JOURNAL OF
CHROMATOGRAPHY
INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

Vol. 165 No. 3 October 1, 1979

CHROMATOGRAPHIC REVIEWS (Vol. 23, No. 3)

edited by

Michael Lederer

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PUBLICATION SCHEDULE FOR 1979

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1978	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	166/1 166/2 167	168/1 168/2	169 170/1	170/2	171 172	173/1 173/2	174/1	174/2 175/1 175/2	176/1 176/2	176/3 177/1 177/2	178/1 178/2	179/1 179/2 180/1	180/2
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Biomedical Applications		162/1	162/2	162/3	162/4	163/1	163/2	163/3	163/4	164/1	164/2	164/3	164/4

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VOL. 165, NO. 3 JOURNAL OF CHROMATOGRAPHY OCTOBER 1, 1979

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Recent Developments in Chromatography and Electrophoresis

Proceedings of the 9th International Symposium on Chromatography and Electrophoresis, Riva del Garda, 15-17 May, 1978

edited by **A. FRIGERIO** and **L. RENOZ**

CHROMATOGRAPHY SYMPOSIA SERIES, Volume 1

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This work, reflecting current developments in the use of chromatography and electrophoresis, will be of value to research workers in chemistry, biochemistry, medicine, toxicology, drug metabolism, forensic science, clinical chemistry and pollution studies.

March 1979 x + 358 pages US $58.50/Dfl. 120.00 ISBN 0-444-41785-0

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This book has been written in response to the great interest currently being shown in modification of some the older, and many newer antibiotics to improve upon existing, naturally produced compounds.

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Aug. 1978   x + 772 pages   US $84.75/Dfl. 195.00   ISBN 0-444-41727-3

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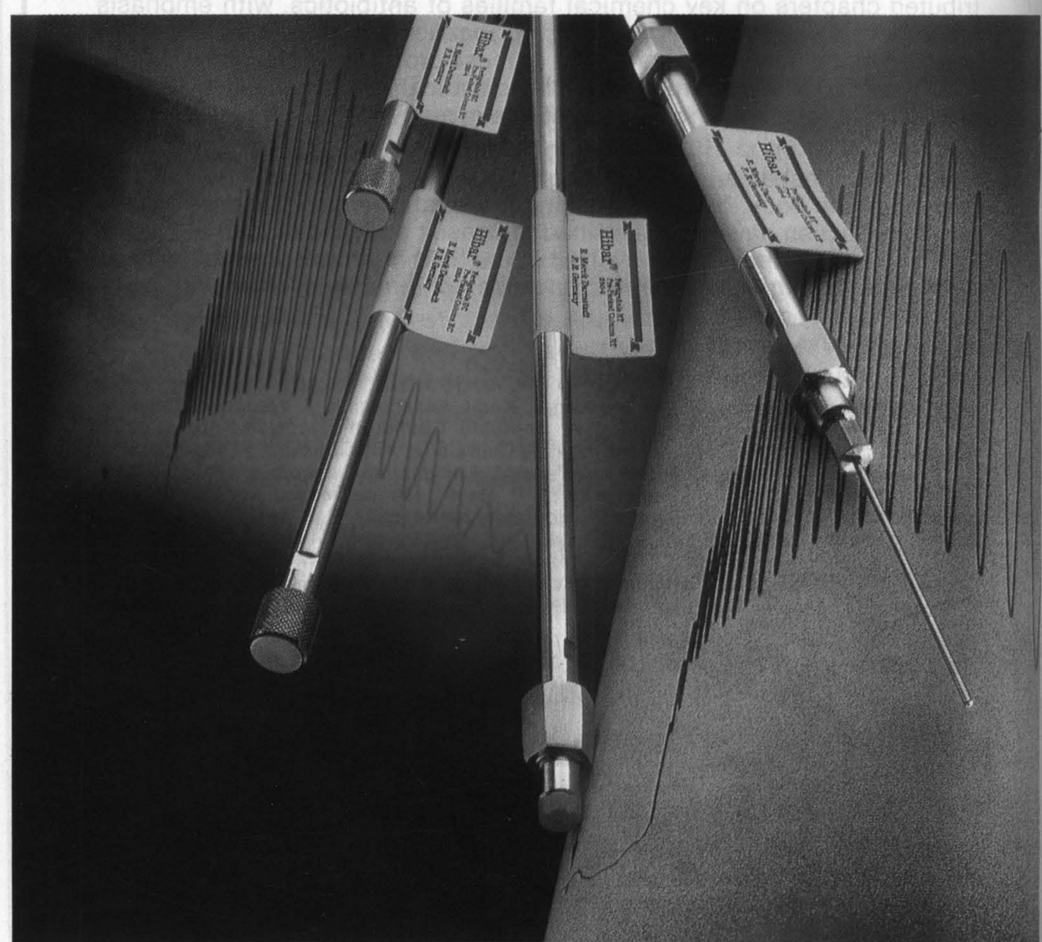

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Journal of Chromatography, 165 (1979) 207–233
Chromatographic Reviews
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CHREV. 120

COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY: A POWERFUL TOOL IN ANALYTICAL CHEMISTRY*

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(First received December 19th, 1978; revised manuscript received March 20th, 1979)

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1. INTRODUCTION

The combination of gas chromatography with mass spectrometry (GC–MS) is one of the most powerful tools available in analytical chemistry. The coupling of these two techniques enables the chemist to detect and identify very small amounts of organic compounds in complex mixtures. GC was introduced in 1952 but it was soon found that, like all analytical techniques, it had various shortcomings. It became clear that it was impossible to identify the hundreds of compounds present in fruit extracts, cigarette smoke, urine, etc., from GC data alone. The solution to this problem was obvious: a detection system was needed with a unique response to any component eluted from the GC column. Such a detector, the mass spectrometer, existed long before the chromatograph was introduced.

* Plenary Lecture presented at the *12th International Symposium on Chromatography, Baden-Baden, September 25–29th, 1978*. The majority of plenary Lectures and Reviews presented at this symposium has been published in *J. Chromatogr.*, Vol. 165, No. 1 (1979).

Mass spectrometry has its origin in the mass spectrometers first designed by Aston[1] and Dempster[2]. It separates chemical elements into their isotopes and is based on the principal that ions accelerate to a certain kinetic energy by an electric field describe, in a subsequent magnetic field, paths that differ according to their mass-to-charge ratios. Nowadays mass spectrometers exist with mass analysers other than the magnetic type, such as quadrupole and time-of-flight mass spectrometers. In the ion source of the mass spectrometer the organic molecules are bombarded in a vacuum by energy-controlled electrons emitted from a heated filament. In the ionization process not only molecular ions are formed, but also a large number of different fragments.

The fragmentation pattern found is characteristic of the identity of the organic molecules ionized, and this was the basis for the application of mass spectrometry in the identification of organic compounds[3–6]. The application of mass spectrometry was satisfactory when applied to pure compounds, but failed when the compounds being analysed were not pure. As GC is an ideal separation tool and the mass spectrometer, as a very sensitive and characteristic detector, was available, it was obvious that these complementary techniques should be combined. The coupling of these two instruments, however, was not easy, owing to their incompatibility, the gas chromatograph operating at atmospheric pressure and the mass spectrometer at high vacuum.

In the early days of the application of the two techniques, each component was trapped as it emerged from the GC column and was transferred manually to the batch inlet system of the mass spectrometer. This procedure had the advantage that both instruments could operate without affecting each other's properties. This, however, was overshadowed by some major disadvantages. The trapping of, *e.g.*, 100 components as found in fruit extracts, for example, from a column is very time consuming and difficult. Even if they can be collected, many of the components will not be pure and there is the possibility that they will be hydrolysed, oxidized or decomposed by some other mechanism before they are analysed in the mass spectrometer. In order to overcome these problems, coupling of the two instruments was necessary. With the advent of combined GC–MS two different types of interfaces were developed: the frit separator introduced by Watson and Biemann[7], and the jet separator introduced by Becker[8] and Ryhage[9]. During the last 15 years, many interface devices have been developed[10,11].

2. REQUIREMENTS FOR OBTAINING AN OPTIMAL GC–MS COMBINATION

2.1. Chromatographic separation

In order to obtain useful information from the mass spectrum, it is necessary for the substance to be introduced into the mass spectrometer to be pure. This means that the separation power of the GC column should be optimal. If complex mixtures have to be analysed, high-resolution capillary columns should be used. Stationary phases are chosen, depending on the problems to be solved. Fractionation of complex mixtures is often also needed, as trace compounds have to be concentrated and separated from interfering main compounds.

However, when the characteristic masses of two compounds differ from each other, interpretation of the mixed spectra is possible, as will be shown later.

The stationary phase should have a low volatility and good thermal stability[12] in order to yield stable baselines and low bleed intensity so that the spectra will not be mixed up with background masses. Frequently excessive bleeding decreases the detection limit for the determination of trace compounds as their low-intensity masses interfere with those of the stationary phase. Continuous bleeding of, for instance, silicone vapours contaminate the ion source and the mass analyser. This may result in severe decreases in sensitivity, resolution and stability.

2.2. Requirements for the mass spectrometer

The requirements for the mass spectrometer in a GC-MS combination are more complicated. They have been described in the literature[10,13].

2.3. Vacuum system

The mass spectrometer must be kept under high vacuum for proper functioning, so that ion–molecule reactions and peak broadening can be avoided. Therefore, it has to be equipped with a differential pumping system, with one pump for the mass analyser and a second for the ion source. The latter is the most critical, as it has to pump off the entering carrier gas while maintaining an acceptable vacuum (10^{-5} Torr). Flow-rates accepted by existing commercial mass spectrometer vary from 0.5 to 10 ml/min of helium. Very good pumping capacities are achieved with turbomolecular pumps; nowadays these are being increasingly used in mass spectrometers. Pumping capacity-reducing cooling baffles and vacuum valve systems can be omitted.

2.4. Dynamic range

In order to cover the ranges of sample amounts used in GC, the dynamic range of the mass spectrometer should be about six orders of magnitude. Actually, the maximal ion current that can be recorded by the detector without saturation effects should be 10^6 times larger than the minimal ion current discernable above the noise level.

2.5. Ion source optics

The pressure linearity and stability of the ion source must be very good. This means that the intensity of the ion current has to vary linearly over a wide range with the amount of sample introduced. The quality of the ion source optics is also very important as it has to yield and maintain a well defined peak shape and resolution during the GC run, which is important in obtaining reliable results in quantitative trace analyses of complex mixtures with single or multiple ion detection.

2.6. Scanning (cycle) time

In order to obtain adequate information on the purity of a GC peak and to obtain representative spectra, the mass spectrometer should be able to record at least three mass spectra per GC peak (0.5–1 sec per mass decade is required for sharp peaks eluting from capillary columns).

2.7. Computer compatibility

For the efficient handling of the large amount of mass spectral information obtained in GC–MS analyses, connection with a computer system is indispensable.

The application of computers to mass spectrometers started with the processing of high-resolution data[14,15] and it soon became a great help for data acquisition, reduction and interpretation[16–19]. This computerization of a GC–MS system saves time and increases efficiency.

2.8. The GC–MS interface

The most critical part of the GC–MS system is the interface. Many different interface systems have been developed during the past 15 years and have been reviewed in the literature[10,11]. When combining GC with MS, there are some major problems: (a) the pressure drop between GC exit and the mass spectrometer; (b) the GC effluent contains only a small concentration of organic compounds, the remainder being carrier gas. In this context, two properties play an important role: the enrichment factor and the efficiency.

The enrichment factor is defined as the relative increase in concentration of the compound in the carrier gas after passing the interface. The efficiency is the percentage of the amount of the compound in the GC effluent entering the mass spectrometer.

The ideal properties of a GC–MS interface can be summarized as follows:

(1) the interface must not affect the properties of the gas chromatograph (separation) and the mass spectrometer (sensitivity, resolution);

(2) the whole of the sample but none of the carrier gas should be transferred to the mass spectrometer;

(3) no chemical changes in the sample should be caused by the interface;

(4) no discrimination against compounds with particular functional groups;

(5) no adsorption and memory effects;

(6) functioning independent of carrier gas flow-rate and temperature;

(7) the system is suitable for all types of columns, both capillary and packed.

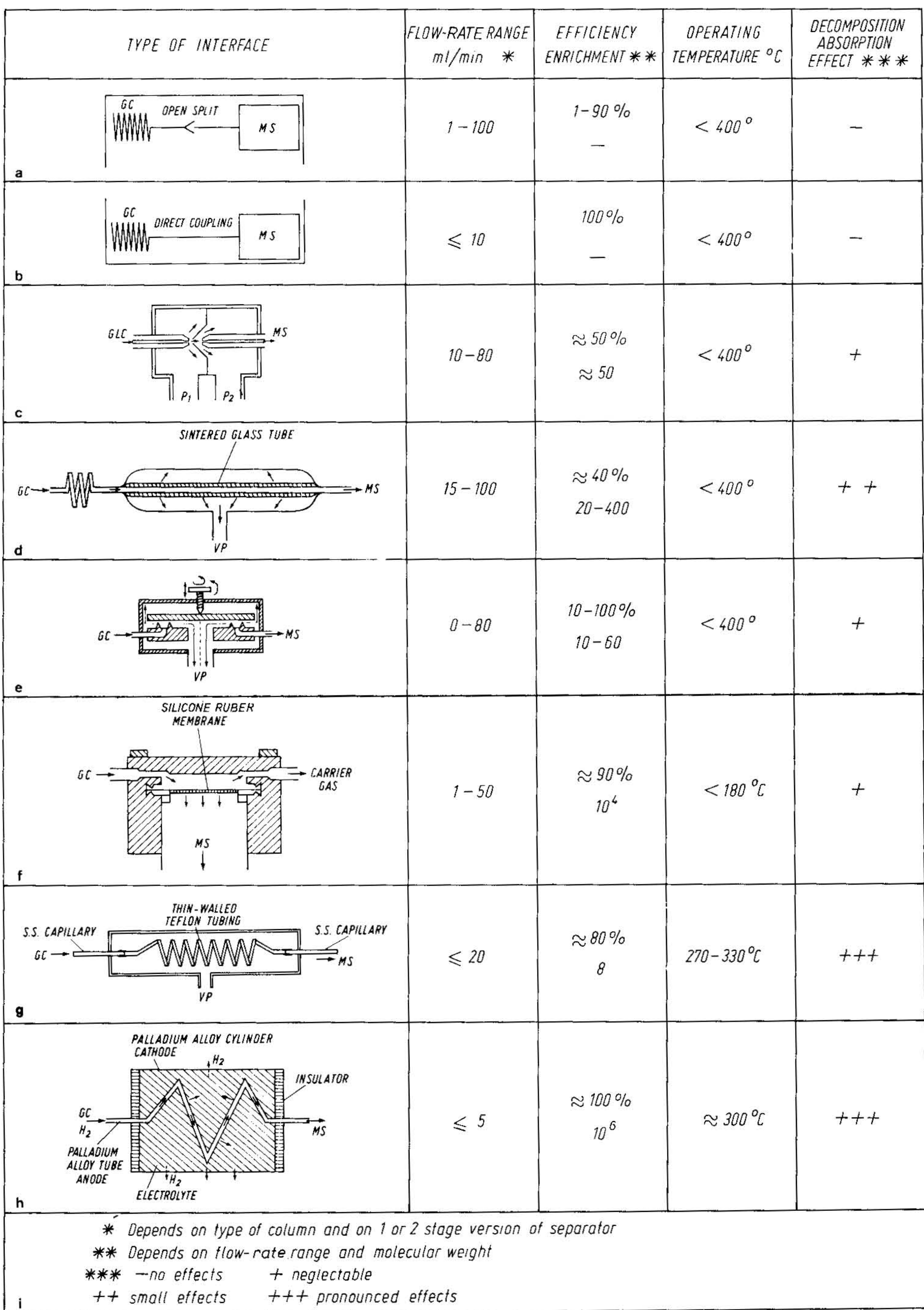
It is obvious that none of these ideal conditions can be achieved by the existing interfaces. Moreover, they all have certain advantages and disadvantages. A brief survey of the most common coupling techniques is given in Fig. 1.

2.9. GC–MS interfacing without a molecular separator

In the early days of GC–MS, the gas chromatograph was connected with the mass spectrometer by means of a splitter system. In this way only a few percent of the GC effluent was admitted to the mass spectrometer. The maximal admissible amount was dictated by the performance of the vacuum system. The mass spectrometer was equipped with a needle valve or narrow inlet capillary so that only 0.1–4 ml of carrier gas (helium) was admitted[20–23] (Fig. 1A).

It is obvious that the efficiency was low (1–10%), whereas no enrichment was achieved. The great advantage of this method is that the GC conditions are not affected; moreover, it is suitable for all types of columns. It is a very simple and cheap device. However, for a long time this system was not very popular, owing to the low efficiency and easy blocking of the inlet capillary, correlated with the poor vacuum systems of the mass spectrometers.

With improvements in the MS vacuum systems and ion sources, open split coupling gained enormously in popularity, and at present it may be said that it is the most recommendable interfacing technique[24–28]. Another type of GC–MS coupling



TYPE OF INTERFACE	FLOW-RATE RANGE ml/min *	EFFICIENCY ENRICHMENT **	OPERATING TEMPERATURE °C	DECOMPOSITION ABSORPTION EFFECT ***
a (GC, OPEN SPLIT, MS)	1 – 100	1 – 90 % / —	< 400°	–
b (GC, DIRECT COUPLING, MS)	≤ 10	100 % / —	< 400°	–
c (GLC, MS, P_1, P_2)	10 – 80	≈ 50 % / ≈ 50	< 400°	+
d (SINTERED GLASS TUBE, GC, MS, VP)	15 – 100	≈ 40 % / 20 – 400	< 400°	+ +
e (GC, MS, VP)	0 – 80	10 – 100 % / 10 – 60	< 400°	+
f (SILICONE RUBER MEMBRANE, GC, CARRIER GAS, MS)	1 – 50	≈ 90 % / 10^4	< 180 °C	+
g (THIN-WALLED TEFLON TUBING, S.S. CAPILLARY, GC, S.S. CAPILLARY, MS, VP)	≤ 20	≈ 80 % / 8	270 – 330 °C	+++
h (PALLADIUM ALLOY CYLINDER CATHODE, H_2, INSULATOR, GC, H_2, MS, PALLADIUM ALLOY TUBE ANODE, H_2, ELECTROLYTE)	≤ 5	≈ 100 % / 10^6	≈ 300 °C	+++

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* Depends on type of column and on 1 or 2 stage version of separator
** Depends on flow-rate range and molecular weight
*** — no effects + neglectable
++ small effects +++ pronounced effects

Fig. 1. Schematic survey of the different interfacing techniques used in GC–MS. (A) Open split coupling; (B) vacuum coupling; (C) jet separator; (D) frit separator; (E) slit separator; (F) membrane separator; (G) Teflon separator; (H) electrolytic silver–palladium separator.

was accomplished by selecting columns for minimal carrier gas flow. The columns could then be connected directly to the ion source (Fig. 1B).

A narrow-bore capillary column (50 m × 0.25 mm I.D.) can be coupled directly to the mass spectrometer through a capillary restriction. The total GC effluent enters the mass spectrometer. This means that the efficiency is 100%. It is a disadvantage that the resolution properties of the column may be affected by the vacuum at the exit of the column. The system is simple and cheap, but is restricted to columns with flow-rates of 1–5 ml/min of helium, depending on the pumping capacity of the mass spectrometer. Vacuum-tight connections, especially at high temperatures, also cause problems, and it is not always easy to change the columns.

2.10. Molecular separators

The interfaces described above were not suitable for every GC–MS application. For the achievement of other coupling systems, enrichment devices, or so-called molecular separators, were constructed. The enrichment process can be divided into three categories:

(a) fractionation of gases in an expanding jet stream[8,9,29,30];

(b) selective effusion through fine pores or through a narrow slit[7,31–34];

(c) preferential diffusion of carrier gas or sample through a semi-permeable membrane (Teflon membrane separator, palladium–silver separator and silicone membrane separator)[35–42].

Today a variety of enrichment devices exist and are frequently applied.

2.10.1. Jet separator (Fig. 1C)

The jet separator, developed by Becker[8] and Ryhage[9], is very widely used. It consists of two- and one-stage designs made of glass and steel. The performance of this type of separator can be summarized as follows:

(1) good efficiency, 50% at a molecular weight of 200 and an enrichment factor of about 50;

(2) performance varies with flow-rate. Flow-rates from 10 to 80 ml/min of helium may be used;

(3) temperatures up to 400° can be applied;

(4) no adsorption or decomposition effects have been reported (hardly any active surface);

(5) separate heating and vacuum pumping are required;

(6) no discrimination against compounds with particular functional groups;

(7) no influence on the GC separation properties.

The single-stage version of this interface is suitable for use with capillary columns (low flow-rates) and the two-stage version for packed columns with flow-rates above 15 ml/min of helium.

2.10.2. Effusion-type separator

The effusion-type separator exists in many different versions. The most commonly used types are those in which the effusion takes place through the pores of a sintered glass frit, designed by Watson and Biemann[7], or through a variable slit between two sharp edges in the variable conductance separator designed by Brunnee

et al.[34]. In other investigations a stainless-steel sinter[43] or porous silver frits[32,33] were used.

Fig. 1D shows the Watson and Biemann separator, made out of glass and consisting of one- and two-stage designs[7,31]. It is widely used. The performance may be summarized as follows:

(a) yields of 50% at a molecular weight of 200 can be obtained as well as an enrichment factor varying from 50 to 400 for the one- and the two-stage version, respectively[31];

(b) for the two-stage version the performance is not affected at flow-rates between 15 and 80 ml/min of helium; at flow-rates less than 15 ml/min a single-stage separator is recommended[31];

(c) temperatures up to 400° can be applied;

(d) adsorption and decomposition effects have been reported[11,31]; these effects, observed for polar compounds, could mostly be eliminated by means of deactivation of the active surface of this type of separator by a silanization procedure[31];

(e) heating and pumping of the interface should be carried out separately;

(f) no discrimination against compounds with particular functional groups;

(g) loss of GC separation properties due to peak broadening has been observed with capillary columns[31].

The properties of the other frit separators with respect to adsorption, decomposition and discrimination effects are poor, which makes them unsuitable for application in organic chemistry.

The properties of the variable slit separator are similar to those of the Watson and Biemann separator. No adsorption or decomposition effects have been reported. However, they may possibly occur due to the metallic surface. The method can be optimized for a wide range of flow-rates from 1 to 100 ml/min of helium. Solvent or high concentrations of other compounds can be diverted from the ion source (Fig. 1E)[34].

2.10.3. Membrane separator

The silicone membrane separator is widely used. The carrier gas (helium) bypasses the membrane and the organic compounds diffuse through it and enter the mass spectrometer. The interface is easy to construct (Fig. 1F). One-[38] and two-stage versions[37] exist. The one-stage version, which was developed later, is the most commonly used because no separate pumping in the second stage is needed.

The performance of this type of interface may be summarized as follows:

(1) efficiencies of up to 95% can be reached and very high enrichment factors can be obtained (10^5 with the two-stage version);

(2) a wide range of carrier gas flow-rates (1–80 ml/min of helium) may be used;

(3) performance is temperature-dependent and limited to 200°; the operating temperature should be the same as the column temperature used for the compounds to be analysed;

(4) no chemical changes to organic compounds have so far been observed;

(5) no separated pumping is necessary (single-stage version); the separator can be built in the GC oven for heating; separate heating is recommended;

(6) no discrimination against compounds with particular functional groups is observed;

(7) GC separation properties are affected, owing to peak broadening[38,44];

(8) saturation and memory effects can be caused by working with large amounts of sample.

In the two other types of membrane separators, the carrier gas diffuses through the membrane instead of the organic compound. The Teflon separator, which was first introduced by Lipsky *et al.*[35], preferentially removes the helium through a very thin Teflon capillary mounted in a vacuum pumped chamber (Fig. 1G). The performance is as follows:

(a) yields up to 80% and enrichment factor 8[36];

(b) carrier gas flow-rate limited to 20 ml/min of helium;

(c) the interface can be operated only in a small temperature range (270–330°);

(d) decomposition of thermally unstable compounds, owing to the high operating temperature;

(e) separate pumping and heating are necessary;

(f) discrimination against functional groups[45];

(g) GC separation properties are affected by peak broadening[45].

As the performance of this interface is poor, it is no longer used in GC–MS systems.

2.10.4. Silver–palladium separator

This interesting interface was first proposed by Lucero and Haley[39], and developed by Simmonds and co-workers[40,41]. It is based on the unique property that a palladium–silver membrane is highly permeable to hydrogen but totally impermeable to other gases and organic compounds at 250°. In later versions it was designed as an electrolytic cell[46,47] and proposed for use in extraterrestrial GC–MS systems[42]. The electrolytic cell, filled with potassium hydroxide–lithium hydroxide, consists of two thin palladium–silver tubes. The inner tube transports the carrier gas and is the anode, and the outer tube is the cathode (Fig. 1H). The fundamental process was described by Lucero[47]. The hydrogen carrier gas is completely removed through the electrolytic cell, while the organic compounds and other gases continue into the mass spectrometer.

The performance of this interface is as follows:

(1) yields of 100%; enrichment factor infinite;

(2) flow-rate range strongly dependent on the interface design;

(3) operating temperature between 200° and 250°;

(4) chemical changes of organic compounds have been reported[40];

(5) pumping of the interface not necessary;

(6) no peak broadening and memory effects;

(7) only hydrogen can be used as the carrier gas;

(8) poisoning of the palladium–silver surface by sulphur and iodine compounds reduces the hydrogen removal.

Owing to the catalytic effects that may occur with unknown organic compounds, which are the most commonly analysed substances in GC–MS applications, this type of separator is not very popular in organic analytical laboratories.

From the many publications on GC–MS applications, it can be concluded that the open split, direct coupling, jet separator, glass frit, slit separator and silicone membrane separator are the most commonly used interfaces. An ideal interface

system which meets all requirements does not exist; for each investigation the best type has to be chosen to solve the particular problems involved.

3. METHODS AND APPLICATION IN GC–MS

In GC–MS applications, two kinds of problems may be distinguished: (a) the investigator wants to know what compounds are present in a sample or extract, and (b) the investigator wants to know if a certain compound or group of compounds is present in an extract. The latter mostly involves trace analysis of complex mixtures.

In the first instance the mass spectroscopist may be overwhelmed by a tremendous amount of information. In the second instance, GC retention times and mass spectra of the compound to be determined are known and the search can be a direct one, as in general it will be sufficient to confirm only the presence of certain masses, characteristic of the compounds at their particular retention times. A common problem which causes difficulties in both instances is that the peaks emerging from the column are not pure, but contain more than one compound. This leaves the spectroscopist with the difficult task of interpreting complex mass spectra and solving interference problems in single or multiple ion detection. To establish whether a GC peak contains more than one compound, it is necessary to record more spectra from every GC peak and then study these with changes in the fragmentation pattern.

Fig. 2 shows a capillary chromatogram with mass spectra taken at the two flanks and the top of the GC peak. The first three spectra are identical, the next three spectra are not identical and there is a change in fragmentation pattern. In the first spectrum the masses of ethylbenzene are dominant, then the masses of amyl alcohol appear and in the last spectrum the masses 106 and 81 of ethylbenzene have decreased. This example is not complicated, but it illustrates well what may happen and the necessity for taking more than one spectrum of a GC peak, in order to obtain information on its purity.

It is clear that many problems exist when a complex chromatogram must be analysed. Such an analysis, resulting in several hundred spectra that have to be calibrated and examined, is an enormous task. The only way of solving the problem of handling such large amounts of data is to connect a computer to the GC–MS system.

Fig. 3 shows schematically a computerized GC–MS combination. The MS yields two signals, one representing the total ion current and the other the MS signal. Both signals are digitized and processed by the computer. The calibrated mass spectral information is stored on magnetic tape or disc. The mass spectrometer can operate in a cyclic scan mode or with the scan controlled by the computer, which means that, for example, every second a mass spectrum is recorded from the GC effluent, so that the GC effluent is sampled mass spectrometrically every second and the complete MS information is available to the investigator after having completed the analysis. This can be of great value, especially when the analyses cannot be repeated. The benefits of such a system are that it will produce the total ion current chromatogram, any individual mass spectrum at any retention time of interest and the mass fragmentogram for any chosen m/e value that is of interest.

The so-called computer mass fragmentography is a very important technique, which enables the investigator to localize any selected masses characteristic of the

Fig. 2. Capillary gas chromatogram and mass spectrum of a mixture of *sec*.-butanol, isoamyl alcohol, ethylbenzene and *n*-amyl alcohol

compounds or group of compounds under investigation. This means that it is very useful for the evaluation of GC peaks that contain more than one compound. Further, spectrum refining can be performed by subtracting mass spectra from each other in order to eliminate interfering masses from neighbouring or overlapping GC peaks

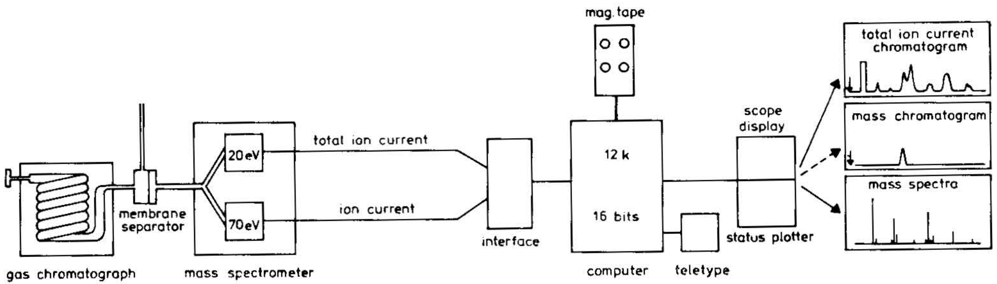

Fig. 3. Schematic diagram of a computerized GC–MS system.

and column bleeding. Library searches of spectra in a reference library and filing of GC–MS analyses are other useful features.

Secondly, knowing the characteristics (retention time and masses) of the compound to be determined, it is not necessary to have all mass spectrometric information available.

This type of analysis can be performed by single or multiple ion detection so that the mass spectrometer is degraded to a very selective and expensive GC detector. The mass spectrometer is equipped with a so-called peak selector, which instructs the mass spectrometer to measure only pre-selected masses. This method is much more sensitive than mass fragmentography performed with a computer and mass spectrometer in the cyclic scan mode. However, this needs careful handling so as to prevent misinterpretations while performing trace analyses in a complex matrix, especially when quantitative results have to be obtained.

The situation is simple when the masses to be searched for are relatively characteristic of those of the matrix in which the trace compounds are hidden. If not, interference of the characteristic masses with the same nominal masses from other compounds occurs. To decrease the chances of interference, increasing the resolving power of the mass spectrometer is a great help because then one can tune the instrument not only to the nominal mass of interest but even to the elemental composition of the *m/e* value one is searching for. For example, if there is a nominal mass 114 characteristic of a compound with the elemental composition $C_5H_{10}N_2O$, there can be interference with the molecular ion of octane, which has the elemental composition

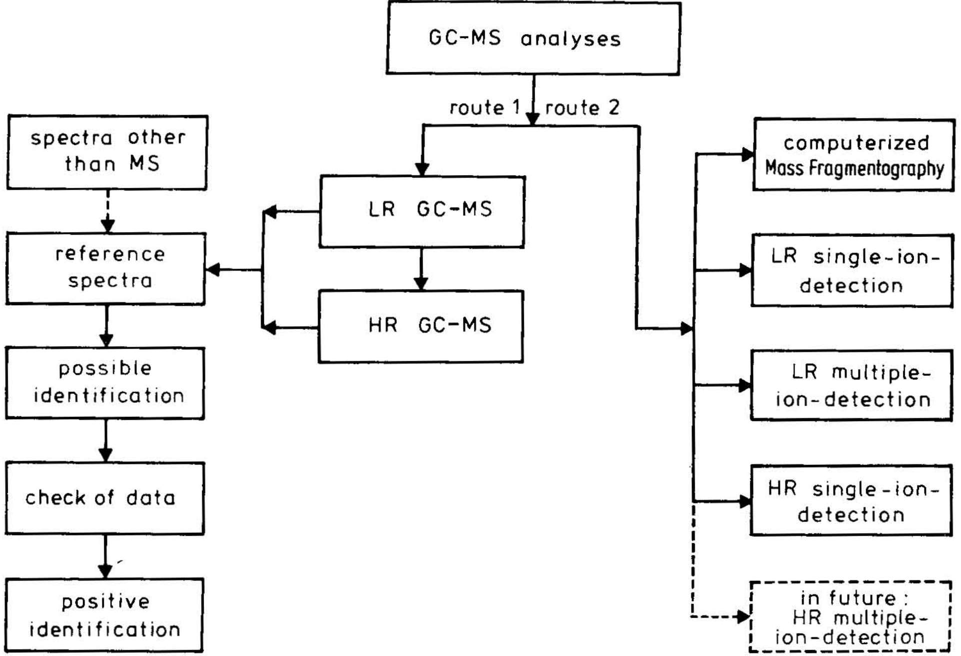

Fig. 4. Flow chart of the different procedures applied in GC–MS analyses. Route 1, multi-component analysis of complex mixtures; route 2, qualitative and quantitative trace analysis of complex mixtures.

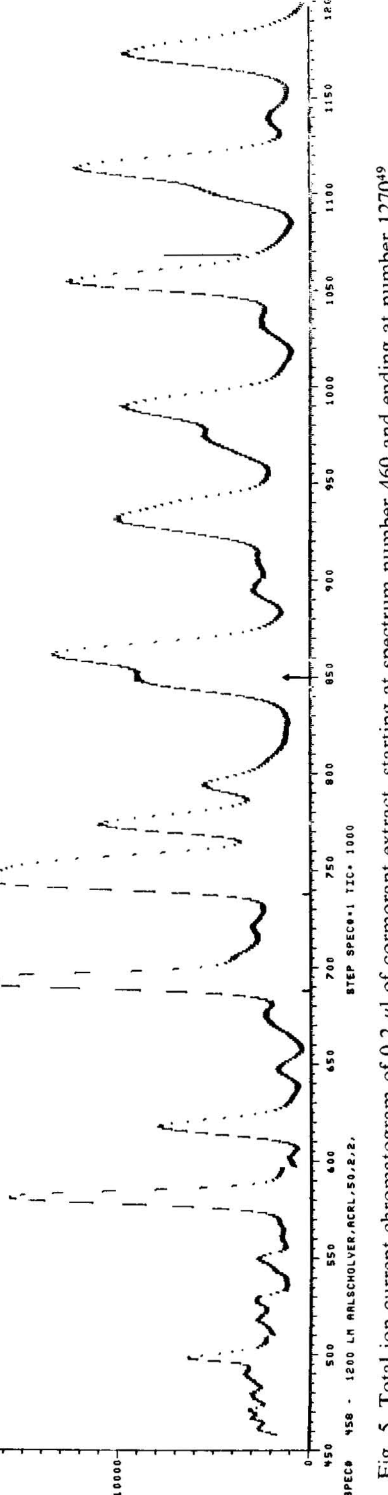

Fig. 5. Total ion current chromatogram of 0.2 μl of cormorant extract, starting at spectrum number 460 and ending at number 1270[49].

C_8H_{18}. The difference is only 0.06 mass units, so that a resolution of 2000 would be sufficient to eliminate this interference. Resolution is obtained with an ideal peak shape that is triangular.

Fig. 4 summarizes the different procedures which can be followed in GC–MS applications[48].

To illustrate route 1, the following example has been chosen. Extracts of total body homogenates of cormorants, found dead in the field, were analysed by GC–MS in order to identify the many different compounds present in these extracts[49]. Fig. 5 shows the total ion current chromatogram obtained from a computerized GC–MS combination operating in the cyclic scan mode, each number on the *x*-axis corresponding to a mass spectrum. Most of the compounds in the extract were identified as polychlorobiphenyls (PCBs). However, at the location marked with an arrow, spectrum number 850, a mass spectrum is found that contains chloro isotope clusters at *m/e* 376, 341 and 306 belonging to an (at that time) unknown compound, and at *m/e* 290 and 220 characteristic of a polychlorobiphenyl (Fig. 6).

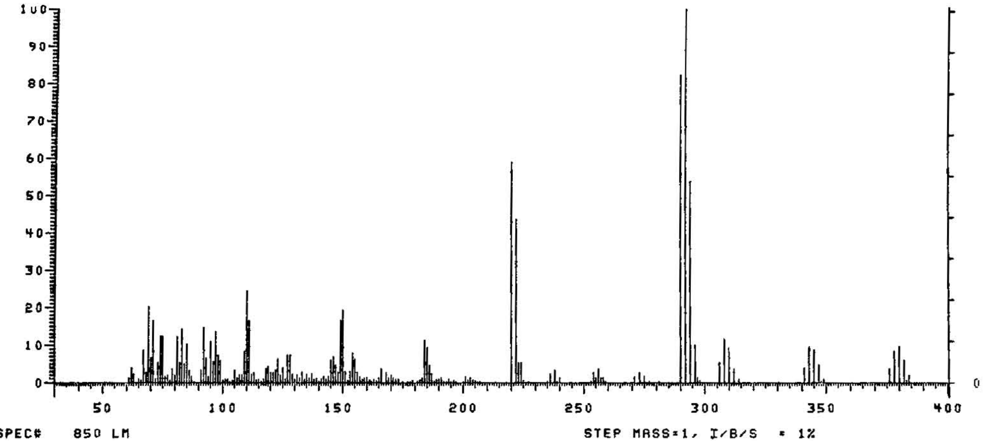

Fig. 6. Uncorrected mass spectrum at location 850 in the total ion current chromatogram.

To find the exact location in the chromatogram and the spectrum that could be subtracted in order to eliminate the PCB masses, the computer was instructed to produce a mass fragmentogram of the masses 376, 341 and 306, as is shown in Fig. 7. The three selected masses coincide fairly well, which justifies the conclusion that they belong to one compound.

To obtain a mass spectrum corrected for the interference with the PCB compound of molecular weight 290, the neighbouring spectrum which does not contain the masses of the unknown compound is subtracted from this main spectrum, shown in Fig. 6. Fig. 8 shows the result of this subtraction procedure and indicates that the PCB masses are eliminated. The compound was identified as octachlorostyrene.

After identification of this compound, the question arose of whether heptachlorostyrenes were present in the extract. The characteristic masses for these compounds were easy to predict, and the computer was ordered to search for their presence. At two locations in the chromatogram, spectrum number 690 and 738, these

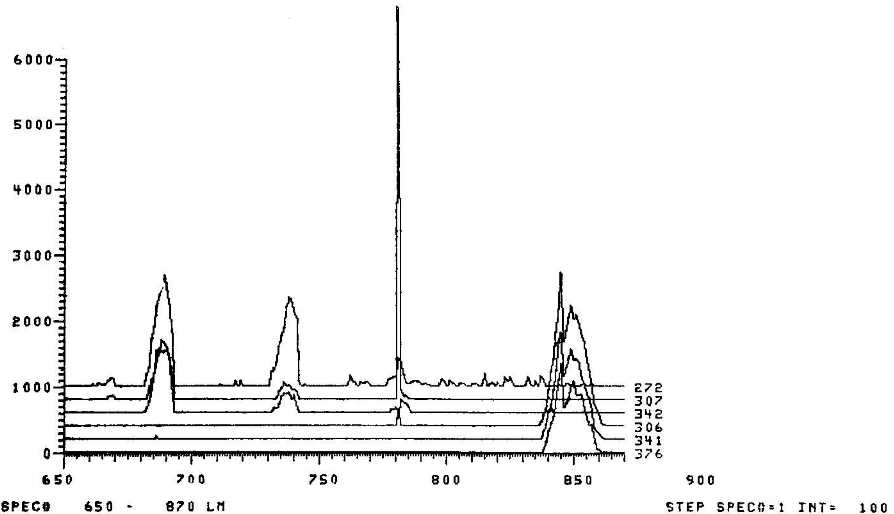

Fig. 7. Selected mass plot section of spectrum 670–870 for the masses *m/e* 376, 341, 306, 342, 307 and 272[49].

masses appeared. They proved to be correct for the isomers of heptachlorostyrene (Fig. 7). These two compounds were completely overlooked during the evaluation of the different mass spectra, as these masses were overshadowed by those of the PCBs. As all MS information was still available on magnetic tape, it was not necessary to prepare a new sample or to repeat the analyses. This example illustrates the benefit of a computerised GC–MS combination in a multi-component analysis of a complex mixture.

Identification from low-resolution mass spectra only is not always successful. High-resolution GC–MS data which also yield the elemental compositions of the masses can be most helpful in identifying completely unknown compounds. The following example[50] demonstrates that it is possible to obtain useful elemental com-

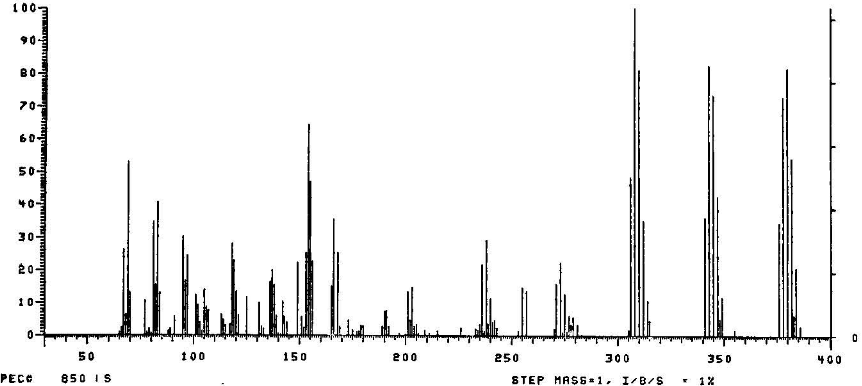

Fig. 8. Corrected mass spectrum number 850 LS, obtained by subtracting spectrum number 860 from 850[49].

positions of compounds eluting from a capillary column connected to a medium-resolution double-focusing mass spectrometer operating at a resolution of 3300 and scanning at 2 sec per mass decade.

Fig. 9 shows a computer-reconstructed gas chromatogram of a mixture of anthracene, pyrene, *p*-terphenyl and triphenylene. Fig. 10 shows the elemental composition obtained from the last peak. Here the molecular ion 228 is measured with a 0.0015 mass unit deviation from the theoretical value ($C_{18}H_{12}$). These data were obtained from only 40 ng of triphenylene.

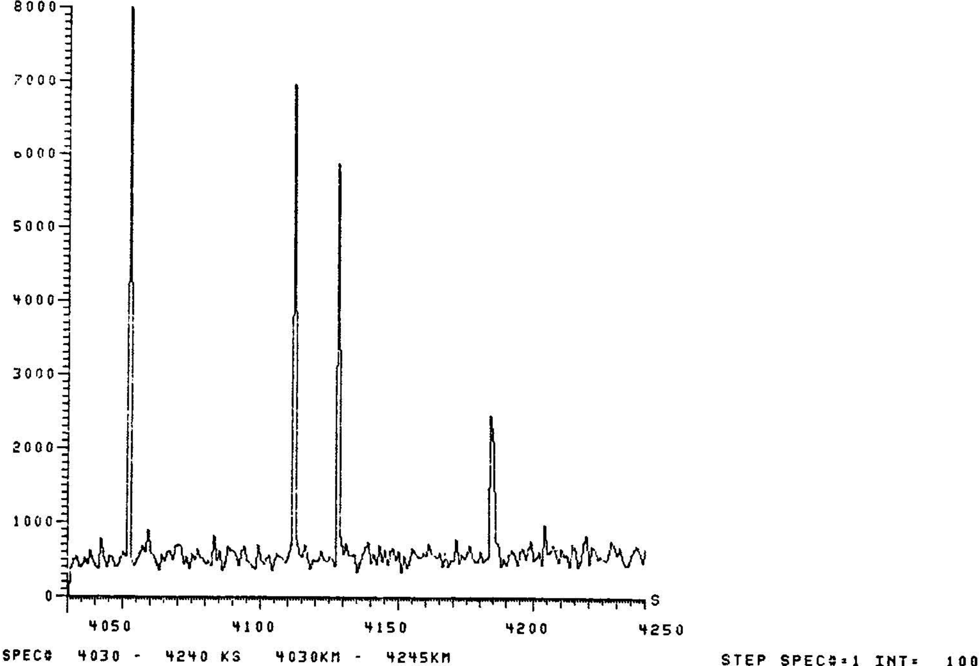

Fig. 9. Total ion current chromatogram of a mixture of anthracene, pyrene, *p*-terphenyl and triphenylene[50].

The following examples deal with problems that can be solved by means of route 2 (Fig. 4). The application of mass spectrometry in the quantitative trace analysis of complex mixtures is increasing, especially in the fields of clinical chemistry, toxicology, forensic medicine and off-flavour research.

Fig. 11 shows a total ion current chromatogram together with some mass fragmentograms of a concentrate of the volatile compounds of white beans. These beans, which are used in the confectionary industry as raw material, had a musty taint which, from experience with previous off-flavour problems, could be caused by chloroanisoles[51,52]. A direct search for these compounds was performed, in this instance at masses 210 and 212 for trichloroanisole and 244 and 246 for tetrachloroanisole. Only one location was found with a retention time corresponding to 2,4,6-trichloroanisole and none for the tetrachloroanisoles.

For this application it is not necessary to have all mass spectrometric infor-

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BASE PEAK 7019 / MASS 228

PEAK	I/BASE	MASS		DIFF	C/C' 12/13	H 1
5	2.36 %	41.0355	*	-3.6	3/0	5
				0.9	2/1	4
9	3.21 %	56.0679		5.3	4/0	8
16	1.75 %	75.0267		3.2	6/0	3
17	1.93 %	76.0271		-4.1	6/0	4
				0.3	5/1	3
22	1.33 %	87.0262		2.7	7/0	3
23	4.55 %	88.0266		-4.6	7/0	4
				-0.2	6/1	3
34	11.76 %	113.0313		-7.8	9/0	5
				-3.3	8/1	4
36	8.09 %	114.0527		5.7	9/0	6
62	1.86 %	188.0545		-3.6	14/1	7
78	6.16 %	225.0657		-4.7	18/0	9
79	26.49 %	226.0781		-0.1	18/0	10
				4.3	17/1	9
80	24.10 %	227.0872		1.1	18/0	11
				5.6	17/1	10
81	100.00 %	228.0924		-1.5	18/0	12
				3.0	17/1	11
83	16.69 %	229.0935		-3.6	17/1	12

Fig. 10. Computer-printed elemental composition of triphenylene[50].

mation available, so one can consider the use of a cheaper multiple ion selector. The method is more sensitive and may reveal the presence of the other chloroanisoles. The result obtained with a four-channel ion selector from the same extract is shown in Fig. 12. The upper trace represents the total ion current and the lower four traces are the selected masses. By applying this method it was possible to detect trace amounts of one other trichloroanisole and two tetrachloroanisoles at the correct retention times.

In the above examples, chlorinated compounds that possess masses very different from those of the compounds in the matrix had to be detected; there were not many chances of misinterpretation. As mentioned before, difficulties will arise when the masses of the compounds to be searched for are not so characteristic. An example is the presence of nitrosamines in cigarette smoke and meat products. Nitrosamines are very toxic and carcinogenic compounds and should not be present in food.

Fig. 13 shows the result of an analysis obtained with a computerized GC–MS system. Cigarette smoke (3 ml) was injected on to a 150-m capillary column coated with Ucon B[43,53]. The upper trace represents the total ion current and the other five the characteristic masses of nitrosopiperidine (30, 42, 55, 56 and 114). None of them are very characteristic ions. For instance, mass 30 can also be the isotope peak of a large mass 29 fragment.

In the retention time range of nitrosopiperidine, all five masses are present and coincide. However, to draw the conclusion from this information that nitrosopiperidine is present is very risky and may lead to misinterpretations.

In order to investigate the value of the information obtained, it is necessary to

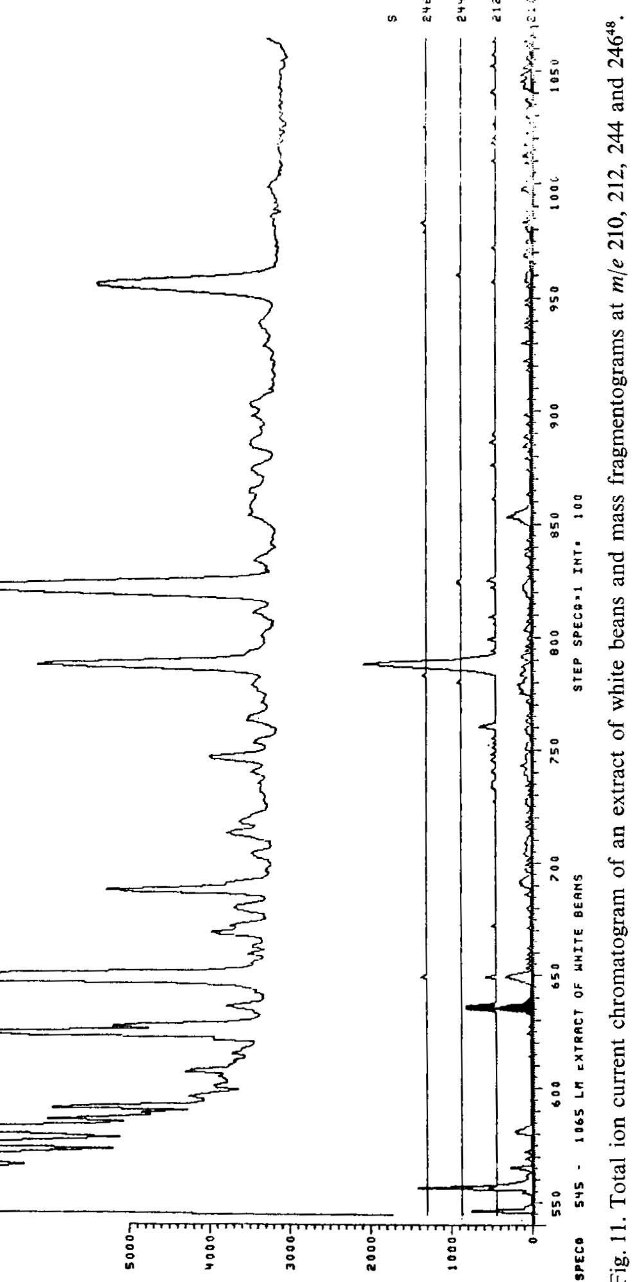

Fig. 11. Total ion current chromatogram of an extract of white beans and mass fragmentograms at *m/e* 210, 212, 244 and 246[48].

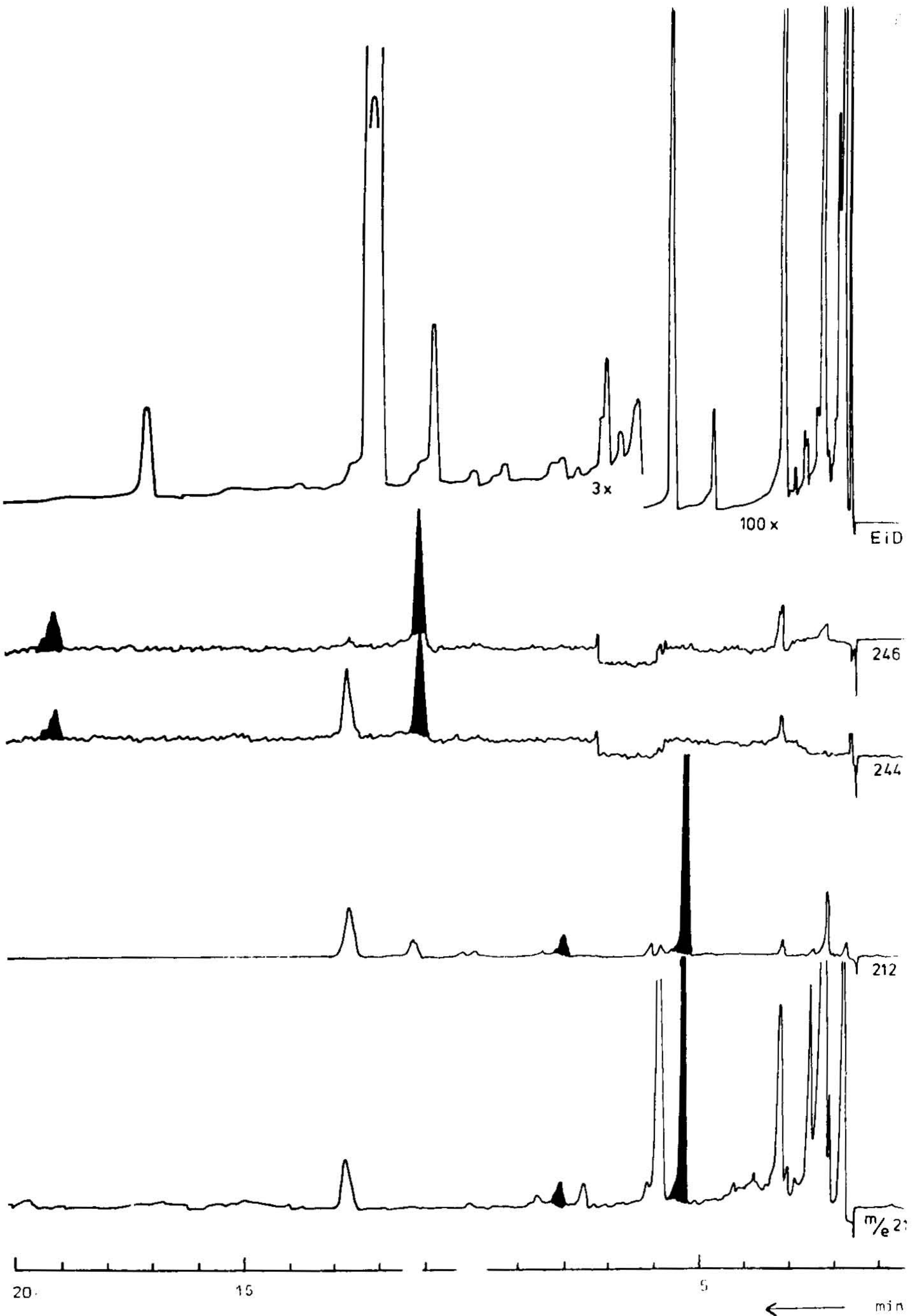

Fig. 12. Total ion current chromatogram and mass fragmentograms of an extract of white beans obtained from a Varian-MAT 112S double-focusing mass spectrometer equipped with a four-channel ion selector.

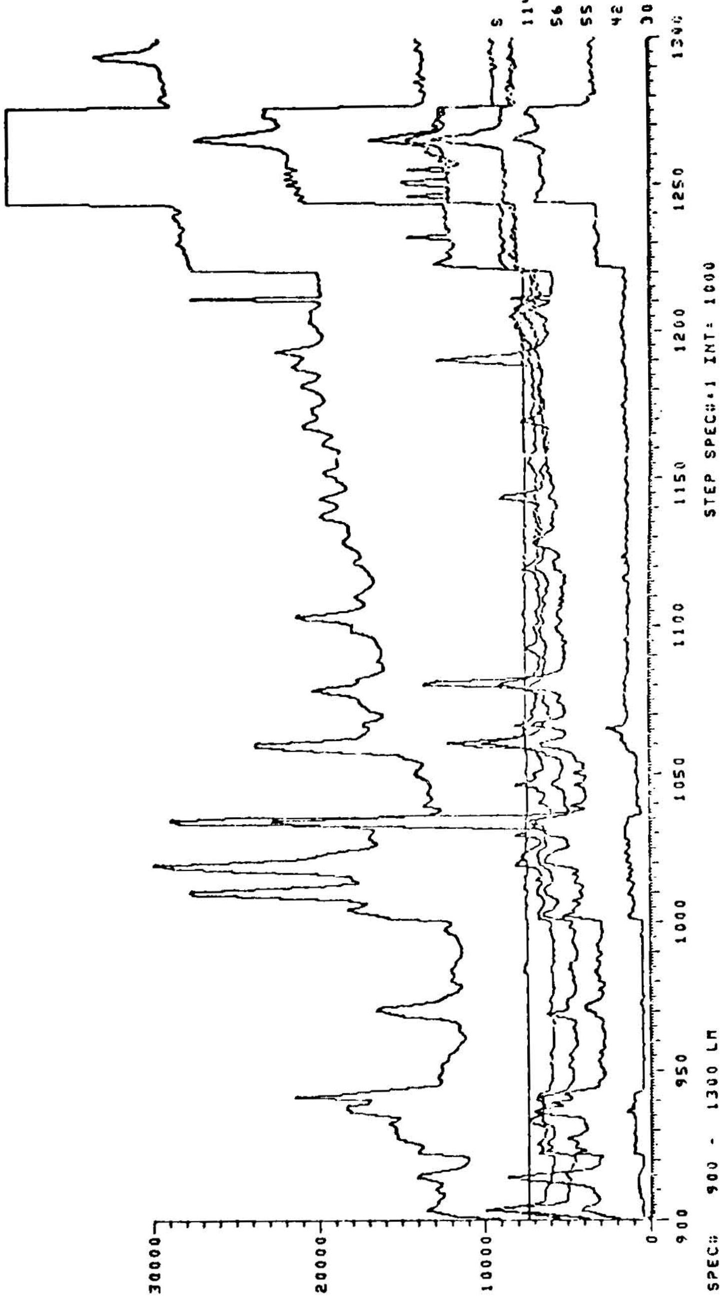

Fig. 13. Selected mass plots and total ion current chromatogram of cigarette smoke. The masses *m/e* 30, 42, 55, 56 and 114 are characteristic of nitrosopiperidine.

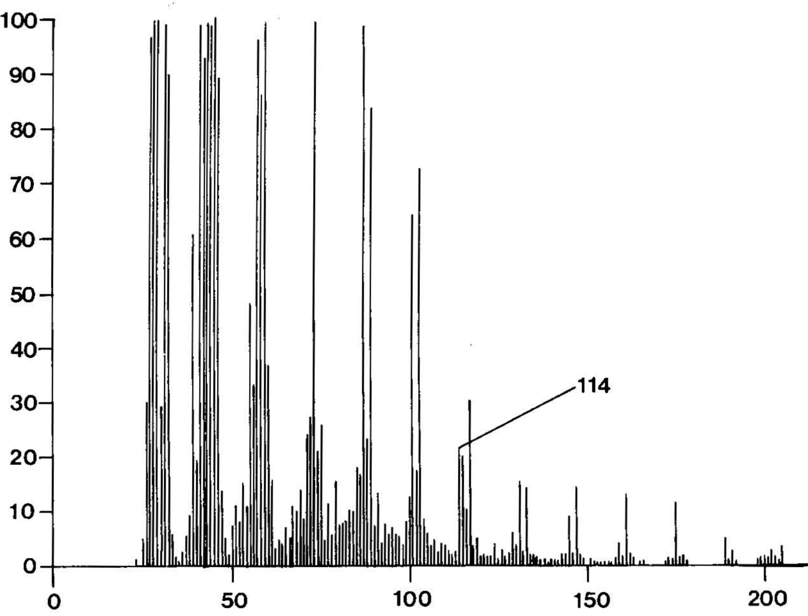

Fig. 14. Uncorrected spectrum at location 1265 LM.

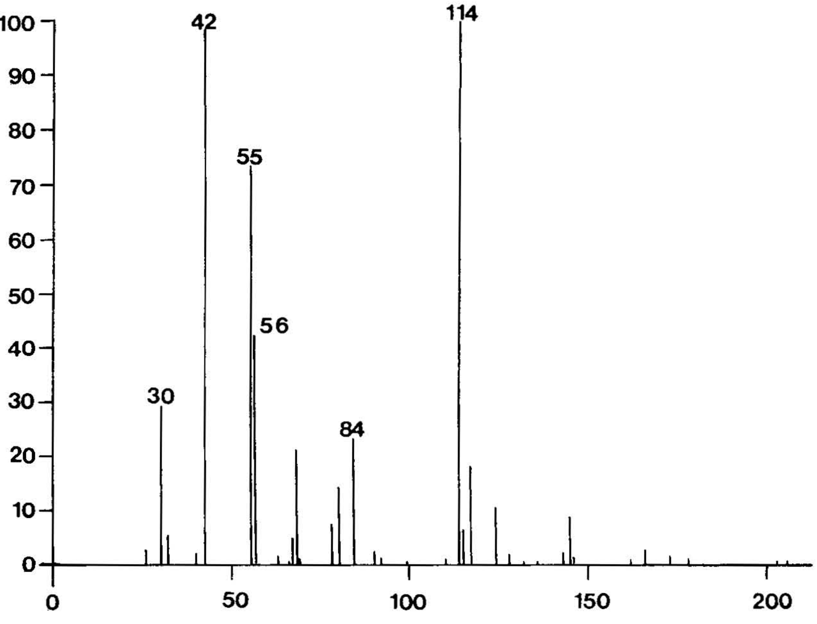

Fig. 15. Mass spectrum of nitrosopiperidine in cigarette smoke, obtained from spectrum 1265 LM by subtraction of a neighbouring background spectrum.

examine the mass spectrum location 1264 derived from the mass fragmentogram. This mass spectrum (see Fig. 14) looks like a forest from which no significant information can be retrieved; even the molecular ion 114 of nitrosopiperidine is completely hidden between other fragment ions. However, a subtraction procedure applied to this spectrum produces a clearer picture, as shown in Fig. 15. This spectrum corresponds well with the reference spectrum of nitrosopiperidine. Only for these particular compounds it was possible to obtain any significant information; for other nitrosamines the interference with the other masses could not be eliminated and no useful information could be obtained.

In order to obtain reasonably quantitative results, any contribution of an interfering mass to the selected mass on which calculations are performed results in

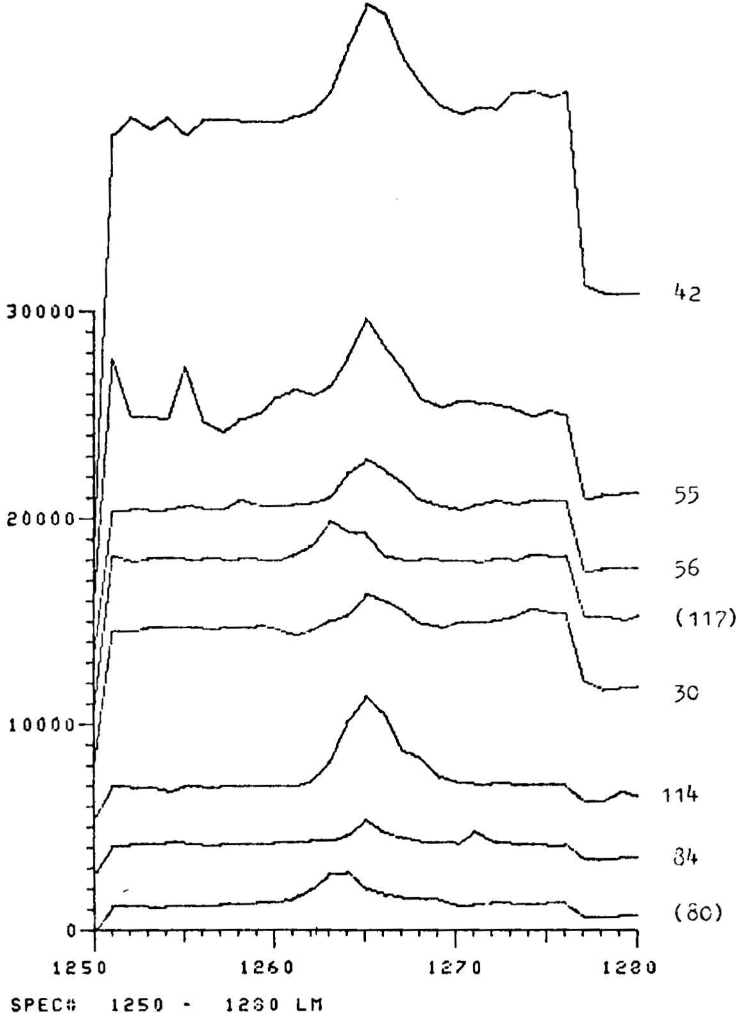

Fig. 16. Selected mass plots of masses m/e 30, 42, 55, 56 and 114, characteristic of nitrosopiperidine. and m/e 117, originating from an interfering compound.

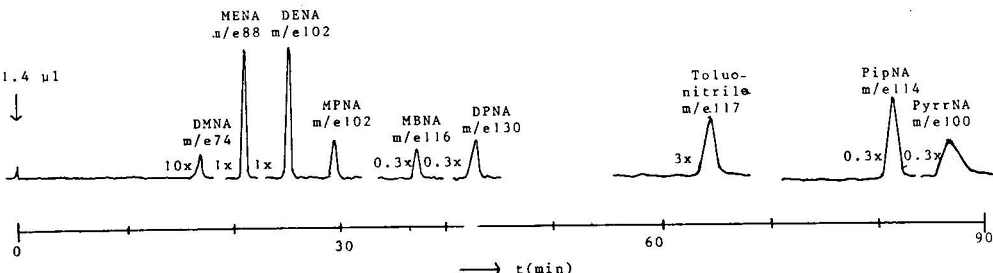

Fig. 17. Mass chromatogram of the molecular ion masses of DMNA (*m/e* 74.05), MENA (88.06), DENA (102.08), MPNA (102.08), MBNA (116.10), DPNA (130.11), PipNA (114.08) and PyrrNA (100.06) of a 10 ppm nitrosamine standard solution at resolution 4000. DMNA = dimethylnitrosamine; MENA = methylethylnitrosamine; DENA = diethylnitrosamine; MPNA = methylpropylnitrosamine; MBNA = methylbutylnitrosamine; DPNA = dipropylnitrosamine; PipNA = nitrosopiperidine; PyrrNA = nitrosopyrrolidine.

higher values of the concentration of the trace compounds that have to be determined. In the above spectrum masses other than those of nitrosopiperidine are present, *e.g.*, mass 117. To find out how this mass is located in relation to those of the nitrosopiperidine, a mass fragmentogram is produced (Fig. 16) by the computer, showing that *m/e* 117 is shifted slightly from those of the nitrosamine. However, it is obvious that it cannot be eliminated by means of a subtraction procedure.

As has already been stated, there was no possibility of obtaining any reliable results for other nitrosamines, owing to the heavy interference from other masses. Increasing the MS resolution might solve the problem. The benefit of increasing the resolution of the mass spectrometer is demonstrated below for the analysis of eight nitrosamines in smoked horse meat by GC–MS medium-resolution single-ion detection[43].

A 150-m wide-bore capillary was connected to a high-resolution mass spectrometer, which was tuned to a resolution of 4000 with a trapezium-shaped peak. This means that the mass spectrometer was first tuned to a resolution of about 8000, then the exit slit was opened in order to obtain a trapezium-shaped peak to perform the measurements under more stable conditions. Perfluorokerosene was led in continuously to generate reference masses in order to calibrate and control the exact mass tuning of the selected mass corresponding to a particular elemental composition. After the

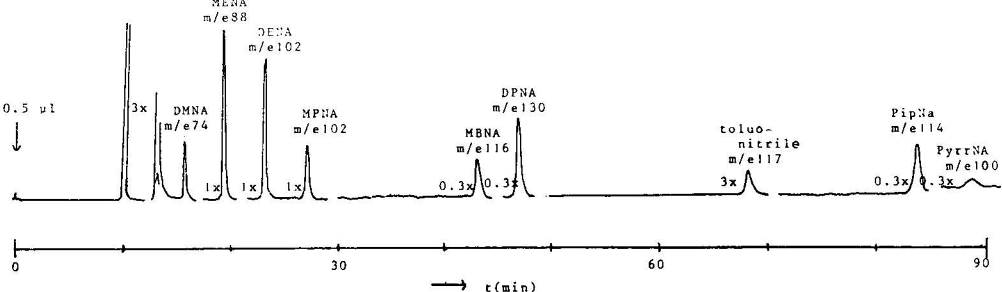

Fig. 18. Mass chromatogram of the molecular ion masses of DMNA, MENA, DENA, MPNA, MBNA, DPNA, PipNA and PyrrNA of an extract of spiked horse meat (cured and smoked) at resolution 4000.

retention time of each nitrosamine, the exact mass was tuned to the exact mass of the next nitrosamine. Switching from mass to mass takes about 1 min, whereas the intervals between peaks are 3 min or more.

Fig. 17 shows the the fragmentograms of a 10 ppm standard solution of the eight nitrosamines (14 ng per peak). *o*-Toluonitrile was added as an internal standard. The efficiency of the isolation and concentration procedures was determined by analysing smoked horse meat spiked with appropriate amounts of nitrosamine standard solution so as to obtain concentrations in the product of 25, 50 and 100 μg/kg.

The fragmentograms of such a recovery test are shown in Fig. 18; the number of non-nitrosamine peaks is very small. When the same analysis is performed with a resolution of 800, a complex and overloaded chromatogram is obtained, from which no reliable conclusion can be drawn as to the presence of nitrosamines at the micrograms per kilogram level.

Fig. 19 gives the results of an analysis of unspiked horse meat. In this product 3 μg/kg of DMNA and 90 μg/kg of DENA were detected and none of the other nitrosamines (no interference from other compounds occurs). Under unfavourable GC–MS conditions, higher concentrations might be suggested, owing to interference with the isotopic masses from the trimethylsilyl ion at *m/e* 73 belonging to some silicone compounds in the extract[43,54,55]. To distinguish the molecular mass 74.048 of DMNA from the two isotope masses of silicon (29) and carbon (13) from the trimethylsilyl ion at *m/e* 73, which differ by 0.0011 and 0.0027 mass units, respectively, a resolution of about 60,000 is required.

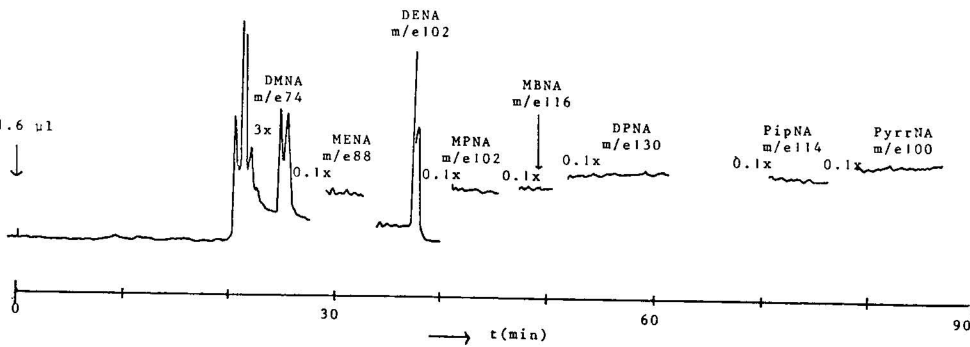

Fig. 19. Mass chromatogram of the molecular ion masses of DMNA, MENA, DENA, MPNA, MBNA, DPNA, PipNA and PyrrNA of an extract of unspiked horse meat (cured and smoked) at resolution 4000.

Fig. 20 shows a high-resolution plot obtained from the peak match display at a resolution of 70,000 at *m/e* 74. Peak 1 belongs to a perfluorokerosene ion, peak 2 represents a silicon impurity and belongs to the silicon isotope of the trimethylsilyl ion at *m/e* 74.0469, $C_3^{12}H_9^1Si^{29}$. Peak 3 belongs to the molecular ion at *m/e* 74.048 of DMNA with elemental composition $C_5H_{10}N_2O$. Peak 4 represents the carbon-13 isotope of the trimethylsilyl ion at *m/e* 74.0507, $C_2^{12}C^{13}H_9^1Si^{28}$. This high-resolution measurement confirmed the presence of DMNA in smoked horse meat. The example above illustrates well how complicated the situation can be and how careful the investigation should be so as not to draw wrong conclusions, especially in quantitative

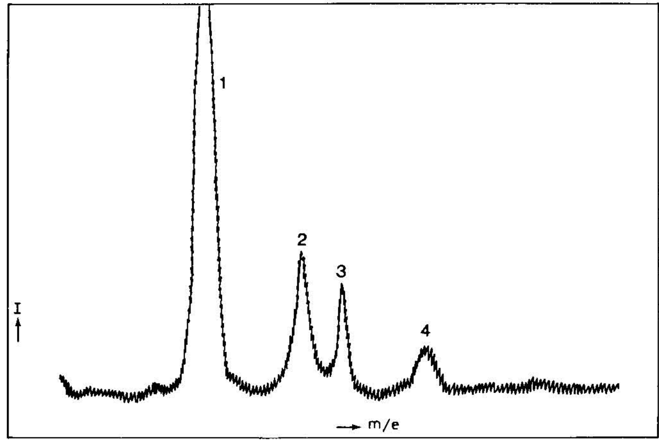

Fig. 20. High-resolution display at a resolution of 70,000 at *m/e* 74 obtained from smoked horse meat. Peaks: 1 = perfluorokerosene; 2 = $C_3^{12}H_9^1Si^{29}$ (74.0469); 3 = $C_5H_{10}N_2O$ (74.048); 4 = $C_2^{12}C^{13}H_9^1Si^{28}$ (74.0507).

analyses, where interference with other masses may lead to a higher concentration value for a certain compound than is actually present in the extract.

The application of chemical ionization is an other promising technique for decreasing interferences in trace analysis. By means of this technique the fragmentation pattern of the spectrum is considerably reduced and new intense pseudo-molecular ions are created, which can be seen as some kind of derivatization of organic compounds in the ion source of the mass spectrometer. With this technique interference problems can also be reduced and the selectivity increased.

Fig. 21 illustrates the electron-impact mass spectrum of caryophyllene oxide,

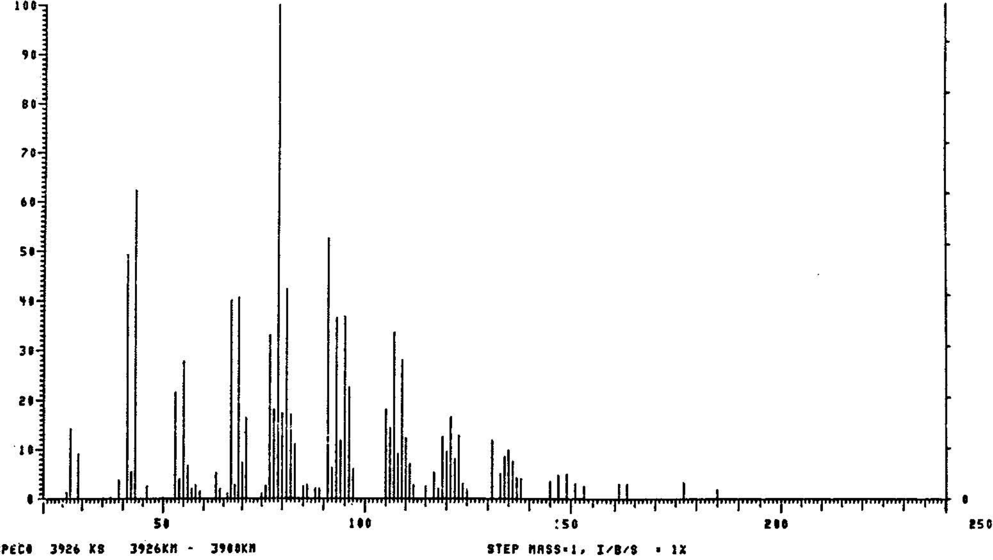

Fig. 21. Electron-impact mass spectrum of caryophyllene oxide

which shows no molecular ion at *m/e* 220 but only a large number of uncharacteristic fragment ions. To detect such a compound by mass fragmentography is almost impossible.

Fig. 22 shows the spectrum of the same compound obtained with chemical ionization. The fragmentation is strongly reduced and new intense masses appear at *m/e* 203 and 221, which is the M + H ion. These masses yield good responses in mass fragmentography.

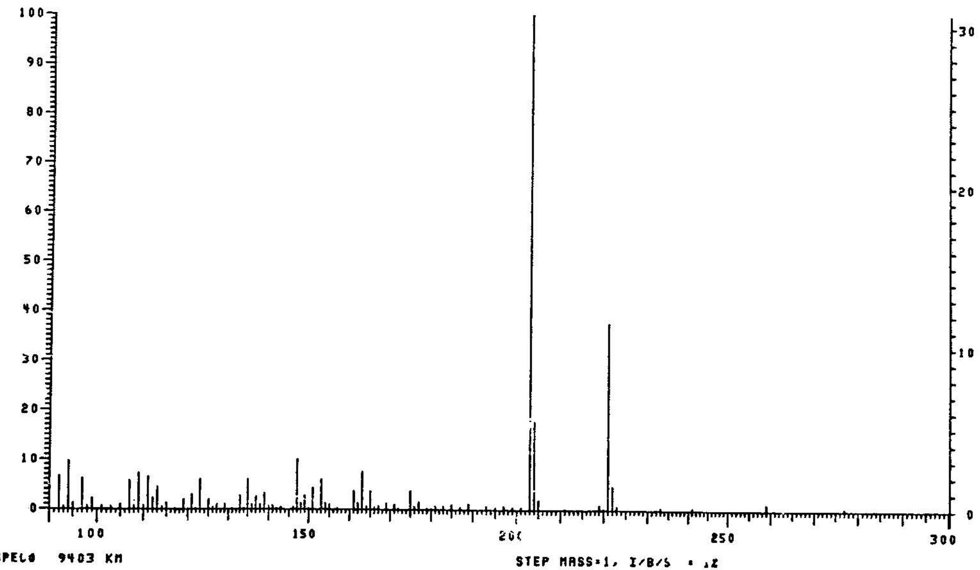

Fig. 22. Chemical-ionization mass spectrum of caryophyllene oxide, obtained with isobutane.

4. CONCLUSION

The application of GC–MS in analytical chemistry is still increasing in spite of the high investment costs. This is due to the fact that the time required for confirming and identifying compounds in complex mixtures can be reduced to a few hours instead of several weeks.

The rapid analytical response of such a system not only yields economic benefits, but is also very important in, *e.g.*, clinical chemistry, where a rapid response from the laboratory may save a patient's life[56].

A subject that has not been discussed in this paper is the combination of high-performance liquid chromatography with mass spectrometry (HPLC–MS). Although many different interfacing techniques are being developed, there is still a long way to go before useful HPLC–MS systems will become available[57–60]. The technology of interfacing and the techniques of ionization required are much more complicated than in GC–MS. The introduction of capillary columns in HPLC might be helpful in realizing a simple interfacing technique so that the application of HPLC–MS could be achieved.

5. SUMMARY

A brief historical survey is given, together with a review of the main requirements for obtaining an optimal GC–MS system. Special attention is paid to the many different coupling techniques that have been developed during the last 15 years. The necessity for computerization and the various operational techniques applied in GC–MS are discussed and illustrated. Examples derived from the daily practice of an analytical laboratory are used to illustrate the different methods applied in GC–MS analyses of complex mixtures, such as low- and high-resolution multi-component GC–MS analyses, computer-aided mass fragmentography, spectrum subtraction procedures, interference problems occurring in multiple and single ion detection (low and medium resolution) and chemical ionization.

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Journal of Chromatography, 165 (1979) 235–256
Chromatographic Reviews
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHREV. 123

THE NOMENCLATURE OF CHROMATOGRAPHY

I. GAS CHROMATOGRAPHY

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(Received March 13th, 1979)

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1. INTRODUCTION

Today, chromatography is the most widely used and probably also the best documented analytical technique: the number of papers dealing with various aspects of chromatography probably exceeds 100,000. At present, gas chromatography still represents the major chromatographic technique but predictions show that liquid chromatography will surpass it within about 5 years.

The evolution of gas chromatography has a unique characteristic: there has probably not been any other analytical technique where an intensive international cooperation played such a vital role. This cooperation started at the inception of the technique and manifested itself by the frequent meetings, symposia and informal and formal get-togethers in both Europe and the United States.

One of the important subjects of these meetings was the nomenclature of gas chromatography. Discussions on this subject actually started before the first International Symposium held in London in 1956 and represented a major topic there. This discussion was further extended in subsequent years, involving the newly formed Gas Chromatography Discussion Group, an American *ad hoc* Committee and

Committee D-2 of the American Society for Testing and Materials (ASTM)*. Soon, the British Standards Institution (BS) and the Division of Analytical Chemistry of the International Union of Pure and Applied Chemistry (IUPAC) also became involved in these activities. As a result of this extensive work, three major documents were compiled: the recommendations of the IUPAC first published in 1960[1,2] and then finalized in 1964[3]; the *Glossary of Terms* of BS, first published in 1963 with the presently valid text dated 1969[4]; and the *Gas Chromatography Terms and Relationships* of ASTM E-19, first published in 1968 with the present text approved in 1977[5]. The IUPAC Committee continued its work, expanding it to chromatography in general; the draft of a generalized nomenclature was first published in 1972[6] and finalized in 1973[7].

The standardization activities were not restricted to nomenclature, terms and symbols only. ASTM in particular has developed three important standards: one dealing in general with gas chromatographic procedures[8] and two dealing with the testing of thermal conductivity[9] and flame-ionization[10] detectors. A similar text on the electron-capture detector was recently completed and will be published later in 1979.

This brief discussion shows that in gas chromatography, well developed, internationally accepted nomenclatures exist. Thus, there is no reason for individuals to invent new symbols and definitions for well established terms. This might be justified in a new field without sufficient international agreements, but certainly not in gas chromatography. Here, individualistic usage is similar to somebody building up his own spelling system, making the reading of publications impossible without a personalized dictionary. We can see examples of this in almost every issue of our scientific and technical journals. Unfortunately, the editors of journals do not prevent this either.

The purpose of this paper is to help to change this situation. It presents these standards, particularly the symbols and key terms specified in them, in detail, and demonstrates their similarities and the few existing differences. We shall deal only with those terms which are in English, although it should be noted that, naturally, standard nomenclatures also exist in other languages. We shall start with a detailed discussion of the evolution of this nomenclature, for two reasons: first, because in general this is necessary in order to understand how the present-day system became established, and secondly, to illustrate that these terms, symbols and definitions are based on extensive cooperation within an international community carried out over more than a decade. After this discussion, we shall deal with the internationally accepted terms and symbols. Finally, we shall discuss the other existing ASTM standards dealing with the specifications of important terms related to the two most frequently used types of gas chromatographic detectors. However, before we start this discussion, two general remarks are necessary.

The first concerns *liquid chromatography*. Although it is the older technique,

* Committee D-2 on Petroleum Products and Lubricants was the standardization group most closely related to gas chromatography at that time. This is the reason why Research Division IV of this ASTM Committee accepted the original responsibility for this task. These activities were later transferred to ASTM Committee E-19 on Gas Chromatography formed in 1961 which, in 1969, changed its name to Committee on Chromatography, now encompassing all forms of the technique. Naturally, however, the development of standard methods for the analysis of certain samples in which chromatography is used as a method remained the responsibility of the individual ASTM committees.

unification was attempted less in liquid than in gas chromatography. Recently, however, this situation has changed. As already mentioned, the IUPAC Committee accepted a unified chromatographic nomenclature and it also proposed a nomenclature for ion-exchange chromatography and for certain similar techniques; also, at the time of writing this paper, the very detailed nomenclature recommendations of liquid (column) chromatography prepared by ASTM Committee E-19 is being approved, with publication to be expected later in 1979*. It should be noted that a standardized nomenclature of gel-permeation chromatography developed by ASTM Committee E-20 on Plastics has been in existence since 1972. It is planned to extend the present discussion to the field of liquid chromatography in a later publication.

The second remark is specifically related to the names of certain terms which sometimes are called outdated by certain "innovators". Nobody questions that there is some validity in these remarks; however, the situation is not dissimilar to many other segments of life. After all, almost everything, from spelling to national boundaries, has undergone a historical evolution and the same is also true of terms, definitions and symbols. In this evolution, they often lose their original meaning, but this fact, in itself, does not warrant discarding them. After all, we are still speaking about "chromatography" although (except maybe in TLC) our analyses no longer have anything to do with "color writing". Also, nobody questions that terms, names and definitions originally adopted over 20 years ago, based on a somewhat limited knowledge, might be formulated differently today. For example, if our nomenclature were to be compiled now from scratch, we would probably find better expressions than "adjusted retention time" or "capacity ratio". After all, the former is not "adjusted" but represents the actual retention by the stationary phase and the latter really does not describe the "capacity" of a column (*i.e.*, the amount of sample that can be introduced while still remaining in the linear range). Still, these terms, names and definitions were developed historically and used in thousands of publications, together with the corresponding symbols which were also logically developed, in the context of the whole nomenclature, and, what is even more important, well understood. Thus, there is no real justification to disregard them and create one's own system. Or shall we change the name of America to something else because today, we know that Amerigo Vespucci really became only through a misunderstanding the patron saint, giving his first name to the New World?

2. THE EVOLUTION OF GAS CHROMATOGRAPHIC NOMENCLATURE

A. The activities of various ad-hoc committees

As already mentioned, our present-day chromatographic nomenclature is the result of a historical evolution, involving various groups, with members from many countries, and intensive discussions among them. The nomenclature discussion actually started before the 1956 London Symposium and then there a Committee consisting of D. H. Desty, E. Glueckauf, A. T. James, A. I. M. Keulemans, A. J. P. Martin

* *Liquid Chromatography Terms and Relationships, ASTM E 682-79* had been approved on March 30, 1979 by the Membership of Committee E-19 and published recently in Vol. 42 of the *1979 ASTM Books of Standards* (American Society for Testing and Materials, Philadelphia, Pa., 1979).

and C. G. S. Phillips was formed to formulate some recommendations. In their report[11] they recommended the use of *retention volumes* and established the term which we call today the *specific retention volume* (V_g):

$$V_g = \frac{V_N}{W_L} \cdot \frac{273.15}{T_c} \tag{1}$$

i.e., the net retention volume per gram of liquid phase, reduced to 0°C*. They also specified the *compressibility (pressure drop) correction factor* (calling it then the "reducing factor"), already included in the original 1952 publication of James and Martin[12], and the *number of theoretical plates* calculated on the basis of the peak width at base obtained by drawing tangents to the points of inflexion of the peak. In this report, the number of theoretical plates was considered as an expression of "column resolution", but the change of this term to *column efficiency* was recommended one year later, by the *ad hoc* Committee of Nomenclature formed at the 1957 Lansing (U.S.A.) Symposium and consisting of W. L. Jones (Chairman), S. Dal Nogare, D. H. Desty, M. J. E. Golay, A. I. M. Keulemans, A. J. P. Martin, S. Ober, C. S. G. Phillips, J. Thoburn and E. Williams[13]. This Committee also established the term *separation factor*, α, to express the relative position of two adjacent peaks:

$$\alpha = t'_{R2}/t'_{R1} \tag{2}$$

At the 1958 Amsterdam Symposium, a Committee consisting of A. J. P. Martin (Chairman), D. Ambrose, W. W. Brandt, A. I. M. Keulemans, R. Kieselbach, C. S. G. Phillips and F. H. Stross[14] also endorsed the change to "column efficiency" and established a separate *peak resolution* term to express the separation of two adjacent peaks as the distance between the two peak maxima divided by the mean peak width at base:

$$R_s = \frac{t_{R2} - t_{R1}}{\bar{w}_b} = \frac{2(t_{R2} - t_{R1})}{w_{b1} + w_{b2}} \tag{3}$$

It should be noted that a similar term**:

$$R_s = \frac{t_{R2} - t_{R1}}{w_{b1}} \tag{3a}$$

has already been mentioned in the report of the Lansing Committee[13], as a proposal of M. J. E. Golay, to express resolution. The two expressions are identical if we assume that $w_{b1} = \bar{w}$, in other words, that the base width of the *first* peak is identical with the mean base width of the two peaks. For some reason, not the first but the *second* base width was later accepted by convention as assumed to be equal to the mean peak width at base; thus, eqn. 3 was simplified to

$$R_s = \frac{t_{R2} - t_{R1}}{w_{b2}} \tag{3b}$$

This duality can also be seen in the evolution of ASTM E-355[5] concerning the term

* The symbols used in the text of this report correspond to those recommended by ASTM; see Table 1.

** In the Lansing proposal[13], no symbol was used for this term; the symbol R_s is used here for the sake of simplicity.

number of theoretical plates required (n_{req}), to result in a given resolution of peaks 1 and 2 ($t_{R2} > t_{R1}$). This term is expressed by different equations depending on whether we assume eqn. 3a or 3b. If we assume eqn. 3a, the expression for n_{req} is

$$n_{req} = 16R_s^2 \left(\frac{1}{\alpha - 1}\right)^2 \left(\frac{k_1 + 1}{k_1}\right)^2 \tag{4a}$$

and the equation included in the original 1969 text of E-355 (although looking somewhat different) was derived along this line. On the other hand, if we assume eqn. 3b, then the expression for n_{req} becomes

$$n_{req} = 16R_s^2 \left(\frac{\alpha}{\alpha - 1}\right)^2 \left(\frac{k_2 + 1}{k_2}\right)^2 \tag{4b}$$

and this is the equation included in the 1977 version of E-355.

B. Papers on nomenclature

According to the report of the 1958 Amsterdam Committee, they discussed in detail the contents of two manuscripts, those of Ambrose, Keulemans and Purnell and of Johnson and Stross, to be published in *Analytical Chemistry* and, as a result of this discussion, the authors agreed to certain alterations in order to bring "their recommendations, symbols and nomenclature into line". These two papers were published in the October 1958 issue of the journal; the first[15] was actually prepared at the request of the Committee of the Gas Chromatography Discussion Group, while the second[16] represented the conclusion of the activities of a working group appointed by Research Division IV of ASTM Committee D-2 on Petroleum Products and Lubricants. A third paper by Jones and Kieselbach[17], published in the same issue of *Analytical Chemistry*, proposed a few additional basic terms; of these, however, only the *average linear carrier gas velocity* became universally accepted.

Almost simultaneously, *Nature* published an important paper by M. J. E. Golay[18] on *Gas Chromatographic Terms and Definitions*, which also addressed itself to the problem of the proper terms. As pointed out by the author, his discussions with workers in the field of gas chromatography convinced him that "much clarification remains to be achieved in nomenclature and definitions of terms" and that particularly two cases create some confusion: the ambiguity of plate number and the HETP for cases when the retention time is not large compared with the gas hold-up time (in other words, for open-tubular columns where the capacity ratio is relatively small), and the resolving power of a column derived from the number of theoretical plates (or HETP).

Concerning the number of theoretical plates, Golay pointed out that for cases when the capacity ratio is not too large, the equation*

$$n_0 = 5.545 \cdot \frac{t_R t_R'}{w_h^2} \tag{5a}$$

should be preferred to the usual

$$n = 16 \left(\frac{t_R}{w_b}\right)^2 = 5.545\left(\frac{t_R}{w_h}\right)^2 \tag{5b}$$

* This expression had already been given by Golay in his paper presented at the American Chemical Society Symposium in Dallas, Texas, in 1956 and published in 1957[19].

relationship advocated in other publications and also in Keulemans' basic textbook[20]. It should be pointed out that while this expression did not replace the well-established theoretical plate expression (eqn. 5b), it certainly is closer to reality than the so-called *number of effective plates* advocated recently by a number of workers in the field of open-tubular columns.

The second question to which Golay addressed himself in his paper is the "resolving power of a column". He pointed out that in the efficiency of a column the t'_R/w_h term, which is the inverse of the "relative band width", has an important role. It is interesting to note that Golay's

$$\frac{t'_R}{w_h} = \left(\frac{k}{k+1}\right)\frac{t_R}{w_h} \tag{6}$$

equation, if properly modified (raising to the second power and multiplying each side by 5.545), yields the well known relationship between the number of theoretical plates (n) and the number of effective plates (N):

$$N = \left(\frac{k}{k+1}\right)^2 n \tag{7}$$

derived later in the literature.

Golay's plate number expression (*cf.* eqn. 5a), like his *performance index*[21], was not generally accepted; however, just like the others, this paper also contributed significantly to our understanding of the various relationships that make a column "better".

C. The activities of the IUPAC committees

Except for Golay's, most of the early nomenclatures expressed retention primarily with respect to volume rather than time, although the corresponding time terms were also mentioned. For some time, there was some confusion concerning the names and symbols of the following terms (using present-day nomenclature and symbols):

the *retention volume* (V_R), measured from start;

the *gas hold-up volume* (V_M), corresponding to the retention volume of an unretained peak;

the *adjusted retention volume* (V'_R), measured from the unretained peak:

$$V'_R = V_R - V_M \tag{8}$$

the *corrected retention volume* (V°_R), representing the retention volume multiplied by the compressibility correction factor (j):

$$V^\circ_R = V_R j \tag{9}$$

the *net retention volume* (V_N), representing the adjusted retention volume multiplied by the compressibility correction factor:

$$V_N = V'_R j = (V_R - V_M) j \tag{10}$$

This confusion even exists in the three papers published in the October 1958 issue of *Analytical Chemistry* already cited, and *e.g.*, Ambrose, Keulemans and Purnell[15] admit that some of their symbols are against "general opinion and usage". It was thus clear that the original intention of the discussion at Amsterdam

to eliminate the inconsistencies was not fully accomplished. Therefore, Ambrose and Stross, the Chairmen of the two groups, approached the Analytical Chemistry Division of IUPAC to set up a special group consisting of people from both groups, which would then issue recommendations for a standard terminology in gas chromatography. This group, consisting of D. Ambrose (Chairman), A. T. James, A. I. M. Keulemans, E. Kováts, H. Röck, C. Rouit and F. H. Stross, was duly formed and published the preliminary text of their recommendations in 1960[1,2]. This nomenclature unequivocally established the meaning of certain symbols, adjectives and superscripts. Thus the prime ($'$), as in V_R' and t_R', and the adjective "adjusted" (*adjusted retention volume* and *time*) have the meaning that the measurement was made from the unretained peak, while the superscript $^\circ$, such as in V_R° and t_R°, and the adjective "corrected" (*corrected retention volume* and *time**) indicate that the compressibility correction factor was applied. In *net retention volume* (and *time**) both superscripts should be used simultaneously; however, because this would be unduly cumbersome, a new symbol, V_N, was accepted. This recommendation also lists the *specific retention volume* (V_g) and the *number of theoretical plates* (n), and the terms related to two peaks: *peak resolution* (R_s) and *relative retention* (r_{12}), the last representing a generalized form of the *separation factor*, component 2 always referring to the standard.

The preliminary recommendations, originally completed in July 1959 and published in 1960, were finalized as the standard terminology of IUPAC in 1964[3]. In the period 1959–64 the composition of the Committee changed slightly: H. Röck and C. Rouit resigned and were replaced by E. Bayer and, in a consulting capacity, P. Chovin. There are two basic differences between the preliminary and the final text. The first is the introduction of a new term, $V_g^\ominus$: this is equal to the net retention volume divided by the amount of liquid phase at column temperature; from eqn. 1 it can be expressed as

$$V_g^\ominus = \frac{V_N}{W_L} = \frac{V_g T_c}{273.15} \qquad (11)$$

We should mention that this term did not become accepted in general usage and it is no longer included in the generalized chromatographic nomenclature of IUPAC[6,7].

The second difference is related to the presentation of data. This subject has been discussed in considerable detail in every committee report since 1956 and various ways and standard components have been suggested. The preliminary recommendations[1,2] still recommended listing the specific retention volume or the partition coefficient, or relative retention data based on certain standards, and eight substances were given as preferred standards. This was changed, however, in the final text: the list of preferred standards was deleted and it was noted that the *retention index system* of Kováts[22] or the *theoretical nonane units* of Evans and Smith[23] are preferred.

* It should be mentioned that corrected and net retention times have no real physical meaning because one does not apply pressure correction to time. These terms are listed in the ASTM nomenclature[5] simply to represent all of the possible expressions, accepting the definition that retention volumes are obtained by multiplying the respective retention times by the flow-rate. The more correct definition would be that retention volumes are obtained by multiplying the respective retention times by the flow-rate and, if necessary, by the compressibility correction factor. Thus, only three retention times, t_M, t_R and t_R', have real physical meaning.

Since 1964, the Commission on Nomenclature of the Division of Analytical Chemistry of IUPAC has been engaged in producing a unified nomenclature applicable to all forms of separation processes. As part of this activity, a Committee consisting of D. Ambrose, E. Bayer and O. Samuelson also considered the possibility of preparing a unified nomenclature encompassing all forms of chromatography. The preliminary recommendations were first published in 1972[6], then approved in a slightly modified form in 1973 and published in 1974[7]. The major part of these recommendations consists of definitions describing the various terms related to classifications, the separation process, methods, techniques, the visual record obtained and the apparatus used. The differences from the earlier gas chromatographic nomenclature are minor and mostly represent additions of new terms. There are, however, two basic differences compared with both the former IUPAC GC nomenclature and all other nomenclatures: these are related to the partition coefficient and the capacity ratio.

In the case of the *partition coefficient*, the new nomenclature recognized its general use in the literature and thus, while proposing to change its name to "distribution constant" it still retains its symbol although now with the subscript D (K_D). At the same time, however, the new nomenclature introduces a number of other *distribution coefficient* terms and proposes to use the symbol D (with various subscripts) for them (see later, in Table 2). In the opinion of this author, while the names might be accepted, the symbols should not be because D has been universally established for the *diffusion coefficient* in gas and liquid phases (D_G and D_L, respectively). Concerning the *capacity ratio*, in the new nomenclature it is proposed to change its name to the "mass distribution ratio" and its symbol to D_m. Again, while the name-change might be accepted, the new symbol has the same problem as mentioned above: it is too similar to the symbol for the diffusion coefficient in the gas phase (for which a number of authors use m subscript for the *mobile phase*).

D. Nomenclatures of the standardization groups

The work of the early Committee was continued by the two major English-speaking standardization groups, the British Standards Institution (BS) and the American Society for Testing and Materials (ASTM). The *Glossary of Terms* of the former was first published in 1963 and the presently valid text is dated 1969[4]. As mentioned, early work by ASTM on setting up a consistent and logical system started in Committee D-2 on Petroleum Products and Lubricants, and was continued in Committee E-19 on Gas Chromatography established in 1961, which, in 1969, changed its name to the Committee on Chromatography. The compilation *Gas Chromatography Terms and Relationships* was completed in the second part of 1967 and approved on March 26th, 1968; it was modified in 1977[5], eliminating some inconsistencies and considering the terms of the generalized IUPAC nomenclature.

3. COMPARISON OF NOMENCLATURE RECOMMENDATIONS

A. Symbols

Table 1 compares the symbols used by BS[4], ASTM[5], IUPAC GC[3] and IUPAC generalized[7] nomenclatures. It is evident that all four are almost identical: the only possible misunderstanding could arise with V_S, which is the solid (active solid

and/or support) volume in the column in the BS and the volume of the stationary phase in the generalized IUPAC nomenclature. It is true that the subscript S would be better suited to express the *stationary* phase and the ASTM nomenclature on liquid chromatography now being finalized is considering this; however, in GC, the term *liquid phase* is so well established that there is no reason to change it.

TABLE 1

GAS CHROMATOGRAPHIC SYMBOLS AND TERMS

Parameter	*BS*	*ASTM*	*IUPAC**	*Notes***
Capacity (partition) ratio; capacity factor; mass distribution ratio	K'	k	D_m	See Note 1
Column				
Length	L	L		
Inside diameter		d_c		
Average diameter of solid particles in column		d_p		
Average liquid phase film thickness		d_f		
Interparticle porosity		ε	ε_1	See Note 2
Phase ratio	β	β		See Note 3 $\beta = V_G/V_L$
Volume of mobile phase in column (interstitial volume)	V_G	V_G	V_G, V_I	See Note 4
Dead volume			V_d	See Note 5
Solid volume in column	V_S			See Note 6
Specific permeability		B_o		
Height equivalent to one effective plate (HEETP)	HEETP	H	H	
Height equivalent to one theoretical plate (HETP)	HETP	h	h	
Liquid phase				
Density	ϱ_L	ϱ_L	ϱ_L	
Volume	V_L	V_L	V_L, V_S	See Note 7
Weight (mass)	w_L	W_L	w_L	
Mobile phase (volumetric) flow-rate				
At column outlet and ambient temperature		F_a	F	
Same as above but corrected to dry gas conditions		F_o		$F_o = F_a \cdot \frac{P_a - P_w}{P_w}$
At column outlet corrected to column temperature	F_c	F_c	F_c	See Note 8
Mobile phase (interstitial) velocity				
At column outlet		u_o	u_o	
Average in column	$\bar{u}$	$\bar{u}$	$\bar{u}$	$\bar{u} = L/t_M = u_o j$
Optimum average		$\bar{u}_{opt}$		
Mobile phase viscosity		η		
Number of effective plates	N	N	N	See Note 9 $N = 16(t'_R/w_b)^2 = 5.545(t'_R/w_h)^2$
Number of theoretical plates	n	n	n	$n = 16(t_R/w_b)^2 = 5.545(t_R/w_h)^2$

(*Continued on p. 244*)

TABLE 1 *(continued)*

Parameter	*BS*	*ASTM*	*IUPAC**	*Notes***
Number of effective plates required for a given resolution of peaks 1 and 2		N_{req}		$N_{req} = 16R_s^2 \left(\frac{\alpha}{\alpha - 1}\right)^2 = n_{req} \left(\frac{k_2}{k_2 + 1}\right)^2$ See Note 10
Number of theoretical plates required for a given resolution of peaks 1 and 2		n_{req}		$n_{req} = 16R_s^2 \left(\frac{\alpha}{\alpha - 1}\right)^2 \left(\frac{k_2 + 1}{k_2}\right)^2$ See Note 10
Partial pressure of water at ambient temperature		P_w	p_w	
Partition coefficient (distribution constant)	K	K	K, K_D	See Note 11
Peak resolution	R	R_s	R_s	$R_s = \frac{2(t_{R2} - t_{R1})}{w_{b1} + w_{b2}}$
Peak width				
At base		w_b		See Note 12
At half-height		w_h		See Note 12
At inflexion points		w_i		
Pressure				
Column inlet	p_i	P_i	p_i	
Column outlet	p_o	P_o	p_o	
Column, relative		P		$P = P_i/P_o$
Factor relating pressure drop and column permeability		j'		$j' = \frac{3}{4} \cdot \frac{P^2 + 2P + 1}{P^2 + P + 1}$
Pressure gradient (compressibility) correction factor	j	j	j	$j = \frac{3}{2} \cdot \frac{P^2 - 1}{P^3 - 1}$
Pressure drop		Δp		$\Delta p = P_i - P_o$
Ambient		P_a	p	
Retention index	—	I	I	No symbol is given in BS system
Relative retention	r_{12}	r_{is}	r_{12} $r_{A/B}$	See Note 13
Retention times				
(Gas) hold-up time	t_M	t_M	t_M	
Retention time	t_R	t_R	t_R	From start
Adjusted retention time	t_R°	t'_R	t'_R	$t'_R = t_R - t_M$
Corrected retention time		t_R°		
Net retention time		t_N		
Retention volumes				
Gas hold-up volume	V_M	V_M	V_M	$V_M = t_M F_c$
Corrected gas hold-up volume		V_M°		$V_M^{\circ} = V_M j$
Retention volume (total retention volume)	V_R	V_R	V_R	See Note 14 $V_R = t_R F_c$
Adjusted retention volume	V'_R	V'_R	V'_R	$V'_R = t'_R F_c = (t_R - t_M) F_c$
Corrected retention volume		V'_R	V_R°	See Note 15
Net retention volume	V_N	V_N	V_N	$V_N = V'_R j = {}_{8R}F_c j$
Specific retention volume	V_g	V_g	V_g	$V_g = \frac{V_N}{W_L} \cdot \frac{273.15}{T_c}$
Net retention volume per gram of liquid phase at column temperature			V_g^{Θ}	$V_g^{\Theta} = \frac{V_N}{W_L} = \frac{V_g T_c}{273.15}$ See Note 16
Separation factor (relative retention)	α	α	$\alpha_{A/B}$	Always refers to two *adjacent* peaks
Temperature (absolute)				
Ambient		T_a	T_m	
Column	T	T_c	T	

* If there is any conflict between the two IUPAC nomenclatures (GC and generalized), this is given in a Note.

** In equations the ASTM symbols are always used.

Notes:

1. The symbol D_m is used in the generalized IUPAC nomenclature. The IUPAC GC nomenclature did not include this term.
2. The term "interstitial fraction" is used by IUPAC in both nomenclatures.
3. IUPAC recognizes this term but does not specify any symbol.
4. The IUPAC GC nomenclature is using the symbol V_G while the generalized chromatography nomenclature is using V_I (subscript I for "interstitial").
5. The generalized IUPAC nomenclature defines "dead volume" as *the volume between the effective injection point and the effective detection point, less the column volume.*
6. BS is using this symbol to express the effective volume occupied by the active solid and/or the solid support in the column. IUPAC GC nomenclature specifies this term as *the volume occupied by the solid support or the active solid in the column*, however, without using any specific symbol.
7. The IUPAC GC nomenclature is using the symbol V_L (subscript L for "liquid" phase), while the generalized IUPAC chromatography nomenclature is using V_S (subscript S for "stationary" phase). There is a conflict between the symbol V_S used by BS for the *solid volume in the column* and the IUPAC symbol V_S for the *volume of the stationary phase in the column.*
8. The BS definition is ambiguous: it refers to "column outlet temperature", which may mean either column temperature or ambient temperature. On the other hand, at other points, it is clear that column temperature is considered here.
9. IUPAC, in the generalized nomenclature, calls it the "effective theoretical plate number".
10. In the equations for n_{req} and N_{req}, $\alpha = t'_{R2}/t'_{R1}$; $k_2 = t'_{R2}/t'_M$ and $t'_{R2} > t'_{R1}$.
11. The K_D symbol is proposed in the generalized IUPAC nomenclature as a compromise between the old K and the newer proposal for suggesting D for the *distribution constant.*
12. BS and IUPAC are using the appropriate term without any specific symbol. "Peak width" as such automatically means in both the BS and IUPAC nomenclatures the peak width at base. One should always avoid the term "half-width": it is not half of the width (as one would expect from this expression) but the full width at 50% of the maximum peak height.
13. While the symbol α always refers to two adjacent peaks, the symbol r refers to any two peaks in a chromatogram. The difference in the meaning of the subscripts is explained in the text.
14. The expression "total retention volume" is included in the generalized IUPAC nomenclature for further clarification.
15. From the two IUPAC nomenclatures this term exists only in the GC nomenclature.
16. This term exists only in the IUPAC GC nomenclature.

Below, a few remarks are made about the symbols listed in Table 1*.

(a) First, it should be noted that in all three nomenclatures great care was taken to avoid any ambiguity. Thus, since the subscript g has already been assigned to the specific retention volume, V_g (g for "gram of liquid phase"), the subscript G is used for the *gas phase*; hence, the equivalent subscripts M and L are also used for the mobile and the liquid phase, respectively. Another reason for this decision was the ambiguity that lower case l can be easily mistaken for the numeral "one". Thus, lower case l is *never* used, either as a symbol or as a subscript: column length is L and liquid phase is characterized by the subscript L and not l.

Naturally, this rule also means that the same considerations should also be valid for other symbols, *e.g.*, the respective diffusion coefficients in the gas and liquid phases should be D_G and D_L and not D_g and D_l, as one often finds in publications.

(b) Similarly, as r represents relative retention, the subscript c is always used in the symbol for column radius (r_c); furthermore, r should never be used as a sub-

* Having served for many years as both a member and the Chairman of the Subcommittee of Nomenclature of ASTM E-19, the author of this review is one of the most authoritative persons to attest to the validity of this explanation.

script: the subscript for "retention" is R, as in t_R and V_R. Similarly, the symbol for "peak resolution" is R or, even better, R_s.

(c) All of the other subscripts are lower case letters, such as in V_g, d_c and d_f.

(d) In the nomenclatures care was taken *never* to use composite symbols consisting of two letters. Thus, proposals for symbols such as *tm*, *ts* and *tms* are not only against the words but also against the spirit of the official, carefully compiled nomenclatures, irrespective whether lower case or capital letters are used. The only exception is HETP, which, however, is not a "symbol" (the corresponding symbol is h) but an acronym.

(e) There is a specific reason why in gas chromatography both the symbols for the gas phase (G) and the mobile phase (M) are used, although they obviously mean the same: G refers to the gas volume irrespective of compressibility (*i.e.*, the geometric volume), while M refers to a *flowing* mobile phase with a higher inlet than outlet pressure, thus automatically considering the compressibility factor. In other words, V_G is the volume of the gas (mobile) phase in the column under static conditions whereas V_M is the gas hold-up volume in an analysis and, under ideal conditions (*i.e.*, no extra-column "dead" volume):

$$V_G = V_M j = V_M^{\circ} \tag{12}$$

In fact, the difference $(V_M^{\circ} - V_G)$ indicates the extra-column volume of the system.

(f) Note that *all four nomenclatures* are clear in using n for the number of *theoretical* plates and N for the number of *effective* plates, and that both ASTM and IUPAC are also clear in using h and H for HETP and HEETP, respectively. Thus, there is no excuse for mixing them up, *e.g.*, by using N for the theoretical plate number. Another clear agreement is in the symbols for velocity: u or $\bar{u}$. One can often find symbols such as v, w or μ in the literature, and again, there is no excuse for it. Similarly, there is a clear agreement in using β for the *phase ratio* and α for the *separation factor*.

(g) The ASTM nomenclature uses P in general for *pressure* while the others use p; also, ASTM uses W_L for the *weight of liquid phase in the column*, compared with w_L by the others. There is no possibility of misinterpreting the former and thus this does not represent any problem; W_L was deliberately selected by ASTM instead of w_L because the symbol w generally refers to the *peak width*.

There are two areas where disagreements between the nomenclatures may result in misinterpretation of certain data: these refer to the capacity ratio and certain subscripts referring to specific peaks.

From the very beginning, gas chromatographers were almost equally divided in the symbol for the *capacity ratio*: in the United States, k has always been used (and K for the *partition coefficient*), while European scientists used k' or K' for the capacity ratio and k or K for the partition coefficient. Unfortunately, this discrepancy still exists and one may have equal arguments for either usage.

Finally, a few notes are necessary concerning the *subscripts* used in the symbol for the separation factor and the relative retention, denoting the individual peaks. Here, ASTM is very clear: if the subscripts 1 and 2 are used, then $t_{R1} < t_{R2}$, *i.e.*, 2 refers to the peak emerging *later*. Thus, the separation factor as expressed by eqn. 2 in this report is, by definition, always larger than unity. The same is also

true of the generalized IUPAC nomenclature where $\alpha_{A/B} = K_{D(A)}/K_{D(B)}$ and $K_{D(A)} > K_{D(B)}$. On the other hand, in the BS nomenclature, although the symbol is the same and $\alpha = K_A/K_B$, the meaning of the subscripts is reversed ($K_B > K_A$). Thus, as interpreted by BS, $\alpha < 1$ because K_A is the partition coefficient of the *first* peak.

In the case of relative retention, the *definition* is the same in all four cases: the adjusted retention time, volume or the partition coefficient of the substance of interest divided by the same term for the standard. However, the subscripts are not the same, creating the possibility of confusion. In ASTM, the subscripts of i and s are used for the peak of interest and the standard, respectively; on the other hand, both BS and the IUPAC GC nomenclature use *1* for the peak of interest and *2* for the standard, and the generalized IUPAC nomenclature uses *A* for the substance of interest and *B* for the standard.

Finally, a few recommendations included only in the ASTM nomenclature should be mentioned, such as d_f for the *average liquid phase film thickness* in open-tubular columns, d_c for the *inside column diameter* (and hence, logically, r_c for the *inside column radius*) and B_0 for the *specific column permeability*. Particularly the last-mentioned symbol is noteworthy: one may find in the literature about as many symbols for this term as there are publications!

B. *Terms and definitions*

As already mentioned, all these nomenclatures also include definitions of various terms. Table 2 lists these terms (except those already included in Table 1) for which clear definitions are given in these nomenclatures. It is interesting to quote the definition of chromatography from the generalized IUPAC nomenclature:

A method used primarily for separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary while the other moves. The stationary phase may be a solid, or a liquid supported on a solid, or a gel. The stationary phase may be packed in a *column*, spread as a *layer*, or distributed as a *film*, etc.; in these definitions *chromatographic bed* is used as a general term to denote any of the different forms in which the stationary phase may be used. The mobile phase may be gaseous or liquid.

As there is practically no ambiguity in the definitions given in these standards, they are not discussed individually here, except for one. As can be seen in Table 2, three of the four standards are very clear in defining the columns first described by M. J. E. Golay in 1957 as *open-tube* or *open-tubular* columns and *not* as *capillary* columns. Open-tubular columns are very clearly defined in the standards as

a column containing stationary phase but having an unobstructed axial channel (*BS*);
(*having*) unobstructed central gasflow channels (*ASTM*);
a column, usually of capillary dimensions, in which the column wall, a liquid or an active solid supported on the column wall acts as the stationary phase (*IUPAC*).

As can be seen, "capillary" is simply given as an indication of the usual diameter but not as a characterization of the column type. This is even clearer in the BS nomenclature where, in addition to "open-tubular columns", we also find a separate definition for "capillary columns":

A column of capillary dimensions, generally less than 1 mm internal diameter.

TABLE 2

TERMS (OTHER THAN THOSE IN TABLE 1) WHICH ARE DEFINED IN THE STANDARD NOMENCLATURES

Term	BS	ASTM	IUPAC GC	IUPAC* general	Notes**
Absolute detector	+				
Active solid	+	+	+	+	
Adsorption chromatography				+	
Adsorption column	+				
Air peak			+		
Auxiliary gas	+				
Backflush	+				
Baseline	+	+	+	+	
Bypass injector	+	+	+	+	
Capillary column	+				
Carrier gas	+	+	+	+	See Note 1
Chromatogram	+	+	+	+	
Chromatograph (verb)				+	
Chromatograph (noun)				+	
Chromatography				+	
Column	+	+	+	+	
Column chromatography				+	
Column packing		+			
Column performance (= number of theoretical plates)			+	+	
Concentration distribution ratio, D_c				+	See Note 2
Concentration-sensitive detector	+				
Conditioning	+				
Detection				+	
Detector	+	+	+	+	
Differential chromatogram				+	
Differential detector	+		+	+	
Differential (concentration) detector		+			
Differential (mass) detector		+			
Distribution coefficients					
D_g				+	See Note 3
D_v				+	See Note 3
D_s				+	See Note 3
Displacement chromatography				+	
Dynamic range	+				
Dynamic range, linear	+				
Dynamic ratio	+				
Effluent	+			+	
Eluate				+	
Eluent				+	
Elute (verb)				+	
Elution band				+	See Note 4
Elution chromatography				+	
Final temperature				+	
Flow-programmed chromatography				+	
Frontal chromatography				+	
Fronting	+				
Gas chromatograph	+				

(Continued on p. 249)

TABLE 2 *(continued)*

Term	*BS*	*ASTM*	*IUPAC GC*	*IUPAC* general*	*Notes***
Gas chromatography (GC)	+	+	+	+	
Gas–liquid chromatography (GLC)	+	+	+	+	
Gas–solid chromatography (GSC)	+	+	+	+	
Gas-displacement chromatography		+			
Gas-elution chromatography		+			
Gas-frontal chromatography		+			
Initial temperature				+	
Injection point				+	
Injection temperature				+	
Integral chromatogram				+	
Integral detector	+	+	+	+	
Integrator	+				
Internal standard	+		+	+	
Isothermal gas chromatography				+	
Linear dynamic range	+				
Liquid phase	+	+	+		
Liquid phase loading		+			
Liquid volume			+		See Note 5
Marker	+			+	
Mass distribution ratio, D_m				+	See Note 6
Mass-sensitive detector	+				
Mobile phase	+		+	+	
Modified active solid				+	
Modified sorbent	+				
Normalization	+				
Open-tube chromatography				+	
Open-tube (open-tubular) column	+	+		+	
Partition chromatography				+	
Packed column	+	+		+	
Packing	+			+	
Partition column	+				
Peak	+	+	+	+	
Peak area	+	+	+	+	
Peak base	+	+	+	+	
Peak height	+		+	+	
Peak maximum	+			+	
Peak integration	+				
Porous-layer open-tube (PLOT) column		+			
Programmed-flow gas chromatography	+	+			
Programmed-pressure gas chromatography	+				
Programmed-temperature gas chromatography (PTGC)	+	+			
Pyrolysis–gas chromatography		+			
Reaction–gas chromatography		+			
Relative response factor	+				
Response	+				
Response factor	+				
Retention temperature				+	
Sample injector	+		+	+	

(Continued on p. 250)

TABLE 2 *(continued)*

Term	*BS*	*ASTM*	*IUPAC GC*	*IUPAC* general*	*Notes***
Sample inlet system		+			
Separation temperature				+	See also retention temperature
Solid, active	+	+			
Solid support	+	+	+	+	
Solid volume			+		See Note 7
Solvent (liquid phase)		+	+		
Solute		+	+		
Stationary phase	+	+	+	+	
Step	+	+	+	+	
Step height	+	+	+	+	
Support, solid	+	+	+	+	
Support-coated open-tube (SCOT) column		+			
Tailing	+			+	
Temperature-programmed chromatography				+	
Traps		+			
Unresolved peak				+	
Unretained substance		+			
Wall-coated open-tube (WCOT) column		+			
Zone				+	

* Those terms which refer only to chromatographic methods other than gas chromatography are not listed here.

** *Notes:*

1. In GC, this term is used for "eluent" or the "mobile phase".

2. The concentration distribution ratio:

$$D_c = \frac{\text{amount of component in stationary phase per cm}^3 \text{ of stationary phase}}{\text{amount of component in mobile phase per cm}^3 \text{ of mobile phase}}$$

is a generalized name for the partition coefficient, now called distribution constant, for which the symbol K_D is also permitted.

3. The generalized IUPAC nomenclature recognizes three distribution coefficients:

$$D_g = \frac{\text{amount of component in stationary phase per gram of dry stationary phase}}{\text{amount of component in mobile phase per cm}^3 \text{ of mobile phase}}$$

$$D_v = \frac{\text{amount of component in stationary phase per cm}^3 \text{ of bed volume}}{\text{amount of component in mobile phase per cm}^3 \text{ of mobile phase}}$$

$$D_s = \frac{\text{amount of component adsorbed per m}^2 \text{ of surface}}{\text{amount of component in mobile phase per cm}^3 \text{ of mobile phase}}$$

Of these, D_g is applicable in ion-exchange and gel chromatography, where swelling occurs, and in adsorption chromatography with adsorbents of unknown surface area; D_v is applicable when it is not practicable to determine the weight of the solid phase; D_s is applicable in adsorption chromatography with a well characterized adsorbent of known surface area.

4. Synonymous with "peak".

5. Defined as the volume of the liquid phase in the column.

6. The term

$$\frac{\text{amount of component in stationary phase}}{\text{amount of component in mobile phase}}$$

existing in all four expressions (D_c, D_g, D_v and D_s) is called the *mass distribution ratio* (D_m) instead of *capacity factor* or *capacity ratio* (see Table 1).

7. Defined as the volume of the support or active solid in the column.

This clear distinction between the two terms is very important, as not every open-tubular column is a capillary column and not every capillary column is an open-tubular column! Without making this clear distinction, we might create confusion, particularly in liquid chromatography where recently both open-tubular columns of capillary dimensions and packed capillary columns have been described for improving column efficiency.

Recently, interest in open-tubular columns has greatly increased and we even find biannual international symposia dealing with advances in this field and, in the name of these symposia, the expression "capillary column" is used. This is, of course, against all the accepted international nomenclatures.

4. OTHER GAS CHROMATOGRAPHY STANDARDS

ASTM has also developed four other standards related to gas chromatography as a technique*. The first is E-260, which specifies the general instrumentation and the information needed to describe a chromatographic method exactly[8]; two others then specify how to test a thermal conductivity[9] and a flame-ionization[10] detector. A standard dealing with the electron-capture detector is in the final approval stage at the writing of this review.

A. Recommendations on the general procedure

E-260 had been developed at a relatively early stage of gas chromatography; in fact, the idea of its compilation originated in Committee D-2, before the organization of Committee E-19, and it originally had a specific purpose. It was around 1960 that analytical procedures utilizing gas chromatography started to be developed by a number of ASTM Committees (and, in fact, also by other groups) active in various fields. At that time, it was felt that a detailed recommendation was needed to describe the individual parts of a GC equipment and to specify what represents a proper gas chromatographic system**; to detail the manipulations (*e.g.*, preparation of column packing) an average gas chromatographer must carry out in a routine analytical laboratory; and to enumerate the individual information which should be included and specified in an ASTM "standard method". There is no question that today this standard is outdated in many respects (*e.g.*, of the eight detector types discussed only three have today any importance), and the compilation of a greatly modified text is in progress. For this reason, we shall not deal with these recommendations but will

* Naturally, there are a large number of ASTM and BS standards in which gas chromatography is used as the analytical method. However, here we deal only with those the subject of which is the technique itself.

** Generally, an ASTM Method also specifies the basic equipment to be used for the given measurement.

mention only that there are still a number of valid points in the existing text, particularly those listing the conditions that one must specify in a GC analytical method.

B. Terms related to detectors

The two other ASTM standards quoted specify the methods of testing thermal conductivity (TCD) and flame-ionization (FID) detectors. Both describe in detail how to use the exponential dilution flask, permeation tubes or the dynamic method for testing and give definitions of sensitivity, minimum detectability, linear range, response time, noise and drift.

Noise is the amplitude expressed in microvolts (TCD) or amperes (FID) of the envelope of the baseline which includes all random variations of the detector signal of a frequency on the order of one or more cycles per minute. As explained, this corresponds to the observed noise: the *actual* noise of the system may be larger or smaller than the observed value, depending on the method of data collection or signal monitoring from the detector.

Drift is the average slope of the noise envelope expressed in microvolts (TCD) or amperes (FID) per hour as measured over $^1/_2$ h. Fig. 1 illustrates both noise and drift.

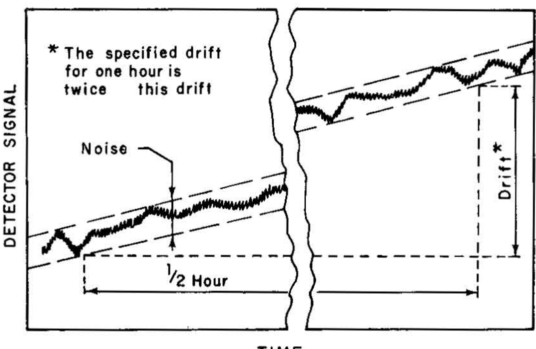

Fig. 1. Example of the measurement of the drift and noise level of GC detectors. From ASTM E 516 and E 594.

Response time is primarily important for thermal conductivity detectors. It is the time required for the output signal to reach 63.2% of the new equilibrium value when the composition of the gas entering the detector is changed in a stepwise manner, within the linear range of the detector. The internal volume and geometry of the detector have to be stated in connection with the response time specification. Response time can be determined and specified at various flow-rates. Fig. 2 shows the graphical determination of the response time from the recorder chart obtained in a high-speed recorder*.

In the case of the flame-ionization detector, response time is not important because the time constant of ionization is negligible and the transit time of the sample

* This method was first described by Schmauch[24].

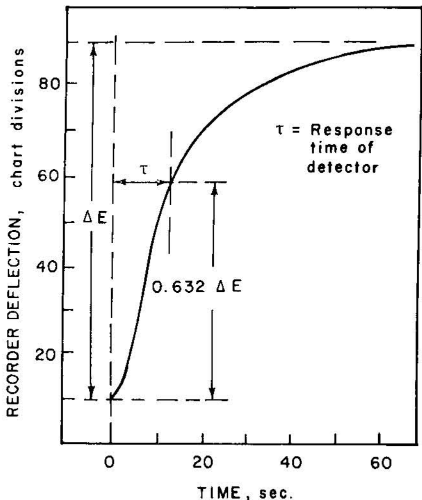

Fig. 2. Example of the measurement of response time of a thermal conductivity detector. From ASTM E 516.

through the detector has little influence. In fact, in a typical FID, not the detector itself, but the electrometer/amplifier is the limiting factor.

The basic expression of *sensitivity* is the same, but in detail it depends on the type of detector, whether it is based on concentration (TCD) or mass flow-rate (FID). Thus, sensitivity is the signal output (peak height) per unit concentration (TCD) (eqn. 13) or unit mass (FID) (eqn. 14) of a test substance in the column effluent:

$$S_{\mathrm{TCD}} = \frac{(P.H.)}{W_s/w_v} = \frac{(P.H.)\,w_v}{W_s} \tag{13}$$

$$S_{\mathrm{FID}} = \frac{(P.H.)}{W_s/w_t} = \frac{(P.H.)\,w_t}{W_s} \tag{14}$$

where $P.H.$ is the peak height at maximum, in millivolts (TCD) or amperes (FID), and W_s is the weight of the test substance in the sample, in grams or milligrams; w represents the peak width at half-height expressed either as time (w_t) (minutes for the TCD and seconds for the FID) or in volume (w_v) (in milliliters). The latter can be calculated as

$$w_v = w_t F_c \tag{15}$$

where w_t is expressed in minutes and F_c, the flow-rate of the carrier gas corrected to column temperature, in milliliters per minute.

Detector sensitivity can also be calculated from the peak area determined either manually or by electronic integration, but the area value obtained in square centimeters or square millimeters, or counts, must be corrected into the units specified below. In this case,

$$S_{TCD} = \frac{AF_c}{W_s} \tag{13a}$$

$$S_{FID} = \frac{A}{W_s} \tag{14a}$$

where A is the peak area in mV·min (TCD) or A·sec (FID). Thus, the respective dimensions of S_{TCD} and S_{FID} are mV·min·g^{-1} or A·sec·g^{-1}. It should be noted that the sensitivity values calculated from eqn. 13 *vs.* eqn. 13a and eqn. 14 *vs.* eqn. 14a will differ by 6%, the difference between peak area calculated using the peak width at half-height and the true peak area.

Minimum detectability is the concentration (TCD) or mass flow (FID) of the test substance in the carrier gas that gives a detector signal equal to twice the noise level, and can be calculated from the measured sensitivity and noise level:

$$M.D. = 2\,(N.L.)/S \tag{16}$$

Here, $M.D.$ refers to the minimum detectability, $N.L.$ is the noise level (in millivolts for the TCD and amperes for the FID) and S is the calculated detector sensitivity, in ml·ml·g^{-1} (TCD) or A·sec·g^{-1} (FID).

The *linear range* of a detector can generally be expressed in three ways. In the traditional way, it is shown as log–log plots, plotting peak area (or height) against concentration (in milliliters per minute, for the TCD) or mass flow-rate (in grams per second, for the FID) of the sample in the column effluent. The shortcomings of this presentation are that it is almost impossible to investigate the variation (randomness) within the range* and the end-point of the range cannot be exactly determined. Therefore, both ASTM standards advocate the presentation of the linear range as the plot of sensitivity** *vs.* the values representing the amount of sample, *i.e.*, the concentration (in milligrams per milliliter, for the TCD) or the mass flow-rate (in grams per second, for the FID) in the column effluent at the detector. Fig. 3 shows a typical presentation; as can be seen, these are semilogarithmic plots. The linearity plot represents, by specification, the smooth line through the data points. This line must be within ±5% of the constant value obtained by a least-squares fit of the lower decades (four specified for the FID). At the end of the linear range the plot will curve downwards. The end of the linear range is the point where the plot crosses the −5% envelope.

The third way of presenting the linear range is simply to give the ratio of the end of the linear range and the minimum detectable limit. For example, if the respective values are $1 \cdot 10^{-5}$ and $4 \cdot 10^{-12}$ g/sec, then the linear range is $2.5 \cdot 10^{6}$. Needless to say, if the linear range is specified in this way, the minimum detectable limit must also be given.

* Except with help of linear regression analysis, giving the regression coefficient.

** As the sensitivity value for a TCD is directly proportional to A/W_s (*cf.*, eqn. 14a), one can simply use A/W_s instead of sensitivity.

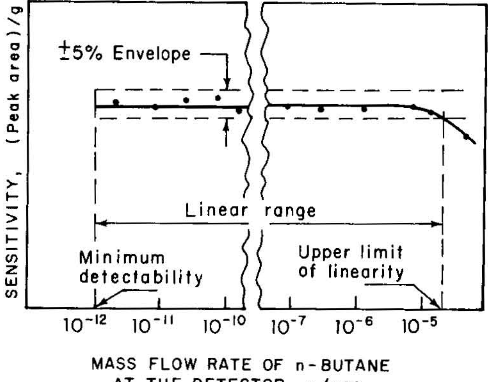

Fig. 3. Example of the linearity plot of a GC detector. From ASTM E 594. The values on the abscissa are typical of a flame-ionization detector. A similar plot would be obtained for a thermal conductivity detector, plotting the concentration of the sample in the column effluent at the detector.

5. ACKNOWLEDGEMENT

I express my appreciation to the American Society for Testing and Materials for permitting the reproduction of Figs. 1–3.

6. SUMMARY

The nomenclature of gas chromatography is reviewed with special emphasis on its evolution and recommendations by early *ad-hoc* committees which represented the basis of the very detailed nomenclatures developed and published by the British Standards Institute, the American Society for Testing and Materials, and the International Union of Pure and Applied Chemistry. The terms and symbols included in the existing four standards (BS 3282; ASTM E 355–77; IUPAC GC and general chromatography nomenclatures) are compared and some additional existing rules outlined. Finally, other existing standards related to gas chromatography as a technique are also discussed.

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Journal of Chromatography, 165 (1979) 257–282
Chromatographic Reviews
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHREV. 124

GAS CHROMATOGRAPHIC ANALYSIS OF ORGANOSILICON COMPOUNDS

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1. INTRODUCTION

The high thermal stability of organosilicon compounds (OSC) is one of the main factors accounting for their wide application in gas–liquid chromatography (GLC). Their main uses are as stationary phases and as reagents for derivatization in the gas chromatography of various organic compounds. For instance, a Handbook of Gas Chromatography[1] lists 70 organosilicon stationary phases (SP) employed in half of the total number of GLC separation procedures. Another important property of OSC is their capacity to reduce the polarity of chromatographic supports by reaction with the surface hydroxyl groups. Similar procedures may be employed to increase the thermal stability and volatility of a number of polar organic compounds, such as carboxylic acids, steroids, carbohydrates and amino acids. Over 1000 publications have been reviewed[2–6] that deal with the problem of the silylation of organic compounds and the analysis of trimethylsilyl (TMS) derivatives.

On the other hand, organosilicon compounds lend themselves readily to separation by GLC. However, only about 250 publications (less than 1% of the total number of publications on GLC) are connected to varying degrees with the gas chromatography of OSC. Several reviews[3,7–10] have surveyed the problems of sampling, the choice of stationary and mobile phases for the separation of some typical mixtures (mostly monomers) and the detection and pyrolysis of OSC.

However, there is no complete survey available of the methods used for OSC separation. The applicability of GLC in combination with other techniques and the gas chromatographic properties of OSC have also been omitted from most

reviews. To fill this gap, we have attempted to review the experimental data bearing on these problems that had been published up to the middle of 1978.

2. RELATIONSHIPS BETWEEN RETENTION PARAMETERS OF ORGANOSILICON COMPOUNDS AND THEIR PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE

The relationships between the structure and properties of substances is a matter of lasting interest in chromatography. Most studies on gas chromatography, at least to some extent, pertain to the problem of the chromatographic behaviour of organic compounds. The chromatography of OSC is not so well documented, the data being scattered in the literature.

The retention of a solute in gas chromatography can be described by using various parameters, such as retention time, retention volume and relative retention values. Retention indices are also frequently used[11–15]. These methods are interrelated[16], because the physical entity determining the chromatographic properties of substances is invariable, regardless of the type of retention parameter applied.

Several retention parameters analogous to Kováts retention indices have been proposed in the study of OSC. Preisler[17] proposed the use of the homologous series of dimethylcyclopolysiloxanes as a reference series:

$$i = 100n + 100\left(\frac{\log V_{R_i} - \log V_{R_n}}{\log V_{R_{n+1}} - \log V_{R_n}}\right) \tag{1}$$

where i applies to the substance being studied, and n and $n+1$ to cyclic polydimethylsiloxanes containing n and $n+1$ silicon atoms, respectively.

Garzo and Alexander[18] introduced the so-called dimethylsiloxane number:

$$DN_x = 100\left(\frac{\log t'_x - \log t'_{D_m}}{\log t'_{D_{m+1}} - \log t'_{D_m}} + m\right) \tag{2}$$

where x is the substance being studied and D_m and D_{m+1} refer to polydimethylsiloxanes with m and $m+1$ silicon atoms, respectively. The use of linear polydimethylsiloxanes as a reference series was reported by Wurst and Churaček[19]:

$$I_{S_i} = y + m\left(\frac{\log V_{N(x)} - \log V_{N(y)}}{\log V_{N(y+m)} - \log V_{N(y)}}\right) \tag{3}$$

where x corresponds to the substance being studied and y and $y+m$ to linear polydimethylsiloxanes containing y and $y+m$ silicon atoms, respectively.

Logarithms of retention volumes and retention indices for homologous series of organic substances are known to be correlated with the boiling temperatures of the solutes:

$$R = a_0 + a_1 T_b \tag{4}$$

where R is the retention index or the logarithm of the absolute or relative retention volume and T_b is the boiling temperature.

There is a fairly good correlation between these parameters, not only for homologues, but also for a much wider range of substances with similar structures. The same relationship is observed in the series of OSC. Bortnikov[20] demonstrated that eqn. 4 is valid for a number of organic derivatives containing silicon and germanium. The equations obtained enable to determine the boiling point with 3% accuracy on the basis of gas chromatographic data. Palamarchuk *et al.*[21] investigated the relationship between the relative retention volumes and the boiling points of the compounds formed during the synthesis of ethylchlorosilane. The resulting diagrams were further used to determine the boiling points of unknown compounds. The departures from literature data never exceeded 4°C. Kirichenko and co-workers[22–24], on the basis of temperature dependence data, found the heats of solution of dimethylcyclosiloxanes, alkoxychlorosilanes and ethoxyfluorosilanes and showed them to be correlated with the boiling temperatures. Nickless and co-workers[15–30] reported on the redistribution reactions of organo-element compounds.

The same linear correlation was found between the logarithms of retention volumes and boiling temperatures for silicon- and tin-containing derivatives. However, with aromatic derivatives, even for the OSC, the experimental points fail to fit the same line[31]. Rumyantseva and co-workers[32–35] studied alkyl-, aryl-, halogen-, chloro- and alkylsilanes, polysiloxanes and other OSC and demonstrated the possibility of determining, on the basis of the boiling temperatures, the logarithms of retention volumes with approximately 2% error. The boiling points of alkoxysilanes[36] and alkylsilanes[37] can be determined with ± 4°C accuracy using chromatographic data, as reported by Peetre and co-workers. Garzo *et al.*[38] also observed the retention indices of chloro-, alkyl-, aryl- and alkoxysilanes to be linearly correlated with their boiling temperatures. Ainshtein and Shulyatieva[39] found the retention indices of 56 alkyl- and arylchlorosilanes to be correlated with the boiling temperatures, but in this instance considerable departures from linearity were observed. Brown and Mazdiyasni[40] reported that isopropoxides of the group IV elements were eluted from the chromatographic column in order of decreasing volatility.

A linear correlation between logarithms of retention volumes and molar volumes was demonstrated by Wurst and co-workers[41,42] for OSC:

$$\log V_g = V^M K_1 + K_2 \qquad (5P)$$

where K_1 and K_2 are constants characteristic of particular classes of organic and organosilicon compounds. Retention may also be represented by:

$$\log V_g = \frac{V_i^M(\delta_L^2 + 2\delta_i\delta_L)}{2.3\ RT} + \text{constant} \qquad (6)$$

where V_i^M and δ_i are molar volume and solute solubility parameters, respectively, and δ_L is the solubility parameter of the stationary phase.

The relationship between molar refraction (MR) and the logarithms of retention parameters was investigated by Wurst and co-workers[43–46], who found that for the compounds under study the equation

$$\log V_g = a_0 + a_1 MR \qquad (7)$$

was valid, where a_0 and a_1 are coefficients specific for homologous series. Supporting evidence demonstrating the validity of this equation was provided by Ellrén *et al.*[36] for alkoxysilanes. Kirichenko *et al.*[22] also found that the heat of solution in alkoxychlorosilanes used as stationary phases was well correlated with molar refraction. Ellrén *et al.*[47] proposed a "refraction number", which is analogous to retention index:

$$N_{D(A)} = 100p + \left(\frac{n_{D(A)}^{20} - n_D^{20}(C_p)}{n_D^{20}(C_{p+1}) - n_D^{20}(C_p)}\right) \cdot 100 \quad (8)$$

where n_D is refractive index and C is the number of carbon atoms in an n-alkane. The indices (A), (p), $(p+1)$ refer to the test-compound and n-alkanes containing p and $p+1$ carbon atoms, respectively. The N_D values were shown to be correlated with retention indices. The above correlation can be employed for the group identification of alkyl- and alkoxysilanes.

Wurst[43,44,46] demonstrated a linear correlation between parachor and the logarithms of retention indices for polysiloxanes and alkylchlorosilanes. The coefficients in the equation

$$\log V_g = a_0 + a_1 P \quad (9)$$

are characteristic of the homologous series.

In cases when specific interactions within a particular group of compounds contribute to retention to the same extent, a linear correlation between retention values and molecular mass is observed. Bortnikov *et al.*[48] reported on the properties of substances in Et_3MH and Et_4M series; it was established that retention volumes tend to increase when the atomic weight of the element M increases. The adsorption and solution properties of organo-element compounds studied by gas chromatography[20,49,50] revealed a linear correlation between the chromatographically determined values of the heat of solution and adsorption and molecular mass of the solute. A linear relationship between logarithm of retention volume and molecular mass was demonstrated by Preisler[17] for polydimethylcyclosiloxane, methylhydrocyclopolysiloxane and methylethylhydrocyclopolysiloxane series. A similar correlation was noted for nitrogen-containing OSC[51]. Similarly, retention indices determined within series of N-substituted saturated nitrogen-containing heterocycles were shown to have the same type of relationship[52,53]. Retention indices of methyl(2-furyl)silanes, -germanes, -stannanes and -plumbanes of the formula $Me_{4-n}MFu_n$ have also been found to increase in parallel with the atomic weight of the element M[54].

Several studies have reported temperature dependences of retention values in OSC. Nagy *et al.*[55] present evidence of a linear correlation between logarithm of effective retention volume measured for members of the same homologous series on the same column at various temperatures with programming of the carrier gas flow-rate. Similar evidence was presented by Nickless and co-workers[25–30]. Wurst and co-workers[44,56] investigated the temperature dependence of the retention parameters of vinylethoxysilanes and polydimethylsiloxanes and proposed the equation

$$\log V_g = A + \frac{B}{T} \quad (10)$$

where A and B are constants.

The temperature dependence of retention indices of OSC was studied by Kirichenko and co-workers[22,57–59]. The retention indices were found to decline with increasing temperature, the temperature coefficient $\partial I/\partial T$ varying with the size of the polydimethylsiloxane ring. Temperature dependence can be used for assigning chromatographic peaks to particular homologous series.

The temperature dependence of retention volumes can also be employed to determine heats of solution in the stationary phase. The dependence of the relative molar heats of solution on the relative molar entropy of solution was studied by Chernoplekova *et al.*[60]. The dependences were found to be identical for silicon, germanium and tin derivatives on non-polar stationary phases, but they varied on polar stationary phases. This finding enables one to discriminate between derivatives of these elements, and to assess the degree of molecular asymmetry and polarity.

Some other physico-chemical properties of solutes have been found to influence retention values. For instance, chromatographic data can be useful in assessing electron polarizability[20,48–50]. Heat of adsorption has been reported to be linearly correlated with chemical shifts in the NMR spectra of OSC[20,48–50].

The dipole moments of solutes are known to be responsible for their capacity for orientational interactions with stationary phase molecules. Ainshtein and Shulyatieva[39] presented evidence of correlations between ΔI values and dipole moments for methyl- and phenylchlorosilanes.

The retention indices of a series of C- and Si-substituted silatranes (I) have been measured[248]. These compounds are characterized by unique ΔI values (as high as

(I)

1517 units for phenoxysilatrane on OV-225). The inter-relations between the ΔI values and dipole moments of these compounds can be described by

$$\Delta I = a_0 + a_1\mu \qquad (11)$$

This equation permits the evaluation of dipole moments with $\pm 5\%$ accuracy using chromatographic data. The chromatographic properties of silatranes can also be described by using modified Taft equation:

$$\Delta I = a_0 + a_1\sigma^* + a_2E_s^0 \qquad (12)$$

Retention is shown to decline with increasing number of substituents on carbon atoms in the α-position with respect to oxygen, the resolution of diastereomers being attainable even on packed columns. The structural element $\equiv N \rightarrow Si \equiv$ provides for 430, 640 and 900 units (Apiezon L, OV-17 and OV-225, respectively). The ex-

tremely high values of the retention indices are probably due to enhanced interaction of oxygen atoms with electron-acceptor groups on the stationary phase as a result of transannular $\equiv N \rightarrow Si \equiv$ bond formation.

We also studied the induction effect of the substituent on the appropriate values of 1-(trimethylsilylalkyl)pyrrolidines, -piperidines and -perhydroazepines[52,53]. It was found that variation in basicity within these series of compounds does not lead to any appreciable changes in the retention values.

The space orientation of fragments in OSC molecules has also been found to affect the retention values. For instance, branched silicon- and germanium-containing organic compounds are characterized by lower retentions than their analogues with normal structures[61]. With polydimethylsiloxane pyrolysis products, Garzo *et al.*[62] demonstrated faster elution for compounds that possessed more compact structures (branched or cyclic) for an equal number of silicon atoms. This observation permitted the preliminary identification of a number of polycyclic siloxanes.

To assess the retention of OSC, one can consider methods of comparative calculations. There have been reports demonstrating that retention indices of alkylchlorosilanes[63] and nitrogen-containing OSC[51] can be calculated *a priori* by using the first method of Karapetyantz's comparative calculations[64].

Several workers[65–69] have discussed the correlation betweed retention parameters measured on different sorbents varying in selectivity. It was found that the homologous series tended to comply with the following equation for non-polar (A) and polar (B) stationary phases:

$$\log V_{g_A} = a_0 + a_1 \log V_{g_B} \tag{13}$$

ΔI = constant

The elution of polydimethylsiloxanes on two stationary phases was studied by Garzo and co-workers[66–69]. The two phase diagram allows one to discriminate between "isomer lines" and "sub-isomer lines", which can be used to clarify the structure of the components of the mixture. The proposed method for the qualitative analysis of polycyclic organosilicon compounds is also applicable to the gas chromatography of TMS esters of silicic acid[69]. According to Ellrén *et al.*[36], retention indices on XE-60 can be estimated using retention indices determined on a non-polar stationary phase (Apiezon L):

$$I^{XE} = kI^{APL} + l \tag{14}$$

Franc *et al.*[65] studied the chromatographic properties of phenylsiloxanes on polar (A) and non-polar (B) stationary phases. It was demonstrated that in the plots of number of phenyl groups *versus* $\log t_{RB}/\log t_{RA}$, the points corresponding to the same number of phenyl radicals tended to fall closely together, the value $\log t_{RB}/\log t_{RA}$ declining with increasing number of phenyl radicals.

It is known that the introduction of the same structural elements into molecules of different solutes may result in similar increments of the logarithm of retention volumes or retention indices. This accounts for the correlation observed between retention parameters and the number of particular atoms or structural fragments present in the molecule. For instance, the number of silicon atoms in silanes is linear-

ly correlated with the logarithm of specific retention volume[61]. The same relationship holds for germanium derivatives. A linear correlation between logarithm of retention volume or retention indices and the number of siloxane units has been reported for polydimethylsiloxanes by Wurst[43], Wurst and Dusek[56] and Rumyantseva *et al.*[32,33,35]. Kirichenko *et al.*[59] studied the influence of the ring size on the retention indices of various organocyclosiloxanes and were able to identify compounds containing up to 12 silicon atoms.

Luskina *et al.*[70] reported that an increase in log V_g values was linearly related to the increasing size of the ring in the homologous series of oligodimethyl(methyldichlorophenyl)cyclosiloxanes. α,ω-Dihydrodimethylsiloxanes were studied by Kochetov *et al.*[71]. The increments of I determined for $-O-SiMe_2-$ fragments were used to calculate retention parameters for a number of compounds. The accuracy of the calculations was about ± 5 index units. Nomograms for the identification of components in complex mixtures of structurally related compounds were prepared by Yudina and co-workers[72,73], based on the finding that methylpropylsiloxane and methyltrifluoropropylsiloxane groups affect retention in a standard manner. Peetre *et al.*[37] demonstrated that the retention indices of mixed tetraalkylsilanes and structurally identical tetraalkoxysilanes may be connected by the following equation:

$$I_{\text{alkyl}} = k_1 I_{\text{alkoxy}} + k_2 \tag{15}$$

the correlation coefficient exceeding 0.999. Thus, I values for alkylsilanes can be calculated by using the I values for alkoxysilanes, or *vise versa*, with an accuracy of ± 1–2 units. A linear correlation between the logarithms of the effective retention volumes of two homologous series studied under the same experimental conditions was reported by Nagy *et al.*[55]. The stability of the effects exerted on retention by various structural fragments allowed Berezkin and co-workers[74–76] to express the logarithm of the relative retention time of OSC as the sum of contributions by particular bonds or structural elements:

$$\log \tau_i = \Sigma n_{gj} \Gamma_{gj} \tag{16}$$

where τ_i denotes relative retention time, Γ_{gj} is $\log \tau$ corresponding to the particular set of bonds (structural element) and n_{gj} is the number of such structural elements in the molecule. The increments contributed by the fragment $-CH_2-CH_2-$ were shown to be virtually identical for alkanes and alkylsilanes, which makes it possible to predict the chromatographic properties of OSC on the basis of retention data found for the carbon analogoues. Similarly, Rumyantseva and co-workers[32–35] expressed the retention values of a number of alkyl-, aryl- and halogensilanes, chloroalkylsilanes and polysiloxanes as the sum of individual contributions. Wurst and Churaček[19] measured increments of I_{S_i} values and Garzo and co-workers reported increment vaues found for retentiõn indices[38] and for DN_X[66–68]. Kochetov [77] described retention parameters as the sum of individual contributions. We also demonstrated[54] that the retention of compounds of the general formula $(\text{C}_4\text{H}_3\text{O})_n\text{M(CH}_3)_{4-n}$ can be described by

$$I = a_0 + a_1 X_1 + a_2 X_2 \tag{17}$$

where X_1 is the atomic weight of the element M and X_2 is the number of furan rings per molecule. The use of structural increments to determine I values for nitrogen-containing OSC was discussed by Kochetov *et al.*[51]. Nagy[5], Chernoplekova *et al.*[60] and Ellrén *et al.*[36] reported retention values to be linearly correlated with the number of carbon atoms in various groups of OSC. Kirichenko and Markov[22] and Preisler[17] demonstrated a linear correlation between retention values and the number of chlorine atoms in solutes.

On the other hand, it is not infrequent to observe a departure from the additivity principle due to the mutual effects of the structural fragments. For instance, such evidence was presented by Ainshtein and Shulyatieva[39] for alkyl- and arylchlorosilanes. Semlyen and co-workers[78,79] demonstrated that the retention values found for chlorosilanes and chlorogermanes on the basis of additive calculations may differ significantly from the experimental data. The differences between the calculated and experimental values were found to depend on the length of the hydrocarbon radicals attached to the silicon atom. Likewise, the retention indices calculated for silatranes fail to comply with the additivity scheme, because the contribution of the structural fragment $\equiv N \rightarrow Si$ is fairly large and it lacks stability.

In many instances, the observed departures from additivity can be taken into account and the appropriate corrections can be made, but this usually results in the creation of too cumbersome models. Kirichenko *et al.*[80] found increments of retention indices that correspond to particular structural fragments in alkylchlorosilanes and attained a fairly high accuracy in their calculations, as they took account of the effect exerted by the number of carbon atoms in hydrocarbon radicals (m) and the total number of alkyl groups (n):

$$I = \delta I_{SiCl_n} + n(100m)(4-n) \quad (18)$$

It has been established that in methyl- and ethylcyclopolysiloxanes the contributions of MeHSiO and EtHSiO fragments to retention indices are not constant and vary with the size of the ring[81]. The relationship between retention indices and the number of members (n) in the ring was described as follows:

$$I = a_0 + a_1\sqrt{n} + a_2 n \quad (19)$$

The accuracy of the calculations was ± 5 units. Takacs *et al.*[82] proposed a method for *a priori* calculations of retention indices for methylchlorosilanes, allowing the assessment of the contributions of atoms and bonds and the interactions with stationary phases[83,84].

Peetre and co-workers[36,37,47,85–88] made a detailed analysis of the chromatographic properties of a large number of tetraalkyl- and tetraalkoxysilanes. A method and an improved computer program adapted for the analysis of complex mixtures when the addition of all necessary n-alkanes is impossible were proposed for the calculations of retention indices[88]. Retention indices of mixed tetraalkoxysilanes were studied[36] and it was demonstrated that these compounds are characterized by higher values of I than those calculated using the additivity principle on the basis of their symmetrical analogous. This discrepancy was found to be dependent on the length of

the shortest alkyl chain and on the difference in length between the shortest and the longest chains. The following equation was proposed for the calculation of I:

$$I_{(RO)_4Si} = \Sigma I_{ROSi} + \Sigma(ndk)_{RO-RO} \tag{20}$$

where I_{ROSi} is one quarter of the retention index of $(RO)_4Si$; n is a combination number that is found by multiplying the numbers of the various alkoxy groups attached to the silicon atom; d is the difference in length between the carbon chains of two alkoxy groups; and k is a constant determined by the length of the shortest carbon chain.

The retention indices calculated on the basis of this equation differed from the experimental values, on average, by 1.2 units. The value of the correction term ndk was shown to be determined by the interaction of carbon atoms in various alkoxy groups. The accuracy of the calculations can be further enhanced for alkoxysilanes by using an improved equation[86] that permits an *a priori* determination of the temperature increment of the retention index. The procedures developed were later extended to tetraalkoxysilanes with branched chains[87]. The increase in the retention indices with the increasing chain length was found to depend on the charge sign of the terminal carbon atom, as revealed by a study with tetraalkylsilanes[37]. Further, it was assumed that charge alternation takes place in the carbon chain attached to the silicon atom[89]. The introduction of a β-CH_2– group into a tetramethylsilane molecule resulted in an increase of 125.6 units in the I value, whereas with a γ-CH_2– group it was only 67.6 units. Thus, the anomalously low value of I of tetramethylsilane may be due to the negatively charged peripheral part of the molecule hindering its dissolution in the stationary phase. Transition from methoxysilane to ethoxysilane results in a small increase in the I value (by 25 units on OV-17). Apparently in this instance also ethoxysilane shows anomalously low solubility in the stationary phase due to the negatively charged outer surface of the molecule.

The above facts serve to illustrate the existence of various correlation patterns between the structure of OSC and their retention values obtained by GLC. A knowledge of these correlation patterns is of the utmost importance for several reasons. It provides a compact representation of retention data for various classes of substances and helps to predict retention parameters for chromatographically unexplored compounds, to identify components of complex mixtures and to gain an insight into the physico-chemical processes that determine the interactions between solutes and stationary phases. Although *a priori* methods used to calculate retention parameters are not sufficiently accurate to provide unequivocal identification of components in multi-component mixtures, as compared with experimentally obtained data, they are useful in helping to substantiate or disprove hypotheses bearing on the structure of components in test mixtures or, at least, to narrow significantly the scope of the available concepts.

3. DETECTORS FOR GAS CHROMATOGRAPHY OF ORGANOSILICON COMPOUNDS

Thermal conductivity and flame-ionization detectors are the most common detector devices currently used in gas chromatography. The former is conspicuous for its simple construction and reliability in operation. Its applicability to the analysis

of both organic and inorganic compounds offers certain advantages, especially with alkylchlorosilane mixtures, which normally contain trace amounts of inorganic substances that cannot be detected with a flame-ionization detector. An important property of the katharometer is that the separated compounds are not destroyed, which allows fractions to be collected for further investigation, for example, by spectroscopic methods. Frequent cleaning the detector is also avoided. On the other hand, the sensitivity is low and its response is slow, which makes it inapplicable to the analysis of impurities and in capillary chromatography. Like most other detectors, katharometers exhibit a wide variation in sensitivity towards different chemical substances, which cannot be readily predicted on the basis of theoretical assumptions. Response factors found for a katharometer (with helium as the carrier gas) were presented by Hanneman[10] for polydimethylsiloxanes and methyl- and phenylchlorosilanes. The response factors increased (and the sensitivity therefore decreased)with increasing number of units in siloxane polymers and with increasing content of chlorine in chlorosilanes. A similar variation in katharometer sensitivity was noted for methylchlorosilanes by Rotzsche[91]. Dearlove *et al.*[91] provided molar and weight response factors to be used for alkoxysilane series and presented some guidelines for the prediction of detector sensitivity with respect to compounds unavailable in a pure form.

Calibration of katharometer becomes superfluous when the apparatus is fitted with a combustion chamber for elemental analysis filled consecutively with copper oxide and silver and which is placed between the column and the detector[92]. The sample components passing through this device are converted into hydrogen, which is registered by the detector. The appropriate calibration coefficients can be calculated theoretically on the basis of the hydrogen content of the component being analysed. This detector has been used in the analysis of polyorganosiloxanes[64], phenylchlorosilanes[93], phenylalkoxysilanes[94]. Its application appears to be extremely advantageous, especially when standard compounds are not available, but its response is still slower than that of the katharometer and the degree of combustion of OSC must be determined in each instance.

The katharometer was shown to exhibit selective sensitivity towards organoelement compounds[95]. For instance, the sensitivity to OSC was approximately 3-fold higher (6-fold to germanium and 9-fold to lead compounds) than to the carbon-containing analogues.

The use of flame-ionization detectors (FID) for OSC analysis is associated with various anomalies. Luskina *et al.*[96] demonstrated that the sensitivity of the FID was dependent on the C:Si ratio in the sample. Pollard *et al.*[25] demonstrated that the FID is easily overloaded with OSC, which results in flattened peaks after reaching a certain threshold value of the sample volume. To avoid this, they recommended reducing the size of the sample or installing an effluent splitter between the detector and the column. Peak inversion was registered for OSC by Fritz *et al.*[97] using an FID. The intensity of the detector signal plotted against mass of silicon produced a curve with a maximum. The effect of gas flow through the detector on the above phenomenon was studied. Infusion of acetylene into the hydrogen flame caused complete inversion of OSC peaks, the magnitude of the observed effect being correlated with the C:Si ratio, which makes it applicable to the qualitative analysis of siloxanes. Background current can be attained in FID by adding methane[98,99]. It has been suggested

that in this mode of operation negatively charged ions are generated by OSC, whereas positively charged ions in considerably smaller amounts are produced by organic compounds. Under normal operating conditions, the sensitivity of such a detector ranges from 80 to 200 nC per gram of silicon, and the lowest detectable amount is $1 \cdot 10^{-9}$ g/sec. The parameters of the detector were studied using an *X–Y* recorder by comparing the signal of a standard detector with that of the detector being studied[100]. Selective features of the detector permitted its subsequent application for the quantitative analysis of the decomposition products of organosilicon polymers[101].

The applicability of an alkali flame-ionization detector to the analysis of organo-element compounds was investigated by Dressler *et al.*[102]. They found it to be more sensitive to OSC than the ordinary flame-ionization detector. Also, it generated a positive signal in response to OSC, which did not occur with tin- and lead-containing compounds. The thermionic detector can be used for the analysis of HCl, $COCl_2$ and methyl- and ethylchlorosilane impurities in silicon tetrachloride[103].

Hill and Aue[104] proposed a flame-ionization detector operating in a hydrogen atmosphere as a selective detector for OSC. Its sensitivity to alkyl- and arylsilanes is 2–3-fold higher than that to hydrocarbons. The addition of small amounts of ferrocene to the flame causes inversion of OSC peaks and increases the sensitivity to these compounds by one order of magnitude. Although the mechanism of such selectivity is unknown, this detector may prove very useful for the analysis of OSC in the presence of a multi-component mixture of organic compounds. However, the detector possesses a comparatively low absolute sensitivity, of the order of tens of nanograms for tetramethylsilane.

To determine methylchlorosilanes, Garzo and co-workers[105–107] used a cell in which the electrical conductivity of a hydrolysate was measured continuously.

An argon ionization detector has also been proposed for OSC analysis[108], but it appears to be unsuitable because of its extremely low sensitivity to OSC, which would decrease with the increasing chlorine content in the sample.

Fritz and Ksinsik[109] designed a device consisting of two flow-sensitive katharometer cells. The device resembles closely the gas density balance proposed by Martin and James [110] and can be applied to quantitative analysis and molecular weight determinations with 5% accuracy. A similar detector was reported by Wurst[111,112]. There have also been reports on ^{14}C radioactivity detectors used for OSC analysis[113]. Selective OSC determinations can also be carried out using various spectral methods. The chromatograph can be connected with a flame emission or atomic-absorption spectrometer[114–116]. A UV detector[117] permits the detection of impurities in OSC at the appropriate wavelength for each impurity.

Of the variety of detection systems used for the analysis of OSC and organic compounds, the katharometer and FID are the only detectors with a wide range of application, and the other systems may be advantageous only in more specific analytical determinations.

4. STUDY OF ORGANOSILICON COMPOUNDS BY GAS CHROMATOGRAPHY IN COMBINATION WITH OTHER METHODS

The general aspects of GC in conjunction with chemical reactions and spectral analysis have been the subject of numerous publications, which were summarized by

Berezkin[118] and Ettre and McFadden[119]. In OSC chemistry, reaction gas chromatography is mainly applied to functional analysis[120].

The use of reaction GC to identify unknown compounds as chlorosilanes was reported by Palamarchuk *et al.*[21]. Chlorosilane absorption was performed in the upper section of the chromatographic column with solid potassium hydroxide on an INZ-600 support. The chlorosilanes were completely absorbed by the sorbent, and the absorbent layer involved in the reaction had no effect on the retention values.

Reaction GC is applicable to the analysis of substances that cannot be separated by conventional GC. Difficulty is experienced, for example, in applying the direct method to the determination of trace amounts of phenyltrichlorosilane in methylphenyldichlorosilane. A pre-column containing sodium fluorosilicate installed prior to the chromatographic column permits the conversion of chlorine-containing silanes to the appropriate fluoro analogues, which can be separated more readily [70,121]. Reaction GC can also be useful for the determination of relative amounts of $(CH_3)_3Si$-, $(CH_3)_2Si=$ and $CH_3Si\equiv$ groups in methylsiloxane polymers. The procedure consists in decomposing the polymers with an ethereal solution of boron trifluoride[122] or sodium bifluoride[123] in a closed system and with subsequent GLC analysis of the resulting methylfluorosilanes either on a capillary column coated with SE-30[122] or on a column packed with fluorosilicone FS-16[123].

A reaction GC device designed for group determination in OSC through Si–C bond hydrolysis in C-halogenated compounds has been reported [124]. The stability of Si–C bonds in β-trimethylsilylpropionic and β-dimethylbutylsilylpropionic acid towards sulphuric acid was determined by measuring methane and butane generated in the reaction[125]. A simple micro-scale method for Si–H bond determination was described by Franc and Mikes[126], which involved decomposition of OSC on a moist mass impregnated with potassium hydroxide, the hydrogen evolved being accumulated in a syringe and its amount measured using an activated carbon column after completion of the reaction.

Several studies have dealt with the problem of vinyl group determination in siloxane polymers. Conversion of vinyl groups into ethylene can be achieved through reaction with 90% sulphuric acid[127] or phosphorus pentoxide[128,129], by polymer fusion with potassium hydroxide[130] and by pyrolysis[131]. The last procedure also permits the determination of alkoxy groups by measuring the yield of alcohols, both vinyl and alkoxy groups being detectable at the 0.1–0.01% level. Combined methods for the qualitative analysis of OSC by means of TLC with subsequent application of reaction GC have also been developed[132,133]. In this instance the material from the thin-layer chromatograms is transferred into a micro-reactor to be further treated with boron trifluoride, followed by chromatographic analysis of the reaction products.

Franc and co-workers[134–136] presented a systematic method for OSC analysis based on reaction GC. Detection of alkoxy and phenyl groups is carried out by reaction with boron tribromide; ethylene oxide and propylene oxide rings can be detected by measuring the acetaldehyde or propionaldehyde produced in the reaction of OSC with potassium hydrogen sulphite. Treatment with potassium persulphate allows the identification of C=C double bonds, whereas reaction with solid potassium hydroxide at 200° is employed for the determination of the terminal $-Si(CH_3)_3$ and $\equiv SiC_6H_5$ groups. Sulphur can be detected in OSC by measuring the output of sulphur dioxide following treatment with boron trifluoride. The presence of carbon

chains bonded to a silicon atom is revealed through catalytic conversion on aluminosilicate modified with tungsten and molybdenum oxides. Acetyl groups are determined by hydrolysis with subsequent analysis of acetic acid. Chromatographic parameters for the determination of volatile products have also been elaborated.

A systematic approach to the analysis of functional groups and some bond types in OSC was described by Franc and Dvoracek[137]. The compounds to be analysed were decomposed in a micro-reactor using sulphuric acid, hydriodic acid, potassium hydroxide or other reactants and the volatile reaction products were passed through a chromatographic column. The procedure is applicable to alkyl, chloroalkyl, vinyl, alkoxy and phenyl groups and also to Si–Si and H–Si fragment determinations.

Thus, reaction gas chromatography offers fairly wide possibilities for the study of OSC. However, most reactions employed in OSC determinations are time consuming and require a very long time for their completion compared with peak elution from chromatographic columns. This advantage limits considerably the applicability of reaction GC to the analysis of multi-component mixtures.

GLC is inapplicable to the direct analysis of siloxane polymers. Nevertheless, some information about the properties of these substances can be obtained by determining the amounts and composition of some comparatively volatile impurities that can be detected by conventional GLC[138].

Gas chromatography–mass spectrometry has been applied to the identification and structural analysis of silsesquioxanes [139] and the analysis of the pyrolysis products of polydimethylsiloxanes[61,140]. Reaction GC of OSC using preliminary fusion has been also studied[141].

The method for the determination of trimethylsilyl groups in amino and carboxylic acid derivatives is based on the gas chromatographic analysis of trimethylphenoxysilane formed on exposure of the sample to phenol[142].

A method of rapid stationary phase selection for siloxane resolution has been described[143] in which relative retention parameters are estimated as follows:

$$\log V_{\mathrm{rel}} = E\Delta\nu_i^{\mathrm{ASS}} + F \tag{21}$$

$$\Delta\nu_i^{\mathrm{ASS}} = \nu_{i(\mathrm{squalane})} - \nu_{i(\mathrm{polar\ phase})} \tag{22}$$

where ν_i denotes the shift in Si–H stretching frequency, and E and F are constants.

GLC can also be used to measure the vapour pressure of OSC[144] and to choose potential separating agents for extractive distillation of methylchlorosilanes[145].

5. GAS CHROMATOGRAPHY OF ORGANOSILICON COMPOUNDS AND MIXTURES THEREOF

The conditions required for OSC analysis are summarized in Table 1. Stationary phases of general usage, such as Apiezons and siloxane polymers characterized by low or medium polarity, are most frequently employed for the analyses. Only with alkylchlorosilanes more polar phases such as phthalates, nitrobenzene and tricresyl phosphate are used. The amount of stationary phase loaded is dependent primarily on the volatility of the sample components and ranges from 5 to 33%. Capil-

TABLE 1

CONDITIONS FOR THE GAS CHROMATOGRAPHIC ANALYSIS OF ORGANOSILICON COMPOUNDS AND THEIR MIXTURES

Compounds	*Composition of packing*	*Column dimensions [mm × mm or (ft. × in.)]*	*Column temperature (°C)*	*Reference*
Halogenosilanes				
Volatile silanes	DC-702 on Celite			69
Chlorosilanes, methylchlorosilanes	SF-96, QF-1			246
Methylchlorosilanes, silicon tetrachloride	Nitrobenzene, dibutyl phthalate, α-chloronaphthalene on Termalite, Sterchamol, Celite	1600	27	107
Alkylchlorosilanes (impurities in $SiCl_4$)	16% E-301 on Chromaton N AW HMDS			103
Methylchlorosilanes	10% dioctyl phthalate on Celite, 80–100 mesh		20–25	108
	Nitrobenzene (30%)	1300 × 4	25	247
	15% FS-16 on INZ-600	3000 × 4	40	146
	10% diethyl phthalate on Celite, 80–100 mesh			98
Methylchlorosilanes	Two columns in series: paraffin oil and insulator oil in firebrick	4000 × 5	50–70	147
	Tricresyl phosphate and dioctyl phthalate			148
	Diethyl phthalate and silicone DC-200/350 on Kieselguhr (60:20:100)	2400	60	149
	α-Chloronaphthalene, dibutyl phthalate, nitrobenzene on Celite, Sterchamol, Termolite			106
	20% nitrile silicone OE-4178		35	90
	Paraffin oil, insulator oil on C-22, 30–60 mesh			150
	15% FS-16 on INZ-600	3000 × 6	70	151
	Polydimethylsiloxane on silanized silica gel		230	152
	Benzophenone		52	153
Methylchlorosilanes	In series: 15% PFMS-4 on Celite and	1000 × 3.5	40	154
(reaction mixture from synthesis)	15% vaseline oil on Celite	3000 × 4		
	20% PFMS-3	4000 × 4	40	154
Methyltrichlorosilane (purification from traces of P and metals)	20% DC-701 on INZ-600	3600 × 20	50	155
Methylchlorosilanes (impurities: $POCl_3$, PCl_3)	Vaseline oil, siloxanes VKZL-94, DC-701, fluorosiloxane 169, dinonyl phthalate (20%)	1000 × 6	50	156
Methylchlorosilanes, phenylchlorosilanes, lower methylpolysiloxanes	Silicon gum, liquid paraffin			157
Methylchlorosilanes (analysis of impurities)	Dibutyl phthalate and tricresylphosphate on INZ-600	3000 × 4	40	158

Dimethyldichlorosilane (analysis of impurities)	20% VKZ-94 on INZ-600		70, 30	159
Methylchlorosilanes (high-boiling by-products)	Polymethylphenylsiloxane oil		130	160
Ethylchlorosilanes	15% FS-16 on INZ-600	3000	80	21
Ethylchlorosilanes (reaction mixture from synthesis)	10% MSO-150		90	161
Ethylchlorosilanes	PFMS-4 on Celite 545,	1000 × 4	90	32
(product of chlorination)	E-301 on TND-TSM	2000 × 4	95	32
Alkylchlorosilanes, low-boiling	DC-200/350 and dibutyl phthalate on Kieselguhr (3:1:5)	1300	30–60	162
Alkylchlorosilanes, high-boiling	Silicone on Kieselguhr (3:10)	4100	150	162
Alkylchlorosilanes	Nitrotoluene, XF-1112, QF-1, SE-30 (15%) on silanized Chromosorb, 60–80 mesh	2000 × 4	40–60	38
2- and 3-pentylchlorosilanes	Apiezon L on Chromosorb	2500	200	163
Chloroalkyltrichlorosilanes	15% PFMS-4 or 3% FS on silanized diatomite brick	2000 × 4		164
Tris-γ-trifluoropropylchlorosilane (analysis of impurities)	15% fluorosiloxane 169 on INZ-600		160	165
Methoxy- and ethoxychlorosilanes	SE-30, FS-16, Carbowax 6000, Apiezon L,	1000 × 3		166
	DC-550 on Rysorb or Chezasorb	2000 × 4		
Diethoxylmethylchlorosilane	Methylsiloxane oil (Perkin-Elmer C)		125	167
Alkoxychlorosilanes	Apiezon, PMS-100, FS-16, DC-550 on Chezasorb,	1000 × 3		168
$[Cl_nSi(OC_mH_{2m+1})_{4-n}; n = 1 \text{ or } 4, m = 1\text{–}4]$	Rysorb 0.2–0.36 mm			
Alkoxychlorosilanes	Apiezon L, SE-30, FS-16 on silanized Chezasorb	1000 × 3		62
Phenylchlorosilanes	20% E-301 on Chromosorb		240	93
(reaction mixture from synthesis)	Polymethylphenylsiloxane on Rysorb BLK	1350 × 5	210	117
Phenylchlorosilanes	15% FS-303 on INZ-600	3000 × 4	130	146
Tolylphenylchlorosilanes	5% E-301 on Porovina		170, 197	169
Methylphenyldichlorosilane	10% PFMS-4 on INZ	4700 × 6		170
(impurities of dimethylphenylchlorosilane and phenyltrichlorosilane)	20% SKTFT-50 on Celite 545	2700	100	171
Vinylchlorosilane	Dibutyl phthalate and tricresylphosphate on INZ	3000 × 4	40	158
Vinylchlorosilanes,		1800 × 4	25,40	172
methylvinylchlorosilanes	Nitrobenzene on Kieselguhr (3:10)	1600 × 5		
Methylvinylchlorosilane, vinyltrichlorosilane	Dibutyl phthalate on Celite		27	105
Methylvinylchlorosilanes	Nitrobenzene on Kieselguhr, silicone elastomer on Celite, Dibutyl phthalate on Rysorb BLK		40, 25	173
Alkenyldichlorosilanes	SE-30	(9 × 3/8)		174
cis–trans isomers	Apiezon L + polyphenyl ether	Capillary		174

(Continued on p. 272)

TABLE 1 *(continued)*

Compounds	*Composition of packing*	*Column dimensions [mm × mm or (ft. × in.)]*	*Column temperature (°C)*	*Reference*
Methylfluorosilanes	SE-30 on capillary columns or squalane on Teflon 6			122
Phenylfluorosilanes, cycloalkylfluorosilanes	30% UCW-98 on Chromosorb W			121
Perfluorophenylsilane	SE-30 on Chromosorb W	(1.5 × 1/4)		175
Phenylhalogenosilanes	Perkin-Elmer O	3000	150, 205	176
	DC-200 or mixture of diethyl phthalate and silicone oil		100, 125	177
Ethoxyfluorosilanes	Apiezon, DC-550, FS-16, XE-60 (5%) on silanized Rysorb and Chezasorb (0.3–0.36 mm)		70–100	24
Alkoxysilanes				
Methylethoxysilanes (deuterated) (preparative GC)	20% SKFT-50 on C-22	4800 × 17		178
Methylethoxysilanes	Apiezon L, linear siloxane polymer			108
Methyltriethoxysilane, chlorobenzene	2,4,7-Trinitrofluorenone on Chromosorb			181
Trimethylethoxysilane	Diisodecyl phthalate		75	179
Butoxyethoxysilanes	E-301 on Celite 545		170	180
Vinylethoxysilanes	E-301 or silicone 7100 on Chromosorb (20:100)	1450 × 5	150–240	56
Methylphenylethoxysilanes	Silicone elastomer on Chromosorb or Kieselguhr (20:100)	1450 × 5	175, 240	181
Diphenyl(methyl)ethoxysilane, phenyl(diethoxy)methoxysilane	Silicone elastomer 7100		180	94
tert.-Butylphenyldimethoxysilane	Didecyl phthalate (Perkin-Elmer A)	2000		182
Phenylethoxysilanes (reaction mixture from synthesis)	10% SE-30 on Chromosorb		160–310	185
Trimethylalkoxysilanes	15% SE-30 on Chromosorb W, 30–60 mesh	1000 × 6	82	55
Ethylpropylsilicates	20% Triton X-100 on Chromosorb P, 60–80 mesh	(4 × 1/8)	80–160	183
Tetraalkoxysilanes, *tert.*-butyltrialkoxysilanes	Didecyl phthalate (Perkin-Elmer A)	2000		182
Tetraethoxysilane (products of thermal degradation)	4.14% polypropylene glycol on Chromosorb P, 45–60 mesh	1000 × 6	40	186
Tetraalkoxysilanes and phenyltriethoxysilane (products of reaction with *tert.*-butyl chloride)	Didecyl phthalate	2000	115–165	182
Alkoxysilanes	1% Apiezon L on Chromosorb W	(1 × 0.25)	60–150	40
Alkoxysilanes	Apiezon L, QF-1, SE-30 (10%) on Chromosorb, 60–80 mesh	2000 × 4 2000 × 2	60–240	38

Alkoxysilanes, siloxanes	5% silicone elastomer on Chromosorb P	2000 × 4	134	184
Methylphenoxysilanes	Polydimethylsiloxane on silanized silica gel		230	152
Trimethylacetoxysilane	Diisodecyl phthalate		75	179
Siloxanes				
Hexamethyldisiloxane	Silicone		100	188
Hexamethyldisiloxane, technical grade	Dibutyl phthalate on Chromosorb A (2:10)		35	41
	Lucoprene 1000 on Chromosorb A (1:10)		120	41
Dimethyloligosiloxanes (linear and branched)	10% PMS-100 on silanized INZ-600		130–170	189
Siloxanes (preparative GC)	20% SKTFT-50 on C-22	4800 × 17		190
Siloxanes, high-boiling (preparative GC)	20% SKTFT-50 on C-22	2000 × 14	200–220	191
Organosiloxanes, polysiloxanes	UCW-98, Apiezon L, QF-1 on Chromosorb W (30%)			192
Alkylhydrosiloxanes and amino alcohols (preparative GC of reaction products)	20% SKTFT-50 on C-22	2100 × 14	200–220	193
Methyl- and ethylcyclosiloxanes	Apiezon L, SE-30, PMS-100 on silanized Chezasorb (10%)	2000 × 3	100–300	81
Octamethylcyclotrisiloxane (analysis of impurities)	23% of linear polydimethylsiloxane of mol.wt. 80,000 on Chromosorb W, 0.2–0.3 mm	3650 × 5.3		194
Methylhydrocyclosiloxanes	20% DC-703 on Sterchamol	1600 × 8	125	195
Methyl(propyl)dimethylcyclosiloxanes	20% SKTFT-50 on Celite 545	2700		73
Methyl(propyl)dimethylcyclosiloxanes, methyl(trifluoropropyl)cyclosiloxanes	20% of siloxane on Celite			72
Trifluoroprolyl(methyl)dimethyl-cyclosiloxanes	CKTFT-50 on Celite 545	1740 × 6	130–203	196
2,6-*cis*-Diphenylhexamethylcyclotetra-siloxane	2% cyclohexanedimethanol succinate on silanized Gas-Chrom P AW		150	197
Methyl(phenyl)dimethylcyclosiloxanes	Dimethylsiloxane–carborane copolymer	3000 × 4	235	198
Methylphenylcyclosiloxanes (isomers)	Apiezon L or silicone gum on Chromosorb	(2)		199
Alkyl-(β-cyanoethyl)cyclosiloxanes	Nitrilesiloxanes NPS-50DF and NPS-100DF			200
	10% PMS-100 or 12% 139-15 liquid on Chromaton N	1000 × 4 2000 × 4	180–390	201
Oligodimethyl(methyldichlorophenyl) cyclosiloxanes	10% PMS-100 on silanized INZ-600	1000 × 4 2000 × 4	175–300	70
Non-geminal divinylhexamethylcyclo-tetrasiloxanes	33% triethylene glycol, saturated with silver nitrate, on Chromosorb P, 100–120 mesh	1500 × 4	65	202
Siloxanes in atmosphere of Skylab	Emulphor ON-870 on capillary	(600 × 0.02)	Program	203
Trimethyltriphenylcyclotrisiloxane (analysis of impurities)	11.3% PMS-100 on Chromaton N AW HMDS (0.25–0.315 mm)	3000 × 4	277	204

(*Continued on p. 274*)

TABLE 1 *(continued)*

Compounds	*Composition of packing*	*Column dimensions [mm × mm or (ft. × in.)]*	*Column temperature (°C)*	*Reference*
Siloxanes (b.p. range 400°)	PMS-100 on silanized INZ-600	250 × 2 1000 × 2	100–340	205
Polymethylsiloxanes (linear and cyclic, $n = 15$)	10% LP-122 on Chromosorb P, 60–80 mesh	610 × 6.4	50–350	206
Polydimethylsiloxanes [linear ($n = 2$–7) and cyclic ($n = 3$–8)]	Lucoprene on Rysorb BLK (20:100), 0.2–0.4 mm	1700 × 4	150, 195	45, 173
Polydimethylsiloxanes (linear and cyclic)	Lucooil M on Celite 545 (1:10)		190	46
	Lucoprene on Rysorb BLK	1900 × 4	150–185	43
Oligosiloxanes (preparative GC)	Percentage loading variable	Variable	Variable	207
Polymethylsiloxanes [linear and cyclic ($n = 40$)]	9% diphenyldimethylsiloxane polymer on Chromosorb W	(2)	100–300	208
Methylphenylpolysiloxanes ($n = 1$–4)	5% polydimethylsiloxane on INZ-600	1000	246	209
Polyethoxysiloxanes	SKTFT-50		175, 235	210
Trimethylsiloxytrimethylgermane	Didecyl phthalate		100–150	187
Bis-triethylsilyl sulphide	Apiezon L on Chromosorb W		254	213
N Containing organosilicon compounds				
N-Alkylcyclotrisolazanes	12% SE-30 on Celite	(4 × 1/4)	180	28
Silazanes and disilazanes	Silicone	(5)		214
trans-(N-Alkyl)hexamethylcyclotrisilazanes (products of redistribution reaction)	12% SE-30 on Chromosorb W	(6 × 1/4)	180	29
Silatranes	Apiezon L, OV-17, OV-225 on Gas-Chrom Q AW BW DMCS (5%)	2400 × 3	200	211
Cyclic siloxazanes	Silicone gum rubber	(2)	100–250	212
Isocyanatosilanes	5% of silicone gum on Haloport F			215
Bis(trimethylsilyl)acetamide	OV-101, SE-30			216
Organylsilanes				
Methylsilanes	25% of nitrodiphenyl on Embacel	(4)	40	217
Dimethylsilyl and trimethylsilyl-substituted methane, ethane and ethylene	Polypropylene glycol (Perkin-Elmer R)	8500	150	218
Silane, methylsilanes	Tetraisobutylene		30	219
Tetramethylsilane	Apiezon	(8)		95

Ethylsilanes and other compounds of Group IVB elements	20% Apiezon L or 15% Carbowax 20M on Chromosorb W	1000 × 3	120	48
Isopropyl- and isobutylsilanes (sterically hindered)	15% E-301 on Silocel, 36–60 mesh	2000 × 5		30
Hexyltrimethylsilanes	Liquid paraffin NF on capillary	(183 × 0.02)	40	220
Alkylsilanes	Apiezon L on Chromosorb W	(5 × 0.5)		221
	13% Squalane on Embacel, 60–80 mesh			79
	Didecyl phthalate	2000	30	219
Alkylsilanes, -stannanes, -germanes	E-301		80–140	25
Trimethylheptafluoroisopropylsilane	Silicone elastomer		134	222
Di(trimethylsilyl)alkanes and -alkenes	Polypropylene glycol (Perkin-Elmer R)	6000	150	223
α,ω-Di(trimethylsilyl)alkanes	Polymethylphenylsiloxane oil			74
Dichlorosilyltrichlorosilylmethane, bistrichlorosilylmethane	Silicone C 200/350 on Kieselguhr 1200 (20:100)	1200	150	149
Triethylvinylsilane	Silicone gum (5%)			224
Vinyl- and allylsilanes	PFMS on INZ (20:100); Polyethylene glycol 1500 on INZ (20:100)	2000 × 6	125–175	75
Stereoisomers of Si and Ge derivatives of ethylene	GSC on graphitized carbon black, Apiezon L on Chromosorb W	1000	100–200	225
cis- and *trans*-1-trimethylsilylhexene-1	Apiezon L on Chromosorb	2500	200	163
Products of hydrosilylation of:				
hexene-1 and styrene	18% FS-1265 or 14.6% E-302 on Chromaton N	4000 × 4 2000 × 4	105–170	226
phenylalkenes	23% DC-200 on Chromosorb	(4 or 10)		227
hexene-1	23%QF-1 on Chromosorb P	(15 × 1/4)		228
	25% SF-96 on Chromosorb P	(5 × 1/4)		
alkynes	10% PEG 10000 on TND		180	229, 230
monosubstituted acetylenes	10% PEG 10000 on TND, 15% 1,2,3-tris-(β-cyanoethoxy) propane on Chromosorb W		130–180	231
Products of pyrolysis of phenyl-dimethylsilane	20% Apiezon L, 20% E-301, Apiezon L + Bentone 34 on Celite 545	1050, 2000	250	232
Chlorophenyltrimethylsilanes	7.5% Apiezon L + 7.5% Bentone 34	1800	120	233, 234
Chlorobenzylsilanes	10% Apiezon L, E-302	1100	150	
Triethylsilylethanol, triarylsilylethanols	Silicone gum (5%)	(3)		224
Products of silylation of polyalcohols	13% polysebacinate on Teflon		110, 155	235

(*Continued on p. 276*)

TABLE 1 *(continued)*

Compounds	*Composition of packing*	*Column dimensions [mm × mm or (ft. × in.)]*	*Column temperature*	*Reference*
Products of synthesis of 1,4′-bis(dimethylsilyl)benzene	5% poly-(4-dimethylsilyl-4-dimethylsiloxyphenyleneoxide) on INZ-600	6000 × 4	140	236
Sterically hindered Si-containing phenols and products of their oxydation	Apiezon L, E-301, Carbowax 20M, OV-17 on Chromosorb W		200	237
Bis(dialkylsilyl)arenes, arylsilanes	5% Arylenesiloxane elastomer	6000	141–146	243
Organosilicon derivatives of piperidine, pyrrolidine and perhydroazepine	Apiezon M, Carbowax 20M (20%) on Chromosorb W AW DMCS	3000 × 2	130	52, 53
2-Furylsilanes, germanes, stannanes, plumbanes	Apiezon L, Versamid 900, Carbowax 20M (10%) on Chromosorb W AW DMCS	2400 × 2.2	180	54
Si-substituted thiophenes	GSC on Spherochrom-1, 10% Apiezon L on Chromosorb W, 10% Reoplex 400 on Chromosorb W	1000 × 4		49
1,1-Dimethylsilacycloalkanes	Polymethylphenylsiloxane oil, polyethylene glycol 1540			74
	20% PFM on INZ	2000 × 6	120	238
products of dehydrogenation	Silicone TK-055	1800	130	239
products of chlorination	Silicone	5000	135	240
		(5)		241
Silacycloalkanes	Polypropylene glycol on Celite 545, SE-52 on Celite 545	9600 × 8	160–250	242
Hexaethyldisilane, triethyl(triethylsilyl) germanium	GSC on graphitized carbon black, Apiezon L and Carbowax 20M on Chromosorb W		150–250	50
	20% UCW-95 on Chromosorb W	2440 × 16	50–200	244

lary columns are seldom used. There are no special requirements for the carrier gases, except for chlorosilane analysis, when they have to be thoroughly dried. Undoubtedly, many of the stationary phases proposed by different investigators have very similar properties. Valuable information on their interchangeability is given in refs. 1 and 245.

6. SUMMARY

The present review deals with some problems that may be of interest to chromatographers studying organosilicon compounds (OSC). It is shown that correlations between structure and retention values of OSC help to predict retention parameters of unexplored compounds and help to gain insight into the physicochemical processes in the gas chromatography of OSC. The detectors used for OSC analysis are discussed. A combination of gas chromatography with other methods is shown to be useful in functional group analysis as well as in the identification of separated compounds. The conditions of 160 analytical methods are tabulated.

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Journal of Chromatography, 165 (1979) 283–299
Chromatographic Reviews
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHREV. 121

HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF STEROIDS

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(Received April 23rd, 1979)

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1. INTRODUCTION

As recently as 1975, when the chromatography of steroids was last reviewed[1], there were only a few attempts to introduce high-pressure liquid chromatography (HPLC) into the steroid field. The prediction "that these accomplishments will be considerably improved upon by the time this book has been published" (ref. 1, p. 100) has come true, and the time is now ripe to evaluate the progress which has been made and to point out directions which future research might profitably take. As far as we know, the HPLC of steroids has not been surveyed previously, except for a review of HPLC limited to the steroid hormones[2].

The steroids are quite narrowly defined chemically as substances containing the cyclopentanoperhydrophenanthrene nucleus, but their physical characteristics and chromatographic properties run the gamut from the lipophilic sterols to the hydrophilic cardiac glycosides. Fortunately, most analyses are confined to only one of the many classes of steroids, but even so, there may be a large difference in polarity between various members, *e.g.*, in the pregnane series between progesterone and aldosterone.

Chromatographic systems must be designed not only to perform well in the polarity range of the class of steroids one wishes to fractionate, but also to provide the capacity and sensitivity called for by biological extracts. One of the difficulties inherent in the analysis of steroids in biological extracts is that they are usually present not only in a large excess of other lipids, but also in mixtures of closely

related analogs. Some of these are present in very low concentration, *e.g.*, using the same example, aldosterone occurs in urine in a 1000-fold excess of other pregnane derivatives. A chromatographic system capable of detecting 1 μg of aldosterone would therefore have to have enough capacity to handle 1 mg of analogous metabolites or several milligrams (depending on the extent of prefractionation) of crude extract.

One of the major attractions of liquid column chromatography (LC) is the relatively large capacity. This means that it can handle biological extracts with a minimum of preliminary purification. For instance, the application of gas–liquid chromatography (GLC) to the analysis of pharmaceutical preparations of vitamin D requires preliminary purification by thin-layer chromatography (TLC), whereas HPLC accomplishes this analysis directly and more accurately in less time[3].

Large capacity also allows HPLC to be used in the preparation of adequate amounts of material for further testing. Preparative HPLC columns can typically handle sample weights up to one-thousandth of the sorbent weight[4–6]. Such columns are conveniently prepared by axial compression[7]. The efficiency of preparative columns depends on the sample volume, as demonstrated with plant extracts containing cardiac glycosides[8]. Chromatographic systems suitable for preparative HPLC may be derived from preliminary tests by TLC[9–11]. Sometimes, a preparative column may be coupled with an analytical column, as, *e.g.*, in the analysis of corticosteroids in serum, where a pre-column packed with porous styrene–divinylbenzene copolymer particles was used for concentrating and purifying the steroids[12].

Another attractive feature of HPLC is that compounds, such as aldosterone, which are not very stable can be handled without derivatization or exposure to heat, light, and air. The effluent from the chromatographic column may be continuously monitored and assayed, or fractions may be collected and either subjected to any suitable physical, chemical, or biological test or battery of tests, or they may be accumulated for future uses.

LC has a greater potential for accomplishing difficult separations than GLC, because it can take advantage of a larger array of sorption mechanisms[13], *viz.* adsorption, partition, reversed-phase partition, chemisorption, gel permeation, ion exchange, and ion-pair formation. The sorbents enlisted for HPLC of steroids include the adsorbents silica and alumina and the permanently bonded octadecylsilane, which usually supports the less polar phase of so-called reversed-phase partition systems.

Silica is supplied either in the form of porous particles of a narrow size range down to 5 μm or in pellicular form, coating inert support beads. The activity of alumina[14] or silica[15] changes with the water content of the eluent, and the character of the chromatographic system gradually changes from adsorption to partition as the water content is increased[16]. Silica columns may be prepared for partition chromatography by loading them either before[17] or after [18] packing with a stationary phase, *e.g.* formamide, which has been diluted with a less viscous solvent. An interesting way to apply argentation chromatography to steroids is the incorporation of silver nitrate in the eluent[19].

A large array of proprietary packing materials for reversed-phase partition chromatography is available. They may be minute beads of polymers[20] or, frequently, pellicular coats of hydrophobic material, which is permanently bonded to beads of silica or other inert materials. Products of different manufacturers may

exhibit considerable differences in selectivity towards steroids[21]. Hydrophobic supports coated with a non-polar stationary phase usually give combined lipophilic adsorption and reversed-phase partition effects[22].

The rational design of two-phase solvent partition systems for HPLC from ternary mixtures was demonstrated by Hesse and Hövermann[23]. Jandera *et al.*[24] have developed a theoretical approach to the selection of the optimal composition of the mobile phase and have applied it to sterols. Following a discussion of solvent strength and solvent selectivity, as applied to the liquid–solid chromatography of steroids[25], Hara and Hayashi[26] have made an extensive study of the retention behavior of 43 steroids in adsorption (Corasil II*) and reversed-phase partition (Bondapak C_{18}) HPLC. From these data they have derived a series of retention parameters for the functional groups of steroids in binary solvent systems. Hara and co-workers[27,28] later based a systematic design of such solvent systems on the retention behavior of mono- and difunctional steroids on silica gel columns. O'Hare *et al.*[29] have devised gradient-elution schemes for the separation of a large array of steroids by reversed-phase partition chromatography. In addition to composition and flow-rate, the temperature is a variable which can be exploited to improve the resolution[30].

HPLC differs from other methods of LC mainly in the use of ultrafine particles of sorbents. This accounts for the high efficiency of this separation method, but this also necessitates the application of pressure to force solvents through packed columns. Finely powdered sorbents will yield highly efficient separations only if they are properly packed, and because packing them properly is so difficult, most researchers either buy packed columns from supply houses or decide to pack their columns with somewhat coarser particles. The limited selection and expense of ready-made columns is one of the obstacles to the development and acceptance of new methods of separating steroids by HPLC.

Other obstacles are the capital investment in equipment, which may be considerable when complete liquid chromatographs with luxury features are purchased, and the inadequacy of commercially available detectors. Practically the only detectors suitable for steroids are the ultraviolet (UV) and the refractive index (RI) detectors, which may be used either separately or in tandem[31]. Until the variable-wavelength detector[32] made the far UV accessible, it was common practice to make those steroids which do not exhibit appreciable absorption at 254 or 280 nm, where the fixed-wavelengths detectors operate, UV-absorbing by derivatization. Thus, *e.g.*, hydroxy-steroids have been esterified to form benzoates or *p*-nitrobenzoates[33], and keto-steroids have been converted to 2,4-dinitrophenylhydrazones[34,35].

By post-column reaction with isonicotinylhydrazine in methanolic aluminum salt solution Δ^4-3-ketones may be converted to hydrazones, which fluoresce owing to complex formation with aluminum ions[36]. This permits the detection of picomole quantities of steroids with greater specificity than by UV spectrometry. Similarly, cardiac glycosides react with concentrated hydrochloric acid to yield fluorescent products which may be exploited for post-column derivatization and an automated, highly sensitive fluorometric assay[37].

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* For chemical composition and manufacturers of commercial products the reader is referred to p. X, ref. 1. Reference to a company and/or product named by the Department is only for the purpose of information and does not imply approval or recommendation of the product named to the exclusion of others which may also be suitable.

Moving-wire flame-ionization detectors (FID), although quite expensive and temperamental, are beginning to find acceptance in lipid research[38,39]. A heated thin-layer plate, moving slowly past the column outlet, has been advocated as an alternate transport detector[40]. Although off-line monitoring by mass spectrometry[41] is now common, field desorption[42] and atmospheric pressure ionization[43] mass spectrometry have rarely been coupled with HPLC in steroid analysis. More use of the infrared and light-scattering[44] detectors could be made in the future.

HPLC, like all new methods, must overcome the competition of highly developed and generally accepted methods of steroid chromatography: GLC and TLC. For many applications, GLC will remain the method of choice, because it is extremely efficient, sensitive, rapid, and convenient. However, these are advantages which are gradually becoming the attributes of HPLC, and the gap between the two competitive methods is narrowing. For instance, completely automatic HPLC systems for pharmaceutical analysis are now available[45,46]. The two main advantages of TLC are that several samples or reference standards can be chromatographed simultaneously and that the relatively unreactive steroids can be subjected to a large array of detection methods, most of which require corrosive reagents. Adding the modest cost and portability to its attractive features, we may venture to predict that in steroid research TLC will probably survive GLC and HPLC in the long run[47].

2. STEROLS

This section deals with C_{27}, C_{28}, and C_{29} steroids with one or two oxygen functions. The *seco*-steroids are discussed in section 4 on vitamins D and the more highly oxygenated sterols with insect-molting hormone activity are treated in section 3 on ecdysteroids. No reports on the behavior of bile alcohols in HPLC have come to our attention. The natural sterols have an oxygen function, usually a hydroxyl group, at C-3 and a hydrocarbon chain at C-17. They differ from each other in the way rings A and B fused, in the presence and location of double bonds, and in the presence, nature, and orientation of the alkyl group at C-24. Additional oxygen functions may occur in the nucleus as well as in the side chain.

Smith and Hogle[48] separated the benzoates of C_{27} sterols in the order of increasing number of double bonds by the use of a column of Corasil II, containing 5% silver nitrate. Gradient elution with increasing concentrations of dichloromethane in light petroleum produced the sequence cholestanol, cholesterol, 7-dehydrocholesterol, and ergosterol. At the same time, Rees *et al.*[49] chromatographed steryl acetates and benzoates on a μBondapak C_{18} column and monitored the effluent with a RI detector. When mixtures of methanol, chloroform, and water were used as eluents, the esters emerged in the order of decreasing polarity, *e.g.*, cholesteryl benzoate, followed by a mixture of stigmasteryl and campesteryl benzoates, and then sitosteryl benzoate. In another chromatogram, ergosteryl acetate was eluted before brassicasteryl acetate, which was followed by the acetates of campesterol and then sitosterol. In a third example, desmosteryl acetate was followed by 7-dehydrocholesteryl and lathosteryl acetates.

This method has been applied to marine invertebrates[50] and yeast mutants[51]. In a study of the *in vitro* biosynthesis of cholesterol, Thowsen[52] chromatographed the acetates of various C_{27} sterols on a μPorasil column and monitored the

effluent with a RI detector. Using hexane–benzene (9:1) as the eluent, she obtained the following order of elution: Δ^0, Δ^5, $\Delta^{8(14)}$, Δ^8, Δ^7, $\Delta^{5,7}$, $\Delta^{8,14}$ and $\Delta^{7,14}$, *i.e.* the acetates were in the order of increasing polarity.

Rees *et al.*[49] have also chromatographed the free sterols in their reversed-phase system, which was later modified by Hansbury and Scallen[53]. Similarly, Kikuchi and Miki[54] have employed a Zorbax ODS column with acetonitrile–water–0.1 *M* sodium acetate (90:10:1) as the eluent to separate some of the free sterols obtained from dates. In this case, the absorbance at 210 nm was used for monitoring the effluent, which yielded, in sequence, cholesterol, stigmasterol, campesterol, and sitosterol. For the specific analysis of ergosterol in fungus-infested grains, Seitz *et al.*[55] have chosen to set their detector to 282 nm, where other sterols do not interfere.

In a systematic study of the behavior of the ketonic C_{27} sterols in HPLC, we took advantage of their UV absorption at 254 and 280 nm[56]. Using two UV detectors in series, we followed the separation of eleven C_{27} ketones, separated on a 50-cm column of LiChrosorb Si 60–10, with dichloromethane–*n*-hexane–ethyl acetate (94:5:1) as the eluent. Under these conditions, the elution sequence was: 5β-cholestan-3-one, 5α-cholestan-3-one, 5-cholesten-3-one, and 4-cholesten-3-one. With dichloromethane–ethyl acetate (99:1) as the eluent, it was: 3,5-cholestadien-7-one, 5α-cholest-7-en-3-one, 4-cholestene-3,6-dione, 4,6-cholestadien-3-one, 1,4-cholestadien-3-one, 3β-hydroxy-5α-cholestan-7-one, and 3β-hydroxy-5α-cholestan-6-one. Generally, the A/B-*trans* steroids are more strongly adsorbed than A/B-*cis* steroids[57]. Thus, 5α-cholestan-3-one follows 5β-cholestan-3-one. The saturated steroids are less adsorbed than unsaturated steroids, and among the unsaturated steroids the ones containing conjugated double bonds are more strongly adsorbed than the ones with isolated double bonds. Thus, 4-cholesten-3-one follows 5-cholesten-3-one.

For the separation of various sterols in the free form, we[58] chose to use Bondapak C_{18}–Porasil B as an adsorbent rather than as the carrier of the less polar phase in a reversed-phase partition system. When 0.5% 2-propanol in *n*-hexane was used as the eluent, the sterols, detected by their UV absorption at 205 nm, emerged in the order of increasing polarity: ergocalciferol, sitosterol, stigmasterol, campesterol, cholesterol and finally, ergosterol. In another chromatogram, cholecalciferol was followed by cholesterol, then lathosterol and, finally, 7-dehydrocholesterol. The glass columns with an I.D. of 1/8 in. were suitable for preparative chromatography, allowing the isolation of 18 μg cholesterol from a 10-mg sample of commercial sitosterol. In the same year, Cortesi *et al.*[59] separated the sterols in vegetable oils by HPLC on Micropak Si-10, using hexane–diethyl ether (4:1) as a developer and the UV absorption at 210 nm for detection.

An outstanding example of the power of HPLC is the resolution of the epimeric 26-hydroxycholesterols[60]. By recycling 2.5% ether in *n*-hexane through a 60-cm Microporasil column, Redel and Capillon[61] succeeded in separating 25β(*S*)-26-hydroxycholesterol from the 25α(*R*)-epimer, which is slightly more polar.

3. ECDYSTEROIDS

Owing to the presence of many hydroxyl groups, the molting hormones are quite polar. They are easily detected by their UV absorption at 254 nm, which is due to their α,β-unsaturated carbonyl group. Although ecdysteroids are easily

separated by HPLC, surprisingly little work has been reported on the use of modern sorbents, perhaps because reference compounds are not easily obtainable.

Nigg *et al.*[62] have demonstrated the ability of Corasil II, in combination with mixtures of chloroform and 95% ethanol, to separate various analogs of insect-molting hormones. Gilgan[63] used this method to isolate, in the order of emergence: ecdysone, makisterone A, and 20-hydroxyecdysone plus inokosterone, which were unresolved. By reversed-phase partition with Bondapak Phenyl-Corasil as the column material and a linear gradient from water to ethanol as the eluent, he obtained, in sequence: 20-hydroxyecdysone, inokosterone plus makisterone A, ecdysone, and ponasterone A. More recently, Ogawa *et al.*[64] determined 20-hydroxyecdysone and inokosterone separately by HPLC on a Permaphase ETH column, which was developed with *n*-hexane–ethanol (9:1) at 50°.

4. VITAMINS D

HPLC is an ideal method of analysis for the vitamin D analogs, because they are light- and heat-sensitive and it is difficult to convert them quantitatively to derivatives. These UV-absorbing lipids are readily handled by LC, but the separation of closely related analogs is sometimes difficult. Adsorption chromatography on silica[65,66] or alumina[67] does not adequately separate vitamin D_2 (D_2) from vitamin D_3 (D_3), but it is very effective for the fractionation of their metabolites and photoisomers, which are usually detected at 254 nm.

Jones and DeLuca[68] used a Zorbax SIL column and 2.5% 2-propanol in Skellysolve B to obtain the following elution sequence: D_2 plus D_3, 24-hydroxy-D_2, 24-hydroxy-D_3, 25-hydroxy-D_2 and 25-hydroxy-D_3. For the mono- and dihydroxy-vitamins D, they used a 10% instead of a 2.5% 2-propanol solution to elute, in order: D_3, 25-hydroxy-D_3, 24,25-dihydroxy-D_3, 1α-hydroxy-D_3, 25,26-dihydroxy-D_2, 25,26-dihydroxy-D_3, 1,25-dihydroxy-D_2 and 1,25-dihydroxy-D_3.

Similarly, Ikekawa and Koizumi[69] used a Zorbax SIL column and either 2% methanol in dichloromethane or a gradient from 0.02 to 6% methanol in dichloromethane for the separation of D_3 metabolites. The polarity, which depends on the number and position of the hydroxyl groups, increased in the following order $24 < 25 < 1\alpha < 24{,}25 < 1{,}24 < 1{,}25 < 1{,}24{,}25$. For the most difficult separations, they converted the metabolites to trimethylsilyl derivatives and eluted with 2% dichloromethane in *n*-hexane: first 24β(*S*),25-dihydroxy-D_3 and then 24α(*R*),25-dihydroxy-D_3, but with 3.5% methanol in dichloromethane: first 1α,24α,25-trihydroxy-D_3 and then 1α,24β,25-trihydroxy-D_3.

For the determination of the photoisomers of D_2, Tsukida *et al.*[70] developed a Zorbax SIL column with pentane–diethyl ether–methanol (2000:40:3) and obtained, first, a mixture of 5,6-*trans*-D_2 and iso-D_2, then pre-D_2, lumisterol$_2$, isotachysterol$_2$, D_2, tachysterol$_2$, and ergosterol. With chloroform–pentane (11:9), iso-D_2 was eluted before 5,6-*trans*-D_2. Similarly Tartivita *et al.*[71] eluted a column of μPorasil with chloroform–*n*-hexane–tetrahydrofuran (70:30:1) to determine, individually and in sequence, *trans*-D_3, pre-D_3, lumisterol$_3$, isotachysterol$_3$, tachysterol$_3$, D_3, and 9,7-dehydrocholesterol. Analogous results were obtained by the Vanhaelens[72]. An earlier method employed a Vydac column for the separation of precalciferol from D[73].

The determination of D in biological specimens, where the concentration

is in the ppb (ng/g) range, by UV absorption requires some prefractionation. Columns of Sephadex LH-20[74,75] and hydroxyalkoxypropyl Sephadex[76] have been used for the analysis of blood and milk, respectively. Kosky and VanDerSlik[77] used a partition column of Celite 545 for this purpose. The mobile phase was *n*-pentane and the stationary phase was 80% aq. methanol. Low-pressure[78] and high-pressure[79] silica chromatography have also been utilized as prefractionation procedures.

The earliest HPLC method for D employed a Permaphase ODS column and an eluent of 78% aq. methanol, which produced a partial separation of D_2 and D_3[80]. Later, a linear elution gradient from 30 to 80% aq. methanol was used in this method[81]. When a μBondapak C_{18} column was developed with a solvent composed of 865 ml methanol, 135 ml water, and 2.4 g silver nitrate, D_2 was eluted ahead of D_3[19]. Complete separation of the two vitamins was also accomplished by developing a Vydac 201TP column with 90% aq. methanol[82]. Other octadecylsilane columns used for D analogs were developed with methanol–1% aq. ammonium carbonate (19:1)[83] or methanol–acetonitrile (1:1)[84]. For the preliminary isolation of D from blood, Koshy and VanDerSlik first used low-pressure chromatography on silica gel alone[85], but later they combined it with partition chromatography on diatomaceous earth[86]. The purified material was then analyzed for D by reversed-phase chromatography on Zorbax ODS with acetonitrile–methanol–water (94:3:3), but adsorption chromatography on Zorbax SIL with *n*-hexane–2-propanol (97:3) was used for the separation of 25-hydroxy-D_2 from 25-hydroxy-D_3[87].

The plasma analysis scheme of Lambert *et al.*[88] involves a preliminary chromatography on Sephadex LH-20 with *n*-hexane–chloroform–methanol (9:1:1), yielding fractions of D, monohydroxy-D, and dihydroxy-D, which are separately chromatographed on μBondapak C_{18} with 90, 87 and 80% aq. methanol, respectively, but a fraction containing 1α,25-dihydroxy-D was resolved on μPorasil with *n*-hexane–2-propanol (22:3). Jones[89] first obtained two fractions by low-pressure silica gel chromatography, using hexane with stepwise increasing 2-propanol concentrations for elution. He later accomplished this preliminary purification by HPLC on Zorbax SIL, which yielded a D fraction and a 25-hydroxy-D fraction[90]. Both fractions were chromatographed on a Zorbax ODS column, the former with 98.5% aq. methanol, the latter with 91% aq. methanol.

5. STEROIDAL SAPOGENINS AND ALKALOIDS

So far, only one publication on HPLC of steroidal sapogenins has come to our attention. Higgins[91] has applied reversed-phase chromatography on LiChrosorb RP-8 to the quantitative determination of sapogenins isolated from *Agave*. The sapogenins, in the form of benzoates, were eluted with 80% aq. acetonitrile and detected at 235 nm. The elution sequence was 9(11)-dehydrohecogenin, hecogenin, 9(11)-dehydrotigogenin and tigogenin in one chromatogram, and diosgenin followed by sarsasápogenin in another chromatogram.

The separation of steroidal alkaloids by HPLC has apparently also been reported only once. We[92] observed the following order of elution from a Porasil A column: tomatidine, solanidine, solasodine, rubijervine, veratramine, and jervine. The eluent was changed from acetone–*n*-hexane (2:1) to 97% aq. acetone between solasodine and rubijervine. In the absence of a detector, the effluent was analyzed by

TLC. The relatively large column (3/8 in. O.D. × 8 ft.) was suitable for fractionating 2 g of a crude alkaloid extract.

6. WITHANOLIDES

The withanolides are a class of plant steroids characterized by an α,β-unsaturated carbonyl and an α,β-unsaturated δ-lactone group, both of which give rise to appreciable UV absorption. Because the withanolides have been discovered rather recently and reference material is not generally available, little is known about their chromatographic properties.

Gustafson *et al.*[93] have separated five synthetic derivatives and two microbial metabolites of withaferin A on a μPorasil column with ethyl acetate or ethyl acetate–hexane (5:1), monitoring with a RI detector. Using the UV absorption at 225 nm for detection, we[94] have chromatographed 12 withanolides on a 12-ft. coiled column of Porasil A. Elution with *n*-hexane–2-propanol (9:1) yielded, in sequence, withanolides G, J, 27-deoxwithaferin A, withanolide D, 20-hydroxywithanone and withanolide E. Elution with *n*-hexane–2-propanol (3:2) separated, in sequence, $4\beta,7\beta$-dihydroxy-8,14-dihydrowithanolide G, withanone, 4β-hydroxywithanolide E, and withaferin A. Thus, the effect of hydroxyl groups on adsorption is in the order primary > secondary > tertiary.

7. PREGNANE DERIVATIVES

Most of the published applications of HPLC to pregnane derivatives deal with the adrenocortical hormones. This is partly due to their medical importance and partly to the fact that the natural and synthetic corticosteroids (as well as some other hormones) are characterized by a Δ^4-3-keto group, which absorbs UV light and is easily detected at 254 nm.

In one of the earliest methods, silica was used as an adsorbent[95], but soon investigators began to take advantage of its ability to act as a carrier of the more polar phase in partition systems, either consciously, by presaturating the silica column[96] and using the organic phase of biphasic solvent systems, such as dichloromethane–ethanol–water (948:35:17)[97–99], as eluent, or by simply developing the silica column with water-containing solvents, such as dichloromethane–95% ethanol (19:1)[100], chloroform–methanol–water (983:150:2)[101], chloroform–methanol (197:3)[102], or dichloromethane–ethanol–water–methanol (963:20:12:5)[103]. Excellent separations can be obtained in this way, the corticosteroids emerging from the columns in increasing order of polarity and aldosterone being eluted between cortisone and cortisol. Gradient elution may be used for this application[104,105].

Hydrophobic packing materials can be used as adsorbents with non-aqueous eluents[105,106], but more commonly, such nonpolar groups as octadecylsilane[107–109], cyanoethylsilane[110], cyanopropylsilane[111], nitro[112], or phenyl[113–115] and others[116,117], bonded to silica, selectively hold an organic solvent stationary. The mobile phase, which is more polar, is a water-containing organic solvent, such as methanol[110,113,118], 2-propanol[107], acetonitrile[113,114], or tetrahydrofuran[109]. The corticosteroids emerge from such reversed-phase columns in the order of decreasing polarity, giving very sharp and symmetrical elution peaks. Burgess[119] has shown that the optimum flow-

rate for such columns may be doubled when the temperature is raised from ambient to 60°.

HPLC is now widely used for the analysis of synthetic corticoid drugs. Silica columns have been recommended for prednisone and prednisolone[120,121], dexamethasone[121,122], and triamcinolone acetonide[123], but non-polar packing materials, especially those containing octadecylsilane groups, are more popular. The latter have found use in the analysis of fludrocortisone acetate[124], triamcinolone acetonide[125], prednisolone[126,127], methylprednisolone[101,128], and other steroid drugs[129]. For the corticosteroid phosphates, buffers are suitable eluents, because they control ionization[130,131]. The resolution of epimeric corticoid drugs by reversed-phase HPLC on Bondapak C_{18} deserves special mention. The epimers of ethynodiol diacetate[132] and of budesonide[133,134] could not have been separated as efficiently by any other method.

8. ANDROSTANE DERIVATIVES

In contrast to the HPLC of the pregnane derivatives, the HPLC of androstane derivatives has received surprisingly little attention. In order to make androstane derivatives detectable at 254 nm, the pioneers of this application, Fitzpatrick and co-workers, converted them to benzoates or *p*-nitrobenzoates[33] or 2,4-dinitrophenylhydrazones[35]. From a Permaphase ODS column, the esters were eluted by 66% aq. methanol in the order androsterone, dehydroepiandrosterone and epiandrosterone. The 2,4-dinitrophenylhydrazones were eluted with isooctane from a column of Zipax, which had been coated with β,β'-oxydipropionitrile, in the following sequence: epietiocholanolone, androsterone, epiandrosterone, etiocholanolone and dehydroepiandrosterone.

Using a RI detector, Lafosse *et al.*[135] separated these steroids, as well as their sulfates and glucuronides, by reversed-phase partition chromatography on Micropak CH with methanol–water mixtures. The order of elution for the free and conjugated 17-ketosteroids was dehydroepiandrosterone, epiandrosterone, etiocholanolone, and androsterone. Thus, the equatorial hydroxyl group makes the first three steroids more polar than the axial hydroxyl of androsterone. Among the steroids with equatorial hydroxyl groups, the most polar one is dehydroepiandrosterone with a double bond at C-5, followed by the A/B *trans*-compound epiandrosterone, and then the A/B *cis*-compound etiocholanolone (*cf.* ref. 57). A μBondapak C_{18} column was used to isolate labeled 19-hydroxyandrostenedione from sow ovaries[136] and to follow the enzymatic dehydrogenation of Δ^4-3-ketosteroids at C-1[137].

Synthetic androgens have been analyzed by reversed-phase partition chromatography on columns of ODS Permaphase[138], Nucleosil CN, Nucleosil C_{18}, Bondapak CN, and Hibar RP-8[139–141], using a concave gradient of methanol in water for elution. The drugs emerged in the following order: methandione, testosterone, methyltestosterone, Embadol, Ultandren, nortestosterone, and ethisterone. As little as 1 ng could be detected by the UV absorption at 254 nm. Higgins[32] recently chromatographed the acetates of some 19-norsteroids on LiChrosorb RP-8 with 50% aq. acetonitrile as the eluent and a detector, made from a Beckman DB spectrophotometer, which was set at 260 nm. Adsorption chromatography on silica gel columns with *n*-hexane–2-propanol–1, 2-dichloroethane (82:15:3) as the eluent gave the elution

sequence methyltestosterone, methandrostenolone, 6β-hydroxymethandrostenolone, and 6α-hydroxymethandrostenolone[142].

9. ESTROGENS

Being phenolic steroids, the estrogens are easily detected by their strong UV absorption. Therefore, work on HPLC of estrogens started early and has been extensive. In 1971, Huber *et al.*[143] reported the quantitative analysis of urinary estrogens, based on partition chromatography with a combination of a diatomaceous earth column and solvent systems of 2,2,4-trimethylpentane–ethanol–water. In 1973, Butterfield *et al.*[144] succeeded in separating the equine estrogens by hydrophobic adsorption chromatography with a column of Permaphase ETH. Elution with *n*-hexane–tetrahydrofuran (49:1) produced the following sequence: estrone, equilin, equilenin, 17α-estradiol, 17β-estradiol, 17α-dihydroequilin, 17β-dihydroequilin, 17α-dihydroequilenin and 17β-dihydroequilenin. Thus, the polarity increases with the number of double bonds and hydroxyl groups, and the 17β-hydroxysteroids are more polar than the 17α-epimers. Elution with *n*-heptane–2-propanol (99:1) gave similar results[145].

For the separation of iodinated estradiols, a column of μPorasil was developed with chloroform[146]. The 2,4-diiododerivative was followed by 4-iodo-, then 2-iodo-estradiol, and finally estradiol. Dolphin[147] analyzed the urinary estrogens by adsorption chromatography on a Corasil I column, eluted with ethanol hexane mixtures. He later used the Partisil 5/*n*-heptane–ethanol (19:1) adsorption system as well as the Partisil 10-ODS/methanol–0.1% aq. ammonium carbonate (11:9) reversed-phase system[148]. Aqueous methanol in combination with the reversed-phase packings Zorbax ODS[149] and μBondapak C_{18}[150] have also been used for estrogen chromatography. The resolution may be increased by adding silver nitrate, which forms complexes with the estrogens, to the mobile phase[19].

Van der Wal and Huber[151] have made a detailed study of the separation of estrogen conjugates by HPLC on ion-exchange cellulose columns. The anion exchangers, Cellex E, B 300, and ET 41, developed with perchlorate–phosphate buffers, separated the glucuronides (G) in the following sequence: estriol 3-G, estrone G, estradiol 3-G, estriol 17-G, estriol 16-G, and estradiol 17-G. For the sulfates (S) the following sequence was obtained: estriol 3-S, estrone S, equilin S, 17α-dihydroequilin 3-S, equilenin S, and 17α-dihydroequilenin 3-S[152].

Experiments with other stationary phases showed that cellulose and polystyrene anion exchangers have the greatest selectivity for the site of conjugation, but hydrophobic adsorption chromatography on LiChrosorb RP-18 or RPZ and on RP-18, coated with a liquid anion exchanger, are also suitable for this fractionation[153]. Musey *et al.*[154] devised a similar scheme, with μPartisil 10-SAX as the strong anion exchanger and 0.1 *M* NaCl at pH 4.8 or 0.01 *M* KH_2PO_4 at pH 4.2 as the developer, but the order of elution was different: estriol 17-G, estriol 3-G, estradiol 17-G, estrone G, estradiol 3-S and estrone S.

A different principle was applied to this problem by a Swedish group. In liquid–liquid ion-pair chromatography, the glucuronides or sulfates are selectively transferred from an aqueous phase to an organic phase by addition of a cation. Fransson *et al.*[155] chose tetraethylammonium ion as this counter ion, which was held stationary in the

form of its bromide on a support of LiChrospher Si-100. The ion pairs, extracted by a mobile phase of dichloromethane–1-pentanol (9:1), emerged in the order estradiol 3-S, estradiol 17-S, estriol 17-S, and estriol 3-S. Hermansson[156] used reversed-phase ion-pair chromatography with 1-pentanol, held stationary on a column of LiChrosorb RP-2 or RP-18, and tetrapropylammonium as the counter ion in a mobile phase containing phosphate buffer at pH 6.4. The following elution sequence was obtained: estriol 17-G, estrone G, estradiol 3-G, and estradiol 17-G.

The following reversed-phase partition systems have been used for HPLC of estrogens analogs: LiChrosorb RP-8/methanol–0.01 *M* phosphate buffer at pH 7 (3:1) for mestranol and norethisterone[157], Spherisorb S5-ODS/aq. methanol for ethinylestradiol[158], and LiChrosorb RP-8-10A/acetonitrile–water (11:9) for mibolerone[159].

10. BILE ACIDS

The bile acids, which cannot be detected with fixed-wavelength detectors, have in many instances been monitored with the RI detector. Although the RI detector is not very sensitive, the concentrations of bile acids in most biological fluids are sufficiently large for this instrument. Free bile acids are best chromatographed by reversed-phase partition, but after their conversion to esters, both partition and adsorption chromatography give satisfactory results. Certain esters also facilitate detection by UV absorption. One of the advantages of analyzing bile acids by HPLC rather than GLC is that their conjugates do not need to be hydrolyzed or derivatized.

Parris[160], who had earlier used a Zorbax SIL column, which was developed with pentane–2-propanol (7:3), for the chromatography of free bile acids, reported better results with a Zorbax ODS column, which he developed with methanol–phosphoric acid solution of pH 2 (4:1). However, cholic and chenodeoxycholic acids could not be separated. When Jefferson and Chang[161] applied adsorption chromatography on μPorasil with ethyl acetate–hexane (7:3) as the eluent to the separation of bile acid methyl esters, partial resolution of several epimeric pairs was achieved.

The phenacyl esters[162] of the bile acids were eluted from a Partisil ODS column by *n*-heptane–dioxane–2-propanol (14:5:1) in the following order: lithocholic, chenodeoxycholic, ursodeoxycholic, hyodeoxycholic, and cholic acid esters. Excess reagents and biological contaminants were removed by prior elution with *n*-heptane–dioxane (9:1). The phenacyl esters as well as the *p*-nitrobenzyl esters[163] are easily detected at 254 nm. The latter were chromatographed on Partisil 10 with isooctane–2-propanol (49:1)[163] or on MicroPak-NH_2 with an elution gradient from isooctane–dichloromethane (1:1) to dichloromethane–2-propanol (9:1)[164]. For the *p*-chlorobenzoyl esters of methylated bile acids, Porasil T and isooctane–diisopropyl ether (19:1) proved to be effective[163].

Okuyama *et al.*[164] chromatographed the glycine conjugates of bile acids in the form of *p*-nitrobenzyl esters, but for the taurine conjugates, which are detectable at 210 nm, they used μBondapak C_{18}, eluted with methanol–0.01 *M* KH_2PO_4 (3:1). Taurodeoxycholic and taurochenodeoxycholic acids were not separated from each other. Conjugated bile acids have also been analyzed by adsorption chromatography on Corasil II with 2-propanol–ethyl acetate–water–7 *N* ammonium hydroxide

(260:60:50:3) as the eluent[165] or on Vydac or Perisorb A columns by elution with a convex gradient from chloroform to ethyl acetate–ethanol (8:3)[166].

More commonly, however, reversed-phase partition chromatography and detection by refractometry have been applied to the conjugated bile acids[167–169]. One approach to the simplification of the complex mixture obtained by extraction of bile is the preliminary fractionation into free bile acids, glycoconjugates, and tauroconjugates by TLC[170]. Another approach is based on ion-exchange chromatography[171]. A column of piperidinohydroxypropyl Sephadex LH-20 is first washed with 90% aq. ethanol to remove lipid contaminants, then free bile acids are eluted with 0.1 *M* acetic acid in 90% ethanol. Later, the glycoconjugates are eluted with 0.2 *M* formic acid in 90% ethanol, and finally the tauroconjugates are eluted with 0.3 *M* acetic acid–potassium acetate buffer at pH 6.3 in 90% ethanol. Each group is then resolved on a column of μBondapak C_{18}. Elution with 0.3% ammonium carbonate–acetonitrile (9:4) gives a mixture of cholate and ursodeoxycholate, followed by chenodeoxycholate, deoxycholate, and finally lithocholate. A second chromatogram with an 11:4 mixture of 0.3% ammonium carbonate and acetonitrile separates ursodeoxycholate from cholate, which is eluted later.

Reversed-phase partition chromatography was also used by Shaw and co-workers[172,173], who separated conjugated bile acids on a Waters Assoc. fatty-acid analysis column by elution with 2-propanol–8.8 m*M* potassium phosphate buffer at pH 2.5 (8:17) in the following order: tauro-α-muricholate, taurocholate, taurochenodeoxycholate, glycocholate, taurolithocholate, glycochenodeoxycholate, and glycodeoxycholate. However, for the resolution of $5\alpha/5\beta$ epimers, Shaw and Elliot[173] preferred adsorption chromatography on Corsail II or on μPorasil with recycling of acetonitrile–acetic acid (40:1).

Sulfated bile acids[174], which were detected by their absorption at 210 nm, emerged from an ODS SC-02 column, eluted with 0.5% ammonium carbonate–acetonitrile (13:4) in the sequence cholate, glycocholate, taurocholate, chenodeoxycholate, deoxycholate, glycochenodeoxycholate, glycodeoxycholate, taurochenodeoxycholate, and taurodeoxycholate. From the same column, eluted with 0.5% ammonium carbonate–acetonitrile (5:2), they emerged in the order taurodeoxycholate, lithocholate, glycolithocholate, and taurolithocholate. Thus, the polarity depends not only on the number of hydroxyl groups but also on the nature of the conjugate, the free bile acids being more polar and the tauroconjugates being less polar than the glycoconjugates.

11. CARDIAC GENINS AND GLYCOSIDES

One of the advantages of HPLC over GLC in the application to the cardiac glycosides is that they may be chromatographed without hydrolysis or derivatization. The unsaturated lactone ring makes the cardenolides and bufadienolides relatively unstable, but facilitates their detection by UV absorption.

Evans[175] in 1974 first reported the HPLC of cardiac glycosides on a SCX (strong cation-exchange) column at 45°. With 4% aq. amyl alcohol, he eluted, in sequence: digitoxigenin, its mono-, bis- and tris-digitoxoside (digitoxin), and lanatoside A. An analogous elution sequence was observed by Cobb[176] for the digoxigenin glycosides in adsorption chromatography on LiChrosorb Si 60-10 with cyclohexane–

ethanol–acetic acid (60:9:1) as eluent. This system separates the cardiac genins in the order digitoxigenin, gitoxigenin, and the cardiac glycosides in the order: digitoxin, gitaloxin, gitoxin, digoxin, and diginatin. A chromatographic method for the determination of gitoxin glycosides in *Digitalis* preparations is based on a silica–methanol adsorption system[177].

Using LiChrosorb Si 60-10 as a carrier for the stationary phase, Lindner and Frei[178] devised two partition systems for *Digitalis*. For the genins and less polar glycosides (digitoxigenin to diginatin, see above), they used isooctane–1-pentanol–acetonitrile–water (124:35:12:2). Heptane–*tert*.-butyl alcohol–acetonitrile–water (3560:1020:465:52) was used to elute the more polar glycosides in the following order: digitoxin, gitoxin, digoxin, diginatin, then lanatosides A, B, C, and D, followed by the desacetyl lanatosides A, B and C. The sensitivity of detection by UV absorption of 254 nm is greatly increased by converting cardiac aglycones or glycosides to the fully substituted 4-nitrobenzoates[179]. In experiments with Merckosorb Si 60-5 and either *n*-hexane–chloroform–methanol (20:2:1) or *n*-hexane–chloroform–acetonitrile (10:3:3) as eluents, as little as 20 ng/ml of the 4-nitrobenzoates could be detected[180]. The order of elution was the same as that observed for the underivatized compounds.

Comparison of partition and reversed-phase partition HPLC of *Digitalis* glycosides showed not only, as expected, that the order of elution was reversed, but also that the resolution is improved when the reversed-phase system Nucleosil C_{10}/37% aq. acetonitrile is used[181]. For the analysis of digoxin and digitoxin glycosides, Castle[182] applied an elution gradient from 25 to 40% aq. acetonitrile to a column of μBondapak C_{18}. As expected, the digoxigenin glycosides emerged before the digitoxigenin glycosides, but oddly within each group the genin was eluted first, followed by the mono-, then the bis-, and finally the trisglycoside. A similar chromatographic system has been used for the cardiac glycosides in milkweed plants and Monarch butterflies[183].

So far, only one group of investigators[184,185] has published data on the application of HPLC to bufadienolides. Best results were obtained with a column of μBondapak C_{18} and either 40% aq. tetrahydrofuran or 66.6% aq. methanol as the mobile phase. The order in which the functional group in bufadienolides and cardenolides retard the elution from that column is: 11α-OH $<$ 12β-OH $<$ 16β-OH $<$ 5β-OH $=$ 16β-OAc. The homologs of gamabufalitoxin emerged in the order gamabufotalin 3-succinoylarginine, 3-adipoylarginine and 3-pimeloylarginine esters, and, finally, gamabufalitoxin. The epimeric 14,15-epoxides were best resolved by adsorption chromatography on a Corasil I column with hexane–tetrahydrofuran (3:1) as the eluent.

12. SUMMARY

After a brief discussion of the merits and limitations of high-pressure liquid chromatography (HPLC) relative to other chromatographic methods, special problems in the application to steroids are discussed. Publications on HPLC of steroids are then discussed under the headings of individual classes, arranged generally in the order of increasing polarity.

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Journal of Chromatography, 165 (1979) 301–319
Chromatographic Reviews
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHREV. 122

PROTEIN PURIFICATION USING IMMOBILISED TRIAZINE DYES

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(Received April 9th, 1979)

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1. INTRODUCTION

Dextran conjugates of Cibacron blue F3GA* (Fig. 1) have been used for many years to measure the void volume in gel filtration[1]. It was discovered that dye conjugates when co-chromatographed with proteins selectively interacted with various enzymes. Furthermore, it was shown in 1968 that the dye chromophore rather than the dextran is responsible for the interaction with enzymes[2,3].

Fig. 1. Structure of Cibacron blue F3GA. The dye is a mixture of two forms: one with the $-SO_3H$ group in moiety D meta to the -NH- bridge, the other with this group para to the -NH- bridge. In Blue Dextran the chlorine atom in ring C is replaced by O-Dextran 2000.

Since this discovery, triazine dye "affinity" adsorbents have been shown to bind a wide variety of different proteins[4–118] (Table 1). In addition to Cibacron blue, one other dye has attracted interest recently, namely Procion red HE 3B, the structure for which is given in Fig. 2[6,10,94,117,118].

Fig. 2. Structure of Procion red HE 3B.

This article sets out to review (a) the chemistry of dye immobilisation; (b) the applications of immobilised triazine dyes; (c) ways of modifying the binding of enzymes to triazine dye columns and (d) the mechanism of action of immobilised dye-protein interactions.

Also known as Procion blue H-B, colour index 61211 (ref. 33).

TABLE 1

INTERACTION OF PROTEINS WITH IMMOBILISED TRIAZINE DYES

Eluent: N = nucleotide; S = salt; Su = substrate; SuA = substrate analogue; P = product; D = Cibacron blue F3GA; N–Su = nucleotide–substrate adduct; A = aprotic solvent; E = electrophoretic desorption; X = not stated; O = other. Adsorbent: BDA = Blue Dextran–Agarose; CBA = Cibacron Blue F3GA–Agarose; PRA = Procion red HE 3B–Agarose; BD-X, CB-X, PR-X = Blue Dextran, Cibacron blue F3GA or Procion red HE 3B, respectively, coupled to other support; BD + X = free Blue Dextran chromatographed on gel filtration medium.

Protein	*Source*	*Eluent*	*Adsorbent*	*Reference*
Dehydrogenases and reductases				
Alcohol dehydrogenase	Horse liver	N	BDA, BX-X	4, 5
	Human	N	CBA	164
	Yeast	S	CBA, PRA	6, 151
	Rainbow Trout	N	CBA	7
	Ox brain	S	PRA	119
	Cotton seeds	N	BDA	8
	Rape seed	E	BD-X	9
Aldehyde reductase	Rat liver	S	CBA, PRA	10
	Sacch. cerevisiae	S	CBA	11
	Sacch. cerevisiae	S	BDA	12
	Ox brain	S	PRA	119
Dihydrofolate reductase	*L. casei*	S	CBA, PRA	6, 151
		E	CB-X, PR-X	
	Ox brain	N	PRA	118
Dihydropteridine reductase	Ox brain	N	PRA	118
Glucose-6-phosphate dehydrogenase	Yeast	S	BDA, BD-X	4
	Yeast	E	BD-X	9
	Yeast, beef liver	S	BDA	13
	Yeast	S	CBA, PRA	6, 151, see 167
	Heterolactic bacteria	S, N	BDA	14
	B. stearothermophilus	S	PRA	121
	A. niger	—	BDA	171
	L. buchneri	S, E	BDA	15
Glutamate dehydrogenase	Beef liver	S, E	CBA, PRA, CB-X, PR-X	6, 151
	Neurospora crassa	S	CBA, PRA	
	Yeast	S	CBA, PRA	
	A. niger	—	CBA	171
Glutathione reductase	Yeast	S	CBA, PRA	6, 151
	Human erythrocytes	X	BX + X	16
Glyceraldehyde-3-phosphate dehydrogenase	Rabbit muscle	N	BDA, BD-X	4
	Rabbit muscle	S	BDA	17
	A. niger	—	BDA	171
Glycerol-3-phosphate dehydrogenase	*E. coli*	X	BDA	18
3-Hydroxyl-3-methyl glutaryl CoA reductase	Chicken liver	S	BDA	41
	Rat liver	S	BDA	42, 160
3(17)β-Hydroxy steroid dehydrogenase	Rat erythrocytes	N	CBA	47
Isocitrate dehydrogenase	Human heart	N + A	BDA	19
	A. niger	—	BDA	171
	B. stearothermophilus	O	BDA	20
	Pig heart	S	CBA, PRA	6, 151

(*Continued on p. 304*)

TABLE 1 *(continued)*

Protein	*Source*	*Eluent*	*Adsorbent*	*Reference*
9-Ketoprostaglandin dehydrogenase	Human placenta	S	CBA	21
Lactate dehydrogenase	Rabbit muscle	N	BDA, BD-X	4, 5, 22, 29, see also 167
	Beef heart	E, N	BD-X	9, 129
	Beef heart			
	Rabbit muscle	N	BDA	13
	Pig heart	S	CBA, PRA	6, 151
	Beef muscle	E	CB-X, PR-X	
	Rat heart	N	CBA, PRA	10
	Rat liver			
	Rat hepatoma	N	BDA	23, 24
	Soya bean	X	BDA	25
	Isoenzymes M + H	N	BDA	168
	A. niger	—	BDA	171
	B. Thermus aquaticus	N	BDA	27
	L. casei	N–Su	CBA	26
	Heterolactic bacteria	S	BDA	17
	Mouse, dog, rat, amphibia, fish tissues	N, S, P, Su, SuA	BDA	28
	Mustelus canis, whiting, skate tissues	N, S	BDX	29–31
Malate dehydrogenase	Pig heart	N	BDA	5, 13, see 167
	Rat heart	N	CBA, PRA	10
	Mustelus canis	N	BD-X	29
	Sacch. cerevisiae	N, S	BD-X	144
	Yeast	S	CBA, PRA	6, 151
		E	CB-X, PR-X	
	Yeast	X	BD-X	32
	A. niger	—	BDA	171
Methylene tetrahydrofolate reductase	Bovine liver	S	CBA, PRA	10
	Ox brain	S	PRA	118
NADH-cytochrome b_5 reductase	Rabbit liver	A	BDA	33
Nitrate reductase	*Chlorella fusca*	N	BDA	34
	N. crassa	N	BDA	35
	N. crassa	N	BDA	39
	Rhodotorula glutinis	N	BDA	36
	Spinacea oleracea	S	BDA	31, 37
	Chloracea	N	BDA	38
2-Oxoaldehyde reductase	Rat liver	S	BDA	40
15-Hydroxyprostaglandin dehydrogenase	Human placenta	S	BDA	43
	Human placenta	N	BDA	44
Pyruvate dehydrogenase complex	*Azotobacter vinelandii*	S	BDA	45, 46
6-Phosphogluconate dehydrogenase	Yeast	N	BDA	4, 5, 13
	Yeast	S	CBA, PRA	6, 149, 151
	B. stearothermophilus	S	PRA	121
	A. pseudoplatanus	S	PRA	121
	A. niger	—	BDA	171
	Heterolactic bacteria	S	BDA	14

TABLE 1 *(continued)*

Protein	*Source*	*Eluent*	*Adsorbent*	*Reference*
Shikimate dehydrogenase	*Physcomitrella patens*	S	CBA	48
	N. crassa	N	CBA	49
Kinases				
Adenylate kinase	Rabbit muscle	N	BDA	5
	Pig heart	S	BDA	50
cAMP-dependent protein kinase	Beef brain	N, S	BDA	51
cGMP-dependent protein kinase	Calf lung	S	BDA	52
Creatine kinase	Rabbit muscle	E, N	BD-X	136, 168
	Snake venom	X	BD-X	53
Cytidylate kinase	Human erythrocytes	N, S	BDA, CBA	136, 54
Deoxynucleoside kinases	*L. acidophilus*	X	CBA	55, 56
	Various mammalia	N	CBA	57
Hexokinase	Rat brain	X	BDA	13
	Yeast	X	CBA	4
	A. niger	-	CBA	171
Myokinase	Rabbit muscle	E	BD-X	9
NAD kinase	Pigeon liver	D	BDA, CBA	58
Phosphofructokinase	*E. coli*	N	BDA	3, 5, 59, 61
	L. plantarum	N	CB-X	62
	L. acidophilus			
	Thermus X-1	N	BDA	60
	Yeast	N	BD-X, BD + X	63–65
		Su	CB-X	66, see 167
		X	CB	163
	A. niger	X	BDA	171
	Sacch. carlsbergensis	S	CBA	67
	Pig kidney	S	CB-X	68, 63
	Human erythrocytes muscle, yeast		CB-X	69, 156
Phosphoglycerate kinase	*B. vulgaris*		CBA	158
	Yeast	N	BDA	70
	Yeast	N, Su	BDA	22, 33
		S	BDA	129
	Horse erythrocytes	N, Su	BDA	71
	A. niger	—	BDA	171
Pyruvate kinase	*Phaseolus aureus*	X	BDA	72
	Yeast	S	BD + X	2, 3
		S	CB-X	73
		N	BDA	70
	Rabbit muscle	Su	BDA	22
	Rabbit muscle	E	BD-X	9
	Human kidney	N	CBA	74
	Human erythrocytes	S, Su	BD + X, BDA	70, 75, 76
	Human liver	Su	BDA	77
		S	BDA	168
	A. niger	-	BDA	171
Other proteins				
Adenylate cyclase	Beef brain	N	BDA	33, 78

(Continued on p. 306)

TABLE 1 *(continued)*

Protein	*Source*	*Eluent*	*Adsorbent*	*Reference*
Adenylate kinase	Rabbit muscle	S	BDA	5
Nucleoside diphosphokinase	Human erythrocytes	N, S	CBA	54
Adenyl succinate synthetase	*Az. vinelandii*	N	BDA	79
Albumin	Chicken egg	S	CBA, PRA	6
	Chicken egg	E	BD-X	9
	Human serum	O, E	CBA, CB-X	6, 80
	Human serum	S	BDA, CBA	82, 83
	Human serum	S	CBA	85
	Human serum	O	CBA	84, 148
	Bovine serum	E	BD-X	9
Aldolase	Rabbit muscle	S	BDA	22
	Rabbit muscle	E	BD-X	9
	A. niger	—	BDA	171
Aminoacyl tRNA synthetase	*Sacch. cerevisiae*	S	BDA	86
	E. coli	N, S	BDA	87, 88
Arom multienzyme complex	*N. crassa*	S	CBA	89
Arylsulphatase	Rat brain, liver Human urine Sheep brain	O	CBA	90
Blood clotting factors	Human	S	BD + X	91
	Human	S	BDA	92
C-3 Factor of complement	Human	X	BDA	148
cAMP phosphodiesterase	X	S	BDA	33
Ceruloplasmin	Human blood	X	BDA	148
Cyclic nucleotide phosphorylase	Beef heart	N	CB-X	103
Carboxypeptidase G	*Pseudomonas* ATCC 25301		PRA	94
Choline acetyl transferase	Human brain, placenta	S	BDA	95
Chymotrypsin } Chymotrypsinogen }	X	N	BDA, BD-X	4, 168
Citrate synthease	X	S	BDA	33
	A. niger	—	BDA	171
Cytochrome *c*	Horse heart	N, S	BDA, BD-X	4, 5
3-Dehydroquinate hydrolase	*Physcomitrella patens*	S	CBA	48
DNA-polymerase I	*E. coli*	N	BDA	5
	Hela cells	S	BDA, BD-X	5, 96
Enolase	Rabbit muscle	Su, P, S	BDA	22
α-Fetoprotein	Human	S	CBA	85
Flavocytochrome *c*	Yeast	S, A, N	BDA	93
Fructose diphosphatase		N	BDA	5, 22, 168
Follicle stimulating hormone	Human	X	BD + X	97, 98
Glyoxylase I	Sheep liver	X	BD	157
Haptoglobulin	Human blood	X	BDA	148

TABLE 1 *(continued)*

Protein	*Source*	*Eluent*	*Adsorbent*	*Reference*
Interferon	Human fibroblasts	A	BDA	138, 81
	Human leukocytes	S	BDA	81
	Mouse	O	BDA	162
	Mammalian	S	BDA	169
Macroglobulin	Human blood	X	CBA	148
Malic enzyme	*A. niger*	S, N	CBA	171
Methylmalonyl CoA isomerase	X	S	BDA	33
NAD$^+$-glycohydrolase	Calf spleen	N, Su, S	BDA	100, 128
Orotate decarboxylase	Human		BDA	102
Orotate phosphoribosyl transferase	Yeast	S, Su, P	DBA	101
Phosphoglucomutase } Phosphoglyceromutase }	Rabbit muscle	Su	BDA	5, 22
Phosphorylase a	Rabbit muscle	N	BDA	22
Phosphodiesterase	Beef heart	N, S, S + N	CB-X	103
	Snake venom	S	BDA, CBA	53
Poly(A)DPR polymerase	Calf thymus	S	BDA	104
Polynucleotide phosphorylase	*E. coli*	N	BDA	105
Polynucleotide kinase	T4 phage	S + N	BDA	106
Pregnancy protein SP_1	Human	O	CBA	148
Propyl hydroxylase	Neonatal rat dermis	S	CBA	107
Pyruvate carboxylase	*A. niger*	S, N	CBA	171
R. enzyme	Sweet corn	S	BD + X	108
Restriction endonucleases	Various bacteria	S	CBA	109, 110
Retinol-binding protein	Human blood	X	CBA	148
RNA-polymerase II	Wheat germ	X	CBA	111
RNA-polymerase, β-subunits	*B. subtilis*	S	BDA	112
Ribonuclease type I-A	Beef pancreas	N, S	BDA	4, 5
Sex hormone binding globulin	Human		CBA	113
Succinyl CoA transferase	Pig heart	X	BD + X	114, 115
Succinyl CoA transferase	Sheep kidney	S	CBA	116
Thiosulphate sulphur transferase	Bovine liver	Su	CBA	170
Transferrin	Human	O	CBA	148
Trypsin	X	N	CBA	168

2. IMMOBILISATION OF DYES — THE SUPPORT MATRIX

As with many affinity chromatographic ligands, triazine dyes have been immobilised to a wide variety of support matrices in the search for an ideal system. Some of the supports that have been examined in this way include agarose, dextrans (cross-linked or uncross-linked in either case), polyacrylamide, agarose–polyacrylamide copolymers, cellulose and glass[30,80,94].

Whilst Cibacron blue has mainly been used when attached to a solid support, several applications have been described where a high-molecular-weight (but soluble) Blue dextran–protein complex was separated from low-molecular-weight material by

TABLE 2

PROTEINS WHICH HAVE BEEN OBSERVED NOT TO INTERACT WITH IMMOBILISED CIBACRON BLUE F3GA

Proteins shown to interact by other workers (see Table 1)

Adenylate cyclase[10], albumin (chicken egg)[4,81], alcohol dehydrogenase[73], aldolase[63], 4-aminoacyl-t-RNA synthetase[86], cytochrome *c* (horse heart)[9], dihydrofolate reductase (bacterial)[5], glycéraldehyde-3-phosphate dehydrogenase[63], phosphofructokinase[64], pyruvate kinase[63].

No references showing otherwise

Acetate kinase[15], rabbit, chicken, bovine serum albumins[80,92], apoflavodoxin[5,11], aspartate transcarbamylase[22], cytidine kinase[136], guanylate kinase[114], hemoglobin[4], heterolactic bacterial and yeast hexokinases[15,22], *N. crassa* NAD^+-glycohydrolase[100], potato lactate dehydrogenase[117], micrococcal nuclease[5,13], phosphate acetyl transferase[15], yeast phosphoglucoisomerase[22], yeast phosphoglucomutase[22], subtilisin[5,11], superoxide dismutase[83], thermolysin[33], thyroglobulin[4], triosephosphate isomerase[22], uridine kinase[54].

gel filtration[2,3,63,73,91,92]. Blue dextran has also been used as an "entrapped" ligand in polyacrylamide[63,66].

Rather few reports actually compare different support matrices[80,82]. When viewed simply from the standpoint of capacity of the column (either moles of protein bound per column volume or per mole of ligand) it has been shown that agarose is superior to cellulose[80], polyacrylamide[69] and Ultrogel[80]. Sephadexes have been claimed either to be at least as good as agarose[94] or inferior[33]. Blue dextran coupled to porous glass or silica has been successfully operated at higher capacities when compared with AMP-glass or agarose[30].

Table 3 shows a comparison of adsorbent capacities for 6-phosphogluconate dehydrogenase from *Acer pseudoplatanus* L. on a range of different matrices containing immobilised Procion red HE 3B (ref. 120). In this study only three supports were of high working capacity. These were: uncross-linked Sepharose, cross-linked Matrex gel (see Table 3) and Sephadex G-200. In contrast to previous work, there did not appear to be any correlation between the concentration of KCl coincident

TABLE 3

CAPACITIES OF IMMOBILISED PROCION RED HE 3B FOR 6-PHOSPHOGLUCONATE DEHYDROGENASE (6PGDH) FROM *ACER PSEUDOPLATANUS*[120]

Support matrix	*Ligand concentration (mg/g wet weight)*	*Capacity for 6PGDH (Units/g wet weight)*
Matrex gel (Amicon)	3.6	62
4% Agarose (IBF)	0.5	2
6% Agarose (IBF)	0.3	5
Sepharose 6B (Pharmacia)	2.6	44
Sepharose C16B (Pharmacia)	3.7	16
Sephadex G-200 (Pharmacia)	1.0	50
Sephacryl S-200 (Pharmacia)	2.4	15
Ultrogel AcA 54	0.8	0.2
Agarose (marine colloids)	2.8	14
Spheron (Hydron)	3.9	1
Cellulose (Whatman)	2.6	0.1

(used by some workers as a measure of binding "affinity") with the peak of eluted enzyme and the matrix capacity (see ref. 6).

In a separate study[121] with thermophilic 6-phosphogluconate dehydrogenase from *B. stearothermeophilus*, Sephadex-immobilised Procion red HE 3B is able to bind the enzyme only when the Sephadex has a larger pore size than G-50. But above the latter (G-50), capacities were similar within the Sephadex series and were proportional to the ligand concentration. In addition, ligand effectiveness was superior to many forms of agarose. In one case (Matrex gel red) higher capacities were obtained probably because of the greater ligand concentration obtainable with this gel.

High ligand concentrations alone do not necessarily determine the potential capacity of a support for a protein. For example, cellulose is more highly substituted with dye than agarose and dye concentrations in agarose are higher than in Ultrogel (an agarose–polyacrylamide copolymer) but only the agarose based dye is effective[80]. In instances where the dye couples less readily to a matrix, polyethyleneimine (or polylysine) can improve substitution levels[6].

The combination of affinity chromatography with gel filtration (affinity gel filtration[12]) remains an attractive proposition. Much of the early work was hampered by the fact that cyanogen bromide adversely affects cross-linked dextran gels. However, several efficient triazine dye columns have been prepared using G-100[94] and G-200[120] (Table 3), Sephadex or Sephacryl[80] (see also Table 4). The successful use of cross-linked dextrans as matrices for triazine dyes is probably due to the mild nature of the reaction between the triazine moiety and the polysaccharide. Wood and co-workers[125,141] have advocated the general use of triazines to immobilize ligands. One prediction which is now apparent from the above is that affinity gel filtration could be performed on dextran-based gels with any of the usual affinity ligands provided that the cyanogen bromide activation step is replaced by a triazine-based reaction either by preassembling the ligand plus triazine (or less satisfactorily by prior attachment of the triazine followed by reaction with the ligand).

In summary, it is likely that triazines will represent a considerable improvement in the methods available for the attachment of ligands to support and that preassembled defined molecular species will be more accessible by this route. Another

TABLE 4

SOME SUPPORT MATRICES

Support	*Trade name*	*Company*
Crosslinked 6% Agarose	Matrex gel red	Amicon
Agarose	Matrex gel blue	
Agarose	Affigel blue	Biorad
Polyacrylamide-Agarose copolymer	Ultrogel	LKB
2,4,6% Agarose	Sepharose	Pharmacia
2,4,6% Agarose	CL Sepharose	
Cross-linked Agarose	CL Sepharose	
Cross-linked 6% Agarose	Blue Sepharose	
Cross-linked dextran	Sephadex	
Cross-linked dextran (polyacrylamide bridges)	Sephacryl	Whatman
Cellulose	Ultrogel	Whatman

prediction is that more precise affinity gels can be constructed where non-uniform ligand distribution is hopefully eliminated (see below).

3. TRIAZINE DYES AND SPACERS

Two questions seem relevant regarding spacers: (a) Does the addition of a hydrophobic spacer arm to a "detergent" or "hydrophobic" ligand have any significant role? (b) Are dextrans or other soluble polysaccharides true spacers when coupled to triazine dyes prior to their immobilisation?

Table 1 shows that about 60% of the cited examples use dextran conjugates of Cibacron blue. The conjugates have been immobilised either (a) via the dextran moiety using periodate oxidation[31]; (b) via cyanogen bromide activation of the dextran[30]; (c) via the anthraquinone amino group using cyanogen bromide-activated adsorbents (this method presumably not only cross-links the agarose but also links the conjugate to the agarose by the same sort of bridges)[23] or (d) via carboxyl groups previously attached to the matrix[58] (Fig. 3). In the examples (a) and (b) the dextran can be viewed as a spacer whilst in (c) and (d) the dye may act in part as a bridge between two much larger polysaccharide species (dextran and agarose). In the latter case the dextran is more likely to operate in a restricting sense making the dye less available to some proteins on the basis of size or accessibility of dye binding sites. Alternatively, since

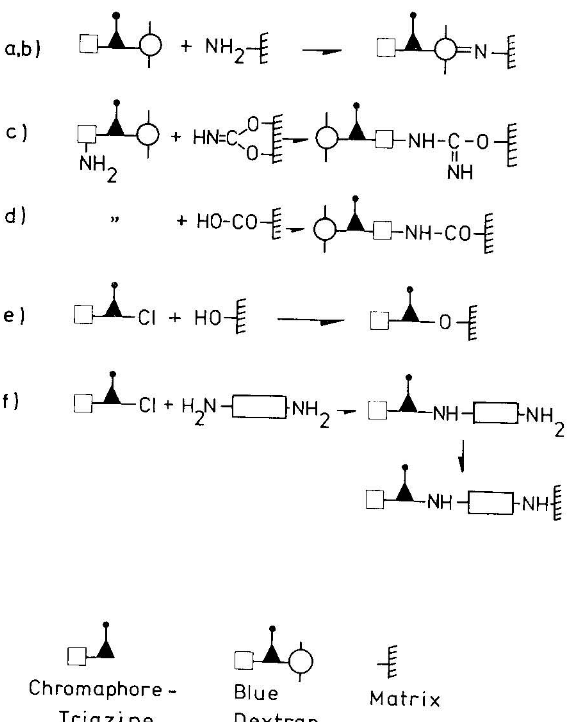

Fig. 3. Structure of some Cibacron blue conjugates. The structure of Blue Dextran indicates multiple sites of attachment of the dye to the dextran polymer.

presumably several dye molecules are bound to each dextran chain and fewer dye molecules react with the activated agarose, the dextran in these cases could also act as a spacer for a proportion of dye molecules.

On the other hand, triazine dyes can be bound directly via the triazine ring to polysaccharide supports ((e) in Fig. 3)[47,69,73,83,123] or via polyethyleneimine ((f) in Fig. 3)[6,124] by prior coupling of the dye to the spacer and the assembled conjugate is then coupled to a cyanogen bromide- (or triazine-) activated matrix.

There have been a few studies comparing the properties of triazine dyes immobilised in different ways. These studies show that directly coupled dye is less selective than the dye coupled via polyethyleneimine, dextran or hexyl spacers[6,13,58,83].

Whether the dye is immobilised via a spacer arm or polymer, or which type of support matrix is used is probably best determined separately for each application. The above discussion can only be used as a guideline (see 152).

However, it is desirable that adsorbents should be compared at similar immobilised ligand concentrations[30]. In parallel studies with immobilised nucleotides[127] and hydrophobic ligands it has been shown that variation of the ligand concentration considerably alters the properties of the column.

Lang *et al.*[125] have made an observation of note concerning hydrophobic spacer arms for immobilization of oxamate. If the uncertainties of cyanogen bromide chemistry are replaced by the triazine linkage then the involvement of the hexamethylene spacer, with or without the oxamate becomes clearer. Oxamate can only recognise lactate dehydrogenase when both a hydrophobic arm and NAD(H) are present —replacement of hexyl spacer by two three carbon chains joined by an amino group abolishes binding— in O'Carra's words[142]: "It is clearly necessary to exercise great care in distinguishing bio-affinity from non-specific adsorption".

4. DETERMINATION OF DYE CONCENTRATIONS

Determinations of triazine dye concentrations by spectrophotometric means are complicated by several factors (a) deviations from Lambert-Beer's law due to association of dye molecules at higher concentrations[126]; (b) many commercial dye preparations are multi-component; (c) a concentration-dependent red shift is observed with Cibacron blue[143] and is likely to exist for dyes of similar structure.

Immobilised dye concentrations have been measured by spectrophotometric measurements of (a) the gel[80] (b) the dye released by digesting the matrix with 3 *M* hydrochloric acid or 50% acetic acid[80,118,159], or (c) by the difference between added and unbound dye at the time the gel is made. For Cibacron blue, extinction coefficients of 12.3 to $13.6 \cdot 10^3\ l \cdot mol^{-1} \cdot cm^{-1}$ at 610 nm (pH 7) have been measured[58,118,126,129,137] and for Procion red the extinction coefficient is reported as 1 to $3 \cdot 10^4\ l \cdot mol^{-1} \cdot cm^{-1}$ at 522 nm (pH 7) at 18° (Ref. 121).

Very few studies have incorporated prior purification of the commercial dye preparations. Nevertheless, contaminants may be removed by solvent precipitation[58], cellulose chromatography[128] or by isoelectrofocusing (using the latter technique many distinguishable bands are observed) (see also ref. 167).

5. MECHANISM

The mechanism of the binding of triazine dyes to enzymes is not simple. The above considerations hardly touch on some of the complications that can arise in such mechanistic studies. Many of the analyses of such interactions have been based upon biospecific elution (low substrate or nucleotide concentrations elute the retarded protein whereas equivalent or higher (50 m*M*) salt concentrations have no effect). This has been taken to indicate a "biospecific" interaction (*i.e.* via a nucleotide binding domain[5]). However, Yon[139] has shown that several enzymes, including dehydrogenases, may be eluted from "detergent" adsorbents under these conditions. Whilst biospecific elution is a useful tool both in affinity and other types of adsorption chromatography[139], information relating to the adsorption phase (and its mechanism) is not obtained by studying desorption[127]. One concludes that the elution of dehydrogenases by nucleotides from immobilised Cibacron blue does not imply any biospecificity in the mechanism of adsorption.

It is nevertheless possible that Cibacron blue, and other polyaromatic sulphonates in general, can mimic certain aspects of the binding of nucleotides to the nucleotide binding sites of proteins[5,33,78,129,130] but more elaborate claims for the use of the dye to test for the dinucleotide fold[33,131,132] must be viewed with caution. Indeed, Stellwagen has recently carried out a careful analysis of the interaction between Cibacron blue and a variety of enzymes and concludes "we find that Cibracon blue F3GA has suffered the fate of many biological inhibitors namely to appear less specific as its use becomes more widespread"[143]. This is in agreement with other analyses[63,69,126].

6. METHODS FOR MODIFICATION OF PROTEIN BINDING TO TRIAZINE DYE COLUMNS

6.1. Decreasing protein binding

In order to operate any chromatographic separation successfully, attention should be paid to the correct selection of a number of variables. These include (apart from the choice of support matrix, spacer and ligand which have been discussed above) column dimensions[145], flow-rate[6], sample size in relation to column capacity[145], sample buffer, pH and ionic strength[146] and temperature[147].

In our hands, the operation of triazine dye columns does not differ very widely from similar separations carried out using immobilised nucleotides. However, several points can be made to underline some important differences.

6.1.1. pH. The correct choice of adsorption pH is essential. Proteins tend to bind to triazine dyes more tightly at lower pH values, presumably because of a contribution from ionic interactions favoured by increasing positive charges on interacting protein molecules. However, this change in binding is not necessarily determined by the p*I* of each protein. Thus the p*I*s of emergent proteins were not related to the elution of plasma proteins from Cibacron blue–agarose[148]. Conversely, the desorption pH can be critical: gradients to higher pH values are successful in separating contaminating yeast proteins from 6-phosphogluconate dehydrogenase on Procion red HE 3B-agarose[149].

6.1.2. Ligand concentration. Careful adjustment of the triazine dye ligand concentration can result in useful alterations in the purification factors achieved with these columns.

This adjustment can be made (a) uniformly *i.e.* each bead has the same ligand concentration or (b) with unsubstituted beads[50].

The application of the methods described in (a) and (b) should be considered when a protein binds more tightly than is desirable. Instances where glucose-6-phosphate dehydrogenase has bound apparently irreversibly have been resolved by altering the adsorption pH to higher values, by lowering the ligand concentration or changing the support matrix from agarose (6%) to crosslinked dextran (G-200 Sephadex)[120]. Electrophoretic desorption may also resolve this problem[6].

6.1.3. Choice of eluent. KCl or nucleotides have been widely used to selectively elute enzymes from immobilised nucleotides. Indeed one of the advantages of the latter would seem to be the wide range of different elution procedures available[140]. In our hands, nucleotides such as NADP are not particularly successful as eluents in the purification of various dehydrogenases from sheep liver, yeast, *B.stearothermophilus*, *A.pseudoplatanus* L. Either KCl or pH elution seem to produce sharper peaks of higher specific activity[120,121,149,155]. Although these observations have been made both with Cibacron blue F3GA and Procion red HE 3B columns it is probably best to investigate each application separately.

6.1.4. Temperature. Few studies have examined the effects of adsorption and desorption temperature in affinity processes[147,151]. The salt-dependent binding of a thermophilic enzyme (6-phosphogluconate dehydrogenase from *B.stearothermophilus*) has been compared with that of the same enzyme from a mesophile (*S.cereviseae*)[121]. The salt concentration required to elute both enzymes from either Cibacron blue F3GA or Procion red HE 3B columns increased with increasing temperature up to 45°. Whilst this effect was observed for thermophilic enzymes on AMP–agarose[151] the opposite was found for mesophilic enzymes[147].

6.2. *Increasing protein binding*

Often all that is needed is an increase in ligand concentration: this may require a change of support matrix (4–6% agarose) or the use of a different type of agarose (Ref. 150 and Table 4).

Lowering the pH of adsorption has also been found to succeed in increasing protein binding[148]. Raising the temperature favours an increase in binding in our experience[121].

Finally, increasing the applied enzyme concentration has been shown to give greater retardation[152].

7. SCOPE OF APPLICATIONS AND CHOICE OF DYE

At present the majority of applications of triazine dyes have involved Cibacron blue F3GA because of its early availability. The wide variety of applications is reflected in the number of papers referred to in Table 1. These include the resolution (a) of isoenzymes[28,59,61,90,144], (b) of subunits of protein aggregates[51,52], (c) of wild type from mutant enzymes[6,18,70] and (d) of enzymes from nucleotide enzyme complexes[17]. Many straightforward protein purifications have been described

and include even multienzyme complexes[45,46,89]. Purification factors for triazine dye steps in isolation procedures are often in excess of ten-fold and may be in excess of fifty-fold[5,14,15,22,31,41,56,60,78,87,111,119,144].

Preliminary comparisons of Cibacron blue and Procion red would suggest that the former is better suited to the purification of NAD^+-dependent dehydrogenases whilst the latter is more selective for $NADP^+$-linked enzymes[6,10,121,155]. This generalisation is likely to be a guideline worth following although many proteins other than dehydrogenases are retarded by immobilised Cibacron blue F3GA and Procion red HE 3B (Table 1 Kinases and Other proteins). We have found it beneficial to run columns of both types[121] during the purification of any one dehydrogenase.

There are many other triazine dyes besides the two discussed above. Screening programmes are already under way in several laboratories to attempt to find useful applications for these dyes.

In addition to the triazine dyes, two recent dyes have been described for base-specific fractionation of nucleic acids[153,154]. These are: A-T specific malachite green and G-C specific phenyl neutral red. A comparison of these dyes with the triazine dyes already mentioned suggests that useful triazine dyes for the future might contain positively charged substituents in place of negative sulphonate groups (see ref. 167).

8. DISADVANTAGES

Many triazine dye preparations are multicomponent and although fillers and minor impurities are removable this aspect has received scant attention.

9. ADVANTAGES

9.1. Stability

Triazine linkages are less prone to ligand leakage than cyanogen bromide-activated polysaccharides. Thus unlike the latter, triazine dye columns can be operated in glycine, Tris or ethanolamine buffers (which can displace ligands from cyanogen bromide-activated supports by nucleophilic attack).

9.2. Uniformity

At commonly used cyanogen bromide concentrations it has been shown that ligands are probably unsymmetrically distributed in agarose beads[155]. The same has not been proven for triazine-linked systems and if prior assembly of the triazine-ligand is carried out, cross-linking should not occur and the problem of asymmetric coupling therefore unlikely to be encountered. Furthermore one can avoid the hazards of cyanogen bromide activation.

9.3. Ease of preparation

The widespread availability of many triazine dyes and their ease and speed of coupling is a major advantage when comparing these ligands with defined nucleotide affinity adsorbents. The low cost of these dyes is also a major consideration when designing large scale systems[6,30].

9.4. Capacity

The binding capacities for proteins of triazine dye columns is far higher than the values obtained for immobilised nucleotides[4,5,6,30,31].

9.5. Special properties

Apart from the facile identification of dye columns from their colour, the red shift which is observed when proteins adsorb to triazine dye columns, is a potentially useful indicator of a successful choice of adsorption conditions and in addition can be used to examine the interaction between protein and immobilized dye (using wavelengths that are well away from absorbances associated with the protein, linkage and matrix).

9.6. Choice of eluent

Triazine dye columns are more effectively eluted with salt or pH changes which are cheaper than with substrates and hence could be important for larger scale separations.

9.7. Ease of storage

Less care is needed to prevent ligand leakage. No evidence of bacterial or other contamination has been observed when dye-agarose columns are stored in the cold room for two years[4,8,31].

9.8. Selectivity and usefulness of triazine chemistry

The rates of reaction of the three chlorine atoms in *s*-trichlorotriazine (cyanuric chloride) are markedly different[125,167]. This enables one to produce precisely coupled ligand assemblies.

10. OPTIMIZED COUPLING PROCEDURES

10.1. Monochlorotriazine dyes (e.g. Cibacron blue, Procion red HE 3B and Procion H dyes in general)

Agarose (either Matrex gel or Sepharose 6B; 20 g moist weight) is suspended in water (70 ml) and a solution of the dye (200 mg) in water (20 ml) is added. The mixture is placed in a rotary mixer (Coulter) for 5 min after which 20%(w/v) sodium chloride (10 ml) is added*. Mixing is continued for 30 min at room temperature. A solution of 5 *M* NaOH or 1 *M* Na_2CO_2 (0.5 ml) is added**. (Any other base may be used to elevate the pH at this stage providing it does not contain nucleophiles such as amino groups[167].) After the addition of base the mixture is incubated for three days, filtered and extensively washed with water, 1 *M* sodium chloride, 4–8 *M* urea and water.

10.2. Dichlorotriazines (Procion MX series)

The above procedure is exactly repeated except that the final incubation is for 1 h at room temperature. The washing procedure is the same.

* This salt step is essential to "salt" the dye into the matrix; as a result faster reaction times result and less hydrolysis of the triazine moiety occurs[167].

** NaOH tends to lead to higher ligand concentrations[167].

11. ACKNOWLEDGEMENTS

The authors would like to thank Drs A. Atkinson and V. Stead for stimulating discussions, ICI (Organics division) for generous gifts of dyes. Professor E. Stellwagen for making unpublished data available.

12. SUMMARY

This review attempts to identify proteins which selectively interact with immobilised triazine dyes such as Cibacron blue F3GA and Procion red HE 3B. Different support matrices are compared by examining the capacities of these dyes for proteins. Various approaches to the immobilisation of triazine dyes are considered together with the use of spacers.

Some theories of the mechanism of protein retardation by immobilised dyes are discussed. A number of methods are suggested for the measurement of dye concentrations and for the modification of the binding of proteins to dye columns. The variety of elution methods is compared with a view to optimizing purifications. The scope of applications is reviewed as well as the choice of dye. Some advantages of triazine dyes over other affinity ligands are given.

It is concluded that although no satisfactory mechanism for the binding of triazine dyes to proteins has yet been proposed, these dyes possess considerable potential for protein purification, particularly when applied on the large scale.

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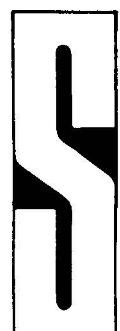

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