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DEGRADATION OF HIGH MOLECULAR WEIGHT POLYSTYRENES DURING THE SEC SEPARATION PROCESS, AS DEMONSTRATED BY SEC COUPLED WITH LALLS AND BY STATIC LIGHT SCATTERING

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ABSTRACT

In order to investigate the phenomenon of polymer degradation during the SEC separation process, we compared the weight-average molecular weights of a series of polystyrene (PS) samples with M_w going from 2.3×10^3 to 1.84×10^7, which were determined by static light scattering (LS) and by SEC-LALLS using various crosslinked PS gels.

For all the gels considered, there was a good agreement between LS and SEC-LALLS values for M_w up to $2x10^6$. For higher M_w, the values given by SEC-LALLS were lower than those given by LS; the decrease in M_w was different in various SEC columns. Since the same chromatographic equipment was used for all the SEC-LALLS measurements, we presumed that the decrease in M_w reflected the degree of shear degradation of PS molecules during their elution through the SEC columns. It depended on the origins of gels, the size of gel particles, the porosity of frits at column ends, the eluent flow rate, the usage of a pre-column filter, and of the third detector, a capillary viscometer.

INTRODUCTION

Size exclusion chromatography (SEC) is a widely used technique for determining the molecular weight (MW) averages of polymers and their molecular weight distributions (MWD), which are very important characteristics to understand the physical, rheological and mechanical properties of polymeric materials. These parameters are obtained with SEC, coupled either with a concentration detector alone (a differential refractometer or a UV spectrophotometer) or also with a molecular weight detector (a light scattering photometer).

In the former case, the columns have to be calibrated with monodisperse polymer standards, most often polystyrenes (PS) with the MW range going from 500 to $1x10^7$.[1-3] Owing to the differences in chemical nature between the samples and PS standards, the calculated MW averages are only relative values. In addition, the calibration curves of log M versus retention volume (V) are often non-linear in the region of high molecular weights (HMW). This may be a consequence of the reduced ability of gels in the SEC columns to separate large molecules effectively, or, as it was shown by some authors, it may be due to a shear degradation of HMW-PS molecules occurring during the SEC separation process.[4-14]

The degradation of some other polymers (polyisobutylene, polyethylene, polyisoprene, polymethyl methacrylate and others) during the SEC analysis was also reported.[7,11,12,15-18] With the introduction of high-performance gels with lower particle sizes, special attention should be turned to the SEC analysis of HMW polymers. Shear degradation during SEC depends on a variety of parameters: shear rate, elongation strain rate, the nature of the solvent, and the chemical nature and concentration of the polymer. Besides, the tight parts of a

chromatographic system (capillary tubing, sample loops, column frits) may generate shear forces contributing to polymer degradation. Some other parameters, such as concentration effects, increased peak dispersion, and ultrafiltration of high molecular weight molecules may also contribute to decrease in MW.[11-14]

The aim of this work was to investigate the phenomenon of PS degradation during the SEC separation process on various crosslinked PS gels with tetrahydrofuran (THF) as eluent. For this purpose, size exclusion chromatography (SEC), coupled with a low-angle laser light scattering (LALLS) photometer was used because it allowed the determination of the absolute weight-average molecular weights (M_w) of PS standards. The following common experimental parameters, which could have an influence on the degree of degradation, were considered: the origins of gels (from various manufacturers), the size of gel particles, the porosity of frits at column ends, the eluent flow rate, the usage of a pre-column filter and of the third detector - a capillary viscometer. To evaluate the degradation degree of a series of PS, we compared the M_w obtained by SEC-LALLS to the M_w determined by static light scattering (LS).

MATERIALS AND METHODS

For this study, monodisperse polystyrenes (PS) with molecular weights going from $2.3x10^3$ to $1x84{\cdot}10^7$ were used (Table 1). PS were of different origins: some of them were synthesized by anionic polymerization at the Institute Charles Sadron, and others were from Waters Associates and Polymer Laboratories.

The refractive index increment of PS in THF (dn/dc=0.186 mL/g) was determined by using a Brice-Phoenix differential refractometer at the same wavelength (632.8 nm) as used with the LALLS photometer.

The LS measurements were performed with a laser light scattering photometer SEM 633 (λ=632,8 nm). Most samples for LS measurements were purified by centrifugation except for the three PS with the highest molecular weights, which were purified by filtration.

The SEC-LALLS measurements were performed on a Waters 150C chromatograph, coupled with two or three detectors in series, a home-made continuous viscometer (CVM),[19] a low-angle laser light scattering (LALLS) photometer Chromatix CMX-100, and a standard Waters differential refractometer (DR); a filter with a pore size of 2 μm was placed at the inlet of a

Table 1

Weight-Average Molecular Weights of Polystyrene Standards Determined by Static Light Scattering (LS) and by Size Exclusion Chromatography Coupled with a Low-Angle Laser Light Scattering Photometer and a Differential Refractometer (SEC-LALLS)

Polystyrene M_w (LS)	Columns A*	Columns B*	Columns C*	Columns D*	Columns E**
$2.3x10^3$	$2.6x10^3$	$2.4x10^3$	$2.4x10^3$	$2.4x10^3$	---
$4.0x10^4$	$4.1x10^4$	$4.2x10^4$	$4.2x10^4$	$4.2x10^4$	---
$9.8x10^4$	$9.8x10^4$	$9.9x10^4$	---	$1.0x10^5$	---
$4.7x10^5$	$4.5x10^5$	$4.5x10^5$	$4.3x10^5$	$4.5x10^5$	---
$6.7x10^5$	$6.5x10^5$	$6.6x10^5$	$6.8x10^5$	$6.6x10^5$	---
$2.7x10^6$	$2.7x10^6$	$2.5x10^6$	$2.3x10^6$	$2.6x10^6$	---
		$2.5x10^6$**	$2.6x10^6$**		
$3.2x10^6$	---	---	---	---	$2.8x10^6$
$3.8x10^6$	$3.4x10^6$	$3.0x10^6$	---	$3.6x10^6$	---
$5.8x10^6$	$5.8x10^6$	$3.3x10^6$	$2.1x10^6$	$6.1x10^6$	---
		$3.6x10^6$**	$5.1x10^6$**		
$6.8x10^6$	$5.0x10^6$	$3.0x10^6$	$2.3x10^6$	$6.0x10^6$	$4.1x10^6$
		$3.8x10^6$**	$4.4x10^6$**		
$8.4x10^6$	---	---	---	$3.3x10^6$**	$4.5x10^6$
$1.47x10^7$	---	---	---	$2.9x10^6$**	$3.8x10^6$
$1.84x10^7$	---	---	---	---	$4.1x10^6$

* With a pre-column filter with a pore size of 2 μm, placed between an injector and the SEC columns.

** Without a pre-column filter.

LALLS photometer to prevent spikes from micro particles possibly present in the eluent. The mobile phase was THF with flow rates going from 0.4 to 2.3 mL/min at 25°C; a flow rate of 1.0 mL/min was used regularly. The concentrations of PS samples in THF varied with MW, and for HMW-PS with MW over 10^6 the latter was 3 - $1x10^{-3}$ g/mL; the injection volume was 100 μL. THF was distilled over sodium wire and filtered over a Millipore filter FGLP with pore size 0.2 μm. Five sets of SEC columns with the crosslinked PS gel of various manufacturers were used; details are described in Table 2. All the columns were tested for the possible adsorption of HMW-PS on gel particles

Table 2

Characteristics of SEC Columns

Columns	Manufacturer	Set of Columns	Particle Size, μm	Porosity of Column Frits, μm
A*	I	10^6, 10^5, 10^4, 10^3	35-45	10
B*	I	10^6, 10^5, 10^4, 10^3	≈10	2
C*	I	10^6, 10^5, 10^4, 10^3	<10	5
D*	II	10^6, 10^5, 10^4, 10^3	8	3
E	III	10^6, 10^5, 10^4, 10^3, $5x10^2$	10	5

* A filter with a pore size of 2μm was placed between an injector and the SEC columns. I - Waters Assoc.; II - Showa Denko; III - Polymer Laboratories. **A** - Styragel; **B** - μ-Styragel; **C** - Ultrastyragel; **D** - Shodex (A-800 series); **E** - Plgel.

by comparing the injected amounts and corresponding areas under DR curves of HMW-PS and PS with low M_w (123,000), which did not degrade during the SEC separation. Differences were of the order of experimental error and did not exceed ±3%.

RESULTS AND DISCUSSION

Since laboratories running the routine SEC characterization of polymers have been usually employing sets of columns covering a broad range of MW, the sets chosen for the present study consisted of four or five columns in series with comparable exclusion limits and MW working range. The columns differed in gel particle size and in the porosity of frits at the column ends, the biggest being for columns *A* (Table 2); they appeared first on the market and are very rarely used for routine SEC analyses at the present time.

According to gel particle size and to frit pore size, columns *C* and *E* could be classified in one group (10 μm, 5 μm), and columns *B* in the other (10 μm, 2 μm). Columns *D* differed from the previous ones, both in gel particle size and in frit pore size (8 μm, 3 μm). Besides, the sets of columns *A*, *B*, *C*, and *D* were used with a pre-column filter with pore size 2 μm.

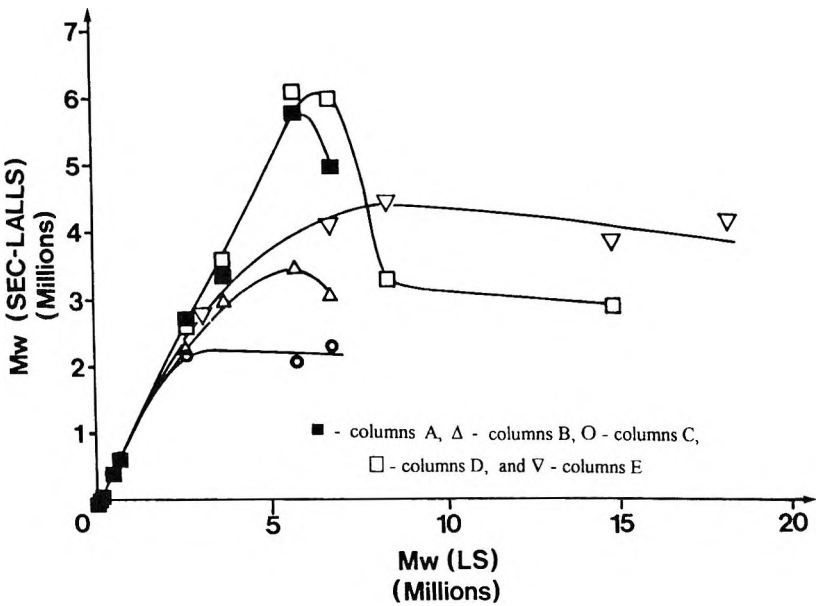

Figure 1. Correlation between the weight-average molecular weights of polystyrene standards determined by LS and SEC-LALLS in THF, 1mL/min; ■ - columns A, Δ - columns B, ○ - columns C, □ - columns D, and ∇ - columns E.

The M_w values of PS standards obtained by LS and SEC-LALLS with various sets of columns are listed in Table 1. We can see that, for PS with M_w up to $2x10^6$, agreement between the two M_w values is very good irrespective of the type of column. Differences become evident especially for PS with M_w over $2x10^6$, where the type of column plays an important role.

The correlation between $M_{w,LS}$ and $M_{w,SEC\text{-}LALLS}$ on different sets of columns are presented in Figure 1, while the decrease in M_w in the MW region of over $6.7x10^5$ is shown in Figure 2. The decrease in M_w is given as the percent ratio of the two M_w values, of $M_{w,SEC\text{-}LALLS}$ (after SEC separation) to $M_{w,LS}$ (without SEC separation).

On all types of columns, no degradation has been observed below $M_w = 2x10^6$, even in the presence of a pre-column filter. Above this value, and with a pre-column filter, it proceeds more rapidly on columns *C* (for $M_w = 6.8x10^6$, $M_{w,SEC\text{-}LALLS}$ is 34% of $M_{w,LS}$) than on columns *B*. Without a pre-column filter, the degradation is stronger on columns *B* with a low frit pore size (2 μm) and comparable for columns *C* and *E* with the same frit pore size (5 μm).

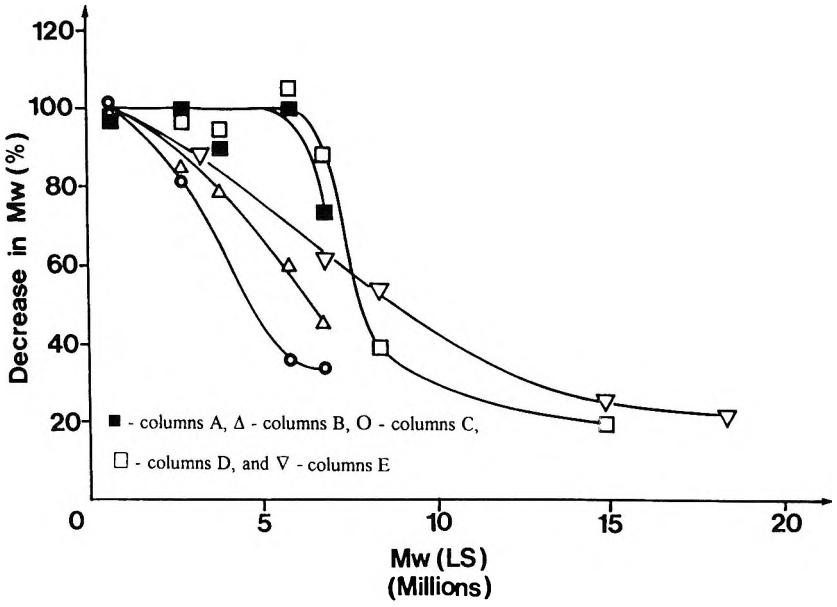

Figure 2. Decrease in M_w of polystyrene standards during the SEC separation process in THF, 1mL/min; ■ - columns A, Δ - columns B, ○ - columns C, □ - columns D, and ∇ - columns E.

The observations are quite different for the sets of columns *A* and *D*, where degradation starts only at very high M_w, around $6x10^6$, and increases with increasing M_w: in the case of the set of columns *D*, for $M_w = 8.4x10^6$, $M_{w,SEC\text{-}LALLS}$ is 39% of $M_{w,LS}$, and for $M_w = 1.47x10^7$, $M_{w,SEC\text{-}LALLS}$ is 20% of $M_{w,LS}$.

This high critical M_w for columns *D* is rather unexpected since they have the smallest particle size among all the columns used (8 μm) and a small frit pore size (3 μm). On the contrary, a high critical M_w could be anticipated for columns *A*,[4,7] since they have the biggest particle and frit pore size (35-45 μm, 10 μm).

Our findings agree well with the data reported in the literature. Some data were obtained by SEC-LALLS[8,10,11] and some of them by SEC with common PS calibration and/or by viscometric or LS measurements of PS samples before and after the SEC separation.[4,6,7] The critical M_w for the PS degradation on the columns of type *A* was estimated to be $1x10^7$,[4,7] and on the columns of type *B* lower than $8x10^6$.[7] However, these results were obtained on

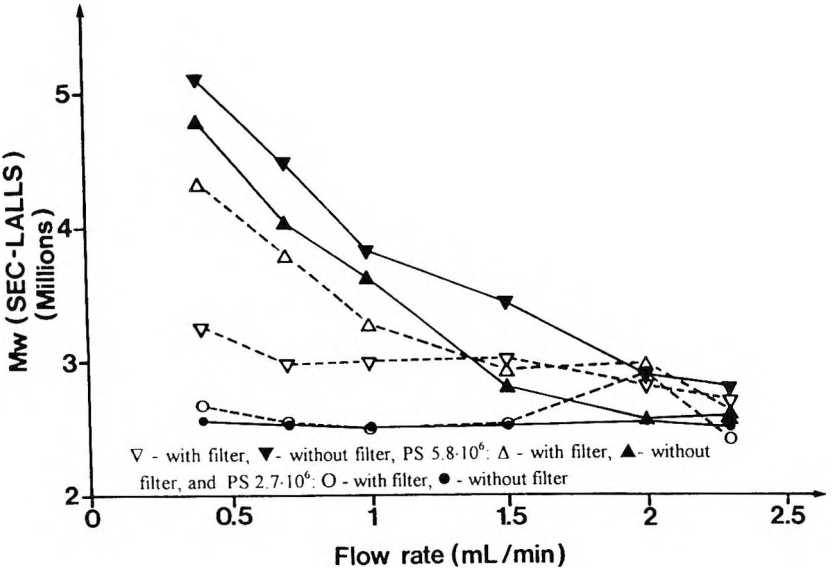

Figure 3. M_w (SEC-LALLS) of polystyrene standards as a function of flow rate, columns *B;* PS 6.8·10^6: ∇ - with filter, ▼- without filter, PS 5.8·10^6: Δ - with filter, ▲- without filter, and PS 2.7·10^6: ○ - with filter, ● - without filter.

5- or 7-column sets covering a high MW working range (from 5x10^4 to 5x10^6) and without using a pre-column filter. Our critical M_w for the PS degradation on columns *D* (6x10^6) can be only compared to the similar value obtained on a mixed bed column from the same manufacturer.[6]

Regarding the columns of type *E*, V. V. Guryanova et al.[10] observed on the series of columns 10^4, 10^5 and 10^6 Å in chloroform no degradation of PS 3.3x10^6, but a severe degradation of PS 6.6x10^6; the decrease in M_w was much higher than in our case in THF ($M_{w,SEC\text{-}LALLS}$ in $CHCl_3$ is only 27% of $M_{w,LALLS}$) and did not change after reducing the flow rate from 1.0 mL/min to 0.2 mL/min. The findings of McIntyre et al.[7] may explain this discrepancy in the degradation degree of PS 6.6x10^6: they have shown that in a non-swelling solvent for the packing the degradation is enhanced because of the lower pore diameters of gel particles.

We have also examined the effects of flow rate on PS degradation. For this purpose, three HMW-PS and the sets of columns *B* and *C* were selected; results are summarized in Tables 3 and 4 and shown in Figures 3 and 4.

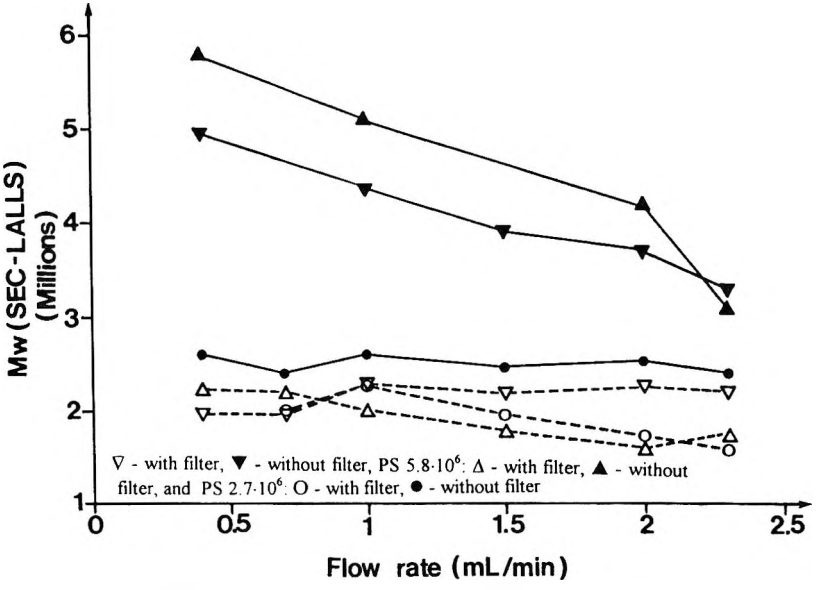

Figure 4. M_w (SEC-LALLS) of polystyrene standards as a function of flow rate, columns *C*; PS 6.8·10⁶: ∇ - with filter, ▼ - without filter, PS 5.8·10⁶: Δ - with filter, ▲ - without filter, and PS 2.7·10⁶: ○ - with filter, ● - without filter.

Table 3

Weight-Average Molecular Weights of Three Polystyrene Standards Determined by SEC-LALLS as a Function of Flow Rate*

Flow Rate	----- 10^{-6} x M_w (SEC-LALLS) -----					
	PS 2.7x10⁶		PS 5.8x10⁶		PS 6.8x10⁶	
mL/min	Without	With	Without	With	Without	With
0.4	2.56	2.67	4.79	4.32	5.10	3.25
0.7	2.52	2.54	4.04	3.79	4.47	2.97
1.0	2.51	2.49	3.62	3.27	3.82	2.98
1.5	2.51	2.53	2.80	2.92	3.43	3.00
2.0	2.54	2.90	2.56	2.97	2.88	2.80
2.3	2.50	2.42	2.60	2.62	2.79	2.70

* Columns B, with or without a pre-column filter (2μm).

Table 4

Weight Average Molecular Weights of Three Polystyrene Standards Determined by SEC-LALLS as a Function of Flow Rate*

Flow Rate	----- 10^{-6} x M_w (SEC-LALLS) -----					
	PS $2.7x10^6$		PS $5.8x10^6$		PS $6.8x10^6$	
mL/min	Without	With	Without	With	Without	With
0.4	2.60	---	5.8	2.23	4.95	1.96
0.7	2.40	2.00	---	2.19	---	1.95
1.0	2.60	2.26	5.1	2.10	4.35	2.28
1.5	2.46	1.96	---	1.78	3.90	2.18
2.0	2.53	1.73	4.2	1.60	3.7	2.25
2.3	2.40	1.58	3.1	1.76	3.3	2.20

* Columns C, with or without a pre-column filter (2 μm).

Once again, the difference in performance of the two types of columns is quite evident. Without a pre-column filter, $M_{w,SEC\text{-}LALLS}$ of PS $6.8x10^6$ and PS $5.8x10^6$ decrease continuously with increased flow rate for both sets of columns, but, for columns *C*, the M_w values of PS $6.8x10^6$ are lower than those of PS $5.8x10^6$, which indicates a stronger degradation of PS chains higher than $6.0x10^6$. With a pre-column filter, the degradation is stronger on columns *C* and practically does not change with flow rate.

On columns *B*, the same observation is valid for PS $6.8x10^6$, while M_w of PS $5.8x10^6$ gradually decreases with the flow rate up to 1.5 mL/min. For PS $2.7x10^6$, the increase in flow rate and the use of a pre-column filter do not affect M_w on columns *B*, while on the *C* columns, M_w is lower with a pre-column filter.

The change in flow rate is related to the shear rate in columns and, at higher flow rates, it contributes to the stronger degradation of HMW polymers, which is also demonstrated by our results. Moreover, the influence of a pre-column filter on PS degradation is surprisingly great for MW over $3x10^6$, which indicates a noticeable contribution of the pre-column filter to the increase in shear rate.

Finally, we examined how the capillary viscometer (CVM) affected the degradation of HMW-PS with M_w over $8.4x10^6$ using the set of columns *E* (Table 5). Owing to viscometer design (capillary and coil diameter),

Table 5

Weight-Average Molecular Weights of Three Polystyrene Standards Determined by LS, SEC-LALLS, and SEC-CVM, LALLS*

Polystyrene M_w (LS)	10^{-6} x M_w SEC-LALLS	SEC-CVM, LALLS
$8.4x10^6$	4.5	3.1
$1.47x10^7$	3.8	2.6
$1.84x10^7$	4.1	4.3

* Columns E without a pre-column filter

degradation might be expected for polymers with very high MW.[20] Actually, when we used SEC coupled with the additional third detector (CVM), the degradation degrees of PS $8.4x10^6$ and PS $1.47x10^7$ were higher as compared to SEC-LALLS measurements. For PS $1.84x10^7$ with the highest M_w, the degradation degree did not increase after coupling SEC with the third detector. It appears that the degradation was already very strong when only two detectors were used ($M_{w,SEC\text{-}LALLS}$ is approx. 20% of $M_{w,LS}$), and, for this reason, the effect of the third one could not be observed.

CONCLUSIONS

The phenomenon of high molecular weight PS degradation during the SEC separation process was studied on various SEC columns with the crosslinked PS gel packings. The weight-average molecular weights (M_w) for a series of monodisperse PS in THF were first determined by static light scattering (LS) and then, after the SEC separation, by SEC-LALLS. Critical M_w and degradation degree depended primarily on the gel origin; however, they were also influenced by the size of gel particles, by the porosity of column frits, and by common experimental parameters: the eluent flow rate, the use of a pre-column filter and of the capillary viscometer.

The authors did not intend to distinguish between "good" or "bad" SEC columns; they simply wanted to demonstrate the performance of several types of SEC columns in the high molecular weight region. They also wished to direct the attention of polymer analysts to the fact that, in order to obtain accurate molecular weight averages by SEC, it is of utmost importance

to consider the possible degradation of high molecular weight polymer standards and samples on the chosen sets of columns, and to take into account the experimental parameters used for the particular SEC analysis.

REFERENCES

1. W. W. Yau, J. J. Kirkland, D. D. Bly, "Size-Exclusion Liquid Chromatography" in **High Performance Liquid Chromatography**, P. R. Brown, R. A. Hartwick, eds., John Wiley & Sons, Inc., New York, 1988, pp. 277-316.

2. M. G. Styring, A. E. Hamielec, "Determination of Molecular Weight Distribution by Gel Permeation Chromatography", in **Determination of Molecular Weight**, A. R. Cooper, ed., John Wiley & Sons, Inc., New York, 1989, pp. 263-300.

3. S. T. Balke, "Characterization of Complex Polymers by Size Exclusion Chromatography and High-Performance Liquid Chromatography," and G. R. Meira, "Data Reduction in Size Exclusion Chromatography of Polymers," in **Modern Methods of Polymer Characterization**, H. G. Barth, J. W. Mays, eds., John Wiley & Sons, Inc., New York, 1991, pp. 1-66 and 67-101.

4. E. L. Slagowski, L. J. Fetters, D. McIntyre, Macromolecules, 7, 394-396 (1974).

5. W. G. Rand, A. K. Mukherji, J. Polym. Sci.: Polym. Lett. Ed., **20**, 501-508 (1982).

6. Y. Mei-Ling, S. Liang-He, J. Liq. Chromatogr., **5**, 1259-1267 (1982).

7. D. McIntyre, A. L. Shih, J. Savoca, R. Seeger, A. MacArthur, ACS Symp. Ser., 245, 227-240 (1984).

8. Z. Grubišić-Gallot, Australian Polymer Symposium, Ballarat, 1984.

9. E. V. Chubarova, V. V. Nesterov, J. Liq. Chromatogr., **13**, 1825-1847 (1990).

10. V. V. Guryanova, N. G. Podosenova, A. V. Sysoev, P. E. Ilmenev, O. M. Karanyan, A. G. Morozov, I. A. Gritskova, A. V. Pavlov, Vysokomol. Soedin., **35**, 1341-1344 (1993).

11. J. G. Rooney, G. Ver Strate, "On Line Determination by Light Scattering of Mechanical Degradation in the GPC Process", in **Liquid Chromatography of Polymers and Related Materials III**, J. Cazes, ed., Marcel Dekker, New York, 1981, pp. 207-235.

12. J. C. Giddings, Adv. Chromatogr., **20**, 217-258 (1982).

13. H. G. Barth, F. J. Carlin, Jr., J. Liq. Chromatogr., 7, 1717-1738 (1984).

14. H. G. Barth, ACS Symp. Ser., **352**, 29-46 (1987).

15. C. Huber, K. H. Lederer, J. Polym. Sci.: Polym. Lett. Ed., **18**, 535-540 (1980).

16. V. Grinshpun, K. F. O'Driscoll, A. Rudin, J. Appl. Polym. Sci., **29**, 1071-1077 (1984).

17. A. W. deGroot, W. J. Hamre, J. Chromatogr., **648**, 33-39 (1993).

18. V. V. Nesterov, E. V. Chubarova, B. G. Belenkii, Vysokomol. Soedin., **31**, 653-657 (1989).

19. A. Halbwachs, Z. Grubišić-Gallot, Macromol. Chem., Rapid Commun., **7**, 709-712 (1986).

20. J. Lesec, D. Lecacheux, G. Marot, J. Liq. Chromatogr., **11**, 2571-2591 (1988).

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POLYDISPERSITY STUDY OF NARROW POLYSTYRENE STANDARDS USING LIGHT SCATTERING

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ABSTRACT

Manufacturers of narrow polystyrene standards for use as column calibrants with size exclusion chromatography (SEC) often label the corresponding polydispersity (M_w/M_n) as $< \sim 1.05$. These values appear to reflect more the errors associated with the measurement than the true narrow polydispersity. Ideally, we would measure polydispersity by a light scattering measurement of M_w and a membrane osmometry measurement of M_w. The ratio of such measurements would be expected to have an error on the order of 5%– a number comparable to the difference of the measured polydispersity and unity! Multi-angle light scattering (MALS) allows the absolute determination of the rms radius independent of the separation technique and any other detector.

Using this information and following the method of Shortt,[1] column band broadening effects may be corrected and a far more precise determination of polydispersity made. A variety of standards from several manufacturers were measured, and the polydispersity of each was determined to be far smaller than stated, further confirming their suitability for column calibration purposes.

INTRODUCTION

Polystyrene standards have been used for column calibration in traditional size exclusion chromatography (SEC). A calibration curve is constructed by plotting the logarithm (base 10) of the peak molar mass (MM) of each selected standard against its corresponding elution volume. A curve is then fit to the data. This so-called "calibration" curve, is usually fit to a polynomial in elution volume, V, of order 3 to 5 and sometimes higher. The linear portion of such a curve for a typical column set is shown in Fig. 1.

Figure 2 shows the differential refractive index (DRI) detector chromatogram of a narrow 200K MM standard. Were we to compare each fraction against the calibration curve, we would seem to have a distribution of masses present no matter how narrow the standard actually was. Such broadening is due almost entirely to band broadening in the column. If we could correct adequately for this broadening, we should be able to calculate the actual sample polydispersity.

In a 1970 experiment which showed an improved resolution of a SEC column following a recycled injection, Waters[2] showed that on each pass of a sample through the column, in the limit of an infinite number of passes, the true MM variance could be calculated. For his sample, he obtained a polydispersity on the order of 1.002, considerably smaller than previously estimated. However, the recycle technique is time consuming and has been rarely used since then.

A great amount of theoretical speculation concerning means to correct for such broadening has filled the literature since Waters' experiments, but more recently, with the advent of multi-angle light scattering (MALS) combined with SEC,[3] a new experimental approach has been developed that, for many examples, may obviate the need to rely upon such sketchy theoretical approaches.

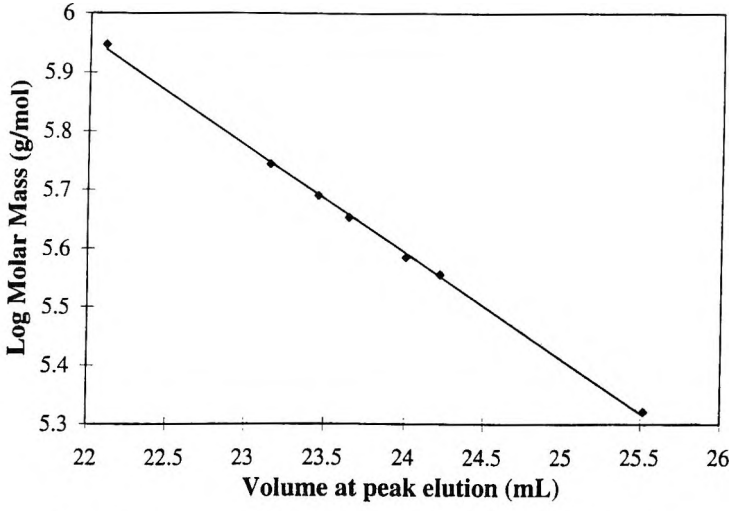

Figure 1. Typical SEC calibration curve in the vicinity of its linear behavior.

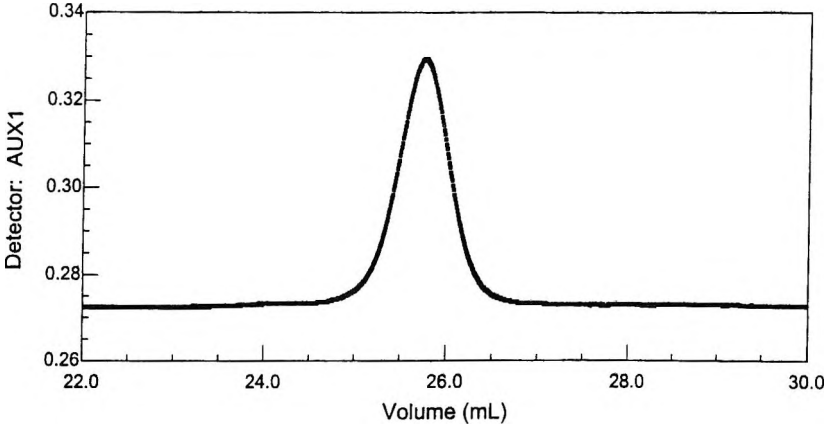

Figure 2. 200K narrow standard differential refractive index chromatogram.

Using MALS combined with SEC, it has become possible to measure MM directly without appeal to a calibration curve. In a recent paper by Wyatt[4] it was pointed out that in the limit of vanishingly small concentrations, the molecular root mean square (rms) radii, r_g can be determined directly from

light scattering measurements for homopolymers independent of any concentration data. Appealing to the nearly flat variation of r_g versus elution volume, the paper stated that the standards must be much less polydisperse than currently estimated since the real MM must be directly proportional to some function of r_g and, therefore, should show a similar behavior with elution volume. The remaining question was, "Can this rms radius information be used to calculate more precisely the actual molar mass distribution?" A recent article by Shortt[1] showed how this could be achieved.

MEASUREMENT OF TRUE POLYDISPERSITY

The ideal determination of a sample's polydispersity would consist of measuring the weight average MM using light scattering and the number average MM using membrane osmometry. The polydispersity is defined as the ratio of these values, or

$$\text{Polydispersity} = \frac{M_w}{M_n}$$

Even under ideal conditions, the errors associated with M_w and M_n will be, at best, on the order of 2% and 4%, respectively. Accordingly, the error of the calculated polydispersities of a "narrow standard" based on such direct measurement will itself be comparable to the traditional manufacturer's stated deviation of the sample's polydispersity from unity, *viz.*, about 5%.

Shortt's paper, referenced above, described a new technique for determining M_w/M_n using a method based upon measurement of r_g from MALS coupled with SEC. This is the technique we follow in the present study to determine the polydispersity of various narrow polystyrene standards.

In his paper, Shortt assumed, at least for the central part of the band broadened peak, that the peak had a gaussian distribution. On this basis, he then showed that the polydispersity is given approximately by

$$\frac{M_w}{M_n} \cong 1 + 0.96B^2 w_0^2 \qquad (1)$$

where B is the slope of the calibration curve in the linear region of the column set, *i.e.* where

$$\log_{10} M = A + BV \qquad (2)$$

Table 1

Reported Polydispersities of Various Standards

Manufacturer	Nominal Molar Mass (g/mol)	Reported Polydispersity
Pressure Chemical	2000,000	≤1.05
Pressure Chemcial	400,000	≤1.06
Pressure Chemical	575,000	≤1.06
Pressure Chemical	900,000	≤1.06
Polymer Labs	350,000	≤1.07
TSK	422,000	≤1.04
PSS	455,000	≤1.04

A is a constant, and w_0 is the full width at half maximum (FWHM) of the unbroadened peak. As it is impossible to measure this value directly, we examine the r_g calibration curve which, as mentioned earlier, may be established independent of a secondary detector. For r_g we have a relation similar to Eq. (2) involving the constants C and D,

$$\log_{10} r_g = C + DV \tag{3}$$

The slope D is related to the observed slope of the specific sample, D_{obs}, by the relation

$$D_{obs} = Dw_0^2/w_{obs}^2 \tag{4}$$

where w_{obs} is the measured broadened FWHM, w_0 is the unbroadened FWHM as before, D is the measured slope of the r_g calibration curve using the peak radius value for each standard. The observed slope of the r_g values across the peak for a given sample is D_{obs}. Equation (4) can then be solved to obtain a value for w_0, which can be substituted into Eq. (1) to obtain the true value of the polydispersity.

EXPERIMENTAL

Data were collected for various standards (the standards used as well as the values of their polydispersities as reported by the manufacturers are presented in Table 1) from a SEC/MALS system. The SEC system consisted of

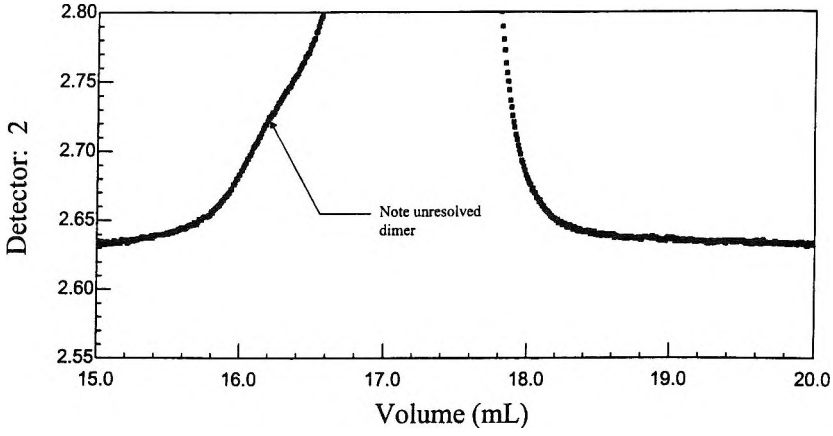

Figure 3. 200K polystyrene standard as separated by two columns (of 10^4Å and 10^6Å pore size). These columns were unable to resolve the dimer from the main peak.

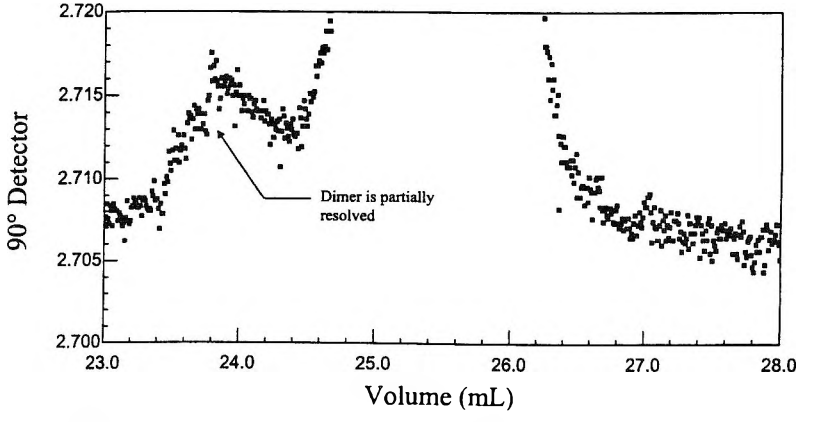

Figure 4. 200 K Polystyrene standard as separated by 3 columns (one additional 10^6Å pore size column was added on this run). The dimer is seen partially resolved from the main peak.

two Styragel columns (each 300 mm x 7.8mm) of 10^4 Å and 10^6 Å pore size, respectively. After each sample was separated, it passed through a Wyatt Technology mini-DAWN laser light scattering detector followed by Waters Model 410 differential refractometer. Mobile phase was toluene, 1 mL/min.

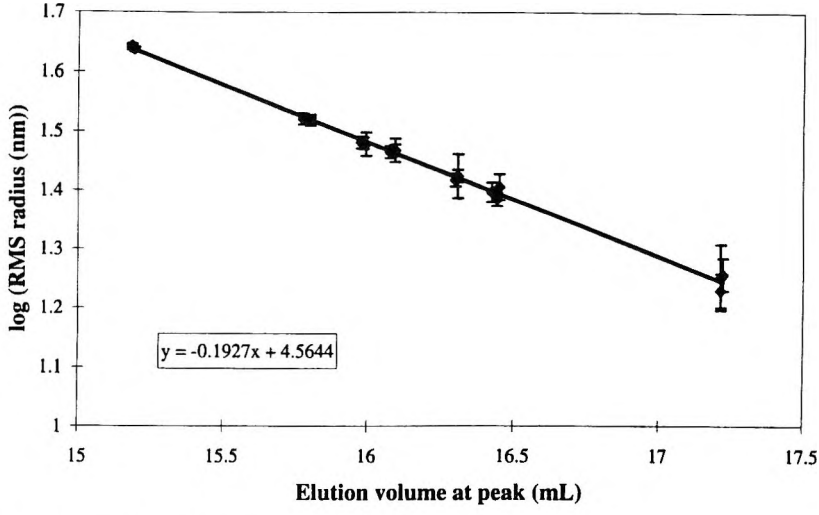

Figure 5. RMS radius calibration curve.

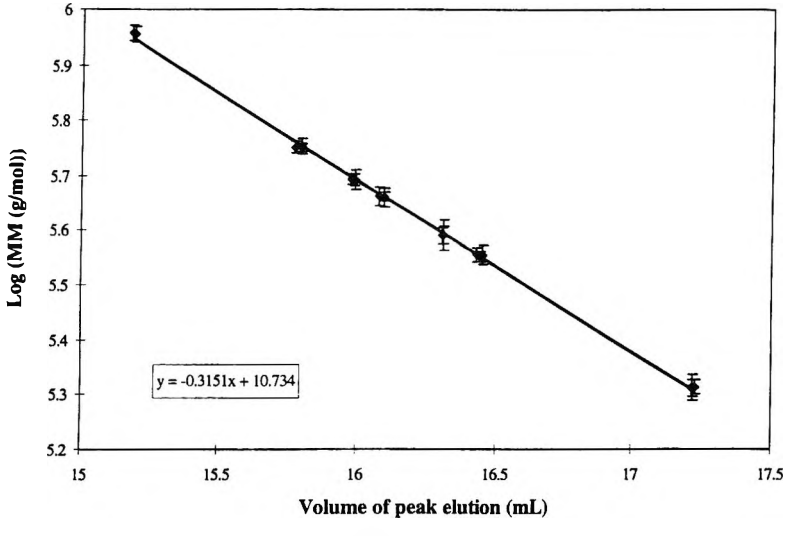

Figure 6. Molar Mass calibration curve. The slope value of the linear fit yields B.

The first two runs of standards were done with the two above mentioned columns, however these did not have the resolving power of those used by Shortt in his study. This configuration was unable to resolve the dimer (see Fig. 3) in some of the samples which led to larger values of D_{obs} and, thence, larger corresponding polydispersities. On the final run another 10^6 Å column was added, and the dimer can be seen partially resolved in Fig. 4 which allows for higher precision in the polydispersity calculation. Shortt was able to obtain baseline resolution of this aggregate component.

PROCEDURE FOR OBTAINING THE POLYDISPERSITIES

Data were collected and analyzed using Wyatt Technology's ASTRA® chromatography software to obtain the values of MM and r_g near the peaks for all of the standards used. The logarithm of these values were plotted against the elution volume for each peak value. A linear fit was applied to each of these curves to obtain calibration constants D and B. The calibration curves used for the second set of measurements (see RESULTS) are shown in Fig. 5 and Fig. 6.

Once values for B and D are obtained from the above plots, each data file must be analyzed individually to obtain unique values of w_{obs} and D_{obs} for each standard. This was done by measuring the 90° light scattering signal, as shown in Fig. 7, from which w_{obs} may be calculated directly. The FWHM is calculated by finding the maximum signal value from the LS chromatograph. This value is then halved and elution volume points with corresponding signal values are found to yield w_{obs}.

The weighted linear fit of the r_g values across the peak yields D_{obs} (Fig.8). Once values are obtained for D_{obs}, D and w_{obs}, one can solve Eq. (4) to find a value for w_0. Using the value of B previously obtained, Eq. (1) may now be evaluated and the polydispersity derived.

RESULTS

The samples measured all yielded values of polydispersity far lower than reported by their manufacturers. The resulting values were averaged over the three runs and are reported in Fig. 9 as a plot of $M_w/M_n - 1$ against the manufacturer's reported nominal MM. The values for the individual runs are detailed in Table 2.

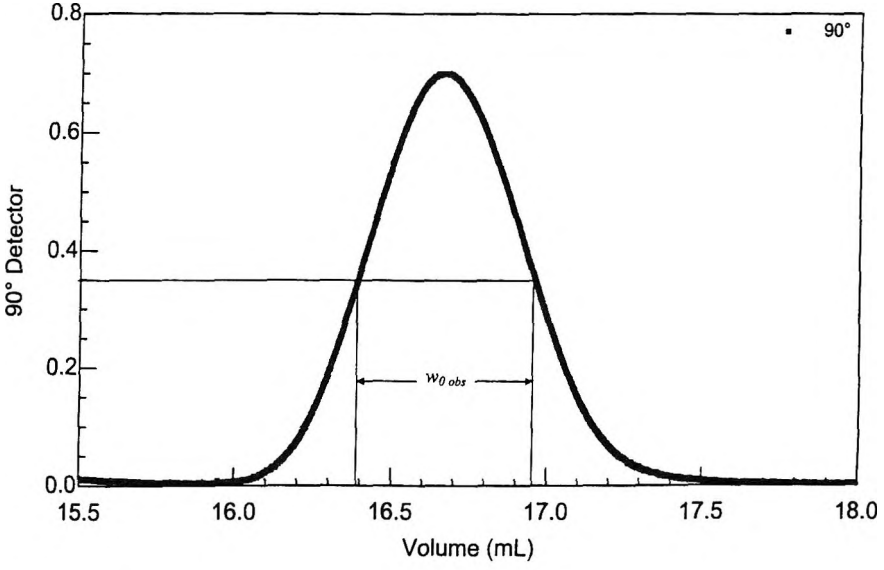

Figure 7. FWHM determination.

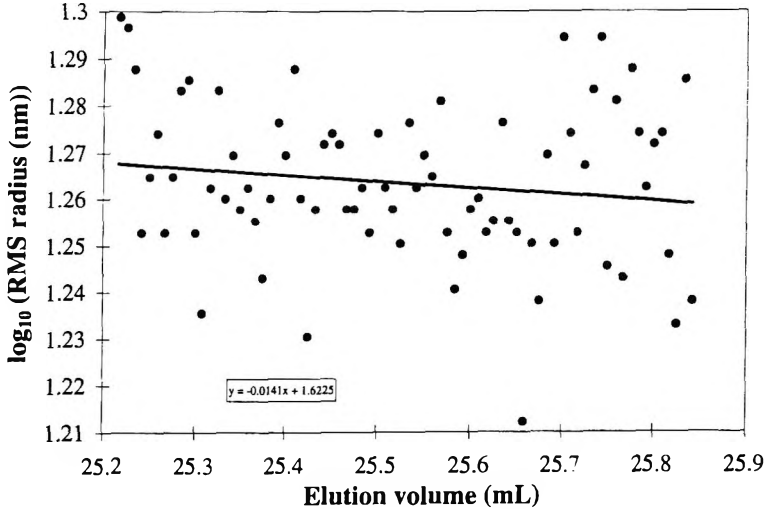

Figure 8. Curve for the determination of D_{obs}, the slope of the weighted linear fit.

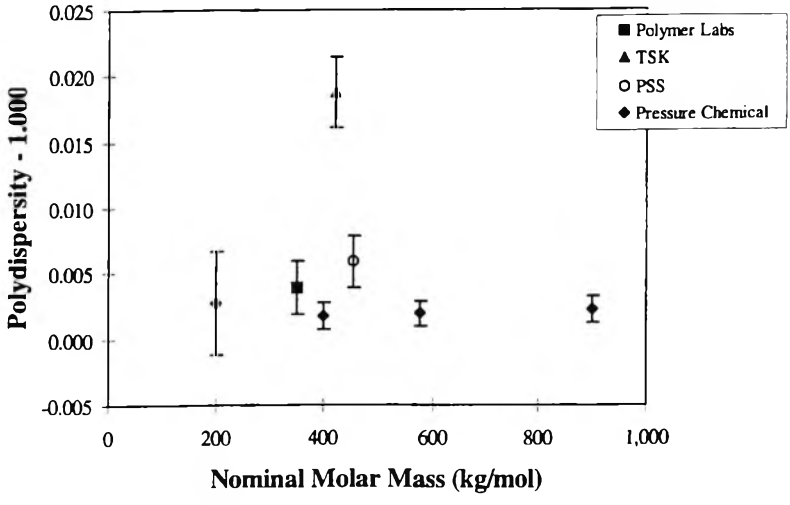

Figure 9. Polydispersity values with associated errors for various calibration standards.

Table 2

Polydispersities of Several Polystyrene Standards

Manufacturer	Nominal MM (kg/mol)	Run #1 Polydispersity	Run #2 Polydispersity	Run #3 Polydispersity
Pressure Chemical	200	1.003 ± 0.003	1.005 ± 0.005	1.001 ± 0.004
Pressure Chemical	400	1.003 ± 0.001	1.002 ± 0.002	1.000 ± 0.001
Pressure Chemical	575	1.003 ± 0.001	1.001 ± 0.001	1.002 ± 0.001
Pressure Chemical	900	1.003 ± 0.001	1.002 ± 0.001	1.001 ± 0.001
Polymer Labs	350	1.005 ± 0.002	1.004 ± 0.002	1.002 ± 0.002
TSK	422	1.025 ± 0.005	1.018 ± 0.002	1.014 ± 0.001
PSS	455	1.010 ± 0.002	1.006 ± 0.002	1.002 ± 0.002

CONCLUSIONS/DISCUSSION

Multiangle light scattering (MALS) is a powerful tool for the characterization of polymer mass and root mean square radius, r_g. For polymers of sufficient size ($r_g \sim 10$ nm or larger), r_g values can be determined in the limit of vanishingly small concentration independently of any concentration detector. (This is true for all homopolymers and homogeneous co-polymers.).

An important assumption of the method, at least in terms of simplifying the calculations, is that the samples are gaussian in their distributions. Most polystyrene standards include a contaminant, usually a dimer, associated with the termination process following polymerization. A good column set, such as selected by Shortt, will separate this aggregate from the main polymer peak leaving the latter indistinguishable from a gaussian and, thereby, assuming that treatment of the peak as a gaussian is valid.

It may be argued, however, that since the standard as provided by the manufacturer contains this termination contaminant, the stated polydispersity should include all of this fraction. We do not agree with this concept especially since the contaminant is often separated from the nominal peak during SEC. The polydispersity on the manufacturer's label invariably describes the product to be used for calibration.

Perhaps manufacturers might add a comment to the effect that a small residual dimer or aggregate is also present, but may be ignored. These dimer fractions are often found at fractions of less than 10^{-6} of the total, so their importance, especially for column calibration purposes, is negligible. Absent these contaminants, the appearance of the eluting standards peaks within the linear range of the column set is virtually indistinguishable from a gaussian.

Most of the analyzed standards were found to be very narrow and thus well suited for calibration purposes. Generally, polydispersities were found with values much lower than 1.01 over the full width at half maximum, which is the most important for calibration.

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REFERENCES

1. D. W. Shortt, J.of Chromatography A, **686**, 11 (1994).

2. J. L. Waters, J. Poly. Sci. A-2, **8**, 411 (1970).

3. P. J. Wyatt, Anal. Chem. Acta., **272**, 1 (1993).

4. P. J. Wyatt, J.of Liquid Chromatography, **14**, 2351 (1991).

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EFFECTS OF ALCOHOL MODIFIERS ON THE SEPARATION OF 1-ALKOXYCARBONYL-ALKYL-PYRROLIDINE-3-CARBOXYLIC ACID ALKYL ESTER ENANTIOMERS ON POLYSACCHARIDE-BASED STATIONARY PHASES

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ABSTRACT

1-Alkoxycarbonylalkyl-pyrrolidine-3-carboxylic acid alkyl esters (**1**, **2**, **3** and **4**, see Figure 1 for structures) are the synthetic intermediates used for the large-scale synthesis of PD 151832. PD 151832 is a highly potent m1 subtype selective muscarinic agonist expected to be useful for patients with Alzheimer's disease. The mobile phase consisting of hexane/2-propanol/diethylamine has been previously shown to resolve the enantiomers of compounds **1**, **2** and **4** on a Chiralpak AS column and compound **3** on a Chiralpak AD column.[1]

In the current study, the nature of the alcohol modifier in mobile phase was varied and the resulting change in stereoselectivity was found to depend on compound and column type. Superior separations can often be achieved by using an

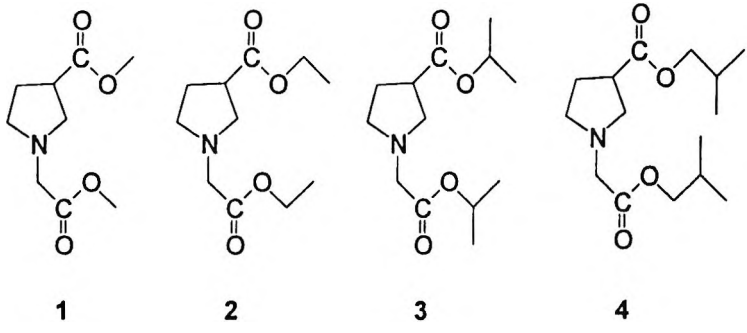

Figure 1. Structures of 1-Alkoxycarbonylalkyl-pyrrolidine-3-carboxylic Acid Alkyl Esters (**1**, **2**, **3** and **4**).

alcohol modifier other than the commonly used 2-propanol or ethanol on the same column. The use of different alcohol modifiers in mobile phase to enhance the enantiomeric resolution can provide useful and less expensive alternatives to the approach of using multiple columns in chiral methodology development.

INTRODUCTION

The HPLC separation of chiral compounds is increasingly important with a large number of new potential chiral drugs. Useful HPLC separations of racemic mixtures were developed by testing columns with different chiral stationary phases. This way of approaching chiral methods development requires considerable effort and can become extremely expensive.

Polysaccharide-based stationary phases have found many successful applications and are among the most widely used stationary phases for enantiomeric separations with the commonly recommended hexane/2-propanol or hexane/ethanol as the mobile phase.[2,3] The effects of mobile phase modifiers, particularly the alcohol on the stereoselectivity of the polysaccharide-based stationary phases, have been demonstrated.[4-8]

It was found that an alcohol modifier other than 2-propanol or ethanol can be superior. 1-Alkoxycarbonylalkyl-pyrrolidine-3-carboxylic acid alkyl esters are important intermediates towards synthesis of PD 151832, a highly potent

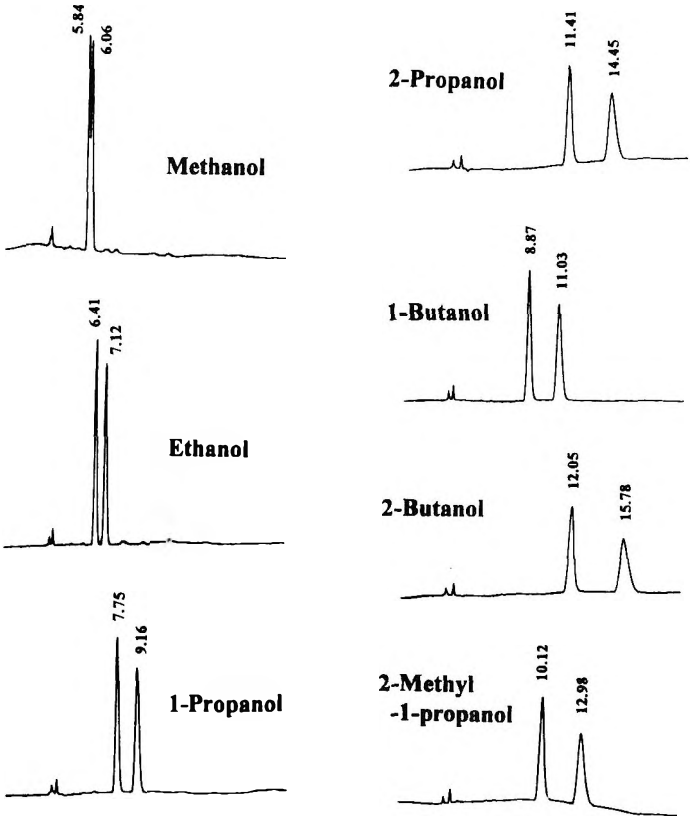

Figure 2. Separation of a Racemic Mixture of Compound **1** using Various Alcohol Modifiers; Column: Chiralpak AS, Mobile Phase: Hexane/Alcohol/DEA (950/50/1), Flow Rate: 1.0 mL/min, Detection: UV @ 230nm, Sample Amount Injected: ~21μg.

m1 subtype selective muscarinic agonist potentially useful for the treatment of neurodegenerative disorders.[9] It was our desire to resolve these early intermediates. In our previous work,[1] two columns were successfully employed to perform the chiral separation for all four compounds using hexane/2-propanol/diethylamine.

It would be advantageous, if the desired chiral separation can be accomplished with one column for all four compounds by simply changing the alcohol modifier.

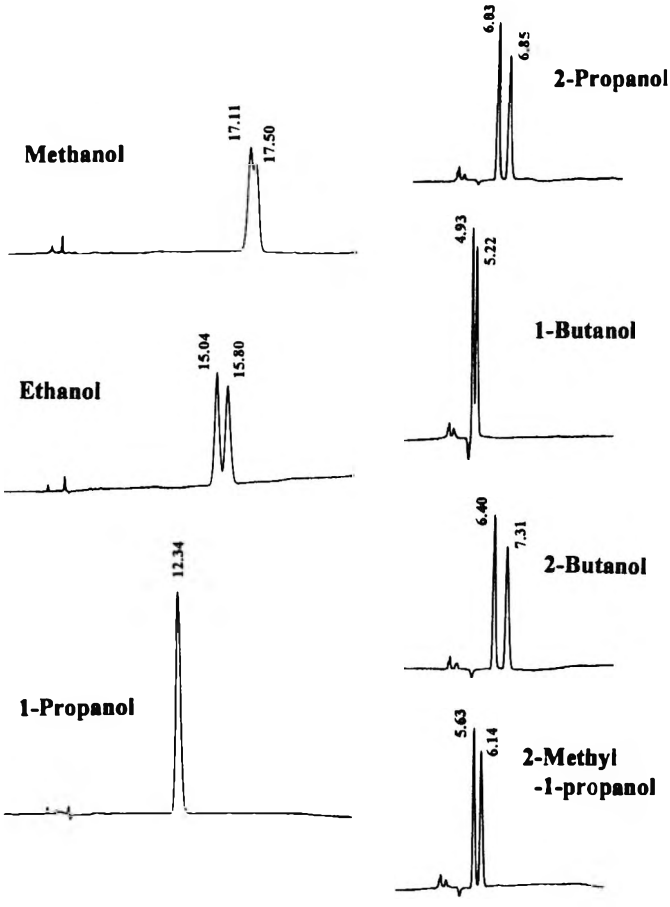

Figure 3. Effect of Alcohol Modifiers on the Separation of a Racemic Mixture of Compound **1** using a Chiralpak AD column; Mobile Phase: Hexane/Alcohol/DEA (980/20/1), Flow Rate: 1.0 mL/min, Detection: UV @ 230 nm, Sample Amount Injected: ~21 μg.

EXPERIMENTAL

Equipment

The liquid chromatographic system consisted of a Hitachi L-6200 intelligent pump, a Micromeritics 728 autosampler, a Valco injector with a 20 μL loop, a Hitachi L-4000 variable wavelength UV detector, a Waters 410

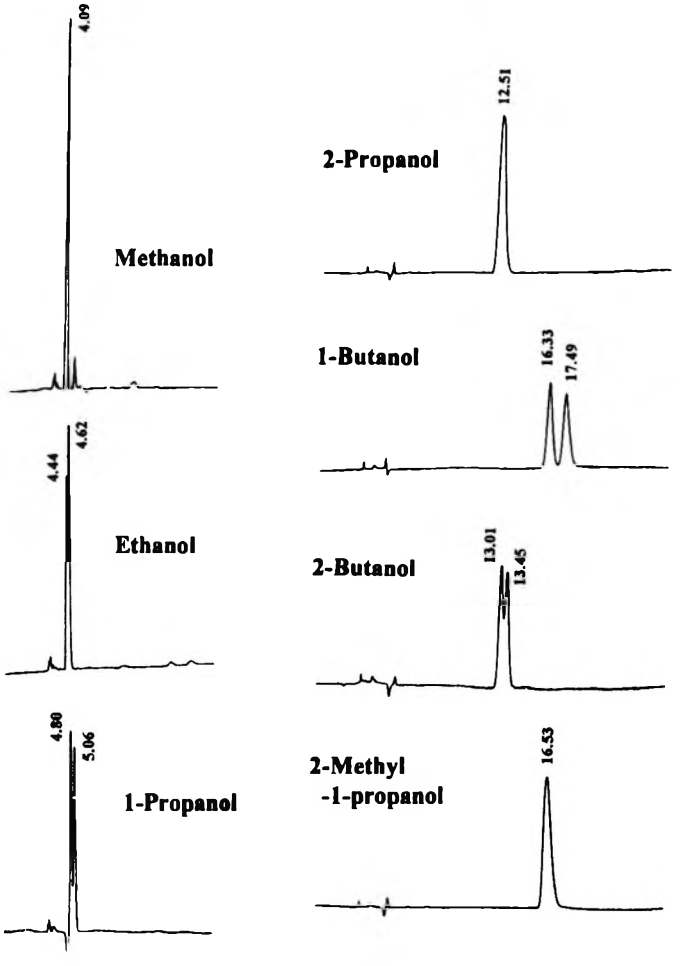

Figure 4. Effect of Alcohol Modifiers on the Separation of a Racemic Mixture of Compound **4** using a Chiralpak AS column; Mobile Phase: Hexane/Alcohol/DEA (980/20/1), Flow Rate: 1.0 mL/min, Detection: UV @ 230 nm, Sample Amount Injected: ~21 μg.

Differential Refractometer equipped with a column oven, and a Hitachi D-2500 Chromato-integrator. The analytical columns were Chiralcel OD-H, OJ, Chiralpak AS and AD. All of the columns were 250 x 4.6 mm I.D., and 10 microns in particle size except OD-H which was 5 microns. They were purchased from Chiral Technologies, Inc, Exton, PA.

Table 1

Effects of Mobile Phase Alcohol Modifiers on the Enantiomeric Separations of Compounds 1, 2, 3, and 4 on Various Columns with a Flow Rate of 1.0mL/min and a Mobile Phase of Hexane/Alcohol/DEA (950/50/1)

Alcohol Modifier	Compound 1			Compound 2			Compound 3			Compound 4		
	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s
Chiralpak AD												
methanol	no separation			----	----	<0.5	no separation			no separation		
ethanol	----	----	<0.5	----	----	<0.5	----	-----	<0.5	0.76	1.11	0.96
1-propanol	no separation			1.02	1.06	0.61	0.55	1.08	0.54	0.61	1.16	1.63
1-butanol	2.05	1.13	1.88	no separation			----	----	<0.5	0.81	1.07	0.64
2-propanol	----	----	<0.5	0.93	1.10	1.19	0.55	1.11	1.09	0.62	1.13	1.07
2-butanol	1.40	1.06	0.73	1.04	1.11	1.26	0.64	1.13	1.14	0.73	1.12	1.22
2-methyl-1-propanol	----	----	<0.5	no separation			----	----	<0.5	0.82	1.06	0.52
Chiralpak AS												
methanol	0.90	1.09	0.80	no separation			no separation			no separation		
ethanol	1.10	1.21	2.29	0.55	1.12	0.80	no separation			no separation		
1-propanol	1.43	1.30	3.47	0.67	1.16	1.31	no separation			0.30	1.14	0.52
1-butanol	1.79	1.39	4.03	0.77	1.22	1.79	no separation			0.31	1.18	0.74
2-propanol	2.80	1.34	4.01	1.28	1.22	2.23	no separation			0.56	1.25	1.65
2-butanol	3.00	1.46	4.79	1.41	1.34	3.28	----	----	<0.5	0.64	1.27	2.00
2-methyl-1-propanol	2.36	1.44	3.06	1.01	1.33	1.87	no separation			0.43	1.22	1.20
Chiralcel OJ												
methanol	no separation			----	----	<0.5	no separation			no separation		
ethanol	no separation			no separation			no separation			no separation		
1-propanol	2.72	1.05	0.61	no separation			no separation			0.39	1.22	1.35
1-butanol	3.15	1.07	0.94	1.43	1.06	0.50	no separation			0.49	1.14	0.85
2-propanol	----	----	<0.5	no separation			no separation			no separation		
2-butanol	2.87	1.06	0.77	----	----	<0.5	no separation			no separation		
2-methyl-1-propanol	2.97	1.06	0.73	no separation			no separation			0.41	1.24	1.03
Chiralcel OD-H												
methanol	no separation			no separation			no separation			no separation		
ethanol	no separation			no separation			no separation			no separation		
1-propanol	----	----	<0.5	no separation			no separation			no separation		
1-butanol	----	----	<0.5	0.78	1.08	0.63	no separation			no separation		
2-propanol	1.51	1.07	0.85	----	----	<0.5	no separation			no separation		
2-butanol	1.86	1.06	0.80	----	----	<0.5	no separation			no separation		
1-methyl-1-propanol	----	----	<0.5	----	----	<0.5	no separation			no separation		

Table 2

Effects of Mobile Phase Alcohol Modifiers on the Enantiomeric Separations of Compounds 1, 2, 3, and 4 on a Chiralpak AD Column with a Flow Rate of 1.0mL/min

Alcohol Modifier	Compound 1			Compound 2			Compound 3			Compound 4		
	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s
Hexane/Alcohol/DEA (950/50/1)												
methanol	no separation			----	----	<0.5	no separation			no separation		
ethanol	----	----	<0.5	----	----	<0.5	----	----	<0.5	0.76	1.11	0.96
1-propanol	no separation			1.02	1.06	0.61	0.55	1.08	0.54	0.61	1.16	1.63
1-butanol	2.05	1.13	1.88	no separation			----	----	<0.5	0.81	1.07	0.64
2-propanol	----	---	<0.5	0.93	1.10	1.19	0.55	1.11	1.09	0.62	1.13	1.07
2-butanol	1.40	1.06	0.73	1.04	1.11	1.26	0.64	1.13	1.14	0.73	1.12	1.22
2-methyl-1-propanol	----	----	<0.5	no separation			----	----	<0.5	0.82	1.06	0.52
Hexane/Alcohol/DEA (980/20/1)												
methanol	----	----	<0.5	----	----	<0.5	no separation			no separation		
ethanol	4.46	1.04	0.90	----	----	<0.5	1.17	1.07	1.04	1.39	1.11	1.31
1-propanol	no separation			2.05	1.06	0.98	1.06	1.10	1.18	1.20	1.19	2.41
1-butanol	3.94	1.12	2.30	no separation			1.15	1.10	1.42	1.43	1.09	1.09
2-propanol	no separation			1.67	1.09	1.39	1.00	1.11	1.26	1.13	1.11	1.38
2-butanol	----	----	<0.5	2.26	1.09	1.58	1.41	1.12	1.55	1.62	1.11	1.73
2-methyl-1-propanol	no separation			no separation			1.49	1.06	0.81	1.85	1.06	0.63

Chemicals

Hexane, methanol, 2-propanol, and 2-butanol (HPLC grades) were obtained from EM Science, Gibbstown, NJ. Ethanol (absolute) was purchased from Aaper Alcohol and Chemical Company, Shelbyville, KY. 1-Propanol, 1-butanol, 2-methyl-1-propanol (HPLC grades), and diethylamine (redistilled, 99.5%) were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. Racemic 1-alkoxycarbonylalkyl-pyrrolidine-3-carboxylic acid alkyl esters were synthesized in the Chemical Development Department, Parke-Davis Pharmaceutical Research Division, Holland, MI.

Chromatographic Conditions

The mobile phase was hexane/alcohol/diethylamine (DEA) in an appropriate volume ratio. The flow rate was either 1.0 or 0.6 mL/min. The

Table 3

Effects of the Flow Rate on the Enantiomeric Separations of Compounds 1, 2, 3, and 4 on a Chiralpak AD Column Using a Mobile Phase of Hexane/Alcohol/DEA (980/20/1)

Alcohol Modifier	Compound 1			Compound 2			Compound 3			Compound 4		
	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s
1.0 mL/min												
methanol	----	----	<0.5	----	----	<0.5	no separation			no separation		
ethanol	4.46	1.04	0.90	----	----	<0.5	1.17	1.07	1.04	1.39	1.11	1.31
1-propanol	no separation			2.05	1.06	0.98	1.06	1.10	1.18	1.20	1.19	2.41
1-butanol	3.94	1.12	2.30	no separation			1.15	1.10	1.42	1.43	1.09	1.09
2-propanol	no separation			1.67	1.09	1.39	1.00	1.11	1.26	1.13	1.11	1.38
2-butanol	----	----	<0.5	2.26	1.09	1.58	1.41	1.12	1.55	1.62	1.11	1.73
2-methyl-1-propanol	no separation			no separation			1.49	1.06	0.81	1.85	1.06	0.63
0.6 mL/min												
methanol	----	----	<0.5	----	----	<0.5	no separation			no separation		
ethanol	4.55	1.04	0.96	2.69	1.04	0.63	1.20	1.07	1.00	1.43	1.11	1.64
1-propanol	no separation			2.03	1.06	1.28	1.04	1.10	1.42	1.18	1.19	2.60
1-butanol	3.92	1.12	2.33	no separation			1.13	1.10	1.49	1.41	1.08	1.38
2-propanol	no separation			1.73	1.09	1.48	1.04	1.11	1.50	1.17	1.11	1.52
2-butanol	2.97	1.03	0.56	2.24	1.09	1.70	1.39	1.12	1.85	1.60	1.11	2.00
2-methyl-1-propanol	no separation			no separation			1.48	1.06	0.89	1.84	1.06	1.00

detection was UV @ 230 nm. The column temperature was maintained at 30°C. The sample was dissolved in mobile phase. The amount of sample injected was 9 to 22 μg unless otherwise stated. The capacity factor of the first eluted peak, k_1', the separation factor, α, and the resolution factor, R_s, were calculated as follows: $k_1' = (t_1 - t_0)/t_0$; $\alpha = (t_2 - t_1)/(t_1 - t_0)$; $R_s = 2(t_2 - t_1)/(w_1 + w_2)$; where t_0 is the time at void volume, t_1 is the retention time of the first eluted peak, t_2 is the retention time of the second eluted peak, w_1 and w_2 are the widths at baseline for the first and second eluted peaks, respectively, and they were obtained by extrapolating the relatively straight sides of the peaks to the baseline.

RESULTS AND DISCUSSION

Table 1 gives k_1', α and R_s for compounds **1**, **2**, **3**, and **4** using different alcohols in the mobile phase on Chiralcel OD-H, OJ, Chiralpak AD, and AS

columns, respectively. The Chiralpak AS column gave the best enantiomeric separation for compounds **1** & **2** no matter what alcohol was used. Among the alcohols studied, 2-butanol was more efficient than 2-propanol as the alcohol modifier on this column. The enantiomeric separation of compound **1** using various alcohol modifiers is shown in Figure 2. The Chiralpak AS column afforded better separation for compound **4** using either 2-propanol, 2-butanol or 2-methyl-1-propanol while Chiralpak AD and Chiralcel OJ columns were better when 1-propanol was used. It was very interesting to note from Table 1 that the Chiralpak AD column was the only column which gave reasonable separation for compound **3** and 2-butanol was slightly better than 2-propanol as the alcohol modifier.

Both the flow rate and alcohol amount can be used to enhance enantiomeric separation for all four compounds. The nature of alcohol does not seem to change this aspect. The resolution increased with a reduced amount of alcohol in mobile phase (Table 2) and/or a reduced flow rate (Table 3).

The effects of alcohol modifiers on the separations of compound **1** on a Chiralpak AD column and compound **4** on a Chiralpak AS column using a mobile phase of hexane/ alcohol/DEA (980/20/1) and a flow rate of 1.0 mL/min are illustrated in Figures 3 & **4**, respectively. In the case of separating compound **1** on a Chiralpak AD column, 1-butanol clearly was the choice of alcohol modifiers. For separating compound **4** on a Chiralpak AS column, the best alcohol modifier was either 2-butanol or 2-propanol. However, 2-methyl-1-propanol also worked.

Finally, by varying the mobile phase alcohol modifiers, the separations of all four compounds on a Chiralpak AD column could be achieved using 1-butanol for compound **1**, 2-butanol for compounds **2** & **3**, and 1-propanol for compound **4**, respectively. These results are shown in Figure 5.

CONCLUSIONS

For chiral HPLC method development, the choice of the right chiral column often dictates the success of the methodology. The results from this study not only confirm this but also suggest that better separation can be obtained via a change of alcohol modifiers in mobile phase. Although the use of a Chiralpak AS column gave the best separations for compounds **1**, **2** & **4**, the separation of compound **3** required a second chiral column.

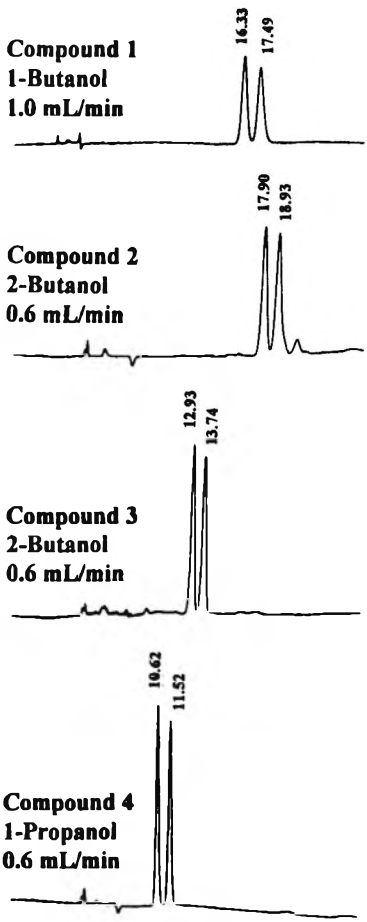

Figure 5. Separations of Racemic Mixtures of Compounds **1**, **2**, **3** & **4** on a Chiralpak AD column by varying the alcohol modifier.
Mobile Phase: Hexane/Alcohol/DEA (980/20/1), Detection: UV @ 230 nm, Sample Amount Injected: ~21 μg.

The change of alcohol modifiers in mobile phase allowed us to separate all four compounds on a Chiralpak AD column. These methods have been routinely employed for screening large-scale resolution conditions for all four compounds in our laboratory.

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REFERENCES

1. S. Lin, C. E. Engelsma, N. J. Maddox, B. K. Huckabee, D. M. Sobieray, "Chiral HPLC Separations of 1-Azabicyclo[2.2.1]heptan-3-one and 1-Alkoxycarbonylalkyl-pyrrolidine-3-carboxylic Acid Alkyl Ester Enantiomers on Polysaccharide-Based Stationary Phases," J. Liq. Chrom. & Rel. Technol., 20 (1997), in press.

2. T. Shibata, I. Okamoto, K. Ishii, "Chromatographic Optical Resolution on Polysaccharides and their Derivatives," J. Liquid Chromatogr., **9**, 313-340 (1986).

3. K. Oguni, H. Oda, A. Ichida, "Development of Chiral Stationary Phases Consisting of Polysaccharide Derivatives," J. Chromatogr., A, **694**, 91-100 (1995).

4. I. W. Wainer, M. C. Alembik, E. Smith, "Resolution of Enantiomeric Amides on a Cellulose Tribenzoate Chiral Stationary Phase: Mobile Phase Modifier Effects on Retention and Stereoselectivity," J. Chromatogr., **388**, 65-74 (1987).

5. M. H. Gaffney, R. M. Stiffin, I. W. Wainer, "The Effect of Alcoholic Mobile Phase Modifiers on Retention and Stereoselectivity on a Commercially Available Cellulose-Based HPLC Chiral Stationary Phase: an Unexpected Reversal in Enantiomeric Elution Order," Chromatographia, **27**, 15-18 (1989).

6. K. M. Kirkland, "Optimization of Chiral Selectivity on Cellulose-Based High Performance Liquid Chromatographic Columns using Aprotic Mobile-Phase Modifiers," J. Chromatogr., A, **718**, 9-26 (1995).

7. A. Kunath, F. Theil, K. Jahnisch, "Influence of the Kind of the Alcoholic Modifiers on Chiral Separation on a Chiralpak AD Column," J. Chromatogr., A, **728**, 249-257 (1996).

8. Y. Tang, "Significance of Mobile Phase Composition in Enantioseparation of Chiral Drugs by HPLC on a Cellulose-Based Chiral Stationary Phase," Chirality, **8**, 136-142 (1996).

9. J. Jaen, S. Barrett, M. Brann, M. Callahan, R. Davis, P. Doyle, D. Eubanks, D. Lauffer, L. Lauffer, W. Lipinski, D. Moreland, C. Nelson, C. Raby, R. Schwarz, C. Spencer, H. Tecle, "In Vitro and In Vivo Evaluation of the Subtype-Selective Muscarinic Agonist PD 151832," Life Sciences, **56**, 845-852 (1995).

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DETERMINATION OF ALIPHATIC AMINES IN WATER USING DERIVATIZATION WITH FLUORESCEIN ISOTHIOCYANATE AND CAPILLARY ELECTROPHORESIS/LASER-INDUCED FLUORESCENCE DETECTION

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ABSTRACT

Detection-oriented derivatization of aliphatic amines and amine functional groups in compounds of environmental interest was studied using fluorescein isothiocyanate (FITC) with separation/determination by capillary electrophoresis/laser-induced fluorescence. Determinative levels down to 10 ppb were studied in deionized and environmental waters. Practical detection limits of 30 to 100 ppb or more were realized, depending on analyte and water source. Criteria were developed to compare results to desired performance in a derivatization method. FITC was judged to be a moderately successful reagent with drawbacks including extensive by-products, lack of specificity to analyte class, requirement to study each potential analyte carefully, and derivatization limits near 30 ppb. Advantages include low cost of reagent, ease in handling, and synthesis of an ionized derivative for free zone electrophoresis.

INTRODUCTION

Various aliphatic amines are of environmental interest due to their toxicity, reactivity, and likely occurrence as a result of their industrial uses.[1] The U.S. EPA, Environmental Sciences Division (CRD-LV), maintains a continuous interest in analytical methods for amines because of their wide occurrence and often problematic analyses. We have recently studied aliphatic amines using indirect UV detection in free zone capillary electrophoresis (CZE).[2] Generally, lower aliphatic amines are considered nonextractable, nonpurgeable volatiles in U.S. EPA methods.[3,4]

Aliphatic amines can present chromatographic problems due to their reactivity and extreme basicity. Derivatization has, therefore, been frequently employed. However, derivatization (e.g., Method 8042[3]) has not achieved as wide adoption in environmental analysis as it has in biological and pharmaceutical analysis. Part of the reason may lie in the tremendous variability of environmental matrices with a real concern for artifact formation from coextractives. Another reason is the desire to determine the analyte directly in the form that occurs in the sample with minimal sample manipulations. For amines, this could encompass primary through quaternary forms.

Gas chromatography (GC) is frequently used to separate amines using a variety of derivatizing agents.[5] Benzenesulfonyl chloride derivatives,[6] imine derivatives derived from pentafluorobenzaldehyde,[7] and isobutyl chloroformate derivatives[8] are representative.

Other techniques used to separate amines include supercritical fluid chromatography,[9] thermospray LC/MS,[10] and ion chromatography.[11] Liquid chromatography has often been the separation technique of choice for amines due to the variety of derivatizing agents available to introduce chromophores for UV detection or fluorophores for fluorescence detection.[5] Dansyl chloride derivatives are probably the most familiar[8-11] along with related compounds such as dabsyl and debsyl chlorides.[12] Other derivatives and reagents commonly used include benzoyl chloride[13], m-toluoyl chloride,[14,15] fluorescamine,[16] phenyl isothiocyanate and p-toluenesulfonyl chloride,[17] o-phthaldialdehyde,[18] 3,5-dinitrobenzoyl derivatives,[19] dichlorotriazinyl-fluorescein,[20] acetylacetone,[21] 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-chloride),[22,33] 2,4-dinitrophenyl derivatives,[24] and sodium benzoxazole-2-sulfonate derivatives.[25] More recently, a special reagent, 3-(p-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQ), has been developed for use in capillary electrophoresis.[26] Applications of CE/LIF for analyses of phenoxy acid herbicides have appeared.[27,28] Applications of capillary electrophoresis to

environmental analysis have been growing at a moderate rate and reviews of environmentally related work have been published.[29,30] Applications originally developed for HPLC applications[31] may have a direct counterpart in CE/LIF detection.[32] Recent work involving comprehensive two-dimensional separations has used fluorescein isothiocyanate (FITC)[33] and related compounds with characterization of some of the by-products.[34]

In this work, derivatization of aliphatic amines and amine functions in compounds of environmental interest was studied using FITC with separation/determination by capillary electrophoresis/laser-induced fluorescence. Determinative levels down to 10 ppb were studied in deionized and environmental waters. Criteria were developed to compare results with desired performance in a derivatization method.

EXPERIMENTAL SECTION

Chemicals

All organic compounds were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA) unless otherwise specified. Other chemicals were from standard sources of supply, and all were used as received. Deionized water (ASTM Type II) was used for all aqueous solutions. Solutions were freshly prepared for each experiment. Liquid amines were measured gravimetrically for preparation of standard solutions.

Capillary Electrophoresis

A P/ACE Model 2050 Capillary Electrophoresis System (Beckman Instruments, Fullerton, CA, USA) was used for all capillary electrophoretic experiments. The instrument was fitted with a capillary 57 cm X 75-μm I.D., 50 cm to the detector, with LIF detection using the 488-nm line of the Ar ion laser and a 520DF10 emission filter as well as a notch filter at 488 nm. The temperature of the capillary was 25°C, and electrophoretic runs were about 20 minutes at 20 or 25 kV using a 40 mM phosphate buffer at pH 7.0 with 25% methanol. The capillary was equilibrated with running buffer at the start of each experiment, and washed extensively between runs with 0.1 M sodium hydroxide, water, and running buffer. Migration times, peak widths, and detection limits were estimated directly from the monitor of the data system (software System Gold, Ver. 8.1). Corrected peak areas, as computed by the data system (peak area multiplied by the velocity of the ion [length to the

detector divided by time]), were normalized to the corrected peak area of the internal standard (fluorescein, disodium salt) as a control for the small variations in the nominal volumes of the pressure injections (ca. 5 nL from 1-s injections).

Derivatization and Sample Handling

Derivatization conditions were as follows: 0.1 mL to 1.0 mL of sample (amine in water) ranging from about 10μg to 30 ng (i.e., 30 ppb) was added to a 4 mL vial to which was added 0.1 mL of the derivatizing solution. The derivatizing solution was prepared daily and consisted of 7 to 10 mg of FITC, 300 mg of sodium carbonate (about 300 mM), and diluted to volume in a 10 mL volumetric flask. This solution was kept in the refrigerator when not in use. The reaction mixture was place on a stirrer plate with micro stirrer and capped; the reaction vial was kept in the dark and allowed to react a minimum of 16 hr up to a maximum of 48 hr at room temperature.

The reaction mixture was worked up by transferring the contents of the reaction vial to a 10 mL volumetric flask and diluting to 10 mL with deionized water. A 1-mL aliquot of this solution was added to 1 mL of internal standard, 1 mL of acetonitrile, 1 mL of phosphate buffer, and 1 mL of deionized water. This 5 mL sample was filtered through a 0.2 μm pore filter into the sample vials of the CE instrument.

The phosphate buffer was 40 mM phosphate, pH 7.0 and the internal fluorescein standard was 1×10^{-6} M which then resulted in about a 2×10^{-7} M solution in the prepared sample. The running buffer for CE was prepared using the 40 mM phosphate buffer but was diluted with methanol to achieve a 25 % level (v/v) in methanol resulting in a phosphate concentration of about 30 mM.

RESULTS AND DISCUSSION

Derivatization Issues

In real environmental samples the contamination level will not be known in advance. Therefore, a concentration in the mM range was chosen for the reagent in order to maintain reagent excess from the 10 ppb level up to the 10 ppm level. A constant level of reagent will also ensure that pseudo first-order rate kinetics will be followed with a half-life independent of initial concentration.[35] The desired detection level in environmental waters was set at

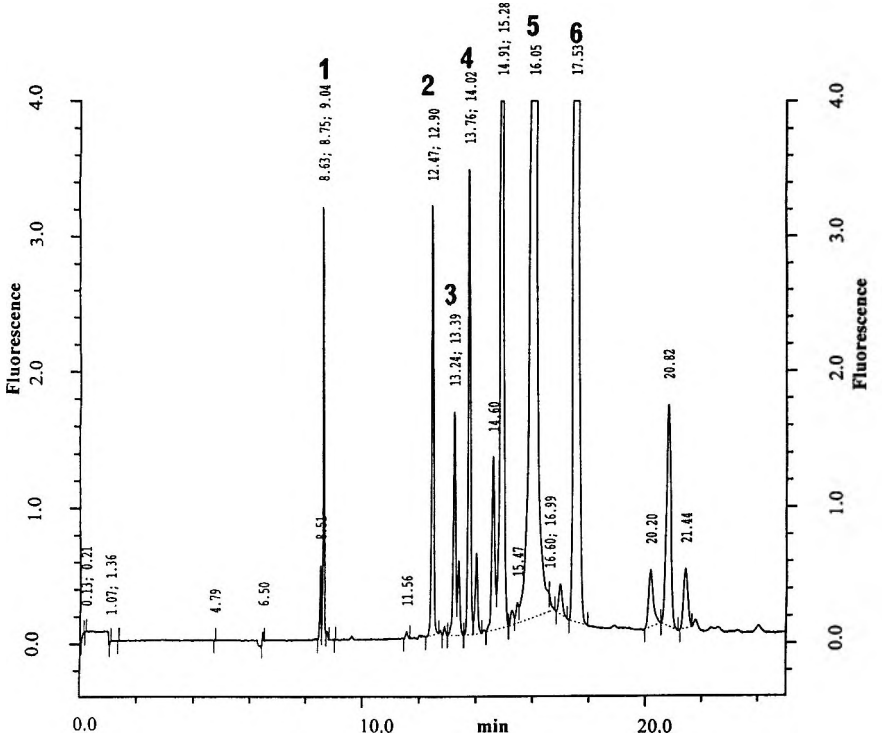

Figure 1. Derivatization of aliphatic amines at 100 ppb in deionized water. Identification of peaks as fluorescein isothiocyanate derivatives: **1**, 1,5-pentanediamine; **2**, 2-[2-aminoethyl]pyridine; **3**, diethylamine; **4**, propylamine; **5**, FITC; **6**, fluorescein internal standard.

10 ppb or lower, if possible. The criteria for the derivatization include: specific for the class of compounds; few or limited side reactions/by-products; separation of products and by-products; low detection limits of desired products (10^{-9} M or below); inexpensive reagent; simple and reproducible reaction conditions; and manageable derivatization of matrix components with separation from target products.

Separation Conditions

In cases where the derivative is ionic, free zone CE provides a convenient separation of analytes. In our work, free zone CE was carried out at pH 7.0

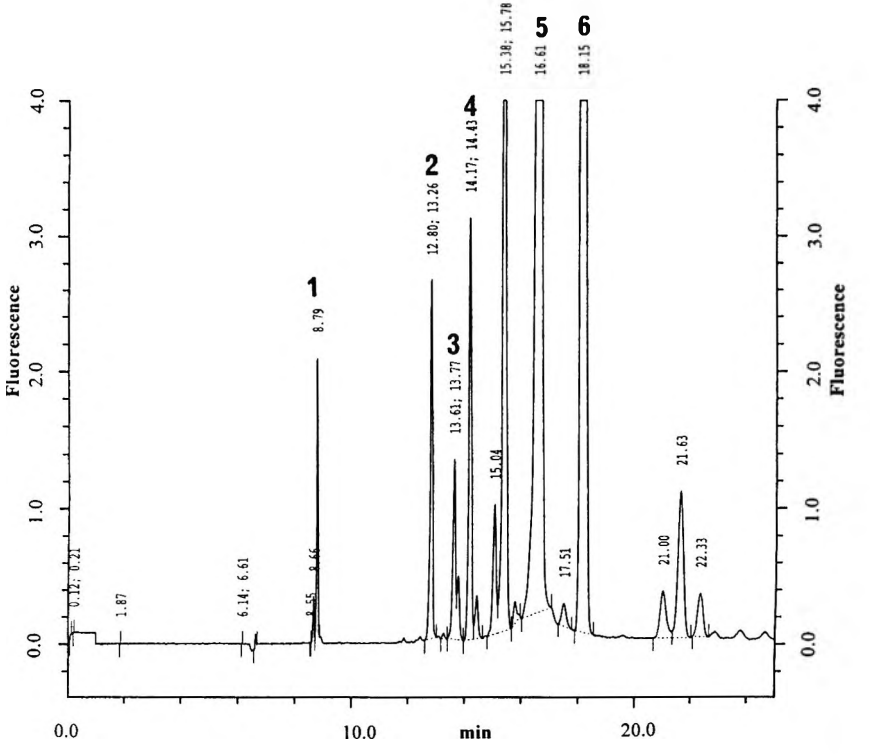

Figure 2. Derivatization of aliphatic amines in spiked ground water at 100 ppb. Identifications as in Fig. 1.

with phosphate buffer and 25 % methanol additive as an electroosmotic (EO) flow suppressor. Micellar electrokinetic chromatography (MEKC) has been used to separate fluorescein-based derivatives of herbicides[28] and amines.[29] In our work, fluorescein disodium salt was a convenient internal standard and migrated after FITC as well as the bulk of the by-products and the desired products. There were, however, later eluting peaks as well.

Detection Levels Achieved in DI Water and Environmental Waters

Standards were derivatized at 10, 30, 100, and 300 ppb in aqueous matrices. Standards representing 10 μg and 1 μg were also subjected to derivatization, representing the amount of material available at 10 and 1 levels. Figure 1 presents results of derivatization at 100 ppb in deionized water

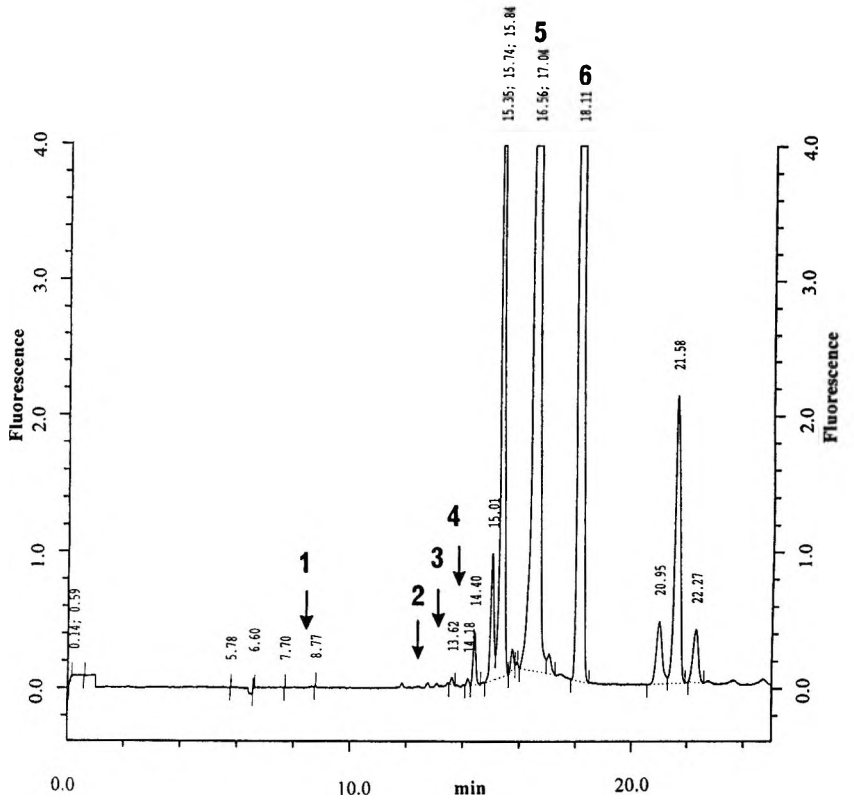

Figure 3. Derivatization of ground water blank. Identifications as in Fig. 1 with indication of where compounds would elute.

for comparison with Fig. 2 and Fig. 3 which represent 100 ppb and blank levels in ground water, respectively. The 100 ppb level appears to be readily achievable in relatively clean water samples, and this is confirmed by the results of ground water spiking. Derivatization at the 30 ppb level in deionized water represents about the lowest level of reliable detection of the amines and is illustrated in Fig. 4.

Results of experiments not reported here suggest that many amine substrates produce more than one product and others react slowly or do not react at all. In view of the known reactivity of isothiocyanates to other

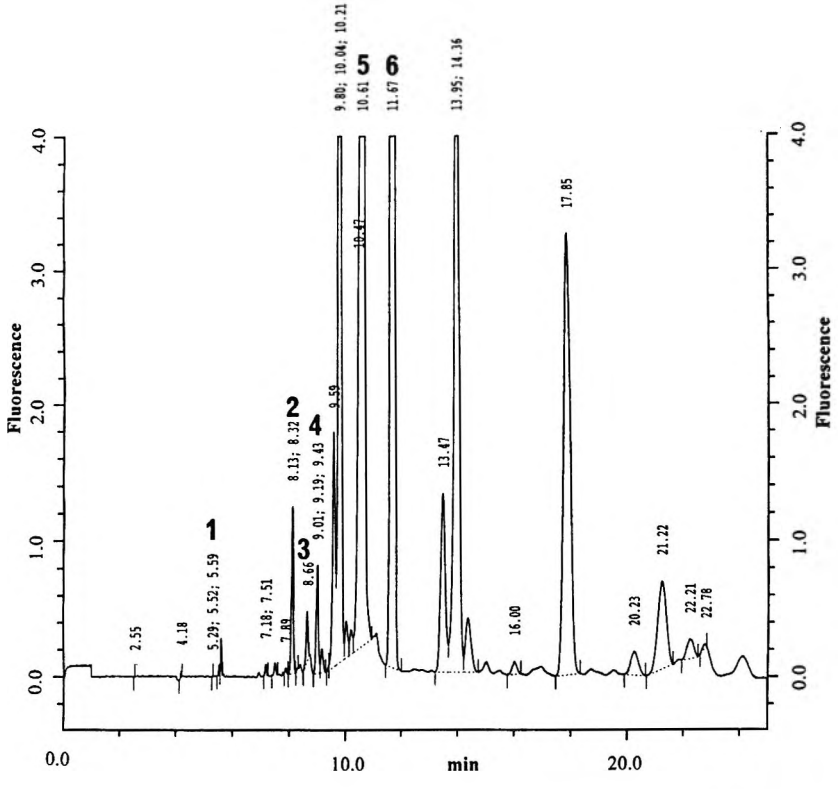

Figure 4. Derivatization of aliphatic amines at 30 ppb in deionized water. Identifications as in Fig. 1.

functional groups such as thiols, care is required in employing such derivatization schemes for real samples. This may explain why much of the literature on CE/LIF with derivatization has been infrequently applied to real samples.

Variations in Migration Times

Under free zone electrophoresis, variations in migration times (MTs) of target ions are principally due to variation in the EO flow. Corrections to this variation are possible based on the MT of an internal standard, and this is an

Table 1

Observed and Corrected MT's for the Compounds of Figures 1-4 with Percentage Differences Between Observed/Calculated MT's

Cpd.	Fig. 1(std) MT (min)	Fig. 2 MT/corr MT	Fig. 3 MT/corr MT	New std MT	Fig. 4 MT/corr MT	% error MT Fig. 1,2/ std, Fig.4
1	8.63	8.79/8.64	----/8.53	5.62	5.59/5.60	-0.1/0.3
2	12.47	12.80/12.49	----/12.51	8.17	8.13/8.15	-0.1/0.2
3	13.24	13.61/13.26	----/13.27	8.70	8.66/8.69	-0.1/0.1
4	13.76	14.17/13.79	----/13.81	9.06	9.01/9.04	-0.2/0.2
6	17.53	18.15/17.53	18.11/17.53	11.72	11.67/11.72	0/0

excellent reason for using an internal standard. Other reasons include the quality control nature of a response that indicates that all went well with injection, separation, and detection phases. These reasons are in addition to the quantitative use of internal standards.

In a previous paper,[37] we reported a tool for correcting MTs of analytes in subsequent runs to reference MTs of a standard run. The approach is based on the reciprocal relationship between MT and apparent mobility. Apparent mobility is the sum of the intrinsic mobility of the analyte ion and the mobility of the EO flow. Using the MT of an internal standard provides a means to correct for EO flow variations from run to run. Variations due to other factors such as wall interactions, temperature effects, and effects due to coextractives are not accounted for by this procedure.

Table 1 shows observed and corrected/predicted values of MT for four derivatives and the internal standard. The first two columns yield correlations of 0.1 % to 0.2 % for runs performed on the same day (reference last column, first number). Day to day variations may be greater than within the day and effects from using new capillaries predicate a new standard run for a new reference set of data. The last two columns indicate that, a different capillary operating on a different day, the same level of agreement is possible for runs on a single day (reference last column, 2nd number). It has been our experience that MT's can agree within 1% and often in the range from 0.1% to 0.3% as a qualitative tool of identification of peaks (i.e., corresponds to agreement within 0.6 sec to 1.8 sec for a MT of 10 min). This reproducibility is closely comparable to that obtained for retention times with HPLC and capillary GC.

Advantages/Disadvantages of FITC

Under our conditions, FITC was found to be unsuitable at 10 ppb levels in DI water because of the relatively high background levels of by-products. Levels of 30 to 100 ppb in DI water and ground water appeared to be feasible. The limiting features of FITC included large by-product peaks induced by the analytes but not present in pure reagent blanks with analyte. Real samples induced production of even more background peaks. Although the derivatives themselves are detectable at levels of 10^{-9} M, background produced by the reagent with analyte and matrix prevented achievement of going below the 30 ppb level (about 10^{-7} M) in actual samples.

CONCLUSIONS

Unforeseen problems arising from more complex matrices must be assumed. Therefore, more extensive testing of derivatization approaches with potential matrices is certainly necessary. The distinct likelihood of unknown components in environmental matrices further complicates any scheme based on derivatization approaches. One possible approach to the problem of specificity is to employ multidimensional separations to the derivatives[33,34] or CE/MS.[35] This, of course, adds complexity to the analysis and may be reserved for positives in order to remove false positive findings.

NOTICE

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development, funded and performed the research described here. This work has been subjected to the Agency's peer review and has been approved as an EPA publication. The U.S. Government has the right to retain a non-exclusive, royalty-free license in and to any copyright covering this article. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

REFERENCES

[†] Enrollee in the Senior Environmental Employee Program, assisting the U.S. Environmental Protection Agency under a cooperative agreement with the National Association for Hispanic Elderly.

1. **Kirk-Othmer Encyclopedia of Chemical Technology**, Fourth Ed., J. Wiley & Sons, New York, USA,1991, Vol. 2.

2. W. H. Matchett, W. C. Brumley, J. Liq. Chromatogr., accepted, 1996.

3. **Test Methods for Evaluating Solid Waste (SW-846)**, Vol. 1B, U.S. Environmental Protection Agency, Washington, D.C., USA, 3rd ed., November 1986.

4. **Code of Federal Regulations**, 40 CFR Pt. 136, App. A, Method 624, July 1, 1988.

5. D. R. Knapp, **Handbook of Analytical Derivatization Reactions**, Wiley-Interscience, New York, USA, 1979.

6. H. Kataoka, S. Ohrui, Y. Miyamoto, M. Makita, Biomed. Chromatogr., **6**, 251-254 (1992).

7. M. J. Avery, G. A. Junk, Anal. Chem., **57**, 790-792 (1985).

8. T. Lundh, B. Aakesson, J. Chromatogr., **617**, 191-196 (1993).

9. Z. Wang, H. Xu, C. Fu, Sepu, **8**, 325-327 (1990) (Chinese).

10. M. -L. Henriks-Eckerman, T. Laijoki, J. Chromatogr., **333**, 220-224 (1985).

11. M. C. Gennaro, E. Mentasti, C. Sarzanini, V. Porta, Chromatographia, **25**, 117-124 (1988).

12. J. -K. Lin, S.-S. Wu, J. Chin. Biochem. Soc., **14**, 10-19 (1985).

13. K. D. Duong, H. Kolodziejczyk, D. D. Blanco-Gomis, R. Rosset, Analusis, **19**, 103-106 (1991).

14. K. D. Duong, H. Kolodziejczyk, R. Rosset, *Analusis*, **19**, 154-157 (1991) (French).

15. P. Simon, C. Lemacon, Anal. Chem., **59**, 480-484 (1987).

16. K. Hunter, D. Lindsay, Pestic. Sci., **12**, 319-324 (1981).

17. J. Lehotay, V. Rattay, E. Brandsteterova, D. Oktavec, J. Liq. Chromatogr., **15**, 307-318 (1992).

18. E. Mentasti, C. Sarzanini, O. Abollino, V. Porta, Chromatographia, **31**, 41-49 (1991).

19. A. J. Bourque, I. S. Krull, J. Chromatogr., **537**, 123-152 (1991).

20. R. Siegler, L. A. Sternson, J. Pharm. Biomed. Anal., **6**, 485-492 (1988).

21. Y. Nishikawa, J. Chromatogr., **392**, 349-360 (1987).

22. C. J. Elskamp, G. R. Schultz, Am. Ind. Hyg. Assoc. J., **47**, 41-49 (1986).

23. G. M. Murray, M. J. Sepaniak, J. Liq. Chromatogr., **6**, 931-938 (1983).

24. S. Baba, Y. Watanabe, F. Gejvo, M. Arakawa, Clin. Chim. Acta, **136**, 49-56 (1984) .

25. O. R. Idowu, G. O. Adequyi, J. Liq. Chromatogr., **16**, 2501-2518 (1993).

26. E. A. Arriaga, Y. Zhang, N. J. Dovichi, Anal. Chim. Acta, **299**, 319-326 (1995).

27. Y. Mechref, Z. El Rassi, Anal. Chem., **68**, 1771-1777 (1996).

28. M. Jung, W. C. Brumley, J. Chromatogr. A, **717**, 299-308 (1995).

29. W. C. Brumley, LC.GC, **13**, 556-568 (1995).

30. W. C. Brumley, J. Chromatogr. Sci., **33**, 670-685 (1995).

31. C. J. Miles, H. A. Moye, Anal. Chem., **80**, 220-226 (1988).

32. S. D. Gillman, A. G. Ewing, Anal. Chem., **67,** 58-64 (1995).

33. A. V. Lemmo, J. W. Jorgenson, Anal. Chem., **65**, 1576-1581 (1993).

34. W. D. Cole, R. R. Holloway, C. A. Keely-Templin, D. McManigill, V. K. Smith, T. A. Van d Goor, H. Yin, "Two-Dimensional Separations Using On-Line Liquid Chromatography-Capillary Electrophoresis,", paper presented at the 20th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Jun 16-21, 1996, San Francisco, CA, L2604.

35. K. J. Laidler, **Chemical Kinetics**, McGraw-Hill, New York, 1950, pp.56-57.

36. W. C. Brumley, W. Winnik, "Applications of Capillary Electrophoresis/ Mass Spectrometry to Environmental Analysis", in **Applications of Liquid Chromatography/Mass Spectrometry in Environmental Chemistry**, D. Barceló, ed., Elsevier, Amsterdam, 1996, C12, pp. 481-527.

37. W. C. Brumley, C. M. Brownrigg, J. Chromatogr., **646**, 377-389 (1993).

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DETERMINATION OF TOLUENE DIISOCYANATE IN AIR USING DI-n-BUTYLAMINE AND 9-N-METHYL-AMINOMETHYL-ANTHRACENE AS DERIVATIZATION REAGENTS

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ABSTRACT

Toluene diisocyanate (TDI) air levels were studied at two TDI-polyurethane (TDI-PUR) factories. One plant was manufacturing TDI-PUR foam and the other produced flame laminated textiles. Sampling was performed with impinger flasks containing 9-N-methyl-aminomethyl-anthracene in toluene as the isocyanate-derivatization reagent, followed by LC-UV, and impinger flasks containing di-n-butylamine (DBA) in toluene as the isocyanate-derivatisation reagent, followed by the derivatisation of amines with ethylchloroformate and determination using LC-UV-MS. The assessment of isocyanates in air using conventional LC-methods, involves a risk for underestimation of the air concentrations. Structural information about isocyanates and amines were obtained using the DBA-method with MS. In the samples, isocyanate concentrations up

to 57 mg m^{-3} of TDI were observed. TDA was found in air at concentrations up to 70 μg m^{-3}. The data of the high air concentrations observed at the two PUR-factories in this study is not part of an exposure assessment but they greatly stress the need to use fail-safe work environment hygiene.

INTRODUCTION

Exposure to isocyanates is a well-known occupational hazard. Exposure to high concentrations of isocyanates is known to produce irritations of the mucous membranes, and they have sensitising properties and may cause asthma and possibly a progressive impairment of pulmonary function.[1]

Isocyanates are reactive compounds that cannot feasibly be analysed as such. For the last 25 years the determination of isocyanates has been based essentially on derivatisation with various amine reagents due to the high reaction rate between isocyanates and amines. New amine reagents have been presented with the purpose of improving the chromatography, detection limits, and selectivity and for the determination of total reactive isocyanate groups.[2-6] Sampling has mainly been performed using impinger flasks or impregnated filters. At present, methods based on sampling on impregnated filters are used in many countries, as sampling and handling are simplified.

Comparative measurements of isocyanates in air have focused upon the comparison between wet and solvent-free sampling.[7-10] For particles collected on filters, there are risks for competitive reactions between the isocyanate and the amine reagent and other compounds present in the aerosol particle.[11] When sampling, using impinger flasks, it is known that some particle sizes are not efficiently collected.[12] The most frequently used isocyanate reagents have been compared to other reagents and about the same results are found using the same sampling technique. The comparisons have, however, most often been made for vapours or aerosols of monomeric isocyanates. The derivatisation yield, in complex isocyanate atmospheres and work atmospheres containing interfering compounds, has only sparsely been studied. The influence of several interfering compounds, known to be present in the work environment, on the derivatisation reaction in toluene spiked with TDI for two amine reagents, 9-N-methyl-aminomethyl-anthracene (MAMA) and 1-(2-methoxy-phenyl) piperazine (MOP), showed severe losses.[13]

In a series of papers[14-16] we have described the use of di-n-butylamine (DBA) as derivatisation reagent for isocyanates with further selective derivatisation of amines with ethyl chloroformate (ET) for determination of

isocyanates, aminoisocyanates and amines in a complex mixture in the work environment. DBA was found to be a robust reagent reacting fast with the isocyanate to protect the isocyanate groups and without known interferences.

In this paper, toluene diisocyanate (TDI) air levels at two polyurethane (PUR)-factories were studied using DBA as the derivatisation reagent. Comparative measurements were made with the MAMA reagent.

MATERIALS AND METHODS

Apparatus

LC-MS: A Quattro quadrupole mass spectrometer (MS) (VG-Organic, Altrincham, Cheshire, England) was used in the electrospray (ESP) mode, monitoring positive ions. For ESP the cone voltage was 50 V and the temperature of the ion source was 140°C. The MS was connected to a Rheos 4000 HPLC solvent delivery system (Flux Instruments, Karlskoga, Sweden). The flow of the pump was 400 μL min^{-1} connected to an Accurate splitter (LC Packings Amsterdam Netherlands) which gave a flow of 40 μL min^{-1} through the column. The column was a Sephasil C_{18} 1 x 250 mm with 5 μm particles (LC Packings Amsterdam Netherlands). A Kontron 433 capillary UV-detector with a 5μL cell volume (Kontron, Basel, Switzerland) was connected in series with the LC-MS system and the UV signal (λ = 240 nm) was recorded. One μL loop injection was made with a CMA/200 Refrigerated Autosampler (Carnegie Inc., Stockholm, Sweden). The gradient elution consisted of solution A 95/5 acetonitrile/water and B 95/5 water/acetonitrile. The elution started with 80% A and 20% B. After one minute the composition was linearly raised to 95% A and 5% B for 20 minutes and, was thereafter, isocratic for 20 minutes. Isocratic elution was performed with 95% A and 5% B. With the isocratic elution the $(M+1)^+$ ions of TDI and MDI, as internal standard, were monitored. For the gradient elution, 8 ions were monitored (m/z= 267, 350, 415, 433, 498, 582, 645, and 730 amu; dwell time 0.2 s), among those the $(M+1)^+$ ions of TDI, toluene aminoisocyanate (TAI), toluene diamine (TDA), and dimers and trimers of isocyanates and aminoisocyanates.

LC-UV: For the determination of MAMA-derivatives, a Millipore Waters (Millipore-Waters, Milford, MA, USA) 600 Multisolvent Delivery System, a Waters 712 WISP with variable injection volume, and a Waters 490 Programmable Multiwavelength detector (254 nm) was used. The LC column was a Hypersil BDS C_{18} 4.6 x 150 mm with 5 μm particles. Chromatograms were evaluated using a MAXIMA 820 Chromatography Workstation

(Millipore, Milford, MA, USA). The mobile phase (1.0 mL min^{-1}) consisted of 70% acetonitrile and 30% water with 3% triethylamine adjusted to pH 3 with phosphoric acid.

The samples were evaporated at 40°C in a Speed-Vac 290 centrifuge (Savant, Farmingdale, NY, USA).

Chemicals

Chemicals used were isooctane, toluene, and HPLC grade acetonitrile from Lab-Scan (Dublin, Ireland). Pyridine and $NaHCO_3$ were from E. Merck (Darmstadt, Germany); technical grade TDI (80/20 2,4-TDI/2,6-TDI) and ET from Janssen Chimica (Beerse, Belgium); MAMA and DBA from Aldrich Chemie (Steinham, Germany); 2,4- and 2,6-TDI derivatives of MAMA from Synthelec (Lund, Sweden); 2,4- and 2,6-TDI-DBA and 4,4′-methylenediphenyl diisocyanate-DBA (MDI-DBA) derivatives were synthesised at our laboratory.[14]

Standard Solutions

A standard solution of TDI was freshly prepared by dissolving accurately weighed amounts in isooctane. The solutions were further diluted in toluene to the appropriate concentrations. MDI-DBA was dissolved in acetonitrile and further diluted to appropriate concentrations.

Sampling and Storage

Air sampling was performed using an SKC universal sampler model 224 (SKC, PA USA). Thirty mL all glass midget impinger flasks, containing 10 mL of 0.01 mol l^{-1} DBA or 0.0001 mol l^{-1} MAMA in toluene were used. Air flows (1 l min^{-1}) were measured, both before and after each sampling, with a bubble flow meter, Gilian Gilibrator (Gilian Instr. Corp., USA). New MAMA solutions were prepared about one week before the comparative measurements were made. Samples derivatised with MAMA were stored in darkness in a refrigerator.

Work-up Procedure

DBA: After sampling, the solution, containing isocyanate urea derivatives and aromatic amines was evaporated to dryness in a vacuum centrifuge. The

dry residue was dissolved in 2 mL toluene. Carbamate esters were formed in a two phase derivatisation procedure by the addition of 1 mL of a 2 M carbonate buffer (pH 9.5), 50 µl ethyl chloroformate and 10 µl pyridine. The mixture was shaken for 5 min and 1.5 mL of the organic phase was then evaporated to dryness and dissolved in 1 mL acetonitrile containing MDI-DBA. The solution was then injected into the LC-UV-MS system.

MAMA: Samples containing the MAMA-derivatives were evaporated and dissolved in mobile phase and then injected into the LC-UV system.

Quantification

Calibration curves were obtained by adding known amounts of an 80/20 TDI mixture in the range of 21.8-2180 ng to toluene solutions of DBA and MAMA. Duplicate work-up, with double injections, were made for each concentration and the peak areas were calculated. Calibration curves were also obtained for the DBA and MAMA-derivatives of TDI. Samples expected to represent high concentrations of TDI were diluted before the first analysis. Samples outside the calibration curve were further diluted in mobile phase and reanalysed. LC-MS quantification was performed monitoring the $(M+1)^+$ ions of 2,4- and 2,6-TDI-DBA using MDI-DBA as internal standard. For the isocratic chromatographic conditions used, the sum of 2,4-and 2,6-TDI was obtained, as the 2,4- and 2,6-TDI-DBA peaks co-eluted. TAI and TDA were quantified in a few samples by LC-UV. In the LC-UV chromatograms the 2,4- and 2,6-TDI-MAMA peaks were well separated and quantified using external standards. For comparison between the DBA and MAMA methods the sum is given in the results.

Field Studies

Air samples were taken at two industrial PUR-plants. Plant A manufactured flexible polyurethane foam in continuous foam blocks. TDI was used in an 80:20 mixture of 2,4- and 2,6-TDI. TDI was mixed with a polyol component and was poured, with a foaming nozzle, onto moving kraft paper. In the well-ventilated curing tunnel a thin fog was observed and the light was slightly scattered. Eight workers were involved in the production. Personal respiratory devices were used at procedures expected to release high concentrations of TDI. Biomarkers and air levels of TDI have earlier been reported at this plant.[17]

Plant B used the PUR-foam produced in plant A and coated it with textile fabric by flame lamination. The surface of the PUR foam was partly melted by an open flame, immediately thereafter the textile fabric was applied. In the same room a control station was located where the workers controlled the ready made product and cut it in smaller pieces. TDI-PUR foam slices were occasionally joined by welding and in a laboratory the product was controlled for, among many things, its flammability. The lamination apparatus was rinsed and cleaned a few times every week. Biomarkers and air levels of TDI have earlier been reported at this plant.[18]

Sampling was simultaneously performed with two impingers placed next to each other, one containing DBA and the other one MAMA. The samples were taken at different locations at the two plants, even in places where no workers were normally present without personal respiratory devices and were not representative for exposure assessment. In plant A samples were taken at different locations in the curing tunnel (CT), one meter from the foaming nozzle (FN), in the "working area" (WA), and where they cut (CUT) the foam blocks into smaller pieces. In plant B samples were taken around the lamination apparatus (LA) and in the smoke produced during the lamination (SMOKE), in the control area (CA), and in the laboratory (LAB). Samples were also taken during lamination and cleaning (CL) and during welding work (WE). The sampling periods varied between 5-14 minutes. In plant A 22 sets of samples were taken and in plant B, 11.

RESULTS AND DISCUSSION

Quantification

Virtually linear calibration curves were obtained for TDI-DBA and TDI-MAMA in the range 21.8-2180 ng mL^{-1}. The correlation coefficients were 0.9998 (n=20) for MAMA using UV-detection and 0.9984 (n=12) for LC-MS determinations using MDI-DBA as IS. The calibration curves for diluted TDI-DBA- and TDI-MAMA- derivatives were virtually the same as calibration curves for TDI spiked DBA and MAMA toluene solutions.

Field study

The estimated air levels in plant A and plant B using DBA- and MAMA-reagents are displayed in Table 1 (Plant A) and 2 (Plant B) and Figures 1 and 2. The difference in concentrations between samples taken at the same place

Table 1

Comparative Measurements at Plant A

Location	MAMA-Measurements (TDI)	DBA-Measurements (TDI)
CT (2m)	0.12 mg m^{-3}	0.22 mg m^{-3}
CT (4m)	2.1 mg m^{-3}	8.9 mg m^{-3}
CT (4m)	2.0 mg m^{-3}	7.1 mg m^{-3}
CT (8m)	1.6 mg m^{-3}	9.7 mg m^{-3}
CT (8m)	0.17 mg m^{-3}	0.25 mg m^{-3}
CT (10m)	2.4μg m^{-3}	25 μg m^{-3}
CT (10m)	0.16 mg m^{-3}	4.5 mg m^{-3}
CT (10m)	16 μg m^{-3}	41 μg m^{-3}
CT (10m)	69 μg m^{-3}	44 mg m^{-3}
FN	18 μg m^{-3}	28 μg m^{-3}
FN	24 μg m^{-3}	36 μg m^{-3}
FN	0.61 mg m^{-3}	0.52 mg m^{-3}
FN	0.50mg m^{-3}	0.25 mg m^{-3}
2m from FN	4.2 μg m^{-3}	7.7 μg m^{-3}
FN and WA	42 μg m^{-3}	40 μg m^{-3}
WA	3.7 μg m^{-3}	8.1 μg m^{-3}
WA	3.6 μg m^{-3}	4.2 μg m^{-3}
WA	2.3 μg m^{-3}	2.9 μg m^{-3}
WA	1.2 μg m^{-3}	2.2 μg m^{-3}
WA	5.2 μg m^{-3}	6.1 μg m^{-3}
WA	0.5 μg m^{-3}	1.6 μg m^{-3}
CUT	8.7 μg m^{-3}	12 μg m^{-3}

CT - curing tunnel, the distances given are in metres from the foaming nozzle.
FN - foaming nozzle.
WA - working area.
CUT - cutter.

but at different times in plant A may be due to several factors. The TDI concentrations differ greatly depending on where the samples were taken, e.g. distance from the foaming nozzle and distance from the centre of the curing tunnel. Different foams types (densities) were produced and the emission of TDI will most likely be affected.

Table 2

Comparative Measurements at Plant B

Location	MAMA-Measurements (TDI)	DBA-Measurements (TDI)
LA	2.8 $\mu g\ m^{-3}$	1.2 $\mu g\ m^{-3}$
LA	2.6 $\mu g\ m^{-3}$	6.5 $\mu g\ m^{-3}$
LA	10 $\mu g\ m^{-3}$	9.6 $\mu g\ m^{-3}$
LA	17 $\mu g\ m^{-3}$	35 $\mu g\ m^{-3}$
LA	31 $\mu g\ m^{-3}$	25 $\mu g\ m^{-3}$
LA and CL	2.2 $\mu g\ m^{-3}$	3.5 $\mu g\ m^{-3}$
CA	1.0 $\mu g\ m^{-3}$	1.5 $\mu g\ m^{-3}$
CA	6.4 $\mu g\ m^{-3}$	4.8 $\mu g\ m^{-3}$
WE	27 $\mu g\ m^{-3}$	20 $\mu g\ m^{-3}$
LAB	2.8 $\mu g\ m^{-3}$	2.9 $\mu g\ m^{-3}$
SMOKE	2.4 $mg\ m^{-3}$	57 $mg\ m^{-3}$

LA - laminating apparatus.
CL - cleaning of laminating apparatus.
CA - control area.
WE - welding.
LAB - laboratory.
SMOKE - collected in the smoke during flame lamination

The foaming process varied between 1.5-2.5 hours and several foam qualities were produced in the same block foaming process. Typically one quality was produced for about 10-30 minutes. In plant B the work-procedures were about the same from one day to another. This is reflected by the smaller differences in estimated air concentrations for samples taken at the same place.

All samples (n=33) were divided into 2 groups, one with concentrations up to the Swedish occupational exposure limit (OEL) and the second with concentrations above the OEL. For the samples with the lower concentrations the DBA-method gave a significantly higher concentration (two tailed paired t-test 95% confidence level; n=23) as compared to the MAMA method (Figure 1). The same result was obtained when tested with a non-parametric test.

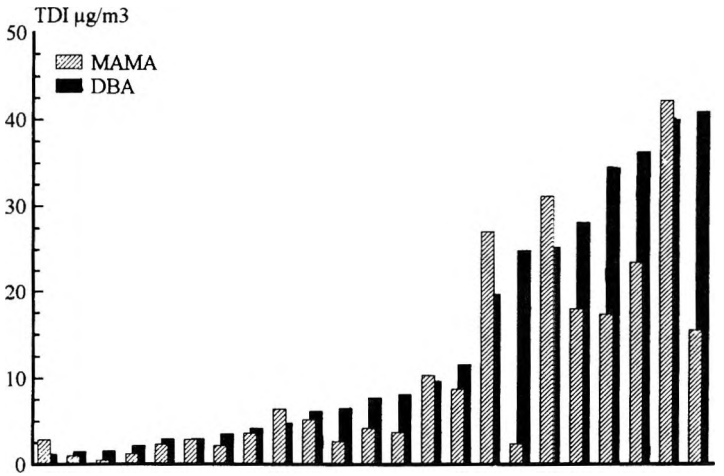

Figure 1. The results for the concentrations below the OEL for the DBA- and MAMA-reagent in impingers.

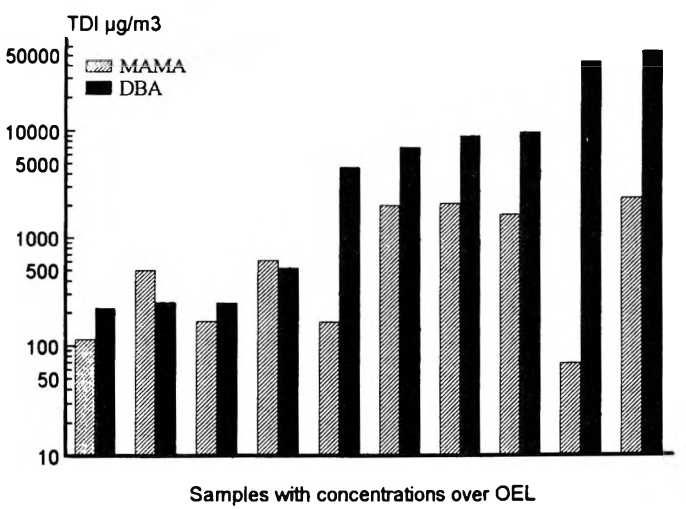

Figure 2. The results for the concentrations over the OEL for the DBA- and MAMA-reagent in impingers. Observe the logarithmic y-scale.

For the second group (Figure 2), no significant difference was seen when using the two tailed paired t-test at a 95% confidence level, probably due to the high standard deviation. By using the Wilcoxon signed rank test (two-tailed) a significant difference was seen.

When performing sampling followed by the immediate derivatisation of isocyanates, which is the basis of all isocyanate methods nowadays, it is of crucial importance that the amount of reagent is sufficient to derivatise and protect all isocyanate groups present. As we have earlier reported, the derivatisation reaction involving, e.g. MAMA or MOP is not instant and it may take hours to complete. The reaction rate depends on the concentration and properties of the reagent used.

If the amount of isocyanates greatly exceeds the amount of reagent, only a very small proportion will form the di-derivatised TDI-reagent-derivative and the mono-derivatised TDI-reagent-derivative and underivatised TDI will dominate. This will obviously result in a great underestimation of the TDI air concentration. Increasing the amount of the reagent will increase the concentration range that can be measured. This results, however, in problems due to the solubility of the derivatives formed and artefacts in the chromatograms. When using DBA, a higher concentration of the reagent can be used compared to other methods as the excess reagent is evaporated in the work-up procedure and will not disturb the chromatographic run. This results in faster reaction rates and a larger linearity of the method.

It is, therefore, not surprising that we saw high concentrations of TDI in some of the higher samples using DBA, where the MAMA samples showed low concentrations as the MAMA-reagent was present in too low concentrations. For the DBA samples indicating lower concentrations more similar results, but still significantly different, were obtained. The lower amounts of TDI-MAMA, as compared to TDI-DBA, indicate the presence of interfering compounds.

When derivatising isocyanates with DBA and amines with ET followed by LC-MS measurements, information of other kinds of isocyanates and amines found in the working atmospheres is also obtained. This is clearly demonstrated for the samples taken at the flame lamination plant where thermal degradation products of TDI-PUR dominated and isocyanate- and amine- containing compounds, other than TDI, were found. In one sample, taken when the workers were cleaning the flame lamination equipment, TDI was found in the magnitude of 3 $\mu g\ m^{-3}$, TAIs about 0.1 $\mu g\ m^{-3}$ and TDA about 70 $\mu g\ m^{-3}$ (Figure 3).

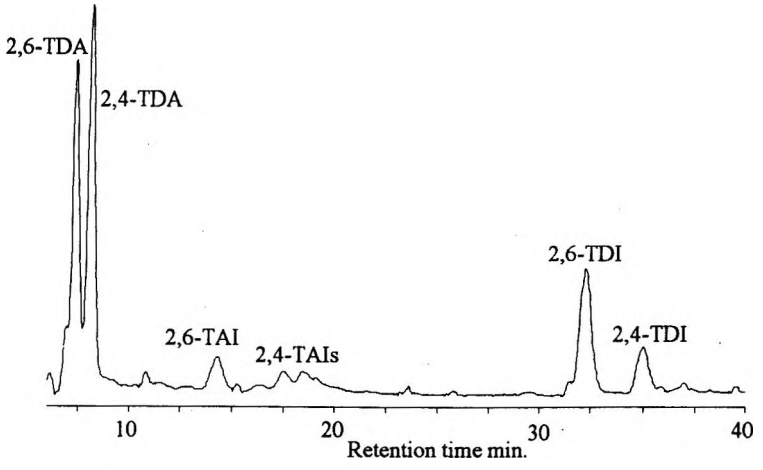

Figure 3. A total ion current chromatogram, when using gradient LC-elution and monitoring 8 ions, for a sample collected at plant B where the exposure was to thermal degradation products of TDI-PUR. This actual sample was collected when the workers were cleaning the flame lamination apparatus. The peaks correspond to about 3μg/m^3 TDI, 0.1 μg/m^3 TAI and 70 μg/m^3 TDA.

CONCLUSION

The assessment of isocyanates in air using conventional LC-methods involves the risk of underestimation of the air concentrations. As DBA can be used in much higher concentrations the risk of underestimating the air concentrations is much less and the linearity increases. Also the reaction rate increases several orders of magnitude.

The DBA method gives, in addition, structural information for other kinds of isocyanates and amines. The high air concentrations observed at the two PUR-factories in this study greatly stresses the need to use fail-safe work environment hygiene.

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REFERENCES

1. A. L. Kennedy, W. E. Brown, "Isocyanates and Lung Disease," in **Occupational Medicine: State of the Art Reviews**, Hanley & Belfus, Philadelphia, 7, 301-329 (1992).

2. J. Keller, K. L. Dunlap, R. L. Sandridge, Anal. Chem., **46**, 1845-1846 (1974).

3. H. L. Hardy, R. F. Walker, Analyst, **104**, 890-891 (1979).

4. C. Sangö, E. Zimerson, J. Liq. Chromatogr., **3**, 971-990 (1980).

5. C. J. Warwick, D. A. Bagoon, C. J. Purnell, Analyst, **106**, 676-685 (1981).

6. W. S. Wu, M. A. Nazar, V. S. Gaind, L. Calovini, Analyst, **112**, 863-866 (1987).

7. C. Rosenberg, P. A. Pfäffli, Am. Ind. Hyg. Assoc. J., 43,160-163 (1982).

8. K. Andersson, A. Gudéhn, J-O. Levin, C-A. Nilsson, Am. Ind. Hyg. Assoc. J., **44**, 802-808 (1983).

9. H. E. Myer, S. T. O'Block, V. Dharmarajan, Am. Ind. Hyg. Assoc. J., **54**, 663-670 (1993).

10. A. Maitre, A. Leplay, A. Perdrix, G. Ohl, P. Boinay, S. Romazini, J. C. Aubrun, Am. Ind. Hyg. Assoc. J., **57**, 153-160 (1996).

11. H. L. Hardy, Am. Ind. Hyg. Assoc. J., **45**, 30-32 (1984).

12. K. S. Booth, V. Dharmarajan, R. D. Lingg, W. C. Darr, "Polyurethane-Marketing and Technology-Partners in Progress," Proceedings of the SPI 28th Annual Technical/Marketing Conference pp10-16 (1984).

13. T. Brorson, C. Sangö, G. Skarping, L. Renman, Intern. J. Environ. Anal. Chem., **38**, 399-413 (1990).

14. M. Spanne, H. Tinnerberg, M. Dalene, G. Skarping, Analyst, **121**, 1095-1099 (1996).

15. H. Tinnerberg, M. Spanne, M. Dalene, G. Skarping, Analyst, **121**, 1101-1106 (1996).

16. H. Tinnerberg, M. Spanne, M. Dalene, G. Skarping, Analyst, **122**, 275-278 (1997).

17. H. Tinnerberg, M. Dalene, G. Skarping, Am. Ind. Hyg. Assoc. J. **58**, 229-235 (1997).

18. P. Lind, M. Dalene, H. Tinnerberg, G. Skarping, Analyst, **122**, 51-56 (1997).

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF CHLORPHENIRAMINE MALEATE IN TABLET FORMULATIONS

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ABSTRACT

A simple, rapid, specific, and reliable high performance liquid chromatographic assay of chlorpheniramine maleate in tablets has been developed. Reverse phase chromatography was conducted using a mobile phase of 0.05 M ammonium acetate and acetonitrile, (60%, v/v) pH 3.5 with UV detection at 265 nm. The % recovery and coefficient of variation from six placebo tablets containing 4 mg of chlorpheniramine maleate were 100.2, 98.25, and 0.4, 2.2 by the HPLC and B.P. 93 methods, respectively. Replicate regression analyses of three standard plots in the concentration range 0.5 - 20 mcg/mL obtained on three different days gave a correlation coefficient >0.9998 and a coefficient of variation of the slopes <1.54%.

The assay was precise within day and between days as indicated by ANOVA test. The average percentage recoveries from 10 replicate tablets of chlorpheniramine maleate was 101.35 and 100.464 of the label amount, and their coefficients of variation were 0.8 and 4.6 by the HPLC and B.P. 93. methods, respectively.

A comparison of the proposed HPLC and the B.P. 93. method, indicated that the HPLC method is more rapid, simple and reproducible. It is suggested that the proposed HPLC procedure could be used for routine quality control and dosage form assay of chlorpheniramine maleate.

INTRODUCTION

Chlorpheniramine maleate, is an antihistaminic agent which is effective in allergic and vasomotor rhinitis, allergic conjunctivitis, mild urticaria, angioedema and as adjunct therapy in anaphylactic shock.[1] It is widely used as an ingredient in proprietary antitussive formulations.[1]

Several methods for the quantitation of chlorpheniramine maleate in pharmaceutical dosage forms have been described, such as Proton NMR Spectroscopy,[2] second derivative Photoiodide-array spectroscopy,[3] near-infrared reflectance spectroscopy,[4] and various HPLC methods employing ion pairing and buffering.[5-12]

Most of the described HPLC procedures focused on the improvement of the separation of chlorpheniramine from other drug substances, rather than on the drug resolution from additives in various formulations containing the drug.

The B.P. 93 for the assay of chlorpheniramine maleate in tablets entails an ether-based extraction, followed by a UV spectrophotometric determination. All of these approaches require either lengthy sample preparation steps and/or non-specific quantitation.

The purpose of the present work was to develop a simple and direct HPLC assay for the quantitation of chlorpreniramine maleate in tablet formulations. The developed HPLC and the B.P. 93 procedures were compared with respect to their accuracy, simplicity and reproducibility.

EXPERIMENTAL

Chemicals and Reagents

Chlorpheniramine maleate[13] and propyl paraben[14] were used without further purifications. Acetonitrile,[15] methanol[15] and water were of HPLC grade. All other chemicals and reagents were of U.S.P. or A.C.S. quality and were used as received.

Instrumentation

A Waters HPLC system[16] was utilized consisting of the following components: Model 45 pump, the WISP model 710 B autosampler, the model 481 UV detector set at 265 nm at 0.02 AUFS, the model 730 data system. Chromatographic separation was accomplished using C_{18} column, 8 mm i.d. x 10cm μ Bondapack C_{18} column with 10μm packing.

Chromatographic Conditions

The eluting medium consisting of 0.05 M ammonium acetate and acetonitrile (60% v/v) adjusted to pH 3.5 with glacial acetic acid, was prepared and degassed by bubbling helium gas for 5 min. prior to use. Column equilibrium with the eluting solvent was established by pumping the mobile phase at a rate of 0.2 mL/min. overnight. The flow rate was set at 1.8 mL/min. during analysis. The chromatogram was recorded and integrated at a speed of 0.3 cm/min.

Internal Standard

A stock solution of propyl paraben containing 10 mg in 100 mL methanol was prepared weekly and stored at 4°C.

Standard Solution of Chlorpheniramine Maleate

A stock solution of chlorpheniramine maleate was prepared by dissolving 10 mg of chlorpheniramine maleate in 10 mL water. Ten aliquots equivalent to 0.5, 1, 2, 4, 6, 8, 10, 12, 15 and 20 mcg of chlorpheniramine maleate were added to 1 mL volumetric flask. After an aliquot of the internal standard

equivalent to 2 mcg were added, the flasks were brought to volume by water and thoroughly mixed. Twenty μL of the standard solutions were injected onto the column for analysis. The peak area ratio of the drug internal standard was plotted against the standard chlorpheniramine maleate concentrations. Least square linear regression analysis was performed to determine the slope, y-intercept, and the correlation coefficients of the standard plots.

Sample Preparation

Individual tablets were pulverized using a mortar and pestle, and completely transferred to 100 mL volumetric flask. The volume was adjusted with water and the flask was mechanically shaken for five min. Five mL of the solution were centrifuged at 3000 r.p.m. in a centrifuge tube for 5 min. Three hundred μL were transferred to a one mL volumetric flask containing 20 μL of propyl paraben stock solution, and diluted to the volume with acetonitrile. Twenty μL were loaded into the sample loop for chromatography. Ten replicate commercial tablets of chlorpheniramine maleate were analyzed for statistical evaluation of the assay.

Quantitation

The amount of chlorpheniramine maleate per tablet was determined from the following equation:

$$Q = [R/A + B] \times \text{dilution factor}$$

were Q is the mg chlorpheniramine maleate per tablet, R is the peak area ratio (drug/internal standard), A is the slope of the calibration curve and B is the y-intercept.

Recovery of Chlorpheniramine Maleate from the Fabricated Placebo Tablets

Placebo samples containing 4 mg of chlorpheniramine maleate and 50 mg each of starch and lactose were prepared and subjected to the described HPLC assay and the B.P 93 method to compare the accuracy and precision of the methods.

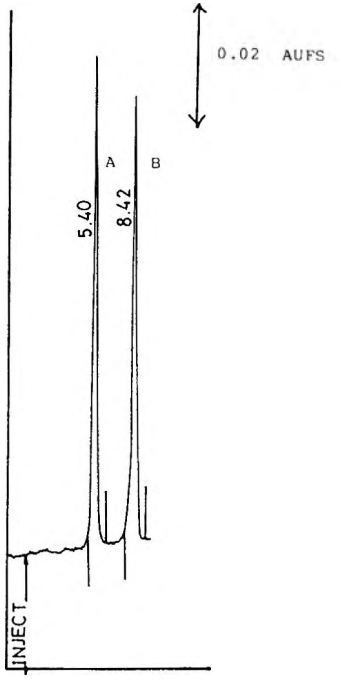

Figure 1. Chromatogram of chlorpheniramine maleate tablet.
Key: A- Chlorpheniramine maleate, B- Propyl paraben

RESULTS AND DISCUSSION

Figure 1, shows a typical chromatogram obtained following analysis of chlorpheniramine maleate in tablets. Using the chromatographic conditions described, chlorpheniramine maleate and the internal standard, propyl paraben were well separated and their retention times were 5.42 and 8.45 min., respectively. For both compounds, sharp and symmetrical peaks were obtained with good baseline resolution and minimal tailing, thus facilitating the accurate measurement of the peak area ratio. No interfering peaks were found in the chromatogram due to tablet excipients. Figure 2, shows a calibration plot for the peak area ratio of varying amounts of chlorpheniramine maleate (0.5-20 mcg/mL) to a constant amount of propyl paraben (2 mcg/mL). The plots were linear ($r = 0.99985$) and the regression analysis of the data gave the slope and intercept as:

$$Y = 0.0677 x - 0.0033$$

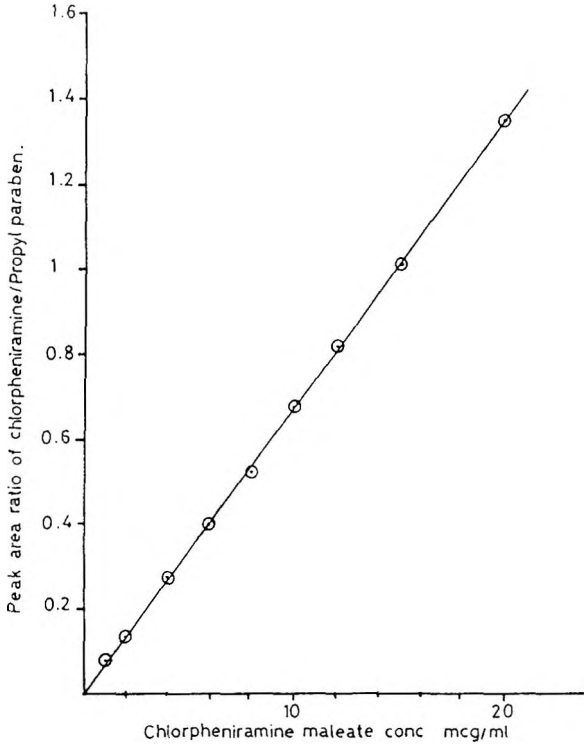

Figure 2. Standard calibration plot of chlorpheniramine maleate.

where Y and X are the peak area ratio and chlorpheniramine maleate concentration, respectively. Three replicate analyses of chlorpheniramine maleate at a concentration of 0.5 - 20 mcg/mL were performed at three different days over one week period. The results of this evaluation are summarized in Table 1.

The average correlation was higher than 0.9998 and the coefficient of variation of the slopes of the three lines was <1.54%. Analysis of variance of the data showed no detectable difference in the slopes of the three standard plots ($F=3.2$, $P > 0.01$). The similarities in the slopes and the high correlation coefficients indicate that the assay possesses excellent reproducibility and linearity. Thus, the method should be accurate and precise within the assay day as well as between assay days.

Table 1

Regression Analysis of the Three Standard Plots of Chloropheniramine Maleate

Standard [a]	Slope [b]	Intercept[b]	Correlation Coefficient[b]
1	0.06777	- 0.00330	0.99985
2	0.07024	- 0.00450	0.99977
3	0.06976	- 0.00399	0.99989

[a] Obtained in 3 different days

[b] The mean of 3 determinations at each drug concentration.

Precision and Accuracy

Six placebo tablets containing 50 mg each of lactose and starch and 4 mg chlorpheniramine maleate were assayed for four consecutive days for intra- and interday precision studies. The average recovery shown in Table 2 was (4.008 mg) with the coefficient of variation 1.3156%. Estimation of day to day and within day precision were calculated by ANOVA test. The calculated F values, $F_{0.05}$ (5, 15) = 0.1873 and $F_{0.05}$ (3, 15) = 2.003 were smaller than the table values $F_{0.05}$ (5, 15) = 2.44 and $F_{0.05}$ (3, 15) = 2.24 respectively.

Thus, it was concluded that, there was no significant difference for the assay which was tested within day and between days.

Recovery from Placebo Samples

Table 3 compares the average recoveries of chlorpheniramine maleate from placebo samples containing 4 mg chlorpheniramine maleate and 50 mg each of lactose and starch, using the HPLC and the B.P. 93 methods. The average recoveries were 4.008 and 3.93 for the HPLC and the B.P. 93 methods, respectively, and their respective relative standard deviations were 0.4 and 2.2.

The values obtained by the HPLC method compared favorably with those obtained by the B.P. 93 procedure. The difference may have been caused by a loss of sampling during several extraction steps in the B. P method.

Table 2

Analysis of Variance for Intra- and Inter day Precision

Day/Assay	1	2	3	4	5	6
1	3.97	4.02	4.03	4.08	3.94	4.08
2	3.96	4.03	4.06	4.01	3.92	4.02
3	3.91	4.02	4.03	4.05	4.03	4.07
4	4.012	3.94	4.05	3.96	4.08	3.93

Mean = 4.008 mg
SD = 0.052735
C.V.% = 1.3156

ANOVA Test

Source of Variation	DF	Sum of Squares	Mean of Squares	F Ratio	P
Within day	5	0.0028471	0.000569	0.1873	0.05
Between day	3	0.0182758	0.00609	2.003	0.05
Error	15	0.0456209	0.00304		
Total:	23				

Table 3

Average Recoveries of Chlorpheniramine Malete from Spiked Placebo Samples by the HPLC and B.P. 93 Procedures

Method	n	Amount Added mg	Amount Recovered mg	CV%
B.P. 93	6	4	3.930	2.2
HPLC	6	4	4.008	0.4

Table 4

Recovery of Chlorpheniramine Maleate from Commercial Tablets by HPLC and B.P. 93 Procedures

Method	n[a]	Mean % Recovery	SD	%CV
B.P. 93	10	100.464	4.6200	4.6
HPLC	10	101.350	0.8108	0.5

[a] Number of replicates

The B.P. 93 assay for chlorpheniramine maleate is very time consuming, as it requires shaking with 0.05 M sulphuric acid for 5 min, then extraction with ether; the etherial layer is extracted twice with sulphuric acid, then the acidic extract is rendered alkaline and re-extracted twice with ether. The etherial layer is washed and re-extracted 3 times with sulphuric acid, followed by spectrophotometric determination at 265 nm. As such, the method requires many hours of analytical time to analyze 6 tablets, compared to <1 hr by the proposed HPLC procedure.

Analysis of Chlorpheniramine Maleate Tablets

Table 4 presents the results comparing mean % recoveries, % CV, and SD of chlorpheniramine maleate tablets by the HPLC and the B.P. 93 assay procedure.

The average % recovery and coefficients of variation were 101.350, 100.464, and 0.8, 4.6 for the HPLC and B.P 93 procedures, respectively. The requirements for content uniformity of chlorpheniramine maleate tablets in the B.P. 93, specify that the potency must fall within 92.5 - 107.5% of the label claim. Thus, the tablets selected randomly in this determination met the B.P. 93 requirement for the content uniformity.

The stability indicating nature of the assay has not been demonstrated in this study, since no sign of degradation was observed by TLC after subjecting the drug solution (pH 3 and 9) at 70°C for 2 hr, which was also evident from the absence of any additional peaks in the chromatogram.

CONCLUSION

The HPLC method developed in this study has the advantage of simplicity, precision, and reliability. It allows for the direct determination of chlorpheniramine maleate bypassing several tedious steps involved in the B.P. 93 method. It should be useful for routine analytical and quality control assay of chlorpheniramine maleate in dosage forms.

REFERENCES

1. **Remington's Pharmaceutical Sciences**, 18th Ed., Mack Publishing, Easton, PA, 1990, p.1125.

2. G. M. Hanna, C. A. Lawcam, J. Pharm. Biomed. Anal., **11(9)**, 855-859 (1993).
3. J. L. Murtha, T. N. Julian, G. W. Radebaugh, J. Pharm. Sci., **77(8)**, 715-718, (1988).

4. E. W. Ciurcazk, R. P. Torlini, Spectroscopy, **2(3)**, 41-43, (1987).

5. N. R. Raju, P. Srilakshimi, U. M. Krishna, Indian-Drugs, **29(9)**, 408-411 (1992).

6. S. Akiyama, K. Nakashima, K. Yamada, N. Shirakawa, Bull. Chem. Soc. Japan., **64(10)**, 3171-3172 (1991).

7. A. W. Lau, K. Chan, Y. K. Lau, W. C. Wong, J. Pharm. Biomed. Anal., **7(6)**, 725-736 (1989).

8. T. A. Biemer, J. Chromatogr., **410(1)**, 206-210 (1987).

9. S. I. Sa-Sa, K. A. Momani, I. M. Jalal, Microchem. J., **36(3)**, 391-398 (1987).

10. D. R. Leidemann, LC-GC, **5(6)**, 488-489 (1987).

11. V. Dasgupta, J. T. Jacob, Drug Dev. Ind. Pharm., **13(1)**, 113-126 (1987).

12. S. B. Mahato, N. P. Sahu, S. K. Maitra, J.Chromatogr., **351(3)**, 580-584 (1986).

13. Sankyo Co., Tokyo, Japan.

14. Eastman Kodak Co., Rochester, N.Y., U.S.A., p. 14650.

15. J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.

16. Waters Associates, Milford, MA., 01757, U.S.A.

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SEPARATION OF ALL ISOMERS OF PYRIDINEDICARBOXYLIC ACIDS BY ION-PAIRING CHROMATOGRAPHY

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ABSTRACT

The separation and quantitation of the six isomers of pyridinedicarboxylic acids (PDAs) can be achieved rapidly utilizing High Pressure Liquid Chromatography (HPLC). Optimal separation is accomplished using reverse phase chromatography with an aqueous mobile phase mantained at a pH of 7.3 by 153.2 mM phosphate buffer, containing 15 mM tetrabutylammonium phosphate as ion-pairing agent and 2 mM EDTA as mobile phase additive.

The influence of the eluent parameters on retention of PDAs has been investigated in order to elucidate the separation mechanisms involved in the ion pair chromatography of these ionizable substances.

INTRODUCTION

The pyridine derivatives, whose separation is described in this paper, are very important in biochemistry. The six isomers (2,3-; 2,4-; 2,5-; 2,6-; 3,4-; 3,5-PDAs) have been studied as a class of compounds in order to investigate their role in the neurotransmission process as triggers of burst firing,[1] and in altered physical state of erythrocyte membrane proteins responsible for hippocampal neurotoxicity;[2] 2,5- and 2,3-PDA's are reported to have occurred as a result of the putrefaction of human viscera.[3]

Moreover, 2,3-; 2,5-; 2,6-; 3,5-PDA's are important compounds obtained by oxidative degradation of humic acids derived from coal.[4]

Earlier analytical work mostly regarded quinolinic acid (2,3-PDA), whose presence in biological samples was determined in order to monitor altered aromatic acids metabolism. The determination of quinolinic acid has been carried out by gas chromatography,[5,6] gas chromatography-mass spectrometry,[7,8] ion-exchange liquid chromatography,[9] liquid chromatography with fluorometric detection,[10] thin layer chromatography,[11] and radioenzymatic assay.[12] 2,6-PDA has been characterized as a fungal metabolite by HPLC.[13]

Previous attempts to separate some of the isomers relied on the use of ion-exchange chromatography on anionic resins[4] and on the use of HPLC with an amino stationary phase,[14] but no attempts to separate all the isomers have ever been made.

Here, a simple and rapid separation procedure, based on ion-pairing in reverse phase HPLC with a buffered isocratic elution is reported.

The chromatographic behaviour of PDA isomers was studied in order to elucidate their separation mechanism.

MATERIALS AND METHOD

A 1090 series II Hewlett Packard high pressure liquid chromatograph equipped with a Rheodyne sample valve injector with 25 mL loop (Model 7125) was used.

The analyses were run at room temperature under isocratic elution condition. The eluent flow-rate was 0.9 mL/min. The detector was operated at 254 nm.

All experiments were carried out with a commercial stainless steel column (25 cm x 4.6 mm I.D.), packed with 5 μm Res Elut 5 C_{18}, for reverse phase chromatography, purchased from Varian.

A Wescan conductivity detector (Model 213-505) monitored the eluent conductivity.

All the isomers of pyridinedicarboxylic acid, uracil, EDTA disodium salt, tetraethyl ammonium bromide, and tetrabutylammonium phosphate were purchased from Aldrich; potassium dihydrogen phosphate and disodium monohydrogen phosphate were purchased from Merck; all chemicals were of the best available quality and used without further purification. Water was produced by a Milli Q 185 system (Millipore).

The best chromatographic performance was obtained with an aqueous mobile phase containing 15 mM tetrabutylammonium phosphate and 2 mM EDTA; the pH was manteined at 7.3 by 153.2 mM phosphate buffer. Eluent parameters were varied and the k' values of PDA's in each system determined (uracil retention time was used as column hold-up time).

All isomers were dissolved in mobile phase at a final concentration of 0.20 μg/μl each for 3,4-; 2,5-; 2,6-; 3,5-PDA's, 0.25 μg/μl for 2,3-PDA and 0.30 μg/μl for 2,4-PDA.

All solutions were filtered through a 0.2 μm pore size cellulose nitrate filter (Whatman).

Prior to use, the reverse phase column was equilibrated with the solvent system to be used in the separation for 30 min. Equilibration was established by obtaining similar results in duplicate runs at a 15 min interval.

RESULTS AND DISCUSSION

Preliminary attempts to separate PDA isomers by reverse phase ion pair chromatography, without EDTA as mobile phase additive, met with difficulty due to the poor peak shape of some isomers. The chromatographic peak of 2,4-PDA was the most tailed and asymmetric (AF_{10} = 10). A severe retention time increase upon dilution was observed for this isomer, so that simultaneous quantitation of all the PDAs was impossible. The tailing bettered with increasing medium basicity but was still very unsatisfactory at pH 7.3. The pH of the buffered mobile phase was chosen to be 7.3 as a compromise between the necessary predominance of the dianionic protolytic

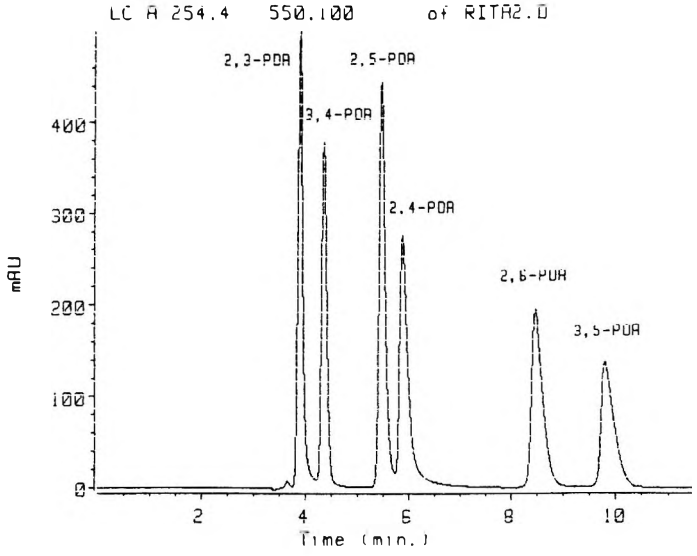

Figure 1. Separation of all PDAs isomers. Condictions: column, 5 μm Res Elut 5 C_{18} (25 cm x 4.6 mm I.D.); mobile phase: 153.2 mM phosphate buffer, pH 7.3 , 15 mM tetrabutylammonium phosphate and 2 mM EDTA.

species[15-16] and the pH compatibility of silica. A pH higher than 7;3 is not advisable for routine analysis because quaternary ammonium salts in an alkaline medium tend to be harmful for silica of the bonded stationary phase base.

Figure 1 illustrates the excellent separation of the isomers achieveable in the presence of tetrabutylammonium phosphate 15mM and EDTA 2mM: all of the isomers were chromatographed in a matter of minutes. Since some of the PDA's isomers readly form complexes with metal impurities in the chromatographic system, EDTA was included in the mobile phase in order to minimize tailing[17-19] and retention time increase upon dilution. Its addition to the mobile phase improves the partition isotherms parameters and this results in a dramatic improvement of the chromatographic performance.

Mobile phase buffer and tetrabutylammonium concentration were important parameters for optimizing the chromatographic performance; the effect of their variation on capacity factors evidenced the complex nature of ion-pair equilibria.

Table 1

Effect of Ion-Pair Reagent Concentration on the Capacity Ratio of PDAs

	Tetrabutylammonium Concentration		
	15mM	**10mM**	**5mM**
k' 2,3-PDA	0.202	0.168	0.123
k' 3,4-PDA	0.346	0.292	0.227
k' 2,5-PDA	0.687	0.586	0.476
k' 2,4-PDA	0.814	0.679	0.562
k' 2,6-PDA	1.616	1.459	1.226
k' 3,5-PDA	2.029	1.810	1.48

Table 1 details the dependence of k' at a fixed EDTA (2mM) and buffer concentration (152.2 mM) upon tetrabutylammonium concentration. The regular increase of the capacity factors with tetrabutylammonium concentration points to the formation of ion pairs between analyte dianions and the lipophilic reagent. Since the dependence of k' upon its concentration is not altered by removing EDTA from the mobile phase, it follows that the ion pairing between PDA isomeric ions and tetrabutylammonium is operating with or without EDTA in the mobile phase at a pH of 7.3.

The best selectivity was achieved with the addition of 15 mM tetrabutylammonium phosphate to the mobile phase (Figure 1). Higher concentration of the lipophilic reagent did not appreciably improve chromatographic retention, hence 15mM represents a kind of saturation concentration. The advantage of working at this saturation level is that we have very high capacity factors, which are not very sensitive to small errors in tetrabutylammonium concentration.

Tetrabutyl ammonium was selected as ion pair agent because its high hydrofobicity served to increase retention. When tetrabutyl ammonium was replaced by tetraethyl ammonium, only three peaks could be resolved, thereby indicating that some isomers co-eluted as a result of lower retention due to the lesser lipophilicity of such an ion pairing agent.

Table 2 shows that k' values for PDAs, at fixed tetrabutylammonium (15mM) and EDTA (2mM) concentrations, decrease regularly with increasing buffer concentration. This can be explained by assuming that a stationary phase is being increasingly blocked by ion-pairs from

Table 2

Effect of Phosphate Buffer Concentration on the Capacity Ratio of PDAs

	Phosphate Concentration		
	76.1 mM	**152.2 mM**	**228.3 mM**
k' 2,3-PDA	0.323	0.202	0.158
k' 3,4-PDA	0.483	0.346	0.295
k' 2,5-PDA	0.973	0.687	0.614
k' 2,4-PDA	1.018	0.814	0.719
k' 2,6-PDA	2.070	1.616	1.403
k' 3,5-PDA	2,417	2.029	1.855

tetrabutylammonium and buffer ions. The experimental results can be explained by the physical-chemical retention model proposed by Bidlingmeyer,[20-21] according to which the lipophilic reagent dynamically adsorbs at the surface of stationary phase, forming an electrical double layer. The retention of the sample results from both electrical and Van der Waals forces.

High phosphate concentration limits the formation of ion-pairs between analyte and tetrabutylammonium because buffer ions are involved in a competing equilibrium with the analyte ones for adsorbed lipophilic ions, and this causes a retention time decrease as is seen from Table 2. Adsorption of the lipophilic reagent at the surface of stationary phase is consistent with the long time the eluent conductivity took to decrease when the column was washed with Milli Q water after use: where tetrabutylammonium phosphate was not present in the same mobile phase, the washing time was considerably shorter.

The final parameter investigated was the limit of detectability via absorption of UV light. By calibrating the integrator with known concentrations of the various compounds and obtaining peak height for 2,4-PDA and peak area for all the other isomers it was possible to estimate the ultimate limits of detection. For 2,4-PDA the level of detection was 8.0 ng; for 3,5-PDA this value was 2.5 ng, while for 2,3-; 3,4-; 2,5-; 2,6-PDA it was 1.5 ng.

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REFERENCES

1. M. J. Peet, K. Curry, D. S. K. Magnuson, H. McLennan, Neuroscience, **22**, 563-571 (1987).

2. A. J. Nonneman, T. Elder, B. T. Farmer,II, D. A. Butterfield, Biochem. Arch., **4**, 209-215 (1988).

3. T. H. Batchelor, H. M. Stevens, J. Forens. Sci. Soc., **18**, 209-229 (1978).

4. C. Davies, R. D. Hartley, G. J. Lawson, J. Chromatogr., **18**, 47-52 (1965).

5. P. A. Toseland, Clin. Chim. Acta, **25**,185-186 (1969).

6. B. A. Chamberlin, C. C. Sweeley, Clin. Chem., **33 (4)**, 572-576 (1987).

7. G. Lombardi, G. Moneti, V. Carlà, F. Moroni, Ann. Ist. Super. Sanità, **20**, 79-84 (1984).

8. J. D. Shoemaker, W. H. Elliott, J. Chromatogr., **562**, 125-138 (1991).

9. J. I. Patterson, R. R. Brown, J. Chromatogr., **182**, 425-429 (1980).

10. K. Mawatari, K. Oshida, F. Iinuma, M. Watanabe, Anal. Chim. Acta, **302**, 179-183 (1995).

11. H. Taguchi, S. Koyama, Y. Shimabayashi, K.Iwai, Anal. Biochem., **131**, 194-197 (1983).

12. A. C. Foster, E. Okuno, D. S. Brougher, R. Schwarcz, Anal. Biochem., **158**, 98-103 (1986).

13. J. C. Frisvad, U. Thrane, J. Chromatogr., **404**, 195-214 (1987).

14. J. Královsky, M. Kalhousová, K. Placek, Chem. Prum., **10**, 537-540 (1987).

15. L. Thunus, Il Farmaco - Ed. Sc., **24**, 1082-1104 (1969).

16. L. Thunus, J Pharm. Belg., **22**, 379-386 (1967).

17. J. H. Knox, J. Jurand, J. Chromatogr., **186**, 763-782 (1979).

18. G. D. Mack, R. B. Ashworth, J. Chromatogr. Sci., **16**, 93-101 (1978).

19. G. Chevalier, C. Bollet, P. Rohrbach, C. Risse, M. Claude, R. Rosset, J. Chromatogr., 124, 343-349 (1976).

20. B. A. Bidlingmeyer, S. N. Deming, W. P. Price, Jr., B. Sachok, M. Petrusek, J. Chromatogr., **186**, 419-434 (1979).

21. B. A. Bidlingmeyer, J. Chromatogr. Sci., **18**, 525-539 (1980).

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A SENSITIVE AND SPECIFIC METHOD FOR ASSAY OF SERTINDOLE AND ITS METABOLITES IN HUMAN, RAT, DOG, AND MOUSE PLASMA USING HPLC WITH TANDEM MASS SPECTROMETRIC DETECTION

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ABSTRACT

A sensitive and specific assay was developed for the concurrent determination of sertindole, a new antipsychotic agent, and its two major metabolites, Lu 28-092 and Lu 25-073, in plasma. The method employs reverse-phase high performance liquid chromatography (HPLC) with tandem mass spectrometric detection. Chromatographic separation is necessary because sertindole and the Lu 28-092 metabolite differ by only two mass units. Results comparable to those found in human plasma were obtained in dog, rat, and mouse plasma, indicating the method is robust and relatively insensitive to matrix constituents.

INTRODUCTION

Sertindole is a new antipsychotic drug with demonstrated efficacy in patients with schizophrenia, but without the adverse effects associated with conventional therapeutic agents. Efficacy is thought to be derived from the drug's selective effects on mesolimbic but not nigrostriatal dopaminergic neurons.[1-5] Two major metabolites of sertindole are formed *in vivo* after oral administration of the drug: dehydro-sertindole (Lu 28-092) and nor-sertindole (Lu 25-073). Because of the importance of the pharmacokinetic characteristics of these metabolites, they were included in the development of an assay to be used in toxicological studies and clinical trials.

Due to its high potency, sertindole is administered at relatively low doses, resulting in low circulating levels of the parent compound and its metabolites. Therefore, a method with high sensitivity and selectivity is required for the quantitation of these analytes. Initially, a reversed-phase HPLC method with ultraviolet detection was developed. The detection limit was approximately 1 ng/mL, for 1 mL of plasma extracted. Multiple liquid/liquid extraction steps were required for sample clean-up and the method suffered from poor reproducibility and tedious work-up procedures. Later, a major improvement in detection limits was achieved using normal-phase HPLC separation with fluorometric detection.

This method was further improved by Tzeng et al.,[6] using a simplified solid-phase extraction step; the fluorometric method had a lower limit of quantitation of 25 pg/mL, with a linear dynamic range up to 4 ng/mL. However, the method was susceptible to interferences from the serum or plasma matrix. Moreover, the Lu 28-092 metabolite eluted as a broad peak in this system, and the Lu 25-073 metabolite was a nonquantifiable late eluting peak.

The method described in this paper was developed in order to measure all three analytes in a single chromatographic run. The method was validated in human plasma and that of three animal species, the rat, dog, and mouse. The assay is based on reverse-phase HPLC with tandem mass spectrometric (MS/MS) detection. The sample clean-up procedure is similar to that described by Tzeng et al.,[6] but includes modifications to permit the co-extraction of the two metabolites as well as the parent compound.

Our experiments demonstrate that atmospheric pressure ionization with tandem mass spectrometry as a detection technique results in good sensitivity for the analytes, along with a short analytical run time which provides high sample throughput capability.

MATERIALS AND METHODS

Reagents and Chemicals

Absolute ethanol was obtained from McCormick (Weston, MO). Glacial acetic acid 99.99% was obtained from Aldrich (Milwaukee, WI). All other chemicals and solvents were HPLC grade obtained from EM Science (Gibbstown, NJ). Eluent and reconstitution solvents were filtered through a 0.2 μm-pore nylon membrane before use. Reference materials: Sertindole was synthesized by Abbott Laboratories; Lu 28-092, Lu 25-073, and Lu 26-009 (internal standard) were obtained from Lundbeck A/S (Copenhagen).

Extraction

Analytes were extracted from plasma using solid-phase extraction. The C_8 solid-phase extraction column (Bond Elut 200 mg, Varian Associates, Harbor City, CA or Isolute IST-EC, Jones Chromatography, Lakewood, CO) was preconditioned with two washes of acetonitrile (2 mL each), then three washings of methanol (3 mL each), followed by two washes of purified water (2 mL each) (MilliQ UV system, Millipore Corp., Bedford, MA). Care was taken to avoid drying the bed, which can disrupt analyte/ solid-phase binding interactions. Plasma was pipetted into a clean borosilicate test tube; extraction volumes were 1000, 500, 200, and 100 μL for human, dog, rat, and mouse plasma, respectively. Samples were supplemented with a phosphate buffer (10 mM dibasic potassium phosphate; pH 8.5) to aid in sample transfer. Then 50 μL of a 100 ng/mL solution of the internal standard (Lu 26-009) in ethanol was added. The tube was vortexed briefly to blend the internal standard and matrix.

The sample was then loaded onto the preconditioned solid-phase column using a gentle vacuum (1 to 2 min residence of matrix on the column). The extraction column was washed with three rinses of purified water (2 mL each) followed by three rinses of acetonitrile (1 mL each). The column was then dried by continuous application of a vacuum, drawing air through the column for 1 to 2 min. Analytes trapped onto the column were eluted with two 1-mL aliquots of acidified methanol (2% glacial acetic acid, 98% methanol; v/v). The eluate was collected into a silanized borosilicate tube and evaporated to dryness under a gentle stream of air or nitrogen, in a water bath maintained at 25° to 35°C. The dried residues were reconstituted into 50 μL of the mobile phase, transferred to a 300-μL silanized micro vial, capped, and centrifuged at approximately 3000 rpm for about 5 min. The reconstituted extract was maintained at 5°C or less until injection.

Chromatography

Analytes were separated by reverse-phase chromatography on a YMC basic column (150 x 2.1 mm; 5 micron, YMC Inc., Wilmington, NC). The analytic column was protected by a guard cartridge. The mobile phase was a mixture (v/v) of 42% acetonitrile and 58% of a 100 mM solution of ammonium acetate in purified water (MilliQ UV). The pH of the mobile phase was adjusted to 6.8 with glacial acetic acid. The mobile phase was pumped using a syringe pump (Model 500D, Isco, Lincoln, NE) at a flow rate of approximately 400 μL/min. Column back pressure ranged between 1500 and 2000 psi at this flow rate. Samples (20 μL) were injected using a CMA-200 refrigerated microsampler (CMA/Microdialysis AB, Stockholm, Sweden). Samples were maintained at a temperature of approximately 5 5°C until injection. Run time was approximately 6 min.

Mass Spectrometric Detection

Analytes in the HPLC effluent were detected and measured using the API III mass spectrometer (SCIEX, Thornhill, Ontario, Canada). The chromatographic system was coupled to the mass spectrometer using the atmospheric pressure ionization source (Heated Nebulizer, SCIEX). Analytes were ionized in the positive ion mode at a source temperature of approximately 450°C. Nitrogen was used both as the nebulizing gas (approximate pressure 80 psi) and as the auxiliary sheath gas (flow rate 2 to 3 L/min). Nitrogen for nebulizing and auxiliary flow was generated by a gas generator (Nitrox NG-4000, Peak Scientific, Buffalo Grove, IL). The purity of the nitrogen was 95% to 99%, depending on the flow rate. The curtain gas was ultra high purity nitrogen (99.999%, AGA Gas Inc., Maumee, OH), at a flow rate of approximately 1.8 L/min. Analyte specific detection was performed in the multiple reaction monitoring mode. Ultra high purity argon (99.999%, AGA Gas Inc.) was used as the reagent gas for collision-activated decomposition of selected parent ions. Data was acquired using a dwell time of 200 ms per channel, which permitted about 20 to 30 scans across chromatographic peaks which generally had peak widths of 40 to 60 seconds. Before each assay batch was loaded in the autosampler for analysis the instrument was checked for proper performance by injecting a reference standard containing all the analytes of interest (approximately 2 ng each injected on the column).

Preparation of Standard and Quality Control Samples

Reference solutions of sertindole and Lu 28-092 were prepared as ethanolic solutions at approximately 1.0 mg/mL. The reference solution of Lu 25-073 (supplied as the fumarate salt) was prepared in methanol because of the poor

solubility of the salt in ethanol. Appropriate volumes of each solution were combined and diluted with methanol to yield a spiking solution containing approximately 10 μg/mL each of sertindole, Lu 28-092, and Lu 25-073. Spiking solutions were prepared independently for standards and quality control (QC) samples.

For the standards, a stock solution of analytes in human plasma (approximately 200 ng/mL) was prepared by spiking the matrix with the reference solution. This stock solution in plasma was then serially diluted to obtain about 10 standard levels, ranging in concentration from 0.1 to 50 ng/mL. QC samples were prepared similarly from a separate weighing, at three concentration levels: approximately 0.7, 10, and 26 ng/mL in human plasma; they were designated QC low, QC mid, and QC high, respectively. The concentrations of standards and quality control samples in the animal matrices were different, reflecting differing extraction volumes and other analytical considerations. (Actual concentrations of the QCs in each matrix appear in Table 2 footnotes.) The volume of plasma extracted was different for the animal species because generally less plasma is available for analysis.

Quantitation Method

Peak areas of sertindole, Lu 28-092, Lu 25-073, and the internal standard were determined using McQuan software (version 1.2, SCIEX). Calibration curves for each analyte were derived from the ratio of analyte peak area to internal standard area and using least-squares linear regression to obtain the relationship of this ratio to the theoretical concentration at each standard level. A weighting of $1/x$ (where x was the concentration of a given standard level) was generally found to give an optimal fit to the concentration/response data; in some case a weighting of $1/x^2$ gave a better fit. The residuals were examined for outliers, which were excluded, and the curve was recomputed to obtain a better fit. Concentrations of the QC samples were calculated from the regression curve using the observed response ratio.

RESULTS AND DISCUSSION

Choice of Analyte-Specific Reactions

A single-stage scan of the ions produced from an infusion of sertindole, Lu 28-092, Lu 25-073, and Lu 26-009 showed only the protonated molecular ions and the characteristic chlorine isotope pattern for these substances. The product ion spectra

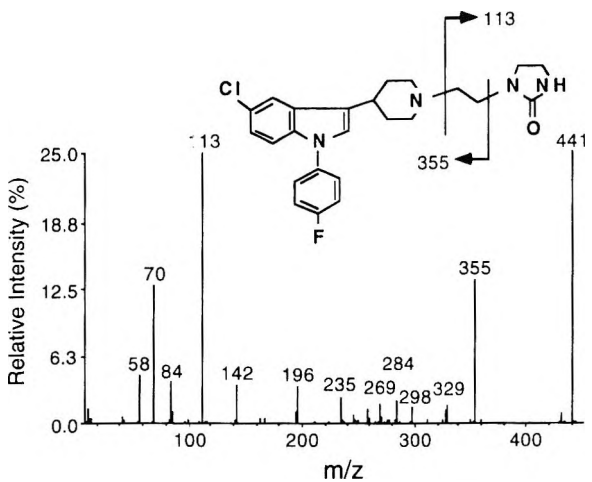

Figure 1. Fragmentation of the $[M + H]^+$ ion (m/z 441) of sertindole.

(MS/MS) of these protonated molecular ions were obtained using argon reagent gas for collision-activated decomposition. Figure 1 shows the product ion spectrum of sertindole. The major fragmentation pathway is the retention of the imidazolidinone moiety (m/z 113 for sertindole). Fragmentations for Lu 28-092 and the internal standard Lu 26-009 were similar (spectra not shown), with appropriate shifts in parent and product ion masses. The fragmentation for Lu 25-073 is different because the molecule does not contain the imidazolidinone moiety. The major fragment ion is at m/z 84. This fragment is thought to arise from opening of the piperazine ring. The following channels were chosen in the multiple reaction monitoring mode (MRM) for peak detection and quantitation: m/z 441→113 for sertindole, m/z 439 →111 for Lu 28-092, m/z 329→84 for Lu 25-073, and m/z 437 →113 for the internal standard. The resolution of the mass filters was reduced for the MRM mode in order to improve ion transmission efficiency.

Specificity

Chromatograms obtained from the injection of a reference solution containing approximately 100 ng/mL of each analyte revealed small peaks in the Lu 28-092 channel. The peaks correspond to the cross-channel talk from the internal standard and sertindole peaks. Sertindole, Lu 28-092, and the internal standard differ by only two mass units; therefore, under the lower resolution conditions employed in the MRM mode there is a small degree of cross talk. Specificity accomplished purely by

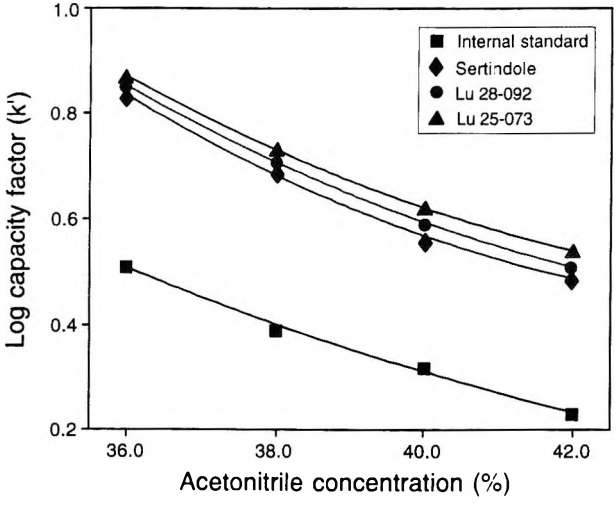

Figure 2. Effect of organic strength on resolution of Lu 26-009 (internal standard), sertindole, Lu 28-092, and Lu-073 at pH 6.0.

mass selection (increasing resolution) would be achieved at the cost of sensitivity. Therefore, these compounds needed to be separated chromatographically. To achieve rapid chromatographic separation of the analytes, we examined the effect of solvent strength and pH on resolution of the analytes on the YMCbasic chromatographic column.

Figure 2 shows the change in the log capacity factor (k') as a function of organic solvent content in the mobile phase at a constant pH of 6.0. The plot demonstrates that increasing the organic solvent strength to about 42% decreased the run time without affecting resolution. A solvent strength of 42% acetonitrile and 58% aqueous 100 mM ammonium acetate was chosen as the optimal concentration to achieve short run times without the peaks eluting too close to each other.

Figure 3 shows a plot of the retention time of the four analytes as a function of mobile phase pH. The organic strength of the mobile phase was held constant at 42% acetonitrile. The pH of the mobile phase had a significant effect on the resolution and elution order of the analytes as demonstrated in the plot. A pH of 6.8 was selected, as it enabled optimal resolution of all the analytes while maintaining a short chromatographic run time (less than 6 min).

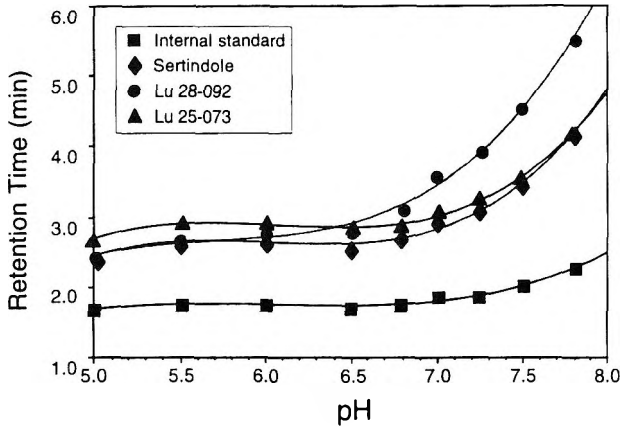

Figure 3. Effect of pH of mobile phase on resolution of Lu 26-009 (internal standard), sertindole, Lu 28-092, and Lu 25-073 at constant organic strength. Composition of the mobile phase was 42% acetonitrile and 58% 100 mM aqueous ammonium acetate in water (v/v).

Ion Current Stability

Multiple injections of a standard reference solution containing 100 ng/mL of each analyte were made to investigate signal stability and the reproducibility of the atmospheric ionization process. The relative standard deviation (RSD) for the ratio of analyte to internal standard (determined from 25 injections) was 7.5% for sertindole, 7.9% for Lu 28-092, and 10.2% for Lu 25-073. No significant trend or drift in the ratio of analyte to internal standard was observed; however, the signal intensity for all analytes decreased after multiple injections. The reason for this drop in signal intensity over time is not apparent.

Limit of Quantitation

The limit of quantitation (LOQ) was determined from standard curves obtained in a study containing eight assay batches and spanning a period of approximately 6 months. The sertindole peak for standard level-1 (approximately 0.1 ng/mL) was detected in all eight calibration curves. The Lu 28-092 peak was detected in seven of the eight curves. However, the Lu 25-073 peak for standard level-1 was not observed in three out of the eight calibration curves, so a higher standard level (level-2, approximately 0.2 ng/mL) was considered as the LOQ for Lu

25-073. The area of the sertindole peak for standard level-1 had an average signal-to-noise (S/N) ratio of 17, with an RSD of 21%. The Lu 28-092 peak for standard level-1 had an average S/N of 12.6 and an RSD of 21%. The Lu 25-073 peak at standard level-2 (0.2 ng/mL) had an average S/N of 18 and an RSD of 21%. The relatively high RSD of the signal near the LOQ suggests that it was prudent to allow a generous S/N (greater than 10) at LOQ when using mass spectrometry for quantitation.

The precision and accuracy for these levels (0.1 ng/mL for sertindole and Lu 28-092, and 0.2 ng/mL for Lu 25-073), were found to be acceptable, indicating that these concentrations could be supported as the LOQ of the assay (1 mL of plasma extracted). The average recalculated concentration (expressed as percent of the theoretical concentration) was 93.8%, 101.0%, and 89.5%, for sertindole, Lu 28-092, and Lu 25-073, respectively. The respective RSDs were 10.7%, 10.3%, and 16.4%. Figure 4 shows a typical chromatogram for the 0.1 ng/mL standard level. Because the Lu 25-073 peak was not sufficiently distinguished from the background noise in some batches, the higher standard level of 0.2 ng/mL was adopted.

Results in dog, rat, and mouse plasma were comparable, with the LOQ for sertindole and Lu 28-092 ranging from 1 to 2 ng/mL (smaller volumes of plasma extracted as noted above). The LOQ for Lu 25-073 ranged from 1 to 4 ng/mL. The variation in limits of quantitation between the animal matrices after accounting for different extraction volumes might be due to slight differences in the efficiency of extraction from the different matrices.

Linearity

For sertindole and its metabolites, standard curves of the internal standard ratio versus concentration of the analyte were linear over greater than two orders of concentration, ranging from the LOQ to approximately 50 ng/mL in human plasma and from the LOQ to approximately 200 ng/mL in dog, rat, and mouse plasma. Nonlinearity (mean deviation exceeding 20% of the theoretical concentration at either end of the curve), was observed when the range of the calibration curve was increased substantially above this value (i.e. three orders of magnitude or more).

A weighting scheme of $1/x$ or $1/x^2$ (where x is the nominal concentration of the standard level) was necessary to obtain good accuracy at the low end of the calibration curves. The inter-assay accuracy for recalculated standard concentrations in human plasma ranged from 91.3% to 106.6% for sertindole, 90.8% to 116.6% for Lu 28-092, and 89.5% to 105.2% for Lu 25-073.

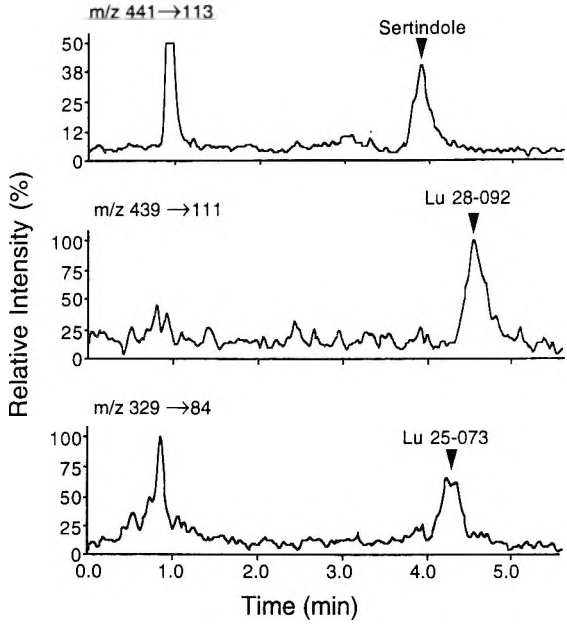

Figure 4. Chromatogram obtained for the 0.1 ng/mL standard in human plasma. This standard level represented the limit of quantitation for sertindole and Lu 28-092. While clearly seen in this chromatogram the peak for Lu 25-073 was not readily identifiable from background in other batches.

Residuals were randomly distributed across the range of the concentration curve, with no significant trend or bias apparent. The RSDs of the recalculated standard concentrations for sertindole, Lu 28-092, and Lu 25-073 averaged 7%, 16%, and 13%, respectively (the mean of RSDs for each standard level).

Precision and Accuracy

Precision and accuracy were determined from the observed concentrations of quality control samples that were assayed along with pharmacokinetic samples from an in house clinical study. Samples were assayed in eight analytical batches spanning a 6-month period. Each batch contained standards, QC samples, and unknowns, with the total number ranging from 80 to 110 samples per assay batch. QC samples at each of the three concentration levels were assayed in triplicate (the first batch had four replicates at each level). Intra-assay statistics were computed from the within-day determination of concentration for the replicate QC samples.

Table 1

Accuracy and Precision of Assays for Sertindole and Metabolites in Human Plasma

	QC Low	QC Mid	QC High
Intra-day accuracy*			
Sertindole	94-122	84-113	88-116
LU 28-092	79-124	71-113	95-145
Lu 25-073	98-127	83-132	92-139
Intra-day precision**			
Sertindole	5.3	5.3	4.5
LU 28-092	15.0	14.0	12.7
Lu 25-073	8.9	9.5	11.2
Inter-day accuracy			
Sertindole	107.7	100.5	104.2
LU 28-092	103.7	95.4	111.4
Lu 25-073	111.9	104.6	111.3
Inter-day precision			
Sertindole	9.2	9.9	8.5
LU 28-092	20.3	19.8	17.2
Lu 25-073	12.6	16.5	16.6

Note: Nominal concentrations of quality control samples were: 0.65 ng/mL for QC low, 10 ng/mL for QC mid, and 26 ng/mL for QC high. There were small differences in the concentration for each compound.
* Range of intra-day means.
** Average of intra-day RSDs.

QC results from all assay batches were pooled to obtain inter-assay (between-day) statistics. Accuracy is reported as the mean concentration of replicate QC determinations, and is expressed as percent of the theoretical concentration. Precision is reported as the RSD of replicate quality control measurements. Table 1 shows the precision and accuracy of the assay for sertindole, Lu 28-092, and Lu 25-073 in human plasma.

The intra-assay accuracy of the method was better for sertindole than for Lu 28-092 and Lu 25-073. Lu 25-073 had the largest within-day deviations from the true mean, possibly due to nonspecific interferences from matrix constituents. The ion channel chosen for Lu 25-073 has a smaller fragment ion (m/z 84) than the other analytes and the molecular weight of the parent ion is also smaller than the other analytes. In the low molecular weight region, there is significantly more noise (higher background chemical noise) than at the higher molecular weights. This high background noise would contribute both to a higher quantitation limit and to larger variability in day to day accuracy. Sertindole also had the best intra-assay precision, averaging approximately 5%. Precision for the two metabolites was not as good as the parent compound; RSDs were approximately 14% for Lu 28-092, and 10% for Lu 25-073. The reason for the larger variation in the assay for Lu 28-092 is thought to be due to the relative instability of the molecule during the sample preparation steps. Sensitivity to laboratory lighting was observed in other in-house studies for this analyte (results not shown).

The pooled mean for all the assays performed over the 6-month period and the associated RSD provided an estimate of the inter-assay accuracy and precision of the method. The accuracy for all three analytes was within 12% of the theoretical concentration for all three concentration levels, indicating that the method has excellent specificity. No distinguishable trends were observed over time, as shown by the control chart for sertindole (Figure 5). Results for Lu 28-092 and Lu 25-073 were similar. Inter-assay precision has a trend similar to intra-assay precision, but is larger in magnitude because this measure also incorporates variation arising from day-to-day implementation of the method (such as: changes in instrument performance, technique differences between operators, effects of multiple freeze/thaw cycles on the sample, etc.). The inter-assay precision for sertindole was again the best, with a RSD of approximately 9% for the three QC concentration levels. The variation for Lu 28-092 was the largest, with a RSD of approximately 19%; as mentioned above, this is suspected to be due to relative instability of the molecule to processing. The inter-assay RSD for Lu 25-073 was approximately 15%.

The inter-assay accuracy and precision for the three analytes indicates that the method is sufficiently robust for GLP compliant production runs. The following criterion was adopted for accepting an analytical batch for GLP-regulated assignments: a QC sample was acceptable if its observed concentration deviated by less than or equal to 20% of the theoretical value. At least two thirds of the measured QCs in the batch were required to be acceptable; otherwise the whole batch and the resulting values for the unknowns were rejected. The expected batch rejection rate based on this criterion, given the accuracy and precision of the method, would be 15% to 20%.

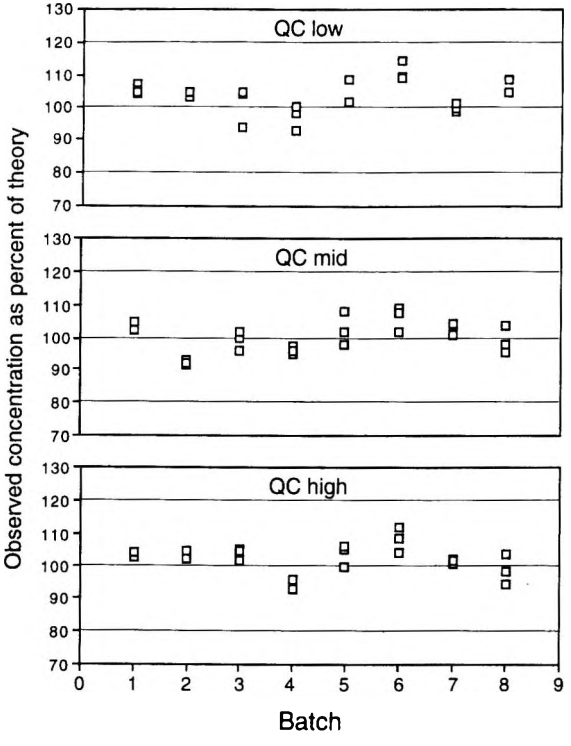

Figure 5. Variation in concentration of sertindole in quality control samples from eight assay batches over a 6 month period. No distinguishable trends were observed over time.

The inter-assay precision and accuracy for sertindole, Lu 28-092, and Lu 25-073 in dog, rat, and mouse plasma are shown in Table 2. The numbers appear to be better than those in human plasma. This may be due to the fact that the experiments in animal plasma were conducted over a shorter time span. Also, the smaller volume extracted for the animal studies would be expected to reduce the effect of matrix on the HPLC column and the ionization interface, such as the buildup of residue and non-eluting compounds on the HPLC column, buildup of non-volatiles on the ionization interface, late eluting peaks that are co-extracted from the matrix, etc. In general, the method was found to perform similarly in the different matrices, and there were no additional issues of stability or interferences that needed to be taken into consideration.

Table 2

Inter-Assay Accuracy and Precision of Assays for Sertindole and Metabolites in Dog, Rat, and Mouse Plasma

	Dog	Rat	Mouse
		Sertindole	
Accuracy*			
QC low	99.8	101.3	104.0
QC mid	100.3	94.7	102.0
QC high	98.4	101.6	100.7
Precision**			
QC low	5.6	7.3	10.0
QC mid	7.0	11.0	6.0
QC high	5.5	6.6	7.2
		Lu 28-092	
Accuracy			
QC low	93.8	106.8	103.8
QC mid	109.6	88.6	104.7
QC high	101.7	98.9	100.9
Precision			
QC low	12.9	6.2	13.2
QC mid	6.7	3.4	12.2
QC high	6.3	10.2	8.8
		Lu 25-073	
Accuracy			
QC low	98.9	104.6	105.3
QC mid	98.7	97.0	104.1
QC high	90.7	98.5	104.0
Precision			
QC low	7.8	10.6	10.5
QC mid	8.8	8.7	11.7
QC high	9.8	11.2	9.0

Note: In dog plasma, the nominal concentrations for low, middle, and high QC levels were 3.4, 16, and 64 ng/mL, respectively. In rat plasma, they were 5.0, 28, and 127 ng/mL respectively. In mouse plasma, they were 6.3, 25, and 127 ng/mL, respectively.

* Mean concentration, expressed as pct. of the theoretical concentration.

** RSDs for replicates were analyzed on different days.

Table 3

Long-Term Frozen Stability of Sertindole and Metabolites

Storage		Sertindole	Lu28-092	Lu 25-073
1 day	High	93.5	94.7	103.6
	Low	103.5	96.2	111.9
6 months	High	88.9	84.9	97.9
	Low	99.4	85.9	98.5
1 year	High	80.4	79.3	101.3
	Low	89.1	75.9	103.1
18 months	High	76.4	76.1	94.6
	Low	84.8	78.3	97.8
2 years	High	75.2	68.2	92.4
	Low	78.0	62.3	95.0

Note: Nominal concentrations for the low and high QC levels were 20 and 100 ng/mL. Observed concentrations were the mean values from five replicate samples at each concentration level.

Long-Term Frozen Stability of Sertindole and Its Metabolites

Because some clinical studies are of long duration and necessitate extended sample storage under frozen conditions, we investigated the stability of the three analytes by storing QC samples prepared in human serum for over a period of 2 years at -20°C. Serum was used for this experiment because some of the earlier clinical trials were conducted using serum as a matrix. Experiments have shown that there is no significant difference in the behavior of the analytes in serum or plasma.[6] The QC samples were aliquoted before storage so that each sample was thawed only once when it was withdrawn for analysis. Representative samples were withdrawn at various time intervals over a two year period and assayed against freshly prepared standards. Samples were assayed in replicates of five to obtain good estimates of the stability over this time period. Two concentration levels were examined: approximately 20 and 100 ng/mL (Table 3). For up to 6 months of frozen storage, all analytes were fairly stable; the decrease was less than 15% of theoretical concentration.

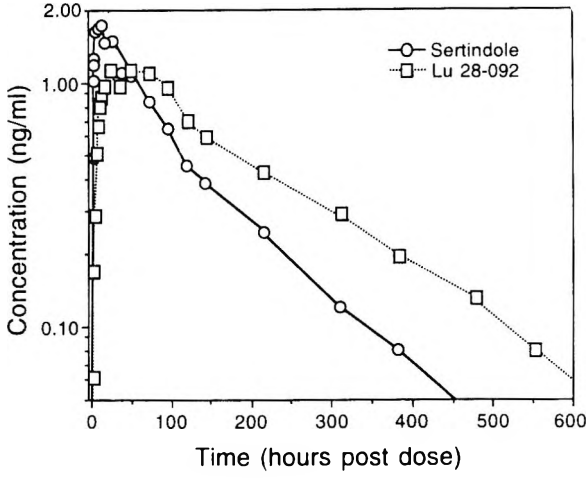

Figure 6. Example of the results obtained in clinical studies using the method. Mean concentration profile of sertindole and Lu 28-092 in a pharmacokinetic study in healthy male subjects.

However, over longer periods, there is an appreciable decrease in the observed concentration of Lu 28-092 and to a smaller extent, sertindole. The Lu 25-073 metabolite seems to be stable and could be stored for extended periods without perceptible loss of analyte.

The assay described above has been used in various clinical and preclinical studies. Figure 6 is an example of the results obtained in such a study. Figure 6 is a plot of the mean concentration profile of sertindole and Lu 28-092 from a clinical study in healthy adult males. Subjects were administered a fairly low oral dose of sertindole (4 mg). The maximal concentration (C_{max}, mean of 16 subjects) was less than 2 ng/mL for both sertindole and Lu 28-092, however, the concentration profiles for both compounds are properly characterized as shown in the figure. The concentrations of Lu 25-073 for the single 4-mg dose were generally below the detection limit of 0.2 ng/mL. Lu 25-073 concentrations have been measured in other studies where subjects are administered higher or multiple doses of sertindole.

CONCLUSION

The HPLC MS/MS assay method for sertindole and its metabolites was found to be sensitive and robust. Results from the human plasma assay were comparable with those from the dog, rat, and mouse plasma assays, indicating that the method is

not significantly affected by compounds co-extracted from the various matrices. The linear dynamic range of the method appears to be smaller (less than three orders of magnitude) relative to other spectroscopic techniques. Correct choice of standard levels and weighting can be used to obtain good accuracy at either the low or high end of the standard curve when working within this limited dynamic range.

REFERENCES

1. B. S. Bunney. TINS June, 212-215 (1984).

2. M. R. Trimble, Zarifian E. British Association for Psychopharmacology Monograph No. 5, Oxford University Press, (1985).

3. T. Skarsfeldt, J. Perregaard, Eur. J. Pharmacol., **182**, 613-614 (1990).

4. T. Skarsfeldt, Synapse, **10**, 25-33 (1992).

5. J. Hyttel, J. B. Nielsen, G. Nowak, J Neural Transm: Gen. Sect., **89**, 61-69 (1992).

6. T. B. Tzeng, G. Stamm, S. Y. Chu, J. Chromatogr. B Biomed. Appl., **661**, 299-306 (1994).

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DIHYDROSTREPTOMYCIN SULFATE IN KIDNEY AND MEAT USING POST COLUMN DERIVATIZATION

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ABSTRACT

A high performance liquid chromatographic method for the determination of dihydrostreptomycin sulfate in kidney and meat from cow, and swine, has been developed. The sample was treated with trichloroacetic acid, and the supernatant clean-up was performed using a Bond Elut Certify II pretreatment column. The lower limit of quantification was 40ng/g and the limit of detection close to 20ng/g. The recovery of dihydrostreptomycin sulfate varyied from 73.2 to 73.5% and from 80.0 to 82.9% for kidney and muscle, respectively.

INTRODUCTION

Dihydrostreptomycin (DHS) is a semi-synthetic aminoglycoside antibiotic approved for use in food-producing animals in most countries. It is available in various formulations for the treatment of a wide range of Gram-negative and some Gram-positive bacteria.[1-4]

In veterinary medicine, the combination of penicillin and DHS is widely used for the treatment of bacterial infections in cattle, pigs and sheep. This represents a potential hazard to consumers due to residues in the meat.[5-7] DHS is potentially toxic, causing damage in vestibular and auditory function.[8]

Numerous chemical and physical methods have been reported for the analysis of streptomycin (STR)and DHS, including paper, thin-layer, and column chromatography, electrophoresis, spectrophotometry, and colorimetry, titrimetry, and polarography.[9] These methods are time-consuming and have poor specificity, sensivity, and precision.

A post column derivatization system with β-naphthoquinone-4-sulfonate as the fluorigenic reagent in the mobile phase, has also been developed for the fluorimetric determination of guanidino compounds by HPLC.[10] A method for the determination of both DHS and STR in pork and bovine muscle and kidney using on-line sample enrichment liquid chromatography was published in 1994.[11] More recently, a HPLC method for the determination of both DHS and streptomycin in milk[12] has been published.

The purpose of the present study was to develop a simple, rapid, and sensitive HPLC method for the routine analysis of DHS in kidney and meat.

MATERIALS AND METHODS

Materials and Reagents

Fresh kidney and meat from cows obtained from the local slaughterhouse, were used as control material and for spiking with DHS to conduct recovery experiments. The samples were stored frozen (-20°C).

1-Octanesulfonic acid and 1-heptanenesulfonic acid were obtained from Supelco Inc. (Supelco Park, Bellefonte. USA.). DHS and STR was supplied by Sigma Co. (St. Louis, MO, USA).

All chemicals and solvents were of analytical and HPLC grade. DHS and STR stock solution and working standards were prepared by dilution with solution A, consisting of 0.02 M 1-heptanesulfonic acid (sodium salt) and 0.01 M di-sodium hydrogenphosphate-2-hydrate,(Ferax, Berlin, Germany), made by dissolving 4.45 g/L heptane sulfonate and 1.8 g/L di-sodium hydrogenphosphate in approx. 750 mL of water when making 1 litre of solution. The pH was then adjusted to c. 5.9 with 5 M phosphoric acid and

then to 5.5 with 0.5 M phosphoric acid, and the solution diluted with water to 1 litre and the pH again adjusted to 5.5 with 0.5 M phosphoric acid. The stock solution and working standards were stored in a refrigerator.

1,2-Naphthoquinone-4-sulfonic acid (NQS), potassium salt, tech. 90% was obtained from Aldrich Chemical Co. (Germany), trichloroacetic acid (TCA) by Ferax (Laborat GMBH, Berlin, Germany) and sodium hydroxido p.a. by R.P. Normapur A. R. (France). Bond Elut Certify II cartridges for solid phase extraction, 6 cc/500 mg, were supplied by Varian (U.S.A.).

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery system, and a ISS 100 sampling system equipped with a Lauda RMT6 cooler (14°C) from Messgueräte Werk Lauda, (Lauda Königshafen, Germany). The integration was carried out using the software programme Turbochrom 4.0 (Perkin-Elmer), which was operated on a Brick personal computer connected to a BJ-330 printer (Canon). The analytical column (operated at 31°C; stainless steel, 150 x 4.6 mm I.D.) and guard column (stainless steel, 2.0 cm x 4.6 mm I.D.) were packed with 5-μm particles of Supelcosil LC-ABZ + Plus (Supelco, Bellefonte, PA, USA). The guard column was connected to an A. 318 precolumn filter with an A-102X frits (Upchurch Scientific , USA).

The mobile phase consisted of a mixture of two solutions, B and C (68 : 32). Solution B consisted of 0.04 M 1-octanesulfonic acid and 0.4 mM NQS at pH c. 3.24, and was prepaired by dissolving 8.65g/L octanesulphonic acid and 110 mg/L NQS in c. 750 mL water. The solution was diluted to 1 litre by addition of water and filtered through a 0.45 μm membrane filter. The pH was adjusted with 1 mL acetic acid. This mobile phase was prepared daily and stored in an amber flask during use. Solution C was acetonitrile. The flow rate was 0.6 mL/min. for 0.5 min., 0.9 mL/min. for 4 min. and finally 0.6 mL/min. for 9 min. The samples were injected at intervals of 14 min. For the determination of DHS aliquots of 25 μL were injected onto the column.

The column effluent was introduced into a vortex mixer from a heater system for HPLC post column reactions (PCRS 520 - Kratos) equipped with a heat exchanger (vortex mixer is not a mixing tee, but a low volume (1.2 μl) mixing device for two fluid streams).

A Series 250 Liquid Chromatograph (Perkin-Elmer) was used, which included an extra pulse-dampened pump (Scientific System inc., USA) and two pump back pression regulators (2 x 250 PSI., Perkin Elmer) with a mobile phase of 0.3 M NaOH at a flow rate of 0.3 mL/min. This was coupled to the vortex mixer, and a reaction coil (Aura Industries, Inc., USA) KRC 15-50 Knitted Reactor Coil, 15 m x 0.50 mm I.D. The reaction temperature was 40°C. The solvent stream was then cooled using a heat exchanger (operated at room temperature), to prevent gas emission in the detector. Flourescence was detected using a LC 240 fluorescence detector (Perkin-Elmer, Norwalk, Conn., USA) with an excitation wavelength of 375 nm and emission wavelength of 420 nm, with a response of 5 and a factor of 512.

Sample Pretreatment

To 8 g kidney or meat, a 5 mL solution A (or standard) was added. The total volume added in this step should amount to 5 mL. One mL 85% TCA in water was then added. The mixture was homogenized for approx. 6 sec. in an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Ika Werk, Staufen, F. R. G.). After centrifugation for approximately 3 min. (5000 rpm), 2 mL dichloromethane was added. The sample was then mixed for 6 s. and centrifuged for 5 min. (5000 rpm). Seven mL of the supernatant (corresponding to 4g kidney/meat) was pipetted into a graduated glass-stoppered centrifuge tube, 0.9 mL 4 M NaOH was added and the mixture then blended. The homogenate was centrifuged for 5 min. (4000 rpm). The upper layer was transferred to a clean tube, 0.9 mL of 0.5 M phosphoric acid added, and the pH adjusted to between 5.5 to 5.8 with 1 M NaOH or 0.5 M phosphoric acid. After 2.5 mL solution D had been added, the sample was mixed and loaded onto a conditioned 500 mg Certify II column. Solution D was made in the same manner as solution A, but the 1-heptanesulfonic acid concentration was 0.06 M (13.35 g/L).

Clean-up on SPE-column

The column was activated with 3mL acetonitrile, followed with 1mL water and 3x1 mL solution D, prior to application of the extract. It is important not to allow the sorbent to dry before applying sample. The aqueous extract was applied to the column and slowly (c. 1mL/min) suctioned through, using a VacMaster system (International Sorbent Technology). The glass stoppered tube was rinsed with 1 mL solution A; this solution was also loaded onto the column. The column was washed and then suctioned to dryness for c. 2 sec.,(with a vacuum of -5 in. Hg.) between every washing, the wash was with

2x5 mL solution A, followed with 3x5 mL NH3 (25%) and 3x1 mL water. The column was suctioned to dryness for 10 sec. (at a vacuum of -10 in. Hg.) and eluted with 2x1 mL 20% formic acid in methanol. The collected eluates were evaporated to dryness under a stream of nitrogen using a Reacti-Therm heating module at 60°C and a Reacti-Vap evaporating unit (Pierce, Rockford. IL, USA). Two hundred μL methanol was added, the sample was mixed for 3-4 s. and evaporated to dryness. The dry residue was dissolved in 400 μl solution A, after which 200 μL chloroform were added. The extract was mixed vigorously for 10 s. followed by centrifugation for approximately 3 min. The water layer was then filtered through a Costar Spin X centrifuge filter unit with 0.22 μm nylon membrane, by centrifugation for 2 min. at 10000 rpm. (5600 g). Aliquots of the aqueous layer (25 μl) were injected onto the column at intervals of 14 min. for the determination of DHS.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for DHS were determined by spiking meat and kidney samples with standard solutions to yield 40, 50, 100, 200, 300, and 400 ng DHS per gram of sample, respectively. Duplicate samples were used. The recovery rates were determined by comparing analysis of spiked meat and kidney, with those of standard solutions. The linearity of the standard curves for DHS in meat and kidney were calculated using peak height measurements.

RESULTS AND DISCUSSION

Chromatograms of clean and spiked kidney samples from cow and swine with DHS are shown in Figure 1 and 3. Chromatograms of extract of blank and spiked samples from cow meat with DHS and STR are shown in Figure 2.

The standard curves are linear in the investigated area (40 - 400 ng/g) for DHS in meat and kidney. The corresponding correlation coefficients were $r = 0.996$ and 0.999 in kidney and meat, respectively. Table 1 shows the recovery and repeatabilities of DHS from kidney and meat from cows:

The recovery of DHS from kidney and meat varied from 73.2 to 73.5% and from 80.0 to 83.0%, respectively. The precision of these recovery studies varied from 1.5 to 3.1% for kidney and from 0.3 to 1.3% for meat.

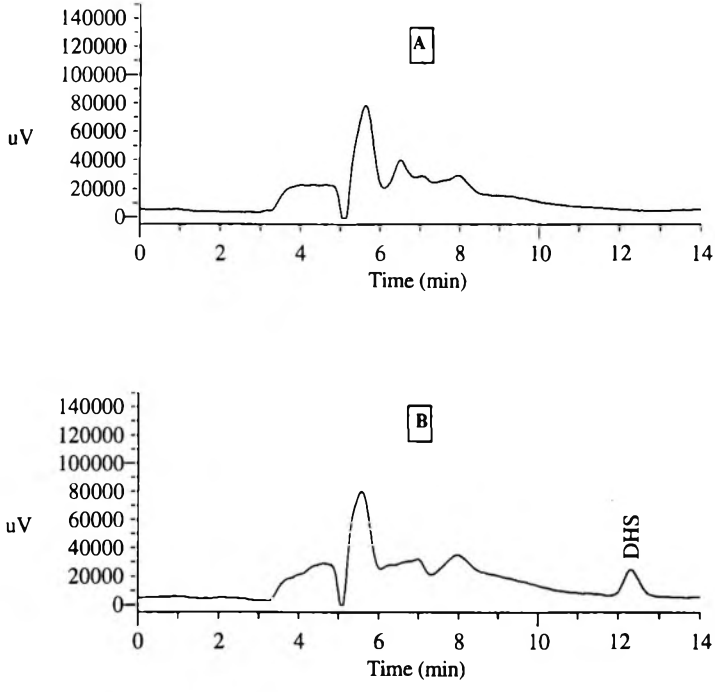

Figure 1. Chromatograms of extracts from cow kidney. **A**: drug-free kidney, **B**: kidney spiked with DHS (400ng/g).

Table 1

Recovery and Repeatability for Dihydrostreptomysin Sulfate from Spiked Samples of Meat and Kidney

Sample	No. of Samples (μg/g)	Amount of DHS in Spiked Samples	Recovery % DHS Mean	SD*
Meat	8	0.1	83.0	1.3
8/g	8	0.4	80.0	0.3
Kidney	8	0.1	73.5	1.5
	8	0.4	73.2	3.1

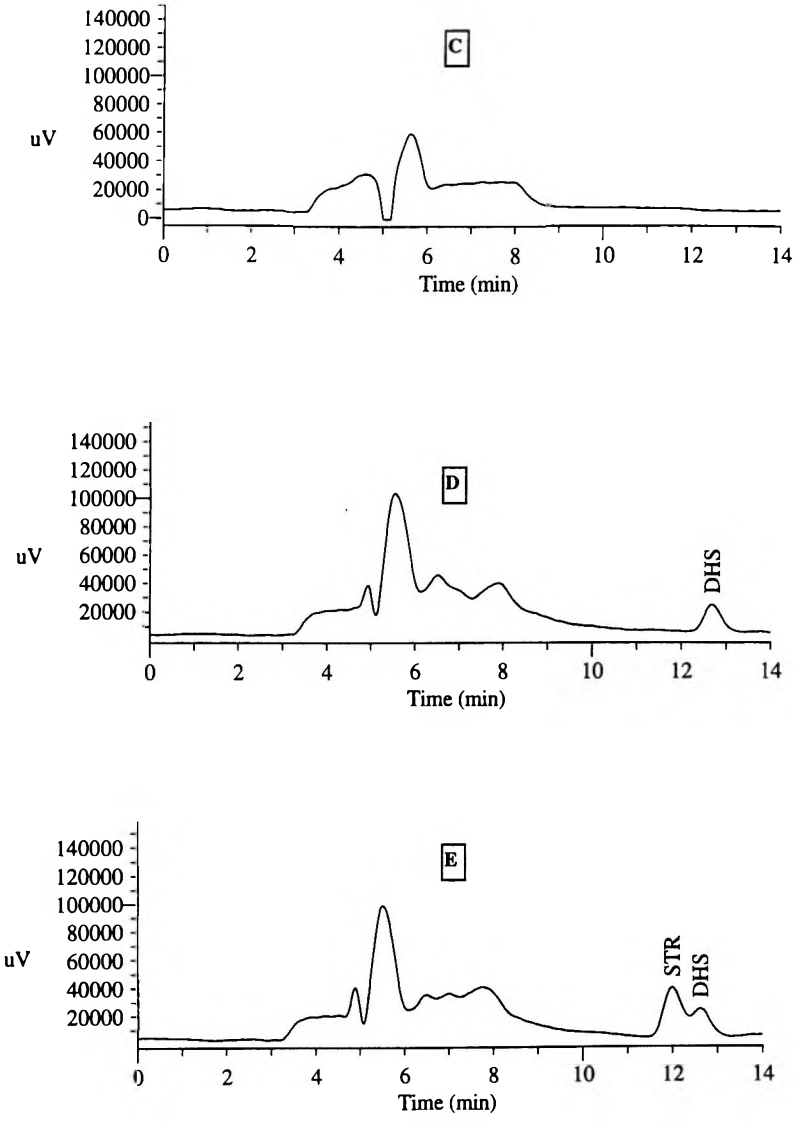

Figure 2. Chromatograms of extract from cow meat. **C**: drug-free meat, **D**: meat spiked with DHS (400 ng/g), **E**: meat spiked with DHS and STR (400 ng/g).

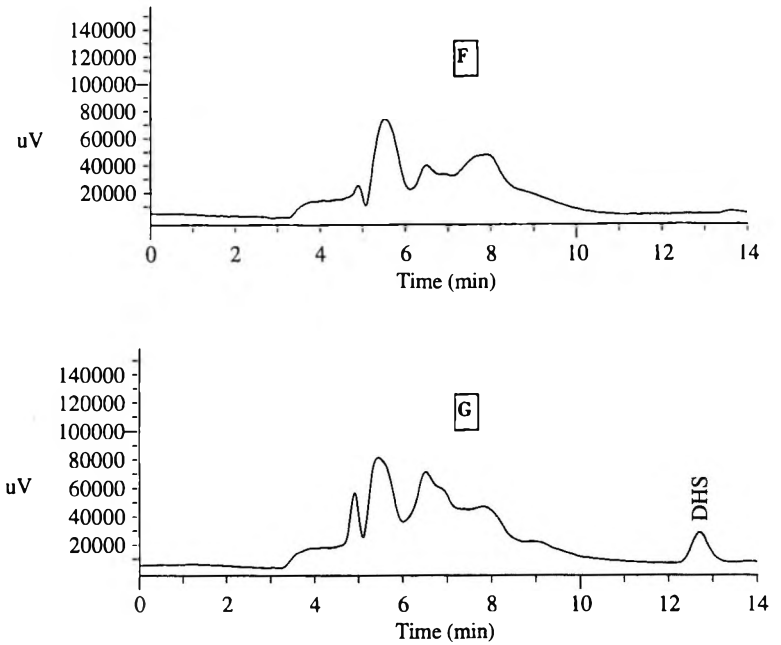

Figure 3. Chromatograms of extracts from swine kidney. **F**: drug-free kidney, **G**: kidney spiked with DHS (400ng/g).

NQS forms high intensity fluorophors with guanidino compounds in alkaline media.[10] DHS, which has two guanidino groups, yields similar fluorophors. In our preliminary studies, non-polar sorbent materials such as tC_{18} SPE-cartridges from Waters, Bond Elut Certify, C_{18} and Bond Elut Certify II from Varian were tested and acceptable recovery were obtained for DHS in meat and kidney pretreated on Bond Elut Certify II. The capacity of the Certify II sorbent material was also tested. If the columns contained 200 mg sorbent material, the recovery of DHS from the kidney and meat homogenates was variable. Increasing the amount of Certify II material in the columns to 500 mg resulted in the analyses of spiked tissue showing good reproducibility.

t-C_{18} from Waters yielded a successful result for determination of DHS in milk,[12] but in meat and kidney the elution already started during washing after 7mL with 30% methanol in water,[12] this is not sufficient to eliminate all interfering peaks near the retention time of DHS in meat and kidney.

Different eluting agents were tested, and the eluting solvents methanol / formic acid (8 : 2, v/v) gave acceptable recovery. The reaction coil and the analytical column must be maintained at a steady temperature and the mobile phases were degassed thorough with helium before use (during analysis we used a solvent system kits from Perkin Elmer), and must be free from fluctuation or an unstable baseline will result.

After all samples and standards had been injected, the analytical column was washed with water-acetonitrile (1:1) in 15 min., thereafter 5 min. gradient to methanol (100%), and 25 min. with methanol. The flow was 0.9 mL/min. The post-column reaction system was washed with water-methanol (9:1) at 0.5 mL/min for 10 min. We used a vortex mixer, but it is not a problem to use a low volume mixing tee instead.

The chromatographic system appeared to be efficient for the determination of DHS in kidney and meat, the limit of quantification being 40 ppb and the limit of detection close to 20 ppb. The detection limits are calculated as 3 times the baseline noise from a drug-free tissue. No interference was seen during analysis, when calibrating the curves, or when performing recovery studies. The method presented in this paper is selective, robust, sensitive, and accurate. As shown in Figure 2 (E) the method can also be used for the determination of STR in kidney and meat. The chromatogram of a spiked meat sample with standard solution, to yield 400 ng/g of DHS and STR, shows that baseline resolution of the two drugs is not achieved. Changing the mobile phase composition to water-acetonitrile (70 : 30) produced baseline resolution, but also resulted in retention times of 16.2 and 17.3 min. for DHS and STR, respectively. Because it was considered unlikely that both drugs would be encountered in the same sample, a mobile phase was chosen that allowed identification of each drug while minimizing analysis time. The precision, recovery, and linearity of STR were not validated in this report.

ACKNOWLEDGMENT

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REFERENCES

1. D. A. Cox, K. Richardson, B. C. Ross, in P. G. Sammes (Editor), **Topics in Antibiotics Chemistry**, Vol. 1, Ellis Horwood, Chichester, 1977, pp. 5-15, 32, 41-43, 51.

2. **AMA Drug Evaluations**, Fourth Edition. American Medical Association/American Society for Clinical Pharmacology and Therapeutics, Publishing Sciences Group, Litleton, MA, 1980,pp. 767-768, 803-804, 963-964.

3. U. Hollstein, in M. E. Wolff (Editor), **Burger`s Medicinal Chemistry. Part II, Wiley**, New York, Chichester. Brisbane, Toronto, 4 th ed., 1979, pp. 219-222.

4. P. Sensi, G. Gialdroni-Grassi, in M.E. Wolff (Editor), **Burger`s Medicinal Chemistry, Part II**, Wiley, New York, Chichester, Brisbane, Toronto, 4th ed., 1979, pp. 311-315.

5. G. Ziv, F. G. Sulman, Res. Vet. Sci., **17**, 68-74 (1974).

6. C. Jayachandrani, M. K. Singh, S. D. Singh, N. C. Banerjee, Vet. Res. Commun., **11**, 353-358 (1987).

7. J. G. Manners, R. Stewart, Aust. Vet. J., **58**, 203-204 (1982).

8. B. Shaikh, E. H. Allen, J. Assoc. Off. Anal. Chem., **68**, 1007-1013 (1985).

9. D. W. Hughes, A. Vilim, W. L. Wilson, Can. J. Pharm. Sci., **13**, 21 (1978).

10. F. Faure, P. Blanquet, Clin. Chim. Acta, , **9**, 292-300 (1964).

11. G. C. Gerhardt, C. D. C. Salisbury, J. D. Mac Neil, Journal of AOAC International, 77**(2)**, 334-337 (1994).

12. V. Hormazábal, M. Yndestad, J. of Liq. Chrom., **18(13)**, 2695-2702 (1995).

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J. LIQ. CHROM & REL. TECHNOL., 20(14), 2269–2280 (1997)

POST-COLUMN ELECTROCHEMICAL OXIDATION OF LEUCO MALACHITE GREEN FOR THE ANALYSIS OF RAINBOW TROUT FLESH USING HPLC WITH ABSORBANCE DETECTION

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ABSTRACT

Malachite green is a dye used in aquaculture as a parasiticide. A method is described for the quantitation of residues of malachite green and its colourless reduced form, leuco malachite green, in the flesh of rainbow trout. The analytes are extracted with solvent and purified using a C_{18} solid phase extraction cartridge. Determination of both compounds by reversed phase HPLC with absorbance detection at 610 nm is achieved following post-column oxidation of the leuco form to malachite green using an electrochemical detector cell. The limits of detection for malachite green and leuco malachite are 6 ng/g and 3 ng/g, respectively. At concentrations from 25-200 ng/g, recoveries in the range 73-87% were achieved for malachite green and 89-98% for leuco malachite green. Oxidation of leuco malachite green using activated charcoal is also described as as an alternate manner of determining total malachite green.

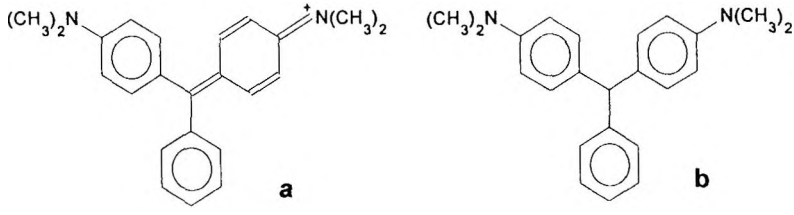

Figure 1. Structures of (a) malachite green, (b) leuco malachite green

INTRODUCTION

Malachite green oxalate (C.I. 42000) is a triarylmethane dye commonly applied to newly laid fish eggs to inhibit fungal and protozoal infections.[1] The reduction of malachite green (**MG**) to its colourless leuco base (**LMG**) is a facile process which has been reported to occur in fish flesh.[2] To monitor good aquacultural practice and prevent exposure of consumers to residues of these suspected carcinogens[1], an efficient method for the detection of malachite green residues in rainbow trout was required (see Figure 1).

Methods employing TLC[3] or HPLC[4] have previously been described for the detection of **MG** itself in fish. Determination of **MG** alone was considered unsuitable for monitoring residues in fish flesh. It has been found that up to 90% of the total malachite green species present in fish were in the leuco form.[2] This is corroborated by the authors' observations. Several HPLC methods for determining both **MG** and **LMG**, in fish flesh,[2,5-8] plasma,[9] and in waters,[10,11] have also been published. While diol, cyano, or cation exchange solid phase extraction (SPE) cartridges, alone, were found to be effective for the relatively simple concentration and clean-up of the analytes during the analysis of water or plasma, methods for analysis of fish tissue have employed either solvent partitioning and washing to separate interferences,[7] or a combination of alumina and cation exchange SPE.[8]

For GC/MS confirmation of **LMG** in catfish tissue, a third clean-up on a cyano column was added.[12] Due to losses of the less polar leuco form into the organic phase, recoveries of **LMG** were often less than 30% in our hands when a solvent partitioning clean-up was attempted. To obtain high yields for both forms of the analyte simultaneously, a simple and efficient SPE protocol using a single C_{18} cartridge was developed and is described here.

Previous HPLC methods for the determination of these compounds have taken advantage of the strong and distinctive visible absorption of **MG** for detection and quantitation of both it and **LMG** (after oxidation to **MG**). In methods where the analytes are individually determined, oxidation of **LMG** can be performed in a post-column reactor containing lead dioxide. As an alternative to the use of absorbance, electrochemical detection has been described in the analysis of water.[11] We found that in our analysis of fish tissue extracts the selectivity and stability of the absorbance detector operating at 610 nm gave the best results for reliable quantitation. However, the use of a coulometrically efficient elecrochemical cell to oxidize **LMG** to **MG** between the outlet of the HPLC column and the inlet of the visible detector was found to be an effective way to overcome the problems associated with packing and maintaining a PbO_2 post-column reactor, while avoiding the band-broadening such reactors are liable to produce.

In previous procedures in which both compounds have been analysed without separation, the leuco form has generally been oxidised to the coloured form by oxidation with a lead (IV) oxide slurry after sample clean-up. The extent of this oxidation tends to be rather variable, depending upon the age, activity, and rate of addition of the oxidant.[1] We also found that the PbO_2 could further oxidise **MG** to a derivative which was not easily quantitated. The degree of further oxidation was found to differ between standards and samples (perhaps due to sacrificial oxidation of other components of the fish extract). Consequently, the use of PbO_2 slurries gave recoveries of **LMG** which were low and variable. Our observations, on the behavior of the carbon electrode in the amperometric detector, led to some investigations which demonstrate that activated charcoal may be superior to lead dioxide for pre-separation oxidation of **LMG** to **MG**.

MATERIALS AND METHODS

Standards and Reagents

L.R grade standards were obtained from E. Merck (Melbourne, Australia). Stock standards of **LMG** and **MG** were prepared fresh weekly in methanol at approximately 0.5 mg/mL and stored below -10°C. Working standards were a 1:125 dilution of the stock in water to give 4 μg/mL. HPLC standards (a 1:40 dilution of working standard in HPLC mobile phase) had a final concentration of 0.1 μg/mL.

Acetonitrile, methanol, and dichloromethane (HPLC grade) were obtained from Mallinkrodt. Water was purified with a Waters Milli-Q system. Perchloric acid (70%) was May and Baker Pronalys (AR) grade and pentane sulfonic acid (HPLC grade) was from Sigma. Bakerbond C_{18} cartridges (500 mg) (J.T.Baker) were employed for SPE clean-ups. Activated charcoal (decolourising powder) was supplied by Ajax Chemicals (Melbourne, Australia).

Chromatographic System

The HPLC system consisted of an LKB 2248 pump / LKB 2157 auto sampler coupled to a 25 cm x 4.6mm Alltech 5μm Econosphere C18 column. The mobile phase was 0.01M pentane sulfonic acid in acetonitrile containing 6% 0.05M aqueous phosphoric acid. It could be recycled 2-3 times, then renewed. Absorbance at 610nm was measured using an Activon Linear UVIS 204 variable wavelength detector. An ESA Coulochem model 5100A electrochemical detector with ESA 5010 analytical cell operating at a potential of 0.45V relative to the in-built proprietary reference electrode was coupled between the HPLC column and the visible detector.

Selection of the operating potential was acheived by performing repeated 10 microlitre injections of standard solutions of **LMG** or **MG** in mobile phase at a flow rate of 0.1mL/min after removal of the analytical column. The response of the visible detector was recorded at a range of potentials at the electrode of the electrochemical detector to determine an optimum voltage for the analysis.

Sample Extraction

Each fish to be tested was filleted and the flesh blended in a food processor before weighing 9g of tissue into a 50 mL polypropylene centrifuge tube. After homogenising for 1 minute at high speed with 2 mL dichloromethane, 16 mL acetonitrile, and 1 mL 0.4M perchloric acid in acetonitrile, the extraction was continued in the dark for 3 hours at 60 rpm on a rotary mixer .

After centrifuging at 3200 rpm for five minutes, 15 mL of the supernatant was transferred to a graduated glass tube and evaporated to 5 mL at 60°C using nitrogen. This extract was diluted with 20 mL of water, before being loaded

onto a C_{18} SPE cartridge which had been pre-conditioned with 10 mL of 20% acetonitrile in H_2O. The cartridge was dried with a stream of air for 10 minutes and the analytes were slowly eluted with 2 mL of HPLC mobile phase of which 25 μL was injected into the HPLC.

Alternative Oxidation and Clean-up using Activated Charcoal

As an alternative to the SPE clean-up, after centrifuging the initial dichloromethane/acetonitrile/perchloric acid extract of the sample, 15mL of supernatant was transferred to a graduated glass tube and evaporated to approximately 0.5 mL at 60°C using nitrogen. This extract was diluted to 2 mL with HPLC mobile phase, in preparation for clean-up and oxidation with charcoal conditioned as follows.

Approximately 1g of finely powdered charcoal was shaken vigorously with 40 mL of 5M nitric acid for 1 minute. After standing for five minutes the acid was removed by centrifuging at 1500 rpm. The remaining paste was rinsed twice with 40 mL of water and then with HPLC mobile phase before being diluted to 20mL with mobile phase.

To oxidise **LMG** in the sample extracts, 100 μL aliquots from the stirred charcoal slurry (5 mg of carbon) were swiftly pipetted into all sample tubes. The samples were then vortexed for 30 seconds, centrifuged at 2000 rpm for 1 minute and 25 μL aliquots analysed by HPLC.

RESULTS AND DISCUSSION

The electrode in the ESA 5010 analytical cell is a porous carbon frit through which the column eluant flows. This design results in efficient oxidation of almost all the analyte passing through the electrode. Consequently, excellent sensitivity can be acheived in subsequent Absorbance detection (at 610 nm) of **LMG** which has been oxidized to **MG**. An oxidation potential of 0.45V was chosen for the analysis of samples in this study since it gave maximal oxidation of **LMG** whilst minimizing the degradation of **MG**. As can be seen from the data in Figure 2, at an applied potential of 0.45V the oxidation efficiency for **LMG** is at a maximum of 57%, while only 3% of **MG** is degraded.

The use of a coulometrically efficient electrochemical cell at the exit of the HPLC column was found to be a more reliable oxidation method for **LMG** than pre-column treatment with lead dioxide. The chromatographic separation of

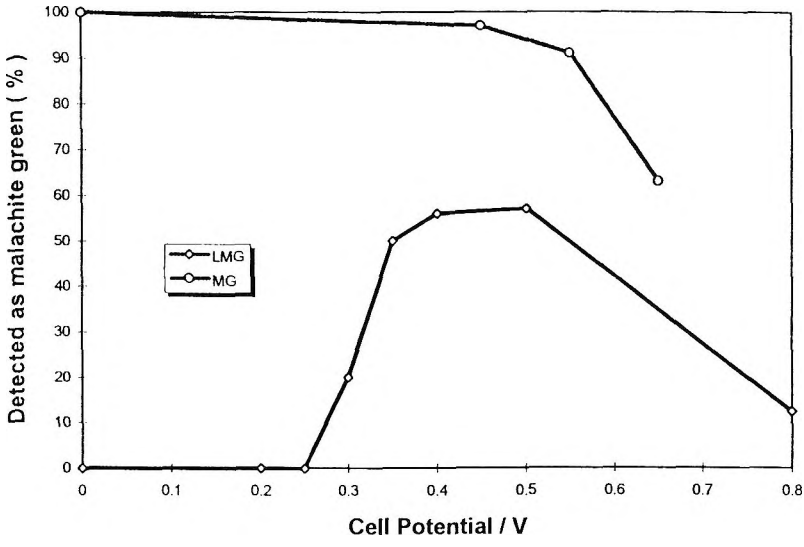

Figure 2. Response of MG and LMG to change in cell potential. Visible response at 610nm following direct injection of malachite green (MG) or leuco-malachite green (LMG) solutions (0.1 µg/mL) into the electrochemical detector.

LMG from other oxidizable co-extractives in the fish extracts, prior to its oxidation enhances the consistency of the oxidation. As oxidation is now fully automated the method requires fewer manipulative steps and this can be expected to increase repeatability of results as well as save time. In addition, the unreliable and unpleasant use of PbO_2, a suspected carcinogen, is avoided. Another benefit of the use of the electrochemical cell for oxidation is that it allows speciation of the two major forms of malachite green by chromatographically separating them prior to detection.

Sample Extraction

A range of extraction systems was examined, including several solvent/ homogenisation and percolation techniques. Their effectiveness appeared to be limited by the strong binding of the dyes to the fish flesh, and its oil content (10-15%) which causes emulsion formation. Long extraction times have been shown to be necessary for extraction of malachite green residues from fish tissue.[4] Three hours was found to be sufficient for fortified samples with the system described here. Acidification of the extraction solvent was vital for good yields.

In studies of various eluants for the HPLC system, it was found the analytes were strongly retained against elution by even traditionally powerful reverse phase solvents such as methanol, acetonitrile, acetone, and dichloromethane. This distinctive selectivity of the HPLC mobile phase for **MG** and **LMG** forms the basis of our SPE clean up. Use of mobile phase as the eluant means that samples are immediately ready for injection and since the SPE cartridge used has a similar chemistry to the HPLC column (ODS), interferences likely to cause column damage are removed.

Analysis of Fish Tissue

Rainbow trout samples were analysed using this method both before and after fortification with the analytes (Figure 3). Apart from one batch of fish (discussed below), no significant interferences were observed in the traces produced by the visible detector at 610nm. Both compounds were readily detected at spiking levels down to 25 ng/g (ppb) with coefficients of variation below 10% at this level (Table 1). Based on three times the standard deviation of the results for the lowest fortification level studied (an approximation of the standard deviation of the blank), the limit of detection for the method is 3 ng/g for MG and 6ng/g for LMG. The limit of quantition for MG and LMG is 6ng/g and 12 ng/g respectively.[13,14]

Chromatographic performance was good. More than 1500 injections of standards and samples were performed during method development, with no decrease in column performance. Maintenance was limited to weekly washing with methanol and 1:1 methanol/dichloromethane and occasional regeneration of the electrochemical cell using 5M nitric acid. Both the visible response and the electrochemical oxidation were consistent and linear within a batch .

Confirmation of Positive Results

The detector wavelength used confers a fairly high degree of selectivity on the analysis when coupled with retention time information. However, the on-line electrochemical oxidation allows additional confidence in the identity of a peak eluting at the correct retention time for **LMG**. If the output of the electrochemical cell is monitored during the analysis, current flow reflecting the passage of an electro-active species (**LMG**) will be observed shortly prior to detection of the compound at the visible detector. This would not occur if the peak was due to an already coloured co-extractive. Secondly, the size of the suspected **LMG** peak (in the visible chromatogram) should vary in a similar manner to that of a leuco standard when the electrode potential is manually

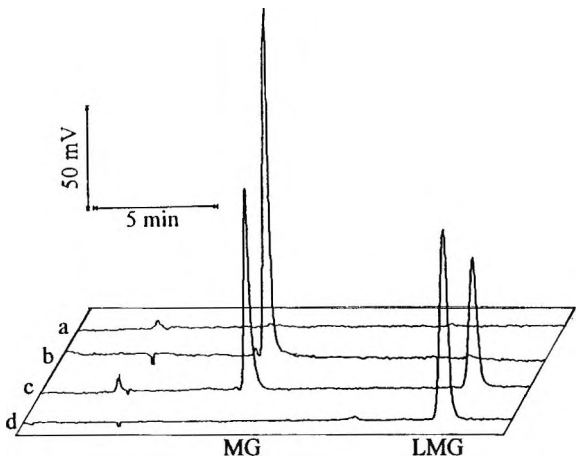

Figure 3. Representative chromatograms; (a) blank rainbow trout sample, (b) 100 ppb MG std, (c) rainbow trout sample fortified at 50 ppb each MG and LMG, (d) 50 ppb LMG std

Table 1

Recovery of Leuco Malachite Green (LMG) and Malachite Green (MG) from Fortified Rainbow Trout Flesh Using the Electrochemical Method

	MG Spiked Samples Spiking Level (ppb)				LMG Spiked Samples Spiking Level (ppb)		
	25	**50**	**100**	**200**	**25**	**100**	**200**
No. of samples	7	6	5	6	7	6	6
Recovery %	87	73	76	84	98	95	89
C.V %	8.0	6.5	12.5	5.8	4.0	7.4	6.9

altered. This would provide strong confirmation of the identity of the unknown as it would seem very unlikely that any co-extracted compound would have the same retention time, hydrodynamic voltammogram, and coloured oxidation product as leuco malachite green.

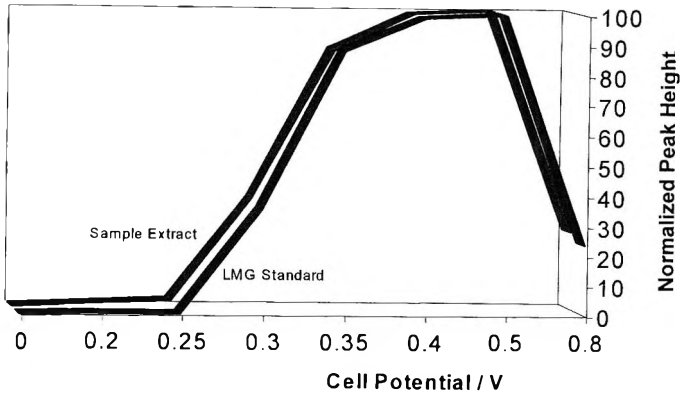

Figure 4. Oxidation of (a) leuco malachite green standard and (b) unfortified rainbow trout sample using the HPLC electrochemical cell.

The usefulness of this technique was strikingly demonstrated on one batch of fish samples that were obtained for use in validation. When a blank sample was analysed, it contained what appeared to be a large interference at the retention time of **LMG**. The sample was re-analysed at a range of electrode potentials, and the results are shown in Figure 4.

When the sample was injected 8 times with different applied voltages in the electrochemical cell, the 'interference' showed an identical hydrodynamic voltammogram to that of a **LMG** std, as well as having the same retention time on two different HPLC columns. It therefore appears that the 'interference' was incurred **LMG**, present in some of the samples at more than 100 ng/g.

Use of Activated Charcoal for Oxidation

A decrease in the efficiency of the oxidation of **LMG** by the electrochemical cell after processing several batches of samples was observed as a reduction in the peak height of standards. This was probably due to poisoning of the carbon electrode by contaminants in the fish extract, as it could be reversed by washing the electrochemical oxidation cell with 10mL of 5M nitric acid. After this cleaning process it was necessary to re-condition the cell with several injections of **LMG** standard, otherwise oxidation would occur in the absence of an applied potential.

Table 2

Oxidation of LMG (200 ng/mL Solution) Using Charcoal

Sample	Charcoal/mg	% Oxidation Efficiency
LMG Standard	1[a]	99
LMG Standard	5[a]	87
LMG Standard	5[b]	0
LMG Spiked fish extract	1[a]	60
LMG Spiked fish extract	5[a]	91

[a] Activated with 5M nitric acid as described in Experimental Section.
[b] Not activated.

Table 3

Effect of Treatment with Acid-Activated Charcoal on Malachite Green*

	Charcoal/mg	% Malachite Green Loss
MG Standard	1	0
MG Standard	5	3
MG Standard	25	43

* 140 ng/mL solution.

The observation of oxidation of **LMG** by a freshly washed carbon electrode in the absence of an applied voltage, led us to examine the use of activated charcoal treated with 5M HNO_3 as an alternative method to PbO_2 slurries for pre-HPLC oxidation. It was found that the leuco base was very efficiently oxidised to **MG** by charcoal slurries freshly activated with nitric acid (Table 2). The use of 5 mg of charcoal gave almost complete oxidation of **LMG** in both standards and sample extracts, without compromising the recovery of **MG**. Charcoal prepared three hours prior to use had only 50% of the activity of the fresh material. This method results in minimal destruction of **MG** as shown in Table 3. It also appears to simultaneously remove many interferences from the sample extract and could form the basis of a simpler and more repeatable method of quantitation of total malachite green residues than current procedures based on lead dioxide slurries.

CONCLUSIONS

Leuco malachite green and malachite green can be simultaneously and independantly determined in rainbow trout flesh, in a fast, efficient, and cost effective procedure. The method described offers repeatable recoveries of more than 75% and 89% in the range 25-200 ng/g for **MG** and **LMG** with detection limits of 6 and 3 ng/g, respectively. Excellent confirmation of the identity of **LMG** residues can be obtained using its electrochemical oxidation profile. This convenient and effective on-line oxidation using an electrochemical cell, can also be expected to be applicable in other liquid chromatographic applications where oxidation of analytes is required prior to detection by absorbance or fluorescence.

REFERENCES

1. M. Grayson, **Kirk-Othmer Encyclopaedia of Chemical Technology,** 2nd Edition, John Wiley and Sons, NY, 1978, Vol 23, p.402; Vol 3, p. 211.

2. K. Bauer, H. Dangschat, H. Knoppler, J. Neudegger, Arch. Lebensmittelhyg., **39**, 97-102 (1988).

3. M. Edelhauser, E. Klein, Dtsch. Lebensm.-Rundsch., **82**, 386 (1986)

4. V. Hormazabál, I. Steffenak, M. Yndestad, J. Liq. Chrom., **15**, 2035-2044 (1992).

5. O. Dafflon, H. Gobet, H. Koch, Mitt. Geb. Lebensmittelunters. Hyg., **83**, 215 (1992).

6. W. Fink, J. Auch, Dtsch. Lebensm. Rundsch., **89**, 246 (1993).

7. J. L. Allen, J. E.Gofus, J. R.Meinertz, J. Assoc. Off. Anal. Chem., **77**, 553-557 (1994).

8. J. E. Roybal, A. P. Pfenning, R. K. Munns, D. C. Holland, J. A.Hurlbut, A. R. Long, J. Assoc. Off. Anal. Chem., **78**, 453-457 (1995).

9. C. A. J. Hajee, N. Haagsma, J.Chromatogr. B, **669**, 219-227 (1995).

10. J. L. Allen, J. Meinertz, J. Gofus, J. Assoc. Off. Anal. Chem., **75**, 646 (1992).

11. K. Sagar, M. R. Smyth, J. G. Wilson, K. McLaughlin, J. Chromatogr. A, **659**, 329-336 (1994).

12. S. B.Turnipseed, J. E. Roybal, J. A. Hurlbut, A. R. Long, J. Assoc. Off. Anal. Chem., **78**, 971-977 (1995).

13. IUPAC, Analytical Chemistry Div., Anal. Chem., **48**, 2294-2296 (1975).

14. H. Kaiser, A. G. Menzies, **The Limit of Detection of a Complete Analytical Method**, Hafner, New York, 1969, p.29.

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ANALYSIS OF HONEY PHENOLIC ACIDS BY HPLC, ITS APPLICATION TO HONEY BOTANICAL CHARACTERIZATION

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ABSTRACT

In this work we described an analytical technique that allowed the identification of 12 phenolic acids in honey samples, with emphasis on those phenolics acids which are markers of the botanical origin. After optimization of the HPLC conditions, this was applied to the phenolic acids analysis of 20 *Erica* sp. (heather) and 20 *Lavandula stoechas* (lavander) portuguese honeys.

A close correlation between the phenolic acids patterns and the botanical origin of honey has been found. *Erica* sp. honeys are characterized by ellagic, *p*-hydroxybenzoic, syringic and *o*-coumaric acids, and *Lavandula stoechas* honeys by gallic acid.

INTRODUCTION

The botanical origin of honey is one of its main quality parameters, and its price is very often related to this floral origin.[1] Recent studies have revealed that the analysis of phenolic compounds constitute a very promising technique to study the geographical and floral origin of honey.[2-7]

Reversed-phase HPLC is considered as the method of choice in phenolic compounds analysis,[8,9] however, HPLC of honey phenolic acids has rarely been reported. Thus, Amiot et al.[10] analysed flavonoids and phenolic acids in floral French honeys.

The aim of the present work is the analysis of honey phenolic acids by HPLC, and this analysis will be applied to the study of the phenolic acids present in some selected honey samples of different floral origin.

MATERIALS AND METHODS

Honey Samples

The *Erica* sp. (heather) and *Lavandula stoechas* (lavander) honey samples used in this study came from the Serra da Lousã and Trás-os-Montes regions (Portugal), respectively, and were directly provided by the beekeepers. The honey samples had not been industrially processed. To minimise any alterations, the samples were stored at -20°C.

Sample Preparation

Phenolic compounds for HPLC analysis were extracted from honey as reported previously.[11-13] The available honey samples (ca. 50 g) were thoroughly mixed with five parts of water (pH 2 with HCl) until completely fluid and filtered through cotton to remove solid particles. The filtrate was then passed through a column (25 x 2 cm) of Amberlite XAD-2 (Fluka Chemie: pore size 9 nm, particle size 0.3-1.2 mm). The phenolic compounds present in honey

remained in the column while sugars and other polar compounds were eluted with the aqueous solvent. The column was washed with acid water (water pH 2 with HCL, 100 mL) and subsequently with distilled water (ca. 300 mL). The whole phenolic fraction was eluted with methanol (ca. 300 mL). This fraction was concentrated under reduced pressure and purified by dissolving them in methanol and passing the solution through a Sephadex LH-20 column (15 x 1 cm). Phenolic fraction was clearly visualized under UV light (360 nm). The phenolic fraction was evaporated to dryness under reduced pressure (40°C), redissolved in methanol (0.5 mL) and analysed by HPLC.

HPLC Analysis of Honey Flavonoids

HPLC analysis was carried out in a Gilson system (Gilson Medical Electronics, Villiers le Bel France) equipped with a type 305 pump, a type 302 pump and a type 7125 Rheodyne Injector with a 20 μL loop. The chromatographic separation was achieved with a Merck Lichrospher 100 RP-18 (125x3 mm; 5 μm particle size) column, using water-formic acid (19:1) (solvent A) and methanol (solvent B) as solvents.

After trying different solvent gradients, the best resolution was obtained at a solvent flow rate of 0.4 mL/min, starting with 5% methanol and installing a gradient to obtain 15% B at 10 min, 30% B at 15 min., 35% B at 25min., 80% B at 40 min, and which then became isocratic until 45 min. Detection was achieved with a diode array detector, and chromatograms were recorded at 320 and 280nm.

The different phenolics compounds were identified by their UV spectra recorded with the diode array detector and by chromatographic comparisons (retention times) with authentic markers.

Phenolic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards of phenolic acids with detection at 320nm for chlorogenic and caffeic acids and 280nm for the others.

RESULTS AND DISCUSSION

The technique presented here for the analysis of phenolic acids in honey is quite useful. The use of Amberlite XAD-2 allows the elimination of sugars and polar compounds from honey, yielding a phenolic acid fraction which also contains other phenolic compounds (flavonoids).[11] As we need a phenolic acid

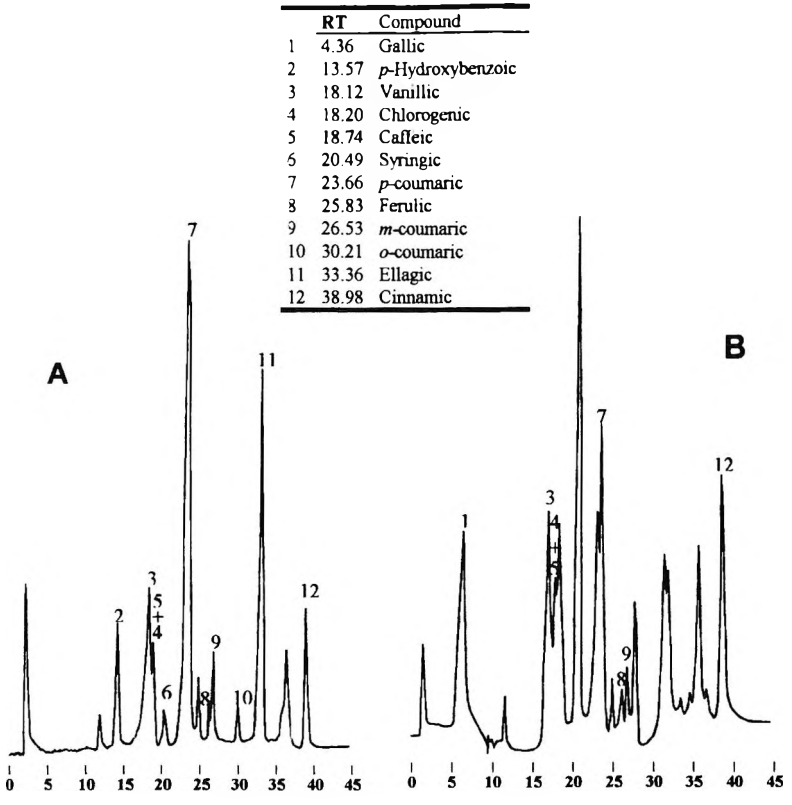

Figure 1. HPLC of honey phenolic acids. (A) Heather honey; (B) Lavender honey. Detection at 280 nm.

fraction which is sufficiently purified to permit phenolic acid analysis and quantification of the various compounds without problems of interference from other UV-absorbing substances (other phenolics), a purification through Sephadex LH-20 with methanol becomes necessary.

The phenolic acids present in the honeys samples were extracted and analysed by HPLC and the results are summarised in Tables 1 and 2. All the analysed samples contained a common phenolic acid profile including vanillic, chlorogenic, caffeic, *p*-coumaric, ferulic, *m*-coumaric, and cinnamic. On the other hand, some compounds were only detected in only one unifloral honey type, and could be considered as potential floral markers. Thus, gallic acid and an unidentified peak (RT 20.71 min) seems to be characteristic of *Lavandula*

Table 1
Phenolic Acid Content of *Lavandula Stoechas* Honeys*

Samples	Gallic	p-OH-benzoic	Vanillic	Chlorogenic	Caffeic	Siringic	p-Coumraric	Ferulic	m-Coumaric	o-Coumaric	Ellagic	Cinnamic
1	237.20	---	0.32	0.49	0.05	---	0.03	0.12	0.05	---	---	0.08
2	209.90	---	0.54	0.51	0.08	---	0.07	0.31	0.06	---	---	0.09
3	195.90	---	0.44	0.47	0.06	---	0.02	0.35	0.07	---	---	0.09
4	212.85	---	0.65	0.55	0.05	---	0.06	0.10	0.04	---	---	0.10
5	202.89	---	0.38	0.45	0.06	---	0.04	0.12	0.07	---	---	0.10
6	209.34	---	0.47	0.59	0.07	---	0.04	0.18	0.04	---	---	0.08
7	211.63	---	0.35	0.43	0.06	---	0.04	0.21	0.07	---	---	0.07
8	199.57	---	0.52	0.56	0.07	---	0.06	0.36	0.08	---	---	0.08
9	214.76	---	0.58	0.44	0.05	---	0.01	0.11	0.06	---	---	0.08
10	221.51	---	0.40	0.60	0.06	---	0.05	0.27	0.06	---	---	0.07
11	202.31	---	0.51	0.53	0.05	---	0.07	0.16	0.07	---	---	0.09
12	199.98	---	0.63	0.48	0.05	---	0.03	0.10	0.05	---	---	0.09
13	226.32	---	0.49	0.52	0.05	---	0.03	0.24	0.06	---	---	0.08
14	216.57	---	0.52	0.44	0.06	---	0.01	0.11	0.08	---	---	0.06
15	183.57	---	0.62	0.61	0.04	---	0.04	0.21	0.05	---	---	0.08
16	225.94	---	0.45	0.58	0.07	---	0.07	0.23	0.06	---	---	0.08
17	232.64	---	0.55	0.43	0.08	---	0.06	0.15	0.05	---	---	0.07
18	219.31	---	0.56	0.46	0.07	---	0.03	0.24	0.04	---	---	0.07
19	209.57	---	0.53	0.54	0.05	---	0.03	0.12	0.07	---	---	0.08
20	185.97	---	0.48	0.40	0.09	---	0.04	0.15	0.05	---	---	0.09
$\overline{X}$	210.89	---	0.50	0.50	0.06	---	0.04	0.19	0.06	---	---	0.08
sd	14.23	---	0.09	0.06	0.01	---	0.02	0.08	0.01	---	---	0.01
V_{min}	183.57	---	0.32	0.40	0.04	---	0.01	0.10	0.04	---	---	0.06
V_{mix}	237.20	---	0.65	0.061	0.09	---	0.07	0.36	0.08	---	---	0.10

*mg phenolic acid/100 g honey

$\overline{X}$ - mean; sd - Standard deviation; V_{min} - Minimum value; V_{max} - Maximum value.

Table 2
Phenolic Acid Content of *Erica sp.* Honeys*

Samples	Gallic	p-OH-benzoic	Vanillic	Chlorogenic	Caffeic	Siringic	p-Coumraric	Ferulic	m-Coumaric	o-Coumaric	Ellagic	Cinnamic
1	---	0.15	0.11	0.07	0.00	0.00	0.94	0.01	0.06	0.05	0.19	0.08
2	---	0.05	0.16	0.47	0.15	1.25	0.54	0.11	0.01	0.01	0.44	0.31
3	---	0.19	0.18	1.00	0.02	0.16	1.48	0.36	0.11	0.01	0.40	0.40
4	---	0.38	0.29	0.34	0.07	0.15	0.80	1.42	0.14	0.10	0.13	0.39
5	---	0.36	0.73	1.03	0.11	0.10	2.65	1.10	0.30	0.05	0.19	0.13
6	---	0.98	0.80	0.54	0.16	0.32	1.39	1.12	0.27	0.02	0.24	0.27
7	---	0.13	0.21	0.33	0.04	0.09	0.84	0.01	0.01	0.03	0.17	0.06
8	---	0.27	0.20	0.14	0.01	0.10	2.05	0.44	0.03	0.02	0.55	0.07
9	---	0.05	0.04	0.22	0.01	0.04	1.59	0.04	0.00	0.02	0.40	0.04
10	---	0.09	0.12	0.34	0.05	0.15	1.59	1.30	0.21	0.02	0.43	0.40
11	---	0.74	0.90	0.54	0.16	0.32	1.39	1.18	0.27	0.02	0.55	0.27
12	---	0.50	0.99	0.27	0.03	0.14	1.51	1.01	0.28	0.48	0.61	0.29
13	---	0.63	0.69	0.90	0.05	0.04	1.64	0.04	0.01	0.02	0.09	0.54
14	---	0.48	0.82	0.28	0.02	0.12	1.44	1.18	0.09	0.11	0.15	0.27
15	---	0.09	0.01	0.08	0.02	0.05	2.69	0.39	0.02	0.02	0.12	0.15
16	---	0.17	0.14	0.10	0.03	0.04	0.99	0.22	0.01	0.01	0.19	0.12
17	---	0.34	0.04	0.24	0.01	0.04	1.59	0.04	0.00	0.02	0.15	0.04
18	---	0.25	0.20	0.48	0.02	0.28	1.83	0.09	0.01	0.03	0.13	0.06
19	---	0.57	0.21	0.88	0.02	0.08	1.29	0.11	0.13	0.09	0.53	0.25
20	---	0.15	0.13	0.89	0.02	0.06	0.93	0.11	0.06	0.09	0.17	0.11
$\overline{X}$	---	0.33	0.35	0.46	0.05	0.12	1.49	0.54	0.11	0.06	0.29	0.21
sd	---	0.25	0.33	0.32	0.05	0.09	0.52	0.52	0.10	0.10	0.18	0.15
V_{min}	---	0.05	0.01	0.07	0.00	0.00	0.80	0.01	0.00	0.01	0.09	0.04
V_{max}	---	0.98	0.99	1.03	0.16	0.32	2.69	1.42	0.30	0.48	0.61	0.54

* mg phenolic acid/100 g honey.

$\overline{X}$ - Mean; sd - Standard deviation; V_{min} Mininum value; V_{max} Maximum value.

stoechas honey, and *Erica* sp. honey is characterised by the presence of *p*-hydroxybenzoic, syringic, *o*-coumaric and ellagic acids (Figure 1). The presence of ellagic acid (a dimeric derivative of gallic acid) in *Erica* sp. honeys agrees with previous reports in which this phenolic acid was suggested as a marker for the floral origin of *Erica* sp. honeys.[14,15]

It seems that the relative amount of one individual phenolic acid could be related to the floral origin of honey. Thus, *Erica* sp. honey (Table 2) contains a considerable amount (around 39%) of *p*-coumaric.

These results are very promising, but more detailed studies are necessary to confirm which phenolic compounds could be useful floral markers for a particular monofloral honey.

To conclude, this study suggests that the technique presented here for the analysis of phenolic acids in honey is quite useful, since allows the separation of the main honey phenolic acids with a single analysis, with emphasis on those phenolic acids which could be markers of the floral origin.

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REFERENCES

1. F. A. Tomás-Barberán, F. Ferreres, F., A. Ortiz-Valbuena, M. C. Fernandez-Maeso, **Estudio Sobre el Contenido en Flavonoids de las Mieles de la Alcarria**, CSIC, Madrid, 1994.

2. S. Sabatier, M. J. Amiot, M. Tacchini, S. Aubert, J. Food Sci., **57**, 773-777 (1992).

3. F. Ferreres, A. Ortiz, C. Silva, C. Garcia-Viguera, F. A. Tomás-Barberán, F. Tomás-Lorente, Z. Lebensm. Unters.-Forsch., **194**, 139-143 (1992).

4. F. A. Tomás-Barberán, F. Ferreres, C. Garcia-Viguera, F. Tomás-Lorente, Z. Lebensm. Unters.-Forsch., **196**, 38-44 (1993).

5. F. A. Tomás-Barberán, F. Ferreres, M. A. Blásquez, C. Garcia-Viguera, F. Tomás-Lorente, J. Chromatogr., **634**, 41-46 (1993).

6. C. Soler, M. I. Gil, C. Garcia-Viguera, F. A. Tomás-Barberán, Apidologie, **26**, 53-60 (1995).

7. F. Ferreres, P. Andrade, F. A. Tomás-Barberán, Z Lebensm. Unters.-Forsch., **199**, 132-137 (1994).

8. K. V. Casteele, H. Geiger, C. F. V. Sumere, J. Chromatogr., **347**, 81-94 (1982).

9. T. Krzaczek, A. Bogucka-Kocka, R. Sniezko, Acta Societatis Botanic. Pol., **1**, 41-44 (1995).

10. M. J. Amiot, S. Aubert, M. Gonnet, M. Tacchini, Apidologie, **20**, 115-125 (1989).

11. F. Ferreres, F. A. Tomás-Barberán, M. I. Gil, F. Tomás-Lorente, J. Sci. Food Agric., **56**, 49-56 (1991).

12. F. Ferreres, F. A. Tomás-Barberán, C. Soler, C. Garcia-Viguera, A. Ortiz, F. Tomás-Lorente, Apidologie, **25**, 21-30 (1994).

13. P. Andrade, **Tipificação de méis Portugueses de *Erica* sp. da Região da Serra da Lousã**- Doctoral Thesis, Coimbra, (1995).

14. F. Ferreres, P. Andrade, M. I. Gil, F. A. Tomás-Barberán, Z. Lebensm. Unters.-Forsch., **202**, 40-44 (1996).

15. P. Andrade, F. Ferreres, M. I. Gil, F. A. Tomás-Barberán, Food Chem., in press.

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SOFTWARE REVIEW

Origin, Version 4 (with 3D, Contour, and Peak Fitting Modules), Microcal Software, Inc., One Roundhouse Plaza, Northampton, MA 01060, USA, $545.

Origin is a software package intended for data analysis and curve plotting. It is aimed at the technical and scientific part of the market. Origin makes it very easy to manipulate columns of numbers (data) and to display these numbers in a useful, graphical manner. Charts are created very easily with Origin. Basic features, such as multiple graphs on a single chart, axis breaks, error bars, log-log and semi-log plots are available, and they work quite well. Applying linear, polynomial and sigmoidal fits is straightforward and the output includes a table with statistical data concerning the goodness of the fit.

The user can plot confidence limits as well as prediction limits of the fitted line. It is a simple manner to add user-defined functions to a data graph. Over and above the standard features, Origin includes analysis tools that make data manipulation easy to perform. For example, data plots can be smoothed (using one of three filters, including FFT). Spectra, chromatograms or any other instrumental output can be imported into Origin, plotted and fitted to several peak shape models; peaks can be located and their areas determined.

Graphs are handled as a collection of objects. The properties of these objects are easily accessible by double-clicking on the objects, so the user can customize the charts according to his specific needs. A chart can include more than one data set, i.e., more than one curve. However, all the plots on a single chart relate to the same axes values. To have two independent graphs on the same chart, the user needs to use the layering system of Origin. Using different layers for different data sets (different columns of data on the same work sheet), the user can control each graph in an independent manner, including plotting in different axes ranges. The graphs can be printed either on a full page or on a part of the page. The user has control on the exact size of the printed plot.

Provided that you have the *3D & Contour* module, both 2D and 3D plots can be obtained. This reviewer found the data entering stage for 3D plots a bit cumbersome. A 3D plot has a large set of tools associated with it; e.g., tilting,

rotation, prospective changes, etc. As with the 2D plots, all the properties of the objects comprising the chart can be adjusted by the user. However, this reviewer did find that, after some rotation steps, pressing the RESET ROTATION button in the 3D toolbar does restore the figure to its original position, but one of the axis labels and the tick marks are completely misplaced. The labels can be restored to their rightful place only by manually doing one or two rotation steps.

My only major criticism is the very poor UNDO feature associated with the graphs. Most of the changes which the user can make, such as changing the axis scale and adding a line to connect data points, are not UNDOable. To restore to the original situation the changes have to be corrected manually. The lack of a complete UNDO feature is really unacceptable with present generation software.

The data to be plotted is entered in a spreadsheet-like table called a worksheet. The factory installed default table has two columns. However, the user can increase the number of columns, either temporarily for a given session, or on a permanent basis. The latter is accomplished by saving a worksheet template after adding as many columns as desired. The columns have properties, such as width, label, and data type that are user-definable. Data types are numerical, time, date, month, and day of week. The format of numerical entries is, of course adjustable. Column and row statistics are available.

Very rudimentary matrix operations are also available. There is a single level UNDO feature which is accessible only through the Menu bar and not via the Tool bar. While the user can carry out mathematical manipulation on columns of data, or on ranges of data in a column, there is no back history listing of the changes made. Since the results of the manipulation are overwritten on the previous data, it is easy to lose track on the action taken after several cycles of manipulation. In such a case, it is difficult to reconstruct the original data. It is, therefore, a good idea to copy the original data to a new column before beginning to carry out the manipulations.

Scrolling the data with the help of the scroll bar allows you to move continuously through the rows of data in the table. However, scrolling with the cursor down a column in the table is not continuous, but rather proceeds with a complete change of a group of rows or even a complete screen. I found this scroll mode to be annoying, since I did not have a continuous view of my data.

For users who want analyze peaks obtained experimentally, the *Peak Fitting module (PFM)* is well worth having. It allows the user to fit his data to quite a few peak models and to obtain several important characterizing properties such moments, and moments-related quantities (i.e., variance, skew, and excess). Goodness of fit parameters are also available. A report which can be obtained after the fit gives the type of peak which was used in the fit routine, the center of gravity of the peak, its area and width, as well as the width at half the height. The only major shortcoming which I found with the PFM is its inability to handle, in a straight and forward manner, negative peaks.

In summary, Origin is a solid technical and scientific graphics software package. It is flexible enough to handle almost any charting and graphing tasks. If you have been relaying on your spreadsheet for plotting your data, Origin will give you many more degrees of freedom in manipulating your graphs.

Reviewed by:
Eli Grushka
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Jerusalem, Israel

J. LIQ. CHROM. & REL. TECHNOL., 20(14), 2293–2299 (1997)

THE BOOK CORNER

HANDBOOK OF CAPILLARY ELECTROPHORESIS, Second Edition, J. P. Landers, ed., CRC Press, Boca Raton, Florida, 1996, 894 pages.

The Second Edition of the Handbook of Capillary Electrophoresis is a massive volume which is made up of 30 chapters totaling 894 pages, and contains 400 figures and 65 tables. The editor writes "This fully updated Second Edition covers all areas of interest in the field of capillary electrophoresis (CE);" however, he failed to tell those who own the First Edition, why they should pay a handsome amount of money to invest in a Second Edition of a book they already have. It is a normal procedure that an editor or author who updates a book would tell the interested prospective buyer of another copy why he should buy it and what he would miss if he did not buy the new volume.

A quick glance would tell us the Second Edition is different from the First Edition in the following obvious points: (a) the Dedication is different; (b) the Foreword to the First Edition was written by Professor Karger while, for the Second Edition it is written by Professor Hjerten, and the two Forewords are different. The First Edition was divided into 23 chapters, i.e., seven chapters and about 200 pages less than the Second Edition. Other obvious changes are: the disappearance, in the Second Edition, of CE: Historical Perpectives, and changes in authors of some of the chapters. The First Edition, in addition to Chapter 1, was divided into: (I) Modes of CE (Chapters 2-6); (II) Detection (Chapters 7-8); (III) General Applications (Chapters 7-14); (IV) Specialized Applications (Chapters 15-17); and (V) Practical and Theoretical Considerations (Chapters 18-23). The Second Edition is divided into: (I) Modes (Chapters 1-5); (II) Analyte (Chapters 6-11); Essential Aspects (Chapters 12-16); (IV) Applications (Chapters 17-23); and (V) Specialized Aspects of CE (Chapters 24-30).

The Second Edition is definitely an improved and more informative book than the First Edition. The topics in the present edition are well selected, of general interest, up to date, and are written by experts in their respective areas.

The book is a welcome addition to the CE Library. It is a useful reference for those involved in biotechnology and clinical chemistry, as well as the pharmaceutical, bioscience, chemical, and instrument-manufacturing industries.

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Reviewed by
Haleem J. Issaq, Ph.D.
Editor, The Book Corner

CAPILLARY GAS ADSORPTION CHROMATOGRAPHY, V. G. Berezkin, J. de Zeeuw, Huthig GmbH, Heidelberg, Germany, 1996, 320 pages.

The Chromatographic Methods Series by Huthig has proven to be a successful and useful one. The published books deal with the selected topic effectively and simply, without any complications. We, at this Journal, have reviewed quite a few of these books and the reviewers have liked them and praised them. The present volume, Capillary Gas Adsorption Chromatography, by Berezkin and de Zeeuw, is no different. Capillary gas chromatography is probably the most widely used separation technique. This book deals with all aspects of GC.

The book is divided into seven chapters and a conclusion. It is well illustrated with 164 figures and 32 tables. The authors state, in the Preface, that they followed the proverb "A picture is worth a thousand words." But, they did not tell how many words a table is worth.

The first chapter is an introduction which discusses the advantages and limitations of gas-solid chromatography, which continues into the second chapter. Chapter 3 deals with the fundamentals of gas solid chromatography. The discussion in this chapter is very good and straightforward. Adsorbents (carbon, silica gel, alumina, molecular sieves, . . .) are discussed in Chapter 4, and modified adsorbents (chemically and dynamically) are discussed in Chapter 5. Preparation of adsorbent layer open tubular columns is discussed in detail in Chapter 6. Chapters 4-6 are very useful for those who want to prepare their own columns; they are helpful in optimizing the separation. Chapter 7 deals with applications and discusses the use of carrier gas, pre-columns, particle traps, separation of gases, hydrocarbons, polar volatiles, halogenated hydrocarbons and others.

Overall, the book is well written and definitely well illustrated. It is recommended to all those interested in using GC.

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Reviewed by
Haleem J. Issaq, Ph.D.
Editor, The Book Corner

ADVANCES IN CHROMATOGRAPHY, Volume 37, P. R. Brown, E. Grushka, eds., Marcel Dekker, Inc., New York, 1997, 462 pages. $195.00.

The explosive growth of chromatography and capillary electrophoresis has made it difficult for any individual to maintain a coherent view of progress in the field. Individual investigators trying to stay abreast of advances must rely on authoritative surveys, rather than attempt to read the avalanche of original research papers.

Volume 37 of this continuing series, which presents current, critical reviews of important developments in separation science, is an excellent example. The current volume is made up of eight sections, each comprising a critical and useful review of the topic.

The subject matter, as in past volumes, is widely different, from assessment of peak purity, to carbon packed materials for HPLC to SFC-GC applications, to CE of proteins and analysis of derivatized peptides by HPLC and CE (see Table of Contents below).

Each chapter in the book includes a brief and informative introduction, followed by discussion of the selected topic. It is worth noting here that each chapter in Volume 37 is written by experts in their areas, which makes this volume a useful one, interesting reading, and a good reference. As usual, this volume is no different from the other 36 volumes in this series; it is well edited and free of typographical and scientific errors. The book is recommended to all those using separation science to achieve their analytical chemistry objectives.

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Reviewed by
Haleem J. Issaq, Ph.D.
Editor, The Book Corner

J. LIQ. CHROM. & REL. TECHNOL., 20(14), 2301 (1997)

EDUCATION ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC AND HPLC SYSTEM TROUBLESHOOTING

A Two-Day In-House Training Course

The course, which is offered for presentation at corporate laboratories, is aimed at chemists, engineers and technicians who use, or plan to use, high performance liquid chromatography in their work. The training covers HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC hardware module and system problems.

The following topics are covered in depth:

- Introduction to HPLC Theory
- Modes of HPLC Separation
- Developing and Controlling Resolution
- Mobile Phase Selection and Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration and Quantitation
- Logical HPLC System Troubleshooting

The instructor, Dr. Jack Cazes, is founder and Editor-in-Chief of the Journal of Liquid Chromatography & Related Technologies, Editor of Instrumentation Science & Technology, and Series Editor of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 35 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on GPC, and has taught at Rutgers University. He is currently Visiting Scholar at Florida Atlantic University.

Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097. Tel.: (954) 570-9446; E-Mail: jcazes@icanect.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(14), 2303-2311 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

SEPTEMBER 2 - 5: 12th International Bioanalytical Forum, Univ. of Surrey, Guildford, UK, sponsored by the Chromatographic Society (U.K.). Contact: Dr. E. Reid, 72 The Chase, Guildford GU2 5UL, U.K. Tel/FAX: (0) 1483-565324; Email: D.Stevenson@surrey.ac.uk.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

SEPTEMBER 8 - 10: 1997 PrepTech Conference, Hyatt Regency Hotel, Orlando International Airport, Florida. Contact: S. Galla, ISC Technical Conferences, Inc., 30 Controls Dr., Box 559, Shelton, CT 06484-0559, USA. Tel: (203) 926-9300; FAX: (203) 926-9722.

SEPTEMBER 14 - 17: International Ion Chromatography Symposium, Westin Hotel, Santa Clara, California. Contact: Janet Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052-0493, USA. Tel: (508) 359-8777; FAX: (508) 359-8778; Email: century@ixl.net.

SEPTEMBER 14 - 19: ACS Int'l Symposium on Systems Approach to Service Life Prediction of Organic Coatings, Breckenridge, Colorado. Contact: J. Martin, NIST, Bldg. 226, Rm B-350, Gaithersburg, MD 20899, USA. Email: jmartin@nist.gov.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

SEPTEMBER 22 - 25: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Fairmont Hotel, San Francisco, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

SEPTEMBER 30 - OCTOBER 2: 53rd Southwest ACS Regional Meeting, Tulsa, Oklahoma. Contact: F. B. Growcock, Amoco Corp., E&PT, P. O. Box 3385, Tulsa, OK 74012, USA. Tel: (918) 660-4224; Email: fgrowcock@amoco.com.

OCTOBER 5 - 8: Conference on Formulations & Drug Delivery, La Jolla, California, sponsored by the ACS Div. of Biochem. Technol. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036. Tel: (202) 872-6286; FAX: (202) 872- 6013; Email: miscmtgs@acs.org.

OCTOBER 6 - 10: Validation d'une Procedure d'Analyse, Qulification de l'Appareillage; Application a la Chromatographie Liquide, Montpellier, France. (Training course given in French) Contact: Prof. H. Fabre, Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ-montpl.fr

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 23: Sensors Expo: Conference on Exposition of Sensors, Detroit, Michigan. Contact: Expocon Mgmt. Assoc., 3363 Reef Rd, P. O. Box 915, Fairfield, CT 06430-0915, USA. Tel: (203) 256-4700; Email: sensors@expo.com.

OCTOBER 21 - 23: Biotechnica Hannover '97: Int'l. Trade Fair for Biotechnology, Hannover, Germany. Contact: D. Hyland, Hannover Fairs USA, Inc., 103 Carnegie Center, Princeton, NJ 08540, USA.

OCTOBER 21 - 24: 152nd Fall Technical Meeting & Rubber Expo'97, Cleveland, Ohio, sponsored by ACS Div. of Rubber Chem. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036. Tel: (202) 872-6286.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.org.

OCTOBER 25 - 30: 24th Annual Conference of the Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Providence, Rhode Island. Contact: ACS Div. of Anal. Chem., Tel: (301) 846-4797; FAX: (301) 694-6860; Internet: http://FACSS.org/info.html.

OCTOBER 26 - 29: ISPPP'97 – 17th International Symposium on the Separation of Proteins, Peptides, and Polynucleotides, Doubletree Hotel, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. FAX: (301) 898-5596.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033; Email: reglmtgs@acs.org.

NOVEMBER 2 - 6: AAPS Annual Meeting & Expo, Boston, Mass. Contact: AAPS, 1650 King Street, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000.

NOVEMBER 2 - 6: 11th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Klyczynski, Electrosysthesis Co., 72 Ward Rd., Lancaster, NY 14086, USA.

NOVEMBER 6 - 8: 2nd North American Research Conference on Emulsion Polymers/Polymer Colloids, Hilton Head Is., SC. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561, USA.

NOVEMBER 7 - 8: 6th Conference on Current Trends in Computational Chemistry, Jackson, Miss. Contact: J. Leszcynski, Jackson State Univ., Chem. Dept., 1400 J. R. Lynch St., Jackson, MS 39217, USA. Tel: 601) 973-3482; Email: jersy@iris5.jusms.edu.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

NOVEMBER 16 - 21: AIChE Annual Meeting, Westin Bonaventure/Omni Los Angeles, Los Angeles, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218.

NOVEMBER 20 - 21: New Horizons in Chemistry Symposium, Los Angeles. Contact: J. May, Tel: (213) 740-5962; Email: jessy@methyl.usc.edu.

1998

FEBRUARY 28 - MARCH 1: 28th Annual Spring Prog. in Polymers: Degradation & Stabilization of Polymers, Hilton Head Is., South Carolina. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561. Email: ims@mhv.net.

MARCH 1 - 6: PittCon '98, New Orleans, Louisiana. Contact: PittCon '98, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 8 - 12: AIChE Spring National Meeting, Sheraton, New Orleans. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA.

MARCH 15 - 18: 14th Rocky Mountain Regional Meeting, Tucson, Arizona. Contact: R. Pott, Univ of Arizona, Tucson, AZ 85721.

MARCH 23 - 25: 5th Meeting on Supercritical Fluids: Materials and Natural Products Processing, Nice, France. Contact: F. Brionne, Secretariat, ISASF, E.N.S.I.C., 1 rue Grandville, B.P. 451, F-54001 Nancy Cedex, France. Email: brionne@ensic.u-nancy.fr.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

MAY 3 - 8: HPLC'98 – 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques, Regal Waterfront Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; EMail: Janetbarr@aol.com.

MAY 20 - 22: 32nd Middle Atlantic ACS Regional Meeting, Wilmington, Delaware. Contact: G. Trainor, DuPont Merck, P. O. Box 80353, Wilmington, DE 19880-0353. Email: reglmtgs@acs.org.

MAY 26 - 29: VIIIth International Symposium on Luminescence Spectrometry in Biomedical and Environmental Analysis: Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Las Palmas de Gran Canaria (Canary Islands), Spain. Contact: Dr. Jose Juan Dantana Rodriguez, University of Las Palmas of G. C., Chem. Dept., Faculty of Marine Sci., 35017 Las Palmas de G. C., Spain. Tel: 34 (9) 28 45.29.15 / 45.29.00; FAX: 34 (9) 28 45. 29.22; Email: josejuan.santana@quimica.ulpgc.es.

MAY 27 - 29: 30th Central Regional ACS Meeting, Ann Arbor, Michigan. Contact: D. W. Ewing, J. Carroll Univ., Chem. Dept., Cleveland, OH 44118. Tel: (216) 397-4241. Email: ewing@jevaxa.jeu.edu.

JUNE 1 - 3: 31st Great Lakes Regional ACS Meeting, Milwaukee, Wisconsin. Contact: A. Hill, Univ. of Wisc., Chem. Dept., Milwaukee, WI 53201, USA. Tel: (414) 229-4256; Email: reglmtgs@acs.org.

JUNE 2 - 5: Third International Symposium on Hormone and Veterinary Drug Residue Anbalysis, Congres Centre Oud Sint-Jan, Bruges, Belgium. Contact: C. Van Peteghem, Dept. of Food Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel: (32) 9/264.81.34;

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483.

JULY 12 - 16: SFC/SFE'98: 8th International Symposium & Exhibit on Supercritical Fluid Chromatography & Extraction, Adams Mark Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; Email: Janetbarr@aol.com.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS, 1155 16th Street, NW, Washington, DC 20036, USA.

AUGUST 31 - SEPTEMBER 3: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Charleston Place, Charleston, South Carolina. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

SEPTEMBER 13 - 18: 22nd International Symposium on Chromatography, ISC'98, Rome, Italy. Contact: F. Dondi, Chem. Dept., Universita di Ferrara, Via L. Borsari, 46, I-44100 Ferrara, Italy. Tel: 39 (532) 291154; FAX: 39 (532) 240707; Email: mo5@dns.Unife.it.

SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania. Contact: Dr. G. L. Radu, S.C.A.R., Faculty of Chemistry, University of Bucharest, 13 Blvd. Reepublicii, 70346 Bucharest - III, Romania.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Res. Triangle Pk, North Carolina. Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh, NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

NOVEMBER 15 - 20: AIChE Annual Meeting, Fontainbleu/Eden Roc, Miami Beach, Florida. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 5 - 10: PittCon 2000, Chicago, Illinois. Contact: PittCon 2000, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Seattle, Washington. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

DECEMBER 14 - 19: 2000 Int'l Chemical Congress of the Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th St., NW, Washington, DC 20036, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: pacific@acs.org.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 25 - 31: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs @acs.org.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2003

MARCH 23 - 28: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 12: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 2: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC.

AUGUST 22 - 27: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

2005

MARCH 13 - 18: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 2: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2006

MARCH 26 - 31: 231st ACS National Meeting, Atlanta, GA. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 10 - 15: 232nd ACS National Meeting, San Francisco, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2007

MARCH 25 - 30: 233rd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 24: 234th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs @acs.org.

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F. D. Pierce, H. R. Brown

Utah Biomedical Test Laboratory
520 Wakara Way
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1. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, John Wiley & Sons, Inc., New York, 1979.

Chapter in a Book:

1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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