MS 5A (30/60) 1.8 m × 3.2 mm	90	N_2 50	FID		CO Sat	195
Tissue		H_2SO_4	Vial	MA 30 min 65°C	Carbosieve S-II 1.8 m × 2 mm	35 (5) 25/min 190	He 22	MS	0.5 μg/ml	Fe (AA)	135
BL		H_3PO_4	Vial	PN 70°C	PP Q (80/100) 3 m × 0.9 mm	80	N_2 30	FID		CO gas	197

[a] For manual HS injection method (MA), HS equilibrium time (min) and temperature (°C) is cited. For sweeping method (SW), time (min) for CO liberation before sweeping vapour is cited.
[b] Method for obtaining 100% CO-Hb value as reference is cited as follows: CO Sat, sample blood is saturated with CO gas and used as reference; Hi-CN, total Hb concentration is measured by spectrophotometric cyanomethaemoglobin method; Fe (AA), total iron content is measured by atomic absorption spectrometry; CO-Ox, total Hb concentration is measured using commercial CO-Oximeter.

[108,41]. Polar stationary phases are used as column packings material, *e.g.*, polyethylene glycol, Carbowax and porous polymer adsorbents. Anthony *et al.* [213] evaluated the selectivity of the column supports Carbopack B and Carbopack C for the determination of a wide range of low-molecular-mass volatiles [213]. Two- and three-column systems have been used to verify ethanol determination [212,214,215]. FID is the most popular mode of detection. Importantly, ethanol is oxidized to acetaldehyde by oxyhaemoglobin during storage or HS equilibrium [173,183] unless special precautions are taken. Although ethanol has a high partition coefficient (*ca.* 2000), the HS-GC method offers suitable sensitivity for most forensic determinations; the legal blood ethanol concentration for motorists is *ca.* 0.5 mg/ml and the background level in normal individuals is 0.001–0.0025 mg/ml [216]. The reliability of the HS-GC method has been verified in comparison with other methods [35,217–219]. Detailed HS-GC conditions for the determination of ethanol are presented in Table 2.

The determination of the level of oxidative metabolites of alcohols is important in forensic science, because aldehydes and organic acids are thought to produce much of the toxicity caused by alcohol consumption. Non-volatile organic acids can be determined after conversion into their ester derivatives in the presence of a strong acid and methanol [111–117]. Formaldehyde can also be converted into an ethoxy derivative [229].

Aldehyde levels can be detected by employing the HS-GC method used for alcohol and the assay is more sensitive because the aldehyde partition coefficient is considerably smaller. However, determination of the acetaldehyde level in blood has serious methodological difficulties. First, because of its high chemical reactivity, acetaldehyde shows rapid disappearance [29] during HS incubation at elevated temperature, owing to the chemical reaction with biological components in samples and/or further oxidation. In order to prevent this disappearance, deproteinization procedures have been conducted [29]. Supernatants from deproteinized blood samples have been used as HS samples. Second, especially in the presence of ethanol, significant artifactual acetaldehyde formation has been observed during the deproteinization procedure or HS incubation [29,184–186,188,189], owing to non-enzymatic oxidation of ethanol by either ascorbic acid [230] and/or erythrocytes [231,232]. Although oxidation by ascorbic acid can be completely prevented by addition of thiourea [233], oxidation by erythrocytes cannot be prevented by thiourea [231,232]. Effective acetaldehyde determinations have been conducted with a rapid deproteinization procedure at low temperature using elaborate additives: perchloric acid and saline [188,232], nitric acid and sulphosalicylic acid [234] and perchloric acid, thiourea, saline and sodium azide [224]. Detailed HS-GC conditions for the determination of alcohols and their metabolites are presented in Table 3.

4.4. Non-halogenated organic solvents

The analysis of biological samples for levels of non-halogenated organic solvents has been performed for the diagnosis of solvent abuse and the monitoring of industrial exposure [239]. Organic solvents are easily metabolized in the body, and in many instances the non-metabolized parent compound is only a minor fraction of total body burden. Sensitive HS-GC methods make it possible to detect trace amounts of non-metabolized compounds. Various kinds of stationary phases have been used, depending on the polarity of the analyte. Polar materials such as polyethylene glycol, Carbowax and porous polymers are common stationary phases, although non-polar and intermediate-polarity phases have also been used, especially in capillary analysis. FID and MS are the major detection methods. Detailed HS-GC conditions are presented in Table 4.

4.5. Halogenated aliphatic hydrocarbons

Halogenated aliphatic hydrocarbons are frequently used as industrial solvents and have been used as anaesthetics in the past. The determination of these substances in biological samples

Table 2
Ethanol

Specimen[a]	Additive	I.S.	HS temperature (°C)	HS time (min)	HS container	HS injection	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit	Ref.
BL (AcH)	$ZnSO_4$ $Ba(OH)_2$		55	15	Bottle	MA	5% CW 1500 HP 60-F 1.2 m × 6.4 mm	100	He 50	FID		29
BL, SR, UR		AcMe AcEt	60		Bottle		15% PEG 1500 (60/80) Celite 545 15% PEG 1540 (80/100) CS W	100	N_2 88	FID		25
BL, UR	NaCl		30	5	Flask	MA	1.5% Flexol 8N8, 1.5% DIP, 1.5% PEG 600 (100/120) CS P 1.8 m × 3.2 mm	75	N_2 19	FID		100
BL		nPrOH	55	15	Vial	MA	25% PEG 1000 (6000) (60/80) Shimalite 0.75 m × 4 mm	90	N_2 90	FID		217
BL			60	10	Vial	MA	20% CW 2M (60/80) firebrick AW (CS P HMDS) 3 m × 3.2 mm	100	N_2 17 12.3	FID		35
SR	$CuSO_4$		80	2	Tube	SW 2 min	15% Flexol 8N8, 10% DIP, 3% PEG 600 (42/60) C-22 firebrick 3 m × 4.8 mm	100	He 75	FID	1 μg/ml	53
BL, UR, SL		nPrOH	55	15	Vial	MA	25% PEG 1000 (60/80) SL	90	N_2 70	FID		26
BL	NaF		27	15	Bottle	MA	15% Flexol 8N8, 10% DIP, 3% PEG 600 (42/60) C-22 firebrick 1.8 m × 6.4 mm	100	He 50	FID		108
BL, UR			27	15	Bottle	LP	PP S (80/100) 1.2 m × 3.2 mm	132	He 50	TCD		41
BL	NaCl	1,4-Dioxane	85	18	Bottle	MA	15% Flexol 8N8, 10% DIP, 3% CW 600 (40/60) firebrick 2.4 m × 6.4 mm	100	He 40	TCD	0.01% (w/v)	218
BL, UR	NaF Filter-paper		35	15	Bottle	MA	5% CW 1500 (30/60) Haloport 60F 1.2–1.8 m × 3 mm	75 ~ 100	N_2 30	FID		220
BL, tissue		nPrOH	55		Bottle	MA	25% PEG 1000 (60/80) 1 m × 4 mm	90	N_2 30	FID		151
BL		nPrOH	27		Flask	MA	PP S (100/120) 1.8 m × 3.2 mm	160	He 50	FID		221
BL		tBuOH	60		Vial	PN	15% CW 1500 Celite 2 m	70	N_2 25	FID		42
BL		nPrOH	62		Vial	PN	PP Q 1.5 m	140	N_2	FID		173
BL		tBuOH					0.4% PEG (60/80) graphite	105		FID		209
BL	NaF	nPrOH	60	3	Vial	MA	PP Q (80/100) 1.8 m × 3.5 mm	150	N_2 30	FID	3 μg/ml	109
BL	PCA[b]	tBuOH	60	20	Vial	PN	15% CW 1500 (60/80) Celite 2 m × 2.7 mm SP-1000 25 m × 0.35 mm split	80 52	N_2 1.5 N_2 0.6 kg/cm^2	FID		55
BL (AcH)	Thiourea PCA[b]	nPrOH	60	30	Vial	MA	5% CW 20M (30/60) HP F 1.8 m × 4 mm	90	He 30	FID	5 μg/ml (EtOH) 0.25 μg/ml (AcH)	222
LV		nPrOH	60	30		PN	5% CW 20M (60/80) CP B 1.83 m × 3.2 mm	65 110	N_2 30	FID	3.125 mg/dl	213
BL, LV (AcH)	Thiourea PCA[b]	iPrOH	65	30	Flask	MA	PP Q (100/120) 1.8 m × 2 mm	130	He 37	FID	2.7 μmol (EtOH) 23 nmol (AcH)	150

BL, BR, LV, ML		tBuOH	60	15	Bottle	PN	15% CW 1500 (60/100) Celite 2 m × 2.7 mm		N_2 35	FID		90
BL		nPrOH	40–60	10–120	Vial	LP	PP Q 2 m × 4 mm	160	N_2 30	FID		182
BL, UR, VH		nPrOH				PN	5% CW 20M CP B					223
BL	NaCl (K_2CO_3)		60	30	Vial	MA	PP Q (80/100) 2 m × 2 mm	120	N_2 20	FID		118
(EtONO)[c]	$NaNO_2$ H_2SO_4	EtOH-d_3(d_5)	5	10			Tenax GC (30/60) 2 m × 2 mm	60		FID MS		
BL, SR			25	30	Flask	MA	PP Q 0.305 m × 3 mm	100	H_2–N_2 6:4	FID		97
BL	$AmSO_4$ Dithionite	nPrOH	60	30	Vial	PN	5% CW 20M CP B 1.8 m × 2 mm	65	N_2 30	FID	0.001% (w/v)	101
BL (AcH)	PCA, NaN_3 Saline Thiourea[b]	nPrOH	60	15	Vial	MA	5% CW 20M (30/60) HP F 1.8 m × 4 mm	90	N_2 30	FID	6 μg/ml (EtOH) 0.1 μg/ml (AcH)	224
BL	NaCl	nPrOH	RT	30	Vial	MA	0.2% CW 1500 (80/100) CP C 2 m × 2 mm	125	N_2 20	FID	2 μg/ml	219
Heart & femoral BL	NaF	nPrOH		30	Vial	PN	0.2% CW 1500 (80/100) CP B 1.8 m × 3.2 mm	130		FID		225
BL		nPrOH	55	12	Vial	PN	DB-1(3 μm) 30 m × 0.53 mm DB-WAX (1 μm)	45	He 7.5	FID		214
BL		nPrOH				PN	0.2% CW 1500 (80/100) CP C 5% CW 20M CP B 15% CW 20M CP W	100 100 120		FID		226
BL	NaF EDTA	nPrOH	40	18	Vial	PN	0.2% CW 1500 (80/100) CP C 5% CW 20M (60/80) CP B 15% CW 20M CS W 2 m × 3 mm	100 100 120	N_2 20	FID		212
BL, UR		nPrOH	60	10		PN	0.2% CW 1500 (80/100) CP C 2 m × 3.2 mm 10% CW 400 (80/100) CS WHMDS	90	30			227
BL, VH		nPrOH	60	35	Vial	PN	0.2% CW 1500 (80/100) CP C 3 m × 3.2 mm	120	N_2 30	FID		228
BL, SR, PL		nPrOH			Vial	PN	CW 1540 CS W 2 m × 3 mm	100	N_2 20	FID		96

[a] Substances in parentheses were also analysed.
[b] HS sample is a supernatant fraction of acidified sample.
[c] The substance is converted by derivatization from non-volatile analytes.

Table 3
Alcohols and their metabolites

Substance[a]	Specimen	Additive[b]	HS temperature (°C)	HS time (min)	HS container	HS injection	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit	Ref.
EtOH, AcMe, MeOH, iPrOH	BL	$CuSO_4$	50	3	Vessel	SW	5% HC M-18 (5% CW 1540) (35) Teflon 6 4.5 m × 2.16 mm	56	He 20	TCD		40
EtOH, AcH, nPrOH	BL		55	15	Vial	MA	25% PEG 1000 (60/80) SL 0.75 m × 4 mm	90	N_2 40	FID		172
AcH	BL	$ZnSO_4$[c], $Ba(OH)_2$, saline	55	60	Vial	MA	PP Q (50/80) 1.8 m × 3.2 mm	85	He 30	FID MS		184
AcH, EtOH	LV	PCA, thiourea, (tBuOH)	65	15	Vial	PN	15% PEG (60/100) Celite			FID		233
AcH	BL, LV	PCA, (tBuOH)	65	15	Vial	PN	15% PEG (60/100) Celite	75	N_2 35	FID		230
AcH	BL	(nPrOH)[a] Saline	55	20	Vial	MA	15% PEG 1500 (60/80) CS W 3 m × 4 mm	100	N_2 50	FID		185
EtOH							2 m × 4 mm					
EtOH, AcMe, MeOH, nPrOH	AQ				Vial	MA	PP P (80/100) 1.8 m × 3 mm	120 130	N_2 30	FID		132
AcH	BL, LV, BR	$HClO_4$[c]	65	15	Vial	PN	15% PEG (60/80) Celite	75	N_2 35	FID	0.5 μM	231
AcH	BL		50	20	Vial	MA	PP S (80/100) 0.9 m × 2 mm	110		FID		235
AcH, MeOH, EtOH	LV	(nPrOH)	60	30		PN	5% CW 20 M (60/80) CP B 1.83 m × 3.2 mm	65 110	N_2 30	FID		213
HCOOH (ester)	BL, PL, UR	H_2SO_4, MeOH (MeCN)	RT	20	Syringe	MA	PP Q 1.8 m × 3 mm	124	N_2 24	FID	1 mM	111
AcH	BL	(iPrOH) PCA[c]	60	30	Vial	MA	CS 101 (60/80) 1.5 m × 4 mm	120	N_2 50	FID		187
EtOH			55	20			15% PEQ 1500 CS WAW (60/80) 2 m × 4 mm	110				
AcH	BL	$NaNO_2$, SSA[c] (IBA)	60	10	Vial	MA	PP Q (80/100) 1 m × 2 mm	110	He 40	FID	0.4 μM	234
HCOOH (ester)	BL	H_2SO_4, MeOH (MeCN)	45	20	Vial	MA	PP Q (80/100) 2 m × 3 mm	135	N_2 48	FID	5 mg/l	113
nPrOH, EtOH	BL, MS		55	15	Vial	MA	25% PEG 1000 (60/80) SL	90	N_2 70	FID		98
HCOOH (ester)	FB	TSA, MeOH	80	30	Beaker	MA	PP Q (QS) 1.5 m × 4 mm	100	He 30	FID	20 μg/ml	112
AcH	BL	(iBuOH)[c] PCA, saline	65	30	Vial	PN	CS 101 (60/80) 1 m × 2.4 mm	130	N_2 40	FID		188
EtOH		(nPrOH)	55	30	Vial	MA	15% PEG 600 (60/80) CS W AW 2 m × 3 mm	130	N_2 55	FID		
AcH	BL	PCA, saline Thiourea[c]	65	15	Vial	PN	15% PEG (60/80) Celite	75	N_2 35	FID	0.1 μM	232

HCHO MeOH	BL	(nPrOH)	60	15	Vial	MA	25% PEG 1000, 2% KOH (60/80) CS WAW-DMCS	80	N_2 60	FID	0.2 mg/ml	236
AcOH (ester)	BL	H_2SO_4, MeOH (MeCN)	55	15	Vial	MA	PP Q (80/100) 2 m × 3 mm	140	N_2 40	FID	0.2 μg/ml	114
MeOH	SR	(MeOH-d_4)	65	30	Vial	MA	CPWAX 57CB 26 m × 0.22 mm split 1:20	50	He 4 p.s.i.	MS	12 μg/ml	129
AcOH (ester) EtOH	PL	H_2SO_4 MeOH	55	30	Vial	PN	PP QS (80/100) 1.35 m × 4 mm	140	N_2 95 kPa	FID	50 μ*M* (AcOH) 1.61 m*M* (EtOH)	115
EtOH, AcH, AcMe, MeOH, iPrOH, nPrOH, tBuOH	BL	NaCL	RT	30	Vial	MA	DB-WAX (1 μm), DB-1 (3 μm), DB-1 (5 μm) 15 m × 0.53 mm DB-1 (1.5 μm) 30 m × 0.53 mm	30~ 40	H^2 25	FID	10 μ*M*	58
EtOH, AcMe, tBuOH, nPrOH, MeCN, iPrOH, MeOH	BL, PL, SR	NaCl	25 37	45	Tube	MA	PP S (80/100) 1.8 m × 3.2 mm	165	N_2 45	FID		92 126
MeOH	BL	K_2CO_3	60	30	Vial	PN	0.2% CW 1500 CP C	80		FID		226
HCHO	BL	HCl, EtOH (nPrOH) (tBuOH)	56	60	Vial	MA	2 m × 3 mm 25% PEG 1000 (60/80) SL 25% PEG 1000 (60/80) SL	 90 90	 N_2 40 H_2 30	 FID MS	10 μ*M*	229
HCOOH (ester) MeOH	BL, SR	H_2SO_4, MeOH (propionic acid)	35	30	Vial	MA	5% HC M-18 (0.5% CW 600) (40/60) Teflon 6	75		FID	25 μg/ml	116
EtOH, MeOH, AcMe, iPrOH, tBuOH, AcEt	BL	K_2CO_3 (PrOH)					0.2% CW 1500 CP C (80/100)	80		FID		237
Organic acid (ester)	FB	$NaHSO_4$ MeOH	120	20	Vial	PN	Restek Stabilwax DA (0.25 μm) 30 m × 0.32 mm	70 (3) 6/min 120	N_2 30	FID		117
MeOH	KD, LV, UR, BL, HE, VH, CF, SC	(nPrOH)	60	30	Vial	PN	15% CW 15000 (80/100) CS WAW 2 m × 1.6 mm	100	N_2 30	FID		238

[a] Substances in parentheses are those which are converted by derivatization from non-volatile analytes.
[b] Substances in parentheses are internal standards.
[c] HS sample is a supernatant fraction of acidified sample.

Table 4
Non-halogenated organic solvents

Substance	Specimen	Additive	I.S.	HS temperature (°C)	HS time (min)	HS container	HS injection	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit	Ref.
Bz, MeBz, mXyl	BL			37	1–2 h	Vial Bottle	MA	10% PEG 400 Celite	70	N_2 70	FID		28 240 46
MeBz	BL, LV, BR		EtBz	50	20	Bottle	MA	CS 102 (60/80) 0.375 m × 3 mm	150	N_2 1 kg/cm^2	FID	0.2 mg/g	241
MeBz, AcMe	BL		Bz					5% APL (80/100) Diasolid M 3 m × 3 mm	50 (75)	N_2 35	FID		242
Bz, MeBz	BL			37	30	Syringe	MA	10% PEG 400 Celite 545 2 m	80	N_2 70	FID		47
MeBz	UR			50–55 (RT)[a]	10[a]	Flask	MA	2% PEG 1500 (60/80) CS W 3 m × 4 mm	70	N_2 25	FID		243
MeBz, Xyl	BL			90	30			10% UC-W98 (80/100) CS W AW-DMCS 0.5 m × 3.2 mm	75			0.005 ppm (w/v)	244
MeBz EtOH, AcOEt	BL, BR, LV		EtBz nPrOH	50	20	Vial	MA	CS 102 0.555 m × 3 mm PP Q (50/80) 0.75 m × 3 mm	170 180	N_2 1 kg/cm^2	FID		245
Bz	ML			40	10	Flask	MA	PP P (80/100) 1.8 m × 3 mm	165		FID		133
AcOEt	LV, BR	H_2SO_4	nPrOH	50	20	Bottle	MA	PP Q (50/80) 0.75 m × 3 mm	180	N_2 37	FID		107
Aromatic hydrocarbon, ketone	BL, Oil, AQ			37	0.5–2 h	Vial	MA	PEG 400 or SE-30 2 m	80	N_2 70	FID		72
AcOEt EtOH	BL, BR, LV FT	H_2SO_4	nPrOH	50	20	Vial	MA	PP QS (80/100) 1 m × 2.6 mm CS 102 (60/80) 0.8 m × 3 mm	170 205	He 20 N_2 38.8	MS FID		160
MeBz	UR		EtBz	50	20	Vial	MA	CS 102 (60/80) 0.5 m × 3 mm	200	N_2 30	FID		175
MeBz	BL, UR		EtBz	50	15–30		MA	5% PEG 1500 (80/100) Celite 545 2 m × 3 mm 5% AG L (60/80) CS W 3 m × 3 mm	55	N_2 35	FID		158
Phenyl conjugates	UR	Sulphatase		90	20		PN	10% CW 20M, 2% H_3PO_4 (100/120) CS W 2 m × 2 mm	165	He 35	FID		121
AcEt, MeBz, AciBu, Xyl	BL, LV, BR LN	NaCl		37	20	Vial	MA	15% SP-1000 (80/100) CS W AW 1.8 m × 2 mm		He 20	MS		246
MeBz	BL, BR, LV LN, KD		iBuOH	55	15	Vial	MA	25% PEG 6000 (60/80) SL 1 m × 3 mm	80		FID		159
MeBz	BL		EtBz, $CHCl_3$	35	20	Vial	MA	20% DOS (60/80) UP B 1 m × 3 mm	100	N_2 30	FID		247
MeBz	BL, BR, LV		AcOEt AcMe	60	30	Bottle	MA	15% CW 20M (85/100) 0.2% CW 1500 (80/100) CP C 1.8 m × 1.6 mm	80 80 (130)	N_2 50 20	FID		248
MeBz	LV	Olive oil NaOH, TX MeOH	EtBz	35	2.5 h	Vial	MA	20% DOS (60/80) UP B 1 m × 3 mm	100	N_2 30	FID		89
Methyl methacrylate	BL	NaCl		70	20	Vial	LP	SE-54 25 m × 0.23 mm	0 30/min 100		FID	0.02 μg/ml	110

iPrOH, AcMe	BL, organ	PCA	tBuOH	65	15	Vial	PN	10% PEG 600 CS W 4 m × 3.2 mm	75	N_2 40	FID	163
AcEt, sBuOH			nPrOH	75	10			UB A 2 m × 3.2 mm	90			
MeBz	BL, LN, LV, BR		EtBz	60	20	Vial	MA	CS 101 (60/80) 1.5 m × 2.6 mm	180	N_2 2 kg/cm^2	FID	249
THF	LV, KD, BR UR, FT, SP MS	NaCl	THP THF-d_8	40	60	Vial	MA	PP P (80/100) 1 m × 3 mm	140	N_2 40 He 30	FID MS	1 μg 0.1 μg	125
MeBz	LV		MeBz-d_8	60	30	Vial	MA	PP P (80/100) 1 m × 3 mm	170	He 30	MS		130
MeBz	BL		EtBz	60	30	Vial	PN	0.3% CW 20M (80/100) CP C 2 m × 1.8 mm	175	N_2 30	FID		176
Gasoline Kerosene	BL		MeBz-d_8	40	20	Vial	MA	PP P 80/100 1 m × 3 mm 10% OV-17 CS W HP 2 m × 3 mm	180 100		MS	0.01 μg	250
Bz, MeBz	BL		FBz, oXyl	80	23	Vial	PN	HP-1 + HP-5 (2.65 μm) (30 + 30) m × 0.53 mm	50(6) 10/min 60 30/min 100 10/min 200	He 12.5	PID	5 n*M*	251
MeBz	Body fluids Tissues		tBuOH				MA	25% PEG 1000 (60/80) SL 2 m × 3 mm 2% OV-17 (60/80) CS W 1.1 m × 2.6 mm	90 90	N_2 38 He 20	FID MS		252
MeBz	BL		iBuOH	62	40	Vial	PN	5% CW 20M (60/80) CP B 1.8 m × 3.2 mm 0.5% CW 600, 3.8% HC M18 (40/60) CS T 2 m × 3.2 mm	130 85	N_2 60 N_2 30	FID		177
MeBz	BL, UR		EtBz	55	20	Vial	MA	BX-20 2.1 m × 3 mm	90	N_2 40	FID	0.3 μg/g (BL) 0.06 μg/g (UR)	253
Hydrocarbon	BL		MeBz-d_6, indan	60 (RT)	20	Vial	MA	DB-5 (1.5 μm) 15 m × 0.53 mm	40 4/min 90	He 10	MS	0.01 μg/g	254
MeBz AcOEt iBuOH	BL	NaCl	oXyl	55	20	Vial	MA	10% PEG-1000 (60/80) UP HP 2 m × 3 mm	80	N_2 60	FID	50 ng 50 ng 200 ng	102
Paint thinner	BL, BR, LV KD, LN, MS	NaCl	oXyl	55	20	Vial	MA	10% PEG-1000 (60/80) UP HP 2 m × 3 mm	80	N_2 60	FID		161
MeBz	BL	NaCl	nPrOH	80	60	Vial	PN	0.2% CW 1500 (80/100) CP C 1.8 m × 2.0 mm HP-1 25 m	80 70		FID MS		178
MeBz, AcOEt EtOH, iBuOH	BL, LN, KD LV, BR, MS	NaCl	oXyl	55			MA	10% PEG 1000 (60/80) UP HP 2 m × 3 mm	80	N_2 60	FID		162
Gasoline Kerosene	BL	NaCl	MeBz-d_8	55	20	Vial	MA	HP-1 (2.65 μm) 10 m × 0.53 mm	50 (2) 10/min 200	He 15	MS	50 pg (meBz) 1 ng (TMB)	255
MeCN EtOH	BL, UR, SC		tBuOH nPrOH			Vial	PN	10% CW 20M CP B 15% CW 20M CS W 2 m × 3 mm	80 110		FID		215

[a] After HS incubation as described, the temperature of the HS vial is returned to room temperature, and HS vapour is injected into the GC system.

is important in industrial hygiene (exposure monitoring), environmental sciences (monitoring of contamination), anaesthesiology (pharmacokinetic information) and forensic sciences (determining cause of death). The analyses are normally done using columns packed with polar materials such as polyethylene glycol, Carbowax and porous polymers, but non-polar and intermediate-polarity phases have also been used. Because it is halogen specific and very sensitive, ECD is the most frequently used method of detection. FID, MS and Hall electrolytic conductivity detection (HECD) have also been used as detection methods.

Halogenated aliphatic substances are also susceptible to biological transformation. Trichloroethylene and tetrachloroethylene, common environmental pollutants, are metabolized to TCA and TCE. In fact, these compounds are the main substances found after exposure and can be used as indicators [181]. The terminal metabolite, TCA, has been converted into its volatile ester derivatives by addition of dimethyl sulphate [93,94,123,124]. The glucuronide conjugate of trichloroethanol has been determined after hydrolysis by β-glucuronidase treatment and measurement of the resulting TCE by HS-GC [119]. Chloral hydrate has been determined after conversion into TCE [123] and, likewise, TCA has been converted into volatile chloroform by heating [181,256,257]. Detailed HS-GC conditions are presented in Table 5.

4.6. Gases

Gases are obviously the most volatile organic pollutants and in general have only limited water solubility. The use of liquified petroleum gas as a domestic fuel can sometimes lead to gas intoxication and therefore forensic analysis for these lower hydrocarbons may be necessary. Victims of these accidents will retain evidence of exposure in the blood [279]. The analysis involves only warming the samples in a closed HS container (needed owing to the low k values). The only major precautions that must be taken are during the sampling and storage of the samples. Porous polymer adsorbents are used as column packing materials and FID is commonly used in detection. Detailed HS-GC conditions are presented in Table 6.

4.7. Hydrogen cyanide

Cyanide determination in blood has been conducted as evidence of cyanide intoxication in unnatural deaths and in the diagnosis of nitroprusside prescription. In fire accidents, blood cyanide is a biological reflection of exposure to hydrogen cyanide gas, as a result of combustion of nitrogen-containing materials. Determination of cyanide levels in biological samples has usually been performed through pretreatment by distillation and microdiffusion followed by the Könic spectrophotometric determination. HS-GC determination is a more rapid analysis and displays high sensitivity and specific detection. The procedure involves the liberation of cyanide from the met-Hb complex and conversion into the volatile protonated form by the addition of acid. Sulphuric acid, phosphoric acid and acetic acid are all used as reagents. The partition coefficient of hydrogen cyanide (HCN) is relatively high (*ca.* 100) [33,286], necessitating sensitive GC detection methods such as FTD (NPD) or ECD (after precolumn conversion into cyanogen chloride with chloramine T) [287,288]. Porous polymer adsorbents are used as stationary phases. Although acetonitrile is often used as an internal standard [289,290], Seto *et al.* [33] have shown that acetonitrile displays a different HS behaviour to HCN and a small amount of acetonitrile might be present within the background owing to environmental contamination. In addition, the HS-GC method has the unavoidable disadvantage of thiocyanate interference; thiocyanate is converted into HCN in the presence of Hb under acidic conditions [33]. Detailed HS-GC conditions are presented in Table 7.

4.8. Endogenous volatile metabolites

Endogenous volatile metabolites are normal by-products of intermediate metabolites, and are therefore compounds of similar chemical struc-

Table 5
Halogenated hydrocarbons

Substance	Specimen	Additive	I.S.	HS temperature (°C)	HS time (min)	HS container	HS injection	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit	Ref.
Halothane	BL			30	45	Tube	MA	20% SE-30 CS 0.6 m × 6.4 mm	100	Ar 45	FID		2
CCl_4	BL, UR, ML	Na_2SO_4		60	3		MA				FID		87
Chlorinated aliphatic hydrocarbon	BR	$AmSO_4$				Flask	MA	25% CW 1540 Diatoport ST 116 1.8 m × 6.4 mm 20% AGL CS 1.8 m × 3 mm	132	N_2 1.1 atm	FID		258
CH_2Cl_2	BL, UR			75–80	5	Flask	LP	20% CW 20M (80/100) Gas Chrom Q 1.8 m	25	N_2 90	FID	22 μg/l	259
TCE	BL, UR	Pb, AcOH		60	3 h			10% OV-17 (80/100) Gas Chrom Q 1.8 m × 3 mm	125	N_2 20	ECD	0.5 μg/ml (CH TCE) 0.1 μg/ml (TCA)	123
CH, TCA		H_2SO_4											
TCE, TCA C_2HCl_3	BL	H_2SO_4 Me_2SO_4		60	4 h	Vial	MA	5% OV-17 (80/100) CS G 2 m × 3.8 mm	120	N_2 40	ECD	3 μg/l (C_2HCl_3) 60 μg/l (TCE) 30 μg/l (TCA)	124
CH_2Cl_2 $CHCl_3$, CCl_4 1,2-DCE	BL, UR		1-Chlorobutane	90	5	Tube	MA	PP Q (100/120) 1.8 m × 4 mm	175 (2) 5/min 235	He 25	HECD	0.1 m*M*	260
$C_2H_3Cl_3$	BL, BR, MF		nPrOH					PP Q (50/80) 0.75 m × 3 mm	180	N_2 37	FID		261
								PP P (80/100) 1 m × 3 mm	140	He 35	MS		
Fron	BL, LV,			37	20	Vial	MA	PP Q (100/120) 1.8 m × 2 mm	150	N_2 30	FID	2 n*M* (BL) 10 n*M* (LV)	262
Chlorinated aliphatic hydrocarbon	BL, BR, FT		cisDCE	40	60	Vial	MA	PP P (80/100) 1 m × 3 mm	155		MS	10-20 pg	263
CCl_4, $CHCl_3$, $CHBrCl_2$, $CHBr_2Cl$, $CHBr_3$, C_2HCl_3	SR AP			115	30 20		DN	n-Octane (100/120) Porasil C 1.8 m × 6.4 mm	60 7/min 140	N_2 30	HECD		264
Environmental pollutant	BL, UR, ML AT			106			DN	1% SE-30, 0.32% Tullanox SCOT 80 m × 0.4 mm	30 4/min 220	He 1.5	FID		265
1,2-DCE	BL, LV, LN, SP, BR, KD, AP	Citric acid	CH_2Cl_2	90	30	Vial	MA	Tenax GC 4 m × 4 mm	150	N_2 43	FID	ng/ml 25 (BL) 50 (Tissue)	266
$CHCl_3$	BL, BR, LV GC		nPrOH					PP Q	160 175		FID		267
C_2HCl_3	LV, BR, KD AT			60			DN	0.2% CW 1500 (80/100) CP C 1.8 m × 2 mm	60 2/min 100	He 35	HECD	50 pg	268
CCl_4	BL, LV, SR			90	14	Vial	PN	UCON LB 550X (80/100) CS G 3.6 m × 3.2 mm	70	N_2 30	FID	2 ng/ml	179
1,1-DCE	BL			55	30	Vial	MA	Durapak 1.8 m × 3.2 mm	80	N_2 25 ~ 28	ECD		269
CCl_4, CH_2Cl_2 $C_2H_3Cl_3$, C_2HCl_3	BL, UR, GC BR, LN, LV			80	15	Vial	PN	BP-1 25 m	45 (5) 10/min 150 (5)		FID MS		270
TCA[a]	PL	$MgSO_4$		30	60	Vial	MA	10% OV-1 (100/120) Supelcoport 2 m × 2 mm	50	N_2 15–20	ECD	2 μg/l	181

(Continued on p. 48)

Table 5 *(continued)*

Substance	Specimen	Additive	I.S.	HS temperature (°C)	HS time (min)	HS container	HS injection	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit	Ref.
C_2HCl_3	FB	SDS	DCPOH	37	60	Vial	MA	GSB/SP 1000 SCOT 27 m × 0.5 mm	100	N_2 10	ECD	0.3 μM	119
TCA[a]	UR		nBuOH	45	20	Vial	MA	10% CW 20M (100/120) CS W AW 2.5 m × 3 mm	130	N_2 70	FID	2 mg/l	256
CF_2BrCl	BL, KD							OV-101 25 m	RT		MS		271
TCA (ester)[b] TCE	UR	H_2SO_4 Me_2SO_4 MeOH		75	30	Vial	PN	PEG 20M 1.8 m	130	N_2 30	ECD		93
TCA (ester)[b] TCE	UR	H_2SO_4 Me_2SO_4 MeOH		75	60	Vial	PN	DB-WAX 30 m split 30:1	140	N_2 1	ECD	0.1 mg/l (TCA) 0.3 mg/l (TCE)	94
$CHCl_3$	BL, BR, KD, LV, Bile, GC		nPrOH	45	30	Vial	MA	5% CW 20M (60/80) CP B 1.8 m × 2 mm	80	He 40	FID		272
$Br^-(CHBr_3)$[b]	AQ	Citrate $KMnO_4$ MnO_2 H_2SO_4		20	40	Vial		20% DC-550 (80/100) CS W AW DMCS 3 m × 3.4 mm	150	N_2 80	ECD		122
$CHCl_3$	PL	$MgSO_4$		30	60	Vial	MA	10% OV-1 (80/100) CS W AW 3 m × 3.2 mm	70	N_2 30	ECD	22.5 ng/l	273
Vinylidene fluoride	BL			55	90	Vial	MA	Unipack IA 1.5 m	40	H^2 30	MS	6 ng/ml	274
Halothane	BL	EDTA		56	30	Vial	MA	PP P (80/100) 2.5 m × 3 mm	150	N_2 35	FID		88
TCA (ester)[b] TCE, C_2HCl_3	BL, UR	βG H_2SO_4	BCE	90	90	Vial	LP	SE-30 (3 μm) 25 m × 0.31 mm split 1:5	70 (1) 20/min 140 (2)	He 2	ECD		257
CH, TCE	LV	βG $AmSO_4$	$iC_2H_3Cl_3$	60	90	Vial	LP	SE-30 (3 μm) 25 m × 0.31 mm split 1:5	70 (1) 20/min 140 (2)	He 2	ECD		120
TCA (ester)[b]		H_2SO_4		90									
$CHCl_3$	BL		nPrOH iPrOH	40		Vial	PN	0.2% CW 1500 (80/100) CP C 1.8 m × 1.6 mm	90	N_2	FID		275
								10% CW 1500 (80/100) CS W 1.8 m × 1.6 mm	85	N_2	FID		
								PP Q 1.5 m	150 (165)	N_2 He	FID MS		
$C_2H_3Cl_3$	BL			80		Vial	PN	FFAP (80/100) CS W-AW 2.4 m × 3.2 mm	85	Ar–CH_4 (1:19) 40	ECD		276
Enflurane	BL, BR, FT		Isoflurane	63	2 h	Vial	PN	15% CW 1500 (80/100) CS W NAW 1.8 m × 3.2 mm	70	N_2 147 kPa	FID MS		277
C_2 halocarbon	Tissue	Isooctane		90	10		PN	10% FFAP	110	Ar–CH_4	ECD		149
				100			LP	3% OV-17	150	60			
				55				3% SP-1000 30 m × 3.2 mm	60				
C_2H_5Cl	BL, VH	NaCl, NaF	nPrOH	37	15	Vial	MA	PP S 2 m	195		FID		278

[a] TCA is converted into chloroform by heating.
[b] Substances in parentheses are those which are converted by derivatization from non-volatile analytes.

Table 6
Gases

Substance	Specimen	Additive[a]	HS temperature (°C)	HS time (min)	HS container	HS injection	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit	Ref.
O_2, N_2	Biological fluids		RT		VS	VE	MS 5A 1.2 m × 0.64 mm	70 100	He	TCD		22
C_2H_2 Cyclopropane C_2H_4 N_2O	BL	*n*-Octanol Pyrogallate	RT			VE	CS P (60/80) 0.51 m × 6.4 mm + 30% BMEEE (60/80) CS P 1.8 m × 6.4 mm 30% BMEEE (60/80) CS P 3.6 m × 6.4 mm Silica gel (60/200) 3.6 m × 4.8 mm 30% HMPA (60/80) CS P 3.6 m × 6.4 mm		He 50	TCD		23
O_2, CO_2	BL	Sapoinin Acid $K_3Fe(CN)_6$	RT			SW	Hexamethylphosphoramide + MS 13X		He 60	TCD		24
Propane Butane	BL, LV, KD UR, GC		50 (RT)[b]	60[b]	Bottle	LP	PP Q (80/100) 1.125 m × 3 mm PP T (150/200) 0.375 m × 3 mm	100	N_2 2 kg/cm^2	FID	10 nl	280
N_2O	BL		25	30	Vial	MA	PP Q 4.2 m × 2.9 mm	50	H_2 120	TCD		281
Butane Propane	BL, MS, MF		RT	60	Bottle	LP	PP Q (80/100) 2 m × 3 mm	120	N_2 80	FID	1 nl	282 283
Gasoline	BL	(ClBz)	60	20	Bottle	MA	2% OV-17 (60/80) CS W 2 m × 3 mm	85	He 20	MS		284
Propane	LV, BL, BR	H_2SO_4 (AcMe)	RT	60	Bottle	MA	PP Q (80/100) 2 m × 3 mm	120	He 30	MS		285
Propane Butane	BL, organ	(nPrOH)	75	10	Vial	PN	UB A 2 m × 3.2 mm	90	N_2 40	FID		163
Propane	BL		40	30	Vial	MA	SPB-1 100 m × 0.25 mm split 50:1	40	He 2.3 cm/s	FID	1.5 μl/l	279

[a] Substances in parentheses are internal standards.
[b] After HS incubation as described, the temperature of the HS vial is returned to room temperature, and HS vapour is injected into the GC system.

Table 7
Hydrogen cyanide

Specimen	Additive[a]	HS temperature (°C)	HS time (min)	HS container	HS injection	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit (μg/ml)	Ref.
BL	H_3PO_4	60	60	Vial	MA	PP QS 1.8 m × 3.2 mm	80	He 30	FTD	0.05	286
AQ	H_3PO_4	80	10	Vial	PN	PP Q (80/100) 2 m × 3 mm	100	N_2 40	NPD	0.01	291
AQ	H_3PO_4, Br_2, phenol	70	10	Vial	PN	PP Q (80/100) 2 m × 3 mm	120	N_2 50	ECD	0.005	292
BL	AcOH	RT	30	Vial	MA	PP Q (100/120) 1.8 m × 2 mm	110	N_2 20	NPD	0.05	293
BL	AcOH, (MeCN)	RT	30	Vial	MA	PP Q (100/120) 1.8 m × 2 mm	120	He 20	NPD	0.05	289
BL	H_3PO_4, (AcH) (MeCN)	50	30	Vial	MA	PP QS (50/80) 2.1 m × 3 mm	80	N_2 50	FID FTD	0.8 0.2	290
BL	H_2SO_4	50 (RT)[b]	30[b]	Vial	MA	PP Q (80/100) 1.5 m × 3 mm	120	He 60	FTD	0.2	294
BL	$H_3PO_4(CH_2Cl_2)$	50	10	Vial	MA	7% HC M-18 (80/100) CS W AW-DMCS 3 m × 3 mm	55	N_2 30	ECD	0.05	287 288
BL	H_3PO_4, $NaNO_3$	60	30	Vial	PN	PP Q			NPD		180
BL	H_3PO_4	50	30	Vial	MA	GS-Q 30 m × 0.53 mm split 1:5	110	He 4.7	NPD	0.0004	33

[a] Substances in parentheses are internal standards.
[b] After HS incubation as described, the temperature of the HS vial is returned to room temperature, and HS vapour is injected into the GC system.

Table 8
Endogeneous volatile metabolites

Substance	Specimen	Additive	HS temperature (°C)	HS time (min)	HS container	HS injection	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit	Ref.
AcH, EtCHO, AcMe, AcEt	ML	Na_2SO_4	60	3	Vial	MA	20% CW 20M (60/80) CS W 3 m × 3.2 mm	100	N_2 20	FID	0.1 ppm (w/v)	87
EtOH, AcMe, AcEt, AcH, AcPr, Me_2S	UR, BL, ML, AQ	Na_2SO_4	60	15	Vial	MA	20% CW 20M (60/80) CS P AW-HMDS 3 m × 3.2 mm	100	N_2 15.4 (17.4)	FID		83
AcMe, BKB[a]	SR		RT		Bottle	MA	20% HC (60/80) CS W 1.8 m × 4 mm	60	N_2 40	FID		297
AcMe, EtOH, MeOH, AcOAc, aldehydes			RT		Vial	MA	20% HC (60/80) CS W 1.8 m × 4 mm	80	N_2 70	FID		99
AcMe ACA, BHB	BL, LV	KOH PCA $K_2Cr_2O_4$ H_2SO_4	65	10	Vial	PN	15% PEG (60/100) Celite	75	N_2 35	FID	1 μM	295
Volatile metabolites	UR	$AmSO_4$	100			DN	Emulphor ON 870 100 m × 0.5 mm			FID FPD		298
Volatile compounds	UR	Phosphate	80			DN	5% Igepal, 95% SF-96-50 300 m × 0.76 mm	50 (30) 0.5/min 150 (6 h)	He 25	FID		299
Volatile compounds	UR	Phosphate	85	60		DN	5% Igepal CO-880, 95% SF 96(50) 305 m × 0.76 mm	25 0.25/min 30 1.16/min 172 (20)	N_2 20 p.s.i.	MS		300
Volatile organic metabolites	UR	$AmSO_4$	90	60		DN	Emulphor ON-87 100 m × 0.5 mm	60 (16) 2/min 175	N_2 5	MS		301
AcMe ACA BHB	UR, PL, body fluid	H_3PO_4 K_2SO_4 (butanone) KOH H_3PO_4 $K_2Cr_2O_7$	70	15	Bottle	MA	PP R (100/120) 1.2 m × 3 mm	180	N_2 57	FID		296
Organic volatile compounds	Body fluid	Porasil E				DN	Witconel LA-23 100 m × 0.25 mm	50 (10) 1.5/min 160 (80)	He 1.5	FID ECD		302
							Silar 10C 100 m × 0.25 mm	40 (6) 2/min 180 (30)				

(Continued on p. 52)

Table 8 *(continued)*

Substance	Specimen	Additive	HS temperature (°C)	HS time (min)	HS container	HS injection	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit	Ref.
AcMe, Me_2S, AcPr, Phenol	UR	NaCl	85			DN	CW 20M 50 m × 0.32 mm	85 (6) 4/min 180 65 (3) 6/min 180	He 1	FID		303
iPrOH AcMe	BL, BR, LV, KD,		37	20	Vial	MA	5% CW 20M (60/80) CP B 1.8 m × 2 mm	85 95	N_2 30 He 20	FID MS		304
AcMe ACA BHB	PL, KD, LN, LV	NaOH PCA $K_2Cr_2O_7$ H_3PO_4	60	90	Vial	MA	CW 1500 2 m	75	N_2 40	FID	1.19 ppm (w/v)	305
EtOH, MeOH, iPrOH, nPrOH, iBuOH, AcH, AcMe, AcEt, AcOEt	BL	NaCl	50	30	Vial	LP	DB-WAX (0.25 μm) 30 m × 0.25 mm	40 (4) 10/min 150	He 18	MS	5 p*M* (AcOEt) 1.5 n*M* (MeOH)	306
AcMe	BL	NaF, NaCl	40	18	Vial	PN	0.2% CW 1500 CP C 2 m × 3 mm	100	N_2 20	FID	0.01 mg/l	91
Short-chain fatty acids	Faeces	HCOOH, Li_2SO_4 (2-ethylbutyric acid)	90	30	Vial	LP	BP-20 (1.0 μm) 25 m × 0.53 mm split 1:7	50 (2) 40/min 100 (0.1) 8/min 215 (1)	He 3	FID	0.7 (C_7)–17.9 (C_1) μmol per vial	45

[a] After heating at 90°C for 1 h, converted acetone is measured.

Table 9
Unique applications of HS-GC

Substance[a]	Specimen	Additive[b]	HS temperature (°C)	HS time (min)	HS container	HS injection	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit	Ref.
Methanethiol	UR		60		Tube	MA	20% DC-200 (60/80) CS P AW 2.4 m × 6.4 mm	50–60	N_2 75	FID		95
CH_3SH Me_2S_2 EtOH	UR FB	K_2CO_3	60			PN	0.4% CW 1500 (60/80) graphite 2 m × 3 mm	115	N_2 180 kPa	FID	0.22 m*M* 0.44 m*M* 2.4 m*M*	310
Acetylcholine (Me_3N) EtOH AcOEt, nPrOH	UR	K_2CO_3	60	5	Vial	MA	0.4% CW 1500 (60/80) graphite 2 m × 3 mm	110	N_2 12	FID	0.22 m*M* (EtOH) 0.1 m*M* (Me_3N)	103
MeHgI	LV, KD	IAA, NaSCN	80	5	Vial	PN	10% AT-1000 (80/100) CS W AW 1 m × 3 mm	150	Ar 100	MIPE	1.5 ng/ml	307
Methamphetamine Amphetamine	UR	K_2CO_3 KOH	80	20	Tube	MA	DB-17 (0.5 μm) 30 m × 0.32 mm	130	N_2 50 cm/s	FID	0.02 μg/ml 0.05 μg/ml	104
Methamphetamine Amphetamine[c]	UR	K_2CO_3	80	20	Tube	MA	DB-1 (0.25 μm) 30 m × 0.32 mm	130	N_2 50	FID	0.03 μg/ml	105
MeHgI	AQ	IAA	80	4.5	Vial	PN	10% AT-1000 (80/100) CS W AW 1 m × 3 mm	150	Ar 3.5	MIPE	0.5 ppm MeHgI	31
Methamphetamine Amphetamine	UR	K_2CO_3	RT		Tube	MA	DB-17 (0.25 μm) 30 m × 0.32 mm	150	H_2 3.5	FID	1.0 μg/ml 1.5 μg/ml	106
MeHgI	Dogfish tissue	IAA, H_2SO_4	80	4.5	Vial	PN	10% AT-1000 (80/100) CS W AW 1 m × 3 mm	150	Ar 100	MIPE	20 ng/g	308
MeHgCl, EtHgCl, PheHgCl	Mussel	Solvent			Vial	PN	10% AT-1000 CS WAW 2 mm × 2 m CS 101–108 2 m × 2 mm Superox-FA (2 μm) 10 m × 0.53 mm RSL-300 (2 μm) 10 m × 0.53 mm	150 155 150 100	100 20 25 25	MIPE ECD	0.5 μg/l	309
Me_2S	BL AP		60 (RT)[d]	4 h[d]	Vial	MA	10% Polyphenyl ether OS-124 (60/80) SL TPA 3 m × 3 mm	60	N_2 100	FPD	2.5 μg (BL) 5 μg (AP)	311
EtSH	BL		60 (RT)[d]	11[d]		DN	DB-624 30 m × 0.53 mm	30 (2) 8/min 250	He 27.6 kPa	MS	0.1 μg/l	279
Et_2NH EtOH, AcH	BL, SC, LV, bile	(Bu_2NH) (nPrOH)					20% AGL, 5% KOH CS G 5% CW 20M CP	70 80				312

[a] Substances in parentheses are those which are converted by derivatization from non-volatile analytes.
[b] Substances in parentheses are internal standards.
[c] Trifluoroacetyl derivatives converted through in-column reaction are measured.
[d] After HS incubation as described, the temperature of the HS vial is returned to room temperature, and HS vapour is injected into the GC system.

Table 10
Complex mixtures of analytes

Substance	Specimen	Additive[a]	HS temperature (°C)	HS time (min)	HS container	HS injection	Column	Oven temperature (°C)	Carrier gas (ml/min)	Detection	Limit	Ref.
Carbonyl, sulphide ester, alcohol	AQ	Na_2SO_4	60	5	Vial	MA	20% CW 20M (60/80) firebrick AW 3 m × 3.2 mm	100	N_2 20	FID	1 ppm (w/v)	5
Ether, halothane, $CHCl_3$, CCl_4, EtOH, AcMe, C_2HCl_3, paraldehyde	BL	K_2CO_3	70	1	Vial	MA	10% Silicone oil (60/80) Embacel 3.6 m	RT	H_2	FID		4
Sulphide, ketone, alcohol, ester, aldehyde	AQ	Na_2SO_4	60	5	Vial	MA	20% CW 20M (60/80) CS W AW 2.9 m × 3.2 mm	100	N_2 20	FID	0.01–1 ppm (w/v)	315
Solvents	BL		25			MA	15% Flexol 8N8, 10% DIP, 3% PEG 600 (42/60) C-22 firebrick 1.8 m × 3 mm	95		βID		27
N_2O	BL		25	1–2 h	Vial	MA	Active carbon 1 m	35	N_2 100	TCD		316
Cyclopropane							25% PEG–Celite 2 m	24	50	FID		
Ether							25% PEG–Celite 1.5 m	40	50	FID		
Fluothane							25% PEG–Celite 1.5 m	60	50	FID		
Penthrene							25% PEG–Celite 1 m	80	50	FID		
Cyclopropane	BL		25	1–2 h	Vial	MA	25% PEG–Celite 2 m	24	N_2 50	FID		19
Ether							25% PEG–Celite 1.5 m	40	50	FID		
Halothane							25% PEG–Celite 1.5 m	60	50	FID		
Methoxyflurane							25% PEG–Celite 1 m	80	50	FID		
N_2O							Activated charcoal 1 m	35	100	TCD		
Hydrocarbon anaesthetics	BL		37	15	Bottle	MA	5% Silicone gum rubber 60/80 CS W 1.5 m × 3.2 mm	100	He 25	FID		20
Anaesthetics	BL		RT	15	Syringe	MA	5% SE 30 (50/80) CS W DMCS 1.5 m × 3.2 mm	85	He 25	FID		21
Volatile compounds	PL		95			DN	10% GE SF-96, 1% Igepal CO 880 9.1 m × 0.5 mm		He 8	FID MS		317
Volatile organic compounds	BL, UR					DN		−20 (10) 25 (72) 2/min 170				318
	BL, UR, tissue		RT		Jar	MA	PP Q (120/150) 3 m × 3.2 mm	−90 10/min 250	He 300	MS		319

Paraldehyde Propane MeBz CH_2Cl_2	BL, PL, UR		60	30	Vial	PN	0.2% CW 1500 CP C 1.8 m × 3.2 mm	120 65 145 80	N_2 15 20 20 15	FID		320
Volatile compounds	SR, UR	(CHClBr) ($ClCH_2BrHCH_3$)		15		DN	2% CW 1500 CP C	50 8/min 180	He 25	MS		321
	Tissue Fluid				Vial	PN	5% CW 20M (60/80) CP B 4 m × 2 mm	70 5/min 170				1
Environmental pollutants	ML					DN	SE-30 WCOT 80 m	30 (2) 4/min 240	He 1.7	MS		322
Volatile organic compounds	BL	($iC_2H_3Cl_3$) (EtBz)	65	15	Vial	MA	0.3% CW 20M (80/100) CP C 2 m × 2 mm	35 (2) 5/min 175 (8)	N_2 30	FID ECD		156
Volatile organic compounds	BL, LV, BR	EtBz	65	45	Vial	MA	(30/50) Tenax GC 10% CW 400 (80/100) CS W 1.5 m × 6.4 mm	170 140	N_2 40	FID		313
Organic pollutant	BL	(xyl-d_{10})	40–50	15		DN	SE-54 50 m × 0.3 mm	−20 (6) 4/min 130		MS	0.5 ng/ml	323
Solvents	Biologial materials		80	30	Vial	LP	5% CW 20M (60/80) CP B 1.3 m × 2 mm 0.3% CW 20M (80/100) CP C	65(3) 5/min 165 50 (2) 8/min	He 10 He 15, 4	FID		314
Low-molecular-mass volatile compounds	BL, LR, MS, FT		37	1–3 h	Vial	MA	1.3 m × 4(2) mm	195 (5)		FID ECD		75
Volatile substance abuse	BL, UR, tissue	($iC_2H_3Cl_3$) (EtBz)	65	15	Vial	MA	SPB-1 (5 μm) 60 m × 0.53 mm	40 (6) 5/min 80 10/min 200	He 8.6	FID ECD		59

[a] Substances in parentheses are internal standards.

ture (alcohols, ketones and acids). Clinical or pathological analysis of urine or blood for these substances is important in the diagnosis of disease states. In addition, the occurrence of acetone in blood and breath has interested both forensic toxicologists and clinicians [91]. An example of HS-GC analysis applied to these metabolites is in the determination of ketone bodies. Individual ketone compounds are measured separately. Acetoacetic acid levels are determined after thermal conversion into acetone by acid, and β-hydroxybutyric acid levels are determined after oxidative conversion into acetone by the addition of potassium dichromate. Acetone levels are determined in the presence of potassium hydroxide, to prevent the degradation of the other ketone bodies [295,296]. The concentration of acetone in blood did not differ appreciably among subject groups such as drunk drivers, patients with type I diabetes mellitus and healthy blood donors [91]. Dynamic HS-GC methods have also been used in these types of analyses. Detailed HS-GC conditions are presented in Table 8.

4.9. Unique applications of HS-GC

Several unique applications of HS-GC methodology for the analysis of biological samples have been reported. Levels of certain stimulants (amphetamine and methamphetamine) were determined by the addition of potassium carbonate to convert the amine form of the stimulant into the volatile deprotonated form, thus increasing the vapour pressure [104–106]. The determination of methylmercury is applicable in the study of environmental mercury pollution, and a special GC detector (microwave-induced plasma emission) was used after conversion of this compound into the volatile iodide [307–309]. Detailed HS-GC conditions are presented in Table 9.

4.10. Complex mixture of analytes

The diagnosis of substance abuse can be confirmed by detecting a mixture of volatile substances through the use of HS-GC [59,156,313] as a general screening procedure [254,314]. This system is qualitative, with semi-quantitative evaluation of various constituents. The split detection mode of FID and specific ECD can be utilized as a simple method of screening for a wide range of volatile substances within biological fluids [59]. Mass spectrometry is another potential mode for multiple detection of various kinds of volatile substances [254]. Detailed HS-GC conditions are presented in Table 10.

5. Dynamic HS-GC and other analytical methods

The application of dynamic HS-GC to the determination of volatile substances in biological samples has offered improved sensitivity over static HS-GC methods. This method is used mainly in the determination of endogenous volatile metabolites and various instruments for this technique have been developed [63,298–303,317,318]. Environmental pollutants have also been measured using dynamic HS-GC [264,265,268,321–325]. Michael *et al.* [265] described a standard procedure for dynamic HS-GC analysis of volatile pollutants in biological samples. Variations of the dynamic HS-GC method include sample purging (where the analyte is bubbled or swept with a carrier gas), sample trapping [where the purged analyte is trapped by physical (cold) or chemical adsorption (using various adsorbents, *e.g.*, Tenax GC)] and the relative temperatures of purging, trapping and desorption.

Early anaesthetic agents, halothane [326] and diethyl ether [327], in the gas phase extracted from blood samples were determined by infrared and mass spectrometry, respectively. Volatile substances extracted from the gas phase of biological samples have also been determined directly by mass spectrometry [328] and Fourier transform infrared spectrometry [319].

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Joint use of retention index and mass spectrum in post-mortem tests for volatile organics by headspace capillary gas chromatography with ion-trap detection

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Abstract

A method for an unbiased search for volatile organics is described. It is based on direct headspace extraction, capillary GC separation on an apolar stationary phase and ion-trap detection. By automatic reconstruction of the chromatogram with each ion in the scanned mass range (29–199 u), peaks that did not appear on the total ion chromatogram could be spotted. The concentration of some organics at the limit of detection was in the range 0.03–8 μmol/l in blood. Owing to the high sensitivity, the mass spectrum of a peak was not reliable as the sole proof of a substance present at a low concentration. The identification was therefore made on the basis of joint data from mass spectra, searched on-line in a library, and retention indices, retrieved from the literature. To show the value of the method, examples are given of some scarce intoxicants found in post-mortem samples.

1. Introduction

To reveal a toxin in post-mortem samples is a challenge. A reason for this is the common lack of information as to possible agents involved, and in these instances a search for the "general unknown" has to be made. It is true that a number of efficient methods are now available for screening body fluids or tissues for alien organics. However, these tests are mainly applicable to spotting substances derived from drugs or narcotics. Methods for an unbiased search for volatile organics are more sparse, and to some extent also more demanding. The broad range of polarity and the complex nature of the organic volatile fraction with components often present at low concentrations are circumstances that contribute to detection and analysis problems.

Most of the current methods for screening biological materials for volatile organics are based on headspace (HS) extraction and gas chromatographic (GC) separation. Even though the huge number of volatile substances necessitate separation by high-resolution capillary GC [1], most laboratories involved in toxicological analysis still prefer packed columns for screening purposes [2–7]. The choice of extraction method, notably direct HS or purge and trap of the HS fraction, depends on the detection method being used. With flame ionization (FID) or electron-capture detection (ECD), both of which yield high sensitivity, direct HS has been used [3–9]. When the substance search was done by mass spectrometry (MS) with a quadrupole [2] or magnetic [10–12] instrument run in the full-scan mode, on the other hand, the more efficient purge and trap extraction had to be employed.

Unlike these "conventional" MS methods, ion-trap detection (ITD) offers nearly the same sensitivity with the mass scanning set over a wide

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range as tuned on a single selected ion. Along with direct HS extraction of blood before GC separation on a polar capillary (DB-WAX), this detection mode has also been found useful for spotting endogenous polar volatile organics in human samples [13]. However, low-molecular-mass volatile substances often give unspecific mass spectra and also occur in a biological sample only at low concentrations. It can be difficult, therefore, to judge whether an ion comes from a target substance or from some interfering ion that by chance has the same mass. Also, compounds in the same series, *e.g.*, the hydrocarbons, may generate very similar mass spectra. To single out a volatile "general unknown" based only on its mass spectrum is, hence, often uncertain, and the search for a match may result in a number of candidates.

The retention time in a GC capillary may also be a fair, but not perfect, marker for a substance. One advantage is that this parameter is easily proved in a reconstituted mass chromatogram, even if the substance concentration is low. The versatile use of the retention time as an identification tool in an unbiased search for the "general unknown" requires, however, access to literature data on retention indices, and that these be transferable between GC systems. From the works of Kováts seven rules arose. Two of these stated that the retention indices of an apolar compound (alkane) assayed on various types of stationary phases, or the retention indices of any substance assayed on various apolar stationary phases, should be close to each other [14]. This means that the most versatile use of retention indices reported in the literature may be gained by a screening test with GC separation on an apolar stationary phase.

Based on mass spectra obtained with ITD, and on retention indices determined by capillary GC with an apolar stationary phase, a comprehensive, coupled HS–GC–ITD method for an unbiased search of post-mortem samples for the volatile "general unknown" is described here. Some examples of its use with post-mortem samples for spotting, identifying and determining solvents or gases rarely seen in intoxications are also given.

2. Experimental

2.1. Chemicals

Propane and *n*-butane were purchased from Aga Gas (Sundbyberg, Sweden). The other *n*-alkanes (C_5–C_{12}), used for indexing the retention times, and all reference substances, used for the final proof of a detected substance, were obtained from Aldrich-Chemie (Steinheim, Germany) or Merck (Darmstadt, Germany).

2.2. Instrumentation and software

HS extraction was carried out with a Hewlett-Packard Model 19395A autosampler together with an 18906B accessory kit for constant heating time. The gas chromatograph was a Hewlett-Packard Model 5890 with a DB-1 capillary from J&W Scientific (Folsom, CA, USA). It was inserted without a flow restrictor directly into the ion source of a Finnigan MAT ITD800 ion-trap detector. The tuning of the latter was done manually to resolve the m/z 69 and 70 peaks and the m/z 131 and 132 peaks. Evaluation of the raw data was carried out with a Datamaster II (program version 1.3; Finnigan MAT).

2.3. Specimens and sample preparation

The samples to be searched for volatile organics were post-mortem samples sent to our laboratory for routine toxicological analysis. To determine the limit of detection or to construct the calibration graphs, blood from blood donors was used. The body fluids were collected in a 25-ml polystyrene container with a polyethylene screw stopper (3-64211 universal container; Nunc, Denmark). After potassium fluoride, at a final concentration of about 1%, had been added to the body fluids as a preservative, the samples were sent by mail to the laboratory. The analyses were carried out on aliquots of 1.5 ml of fluids added to a 20-ml HS vial (No. 092357; Apodan, Copenhagen, Denmark) which contained 1.8 g of sodium chloride.

2.4. Analytical procedures

The analytical process, generating the raw data, was fully automated with a capacity of 21 samples per batch and a turnover time for each sample of 33 min. The parameters used for the chemical analysis and the instrumental operations are given in Table 1.

For detecting a "general unknown", the total ion current of the mass chromatogram was reconstituted with each single ion in the recorded mass range (29–199 u). This first step of the search was carried out automatically by a data program, written in the "procedure language" (version 2.01) of Datamaster II. The chromatograms, formed and frozen in a spooler file (171 chromatograms on top of one another on 22 screen pages), could then easily be surveyed for random peaks. To be able to judge whether a peak arose from the background noise or from a "general unknown", the limit of detection, *i.e.*, a signal response equal to three times the standard deviation of the gross blank signal, was measured according to the method of Knoll [15]. This was done on control samples at the proper mass number or combination of added mass numbers.

To identify a spotted "general unknown", the library retrieval program of Datamaster II was used. This is a forward library search, in which the spectrum of the "general unknown" is compared with a number of library entries [National Bureau of Standards/National Institute of Health/Environmental Protection Agency (NBS)], and the ten best matches along with their Chemical Abstracts Service (CAS) Registry Numbers are reported. Normally, the retrieval was done with the molecular mass set at 0–220. To obtain a "purity" index also of a compound that was suspected but not among the ten candidates, the molecular mass range was narrowed until the substance was listed. Before searching for "purity" matches of a low-intensity mass spectrum, which might be partly obscured by background ions, it was edited based on the peak height as measured in the reconstituted mass chromatograms.

The retention index was measured and calculated according to the equation [16]

$$I_{calc} = 100(t_{R(unknown)} - t_{R(z)})/(t_{R(z+1)} - t_{R(z)}) + 100z$$

where I_{calc} is the retention index of the "general unknown", $t_{R(unknown)}$ is the total retention time for the "general unknown", $t_{R(z)}$ and $t_{R(z+1)}$ are the total retention times for the *n*-alkanes that bracket the "general unknown" and z is the number of carbon atoms in the *n*-alkane standard that elutes just before the "general unknown".

To be able to compare I_{calc} with literature data, references that show the retention index (I_{lit}) of a "general unknown" was searched in the ESA–IRS database "Chemabs" using the CAS number along with the search word "retention index". The I_{lit} values referred to in this paper were drawn from the work of Streete *et al.* [9] or from different papers quoted in the review by Evans and Haken [14].

If the I_{lit} was not found, an estimate on a substance's retention index was obtained by using the equation [17]

$$I_{bp} = 10^{0.00134052T(bp)+2.558916} - 440.5$$

where I_{bp} is the boiling point index in retention index units and T(bp) is the boiling point of the compound in kelvin at atmospheric pressure.

As a final proof of the identity of an organic compound, its chromatographic and mass spectrometric traits were compared with those of the matching reference substance. If the signal response of an identified substance exceeded ten standard deviations of the background noise [15] and if relevant for the toxicological survey, a quantitative assay according to the external standard method was carried out on the blood. Calibration graphs were constructed using normal blood spiked with suitable concentrations of the analyte.

3. Results

The above-described method for screening post-mortem samples for low-molecular-mass

Table 1
Experimental conditions

Headspace extraction		Gas chromatography		Ion-trap detection	
Equilibration temperature	50°C	Capillary dimensions	30 m × 0.25 mm I.D.	Sample introduction	Direct coupling
Equilibration time	33 min	Coating	1 μm of methylsiloxane	Transfer line temperature	220°C
Valve/loop temperature	54°C	Carrier gas (He) flow-rate	18 ml/min	Ionization mode	Electron impact (50–80 eV)
Auxiliary gas pressure	130 kPa	Column head pressure	75 kPa	Multiplier voltage	1900 V
Vial pressurization time	15 s	Injector temperature	60°C	Automatic gain control	On
Sample loop volume	1 ml	*Oven temperature programme*		Background mass ejected	<29 u
Sweep gas (He) flow-rate	70 ml/min	Initial value	40°C	Analytical scan rate	0.5 s per scan
Injection mode	Split	Initial hold	4 min	Microscans/analytical scan	4
Vent/loop fill time	1 s	Ramp to 200°C	10°C/min	Acquisition time	1.45–20 min
Injection time	2 s	Ramp to 250°C	50°C/min	Mass range examined	29–199 u

volatile organics has been in routine use for about 8 months. During this period 43 different substances were detected and identified, and in some instances also determined. They constituted different types of compounds, *e.g.*, hydrocarbons, alcohols, aldehydes, ketones, esters and ethers. The I_{calc} values ranged from less than 350 to over 1100, and the I_{calc}/I_{lit} ratio was 0.997 ± 0.010 (mean ± S.D.; $n = 40$). The I_{calc} values for the hydrocarbons were also related to the boiling points, with $I_{calc}/I_{bp} = 1.008 \pm 0.016$ (mean ± S.D.; $n = 19$). For the non-hydrocarbons, on the other hand, there seemed to be no simple relationship between I_{calc} and the boiling points, as indicated by the I_{calc}/I_{bp} ratio of 0.874 ± 0.121 (mean ± S.D.; $n = 24$).

Fig. 1 shows the chromatograms of volatile organics found in samples from two subjects who had died following the intake of solvents. The upper chromatogram reveals the findings in the blood of a 35-year-old diabetic and alcoholic. The pathologist found no direct cause of the death, but as the victim was also a solvent thinner sniffer, a screen for solvents was asked for. The total ion current of the mass chromatogram showed two main peaks, which held methanol and nitromethane. The concentration of methanol in the blood was 28.1 m*M* (measured by the routine method of the laboratory) and that of nitromethane was 3.4 m*M* (determined from a calibration graph generated at four concentrations in the range 2.5–25 m*M*, $r = 0.999$). The lower chromatogram in Fig. 1 shows the data from the study of the gastric contents in a 78-year-old man who died suddenly without a previous history of illness. The pathologist found no direct cause of the death, but he noticed a sweet smell when opening the victim's stomach, and, therefore, asked for a screen of its contents for volatile compounds.

Fig. 2 shows the chromatograms of volatile organics found in the blood from two subjects following death caused by inhalation of gases. The upper mass chromatogram is from a 34-year-old man who had inhaled town gas and the lower chromatogram is from a 23-year-old woman who had inhaled car exhaust gas. In both instances over 70% of carbon monoxide–haemoglobin was also measured along with 15–26 m*M* of ethanol (measured by the routine method of the laboratory).

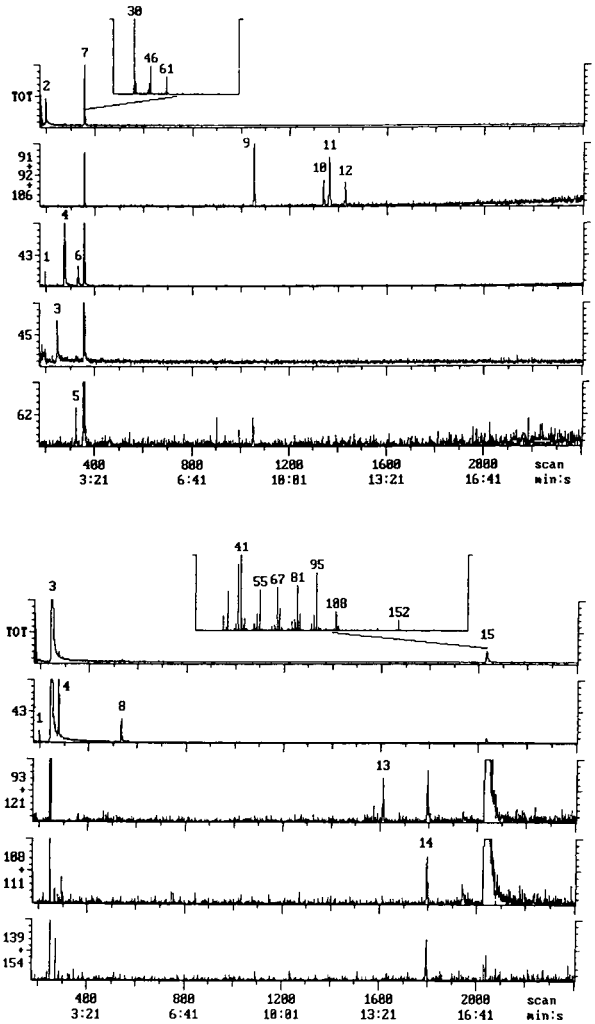

Fig. 1. Total ion current and reconstituted mass chromatogram of volatile organics in post-mortem samples from two deaths caused by intake of solvents. Peaks: 1 = acetaldehyde; 2 = methanol; 3 = ethanol; 4 = acetone; 5 = methyl sulphide; 6 = methyl acetate; 7 = nitromethane; 8 = ethyl acetate; 9 = methylbenzene; 10 = ethylbenzene; 11 = 1,3- and/or 1,4-dimethylbenzene; 12 = 1,2-dimethylbenzene; 13 = camphene; 14 = eucalyptol; 15 = camphor.

As seen in both Figs. 1 and 2, most of the substances were present in such low concentrations that they showed up as peaks only on the chromatograms that had been reconstituted

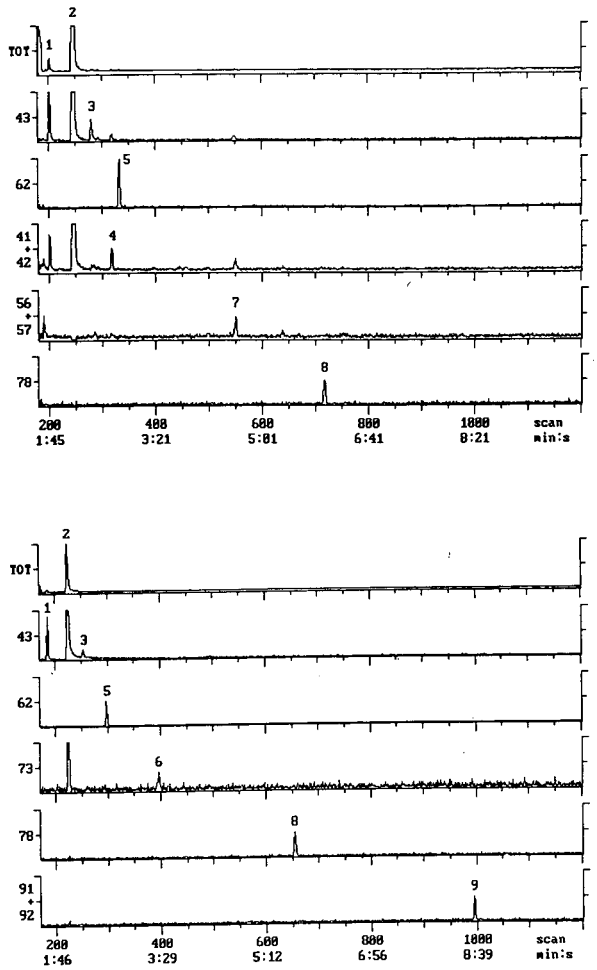

Fig. 2. Total ion current and reconstituted mass chromatogram of volatile organics in post-mortem samples from two deaths caused by inhalation of gases. Peaks: 1 = acetaldehyde; 2 = ethanol; 3 = acetone; 4 = pentane; 5 = methyl sulphide; 6 = methyl *tert.*-butyl ether (MTBE); 7 = hexane; 8 = benzene; 9 = methylbenzene.

with the proper mass number, but not in the total ion current. A peak height exceeding three times the standard deviation of the gross blank signal was judged to indicate a spotted "general unknown". Table 2 gives the concentrations of some of the compounds at this limit of detection.

Table 3 shows the data from the process of identifying the volatile organics in Figs. 1 and 2. As can be seen, the "purity" of a target substance's mass spectrum was in most instances not high enough to be used as the sole proof of identification. Except for acetone, MTBE and hexane, the analyte was listed among the ten most probable alternatives. The I_{calc} values, used as a paired identification proof, agreed reasonably well with the I_{lit} values for all substances, and also with the I_{bp} values for the hydrocarbons.

4. Discussion

Exposure to volatile substances causes numerous deaths each year [9]. The variety of this type of compound in trade products used in daily life is also abundant [18], and a laboratory carrying out toxicological analyses should therefore have the means for the efficient search and assay of such organics.

Packed columns are today the most commonly used separation tool in the search for volatile organics [2–7]. To widen the range of detectable substances, two columns with different polar stationary phases have been used [6], and also recently recommended for routine toxicological work [7]. The single capillary with an apolar stationary phase used in the work presented here seemed to serve well to separate organics with a broad range of boiling points. This is in accord with a newly reported method, which allows the screening of blood or urine for 244 substances with a wide-bore apolar capillary [9]. However, methanol and acetaldehyde, two organics often found in post-mortem samples, were not separated on the apolar phase. With the present method they could be easily separated based on their different fragmentation patterns.

Another means of widening a GC screening for volatile organics is to use two detectors, FID and ECD, coupled to a packed column [5] or to a wide-bore capillary [9]. In this context it is worth mentioning that ITD can be regarded as a multiple detection method, with each mass number combination as a single detector. At the same time ITD gives high sensitivity, as shown in Table 2 for some intoxicants and also reported previously for a number of alcohols, ketones and esters [13].

As can be seen in Figs. 1 and 2, a number of

Table 2
Limits of detection (LOD) of volatile organics in blood

Substance	LOD (μmol/l)	Substance	LOD (μmol/l)
Pentane	0.10	*m*-/*p*-Xylene	0.05
Methyl sulphide	0.19	Styrene	0.13
Nitromethane	1.44	*o*-Xylene	0.08
Methyl *tert.*-butyl ether (MTBE)	0.03	Propylbenzene	0.74
Hexane	0.11	Camphene	1.24
Benzene	0.07	Eucalyptol	3.11
Methylbenzene	0.04	Camphor	8.33
Ethylbenzene	0.06		

The LOD, equal to three times the standard deviation of the background noise, was calculated according to Knoll [15].

Table 3
Summary of identification criteria of substances shown in Figs. 1 and 2

Substance	Mass spectrum library search: purity[a] (rank number)[b]	Retention index		
		I_{calc} [c]	I_{lit} [d]	I_{bp} [e]
Acetaldehyde	903(1), 659(1), 845(1)	342	352	457
Methanol	821(2)	348	353	588
Ethanol	578(1), 547(1), 813(1), 798(1)	428	427	633
Acetone	661(>10), 360(>10), 499(>10), 488(2)	465	460	562
Pentane	592(2)	501	500[f]	500
Methyl sulphide	451(1), 761(1), 740(1)	506	508	499
Methyl acetate	451(1)	507	512	562
Nitromethane	803(1)	521	526	511
MTBE	156(>10)	556	560	556
Ethyl acetate	745(1)	600	598	626
Hexane	540(>10)	601	600[f]	600
Benzene	591(2), 556(4)	651	655	636
Methylbenzene	741(2), 558(6)	758	763	744
Ethylbenzene	552(1)	854	861	839
m-Xylene	421(4)	863	869	851
p-Xylene	421(3)	863	870	847
o-Xylene	596(2)	886	892	871
Camphene	418(8)	954	944	934
Eucalyptol	506(1)	1031	–	1001
Camphor	708(2)	1142	1160	1163

[a] Measure of the mass spectral resemblance rated from zero (no peaks in common) to 1000 (identical library and target substance mass spectra).
[b] The order of the target substance among the top ten matches based on the purity criterion.
[c] Measured data.
[d] Literature data.
[e] The boiling point index in retention index units.
[f] Value by definition.

volatile "general unknowns" were present only in trace amounts and, therefore, never showed up on the total ion chromatogram. A general problem, then, was to activate the right "detector" to make them visible. A useful help in the search for hidden peaks was the data program for automatic reconstitution of the chromatogram with each of the recorded 171 mass numbers. These data formed the basis for further manual processing, by which the sum of two or three of the mass numbers in any combinations could be made up and used to fine tune the reconstruction of the mass chromatogram.

To identify a volatile "general unknown" that had been spotted on the total ion chromatogram or in the reconstituted total ion current, a library search for mass spectrum matches was first done. However, as shown in Table 3, the spectrum resemblances were often far from ideal, resulting in a number of possible candidate target substances. One reason for this was difficulties in sorting out mass fragments of background compounds from fragments of an analyte present in trace amounts. Another was the, under some experimental conditions, inherited ITD problem with distortion of electron impact mass spectra, giving rise to enhanced $[M+1]^+$ peaks. As reported while this work was in progress, a decrease in the ITD manifold temperature or in the helium flow-rate through the capillary may, however, improve the same spectrum [19].

To narrow the number of possible target substances listed in the mass spectrum search, the I_{calc} value of the unknown peak was compared with the I_{lit} values for the different candidates. A similar approach, but with in-house retention indices, has been used by other workers to identify drugs in body fluids of comatose patients [20], polycyclic compounds of environmental interest [21] and hydrocarbons in aviation fuels [22]. As shown in this paper, the I_{calc} and I_{lit} values agreed well enough to be used as a complementary identification marker. Also, the I_{bp} values are a useful tool for the hydrocarbons, should no reference with the key information on I_{lit} be found.

The method described has served in routine toxicology, and to show its potential some findings of scarce intoxicants are presented. The detected compounds nitromethane, camphene, eucalyptol and camphor shown in Fig. 1, seem not to have been reported earlier in any post-mortem body fluid. Even though accidental ingestions of camphor are not rare, the compound has so far only been shown in the serum of two hospital patients [23].

Town gas, which caused the death represented in the upper trace in Fig. 2, contains over 90% of hydrogen, methane, carbon dioxide and carbon monoxide. None of these, though, can be seen by the present method. However, the gas also contains some unspecified hydrocarbons, which according to the producer amount to no more than 2%. By analysis of town gas, sampled in an evacuated HS vial, nine hydrocarbons, which eluted with I_{calc} values in the range 472–651, were identified. Of these, pentane, hexane and benzene were spotted in the blood of the deceased. The other subject represented in Fig. 2, killed by inhalation of car exhaust gas, had two hydrocarbons in the blood along with the octane booster MTBE, which is an additive in motor gasoline. These organics do not seem to have been reported previously in post-mortem samples from deaths caused by inhalation of car exhaust gas.

The method has been applied to blood samples from living persons, but owing to contaminants freed from the rubber septum of the vacutainer tubes used for specimen sampling, the results were difficult to assess. In the HS of one septum type, about fifteen substances were spotted at m/z 57 with I_{calc} values in the range 557–1189. Some of these organics were identified as 3-methylpentane, hexane, methylcyclopentane, cyclohexane, isooctane and toluene. From another brand of septa, *tert.*-butanol, MTBE, methylcyclopentane and cyclohexane were released into the HS. It is therefore urgent that the sampling tools be tested for contaminants that may appear as the "general unknown".

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Review

Confirmatory tests for drugs in the workplace by gas chromatography–mass spectrometry

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Abstract

The Mandatory Guidelines for Federal Workplace Drug Testing Programs require the use of gas chromatography–mass spectrometry (GC–MS) for the confirmation of presumptive positive urine specimens. This review focuses upon GC–MS methods developed specifically for forensic confirmation of amphetamine, methamphetamine, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-acid), benzoylecgonine, morphine, codeine and phencyclidine in urine for purposes of workplace drug testing. In addition, current laboratory issues pertaining to each drug class are reviewed. Generally, drug assays utilized either liquid–liquid or solid-phase extraction methods, derivatization if necessary, and GC–MS detection operating in the selected ion monitoring mode or by full scan acquisition.

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1. Introduction

The abuse of drugs often leads to medical emergencies as a result of acute toxic reactions, increased susceptibility to potentially life-threatening infections, injury from accidents and violence, other health-related problems and to death [1]. Data collected by the United States Substance Abuse and Mental Health Services Administration, Drug Abuse Warning Network, indicates that abused drugs such as heroin, cocaine and ethanol account for a significant number of drug-related illnesses, injuries and deaths in the USA [2,3].

Concerns regarding the proliferation of urine drug testing in the mid-1980s led to the development of standards by the United States Department of Health and Human Services for laboratories conducting forensic urine drug tests. The final version of the standards was published in guideline format on April 11, 1988 in the *Federal Register*. The Mandatory Guidelines for Federal Workplace Drug Testing Programs require the use of immunoassay techniques for initial drug tests, and a confirmatory test by gas chromatography–mass spectrometry (GC–MS) [4]. The confirmation cutoff concentrations for the applicable drugs are shown in Table 1. Since considerable differences exist between regulated and non-regulated confirmatory testing, cutoff concentrations for both levels of testing are included.

Table 1
Regulated and non-regulated confirmation cutoff concentrations

Analyte	Regulated confirmation cutoff concentration (ng/ml)	Non-regulated confirmation cutoff concentration (ng/ml)
Amphetamines		
Amphetamine	500	300
Methamphetamine	500[a]	300
THC-acid	15	10
Benzoylecgonine	150	75
Opiates		
Morphine	300	200
Codeine	300	200
6-Acetylmorphine	Variable[b]	10
Phencyclidine	25	10

[a] To report a positive methamphetamine result, the sample must also contain amphetamine at a concentration greater than or equal to 200 ng/ml.
[b] Cutoff concentration determined by laboratory.

The purpose of this review is to provide a compilation of GC–MS procedures that can be employed for forensic confirmation of amphetamine, methamphetamine, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-acid), benzoylecgonine, morphine, codeine and phencyclidine in urine for purposes of workplace drug testing. In addition, current laboratory issues pertaining to each drug class will be discussed.

2. Amphetamines

2.1. Amphetamine and methamphetamine

Amphetamine and methamphetamine are extensively metabolized and can be detected in urine specimens for up to 72 h. Amphetamine and methamphetamine are the primary analytes found in urine following methamphetamine ingestion. The chemical structures of amphetamine and methamphetamine are shown in Fig. 1. Table 2 provides a summary of GC–MS methods

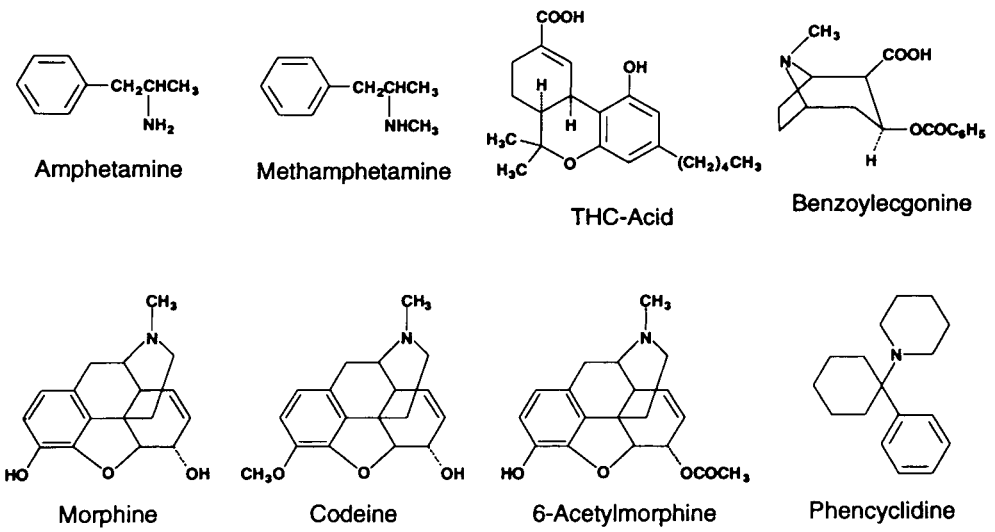

Fig. 1. Chemical structures of abused drugs.

for the analysis of amphetamine and methamphetamine in urine.

Mule and Casella [5] utilized liquid–liquid extraction to isolate amphetamine and methamphetamine prior to GC–MS analysis. Alkalinized samples were extracted with a chloroform–isopropanol solution (9:1, v/v), and the extract was reacted with trifluoroacetic anhydride forming the trifluoroacetyl derivatives of amphetamine and methamphetamine. Phenylcyclohexylamine was utilized as an internal standard. The extraction efficiencies for amphetamine and methamphetamine were approximately 61 and 53%, respectively.

Hornbeck and Czarny [6] compared the trichloroacetyl derivatives of amphetamine and methamphetamine with other common derivatives including trifluoroacetyl and heptafluorobutyryl derivatives for analysis by GC–MS. The extraction procedure utilized an initial liquid–liquid extraction with dichloromethane, a liquid–liquid back-extraction with 1-chlorobutane and derivatization with dimethylaminopyridine and trichloroacetic anhydride. Compared to other

Table 2
GC–MS analysis of amphetamine and methamphetamine in urine

Extraction technique	Derivative	GC column	Detection mode	Detection limit (ng/ml)	Ref.
Liquid–liquid	Trifluoroacetyl	100% Dimethylpolysiloxane	SIM[a]	25	5
Liquid–liquid	Trichloroacetyl	5% Diphenyl/95% dimethylpolysiloxane	SIM	NR[b]	6
Liquid–liquid	Carbethoxy-hexafluorobutyryl	5% Diphenyl/95% dimethylpolysiloxane or 100% dimethylpolysiloxane	SIM	10	7
Solid-phase	Heptafluorobutyryl	5% Methyl/95% dimethylpolysiloxane	SIM	35	8
Solid-phase	Trichloroacetyl	5% Diphenyl/95% dimethylpolysiloxane	SIM	50	9
Solid-phase	Heptafluorobutyryl	5% Diphenyl/95% dimethylpolysiloxane	Scan	50	10

[a] SIM = Selected ion monitoring mode.
[b] NR = Not reported.

derivatives, the trichloroacetyl derivatives demonstrated lower volatility, abundant mass ions and good chromatographic resolution. Utilizing similar extraction conditions, Czarny and Hornbeck [7] also studied the carbethoxyhexafluorobutyryl derivatives of amphetamine and methamphetamine. The carbethoxyhexafluorobutyryl derivatives demonstrated low volatility, high mass fragmentation and good chromatographic resolution at high column temperatures under isothermal conditions.

Taylor *et al.* [8] utilized solid-phase extraction (modified XAD-2 resin) to isolate amphetamine and methamphetamine. Analytes were eluted with a solution of 1% hydrochloric acid in methanol and the final extracts were reacted with heptafluorobutyric anhydride forming the heptafluorobutyryl derivatives of amphetamine and methamphetamine. The extraction efficiencies for amphetamine and methamphetamine were approximately 78 and 87%, respectively. With *n*-propylamphetamine as an internal standard, the assay was linear to 7000 ng/ml.

Gan *et al.* [9] developed a method employing solid-phase extraction (hydrophobic cation exchange) for the isolation of amphetamine and methamphetamine. Analytes were isolated in a solution of 2% ammonium hydroxide in ethyl acetate. The extracts were back-extracted into chlorobutane and reacted with 4-dimethylaminopyridine and trichloroacetic anhydride forming the trichloroacetyl derivatives of amphetamine and methamphetamine. The extraction efficiencies for amphetamine and methamphetamine were approximately 66 and 81%, respectively. The lower recovery obtained for amphetamine was attributed to loss during evaporation. With deuterated internal standards, the assay was linear to 4000 ng/ml.

Wu *et al.* [10] developed an assay for the analysis of amphetamine and methamphetamine employing GC–MS operated in the full scan acquisition mode. Samples were extracted by solid-phase extraction (hydrophobic cation exchange) with [$^2H_8$]methamphetamine as the internal standard. Analytes were isolated in a solution of 2% ammonium hydroxide in ethyl acetate. The extracts were reacted with heptafluorobutyric anhydride forming the heptafluorobutyryl derivatives of amphetamine and methamphetamine.

2.2. Current issues

2.2.1. Other sympathomimetic amines

Because of similarities in chemical structure to amphetamine and methamphetamine, amphetamine assays should be evaluated for interference from other sympathomimetic amines like ephedrine, hydroxynorephedrine, norephedrine, norpseuodephedrine, phentermine, phenylephrine, phenylpropanolamine, propylhexedrine and pseudoephedrine. The evaluation should include the potential for co-elution, and similarity of mass ions and ion ratios to amphetamine and methamphetamine. In addition, chromatograms should be examined for the presence of extraneous chromatographic peaks which may prevent definitive identification.

Thurman *et al.* [11] utilized some of these techniques in a study of the carbethoxyhexafluorobutyryl and heptafluorobutyryl derivatives of amphetamine, methamphetamine and other related sympathomimetic amines. Samples were subjected to liquid–liquid extraction with Toxi-Lab® Toxi-A extraction tubes. Extracts were derivatized with either 4-carbethoxyhexafluorobutyryl chloride or heptafluorobutyric anhydride. [$^2H_3$]Amphetamine and [$^2H_5$]methamphetamine were utilized as internal standards. Based upon the data obtained in full scan acquisition mode, a list of mass ions for selected ion monitoring were developed for all analytes that distinguished amphetamine and methamphetamine from potentially interfering sympathomimetic amines.

2.2.2. Chirality

GC–MS methods utilizing non-chiral derivatives and non-chiral chromatographic liquid phases cannot distinguish between licit (*l*-isomer) and illicit (*d*-isomer and racemic mixtures) forms of methamphetamine. With chiral derivatizing reagents such as N-trifluoroacetyl-l-prolyl chlo-

ride and (−)-menthyl chloroformate, methamphetamine diastereomers can be readily separated with a non-chiral chromatographic system. Typically, isolation of methamphetamine diastereomers is accomplished with routine extraction methods, and extracts are derivatized with the chiral derivatizing reagent. Baseline separation of the *d*- and *l*-isomers is normally achieved allowing identification of the probable source of methamphetamine [12,13].

2.2.3. False positives

Recently, it was discovered that several laboratories engaged in forensic urine drug testing reported false positive methamphetamine results for specimens that contained high concentrations of either ephedrine or pseudoephedrine. Studies performed by Hornbeck *et al.* [14] indicated that methamphetamine was produced by ephedrine or pseudoephedrine derivatized with 4-carbethoxyhexafluorobutyryl chloride, heptafluorobutyric anhydride or N-trifluoroacetyl-l-prolyl chloride. The formation of methamphetamine by thermoconversion was directly related to the temperature of the injection port and the presence of high concentrations of ephedrine or pseudoephedrine. Lowering the injection port temperature, coupled with other preventive measures, eliminates the production of methamphetamine.

To eliminate the possibility of false positive results, ElSohly *et al.* [15] studied the effectiveness of periodate addition to samples containing ephedrine, pseudoephedrine, phenylpropanolamine and norpseudoephedrine. Samples were reacted with a solution of 0.35 *M* sodium periodate for 10 min at room temperature, then subjected to liquid–liquid extraction with chloroform and GC–MS analysis. The data indicated that ephedrine and related compounds are oxidized in the presence of the periodate ion while leaving amphetamine and methamphetamine intact. This process effectively removed the interfering amines and allowed successful analysis of amphetamine and methamphetamine by GC–MS.

3. Cannabinoids

3.1. THC-acid

The major metabolite of tetrahydrocannabinol is THC-acid which is present in urine in both conjugated and unconjugated forms. THC-acid may be detected in urine specimens for variable periods of time depending upon the frequency of use. The chemical structure of THC-acid is shown in Fig. 1. Table 3 provides a summary of GC–MS methods for the analysis of THC-acid in urine.

A comprehensive study of methods for confirmation of THC-acid in urine was reported by Baker *et al.* [16]. The work included an evaluation of hydrolysis, extraction and derivatization procedures. The selected procedure utilized base hydrolysis with 1.0 *M* potassium hydroxide followed by acidification and liquid–liquid extraction with a hexane–ethyl acetate (7:1, v/v) solution. Final extracts were treated with a solution of bis(trimethylsilyl)-trifluoroacetamide and 1% trimethylchlorosilane which formed the trimethylsilyl derivative of THC-acid. The extraction efficiency was 85%, and the procedure was linear to 200 ng/ml.

McCurdy *et al.* [17] developed a method employing C_{18} bonded-phase adsorption columns to isolate THC-acid. Following base hydrolysis, samples were acidified and added to the extraction columns. THC-acid was isolated in methanol, the extract was evaporated and reconstituted in 0.1 *M* hydrochloric acid. This solution was extracted with a hexane–ethyl acetate solution (7:1, v/v), and following evaporation, the final extracts were reacted with a solution of trimethylanilinium hydroxide and iodopropane forming the propyl derivative of THC-acid.

Paul *et al.* [18] developed a method employing a strongly basic anion-exchange resin to isolate THC-acid from base-hydrolyzed urine samples. THC-acid was isolated in ethyl acetate–methanol–acetic acid (90:10:1, v/v/v). Following evaporation, the final extracts were methylated with a mixture of tetramethylammonium hydroxide and iodomethane, acidified and extracted

Table 3
GC–MS analysis of THC-acid in urine

Extraction technique	Derivative	GC Column	Detection mode	Detection limit (ng/ml)	Ref.
Liquid–liquid	Trimethylsilyl	3% SP-2250 on Supelcoport	SIM[a]	10	16
Solid-phase and liquid–liquid	Propyl	100% Dimethylpolysiloxane	SIM	<10	17
Solid-phase	Dimethyl	5% Diphenyl/95% dimethylpolysiloxane	SIM	2	18
Liquid–liquid	Trimethylsilyl	100% Dimethylpolysiloxane	SIM	10	5
Liquid–liquid	Dimethyl	100% Dimethylpolysiloxane	SIM	15	5
Liquid–liquid	Pentafluoropropionyl	5% Diphenyl/95% dimethylpolysiloxane	SIM	1.8	19
Solid-phase	Trimethylsilyl	5% Diphenyl/95% dimethylpolysiloxane	SIM	NR[b]	20
Solid-phase	Dimethyl	100% Dimethylpolysiloxane	Scan	NR	21
Solid-phase	Dimethyl	NR	Scan	2.5	22
Solid-phase	Dimethyl	5% Diphenyl/95% dimethylpolysiloxane	Scan	2.5	10
Liquid–liquid	Dimethyl	5% Diphenyl/95% dimethylpolysiloxane	SIM	2	23
Liquid–liquid	Dimethyl	5% Diphenyl/95% dimethylpolysiloxane	Scan	4	23
Solid-phase	Dimethyl	100% Dimethylpolysiloxane	SIM	NR	24
Liquid–liquid	*tert.*-Butyldimethylsilyl	100% Dimethylpolysiloxane	SIM	1	25
Solid-phase	Trimethylsilyl	5% Diphenyl/95% dimethylpolysiloxane	Scan	1	26

[a] SIM = Selected ion monitoring mode.
[b] NR = Not reported.

into isooctane. The extraction efficiency was approximately 50–60%. The methylated derivative was stable at room temperature for a minimum of 72 h.

Mule and Casella [5] developed a method employing liquid–liquid extraction for the isolation of THC-acid. In addition, two derivatives of THC-acid were studied. Samples were hydrolyzed under basic conditions, acidified and subjected to extraction with a hexane–ethyl acetate solution (7:1, v/v). The final extracts were silylated with N-methyl-N-trimethylsilyl-trifluoroacetamide or methylated with a mixture of tetramethylammonium hydroxide and iodomethane, acidified and subjected to back-extraction in hexane. Mule and Casella [5] noted that both THC-acid derivatives were acceptable; however, the silyl derivative readily contaminated the ion source in the mass spectrometer and was less acceptable than the methyl derivative.

Joern [19] developed a method in which samples were initially hydrolyzed with potassium hydroxide–methanol (1:4, v/v). The hydrolyzed samples were subjected to liquid–liquid extraction with hexane–isoamyl alcohol (98.5:1.5, v/v), followed by acidification and liquid–liquid extraction with hexane–ethyl acetate (5:1, v/v). The final solution was reacted with pentafluoropropionic acid and pentafluoropropanol and evaporated. The extraction efficiency was 70%, and the procedure was linear to 250 ng/ml.

Parry *et al.* [20] developed an assay for THC-acid utilizing hydrophobic anion-exchange chromatography for the isolation step. Following base hydrolysis, the samples were acidified and added to the extraction columns. THC-acid was eluted with methanol. After evaporation, extracts were reacted with bis(trimethylsilyl)-trifluoroacetamide forming the trimethylsilyl derivative of THC-acid. The extraction efficiency was greater than 85%.

Nakamura *et al.* [21] developed a method employing C_{18} bonded-phase adsorption columns to isolate THC-acid. Following base hydrolysis, samples were acidified and added to the ex-

traction columns. THC-acid was isolated in methanol. The final extract was methylated with a mixture of tetramethylammonium hydroxide and iodomethane, acidified and back-extracted into cyclohexane. Meclofenamic acid was employed as a non-isotopic internal standard. The extraction efficiency was greater than 90%, and the methylated derivative was stable at room temperature for several days.

Wimbish and Johnson [22] and Wu *et al.* [10] utilized solid-phase extraction (hydrophobic anion exchange) to isolate THC-acid from base-hydrolyzed urine samples. Following hydrolysis, samples were acidified and transferred to extraction columns. THC-acid was isolated in a solution of hexane–ethyl acetate (75:25, v/v) with 1% acetic acid. The dimethyl derivatives of THC-acid and its corresponding [$^2H_6$]THC-acid internal standard were prepared by reaction with tetramethylammonium hydroxide and iodomethane. The analysis was performed by GC–MS operating in the full scan acquisition mode. Both assays were linear to 1000 ng/ml.

Wimbish and Johnson [22], Joern [23], and ElSohly *et al.* [24] evaluated the use of [$^2H_6$]THC-acid as an internal standard in GC–MS assays for THC-acid. In all reports, final extracts of THC-acid were methylated, acidified and subjected to back-extraction. It was concluded that the use of a highly deuterated internal standard compound increased the dynamic range for GC–MS detection of THC-acid.

Clouette *et al.* [25] modified the method of Baker *et al.* [16] by derivatizing the final extract with N-methyl-N-(*tert.*-butyldimethylsilyl)-trifluoroacetamide in order to form the *tert.*-butyldimethylsilyl derivative of THC-acid. The method provided unusually stable derivatives of THC-acid that demonstrated no apparent deterioration over a period of 10 days.

A newly developed solid-phase C_{18} extraction disc was evaluated by Wu *et al.* [26]. Following base hydrolysis, samples were acidified and applied to the disc. The disc was washed with 20% acetic acid. After drying, the THC-acid was simultaneously eluted and derivatized with N-methyl-N-trimethylsilyl-trifluoroacetamide. The method was linear to 250 ng/ml, and the extraction efficiency was 56%. The new procedure was rapid, produced cleaner extracts compared to ordinary solid-phase extraction minicolumns, and did not require the use of organic solvents.

Two recent reports from the United States Department of Defense described potential sources of interference with the methyl derivative of [$^2H_3$]THC-acid. Depending upon the chromatographic system utilized, ritodrine metabolites [27] and a secondary THC metabolite [28] co-eluted with the prominent mass ions of [$^2H_3$]THC-acid. The presence of these interferences caused the assay to fail quality control criteria; thus, producing false negative results.

3.2. Current issues

3.2.1. Marijuana in foodstuffs

When marijuana is cooked in foodstuffs and ingested, a substantial amount of tetrahydrocannabinol is absorbed and metabolized to THC-acid. The profile of THC-acid excretion in urine is similar to that obtained with a marijuana smoker. Cone *et al.* [29] analyzed consecutive urine samples collected from subjects following the ingestion of one or two marijuana cigarettes cooked in brownie cookies. The samples were extracted with a Prep I extractor (DuPont) and derivatized with tetramethylammonium hydroxide and iodomethane. The analysis was performed by GC–MS operating in the selected ion monitoring mode. Following the ingestion of marijuana in brownies, THC-acid was detectable in urine for 72–346 h.

3.2.2. Passive inhalation

The presence of cannabinoid metabolites in urine specimens due to passive inhalation is a function of environmental conditions, duration and frequency of exposure and tetrahydrocannabinol content of the smoked marijuana. Cone *et al.* [30] studied the effects of passive marijuana smoke in six human subjects who were exposed to smoke of 4 and 16 marijuana cigarettes in a small unventilated room. Consecutive urine samples were collected after passive exposure and analyzed by GC–MS operating in the selected

ion monitoring mode. The GC–MS analysis established that only traces of tetrahydrocannabinol were absorbed by the subjects who were exposed to the smoke of 4 marijuana cigarettes; however, significant amounts of THC-acid were measured after exposure to the smoke of 16 cigarettes. Peak concentrations of THC-acid after the 16 marijuana cigarette exposure ranged from 10 to 87 ng/ml in seven individuals. The results indicated that it was unlikely that individuals exposed to marijuana cigarette smoke would test positive for cannabinoids.

4. Cocaine metabolite

4.1. Benzoylecgonine

Cocaine is rapidly hydrolyzed to benzoylecgonine and ecgonine methyl ester by chemical and metabolic reactions. Benzoylecgonine, the primary cocaine metabolite, may be detected in urine for several days following drug administration. The chemical structure of benzoylecgonine is shown in Fig. 1. Table 4 provides a summary of GC–MS methods for the analysis of benzoylecgonine in urine.

Benzoylecgonine can be extracted readily with either liquid–liquid or solid-phase techniques. Joern [31] adapted the procedure of Graas and Watson [32] to simultaneously extract and derivatize benzoylecgonine employing an extractive alkylation solvent solution of methylene chloride–1-iodopropane (99:1, v/v). Utilizing N-butylbenzoylecgonine as an internal standard, the modified method was linear to approximately 1250 ng/ml.

Taylor *et al.* [33] described a method for the isolation of benzoylecgonine utilizing solid-phase extraction with Amberlite XAD-2 extraction material. Following isolation in *n*-butylchloride–acetonitrile–methanol solution (40:50:10, v/v/v), the final extract was evaporated and reacted with N-methyl-N-trimethylsilyl-trifluoroacetamide forming the trimethylsilyl derivative of benzoylecgonine. Utilizing [$^2H_3$]benzoylecgonine as an internal standard, the method was linear at concentrations ranging from 50 to 4000 ng/ml. The extraction efficiency was approximately 75–80%.

Mule and Casella [34] developed an assay in which an alkalinized sample was extracted with a chloroform–isopropanol (9:1, v/v) solution. Following a water wash, the organic solvent was evaporated, and the extract was reacted with a solution of pentafluoropropionic anhydride and pentafluoropropanol forming the pentafluoropropyl derivative of benzoylecgonine. Ketamine was utilized as an internal standard. The extraction efficiency for benzoylecgonine was 76%.

Gerlits [35] recently described the formation of a unique *tert.*-butyldimethylsilyl derivative of benzoylecgonine. Following extraction, extracts were reacted with a solution of N-methyl-N-*tert.*-butyldimethylsilyl-trifluoroacetamide and 1% *tert.*-butyldimethylchlorosilane.

Wu and co-workers [10,36] developed an assay for the analysis of benzoylecgonine employing

Table 4
GC–MS analysis of benzoylecgonine in urine

Extraction technique	Derivative	GC column	Detection mode	Detection limit (ng/ml)	Ref.
Liquid–liquid	*n*-Propyl	5% Diphenyl/95% dimethylpolysiloxane	SIM[a]	35	31
Solid-phase	Trimethylsilyl	100% Dimethylpolysiloxane	SIM	50	33
Liquid–liquid	Pentafluoropropyl	100% Dimethylpolysiloxane	SIM	12.5	34
Liquid–liquid	*tert.*-Butyldimethylsilyl	100% Dimethylpolysiloxane	SIM	NR[b]	35
Liquid–liquid	Pentafluoropropyl	5% Diphenyl/95% dimethylpolysiloxane	Scan	37	10, 36
Solid-phase	Trimethylsilyl	100% Dimethylpolysiloxane	SIM	5	37

[a] SIM = Selected ion monitoring mode.
[b] NR = Not reported.

GC–MS operated in the full scan acquisition mode. Samples were extracted using solid-phase extraction (hydrophobic cation exchange) with difluorobenzoylecgonine as the internal standard. Benzoylecgonine was isolated in a solution of methylene chloride–isopropanol (80:20, v/v) in 2% ammonium hydroxide. The final extracts were reacted with pentafluoropropionic anhydride and pentafluoropropanol forming the pentafluoropropyl derivative of benzoylecgonine.

Taylor and Le [37] developed a fully automated procedure for the analysis of cocaine and benzoylecgonine utilizing a laboratory robotic system. The system was capable of performing precise sample aliquot transfers, solid-phase extraction and derivatization. The method was linear at concentrations ranging from 5 to 100 000 ng/ml. With this system, within-run and between-run precision studies produced relative standard deviations less than 10%.

4.2. Current issues

4.2.1. Coca tea

Consumption of tea prepared with coca leaf (*e.g.* "Health Inca Tea") can produce detectable concentrations of benzoylecgonine in urine. El-Sohly *et al.* [38] and Jackson *et al.* [39] reported peak urinary benzoylecgonine concentrations exceeding 1000 ng/ml following ingestion of a single cup of tea. Both studies utilized GC–MS analysis to confirm the presence of benzoylecgonine in the urine specimens of tea drinkers.

4.2.2. Passive inhalation

Cocaine base ("crack") is readily vaporized by heating resulting in efficient delivery of drug to the lungs and circulatory system of "crack" smokers. During the smoking process, some of the vaporized cocaine is released into the atmosphere. Individuals in the vicinity of a "crack" smoker could passively breathe in cocaine vapor. Cone *et al.* [40] measured cocaine and benzoylecgonine in urine of persons passively exposed to cocaine vapor by GC–MS analysis. Although benzoylecgonine concentrations were insufficient to be reported positive by the Department of Health and Human Services guidelines, there were sufficient amounts excreted to indicate that significant absorption of cocaine had occurred as a result of passive inhalation.

5. Opiates

5.1. Morphine and codeine

Following administration, heroin is rapidly metabolized to 6-acetylmorphine and then to morphine by chemical and enzymatic processes. Morphine is further metabolized by conjugation to morphine-glucuronide and by demethylation to normorphine. Morphine and conjugated morphine are the primary heroin metabolites found in urine, but heroin and 6-acetylmorphine may also be present for a short period after drug administration. Codeine is metabolized by conjugation to codeine-glucuronide and by demethylation to morphine and norcodeine. Codeine and morphine are the primary analytes found in urine following codeine ingestion. Morphine and codeine may be detected in urine for 2–4 days following drug use. The chemical structures of morphine, codeine and 6-acetylmorphine are shown in Fig. 1. Table 5 provides a summary of GC–MS methods for the analysis of morphine and codeine in urine.

The initial methods developed for the simultaneous analysis of opiates in urine were based upon liquid–liquid extraction techniques. Paul *et al.* [41] developed an assay where an acid-hydrolyzed sample was alkalinized and extracted with methylene chloride–isobutanol (9:1, v/v) solution. This was followed by acid–base extraction and re-extraction into organic solvent. The extract was evaporated and derivatized with acetic anhydride and pyridine. The acetyl derivatives of morphine and codeine were compared to those formed by reaction with trifluoroacetic anhydride, pentafluoropropionic anhydride or heptafluorobutyric anhydride. The acetyl derivative demonstrated the most acceptable chromatographic properties. Nalorphine was utilized as an internal standard. The extraction efficiencies for morphine and codeine were 40 and 58%, respectively.

Table 5
GC–MS analysis of morphine and codeine in urine

Extraction technique	Derivative	GC column	Detection mode	Detection limit (ng/ml)	Ref.
Liquid–liquid	Acetyl	5% Diphenyl/95% dimethylpolysiloxane	SIM[a]	25	41
Liquid–liquid	Perfluoroester	100% Dimethylpolysiloxane	SIM	50	5
Liquid–liquid	Acetyl	100% Dimethylpolysiloxane	SIM	10	42
Solid-phase	Acetyl	5% Diphenyl/95% dimethylpolysiloxane	Scan	10	43
Solid-phase	Perfluoroester	5% Diphenyl/95% dimethylpolysiloxane	Scan	50	10
Solid-phase	Trimethylsilyl	100% Dimethylpolysiloxane	SIM	NR[b]	44

[a] SIM = Selected ion monitoring mode.
[b] NR = Not reported.

Mule and Casella [5] developed an assay in which an acid-hydrolyzed sample was alkalinized and extracted with a chloroform–isopropanol (9:1, v/v) solution. After a water wash, the organic solvent was evaporated, and the extract was reacted with a solution of pentafluoropropanol and pentafluoropropionic anhydride forming the perfluoroester derivative of morphine and codeine. Nalorphine was utilized as an internal standard. The extraction efficiencies for morphine and codeine were 96 and 91%, respectively.

Bowie and Kirkpatrick [42] developed a method for the determination of morphine, codeine, 6-acetylmorphine and other 6-keto-opioids. Following enzymatic hydrolysis, alkalinized samples were extracted with a chloroform–isopropanol (9:1, v/v) solution. After a water wash, the organic solvent was evaporated, and the extract was acetylated by reaction with [$^2H_6$]acetic anhydride and pyridine. The extraction efficiency for morphine and codeine was greater than 95%.

The recent development of copolymeric bonded-phase extraction cartridges has improved the efficiency and ease of simultaneous opiate extraction. For example, Huang *et al.* [43] developed a method for the analysis of morphine, codeine, hydromorphone, hydrocodone and oxycodone utilizing hydrophobic cation-exchange solid-phase extraction cartridges. Samples were subjected to enzyme hydrolysis, followed by solid-phase extraction. Opiates were isolated in a solution of methylene chloride–isopropanol (80:20, v/v) with 2% ammonium hydroxide. The final extracts were reacted with acetic acid and pyridine forming acetyl derivatives of morphine and codeine. GC–MS was operated in the full scan acquisition mode. Using nalorphine as an internal standard, the assay was linear for all analytes between 50 and 1000 ng/ml. The extraction efficiencies for morphine and codeine were greater than 80%.

Wu *et al.* [10] developed an assay for the analysis of opiates employing GC–MS operated in the full scan acquisition mode. Following enzyme hydrolysis, samples were extracted using solid-phase extraction (hydrophobic cation exchange) with deuterated dihydromorphine and dihydrocodeine as internal standards. Opiates were isolated in a solution of methylene chloride–isopropanol (80:20, v/v) with 2% ammonium hydroxide. The final extracts were reacted with pentafluoropropionic anhydride forming the perfluoroester derivatives of morphine and codeine.

Vidal *et al.* [44] developed a fully automated procedure for the analysis of morphine and codeine utilizing a laboratory robotic system. The system was capable of performing precise sample aliquot transfers, enzyme hydrolysis, solid-phase extraction and derivatization. With this system, the within-run and between-run precision studies produced relative standard deviations of approximately 10%.

The stability of derivatives commonly utilized for the determination of morphine and codeine by GC–MS was evaluated by Chen *et al.* [45].

The study examined derivatives formed by reaction of unextracted morphine and codeine standards with pentafluoropropionic anhydride, heptafluorobutyric anhydride, N-methyl-bis-trifluoroacetamide, bis(trimethylsilyl)-trifluoroacetamide, and acetic anhydride and pyridine. Of the five different derivatives evaluated, the trimethylsilyl and acetyl derivatives produced the most stable mass spectra for GC–MS quantitation.

The stability, chromatographic characteristics, potential interference, and suitability of major fragment ions for GC–MS analysis of morphine and codeine perfluoroester and acetyl derivatives of morphine and codeine were evaluated further by Grinstead [46]. All of the derivatives were found to suffer from some limitations. The perfluoroester derivatives of morphine and codeine showed acceptable stability and lack of interference from other opiates; however, the codeine derivative demonstrated poor chromatography and its mass spectrum consisted of only two ions suitable for analysis by GC–MS operated in the selected ion monitoring mode. The acetyl derivatives were stable, demonstrated acceptable chromatography and produced suitable mass spectra; however, problems with incomplete derivatization and side reactions limited its usefulness. Also, morphine was indistinguishable from 6-acetylmorphine with this derivative since both analytes form diacetylmorphine.

5.2. Current issues

5.2.1. Other opiates

Because of similarities in chemical structure to morphine and codeine, opiate assays should be evaluated for interference from opiate metabolites and semi-synthetic 6-keto-opioids, including commonly prescribed analgesics such as hydromorphone, hydrocodone, oxymorphone and oxycodone. For example, the mass spectra of the trimethylsilyl derivative of hydromorphone resembles the trimethylsilyl derivative of morphine. In addition, the mass spectra of the trimethylsilyl derivative of morphine is similar to the trimethylsilyl derivative of norcodeine.

5.2.2. 6-Acetylmorphine

Recently, assays designed to detect 6-acetylmorphine in urine have been developed to provide information regarding the nature of opiate drug ingestion. The extraction of 6-acetylmorphine in urine can be accomplished readily by liquid–liquid or solid-phase extraction techniques. Mule and Casella [47] utilized a chloroform–isopropanol (9:1, v/v) solution to isolate 6-acetylmorphine. Following derivatization, the trimethylsilyl derivative of 6-acetylmorphine was assayed by GC–MS. The method was sensitive to 10 ng/ml of 6-acetylmorphine with 0.5 ml sample volume.

Paul *et al.* [48] reported a highly sensitive method for 6-acetylmorphine in urine utilizing an initial solvent extraction with 10% isobutanol in methylene chloride, followed by acid–base organic extraction or solid-phase (LC-CN columns) purification. The final extract was reacted with propionic anhydride and pyridine forming propionylated 6-acetylmorphine. The limit of detection of the method was 0.81 ng/ml, and the approximate extraction efficiencies were 80% and greater than 90% for the liquid–liquid and solid-phase extraction methods, respectively. Romberg and Brown [49] subsequently reported an improvement of the methodology of Paul *et al.* [48]. The solid-phase purification step was replaced with an acidic sodium acetate back-extraction, followed by an additional alkaline extraction into 10% isobutanol in methylene chloride. An increase in 6-acetylmorphine recovery, and elimination of extraneous peaks were observed with the modified procedure.

Procedures for the simultaneous determination of 6-acetylmorphine and other opiate analytes have been reported by Bowie and Kirkpatrick [42], Fuller and Anderson [50] and Goldberger *et al.* [51]. The assays utilized either liquid–liquid or solid-phase extraction techniques, followed by derivatization and GC–MS analysis. In order to obtain accurate measurements of 6-acetylmorphine, techniques were developed that avoided chemical and enzymatic hydrolysis. In addition, optimum chemical stability of 6-acetylmorphine was achieved with extraction procedures that utilized neutral pH conditions [51].

5.2.3. Poppy seeds

Morphine and codeine occur naturally in poppy seed. As a result, morphine and codeine are excreted in urine specimens of individuals who have recently ingested foodstuffs prepared with poppy seeds. The concentration of morphine and codeine in urine generally reaches a maximum within 2 to 4 h following ingestion, and declines over a period of 24–48 h. Consequently, urine specimens from poppy seed consumers resemble those obtained from heroin users. In order to differentiate poppy seed ingestion from codeine, morphine and/or heroin use, ElSohly and Jones [52] proposed the following guidelines based upon GC–MS analysis of opiates in urine: morphine concentration greater than 5000 ng/ml; codeine concentration greater than 300 ng/ml with a morphine-to-codeine ratio less than 2; or the presence of 6-acetylmorphine. Presently, these guidelines are being evaluated to determine their validity in differentiating heroin users from poppy seed eaters.

6. Phencyclidine

Phencyclidine undergoes oxidative metabolism forming monohydroxy and dihydroxy metabolites. The hydroxylated metabolites are excreted in urine as glucuronide conjugates. Approximately 10% of a phencyclidine dose is excreted unchanged in urine. Phencyclidine may be detected in urine for several weeks following drug use. The chemical structure of phencyclidine is shown in Fig. 1. Table 6 provides a summary of GC–MS methods for the analysis of phencyclidine in urine.

A limited number of methods have been published describing the analysis of phencyclidine in urine utilizing GC–MS detection. Mule and Casella [5] reported a highly sensitive method employing only 0.2 ml of urine. Urine samples were alkalinized and extracted with a chloroform–isopropanol (9:1, v/v) solution. Ketamine was used as an internal standard. The assay was linear over a range of 10–100 ng/ml with an extraction efficiency of 87%.

Stevenson *et al.* [53] and Chan *et al.* [54] employed solid-phase extraction (hydrophobic cation exchange) for the analysis of phencyclidine. [$^2H_5$]Phencyclidine was employed as the internal standard in both methods. To isolate phencyclidine, Stevenson *et al.* [53] and Chan *et al.* [54] utilized a solution of 2% ammonium hydroxide in methanol or ethyl acetate, respectively.

Wu *et al.* [10] developed an assay for phencyclidine utilizing solid-phase extraction (hydrophobic cation exchange) with difluorophencyclidine employed as an internal standard. GC–MS was operated in the full scan acquisition mode. The method was linear to 500 ng/ml, and the extraction efficiency was approximately 90%.

7. Conclusions

Although current methods for GC–MS confirmation of abused drugs have been adequate for the certification of approximately ninety laboratories under the Mandatory Guidelines for

Table 6
GC–MS analysis of phencyclidine in urine

Extraction technique	Derivative	GC column	Detection mode	Detection limit (ng/ml)	Ref.
Liquid–liquid	NA[a]	100% Dimethylpolysiloxane	SIM[b]	10	5
Solid-phase	NA	5% Diphenyl/95% dimethylpolysiloxane	SIM	0.5	53
Solid-phase	NA	100% Dimethylpolysiloxane	SIM	10	54
Solid-phase	NA	5% Diphenyl/95% dimethylpolysiloxane	Scan	0.25	10

[a] NA = Not applicable.
[b] SIM = Selected ion monitoring mode.

Federal Workplace Drug Testing, many laboratories continue to improve their methods of extraction and derivatization, and their instrumental techniques. For example, the present trend towards the utilization of solid-phase extraction permits rapid isolation of drug and/or metabolite for GC–MS analysis. This has resulted in cleaner extracts and increased throughput. New isotopic and non-isotopic compounds for use as internal standards have been recently introduced eliminating some of the interferences produced with earlier internal standards. These modifications in chromatographic and spectrometric techniques continue to improve assay specificity and sensitivity. It is expected that future changes in confirmatory tests for drugs in the workplace will focus upon development of new assays for additional drugs of abuse and the use of new hyphenated methods for sample introduction into the mass spectrometer.

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Toxicological analysis of whole blood samples by means of Bond-Elut Certify columns and gas chromatography with nitrogen–phosphorus detection

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Abstract

The application of Bond-Elut Certify solid-phase extraction columns to the systematic toxicological analysis of whole blood was evaluated. The reproducibility of the extraction was tested with thirteen drugs varying in physico-chemical properties. Analysis was performed with capillary gas chromatography with nitrogen-selective detection. The recoveries were reproducible, as long as other limiting factors, *e.g.*, chromatographic behaviour or volatility, do not play a significant role. The effect of limiting chromatographic behaviour was studied in more detail with the more sensitive mass spectrometry with selected-ion monitoring after converting the extracted morphine into its ditrimethylsilyl derivative.

1. Introduction

Solid-phase extraction (SPE) has mostly been applied to the highly selective extraction of individual analytes or groups or closely related analytes. Because the number of relevant parameters governing the extraction is larger than with liquid–liquid extraction, higher selectivity can be obtained. In the analytical screening for analytes that differ widely in physico-chemical properties such as acidic–basic nature, pK_a and polarity, SPE has not been considered the first choice.

The development of new types of stationary phases, *e.g.*, Bond-Elut Certify, has opened up new possibilities in this field. These columns possess both cation-exchange and hydrophobic properties [1,2].

After the reported good results with matrices such as plasma and urine [1,3] we considered the application to whole blood. Haemolysed whole blood is a matrix encountered in most cases in forensic toxicology. The preparation of serum or plasma from this blood is hardly ever possible. Pretreatment of whole blood before SPE is carried out either by precipitation of the proteins with a reagent such as zinc sulphate solution or trichloroacetic acid [4] or by dilution of the blood with an appropriate buffer solution. [1–3,5]. The dilution procedure has proved to yield better results and was employed in this study.

For this study, the dilution method was used followed by gas chromatography with nitrogen–phosphorus detection (NPD). Capillary GC–

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NPD combines high separation power, flexibility and selectivity. The last factor is based on the fact that most drugs encountered in forensic case work contain nitrogen. Gas chromatography–mass spectrometry is used for problems that cannot be solved using GC–NPD.

Whole blood was spiked with a mixture of drugs with various physico-chemical properties and GC behaviour. The individual substances chosen are listed in Table 1. Extraction recoveries were measured with the use of procaine as a chromatographic standard. For drugs with unfavourable chromatographic behaviour, morphine was selected as a model substance and recoveries were determined after silylation with N,O-bistrimethylsilyltrifluoroacetamide (BSTFA). Detection in this instance was performed using mass spectrometry in the selected-ion monitoring mode (MS-SIM).

2. Experimental

2.1. Materials

The extraction columns used were Bond-Elut Certify (Analytichem International/Varian, Harbor City, CA, USA), 3 ml, containing 130 mg of stationary phase. The packing material exhibits both hydrophobic and ion-exchange properties. The structural basis is silica.

The blood employed for experiments was outdated citrated whole blood from a blood bank and was stored at −18°C until used.

Methanol (HPLC grade) and chloroform and dichloromethane (glass-distilled grade) were obtained from Rathburn (Walkerburn, UK), acetone (Nanograde) from Prochem (Wesel, Germany), ethyl acetate, 2-propanol, 25% ammonia solution, potassium phosphate and potassium hydroxide (analytical-reagent grade) from Merck (Darmstadt, Germany) and BSTFA from Chrompak (Middelburg, Netherlands). Water was purified with a Milli-Q/Organex-Q system (Millipore, Milford, MA, USA). Amphetamine sulphate, codeine hydrochloride (dihydrate), morphine hydrochloride (trihydrate) and cocaine hydrochloride were purchased from Brocacef (Maarsen, Netherlands), phenobarbitone from Interpharm ('s-Hertogenbosch, Netherlands) and diphenhydramine hydrochloride, methaqualone and methadone hydrochloride from OPG (Utrecht, Netherlands). 6-Monoacetylmorphine hydrochloride was synthesized in our laboratory. Nalorphine hydrobromide and haloperidol were obtained from Bufa (Uitgeest, Netherlands) and strychnine from Merck. Oxazepam was kindly donated by Hoffman-La Roche (Mijdrecht, Netherlands).

2.2. Instruments and chromatographic conditions

GC–NPD was carried out using an HP 7673 autosampler and an HP 5890-II gas chromatograph equipped with a nitrogen–phosphorus detector and linked to an HP 5970C Chemstation (all from Hewlett-Packard, Waldbron, Germany).

Aliquots of 1 μl were injected in the split mode (1:9) on to an Ultra-1 column (12 m × 0.2 mm I.D.; 0.33 μm film thickness) (Hewlett-Packard). Helium was used as the carrier gas (2 ml/min). The detector and injector temperatures were 280°C. The oven temperature was programmed from 100 to 280°C at 5°C/min and kept at 280°C for 10 min. The bead current was adjusted to maintain a collector current of 20 pA at a 100°C oven temperature.

GC–MS was performed on a Model 5971A mass-selective detector (Hewlett-Packard). The autosampler, gas chromatograph, column and data system were as mentioned above. The temperature programme was as mentioned above, except that the programming rate was 10°C min. The injection port, transfer line and ion source temperatures were maintained at 280°C. Aliquots of 1 μl were injected in the splitless mode (purge time 0.5 min). The mass spectrometer was operated in the SIM at m/z 429.5 and 236.35 at the retention time of morphine and m/z 235.2 and 250.2 at the retention time of methaqualone.

2.3. Sample preparation and treatment

Spiking was done, on the day of analysis, in polypropylene tubes. The solutions of the drugs

in methanol were pipetted into the tube. Care was taken that the resulting spiked blood never contained more than 2% (v/v) of methanol. If necessary, the methanolic solution was evaporated to a sufficiently small volume. Before adding the blood to the contents of the tube, the methanolic solution was diluted with the same volume of water. In this way coagulation of the proteins by the methanol was prevented.

Samples were processed on a Vac Elut SPS 24 vacuum manifold (Analytichem/Varian). The vacuum pressure was adjusted to at least 15 mmHg and the flow through the columns was regulated with PTFE valves.

The columns were preconditioned with 2 ml of methanol followed by 2 ml of 0.1 *M* phosphate buffer (pH 6.0).

The vacuum was adjusted in such a way that a flow of about 2.0 ml/min was obtained through the columns. The columns were not allowed to dry before the sample was applied.

A volume of 1.00 ml of blood was mixed with 6.0 ml of 0.1 *M* phosphate buffer (pH 6.0) and the mixture was sonicated for 15 min and centrifuged for 15 min at 2000–3000 rpm (500–750 *g*). The supernatant was passed through the pretreated column at a flow-rate of about 1.5 ml/min. Care was taken that no precipitate was transferred. The columns were rinsed with 1.0 ml of water and 0.5 ml of 0.01 *M* acetic acid (pH 3.3). The columns were dried by suction of air at the maximum vacuum (>15 mmHg; 1 mmHg = 133.322 Pa) for 5 min. A 50-μl volume of methanol was added to the column and air (>15 mmHg for at least 1 min) was passed through in order to remove the last remaining liquid. Before elution the columns were placed on a clean outlet valve.

Acid–neutral fraction

The acid–neutral drugs were eluted with two portions of 2.0 ml of acetone–chloroform (1:1) in about 5 min.

Basic fraction

The basic drugs were eluted with 3 ml of ethyl acetate containing 2% (v/v) of 25% ammonia solution (freshly prepared before use). This solution passed through the column by gravity only (in about 4 min).

The two fractions were collected separately in silanized conical glass tubes and evaporated to dryness at 40°C under a stream of nitrogen. The residues were dissolved in 50 μl of a solution of procaine in methanol (5 μg/ml). The extracts were analysed by GC–NPD (standard procedure).

For the study of the recovery of morphine, the basic fraction was submitted to derivatization (see *Recovery of morphine*).

2.4. Determination of recoveries

Standard procedure

Ratios of the peak heights of the respective substances to the peak height of procaine were used for calculations. These ratios were compared with those obtained from injections of a mixture of the pure drugs and procaine (chromatographic standard) in methanol at known concentrations. The concentrations of the drugs in this solution were chosen in such a way that they equalled the concentrations in the reconstituted extracts when the recoveries would be 100%. This procedure was repeated five times for each concentration level (500, 250, 100 and 50 ng/ml) of the drugs in whole blood.

Recovery of morphine

In order to be able to discriminate between poor extraction recoveries and poor results originating from poor GC behaviour, a derivatization experiment was carried out. Morphine was chosen as an example of a substance with difficult GC behaviour.

Morphine was added to blood at concentrations of 500, 50, 10.0 and 5.0 ng/ml and the blood was extracted according to the standard procedure described above, with the following modifications. The basic fraction was evaporated to dryness after the addition of 250 ng (highest levels) or 10 ng (lower levels) of methaqualone dissolved in methanol as a chromatographic standard. To the residue 40 μl of acetone were added and the tube was vortex mixed for 15 s. Then 10 μl of BSTFA were added and after mixing the solution was transferred into a 100-μl

insert of an autosampler vial. The vial was sealed with a PTFE-lined septum and heated at 70°C for 30 mins, then 1.0 μl was injected splitless into the GC–MS system.

Recoveries were calculated by means of the peak areas with the ions of *m/z* 236.35 for morphine-di-TMS and *m/z* 235.2 for methaqualone, the so-called target ions. The qualifier ions of *m/z* 429.5 (for morphine-di-TMS) and 250.2 (for methaqualone) were used to confirm the identities of the substances. Only the chromatograms of extracts that showed satisfactory ion chromatograms of the qualifier ions were used for calculating the recovery of morphine. The criterion used was that the ratio of the peak areas of the target ion and the qualifier ion at the retention time of morphine-di-TMS should differ by not more than three times the standard deviation of the mean ratio obtained with the extracts of the spiked samples. An analogous criterion was used for the target/qualifier ratio of the internal standard.

The optimum conditions for the silylation were established as follows: a methanolic solution containing 0.5 μg of morphine and imipramine was evaporated to dryness in a silanized conical test tube. A 40-μl volume of acetone was added, followed by sonication for 5 s and then 10 μl of BSTFA were added. After vortex mixing, the mixture was transferred into an autosampler vial. The sealed vials were kept at temperatures of 40, 60, 80 and 100°C. The oven temperature was set isothermal at a temperature that separated morphine, morphine-di-TMS and the chromatographic standard (imipramine) within 10 min. The reaction can be monitored by injecting 1-μl aliquots into the GC–NPD system at 10-min intervals and calculating the ratio of the heights of the peaks of morphine-di-TMS and of the non-reacting imipramine.

3. Results and discussion

3.1. Standard procedure

The chromatograms of blank blood samples are very clean. The extracts of blank blood

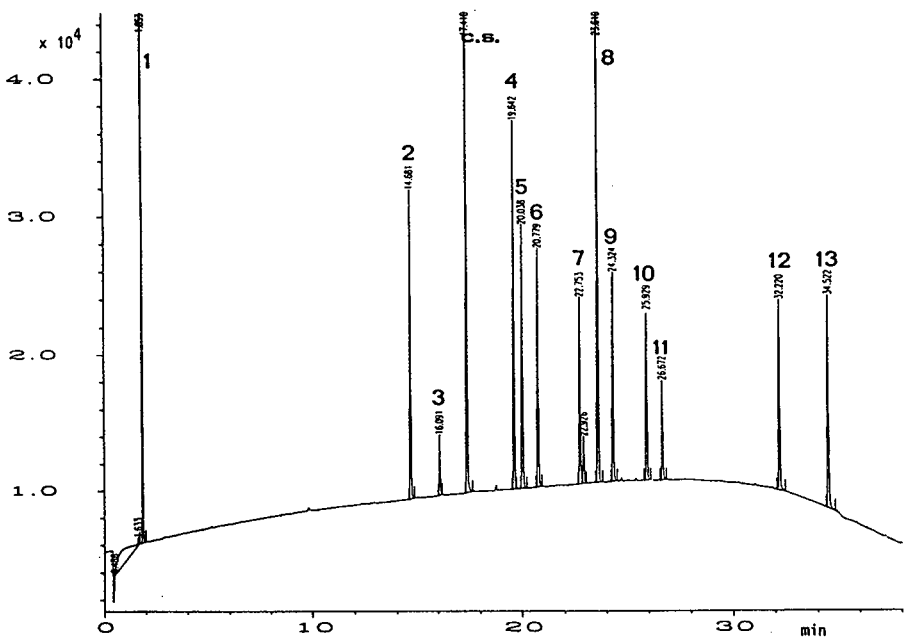

Fig. 1. GC–NPD of a solution in methanol containing the tested drugs and procaine. Volume injected, 1 μl containing 25 ng of each component. Peak numbers refer to the compounds in Table 1. C.S. = Chromatographic standard = procaine.

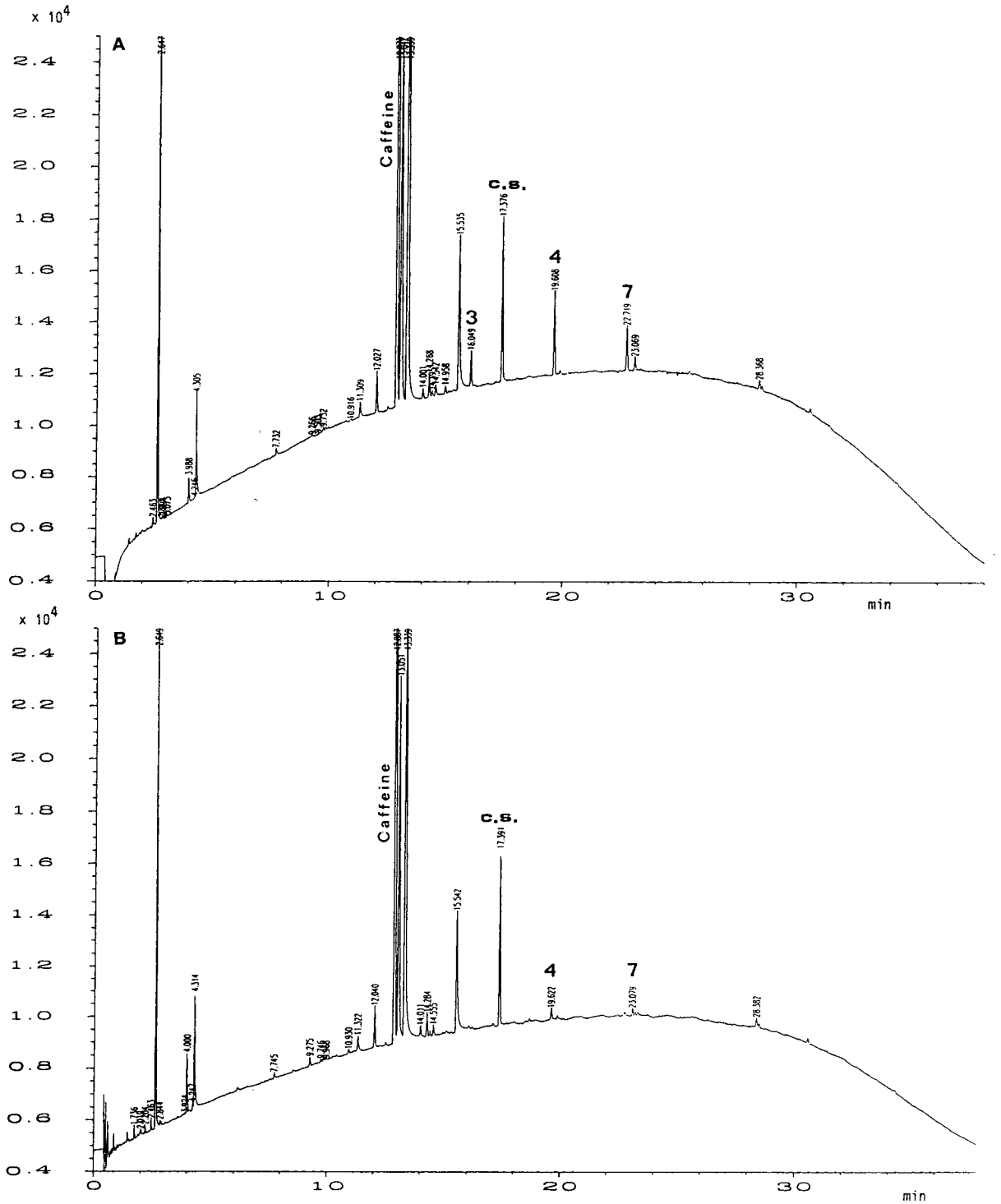

Fig. 2. GC–NPD of the acid–neutral fraction from whole blood spiked with (A) 500 ng of each drug and (B) 50 ng/ml. Peak numbers refer to the compounds in Table 1. C.S. = Chromatographic standard = procaine.

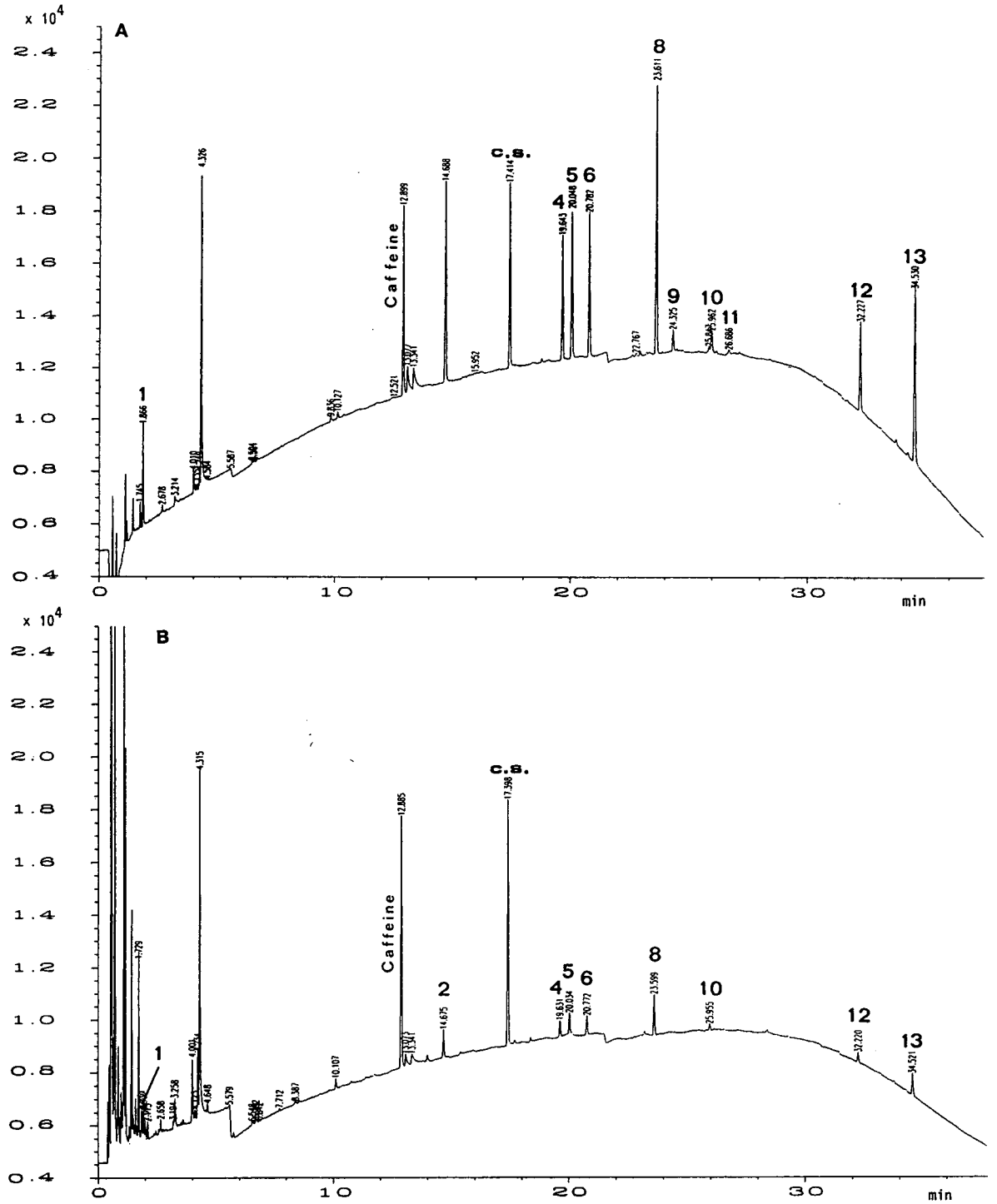

Fig. 3. GC–NPD of the basic fraction from whole blood spiked with (A) 500 ng of each drug and (B) 50 ng/ml. Peak numbers refer to the compounds in Table 1. C.S. = Chromatographic standard = procaine.

Table 1
Extraction recoveries (means of five determinations) of the drugs extracted from spiked whole blood

No.	Compound	500 ng/ml				250 ng/ml				100 ng/ml				50 ng/ml			
		A/N (%)	B (%)	Total (%)	R.S.D. (%)	A/N (%)	B (%)	Total (%)	R.S.D. (%)	A/N (%)	B (%)	Total (%)	R.S.D. (%)	A/N (%)	B (%)	Total (%)	R.S.D. (%)
1	Amphetamine	ND	47.9	47.9	10.8	ND	68.3	68.3	7.8	ND	72.1	72.1	10.2	ND	50.4	50.4	13.3
2	Difenhydramine	ND	80.8	80.8	2.3	ND	94.4	94.4	3.5	ND	88.4	88.4	1.7	ND	93.6	93.6	6.1
3	Phenobarbitone	138.2	ND	138.2	10.3	117.0	ND	117.0	15.8	–[a]	ND	–	–	–[a]	ND	–	–
4	Methaqualone	42.8	49.8	92.6	3.9	49.7	50.5	100.2	8.0	48.5	52.5	101.0	3.1	40.5	54.8	95.3	7.7
5	Methadone	ND	85.7	85.7	3.9	ND	95.8	95.8	3.0	ND	85.6	85.6	2.7	ND	89.5	89.5	6.6
6	Cocaine	ND	88.8	88.8	3.5	ND	95.1	95.1	3.1	ND	84.8	84.8	3.0	ND	88.5	88.5	5.9
7	Oxazepam	63.0	16.4	79.4	12.0	55.5	29.3	84.8	7.6	47.5	45.9	93.4	5.0	41.0	–[a]	41.0	5.5
8	Codeine	ND	83.9	83.9	4.6	ND	81.6	81.6	3.2	ND	86.4	86.4	4.4	ND	93.0	93.0	11.8
9	Morphine	ND	171.3	171.3	66.0	ND	56.3	56.3	26.8	ND	–[a]	–	–	ND	–[a]	–	–
10	6-Acetylmorphine	ND	57.6	57.6	33.3	ND	26.4	26.4	3.4	ND	58.3	58.3	16.6	ND	90.7	90.7	20.7
11	Nalorphine	ND	135.2	135.2	120.2	ND	–[a]	–	–	ND	–[a]	–	–	ND	–[a]	–	–
12	Haloperidol	ND	80.1	80.1	8.7	ND	98.3	98.3	4.3	ND	94.0	94.0	5.5	ND	112.4	112.4	8.1
13	Strychnine	ND	87.2	87.2	6.3	ND	114.6	114.6	3.0	ND	107.0	107.0	7.8	ND	103.3	103.3	11.1

A/N = Acid–neutral fraction; B = basic fraction; ND = not detected.
[a] Not detected owing to limiting chromatographic behaviour.

contain only the peaks not labelled with the numbers referring to the spiked substances in Figs. 2 and 3, except for caffeine, which is present in almost all blood samples. A chromatogram of a mixture of the pure drugs is shown in Fig. 1.

The extraction recoveries at the 500, 250, 100 and 50 ng/ml levels are given in Table 1. They are high, reproducible and independent of the concentration provided that the chromatographic behaviour (morphine), volatility (amphetamine) and stability (6-monoacetylmorphine, oxazepam) are not limiting in the determinations. Drugs for which the chromatographic behaviour is not the limiting factor in detection can be identified on the chromatogram when present in the blood at concentrations down to at least 50 ng/ml (Fig. 2) using GC–NPD.

Substances that exhibit problematic behaviour during GC (*e.g.*, morphine) cannot always be detected at toxicologically relevant concentrations using the standard procedure, *i.e.*, without derivatization.

The procedure is at least suitable for reliable qualitative analysis, as can be concluded from Table 1.

3.2. Recovery of morphine

The study of the extraction of morphine with derivatization clearly shows that the analytical technique rather than the SPE limits the detectability in the concentration range studied. The results are presented in Table 2.

Blank blood does not show significant interfering peaks down to at least 5 ng of morphine per millilitre of blood. Dissolution in acetone of the dry residue before adding BSTFA is necessary in order to obtain a sufficient reaction rate. Also, the BSTFA burden on the NPD bead is limited by the diluting effect of acetone. The conversion of morphine into the di-TMS derivative is complete after 30 min at 70°C. Benzoylecgonine is not detected with the GC–MS-SIM procedure.

Table 2
Recoveries (means of five determinations) of morphine by GC–MS after silylation

Concentration of morphine added (ng/ml)	Recovery (%)	R.S.D. (%)
500	95.4	6.6
50	74.8	10.8
10	86.2	12.0
5	92.4	11.5

Probably a more polar solvent is needed for elution. This is in accordance with the results of Wernly and Thormann [6], who prepared three fractions, including a further more polar basic fraction with a more polar eluent in which benzoylecgonine was detected. Addition of an extra fraction and/or replacement of the eluent with a more polar eluent for the basic fraction is currently being studied.

Case example

Fig. 4 shows the chromatogram of the basic fraction of a blood sample obtained at a forensic autopsy (suicide). After screening with the method described here, strychnine (at the lethal concentration of 6.0 mg/l), amitriptyline and nortriptyline (at a therapeutic concentration, 0.1 mg/l) were detected, quantified and confirmed using HPLC–scanning UV detection, as described previously [7].

4. Conclusions

Clean extracts are obtained by the procedure described without the need for back-extractions from the organic phase into acid or base and re-extraction into an organic phase.

High and reproducible recoveries, independent of the concentration in the range under study, make this method very suitable for qualitative and/or semi-quantitative applications.

The extracts do not contain much material that contaminates the injection port of the gas chromatograph, thus making this method very suitable for routine work.

Our experience with benzoylecgonine indicates that not all substances of potential interest are recovered. Further validation and fine-tuning of the procedure will be undertaken.

This relative simple solid-phase extraction method shows the potential and reliability of the

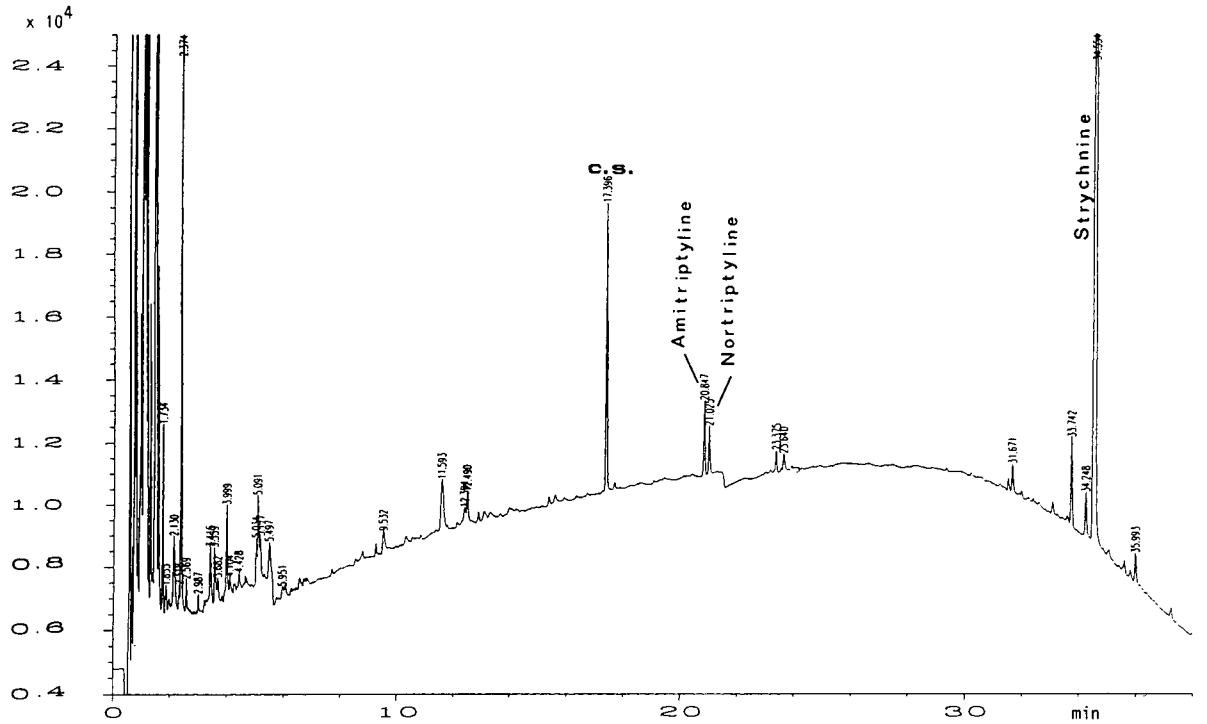

Fig. 4. GC–NPD of an extract of blood obtained at a forensic autopsy (suicide). Detected are strychnine (6 mg/l), amitriptyline and nortriptyline (each *ca.* 0.1 mg/l).

most elaborate comprehensive extraction procedures available today.

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JOURNAL OF CHROMATOGRAPHY A

Reversed-phase high-performance liquid chromatographic database of retention indices and UV spectra of toxicologically relevant substances and its interlaboratory use

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Abstract

An HPLC identification system based on a 1-nitroalkane retention index scale and secondary retention index standards is described. The retention index values and spectral data for 383 toxicologically relevant compounds (therapeutic and illicit drugs, environmental toxins and endogenous compounds) are given. The retention data may be directly applied in any laboratory using any reversed-phase, base-deactivated column, provided that the standardized procedure is observed.

1. Introduction

The identification of toxic compounds in biological material presents a challenging task for an analytical toxicologist and is of the utmost importance in clinical toxicology wards and in forensic institutes. From the clinical point of view, the identification of a toxic substance, which always precedes a proper determination, may influence the therapeutic means applied in the case of acute poisoning. On the other hand, the forensic relevance of the correct and unequivocal identification of a poison is obvious.

Toxicologists have developed several methodical approaches that are particularly useful for the identification of toxic substances, under the common name of "systematic toxicological analysis" (STA). A method applicable for STA should meet the following requirements: it should cover a broad range of relevant substances in one analytical run; it should be universally applicable and the data should be exchangeable between any laboratories; and it should use several uncorrelated identification parameters.

The STA Committee of the International Association of Forensic Toxicologists (TIAFT) has adapted and optimized several identification procedures that may fulfil the above requirements. So far chromatographic methods such as thin-layer chromatography (TLC) and gas chromatography (GC) have appeared especially useful for the identification of unknown toxic substances. TLC and GC procedures have been standardized and comprehensive, multi-laboratory databases for about 6000 compounds have been established [1–3]. Particularly valuable is the combination of mass spectrometry with standardized GC retention parameters, applied after standardized sample preparation [4].

High-performance liquid chromatography (HPLC), applied as an identification method, shows similar sensitivity and selectivity to GC.

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The advent of the diode-array detector brought a potentially very powerful identification system, combining retention parameters and UV spectra. It appeared, however, that the nominally identical column packings of various commercial origin show strikingly different selectivities and these differences were difficult to compensate through standardization. Therefore, a number of toxicologists have developed their own databases, but the exchange of data was hardly feasible owing to the use of non-standardized retention parameters [5–15].

In previous studies, we have developed a standardized HPLC identification system, based on retention parameters and UV spectra, recorded by means of a diode-array detector. The retention and spectral data for 225 toxicologically relevant substances have been published [16]. The retention was expressed on a 1-nitroalkane retention index scale, using selected drugs as secondary retention index standards. The evolution of this concept and its final form have been described elsewhere [17–22]. This system allows comparable results to be obtained using different reversed-phase columns, also among different laboratories [23,24].

The purpose of this study was to establish a database of toxicologically relevant substances (therapeutic and illicit drugs, pesticides and endogenous compounds), which may be used in various toxicological laboratories using different reversed-phase (octyl- or octadecylsilica) columns and the same, standardized procedure. The database can be expanded in the future, and the data may be exchanged.

2. Experimental

2.1. Standard substances

A range of 365 toxicologically relevant substances, obtained from various manufacturers in pure form, were diluted in acetonitrile–water (1:1) to a concentration of 0.1 mg/ml. Ten benzodiazepine derivatives were subjected to acid hydrolysis and extracted according to Schütz [25]. The hydrolysis products obtained were diluted in acetonitrile–water (1:1) to a concentration corresponding to 0.1 mg/ml of parent drug. The retention and spectral data of all pure substances were stored in the HPLC library.

2.2. Biological extracts

Serum and autopsy blood and liver samples were checked for the absence of drugs and extracted with diethyl ether at pH 4.6 or with dichlormethane–2-propanol (9:1) at pH 8.5, with and without an acid hydrolysis step. Retention and spectral data of three peaks found in acidic extracts and of five peaks found in basic extracts were included in the HPLC database. The detailed conditions and results of these experiments have been described elsewhere [26].

2.3. Chromatographic conditions and equipment

Gradient elution with a mixture of acetonitrile and triethylammonium phosphate buffer (25 m*M*, pH 3.0), as in our previous studies [16,24], was applied. Triethylammonium phosphate buffer (1 *M*, pH 3.0) was supplied by Fluka (Buchs, Switzerland) and diluted with HPLC-grade water before use. The gradient profile was 0–70% acetonitrile in 30 min and 5 min at 70% acetonitrile. The flow-rate was 1 ml/min and the injection volume was 5–10 μl. The equilibration time between two consecutive injections was 10 min.

All experiments were performed using a low-pressure gradient system, consisting of a Type L 6200 gradient pump, AS 2000 autosampler (both from Merck, Darmstadt, Germany) and a Type 990 diode-array detector (Waters, Eschborn, Germany). Three Superspher 100 RP-18, 4-μm particle size, fully end-capped columns (125 × 4 mm I.D.) were supplied by Merck. These columns were used throughout the study.

2.4. Standardization procedure and parameters collected

Retention times and UV spectra of all substances were stored as primary identification parameters. As secondary retention parameters,

Table 1
Retention indices of secondary retention index markers

Acidic mixture		Basic mixture	
Compound	*I*	Compound	*I*
Paracetamol	234	Morphine	198
Barbital	287	Chloroquine	265
Brallobarbital	359	Benzoylecgonine	295
Pentobarbital	405	Cocaine	336
Secobarbital	437	Diphenhydramine	385
Clobazam	484	Haloperidol	409
Indomethacine	610	Amitriptyline	446
Prazepam	648	Thioridazine	504
		Meclozine	601
		Amiodarone	762

the retention indices of all compounds were calculated from their retention times by linear interpolation between standard drugs, whose retention indices had been previously determined on the 1-nitroalkane scale [16]. Separate sets of standard drugs were used for acidic/neutral and basic drugs (Table 1, Figs. 1 and 2).

Standard drug mixtures were examined with every series of compounds examined. As a rule, the retention times of these standards were very stable for the same column. The calculated retention index values were stored in the library together with corresponding retention times and UV spectra. The HPLC software of the Type 990 detector allows a compound to be identified by means of the UV spectrum and selected retention parameter (retention time or retention index). All measurements were performed in duplicate, on different days.

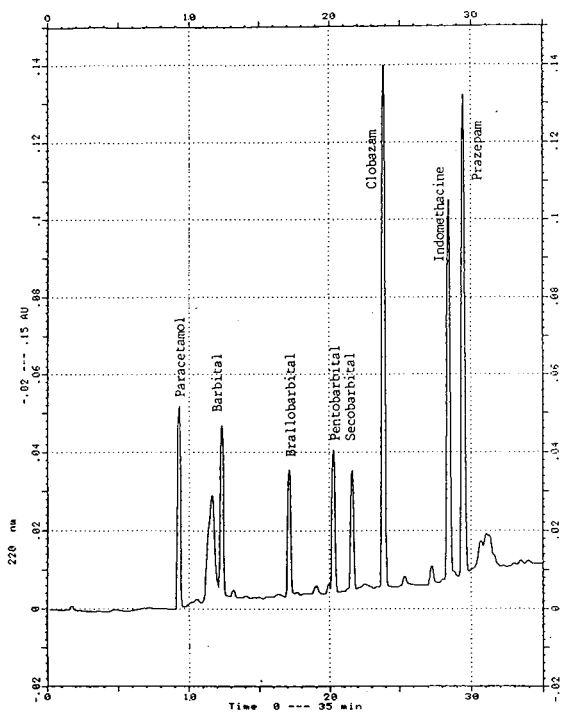

Fig. 1. Chromatograms of secondary retention index standards (acidic/neutral mixture).

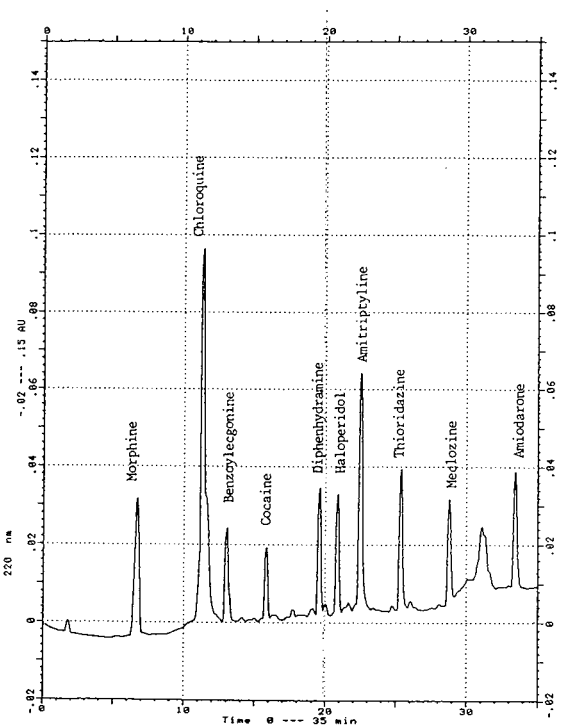

Fig. 2. Chromatograms of secondary retention index standards (basic mixture).

3. Results

Tables 2 and 3 give the data for all 383 substances examined. The absorbance values (A_1^1) given an insight into the prospective detectability of substances by means of a spectrophotometric detector. These data, and UV maxima, are given only for substances showing distinct absorption maxima under the applied conditions. Several compounds (*e.g.*, barbiturates) show only gradually decreasing UV absorbance. For these compounds no absorbance data are given. The last column gives information on whether the retention index values were calculated using acidic/neutral or basic drugs as secondary retention index standards. The collection of I values follows a normal distribution, showing the majority of results in the region between 300 and 500, *i.e.*, in a time span of *ca.* 13 min.

In a previous study [16], we examined the I values of 225 compounds under the same con-

Table 2
Substances examined, in alphabetical order, with their I values, UV maxima, A_1^1 values and scale used

No.	Substance	I	UV	A_1^1	Scale	No.	Substance	I	UV	A_1^1	Scale
1	ABP, 1st peak	461	327	0	ACN	37	Benzoylecgonine	295	274	30	BAS
2	ABP, 2nd peak	495	237	0	ACN	38	Benztropine	461	0	0	BAS
3	ACB	673	236	310	ACN	39	Betaxolol	371	275	76	BAS
4	Acebutolol	311	233	655	BAS	40	Bisacodyl	483	264	270	ACN
5	Acecarbromal	422	0	0	ACN	41	Brallobarbital	359	0	0	ACN
6	Acepromazine	399	278	570	BAS	42	Bromazepam	378	317	65	ACN
7	Acetanilide	316	239	815	ACN	43	Bromisoval	354	0	0	ACN
8	Acetazolamide	249	264	475	ACN	44	Bromophos-ethyl	800	0	0	ACN
9	ADB	704	233	0	ACN	45	Bromoxynil	464	255	380	ACN
10	Ajmaline	328	287	90	BAS	46	Brompheniramine	355	263	250	BAS
11	Alimemazine	437	252	0	BAS	47	Buflomedil	324	276	460	BAS
12	Alimemazine sulphoxide	334	338	0	BAS	48	Bupivacaine	355	0	0	BAS
13	Allobarbital	340	0	0	ACN	49	Bupranolol	373	276	78	BAS
14	Allopurinol	190	251	563	ACN	50	Buprenorphine	386	288	30	BAS
15	Alprazolam	443	276	938	ACN	51	Buspirone	353	238	552	BAS
16	Alprenolol	381	270	69	ACN	52	Butalbital	380	0	0	ACN
17	Amiloride	233	363	800	BAS	53	Butaperazine	496	278	540	BAS
18	Aminophenazone	243	257	443	ACN	54	Butobarbital	365	0	0	ACN
19	Amiodarone	762	240	1200	BAS	55	Caffeine	265	272	504	BAS
20	Amitriptyline	446	239	500	BAS	56	Camazepam	566	314	80	ACN
21	Amobarbital	415	0	0	ACN	57	Carazolol	354	332	438	BAS
22	Amoxapine	372	299	236	BAS	58	Carbamazepine	380	284	490	BAS
23	Amphetamine	241	257	14	BAS	59	Carbaryl	452	280	351	ACN
24	ANB	549	365	0	ACN	60	Carbromal	400	0	0	ACN
25	ANCB	580	356	0	ACN	61	Carbutamide	369	267	149	ACN
26	ANFB	559	357	0	ACN	62	Carpipramine	399	251	184	BAS
27	Aprobarbital	347	0	0	ACN	63	CCB	999	237	0	ACN
28	Ascorbic acid	90	244	556	ACN	64	CCMK	900	271	0	ACN
29	Aspirin	326	274	68	ACN	65	Chlordiazepoxide	357	306	316	ACN
30	Atenolol	224	275	48	BAS	66	Chloridazone	324	285	420	ACN
31	Atrazine	451	264	180	ACN	67	Chlormezanone	382	227	25	BAS
32	Atropine	287	259	17	BAS	68	Chloroquine	265	342	625	BAS
33	Barbital	287	0	0	ACN	69	8-Chlorotheophylline	295	267	604	BAS
34	Benperidol	371	278	230	BAS	70	Chlorpromazine	466	254	1030	BAS
35	Benzocaine	380	284	930	BAS	71	Chlorprothixene	476	267	420	BAS
36	Benzoctamine	370	271	57	BAS	72	Chlorpyrifos	745	288	155	ACN

Table 2 *(continued)*

No.	Substance	I	UV	A_1^1	Scale	No.	Substance	I	UV	A_1^1	Scale
73	Chlorthalidone	359	275	58	ACN	125	Doxylamine	274	263	335	BAS
74	Cimetidine	229	0	0	BAS	126	Droperidol	369	278	190	BAS
75	Cinchocaine	430	327	190	BAS	127	Edifenphos	660	0	0	ACN
76	Clobazam	484	293	75	ACN	128	Ephedrine	224	0	0	BAS
77	Clomethiazole	369	251	270	BAS	129	Estazolam	424	0	0	ACN
78	Clomipramine	471	254	250	BAS	130	Etenzamide	350	293	220	ACN
79	Clonazepam	451	309	350	ACN	131	Ethacrynic acid	516	276	100	ACN
80	Clonidine	237	269	20	BAS	132	Ethosuximide	284	0	0	ACN
81	Clopamide	356	240	346	ACN	133	Ethylmorphine	283	282	48	BAS
82	Clopenthixol	456	270	370	BAS	134	Etilefrine	183	273	100	BAS
83	Clorazepate	464	311	55	ACN	135	Etomidate	466	237	450	ACN
84	Clotiapine	397	299	174	BAS	136	Euphylline	270	271	399	BAS
85	Clotiazepam	551	243	580	ACN	137	Fenbufen	522	285	835	ACN
86	Clozapine	330	294	650	BAS	138	Fenethylline	325	273	240	BAS
87	Cocaine	336	275	30	BAS	139	Fenfluramine	364	264	22	BAS
88	Codeine	243	284	55	BAS	140	Fenoprofen	587	0	0	ACN
89	Colchicine	357	351	420	BAS	141	Fentanyl	377	0	0	BAS
90	Coumarin	368	315	607	ACN	142	Flecainide	410	298	294	BAS
91	Cyclobarbital	374	0	0	ACN	143	Fluanisone	398	244	550	BAS
92	Cyclopentobarbital	377	0	0	ACN	144	Flumazenil	362	245	914	ACN
93	DCFB	458	236	0	ACN	145	Flunarizine	571	252	599	BAS
94	Demeton-S-methyl	402	0	0	ACN	146	Flunitrazepam	459	309	330	ACN
95	Demeton-S-methylsulphone	660	0	0	ACN	147	Flunitrazepam, 3-hydroxy	418	311	0	ACN
96	Demoxepam	388	308	600	ACN	148	Flunitrazepam, 7-amino	336	236	0	ACN
97	Desalkylflurazepam	459	230	0	ACN	149	Flunitrazepam, nor	424	312	0	ACN
98	Desipramine	421	251	308	BAS	150	Fluoxetine	450	263	130	BAS
99	Dexchlopheniramine	342	263	0	BAS	151	Flupenthixol	487	267	294	BAS
100	Dextromethorphan	370	281	70	BAS	152	Fluphenazine	480	307	85	BAS
101	Dextromoramide	440	0	0	BAS	153	Flurazepam	392	309	50	ACN
102	Dextropropoxyphene	438	255	12	BAS	154	Fluvoxamine	412	252	340	BAS
103	1,4-Diaminobutane	80	0	0	BAS	155	Furosemide	414	275	600	ACN
104	1,5-Diaminopentane	90	0	0	BAS	156	Glafenine	322	345	467	ACN
105	Diamorphine	327	276	46	BAS	157	Glibenclamide	623	302	63	ACN
106	Diazepam	529	229	1100	ACN	158	Gliclazide	538	261	20	ACN
107	Diazinon	687	247	35	ACN	159	Glipizide	464	275	230	ACN
108	Diazoxide	345	264	436	ACN	160	Gliquidone	723	311	50	ACN
109	Dibenzepin	349	0	0	BAS	161	Glisoxepide	453	230	70	ACN
110	Diclofenac	630	275	320	ACN	162	Gluthetimide	430	0	0	ACN
111	Digoxin	389	0	0	BAS	163	Guaifenesine	307	275	140	ACN
112	Dihydrocodeine	237	281	47	BAS	164	Haloperidol	409	245	340	BAS
113	Dilthiazem	392	238	661	BAS	165	Harmane	301	299	0	BAS
114	Dimenhydrinate	100	276	268	BAS	166	Heptabarbital	404	0	0	ACN
115	Dimethindene	334	260	613	BAS	167	Hexobarbital	404	0	0	ACN
116	Dimethoate	336	0	0	ACN	168	Hydrochlorothiazide	275	270	640	ACN
117	Diphenhydramine	385	254	17	BAS	169	Hydrocodone	262	282	50	BAS
118	Dipyridamole	387	284	650	BAS	170	Hydromorphone	231	282	50	BAS
119	Disopyramide	327	261	195	BAS	171	Hydroxyzine	428	230	420	BAS
120	Disulfiram	730	279	680	BAS	172	Hyoscine butyl bromide	338	0	0	BAS
121	Disulfoton	752	0	0	ACN	173	Ibuprofen	650	0	0	ACN
122	Diuron	468	250	900	ACN	174	Idobutal	395	0	0	ACN
123	Dothiepin	428	260	260	BAS	175	Imipramine	434	251	300	BAS
124	Doxepin	401	294	230	BAS	176	Indole	448	270	0	BAS

(Continued on p. 102)

Table 2 *(continued)*

No.	Substance	I	UV	A_1^1	Scale	No.	Substance	I	UV	A_1^1	Scale
177	Indomethacin	610	317	180	ACN	230	Midazolam	386	250	360	ACN
178	Isoniazid	132	264	390	ACN	231	Minaprine	278	266	392	BAS
179	Isosorbide dinitrate	445	0	0	BAS	232	MNFB, 1st peak	525	230	0	ACN
180	Ketamine	294	270	25	BAS	233	MNFB, 2nd peak	630	231	0	ACN
181	Ketazolam	583	242	500	ACN	234	Molsidomine	272	296	318	BAS
182	Ketobemidone	295	273	80	BAS	235	6-Monoacetylmorphine	276	283	50	BAS
183	Ketotifene	365	299	480	BAS	236	Monolinuron	455	246	1130	ACN
184	Labetalol	350	303	100	BAS	237	Moperon	387	245	564	BAS
185	Levomepromazine	446	305	140	BAS	238	Morazone	344	261	200	BAS
186	Levorphanol	308	281	80	BAS	239	Morphine	198	285	50	BAS
187	Lidocaine	285	0	0	BAS	240	Nadolol	271	269	40	BAS
188	Linuron	559	249	900	ACN	241	Naftidrofuryl	460	282	141	BAS
189	Loprazolam	379	330	900	ACN	242	Nalorphine	237	284	50	BAS
190	Lorazepam	422	230	1100	ACN	243	Naloxone	251	282	40	BAS
191	Lormetazepam	474	229	1030	ACN	244	Naproxene	476	262	200	ACN
192	Loxapine	399	296	184	BAS	245	Nicotinamide	146	261	450	ACN
193	LSD	358	315	0	BAS	246	Nicotine	100	260	200	BAS
194	MACB	826	236	0	ACN	247	Nifedipine	503	275	0	ACN
195	MAFB	339	235	0	ACN	248	Niflumic acid	586	288	800	ACN
196	Matrotiline	440	0	0	BAS	249	Niketamide	280	260	280	ACN
197	MATRAC1	379	0	0	ACN	250	Nimodipine	641	237	0	ACN
198	MATRAC2	720	279	0	ACN	251	Nitrazepam	430	311	364	ACN
199	MATRAC3	404	323	0	ACN	252	Nitrendipine	610	236	1340	ACN
200	MATRBAS1	520	279	0	BAS	253	Nitrofurantoin	307	366	770	ACN
201	MATRBAS2	226	0	0	BAS	254	Nitroglycerine	531	0	0	ACN
202	MATRBAS3	344	258	0	BAS	255	Nizatidine	221	320	748	BAS
203	MATRBAS4	384	260	0	BAS	256	Nomifensine	346	237	250	BAS
204	MATRBAS5	270	279	0	BAS	257	Nordiazepam	464	230	1200	ACN
205	MDA	261	285	200	BAS	258	Norephedrine	190	0	0	BAS
206	MDB	750	265	0	ACN	259	Norfenefrine	80	273	171	BAS
207	MDE	244	285	200	BAS	260	Normethadone	417	0	0	BAS
208	MDMA	280	275	215	BAS	261	Norpethidine	339	0	0	BAS
209	Meclozine	601	228	390	BAS	262	Nortriptyline	418	239	898	BAS
210	Medazepam	395	254	860	ACN	263	Noscapine	354	312	85	BAS
211	Melperone	327	248	415	BAS	264	Opipramol	387	254	870	BAS
212	Mephenytoin	382	0	0	ACN	265	Orciprenaline	180	278	140	BAS
213	Mepivacaine	300	0	0	BAS	266	Orphenadrine	416	0	0	BAS
214	Mescaline	255	269	35	BAS	267	Oxazepam	441	228	1200	ACN
215	Metamizol	289	0	0	ACN	268	Oxazolam	340	240	698	ACN
216	Metapramine	358	270	181	BAS	269	Oxprenolol	332	0	0	BAS
217	Methadon	443	0	0	BAS	270	Oxycodone	260	279	40	BAS
218	Methamphetamine	255	255	15	BAS	271	Oxydemeton-S-methyl	269	0	0	ACN
219	Methaqualone	455	266	350	BAS	272	Oxyphenbutazone	503	237	1400	ACN
220	Methohexital	503	0	0	ACN	273	Papaverine	346	249	1800	BAS
221	Methomyl	270	234	0	ACN	274	Paracetamol	234	243	680	ACN
222	Methyl phenidate	316	0	0	BAS	275	Paraoxon	479	270	249	ACN
223	Methylphenobarbital	420	0	0	ACN	276	Parathion-methyl	621	275	238	ACN
224	Metipranolol	363	278	40	BAS	277	Paroxetin	385	294	0	BAS
225	Metoclopramide	308	309	420	BAS	278	Pecazine	440	252	900	BAS
226	Metolachlor	618	0	0	ACN	279	Pemoline	281	0	0	BAS
227	Metoprolol	317	273	50	BAS	280	Penfluridol	716	270	50	BAS
228	Metronidazole	236	320	520	ACN	281	Pentazocine	357	278	70	BAS
229	Mianserine	390	278	75	BAS	282	Pentobarbital	405	0	0	ACN

Table 2 *(continued)*

No.	Substance	*I*	UV	A_1^1	Scale	No.	Substance	*I*	UV	A_1^1	Scale
283	Pentoxifylline	320	275	314	BAS	334	Spironolactone	594	287	570	ACN
284	Perazine	418	252	900	BAS	335	Strychnine	292	254	370	BAS
285	Periciazine	405	269	780	BAS	336	Sulphadiazine	260	267	140	ACN
286	Perphenazine	438	255	800	BAS	337	Sulpiride	240	291	70	BAS
287	Pethidine	334	0	0	BAS	338	Sulthiam	321	246	390	ACN
288	Phenacemide	312	0	0	ACN	339	Suprofen	156	273	0	ACN
289	Phenacetin	356	246	650	ACN	340	Tebuconazole	607	0	0	ACN
290	Phenazone	303	256	450	ACN	341	Temazepam	466	228	1100	ACN
291	Phencyclidine	281	0	0	BAS	342	Terbutryn	494	260	370	ACN
292	Phenelzine	377	0	0	BAS	343	Terfenadine	567	0	0	BAS
293	Pheniramine	279	0	0	BAS	344	Tetracaine	381	314	600	BAS
294	Phenobarbital	357	0	0	ACN	345	Tetrazepam	538	228	1100	ACN
295	Phenprocoumon	609	310	450	ACN	346	Tetroxoprim	286	272	113	BAS
296	Phentermine	277	0	0	BAS	347	Δ^9-THC	999	279	40	ACN
297	Phenylbutazone	651	237	450	ACN	348	Thebaine	324	285	250	BAS
298	1-Phenylethylamine	199	257	0	BAS	349	Theobromine	224	272	560	BAS
299	2-Phenylethylamine	198	257	0	BAS	350	Theophylline	239	270	540	BAS
300	Phenytoin	415	0	0	ACN	351	Thiabendazole	283	302	1120	ACN
301	Pindolol	277	263	300	BAS	352	Thiopental	481	285	790	ACN
302	Pipamperone	286	248	315	BAS	353	Thioridazine	504	261	1000	BAS
303	Piracetam	178	0	0	ACN	354	Tiaprofenic acid	475	306	570	ACN
304	Piribedil	301	286	203	BAS	355	Tilidine	337	0	0	BAS
305	Piritramid	395	0	0	BAS	356	Timolol	297	297	279	BAS
306	Piroxicam	425	363	420	ACN	357	Tocainide	251	0	0	ACN
307	Prajmaline	390	246	198	BAS	358	Tolbutamide	470	228	581	ACN
308	Prazepam	648	227	1800	ACN	359	Tramadol	314	270	70	BAS
309	Primidone	308	0	0	ACN	360	Trancylpromine	241	261	20	BAS
310	Probenecid	560	249	319	ACN	361	Trazodon	358	249	320	BAS
311	Procainamide	202	279	700	ACN	362	Triamterene	291	359	860	BAS
312	Procaine	236	291	950	BAS	363	Triazolam	452	220	1100	ACN
313	Prochlorperazine	462	257	880	BAS	364	Trichlorfon	800	0	0	ACN
314	Promazine	418	252	1060	BAS	365	Trichlormethiazide	377	267	466	ACN
315	Promethazine	411	249	1050	BAS	366	Trifluoperazine	491	255	750	BAS
316	Propafenone	408	304	107	BAS	367	Trifluperidol 1	350	257	308	BAS
317	Propoxur 1	540	264	84	ACN	368	Trifluperidol 2	436	246	0	BAS
318	Propoxur 2	624	264	84	ACN	369	Triflupromazine	505	257	992	BAS
319	Propanolol	370	290	220	BAS	370	Trifluralin	761	274	248	ACN
320	Propyphenazone	422	272	400	ACN	371	Trimethoprim	345	270	220	ACN
321	Protriptyline	424	290	530	BAS	372	Trimipramine	451	249	300	BAS
322	Proxibarbal	255	0	0	ACN	373	Tripelenamine	337	311	250	BAS
323	Quinidine	316	245	950	BAS	374	Tripolidine	360	280	292	BAS
324	Quinine	308	246	950	BAS	375	Tryptamine	233	278	350	BAS
325	Ranitidine	240	317	500	BAS	376	Tyramine	124	275	110	BAS
326	Reserpine	473	294	200	BAS	377	Verapamil	454	278	130	BAS
327	Saccharin	254	269	80	ACN	378	Viloxazine	321	272	90	BAS
328	Salicylamide	305	299	270	ACN	379	Vinylbarbital	410	0	0	ACN
329	Salicylic acid	331	299	270	ACN	380	Warfarin	555	305	450	ACN
330	Scopolamine	288	0	0	BAS	381	Yohimbine	333	273	220	BAS
331	Secutobarbital	365	0	0	ACN	382	Zolpidem	343	296	482	BAS
332	Secobarbital	437	0	0	ACN	383	Zopiclon	314	304	177	BAS
333	Sotalol	273	270	16	BAS						

Table 3
Substances examined, in order of elution, with their I values, UV maxima, A_1^1 values and scale used

No.	Substance	I	UV	A_1^1	Scale	No.	Substance	I	UV	A_1^1	Scale
1	Norfenefrine	80	273	171	BAS	53	Hydrocodone	262	282	50	BAS
2	1,4-Diaminobutane	80	0	0	BAS	54	Caffeine	265	272	504	BAS
3	Ascorbic acid	90	244	556	ACN	55	Chloroquine	265	342	625	BAS
4	1,5-Diaminopentane	90	0	0	BAS	56	Oxydemeton-S-methyl	269	0	0	ACN
5	Nicotine	100	260	200	BAS	57	Methomyl	270	234	0	ACN
6	Dimenhydrinate	100	276	268	BAS	58	Euphylline	270	271	399	BAS
7	Tyramine	124	275	110	BAS	59	MATRBAS5	270	279	0	BAS
8	Isoniazid	132	264	390	ACN	60	Nadolol	271	269	40	BAS
9	Nicotinamide	146	261	450	ACN	61	Molsidomine	272	296	318	BAS
10	Suprofen	156	273	0	ACN	62	Sotalol	273	270	16	BAS
11	Piracetam	178	0	0	ACN	63	Doxylamine	274	263	335	BAS
12	Orciprenaline	180	278	140	BAS	64	Hydrochlorothiazide	275	270	640	ACN
13	Etilefrine	183	273	100	BAS	65	6-Monoacetylmorphine	276	283	50	BAS
14	Norephedrine	190	0	0	BAS	66	Phentermine	277	0	0	BAS
15	Allopurinol	190	251	563	ACN	67	Pindolol	277	263	300	BAS
16	2-Phenylethylamine	198	257	0	BAS	68	Minaprine	278	266	392	BAS
17	Morphine	198	285	50	BAS	69	Pheniramine	279	0	0	BAS
18	1-Phenylethylamine	199	257	0	BAS	70	MDMA	280	275	215	BAS
19	Procainamide	202	279	700	ACN	71	Nikethamide	280	260	280	ACN
20	Nizatidine	221	320	748	BAS	72	Phencyclidine	281	0	0	BAS
21	Ephedrine	224	0	0	BAS	73	Pemoline	281	0	0	BAS
22	Theobromine	224	272	560	BAS	74	Thiabendazole	283	302	1120	ACN
23	Atenolol	224	275	48	BAS	75	Ethylmorphine	283	282	48	BAS
24	MATRBAS2	226	0	0	BAS	76	Ethosuximide	284	0	0	ACN
25	Cimetidine	229	0	0	BAS	77	Lidocaine	285	0	0	BAS
26	Hydromorphone	231	282	50	BAS	78	Tetroxoprim	286	272	113	BAS
27	Amiloride	233	363	800	BAS	79	Pipamperone	286	248	315	BAS
28	Tryptamine	233	278	350	BAS	80	Barbital	287	0	0	ACN
29	Paracetamol	234	243	680	ACN	81	Atropine	287	259	17	BAS
30	Procaine	236	291	950	BAS	82	Scopolamine	288	0	0	BAS
31	Metronidazole	236	320	520	ACN	83	Metamizol	289	0	0	ACN
32	Dihydrocodeine	237	281	47	BAS	84	Triamterene	291	359	860	BAS
33	Clonidine	237	269	20	BAS	85	Strychnine	292	254	370	BAS
34	Nalorphine	237	284	50	BAS	86	Ketamine	294	270	25	BAS
35	Theophylline	239	270	540	BAS	87	8-Chlorotheophylline	295	267	604	BAS
36	Ranitidine	240	317	500	BAS	88	Ketobemidone	295	273	80	BAS
37	Sulpiride	240	291	70	BAS	89	Benzoylecgonine	295	274	30	BAS
38	Amphetamine	241	257	14	BAS	90	Timolol	297	297	279	BAS
39	Trancylpromine	241	261	20	BAS	91	Mepivacaine	300	0	0	BAS
40	Codeine	243	284	55	BAS	92	Piribedil	301	286	203	BAS
41	Aminophenazone	243	257	443	ACN	93	Harmane	301	299	0	BAS
42	MDE	244	285	200	BAS	94	Phenazone	303	256	450	ACN
43	Acetazolamide	249	264	475	ACN	95	Salicylamide	305	299	270	ACN
44	Naloxone	251	282	40	BAS	96	Nitrofurantoin	307	366	770	ACN
45	Tocainide	251	0	0	ACN	97	Guaifenesine	307	275	140	ACN
46	Saccharin	254	269	80	ACN	98	Quinine	308	246	950	BAS
47	Methamphetamine	255	255	15	BAS	99	Levorphanol	308	281	80	BAS
48	Proxibarbal	255	0	0	ACN	100	Metoclopramide	308	309	420	BAS
49	Mescaline	255	269	35	BAS	101	Primidone	308	0	0	ACN
50	Sulphadiazine	260	267	140	ACN	102	Acebutolol	311	233	655	BAS
51	Oxycodone	260	279	40	BAS	103	Phenacemide	312	0	0	ACN
52	MDA	261	285	200	BAS	104	Tramadol	314	270	70	BAS

Table 3 *(continued)*

No.	Substance	*I*	UV	A_1^1	Scale	No.	Substance	*I*	UV	A_1^1	Scale
105	Zopiclon	314	304	177	BAS	157	Brompheniramine	355	263	250	BAS
106	Acetanilide	316	239	815	ACN	158	Bupivacaine	355	0	0	BAS
107	Methyl phenidate	316	0	0	BAS	159	Phenacetin	356	246	650	ACN
108	Quinidine	316	245	950	BAS	160	Clopamide	356	240	346	ACN
109	Metoprolol	317	273	50	BAS	161	Phenobarbital	357	0	0	ACN
110	Pentoxifylline	320	275	314	BAS	162	Colchicine	357	351	420	BAS
111	Viloxazine	321	272	90	BAS	163	Chlordiazepoxide	357	306	316	ACN
112	Sulthiam	321	246	390	ACN	164	Pentazocine	357	278	70	BAS
113	Glafenine	322	345	467	ACN	165	LSD	358	315	0	BAS
114	Thebaine	324	285	250	BAS	166	Metapramine	358	270	181	BAS
115	Chloridazone	324	285	420	ACN	167	Trazodon	358	249	320	BAS
116	Buflomedil	324	276	460	BAS	168	Brallobarbital	359	0	0	ACN
117	Fenethylline	325	273	240	BAS	169	Chlorthalidone	359	275	58	ACN
118	Aspirin	326	274	68	ACN	170	Tripolidine	360	280	292	BAS
119	Disopyramide	327	261	195	BAS	171	Flumazenil	362	245	914	ACN
120	Melperone	327	248	415	BAS	172	Metipranolol	363	278	40	BAS
121	Diamorphine	327	276	46	BAS	173	Fenfluramine	364	264	22	BAS
122	Ajmaline	328	287	90	BAS	174	Secbutobarbital	365	0	0	ACN
123	Clozapine	330	294	650	BAS	175	Ketotifene	365	299	480	BAS
124	Salicylic acid	331	299	270	ACN	176	Butobarbital	365	0	0	ACN
125	Oxprenolol	332	0	0	BAS	177	Coumarin	368	315	607	ACN
126	Yohimbine	333	273	220	BAS	178	Droperidol	369	278	190	BAS
127	Pethidine	334	0	0	BAS	179	Carbutamide	369	267	149	ACN
128	Alimemazine sulphoxide	334	338	0	BAS	180	Clomethiazole	369	251	270	BAS
129	Dimethindene	334	260	613	BAS	181	Dextromethorphan	370	281	70	BAS
130	Flunitrazepam, 7-amino	336	236	0	ACN	182	Benzoctamine	370	271	57	BAS
131	Cocaine	336	275	30	BAS	183	Propranolol	370	290	220	BAS
132	Dimethoate	336	0	0	ACN	184	Benperidol	371	278	230	BAS
133	Tripelemaine	337	311	250	BAS	185	Betaxolol	371	275	76	BAS
134	Tilidine	337	0	0	BAS	186	Amoxapine	372	299	236	BAS
135	Hyoscine butyl bromide	338	0	0	BAS	187	Bupranolol	373	276	78	BAS
136	Norpethidine	339	0	0	BAS	188	Cyclobarbital	374	0	0	ACN
137	MAFB	339	235	0	ACN	189	Trichlormethiazide	377	267	466	ACN
138	Allobarbital	340	0	0	ACN	190	Phenelzine	377	0	0	BAS
139	Oxazolam	340	240	698	ACN	191	Fentanyl	377	0	0	BAS
140	Dexchlopheniramine	342	263	0	BAS	192	Cyclopentobarbital	377	0	0	ACN
141	Zolpidem	343	296	482	BAS	193	Bromazepam	378	317	65	ACN
142	MATRBAS3	344	258	0	BAS	194	Loprazolam	379	330	900	ACN
143	Morazone	344	261	200	BAS	195	MATRAC1	379	0	0	ACN
144	Diazoxide	345	264	436	ACN	196	Butalbital	380	0	0	ACN
145	Trimethoprim	345	270	220	ACN	197	Carbamazepine	380	284	490	BAS
146	Nomifensine	346	237	250	BAS	198	Benzocaine	380	284	930	BAS
147	Papaverine	346	249	1800	BAS	199	Tetracaine	381	314	600	BAS
148	Aprobarbital	347	0	0	ACN	200	Alprenolol	381	270	69	ACN
149	Dibenzepin	349	0	0	BAS	201	Mephenytoin	382	0	0	ACN
150	Labetalol	350	303	100	BAS	202	Chlormezanone	382	227	25	BAS
151	Trifluperidol 1	350	257	308	BAS	203	MATRBAS4	384	260	0	BAS
152	Etenzamide	350	293	220	ACN	204	Diphenhydramine	385	254	17	BAS
153	Buspirone	353	238	552	BAS	205	Paroxetin	385	294	0	BAS
154	Bromisoval	354	0	0	ACN	206	Midazolam	386	250	360	ACN
155	Carazolol	354	332	438	BAS	207	Buprenorphine	386	288	30	BAS
156	Noscapine	354	312	85	BAS	208	Moperon	387	245	564	BAS

(Continued on p. 106)

Table 3 *(continued)*

No.	Substance	*I*	UV	A_1^1	Scale	No.	Substance	*I*	UV	A_1^1	Scale
209	Opipramol	387	254	870	BAS	262	Imipramine	434	251	300	BAS
210	Dipyridamole	387	284	650	BAS	263	Trifluperidol 2	436	246	0	BAS
211	Demoxepam	388	308	600	ACN	264	Secobarbital	437	0	0	ACN
212	Digoxin	389	0	0	BAS	265	Alimemazine	437	252	0	BAS
213	Mianserine	390	278	75	BAS	266	Perphenazine	438	255	800	BAS
214	Projmaline	390	246	198	BAS	267	Dextropropoxyphene	438	255	12	BAS
215	Dilthiazem	392	238	661	BAS	268	Pecazine	440	252	900	BAS
216	Flurazepam	392	309	50	ACN	269	Dextromoramide	440	0	0	BAS
217	Medazepam	395	254	860	ACN	270	Maprotiline	440	0	0	BAS
218	Idobutal	395	0	0	ACN	271	Oxazepam	441	228	1200	ACN
219	Piritramid	395	0	0	BAS	272	Methadon	443	0	0	BAS
220	Clotiapine	397	299	174	BAS	273	Alprazolam	443	276	938	ACN
221	Fluanisone	398	244	550	BAS	274	Isosorbide dinitrate	445	0	0	BAS
222	Carpipramine	399	251	184	BAS	275	Amitriptyline	446	239	500	BAS
223	Acepromazine	399	278	570	BAS	276	Levomepromazine	446	305	140	BAS
224	Loxapine	399	296	184	BAS	277	Indole	448	270	0	BAS
225	Carbromal	400	0	0	ACN	278	Fluoxetine	450	263	130	BAS
226	Doxepin	401	294	230	BAS	279	Atrazine	451	264	180	ACN
227	Demeton-S-methyl	402	0	0	ACN	280	Clonazepam	451	309	350	ACN
228	Hexobarbital	404	0	0	ACN	281	Trimipramine	451	249	300	BAS
229	Heptabarbital	404	0	0	ACN	282	Triazolam	452	220	1100	ACN
230	MATRAC3	404	323	0	ACN	283	Carbaryl	452	280	351	ACN
231	Periciazine	405	269	780	BAS	284	Glisoxepide	453	230	70	ACN
232	Pentobarbital	405	0	0	ACN	285	Verapamil	454	278	130	BAS
233	Propafenone	408	304	107	BAS	286	Monolinuron	455	246	1130	ACN
234	Haloperidol	409	245	340	BAS	287	Methaqualone	455	266	350	BAS
235	Flecainide	410	298	294	BAS	288	Clopenthixol	456	270	370	BAS
236	Vinylbarbital	410	0	0	ACN	289	DCFB	458	236	0	ACN
237	Promethazine	411	249	1050	BAS	290	Flunitrazepam	459	309	330	ACN
238	Fluvoxamine	412	252	340	BAS	291	Desalkylflurazepam	459	230	0	ACN
239	Furosemide	414	275	600	ACN	292	Naftidrofuryl	460	282	141	BAS
240	Amobarbital	415	0	0	ACN	293	ABP, 1st peak	461	327	0	ACN
241	Phenytoin	415	0	0	ACN	294	Benztropine	461	0	0	BAS
242	Orphenadrine	416	0	0	BAS	295	Prochlorperazine	462	257	880	BAS
243	Normethadone	417	0	0	BAS	296	Clorazepate	464	311	55	ACN
244	Promazine	418	252	1060	BAS	297	Bromoxynil	464	255	380	ACN
245	Flunitrazepam, 3-hydroxy	418	311	0	ACN	298	Glipizide	464	275	230	ACN
246	Perazine	418	252	900	BAS	299	Nordiazepam	464	230	1200	ACN
247	Nortriptyline	418	239	989	BAS	300	Etomidate	466	237	450	ACN
248	Methylphenobarbital	420	0	0	ACN	301	Chlorpromazine	466	254	1030	BAS
249	Desipramine	421	251	308	BAS	302	Temazepam	466	228	1100	ACN
250	Propyphenazone	422	272	400	ACN	303	Diuron	468	250	900	ACN
251	Lorazepam	422	230	1100	ACN	304	Tolbutamide	470	228	581	ACN
252	Acecarbromal	422	0	0	ACN	305	Clomipramine	471	254	250	BAS
253	Protriptyline	424	290	530	BAS	306	Reserpine	473	294	200	BAS
254	Estrazolam	424	0	0	ACN	307	Lormetazepam	474	229	1030	ACN
255	Flunitrazepam, nor	424	312	0	ACN	308	Tiaprofenic acid	475	306	570	ACN
256	Piroxicam	425	363	420	ACN	309	Chlorprothixene	476	267	420	BAS
257	Hydroxyzine	428	230	420	BAS	310	Naproxene	476	262	200	ACN
258	Dothiepin	428	260	260	BAS	311	Paraoxon	479	270	249	ACN
259	Cinchocaine	430	327	190	BAS	312	Fluphenazine	480	307	85	BAS
260	Nitrazepam	430	311	364	ACN	313	Thiopental	481	285	790	ACN
261	Gluthetimide	430	0	0	ACN	314	Bisacodyl	483	264	270	ACN

Table 3 *(continued)*

No.	Substance	I	UV	A_1^1	Scale	No.	Substance	I	UV	A_1^1	Scale
315	Clobazam	484	293	75	ACN	350	Tebuconazole	607	0	0	ACN
316	Flupenthixol	487	267	294	BAS	351	Phenprocoumon	609	310	450	ACN
317	Trifluoperazine	491	255	750	BAS	352	Nitrendipine	610	236	1340	ACN
318	Terbutryn	494	260	370	ACN	353	Indomethacin	610	317	180	ACN
319	ABP, 2nd peak	495	237	0	ACN	354	Metolachlor	618	0	0	ACN
320	Butaperazine	496	278	540	BAS	355	Parathion-methyl	621	275	238	ACN
321	Oxyphenbutazone	503	237	1400	ACN	356	Glibenclamide	623	302	63	ACN
322	Nifedipine	503	275	0	ACN	357	Propoxur 2	624	264	84	ACN
323	Methohexital	503	0	0	ACN	358	Diclofenac	630	275	320	ACN
324	Thioridazine	504	261	1000	BAS	359	MNFB 2nd peak	630	231	0	ACN
325	Triflupromazine	505	257	992	BAS	360	Nimodipine	641	237	0	ACN
326	Ethacrynic acid	516	276	100	ACN	361	Prazepam	648	227	1800	ACN
327	MATRBAS1	520	279	0	BAS	362	Ibuprofen	650	0	0	ACN
328	Fenbufen	522	285	835	ACN	363	Phenylbutazone	651	237	450	ACN
329	MNFB, 1st peak	525	230	0	ACN	364	Demeton-S-methylsulphone	660	0	0	ACN
330	Diazepam	529	229	1100	ACN	365	Edifenphos	660	0	0	ACN
331	Nitroglycerine	531	0	0	ACN	366	ACB	673	236	310	ACN
332	Tetrazepam	538	228	1100	ACN	367	Diazinon	687	247	35	ACN
333	Gliclazide	538	261	20	ACN	368	ADB	704	233	0	ACN
334	Propoxur 1	540	264	84	ACN	369	Penfluridol	716	270	50	BAS
335	ANB	549	365	0	ACN	370	MATRAC2	720	279	0	ACN
336	Clotiazepam	551	243	580	ACN	371	Gliquidone	723	311	50	ACN
337	Warfarin	555	305	450	ACN	372	Disulfiram	730	279	680	BAS
338	ANFB	559	357	0	ACN	373	Chlorpyrifos	745	288	155	ACN
339	Linuron	559	249	900	ACN	374	MDB	750	265	0	ACN
340	Probenecid	560	249	319	ACN	375	Disulfoton	752	0	0	ACN
341	Camazepam	566	314	80	ACN	376	Trifluralin	761	274	248	ACN
342	Terfenadine	567	0	0	BAS	377	Amiodarone	762	240	1200	BAS
343	Flunarizine	571	252	599	BAS	378	Bromophos-ethyl	800	0	0	ACN
344	ANCB	580	356	0	ACN	379	Trichlorfon	800	0	0	ACN
345	Ketazolam	583	242	500	ACN	380	MACB	826	236	0	ACN
346	Niflumic acid	586	288	800	ACN	381	CCMK	900	271	0	ACN
347	Fenoprofen	587	0	0	ACN	382	Δ^9-THC	999	279	40	ACN
348	Spironolactone	594	287	570	ACN	383	CCB	999	237	0	ACN
349	Meclozine	601	228	390	BAS						

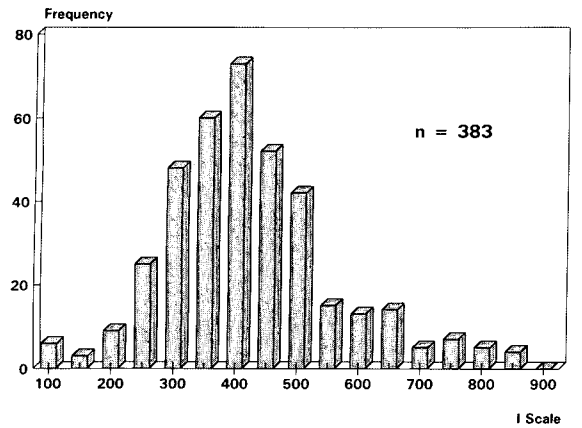

Fig. 3. Distribution of retention index values of all substances in the HPLC database.

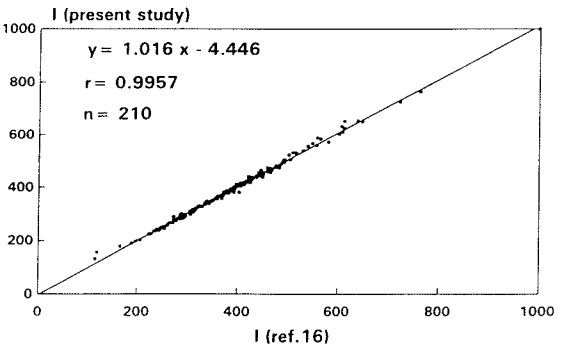

Fig. 4. Correlation of retention index values of 210 compounds obtained in two laboratories under standardized conditions.

ditions and using the same make of column, but in another laboratory and by means of different instrumentation; 210 of these substances were also examined in this study. This gave an opportunity to compare the I values, which were very consistent. The differences between the I values obtained for the same substances in two laboratories were, as a rule, less than 10 units. Similar reproducibility was observed for duplicate measurements in a single laboratory. The correlation graph of these data is shown in Fig. 3.

The UV spectra for 190 selected drugs are shown in Figs. 4 and 5. The reason for present-

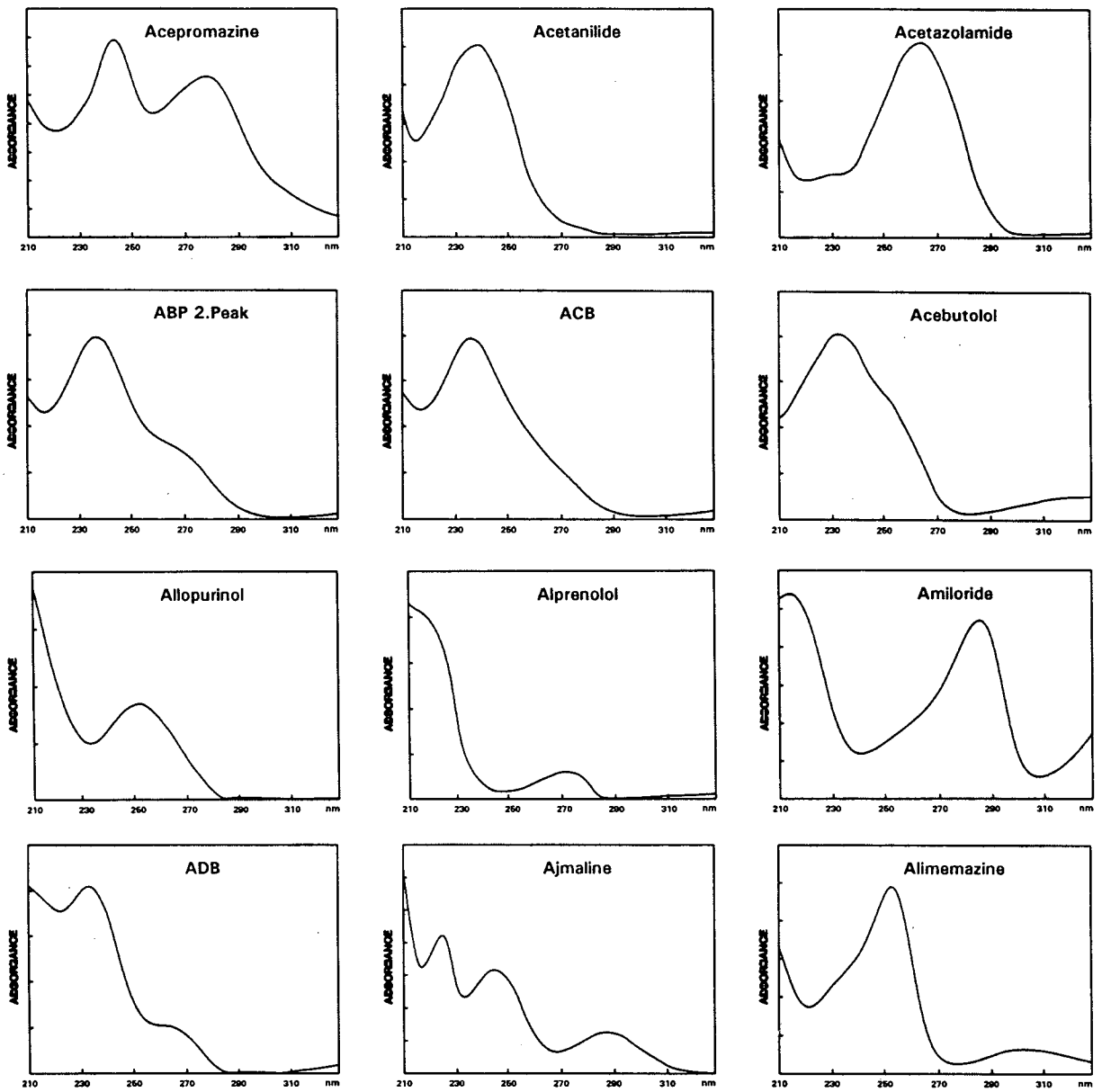

Fig. 5. UV spectra of selected drugs recorded during chromatographic runs and stored in the HPLC database.

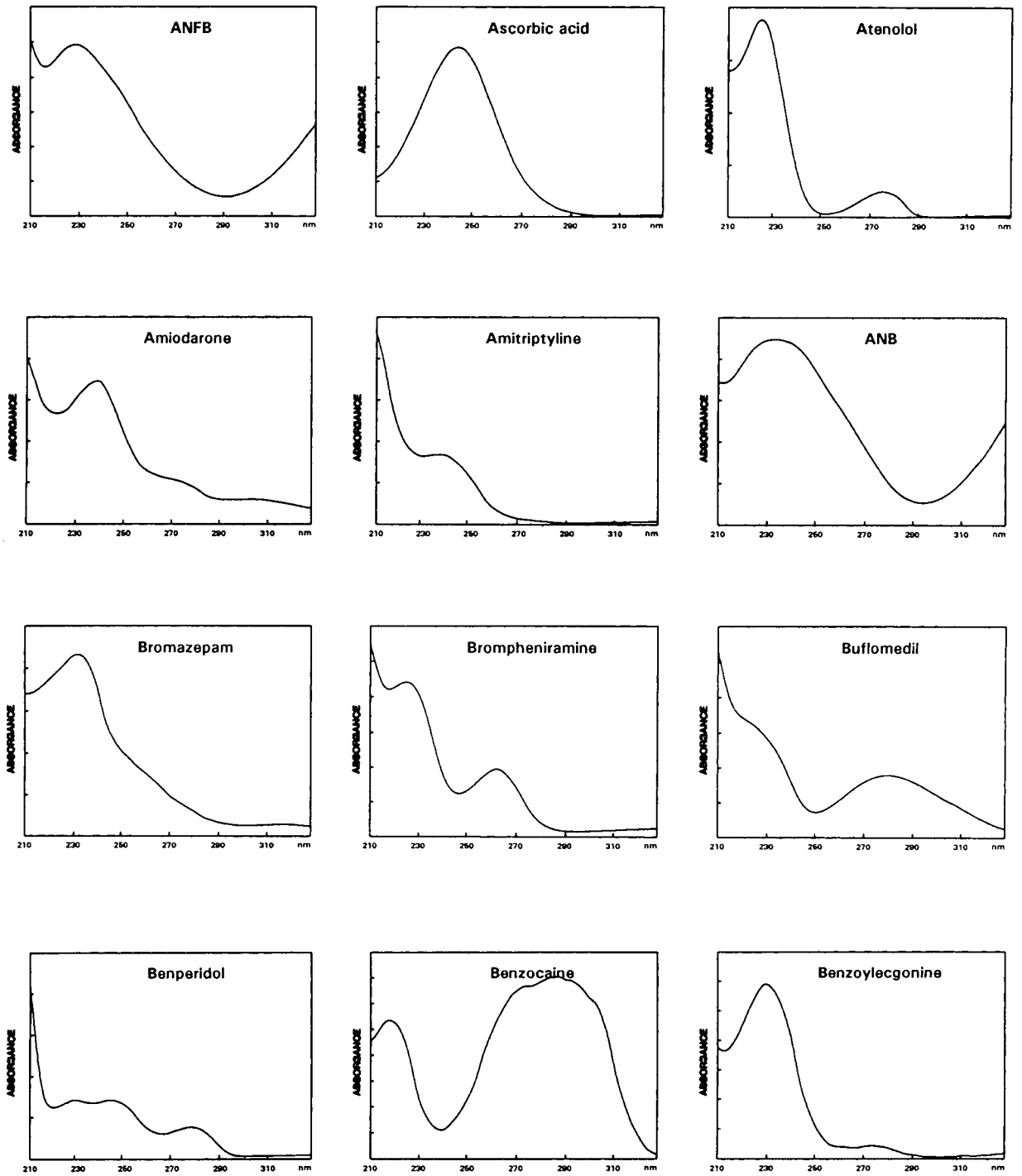

Fig. 5. *(Continued on p. 110)*

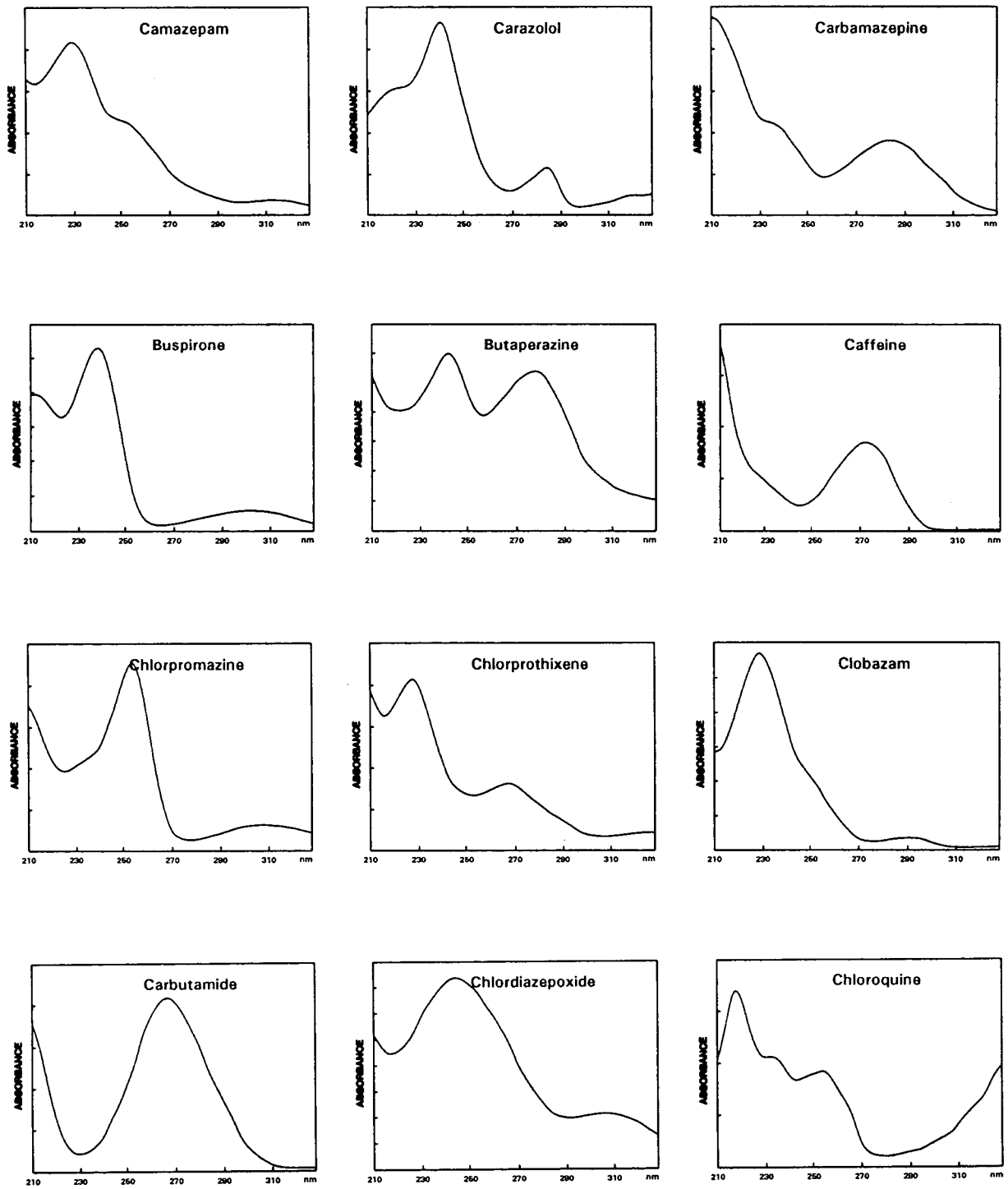
Camazepam
Carazolol
Carbamazepine
Buspirone
Butaperazine
Caffeine
Chlorpromazine
Chlorprothixene
Clobazam
Carbutamide
Chlordiazepoxide
Chloroquine
ABSORBANCE
210 230 250 270 290 310 nm

Fig. 5.

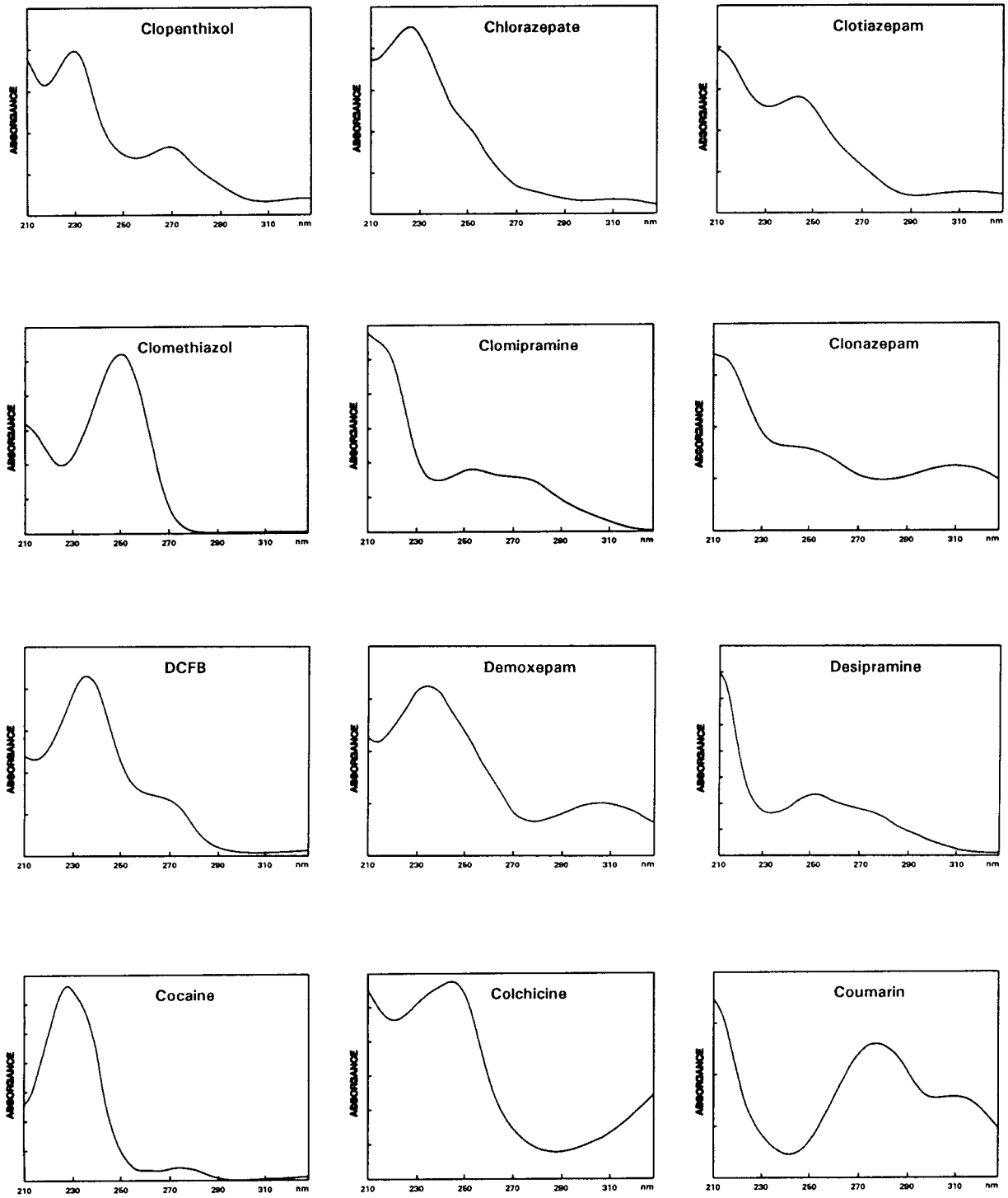

Fig. 5. *(Continued on p. 112)*

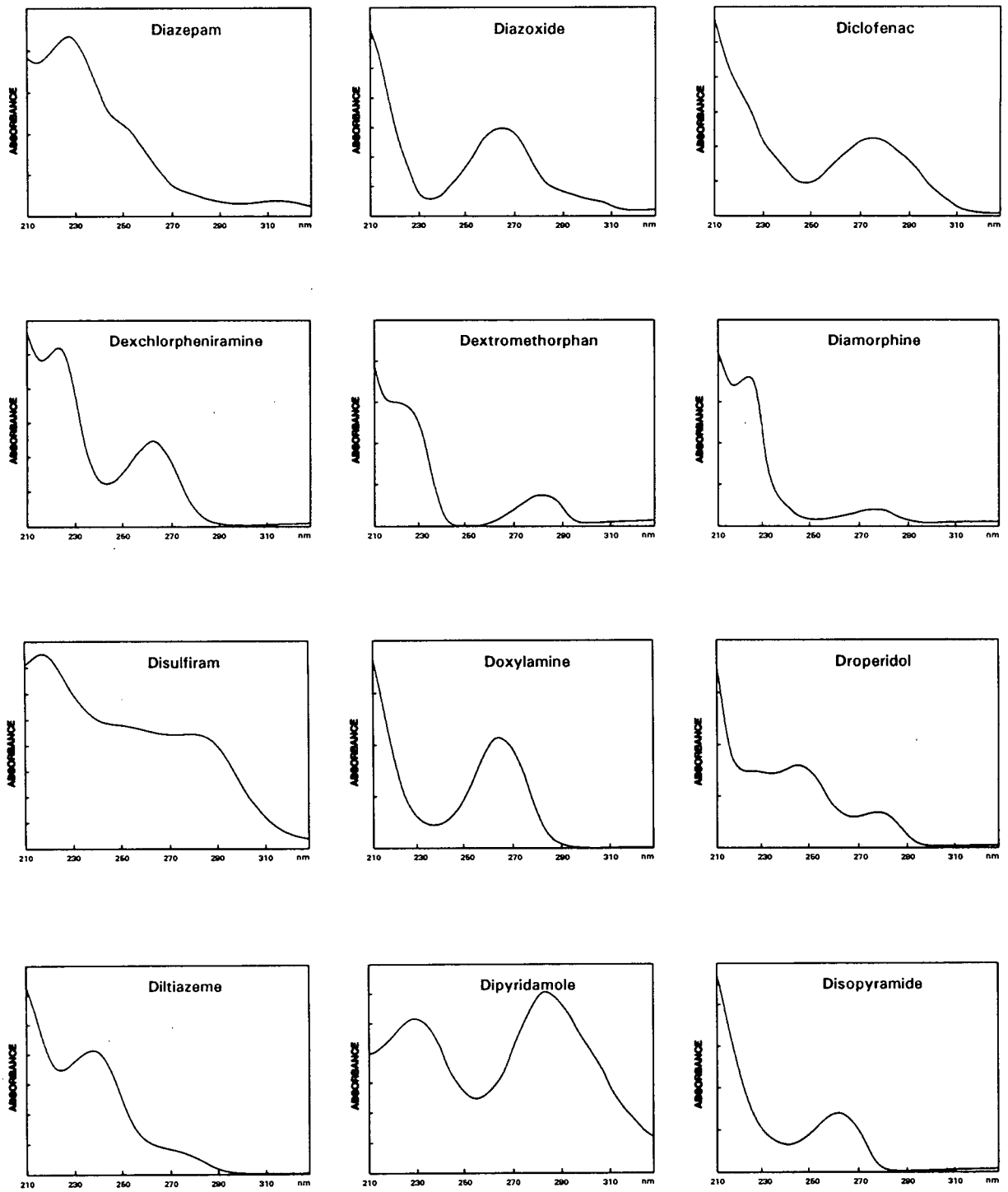
Diazepam
Diazoxide
Diclofenac
Dexchlorpheniramine
Dextromethorphan
Diamorphine
Disulfiram
Doxylamine
Droperidol
Diltiazeme
Dipyridamole
Disopyramide
ABSORBANCE
210 230 250 270 290 310 nm

Fig. 5.

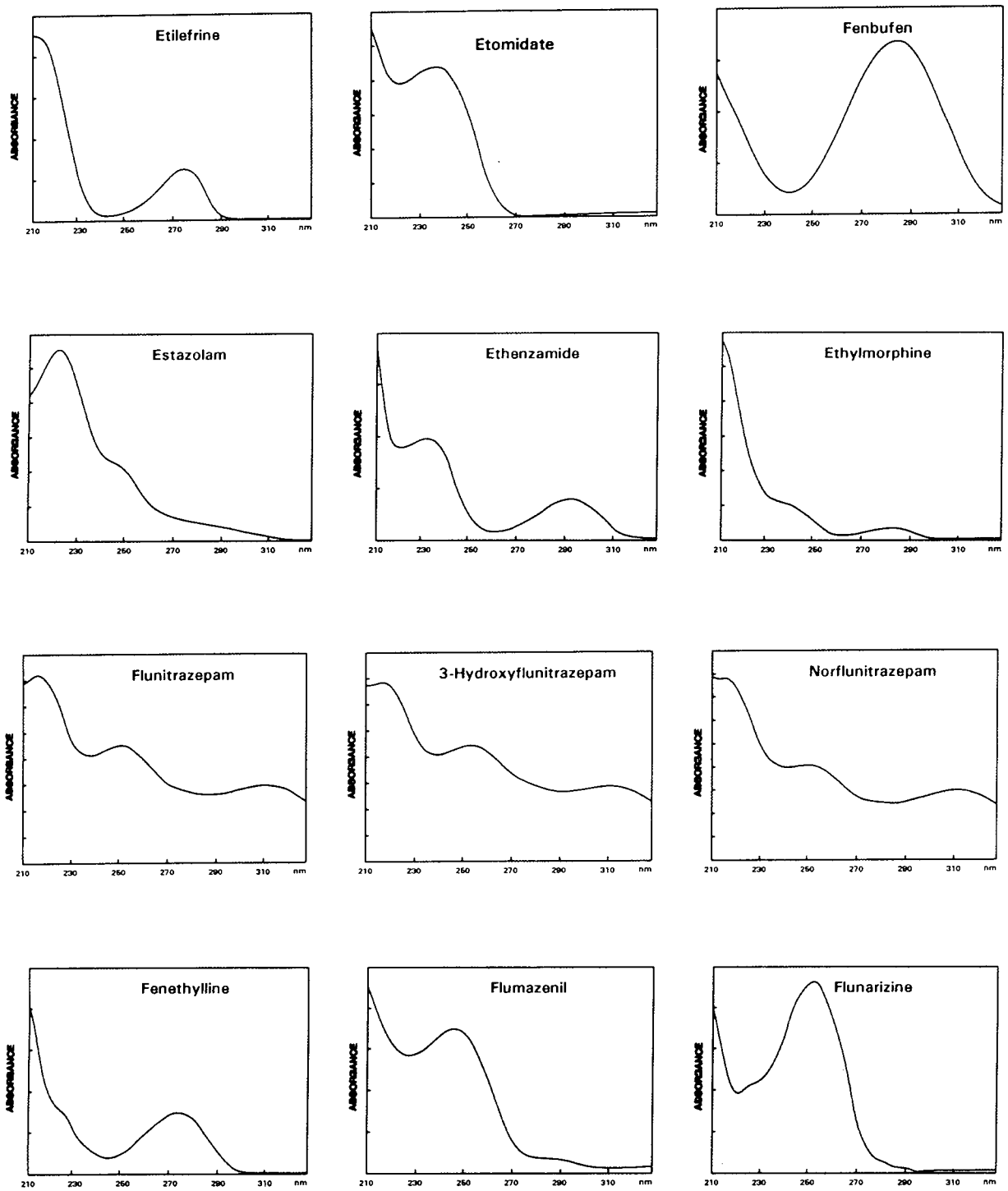

Fig. 5. *(Continued on p. 114)*

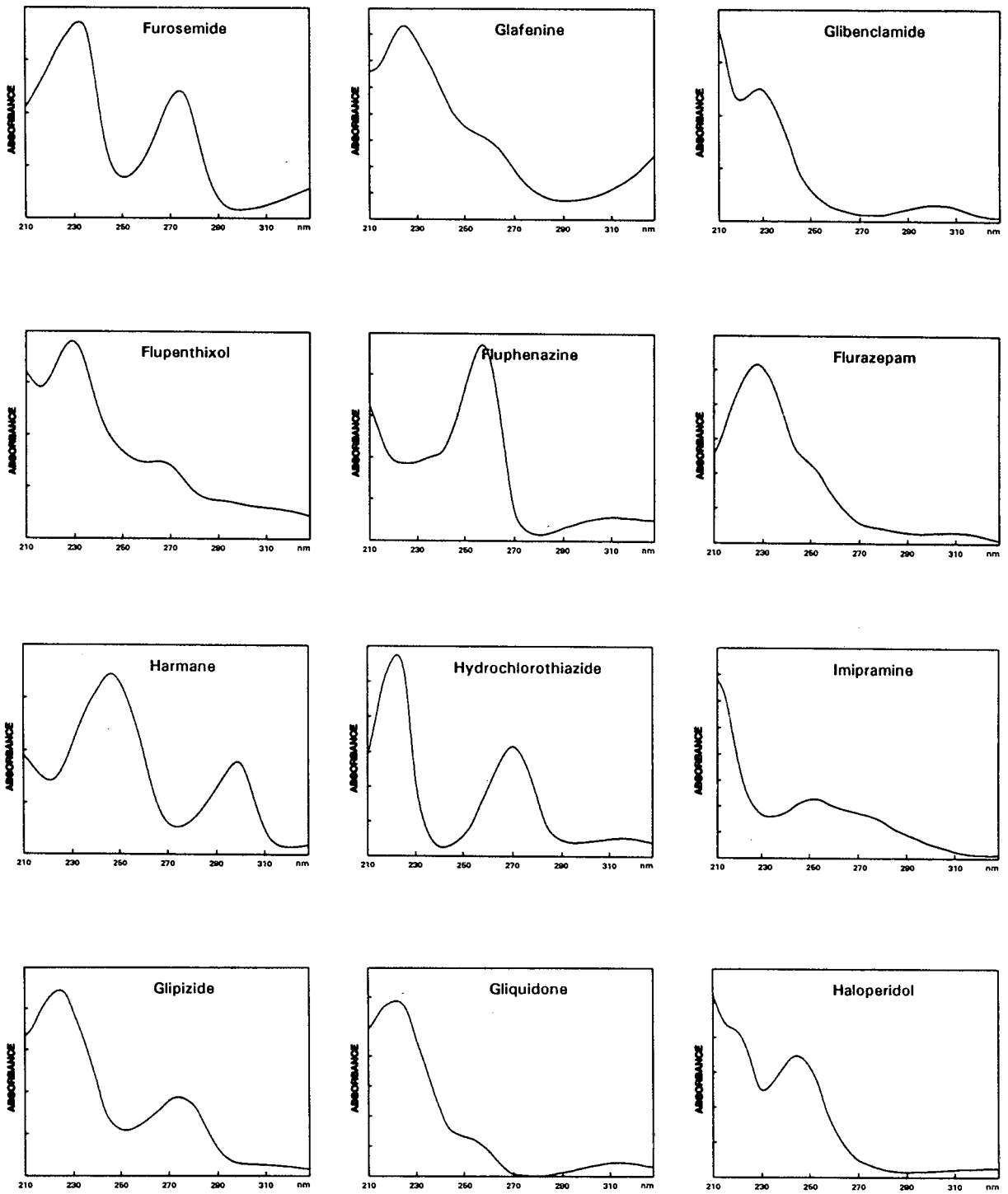
Furosemide
Glafenine
Glibenclamide
Flupenthixol
Fluphenazine
Flurazepam
Harmane
Hydrochlorothiazide
Imipramine
Glipizide
Gliquidone
Haloperidol
ABSORBANCE
210 230 250 270 290 310 nm

Fig. 5.

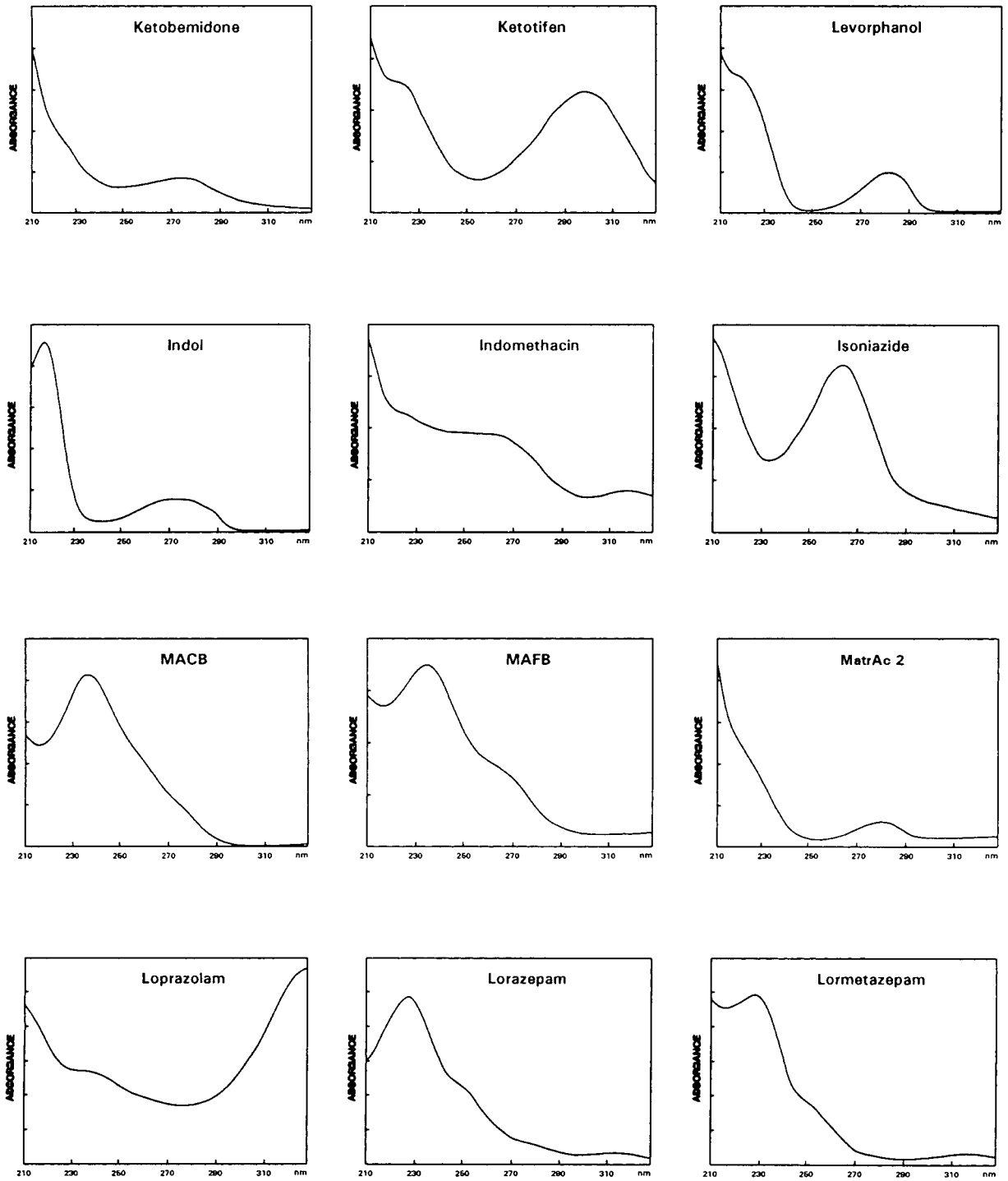

Fig. 5. *(Continued on p. 116)*

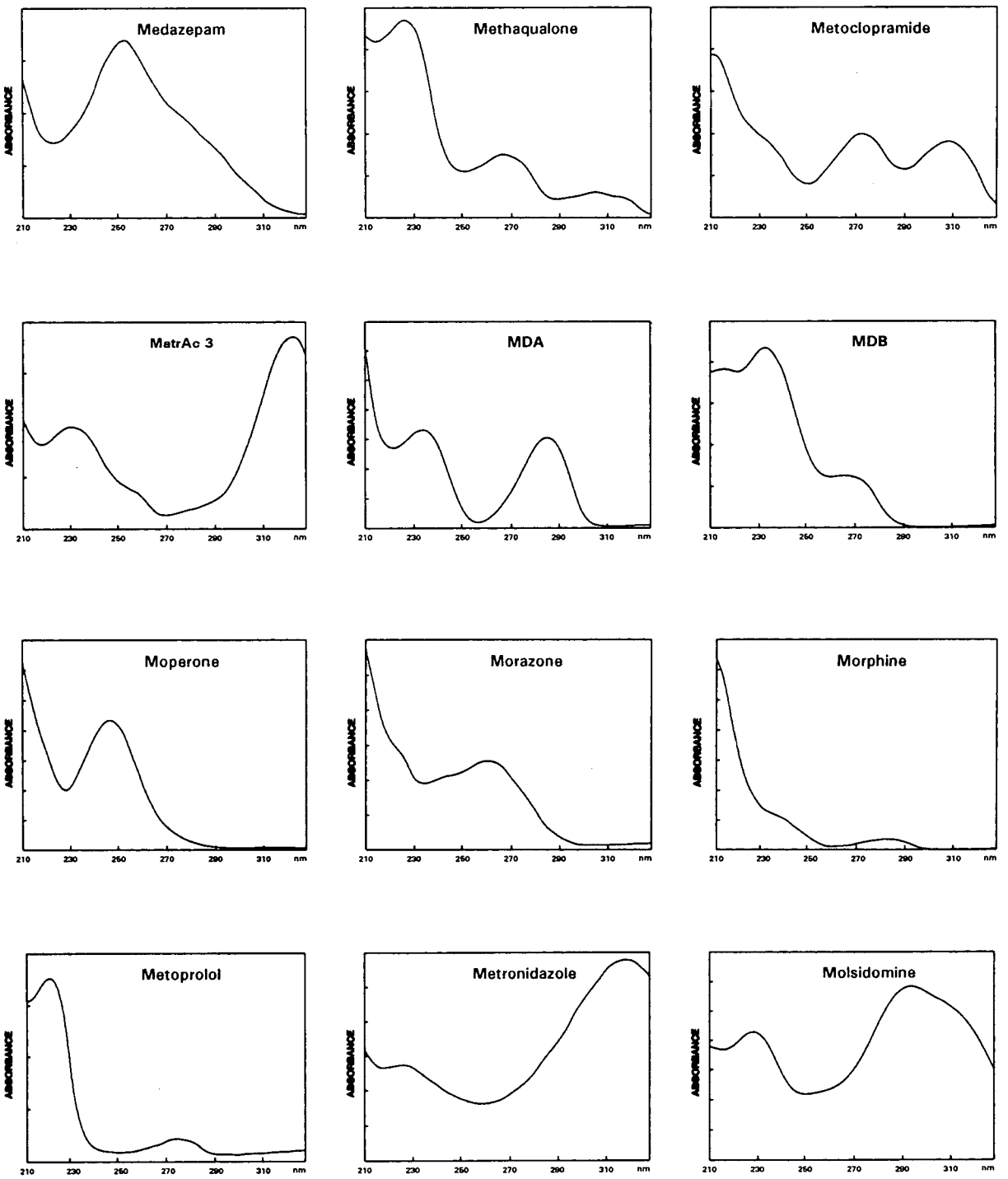
Medazepam
Methaqualone
Metoclopramide
MetrAc 3
MDA
MDB
Moperone
Morazone
Morphine
Metoprolol
Metronidazole
Molsidomine
ABSORBANCE
210 230 250 270 290 310 nm

Fig. 5.

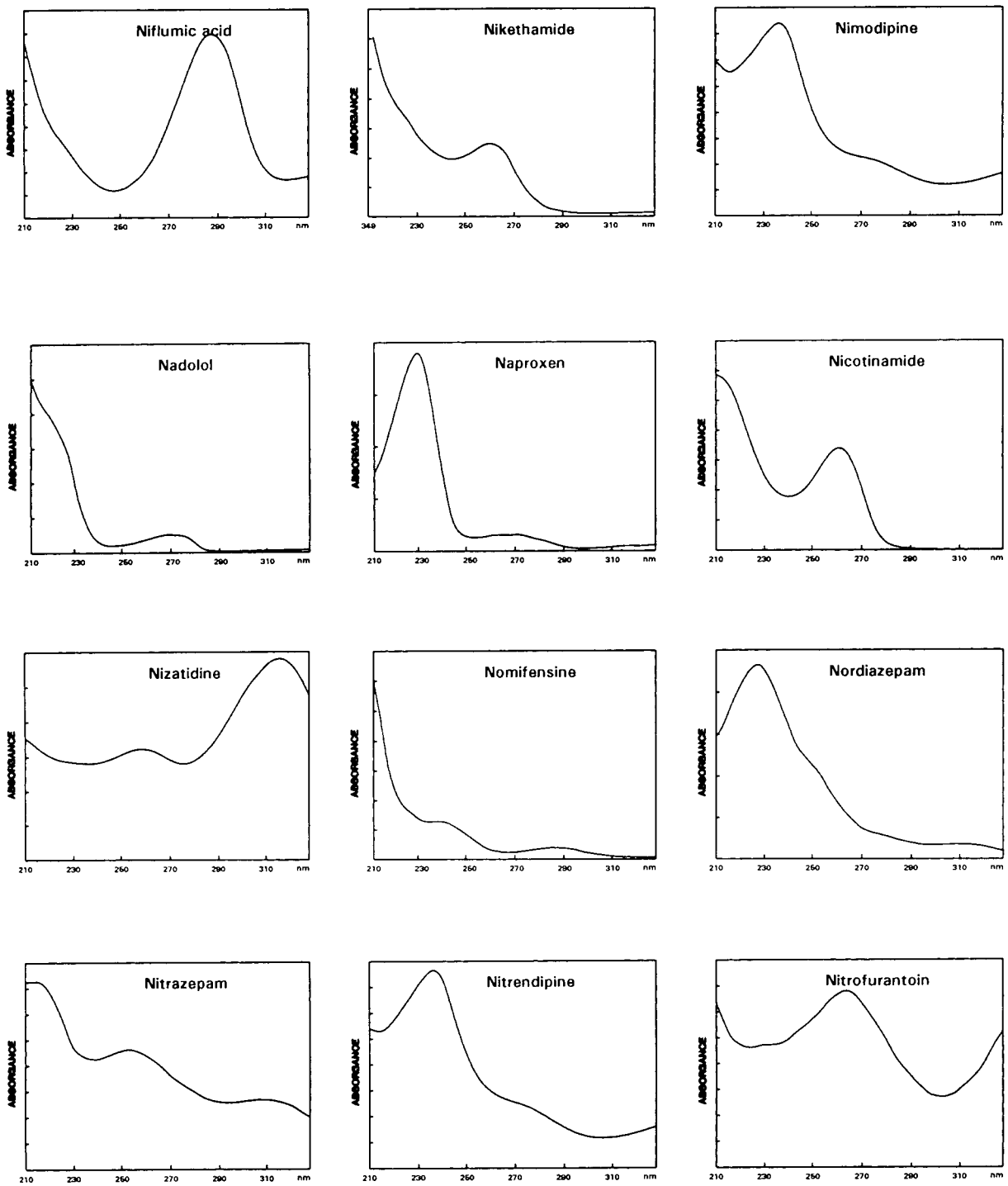

Fig. 5. *(Continued on p. 118)*

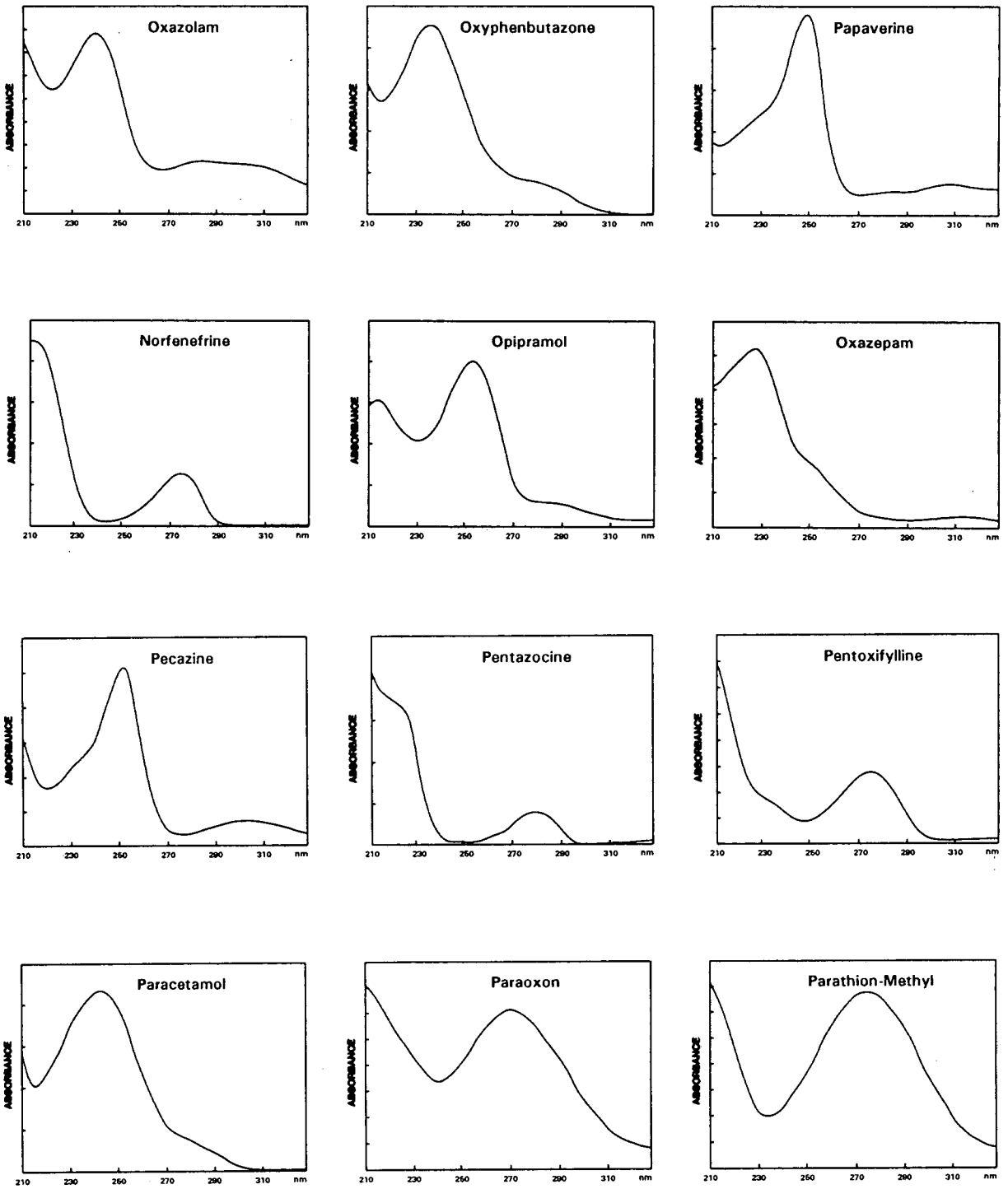
Oxazolam
Oxyphenbutazone
Papaverine
Norfenefrine
Opipramol
Oxazepam
Pecazine
Pentazocine
Pentoxifylline
Paracetamol
Paraoxon
Parathion-Methyl
ABSORBANCE
210 230 250 270 290 310 nm

Fig. 5.

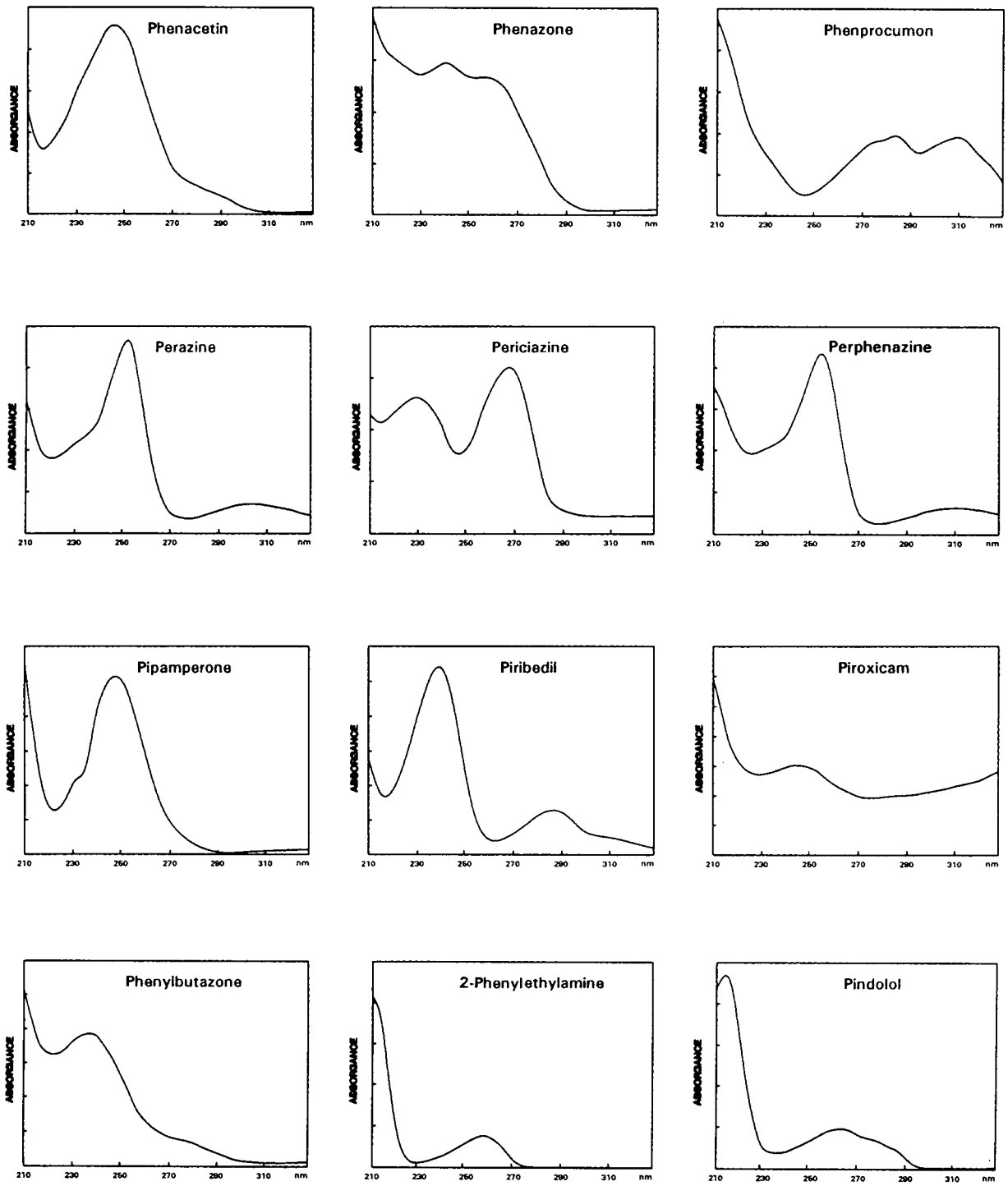

Fig. 5. *(Continued on p. 120)*

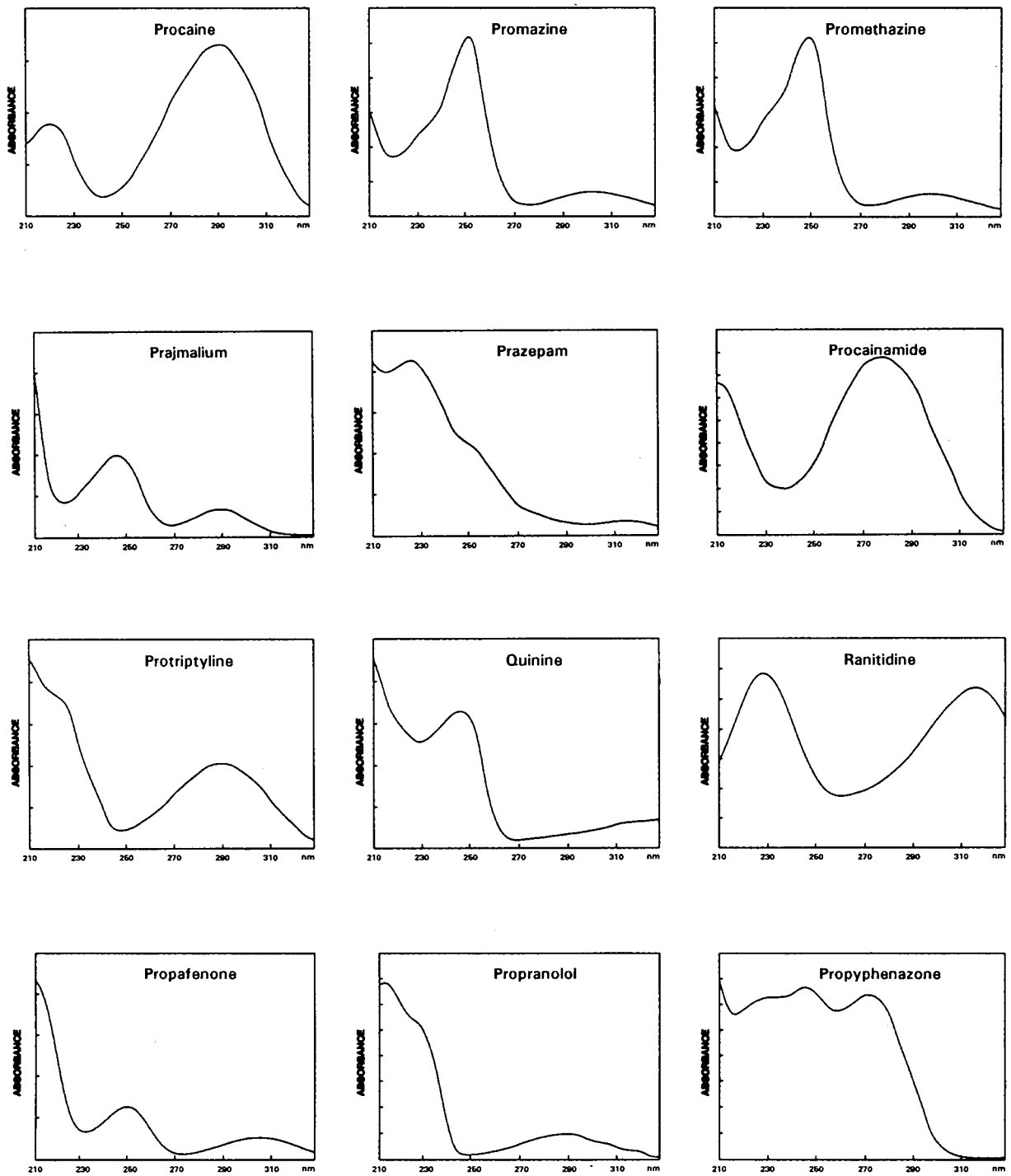
Procaine
Promazine
Promethazine
Prajmalium
Prazepam
Procainamide
Protriptyline
Quinine
Ranitidine
Propafenone
Propranolol
Propyphenazone
ABSORBANCE
210 230 250 270 290 310 nm

Fig. 5.

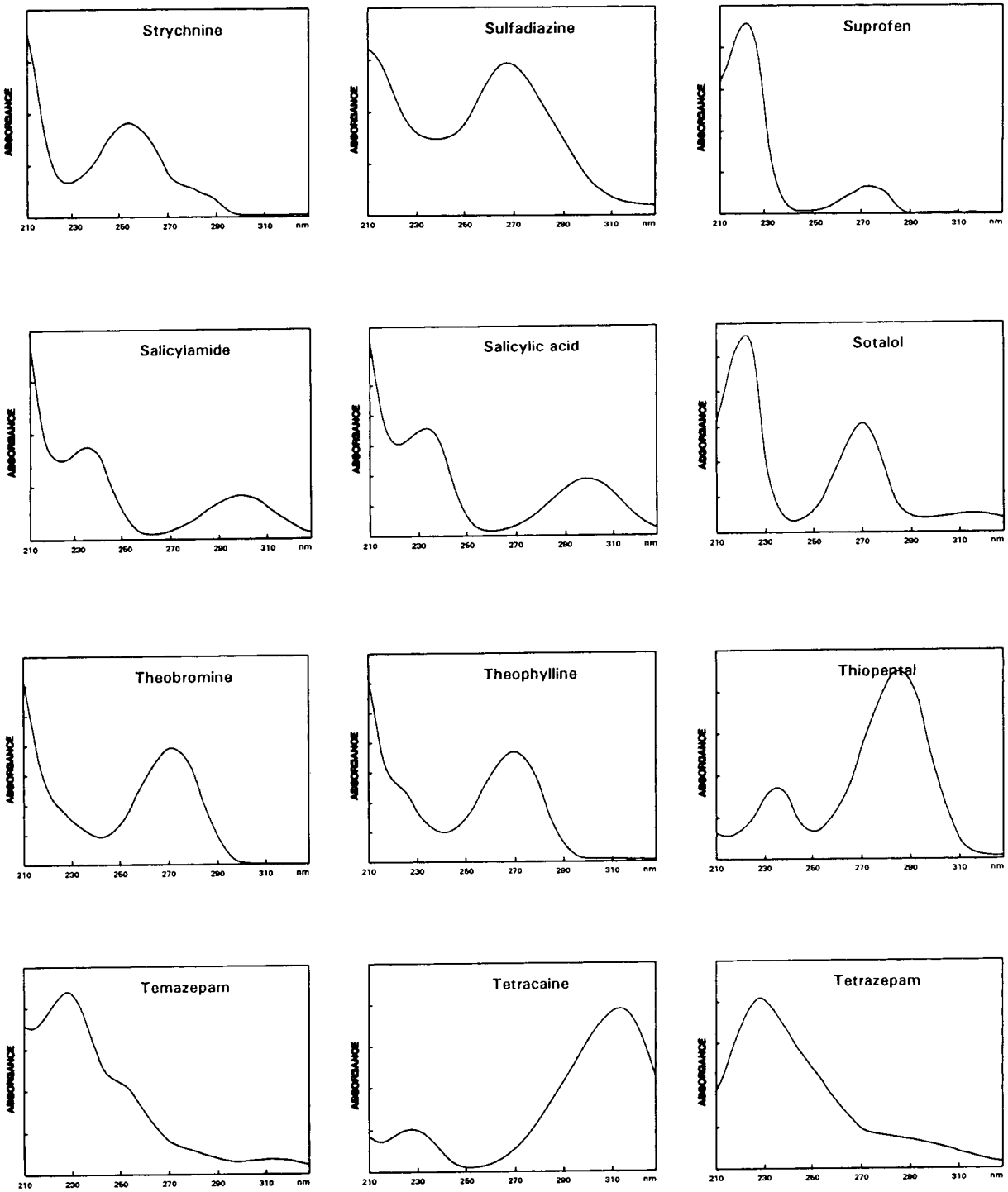

Fig. 5. *(Continued on p. 122)*

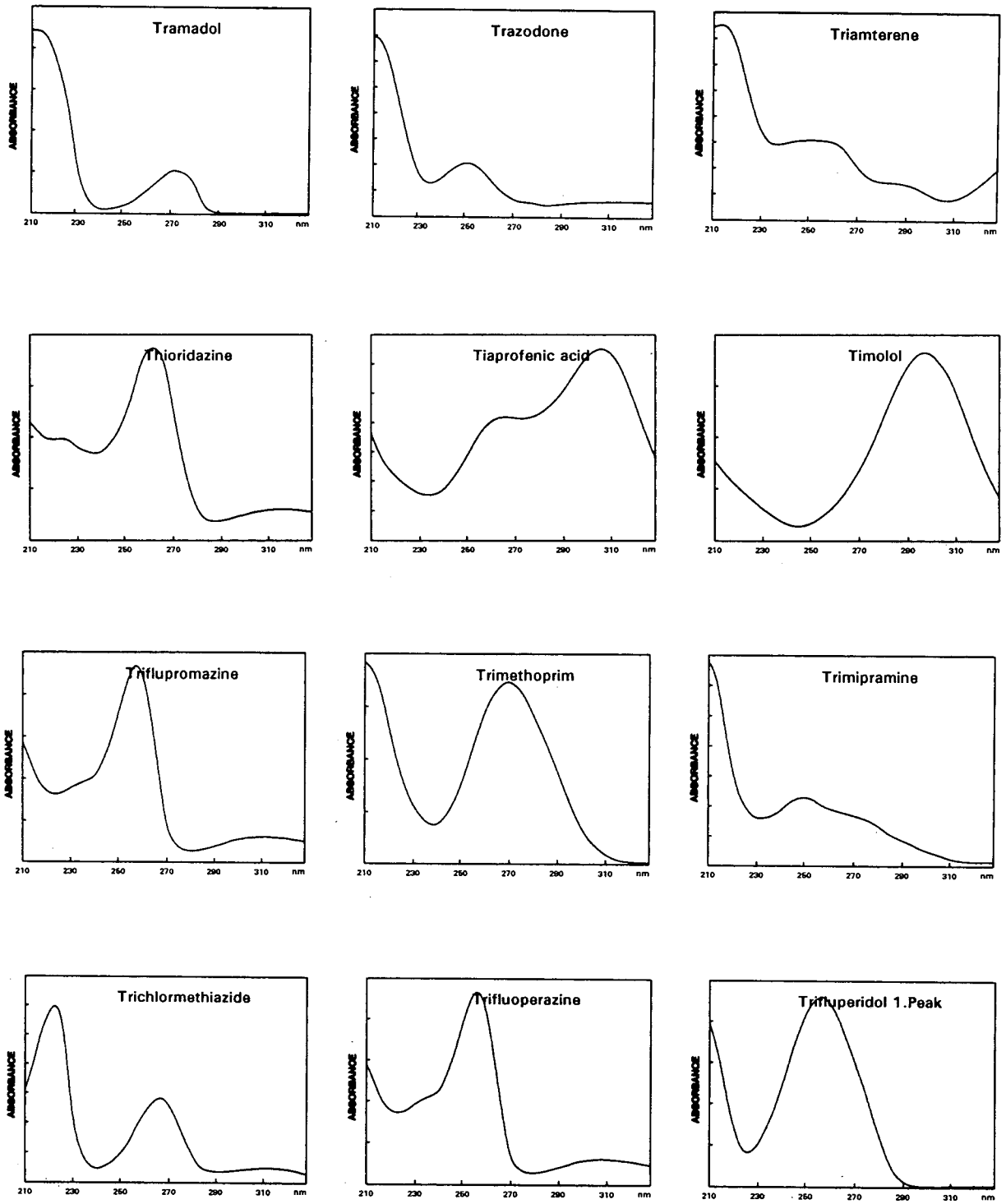
Tramadol
Trazodone
Triamterene
Thioridazine
Tiaprofenic acid
Timolol
Triflupromazine
Trimethoprim
Trimipramine
Trichlormethiazide
Trifluoperazine
Trifluperidol 1.Peak
ABSORBANCE
210 230 250 270 290 310 nm

Fig. 5.

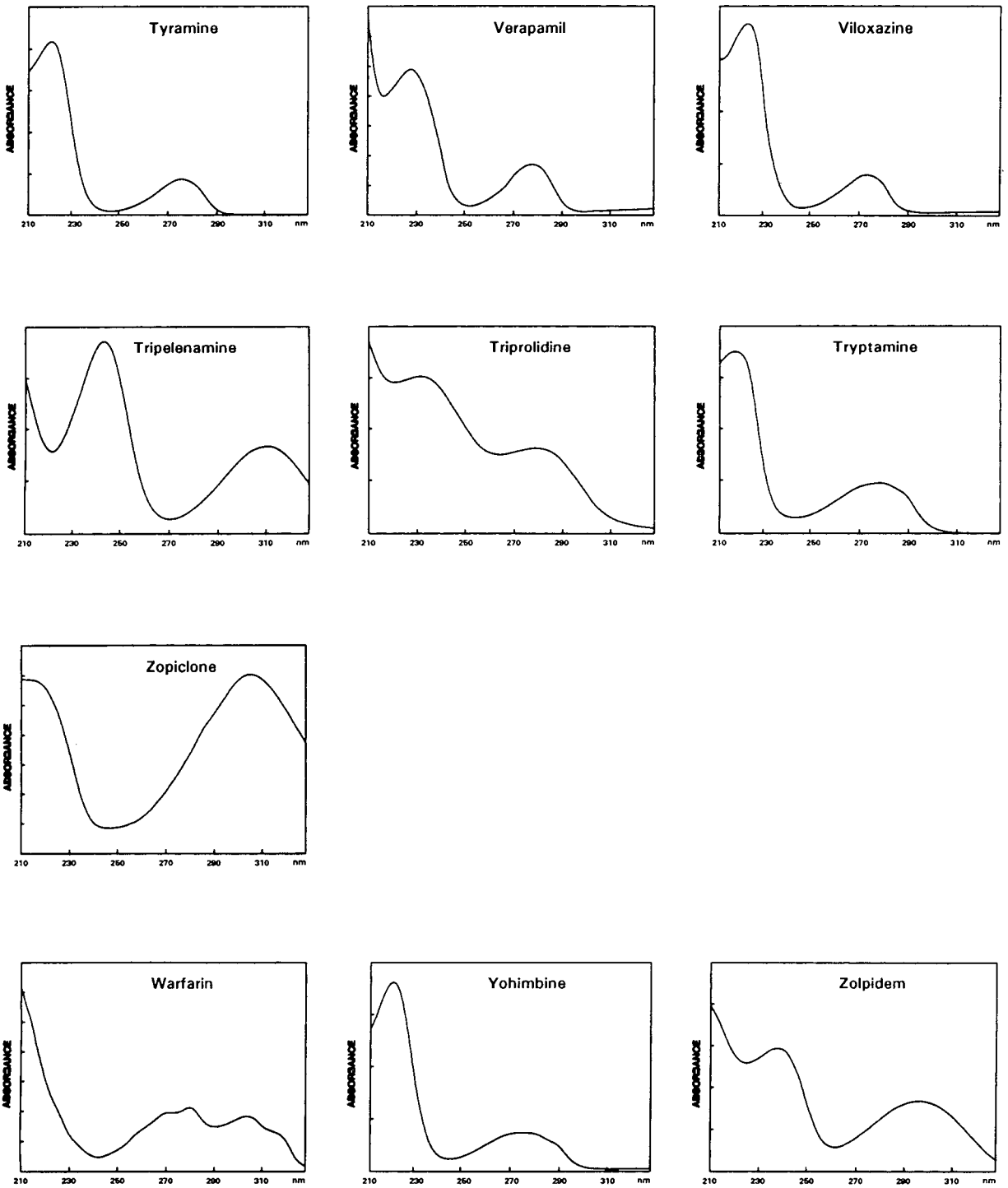
Tyramine
Verapamil
Viloxazine
Tripelenamine
Triprolidine
Tryptamine
Zopiclone
Warfarin
Yohimbine
Zolpidem
ABSORBANCE
210 230 250 270 290 310 nm

Fig. 5.

ing the spectra is that not all spectra which were measured at pH 3.0 are identical with the spectra measured in acidic solution, *e.g.*, in 0.1 *M* HCl. Comparison of several spectra with those published in a standard source [27] showed that some of them correspond to the spectra measured in alkaline medium. The "alkaline" spectra were observed for benzodiazepine derivatives (clonazepam, clorazepate, diazepam, flurazepam, nitrazepam, nordiazepam, oxazepam, prazepam, temazepam and tetrazepam), for some local anaesthetics (benzocaine, cinchocaine, procaine), antidepressant drugs (amoxapine, loxapine, nomifensine) and others (clomethiazole, clopamide, gliclazide, metronidazole, morazone, niflumic acid, propyphenazone, salicylic acid). Additionally, numerous substances produced spectra that were intermediate between acidic and alkaline types. These differences were caused by two factors: the different pH values of the mobile phase and of 0.1 *M* HCl and the influence of triethylammonium ion. Koves and Wells [14] also observed "alkaline" spectra for diazepam and nordiazepam using a mobile phase of pH 3.4 and containing triethylamine.

4. Discussion

The various factors relevant for the successful application of HPLC identification system are discussed in turn.

4.1. Intra- and interlaboratory reproducibility of results

The reproducibility of retention parameters, stored in the database, determines the identification potential of an identification system [28]. Therefore, various aspects of the reproducibility of the presented HPLC identification system were subjected to separate studies. Comparison of *I* values of 39 acidic and 109 basic drugs, obtained in two laboratories using two different ODS columns and different instrumental sets, demonstrated good reproducibility of the results, with mean standard deviations of 6.9 and 8.4 *I* units for acidic and basic drugs, respectively [29]. The interlaboratory study with application of seven different reversed-phase columns (octyl- and octadecylsilica) revealed an interlaboratory mean standard deviation of 10.3 *I* units [24]. This level of reproducibility was comparable with values observed for the same column packing in different laboratories, as shown by the comparison of the present results with those observed in the previous study [16].

The identification potential of the presented HPLC system was assessed by means of the "discriminating power" [30] and "mean list length" [31] approaches. It was demonstrated that the combination of retention indices and UV data shows a similar identification potential to the combination of capillary GC and off-line UV spectrophotometry [32].

4.2. Applicability of HPLC library to biological samples

The present HPLC database was established with pure drugs. It was therefore important to know whether the co-extracted biological matrix might influence the chromatographic behaviour of drugs in the extract, and to what extent the detectability of toxicologically relevant substances might be affected by endogenous matrix substances. This problem was investigated by Neidl-Fischer [33], who demonstrated that the *I* values of barbiturates and of doxepin, extracted from biological material in authentic cases of poisoning, were virtually identical with the listed values. Also, our own study showed that the chromatographic mobility of selected acidic and basic drugs is not significantly affected by endogenous material, extracted from serum, blood or liver [34]. It is therefore possible to use the *I* values or UV spectra taken from pure compounds for the identification of extracted substances. Biological extracts may, however, contain various endogenous substances of high UV absorbance and similar chromatographic mobility to various drugs. These substances may over-

lap the elution regions of toxicologically relevant substances and affect the identification potential. Therefore, the chromatographic purity of extracts is extremely important for identification and several workers have developed isolation methods that are particularly suitable for HPLC screening with diode-array detection [12–14]. The database contains some identified endogenous compounds, such as indole, tryptamine and phenylethylamine, and unidentified matrix substances of acidic or basic character.

4.3. Selectivity and sensitivity of detection

It is obvious that the identification potential of the HPLC system depends on the chromatographic quality of the column used. In previous studies we demonstrated that fully end-capped reversed-phase columns (octyl- or octadecylsilica) are very suitable for this kind of analysis [24,34]. Nevertheless, the possible future development of capillary HPLC, together with suitable detection systems, may bring new dimensions to the identification power of HPLC. The detection limit of drugs depends on the efficiency of the isolation method applied and the spectral properties of particular compounds and ranges between 0.01 and 1 mg/l. These values are higher than those observed using UV spectrophotometric detectors. The problem of the sensitivity of detection seems to be a limiting factor at the moment, and therein lies the greatest potential for technical improvement.

4.4. Possibility of further expansion of database and exchange of data

The feasibility of standardization of retention data with the retention index scale applied made possible the interlaboratory use and expansion of the HPLC database. On the other hand, it should be stated that the standardization and the possibility of exchange of spectral data remain to be solved. Various manufacturers of diode-array detectors have developed different software systems that are not compatible and do not allow the transfer of spectral data from one library to another.

5. Abbreviations

A_1^1	absorbance of 1% solution in a 1-cm cuvette
ABP	(2-amino-5-bromphenyl)(pyridin-2-yl)methanone, hydrolysis product of bromazepam or 3-hydroxy-bromazepam
ACB	2-amino-5-chlorobenzophenone, hydrolysis product of chlordiazepoxide, clorazepate, demoxepam, nordiazepam, oxazepam or oxazolam
ACN	acidic/neutral drugs scale
ADB	2-amino-2′,5-dichlorbenzophenone, hydrolysis product of lorazepam
ANB	2-amino-5-nitrobenzophenone, hydrolysis product of nitrazepam
ANCB	2-amino-2′-chloro-5-nitrobenzophenone, hydrolysis product of clonazepam
ANFB	2-amino-2′-fluoro-5-nitrobenzophenone, hydrolysis product of norflunitrazepam
BAS	basic drugs scale
CCB	5-chloro-2-[(cyclopropylmethyl)-amino]benzophenone, hydrolysis product of prazepam or 3-hydroxyprazepam
CCMK	1-cyclohexenyl 5-chloro-2-methylaminphenyl ketone, hydrolysis product of tetrazepam
DCFB	2-[2-(diethylamino)ethylamino]-5-chloro-2′-fluorobenzophenone, hydrolysis product of flurazepam
LSD	lysergic acid diethylamide
MACB	2-methylamino-5-chlorobenzophenone, hydrolysis product of camazepam, diazepam, ketazolam or temazepam
MAFB	5-amino-2′-fluor-2-(methylamino)-benzophenone, hydrolysis product of 7-aminoflunitrazepam
MATRAC	biological matrix peak in acidic extract
MATRBAS	biological matrix peak in basic extract

MDA	3,4-methylenedioxyamphetamine
MDB	2′,5-dichloro-2-(methylamino)-benzophenone, hydrolysis product of lormetazepam
MDE	3,4-methylenedioxyethylamphetamine
MDMA	3,4-methylenedioxymethylamphetamine
MNFB	2′-fluoro-2-(methylamino)-5-nitrobenzophenone, hydrolysis product of flunitrazepam
	retention index
THC	tetrahydrocannabinol

6. Acknowledgements

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7. References

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Review

On-line high-performance liquid chromatography–fast atom bombardment mass spectrometry in forensic analysis

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Abstract

Various types of on-line high-performance liquid chromatography (HPLC)–mass spectrometry (MS) have been developed for polar, non-volatile or thermolabile compounds unsuitable for gas chromatography (GC)–mass spectrometry (MS). On-line systems of HPLC–fast atom bombardment (FAB) MS are now gaining popularity in forensic laboratories. In this paper, HPLC–FAB-MS systems are classified according to the inner diameter (I.D.) of the HPLC columns used, and each group is reviewed with respect to analyses for drugs, poisons or their metabolites. The coupling of capillary (I.D. 0.2–0.5 mm) HPLC with FAB-MS is especially recommended, because a high level of sensitivity can be achieved as splitting is not necessary. A special column-switching device with a large-volume (500 μl) injection loop before introduction to the capillary HPLC column is useful for obtaining even higher sensitivity. Such a combination has allowed the identification of very low (therapeutic) levels of drugs or their metabolites in serum or plasma. Octadecyl reversed-phase cartridges are used for clean-up of samples with high protein contents. In the FAB-MS system, both an internal quasi-molecular peak and adequate numbers of fragment peaks, which are useful for the determination of molecular mass or for the final identification of a compound, respectively, can be obtained from many polar compounds. This is one of the significant advantages of HPLC–FAB-MS analysis over most other HPLC–MS systems. In addition, this paper briefly mentions some other HPLC–MS interfaces, such as thermospray, monodisperse aerosol generation interface, atmospheric pressure chemical ionization and electrospray.

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1. Introduction

The use of fast atom bombardment (FAB) as an ionization technique to obtain the mass spectra of polar, involatile or thermolabile compounds has become universally accepted in the short time since its development in 1981 for a quadrupole mass spectrometer [1] and for a double-focusing magnetic mass spectrometer [2]. The combination of high-performance liquid chromatography (HPLC) with FAB mass spectrometry (MS) has been a very challenging and difficult task because FAB-MS can accept a rate of introduction of HPLC column effluent of not more than 10 μl/min. Off-line methods [3–10], *i.e.*, concentration of HPLC fractions followed by direct inlet FAB-MS, have been widely used in analyses for polar or thermolabile compounds in various fields. However, collecting and drying HPLC fractions in off-line measurements is time consuming and often results in a considerable loss of sample. On-line HPLC–FAB-MS of a compound provides significant advantages over off-line methods in that mass spectral analysis of a compound can be completed within the time of its HPLC separation, and intense peaks due to the matrix can be subtracted by the use of a computer system.

Many efforts have been made to establish on-line HPLC–FAB-MS. In particular, two forms of interfaces coupling them have been developed: "frit-FAB" [11–17] and "continuous-flow FAB" (CF-FAB) [18–27]. Both interfaces utilize a fixed silica capillary to transport the effluent from a HPLC column to its tip. In the former, the capillary end is attached to a stainless-steel mesh, or frit, used as a FAB target [11]; that of the latter terminates without any cap in the ionization chamber of the mass spectrometer with a special device [18].

Most applications of HPLC–FAB-MS using either interface have been made for analyses of biological materials such as peptides [15,16,18–21,23–25], bile acids [11,12] and oligosaccharides [13]. Recently some applications of the system have been reported for drugs [22,23,25], poisons [16,26] and their metabolites [17,27].

We have been establishing highly sensitive analyses for drugs and their metabolites in human sera or plasma by capillary HPLC–frit-FAB-MS utilizing a special column-switching device for injection and concentration of samples [28–32]. Hattori *et al.* [33] devised a similar system and applied it to the determination of quinolone antimicrobials in human plasma. Thus the application of HPLC–FAB-MS in forensic analysis has only recently started.

In this paper, we classify HPLC columns into five categories according to their inner diameters (I.D.) as described in the next section and review each category of HPLC coupled with FAB-MS using either a frit-FAB or CF-FAB interface,

especially for analyses for drugs, poisons and their metabolites. In addition, we briefly review some other forms of interfaces that directly couple HPLC with MS. The usefulness of various types of HPLC–MS systems in forensic analysis is also discussed.

2. Interfaces for high-performance liquid chromatography–fast atom bombardment mass spectrometry

2.1. Frit interface

In 1985, Ito *et al.* [11] first developed an HPLC to FAB-MS interface coupling, in which a porous stainless-steel frit was used as a FAB target. This form of interface was named "frit-FAB" [12–14].

Fig. 1 shows the tip parts of the frit-FAB interface used in a JEOL (Tokyo, Japan) HPLC–MS system [11–14]. The solvent of the HPLC column effluent (3–7 μl/min) is immediately vaporized on the surface of the frit where the solute and matrix are concentrated, and bombarded with energized Xe atoms [11–14]. In this way, a conventional FAB process takes place continuously in the thin liquid film formed on the target. The frit is warmed sufficiently to prevent freezing, and the ion source temperature is maintained at 50–60°C to produce stable ion currents. Glycerol is the most common matrix; thioglycerol or other viscous fluids are also used depending on the analyte compound.

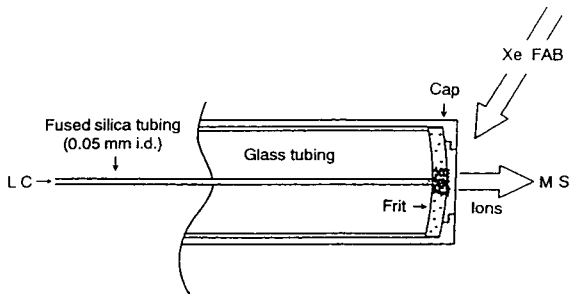

Fig. 1. Schematic illustration of ion generation in frit-FAB interface used in JEOL MS instruments (from Dr. K. Matsuura, with permission of JEOL).

2.2. Continuous-flow interface

In 1986, Caprioli *et al.* [18] developed a sample probe that allowed a continuous flow of solution to be introduced into a FAB ion source and applied it to combination of HPLC with FAB-MS [19]. Some improvements [20,21] were subsequently made and this form of interface was termed "CF-FAB".

Fig. 2 shows a schematic illustration of the CF-FAB probe used in a Shimadzu (Kyoto, Japan)–Kratos system, which is based on the probe of Caprioli *et al.* [18]. The effluent containing the solute and matrix does not evaporate in the capillary tube but forms a small droplet on the "target" at the end of the capillary in the ion source of the mass spectrometer (Fig. 2). The energized Xe atoms directly bombard the liquid droplet and a conventional FAB process takes place [19]. The source temperature is *ca.* 50°C.

Recently, Iida [34] indicated that collection of the remainder of the matrix causing chromatographic broadening occurred at the tip of the customary CF-FAB probe without any cap (Fig. 2), and that altering the source temperature and solvent flow did not eliminate the problem of tailing. Hence she presented a modified probe, shown in Fig. 3. This modification, in which a metallic mesh with a stainless-steel cap was fixed at the tip of the capillary, minimized broadening of the peak significantly [34]. Some other researchers [25,26] also indicated the same problem for the customary CF-FAB interface and modified it for their own uses. Shimadzu–Kratos applied the new probe modified by Iida [34] to

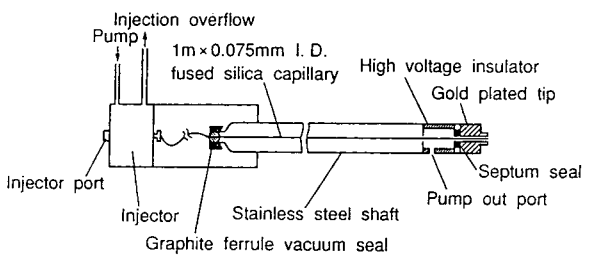

Fig. 2. CF-FAB probe used in Shimadzu–Kratos MS instruments (from Dr. J. Iida, with permission of Shimadzu and Kratos).

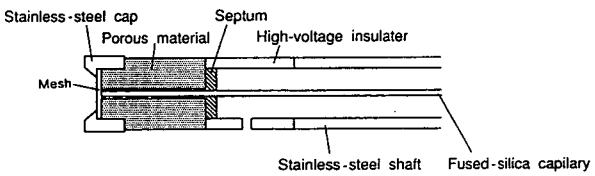

Fig. 3. New CF-FAB probe used in Shimadzu–Kratos MS instruments (from Dr. J. Iida, with permission of Shimadzu and Kratos).

their HPLC–CF-FAB-MS system. Interestingly, the new probe resembles the frit-FAB interface (Fig. 1) in that the capillary end is attached to a metallic mesh.

3. Classification of high-performance liquid chromatographic columns

In HPLC analysis, 4–6 mm I.D. columns packed with 3–30-μm materials have mostly been employed. However, research on miniaturization of the column dimensions in HPLC is continuing [35]. Thus, various dimensions of HPLC columns are now commercially available, and a suitable size for each purpose can be selected. Ishii *et al.* [35] classified HPLC columns into five categories according to their I.D., as listed in Table 1.

In recent reports [17,19,21,23,27], 1 mm I.D. columns have been called "microbore", although Ishii *et al.* [35] termed them "semi-micro". Similarly, 0.25–0.5 mm I.D. columns have been termed "capillary" [15,23,28–33]. Also, Moseley *et al.* [25] called their 0.05 mm I.D. column a "packed microcapillary" column. According to these papers, we classified HPLC systems into five groups to avoid confusion in this paper, and these are also listed in Table 1.

4. Conventional high-performance liquid chromatography–fast atom bombardment mass spectrometry

In analysis by on-line HPLC–FAB-MS, the flow-rate of the mobile phase introduced into the mass spectrometer is limited to 1–10 μl/min. When conventional HPLC is coupled with FAB-MS, most HPLC effluents must be split, and only a small fraction of the effluent can be introduced to the mass spectrometer, causing a marked sample loss. Kondo *et al.* [16] applied conventional HPLC–frit-FAB-MS to the separation and identification of microcystins in cyanobacteria. Microcystins RR, YR and LR were isolated and identified from lake surface blooms. The detection limits were reported to be 1 μg in injected amounts. This relatively low sensitivity originates in the postcolumn splitting (4:500); nevertheless, this application made it possible to shorten analysis times greatly.

5. Microbore high-performance liquid chromatography–fast atom bombardment mass spectrometry

Microbore (1 mm I.D.) HPLC–FAB-MS has been used mostly in molecular biology [19–21,23], with the CF-FAB interface. However, this does not mean that only the CF-FAB inter-

Table 1
Classification of HPLC columns according to their I.D.

Classification by Ishii *et al.* [35]	I.D. (mm)	Classification in this paper	I.D. (mm) cited here	Refs.
Densely packed columns:				
Conventional column	4–6	Conventional column	4	16
Semi-micro column	1–2	Microbore column	1	17, 19–21, 23, 27
Micro column	0.2–0.5	Capillary column	0.25–0.5	11–15, 23, 28–33
Loosely packed column	0.05–0.2	Microcapillary column	0.075	26
Open-tubular column	0.01–0.05	Open-tubular column	0.01	25

face is suitable for such analyses; the combination of microbore HPLC column with frit-FAB-MS is possible with postcolumn splitting. No reports dealing with forensic analysis by microbore HPLC–FAB-MS are available, to our knowledge. In other fields (pharmacological and environmental), a few studies have been conducted in recent years dealing with analyses of drugs or metabolites using HPLC–MS [17,23, 27].

Although Teffera *et al.* [5] succeeded in the FAB-MS of benzo[*a*]pyrene sulphate conjugates from benzo[*a*]pyrene-treated cells using the off-line method, they could not obtain good-quality full-scan mass spectra for the same compounds by on-line microbore HPLC–CF-FAB-MS using 1:6 splitting [27]. Fujiwara *et al.* [17] reported that some *in vitro* metabolites of chloroacetanilide by rat liver enzyme gave quasi-molecular peaks in microbore HPLC–frit-FAB-MS using a 1:10 split, but that structural analysis was not possible owing to the minimal fragmentation obtained. The postcolumn splitting results in a significant loss of sensitivity even in microbore HPLC–FAB-MS.

6. Capillary high-performance liquid chromatography–fast atom bombardment mass spectrometry

Until recently, capillary (0.25–0.5 mm I.D.) HPLC–frit-FAB-MS has been used mostly in molecular biology [11–13,15]. In this section, applications of capillary HPLC–frit-FAB-MS to drug analyses by our group [28–32] and Hattori *et al.* [33] are described in detail. Both groups utilize an on-line high-pressure precolumn concentration method and a special column-switching device for highly sensitive analysis.

6.1. Capillary high-performance liquid chromatographic system

An outline of the capillary HPLC system used by our group [28–32] is shown in Fig. 4. This system is a modification of the method of Takeuchi *et al.* [36].

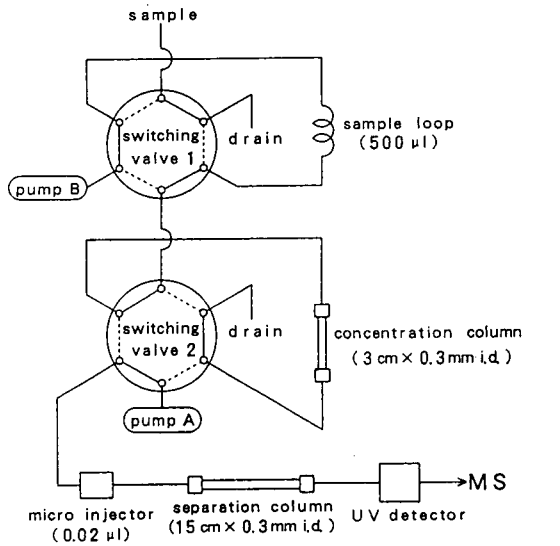

Fig. 4. Capillary HPLC system used in our research (from ref. 28, with permission of the Japanese Association of Forensic Toxicology).

Our system consists of two pumps, A and B. Pump A (micrometric pump; LDC/Milton Roy, Riviera Beach, FL, USA) is used to deliver mobile phase A at a rate of 4 µl/min to the capillary separation column. Pump B, a conventional pump (880-PU; Jasco, Tokyo, Japan), is used to send sample fluid to the concentration column with mobile phase B at a rate of 0.05–0.1 ml/min. A capillary separation column packed with 5-µm Develosil ODS or 5-µm Develosil PhA packing material and a concentration column packed with 15–30-µm Develosil ODS or 10-µm Develosil PhA packing material were purchased from Nomura Chemical (Seto, Aichi, Japan). The composition of mobile phases A and B varied from sample to sample [28–32]. Both mobile phases always contained 0.5% glycerol as matrix. This matrix concentration was much lower than that in most previous reports (5–20%).

The injection system (Model 7012; Rheodyne, Cotati, CA, USA) incorporates a 500-µl fixed load with a tubing dead volume of 50 µl. Accurate volumes of analyte (100–500 µl) are injected into the sample loop followed by deionized water (at least 50 µl) to fill the dead

volume. The compound to be analysed in the 100–500-μl sample is sent to the separation column without any loss after the purification and concentration process by our system, and the entire effluent is introduced to the frit-FAB interface without any splitting. All the tubing used to connect various sections of the HPLC system are of 0.05 mm I.D. A variable-wavelength UV detector (Jasco 875-UV) monitors the effluent prior to its introduction to the MS instrument. In this HPLC system, some cephalosporins could be determined with a tenfold increase in sensitivity [37] over our conventional HPLC procedure [38].

The HPLC system devised by Hattori *et al.* [33] is similar to ours except that it utilizes precolumn splitting for gradient formation. The details of the system have been described in their recent paper [33].

6.2. Mass spectrometric conditions

Both our group and Hattori *et al.* use a JMS-DX303 double focusing MS instrument fitted with a FAB ion source (MS-FAB 09A) and a JMA-5000 computer-controlled data analysis systems (JEOL, Tokyo, Japan). Xenon is used for generation of the fast atom beam with a gun current of 10 mA and a voltage of 3 keV. The mass spectrometers are scanned over the mass range m/z 50–800 for 5 s. Determination is made in the positive mode.

6.3. Application of capillary high-performance liquid chromatography–frit-fast atom bombardment mass spectrometry

6.3.1. Benzodiazepines

Benzodiazepines, which have hypnotic, tranquillizing and antiepileptic properties, are used worldwide, and are therefore very frequently encountered in forensic analysis.

Chlordiazepoxide is one of the typical and classical benzodiazepines, and is still used extensively. This drug, after its absorption is rapidly metabolized by demethylation and deamination to desmethylchlordiazepoxide and demoxepam, respectively. The latter is further metabolized to desmethyldiazepam, followed by hydroxylation to oxazepam [39]. Chlordiazepoxide and its metabolites were reported to be thermolabile and decomposed during capillary gas chromatography (GC) [40], and thus they have been mainly determined by HPLC [39].

We have reported that chlordiazepoxide and its metabolites could be identified in human serum after oral administration of chlordiazepoxide by capillary HPLC–frit-FAB-MS (Fig. 4) with high sensitivity [29]. A blood sample was drawn from a psychiatric in-patient receiving 30 mg of chlordiazepoxide and 500 mg of carbamazepine four times daily, in the morning before breakfast (12 h after the last administration of the drugs). Chlordiazepoxide and its metabolites were isolated from 0.5 ml of serum using a Bond-Elut C_{18} cartridge (100 mg/ml) (Varian, Harbor City, CA, USA) according to the method of Good and Andrews [41] with a minor modification [28]. The recoveries of chlordiazepoxide, desmethyldiazepam and oxazepam that had been added to blank sera were more than 90% with the Bond-Elut C_{18} isolation procedure.

Fig. 5 shows mass chromatograms with channels at m/z 271, 286, 287 and 300 for a patient's serum sample. The location of drug peaks is also indicated. The peaks due to both chlordiazepoxide and desmethylchlordiazepoxide appeared incompletely separated at a retention time of 17–18 min on the channel at m/z 286. A large peak

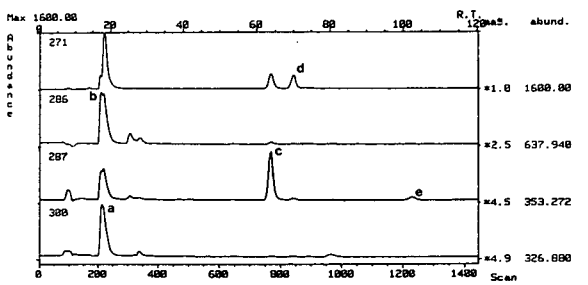

Fig. 5. FAB mass chromatograms for the extract from a 0.5-ml serum sample from a patient receiving 30 mg of chlordiazepoxide daily. (a) Chlordiazepoxide; (b) desmethylchlordiazepoxide; (c) demoxepam; (d) desmethyldiazepam; (e) oxazepam (from ref. 29, with permission of the Japanese Association of Forensic Toxicology). R.T. = Retention time in min.

appeared on the channel of m/z 271 at a similar retention time to that of chlordiazepoxide. By mass spectral measurements (data not shown), the peak was identified as a diol metabolite of epoxycarbamazepine, the major metabolite of carbamazepine [42]. Demoxepam, desmethyldiazepam and oxazepam were well separated under our conditions.

Fig. 6 shows the results of FAB-mass spectral measurements at each retention time, obtained from the serum of the patient, after appropriate subtraction of the background and interfering peaks appearing in the mass chromatograms (Fig. 5) by the use of a computer system. Chlordiazepoxide, desmethyldiazepam and oxazepam in the sample could finally be identified by comparison with the corresponding mass spectra of the authentic compounds [29]. For desmethylchlordiazepoxide and demoxepam, their identity was obvious because of the appearance of $[M+H]^+$ and $[MH-O]^+$ peaks together with each isotopic peak due to the chlorine group, although their authentic compounds were not available. The analysis of the fragmentation, shown in Fig. 7, also supports their identity in the sample.

The detection limits with respect to the mass spectral measurements were 2–5 ng on-column (corresponding to 4–10 ng/ml in serum) for chlordiazepoxide, 0.5–1 ng (2–4 ng/ml) for desmethyldiazepam and 1–2 ng (2–4 ng/ml) for oxazepam. Plasma concentrations of chlordiazepoxide and its metabolites in a patient receiving 30 mg of chlordiazepoxide daily were reported to be 100–200 ng/ml for chlordiazepoxide, desmethylchlordiazepoxide, demoxepam and desmethyldiazepam and 20–60 ng/ml for oxazepam [39]. Hence our system proved to be sufficiently sensitive to determine chlordiazepoxide and its metabolites in human serum or plasma.

Triazolam is a relatively new type of short-acting benzodiazepine hypnotic that is widely used. Its therapeutic doses are as low as 0.125–0.25 mg and hence its therapeutic concentration in serum was reported to be 2–20 ng/ml [43]. With our capillary HPLC–frit-FAB-MS system, triazolam could actually be identified by mass spectral measurements in the extracts from 0.5 ml of serum from two adults who had taken only one 0.25-mg tablet of the drug 1 h before sampling [28]. The recovery of triazolam, which had been added to blank serum, was more than 95% with the Bond-Elut C_{18} isolation procedure. The detection limit was 1–2.5 ng on-column, corresponding to 2–5 ng/ml in serum [28].

Our HPLC–frit-FAB-MS system, with a capillary column of 0.3 mm I.D. and a large injection capacity (Fig. 4), has permitted mass spectral measurements of as little as 0.5–5 ng (on-column) of a benzodiazepine and/or its metabolites. The sensitivity of HPLC–FAB-MS was almost comparable to that of the GC–MS with selected-ion monitoring (SIM) procedure of Hattori *et al.* [44], in which a wide-bore capillary column was used instead of a medium-bore capillary column to prevent thermolabile benzodiazepines from decomposing.

6.3.2. Cephalosporins

Medical accidents due to shock following the use of cephalosporin antibiotics have been reported [45], although such cases are becoming less frequent nowadays. Cephalosporins are polar, involatile, thermolabile and thus not suitable for analysis by conventional GC–MS in their underivatized forms. FAB-MS analyses of cephalosporins using direct inlet methods have been reported [46,47].

We have reported that cefaclor, one of the most popular cephalosporins, can be identified in human serum after oral administration by capillary HPLC–frit-FAB-MS (Fig. 4) [31]. Blood samples were taken from two adults 2 h after oral administration (with food) of only one 250-mg capsule of the drug. Cefaclor was isolated from the 0.5 ml of serum using Bond-Elut C_{18} cartridges (200 mg per 3 ml) (Varian) according to the manufacturer's manual with a minor modification [31]. The recovery of the drug, which had been added to blank serum, was $101.4 \pm 5.2\%$ for the isolation procedure. Both mass spectra obtained from sera from two different subjects were almost identical with that of authentic cefaclor. In another paper, we reported that cephalexin and cephaloridine, two of the most common cephalosporins, could also be

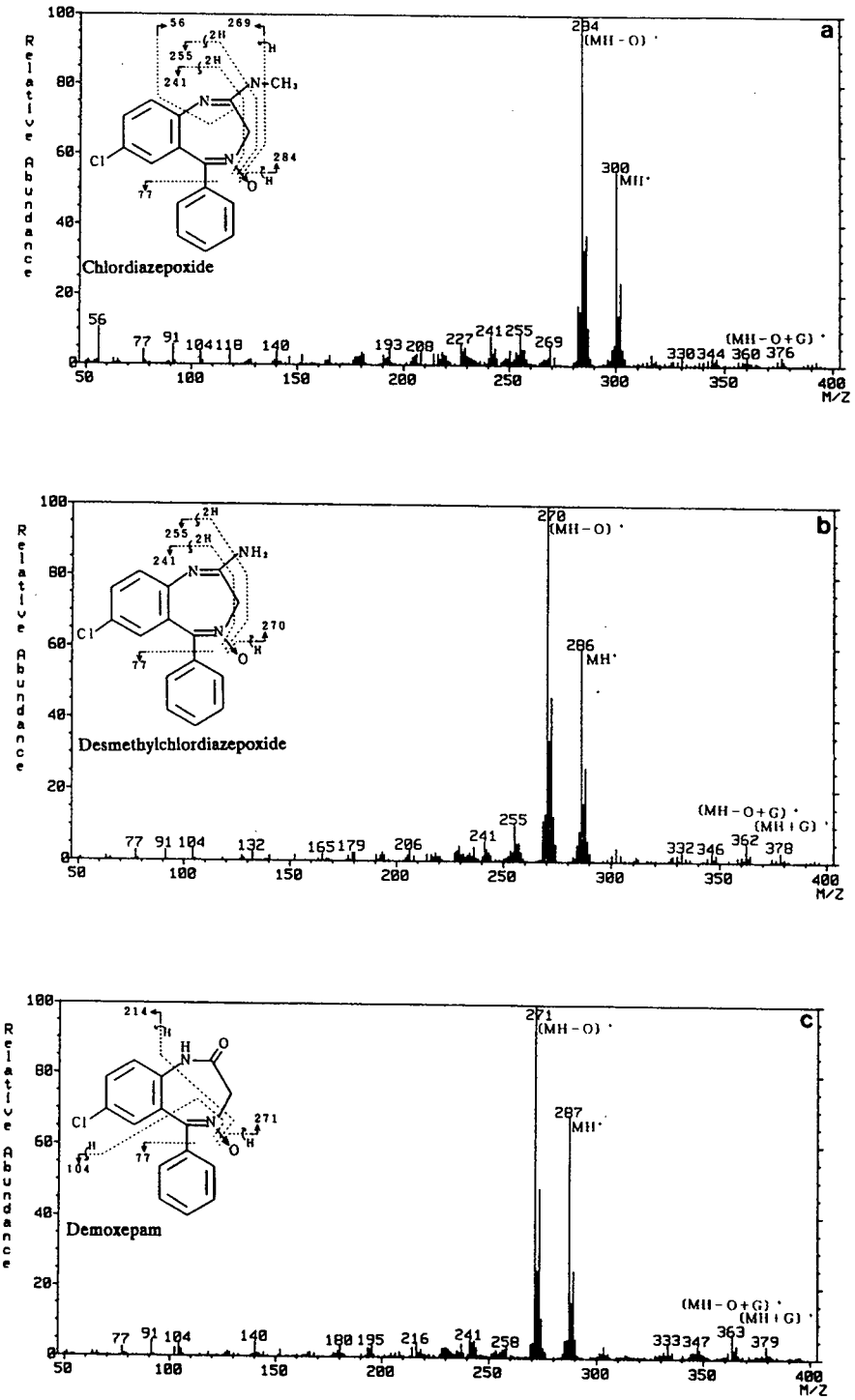
a
Chlordiazepoxide
294 (MH-O)·
300 MH·
(MH-O+G)·
b
Desmethylchlordiazepoxide
270 (MH-O)·
286 MH·
(MH-O+G)·
(MH+G)·
c
Demoxepam
271 (MH-O)·
287 MH·
(MH-O+G)·
(MH+G)·
Relative Abundance
M/Z

Fig. 6.

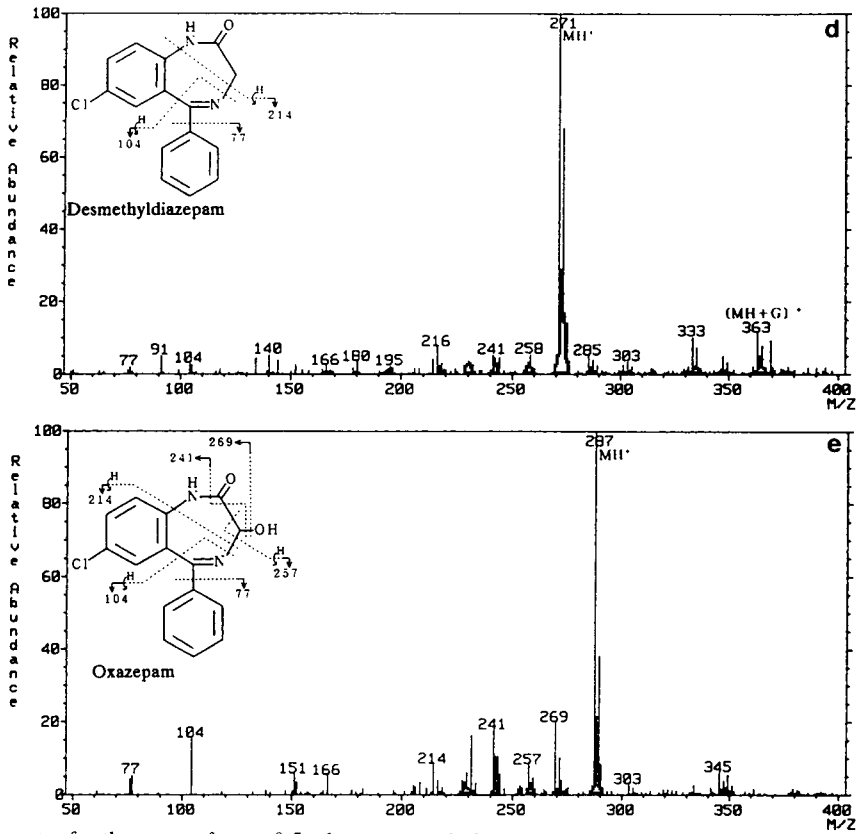

Fig. 6. FAB mass spectra for the extract from a 0.5-ml serum sample from a patient receiving 30 mg of chlordiazepoxide daily. (a) Chlordiazepoxide (M_r 299); (b) desmethylchlordiazepoxide (M_r 285); (c) demoxepam (M_r 286); (d) desmethyldiazepam (M_r 270); (e) oxazepam (M_r 286) (from ref. 29, with permission of the Japanese Association of Forensic Toxicology).

identified in human plasma spiked with 2 μg/ml of each drug by the same system [30]. For the three compounds examined in both studies [30,31], the quasi-molecular $[M+H]^+$ ions appeared as intense peaks; cations due to cleavage of the side-chains in the α-position to the carbonyl group gave the base peaks (m/z 106 for cefaclor and cephalexin and m/z 97 for cephaloridine). For cephaloridine, an intense peak due to liberation of the pyridinyl ring was also observed.

The detection limits for their mass spectral measurements were 10–20 ng on-column for cephalexin [30], 25–50 ng on-column for cefaclor [31] and 50–100 ng for cephaloridine [30]. The sensitivity for the cephalosporins (10–100 ng on-column detection limits) is almost comparable to that for quinolone antimicrobials (10–20 ng on-column) reported by Hattori *et al.* [33], but is one order of magnitude lower than that for the benzodiazepines (0.5–5 ng on-column) [28,29] or that for seventeen phenothiazines (0.25–10 ng on-column) [32]. This may be due to the difference in cation formation between basic and acidic compounds [30].

6.3.3. *Phenothiazines*

Many phenothiazine derivatives have been synthesized and are widely used as antipsychotics, antiparkinsonian drugs or antihistaminics. These drugs are also often encountered in forensic analyses. Recently, Ishikawa *et al.* [48] reported determinations of nineteen phenothiazines by GC–MS utilizing a wide-bore capillary column, and found that six of the compounds were not detectable. Most of the ther-

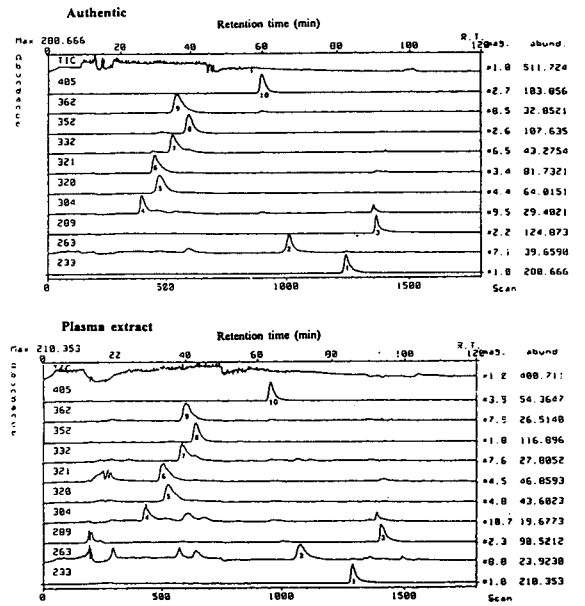

Fig. 7. FAB mass chromatograms for the authentic quinolone antimicrobials (top) and for spiked plasma (bottom). 1 = Nalidixic acid; 2 = cinoxacin; 3 = piromidic acid; 4 = pipemidic acid; 5 = norfloxacin; 6 = enoxacin; 7 = ciprofloxacin; 8 = lomefloxacin; 9 = ofloxacin; 10 = tosufloxacin. The mixture of the ten quinolone antimicrobials (1 μg of each) was added to 1 ml of plasma; a 300-μl aliquot including about 100 ng each drug of the final supernatant was injected (from ref. 33, with permission of the Japanese Association of Forensic Toxicology).

molabile phenothiazines possess long piperazinyl side-chains and thus seem unsuitable for GC–MS analysis.

We have reported a preliminary study of the determination of seventeen phenothiazines using our system [32]. Quasi-molecular peaks $[M+H]^+$ along with adequate fragment peaks were detected for all compounds with detection limits of 0.25–10 ng on-column.

Human sera obtained from four different subjects receiving chlorpromazine, levomepromazine, promethazine and propericiazine, four of the most common phenothiazines, were also analysed with our system [32]. Bond-Elut C_2 cartridges (100 mg/ml) (Varian) were effective for the isolation of the drugs and their metabolites from the sera. For all four drugs including propericiazine, having long piperazinyl side-chains, the parent drugs and their oxide compounds could be identified in the extracts from 0.5 ml of sera after oral administration of therapeutic amounts of the drugs.

6.3.4. *Quinolones*

Quinolone carboxylic acid antimicrobials are one of the most popular drug groups for the treatment of bacterial infection. They are usually determined by HPLC because of their high polarity and thermolability [49,50]. It has been reported that concomitant administration of enoxacin and fenbufen induced severe convulsions in several cases [51]. This may be true for other quinolones if they are administered together with fenbufen.

Hattori *et al.* [33] presented a detailed procedure for the detection of ten quinolone antimicrobials in human plasma by capillary HPLC–frit-FAB-MS, in which the capillary HPLC system utilized preinjection splitting for gradient formation. For extraction of the drugs from spiked plasma (1 μg/ml), perchloric acid precipitation was used.

When FAB mass spectra of the ten antimicrobials were determined, the $[M+H]^+$ quasi-molecular ions constituted the base peaks for all compounds. The $[M+2]^+$ peaks were also relatively intense for all compounds. The peaks at m/z M − 17 and M − 43, which probably correspond to MH − H_2O and MH − CO_2, respectively, were also observed for most compounds [33].

Fig. 7 shows mass chromatograms obtained with their capillary HPLC–frit-FAB-MS system for the authentic quinolone drugs (1 μg each) and a plasma sample spiked with the same amount of drugs, with use of each quasi-molecular base peak. With their gradient programme, all drugs could be detected within 100 min.

To check for the quantitative recovery of the drugs by the method, peak-area intensities were plotted against the amounts of drugs on-column for nalidixic acid, tosufloxacin and lomefloxacin as shown in Fig. 8. The calibration graphs were not sufficiently linear, but semi-quantitative. The detection limits for ten drugs were 0.1–0.2 μg/ml in plasma (*ca.* 10–20 ng on-column).

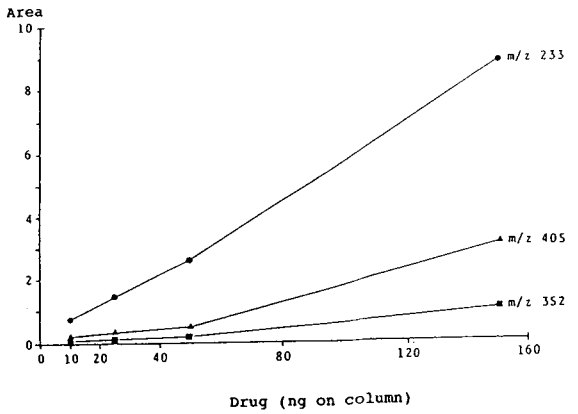

Fig. 8. Calibration graphs for (●) nalidixic acid, (▲) tosufloxacin and (■) lomefloxacin of peak-area intensity *versus* amount of drug on-column obtained by mass chromatography (from ref. 33, with permission of the Japanese Association of Forensic Toxicology).

7. Microcapillary high-performance liquid chromatography–fast atom bombardment mass spectrometry

A microcapillary (0.0075 mm I.D.) HPLC column is loosely packed with 10–30-μm diameter particles embedded inside the column of 0.05–0.2 μm I.D. [35]. Only a few reports [26,52] dealing with coupling of microcapillary HPLC with MS have been found in recent years.

Pawlosky and Mirocha [26] reported the mass spectral analysis and fragment ion structure of fusarochromanone, a toxin produced by the fungus *Fusarium equiseti*, by microcapillary HPLC–CF-FAB-MS. The detection limit of the toxin was 500 pg on-column in the SIM mode; the calibration graph was not sufficiently linear, but semi-quantitative. A relatively good-quality FAB mass spectrum with a thioglycerol matrix was also obtained although no mention was made of the detection limit in the full-scan mode [26].

As this microcapillary HPLC is operated at a flow-rate of 5 μl/min, similar to that of the capillary HPLC system, the coupling of the microcapillary column with our column-switching device (Fig. 4) is possible. This coupling will give even higher sensitivity than that of our own capillary system, because the resolution capability increases with decreasing HPLC column I.D. [35]. However, difficulty in achieving a smooth flow through such narrow columns is to be expected.

8. Open-tubular liquid chromatography–fast atom bombardment mass spectrometry

Open-tubular (OT) columns were originally developed for GC analysis and are now widely used. Similar columns have been tested for HPLC [35]; an OT-LC column is coated with a stationary phase, such as a liquid or finely dispersed solid, on the inside wall without any packing material. Efficient separation requires not only long columns (1–10 m) and very narrow I.D.s (1–20 μm), but also operation at very low flow-rates (5–250 nl/min) [53] with a special device such as a gas pressure regulator connected with mobile phase reservoirs [54]. The low flow-rate permits direct liquid introduction of the effluent into the ionization chamber [53,55]. The direct coupling of OT-LC with MS could be made in the conventional electron impact (EI) and chemical ionization (CI) modes. However, the applicability of these approaches was limited to volatile or less polar compounds [53–56].

In 1988, De Wit *et al.* [24] developed a method to couple OT-LC with CF-FAB using a coaxial CF-FAB probe. This coaxial "capillary within a capillary" interface utilized two fused-silica capillary tubes to deliver the LC effluent and the FAB matrix independently to the CF-FAB probe tip. They applied this interface to couple OT-LC (1.5 m × 10 μm I.D. column) with CF-FAB-MS for analyses of biomolecules, and reported that full-scan mass spectra of corticosterone and glutathione were obtained from 2.2 to 4.4 ng of the authentic compounds [25]; this OT-LC system was operated at a flow-rate of 53 nl/min with an injection volume of 4.4 nl.

As OT-LC should be performed at very low flow-rates (5–250 nl/min), extremely small volumes of samples can be applied. To our knowledge, no forensic application of this method has

been reported; further studies are required to assess its utility.

9. Other high-performance liquid chromatography–mass spectrometry systems

To date, many forms of interfaces besides frit-FAB and CF-FAB have been developed for directly coupling HPLC with MS. In this section, we briefly review some of them which are widely used, or expected to be useful, in forensic analysis. Excellent reviews [53,57,58] dealing with on-line HPLC–MS (not from forensic viewpoints) have been published recently, and details of other interfaces can be found in them.

9.1. Thermospray

In the early 1980s, Blakley *et al.* [59–61] developed and improved the thermospray (TSP) interface for coupling conventional HPLC with MS. In the TSP interface probe, the HPLC column effluent passes through a heated vaporizer, forming a supersonic jet of vapour with entrained fine droplets. These droplets are electrically charged while travelling through the heated source. As the size of the droplets decreases, the electric field at the liquid surface increases until the ions present in the droplet are ejected. The ions formed in this way subsequently undergo ion–molecule reactions in a similar way to conventional CI [57]. For the TSP process to be efficient, the mobile phase should contain a high percentage of water and electrolyte.

Because TSP is capable of handling flow-rates of HPLC column effluent of 1–2 ml/min, it became the first popular HPLC–MS interface. In spite of the rapid heating in the interface, it usually yields molecular ion adducts with little or no fragmentation even for some of thermolabile compounds [59–61].

Subsequently, the combined use of a discharge electrode and a repeller electrode in the ion source was devised to produce collision-induced dissociation (CID) [62]. In this device, only molecular ion adducts were observed at low repeller voltages (0–65 V), but significant fragmentation could be produced at higher repeller voltages (65–190 V). With such improvements, the TSP interface is still most popular among various HPLC–MS interfaces. Many reports dealing with analyses of relatively high-molecular-mass compounds, such as peptides and phospholipids, by HPLC–TSP-MS have been published in the molecular biology field. Several reports [17,63–65] on analyses of drugs, poisons or their metabolites by the system are also available.

However, some disadvantages of the TSP technique have been indicated [53,57,58]. Careful physical optimization of many parameters in the interface is required to achieve maximum sensitivity. The sensitivity of the technique is compound dependent even when careful optimization is made. Fujiwara *et al.* [17], in analyses of metabolites of a fluorinated herbicide, reported that the amount of the metabolites required for their HPLC–TSP-MS was normally 10–100 times more than that for HPLC–FAB-MS, and that polar compounds often degraded during analysis by HPLC–TSP-MS. In addition, it is not straightforward to use TSP with gradient elution because of the dependence of ion production on solvent composition [58], although this has been eased by the use of either the discharge electrode or a filament in the isocratic mode [53,57,58].

9.2. Monodisperse aerosol generation interface

In 1984, Willoughby and Browner [66] reported the design of a new type of monodisperse aerosol generation interface for chromatography (MAGIC). In this interface, the HPLC effluent passes into the desolvation chamber through a glass orifice to form a liquid jet. This jet is broken up into small droplets due to an orthogonal gas flow, formed by the monodisperse aerosol generation, at atmospheric pressure, at which heat transfer is more efficient. As the drops pass through the desolvation chamber, the solvent rapidly evaporates from the drops. In this way, a high-velocity particle beam having uniform size is formed; this is the basis of the alternative term for the technique, HPLC–par-

ticle beam MS. An aerosol beam separator connects the desolvation chamber to the mass spectrometer ion source. The analyte particles strike the heated source and are then vaporized. The resultant source pressure is approximately 10^{-6} Torr (1 Torr = 133.322 Pa). This type of interface is capable of handling HPLC-flow-rates of up to 1 ml/min, following their improvement [67].

The low source pressure enables the analyst to obtain standard EI spectra which can be compared with spectra in the library of the National Bureau of Standards. CI spectra can also be obtained by standard CI procedures. In these respects, MAGIC will be useful for analyses for volatile compounds in forensic laboratories. Winkler *et al.* [67] reported that the detection limits were 10 ng on-column in the full-scan EI mode and 1 ng for SIM even for a relatively involatile compound (retinol acetate) with their improved HPLC–MAGIC-MS system. However, flash vaporization of the analyte in the source is part of the ion formation process, and hence its sensitivity appears to be low for non-volatile compounds at present [53,57]. As coupling of MAGIC with FAB-MS is now being tried, increased sensitivity even for non-volatile compounds may be realized in the near future.

9.3. Atmospheric pressure ionization

As the name indicates, the source of atmospheric pressure ionization (API) instruments is maintained at atmospheric pressure. Although various interfaces utilizing API-MS have been developed, they are divided into two groups in this paper: atmospheric pressure chemical ionization (APCI) and electrospray (ESP).

9.3.1. Atmospheric pressure chemical ionization

Pioneering work by Horning *et al.* [68], demonstrating a kind of HPLC–APCI-MS, was first described in 1973. This method utilized ion–molecule reactions which occur in pure nitrogen irradiated by beta particles from ^{63}Ni foil in the ion source, but yielded significant amounts of cluster ions, which interfered with MS analysis.

In 1982, Thomson *et al.* [69] devised HPLC–liquid ion evaporation MS in which a curtain of nitrogen gas prevented non-ionized interferents from entering the mass spectrometer and also disrupted cluster ions. In this device, nebulization of the HPLC effluent without heating is used to produce a fine mist of solvent droplets. When these droplets pass through a needle held at a high voltage, a corona discharge occurs and produces charged droplets, which evaporate and emit ions from the surface. These are then electrically focused through a 100-μm orifice into the high-vacuum analyser region, where the orthogonal curtain of nitrogen gas disrupts cluster ions prior to mass analysis. As ionization occurs in a high-pressure region, CI spectra are obtained. All preionized species appear as protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ions, with essentially no fragmentation. Many neutral compounds, such as the anabolic steroid dianabol [70], also appear as protonated ions. This interface is capable of handling the liquid flow-rate of 1 ml/min at which a conventional HPLC system is operated. The sensitivity of the device is reflected by the fact that a single dose of dianabol could be detected as the epi-dianabol metabolite for 16 days [70]. However, this device seems unsuitable for forensic identification because no fragmentation is obtained.

In 1988, Sakairi and Kambara [71] reported a modification of HPLC–APCI-MS that permitted CID analysis in a relatively high-pressure region of a mass spectrometer. In this device, a drift voltage between two apertures in the intermediate vacuum region was applied to increase the efficiency of transmission of ions into the ion source housing and to dissociate cluster ions into quasi-molecular ions. At a low applied voltage (60–100 V), abundant pseudo-molecular ions with a little fragmentation were observed, but significant fragmentation resulting from CID could be produced at a high voltage (130–200 V).

Instead of the pneumatic nebulizer without heating, a heated nebulizer, the temperature of which was controlled up to 400°C, was also used to treat a wide range of HPLC effluent flows (0.1–2.0 ml/min). This system could produce

pseudo-molecular ions of many non-volatile compounds including amines, peptides, antibiotics, steroids, vitamins and alkaloids. The detection limit of the system in the SIM mode was 5 pg for some compounds such as theophylline and caffeine. In addition, the isomers of kanamycin could be differentiated from each other on the basis of their mass spectra obtained at relatively higher drift voltages [71].

Kawasaki *et al.* [72] established the screening and identification of 21 organophosphorus pesticides in blood from patients suffering from acute agricultural chemical toxicity, using a further improved version of the HPLC–APCI-MS system developed by Sakairi and Kambara [71]. In comparison with a GC–MS method, the chemicals indicated a similar specificity and were within equivalent detection limits (100–1000 ng in the full-scan mode, 2–50 ng in the SIM mode).

From the results of Kawasaki *et al.* [72] and Sakairi and Kambara [71], the sensitivity of HPLC–APCI-MS also seems to be compound dependent. This may be partly due to the heating of a sample by the heated nebulizer, which is usually operated around 250°C [73]. In addition, a recent excellent review [73] describing HPLC–APCI-MS in detail, has indicated that APCI-MS still has the propensity to form low-molecular-mass cluster ion adducts even with the use of the CID technique, and that they can interfere with the trace detection of low-molecular-mass compounds ($M_r < 300$). Further studies are needed to show the applicability of HPLC–APCI-MS to forensic analyses, although the usefulness of the system can be expected in the near future.

9.3.2. Electrospray

The production of a fine mist of charged droplets by ESP was first described by Zeleny [74] in 1917. Dole and co-workers described the usefulness of ESP as an HPLC–MS interface in patents [73,75], but the first literature report was published by Yamashita and Fenn in 1984 [75].

The sample is injected into nitrogen gas at atmospheric pressure through a metal capillary tube that is at a potential of several kilovolts relative to the surrounding chamber walls. Charge is deposited on the surface of the emerging liquid, resulting in the production of Coulomb repulsion forces that are sufficient to overcome surface tension. Hence the liquid is dispersed in a fine spray. Ions emitted from charged droplets are transferred into the vacuum chamber of a mass spectrometer and are mass analysed.

As ESP operates without the input of heat into the spray-ionization step, polar and thermolabile compounds are ionized without thermal degradation [76]. The technique works best with flow-rates in the range of 5–10 μl/min [75,76]. In 1987, Bruins *et al.* [77] successfully combined the electrostatic charging atomization of ESP and the pneumatic nebulization used in liquid ion evaporation [69] to produce the ionspray (ISP) interface. In this approach, the capillary itself is charged to several kilovolts and a coaxial nitrogen gas flow is used to assist atomization. This allows the use of flow-rates as great as 100 μl/min, which make the technique compatible with microbore HPLC.

The absence of heat in these interfaces makes possible the detection of thermolabile species, such as sulphate conjugates of drugs. For example, the ISP interface was used to detect picogram amounts of the thermolabile anabolic steroid metabolite boldenone sulphate in the SIM mode. This allowed the detection in urine of the metabolites from a single dose of boldenone in a horse 45 days after administration [78]. The sensitivity of the system shown by these results was almost in agreement with that given by Bruins *et al.* [77], who reported that the full-scan detection limit appeared to be about 10 ng for ionic or selected neutral compounds and about 10 pg under SIM conditions with their HPLC–ISP-MS system. In addition, CID reactions generated fragment ions corresponding to structural units with their system [77].

On the other hand, there is a report [79] that very few fragment ions due to CID were observed in the analysis on glycine conjugates of carboxylic acids by HPLC–ESP-MS even when the drift voltage was varied. Further studies are also needed for applying HPLC–ESP (or ISP)-MS in forensic analysis.

In addition, it should be noted that multiply

charged ions appear in the corresponding mass spectra obtained with HPLC–ESP (or ISP)-MS. The ability to produce multiply charged molecular ions for biomolecules allows mass spectrometers to measure compounds with molecular masses exceeding the mass-to-charge (m/z) range of the instrument by a factor equal to the molecule's charge state [73]. In fact, proteins with molecular masses in excess of 130 000 were successfully determined by HPLC–ESP-MS with a quadrupole mass spectrometer of limited m/z range (1700) [80,81]. Although multiply charged ions produced in HPLC–ESP (or ISP)-MS are useful in the molecular biology field, they seem not always helpful in forensic analyses because of their complicated mass spectra.

10. Conclusions

HPLC–MS techniques have recently been introduced in forensic toxicology. In this review, we have mainly presented HPLC–FAB-MS systems, in which we are engaged, together with brief mentions of other types of HPLC–MS. Examples of analyses for compounds of forensic interest by HPLC–FAB-MS, arranged according to HPLC column types, are given in Table 2.

The interfaces utilizing heat for the ionization of liquid compounds are usually capable of handling liquid flow-rates of 0.5–2 ml/min, at which a conventional HPLC system is operated, but sometimes cause decomposition of thermolabile compounds [53,73]; TSP, MAGIC and APCI with a heated nebulizer have such disadvantages, although each interface has its own individual advantages, as described before. APCI with a pneumatic nebulizer has a stronger propensity to form low-molecular-mass cluster ion adducts than that of the heated nebulizer [73]. The application of ESP or ISP is limited to highly polar or ionic compounds.

Frit-FAB and CF-FAB require no heating during analysis of a sample and can handle low- to high-polarity compounds by selection of a suitable matrix [82]. HPLC–FAB-MS is also suitable for non-volatile and thermolabile compounds that are unsuitable for GC–MS analysis. On-line HPLC–FAB-MS gives clean mass spectra free of dirty peaks due to the matrix and gives intense quasi-molecular peaks together with an adequate fragment peaks (Fig. 6), which are useful for both screening and identification of drugs or poisons.

Capillary HPLC without any splitting of effluents, and the special column-switching device allowing a 500-μl injection volume (Fig. 4), contributed greatly to enhancing the sensitivity of HPLC–FAB-MS. Capillary HPLC–FAB-MS combined with the special column-switching device seems highly recommendable for forensic analysis by HPLC–MS for the above reasons, although it gives semi-quantitative results without stable isotope internal standards.

Tandem mass spectrometry (MS–MS) was developed to obtain structural information of molecules mainly in organic chemistry [26,52, 83]. As the first MS instrument can be utilized for the separation of a target compound from impurities, crude samples can usually be analysed by the direct-inlet method on the second mass spectrometer. Trial use of MS–MS in forensic chemistry has begun. Recently, even the combination of HPLC with MS–MS has been tried [84–86]. The balance between the utility and the high cost of MS–MS instruments should be evaluated.

11. Abbreviations

APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
CF	Continuous-flow
CI	Chemical ionization
CID	Collision-induced dissociation
EI	Electron impact
ESP	Electrospray
FAB	Fast atom bombardment
GC	Gas chromatography
HPLC	High-performance liquid chromatography
ISP	Ionspray
LC	Liquid chromatography

Table 2
Analyses of drugs, poisons or their metabolites by HPLC–FAB-MS

Analytical system	Compound	Sample	Work-up	Analytical column packing material and dimensions	Injection volume	Splitting ratio	Detection limits[a]		Refs.
							Injected amount	Sample concentration	
Conventional HPLC–frit-FAB-MS	Microcystins (RR, YR and LR)	Lake surface blooms	ODS cartridge (Baker)	Chromatorex ODS-5, 5 μm (25 cm × 4.0 mm I.D.)	–[b]	4:500	1 μg	–[b]	16
Microbore HPLC–frit-FAB-MS	Metabolites of chloroacetanilide	Incubation mixture	Methanol	Brownlee RP5, C_{18} (25 cm × 1.0 mm I.D.)	3 μl	1:10	5–100 ng	1.7–33 ng/ml	17
Microbore HPLC–CF-FAB-MS	Teicoplanin (A2-2)	Authentic	–	Spheri S5, ODS2 (25 cm × 1.0 mm I.D.)	0.5 μl	3:100	28 μg	56 mg/ml	23
	Benzo[*a*]pyrene (BP), BP conjugates, BP sulphate and BP glucuronide	Cell culture medium	Chloroform–methanol Sep-Pak C_{18}	C_{18}, 5 μm (25 cm × 1.0 mm I.D.)	20 μl	1:6	0.45–18 ng[c]	22.5–900 ng/ml[c]	27
Capillary HPLC–frit FAB-MS	Benzodiazepines (chlordiazepoxide, desmethyldiazepam, oxazepam and triazolam)	Serum	Bond-Elut C_{18} (100 mg/ml)	Develosil ODS, 5 μm (15 cm × 0.3 mm I.D.)	100–500 μl	1	0.5–5 ng	1–10 ng/ml	28, 29
	Cephalosporins (cephalexin, cefaclor and cephaloridine)	Serum or plasma	Bond-Elut C_{18} (200 mg per 3 ml)	Develosil ODS, 5 μm (15 cm × 0.3 mm I.D.)	100–500 μl	1	10–100 ng	20–200 ng/ml	30, 31

	Phenothiazines (chlorpromazine levomepromazine, promethazine and propericiazine)	Serum	Bond-Elut C_2 (100 mg/ml)	Develosil PhA, 5 μm (15 cm × 0.5 mm I.D.)	100–500 μl	1	0.25–2.5 ng	0.5–5 ng/ml	32
Capillary HPLC–frit-FAB-MS (precolumn splitting system)	Quinolones (cinoxacin, ciprofloxacin, enoxacin, lomefloxacin, nalidixic acid, norfloxacin, pipemidic acid, piromidic acid and tosufloxacin)	Plasma	Perchloric acid	Develosil PhA, 5 μm (25 cm × 0.5 mm I.D.)	300 μl	1	10–20 ng	0.1–0.2 ng/ml	33
Capillary HPLC–CF-FAB-MS	Teicoplanin (A2-2)	Authentic	–	Spherisorb ODS, 5 μm (25 cm × 0.32 mm I.D.)	0.5 μl	1	940 ng	1.9 mg/ml	23
Microcapillary HPLC–CF-FAB-MS	Fusarochromanone	Ground corn samples	Sep-Pak silica cartridge	C_8-bonded micro capillary column (3 m × 75 μm I.D.)	0.5 μl	1	500 pg[d]	1 μg/ml[d]	26
OT-LC–CF-FAB-MS	Corticosterone and glutathione	Authentic	–	OTLC column without any coating (1.5 m × 10 μm I.D.)	4.4 nl	1	2.2–4.4 ng	0.5–1.0 mg/ml	25

[a] Detection limits described here are for full-scan mass spectra unless specified otherwise.
[b] No mention made.
[c] Detection limits in SIM mode were described, but full-scan mass spectra of extracts of cell culture medium were of low quality.
[d] Detection limits in SIM mode were described, but no mention was made for those for the full-scan mode.

MAGIC	Monodisperse aerosol generation interface for chromatography
MS	Mass spectrometry
MS–MS	Tandem mass spectrometry
OT-LC	Open-tubular liquid chromatography
SIM	Selected-ion monitoring
TSP	Thermospray
UV	Ultraviolet

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JOURNAL OF CHROMATOGRAPHY A

Identification of drugs in autopsy liver samples by instrumental qualitative thin-layer chromatography

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Abstract

An instrumental thin-layer chromatographic (TLC) procedure is described for the identification of drugs with use of corrected R_F values (hR_F^c) and *in situ* ultraviolet spectra. One hundred and eleven successive autopsy liver samples received from medical examiners were investigated by this technique and the results were compared to those obtained with a reference method. From the nineteen findings by the reference method, sixteen (84%) were correctly identified by the instrumental TLC method, using an hR_F^c pre-search with a window size of +/−7 units followed by a correlation search. In addition, one drug was identified correctly by correlation search only. There was no serious interference from endogenous substances, and a correlation value of about 0.9 is suggested as a limit to cut the hit list of candidates.

1. Introduction

Thin-layer chromatography (TLC) belongs to the basic method arsenal of forensic drug analysts, and it has been stated that TLC should be the first test in any drug analysis protocol [1]. During the last decade, the development of instrumental TLC [2] has created new possibilities for the quantitative analysis of drugs. However, qualitative TLC still largely relies on manual methods which, although often efficient, are usually too much dependent on individual analysts' skills in interpreting the chromatograms.

Recently, a new concept for qualitative analysis by instrumental TLC was reported: correction of R_F values by the polygonal method with several standards, and searching libraries using the corrected R_F values (hR_F^c) and UV spectra. A study involving five laboratories proved that drug libraries consisting of hR_F^c values and UV spectra can be used for identification on an interlaboratory basis, and promising results were obtained in the analysis of selected biological samples for drugs [3].

In the present study, instrumental qualitative TLC is evaluated for the identification of basic drugs in autopsy cases. A series of one hundred and eleven successive liver samples received from medical examiners were analysed during an eight-day period, and the results were compared with those obtained with a reference method.

2. Experimental

2.1. Chemicals

Trypsin of type IX was purchased from Sigma (St. Louis, MO, USA). Bis(2-ethylhexyl)-

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hydrogen phosphate (HDEHP) was from Aldrich (Steinheim, Germany).

2.2. Chromatography

The TLC plates were 10 × 20 cm glass plates precoated with 0.25 mm Silica Gel 60 F_{254} (Merck, Darmstadt, Germany). The plates were developed to a distance of 7 cm in an automatic developing chamber (ADC) (CAMAG, Muttenz, Switzerland). The mobile phase (12 ml) was toluene–acetone–94% ethanol–25% ammonia (45:45:7:3, v/v) [4], and presaturation (1 min) was carried out with the mobile phase without ammonia.

2.3. Densitometric evaluation

The scanning densitometer was a TLC Scanner II operated with CATS 3.16 software (CAMAG). The plates were scanned by absorbance at 220 nm, the spots were integrated and the spectra were recorded in the range of 200–400 nm with 5 nm wavelength increments. The R_F values were corrected by the polygonal method [5] using four reference compounds [6]: codeine ($hR_F^c = 16$), promazine (36), clomipramine (49) and cocaine (66).

2.4. Drug library

A library of 100 basic and quaternary drugs was created by chromatographing pure drug standards (4 μg) using the mobile phase described above with a saturated twin-trough chamber. The hR_F^c values were taken from existing data [4].

2.5. Sample preparation

The enzyme digestion and ion-pair extraction procedure for liver samples is shown in Fig. 1.

2.6. Reference method

The reference method for the analysis of liver samples was a combination of the present TLC system and an RP-18 system [4] using several post-chromatographic visualization reagents. Normal phase chromatography was carried out on three plates to allow undisturbed use of various reagents. The liver findings were supported by blood analysis by dual-column capillary gas chromatography with nitrogen specific or electron-capture detection.

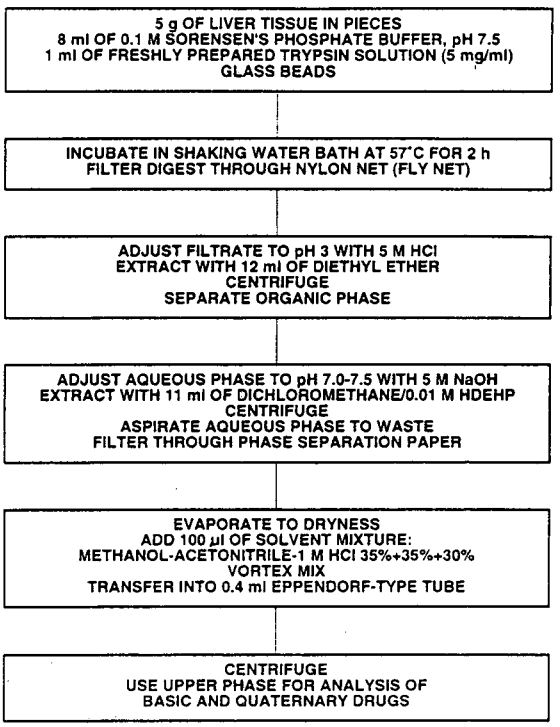

Fig. 1. Extraction procedure of liver samples for TLC.

3. Results and discussion

3.1. Description of the spectrum library option

The following example shows the function of the spectrum library option of CATS software. Thirteen autopsy liver extracts are run parallel with a set of four correction standards on a separate track (Fig. 2). The user assigns the standards on the monitor screen by clicking with the mouse. The programme calculates the hR_F^c

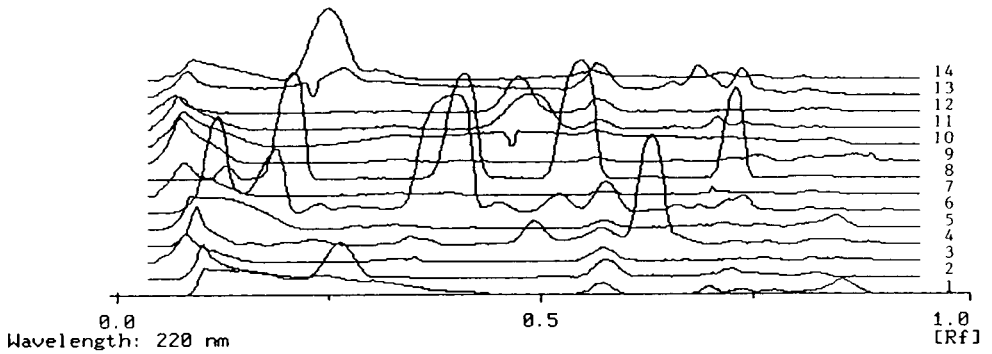

Fig. 2. Thirteen liver extracts chromatographed parallel with a set of four correction standards on track 8.

values of the unknowns based on the standards, using the origin and the solvent front as additional correction points [5]. The hR_f^c values are then listed together with track numbers, possible substance names and UV maximum wavelengths.

To identify an intensive spot on track 6 at hR_F^c 35, the respective position in the list is clicked and the spectrum is displayed. By using an hR_F^c pre-search with a window size of +/−7 hR_F^c units prior to the correlation search, a hist list is obtained that ranks promazine first with a correlation value of 0.998. Superimposing the sample spectrum with the best library match shows considerable similarity (Fig. 3).

3.2. Systematic analysis of liver samples

One hundred and eleven successive autopsy liver samples received from medical examiners were analysed for basic and quaternary drugs by the instrumental TLC method and by the reference method. There were nineteen findings by the reference method for such drugs that were included in the present drug library of one hundred compounds (Table 1). In addition, carbamazepine, citalopram, moclobemide, oxazepam, pentobarbital, phenytoin, salicylate, temazepam and triamterene, not included in the present library, were found once or more often by the reference method.

Table 1 shows the library search results obtained by the instrumental TLC method for each of the nineteen findings. The average correlation value of the sample spectrum with the correct library spectrum was 0.95 (S.D. 0.08). The correct drug was ranked first in sixteen cases out of nineteen (84%) by the combination hR_F^c presearch and correlation search. In addition, in one case (3138/93) the drug was out of the hR_F^c search window because of its very high concentration and partial co-eluting with a metabolite but was ranked first by the correlation search only.

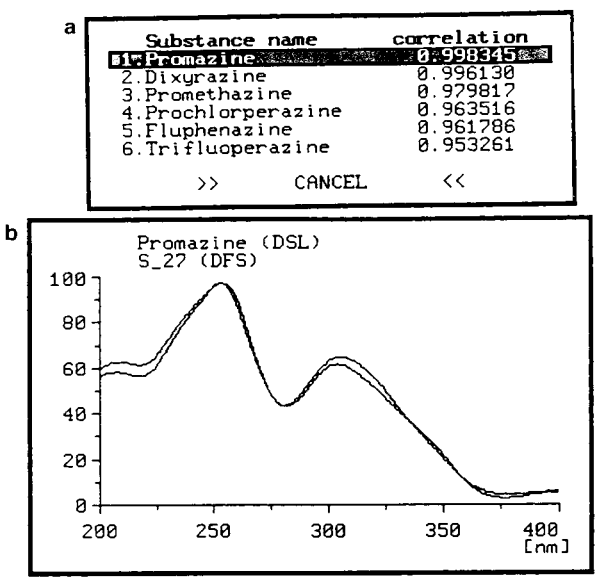

Fig. 3. Library search hit list obtained by hR_F^c + correlation for the spot at hR_F^c 35 on track 6 (a), and the best match superimposed with the sample spectrum (b).

Table 1
Drug findings from 111 successive autopsy liver samples received from medical examiners

Drug	Case No.	hR_F^c found	hR_F^c libr.	Correl. value	Hit list position[a]	Blood conc. (mg/l)
Levomepromazine	3031/93	56	60	0.983	1st	2.9
Clomipramine	3032/93	42	49	0.990	1st	30
Thioridazine	3035/93	43	45	0.917	1st	0.5
Promazine	3037/93	35	36	0.998	1st	13
Amitriptyline	3052/93	40	46	0.937	1st	0.8
Promazine	3053/93	34	36	0.976	3rd	4.0
Trimipramine	3053/93	65	65	0.651	–	0.6
Dextropropoxyphene	3067/93	64	67	0.990	1st	2.9
Promethazine	3102/93	46	41	0.979	1st	3.4
Dextropropoxyphene	3104/93	66	67	0.993	1st	0.2
Levomepromazine	3108/93	60	60	0.995	1st	1.0
Clomipramine	3122/93	46	49	0.963	1st	4.5
Levomepromazine	3122/93	60	60	0.941	1st	2.9
Chlorprothixene	3123/93	58	56	0.998	1st	1.2
Amitriptyline	3136/93	44	46	0.963	1st	2.0
Nortriptyline	3136/93	19	20	0.948	1st	–
Trazodone	3138/93	64	53	0.995	1st[b]	23
Nortriptyline	3149/93	20	20	0.960	1st	0.7[c]
Thioridazine	3149/93	45	45	0.953	1st	4.1

[a] Hit list generated by hR_F^c pre-search (+/−7 units) followed by correlation search.
[b] Library search by correlation only.
[c] Concentration for amitriptyline.

Table 2 shows the best library matches and the corresponding spectrum correlations for all spots in thirteen successive liver samples. There were only few endogenous substances that could cause false interpretation. One such compound was observed at hR_F^c 20–22 in some cases (tracks 2 and 14), giving a metoprolol-like spectrum. Inclusion of this compound or other interfering compounds in the drug library would greatly facilitate the interpretation of the results. Investigation of all the 111 cases of this study revealed that a correlation value of about 0.9 could be used as a limit to cut the hit list of candidates.

Drug metabolites often produce UV spectra very similar to that of the parent drug. This can be seen on track six where promazine and its metabolite give UV spectra that resemble each other (Fig. 4). Inclusion of the most important metabolites in the drug library would prevent misinterpretations and aid the identification of at least antipsychotic and antidepressant drugs.

3.3. Requirements for comprehensive drug screening

No single TLC method, not even an instrumental one, can positively identify all the hundreds of drugs available. However, if a second TLC system with a low correlation is added to the first one, the situation is better. The combination of an RP-18 reversed-phase system with the present TLC system has proved to be very efficient in the general screening of basic and quaternary drugs [4,6,7]. One of the most characteristic features of TLC is the possibility to utilize post-chromatographic off-line derivatization. The many visualization reactions available provide a means for further confirmation of findings that, *e.g.*, high-performance liquid chromatography is lacking.

Post-chromatographic visualization reactions can be utilized satisfactorily even if the separation of analytes is not optimal. *In situ* UV spectra are more disturbed by co-eluting of

Table 2
Best library matches obtained for all spots of the 13 successive liver samples of Fig. 2

Track No.	Case No.	hR_F^c found	Best library match[a]	Correl. value
1	2979/93	8	Maprotiline	0.875
1		52	Clomipramine	0.789
1		81	Meclozine	0.666
2	3033/93	8	Maprotiline	0.705
2		22	Oxprenolol	0.978
2		52	Clomipramine	0.788
3	3034/93	6	Maprotiline	0.797
3		51	Clomipramine	0.716
4	3035/93	8	Maprotiline	0.862
4		43	Thioridazine	0.917[b]
4		51	Clomipramine	0.801
4		57	Pitofenone	0.921
5	3036/93	7	Maprotiline	0.848
5		8	Maprotiline	0.870
5		81	Meclozine	0.644
6	3037/93	9	Acebutolol	0.848
6		14	Opipramol	0.900
6		35	Promazine	0.998[c]
6		46	Promethazine	0.900
6		52	Clomipramine	0.806
6		67	Levomepromazine	0.812
7	3038/93	6	Maprotiline	0.593
8[d]				
9	3039/93	6	Maprotiline	0.851
10	3040/93	0	Maprotiline	0.756
10		40	No match	
10		42	No match	
11	3041/93	5	Maprotiline	0.830
11		43	Amitriptyline	0.886
12	3042/93	5	No match	
12		42	Periciazine	0.894
12		51	Clomipramine	0.664
13	3043/93	7	No match	
13		17	No match	
13		22	Pindolol	0.799
13		51	Clomipramine	0.797
13		62	Pentazocine	0.842
13		67	Levomepromazine	0.896
14	3044/93	7	Maprotiline	0.774
14		20	Metoprolol	0.987

[a] Hit list generated by hR_F^c pre-search (+/−7 units) followed by correlation search.
[b] Correct result (see Table 1, case 3035/93).
[c] Correct result (see Table 1, case 3037/93).
[d] Standard track.

substances, and consequently the use of high-performance thin-layer chromatography (HPTLC) plates to improve resolution may prove to be feasible. However, to avoid overloading in HPTLC a scale-down is probably necessary, and further studies are needed to estimate the applicability of spectrum libraries at the lower concentration range.

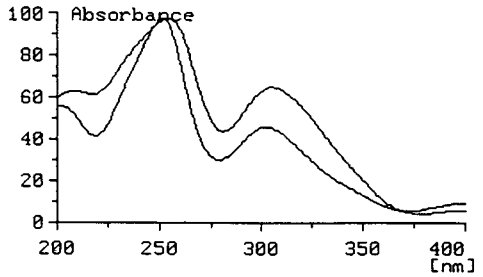

Fig. 4. Spectra of promazine and its metabolite superimposed.

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JOURNAL OF CHROMATOGRAPHY A

Chemical profiling of pharmaceuticals by capillary electrophoresis in the determination of drug origin

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Abstract

Capillary electrophoresis has been utilized to detect trace components in bulk pharmaceutical products, with emphasis on the identification of differences among manufacturers that can be used for source verification in suspect/counterfeit cases. Micellar electrokinetic capillary chromatography with sodium dodecyl sulfate was used in the analyses of β-lactam antibiotics. The aminoglycoside clindamycin phosphate and the macrolide erythromycin stearate were analyzed using borate buffers with direct UV detection. Methyl-β-cyclodextrin was used as a buffer additive in the erythromycin studies. Determination of product potency using peak area ratios has been demonstrated for ampicillin and clindamycin phosphate.

1. Introduction

The use of capillary electrophoresis (CE) and micellar electrokinetic capillary chromatography (MECC) to detect trace components, drug-related impurities, and degradation products within a single pharmaceutical product has been demonstrated for a variety of compounds [1–11]. During the course of sample analysis at the National Forensic Chemistry Center, we may be called upon to determine whether a finished product contains a drug supplied by one manufacturer or another, particularly in the area of counterfeit pharmaceuticals. The Food and Drug Administration has legal and scientific processes, such as the New Drug Application (NDA) and Abbreviated New Drug Application (ANDA), by which it approves new and generic drugs. However, the agency must ensure that drugs are produced only by approved manufacturers, and that the manufacturer adheres to the processes, formulations, and source of raw materials that have been approved.

The development of protocols that identify characteristic components in a bulk drug is essential in this task. In cases concerning β-lactam antibiotics, these procedures may utilize simple modifications of previously published MECC methods [4,12]. In other cases the pharamaceuticals of interest have not been studied by CE or MECC, except as the means by which compounds such as macrolide antibiotics have been introduced into a mass spectrometer [13].

The work presented in this paper summarizes efforts to detect differences in bulk products in order to distinguish among manufacturers. Fig. 1 shows the structures of the three β-lactam antibiotics, the aminoglycoside clindamycin phosphate, and the macrolide erythromycin stearate

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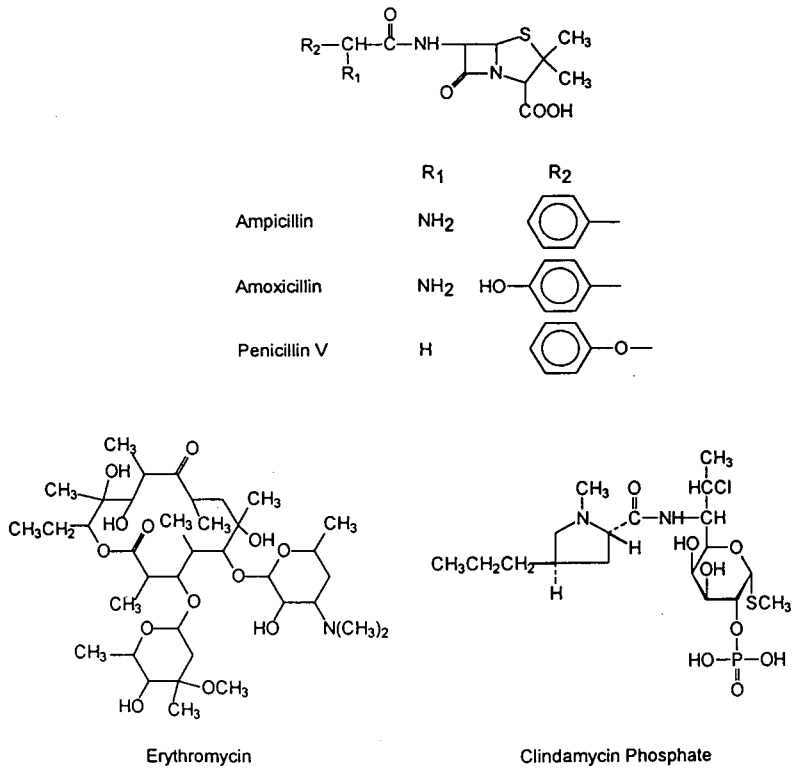

Fig. 1. Structures of antibiotics used in these studies.

that were the focus of studies being conducted in this laboratory. In the analyses of clindamycin phosphate and erythromycin stearate, the development of new separation protocols was required to enhance the minor differences observed among sample manufacturers.

2. Experimental

2.1. Reagents

Amoxicillin trihydrate (AMOX; lot H-1), penicillin V potassium (PENV; lot F-2), clindamycin phosphate (CLIPHOS; lot H), clindamycin hydrochloride (CLIND; lot G-1) and erythromycin stearate (ERYTHST; lot G-1) standards were purchased from US Pharmacopeial Convention (Rockville, MD, USA). Lincomycin hydrochloride (LINC; lot 51H05735), penicillin G potassium (PENG; lot 12H0275), penicillin V potassium (lot 20H0290), ampicillin anhydrous (lot 71H0594), and 6-aminopenicillanic acid (AMINOPEN; lot 30H3498) standards were purchased from Sigma (St. Louis, MO, USA). HPLC-grade isopropanol and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Methyl-β-cyclodextrin (Me-β-CD) was obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were reagent grade. Distilled, deionized water was obtained in the laboratory from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Buffers were filtered through 0.2-μm nylon 66 filters (Alltech, Deerfield, IL, USA), and were degassed under aspirator vacuum. Other bulk drug samples used in these studies were available in the laboratory.

Fused-silica capillaries of 50 μm I.D. $\times$ 360 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA), and Isco (Lincoln, NE, USA).

2.2. Instrumentation

For the study of the β-lactam antibiotics, a Dionex capillary electophoresis system with an Advanced Computer Interface (Dionex, Sunnyvale, CA, USA) was used, and data collection and processing were accomplished with the AI-450 chromatography software package. CLIPHOS and ERYTHST studies utilized an Isco Model 3140 Electropherograph. Data collection and processing were accomplished with the Isco capillary electrophoresis software package.

2.3. Sample analyses

β-Lactam antibiotics

Stock solutions of standards and samples were prepared in 1% phosphate buffer, pH 6, to concentrations of 2.0 mg/ml, and were diluted with the phosphate buffer to appropriate levels as necessary. A 0.20 mg/ml mixture of AMOX, ampicillin, PENG, AMINOPEN and PENV was used to standardize the system in terms of migration times and peak areas. Due to sample deterioration, all solutions were used within 3 h of preparation.

Samples were introduced into the capillary by gravity injection at a height of 100 mm for 15 s. Separations took place in an 80 cm capillary (75 cm to detector), using a 0.050 *M* sodium dodecyl sulfate (SDS)–0.10 *M* NaH_2PO_4–0.05 *M* $Na_2B_4O_7$, pH 8 buffer at +18 kV with 205 nm direct detection. Analyses were accomplished at ambient temperatures. The capillary was rinsed for 3 min between analyses with buffer.

For the profiling experiments, all samples were analyzed initially without spikes. The relative percentage composition of a component within a sample was calculated after normalizing the component peak areas with respect to their migration times [1]. Ampicillin samples of 50 μg/ml were quantitated with 20 μg/ml AMOX as an internal standard. Potency is given in terms of micrograms of anhydrous ampicillin per milligram of solid, so the appropriate adjustment in concentration was calculated if the sample was in the trihydrate form. A ratio of normalized peak areas of ampicillin/AMOX was used as a single point comparison between the Sigma ampicillin standard (920 μg/mg potency) and the sample.

Clindamycin phosphate

Sucrose was used as an internal standard in these studies. Samples of CLIPHOS and sucrose were dissolved in distilled water such that the sucrose concentration was 15 mg/ml and CLIPHOS was 0.18 mg/ml. Samples were introduced into the capillary by vacuum injection at 25.0 kPa s.

Separation was accomplished at 34°C with a 75 m*M* $Na_2B_4O_7$ buffer, pH 9, at +15 kV in a 70 cm capillary (45 cm to detector). Direct detection took place at 195 nm. The capillary was rinsed with buffer for 3 min between analyses. For the profiling experiments, all samples were analyzed initially without spikes. The potency of the CLIPHOS samples was determined with single point comparisons of the ratio of normalized peak areas of CLIPHOS/sucrose to the US Pharmacopeia (USP) CLIPHOS standard (799 μg clindamycin/mg clindamycin phosphate).

Erythromycin stearate

Samples were dissolved in isopropanol, and were diluted with distilled water to produce a 4.0 mg/mL ERYTHST solution in isopropanol–water (50:50). Due to sample deterioration, solutions were prepared daily. The buffer used was 60 m*M* $Na_2B_4O_7$–10 m*M* Me-β-CD–5% methanol, pH 9, and was prepared daily. Samples were introduced into the capillary by vacuum injection at 25.0 kPa s. Separation was accomplished over a 90 cm capillary (65 cm to detector) at +22 kV, with 205 nm direct detection at 34°C. The capillary was rinsed with buffer for 4 min between analyses.

3. Results and discussion

3.1. β-Lactam antibiotics

This family of antibiotics is perhaps the most widely used of all antibiotics available. Members of this family are also easily detected at UV wavelengths, and have been studied extensively

by CE and MECC as separate standards [4,12,14,15], in tablets and injectable solutions [7,15], in gastric contents [16] and in human plasma [17]. The method used in the present studies was a modification of the MECC buffers previously used [4,12]. Table 1 demonstrates the reproducibility of the separation of the standard mixture over six consecutive injections. The relative standard deviations (R.S.D.s) given for the migration times of the five standards are indicative of those observed with the components discussed below.

Fig. 2 shows a qualitative comparison of AMOX samples from three different manufacturers, and Table 2 summarizes the relative percentage compositions of the trace components of interest. The peak identified in Fig. 2 as a degradation product differs in initial quantity from one sample to another, and is essentially absent from manufacturer C (Fig. 2D). However, the intensity of this peak increases with solution age, and therefore cannot be used reliably as a point of differentiation. The presence of component 2 is an interesting feature of manufacturer C; the small peak present in the sample from manufacturer A at a similar migration time was not present consistently and was not evaluated. Additionally, the baseline distortion seen at 21 min represents a unique aspect of the amoxicillin samples obtained from manufacturer C.

Although samples from all manufacturers test-

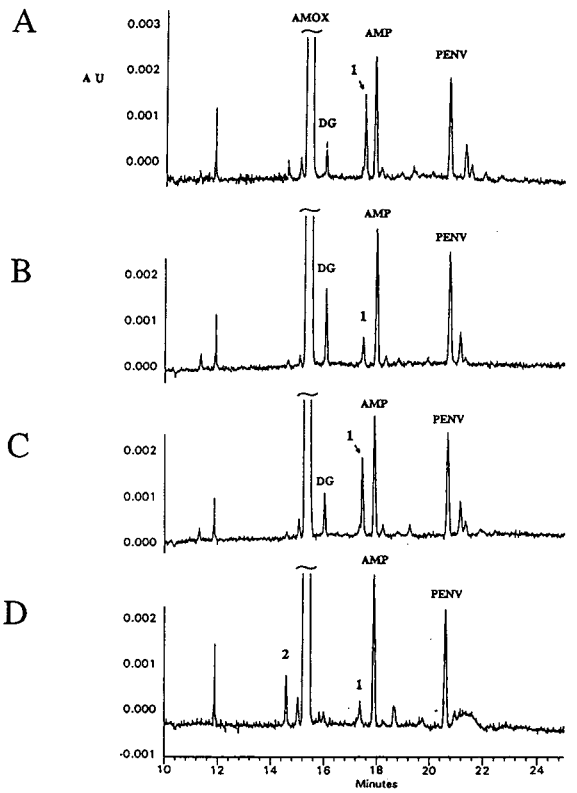

Fig. 2. Capillary electrophoretic analysis of 2.0 mg/ml AMOX samples: (A) manufacturer A; (B) manufacturer B, sample 1; (C) manufacturer B, sample 2; (D) manufacturer C. Separation conditions are given in text. DG = Degradation product; AMP = 20 μg/ml ampicillin spike; PENV = 20 μg/ml penicillin V potassium spike. Percentage composition of components 1 and 2 are given in Table 2.

Table 1
Reproducibility of β-lactam antibiotic standard mixture ($n = 6$)

	AMOX	Ampicillin	PENG	AMINOPEN	PENV
Migration time (min)					
Average	15.63	17.97	18.52	20.02	20.91
S.D.	± 0.12	± 0.12	± 0.12	± 0.14	± 0.14
R.S.D. (%)	0.78	0.67	0.65	0.68	0.68
Peak area					
Average	12 259	13 913	12 609	6552	12 363
S.D.	± 242	± 229	± 197	± 98	± 160
R.S.D. (%)	1.97	1.65	1.56	1.50	1.29

Table 2
Comparison of relative percentage composition of components in amoxicillin by manufacturer

	A[a]	B	C[b]
Component 1			
Average	0.544	0.0947[c]	0.160
S.D.	±0.050	± 0.0198	± 0.018
R.S.D. (%)	9.2	20.9	11
Average		0.904[d]	
S.D.		± 0.072	
R.S.D. (%)		8.0	
Component 2			
Average			0.342
S.D.			±0.030
R.S.D. (%)			8.8

Component numbers correspond to those given in Fig. 2.
[a] $n = 12$; three lots, four repetitions each.
[b] $n = 17$; repetitions from same lot.
[c] $n = 7$; two lots, four repetitions each; value of 0.042 discarded.
[d] $n = 12$; three lots, four repetitions each.

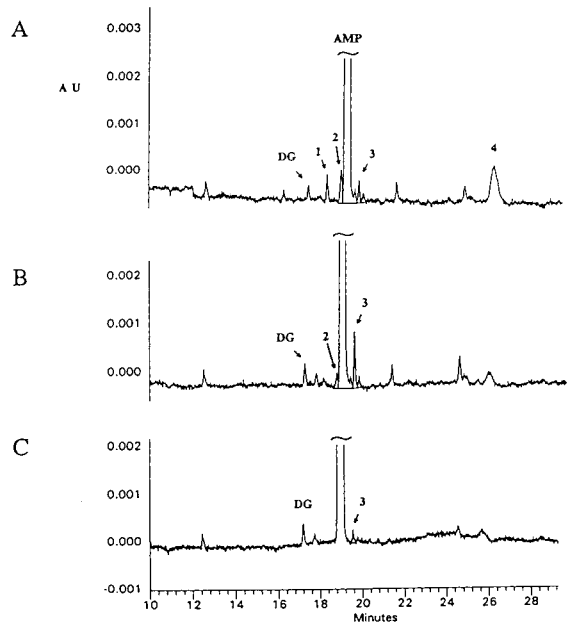

Fig. 3. Capillary electrophoretic analysis of 2.0 mg/ml ampicillin (AMP) samples: (A) manufacturer E; (B) manufacturer D; (C) manufacturer F. Separation conditions are given in text. DG = Degradation product. Percentage composition of components 1–4 are given in Table 3.

ed contain components corresponding to component 1 (Fig. 2), the differences in relative abundances between manufacturers are outside the standard deviations determined during analyses. They are sufficiently significant that they can be used as markers to distinguish between the lots analyzed from manufacturer A and the two sets of lots analyzed from manufacturer B (see Table 2).

Similar qualitative and quantitative comparisons can be made among ampicillin samples (Fig. 3 and Table 3) and PENV samples (Fig. 4 and Table 4) to facilitate the identification of a particular manufacturer. As was the case with manufacturer B, samples of ampicillin that were obtained from manufacturer E show some variation among lots. However, by utilizing the relative abundances of both components 2 and 3, the two sets of lots from manufacturer E can be distinguished from manufacturer D and from one another. In the case of the penicillin V samples, the relative cleanliness of the sample (Fig. 4C) as compared to the standards (Fig. 4A and B) can be used as identification.

The R.S.D.s reported in Tables 2–4 are not as small as those reported for components having relative abundances of ≤1% [1] or ≤0.1% [4] in other pharmaceutical preparations. Solutions of all three β-lactam antibiotics demonstrated nonlinear behavior at concentrations above 1.2 mg/ml, so the use of 2.0 mg/ml solutions could contribute to the poor R.S.D.s. However, the qualitative differences among samples were not as pronounced in solutions of 1.0 mg/ml, because the smaller peaks could not be distinguished from baseline fluctuations with confidence. For the purposes of this series of experiments, the relatively poor R.S.D.s were accepted, because differences between samples were outside the range of standard deviations established.

Quantitation of ampicillin using amoxicillin as an internal standard is given in Table 5. The USP method for potency determination is an HPLC procedure [18]. Both the USP and CE methods are straightforward and involve the use of peak area ratios. The potency values calcu-

Table 3
Comparison of relative percentage composition of components in ampicillin by manufacturer

	E	D	F
Component 1			
Average	0.313		
S.D.	± 0.026		
R.S.D. (%)	8.3		
Component 2			
Average	0.441	0.248	
S.D.	± 0.018	± 0.027	
R.S.D. (%)	4.1	11	
Average	0.192[a]		
S.D.	± 0.030		
R.S.D. (%)	16		
Component 3			
Average	0.191	0.564	0.077
S.D.	± 0.022	± 0.027	± 0.026
R.S.D. (%)	12	4.8	34
Average	0.368[a]		
S.D.	± 0.017		
R.S.D. (%)	4.6		
Component 4			
Average	1.143		
S.D.	± 0.106		
R.S.D. (%)	9.27		

Component numbers correspond to those given in Fig. 3; $n = 3$; repetitions from one sample lot, unless otherwise noted.
[a] $n = 6$; two lots, three repetitions each.

lated with the CE normalized peak area ratios are within 7% of the declared potency, as given by the supplier of the antibiotic using the USP procedure. Reproducibility of this method is given in Table 6, with assays performed over four days with fresh preparations of standards and unknowns, and two buffer preparations. It is interesting that the potency values obtained for the trihydrate preparations were consistently higher than their declared values, and that those for the anhydrous preparation were consistently lower. It is not known whether this behavior is indicative of a general trend, or whether it simply occurred with the three samples used in this study.

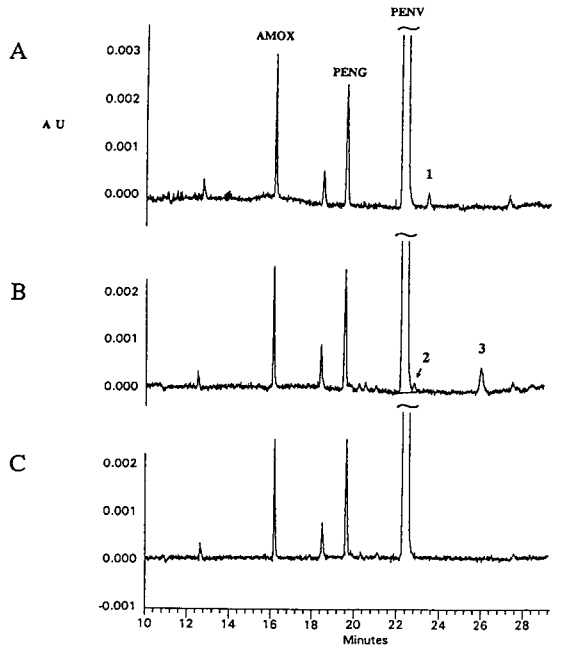

Fig. 4. Capillary electrophoretic analysis of 2.0 mg/ml PENV samples: (A) USP Standard; (B) Sigma standard; (C) manufacturer G. Separation conditions are given in text. AMOX = 20 μg/ml amoxicillin spike; PENG = 20 μg/ml penicillin G potassium spike. Percentage composition of components 1–3 are given in Table 4.

Table 4
Comparison of relative percentage composition of components in penicillin V potassium by supplier

	USP	Sigma
Component 1		
Average	0.126	
S.D.	±0.010	
R.S.D. (%)	7.9	
Component 2		
Average		0.095
S.D.		± 0.026
R.S.D. (%)		27
Component 3		
Average		0.424
S.D.		± 0.014
R.S.D. (%)		3.3

Component numbers correspond to those given in Fig. 4. $n = 4$.

Table 5
Quantitation of ampicillin

	Normalized peak areas ampicillin/AMOX	Quantitation (potency)[a]	
		Declared[b]	Calculated
Sigma Standard[c]			
Average	2.538	920	
S.D.	±0.066		
R.S.D. (%)	2.6		
Manufacturer D[d]			
Average	2.096	849	891
S.D.	±0.054		
R.S.D. (%)	2.6		
Manufacturer E[d]			
Average	2.164	867	914
S.D.	±0.071		
R.S.D. (%)	3.3		
Manufacturer F[c]			
Average	2.594	996	931
S.D.	±0.042		
R.S.D. (%)	1.6		

[a] Potency given in terms of micrograms of ampicillin per milligram of solid.
[b] Based on USP protocol [18] utilizing HPLC procedure.
[d] $n = 4$; preparation is anhydrous form.
[d] $n = 6$; preparation is trihydrate form.

3.2. Clindamycin phosphate

Clindamycin phosphate is the water-soluble ester of clindamycin, which, in turn, is a semi-synthetic derivative of lincomycin, produced by *Streptomyces lincolnensis* [19]. Clindamycin phosphate is used in the treatment of serious infections that are caused by susceptible anaerobic bacteria and strains of streptococci, pneumococci and staphylococci [20], and is reserved for penicillin-allergic patients. As stated by Ackermans *et al.* [21], CE studies of aminoglycoside

Table 6
Reproducibility of ampicillin potency determination

	Manufacturer		
	D	E	F
	Average potency values (μg/mg)[a]		
	891, 903, 884, 961[b]	914, 935, 921, 903	931, 970, 964, 947
Average	893	918	953
S.D.	± 10	± 13	± 18
R.S.D. (%)	1.1	1.4	1.9

[a] n between 4 and 6 per day.
[b] Value excluded.

antibiotics as a group have been avoided, because these antibiotics in general contain no chromophores, so direct detection is difficult. The use of imidazole for indirect detection and a cationic surfactant at low pH for analysis in an anodic mode has met with considerable success with regard to detection sensitivity and separation selectivity [21]. Unfortunately, for the work that we are called to do, this approach may not accentuate differences among manufacturers of the same antibiotic.

Previous work with mono-, di- and oligosaccharides [22,23] has demonstrated that borate buffers form UV-detectable complexes with hydroxyl moieties. As seen in Fig. 1, CLIPHOS contains a *cis*-1,2-diol configuration on the pyranose ring and should form a particularly stable complex [22]. The negatively charged complex should also alleviate interactions with the capillary wall, and permit analyses in the cathodic mode that were not previously successful [21].

Fig. 5 (trace A) demonstrates the detection of the USP standard CLIPHOS and the internal standard sucrose (peak 1), with efficiencies for CLIPHOS on the order of 200 000 plates/m. Similar behavior is seen with the sample from manufacturer H (Fig. 5, trace B). The migration times of sucrose (9.307 ± 0.020 min, $n = 14$) and

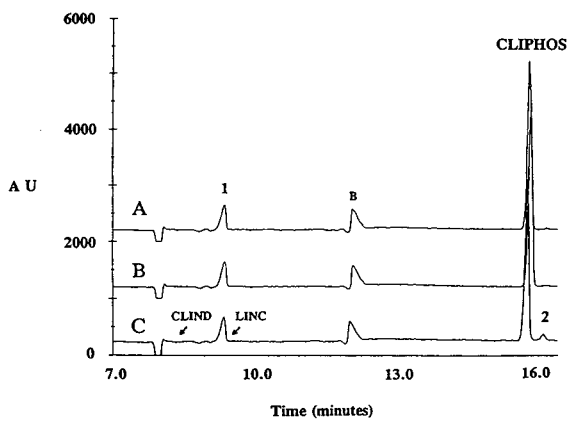

Fig. 5. Separation of internal standard sucrose (15 mg/ml; peak 1), CLIPHOS (0.18 mg/ml), and unknown (peak 2) from (A) USP Standard, (B) manufacturer H and (C) manufacturer J. Separation conditions are given in text. B = Buffer blank.

CLIPHOS (15.730 ± 0.019 min, $n = 14$) yield R.S.D.s of approximately 0.2%. Analysis of a sample from manufacturer J reveals the presence of an unidentified component (peak 2, Fig. 5, trace C) with a migration time of 16.053 ± 0.006 min ($n = 4$). Two reasonably expected impurities, clindamycin and lincomycin, migrate as indicated by the arrows in Fig. 5 (trace C). Although LINC and sucrose co-migrate under these buffer conditions, separate injections of the CLIPHOS samples without added sucrose do not indicate the presence of lincomycin. Two samples from different lots from manufacturer J yielded relative percentage compositions of the minor peak as 4.614 ± 0.121% and 4.024 ± 0.069%. There are indications of the presence of this unknown in the other samples, but it is not readily quantitated.

Table 7 summarizes the quantitation of CLIPHOS based on the use of sucrose as an internal standard. The USP method for potency determination of clindamycin phosphate is an HPLC procedure [24]. For the bulk drug, current USP guidelines require not less than 758 μg of clindamycin per mg of clindamycin phosphate, calculated on the anhydrous basis. As seen in Table 7, this CE methodology is 5% from the manufacturers' declared potency. The acceptable range for injectable solutions is 90–120% of the potency given on the product label, and 90–110% for topical solutions [24]. As was the case with ampicillin, the CE method given here is well within USP guidelines for product acceptance.

3.3. Erythromycin stearate

Erythromycin stearate is the stearic acid salt of the macrolide antibiotic erythromycin, which is used against many gram-positive bacteria [20]. Fig. 6 illustrates the presence of minor components within the USP standard and samples from two manufacturers. The migration times decreased over the course of the day, presumably due to the changing concentration of methanol at the elevated temperatures used in these experiments. However, the buffer is sufficiently stable to verify the presence of ERYTHST and

Table 7
Quantitation of clindamycin phosphate in terms of micrograms of clindamycin per milligram of clindamycin phosphate

	Peak area[a]		Area ratio CLIPHOS/Sucrose	Quantitation[b]	
	Sucrose	CLIPHOS		Declared	Area ratio
USP Standard					
Average	12 120	42 678	3.5255	799	
S.D.	± 240	± 310	±0.0732		
R.S.D. (%)	1.98	0.726	2.08		
Manufacturer H					
Average	12 382	46 164	3.7323	845	856
S.D.	± 299	± 513	±0.0785		
R.S.D. (%)	2.41	1.11	2.10		
Manufacturer J, sample 1					
Average	11 492	39 806	3.4661	828	789
S.D.	± 326	± 395	±0.0964		
R.S.D. (%)	2.84	0.992	2.78		
Manufacturer J, sample 2					
Average	11 667	41 626	3.5690	846	817
S.D.	± 228	± 275	±0.0738		
R.S.D. (%)	1.95	0.661	2.07		

$n = 8$ for all samples.
[a] Peak areas normalized during data acquisition.
[b] Calculated as ratio of USP response to sample.

to perform identification experiments. Component 1 is used primarily as a qualitative indicator, because the peak shape is too poor to give reproducible relative abundances, as indicated in Table 8. However, the absence/presence of component 1 definitely differentiates between the supplier of the USP product and the other manufacturers. Component 2 demonstrates good

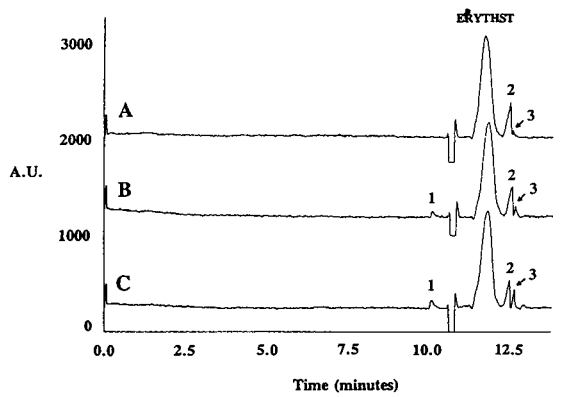

Fig. 6. Capillary electrophoretic analysis of 4.0 mg/ml ERYTHST samples: (A) USP Standard; (B) manufacturer K; (C) manufacturer L. Separation conditions are given in text. Percentage composition of components 1–3 are given in Table 8.

Table 8
Comparison of relative percentage composition of components in erythromycin stearate by manufacturer

	USP[a]	K[b]	L[b]
Component 1			
Average		3.570	1.936
S.D.		±0.190	± 0.293
R.S.D. (%)		5.3	15
Component 2			
Average	11.51	7.471	10.83
S.D.	± 0.32	±0.377	± 0.37
R.S.D. (%)	2.8	5.0	3.4
Component 3			
Average	1.27	3.450	2.233
S.D.	± 0.10	±0.296	± 0.173
R.S.D. (%)	7.9	8.6	7.8

[a] $n = 5$ from one lot.
[b] $n = 4$ from one lot.

reproducibility and can be used to distinguish among the USP supplier and manufacturers K and L, particularly when coupled with the relative abundance of component 3. Two additional lots from manufacturer L were studied. The relative abundances for component 2 were 11.27 and 11.15%, and for component 3, 4.148 and 3.038%. Using component 2, samples from manufacturers K and L can still be distinguished, despite the fact that component 3 is more variable and cannot be used in this instance.

4. Conclusions

The studies presented in this paper have demonstrated the ability to utilize the efficiency and flexibility inherent to CE and MECC to facilitate the differentiation among manufacturers of bulk pharmaceutical products. Reproducibility of these methods allows both qualitative and quantitative comparisons in terms of the relative percentage abundances of minor components. It is also possible to utilize CE methods to determine product potency, circumventing the need for multiple identification and quantitation assays called for in USP guidelines. As a matter of course, it will be necessary to determine whether the qualitative and quantitative aspects of this work will be applicable to finished products such as tablets, injectables, and ointments, which contain excipients that may interfere with some analyses.

The comparisons presented here are by no means exhaustive of all approved manufacturers of each of these pharmaceuticals. It is recognized that further refinements of the methods and data manipulations used may be required as the data base increases. With regard to differences that appear within samples from one manufacturer, we will need to establish a possible pattern between trace component profiles and dates of production, amended preparation procedures, etc. This pattern recognition would be facilitated by the identification of the impurities themselves. Additionally, verification will be necessary by comparing the presumed manufacturer's sample to an authentic sample, particularly to account for variations due to buffer preparation, capillary age, ambient temperature, and differences among lots from the same manufacturer. Although much work has yet to be done, the work presented here demonstrates that capillary electrophoretic methods present a viable approach to the cataloging of differences among manufacturers of bulk pharmaceuticals.

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JOURNAL OF CHROMATOGRAPHY A

Review

In-depth chromatographic analyses of illicit cocaine and its precursor, coca leaves

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Abstract

Chromatographic methodology used for the in-depth alkaloid analyses of coca leaves and for the characterization of alkaloidal impurities and manufacturing by-products in illicit refined cocaine samples is reviewed. This includes liquid–liquid partition and liquid–solid adsorption column chromatography, packed- and capillary-column gas chromatography with flame-ionization, electron-capture, nitrogen–phosphorous and mass spectrometric detection, and high-performance liquid chromatography with ultraviolet detection. The rationale supporting the presence and determination of processing impurities/by-products in cocaine samples is discussed, and chromatographic methodology used for the development of drug impurity signature profiles is presented.

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1. Introduction

Chromatography has played a dominant role in forensic drug chemistry over the past 25–30 years. This not only pertains to routine drug analyses but, more recently, to the comprehensive characterization of trace-level manufacturing impurities in illicit drugs. This review focuses upon chromatographic methodology reported for the detection, characterization and determination of manufacturing impurities in one of those drugs, illicit refined cocaine. Also presented are procedures that have been reported recently for the development of cocaine impurity signature profiles. Since illicit cocaine is derived primarily from the South American coca leaf, methods describing the analysis of both known and recently identified alkaloids in that matrix are also discussed. Concomitantly, the relationship between cocaine manufacturing impurities and alkaloids of the coca leaf is presented. For comparison purposes, the analyses of cocaine-bearing cultivars grown in a greenhouse environment is also reviewed.

As a preface to this review, the rationale behind the characterization of drug manufacturing impurities will be presented, along with a brief discussion of how these impurities are generated. Also included will be an overview of both classical and state-of-the-art chromatographic techniques utilized in forensic drug chemistry. The preface will conclude with a cursory review of both chemical derivatization and artifact formation and the impact they have

upon alkaloid analyses and in the development of drug impurity signature profiles.

2. Background

One of the major goals in forensic drug chemistry/research is the full characterization of manufacturing impurities and by-products in illicit drugs. This includes the structural elucidation of these compounds, their quantitative determination, a rationalization for their presence in drugs, investigation of their chemical stability and an evaluation of chemicals/solvents used in the process. If the drug has a natural product origin, such as cocaine, then the in-depth analysis of the plant may also be warranted. The drugs most frequently studied with regard to manufacturing impurities/by-products have been cocaine [1–38], heroin and amphetamine-type compounds.

A thorough analysis of drug processing impurities can provide both tactical and strategic intelligence data to drug enforcement personnel. These include: (1) the ability to compare drug seizures from similar or disparate areas to determine if they were derived from a common source; such information is useful in the development of drug conspiracy cases for eventual criminal prosecution; (2) data derived from processing impurities may allow for the determination of the drug's geographic origin; this applies especially to drugs that have natural product origins, such as cocaine and heroin; (3) tracing of drug distribution routes; (4) an evaluation of the drug's precursor and/or intermediate chemicals used in its manufacture/synthesis; this allows for the monitoring of such chemicals to determine if their use is for legitimate or illicit purposes; (5) the possibility, in some cases, to differentiate between a drug that was manufactured illicitly and that same drug diverted from legitimate pharmaceutical sources; and (6) some illicit drug manufacturing impurities may have serious health implications, even more so than the parent drug; the characterization of such compounds could assist in understanding their toxicology, as a prelude to medical treatment.

3. Origin of manufacturing by-products and impurities in illicit refined cocaine

During the clandestine manufacture of illicit refined cocaine from coca leaves, manufacturing by-products are created that can be detected in the final product. These by-products can be formed by a variety of chemical processes. One of the most common is hydrolysis of the parent drug, which can occur during the manufacture/synthesis or while in storage. Hydrolysis usually occurs at ester linkages; thus, hydrolysis of cocaine yields benzoylecgonine, ecgonine methyl ester, ecgonine and benzoic acid [2,3,38]. Minor alkaloids co-extracted from the coca leaf along with cocaine can also undergo hydrolysis at ester sites. These alkaloids include the cinnamoylcocaines, tropacocaine and the truxillines, among others. Other impurities are the result of oxidative processes. For example, the action of strong oxidants such as peroxides, present as impurities in solvents such as diethyl ether, may cause some tertiary N-methyl amine drugs to undergo N-demethylation. This creates a class of impurities sometimes referred to as an "N-nor" series [39]. The use of potassium permanganate during the processing of illicit cocaine can result in the oxidation of cocaine to yield trace levels of N-formylnorcocaine [24,28]. Other manufacturing by-products are the direct result of epimerization reactions. Thus, ecgonine and ecgonine methyl ester may undergo epimerization at their C-2 positions in the presence of strong base to form their pseudo-epimers [22,40]. Finally, rearrangement reactions may occur during the manufacturing process. Thus, the presence of N-benzoylnorecgonine methyl ester in cocaine samples is believed to be the result of the base-assisted benzoyl migration from the C-3 position to the nitrogen site of N-norcocaine [28]. The levels of different manufacturing by-products in refined cocaine samples vary significantly, about 2–3

orders of magnitude, with the hydrolytes of cocaine being the most abundant.

In addition to manufacturing by-products, refined drugs that have natural product origins are usually contaminated with alkaloidal impurities. When cocaine is extracted from the South American coca leaf, minor alkaloids that are also present in the leaf "survive" the manufacturing process and can be detected in refined samples. These include the cinnamoylcocaines, truxillines, tropacocaine, hydroxycocaines and trimethoxy-substituted alkaloids [1,14,33,36,38]. These alkaloidal impurities vary widely in concentration in illicit refined cocaine samples, from as high as 5–10% to below 10^{-3}% (relative to cocaine).

Yet another source of illicit cocaine contamination is due to processing solvents. The solvent residues that are present at trace levels in illicit refined cocaine samples are believed to appear during the conversion of cocaine base to cocaine hydrochloride. Using nuclear magnetic resonance spectroscopic techniques and headspace gas chromatography–mass spectrometry, solvents that have been detected and quantified include acetone, methyl ethyl ketone, toluene and diethyl ether, among others [41–44].

4. Chromatographic analysis of drug manufacturing impurities and by-products

Chromatography is an integral part of methodology developed for the in-depth analyses of manufacturing impurities and by-products in illicit drugs. This encompasses classical techniques, including various types of column chromatography, and modern applications using capillary gas chromatography (cGC), high-performance liquid chromatography (HPLC) and, most recently, capillary electrophoresis (CE).

4.1. Column chromatography

Classical chromatographic techniques, such as column chromatography, have been utilized over the years for the analyses of many and varied complex matrices. Two types of column chromatography have been most relevant in forensic drug research, namely, liquid–liquid partition and liquid–solid adsorption chromatography. The former type is usually a combination of ion-pairing, "trap" and liquid–liquid partition column chromatography. Typically, cylindrical glass columns (of widely varying lengths and diameters) are packed with a mixture of an acidic or basic aqueous phase and a stationary support of Celite 545, an inert diatomaceous earth. In ion-pairing chromatography the stationary phase is generally a mixture of dilute hydrochloric or nitric acid and Celite 545, while the mobile phase is halogenated and includes chloroform or methylene chloride. Typically, in ion-pairing chromatography an amine drug or basic impurity is eluted quantitatively from the chromatographic column as a hydrochloride or nitrate ion pair. In "trap" chromatography the drug or by-product is quantitatively retained, or "trapped", on the column. For this to occur the compound must be basic or acidic in character. Typical column packings for trap chromatography include mixtures of Celite 545 with dilute inorganic or organic acids or bases, *e.g.*, sulfuric, hydrochloric, nitric, citric and tartaric acids; bases include sodium hydrogencarbonate, sodium carbonate and sodium hydroxide. Neutral compounds, depending upon their polarity and/or solubility characteristics, can also undergo liquid–liquid partition column chromatography. In practical terms, most parent drug/manufacturing impurity separations using Celite column chromatography are a combination of ion-pairing, trap and liquid–liquid partitioning chromatography. Typical mobile phases must be water-immiscible and include chloroform, methylene chloride, diethyl ether, light petroleum and toluene, among others. Ion-pairing and related column chromatography methodology, as described by Doyle and Levine [45,46], have been utilized extensively for the resolution of legitimate pharmaceutical drug mixtures for quantitation by ultraviolet spectroscopy. Forensic chemists have adapted this methodology for the isolation of alkaloidal impurities and manufacturing by-products from illicit cocaine and in the isola-

tion of alkaloids from coca leaf and other coca alkaloids [1,35,36].

In liquid–solid adsorption column chromatography, the stationary phase consists of activated adsorbents such as alumina, silica gel and Florisil; mobile phases are organic and vary widely in polarity. Typical mobile phases include chloroform, acetone, hexane, diethyl ether, methylene chloride, methanol and acetonitrile, among others. Separations are based, in part, on the relative polarities, solubility characteristics and/or molecular mass of the components in the drug matrix and upon the level of activity of adsorbent and polarity of mobile phase. The separation mechanisms are roughly analogous to those occurring in flat-bed chromatography, *e.g.* thin-layer chromatography, except in adsorption column chromatography the conditions are usually anhydrous. In fact, some types of adsorption column chromatography might be viewed as thin-layer chromatography in a column. A major advantage of this type of column chromatography, as was the case with Celite column chromatography, is the capability of fraction collection and the ease of varying the polarity of the mobile phase. These advantages have proven useful both in the isolation of manufacturing impurities in illicit, refined cocaine [14] and in the separation isolation of trace-level alkaloids from the bulk cocaine matrix in coca leaves [33,35]. A disadvantage that has been associated with this type of chromatography is a lack of reproducibility between different manufacturing batches of a given adsorbent.

In some instances liquid–liquid partition and liquid–solid adsorption column chromatography are used for gross separations, as a prelude to more refined isolation using higher-resolution techniques such a preparative HPLC.

4.2. Gas chromatography

Gas chromatography (GC) has been the most commonly used technique for generating chromatographic profiles of drug manufacturing impurities and by-products. This chromatography has been referred to as "impurity signature profiles", "geographic signature profiles" or "sample comparison profiles", among other names. Impurity signature methodology developed in the early-to-mid-1970s made use of packed columns for GC analyses. However, given the complex matrices associated with drug manufacturing impurities, packed columns often did not provide sufficient resolution. With the advent of fused-silica capillary columns, a marked improvement was realized in column efficiency and handling, providing the forensic chemist with a powerful analytical tool. Thus, since about 1980 the literature has seen a marked increase in reported methodology using cGC in the development of impurity signature profiles.

A plethora of capillary columns are now commercially available for use in the GC analyses of complex matrices. Furthermore, column selection has been greatly simplified with the advent of fused-silica capillary columns. A considerable body of experience in illicit drug analyses and in the characterization of alkaloidal impurities and manufacturing by-products indicate that columns of 15 m and 30 m in length, with an inner diameter of 0.25 mm, are suitable for most applications. For method development, column substrates covering a wide range of polarities have been investigated. Three of the most common are commercially available (J & W Scientific) DB-1 (non-polar), DB-5 (moderately polar) and DB-1701 (polar), all at a film thickness of 0.25 μm.

Several detection methods have been used in conjunction with the GC generation of impurity signature profiles. The most commonly used is flame-ionization detection (FID). It has sufficient sensitivity and dynamic range for the detection and determination of numerous drug manufacturing impurities and by-products. Typically, the on-column detection of nanogram levels of impurities is possible using FID. Other positive features of FID are its universal response to organic compounds and its ruggedness.

For the determination of impurities present at ultra-trace levels, FID, however, does not possess sufficient sensitivity. For these applications the use of electron-capture detection (ECD) may be warranted. When used with cGC, ECD is capable of routinely providing on-column detec-

tion levels in the low picogram and sometimes femtogram range for highly electrophilic compounds. Methodology using cGC–ECD generally requires a derivatization step that introduces an electrophilic group, such as a heptafluorobutyryl moiety or other halogenated group, into the impurity's structure [31].

Nitrogen–phosphorous detection (NPD) has been used sparingly in the cGC analysis of manufacturing impurities. For compounds containing one nitrogen atom, NPD exhibits a modest enhancement in sensitivity when compared to FID. However, it is markedly less sensitive compared to the ECD analysis of appropriately derivatized compounds. The less-than-rugged characteristic of NPD has probably limited its widespread use in signature analyses. Furthermore, the injection of certain solvents, such as silylating reagents, preclude the use of NPD. Such injections would coat the rubidium source, diminishing the concentration of this element in the flame plasma. An obvious advantage of NPD is its selectivity towards nitrogenous compounds.

When a mass spectrometer is used as GC detector, two primary advantages are realized. First, and clearly most significant, is the potential to structurally characterize the processing impurities and by-products. Although impurity signature profiles for geographic origin or sample comparison determinations can be accomplished without identification of all chromatographic components, their full structural characterization is desirable. Only in this manner can one fully rationalize the presence of impurities/by-products and discount their presence as artifacts. Second, the use of a mass spectrometer allows the incorporation of deuterated analogues of processing impurities as internal standards; such deuterated analogues are generally recognized as the ideal internal standards for the optimization of quantitative results and reproducibility.

4.3. *High-performance liquid chromatography*

The second most common technique for producing drug impurity signature profiles is HPLC. Because of its lower column efficiencies, HPLC is at a disadvantage, *vis-a-vis* cGC, in the analysis of the more complex impurity matrices. However, this limitation can be partly overcome by the use of multiple detectors. In terms of on-column detection levels, GC systems, in general, are more sensitive than those used in HPLC. This disadvantage can be partially compensated for by the fact that HPLC systems can accommodate much larger sample injections. A major attribute of HPLC is that compounds that do not chromatograph by GC because of their polarity, low volatility, or high molecular mass, are often amenable to HPLC analyses. This is perhaps best illustrated by considering the isomeric truxillines, which are alkaloids present in the coca leaf and in illicit refined cocaine samples. Having a molecular mass of 658, direct GC analysis of these alkaloids for analytical purposes is difficult because of thermal degradation in the injection port. However, direct chromatography of intact truxillines in illicit refined cocaine samples using HPLC has been successful and recently reported [15,21]. There is also some evidence to suggest that there are other high-molecular-mass alkaloids in coca leaf, heretofore unreported, that could be more suitable to HPLC rather than GC analysis.

A survey of the literature revealed that reversed-phase chromatography was favored for the HPLC analyses of drug manufacturing impurities, and for drug analyses in general. For these analyses, commercial columns included HS-5 C_{18}, μBondapak C_{18}, LC-8, RP-8 Spheri 10 and LiChrosorb RP-2 [8,10,15,20,21,25]. Typical mobile phases were usually binary and tertiary solvent mixtures that included acetonitrile, methanol and aqueous buffers, among others.

HPLC detection systems have been discussed for use in forensic drug analyses [31,47] and include dual variable-wavelength UV, diode-array UV, fluorescence and electrochemical detectors [47]. An inherent advantage of these detectors is the capability of interfacing them serially [47]; this is not possible with most cGC detection systems, such as FID and NPD, which are organic-compound destructive. The most commonly used detector for the analysis of cocaine and its manufacturing impurities/by-products are UV and diode-array UV detectors.

The latter detection system has the advantage of being able to monitor multiple wavelengths simultaneously, and in real time, thus providing some structural information.

The reported interfacing of HPLC with mass spectrometry (MS) for use in forensic drug analyses has been a rare occurrence. A major reason is that the majority of illicit drugs submitted for routine analyses are readily characterized by cGC–MS using electron ionization. Furthermore, if HPLC is interfaced with thermospray (TSP) MS, few data beyond molecular mass are obtained. Nonetheless, the use of TSP-MS has been reported for the HPLC analyses of a number of drugs of forensic interest [48,49]. Although no literature references were found for the application of TSP-MS in the analysis of drug manufacturing impurities, this technique holds promise for such. This is especially so for drugs that could be contaminated with high-molecular-mass compounds ($M_r > 600$) which are not amenable to cGC–MS analyses, especially those derived from natural products.

4.4. Micellar electrokinetic capillary chromatography

A new technique that appears very promising for forensic drug analyses is micellar electrokinetic capillary chromatography (MECC). First described in 1984 [50,51], MECC is a subclass of CE and offers significantly greater efficiency, selectivity, peak symmetry and speed, compared to HPLC. Detectors used in MECC include UV, fluorescence and electrochemical. The advantages of MECC over HPLC, *vis-a-vis* forensic drug analyses, have been described recently [52,53]. In those reports it was shown that drugs that chromatographed poorly by HPLC, or not at all by GC, exhibited good chromatographic behavior using MECC. The applicability of MECC for generating impurity signature profiles of illicit drugs such as cocaine and heroin is currently being investigated. As with TSP-MS the feasibility of determining high-molecular-mass impurities is good, as MECC has been previously reported for the analysis of nucleosides, peptides, porphyrins and antibiotics. A recognized deficiency of MECC is sensitivity, which is below that for HPLC–UV [52,54]. This could limit the use of MECC in the analysis of ultra-trace levels of manufacturing impurities.

5. Chemical derivatization

An invaluable adjunct of chromatographic analyses is chemical derivatization (ChD). This technique has played an integral role in the development of impurity signature profiles for manufacturing impurities/by-products in illicit cocaine, as well as heroin [31]. It is not the intent of this review to discuss ChD in detail, as it has been covered in-depth for general applications [55] and, specifically, for forensic drug analyses [31]. Briefly, the major reasons for the application of ChD in chromatographic analyses are to improve chromatographic behavior of target compounds and/or to enhance their detectability. Drugs and manufacturing impurities/by-products most likely to undergo derivatization have functional groups with labile protons, including RNH_2, R_1R_2NH,–OH,–COOH and–SH. There have also been reports of very unusual high-yield acylation at carbon sites in some drugs and their manufacturing impurities [31]. The most common derivatization reactions pertaining to illicit drugs include silylation, acylation and alkylation. Although ChD has been applied sparingly in routine illicit drug analyses, its greater impact is upon the analysis of manufacturing impurities, especially in lowering their minimum detectable levels. Although ChD is more widely used in cGC analyses, it can also be useful in HPLC, especially for the introduction of chromophoric or fluorophoric groups into the drugs's structure for enhanced UV or fluorescence detection.

6. Artifact formation

During the legitimate in-depth analyses of illicit drugs and natural products, especially for intelligence purposes, the chemist should be aware of the inadvertant creation of artifactual compounds during the work-up of samples. This is especially applicable to trace analyses. These artifacts are usually formed by the interaction of

bona fide alkaloids in the plant or refined drug with chemicals, solvents, and impurities in solvents as well as by the application of heat during the alkaloid isolation process. These interactions can result in epimerization, hydrolysis and N-demethylation of plant alkaloids. Thus, in coca leaf analyses using conventional extraction techniques, the detection of cocaine hydrolysis products, *e.g.*, benzoylecgonine and ecgonine, should be viewed cautiously. Likewise, the presence of trace levels of N-norcocaine in a coca leaf extract may be the result of the peroxide-assisted N-demethylation of cocaine *vs.* its presence as a *bona fide* alkaloid. Peroxides are common in some solvents, especially diethyl ether. Epimerization at the C-2 site in the tropane moiety can occur if the alkaloid work-up includes the use of a strong base. Finally, as will be discussed in a subsequent section of this review, it is suspected that the reported presence of low levels of hygrine in coca may in fact be due to the degradation of cuscohygrine during coca leaf analysis. From the foregoing, it is apparent that, in some cases, artifact formation during the legitimate analyses of coca and cocaine closely mirrors the creation of by-products during the clandestine manufacture of refined cocaine from coca leaf.

Artifact formation can also occur during chromatographic analyses of an alkaloid extract, especially if GC is used. For example, ecgonidine methyl ester has been incorrectly reported as a *bona fide* coca leaf alkaloid. Actually, this compound is an artifact formed in the heated injection port of the gas chromatograph, from the thermal degradation of cocaine [6] and/or the truxillines [14]. In a recent GC study, it was demonstrated that cocaine, in the presence of base, can undergo hydrolysis, transesterification and epimerization in the injection port [56].

7. Chromatographic analyses of alkaloids in coca leaf

From a forensic perspective, the in-depth analyses of alkaloids in the coca leaf are important, because these endogenous compounds, and the by-products derived from them, appear in illicit cocaine samples. As discussed in the introduction of this review, such data can be used for tactical and strategic law enforcement purposes. Furthermore, the structural elucidation of these alkaloids in the leaf allows for their more facile characterization in illicit, refined cocaine; this is important because, in some instances, these alkaloids experience a marked diminution in the processing of leaf to refined cocaine. Finally, the only unequivocal method for determining if an impurity detected in illicit cocaine is a *bona fide* leaf alkaloid, a manufacturing by-product, or both, is after careful examination of the alkaloid content of the leaf.

Virtually all illicit cocaine is derived from South American coca leaf. There are four recognized coca varieties derived from two species and a single genus, that contain significant levels of cocaine [57,58]. These are *Erythroxylum coca* var. *coca* (ECVC), *Erythroxylum coca* var. *ipadu* (ECVI), *Erythroxylum novogranatense* var. *novogranatense* (ENVN) and *Erythroxylum novogranatense* var. *truxillense* (ENVT). Historically, ECVC is the cultivar that has been used for the manufacture of illicit cocaine. This is doubtless because this variety grows over a much larger geographic area than do the other cultivars. Also, the cocaine content of ECVC is similar to or higher than in the other varieties. Finally, varieties such as *novogranatense* and *truxillense* contain greater levels of other alkaloids that are difficult to remove from cocaine, via current illicit production methods, thus contaminating the refined cocaine product.

Most South American coca leaf is cultivated on the eastern slopes and valleys of the Andes mountains ranging from Colombia to Bolivia. Most of the ECVC cultivated for illicit cocaine manufacture is done so in the Upper Huallaga Valley of Peru and the Chapare region of Bolivia. The cocaine content of this leaf is usually in the 0.5–0.8% range (relative to dry mass leaf) [59]. The processing of ECVC coca to yield refined cocaine has been described adequately elsewhere [60,61] and will not be discussed in detail here. Occasional reference to this methodology will be necessary, however, in relating components of the coca leaf to alkaloidal

impurities and manufacturing by-products in illicit refined cocaine.

In addition to cocaine, coca leaves contain numerous other alkaloids, including among others *cis*- and *trans*-cinnamoylcocaine, tropacocaine, the isomeric truxillines, cuscohygrine and hygrine [38]. The structures for some of these alkaloids are seen in Figs. 1, 4 and 7. Until

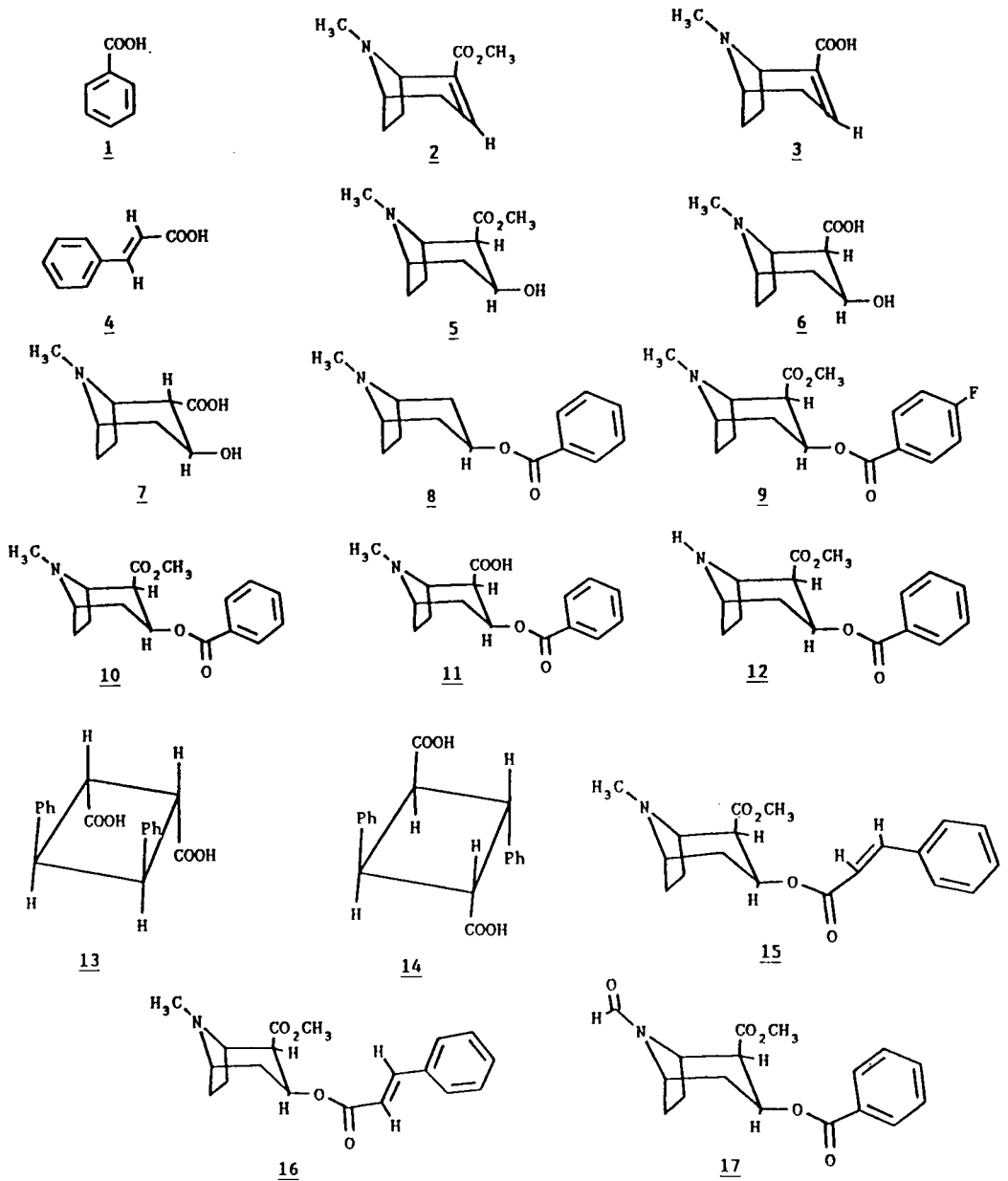


Fig. 1. Structures of some alkaloids in coca leaf and alkaloidal impurities and manufacturing by-products in illicit refined cocaine: 1 = benzoic acid; 2 = ecgonidine methyl ester; 3 = ecgonidine; 4 = *trans*-cinnamic acid; 5 = ecgonine methyl ester; 6 = ecgonine; 7 = pseudoecgonine; 8 = tropacocaine; 9 = *p*-fluorococaine internal standard; 10 = cocaine; 11 = benzoylecgonine; 12 = N-norcocaine; 13 = β-truxinic acid; 14 = α-truxillic acid; 15 = *cis*-cinnamoylcocaine; 16 = *trans*-cinnamoylcocaine; 17 = N-formylnorcocaine. From ref. 26.

recently, the only quantitative data available for South American ECVC and ENVN coca leaf alkaloids, as well as alkaloids from greenhouse-cultivated coca plants, pertained primarily to cocaine and *cis*- and *trans*-cinnamoylcocaine. Recently, however, quantitative data have been reported for other coca alkaloids, including the isomeric truxillines, tropacocaine, cuscohygrine and hygrine [34,35]. This was accomplished not only for South American ECVC and ENVN, but also for greenhouse- and non-South American tropical-cultivated ECVC, ENVN and/or ENVT.

7.1. Cocaine and the cinnamoylcocaines

In one of the early studies of forensic interest, Moore [1] applied ion-pairing column chromatography for the partial resolution of *cis*- and *trans*-cinnamoylcocaine, tropacocaine and cocaine in a coca leaf extract. Those alkaloids were eluted as hydrochloride ion pairs from a dilute hydrochloric acid/Celite column with water-saturated chloroform. This chromatography, seen in Fig. 2, provided sufficient resolution to allow, for the first time, structural characterization of the isomeric cinnamoylcocaines by UV, IR, MS and ^{1}H NMR spectroscopy. No quantitative data for the cinnamoylcocaines was provided in that study.

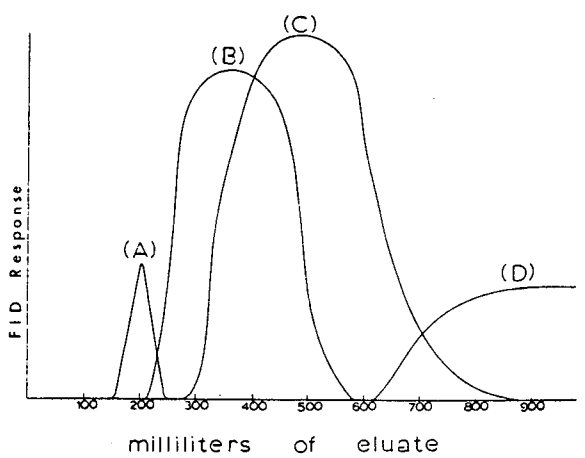


Fig. 2. Column partition chromatographic (ion-pairing) elution pattern of (A) tropacocaine, (B) *trans*-cinnamoylcocaine, (C) *cis*-cinnamoylcocaine and (D) cocaine. From ref. 1.

There have been a number of subsequent reports describing the quantitation of cocaine and *cis*- and *trans*-cinnamoylcocaine in coca leaves [58,62–67]. In one of the most significant of these, Plowman and Rivier [58] reported the cocaine and *cis*- and *trans*-cinnamoylcocaine content of ECVC, ECVI, ENVN and ENVT cultivated in Colombia, Ecuador, Bolivia and Peru. Using a stable-isotope dilution method and GC–MS with selected-ion monitoring, they reported a mean cocaine content of 0.63% and a mean *cis*- + *trans*-cinnamoylcocaine content of 10.8% for ECVC leaf (relative to cocaine). The *cis*-/*trans*-ratio for the ECVC coca had a mean value of 2.7. Table 1 summarizes the data for all four coca varieties.

In a packed column GC–FID study of ECVC leaves from Peru, Turner *et al.* [62] determined the cocaine content of samples collected from three disparate geographical regions. They reported cocaine levels in air-dried leaf of 0.60, 0.57 and 0.60% from leaves harvested in Cuzco, Trujillo and Tingo Maria, respectively. In a follow-up study by the same group [63], Peruvian ECVC leaves from three locations were analyzed for both cocaine and cinnamoylcocaine content. Differences were observed in the cinnamoylcocaine levels from each site and the relative ratios of total cinnamoylcocaines to cocaine varied with sample origin. In this latter study, coca leaf extracts were also examined by GC–NPD and GC–MS.

In an investigation using packed-column GC–MS, Holmstedt *et al.* [64] examined 13 South American species of *Erythroxylum* for cocaine content. Cocaine was found in only ECVC, ENVT and ENVN leaf from Bolivia, Peru and Colombia. The average dried-leaf cocaine content of 12 ECVC samples from Peru was 0.73% while two samples of Bolivian ECVC leaf had cocaine levels of 0.70 and 0.74%. Leaves of 10 ENVN samples collected in Colombia varied in cocaine content from 0.17 to 0.76%, with an average of 0.47%. A single sample of Peruvian ENVT leaf had a cocaine content of 0.71%. The quantitative accuracy of the foregoing results was

Table 1
Summary of cocaine and cinnamoylcocaine content of dried leaves of the cultivated cocas *E. coca* and *E. novogranatense*

	Total							
	Cocaine		Cinnamoylcocaines		*cis*		*trans*	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
E. coca var. coca (30 samples)	0.23–0.96	0.63	0.0011–0.532	0.068	0–0.44	0.05	0–0.11	0.0183
E. coca var. ipadu (6 samples)	0.11–0.41	0.25	0–0.0084	0.005	0–0.008	0.005	0	0.0
E. novogranatense var. novogranatense (3 samples)	0.55–0.93	0.77	0.107–0.65	0.379	0.072–0.53	0.287	0.035–0.12	0.092
E. novogranatense var. truxillense (14 samples)	0.42–1.02	0.72	0–0.93	0.231	0–0.68	0.154	0–0.43	0.0775

Data reported as mg 100 mg^{-1} dry mass. From ref. 58.

enhanced by the use of a deuterated cocaine standard.

Amazonian coca, *i.e.* ECVI, has been reported to have consistently lower cocaine content compared to Andean coca [58]. In a study of freshly air-dried leaves of Amazonian coca, the cocaine content ranged from only 0.11 to 0.39% [65] (authors' note: like South American ENVN and ENVT coca, leaves of ENVI are not believed to be a major factor in illicit cocaine production).

In a cGC–MS study by Rivier [66], using deuterated cocaine as an internal standard, the intra-plant variation of cocaine and the cinnamoylcocaines in coca leaves was found to be significant. It was pointed out that the variation of alkaloid levels in coca leaf can depend upon environmental conditions and leaf age. It was concluded that the variation of the alkaloid content in individual leaves was too great to allow the use of the ratio of cocaine to the cinnamoylcocaines as a taxonomic marker [authors' note: Rivier's study should encourage others to use sufficient sample size in the determination of coca leaf alkaloids, so as to render intra-and inter-plant (from the same plot) variations a non-factor when evaluating data].

In unique, non-chromatographic methods by Youssefi *et al.* [67] and Cooks *et al.* [68], cocaine/cinnamoylcocaine ratios were determined in individual coca leaves using mass-analyzed ion kinetic energy spectrometry (MIKES). They determined that coca plants from different geographic regions could be distinguished on the basis of alkaloid content. They also confirmed the findings of Rivier [66] relating to the significant intra-plant leaf variation *vis-a-vis* the cocaine/cinnamoylcocaine ratios. Among the purported advantages of MIKES were small sample size (1 mg), reduced analysis time and minimization of sample preparation, in particular the avoidance of solvent extractions. The latter is especially advantageous because of the problem of artifact formation during solvent extractions of natural products.

In a coca leaf method described by Solon and Sperling [69], and in current use by the US Drug Enforcement Administration, cocaine was quantitated by cGC–FID after its extraction from the leaf with warm (75°C) methanol. As will be discussed subsequently, this method, though not touted as such, also appeared suitable for the determination of *cis*- and *trans*-cinnamoylcocaine [35].

7.2. Cocaine, cinnamoylcocaines, tropacocaine, truxillines, ecgonine methyl ester, cuscohygrine and hygrine (see Figs. 1, 4 and 7)

In the most in-depth coca leaf/alkaloid study to date, Moore *et al.* [35] recently reported the quantitative alkaloid analyses of cocaine-bearing plants from the field in South America, a greenhouse in the USA and from a tropical site other than South America. The samples examined included ECVC and ENVN from Bolivia, Peru, Ecuador and Colombia, greenhouse-cultivated ECVC, ENVN and ENVT and non-South American tropically grown ECVC and ENVN cultivars. In that study the rationale behind the analyses of greenhouse cultivars and coca leaf other than ECVC was three-fold. First, such in-depth data had never been previously reported. Second, it was of forensic interest to know whether coca leaf of a species/variety other than ECVC yielded similar or markedly different alkaloidal profiles. Third, it was hoped that uncharacterized trace-level alkaloids in ECVC might be present at significantly higher levels in greenhouse-cultivated coca leaves, allowing for their more facile isolation and structural elucidation.

In the methodology referenced above [35], dried coca leaves were reduced to a powder, basified and the alkaloids extracted in high yield from the leaf using water-saturated toluene. Cocaine and other alkaloids were isolated from interfering leaf components in the toluene extract by retaining the former on a dilute sulfuric acid/Celite column. After the alkaloids were liberated from the column, they were quantitated using cGC–FID and cGC–ECD. In addition to the determination of cocaine and *cis*- and *trans*-cinnamoylcocaine, the quantitation of the alkaloids ecgonine methyl ester, tropacocaine, the isomeric truxillines, cuscohygrine and hygrine were reported for the first time. The accuracy and reproducibility of this methodology were enhanced by the use of structurally related internal standards [35]. The quantitative results for ecgonine methyl ester, cuscohygrine, tropacocaine, cocaine and *cis*- and *trans*-cinnamoylcocaine in South American, greenhouse and non-South American tropical-cultivated coca are found in Tables 2 and 3. The cGC–FID chromatogram for the determination of selected alkaloids in greenhouse-cultivated ENVN coca is illustrated in Fig. 3. A brief review of the results from the Moore *et al.* method is given below.

7.2.1. Cocaine and the cinnamoylcocaines

The cocaine and cinnamoylcocaine content for Bolivian and Peruvian ECVC leaf (Table 2) were in reasonable agreement with other studies. The much higher cinnamoylcocaine content for Colombian leaf was believed related to its taxonomy, which was suspected to be ENVN coca, and not due to environmental conditions. The markedly higher cinnamoylcocaine levels for greenhouse- and tropical-cultivated ENVN and ENVT coca *vs.* ECVC (Table 3) were also due to taxonomic differences. When the *trans*/*cis*-cinnamoylcocaine ratios for the greenhouse cul-

Table 2
Quantitative results for cocaine and other coca alkaloids in South American cultivated coca leaves (see ref. 35)

Country	Cocaine	Cinnamoylcocaine		Tropacocaine	Cuscohygrine
		cis	*trans*		
Bolivia	0.70	8.6	6.0	0.34	78
Peru	0.72	5.8	2.9	0.25	51
Ecuador	0.36	6.6	7.4	1.6	11
Colombia	0.44	28	33	4.9	33

All cocaine results are % (w/w) and are calculated relative to dry leaf mass. Results for all alkaloids, excepting cocaine, are % (w/w) and are calculated relative to cocaine content.

Table 3
Quantitative results for cocaine and other coca alkaloids in greenhouse- and tropical-cultivated coca leaves (see ref. 35)

Alkaloid	ECVC (G)[a]	ENVT (G)[b]	ENVN (G)[c]	ENVN (T)[d]	ECVC (T)[e]
Cocaine	0.54	0.60	0.37	0.43	0.67
Ecgonine methyl ester	57	38	63	29	47
Cuscohygrine	57	3.8	11	5.8	61
Tropacocaine	0.3[f]	1.4	4.6	3.8	0.16
cis-Cinnamoylcocaine	7.2	25	50	53	18
trans-Cinnamoylcocaine	18	46	98	170	22

All cocaine results are % (w/w) and are calculated relative to dry leaf. Results for all alkaloids, excepting cocaine, are % (w/w) and are calculated relative to cocaine content.
[a] Greenhouse-cultivated *E. coca* var. *coca*.
[b] Greenhouse-cultivated *E. novogranatense* var. *truxillense*.
[c] Greenhouse-cultivated *E. novogranatense* var. *novogranatense*.
[d] Tropical-cultivated *E. novogranatense* var. *novogranatense*.
[e] Tropical-cultivated *E. coca* var. *coca*.
[f] Sample also contained benzoyltropine, an epimer of tropacocaine, at a level of 1.1% (relative to cocaine content).

tivars in Table 3 were compared with the same ratios for South American coca in Table 2, it was seen that the ratios of the former were significantly higher. The authors attributed this to the fact that less UV light is available in the greenhouse environment to convert the *trans* isomer to its *cis* counterpart [70].

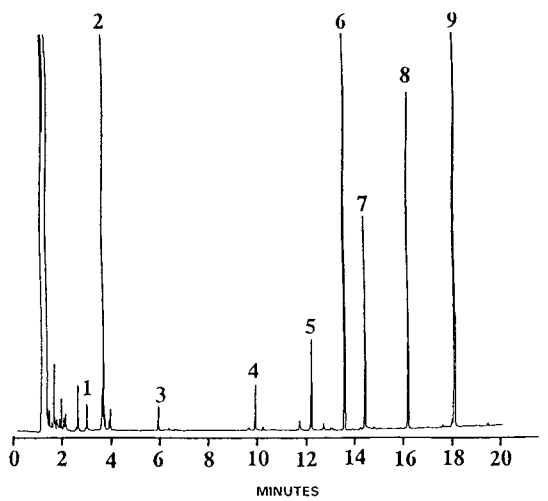


Fig. 3. The cGC–FID chromatogram of major coca alkaloids isolated and determined in greenhouse-cultivated *E. novogranatense* var. *novogranatense*. Peaks: 1 = ecgonidine methyl ester; 2 = ecgonine methyl ester; 3 = cuscohygrine; 4 = tropacocaine; 5 = 1-hydroxytropacocaine; 6 = cocaine; 7 = cocaethylene internal standard; 8 = *cis*-cinnamoylcocaine; 9 = *trans*-cinnamoylcocaine (see ref. 35).

7.2.2. Tropacocaine

With one exception, the tropacocaine levels seen in Tables 2 and 3 were unremarkable. As was the case for the cinnamoylcocaines, the tropacocaine content for the ECVC coca was markedly lower than that of ENVN and ENVT leaf, again believed due to differences in taxonomy. One of the most interesting result reported by the authors was for the greenhouse-cultivated ECVC leaf. As seen in Table 3, the epimer of tropacocaine, namely benzoyltropine, was 3 × higher in concentration. This compares with Peruvian and Bolivian coca leaf in which tropacocaine is readily detectable, but benzoyltropine, when present, is at significantly reduced levels

7.2.3. Truxillines

The coca leaf extract described above also contained the isomeric truxillines, seen in Fig. 4. These alkaloids had been previously characterized in illicit refined cocaine samples [14,18]. After isolation from the leaf, the truxillines were reduced with lithium aluminum hydride to their respective diols, which were then derivatized with heptafluorobutyric anhydride (HFBA) to

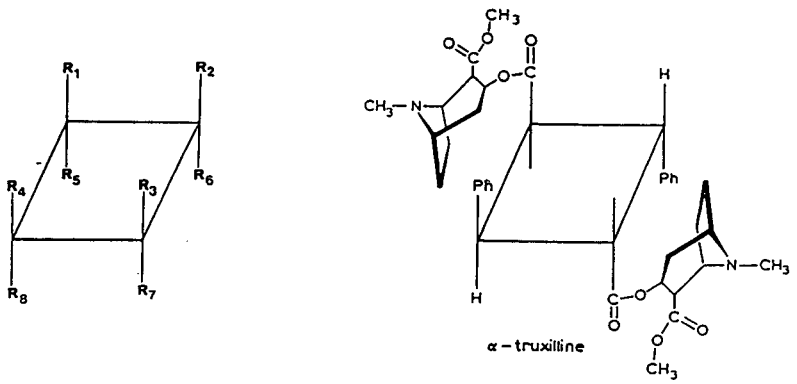


Fig. 4. Structures for the isomeric truxillines. (Left) General structure. (1) α, $R_1 = R_7$ = methyl ecgonine ester, $R_4 = R_6$ = phenyl, $R_2 = R_3 = R_5 = R_8 = H$; (2) β, $R_5 = R_6$ = methyl ecgonine ester, $R_3 = R_4$ = phenyl, $R_1 = R_2 = R_7 = R_8 = H$; (3) δ, $R_2 = R_5$ = methyl ecgonine ester, $R_4 = R_7$ = phenyl, $R_1 = R_3 = R_6 = R_8 = H$; (4) ϵ, $R_5 = R_7$ = methyl ecgonine ester, $R_2 = R_4$ = phenyl, $R_1 = R_3 = R_6 = R_8$; (5) μ, $R_1 = R_6$ = methyl ecgonine ester, $R_4 = R_7$ = phenyl, $R_2 = R_3 = R_5 = R_8 = H$; (6) γ, $R_1 = R_3$ = methyl ecgonine ester, $R_4 = R_6$ = phenyl, $R_2 = R_5 = R_7 = R_8 = H$; (7) neo, $R_2 = R_5$ = methyl ecgonine ester, $R_3 = R_4$ = phenyl, $R_1 = R_6 = R_7 = R_8 = H$; (8) ζ, $R_5 = R_6$ = methyl ecgonine ester, $R_4 = R_7$ = phenyl, $R_1 = R_2 = R_3 = R_8 = H$; (9) *epi*, $R_1 = R_7$ = methyl ecgonine ester, $R_2 = R_4$ = phenyl, $R_3 = R_5 = R_6 = R_8 = H$; (10) *peri*, $R_1 = R_3$ = methyl ecgonine ester, $R_2 = R_4$ = phenyl, $R_5 = R_6 = R_7 = R_8 = H$; (11) ω, $R_1 = R_2$ = methyl ecgonine ester, $R_3 = R_4$ = phenyl, $R_5 = R_6 = R_7 = R_8 = H$. (Right) Structure of α-truxilline, Ph = phenyl (see refs. 14, 18 and 35).

yield ECD-sensitive diheptafluorobutyryl (di-HFB) derivatives. This reaction is shown in Fig. 5. The di-HFB derivatives were determined by cGC–ECD using a 30-m fused-silica capillary column coated with DB-1701. This chromatography is illustrated in Fig. 6 for a coca leaf sample from Peru. The accuracy and reproducibility of the truxilline quantitative data were enhanced by the use of a structurally related internal standard, namely, μ-truxinic acid. The quantitative results for truxillines in South American coca are presented in Table 4. The much higher levels of truxillines in the Colombian sample was again believed due to differences in plant taxonomy. Truxilline data from the greenhouse coca were not reported.

7.2.4. Ecgonine methyl ester

The quantitative levels of this alkaloid were reported only for the greenhouse and non-South American tropical coca cultivars. As seen in Table 3, ecgonine methyl ester was present in those leaves at unexpectedly high levels (relative to cocaine). Whereas the presence of this compound in illicit refined cocaine samples is attributed to cocaine hydrolysis, the values for ecgonine methyl ester in Table 3 were due to *bona fide* alkaloid, *i.e.*, present in the leaf itself.

7.2.5. Cuscohygrine and hygrine

Unlike most coca alkaloids, cuscohygrine and hygrine, seen in Fig. 7, do not contain the tropane moiety. These N-methylpyrrolidine alkaloids, especially cuscohygrine, vary widely in quantitative levels between cultivars. There has also been some evidence that hygrine may be created as an artifact. As seen in Tables 2 and 3, cuscohygrine was present at the highest concentrations in all four of the ECVC cultivars, with an average of 58% (relative to cocaine). Conversely, the cuscohygrine content of the ENVN and ENVT leaf (Tables 2 and 3) averaged only 13%. It is not clear if these wide differences were also related to leaf taxonomy.

Hygrine has been reported previously as an alkaloidal component in South American coca [38]. In the Moore *et al.* study [35], the apparent hygrine content of coca leaf from Peru, Bolivia, Ecuador and Colombia was reported to be 1.4, 2.3, 4.2 and 24%, respectively (relative to cocaine). It was also observed by the authors that, over the course of several weeks, the

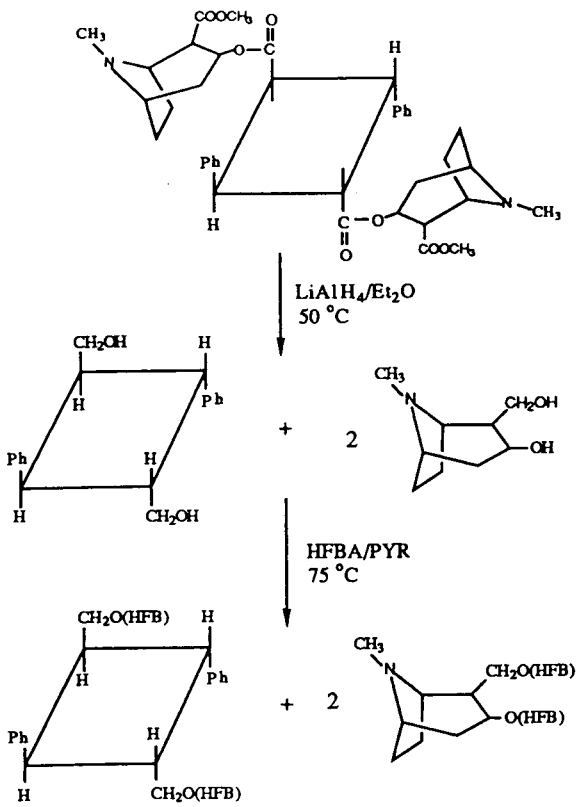


Fig. 5. Lithium aluminum hydride reduction of α-truxilline and derivatization with heptafluorobutyric anhydride (see refs. 14, 18 and 35).

cuscohygrine content of powdered Peruvian and Bolivian leaf, stored at room temperature, gradually decreased while, concomitantly, their hygrine content increased. This suggested that the hygrine content of that leaf might be in part, or in whole, artifactual. There was also suspicion that during the work-up of cuscohygrine, its partial degradation to hygrine may occur.

8. New coca leaf alkaloids

8.1. Hydroxycocaines

In a recent study by Moore and Cooper [33], 4–7 hydroxycocaines were presumptively identified at trace levels in illicit refined cocaine samples. Mass spectral analysis of these alkaloids

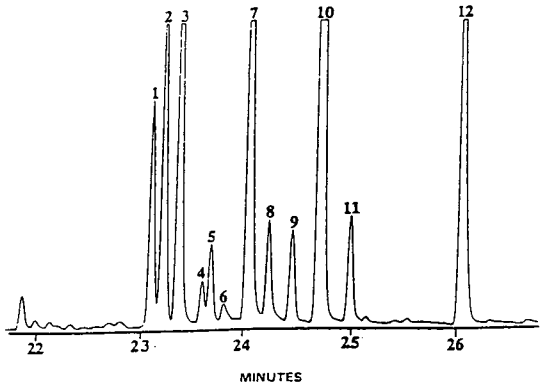


Fig. 6. The cGC–ECD chromatogram (30 m × 0.25 mm I.D. DB-1701) of the truxillines isolated from Peruvian coca leaf. Pertinent chromatographic peaks are the result of lithium aluminum hydride reduction of the truxilline followed by derivatization with heptafluorobutyric anhydride to yield a di-O-heptafluorobutyryl derivative. Peak identification of truxilline isomers: 1 = ϵ-; 2 = δ-; 3 = β-; 4 = *peri*-; 5 = neo-; 6 = *epi*-; 7 = α-; 8 = ω-; 9 = γ-; 10 = μ- (added as internal standard); 11 = ζ-; 12 = heneicosanol internal standard (chromatographed as O-HFB derivative) (see Figs. 4 and 5 and refs. 14, 18 and 35).

Table 4
Isomeric truxilline content of South American cultivated coca leaves (see ref. 35)

Truxilline	Bolivia	Peru	Ecuador	Colombia
α	0.74	0.87	3.51	20.4
β	0.62	0.74	3.22	14.5
δ	0.46	0.50	1.82	9.2
ϵ	0.30	0.35	1.36	6.2
ω	0.11	0.15	0.84	2.8
γ	0.11	0.15	0.64	2.5
neo	0.09	0.11	0.61	2.4
peri	0.05	0.05	0.33	1.4
ζ	0.03	0.05	0.50	1.2
epi	0.02	0.02	0.21	0.64
Total	2.53	2.99	13.04	61.2

All isomeric truxilline results calculated using the μ-isomer as reference standard. All data presented as % (w/w) relative to cocaine.

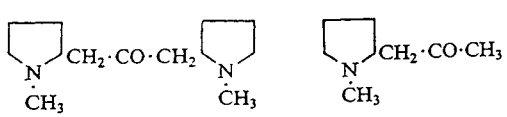


Fig. 7. Structures for cuscohygrine (left) and hygrine (right).

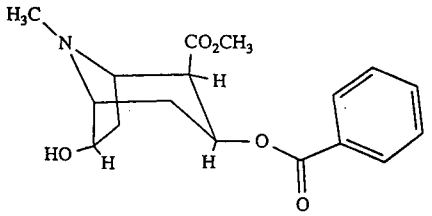


Fig. 8. Structure for 6-hydroxycocaine (see refs. 33 and 35).

suggested hydroxy substitution on the tropane ring. A preliminary investigation of South American coca leaf indicated the presence of at least four of the hydroxycocaines at very low levels [33,71]. One of these, believed to be 6-hydroxycocaine, is illustrated in Fig. 8.

8.2. Trimethoxy analogues of cocaine, cinnamoylcocaines and tropacocaine

In a modification of the Moore *et al.* method [35] described above, Casale and Moore [36] used toluene extraction of basified South American coca leaf, followed by trap and ion-pairing column chromatography, to isolate four previously unknown coca alkaloids in South American coca leaf. The structural characterization of 3′,4′,5′-trimethoxycocaine, the *cis* and *trans* isomers of 3′,4′,5′-trimethoxycinnamoylcocaine and 3′,4′,5′-trimethoxytropacocaine, all seen in Fig. 9, was accomplished by the comparison of their electron ionization mass spectra with synthesized standards. Quantitative data were provided by cGC–FID; the accuracy of this method was enhanced by using 3′,4′,5′-trimethoxycocaethylene as a structurally related internal standard. The quantitative data for a limited number of coca leaf samples from Brazil, Colombia, Bolivia and Peru are reported in Table 5. It was interesting to note that the total trimethoxycinnamoylcocaine content for the Peruvian and Bolivian leaf samples was 4–5 × higher than their respective levels of trimethoxy-

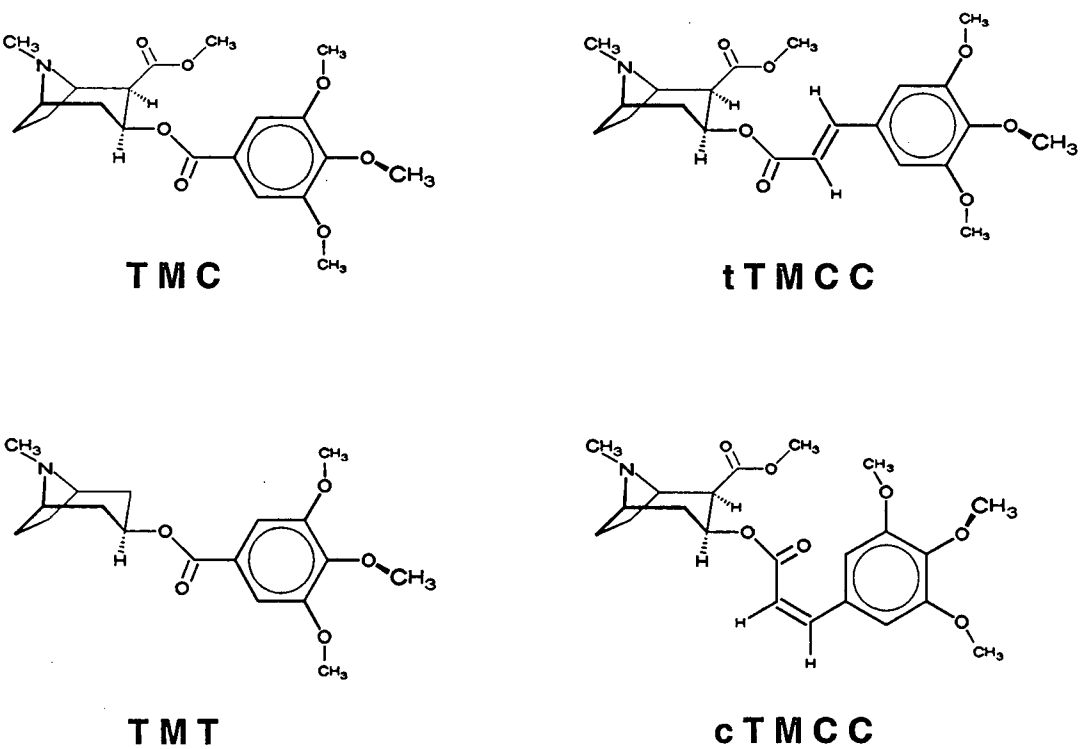


Fig. 9. Structures for some trimethoxy-substituted tropane alkaloids in South American coca: TMC = 3′,4′,5′-trimethoxycocaine; tTMCC = 3′,4′,5′-trimethoxy-*trans*-cinnamoylcocaine; cTMCC = 3′,4′,5′-trimethoxy-*cis*-cinnamoylcocaine; TMT = 3′,4′,5′-trimethoxytropacocaine. From ref. 36.

Table 5
Trimethoxy-substituted alkaloid content in South American coca leaf

Country	TMT[a]	TMC[b]	cTMCC[c]	tTMCC[d]
Brazil	–	1.12	–	0.15
Colombia	–	–	–	–
Bolivia-1	–	0.14	0.13	0.74
Bolivia-2	–	0.22	0.11	0.95
Peru	0.18	0.24	0.16	0.83

Results calculated using 3′,4′,5′-trimethoxyethylcocaine as the internal standard. All data are presented as % (w/w) relative to cocaine. From ref. 36.
[a] 3′,4′,5′-Trimethoxytropacocaine.
[b] 3′,4′,5′-Trimethoxycocaine.
[c] 3′,4′,5′-Trimethoxy-*cis*-cinnamoylcocaine.
[d] 3′,4′,5′-Trimethoxy-*trans*-cinnamoylcocaine.

cocaine. These data were contrasted by the cocaine content of leaf from Peru and Bolivia being 8–10 × greater than the cinnamoylcocaine levels. The authors of this study cautioned that many more samples needed to be examined before determining if this data are representative.

8.3. Other alkaloids

In addition to the coca leaf alkaloids described above, Moore *et al.* [35] reported the presumptive presence of many additional previously uncharacterized trace level alkaloids in Bolivian and Peruvian coca leaf. After the toluene extraction of basified and powdered coca leaves, the extract was subjected to trap and ion-pairing column chromatography as well as extractions with a pH 4 buffer. Additional resolution of the complex alkaloid mixture was provided by column chromatography on basic alumina using an elutropic series of chloroform, chloroform–acetone, acetone, acetone–methanol and methanol [71]. All fractions were subjected to trimethylsilylation followed by cGC–MS analyses. Of the 100 or so suspected alkaloids, more than half yielded mass spectra that exhibited fragment ions at m/z 82 and 182, indicating the presence of a carbomethoxytropane moiety, such as that found in cocaine. Furthermore, the majority of these compounds possessed derivatizable functional groups, an important consideration if sensitive cGC–ECD analysis of these alkaloids is contemplated. The individual levels of all of these new alkaloids were well below 0.1% (relative to cocaine).

9. In-depth analysis of illicit refined cocaine

The manufacture of illicit refined cocaine from South American coca leaf is a somewhat unsophisticated and crude, albeit effective, process. Although there are many clandestine cocaine laboratories throughout the coca-producing regions of South America, they all use methodology that is similar in their basic elements. Briefly, after harvesting, the coca leaves are basified with an inorganic salt and extracted with kerosene or alternatively, extracted directly with dilute sulfuric acid. The resulting extract is then subjected to additional liquid–liquid extractions, precipitations, a potassium permanganate oxidation step and, finally, conversion of cocaine base to cocaine hydrochloride. A more detailed account of the cocaine manufacturing process is discussed elsewhere [60,61].

Because of the unsophisticated nature of the cocaine manufacturing process, a multitude of trace-level alkaloidal impurities are present in illicit refined cocaine. Many of these impurities are *bona fide* alkaloids that cohabit the leaf with cocaine and are "carried through" the manufacturing process. In addition, and as discussed previously, cocaine samples are also contaminated with a variety of manufacturing by-products, *i.e.* compounds resulting from alkaloid degradation. As a result, the cocaine content of unadulterated samples rarely exceeds 98–99%. In most unadulterated samples, cocaine levels range from 80–97%. A review of published reports describing the presence of alkaloidal impurities and manufacturing by-products in illicit refined cocaine is presented below.

9.1. Alkaloidal impurities in illicit refined cocaine

A comparison of alkaloids in coca leaf and alkaloidal impurities that have been reported in

illicit refined cocaine is presented in Table 6. As seen, there is significant "carryover" of the tropane alkaloids from the leaf to refined cocaine during the manufacturing process. It is also apparent that some alkaloids are at least semi-quantitatively carried through to the final product while others experience a more marked decline in concentration (relative to cocaine) in proceeding from the leaf to refined cocaine.

9.1.1. Cinnamoylcocaines

First reported in illicit cocaine by Moore in 1973 [1], *cis*- and *trans*-cinnamoylcocaine (Fig. 1) are almost always found together when present in illicit refined cocaine samples. They are readily detected in the vast majority of cocaine samples by either GC–FID or HPLC–UV methodology. In the first study of its kind, Clark [4] used packed column GC–FID to quantitate and report the cinnamoylcocaine content of 40 cocaine samples. The *cis*- + *trans*-cinnamoylcocaine levels varied from less than 0.10% to 5.17%, with a mean content of 1.45% (relative to cocaine). The *cis*-/*trans*-ratio for 32 of the samples having quantifiable levels of the cinnamoylcocaines had a mean value of 1.4. Jane *et al.* [8] are believed to be one of the first to describe the HPLC–UV analysis of illicit cocaine samples. In that study, the cinnamoylcocaines were detected in only 126 cocaine samples out of

Table 6
Selected coca alkaloids detected in South American coca leaves and refined illicit cocaine

Alkaloid	Coca leaf	Refined cocaine	Comments[a]
cis-Cinnamoylcocaine	Yes	Yes	0–53% in leaf; 1.9%[b] in refined cocaine
trans-Cinnamoylcocaine	Yes	Yes	0–170% in leaf; 1.2%[b] in refined cocaine
Truxillines	Yes	Yes	2–60% in leaf; 2–13% in refined cocaine
Tropacocaine	Yes	Yes	0.2–5% in leaf; 0.02%[b] in refined cocaine
Ecgonine methyl ester	Yes	No	30–60% in leaf; < 3% in refined cocaine, due to cocaine hydrolysis?
Hydroxycocaines	Yes	Yes	4 detected in leaf, at < 0.01%; 4–7 detected in refined cocaine, at < 0.01%
3′,4′,5′-Trimethoxycocaine + 3′,4′,5′-trimethoxy-*cis*-cinnamoylcocaine + 3′,4′,5′-trimethoxy-*trans*-cinnamoylcocaine + 3′,4′,5′-trimethoxytropacocaine	Yes	Yes	0.1–1% in leaf; 0–0.1% in refined cocaine
Cuscohygrine	Yes	No	10–80% in leaf; not reported in refined cocaine
Hygrine	Yes	No	1–25% in leaf; not reported in refined cocaine

[a] All coca leaf % values are based upon the analyses of a limited number of samples. All % values are relative to cocaine content. All % results for coca leaf included the four major coca varieties (even though ECVC is the cultivar reportedly used most often in clandestine cocaine manufacture); all data were obtained from Tables 1-5 and 7 as well as refs. 26, 35, 36 and 76.
[b] Average of 4000 analyses (see ref. 26).

a total of 336 examined. This could have been due to the lesser sensitivity of HPLC–UV *vs.* GC–FID, or the implementation of a high attenuation during generation of the chromatographic profiles. Shortly after the study by Jane *et al.*, Noggle and Clark [10] also reported the detection of the cinnamoylcocaines using HPLC–UV methodology. Lurie *et al.* [15] used HPLC and photodiode-array UV detection for the analysis of the cinnamoylcocaines as well as other alkaloidal impurities and manufacturing by-products. Using packed-column GC–FID, Ensing *et al.* [30] reported the presence of the cinnamoylcocaines in about 85% of 71 cocaine samples examined. cGC–FID, cGC–MS and HPLC–UV were employed by LeBelle and co-workers [20,25] to provide quantitative data for the cinnamoylcocaines. The intra- and inter-sample variability of these alkaloids as well as their stability was also investigated.

In the most comprehensive study done to date, conducted from 1989 to 1992, Casale and Waggoner [26] determined the *cis*- and *trans*-cinnamoylcocaine content of about 4000 illicit refined cocaine seizures using cGC–FID. The data revealed that the cinnamoylcocaines were detected in $>95\%$ of samples and varied between about 0.1 and 9% (relative to cocaine), with a mean total cinnamoylcocaine level of 3.2%. As in the Clark study [4], the *cis* predominated over the *trans* isomer, with an average ratio of 1.56.

Although the average total cinnamoylcocaine content of South American ECVC coca leaf is in the range of 8–12%, the mean content in illicit refined cocaine samples is below 4% (relative to cocaine). There is little doubt that this marked decline in going from leaf to refined sample is due to the use of potassium permanganate in the clandestine cocaine manufacturing process. Potassium permanganate is used to oxidize the cinnamoylcocaines and thereby diminish their presence so as to allow for more effective crystallization in the cocaine base-to-cocaine hydrochloride conversion step.

9.1.2. Tropacocaine

As seen in Table 2, tropacocaine is present in Peruvian and Bolivian coca leaf at much lower levels than the cinnamoylcocaines and is therefore less readily detected in refined cocaine samples. Janzen *et al.* [29] used cGC–NPD to detect tropacocaine in each of a representative set of 10 cocaine samples. In a survey of 71 cocaine samples using packed column GC–FID, Ensing *et al.* [30] reported tropacocaine in about 75% of them. Neither of these two studies provided absolute quantitative data for tropacocaine. In the study by Casale and Waggoner [26,72] of 4000 cocaine samples, tropacocaine was detected and quantitated in over half of the exhibits examined, with levels varying from 0.01% to as high as 0.9% (relative to cocaine), with most samples rarely exceeding 0.1%. The mean tropacocaine content for the 4000 samples was found to be 0.02%.

From Table 2 and other data [72], there appears to be more than an order of magnitude decline in the tropacocaine content from the processing of leaf to refined cocaine. The reason for the marked decrease in this alkaloid is not clear, but it may occur during the potassium permanganate oxidation step, or more likely, due to its solubility characteristics.

9.1.3. Truxillines

Although the presence of α- and β-truxilline in coca leaf was first described by Hesse [73] and Liebermann [74] in the late 19th century, it was not until 1987 that Moore *et al.* [14] first reported them and nine related isomers in illicit refined cocaine samples (Fig. 4). The structural elucidation of the truxillines was accomplished by first removing them from the bulk cocaine matrix using alumina column chromatography. The combined truxillines were acid hydrolyzed to their corresponding truxillic/truxinic acids and then converted to their respective dimethyl esters. The mixture of dimethyl esters were then resolved by preparative HPLC [14] and characterized by cGC–MS and ^{1}H NMR. Because of their considerable mass ($M_r = 658$), the truxillines are difficult to chromatograph directly by GC, usually undergoing thermal degradation in the injection port to yield ecgonidine methyl ester and the truxillic/truxinic acids. In order to determine the truxillines by GC, they were first

reduced and then derivatized as previously described for coca leaf (Fig. 5). As in the coca leaf analysis, the truxillines were then determined in refined samples by cGC–ECD. In a follow-up investigation, Moore [18] determined the total truxilline content of about 130 refined cocaine samples by cGC–ECD and found that their levels varied from 0.2% to 12% (relative to cocaine). The accuracy and reproducibility of that methodology was enhanced by the incorporation of μ-truxinic acid as a structurally related internal standard (note: it had been previously determined by the author that the μ-truxilline content of illicit refined cocaine samples to be negligible, thus allowing the use of this isomer as an internal standard). Table 7 gives the total individual truxilline content for 10 representative illicit cocaine samples seized in South America.

Preliminary truxilline data from Peruvian and Bolivian coca leaf (Table 4) and recent truxilline data from the analysis of about 50 refined illicit cocaine samples from Peru, Bolivia and Brazil [71] suggested that, in the processing of coca leaf to refined cocaine, the truxillines experienced a much higher carry-through than either the cinnamoylcocaines or tropacocaine. It was emphasized by the authors [18,35,71], however, that the truxilline content of many more Peruvian and Bolivian coca leaf samples need to be determined to confirm those preliminary findings.

To circumvent the thermal degradation associated with the GC analysis of the truxillines, Lurie *et al.* [15,21] presented methodology involving HPLC with photodiode-array UV detection. In one of those methods [15] the truxillines were chromatographed intact after direct injection of the cocaine sample on an HS-5 C_{18} column followed by gradient elution with a solvent mixture of acetonitrile–phosphate buffer (0.02 *M* dodecyl sulfate, pH 2.0). Fig. 10 (top) illustrates this chromatography. It is noted that, in addition to the truxillines, the cinnamoylcocaines and several manufacturing by-products were also detected by this methodology. In Fig. 10 (bottom) the truxillines were isolated via size-exclusion chromatography [21]. Recently, Ensing and De Zeeuw [27] described the thin-layer chromatographic separation and mass spectral identification of five truxillines in illicit cocaine samples seized in the Netherlands Antilles.

Table 7
Individual and total truxilline content ("intact" truxillines + truxilline hydrolysis products) of ten representative refined illicit cocaine hydrochloride samples seized in South America (see ref. 18)

Truxilline	Sample numbers									
	1	2	3	4	5	6	7	8	9	10
ϵ	0.84	0.64	0.49	0.36	0.16	0.15	0.12	0.14	0.11	0.03
δ	1.06	0.81	0.72	0.48	0.15	0.22	0.18	0.23	0.17	0.06
β	2.11	1.48	1.60	0.94	0.60	0.55	0.54	0.42	0.41	0.04
peri + neo-	0.48	0.40	0.36	0.19	0.08	0.09	0.06	0.06	0.04	0.02
epi	0.05	0.08	0.05	0.02	0.01	0.02	0.01	0.01	0.01	0.00
α	3.13	2.68	1.80	1.00	0.75	0.51	0.31	0.38	0.30	0.06
ω	0.69	0.41	0.33	0.17	0.15	0.06	0.03	0.05	0.03	0.01
γ	0.50	0.42	0.28	0.14	0.12	0.05	0.03	0.04	0.02	0.01
μ	–	–	–	–	–	–	–	–	–	–
ζ	0.15	0.09	0.13	0.07	0.06	0.04	0.04	0.03	0.03	0.02
Total	9.01	7.01	5.76	3.37	2.08	1.69	1.32	1.36	1.12	0.25

A total of 130 illicit cocaine hydrochloride samples from South America were subjected to total truxillines quantitation, of which 10 sample results were selected at random. All results are % (w/w), relative to cocaine. An internal standard of μ-truxinic acid was used as a reference standard for all calculations, and similar molar ECD responses and concentration/response linearity is assumed for all truxillines.

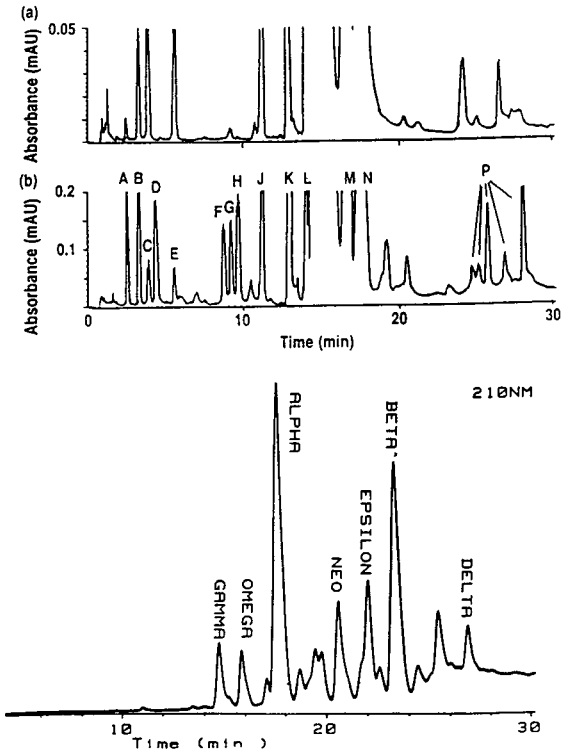


Fig. 10. (Top) HPLC chromatograms obtained at (a) 277 nm and (b) 215 nm from the separation of a cocaine hydrochloride sample. Peaks: A = meconin (internal standard); B = benzoic acid; C = *cis*-cinnamic acid; D = α-truxillic acid; E = *trans*-cinnamic acid; F = ϵ-truxillic acid; G = β-truxinic acid; H = δ-truxinic acid; J = *n*-butyrophenone (internal standard); K = benzoylecgonine; L = cocaine; M = *cis*-cinnamoylcocaine; N = *trans*-cinnamoylcocaine; P = truxillines. From ref. 47. (Bottom) HPLC of illicit cocaine impurities including isomeric truxillines isolated via size-exclusion chromatography (see ref. 21).

9.1.4. Hydroxycocaines

In a 1993 report, Moore and Cooper [33] described for the first time the detection of 4–8 suspected hydroxycocaines in refined cocaine samples. After their isolation from the bulk cocaine matrix using alumina column chromatography, the hydroxycocaines were presumptively identified by MS as HFB and trimethylsilyl derivatives. 6-Hydroxycocaine, seen in Fig. 8, is suspected of being one of the hydroxycocaines present in cocaine samples. Over 100 unadulterated and refined cocaine hydrochloride samples from South America were subjected to derivatization with HFBA, and the resultant HFB derivatives of the hydroxycocaines, and other derivatizable compounds, were subjected to cGC–ECD analyses. The hydroxycocaines were detected in all samples examined, with most at individual concentration levels below 0.01% (relative to cocaine). Partial chromatograms of the hydroxycocaines and other impurities/by-products present in two illicit cocaine samples are illustrated in Fig. 11 [33]. The chromatography

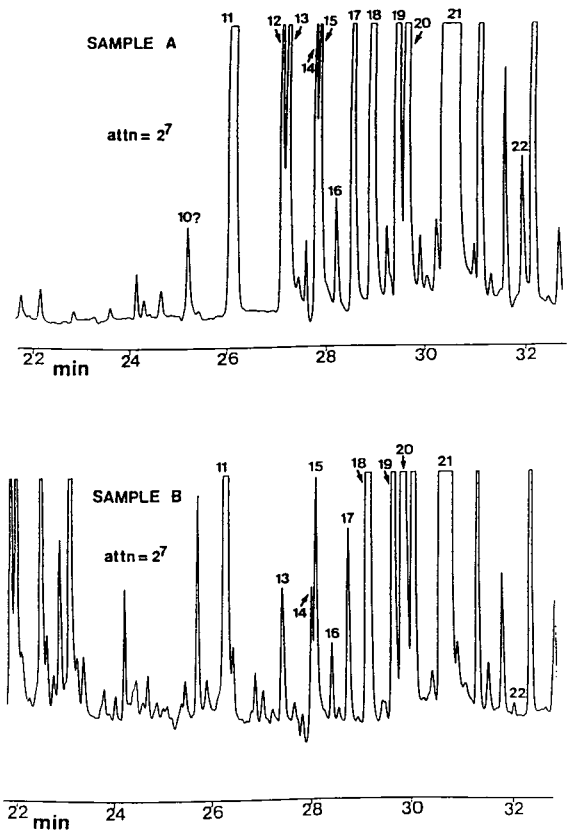


Fig. 11. Partial cGC–ECD chromatograms of heptafluorobutyryl derivatives of some cocaine manufacturing impurities/by-products in two unrelated and unadulterated illicit cocaine hydrochloride samples (30 m × 0.25 mm DB-1701). Peaks: 11 = heneicosanol internal standard (as O-HFB derivative); 12–18, 20 = suspected isomeric hydroxycocaines (as O-HFB derivatives; *e.g.*, see Fig. 8); 19 = N-benzoylnorecgonine methyl ester (as O-HFB derivative; see Fig. 13); 21 = N-norcocaine (as N-HFB derivative; see Fig. 1). From ref. 33.

was done on a 30 m × 0.25 mm fused-silica column coated with DB-1701.

9.1.5. 3',4',5'-Trimethoxy-substituted coca alkaloids

Using ion-pairing column chromatography, with 1 *M* HCl–2 *M* NaCl/Celite 545 as the stationary phase and water-saturated chloroform as the eluent, Casale and Moore [36] quantitatively isolated trace levels of the trimethoxy-substituted analogues of tropacocaine, cocaine and the cinnamoylcocaines, along with N-norcocaine and tropacocaine, from 15 South American coca paste and refined cocaine base and hydrochloride samples. These alkaloidal impurities were determined using cGC–FID and a 30-m DB-1 fused-silica capillary column. Of the eight refined cocaine base and hydrochloride samples, the levels of trimethoxycocaine were all below 0.05% (relative to cocaine), with most below 0.005%; trimethoxycocaine was not detected in two Peruvian cocaine hydrochloride samples. It was also interesting to note that of the 13 illicit refined and paste samples with measureable levels of the trimethoxy compounds, eight had *cis*- + *trans*-cinnamoylcocaine levels higher than trimethoxycocaine. This was also in agreement for three of the South American coca leaf samples, in which the total trimethoxycinnamoylcocaines/trimethoxycocaine ratio was about 5 (Table 5). However, this was in sharp contrast to the average cocaine/*cis*- + *trans*-cinnamoylcocaine ratio of 40 for illicit refined samples. Overall, there were sharp declines in the concentrations of trimethoxy-containing alkaloids in the processing of leaf to refined cocaine, in some cases more than an order-of-magnitude decrease. The cGC–FID chromatogram of trimethoxy-substituted alkaloidal and other known and unknown impurities in a refined South American cocaine base sample is illustrated in Fig. 12.

9.1.6. Cuscohygrine and hygrine

Although methodology has been recently developed for the detection of cuscohygrine and hygrine in refined cocaine samples [34], there have been no reports of their presence in that

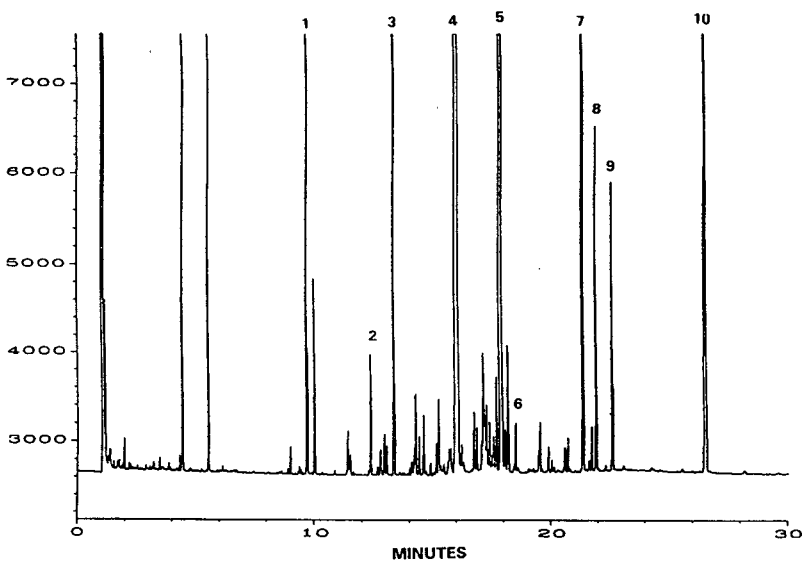


Fig. 12. The cGC–FID chromatogram (30 m × 0.25 mm I.D. DB-1) of ion-pair eluate from a refined cocaine base sample containing trimethoxy-substituted alkaloidal impurities and other compounds. See Figs. 1 and 9 for structures. Peaks: 1 = tropacocaine; 2 = N-norcocaine; 3 = cocaine; 4 = *cis*-cinnamoylcocaine; 5 = *trans*-cinnamoylcocaine; 6 = 3',4',5'-trimethoxy-tropacocaine; 7 = 3',4',5'-trimethoxycocaine; 8 = 3',4',5'-trimethoxycocaethylene (internal standard); 9 = 3',4',5'-trimethoxy-*cis*-cinnamoylcocaine; 10 = 3',4',5'-trimethoxy-*trans*-cinnamoylcocaine (see Fig. 9). From ref. 36.

matrix. It is suspected that their levels in refined cocaine would be very low because of their high solubility in aqueous solutions and higher volatility, resulting in substantial losses during the manufacturing process.

9.2. Manufacturing by-products in illicit refined cocaine

9.2.1. Alkaloid hydrolysis products

Varying amounts of manufacturing by-products are present in virtually all illicit refined cocaine samples. The most readily detected by GC–FID and/or HPLC–UV are cocaine hydrolysis products, *i.e.*, benzoylecgonine, ecgonine methyl ester, ecgonine and benzoic acid (Fig. 1) [2,3,6,8,10,15,20,26]. Additional hydrolysis by-products include the cinnamic acids (Fig. 1) [26], which are derived from the cinnamoylcocaines, and a multitude of other trace level products from the hydrolysis of the isomeric truxillines, including truxillic and truxinic acids (Fig. 4) [14,26]. Most of the aforementioned hydrolytes are probably produced during the manufacturing process; however, some, especially those derived from the truxillines, can be formed during prolonged storage [18].

9.2.2. Cocaine oxidation by-products

Other cocaine manufacturing by-products detected by GC–FID and/or cGC–ECD at trace levels in refined samples include N-norcocaine (Fig. 1) [17,26,28,29,33], N-formylnorcocaine (Fig. 13) [24,28,30], N-benzoylnorecgonine methyl ester (Fig. 13) [28,30,33] and N-norecgonine methyl ester [33]. Ensing and Hummelen [28] have attributed the first three of these by-products to the use of potassium permanganate in the cocaine manufacturing process. These authors also described the relationship between N-norcocaine and N-benzoylnorecgonine methyl ester as being pH dependent. It is also believed that N-norcocaine can be formed by the peroxide-assisted N-demethylation of cocaine [33]. Peroxides are often present in certain solvents, notably diethyl ether, used in the manufacture of cocaine and other drugs. N-Norecgonine methyl ester, which has been detected by cGC–ECD [33], is probably the result of N-norcocaine hydrolysis.

The quantitative levels of the above by-products can vary by several orders of magnitude in illicit refined cocaine samples. Cocaine hydrolysis products are often present in samples at levels over 1% and are readily detected by either cGC–FID or HPLC–UV. The other manufacturing by-products are usually detected by cGC–FID, and especially by cGC–ECD, at levels well below 1%.

9.2.3. Solvent residues

The analysis of solvents as contaminants/by-products in illicit refined cocaine samples has been only recently reported. Methodology that has been applied in the detection of solvent residues have utilized ^{1}H NMR spectroscopy and headspace GC. In 1991, Avdovich *et al.* [41] described the ^{1}H NMR analysis of over 150 illicit refined cocaine exhibits and reported the presence of seven solvents in those samples. By far the two most commonly seen solvent residues were acetone and methyl ethyl ketone, followed by diethyl ether, benzene and toluene. The minimum detectability of selected solvents, based upon a 200-mg sample mass, was benzene (100 ppm), toluene (150 ppm), acetone (100 ppm), diethyl ether (250 ppm) and methyl ethyl ketone (200 ppm). Quantitative estimations of

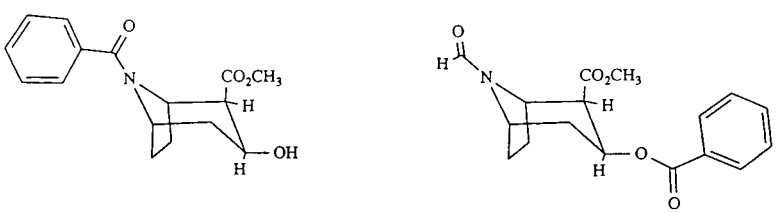


Fig. 13. Structures for N-benzoylnorecgonine methyl ester (left) and N-formylnorcocaine (see refs. 28 and 33).

solvents in 36 samples revealed ppm residue ranges from 400–11000 (acetone), 1600–7700 (methyl ethyl ketone), 700–1200 (diethyl ether), 100–600 (benzene) and 800–2300 (toluene).

Kram [42] also utilized ^{1}H NMR for solvent analyses and reported for the years 1986–1990 similar solvents as described by Avdovich *et al.* [41]. However, Kram [42] listed somewhat lower minimum detectability levels of 10–70 ppm for the most common solvent residues. It is interesting to note in the Kram study, that from 1985 to 1990 the detection of acetone and diethyl ether in cocaine samples declined markedly, while solvents such as methyl ethyl ketone, aliphatic hydrocarbons, toluene, methyl chloride and ethyl acetate were seen with much greater frequency.

In the most comprehensive solvent residue study done to date, Fortuna [43] compiled the headspace GC qualitative results of 1702 cocaine samples received from forensic laboratories during the period of 1986–1991. The data revealed that methyl ethyl ketone was the most frequently detected residue (74.1% of samples), followed by toluene (57.4%), methylene chloride (49.1%), ethyl acetate (36.6%), aliphatic hydrocarbons (33.3%), acetone (25.8%), benzene (18.8%), methyl acetate (14.1%) and diethyl ether (3.9%). Other solvent residues were also present, but were encountered with less frequency.

In what is perhaps the most sensitive solvent residue method to date, Morello and Myers [44] reported quantitative data for solvents in illicit refined cocaine samples using headspace GC–MS with ion-trap detection. Twenty-five of the most commonly encountered solvents provided linear responses over significantly different ranges, with minimum detection limits ranging from 0.1 to 5.7 μg (present in headspace chamber), depending upon the solvent. Minimum detection limits (based upon 45 mg sample mass) for several commonly encountered solvents occluded in cocaine hydrochloride samples included hexane 0.5 ppm, methylene chloride 1 ppm, toluene 1 ppm, diethyl ether 3 ppm, methyl ethyl ketone 13 ppm, ethyl acetate 14 ppm and acetone 48 ppm. The use of deuterated solvents as internal standards in this methodology enhanced its quantitative accuracy and reproducibility. Recently, this method was used successfully in a cocaine sample comparison case.

9.3. Electron ionization mass spectra of coca alkaloids and by-products present in coca leaf and/or refined illicit cocaine

Found in the Appendix are the electron ionization mass spectra (mass-selective detection) of old and recently-reported alkaloids, as well as manufacturing by-products reported to be present in ECVC coca leaves and/or illicit refined cocaine samples. All mass spectra were generated at the US Drug Enforcement Administration's Special Testing and Research Laboratory.

10. Cocaine impurity signature profiles

Since about 1988 there has been a spate of chromatographic methods reported for the simultaneous detection and determination of alkaloidal impurities and manufacturing by-products in illicit refined cocaine samples. These so-called "impurity signature profiles" were usually developed for the comparative analyses of cocaine seizures. This was accomplished primarily by packed-column or cGC–FID, cGC–NPD, cGC–ECD, cGC–MS and HPLC–UV.

10.1. Methods 1–4

The earliest reports of a cocaine impurity signature profile were by Moore in 1974 [2] and again in 1978 [3]. In those procedures, unadulterated cocaine samples were subjected to derivatization with N,O-bis-(trimethylsilyl)-acetamide (BSA) followed by packed-column GC–FID analysis. The impurities and by-products detected included the cocaine hydrolysis products (Fig. 1), all chromatographed as trimethylsilyl (TMS) derivatives, and the cinnamoylcocaines. Soon after, Clark [4] described the first packed-column GC-FID quantitation of the cinnamoylcocaines, ecgonine methyl ester and ecgonine in a cocaine impurity signature

profile. The cinnamoylcocaines were hydrolyzed to their respective cinnamic acids, which were then determined as methyl esters, while in a separate analysis, ecgonine methyl ester and ecgonine were quantitated as TMS derivatives. In a subsequent study, done without chemical derivatization, Lukaszewski and Jeffery [6] chromatographed some of these same compounds on a packed column with MS detection. The authors identified the presence of methylecgonidine and ethylecgonine in the chromatographic profiles as artifacts, created in the injection port of the GC.

10.2. Method 5

The isomeric truxillines have also been used successfully in impurity signature profiles of refined cocaine samples [18,75]. This group of compounds is attractive for sample comparison analyses because of the great number of absolute and relative mathematical permutations of the generated data that are possible. This is because the total truxilline content of refined illicit cocaine samples can vary from 1–2 orders of magnitude [71]. Furthermore, there are 10 easily detected individual truxilline isomers that can provide numerous intra-truxilline ratios. The use of μ-truxinic acid as a structurally related internal standard allows for enhanced reproducibility and accuracy [18]. Recently, the truxilline methodology was instrumental, in part, for a successful federal criminal prosecution of a cocaine comparison case [75].

10.3. Method 6

In the most comprehensive study done to date, Casale and Waggoner [26] markedly improved upon Moore's methodology [3] by using a capillary column, in lieu of a packed column, for the development of a cocaine impurity signature profile. After BSA derivatization of the cocaine sample, it was chromatographed on a 30-m fused-silica capillary coated with DB-1701 (0.26 μm) with detection by FID. The capillary column allowed for improved resolution and increased sensitivity and resulted in the detection of many more compounds, including all those seen in Fig. 1 (excepting *p*-fluorococaine, which was used as an internal standard). Two cGC–FID chromatograms illustrating the chromatography of an illicit refined cocaine hydrochloride and cocaine base samples are presented in Fig. 14. The reproducibility and quantitative accuracy of the profiles were enhanced by the incorporation of a structurally related internal standard, *p*-fluorococaine (Fig. 1). Over 4000 samples were analyzed using this methodology and the data compiled in a computerized data base [26,72,76]. A detailed discussion of statistical analyses supporting the impurity chromatographic profiles, including principal component analyses, were provided by the authors. The ultimate goal was to build a computerized data base of samples to be searched against new exhibits for possible "matches". The authors reported that a new neural network pattern recognition analysis appeared promising for the establishment of a common source identity for each batch of cocaine [76]. This methodology, and a subsequent modification, have both been recently accepted by two federal courts in the successful criminal prosecution of a cocaine comparison case involving cocaine traffickers [75].

10.4. Methods 7 and 8

In another cocaine impurity profile study, LeBelle *et al.* [20] utilized HPLC–UV and cGC–MS (ion-trap detection) for the detection and quantitation of components in illicit cocaine exhibits. Using a dual-wavelength detector (220 and 280 nm), the HPLC on-column minimum detectable levels for benzoylecgonine and *trans*-cinnamoylcocaine were 10 and 15 ng, respectively. The authors described the utility of their methodology for the examination and comparison of cocaine exhibits. In a follow-up study, LeBelle *et al.* [25] used HPLC–UV to determine the alkaloidal ratios of cocaine and *cis*- and *trans*-cinnamoylcocaine for sample comparison purposes. In the same study, cocaine samples were subjected to an accelerated stability study to determine if the ratios of the three target alkaloids changed markedly. The samples were stored for two weeks under controlled humidity

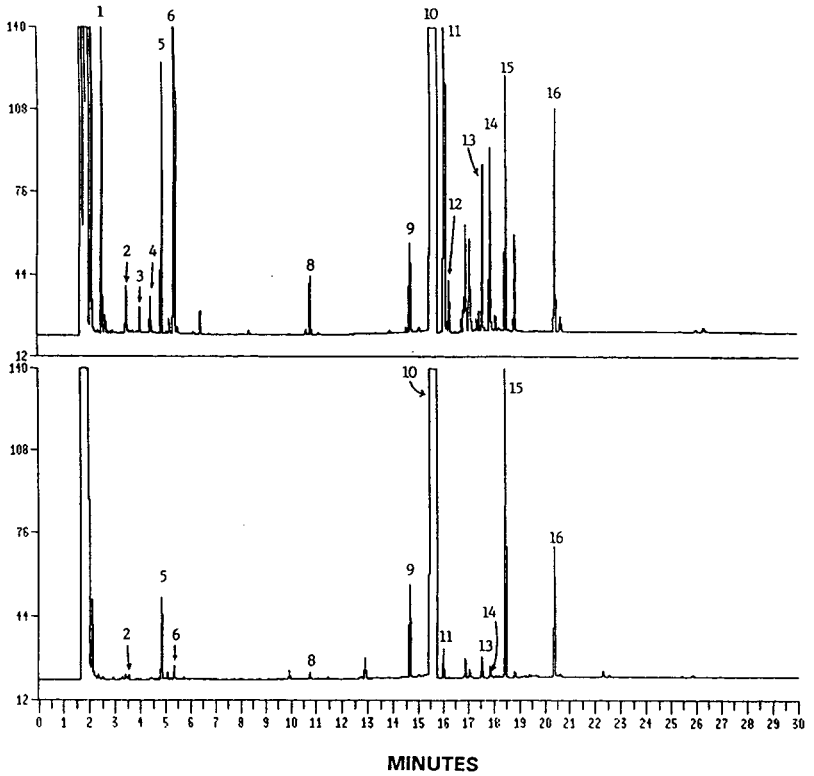


Fig. 14. cGC-FID (30 m × 0.25 mm I.D. DB-1701) of alkaloidal impurities and manufacturing by-products in (top) illicit refined cocaine hydrochloride sample and (bottom) illicit refined cocaine base sample. See Fig. 1 for peak identification and structures. From ref. 26.

of 50 and 75% and at a temperature of 60°C. No significant variance in ratios was noted. For more reliable sample comparisons, the alkaloidal ratios were complemented by the cGC–MS and cGC–FID analysis of acidic and neutral manufacturing by-products in cocaine samples. These by-products were isolated from the bulk cocaine matrix by extraction from a dilute acid solution into toluene. The cGC–MS analyses of these compounds revealed that some of them exhibited cocaine-like fragment ions; however, no structures were proposed.

10.5. Method 9

In an investigation using cGC–NPD, Janzen *et al.* [29] developed cocaine impurity signature profiles by computing the ratios of the peak area for cocaine to the peak areas for tropacocaine, N-norcocaine and *cis*- and *trans*-cinnamoylcocaine. Stability studies conducted under a variety of conditions suggested, and in agreement with LeBelle *et al.* [25], that the area ratios of cocaine to the four target alkaloids were stable. Furthermore, the ratios of the target alkaloids to cocaine were reproducible for same-batch samples and varied widely in unrelated samples. This is a desirable condition when developing methodology for impurity signature profiles. Also examined was the intra-sample variability of kilogram batches of cocaine; it was concluded such variations were small when compared with the variability in the general population. For the evaluation of data, a computer program was written to evaluate the Euclidian distances between a test sample and those contained in a library and to

locate those library samples that were closest to the test sample.

10.6. Method 10

Ensing *et al.* [30] applied packed-column GC–FID in the "fingerprinting" of illicit cocaine samples seized in the Netherlands Antilles. The impurity profiles were based on the presence or absence of six congeners, namely, tropacocaine, N-norcocaine, N-benzoylnorecgonine methyl ester, N-formylnorcocaine, and *cis*- and *trans*-cinnamoylcocaine. This method utilized the relative ratios of these compounds in the analyses of over 70 unrelated cocaine samples. The data revealed that a great variation was observed in the parameter composition and could be expressed numerically or graphically in the form of pictograms for easy visual comparison.

10.7. Method 11

In the most recent report of a cocaine impurity signature profile, Moore and Cooper [33] described the chemical derivatization of unadulterated cocaine samples with HFBA followed by cGC–ECD analyses. This methodology, referred to as the N-norcocaine method because of the usual dominance of the N-norcocaine chromatographic peak, is by far the most sensitive to date for the chromatographic detection of selected alkaloidal impurities as well as manufacturing by-products. In the analysis of over 100 illicit refined cocaine samples, N-norcocaine was easily detected in all samples, always as an off-scale peak at nominal sensitivity. Likewise, the hydroxycocaines, which were presumptively identified by MS, were detected in all samples, but at significantly lower levels than for N-norcocaine. The hydroxycocaines (*e.g.* Fig. 8) were believed to represent the most significant impurities in terms of sample discrimination. Unlike the truxillines, in which 10 of the 11 isomers were detected in all cocaine samples and had somewhat similar inter-isomer ratios, some of the hydroxycocaine isomers were present in some samples and not in others and varied more widely in inter-isomer ratios. Among other compounds that could be detected by this method were N-benzoylnorecgonine methyl ester, ecgonine methyl ester, N-norecgonine methyl ester, ψ-tropine, N-nor-ψ-tropine and other hydroxy-containing tertiary amine impurities and other N-nor compounds. Two partial cGC–ECD chromatograms of unrelated cocaine samples, illustrating the most discriminatory retention time windows, are presented in Fig. 11. As seen in Fig. 15, this method demonstrated excellent reproducibility. This methodology also played a prominent role in a federal prosecution of a cocaine comparison case [75].

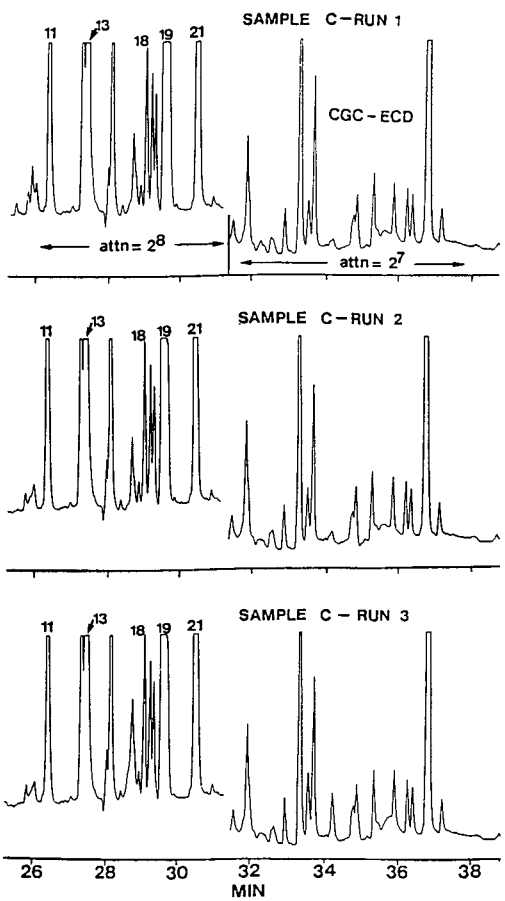


Fig. 15. The repetitive heptafluorobutyric anhydride derivatization and cGC-ECD analysis of an illicit refined cocaine hydrochloride sample (30 m × 0.25 mm I.D. DB-1701). For peak identification see Fig. 11. From ref. 33.

11. Signature profiles of adulterated and diluted cocaine samples

Virtually all of the foregoing profiling methods were applied to unadulterated and undiluted cocaine samples. However, the detection and quantitation of adulterants/diluents in illicit drugs can also be useful in sample comparison analyses and, in some instances, geographic origin determinations. For example, the presence of citric acid in two recent illicit cocaine seizures was so unusual that it proved to be a significant factor in a cocaine comparison case [75]. The most common adulterants and diluents of illicit cocaine include procaine, benzocaine, lidocaine, caffeine, boric acid, talc and sugars, such as inositol, mannitol, dextrose and lactose. Although a comparison of cocaine samples can be achieved by reporting only the qualitative presence of adulterants/diluents, these analyses are markedly enhanced by including quantitative data.

The development of chromatographic signature profiles using cocaine manufacturing impurities/by-products for adulterated and diluted cocaine samples can be problematic, particularly if chemical derivatization is used. That this is so is because cocaine samples are frequently "cut" with substances that possess labile protons, thus rendering them susceptible to derivatization. This can result in the appearance of spurious and interfering peaks in the chromatographic profile and/or total quenching of the derivatization reagent. Even adulterants/diluents that do not derivatize may interfere with pertinent chromatographic peaks of cocaine manufacturing impurities/by-products. Caution should also be excercized when attempting to isolate cocaine manufacturing impurities/by-products from sample adulterants and diluents, especially when using liquid–liquid extraction techniques; such treatment may create artifacts that subsequently appear in the chromatographic profile. Thus, some manipulations may cause unwanted hydrolysis of target compounds. Other impurities/by-products may exhibit anomalous solubility characteristics, thus discriminating against the efficient recovery for some of them. In other instances impurities in solvents, such as peroxides, may result in the N-demethylation of some amine compounds.

12. Internal standards, accuracy and reproducibility

In the development of impurity signature profiles using chromatographic methodology, the judicious selection of an internal standard can enhance accuracy and reproducibility of the data, especially if absolute quantitative data is desired. This is of particular importance in sample comparison cases that may result in court testimony by the chemist. Probably the most ideal internal standards for both accuracy and reproducibility are the isotopic analogues of the impurities under study for use with MS quantitation. Unfortunately, this requires a level of sophistication most forensic drug laboratories do not possess. In any case, other types of internal standards are available for use in impurity signature profiles. Although straight-chain hydrocarbons have been reportedly used, they are inferior to drug compounds that are structurally related to the manufacturing impurities under study. A close structural relationship can include compounds such as homologues, positional and geometric isomers. These kinds of internal standards are especially warranted for derivatization methodology or procedures involving liquid–liquid partition chromatography. In this review, four methods that incorporated structurally related internal standards were discussed; these included μ-truxinic acid [18], *p*-fluorococaine [26], cocaethylene [35] and 3′,4′,5′-trimethoxycocaethylene [36]. The use of structurally related internal standards usually results in improved method reproducibility, as evidenced by typical relative standard deviation values of $\pm$ 2–5% for most impurities and by-products. Structurally related internal standards are also strongly recommended when optimum quantitative accuracy is required.

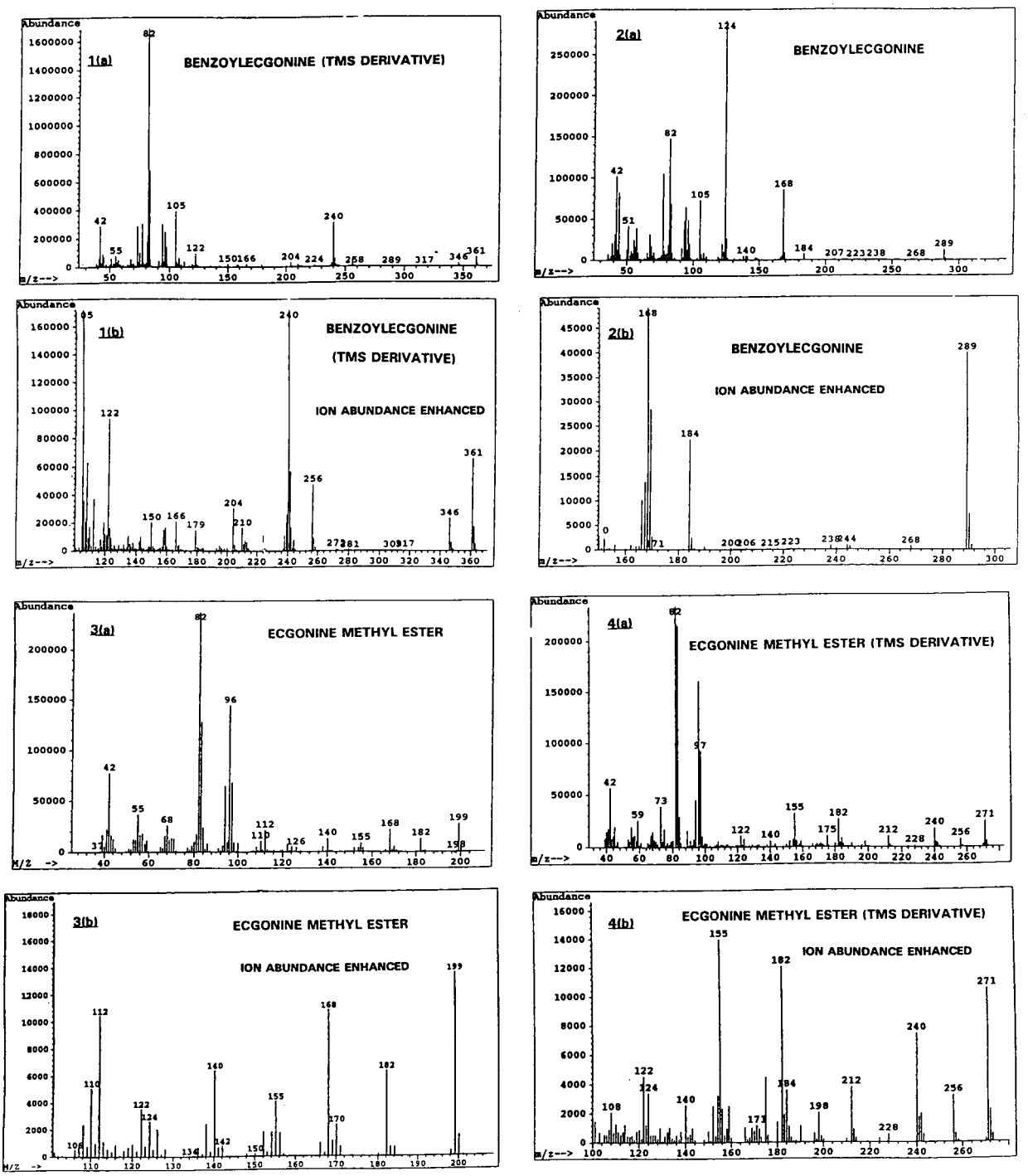


Fig. 16. Electron ionization mass spectra of alkaloids and manufacturing by-products detected in the leaves of South American *E. coca* var. *coca* and/or illicit refined cocaine samples.

(Continued on p. 194)

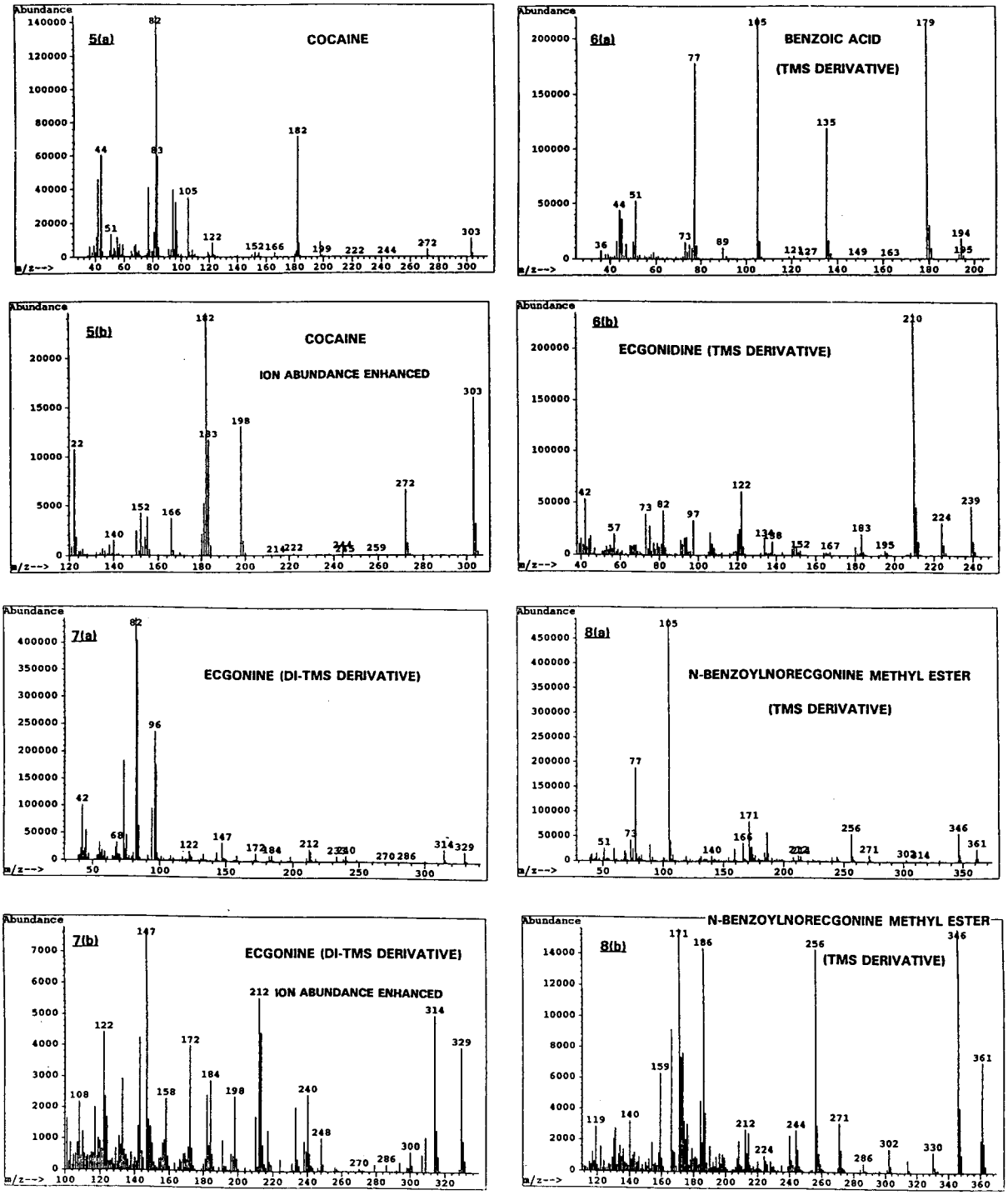

5(a)
COCAINE
6(a)
BENZOIC ACID
(TMS DERIVATIVE)
5(b)
COCAINE
ION ABUNDANCE ENHANCED
6(b)
ECGONIDINE (TMS DERIVATIVE)
7(a)
ECGONINE (DI-TMS DERIVATIVE)
8(a)
N-BENZOYLNORECGONINE METHYL ESTER
(TMS DERIVATIVE)
7(b)
ECGONINE (DI-TMS DERIVATIVE)
212 ION ABUNDANCE ENHANCED
8(b)
N-BENZOYLNORECGONINE METHYL ESTER
(TMS DERIVATIVE)
Abundance
m/z-->

Fig. 16.

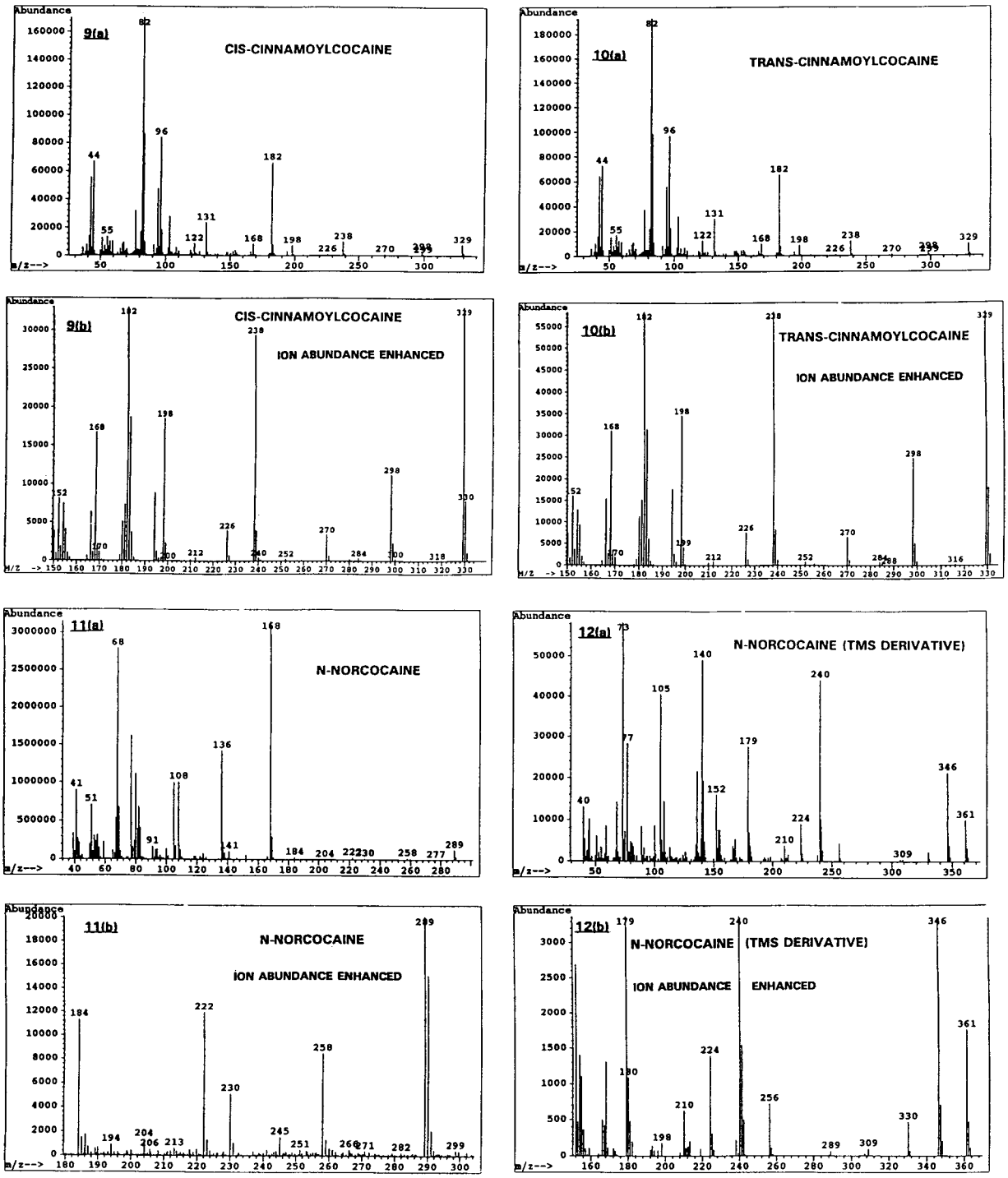


Fig. 16. *(Continued on p. 196)*

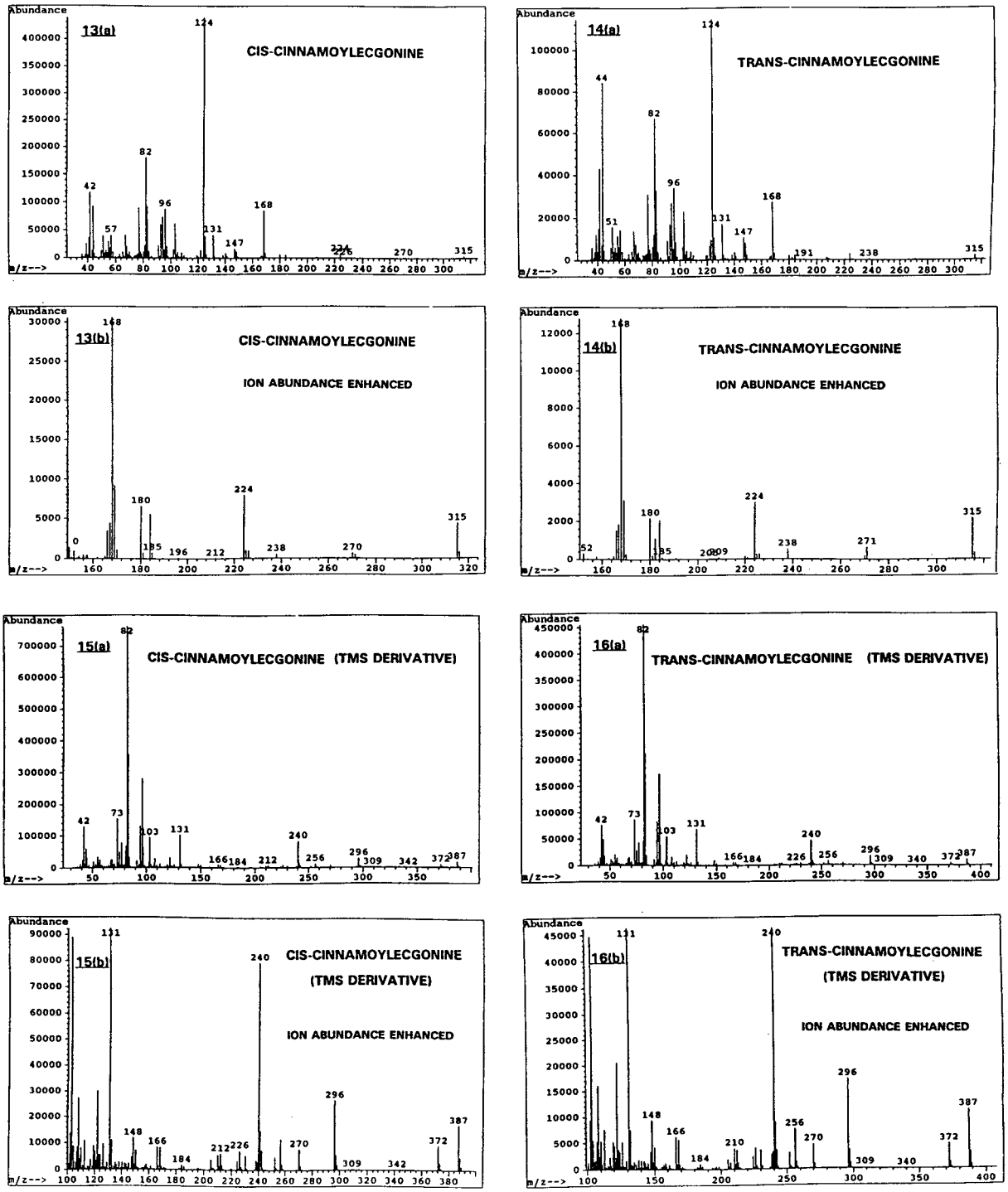
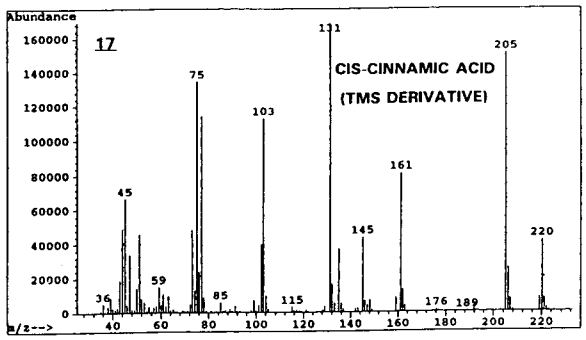
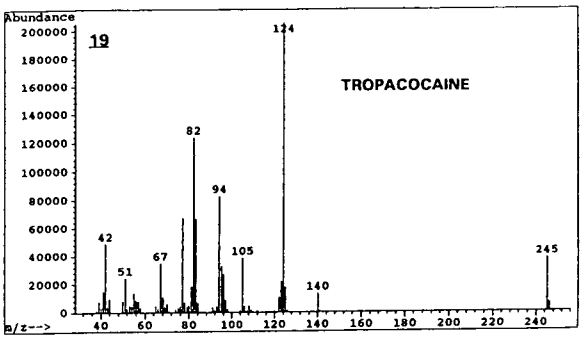
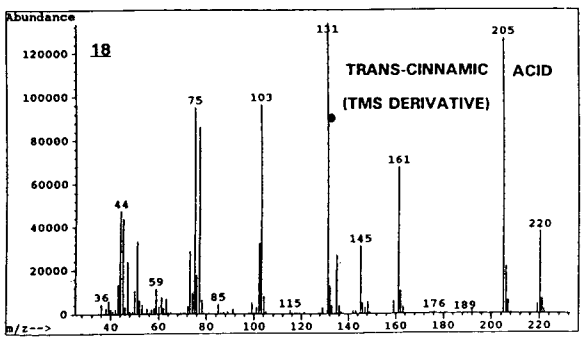
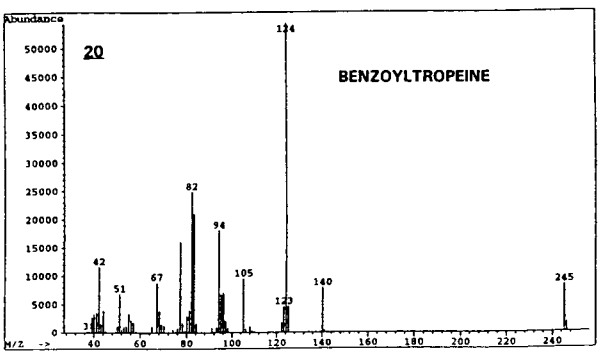

13(a)
CIS-CINNAMOYLECGONINE
14(a)
TRANS-CINNAMOYLECGONINE
13(b)
CIS-CINNAMOYLECGONINE
ION ABUNDANCE ENHANCED
14(b)
TRANS-CINNAMOYLECGONINE
ION ABUNDANCE ENHANCED
15(a)
CIS-CINNAMOYLECGONINE (TMS DERIVATIVE)
16(a)
TRANS-CINNAMOYLECGONINE (TMS DERIVATIVE)
15(b)
CIS-CINNAMOYLECGONINE
(TMS DERIVATIVE)
ION ABUNDANCE ENHANCED
16(b)
TRANS-CINNAMOYLECGONINE
(TMS DERIVATIVE)
ION ABUNDANCE ENHANCED
Abundance
m/z-->

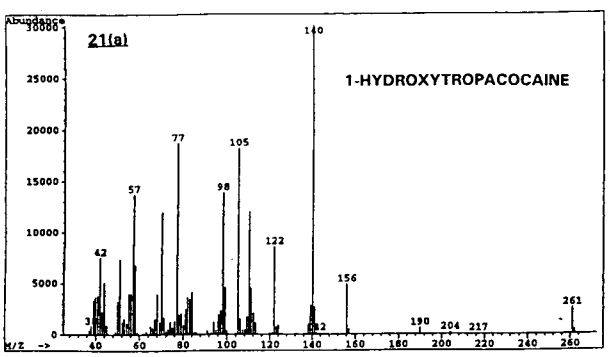
Fig. 16.

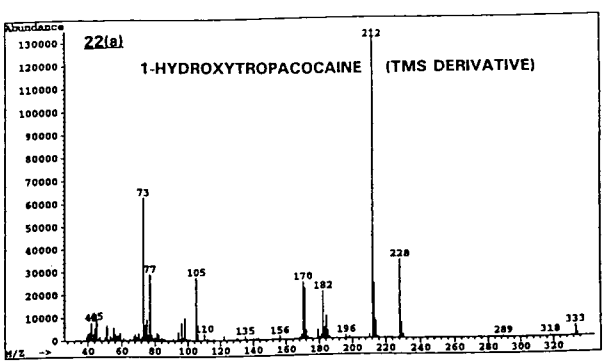


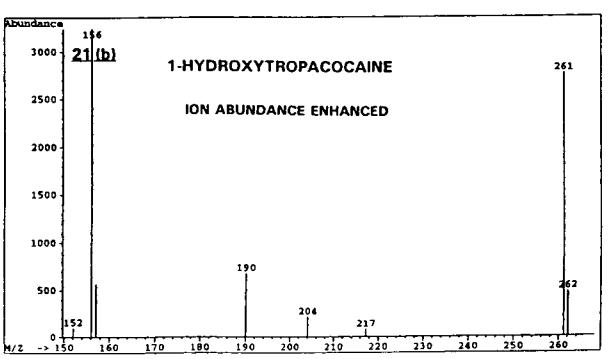












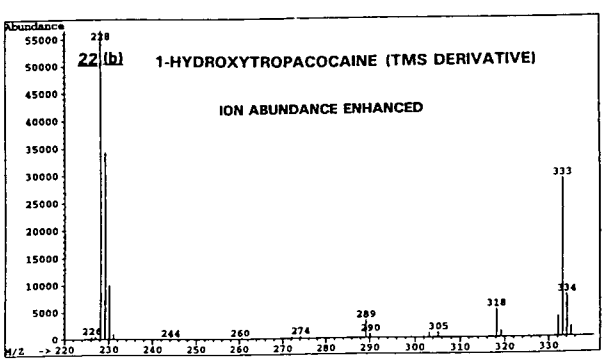


Fig. 16. *(Continued on p. 198)*

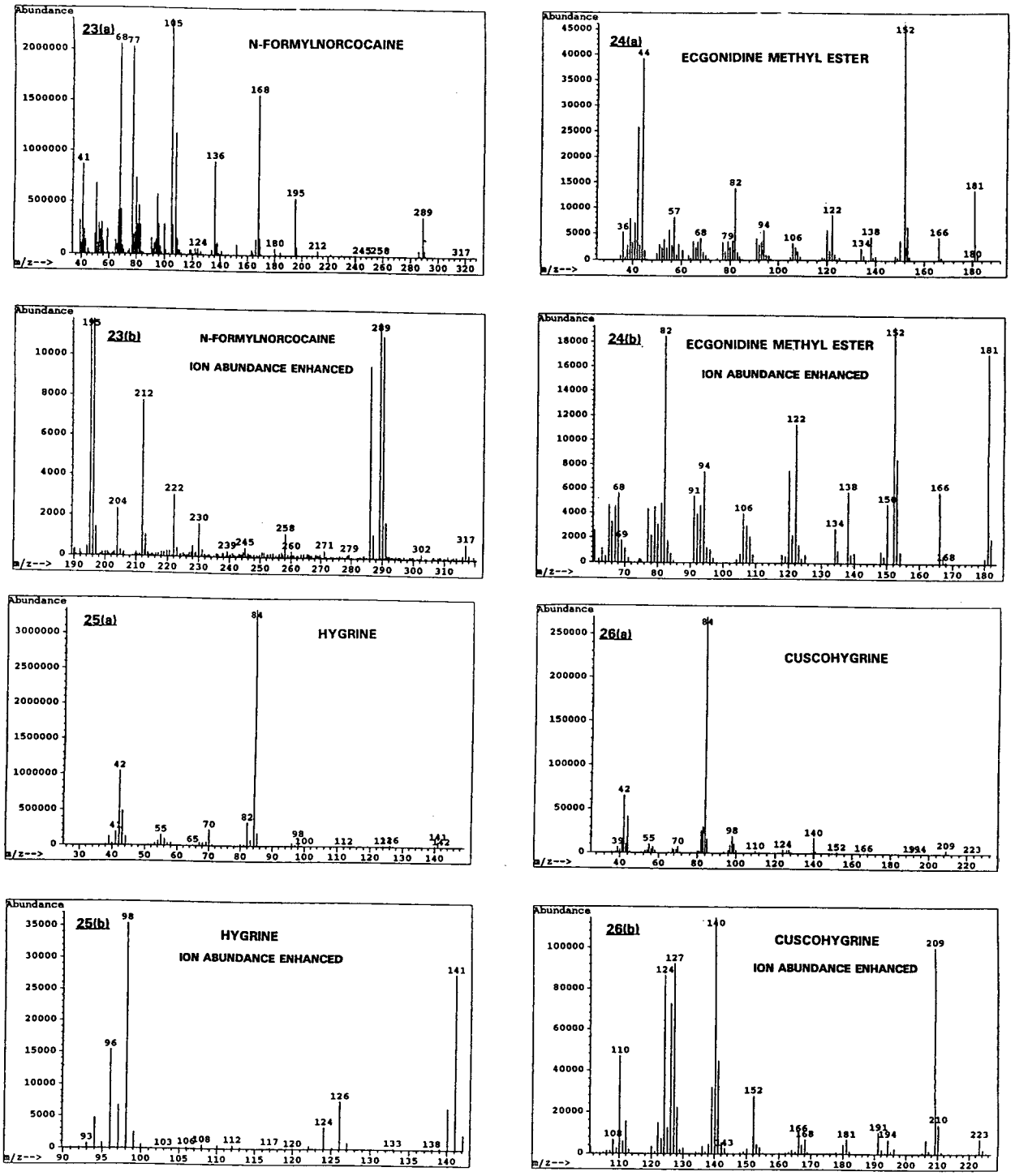

23(a)
N-FORMYLNORCOCAINE
24(a)
ECGONIDINE METHYL ESTER
23(b)
N-FORMYLNORCOCAINE
ION ABUNDANCE ENHANCED
24(b)
ECGONIDINE METHYL ESTER
ION ABUNDANCE ENHANCED
25(a)
HYGRINE
26(a)
CUSCOHYGRINE
25(b)
HYGRINE
ION ABUNDANCE ENHANCED
26(b)
CUSCOHYGRINE
ION ABUNDANCE ENHANCED
Abundance
m/z-->

Fig. 16.

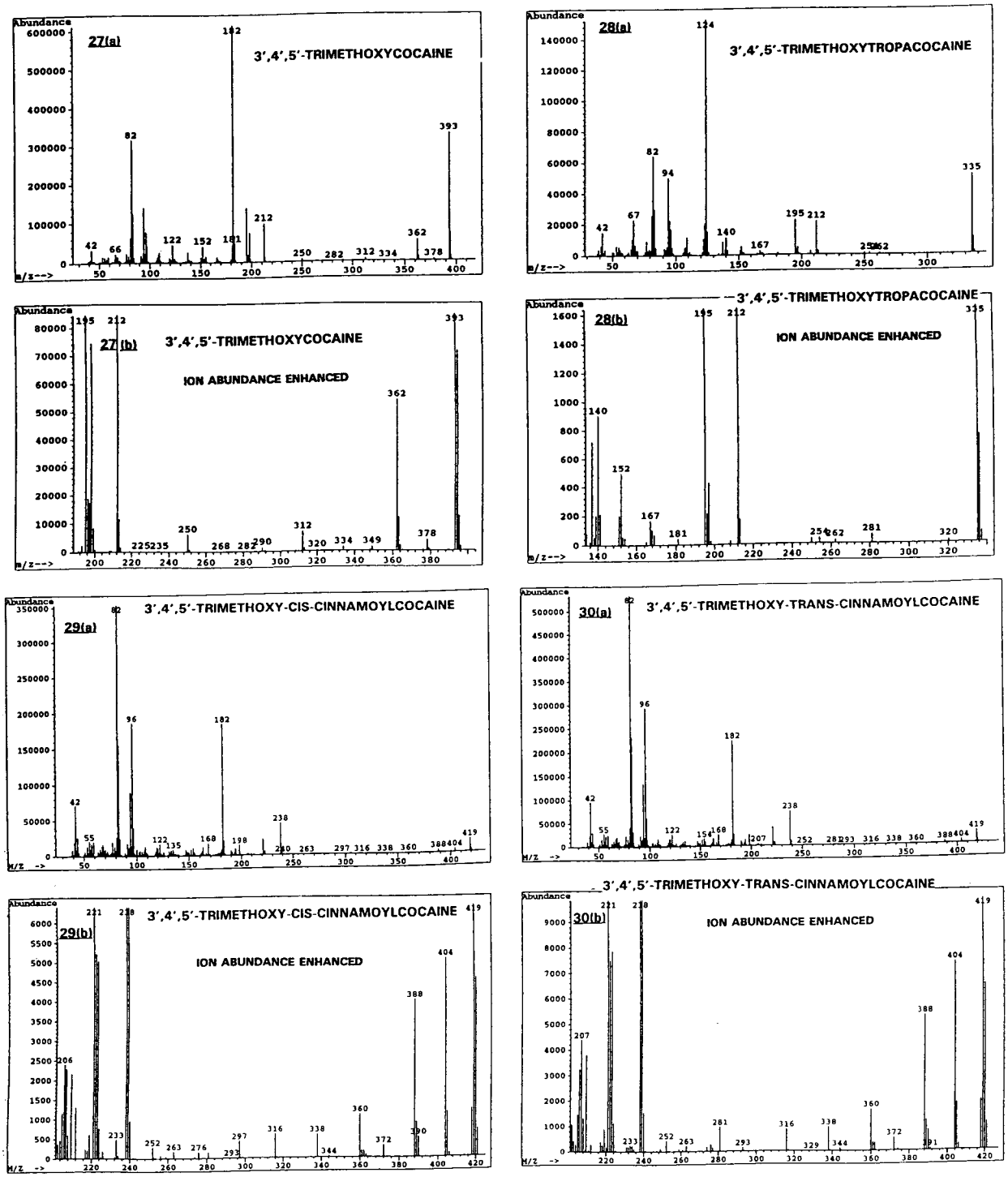


Fig. 16. *(Continued on p. 200)*

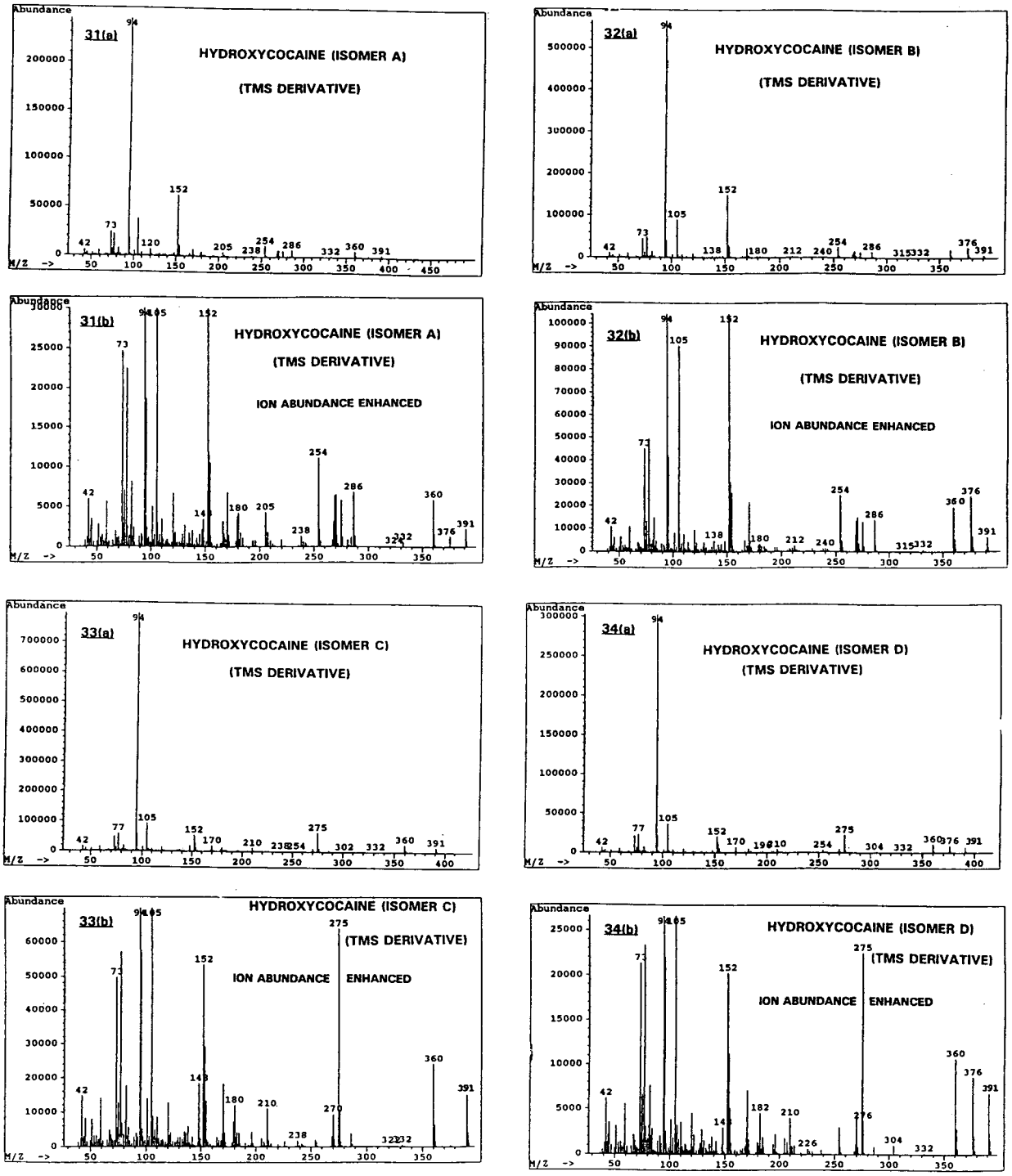

31(a)
HYDROXYCOCAINE (ISOMER A)
(TMS DERIVATIVE)
32(a)
HYDROXYCOCAINE (ISOMER B)
(TMS DERIVATIVE)
31(b)
HYDROXYCOCAINE (ISOMER A)
(TMS DERIVATIVE)
ION ABUNDANCE ENHANCED
32(b)
HYDROXYCOCAINE (ISOMER B)
(TMS DERIVATIVE)
ION ABUNDANCE ENHANCED
33(a)
HYDROXYCOCAINE (ISOMER C)
(TMS DERIVATIVE)
34(a)
HYDROXYCOCAINE (ISOMER D)
(TMS DERIVATIVE)
33(b)
HYDROXYCOCAINE (ISOMER C)
(TMS DERIVATIVE)
ION ABUNDANCE ENHANCED
34(b)
HYDROXYCOCAINE (ISOMER D)
(TMS DERIVATIVE)
ION ABUNDANCE ENHANCED

Fig. 16.

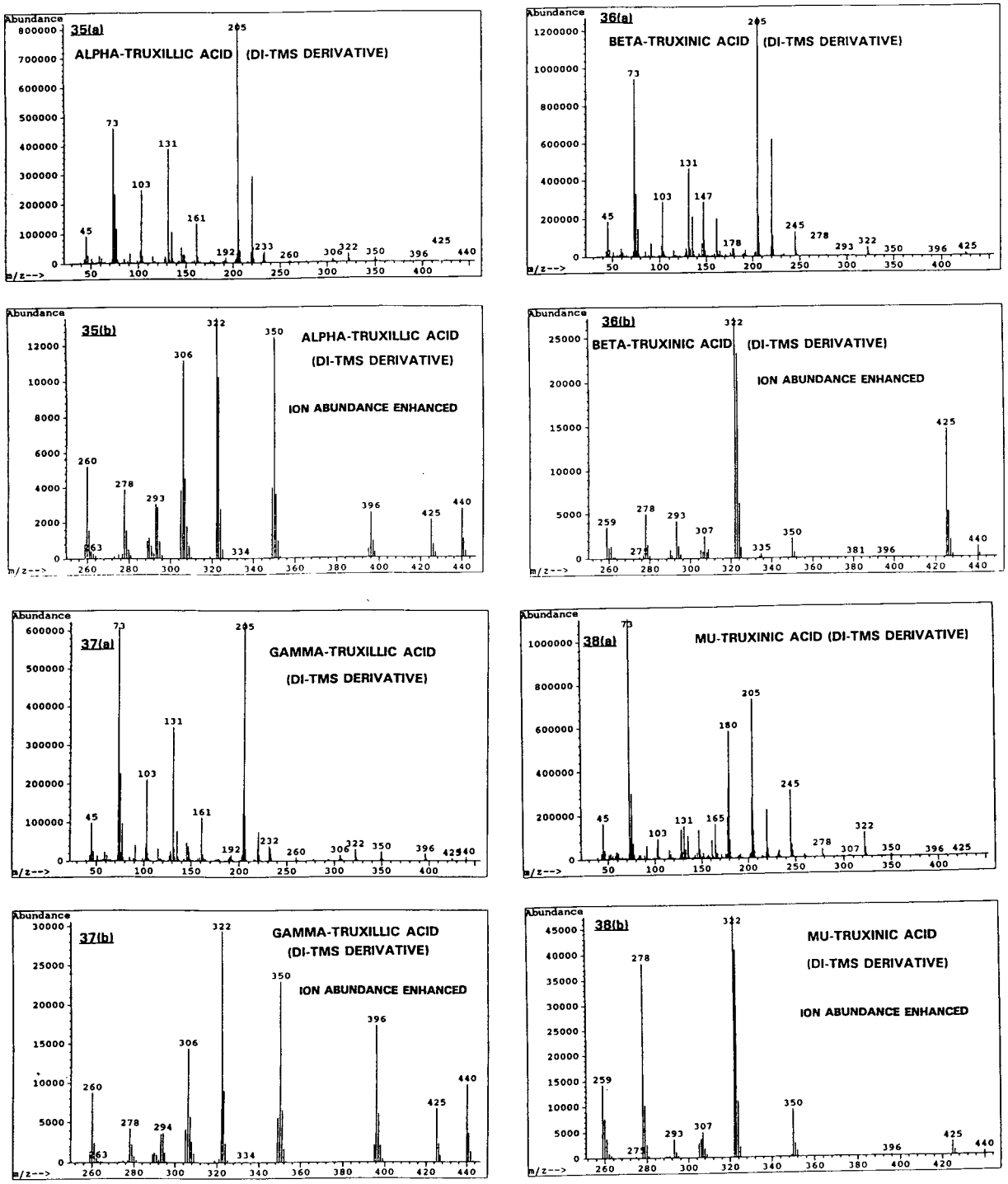


Fig. 16. *(Continued on p. 202)*

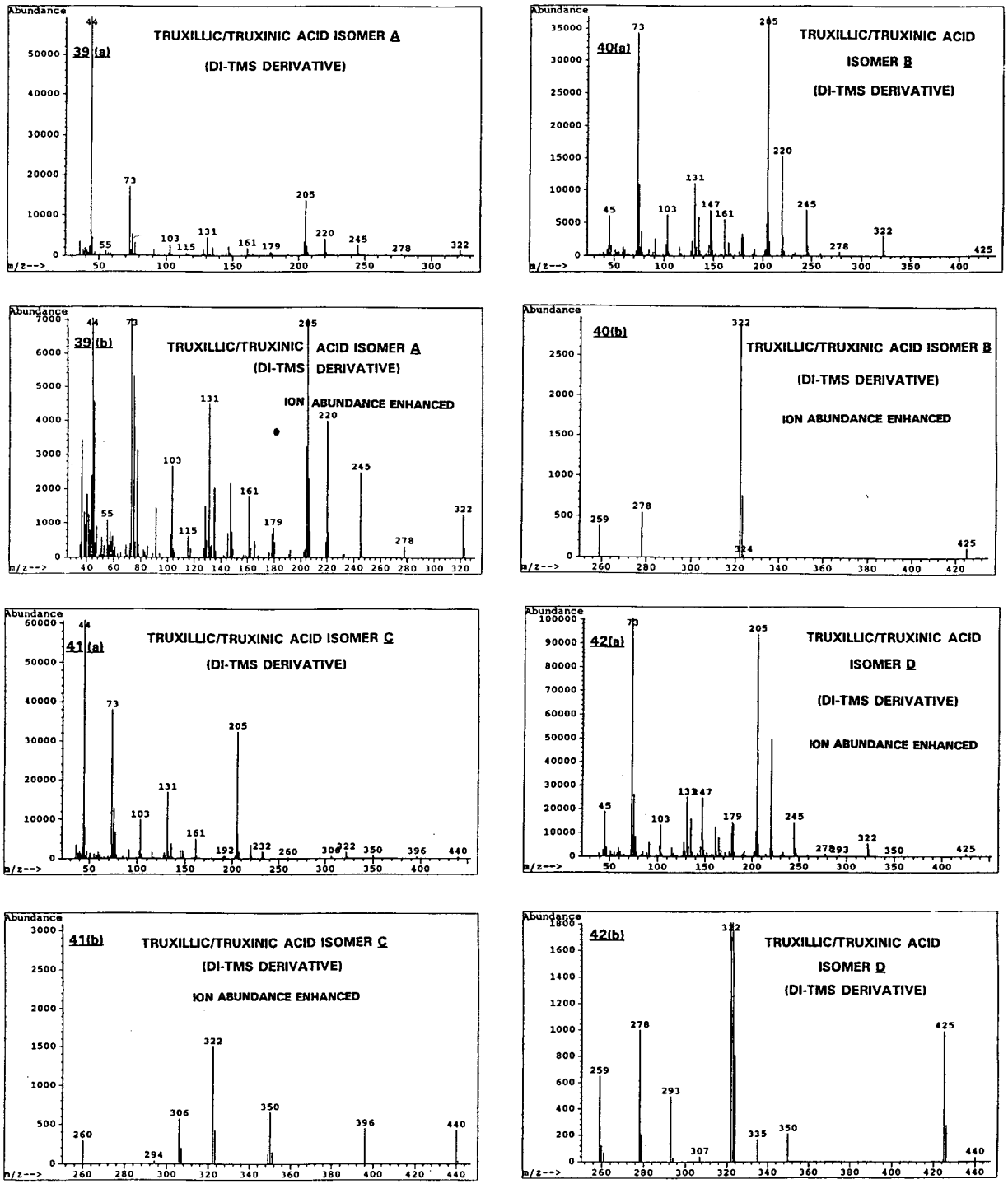

39 (a)
TRUXILLIC/TRUXINIC ACID ISOMER A
(DI-TMS DERIVATIVE)
40(a)
TRUXILLIC/TRUXINIC ACID ISOMER B
(DI-TMS DERIVATIVE)
39 (b)
TRUXILLIC/TRUXINIC ACID ISOMER A
(DI-TMS DERIVATIVE)
ION ABUNDANCE ENHANCED
40(b)
TRUXILLIC/TRUXINIC ACID ISOMER B
(DI-TMS DERIVATIVE)
ION ABUNDANCE ENHANCED
41 (a)
TRUXILLIC/TRUXINIC ACID ISOMER C
(DI-TMS DERIVATIVE)
42(a)
TRUXILLIC/TRUXINIC ACID ISOMER D
(DI-TMS DERIVATIVE)
ION ABUNDANCE ENHANCED
41(b)
TRUXILLIC/TRUXINIC ACID ISOMER C
(DI-TMS DERIVATIVE)
ION ABUNDANCE ENHANCED
42(b)
TRUXILLIC/TRUXINIC ACID ISOMER D
(DI-TMS DERIVATIVE)
Abundance
m/z-->

Fig. 16.

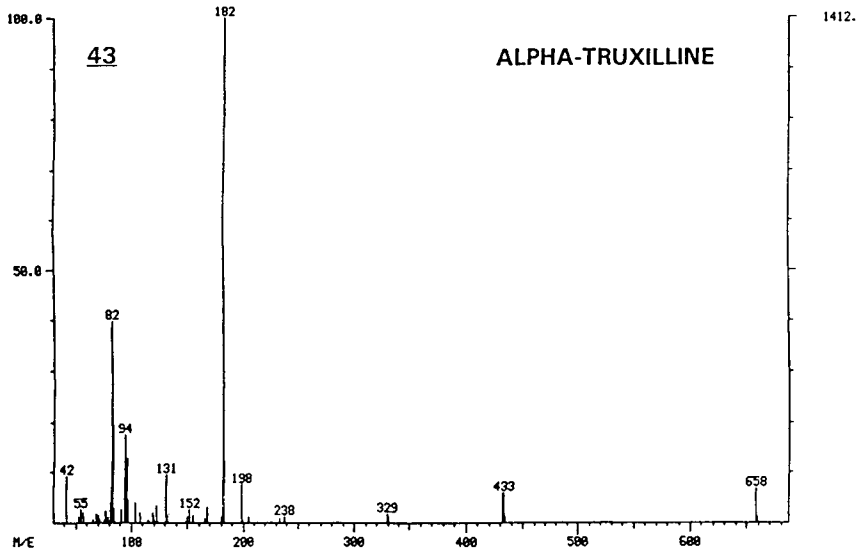


Fig. 16.

13. Acknowledgement

We are grateful to Donald A. Cooper for providing the electron ionization mass spectrum of α-truxilline.

14. Appendix: Electron ionization mass spectra of alkaloids and manufacturing by-products detected in the leaves of South American *E. coca* var. *coca* and/or illicit refined cocaine samples

All spectra (Fig. 16), excepting that for α-truxilline, were acquired on a Hewlett-Packard Model 5971 quadrupole mass-selective detector interfaced with a Hewlett-Packard 5890 Series II gas chromatograph. The mass-selective detector was operated in the electron ionization mode with an ionization potential of 70 eV, a secondary electron multiplier value of 1541 and 1.2 scans/s. The gas chromatograph was fitted with a 30 m $\times$ 0.25 mm I.D. fused-silica capillary column coated with DB-1 at a film thickness of 0.25 μm. The mass spectra of impurities/by-products with labile protons were also acquired as their trimethylsilyl derivatives. Derivatized sites include carboxyl, hydroxyl and secondary amine moieties. The spectra of four isomeric hydroxycocaines, as trimethylsilyl derivatives (mass spectra 31–34), were acquired from the cGC–MS of a concentrated coca leaf extract. Whereas the hydroxy groups of these isomers can be assigned to the tropane moiety (*e.g.*, see Fig. 8), their positional assignments are equivocal. The spectra of the isomeric truxillic/truxinic acids A–D, as their di-trimethylsilyl derivatives (seen in mass spectra 39–42), are represented by some of the structures illustrated in Fig. 4 (except the α, β, γ and μ isomers).

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Journal of Chromatography A, 674 (1994) 207–215

JOURNAL OF CHROMATOGRAPHY A

Reversed-phase high-performance liquid chromatographic determination of cocaine in plasma and human hair with direct fluorimetric detection

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Abstract

A simple, but sensitive and specific, high-performance liquid chromatographic assay for cocaine with direct fluorimetric detection, particularly intended for the routine analysis of hair and blood samples, is described. Benzoylecgonine, eluting before cocaine in a completely resolved peak, is also detectable. Detection is based on the weak native fluorescence of cocaine and benzoylecgonine, depending on the benzene ring present in both molecules. Hair samples (20–200 mg) were incubated overnight in 2 ml of 0.25 *M* HCl at 45°C and extracted with a commercial liquid–liquid method; the dried residue reconstituted with 500 μl of 0.05 *M* NaH_2PO_4 (pH 5.2) was injected. Blood plasma samples (200 μl) were mixed with 150 μl of 0.1 *M* Na_2HPO_4 (pH 8.9) and extracted with 5 ml of chloroform–2-propanol (9:1); the organic phase was evaporated and the residue dissolved and injected as above. Isocratic reversed-phase liquid chromatography was carried out on a column (150 × 4.6 mm I.D.) packed with spherical 5-μm poly(styrene–divinylbenzene) particles; the mobile phase was 0.1 *M* potassium phosphate (pH 3)–methanol–tetrahydrofuran (70:25:5). The excitation and emission wavelengths were set at 230 and 315 nm, respectively. Under the described conditions, cocaine eluted in a symmetrical peak with a capacity factor of about 5. The limit of detection was about 1 ng/ml (0.2 ng injected), with a signal-to-noise ratio of 3. Using external standardization and partial loop filling, the intra-assay precision of the assay was characterized by R.S.D.s of 5.0 and 3.6% ($n = 6$) for cocaine concentrations of 10 and 100 ng/ml, respectively, and in inter-assay tests ($n = 6$) R.S.D.s of 7.5 and 5.2% were achieved for the same cocaine levels. The linearity of the method was fairly good in the concentration range 1.5–500 ng/ml ($r^2 = 0.9998$). Possible interferences from as many as 90 therapeutic and/or illicit drugs were excluded.

1. Introduction

Cocaine has long been the major abused illicit drug in the USA and, in recent times, it has also flooded into Europe [1]. Differently from opiates, fatal acute intoxications from cocaine are relatively rare, but it is still a frequently mentioned drug by emergency rooms in the USA [2]. In addition, as one of the most potent central nervous system stimulants, cocaine use is often related to road accidents and diverse violent crimes [3]; its use as doping agent has also been reported [4].

Several methods have been developed for the determination of cocaine and its metabolites in biological fluids (*e.g.*, serum, plasma, urine and vitreous humour) and in tissues, among which hair has recently received particular attention [5].

Notwithstanding problems posed by its poor

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stability [6,7] and rapid metabolism to biologically inactive hydrophilic products (ecgonine methyl ester, benzoylecgonine, ecgonine) [8], several immunometric methods (*i.e.*, radioimmunoassays, enzyme immunoassays, fluorescence polarization immunoassays) and chromatographic methods of analysis, as specified later, are now available. However, it is well known that most immunoassays, because of insufficient selectivity, are not suitable for the determination of cocaine in the presence of its metabolites; this is particularly important in clinical and forensic toxicology, if one considers that only cocaine (excluding the minor metabolite norcocaine) is biologically active and that its major metabolites have longer half-lives [8]. Further, results from immunoassays need, in principle, confirmation with alternative (chromatographic) techniques to achieve legal defensibility [9].

Several gas chromatographic (GC) methods for the determination of cocaine and its metabolites in biological fluids and tissues have been reported, using flame ionization [10] or, more effectively, nitrogen-selective [11–13] or electron-capture [13,14] detection. More recently, gas chromatography–mass spectrometry (GC–MS) has achieved wide acceptance, becoming almost a "gold standard" [15,16] (for a review, see ref. 17).

Tandem mass spectrometry [18] and ion-trap mass spectrometry [19] have also proved to be excellent, although very expensive, spectrometric tools for, mainly qualitative, investigations of cocaine in complex biological matrices, such as human hair.

Reversed-phase high-performance liquid chromatography (HPLC), inherently more compatible with biological matrices than GC, has also been applied to the determination of cocaine and its more polar metabolites, especially for emergency toxicology purposes. However, as almost all the methods reported in the literature use UV detection around 230 nm [20–32], some sensitivity and specificity problems still remain unresolved. This means that sample preparation needs special care.

The use of electrochemical detection, in principle more sensitive and selective, was first reported by Selavka *et al.* [33] and applied to plasma by Miller and DeVane [34], but the high potentials required limited both sensitivity and selectivity in practice.

Also the HPLC method with fluorescence detection (HPLC–FL) proposed by Roy *et al.* [35], although aimed specifically at the determination of the non-UV-absorbing metabolite ecgonine methyl ester, did not improve the sensitivity of cocaine determination and required a complex system of postcolumn ion-pair derivatization and in-line extraction.

Recently, capillary electrophoresis has also been reported as an alternative method for assaying drugs of abuse, including cocaine, in biological samples, but problems of sensitivity and reliability still exist [36,37].

The aim of this work was to develop a simple but very sensitive and specific HPLC assay for cocaine, based on the weak native fluorescence of the drug (related to its benzene ring), intended for application in forensic and clinical toxicology and, particularly, in the routine analysis of hair and blood samples.

2. Experimental

2.1. Reagents and standards

Toxi-tubes A (Analytical Systems, Laguna Hills, CA, USA) and Isolute Confirm HCX (IST, Hengoed, UK) were used for liquid–liquid and liquid–solid extraction, respectively.

Stock standard solutions of cocaine (Sigma, St. Louis, MO, USA) were prepared in methanol at 1 mg/ml and stored at −18°C. Working standard solutions at suitable concentrations were prepared every day in water or drug-free extracts of plasma or hair, as needed.

The chromatographic peak of benzoylecgonine was identified using standards of benzoylecgonine (about 10 μg dried on glass microfibre discs impregnated with silicic acid) enclosed in the Toxi Disc Library (Analytical Systems). Larger amounts of benzoylecgonine were obtained from cocaine by mild hydrolysis in 0.01 *M* borate buffer (pH 8.6) at room temperature; the conversion was monitored by this HPLC method and the benzoylecgonine peak was collected.

However, lacking a certified standard, no precise titration of benzoylecgonine was carried out.

Standards of 90 therapeutic drugs and drugs of abuse (10 μg each), supplied desiccated on glass microfibre discs impregnated with silicic acid, were obtained with the Toxi Disc Library.

Water and other solvents, all of HPLC grade, and chemicals (RPE grade) used for extraction and liquid chromatography were purchased from Carlo Erba (Milan, Italy).

Citrate–phosphate–dextrose solution (Sigma) was used as anticoagulant, according to the producer's guidelines (anticoagulant:whole blood ratio equal to 1.4:10).

2.2. HPLC instrumentation and analytical conditions

The isocratic HPLC system used consisted of a single-piston high-pressure pump (Model 302; Gilson, Villiers-le-Bel, France), a pulse damper (Model 802 C; Gilson), a manual injector (Model 7125; Rheodyne, Cotati, CA, USA) with a 200-μl loop and a double monochromator spectrofluorimeter (Model 821 FP; Jasco, Tokyo, Japan). The excitation and the emission wavelengths were routinely set at 230 and 315 nm, respectively. The detector signal was recorded with a data system (Model 620; Gilson).

The column (150 × 4.6 mm I.D.) was packed with spherical 5-μm poly(styrene–divinylbenzene) (Bio-Gel PRP 70-5; Bio-Rad Labs., Brussels, Belgium). The mobile phase, pumped at a flow-rate of 0.5 ml/min, was 0.1 *M* potassium phosphate (pH 3)–methanol–tetrahydrofuran (70:25:5).

Usually, 100 μl of sample were injected with partial loop filling but, when sensitivity was crucial, full-loop injections were accomplished. In this event, the loop was flushed with a sample volume at least three times greater than that of the loop itself (200 μl).

Lacking a suitable internal standard, external standardization was adopted for quantification.

Using a feature of the Jasco spectrofluorimeter, rough fluorescence spectra of 10 μg/ml cocaine dissolved in water and in 50 m*M* phosphate buffers in the pH range 1.5–9.2 were obtained. For this purpose, the flow cell of the instrument was filled with cocaine solutions using an intradermal syringe. The width of the excitation and emission slits was 18 nm.

2.3. Sample preparation

Hair samples (20–200 mg), cut close to the scalp, were washed with two 20-ml volumes of 0.3% Tween 20 (Sigma) solution in water and then thoroughly rinsed with tap water. After drying at 37°C, the hair samples were cut manually into small fragments and incubated overnight in 2 ml of 0.25 *M* HCl at 45°C. The resulting mixtures were neutralized with equimolar amounts of 1 *M* NaOH and extracted twice into the organic phase with ready-to-use Toxi-Tubes A. The pooled organic layers were evaporated to dryness and the residue was usually reconstituted with 500 μl of 0.05 *M* NaH_2PO_4 (pH 5.2).

Blood bank plasma samples (with citrate–phosphate–dextrose) were assayed in addition to plasma and cadaveric blood from intoxicated subjects. The addition of NaF to blood to prevent cocaine hydrolysis was omitted because the plasma was soon extracted after blood centrifugation (at 4°C) or, otherwise, frozen at −20°C. Amounts of 200 μl plasma were mixed with 150 μl of 0.1 *M* Na_2HPO_4 (pH 8.9) and 5 ml of chloroform–2-propanol (9:1); after vortex mixing for 2 min and centrifuging at 3500 rpm (about 700 *g*) for 10 min, 4 ml of the organic phase were evaporated to dryness under air stream and the residue was dissolved as above.

With "dirty" biological matrices, such as some hair samples, a solid–liquid extraction (SPE) step was added to the liquid–liquid extraction. In this case, the extracts in phosphate buffer (pH 5.2) were loaded in "double-mechanism" (reversed-phase and cation-exchange) SPE cartridges (Isolute Confirm HCX, 80 mg), previously conditioned with 2 ml of methanol and 3 ml of 0.05 *M* NaH_2PO_4 (pH 5.2). After washing with 3 ml of 0.1 *M* HCl and 3 ml of methanol, cocaine was eluted with 3 ml of a methylene chloride–2-propanol (4:1) containing 2% of ammonia. The eluate was then evaporated to dryness and the

residue was usually dissolved in 500 μl of 0.05 *M* NaH_2PO_4 (pH 5.2).

Recovery studies were carried out by adding known amounts to blank plasmas or blank hair acid extracts, which were then processed according to the specific extraction procedures. The extracts were then injected and peaks measured *versus* the corresponding standards directly injected.

3. Results and discussion

3.1. Cocaine native fluorescence

Cocaine in water showed a fluorescence band with a maximum at 315 nm when irradiated at an excitation wavelength of 230 nm. No influence of pH on the fluorescence characteristics was observed.

3.2. HPLC determination

Under the described conditions, cocaine eluted with a capacity factor (k') of about 5. The adoption of a polymeric column, avoiding the silanol-related problems, common in the analysis of basic drugs such as cocaine, allowed us to obtain symmetric peaks (asymmetry factor = 1) without the use of any additives.

Benzoylecgonine also proved to be fluorescent and, according to its more polar nature, it eluted before cocaine in a symmetric peak, with $k' = 3.0$. However, because of the lack of a certified standard, poor extraction from aqueous solutions with our routine liquid–liquid methods and considering the scope of this work aimed at cocaine assay, its analytical optimization has been overlooked.

The efficiency of cocaine separation was about 20 000 plates/m.

The use of fluorescence detection, although based on the simple aromatic ring of cocaine, allowed an important increase in sensitivity in comparison with UV detection. In reality, the limit of detection (LOD) of the present method was about 1 ng/ml (0.2 ng injected), with a signal-to-noise ratio of 3. This result is more than five times better than the LODs recently reported using HPLC–UV detection [24–26,28, 29,34]. Only one HPLC–UV method, by Lampert and Stewart [23], gave a comparable sensitivity, but using a fixed-wavelength detector (cadmium lamp, 228 nm), which is inherently less selective. In addition, the instrument was fitted with a "laboratory-made preamplifier/analogue

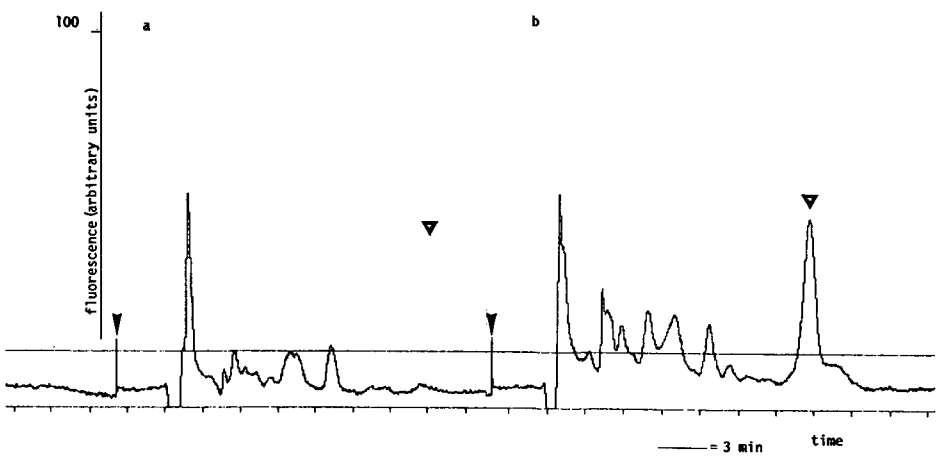

Fig. 1. Typical chromatograms of (a) extract (with Toxi-tubes) of blank hair (25 mg) and (b) hair sample (25 mg) from a very light cocaine user containing 0.2 ng/mg of cocaine (the cut-off limit is 0.1 ng/mg). Volume injected, 100 μl, corresponding to 1 ng of cocaine, in case b. The arrow heads indicate injections and triangles indicate cocaine retention time. Chart speed, 4 mm/min. For other analytical conditions, see text.

filter", which can hardly be reproduced in other laboratories.

Moreover, the main advantage of the proposed method is the possibility of dealing with biological matrices, such as plasma and hair, without complex sample pretreatments. In fact, a simple and rough liquid–liquid extraction was sufficient in the most instances for obtaining an extract suitable for injection, even at the highest sensitivity. Only in few cases did hair samples, which were still "dirty" after the liquid–liquid extraction, need further purification with SPE. Fig. 1 shows typical chromatograms of blank hair and hair from a cocaine user; in Fig. 2 chromatograms of blank plasma and spiked plasma, containing 10 ng/ml of cocaine, are depicted. In Fig. 3 the chromatogram of partially putrefied cadaveric blood (containing 1200 ng/ml of cocaine) from a subject who had died from intravenous overdosage of cocaine is shown (an important concentration of benzoylecgonine coexists with cocaine). All these samples were extracted with the simple liquid–liquid procedures reported for hair and plasma.

The effect of the application of SPE after Toxi-tube extraction to a very complex hair sample is shown in Fig. 4.

Owing to the high selectivity of the method, no suitable internal standard has yet been found.

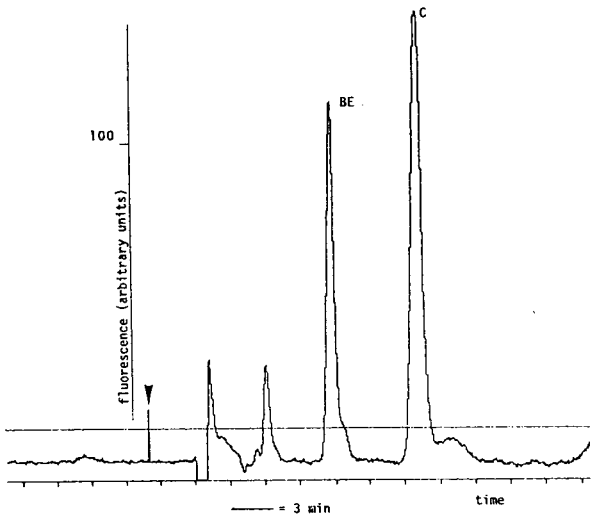

Fig. 3. Chromatogram of a sample of partially putrefied cadaveric blood (containing 1200 ng/ml of cocaine) from a subject who had died from an intravenous overdosage of cocaine (an important concentration of benzoylecgonine coexists with cocaine). Peaks: C = cocaine; BE = benzoylecgonine. Analytical conditions as in Fig. 1.

However, the use of the external standard method for quantification proved suitable for application in real cases.

Using Toxi-tubes, the average overall recovery of the method, from 100-mg blank hair samples

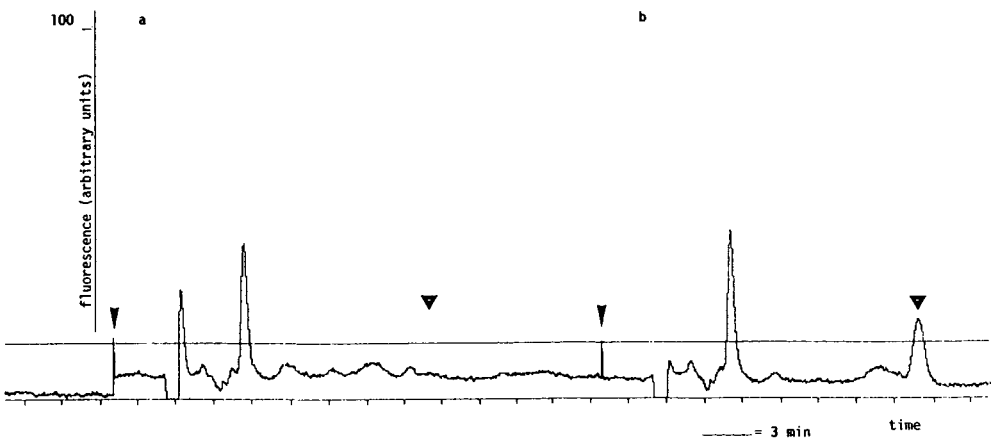

Fig. 2. Typical chromatograms of (a) extract (liquid–liquid method) of blank plasma and (b) plasma spiked with 10 ng/ml of cocaine (100 μl of extract injected, corresponding to 0.4 ng of cocaine). The arrow heads indicate injections and triangles indicate cocaine retention time. Analytical conditions as in Fig. 1.

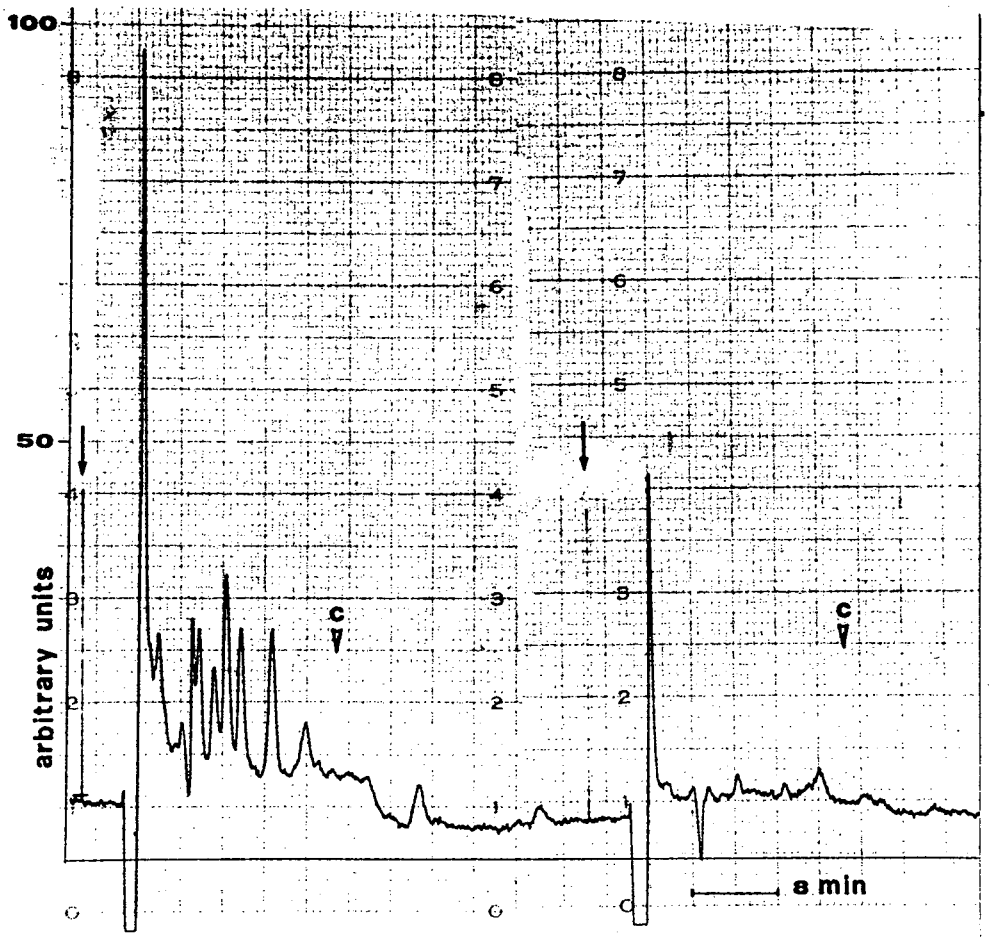

Fig. 4. Chromatograms of the same "dirty" blank hair sample (100 mg, 100 μl of extract injected) extracted only with Toxi-tubes (left) and with Toxi-tubes followed by SPE (Isolute Confirm HCX, 80 mg) (right). Arrows indicate injections; c indicates the elution time of cocaine. Chart speed, 2.5 mm/min. For other analytical conditions, see text.

with 50 ng of cocaine added, was 94.9% with a relative standard deviation (R.S.D.) of 2.2% ($n = 10$).

With the chloroform–2-propanol extraction, the recovery of cocaine from blank plasma samples spiked with the drug to a final concentration of 100 ng/ml was 101.0% (R.S.D. = 4.8%; $n = 10$) and 98.5% (R.S.D. = 3.1%; $n = 10$) for a concentration of 500 ng/ml.

For economic reasons, we used SPE only in series with one of the two liquid–liquid methods, when specifically needed. With this two-step procedure, the recovery of cocaine from hair extracts (spiked as above) was 91.0% (R.S.D. = 2.9%; $n = 6$) and 95.2% (R.S.D. = 5.2%; $n = 6$) from plasma (cocaine concentration 100 ng/ml).

Unfortunately, with both of the liquid–liquid methods presented the recovery of benzoylecgonine, tested in the concentration range 90–540 ng/ml, was unsatisfactory, varying from 13 to 27% with the Toxi-Tube method and from 15 to 23% with the chloroform–2-propanol method. On the other hand, with SPE the recovery of benzoylecgonine was very good, as reported by other workers [15,35], being 93.8% (R.S.D. = 2.9%; $n = 6$) for a level of 100 ng/ml.

Notwithstanding the better performance of SPE with benzoylecgonine, for our purposes we retained the liquid–liquid extractions because these methods allowed the HPLC determination of morphine in the same extracts [38], which with SPE would have required a specific protocol.

The linearity of the method for cocaine was fairly good in the concentration range 1.5–500 ng/ml (corresponding for hair to 0.015 and 5 ng/mg, respectively), being described by the equation $y = 0.395x - 0.028$, $r^2 = 0.9998$ (where x = cocaine concentration in ng/ml and y = fluorescence response).

The intra-assay precision was characterized by R.S.D.s of 5.0 and 3.6% ($n = 6$) for cocaine concentrations of 10 and 100 ng/ml, respectively; in inter-assay tests ($n = 6$), R.S.D.s of 7.5 and 5.2% were achieved for the same cocaine levels in plasma. A comparable precision was also observed in the analysis of hair extracts.

The use of full-loop injection allowed the precision to be increased to R.S.D.s down to less than 3% intra-assay and 4% inter-assay; however, in order to ensure reproducible full-loop filling, a volume of sample at least three times larger had to be used.

Possible interferences were studied by injecting mixtures of common therapeutic and illicit drugs contained in the Toxi Disc Library (Table 1) at a concentration of 20 μg/ml, with the fluorimeter at a sensitivity range allowing the determination of 2 ng/ml of cocaine. Under these conditions, none of the 90 drugs gave any significant peak eluting at the retention time of cocaine. Of course, as the test was carried out *in vitro*, we could not exclude possible interferences from drug metabolites.

Table 1
Drugs investigated in order to exclude interferences in cocaine determination

Type	Drugs			
Opiates and antagonists	Codeine	Dextromethorphan	Dihydrocodeine	Diphenoxilate
	Ethylmorphine	Hydrocodone	Hydromorphone	Meperidine
	Morphine	Methadone	Naloxone	Oxicodone
	Papaverine	Propoxyphene	Terpin hydrate	
Central nervous system active drugs	Amphetamine	Amitriptyline	Benztropine	Carbamazepine
	Caffeine	Chlorprothixene	Chlorpromazine	Diazepam
	Diphenylhydantoin	Doxepin	Ethinamate	Flurazepam
	Imipramine	Loxapine	Meprobamate	Methamphetam.
	Methaqualone	Methylphenidate	Nordiazepam	Nortriptyline
	Pentobarbital	Phenmetrazine	Phentermine	Phencyclidine
	Phenobarbital	Phenytoin	Phetidine	Prazepam
	Protriptyline	Secobarbital	Strychnine	Thioridazine
	Thiothixene	Trifluperazine	Triflupromazine	Amobarbital
	Aprobarbital	Butabarbital	Barbital	
Miscellaneous	Acetaminophen	Atropine	Benzoylecgonine	Carisoprodol
	Chlorpheniramine	Cimetidine	Diphenhydramine	Disopyramide
	Doxylamine	Emetine	Erythromycin	Glutethimide
	Hydrocortisone	Hydroxyzine	Lidocaine	Methapyrilene
	Methocarbamol	Nicotine	Orphenadrine	Pentazocine
	Phenacetin	Pyrilamine	Phenolphthalein	Phenylpropanola.
	Propranolol	Procaine	Procainamide	Pseudoephedrine
	Quinine	Salicylamide	Spironolactone	Triamterene
	Triexyphenidyl	Trimeprazine	Trimetobenzamide	Trimethoprim

No interferences were observed up to levels of 20 μg/ml of each drug.

4. Conclusions

In the investigations of drugs of abuse, high costs, low productivity and/or insufficient sensitivity of the chromatographic methods (TLC, GC, GC–MS) customarily adopted for confirmation of the results from immunometric screening assays are often disadvantages. These limits are crucial in epidemiological surveys, in which high sensitivity and low cost per analysis, together with a high degree of automation, are mandatory features. Also, the traditional chromatographic approaches show limits in clinical toxicology, requiring rapidity, simplicity and ruggedness.

The HPLC–FL method here reported is, to the best of our knowledge, the first based on the direct fluorescence of the cocaine molecule. It is suitable also for the determination of benzoylecgonine. The sensitivity achieved for cocaine is very good, being higher than that reported with GC–MS [15]; the accuracy and precision are comparable to those of other chromatographic methods and the sample pretreatment needed is very simple. In fact, the selectivity of fluorescence detection permitted cocaine to be determined also in the presence of the many UV-absorbing coextractives, which the rapid but rough extraction procedures adopted do not eliminate. Owing to its simplicity, the method is rugged and intrinsically susceptible to automation.

Hence the present HPLC–FL method seems to be important for the determination of cocaine and, tentatively, benzoylecgonine not only in forensic and clinical toxicology, but also in epidemiological surveys on the spreading of drugs of abuse based on hair testing, a purpose for which it is currently applied in our laboratory.

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JOURNAL OF CHROMATOGRAPHY A

Cocaine-related deaths

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Abstract

Cocaine availability has been increasing in Spain in the past few years. A review of all the toxicological analyses carried out at the Madrid Department of the Instituto Nacional de Toxicología, with subjects who had died of drugs from 1990 to 1992, found 533 persons who had cocaine in their blood and/or tissues; 450 (84%) deaths involved cocaine and heroin together whereas 83 (16%) deaths involved cocaine with an absence of heroin. This paper reports the circumstances, cocaine and benzoylecgonine concentrations in the blood and other toxicological findings for the two major groups of deaths where cocaine was found with an absence of heroin, *i.e.*, possible overdose cases (35 cases) and traffic accidents (23 cases).

1. Introduction

Spain has rapidly become a major port of entry into Europe for South American cocaine, owing not only to its geographical situation but also to its linguistic links and the historical bonds between the two countries. Although it is alleged that Spain is only the port for the introduction of cocaine into Europe, this traffic has resulted in cocaine becoming a leading abused drug in Spain. Cocaine is used not only along with heroin but also alone, resulting in an increase in cocaine-related deaths.

Fatalities from cocaine overdose were reported as early as 1972 [1] and 1973 [2]. Some studies tried to assess the blood concentration levels of cocaine in fatal cocaine overdoses [3]. Subsequent studies on cocaine-related deaths included the description of fatal cocaine poisonings [4], cocaine suicides [5] and cocaine deaths where the drug itself was not the primary cause but a contributory factor [6,7]. Other studies focused on cases where cocaine use was directly associated with cardiovascular and neurological deaths [8]. It has also been reported that the number of violent deaths involving cocaine was significant [9]. The association between cocaine and violence has been pointed out [10].

All but three of the above-mentioned studies reported blood concentrations of the parent drug, cocaine. One study calculated cocaine and benzoylecgonine together in the blood using radioimmunoassay [5] whereas only two of the studies calculated cocaine and benzoylecgonine in the blood separately, one of them suggesting that in the cases where the cause of death was not directly attributable to cocaine, only benzoylecgonine was found in the blood.

The major cocaine (COC) metabolites reported in the blood are benzoylecgonine (BE) and ecgonine methyl ester (EME), although relatively few quantitative data on these metabolites exist. Benzoylecgonine has a relatively long half-life (5–7 h) [11] in blood compared with cocaine (1 h) [12]. Consequently, BE is a much better marker for long-term exposure to cocaine

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and hence the amount of cocaine and benzoylecgonine in blood, calculated separately, may provide useful data for the interpretation of the cause of death. Ecgonine methyl ester is not considered so useful a marker as it is produced not only *in vivo* but also *in vitro* by hydrolysis of cocaine in the blood [13].

This paper presents the results of a systematic study of all the cases brought to the Madrid Department of the Instituto Nacional de Toxicologia, where cocaine and/or metabolites were found in the deceased upon toxicological analysis. The same methodology was applied to all the cases: after a preliminary immunoassay of blood and urine, a comprehensive screening of all the available samples was performed by gas chromatography with nitrogen–phosphorus detection (GC–NPD). The extracts of the samples used for GC were derivatized and subjected to gas chromatography–mass spectrometry (GC–MS) for confirmation and quantification of cocaine, its metabolites and heroin metabolites, if present. The major advantage of the method over other published methods is that only one extraction of the blood and one derivatization step are needed for the confirmation and quantification of heroin metabolites, cocaine and benzoylecgonine. The cases were classified into several groups according to the possible cause of death, and cases where cocaine and/or metabolites were found in the absence of heroin are described. We attempt to explain some of the cocaine-related deaths and try to present data that could aid in the forensic interpretation of those deaths.

2. Experimental

2.1. Samples

Blood, urine and tissues from all the cases suspected to be drug related were obtained during routine autopsies by various pathologists over a 3-year period. Information was provided by the police reports and the judicial inquests.

2.2. Drugs and chemicals

Cocaine hydrochloride, pentafluoropropionic anhydride and hexafluoro-2-propanol were obtained from Sigma. Benzoylecgonine and ecgonine methyl ester were prepared from cocaine according to a published procedure [14]. Columns packed with Extrelut silica were purchased from Merck (Scharlau, Germany) and Sep-Pak C_{18} cartridges from Waters. All other chemicals and solvents were of the purest grade available (Merck).

2.3. Immunoassays

Immunoassay screenings were performed on a Syva (Palo Alto, CA, USA) ETS system using original reagents for opiates, cocaine, benzodiazepines, methadone, propoxyphene and amphetamines.

Urine samples were filtered and the pH was adjusted to 6.5 with 0.1 *M* HCl or 0.1 *M* NaOH.

Blood samples (0.5 ml) were extracted in Sep-Pak C_{18} cartridges following procedures already described [15]. The residue was reconstituted with 0.5 ml of the buffer supplied with the EMIT reagents (0.055 *M* Tris buffer, pH 8). The results from both urine and blood provided rapid information on the possible presence of drugs, which was confirmed later.

2.4. Extraction procedure for blood and urine

To 5 ml of whole blood, 100 μl of nalorphine solution (8 μg/ml) were added as an internal standard. The pH was then adjusted to 9 with borate buffer. The sample was vortex mixed for 10 min and poured into an extraction column packed with Extrelut silica. After 10 min, elution was carried out with 20 ml of a 2-propanol–dichloromethane (10:90). The eluate was evaporated under nitrogen.

The same procedure was applied to 5 ml of urine, but adding 0.1 ml of nalorphine (80 μg/ml).

2.5. Extraction procedure for viscera

To 5 g of lung, liver or kidney, 0.5 ml of the nalorphine solution (80 μg/ml) was added as an internal standard, then 2.5 ml of 10% phosphoric acid and 15 ml of saturated ammonium chloride solution were added. The mixture was heated in a water-bath for 10 min. After cooling, it was filtered and the filtrate was extracted as for urine.

2.6. Gas chromatographic analysis

The extraction residues of blood, urine, etc., were reconstituted with 0.3 ml of methanol, shaken and poured into GC autosampler vials. They were injected into the chromatograph for general screening of drugs and metabolites [16].

The instrument was a Hewlett-Packard (HP) (Avondale, PA, USA) Model 5890 gas chromatograph equipped with a nitrogen–phosphorus detector, a Model 7673A automatic sampler and a split–splitless capillary inlet system. A 25 m × 0.20 mm I.D. fused-silica capillary column coated with cross-linked methylsilicone (0.11-μm film thickness) was used. The operating conditions were as follows: linear velocity of helium, 53 cm/s; detector and injection port temperatures, 300°C; and splitting ratio, 1:30. The column temperature was initially held at 180°C for 1 min and then increased to 300°C at 10°C/min.

2.7. Gas chromatographic–mass spectrometric analysis

The GC autosampler vials containing the extracts were evaporated to dryness under nitrogen. A 100-μl volume of pentafluoropropionic anhydride (PFPA) along with 50 μl of hexafluoro-2-propanol (HFIP) were added to each vial; after sealing, the vials were incubated for 30 min at 60°C. They were then decapped and carefully evaporated to dryness under nitrogen. The dried extracts were taken up in 100 μl of ethyl acetate before injection into the GC–MS system. The instrument was an HP Model 5890 gas chromatograph coupled to an HP Model 5791 mass selective detector, controlled by MS Chemstation (DOS series) software. The column was identical with that used for GC. The operating conditions for the GC–MS system were as follows: injection port, oven temperature, linear velocity of helium and splitting ratio as for GC; ionization energy, 70 eV; MS temperature, 190°C; and operating mode, selected-ion monitoring (SIM). A user macro was created to automate the analysis. The selected ions for cocaine (COC) and its derivatized metabolites ecgonine methyl ester pentafluoropropionyl derivative (EMEPFP), benzoylecgonine hexafluoroisopropyl ester (BEHFIP) and the heroin derivatized metabolites 3-monoacetylmorphine pentafluoropropionyl derivative (MAMPFP), codeine pentafluoropropionyl derivative (CODPFP) and morphine pentafluoropropionyl derivative (MORFPFP) are given in Table 1.

Cocaine and benzoylecgonine concentrations in the blood were determined in all cases where this sample was available. Quantification was performed by GC–MS in the SIM mode using calibration graphs obtained with nalorphine as internal standard and derivatization as described for the samples. The procedure was achieved by application of linear regression analysis to the peak-area ratios of ions of m/z 182 to 440 (for COC) and m/z 318 to 440 (for BEHFIP) for a blood sample and the three-point calibration graph prepared at the beginning of the batch.

Table 1
Selected ions for GC–MS

Compound[a]	m/z[b]
Ecgonine methyl ester-PFP	182, 345, 314
Cocaine	182, 303, 272
Benzoylecgonine-HFIP	318, 439, 334
Morphine-$(PFP)_2$	414, 577, 430
Monoacetylmorphine-PFP	414, 473, 361
Codeine-PFP	282, 445, 388
Nalorphine-PFP	440, 456, 357

[a] PFP = pentafluoropropionic derivative; HFIP = hexafluoro-2-propyl ester;
[b] The first ions listed are selected for quantification.

The calibration standards were prepared from spiked negative blood to provide concentrations of 0.1, 0.8 and 2.5 μg/ml for each compound. The equations for the graphs were $y = 1.02x - 0.157$ ($r = 0.998$) for COC and $y = 3.88x - 0.18$ ($r = 0.999$) for BEHFIP, where y is response ratio (area of COC or BHFIP/area of nalorphine-PFP) and x is amount ratio (concentration of COC or BE/concentration of nalorphine). Two controls of 0.05 and 5 μg/ml were then analysed to verify the linearity of each graph. The limit of quantification was 0.01 μg/ml for both COC and BEHFIP. Samples with a higher concentration than the highest standard were diluted so as to fall within the stated levels before repeating the analysis. The precision of the method was determined by analysing quality control samples that contained COC and BE at known concentrations. At the level of 0.1 μg/ml, the relative standard deviations (R.S.D.s) were 7% for COC and 5% for BEHFIP ($n = 6$). At the level of 10 μg/ml, the R.S.D.s were 5% for COC and 3% for BEHFIP ($n = 6$). The recovery for COC and BE was established using spiked negative blood: for samples spiked at 0.1 μg/ml the mean recovery was 85% for COC and 72% for BE. At higher concentration (10 μg/ml) the mean recovery was 80% for COC and 68% for BE. Fig. 1 shows a typical SIM chromatogram for the determination of COC and BE in a sample of blood.

Table 2
Cocaine-related deaths (in absence of heroin)

Cause of death	No.
Possible cocaine overdose	35
Traffic accidents	23
Suicide by hanging	7
Gunshot (homicide or suicide)	6
Stab wound (homicide)	3
Accidental	9
Total	83

Ecgonine methyl ester was always investigated but not routinely determined.

3. Results and discussion

A total of 533 deaths involved cocaine in the period 1990–92. Of these, 450 (84%) were found to contain heroin plus cocaine and only 83 cases (16%) contained cocaine with no presence of heroin. Table 2 presents these cases classified according to the possible cause of death. Tables 3 and 4 show the circumstances of death, toxicological findings and cocaine and benzoylecgonine concentrations in the blood of the two major groups of deaths that involved cocaine in absence of heroin.

We purposely avoided the study of the 450 deaths involving cocaine plus heroin because it is

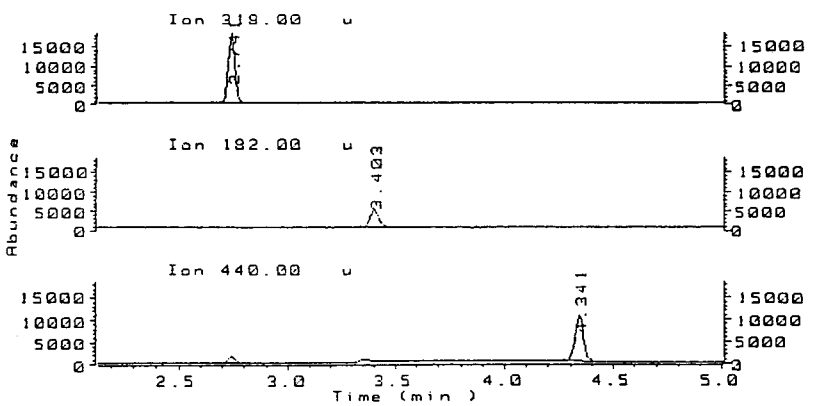

Fig. 1. Single-ion profile of the molecular ions for each drug in a 0.05 μg/ml blood control: benzoylecgonine hexafluoroisopropyl ester, m/z 318; cocaine, m/z 182; nalorphine pentafluoropropionyl derivative, m/z 440.

Table 3
Cocaine-related deaths: possible cocaine overdose

Case/age/sex	Administration route	Circumstances	COC/BE concentration in blood (μg/ml)	Other drugs in blood (μg/ml)
1/23/M	Unknown	Emergency room Heart attack Cardiomegalia Hepatitis AIDS	0.95/0.5	None
2/30/M	Unknown	Emergency room Suspected heroin O.D.	N.D./0.18	None
3/30/M	Unknown	Emergency room Suspected heroin O.D.	9/16	Lidocaine: 0.01
4/30/M	Oral	Hospital Five packages in bowel	N.D./7	Dipyrone: 17.4 Theophylline: 1.7
5/35/M	Unknown	Emergency room Heart attack Chronic hepatopathy	N.D./0.4	Nordiazepam: 0.5 Ethanol: 0.4 g/l
6/28/M	Unknown	Emergency room	N.D./0.4	None
7/22/M	Unknown	Home Heart attack Hepatitis AIDS	0.1/1	Phenobarbital: 5 Naproxen: 0.13
8/30/M	Unknown	Prison Heart attack	1.5/1	Nordiazepam: 1
9/33/F	Unknown	Home Cocaine user Diabetic	N.D./4.6	None
10/37/M	Unknown	Emergency room Cocaine user Myocardial failure AIDS	0.5/5.18	None
11/28/M	Unknown	Sudden death Anoxia Cardiomegalia	N.D./0.10	None
12/29/M	Intravenous injection	Home Lung acute oedema	1.2/3.9	None
13/?/M	Intravenous injection	Open space Suspected heroin O.D.	0.25/5.7	None
14/50/M	Oral	Airport; 85 packages in stomach	10/1.2	None
15/35/M	Oral	Prison hospital; 98 packages in digestive tract	11.8/0.67	Dipyrone: 0.41
16/20/M	Unknown	Discoteque Sudden death after seizures	0.6/14	Ethanol: 0.4 g/l

(Continued on p. 222)

Table 3 *(continued)*

Case/age/sex	Administration route	Circumstances	COC/BE concentration in blood (μg/ml)	Other drugs in blood (μg/ml)
17/41/M	Inhaled	Home Heroin seller Brain and lung oedema	1.24/0.05	Ethanol: 0.84 g/l
18/40/M	Unknown	Transvestite Death after vomiting and abdominal cramps	N.D./0.21	Ethanol: 1.10 g/l
19/30/M	Unknown	Home Cardiorespiratory failure	B.N.A.	None
20/30/M	Unknown	Heroin and cocaine user Heart attack Lung oedema	N.D./0.2	Methadone: 0.1 Levomepromazine: 0.56 Nordiazepam: 1.8
21/?/M	Oral	Death in hospital after 4 days	N.D./4.5	Dipyrone: 4
22/28/F	Unknown	Recent injection	N.D./24	Naproxen: 45
23/36/M	Unknown	Home	6/24	None
24/35/M	Unknown	Sudden death after seizures	0.6/7.4	None
25/34/M	Snorted	Cocaine user Suspected cocaine O.D.	0.65/3.5	None
26/23/M	Unknown	Emergency room Vomiting, bronchoaspiration and heart attack	3.6/20	None
27/32/M	Unknown	Death on arrival at the hospital	0.7/4	None
28/25/M	Intravenous injection	Death with syringe affixed	1.7/16	None
29/32/M	Intravenous injection	Suspected cocaine O.D.	2.1/39	None
30/60/M	Oral	Hospital Packages in digestive tract	66/40	None
31/30/M	Oral	Police station. Ingested the drug	0.38/15	None
32/22/M	Oral	Police station. Ingested the drug	6/129	None
33/29/M	Intravenous injection	Home	B.N.A.	None
34/26/M	Unknown	Open space	N.D./0.02	MDMA: 0.41 Lidocaine: 1.9 Nordiazepam: 0.13
35/33/M	Intravenous injection	Death on arrival at the hospital after seizures	N.D./0.18	Nordiazepam: 0.18

Abbreviations: M = male; F = female; N.D. = not detected; B.N.A. = blood not available; O.D. = overdose

Table 4
Cocaine-related deaths: traffic accidents

Case/age/sex	COC/BE concentration in blood (μg/ml)	Other drugs in blood (μg/ml)
36/26/M	0.34/43	None
37/34/M	N.D./0.04	None
38/26/M	N.D./0.3	Ethanol: 1.2 g/l
39/?/M	0.01/1.6	Ethanol: 0.3 g/l
40/22/M	B.N.A.	None
41/?/M	0.02/2	None
42/27/M	0.07/3.5	None
43/25/M	N.D./0.13	Ethanol: 0.93 g/l THC: 4
44/26/M	N.D./0.3	Ethanol: 1.14 g/l
45/?/F	N.D./0.7	Ethanol: 0.56 g/l
46/36/M	N.D./0.05	Ethanol: 1.37/g/l
47/26/M	N.D./0.20	Ethanol: 0.51 g/l
48/26/M	N.D./0.20	None
49/24/M	B.N.A.	None
50/31/M	N.D./13	None
51/35/M	B.N.A.	None
52/33/M	N.D./0.07	None
53/25/M	N.D./0.05	Ethanol: 2.08 g/l
54/19/M	0.2/3.7	Ethanol: 1.55 g/l
55/27/M	N.D./0.03	Ethanol: 1.91 g/l
56/24/M	0.03/31	Ethanol: 1.7 g/l
57/29/M	B.N.A.	None
58/?/M	N.D./3.8	None

Abbreviations: M = male; F = female: N.D. = not detected; B.N.A. = blood not available

very difficult to evaluate the contributions of each of the two drugs to the deaths. The risk of acute overdose reaction increases with the mixture of both drugs [17]. We concentrated on deaths where only cocaine was involved as a major drug and we present data for the two largest groups: the first where cocaine appeared to be the cause of death and the second, traffic accidents, where cocaine may have contributed to the deaths.

In both groups, women made up a small number of cases (5%), which is in accordance with published data on the distribution of gender of cocaine users [18].

The median of age at the time of death was 31 years for the first and 27 years for the second group. The ages ranged from 20 to 60 years in the first group and from 19 to 36 years in the second group.

In the first group the major route of intake, when known, was by oral ingestion followed by intravenous injection. Of the seven cases of oral ingestion, five corresponded to body-packers. One of them died in the airport, two others lived for a short time until arrival at the hospital and the other two survived for 2 days (case 4) and 4 days (case 21) in the hospital. The other two cases of oral intake corresponded to cocaine sellers who after having been arrested and taken to the police station, ingested the drug to avoid being caught when searched. Except for cases 4 and 21 these cases showed high values of cocaine or benzoylecgonine in blood. Owing to the shorter half-life (1 h) of cocaine in blood compared with that of BE (5–7 h), the survival time determines which is higher, the level of the parent drug or the level of the metabolite in the blood. Fifteen more cases in this group also had both cocaine and benzoylecgonine in the blood: five died on arrival at the hospital or emergency

room, two died suddenly after seizures, three were suspected drug (cocaine or heroin) overdoses, two died of a heart attack, one died with a syringe still attached to the arm and two died at home of lung oedema. In the other thirteen cases in this group (35%), only benzoylecgonine was found in the blood, thus suggesting longer survival periods for most of them or the contribution of other drugs to the cause of death (cases 5, 18 and 35). A wide range was noted for both cocaine and benzoylecgonine concentrations in the blood: from 0.1 to 66 μg/ml for cocaine and from 0.02 to 129 μg/ml for benzoylecgonine. The cocaine concentrations are in agreement with previously published data [19–21]. For benzoylecgonine this wide variation has also been described [9]. Ecgonine methyl ester was found positive in all the cases, which confirms the *in vivo* or *in vitro* hydrolysis of cocaine in the blood [13]. Ethanol was found only in four of the 35 cases in this group and some of the other drugs found in blood could be attributed to medical treatment (dipyrone, theophylline).

In the second group, traffic accidents (Table 4), the presence of ethanol in the blood was very significant: eleven of the nineteen blood samples analysed (57%), at concentrations ranging from 0.3 to 2.08 g/l with a mean value of 1.21 g/l. Only six cases in this group (26%) had detectable levels of cocaine in the blood, ranging from 0.01 to 0.34 μg/ml. One of them (case 56) was found in the involved vehicle with a syringe still attached to the arm. For the rest, only benzoylecgonine was found at the time of the death at concentrations ranging from 0.03 to 43 μg/ml. Those ranges, albeit also wide, are lower than those obtained for the group in Table 3. In all the cases involving traffic accidents ecgonine methyl ester was also found.

4. Conclusions

In conjunction with the availability and popularity of cocaine, this drug is becoming responsible for a certain number of deaths, either directly (overdoses) or indirectly (traffic accidents, homicides, etc.). In this study it has been shown that the amounts of cocaine and benzoylecgonine in the blood should be calculated separately as their presence and relative concentrations prove useful in determining the cause of death and possible survival time after administration.

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Review

Gas chromatographic–mass spectrometric procedures used for the identification and determination of morphine, codeine and 6-monoacetylmorphine

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Abstract

An overview of the analysis of opiates by gas chromatography–mass spectrometry (GC–MS) is presented. The review is focused on the hydrolysis, extraction and derivatization procedures most widely used for the identification and determination by GC–MS of legal and illegal opiates in various biological fluids.

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1. Introduction

Opioid analgesics comprise a large group of substances. Some compounds have long been used for their therapeutic qualities as analgesics (morphine) or antitussive agents (codeine and the semi-synthetic derivatives, dihydrocodeine, oxycodone, etc.). Opiates are also found in opium poppy seeds, an ingredient of bakery products. With the rise in the use of illegal drugs there is increasing pressure to identify illegal drug consumption. Consequently, toxicology laboratories, especially those testing for substance abuse, must have specific and sensitive techniques to discriminate between the legal and illegal intake of opiates.

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Different methods have been developed for the detection and determination of opiates in body fluids, including thin-layer chromatography [1,2], gas chromatography with flame ionization [3,4] or electron-capture detection [5,6] and liquid chromatography [7–9]. However immunological methods are now widely adopted as the initial screening test to detect opiates in urine because they have adequate sensitivity and pretreatment of samples is not required [10,11]. These immunoassays are reliable for differentiating specimens containing opiate metabolites above cut-offs specified in the Mandatory Guidelines for Federal Workplace Drug Testing Programs, Notice Fed. Reg. 53 (1988) 11970 (300 ng/ml for total morphine and 25 ng/ml for free morphine). Some radioimmunoassay (RIA) kits are highly specific for free morphine [12], but generally the immunoassays are not very specific as legal and illegal opiates give substantial cross-reactivity. Therefore, presumptive positive specimens need to be retested and gas chromatography coupled with mass spectrometry (GC–MS) has been designated as the only acceptable confirmation technique [13].

This review is focused on sample preparation, derivatization and GC–MS procedures commonly used for confirmatory analysis and determination of opiates. The number of protocols proposed in the literature is relatively limited and numerous minor variations have been described, rendering the choice of a method difficult.

2. Opiate metabolism

The opiate substances the most frequently detected and determined in biological fluids are codeine, morphine and 6-monoacetylmorphine (6-MAM), a metabolite of heroin.

2.1. Morphine

In man, morphine metabolism depends largely on the route of drug administration. After oral administration, morphine is quickly absorbed from the gastrointestinal tract and is rapidly conjugated in the cells of the intestinal mucosa and in the liver, hence no free morphine appears in the plasma [14]. After an intravenous injection, the morphine level rises to a maximum in the plasma during the first 5 min and declines rapidly during the next 12 h. However, it can still be found in plasma 48 h after injection [15]. A half-time of 1.9–3.1 h and a detection time of 10–44 h have been established [16]. The mean half-life for free morphine is reported to range from 4.3 to 8.1 h and that for conjugate morphine is between 6.4 and 9.7 h [17,18].

In plasma, morphine is partly bound to proteins, preferentially to albumin [19,20]. The binding to albumin explains why morphine is still found in plasma 48 h after injection. The distribution of morphine to the tissues, principally the liver, kidneys, lungs and brain, then proceeds very rapidly.

Morphine is converted into the 3-glucuronide (M3G) and to a lesser extent into the 6-glucuronide (M6G) and 3,6-diglucuronide (M3,6G). More than 50% of the administered morphine is eliminated as M3G [21,22]. The level of M6G in the urine could reach 10% of that of M3G [22]. M6G has potent analgesic activity [23]. Physiologically, this metabolite does not accumulate in plasma, but it may be present in the plasma of patients with renal failure, resulting in side-effects [24] such as respiratory depression [25] or brain syndrome [26].

About 5–10% of administered morphine is converted into the 3-ether sulphate [27,28] and 3–5% into normorphine [29]. Codeine was previously reported as a metabolite of morphine [14,15] but recently Mitchell *et al.* [17] demonstrated unequivocally the absence of codeine as a metabolite of morphine.

The water-soluble conjugates are mainly excreted via the kidneys and very little is eliminated via bile and faeces [15].

2.2. Heroin

The route of heroin administration is generally intravenous, resulting in a transient high drug

concentration in the blood. Heroin quickly disappears from the blood, its half-life being estimated to be *ca.* 2 min [30,31]. Heroin is rapidly deacetylated, first to 6-MAM, which is further hydrolysed to morphine. The pharmacological effects of heroin and 6-MAM are equipotent. 6-MAM is rapidly excreted in the urine within 1–4 h whereas the peak of free morphine occurs within 4 h (half-life 0.6 h) and that of total morphine within 8 h [18]. Hence the detection time is a very important parameter for interpreting results. The detection time for free morphine is substantially shorter than that for total morphine.

2.3. Codeine

Codeine is generally administered orally. The mean half-life of codeine in plasma ranges from 1.6 to 2.4 h according to the dose administered [18]. It is extensively metabolized in the liver, mainly by conjugation with glucuronic acid, and minor routes involve N-demethylation to norcodeine (about 10%) and O-demethylation to morphine. Codeine and its metabolites are eliminated in the urine.

Fig. 1 summarizes the main routes of opiate biotransformation. It is noted that morphine is a metabolite of both heroin, an illegal drug, and codeine, used in prescription medication. The evaluation of the percentages of urinary metabolites of opiates and the presence of M6M, which is solely attributed to heroin, allow the differentiation of heroin abuse from the consumption of legal drugs [13]. Table 1 gives the urinary percentages of opiate metabolites after administration of heroin, codeine and morphine.

3. Assays of opiates by GC–MS

3.1. Choice of samples

Many biological specimens can be used for substance abuse testing. Each type has advantages and disadvantages with respect to its availability and the information that its analysis can supply.

Saliva is readily available but has low drug concentrations and the drug level rapidly declines [34,35]. Hair is also easily available but requires sample pretreatment [36,37]. Tissues [38,39] and vitreous humor [40] are sometimes used. However, blood (or serum or plasma) and urine are the specimens preferentially used for the detection and determination of opiates. A blood sample often offers the advantage of acquiring the parent drug, but its collection requires invasive venous puncture and the drug concentrations decline rapidly. Blood samples are preferred for the follow-up of analgesic treatments. However, urine is generally accepted as the specimen of choice for drug abuse testing or doping analysis. Its collection is non-invasive, the volume obtained can be large and the concentrations of drugs or metabolites are often high, but the drug concentration can vary widely with dose absorbed, time elapsed since administration, etc. [13]. Hence the choice of sample depends on the aim of the analysis.

3.2. Hydrolysis

The opiates are partly conjugated with glucuronic acid and sulphate prior to urinary excretion. Hence total opiates can be recovered after hydrolysis. However, 6-MAM, a marker of heroin abuse, is degraded by acid hydrolysis and morphine can also be partially destroyed [41], so many laboratories do not routinely hydrolyse samples.

Urine is hydrolysed with concentrated hydrochloric acid at 115–120°C [15 p.s.i.; 1 p.s.i. = 6894.76 Pa] for 15 min [42,43,44] or with various concentrations of β-glucuronidase at 37°C for 1 h [45,46] or 24 h [47]. β-Glucuronidase has also been used in combination with arylsulphatase for 1 h at 60°C [40]. The hydrolysis can be performed with *Helix pomatia* juice for 2 h at 56°C [48] or 24 h [49]. In a recent study, acidic hydrolysis was compared with enzymatic hydrolysis and a highest codeine metabolite recovery was obtained with enzymatic hydrolysis [50] even though it is known that β-glucuronidase cannot completely hydrolyse codeine 6-glucuronide. To overcome problems relating to hydrolysis, in our

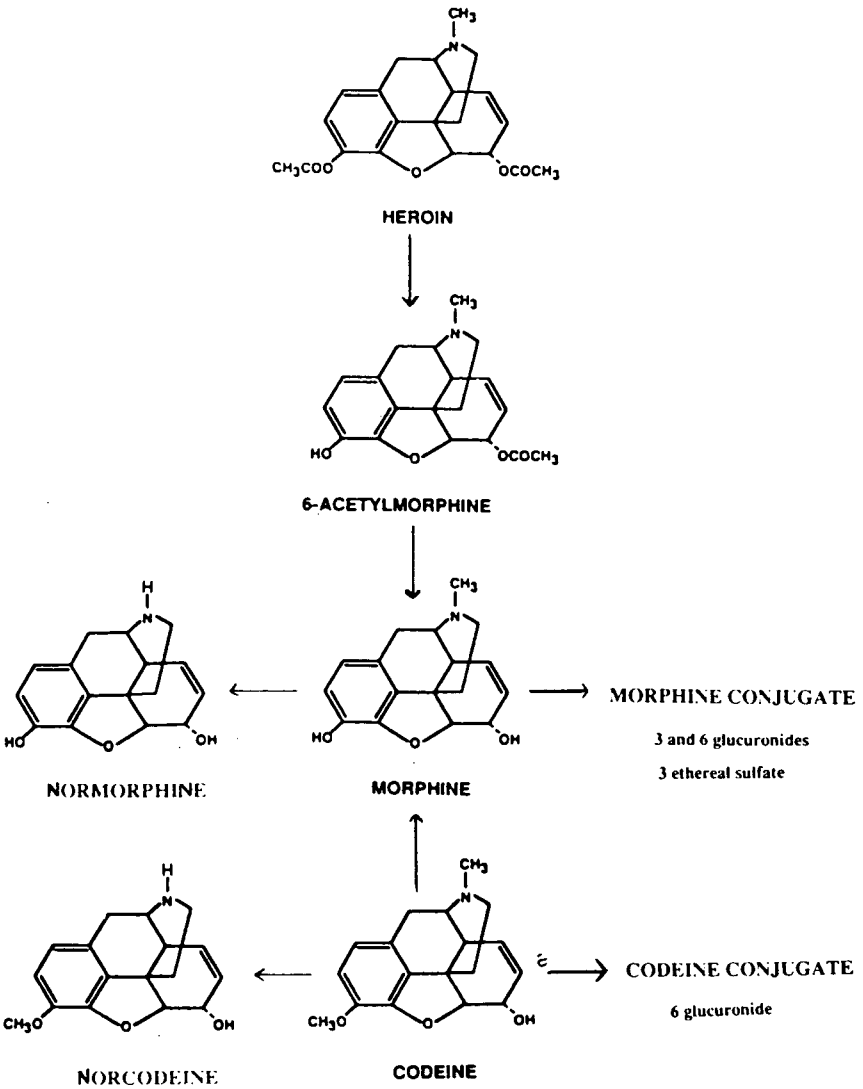

Fig. 1. Main steps of opiate biotransformation.

laboratory we usually assay both acid-hydrolysed and unhydrolysed urine.

3.3. Extraction

The opiates are extracted from biological fluids or tissue homogenates prior to detection and determination. Extraction with organic solvents is frequently used. In this case the samples are previously made alkaline (pH $\approx$ 9) with 1 *M* ammonia solution [3], 1.5 *M* sodium carbonate buffer [46], a mixture of 12 *M* sodium hydroxide and 7.3 *M* ammonium chloride [43] or borate buffer [45]. Then they are extracted with organic solvents: chloroform–2-propanol (9:1) [46,51,52], (4:1) [49] or (3:1) [38], dichloromethane–methanol (9:1) [3], dichloromethane–2-propanol (9:1) [47], isobutanol–dichloromethane (1:9) [43,53], ethyl acetate [39], or toluene–dichloromethane–isobutanol (6:3:1) [54].

The organic phase is evaporated and directly derivatized [46,49,55] or purified by the acid-

Table 1
Urinary metabolites of codeine, morphine and heroin expressed as percentage of administered dose

Metabolite	Codeine	Morphine	Heroin
Free	4.9–8.2 [18]	2–12 [14,15,17]	–
6-Glucuronide	25–56 [18]	<1–10% [21–23]	–
3-Glucuronide	–	20–74 [15,17,23]	–
3-Ether sulphate	–	0.5–10 [23,28]	–
6-Monoacetylmorphine	–	–	0.1–2.8 [27,29]
Morphine:			
Free	~0.1% [18]	–	3.1–7.7 [27,29,32]
3-Conjugate	2–9% [18]	–	34–67 [23,27,29,33]
Normorphine:			
Free	Traces	0.5–1.5	–
Conjugate	–	3–5	–
Norcodeine:			
Free	–	–	–
Conjugate	–	–	–

base method [3,38,43,48,53], by organic phase partitioning [54] or by the solid-phase method on cartridges containing cyanopropyl- or propylamine-modified silica [53]. Paul *et al.* [53] compared solid-phase and acid-base purification and preferred the latter method because it provides between-run consistency in drug recovery.

When a solid-phase extraction technique is used directly, the samples are first made alkaline and passed through a C_{18} reversed-phase column [40,56], C_{18} Bond Elut column [57–60] or Extrelut column [4]. The effect of sample pH on retention has been investigated. Huang *et al.* [59] found the recovery of compounds such as morphine, codeine and hydrocodone from urine to be independent of pH, whereas a better recovery for morphine, codeine and to a lesser extent M3G and M6G was obtained at pH 9 by Pawula *et al.* [61]. On increasing the pH, the ionization of the basic nitrogen group ($pK_a = 8$) is suppressed, thus making morphine and normorphine interact more with the C_{18} group, whereas the glucuronides remain completely ionized at high pH, so their retention times are not increased. The optimum pH for extraction of 6-MAM was found to be 8–8.5 [53]. The opiates are eluted from the column with dichloromethane–acetone (1:1) [56,58], chloroform–2-propanol (9:1) [52] or (4:1) [39], methanol [40], ethyl acetate [4] or dichloromethane–2-propanol (4:1) [50]. Solid-phase extraction gives the best sample purification [40], yielding a low GC–MS background which enhances the mass spectral characteristics and permits a better identification of drugs and metabolites, but it is more expensive than liquid extraction.

3.4. Derivatization

Some investigators do not derivatize the opiates before chromatography [6,62]. However, the underivatized opiates show poor chromatographic properties. The derivatization process converts the polar hydroxyl groups into a non-polar derivative, improving the chromatographic resolution and increasing the sensitivity. Several methods are available for obtaining derivatives: acetylation, propionylation (propionic anhydride) and the formation of trimethylsilyl or perfluoroester derivatives. Although the number of derivatizing agents described in the literature is relatively limited, there is great variability in the experimental conditions. The main protocols are summarized in Table 2.

Maurer and Pfleger [63] described a screening procedure for the detection of 56 opioids, analgesics and their metabolites. However, most reports concern methods for the identification of morphine, codeine and 6-MAM because the purpose is to identify illegal drug abuse. The

Table 2
Derivatizing agents and derivatization conditions described in the literature for the identification and determination of opiates

Method or derivatives	Sample	Derivatizing agent	Volume of derivatizing agent (μl)	Conditions		Solvent for reconstitution of derivatives	Ref.
				Temperature (°C)	Time (min)		
Acetylation	Purified products Urine	Acetic anhydride–pyridine (1:1)	100–400	50–100	15–50	Chloroform, acetone, acetyl acetate	43,55,64
	Vitreous humor	Acetic anhydride–pyridine (2:1)	150	60	30	–	40
	Urine	[$^2H_6$]Acetic anhydride–pyridine 1:1	200	60	15	Ethyl acetate	46
	Urine	Acetic anhydride–4-dimethylamino-pyridine (9:1)		50	15–30	–	44
Propionylation	Urine	Propionic anhydride–pyridine (1:1)	100	50–70	30	Toluene	53
Trimethylsilyl derivatives	Urine	BSA	25	60	60	Chloroform	49
	Urine	BSTFA–acetonitrile (1:2)	80	60	15	Butyl acetate	3[a]
	Urine	BSTFA–TMCS (99:1)	25–50	Room temperature to 70	15–30	Ethyl acetate, dichloromethane	46,64,65
Trifluoroacetyl derivatives	Urine	TFA	50–100	60	20–30	Dichloromethane, ethyl acetate, chloroform	43,48[a],66
	Blood, pure compounds, hair	MBTFA	40–50	60–70	20–30	Dichloromethane	64,70
Pentafluoropropionyl derivatives	Blood, urine	PFPA	25–80	60–70	15–30	Ethyl acetate, dichloromethane, butyl acetate, heptane	3[a],43,47[a],56, 58,60,64,68
		PFPA–ethyl acetate (1:1)	100	90	15	Ethyl acetate	39
		PFPA–PFP-OH (2:1)	75	70–90	15–20	Ethyl acetate	55,67
Heptafluorobutyryl derivatives		HFBA	50–100	60–70	30	Dichloromethane	43,64,71[a]

Abbreviations: BSA = bis(trimethylsilyl)acetamide; BSTFA = bis(trimethylsilyl)trifluoroacetamide; HFBA = heptafluorobutyric anhydride; MBTFA = methylbistrifluoroacetamide; PFPA = pentafluoropropionic anhydride; TMCS = trimethylchlorosilane; TFA = trifluoroacetic anhydride. Detection by GC–MS except where indicated otherwise.
[a] Detection by GC.

determination of these compounds requires the addition of an internal standard to the samples before the extraction procedure. In general, nalorphine or corresponding deuterated products ([$^2H_3$]morphine, [$^2H_3$]codeine, [$^2H_3$]6-MAM) are used as internal standards, the latter permitting the recovery problem to be overcome. At least two ions are monitored for the identification of each opiate. The ions most often used for morphine are those at m/z 327 and 353 (acetylation), 364 and 477 (TFA), 414 and 577 (PFPA), 464 and 207 (HFBA); for codeine 282 and 229 (acetylation), 292 and 395 (TFA), 282 and 445 (PFPA), 282 and 495 (HFBA); for nalorphine 353 and 395 (acetylation), 390 and 503 (TFA), 440 and 603 (PFPA), 207 and 490 (HFBA); and for 6-monoacetylmorphine 204, 328 and 372 (acetylation), 364 and 423 (TFA), 414 and 473 (PFPA). Table 3 gives a more complete list of ions used for opiate identification.

The acetyl derivatives are stable for up to 72 h when stored at room temperature in ethyl acetate [46,56]. However, the acetylation protocol (70°C, 20 min) results in incomplete derivatization, and in addition to the major derivatization product diacetylmorphine a small amount of 3-monoacetylmorphine (3-MAM) is also produced [55].

The m/z 285 ion is found in the mass spectrum of both 3-MAM and [$^2H_3$]acetylcodeine, making this ion unsuitable as a specific ion for [$^2H_3$]codeine. Morphine and 6-MAM are both converted into diacetylmorphine, and therefore acetyl derivatives do not permit morphine and 6-MAM to be distinguished [55].

Derivatization with BSTFA is quantitative and each opiate gives only one derivative. However, TMS derivatives of codeine and norcodeine coelute and 6-MAM gives an additional peak, eluting at the retention time of morphine, which increases when the 6-MAM derivative is stored at room temperature for more than 3 h [3]. The TMS derivatives are known as to be moisture sensitive [64].

PFP derivatives are also sensitive to moisture, but no breakdown products are detected after storage for 24 h in good conditions [55]. The addition of PFPOH improves the yield of the derivatives. Christophersen *et al.* [3] obtained only one PFP derivative for each opiate with the derivatization protocol described (60°C, 15 min), whereas Paul *et al.* [43] found two derivatives for morphine (3,6-di-PFP-morphine and 6-PFP-morphine). In addition, the morphine and the 6-MAM can be clearly detected [56,58]. In spite of their disadvantages, acetyl and PFP derivatives are widely used for the identification and determination of opiates.

3.5. GC–MS procedures

Some investigators use chemical ionization mass spectrometry for the identification and determination of opiates with methane [49,42,61] or ammonia–methane (1:5) [57] as the reactant gas. However, in recent studies the electron impact mode is chosen, generally at 70 eV. The chromatographs are equipped with a 12- or 15-m fused-silica capillary column with apolar stationary phases of cross-linked dimethylsilicone, phenylmethylsilicone or 95% dimethyl–5% polysiloxane [46,56]. The oven temperature can be maintained in the isothermal mode at 230°C [52,45], but in general temperature programming is used with an initial temperature between 50°C [64] and 160°C [40] and a final temperature from 240°C [40] to 280°C [37], the rate of increase being from 10°C/min [31] to 50°C/min [64].

4. Conclusions

Various GC–MS methods have been described for the identification and determination of opiates. As morphine and codeine are conjugated before being excreted in the urine, hydrolysis is required to recover these two compounds totally. Acidic hydrolysis is more rapid and easier than enzymatic hydrolysis. However, 6-MAM can be destroyed during this process. The assays of opiates include an extraction step, first performed with organic solvents but now often replaced by solid-phase extraction. The latter technique has the advantage of decreasing the background noise, which improves the identification of the drugs. Further, this pro-

Table 3
Principal ions (m/z) used for the detection and identification of opiates

Product	Acetylation	Propionylation	TMS	TFA	PFPA	HFBA
Morphine	395, 375, 369, 353 310, 268, 216		429, 414, 369, 287, 268, 236	477, *363*, 263	*577*, *414*, 361, 357	677, 464, 210, 207
[$^2H_3$]Morphine	378, 372, 334, 330, 313			480, *367*	580, *417*	
Codeine	395, 353, *344*, 341, *282* 229		371, 234, 229	*395*, *282*	*445*, 283, *282*	495, 282, 225
[$^2H_3$]Codeine	344, *285*, 232			*398*, *285*		
Nalorphine	401, 395, 357, 353, 336		455, 414, 324, 260	503, 391, 390	*603*, 440, 207	491, 490, 207
6-MAM	372, 328, 204	384, *383*, 324	390, 340, 287, 204	423, *364*, 426, 367	*473*, 414, 361, 476, 417	
[$^2H_3$]6-MAM		*389*				
Hydrocodone	299, 242, 185					
Oxycodone	360, 257, 314, 298					
Hydromorphone:						
Monoacetyl	327, 285, 229			381, 325		
Diacetyl	369, 327, 310					
Oxymorphone	391, 221					
Ref.	44, 46, 52, 55, 64	53	64, 65	37, 43, 66	3, 34, 37, 43, 55, 56, 58, 60, 64, 67, 68	43, 64

The ions with m/z values in italics were the most commonly used for determination.

cedure reduces the handling of organic solvents, but it is more expensive.

The derivatization agents most often used are on the one hand an acetylating agent which gives stable derivatives and on the other PFPA, the derivatives of which are sensitive to moisture, and in some circumstances two peaks can be obtained; however, it gives a clean mass spectrum and good results for determination using the selected-ion monitoring mode.

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Analysis of drug seizures of heroin and amphetamine by capillary electrophoresis

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Abstract

Capillary electrophoresis has been used to separate heroin and amphetamine from structurally related compounds and commonly occurring adulterants in drug seizures. The method was based on micellar electrokinetic chromatography with a running buffer of pH 9.0 containing sodium dodecyl sulphate as surfactant and acetonitrile as organic modifier. The drugs were dissolved in running buffer containing crystal violet. Crystal violet was used to calculate relative migration times for drug identification and as internal standard for quantitative analysis. Both qualitative and quantitative analysis was shown to be reproducible. Because of the speed and resolving power of the method it is a powerful alternative to the high-performance liquid chromatographic and gas chromatographic methods in current use for the analysis of illicit drugs.

1. Introduction

Seizures of clandestinely manufactured drugs such as heroin and amphetamine can be highly complex. Heroin is produced by a variety of batch processes from a variable natural product and thereafter altered for trafficking purposes. The physical appearance varies widely, ranging from almost pure-white heroin hydrochloride to crude and impure heroin containing manufacturing impurities and various adulterants. Illicit amphetamine varies in colour from white to pink to yellow to brown depending upon the type and amount of impurities and adulterants. Illicit heroin and amphetamine may consist of a mixture of neutral, acidic and basic compounds that can be non-polar and/or polar.

Many methods are available for analysis of illicit heroin and amphetamine, and approaches to select a technique appropriate to the sample being examined are available to national narcotics laboratories [1,2]. At least two independent analytical parameters should be used to establish the identity of the drug, and infrared spectroscopy and thin-layer chromatography are widely used for this purpose [3]. Quantitation is usually carried out by gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC). These methods are in general able to separate heroin from other opiates, amphetamine from structurally related drugs and common adulterants [3,4].

Capillary electrophoresis and micellar electrokinetic chromatography (MEKC) has attracted much attention as an efficient separation technique in many areas. MEKC provides the possibility to separate both neutral and charged molecules in a single run [5–7]. Owing to its speed and high resolving power, MEKC has

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been shown to be well suited for drug screening [8].

Heroin and amphetamine are two of the most widely abused drugs in Norway. A method which is able to analyse heroin and amphetamine seizures more rapidly and with greater resolving power than the HPLC and GC methods in use is highly desirable. A method based on MEKC for analysis of heroin and amphetamine seizures was developed and evaluated through analysis of a series of drug seizures collected over the last 2 years.

2. Experimental

2.1. Equipment

Capillary electrophoresis was performed using a Dionex Capillary Electrophoresis System I (Dionex, Sunnyvale, CA, USA). This apparatus features automated sampling and execution of the electrophoretic run. For these experiments it was equipped with a 375 μm O.D. × 50 μm I.D. fused-silica capillary of 50 cm effective separation length. Automated gravity injection was performed for all runs. The sample was held 5 cm above the level of the downstream buffer for 10 s. All separations were run at 20 kV. The current did not exceed 40 μA. On-column UV detection was employed with the wavelength set at 214 nm. The detector signals were collected and analysed using The Dynamax HPLC Method Manager Programme (Rainin, Woburn, MA, USA) and a Macintosh LC computer.

The column was etched with 0.1 *M* NaOH for 30 min at the start of each day. Water was used to rinse the column and then the running buffer was introduced and allowed to equilibrate with the silica capillary for 10 min. Samples were then injected every 13 min with new running buffer automatically filled into the source vial, destination vial and column. The flush time of new running buffer between injections was 2 min.

2.2. Chemicals

Sodium dodecyl sulphate (SDS) from Sigma (St. Louis, MO, USA) was used as received. Sodium dihydrogenphosphate, sodium monophosphate and sodium hydroxide were of analytical grade from E. Merck (Darmstadt, Germany). Crystal violet was from Sigma. Deionized water from a Milli-Q system (Millipore, MA, USA) was used to prepare all buffers. HPLC-grade acetonitrile was supplied by Rathburn (Walkerburn, UK). The drug standards used to prepare the test solution were supplied either by The Norwegian Medicinal Depot (Oslo, Norway) or as gifts from The National Institute of Forensic Toxicology (Oslo, Norway). Drug seizures were supplied by The Bureau of Crime Investigation (Oslo, Norway).

2.3. Running buffer

The running buffer was prepared by mixing 25 m*M* SDS, 10 m*M* NaH_2PO_4, 10 m*M* $Na_2B_4O_7$, adjusted to pH 9.0, 5% acetonitrile.

The running buffer was degassed and filtered through a 0.45-μm membrane filter prior to use.

2.4. Test solution

The test solution used for method development contained 0.1 mg/ml nicotinamide, caffeine, paracetamol, phenacetin, phenemal, morphine, 6-monoacetylmorphine, codeine, procaine, heroin, acetylcodeine, papaverine and noscapine dissolved in running buffer. Crystal violet used as internal standard was added to the test solution at a concentration of 0.05 mg/ml.

2.5. Sample preparation

Drug seizures were homogenized to a fine powder and dissolved in running buffer at a concentration of 0.5 mg/ml. To the running buffer were added 0.05 mg/ml of crystal violet. The sample solution was filtered through a 0.45-μm syringe filter before analysis.

2.6. Quantitative analysis

Crystal violet was used as internal standard for quantitative measurements. The standard curves, accuracy and precision of the method were evaluated by analysis of the drug standards and

drug seizures in running buffer. The calibration graphs were based on peak-area ratios relative to the internal standard.

3. Results and discussion

3.1. MEKC

The test solution used to develop the running buffer was a mixture of the major alkaloids found in most heroin samples and adulterants. Crystal violet was added as internal standard. The criteria used to develop the running buffer were separation of all components in the test solution in as short an analysis time as possible, preferably less than 15 min. In addition, accurate and reproducible migration times and quantitative analysis should be obtained.

Several reports have shown the effect of surfactants, surfactant concentration and pH on the MEKC separation of various classes of drugs [5–7], and a general strategy for improving separation in MEKC has been presented [9]. Weinberger and Lurie [8] separated bulk heroin on a 25 cm × 50 μm I.D. capillary using 85 m*M* SDS, 8.5 m*M* phosphate, 8.5 m*M* borate, 15% acetonitrile, pH 8.5, as running buffer. At 40°C and 20 kV the last-eluted compound in the sample, noscapine, had a migration time of 13.5 min. A model mixture of eighteen drugs were separated with this system in 40 min. Wernly and Thormann [10] separated model mixtures of illicit drugs in 25 min with 75 m*M* SDS, 6 m*M* $Na_2Ba_4O_7$, and 10 m*M* Na_2HPO_4 (pH about 9.1) as running buffer.

In order to separate the compounds in the test solution in less than 15 min a MEKC separation with SDS as surfactant was investigated. The selectivity was optimized by altering the pH and by addition of organic modifier to the buffer. Short analysis times were obtained with low surfactant concentrations and low ion strength buffers. The electroosmotic flow decreases as the buffer concentration increases [11]. The desired resolution was obtained with an effective column length of 50 cm. The pH was found to be a critical factor affecting resolution of the present test mixture and an adequate separation was obtained at pH 9. With the exception of opiates with a phenolic group the migration times of the alkaloids increased by lowering the pH. The phenolic group of, *e.g.*, morphine is partly ionized at pH 9.0, which causes a decreased interaction with the micelle. Morphine is therefore the first eluted of the opiates.

Fig. 1 shows the effect of organic modifier and SDS concentration on the separation of the compounds in the test mixture. Complete separation of all components in the test mixture was not possible without addition of organic modifier. Both noscapine and papaverine as well as caffeine and paracetamol were unresolved with-

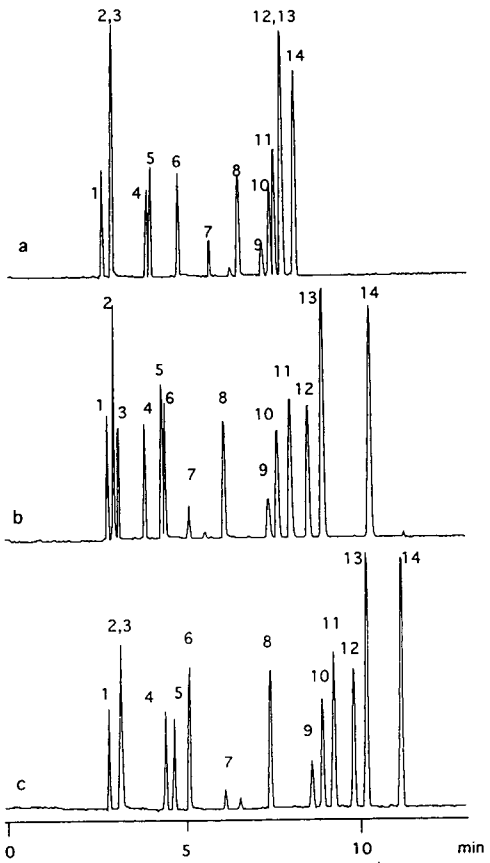

Fig. 1. Electropherograms of the test mixture separated with a running buffer consisting of 25 m*M* SDS, 10 m*M* NaH_2PO_4, 10 m*M* $Na_2B_4O_7$, pH 9.0 (a), 25 m*M* SDS, 10 m*M* NaH_2PO_4, 10 m*M* $Na_2B_4O_7$, pH 9.0, 5% acetonitrile (b), and 50 m*M* SDS, 10 m*M* NaH_2PO_4, 10 m*M* $Na_2B_4O_7$, pH 9.0, 5% acetonitrile (c). Further details see text. Peak numbers as in Table 1.

out organic modifier. The addition of acetonitrile greatly improved resolution and complete separation was achieved in 10 min with 25 m*M* SDS and 5% acetonitrile. The improved resolution obtained by addition of organic modifier was due to a decrease in the electroosmotic flow [11] and to the alteration of the partition coefficient. A decreased resolution was observed for phenemal and morphine and an increased resolution was observed for caffeine and paracetamol under the given conditions. Increasing the SDS concentration to 50 m*M* prolonged the analysis time and the resolution of caffeine and paracetamol was lost. Both caffeine and paracetamol have been found in recent drug seizures, while phenemal is more rarely detected. Priority was therefore given to the resolution of caffeine and paracetamol. The running buffer consisting of 25 m*M* SDS, 10 m*M* NaH_2PO_4, 10 m*M* $Na_2B_4O_7$, pH 9.0, and 5% acetonitrile was therefore selected for analysis of the drug seizures. This running buffer was also able to separate drugs structurally related to amphetamine, as shown in Fig. 2. The number of theoretical plates was in the range 120 000–290 000.

3.2. Analysis of illicit heroin and amphetamine

For the analysis of illicit samples, an internal standard, crystal violet, was added to the sample solution for calculation of relative migration

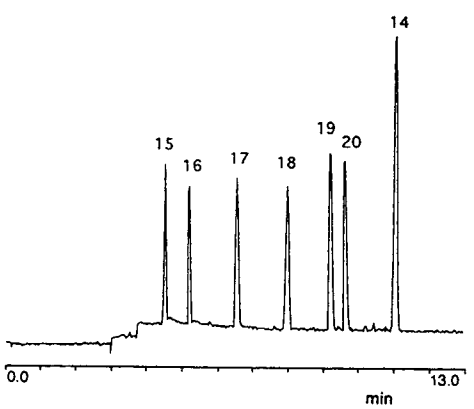

Fig. 2. Electropherogram of drugs similar in structure to amphetamine. Running buffer: 25 m*M* SDS, 10 m*M* NaH_2PO_4, 10 m*M* $Na_2B_4O_7$, pH 9.0, 5% acetonitrile. Further details see text. Peak numbers as in Table 1.

times. Crystal violet was also used as internal standard in quantitative analyses. The selection of the internal standard was based on the requirement that there should be no possibility of its presence in any drug seizure. Several substances were tested and crystal violet was selected because it is not found in any drug seizure, it is highly soluble in the running buffer and it is eluted from the column with a longer migration time than the basic drugs found in illicit samples. The migration time of crystal violet was similar to Sudan III, which is often used as tracer for the micelle but is poorly soluble in the running buffer. Substances present in a drug seizure should therefore be eluted with a migration time shorter or equal to that of crystal violet.

Table I shows the migration times for substances tested relative to crystal violet and their within-day and between-day coefficient of variations. In addition to a positive identification by at least another analytical method, these relative migration times were used to verify identity. The migration reproducibility depends on several operational factors such as ionic strength of the buffer, age of capillary, previous capillary treatment, applied voltage and external capillary temperature [12]. With the apparatus used in this study no control over the capillary temperature was possible and the migration times decreased as the room temperature increased. The within-day relative standard deviations (R.S.D.s) on the migration times were in the range 2.5–4.0% and the between-day R.S.D. values were 3.5–6.0% when the room temperature varied in the range 20–23°C. However, by using relative migration times instead of absolute migration times identification problems were reduced. As shown in Table I, reproducibility of the relative migration times was satisfactory with a within-day R.S.D. in the range 0.5–1.9% and a between-day R.S.D. ranging from 0.89 to 2.23%. The capillary was in general replaced after 500 analyses to maintain reproducibility.

Fig. 3 shows electropherograms of a typical heroin seizure and a typical amphethamine seizure. In general, no problems were encountered in identification of the illicit drugs even if some heroin seizures were highly complex.

Table 1
Relative migration times and within-day relative standard deviations and between-day relative standard deviations

Peak No.	Drug	Relative migration time	Within-day R.S.D. (%) ($n = 6$)	Between-day R.S.D. (%) ($n = 5$)
1	Nicotinamide	0.280	1.90	2.04
2	Caffeine	0.295	1.84	2.03
3	Paracetamol	0.310	1.82	1.96
4	Phenacetin	0.378	1.60	2.01
5	Phenemal	0.422	1.66	2.23
6	Morphine	0.439	1.54	1.58
7	6-monoacetylmorphine	0.495	1.55	2.23
8	Codeine	0.589	1.41	2.30
9	Procaine	0.714	0.96	2.16
10	Heroin	0.378	0.98	1.91
11	Acetylcodeine	0.772	0.94	1.73
12	Papaverine	0.823	0.69	1.25
13	Noscapine	0.867	0.49	0.89
14	Crystal violet	1.000	–	–
15	Phenylephrine	0.403	0.88	–
16	Etilefrine	0.461	1.02	–
17	Phenylpropanolamine	0.574	1.59	–
18	Ephedrine	0.713	0.67	–
19	Amphetamine	0.827	0.39	–
20	Methamphetamine	0.865	0.26	–

3.3. *Quantitative analysis*

The standard curves were based on peak-area measurements relative to crystal violet. Standard curves were set up for morphine, heroin, codeine, papaverine, paracetamol, metamphetamine and amphetamine. All standard curves were linear in the concentration range 0.02–0.5 mg/ml with correlation coefficients (r) in the range 0.997–0.999. The method was validated by analysis of ten heroin seizures and ten amphetamine seizures. The content of heroin in these seizures was in the range 12–51% and the content of amphetamine was in the range 10–83%. The relative standard deviations were in the range 2.0–4.3% ($n = 6$). It is known that small changes in electroendosmosis can lead to changes in peak area. To overcome this problem peak areas can be normalized to one of the migration times in each experiment [13]. The

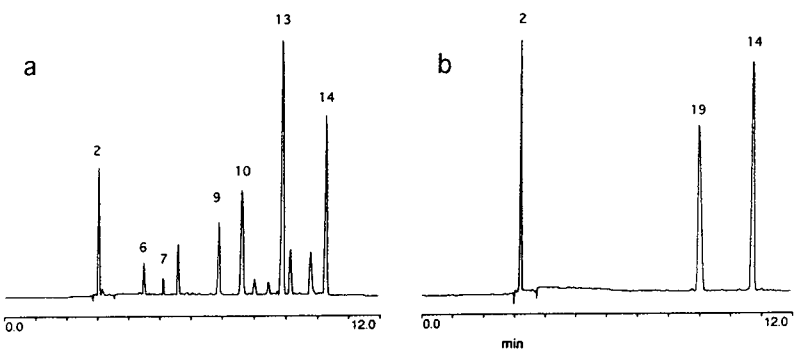

Fig. 3. Electropherograms of a heroin seizure (a) and of an amphetamine seizure (b). Running buffer: 25 mM SDS, 10 mM NaH_2PO_4, 10 mM $Na_2B_4O_7$, pH 9.0, 5% acetonitrile. Further details see text. Peak numbers as in Table 1.

relative standard deviations obtained in this investigation were considered satisfactory without using normalized peak areas.

4. Conclusions

The advantages of using capillary electrophoresis for the analysis of illicit drugs with MEKC is the speed and resolving power of the method. The drug seizures are dissolved in the running buffer and new samples were injected every 13 min. Capillary electrophoresis is a valuable complement to the HPLC and GC methods in current use for the analysis of illicit drugs.

Acknowledgements

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Determination of nine β-blockers in serum by micellar electrokinetic capillary chromatography

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Abstract

β-Adrenergic blocking agents are used for the treatment of angina pectoris, cardiac arrythmia, hypertension, anxiety attacks, thyrotoxicosis, migraine and glaucoma. Owing to their sedative effect, they are also used as doping agents in sport. All β-blockers have an alkanol amine side chain terminating in a secondary amino group in their structure. The pK_a values vary from 9.2 to 9.8. Because some β-blockers are hydrophilic and some lipophilic, simultaneous determination is difficult. In this work, a method based on micellar electrokinetic capillary chromatography (MECC) was developed for the separation and determination of β-blockers in serum. The phosphate buffer 0.08 *M* (pH 6.7) solution contained 15 m*M* N-cetyl-N,N,N-trimethylammonium bromide. Nine parent β-blockers could be separated in a single run and the concentrations determined by internal standard (ephedrine) method. The simple clean-up procedure consisted of enzyme hydrolysis (*Helix pomatia*), protein precipitation, and filtration through 0.5-μm PTFE membranes. The MECC method exhibited good repeatability and a linear range of 75–300 μg/ml. The method was successfully applied after concentration to the determination of propranolol in real samples.

1. Introduction

Micellar electrokinetic capillary chromatography (MECC), which is a form of capillary zone electrophoresis (CZE) relying on an ionic micellar solution system, has lately become recognized as a technique suitable for the separation and determination of small neutral molecules and charged compounds [1]. In MECC, solutes are distributed between the micelles and aqueous phase and simultaneously separated electrophoretically according to their mobilities. MECC is the most widely used capillary electrophoresis method for drug analysis in biological matrices [2–8].

β-Adrenergic blocking agents are clinically used to treat angina pectoris, cardiac arrythmia, hypertension, anxiety attacks, thyrotoxicosis, migraine and glaucoma [9]. Owing to their sedative effect, they are also used as doping agents by athletes [10]. Current methods for determining and identifying β-blockers have been developed over a period of several decades. Optical [11], GC [12,13] and GC–MS [14], HPLC [15], TLC [16], immunological [17] and radioreceptor assay [18,19] methods are the conventional approaches to determining β-blockers in biological fluids. In most cases a single compound is determined, with another β-blocker used as internal standard [17], which is a realistic approach if it can be assumed that two β-blockers are not ingested at the same time. Recently, it has become of interest to determine several parent β-blockers simultaneously [1,20].

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Some β-blockers are hydrophilic and others lipophilic. In addition, their relatively high pK_a values (pK_a 9.2–9.8) complicate the sample pretreatment and analysis. At physiological pH (pH 7.4), β-blockers exist as single cations [15], which enables their separation and determination by methods exploiting the different mobilities of analytes in an electrical field. The structures and pK_a values [21] of the studied β-blockers are presented in Fig. 1.

The general procedure for the separation of β-blockers from blood samples was introduced for propranolol in 1965 [22]. The sample was made alkaline with sodium hydroxide solution and extracted with an organic solvent, heptane with 1% (v/v) ethanol. After an acidic back-extraction the amount of the β-blocker was measured by fluorescence spectrometry [21]. The sample pretreatment method for GC that Walle

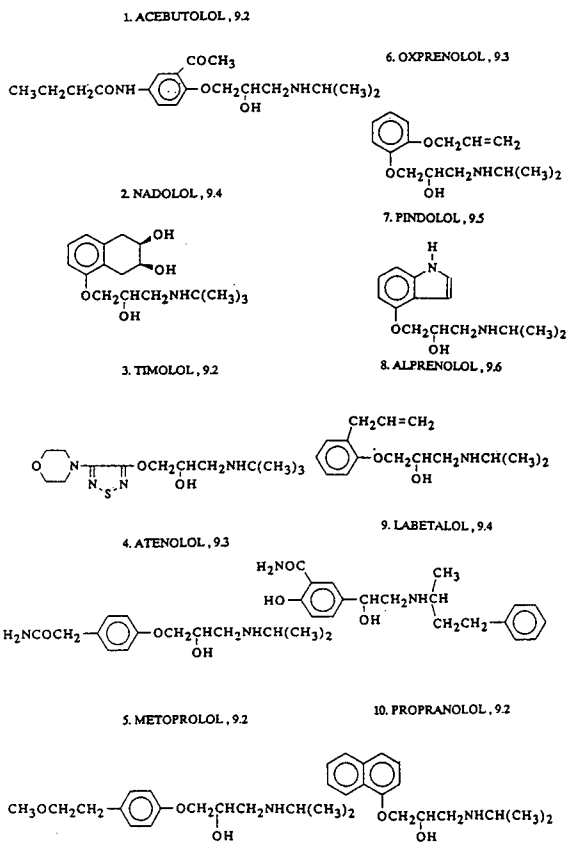

Fig. 1. Structures and pK_a values of β-blockers [14].

et al. [23] published in 1975 introduced some changes in the materials used but not in the procedure. The same procedure was also adopted in 1986 for use in an HPLC method [24]. This means that the sample pretreatment method has remained virtually unchanged for almost thirty years, while at the same time analytical techniques and equipment have developed dramatically.

Urine is not so complex a matrix as plasma or serum as it does not normally contain significant amounts of proteins which tend to adsorb on to the capillary wall and interfere with the analysis. In our laboratory, β-blockers have been determined in human urine samples by MECC, with only dilution and filtration employed in the sample pretreatment [2].

Earlier we have studied the effects of instrumental parameters [25], pH [26] and organic modifiers [27] of the buffer solution on the separation and resolution of β-blockers in MECC. The results from these previous studies [25–27] are exploited in the optimization of the method we now describe for determining, in serum, nine parent β-blockers: acebutolol, alprenolol, atenolol, metoprolol, nadolol, oxprenolol, pindolol, propranolol and timolol. The spiked β-blockers were separated in a single run, after enzyme hydrolysis and protein precipitation. The method was validated for eventual application to real samples, obtained after oral administration of a β-blocker.

2. Experimental

2.1. Apparatus

MECC was performed in a 580 mm × 0.050 mm I.D. fused-silica capillary tube (Polymicro Technologies, White Associates, Pittsburgh, PA, USA) where 500 mm was the length from injector to detector. A Waters Quanta 4000 capillary electrophoresis system (Millipore, Waters Chromatography Division, Milford, MA, USA) with laboratory-made temperature control unit was employed for the analyses. All experiments were done at 35°C. UV detection was at 214 nm.

Injections were carried out hydrostatically for 20 s and the running voltage was −27 kV at the injector end of the capillary. The data (peak height and migration times) were collected with an HP 3396A integrator (Hewlett-Packard, Avondale, PA, USA).

2.2. Materials

The β-blockers were acebutolol hydrochloride, alprenolol hydrochloride, atenolol, labetalol hydrochloride, (±)-metoprolol (+)-tartrate, nadolol, oxprenolol hydrochloride, pindolol, (*S*)-(−)-propranolol hydrochloride, timolol maleate, ephedrine hydrochloride (internal standard) and control serum type 1-A, all from Sigma (St. Louis, MO, USA). Acetonitrile, sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dihydrate, and N-cetyl-N,N,N-trimethylammonium bromide (CTAB) were from E. Merck (Darmstadt, Germany). The β-glucuronidase (EC 3.2.1.31) type H-1 was from *Helix pomatia* (416 800 I.U./g) (Separacor, France). Other reagents were of analytical grade and were used as received. A Water-I system from Gelman Sciences (Ann Arbor, MI, USA) was used for ion exchange of the distilled water. Samples and other solutions were filtered through Millex filters of 0.5 μm pore size from Millipore (Nihon Millipore, Kogyo K.K. Yonezawa, Japan). All the micellar buffer solutions were filtered through 0.45-μm membrane filters (Millipore, Molsheim, France) and degassed before use.

2.3. MECC buffer

The buffer was prepared from 0.08 *M* disodium hydrogenphosphate and 0.08 *M* sodium dihydrogenphosphate solutions containing 15 m*M* of CTAB. The pH of the buffer solution was adjusted to 6.7.

2.4. MECC procedure

To ensure a reproducible separation, before each injection the capillary was purged for 0.2 min with 5% (v/v) phosphoric acid, 0.5 min with water and 10 min with buffer solution.

2.5. Preparation of the serum samples

A 1-ml volume of serum was spiked with a solution containing an accurate amount of each β-blocker. The serum samples were hydrolyzed with β-glucuronidase enzyme at 80°C for 30 min. Proteins were precipitated by adding 700 μl acetonitrile to the samples, vortex-mixed for 15 min and centrifuged at 2004 *g* for 10 min. Ephedrine (165 μg/ml) was added as internal standard. The serum samples were passed through filters of 0.5 μm pore size and then analyzed.

Real human serum sample (2 ml) taken after 3 h after ingestion was prepared as described above except 900 μl of acetonitrile were added.

3. Results and discussion

Earlier studies have shown that MECC is a suitable technique for the determination of β-blockers in urine [2], and the use of organic buffer modifiers improves the separation [27]. Labetalol and propranolol were coeluted in the MECC method for serum described here, but with the addition of 1% (v/v) 2-propanol to the buffer solution they could be separated, too, so that altogether ten parent β-blockers were separated (Fig. 2). However, the resolution between atenolol and metoprolol was decreased by the addition of 2-propanol, and at higher concentrations labetalol and propranolol could not be quantified at the same time because they were again coeluted. The present study nevertheless clearly demonstrates that β-blockers can be determined in serum samples by MECC: Fig. 3 shows the baseline separation of nine β-blockers in 18 min. The interferences in the electropherogram are attributable to the serum background.

Like many other drugs, β-blockers tend to bind to the serum proteins and need to be released from them during the sample preparation. This can be done by enzyme hydrolysis, which is a better method than acidic or basic

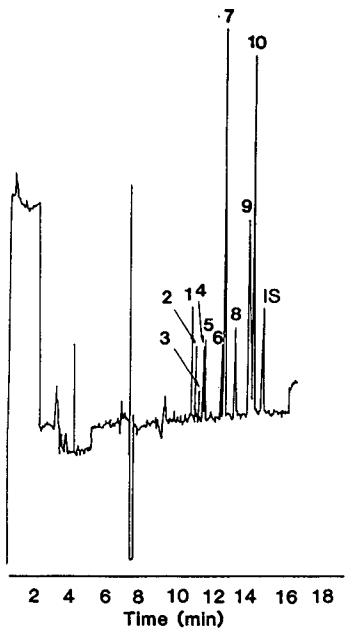

Fig. 2. Electropherogram of ten β-blockers at concentration 100 μg/ml (except for timolol 200 μg/ml) and ephedrine (internal standard, IS) at 165 μg/ml in 1% (v/v) 2-propanol modified buffer. Compound numbers can be found in Fig. 1 and separation conditions in the Experimental section.

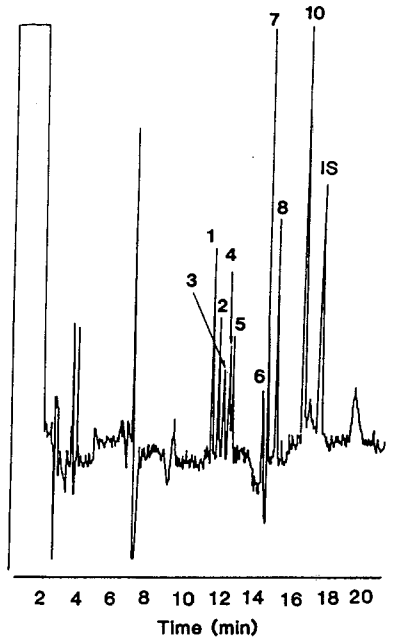

Fig. 3. Electropherogram of a serum sample containing nine β-blockers at concentration 150 μg/ml (except for timolol 300 μg/ml). For the identification of compounds see Fig. 1. Ephedrine (IS) is present at a concentration of 165 μg/ml. Separation conditions can be found in the Experimental section.

hydrolysis since the latter tend to decompose β-blockers. Sample clean-up is further important because the serum proteins tend to adsorb to the capillary wall and disturb the analysis. The simplest way to overcome this problem is to add organic solvent or sodium chloride to the sample. Acetonitrile was successfully used to precipitate the proteins from serum samples. Despite the requirement for two sample pretreatment steps, the method we describe is simpler and less time consuming than earlier methods [21,23,24]. Recovery for the β-blockers at 150 μg/ml (except for timolol 300 μg/ml) varied from 49% (nadolol) to 80% (alprenolol), as shown in a comparison of the peak height/migration time values of standard samples extracted from serum with the peak height/migration time values of unextracted standards.

Linearity was tested over the range 75–300 μg/ml. The correlation coefficients of the linearity curves varied from 0.986 to 0.997 (Table 1) and can be regarded as satisfactory.

Table 1
Linearity of the method in the range 75–300 μg/ml (timolol 150–600 μg/ml)

Compound	*r*	*a*	*b*
Acebutolol	0.986	0.22	0.01
Nadolol	0.991	0.17	0
Timolol	0.996	0.15	0
Atenolol	0.997	0.16	0
Metoprolol	0.997	0.05	0.01
Oxprenolol	–	–	–
Pindolol	0.997	1.03	0.04
Alprenolol	0.995	0.01	0.01
Propranolol	0.997	−0.20	0.03

Analyses were performed using 165 μg/ml of ephedrine as the internal standard. The concentrations of β-blockers used in the determination of the linear range were 75, 100, 125, 175, 250 and 300 μg/ml (for timolol 150, 200, 250, 350, 500 and 600 μg/ml). *r* = Correlation coefficient. The equation for the straight line is $y = bx + a$, where *a* is the intercept of the *y* axis and *b* the slope.

The linearity values for oxprenolol are missing because it sometimes coeluted with pindolol. Furthermore, the baseline penetration in the electropherogram caused by the serum matrix disturbed the measurement of oxprenolol (Fig. 3). The detection limit varied from 1 μg/ml for propranolol to 50 μg/ml for timolol (determined as $3 \times S/N$). The large difference between these values is explained by the considerable variation in the UV-absorption properties of β-blockers.

Repeatability of the method was determined at two levels: 150 and 250 μg/ml. Relative standard deviations (R.S.D.s) varied from 4.5 to 15.8% ($n = 6$) at the 150 μg/ml and from 4.2 to 12.3% ($n = 6$) at the 250 μg/ml level (Tables 2 and 3). The relatively high R.S.D.s are thought to be due to the injection technique and the complex serum matrix, but the values are still at acceptable level. The repeatability of the serum method is not as good as the corresponding method for urine [2], even though the urine samples were analyzed without temperature control.

The method can be applied to real samples. Propranolol was separated from a real human serum sample at μg/ml level (Fig. 4). Since the therapeutical blood level of propranolol is generally 1–500 ng/ml [28], the method is not sensitive enough as such for routine analysis. However, a concentration step can easily be included in the sample pretreatment procedure [*e.g.*, the acetonitrile portion used for protein precipitation can be evaporated to a smaller volume (even to 30 μl)], so that therapeutical serum samples

Table 2
Repeatability of the method at the level 150 μg/ml (timolol 300 μg/ml)

Compound	χ	S.D.	R.S.D. (%)
Acebutolol	1.42	0.01	7.0
Nadolol	0.93	0.04	4.5
Timolol	0.60	0.07	11.8
Atenolol	0.74	0.09	11.8
Metoprolol	0.95	0.10	10.4
Oxprenolol	0.67	0.05	7.3
Pindolol	10.1	0.98	9.7
Alprenolol	1.32	0.21	15.8
Propranolol	4.45	0.57	12.9

Analyses were performed using 165 μg/ml of ephedrine as the internal standard. χ = Mean ($n = 6$) of (peak height/migration time of compound)/(peak height/migration time of internal standard); S.D. = standard deviation; R.S.D. = relative standard deviation.

Table 3
Repeatability of the method at the level 250 μg/ml (timolol 500 μg/ml)

Compound	χ	S.D.	R.S.D. (%)
Acebutolol	1.74	0.12	6.7
Nadolol	1.11	0.08	6.8
Timolol	0.71	0.07	9.2
Atenolol	0.93	0.04	4.2
Metoprolol	1.29	0.05	3.9
Oxprenolol	0.59	0.07	12.3
Pindolol	11.20	0.69	6.1
Alprenolol	2.08	0.15	7.3
Propranolol	6.25	0.48	7.8

See Table 2.

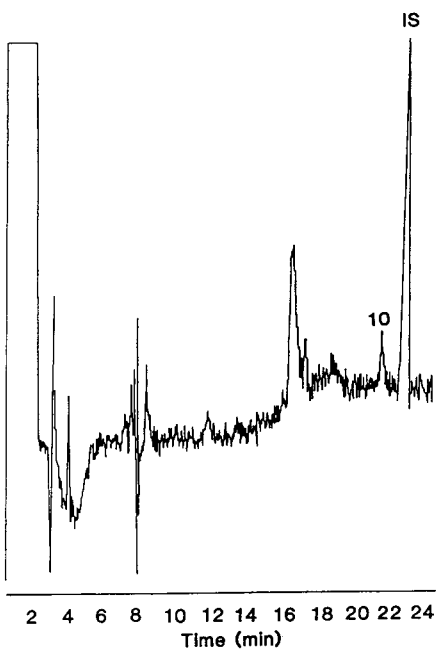

Fig. 4. Electropherogram of a real human serum sample containing propranolol (10). The sample was taken 3 h after ingestion. IS is ephedrine (165 μg/ml). Separation conditions can be found in the Experimental section.

containing β-blockers can be analyzed with the described MECC method.

4. Acknowledgement

A grant from the Leiras Research Foundation (P.L.) is gratefully acknowledged.

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JOURNAL OF CHROMATOGRAPHY A

Application of high-performance thin-layer chromatography and gas chromatography–mass spectrometry to the detection of new anabolic steroids used as growth promotors in cattle fattening

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Abstract

The misuse of natural and synthetic hormones as growth promotors in cattle fattening, although forbidden within the European Community, is well known. Frequently these hormones are injected into the animal as highly concentrated mixtures (the so-called "hormone cocktails"), which usually stay locally at the site of injection from where they are distributed by a slow diffusion process. The analysis of these injection sites by an HPTLC method following a simple and unselective extraction yields a good picture of the compounds which are misused. Although almost 40 hormone reference standards are run with the sample, unknown spots regularly appear on the HPTLC plates, demonstrating that attempts are continuously made to bypass the laboratory controls by introducing new products on the black market. By continuously gathering HPTLC data for a broad range of hormones which were not yet known to be used in cattle fattening, it was possible to elucidate quickly the identities of two new spots that appeared on the chromatogram in the period between the end of 1992 and early 1993. These new compounds that were found in injection sites were the gestagens delmadinone acetate and algestone acetophenide. Their identities were confirmed by GC–MS analysis.

1. Introduction

The misuse of natural and synthetic hormones as growth promotors in cattle fattening, although forbidden within the European Community (EC), is well known. Among these xenobiotics, orally administered compounds have gained popularity, as they leave no injection sites. Nevertheless, during controls of the cattle carcasses in the slaughterhouses, inspectors of the Ministry of Public Health still frequently encounter injection sites, which generally consist of a piece of inflamed muscle.

The analysis of these injection sites by an HPTLC method following a simple and unselective extraction yields a good picture of the compounds which are misused [6]. Usually amounts in the order of milligrams or more of hormones in an oily matrix, sometimes as mixtures in the so-called "hormone cocktails", are injected into the animal. These large amounts remain at the site of injection, which can be the neck, the back, the udder and even behind the eye socket [1], from where they are distributed by a slow diffusion process (long-term effect). Sometimes, as a result of encapsulation, injected hormones can be found even months after application. Owing to these large amounts of hormon-

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al substances, a considerable risk to the consumer persists [2,3].

Despite the fact that during the analysis almost 40 reference standards are run together with the sample, unknown spots regularly appear on the HPTLC plate, which indicates that continuous attempts are made to bypass the laboratory controls by introducing new products. Therefore, a bank of HPTLC data for a broad range of hormones was collected. By comparing the R_F values and colours with the stored data, the identities of two unknown spots were recently elucidated. Conformation of the identities was carried out by gas chromatography–mass spectrometry (GC–MS).

2. Experimental

2.1. Chemicals, glassware and solvents

Reference materials of 37 anabolics were obtained from different sources. The standards were divided into six mixtures based on the R_F values (see Table 1), in such a way that all constituents of each mixture were clearly resolved in both eluents.

Stock standard solutions of 1 mg/ml of the different reference standards were prepared in methanol and were stored at 4°C. For progesterone, a stock standard solution of 10 mg/ml was used because of its low detectability on the plate. Algestone acetophenide was obtained from Diosynth (Kremer and Louward, Eigenbrakel, Belgium) and delmadinone acetate was a gift from SmithKline Beecham (Louvain-La-Neuve, Belgium).

Methanol, cyclohexane, sulphuric acid, fuming hydrochloric acid and ammonium iodide were purchased from Merck (Darmstadt, Germany), diethyl ether, acetone, chloroform and ethyl acetate from Janssen Chimica (Geel, Belgium), heptafluorobutyric acid anhydride (HFBAA) from Macherey–Nägel (Düren, Germany) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and dithioerythritol (DTE) from Aldrich (Milwaukee, WI, USA), were used as received.

2.2. HPTLC analysis

The analysis was based on previously described work [4–6].

Sample preparation

The injection site was cut into pieces with a disposable scalpel and placed in a plastic bag. After adding 5 ml of methanol the bag contents were blended in a Stomacher (Colworth 5) for 5 min. The methanolic extract was then transferred into a glass tube for centrifugation for at least 5 min at 1000 *g*. If the supernatant was clear, it was evaporated under a stream of nitrogen at 40°C. However, if the supernatant apparently contained blood, this supernatant was additionally extracted twice with 5-ml portions of diethylether. After centrifugation, this supernatant was evaporated under a stream of nitrogen at 40°C. The residue was dissolved in 100 μl of methanol.

HPTLC conditions

Separations were carried out on Kieselgel 60 HPTLC plates (10 × 10 cm) (Merck). A 0.75-μl volume of each reference standard mixture was applied to the plate, 1 cm from the edge, on the upper and lower sides of the plate. Of the sample extract, volumes of 0.75 and 1.50 μl were spotted. Development was carried out in one direction in a Camag twin-through chamber at ambient room temperature with 5 ml of chloroform–acetone (90:10, v/v) (solvent system 1) over a distance of 4 cm. After drying the plate in a cold air stream, the spots on the opposite side were developed in 5 ml of cyclohexane–ethyl acetate–methanol (58.5:39.0:5.5, v/v/v) (solvent system 2) over a distance of 4 cm.

Detection

After the second elution, the plate was dried under a cool air stream and sprayed with 10% H_2SO_4 in methanol, then heated for 10 min at 95°C in an oven and examined in daylight and under UV light at 366 nm.

In cases of persistent doubt about the presence of a certain compound in the sample or when the separation between two components was not

sufficient, two-dimensional cochromatography was carried out, as described by De Brabander *et al.* [7].

2.3. GC–MS analysis

Heptafluorobutyrate (HFB) derivatization

A 5-μl volume of the injection site extract was placed in a derivatization vial and concentrated to dryness at 40°C under a stream of nitrogen, then 200 μl of benzene and 50 μl of HFBAA were added. The mixture was heated for 1 h at 60°C and then concentrated to dryness at 40°C under a stream of nitrogen. The final residue was dissolved in 50 μl of hexane and 1 μl was injected into the GC–MS system.

Trimethylsilyl (TMS) derivatization

A 5-μl volume of the injection site extract was placed in a derivatization vial and evaporated to dryness, then 50 μl of a solution containing 20 mg of NH_4I, 40 mg of DTE and 10 ml of MSTFA were added. The mixture was heated for 0.5 h at 60°C. After cooling, 1 μl was injected into the GC–MS system.

Procedure

The analyses were carried out on an HP 5970 mass-selective detector (Hewlett-Packard, Palo Alto, USA) linked to an HP 5890 gas chromatograph equipped with an HP Ultra-2 (5% phenyl–methylsilicone) fused-silica capillary column (25 m × 0.2 mm I.D., film thickness 0.33 μm) and an all-glass moving-needle injection system. The carrier gas was high-purity helium (L'Air Liquide, Liege, Belgium) at a flow-rate of 0.5 ml/min. The injector and interface temperature were maintained at 290°C. The oven temperature was programmed from 200 to 280°C at 5°C/min, the final temperature being held for 10 min (programme 1). To shorten the analysis time, an alternative temperature programme was used, *i.e.*, from 250 to 280°C at 10°C/min, the final temperature being held for 20 min (programme 2). The analyses were performed in the electron impact (EI) mode and the ionization voltage was fixed at 70 eV.

3. Results

The injection sites are routinely screened for the presence of 37 hormones. Their R_F values and the appearance of the spots in daylight and under UV light (366 nm) are listed in Table 1. Although a broad spectrum screening is carried out, new unknown spots sometimes appeared on the HPTLC plates. By continuously collecting HPTLC data for a wide range of hormones which were not yet known to be used in cattle fattening, it was possible to elucidate quickly the identities of two new spots that appeared on the HPTLC plates in the period between the end of 1992 and early 1993. These new compounds that were found in injection sites were the gestagens delmadinone acetate and algestone acetophenide.

Delmadinone acetete (1,6-bisdehydro-6-chloro-17α-acetoxyprogesterone) is a synthetic steroid which possesses progestin activity. In Belgium it is commercially available as an injectable solution for the treatment of hypersexuality of cats and dogs (Tardak; Syntex). Its structure is shown in Fig. 1.

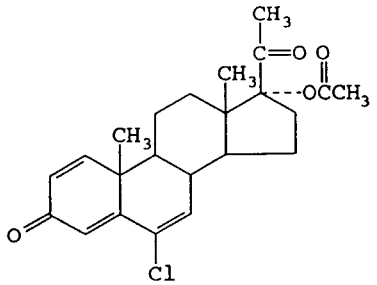
The R_F values relative to β-testosterone and the appearance of the spots in daylight and under UV light (366 nm) are given in Table 2. A study of spots obtained with decreasing amounts showed that the first visible signal was observed with 6 ng per spot on the plate.

The structure of the gestagen algestone acetophenide (16α,17α-dihydroxyprogesterone acetophenide) is shown in Fig. 2 and the HPTLC data are given in Table 2. The detectability on the plate was very low (detection limit 2.5 μg per spot). Therefore, in further routine analysis, 1.5 μl of a standard solution of 25 mg/ml in methanol was spotted.

The identities of these compounds were confirmed by GC–MS. For delmadinone acetate, a mono-HFB derivative was obtained with a molecular ion of $m/z = 598$ and a methylene unit value of 32.13. The spectrum with the presumed molecular formula of the HFB derivative is shown in Fig. 3. The presence of delmadinone acetate in the extract of an injection site was confirmed by GC–MS. The spectrum of the HFB

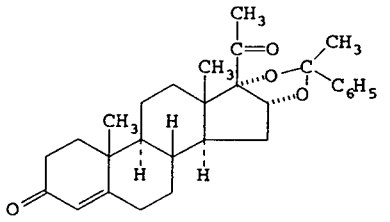
Table 1
Composition of the reference standard mixtures, R_F values relative to β-testosterone and appearance of the spots in daylight and under UV light (366 nm)

Mixture No.	Components	R_F		Colour	
		Solvent 1	Solvent 2	Daylight	UV (366 nm)
1	Chlorotestosterone	1.33	1.24	Blue	Dark spot with yellow edge
	4-Chlorotestosterone-17-acetate	2.02	1.93	Blue	Dark spot with yellow edge
	β-Estradiol-3-benzoate	1.42	1.54	Orange	Orange
	17β-Trenbolone	1	0.88	Fluorescent yellow	Fluorescent green
	17β-Trenbolone acetate	1.71	2.22	Fluorescent yellow	Fluorescent green
	Mestranol	1.73	1.90	Pink	Light brown
	β-Nortestosterone laurate	1.95	2.09	Brown with blue edge	Red-brown with yellow edge
2	β-Estradiol	0.87	1.22	Orange	Orange
	β-Estradiol-17β-cypionate	1.82	2.02	Orange	Orange
	Medroxyprogesterone acetate	1.73	1.34	Dark blue with yellow edge	Dark brown
	Norethisterone acetate	1.82	2.22	Light-grey-purple	Light orange
	Diethylstilbestrol	1.20	1.63	Grey-purple	Purple
	β-Testosterone-17β-cypionate	1.96	1.93	Purple	Red with yellow edge
3	17β,19-Nortestosterone	0.93	0.90	Brown with blue edge	Red-brown with yellow edge
	17α-Methyltestosterone	1.04	1.02	Yellow-orange	Fluorescent yellow with orange core
	Megestrol acetate	1.73	1.29	Heavy green	Dark green with yellow edge
	Progesterone	1.89	1.63	Yellow	Fluorescent green
	Testosterone isocaproate	2.04	2.05	Purple	Red with yellow edge
	17β,19-Nortestosterone-17-decanoate	2.07	2.12	Brown with blue edge	Red-brown with yellow edge
4	Boldenone	0.89	1.29	Brown	Chestnut brown
	Chlormadinone acetate	1.89	1.34	Petroleum green	Dark orange
	Diethylstilbestrol dipropionate	1.33	1.66	Light purple	Faded purple
	Testosterone propionate	2	1.83	Purple	Red with yellow edge
	Testosterone enanthate	2	1.95	Purple	Red with yellow edge
	Estradiol phenylpropionate	1.98	2	Orange	Orange
5	Stanozolol	0.38	0.49	Purple-brown	Purple
	β-Testosterone	1	1	Purple	Red with yellow edge
	Norethisterone	1.29	1.24	Light grey	Light orange
	Ethinylestradiol	1.18	1.56	Bright pink	Dark with orange edge
	Testosterone phenylpropionate	1.93	1.85	Purple	Red with yellow edge
	Estradiol valerate	1.82	2	Orange	Orange
6	Fluoxymesterone	0.27	0.41	Light grey-brown	Chestnut brown
	Methylboldenone	0.62	0.51	Red-purple	Chestnut brown
	17α-Acetoxyprogesterone	1.64	1.15	Purple with yellow edge	Bright red with yellow edge
	Melengestrol acetate	1.73	1.29	Brown	Brown with yellow edge
	Vinyltestosterone	1.33	1.37	Grey-brown	Fluorescent yellow
	17α-Hydroxyprogesterone caproate	1.89	1.76	Purple with yellow edge	Bright red with yellow edge



Delmadinone acetate

Fig. 1. Structure of delmadinone acetate.



Algestone acetophenide

Fig. 2. Structure of algestone acetophenide.

derivative is shown in Fig. 4 and corresponds to that of the reference standard.

Algestone acetophenide could be injected into the GC–MS system without derivatization but derivatization was preferred because of the higher specificity. Derivatization with HFBAA yielded a mono-HFB derivative, the spectrum and presumed molecular structure of which are shown in Fig. 5. The molecular ion was at m/z = 644 and the retention times were 26.66 and 16.87 min for temperature programmes 1 and 2, respectively. Trimethylsilyl derivatization yielded a mono-TMS derivative. The spectrum and the presumed structure of the derivative are shown in Fig. 6. The molecular ion was at $m/z = 520$ and the retention times were 34.82 and 24.76 min for temperature programmes 1 and 2, respectively. Methylene unit values of these derivatives could not be determined because no suitable alkanes ($>C_{34}$) were available.

The presence of algestone acetophenide in the extract of an injection site was confirmed by GC–MS. The spectra of algestone acetophenide-mono-TMS and algestone acetophenide-mono-HFB in a routine sample are shown in Figs. 7 and 8, respectively.

The use of delmadinone acetate and algestone

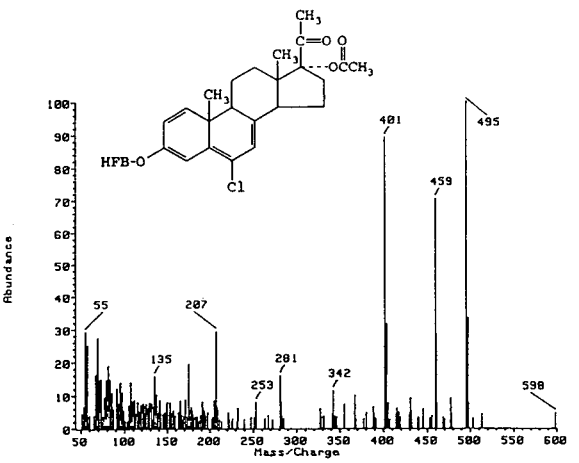


Fig. 3. Spectrum of the HFB derivative of delmadinone acetate.

Table 2
HPTLC data for delmadinone acetate and algestone acetophenide

Compound	R_F [a]		Colour	
	Solvent 1	Solvent 2	Daylight	UV (366 nm)
Delmadinone acetate	1.59	1.19	Brown	Chestnut brown
Algestone acetophenide	1.99	1.83	Faded yellow	Faded beige with blue shine

[a] Relative to β-testosterone.

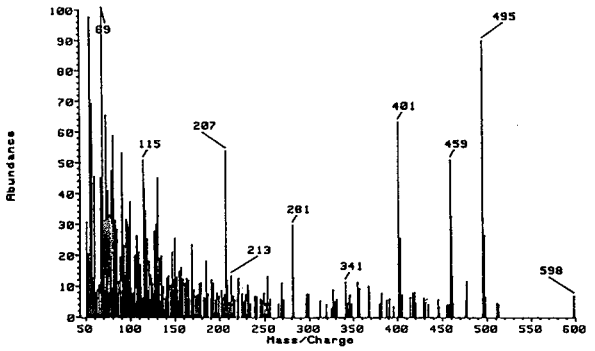


Fig. 4. Spectrum of HFB derivative of delmadinone acetate in the extract of an injection site.

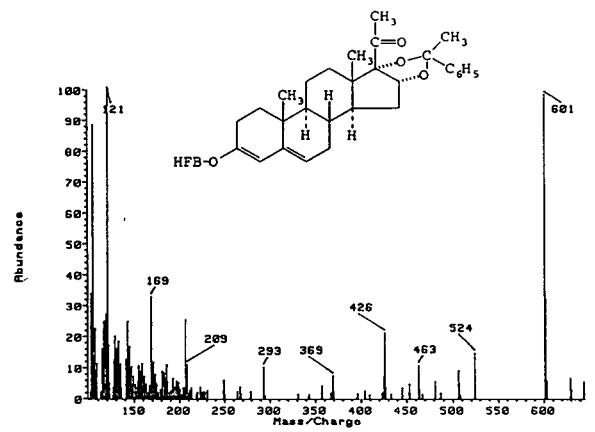


Fig. 5. Spectrum of the HFB derivative of algestone acetophenide.

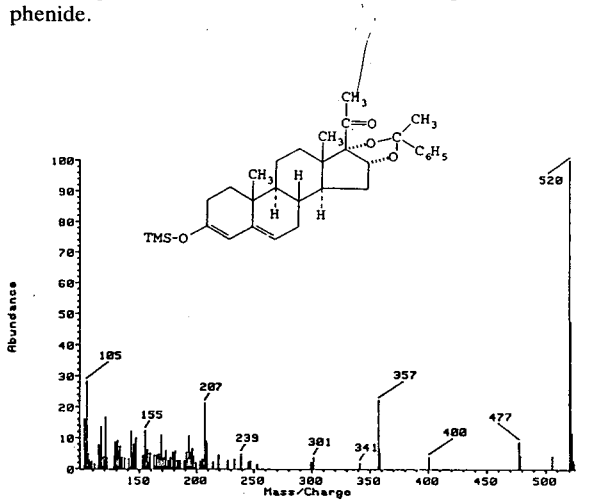


Fig. 6. Spectrum of the TMS derivative of algestone acetophenide.

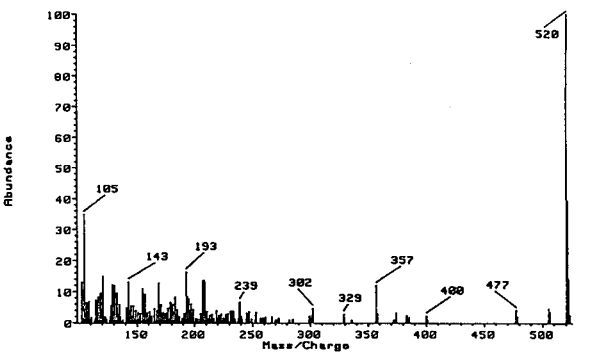


Fig. 7. Spectrum of TMS derivative of algestone acetophenide in the extract of an injection site.

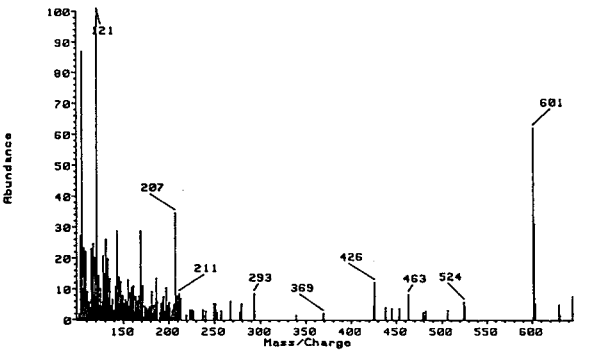


Fig. 8. Spectrum of HFB derivative of algestone acetophenide in the extract of an injection site.

acetophenide as cattle fattening agents has not yet been reported.

4. Conclusions

In the routine analysis of injection sites, HPTLC has been shown to be a very efficient multi-screening method for the presence of anabolic agents. This technique has also been shown to be very useful for the detection of new products administered illegally. By comparing the HPTLC data for new spots with a data bank of a large number of hormones, rapid information can be obtained as to the identity of new products. Confirmation by GC–MS gave decisive answers about the identities of the compounds.

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JOURNAL OF CHROMATOGRAPHY A

Forensic investigation of gentamicin sulfates by anion-exchange ion chromatography with pulsed electrochemical detection

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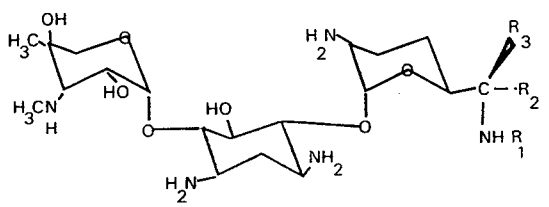
Abstract

Many methods exist for the separation of gentamicin C complex components, C_1, C_{1a}, C_2 and C_{2a}. In an investigation of possible counterfeit suppliers of gentamicin sulfate, a new method utilized high-pH anion-exchange separation on a carbohydrate column, with pulsed electrochemical detection on a gold electrode. Component ratios and the presence and/or absence of additional peaks were used to link or dissociate forensic samples.

1. Introduction

The aminoglycoside antibiotic gentamicin is produced by the growth of *Micromonospora purpurea*, and consists of four major components



Gentamicin	R_1	R_2	R_3	M_r
C_1	CH_3	H	CH_3	477
C_{1a}	H	H	H	449
C_2	H	H	CH_3	463
C_{2a}	H	CH_3	H	463
C_{2b}	CH_3	H	H	463

Fig. 1. Structures of major gentamicin C complex components.

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C_1, C_{1a}, C_2 and C_{2a} [1]. As many as four minor components have also been identified [2,3]. Numerous methods for the analysis of gentamicin components have been published including: thin-layer paper chromatography [4], high-performance liquid chromatography (HPLC) [1,5–7], and HPLC–thermospray mass spectrometry [8]. The official Food and Drug Administration method for the determination of percent composition is a paper chromatographic separation followed by microbiological assay [9]. Current United States Pharmacopeia (USP) protocol specifies an HPLC separation of pre-column derivatized components for the determination of percent composition and a microbial assay for potency [1]. The lack of strong UV chromophores in gentamicin (refer to Fig. 1) makes direct detection difficult. The hydroxyl moieties, however, present the possibility of electrochemical detection as used for carbohydrate analysis. Aminoglycosides are anionic at high pH and therefore are retained on an anion-exchange column. The aminoglycosides are then oxidized at high pH on a gold electrode [10]. High-performance anion-exchange chromato-

graphic (HPAEC) separation was used with pulsed amperometric detection on a gold electrode (HPAEC–PAD) by Statler for the determination of tobramycin [11], an aminoglycoside antibiotic similar to gentamicin.

Forensic investigations of bulk drugs and injectable preparations at the National Forensic Chemistry Center (NFCC) attempted to link or dissociate various sources of gentamicin. Although studies by other researchers have shown the considerable variation in the percent composition of commercial gentamicin, most gentamicin tended to be within the USP allowable range [6]. USP allowable ranges are: $C_1 = 25–50\%$; $C_{1a} = 10–35\%$; $C_2 + C_{2a} = 25–55\%$, where percent of each component is calculated as the peak responses of each peak divided by the sum of peak responses for all four peaks [1]. A method was sought which could determine C_1, C_{1a}, C_2 and C_{2a}, and which might also be sensitive enough to detect trace components in order to compare samples. Analysis of contaminants in bulk drugs may be used as a "chemical fingerprint" to track drug sources since various manufacturing processes may contribute characteristic residual chemicals to the fingerprint. This study reports the use of HPAEC–PAD for the comparison of gentamicin from various sources.

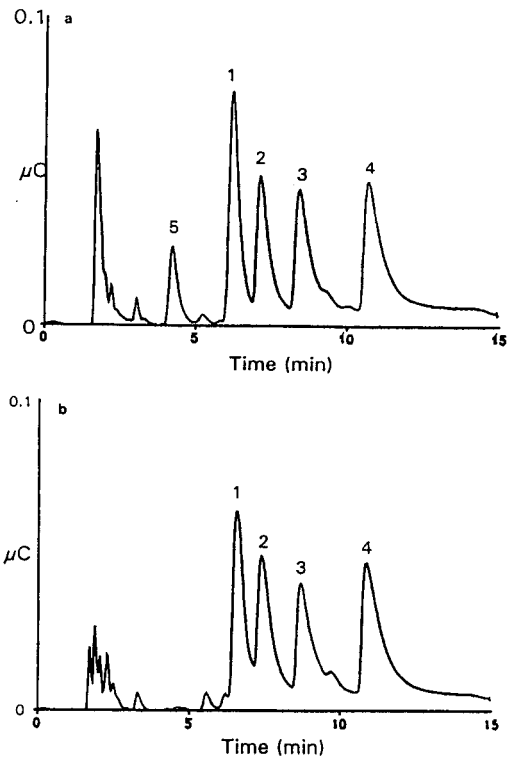

Fig. 2. Comparison of bulk gentamicin sulfate sources. (a) Manufacturer A; (b) manufacturer B. Peaks: 1 = C_{1a}; 2 = C_2; 3 = C_{2a}; 4 = C_1; 5 = fifth peak. Column: Carbopac PA-1; flow-rate: 1.0 ml/min; injection volume: 20 μl. Post column reagent: 0.5 *M* NaOH at 0.5 ml/min. Gradient program: eluent 1: 18 MΩ water; eluent 2: 10 m*M* NaOH.

Time(min)	Eluent 1 (%)	Eluent 2 (%)	Comments
0.0	70	30	Re-equilibration
5.0	70	30	
5.1	70	30	Injection occurs
20.0	50	50	

2. Experimental

2.1. Reagents and standards

Gentamicin sulfate USP reference standard lot I-1 was obtained from United States Pharmacopeial Convention (Rockville, MD, USA) with a labeled potency of 682 μg gentamicin base/mg standard. Eluent and post column reagent were prepared using 50% (w/w) sodium hydroxide (Fisher Scientific, Fair Lawn, NJ, USA). Care was taken to minimize carbonate contamination of the eluent since carbonate would alter the eluent strength. Deionized distilled water (18 MΩ), purified using a Milli-Q water system (Millipore, Milford, MA, USA), was sparged with helium to eliminate CO_2, prior to the addition of hydroxide. Sisomicin sulfate, kanamycin A, tobramycin sulfate, clindamycin hydrochloride, cloxacillin sodium salt, cefazoline sodium salt, penicillin G potassium salt, and neomycin sulfate standards were obtained from Sigma (St. Louis, MO, USA).

2.2. Apparatus and chromatography

The instrumentation used was a Dionex 4500 ion chromatograph (Dionex, Sunnyvale, CA, USA) consisting of a gradient pump (GPM-1); a

Rheodyne 9126 injector equipped with a 20-μl sample loop; a pulsed electrochemical detector (PED-1) with gold working electrode operated in the integrated amperometry mode; an automated sampler (ASM-1); a reagent-delivery module (RDM) consisting of a pressurized reservoir, mixing tee, and reaction coil; and an AI-450 software program for instrument control and data collection.

An anion-exchange column, Dionex Carbopac PA-1 analytical column (250 × 4 mm), and PA-1 guard (50 × 4 mm) were used. Gradient conditions are listed with Fig. 2.

Eluent flowed through the column to the mixing tee, where 0.5 *M* NaOH was added from the pressurized reservoir, and mixed in the reaction coil prior to the electrochemical cell. The post-column addition of base was necessary to raise the pH of the mobile phase to approximately 13 to improve sensitivity and stabilize the gradient baseline [12]. The detector settings were potential $E_1 = 0.10$ V, time $t_1 = 300$ ms; $E_2 = 0.60$ V, $t_2 = 120$ ms; $E_3 = -0.80$ V, $t_3 = 300$ ms. The pulse sequence cleans the electrode surface on a continuous basis, resulting in improved reproducibility and lessened electrode fouling.

Percent composition was determined by the following formula: % composition = area of component peak/sum of areas of four peaks $(C_{1a} + C_2 + C_{2a} + C_1) \times 100$. Potency was calculated based on total area of the four peaks compared to USP standard of known potency. Samples of gentamicin were diluted in water to a concentration of approximately 100 to 200 μg gentamicin sulfate/ml.

3. Results and discussion

The separation of gentamicin components C_{1a}, C_2, C_{2a} and C_1 by gradient HPAEC–PAD is shown in Fig. 2. Peak identifications were made based upon comparison of the percent composition calculated for the USP standard compared to the reported values based upon HPLC. In assigning C_2 as the peak prior to C_{2a}, since the USP did not report values for C_2 and C_{2a} individually, it was assumed that positional isomers would elute near one another. The first peak was assigned as C_2 since the area of that peak was larger and C_2 is generally present in higher proportions than C_{2a}. These peak assignments make sense chromatographically concerning size and steric hindrance but do not take into account effects on pK_a. Retention time and area response reproducibility of ten replicate injections on the same day, as well as linear range data are presented in Table 1. The minimum detection limit for gentamicin sulfate is 20 ng on column, defined as three times the standard deviation of a blank divided by peak response factor. The detection limit calculated based on the smallest peak in the USP standard, C_{2a}, was used as the detection limit of gentamicin sulfate. The detection limits reported for other methods include: 10 ng on column for HPLC with fluorescence of derivitized components [13]; approximately 10 μg on column for HPLC–UV with derivatization [14]; 16 μg on column for HPLC–electrochemical detection [15]; and 400 ng on column for HPLC–thermospray MS [8]. Several antibiotics which are used

Table 1
Short-term reproducibility of area response and retention time for 190 μg/ml gentamicin sulfate

Peak	t_R (min)	R.S.D. (%)[a]	R.S.D. (%)	Linearity 5–200 μg/ml	
		t_R	Area	Slope	Correlation coefficient
C_{1a}	5.91	0.8	1.1	3 113 258	0.9998
C_2	6.66	0.7	1.5	5 304 894	0.9999
C_{2a}	8.04	0.7	4.0	2 918 240	0.9999
C_1	10.05	0.7	4.0	4 565 322	0.9999

[a] $n = 10$.

Table 2
Comparison of gentamicin component values determined by ion chromatography with manufacturer's certified values

Manufacturer	Lot	Ion chromatography values[a]					Manufacturer's values[b]		
		C_{1a} (%)	C_1 (%)	$C_2 + C_{2a}$ (%)	C_2 (%)	C_{2a} (%)	C_{1a} (%)	C_1 (%)	$C_2 + C_{2a}$ (%)
A	Feb 90	30.1 (0.5)	22.8 (0.7)	41.1 (0.2)	27.7 (0.4)	13.4 (1.2)	NA[c]	NA	NA
	May 91	22.8 (0.4)	29.0 (1.0)	48.3 (0.5)	32.7 (0.4)	15.5 (0.6)	22	31	48
	Aug 91	32.4 (1.5)	26.3 (1.8)	41.4 (0.2)	22.7 (1.8)	18.6 (2.1)	30	31	46
	Dec 91	25.6 (0.8)	29.9 (1.7)	44.5 (0.7)	24.0 (1.7)	20.6 (0.4)	23	34	46
	Feb 92	24.3 (2.0)	32.5 (0.6)	43.2 (0.7)	22.3 (0.4)	20.8 (1.4)	26	32	42
B	Sept 91	29.4 (0.3)	30.0 (0.7)	40.6 (0.5)	24.5 (0.4)	16.1 (1.0)	30.2	37.5	32.3[d]
	Feb 92	21.7 (0.5)	30.8 (1.0)	47.5 (0.4)	33.4 (0.6)	14.0 (0.7)	21.9	30	48.1[e]
USP	I-1	20.5 (1.0)	28.3 (1.2)	51.2 (0.9)	33.8 (1.0)	17.5 (1.0)	21	29	50

[a] Averages of four analyses. Sum of four components taken as 100%. Numbers in parentheses are of component ratios. R.S.D. (%).
[b] HPLC analysis, qualified to USP XXII.
[c] NA = Not available.
[d] $C_2 = 18.5$; $C_{2a} = 13.9$.
[e] $C_2 = 33.5$; $C_{2a} = 14.6$.

concomitantly with gentamicin or are structurally similar (kanamycin, tobramycin, clindamycin, cloxacillin, cefazoline, penicillin G, and neomycin) were injected onto the ion chromatograph and did not interfere with the determination of gentamicin.

Table 3
Comparison of percent C_2 to C_{2a} ratios and presence of fifth peak in bulk drugs, as determined by ion chromatography

Manufacturer	Lot	$C_2 + C_{2a}$ (%)	C_2 (%)	C_{2a} (%)	C_2 (%)/C_{2a} (%)	Area 5th peak[a]
A	Feb 90	41.1	27.7	13.4	2.07	ND[b]
	May 91	48.3	32.7	15.5	2.11	ND
	Aug 91	41.4	22.7	18.6	1.22	1.92E9
	Dec 91	44.5	24.0	20.6	1.16	2.10E9
	Feb 92	43.2	22.3	20.8	1.07	1.39E9
B	Sept 91	47.5	33.4	14.0	2.38	6.3E4
	July 92	40.6	24.5	16.1	1.52	ND
USP	I-1	51.2	33.8	17.5	1.93	ND

[a] Retention time = 4.3 min.
[b] ND = Not detected.

3.1. Bulk product

Several samples of gentamicin sulfate from two manufacturers were analyzed by HPAEC–PAD. The percent composition of the components as determined by ion chromatography was compared to the manufacturers' certified values obtained by HPLC according to USP protocol (values were obtained from manufacturers' certificates of analysis). Reproducibility of percent composition by ion chromatography ranged from 0.2 to 2.0% relative standard deviation for replicate injections. The values determined by ion chromatography generally agreed well with the manufacturer's values (refer to Table 2). No distinct pattern to distinguish between manufacturers was detected using the percent composition as reported by USP protocol: C_1, C_{1a}, and $C_2 + C_{2a}$. The ratio between C_2 and C_{2a} was then calculated (refer to Table 3). Differences between lots were then noticed. For manufacturer A, whenever the ratio C_2/C_{2a} was approximately 1, an additional peak at 4.3 min was detected (labeled as fifth peak). For C_2/C_{2a} ratio approximately 2, no fifth peak was detected. In manufacturer B samples, an opposite trend was noted. Fig. 2a and 2b show representative chromatograms for manufacturer A (with fifth peak) and manufacturer B, respectively. Distinct differences between manufacturer A and B were seen in the early part of the chromatograms. The patterns of the early peaks were the same for all lots of the same manufacturer. An additional peak can be seen in Fig. 2b after C_{2a} which may be the minor component C_{2b}. Although these peaks have not yet been identified, they still form useful patterns with which manufacturers can be distinguished.

3.2. Injectables

Four injectable solutions of gentamicin sulfate were also analyzed by HPAEC–PAD. The samples consisted of a control (with labeled potency of 100 mg gentamicin base/ml), and three unknowns. The four solutions had already been analyzed using capillary electrophoresis for potency and percent C_1, C_{1a} and $C_2 + C_{2a}$ and compared to microbial assay [16]. Analysis was required to determine if unknowns 1, 2 and 3 were all from the same source. Results of the ion chromatographic analysis is presented in Table 4. There was very little variation in the percent composition of C_1, C_{1a}, and $C_2 + C_{2a}$. Once again, as with the bulk samples, the ratio C_2/C_{2a} was calculated. The ratios for unknown 1 and 2

Table 4
Analysis of gentamicin in injectable solutions

Sample[a]	C_{1a} (%)	C_1 (%)	C_2 (%)	C_{2a} (%)	$C_2 + C_{2a}$ (%)	Total peak area[b]	Gentamicin base mg/ml
USP[c]	20.5 (1.0)	28.3 (1.2)	33.8 (1.0)	17.5 (1.0)	51.2 (0.9)	2260599 (4.9)	0.0658
Control[d,e]	20.5 (1.2)	26.6 (1.8)	36.6 (0.9)	16.3 (0.5)	52.9 (0.5)	3275903 (1.1)	95.4 ± 1.1
Unknown 1[e]	20.2 (0.7)	27.2 (0.6)	36.2 (0.2)	16.3 (0.7)	52.5 (0.1)	3689941 (0.5)	107.4 ± 0.6
Unknown 2[e]	20.3 (0.2)	27.0 (0.5)	36.3 (0.3)	16.3 (0.9)	52.6 (0.3)	3493696 (0.9)	101.7 ± 0.9
Unknown 3[e]	22.8 (0.5)	25.8 (0.2)	28.4 (0.2)	23.0 (0.5)	51.4 (0.1)	3322589 (0.5)	96.7 ± 0.5

[a] $n = 3$.
[b] Number in parentheses = % relative standard deviation.
[c] 0.0965 mg gentamicin sulfate USP standard/ml × 0.682 mg gentamicin base/mg USP standard.
[d] Labeled as 100 mg gentamicin base/ml.
[e] Dilution = 1000.

were the same (2.22) and different from unknown 3 (1.23). Also, as was found for the bulk product, there were differences in the early part of the chromatograms prior to C_{1a} which distinguished unknowns 1 and 2 as from the same source, and different from unknown 3 (refer to Fig. 3a–c).

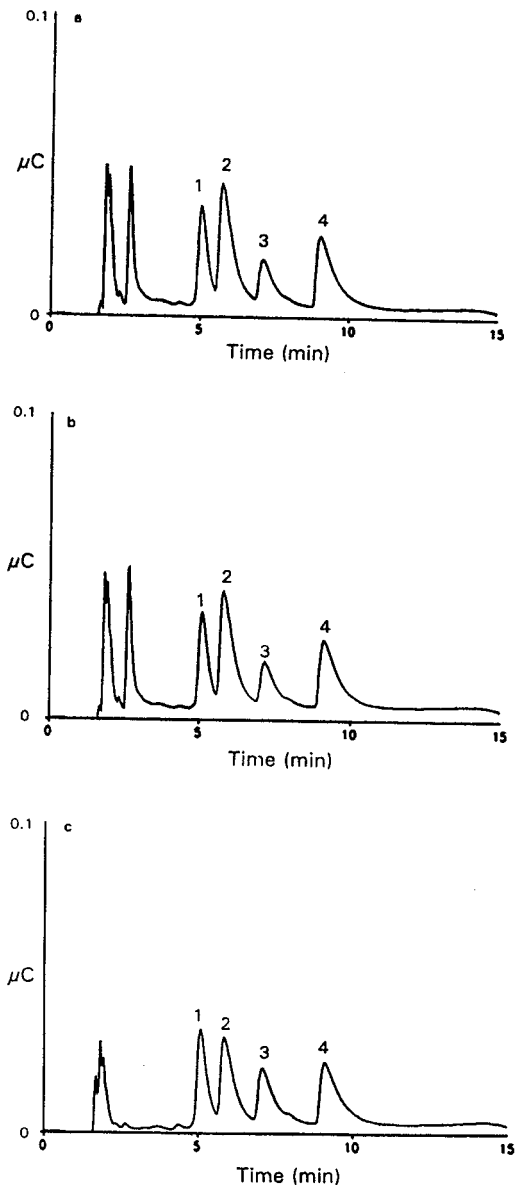

Fig. 3. Comparison of three gentamicin injectable solutions. (a) unknown 1, (b) unknown 2, and (c) unknown 3. Peaks: $1 = C_{1a}$; $2 = C_2$; $3 = C_{2a}$; $4 = C_1$.

The potency of the injectables was calculated using the total peak area of C_1, C_{1a}, C_2 and C_{2a}. The control was found to contain 95.4 ± 1.1 mg gentamicin base/ml compared to the labeled value of 100. The potencies of the unknowns were compared to results of microbial assay: unknown 1, 107.4 ± 0.6 by ion chromatography compared to 116 by microbial assay; unknown 2, 101.7 ± 0.9 by ion chromatography compared to 111; and unknown 3, 96.7 ± 0.5 by ion chromatography to 90.2. Based on the limited analysis of the injectables, ion chromatography appears to be a viable technique for the determination of potency for gentamicin.

4. Conclusions

The quantitative analysis of the major components of gentamicin sulfate has been accomplished using high-pH anion-exchange ion chromatography with pulsed electrochemical detection. Although a gradient run is required, the total analysis time is only 20 mins. The components were detected without derivatization with a detection limit of 20 ng gentamicin sulfate on column. The qualitative analysis of other unidentified components or impurities in the early portion of the chromatograms was useful in investigative comparisons of both bulk product and injectable solutions.

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Separation of acid, basic and dispersed dyes by a single-gradient elution reversed-phase high-performance liquid chromatography system

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Abstract

A gradient elution high-performance liquid chromatography system with an end-capped narrow-bore reversed-phase column and photodiode-array detection has been developed for the separation and characterisation of acid, basic and dispersed dyes. The system exhibits good reproducibility, efficiency and sensitivity. Using a range of commercial dyes, a database of relative retention times and spectral data has been produced. The HPLC method has been applied to the analysis of dyes extracted from single fibres.

1. Introduction

The examination of textile fibres forms a major part of casework at the Northern Ireland Forensic Science Laboratory. An important element of fibre analysis is the comparison of extracted dyes by thin-layer chromatography (TLC). While TLC is a good discriminating technique it does have the following limitations. Relatively large amounts of extracted dyes are required for the comparison of all components. Various dye classes require different eluent systems. Unless TLC conditions are strictly controlled the reproducibility of the R_F values can be poor [1]. TLC is essentially a qualitative technique.

An alternative chromatographic method is high-performance liquid chromatography (HPLC). HPLC systems have been developed for the analysis of disperse dyes extracted from polyester fibres [2,3], acid dyes from wool fibres [4], basic dyes from acrylic fibres [5,6] and natural and early synthetic dyes from archaeological textiles [7]. Comparisons of HPLC and TLC have concluded that HPLC offers better sensitivity and resolution [2–6]. Detection limits for the HPLC analysis of basic dyes [6] and disperse dyes [3] are 25 and 200 picograms respectively.

A limitation of previous HPLC systems, as with TLC is that each dye class requires different running conditions. Other workers have developed a general gradient elution system for the analysis of a range of dye classes using a reversed-phase column and ion-pairing agent but because of practical difficulties it was not recommended for use [8].

In this paper the separation and characterisation of acid, basic and dispersed dyes by gradient elution with a narrow-bore, end-capped reversed-phase column and photodiode-array (PDA) detector was investigated. The development of a database using standard dyes for the identifica-

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tion of dyes extracted from casework size fibres was also investigated.

2. Experimental

2.1. HPLC equipment

The equipment consisted of a Waters 600E multisolvent delivery pump, 717 Wisp autosampler and 996 photodiode-array detector (Millipore, Watford, UK). Analysis of data, gradient control and sample injection controlled by Millipore Millennium 2110 chromatography manager on a NEC 486–33 MHz personal computer with a Hewlett-Packard 550C deskjet printer. Real time display and printed chromatograms are Maxplots. This plots each dye at its maximum absorbance wavelength.

The PDA was operated over the range 380 to 600 nm, resolution 4.8 nm and data acquisition rate 1 spectrum per second. PDA flow cell 8 μl and path length 10 mm. Sample injection size 5 μl. System dead volume kept to a minimum. Analysis run time 25 min with 10 min equilibration time between injections.

2.2. HPLC column

Inertsil ODS-2, 150 × 2.1 mm I.D., 5 μm particle size and 150 Å pore size. (GL Sciences Inc., Tokyo, Japan).

2.3. HPLC eluent

Eluent A consisted of water deionised using a Milli-Q plus 185 with Milli-RO_{10} pretreatment pack. Eluent B consisted of HPLC grade acetonitrile. Both eluents were acidified to pH 2.5 by the addition of 1 ml per litre of 2.5 *M* sulphuric acid (analar grade). Gradient flow-rate 0.5 ml/min. The column was washed with 25 ml of HPLC grade methanol at the end of each batched analysis run. Eluents were continuously sparged with helium at 25 ml/min.

2.4. Standards

Samples of commercial acid, basic and dispersed dyes were obtained from Bayer UK and Ciba-Geigy UK. The dyes are listed in Table 1. Internal standard Rhodamine B (80%) C.I. 45170 was obtained from Aldrich Chemicals (Gillingham, UK). Rhodamine B is a basic dye not used in the dyeing of textile fibres.

For method development 0.01% (w/v) solutions of acid, basic and internal standard were prepared by dissolving 1 mg of dye in 10 ml of acidified acetonitrile–water (1:1). 0.01% solutions of dispersed dyes were prepared by first dissolving 10 mg of dye in 10 ml of methanol (0.1%) with a further dilution of 1:10 in acidified acetonitrile–water (1:1) to give a final concentration of 0.01% (w/v).

Pattern cards of known acid dyes on wool, basic dyes on acrylic and dispersed dyes on polyester fibres were obtained from Ciba-Geigy. Other fibres were obtained from the laboratory reference collection.

2.5. Extraction of fibre dyes

Basic dye extracts were obtained by placing single acrylic fibres of 10 mm or less in a capillary tube with 5 μl of formic acid–water (1:1). After sealing, the tube was heated to 100°C for 20 min. The dye extract was removed from the tube with a GC syringe and further diluted with 10 μl of eluent containing internal standard at a concentration of 0.7 ng per 5 μl injection.

Disperse dyes were extracted from polyester fibres using the procedure recommended by Wheals et al. [3]. Single fibres of 10 mm or less were placed in a capillary tube with 5 μl of chlorobenzene. The tube was sealed and heated in an oven to 130°C for 30 min. The tube was then shaken to separate the dye extract from the fibre. The end containing the fibre was removed. The dye extract was evaporated to dryness at 130°C for 30 min. The dye extract was redissolved in 15 μl of acidified acetonitrile containing internal standard at a concentration of 1 ng per 5 μl injection.

Table 1
List of commercial dyes with absorption maxima (λ_{max}), limits of detection (LOD) in picograms per 5 μl injection and relative retention times (RRT) with respect to Rhodamine B

Dye (CI name)	Manufacturer's name	Manufacturer	Dye class	RRT	λ_{max} (nm)	LOD (pg/5 μl)
Acid Red 299	Erionyl Bordeaux 5BLF	Ciba-Geigy	Disazo	1.098	520	230
Acid Red 361	Tectilon Red 2B	Ciba-Geigy		1.196	515	320
Acid Blue 225	Polar Blue RLS	Ciba-Geigy		1.244	605	640
Acid Blue 227	Tectilon Blue 4R-01	Ciba-Geigy	Anthraquinone	1.261	588	470
Acid Yellow 219	Tectilon Yellow 4R	Ciba-Geigy		1.285	396	375
Acid Red 127	Erionyl Red 2B	Ciba-Geigy	Monoazo	1.304	515	500
Acid Blue 260	Erionyl Blue RL	Ciba-Geigy	Anthraquinone	1.373	601	1250
Acid Orange 67	Erionyl Yellow RXL	Ciba-Geigy		1.401	435	235
Acid Red 260	Polar Red RLS	Ciba-Geigy	Disazo	1.492	527	1200
Basic Red 109	Maxilon Red M-4GC	Ciba-Geigy		0.107	506	110
Basic Blue 124	Maxilon Blue M2G	Ciba-Geigy		0.116	623	500
Basic Yellow 87	Maxilon Yellow M-4GL	Ciba-Geigy		0.152	412	170
Basic Yellow 91	Maxilon Yellow M-3RL	Ciba-Geigy		0.158	422	165
Basic Red 51	Maxilon Red M-RL	Ciba-Geigy	Azo	0.168	526	250
Basic Blue 151	Maxilon Blue M-G	Ciba-Geigy		0.201	625	800
Basic Red 46	Astrazon Red FBLN	Bayer	Monoazo	0.819	531	250
Basic Blue 3	Maxilon Blue 5G	Ciba-Geigy	Oxazine	0.821	650	70
Basic Yellow 28	Maxilon Golden Yellow	Ciba-Geigy	Methine	0.874	444	60
Basic Red 14	Maxilon Red 49	Ciba-Geigy	Cyanine	0.881	519	400
Basic Yellow 21	Astrazon Yellow 7GLL	Bayer	Polymethine	0.928	420	71
Basic Blue 147	Astrazon Blue F2RL	Bayer		0.942	584	75
Basic Red 27	Maxilon Pink B	Ciba-Geigy	Methine	0.943	531	40
Basic Red 18:1	Maxilon Red 2GL-N	Ciba-Geigy	Monoazo	0.978	480	120
Basic Violet 16	Astrazon Red Violet 3RN	Bayer	Methine	0.982	547	50
Basic Violet 21	Astrazon Violet F3RL	Bayer	Polymethine	1.038	558	180
Dispersed Yellow 82	Terasil Flavine 8GFF	Ciba-Geigy	Methine	0.859	475	460
Dis Violet 95	Terasil Bordeaux 2B	Ciba-Geigy	Azo	1.164	520	330
Dis Orange 45	Terasil Orange 5RL	Ciba-Geigy	Monoazo	1.211	459	380
Dis Yellow 211	Terasil Yellow 4G	Ciba-Geigy	Monoazo	1.221	445	80
Dis Blue 125	Terasil Navy SGL	Ciba-Geigy	Monoazo	1.254	584	195
Dis Red 151	Terasil Red 4G	Ciba-Geigy	Disazo	1.266	503	750
Dis Violet 57	Terasil Violet BL	Ciba-Geigy	Anthraquinone	1.285	541	1280
Dis Blue 165	Terasil Blue BG-01	Ciba-Geigy	Monoazo	1.326	610	110
Dis Red 349	Terasil Red 3GS	Ciba-Geigy	Azo	1.355	497	210

Due to a technical problem with the PDA detector it was not possible to analyse extracted acid dyes.

3. Results and discussion

3.1. Choice of column

The Inertsil-ODS2 column consists of octadecyl groups bonded onto ultra-pure silica. The bonded phase has been extensively end-capped to remove silanol group interferences, enabling the separation of basic and other polar compounds without the addition of ion-pairing agents. Using an Inertsil ODS-2 column, an acetonitrile water gradient HPLC system has been developed for the analysis of acid, basic and neutral drugs [9]. As the chemical structures of drugs and dyes are similar the application of the column to the separation of acid, basic and dispersed dyes has been investigated.

3.2. Optimisation of gradient

Mixtures of standard acid, basic and dispersed dyes were initially chromatographed with a linear gradient of 2% to 98% acidified acetonitrile for 20 min and held at 98% for 5 min. This was to take into account the wide range of polarities of the dye mixtures. The gradient was repeated over a range of pH values and flow-rates. The optimum eluent pH value was 2.5 and the flow-rate was 0.5 ml/min.

All chromatograms of mixtures exhibited symmetrical peak shape and good efficiency. The gradient range was further optimised (20–98% acidified acetonitrile) to remove the initial portion of the gradient at which no peaks were eluted. The linear gradient time was reduced from 20 min to 10 min with minimal effect on resolution. Table 2 lists the optimum gradient profile.

Although the gradient run was completed in 15 min it was a further 10 min before the last peak was eluted. The cause of the lag can be explained by an inherent dead volume in the HPLC pump due to the pulse dampers (volume 2.5 ml) and the tubing between the low pressure mixing chamber and the pump (volume 2.5 ml). The system was therefore operating isocratically at the initial gradient conditions for the first 10 min of the chromatographic run. The pulse dampers can be removed, but since this would increase baseline noise and because the chromatographic runs were reproducible this was not done.

Table 2
Optimum gradient profile

Time (min)	Flow-rate (ml/min)	Eluent		
		%A	%B	%C
Initial	0.5	80	20	0
10	0.5	2	98	0
15	0.5	2	98	0
20	0.5	80	20	0
40	0.1	0	0	100

Eluent A acidified water, eluent B acidified acetonitrile, eluent C methanol.

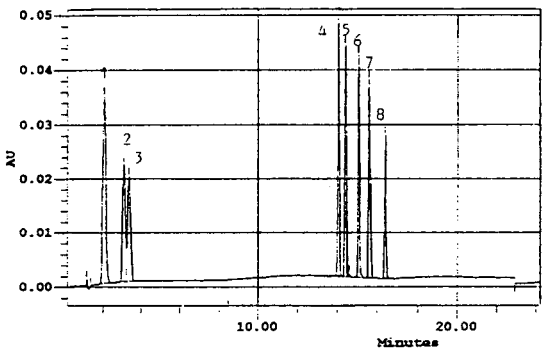


Fig. 1. Maxplot HPLC chromatogram of basic dye mix 1. Chromatographic conditions: Inertsil ODS-2 column, 150 × 2.1 mm I.D.; eluent, acidified acetonitrile–water pH 2.5; gradient 20–98% acidified acetonitrile over 10 min; flow-rate 0.5 ml/min; PDA detector operating over the range 380 to 600 nm; concentration approx. 100 ng/μl; Peaks: 1 = Basic Red 109; 2 = Basic Yellow 87; 3 = Basic Yellow 91; 4 = Basic Blue 3; 5 = Basic Yellow 28; 6 = Basic Blue 147; 7 = Basic Red 18:1; 8 = Basic Violet 21.

Figs. 1–7 show representative chromatograms of the dye mixtures (concentration approx. 100 ng/μl). In the legends the components of the dye mixtures are listed with their corresponding peak.

3.3. Reproducibility of the HPLC system

Basic dye mixture 2 was injected 10 times with 10 min equilibration time at initial conditions at the start of each run. The relative standard

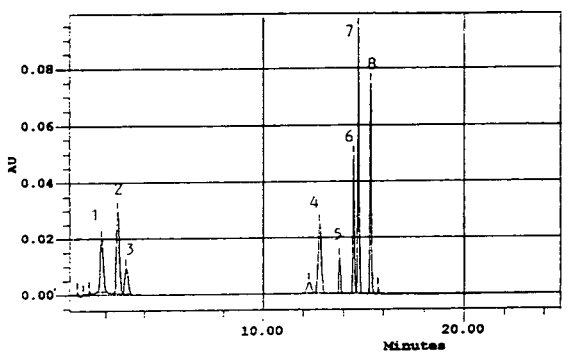


Fig. 2. Maxplot HPLC chromatogram of basic dye mix 2. Chromatographic conditions as in Fig. 1. Concentration approx. 100 ng/μl. Peaks: 1 = Basic Blue 124; 2 = Basic Red 51; 3 = Basic Blue 151; 4 = Basic Red 46; 5 = Basic Red 14; 6 = Basic Yellow 21; 7 = Basic Red 27; 8 = Basic Violet 16.

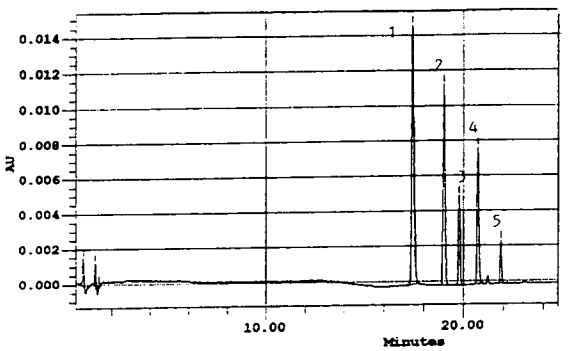


Fig. 3. Maxplot HPLC chromatogram of acid dye mix 1. Chromatographic conditions as in Fig. 1. Concentration approx. 100 ng/μl. Peaks: 1 = Acid Red 299; 2 = Acid Red 361; 3 = Acid Blue 225; 4 = Acid Red 127; 5 = Acid Blue 260.

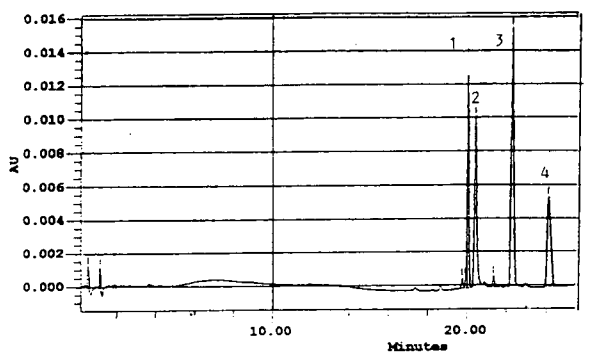


Fig. 4. Maxplot HPLC chromatogram of acid dye mix 2. Chromatographic conditions as in Fig. 1. Concentration approx. 100 ng/μl. Peaks: 1 = Acid Blue 227; 2 = Acid Yellow 219; 3 = acid Orange 67; 4 = Acid Red 260.

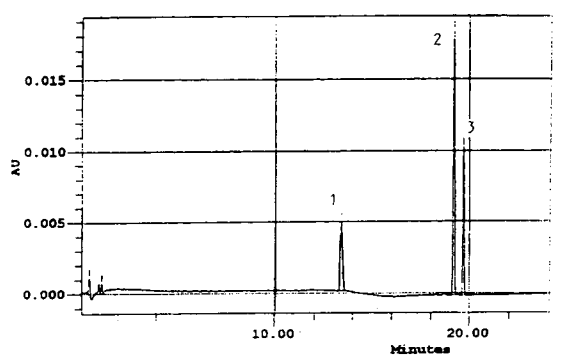


Fig. 5. Maxplot HPLC chromatogram of disperse dye mix 1. Chromatographic conditions as in Fig. 1. Concentration approx. 100 ng/μl. Peaks: 1 = Dispersed Yellow 82; 2 = Dispersed Yellow 211; 3 = Dispersed Blue 125.

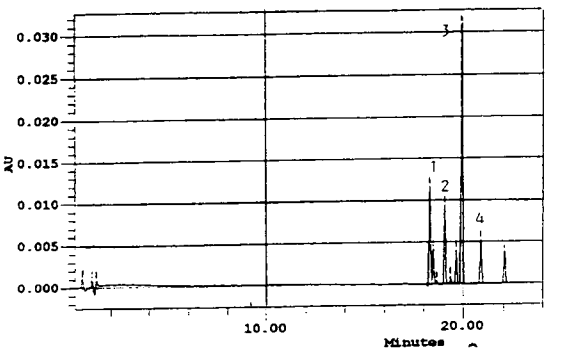


Fig. 6. Maxplot HPLC chromatogram of disperse dye mix 2. Chromatographic conditions as in Fig. 1. Concentration approx. 100 ng/μl. Peaks: 1 = Dispersed Violet 95; 2 = Dispersed Orange 45; 3 = Dispersed Blue 165; 4 = Dispersed Red 151.

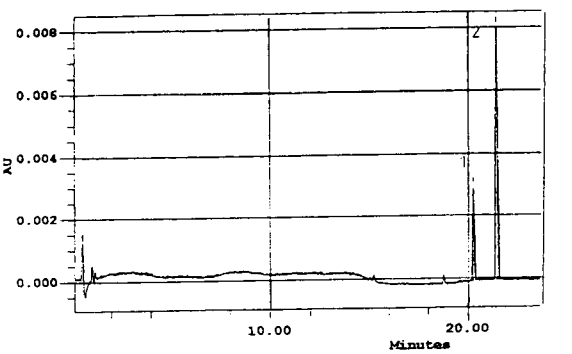


Fig. 7. Maxplot HPLC chromatogram of disperse dye mix 3. Chromatographic conditions as in Fig. 1. Concentration approx. 100 ng/μl. Peaks: 1 = Dispersed Violet 57; 2 = Dispersed Red 349.

deviation (R.S.D.) of the relative retention time (with respect to Rhodamine B) of the first eluted peak (basic blue 124) was 0.76%. The R.S.D. for the last peak basic violet 16 was 0.2%. Increasing the equilibration time between injections did not reduce the R.S.D.s further. The Inertsil ODS-2 column was used daily over a period of 10 weeks, with no deterioration in dye peak shapes.

3.4. Detection limits of PDA detector

Concentrations of the five standard dye mixtures ranging from 25 to 0.5 ng per 5 μl injection were analysed to determine the limit of detection

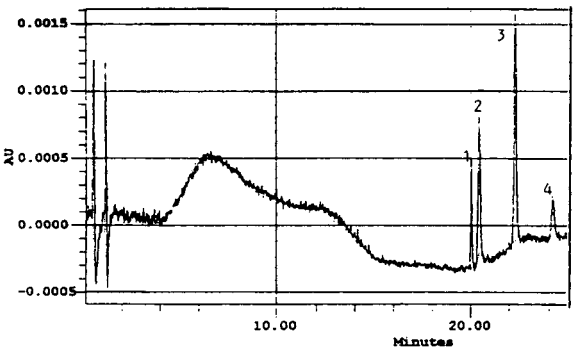


Fig. 8. Example of a Maxplot HPLC chromatogram of acid dye mix 2, used for the determination of limits of detection. Chromatographic conditions as in Fig. 1. Concentration 2000 pg/5 μl injection. Peaks: 1 = Acid Blue 227; 2 = Acid Yellow 219; 3 = Acid Orange 67; 4 = Acid Red 260.

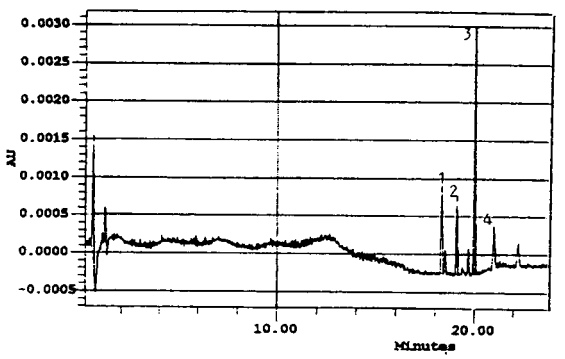


Fig. 10. Example of a Maxplot HPLC chromatogram of dispersed dye mix 2, used for the determination of limits of detection. Chromatographic conditions as in Fig. 1. Concentration 2000 pg/5 μl injection. Peaks: 1 = Dispersed Violet 95; 2 = Dispersed Orange 45; 3 = Dispersed Blue 165; 4 = Dispersed Red 151.

for each individual dye. The limit of detection (LOD) was calculated on a signal to noise ratio S/N of 3:1.

The LOD for the standard basic dyes ranged from 40 pg for basic red 27 to 800 pg for basic blue 151. For standard acid dyes the range was 230 pg for acid red 299 to 1250 pg for acid blue 260. For standard dispersed dyes the range was 80 pg for dispersed yellow 211 to 1280 pg for dispersed violet 57. The majority of the dyes had an LOD between 50 and 500 pg. All figures quoted are per 5 μl injection. For examples see Figs. 8–10.

Studies in this laboratory indicate the concentration of dyed fibres to be of the order of a few nanograms or more per 10 mm depending on the percentage dyeing.

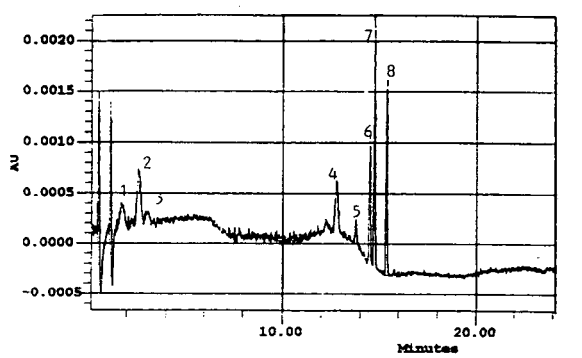


Fig. 9. Example of a Maxplot HPLC chromatogram of basic dye mix 2, used for the determination of limits of detection. Chromatographic conditions as in Fig. 1. Concentration 500 pg/5 μl injection. Peaks: 1 = Basic Blue 124; 2 = Basic Red 51; 3 = Basic Blue 151; 4 = Basic Red 46; 5 = Basic Red 14; 6 = Basic Yellow 21; 7 = Basic Red 27; 8 = Basic Violet 16.

3.5. Baseline stability

Refractive index (RI) disturbances at the PDA detector flow cell were minimised by the low pressure mixing of eluents A and B by the Waters 600E multisolvent delivery system. The baseline at high detector sensitivity (<0.002 AUFS) is acceptable, although not as smooth as an isocratic HPLC chromatogram. A previous attempt to develop a gradient system using high pressure mixing of eluents with two pumps, T-piece and a dynamic mixer produced excessive baseline drift and was unsuitable for detecting low levels of dyes.

3.6. Characterisation of dyes extracted from textile fibres

Spectral data of the standard commercial dyes separated by the HPLC system, together with their relative retention times with respect to Rhodamine B (as listed in Table 1) were stored

in a library created using the Millennium PDA software. Chromatograms of unknown fibre dye extracts can be searched against this library. A display of the best library match is overlaid onto the spectrum of the unknown dye. The system was tested using 10 fibre dye extracts.

Five known dispersed dyed fibres and five known basic dyed fibres 10 mm in length were removed from Ciba-Geigy pattern cards. A range of colours were chosen. The dyes were extracted according to the procedures described in the experimental section. The five dispersed dyed fibres contain blue 125, yellow 82, orange 45, red 151 and violet 57. The five basic dyed fibres contain blue 147, blue 124, yellow 21, yellow 87 and red 51.

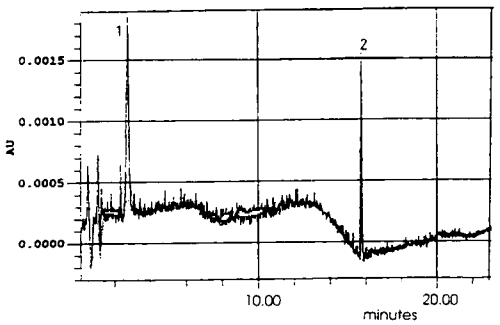
In all cases the extracted dyes gave identifiable peaks. All extracted dyes were matched with their respective standard library dye on the basis of relative retention time with respect to Rhodamine B and visible spectrum match.

The amount of dye extracted from the fibres was determined from their peak heights. This value was further multiplied by 3 to account for injecting 5 μl from a total fibre extract volume of 15 μl. Table 3 lists the dyes with their relative retention times and quantity of dye in 10 mm of fibre. See Fig. 11 for an example of a chromatographed fibre dye extract.

Table 3
Identification of dyes extracted from 10-mm fibres

Dye	RRT	Standard dye RRT	Quantity of dye in fibre (ng)
Basic Blue 147	0.947	0.942	2.1
Basic Blue 124	0.118	0.116	6.0
Basic Yellow 21	0.922	0.928	1.2
Basic Yellow 87	0.155	0.152	5.1
Basic Red 51	0.161	0.168	2.6
Dis Blue 125	1.259	1.254	4.5
Dis Yellow 82	0.850	0.859	8.9
Dis Orange 45	1.203	1.211	5.8
Dis Red 151	1.269	1.266	12.2
Dis Violet 57	1.277	1.285	10.6

Relative retention time (RRT) with respect to Rhodamine B, the internal standard.



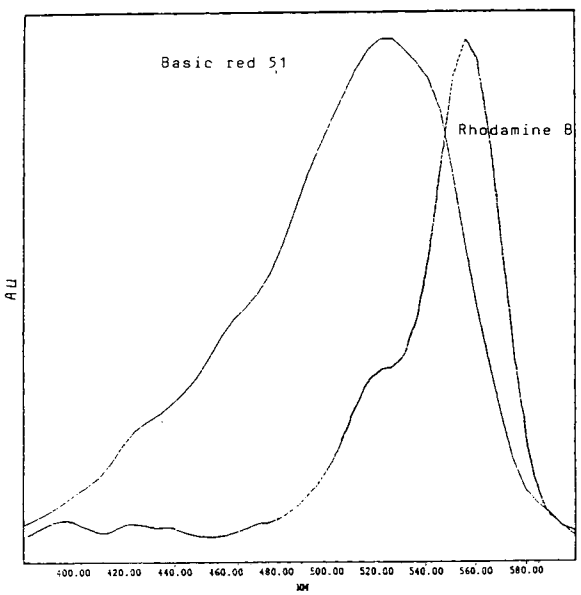


Fig. 11. Example of a Maxplot HPLC chromatogram and visible spectrum of a 10 mm fibre dye extract. Fibre dyed with basic red 51. Chromatographic conditions as in Fig. 1. Peaks: 1 = Basic Red 51; 2 = Rhodamine B (internal standard).

4. Conclusions

A single-gradient HPLC system has been developed for the separation of acid, basic and dispersed dyes. The Inertsil ODS-2 column has minimal residual silanol groups enabling the separation of a range of dye classes with good efficiency and peak shape without the need for ion-pairing agents. The HPLC system with a

PDA detector has detection limits typically less than 1 ng. Basic and dispersed dyes extracted from 10 mm fibres were qualitatively and quantitatively identified using a database of relative retention times and spectral data of standard commercial dyes. The database library will be upgraded with more commercial dyes.

Further work is in progress comparing the HPLC system with TLC and high-performance TLC in the analysis of casework size fibres. The results of this work will decide if the HPLC system is to be used in forensic casework.

A more detailed study relating the chemical structures of the dyes to their separation by the HPLC system is also ongoing.

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An improved high-performance liquid chromatography system for the analysis of basic dyes in forensic casework

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Abstract

Acrylic fibres are frequently encountered as physical evidence in casework in the Northern Ireland Forensic Science Laboratory. Separation and analysis of the basic dyes, used in the garment industry for the dyeing of acrylic fibres, was carried out by isocratic HPLC with a narrow bore column. The HPLC eluent used was a mixture of methanol and aqueous ammonium acetate solution (pH 9.76) in a ratio of 9:1. The sensitivity of the system is such that the dyes extracted from 0.2 cm of a black acrylic fibre could be detected using three injections on a single channel detector at wavelengths of 400, 500 and 600 nm (for yellow, red and blue dye components respectively).

1. Introduction

Textile fibres contain a number of dyes which may be mixed in different proportions to give varying shades and colours. Methods used by forensic scientists for the comparison of fibres are: (a) an identification of the fibre type by polarising and infrared spectroscopy and (b) examination of the dye components by comparison microscopy, microspectrophotometry and thin-layer chromatography (TLC) [1].

High-performance liquid chromatography (HPLC) systems have been described for the analysis of acid, disperse [2], direct and pigment dyes in forensic science. These include the analysis of fibres [3–7], lipstick smears [8,9], inks [10,11], illicit tablets [12], and counterfeit notes [13].

Acrylic fibres are one of the most frequently occurring fibre types encountered in casework in the Northern Ireland Forensic Science Laboratory. These are found particularly in terrorist incidents where masks and gloves have been used. Basic (cationic) dyes are commonly used in the dyeing of these acrylic fibres.

A few HPLC systems have previously been reported for the analysis of basic dyes. Individual [14,15] or a narrow range of basic dyes [16,17] can be analysed with these systems.

An HPLC method for basic dyes was described previously by this laboratory [18]. It consisted of gradient elution with mixtures of methanol and aqueous ammonium acetate solutions on a normal-phase silica column (160 × 4.5 mm I.D.) and was found to provide acceptable separation of twenty-one basic dyes. However, baseline drift was found to occur in the gradient system due to refractive index changes. This was most noticeable at visible wavelengths, when high sensitivity (0.0005 AUFS) was required for the analysis of the small quantities of extracted fibre dyes available in forensic science casework.

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An isocratic HPLC system was developed in this laboratory [5] for the analysis of basic dyes. This gave acceptable separation of seventeen of the standard dyes analysed.

Acrylic fibres were obtained from local manufacturers, dye pattern cards and casework. The dyes were extracted from the fibres and analysed on the HPLC system using a single channel detector and an autoinjector. Three injections were required to detect the dyes at 400, 500 and 600 nm. Using this system picogram levels of basic dyes were detected and dyes extracted from less than 0.2 cm of heavily dyed fibres could be detected.

The stability of the dyes was assessed during extraction, after extraction and over a six day period. Dye batch variations were examined on fibres from different areas of the garments and over the length of a single fibre.

2. Experimental

The equipment consisted of Waters Assoc. Model 510 HPLC pump, Model 680 gradient controller and a Severn Analytical 6504 absorbance detector. A Waters 990 photodiode array detector was used, coupled to a NEC personal computer. The photodiode array detector had 512 diodes and a wavelength range of 190 to 800 nm with a resolution of 1.4 nm. The cell had a volume of 8 μl and a 10 mm pathlength. The results were recorded either with a Phillips PM 8251 single-pen recorder or on a Tandon microcomputer utilising the Drew Scientific Roseate chromatography system for data capture and manipulation. The autoinjector consisted of a Waters Associates Model 712 Wisp instrument. This was electronically linked to the Severn detector and the Tandon computer.

A Shandon stainless-steel column (250 × 4.5 mm I.D.) was packed with Phase-Sep Spherisorb silica (5 μm). A flow-rate of 2 ml/min was used. A Jones Chromatography stainless-steel column (250 × 2.1 mm I.D.) or a Phase-Sep (250 × 2.0 mm I.D.) column packed with Spherisorb W silica (5 μm) was used at a flow-rate of 0.46 ml/min. A Rheodyne multiloop injector was used for all manual injections (10 or 20 μl loop) and a Waters Guard-Pack precolumn module (with a silica precolumn insert) was fitted.

The eluent consisted of a solution of methanol–aq. ammonium acetate (pH 9.76) (9:1, v/v). This was prepared by adding 94 ml of concentrated ammonia and 22 ml of glacial acetic acid to 884 ml of deionised water. The pH was adjusted to 9.76 by addition of either a few drops of ammonia or of glacial acetic acid. This was then diluted with methanol (1:9) before use. All solvents were filtered under vacuum through a Gelman Science 0.2 μm FP Vericel TM membrane. The deionised water and methanol were degassed for 15 min using helium displacement of air.

The column was conditioned for 40 min in the eluent before use. To maintain reproducibility of retention times and separation it was necessary to clean out the column with methanol–water (9:1) for at least 1 h after use. This was mainly because of the pH of the eluent degrading the silica despite the use of an in-line filter (precolumn). For the same reason the eluent should not be left static in the column.

2.1. Standards

Chromatographic performance of the HPLC column was monitored by injecting a mixture of basic yellow 21, basic red 14, and basic blue 45 (0.002%) at the beginning of each day and at intervals throughout the day using a detecting wavelength of 280 nm.

Samples of basic dyes were obtained from Bayer UK and from Ciba Geigy UK and are: Maxilon yellow M-4GL (basic yellow 87), Astrazon Golden yellow Gl-E (basic yellow 28), Maxilon yellow M-3RL (basic yellow 91), Maxilon brilliant flavine 10GFF (basic yellow 40), Astrazon yellow 7GLL (basic yellow 21), Astrazon blue FGRL, Maxilon blue M-G (basic blue 151), Astrazon blue BG (basic blue 3), Maxilon blue M2G (basic blue 124), Astrazon red GTLN (basic red 18:1), Maxilon red M-4GL (basic red 109), Maxilon brilliant pink B (basic red 27), Maxilon red GRL Pearls (basic red 46), Maxilon Brilliant red 4G liquid (basic red 14),

Maxilon red M-RL (basic red 51), Maxilon red B-LN liquid (basic red 22), Astrazon red violet 3RN (basic violet 16).

Solutions of these dyes were prepared by dissolving 10 mg of dye in 10 ml of methanol and further diluting to the required concentration.

Fibre samples were obtained from casework and from a laboratory reference collection. Two Courtaulds fibres I and II were analysed. A Ciba Geigy pattern card which contained known dyes on acrylic fibre was used, and fibres were obtained from three local manufacturers.

2.2. Extraction of dyes

This was carried out using a 1:1 solution of HPLC-grade formic acid and deionised water. Each fibre was inserted into a capillary tube in which one end had been sealed with a bunsen burner. The fibres were pushed to the bottom of the tube. A volume of 3 μl of formic acid–water was added and the tube sealed in an oven at 100°C for 20 min. The extracts were then removed from the tubes using a 5-μl GC syringe. The solution was spotted onto a TLC plate or was diluted with 60 μl of the HPLC eluent. Aliquots of 15–20 μl were injected onto the HPLC column. TLC analysis was carried out on Kieselguhr DC Alufolien silica gel 60 F254 plates. The fibre extracts were spotted onto the TLC plate and eluted with a mixture of chloroform–methyl ethyl ketone–acetic acid (glacial)–formic acid (8:6:1:1, v/v).

3. Results and discussion

An eluent compositon was required which would separate a mixture of unknown dyes in a maximum run time of 15 min per sample injection. Initial investigations were carried out on a standard normal-phase silica HPLC column (250 × 4.5 mm I.D.). The eluent composition was determined by varying the methanol–aq. ammonium acetate (pH 9.76) ratio and the concentration of the ammonium acetate solution. A 9:1 ratio of methanol–aq. ammonium acetate (0.71:0.34 mol/l) solution was found to give the best separation and resolution of the commercial basic dyes.

Three dyes (basic yellow 21, basic red 14 and basic blue 45) were used to check the performance and reproducibility of the column. These dyes were injected at the start of each day and at intervals throughout the day. The wavelength chosen for the detection of these standards was 280 nm as most of the commercial dyes display a broad absorption at this wavelength.

UV–Vis spectroscopy was used to determine the wavelength maximum of the individual dyes. Wavelengths of 400, 500 and 600 nm were chosen for the detection of yellow, red and blue basic dyes, respectively. Some of the dyes were detected at two wavelengths due to the broad absorption spectrum of the dyes. Unknown samples such as in fibre extracts may need to be reanalysed at other wavelengths. The wavelengths for reanalysis can be determined from the visible spectrum of the fibres using microspectrophotometry, for example (Fig. 1) analysis at 450, 550 and 620 nm would be required to detect the dye components.

A variable-wavelength detector was used but only one wavelength could be sampled at any one time. This is a lengthy process requiring a

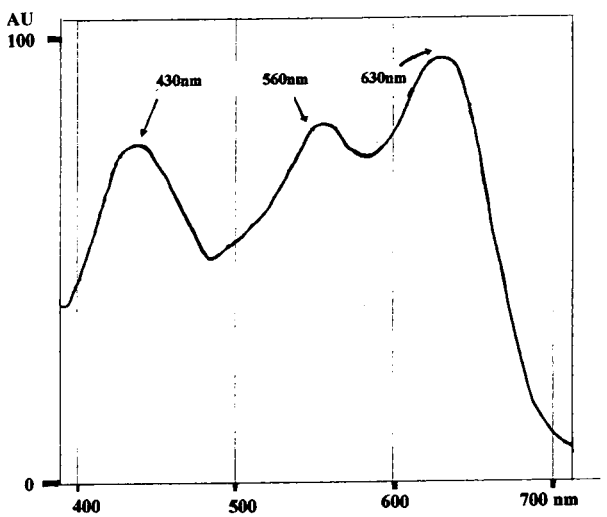

Fig. 1. Visible spectrum (400–700 nm) of a pale coloured casework fibre (Fibre 7) showing the wavelength maxima at approximately 430, 560 and 630 nm.

minimum of 45 min per fibre analysed. Attempts to change the wavelengths throughout the analysis were not feasible especially when an unknown dye mixture is present in the fibres. An autoinjector was incorporated into the HPLC system and the detector was programmed to change automatically the wavelength every 17 min. The dye extract was injected every 17 min in conjunction with the wavelength changes for the analysis of the yellow, red and blue dye components in the dye mixture. The program was repeated after every third injection.

A narrow bore HPLC column (250 × 2.1 mm I.D.) was then investigated to see if the resultant increase in sensitivity would facilitate the use of a diode array detector. The mixture of methanol–ammonium acetate (9:1) already described was used as the eluent at a flow-rate of 0.46 ml/min. The commercial basic dyes were injected onto this HPLC column individually and as a mixture of yellow, red and blue dyes. The retention times of the dyes on the HPLC column are shown in Table 1. The separation of the dye mixture is shown in Fig. 2.

The majority of the dyes were eluted between 3 and 6 min. It was found that the dyes were not all resolved by the HPLC system but if the coeluting components were of a different colour they could in most cases be identified by a combination of retention time and detecting wavelength (colour). If, however, the coeluting dyes are the same colour (basic yellow 87 and 91) then the use of a diode array detector, which has the capability of giving both retention and spectral data, would facilitate the identification of these dyes (Fig. 3). The ability of the diode array detector to detect all wavelengths at one time means that only one injection would be required and a more concentrated solution of the dye extract would be available.

A comparison of the limits of detection (LOD) of the single channel detector and the diode array detector was carried out on the narrow bore column. The lowest detectable limit (signal-to-noise, S/N, of at least 3:1) was determined for each dye.

A 0.002% stock solution of the dyes was prepared (not corrected for the pure dye content of the commercial dye) and diluted such that 4 ng to 0.02 ng of dye in 10 μl of solution was injected onto the HPLC column. The absorbances of the dyes were measured and when plotted against concentration were found to obey Beer's law.

Table 1
Retention times of standard dyes on the 4.5 mm I.D. and 2.1 mm I.D. HPLC columns

Dyes	4.5 mm I.D.	2.1 mm I.D.
Yellow 21	3.5	3.9
Yellow 28	4.8	5.1
Yellow 40	4.8	[a]
Yellow 87	9.3	9.6
Yellow 91	9.2	9.4
Red 14	4.5	4.3
Red 18:1	7.4	7.0
	16.1	14.7
Red 27	3.8	4.0
Red 46	3.7	3.9
	6.5	6.6
Red 51	7.0	6.3
Red 109	5.4	4.9
Red 22	4.2	[a]
Blue 3	4.2	3.8
Blue 124	4.8	4.6
Blue 151	3.3	4.0
Blue 45	12.5	11.2

Conditions: eluent, methanol–aq. ammonium acetate (9:1); sensitivity, 0.005 AUFS; flow-rate, 0.46 ml/min.
[a] Not analysed.

The average LOD at 280 nm (on the single channel detector, 0.0005 AUFS) was found to be 1 ng whereas at 400, 500 and 600 nm the LOD was found to be between 12 and 25 pg using the narrow bore (2.1 mm I.D.) column and 100 pg for the wide bore (4.5 mm I.D.) column. These limits would be improved by analysis at the wavelength maxima of each dye. The LOD for the diode array detector (0.002 AUFS) was found to be 3.4 ng (yellow 21), 3.2 ng (red 14) and 1.9 ng (blue 45). To obtain a whole visible spectrum of the dyes more dye than this would be required. This quantity is not usually available in most of the fibres (less than 1 cm in length) found in forensic casework.

Fibres (5 mm in length) were removed from pattern cards. The dyes were extracted (formic

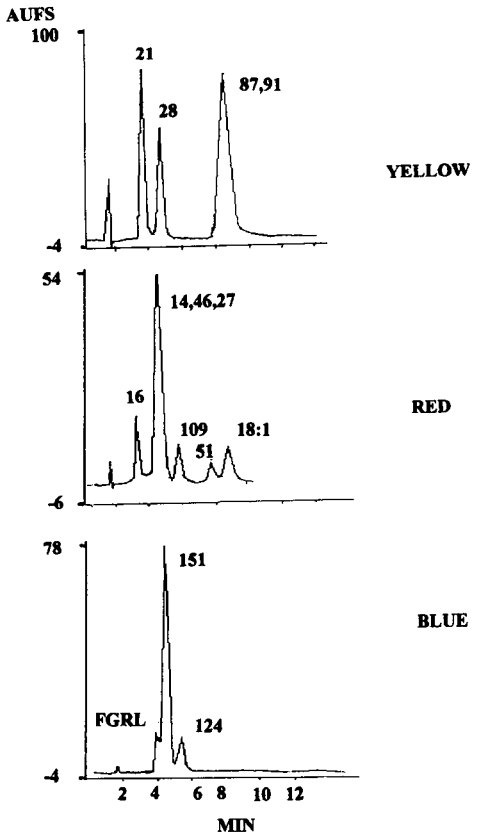

Fig. 2. HPLC analyses showing the separation of a mixture of yellow, red, and blue standard basic dyes using detecting wavelengths of 400, 500 and 600 nm respectively. Eluent, methanol–aq. ammonium acetate (9:1); sensitivity, 0.005 AUFS; column, 2.1 mm I.D.; flow-rate, 0.46 ml/min.

acid–water) and analysed by HPLC. The peak heights of the dye components were compared with a known quantity (1 ng in 10 μl) of the standard dye. The results are shown in Table 2.

Problems could arise with the use of the autoinjector if the extracted dyes were to decompose prior to injection as some samples will then be analysed after 24 h.

Some of the basic blue dyes are known to degrade in the pyridine and water extracting solvent [19,20] giving a different coloured solution. This is possibly due to deprotonation of the dye. A similar mechanism may be occurring in formic acid and water. It was found that for some of the black fibres examined pink solutions

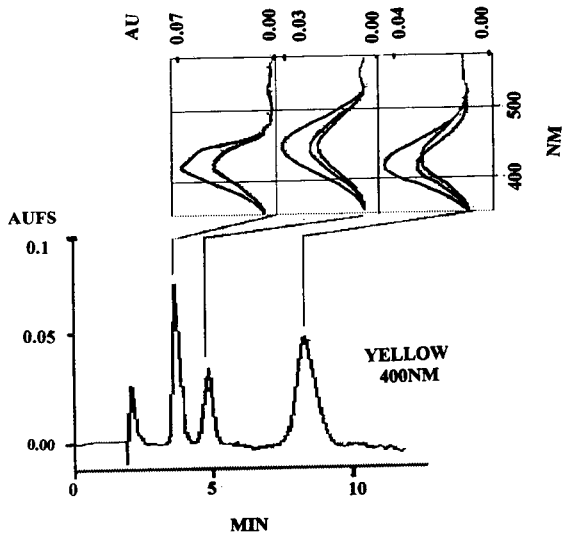

Fig. 3. HPLC analysis and visible spectra obtained using a diode array detector. Eluent, methanol–aq. ammonium acetate (9:1); sensitivity, 0.005 AUFS; column 2.1 mm I.D.; flow-rate, 0.46 ml/min.

were obtained on extraction. The colour changed to blue when the extract was spotted onto a TLC plate or when diluted with the HPLC eluent. Other studies in this laboratory [18] have shown that basic blue 151 decomposed in solution to a red dye over a period of one week.

The time and temperature of extraction, the

Table 2
Quantities of dyes in pattern card fibres

Dye	Peak height standard	Peak height fibre/mm	Amount of dye (ng)
Blue 151	76.86	42.36	1.1
Blue 124	98.77	51.67	1.0
Blue 3	89.83	59.68	1.3
Red 109	95.77	20.92	0.5
Red 51	61.65	40.92	1.3
Red 46	73.79	81.54	1.1
Red 14	52.04	41.06	1.6
Red 18:1	17.25	12.88	1.5
Yellow 87	39.72	5.08	0.3
Yellow 91	25.87	19.99	1.6
Yellow 28	31.09	13.02	0.9

Conditions: eluent, methanol–aq. ammonium acetate (9:1); sensitivity 0.005 AUFS; column 2.1 mm I.D.; flow-rate, 0.46 ml/min.

time in the formic acid–water after extraction, and the time in the eluent before injection were all examined by HPLC analysis. The degree of extraction (formic acid–water) of the dyes from acrylic fibres was investigated using a blue, a red and a black fibre. All fibres were found to be fully extracted within 20 min at 90°C. Generally extraction was complete after 15 min at 90°C and 10 min at 100°C. There was almost no extraction of the dyes at room temperature even after 10 days. Degradation of the dyes in the extracts was found to occur at elevated temperatures or elongated extraction times (greater than 100°C and 30 min). Fig. 4 shows the chromatograms obtained for a red casework fibre at 100 and 130°C.

The analysis of the extracts after a period of a few min up to 4 h in the formic acid–water extracting solvent showed that no degradation had occurred for seven of the eight fibres analysed. One green fibre did appear to degrade in the formic acid–water after extraction. The solution changed from green to orange after approximately 40 min. Over a 24-h period the fibre extract returned to its original green colour with an increase in the blue dye component in the chromatogram.

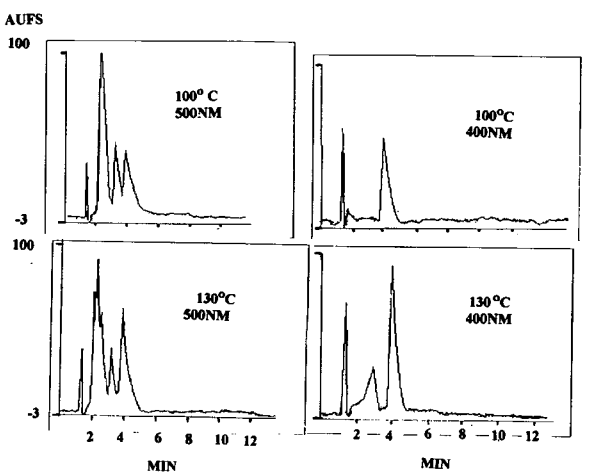

Fig. 4. HPLC analyses at 400 and 500 nm showing the degradation of a red casework fibre using an extraction temperature of 130°C compared to 100°C. Eluent, methanol–aq. ammonium acetate (9:1); sensitivity 0.005 AUFS; column, 2.1 mm I.D.; flow-rate, 0.46 ml/min.

Fibre extracts were stored at room temperature in the HPLC eluent to simulate the queue for the autoinjector. The extracts were analysed every hour for 6 h and then daily for 6 days. None of the fibres analysed showed any signs of degradation in the eluent.

Fibres from pattern cards dyed with the commercial dyes were also analysed. Slight variation in the retention times between the methanol solution of the individual dyes and the extracted or formic acid–water solutions of the dyes was found. It was postulated that ion-pair formation was occurring between the formic acid and the dye. This effect was reduced when the extract was diluted with methanol or eluent. There was also a slight variation observed in the retention times of some of the dyes when injected individually or in a mixture of dyes.

If the crime and control fibres are treated identically then a comparison can be carried out. A control fibre was therefore analysed every third or fourth sample to take account of any degradation that might have occurred.

Fibres (0.5 cm) containing mixtures of known dyes were removed from the pattern cards. Three shades each containing the same three dyes (basic yellow 91, basic red 51 and basic blue 124) were analysed. The results are shown in Table 3. It was found that between 0.1 and 0.8 ng per mm of the dyes were present in the fibres. The ratios of the peak heights indicate the relative amounts of the dyes used on each fibre.

Fibres were obtained from casework and local manufacturers to assess the HPLC system for the

Table 3
Concentration of dyes in three fibres (from the pattern card) which contain different proportions of the same dyes

Colour of fibre	Concentration of dye (ng/mm)		
	Yellow 91	Red 51	Blue124
Blue	0.3	0.1	0.2
Green	0.8	0.2	0.1
Brown	0.7	0.5	0.1

Conditions: eluent, methanol–aq. ammonium acetate (9:1); sensitivity, 0.005 AUFS; column, 2.1 mm I.D.; flow-rate, 0.46 ml/min.

Table 4
Retention times of dyes extracted from samples of fibres from local manufacturers

Manufacturer	Colour of fibre	Retention times (min)		
		400 nm	500 nm	600 nm
A	Green	8.5	6.4	4.4
A	Navy	4.7	3.4, 3.6, 4.7, 6.5	3.3
A	Black	4.7	3.3, 3.5, 4.5, 6.2	3.2
B	Black	4.4	4.3, 5.5	4.4
B	Blue	3.3	3.0, 3.5	3.0
B	Red	3.1, 4.2, 5.5	5.5	3.1
B	Light green	4.2	3.3, 4.2	3.1
B	Dark green	4.2	3.1, 4.4	3.1
C	Black	4.2	3.1, 4.4, 6.1	3.1
C	Orange		5.5	
C	Yellow	4.0, 10.2		
C	Red		4.3	
C	Blue		5.6	4.2
C	Pink		7.3	
C	Purple		4.0	

Conditions: eluent, methanol–aq. ammonium acetate (9:1); sensitivity, 0.005 AUFS; column, 4.5 mm I.D.; flow-rate, 2 ml/min.

separation and detection of mixtures of unknown basic dyes. Fibres (1 cm in length) were extracted and analysed by both TLC and HPLC. The extract for the HPLC analysis was split into three portions for injection at the three wavelengths. The whole extract was used for TLC

Table 5
Retention times of extracted dyes from black casework fibres

Sample	Retention times (min)		
	400 nm	500 nm	600 nm
1B9	3.9, 4.6, 6.5	3.3, 3.8, 4.5, 6.5	2.1, 4.5
1B15	4.5, 6.5	4.5	
2P2	4.6	3.1, 3.6, 4.0, 4.7, 6.9	3.1, 4.6
2U9	4.4, 6.5	2.8, 3.2, 4.5, 6.6	2.9, 4.3
2B13	4.8	3.1, 3.5, 4.0, 4.8, 7.1	3.1, 4.4
3P6	4.4	3.4, 4.2	4.4
3P1	1.5, 2.4, 3.5	2.7, 4.0, 4.7, 5.3, 6.6	
4C5	4.3	3.2	4.1
4U12	4.4	3.3, 5.7	4.1
5C4	1.8, 3.6	3.7	
5C13	1.8	3.7	3.5
6C	4.0	4.2	5.0
6C12	3.9	3.6	
7U11		2.3	
7B11		1.5	3.4
8B8	3.4, 4.6, 14.4	13.7	
8B2	3.5, 4.6, 14.4	3.9, 14.5	

Conditions: eluent, methanol–aq. ammonium acetate (9:1); sensitivity 0.005 AUFS; column, 4.5 mm I.D.; flow-rate, 2 ml/min.

analysis. Fibres from three different manufacturers were analysed. The dye composition of two of these was known; (A) basic yellow 87, basic red 51 and basic blue 124 and (B) basic yellow 28, basic red 46 and basic blue 41. The dyes in these fibres had been mixed in different proportions according to the colour required for the final product. The results are shown in Table 4.

The black fibres were found to contain numerous dyes. On discussion with the manufacturers it was found that in black or dark coloured fibres either a known mixture of dyes is used, or the dye baths from previous dyeing processes may be used. Therefore not every batch of these fibres is the same.

Black casework fibres which had previously been grouped into 8 groups by TLC analysis were extracted and injected onto the HPLC system (Table 5). It was found that more discrimination was found by HPLC and also quantitative differences were detectable (Fig. 5). The black fibres in group 2 were found to be similar in dye content to a sample of a black Courtaulds fibre (I). It is known that the black dye used in this fibre is bought from a supplier and adjusted to the correct shade by the addition of a red or a yellow dye.

Three black masks which were indistinguishable by TLC were also analysed and differences in the dye content detected at 500 nm. Black fibres from different sources (A–R) in one household were also examined. HPLC was found to differentiate between the different black acrylic fibres. Also three fibres (C, I and S) contained peaks similar to those found in a Courtaulds fibre (II). The same 6 or 7 dyes were present in all the fibres suggesting that these fibres were dyed by the Courtaulds process (Table 6).

Pale coloured case fibres (approximately 6 mm in length) which had shown no visible spots on TLC were extracted and analysed. Bulk extracts were prepared from 5 or 6 fibres and these solutions were analysed by TLC to determine the dye components present in the fibres. Some dye components were still not detectable due to the small quantities present in the 6 mm of fibre. HPLC was found to give peaks for all but one of the fibres (Table 7). Analysis of the unextracted fibre by microspectrophotometry indicated that the dye components in the fibres have sharp absorption maxima at 440, 550 and 630 nm and minimum absorption at the detecting wavelengths (Fig. 1). Reanalysis at these wavelength maxima would be necessary.

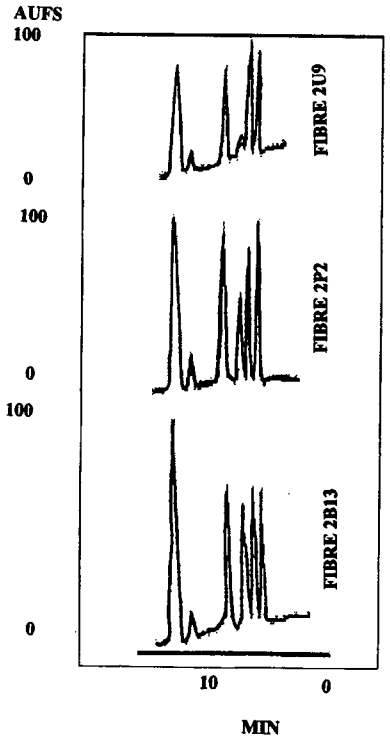

Fig. 5. HPLC analyses (500 nm) of three Group 2 TLC fibres showing the differences in dye ratios. Eluent, methanol–aq. ammonium acetate (9:1); sensitivity 0.005 AUFS; column, 4.5 mm I.D.; flow-rate, 2 ml/min.

Variation of the dyes within one fibre source was investigated. One particular acrylic fibre appears under the microscope to have variation between fibres and along the length of a single fibre. These are the producer dyed fibres made by Courtaulds UK Ltd. These fibres are known as 'Tiger Tail' fibres because of the pattern of dark and light (or coloured) bands which are visible under the microscope, even at low power. The analysis of the dye content of the Tiger Tail fibres (0.5–1 cm in length) was investigated along the length of a fibre, between individual fibres and from different areas of the garment.

Table 6
Retention times of fibres from different sources

Fibre	Retention time (min)		
	400 nm	500 nm	600 nm
Black A	5.7	5.2	
B	4.4, 5.7, 7.8, 8.4	3.0[a], 5.7, 7.6, 8.4	3.0, 6.5
C	4.5, 5.8, 7.8	4.1, 4.5, 5.9, 7.6, 8.5	2.9, 4.1, 6.4
D	4.4, 5.7	4.5, 5.7, 7.2	2.9, 4.5, 6.2
E	5.7, 7.9[a], 8.3[a]	5.7, 7.6, 8.4	2.9, 6.3
F	5.7	5.3	
G	5.8	2.7, 5.2, 5.8	
H	3.5[a]	5.3	2.9[a], 3.7
I	4.4, 5.7, 7.7	4.1, 4.5, 5.8, 7.5, 8.4	2.9, 4.1, 6.5
J	5.8	2.9, 5.8, 7.8	2.9, 6.4
L	7.5, 8.5	7.5, 8.5	4.6
O	4.4, 5.7, 7.0, 8.4[a]	5.8, 7.6, 8.4	3.0, 4.5, 6.2
P	2.9[a]	2.8, 3.4	3.4, 6.5[a]
R	5.7	4.5, 5.3, 5.7, 7.3	2.8[a]
S	4.1, 5.6, 7.8, 8.2	4.0, 4.4, 5.9, 7.9, 8.2	2.9, 4.0, 6.0
Courtaulds II	2.7, 4.0, 5.5	2.7, 2.95, 4.0, 5.5	2.7, 3.5
Blue G74	3.0	2.7, 3.1, 5.2	2.6
Red INH6/90	3.9	2.3, 3.2, 3.9	2.3[a]
Green	3.8		3.5
Blue		2.9, 3.2	2.9
Red	3.8	3.1	3.2
Yellow	3.8	4.3[a]	
Blue	3.5, 4.2	3.1, 5.5	3.6
Pale green	3.8	2.5, 3.0	2.9
Pale blue		3.0	3.8, 2.7
Blue	2.5	2.5, 3.2	2.7
Green	3.8, 12.0	3.7, 12.0	2.7, 3.3

Conditions: eluent, methanol–aq. ammonium acetate (9:1); sensitivity, 0.005 AUFS; column, 2.1 mm I.D.; flow-rate, 0.46 ml/min.
[a] Small peak.

No differences were detected in the three wavelengths for any of the samples analysed.

4. Conclusions

It was found that degradation of the dye components could occur in formic acid–water when the extraction was carried out at temperatures greater than 100°C or for longer than 30 min and also if the extracts were not used within 40 min of removal from the oven. No degradation was noticed when the fibre extracts were diluted with the HPLC eluent and left for up to 48 h before injection onto the HPLC column thus facilitating the use of an autoinjector.

The HPLC system described here has been successfully used to separate the commercial basic dyes known to be used by the garment industry for the dyeing of acrylic fibres. The use of the narrow bore HPLC column has enabled the dyes from 2 mm of coloured acrylic fibres and from 5 mm of pale coloured fibres to be detected and the dye components resolved. The relative quantities of the dye components could be compared. In many fibres the small quantities of minor dye components are not visible on TLC plates and also slight variation between dye batches cannot be detected. The HPLC system

Table 7
Retention times of dyes from pale coloured fibres (6 mm)

Fibre	Colour of dye present[a]	Retention time (min)		
		400 nm	500 nm	600 nm
1	Blue, red, yellow	5.6	3.8[b], 5.0	
2	Blue, red, yellow	5.7	3.8[b], 5.0	4.4[b]
3	Purple, blue, yellow		4.4	5.7
4	Blue, red, yellow	5.7	3.9, 5.1	4.3
5	Blue, red, yellow	5.8	3.8[b], 5.1	4.3
6	Blue, turquoise		6.1[b]	4.0, 6.1
7	Turquoise, pink, orange			
8	Purple, blue		4.4	5.4
9	Blue, pink, blue		4.3	4.2
10	Pink, turquoise, blue		3.1	3.1, 4.1

Conditions: eluent, methanol–aq. ammonium acetate (9:1); sensitivity, 0.005 AUFS; column, 2.1 mm I.D.; flow-rate, 0.46 ml/min.
[a] From bulk extracts of fibres.
Small peaks.

described here has been found to be more discriminating than TLC providing greater sensitivity and resolution for the detection of basic dyes from fibre extracts. Picogram levels of detection were recorded for the dyes analysed.

The diode array detectors have not yet been proven to be sufficiently sensitive for detection of the quantities of dyes present in acrylic fibres. The single channel variable-wavelength detector was found to be more sensitive than the diode array detector although injecting the samples at three different wavelengths is time consuming.

The analysis of different sources of black fibres demonstrated the discriminating power of the HPLC system. It was found that some fibres contained the same dye components. These fibres could be from the same manufacturer and potentially the source of individual fibres could be assessed by this system.

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JOURNAL OF CHROMATOGRAPHY A

Review

High-performance liquid chromatography of food colours and its relevance in forensic chemistry

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Abstract

The forensic interest in the use of food colours is related to health rights and safeguard of the consumer, as established in national legislations. The regulations related to food dyes consider the health of human beings and the adulteration of foodstuffs and economic needs connected with quantitative restrictions on imports.

Efficient analytical methods are required for evaluating toxicity and authenticity or adulteration, in order (a) to determine whether there are synthetic dyes present in foods and whether they are permitted, (b) to determine the levels, (c) to confirm the absence of added dyes in foods where they are not declared and (d) to check on the stability of dyes during processing and storage.

Recent HPLC methods for the identification and determination of natural and synthetic dyes and of carcinogenic amines contained as impurities in synthetic dyes are reviewed.

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1. Introduction

1.1 The use of colour in food

What does a green-coloured fruit-jelly taste of? Most people perceive a mint taste, even if the jelly is orange, lemon or strawberry flavoured. Many wine consumers, if blindfolded, cannot distinguish between red and white wine. These and other tests [1] can show how the colour of food is so important as to organoleptically dominate the flavour. The enjoyment of food is so strictly affected by its appearance that an unappetizing colour was proved to adversely affect digestion. People are so used to buying food with a well coloured aspect that, when recently [1] a leading food firm in the UK put on the market canned garden peas and strawberry jams without added colours, sales decreased dramatically and were slowly restored only when colours were again added.

The addition of dyes replaces natural colours destroyed during processing conditions, gives the expected colour to otherwise colourless food or drinks, enhances natural colour and assures uniformity from batch to batch [1,2].

It would be a mistake to believe that colouring food is a choice of our industrial and consumer society. The practice probably predates the written record. Colours naturally present in vegetables, animals and minerals were used. Plinius the Senior (23–79 B.C.) reports that in Rome in the days of Roman Empire (3rd and 2nd centuries B.C.) wines were coloured with fruit berries and white earth was added to bread. In Athens "meat inspectors" overlooked the forbidden addition of a colour [1,2]. In the twelfth century precious rosy and violet sugars were imported into England from Alexandria [1] and an edict of 1396 in Paris banned colour addition to butter. The intensity of the colour and its stability were the only properties of the dye considered and no attention was paid to secondary, often toxic, aspects. The first attempts to draw public opinion against the indiscriminate use of colour in food came in 1820 from Accum and in 1850 from Hassal, who first proposed tests to evaluate dangerous adulterations and denounced a series of widely diffused toxic applications. Brazil woods, husks, elderberry and bilberry were employed in the manufacture of the so-called "genuine" old Port wine. Tea leaves were coloured with verdigris (copper acetate) and Gloucester cheese with red lead and pickles were boiled with a halfpenny to make them green. Confectionery was coloured with lead chromate, cinnabar [mercury(II) sulphide] or prussian blue mixed with lead chromate.

With the advent of dyestuff manufacture (dating from about 1856), new colours were synthesized and many of them were found to be suitable for food. The introduction into food preparations of the new synthetic colours had great success not only because of the generally greater efficiency and stability to light, temperature and redox agents but also because synthetic colours were believed to be safer. In recent times, with the rediscovery of "natural things",

the opposite holds, even though the use of natural colours cannot guarantee the absence of toxic side effects.

When natural and synthetic dyes began to be suspected of toxicity, many actions were undertaken in different countries. Positive lists (containing only the permitted colours) were preferred to forbidden lists (which indirectly permitted the use of all the dyes not included). These lists are continuously updated with the exclusion or inclusion of different dyes. More information about the use of additives and colours in food and in packaging, food chemistry and food safety can be found in specific publications [1–9]. Let us consider some regulations that are useful to know when planning a chemical analysis devoted to the identification and/or determination in food of toxic or adulterating dyes.

1.2. Forensic implications

Food colours are of forensic interest because of their connection with health rights, as established in national legislations [10–12].

Human health protection includes legislation concerning the use of colourants and safeguards for consumers. Every regulation related to food dyes considers, first, the health of human beings and second adulteration of foodstuffs and economic needs, especially connected with quantitative restrictions on imports.

The disparities between national legislations increase the difficulties in achieving a real free market and a greater uniformity within the community. Therefore such problems do not ease the difficulties of the European Economic Community (EEC), one of whose aims is to harmonize practice among the member countries. It is necessary to prepare a positive list that can be accepted by the largest number of countries and that contains the allowed colours and the purity requirements for food, in addition to the permitted divergences.

In the EEC, the colourants used in foodstuffs are regulated by directives, updated in the last few years, stating that: (a) member countries may authorize the movements of the foodstuffs containing only the permitted colourants listed in the directives; (b) the use of the allowed colourants cannot be completely forbidden; (c) if health protection may be at risk by using allowed dyes in the foodstuffs, each member country can withdraw, for a specific period, the authorization to trade the products, informing the other members and the Commission, to update the positive list; (d) the directives specify the transitory period allowed to conform with the national laws with the Community directives; (e) colourants of natural origin, used in the preparation of foodstuffs, must be excluded from the directives concerning the colourants because of their aromatic, tasty and nutritional properties; (f) listed colourants can be marketed only if regularly labelled, indicating the contained food dyes; in other words, consumers must be protected by adequate labelling; (g) imports within the EEC, applying Community regulations, cannot be forbidden only because of inadequate advertizing. The health authority checks the production and marketing of foodstuffs, executing inspections during processing, and confiscation in factories and stores. If necessary, the confiscated foodstuffs can be destroyed by health authority.

The national rules concerning foods and requiring registration in the positive list of food colourants must also be applied to imported products; this is because even if they are lawfully manufactured and marketed in another member country, there may be uncertainties of scientific research in that particular state or there may not be Community harmonization. Applying the first-mentioned regulation to imported products produced and widely consumed in other member states, national authorities, according to EEC Article 36, based on the principle of proportionality, have to authorize the use of colourant additives if justified by a real need or a nutritional need or in the light of eating habits, considering the possible interaction of one colourant with another or their cumulative or global effect. Therefore, the power of a member state to prohibit imports of products from other member States should be restricted to what is necessary to attain the legitimate aim of protecting health, also taking account of international scientific

research, particularly that of the Community committees.

In the same way, national authorities have to evaluate the risk linked to overcoming the acceptable daily intake of additives contained in foodstuffs, paying attention to eating habits. The absence of Community harmonization shows that European legislation has been very careful about the potential harm from these products. Therefore, member states may decide what degree of protection of health and life of humans is justified. But this is not an absolute discretion, because they must consider the Treaty requirements of the free movement of goods. The Community works through a Scientific Committee for Food whose members are either expert technologists, toxicologists or nutritionists. All are drawn from national specialist committees, to maintain effective contact with policies and action in the individual countries. Even if member states have to take into account the scientific research of international and EEC Committees, the Community suggestions are not mandatory, so that they must be incorporated in a regulation for each country under it own food law.

The use of any additive in manufactured products can be justified only if technologically necessary or because of meeting an economic or technical need. Community regulations about organoleptic and psychological aromas and colours allows a national authorization for selling foodstuffs containing dyes, taking care of public health and of the real need for colours in the manufactured products.

1.3. Some regulations

Directive 78725/EEC states that a dye is suitable for food or pharmaceutical use if it contains no more than 4% [10] of accessory colouring matter (isomers, homologues) and not more than 0.5% of intermediate synthetic products other than free aromatic amines. Owing to the admitted presence of these side-products, dyes are not denominated with the chemical term of the principal component, but often with a casual name and some letters (*e.g.*, CI, FD&C and E) and numbers. CI refers to the Color Index [13], which is a reference book that lists, divided into chemical classes, the colourants used in all kind of industries, each classification number describing a compound, its synthesis or natural origin, the official standard (if available) and relevant references. A second number in parentheses indicates the Index edition year in which the colourant is described.

The US Food and Drug Administration (FDA) nomenclature indicates the dyes with the letters F (food), D (drug) and C (cosmetics), followed by the colour of the dye and a number. A FD&C dye can be used for food, drug and cosmetics, whereas, for example, quinoline (D&C Yellow No. 10) is not admitted for colouring food. The EEC system has a list of purity specifications similar to the FDA and the nomenclature uses a number associated with the letter E. At least the WHO/FAO system (World Health Organization/United Nations Food and Agriculture Organization), mainly used in Australia, makes use of standard names.

The two main political areas in the world active in colourant regulations are the USA and Europe. The admitted colour lists are continuously updated, according the "Delaney clause" of Color Additives Amendment of 1960 to the FDA Act, which requires the banning from use in food of all additives that are carcinogenic at any level in animal tests. Because of suspected carcinogenicity in the 1970s, the dye amaranth (E123, FD&C Red No. 2), which was one of the most widely used, was delisted in the USA, being limited to only a few uses in other countries. In the 1980s FD&C Blue No. 2 (indigo carmine; E132) was also delisted, tartrazine (E102) was subjected to rigorous tests and recently (1990) the use of erythrosine (FD&C Red No. 3, E127 was discontinued). In the EEC, colours are regulated by Council Directives on colouring matters authorized for use in foodstuffs. Of relevant interest are the proposals in 1985 and 1992 in Foodstuff Directives for international harmonization of colourant regulations.

Table 1 lists some of the most widely used natural and synthetic dyes, taking into account

Table 1
Common natural and synthetic dyes

Colour	E No.	FD&C	Name	Comments
Yellow	E100		Curcumin	1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione
	E101		Riboflavine	7,8-Dimethyl-10-(D-ribo-2,3,4,5-tetrahydroxypentyl)isoalloxazine
	E102	FD&C Yellow No. 5	Tartrazine	4,5-Dihydro-5-oxo-1-(4-sulphophenyl)-4-[(4-sulphophenyl)azo]-1*H*-pyrazole-3-carboxylic acid trisodium salt
	E103		Crisoine S	2,4-Dihydroxy-azobenzene-4′-sulphonic acid
	E104	D&C Yellow No. 10	Quinoline Yellow	2-(2-Quinolyl)-1,3-indandione sulphanilic acid disodium salt
Orange	E110	FD&C Yellow No. 6	Sunset Yellow	1-(4′-Sulpho-1′-phenylazo)-2-naphthol-6-sulphonic acid disodium salt
Red	E120		Cochineal	Dried female insect *Coccus cacti* L. enclosing the young larvae
	E121		Orcein	Extract from lichens
	E123	FD&C Red No. 2	Amaranth	3-Hydroxy-4-[(4-sulpho-1-naphthalenyl)-azo]-2,7-naphthalenedisulphonic acid trisodium salt
	E124		Ponceau 4R	1-(4′-Sulpho-1′-naphthylazo)-2-naphthol-6,8-disulphonic acid trisodium salt
	E127	FD&C Red No. 3	Erythrosine	3′,6′-Dihydroxy-2′,4′,5′,7′-tetraiodospiro{isobenzofuran-1(3*H*)-[9*H*] xanthen}-3-one disodium salt
Blue	E130		Blue Anthraquinone	N,N′-Dihydro-1,2,1′,2′-anthraquinonazine
	E132	FD&C Blue No. 2	Indigo Carmine	Indigotin-5,5-disulphonic acid disodium salt
	E133	FD&C Blue No. 1	Brilliant Blue FCF	N-Ethyl-N-{4-[(4-{ethyl[(3-sulphophenyl)methyl]amino}phenyl)(2-sulphophenyl)methylene]-2,5-cyclohexadien-1-ylidene}-3-sulphobenzenemethanaminium hyroxide inner salt, disodium salt
Green	E140		Chlorophyll	See text
Brown	E150		Caramel	
Black	E151		Brilliant Black BN	2-4′(4-Sulpho-1-phenylazo)-7′-sulpho-1-naphthylazo-1-hydroxy-8-acetylaminonaphthalene-3,5-disulphonic acid tetrasodium salt
Yellow, orange, red	E160		Carotenoids	See text
	E161		Xanthophylls	See text
	E162		Betalaines	Beetroot
	E163		Anthocyanines	See text

that in each country it is necessary to make reference to their own list. An important parameter, ADI (acceptable daily intake), was first formulated by the Joint WHO/FAO Expert Committee on Foodstuffs and is defined as the daily intake of a chemical which during the

lifetime appears to be without appreciable risk on the basis of all the known facts at the time.

2. Natural and synthetic colours

Food colours are usually divided into natural or nature-identical (synthetically prepared) and synthetic colours.

2.1. Natural colours

In the last few years, colourants of natural origin have been increasingly used, owing to consumer pressure. However, some of them are made synthetically and are termed nature-identical. World legislation [1] does not distinguish between naturally occurring colours and their chemical equivalents produced by a synthetic route, as long as the purity requirements are met. Anyway, when colours are prepared through chemical synthesis, the presence of toxic impurities must be considered, which can be present in the starting reagents or can be formed as by-products during processing or storage. Natural colours are generally characterized by a lower tinctorial strength with respect to the synthetic colours and, in addition, are generally more sensitive to light, temperature, pH and redox agents.

Natural colours are generally divided into the following classes: anthocyanins, carotenoids, betalaines, chlorophylls, and other colours such as caramel, carminic acid (an animal-source colour), oricel (a vegetable-source colour) and GSE (grape skin extract).

2.2. Synthetic colours

A study in the 1970s estimated that the annual average individual consumption of food in USA was 645 kg [7], 5.5 g of which were represented by synthetic colours and about 85% of this amount was represented by tartrazine, Sunset Yellow FCF and amaranth (which was subsequently banned). According an evaluation done in the UK (ref. 1, Ch. 3), the amount of consumed food which contains added colour is *ca.* 0.5 kg per head per day and represents nearly half of the total diet. The average colour content is less than 50 ppm in these foods and the average amount of synthetic colours consumed is less than 10 g per head per year. Yellow colours (tartrazine and Sunset Yellow FCF) and the red colours (carmoisine, Ponceau 4R and amaranth or Allura Red) were predominantly used.

The kind and the amounts of food and beverage with added colour (in amounts up to 120 mg per kg of product) are surprisingly high. Colour is added [1] to blackcurrant, raspberry, strawberry, plum, soft drinks, sugar and jelly confectionery, desserts, table jellies, canned fruits, jams, baked cakes, gravy mix, wafers, sausages, fish, pastes, broad beans, canned vegetables, drink powder, custard powder, breakfast cereals, haddock fillets, salad cream mayonnaise, piccalilli, meat pastes, etc. Different mixtures of two or more colours are also used for obtaining different shades and hues. Dyes can be used as water-soluble pigments or as the so-called lake form, prepared for precipitation of water-soluble colours with aluminium, calcium or magnesium salt on a substrate. The water-insoluble powder so obtained can be mixed to prepare new shades, can be dispersed in various carriers and assures greater homogeneity of colour, for example in frozen products. A new generation of non-toxic dye is the "polymeric dye", in which the colour is chemically bound to a polymer substrate. The resulting molecule, owing to its size, is able to pass through the gastrointestinal tract and to be excreted without being adsorbed or metabolized [1], provided that it is stable enough and it does not release absorbable and potentially toxic fragments.

Synthetic colours can be divided into classes as a function of their chemical structure: azo dyes, triarylmethane and phenylmethane dyes, xanthene dyes, quinoline dyes, anthraquinones, phenols.

2.3. Impurities

Owing to impurities contained in the reagents or due to formation in side-processes during the chemical synthesis of the dyes, aromatic amines (in particular naphthylamine, benzidine, 4-aminodiphenyl) and aromatic polycyclic hydro-

carbons can be present in many dyes and mainly in azo dyes. Carcinogenicity and toxicity must be checked, therefore, not only for dyes but also for impurities contained therein. Also, the toxicity of the colours used in food packaging must be considered, and also the possibility of their release into the contained food.

2.4. Analyses for dyes

As mentioned, many food dyes show possible carcinogenic effects and colours can be used in the adulteration of food. These considerations underline the importance of dye analysis in forensic science. Ever increasing numbers of forensic science laboratories will have to deal with the analysis of food dyes with respect to the adulteration of food.

Efficient analytical methods are therefore required in food quality control for evaluating toxicity and authenticity or adulteration, in order (a) to determine whether there are synthetic dyes present in foods and whether they are permitted, (b) to determine the levels, (c) to confirm the absence of added dyes in foods where they are not declared and (d) to check on the stability of dyes during processing and storage.

Difficulties are often encountered when a complex mixture has to be analysed, also because standard solutions are not easily available. Reviews and books [2,7,15–17] concerning food chemistry report HPLC methods published up to 1980 and the extraction procedures, which are often a very important and essential step in the analysis [1,2,18–20].

Recent HPLC methods for the identification and determination of natural and synthetic dyes and impurities contained in them are reviewed in this paper.

3. Natural colours

3.1. Anthocyanins

Anthocyanins are water-soluble bluish-red pigments that occur naturally in many plants as glycosides of anthocyanidins, which are characterized by a flavylium cation structure. They are naturally present in cranberry, roselle, hibiscus flowers, miracle fruit and grape or are formed as by-products in the wine and grape-juice industry and are receiving increasing attention after the delisting of amaranth. The most common anthocyanidins are pelargonidine, cyanidine, peonidine, delphinidine, petunidine and malvidine and the sugar moiety can be glucose, rhamnose, galactose, xylose or arabinose. Anthocyanin structures being correlated with genetic factors, their distribution has a taxonomic relevance and can be usefully employed in detecting adulteration.

Pigment extracts are commercially available, grape skin extract (GSE) being the most common. In the USA [21], only two pigments are allowed: GSE (grape skin extract) only for beverages and GCE (grape colour extract) for non-beverage foodstuffs, whereas in EEC countries the anthocyanin extracts from edible vegetables are generally allowed. Fruit juice and concentrates can be used without particular restrictions and represent important ingredients in the manufacture of foods and beverages.

3.1.1. HPLC determination

Many HPLC methods have been reported for the determination of anthocyanins and some of them are covered in a recent review [22]. The use of a reversed-phase C_{18} stationary phase and a water–methanol–formic acid mobile phase with linear gradient elution and photodiode-array detection was proposed by Mazza [23] for the separation of anthocyanins and related compounds in Saskatoon berry, which is native to the southern Yukon and Northwest Territories and has recently received interest in industrial cultivation. The extract is particularly complex because it contains, besides anthocyanin pigments, also gallic acid, cinnamic acid, quercetin, naringenin, rutin and caffeic acid. Fractions from HPLC analysis, re-analysed by paper chromatography and spectrophotometric analysis, permitted the identification of malvidine-3,5-diglucoside, malvidine chloride, cyanidine-3-galactoside, pelargonidine chloride, cyanidine-3-glucoside and cyanidine 3-xyloside. HPLC methods were employed by Hong and Wrolstad [21,24] in the identification of anthocyanin pigments (del-

phinidine, cyanidine, petunidine, pelargonidine, peonidine and malvidine) in blackcurrant, blackberry, black raspberry, elderberry, cherry, plum, grape, bilberry and red cabbage. The anthocyanin composition of many fruits being quite distinctive, the study has chemotaxonomic relevance and at the same time anthocyanin analysis is very useful for detecting adulteration and in product development and quality assurance fields. They also studied the colouring properties of the commercially available anthocyanin colourants. The same authors [25] used a polymer-based reversed phase as the stationary phase and water–acetonitrile–orthophosphoric acid as the mobile phase (pH < 2) for the determination of anthocyanins in their red flavylium cation form, while a reversed-phase C_{18} stationary phase and a water–acetonitrile–acetic acid mobile phase with isocratic elution were used for anthocyanidin determination. Spectrophotometric detection at 520 nm was employed. Derivatives of delphinidine, petunidine and malvidine could be distinguished from derivatives of cyanidine, peonidine and pelargonidine and it was shown that cranberry contains only cyanidine and peonidine, roselle contains delphinidine and cyanidine and strawberry contains pelargonidine as the major pigment and a smaller amount of cyanidine. By a combination of droplet counter-current chromatography and semi-preparative HPLC, Andersen [26] isolated antocyanin pigments from fruits of *Vaccinium uliginosum* L. (bog whortleberry). Fifteen pigments were identified, malvidine-3-glucoside being the major component (35.95% of the total pigment content). The pigments were identified as 3-monoglycosides in which the aglycones malvidine, delphinidine, cyanidine, and petunidine are combined with glucose, arabinose and galactose and peonidine with glucose. The method makes use of photodiode-array detection at 515 nm, a reversed-phase C_{18} column and a mobile phase of formic acid–water–methanol in different proportions with linear gradient elution.

In *Tradescantia pallida*, two major complex anthocyanins were identified with an HPLC method by Shi *et al.* [27]: one was cyanidine-3,7,3′-triglucoside with three molecules of ferulic acid and an extra terminal glucose and the other was similar but without the terminal glucose unit. These pigments have good properties as food colours because they are stable and highly coloured at pH > 4. A reversed-phase C_{18} 10-μm and a semi-preparative polymeric 10-μm stationary phase were used, with water–methanol–acetic acid–formic acid as the mobile phase. The increased demand for red raspberry (*Rubus ideaus* L.) and its relatively high cost make raspberry juice a likely target for adulteration. An HPLC method for anthocyanin composition analysis was developed by Spanos *et al.* [28] with the aim of creating a reference database for the authenticity of red raspberry juice. A reversed-phase C_{18} 10-μm stationary phase was used and the mobile phase was water–methanol–acetic acid with linear gradient elution. Cyanidine-3-sophoroside, cyanidine-3-glucoside, cyanidine-3-glucorutinoside, cyanidine-3-rutinoside and pelargonidine-3-sophoroside were identified, cyanidines being highly predominant. A comparison with the anthocyanin composition in other berries (blackcurrant, sour cherry, blackberry, strawberry) confirmed that the anthocyanin pigment composition depends greatly on variety but also on geographical origin, processing and storage. By HPLC Goiffon *et al.* [29] determined anthocyanins contained in bilberry, blackcurrant, strawberry, blackberry, black cherry, morello cherry, redcurrant, raspberry and elderberry. Identification was performed by semi-preparative chromatography, partial hydrolysis and analysis of fragments. Retention was shown to depend on two factors, one specific to the anthocyanidin and the other to the sugar. Glucose, galactose, arabinose, xylose and L-rhamnose were the sugars considered and cyanidine, pelargonidine, paenidine, petunidine and malvidine the aglycone moieties. The chromatographic profiles obtained for the mentioned fruits can be usefully employed in quality control analysis of commercial juices, sorbets, fruit wines, liquors and jams and can usefully assist in detecting adulteration. The chromatographic conditions are reversed-phase C_{18}-bonded silica (7 μm) as the stationary phase, different compo-

sitions of water–acetonitrile–formic acid (pH 1.9) as the mobile phase with isocratic and gradient elution and spectrophotometric detection at 546 nm.

Using HPLC, Williams *et al.* [30] separated twenty anthocyanin glucosides and diglucosides, making use of a reversed-phase C_{18} analytical column and different water–methanol–acetic acid (for the non-acetylated glucosides) or phosphoric acid (for the acetylated glucosides) mobile phases with isocratic and gradient elution with spectrophotometric detection at 520 nm. In particular, 3-glucosides, 3,5-diglucosides, 3-(6-*o*-coumaryl)glucosides and 3-(6-O-*p*-coumaryl-glucoside)-5-glucosides were separated in grape juice. The effects of temperature and mobile phase pH were also studied. Lunte [31] used a combination of spectrophotometric (photodiode-array detector) and amperometric (dual-electrode electrochemical detector, upstream electrode +1.00 V *vs.* Ag/AgCl, downstream electrode, 0.00 V *vs.* Ag/AgCl) detection to develop an HPLC method able to classify anthocyanidins and flavonoids in wine and grape juice in a single chromatographic run and without the need for component isolation. Gradient elutions with a mobile phase containing ammonium phosphate buffer (pH 2.5) and acetonitrile were performed. A reversed-phase C_{18} (5 μm) stationary phase thermostated at 35°C was used.

An HPLC method was employed by Velioglu and Mazza [32] for the separation of anthocyanins and other flavonoids in the petals of *Rosa damascena*, used to produce rose petal jam and attar of roses. The study proposed the characterization of both anthocyanins and colourless flavonoids (which contribute to the intensification and stabilization of the colour) in order to establish the experimental conditions for improving colour stability during the processing and storage of rose jam. About 25 components were detected and most of them identified. The total anthocyanin content was evaluated as 285 mg/kg of fresh petals. An ion-pair HPLC method was proposed by Drdàk *et al.* [33] for the determination of anthocyanins in red wines, with spectrophotometric detection. A silica-based C_{18} column was used with a mobile phase containing water, methanol, perchloric acid and different alkylamines at pH 1.45, the best separation being obtained with butylamine as ion-pairing agent.

3.2. Carotenoids

Carotenoids can be of natural origin or synthesized. More than 500 naturally occurring carotenoids are known, which contribute to the yellow, orange and red colours in fruits, flowers, seeds and feathers. Carotenoid pigments are generally extracted from carrots, annatto, paprika, saffron, palm oil seed, citrus peel and maize. From reactions with proteins, blue, green and purple colours can also be obtained. Carotenoids are generally water insoluble and soluble in fats; food colours are prepared as dispersions in water. The most important carotenoids generally admitted as food colours are α-, β-, γ- and ζ-carotene, bissine, norbissine, capsantine, capsorubine, licopene, phytoene, phytofluene, β-apo-8′-carotenal (E160). β-Carotene is orange, ζ- is yellow and phytoene and phytofluene are almost colourless. Another group of xanthophylls (E161) includes flavoxanthine, luteine, cryptoxanthine, rubixanthine, violaxanthine, rodoxanthine and cantaxanthine. At concentrations between 1 and 10 ppm carotenoids are added to margarine, fruit juices, salad dressing, ice-cream, cheese, pasta, cakes, icings and soups.

In addition to their colouring properties, the importance of carotenoids is also correlated with their content in vitamin A and their properties as antioxidants. As carotenoids are one of the most abundant micronutrients in cancer-preventive food, methods for their identification and separation are particularly required in the development of epidemiological studies in the area of cancer prevention.

3.2.1. HPLC determination

Many HPLC methods have been developed for the determination of carotenoids, owing also to their antioxidant and radical scavenger properties. Problems associated with analysis have been discussed by Scott [34]. The analysis is

complicated because of the large number of naturally occurring carotenoids, the instability to light, heat, oxygen and acids and the difficulty of obtaining authentic reference standards. A review by Tee [35] was recently published concerning the determination of carotenoids in foods, underlining the importance for health and nutrition.

The ever more sophisticated adulteration methods require the development of ever more sophisticated analytical methods. Perfetti *et al.* [36] developed a method for the characterization of orange juice with the aim of detecting its adulteration by means of computer pattern recognition analysis. The experimental data were obtained with the use of a C_{18} stationary phase and acetonitrile–water–methanol–methylene chloride–acetic acid as the mobile phase, with spectrophotometric detection at 280 nm. The study is further complicated by the fact that fruit juice shows large natural variations induced by variety, growing location, season and processing methods. The presence of β-carotene, cryptoxanthine and zeaxanthine was identified. Principal components and *K*-nearest neighbour analysis were usefully employed to distinguish between authentic and adulterated samples for 99 orange juices.

Czinkotai *et al.* [37] studied the composition of paprika pigments (*Capsicum annuum*) whose dry powder and oleoresins are used as food colours. In addition to carotenoids, pigments also contain xanthines. A reversed-phase C_{18} stationary phase was used and different mobile phase compositions with isocratic and gradient elution were compared. The use of acetonitrile-2-propanol–water (39:57:4) permitted the separation of capsorubin, capsanthine, zeaxanthine and their esters and β-carotene, phytoene and phytofluene. The qualitative and quantitative variation in carotenoid composition of two paprika products was also followed during storage. The data of Czinkotai *et al.* were confirmed in a TLC study by Minguez-Mosquerra *et al.* [38].

Tee and Lim [39] developed an HPLC method for the separation of carotenoids (in particular α- and β-carotene and lycopene) and retinol using a C_{18} (10 μm) stationary phase, acetonitrile–methanol–ethyl acetate as the mobile phase and spectrophotometric detection at 313, 340 and 436 nm. The method was applied to the determination of β-carotene in 40 types of foods and was compared with the AOAC method. β-Carotene contents were determined in eggs (the highest level being in duck egg), fish and seafood (high concentrations were found in canned sardines and dried oysters), meat and meat products (the highest amount was in chicken liver), milk and milk products (butter had the highest content), oils (the highest content in margarine) and other processed foods such as canned baked beans and canned sardines, which also contain lycopene.

Taylor and McDowell [40] developed an HPLC method for the separation and classification of carotenoids and chlorophylls in the fresh leaf of tea (*Camellia sinensis* L.). From a Kenyan tea clone 28 pigments were identified and a correlation was proposed between levels of chlorophylls and/or carotenoids and the quality of black tea. The experimental conditions were a reversed-phase C_{18} (5 μm) column, acetonitrile–water–ethyl acetate as the mobile phase and photodiode-array detection at different wavelengths. Using HPLC, twelve carotenoids were identified in palm oil by Ng and Tan [41], the predominant ones being α- and β-carotene. A reversed-phase C_{18} column and acetonitrile–methanol–methylene chloride flowing as the mobile phase with isocratic elution were used, with UV–Vis diode-array detection.

Lesellier *et al.* [42] studied in dried carrots the separation between *trans* and *cis* isomers of α- and β-carotenes. The use of different reversed-phase silica- and polymer-based stationary phases and different mobile phase compositions was compared, and the effect of temperature was studied. It was shown that the industrial food treatment and cooking causes *trans–cis* isomerization in carotenoids, which in turn leads to a decrease in colour and to decreased provitamin A activity. Resolution of *cis*- and *trans*-α- and β-carotene isomers was achieved using a rapid HPLC method by Chandler and Schwartz [43] which employed a column packed with calcium hydroxide and acetone–hexane mixtures as the

mobile phase with spectrophotometric detection at 340 and 436 nm. In agreement with the results obtained by O'Neil and Schwartz [44], the highest percentages of isomers were found in processed foods. In fresh products, *cis* isomers were found in plums, nectarines and peaches and no isomer in sweet potatoes, carrots and tomatoes. In general, the processing of vegetables by cooking, freezing or canning does not usually cause significant decreases in total carotene content but a 15–35% decrease in vitamin A content.

Simon and Wolff [45] separated carotenes from typical and dark orange carrots (containing high concentrations of provitamin A) in raw and frozen samples; α-, β-, γ- and ζ-carotene and lycopene were identified, carotene accounting for 44–79% of the total while α-, β- and ζ-carotenes accounted for 94–97% of the total carotene. The method makes use of a C_{18} ODS-3 column together with a mobile phase of acetonitrile–methylene chloride–methanol. Phytofluene, β-carotene, phytoene, lycopene and, as minor components, α-carotene, lycoxanthine and *cis*-mutatoxanthine were identified by Tan [46] in tomato paste. An HPLC method was employed using a C_{18} (5 μm) stationary phase and different mixtures of acetonitrile–methylene chloride–methanol as the mobile phase with photodiode-array detection.

Razungles *et al.* [47] determined carotenoids in fruits of *Rosa canina* and *Rosa rugosa* and of chokoberry (*Aronia melanocarpa*). Nine carotenoids, three carotenes (lycopene, ζ-carotene and β-carotene) and six xanthophylls (neoxanthine, *trans*-violaxanthine, *cis*-violaxanthine, 5,6-epoxyluteine, luteine and β-cryptoxanthine) were identified. The fruits studied were characterized by large quantitative differences, *Rosa canina* containing the highest levels of β-carotene. The HPLC conditions were a C_{18} reversed-phase (5 μm) stationary phase, water–acetone as the mobile phase with gradient elution and spectrophotometric detection at 450 nm.

Using HPLC with a C_{18} stationary-phase, methanol–water–ethyl acetate as the mobile phase with convex gradient elution and spectrophotometric detection, carotenoids were determined in different fruits by Philip and Chen [48]. Apricot, mango and cantaloupe contain β-carotene whereas cryptoxanthin predominates in papaya, persimmon and tangerine. Peach, red bell pepper, oranges and red grapefruits contain intermediate levels of provitamin A, whilst tomato, yellow bell pepper, pineapple and watermelon are poor sources. Normal-phase (with a mobile phase of hexane–ethyl acetate) and reversed-phase HPLC (with methanol–ethyl acetate as the mobile phase with gradient elution) were used by the same workers [49] for the determination of carotenoid esters in extracts of red bell pepper (*Capsicum annuum*), marigold flower petals (*Taget erecta*), navel orange peel (*Citrus sinensis*) and tangelo peel and also [50] in commercially processed Valencia and navel orange juice concentrates. An accurate measurement of cryptoxanthine was found to be very important in the evaluation of the provitamin A content in citrus juice and in detecting the adulteration of orange juices with mandarin juice.

Red bell peppers are good sources of carotenoids and paprika extracts are commercially used as colouring and flavoring agents for foods. Gregory *et al.* [51], in an HPLC study with methanol–ethyl acetate as the mobile phase with linear gradient elution and a reversed-phase C_{18} stationary phase, showed that in red bell peppers capsanthin accounts for 60% of the total carotenoids, with the simultaneous presence of β-carotene (around 11%) and capsorubin (about 20%). Khachik and Beecher [52] used an HPLC method with methanol–acetonitrile–methylene chloride as the mobile phase and a C_{18} (5 μm) spherical reversed-phase column for the determination of carotenoids in carrot, sweet potato, pumpkin and red palm oil and showed that the effect of cooking and processing leads to a loss of β-carotene of about 19%, of which about 82% is the *trans* isomer, in agreement with other workers [42–44] and with the results of Kimura *et al.* [53], who studied the effect induced by saponification. Chen and co-workers [54,55] identified and determined the major carotenoids contained in water convolvulus (*Ipomea aquatica*), a vege-

table grown in Taiwan and China, by using an HPLC method with a C_{18} (5 μm) stationary phase and a mobile phase of acetonitrile–methanol–ethyl acetate. With a mobile phase of acetonitrile–methanol–chloroform–hexane up to twelve pigments could be separated.

The determination of ten carotenoids (luteine, zeaxanthine, canthaxanthine, β-apo-8′carotenal, β-cryptoxanthine, echinenone, lycopene, γ-carotene, α-carotene, β- and 15-cis-β-carotene) was performed by Olmedilla *et al.* [56] with a C_{18} (5 μm) column and different mobile phases containing acetonitrile, dichloromethane and methanol in different ratios. In tomato extract, luteine, lycopene, γ-carotene and β-carotene were identified. Daood *et al.* [57] identified carotenoids in *Diospyros kaki* fruits, together with sugars and organic acids. A reversed-phase C_{18} column was used, with acetonitrile–2-propanol as the mobile phase and spectrophotometric detection at 438 nm. γ-Carotene and β-carotene were identified and some esters (mutatoxanthine, zeaxanthine, α-cryptoxanthine, β-cryptoxanthine, γ-carotene, β-carotene, α-cryptoxanthine ester, β-cryptoxanthine ester and zeaxanthine esters) were separated. In virgin olive oil seventeen pigments containing chlorophylls and carotenoids were separated by Minguez-Mosquera *et al.* [58]. The pigment content was shown to be very different for the different varieties so that the method allows routine control analysis. A reversed-phase ion-pair chromatographic method was employed, making use of a C_{18} (5 μm) column and a methanol–acetone mobile phase containing tetrabutylammonium and ammonium acetate, with spectrophotometric detection at 410 and 430 nm. Rouseff *et al.* [59] determined β-carotene and other carotenoids (lycopene, ζ-carotene, phytoene and phytofluene) in a red grapefruit cultivar. A C_{18} column was employed, together with an isocratic mobile phase of acetonitrile–methylene chloride–methanol.

The use of a polymeric C_{18} column and tetrahydrofuran as the mobile phase permitted the optimization of an isocratic separation of carotenoids by Craft *et al.* [60], who studied the influence of multiple solvent modifiers and temperature on the separation of a complex carotenoid mixture. The major carotenoid fatty acids were determined by Philip and Chen [61] in persimmon (*Diospyros kaki*) and papaya (*Carica papaya*) by an HPLC method using a C_{18} stationary phase and methanol–ethyl acetate as the mobile phase with linear gradient elution, with spectrophotometric detection at 465 nm. Khachik *et al.* [62] performed the separation, identification and determination of the major carotenoids and carotenal fatty acid esters in extracts of apricot, peaches, cantaloupe and pink grapefruits using a C_{18} reversed-phase column and isocratic and gradient elution.

A procedure was developed by Philip *et al.* [63] for the detection of adulteration of California orange juice concentrates with externally added carotenoids. The method involves the treatment of the extracted carotenoids with methanolic HCl to convert carotenoids with 5,6-epoxide end-groups into 5,8-epoxides and HPLC measurement with a reversed-phase C_{18} stationary phase, methanol–ethyl acetate as the mobile phase with convex gradient elution and detection at 465 nm. Adulteration can be evidenced by the use of characteristic ratios that can be calculated between the concentrations of the esters of cryptoxanthine and luteine in orange concentrates. Nagy *et al.* [64] developed an HPLC method with a C_{18} stationary phase and water–acetonitrile–tetrahydrofuran as the mobile phase for the separation of browning pigments forming in white grapefruit juice when stored in glass and cans under non-refrigerated conditions. Up to 100 pigments that absorb in the range 382–400 nm are formed.

An HPLC method with a C_{18} column and acetonitrile–methanol–ethyl acetate as the mobile phase permitted Daood *et al.* [65] to identify carotenoids and chlorophylls in vegetables and to show that cooking vegetables with brine or acidic solutions leads to the formation of phephytins from chlorophylls. β-Carotene was determined in green peas, green pepper, cucumber, lettuce, spinach and celery. It is worth emphasizing the high content of β-carotene in celery leaves and the consequent high nutritive value of this vegetable.

3.3. Betalaines

Betalaines are pigments generally contained in beetroot and include two classes of pigments: betacyanines and betaxanthines. The chromophore group is the 1,7-diazaheptamethine. Betacyanines are red and betaxanthines are yellow. The most common is betanine, which accounts [2] for 75–95% of the betacyanine content of beetroot (*Beta vulgaris*) and whose extract is a natural colour listed in the EEC list as E162. The principal betaxanthines are vulgaxanthin I and vulgaxanthin II. Owing to their low stability, betalaines are generally employed to colour food having a relatively short life and not requiring thermal treatment, such as yoghurt, confectionery, ice-cream, jellies, dessert, soups, meat and meat-based products.

A betanine pigment was also extracted by Forni *et al.* [66,67] from the berries of the American pokeberry (*Phytolacca decandra*). This red pigment, known as phytolaccanine, is a betacyanine identical with the betanine of beetroot and can be used as food colour, after its extraction and purification from toxic saponins.

3.3.1. HPLC determination

Pourrat *et al.* [68] developed an HPLC method for the determination of betacyanine and betaxanthine pigments in fermented red beetroot extracts. A reversed-phase C_{18} (10 μm) stationary phase was used and the mobile phase was methanol–water containing phosphate buffer with gradient elution. Five betacyanines (betanine, isobetanine, betanidine, isobetanidine and prebetanidine) were identified at 538 nm and confirmed by enzymatic hydrolysis with β-glucosidase of a solution of *n*-betanine and isobetanine. Small amounts of betaxanthines (identified at 477 nm) and the probable presence of vulaxanthines were also evidenced. Forni *et al.* [69] obtained the separation of betalaines pigment in blood-red *Opuntia ficus indica* by an HPLC method with a reversed-phase RP-18 column and water-phosphate buffer (pH 5) containing different amounts of methanol as the mobile phase, with spectrophotometric detection. A yellow pigment (475 nm) was identified as indicaxanthine and red-violet pigments (538 nm) were identified as betanine, isobetanine and a betalainic glucoside.

3.4. Porphyrin pigments (chlorophylls)

Porphyrins are macrocyclic compounds containing four methine-linked pyrrole rings. Chlorophylls are the most important examples. Chlorophylls (E140) and their copper complexes (E141) are generally admitted green pigments. Copper derivatives are more stable than the natural magnesium derivatives, but free ionizable copper must not exceed 200 ppm. Plant extracts normally contain a mixture of four different chlorophylls.

3.4.1. HPLC determination

A separation method for chlorophylls and pheophytins in fresh and frozen peas was developed by Forni *et al.* [70] with the aim of following the colour changes that take place during storage under frozen conditions. The HPLC method employs an end-capped RP-18 (5 μm) column and acetone–ethanol–water as the mobile phase, with fluorimetric detection (excitation at 413 nm, emission at 669 nm). During storage of peas, the colour can loose its bright greenness, owing to the conversion of chlorophylls into grey-brown pheophytins and the discolouration of frozen peas was therefore followed through chlorophyll composition changes. Garrido and Zapata [71] studied the chlorophyll pigments in algae by HPLC methods with C_{18} reversed-phase silica- and and polymer-based stationary phases. The mobile phases were methanol–ammonium acetate–acetone–acetonitrile mixtures with gradient elution. Minguez-Mosquera *et al.* [72] studied the degradation of chlorophylls in olives during fermentation and conservation processes and their transformation into pheophytins and pheophorbides. Eighteen different pigments, including chlorophylls, carotenoids and degradation products, were separated by reversed-phase ion-pair chromatography with a C_{18} (5 μm) reversed-phase column and a mobile phase of water–methanol–acetone–tetrabutylammoniumm acetate–ammonium acetate.

Photosynthetic pigments from chromophyte marine algae were analysed by Garrido and Zapata [73] by an HPLC method using different reversed-phase C_{18} columns and a mobile phase of methanol–acetonitrile–ammonium acetate–ethyl acetate in different ratios with linear gradient elution. In leaves of spinach (*Spinacia olearia*), Canjura and Schwartz [74] separated and identified chlorophylls, α- and β- chlorophyllides and degradation products by means of photodiode-array detection and the help of mass spectrometry.

3.5. Other natural pigments

Studies of natural non-toxic dyes led to the proposal of extraction of pigments from animal and vegetable sources, *e.g.*, cochineal or carminic acid (E120) extracted from *Coccus cacti*, oricel or orcein (E121) extracted from *Lichen roccelle* or curcumin or annatto extracted from a tree.

3.5.1. Lac colour

Lac colour is a natural colour that derives from a secretion of the insect *Coccus laccae* (*Laccifer lacca* J. Kerr) from India and Southeast Asia, which parasitizes especially on legumes. The colour constituents are laccaic acids (mono- and dicarboxylic acids) and are characterized by an anthraquinone moiety. The colour is water-soluble and is used in Japan to colour tomato ketchup, strawberry jam, candy and beverages. The colour was extracted, treated with diazomethane to produce two reddish-orange markers and then analysed by an HPLC method developed by Yamada *et al.* [75] using a reversed-phase C_{18} (5 μm) column and acetonitrile–water as the mobile phase with detection at 495 nm. The method was employed for the determination of lac colour in colour preparations and in jellies. Concentrations of *ca.* 1.92 μg/g of laccaic D1 acid and 8.53 μg/g of laccaic D2 acid were shown to be present in a jelly, in disagreement with the label declaring no added colour.

3.5.2. Annatto and turmeric

Rouseff [76] developed an HPLC method for determining annatto and turmeric pigments in food using a reversed-phase C_{18} column and water–tetrahydrofuran as the mobile phase with isocratic elution, with spectrophotometric and fluorescence detection. Annatto consists of pigments from the outer seed coat from the tree *Bixa orella*, while turmeric is an extract from the rhizomes of *Curcuma turmeric*. Both extracts are used in the food industry separately or in combination to impart yellow-orange-red hues to a wide variety of products. The major components were shown to be curcumin, dimethoxycurcumin and bismethoxycurcumin.

4. Synthetic colours

4.1. Azo dyes

The chromophoric system is an azo group in association with one or more aromatic systems. Some common example are Allura Red AC, amaranth, Ponceau 4R, Red 2G, Fast Yellow AB, Sunset Yellow 2G and Chocolate Brown HT. The colours can be red, orange, yellow, blue, violet, black and brown. Another azo class of dyes is the azopyrazolines such as tartrazine and Yellow 2G.

4.1.1. HPLC determination

Ion-pair chromatography was employed by Lawrence *et al.* [77] for the separation of twelve primary food colours and the analysis of grape beverages. The use of different mobile phases formed by methanol and water in different ratios and containing tetra-*n*-butylammonium phosphate was compared. Spectrophotometric detection at different wavelengths made possible the separation of Ponceau SX, Fast Red, Benzyl Violet 4B, erythrosine, Skyark (a subsidiary of Sunset Yellow FCF), indigotine, Fast Green FCF, Brilliant Blue FCF, amaranth, tartrazine, Allura Red AC and Benzyl Violet 4B. The method is interference free from natural colours contained in commercial grape soda drinks.

Puttemans *et al.* [78] developed an ion-pair chromatographic method with a C_{18} (10 μm) stationary phase and an aqueous methanol mobile phase with tri-*n*-octylamine added as an ion-pairing agent for the determination of syn-

thetic dyes in soft drinks in the presence of organic acids and saccharin. They analysed 45 commercial samples of soft drinks and 11 lemonade syrups for tartrazine, amaranth, Sunset Yellow and Cochineal Red. Of the fourteen samples of orange soft drink analysed, only two contained synthetic colours (a mixture of E102 and E110) and of the fourteen lemonades one sample contained the same mixture, the six cola samples contained no additive and of twelve soft drinks only a grenadine was coloured (with Cochineal Red).

An isotachophoretic method was proposed by Karovicova *et al.* [79] for the determination of the synthetic colours tartrazine, Sunset Yellow FCF, Cochineal Red, amaranth, azorubine, Patent Blue Brilliant and Black Indigotine. The method was applied to powdered beverages, powdered pudding, hard candies and beverages. A method for the determination of synthetic dyes in sugar-rich foods, such as boiled sweets, fruit gums, lemon curd, jelly, blancmange and soft drinks, was developed by Greenway *et al.* [80] and makes use of HPLC and on-line dialysis for sample preparation. The method was called ASTED (automated sample treatment through enrichment of dialysates). The mobile phase was water–methanol containing ammonium acetate buffer and the column was ODS-2 Spherisorb (5 μm). Spectrophotometric detection was performed at 475 nm. Detection limits obtained for amaranth, Brown FK, Ponceau 4R and Sunset Yellow were 0.64, 1.58, 0.50 and 0.50 μg/ml, respectively. The method was applied to the analysis of blancmange banana and vanilla dessert, lemon curd, yellow and red boiled sweets, jelly, fruit gums and drinks. Using an HPLC method with a spherical C_{18} stationary phase and a water–methanol–phosphate buffer (pH 3.6) mobile phase with gradient elution and spectrophotometric detection at 214 nm, Sunset Yellow was determined in the presence of saccharin, tartrazine, aspartame and benzoic acid [81] in fruit drinks, lemonade, vodka mixer, bitter lemon, strawberry drink, diet tonic, orange and passion fruit drink. Quantification was performed by a multi-level calibration method.

The colour additive Citrus Red No. 2 is principally formed by 1(2,5-dimethoxyphenylazo)-2-naphthol and it is used to improve the colour of the skin of oranges. Its use is permitted up to 2 ppm (based on the mass of whole orange) in fruit that is not to be processed, whereas it is forbidden for orange peel intended for consumption (for flavouring herbal teas and for candies). An HPLC method with detection limits of 28 ppb and recoveries of >93% for dried orange peel and >87% for fresh oranges was developed by Hope and Connors [82]. A C_{18} (5 μm) column was used and the mobile phase was acetonitrile–water mixture, with spectrophotometric detection at 504 nm. No dye was found to be present in all the dried orange peels tested or in the tea blends, whereas among the fourteen fresh oranges randomly purchased in various supermarkets four contained Citrus Red above the limit.

4.2. Xanthene dyes

The chromophore group is xanthene (dibenzo-1,4-pyran with amino hydroxyl groups in the *meta* position), which gives brilliant red or fluorescent yellow colours.

4.2.1. HPLC determination

The determination of erythrosine and subsidiary dye intermediates and side-reaction products (such as fluoresceine and 2′,4′,5′- and 2′,4′,7′-triiodofluoresceine) was performed by Lancaster and Lawrence [83] using an ion-pair chromatographic method. The mobile phase was methanol–water in different ratios and containing tetrabutylammonium phosphate and the stationary phase was C_{18}, with spectrophotometric detection at 249 and 500 nm. The commercial samples of erythrosine analysed were found to contain no detectable amounts of resorcinol and allowable amounts of subsidiary dyes and intermediate or side-reaction products. Thermal decomposition of erythrosine and fluoresceine in sugar solutions and in candies was also investigated. An HPLC method was also developed by Van Liederkerke and De Leenheer [84] for the determination of xanthene dyes and derivatives, namely fluoresceine, 4′,5′-dibromofluoresceine, eosine Y, ethyleosine, 2′,7′-dichlorofluoresceine,

tetrachlorofluoresceine, 4′,5′-diiodofluoresceine, erythrosine B and phloxine B. A polystyrene–divinylbenzene (10 μm) column was used and the mobile phase was water–acetonitrile mixtures containing tetramethylammonium hydroxide and phosphoric acid, with diode-array spectrophotometric detection. In analyses of the purity of different commercial dyes it was found that in many instances the label indications and the actual amounts of dyes present did not correspond. The method can be extended to the preparative scale for the purification of impure commercial dyes.

4.3. HPLC determination of antraquinone dyes

By means of centrifugal partition chromatography, a separation method was developed by Hermans–Lokkerbol *et al.* [85] for the separation of anthraquinones in an extract of *Rubia tinctorum* hairy root culture. Alizarin, alizarin 1-methyl ether, lucidine and nordamncanthal were separated. Okamura *et al.* [86] developed an HPLC method with reversed-phase C_{18} column and water–acetonitrile as the mobile phase with gradient elution for the determination of altersolanol pigments. These are rare but not unique examples of naturally occurring tetrahydroanthraquinones, which are produced by the fungus *Alternaria solani*, a pathogen of early blight disease of tomato and potato.

4.4. HPLC determination of phenol dyes

It was shown by Bailey *et al.* [87] that in black tea two main classes of phenolic pigments are formed during the manufacturing processes, *i.e.*, theaflavins and thearubigins. Whereas theaflavins are compounds of known structure, thearubigins are represented by a non-homogeneous group of phenolic pigments of unknown structure, with different chemical functionalities, probably formed by oxidative degradation of theaflavins. Eight theaflavins could be separated by means of an HPLC method with the comparative use of reversed-phase C_{18} silica-based columns of different particle size and of a styrene–divinylbenzene copolymer. The mobile phase was water–acetonitrile with addition of acetic and citric acid or EDTA disodium salt.

4.5. HPLC determination of toxic impurities contained in dyes

An HPLC method was developed by Richfield-Fratz *et al.* [88] for the determination of ppb levels of aniline, benzidine, 4-aminobiphenyl (4-ABP) and 4-aminoazobenzene (4-AAB) in the colour additive FD&C Yellow No. 6, a synthetic water-soluble, monoazo colour additive, principally composed of the disodium salt of 6-hydroxy-5-[(4-sulphophenyl)azo]-2-naphthalensulphonic acid. The dye is synthesized by coupling diazotized sulphanilic acid with Schaeffer salt and could be contaminated by impurities from the reagents or the intermediates. The mobile phase was ammonium acetate–acetonitrile–water and a reversed-phase C_{18} column was used, with diode-array detection. Aniline was found in all the 34 certified samples of FD&C Yellow No. 6 investigated and produced by different manufacturers, at average levels of 97.6 ppb, the highest concentration being 419 ppb. 4-ABP and 4-AAB were found in 30 and 23 samples, respectively, with maximum levels of 23 and 1098 ppb, while benzidine was not identified in any of the samples. The same authors [89] investigated the presence of 4-nitro-*p*-cresidine and *p*-cresidine (responsible for carcinogenic effects) in FD&C Red No. 40, performing separation and identification by HPLC and GC–MS techniques. In all the 28 certified samples of colour surveyed, both 4 nitro-*p*-cresidine (at average level 1035 ppb, concentration range 165–7526 ppb) and *p*-cresidine (average level 105 ppb, concentration range 4–920 ppb) were found. Aniline was found in thirteen samples (average level 26 ppb, concentration range 3–383 ppb).

An RP-HPLC method with a C_{18} column, ammonium acetate–acetonitrile as mobile phase with gradient elution and spectrophotometric detection was developed by Bailey [90] for the determination of unsulphonated aromatic amines (aniline, benzidine, 2-aminobiphenyl, 4-aminobiphenyl and 4-aminoazobenzene) in the reg-

ulated colour D&C Red No. 33. An RP-HPLC method developed by the same author [91] with ammonia–tetrahydrofuran as the mobile phase permitted the determination of the lower subsidiary colours 5-(phenylazo)-6-hydroxynaphthalene-2-sulphonic acid (ANSC) and 4-[(2-hydroxynaphthalene-1-yl)azo]benezenesulphonic acid (BNSC) in FD&C Yellow No. 6. The analysis of 31 commercial dyes from different manufacturers showed that the levels of BNSC ranged from 0.002 to 0.728% and those of ANSC from 0.002 to 0.521%.

Lancaster and Lawrence [92] developed an ion-pair HPLC method for the identification of impurities in some food colours that can be present as a result of incomplete reaction during commercial colour synthesis. In particular, the presence of the intermediates naphthionic acid and R salt (2-naphthol-3,6-disulphonic acid, sodium salt) were investigated in amaranth, of sulphanilic acid and Schaeffer salt (2-naphthol-6-sulphonic acid, sodium salt) in Sunset Yellow and of sulphanilic acid and 1-(4-sulphophenyl-3-carboxy-5-hydroxypyrazolone) in tartrazine. The stationary phase was C_{18} (10 μm) and the mobile phase was a methanol–water containing tetra-*n*-butylammonium phosphate, with spectrophotometric detection. The method was also employed by the same group [93], with spectrophotometric detection at 522 nm, in the determination of non-sulphonated aromatic amines (mainly 1- and 2-naphthylamine), which are known to be carcinogens. Most are diazotized and coupled during the colour manufacturing process but low levels of unreacted free amines can still be found in most food colours. After treatment with dithionite, dyes were extracted with chloroform and H_2SO_4, diazotized and coupled. Recoveries always greater than 80% were obtained, with detection levels of 8 ng/g. Analyses of nine commercial samples of amaranth from seven manufacturers and three countries gave levels up to 435 μg/g of total 1-naphthylamine and up to 214 μg/g of total 2-naphthylamine, most being bound to the coupling salt and less than 5% being in the free state in the dye.

The presence of various non-sulphonated aromatic amines was also studied by the same workers [94] in other synthetic food colours such as tartrazine, Sunset Yellow and Allura Red, which are manufactured from reagents that contain aromatic amines as impurities. By using ion-pair HPLC recoveries always greater than 85% and detection limits always lower than 32 ng/g were obtained. The analysis of seven commercial samples of tartrazine indicated that benzidine was present in three of them at concentrations up to 326 ng/g, and all the samples contained total aniline in concentrations up to 83.2 μg/g. In some samples 1-naphthylamine, 2-aminobiphenyl and 4-aminobiphenyl were also present, at concentrations lower than 0.1 μg/g. The analysis of nine samples of commercial Sunset Yellow FCF showed high aniline contents for all the samples (maximum concentration 519 μg/g), and one sample also contained 1-naphthylamine, 2-aminobiphenyl and 4-aminobiphenyl. All eight samples of Allura Red investigated contained aniline (maximum concentration 98.7 μg/g) and *p*-cresidine (maximum concentration 35.4 μg/g) and six samples contained 1-naphthylamine (maximum concentration 4.76 μg/g). Most of the aromatic amines are bound to the coupling agent and less than 7% remains as free amine in the dye. Lancaster and Lawrence [95] then applied the results of these studies to the determination of non-sulphonated aromatic amines in finished food products, such as soft drinks and hard candies. The effects of heat and of the presence of sugar and citric acid were studied. The recovery was not affected and the detection limits for aniline, 1- and 2-naphthylamine and 2- and 4-aminobiphenyl were always lower than 0.3 ng/ml. The analysis of commercial samples of soft drinks showed the presence of aniline (up to 12.6 ng/ml) in an orange beverage, of 1-naphthylamine (up to 8.25 ng/ml) and 2-naphthylamine (up to 1.12 ng/ml) in a grape product. It is noteworthy that notwithstanding, on the basis of the manufacturing process, the presence of naphthylamine could be expected in drinks containing amaranth and of aniline in beverages containing tartrazine and Sunset Yellow FCT, aniline was found in a grape drink coloured with amaranth and Brilliant Blue. For hard candies, aniline was unexpectedly de-

tected in a product containing amaranth (concentration 9.2 ng/g) together with 1-naphthylamine (10.6 ng/g).

5. References

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JOURNAL OF CHROMATOGRAPHY A

Identification of unlawful food dyes by thin-layer chromatography–fast atom bombardment mass spectrometry

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Abstract

A thin-layer chromatographic–fast atom bombardment mass spectrometric (TLC–FAB-MS) method incorporating an analyte condensation technique was established for the identification of the 27 food dyes consisting the twelve dyes permitted for use in foods and the fifteen unlawful dyes in Japan. The use of magic bullet [1,4-dithiothreitol–1,4-dithioerythritol (3:1)] as a matrix allowed the measurement of the FAB mass spectra of the food dyes except for Food Blue No. 2 (Indigo Carmine). The separation was performed on a C_{18}-modified silica gel TLC plate using the following two solvent systems: methanol–acetonitrile–5% aqueous sodium sulphate solution (3:3:10) and methyl ethyl ketone–methanol–5% aqueous sodium sulphate solution (1:1:1). The condensation technique for concentration of a diffuse sample spot on the TLC plate improved the detection limit 4–20-fold with good reproducibility. The method was successfully applied to the identification of unlawful dyes in imported foods.

1. Introduction

Over 50 synthetic dyes are used in foods all over the world [1]. In Japan, the following twelve dyes are permitted for use in foods; Amaranth [Color Index (C.I.) No. 16185, R-2), Erythrosin (C.I. No. 45430, R-3), Allura Red AC (C.I. No. 16035, R-40), New Coccine (C.I. No. 16255, R-102), Phloxine (C.I. No. 45410, R-104), Rose Bengal (C.I. No. 45440, R-105), Acid Red (C.I. No. 45100, R-106), Tartrazine (C.I. No. 19140, Y-4), Sunset Yellow FCF (C.I. No. 14982, Y-5), Fast Green FCF (C.I. No. 42053, G-3), Brilliant Blue FCF (C.I. No. 42090, B-1) and Indigo Carmine (C.I. No. 73015, B-2). However, unlawful dyes are also frequently detected in foods [2], so the inspection of dyes in foods has been continued by public health agencies.

The analyses of foods for dyes have been mainly achieved by thin-layer chromatography (TLC), because it is a simple and effective technique for the separation of components in a mixture. However, the only useful information obtained from a TLC plate to identify components is the R_F values, and the identification of the separated components is difficult unless an appropriate spectrometric method such as mass spectrometry (MS) is used. A stepwise operation including separation by TLC and measurement of the individual mass spectra is laborious and time consuming, because it needs extra steps such as extraction of the desired compounds from the TLC plate and elimination of adsorbents. Recently, the direct analysis of TLC spots in a mass spectrometer has been reported, and

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TLC–fast atom bombardment (FAB) and TLC–liquid secondary ion (LSI) MS have been successfully applied to the identification of drugs, their metabolites and dyes [3–9].

In the previous study [10], we successfully applied TLC–FAB-MS with a condensation technique to the identification of the twelve permitted food dyes in Japan. Although this method is highly effective, an evaluation of its applicability to unlawful dyes has not been achieved. As unlawful dyes are frequently detected in foods as mentioned above, we wished to establish an identification method for unlawful dyes using TLC–FAB-MS. In this paper, we report an identification technique for the 27 dyes constituting the above twelve permitted and the following fifteen unlawful dyes using TLC–FAB-MS: Ponceau 3R (C.I. No. 16155, R-1), Ponceau SX (C.I. No. 14700, R-4), Oil Red (C.I. No. 12140, R-5), Ponceau R (C.I. No. 16150, R-101), Eosine (C.I. No. 45380, R-103), Azo Rubine (C.I. No. 14720, R-AZ), Orange I (C.I. No. 14600, O-1), Orange RN (C.I. No. 15970, O-RN), Oil Orange SS (C.I. No. 12100, O-SS), Naphthol Yellow S (C.I. No. 10316, Y-1), Yellow AB (C.I. No. 11380, Y-2), Yellow OB (C.I. No. 11390, Y-3), Guinea Green B (C.I. No. 42085, G-1), Wool Green S (C.I. No. 44090, G-S) and Acid Violet 6B (C.I. No. 42640, V-1). Finally, the established technique was applied to the identification of unlawful dyes in important foods.

2. Experimental

2.1. Chemicals

Magic bullet [a mixture of 1,4-dithiothreitol and 1,4-dithioerythritol (3:1)], glycerol, thioglycerol, diethanolamine, triethanolamine, *o*-nitrophenyl octyl ether, *m*-nitrobenzyl alcohol, R-1, R-4, R-5, R-101, R-103, R-AZ, O-1, O-RN, O-SS, Y-1, Y-2, Y-3, and G-1 were purchased from Tokyo Kasei (Tokyo, Japan), R-2, R-3, R-40, R-102, R-104, R-105, R-106, Y-4, Y-5, G-3, B-1, B-2 and V-1 from San-Ei Gen FFI (Osaka, Japan) and G-S from Aldrich (Milwaukee, WI, USA).

2.2. Thin-layer chromatography

TLC was performed on precoated glass-backed C_{18}-modified silica gel TLC plates (Merck, 15423). The following solvent systems were used: methanol–acetonitrile–5% aqueous sodium sulphate solution (3:3:10) (solvent system A) and methanol–methyl ethyl ketone–5% aqueous sodium sulphate solution (1:1:1) (solvent system B).

2.3. Extraction of unlawful dyes from foods

An amount of 20 g of the sample was dissolved in 50 ml of water and the solution was acidified by the addition of 5 ml of concentrated acetic acid. A piece of pure wool that had been throughly washed in boiling 10% ammonia solution and boiling water was added to the sample solution. The coloured wool was removed, rinsed thoroughly with water and then heated slightly in 3% ammonia solution to remove colour. The solution was evaporated to dryness and the residue was dissolved in methanol.

2.4. Sample condensation technique on TLC plate

The following sample condensation technique was used to concentrate an analyte on the TLC plate before measurement of its FAB mass spectrum (Fig. 1): (1) an area including the desired spot on the developed TLC plate was cut rectangularly; (2) a small volume of methanol was applied around the sample spot on the developed TLC plate; (3) after several tens of seconds, the sample was condensed towards the centre of the spot in a line with penetration of methanol.

2.5. Mass spectrometry

After application of the condensation technique, the TLC plate was placed on the TLC holder, a matrix was applied on the sample spot

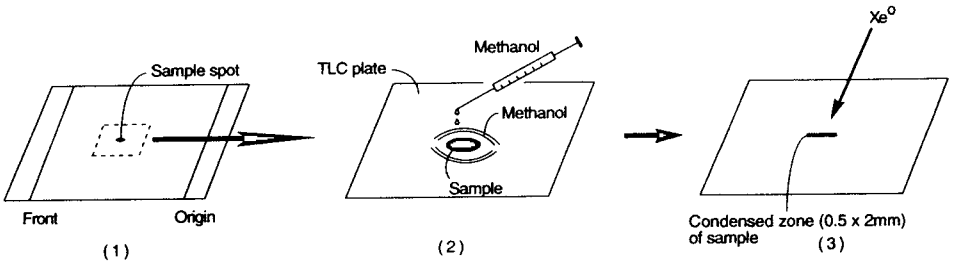

Fig. 1. Procedure for the sample condensation technique.

and the TLC–FAB mass spectrum was measured using a JMS-AX505W mass spectrometer (JEOL, Tokyo, Japan) with a TLC–FAB ion source. The FAB gun was operated with xenon gas at 5 kV using an acceleration voltage of 3 kV for measurement of all spectra.

3. Results and discussion

To apply TLC–FAB-MS successfully to the identification of food dyes, aspects that must be carefully investigated include the selection of a suitable matrix, TLC conditions and sample condensation on the TLC plate. These are discussed below.

3.1. Selection of suitable matrix

Organic dyes possess conjugated chromophores including azo, immonium and quinone groups that are generally fragile and easily reducible. It is well known that these dyes are readily reduced under FAB-MS conditions [11–13]. In our basic studies on FAB and LSIMS of the twelve permitted dyes [7], we observed these phenomena and found that only the use of magic bullet as a matrix depresses the reduction of the dyes and gives correctly molecular ion species. In this study, magic bullet was examined for the TLC–FAB-MS of the fifteen unlawful dyes using a conventional procedure. It depressed the reduction to give the molecular ion species of the dyes with the theoretical isotopic abundances. Therefore, we selected magic bullet as a matrix.

3.2. TLC conditions

For the separation of food dyes by TLC, silica gel, polyamide and C_{18}-modified silica gel have been used as stationary phases [1,14]. TLC–FAB-MS on a normal-phase plate usually provides a higher sensitivity than is observed with a reversed-phase plate [8]. On the other hand, a reversed-phase plate gives better results than normal-phase plates such as silica gel and polyamide plates for the separation of the dyes. Our previously reported C_{18}-modified silica gel TLC method with two solvent systems can separate the twelve permitted dyes completely with no tailing spots and good reproducibility [14,15]. Further, the separation is not affected by coexisting substances from foods and the spots of dyes extracted from foods always give the same R_F values as the authentic standards [15]. Therefore, we applied C_{18}-modified silica gel TLC in this study. The chromatograms gave roundish spots of the dyes with no tailing and good reproducibility. Table 1 gives the R_F values for the 27 food dyes studied. Only five and eight isolated spots of the dyes could be observed using solvent systems A and B, respectively. However, the combined use of both solvent systems enabled us to distinguish nineteen dyes. Although unsatisfactory results were obtained for distinguishing dyes between R-3 and R-103, between R-5 and O-SS and among R-1, R-101, R-AZ and G-S, it is not always necessary to differentiate between overlapping dyes, because these dyes have different molecular masses and they can be identified by the measurement of FAB mass spectra after TLC separation. Therefore, the TLC conditions for TLC–FAB-MS of

Table 1
R_F values of dyes on reversed-phase TLC plates

Dye	R_F	
	Solvent system A[a]	Solvent system B[b]
Ponceau 3R (R-1)	0.16	0.86
Amaranth (R-2)[c]	0.71	1.00
Erythrosine (R-3)[c]	0.00	0.40
Ponceau SX (R-4)	0.21	0.89
Oil Red (R-5)	0.00	0.06
Allura Red AC (R-40)[c]	0.34	1.00
Ponceau R (R-101)	0.22	0.89
New Coccine (R-102)[c]	0.58	1.00
Eosine (R-103)	0.00	0.42
Phloxine (R-104)[c]	0.00	0.15
Rose Bengal (R-105)[c]	0.00	0.19
Acid Red (R-106)[c]	0.04	0.77
Azo Rubine (R-AZ)	0.19	0.88
Orange I (O-1)	0.15	0.75
Orange RN (O-RN)	0.05	0.61
Oil Orange SS (O-SS)	0.00	0.09
Naphthol Yellow S (Y-1)	0.42	0.90
Yellow AB (Y-2)	0.00	0.16
Yellow OB (Y-3)	0.00	0.13
Tartrazine (Y-4)[c]	0.79	1.00
Sunset Yellow FCF (Y-5)[c]	0.45	1.00
Guinea Green B (G-1)	0.03	0.73
Fast Green FCF (G-3)[c]	0.17	1.00
Wool Green S (G-S)	0.22	0.90
Brilliant Blue FCF (B-1)[c]	0.14	1.00
Indigo Carmine (B-2)[c]	0.66	1.00
Acid Violet 6B (V-1)	0.01	0.67

[a] Solvent system A = methanol–acetonitrile–5% sodium sulphate solution (3:3:10).
[b] Solvent system B = methanol–methyl ethyl ketone–5% sodium sulphate solution (1:1:1).
[c] Permitted dyes for use in foods in Japan.

the food dyes were standardized to the use of a C_{18}-modified silica gel plate with two solvent systems as described under Experimental.

3.3. Condensation of spots of dyes on TLC plates

When a matrix is deposited on a sample spot on a TLC plate, diffusion of the sample usually occurs with spreading of the matrix used, so that no satisfactory spectrum is obtained with good sensitivity unless a large amount of sample is applied to the TLC plate. In previous work, to prevent diffusion of the analyte and to obtain higher sensitivity in the TLC–FAB mass spectrum, we developed a sample condensation technique [4,7,8]. Although the diffusion of a sample on a reversed-phase plate is not as great as is observed using normal-phase plates when a matrix is deposited on the spot, no satisfactory spectra were obtained unless more than 20 μg per spot of the dyes was applied to the TLC plate without the condensation technique. To obtain high sensitivity, the developed spot was reconcentrated on the TLC plate using the concentration technique described under Experimental.

Table 2 shows the molecular ion species and the detection limits of the dyes using TLC–FAB-MS. The $[M + H]^+$ ion appeared clearly for all

Table 2
Molecular ion species in the TLC–FAB mass spectra of the dyes and their detection limits

Dye	Molecular ion species[a]				Detection limit (μg per spot)
	$[M+Na]^+$	$[M+H]^+$	$[M-Na+2H]^+$	$[M-2Na+3H]^+$	
Ponceau 3 R (R-1)	517 (38)	495 (100)	473 (31)	ND[c]	5
Amaranth (R-2)[b]	627 (33)	605 (100)	583 (85)	ND	5
Erythrosine (R-3)[b]	903 (19)	881 (100)	859 (73)	837 (35)	1
Ponceau SX (R-4)	503 (24)	481 (100)	459 (30)	ND	5
Oil Red (R-5)	ND	277 (100)	ND	ND	0.5
Allura Red AC (R-40)[b]	519 (31)	497 (100)	ND	ND	5
Ponceau R (R-101)	503 (28)	481 (100)	459 (43)	ND	5
New Coccine (R-102)[b]	627 (39)	605 (100)	583 (51)	ND	5
Eosine (R-103)	715 (20)	693 (100)	671 (87)	649 (73)	0.5
Phloxine (R-104)[b]	853 (30)	831 (100)	809 (47)	787 (37)	0.5
Rose Bengal (R-105)[b]	1041 (14)	1019 (100)	997 (27)	975 (15)	0.5
Acid Red (R-106)[b]	603 (43)	581 (100)	559 (16)	ND	0.03
Azo Rubine (R-AZ)	525 (30)	503 (100)	481 (28)	ND	5
Orange I (O-1)	373 (82)	351 (100)	329 (175)	ND	1
Orange RN (O-RN)	373 (143)	351 (100)	329 (246)	ND	1
Oil Orange SS (O-SS)	ND	263 (100)	ND	ND	0.5
Naphthol Yellow S (Y-1)	381 (30)	359 (100)	337 (20)	ND	5
Yellow AB (Y-2)	ND	248 (100)	ND	ND	0.5
Yellow OB (Y-3)	ND	262 (100)	ND	ND	0.5
Taratrazine (Y-4)[b]	557 (25)	535 (100)	ND	ND	5
Sunset Yellow FCF (Y-5)[b]	475 (37)	453 (100)	ND	ND	5
Guinea Green B (G-1)	713 (89)	691 (100)	669 (48)	ND	1
Fast Green FCF (G-3)[b]	831 (68)	809 (100)	787 (61)	ND	1
Wool Green S (G-S)	599 (68)	577 (100)	555 (25)	ND	1
Brilliant Blue FCF (B-1)[b]	815 (54)	793 (100)	771 (37)	ND	1
Indigo Carmine (B-2)[b]	ND	ND	ND	ND	ND
Acid Violet 6B (V-1)	756 (23)	734 (100)	712 (70)	ND	1

[a] m/z values with relative abundances (%) in parentheses.
[b] Permitted dyes for use in foods in Japan.
[c] ND = not detected.

dyes except for B-2 and some of them also showed $[M+Na]^+$, $[M-Na+2H]^+$ and $[M-2Na+3H]^+$. These molecular ion species are considered to be useful for the identification of the food dyes. The detection limits of the molecular ion species vary with their chemical structures. Xanthene dyes (R-3, R-103, R-104, R-105 and R-106) showed high sensitivity, ranging from 0.03 to 1.0 μg per spot. Triphenylmethane dyes (G-1, G-3, G-S, B-1 and V-1) gave molecular ion species at concentrations of 1.0 μg per spot. Although azo dyes having carboxylate and/or sulphate groups (R-1, R-2, R-4, R-40, R-101, R102, R-AZ, O-1, O-RN, Y-4 and Y-5) showed a lower sensitivity of 1.0–5.0 μg per spot, oil-soluble azo dyes having no carboxylate and/or sulphate groups (R-5, O-SS, Y-2 and Y-3) showed a moderate sensitivity of 0.5 μg per spot. Naphthalene dye (Y-1) gave a low sensitivity of 5.0 μg per spot. Hence the technique can improve the detection limits of dyes 4–20-fold with good reproducibility.

3.4. Identification of unlawful dyes in foods

In order to evaluate the capability of the proposed method, TLC–FAB-MS was finally applied to the identification of unlawful food dyes in imported candy and powdered juice. The dyes were extracted with degreased wool from

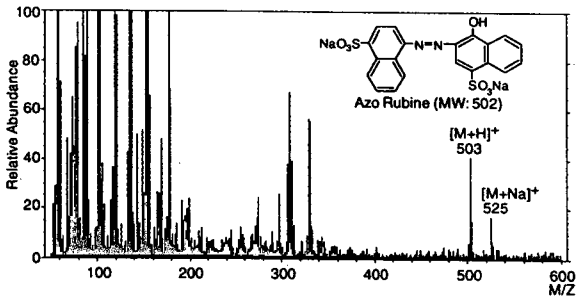

Fig. 2. TLC–FAB mass spectrum of an unknown red dye from a candy.

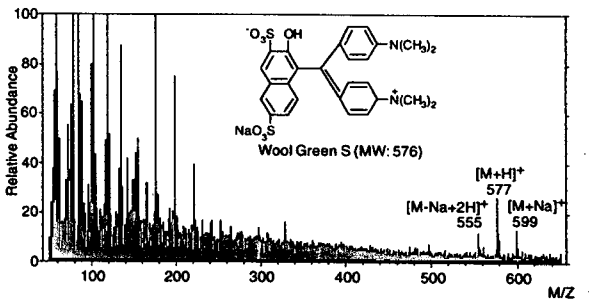

Fig. 4. TLC–FAB mass spectrum of an unknown green dye from powdered juice.

foods as described under Experimental. The residues were dissolved in methanol and the solutions were subjected to TLC–FAB-MS. The candy contained a red food dye which was suspected to be R-1, R-101 or R-AZ from the results of TLC analyses with solvent system A. As shown in Fig. 2, the two molecular ion species at m/z 525 ($[M + Na]^+$) and m/z 503 ($[M + H]^+$) are clearly observed in the TLC–FAB mass spectrum. After comparison with the authentic standard, this dye was identified as Azo Rubine (R-AZ), which is an unlawful dye in Japan. The imported powdered juice contained three dyes, as shown in Fig. 3, and we suspected them to be Y-4, Y-5 and G-S from the R_F values in Table 2. Because G-S is not permitted for use in foods in Japan, we focused on the identification of this green spot. The spectrum obtained is shown in Fig. 4. Three molecular ion species, m/z 599 ($[M + Na]^+$), m/z 577 ($[M + H]^+$) and m/z 555 ($[M - Na + 2H]^+$), clearly appear and this dye was identified as Wool Green S (G-S). Hence the effectiveness of the proposed method for the identification of unlawful dyes in foods was confirmed.

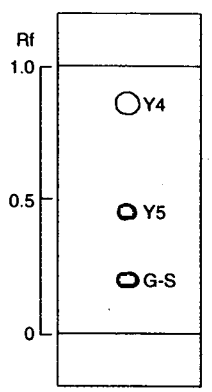

Fig. 3. TLC separation of the dyes extracted from powdered juice. Plate: C_{18}-modified silica gel. Solvent system: methanol–acetonitrile–5% aqueous sodium sulphate solution (3:3:10) (solvent system A).

4. Conclusions

A TLC–FAB-MS method with a sample condensation technique was developed for the identification of 27 food dyes constituting the twelve permitted dyes for use in foods and the fifteen unlawful dyes in Japan. The method was performed under the following conditions: TLC plate, C_{18}-modified silica gel; mobile phases, methanol–acetonitrile–5% sodium sulphate solution (3:3:10, solvent system A) and methanol–methyl ethyl ketone–5% sodium sulphate solution (1:1:1) (solvent system B); matrix, magic bullet; condensation of spot, with methanol. The method has been successfully applied to the identification of unlawful dyes in imported foods.

5. Acknowledgement

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Gas chromatography, liquid chromatography and ion chromatography adapted to the trace analysis of explosives

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Abstract

The potential of gas chromatography, high-performance liquid chromatography and ion chromatography for application to the determination of traces of explosives was investigated. The most important aspect is the combination of the chromatographic technique with selective detection methods. In gas chromatography a chemiluminescence detector is preferred and for high-performance liquid chromatography the advantages of postcolumn derivatization are described. Problems and possible solutions in the trace analysis of explosives which depend on matrix effects and chemical properties are discussed.

1. Introduction

Chromatographic methods are important in the trace analysis of explosives. The utility of chromatography is based on the selective separation of sought compounds from unwanted accompanying substances. Traces of explosives have usually to be determined in very complex matrices, such as debris from a bomb scene or environmental dirt from cars or houses. The chromatographic method can be selected to match the characteristics of the explosives and eliminate interfering substances from the matrix. Also, chromatographic methods can be combined with special detectors for sensitive and specific detection of the explosive constituents. An extensive review on the detection and identification of explosive residues has been written by Beveridge [1]. There is also an excellent book on modern methods and their applications in the analysis of explosives [2].

Gas chromatography (GC), liquid chromatography (LC) and ion chromatography (IC) can be applied to the analysis of explosives depending on the characteristics of each constituent. The application of supercritical fluid chromatography (SFC) has also been reported [3], but this is not discussed in this paper. Gas chromatography is preferred for the organic compounds, which can be vaporized without decomposition. To this group belong the explosive oils, *e.g.* ethylene glycol dinitrate (EGDN), diethylene glycol dinitrate (DEGN) and nitroglycerine (NG), also nitroaromatics such as dinitrotoluene (DNT), trinitrotoluene (TNT) and tetryl (CE), and even high explosives such as pentaerythritol tetranitrate (PETN) and hexogen (RDX).

Gas chromatography has the advantage of a high resolving power. Several isomers of nitroaromatics can be separated with narrow peaks in one run together with explosive oils and high explosives in a reasonable time. The importance of gas chromatography is based on its compatibility with mass spectrometric [4] and chemoluminescence (TEA) [5] detectors. These detectors make the identification of small

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amounts possible by selective detection at reproducible retention times.

Liquid chromatography has a wide range of applications. The trace analysis of explosives is preferably done by high-performance liquid chromatography (HPLC), because it can be coupled to sensitive detectors. Even coupling to a chemiluminescence detector (TEA) is possible [5]. HPLC is important for the determination of thermolabile compounds or explosive constituents that cannot be vaporized. The separation mechanism in LC is totally different from that in GC, and the methods can be used alternatively to confirm the results. Usually HPLC is less sensitive than GC because of the broad peak shape and lower peak heights. Also, the resolution between substances of similar polarity such as isomers is lower than in GC.

Thin-layer chromatography (TLC) can be used for the trace analysis of explosives with the disadvantage of about a 100 times lower sensitivity than GC. However, TLC is an inexpensive technique that can often be applied successfully if the amount of explosive is large enough and the detection is made fairly selective with spray reagents.

Ion chromatography is the method of choice for the ionic constituents of explosives. Preferably IC is used for inorganic anions such as nitrite, nitrate and chlorate [6]. Inorganic cations with the exception of ammonium are not typical components of explosives and cation chromatography is not often applied. Recently capillary electrophoresis has been introduced for the determination of inorganic anions and it seems to be superior to IC because of its higher efficiency and shorter analysis times [7].

The trace analysis of explosives has to be applied to samples from bomb scenes (post-blast analysis), to samples from cars of premises and to samples from suspects. Each sample source has its own characteristics, but accompanying substances having a higher concentration than the explosive itself are the greatest problem for the analysis. Therefore, the adaptation of the chromatographic techniques to the trace analysis of explosives must be focused, in addition to optimization of the chromatographic conditions, on a selective detection method. The detector should only respond to explosives and should give no signal for all substances that are not related to explosives. This requirement cannot be met ideally, because the structural properties of the different explosives are not so unique that other substances cannot interfere. Only the nitroaromatics can be easily identified by mass spectrometry. The structural property that contrasts with most of the compounds in the matrix is the high content of nitro groups in organic explosives. In addition to spectrometric methods, selective detection can also be based on the recognition of nitro groups in the eluate.

This paper describes optimized combinations of chromatographic methods with selective detection techniques for the trace analysis of explosives.

2. Experimental

2.1. Gas chromatography

The gas chromatograph was a Varian 3400 coupled to a TEA detector from Thermedics, as described by Kolla [8]. Injection was performed with a split–splitless injector operated at 170°C. The column was a 10 m DB-5 capillary column. The column temperature was initially 50°C and was increased at 10°C/min to 250°C.

2.2. Liquid chromatography

Liquid chromatography was carried out with modular equipment. An LDC Analytical 3200 pump was used. The column was Lichrospher RP-8 and the eluent was water–methanol (1:1). Postcolumn derivatization was performed as described by Engelhardt *et al.* [9] with a low-pressure mercury lamp behind the column and mixing sulphanilamide and naphthylethylenediammonium chloride sequentially with the eluent. The detector was a Shimadzu UV–Vis detector operated at 540 nm.

2.3. Ion chromatography

Ion chromatography was performed with a Metrohm IC 690 instrument, which operates on

the single-column principle without chemical suppression. The anion-exchange column was a Hamilton PRP-X100 and the eluent was 4 m*M* potassium hydrogenphthalate (pH 4.5).

3. Results and discussion

3.1. Gas chromatography

In general analytical operations, GC is the method of choice because it is easy to handle, the conditions are stable and reproducible, the analysis is fast and many different detectors can be coupled to the system. Therefore, GC is also well suited to the analysis of organic explosives. However, the usual GC systems cannot be applied to explosives without slight modifications, because not every explosive is stable at higher temperatures. In Table 1 the deflagration temperatures of explosives and their vapour pressures at GC temperatures are given. At the deflagration temperature sudden decomposition of the explosive takes place, but partial decomposition starts at much lower temperatures with the lowest stability for nitrate esters. Nitroglycerine shows significant decomposition at 135°C, turning red because of the formation of nitrosic gases. There is a dilemma especially for explosives with very low vapour pressures such as PETN and RDX, which decompose at elevated temperatures. No problems arise for the more stable nitroaromatics (the C–NO_2 bond is more stable than the O–NO_2 or the N–NO_2 bond), which have fairly high vapour pressures.

Table 1
Deflagration temperatures and vapour pressures of explosives

Explosive	Deflagration temperature (°C)	Vapour pressure at 100°C (Torr)[a]	Vapour pressure at 200°C (Torr)[a]
EGDN	200	22.2	2582
NG	200	0.39	78.6
TNT	333	0.069	360
PETN	209	0.0008	26.9
RDX	229	0.00016	0.31

[a] Vapour pressures from ref. 10 were recalculated. 1 Torr = 133.322 Pa.

Also, the explosive oils NG and EGDN with their high vapour pressures can be determined by GC despite their thermal instability. In order to be able to analyse all explosives, the explosive oils and the nitroaromatics together with PETN and RDX, a compromise has to be found for the GC conditions.

3.2. Injection port

The large amounts of compounds of higher molecular mass and salts in the matrices, which cannot be vaporized, prevent the use of on-column injection, otherwise serious contamination of the column would occur. An injection port with an insert that traps the non-vaporizable substances should be used. The easiest system for this purpose is a split–splitless injector with a fritted glass insert. Its inner surface is small and inactive, so that possible decomposition is prevented and it is not adsorptive for explosives. It can be replaced very quickly if it is dirty and the analysis deteriorates. The effect of a dirty insert can be seen when determining PETN and RDX, which evaporate only at higher temperatures and are simultaneously unstable if polar substances are present [8] in the injector (Fig. 1). The PETN peak decreases and the RDX peak tails badly. Often the beginning of the column (about 20 cm) has to be cutoff to restore the original sensitivity. The quality of the separation should be checked periodically with a standard mixture.

The injection temperature is chosen in the range between optimum evaporation and the beginning of decomposition for the most difficult explosives (PETN and RDX). In Fig. 2, peak area *versus* injection temperature plots for some important explosives are shown. To determine the optimum injection temperature, the area of the peaks was calculated. The optimum temperature occurs near 170°C, where the peaks for the explosive oils have already decreased. The injection temperature can even be used for confirmation, *e.g.*, PETN decomposes at a temperature higher than 200°C and a new peak appears at a low retention time. To confirm PETN, the peak at 170°C must decrease with increasing injector temperature and a new peak must be observable (Fig. 3). The injector is operated in

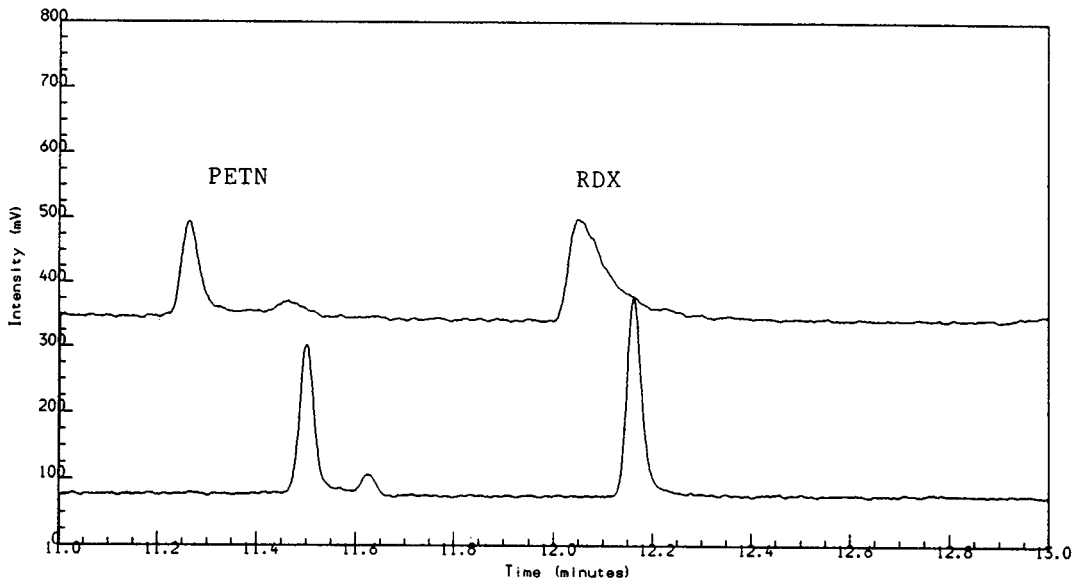

Fig. 1. Worsening of the peak shape of PETN and RDX because of contamination of the injector with polar substances.

the splitless mode in order to avoid any losses of explosives in the sample.

3.3. Capillary column

The column should have only a low polarity because the polar interaction of the nitro group, especially with PETN and RDX, can lead to irreversible adsorption on the stationary phase or decomposition of the explosives if the temperature is increased. However, it is necessary to have some functional groups in the stationary phase for selective interaction. This is because with a pure polymethylsiloxane (OV-1) the separation of PETN and RDX is not possible. A small amount of phenyl groups in the stationary phase such as that found in a polymethylphenyl(5%)siloxane (DB-5) enhances the selectivity of the column for the nitro compounds and good separations are obtained. Another problem is the column length. At the temperature at which PETN and RDX evaporate, the decomposition of these two compounds begins to take place. The decomposition is fairly slow, but these compounds should be eluted from the column as fast as possible if the temperature programme reaches this limit. There are two methods to achieve fast elution: the gas velocity can be increased or the column length can be shortened. Both are applied in the GC of explosives. The inlet pressure in the injector is increased to the point where injection by the usual type of syringe is just possible and the capillary column is not made longer than 10 m for DB-5. Columns of higher polarity should be shorter. In order to have a sufficient number of theoretical plates, *i.e.*, narrow peak shapes, the inner diameter of the columns should be only 250 μm. The film thickness should be small to prevent peak broadening because of low diffusion velocities.

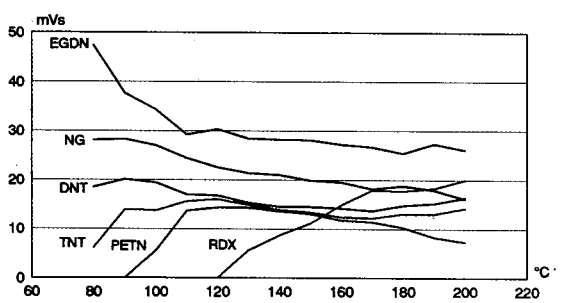

Fig. 2. Peak areas of explosives *vs.* injection temperature.

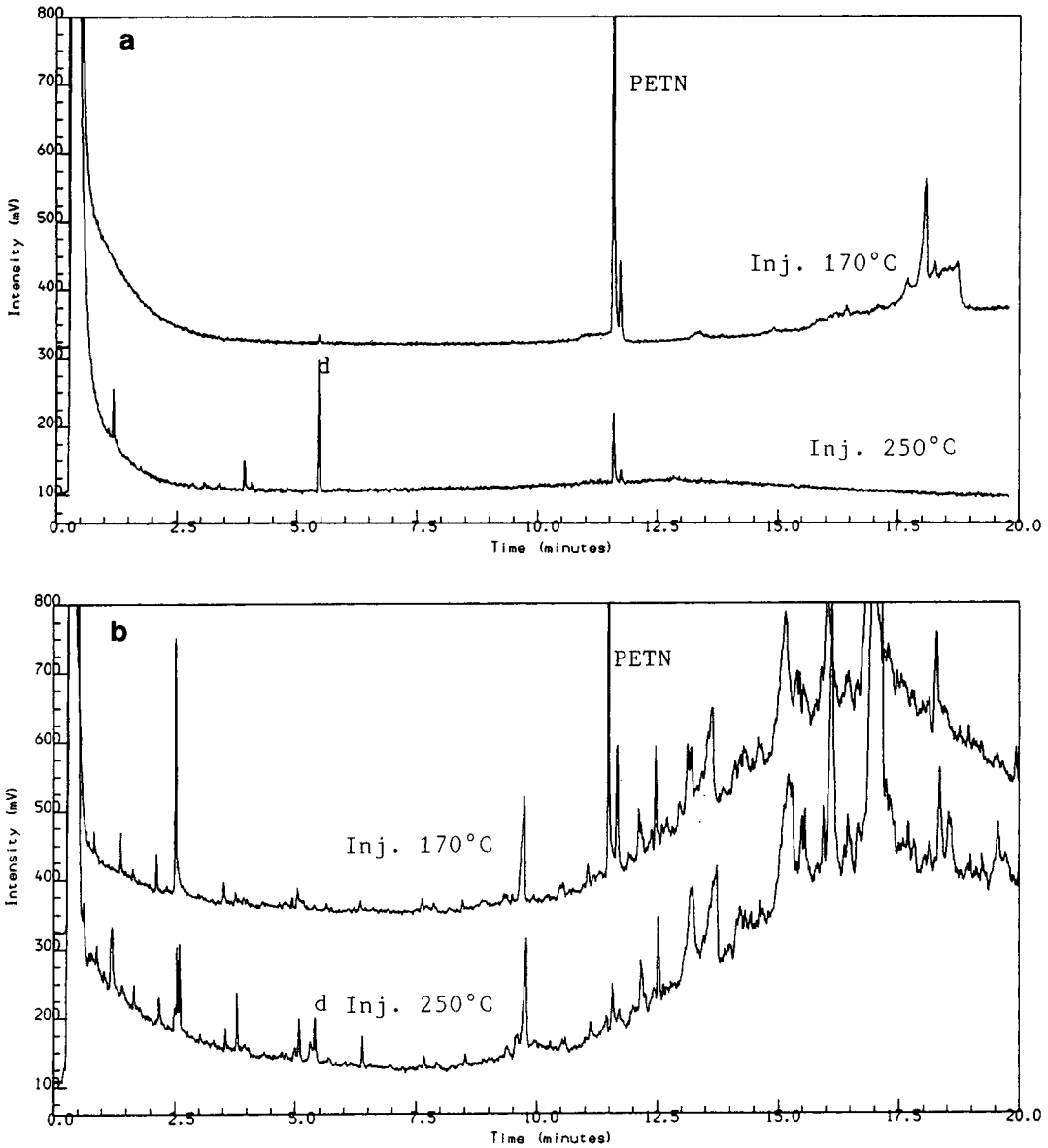

Fig. 3. (a) GC–TEA of pure PETN at injection temperatures of 170 and 250°C. d = Decomposition product of PETN. (b) GC–TEA of a real sample containing PETN traces at injection temperatures of 170 and 250°C.

3.4. GC detector

As described above, the detector has to be selective for explosives because of possible interferences from accompanying substances in the matrix. The mass spectrometer is well suited for the determination of nitroaromatics, especially with negative chemical ionization (NCI) [11]. Problems with MS may arise in the identification of nitrate esters or RDX in complex matrices because the spectra are often not unequivocal if there is co-elution with other substances. In

addition, GC–MS is usually used for many other analytical problems in a laboratory and the system cannot be modified each time for the trace analysis of explosives. As an alternative the TEA, a chemiluminescence detector, has been introduced. This detector responds specifically to nitro and nitroso compounds. The eluting substances are pyrolysed at high temperature and nitrogen monoxide is formed. The reaction of nitrogen monoxide with ozone is followed by the emission of light, which can be measured by a photomultiplier. This operating principle produces only peaks for substances containing nitro or nitroso groups. A commercially available TEA was modified [8,12] to make the TEA compatible with capillary columns with small inner diameters and to enhance significantly both the sensitivity and the resolving power.

Despite the specifity for nitro and nitroso compounds, in some samples, in addition to the explosive constituents, many unidentified peaks appear in the chromatogram (Fig. 4). Therefore, confirmation with at least one different method is necessary. To confirm explosive traces, the alternative analysis must have a similar sensitivity and should have also a selective detector. A simple column change to a stationary phase of different polarity in the GC–TEA system is one way of confirmation. If the identified peak has the same retention time on the new column as the standard, the probability is very high that the peak comes from the same substance. This polarity change of the column can be achieved with a stationary phase of higher phenyl content or with an additional content of cyanopropyl groups in polymethylsiloxane. In the latter column the interaction of the nitro compounds is stronger and retention changes will be observed depending on the chemical structure. The TEA detector can also be coupled to HPLC, which is based on a totally different separation mechanism. The greatest disadvantage of this approach is the lower sensitivity, which results from broad peak shapes and poor resolution.

Confirmation is also possible by GC–MS as already mentioned. Other GC detectors with lower selectivity such as the electron-capture detector or a nitrogen-specific detector can only be used if the matrix is relatively clean. When combined with high concentrations of chloro compounds or nitrogen-containing compounds the explosives peaks are often overlapped by these contaminants and identification of the explosive is impossible. For compounds such as

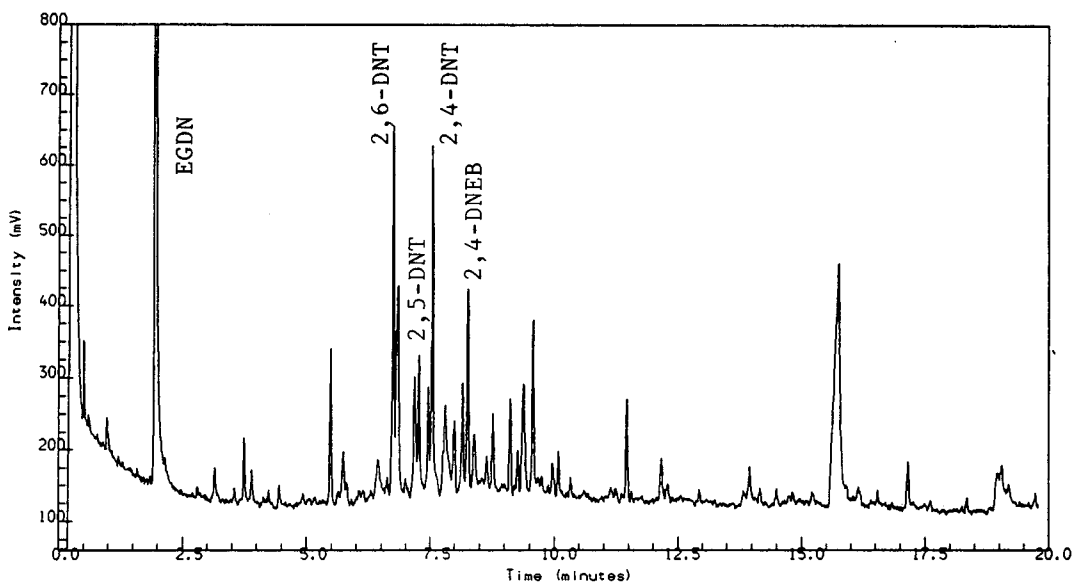

Fig. 4. GC–TEA of a real sample from a scene showing many unknown peaks.

nitrate esters and nitramines an alternative can be found with LC systems as described in the next section.

3.5. Liquid chromatography

The term liquid chromatography generally applies to all chromatographic methods with liquid mobile phases. The evolution of techniques in the last few years has concentrated on HPLC, mainly because of the direct coupling to sensitive detectors. For the trace analysis of explosives, detectors with selective responses are necessary. The mostly commonly used UV detector is non-specific for explosives because nitrate esters have very low chromophoric properties and are only detectable at wavelengths below 230 nm. At these wavelengths most organic substances have fairly high molar absorptivities and interfere with trace explosives. Just as in GC, selective detection must be based on reaction or interaction of the nitro group of the organic explosives. The TEA (chemiluminescence) detector can be applied in HPLC also, but it is more suitable for GC. Other selective HPLC detectors are the hanging mercury drop electrode (HMDE) [13] or UV photolysis followed by electrochemical detection [14]. Neither method has the same selectivity as the TEA and there are many substances that may interfere with the constituents of explosives.

Very specific detection can be achieved with a special postcolumn derivatization (PCD) [9]. The eluate from the HPLC column is subjected to UV irradiation and the nitro compounds split off nitrite ions. The nitrite ions are reacted with sulphanilamide and naphthylethylendiamine in a modified Griess reaction. The dye produced is detected in the visible range at 540 nm. This detection procedure is very specific, because only substances that produce nitrite ions by photolysis give peaks. Other dyes with absorption characteristics at 540 nm usually have a totally different HPLC behaviour from that of explosives. In Fig. 5 a chromatogram of a real sample analysed by HPLC–PCD is shown. Additional confirmation can be easily achieved by turning the UV irradation off. If the peak of the explosive then vanishes, it must be a nitro compound. The PCD detection shows very good sensitivities for nitrate esters (25–50 ppb) and nitramines (30–100 ppb). The sensitivity for nitroaromatics is ten times lower, because the C–NO_2 bond is fairly stable towards UV irradiation. The O–NO_2 and N–NO_2 bonds are so unstable that the NO_2 is separated very early in the light path and the further conversion of nitrite into nitrate must be suppressed by addition of sodium hydroxide to the eluent in order to have high sensitivity.

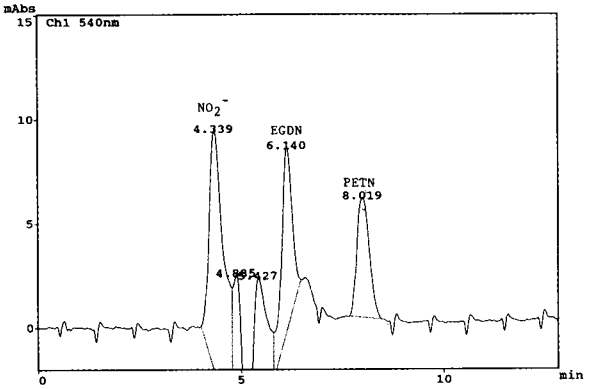

Fig. 5. HPLC–PCD of a sample after the explosion of a car bomb.

HPLC has special advantages in the analysis of very dirty samples because the columns and the eluents are more stable to contamination from accompanying substances in extracts of debris or dust. It is also possible to analyse explosives that cannot be evaporated without decomposition such as octogen (HMX), but the latter seldom appear in real cases. The essential disadvantage of HPLC is the low resolving power and the broad peak shapes. In reversed-phase HPLC, which is the most commonly used phase system, the several isomers of nitroaromatics cannot be completely separated as necessary to allow the calculation of the isomer distribution [15]. This problem can only be solved by using normal-phase HPLC, but with normal-phase systems most of the selective detection methods cannot be used and the column and eluent behaviour is often unstable.

Overall, HPLC is well suited for the trace analysis of explosives, but if GC–TEA is available then HPLC should be used as the second analytical step.

3.6. Ion chromatography

In the trace analysis of explosives, IC is applied to the inorganic part. The determination of inorganic ions is primarily concerned with anions. Anion determination is important if the organic tests reveal that no military high explosive or plastic explosive was involved, or that a dynamite is assumed. With a dynamite, nitrate should be found in the anion chromatographic analysis. If no organic traces are found, a purely salt-based explosive can be presumed and anion determination must be performed with respect to chlorate, nitrate, perchlorate or sulphate.

Cation chromatography is also applicable to the trace analysis of explosives, but usually only ammonium is significant. Instead of modifying the ion chromatograph, ammonium can be specifically detected in small amounts using Nessler's reagent.

The presence of nitrate in the water extract from debris or dust is not specific for explosives. The interpretation of the nitrate content is always very difficult, because there are other sources from which nitrate may have originated, *e.g.*, fertilizers. Nitrate from commercial explosives, especially in post-blast analysis, is at a very low concentration and often in the range of the environmental level, except for low explosives such as flash powder or black powder. Sometimes the ratio to the ubiquitous chloride helps to establish the significance of the nitrate level [16], but in post-blast analysis such determinations are possible only with samples taken at very short distances from the centre of the explosion (Fig. 6). The presence of nitrate can only be related to explosives if there is a fairly large amount and if other analytical results support a nitrate-containing explosive. Other analytical results may be the finding of ammonium; traces of explosive oils and/or nitroaromatics (dynamites/European dynamites); significant amounts of oils and/or waxes (emulsion explosives) [17]; microscopic identification of microballons (emulsion explosives) [18]; unreacted particles of gelatinous nitrocellulose (dynamites) [15]; fragments of explosive wreckage paper (commercial explosives).

One problem with IC is the relatively low

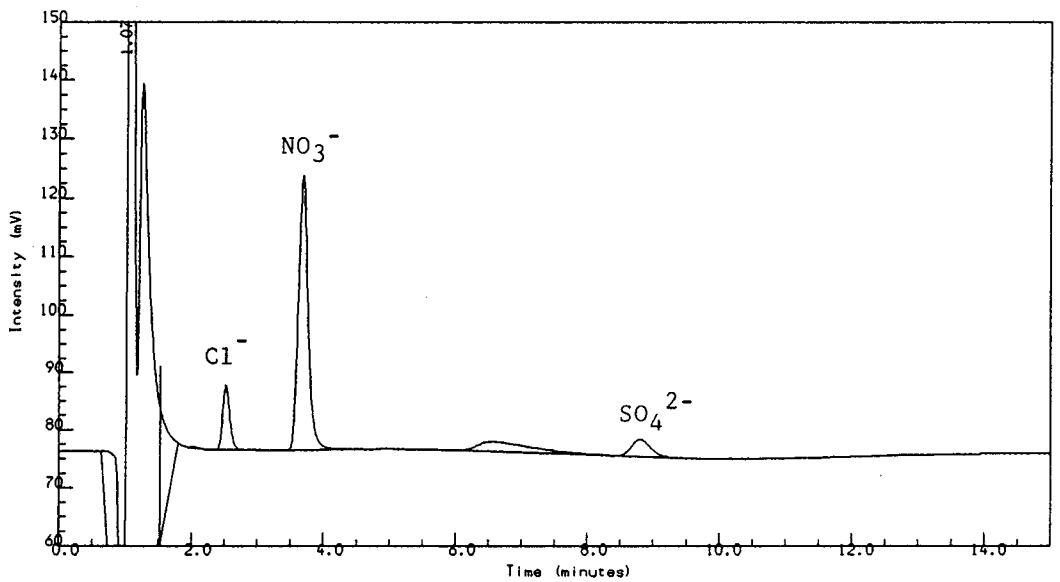

Fig. 6. IC of a swab taken at a 1-m distance after the explosion of 500 g of a dynamite.

sensitivity if it is used as an additional method for the trace analysis of inorganic salts after positive results for explosive oils or nitroaromatics in organic trace analysis. This appearance is typical with European dynamites, which contain several organic nitro compounds in addition to the nitrate salt. The sensitivity of IC with direct conductivity detection is about 1 ppm for nitrate. The injection of 100 μl of the water extract corresponds to 100 ng. Presuming a total amount of 1 ml of concentrated water extract, the limit for a positive result is 1 μg of nitrate in the original sample (assuming 100% recovery). A European dynamite usually contains 5–40% of explosive oils and 2–10% of nitroaromatics. The sensitivity with GC–TEA detection is about 100 pg for an explosive oil. The injection of 1 μl from an extract concentrated to 100 μl corresponds to 10 ng in the original sample (assuming 100% recovery). If there was a very low content of 5% of explosive oil in the dynamite, the nitrate content was 0.2 μg at the most, which is below the limit of detection in IC. The real relationships are often worse for IC.

Inorganic analysis is never successful when explosives traces were found in the organic analysis. The major importance of IC is in the field of home-made mixtures of chlorates, the analysis of residues of black powder or if pyrotechnic mixtures have been used. Mixtures of chlorates with different types of fuels in gas bottles or steel pipes have frequently been used in Europe. In post-blast analysis large amounts of chloride dominate the ion chromatogram. An enrichment of chlorate traces can be achieved by extracting the dried water extract with acetone, and after evaporation of the acetone, making a new water extract [16]. Chlorate salts are more soluble in acetone than chloride salts.

Residues of black powder can be recognized from the unburnt nitrate and the large amount of sulphate [18]. Typical residue components such as thiocyanate are difficult to determine in the residue by simple IC with direct conductivity detection. Pyrotechnic mixtures with fast burning rates usually contain perchlorates and aluminium or magnesium powder. The determination of perchlorate can be performed by IC, but often the eluent has to be changed to a higher ionic strength and perchlorate tends to have a bad peak shape.

Some of the difficulties with IC can be avoided if the ions are determined by capillary electrophoresis (CE). This method permits a very fast, highly efficient and sensitive determination of ions and will certainly be surely helpful in the analysis of explosives residues [7].

4. Conclusions

The trace analysis of explosives places its own demands on the chromatographic methods applied. The complex matrix of the sample that has to be analysed for explosives traces makes a selective analysis inevitable. The chromatographic separation has to be highly efficient and the detection selective for explosives. GC is the method of choice for the determination of the organic constituents of explosives. GC is easy to combine with MS or nitro/nitroso-specific TEA detection. The highly efficient separation with the capillary column permits the analysis of explosive oils, isomers of nitraromatics and the high explosive PETN and RDX in one run. HPLC in combination with selective detection can be used as alternative method to GC for confirming explosives traces. HPLC and TLC are very helpful in the trace analysis of explosives with the disadvantage of lower resolving power and lower sensitivity than GC. IC is used for the determination of the inorganic contituents of explosives. In particular, anion chromatography can be applied to determine characteristic salts used as oxidizers in many commercial and homemade explosives.

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JOURNAL OF CHROMATOGRAPHY A

Evaluation of improved methods for the recovery and detection of organic and inorganic cartridge discharge residues

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Abstract

An efficient vacuuming system for the recovery of organic and inorganic cartridge discharge residues (CDRs) from clothing was developed. Sample extracts for organic CDR analysis were cleaned and concentrated by an automated solid-phase extraction system. Two systems were used for the analysis of organic CDRs, a sensitive gas chromatography–mass spectrometry method and a modified automated high-performance liquid chromatography pendant mercury drop electrode system. Inorganic cartridge discharge residues were analysed by scanning electron microscopy with energy dispersive analysis of X-rays. The combined systems have been applied to firearms casework.

1. Introduction

The analysis of cartridge discharge residues (CDRs) is important in determining if a person has been in contact with, or close to, the discharge of a firearm. At present the method of choice is scanning electron microscopy (SEM) with an energy dispersive analysis of X-rays (EDAX) for the investigation of the morphology and chemical composition of inorganic CDRs from the primer [1–3]. Automation of the system is necessary because the procedure for searching for residues is time consuming [4]. An alternative or complementary approach would be the detection of partial or unburnt propellant (organic CDRs) on hands or clothing.

Modern propellants are composed mainly of the explosive nitrocellulose (single-based). Other explosive ingredients may also be present for example, nitroglycerine (NG) (double-based) or NG and nitroguanidine (triple-based). Propellants also contain stabilizers such as diphenylamine (DPA), ethyl centralite (EC) or methyl centralite (MC), flash inhibitors such as 2,4-dinitrotoluene (2,4-DNT) and plasticisers.

Although there is extensive literature on the identification and detection of propellants [5–7], little work has been devoted to the combined analysis of organic and inorganic CDRs recovered from hands [8] and clothing [9] in forensic casework. The analysis of organic CDRs has concentrated on the detection of NG and 2,4-DNT by high-performance liquid chromatography (HPLC) with a pendant mercury drop electrode (PMDE) [10]. The HPLC–PMDE system requires a clean up and concentration of samples containing organic residues for optimum performance [8].

Initially this laboratory used the technique developed by Lloyd and King [8] for the clean-up and concentration of organic CDRs and explosive residues. A slurry mix of the sample

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and Chromosorb 104 was sucked into a 1 mm I.D. PTFE tube containing Amberlite XAD-4. Organic explosive residues and CDRs were selectively retained on support material. The cleaned organic residues were then eluted from the tubing using acetonitrile–water (25:12, v/v). The technique was found to be time consuming and, because of the large number of samples processed by this laboratory, the extraction system has been adapted and optimised (Harvey and Speers [11]). In this study an efficient vacuuming system was investigated for the recovery of organic and inorganic CDRs from clothing. This was coupled to an automated system for the extraction and clean-up of the organic CDRs using a solid-phase extraction (SPE) system containing Chromosorb 104 and Amberlite XAD-4. A sensitive gas chromatography–mass spectrometry (GC–MS) method has been developed for the analysis of DPA, EC and MC. An existing HPLC–PMDE system [10] has been adapted such that the automated deoxygenation and injection of samples for the detection of NG and 2,4-DNT was achieved. GC–MS and HPLC–PMDE analysis were performed on fractions of the same extract. The system has been applied to routine firearms casework for a trial period to assess its evidential value.

2. Experimental

2.1. Standards

Pure samples of EC and MC were obtained from a munitions manufacturer. NG was extracted from a known composition of Super Dopex explosive (Explosives Chemical Products, UK). DPA, 1,3-dinitrobenzene (1,3-DNB) and 2,4-DNT were obtained from Aldrich (Gillingham, UK).

2.2. Materials

All solvents were HPLC grade unless stated otherwise. All reagents were analytical-reagent grade unless stated otherwise.

2.3. HPLC equipment

The equipment consisted of a 8810 isocratic HPLC pump (Spectra-Physics, Hemel Hempstead, UK) and a 7970/7980 series column block heater operating at 35°C (Jones Chromatography, Hengoed, UK).

The HPLC column was a Zorbax ODS 150 mm × 4.6 mm I.D., 5 μm particle size and 80 Å pore size (Jones Chromatography). The column was cleaned with 50 ml of methanol at the end of each analysis run.

The eluent consisted of methanol–phosphate buffer pH 3.0 (55:45). The flow-rate was 1.2 ml/min.

Phosphate buffer was prepared by adding 11.5 g of 85% (w/v) phosphoric acid to 4 l of deionised water. Anhydrous potassium carbonate was added to the mixture to increase the pH to 3.0.

The eluent was continuously refluxed in a 2-l flask, under an atmosphere of nitrogen, to remove oxygen.

The detector was a Model 420 pendant mercury drop LC electrode connected to a Model 400 electrochemical detector via an external cell cable and 407 module (EG&G Princeton Applied Research, Princeton, USA). The electrode settings were: standing mercury drop electrode (SMDE) and drop size small. The detector settings were: reductive d.c. mode, −1 V, reference electrode 5 *M* aqueous lithium chloride [10]. A new mercury drop was automatically dispensed at the start of each run via a signal from an autoinjector.

The standard contained 1 ng/μl of NG, 1,3-DNB and 2,4-DNT. The detection limits for NG and 2,4-DNT, based on a signal-to-noise ratio of 3, were 50 and 65 pg, respectively, per 10-μl injection.

The detector was connected to a Drew 3040 data capture unit linked to a 286–16 MHz personal computer operating the Drew Scientific Chromatography Roseate software (Drew Scientific, London, UK).

Samples were deoxygenated and injected using a programmable Model 231 autoinjector (Gilson Medical Electronics, France) fitted with a 10-μl sample loop, a Model 401 diluter and a universal

switching valve module with on-line nitrogen. Methanol was used for injector needle wash. The autoinjector was programmed to deoxygenate the sample for 3 min prior to injection, dispense a new mercury drop on the HPLC–PMDE system at the start of each run and commence the data collection on the Drew chromatography software system (a full description of the programme can be obtained from the author).

2.4. Gas chromatography–mass spectrometry

A Trio 2000 quadrupole mass spectrometer with VG Lab base data system (VG Biotech, Manchester, UK), Model HP5890 gas chromatograph and HP7673 autosampler (Hewlett-Packard, Wokingham, UK) was used. The capillary column was a Rtx-1, 15 m × 0.32 mm I.D., 1 μm film thickness (Thames Chromatography, Maidenhead, UK). The conditions of analysis were: helium carrier gas, inlet pressure 5 p.s.i. (1 p.s.i. = 6894.76 Pa); temperature settings 85°C initially then ramped to 250°C at 30°C/min, maintained at 250°C for 5 min; 1 μl splitless sample injection.

Further conditions were: GC–MS interface temperature 250°C; mass spectrometer source temperature 250°C; scan rate 0.9 s; interscan time 0.1 s; masses scanned 45 to 300 u full scan. The samples were analysed with the instrument in electron impact (EI) mode with a setting of 70 eV. The system was set up for selective ion recording (SIR). Samples were initially screened for single masses 169 (DPA), 120 (EC) and 134 (MC) and subsequently reinjected for confirmation based on the masses 77, 167, 168, 169 for DPA; 77, 120, 148 for EC and 77, 106, 134 for MC. The standard contained 1 ng/μl of DPA, EC and MC. Detection limits for all three analytes, based on signal-to-noise ratio of 3, was 10 pg per injection. Prior to injection the acetonitrile extract was blown down to a volume of 20 μl under an atmosphere of nitrogen.

2.5. SEM–EDAX

Inorganic CDRs were analysed using a Camscan series 2 scanning electron microscope (Cambridge, UK) connected to a Link AN 10000 analyser (High Wycombe, UK) and detected by an automatic residue detection system (ARDS) developed at this laboratory [12].

2.6. Recovery of organic and inorganic CDRs from clothing

Suction sampling apparatus used for the recovery of organic and inorganic CDRs from clothing consisted of a 25 mm diameter in-line Deldrin filter holder unit (Gelman product No. 1109, Northampton, UK) with one of the nylon hose barb adapters removed. The filter used was a 25 mm diameter 1 μm fluoropore membrane filter FHLP 02500 (Millipore, Watford, UK). When in use the filter holder is attached to an Edwards E2 M12 vacuum pump (Crawley, UK). An autosampler cap is used to seal the holder before and after use. Wallace and McKeown [13] have described in detail the suction sampling and contamination avoidance procedures.

2.7. Extraction procedure on Millilab 1A workstation

The Millilab 1A workstation (Millipore) is a personal computer-controlled automated robotic system which performs sample extraction from filters and SPE according to user-defined programmes.

Organic residues were extracted from the Deldrin filter holders, then cleaned and concentrated by SPE on the Millilab workstation. The system is fully automated and incorporates wash procedures into its programme. (A full description of the programme can be obtained from the author.)

Deldrin filter holders were adapted for use on the Millilab workstation by the addition of a male and female PTFE luer adapter with 1/8 in. (1 in. = 2.54 cm) NPTF thread. (These were manufactured within the laboratory.)

2.8. Extraction of organic CDRs from Deldrin unit on Millilab 1A workstation

A 400-μl volume of methanol, containing internal standard 1,3-DNB (to monitor extraction efficiency, concentration 0.25 ng/μl), were

pipetted into each Deldrin filter holder unit to wet the filter. After 2 min 500 μl of acetonitrile were added and left for 5 min to dissolve any organic residues on the filter. The Deldrin unit was then purged with nitrogen for 20 s and the extract collected in a disposable glass tube 160 mm × 10 mm. This was repeated with a further 500 μl of acetonitrile. The total extract was cleaned and concentrated using SPE. The filter from the Deldrin holder was processed for inorganic residues (see below).

2.9. SPE of organic CDRs on Millilab 1A workstation

Chromosorb 104, 125–150 μm mesh size, was obtained from Phase Separations (Clwyd, UK) and Amberlite XAD-4 from Sigma (Poole, UK). Prior to use both materials were prepared and cleaned according to the procedure recommended by Lloyd [14]. Amberlite XAD-4 and Chromosorb 104 (10 mg:30 mg) were packed between frits into empty 1.5-ml size SPE tubes (Alltech, Carnforth, UK).

The packed SPE tubes were first rinsed with 2 ml of acetonitrile to remove possible contaminants and then conditioned with 2 ml of deionised water to activate the support material. The acetonitrile–methanol organic CDR extracts from the Deldrin units were diluted 1:9 with deionised water and applied to the SPE columns at a rate of no greater than 4 ml/min. The columns were then washed with 2 ml of acetonitrile–water (1:10) and purged to dryness in an atmosphere of nitrogen. Analytes were eluted from the columns with 300 μl of acetonitrile into tapered 1.1 ml glass vials (Chromacol, Welwyn Garden City, UK). The 300 μl acetonitrile samples were analysed by HPLC–PMDE for NG, 2,4-DNT and 1,3-DNB and by GC–MS for DPA, EC and MC.

2.10. Extraction of inorganic CDRs for SEM–EDAX analysis

After the extraction of organic CDRs by the Millilab workstation, the 1-μm fluoropore filter was removed from the Deldrin holder and placed in a 150-ml glass beaker. The filter holder interior and autosampler cap interior were rinsed with light petroleum (quality over 120°C) into the same beaker and the volume made up to 20 ml. The beaker was ultrasonicated for 10 min and allowed to settle. The suspension was filtered through a 13 mm diameter 25 μm wire-mesh coarse filter housed in a Swinnex holder No. SX 0001300 (Millipore) and then through a 13 mm diameter 1-μm fluoropore filter No. FALP 01300 also housed in a Swinnex holder. After filtration the final 1-μm filter was place on a 13 mm diameter aluminium stub (Agar Scientific, Stansted, UK) using double-sided adhesive tape. The stub was coated with carbon using an automatic vacuum controller E6430 (Bio-Rad Microscience Division, Hemel Hempstead, UK) and analysed by SEM–EDAX for the presence of inorganic CDRs. The Deldrin filter holders and glassware were reused after thorough cleaning according to the procedure published by Wallace and McKeown [13].

3. Results and discussion

3.1. Efficiency of SPE of organic CDRs

Samples containing organic CDRs extracted from Deldrin filter units used for the suction sampling of clothing need to cleaned and concentrated using SPE to maximise the performance of the HPLC–PMDE and GC–MS detection systems. The previous SPE system, using a mixture of Chromosorb 104–Amberlite XAD-4 (10 mg:3.5 mg) in 1 mm I.D. PTFE tubing developed by Lloyd and King [8], was laborious and time consuming in its preparation and execution. An alternative clean-up and concentration system capable of being automated using 1.5-ml SPE columns was investigated.

Commercial reversed-phase (C_{18}) and aminopropyl (NH_2) 100-mg 1.5-ml SPE columns were compared to a 1.5-ml SPE column prepared in the laboratory containing 40 mg Chromosorb 104–Amberlite XAD-4 (30:10). The ability of the different SPE materials to extract and re-

cover organic CDRs from acetonitrile using the Millilab workstation was assessed.

An acetonitrile standard containing 10 ng each of NG, 1,3-DNB, 2,4-DNT, DPA, EC and MC was used to simulate organic CDRs extracted from a Deldrin filter unit. The acetonitrile standard was added to the reversed-phase C_{18}, aminopropyl and Chromosorb 104–Amberlite XAD-4 SPE columns according to the procedure recommended in the Experimental section. To improve the binding of the organic residues to the aminopropyl support the mixed standard was diluted 1:19 with hexane.

The organic CDRs were recovered from the SPE columns in 300 μl of acetonitrile, analysed by HPLC–PMDE and GC–MS and the recovery calculated. The experiments were performed twice to obtain an average recovery. The results are listed in Table 1.

It was demonstrated that the recovery of residues from the Chromosorb 104–Amberlite XAD-4 SPE column prepared in the laboratory was more efficient (greater than 95%) compared to the commercial C_{18} (32–47%) and aminopropyl SPE columns (2–9%). This confirms the work of Lloyd [15] who found that Chromosorb 104 and Amberlite XAD-4 were the most efficient supports for the recovery of organic explosive residues from relatively polar solvents. To reduce the minimum volume required to elute the organic residues from the 1.5-ml SPE columns prepared in the laboratory, 40 mg of support material was used. Using these columns a 1.4-ml extract from the Deldrin unit is cleaned and concentrated to 300 μl.

Table 1

Average recovery of 10-ng standard containing organic CDRs by SPE on the Millilab workstation

Organic CDR	Recovery (%)		
	SPE support material		
	Chromosorb–Amberlite	C_{18}	Aminopropyl
NG	95	47	5
1,3-DNB	96	36	9
2,4-DNT	96	35	9
DPA	98	42	7
EC	95	39	2
MC	96	32	5

The relative standard deviation of the percentage recovery of the organic residues from the Chromosorb 104–Amberlite XAD-4 SPE columns ranged from 3.5 for 1,3-DNB to 5.5% for DPA.

The Chromosorb 104–Amberlite XAD-4 SPE column prepared in the laboratory allows full automation of the extraction process on the Millilab workstation. Subsequent experiments were performed using these SPE columns.

3.2. Assessment of Millilab 1A workstation extraction of organic CDRs

CDRs are recovered from clothing by suction sampling using a Deldrin filter holder (1-μm fluoropore filter) connected to an Edwards vacuum pump. The efficiency of extraction of organic CDRs from the Deldrin unit and subsequent SPE clean-up and concentration using the Millilab workstation was assessed. Three new cotton laboratory coats were vacuumed for a period of 5 min each using the procedure described in the Experimental section. A 400-μl volume of methanol containing 10 ng of NG, 1,3-DNB, 2,4-DNT, DPA, EC and MC were added to each Deldrin holder to simulate the recovery of organic CDRs from clothing. Organic CDRs were extracted from the three Deldrin units and cleaned-up and concentrated by SPE using the Millilab workstation. To test the system for carryover, clean Deldrin units and SPE tubes (blanks) were extracted after each sample. The extracted organic CDRs and blanks were analysed by HPLC–PMDE and GC–MS. The results are listed in Table 2. All blanks were negative.

It was found that the recovery of organic CDRs was reduced when the Deldrin filter unit was used (57–78% recovery compared to 95% recovery from the SPE columns). This may be explained by the presence of garment fibres recovered with the CDRs. The more material present within the Deldrin unit, the more difficult it is to extract the CDRs with a given

Table 2

Recovery of organic CDRs from Deldrin units and subsequent SPE on Millilab workstation

Organic CDR	Extraction efficiency (%)
NG	78
1,3-DNB	72
2,4-DNT	74
DPA	57
EC	60
MC	67

volume of acetonitrile (total extract 1.4 ml). Using a greater volume of acetonitrile poses problems with the subsequent 1:9 dilution of extracts for SPE. The Millilab workstation is limited to using 160 mm × 10 mm tubes for dilution (a total workable volume of 14 ml).

When examining "dirty" garments a number of Deldrin units may be required to cover the entire surface as a result of the fluoropore filter becoming clogged with material, hence reducing the vacuuming efficiency. An attempt to use a 20-μm pre-filter to prevent clogging was abandoned because this resulted in reduced recovery of inorganic CDRs.

3.3. Recovery and analysis of CDRs from clothing (six shots)

The efficiency of the technique to recover and detect CDRs from different types of clothing worn during the firing of six rounds of ammunition from a revolver was assessed.

Three different items of clothing with varying retentive properties for CDRs (laboratory coat, woollen jumper and sweatshirt) were doped with residues from a Colt python .357 Magnum revolver using double-based Winchester .357 ammunition by wearing each garment and firing six shots in still air conditions. The garments were suction sampled for residues within 1 h of the shooting using recommended procedures. The recovered residues were extracted and analysed for organic and inorganic CDRs. Results are listed in Table 3. Precautions were taken to ensure that no contamination of garments with CDRs from other sources occurred. Samples of air within the room where the shooting took place and the hands and clothing of the person prior to performing the shootings were analysed and found to be negative.

CDRs were recovered from all garments, with the sweatshirt and laboratory coat giving better recovery than the woollen jumper. In all cases organic and inorganic CDRs were easily identified. It was assumed at the start of the experiment that the woollen jumper would have the best retention of CDRs but this was not reflected in the results. It is suggested that a reason for this could be the vacuum suction sampling procedure which works best on flat/tight weave garments such as the laboratory coat and sweat shirt.

3.4. Recovery and analysis of CDRs from clothing (one shot)

The suction sampling and analysis techniques were repeated to determine if organic and inorganic CDRs could be detected on clothing worn

Table 3

Analysis of CDRs recovered from clothing (six shots)

Garment	Organic CDR (ng)				Inorganic CDR (No. of particles)			
	NG	2,4-DNT	DPA	EC	Pb, Sb, Ba	Sb, Ba	Pb, Sb	Pb, Ba
Laboratory coat	976	39	4.6	1	3	2	71	3
Sweatshirt	1273	39	7.4	2.2	30	5	175	13
Woollen jumper	730	10	1.7	0.5	1	–	34	6

during the firing of one round of ammunition from a revolver.

Two new laboratory coats were each doped with residues from a single shot using the same revolver and ammunition already described. Precautions were taken to avoid contamination from extraneous sources. The samples were extracted and analysed for CDRs. The results are listed in Table 4.

Detectable quantities of organic and inorganic residues were recovered from the laboratory coats (although no 2,4-DNT or MC was detected). The amounts of NG, DPA and EC detected were well above the detection limits of the systems. A smaller number of inorganic CDR particles were recovered from the laboratory coats compared to the garments doped with six shots. Examples of HLPC–PMDE and GC–MS chromatograms of organic residues recovered from a laboratory coat worn during the firing of a single shot are shown in Figs. 1 and 2.

3.5. Survey of clothing submitted to the laboratory for CDR examination

For a trial period of three months clothing submitted to the laboratory for inorganic CDR analysis were also examined for organic residues. Organic residues detected during the trail period were not used as evidence in criminal proceedings. Thirteen different firearm-related incidents (cases F1–F13) with a total of 186 exhibits were examined. One case F1 accounted for 100 exhibits. The positive results are listed in Table 5.

Only one exhibit, mask (a) in case F13, was positive for inorganic CDRs although no organic CDRs were detected for this item. The indicative

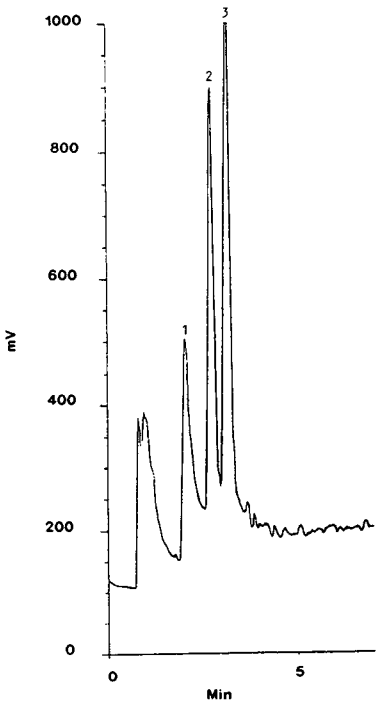

Fig. 1. HPLC–PMDE chromatogram (50 nA full scale) of organic residues recovered from a laboratory coat worn during the firing of one shot. Chromatographic conditions: Zorbax ODS column, 150 × 4.6 mm I.D.; eluent, methanol–phosphate buffer pH 3.0 (55:45), flow-rate 1.2 ml/min. Detector setting −1 V. Peaks: 1 = oxygen; 2 = 1,3-dinitrobenzene; 3 = nitroglycerine.

inorganic particle Pb, Ba was detected in 17 exhibits from four cases but, in the absence of any unique inorganic particles (Pb, Sb, Ba/Sb, Ba), they were reported as negative.

Five exhibit extracts from three different cases were positive for organic CDRs with two of these exhibits also having a single indicative (Pb,

Table 4

Analysis of CDRs recovered from clothing (one shot)

	Organic CDR (ng)			Inorganic CDR (No. of particles)			
	NG	DPA	EC	Pb, Sb, Ba	Sb, Ba	Pb, Sb	Pb, Ba
Laboratory coat 1	775	3.5	2.6	2	1	1	–
Laboratory coat 2	910	8.7	4.3	3	–	7	14

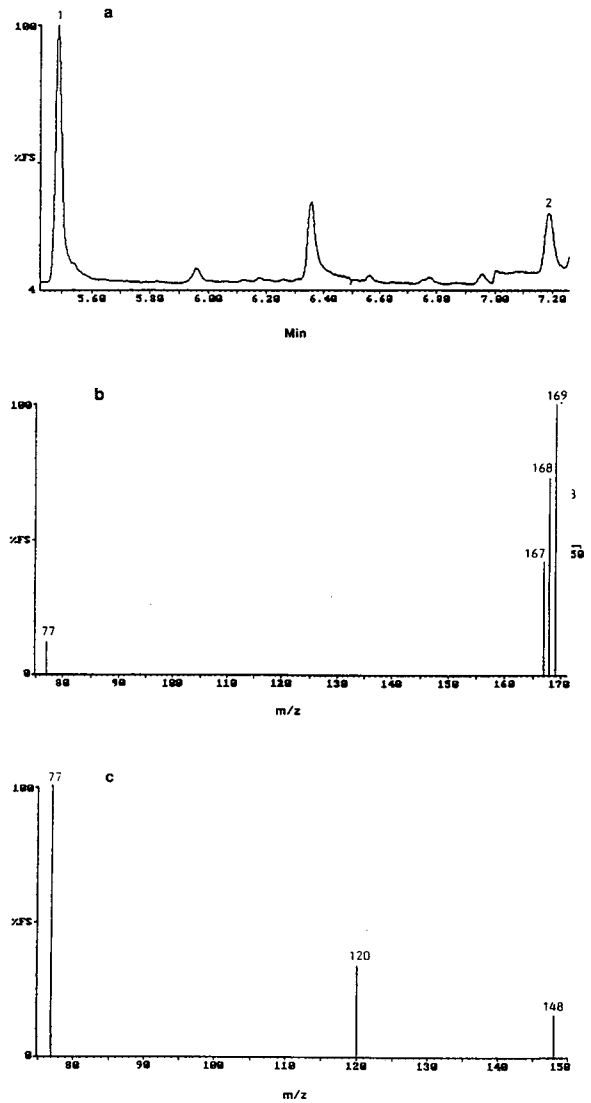

Fig. 2 (a) GC–MS total ion chromatogram of organic residues recovered from a laboratory coat worn during the firing of one shot. Chromatographic conditions: capillary column, Rtx-1, 15 m × 0.32 mm I.D., 1 μm film thickness; helium carrier gas, inlet pressure 5 p.s.i.; temperature 85°C initially then ramped to 250°C at 30°C/min, maintained at 250°C for 5 min. GC–MS interface and source temperature 250°C; scan rate 0.9 s, interscan time 0.1 s; masses scanned 45–300 u full scan; electron impact mode 70 eV. Peaks: 1 = diphenylamine; 2 = ethyl centralite. (b) GC–MS selective ion recording confirmation of diphenylamine recovered from a laboratory coat worn during the firing of one shot. GC–MS conditions as for (a). (c) GC–MS selective ion recording confirmation of ethyl centralite recovered from a laboratory coat worn during the firing of one shot. GC–MS conditions as for (a).

Ba) inorganic particle detected. All three cases would have been reported as negative based on the inorganic CDR results. Unfortunately it was not possible to confirm DPA, EC and MC in the extracts from the 186 items by GC–MS due to a terrorist explosion at the laboratory which resulted in the loss of the samples prior to analysis.

Only 5 out of 186 exhibits were positive for organic CDRs and only 1 exhibit positive for unique inorganic CDR particles. This may be the result of a number of factors:

(1) The suspects may not have fired a weapon.

(2) The suspects in the 13 firearm incidents were not arrested at the scene of the crime. It was therefore a period of time before their clothing was collected and submitted to the laboratory.

(3) Terrorists in Northern Ireland take considerable precautions to avoid the deposition and recovery of CDRs from their clothing (such as the wearing of boiler suits and rubber gloves).

On the basis of this trial the analysis of organic CDRs has greater sensitivity than inorganic CDR analysis. The detection of a unique inorganic CDR particle in the absence of organic residues from case F13 exhibit (a), may be the result of the composition of the ammunition used. A survey of propellants encountered in the British Forensic Science Laboratories found that 2 out of 5 propellants did not contain NG [16]. Using GC–MS analysis increases the range and specificity of organic CDRs that can be detected. Further work analysing unburnt or partially burnt propellant removed from gunshot entrance holes on clothing submitted to the laboratory over period 1991–1992 found that a combination of organic CDRs were identified in 60 out of 61 samples. DPA was identified in 57 propellant samples. More importantly DPA only was found in 34 samples, emphasising the need for all systems —HPLC–PMDE, GC–MS and SEM–EDAX.

4. Conclusions

An efficient vacuuming system for the recovery of organic and inorganic CDRs from

Table 5

Survey of clothing submitted to the laboratory for CDR analysis

Case	Exhibit	Organic CDR (ng)		Inorganic CDR (No. of particles)			
		NG	2,4-DNT	Pb, Sb, Ba	Sb, Ba	Pb, Sb	Pb, Ba
F1	(a) Upper front body/cuffs	124	93	–	–	–	–
F1	(b) Pockets	300	4	–	–	1	–
F3	(a) General outer/body	124	–	–	–	–	–
F3	(b) General outer/body	2068	4	–	–	–	–
F8	(a) Front pocket	1685	–	–	–	1	–
F13	(a) Mask	–	–	1	1	1	1

clothing and an automated system for the clean-up and concentration of organic residues has been developed. Using this system CDRs from a single shot fired under laboratory conditions can be detected on clothing by HPLC–PMDE, GC–MS and SEM–EDAX.

A survey of clothing submitted to the firearms laboratory for examination suggests that the systems used for the detection of organic CDRs are more sensitive than the SEM–EDAX system for the detection of inorganic CDRs.

In the survey five items of clothing were positive for organic CDRs and only one item of clothing positive for inorganic CDRs, emphasising the need to analyse for both types of residue. The automated clean-up technique is to be applied to the recovery of CDRs from hand swabs.

5. Acknowledgements

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Volatiles from carpet: a source of frequent misinterpretation in arson analysis

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Abstract

Carpet and carpet padding are frequently submitted to laboratories for the analysis of residual accelerant in suspect arson cases. Charring and pyrolysis of the above matrixes produces volatiles that can cause interferences with some accelerants, most notably gasoline. A study was conducted to evaluate the interference potential of pyrolysis products obtained from simulated fire debris samples and from samples collected in the field. It was found that variable amounts of alkylbenzenes and naphthalenes are produced, along with large quantities of styrene and alkylstyrenes. Small amounts of higher-molecular-mass aromatic hydrocarbons are also generated but no alkanes or alkenes appeared.

Since aromatic hydrocarbons are the major diagnostic components in gasoline, a potential interference is indicated. Careful observation of patterns within each group of isomers is required to rule out interferences. The distribution of alkylbenzenes in gasoline is quite different from the patterns produced by pyrolysis. Guidelines are proposed to distinguish between petroleum-based aromatic hydrocarbons and those derived from the pyrolysis of carpet/carpet padding. Mass fragmentography was found to be particularly useful toward the discrimination of interferences.

1. Introduction

Chemical analysis of residual accelerant in fire debris is an important investigative tool to determine whether a fire loss can be attributed to natural causes, such as malfunctioning electrical circuits, or is incendiary in nature [1]. One of the tasks of an arson investigator is to examine burn patterns at the fire scene and secure debris samples for later chemical analysis [2]. Among the many materials that can be collected for testing, carpet and carpet padding represent an almost ideal choice from an arson investigator's point of view. Carpet is widely encountered at the fire scene because most modern homes are furnished with carpeting. It is common practice in the building industry to install carpet on padding to provide additional insulation and improve comfort. Since most incendiary fires are started by pouring a petroleum-based liquid along the walls of a structure, there is a good possibility of locating a burn pattern on carpet. The favorable adsorptive properties of carpet and carpet padding make them good candidates for the preservation of residual accelerant. One needs to keep in mind that combustion under such conditions is a gas-phase process that only involves the vapor above the solid. A trace of an accelerant that has deeply penetrated into the matrix may remain in recoverable quantities, even after a severe fire.

It is not surprising that approximately two-thirds of all samples submitted for chemical analysis consist of carpet/carpet padding or, at

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least, contain some of these matrixes [3]. From a chemist's point of view, carpet/carpet padding has a number of disadvantages. Modern carpets generally are composed of synthetic fibers, such as nylon, that are attached to a support consisting of a polypropylene copolymer. Carpet padding is usually composed of a synthetic rubber that sometimes contains fiber for added strength. When heated to a temperature where pyrolysis takes place, a variety of products are generated from the polymers. In contrast to other building materials such as sheet rock, wood and tile, carpet and carpet padding produce copious amounts of volatiles. Combustion studies indicate that carpet padding does not generate hydrocarbons that can be confused with petroleum-based products [4]. The fiber in carpet does not produce significant amounts of hydrocarbons but the carpet backing is a rich source for alkylbenzenes and similar compounds. Aromatic hydrocarbons, including benzene, toluene, ethylbenzene, styrene, and alkylstyrenes have been identified in charred carpet [5–7].

Accelerant analysis is commonly carried out by comparing the gas chromatographic (GC) profile from a sample to chromatograms from standards [8]. Most laboratory analysts keep a library of chromatograms from petroleum-based fuels at hand. Results from round robin tests involving charred carpet show that isolation of residual accelerant and separation of isolated volatiles by GC do not seem to present problems. Serious errors however may occur in the interpretation of chromatographic data. Approximately 10% of all laboratories participating in a large-scale study reported an accelerant in charred carpet which did not contain a flammable substance [9]. In most cases gasoline was reported. This is obviously a very serious error. The situation is compounded because gasoline is the most frequently used accelerant in incendiary fires [10,11].

Gasoline is relatively difficult to recognize in a chromatogram. In contrast to many other accelerant types such as kerosene, gasoline does not produce a pattern of equidistant peaks. A further complication arises from exposure to heat and other environmental factors. While the effects of partial evaporation are easily compensated for in accelerants that are dominated by *n*-alkanes, chromatographic patterns for highly evaporated gasoline are much more difficult to recognize [12]. Only 70% of some 120 laboratories supplied with a 95% evaporated gasoline were able to correctly interprete the chromatographic profile [9]. The variable contribution from different matrixes make analysis of petroleum-based accelerants particularly difficult. In most laboratories, capillary column GC with flame ionization detection is the standard method. This will probably change in the not too distant future. With the large scale introduction of benchtop mass spectrometers, GC–MS is becoming increasingly accepted, even in small laboratories.

Mass fragmentography is an effective method for the reduction of chemical interferences [13]. Petroleum-based fuels contain a large number of individual compounds. Fortunately, the bulk of these substances falls into only a few categories [14–16]. Mass fragments can be assigned that are common to individual categories, *i.e.*, m/z 57, 71, etc. represent alkanes. Examination of mass fragmentograms representing alkylbenzenes shows that carpets produce several of the compounds that are diagnostic indicators for petroleum-based fluids, in particular of gasoline.

In this study, we report on the generation of some alkylbenzene isomers originating from the combustion of carpet/carpet padding. Results from simulated arson samples and from samples obtained in the field are presented. Guidelines are proposed to distinguish alkylbenzenes originating from the pyrolysis of carpet/carpet padding to those found in petroleum-based fluids.

2. Experimental

Carpet and carpet padding for the control experiments were obtained from local sources. Actual arson samples were also processed [17]. The carpet and carpet padding in the control experiments were placed in one-gallon (*ca.* 3.6 l) paint cans and charred on a Bunsen burner under a variety of conditions. In some experiments, charring was extensive to simulate the

effects of a burnout. In others, fires were extinguished by dousing with water or by oxygen starvation. Unburned carpets, both used and fresh from the shelf, were also investigated. Volatiles were collected by a heated headspace method using a scaled down version of conventional charcoal adsorption/CS_2 extraction [18]. Sample extracts were analyzed by GC–MS on a standard benchtop instrument (MSD 5970A, Hewlett-Packard, Avondale, PA, USA). A standard fused-silica capillary, 25 m × 0.21 mm, coated with a 0.33-μm film of methylpolysiloxane was used under temperature-programmed conditions. Data were examined after manual extraction of ion chromatograms as well as by automated data processing [8,13].

3. Results and discussion

Interpretation of chromatographic data from fire debris remains one of the most difficult and ill-defined steps in the analysis of accelerants from suspect arson samples. Interpretation is commonly carried out by visual inspection of chromatograms. The analyst attempts to visually extract an accelerant pattern from the target sample. This is a manual process in which the chromatographic profile from the sample is compared to profiles from a series of accelerant standards. The major difficulties in interpretation originate from the contributions of extraneous components and from changes in accelerant profiles due to environmental factors. Chromatographic resolution, ratio of interferences to accelerant, and methods of chromatogram comparison influence the outcome, to various degrees [12]. A mass spectrometer can be used as a tunable detector to suppress chemical noise from the matrix. The combination of retention data and mass spectral selectivity greatly improves the level of confidence but it can only work if the components generated from pyrolysis differ in mass spectral response characteristics from those produced from the analyte. It is a manual process where the analyst attempts to visually extract an accelerant pattern from a suspect sample and correlate it to a standard. The major difficulties in interpretation originate from the contributions of extraneous components and changes in accelerant profiles due to environmental factors. Chromatographic resolution, ratio of interferences to accelerant, and method of chromatogram comparison influence the outcome, to various degrees [12]. A mass spectrometer can be used as a tuneable detector to suppress chemical noise from the matrix. The combination of retention data and mass spectral selectivity greatly improves the level of confidence but it can only work if the components generated from pyrolysis differ in mass spectral response characteristics from those produced from the analyte.

Fig. 1 shows the total ion chromatograms of charred carpet/carpet padding before and after spiking with a small amount of partially evaporated gasoline. The corresponding gasoline standard is also shown. It is evident that charred carpet contains only a few of the substances found in gasoline. Due to the high level of interferences from pyrolysis products, it is quite impossible to recognize a gasoline pattern in the simulated arson sample. Large amounts of styrene and methylstyrenes suppress the chromatogram scale and many of the substances from the background coelute with the compounds of

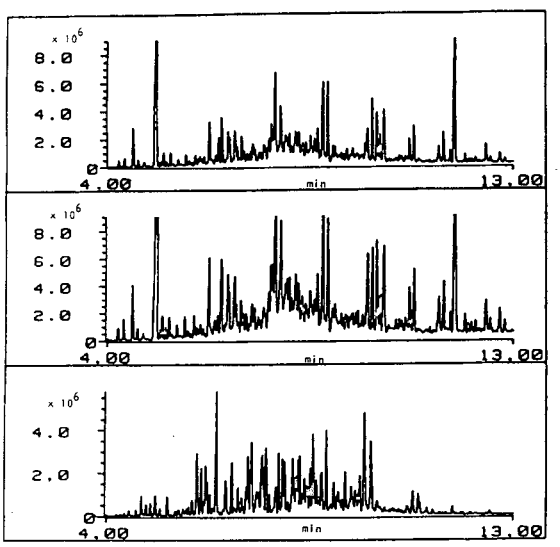

Fig. 1. Total ion chromatograms. Bottom: 50% evaporated gasoline standard; middle: burned carpet/carpet padding, spiked with a small amount of the evaporated gasoline standard; top: burned carpet/carpet padding matrix.

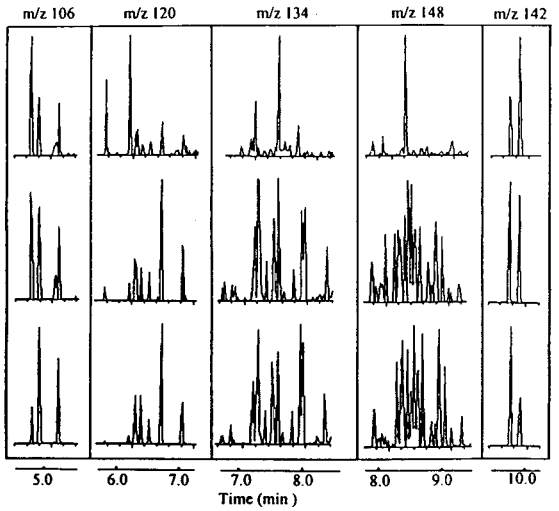

Fig. 2. Selected ion chromatograms of the samples from Fig. 1. m/z 106, 120, 134 and 148 are C_2–C_5 alkylbenzenes, and m/z 142 are methylnaphthalenes.

interest. The major peak at the beginning of the matrix and spiked matrix chromatograms represents styrene. Fig. 2 represents several extracted ion chromatograms from Fig. 1. For brevity, only a few of the target components are shown. The sequence of chromatograms is the same as in Fig. 1. Gasoline contains only small amounts of styrenes whereas this substance class constitutes the major compounds in charred carpet. Fragments m/z 106, 120, 134, 148 and 142 are indicative of C_2–C_5 alkylbenzenes and of the two methylnaphthalene isomers. These substances are considered primary indicators of highly evaporated gasoline. Examination of the fragments shows that the ion profiles between gasoline and charred carpet differ significantly. It can be seen that the matrix contains small amounts of aromatic hydrocarbons. In the charred carpet, one isomer usually dominates in each group. In some cases, more than one isomer may be generated during combustion but the ratio of isomers within a group is always distorted and different from gasoline. Distortions are particularly noticeable in the xylene isomer group where ethylbenzene is often larger than the other isomer. The spiked ion chromatograms show that the characteristic ions for gasoline are present but additional peaks have appeared. Different carpets do not always produce the same interferences. The number of isomers generated by pyrolysis is relatively small. Carpets also produce naphthalene and methylnaphthalenes. The generation of naphthalene is particularly noteworthy. It seems to be correlated to styrene. When styrene is present in large amounts, a large naphthalene peak usually is also visible. The 2-methylnaphthalene isomers are also affected. The ratio between the two methylnaphthalene isomers is about 1:0.5 in favor of the 2-methylnaphthalene isomer which elutes first on a non-polar column. In carpet pyrolysate, these ratios are often reversed and the 1-methylnaphthalene peak is often larger than the 2-methylnaphthalene peak. In contrast, isomer distributions in petroleum-based products are highly reproducible. It should be noted that the diagnostic components of gasoline are also contained in small amounts in straight-run distillates, such as mineral spirits, kerosene and fuel oils. Even though these fuels have significantly different boiling point ranges and physical properties, chromatographic patterns of alkylbenzenes are highly reproducible and resemble those found in gasoline [13]. Peak ratios among each isomer group vary little, even for partially evaporated fuels. A pattern recognition process can, therefore, be instituted that takes into consideration the contributions from pyrolysis products.

The question of "false positives" in accelerant analysis is a matter of continuous discussion in the community of laboratory analysts. The data from this study indicate that some accelerant type compounds can be generated from pyrolysis of carpet/carpet padding but profiles consistent with accelerants are not produced unless the accelerant is already present in the matrix or is adsorbed on the matrix. Under conditions of very high sensitivity, virgin carpet can produce a discernable amount of petroleum-type hydrocarbons. These are likely left over from production or are simply absorbed on the surface [19]. The chemist should always be cautious when the fire debris contains newly manufactured materials. Extraneous contributions from petroleum-based products are a very serious source of interfer-

ences. Treatment of carpets with materials containing a petroleum base, such as a water-proofing agent or an insect spray can generate profiles consistent with gasoline. Interferences due to adsorption from air can easily be dealt with by adjustment of a proper threshold level. Unfortunately, it is far more difficult to eliminate potential artifacts from the second source. It is not possible to reproduce the pyrolysis conditions of a fire in the laboratory. So called control samples are meaningless for background evaluation [20]. Only vigilance on part of both the fire investigator and chemist can minimize such problems.

4. Conclusions

Data from simulated arson samples and from a field study show that the pyrolysis of carpet/carpet padding generates a small number of aromatic hydrocarbons. Even in the presence of these interferences, gasoline can be recognized by setting thresholds for the ratios of the components constituting the various isomer groups. The presence of large amounts of styrene and methylstyrenes always indicates pyrolysis and special caution must be exercised in data interpretation of such cases.

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Capillary Electrophoresis

Principles, Practice and Applications

by **S.F.Y. Li**, *National University of Singapore, Singapore*

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Capillary Electrophoresis (CE) has had a very significant impact on the field of analytical chemistry in recent years as the technique is capable of very high resolution separations, requiring only small amounts of samples and reagents. Furthermore, it can be readily adapted to automatic sample handling and real time data processing. Many new methodologies based on CE have been reported. Rapid, reproducible separations of extremely small amounts of chemicals and biochemicals, including peptides, proteins, nucleotides, DNA, enantiomers, carbohydrates, vitamins, inorganic ions, pharmaceuticals and environmental pollutants have been demonstrated. A wide range of applications have been developed in greatly diverse fields, such as chemical, biotechnological, environmental and pharmaceutical analysis.

This book covers all aspects of CE, from the principles and technical aspects to the most important applications. It is intended to meet the growing need for a thorough and balanced treatment of CE. The book will serve as a comprehensive reference work and can also be used as a textbook for advanced undergraduate and graduate courses. Both the experienced analyst and the newcomer will find the text useful.

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(Detailed *Instructions to Authors* were published in *J. Chromatogr. A*, Vol. 657, pp. 463–469. A free reprint can be obtained by application to the publisher, Elsevier Science B.V., P.O. Box 330, 1000 AH Amsterdam, Netherlands.)

Types of Contributions. The following types of papers are published: Regular research papers (full-length papers), Review articles, Short Communications and Discussions. Short Communications are usually descriptions of short investigations, or they can report minor technical improvements of previously published procedures; they reflect the same quality of research as full-length papers, but should preferably not exceed five printed pages. Discussions (one or two pages) should explain, amplify, correct or otherwise comment substantively upon an article recently published in the journal. For Review articles, see inside front cover under Submission of Papers.

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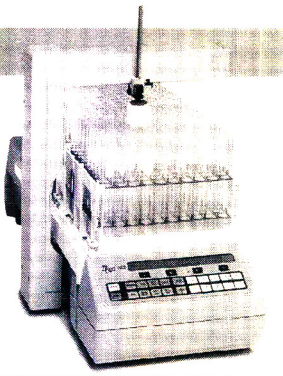

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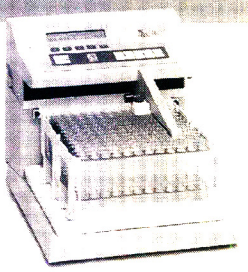

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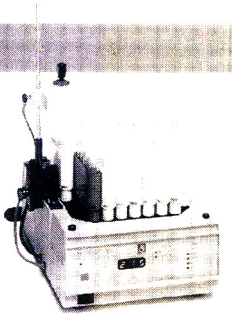

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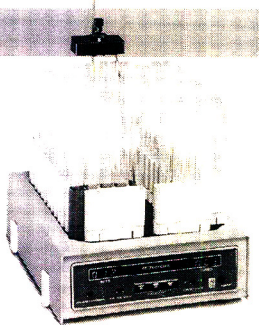

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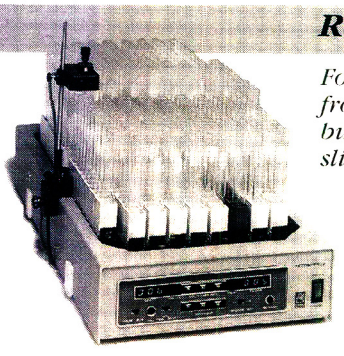

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