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JOURNAL OF CHROMATOGRAPHY

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CONTENTS

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Determination of Beta-Blockers in Biological Material

edited by **V. Marko,** *Institute of Experimental Pharmacology, Centre of Physiological Sciences, Slovak Academy of Sciences, Bratislava, Czechoslovakia*

(Techniques and Instrumentation in Analytical Chemistry, 4C)

This is the third volume of a sub-series entitled *Evaluation of Analytical Methods in Biological Systems*. (The first two were *Analysis of Biogenic Amines* edited by G.B. Baker and R.T. Coutts and *Hazardous Metals in Human Toxicology* edited by A. Vercruysse). This new volume addresses beta-blockers - an area of research for which a Nobel Prize in Medicine was awarded in 1988. It provides an up-to-date and comprehensive coverage of the theory and practice of the determination of beta-blockers in biological material. Two main fields of research are dealt with in this book: analytical chemistry and pharmacology, and, as it deals with drugs used in clinical practice, it is also related to a third area: therapy. Thus, it offers relevant information to workers in all three fields.

Some 50 beta-blockers and nine methods of analysis are discussed. The methods are divided into three groups: optical, chromatographic, and saturation methods. In addition to the analytical methods themselves, sample handling problems are also covered in detail, as is the information content of the analytical results obtained. Special chapters are directed to those working in pharmacology and pharmacokinetics. Finally, as recent evidence points to the increased importance of distinguishing optical isomers of drugs, a chapter on the determination of optical isomers of beta-blockers in biological material is also included. An extensive subject index and two supplements giving retention indices and structures of beta-blockers complete the book.

This is the first book to treat beta-blockers from the point of view of their determination and to discuss in detail the use of analytical methods for beta-blockers. It will thus appeal to a wide-ranging readership.

CONTENTS: Introduction *(V. Marko)*. 1. Recent Developments in Clinical Pharmacology of Beta-Blockers *(M.A. Peat)*. 2. Clinical Pharmacokinetics of Beta-Blockers *(T. Trnovec, Z. Kállay)*. 3. Sample Pretreatment in the Determination of Beta-Blockers in Biological Fluids *(V. Marko)*. 4. Determination of Beta-Blockers by Optical Methods *(W.-R. Stenzel, V. Marko)*. 5. Determination of Beta-Blockers by Chromatographic Methods. GLC of Beta-Blockers *(M. Ahnoff)*. HPLC Determination of Beta-Adrenergic Blockers in Biological Fluids *(J.G. Barnhill, D.J. Greenblatt)*. TLC *(M. Schäfer-Korting, E. Mutschler)*. 6. Determination of Beta-Blockers by Saturation Methods. Immunological Methods for the Determination of Beta-Blockers *(K. Kawashima)*. Radioreceptor Assay of Beta-Blockers (RRA) *(A. Wellstein)*. 7. Determination of Optical Isomers of Beta-Blockers *(T. Walle, U.K. Walle)*. Subject Index. Supplements: Retention Indices of Beta-Blockers. Structures of Beta-Blockers.

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NATURAL PRODUCTS ISOLATION

Separation Methods for Antimicrobials, Antivirals and Enzyme Inhibitors

edited by **GERALD H. WAGMAN** *and* **RAYMOND COOPER,**
Schering-Plough Research, Bloomfield, NJ, USA

(Journal of Chromatography Library, 43)

This new book encompasses, in great detail, the most recent progress made in the isolation and separation of natural products. Written by experts in their respective fields, it covers antibiotics, marine and plant-derived substances, enzyme inhibitors and interferons. The book has extensive isolation schemes, tables, figures and chemical structures. In many instances a short summary of the producing organism, brief chemical description and structure and biological activity of the compounds is presented. Detailed information of extraction, separation and purification techniques follow. Each chapter has an extensive bibliography and, where applicable, an appendix showing sources of materials and equipment. A detailed index to the subject matter is also provided.

The book thus offers the reader: up-to-date reviews (including 1988) of specific topics in the natural products field not to be found elsewhere; information on new chromatographic methods and techniques described in sufficient detail to be utilized by investigators in this area of research; and extensive references to enable the serious researcher to pursue particular information. It will appeal to pharmaceutical and natural products researchers and is a valuable acquisition for university chemistry and biochemistry departments.

Contents: Countercurrent chromatography *(J.B. McAlpine, J.E. Hochlowski)*. HPLC detection methods for microbial products from fermentation broth *(R. Mierzwa et al.)*. Affinity and purification of glycopeptide antibiotics *(R.D. Sitrin, G.F. Wasserman)*. Nikkomycins and polyoxins *(H.-P. Fiedler)*. Saframycins and isoquinolines *(T. Arai)*. New cephalosporins *(S. Harada)*. Monocyclic β-lactam antibiotics *(W.L. Parker)*. Isolation of carbapenems *(K.E. Wilson)*. Avermectins and related compounds *(T. Miller, V.P. Gullo)*. Bioactive compounds from marine organisms and cultivated blue-green algae *(J.S. Mynderse et al.)*. The interferons *(S. Pestka)*. Enzyme inhibitors produced by microorganisms *(H. Umezawa)*. Alkaloidal glycosidase inhibitors from plants *(L.E. Fellows, G.W.J. Fleet)*. Chemical communication and control of development *(C.E. Smith, J.D. Orr, D.G. Lynn)*. Subject Index.

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Influence of adsorption at the gas–liquid interface on the determination of gas chromatographic retention indices on open-tubular columns coated with cyanosilicones

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(First received May 23rd, 1989; revised manuscript received September 25th, 1989)

SUMMARY

The occurrence of adsorption of *n*-alkanes on the surface of polar stationary phases is demonstrated. The Kováts retention index in such instances is strongly dependent on the stationary phase film thickness. When using polar homologues as standards on a cyanosilicone-coated column, the retention index was only weakly dependent on film thickness.

INTRODUCTION

The Kováts retention index is widely used as a means for the uniform presentation of gas chromatographic retention data obtained in isothermal runs[1–3]. This index can be applied to all possible combinations of solutes and stationary phases; the reliability is, however, not equally acceptable in all situations. The reproducibility of retention indices between different laboratories is generally considered to be within one index unit for non-polar columns, whereas the reproducibility on polar columns may extended over several units. The relatively low reproducibility achieved on polar columns may be explained partly by poor reproducibility of the columns; the main problem, however, seems to be adsorption of the *n*-alkane standards at the gas–liquid interface. A high degree of gas–liquid adsorption leads to ill-defined retention times, and it is of course especially detrimental when this concerns the standard substances. By definition, true Kováts retention indices are obtained when retention is due only to dissolution of the solutes in the stationary phase. The use of more polar standards has been suggested to improve the precision of retention indices on polar stationary phases (*e.g.*, refs. 4–10). In a recent paper, we demonstrated the advantages of 2-ketones as standards for open-tubular columns coated with a cyanosilicone[11]. In this paper, the adsorption of *n*-alkanes on the surface of a polar stationary phase and the influence of such an adsorption on Kováts retention indices are demonstrated.

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EXPERIMENTAL

A methyl(biscyanopropyl)silicone gum, 60-CN, synthesized in our laboratory[12], was used. The silicone is similar to polymer 60-CN-6 in ref. 12, and contains 60% cyanopropyl, 37% methyl and 3% vinyl substituents. This stationary phase has been extensively evaluated[11,13–15] and some further calculations based on data from these evaluations have been compiled in this work. In addition, retention data obtained on a dimethylsilicone gum, PS 264 (Petrarch Systems, Bristol, PA, U.S.A.), having 5% phenyl and 0.1–0.3% vinyl substitution[15,16], are included.

Data were obtained on various gas chromatographs. In ref. 13, normal-bore columns were evaluated on a Hewlett-Packard HP 5790 instrument and narrow-bore columns on some different instruments at the Laboratory of Instrumental Analysis, University of Technology, Eindhoven, The Netherlands. The HP instrument was used in ref. 14 and a Carlo Erba Mega instrument in refs. 11, 15 and 16.

Three test mixtures were used. In addition to the substances to be indexed, these contained *n*-alkanes, 2-ketones and fatty acid methyl esters (FAME). C_{13}–C_{20} *n*-alkanes were used for the polar columns and C_{10}–C_{16} *n*-alkanes for the columns coated with PS 264. For indexing in the 2-ketone system, C_{10}, C_{11}, C_{12} and C_{15} ketones were used. C_{10} was 2-decanone, and this substance was assigned a retention index of 1000 i.u. The FAME standard contained C_{10}, C_{11}, C_{12} and C_{14}, C_{10} being methyl decylate, to which a retention index of 1000 i.u. was assigned.

RESULTS

Three types of retention mechanisms are generally involved to different extents in gas–liquid chromatography: dissolution of the solutes in the stationary phase, adsorption at the gas–liquid interface and adsorption at the solid–liquid interface. The retention of a solute can be described by

$$V_N = (K_L V_L) + (K_{AL} A_L) + (K_L K_{AS} A_S) \quad (1)$$

where V_N is the net retention volume, K_L is the partition coefficient, V_L is the volume of stationary phase, K_{AL} is the coefficient of adsorption to the stationary phase surface, A_L is the surface area of the stationary phase, K_{AS} is the coefficient of adsorption to the liquid–support surface and A_S is the surface area of the support[17,18]. In the derivation of eqn. 1, it is assumed that the dissolution and adsorption isotherms are linear in the concentration interval studied, and that the stationary phase properties do not differ from the properties of the polymer in bulk.

When adsorption occurs at both the gas–liquid and the liquid–solid interfaces in open-tubular columns, it may be difficult to distinguish between the two. In this work, the intention was to study the adsorption of *n*-alkanes at the gas–liquid interface, and a system where other types of adsorption can be neglected was therefore selected here. For polar stationary phases, it is reasonable to assume that adsorption at the gas–liquid interface predominates and that adsorption at the liquid–support surface can be neglected[19,20]. Further, 2-methylnaphthalene was chosen as a probe, because it has a negligible adsorption at the gas–liquid and liquid–solid interfaces in the system used here. Observed shifts in retention indices would therefore be due, in principle,

TABLE I

VALUES OF K_L AND K_{AL}, WITH THE 90% CONFIDENCE LIMITS FOR 60-CN AT 100°C, CALCULATED FROM $k\,(V_m)/A_L = K_L\,(V_L/A_L) + K_{AL}$ ($n = 10$)

Compound	K_L	K_{AL}	K_L/K_{AL} *(sign)*	r^2
2-Methylnaphthalene	2635 ± 145	0.040 ± 0.061	±	0.993
C_{17}	670 ± 109	0.085 ± 0.046	+	0.942
C_{20}	2541 ± 666	0.489 ± 0.283	+	0.863

only to surface adsorption of *n*-alkanes. However, when an extremely thin film of stationary phase has been applied, an active support surface may attract polar moieties in the stationary phase, thereby decreasing the observed polarity[21]. The extent of such an orientation depends on the nature of the support surface. The different batches of fused-silica capillary tubing used in this work may have shown some variations in surface properties. These variations, however, were probably evened out by the surface deactivation that was applied here.

By use of eqn. 1, and making the assumption that adsorption occurs only at the gas–liquid interface, it is possible to calculate the adsorption and partition coefficients from[22]

$$V_N/A_L = K_L(V_L/A_L) + K_{AL} \tag{2}$$

and

$$(kV_m)/A_L = K_L(V_L/A_L) + K_{AL} \tag{3}$$

where k is the retention factor and V_m the column dead volume. The pressure correction factor according to James and Martin[23] was assumed to be 1 in all calculations. For open-tubular columns, $V_L/A_L \approx d_f$ when $r \gg d_f$ (d_f = film thickness, r = column radius).

In Table I, the partition and adsorption coefficients at 100°C are listed for

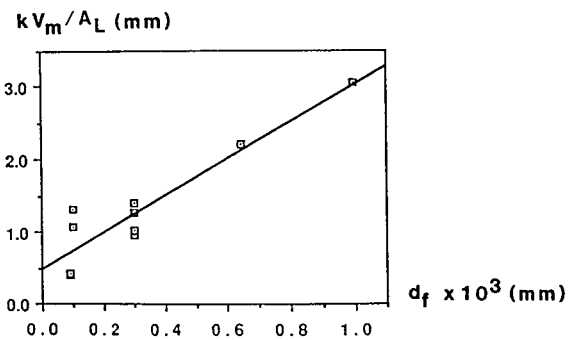

Fig. 1. Plot of kV_m/A_L *versus* d_f for eicosane (C_{20}) at 100°C on the columns listed in Table II.

TABLE II

DATA FROM DIFFERENT COLUMNS COATED WITH 60-CN (T = 100°C)

Column length (m)	*I.D. (mm)*	d_f *(μm)*	*Ref.*	*k*			*I: 2-MN*[a]	$(1/d_f) \cdot 10^{-3}$ (mm^{-1})
				2-MN[a]	C_{17}	C_{20}		
20	0.32	1.00	11	32.8	9.5	38.4	1964	1.00
20	0.32	0.3	14	10.0	3.1	12.8	1948	3.33
19	0.32	0.64	14	22.4	6.5	27.5	1956	1.56
20	0.32	0.09	14	3.6	1.2	4.9	1935	11.1
11	0.10	0.09	14	10.7	3.8	17.3	1905	11.1
20	0.32	0.3	13	11.7	4.2	15.8	1932	3.33
10	0.32	0.3	13	11.9	3.8	17.6	1923	3.33
26	0.32	0.3	13	9.7	2.9	11.9	1953	3.33
4	0.05	0.1	13	21.1	16.4	85.8	1746	10.0
4.5	0.05	0.1	13	19.4	18.2	104.8	1708	10.0

[a] 2-Methylnaphthalene.

2-methylnaphthalene, *n*-heptadecane and *n*-eicosane, and in Fig. 1 is shown the plot of eqn. 3 for *n*-eicosane on the columns listed in Table II. It is worth noting that on the 60-CN coated columns, the adsorption coefficient, K_{AL}, increases more rapidly than the partition coefficient, K_L, with an increase in length of the hydrocarbon chain. Moreover, the value of K_{AL} for 2-methylnaphthalene includes the origin within the 90% confidence limit, which indicates that this compound has a non-adsorptive character on this stationary phase. Experimental factors certainly introduced some errors here, the data being obtained on different instruments and in different laboratories. The results indicate, however, that the adsorption effects are more pronounced with the *n*-alkanes than with 2-methylnaphthalene. A presupposition for the validity of eqn. 1 is that the adsorption isotherm should be linear in the concentration interval studied. The lack of such a linearity for immobilized 60-CN was demonstrated in ref. 14; the columns evaluated here were, however, coated with non-immobilized stationary phase, and retention times for *n*-alkanes were found not to vary with amount injected within the concentration range studied.

Using the same approach, tests were made at 125°C, the data taken from refs. 11

TABLE III

DATA FROM DIFFERENT COLUMNS COATED WITH 60-CN (T = 125°C)

Column length (m)	*I.D. (mm)*	d_f *(μm)*	*k*					*I: 2-MN*[a]	$(1/d_f) \cdot 10^{-3}$ (mm^{-1})
			2-MN[a]	C_{17}	C_{20}	K_{12}[a]	E_{12}[a]		
20	0.32	2.0	23	5.7	19	15	15	2053	0.50
20	0.32	1.0	12	3.0	10	7.6	8.0	2042	1.00
26.5	0.22	0.16	2.8	0.9	3.6	1.8	2.0	1947	6.29
26.5	0.25	0.20	2.9	0.8	2.8	1.9	2.0	2010	5.00
26.5	0.25	0.20	2.9	0.8	2.8	1.9	2.0	2011	5.00

[a] 2-MN = 2-Methylnaphthalene; K_{12} = 2-decanone; E_{12} = methyl dodecylate.

TABLE IV

VALUES OF K_L AND K_{AL}, WITH THE 90% CONFIDENCE LIMITS FOR 60-CN AT 125°C, CALCULATED FROM $k\,(V_m)/A_L = K_L\,(V_L/A_L) + K_{AL}$ ($n = 5$)

Compound	K_L	K_{AL}	K_L/K_{AL} *(sign)*	r^2
2-Methylnaphthalene	925 ± 36	0.009 ± 0.040	±	0.9992
C_{17}	218 ± 10	0.012 ± 0.010	+	0.9988
C_{20}	723 ± 47	0.055 ± 0.050	+	0.9977
2-Decanone	254 ± 12	0.005 ± 0.010	±	0.9988
2-Dodecanone	572 ± 24	0.009 ± 0.020	±	0.9991
Methyl decylate	269 ± 10	0.005 ± 0.010	±	0.9993
Methyl dodecylate	603 ± 21	0.010 ± 0.020	±	0.9993

and 15 being listed in Tables III and IV. The effect of adsorption is more significant for the *n*-alkanes than for the 2-ketones and the FAME. No significant adsorption could be detected with the latter compounds on the 60-CN stationary phase at 125°C.

For comparison, data were obtained from tests made on a non-polar stationary phase. The columns used were the same as those used in refs. 15 and 16. In Table V the column dimensions and k values are listed, and in Table VI the calculated values of the adsorption and partition coefficients are given. The retention index for 2-methylnaphthalene, with the *n*-alkane standards, was found to be independent of film thickness, 1305.1 ± 0.11 ($n = 5$) at 125°C.

For the non-polar columns, the adsorption coefficients are significant only for the polar solutes, *e.g.*, 2-ketones and FAME. The adsorption coefficients for these compounds are, however, much lower than those observed for the *n*-alkanes on the polar cyano phase. The partition coefficient seems to increase more rapidly than the adsorption coefficient with increasing chain length, which indicates that the contribution to retention from adsorption effects will be less pronounced for higher homologues. Possibly, the adsorption in this instance can be attributed to adsorption on the fused-silica surface, rather than at the gas–liquid interface.

TABLE V

DATA OBTAINED ON DIFFERENT COLUMNS COATED WITH PS 264 ($T = 125$°C)

Column length (m)	*I.D. (mm)*	d_f *(μm)*	k							$(1/d_f) \cdot 10^{-3}$ (mm^{-1})
			2-MN[a]	C_{12}	C_{14}	E_{10}[a]	E_{12}[a]	K_{10}[a]	K_{12}[a]	
20	0.32	4.14	54.0	29.9	92.2	–	–	–	–	0.24
20	0.32	2.00	26.3	14.6	45.0	–	–	–	–	0.50
20	0.32	0.93	12.2	6.8	20.8	12.8	39.6	6.2	19.2	1.07
20	0.32	0.49	–	3.7	11.2	–	–	–	–	2.04
20	0.32	0.24	–	1.8	5.4	–	–	–	–	4.16
18	0.32	0.49	6.2	–	–	6.8	21.0	3.3	10.2	2.04
17	0.32	0.24	3.1	–	–	3.4	10.5	1.6	5.1	4.16

[a] 2-MN = 2-Methylnaphthalene; E_{10} = methyl decylate; E_{12} = methyl dodecylate; K_{10} = 2-decanone; K_{12} = 2-dodecanone.

TABLE VI

VALUES OF K_L AND K_{AL}, WITH THE 90% CONFIDENCE LIMITS FOR PS 264 AT 125°C, CALCULATED FROM $k\ (V_m)/A_L = K_L\ (V_L/A_L) + K_{AL}$ (n = 5 FOR THE n-ALKANES AND 2-METHYLNAPHTHALENE AND n = 3 FOR THE 2-KETONES AND FAME)

Compound	K_L	K_{AL}	K_L/K_{AL} *(sign)*	r^2
2-Methylnaphthalene	1009 ± 27	0.026 ± 0.060	±	0.9996
C_{12}	550 ± 14	0.029 ± 0.030	±	0.9997
C_{14}	1703 ± 42	0.080 ± 0.090	±	0.9997
2-Decanone	518 ± 1	0.006 ± 0.001	+	1.0000
2-Dodecanone	1618 ± 8	0.015 ± 0.005	+	1.0000
Methyl decylate	1083 ± 1	0.012 ± 0.001	+	1.0000
Methyl dodecylate	3342 ± 31	0.034 ± 0.020	+	1.0000

Berezkin[24,25] developed from eqn. 1 an equation for the calculation of an invariant retention index, I_0:

$$I_i = I_0 + a_{lLi}(1/V_L) \tag{4}$$

where I_i is the retention index for compound i, I_0 is the independent retention index for compound i, arising from pure partitioning, and a_{lLi} is a constant including, *inter alia*, the adsorption coefficient for compound i at the stationary phase surface. A presupposition for eqn. 4 is that the standards should show no adsorption on the surface of the stationary phase used. For polar phases, it is therefore necessary to use polar standards. The constant a_{lLi} in eqn. 4 also contains the stationary phase surface area A_L, and for columns having different values of A_L, I should be plotted against A_L/V_L, which is *ca.* $1/d_f$ when the column radius $r \gg d_f$. Plots of I *vs.* $1/d_f$ for 2-methylnaphthalene at 100 and 125°C using n-alkanes as standards are shown in Figs. 2 and 3. It seems that column radius would be of no importance here. It should be noted, however, that narrow-bore columns have much smaller sample capacities than normal-bore columns. In order to remain in the solute concentration region applied

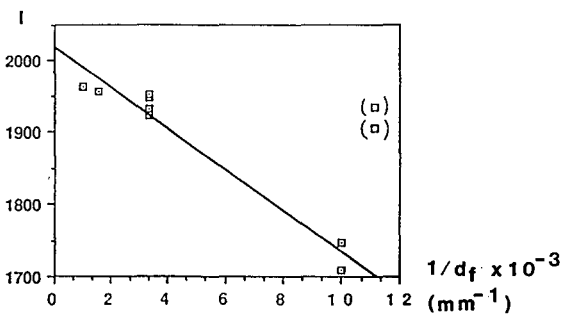

Fig. 2. Dependence of the retention index for 2-methylnaphthalene on $1/d_f$. Conditions: split injections on the columns listed in Table II, isothermal at 100°C. $I = 2019 - 29 \cdot 10^{-3}\ (1/d_f)$; correlation coefficient 0.98, $n = 8$.

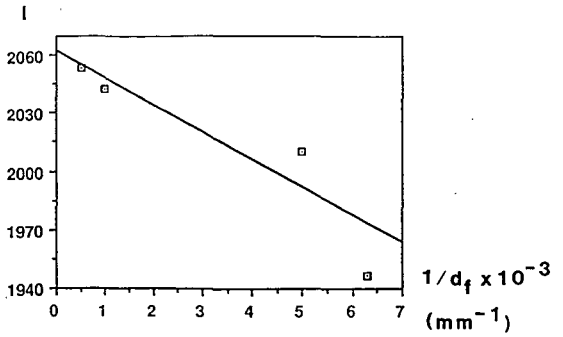

Fig. 3. Dependence of the retention index for 2-methylnaphthalene on $1/d_f$. Conditions: split injections on the columns listed in Table III, isothermal at 125°C. $I = 2063 - 14 \cdot 10^{-3} (1/d_f)$; correlation coefficient 0.89, $n = 5$.

for normal-bore columns, only small sample amounts should be injected on the narrow-bore columns.

Calculation of I_0 at 125°C in the *n*-alkane, 2-ketone and FAME retention index systems from plots of I *vs.* $1/d_f$ resulted in $I_0 = 2063$, 1318 and 1309, respectively[11].

The adsorption coefficients given in Tables I, IV and VI are relatively small, and they do not directly indicate the practical situation. Using eqn. 1 and the data given in Tables I–IV, the contribution of adsorption to the retention can be calculated (Table VII). The percentage contributions given in Table VII show tendencies rather than absolute figures. When the film thickness is increased, the stationary phase volume is increased, but the stationary phase surface area is almost unaffected. The

TABLE VII

PERCENTAGE CONTRIBUTIONS TO THE RETENTION FROM ADSORPTION EFFECTS

Column length (m)	*I.D. (mm)*	d_f *(μm)*	*Ref.*	*Contribution to retention from adsorption effects (%)*		*T (°C)*
				C_{17}	C_{20}	
20	0.32	1.00	11	11	16	100
20	0.32	0.3	14	30	39	100
19	0.32	0.64	14	17	23	100
20	0.32	0.09	14	58	68	100
11	0.10	0.09	14	58	68	100
20	0.32	0.3	13	30	39	100
10	0.32	0.3	13	30	39	100
26	0.32	0.3	13	30	39	100
4	0.05	0.1	13	56	66	100
4.5	0.05	0.1	13	56	66	100
20	0.32	2.0	11	3	4	125
20	0.32	1.0	11	5	7	125
26.5	0.22	0.16	11	26	32	125
26.5	0.25	0.20	11	22	28	125
26.5	0.25	0.20	11	22	28	125

role of gas–liquid adsorption is thus decreased for increased values of d_f. For polar packed columns, a strong influence of column loading on Kováts retention index was demonstrated by Fritz *et al.*[26].

The relationship in eqn. 4 can also be expressed as[25,27]

$$I_i = I_0 + a_{IKi}(1/k_{st}) \tag{5}$$

where k_{st} is the retention factor of a standard compound. Use of this equation was proposed for measurement of the retention index on capillary columns. The factor a_{IKi} includes $1/r$, and eqn. 5 is therefore relevant only for constant column radius.

I_0 can also be expressed as[25]

$$I_0 = 100\,z + 100 \cdot \frac{\log (K_{Ii}/K_{Iz})}{\log [K_{I(z+1)}/K_{Iz}]} \tag{6}$$

where K_{Ii} is the partition coefficient of the substance to be indexed and $K_{I(z+1)}$ and K_{Iz} are the partition coefficients of the standards, whose molecules contain $z + 1$ and z carbon atoms, respectively[25]. Insertion of the data in Table IV into eqn. 6 gives the invariant retention index for 2-methylnaphthalene at 125°C in the three different retention index systems tested. I_0 in the *n*-alkane, 2-ketone and FAME systems were 2062, 1318 and 1306, respectively. Good agreement with the I_0 values calculated from plots of I *vs.* $1/d_f$ can be observed. The I_0 value for 2-methylnaphthalene at 100°C in the *n*-alkane system was 2008 when calculated from eqn. 6. The same I_0 calculated from Fig. 2 was 2019 when the two points at $1/d_f = 11.1$ were excluded. Excluding instead the two points at $1/d_f = 10.0$ resulted in a line $I = 1956 - 3.45 \cdot 10^{-3}\,(1/d_f)$; correlation coefficient 0.71 and $n = 8$. The I_0 and correlation coefficient values and the slope of the line indicate that the data obtained on the two columns having $1/d_f = 11.1$ are anomalous.

When using *n*-alkanes as standards on a polar column, the conditions for the calculation of I_0 are not strictly followed, *i.e.*, that the standard should show no adsorption at the gas–liquid interface. In this work, the probe, 2-methylnaphthalene, is not adsorbed, and here the strong adsorption of *n*-alkanes is demonstrated by the variation in Kováts retention index for 2-methylnaphthalene with $1/d_f$. The unsuitabilty of *n*-alkanes as retention index standards on polar columns is thus demonstrated. It has been shown that when using 2-ketones or FAME as standards, the slope of plots of I for 2-methylnaphthalene *vs.* $1/d_f$ was approximately ten times lower than when *n*-alkanes were used as standards[11]. A comparison of Tables I and IV shows that the adsorption effects decrease, at least for *n*-alkanes, as the temperature increases. This was observed also by Martin[22].

The concept of invariant retention index has been used to demonstrate the adsorption of *n*-alkanes at the gas–liquid interface when using a polar column. Polar standards show only a slight degree of surface adsorption, and, therefore, they would give more precise indexing on than *n*-alkane standards polar columns.

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Displacement chromatography on packed capillary columns[a]

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SUMMARY

A displacement chromatographic microsystem was constructed using packed fused-silica capillary columns and commercially available micro-liquid chromatographic elements. The feasibility of displacement chromatographic separations on the microscale was demonstrated with a reversed-phase system using alkylphenols as test solutes. Successful separations were achieved even with samples as large as 0.1 mg on 200 μm I.D. capillary columns packed with 5-μm particles. UV detection of the separated bands was facile, as sample concentrations in the detector cell reached millimolar levels. Separation conditions were optimized to allow the direct injection of up to 10-μl sample volumes, equivalent to half of the total column volume, without deterioration of the quality of the separation.

INTRODUCTION

Notwithstanding the technical difficulties involved, packed and open-tubular capillary columns are becoming an increasingly accepted means of high-performance liquid chromatographic (HPLC) analysis (for a recent review see, for example, ref. 1). Because of the high mass sensitivity that can be realized, the use of microcolumn liquid chromatograpy systems is especially justified when the amount of sample available is limited. As only nanoliter sample volumes can be injected into packed capillary columns, detectors more sensitive than those currently applied in conventional analytical-scale HPLC systems are needed. The detection problems cannot be eliminated by simply concentrating the sample into a very small volume and injecting all of it into the column because this approach would overload the small sample capacity of the column; resulting in distorted peaks and a decrease in separation efficiency. Thus, in most instances, the bulk of the available sample cannot be utilized for analysis.

Owing to the low flow-rates (μl/min) typically encountered in microcolumn liquid chromatography, the technique is eminently suitable for directly coupled LC–

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mass spectrometric systems. However, as the concentration of the eluted components is low and their amounts are limited, detection may become a problem. Often, comparatively large amounts would be required for post-separation structure elucidation or other sample characterization purposes, or simply for further research on the pure material. Microcolumns as used today are not very effective for micropreparative separations.

Displacement chromatography is a non-linear, preparative-scale separation technique that has attracted considerable attention lately. Although displacement chromatography has been known for many years[2,3], it was revived only recently, when efficient separations were achieved using HPLC equipment[4–6]. Since then, there has been increasing interest in the utilization of displacement chromatography for preparative separations and several groups are pursuing its theoretical [7–13] and practical aspects[14–19].

In displacement chromatography, the column is first equilibrated with the carrier solution which has the least affinity for the stationary phase. Then the sample, whose components are adsorbed more strongly, is introduced, followed by the displacer which has the strongest affinity for the stationary phase. As the front of the displacer moves down the column, it moves the sample components which, in turn, move each other according to their adsorption strength. As the leading front of the displacer is extremely sharp owing to the non-linear nature of its isotherm and as the sample components can move down the column only as they are removed from the adsorption sites by the front of the displacer, the sample bands also assume the very sharp shape of the displacer front (template effect). Eventually, if the components have sufficiently different adsorption strengths and the column has the necessary efficiency, the components will occupy adjacent zones and move with the same velocity in the displacement train. The concentration of each component in the fully developed train depends only on the respective adsorption isotherms and the concentration of the displacer.

Solute concentrations and column loadings orders of magnitude higher than in elution chromatography can be realized in the displacement mode. The sample capacity of the column is better utilized, and higher fraction purities and yields can be achieved than in the alternative elution-mode separations.

Although much has been learned about the role of the operating parameters (column efficiency, capacity, dispersion, mass transfer rate, relative sample loading)[9,10,13], still relatively little is known about the rules of displacer selection and the control of separation selectivity. The lack of well characterized displacers and the lack of knowledge of the adsorption isotherms of both solutes and displacers are the main factors which hamper the wider acceptance of displacement chromatography. Displacer selection is still done by trial-and-error.

Most modern displacement chromatographic separations used either an alkyl-silica-type reversed-phase system to separate small polar molecules, antibotics, oligopeptides and small proteins[12–18], or cyclodextrin silicas to separate chiral components[19].

These considerations, and the need for larger amounts of pure, high-molecular-weight biomolecules in mass spectrometric investigations prompted us to explore the idea of miniaturized displacement chromatographic separation using packed capillary columns. To the best of our knowledge, no such separations have previously been

reported. Our objectives included the construction of an operating microcolumn displacement chromatographic system, the demonstration of the feasibility of the approach by separating model mixtures of phenolic compounds and, if successful, the determination of preliminary performance characteristics and the identification of the main areas for further research.

EXPERIMENTAL

The miniaturized displacement chromatographic system built for this study is shown in Fig. 1. It consists of an SFC 500 micropump, a μLC 10 variable-wavelength UV detector (both from ISCO, Lincoln, NE, U.S.A.), a pneumatically activated, computer-controlled Type 7001 switching valve with a 2-ml loop and a Type 7125 injection valve with 4.3- and 10-μl loops (both from Rheodyne, Cotati, CA, U.S.A.), an 85-cm long fused-silica capillary column packed in our laboratory with 5-μm reversed-phase material, and an NEC Powermate II personal computer (Computerland, College Station, TX, U.S.A.) equipped with a Metrabyte II chromatographic A/D board (Metrabyte, Taunton, MA, U.S.A.).

Columns were made of 200 μm I.D., 280 μm O.D. (Hewlet-Packard, Avondale,

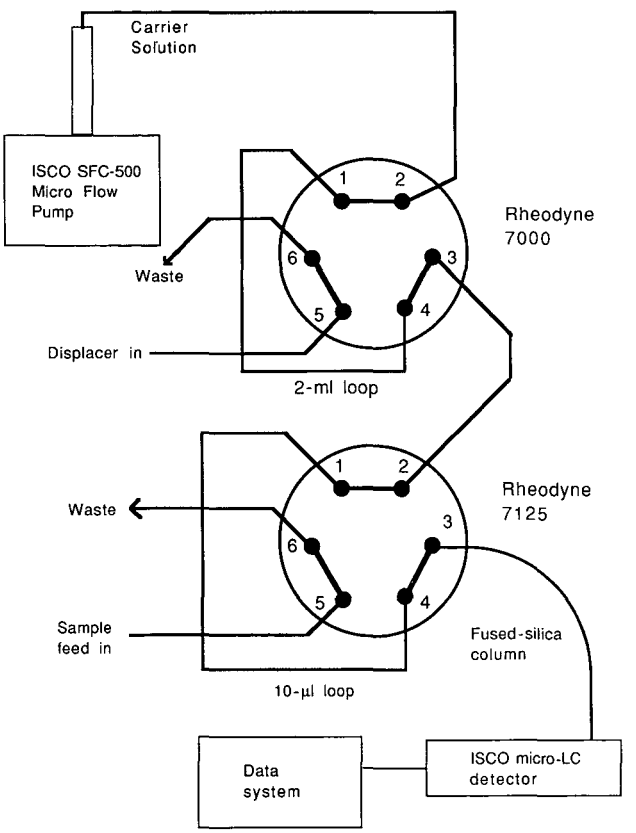

Fig. 1. Schematic diagram of the miniaturized displacement chromatographic system.

PA, U.S.A.) and 250 μm I.D., 340 μm O.D. (Polymicro Technologies, Phoenix, AR U.S.A.) fused-silica tubes. The inner surface of the 200 μm I.D. column was coated with a 0.33 μm thick layer of cross-linked methylsilicone gum[20]. The columns were terminated using a modified version of the method published by Gluckman *et al.*[21] by a Type A/E glass-fiber filter (Gelman, Ann Arbor, MI, U.S.A.) and a piece of 192 μm O.D., 100 μm I.D. fused-silica (Polymicro Technologies) tube glued into the end of the column with a fast-curing epoxy resin (Epo-Tek 353ND; Epoxy Technology, Billerica, MA, U.S.A.).

The columns were slurry packed with 5-μm Nucleosil C_{18} (Macherey, Nagel & Co., Bad Durkheim, F.R.G.) reversed-phase spherical silica. Columns made from uncoated fused-silica tubing were extremly fragile when packed with the reversed-phase silica. Columns coated with the immobilized silicone layer were very stable, and have been used without fracture. A custom-designed 150-μl slurry reservoir and the SFC micropump were used to pack the columns.

The packed capillary columns were connected to a regular Type 7125 Rheodyne injection valve through a 'T' splitter (in the elution mode), or through a common zero-dead volume union (in the displacement mode). Short pieces of a tightly fitting PTFE tube, compressed by segments of a stainless-steel capillary and beads of epoxy glue, were used as ferrules. A 1-mm long section of the polyimine layer was removed from the 100 μm I.D. fused-silica tubing below the column terminator using a laboratory-made electric device. This section was used, together with a 0.25-mm diameter aperture, as the detector cell (replacing the original detector cell of the μLC 10 UV detector). The system was controlled and chromatographic data (in both the elution and displacement modes) were collected by the NEC Powermate II computer, using an interactive system control/data acquisition and evaluation software package developed in our laboratory.

HPLC-grade ChromAr solvents were obtained from Malinckrodt (Paris, KY, U.S.A.). All solutes (1-naphthol, 2-naphthol, 4-isopropylphenol and 3-*tert.*-butylphenol) were of analytical-reagent grade and used as received (Aldrich, Milwaukee, WI, U.S.A.). Eluents were prepared by the weighing method[16] and freshly degassed before use.

The adsorption isotherms of all solutes were determined by the breakthrough method, as described [16]. Elution-mode capacity factors and the adsorption isotherms were used to select the type of displacer and the concentration of its solutions.

RESULTS AND DISCUSSION

Selection of the operating conditions: retention and isotherm data

In order to achieve a good displacement chromatographic separation, the injected sample must accumulate at the top of the column. Thus, a carrier solution composition in which the k' values of the solutes are larger than 10 (even for the least retained components) must first be determined. Therefore, the k' values of the alkylphenol model compounds were determined as a function of the methanol concentration of the eluent, as shown in Fig. 2. The k' values are larger than 10 in the eluent containing 45% (v/v) of methanol. Therefore, methanol–water (45:55, v/v) was selected both as the carrier and as the base solvent of the displacer.

The elution-mode analytical separation of the test solutes is shown in Fig. 3.

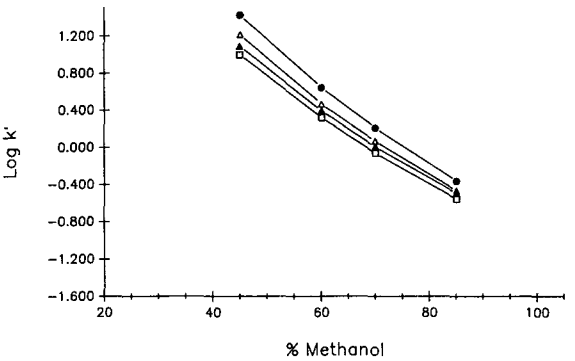

Fig. 2. Logarithm of the capacity factors of the test solutes as a function of the methanol concentration of the eluent on the Nucleosil C_{18} reversed-phase column. ● = 3-*tert.*-Butylphenol; △ = 4-isopropylphenol; ▲ = 1-naphthol; □ = 2-naphthol.

There is adequate, but not excessive, resolution between the first three solutes. The selectivity factors between the neighboring peaks are sufficiently large for analytical-scale separations (1.23 for the 1-naphthol–2-naphthol pair, 1.44 for the 4-isopropylphenol–1-naphthol pair and 1.51 for the 3-*tert.*-buthylphenol–4-isopropylphenol pair), but not large enough to permit easy preparative separations in the elution mode.

For displacement chromatographic separation to occur, the displacer must be sufficiently soluble in the carrier, more retained than the sample and have a convex isotherm that does not intersect the isotherms of the sample components. To select the displacer, the *k' vs.* methanol concentration diagram (Fig. 2) is consulted again. With 45% methanol 3-*tert.*-butylphenol is more retained than any of the other components, and consequently it may prove to be a suitable displacer. (This step can eliminate those components which cannot be used as displacers, but a successful passing of the test does not guarantee that the prospective displacer is actually a suitable one.)

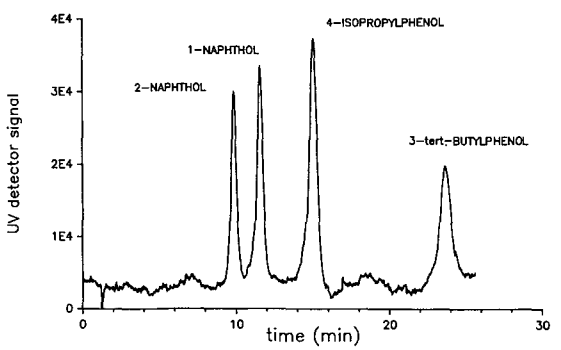

Fig. 3. Elution-mode analytical separation of the test solutes using methanol–water (45:55, v/v) as eluent on the Nucleosil C_{18} reversed-phase column.

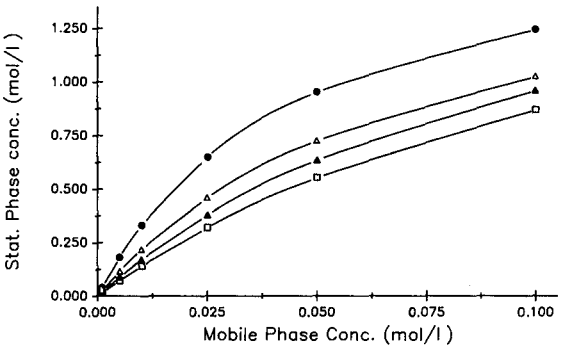

Fig. 4. Adsorption isotherms of the test solutes in methanol–water (45:55, v/v) carrier solution on the Nucleosil C_{18} reversed-phase column. Symbols as in Fig. 2.

Next, the adsorption isotherms of the test solutes and the selected displacer were determined using methanol–water (45:55, v/v) solution (the selected carrier solution composition), as shown in Fig. 4. The individual isotherms can be described well by the Langmuir isotherm equation. As 3-*tert.*-butylphenol fulfils all the requirements listed above, it can be used as a displacer for the other phenolic compounds.

Displacement chromatographic separations

For a displacement chromatographic separation to occur for all components, the operational line must intersect the isotherms of all components. Therefore, the smallest possible displacer concentration is determined by the intersection of the displacer isotherm and the tangent of the isotherm of the least retained solute, 2-naphthol, at infinite dilution. This intersection occurs at *ca.* 80 m*M*, indicating that displacer concentrations above this value may lead to fully developed displacement trains. Therefore, to be on the safe side, a 95.2 m*M* 3-*tert.*-butylphenol concentration was selected as the displacer concentrations to be used throughout the remainder of the experiments.

The displacement chromatogram obtained with direct injection of a large-volume (10 μl), large-mass (0.65 μmol) sample at high flow-rate (1.5 μl/min) is shown in Fig. 5a. (In terms of linear velocity, this flow-rate is equivalent to 0.8 ml/min in a 4.65 mm I.D. column packed with the same stationary phase.) As the UV absorbances of the individual components are different at 280 nm, the wavelength selected for detection, the successive bands can have both higher and lower absorbance signals than the preceding bands, even though the concentrations increase monotonously toward the end of the chromatogram. It can be seen that even though a sample as large as 650 nmol (*ca.* 0.1 mg) was introduced into the capillary column in an injection volume as large as 10 μl (over half of the column volume), the bands of the three solutes are separated from each other. The derivative of the displacement chromatogram is also shown. This helps to locate the inflection points and determine the widths of the fronts. It can be concluded that although the shape of the displacement chromatogram is not yet ideal, separation is possible even with such large-volume, large-mass injections.

In order to improve the shape of the displacement train, the amount of sample

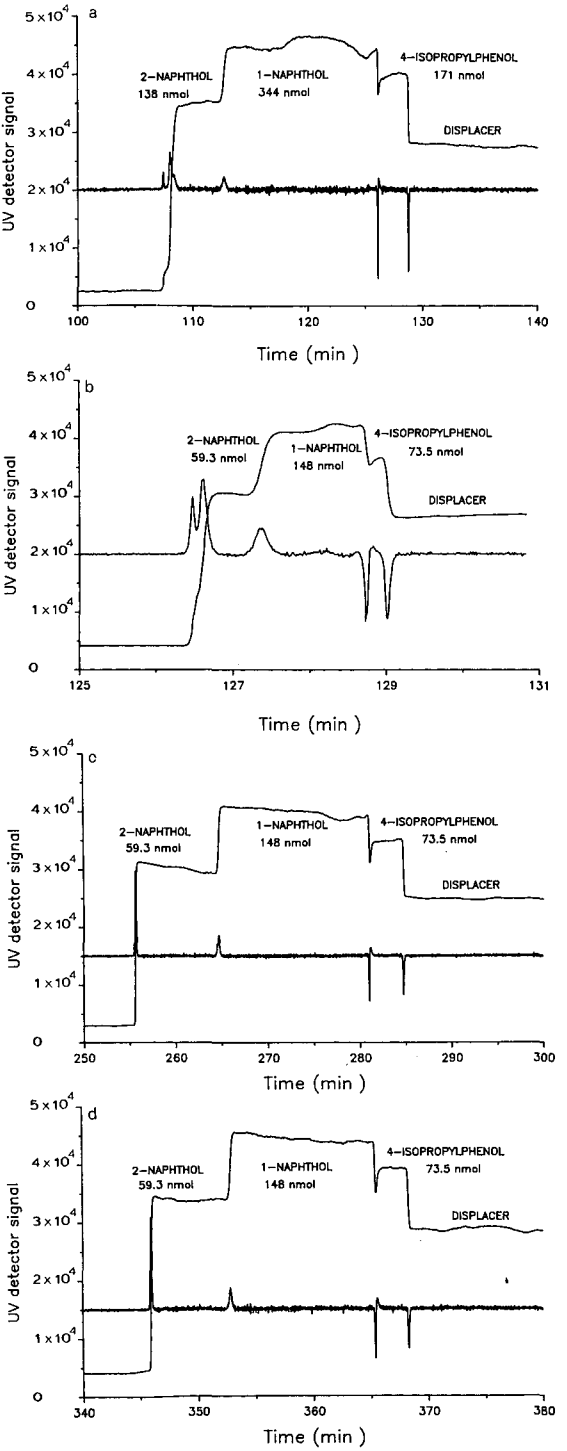

Fig. 5. Displacement chromatograms of the test solutes using a 95.2 m*M* solution of 3-*tert.*-butylphenol in methanol–water (45:55, v/v) as displacer and an 85 cm × 200 μm I.D. packed capillary as separation column. Conditions: (a) injection volume 10 μl, sample mass 65 nmol (0.1 mg), flow-rate 1.5 μl/min; (b) injection volume 4.3 μl; sample mass 280 nmol (43 μg), flow-rate 1.5 μl/min; (c) as (b), except flow-rate 0.5 μl/min; (d) as (c), except carrier solution: methanol–water (20:80, v/v) and displacer solvent methanol–water (45:55, v/v).

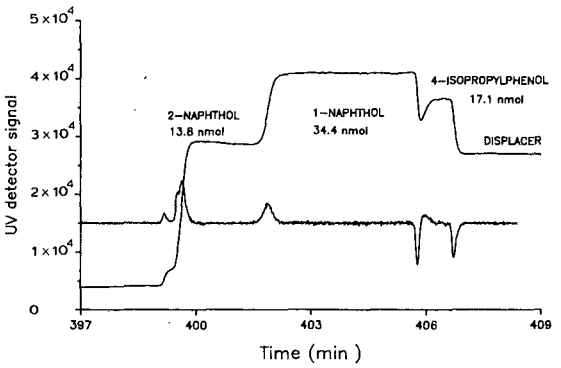

Fig. 6. Displacement chromatogram of the test solutes using a 95.2 m*M* solution of 3-*tert.*-butylphenol in methanol–water (45:55, v/v) as displacer and an 85 cm × 200 μm I.D. packed capillary as separation column. Conditions: injection volume, 10 μl; sample mass, 65 nmol (10 μg); flow-rate, 0.5 μl/min; carrier solution, methanol–water (20:80, v/v).

injected was approximately halved by replacing the manufactured 10-μl sample loop of the Type 7125 Rheodyne valve with a laboratory-made 4.3-μl loop. The displacement chromatogram obtained with the smaller volume (4.3 μl), smaller mass (280 nmol) injection of the same sample solution at the same high flow-rate (1.5 μl/min) is shown in Fig. 5b. It can be concluded that a developed displacement train is present even with the smaller sample amounts, but the sharpness of the front has not improved sufficiently.

Next, we attempted to sharpen the fronts by decreasing the flow-rate of the displacer to one third of the original, *i.e.*, to 0.-5 μl/min. The displacement chromatogram of the same sample as in Fig. 5b is shown in Fig. 5c. The chromatogram clearly shows that at reduced flow-rate the definition of the zones improved significantly.

In trying to improve the band shape even further, the same sample was injected onto the microcolumn after pre-equilibration with methanol–water (20:80, v/v) carrier solution to improve the focusing of the sample on the top of the column. The previous methanol–water (45:55, v/v) solution was still used as the solvent of the displacer. The displacement chromatogram obtained is shown in Fig. 5d. As a result of depositing the sample in a narrow at the top of the column, an ideal displacement train was obtained.

Finally, in order to see if the concentrating effect of the sample solvent is strong enough to cope with the larger injection volume (a case of practical significance), the sample used in Fig. 5d was diluted 10-fold and 10 μl were injected onto the column pre-equilibrated with methanol–water (20:80, v/v) carrier solution. This means that the sample load was now almost five times lower than in Fig. 5d. Nevertheless, the displacement chromatogram in Fig. 6 indicates that a good separation and a good zone shape are obtained even though the dilute sample had an injection volume as large as half of the column volume.

CONCLUSIONS

It has been shown that displacement chromatographic separations are possible on microcolumns. Successful separations were achieved with 200 μm I.D. packed capillary columns and sample loadings as high as 0.1 mg. Injection volumes up to 10 μl, the equivalent of half of the column volume, were successfully used. UV detection of the separated bands was facile, as sample concentrations in the detector cell reached millimolar levels. It is expected that by decreasing the column I.D. to 10 μm and using micropellicular stationary phases, the amounts injected can be decreased significantly without losing band definition or sacrificing millimolar exit concentrations. However, we are still far away from the time when this promising technique will be readily available to life scientists to help them solve their pressing micropreparative separation needs.

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Determination of biopolymer (protein) molecular weights by gradient elution, reversed-phase high-performance liquid chromatography with low-angle laser light scattering detection

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SUMMARY

The determination of molecular weights for certain proteins has been performed. This has involved the on-line coupling of gradient elution, reversed-phase high-performance liquid chromatography (RP-HPLC) with low-angle laser light scattering (LALLS) detection. A new 1.5-μm, non-porous, Monosphere RP-C_8 column has been used in order to perform fast and conventional RP-HPLC gradients (5–45 min). Traditional specific refractive index increment (dn/dc) and refractive index (n) measurements have been performed in order to derive absolute weight-average molecular weight ($\bar{M}_w$) information for ribonuclease A, lysozyme, and bovine serum albumin. Standard mixtures of known concentrations of each protein have been separated using reversed-phase gradients utilizing acetonitrile with on-line LALLS determination of excess Rayleigh scattering factors. Accurate $\bar{M}_w$ data have been obtained for all three proteins, but only under certain, conventional reversed-phase gradient elution conditions. Between 5–10 min of fast gradient elution, each protein appears to exhibit unusual $\bar{M}_w$ values, suggestive of aggregate formations. Methods have been developed to define the nature of such aggregates. The on-line coupling of modern RP-HPLC for biopolymers with LALLS represents a major step forward in the ability of bioanalytical chemists to determine the nature (monomer *versus* aggregate) of such materials. Other classes of biopolymers should prove suitable for studies with the same RP-HPLC–LALLS–UV approaches.

INTRODUCTION

The separation and determination of biopolymers by modern high-performance liquid chromatography (HPLC) has become an area of intense interest within the past few years[1–5]. Separation approaches for biopolymers have advanced rapidly,

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however, methods of detection have lagged somewhat behind these advances[6,7]. Most of the routine detectors for HPLC, such as ultraviolet–visible (UV–VIS), fluorescence (FL), differential refractive index (DRI), and electrochemistry, offer little in the way of biopolymer information or characterization. Most biopolymers show little or no response under conventional electrochemical detection (ED) conditions, in the absence of some type of derivatization. Though UV–FL detectors may provide some information, they rarely provide biopolymer identification or characterization. This is because in UV–FL detection, most biopolymers have very similar absorbance and emission spectra. Thus, in the past, chromatographic performance via capacity factor determination remains the most widely used identification parameter.

We have recently described the use of low-angle laser light scattering (LALLS) with linear diode array (LDA) detection coupled to hydrophobic interaction chromatography (HIC) for alkaline phosphatase enzymes[8] and have had even more success with other chromatographic systems. Even with just the LDA's complete spectral data profile, it remains difficult to identify/characterize proteins or their aggregates without the use of LALLS for $\bar{M}_w$ determination.

LALLS has been utilized in the past for various biopolymers in HPLC, but it has generally been restricted to isocratic conditions, mainly via size exclusion chromatography (SEC)[9–14]. It has rarely been utilized in combination with mobile phase gradient elution conditions[9]. It was our interest to investigate the use of modern chromatographic methods for protein characterization involving the use of solvent gradients. For example, HIC utilizes salt gradient elution conditions for biopolymers[15–19] (ref. 19 contains a special section on HIC, together with excellent reviews). We have recently demonstrated the advantages possible in using gradient elution HIC conditions together with LALLS for biopolymer characterization[8–10].

The use of aqueous–organic gradients in reversed-phase (RP)-HPLC has been widely described and applied in the literature, often showing high resolving capabilities, but usually resulting in the loss of enzymatic activity[20–26]. Conventional gradient elution RP-HPLC for proteins/enzymes has used methanol, acetonitrile, and isopropanol as general organic modifiers. Most RP-HPLC for biopolymers has employed 5–10-μm, wide-pore (*ca.* 300 Å), C_8 or C_{18} bonded phases, of conventional column dimensions (10–25 cm × 2.0–4.6 mm I.D.). There have been descriptions of 3-μm RP supports being used, and this can be one type of approach for fast-RP gradient elution of biopolymers[27–28]. Unger and co-workers[29–34] have recently described a totally non-porous, monodisperse, 1.5-μm bonded silica used in the separation of proteins by HIC and RP-HPLC. Essentially, the pore size here is related to the surface area. Their studies have involved both 1.5-μm HIC, as well as RP (C_8) bonded silica supports for fast gradient elution biopolymer separations. Horváth's group has also published extensively in the area of fast gradient RP-HPLC for proteins[28,35,36]. In addition, several industrial firms have now commercialized special columns for the fast elution of biopolymers, with emphasis on proteins[37].

There is no discussion in the literature wherein any type of aqueous–organic, RP gradient elution separation of biopolymers was coupled in series with on-line LALLS measurements[9]. There have been suggestions that this would be impractical, mainly because of changes in mobile phase refractive index (n) and changes in the specific refractive index increment (dn/dc) of the eluting biopolymer. Such changes were expected to invalidate any on-line LALLS measurements. We have been interested in

demonstrating the ability of using most modern biopolymer HPLC separations with on-line LALLS measurements. Our initial success with salt gradient HIC–LALLS and isocratic SEC–LALLS for bovine alkaline phosphatase[8] and β-lactoglobulin A (β-lact A)[10], led us to consider certain gradient elution RP-HPLC–LALLS-UV methods for other biopolymer systems (*i.e.*, proteins and enzymes).

This paper describes the coupling of RP-HPLC with on-line LALLS–UV measurements while using traditional dn/dc and n determinations with standard computer data acquisition for weight-average molecular weight ($\bar{M}_w$) computations. Gradient elutions were performed with varying ratios of water–acetonitrile over times from 5–45 min, using linear gradients. Efforts have been made to understand the limitations of fast RP-HPLC–LALLS–UV approaches for proteins/enzymes, as well as its advantages and possibilities. Ribonuclease A (RNase A), lysozyme (LYS), and bovine serum albumin (BSA) were the proteins investigated. Results have suggested that this approach should be amenable to many other proteins/enzymes, and conceivably to many other classes of biopolymers.

THEORY

The design of the KMX-6 LALLS photometer differs considerably from that of conventional light scattering photometers. In brief, the red light from a He–Ne laser at 632.8 nm is focused onto the sample cell and solution contained between two silica windows. The light scattered at low-angles, namely 6–7°, determined by a series of annuli and known solvent refractive index, is detected by a photomultiplier tube. The measured quantity is the ratio of the scattered to the transmitted radiant power. Sample and scattering volumes are small, 10 μl and 35 nl, respectively, using the flow through cell. The calibration method is based on geometry, hence the resulting measurements are absolute rather than being referenced to a known scattering standard. The reader is referred to references 8–10, 38–51 and others for a more in-depth review.

The calculated Rayleigh factor of solutions using the Chromatix KMX-6 is simply the quantity

$$R_\theta = \frac{G_\theta}{G_0}(D)(\sigma' l')^{-1} \tag{1}$$

where G_θ = intensity of light scattered from the solution at an angle θ, D = transmittance of the attenuators used in measuring the incident illuminating light beam, G_0 = illuminating light beam transmitted through the sample at the incident angle, and the product $(\sigma' l')^{-1}$ is a function of solution refractive index n', for the available annuli, recommended field stops and cell type. The average of median scattering angle, $\bar{\theta}$, is also constant. The "excess" Rayleigh factor, $\bar{R}_\theta$, is the difference between R_θ for the polymer solution and that for the solvent.

The equation used for the calculation of $\bar{M}_w$ is:

$$\frac{Kc}{\bar{R}_\theta} = \frac{1}{\bar{M}_w} + 2A_2 c \tag{2}$$

where c is the concentration in g/ml, A_2 is the second virial coefficient, $\bar{R}_\theta$ is the excess Rayleigh factor for solvent, and K is the polymer optical constant (for vertically polarized light) where

$$K = \frac{2\pi^2 n^2}{\lambda^4 N}(\mathrm{d}n/\mathrm{d}c)^2 \tag{3}$$

where n is the solvent refractive index, λ is the wavelength, N is Avogadro's number, and $\mathrm{d}n/\mathrm{d}c$ is the change in n per change in concentration or specific refractive index increment. n and $\mathrm{d}n/\mathrm{d}c$ should be values obtained at wavelength λ.

With monochromatic, vertically polarized light (He–Ne laser, 632.8 nm), eqn. 3 reduces to:

$$K = 4.079 \cdot 10^{-6}(n)^2(\mathrm{d}n/\mathrm{d}c)^2 \tag{4}$$

To measure molecular weight one simply requires measuring $\bar{R}_\theta$ at several different concentrations and then extrapolating the function $Kc/\bar{R}_\theta$ to zero concentration. The intercept results in the reciprocal of $\bar{M}_w$, and the second virial coefficient, A_2, may be determined from one half the slope, in ml mol/g^2. This is deduced from eqn. 2.

In the on-line mode of operation, in conjunction with an HPLC or SEC, one may determine the weight-average molecular weight for each incremental elution point across the peak by applying eqn. 2 in the following form:

$$\frac{K_i c_i}{\bar{R}_{\theta_i}} = \frac{1}{\bar{M}_{w_i}} + 2A_2 c_i \tag{5}$$

Noting that for isocratic HPLC operation analyzing homopolymers, K is constant; however, for heteropolymers or in solvent gradient operation, K will change in addition to the solid angle, $(\sigma' l')^{-1}$.

Eqn. 2 is strictly valid for two-component systems[42–50]; however, aqueous protein solutions generally contain at least three components: water (component 1), protein (component 2), and electrolyte/organic modifier (component 3), and in most cases, more than three components, since a buffer is used to control pH. Therefore, the presence of extra components limits the validity of eqn. 2. This point has been extensively discussed in the literature, and the most important conclusions with regard to the validity of eqn. 2 are: first, the value obtained by extrapolation of eqn. 2 to zero protein concentration will be an apparent molecular weight, $\bar{M}_{w_{app}}$, which contains the product of the molecular weight, $\bar{M}_w$, with a compositional parameter α, and a parameter describing thermodynamic interactions between components 2 and 3, Ψ, *i.e.*:

$$\bar{M}_{w_{app}} = \bar{M}_w(1 + \alpha\Psi) \tag{6}$$

Second, the three component system can be reduced to a two component system if the measurement of the specific refractive index increment, $\mathrm{d}n/\mathrm{d}c$, is performed using the dialyzed polymer solution. Thirdly, under certain experimental conditions, it is

possible to apply eqn. 6 and obtain correct values of molecular weight from the extrapolated value, since the values of the compositional parameter α and/or the interaction parameter Ψ are zero or compensate one another. For example, Edsall *et al.*[49] have shown that for dilute electrolyte solutions and high net charge on the protein, the intercept $Kc/\bar{R}_\theta$ *versus* c can be identified with the inverse of the actual molecular weight. However, the slope is greatly affected by the salt concentration.

Gradient elution in HPLC has characteristics that are very stringent for LALLS measurements: there is a constant change in mobile phase composition, the system is a multiple component system, and the detection of the analyte is performed under dynamic conditions. Therefore, it is necessary to investigate the validity of the method under these conditions.

For a three-component system, the relationship between the specific refractive index increment measured under conditions of osmotic equilibrium, $(dn/dc_2)^0_\mu$, and the conventional specific refractive index increment, dn/dc_2 (formerly noted as simply dn/dc), in the limit of low concentration of protein, c_2, is given by[45]:

$$(dn/dc_2)^0_\mu \approx (dn/dc_2) + (dn/dc_3)_{m_2=0}(1 - c_3V_3)\Upsilon_3 \quad (7)$$

where $(dn/dc_3)_{m_2=0}$ is the specific refractive index increment for the electrolyte/organic modifier solution when the concentration of the protein equals zero, c_3 is the concentration of the electrolyte/organic modifier, V_3 is the specific partial volume of the electrolyte/organic modifier, and Υ_3 is the specific interaction parameter, or selective sorption, of the electrolyte/organic modifier with the protein.

From eqn. 7, it is clear that the left side will be equal to dn/dc_2 when either $(dn/dc_3)_{m_2=0}$, $(1-c_3V_3)$, or Υ_3 is near or equal to zero. Therefore, three-component theory can be artificially reduced and manipulated as a two-component system.

This refractive index increment correction becomes less important with diminishing absolute value of the difference of refractive indices of the two solvent components (n_1-n_3), and becomes zero when they are "isorefractive" $(n_1 = n_3)$. When $n_1 = n_3$, the refractive index of the mixed solvent (components 1 and 3 combined) virtually does not change with its composition. Also, when both refractive increments of the macromolecule, $(dn/dc_2)^0_\mu$ and dn/dc_2 are equal, both molar masses, $\bar{M}_w$ and $\bar{M}_{w_{app}}$ assume the same value. In mixtures of two strictly isorefractive solvents, the true molar mass of a macromolecule is measured by light scattering, regardless of the composition of the mixture and of the extent of selective sorption. Therefore, isorefractive solvent systems can be treated in light scattering measurements simply as single solvents using two-component manipulations.

Isorefractivity may now be defined from the viewpoint of the light scattering method (the method itself has about a $\pm 5\%$ accuracy). $\bar{M}_{w_{app}}/\bar{M}_w$ should therefore be less than 1.05 and the relationship (eqn. 8) must hold:

$$(n_1 - n_3) < (0.025)(dn/dc_2)(1/\Upsilon_3) \quad (8)$$

For a system with a typical value of $dn/dc_2 = 0.15$ ml/g and a small $\Upsilon_3 = 0.10$ ml/g, the solvent components can be regarded as isorefractive if the absolute value of $n_1 - n_3 < 0.038$. In a system with strong selective sorption, $\Upsilon_3 = 1.0$ ml/g and equivalent dn/dc_2, solvents are considered isorefractive when the absolute value of $n_1 - n_3 < 0.0038$, which is much more restrictive.

EXPERIMENTAL

Apparatus

The HPLC–UV system was modular in design, and consisted of a Rheodyne (Cotati, CA, U.S.A.) Model 7125 syringe loading injector fitted with a 20-μl loop, two Waters (Waters-Millipore, Milford, MA, U.S.A.) Model M501 high-pressure solvent delivery systems, a Waters Model 660 solvent programmer, an Altex-Hitachi (Rainin, Berkeley, CA, U.S.A.) Model 100-40 variable-wavelength UV–VIS detector, and a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 3380A reporting integrator. The RP column used throughout this research was a 1.5-μm, Monosphere RP-C_8, non-porous material, and had column dimensions of 3.6 cm × 8 mm I.D. (Merck, Darmstadt, F.R.G., c/o EM Science, Cherry Hill, NJ, U.S.A.).

The RP-HPLC–LALLS–UV, SEC–LALLS–UV–DRI, and FIA–LALLS (a modernized instrumental derivation of the traditional static light scattering experiment utilizing flow injection analysis for sample introduction purposes) systems consisted of two modular arrangements.

System I, used for RP-HPLC–LALLS studies performed at ambient temperature (25°C), 4°C, and −5°C, was composed of an LDC Model CM4000 multiple-solvent delivery system, a Rheodyne Model 7125 syringe loading injector equipped with a 20-μl loop, a Chromatix (LDC Analytical/Thermo Instruments, Riviera Beach, FL, U.S.A.) Model KMX-6 LALLS detector set-up for flow analyses, an LDC SM4000 programmable UV–VIS detector, linked to a Soltec (Sun Valley, CA, U.S.A.) Model 1242 chart recorder, and to an IBM PC-AT compatible computer using PCLALLS (LDC Analytical) data software system.

System II, used primarily for SEC–LALLS–UV–DRI, consisted of an LDC ConstaMetric III analytical metering pump (1/3 speed), a Rheodyne Model 7125 injection valve equipped with a 100-μl loop, a TSK SW-3000 size-exclusion column, 30 cm × 8 mm I.D. (Phenomenex, Ranchos Palos Verdes, CA, U.S.A.), a Chromatix KMX-6 LALLS set-up for flow analyses, an LDC SpectroMonitor-D variable UV–VIS and RefractoMonitor IV DRI detector, all linked to both a Soltec Model 1242 chart recorder and an LDC CMX-10A analog-to-digital (A/D) converter. These A/D converters were linked to a DEC (Digital Equipment, Boston, MA, U.S.A.) Micro PDP-11/23+ computer system for digitization of instrumental analog outputs and further graphics/data manipulation. Software for calculating LALLS molecular weight information was from Chromatix, version MOLWT3.

System II was sometimes configured for FIA–LALLS analyses merely by decreasing the flow-rate to a nominal 0.1 ml/min, increasing the injection loop volume to 1.00 ml, removing the chromatographic column, and having flow directly into the KMX-6 LALLS photometer. The analog output from the LALLS photometer was sent to a Soltec chart recorder and the excess Rayleigh scattering factor was then determined for a series of individual biopolymer concentrations.

The dn/dc determinations were performed in bulk solution, with an off-line Chromatix model KMX-16 laser (633 nm) differential refractometer. Samples were analyzed via conventional methods, which theory permits. Protein concentrations were selected in order to produce data that is well within the linear range of the instrument's capabilities, typically 3–5 mg/ml, while keeping minimal sample consumption in mind.

Mobile phases

Two mobile phases were prepared for gradient elution RP-HPLC–LALLS–UV studies. The first consisted of 0.15% trifluoroacetic acid in water (A), and the second consisted of 0.15% trifluoroacetic acid in acetonitrile–water (95:5) (B). Both solvents were adjusted to pH 3.0 with ammonium hydroxide, degassed under vacuum, and filtered through a 0.2-μm hydrophilic Durapore membrane (Millipore, Bedford, MA, U.S.A.). The column was equilibrated overnight, when not in use, with B–A (50:50) at a flow-rate of 0.2 ml/min. Accurate and precise proportions of these two solvent combinations were used in the FIA–LALLS determinations. The modified protein buffer used for the SEC and FIA–LALLS studies consisted of: 18 m*M* HEPES, 7 m*M* imidazole, 1 m*M* EDTA, 3 m*M* sodium azide, 200 m*M* sodium acetate, 0.5 m*M* non-ionic surfactant, octaethylene glycol-mono-N-dodecyl ether, (Nikko, Tokyo, Japan, part No. NIKKOL BL-85Y), refractive index (RI) at 25°C, 633 nm = 1.3355 refractive index units, pH = 7.0.

Chemicals and supplies

Acetonitrile, methanol and water were HPLC Omnisolv grade, trifluoroacetic acid and ammonium acetate were reagent grade, all from EM Science. All solvents were filtered through a 0.2-μm hydrophilic Durapore filter (Millipore). Protein standards, *viz.*, RNase A, LYS, and BSA, were obtained from Sigma (St. Louis, MO, U.S.A.). All proteins were used as received, without further purification.

Procedures

RP–HPLC–LALLS–UV studies. Standard protein mixtures were prepared in mobile phase A at concentrations in the range of 3–12 mg/ml. The mobile phase composition for actual separation/elution of proteins is given in Results and Discussion. All gradient conditions were held constant except for time. Gradient conditions were 1.6 ml/min, 20–55% B (linear) and was used throughout, generating a back pressure of about 2000–2500 p.s.i. For clarification purposes, fast gradients are herein defined as having a solvent change of greater than 2% B/min, while conventional gradients are less than 2% B/min.

One important point that must be considered before performing LALLS analyses was true injected sample mass. A major source of systematic error has been due to inaccurate, but precise, injection volumes claimed by the manufacturers. Each loop was carefully custom fitted onto a particular injector to ensure a minimum dead volume, if any, between the loop itself and the injector assembly. Its volume was gravimetrically determined and contained the specific volume based on the density of water at the experimental temperature (rearrange $\rho_T = m/V$ to $V = m/\rho_T$). One can then calculate true loop volume to within 2% of actual total volume when connected to the injector. To ensure chromatographic reproducibility, each injection was performed at least five times. Linear gradients ranging from 5–45 min were employed, with simultaneous LALLS and UV detection for the RP-HPLC–LALLS–UV studies.

FIA–LALLS studies. FIA–LALLS determinations used the same mobile phase composition (%B–%A) observed at the point of elution (peak apex conditions) for each protein under RP-HPLC–LALLS–UV. These studies were performed under pure isocratic conditions, and measured bulk solution Rayleigh factors. Concentrations of proteins varied, but were usually in the range of 0.20–6.00 mg/ml.

SEC–LALLS–UV–DRI studies. This conventional approach to $\bar{M}_w$ determination for proteins and other biopolymers utilized a bonded diol phase TSK SW-3000 SEC column at constant flow-rate of 0.75 ml/min, using the modified protein buffer as the mobile phase. UV detection was at 280 nm. Each protein was injected individually, chromatographed at least in triplicate, and all data are given as the mean ± standard deviation (S.D.).

Refractive index (n) and specific refractive index increment (dn/dc) determinations. The refractive index and specific refractive index increment determinations were conducted using a modified Abbé refractometer (Milton Roy, Rochester, NY, U.S.A.) using a 632.8-nm narrow bandpass filter (Melles Griot, Rochester, NY, U.S.A.) between the light source and the receiving optics, and the Chromatix KMX-16, a differential laser refractometer. Experimental determinations of n coincided closely with interpolated literature values. Refractive index measurements, with varying percentages of B, were performed in an off-line study over the gradient range of B used in the RP-HPLC separations. Solutions of 0–100% B were prepared volumetrically in final volumes of 10 ml, with the % B increasing successively by 10 in each solution. The refractive indices of these solutions were determined in triplicate, using standard operating procedures provided with the refractometer and described in the manual.

The dn/dc values for each protein were calculated using the laser differential refractometer, Chromatix Model KMX-16, connected to a low-temperature (20-l) water bath (Fisher Scientific, Boston, MA, U.S.A.). The sample cell of the refractometer was frequently cleaned by flushing with water and then methanol, and dried by suction. The system was allowed to equilibrate for at least 30 min between measurements. The cell compartment temperature was set for 25, 4 or −5°C depending on the required temperature and was allowed to equilibrate for at least 30 min prior to taking actual measurements. To check for cell inconsistencies, readings of air *vs.* air (*i.e.*, an empty cell) were recorded. A reading of about ±30 counts indicated that the cell was clean and the system was operating properly. This procedure was followed for each solution.

The mobile phase composition for each eluting protein was calculated in the customary manner incorporating factors such as gradient lag volume, individual peak retention volume/time, flow-rate, gradient change (%B/min), and initial gradient %B composition.

Protein solutions were prepared in the predetermined mobile phase composition. Concentrations ranged from 3–12 mg/ml. The protein was first dissolved in mobile phase A. Once the solutions were homogeneous and at equilibrium, approximately one hour later, appropriate amounts of solvent B were added. These solutions were mildly swirled for about 10 min and then refrigerated at 4°C overnight. Blanks were prepared identically, side by side.

These sample solutions were then placed into the cell assembly and 6–8 readings were recorded for each sample. The refractive index was measured separately for the blank solution. Subsequent solutions, of different concentrations of proteins and blanks, were analyzed in the same manner.

Orthogonal chromatography (RP–RP) of protein mixtures. Rather than perform reinjection experiments where after initial chromatographic separation an individual peak is collected and reinjected into the same system (resulting in even further dilution), orthogonal chromatography was used using two independent chromato-

graphic systems. The first HPLC system employed a 5-min gradient while the second was running a 20-min gradient. The second injector (Rheodyne model 7010) was modified into a switching valve with a 2-ml loop, which allowed efficient and complete transfer of the analyte of interest from one system to the other under dynamic conditions (Fig. 3). The 2-ml loop was used since the elution volume of each peak from the first system was calculated to be just less than 2 ml.

Low-temperature gradient elution RP-HPLC–LALLS–UV. Low-temperature gradient elution RP-HPLC–LALLS–UV was performed under the same identical gradient conditions as at ambient temperature, except that the column and transfer tubings were equilibrated and maintained in an ice–water bath (4°C) or an ice–methanol bath (−5°C).

RESULTS AND DISCUSSION

This study has been designed to understand various phenomena regarding protein separations under normal ($\geq$15 min) and fast (<15 min) gradient elution conditions. This has involved: (1) determination of chromatographic performance criteria with UV detection; (2) determination of $\bar{M}_w$ for each protein as a function of the gradient employed; (3) determination of aggregation, if any, as a function of the gradient; and (4) overall reproducibility, accuracy, and precision of on-line LALLS determinations under certain gradient RP conditions for some proteins. These are the first studies of any protein eluted under gradient RP conditions monitored by on-line LALLS photometry for determination of $\bar{M}_w$.

We believe that other reports of gradient elution, RP separations of proteins, using conventional detection such as UV, DRI, FL, etc., may have never determined, with a high degree of certainty, the precise nature of the eluting species. Though RP-HPLC coupled to mass spectrometry (RP-HPLC–MS) may have been able to identify proteins, it is not clear that aggregates would survive the MS measurement conditions. There are no obvious reports of aggregate determination via RP-HPLC–MS approaches. The results reported here suggest that proteins behave very differently than most investigators have assumed under RP-HPLC conditions, and that their behavior is a function of the nature of the gradient generated, especially with respect to its speed of formation (change in %B/min).

In order for HPLC–LALLS measurements employing three-component systems (see Theory) to be manipulated as a two-component system, eqn. 7[45] or 8[44] need strict consideration. In RP-HPLC using acetonitrile, the refractive index (at 25°C and 633 nm) for water and acetonitrile at pH 3.0 was 1.332 and 1.341, respectively. Their difference being 0.009 refractive index units, well below the 0.025 boundary of eqn. 8. Protein elution occurred in the region where the refractive index of the buffer combination was linear when plotted *versus* % composition (25–45% B). In summary, for gradient elution to be successfully coupled to LALLS for valid molecular weight determinations, the "isorefractivity" issue must be satisfied.

We present several related studies, including: (1) RP–LALLS–UV chromatograms and $\bar{M}_w$ values for three standard proteins (RNase A, LYS, and BSA), using water–acetonitrile linear gradients with a 1.5-μm Monosphere, C_8, non-porous column, eluting within various times (5–45 min); (2) n and dn/dc values for each protein in the mobile phase composition causing elution under RP-HPLC–LALLS–

UV conditions; (3) orthogonal chromatography of 5-min protein peaks introduced into a 20-min gradient for $\bar{M}_w$ determination; (4) low temperature and concentration studies in RP-HPLC–LALLS–UV with the determination of $\bar{M}_w$ under fast gradient elution RP conditions (10 min); (5) SEC–LALLS–UV–DRI determinations of $\bar{M}_w$ with a modified protein buffer; and (6) FIA–LALLS determinations of $\bar{M}_w$ and A_2 using combinations of water–acetonitrile determined from the RP-HPLC–LALLS–UV experiments. These results demonstrate significant aggregate formation, as a function of the gradient formation times. The overall determined experimental errors, expressed in percent relative standard deviation (%R.S.D.), for all reported $\bar{M}_w$ values, were less than 10%, often less than 5% for SEC–LALLS determinations.

RP-HPLC–UV chromatographic performance criteria for typical proteins using water–acetonitrile linear gradients with a 1.5-μm monosphere, C_8, non-porous column

Others have discussed chromatographic performance with respect to proteins using gradient elution RP conditions with a 1.5-μm, non-porous C_8 silica based column[29–34]. In general, peak shapes, capacity factors, efficiencies, resolutions, plate heights, and asymmetries have been better than what has been possible using conventional ($\geqslant$3-μm) RP columns under similar gradient elution conditions. Our results are in general agreement with those already reported by Horváth and co-workers[28,35,36] and Unger and co-workers[29–34] using different approaches to the preparation of their stationary phases.

Table I summarizes our data obtained for RNase A, LYS, and BSA under typical fast-RP (5 min) conditions, using the mobile phase indicated (Experimental). We initially chose a narrow gradient profile for these first studies, namely 28–40% B in A, but later work showed that larger gradient ranges were practical. We chose water–acetonitrile in view of their popularity and similar refractive indices, with the expectation that n would not deviate markedly over the range of solvent combinations desired[42–45]. Table I illustrates reproducibility over time. Other column performance

TABLE I

SUMMARY OF RP-HPLC–LALLS–UV MOLECULAR WEIGHT DETERMINATIONS FOR RIBONUCLEASE A, LYSOZYME AND BOVINE SERUM ALBUMIN AS A FUNCTION OF GRADIENT FORMATION TIMES

Gradient time (min)	*Molecular weight ± standard deviation*[a]		
	Ribonuclease A[b]	*Lysozyme*[b]	*Bovine serum albumin*[b]
5	21 700 ± 700	25 600 ± 1100	249 000 ± 12 600
10	18 400 ± 1600	19 800 ± 1000	152 000 ± 14 400
15	14 400 ± 800	15 400 ± 1000	92 400 ± 1300
20	14 500 ± 1300	13 800 ± 1300	93 500 ± 3900
30	15 000 ± 900	14 900 ± 1100	109 500 ± 7300
45	14 200 ± 900	15 700 ± 900	96 700 ± 6100

[a] Standard deviation using 5 measurements throughout, except 30 and 45 min, where 3 measurements were used.

[b] Literature monomer $\bar{M}_w$ values for RNase A = 13 700, LYS = 14 400, and BSA = 65 000 daltons.

criteria such as capacity factor, theoretical plates, resolutions, plate heights and asymmetries have been determined, but not shown here, and are in agreement with Unger and co-workers[29–34]. In general, there is excellent reproducibility and precision, as indicated by the low %R.S.D. values.

RP-HPLC–LALLS–UV chromatograms and $\bar{M}_w$ for RNase A, LYS, and BSA, eluting under various gradient times (5–45 min)

It was clear that fast gradient RP-HPLC is a practical approach to separate and isolate proteins, but does chromatography alter the biopolymer, or does it remain intact as originally assumed? There have been no discussions in the literature of what effect fast gradient RP conditions might have on the nature of the eluting species, or of what changes might incur when varying gradient formation times. It has generally been assumed that the formation of the gradient and overall time of elution do not affect the nature of the protein species eluted. However, in the absence of $\bar{M}_w$ information, these are indeed, assumed. These studies were designed to determine if LALLS photometry was compatible with on-line gradient RP-HPLC and could the approach be used to indicate/characterize the nature of the proteins eluting as a function of gradient formation time?

First attempts were to couple RP-HPLC with LALLS detection for proteins using a 20-min gradient, Fig. 1. The LALLS–UV signals were obtained free of any baseline anomalies, and background/baseline subtraction or correction procedures were not necessary. Each chromatographic condition was reproduced five times, except where noted, under identical HPLC–LALLS–UV conditions over a long period of time (days to weeks). On processing this data, Table I, it was apparent that the molecular weights obtained were different than those initially expected.

Table I contains the RP-HPLC–LALLS–UV data for these three proteins under identical chromatographic conditions while varying only gradient formation time, with the molecular weights representative of five separate injections under each condition. For gradients operating at 15 min or greater, the first two eluted proteins. RNase A and LYS, agree with literature, with %R.S.D. values often less than 10%. BSA is anomalous, since it shows a much higher $\bar{M}_w$, suggestive of a mixture of species. Careful examination of the LALLS signal of Fig. 1 indicates a shoulder on the BSA tail, which is not as apparent in the UV signal. This is because LALLS responds to the multiplicative factor of concentration and molecular weight. Low concentrations of higher order aggregates are detected more readily with LALLS since it is sensitive to both concentration and $\bar{M}_w$. Bulk concentration detection such as UV, responds to chromophore additivity in addition to concentration. RNase A and LYS, showed no evidence of chromatographic higher order species. At this point in time we were lead to believe that BSA, as received from the vendor, already contained an aggregate, most likely the dimer, perhaps even higher order species. Our studies with this particular lot of BSA, including FIA–LALLS and SEC–LALLS, strongly supported our assumptions and that this particular lot of standard BSA was a mixture of at least monomer and dimer. It is also well known that BSA aggregates readily, and that these results were not uncommon.

At gradient formation times greater than or equal to 15 min, the calculated $\bar{M}_w$ values for all three proteins remained fairly constant and within experimental error when referenced to literature. However, at both 10 and 5 min, there was a marked

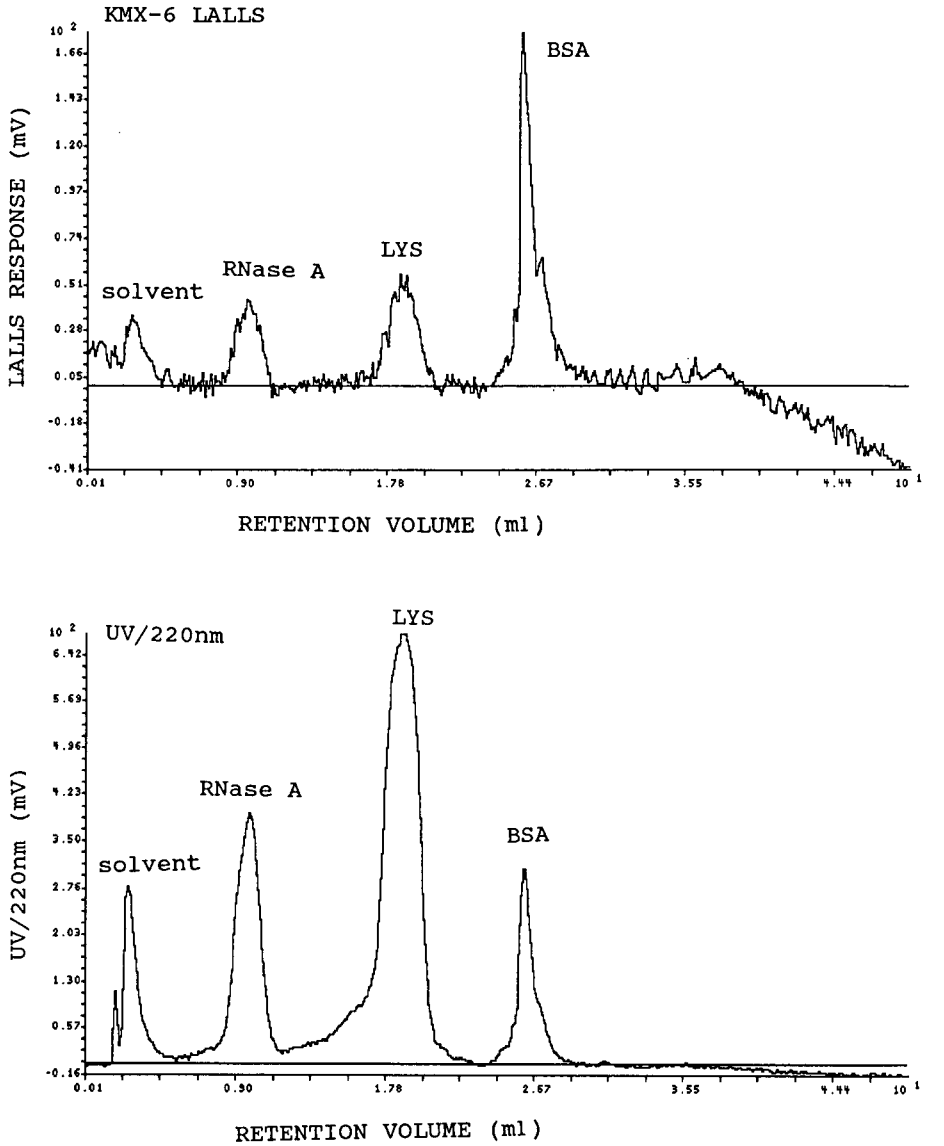

Fig. 1. RP-HPLC–LALLS–UV chromatogram of RNase A, LYS, and BSA, using a conventional 20-min, 20–55% B in A, gradient. HPLC conditions: mobile phase A = 0.15% trifluoroacetic acid, pH 3.0 and B = 0.15% trifluoroacetic acid in 95% acetonitrile–water, pH 3.0; flow-rate = 1.63 ml/min; detectors: UV = 220 nm (1000 mV f.s. = 2 a.u.f.s.) and LALLS = SEC cell, 6–7° annulus, 0.2-mm field stop, G_0 = 320 mV at initial gradient conditions, G_0 = 400 mV with $D = 4.40 \cdot 10^{-9}$; column: 1.5-μm C_8 bonded silica, non-porous, 3.6 cm × 8 mm; order of elution and injected mass = 20 μl of RNase (11.93 mg/ml), LYS (14.73 mg/ml), and BSA (3.93 mg/ml); gradient: 20–55% B in A, linear, in 20 min. Top = LALLS; bottom = UV.

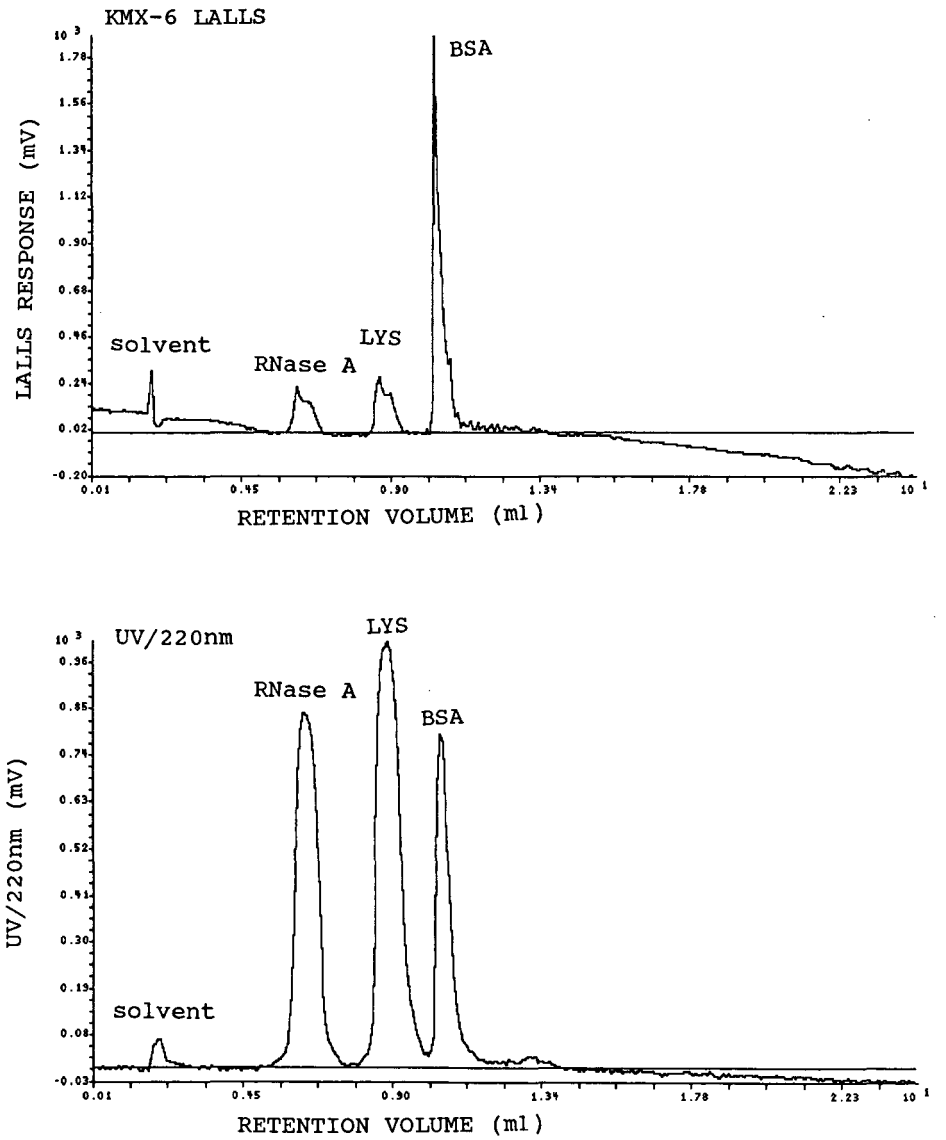

Fig. 2. RP-HPLC–LALLS–UV chromatogram of RNase A, LYS, and BSA, using a fast 5-min, 20–55% B in A gradient. Same conditions as in Fig. 1 but gradient time is now 5 min. Top = LALLS; bottom = UV.

increase in $\bar{M}_w$ for all three proteins, especially that for BSA. $\bar{M}_w$ values were derived using off-line experimentally determined refractive index, n, and specific refractive index increment, dn/dc, for each protein using as the solvent, the mobile phase composition of elution observed for each gradient formation time.

Fig. 2 illustrates typical RP-HPLC–LALLS–UV chromatograms for the three proteins using a gradient formation time of 5 min. The LALLS signal showed increased "apparent" peak splitting for all proteins not evidenced by UV. This

TABLE II

SUMMARY OF 10-MIN GRADIENT RP-HPLC–LALLS–UV STUDIES FOR $\bar{M}_w$ DETERMINATION

Temperature effect on biopolymer/aggregate formation. All $\bar{M}_w$, n, and dn/dc determinations were at 633 nm.

Temperature	*Molecular weight ± S.D. (n) (dn/dc)*		
	RNase A	*LYS*	*BSA*
Ambient (25°C)	18 400 ± 1600 (1.335) (0.172)	19 800 ± 1000 (1.335) (0.182)	152 000 ± 16 400 (1.336) (0.172)
Ice–water (4°C)	19 300 ± 300 (1.339) (0.145)	11 500 ± 600 (1.340) (0.148)	156 900 ± 3700 (1.341) (0.139)
Ice–methanol (−5°C)	38 100 ± 300 (1.340) (0.133)	12 600 ± 600 (1.342) (0.133)	223 800 ± 5300 (1.344) (0.119)

phenomenon was initially thought to be due to unaccounted changes in the LALLS scattering volume giving rise to an obviously invalid LALLS response. However, after manipulating the procedure for proper cell alignment throughout the gradient, peak splitting was determined to be a function of the analyte and the chromatographic production of higher order species due to the speed of gradient formation. All data were reproducible over time to further support our findings.

These data strongly suggested that protein aggregation resulted as a function of the gradient under fast-RP conditions.

n And dn/dc values for proteins using the mobile phase composition causing elution under RP-HPLC–LALLS–UV conditions

Various RP-HPLC mobile phase combinations (%B:%A) were prepared volumetrically, and their refractive indices at 633 nm and at the required temperature were determined. Though not illustrated here, the refractive index at 25°C changed somewhat markedly in going from 0 to about 30% B, 1.333–1.343. It remained fairly linear from 30–80% B in A, 1.343–1.347. These determinations are presented, in part, in Tables II and III. This was reassuring, for it suggested that non-linear changes in n could not be responsible for any observed changes in $\bar{M}_w$.

TABLE III

FIA–LALLS STUDIES FOR $\bar{M}_w$ DETERMINATION USING VARIOUS RP BUFFER COMBINATIONS

All reported numbers are $\bar{M}_w$ ± S.D. S.D. values are standard deviations of the reciprocal of the y-intercept.

Protein	$\bar{M}_w$ ± *S.D.*	A_2 *(ml mol/g²)*	*dn/dc ± S.D. (ml/g)*	*n ± S.D. (633 nm/25°C)*
RNase A[a]	12 800 ± 400	$7.50 \cdot 10^{-2}$	0.172 ± 0.001	1.335 ± 0.001
LYS[b]	14 600 ± 900	$-5.39 \cdot 10^{-2}$	0.182 ± 0.002	1.335 ± 0.001
BSA[c]	92 600 ± 5600	$7.49 \cdot 10^{-2}$	0.172 ± 0.004	1.336 ± 0.001

[a] 25% B:A buffer combination.
[b] 35% B:A buffer combination.
[c] 42% B:A buffer combination.

The dn/dc values for each protein as a function of the water–acetonitrile composition were experimentally determined in the traditional manner, and plotted, though not shown here, for a large number of individual $\Delta n/\Delta c$ *vs.* c points. Tables II and III include summarized dn/dc data, and these values were used to calculate individual protein $\bar{M}_w$ values. It was again reassuring that the individual dn/dc values in various %B:A RP buffer combinations were quite constant for RNase A, LYS, and BSA.

The two most suggestive reasons for the observation of the increasing $\bar{M}_w$ for these proteins, as a function of the speed of the gradient formation, namely unaccounted for changes in n and dn/dc, appeared not to be involved. In eliminating other plausible interpretations, it appeared that the increases in $\bar{M}_w$ were real, and due to biopolymer aggregate formation. However, it remained to unequivocally demonstrate that this was true through additional studies. Several major studies will be described: (1) orthogonal chromatography of protein peaks eluted under 5-min gradients, into a 20-min gradient experimentally determined not to cause aggregate formation; (2) low temperature $\bar{M}_w$ determinations under 10-min gradient conditions, and comparing the above determined sets of $\bar{M}_w$ data for these proteins; (3) concentration related $\bar{M}_w$ determinations under 10-min gradient conditions; (4) SEC–LALLS–UV–DRI determinations of $\bar{M}_w$ using modified protein buffer conditions known to theoretically prevent aggregation and association; and (5) FIA–LALLS determinations of $\bar{M}_w$ and A_2 under various isocratic water–acetonitrile mobile phase conditions.

Orthogonal RP-HPLC–UV–RP-HPLC–LALLS–UV chromatography for individual $\bar{M}_w$ determinations

It was possible that the increased $\bar{M}_w$ observed at fast RP gradient elution times (5–10 min) were yet due to an artifact of the analytical approach. In order to further determine if real aggregates were formed, two additional chromatographic studies were undertaken. In the first, orthogonal chromatography was used for each individual protein eluting under a 5-min gradient, and then this protein was directly valve switched into the start of a 20-min gradient (Fig. 3, Experimental). A UV detector was placed after the first 5-min RP column to indicate protein elution. This protein was then valve switched into the 20-min gradient, at the end of which was the usual series of LALLS–UV detection. Only two plausible outcomes for this study were possible. The protein aggregate, if indeed present and kinetically stable at the initial 5-min chromatographic time scale, *would* or *would not* survive the second 20-min gradient run and elute as the previously observed $\bar{M}_w$ from the direct 5- or 20-min run. It was also possible that, protein dependent, some of the proteins would exhibit one form, or another, of aggregation behavior.

Table IV summarizes the results of this study represented as $\bar{M}_w \pm$S.D. in g/mol or daltons. In the case of RNase A, $\bar{M}_w$ was determined to be 24 700(1700), which was slightly above the 21 700 observed from the direct 5-min run, and substantially above a direct 20-min gradient (14 500). For LYS, the $\bar{M}_w$ was 24 500 (2400), almost identical to the value found for the direct 5-min run alone (25 600). This was again above the value for LYS in a direct 20-min run (13 800). Thus, the first two proteins exhibited behavior under the orthogonal gradient conditions coinciding with the presence of aggregates formed in the 5-min gradient. Therefore, the 5-min gradient formed

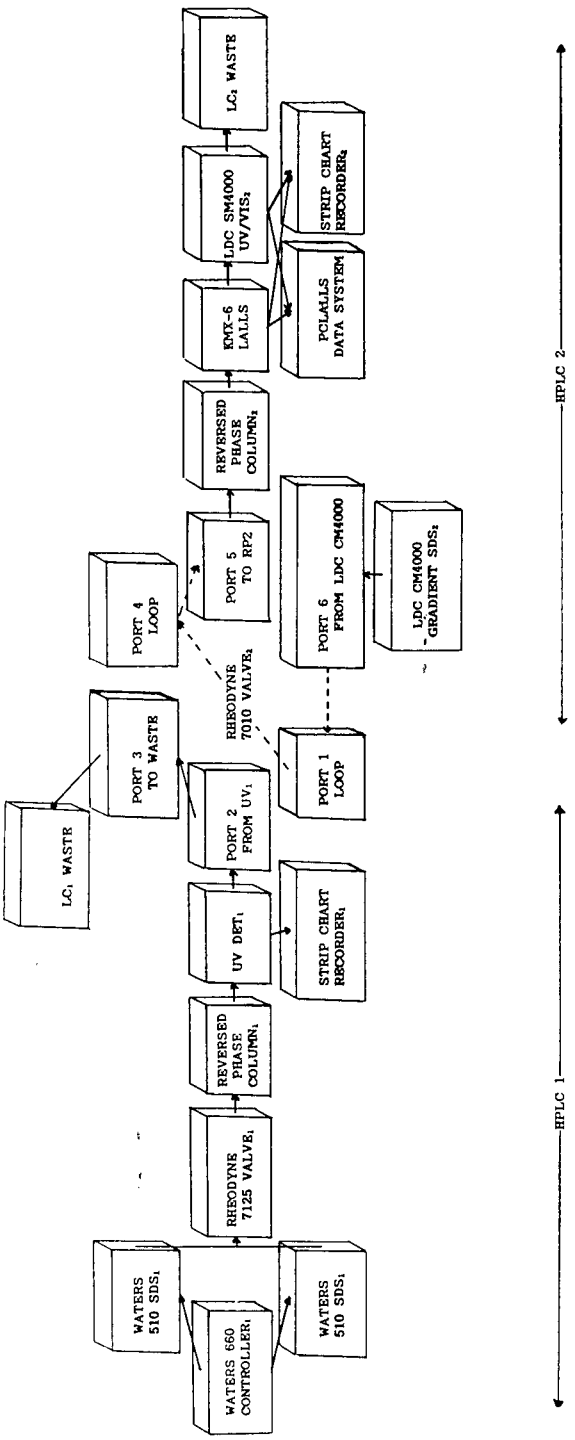

Fig. 3. Schematic block diagram of orthogonal RP-HPLC–UV–RP-HPLC–LALLS–UV instrumental system.

TABLE IV

MOLECULAR WEIGHT DETERMINATIONS FOR ORTHOGONAL CHROMATOGRAPHIC STUDIES WITH RNase A, LYS, AND BSA, AS COMPARED TO OTHER STUDIED RP CHROMATOGRAPHIES

Orthogonal conditions: RP 1: 5-min gradient into RP 2: 20-min identical gradient. All reported numbers are $\bar{M}_w \pm$ S.D.

Protein	*Orthogonal* $\bar{M}_w \pm$ *S.D.*	*Fast 5 min* $\bar{M}_w \pm$ *S.D.*	*Conv. 20 min* $\bar{M}_w \pm$ *S.D.*
RNase A	24 700 ± 1700	21 700 ± 700	14 500 ± 1300
LYS	24 500 ± 2400	25 600 ± 1100	13 800 ± 1300
BSA	85 400 ± 2100	249 000 ± 12 600	93 500 ± 3900

protein aggregate remained essentially intact throughout the second 20-min gradient chromatographic timescale.

In the case of BSA, its $\bar{M}_w$ after the orthogonal analysis was 85 400 (2100) daltons, substantially lower than the value of 249 600 daltons observed in the direct 5-min run alone. This orthogonal chromatography determined value was almost identical, within experimental error, to the value for BSA observed on a direct 20-min run. BSA behaved differently when its aggregates had formed in the 5-min gradient. These species may not have been of sufficient lifetime or stability to elute intact from the 20-min gradient. It seems reasonable to assume that aggregate stability under a 20-min gradient run would be species/protein dependent. The fact that RNase A and LYS behaved similarly, as opposed to BSAs behavior, is again reasonable given the extreme differences in $\bar{M}_w$ values, primary structures, as well as higher order structures.

The above results were all consistent of the assumption that aggregates of at least RNase A and LYS were formed in the 5-min, and most likely the 10-min, RP gradients. It is probable that BSA had also formed aggregates under the 5-min gradient but its orthogonal studies did not support this assumption. It seems unlikely that the observations for BSA were due to experimental artifacts of this approach, but rather had much more to do with the kinetics of aggregate formation and dissociation under the chromatographic conditions utilized.

Fig. 4 illustrates the type of chromatograms obtained for the above orthogonal gradient runs, using RNase A as the illustrative protein. With respect to the LALLS signal, there is evidence of a lower retention volume shoulder co-eluting with the principal peak. This is not as apparent when the UV signal is viewed. Similar observations were made for BSA, suggestive of the presence of a mixture of monomer and dimer, since its $\bar{M}_w$ was always higher than that of monomer alone. This particular BSA sample never yielded a correct $\bar{M}_w$, under any HPLC or FIA–LALLS conditions, suggestive of pure monomer.

Low-temperature studies in RP-HPLC–LALLS–UV for RNase A, LYS and BSA

Determination of $\bar{M}_w$ *under fast gradient RP elution conditions (10 min).* All of the above studies were consistent with the presence of aggregates formed under fast (5–10 min) gradient RP elution chromatography. Another study was suggested[52] that involved determining $\bar{M}_w$ at lower temperatures, using a gradient formation time

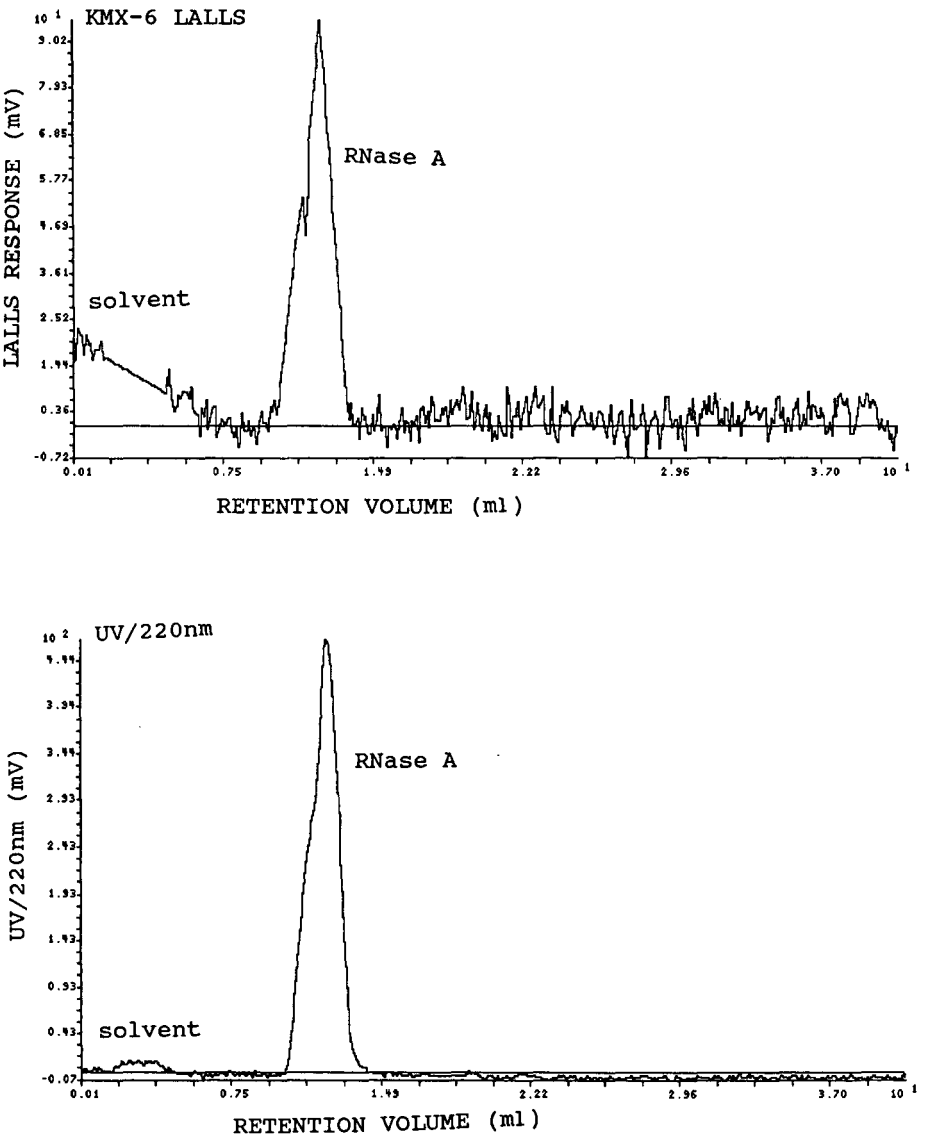

Fig. 4. Orthogonal RP-HPLC–UV–RP-HPLC–LALLS–UV chromatogram for RNase A, first injected into a 5-min gradient from 20–55% B in A, then valve switched into a 20-min gradient from 20–55% B in A. First HPLC conditions: flow-rate = 1.63 ml/min; detector: UV = 220 nm (1000 mV f.s. = 2 a.u.f.s.); column: 1.5-μm C_8 bonded silica, non-porous, 3.6 cm × 8 mm I.D.; order of elution and injected mass = 20 μl of RNase A (10.32 mg/ml), LYS (10.52 mg/ml), and BSA (4.05 mg/ml); gradient: 20–55% B in A, linear, in 5 min. Second HPLC conditions: Same operating conditions as first, except gradient: 20–55% B in A, linear, in 20 min and the addition of LALLS detection post column/pre-UV using the SEC cell, 6–7° annulus, 0.2-mm field stop, G_0 = 320 mV at initial gradient conditions, G_0 = 400 mV with $D = 4.40 \cdot 10^{-9}$. LALLS solvent front peak removed in order to better illustrate the orthogonal RNase A peak. Top = LALLS, bottom = UV.

suggesting the formation of aggregates (*i.e.*, 10 min). If these were really aggregates, and not artifacts of the system/approach, then similar analyses at lower temperatures should form even higher aggregated species, at least theoretically. As protein solubility decreases with lower temperatures, their propensity to aggregate increases accordingly. For most proteins, this is a well known phenomenon. We have performed two low-temperature studies with Table II summarizing the $\bar{M}_w$, n, and dn/dc values for all three proteins in a 10-min gradient run at ice–water temperatures (*ca.* +4°C) and at ice–methanol temperatures (*ca.* −5°C).

At ice–water conditions (4°C) both RNase A and BSA remained unchanged in their $\bar{M}_w$ values over ambient temperature, however, LYS decreased. At ice–methanol (−5°C) temperatures, both RNase A and BSA showed much higher $\bar{M}_w$ values in comparison with both the room temperature and ice–water runs. LYS remained unchanged with respect to ice–water conditions, an apparent anomaly. The LYS peak produced a higher retention volume shoulder, as evidenced from the UV signal, but due to the lack of ample LALLS signal, this shoulder can be assumed to be a much lower $\bar{M}_w$ impurity present in the standard sample. Since LYS is highly protonated at pH 3.0 (its pI = 11.0), and colder temperatures generally promote protein aggregation, one must also consider that LYS could have been partially adsorbed to the column support. This would result in under-estimating $\bar{M}_w$ since eqn. 5 states that $\bar{M}_w$ is inversely proportional to concentration. After inspection of the chromatogram, Fig. 5, it became apparent that both LALLS and UV peak shapes for RNase A were atypical. What were single UV and split LALLS peaks for RNase A, were now more pronounced and easily identifiable as multiple species peaks.

LALLS peak shapes for BSA, as evidenced by UV, were well behaved at both sub-ambient temperatures, though additional peaks appeared at −5°C for RNase A, by both LALLS and UV. It was clear that the first eluting protein, RNase A, had been split into at least three separate species, and BSA (last eluter) had a tailing shoulder, as always. The LALLS signal for LYS at −5°C in a 10-min gradient run had become quite broadened, very different from what was always observed for this protein at higher temperatures. This was also apparent for RNase A from the UV signal. We have seen that LYS apparently behaved quite differently under very low temperature fast gradient conditions, not aggregating like the others.

One other point of information for these low temperature studies was that the retention times for all three proteins had shifted by about +3% B in comparison with ambient temperature. This suggested the increased formation of aggregates (increased number of hydrophobic patches on the surface) under colder column conditions. All of the low-temperature studies were consistent, and that most aggregates increased proportionally at the lower temperatures.

Concentration studies in RP-HPLC–LALLS–UV for RNase A, LYS and BSA

Determination of $\bar{M}_w$ under fast gradient RP elution conditions (10 min). To further support our experimental findings, one last reversed-phase study was undertaken, where all conditions were held constant using fast gradient 10-min elution conditions, while varying biopolymer injected mass. The injected masses were in the range of 40–300 μg depending on which biopolymers were chromatographed (Table V). Accordingly, all retention volumes increased slightly (≈1% B for RNase A and LYS, while ≈2% B for BSA) in addition to their $\bar{M}_w$ values with increasing injected

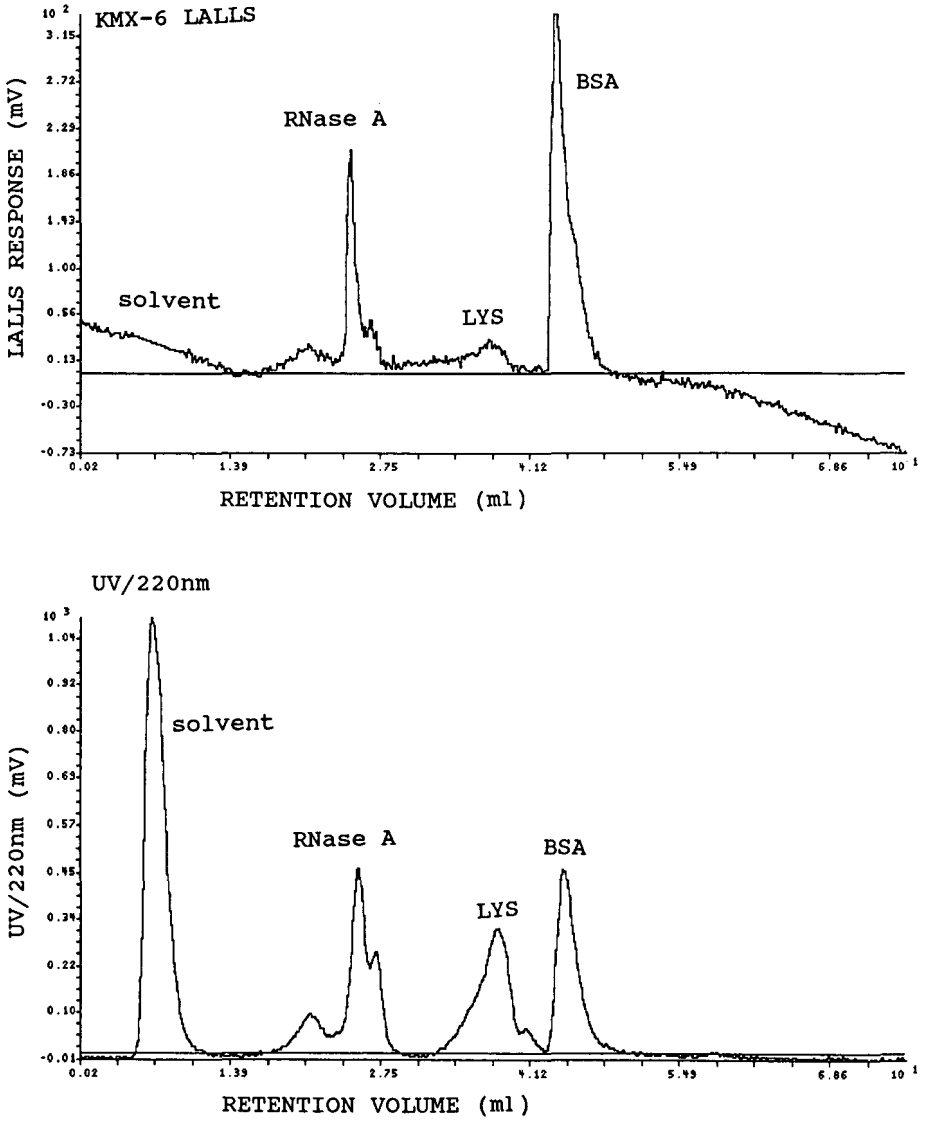

Fig. 5. Cold RP-HPLC–LALLS–UV chromatogram of RNase A, LYS, and BSA, using a 10-min, 20–55% B in A, gradient. Same conditions as in Fig. 1, but temperature is now −5°C. Top = LALLS, bottom = UV.

mass. Though not illustrated here, when $\bar{M}_w$ was plotted *versus* injected mass, and linear regression was performed, the corresponding *y*-intercepts (a condition of infinite dilution) yielded $\bar{M}_w$ values close to that of its monomer. These data further confirm our findings that under certain fast RP-HPLC conditions, proteins have a tendency to aggregate.

TABLE V

SUMMARY OF 10-MIN GRADIENT RP-HPLC–LALLS–UV STUDIES FOR $\bar{M}_w$ DETERMINATION

Concentration effect on biopolymer/aggregate formation.

Injected mass (μg)	*Molecular weight ± S.D. (5 determinations)*		
	RNase A	*LYS*	*BSA*
40	–	–	120 000 ± 4800
60	–	–	135 000 ± 5000
80	–	–	147 000 ± 6100
100	15 800 ± 900	16 000 ± 800	168 000 ± 4900
120	–	–	180 000 ± 6200
150	16 700 ± 1000	16 700 ± 1000	–
200	17 800 ± 1200	17 500 ± 1000	–
250	18 700 ± 700	18 600 ± 900	–
300	20 000 ± 800	19 500 ± 900	–

FIA–LALLS studies in RP-HPLC–LALLS buffers at ambient temperature

In order to better understand the intact nature of the proteins present, as supplied by the vendor, FIA–LALLS studies were performed in various %B:A RP buffer combinations as determined in the previous RP-HPLC–LALLS–UV experiments. Plots of the scattering function $Kc/\bar{R}_\theta$ *versus* concentration were constructed for each protein, as illustrated in Fig. 6. Each protein was prepared in a mixture of %B:A equal to that which resulted in elution under gradient RP conditions. Thus, Table III summarizes all of the $\bar{M}_w$ data obtained for each individual protein, as well as its

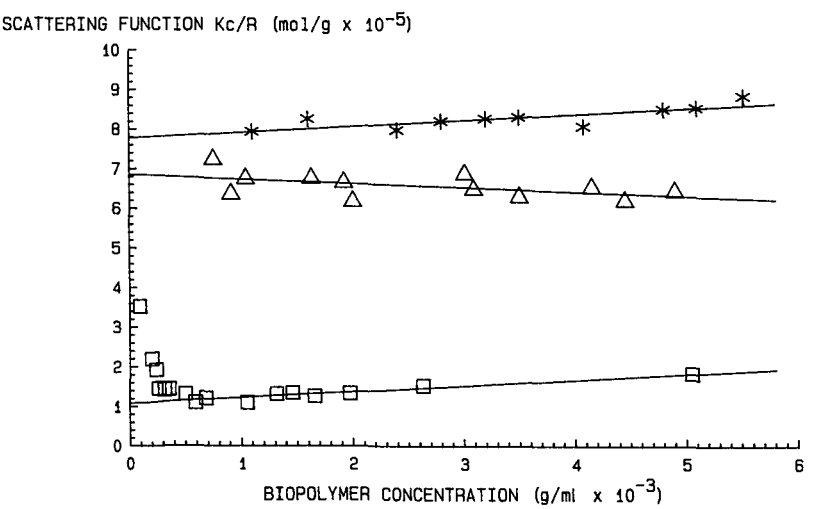

Fig. 6. FIA–LALLS (via Chromatix KMX-6) plots for RNase A, LYS, and BSA. LALLS conditions: 6–7° annulus; 0.2-mm field stop; G_θ = 115 mV; G_0 = 100 mV with $D = 3.67 \cdot 10^{-9}$; flow-rate = 0.1 ml/min (nominal); solvent/carrier = 25% B in A (RNase A), 35% B in A (LYS), and 42% B in A (BSA). * = RNase A ($\bar{M}_w$ = 12 800 ± 400, $A_2 = 7.5 \cdot 10^{-2}$); △ = LYS ($\bar{M}_w$ = 14 600 ± 900, $A_2 = -5.39 \cdot 10^{-2}$); □ = BSA ($\bar{M}_w$ = 92 600 ± 5600, $A_2 = 7.49 \cdot 10^{-2}$).

second virial coefficient, A_2. RNase A exhibited a $\bar{M}_w$ of 12 800 (400) daltons, similar to the accepted literature value of 13 700, BSA's $\bar{M}_w$ was 92 600 (5600), while LYS showed a value of 14 600 (900) daltons.

It should be noted that LYS was, experimentally, the most difficult to analyze via the FIA–LALLS technique, prone to indeterminate error. This could be due to the fact that LYS is in the highly protonated form at pH 3.0. The resultant linear regression analysis was the product of numerous sample concentrations that were statistically treated using the t test with a 95% confidence interval.

Others have described deviations in $\bar{M}_w$values for numerous proteins as a function of inappropriate buffers[42,44,45]. The A_2 values for RNase A and BSA were positive, suggesting good solubility (good protein–buffer interactions). LYS, on the other hand, showed an A_2 value that was negative, but of the same order of magnitude, suggesting poor protein–buffer interaction and more protein–protein interaction. We and others have demonstrated that negative A_2 values are often suggestive of non-ideal protein–buffer interaction.

This particular lot of BSA had never shown itself to be pure monomer, as supplied by the vendor. It has consistently exhibited the contributions of higher order aggregates. As supplied, BSA existed as both a mixture of about 70% monomer and 30% dimer (FIA–LALLS $\bar{M}_w$ = 92 600 daltons), though higher aggregates may have been present. It behaved anomalously, at lower concentrations, as evidenced by plotting the data. Its scattering function $Kc/\bar{R}_0$ (inverse of $\bar{M}_w$) increased exponentially as concentration was lowered below 0.30 mg/ml. This can be interpreted: (1) as concentration decreases, the fraction of BSA containing the dimer dissociates into monomer, or (2) more evidently, as concentration goes below 0.30 mg/ml, errors associated from indeterminate sources have higher probability. In other words, for this particular biopolymer–buffer system studied, we would have exceeded the linear detection limit in FIA–LALLS of approximately 0.30 mg/ml. We therefore extrapolated the data above this concentration, in order to deduce $\bar{M}_w$ in the customary manner.

SEC–LALLS at ambient temperature using a disaggregating buffer system for the determination of protein $\bar{M}_w$

In order to more fully define the exact nature of the protein species present on receipt, and to support the above results on the FIA–LALLS studies, we performed conventional SEC–LALLS–UV–DRI determinations for $\bar{M}_w$ in a special buffer. This particular buffer has been described and used to prevent aggregate formation and dissociation[9,51,53]. Under these particular SEC conditions, using this buffer as the mobile phase, $\bar{M}_w$ values were determined for each protein. Work with LYS under these conditions, at pH 7.0 for the disaggregating buffer, showed adsorption. The basic protein LYS has been shown to be a sensitive probe for the determination of residual silanols represented as SiO^-, as shown by Pfannkoch *et al.*[54]. In buffers whose pH is less than or equal to 7, LYS has a strong net positive charge involving hydrophobic and electrostatic attractive forces between the protein and substrate making it an ideal probe in studying residual SiO^-, but not particularly well suited for true SEC separations utilizing silica stationary phases. This phenomenon is chromatographically undesirable and subsequent $\bar{M}_w$ determination was not possible for LYS under these chromatographic conditions. However, much of the literature on the SEC of

proteins involves derivatized silica packings, and the TSK SW series show the most popularity[55–57], even though LYS was retained.

Assuming a 90% purity for RNase A (claimed by the vendor), its $\bar{M}_w$ was exactly as that in the literature, 13 800(300) *vs.* 13 700 daltons, respectively. In the case of BSA, once again a purity assumption of 95%, claimed by the vendor, yielded a $\bar{M}_w$ of 86 900(2200) daltons, significantly above that reported for the monomer, 65 000. This had always been the case for our BSA. It had always existed as a mixture of BSA-mers. We have now been able to unequivocally characterize each peak present under these SEC–LALLS–UV–DRI conditions, and it is inclusive that this particular lot of BSA consisted of approximately 70% monomer and 30% dimer, having $\bar{M}_w$ values of 67 000(1700) and 138 000(3500) daltons, respectively, with % RSD values of less than 3%.

CONCLUSIONS

From this study, certain interesting observations have been made. After having ruled out factors such as incorrect measurements of refractive index and dn/dc leading to artifacts of the system which would lead to apparent molecular weights, one may conclude that the higher molecular weights are due to on-column aggregation and are protein specific. Though it is not fully understood at this point as to why aggregates would form as a function of the rapidity of the gradient, a certain hypothesis can be made to explain this phenomenon.

Proteins that have been studied in these experiments are hydrophilic. In the presence of hydrophobic solvents the protein molecules have a tendency to attract one another, to a point where precipitation may occur. In case of the 5- and 10-min gradients the percentage of the organic modifier changes very rapidly at the rate of about 5–7% B per min. The sudden increase in the hydrophobicity of the environment could be the reason for the protein molecules to aggregate. Apart from this, it is very difficult to imagine, on a molecular level, the behavior of proteins in a rapidly changing, dynamic environment.

The purpose of this study was to determine if it was possible to couple RP-HPLC with LALLS for doing on-line separation and characterization of proteins in a short period of time. The results obtained with shallower gradients, 15-min (less than a change of 2% B/min), do indicate that it is possible to separate and characterize proteins in a reasonable amount of time. However, using shorter gradients to speed up the analysis has serious drawbacks. In doing future work on separating proteins, optimization of the gradient range and time would be of major consideration. Though this study has established the fact that gradient RP-HPLC is compatible with LALLS under optimum conditions, further work needs to be done to investigate the precise reasons behind the formation of higher order species as a function of the rapidity of the gradient. This would involve some kind of molecular modeling to investigate the changes in the structures of the molecules under certain experimental conditions.

SYMBOLS AND ABBREVIATIONS

A_2	= second virial coefficient in units of ml mole/g^2
a.u.f.s.	= absorbance units full scale
BSA	= bovine serum albumin

c = concentration, usually expressed in mg/ml, or g/ml $\times 10^{-3}$
CMX-10A = "Chromatix Products" 12 bit analog-to-digital converter
D = transmittance of optical attenuators used in measuring the incident illuminating light beam
dn/dc = specific refractive index increment, expressed in ml/g
DRI = differential refractive index, a concentration detection method
ED = electrochemical detection
FIA–LALLS = flow injection analysis coupled to low-angle laser light scattering photometry for determination of $\bar{M}_w$ and A_2 of bulk solutions (a modern derivation of the traditional static LALLS experiment)
FL = fluorescence, concentration sensitive detection method
G_θ = intensity of light scattered from the sample at an angle, θ
G_0 = intensity of laser light beam transmitted through the sample at zero angle
HEPES = N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HIC = hydrophobic interaction chromatography
HPLC = high-performance liquid chromatography
K = the polymer optical constant and for measurements below 7° = $(4.079 \cdot 10^{-6})\,(n)^2(dn/dc)^2$ in units of mol cm^2/g^2
KMX-6 = LDC Analytical/Thermo Instruments, "Chromatix Products" LALLS photometer
KMX-16 = LDC Analytical/Thermo Instruments, "Chromatix Products" instrument used to physically measure dn/dc
LALLS = Low-Angle Laser Light Scattering
LDA = linear diode array, a concentration sensitive detection method (UV)
LYS = lysozyme
-mers = monomer repeat units
MS = mass spectrometry
mV f.s. = millivolts full scale
$\bar{M}_w$ = weight-average molecular weight in units of g/mole or daltons
n = solvent/mobile phase refractive index, determined at 633 nm
PCLALLS and MOLWT3 are software packages available through LDC Division/Thermo Instruments, and are used to process on-line LALLS measurements
RNase A = ribonuclease A
RP-HPLC = reversed-phase high-performance liquid chromatography
R.S.D. = relative standard deviation = S.D./mean, sometimes expressed as %R.S.D.
R_θ = Rayleigh factor
$\bar{R}_\theta$ = excess Rayleigh factor = Rayleigh factor of the sample solution − Rayleigh factor for solvent alone
S.D. = standard deviation using $n-1$ sampling
SEC = size exclusion chromatography
SEC–LALLS–UV–DRI = SEC coupled to LALLS, UV and DRI detection, in series
$(\sigma' l')^{-1}$ = solid angle, scattering volume correction, defined by instrument geometry and solvent refractive index
UV–VIS = ultraviolet–visible, a concentration detection method

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Purification of histone H1 polypeptides by high-performance cation-exchange chromatography

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SUMMARY

Calf thymus histone 1 (H1) was cleaved by chemical and enzymatic methods and the resulting polypeptides were fractionated by high-performance cation-exchange. Up to 1 mg of H1 polypeptides were loaded onto a 50 × 5 mm I.D. cation-exchange column and fractionated to greater than 95% purity in less than 30 min. This is the first report on the separation of H1 polypeptides by a strong cation-exchange matrix. In addition, the high-performance cation-exchange chromatography protocol represents a significant decrease in fractionation time when compared to conventional ion-exchange and gel filtration chromatography. The utility of this procedure is shown when the H1 peptides purified by the protocol were used to define antigenic domains of H1 band by procainamide-induced lupus and idiopathic systemic lupus erythematosus. The majority of the sera tested by enzyme-linked immunoassay (ELISA) reacted to the C-terminal peptides of H1 indicating this to be the major antigenic domain of H1.

INTRODUCTION

Histones are low-molecular-weight nuclear proteins with a high content of arginine or lysine. Histone 1 (H1) is the most variable of the histones and the number of H1 subfractions varies from tissue to tissue and species to species[1]. Although the precise cellular role of H1 remains unknown, an important adjunct to these studies is the ability to isolate and purify various domains of the native protein. These types of studies, pioneered by Crane-Robinson and co-workers[2,3], have shown that H1 can be cleaved into three structural domains. The purification of these peptides has provided an important approach to determine the primary structures and antigenic domains of H1 and its variants[4–6]; to study their role in chromatin structure[7] and the transport of newly synthesized H1 from the cytoplasm to the nucleus[8].

Using H1 peptides prepared by enzymatic or chemical cleavage, it has been demonstrated that the central region, made up of approximately 75 amino acids, contains hydrophobic residues and under physiological conditions assumes a globular structure. This domain locates H1 in the nucleosome at the point where DNA

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strands exit the chromatosome[7]. The cationic N-terminal domain consists of approximately 33 amino acid residues while the randomly coiled C-terminal domain makes up the other half of the intact H1 molecule.

Most protocols call for the separation and purification of the cleavage products of H1 by gel filtration and weak cation-exchange chromatography. Because gel filtration and ion-exchange chromatography of the cleaved H1 polypeptides normally requires extensive fractionation time, other approaches such as high-performance cation-exchange chromatography (fast protein liquid chromatography, FPLC) have been evaluated.

We describe an FPLC protocol that provides a rapid and quantitative method for purifying H1 polypeptides. This method can be used to fractionate milligram quantities of H1 polypeptides by employing a short, cation-exchange column and a linear gradient of sodium chloride in phosphate buffer. The polypeptides can be fractionated to more than 95% purity in 20–30 min. The utility of this approach in preparing peptides that can be used to determine immunodominant domains of H1 that react with systemic lupus erythematosus (SLE) and procainamide induced lupus sera is demonstrated.

MATERIALS AND METHODS

Cleavage of calf thymus H1

H1 was extracted from calf thymus with 5% aqueous trichloroacetic acid and purified by chromatography on a Bio-Gel P-60 column[9]. A schematic representation of H1 and the different peptides derived by the following chemical and proteolytic cleavage is shown in Fig. 1.

N-Bromosuccinimide cleavage of H1. N-bromosuccinimide (NBS) cleaves on the C-terminal side of tyrosine residue 72 of calf thymus H1[10]. An amount of 2.5 mmol of H1 in 10 ml of 50% aqueous acetic acid was digested with 25 mmol of freshly prepared NBS. After 3 h 25 mmol of fresh NBS was added and the reaction terminated after 4 h by lyophilization. This resulted in the production of peptides 1–72 and 73–220 (see Fig. 1).

Chymotryptic digestion of H1. A 50-mg amount of purified calf thymus H1 in 5 ml of 50 mM Tris–HCl (pH 8.0) was cleaved with four units of α-chymotrypsin at 24°C for 15 min to yield peptides corresponding to residues 1–106 and 107–220, and residual intact H1[11]. After the reaction was terminated with 5 m*M* phenylmethylsulfonyl fluoride, the peptides were dialysed (Spectra Por 3, molecular weight cut-off 3500) overnight at 4°C against 5 m*M* acetic acid, lyophilized and the dried powder stored at −20°C in a desiccator.

Thrombin digestion of calf thymus H1. Thrombin cleaves on the C-terminal side of residue 122 of calf thymus H1 at the sequence -(Pro)-Lys-Lys-Ala to produce peptide residues 1–22 and 123–220[12]. Digestion of 50 mg of H1 in 5 ml of 50 m*M* Tris–HCl (pH 8.0) buffer containing 2 m*M* calcium chloride was achieved with 50 units of thrombin for 6 h at 36°C. The reaction was terminated by the addition of 5 m*M* diisopropyl fluoride and the peptides were dialysed against 5 m*M* acetic acid and lyophilized.

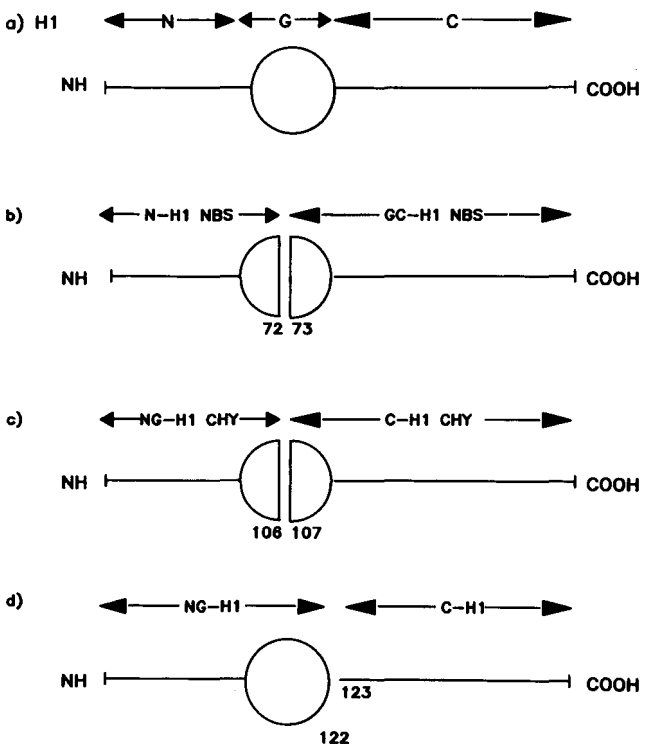

Fig. 1. Calf thymus H1 and peptides obtained by chemical and enzymatic cleavage. (a) Intact calf thymus H1 represented as a tripartate structure with the N-terminus (N), the central globular domain (G) and the C-terminus (C). (b) Peptides generated by NBS cleavage, GC-H1 NBS (residues 73–220) and N-H1 NBS (residues 1–72). (c) Peptides generated by chymotrypsin, C-H1 CHY (residues 107–220) and NG-H1 CHY (1-106). (d) Peptides generated by thrombin digestion C-H1 (residues 123–220) and NG-H1 (residues 1–122).

FPLC of H1 polypeptides

Calf thymus H1 polypeptides generated by digestion with NBS, chymotrypsin and thrombin were separated by FPLC. Lyophilized H1 polypeptides were dissolved at a concentration of 5 mg/ml in 0.1 *M* phosphate buffer (pH 7.0) and 200 ml of sample loaded onto a Mono Q column (HR5/5, 50 × 5 mm I.D., Pharmacia). The progress of chromatography was monitored and controlled by the Pharmacia FPLC LCC-500 computer. The peptides were eluted with a linear gradient of 0 *M* NaCl–0.1 *M* phosphate buffer (pH 7.0) to 1.0 *M* NaCl–0.1 *M* phosphate buffer (pH 7.0) over 20–30 ml. The eluate was monitored at 214 nm and 1-ml fractions were collected. The peptides were precipitated with $\frac{1}{3}$ volume of 100% trichloroacetic acid and washed three times with 100 volumes of acetone–25 m*M* hydrochloric acid. The powder was vacuum-dried and stored at −20°C in a desiccator.

Enzyme-linked immunoassay (ELISA) of H1 polypeptides

ELISA was performed as previously described[5]. The polystyrene plates were coated with 2 nmol of intact H1 and its polypeptides and subsequently tested with patient sera at a dilution of 1:100.

Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels (18%) in sodium dodecyl sulfate (SDS) were cast at a dimension of 120 × 170 × 15 mm and electrophoresis was performed at 30 mA per gel for 6 h[13].

Amino acid analysis

Amino acid analysis of H1 polypeptides purified by FPLC was performed on a Beckman 119 LC amino acid analyzer after hydrolysis in 6 *M* hydrochloric acid (1 ml per mg protein) at 110°C for 24 h *in vacuo* with 1% phenol to avoid excessive degradation of tyrosine. The amino acid composition of the H1 peptides was compared to published reports and has been previously published (see Table I)[8].

RESULTS

FPLC and identification of N-bromosuccinimide-digested H1

Digestion of H1 with NBS produces two major polypeptides which are represented as three bands on the polyacrylamide gels (see Fig. 2B, lane b). The C-terminus of H1 (GC-H1 NBS) is characterized by microheterogeneity and therefore it appears as a doublet (Fig. 2B, lanes b and c). The N-terminal peptide appears as a faint Coomassie blue stained single band (Fig. 2B, lane b).

When H1 was digested with N-bromosuccinimide and fractionated on a Mono S column with a linear gradient of increasing sodium chloride concentration, three peaks were observed. The first peak between 0–0.08 *M* sodium chloride beginning at 1 ml and ending at 4 ml. No polypeptides were identified in this peak. A second peak started eluting at 0.3 *M* sodium chloride at 8 ml, finishing at approximately 13 ml and sodium chloride concentration of 0.55 *M* (Fig. 2A). The second peak, containing N-H1 as determined by amino acid analysis, was more than 95% pure as demonstrated by SDS-PAGE (Fig. 2B, lane d; Table I). A third peak, which eluted between 14 and 21 ml and at a salt concentration between 0.65 and 0.95 *M* sodium chloride, contained GC-H1 NBS (Table I). The purity of this peak was more than 95% as determined by SDS-PAGE (Fig. 2B, lane c). Two bands in the gel (Fig. 2B, lane c) reflect the microheterogeneity in the C-terminal half of the H1 molecule. This doublet was demonstrated in all the C-terminal polypeptides produced by chemical or enzymatic cleavage whereas the N-terminal H1 polypeptide appeared as a single band (see Figs. 2B, 2B and 4B). Because the FPLC fractionation was highly reproducible and performed in less than 30 min, it was possible to chromatograph several aliquots of NBS-digested H1 to obtain milligram amounts of pure GC-H1 NBS and H-H1 NBS.

FPLC and identification of chymotrypsin-digested H1

Chymotrypsin digestion of calf thymus H1 results in three major bands and other minor ones visualized in SDS-polyacrylamide gels (Fig. 3B, lane b). These bands were identified by amino acid analysis (Table I) as C-H1 CHY (residues 107–220) and NG-H1 CHY (residues 1–106).

The FPLC profile of chymotrypsin-digested H1 was similar to that obtained with NBS-digested H1 (Fig. 2A and 3A). Three peaks were observed and the first narrow peak (1 ml) between 0.0 and 0.1 *M* sodium chloride is the solvent front and no

TABLE I

AMINO ACID ANALYSIS OF HISTONE 1 PEPTIDES PRODUCED BY CHEMICAL OR ENZYMATIC CLEAVAGE

Results are expressed as mol % of recovered amino acids.

Amino acid	*N-H1 NBS (1–72)*		*GC-H1 NBS (73–220)*		*NG-H1 CHY (1–106)*		*C-H1 CHY (107–220)*		*NG-H1 (1–122)*		*C-H1 (123–220)*	
	Calc.	*Obs.*	*Obs.*[a]	*Obs.*	*Calc.*	*Obs.*	*Obs.*[b]	*Obs.*	*Calc.*	*Obs.*	*Obs.*[c]	*Obs.*
Aspartic acid	0	0.5	2.2	3.0	1.9	1.1	0.9	1.0	2.4	2.0	0.2	1.0
Threonine	4.2	4.6	5.1	5.4	5.7	5.3	4.5	5.0	4.9	4.3	4.8	4.9
Serine	9.7	9.1	5.0	5.0	11.3	9.4	3.3	4.7	10.7	11.3	2.7	2.4
Glutamic acid	5.6	5.2	2.3	2.7	5.8	5.8	1.0	1.6	5.7	5.7	1.2	1.9
Proline	12.5	12.0	8.7	9.3	8.5	8.0	12.7	11.2	9.0	8.8	10.0	9.3
Glycine	5.6	6.0	7.6	8.1	8.5	9.1	3.2	4.9	8.2	7.6	4.4	3.7
Alanine	29.2	29.0	25.5	25.9	20.8	21.3	32.1	29.6	20.5	20.9	30.9	29.1
Cystine	0	–	–	–	0	–	0	–	0	–	–	–
Valine	5.6	5.0	4.5	–	6.6	7.2	1.7	2.9	5.7	5.1	2.5	2.1
Methionine	0	–	–	–	0	–	0	–	0	–	–	–
Isoleucine	1.4	0.9	0.7	0.9	1.9	1.2	0	0.4	1.6	2.2	0	0.3
Leucine	5.6	5.6	3.5	3.9	7.5	6.8	0.9	0.9	7.4	7.8	0.1	0.6
Tyrosine	0	–	–	–	0.9	–	0	–	0.8	–	0	–
Phenylalanine	0	–	0.7	–	0.9	–	0	–	0.8	–	0	–
Histidine	0	0.6	–	0.5	0	0	0	0	0	0.6	–	0.6
Lysine	16.7	17.5	32.9	33.6	16.0	21.7	39.8	37.6	18.9	19.5	43.2	42.7
Arginine	4.2	4.1	0.7	1.3	3.8	2.8	trace	0.2	3.3	3.5	0	0.4

[a] Ref. 10.
[b] Ref. 11.
[c] Ref. 12.

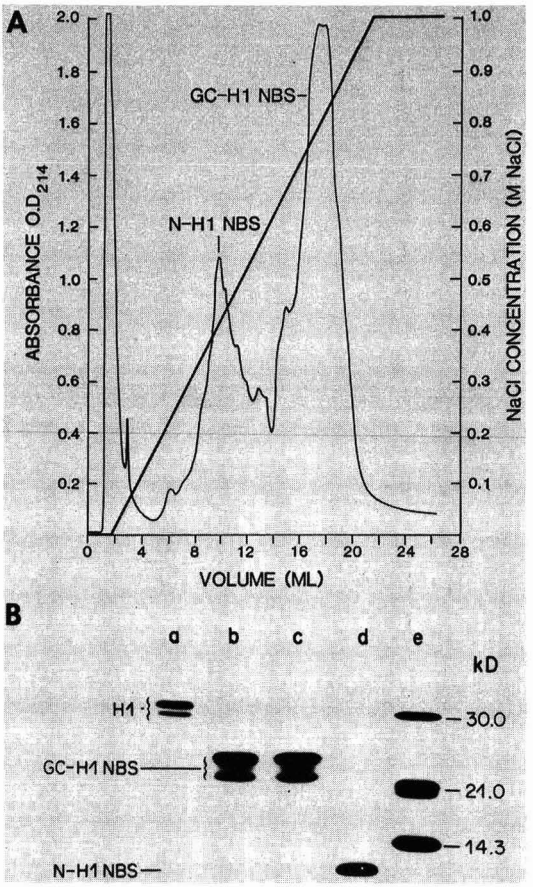

Fig. 2. (A) FPLC profile of histone H1 peptides digested with NBS. A 1-mg amount of histone H1 peptide was loaded on a Mono S column HR5/5 (50 × 5 mm I.D., Pharmacia) and eluted with increasing salt concentration (solid line) in 0.1 *M* phosphate buffer, pH 7.0 at a flow-rate of 1 ml/min. (B) SDS-polyacrylamide gel profile of purified NBS-digested histone H1 peptides. (a) Purified intact calf thymus H1. (b) Total NBS digest of H1. (c) C-Terminal peptide, residues 73–220. (d) N-Terminal peptide, residues 1–72. (e) Molecular weight markers. kD = kilodaltons.

polypeptides were identified. The second peak that eluted between 0.2 and 0.32 *M* sodium chloride and 7–10 ml (Fig. 3A) contained NG-H1 CHY and was represented as a single band on the gel (Fig. 3B, lane d). The third peak, containing C-H1 CHY, eluted between 0.5 and 0.65 *M* sodium chloride in a 2-ml volume between 13.5 and 15.5 ml (Fig. 3A). This peak was greater than 90% pure as assessed by an overloaded Coomassie blue stained gel (Fig. 3B, lane c).

FPLC and identification of thrombin-digested H1

H1 digested by thrombin resulted in three protein bands as seen by SDS-PAGE (Fig. 4B, lane b). These bands were identified as the singlet NG-H1 (residues 1–106) and the doublet C-H1 (residues 107–220).

Fractionation of thrombin-digested H1 by FPLC was slightly different from

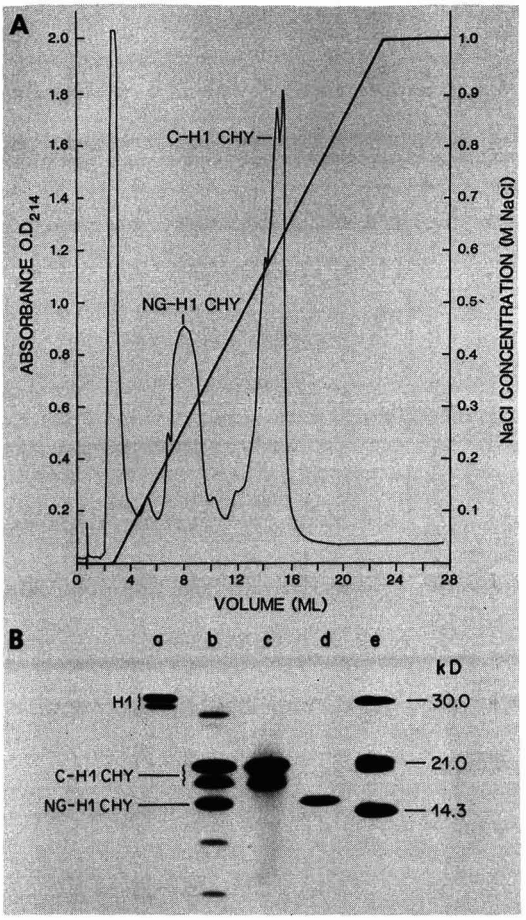

Fig. 3. (A) FPLC profile of histone H1 peptides digested with chymotrypsin. A 1-mg amount of histone H1 peptide was loaded on a Mono S column HR5/5 (50 × 5 mm I.D., Pharmacia) and eluted with increasing salt concentration in (solid line) 0.1 *M* phosphate buffer, pH 7.0 at a flow-rate of 1 ml/min. (B) SDS-polyacrylamide gel profile of purified chymotrypsin-digested histone H1 peptides. (a) Purified calf thymus histone H1. (b) Total chymotryptic digest of H1. (c) C-Terminal peptide 107–220. (d) N-Terminal peptides 1–106. (e) Molecular weight markers.

that of chymotrypsin and NBS-digested H1. Three peaks were seen but the last two large peaks were not homogeneous peaks in that each peak was characterized by one or two shoulders (Fig. 4A). When each of the peaks were analyzed by SDS-PAGE and amino acid analysis, no differences were noted in the peptide profile (Table I). This may reflect different degrees of acetylation or phosphorylation. Therefore, the shoulders in the second peak between volumes of 8–12 ml contained NG-H1 and the two shoulders in the third peak between volumes 12–15 ml contained C-H1 (Table I). SDS-PAGE analysis showed that the polypeptides NG-H1 and C-H1 were fractionated to greater than 95% purity (Fig. 4B, lanes c and d). NG-H1 eluted between 0.3 and 0.4 *M* sodium chloride in 2 ml beginning at 9 and ending at 11 ml. C-H1 eluted between 13–15 ml at a salt concentration of 0.52 and 0.62 *M* sodium chloride (Fig. 4A).

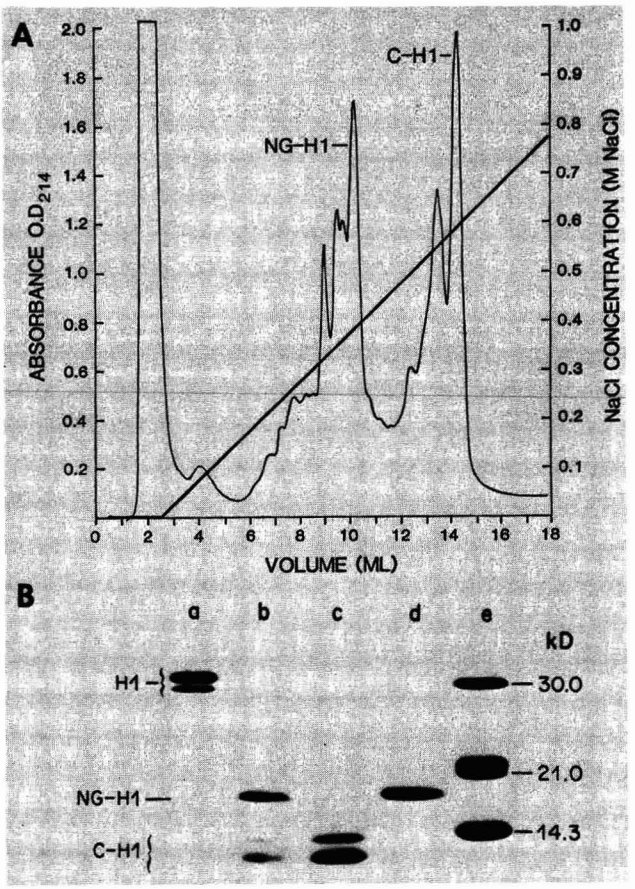

Fig. 4. (A) FPLC profile of histone H1 peptides digested with thrombin. A 1-mg amount of histone H1 peptide was loaded on a Mono S column HR5/5 (50 × 4 mm I.D., Pharmacia) and eluted with increasing salt concentration (solid line) in 0.1 *M* phosphate buffer, pH 7.0 at a flow-rate of 1 ml/min. (B) SDS-polyacrylamide gel profile of purified thrombin digest of H1 peptides. (a) Purified calf thymus histone H1. (b) Total thrombin digest of H1. (c) N-Terminal peptides of H1, residues 1–22. (d) C-Terminal peptides of H1, residues 123–220. (e) Molecular weight markers.

ELISA of H1 polypeptides

The H1 peptides generated by the various chemical and enzymatic digestions and purified by FPLC were used to identify antigenic domains of H1. SLE sera and procainamide-induced lupus sera were reacted with H1 and the H1 peptides bound to Immunolon II microtiter plates (Fig. 5). All six SLE sera (median O.D. 1.59) and all six drug-induced lupus (DIL) sera (median O.D. 1.45) tested bound to intact H1. Predominant reactivity with the H1 peptides was noted only in the C-terminal peptides (73–220, 107–220 and 123–220). The N-terminal peptides were not found to be reactive with DIL sera and minimally reactive with SLE sera. Control sera exhibited no reactivity toward either intact H1 or any of its peptides.

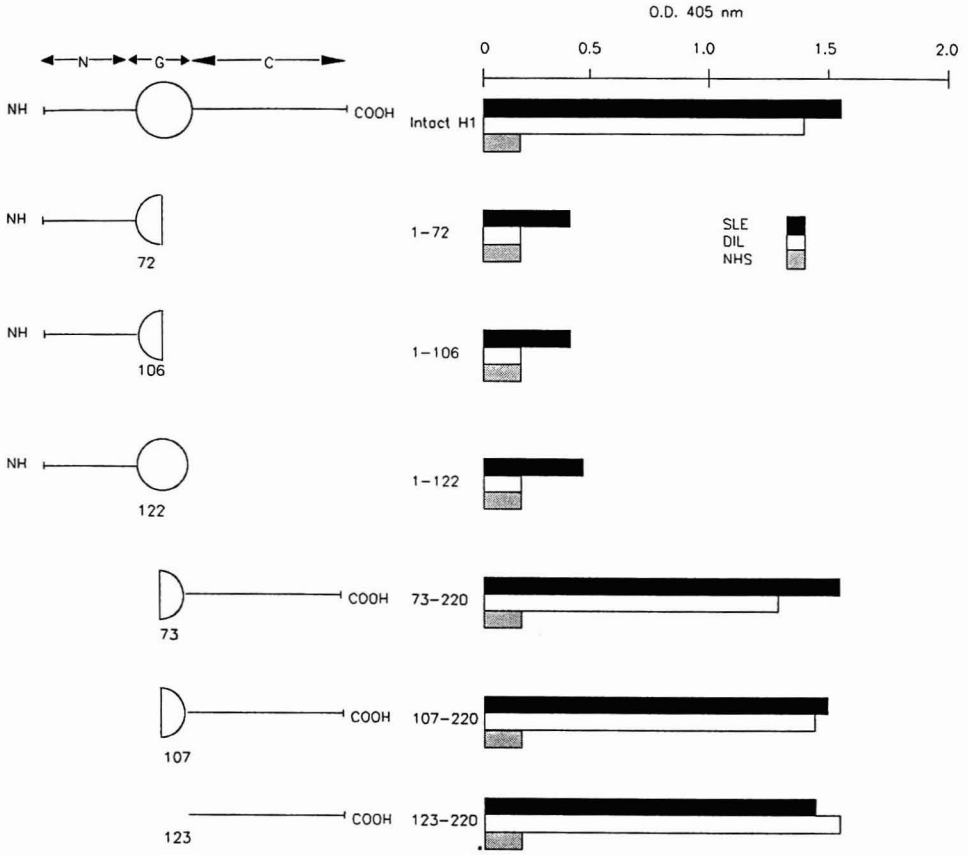

Fig. 5. ELISA of H1 and the cleavage peptides with SLE, DIL and normal human serum (NHS). Of each peptide 2 nmol were coated onto the plates and ELISA was performed as described in Materials and methods.

DISCUSSION

In the past, the fractionation and purification of histones and histone polypeptides has generally relied on gel filtration and ion-exchange chromatography. Two types of gel filtration matrices have been used for fractionating these basic, low-molecular-weight proteins —a cross-linked dextran matrix (Sephadex, Pharmacia) and a polyacrylamide sieve (Bio-Gel P, Bio-Rad).

Cleavage products of H1 have been purified by gel filtration chromatography for various studies. When NBS-digested H1 polypeptides were separated on a Bio-Gel P-60 column (40 × 1.9 cm I.D.) at a flow-rate of 6 ml/h, 6 h were required to separate the amino and carboxy terminal fragments and upon rechromatography of the two peaks, SDS-PAGE showed no cross-contamination[14]. Similarly, 6 h were required to fractionate α-chymotrypsin digested H1. Chromatography on different Sephadex matrices has also been used to separate and purify various H1 polypeptides[15,16]. Like other gel filtration techniques, the time required to resolve the peptides were long ranging from 20 to 50 h. However, despite these drawbacks, gel

filtration is technically quite simple, special buffers and gradients are not required and quantitative amounts of polypeptide can be fractionated.

Ion-exchange chromatography is technically more difficult, requires a buffer gradient and complicated washing steps but quantitative amounts of proteins and peptides can be fractionated. Histones and their polypeptides have generally been fractionated on weak cation-exchange material such as carboxymethyl cellulose (CM-cellulose) or Amberlite IRC-50, a weakly acidic cationic copolymer of methacrylic acid and divinyl benzene. Cellulose ion-exchange material such as CM-cellulose provides a good matrix for ion-exchange since proteins and peptides do not bind tightly to it. Histone fractionation on weak ion-exchange columns has been performed at a pH near neutrality in order to ionize the carboxylate groups. H1 fragments resulting from thrombin digestion have been fractionated on carboxymethyl cellulose columns (Whatman CM-32) at a flow-rate of 30 ml/h but up to 33 h were required to resolve the different peptides[12]. Ishimi *et al.*[17] decreased the separation time by 50% using similar ion-exchange media (Whatman CM-52) and by decreasing the size of the column (12 × 1 cm I.D.). H1 peptides derived from cyanogen bromide digestion have also been separated on Whatman CM-52 columns but once again the elution times were long[18].

We have devised a fast and efficient protocol for the fractionation of H1 polypeptides using a strong cation-exchange matrix and elution with a linear salt gradient. The Mono S (Pharmacia) matrix is a beaded hydrophilic resin with a pore size of 10 m*M*. The charged group on the gel ($-CH_2SO_3^-$) has an ionic capacity of 0.13–0.18 mmol/ml. The elution time for the H1 polypeptides was constantly less than 30 min, and the profile was highly reproducible. With the LCC-500 controller (Pharmacia) the samples are easy to load, elute and collect. The linear salt gradient is automatically created by two pumps controlled by the computer.

In summary, the FPLC protocol described here provides a unique technique for fractionating H1 polypeptides that has advantages of speed and reproducibility when compared to other gel filtration and ion-exchange techniques. We have successfully used the H1 polypeptides purified by the FPLC technique to determine the antigenic domains of H1 bound by SLE and DIL sera. This purification protocol will be useful for investigators wishing to examine the primary structure of H1 variants, the role of H1 and its domains on chromatin structure, and the effect of H1 peptides on enzyme activity.

ACKNOWLEDGEMENTS

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Purification and analysis of infectious virions and native non-structural antigens from cells infected with tick-borne encephalitis virus

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SUMMARY

By employing the techniques of column chromatography and membrane filtration, we have succeeded in purifying flavivirus particles with low particle to infectivity ratios and free from contamination with cellular proteins. Virus particles purified by this method have an average diameter of 53 nm, a particle to infectivity ratio of less than 10, and a K_D (partition coefficient) consistent with a molecular weight of $2.63 \cdot 10^7$. In addition it has been possible to purify the extracellular form of non-structural protein 1 (NS1), which in its native form appears to be a hexamer. It is also apparent from these studies that the slowly sedimenting haemagglutinin particle (or SHA) is an artifact of purification methods using gradient centrifugation. This technology should not only prove useful in the laboratory for studying the detailed structure of these viruses and the proteins encoded by them, but should also prove useful in industrial vaccine manufacture where large volumes of highly pathogenic material are handled.

INTRODUCTION

Flaviviruses cause some of the most important infectious diseases of man; the most widespread being yellow fever and dengue fever. In addition tick-borne encephalitis (TBE) is a significant public health problem in the Soviet Union and many eastern and central european countries. Japanese encephalitis is also widespread in China and the far east[1]. Although there are successful vaccines against some flaviviruses, research on the development of new and improved vaccines has been hampered by difficulties in purifying intact and infectious particles by conventional techniques. Moreover, research on the physical structure of these viruses and their extracellular

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antigens has also been hampered as conventional methods of purification and concentration such as gradient centrifugation and pelleting either distort or disrupt the virus particle, resulting in high particle to infectivity ratios[2].

The procedure described here involves ultrafiltration and gel exclusion chromatography to purify and concentrate viral particles and the major non-structural antigen NS1, whereby non-physiological conditions such as high sucrose concentrations, pellet formation, high salt and pH changes are avoided. These all can and do lead to irreversible changes in the integrity of virus particles and the structure of viral proteins.

A further advantage of this technology is that it is more readily adapted to industrial scale up than is ultracentrifugation. At present several vaccines for human use including that against TBE, use ultracentrifugation to purify the product[3,4]. For the best results it is necessary to handle large amounts of pathogenic material in the centrifuge and if the machine fails, containment of infectious aerosols is difficult and costly. Although some pressurisation occurs during gel chromatography and ultrafiltration these forces are much lower than those generated in a centrifuge and thus containment is easier. Apart from the problems discussed above, some flaviviruses grow to low titres and thus need extensive concentration. In addition cells infected with these viruses overproduce convoluted tubules, and as these tubules are of a similar size and density to virus particles they copurify with them when conventional procedures are used.

We report here the application of these techniques to the purification of flaviviruses which, in the past, have proved to be particularly difficult to prepare. The procedure reported here not only produces intact, pure and infectious virions and extracellular antigens, but is readily adaptable to the high containment conditions necessary for handling pathogenic viruses under laboratory and industrial conditions.

MATERIALS AND METHODS

Virus growth

Seed stocks of the Neudorfl isolate of Central European TBE was grown in suckling mouse brain as previously described[5]. For each batch of virus $3 \cdot 10^{10}$ PS cells were grown in roller culture and infected at a multiplicity of infection of 10. After 42 h in serum-free L15 medium, the supernatant was removed from the cultures and clarified by centrifugation at 2000 *g* and 4°C for 10 min and then at 10 000 *g* and 4°C for 10 min. The resultant supernatant was then made 0.01% (w/v) with respect to sodium azide; and aprotinin was added to a final concentration of 10 units per ml. All further procedures were performed at 4°C.

Virus concentration

Clarified supernatants (2-l samples) were concentrated 10-fold on Minitan PTHK OMT05 filters (molecular weight cut-off, 100 000), and diluted ten times in pH 8.0 phosphate buffer [$Na_2HPO_4 \cdot 12H_2O$ (0.0185 *M*)–K_2HPO_4 (0.0013 *M*)–NaCl (0.13 *M*)]. After a second 10-fold concentration, the virus was diluted 10 times again in phosphate buffer and finally concentrated to 30 ml for column chromatography.

Column chromatography of infected cell supernatants

Aliquots of about 20 ml of clarified virus concentrate were applied to a column (69.5 cm × 2.2 cm I.D.) of Sephacryl S300 at 4°C in phosphate buffer (as defined for virus concentration) at a flow-rate of 72 ml/h. The eluate was monitored at $O.D._{280}$ using an LKB Uvicord S UV monitor and 3-ml fractions were collected. Aliquots for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were immediately boiled for 1 min in electrophoresis sample buffer and stored at −20°C. Those for infectivity assay were stored at −20°C in the presence of 1% bovine serum albumin (BSA). On the basis of infectivity assays and SDS-PAGE, the fractions containing live virus were pooled, concentrated to 10 ml on an Amicon XM300 membrane and applied to a column (79 cm × 1.6 cm I.D.) of Sephacryl S500 at a flow-rate of 46 ml/h. The void volume of the S300 column was determined using Blue Dextran; and that of the S500 column was determined using a formalin-fixed, trypsin treated suspension of *Escherichia coli.*

Purification of SCF antigen

Fractions from the first S300 column which contained the SCF antigen (as determined by SDS-PAGE) were pooled, concentrated to 15 ml on an Amicon XM50 membrane and applied to a second S300 column.

Electron microscopy

A drop of virus suspension was placed on a 400-mesh copper specimen grid coated with a formvar–carbon film. After approximately 30 s the suspension was removed by touching the edge of the grid with moist filter paper. A drop of negative stain (1%, w/v, sodium silicotungstate, pH 6.8, or 0.5% uranyl acetate, pH 4.3) was then placed on the grid and similarly removed after approxamately 10 s. Grids were allowed to air dry before being examined in a Philips EM400T electron microscope operated on a accelerating voltage of 80 kV. Micrographs were recorded on an Ilford EM technical film at 60 000× magnification.

SDS-PAGE

Column fractions were analysed by SDS-PAGE under reducing conditions, as described by Stephenson *et al.*[6]. The gels were fixed in 7% acetic acid and stained using Coomassie blue and/or Bio-Rad silver stain.

Infectivity assays

Infectious viral particles were assayed in monolayers of PS cells as described previously[7].

Sucrose gradient centifugation

Virus particles and extracellular antigens, labelled with ^{35}S methionine were prepared as described previously[8].

Reagents

Sephacryl was obtained from Pharmacia (Milton Keynes, U.K.); Minitan membranes from Millipore (Watford, U.K.) and membranes were obtained from Amicon (Stonehouse, U.K.). [^{35}S]Methionine was from Amersham International (Amersham, U.K.).

RESULTS

Purification of virus particles

Chromatography of infected cell supernatants on Sephacryl S300 resulted in three species of particle being detected by monitoring the column eluate at O.D. $_{280}$ (Fig. 1). Virtually all infectious virus particles were present in the void volume (peak 1) and when this material was analysed by PAGE (Fig. 2) the major virion envelope protein E was clearly seen. Staining of the virion capsid protein C and the minor envelope protein M is very poor and is only uneqivocally seen when column fractions are concentrated 50-fold or more. As peak 1 represents the void volume of the column other particles are found apart from virions. PAGE analysis however (Fig. 2) indicates that very little protein, apart from virus proteins, is present in peak 1. Electron microscopy indicates that these non-virion particles are similar to the heterogenous smooth membranes reported from cells infected with alphaviruses[9]. PAGE analysis also reveals that the major protein in peak 2 is the extracellular form of the non-structural virus specific protein (NS1), previously referred to as the "soluble complement fixing antigen"[10]. Electron microscopy and PAGE analysis both confirm that the material in peak 3 is the amorphous granular cell debris seen in similar samples from cells infected with alphaviruses[9].

Virus particles were further purified by pooling fractions 40–55 and concentrating the preparation with XM300 membranes in stirred cells. Subsequent chromatography on Sephacryl S500 columns (Fig. 3) enabled infectious virus particles to be resolved by the column matrix, eluting with a K_D (partition coefficient) of 0.66. This figure is consistant with a particle of molecular weight of $2.38 \cdot 10^7$, as determined by conventional methods. PAGE analysis shows that fractions 43–55 from this column contain pure virus particles (Fig. 4).

It is clear from the data described above that only two virus-specific particles are released from the cell, but when similar material is analysed by gradient centrifu-

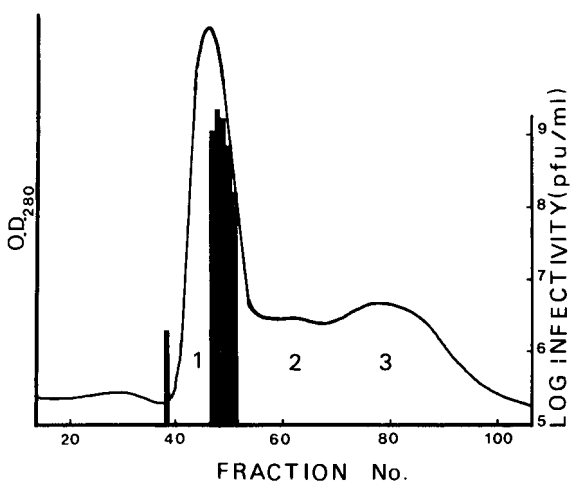

Fig. 1. Purification of TBE extracellular antigens by chromatography on S300. Clarified, concentrated infected cell supernatants were applied to the column and eluted in phosphate buffer as described in methods. —— = O.D.$_{280}$ profile; ■ = infectivity in plaque forming units (pfu)/ml, assayed in PS cells.

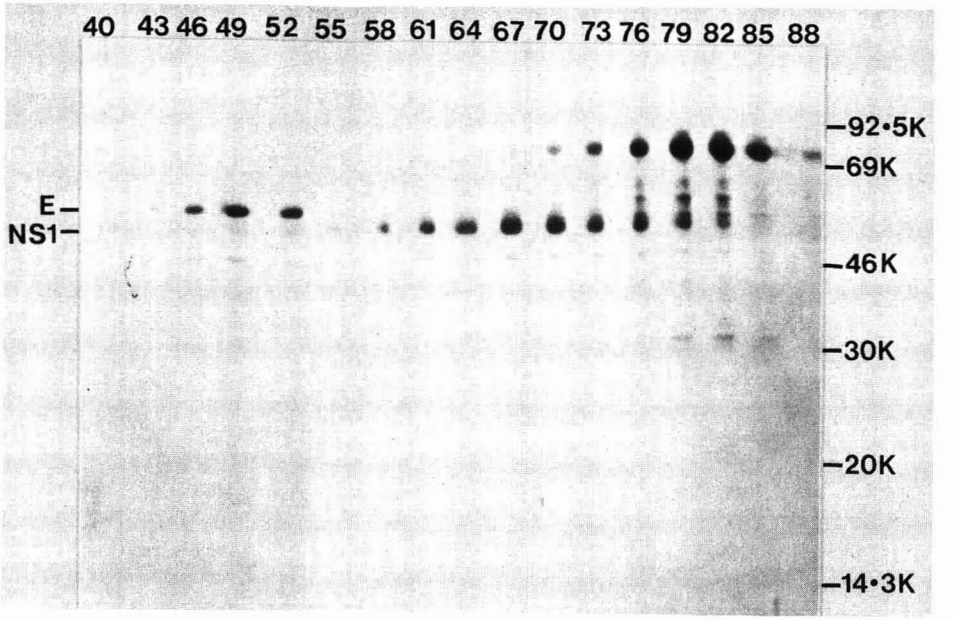

Fig. 2. PAGE of selected fractions from Fig. 1. Aliquots of 20 μl were boiled in sample buffer and analysed as described in Materials and methods. Gels were stained with Coomasie blue. Gel tracks are indicated by the fraction numbers of the samples and the positions of molecular weight markers are indicated on the right hand side. K = Kilodalton.

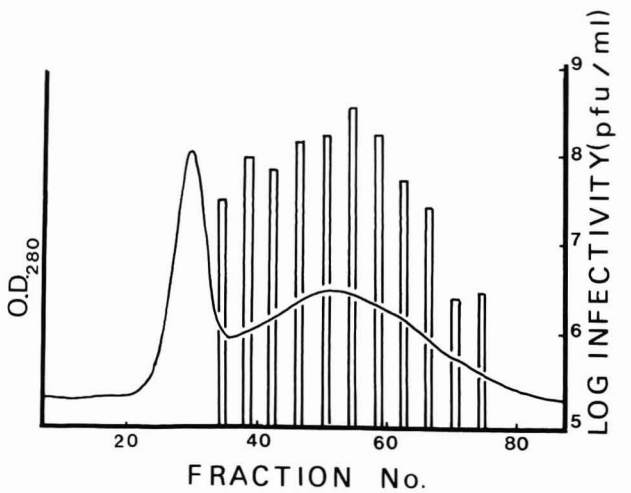

Fig. 3. Purification of TBE virions on S500. Fractions from Fig. 1, containing virus particles, were concentrated and applied to the column as described in Materials and methods. — = $O.D._{280}$ profile; bars indicate infectivity.

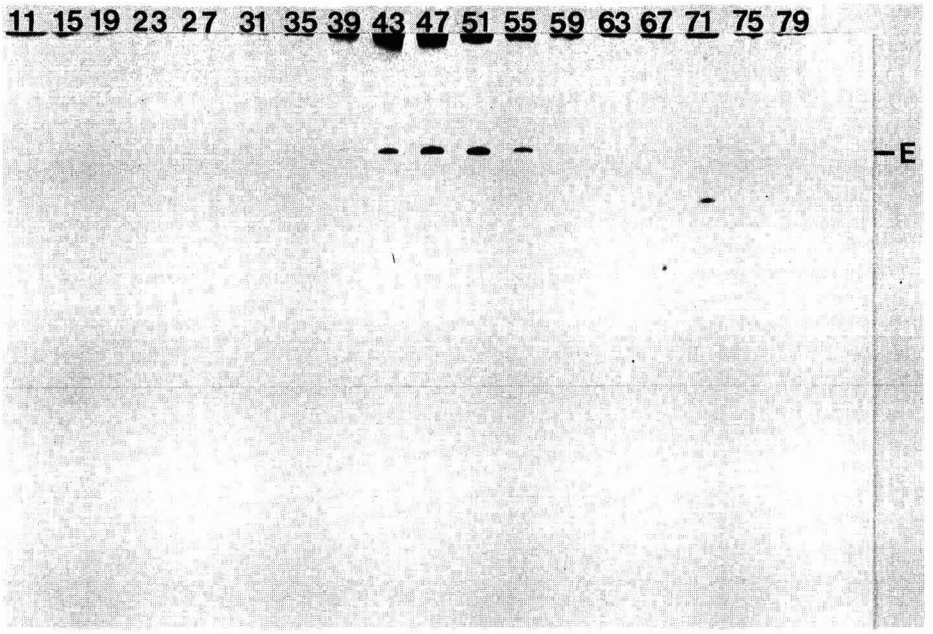

Fig. 4. PAGE of selected fractions from Fig. 3. Samples were prepared and analysed as in Fig. 2. Track designations are as for Fig. 2.

gation (Fig. 5) three species are seen. In addition to infectious virions and NS1 a third moiety, the SHA (slowly sedimenting haemagglutinin) can be observed. This particle contains only the E polypeptide and has been reported for several mosquitoborne flaviviruses as well as TBE[10,11].

Recovery of biological activity

The recovery of infectious virus particles was monitored throughout the purification procedure by assaying infectivity in PS cells, as described above (Table I). Some virus was lost during the initial concentration step and on each chromatography, but the overall recovery rate of 32% is significantly better than that achieved by conventional means and is similar to that reported for alphaviruses[9].

Physical integrity of the virus particles

The physical integrity of the virus particles was examined by electron microscopy. Fig. 6 shows a typical field of virus particles with an average diameter of 53 nm, a figure similar to that reported for other flaviviruses. Although some of the particles appeared damaged most virions were intact. The damaged particles are almost certainly a result of the sample preparation as it has been known for some time that flavivirus particles are very sensitive to disruption by techniques used to prepare samples for electron microscopy.

The particle to infectivity ratio was calculated from similar preparations by the method described in ref. 9 and these calculations gave a value of 6.8, similar to that reported for alphaviruses.

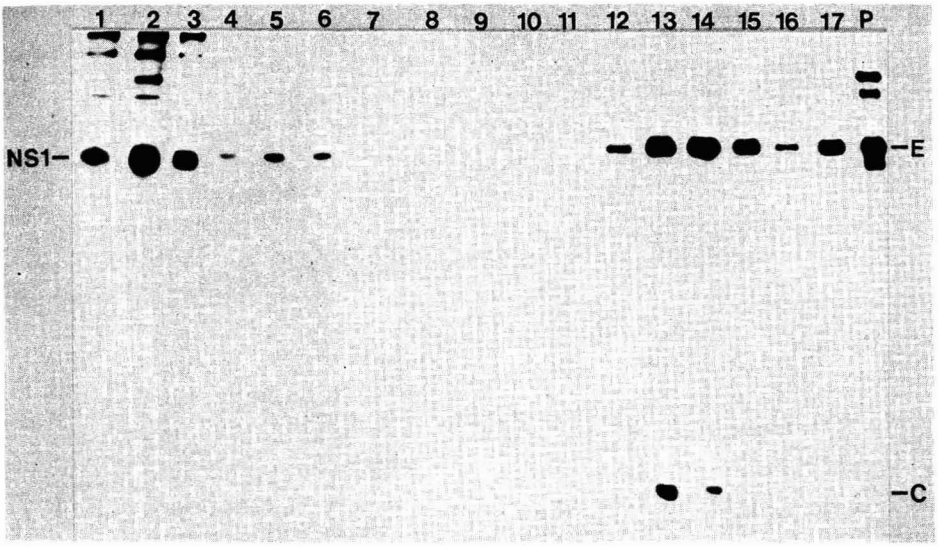

Fig. 5. Sucrose gradient analysis of extracellular antigens from cells infected with TBE virus. Gradient sedimentation and analysis of gradient fractions by autoradiography and PAGE were performed as in Materials and methods with sedimentation being from left to right. Gel tracks are designated by the fraction numbers from the sucrose gradient. P = Pellet from the gradient.

Purification of the extracellular form of NS1

Extracellular NS1 was further purified by pooling fractions 61–70 from peak 2 (Fig. 1) and concentrating the preparation on XM50 membranes. This preparation was reapplied to an S300 column and purified as described in Materials and methods. As can be seen in Fig. 7, the extracellular NS1 elutes with a K_D of 0.22 and is clearly resolved from virus particles, cellular proteins and serum. A typical PAGE analysis of a chromatography run (Fig. 8) shows no evidence of virus particles and thus the $O.D._{280}$ peak 1 in Fig. 7 must represent membranous particles as described above. PAGE analysis also shows some contamination of the NS1 peak fractions by serum albumin; but this can be completely removed if required by further chromatography on S300 (data not shown). By calibrating the S300 column with aldolase, thyroglobulin, ferri-

TABLE I

EFFICIENCY OF RECOVERY OF INFECTIOUS VIRUS DURING PURIFICATION BY ULTRAFILTRATION AND CHROMATOGRAPHY

Sample	*Plaque forming units (total)*	*Recovery (%)*[a]
Clarified supernatant	$2.0 \cdot 10^{11}$	–
Concentrated supernatant	$1.5 \cdot 10^{11}$	75
Pooled peaks from S300 column	$9.5 \cdot 10^{10}$	63
Pooled peaks from S500 column	$6.35 \cdot 10^{10}$	67
Overall recovery	–	32

[a] Apart from the overall recovery these figures represent the yield at each step.

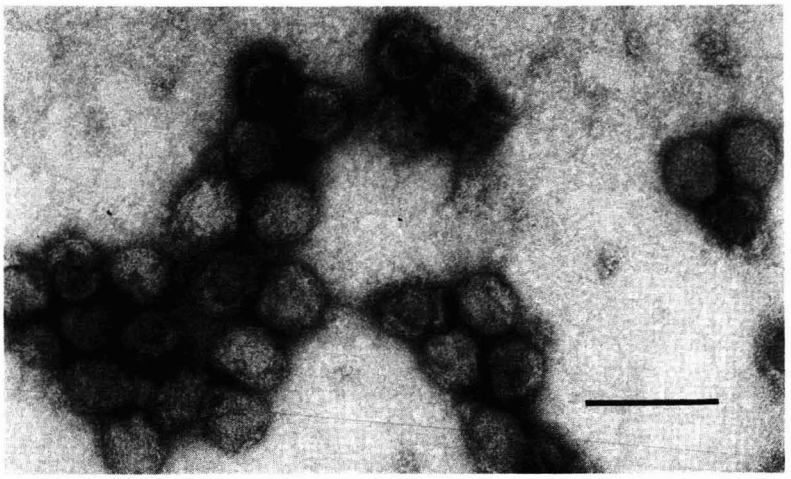

Fig. 6. Electron microscopy of purified TBE virus from Fig. 3; negatively stained with uranyl acetate. Bar marker equals 100 nm.

tin and catalase, the extracellular NS1 protein elutes with an apparant molecular weight of 331 000, consistent with it being in form of a hexamer.

DISCUSSION

Flaviviruses have been responsible for several severe diseases of mankind for over three centuries[12]. Although some successful vaccines have been developed against these viruses, protection against many flavivirus diseases is either poor or non-existant[13]. Research in this area has been hampered by several factors, the pathogenic nature of the agents, poor growth in tissue culture for many isolates and difficulty in purifying virus particles and extracellular antigens.

Conventional methods of virus purification such as sucrose gradient centrifugation, pelleting or ion-exchange chromatography frequently result in loss of infectivity and distortion of viral particles. The techniques reported here do not employ any harsh chemical or physical environments and therefore we have succeeded in purifying TBE virus with high yields and low particle to infectivity ratios.

TBE virus particles prepared by this methodology elute with a K_D of 0.66 which

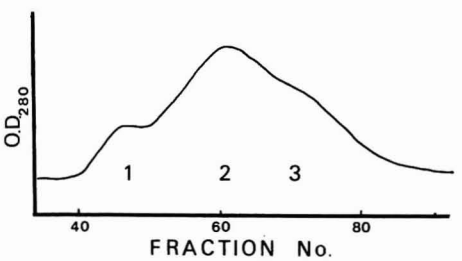

Fig. 7. Purification of extracellular NS1 by chromatography on S300, as described in Materials and methods.

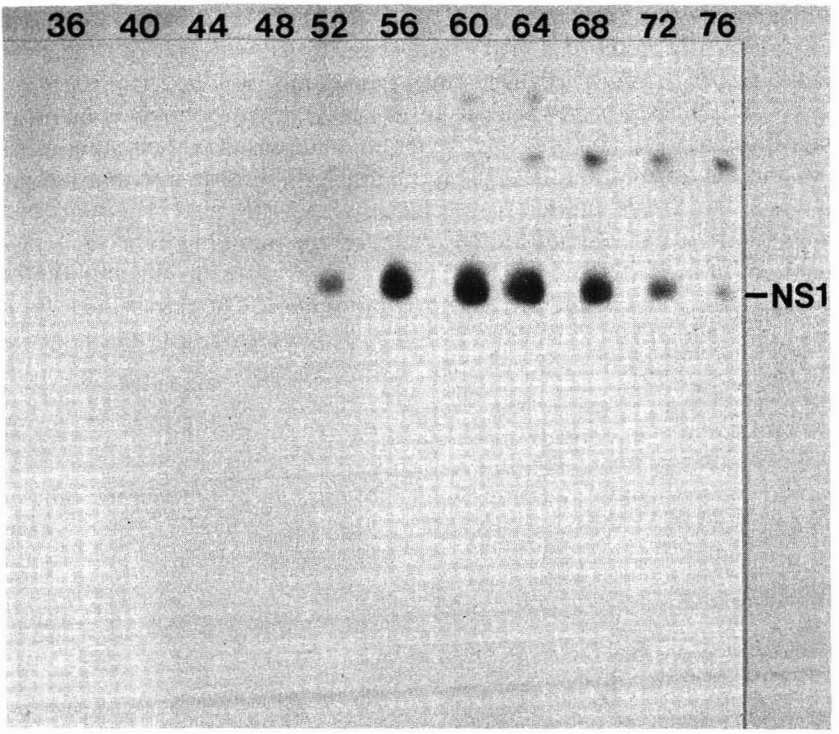

Fig. 8. PAGE analysis of selected fractions from Fig. 7. Samples were prepared and analysed as in Fig. 2. Track designations are as for Fig. 2.

is consistent with the particle diameter of 53 nm observed by electron microscopy and with a molecular weight of $2.63 \cdot 10^7$. As determined by PAGE the virus preparations are clear of any contaminating cell protein and by electron microscopy they also appear to be clear of cell membranes. The material stained with silver, but not coomassie blue, appearing at the top of the gels is thought to be viral nucleic acids.

It is surprising to note that the SHA particles observed when extrallular material is purified on sucrose gradients is not detected during column chromatography. It is possible therefore that the SHA does not represent a true extracellular product from infected cells but is a by-product of the breakdown of virus particles during gradient purification.

Although the extracellular NS1 is not as clearly resolved from cellular proteins as are virus particles, it is still possible to obtain preparations of this protein which are free from detectable contaminants. It is difficult to estimate the yield of this protein as it has no biological property which is not shared by either virion or cellular components. The elution profile of NS1 is consistent with it being present as a hexamer in the extracellular environment. Studies with other flaviviruses however have indicated that NS1 is a dimer[14]. This apparent discrepancy can be resolved if it is remembered that the studies with dengue virus involved exclusively SDS-PAGE analysis of NS1 and thus the protein was at least partially denatured. In addition it has been shown in our laboratory that the extracellular form of NS1 is more heavily glycosylated than

its intracellular counterpart[8] and therefore it is possible that NS1 is indeed a dimer in the infected cell, but the additional glycosylation associated with its export enables three dimers to associate into a hexamer. This hexamer may well be sensitive to low concentrations of SDS as sequence analysis reveals several hydrophobic domains in this protein. Multimeric proteins are much better immunogens than their monomeric counterparts and the observation that NS1 is a hexamer is consistent with it being a good immunogen in animals infected or vaccinated with TBE virus[15].

In conclusion we have demonstrated a method for purifying TBE virus that gives preparations of high titre and free from contaminating proteins. In addition the virus is highly infectious and maintains a native conformation. The extracellular form of NS1 can also be purified by this method and this protein appears as a hexamer in its native state.

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Affinity gel interactions of alcohol and polyol dehydrogenases: anomalous behaviour and structural correlations

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SUMMARY

The initial interaction of horse and sheep liver polyol (sorbitol) dehydrogenase (L-iditol:NAD 2-oxidoreductase, EC 1.1.1.14) with the affinity gel Blue Sepharose CL-6B is anomalous and shown to be driven by an increase in entropy. In all other affinity gel interactions examined using polyol dehydrogenase with AGAMP and AGNAD and horse liver alcohol dehydrogenase (EC 1.1.1.1) with these three gels, an increase in temperature facilitated release of enzyme from the gel to the mobile phase. A more extensive evaluation of polyol dehydrogenase–Blue Sepharose CL-6B interaction points to a spectrum of forces whose individual contributions to complex formation are subject to manipulation by a change in time, temperature and/or solvent. These results can be explained by the two-stage thermodynamic model of protein–ligand association proposed by P. D. Ross and S. Subramanian [*Biochemistry*, 20 (1981) 3096]. Comparisons based on hydropathy plots with secondary and tertiary [J. Jeffery and H. Jornvall, *Adv. Enzymol.*, 61 (1988) 47] structure alignments of polyol dehydrogenase fitted to alcohol dehydrogenase were used to reveal major differences at residues 271, 47 and 202 which could account for the disparate thermal behaviour of these enzymes on Blue Sepharose CL-6B.

INTRODUCTION

Jornvall *et al.*[1] and Eklund *et al.*[2] have demonstrated that close structural similarities exist between sheep liver polyol (*i.e.*, sorbitol) dehydrogenase (EC 1.1.1.14) (SLPDH) and yeast and horse liver alcohol dehydrogenase (EC 1.1.1.1) (YADH and HLADH, respectively). This assessment is derived from secondary structure predictions[1] and projections for the tertiary structure of the polyol dehydrogenase (PDH) subunit based on model building and substrate docking using computer graphics[2].

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Parallel with these structural and mechanistic similarities there is a consensus recognition that significant compositional differences exist between SLPDH and HLADH. Maret and Auld[3], reporting on the characterization of human liver polyol dehydrogenase (ULPDH), have noted several of them: (i) SLPDH is a tetramer rather than a dimer; (ii) it contains one instead of two zinc atoms per subunit; (iii) a cysteine ligand of the catalytic zinc in HLADH is thought to be replaced by a glutamate residue.

Contrasted with these structural differences two recent reports point to the unusual behaviour displayed by PDH when this enzyme is ligated to NAD[4] and Cibacron Blue 3GA[5]. In one study Gronenborn *et al.*[4], using transferred nuclear Overhauser enhancement measurements, reported an unusual conformation of NAD bound to SLPDH *vis-a-vis* the extended conformation found in several other NAD–dehydrogenase complexes, including HLADH.

In another investigation[5], dealing with the purification of horse liver polyol dehydrogenase (HLPDH), it was observed that unlike HLADH the interaction between Blue Sepharose CL-6B (BSCL-6B) and HLPDH is unusual in that an increase of temperature enhances enzyme–gel interaction. This property was exploited in the purification of HLPDH. Harvey *et al.*[6] in a series of thermal affinity gel studies, noted that an increase in temperature decreased the affinity of yeast alcohol dehydrogenase and glycerokinase for N^6-(6-aminohexyl)-5′-AMP-Sepharose. Subsequently this same group reported[7] that an elevation in temperature increased the affinity of *Bacillus stearothermophilus* alcohol dehydrogenase and phosphofructokinase for this gel. Similarly, haptoglobin is bound to immobilized Cibacron Blue at 25°C, whereas at 4°C it is not[8]. The fact that the majority of affinity gel experiments are performed under isothermal conditions could mask the possibility that entropically driven protein–gel interactions are more common than is generally realized.

In this paper we describe the results of our studies on the similarities and differences displayed by PDH towards the affinity gels, AGAMP, AGNAD and BSCL-6B relative to HLADH, using a dual-temperature probe. The structural regions and amino acids within these proteins which could possibly account for their dissimilar thermal behaviour with BSCL-6B are examined.

EXPERIMENTAL

Materials

AGAMP Type 3 (AGAMP/3) and AGNAD Type 1 (AGNAD/1) were procured from PL Biochemicals (now Pharmacia Fine Chemicals). BSCL-6B was obtained from Pharmacia. The ligand of this gel, the textile-dye Cibacron Blue 3GA (CB-3GA), is a mixture of *m*- and *p*-isomers. It has the structure 1-amino-4[[4-[[4-chloro-6-[[3(or 4)-sulfophenyl]amino]-1,3,5-triazin-2-yl]amino]-3-sulfophenyl]amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid (CAS No. 12236-82-7). (The *o*-isomer of this dye contains a terminal 2-amino sulfonate ring and is designated CB-F3GA[9,10].) HLPDH was prepared as described[5]. All other reagents and chemicals, unless noted, were purchased from Sigma.

Methods

Chromatography. Affinity gel chromatography was carried out on column beds

(10.0 cm × 0.9 cm) of gel media previously equilibrated with 20 m*M* Tris–HCl buffer, pH 7.5, 50 m*M* in NaCl and 3.0 m*M* in dithiothreitol (DTT). Calibrated thermistors attached to the columns were used to monitor temperature. Fractions of 5.0 ml were collected (4°C) at a flow-rate of 1.0 ml/min.

Protein measurement. Protein assays were carried out using the method of Bradford[11] or Whitaker and Granum[12].

Enzyme measurements. Dehydrogenase activities were measured at 25°C in 20 m*M* Tris–HCl buffer, pH 9.2, by following the increase in absorbance at 340 nm. A typical assay system contained sorbitol (0.120 mmol) or alcohol (1.7 mmol), NAD (0.0024 mmol) and an aliquot (0.025–1.0 ml) of column effluent as the enzyme source in a total final volume of 3.00 ml. A unit of enzyme activity is reported as the number of micromoles of NADH produced per minute.

Structure comparisons. Hydropathy profiles[13] for HLADH and SLPDH were constructed using the Lotus® 1-2-3® spreadsheet program of Vickery[14].

RESULTS AND DISCUSSION

Thermal affinity gel studies

The dual-temperature release patterns of HLADH and PDH from AGNAD/1, AGAMP/3 and BSCL-6B reveal marked similarities and differences in this two-enzyme, three-gel comparison (Table I). Both enzymes show a similar temperature-insensitive interaction with AGNAD/1 at 4 and 30°C. Although the relative recoveries differ (*ca.* 40% for HLADH, *ca.* 25% for HLPDH), the bulk of the enzyme activity in both cases (*ca.* 60% for HLADH, *ca.* 75% for HLPDH) remains column-bound and is not released with a pulse of NAD.

In contrast, HLADH and HLPDH interactions with AGAMP/3 and BSCL-6B are temperature-sensitive, but with some noteworthy differences. Thus both HLPDH and HLADH interactions with AGAMP/3 are exothermic in that an increase in temperature leads to a higher relative concentration of each enzyme in the mobile

TABLE I

ENZYMATIC RECOVERY PATTERNS FOR HORSE LIVER ALCOHOL AND POLYOL DEHYDROGENASE FROM SPECIFIC AND GENERAL AFFINITY GEL LIGAND GELS AS A FUNCTION OF TEMPERATURE

(1) In the solvent front (*i.e.*, void volume) unbound by the gel after a 30-min stand; (2) subsequently released with a pulse of 10 m*M* NAD; (3) total. Tris–HCl (pH 7.5, 50 m*M* in sodium chloride, 3 m*M* in dithioerythreitol) was used as irrigant/eluent buffer. See text for commentary.

Enzyme	*Temperature (°C)*	*Percentage of enzyme activity recovered (± 7%)*								
		BSCL-6B			*AGAMP/3*			*AGNAD/1*		
		1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
HLADH	30	80	10	90	70	25	95	10	30	40
	4	5	90	95	0–5	80	85	10	30	40
HLPDH	30	5	90	95	70	20	90	0–5	20	25
	4	27	70	97	45	50	95	0–5	20	25

phase[6]. However, the interaction HLADH–AGAMP/3 is the more sensitive of the two (0–5% → 70%, *versus* 45% → 70%), suggesting this adsorption is the more exothermic of the two[6]. In all of these cases recovery of enzyme activity is essentially complete (*i.e.*, ≈85–95%).

The most striking difference in these patterns involves the reactions of HLADH and HLPDH with the BSCL-6B. Here HLADH–BSCL-6B adsorption is exothermic, resembling HLADH–AGAMP/3 interaction. In contrast, HLPDH–BSCL–6B adsorption is endothermic in that an increase in temperature enhances enzyme–gel interaction. Like AGAMP/3, but unlike AGNAD/1, full activity (≈95–97%) is recovered following the pulse of NAD.

In view of the unusual interaction of HLPDH with BSCL-6B,shown in Table I, a dual-temperature study, using SLPDH and HLADH, was carried with this gel (Fig. 1). Addition of SLPDH to BSCL-6B at 30°C shows that the bulk of this protein is bound by the gel. Only a small amount (*ca.* 10%) is recovered in the breakthrough volume. Bound enzyme is subsequently released at 4°C by salt (*ca.* 18%), followed by a pulse of NAD (72%) (Fig. 1A). When added at 4°C, the majority (*ca.* 95%) of the

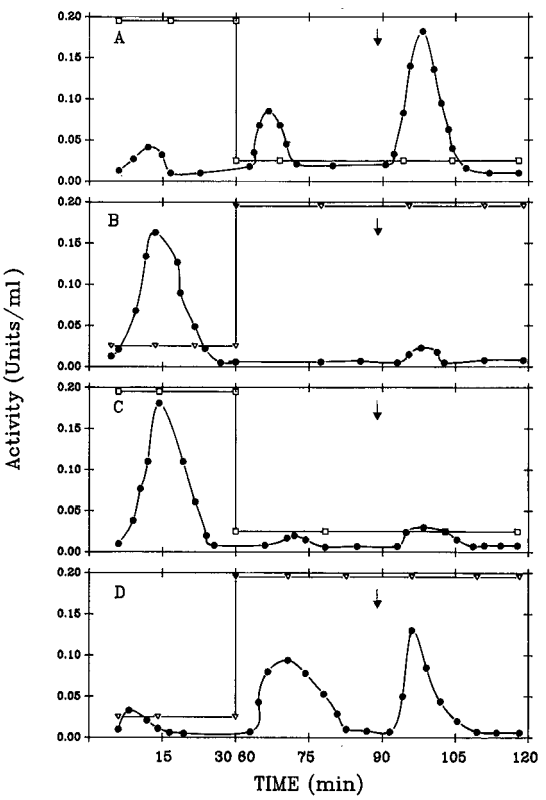

Fig. 1. Chromatography of SLPDH and HLADH on Blue Sepharose CL-6B. Samples were loaded at 30°C (□, incubator, A and C) or 4°C (▽, cold box, B and D) and eluted immediately. The columns were then reequilibrated to the alternate temperature (30 min) and the elution was resumed. The area (%) under each curve (see text) was taken as the measure of activity recovered in the individual peaks. (A and B) SLPDH; (C and D) HLADH. (●) Enzyme activity. (↓) The point of addition of a pulse of NAD.

SLPDH activity appears in the void volume. A residual amount (*ca.* 5%) is released by a pulse of NAD (10 m*M*) (Fig. 1B). Interaction of HLADH with BSCL-6B at 30°C is unlike that of SLPDH (see Fig. 1A). Here the majority of the activity (*ca.* 78%) appears in the breakthrough volume. Bound HLADH is subsequently released at 4°C by salt (*ca.* 4%) followed by a pulse of NAD (17%) (Fig. 1C). If HLADH is loaded at 4°C under the conditions described for SLPDH (see Fig. 1B), *ca.* 5% appears in the void volume. The remainder is released at 30°C by salt (*ca.* 55%) followed by a pulse of NAD (*ca.* 35%) (Fig. 1D).

The same trends described for Tris buffer were observed when the elutions were carried out in phosphate buffer (in both cases the eluting buffer contained 50 m*M* sodium chloride). For these conditions the release patterns are not significantly influenced by the larger $\Delta pK/\Delta T$ of Tris relative to phosphate (-0.028 *versus* -0.003). In all experiments described the enzymes were eluted within 1.5–2.0 h of addition to the columns. Prolonged exposure (4–6 h) led, in all cases, to enzyme entrapment and irreversible binding.

A more extensive evaluation of HLPDH–BSCL-6B interaction points to a spectrum of forces whose individual contributions to complex formation can be manipulated by a change in time, temperature and/or solvent (Table II, experiments 1–5, interacting species A–D).

Experiment 1A. At 4°C HLPDH is not bound by the gel, since *ca.* 90% of the enzyme activity is recovered in the effluent, if eluted immediately. This behaviour mirrors that of SLPDH (Fig. 1B).

Experiment 1B. A 30-min hold at 4°C enhances enzyme–gel interaction since only 31% of the activity appears in the breakthrough volume.

Experiment 1C. At 30°C, with a 30-min hold 50 m*M* sodium chloride releases

TABLE II

THERMAL RELEASE PATTERNS OF HORSE LIVER POLYOL DEHYDROGENASE FROM BLUE SEPHAROSE CL-6B

Experiment No.	*Eluting buffer*[a]			*Percentage of enzyme activity recovered (± 7%)*			
	NAD (mM)	*NaCl (M)*	*Ethylene glycol (%)*	*Protein–gel complex*[b]			
				A	*B*	*C*	*D*
1	0	0.05	0	90	31	3–5	0
2	0	0.05	50	95[c]	95	0	0
3	10	0.05	0	95	95	95	0
4	0	0.20	0	95	95	95	0
5[d]	0	1.00	0	95	95	95	95

[a] Eluting buffer is Tris–HCl (20 m*M*), pH 7.5, 3.0 m*M* in DTT with the additive(s) (NaCl, NAD or ethylene glycol) indicated.

[b] Refers to the interacting species A, B, C or D formed at 4 or 30°C between BSCL-6B and the enzyme. All samples were added to the columns at 4°C. They were then released immediately (A), held for 30 min at the stated temperature, then released [(B) 4°C; (C) 30°C] or released following treatment of C with 50% ethylene glycol at 30°C (D).

[c] Recovery of ≈90–95% or greater is taken to mean full recovery of the enzyme in the effluent.

[d] Recovery here is based on protein measurement, since HLPDH is not active in 1.0 *M* NaCl.

only a negligible amount (*ca.* 3–5%) of the enzyme. The same pattern is seen for SLPDH (Fig. 1A).

Experiment 2B. When ethylene glycol (50%) is incorporated in the eluting solvent at 4°C recovery is boosted to 95%.

Experiment 2C. Exposure of the complex at 30°C (following a 30-min hold) to 50% ethylene glycol profoundly enhances enzyme–gel binding such that the "all" release pattern (95%, Experiment 2B) is changed to "none".

Experiments 1D–5D. This HLPDH–BSCL–6B species was refractory to all subsequent release attempts, except with 1.0 *M* sodium chloride.

The void volume recovery noted for HLPDH at 4°C (27%, Table I) from BSCL-6B compares favorably with the value (31%) cited in Experiment 1B (Table II). Similarly, the recovery reported for SLPDH at 4°C (95%, Fig. 1B) is comparable to that cited for HLPDH (90%, Experiment 1A, Table II). These results demonstrate the profound influence of the contact time variable on the gel–enzyme release patterns at 4 or 30°C.

Release patterns displayed here (Table II) are consistent with the interpretation that ethylene glycol diminishes hydrophobic interaction and strengthens electrostatic interaction; that salt weakens electrostatic interaction and strengthens hydrophobic interaction[15]. Elevated temperature and prolonged exposure time enhance hydrophobic interaction.

The gel–enzyme interactions described in Table II are in accord with the two-step thermodynamic model proposed by Ross and Subramanian[16] to describe protein–ligand associations. Thus A corresponds to the individually hydrated species (in this case HLPDH and BSCL-6B) which in B or C partially interact so that there is a mutual penetration of their hydration layers to form a "hydrophobically associated species". For this transformation (A → B or C) both ΔH^0 and ΔS^0 are positive and ΔG^0 is negative. In step 2 (B or C → D) the presence of ethylene glycol at 30°C weakens the hydrophobic association and, at the same, time enhances "intermolecular interactions" which include ionic, hydrogen bond and Van der Waals forces. The solvent molecules are not thought to play a significant role in this step.

Structure comparison studies

In what follows we compare the results of our thermal affinity gel data and (i) the hydropathic characteristics of these enzymes with, (ii) their tertiary structure alignments[2,17] and (iii) the results derived from X-ray studies dealing with HLADH and CB3GA interaction sites[18]. This assessment points to the fact that only a very few (one to three) key amino acid differences between HLADH and PDH may be sufficient to account for the dissimilar dual-temperature gel interactions reported here.

From hydropathy plots. When the amino acid composition of HLADH[19] is ranked with SLPDH[20] (and ULPDH[3]) on the basis of residue apolarity[21], no differences between these proteins are evident (data not shown). However, some insight to the location of HLADH and SLPDH site differences can be gained from a comparison of Kyte and Doolittle[13] hydropathy plots of these enzymes (Fig. 2).

Thus the amino acid segment *ca.* 45–70 in SLPDH is a hydrophilic region leading up to a weak hydrophobic area. The comparable segment in HLADH begins with a *ca.* 10 amino acid stretch not showing an apolar or polar bias and closes with the build-up of a strong hydrophobic region. Within the sequence *ca.* 145–180 SLPDH

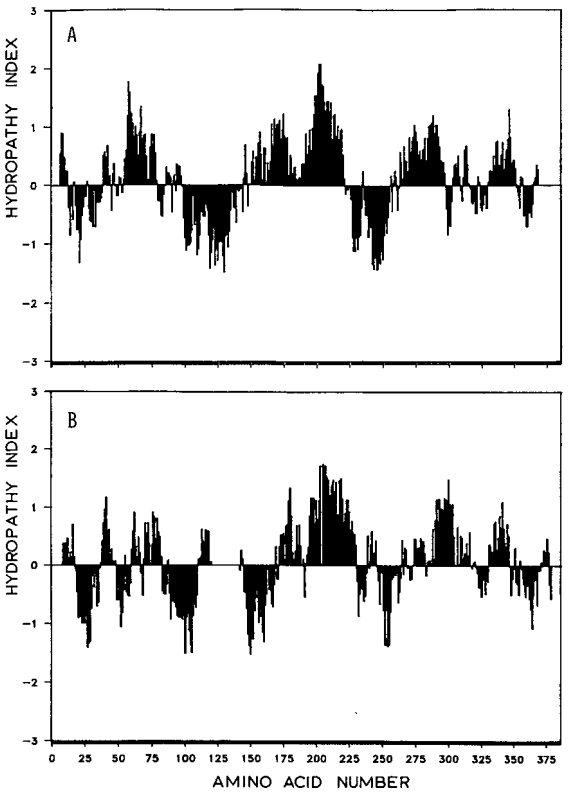

Fig. 2. (A) Hydropathy plot of HLADH showing a 13 amino acid average segment plotted against the amino acid number. Regions above the (universal) ≈zero midline are designated hydrophobic (*i.e.*, interior) spans, those below identify hydrophilic (exterior) spans. The boundary of the catalytic domain is defined by amino acids ≈1–175 and ≈319–374. The coenzyme binding domain is localized over amino acids ≈176–318. (B) Hydropathy plot of SLPDH showing a 13 amino acid average segment plotted against the HLADH amino acid number. The gaps that appear in the SLPDH amino acid profile arise from an aligment utilizing the computer-based model generated from the conserved tertiary structure of HLADH[2]. Regions above the (universal) ≈zero midline are designated hydrophobic (interior), those below hydrophilic (exterior).

is judged to be predominantly hydrophilic, while HLADH is characterized as hydrophobic. A third regional difference is centered on segment *ca.* 260–285. Here HLADH is strongly hydrophobic while the corresponding SLPDH segment is classified as indifferent to weakly hydrophobic.

From tertiary structure alignment. When the individual amino acids in the retained tertiary structure of HLADH are replaced with the corresponding SLPDH amino acids relatively few residues are altered in the NAD coenzyme binding domain[2,22]. These results are summarized in Table III. Major differences are seen to occur within three of the regions identified in the hydropathy plots (at positions 271, 47 and 174) and at residue 202. Within this preserved three-dimensional alignment, amino acids 271 and 202 are found to lie in relatively close proximity to each other

TABLE III

NAD AND CB3GA INTERACTION SITES WITH HLADH AND SLPDH

Amino acid				*Interaction sites*	
Residue No.[a]	*ADH*[b]	*PDH*[b]	*Diff.*[c]	*NAD*[d]	*CB3GA*[e]
224	I	L	m	N1A, C2A, N3A, C4A, C5A, C6A	(D) Anthraquinone ring
271	R	V	M	N6A	(C) Bridging 3-sulfophenyl ring
269	I	T	m	N3A, C4A, C5A, N7A, C8A, O4'A, C3'N, O3'N, C4'N	
223	D	D	o	C2A, N3A C1'A, C2'A, O2'A, O3'A, C3'A, O4'A	
199	G	G	o	N3A, O4'A	
228	K	R	m	O2'A, C3'A, O3'A	
201	G	G	o	O3'A, OP2A, OP2N	
47	R	G	M	OP1A OP3 OP1N, C3'N	(B) Triazinyl ring
202	G	P	M	OP2A, OP2N	
369	R	K	m	OP2N, OP1N	(A) Terminal 4-sulfophenylamino ring
203	V	I	m	OP2N, C5'N O5'N, C5N, C6N	
51	H	H	o	O2'N, O3'N	
48	S	S	o	O2'N	
178	T	V	m	C4N, C5N	
46	C	C	o	OP1N, C5N, C6N	
174	C	E	M	C4N	

[a] Numbers refer to the residues of HLADH or SLPDH that interact with NAD. Alignment is based on computer model fitting to the tertiary structure of HLADH[2].

[b] Single-letter amino acid identities (see Table III) of HLADH and SLPDH with site number referenced to HLADH[2].

[c] Designates residue differences: M = major; m = minor; o = none.

[d] Descriptors of NAD atoms closer than 0.38 nm to HLADH[22] for amino acid residues identified in the first column. See also Fig. 3.

[e] D→A Refer to the Cibacron Blue ring identities cited by Biellmann *et al.*[18] (see Fig. 3).

towards the purine ribose side of the cofactor binding domain (see Fig. 2 of ref. 23 or Fig. 6a of ref. 22).

Though few, these residue substitutions could lead to profound changes in the character of ligand binding. Thus the alteration Arg271 → Val271 leads not only to a loss of positive charge at the rim of the adenine binding pocket[2], but also to a loss of the putative binding site for the bridging 3-sulfophenyl (C) ring of CB3GA[18] (see Fig. 3 and the section *From X-ray studies*).

The transformation Gly202 → Pro202 precludes hydrogen bond formation of the main-chain nitrogen residue 202 with cofactor phosphate oxygen. Similarly, the replacement Arg47 → Gly47 results in the loss in SLPDH of one charged hydrogen

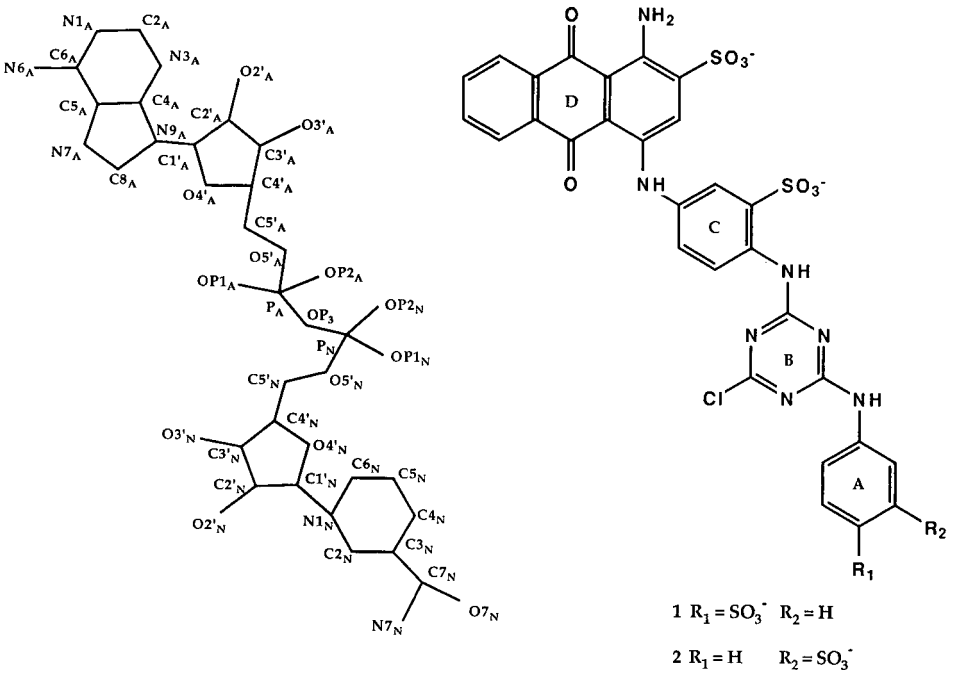

Fig. 3. (Left) Structural display of NAD^+ including the atom descriptors cited in Table III (column 5). Adapted with permission from ref. 22. © (1984), American Chemical Society. (Right) Chemical structure of Cibacron Blue 3GA with the ring identities cited in Table III (column 6). Reproduced with permission from ref. 18. © (1979), Springer-Verlag.

bond interaction between the (Arg) side-chain and one phosphate of NAD. Finally, the change Cys174 → Glu174 arises by default as a consequence of the preserved HLADH conformation at the nicotinamide catalytic site[2]. It is not thought to be involved in the binding described here, as judged by the dye–HLADH X-ray studies[18].

From X-ray studies. These experiments[18] (summarized in Table III and Fig. 3) reveal that the anthraquinone (D) ring of the dye (*p*-isomer) binds in the wide apolar pocket (of HLADH) which at one end binds the adenine segment of the NAD coenzyme. The position of the bridging 3-sulfophenyl (C) ring is such that it could interact with the ADH guanidinum group of Arg271. The triazinyl (B) ring binds in the region where the NAD pyrophosphate moiety binds with the chlorine atom close to the nicotinamide ribose binding site. From then on CB3GA binds quite differently from NAD. The terminal 4-sulfophenylamino (A) ring is bound in the left between the domains with a possible interaction of the sulfonate group with Arg369. The midpoint of this ring differs from the midpoint of bound nicotinamide by about 1 nm. Thus the binding of Cibacron Blue to HLADH partially resembles NAD binding in the ADP part, but differs greatly in the remaining parts[18]. The affinity gel binding patterns of HLADH and PDH described here (Table I and Fig. 1) are in accord with the results from these X-ray studies.

CONCLUSIONS

Among the AGNAD, AGAMP and BSCL-6B affinity gel alcohol or polyol dehydrogenase interactions reported here, the AGAMP and BSCL-6B binding patterns with these enzymes reveal a thermal dependence not evident in the AGNAD–enzyme interactions. This behaviour is indicative of hydrophobic association, since among the various intramolecular interactions, only hydrophobic interactions are strongly temperature-dependent[24]. Further, all interactions are shown to be exothermic, except the BSCL-6B–PDH association, which is driven by an increase in entropy. This binding pattern is similar to the HLADH–CB3GA association revealed by X-ray studies[18] where the anthraquinone ring binds in the hydrophobic pocket reserved for the purine-ribose segment of NAD. The substitutions Lys228 → Arg228 and Arg369 → Lys369, although classed as minor (m, Table III), will undoubtedly contribute to altered hydrogen bond and bound-water patterns in their local environments[25]. However, structure comparisons between SLPDH and HLADH based on alignment studies suggest that the focus of attention for future experiments could best be directed to residues 271, 47 and 202 (based on HLADH amino acid numbers) as the most obvious targets for PDH site-specific mutation experiments designed to account for the differences reported here.

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Use of column switching for the determination of niacinamide in compound feed

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of niacinamide in compound feed. The niacinamide is extracted with 0.2 *M* hydrochloric acid in order to suppress the hydrolysis of the niacinamide to nicotinic acid. The chromatography is carried out with the aid of column switching. An RP-18 column is used for the preseparation, and the analytical separation takes place in a cation-exchange column. With the proposed method, the limit of determination is about 2 ppm of niacinamide.

INTRODUCTION

The provision of amino acids and vitamins, now mostly produced synthetically by large-scale industrial processes, is an important factor in achieving high levels of productivity in intensive livestock husbandry.

One vitamin that is acquiring growing importance is niacin (vitamin B_3). Niacinamide, similarly to nicotinic acid, participates in the form of coenzymes NAD and NADP in all living cells in vital metabolic reactions. A deficiency of niacinamide or nicotinic acid in animal nutrition has adverse effects; *e.g.*, poultry it leads to disorders in the development of feathers, reduced laying activity, decreased hatchability and to delays in growth. With pigs, the deficiency manifests itself in the development of rough skin and determatitis at the ears. Cases of intestinal inflammation can also occur.

The requirement for niacin is partly met through the particular feed diet. Independent of this, however, niacinamide/nicotinic acid is generally supplemented to ensure that the requirement is met. As the rate of supplementation is only a few parts per million, it is necessary to have an efficient method for determining these low levels reliably and quickly.

The quantification of niacinamide/nicotinic acid is usually carried out spectrophotometrically[1–4], by thin-layer chromatography[5,6] or by high-performance liquid chromatography (HPLC)[7,8]. Of these, HPLC is certainly the most efficient. However, the polar structure of niacinamide/nicotinic acid results in most HPLC processes

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being based on ion-pair chromatography[9–14]. Because, in our experience, good retention time stability is not always achieved in ion-pair chromatography, reversed-phase HPLC without ion-pair reagents was employed for the quantification of niacinamide in compound feed. A requirement was that costly preparation of the samples should be avoided.

EXPERIMENTAL

Equipment

The HPLC system consisted of a Hewlett-Packard 1090 liquid chromatograph fitted with a column-switching mechanism and a (Spectra-Physics SP 8450) UV detector. The evaluation was carried out by means of a Spectra-Physics SP 4270 computer–integrator which was connected to a Labnet laboratory data station. The measurements were made at 264 nm at a flow-rate of 1.5 ml/min and at 50°C. The following columns were obtained from Macherey, Nagel & Co. (Düren, F.R.G.): precolumn, RP-18, 5 μm (17 × 4.6 mm I.D.); column A, RP-18, 5 μm (250 × 4.6 mm I.D.); and column B, Nucleosil 5 SA cation-exchange column (250 × 4.6 mm I.D.). The retention time of niacinamide on column A was 13.7 min and the total analysis time was *ca.* 32 min.

The column switching system is presented in Fig. 1 and proceeds as follows: column switching, 0 min CS = 1, 12.9 min CS = 0, 14.5 min CS = 1 and 26 min

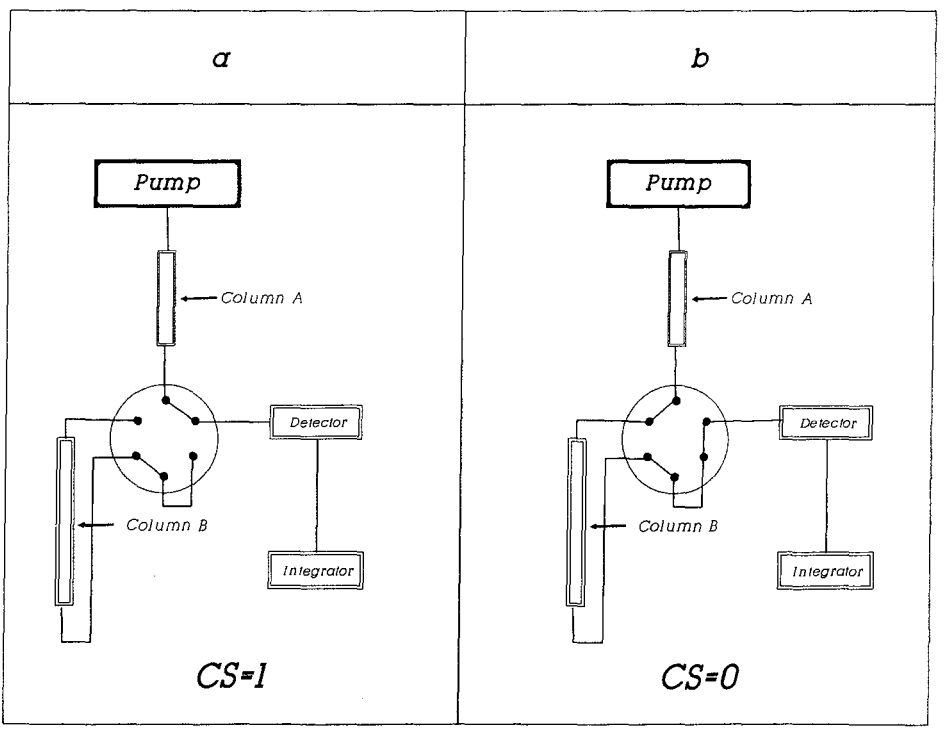

Fig. 1. Flow diagram for the determination of niacinamide. For explanations, see Experimental. (a) Separation only on column A (equal to CS = 1), (b) separation on columns A and B (equal to CS = 0).

CS = 0 (CS = 1, via precolumn and column A; CS = 0, via precolumn, column A and column B); and eluent, 0 min 0.01 *M* KH_2PO_4, 17 min 0.01 *M* KH_2PO_4–acetonitrile (60:40) and 20 min 0.01 *M* KH_2PO_4. The time parameters of the column switching must be checked from time to time and corrected if necessary. The column switching times given above apply for a retention time of niacinamide of 13.7 min on column A.

Materials and solvents

All substances were obtained from Merck (Darmstadt, F.R.G.). The compound feeds used for the feeding of poultry and pigs were obtained from various producers. The eluent was a 0.01 *M* aqueous solution of potassium dihydrogenphosphate (pH 4.80). Before use, it was degassed ultrasonically.

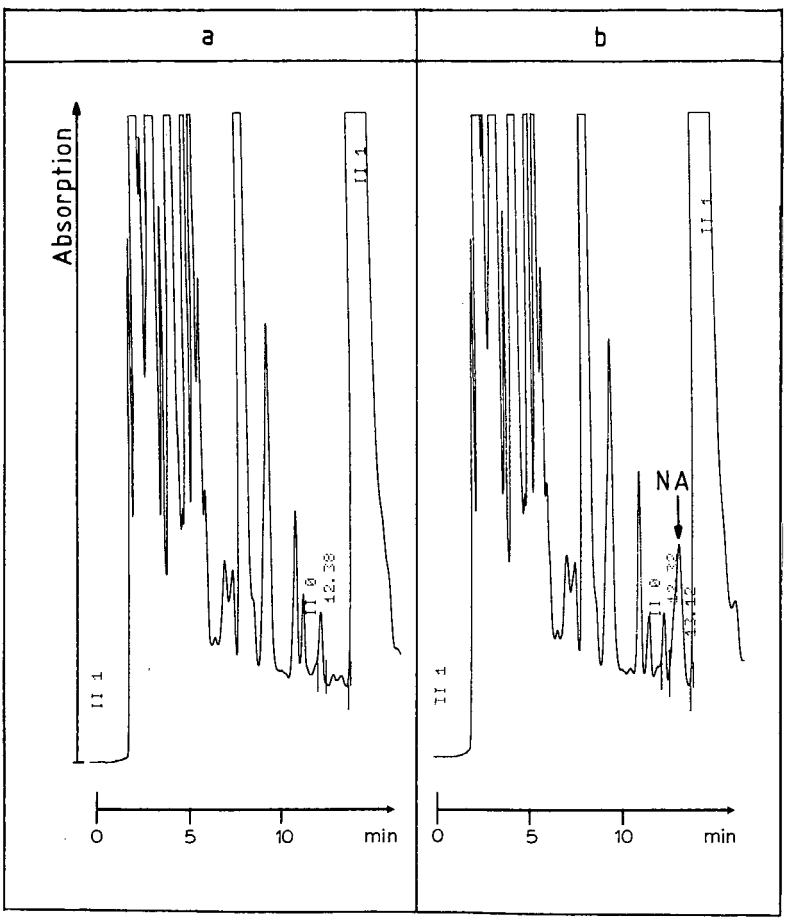

Fig. 2. HPLC of niacinamide (NA) without column switching. (a) Compound feed without niacinamide; (b) compound feed supplemented with 10 ppm of niacinamide. Eluent: 0.01 *M* KH_2PO_4.

Reagent solutions

Solutions of known concentrations of niacinamide were prepared with doubly distilled water, using these calibration solutions, a calibration graph was then plotted, and was linear in the range 5–50 ppm.

Preparation of samples

A 2-g amount of finely ground (0.25 mm) compound feed, weighed exactly, was mixed with 10.0 ml of 0.2 *M* hydrochloric acid and stirred for 10 min. The mixture was centrifuged and filtered through a 0.5-μm membrane filter and the filtrate was used for the HPLC measurement.

Kinetics of niacinamide hydrolysis

The sample was prepared as described above. In addition to 0.2 *M* hydrochloric acid, 0.1 *M* hydrochloric acid and doubly distilled water were used. After filtration, the pH and the niacinamide content of the solution were determined as a function of time.

RESULTS AND DISCUSSION

A compound feed for animal nutrition is a very complex matrix when regarded analytically. It is built up from mostly vegetable raw materials with minerals, amino acids and vitamins added. Consequently, a large number of peaks must be identified even when detection is carried out at 264 nm (Fig. 2).

If one wants to conduct the analysis without ion-pair reagents, it is not possible to maintain a retention time of niacinamide of appreciably more than 10 min with RP-18 phases. Although this is possible with cation-exchange columns, the same problem arises with regard to the number of peaks as with RP-18 columns. As a complicated and therefore time- and cost-intensive preparation of samples was to be avoided, column switching was considered[15,16].

Our positive experience with column switching[17] was continued in the determination of niacinamide. Fig. 2a shows the chromatogram of a compound feed without addition of niacinamide, and Fig. 2b shows that of a compound feed with 10 ppm of niacinamide added. Without the zero diet, however, it is not possible to be certain that the niacinamide peak is free from interferences.

By using column switching, it is now possible to obtain the niacinamide peak virtually free from interferences. Here, preseparation is carried out on an RP-18 column with 0.01 *M* potassium dihydrogenphosphate solution. Then, by means of column switching, the niacinamide fraction is applied to the second column and chromatographed there, also with 0.01 *M* potassium dihydrogenphosphate solution. The second column contains a cation exchanger, which makes it possible to separate the niacinamide cleanly from possible interfering substances present (Fig. 3a and b).

In conjunction with a more extensive study, various compound feeds were analysed for their niacinamide contents. The compound feeds to which no niacinamide was added proved to be virtually interference-free in the niacinamide region. Some vegetable and animal raw materials were likewise studied for their free niacinamide contents and the results are given in Table I.

The reproducibility of the extraction and recovery of niacinamide were determi-

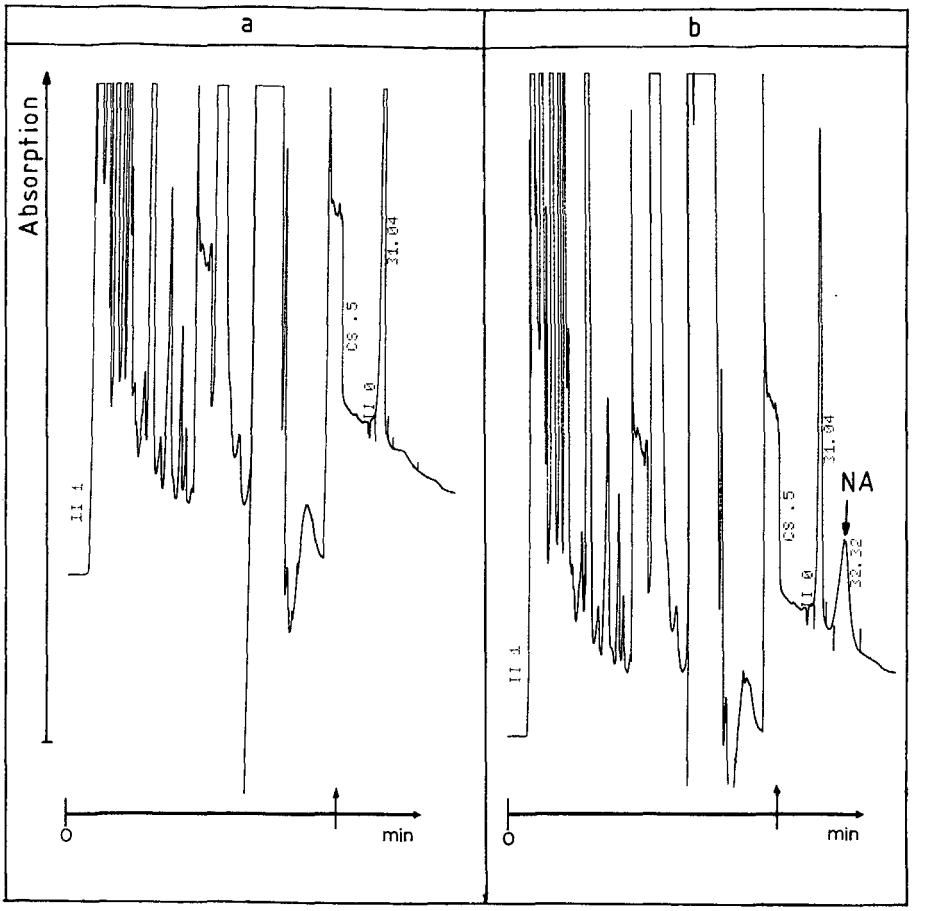

Fig. 3. HPLC of niacinamide (NA) with column switching. (a) Compound feed without niacinamide; (b) compound feed supplemented with 10 ppm of niacinamide. Conditions as described under Experimental. The arrows indicate a change in chart speed from 0.25 to 0.50 cm/min.

TABLE I

FREE NIACINAMIDE CONTENT IN VARIOUS RAW MATERIALS

Sample	*Niacinamide content from extraction with 0.2 M HCl (ppm)*	
Blood meal	2– 3	(n = 2)
Corn	–[a]	(n = 2)
Soybean meal	6– 8	(n = 2)
Fish meal	19–72	(n = 5)
Meat meal	3–36	(n = 5)

[a] <0.5 ppm.

TABLE II

RECOVERY OF SUPPLEMENTED NIACINAMIDE IN COMPOUND FEED

Rate of supplementation (ppm)	*Content from extraction with 0.2 M HCl (ppm)*	*Recovery (%)*
5	5.0	100
	4.9	98
10	10.2	102
	10.1	101
15	15.1	100.7
	14.7	98

ned. The study of the extraction reproducibility was carried out with a sample that contained 10 ppm of niacinamide. The coefficient of variation was 0.86% (n = 10). The recovery was determined for the addition of 5, 10 and 15 ppm niacinamide (Table II).

With the sample preparation described, the limit of determination was about 2 ppm and the detection limit about 0.5 ppm. Both limits can be lowered, especially by changing the range adjustment of the UV detector. However, this is not relevant for the analysis of feedstuffs because niacinamide is supplemented at levels of about 20 ppm upwards.

A parameter of essential importance for the determination of niacinamide in feedstuffs is the pH of the test solution. In extensive studies, the hydrolysis of the niacinamide to nicotinic acid in the test solution was analysed at various pH values and the results are shown in Fig. 4. It can be clearly seen that a reduction in pH is accompanied by an increase in the stability of the niacinamide in the test solution.

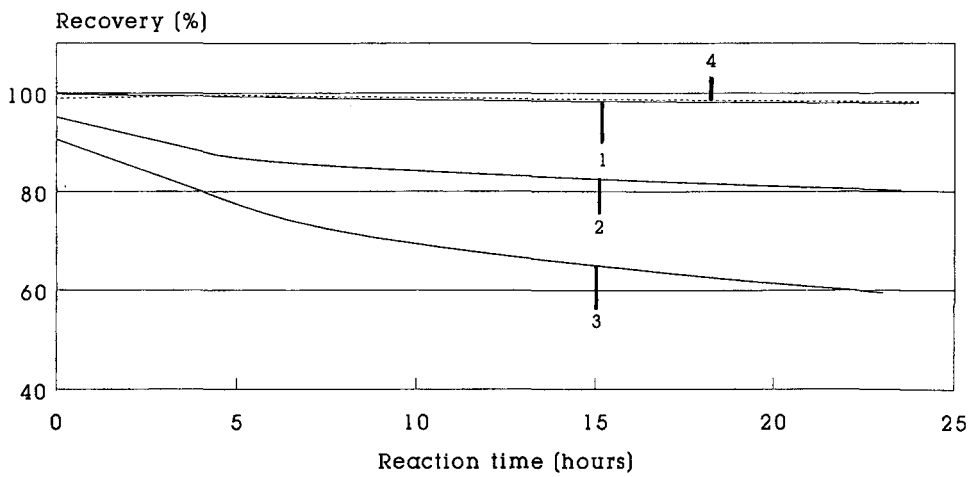

Fig. 4. Hydrolysis of niacinamide. Compound feed (CF) supplemented with 30 ppm of niacinamide (NA). (1) NA in water; (2) CF + NA in 0.1 *M* HCl; (3) CF + NA in water; (4) CF + NA in 0.2 *M* HCl. Actual pH values: (1) 5.5; (2) 5.3; (3) 3.5; (4) 2.1.

However, it is not only the pH, but also the composition of the test solution that is of decisive importance for the hydrolysis of niacinamide.

Niacinamide in water at pH 5.2 shows no notable decomposition even after 24 h. A compound feed sample supplemented with niacinamide, however, was found to contain only about 60% of niacinamide after 24 h. As the feed acts as a buffer, it is therefore advisable to suppress the hydrolysis by lowering the pH of the test solution. As a result, it is possible to analyse several samples overnight with an autosampler without the risk of hydrolysis of niacinamide occurring.

It is not possible to determine nicotinic acid by this procedure, because it co-elutes with a number of other feed components. A separation could not be achieved by column switching, so that one cannot determine both forms of the vitamin with a single injection. However, using this procedure, improved stability of the retention times over ion-pair chromatography could be achieved.

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Method for the isolation and liquid chromatographic determination of eight sulfonamides in milk

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SUMMARY

A method for the isolation and liquid chromatographic determination of eight sulfonamides in milk is presented. Fortified or blank milk samples (0.5 ml) were blended with octadecylsilyl ($C_{18}T$) derivatized silica (2 g). A column made from the sample $C_{18}T$ matrix was first washed with hexane (8 ml) following which the sulfonamides were eluted with methylene chloride (8 ml). The eluate contained sulfonamide analytes which were free from interferences when analyzed by high-performance liquid chromatography (HPLC) utilizing UV detection (270 nm, photodiode array). Standard curve correlation coefficients (range, 0.998 ± 0.002 to 0.999 ± 0.001), average percentage recoveries (73.1 ± 7.4 to 93.7 ± 2.7%), and the inter- (3.9–9.6%) and intra-assay (2.2–6.7%) variabilities, were determined for the concentration range examined (62.5–2000 ng/ml) and resulted in a minimal detectable limit of 1.25 ng on column (62.5 ng/ml, 20 μl injection from a final sample volume of 0.5 ml). Savings in terms of time and solvent make this procedure attractive when compared to classical isolation techniques for sulfonamides.

INTRODUCTION

Sulfonamides are used as antibacterial agents and are commonly administered to domestic agricultural species via medicated feeds. In this manner, these compounds prevent and treat disease, increasing the vigor and the general well being of food producing animals. Legal tolerance levels and withdrawal periods prior to slaughter have been established for these drugs. However, these levels are being continuously re-evaluated based on, for example, the recent evidence implicating sulfamethazine as a possible carcinogenic agent[1].

Sulfonamide residue levels have been monitored by various analytical techniques[2–6]. While these colorimetric, thin-layer chromatographic, high-performance liquid chromatographic (HPLC) and gas chromatographic (GC) techniques find application in sulfonamide analyses, a critical factor which governs their usefulness is the sample extraction procedure.

Residue isolation techniques must be such that they isolate the targeted com-

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pound(s) with relatively high percentages of recovery while simultaneously minimizing interferences which may contribute to high background in the analysis. Sulfonamides are generally extracted from solid samples by homogenizing the sample in an extracting solvent. The particulated sample is then repeatedly extracted to increase recovery. Liquid samples are treated similarly by multiple extraction with organic solvents. For example, sulfonamides in milk have been extracted with an acetone–chloroform mixture according to the method of Tishler *et al.*[7]. This method was originally developed for the determination of sulfamethazine in milk but has since found application for meat tissues. In addition to the need for large volumes of extracting solvent the method requires additional clean-up steps in the form of pH adjustments, further extraction and backwashing. Furthermore, emulsion formation during the extraction procedure complicates sulfonamide isolations. Multiresidue sulfonamide isolation techniques which minimize or eliminate problems inherent in these traditional solvent extraction methods are needed.

Recently we have demonstrated[8–11] that by blending biological samples with C_{18} packing material one can prepare a column from which one can selectively elute targeted residues. This method, which we have named matrix solid phase dispersion (MSPD), eliminates many of the difficulties associated with traditional isolation techniques. Based on this fundamental principle, we report here the first use of the MSPD methodology for the rapid extraction, HPLC separation, photodiode array UV detection and quantitation of sulfanilamide, sulfathiazole, sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfisoxazole and sulfadimethoxine as residues in milk.

EXPERIMENTAL

Chemicals and expendable materials

All standard compounds and solvents were obtained at the highest purity available from commercial sources and were used without further purification. Water for HPLC analyses was double distilled water passed through a Modulab Polisher I (Continental Water Systems, San Antonio, TX, U.S.A.) water purification system. Bulk C_{18} (40 μm, 18% load, endcapped from Analytichem, Harbor City, CA, U.S.A.) was cleaned by making a column (50 ml syringe barrel) of the bulk C_{18} material (22 g) and sequentially washing with two column volumes each of hexane, methylene chloride and methanol. The washed C_{18} was vacuum aspirated until dry. Stock sulfonamide solutions (1 mg/ml) were prepared by dissolving standard compounds with HPLC-grade methanol and diluting to the desired (3.13, 6.25, 12.5, 25.0, 50.0 and 100 μg/ml) levels with methanol. Syringe barrels of 10 ml were thoroughly washed and dried prior to use as columns for sample extraction.

Extraction procedure

Milk samples (Vit. D homogenized, 3.2% butterfat) were obtained from a local market. A 2-g amount of C_{18} material was placed into a glass mortar. Standard sulfonamides (10 μl of 3.13–100 μg/ml stock solutions) and internal standard sulfamerazine (10 μl, of 12.5 μg/ml stock solution) were added to the milk and the samples were allowed to stand for 1 min. Blank milk samples were prepared similarly except that 20 μl of methanol containing no sulfonamides were added to the sample. An

aliquot (0.5 ml) of milk was placed on the C_{18} material and the samples were then gently blended into the C_{18} material with a glass pestle until the mixture was homogenous in appearance. A gentle circular motion with very little pressure was required to obtain a homogenous mixture. The resultant C_{18}–milk matrix was placed into a 10-ml plastic syringe barrel which was plugged with a filter paper disc (Whatman No. 1). The column head was covered with a filter paper disc and the column contents were compressed to a final volume of 4.5 ml with a syringe plunger that had the rubber end and pointed plastic portion removed. A pipette tip (100 μl, plastic, disposable, Eppendorf) was placed on the column outlet to increase residence time of the eluting solvents on the column. The resulting column was first washed with 8 ml of HPLC-grade hexane. Flow through the column was gravity controlled in all cases. If the flow through the column was hindered, positive pressure was applied to the column head (pipette bulb) to initiate gravity flow. When flow had ceased, excess hexane was removed from the column with positive pressure as described above. The sulfonamides were then eluted with 8 ml of methylene chloride as described above for hexane. The methylene chloride extract was dried under a steady stream of dry nitrogen gas under a hood. To the dry residue were added 0.1 ml of methanol and 0.4 ml of 0.017 *M* orthophosphoric acid. The sample was sonicated (5–10 min) to disperse the residue, which resulted in a suspension. This was transferred to a microcentrifuge tube and centrifuged (Fisher Microcentrifuge Model 235, Fisher Scientific, Pittsburg, PA, U.S.A.) at 13 600 *g* for 5 min. The resultant clear supernatant was filtered through a 0.45-μm filter (Bio-Rad Labs., Richmond, CA, U.S.A.) and an aliquot (20 μl) was analyzed by HPLC.

HPLC analysis

Analyses of extracted samples and standard sulfonamides were conducted utilizing a Hewlett-Packard HP1090 HPLC system (HP 79994A Chemstation) equipped with a photodiode array detector set at 270 nm with a bandwidth of 20 nm, spectrum range of 200–350 nm and a reference spectrum of 450 nm with a bandwidth of 100 nm. The solvent system was 0.017 *M* orthophosphoric acid–acetonitrile (90:10, v/v) at a flow-rate of 1 ml/min, for 5 min increasing the flow-rate to 2 ml/min at 5 min for the remainder of the 16-min run. A reversed-phase octadecylsilyl (ODS) derivatized silica column (Supelcosil LC-18, 3 μm, 7.5 cm × 4 mm I.D., Supelco, Bellefonte, PA, U.S.A.) maintained at 45°C was utilized for all determinations.

Peak area ratio curves of standards and samples were obtained by plotting integration areas of generated peaks as a ratio to the area of the internal standard. A comparison of fortified sample peak area ratios to peak areas of pure standards run under identical conditions gave percentage recoveries ($n = 30$; 30 samples, 5 replicates of each concentration). The interassay variability was calculated as follows. The mean of the peak area ratios for five replicates of each concentration (62.5, 125, 250, 500, 1000 and 2000 ng/ml) was calculated. The standard deviation corresponding to each mean was divided by its respective mean and this resulted in the coefficient of variation for each concentration. The mean of these coefficients of variation was calculated along with its standard deviation and defined as the interassay variability, plus or minus the standard deviation. Intra-assay variability was determined as the coefficient of variation (standard deviation of the mean divided by the mean) of the mean peak area ratio of five replicates of an identical sample.

TABLE I

STATISTICAL DATA OF THE EIGHT SULFONAMIDES ISOLATED FROM FORTIFIED MILK SAMPLES

Concentrations: 62.5, 125, 250, 500, 1000 and 2000 ng/ml. Internal standard: sulfamerazine, 250 ng/ml. S.D. = standard deviation; C.V. = coefficient of variation. Linear regression equation: $y = mx + b$, where m = slope and b = y-intercept.

Compound	*Correlation coefficient ($r \pm$ S.D., $n = 5$)*[a]	*Linear regression equation*	*Recovery (%) ($\bar{x} \pm$ S.D., $n = 30$)*[b]	*Inter-assay variability (%, $n = 30$)*[c]	*Intra-assay variability (%, $n = 5$)*[d]
Sulfanilamide	0.999 ± 0.001	$y = 255.48x - 0.05$	73.1 ± 7.4	5.6 ± 3.3	3.2
Sulfathiazole	0.999 ± 0.001	$y = 407.89x - 0.04$	93.7 ± 2.7	6.6 ± 1.1	2.2
Sulfadiazine	0.999 ± 0.001	$y = 198.95x - 0.02$	81.2 ± 4.8	4.8 ± 2.9	2.7
Sulfamerazine	–	–	81.9 ± 4.6	4.5 ± 2.4	2.7
Sulfamethazine	0.999 ± 0.001	$y = 319.66x - 0.04$	92.7 ± 5.6	4.8 ± 2.7	2.3
Sulfamethoxazole	0.999 ± 0.001	$y = 419.67x - 0.08$	89.4 ± 8.3	4.0 ± 2.0	5.1
Sulfisoxazole	0.999 ± 0.001	$y = 479.48x - 0.04$	88.6 ± 11.2	8.1 ± 3.1	6.7
Sulfadimethoxine	0.998 ± 0.002	$y = 562.73x - 0.09$	89.6 ± 8.1	9.6 ± 3.3	2.3

[a] Mean of five standard curves.

[b] Five replicates at each concentration and $n = 6$ levels (62.5–2000 ng/ml).

[c] Calculate (100 · S.D.)/$\bar{x}$ = C.V. for $n = 5$ replicates at each concentration. Average C.V. values at $n = 6$ levels (62.5–2000 ng/ml) result in $\bar{x}$(C.V.) ± S.D. = inter-assay variability.

[d] Replicates ($n = 5$) of an identical sample, (100 · S.D.)/$\bar{x}$ = C.V. (intra-assay variability).

RESULTS AND DISCUSSION

A critical aspect of drug residue isolations, which dictates the usefulness of any analytical technique, is the sample preparation step. While classical extraction techniques utilizing large volumes of organic solvents isolate compounds of interest they may also result in extracts containing many interferences which necessitates further clean-up steps. As a result of multiple sample manipulations, aside from being time consuming, the accuracy, precision and reproducibility of the technique may be affected. In addition, the multi-step procedures may negate multi-residue isolations from a single sample due to losses of compounds within a class as a result of pH adjustments, reextractions and backwashing.

Results presented here overcome some of these aforementioned limitations and are an extension of previous work conducted in this laboratory[4–7]. The milk was evenly dispersed onto the solid (C_{18}) support which distributed the sample over a large surface area (1000 m^2 per 2 g of C_{18} material) and exposed the entire sample to the extraction process. The initial elution of the column with hexane removed lipid materials and, under these conditions, the sulfonamides remained on the column. Methylene chloride was then used to elute the sulfonamides. The high percentage recoveries and small variabilities (Table I) are a result of what can be envisioned as an exhaustive extraction process whereby a large volume of solvent is passed over an extremely thin layer of milk. The theoretical aspects of he MSPD technique have been the subject of previous publications[8–11] for the isolation of different residues from milk and other matrices.

Chromatograms of blank (Fig. 1A) and sulfonamide fortified milk (Fig. 1B) extracts show the blank milk extract to be free of compounds which might have interfered with sulfonamide analysis. A method blank contained no interfering compounds. This can be explained by the manner in which the sulfonamides were eluted from the column. The hexane wash removes lipid and other compounds, such as neutral chromophores, which could otherwise interfere with the sulfonamide analysis. Other more polar chromophores, which are less soluble in methylene chloride remain in the column. Thus, one can selectively elute the compounds of interest while eliminating potentially interfering compounds. While this explanation is simplistic, it serves to underscore the basic principle. Observations made during this work suggest the principles involved may be more complex. If one blends sulfonamide standards, which are dissolved in methanol, directly with C_{18} packing material they are not readily eluted with methylene chloride. However, if sulfonamides are fortified into milk, and blended into the C_{18} material they are eluted with high recoveries as indicated by the data presented here. Milk contains proteins, lipids, carbohydrates, salts, etc. which alters the normal elution profile of the C_{18} column. While the elution of sulfonamides from the C_{18} material alone can be considered a reversed-phase mechanism this cannot be said for the C_{18}–milk matrix where the sample constituents contribute a unique chemical characteristic to the column bed. The hydrophobic, ionic and electrostatic qualities contributed by the sample constituents result in unique but reproducible sulfonamide elution profiles. The theoretical aspects concerning the contribution of sample components to separations by MSPD have been discussed[8–11]. Additionally one cannot simply pass milk through a C_{18} cartridge and obtain consistent recoveries of sulfonamides. Passing milk through an SPE C_{18} col-

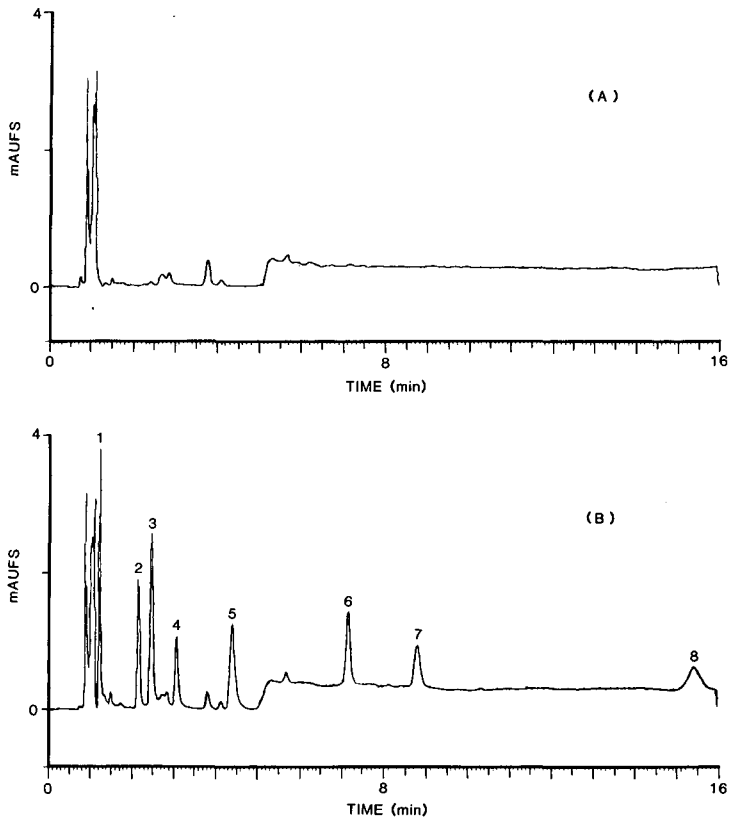

Fig. 1. Representative chromatograms (UV photodiode array, 270 nm) of the methylene chloride extracts of (A) blank milk, and (B) milk fortified with standard sulfonamides (250 ng/ml) and internal standard (250 ng/ml) sulfamerazine. Order of elution is (1) sulfanilamide, (2) sulfathiazole, (3) sulfadiazine, (4) sulfamerazine, (5) sulfamethazine, (6) sulfamethoxazole, (7) sulfisoxazole and (8) sulfadimethoxine.

umn does not appear to sufficiently coat the particles, resulting in inconsistent elution profiles for the sulfonamides, as was experienced in this laboratory. The milk must be mechanically blended into the C_{18} material for consistent results.

As a result of the cleanliness of the milk extracts so obtained a scale-up of this procedure could allow for the determination of sulfonamide levels in the low-ppb range and is presently being pursued. The minimum detectable limit utilizing a photodiode array detector was between 31.25 and 62.5 ng/ml (20 μl injection from 0.5 ml sample volume, 1.25 ng on-column) which reflects the sensitivity characteristics of the detection system utilized and the compounds analyzed in this study. The consistency of recovery at each concentration is reflected in the standard deviation of the average recoveries shown in Table I. The recoveries for eight different sulfonamides underscores the utility of the MSPD approach in terms of multiresidue isolations. Because of the cleanliness of the extract, an increase in sensitivity could be achieved by increasing injection volume and/or dissolving the extract residue in a smaller final volume. Additionally, by extending the theoretical aspects of this method, it is likely

that multi-residue sulfonamide determinations in other milk based products or liquids, as well as tissues or blood components, could be achieved with similar results.

The results presented here are based on fortified samples, such as would be required and obtained for the preparation of standard curves and for conducting recovery studies for the quantitative analysis of drug residues in milk incurred from the administration of a drug. The purpose of the present study was to examine the application of matrix solid phase dispersion for the simultaneous isolation of the eight sulfonamides from a single milk sample, demonstrating the prospect that such methodology may be used to screen for a wide range of drugs in a single sample with the understanding that it is unlikely that more than two compounds may be present in an actual sample. While an examination of milk from animals actually administered these eight sulfonamides would be ideal, such samples were not available to us and is outside the scope and limits of practicality of the present research. Such studies are currently underway, examining incurred residues of individual sulfonamides in milk obtained from animals used in drug depletion studies, with the assistance of the United States Food and Drug Administration.

CONCLUSIONS

The savings in terms of time and solvent requirements, compared to classical extraction techniques[3], make this procedure attractive. For example, the Tishler method[7] requires 50 ml of milk which is extracted several times resulting in a minimum of 600 ml of extracting solvent which must be evaporated. Additional pH adjustments and washing are necessary before the sample is ready for analysis. The method presented here isolates eight sulfonamides simultaneously, requires a 0.5-ml sample, 8 ml of hexane and 8 ml of methylene chloride and requires no extensive extract clean-up steps other than drying the methylene chloride, centrifugation and filtering prior to analysis. Furthermore, use of the MSPD method as outlined here results in extracts containing several sulfonamides which are relatively free from interfering coextractants which could aid in their detection by other more sensitive means, such as immunoassay techniques, by eliminating cross-reacting compounds.

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Separation and determination of individual carotenoids in a *Capsicum* cultivar by normal-phase high-performance liquid chromatography

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SUMMARY

A normal-phase high-performance liquid chromatographic system was developed for the separation of individual carotenoid pigments in a saponified extract of a *Capsicum* cultivar. Eighteen major components were separated, of which sixteen pigments were identified. The chromatographic system described permits a good separation and quantification of pigments. It can be used in physiological studies for the characterization of different varieties and in the food industry.

INTRODUCTION

Carotenoids constitute one of the most important groups of natural pigments and are widely distributed in the plant, animal and protista kingdoms. The pepper, the fruit of *Capsicum annuum* L., is an ideal plant material for the study of carotenoids as they are present in a great quantity and diversity.

Carotenoids are very important and some are useful for man: β-carotene and β-cryptoxanthin are provitamin A[1,2] and capsanthin, capsorubin and cryptocapsin, which are found nearly exclusively in fruits of *Capsicum*, are used as natural colorants. Finally, their metabolism is interesting owing to the spectacular changes that are produced during the ripening process[3,4].

Many methods have been proposed for the isolation and identification of carotenoids in plant material, but their structural similarities make their resolution difficult. In the particular case of *Capsicum* spp. pigments, paper chromatography[5] and thin-layer chromatography (TLC) are useful methods of separation[6–8]. However, the main inconvenience of these chromatographic systems is that they increase the inherent instability of the carotenoids in light, acids or oxygen[9]. Other workers have used a combination of counter-current chromatography and open-column[10,11] or gas

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chromatography[12]. Of these methods, open-column chromatography and TLC fail from the point of view of reproducibility and accurate quantification, whereas gas chromatography provides this last advantage, but cannot be used with thermolabile carotenoids.

Until now, no method has been reported that separates all *Capsicum* spp. pigments with good resolution using any one chromatographic system. Often it has been necessary to rechromatograph fractions, a method that takes several hours, during which time non-natural products are formed, owing to prolonged exposure to adsorbents, eluents, light and oxygen[1].

High-performance liquid chromatography (HPLC) has recently been applied to the qualitative analysis and determination of carotenoids from different plant materials[13–19]. It offers significant advantages over the techniques previously mentioned, namely rapidity, absence of alterations, on-line detection and sensitivity. When this technique is applied to the carotenoids of *Capsicum* spp. it is necessary to take into account that they are mainly esterified. Studies on the pepper fruit reported so far have used reversed-phase HPLC[20,21] and unsaponified extracts. Different peaks were obtained, some of them corresponding to the same carotenoid with different degrees of esterification[22,23].

In this paper, we describe the separation and identification of individual unesterified carotenoids of *Capsicum annuum* using a normal-phase HPLC system in one step. The method is rapid and reproducible. Also, it can be used in physiological studies, for the characterization of different varieties and in the food industry.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Shimadzu Model LC-6A ternary solvent-delivery system equipped with a Shimadzu Model SLC-6A controller, connected to a Perkin-Elmer Model LC-55 UV–VIS detector. The data were processed by means of a Shimadzu Model CR-6A integrator to evaluate the peak area and peak height.

Absorption spectra of isolated components in various solvents were recorded on a Hitachi Model U-3200 UV–VIS spectrophotometer with a double "monochromator". The absorption spectra of the carotenoids were recorded between 300 and 500 nm.

The purity of the chromatographic peaks was evaluated using a Shimadzu Model SPD-M6A photodiode detector. The spectrophotometric data were processed in a PC with hard disk using a specific chromatographic program. This program provides, among other data, the spectrum, the absorbance ratio and purity of a peak.

Columns

Analytical separation was performed on a stainless-steel column (25 cm × 4.6 mm I.D.) of Spherisorb (5-μm spherical particles) (Phase Separations).

Semipreparative separation was carried out on a Waters Assoc. stainless-steel column (30 cm × 7.8 mm I.D.) of μPorasil (10 μm irregular particles). All columns were equilibrated with the initial elution solvent for 15 min prior to analysis.

Materials and reagents

Detection in the visible zone of the spectrum (400–500 nm) allowed the use of ACS-quality reagents; this provides important savings in solvent costs, without a notable decrease in the operational lifetime of column. The solvents used for liquid chromatography were dehydrated (0.3 nm molecular sieve for acetone; sodium for light petroleum), filtered through 0.45-μm filters and degassed in an ultrasonic bath at reduced pressure.

The fruits of *Capsicum annuum* cv. Belrubí were kindly supplied by the Instituto Nacional de Investigaciones Agrarias (experimental field in Torreblanca, Murcia, Spain). This cultivar was developed from "Cornicabra" (Mexican pepper) for greater productivity. The fruit has a bright red colour, is long and contains capsaicin, which makes its hot. The fruits were harvested fully ripened. Pepper samples (3 kg) of uniform size were collected and taken immediately to the laboratory. The peppers were cut open and the seeds removed and discarded. The remainder of the fruits were chopped into small pieces and 25-g subsamples were removed from each of the samples and stored at $-28°C$ under nitrogen until analysed.

Pigment extraction and saponification

Three 25-g samples of peppers were extracted with methanol in a Sorvall Omni-Mixer blender at high speed for about 20 min. The stainless-steel chamber was immersed in an ice-bath. The mixture was filtered with a sintered-glass funnel and the residue repeatedly extracted with methanol–diethyl ether (90:10) until the triturate was colourless. The filtrates were combined and brought to 500 ml with the same extraction solvent.

A 50-ml aliquot of each sample was saponified with aqueous potassium hydroxide (60%) and the non-saponified fraction of carotenoids was re-extracted with diethyl ether employing the method of Candela *et al.*[8]. The ether solution was brought to a final volume of 50 ml.

For HPLC analysis 3-ml aliquots were dried in a nitrogen stream and the residue was dissolved in 300 μl of acetone and filtered through a 0.45-μm filter (Swinnex, Millipore) prior to injection. Acetone was used because it is a very good solvent for all the pigments.

Other plant materials used as sources of some of the carotenoids present in peppers were anthers from *Asphodelus fistulosus* L., maize seeds, citrus (lemon) leaves and spinach leaves. We also used synthetic β-carotene from Hoffmann-La Roche. Extraction of the carotenoids and the saponification conditions were the same as employed with the pepper fruits. All the manipulations were carried out under red light.

Chromatographic procedures

The separations of carotenoids were carried out under two sets of HPLC conditions. In both, two solvents were used: light petroleum (b.p. 40–60°C) (LP) and acetone (AC). The analytical separations were carried out with HPLC system A and the semipreparative separations with system B.

System A. The initial solvent mixture was LP–AC (95:5, v/v). After injection, a stepwise gradient was introduced as follows: (1) the proportion of LP was decreased to 75% in 30 min with a linear gradient (curve 0 of the system controller); (2) LP was

maintained at 75% for 5 min; (3) LP was increased to 95% in 5 min with a convex gradient (curve −10 of the system controller). Other chromatographic conditions were as follows: flow-rate, 1 ml/min; chart speed, 3 mm/min; wavelength, 460 nm; detection limit, 0.02 a.u.f.s.; and injection volume, 4 μl. The injection of this small, highly concentrated volume prevents distortions of the peak and the formation of artifacts in HPLC[24].

System B. This was identical with A except that in the second step LP was maintained at 75% for 15 min and the flow-rate was 4 ml/min. The detection limit was 0.5 a.u.f.s. and the injection volume was 175 μl.

TLC analyses were made on silica gel 60 plates (10 × 20 cm, layer thickness 0.25 mm; Merck) with a concentration zone (2.5 cm). Development was carried out for 75 min in a rectangular TLC tank previously equilibrated with the elution solvent of LP–AC (77:23) for 5 min.

Pigment quantification

The pigments were quantified by evaluating as capsanthin the red carotenoids: capsanthin, capsanthin epoxide, capsorubin, capsorubin isomer and cryptocapsin. The remaining yellow pigments were quantified as β-carotene. Known amounts between 30 and 600 ng and 100 and 2500 ng of β-carotene and capsanthin, respectively, were analysed with system A and their corresponding areas evaluated. The concentration of these standards in benzene was determined by spectrophotometry, using specific extinction coefficients ($E^{1\%}_{1cm}$) of 2337 for β-carotene and 2072 for capsanthin. Six to eight determinations were made for each carotenoid, resulting in a linear calibration graph (area *vs.* concentration). The standard deviation was less than 1.5%.

For the quantification of the problem extracts, a first injection of the standard into the HPLC system was followed by four injections of the corresponding extracts and then the standard.

RESULTS

Separation and identification of carotenoids by semipreparative TLC and HPLC

Concentrated solutions of anthers, maize seeds, citrus (lemon) leaves, spinach leaves and pepper fruit extracts were chromatographed on silica gel thin-layer plates. Of the bands obtained, the following were subsequently analysed by HPLC.

Anthers. Five bands were separated, of which the third in order of chromatographic elution was the largest with $R_F = 0.67$. This band was subsequently purified by semipreparative HPLC (system B), resulting in a retention time (t_R) of 12.31 min and was identified as anteraxanthin. The visible absorption maxima (nm) were hexane, $\lambda_{max} = 421, 443, 473$; benzene, $\lambda_{max} = 433, 457, 486$. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift of 19.5 nm corresponding to a 5,6-epoxy group.

Maize seeds. Three bands were separated, of which the second ($R_F = 0.84$) and the third ($R_F = 0.38$) in order of elution were scraped off. These bands were subsequently purified by semipreparative HPLC (system B) resulting, in t_R of 10.80 and 23.41 min, respectively.

Band 2 was identified as β-cryptoxanthin. The visible absorption maxima (nm; values in parentheses are shoulders) were hexane, $\lambda_{max} = (425), 450, 477$; benzene, $\lambda_{max} = (435), 463, 490$.

Band 3 was identified as zeaxanthin. The visible absorption maxima (nm) were hexane, λ_{max} = (423), 450, 477; benzene, λ_{max} = (435), 462, 490.

Citrus (lemon) leaves. Eleven bands were separated, of which the ninth in order of chromatographic elution with R_F = 0.20 was scraped off. This band was subsequently purified by semipreparative HPLC (system B), resulting in a t_R 31.34 min. It was identified as violaxanthin. The visible absorption maxima (nm) were hexane, λ_{max} = 420, 444, 473; benzene, λ_{max} = 434, 457, 486. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift of 40 nm, converting the violaxanthin to auroxanthin (λ_{max} = 380, 400, 425 nm in hexane) within a few minutes.

Spinach leaves. Four bands were separated, scraped off and collected.

Band 1. This band gave R_F = 0.96 on TLC and was subsequently purified by semipreparative HPLC (system B), where it gave a t_R of 2.90 min. The visible absorption maxima (nm) were hexane, λ_{max} = (425), 449, 476; benzene, λ_{max} = (437), 460, 489. This band was identified as β-carotene.

Band 2. This band gave R_F = 0.57 on TLC and was purified by HPLC (system B), resulting in a t_R of 17.80 min. The visible absorption maxima (nm) were hexane, λ_{max} = 420, 447, 472; benzene, λ_{max} = 433, 456, 486. This band was identified as lutein.

Band 3 was identified as violaxanthin.

Band 4. This band gave R_F = 0.13 on TLC. This band was subsequently purified by semipreparative HPLC (system B), resulting in a t_R of 35.74 min. The visible absorption maxima (nm) were hexane, λ_{max} = 412, 435, 465; benzene, λ_{max} = 422, 447, 477. This band was identified as neoxanthin and was converted to neochrome by the addition of a few drops of ethanolic hydrogen chloride, with a hypsochromic shift of 16–17 nm. The visible absorption maxima (nm) of neochrome were hexane, λ_{max} = 400, 423, 450; benzene, λ_{max} = 407, 431, (460).

Pepper fruits. The TLC of a Belrubí (half ripening) pepper extract gave twelve bands, which were recovered and chromatographed by HPLC with the following results.

Band 1 was identified as β-carotene.

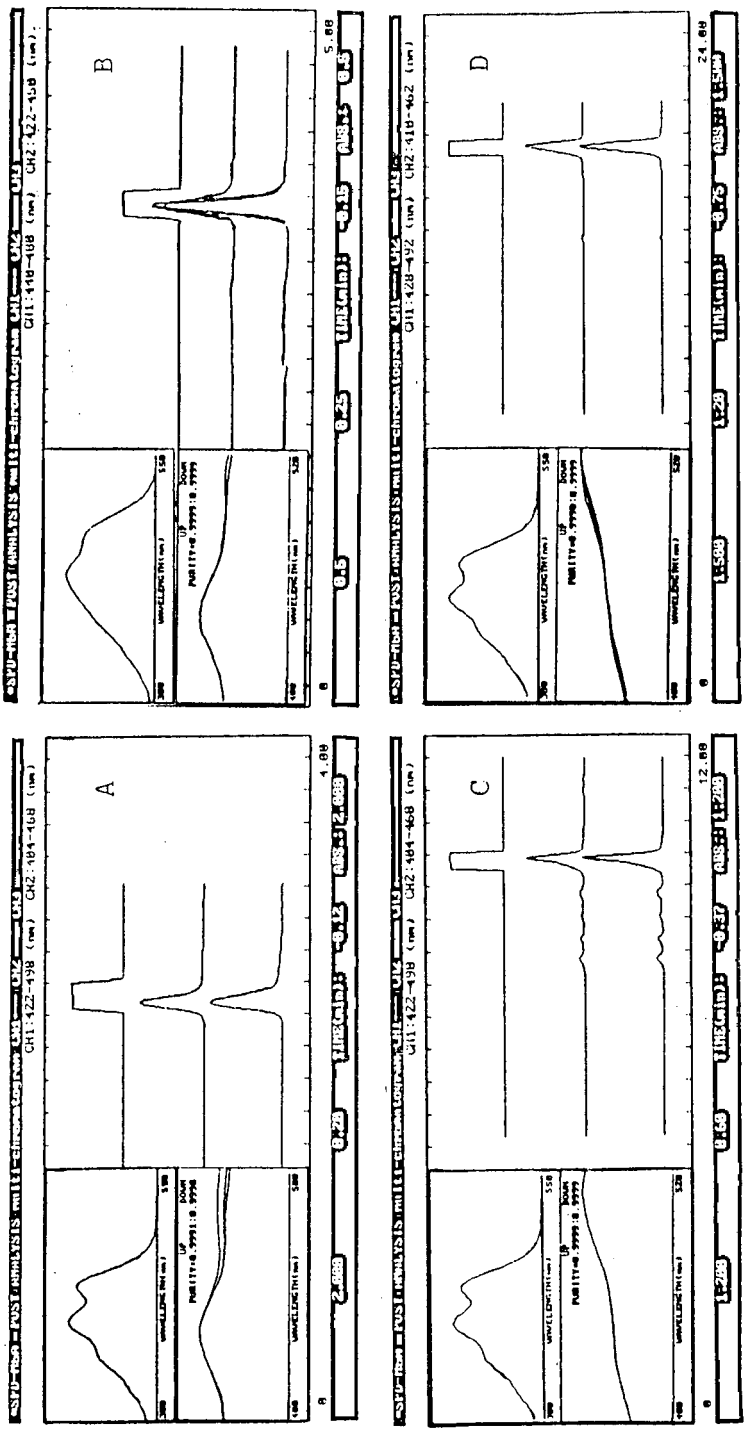
Band 2 had R_F = 0.93 on TLC. When purified by HPLC (system B) it resulted in two peaks with t_R of 3.46 and 4.65 min. The visible absorption maxima (nm) corresponding to the peak of t_R 3.46 min were hexane λ_{max} = (445), 469, 498; benzene, λ_{max} = 483, (518). This band was identified as cryptocapsin[10]. The visible absorption maxima (nm) corresponding to the peak of t_R 4.65 min were hexane λ_{max} = (402), 427, 451; benzene, λ_{max} = (408), 437, 464. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift of 19 nm. This band was identified as criptoflavin[10].

Band 3 was identified as β-cryptoxanthin.

Band 4 was identified as anteraxanthin.

Band 5 was identified as lutein.

Band 6 had R_F = 0.50 on TLC. Analysis by HPLC (system B) resulted in two peaks with t_R of 20.16 and 22.35 min. The visible absorption maxima corresponding to the peak of t_R 20.16 min were hexane, λ_{max} = 422, 444, 473; benzene, λ_{max} = 433, 458, 484; this band was identified as capsolutein. For the peak of t_R 22.35 min the visible absorption maxima (nm) were hexane, λ_{max} = 399, 422, 449; benzene, λ_{max} = 409, 432, 460. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift of 20 nm. This band was identified as luteoxanthin.


A
B
C
D

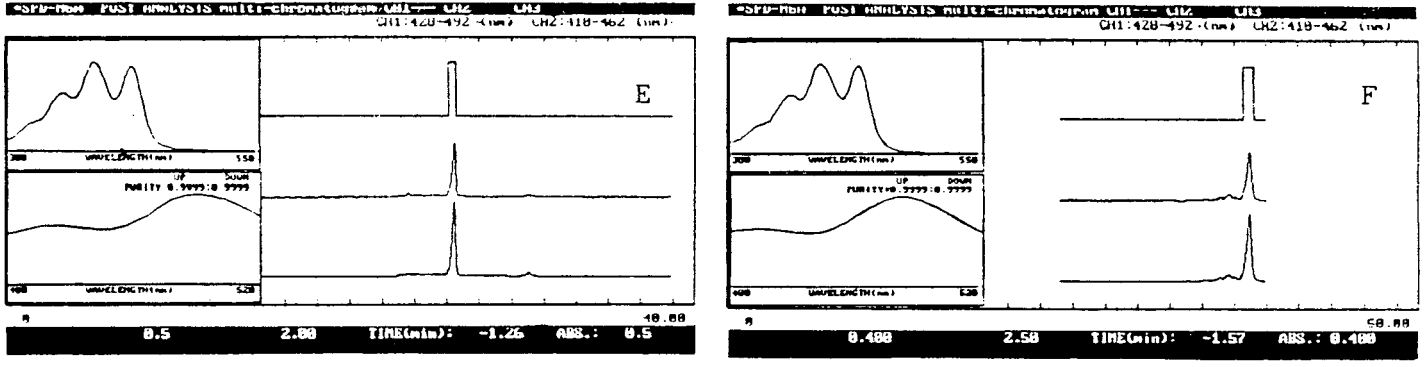


Fig. 1. Absorption spectra, absorbance ratios and purity of some carotenoids. (A) β-Carotene; (B) cryptocapsin; (C) antheraxanthin; (D) zeaxanthin; (E) violaxanthin; (F) neoxanthin.

Band 7 was identified as zeaxanthin.

Band 8 had $R_F = 0.28$ on TLC. Purification by HPLC (system B) resulted in a t_R of 24.82 min and the band was identified as mutatoxanthin. The visible absorption maxima (nm) were hexane, $\lambda_{max} = (406), 427, 453$; benzene, $\lambda_{max} = (415), 439, 466$. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift corresponding to 5,8-epoxy group.

Band 9 had $R_F = 0.24$ on TLC. Purification by HPLC (system B) resulted in two bands with t_R of 29.89 and 30.85 min. The band with $t_R = 29.89$ min was identified as capsanthin. The visible absorption maxima (nm) were hexane, $\lambda_{max} = 470, 497$; benzene, $\lambda_{max} = 483, 511$. The band with $t_R = 30.85$ min was identified as 5,6-epoxy-capsanthin. The visible absorption maxima (nm) were benzene, $\lambda_{max} = 479, (508)$. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift of 20 nm corresponding to 5,6-epoxy group.

Band 10 was identified as violaxanthin.

Band 11 had $R_F = 0.18$ on TLC. Purification by HPLC (system B) resulted in two peaks with t_R of 33.30 and 33.98 min. The band with $t_R = 33.30$ min was identified as capsorubin. The visible absorption maxima (nm) were hexane, $\lambda_{max} = 440, 467, 501$; benzene, $\lambda_{max} = 460, 488, 523$. The peak with $t_R = 33.98$ min had the same spectrum as capsorubin; it was identified as a capsorubin isomer.

Band 12 was identified as neoxanthin.

DISCUSSION

Once separated and purified by TLC and HPLC, the principal carotenoids responsible for the colour of the *Capsicum annuum* cv. Belrubí fruits were used as standards for normal-phase HPLC separation.

When the photodiode detector was used, the chromatographic software provided, among other data, the absorbance spectrum, the absorbance ratio and purity of each peak. Spectra were obtained using solvent system A. Fig. 1 shows the purity criteria for some of the pigments obtained.

To optimize the HPLC conditions, we tried different gradients with the solvents light petroleum (b.p. 40–60°C) and acetone in order to find the most suitable polarity for the separation of the different pigments. We finally adopted the elution gradient described previously (system A). Of all the bands appearing in the chromatogram, only capsanthin with its epoxide and capsorubin with its isomer are insufficiently separated. Nevertheless, the chromatographic integrator quantified them separately. Modifications of the chromatographic gradient in order to reduce the overlapping of these bands leads to very long analysis times with no significant improvement in resolution. Fig. 2 shows the chromatogram corresponding to this separation.

In Table I, the main chromatographic parameters corresponding to the separation defined by Kirkland[25] are summarized.

For products derived from *Capsicum annuum* the elution order of the individual carotenoids in the normal-phase column is almost the reverse of that obtained by other workers using a reversed-phase column[14].

Fig. 3 shows a chromatogram corresponding to the separation of carotenoids of a saponified extract of the fruits of *Capsicum annuum* cv. Belrubí, harvested completely ripened.

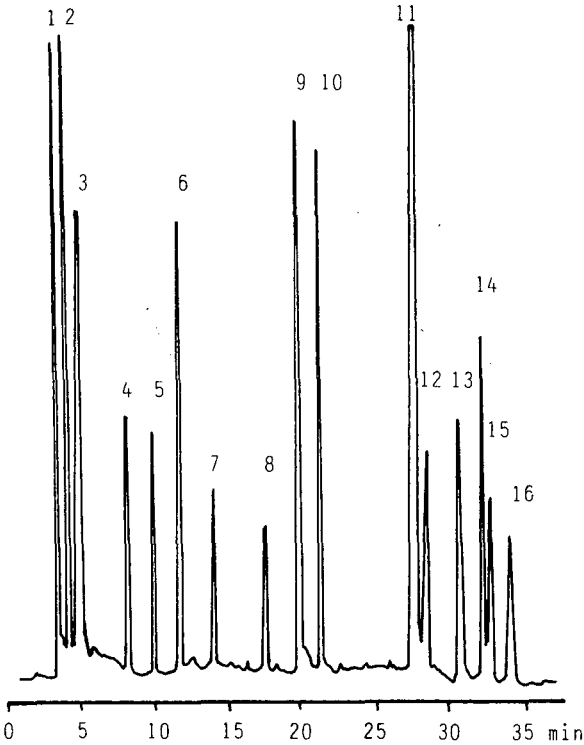


Fig. 2. Separation of carotenoids in a standard mixture (system A). Peak identification: 1 = β-carotene; 2 = cryptocapsin; 3 = cryptoflavin; 4 = β-cryptoxanthin; 5 = antheraxanthin; 6 = lutein; 7 = capsolutein; 8 = luteoxanthin; 9 = zeaxanthin; 10 = mutatoxanthin; 11 = capsanthin; 12 = capsanthin 5,6-epoxide; 13 = violaxanthin; 14 = capsorubin; 15 = capsorubin isomer; 16 = neoxanthin.

TABLE I

MAIN CHROMATOGRAPHIC PARAMETERS CORRESPONDING TO THE SEPARATION OF CAROTENOID PIGMENTS

Retention times were taken from the chromatographic integrator. Symbols: t = retention time (min), t' = reduced retention time ($t' = t - t_0$; dead time t_0 = 1.10 min), ω = band width (min), k' = capacity factor, α = separation factor, R_s = resolution.

Carotenoid	t	t'	ω	k'	α	R_s
β-Carotene	2.75	1.65	0.37	1.51	—	—
Cryptocapsin	3.36	2.26	0.40	2.05	1.36	1.58
Cryptoflavin	4.40	3.30	0.40	3.00	1.46	2.60
β-Cryptoxanthin	7.74	6.64	0.60	6.03	2.01	6.68
Antheraxanthin	9.56	8.46	0.83	7.70	1.27	2.54
Lutein	11.13	10.03	0.73	9.12	1.18	2.01
Capsolutein	13.73	12.63	0.67	11.50	1.26	3.71
Luteoxanthin	17.03	15.93	0.67	14.50	1.26	4.92
Zeaxanthin	19.58	18.48	0.67	16.80	1.16	3.80
Mutatoxanthin	21.39	20.29	0.67	18.45	1.10	2.70
Capsanthin	27.60	26.50	0.67	24.10	1.30	9.27
Capsanthin 5,6-epoxide	28.29	27.19	0.40	24.72	1.03	1.29
Violaxanthin	30.58	29.48	0.73	26.80	1.08	4.05
Capsorubin	32.12	31.02	0.67	28.20	1.05	2.20
Capsorubin isomer	32.48	31.38	0.50	28.53	1.01	0.62
Neoxanthin	33.81	32.71	0.60	29.74	1.04	4.93

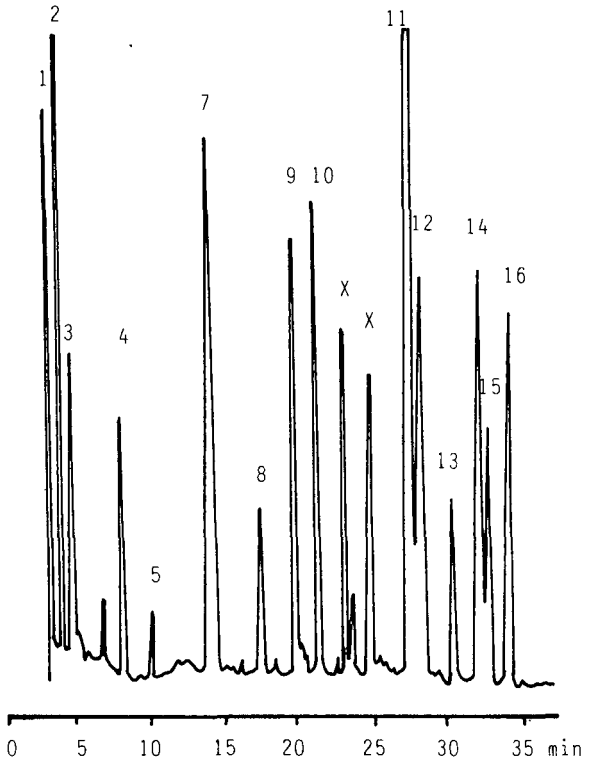


Fig. 3. Normal-phase HPLC of *Capsicum annuum* cv. Belrubí carotenoids. Peak numbers as in Fig. 2; × = unknowns.

Table II lists the distribution of the individual carotenoids, in the manner described in the text. The results obtained demonstrate the high content of carotenoid pigments in the *Capsicum* cultivar studied. It is therefore not surprising that the Belrubí cultivar has been chosen for its intense colour, which in its fresh fruit can be double that of other cultivars now used for the manufacture of paprika.

The proposed chromatographic system permits the identification of capsanthin epoxide, whose presence was questioned by Baranyai and Szabolcs[26], although recognized later when using reversed-phase HPLC[21]. In the pepper cultivars they used, the percentages of carotenoids did not differ essentially from our calculations, except that β-carotene increased at the expense of cryptocapsin, whereas capsorubin corresponded to the total capsorubin and capsorubin isomer calculated with our chromatographic system.

We did not find α-carotene in any of the ripened fruits of *Capsicum annuum*, which is in accordance with the results obtained previously with other varieties[27] and by other workers[28–30]. The previous work confirmed that the essential phenomenon taking place during ripening of pepper fruits is the disappearance of the β,ε-carotene series. The chromatographic system proposed now allows an adequate separation of α-carotene and β-carotene when both isomers are present.

The results obtained by Gregory *et al.*[22], who separated carotenoids acylated

TABLE II

QUANTITATIVE DISTRIBUTION OF *CAPSICUM ANNUUM* cv. BELRUBÍ CAROTENOIDS DETERMINED BY NORMAL-PHASE HPLC

Peak No.	*Carotenoid*	*Concentration (g/g fresh weight × 10^{-4})*	*Proportion (%)*
1	β-Carotene	2.75	6.72
2	Cryptocapsin	8.14	19.90
3	Cryptoflavin	1.09	2.66
4	β-Cryptoxanthin	0.79	1.93
5	Antheraxanthin	0.38	0.93
6	Lutein	–	–
7	Capsolutein	2.13	5.21
8	Luteoxanthin	0.85	2.07
9	Zeaxanthin	1.25	3.06
10	Mutatoxanthin	1.64	4.01
11	Capsanthin	12.07	29.51
12	Capsanthin 5,6-epoxide	2.16	5.28
13	Violaxanthin	0.98	2.40
14	Capsorubin	1.79	4.38
15	Capsorubin isomer	1.38	3.37
16	Neoxanthin	1.74	4.25
Unidentified pigments (as β-carotene)		1.77	4.32
Total		40.91	100.00
Red pigments		25.54	62.43
Yellow pigments		15.37	37.57

with fatty acids, demonstrated a concentration of pigments in the form of capsanthin, capsorubin and β-carotene ester. This suggests that in non-saponified extracts, the lipid part of the molecule predominates in the separation and when the esters of different carotenoids are eluted together, the components present in smaller amount are masked by the larger amounts in such a way that the proportion of the latter appears even greater.

To summarize, the chromatographic system described permits a good separation and quantification of individual carotenoid pigments. The method is applicable to the study of the contents and evolution of these pigments in the fresh fruit of *Capsicum annuum* cv. and their commercial products.

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Polyphenols of *Quercus robur* L.

II[a]. Preparative isolation by low-pressure and high-pressure liquid chromatography of heartwood ellagitannins

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SUMMARY

Heartwood of *Quercus robur* L. contains about 10% (w/w), of hexahydroxydiphenoyl esters (ellagitannins). Five of the most abundant hexahydroxydiphenoyl esters were purified by a combination of chromatography on Sephadex LH-20 and high-performance reversed-phase liquid chromatography. Two of them were identified as castalagin and vescalagin.

INTRODUCTION

Heartwoord of *Quercus robur* L. (pedonculate oak) is known to contain about 10% (w/w) of hexahydroxydiphenoyl esters (HHDP esters)[1]. These HHDP esters, also known as ellagitannins, are responsible for the high durability of this wood[2]. They also contribute to the taste and the colour of brandies and wines aged in oak barrels[3–5].

The two main HHDP esters of *Q. robur* heartwood, castalagin and vescalagin (Fig. 1), were characterized by Mayer *et al.*[6] more than 20 years ago. However, the presence of at least six other HHDP esters in this wood was reported recently[1]. In order to determine their structures, their purification was undertaken.

Several methods have previously been used for the purification of hydrolysable tannins; the most widely used technique has been chromatography on Sephadex LH-20. High-performance liquid chromatography (HPLC) has also been used for the separation of hydrolysable tannins[1,7,8], although seldom on a preparative scale[7,9]. We have shown previously that HHDP esters from oak wood extracts were well

[a] For Part I, see ref. 1

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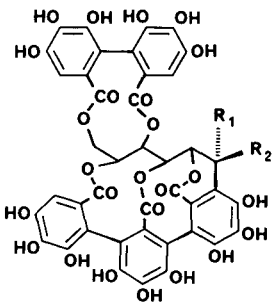

Fig. 1. Structures of castalagin (R_1 = H; R_2 = OH) and vescalagin (R_1 = OH; R_2 = H).

separated by reversed-phased HPLC, although they were poorly resolved by silica chromatography or paper chromatography[1]. For this reason, we undertook their purification by a combination of preparative reversed-phase HPLC and Sephadex LH-20 chromatography. The influence of some parameters on the quality of the separation is detailed.

EXPERIMENTAL

Materials

Heartwood was obtained from a branch 25 cm in diameter, cut from a 100-year-old, freshly felled pedonculate oak (*Q. robur*). The wood was dried at room temperature and ground in a Retch SM 1 rotating-knife grinder (particle size less than 60 mesh).

The water used for chromatography was deionized [Milli-Q (Millipore)]. Methanol was of RPE grade (Carlo Erba) and all other solvents of R. P. Normapur grade (Prolabo); acetone was redistilled before use. For HPLC, all solvents were ultrafiltered (modified cellulose, 0.2 μm; Sartorius) and degassed daily under vacuum.

Solvents were eliminated under reduced pressure in a rotary evaporator at 40°C. Water in concentrated solutions was eliminated by lyophilization, yielding easily recovered powders.

Extraction of HHDP esters

Wood powder (1 kg) was extracted with methanol–water (4:1)[10] (2 l for 8 h, then 1 l for 18 h) with magnetic stirring at room temperature.

The mixture was filtered on a Büchner funnel and methanol was eliminated under reduced pressure. The residual solution was successively extracted with light petroleum (b.p. 35–60°C) (7 × 250 ml), diethyl ether (7 × 250 ml) and ethyl acetate (7 × 250 ml). All the extracts were dried. Yields were typically 0.5, 7.4, 11.9 and 98.3 g for light petroleum, diethyl ether, ethyl acetate and water, respectively. HHDP esters (positive reaction with nitrous acid[11]) were present mainly in the water extract and to a lesser extent in the ethyl acetate extract.

Two-dimensional paper chromatography

Samples were eluted on Whatman No. 1 paper (12.5 × 12.5 cm^2) with 6% acetic acid for the first dimension and *sec.*-butanol–acetic acid–water (14:1:5) for the

second dimension. Polyphenols (*o*-dihydric phenols) were revealed by spraying with $FeCl_3$–$K_3Fe(CN)_6$ reagent[12].

Thin-layer chromatography on silica gel

Silica layers on aluminium foils (60 F_{254}, 7 × 7 cm^2; Merck) were eluted with ethyl acetate–formic acid–water (7:2:1, v/v/v). To reduce streaking, the layer was preconditioned, before application of samples and development, by spraying the eluent and rapid drying with a hair dryer.

Analytical HPLC

A Gilson apparatus composed of two pumps (303 and 305), equiped with 5 SC pumps heads, an 811 dynamic mixer, an 803C manometric module and a Holochrome detector (280 nm; cell volume 11 μl; path length 10 mm) was used with a Merck LiChrospher RP-18e (5-μm) column (25 cm × 4 mm I.D.). The elution conditions were as follows: linear gradient, 0–10% solvent B from 0 to 40 min, solvent A = water–phosphoric acid (990:1, v/v), solvent B = methanol–phosphoric acid (990:1, v/v); flow-rate, 1 ml/min. UV spectra (240–400 nm) were obtained by on-line detection with a Hewlett-Packard 1040A diode-array detector.

Chromatography on Sephadex LH-20

Aqueous extracts (10 or 100 g dissolved in a minimum volume of water, *i.e.*, 20–50 ml) were loaded on an 80 cm × 25 mm I.D. Sephadex LH-20 (100 μm; Pharmacia) column. A peristaltic pump delivered a water–methanol mixture at a flow-rate of 5 ml/min. The concentration of methanol was increased stepwise (20% increments). Ascorbic acid (1 g per litre of water) was added to limit the oxidation of polyphenols. Fractions of 50–500 ml were collected. Each fraction was analysed by high-performance thin-layer chromatography on cellulose (Merck). The same eluents as described for two-dimensional paper chromatography were used. Chromatography was run in a linear development chamber (Camag). Similar fractions were collected, dried under reduced pressure and lyophilized. Their degree of purification was controlled by analytical HPLC and two-dimensional paper chromatography.

Preparative HPLC

The same Gilson equipment was used with 25 SC pump heads and a preparative cell (volume 2 μl; path length 5 mm). The solvents were the same as for analytical HPLC. A Hibar LiChrospher RP-18, 7-μm column (25 cm × 25 mm I.D.) (Merck) was used with a precolumn 3 cm × 4 mm I.D. filled with LiChrospher RP-18, 10-μm (Merck). Samples were dissolved in water and filtered on a Millex SR 0.5-μm filter (Millipore). According to the mixture to be purified, up to 400 mg can be introduced into the 2-ml loop. Elution was conducted in the isocratic mode with 4 or 6% B, depending on the retention time of the compounds on the column. The flow-rate was 20 ml/min. The rising, top and decreasing parts of the peaks were collected separately and their purity was monitored by analytical HPLC.

The fractions obtained were concentrated under reduced pressure to eliminate the methanol, and adsorbed on a small Sephadex LH-20 column (5 cm × 1.5 cm I.D.) to eliminate phosphoric acid which was eluted with water. As soon as the pH was neutral (monitored with pH paper), the adsorbed HHDP esters were eluted with

methanol. The presence of the HHDP esters in the eluent was followed by reaction with $FeCl_3$–$K_3Fe(CN)_6$ reagent. Solutions were dried under reduced pressure and residual water was eliminated through repeated washings with acetone. Samples were solubilized in [$^2H_6$]acetone or, if not soluble enough, in [$^2H_4$]methanol, for ^1H NMR spectroscopic analysis (Cameca 250-MHz or Bruker AM 400-MHz spectrometer).

RESULTS AND DISCUSSION

Analytical HPLC

Compared with other HHDP esters, oak wood tannins are highly polar compounds which cannot be properly separated on silica or cellulose. On the other hand, reversed-phase HPLC affords an easy separation, although several parameters had to be determined in order to maximize the retention on the column, thus allowing a better separation.

Several columns were compared. Retention was higher on RP-18 columns than on RP-8 columns (Fig. 2a and b). Very different performances were obtained depending on the commercial origin of the columns. Lichrospher columns provided the best separations; columns with 5-μm particle size material gave a higher number of peaks

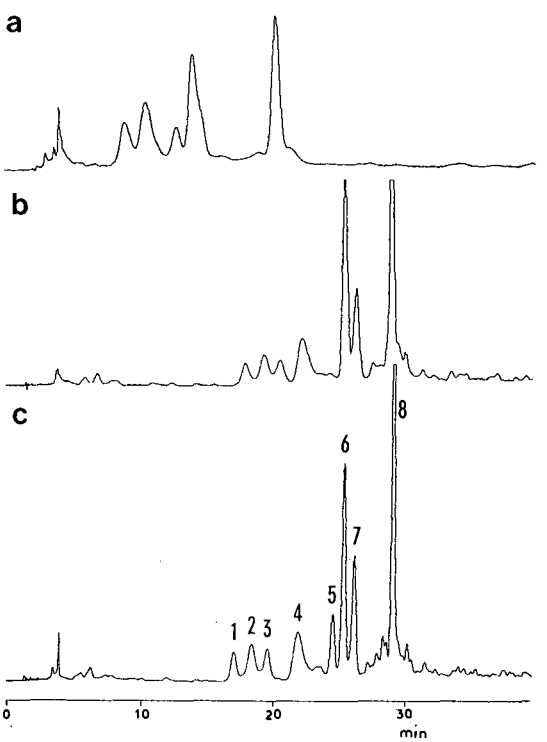

Fig. 2. HPLC of an aqueous extract of *Quercus robur* L. heartwood on different reversed-phase columns (25 cm × 4 mm I.D.). (a) LiChrospher 100-CH8, 10 μm; (b) LiChrospher RP-18, 10 μm; (c) LiChrospher RP-18, 5 μm. See text for elution conditions. Numbers 1–8 refer to the different HHDP esters identified in the extract.

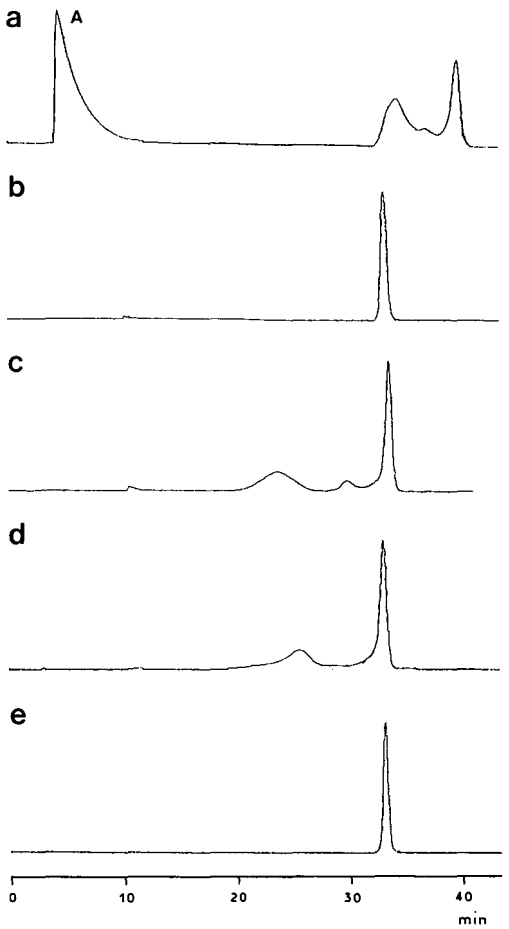

Fig. 3. HPLC of castalagin solubilized in different solvents before injection. (a) Acetone; (b) same sample as (a) dried and resolubilized in water; (c) methanol; (d) same sample as (c) to which is added water (one tenth of its volume); (e) same sample as (c) dried and resolubilized in water. See text for elution conditions. Peak A = acetone.

than 10-μm particle size material, regardless of flow-rate (Fig. 2b and c). The nature of the acid in the eluent also affected the retention times. Phosphoric acid (1%, v/v) doubled the retention times compared with acetic acid (1%, v/v).

The solvent used to solubilize the sample also had a strong influence on the chromatographic profile. When 10 μl of a solution of a pure HHDP ester in acetone (Fig. 3a) or methanol (Fig. 3c) was injected, several peaks were observed. The relative area of the extra peaks was reduced on addition of water to the sample solution (Fig. 3d). If the organic solvents were completely removed by evaporation, and the same sample was then resolubilized in water, a single peak was observed (Fig. 3b and e). Therefore, it was necessary to dissolved these HHDP esters in water and not in organic solvents, in order to obtain an optimum analysis with the present system. These observations could be due to reversible autoassociation of these very polar

tannins, whose solubility in organic solvents is limited. Solvation by water would induce the dissociation of these soluble complexes[13].

Chromatography on Sephadex LH-20

Sephadex LH-20 has been widely used to purify tannins. Ethanol and methanol, usually in mixtures, are the most widely used eluents. However, oak heartwood tannins are not sufficiently soluble in alcohols, and water followed by methanol–water mixtures were preferred. Similar gradients of methanol in water have been used previously for ellagitannins[14] or proanthocyanidins[15]. We encountered difficulties with this purification step, owing to extensive oxidation of the HHDP esters, as the chromatography lasts several weeks and the volume of eluents occasionally exceeded

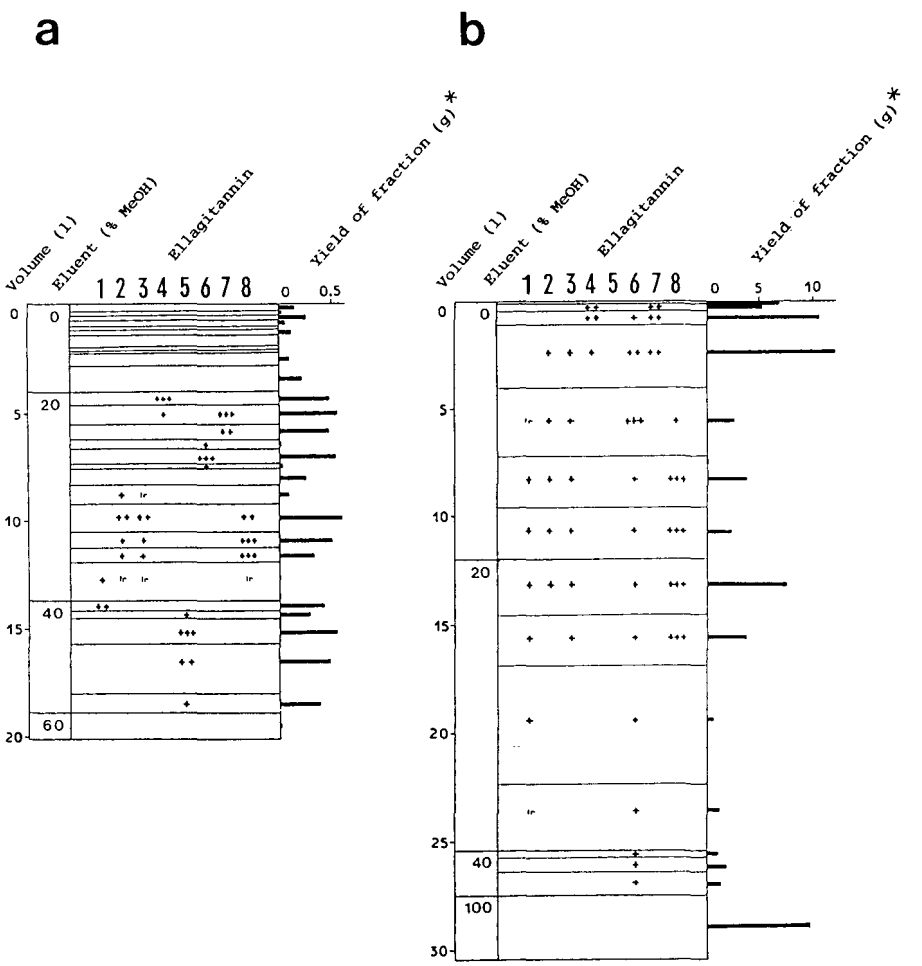

Fig. 4. Chromatography on Sephadex LH-20 of an aqueous extract of *Quercus robur* L. heartwood: (a) 10 g and (b) 100 g of extract loaded on the column. tr (traces), +, + + and + + + refer to the relative abundances of the eight main compounds (1–8, Fig. 2c) as observed by HPLC. (*) Mass of the ascorbic acid in the eluent has been deduced. MeOH = Methanol.

25 l. The addition of ascorbic acid as an antioxidant in the eluent[16] improved the purification yields substantially. Ascorbic acid has the advantage of being easily eliminated by preparative HPLC in the next step of purification, as it is not retained on the reversed-phase column. Further, its presence in the fraction is easily monitored by UV detection (see following section).

Part of the extract (7–9%) was not retained on the column, and was eluted with water before the main HHDP esters (Fig. 4). These fractions were also poorly retained on the reversed-phase HPLC column. They consist of a complex mixture of products and were not further purified.

Most of the extract (60%) was eluted after these compounds and corresponded to the various peaks (1–8, Fig. 2) observed in HPLC (some of the fractions were selected for further purification by preparative HPLC). The quality of the separation of these products was strongly affected by the amount of extract injected (Fig. 4). When 100 g of extract, instead of only 10 g, were loaded on the column, although the order of elution was not modified, the products were more easily eluted with lower concentrations of methanol in the eluent. However, in most instances, the resolution was so low that it became very difficult to obtain pure products from these fractions by preparative HPLC.

Other HHDP esters (3–4% of the extract) were eluted from the gel with concentrations of methanol in the eluent of 60% or higher. These HHDP esters were not resolved by HPLC and appeared as a streak on two-dimensional paper chroma-

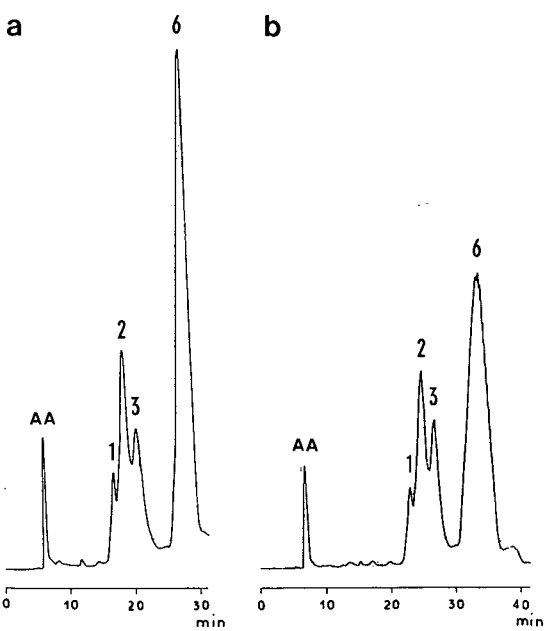

Fig. 5. Preparative HPLC of a fraction obtained after chromatography on Sephadex LH-20. (a) Isocratic elution, 2% B from 0 to 20.4 min, 5% B from 20.4 min; (b) gradient elution, 0 to 5% B from 0 to 40 min. Amount of sample injected, 200 mg. See text for other elution conditions. Numbers refer to the components of the aqueous extract of *Quercus robur* L. heartwood (Fig. 2c). AA = Ascorbic acid.

tograms. These fractions, which were more intensely coloured than the previous ones, probably correspond to partially oxidized (and polymerized) HHDP esters.

The total amount of products recovered in the fractions never exceeded 70% of the extract (Fig. 4). Most of what remained bound to the gel was removed by elution with 0.1 *M* sodium hydroxide solution.

Preparative HPLC

As analytical HPLC with reversed-phase columns afforded good separations of HHDP esters, it was tempting to use this technique on a preparative scale. The same solvents were used and different elution conditions were compared. In difficult separations, no better resolution was obtained with gradient elution than with isocratic

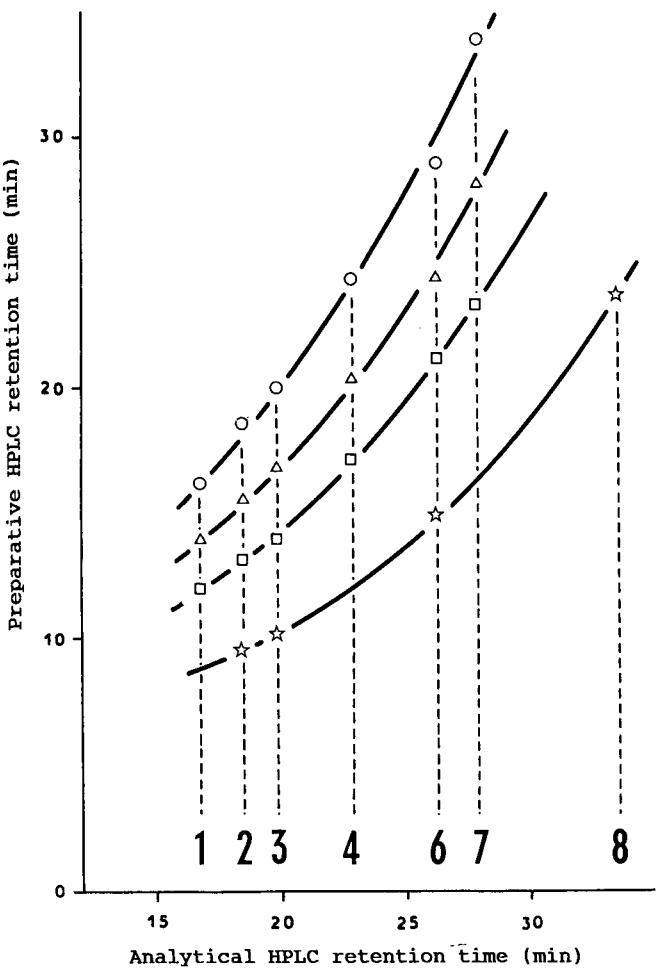

Fig. 6. Dependence of the preparative HPLC retention times on the analytical HPLC retention times for various compositions of the eluent used in preparative HPLC: (○) 2% B; (△) 3% B; (□) 4% B; (☆) 6% B. Amounts of sample injected into the preparative column, 1–2 mg. Numbers refer to the main components of aqueous extract of *Quercus robur* L. heartwood (Fig. 2c). See text for elution conditions.

elution (Fig. 5). Here, gradient elution cannot improve the resolution as it does for analytical HPLC, probably because of the high loading of the column.

Isocratic elution was therefore used and the concentration of methanol in the eluent was chosen so as to obtain a satisfactory separation without increasing the volume of the fractions collected too much. A relationship could be established between the retention times observed by analytical HPLC and preparative HPLC (Fig. 6). The concentration of methanol in the eluent was varied between 4 and 6% in order to obtain retention times that did not exceed 25 min.

As with Sephadex LH-20, the retention times were affected by the amounts of sample loaded on the column. The retention times were reduced by about 20% when

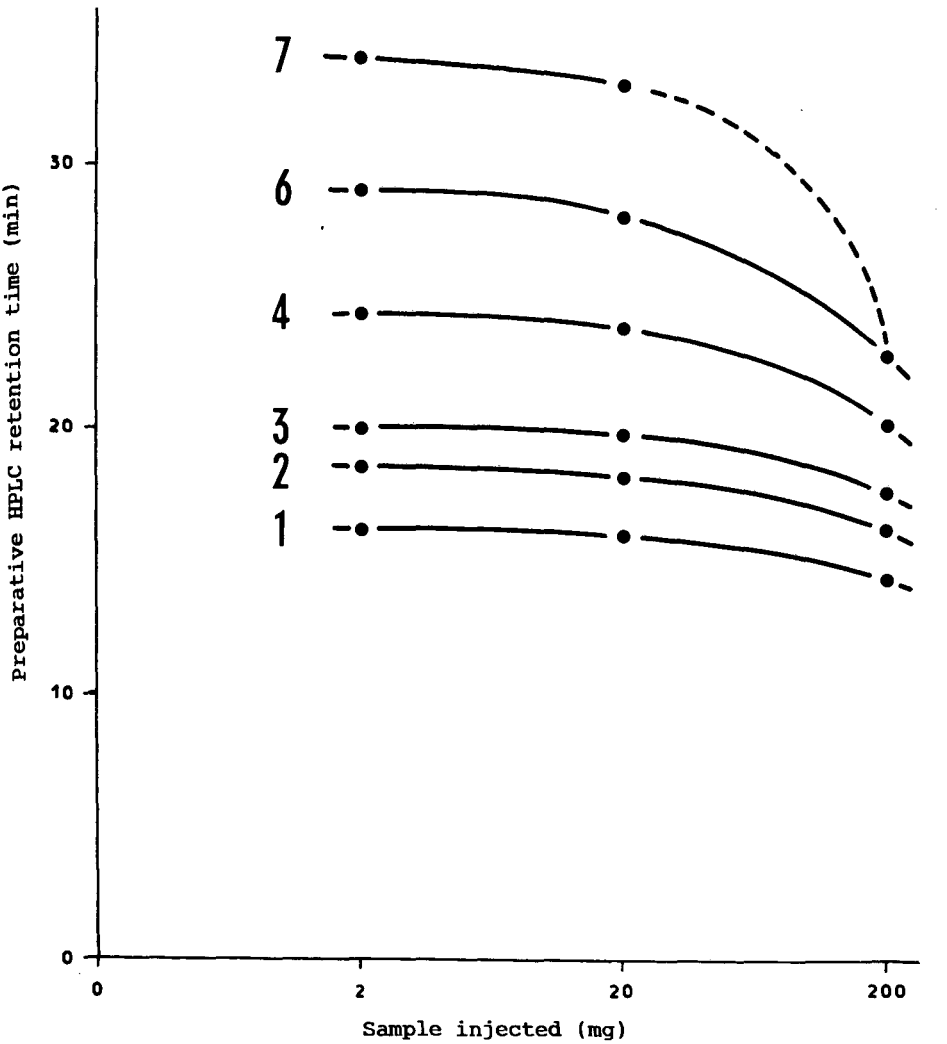

Fig. 7. Dependence of the preparative HPLC retention times on the amount of sample injected. The sample consists of a Sephadex fraction containing compounds 1, 2, 3, 4, 6 and 7 of the aqueous extract of *Quercus robur* L. heartwood (Fig. 2c). Eluent: 2% B.

the sample size was increased from 2 to 200 mg (Fig. 7). In some instances the resolution was severely affected by too high a loading of the column (in the example shown in Fig. 7, peak 7 is no longer resolved from the major peak 6 when the amount of sample injected exceeds 20 mg). Depending on the complexity of the mixture and the ease of separation, between 100 and 400 mg could usually be chromatographed in one run.

As with analytical HPLC, the solvent used to dissolve the sample before injection had a strong influence on the chromatographic profile. The sample had to be introduced in water in order to avoid the formation of multiple peaks.

Addition of an acid to the eluent is recommended in order to avoid ionization of the phenols, unless the peaks are considerably flattened and no reproducible retention times are obtained. The acid in the fractions collected must then be removed before concentration of the sample. Otherwise, the HHDP esters are rapidly hydrolysed and a precipitate of ellagic acid is observed. In order to remove phosphoric acid, HHDP esters were adsorbed on a column. Two different gels were examined: Sephadex LH-20 and Sep-Pak C_{18} (Millipore). The column was washed with water until neutrality was obtained and tannins were desorbed with methanol. HHDP esters were not retained strongly enough on Sep-Pak C_{18} and were partially eluted with water before reaching neutrality (Fig. 8). On the other hand, chromatography on Sephadex LH-20 allowed the total recovery of HHDP esters in methanol.

Purity of the fractions obtained

The purity of the different fractions obtained after preparative HPLC was determined by different chromatographic methods: two-dimensional paper chromatography, thin-layer chromatography on silica and HPLC. After separation, only one spot or one peak was observed for each of the eight main peaks on the HPLC trace of the raw extract (Fig. 9, Table I). The products corresponding to the eight peaks had

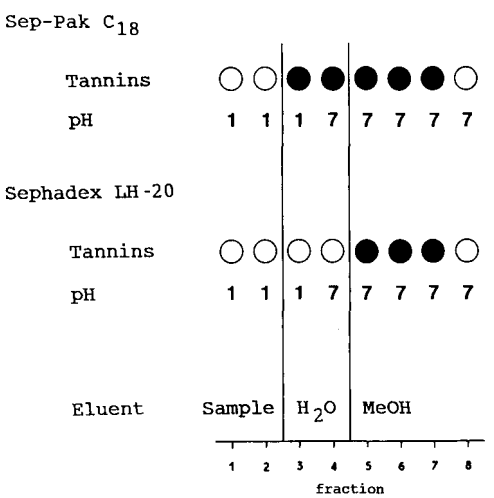

Fig. 8. Adsorption of HHDP esters on Sep-Pak C_{18} and Sephadex LH-20 for elimination of phosphoric acid. The presence (●) or absence (○) of HHDP esters is determined by their reaction with $FeCl_3$–$K_3Fe(CN)_6$ reagent. Volume of fractions, 20 ml. MeOH = Methanol.

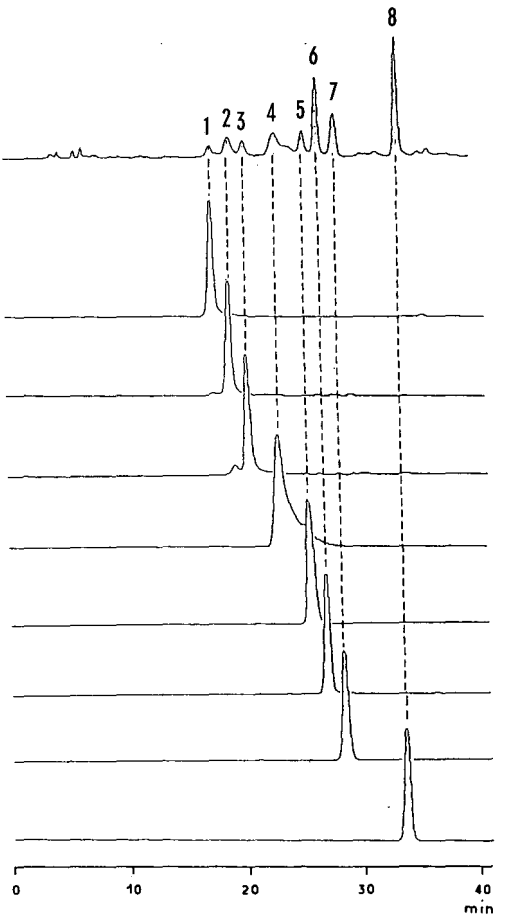

Fig. 9. HPLC of the fractions obtained from aqueous extracts of *Quercus robur* L. heartwood after chromatography on Sephadex LH-20 and preparative HPLC. The corresponding chromatogram of the raw aqueous extract is shown for reference.

TABLE I

YIELDS AND R_F VALUES OF THE COMPOUNDS PURIFIED FROM *Q. ROBUR* HEARTWOOD

Parameter	*Peak No.*[a]							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Yield (g/kg)	0.4	0.3	0.4	3.0	1.3	3.1	1.7	1.1
Paper chromatography, R_F:								
Acetic acid (6%)	0.38	0.45	0.55	0.56	0.37	0.57	0.60	0.43
sec.-Butanol–acetic acid–water (14:1:5)	0.00	0.00	0.00	0.03	0.00	0.06	0.03	0.06
Silica thin-layer chromatography, R_F	0.31	0.18	0.18	0.45	0.37	0.65	0.46	0.65

[a] See Fig. 2c.

identical UV spectra, showing no maxima between 240 and 400 nm, but a shoulder at 280 nm. They all gave the characteristic colour of HHDP esters when sprayed with nitrous acid.

Each fraction was analysed by ^{1}H NMR spectroscopy. Spectra were run in deuterated acetone, or in deuterated methanol if the sample was not soluble enough in acetone (peaks 2 and 3, Fig. 9). Compounds were considered pure when all the integrals of the different protons were similar. This was the case for five of the eight peaks (1, 3, 5, 6 and 8, Fig. 9). Among the pure compounds obtained, two (peaks 6 and 8) had spectra identical with those reported by Mayer *et al.*[6] and could be identified as vescalagin and castalagin, respectively. The other compounds, whose structures have not yet been reported, are in the process of being characterized. The other three peaks (2, 4 and 7) may require further purification as the integrals were not all similar. Chromatography on Fractogel (Merck) or speed flow counter-current chromatography, techniques already used by other workers[17,18], may complete the purification.

The yields calculated for 1 kg of wood extracted are given in Table I. The lower yields (peaks 1, 2 and 3) correspond to the minor products in HPLC trace of the raw extract. Vescalagin (peak 6), which was one of the two most important peaks in the HPLC trace of the raw extract, gave the best yield. However, the yields depended not only on the initial concentration of the compounds in the raw material, but also on the dispersion of each compound in one or several fractions after each purification step. This explains why the yields of some compounds are relatively small compared with what was expected.

CONCLUSIONS

Chromatography on Sephadex LH-20 and preparative HPLC have been shown to be complementary methods for the purification of HHDP esters, even for compounds with similar polarities. Purification of the HHDP esters is no longer limited to the one or two main compounds present in plant materials.

It is now widely accepted that the properties of HHDP ester, notably their astringency, are strongly dependent on their structure[13]. Hence the modern purification methods now available to the biologist should lead to a better understanding of the biological and physico-chemical properties of plant materials rich in HHDP esters which are not necessarily determined by the most abundant compounds.

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Ion chromatography with ultraviolet and amperometric detection for iodide and thiocyanate in concentrated salt solutions

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SUMMARY

An ion chromatographic system with ultraviolet (UV) and amperometric (AMP) detectors is described for the highly sensitive detection of iodide and thiocyanate. Solutions of four inorganic salts as mobile phases and three stationary phases were examined. The optimum separation was achieved by using a polymethacrylate-based anion-exchange column and 0.1 *M* sodium chloride–5 m*M* sodium phosphate buffer (pH 6.7) as the mobile phase. Conditions were established for UV and AMP detection on a electrochemically pretreated glassy carbon electrode. The method, which has the ability to eliminate most interferences from other anions, could be applied to the direct determination of ppb levels of iodide in sea water.

INTRODUCTION

Ion chromatography, introduced by Small *et al.*[1], has become one of the most important methods for the determination of inorganic anions[2]. This method makes possible the rapid separation and highly sensitive measurement of analyte ions. However, for a conventional ion chromatographic system using a polystyrene-based anion-exchange resin and a conductivity detector, both iodide and thiocyanate, with low hydration energies, are generally associated with long retention times and severe tailing of the anion peaks, owing to the strong affinity of the anions to the resin[1–3].

In order to solve this problem, many methods have been considered with combinations of various mobile phases, columns and detectors. The methods can be mainly classified into two groups, as follows. (1) One aims to increase the ionic strength in the mobile phase[4–9]. For single-column ion chromatography with conductivity detection, however, the ionic concentration in a mobile phase is limited to very dilute solutions as the detector shows responses to all ions[2]. For dual-column systems, the ionic strength is limited to the capacity of the suppressor[1–3]. Hence it is necessary to use other detectors with high sensitivity for iodide and thiocyanate and to select a mobile phase with high concentrations that do not show a response on the

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detectors. (2) The other approach is to use different kinds of columns. (a) For ion-exchange chromatography, the use of a silica-based resin does not result in tailing of the anion peaks[10,11]. The use of a polymer-based resin is accompanied by longer retention times, thus requiring an increase in ionic strength in the mobile phase. (b) For ion-interaction chromatography (or ion-pair chromatography)[12–17], sharp peaks with good resolution can be obtained by using reversed-phase alkyl-chain bonded silica or polystyrene where ion-interaction reagents and counter ion additives are added to mobile phases consisting of aqueous or aqueous–organic solutions.

This work concentrates on the first category of method. Ion chromatographic systems combining mobile phases with high ionic strength and ultraviolet (UV) and amperometric (AMP) detectors were studied for the separation and detection of iodide and thiocyanate. The use of such mobile phases has the advantage of almost eliminating the interference of faster eluting species from the analyte ions. The separations were examined for mobile phases of four inorganic salts and three ion-exchange columns. Solutions of nitrate salts were generally used as mobile phases[3,4,8,9]. However, as the solutions, which are very effective for amperometric detection systems, show strong UV absorption, it is desirable to use other mobile phases that do not respond to both ultraviolet and amperometric detectors. We previously reported on ion chromatography with amperometric detection of iodide using 0.1 *M* sodium chloride solution as the mobile phase[7]. The detection of iodide and thiocyanate has been achieved by the use of ultraviolet spectrophotometry[10–13,15–17], conductimetry[14], amperometry [platinum[4], glassy carbon (GC)[7,17] and silver[3,8,9] working electrodes] and potentiometry[5,6]. This paper describes a detection system for iodide and thiocyanate with ultraviolet spectrophotometry and amperometry (GC working electrode), which have high sensitivities for these ions, thus providing the optimum ion chromatographic system.

EXPERIMENTAL

Apparatus

The ion chromatographic system consisted of a computer-controlled pump (CCPM; Tosoh), a Rheodyne 7125 injection valve equipped with a 100-μl sample loop, a UV spectrophotometric detector (L-4200; Hitachi), an amperometric detector (VMD-101A and P-1000; Yanagimoto) and a dual-pen strip-chart recorder. The amperometric detection system uses a three-electrode potentiostat: a thin-layer flow system with a glassy carbon (GC) working electrode, a stainless-steel counter electrode and an Ag/AgCl reference electrode were used.

Three anion-exchange columns (with quaternary ammonium groups) were used: TSKgel IC-Anion-PW (50 × 4.6 mm I.D.) (Tosoh) and, for comparison, TSKgel IC-Anion-SW (50 × 4.6 mm I.D.) (Tosoh) and Yokogawa AX-1 [(50 + 250) × 4.6 mm I.D.] (Yokogawa). The flow-rate was maintained at 1.2 ml/min for TSKgel IC-Anion-PW and TSKgel IC-Anion-SW and at 2.0 ml/min for Yokogawa AX-1. Separations and measurements were carried out at *ca.* 23–25°C.

Reagents and mobile phase

All reagents were of analytical-reagent grade. Standard anion solutions were prepared from stock solutions (10–50 g/l), obtained by dissolution of the correspond-

ing sodium salts. Mobile phases of inorganic salt solutions containing 5 m*M* sodium phosphate buffer (pH 6.7) were prepared from stock solutions of 2 *M* NaCl, 0.6 *M* $NaNO_3$, 1.0 *M* Na_2SO_4, 0.25 *M* Na_2HPO_4, and 0.25 *M* NaH_2PO_4. Mobile phases of sodium phosphates were prepared from equimolar (0.25 *M*) Na_2HPO_4 and NaH_2PO_4. All solutions were prepared in distilled, deionized water and filtered through a 0.45-μm membrane filter (made of cellulose nitrate) before use.

Pretreatment of a GC working electrode

It has generally been recognized that somehow the electrochemical treatment of a GC electrode tends to enhance and stabilize the electrode response of electroactive compounds[18,19]. The following procedures were adopted in this study. The electrode was resurfaced to a mirror-like condition by polishing with a diamond compound (particle size, 1 μm) and rinsed with distilled, deionized water after the removal of residual polishing compound by ultrasonic treatment in a water-bath for at least 5 min. The electrode was then electrochemically treated by repeated anodization at +1.6 V (*vs.* Ag/AgCl) for 5 min followed by cathodization at −1.0 V for 2 min under a flow (0.5 ml/min) of 0.5 *M* sodium phosphate buffer (pH 6.7).

RESULTS AND DISCUSSION

Mobile phase

Fig. 1 shows the variation of the retention volumes of iodide and thiocyanate with the concentration of inorganic salts in the mobile phase. Separation was performed on a TSKgel IC-Anion-PW. The solutions of NaCl, $NaNO_3$ and Na_2SO_4

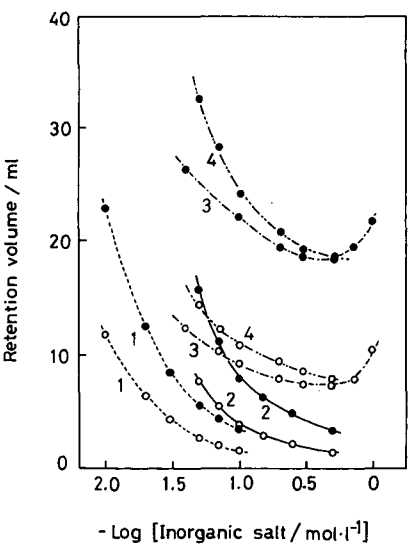


Fig. 1. Retention volumes of (○) I^- and (●) SCN^- as a function of the concentration of inorganic salts in the mobile phase. Mobile phase: 1 = $NaNO_3$; 2 = NaCl; 3 = Na_2SO_4; 4 = NaH_2PO_4–Na_2HPO_4; mobile phases 1–3 contain 5 m*M* sodium phosphate buffer (pH 6.7). Column: TSKgel IC-Anion-PW. Flow-rate: 1.2 ml/min.

contained 5 m*M* sodium phosphate buffer in order to stabilize the response of the amperometric detector. Although the eluting powers are relatively weak for the inorganic salt solutions in this study, it is possible to elute iodide at reasonable retention times using all the mobile phase systems with higher concentrations. The peaks of iodide for NaCl and $NaNO_3$ mobile phases are sharp compared with those for Na_2SO_4 and NaH_2PO_4–Na_2HPO_4. For Na_2SO_4 and NaH_2PO_4–Na_2HPO_4, in spite of an increase in concentration, the chromatograms of thiocyanate have longer retention times and show tailing of peaks, thus making it difficult to determine trace levels of thiocyanate. The effect of added salts in reducing the analyte retention follows the order $NO_3^- > Cl^- > SO_4^{2-} > H_2PO_4^-$–$HPO_4^{2-}$. Further, the effect remains constant over the ionic concentration range studied, except for SO_4^{2-} at high concentration. For SO_4^{2-}, the retention volumes of iodide and thiocyanate show a minimum value in the vicinity of 0.4 *M*. This suggests that the increase in the affinity of iodide and thiocyanate to the resin, due to an increase in the ionic strength of the mobile phase, is larger than the mass-action (ion-exchange) effect of SO_4^{2-} in reducing retention. Thus, reasonable retention times and sharp peaks of the analyte ions were obtained at 0.03 *M* $NaNO_3$ and 0.1 *M* NaCl.

Column

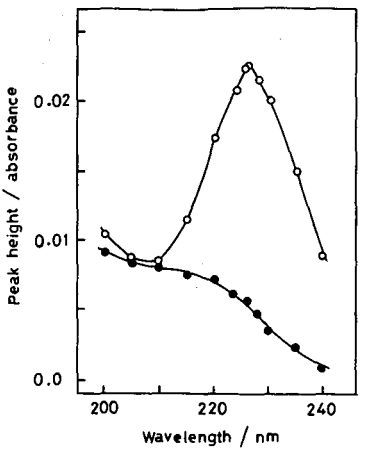
The effect of the stationary phase on analyte retention was studied using a mobile phase of 0.1 *M* NaCl–5 m*M* sodium phosphate buffer (pH 6.7). Table I shows the retention volumes for the three columns. Both TSKgel IC-Anion-PW and TSKgel IC-Anion-SW gave shorter retention volumes and sharper peaks. They also gave the same elution order ($IO_3^- < NO_2^- < NO_3^- < I^- < SCN^-$) with similar retention volumes, although the anion-exchange capacity of the PW- is about one-tenth that of the SW-type column. This can be ascribed to the difference in packing materials: polymethacrylate has a slightly stronger affinity to iodide and thiocyanate than silica, which is very weak in hydrophobicity. Polystyrene with high hydrophobicity (Yokogawa AX-1) has a strong affinity to iodide and thiocyanate, resulting in larger retention volumes. Hence it is preferable to use columns with weak hydrophobicity. In practice, however, TSKgel IC-Anion-PW is effective as the silica-based column in-

TABLE I

RETENTION VOLUMES OF IODIDE AND THIOCYANATE

Mobile phase, 0.1 *M* NaCl–5 m*M* sodium phosphate buffer (pH 6.7). Anion-exchange columns with quaternary ammonium groups; TSKgel IC-Anion-PW [50 × 4.6 mm I.D.; packing material, polymethacrylate; particle size, 10 μm; exchange capacity, 0.03 mequiv./ml (dry)]; TSKgel IC-Anion-SW [50 × 4.6 mm I.D.; packing material, silica; particle size, 5 μm; exchange capacity, 0.4 mequiv./ml (dry)]; and Yokogawa AX-1 [(50 + 250) × 4.6 mm I.D.; packing material, polystyrene; particle size, 10 μm; exchange capacity, 0.02 mequiv./g].

Column	*Retention volume (ml)*	
	I^-	SCN^-
TSKgel IC-Anion-PW	3.7	7.7
TSKgel IC-Anion-SW	4.0	6.2
Yokogawa AX-1	7.8	22.6



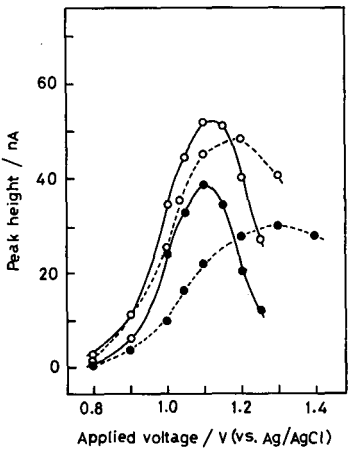


Fig. 2. Effect of wavelength on peak height of (○) I^- and (●) SCN^- (1 mg/l each). Mobile phase: 0.1 *M* NaCl–5 m*M* sodium phosphate buffer (pH 6.7).

Fig. 3. Effect of applied voltage on peak height of (○) I^- and (●) SCN^- (0.1 mg/l each). Mobile phase: solid lines, 0.1 *M* NaCl–5 m*M* sodium phosphate buffer (pH 6.7); dashed lines, 0.03 *M* $NaNO_3$–5 m*M* sodium phosphate buffer (pH 6.7).

dicates a slow decrease in the retention volumes, probably owing to dissolution of silica[11] under the conditions of the mobile phase.

UV and AMP detection

Fig. 2 shows the dependence of peak height (UV absorbance) on the wavelength for the injection of 1 mg/l iodide and thiocyanate solutions. Iodide has maximum UV absorbance at *ca.* 226 nm, where the background absorbance of 0.1 *M* NaCl is low. Thiocyanate has a strong absorbance at lower wavelength. However, as the UV absorbance of 0.1 *M* NaCl increases at that wavelength, it is necessary to measure at a wavelength longer than 210 nm.

Fig. 3 shows the dependence of peak height (AMP response) on applied potential for the injection of 0.1 mg/l iodide and thiocyanate solution. As the potential is increased, the peak height initially increases to a maximum. The reason for this is that the gain in background current at higher potential is larger than that in response to the analyte ions. The potential of the peak current response is *ca.* +1.1 V for iodide and thiocyanate in 0.1 *M* NaCl mobile phase and +1.2 and +1.3 V for iodide and thiocyanate, respectively, in 0.03 *M* $NaNO_3$ mobile phase. The higher peak potential in $NaNO_3$ than in NaCl is ascribed to the lower background current of the $NaNO_3$, probably owing to its lower concentration and stability towards oxidation. The optimum voltage to keep a constant background signal from the mobile phase was *ca.* +1.0 V in 0.1 *M* NaCl. Although a higher voltage can be acceptable in 0.03 *M* $NaNO_3$, the applied potential was similarly selected as +1.0 V in order to suppress the response of other ions.

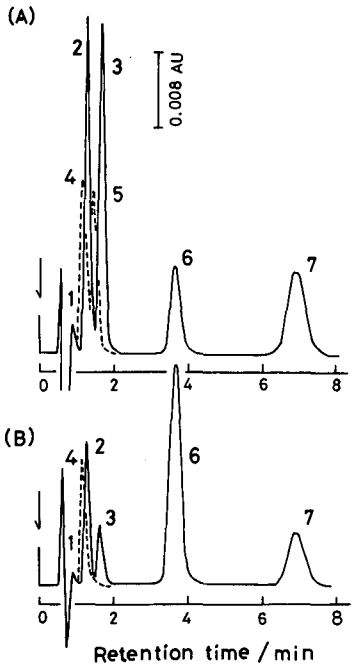


Fig. 4. Ion chromatograms of inorganic anions. 1 = IO_3^- (1 mg/l); 2 = NO_2^- (0.5 mg/l); 3 = NO_3^- (0.5 mg/l); 4 = $S_2O_3^{2-}$ (1 mg/l); 5 = Br^- (1 mg/l); 6 = I^- (1 mg/l); 7 = SCN^- (1 mg/l). Column, TSKgel IC-Anion-PW; mobile phase, 0.1 *M* NaCl–5 m*M* sodium phosphate buffer (pH 6.7); detection, UV absorbance at (A) 210 nm and (B) 226 nm; flow-rate, 1.2 ml/min; sample volume, 100 µl.

Ion chromatogram

Fig. 4 shows chromatograms of a mixture of UV-absorbing ions at 210 and 226 nm. They have two characteristics. (1) Iodide and thiocyanate are completely separated from the other anions; the hydrophilic ions (iodate, thiosulphate, nitrite, nitrate and bromide) are eluted within 2 min. On the other hand, the elution of iodide and thiocyanate with smaller hydration energy is relatively retarded. Hence matrix effects apart from the ion-exchange effect are exerted on the column used, as described under *Column*. (2) The wavelengths of maximum UV-absorbance for the other anions are shifted downwards compared with iodide (*ca.* 226 nm). These results indicate that the use of UV detection and 0.1 *M* NaCl as the mobile phase makes it possible to determine trace levels of iodide and thiocyanate in solutions containing large amounts of other anions.

Fig. 5 shows chromatograms of a mixture of electroactive anions using 0.1 *M* NaCl and 0.03 *M* $NaNO_3$ mobile phases. Although a good separation and highly sensitive detection of iodide and thiocyanate are obtained with both mobile phases, the elution of the faster eluting species with 0.1 *M* NaCl is faster than that with 0.03 *M* $NaNO_3$. Hence it is considered that NaCl is to be preferred to $NaNO_3$ in ion chromatography with amperometric detection. In addition, NaCl can be applied with a UV detection system.

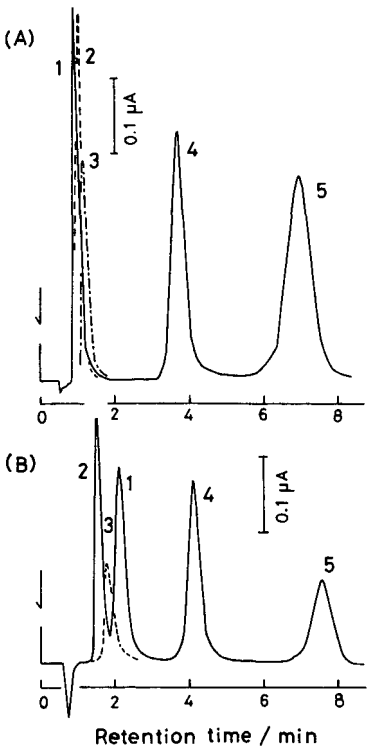


Fig. 5. Ion chromatograms of inorganic anions. 1 = $S_2O_3^{2-}$ (1 mg/l); 2 = NO_2^- (0.5 mg/l); 3 = Br^- (100 mg/l); 4 = I^- (1 mg/l); 5 = SCN^- (1 mg/l). Mobile phase, (A) 0.1 *M* NaCl–5 m*M* sodium phosphate buffer (pH 6.7) and (B) 0.03 *M* $NaNO_3$–5 m*M* sodium phosphate buffer (pH 6.7); detection, amperometry using a GC working electrode (+1.0 V *vs.* Ag/AgCl); other conditions as in Fig. 4.

Calibration graph, detection limit and repeatability

The calibration graphs of iodide and thiocyanate based on peak-height measurements were linear up to a concentration of 20 mg/ml with UV detection. With AMP detection using both mobile phases the upper limit of linearity was about one tenth (2 mg/l) of that for UV detection. Further, at concentrations below 0.1 mg/l the calibration graphs showed downward curvatures. With a 100-μl injection, the detection limits (at a signal-to-noise ratio of 2) were 5 μg/l for I^- (226 nm) and 10 μg/l for SCN^- (210 nm) by the UV method and 5 and 5 μg/l for I^- and SCN^- respectively, by the AMP method using 0.1 *M* NaCl. The reproducibility of five replicate injections of 0.1 mg/l I^- and SCN^- was good; the relative standard deviation (R.S.D.) for UV detection at 210 and 226 nm was 2.8 and 3.0%, respectively, for I^- and 2.0 and 2.2%, respectively, for SCN^-. For AMP detection with 0.1 *M* NaCl and 0.03 *M* $NaNO_3$, the R.S.D. values were 3.1 and 2.1%, respectively, for I^- and 3.6 and 2.7%, respectively, for SCN^-.

Interferences

The effects of eleven inorganic anions (Cl^-, SO_4^{2-}, F^-, HCO_3^-, $H_2PO_4^-$, IO_3^-,

SeO_4^{2-}, Br^-, NO_2^-, $S_2O_3^{2-}$, NO_3^-, 1–10 g/l) on the detection of iodide and thiocyanate (1 mg/l each) were examined using a TSKgel IC-Anion-PW column and 0.1 *M* NaCl as the mobile phase. The peaks of the anions (retention volumes are within 2 ml) are separated completely from iodide (3.7 ml) and thiocyanate (7.7 ml). For UV detection at 210 and 226 nm, the chromatograms (1 mg/l) of the analyte ions were baseline resolved with those of 10 g/l solutions of both UV-transparent ions (Cl^-, SO_4^{2-}, F^-, HCO_3^- and $H_2PO_4^-$) and UV-absorbing ions (IO_3^-, $SeSO_4^-$ and Br^-). For AMP detection, baseline resolution of I^- and SCN^- (1 mg/l) was obtained for 10 g/l solutions of electroinactive Cl^-, SO_4^{2-}, F^-, HCO_3^-, $H_2PO_4^-$, IO_3^- SeO_4^{2-}, NO_3^- and Br^-. However, the peak heights of iodide in each solution (10 g/l) were reduced by *ca.* 5% (Cl^-), 10% (SO_4^{2-}) and 20% (Br^-) for UV detection at 226 nm and by *ca.* 5% (Cl^-), 20% (Br^-) and 25% (NO_3^-) for AMP detection. On the other hand, the effects on the peak height of thiocyanate were small. The retention times were reduced by *ca.* 0.1 min for iodide and 0.2 min for thiocyanate.

The strongly UV-absorbing ions, $S_2O_3^{2-}$ (5 g/l), NO_2^- (1 g/l) and NO_3^- (1 g/l), did not interfere with the baseline separation of iodide at 226 nm, but interfered at 210 nm. The tailing portions of chromatograms of 1 g/l $S_2O_3^{2-}$ and NO_2^-, which are electroactive, partly overlapped with iodide.

Determination of iodide in sea water

The determination of iodide in sea water was examined in order to clarify the highly sensitive detection of trace levels of ions in solutions containing an excess of salts. Fig. 6A shows chromatograms of iodide (0.1 mg/l) in artificial sea water (salinity 35‰) with UV detection. The artificial sea water, which was prepared according to the Lyman and Fleming Formula[20], contains 19 300 mg/kg of Cl^-, 2710 mg/kg of SO_4^{2-}, 142 mg/kg of HCO_3^- and 67 mg/kg of Br^-. A good iodide peak and high sensitivity were obtained at 226 nm compared with 210 nm, mainly because the

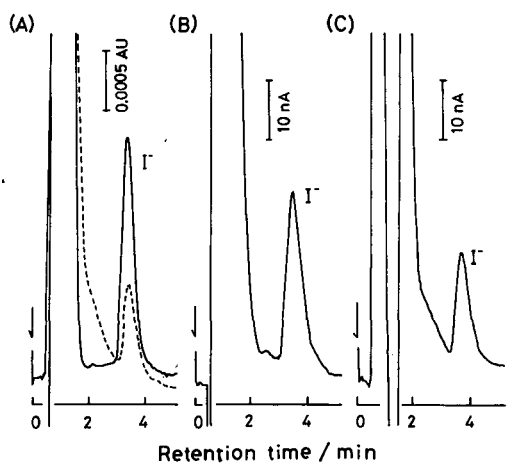


Fig. 6. Ion chromatograms of I^- (0.1 mg/l) in artificial sea water (salinity 35‰). Mobile phase, (A), (B) 0.1 *M* NaCl–5 m*M* sodium phosphate buffer (pH 6.7) and (C) 0.03 *M* $NaNO_3$–5 m*M* sodium phosphate buffer (pH 6.7); detection, (A) UV absorbance at 226 nm (solid line) and 210 nm (dashed line) and (B), (C) amperometry (+1.0 V *vs.* Ag/AgCl); other conditions as in Fig. 4.

TABLE II

ANALYTICAL RESULTS FOR IODIDE (μg/ml) IN SEA WATER

Average results for two determinations.

Sample	*UV detection*	*AMP detection*[7]
Standard sea water (England)	0.008	0.006
Standard sea water (Japan)	0.011	0.010
Sea water (Seto Inland Sea):		
No. 1	0.022	0.020
No. 2	0.030	0.032

absorbance of Br^- at 226 nm is small compared with that at 210 nm, as is shown in Fig. 4. Fig. 6B and C show chromatograms of iodide (0.1 mg/l) with AMP detection. The use of 0.1 *M* NaCl as the mobile phase gives a sufficient resolution of iodide compared with that obtained with 0.03 *M* $NaNO_3$, which elutes bromide slowly, as is shown in Fig. 5.

The NaCl system was applied to the determination of trace levels of iodide in standard sea water (England and Japan) and real sea water samples. The real samples were collected at Hiroshima bay in the Seto Inland Sea (Japan), filtered through a 0.45-μm membrane filter (made of cellulose nitrate) and pretreated by passage through a Sep-Pak C_{18} cartridge (Waters Assoc.). The calibration graphs for iodide (0–0.1 mg/l) in artificial sea water (salinity 35‰) were used for determination, as the peak heights in the artificial sea water were *ca.* 20% low compared with those in deionized water. The results are summarized in Table II. The agreement between the UV and AMP methods was good. Moreover, fairly quantitative recoveries (80–120%) were obtained for the addition of 0.01 and 0.03 mg/l of iodide, although the precision was poor owing to the low concentration of iodide.

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Isoelectric focusing studies of concanavalin A and the lentil lectin

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SUMMARY

Isoelectric focusing (IEF) of metallized and demetallized preparations of concanavalin A (Con A) consisting of either intact or fragmented subunits shows different band patterns. Metallized Con A consisting of intact polypeptide chains (intact Con A) has an isoelectric point (p*I*) 8.35. Metallized preparations consisting of fragmented chains (fragmented Con A) show three bands with p*I* values 8.0, 7.8 and 7.7. Demetallized intact Con A (intact apoCon A) has a p*I* of 6.5, however, it undergoes pH dependent association during IEF under certain conditions, which gives rise to multiple bands. Ampholyte-mediated demetallization of intact and fragmented Con A and subsequent aggregation of the apoprotein results in multiple bands during IEF in the presence of the pH range 3 to 10 ampholytes. However, ampholytes of the pH range 7 to 9 do not demetallize the proteins and show a single band with intact Con A. The p*I* of intact Con A remains essentially the same in the presence of inhibitory sugar. Furthermore, different moleculars forms of Con A, including locked and unlocked conformers of intact apoCon A, and the dimeric and tetramic states of both intact Con A and intact apoCon A have been identified and their p*I* values determined.

IEF of the lentil isoelectins, LcH-A and LcH-B, shows single bands of p*I* 8.5 and 9.0, respectively. However, the native lectin mixture gives rise to an additional band of p*I* 8.8 due to a hybrid protein formed by ampholyte-mediated subunit exchange between the isolectins.

INTRODUCTION

Concanavalin A (Con A), the D-glucose/D-mannose-specific lectin from jackbean seeds[1], has found wide application in biological studies[2,3]. It is a metalloprotein containing Mn^{2+} and Ca^{2+} (ref. 1). The native lectin contains intact polypeptide chains of molecular mass (M_r) 26 000 daltons, and "fragmented" chains of molecular masses 13 000, 11 000 and 10 600 daltons[4–6]. The fragmented chains appear to result from the posttranslation cleavage of a precursor polypeptide chain and the intact

0021-9673/90/$03.50

polypeptide chain is formed by subsequent transpeptidation and reannealing of the two fragments[7].

Controversy still exists in the literature concerning the isoelectric focusing (IEF) pattern of the protein. Entlicher *et al.*[8] reported isoelectric points (p*I*) from 4.5 to 5.5, while others[9–12] found p*I* values from 6.0 to 8.0. In addition, Con A shows multiple bands on IEF which some authors concluded is probably due to the presence of several molecular species formed by different combinations of fragmented and intact subunits[8–10], while others assert it is due to different conformational states of the protein[11].

There are many reports in the literature of artifacts associated with the IEF of proteins (*cf.* ref. 13). These include chelation of metal ions of metalloproteins and metalloenzymes by ampholytes[14], ampholyte binding to proteins[15], and ampholyte mediated subunit exchange[16,17]. In the present study, we demonstrate that the multiple band patterns in IEF of homogeneous, metallized preparations of Con A are due to chelation of the metal ions by ampholytes and consequent pH dependent aggregation of the apoprotein. However, under appropriate conditions intact Con A shows a single band of pI 8.35. We also report pI values of different molecular forms of Con A, including the two conformational states of intact apoCon A —the "locked" and "unlocked" conformers[18]— and the dimeric and tetrameric states of the protein.

We also report IEF studies of the lentil lectin (LcH) which possesses similar monosaccharide specificity as Con A[1]. The native lectin consists of equal amounts of two isolectins, LcH -A and LcH-B[1], which individually show single bands with characteristic p*I* values. The native mixture, however, shows an additional band due to the formation of a hybrid protein in the presence of ampholytes.

MATERIALS AND METHODS

Native Con A was purchased from Miles-Yeda (Rehovot, Israel). LcH native mixture and the isolectins, LcH-A and LcH-B, were purified from locally purchased seeds (*Lens culinaris* sub. *Macrosperma*) as described[19,20]. Ampholyte solutions of different pH ranges were obtained from Pharmacia LKB (Piscataway, NJ, U.S.A.) and Serva Fine Biochemicals (Heidelberg, F.R.G.). Monosaccharides were products of Sigma (St. Louis, MO, U.S.A.) and Pfanstiehl Laboratories (Waukegan, IL, U.S.A.).

Preparation of intact and fragmented Con A

This was done by modification of a previously described procedure[21]. About 250 mg of native Con A was dissolved in 20 m*M* Tris–HCl buffer, pH 7.2, containing 0.1 *M* NaCl, 1 m*M* $MnCl_2$ and 1 m*M* $CaCl_2$ at about 5 mg/ml. The solution was allowed to stand for 30 min, clarified by centrifugation and applied to a Sephadex G-75 column (40 × 2.6 cm) equilibrated at room temperature in 20 m*M* Tris–HCl buffer, pH 7.2, containing 1 m*M* $MnCl_2$ and 1 m*M* $CaCl_2$. The column was eluted with 20 m*M* glucose in the equilibrating buffer at a flow of 8–10 ml/h until the absorbance went down to below 0.1 (about 600 ml). Fractions having absorbance at 280 nm greater than 0.3 were pooled and rechromatographed to get fragmented Con A. Con A enriched in intact subunits was eluted at 20 ml/h with 0.1 *M* glucose in the equilibrating buffer. This fraction was rechromatographed to get intact Con A. The preparations were then dialyzed against water and stored as salt-free lyophilizates.

Preparation of intact apoCon A

This was done by demetallization of intact Con A as described[22].

Preparation of "locked" intact apoCon A

Intact apoCon A in the "locked" conformation was prepared by dissolving the protein in pH 5.3 or 6.4 buffer (10 m*M* sodium acetate, 0.1 *M* NaCl) containing 1 *M* α-MM (methyl α-D-mannopyranoside) and 100 μ*M* EDTA, and allowing the solution to stand for 6 days at room temperature[23].

Preparation of sample for IEF

Metallized Con A samples (native, intact or fragmented) were dissolved in pH 5.3 (10 m*M* sodium acetate, 0.1 *M* NaCl) or 7.2 (10 m*M* Tris–HCl, 0.1 *M* NaCl) buffers each containing 2 m*M* $MnCl_2$ and 2 m*M* $CaCl_2$ at about 10 mg/ml[18]. Solutions at pH 5.3 were allowed to stand overnight at 4°C and diluted 10-fold with ice-cold 10% glycerol immediately before the IEF run. Solutions at pH 7.2 were allowed to stand overnight at 25°C and diluted similarly with the glycerol solution at the same temperature. Intact apoCon A was treated in the same manner except that pH 7.2 buffer was replaced by pH 6.4 buffer (10 m*M* sodium acetate, 0.1 *M* NaCl) to avoid precipitation of the apoprotein[24], and $MnCl_2$ and $CaCl_2$ were replaced by 100 μ*M* EDTA in the buffer.

Isoelectric focusing

IEF was done according to Wrigley[25] using 7.5% gel and 2% ampholyte solutions. For runs in the presence of monosaccharides, the sugar is included in the gel solutions at the appropriate concentrations. Unless stated otherwise, the runs were made at 4°C with the cathode at the top (catholyte: 0.08% NaOH)[26] and anode at the bottom (anolyte: 0.2% H_2SO_4). The bands were stained with 1% Coomassie Blue and their relative intensities measured by a Joyce Loebl Chromoscane 3 gel scanner. The pH gradient was determined by eluting 5 mm thick gel slices with 1 ml CO_2-free glass-distilled water[26]. Whenever possible, complete focusing was ensured by simultaneously running a mixture of cow α-lactoglobulins A and B (the p*I* values[27] of the A and B variants are 5.35 and 5.41, respectively). In other cases, LcH-A (pI 8.5; see later) was used.

Polyacrylamide gel electrophoresis (PAGE)

This was done in alkaline pH essentially as described[28]. The pH of the gel and the tray buffers were adjusted to appropriate values. Gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was done acording to Weber and Osborn[29].

RESULTS

Preparation of intact Con A and fragmented Con A

Attempts at preparing intact Con A by the method of Cunningham *et al.*[6] which involves incubating native Con A with 1% NH_4HCO_3 did not work in our hands. Among other methods reported[4,5,21], the procedure of Williams *et al.*[21] is the simplest and the most convenient. However, the problem of the latter procedure is

relatively poor yield. Overall recovery of the protein from a Sephadex G-75 column was reported to be about 50%[21]. In our modified procedure, native Con A was dissolved and loaded on the column in a buffer containing a higher salt concentration (0.1 *M* NaCl) which stabilized the protein, and then eluted with low ionic strength buffer as described[21]. The overall recovery of the protein increased to 99%.

Elution of the adsorbed protein from the Sephadex G-75 column with 20 m*M* glucose gives a broad peak (approximately 20% of the total load) which is enriched in fragmented polypeptide chains. This fraction was rechromatographed to obtain fragmented Con A preparation which, by SDS gel electrophoresis, was found to contain about 55% of the 13 000-dalton chain and 42% of the 11 000- and 10 600-dalton chains together.

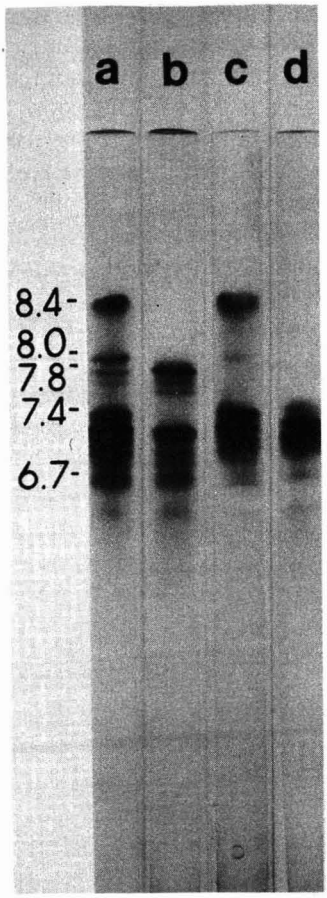
The fraction eluted from the column by 0.1 *M* glucose emerged as a sharp peak (78–80% of the total load), and is composed of approximately 80% intact chains. Rechromatography of this fraction gives a preparation of intact Con A containing about 98% intact polypeptide chain.

Flow-rate during elution with 20 m*M* glucose is an important factor. The results described above were obtained at a flow-rate of 8 ml/h. However, when the flow-rate was increased to 30 ml/h, the proportion of the second peak is reduced to 40% of the total protein, and the absorbance at 280 nm did not go below 0.25 after the first peak had emerged due to "bleeding" of the protein from the column.

IEF of native, fragmented and intact Con A

Studies were carried out with Con A preparations incubated in a pH 5.3 buffer at 4°C. Under these conditions, the protein exists essentially as a dimer[30]. Fig. 1 shows the IEF patterns of native, fragmented and intact Con A as well as intact apoCon A using ampholytes of the pH range 3 to 10. The top most band (pI 8.4) of native Con A (Fig. 1a) is also present in intact Con A (Fig. 1c). The three middle bands of native Con A at pI 8.0, 7.8 and 7.7 correspond to the three top bands of fragmented Con A (Fig. 1b). None of these bands are seen with intact apoCon A (Fig. 1d). Therefore, these bands are due to the fully metallized forms (intact or fragmented) of Con A. The multiple bands seen with native Con A between p*I* 7.4 to 6.7 (Fig. 1a) are also present in fragmented Con A (p*I* 7.2 to 6.7) (Fig. 1b), intact Con A (p*I* 7.4 to 7.0) (Fig. 1c), and intact apoCon A (p*I* 7.4 to 7.0) (Fig. 1d). Thus, these bands are clearly due to the apoproteins. Since native, fragmented and intact Con A samples were preincubated overnight in the presence of Mn^{2+} and Ca^{2+} in order to ensure fully metallized proteins[20], the apoprotein bands are due to demetallization during the run.

Fig. 2 shows the results of IEF studies with native and intact Con A, and intact apoCon A using ampholytes of the pH range 7 to 9. The samples were treated in the same way as above before the runs. Native Con A gave major bands at p*I* 8.35, 8.05 and 7.75, and a minor band at 7.60 (Fig. 2a). Intact Con A focused as essentially a single band at pI 8.35 (a trace of the pI 8.05 band is also seen with intact Con A) (Fig. 2b). Thus, the results using ampholytes of both pH ranges show that the p*I* of fully metallized intact Con A is 8.35. Intact apoCon A shows a broad band at the bottom of the gel (Fig. 2c), as expected from the results using ampholytes of the pH range 3 to 10. Neither native or intact Con A show any band in this region using ampholytes in the pH range 7 to 9, indicating the lack formation of apoproteins. Thus, the bands at



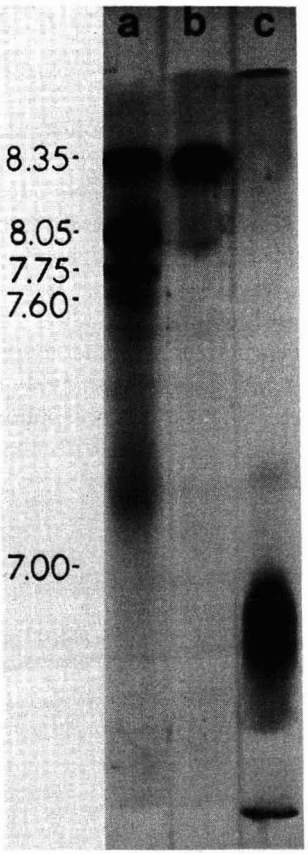


Fig. 1. IEF patterns of (a) native, (b) fragmented, and (c) intact Con A, and (d) intact apoCon A in the pH range 3 to 10. The pH decreases from the top to the bottom of the gels. The direction of migration is also from the top to the bottom. Loads: (a), 150 μg; (b) and (c), 100 μg; and (d), 50 μg.

Fig. 2. IEF patterns of (a) native and (b) intact Con A, and (c) intact apoCon A in the pH range 7 to 9. The pH decreases from the top to the bottom of the gels. The direction of migration is also from the top to the bottom. Loads: (a) and (c), 100 μg; (b), 50 μg.

pI 8.05, 7.75 and 7.6 of native Con A correspond to fully metallized fragmented Con A molecules. These p*I* values of fragmented Con A are essentially the same as those obtained by IEF in the pH range 3 to 10.

IEF in the pH range 3 to 10 in the presence of 0.1 *M* α-MM, a monosaccharide that binds to Con A[1], shows a single band of pI 8.5 with intact Con A and close multiple bands of p*I* 7.8 to 7.5 with fragmented Con A (not shown). Thus, binding to a specific monosaccharide has little effect on the pI of the protein.

Studies were also carried out with intact Con A tetramers (protein incubated in pH 7.2 buffer at 25°C [ref. 30]). IEF at 25°C using ampholytes in the pH range 7 to 9 results in a major band at p*I* 8.15, along with minor bands at the bottom of the gel, which are, therefore, associated with the apoprotein (not shown). The results show that dimeric Con A has a slightly higher p*I* than tetrameric Con A.

IEF of "unlocked" and "locked" forms of intact ApoCon A

In IEF of intact apoCon A, described above (Fig. 1d), the sample treatment results in predominantly the "unlocked" form of the protein in solution[18]. IEF of the same sample of intact apoCon A also showed multiple bands in the pH range 5 to 8 (Fig. 3a). Following reported procedures[25,26], the cathode was placed at the top and the anode at the bottom in this and previous experiments. On the other hand, IEF of the above sample in the same pH range with reversed electrode polarities (anode at the top and cathode at the bottom) showed one major band (approximately 85% of the total load) corresponding to a pI of 6.5 (Fig. 3b). Thus, p*I* of intact apoCon A dimer in the "unlocked" conformation is 6.5.

Fig. 3c shows the results of IEF with anode at the top and cathode at the bottom in the pH range 5 to 8 of intact apoCon A in the "locked" from prepared by incubating the protein with 1 *M* α-MM in pH 5.3 buffer at 4°C (ref. 23). Two major bands of p*I* 6.25 and 6.4 are observed, along with a minor band of pI 6.5. The pI of the latter band corresponds to the "unlocked" form of the apoprotein. Thus, the two major bands appear to be related to the apoprotein in the "locked" conformation.

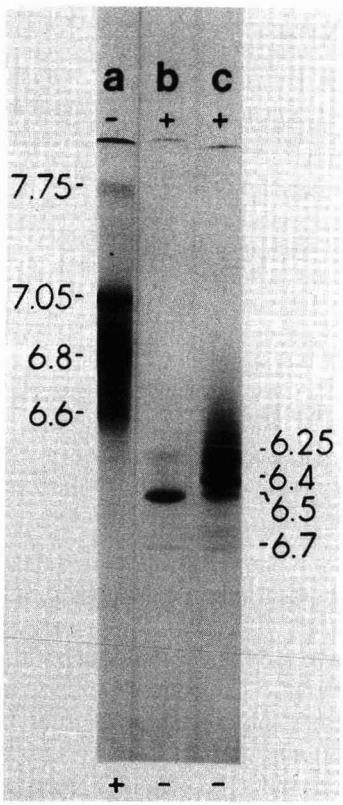


Fig. 3. IEF patterns of intact apoCon A in the pH range 5 to 8 in (a,b) "unlocked" and (c) "locked" conformations. The pH decreases from the top to the bottom in (a), and increases in (b) and (c). The direction of migration is from the top to the bottom for all gels. Gel (c) was run in the presence of 1 *M* α-MM. Loads: (a) and (c), 100 μg; (b), 35 μg.

The intensities of the major bands are approximately equal. These bands are also observed as minor species in Fig. 3b, which is consistent with the presence of smaller amount of the "locked" form under the condition of sample incubation[18]. The presence of two locked apoCon A bands, however, required further experimental evidence to suggest their identity.

"Locked" apoCon A prepared by incubation in 1 *M* α-MM in pH 6.4 buffer at 25°C also showed two major bands of p*I* 6.25 and 6.4 on IEF at 25°C (anode at the top and cathode at the bottom, however, the ratio of the intensities of the bands was approximately 70:30 (not shown). Since Con A tetramer is predominant over the dimer at higher temperature and pH[30], the present results suggest that the p*I* 6.25 band corresponds to the apoprotein tetramer in the "locked" conformation, and the pI 6.4 band corresponds to the dimer.

IEF of LcH

Fig. 4 shows the results of IEF of LcH native mixture and the two isolectins, LcH-A and LcH-B, using ampholytes of the pH range 3 to 10. The patterns of the

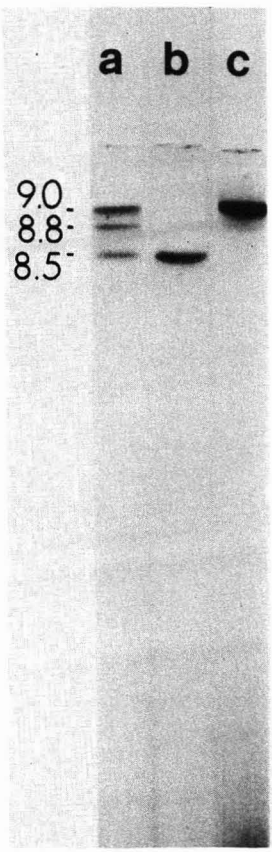


Fig. 4. IEF patterns of (a) LcH native mixture, (b) LcH-A, and (c) LcH-B in the pH range 3 to 10. The pH decreases from top to bottom of the gels. The direction of migration is also from the top to the bottom. Loads: (a), 50 μg; (b) and (c), 30 μg.

native mixture (Fig. 4a) and an equimolar mixture of the two isolectins are similar, both giving three bands, two of which correspond to the two isolectins of p*I* (LcH-A) and 9.0 (LcH-B) (Fig. 4b and c, respectively). The third band which constitutes approximately 25% of the total load appears in the middle and has a p*I* of 8.8. When the middle band is isolated and refocused, it shows a pattern similar to that of the native mixture. IEF of LcH native mixture in the pH range 7 to 10 shows a similar pattern except that the middle band constitutes about 10% of the total load. IEF of the native mixture in the presence of 0.1 *M* α-MM using ampholytes of the pH range 3 to 10 shows the same pattern as in the absence of sugar with no change in p*I* values.

Fig. 5 shows the results of PAGE of LcH native mixture and the two isolectins at pH 9.4. The native mixture (lane c) shows two sharp bands corresponding to LcH-A (lane a) and LcH-B (lane b). Preincubation of the native mixture with 1% ampholyte solution of the pH range 3 to 10 gives the same pattern (lane d) as the native mixture in the absence of ampholytes. Furthermore, the protein of p*I* 8.8 also shows two bands corresponding to LcH-A and LcH-B on PAGE (not shown).

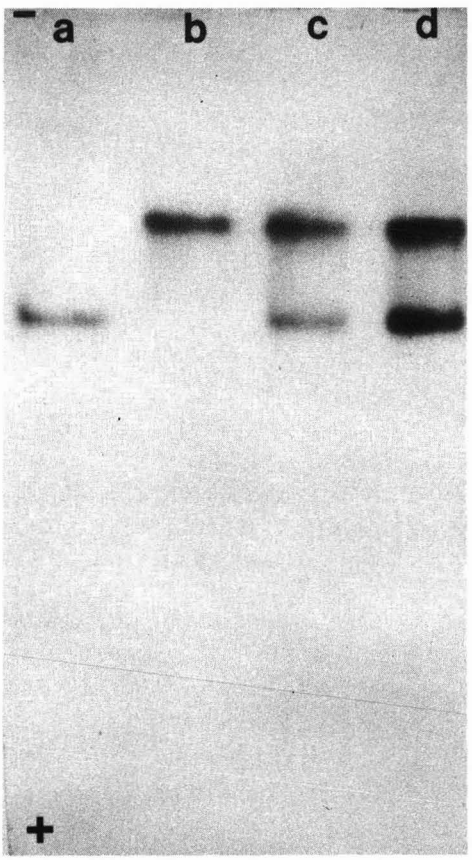


Fig. 5. Polyacrylamide gel electrophoresis at pH 9.4 of LcH-A (lane a, 30 μg), LcH-B (lane b, 30 μg), LcH native mixture (lane c, 60 μg) and LcH native mixture preincubated with 1% ampholyte solution of the pH range 3 to 10 (lane d, 100 μg). The direction of migration is from the top to the bottom of the gel.

DISCUSSION

IEF studies of Con A

The present results show that IEF techniques can be used to characterize not only the fully metallized intact and fragmented Con A but also different molecular forms of the lectin, including the apoprotein, the "locked" and "unlocked" conformers, and the dimeric and the tetrameric states of the protein.

Fig. 1 and 2 show that intact Con A can be focused as a single band, with a p*I* of 8.35 in the presence of ampholytes of pH ranges 3 to 10 and 7 to 9, respectively. Candiano *et al.*[10] recently reported a p*I* of 7.65 for intact Con A using ampholytes of the pH range 7 to 11. However, under these conditions the protein is exposed to a pH above 9 (ref. 31), and undergoes irreversible denaturation[32] with concomitant loss of metal ions[24] to generate apoCon A.

Fragmented Con A shows three major bands in either pH range (excluding the additional bands in the pH range 3 to 10) at p*I* values 8.0, 7.8 and 7.7. SDS-PAGE of fragmented Con A showed that the intact chain was essentially absent in the preparation. Thus, it appears that these bands are due to proteins formed by different combinations of the M_r 13 000-, 11 000- and 10 600-dalton polypeptide fragments.

The pattern of the native Con A appears to be a combination of intact and fragmented Con A (Figs. 1 and 2). The lack of any additional band in native Con A suggests the absence of hybrid protein molecules consisting of both intact and fragmented polypeptide chains.

Native and intact Con A show different IEF band patterns using ampholytes of the pH ranges 3 to 10 and 7 to 9 (Figs. 1 and 2, respectively). Although the metallized proteins are focused essentially at the same pH in the presence of either carrier ampholytes, both protein samples show multiple bands due to formation of apoproteins in the presence of ampholytes of the pH range 3 to 10 but not 7 to 9 range. Since both protein samples were preincubated with Mn^{2+} and Ca^{2+} to ensure full metallization[18], the results demonstrate that formation of apoproteins was mediated by ampholytes specific for the pH 3 to 10 range but not the pH 7 to 9 range. The latter results also show that demetallization of the protein during IEF is not mediated by the applied electric gradient.

IEF of intact and fragmented Con A in the presence of α-MM with ampholytes of the pH range 3 to 10 showed that the specific binding sugar prevents the formation of apoproteins. These results are consistent with tighter binding of the metal ions to the protein in the presence of bound monosaccharides[18]. The results also showed that the p*I* values of intact and fragmented Con A do not change due to monosaccharide binding, although the binding involves a conformational change[32] and masking of titrable carboxyl groups[33] of Con A. The results contradict the conclusion of Akedo *et al.*[9] that the p*I* values of the protein undergo extensive shifts toward alkaline pH due to monosaccharide binding. These authors found essentially the same IEF patterns as the present report using ampholytes of the pH range 3 to 10 in the presence and absence of sugar, however, the bands below pH 7.4 in the absence of sugar were due to the formation of apoprotein.

Con A exists in dimer–tetramer equilibrium, with the dimer predominating below pH 5.6 and at low temperatures, and the tetramer above pH 7.0 and at room temperature[30]. The results with intact Con A and intact apoCon A show that the

dimers have slightly higher p*I* values (by about 0.2 pH units) than the corresponding tetramers. Differences in the p*I* values between the dimeric and tetrameric states of Con A are expected in view of the involvement of Lys 114 and 116, Glu 192, His 51 and 121, and Ser 108 and 117 residues in the dimer–dimer contact region[34].

The results in Figs. 1 and 2 show that intact apoCon A gives multiple bands in the pH range 3 to 10, though the corresponding metallized protein gives a single band. This is not due to cleavage of some of the amide linkages of Con A during demetallization by 0.1 *M* HCl, since SDS-PAGE of intact Con A and intact apoCon A show single bands of M_r 26 000 daltons. There may be two other possibilities. The multiple bands may be formed either due to aggregation of intact apoCon A, since it undergoes association at a pH greater than 6.5 (ref. 24), or due to ampholyte binding to intact apoCon A, as has been observed with certain other proteins[13,15]. To test these possibilities, we performed IEF using ampholytes of the pH range 5 to 8, first placing the cathode at the top of the gel and then reversing the electrode polarities.

In the former run (cathode at the top), pH decreases from the top to the bottom in the gels[13,31]. With pH 5 to 8 range ampholytes, protein entering the gel from the top experiences a pH higher than 6.5 (the pH of 2% ampholyte solution is 6.5), since the ampholyte molecules have higher mobilities than the protein in polyacrylamide gels due to the smaller size of the former[13,31]. Since apoCon A aggregates above pH 6.5, the multiple bands in Fig. 3a appear to be due to aggregation of apoCon A. In the latter run (anode at the top) (Fig. 3b), the pH increases from the top to the bottom of the gels, and the protein experiences a pH lower than 6.5, as it enters the gel. Thus, the results indicate that the multiple bands of intact apoCon A are due to the pH-dependent aggregation of the apoprotein. Had the multiplicity been due to ampholyte binding to the protein, the same, but inverted pattern is expected by the exchange of electrode polarity.

Con A exists as an equilibrium mixture of two conformational states. The "unlocked" state is the predominant conformation (87%) of apoCon A at pH 6.4 and 25°C, which weakly binds both metal ions and saccharides[18]. (The results in Fig. 3b show essentially the same percentage for the major protein band in the "unlocked" apoCon A preparation.) The "locked" conformation tightly binds two metal ions per monomer and, once fully metallized, possesses full saccharide binding activity[18]. Addition of specific sugar to apoCon A solution shifts the overall equilibrium toward the "locked" conformation due to the higher sugar binding activity of the latter[23]. The present findings (Fig. 3b and c) show that IEF techniques can distinguish the p*I* values of the two conformers of intact apoCon A. (Similar studies with the metallized protein are not possible. Addition of metal ions to apoCon A quickly converts the "unlocked" protein to the "locked" conformation[18].) The results also show that intact apoCon A in the "locked" conformation, prepared in the presence of the inhibitory saccharide, exists as a temperature-dependent mixture of dimer and tetramer (Fig. 3c). However, at present we do not know whether the dimer-tetramer equilibrium of "locked" apoCon A is an intrinsic property of the protein, or if the bound saccharide influences this equilibrium.

IEF studies of LcH

Native LcH which consists of two isolectins shows three bands upon IEF, two of which correspond to the two isolectins at p*I* values 9.0 and 8.5 (Fig. 4). The third

band at p*I* 8.8 when isolated gives the same pattern as the native mixture on IEF, and two bands corresponding to the two native isolectins on PAGE. Thus, the third band appears to be a hybrid of the two isolectins formed by subunit exchange between the two isolectins. Furthermore, the native mixture preincubated with 1% ampholyte shows two bands on PAGE corresponding to the two isolectins. Since ampholyte molecules are separated from the protein during PAGE due to their faster mobilities[13,31], the results indicate that the presence of ampholytes is required for the formation and stability of the hybrid band, and that upon removal of the ampholytes dissociation of the hybrid protein to the native isolectins is rapid. Interestingly, the proportion of the hybrid band depends on ampholyte composition, since the intensity of the band is different between the pH 3 to 10 and 7 to 10 IEF runs. Formation of a hybrid protein by ampholyte-mediated subunit exchange between the isolectins has also been reported for the pea lectin[17], which is closely related to LcH and consists of two isolectins[1]. However, contrary to the observation with pea lectin[17], the hybrid LcH is not produced by the prolonged exposure of the protein to alkaline pH.

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Isotachophoretic separation of alkali and alkaline earth metal cations in water–polyethylene glycol mixtures

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SUMMARY

The influence of polyethylene glycol (PEG) on the effective mobilities of alkali and alkaline earth metal cations was investigated. It is shown that appropriate proportions of water and PEG in the leading electrolyte enable a complete separation of this group of cations to be achieved by capillary isotachophoresis (ITP). The high dynamic concentration ranges of the ITP determinations achieved in the operational systems based on water–PEG mixtures are illustrated by practical examples (water samples, urine, aqueous extract of apple flesh).

INTRODUCTION

Considerable attention has been paid to the separation and determination of alkali and alkaline earth metal cations by capillary isotachophoresis (ITP) because ITP is a promising method for their determination in waters[1–3], in pharmaceuticals[4,5], in industrial control[6], in biological materials[7,8] and in plant nutrition[9].

The ionic mobilities of some of the alkali and alkaline metal earth cations in aqueous solutions are close[10], so that complex equilibria play a key role in their ITP separations. In this respect, mainly negatively charged ligands[1,4,9,11] and electroneutral crown ethers[3,6,12–14] are effective in the cationic mode of the separation. The separation of alkaline earth metal cations in the anionic mode has some inherent

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disadvantages (a restricted pH range of the separation[2,15,16] and close effective mobilities of the metal chelates[16]).

Methanol is a suitable solvent in ITP separations of alkali and some of the alkaline earth metal cations[9,17,18], as their ionic mobilities in this solvent differ sufficiently. It was reported recently[7] that addition of a polyoxyethylene ether-type detergent (Triton X-100) to the leading electrolyte improves their separations in this solvent. This effect of Triton X-100 is understandable when the stability constants of linear polyethers with alkali metal cations in methanol are considered[19,20].

Crown ethers are useful in extractions of alkali and alkaline earth metal cations from aqueous solutions into organic solvents (for a review see, *e.g.*, refs. 20 and 21). Considerably cheaper polyethylene glycols (PEG) are also effective in this respect[22]. In both instances, the equilibria involved assume the complexation of the cations with polyethers in the aqueous phase. While the stability constants for crown ethers in the aqueous solutions are known[19–21] the corresponding data for their linear analogues (*e.g.*, PEG) are not available. Hence a reasonable assessment of the potential applicability of PEG in ITP separations of alkali and alkaline earth metal cations in aqueous operational systems was impossible. However, limited data available for linear polyethers in methanol[19,20] allow the speculation that the stability constants of PEG with the cations in aqueous solutions should be $10–10^3$ lower in comparison with the cyclic polyethers. This indicates that a much higher concentration of PEG may be required to achieve effects equivalent to those of crown ethers.

The above facts led us to investigate in detail the influence of the concentration of PEG on the effective mobilities of alkali and alkaline earth metal cations and thus to assess its applicability in the ITP separations of this group of cations. The results of this investigation and relevant ITP separations of model and practical samples are presented.

EXPERIMENTAL

Instrumentation

A CS Isotachophoretic Analyzer (VVZ PJT, Spišská Nová Ves, Czechoslovakia) was used in the column-coupling configuration of the separation unit[23]. The separation unit was assembled from modules supplied by the manufacturer and others made in our laboratory. The lengths of the zones from the conductivity detectors were measured electronically[24].

Chemicals and purification

The chemicals used for the preparation of the leading and terminating electrolytes were obtained from Serva (Heidelberg, F.R.G.), Loba-Chemie (Vienna, Austria), Janssen Chimica (Beerse, Belgium) and Lachema (Brno, Czechoslovakia).

Hydroxyethylcellulose 4000 (Serva), after purification on an Amberlite MB-1 mixed-bed ion exchanger (BDH, Poole, U.K.), was used as an additive to the leading electrolytes.

Water obtained from a Rodem-1 two-stage demineralization unit (OPP, Tišnov, Czechoslovakia) was further purified by circulation through a pair of tandem-coupled 200-ml polytetrafluoroethylene (PTFE) laboratory-made columns packed with an Amberlite MB-1 mixed-bed ion exchanger (BDH).

PEG samples were batch demineralized on an Amberlite MB-1 mixed-bed ion exchanger in a polyethylene vessel. Ammonia and acetic acid were purified by isothermal distillation into demineralized water kept in polyethylene vessels cleaned as described in the literature[25].

To minimize contamination of the leading and terminating electrolyte solutions with alkali and alkaline earth metal cations, they were prepared and stored in quartz or polyethylene vessels.

Samples

In the analysis of demineralized water, the sample was introduced into the injection valve (PTFE-lined) of the ITP instrument with the aid of a thoroughly cleaned disposable polyethylene syringe. The syringe was repeatedly washed with the sample to be analysed immediately before the ITP separation. To eliminate potential contamination of the samples during storage, only samples taken directly from the outlet of the PTFE purification columns were analysed.

Vincentka mineral water was bought in a local supermarket. A precipitate present in the sample was removed by filtration through a dense paper filter (the first 5-ml portion of the filtrate was discarded) and fine particulates in the filtrate were removed by an Anotop 10 0.02-μm disposable syringe filter (Anotec Separations, Banbury, U.K.). The membrane-filtered sample was diluted (1:25) with freshly demineralized water before the ITP analysis.

TABLE I

OPERATIONAL SYSTEMS

Parameter	*System No.*[a]				
	1	*2*	*3*	*4*	*5*
Leading electrolyte					
Solvent	H_2O–PEG	H_2O–PEG	H_2O–PEG	H_2O–PEG	H_2O–PEG
Proportions (v/v)	100–50:0–50	55:45	60:40	55:45	55:45
Leading cation	NH_4^+	NH_4^+	NH_4^+	NH_4^+	NH_4^+
Concentration (m*M*)	10	10	20	20	5
Counter ion	OAc^-	OAc^-	OAc^-	OAc^-	OAc^-
pH_L[b]	5.0	5.0	5.0	5.0	5.0
Additive	HEC	HEC	HEC	HEC	HEC
Concentration (%, w/v)	0.2	0.2	0.2	0.2	0.2
Co-counter ion	–	–	Mal^-	–	–
Concentration	–	–	3	–	–
Terminating electrolyte					
Solvent	H_2O				
Terminating cation	TBA^+ (TEA^+)				
Concentration (m*M*)	3 (5)				
Counter ion	ClO_4^-				

[a] OAc^- = acetate; Mal^- = malate; TBA^+ = tetrabutylammonium; TEA^+ = tetraethylammonium.

[b] The desired amount of PEG was added to a stock aqueous solution of the required electrolyte composition and pH.

An aqueous extract of apple flesh[8] was obtained from the Research Institute of Fruit Production (Bojnice, Czechoslovakia). The extract was diluted (1:5) with freshly demineralized water before the ITP analysis.

RESULTS AND DISCUSSION

Influence of PEG on the effective mobilities of alkali and alkaline earth metal cations

The operational systems used are listed in Table I. Experiments aimed at investigating the influence of the concentration of PEG in the leading electrolyte on the effective mobilities of the cations were carried out in operational system No. 1. In preliminary experiments we found that the molecular weight of PEG (evaluated for molecular weights in the range 200–3000) has no observable influence on the effective mobilities of the cations, and the polymer preparations with average molecular weights in the range 200–300 were arbitrarily used throughout.

To show the effect of PEG on the effective mobilities of alkali and alkaline earth metal cations, their relative step heights as obtained from the response of the conductivity detector in the analytical column plotted against the concentration of the polymer in the leading electrolyte (Fig. 1). Here, the leading ion (NH_4^+) and Li^+ were taken as reference constituents (see ref. 10, p. 307) as they exhibited minimum relative changes in their step heights (when compared with the remainder of the cations) with increasing concentration of PEG.

From the plots in Fig. 1, it is apparent that PEG retarded the alkaline and alkaline earth metal cations in the order Ba > Sr > Ca > Mg, *i.e.*, in order of their extractibilities from the aqueous phase in the presence of the polymer[22]. This retardation order also agrees with that achieved in ITP when 18-crown-6 was used as a complexing agent[12,13]. The plotted data for the alkali metal cations show that PEG is

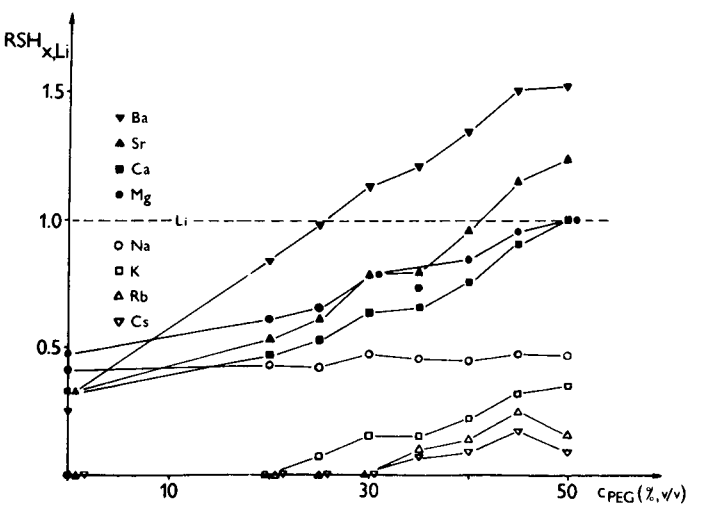

Fig. 1. Dependences of relative step heights ($RSH_{x,Li}$) of the alkali and alkaline earth metal cations on the concentration of PEG in the leading electrolyte. Operational system No. 1 (Table I) was used. The driving current in the analytical column was 45 μA. For further details, see text.

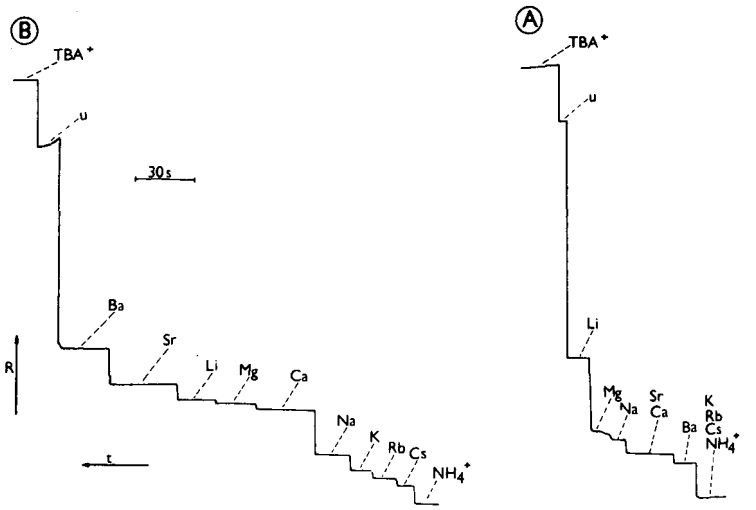

Fig. 2. Isotachopherograms for the separations of alkali and alkaline earth metal cations. Only the records from the analytical column are given. (A) Leading electrolyte without PEG; (B) leading electrolyte containing water–PEG (55:45). The driving currents were 250 and 45 μA in the preparation and analytical columns, respectively. R, t = increasing resistance and time, respectively. u = Unidentified impurity originating from the terminating electrolyte.

responsible also for their differentiation with a relative retardation in the order $K^+ > Rb^+ > Cs^+ > Na^+ \approx Li^+$. Although this is analogous to 18-crown-6[12–14], the extent to which, *e.g.*, K^+ is influenced is lower relative to the effect of the cyclic polyether. This difference can be explained via a macrocyclic effect of the crown ether[20].

From the differences in the relative step heights of the cations (Fig. 1), it is clear that water–PEG mixtures can be effective in separating various groups of alkali and alkaline earth metal cations and that their complete ITP resolution can be expected at 40–45% (v/v) concentrations of PEG in the leading electrolyte. The isotachopherograms in Fig. 2 clearly illustrate such a possibility. To our knowledge, a complete ITP separation of this group of cations by using other separation systems (see Introduction) has not been reported previously.

Anionically migrating constituents forming complexes with alkaline earth metal cations usually retard them in the ITP separations in the order Ca > Sr > Mg $\geqslant$ Ba (see, *e.g.*, ref. 11). The use of such complexing agents in water–PEG separation systems could be effective in enhancing differences in the effective mobilities of alkaline earth metal cations relative to alkali metal cations in general or in changing the ITP migration configurations of the former group of cations. The isotachopherogram in Fig. 3 illustrates such an effect due to the use of malate in the leading electrolyte. Although no detailed study of the applicability of complex equilibria in water–PEG systems was carried out, general rules for their use in ITP (see, *e.g.*, refs. 26 and 27) suggest that they may be useful to complement the differentiating power of PEG in some practical analytical problems.

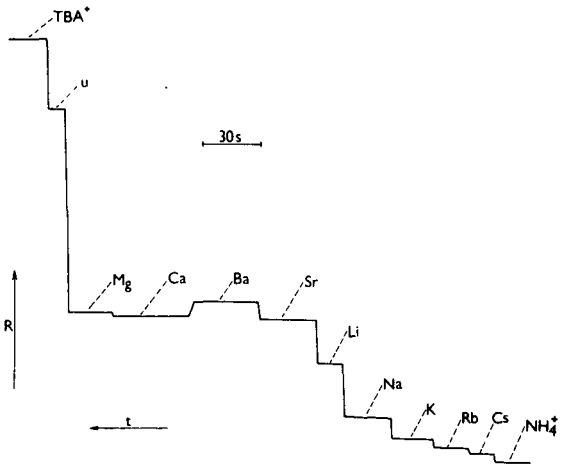

Fig. 3. Isotachopherogram for the separation of alkali and alkaline earth metal cations in a water–PEG electrolyte system containing malate as a complexing co-counter ion (system No. 3, Table I). The driving currents were 350 and 75 μA in the presentation and analytical columns, respectively. Symbols as in Fig. 2.

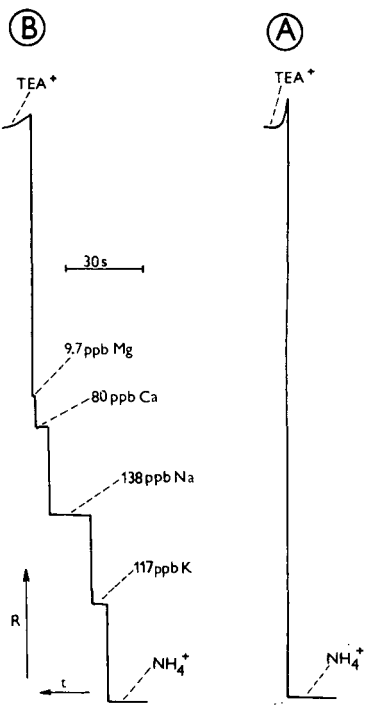

Fig. 4. Isotachopherogram from the analytical control of water demineralization. (A) Blank run (the terminating electrolyte solution was injected); (B) a 300-μl volume of demineralized water further purified by a two-fold recirculation through the columns packed with a mixed-bed ion exchanger (for details, see Experimental). The 300-μl sample volume was injected in 30-μl aliquots (the volume of the internal sample loop of the injection valve) by a 10-fold repetition of the injection. Each sample aliquot was transferred into the preseparation column by applying the driving current (250 μA) for 10 s. Approximately 150 s after the injection of the last aliquot, a steady state was achieved. The driving current in the analytical column was 50 μA. Symbols as in Fig. 2.

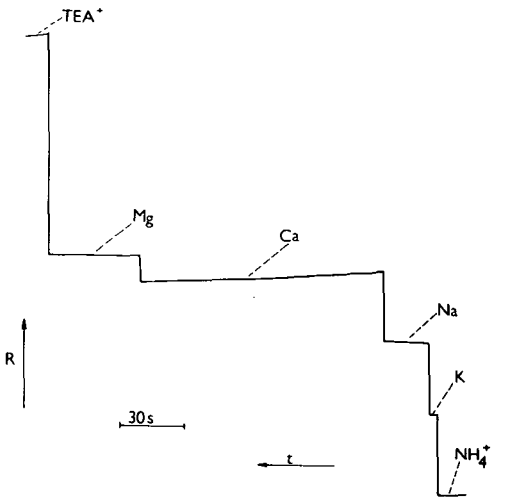

Fig. 5. Determination of K^+, Na^+, Ca^{2+} and Mg^{2+} in tap water. Only the record from the analytical column is given. The sample taken for the analysis was diluted with demineralized water (1:4). The driving currents were 250 and 50 μA in the preseparation and analytical columns, respectively. Symbols as in Fig. 2.

Examples of ITP separations in water–PEG operational systems

The determination of residual alkali and alkaline earth metal cations is often required in the analytical control of water demineralization. ITP has inherent advantages for this type of application, since a low load capacity of the separation

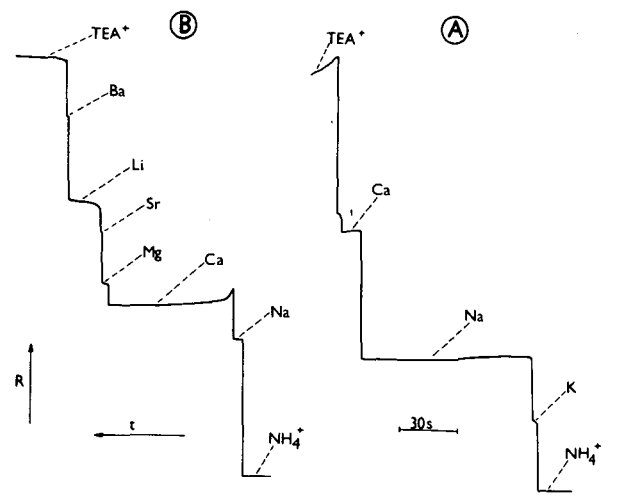

Fig. 6. Isotachopherograms from (A) the preseparation and (B) the analytical columns obtained in the analysis of Vincentka mineral water. A 30-μl volume of the sample pretreated as described under Experimental was taken for analysis. The driving currents were 350 and 25 μA in the preseparation and analytical columns, respectively. For the other details, see the text. Symbols as in Fig. 2.

compartment is sufficient also when a high sample volume is to be injected while a short analysis time is typical[28]. The isotachopherograms in Fig. 4 were obtained in the analytical control of water demineralization carried out as described under Experimental. Here, an analysis time of less than 5 min could easily be achieved for a 300-μl injection volume with operational system No. 2 (Table I). The blank run in Fig. 4A shows that the electrolyte solutions used contained alkali and alkaline earth metal cations at concentrations that were undetectable under our working conditions, so that they did not introduce (to some extent subtractable) systematic analytical errors.

Very reproducible ITP determinations of K^+, Na^+, Ca^{2+} and Mg^{2+} in drinking water (0.5–1.5% relative standard deviations) were possible by using operational system No. 2 (Table I) with an analysis time of less than 10 min (see also Fig. 5).

Alkali and alkaline earth metal cations need to be determined in mineral waters, which represent samples with typically extremely varying concentrations of cations. When their determinations are to be carried out in one run with minimum sample preparation, a high load capacity of the separation compartment needs to be combined with low detection limits attainable by a universal detector, *i.e.*, a high performance index of the separation compartment[29] is necessary. At present, such contradictory requirements in ITP can be best fulfilled by using a column-coupling configuration of the separation unit[23]. The isotachopherograms in Fig. 6 were obtained from the analysis of Vincentka mineral water containing the cations of interest within a $2 \cdot 10^3$–$3 \cdot 10^3$ concentration span (Na^+ *vs.* Sr^{2+} and Ba^{2+}). Here, the analysis was divided into two stages, *i.e.*, the determination of macroconstituents (K, Na, Ca) in the preseparation column (operational system No. 4, Table I), followed by their removal from the separation compartment, while the microconstituents (Mg, Sr, Li, Ba) were evaluated under more favourable detection conditions (operational system No. 5) in the analytical column. Diffusion of ions into the analytical column in the bifurcation block during their removal (K^+, Na^+ and a major part of Ca^{2+}) from the separation compartment was mainly responsible for the presence of Na^+ in this column (the detected zone corresponded to less than 0.1% of the total amount of Na^+ taken for the analysis). Although this undesirable transfer of the ions into the analytical column is difficult to suppress[30], it did not disturb the determination of the microconstituents in the analytical column. For example, in our particular case, the reproducibilities of determinations were characterized by 0.2–7.0% relative standard deviations with the highest values being typical of Sr^{2+} and Ba^{2+} (occurring in the sample at *ca.* 10^3 lower concentrations than Na^+).

It has been reported[7] that determinations of K^+, Na^+, Ca^{2+} and Mg^{2+} in urine may account for about 50% of all routine analyses of urine samples carried out in some clinical laboratories. Possibilities of using ITP in a methanolic operational system in the determination of these cations in urine has been studied[7]. A high dynamic concentration range may be required when all of these cations are to be determined in one ITP run. The isotachopherograms in Fig. 7 obtained in the analysis of a urine sample with a very low concentration of Mg^{2+} illustrate that the ITP analysis in the column-coupling system with the electrolyte system based on a water–PEG mixture can fulfil these extreme analytical requirements. An appropriate sample dilution was sufficient in this instance, so that no precipitation of the anions before the analysis was needed[7].

Determinations of Ca^{2+}, Mg^{2+} and K^+ in apple flesh are important as they

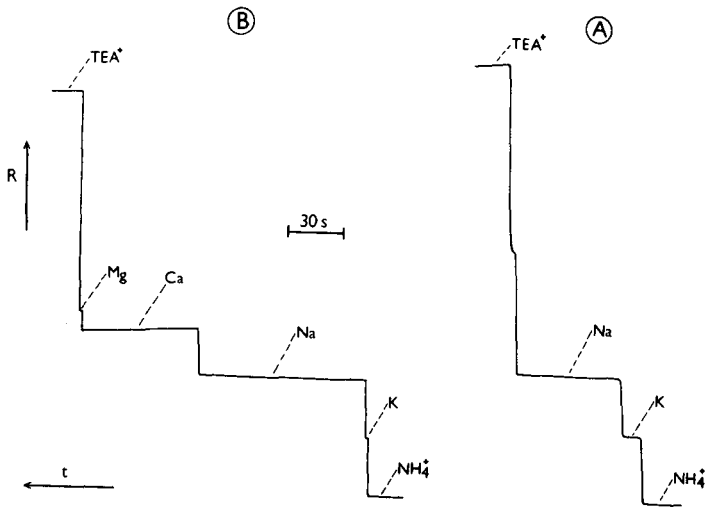

Fig. 7. Isotachopherograms from the analysis of a urine sample. The record from (A) the preseparation and (B) the analytical columns served to evaluate macro- and microconstituents, respectively. The injected sample (30 μl) was diluted (1:100) with demineralized water. The driving currents were 250 and 50 μA in the preseparation and analytical columns, respectively. Symbols as in Fig. 2.

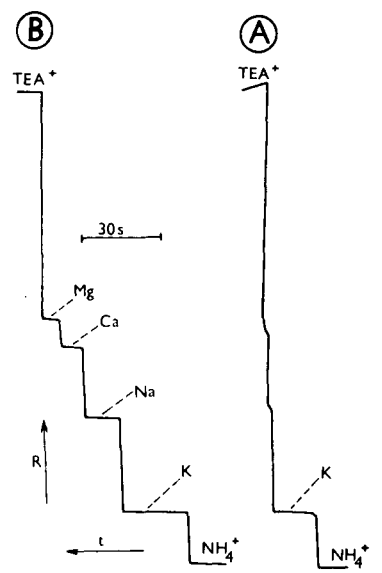

Fig. 8. Isotachopherograms from the analysis of an aqueous extract of apple flesh. K^+ in the extract (see Experimental) was evaluated from the response of the detector in the preseparation column (A) and the remainder of the cations from the response of the detector in the analytical column (B). The driving currents were 250 and 40 μA in the preseparation and analytical columns, respectively. Symbols as in Fig. 2.

provide important data with respect to long-term storage of apples. ITP combined with a simple extraction procedure[8] can be a convenient method for this purpose, especially owing to its high precision in the determination of Ca^{2+} (2% relative standard deviation) since the concentration of this cation is of primary diagnostic value in this respect. The isotachopherograms in Fig. 8 for the analysis of an aqueous extract of apple flesh for alkali and alkaline earth metal cations were obtained with the operational system No. 2 (Table I). Under these conditions, Ca^{2+}, Na^+ and Mg^{2+} could be determined in one run together with K^+ also when K^+ was present in the sample at a 10^3-fold excess.

CONCLUSIONS

This work has clearly shown that water–PEG mixtures are useful for ITP separations and/or determinations of alkali and alkaline earth metal cations. An appropriate ratio of both components in the leading electrolyte enables a complete resolution of this group of cations to be achieved in one ITP run. The favourable price of PEG makes this mixture a convenient alternative to separations based on the use of crown ethers. From the practical point of view, it is also important that the separation need not be performed in the separation compartment made of highly inert materials, as is necessary, *e.g.*, with methanolic operational systems.

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Note

Acylation effects on chiral recognition of racemic amines and alcohols by new polar and non-polar cyclodextrin derivative gas chromatographic phases

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Various derivatives of amino acids and peptides have been used as chiral stationary phases (CSPs) for gas chromatography (GC) since the initial work in the area[1–8]. The most widely used CSP of the genre, today, is Chirasil-Val (*i.e.*, L-valine-*tert.*-butylamide coupled to a siloxane copolymer[9,10]. Also, at that time, a number of investigators were attempting to use cyclodextrins as GC stationary phases[11–16]. Although interesting selectivities were obtained, the crystalline cyclodextrins were sometimes difficult to use and coat and were inefficient. In general, they had to be dissolved or dispersed in another solvent prior to use[11–18]. Of these, the permethylated cyclodextrins dissolved in traditional achiral GC liquid stationary phases seemed to give the best enantiomeric separations[17,18]. The role of cyclodextrins in chromatography paralleled that of other CSPs in that liquid chromatographic developments occurred much more rapidly in the last decade.

Recently, there have been reports on hydrophobic cyclodextrin derivatives that are effective CSPs in GC[19–22]. These liquid derivatives were directly coated on glass capillaries and shown to resolve a number of racemic solutes. We have developed a new polar, hydrophilic cyclodextrin derivative and have compared it to the alkyl derivative[23,24]. Very different properties and enantioselectivities were observed.

Often in chiral, as in achiral, GC analysis, it is necessary to increase the volatility of polar analytes by making less polar derivatives. For example, it is common practice to make acetyl or trifluoroacetyl derivatives of amines and alcohols, or esters from carboxylic acids. These reactions are simple and quantitative and go rapidly to completion under proper conditions. One factor which generally is not appreciated is that these achiral derivative groups can play a significant role in enantioselective separations. As will be shown in this communication, the choice of an acylation reagent can mean the difference between success or failure in obtaining an enantiomeric separation. In addition, an optimal acyl derivative for one CSP may be marginal or poor for another CSP. In a previous work, Charles and Gil-Av[3] found that the resolution of enantiomeric aminoalcohols improved somewhat as the size of the acyl derivative increased from propionyl to pivaloyl.

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EXPERIMENTAL

Materials

Fused-silica capillary tubing (0.25 mm I.D.) was obtained from Alltech. Dipentyl cyclodextrins (DP-CDs) were made as previously reported[22,24]. The heptakis (2,6-di-O-pentyl) cyclodextrins were made by reacting excess 1-bromopentane with 3.0 g of the desired cyclodextrin in 30 ml of dimethyl sulfoxide (DMSO) at 50°C for 2 h, then at room temperature for 24 h. The product was isolated by precipitation with water. The precipitate was washed and dissolved in chloroform. The chloroform solution was washed with water and the chloroform was evaporated to leave the product, which was subsequently vacuum-dried. Permethyl derivatives of O-[(*S*)-2-hydroxypropyl] cyclodextrin (PMHP-CD) mixtures were made in two steps. First, the respective cyclodextrin was dissolved in aqueous sodium hydroxide (5%, w/w) and the solution cooled in an ice-bath; then (*S*)-propylene oxide was slowly added while stirring. After about 6 h in an ice-bath the reaction was allowed to proceed for a day at room temperature, neutralized and dialyzed briefly in order to remove the contam-

TABLE I

COMPARISON OF GC SEPARATION DATA FOR DIFFERENT ACYL DERIVATIVES OF CHIRAL AMINES AND ALCOHOLS

Compound	*Optimal acyl group*[a]	α	R_s	*Range of R_s for other acyl derivatives*[b]	*Column*[c]
1-Aminoindane	$-\overset{\overset{O}{\|}}{C}-CH_3$	1.03	1.6	0–0.9	PMHP-β-CD
1,2,3,4-Tetrahydro-α--naphthylamine	$-\overset{\overset{O}{\|}}{C}-CH_3$ or $-\overset{\overset{O}{\|}}{C}-CF_3$	1.04	1.5	0.7–1.2	PMHP-β-CD
1-Phenylethylamine	$-\overset{\overset{O}{\|}}{C}-CH_3$	1.03	1.3	0–0.9	PMHP-β-CD
1-Methoxy-2-aminopropane	$-\overset{\overset{O}{\|}}{C}-CH_2Cl$	1.07	1.5	0–1.1	PMHP-β-CD
2-Amino-1-propanol	$-\overset{\overset{O}{\|}}{C}-CH_3$	1.04	1.5	0.8–1.2	PMHP-β-CD
1-(α-Naphthyl)-ethylamine	$-\overset{\overset{O}{\|}}{C}-CH_3$	1.02	1.4	0	PMHP-β-CD

(Continued on page 156)

TABLE I *(continued)*

Compound	*Optimal acyl group*[a]	α	R_s	*Range of R_s for other acyl derivatives*[b]	*Column*[c]
α-Amino-γ-butyro-lactone	$-\overset{\overset{O}{\|}}{C}-CH_3$	1.03	1.2	0–0.6	PMHP-β-CD
1,2,3,4-Tetrahydro-α-napthylamine	$-\overset{\overset{O}{\|}}{C}-CH_3$	1.02	1.4	0–0.5	DP-β-CD
2-Amino-1-hexanol	$N-\overset{\overset{O}{\|}}{C}-CH_2Cl$	1.01	1.1	0	PMHP-β-CD
1-Indanol	$-\overset{\overset{O}{\|}}{C}-CF_3$	1.01	1.0	0	PMHP-β-CD
1-Aminoindan	$-\overset{\overset{O}{\|}}{C}-CF_3$	1.31	2.8	1.1–1.3	DP-α-CD
2-Amino-4-methyl-1-butanol	Underivatized	1.11	1.5	0.4–1.3	PMHP-β-CD
trans-Cyclohexane-1,2-diol	$-\overset{\overset{O}{\|}}{C}-CH_2Cl$	1.05	1.4	0–1.0	PMHP-β-CD
1,2,3,4-Tetrahydro-1-naphthol	$-\overset{\overset{O}{\|}}{C}-CH_3$	1.07	1.2	0–0.6	DP-α-CD

[a] The optimal acyl group is the derivative that gave the best separation factor (α) and resolution (R_s) on the indicated column under optimized conditions.

[b] This resolution range was for all the other acyl derivatives tested (see Experimental for complete list)

[c] The abbreviation PMHP-β-CD stands for the permethyl-(*S*)-hydroxypropyl-β-cyclodextrin stationary phase. These columns were 30 m in length. The abbreviation DP-α-CD stands for the 2,6-O-dipentyl-α-cyclodextrin stationary phase. These columns were 20 m in length.

inating salts. The reformed solution was filtered and the product obtained by freeze-drying. Permethylation was achieved by a reaction with methyl iodide after the dissolution of cyclodextrin derivative in a solution of sodium hydride in DMSO[24,25]. Additional data on the make-up and properties of this compound are to be published subsequently. The capillaries were coated via the static method as previously reported[26].

Acetic anhydride, trifluoroacetic anhydride, chloroacetic anhydride, dichloro-

acetic anhydride and trichloroacetic anhydride were obtained from Aldrich. In each case, approximately 1.0 mg of the racemic analyte was dissolved in 0.5 ml of methylene chloride and 200 μl of the desired anhydride were added. After reaction, dry nitrogen was bubbled through the solution to remove excess reagent.

Methods

Both Hewlett-Packard (5710A) and Varian (3700) gas chromatographs were used for all separations. Split injection and flame ionization detection were utilized. The injection port temperature was 200°C and nitrogen was used as the carrier gas.

RESULTS AND DISCUSSION

A comparison of enantiomeric separation data for a variety of acyl-derivatized analytes is given in Table I. In each case the temperature was optimized to produce the highest resolution. It is apparent that enantioselectivity can be very dependent on the type of acylation reagent used. Indeed, in some cases, it can mean the difference between obtaining or not obtaining a separation of enantiomers. For the majority of compounds listed in Table I, there were one or more common racemic acyl derivatives that could not be resolved. Fig. 1 nicely illustrates this phenomenon. In this case, the

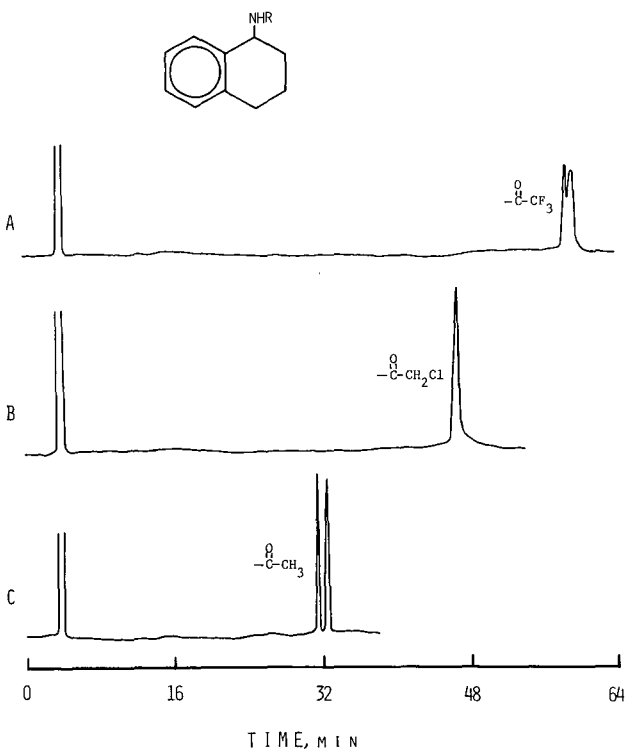

Fig. 1. Chromatograms comparing the resolution of three different acyl derivatives of racemic 1,2,3,4-tetrahydro-1-naphthylamine. The acetyl derivative (A) gave the best resolution while the monochloroacetyl derivative was unresolved (B). All separations were done on a 30-m fused-silica capillary coated with DP-β-CD. The column temperature for (A) was 130°C while it was 180°C for (B) and (C).

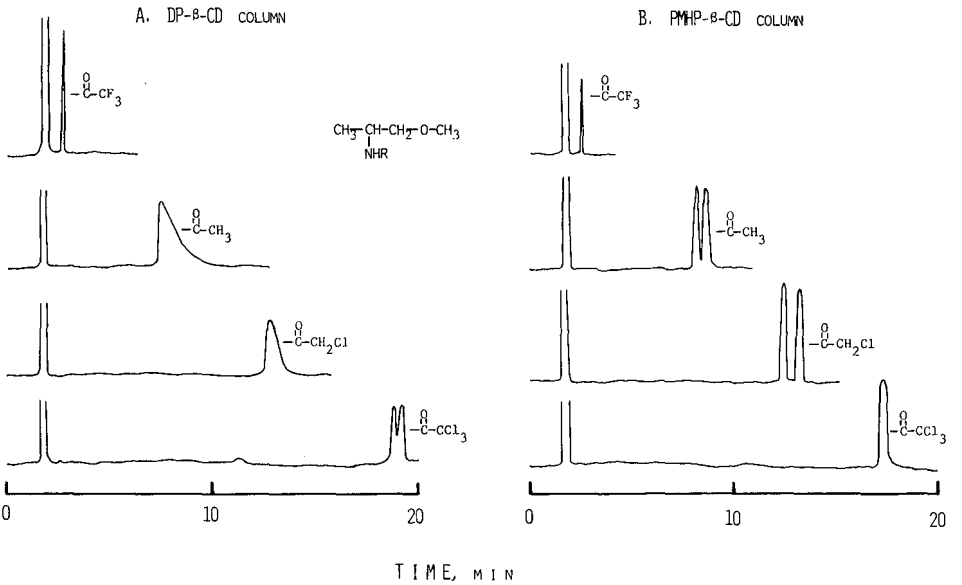

Fig. 2. Chromatograms showing the separation of different acyl derivatives of racemic 1-methoxy-2-aminopropane on two different cyclodextrin CSPs. On column A (DP-β-CD) only the trichloroacetyl derivative shows any resolution. On column B (PMHP-β-CD) only the monochloroactyl and acetyl derivative show resolution. Both columns were 30 m in length. The separations were done at 130°C.

monochloroacetyl-1,2,3,4-tetrahydro-1-naphthylamine was not resolved, while the trifluoroacetyl derivative shows partial resolution and the acetyl derivative is baseline-resolved.

Another interesting fact is that the best derivative of a racemate for one CSP may, or may not, be optimal for another CSP, even if the stationary phases are related as in the case of substituted cyclodextrins. Occasionally, one particular derivative resolves best on different CSPs; see, for example, the separation data for 1,2,3,4-tetrahydro-1-naphthylamine (acetyl derivative) on PMHP-β-CD and DP-β-CD columns (Table I). In other cases, completely different derivatives give better separations on these columns. This trend is shown for racemic 1-methoxy-2-aminopropane in Fig. 2. The acetyl derivative is baseline-separated on the PMHP-β-CD column but is unresolved on the DP-β-CD column. Conversely, the trichloroacetyl derivative is partially resolved on the DP-β-CD phase but is unresolved on the PMHP-β-CD column.

It is clear that when making simple acyl derivatives for chiral GC analysis, volatility cannot be the only consideration. Since one acyl derivative is as simple as another to make, it provides an additional parameter by which these separations can be optimized. Not only can a separation be improved by selecting the best derivative for a particular CSP, but sometimes it can mean the difference in obtaining any resolution. It has been reported that acylation of hydroxy compounds can lead to small separation factors when using the Chirasil-Val column[27]. This was thought to be due to the elimination of the donor functions for hydrogen bonding to the CSP[27]. This does not seem to be the case with the new derivatized cyclodextrin stationary phases. Indeed, there have been a number of separations of racemates that contain no hydrogen bonding groups, on these columns[17–24].

It is interesting to consider the role these closely related acyl groups play in chiral recognition. For example, the trifluoroacetyl and acetyl groups have about the same size and geometry. Any difference in the enantioselectivity of their analogues must be due to differences in polarity and hydrogen-bonding ability. On the other hand, the chlorinated acyl groups are much bulkier and steric factors may be important when they are used. In at least one case (1-methoxy-2-aminopropane, Fig. 2B) the trichloroacetyl derivative gave the best separation, possibly because it decreased the volatility of the solute, allowing it to interact with the CSP longer. In order to better understand these effects as they relate to cyclodextrins, thorough thermodynamic studies must be completed. Plots of retention *versus* the inverse of temperature have yet to show enantiomeric reversals for these CSPs[23,24]. However, one thing is clear. From a practical stand-point, acylation offers the chromatographer a facile way to improve marginal separations when all other parameters have been optimized. Currently, we are further evaluating the role of different acyl groups in chiral recognition using gas phase calorimetry, computer modeling and energy minimization calculations.

ACKNOWLEDGEMENT

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Note

Gas chromatographic determination of polychlorinated dibenzo-*p*-dioxins in wood preservatives

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Xylamit żeglarski, Xylamit stolarski and Antox, the most popular wood preservatives in Poland, were analysed. These oily liquids contain 5–6% of technical pentachlorophenol (PCP), dissolved in 1-chloronaphthalene, in resins or in chlorinated light coal tar. Technical PCP is commonly used as a wood preservative in the Xylamit formulation. Significant amounts of polychlorinated dibenzodioxins (PCDDs) have been found in technical PCP, chlorobenzenes and other chlorinated aromatic compounds[1–4].

Technical PCP has been shown to be contaminated by tetra- to octachlorodibenzodioxins. Recently obtained data on the concentrations of PCDDs in technical PCP indicate that concentration of dioxins varies from 361 to 1723 ppm for octa-(OCDD), from 119 to 562 ppm for hepta-(HPCDD) and from 1 to 38.5 ppm for hexachlorodibenzodioxins (HXCDD)[4].

EXPERIMENTAL

Apparatus

An N-504 gas chromatograph equipped with an electron-capture detector, an SE-30 capillary column (50 m × 0.3 mm I.D.) was used with nitrogen as the carrier gas and helium as make-up gas, a split–splitless injector and a TZ-4620 analog recorder.

A KB-5101 high-performance liquid chromatograph with an ODS (10 μm) column (250 × 4 mm I.D.) was equipped with a 254-nm UV detector.

The pyrolysis reactor for PCDD isomers was based on the design described by Nestrick *et al.*[5]. A glass tube (10 mm I.D.) was inserted in a temperature-controlled furnace (280–300°C, 1°C accuracy). Amounts of 20 mg of potassium pentachlorophenate solutions were injected onto a 2-cm glass plug and processed as described[6] for hexachlorodioxins.

A TH-24 haematological thermostat was obtained from DHN, Poland.

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PCDD standards

A 2,3,7,8-Tetrachlorodibenzodioxin (TCCD) standard solution for quantitative analysis (10 μg/ml) in toluene was obtained (from Supelco (Bellefonte, PA, U.S.A.). Additionally, the compound was synthesized by pyrolysis of 2,4,5-trichlorophenol and reversed-phase high-performance liquid chromatographic (RP-HPLC) purification as described[5,7].

Octachlorodibenzodioxin standard was synthesized by pyrolysis of analytical-reagent grade pentachlorophenol (Aldrich Milwaukee, WI, U.S.A.), purified by precipitation from a crude benzene extract, recrystallized twice from benzene and determined by weighing 10.2 mg OCDD. Then the sample was dissolved in toluene and analysed by capillary gas chromatography with electron-capture detection (GC–ECD).

Heptachlorodibenzodioxin standard was synthesized by photolytic degradation of OCDD standard solution and RP-HPLC separation from lower chlorinated PCDDs. This solution of two HPCDD isomers was treated as a quantitative standard.

Chemicals

Analytical-reagent grade chemicals were used unless indicated otherwise.

Silica gel (50–100 mesh) for chromatography (Suchardt, Munich, F.R.G.), was processed as described by Miles *et al.*[4] and stored in a desiccator over phosphorus pentoxide.

Alumina for chromatography, basic, activity I, was obtained from POCH (Gliwice, Poland). It was Soxhlet extracted with *n*-hexane and methylene chloride, dried and stored under the same conditions as the silica gel.

Anhydrous sodium sulphate (POCH) was purified by the same procedure as for alumina. Sulphuric acid, hydrochloric acid, potassium hydroxide and sodium hydroxide (POCH) were used without further purification. *n*-Hexane (Reachim, U.S.S.R.) was washed with concentrated sulphuric acid and water, refluxed with sodium, and distilled in glass. Methylene chloride, benzene, toluene and methanol were distilled in glass.

The preparations of 22% and 44% concentrated sulphuric acid on silica gel, 33% 1 M sodium hydroxide on silica gel and activated 10% silver nitrate on silica gel were described by Lamparski *et al.*[8]

Procedure

All the samples of Xylamit wood preservatives and three samples of technical PCP were obtained from the Building Research Institute (Warsaw, Poland). For the determination of PCDDs in Xylamit samples, all glassware and parts of the apparatus that could come into contact with any solution were cleaned as described by Lamparski and Nestrick[9]. Immediately before use they were rinsed twice with methylene chloride. The procedure for the determination of PCDDs in Xylamit samples was based on the method described by Hagenmaier and Brunner[10].

Xylamit samples analysis

Two 5-g portions of each kind of Xylamit (Xylamit stolarski, Xylamit żeglarski and Antox) were weighed into 50-ml beakers. After addition of 30-ml portions of

n-hexane, one portion was spiked with native 2,3,3,8-TCDD, HPCDDs and OCDD at concentrations corresponding to 10 ppb[a], 25 ppm and 50 ppm, respectively. Each pair of Xylamit solutions was analysed according to the following procedure.

The solution was passed through the chromatographic column (30 cm × 2.0 cm I.D.) filled with *ca.* 30 g of basic alumina, freshly prewashed with 50 ml of *n*-hexane. When the sample solution had drained, an additional 100 ml *n*-hexane and 200 ml of methylene chloride-*n*-hexane (2:98) were introduced into the column. The column effluent was discarded. Polychlorinated dibenzo-*p*-dioxins were removed from the alumina column by elution with 250 ml of methylene chloride–*n*-hexane (50:50). This effluent was collected in a beaker and concentrated to 5 ml under a stream of specially purified nitrogen[8].

The concentrated dioxin solution was passed through a combined clean-up column to remove potential interferences. This column was filled, from bottom to top, with a glass-wool plug, 2 g of silica gel, 5 g of 33% 1 *M* sodium hydroxide on silica gel, 2 g of silica gel, 10 g of 44% sulphuric acid on silica gel, 10 g of 22% sulphuric acid on silicagel and 2 g of silica gel. After preparation, the column was prewashed with 25 ml of *n*-hexane immediately before use. When the concentrated dioxin solution had drained, the column was rinsed with 50 ml of *n*-hexane and the effluent was concentrated to *ca.* 2 ml under a stream of nitrogen and passed through a silver nitrate column[8]. When it had drained, the column was eluted with 10 ml of *n*-hexane.

The silver nitrate column effluent was evaporated to approximately 2 ml under a stream of purified nitrogen and introduced into another basic alumina column (150 mm × 6 mm I.D.) containing a 1.5-g bed of freshly activated alumina on top of which was a 1-cm layer of sodium sulphate. When the effluent had drained, the column was rinsed with 20 ml of methylene chloride–*n*-hexane (2:98) and the effluent was discarded. PCDDs were eluted with 20 ml of methylene chloride–*n*-hexane (50:50) and collected in a 25-ml conical test-tube.

The methylene chloride–*n*-hexane (50:50) PCDD fraction was evaporated to dryness under a stream of purified nitrogen. During evaporation the tube containing the solution was gently heated to *ca.* 70°C in a TH-24 haematological thermostat. The residue in the tube was dissolved in 5 ml of toluene, transferred to a PTFE-sealed glass vial (Supelco) and stored in a refrigerator until the GC–ECD analysis.

PCP analysis

Three different technical PCP samples (NZPO Organika-Rokita, Dolny Brzeg, Poland, typical components in the Xylamit formulation, were processed as described below.

A 5-g amount of PCP was dissolved in 500 ml of 0.5 *M* potassium hydroxide and the solution was extracted twice with 100-ml portions of *n*-hexane. The *n*-hexane solutions were combined and washed with redistilled water. After drying with sodium sulphate and evaporation in a stream of purified nitrogen to a volume of *ca.* 5 ml, the concentrate was inserted in a basic alumina column (150 mm × 8 mm I.D.) filled with 5 g of freshly activated alumina. This column was first eluted with 50 ml of methylene

[a] Throughout this article, the American billion (10^9) is meant.

chloride–*n*-hexane (2:98) and the effluent was discarded. Subsequently the PCDDs were eluted with 60 ml of methylene chloride–*n*-hexane (50:50) and collected in a 75-ml calibrated conical tube.

This solution was concentrated to 20 ml in a stream of nitrogen, and transferred to a PTFE-sealed amber-glass bottle and stored in a refrigerator as a stock PCDD–PCP solution. A 1-ml volume of this solution was evaporated to dryness in a stream of purified nitrogen and the residue was dissolved in 4 ml of toluene. Additionally, a 5-g portion of PCP spiked with 2,3,7,8-TCDD to a concentration corresponding to 10 ppb was processed.

Selective separation of 2,3,7,8-TCDD

The separation of 2,3,7,8-TCDD from other chlorinated dibenzodioxins and dibenzofurans was carried out using the method described by Hagenmaier *et al.*[2].

Samples of 5 g of Xylamit were dissolved, spiked with 2,3,7,8-TCDD and PCDDs were isolated as described above. The PCDD residue was dissolved in 10 ml of benzene. The 2,3,7,8-TCDD from this solution and from a solution of technical PCP were separated and processed following Hagenmaier *et al.*'s procedure.

The benzene solution was introduced into a column filled with 2.5 g of alumina Woelm B Super I and 2 g of sodium sulphate on the top of the column. A 30-ml volume of methylene chloride–*n*-hexane (20:80) was passed through the column and the effluent was discarded. 2,3,7,8-TCDD was eluted from the alumina column with 25 ml of methylene chloride–*n*-hexane (50:50). This fraction was evaporated to dryness under purified nitrogen and the residue was dissolved in 50 μl of chloroform (Merck, Darmstadt, F.R.G.). This solution was injected into an ODS HPLC column (250 × 4 mm I.D.). The column was eluted with methanol at 2.75 ml/min at 25°C. The retention time of 2,3,7,8-TCDD was 12 min 30 s. The fraction between 11 min 50 s and 13 min 10 s was collected and 2,3,7,8-TCDD was extracted with 2 ml of *n*-hexane. This solution was dried and 100 μl of toluene were added. After evaporation to approximately 50 μl, this sample was analyzed by capillary GC–ECD.

Gas chromatography

Sample aliquots of 1–2 μl in toluene were injected onto the column using the splitting technique (1:120). The oven temperature was programmed from 180°C (held for 2 min) at 5°C/min to 250°C (held) for 2,3,7,8-TCDD analysis or isothermally at 250°C for HPCDD and OCDD. The detector temperature was 300°C and the injector temperature 240°C. The carrier gas was chromatographic-grade nitrogen (ZLE Różу Luksemburg, Warsaw, Poland) and the make-up gas was helium. Retention times were 17.2 min for 2,3,7,8-TCDD and 80.6 min for OCDD. The limit of detection for 2,3,7,8-TCDD (standard solution) was 100 pg and that for HPCDDs and OCDD was 750 pg.

RESULTS AND DISCUSSION

The three wood preservative samples contain a 5–6% solution of technical PCP in light coal tar. The PCDD analytical data for each sample are given in Table I. The 1-ppb limit of detection for 2,3,7,8-TCDD was defined as 2.5 times the peak-to-valley noise close to the 2,3,7,8-TCDD retention time.

The limits of detection of HPCDD and OCDD were not calculated because of their very high concentrations in all of the samples.

The recoveries of 2,3,7,8-TCDD from the Xylamit and PCP samples were 45% and 65%, respectively, and those of HPCDD and OCDD isomers were 70% for Xylamit samples and 90–95% for PCP. This evaluation was carried out by GC–ECD determination of these compounds in each of the spiked and non-spiked final extracts by comparing the peak-height responses at their retention times with those of standards injected after the injection of the extract.

Gas chromatograms of the three Xylamit formulations are given in Fig. 1a–c and for technical PCP in Fig. 1d.

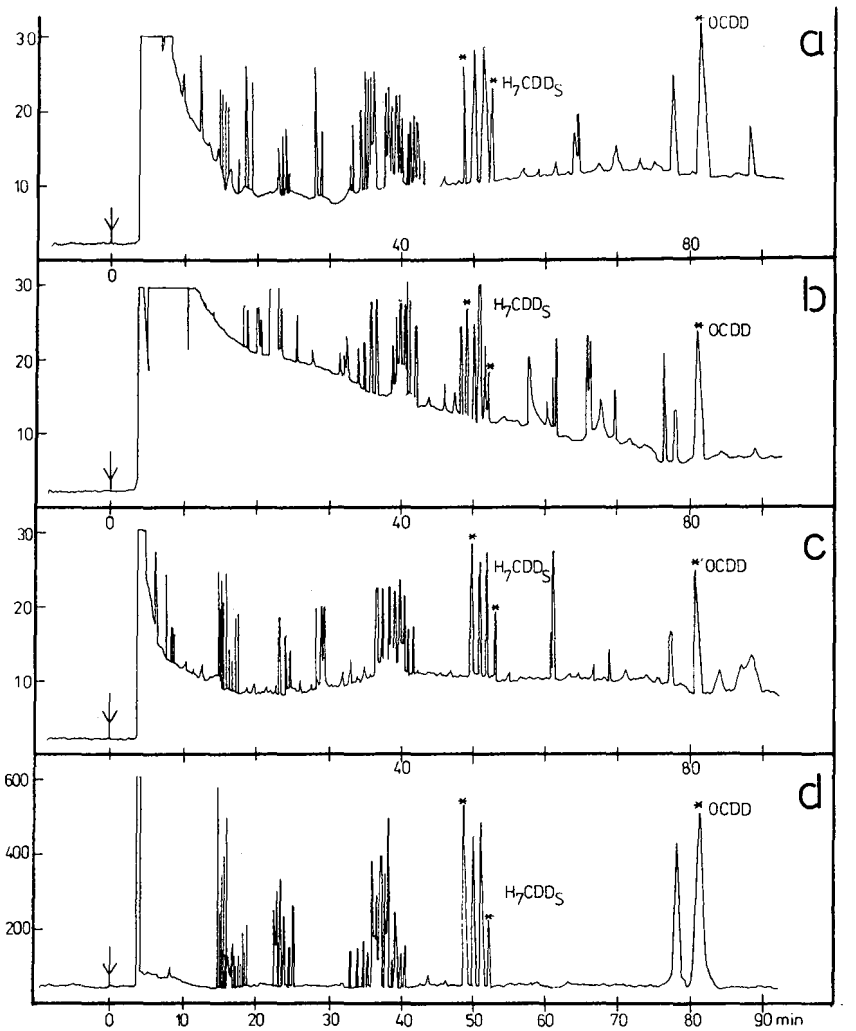

Fig. 1. Gas chromatograms of PCDD–polychlorinated dibenzofuran (PCDF) fractions from: (a) Xylamit stolarski, (b) Xylamit żeglarski, (c) Antox and (d) technical PCP.

TABLE I

RESULTS OF GC ANALYSIS OF WOOD PRESERVATIVE SAMPLE

Results are from three parallel analyses.

Sample	*2,3,7,8-TCDD*[a]	*HPCDDs (ppm)*	*OCDD (ppm)*
Xylamit stolarski	n.d.	9.2– 13.1	27.8– 33.9
Xylamit żeglarski	n.d.	7.7– 12.9	17.4– 21.8
Antox	n.d.	8.5– 10.1	19.9– 23.7
PCP 1	n.d.	166.1–236.2	547.3–712.0
PCP 2	n.d.	188.8–277.0	590.3–606.1
PCP 3	n.d.	117.3–119.3	435.6–486.9

[a] n.d. (Not detected) indicates that the compound was not detected within a detection limit of 1 ppb.

[b] HPCDDs is the sum of 1,2,3,4,6,7,8- and 1,2,3,4,6,7,9-heptachlorodibenzo-*p*-dioxin.

OCDD was the main PCDD isomer obtained in all the Xylamit and technical PCP samples. Two HPCDDs were determined as a sum. Polychlorinated dibenzofurans and some other unknown compounds that were found were not determined.

The results of the capillary GC–ECD analysis of the Antox 2,3,7,8-TCDD

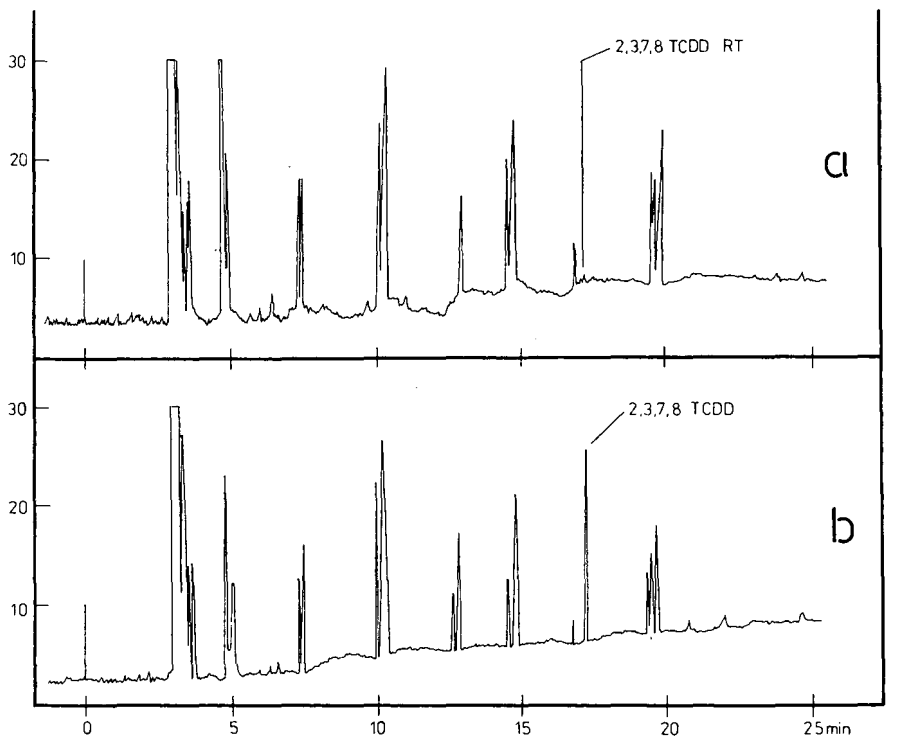

Fig. 2. Gas chromatograms of (a) Antox 2,3,7,8-TCDD fractions from alumina Woelm B Super I and (b) the same sample spiked with 2,3,7,8-TCDD standard solution to a concentration of 10 ppb. RT = Retention time.

fraction from alumina Woelm B Super I are shown in Fig. 2a and those for an Antox sample spiked with 2,3,7,8-TCDD standard solution (to obtain a concentration of *ca.* 10 ppb) in Fig. 2b.

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Note

Rapid gas chromatographic determination of volatile degradation products of glucosinolates in rapeseed oil

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Rapeseed, similarly to other *Brassicaceae* seeds and plants, contains up to 5% of sulphur heterocyclic compounds, called glucosinolates, which are partially decomposed during rapeseed processing or storage. The most important degradation products are isothiocyanates, vinyloxazolidinethione and nitriles, which contaminate crude rapeseed oils, impairing their refining, hydrogenation, transesterification, etc.

Volatile degradation products of glucosinolates are usualy determined by gas chromatography with flame ionization detection (FID) and the international standard method[1] is based on this principle. Compared with the content in seeds and extraction meals, the content of glucosinolate degradation products in oils is one (in crude oils) or several (in refined or hydrogenated oils) orders of magnitude lower so that an isolation and concentration step is necessary if FID is being used. Therefore, we have applied more selective and more sensitive detectors for this determination.

EXPERIMENTAL

Material

Plant-scale-produced crude and refined rapeseed oils were analysed. Butyl isothiocyanate, used as an internal standard, was synthesized according to Franzke *et al.*[2].

Apparatus

A Hewlett-Packard Model 5880 A apparatus equipped with flame photometric and nitrogen–phosphorus-specific detectors and a glass column (8 ft. × 1/16 in. I.D.) packed with 10% Carbowax 20M on Chromaton N AW DMCS (particle size 0.125–0.160 mm) was used. The carrier gas was nitrogen at a flow-rate of 30 ml min^{-1}. Linear programming from 100 to 200°C at 6°C min^{-1} [flame photometric detection (FDP)] or a constant temperature of 150°C [nitrogen–phosphorus detection (NPD)] was applied. The injection temperature was 220°C in both instances.

Procedure

About 3 g of rapeseed oil were weighed to the nearest 0.01 g, dissolved in 5 ml of

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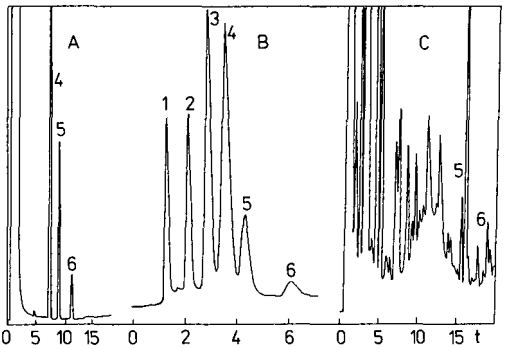

Fig. 1. Separation of volatile glucosinolate degradation products in rapeseed oil. Conditions as described in the text (FPD, NPD). FID: conditions according to ISO[1]. Injection temperatures 220°C in all instances. t = retention time (min). Detection: (A) FPD; (B) NPD; (C) FID. Degradation products: 1 = 1-cyanopropane; 2 = 1-cyano-3-butene; 3 = 1-cyano-4-pentene; 4 = butyl isothiocyanate; 5 = 3-butenyl isothiocyanate; 6 = 4-pentenyl isothiocyanate.

heptane, 1 ml of a standard heptane solution containing 10 μg of butyl isothiocyanate and 10 μg of 1-cyanopropane was added and 6 μl of the mixture were injected for the analysis. The column performance remains constant for 60 injections, and the column should then be replaced, if the sample is being injected directly into the column. The column performance may be prolonged if the sample is injected into the injection port, and the non-volatiles are removed after 60 injections.

RESULTS AND DISCUSSION

Well resolved peaks of isothiocyanates were obtained using FPD and well resolved peaks of both isothiocyanates and nitriles were obtained with NPD (Fig. 1). FPD gave results similar to those reported by Daun and Hougen[3], but the content of less volatile sulphur compounds was lower than those reported. Relative retention times of the most important volatile rapeseed glucosinolate degradation products are given in Table I.

The average contents of nitriles and isothiocyanates were 10–18 and 6–9 mg kg^{-1} in crude oils and 0.01–0.10 and 0.04–0.15 mg kg^{-1} in refined oils, respectively. The

TABLE I

RETENTION DATA AND SENSITIVITIES OF THE GAS CHROMATOGRAPHIC DETERMINATION OF VOLATILE GLUCOSINOLATE DEGRADATION PRODUCTS USING FID

Analysed substance	*Relative retention time*[a]	*Limit of determination (mg kg^{-1})*	*Limit of identification (mg kg^{-1})*
1-Cyano-3-butene	0.634	0.04	0.00138
1-Cyano-4-pentene	0.837	0.03	0.00046
3-Butenyl isothiocyanate	1.186	0.03	0.00034
4-Pentenyl isothiocyanate	1.464	0.01	0.00009

[a] Butyl isothiocyanate = 1.00.

TABLE II

REPEATABILITIES OF THE DETERMINATION OF VOLATILE GLUCOSINOLATE DEGRADATION PRODUCTS IN RAPESEED OIL

Concentration range (mg kg^{-1})	*Mean concentration (mg kg^{-1})*	*n*	*Repeatability (mg kg^{-1})*	*Standard deviation of repeatability (mg kg^{-1})*	*Relative standard deviation of repeatability (%)*
1-Cyano-3-butene					
13–81	40.0	6	0.260	0.092	0.23
3.0–9.0	5.43	20	0.051	0.018	0.33
1.9–2.9	2.40	20	0.036	0.013	0.53
0.32–0.71	0.489	22	0.021	0.007	1.48
0.11–0.23	0.161	16	0.025	0.0089	5.51
0.027–0.082	0.052	12	0.0112	0.0039	7.60
0.010–0.020	0.013	12	0.0075	0.0025	19.00
1-Cyano-4-pentene					
16–41	24.8	8	0.090	0.032	0.13
3.0–5.6	4.01	24	0.051	0.018	0.45
1.3–2.9	1.82	14	0.048	0.017	0.94
0.33–0.97	0.500	32	0.022	0.008	1.59
0.10–0.28	0.195	16	0.012	0.0040	2.16
0.030–0.084	0.052	10	0.0060	0.0020	3.85
0.010–0.021	0.019	10	0.0064	0.0023	11.89
3-Butenyl isothiocyanate					
2.0–12.7	5.07	24	0.031	0.011	0.22
0.32–0.84	0.68	22	0.045	0.016	2.34
0.10–0.28	0.157	22	0.016	0.006	3.60
0.031–0.089	0.0451	24	0.0090	0.0032	7.08
0.010–0.022	0.019	12	0.0080	0.0028	14.89
4-Pentenyl isothiocyanate					
1.3–2.8	1.69	12	0.037	0.013	0.78
0.9–1.2	1.07	10	0.063	0.022	2.06
0.26–0.50	0.33	20	0.047	0.016	4.95
0.044–0.092	0.071	14	0.0046	0.0016	2.31
0.010–0.036	0.022	22	0.0033	0.0012	5.50

detection limits according to Kaiser[4] and limits of determination[5] are given in Table I, and show that the method was sufficiently sensitive for the analysis of crude rapeseed oils and for the evaluation of their refining. However, the procedure is not sufficiently sensitive for the determination of trace amounts in deodorized oils. Improper deodorization can be detected with satisfactory sensitivity.

Repeatabilities of the determination of the most important isothiocyanates and nitriles are given in Table II for several concentration levels. They were calculated from large series of duplicate analyses following the standardized procedure[6].

The main advantages of the above procedure are its simplicity and speed; the duration of an analysis, including the sample preparation, does not exceed 10 min, whereas with FID the samples have to be distilled with water vapour, the distillate extracted with diethyl ether and the extract concentrated before the analysis. Many

other compounds, mainly volatile oxidation products, interfere when FID is used[7] (Fig. 1C); hence NPD is more reliable than FID.

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Note

Use of an activated nylon membrane (Immunodyne) as an affinity adsorbent for the purification of phosphofructokinase and phosphoglycerate kinase from yeast

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Affinity chromatography has been widely applied to the isolation of biological macromolecules either as a single-step procedure or in combination with other purification techniques[1,2]. In principle, a ligand immobilized to a solid matrix interacts with binding sites on proteins such as active or regulatory sites of enzymes, therefore discriminating between proteins exhibiting such binding sites or not. Further conditions for the subsequent desorption of bound proteins can be chosen which allow a selective elution.

Despite its simple concept and its successful application, problems are often connected with affinity chromatography such as the rigidity and physical shape of the affinity matrix used, its insolubility and chemical stability, the ease of derivatization, its reuseability and, of course, the overall costs.

This paper demonstrates the reversible binding of yeast phosphofructokinase (EC 2.7.1.11) and yeast phosphoglycerate kinase (EC 2.7.2.3) to commercially available Immunodyne nylon membrane and the use of this membrane as an affinity matrix for the enrichment of these two enzymes from yeast cell homogenates. The technique applied is extremely simple and does not require sophisticated equipment.

EXPERIMENTAL

Immunodyne, Biodyne A and Loprodyne nylon membranes were obtained from Pall Filtrationstechnik (Dreieich, F.R.G.). The membranes were cut with scissors to the appropriate size. Fructose-6-phosphate, 3-phosphoglycerate, ATP, AMP, NAD, NADP and auxiliary enzymes (aldolase, glycerolphosphate dehydrogenase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase) were obtained from Boehringer (Mannheim, F.R.G.). Reagents for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), phenylmethylsulphonyl fluoride (PMSF) and molecular weight markers were purchased from Serva (Heidelberg, F.R.G.). Buffer substances were of analytical-reagent

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grade from Sigma (St. Louis, MO, U.S.A.) and VEB Laborchemie (Apolda, G.D.R.). Baker's yeast was supplied by VEB Backhefe (Leipzig, G.D.R.).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE was carried out at 8% (w/v) acrylamide according to Laemmli[3]. Treatment with SDS prior to electrophoresis was done by mixing equal volumes of sample and incubation buffer [110 m*M* Tris–HCl (pH 6.8), 16% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue] and subsequent heating of the mixture at 100°C for 10 min.

Enzyme assays

Phosphofructokinase activity was assayed at 3.0 m*M* fructose-6-phosphate, 0.6 m*M* ATP and 1 m*M* AMP in imidazole buffer (pH 7.2) according to Hofmann and Kopperschläger[4].

Phosphoglucose isomerase activity was assayed as described by Clifton *et al.*[5] at 1.4 m*M* fructose-6-phosphate in triethanolamine buffer (pH 7.6).

Phosphoglycerate kinase activity was determined at 6 m*M* 3-phosphoglycerate and 1 m*M* ATP in triethanolamine buffer (pH 7.6) according to Kulbe and Bojanovski[6].

Protein determination

Protein concentration was determined according to Bradford[7] using dried bovine serum albumin for calibration.

Preparation of yeast cell homogenates and extracts

Yeast cells were washed several times with buffer A [50 m*M* sodium phosphate (pH 7.2), supplemented prior to use with 0.5 m*M* PMSF and 1 m*M* 2-mercaptoethanol]. A 1-g portion of the cell pellet after the last centrifugation was swirled with 3 g of acid-washed glass beads (0.5 mm diameter) in 1 ml of buffer A for five 1-min cycles. The tubes were kept on ice during 30-s interruptions. The supernatant above the fast-settling glass beads was used as a cell homogenate. It was centrifuged at 25 000 *g* to remove cell debris to give a cell-free extract.

Preparation and handling of the nylon membranes

The membranes were cut into 50 × 110 mm pieces and transferred to polyethylene bottles (diameter 45 mm, height 70 mm). The bottles were filled with 10 m*M* sodium hydroxide solution, which causes the membranes to adhere loosely to the wall of the bottles. After 1 h the bottles were emptied, and 5 ml of fresh 10 m*M* sodium hydroxide solution were added; 5 ml was always the standard volume used in washing and regeneration steps. The bottles were then fixed with rubber bands to a rotating shaft in horizontal positions (Fig. 1). This arrangement allowed the use of small volumes and utilization of the whole membrane area. To replace solutions the bottles could be easily removed and refixed and it was also very convenient to handle several bottles simultaneously. The membranes were treated in this way three times with 10 m*M* sodium hydroxide solution for 60 min each time and then equilibrated with buffer A. Binding of proteins to the nylon filters was achieved by incubation of the cell homogenates or extracts with the membranes with continuous rotation for the

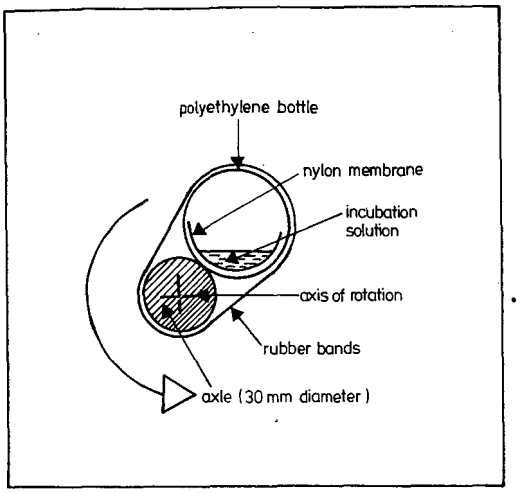

Fig. 1. Treatment of the nylon filters with a small volume of incubation solution within a bottle fixed to a rotating shaft.

indicated times. Subsequently the membranes were washed six times with buffer A. Desorption was performed by exposing the membranes with the desorption buffers indicated in the text. All incubations were performed at cold room temperature (4–6°C).

To regenerate the membranes they were washed three times before each use with 10 m*M* sodium hydroxide solution in 15-min cycles and re-equilibrated. The membranes were stored in 15% methanol for periods up to 1 week or air-dried after regeneration and equilibration with water.

RESULTS AND DISCUSSION

Immunodyne membranes are pre-activated nylon membranes developed for the covalent fixation of proteins via hydroxyl, carboxyl or amino groups (see the manufacturer's instructions for the use of these membranes). An unexpected property of this membrane after alkaline inactivation of the reactive group is its ability for selective binding of phosphofructokinase and phosphoglycerate kinase from a yeast cell extract (Fig. 2). During incubation of the yeast extract with the membrane, the phosphofructokinase activity decreased within 2 h from 7.5 to 0.4 U/ml and the phosphoglycerate kinase activity decreased from 83 to 50 U/ml. In contrast, the phosphoglucose isomerase activity remained constant over the incubation period, indicating the selectivity in the binding of phosphofructokinase and phosphoglycerate kinase. The total protein content in the extract decreased within the first 30 min of incubation from 16.6 to 15 mg/ml and remained at this level during further incubation. In a control incubation of the extract without the membrane (data not shown), no inactivation of the two enzymes was observed, indicating that the decrease in the activities of phosphofructokinase and phosphoglycerate kinase is due to adsorption of the two proteins to the membrane.

The binding of phosphofructokinase is restricted to the Immunodyne membrane

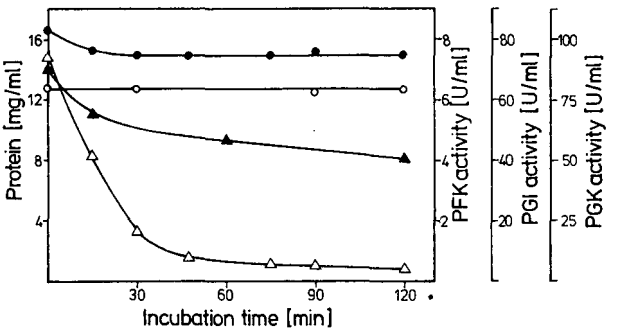

Fig. 2. Incubation of yeast cell extract with the Immunodyne membrane. Aliquots of 3 ml of yeast cell extract were rotated with the Immunodyne membrane and aliquots were taken at the indicated times for the determination of total protein content (●) and the activities of phosphofructokinase (PFK) (△), phosphoglycerate kinase (PGK) (▲) and phosphoglucose isomerase (PGI) (○).

(Table I). Incubation of a yeast cell homogenate with different nylon membranes (Immunodyne, Biodyne, Loprodyne) caused a decrease in the phosphofructokinase activity in the homogenate only with the Immunodyne membrane. The selectivity in the binding is underlined by the finding that the specific activity of phosphofructokinase decreased in the cell homogenate after incubation with the Immunodyne membrane, whereas with the other two nylon filters it remained equal to the control value.

Therefore, the group used for substitution of the nylon matrix of Immunodyne to preactivate this membrane seems to act as an affinity ligand. The two enzymes for which an interaction with the membrane has been demonstrated (Table I) are both kinases with the substrate ATP in common and both are known to interact with the affinity ligand Cibacron Blue F3G-A[4,6], which has been shown to mimic adenine nucleotides[1,8]. Assuming a binding mechanism due to the presence of the nucleotide binding site on the two enzymes, an affinity elution with several adenine nucleotides (ATP, AMP, NAD, NADP) was performed (Table II, Fig. 3). Together with some other proteins the two kinases are desorbed but the specific activities of the two

TABLE I

TIME-COURSE OF PHOSPHOFRUCTOKINASE BINDING TO DIFFERENT NYLON MEMBRANES

Aliquots of 6 ml of a yeast cell homogenate were incubated with the respective nylon membranes and assayed for phosphofructokinase (PFK) activity at the indicated times. An incubation without a membrane was performed as a control. Values in parentheses are the specific activities at the end of incubation determined in the supernatant from a 25000 *g* spin.

Time of incubation (min)	*PFK activity (U/ml) in the cell homogenate*			
	Control	*Immunodyne*	*Biodyne A*	*Loprodyne*
0	15.7	–	–	–
60	14.2	7.6	14.7	15.3
120	14.9	4.8	14.2	15.8
	(0.42)	(0.14)	(0.46)	(0.42)

TABLE II

DESORPTION OF PHOSPHOFRUCTOKINASE AND PHOSPHOGLYCERATE KINASE BOUND TO IMMUNODYNE NYLON MEMBRANE BY DIFFERENT ADENINE NUCLEOTIDES

Aliquots of 3 ml of a yeast cell homogenate were incubated with the Immunodyne membranes. Unbound material was removed by washing the membranes with buffer A. Subsequently the membranes were rotated for 20 min with 3 ml of buffer A, containing 10 m*M* of the respective nucleotide. The activities of phosphofructokinase (PFK) and phosphoglycerate kinase (PGK) and the protein content in the eluates were determined.

Enzyme	*Specific activity (U/mg) of PFK and PGK in the elution buffer containing*			
	ATP[a]	*AMP*[b]	*NAD*[b]	*NADP*[b]
PFK	16.5 (±3.5)	19.1 (±2.6)	0.55 (±0.2)	25.7 (±3.9)
PGK	198 (±16)	156 (±12)	117 (±12)	265 (±22)

[a] Values are the means of fifteen experiments.
[b] Values are the means of four experiments.

enzymes after elution and the electrophoretic pattern of the desorbed proteins indicate differences in the action of the nucleotides. All the adenine nucleotides used caused elution of phosphoglycerate kinase, although with different effectiveness. Such differences are more obvious with phosphofructokinase, where almost no elution could be achieved by NAD. With this exception, the other nucleotides (ATP, AMP, NADP) were able to desorb phosphofructokinase from the Immunodyne membrane again, indicating a selective interaction of the two enzymes with the Immunodyne membrane. The electrophoretic patterns of the protein desorbed by these nucleotides are similar. With NADP the two unidentified prominent bands in the low-molecular-weight range are missing, which explains the higher specific activities of the two enzymes in this case (last column in Table II). The adsorption and ATP elution also worked well with the cell homogenate of a different yeast strain (DFY1 in Fig. 3), although differences in the protein distribution are observed. The genetic background and/or different growth conditions might account for these differences.

Using the approach of selective binding and affinity elution with ATP for the partial purification of phosphofructokinase and phosphoglycerate kinase from yeast cell extracts, purification factors of 46.5 and 55.7 were obtained, respectively (Table III). Obviously, there is no difference in the resulting specific activities whether a crude cell homogenate (first column of data in Table II) or the cell extract free of cell debris (Table III) was used in the adsorption step. For purification purposes it might even be advantageous to use the cell homogenate, for the centrifugation step to clear the extract can be omitted.

The capacity of the Immunodyne membrane for the binding of phosphofructokinase and the recovery were determined with purified yeast phosphofructokinase. At a concentration of 1 mg of phosphofructokinase per millilitre of buffer A a maximum of 22 μg of the enzyme was bound per square centimetre of the membrane and was desorbed by a one-step elution with 10 m*M* ATP with a recovery of 72%.

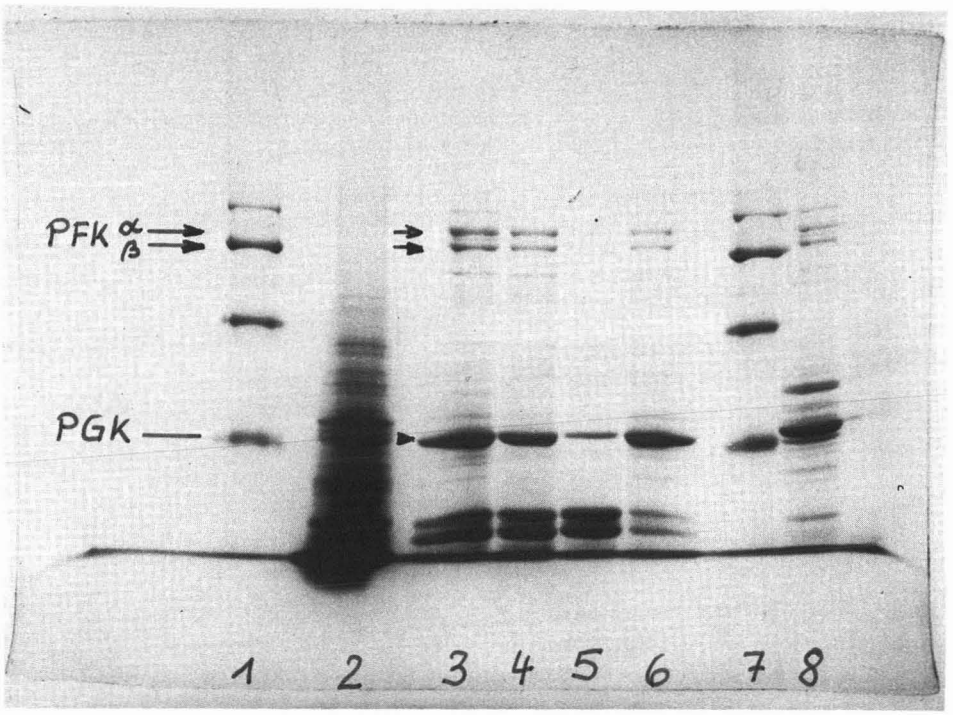

Fig. 3. SDS-PAGE of yeast proteins eluted from the Immunodyne membrane with different adenine nucleotides. Aliquots of 3 ml of yeast cell homogenate were incubated for 60 min with the Immunodyne membranes. Unbound material was removed by washing the membranes with buffer A and the membranes were treated for 20 min with 5 ml of buffer A containing 10 m*M* of the respective adenine nucleotide. Volumes of 50 μl of the eluates and 5 μl of the original cell homogenate were subjected to SDS-PAGE: ATP (lane 3), AMP (lane 4), NAD (lane 5), NADP (lane 6), original cell homogenate (lane 2). The arrows indicate the positions of the two subunits[8] of phosphofructokinase (PFK_β and PFK_γ) and of phosphoglycerate kinase (PGK). They were identified by immunoblotting (not shown). Lanes 1 and 7 are molecular-weight standards: β-galactosidase (116 000), phosphorylase *b* (92 000), bovine serum albumin (69 000) and ovalbumin (45 000). The same experiment (ATP elution) as with the cell homogenate of the commercial yeast in lane 3 was performed with a different strain of *Saccharomyces cerevisiae* (lane 8): DFY1[5] was grown on rich glucose medium to stationary phase and the cell homogenate used for the incubation with the Immunodyne membrane.

TABLE III

PARTIAL PURIFICATION OF PHOSPHOFRUCTOKINASE AND PHOSPHOGLYCERATE KINASE BY SELECTIVE ADSORPTION–DESORPTION WITH IMMUNODYNE NYLON MEMBRANES

A 3-ml volume of yeast cell extract was incubated for 60 min with the Immunodyne nylon membrane. The membranes were washed with buffer A and treated for 20 min with 3 ml of buffer A, containing 10 m*M* ATP.

Sample	*Specific activity (U/mg)*[a]	
	Phosphofructokinase	*Phosphoglycerate kinase*
Cell extract	0.4 ± 0.18	3.52 ± 0.43
Product	18.6 ± 3.2	195 ± 35

[a] Values are the means of six experiments.

The membrane is easily regenerated with sodium hydroxide and its capacity did not decrease after twenty cycles of binding and regeneration. Considering the ease of handling the nylon membrane, the potential use of particle-containing cell homogenates and the overall time to obtain a 50-fold enriched enzyme preparation, affinity sorption on Immunodyne membranes may be superior to other methods as a first step for the extraction of phosphofructokinase and phosphoglycerate kinase from yeast, at least on a small scale of up to a few milligrams.

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Note

Phthalimidylbenzenesulphonyl chlorides as fluorescence labelling reagents for amino acids in high-performance liquid chromatography

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Benzenesulphonyl chloride, which reacts with amines to give the corresponding sulphonamides, is a useful reagent for separating primary and secondary amines[1,2]. N,N-Dimethylamino-*p*-aminobenzeneazosulphonyl chloride[3,4] and dimethylaminonaphthalenesulphonyl chloride (DNS-Cl)[5] have been used as labelling reagents for amino acids in high-performance liquid chromatography (HPLC) with visible and fluorescence detection, respectively. Previously, we developed some fluorescence derivatization reagents having phenylphthalimidine as a fluorophore for hydroxyl and amino compounds[6] and for thiol compounds[7].

This paper deals with the preparations of 4-(N-phthalimidyl)benzenesulphonyl chloride (Phisyl-Cl) and 2-methoxy-5-(N-phthalimidyl)benzenesulphonyl chloride (M-Phisyl-Cl) (Fig. 1) as fluorescent derivatization reagents for amines and amino acids and their reactivities towards amino compounds using thin-layer chromatography (TLC) and HPLC.

EXPERIMENTAL

Chemicals and solvents

All chemicals were of analytical-reagent grade, unless stated otherwise. *n*-Propylamine, piperidine and aniline were purchased from Wako (Osaka, Japan) and amino acids from Kyowa Hakko (Tokyo, Japan). Amines were dissolved in acetone, and amino acids (alanine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, ornithine, phenyl-

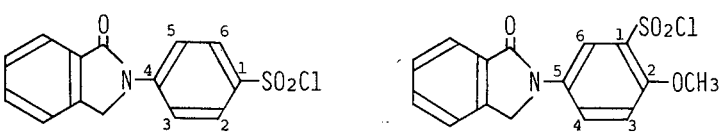

Fig. 1. Structures of Phisyl-Cl and M-Phisyl-Cl.

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alanine, proline, serine, threonine and valine) in 0.01 *M* hydrochloric acid, except for cystine and tyrosine (in 0.05 *M* sodium hydroxide solution). Organic solvents were purified by distillation prior to use.

Synthesis of Phisyl-Cl. *o*-Phthalaldehyde (2.68 g, 20 mmol) in diethyl ether (100 ml) and aniline (1.86 g, 20 mmol) in diethyl ether (20 ml) were mixed. After overnight stirring at room temperature, the precipitate of phenylphthalimidine was filtered off, washed with diethyl ether and recrystallized from methanol (yield 3.30 g). Chlorosulphonic acid (6.6 g, 50 mmol) was dropped onto crystals of N-phenylphthalimidine (2.09 g, 10 mmol) in an ice-bath over 20 min with vigorous stirring, followed by heating at 60°C for 2 h. On adding crushed ice (*ca.* 30 g) to the resulting mixture, a white precipitate of Phisyl-Cl was obtained (yield 1.21 g). Recrystallization from benzene gave fine colourless needles, m.p. 186–187°C. Calculated for $C_{14}H_{10}NO_3SCl$, C 54.63, H 3.28, N 4.55; found, C 55.22, H 3.30, N 4.71%. Mass spectrum: *m/z*, 307 (M^+). ^1H NMR (C^2HCl_3–[$^2H_6$] DMSO): δ, 4.94 (2H, singlet, CH_2), 7.44 (4H, multiplet, phthalimidyl ArH), 8.10 (2H, doublet, J = 10 Hz, 3- and 5-H), 8.18 ppm (2H, doublet, J = 10 Hz, 2- and 6-H).

Synthesis of M-Phisyl-Cl. *p*-Anisidine was used instead of aniline as above in the synthesis of Phisyl-Cl. The reaction with *o*-phthalaldehyde was carried out for 3 days with stirring. The precipitate of N-(4-methoxyphenyl)phthalimidine was collected, washed with diethyl ether and recrystallized from methanol. Chlorosulphonic acid (*ca.* 0.8 ml) was dropped onto crystals of N-(4-methoxyphenyl)phthalimidine (0.3 g, 1.3 mmol) in an ice-bath. After standing at 60°C for 5h, crushed ice was added to the reaction mixture. The resulting precipitate was collected and extracted with chloroform. After evaporation of the solvent, crude M-Phisyl-Cl (yield 0.11 g) was recrystallized from chloroform–carbon tetrachloride as colourless plates, m.p. 215–217°C (decomp.). Calculated for $C_{15}H_{12}NO_4SCl$, C 53.33, H 3.58, N 4.15; found, C 52.64, H 3.65, N 3.94%. Mass spectrum: *m/z*, 337 (M^+). ^1H NMR (C^2HCl_3–[$^2H_6$]DMSO): δ, 4.08 (3 H, singlet, OCH_3), 4.88 (2H, singlet, CH_2), 7.20 (1H, doublet, J = 8 Hz, 3-H), 7.58–7.91 (4H, multiplet, phthalimidyl ArH), 8.05 (1 H, doublet, J = 3 Hz, 6-H), 8.82 ppm (1H, quadruplet, J = 8 and 3 Hz, 4-H).

Derivatization procedure

To a test solution of amine or amino acid (0.05–10 m*M*, 5 μl), sodium hydroxide (0.1 *M*, 20 μl) and reagent solution (5 m*M* in acetone, 150 μl) were successively added and mixed well. The mixture was heated at 50°C for 15 min.

Thin-layer chromatography

An aliquot of the resulting mixture was applied to a silica gel plate (Analtech, Newark, DE, U.S.A.) and developed at *ca.* 25°C with the solvent systems S_1 (benzene–acetone, 8:2, v/v) for amines and S_2 (*n*-propanol–ammonia, 8:2, v/v) for amino acids. Visual detection of the fluorescent spots on the silica gel plate was achieved with a UV cabinet II (Camag, Muttenz, Switzerland).

High-performance liquid chromatography

The apparatus used was a Bip-1 HPLC system equipped with a Model 880-51 two-line degasser (Jasco, Tokyo, Japan), a Rheodyne 7161 injector (20-μl loop) and an F-1000 fluorescence detector (Hitachi, Tokyo, Japan). An ERC-ODS-1161 (3 μm)

column (100 mm × 6 mm I.D.) (Erma, Tokyo, Japan) was used with a gradient system of 2.5 m*M* phosphate buffer (pH 7.3)–methanol in order to examine the reaction conditions of Phisyl-Cl and M-Phisyl-Cl with amino acids. The separation was carried out with a linear gradient from 25 to 75% methanol over 20 min, followed by 75% methanol for 5 min, at a flow-rate of 1.0 ml/min. The separation of a reaction mixture of twenty amino acids with Phisyl-Cl was carried out by using a YMC AM-303 packed column of 5-μm ODS (C_{18}) (250 mm × 4.6 mm I.D.) (Yamamura, Kyoto, Japan). The flow-rate was 0.6 ml/min stepwise elution using solvent systems A [acetonitrile–30 m*M* Tris buffer (pH 6.5) (10:90)] and B [acetonitrile–30 m*M* Tris buffer (pH 6.5). (75:25)]. The elution programme was as follows (%B): 0% (8 min)–10% (12 min–20% (18 min)–30% (10 min)–45% (5 min)–60% (7 min)–100% (5 min)–0% (15 min). A C-KGC-324C-S guard column of 5-μm ODS (C_{18}) (25 mm × 4 mm I.D.) (Yamamura) was used together with each column. A 20-μl volume of the sample prepared from the reaction mixture by ten-fold dilution with eluent was injected. Fluorescence was detected with excitation at 295 nm and emission at 425 nm for Phisyl derivatives and excitation at 298 nm and emission at 445 nm for M-Phisyl derivatives.

RESULTS AND DISCUSSION

N-Phenylphthalimidine prepared from the reaction of *o*-phthalaldehyde with aniline showed strong fluorescence[8] and was easily converted to Phisyl-Cl by treatment with chlorosulphonic acid. The methoxy derivative, M-Phisyl-Cl, was obtained from *p*-anisidine in a similar manner.

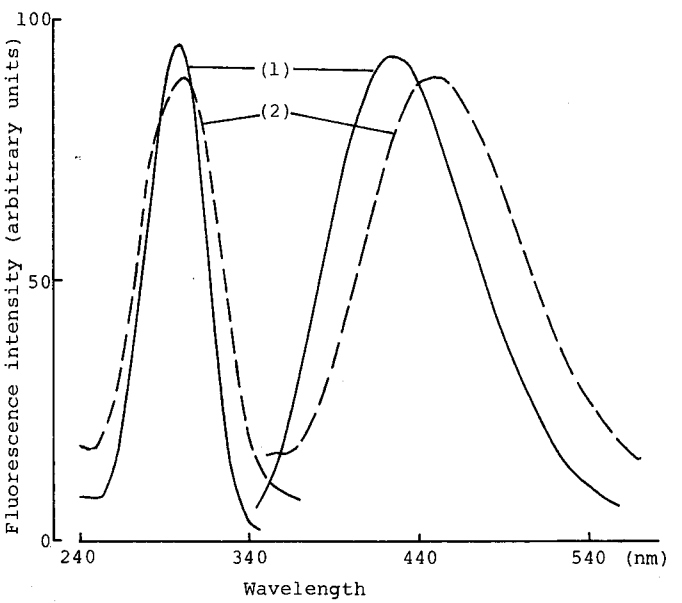

Fig. 2. Fluorescence spectra of the derivatives of alanine with Phisyl-Cl and M-Phisyl-Cl. (1) Phisyl-alanine (λ_{ex} 295 nm, λ_{em} 422 nm); (2) M-Phisyl-alanine (λ_{ex} 298 nm, λ_{em} 445 nm).

TABLE I

FLUORESCENCE WAVELENGTHS OF AMINO COMPOUNDS LABELLED WITH PHISYL-Cl AND M-PHISYL-Cl

Compound	*Phisyl-labelled*		*M-Phisyl-labelled*	
	λ_{ex} *(nm)*	λ_{em} *(nm)*	λ_{ex} *(nm)*	λ_{em} *(nm)*
n-Propylamine	298	407	303	435
Piperidine	297	404	304	431
Aniline	288	404	302	432
Alanine	295	422	298	445

The reactivities of Phisyl-Cl and M-Phisyl-Cl were examined with respect to amines (*n*-propylamine, piperidine and aniline) and an amino acid (alanine) by use of TLC. Reactions of the reagents with amino compounds mostly proceeded in the presence of sodium hydroxide to give the fluorescent derivatives, which were separated on a silica gel plate with solvent system S_1 for amines and S_2 for alanine. Fluorescence spectra of the derivatives of amino compounds with Phisyl-Cl and M-Phisyl-Cl, extracted with methanol from the fluorescent spots on the TLC plate, were measured. Fig. 2 shows the fluorescence spectra of the derivatives of alanine with the reagents. The fluorescence maxima of the extracted derivatives are given in Table I. The emission spectra of the derivatives in methanol–water (1:1) were shifted to a slightly longer wavelength (*ca.* 4 nm) than those in methanol. However, the fluorescence was not affected in the pH range 4–8 in methanol–water (1:1). The wavelengths of the emission maxima of Phisyl derivatives were about 25 nm shorter than those of M-Phisyl derivatives. Lysine and histidine, with more than two amino groups, and tyrosine, with a phenolic hydroxyl group, showed one fluorescent spot on the TLC plate with solvent system S_2 when treated with an excess of the reagents, although two or three fluorescent spots were observed with the reactions of equimolar amounts of reagents and amino acids.

The reaction conditions for Phisyl-Cl and M-Phisyl-Cl were studied with five amino acids (alanine, histidine, phenylalanine, proline and tyrosine, 0.25 m*M* each) by HPLC using an ERC-ODS-1161 column. Peaks of Phisyl and M-Phisyl derivatives of amino acids were clearly separated by the gradient system described under Experimental. The fluorescence maxima of each peak eluate were λ_{ex} 294–296 nm and λ_{em} 422–426 nm for Phisyl-amino acids, and λ_{ex} 296–299 nm and λ_{em} 444–445 nm for M-Phisyl-amino acids. The reaction temperature affected the derivatization reactions. At 25°C the reactions were incomplete and decomposition of Phisyl derivatives of histidine, proline, phenylalanine and tyrosine took place at 100°C. As shown in Fig. 3, maximum peak heights of Phisyl-amino acids were obtained at 50°C in 15 min. The Phisyl derivatives were stable for at least 1 week at room temperature. M-Phisyl-Cl gave similar results. However, the peaks of Phisyl derivatives were about five to twenty times higher than those of M-Phisyl derivatives. This suggests that Phisyl-Cl is suitable for the determination of small amounts of amino acids.

The fluorescence intensities (peak heights) of the Phisyl-amino acids (glycine, methionine and proline) were compared with those of DNS-amino acids under the same HPLC conditions (ERC-ODS-1161 column) as described above. The reactions

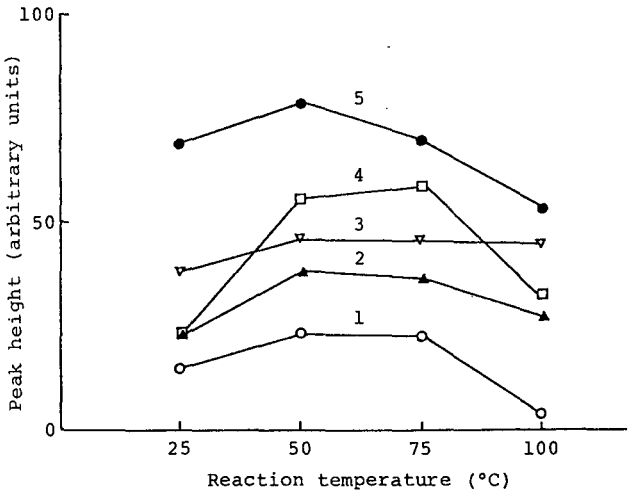

Fig. 3. Effect of reaction temperature on the peak heights of amino acids labelled with Phisyl-Cl. 1, Proline; 2, phenylalanine; 3, alanine; 4, histidine; 5, tyrosine.

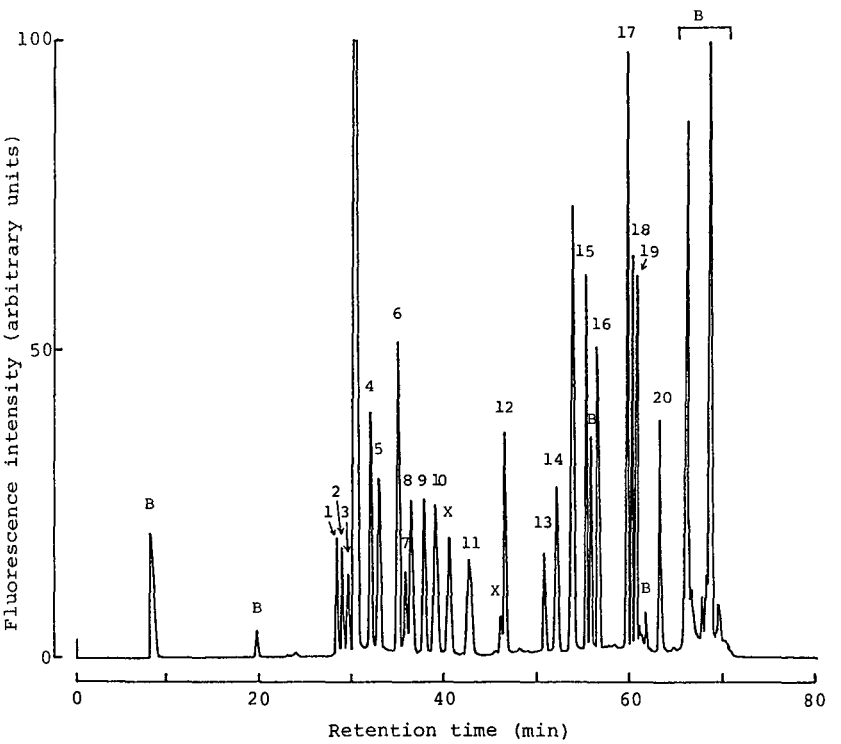

Fig. 4. Separation of amino acids labelled with Phisyl-Cl. A mixture of twenty amino acids (0.25 m*M* each) was treated according to the procedure (each corresponding to 14.2 pmol per injection). Peaks: 1 = cysteine; 2 = aspartic acid; 3 = glutamic acid; 4 = hydroxyproline; 5 = asparagine; 6 = serine; 7 = methionine; 8 = threonine; 9 = glycine; 10 = alanine; 11 = proline; 12 = valine; 13 = isoleucine; 14 = leucine; 15 = phenylalanine; 16 = cystine; 17 = ornithine; 18 = lysine; 19 = histidine; 20 = tyrosine; B = reagent blank; X = unidentified.

of DNS-Cl with amino acids were run according the method of Tapuhi *et al.*[9], and the fluorescence was detected at λ_{ex} 350 nm and λ_{em} 530 nm. The ratios of the peak heights of Phisyl-amino acids to those of DNS-amino acids were >3.

As an example, the separation of a reaction mixture of twenty amino acids with Phisyl-Cl is shown in Fig. 4. The twenty amino acids were successfully separated by using a YMC AM-303 packed column with stepwise elution as described under Experimental. Each peak was assigned by the overlapping method. The sensitivities (signal-to-noise ratio = 2) were less than 0.2 pmol per injection. The peak heights of Phisyl-amino acids were linear at amino acid concentrations up to at least 10 nmol per test-tube. The coefficients of variation (n = 8) for the twenty Phisyl-amino acids were in the range 3.5% (hydroxyproline)–8.2% (histidine) at a concentration of 1.25 nmol per test-tube.

Physil-Cl was stable for more than 1 year at room temperature and the acetone solution was also stable for 1 day in a stoppered vial.

The application of Phisyl-Cl to the simultaneous determination of amino acids in biological fluids is currently under investigation.

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CHROM. 22 115

Note

Assay of methylthiolincosaminide in fermentations by high-performance liquid chromatography with fluorescence detection

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Lincomycin[1], a clinically important antibiotic produced by *Streptomyces lincolnensis*, consists of a unique aminooctose moiety, α-methylthiolincosaminide (MTL), attached via an amide linkage to a propylhygric acid unit. Biosynthetically, it has been proposed that MTL is coupled to propylproline to form N-demethyllincomycin which is then N-methylated to become lincomycin (Fig. 1)[1]. The existence of MTL and its precursors in the pathway was inferred from labeling studies using α-D-[$^{13}C_6$]glucose. Our intention in developing an assay for MTL was to demonstrate the presence of MTL in fermentation beers of *S. lincolnensis* and to provide a quick, sensitive and specific method to aid our studies of lincomycin biosynthesis.

Since MTL does not possess a chromophore for detection and since fermentation broths contain a rich abundance of primary and secondary metabolites, unconsumed media and cellular debris, derivatization of MTL with a fluorescent tag and high-performance liquid chromatography (HPLC) separation of the derivatized MTL from the other broth constituents was employed. In the method described below, we used 4-chloro-7-nitrobenzofurazan[2] as the derivatizing agent based on a number of considerations discussed in the text.

EXPERIMENTAL

Fermentation

The lincomycin producing culture, *Streptomyces lincolnensis* UC® 5124 was grown on Hickey and Tresner agar (BBL, Cockeysville, MD, U.S.A.) and stored above liquid nitrogen as agar plugs. The seed flasks (100 ml medium in 500-ml wide mouth Erlenmeyer flasks) were inoculated with four agar plugs of the stock culture and incubated at 28°C for $2\frac{1}{2}$ days on a rotary shaker. The seed medium consisted of yeastolac (Vico Products, Decatur, IL, U.S.A.; 10 g/l), NZ-amine B (Sheffield Chemical, Norwich, NY, U.S.A.; 5 g/l) and glucose monohydrate (cerelose; 20 g/l). Presterilization pH of the medium was adjusted to 7.2. The fermentation flasks (100 ml medium in 500-ml wide mouth Erlenmeyer flasks) were inoculated with 5 ml of the

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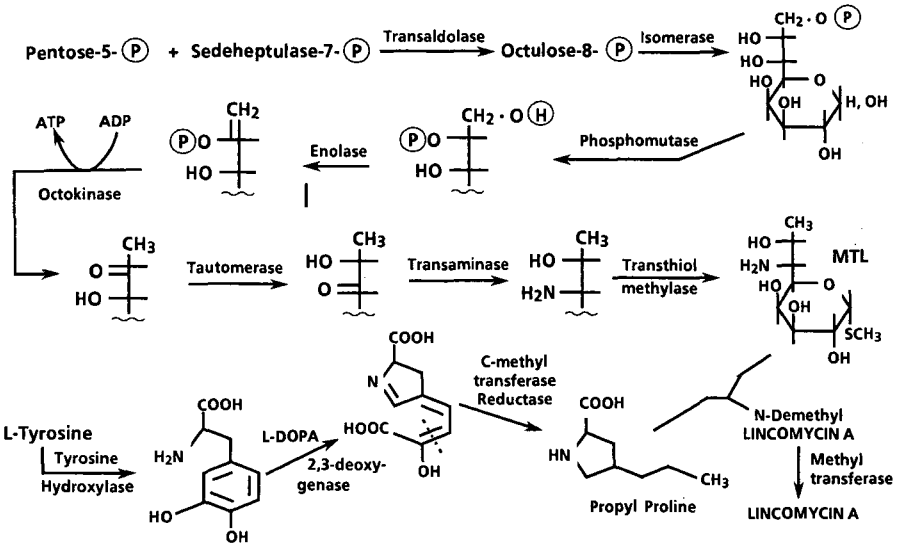

Fig. 1. Proposed biosynthetic pathway to lincomycin A.

seed culture and incubated at 28°C on a rotary shaker for 4 days. Fermentation beers were then pooled and kept frozen until use. The production medium F-13 consisted of glucose monohydrate (15 g/l), black strap molasses (Pacific Molasses, San Francisco, CA, U.S.A.; 20 g/l), hi-starch (Lauhoff Grain, Danville, IL, U.S.A.; 40 g/l), cotton seed hydrolysate (Pharmamedia, Traders Oil Mill, Fort Worth, TX, U.S.A.; 25 g/l), calcium carbonate (8 g/l), potassium sulfate (2 g/l) and antifoam (Ucon LB625, Union Carbide; 3 drops per 100 ml). Presterilization pH was adjusted to 7.2.

Derivatization

Whole beer was diluted 1:1 with water and samples were sonicated (Fisher sonicator, small tip, setting of 25 for 5 min) in centrifuge tubes on ice to break open cells. The pH was adjusted to 8.5 with 10 μl 1 *M* sodium hydroxide per ml beer. The sonicated mixture (400 μl) was placed in an autosampler vial and 100 μl of 1 *M* sodium hydrogencarbonate and 500 μl of a 30 mg 4-chloro-7-nitrobenzofurazan per ml methanol stock solution were added. Sodium hydroxide and sodium hydrogencarbonate were obtained from Mallinckrodt while 4-chloro-7-nitrobenzofurazan was purchased from Aldrich. Vials were sealed and heated in a water bath at 60°C for 4–6 h. Vials were shaken periodically to mix. Samples were then cooled on ice and filtered prior to injection. For testing the linearity and recovery from beer, the final reaction volume was made to contain 0, 2, 4, 10, 20 and 40 μg MTL per ml by adding appropriate amounts of a 0.2 mg MTL per ml stock solution to the water used to dilute the whole beer prior to sonication. (MTL was obtained by cleavage of lincomycin with base[1].) To determine the efficiency of derivatization, water was substituted for beer. The precision was determined by derivatizing nine replicates of a beer sample spiked with MTL to yield a concentration of 10 μg MTL per ml in the final reaction volume.

Chromatography

HPLC was performed using a Varian 8055 autosampler that filled a 10-μl loop on a Varian 5500 HPLC system. Fluorescent detection was performed by an EM Science Model F1000 fluorescence detector set at an excitation frequency of 420 nm and an emission frequency of 525 nm. The signal was integrated by a Hewlett-Packard 3392A integrator. An Alltech Econosphere cartridge column with a guard cartridge was utilized to provide the separation (C_{18}, 5 μm, 250 $\times$ 4.6 mm I.D.). Gradient elution was used employing a linear ramp from 10 to 33% methanol and 90 to 67% 2.2 m*M* NaH_2PO_4, pH 6.9, in 5 min followed by a linear gradient from this point to a 50:50 mixture at 20 min. A wash with 100% methanol for 10 min followed by an equilibration for 8 min at starting conditions was employed to recycle the column prior to the next injection.

RESULTS AND DISCUSSION

Assay of samples as complex as a fermentation beer requires great selectivity in the method of identification of the compound of interest. Fluorescence derivatization offers this selectivity as well as high sensitivity. The choice of the derivatization reagent must also consider the complexity of the sample and be as specific as possible to the compound of interest. The MTL amino group lends itself to derivatization with many fluorescent tags. 4-Chloro-7-nitrobenzofuran was selected as the reagent of choice since it produces highly fluorescent products only on reaction with amino groups resulting in less background fluorescence and a smaller hydrolysis product peak. A final 4-chloro-7-nitrobenzofuran concentration of 15 mg/ml in the reaction vial was determined to give the most effective derivatization of added MTL. Since the reaction produces hydrochloric acid as a by-product, a concentration of 0.1 *M* sodium hydrogencarbonate was necessary to maintain the slightly alkaline conditions necessary for derivatization.

The reaction was complete after 4 h at 60°C with no change up to 6 h. Variation in the observed peaks occurred with the fermentation organism, media used and date of sampling. However, the MTL peak is still resolved from any large interferents observed thus far. The HPLC conditions may have to be modified for individual fermentations. Buffer concentration and pH are used to modify the chromatogram in the reversed-phase mode. Gradient elution provides the separation with good peak shape. Fig. 2A shows a chromatogram of the derivatization of 10 μg/ml of MTL in water. Fig. 2B is an elution of a derivatized beer sample spiked with 10 μg MTL per ml. Fig. 2C is a blank beer sample. Fig. 3 is the HPLC trace for another lincomycin producing strain that has a larger amount of MTL. Many small peaks are seen to elute in the vicinity of the MTL derivative in the beer sample. This puts the lower limit on the detection of MTL in fermentation beers at 1 μg/ml.

A linear relationship ($r^2 = 0.9999$) was obtained for the assay of 0–40 μg MTL per ml in aqueous solution. The linear regression equation was $y = 1.60 \cdot 10^6 x - 3.58 \cdot 10^4$. The precision of the assay was determined for beer with added MTL. Nine samples spiked with MTL to give a final concentration of 10 μg/ml were assayed. A value of 8.44 $\pm$ 0.14 μg/ml relative standard deviation (R.S.D.) was obtained. The accuracy was tested by adding MTL to beer samples in the concentration range 0–40 μg/ml. The recovery was 73 $\pm$ 1.7% (R.S.D.). Regression analysis showed a linear

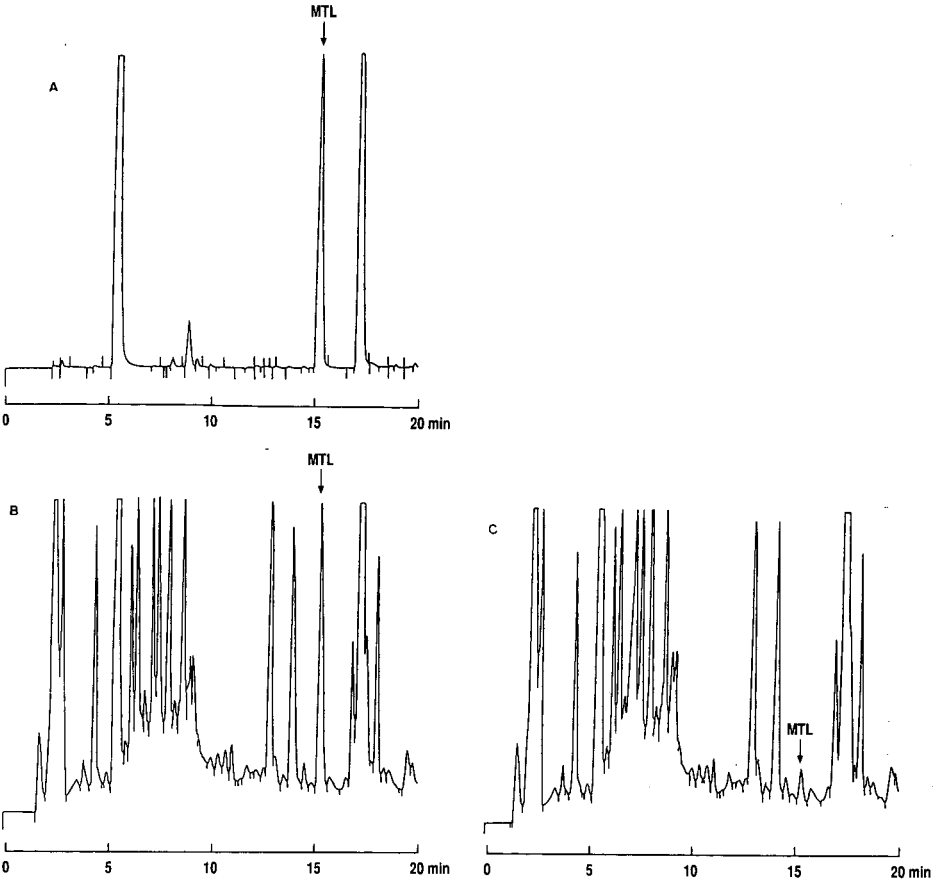

Fig. 2. (A) HPLC trace of an MTL sample in water after derivatization. Gradient elution from 10 to 33% A and 90 to 67% B in 5 min followed by a gradient to A–B (50:50) in 15 min. Solvent A, methanol; solvent B, 2.2 m*M* NaH_2PO_4, pH 6.9. (B) HPLC trace of a beer sample with added MTL after derivatization. Elution conditions as in (A). (C) HPLC trace of a derivatized beer sample. Elution conditions as in (A).

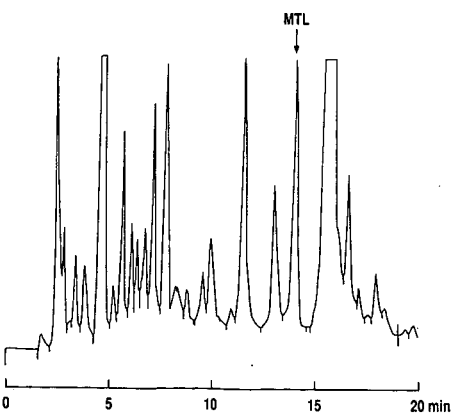

Fig. 3. HPLC trace of derivatized beer sample from another lincomycin producing strain. Elution conditions as in Fig. 2A.

relationship ($r^2 = 0.999$) between added and recovered MTL: $y = 0.742x - 0.74$. Since the recovery is not 100%, a standard curve generated in the fermentation beer must be used to determine the MTL concentration in a beer sample. A standard curve for MTL added to whole beer is linear ($r^2 = 0.999$) with an equation of: $y = 1.18 \cdot 10^6 x + 1.07 \cdot 10^6$. The y-intercept reflects the amount of MTL in an unspiked fermentation and is equivalent to 0.8 μg MTL per ml in the derivatization mixture or 4 μg/ml in the fermentation broth.

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Note

Separation and simultaneous determination of bamipine and salbutamol in dosage forms by high-performance liquid chromatography

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Antihistamines, available by prescription and over-the-counter as single-entity preparations and in mixtures, are among the most widely used drugs. Bamipine [N-benzyl-N-(1-methyl-4-piperidyl)aniline] is an antihistamine and also has sedative effects. As it is well known that antihistamines offer weak protection against bronchospasm, bamipine can be combined with salbutamol {α^1-[(*tert.*-butylamino)methyl]-4-hydroxy-*m*-xylene-α,α'-diol}, which has a selective action on β_2-receptors. Both are administered in tablet form, whereas salbutamol is also available in aerosol spray and as an injection.

Although several methods have been described for the quantification of bamipine and salbutamol in pharmaceutical dosage form, none is suitable for the simultaneous determination of both drugs[1–7]. In this paper, we describe a high-performance liquid chromatographic (HPLC) procedure in which both compounds can be quantified simultaneously under the same chromatographic conditions.

EXPERIMENTAL

Chemicals

Analytical-reagent grade acetonitrile and methanol (Ferak, Berlin, F.R.G.) were used. Ammonium acetate was of zur Analyse grade from Merck (Darmstadt, F.R.G.). Water was purified with a Millipore filtration unit (deionized, < 10 $\mu\Omega$).

Reference standards and standard solutions

Bamipine hydrochloride and salbutamol sulphate were kindly donated by Knoll (Ludwigshafen, F.R.G.) and Glaxo (Krioneri, Athens, Greece), respectively.

Two series of standard solutions, 3.75, 5.62, 7.49, 9.36, 11.23 and 13.11 μg/ml and 2.24, 3.36, 4.48, 5.60, 6.72, 7.84 μg/ml, were prepared for salbutamol sulphate and bamipine hydrochloride, respectively, using the mobile phase as a solvent.

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Apparatus

A Perkin-Elmer Series 3B high-performance liquid chromatograph, a Rheodyne 7010 20-μl loop injector valve and an LC wavelength system was used. The spectrophotometer was operated at 0.04 a.u.f.s. (1-cm path length). The wavelength was set at 264 nm. The chromatographic peaks were recorded by employing an LKB (Bromma, Sweden) 2210 potentiometric recorder connected to the spectrophotometer, with an operating voltage of 10 mV and chart speed of 2 mm/min. A 250 × 2.1 mm I.D. stainless-steel column containing Polygosil®-60, C_{18}, 10 μm (Macherey-Nagel, Düren, F.R.G.) was employed. A flow-rate of 0.6 ml/min eluted salbutamol and bamipine in 4.00 and 5.42 min, respectively. All analyses were performed at room temperature.

Mobile phase and stability of chromatographic system

The mobile phase was acetonitrile–methanol–0.015 *M* aqueous ammonium acetate (85:10:5, v/v/v), degassed by vacuum filtration through a 0.2-μm Sartorius S 11807 PTFE membrane filter while the container (flask) was in an ultrasonic bath. To the mobile phase *ca.* 0.6% acetic acid was added to adjust the pH to *ca.* 5.72, the optimum for the chromatographic system. The column was equilibrated with mobile phase at a flow-rate of 0.6 ml/min. The relative standard deviation (R.S.D.) of six replicate injections of a standard was not more than 2.0%, as defined in the USP XXI under 'System suitability for HPLC'.

Sample preparation

For tablets, not less than ten were weighed and the average weight determined. The tablets were finely powdered and a portion of powder equivalent to one average tablet weight was weighed and transferred quantitatively into a 50-ml volumetric flask. A 25-ml volume of mobile phase was added and the dispersion was shaken for 40 min on a mechanical shaker. After ultrasonication for 20 min, the solution was diluted to volume with mobile phase and left to precipitate. Appropriate dilutions were made from the clear supernatant solution, so that the concentration of each sample solution approached the concentration of that in the middle of the standard solution range. Filtration kits (Millipore) for the sample preparations were used to ultraclean the solutions of particles 0.5 μm or greater.

For injections, a 10-ml volume containing 0.5 mg/ml was carefully transferred into a 25-ml volumetric flask, which was then placed in a desiccator under vacuum for 24 h. Mobile phase was added to the dried formulation, then shaken and made up to volume. Appropriate dilutions with mobile phase similar to those of the standard solutions were made.

For oral inhalation aerosol, using the manufacturer's directions, twenty metered doses (each containing 0.1 mg of salbutamol) were collected in a 250-ml conical flask. Portions of mobile phase were added and transferred into a 50-ml volumetric flask, then appropriate dilutions were made.

RESULTS AND DISCUSSION

Fig. 1 shows typical standard and sample chromatograms obtained using the above procedure. Although other HPLC methods with biomedical applications have

been described for the quantification of salbutamol (with electrochemical[8], amperometric[9] and fluorimetric[10] detection), the method proposed here proved suitable for the simultaneous determination of salbutamol and bamipine. Salbutamol sulphate and bamipine hydrochloride were eluted in 4.00 and 5.42 min, respectively, from standard and sample solutions.

The relatively small amount of buffered aqueous solution in the mobile phase and the easy adjustment of the pH to *ca.* 5.72 were necessary in order to achieve the optimum chromatographic conditions. The separation and elution were also affected by the increase in methanol concentration in the mobile phase. Therefore, a combination of both pH and polarity was critical for the development of the chromatographic system.

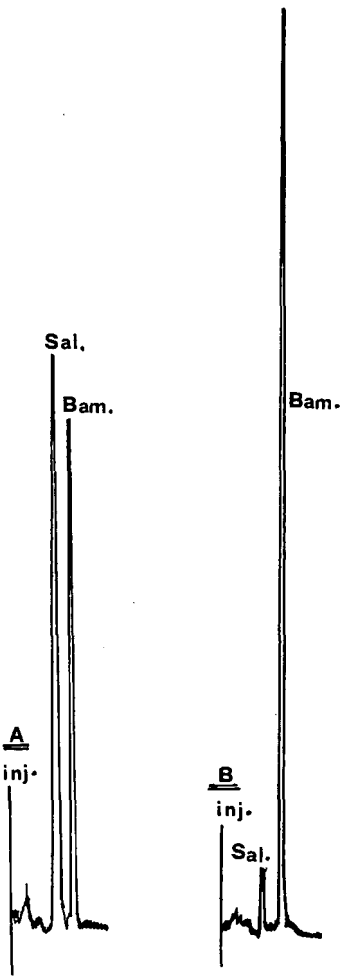

Fig. 1. Typical chromatograms of the separation of salbutamol (Sal.) from bamipine (Bam.) at 264 nm. Left: standard, Sal. 10.61 μg/ml, Bam. 4.34 μg/ml; right: tablet extract, Sal. 0.64 μg/ml, Bam. 8.00 μg/ml. Retention times: 4.00 min (Sal.) and 5.42 min (Bam.).

TABLE I

PRECISION OF METHOD IN ANALYSIS OF PHARMACEUTICAL FORMULATIONS FOR SALBUTAMOL AND BAMIPINE

Samples were purchased in different European countries.

Commercial dosage form	*Active ingredients*	*Labelled amount*[a]	*HPLC results*[b]	*Coefficient of variation (%)*	*Found (%)*
Tablets	Salbutamol sulphate	2	1.97	0.97	98.4
Tablets	Salbutamol sulphate	4	3.95	0.46	98.8
Injection	Salbutamol sulphate	0.5	0.50	0.24	100.4
Injection	Salbutamol sulphate	0.5	0.50	0.32	100.5
Aerosol	Salbutamol	0.1	0.102	0.42	102.4
Film-coated tablets	Bamipine hydrochloride	50.0	49.15	1.04	98.3
Sugar-coated tablets	Bamipine hydrochloride	25.0	24.83	0.96	99.3
	Propylhexedrine	–	–	–	–
Tablet placebo I	Salbutamol sulphate	2	1.97	0.92	98.5
	Bamipine hydrochloride	50	49.05	1.11	98.1
Tablet placebo II	Salbutamol sulphate	4	3.95	0.54	98.7
	Bamipine hydrochloride	50	49.10	1.02	98.2

[a] Tablets, mg per tablet; injection, mg/ml; aerosol, mg per metered dose.
[b] Means of four replicates.

Calibration graphs were constructed of peak height *versus* concentration. Linear regression and correlation showed that the correlation coefficient, intercept and slope were 0.9999, 0.22 and 9.60, respectively, for salbutamol sulphate and 0.9995, 0.23 and 17.48, respectively, for bamipine hydrochloride.

The results of the quantification of salbutamol and bamipine in different pharmaceutical formulations are shown in Table I. These were in agreement with the labelled amounts. No interference from the excipients was observed in the chromatograms. The coefficient of variation for both compounds was in the range 0.24–1.11%.

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Note

High-performance liquid chromatographic determination of free formaldehyde in cosmetics preserved with Dowicil 200

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(Received July 31st, 1989)

The EEC Council Directive[1] allows the use of formaldehyde as a preservative in cosmetic products at a maximum concentration of 0.2%. If the concentration exceeds 0.05%, the addition of formaldehyde must be declared on the label.

The official EEC method for formaldehyde determination is based on condensation of free formaldehyde with ammonium acetate and acetylacetone, to form the fluorescent 3,5-diacetyl-1,4-dihydrolutidine compound which is selectively detectable[2]. Although the method is sensitive, it is not suitable when formaldehyde donors are present in the cosmetic formula, because additional formaldehyde is released during analysis.

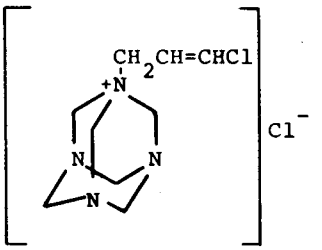
We recently reported a rapid and reliable method for the determination of free formaldehyde in cosmetics[3]; the procedure is based on sample dilution with a tetrahydrofuran (THF)–water solvent mixture[4], followed by precolumn derivatization with 2,4-dinitrophenylhydrazine (2,4-DNPH)[5] and direct high-performance liquid chromatographic (HPLC) analysis[6].

Our studies showed the applicability of this method when preservative donors are present in the cosmetic formula: only Dowicil 200 [*cis*-1-(chloroallyl)-3,5,7-triaza-1-azoniaadamantane chloride] is not compatible with the procedure, because of its instability in the acidic media[7].

This preservative, increasingly employed for antimicrobial protection of personal care formulations[8], is a quaternary derivative of urotropine which releases formaldehyde by a hydrolytic process[9].

To overcome this problem, the method was modified by incorporating a precolumn step before 2,4-DNPH derivatization. In this way, Dowicil 200 is completely retained on a cationic stationary phase, while free formaldehyde is eluted and derivatized without memory effects[10,11]. The procedure allows the rapid and reliable determination of free formaldehyde released by the preservative in untreated cosmetic products, simply diluted with a THF–water (9:1) solvent mixture.

0021-9673/90/$03.50



Dowicil 200

EXPERIMENTAL

Materials

Formaldehyde (40% RPE) and 2,4-DNPH were obtained from Carlo Erba (Milan, Italy), Dowicil 200 from Dow Chemical (Midland, MI, U.S.A.) and analytical-reagent grade reagents and solvents from Merck (Darmstadt, F.R.G.). Bond Elut SCX columns (500 mg of sorbent, 2.8 ml) (silica functionalized with benzenesulphonylpropyl cation-exchange groups) were supplied by Analytichem International (Harbor City, CA, U.S.A.).

Apparatus

A Perkin-Elmer Series 410 liquid chromatograph equipped with a Rheodyne 7125 valve, UV LC-95 detector and LCI-100 data station was used. The LiChrosorb RP-8 column (250 mm × 4 mm I.D., 10 μm) (Merck) was eluted with acetonitrile–water (50:50) at a flow-rate of 1 ml/min and with UV detection (345 nm).

2,4-DNPH solution (0.1%)

2,4-DNPH (0.25 g) was dissolved in 40 ml of 32% HCl with heating in a 250-ml volumetric flask and then diluted to volume with water.

Standards

Formaldehyde solution (40%), iodimetrically controlled, was diluted to the range 0.004–0.001% with THF–water (9:1). The solution was freshly prepared and stored in a refrigerator. Dowicil 200 aqueous standard solution (0.2%) was stored at room temperature in darkness and diluted 1:100 before analysis.

Samples

About 1 g of each commercial cosmetic sample, carefully weighed, was diluted 1:100 with THF–water (9:1) in a volumetric flask and stirred in a vortex mixer until completely homogeneous.

SCX column step

The column[1,2] was connected with a syringe by means of a PTFE tube and rinsed with 2–3 volumes of THF–water (9:1). A 1-ml volume of standard or sample solution was added to the top of the column and the eluate was collected by light suction; the

column was then washed with 1 ml of solvent mixture (THF–water, 9:1) and the eluate was added to the preceding one.

Derivatization procedure

A 1-ml volume of standard or sample solution was added to 0.4 ml of 0.1% 2,4-DNPH, stirred for 60 s in a vortex mixer and allowed to stand for 2 min at room temperature. The solution was then stabilized by adding 0.4 ml of 0.1 *M* phosphate buffer (pH 6.8) and 0.7 ml of 1 *M* sodium hydroxide solution. Aliquots of 6 μl were injected into the HPLC system.

RESULTS AND DISCUSSION

Dowicil 200 is a highly unstable molecule in acidic media (pH < 4) in which formaldehyde is quickly released[7]. For this reason, we studied a new approach to the quantitative evaluation of free formaldehyde in its solutions; determination is performed in the absence of the preservative, which is captured from the solution before analysis.

Fig. 1 shows the simple apparatus employed for this purpose, with a polypropylene column filled with silica functionalized with benzenesulphonylpropyl cation-exchange groups (SCX).

Formaldehyde analysis

We first verified the compatibility of the system with the analysis of standard formaldehyde solutions. Fig. 2 shows the analysis of a standard solution of formaldehyde with and without the SCX column step in comparison with blanks. The system does not absorb or release formaldehyde, as also confirmed by the correlations

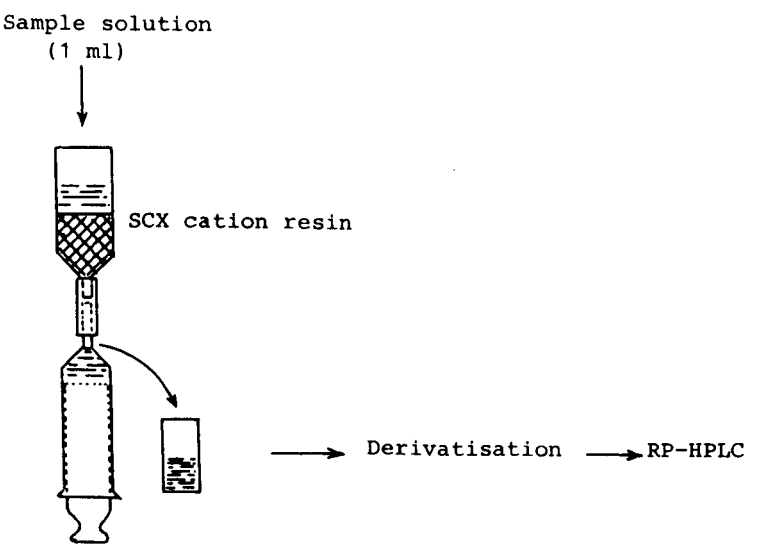


Fig. 1. Apparatus for determination of released formaldehyde.

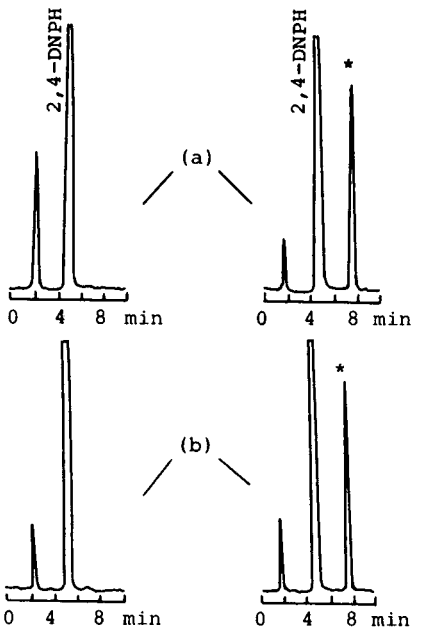


Fig. 2. HPLC of a derivatized formaldehyde standard solution (10 μg/ml) obtained (a) with and (b) without the SCX column step in comparison with blanks. The asterisks (in all figures) indicate the formaldehyde derivative peaks.

obtained with the same formaldehyde solutions (concentration range 4–60 μg/ml) before ($y = 18.61x + 34.15, r = 0.999$) and after the column step ($y = 18.74x + 33.02$, $r = 0.999$).

We also verified the applicability of the column system to the determination of formaldehyde in cosmetic samples. Fig. 3 shows the chromatographic patterns of a cosmetic emulsion, simply diluted in a THF–water (9:1) solvent mixture, containing formaldehyde, derivatized (a) before and (b) after the SCX column step, in comparison

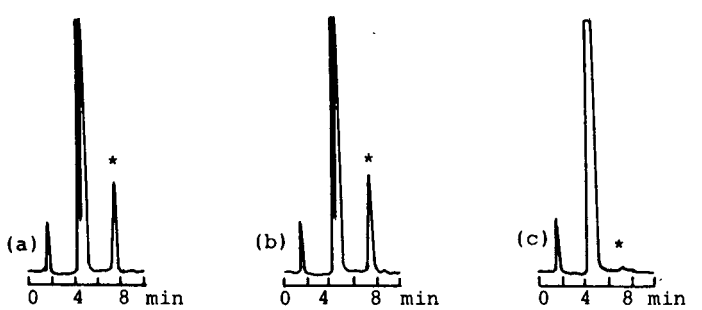


Fig. 3. Chromatographic patterns of a cosmetic emulsion containing formaldehyde derivatized (a) before and (b) after the SCX column step, in comparison with (c) the same sample without added formaldehyde.

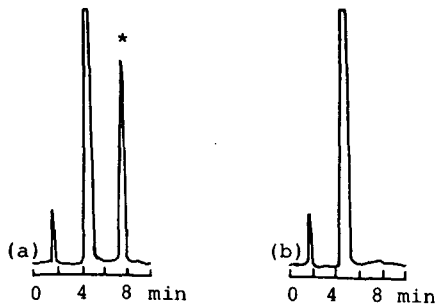


Fig. 4. HPLC of a freshly prepared Dowicil 200 standard solution derivatized (a) directly and (b) after elution from an SCX column.

with the same sample without added formaldehyde. No matrix effect and no interference of the SCX column in the quantitative evaluation of formaldehyde resulted.

Dowicil 200 analysis

A freshly prepared Dowicil 200 solution does not contain formaldehyde, the release of which is due to a time-dependent hydrolytic process. Fig. 4 shows the HPLC analysis of a freshly prepared standard solution of Dowicil 200 derivatized (a) directly and (b) after passage through the SCX column. In the first instance we found a large amount of formaldehyde because of the instability of the molecule in the acidic reaction medium, whereas in the latter instance no formaldehyde was detectable, as expected. Clearly, the preservative is not hydrolysed during the column step, although a small amount of H^+ is released into the medium. Under these experimental conditions, the pH of the solution remains stable at about 4.5–5. In order to ascertain that the preservative was completely blocked on the cation-exchange resin, we repeatedly derivatized each eluate; the good reproducibility (relative standard deviation = 1.2%) of the data obtained confirmed our hypothesis.

It is important to stress that the column must be used for only one sample; however, it is possible to regenerate the resin by the following washing sequence: 1 volume of 0.5 *M* methanolic hydrochloric acid, 1 volume of water, 1 volume of methanol and 1–2 volumes of water to neutrality. No significant variation in quantitative measurements was found using regenerated columns.

Fig. 5 shows the chromatographic patterns of a cosmetic emulsion preserved with Dowicil 20 analysed (a) at zero time and (b) after 24 h storage. No matrix effect was detected.

Fig. 6 shows the kinetics of release of formaldehyde from a 0.2% solution of Dowicil 200, stored at room temperature in darkness for 200 days and performed with the described procedure: the plateau formaldehyde concentration is about 300 μg/ml.

Starting from these results we studied the applicability of the procedure to the evaluation of free formaldehyde in different cosmetic products preserved with Dowicil 200. First we considered the influence of formulation components such as salts and other cationic moieties, on the blocking step on the cation-exchange column, and in particular on the amount of H^+ exchanged with the resin. The results showed that with

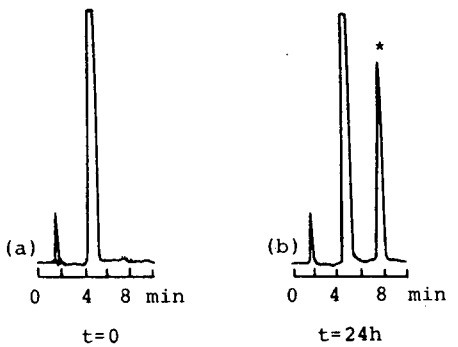


Fig. 5. Chromatographic patterns of a cosmetic emulsion preserved with 0.2% Dowicil 200 analysed (a) at zero time and (b) after 24-h storage.

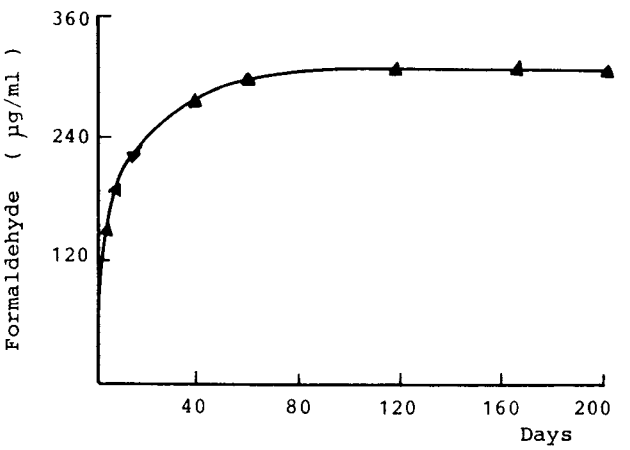


Fig. 6. Kinetics of formaldehyde release in a 0.2% Dowicil 200 aqueous solution stored at room temperature in darkness.

TABLE I

FORMALDEHYDE RELEASED IN COSMETICS PRESERVED WITH DOWICIL 200 STORED FOR 1 MONTH AT ROOM TEMPERATURE IN DARKNESS

Sample	*Dowicil 200 added (%)*	*Formaldehyde (µg/ml)*	*Relative standard deviation (%) (n=5)*
Bath foam	0.2	398.5	4.3
Cleansing milk	0.2	481.6	0.9
Lotion	0.2	525.2	2.4
Anti-dandruff shampoo	0.2	310.0	2.7
Sunscreen emulsion	0.2	405.5	3.0
Standard solution	0.2	250.0	2.5

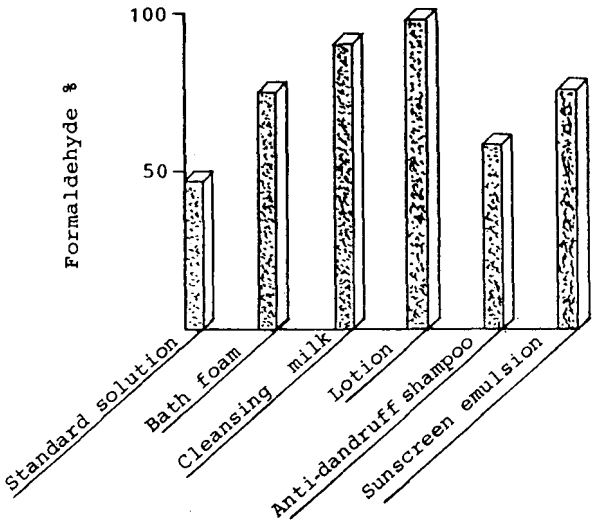


Fig. 7. Formaldehyde levels in Dowicil 200 standard solution (0.2%) and in different cosmetic samples preserved with Dowicil 0.2%, stored for 1 month at room temperature in darkness.

the dilution conditions employed (1:100), the pH of the eluate remained at *ca.* 4.5–5 for all kinds of formulations considered (emulsions, lotions, shampoos, bath foams, gels tonics, balsams, tanning and suncare products, etc.) and no interference was found in formaldehyde evaluation.

The applicability of the method was studied on five different commercial formulations, free of formaldehyde but with Dowicil 200 added (0.2%). Table I summarizes the results obtained. The samples were analysed after storage for 1 month at room temperature in darkness.

Fig. 7 shows the amount of formaldehyde found in each cosmetic sample compared with the value for a Dowicil 200 aqueous standard solution at the same concentration and stored under the same conditions for the same period of time. It is clear that the formulation significantly influences the hydrolysis of the molecule and the consequent release of formaldehyde.

ACKNOWLEDGEMENT

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CHROM. 22 113

Note

High-performance liquid chromatographic determination of the herbicide glyphosate and its metabolite (aminomethyl)phosphonic acid and their extraction from cyanobacteria

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Glyphosate[a] [N-(phosphonomethyl)glycine] is a very broad spectrum, post-emergence herbicide. The free acid (PMG) has strong herbicidal properties, although it is the isopropylamine salt (IPA) rather than PMG which is the active ingredient of Roundup® for soil use and of Rodeo® for use in water. Once applied to plants, by foliar spray, it is absorbed and translocated to other plant tissues where its primary effect is to interrupt aromatic amino acid biosynthesis by inhibition of the enzyme 5-enol-pyruvylshikimate-3-phosphate (EPSP) synthase[1,2]. Glyphosate may also directly affect the enzyme 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase[3]. Many secondary effects of glyphosate have been described, including increased metabolism of indole-3-acetic acid[4] and increased levels of both hydroxybenzoic acids[5] and phenolic compounds[6].

Glyphosate itself is apparently non-toxic to animals although some toxic effects have been attributed to the non-herbicidal ingredients present in commercial formulations[7]. Residual herbicide in the soil is readily adsorbed to soil particles and subsequently undergoes microbial degradation to ammonia and carbon dioxide[8,9]. The major degradative metabolite of PMG is (aminomethyl)phosphonic acid (AMPA) although degradation via sarcosine and glycine has also been described[10].

A variety of methods for the analysis of glyphosate and AMPA derivatives have been described including gas chromatography[11,12], thin-layer chromatography[13,14] and high-performance liquid chromatography (HPLC) with both pre-column[15–17] and post-column[18,19] derivatisation. Detection of underivatised glyphosate following HPLC has also been described[20]. These methods generally involve the use of extensive, laborious clean-up procedures including ion-exchange chromatography and gel chromatography, which may result in sample loss and reduced reproducibility, especially when no internal standard is used.

[a] Glyphosate is used to describe any form of the herbicide; N-(phosphonomethyl)glycine (PMG), PMG isopropylamine salt (IPA) and Roundup®, are used to specify free acid, IPA salt and commercial formulation, respectively.

0021-9673/90/$03.50

This paper describes a method for the detection of phenylthiocarbamyl (PTC) derivatives of glyphosate and AMPA using the Waters Pico Tag™ amino acid analysis method[21] modified by us for use with a radially compressed column. The method is sensitive, reproducible and rapid and can also be combined with amino acid detection and quantification, giving significant advantages over other previously described methods. A method for sample preparation for the detection of glyphosate and the pool of free amino acids from cyanobacteria is also described.

MATERIALS AND METHODS

Instrumentation

A Waters HPLC system was used, consisting of two Waters 510 pumps, a 10-μl fixed volume sample loop with Rheodyne valve Model 7125 and a valve position sensing switch Model 7161. The column was a Waters Nova-Pak C_{18} (100 mm × 8 mm I.D., 4 μm particle size) Radial-Pak cartridge fitted in an RCM 8 × 10 radial compression module at 17.2 MPa and at ambient temperature. For full physiological amino acid analysis a Waters Pico Tag column (300 mm × 3.9 mm I.D.) was used and the temperature was maintained at 46°C with a column heater–temperature control module. The PTC derivatives were detected using a Waters LC Model 455 variable-wavelength spectrophotometer set at 254 nm. The system was controlled, data collected and analysed using a System Interface Module, a NEC APCIV computer and Waters Maxima 820 software. Samples were prepared using a Waters Pico Tag work station with a Trivac D16B pump. Fractions from samples containing ^{14}C-labelled PMG were collected with a Pharmacia Frac100 fraction collector and counted on a Packard Tricarb 4000 scintillation counter.

Sample preparation and derivatisation

Samples of up to 200 μl were dispensed into Corning sample tubes, 50 mm × 6 mm I.D., and placed in a reaction vial with a capacity of 14 tubes. Using a Pico Tag workstation, samples were dried under vacuum to less than 9.3 Pa and then resuspended in 10 μl of a mixture of methanol–1 *M* sodium acetate–triethylamine (2:2:1, v/v/v) and redried to less than 9.3 Pa. A 20-μl aliquot of reaction mix which consisted of methanol–phenylisothiocyanate (PITC)–triethylamine–water (7:1:1:1, v/v/v/v) was added to each sample. Following a 20-min incubation at room temperature, the samples were redried to less than 9.3 Pa. For analysis, samples were resuspended in 100 μl of loading buffer [5%, (v/v) acetonitrile in 5 m*M* Na_2HPO_4, pH 7.4], and centrifuged at 10 000 *g* for 1 min prior to injection.

Mobile phase

Eluents A and B consisted of 2.5% (v/v) acetonitrile in 70 m*M* sodium acetate trihydrate pH 6.45 and acetonitrile–water–methanol (45:40:15, v/v/v), respectively. The eluents were filtered through a 0.22-μm Durapore (Millipore) membrane filter and degassed by sonication in a Cole–Parmer sonicating water bath before use. All reagents were of HPLC grade and Milli-Q (Millipore) water was used throughout. National Diagnostics Ecoscint A was used for liquid scintillation counting.

Glyphosate determination

In order to determine the retention times and linearity of the peak response to glyphosate and AMPA derivatives, samples were prepared such that between 0.05 and 10 nmol of PMG, IPA, commercial Roundup or AMPA were applied to the Radial-Pak™ column. Each sample also contained 1 nmol norleucine or taurine as an internal standard. The retention times of other possible metabolites of glyphosate, namely sarcosine and glycine, were also determined. The identity of the PTC–glyphosate peak was verified using N-phosphono-[^{14}C]methylglycine (^{14}C-PMG). Fractions eluting from the column were collected for liquid scintillation counting.

Preparation of cyanobacterial samples

Synechocystis PCC 6803 cultures were grown in 100 ml of BG11 medium[22], on an orbital shaker, at room temperature and a photon fluence rate of 30–50 μmol m^{-2} s^{-1}. Chlorophyll *a* was determined by the method of Mackinney[23] following methanol extraction of cells. Cells from cultures containing between 1 and 20 μg chlorophyll *a* per ml, were harvested by centrifugation at 10 000 *g* for 10 min and were washed twice by resuspension of the cell pellet in BG11. The cell pellet was then resuspended in 200 μl of 70% (v/v) ethanol acidified to pH 3 with hydrochloric acid, and extracted at −20°C for at least 12 h. The internal standard was added to the sample during the ethanol extraction. Extracts were centrifuged at 10 000 *g* for 10 min and then prepared for HPLC analysis as described above. In order to determine the recovery of glyphosate, samples from glyphosate-free cultures of the cyanobacterium *Synechocystis* PCC 6803 were fortified with 2–20 nmol of glyphosate (as Roundup) or AMPA, and prepared such that 0.1–1 nmol was applied to the column, assuming a recovery of 100%.

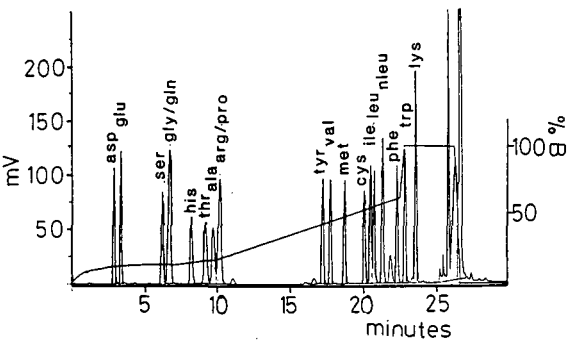
Chemicals

All chemicals were of the highest grade available and were purchased from BDH (Poole, U.K.), Millipore (Watford, U.K.) (for Waters products), Sigma (Poole, U.K.) or Rathburn Chemicals (Walkerburn, U.K.). Glyphosate (96.7% pure) andIPA (62% pure) were gifts from Monsanto (St. Louis, MO, U.S.A.). Roundup which contains 36% (w/v) glyphosate was obtained from a farm supplier. ^{14}C-PMG was purchased from Amersham International (Amersham, U.K.).

RESULTS AND DISCUSSION

Peak identification

Fig. 1 shows the gradient profile of percentage of eluent B developed for the maximal separation of Pierce H standard amino acids supplemented with norleucine, glutamine and tryptophan on the Radial-Pak column. This gradient was used for the analysis of glyphosate combined with analysis of the pools of free amino acids. HPLC elution profiles of samples containing 1 nmol of PMG, IPA or Roundup and 1 nmol of norleucine are shown in Fig. 2, together with the elution profile of ^{14}C-PMG. From the ^{14}C-labelling pattern, peak A was identified as PMG. Peak B, which has an area of up to 0.5% of that of peak A, coeluted with AMPA while peak C, with an area of 3% of that of peak A did not coelute with either glycine or sarcosine. As the retention times obtained by reversed-phase chromatography of similar compounds



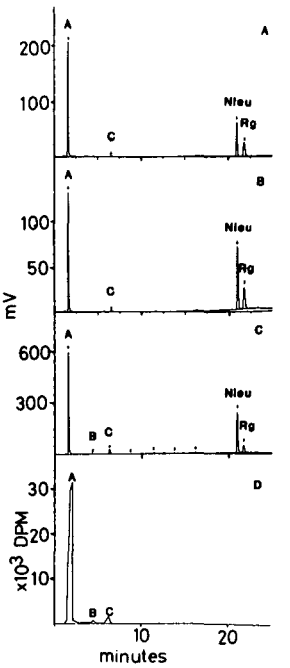


Fig. 1. Elution profile of PTC derivatives of Pierce H standard amino acids supplemented with norleucine (nleu), glutamine and tryptophan (1 nmol of each) from the Radial-Pak column using a 30-min run time. The profile of percentage of eluent B used for separation is also shown.

Fig. 2. Elution profiles of PTC derivatives of glyphosate and norleucine (1 nmol of each) from the Radial-Pak column using a 30-min run time. (A) PMG and norleucine. (B) IPA and norleucine. (C) Roundup and norleucine. (D) Profile of DPM recovered from 0.04 μCi ^{14}C-PMG. Peaks A, B and C have retention times of 1.6, 4.3 and 6.3 min, respectively. Rg denotes a reagent peak. Nleu = Norleucine.

are dependent primarily on their polarity, it can be argued that peak C was N-methylaminomethylphosphonic acid (MAMPA). MAMPA is more polar than glycine, having a phosphonic acid residue rather than a carboxylic acid residue and less polar than AMPA, having an extra methyl group. Peak C, tentatively identified as MAMPA, also contains a small fraction of the ^{14}C label from the ^{14}C-PMG. Peaks B and C were probably breakdown products of glyphosate which may have been formed during the derivatisation reaction. The major product of glyphosate degradation is AMPA although MAMPA has also been found[8] at much lower levels than AMPA. Extra peaks detected on analysis of the Roundup sample (Fig. 2C) may have been due to other UV absorbing or PTC derivatising compounds in the formulation.

As Fig. 2 shows, PMG had a short retention time, resulting in the separation of the herbicide from other components in physiological samples (Fig. 3). Any interference by other amino acids, which might lead to inaccuracies in the quantification of glyphosate was, therefore, minimised.

Response and recovery

Over the range of 0.05 to 10 nmol, for PMG (peak A) from PMG, IPA and Roundup, and with both the Radial-Pak cartridge and the Pico Tag column, a linear

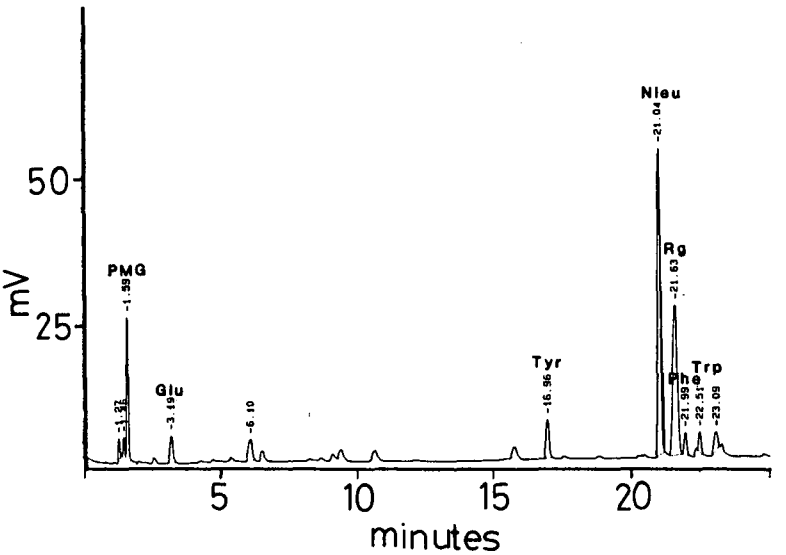


Fig. 3. Elution profiles of PTC derivatives of glyphosate and free-pool amino acids extracted from *Synechocystis* PCC 6803 grown in BG11 in the presence of 10 m*M* IPA and 1 m*M* tyrosine, phenylalanine and tryptophan. Rg denotes a reagent peak. Nleu = Norleucine.

response to the amount of PMG in the sample was obtained. Responses were calculated relative to 1 nmol norleucine. When data from all sources were collated, the same response (85.8 ± 5.5%) relative to the response for norleucine was obtained, showing no difference between either the various sources of glyphosate or between the two columns (Table I). Samples containing AMPA also gave a linear response of 59 ± 3% relative to the response for norleucine which was different to that obtained for PMG.

The high levels of reproducibility obtained were facilitated by the use of an internal standard which, when added during extraction, corrected for variation in the efficiency of derivatisation, sample preparation and injection. The sensitivity and

TABLE I

LINEARITY OF RESPONSE TO PTC–PMG AND PTC–AMPA FOLLOWING SEPARATION ON PICO TAG AND RADIAL-PAK COLUMNS

Samples containing between 0.05 and 10 nmol of glyphosate were applied to the columns.

Compound	*Column*	*Number of samples*	*Correlation coefficient*
PMG	Pico Tag	10	0.997
PMG	Radial-Pak	7	0.998
IPA	Pico Tag	6	0.995
IPA	Radial-Pak	8	0.998
Roundup	Radial-Pak	5	0.996
Collated data (above)		36	0.994
AMPA	Radial-Pak	7	0.986

reproducibility of this method may allow its use for the accurate determination of picomol levels of glyphosate, it may be especially useful when analysis of other amino acids is also required. A 10-μl water sample containing 1 ppm of glyphosate could readily be quantified.

Recovery of glyphosate from fortification experiments was similar to, or greater than that obtained by other methods. Recovery of PMG from five samples of the cyanobacterium *Synechocystis* PCC 6803 fortified with Roundup was 78 ± 8.49% and the recovery of AMPA from six fortified samples of the cyanobacterium *Synechocystis* PCC 6803 was 77 ± 12.6%. These values compare favourably with those obtained for fortified blackberries (83 ± 14%)[18], asparagus (89 ± 36%) and kiwi fruit (87 ± 7.1%)[24].

The method of sample preparation developed for cyanobacteria, which did not require deproteination prior to analysis, should be easily adaptable for use with other tissues of both plant and animal origin. A simple maceration step prior to ethanol extraction may be required for such tissues.

Rapid analysis

The method described above allowed the determination of glyphosate as well as other amino acids which may be of use in studies of herbicide action. A more rapid analytical method can be used if other amino acid data are not required. An analysis time of 15 min including column reequilibration is possible with taurine (retention time 6.1 min) used as the internal standard (Fig. 4). The response relative to taurine was linear with a correlation coefficient of 0.994 (n = 10) over the range 0.1 to 10 nmol of glyphosate.

Sample storage

Samples were stable during long-term storage, at least three months, in 70%

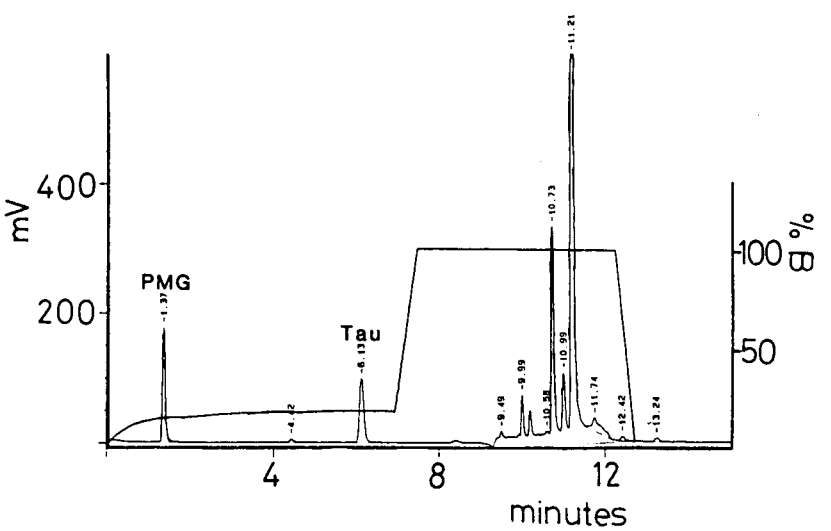


Fig. 4. Elution profile of PTC–glyphosate and PTC–taurine from the Radial-Pak column using a 15-min run time showing the gradient profile of eluent B percentage.

(v/v) acidified ethanol at −20°C prior to vacuum desiccation. Once dried and treated with drying solution, samples could be stored for at least five days under vacuum or nitrogen at −20°C. Following derivatisation samples are only stable for 24 h under vacuum at −20°C.

The above method of PMG and AMPA analysis is sensitive, reproducible and rapid. In contrast to some previously published methods, sample preparation was accomplished with relative ease. Up to 14 samples were prepared simultaneously and their stability prior to derivatisation allowed them to be prepared in advance. Analysis of 30 samples a day was achieved and the use of an autosampler would allow the analysis of even greater numbers each day.

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Note

High-performance liquid chromatographic separation and determination of quinalphos in technical and commercial formulations[a]

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O,O-Diethyl O-2-quinoxalinyl phosphorothioate (quinalphos) is a pesticide used effectively against caterpillars on cotton, groundnuts, vegetables and rice and its widespread use necessitates a simple, specific and rapid method for its determination in formulations. Many methods based on gas chromatography (GC)[1–4] and thin-layer chromatography (TLC)[5–8] have been reported for the determination of organophosphorus pesticides. GC methods for the determination of quinalphos lack not only precision but also accuracy as the compound is highly sensitive to heat and often decomposes in the column before it is detected and quantified. The separation of quinalphos by paper chromatography followed by its combustion for spectrophotometric determination is an accurate procedure[9], but it is tedious and time consuming. Chemical methods[10,11] in which the quinalphos content is measured by phosphorus or nitrogen determination suffer from interference from O,O-diethyl phosphorochloridothioate, ethyl phosphorodithioate and 2-ethoxyquinoxaline. Rastogi *et al.*[12] described a gravimetric method for the determination of quinalphos using copper(I) chloride as a precipitating agent. However, it has the drawback that 2-hydroxyquinoxaline and the emulsifier interfere during precipitation. Therefore, a rapid and reliable method for the determination of quinalphos in formulations is still needed.

High-performance liquid chromatographic (HPLC)[13–16] methods for the separation of several organophosphorus pesticides have been reported, but these are neither specific nor selective for quinalphos in formulations. We report here a simple and selective HPLC method for the separation and determination of quinalphos in technical and commercial formulations. The proposed method is also applicable to the analysis of food and agricultural products for residues of quinalphos.

EXPERIMENTAL

Materials and reagents

All reagents were of analytical-reagent grade unless stated otherwise. HPLC-

[a] IICT Communication No. 2404.

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grade 2,2,4-trimethylpentane and 2-propanol were obtained from Spectrochem (Bombay, India) and 1,3-dinitrobenzene from BDH (Poole, U.K.).

Quinalphos was prepared by condensing 2-hydroxyquinoxaline with O,O-diethyl phosphorochloridothioate in the presence of anhydrous potassium carbonate and acetonitrile according to Schmidt and Hammann[17]. The product was stabilized by adding epichlorohydrin and diluted with *o*-xylene to obtain a sample of technical-grade quinalphos. Commercially formulated samples, Ekalux and Suquin (25% emulsifiable concentrate) and dust powder (1.5% quinalphos), manufactured by Sandoz (Bombay, India) and Sudarshan Chemicals (Poona, India), respectively were used.

Apparatus

A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) with a 20-μl loop injector having an high-pressure six-way valve was used. A Model SPD-6AV variable-wavelength UV–VIS spectrophotometric detector (Shimadzu, Kyoto, Japan) was connected after the column. A Zorbax SIL (DuPont, Wilmington, DE, U.S.A.) column (250 mm × 4.6 mm I.D.; 5 μm particle size) was used for separation. The chromatograms and the integrated data were recorded by a Chromatopac C-R3A processing system.

Chromatographic conditions

The mobile phase was 2,2,4-trimethylpentane–2-propanol (9:1, v/v). Samples were dissolved in the mobile phase. The analysis was carried out under isocratic conditions at a flow-rate of 1 ml/min and a chart speed of 5 mm/min at room temperature (27°C). Chromatograms were recorded at a wavelength of 245 nm.

Sample preparation

Samples of quinalphos (50 mg) and 1,3-dinitrobenzene (10 mg) used as the internal standard (I.S.) were dissolved in the mobile phase (25 ml).

Analytical procedure

Standard mixtures containing 2–10 mg of internal standard, 5–20 mg of quinalphos, 15–50 mg of *o*-xylene and 1–2 mg of epichlorohydrin were prepared by dissolving known amounts of the compounds in 10 ml of the mobile phase. A 5-μl volume of each standard mixture was injected and chromatographed under the above conditions. From the areas of the peaks the response factor of quinalphos with respect to the internal standard was calculated.

Synthetic mixtures and commercial formulations were analysed under identical conditions. Quinalphos of technical grade or a commercial formulation (50 mg) together with the internal standard (10 mg) was dissolved in 25 ml of mobile phase and chromatographed. The percentage of quinalphos was calculated from the peak areas.

RESULTS AND DISCUSSION

The HPLC separation of quinalphos, epichlorohydrin, *o*-xylene and 1,3-dinitrobenzene is shown in Fig. 1. The peaks were identified by injecting the individual compounds. It can be seen that quinalphos is not only resolved from its formulation

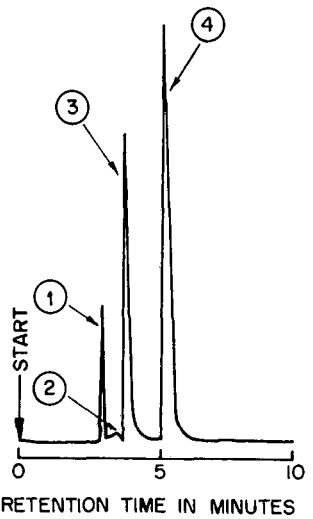

Fig. 1. Typical chromatogram of technical-grade quinalphos (30 μg). For conditions, see text. Peaks: 1 = *o*-xylene; 2 = epichlorohydrin; 3 = quinalphos; 4 = 1,3-dinitrobenzene (10 μg).

additives but also from 1,3-dinitrobenzene. The λ_{max} and retention time data for all the compounds are given in Table I. A wavelength of 245 nm, at which all the compounds under investigation absorb UV light, was selected for analysis not only because detection is ensured but also a good linearity between mass and integral response is obtained. When the UV detector is set at 0.001 a.u.f.s. the limit of detection for quinalphos is $3 \cdot 10^{-9}$ g with a signal-to-noise ratio of 4.0. Response factors for quinalphos were determined in the range 25–75% of quinalphos and are recorded in Table II. They were found to remain constant throughout this range.

Standard mixtures containing different amounts of quinalphos were prepared and analysed by HPLC. The results are given in Table III. The accuracy of the method was determined by the standard addition technique. Subsequent additions of quinalphos were accurately reflected in the peak heights. The measured amounts of quinalphos agreed well with the actual values to within 1.88%. Linear regression analysis of the data yielded the line $y = 0.9978x + 0.1999$ with a correlation coefficient of 0.9998.

TABLE I

RETENTION DATA

Compound	*Retention time (min)*	*Relative retention time*	λ_{max} *(nm)*
1,3-Dinitrobenzene	5.61	1.00	233
Quinalphos	4.12	0.73	237
Epichlorohydrin	3.57	0.64	210
o-Xylene	3.25	0.58	263

TABLE II

DETERMINATION OF RESPONSE FACTORS FOR QUINALPHOS

Sample No.	*Weight of quinalphos (mg)*	*Weight of I.S. (mg)*	*Area of quinalphos peak*	*Area of I.S. peak*	*Response factor*[a]
1	16.53	8.10	15 557	13 902	1.8236
2	16.53	8.10	15 814	14 068	1.8154
3	16.53	5.40	15 505	9271	1.8303
4	16.53	5.40	15 721	9627	1.8745
5	12.40	5.40	11 813	8907	1.7314
6	12.40	5.40	11 915	9639	1.8577
7	8.27	5.40	7783	8957	1.7625
8	8.27	5.40	7515	8770	1.7872
9	8.27	2.70	7768	4514	1.7799
10	8.27	2.70	7724	4469	1.7722
Mean					1.8035
S.D.					0.0423
R.S.D.[b] (%)					2.4

[a] Response factor of quinalphos = [A (I.S.)/W (I.S.)] · [W (quinalphos)/A (quinalphos)], where A = peak area and W = weight.

[b] Relative standard deviation.

Technical and commercial formulations of quinalphos were analysed by the proposed method and also by the Indian Standards Institution procedure[9]. The results (Table IV) are in good agreement, with an error of 1.56%.

Vegetables spiked with quinalphos at the level of 1 mg kg^{-1} were been crushed and dried, extracted with acetone and analysed using the developed method. The chromatograms of a cabbage blank and the same sample spiked with quinalphos are shown in Fig. 2. Even at the lowest detector attenuation setting, the blank showed a fairly flat baseline in the region where quinalphos and 1,3-dinitrobenzene elute and the limit of detection for quinalphos in spiked vegetables was found to be 0.05 mg kg^{-1}. The results are given in Table V.

It is concluded that the proposed method is not only suitable for the routine determination of the quinalphos in technical and commercial formulations but may

TABLE III

DETERMINATION OF QUINALPHOS IN STANDARD MIXTURES

Sample No.	*Quinalphos (%)*		*Error (%)*
	Taken	*Found*[a]	
1	20.36	20.72	+1.77
2	25.07	25.54	+1.88
3	29.14	28.69	−1.54
4	65.20	65.09	−0.17
5	70.05	70.32	+0.39

[a] Averages of triplicate analyses.

TABLE IV

DETERMINATION OF QUINALPHOS IN TECHNICAL AND COMMERCIAL FORMULATIONS

Sample No.	*Formulation*	*Quinalphos present (%)*	*Quinalphos found[a] (%)*		*Mean ± R.S.D. (%)*		*Error (%)*
			By HPLC	*By ISI method[9]*	*By HPLC*	*By ISI method[9]*	
1	Ekalux	25.00	24.73	26.36	25.03 ± 1.33	24.82 ± 4.38	+0.85
			25.49	24.15			
			24.86	23.96			
2	Suquin	25.00	24.51	25.92	24.81 ± 1.23	25.17 ± 2.69	−1.43
			24.70	25.31			
			25.23	24.28			
3	Technical I	65.00	63.79	65.45	64.58 ± 1.36	65.59 ± 1.20	−1.54
			65.80	66.62			
			64.15	64.70			
4	Technical II	70.00	71.36	68.57	70.96 ± 1.03	69.87 ± 1.37	+1.56
			71.59	70.84			
			69.94	70.20			
5	Dust	1.50	1.53	1.43	1.48 ± 6.47	1.46 ± 6.83	+1.37
			1.57	1.35			
			1.35	1.59			

[a] Average values obtained from duplicate analyses.

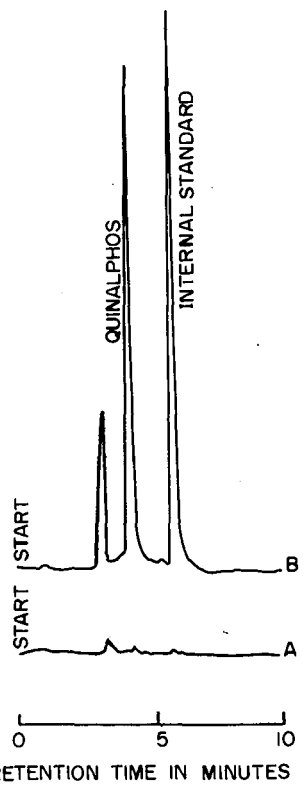

Fig. 2. Chromatograms of (A) a cabbage blank and (B) the same cabbage spiked with 7.3 mg kg^{-1} of quinalphos and 10 mg of internal standard. For conditions, see text.

TABLE V

DETERMINATION OF TRACE AMOUNTS OF QUINALPHOS IN VEGETABLES

Sample	*Amount of sample (g)*	*Content of quinalphos*	
		mg	*ppm*
Cabbage	125.70	0.92	7.32
Cauliflower	104.52	0.50	4.78
Potato	139.86	0.17	1.26

also be useful for the analysis of environmental samples and agricultural products for its residues. It is simple, specific, rapid and inexpensive compared with methods reported earlier[9–12]. Moreover, impurities such as O,O-diethyl phosphorochloridothioate and 2-hydroxyquinoxaline, which are generally present in quinalphos, do not interfere because they elute at 3.43 and 8.95 min, respectively.

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Note

High-performance liquid chromatographic determination of dibutyldithiocarbamate degradation products in cucumbers and soil

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Metal dialkyldithiocarbamates are used as active fungicides in agriculture (ferbam, ziram) and also as additives and antioxidants in rubber and plastic materials. Therefore, direct monitoring of their purities and their residues in the foods and agricultural products is necessary.

High-performance liquid chromatography (HPLC) of metal complexes of N-disubstituted dithiocarbamic acids (dimethyl and dibutyl derivatives) was described in previous papers[1–4]. The results confirmed that all these complexes are unstable and decompose rapidly.

The effects of UV radiation, temperature and time on the degradation of iron(III) dibutyldithiocarbamate were studied[5] and the use of HPLC and mass spectrometry (MS) for the identification and determination of this complex and the products of its transformation was discussed. Adsorption and reversed-phase chromatography were investigated.

Ethylenethiourea (ETU) was determined as the main degradation product of ethylene bis dithiocarbamate in some fungicides by HPLC[6]. The identification of some mixed-ligand dithiocarbamate complexes has been described[7,8] and the rate constants for ligand-exchange reactions evaluated[9].

Dithiocarbamate complexes are known to have toxicological and mutagenic effects and their degradation products have been found to be more biologically effective than the original chelates[10–12]. The conversions of dimethyldithiocarbamates into tetramethylthiuram disulphide (TMTD) and its mechanism for ferbam [iron(III) dimethyldithiocarbamate] and ziram [zinc(II) dimethyldithiocarbamate] have been illustrated[13].

Some papers have discussed in detail toxicological aspects of dithiocarbamates (DTCs) and related compounds[14,15]. The short-term toxicity of 26 DTCs was

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determined in tests with various organisms. It was found that dialkyl-DTCs were generally more toxic than ethylene bis-DTCs. The application of heavy metal DTCs is questionable as these compounds are generally more toxic than the metals.

Liquid–liquid extraction of some dialkyl-DTCs has been studied with various solvent combinations in order to establish reagent interferences in the final determination[16].

Liquid chromatography has been applied to the identification and determination of some DTCs in vulcanized rubber products. After conversion of zinc DTCs into the corresponding cobalt(III) DTCs, the latter were separated by a reversed-phase method with methanol–water using UV detection[17]. HPLC with amperometric detection on a copper electrode has been used for the determination of ethylenethiourea in beverages without sample pretreatment; the limit of detection was 3 ng/ml[18].

The aim of this work was to use the optimum separation conditions for the simultaneous HPLC separation of iron(III) dibutyldithiocarbamate [Fe(III) DB DTC] and its degradation products in some agricultural samples grown on plastic foil containing dithiocarbamates as antioxidants. It is known that such foil is degraded during the growth period under the influence of UV radiation, temperature and oxidizing agents, and the degradation products can penetrate into the soil or crop grown in contact with the foil. For these reasons, it is necessary to monitor the residues of dithiocarbamates and their degradation products.

EXPERIMENTAL

Apparatus

All experiments were carried out on Packard Model 8200 HPLC system with a UV detector (254 nm). Stainless-steel columns (250 × 2.2 mm I.D.) packed with LiChrosorb Si 60 (particle diameter 5 μm) and LiChrosorb RP-18 (5 μm) were applied. Chloroform–*n*-heptane (20:80) and water–isopropanol (25:75) were used as mobile phases.

Some gas chromatographic–mass spectrometric (GC–MS) measurements were performed using a Finnigan 4000 instrument. Helium was used as the carried gas. A glass "falling needle" device was used as an injector. All experiments were carried out under the following GC conditions: inlet pressure 2.0 bar (absolute), injection temperature 240°C, column temperature 230°C and ion-source temperature 250°C.

The mass spectrometer was operated in the electron-impact (EI) mode under the following conditions: electron energy 70 eV, electron current 0.25 mA, multiplier voltage 1.70 kV, mass range 40–550 and scan speed one spectrum per second.

A Model 1102 elemental analyser (Carlo Erba, Milan, Italy) was used to establish the purities of the standards.

Chemicals

Iron(III) dibutyldithiocarbamate [Fe(III) DB DTC] and tetrabutylhiuram disulphide (TBTD) were synthesized by literature procedures[11,19]. A standard Fe(III) DB DTC was obtained as an antioxidant used in the plastics industry (VUAPL, Research Institute of Plastic Materials, Nitra, Czechoslovakia). The identities and purities of the standards were confirmed by elemental analysis and mass spectrometry.

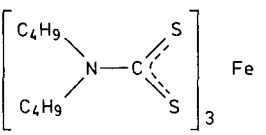
All organic solvents were of analytical-reagent grade (Lachema, Brno, Czechoslovakia) and were dried over magnesium perchlorate and redistilled.

Procedures

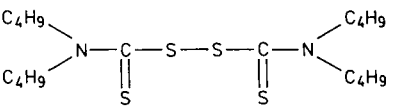
Chloroform was applied for the extractions of real samples (cucumbers and soil). Amounts of 500 g of the soil (cucumbers) were extracted gradually at room temperature with 3 × 300 cm^3 of chloroform in the discontinuous mode. Each extraction was performed for 10 min. After evaporation of the solvent, dissolution of the residues in the mobile phase (1 cm^3) and filtration through glass-fibre filters (Tessek, Prague, Czechoslovakia), the extracts were injected onto the chromatographic column in volumes of 10 μl.

RESULTS AND DISCUSSION

The optimum separation conditions for the simultaneous HPLC separation of Fe(III) DB DTC and TBTD have been published[5]. Reversed-phase HPLC with a LiChrosorb RP-18 column and isopropanol–water as the mobile phase was recommended.



Fe(III) DB DTC



TBTD

Determination of Fe(III) DB DTC and TBTD in cucumbers

Samples of cucumbers grown on foil containing Fe(III) DB DTC as an antioxidant were extracted according to the procedure described. Four different cucumbers extracts were analysed: (1) cucumbers grown without using the foil; (2) cucumbers grown on foil containing 0.6% of Fe(III) DB DTC; (3) cucumbers grown on foil containing 0.3% of Fe(III) DB DTC in combination with another antioxidant; and (4) cucumbers grown on foil without Fe(III) DB DTC.

HPLC separation of cucumber extracts fortified with Fe(III) DB DTC and TBTD is shown in Fig. 1 and HPLC traces for cucumbers extracts of samples 2 and 4 are illustrated in Fig. 2a and b. The extraction recovery of Fe(III) DB DTC was 89% for an Fe(III) DB DTC concentration in cucumbers of 0.05 mg/kg. The standard deviation ($n = 5$) was 2% and linearity achieved at the spiking levels of 0.02–0.1 mg/kg. The limit of detection of Fe(III) DB DTC in cucumber extracts was 0.020 mg/kg. For TBTD the recovery from cucumbers was 90% the and limit of detection of TBTD in extracts was 0.018 mg/kg.

From the chromatogram in Fig. 2a, it is obvious that this extract did not contain residues of Fe(III) DB DTC. However, very low concentrations of TBTD were determined in cucumber samples 2 and 3 (0.036 and 0.022 mg/kg, respectively).

The presence of TBTD in the extracts was verified by GC–MS. The results confirm that dibutyldithiocarbamates are not stable and after several months they could be decomposed completely to their degradation products (*e.g.*, TBTD)[5].

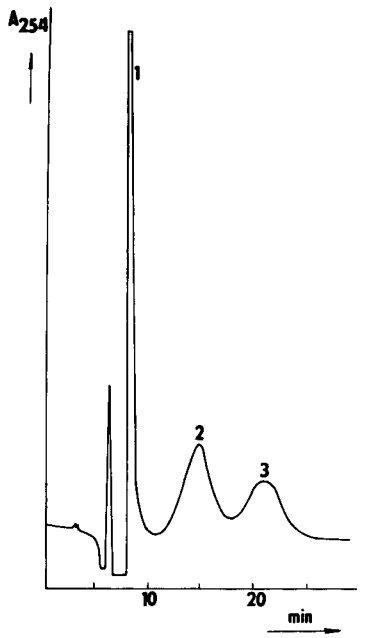


Fig. 1. HPLC separation of cucumber extract fortified with TBTD and Fe(III) DB DTC (0.1 mg/kg). Column, LiChrosorb RP-18, 5 μm (250 × 2.2 mm I.D.); mobile phase, water–isopropanol (25:75); flow-rate, 0.2 cm^3/min; injection volume, 10 μl; attenuation 32. Peaks: 1 = inert; 2 = TBTD; 3 = Fe(III) DB DTC. resolution $R_{2,3}$ = 1.28.

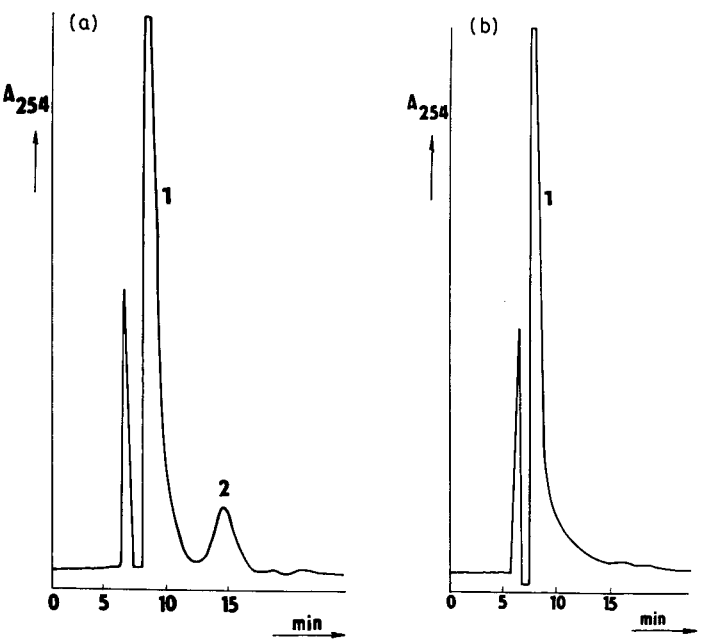


Fig. 2. HPLC traces of cucumber extracts, samples (a) 2 and (b) 4. Separation conditions as in Fig. 1; attenuation 16. Peaks: 1 = inert; 2 = TBTD.

Determination of Fe(III) DB DTC and TBTD in soil

Extracts of soils from cucumber growing (samples 1–4) were analysed in the same way as the cucumbers samples. After filtration the extracts were injected onto the chromatographic column. The separation conditions were the same as for HPLC analysis of cucumber extracts. HPLC separations of the soil extracts (samples 2 and 4) are illustrated in Fig. 3a and b. Both Fe(III) DB DTC and TBTD were identified in soil sample 2 [0.022 mg/kg Fe(III) DB DTC and 0.026 mg/kg and TBTD in sample 3 (0.018 mg/kg). Th results obtained were verified by GC–MS. A gas chromatogram of the soil extract (sample 2) is shown in Fig. 4.

It was discussed previously[5] that TBTD had been decomposed to tetrabutylthiuram monosulphide (TBTM) during GC separation at temperatures above 473 K. For this reason, the peak of TBTM was identified in Fig. 4 in site of the fact that TBTM had not been present in the soil extract. At the temperature of GC separation (503 K) it is certain that TBTD present in the extract would also have been converted into TBTM. Mass spectra of TBTD and TBTM from the soil extract are illustrated in Fig. 5a and b.

The identification of TBTD and TBTM was confirmed at low voltage at 12–16 eV. The recovery of Fe(III) DB DTC in the soil (fortified at 0.05 mg/kg) was 91% and that of TBTD 92%. The detection limits for Fe(III) DB DTC and TBTD were 0.016 mg/kg and 0.014 mg/kg in soil, respectively.

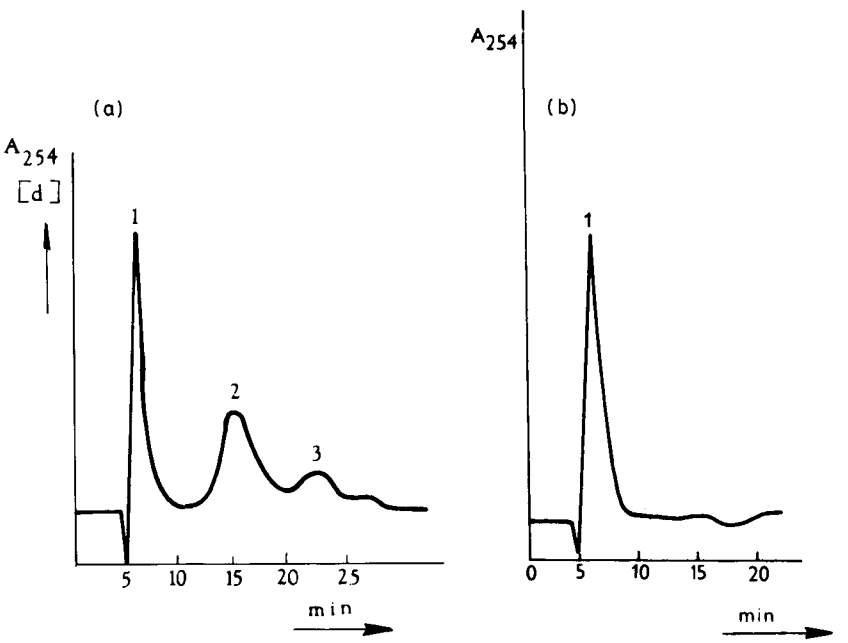


Fig. 3. HPLC traces of soil extracts, samples (a) 2 and (b) 4. Separation conditions as in Fig. 1; attenuation 4. Peaks: 1 = inert; 2 = TBTD; 3 = Fe(III) DB DTC.

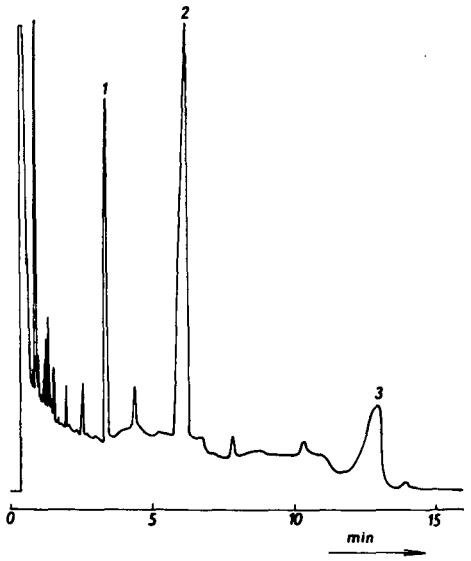


Fig. 4. Gas chromatogram of soil extract, sample 2. Column, SE-30 (30 m × 0.4 mm I.D.); pressure (helium), 200 kPa; temperature, 503 K. Peaks: 1 = unknown; 2 = TBTM; 3 = TBTD.

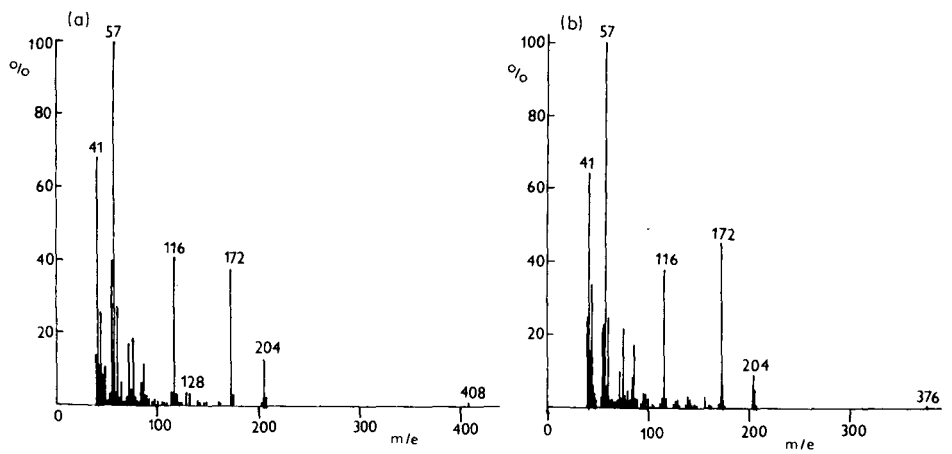


Fig. 5. Mass spectra of (a) TBTD and (b) TBTM.

The proposed method has already been accepted by VU APL Nitra (a producer of plastic foils used in agriculture) and has been applied for the control analysis of DTC antioxidants in rubber and plastic products.

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Note

Studies on the separation of Al^{III}, Co^{III}, Cr^{III} and Fe^{III} complexes with acetylacetone by reversed-phase high-performance liquid chromatography

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The separation and determination of metal ions in the form of chelates of fluorinated analogues of acetylacetone (HAA) by gas chromatography (GC) are well known[1–4], but the application of reversed-phase high-performance liquid chromatography (RP-HPLC) to the separation and determination of simple HAA metal chelates has not yet been successfully demonstrated[5].

The separation of β-diketone metal chelates, including metal acetylacetonates [$M(AA)_n$], by HPLC was first reported by Huber and Kraak[6]. They employed liquid–liquid partition chromatography involving a ternary solvent system consisting of water, methanol and 2,2,4-trimethylpentane. They established the conditions necessary to separate four to six metal chelates of Be^{II}, Al^{III}, Cr^{III}, Fe^{III}, Co^{III}, Ni^{II}, Cu^{II}, Zn^{II}, Co^{II}, Zr^{IV} and Ru^{III}. The first attempt to separate β-diketone chelates by RP-HPLC was reported by Tollinche and Risby[7]. Although their studies demonstrated a successful separation of the chelates by normal-phase HPLC, they were completely unsuccessful in separating the complexes by RP-HPLC.

RP-HPLC was successfully used by Gurira and Caar[8] for the separation of Mn^{II}, Be^{II}, Co^{III}, Cr^{III}, Rh^{III}, Ir^{III}, Pd^{II} and Pt^{II} acetylacetonates with Supelco C_{18} and Ultrasphere C_{18} columns. They obtained good separations of these chelates (4–7 components) using methanol–water or acetonitrile–water mixtures as mobile phases.

This paper reports the separation of mixtures of acetylacetonates of Co^{III}, Cr^{III}, Al^{III} and Fe^{III} by RP-HPLC with columns containing LiChrosorb packings modified with RP-2, RP-8 and RP-18 hydrocarbon bonded phases and methanol–water or acetonitrile–water mobile phases.

EXPERIMENTAL

A Liquochrom Model 2010 high-performance liquid chromatograph (Labor, Budapest, Hungary), equipped with a UV detector (180–440 nm), was used. Eight steel columns (25 or 10 cm × 4.6 mm I.D.) were used with LiChrosorb packings (10, 7 or 5 μm) modified with RP-2, RP-8 or RP-18 hydrocarbon bonded phases (ZOCh,

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TABLE I

THE CHARACTERISTICS OF COLUMNS FOR SEPARATION OF $M(AA)_3$

Column No.	*Bonded stationary phase*	*Column length (cm)*	*Particle size (μm)*	*High equivalent to a theoretical plate (mm)*	*Flow-rate (linear) of mobile phase (cm/s)*	
					CH_3OH–H_2O (40:60)	*CH_3CN–H_2O (30:70)*
1	RP-18	25	10	0.254	0.250	0.228
2	RP-8	25	10	0.285	0.209	0.188
3	RP-2	25	10	0.283	0.245	0.208
4	RP-2	25	7	0.266	0.233	0.227
5	RP-2	25	5	0.290	0.157	0.190
6	RP-18	10	10	0.244	0.247	0.251
7	RP-18	10	7	0.230	0.254	0.220
8	RP-18	10	5	0.247	0.166	0.175

Lublin, Poland). Table I gives the characteristics of the columns. UV spectra of the metal chelates were obtained on a Specord UV–VIS instrument (Carl Zeiss, Jena, G.D.R.).

The reagents used were pure chromium acetylacetonate, $Cr(C_5H_7O_2)_3$ (Serva, Heidelberg, F.R.G.), cobalt acetylacetonate for synthesis, $Co(C_5H_7O_2)_3$ (Merck-Schuchard, Darmstadt, F.R.G.), aluminium acetylacetonate, $Al(C_5H_7O_2)_3$, and iron(III) acetylacetonate, $Fe(C_5H_7O_2)_3$, both prepared by us by the method described by Charles and Pawlikowski[9], spectrally pure methanol (POCh, Gliwice, Poland) and acetonitrile for chromatography (Merck-Schuchard). The mobile phases were methanol–water (50:50 and 40:60, v/v) and acetonitrile–water (50:50, 40:60, 30:70 and 25:75, v/v). The solvents were thoroughly mixed with redistilled water and degassed on an ultrasonic bath under reduced pressure for 10–15 min.

Solutions of $M(AA)_3$ were prepared by dissolving suitable amounts of chelates in methanol or acetonitrile and diluting to concentrations of 10^{-4}–10^{-5} *M*. UV absorption bands (λ_{max}) of the investigated solutions of $Co(AA)_3$, $Cr(AA)_3$, $Al(AA)_3$ and $Fe(AA)_3$ were 256, 332, 294 and 276 nm, respectively, in acetonitrile solutions and 252, 326, 282 and 270 nm, respectively, in methanol solutions. Sample solutions of the complexes were injected onto the column (20 μl) and the absorbances of the mixture complexes was detected at 254, 280 and 310 nm, but the best results in peak height were obtained at 280 nm.

RESULTS AND DISCUSSION

Measurements of the retention times of $Co(AA)_3$, $Cr(AA)_3$, $Al(AA)_3$ and $Fe(AA)_3$ acetylacetonates were carried out on eight columns with methanol–water and acetonitrile–water mobile phases at flow-rates in the range 0.7–2.0 cm^3/min. Depending on the mobile phase composition (see above), these flow-rates forced the slowest component, $Cr(AA)_3$, through the column in less than 15 min. Table II gives the measured retention times of $M(AA)_3$ for acetonitrile–water (30:70) and methanol–water (40:60) as mobile phases. Under the conditions employed, the chelates gave

TABLE II

RETENTION TIMES OBTAINED FOR $M(AA)_3$ IN ACETONITRILE–WATER (30:70) AND METHANOL–WATER (40:60)

Column No.	*Flow-rate of mobile phase (cm^3/min)*	*Retention time (min)*						
		Acetonitrile–water (30:70)			*Methanol–water (40:60)*			
		$Co(AA)_3$	$Al(AA)_3$	$Cr(AA)_3$	$Fe(AA)_3$	$Al(AA)_3$	$Co(AA)_3$	$Cr(AA)_3$
1	1.5	7.75	10.67	13.00	–	–	11.17	15.00
2	1.5	7.50	10.50	12.17	–	–	11.08	14.62
3	1.5	6.58	8.92	10.42	3.50	4.58	10.00	13.00
4	1.5	5.13	6.70	7.72	3.67	4.17	9.82	12.58
5	1.5 (1.0)[a]	6.20	8.12	9.47	5.75	6.33	8.82	10.82
6	1.5	3.45	5.03	6.08	1.93	3.01	5.17	7.17
7	1.5	3.75	5.33	6.50	3.02	4.01	6.42	9.17
8	0.9	4.45	6.18	7.58	3.00	3.67	6.82	9.50

[a] Flow-rate of 1.0 cm^3/min applies to the methanol–water (40:60) eluent.

sharp symmetric peaks, with the exception of $Fe(AA)_3$, which showed slight tailing with both mobile phases, and $Al(AA)_3$, which showed the same phenomenon with methanol–water. According to our results, consistently with those of Huber and Kraak[6], these chelates tend to dissociate in aqueous solutions. The elution order of the $Co(AA)_3$ and $Al(AA)_3$ chelates is influences by the type of mobile phase. In particular, $Al(AA)_3$ elutes first and $Co(AA)_3$ elutes next with methanol–water, but with acetonitrile–water this order is reversed.

Figs. 1 and 2 show chromatograms of $M(AA)_3$ mixtures. The mixtures were well

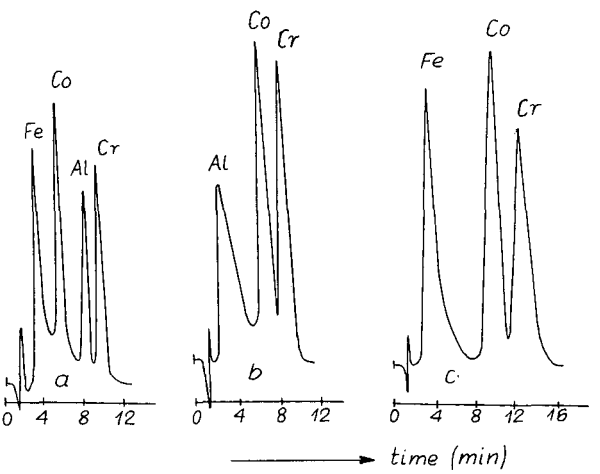

Fig. 1. Chromatograms of $M(AA)_3$ mixtures obtained on columns containing LiChrosorb RP-2. Mobile phase: (a) acetonitrile–water (30:70); (b, c) methanol–water (40:60). Flow-rate: (a, c) 1.5 cm^3/min; (b) 2.0 cm^3/min. (a) $Fe(AA)_3$ + $Co(AA)_3$ + $Al(AA)_3$ + $Cr(AA)_3$ (3:1:4:2); 20-μl sample volume contains $4.21 \cdot 10^{-7}$ g Fe, $2.38 \cdot 10^{-7}$ g Co, $6.74 \cdot 10^{-7}$ g Al and $4.2 \cdot 10^{-7}$ g Cr. (b) $Al(AA)_3$ + $Co(AA)_3$ + $Cr(AA)_3$ (3:1:2); $3.25 \cdot 10^{-7}$ g Al, $2.37 \cdot 10^{-7}$ g Co and $4.23 \cdot 10^{-7}$ g Cr. (c) $Fe(AA)_3$ + $Co(AA)_3$ + $Cr(AA)_3$ (4:1:2); $8.98 \cdot 10^{-7}$ g Fe, $2.37 \cdot 10^{-7}$ g Co and $4.23 \cdot 10^{-7}$ g Cr.

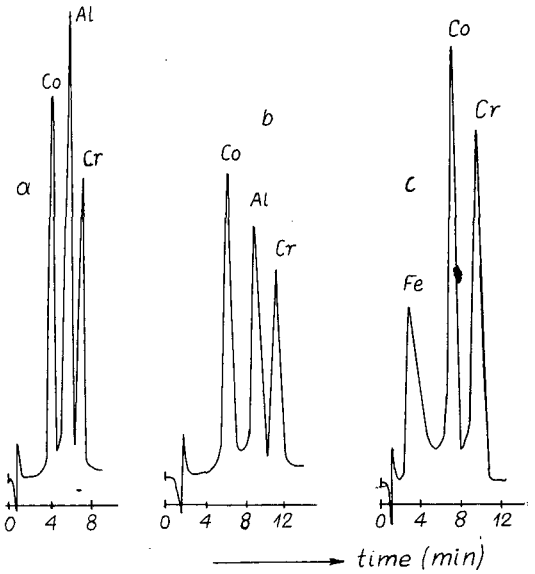

Fig. 2. Chromatograms of $M(AA)_3$ mixtures obtained on columns containing LiChrosorb RP-18. Mobile phase: (a, b) acetonitrile–water (30:70); (c) methanol–water (40:60). Flow-rate: (a, c) 2.0 cm^3/min; (b) 1.5 cm^3/min. (a, b) $Co(AA)_3$ + $Al(AA)_3$ + $Cr(AA)_3$ (1:3:2); $2.38 \cdot 10^{-7}$ g Co, $3.16 \cdot 10^{-7}$ g Al and $4.2 \cdot 10^{-7}$ g Cr. (c) $Fe(AA)_3$ + $Co(AA)_3$ + $Cr(AA)_3$ as in Fig. 1a.

separated with the columns and mobile phases employed. A mixture of $Co(AA)_3$, $Cr(AA)_3$, $Al(AA)_3$ and $Fe(AA)_3$ was separated on column 3 modified with RP-2 using acetonitrile–water (30:70) (Fig. 1). A mixture of $Co(AA)_3$, $Al(AA)_3$ and $Cr(AA)_3$ was successfully separated on all columns using the same mobile phase. Methanol–water (40:60) was used for the separation of another three-component chelate mixture, but the separation of $Co(AA)_3$ and $Al(AA)_3$ was not complete.

TABLE III

COMPARISON OF CAPACITY FACTORS (k') AND RELATIVE RETENTIONS (α) FOR $M(AA)_3$

Column No.	*Column packing*	*Mobile phase*	k'			α		
			$Co(AA)_3$	$Al(AA)_3$	$Cr(AA)_3$	*Al/Co*	*Cr/Al*	*Cr/Co*
1	RP-18	CH_3CN–H_2O (30:70)	3.15	4.82	5.96	1.53	1.24	1.89
2	RP-8		2.40	3.72	4.50	1.55	1.21	1.87
3	RP-2		2.28	3.45	4.19	1.51	1.21	1.84
4	RP-2		1.80	2.65	3.20	1.47	1.21	1.78
5	RP-2		2.27	3.45	4.23	1.52	1.23	1.86
6	RP-18		4.20	6.55	8.15	1.56	1.24	1.94
7	RP-18		3.95	6.10	7.65	1.54	1.25	1.94
8	RP-18		3.70	5.53	6.95	1.50	1.25	1.94
1	RP-18	CH_3CN–H_2O (25:75)	4.68	7.50	9.43	1.60	1.26	2.01
6	RP-18		4.85	7.85	9.85	1.62	1.25	2.03
7	RP-18		5.75	9.60	12.05	1.67	1.25	2.09
8	RP-18		4.10	6.45	8.16	1.57	1.26	1.99

Table III gives the capacity factors (k') and relative retentions (α) calculated according to our results, consistent with Hamilton and Sewell[10]. These parameters were calculated for all columns and all mobile phases, but Table III just gives the data for acetonitrile–water (30:70) for all columns and for acetonitrile–water (25:75) for columns packed with LiChrosorb RP-18. The k' values for column 1 range from 1.4 to 2.2 for acetonitrile–water (50:50) and increase to 4.7–9.4 for acetonitrile–water (25:75). All the k' values for the other columns are limited to the range 1.8–8.15. The k' values are higher for the 10-cm columns (columns 6–8) using acetonitrile–water (25:75).

The relative retention (α) varies slightly, from 1.4 to 2.0 in acetonitrile–water and from 1.25 to 1.40 in methanol–water. The lowest α values of 1.21–1.25 were found for $Cr(AA)_3$ and for $Al(AA)_3$. The α values for $Al(AA)_3$ range from 1.78 to 1.94. Lower α values (1.29–1.43) were obtained with methanol–water (40:60) for $Cr(AA)_3$ and $Co(AA)_3$ chelates.

The separation of $M(AA)_3$ was performed using different particle sizes of the support (columns 4–8) and a 10-cm column (columns 6–8). The retention times and capacity factors found for columns of the same length and particle size (7 or 10 μm) indicate similar separation effects. Retention times obtained on the same support (columns 1 and 6) depends on the column length. In particular, the retention times of $M(AA)_3$ on a 25-cm column are 2.15 times longer than those on a 10-cm column. The separation effects on these shorter columns were consistent with those on the others tested with regard to the chromatograms, k' values and relative retentions.

CONCLUSIONS

Optimum parameters for the separation of $M(AA)_3$ mixtures were established. The resolution time, depending on the column type and flow-rate (1.5–2.0 cm^3/min), was 6–10 min. The effectiveness of the separation is independent of the column length in the range 10–25 cm, but the column length influences the retention time (proportional relationship).

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Note

Resolution of the neuroexcitatory N-methylaspartic acid (2-methylaminosuccinic acid) enantiomers by ligand-exchange thin-layer chromatography

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Excitatory amino acid receptors which are thought to be activated physiologically by L-aspartic acid and/or L-glutamic acid[1–3] in the central nervous system of vertebrates have been classified into different types[3–6]. The N-methyl-D-aspartate receptor is probably the best characterised of all the receptor subtypes[7]. It is activated by N-methyl-D-(−)-aspartic acid (NMDA) and blocked by 2-amino-ω-phosphonoalkanoic acids, such as D-2-aminophosphonoheptanoic acid and D-2-aminophosphonovaleric acid[8]. Activation of the NMDA receptor is thought to play a role in learning, memory and central control of muscle tone. Alzheimer's and Huntington's diseases may also be linked to overactivation of the NMDA receptor, leading to progressive neuronal pathology and death[3].

The L-isomer of N-methylaspartic acid (NMA) is less effective in activating the NMDA receptor than its corresponding D-enantiomer, which is 10–1000 times more potent than L-glutamic acid depending on the type of test preparation[9,10]. Hence it is essential to establish the enantiomeric purity of the NMA enantiomers from commercial sources prior to pharmacological or biochemical studies. To date, the optical purity of these compounds has been ensured by optical rotation measurements. However, this method is insensitive to contamination with small quantities of minor enantiomers arising from racemisation on synthesis or storage.

Recently a ligand-exchange high-performance liquid chromatographic (HPLC) separation has been reported of the NMA enantiomers using a silica-bonded L-proline and a copper(II) acetate mobile phase system[11]. Whereas the separation of the enantiomers was excellent, the method was time-consuming requiring costly equipment. There has also been interest in the enantioselective separation of amino acids and derivatives by thin-layer chromatography (TLC) using a Chiral plate coated with a reversed-phase silica gel and impregnated with a chiral selector (a proline derivative) and copper(II) ions[12–14]. The separation of enantiomers is based on li-

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gand-exchange chromatography as described by Davankov[15]. Therefore it was decided to evaluate the use of ligand-exchange TLC using the commercially available Chiral plates in the development of a convenient and enantioselective assay of NMA enantiomers.

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents were of analytical or HPLC grade. Ultra-pure water was obtained by means of a Milli-Q system (Millipore). NMDA was purchased from the following suppliers: Sigma, Aldrich and Cambridge Biochemical Research. L- and DL-NMA were purchased from Aldrich. Pre-coated TLC plates (Chiral plates®, catalogue No. 811055/056, size 10 cm × 20 cm, layer thickness 0.25 mm) were purchased from Machery–Nagel.

Chromatographic conditions

The Chiral plates were used immediately after activation, which involved heating the plates at 110°C for 15 min and cooling, as recommended by the manufacturers. The mobile phases were made up as described in Table I. The TLC chambers were equilibrated for 1 h prior to the chromatographic development.

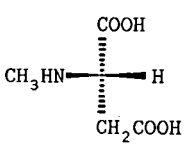
After development (15–18 cm, 30–90 min), the plates were left in the air to dry and subsequently sprayed with a 0.2% (w/v) solution of ninhydrin in acetone. The enantiomers were revealed as red-coloured derivatives on a white background after heating at 100°C for 10 min.

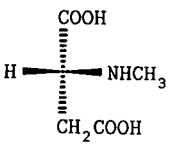
Preparation of the standard NMA solutions

Stock solutions of the individual enantiomers were prepared, freshly each day,

TABLE I

EFFECT OF ELUENT COMPOSITION ON THE R_F VALUES OF THE N-METHYLASPARTIC ACID ENANTIOMERS





Acetonitrile–methanol–water ratio	*R_F NMLA*	*R_F NMDA*
5.0:1.0:1.0	0.42	0.34
4.0:1.0:1.0	0.45	0.39
3.0:1.0:1.0	0.49	0.45
2.0:1.0:1.0	0.52	0.52
1.0:1.0:1.0	0.60	0.66
0.6:1.0:1.0	0.58	0.67
0.0:1.0:1.0	0.60	0.68

in water at a concentration of 10 mg/ml. A 2-μl volume of each solution was applied to the plate and dried with the aid of a cold-blowing hair drier before development.

RESULTS AND DISCUSSION

Ligand-exchange TLC utilising either home-prepared or commercially available Chiral plates which are coated with the chiral selector (2*S*,4*R*,2′*RS*)-4-hydroxy-1-(2′-hydroxydodecyl)proline have been most commonly developed with acetonitrile–methanol–water mixtures, the most popular ratios being 4:1:1 and 0.6:1:1 (v/v/v) mixtures as mobile phases. With the latter system, excellent separation and reproducible R_F values could be obtained for the enantiomers of NMA. The level of detection of the L- in the D-enantiomer was less then 0.5% (w/w) using ninhydrin spray to visualise the enantiomers. The optical purity of the L- and D-enantiomers of NMA from differing commercial sources was shown to be better than 99.5% (no detectable levels of the opposite enantiomer were observed). Use of the 4:1:1 (v/v/v) mobile phase failed to give such tight separation of the enantiomers but surprisingly the elution order of the enantiomers had been reversed, *i.e.* the elution order was L- before D-enantiomer (4:1:1) where, as previously, it had been the D- before the L-enantiomer (0.6:1:1).

To investigate this phenomenon further, acetonitrile–methanol–water mobile phases with ratios varying from 0:1:1 to 5:1:1 (v/v/v) were employed. The results shown in Table I show that at ratios of 3:1:1 to 5:1:1 the L-enantiomer elutes before the D-enantiomer, whereas at ratios of 1:1:1 to 0:1:1 the D-enantiomer elutes before the L-enantiomer. At the latter ratios the R_F values for both enantiomers reached a plateau. A ratio of 2:1:1 resulted in co-elution of the enantiomers.

Brinkman and Kamminga[12] have previously examined the effect of varying the acetonitrile content in the mobile phase on the R_F values for the enantiomers of glutamine, phenylalanine, isoleucine, norleucine and norvaline but failed to observe a reversal in enantioselectivity on changing the acetonitrile content of the mobile phase. The reversal of enantioselectivity observed in this paper may be attributed to acidic amino acids only.

The involvement of hydrophobic interactions in the separation mechanism is thought to be small as the R_F values did not increase on increasing the acetonitrile content of the mobile phase. The change in selectivity of the system on varying the acetonitrile to methanol and water concentration of the eluent is probably a direct consequence of the change in polarity of the resultant eluent. The TLC method described is sensitive, fast and very convenient, the plates being commercial available; it has been reported that the method is amenable to quantification by the use of a densitometer[13].

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Note

Application of high-performance thin-layer chromatography–fluorescence densitometry to the simultaneous determination of reduced and oxidized glutathione

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The tripeptide glutathione (GSH), γ-glutamylcysteinylglycine, is the major non-proteic thiolic compound of living plant and animal cells. Its important role in cellular metabolism includes the detoxification of xenobiotics, regulation of enzyme activity by disulphide interchange, protection against cell damage by ionizing radiation or by active oxygen species and transport of amino acids[1,2]. It is mainly the reduced form of glutathione that is present in tissues. However, the simultaneous determination of reduced (GSH) and oxidized (GSSG) glutathione could be useful for observing possible disorders in glutathione metabolism[3].

There are several analytical methods available for the determination of thiols and disulphides. Enzymatic, chromogenic and fluorogenic reactions as well as electrochemical detection systems preceded by liquid chromatography have been widely reported[4–6]. However, these assays often present serious drawbacks. Enzymatic reactions are mostly specific for a particular thiol or disulphide, whereas in electrochemical systems the selectivity, stability and reproducibility of the electrode may sometimes cause problems.

Based on the high-performance liquid chromatographic system recently described by Toyo'oka *et al.*[6], this paper proposes an alternative high-performance thin-layer chromatographic (HPTLC) system for the simultaneous determination of GSH and GSSG. In combination with fluorescence scanning densitometry and prior fluorescent labelling of the thiol group, a relatively simple, fast and versatile separation method is coupled to a sensitive and selective detection system, avoiding several inconveniences related to column liquid chromatography[7].

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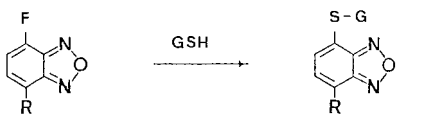
EXPERIMENTAL

Chemicals

The thiol-specific fluorogenic reagents 7-fluoro-4-sulphamoyl-2,1,3-benzoxadiazole (ABD-F) and ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate (SBD-F)[8] (Fig. 1) were purchased from Wako (Neuss, F.R.G.). GSH was obtained from Merck (Darmstadt, F.R.G.) and GSSG from Aldrich (Brussels, Belgium). The reducing agent tributylphosphine (TBP) was dissolved in dimethylacetamide (DMA), both from Janssen Chimica (Beerse, Belgium). Redistilled, deionized water was used throughout. All other chemicals were of analytical-reagent grade and used without further purification.



ABD–F : $R=SO_2NH_2$

SBD–F : $R=SO_3^-NH_4^+$

GSH : $H_2NCHCH_2CH_2CONHCHCONHCH_2COOH$ (COOH; CH_2SH)

Fig. 1. Structures of the fluorogenic reagents ABD-F and SBD-F and reaction with glutathione.

Apparatus

Derivatization reaction. A standard vortex reagent mixer, centrifuge and temperature-controlled water-bath were used.

Chromatography. The derivatized samples were spotted on pre-coated silica gel 60 HPTLC plates (10 × 10 cm) without fluorescence indicator (Merck) using a Nano-Applicator in combination with a Nanomat application system (Camag, Muttenz, Switzerland). Saturated twin-trough chambers (Camag) were used to develop the plates. A standard UV lamp (Camag Type 29 000) was used at 366 nm for viewing the adsorbed fluorescent derivatives.

Fluorodensitometry. In situ quantitative scannings were performed with a PMQ 3 densitometer (Zeiss, Oberkochen/Württemberg, F.R.G.) equipped with micro-optics, in the reflectance mode, using a mercury lamp source at a $\lambda_{exc.} = 365$ nm and an emission cut-off filter for $\lambda_{em.} > 460$ nm (FL 46; Zeiss). The chromatograms were registered with an Ankersmit A40 recorder (Kipp and Zonen, Delft, The Netherlands) installed at the appropriate amplifying voltages. Integration of the chromatograms was done with a Chromatopac C-R3A integrator computer (Shimadzu, Kyoto, Japan). All HPTLC results represent average values of at least ten measurements.

Procedure

Derivatization reaction[6]. To an aliquot of 1.0 ml of a mixed solution containing GSH and GSSG (each at a working concentration of 100 μM) in 0.1 M sodium borate buffer (pH 9.3, containing 2 mM disodium EDTA) was added an equal volume of

ABD-F (1.0 m*M*) in 0.1 *M* sodium borate buffer (pH 9.3, 2 m*M* disodium EDTA). The reaction mixture was vortex-mixed, heated in a water-bath at 60°C for 5 min and cooled in ice. Ethyl acetate (4.0 ml) was then added to the mixture to extract the excess of unreacted ABD-F. The solution was shaken vigorously for 1 min and centrifuged at 1500 *g* for 5 min. A 400-μl volume of the lower aqueous layer was then treated with 550 μl of SBD-F [1.0 m*M* in 0.1 *M* sodium borate buffer (pH 9.3, 2 m*M* disodium EDTA)] and with 50 μl of the reducing agent [10% (v/v) TBP in DMA], followed by heating of the reaction mixture at 60°C for 20 min. An HPTLC analysis was then carried out.

Chromatography[7]. The derivatized solutions, acclimatized to room temperature, were spotted in 200-nl volumes at 5.0-mm intervals and at 1.0 cm from the bottom and borders of an HPTLC silica gel 60 plate. The plates were then developed in saturated twin-trough chambers for about 5.0 cm using diisopropyl ether–methanol–water–acetic acid (45:40:10:5, v/v) as an optimized eluent. The separated ABD and SBD derivatives were subsequently measured by fluorescence scanning densitometry.

RESULTS AND DISCUSSION

Derivatization conditions[6]

Disodium EDTA was added to all sample solutions in order to prevent metal-catalysed thiol oxidation. The thiol-specific fluorogenic reagents ABD-F and SBD-F bearing the fluorobenzoxadiazole structure (Fig. 1) were chosen owing to their high reactivity and selectivity towards thiols, and also because of the optimum stability and luminescence properties of their thiol derivatives[8]. ABD-F was used as the first derivatizing reagent because, in comparison with its homologue SBD-F, the former's reduced water solubility allows easier removal of the unreacted excess of ABD-F reagent (extraction with ethyl acetate). The optimum reaction conditions (solvents, pH, temperature and time of reaction, extraction procedure, reducing agent, etc.) recommended by Toyo'oka *et al.*[6] for the derivatization of thiols and disulphides prior to chromatography were adopted in this work.

Optimization of HPTLC conditions

Different types of eluents (acidic, neutral and alkaline) were tried as developing solvents for the separation of the glutathione ABD and SBD derivatives. The most compact and intensely fluorescent spots for the HPTLC silica gel system formerly described were obtained with the solvent mixture diisopropyl ether–methanol–water–acetic acid (45:40:10:5, v/v), providing R_F values of 0.24 for GSH (corresponding to GSH–ABD) and at 0.15 for GSSG (corresponding to the SBD derivative after TBP reduction of GSSG to the free thiol). The use of other stationary phases such as reversed-phase C_{18} or cellulose HPTLC plates provided, under the same experimental conditions, highly diffuse spots that could not be quantitated, situated at $R_F > 0.90$ (RP-18) and < 0.10 (cellulose).

The influence of increasing proportions of acetic acid and water in the eluent[7] on the R_F values of the ABD and SBD derivatives is shown in Fig. 2. As observed, the addition of acetic acid to the eluent drastically decreases the R_F values of both derivatives, providing more compact and highly fluorescent spots. Apparently, protonation of the sulphonic acid function makes the SBD derivatives migrate at lower

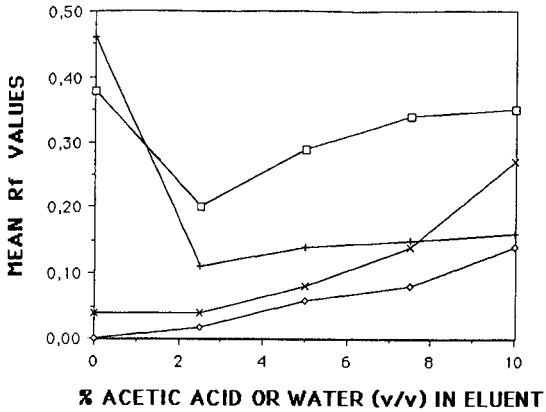


Fig. 2. Influence of acetic acid and water concentrations on R_F values of GSH derivatives (n = 10): □, G-ABD (acetic acid); +, G-SBD (acetic acid); ×, G-ABD (water); ◇, G-SBD (water). G-ABD = GSH derivatized with ABD-F; G-SBD = GSSG reduced with TBP and derivatized with SBD-F.

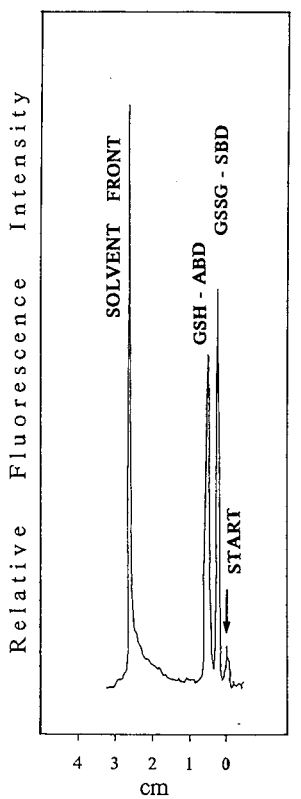


Fig. 3. HPTLC of reduced (GSH-ABD) and oxidized (GSSG-SBD) glutathione (each at a working concentration of 100 μM) following the described method.

R_F values. Similar results were obtained when the influence of eluent pH was studied. Owing to salt formation, a general increase in R_F values was observed with increasing eluent pH, accompanied by diffusion of the spots. Eluent pH values higher than 7 could not be tested owing to solvent inmiscibility problems. The optimum acetic acid content was of 5% (v/v) with a final pH of 2. With respect to the hydrophilicity of the eluent, an increase in the R_F values is clearly observed with increasing water concentration, a 10% (v/v) concentration providing satisfactory separations. Higher water concentrations in the eluent are not recommended as they are not compatible with the silica layer and as they tend to increase the developing times.

Determination of reduced and oxidized glutathione

Fig. 3 shows the chromatogram obtained with the described HPTLC system. The deviation of the baseline towards the solvent front is due to the migration of the hydrolysis product of the excess of fluorogenic reagents.

The detection limits (signal-to-noise ratio $\geqslant 2$) were 18 and 63 pg per spot for GSH and GSSG, respectively, although these values might be lowered by applying a luminescence enhancing treatment[9–13].

Following the proposed method, linear calibration graphs for GSH ($y = -2.536 + 0.268x$, $r = 0.99$) and GSSG ($y = 0.757 + 0.286$x, $r = 0.99$) were obtained for plots of relative fluorescence intensity (peak height, y) of the derivatives *versus* the working concentration of GSH or GSSG (x) over the range 0–100 μM. The reproducibility of the overall derivatizing and chromatographic procedure is of 4.3% ($n = 10$).

The proposed method for the determination of GSH and GSSG is relatively simple, rapid and selective and may be applied to the determination of other thiols and disulphides. The use of an internal standard is recommended for the accurate determination of low levels of analytes in biological samples, and this aspect is currently being investigated.

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CHROM. 22 135

Letter to the Editor

Comments on solute–solvent interactions in quantitative high-performance liquid chromatography

Sir,

We have recently become interested in the causes behind the apparent errors in quantification which have been noted with various solute–solvent combinations in high-performance liquid chromatography (HPLC)[1–5]. Inman *et al.*[1] have carefully examined this phenomenon and have identified the sample injection valve as the cause of this effect.

During the examination of the *in vitro* release characteristics of a novel anxiolytic agent in our laboratories, it was noted that there was an apparent high bias of 8–10% in the recovery of the drug from the aqueous dissolution media at levels of 6 μg/ml using standards prepared from 35% acetonitrile. This bias could be removed when standards were made up using 1% acetonitrile. Most interestingly, and in agreement with the findings of Inman *et al.*[1], the 1% acetonitrile standards gave a response which was larger than the standards prepared in 35% acetonitrile.

This problem seemed similar to those reported in the literature[1–5] and suggested that we might be experiencing the same or a related phenomenon. As sample injection valves are in wide usage, it was of general interest to further examine the problem.

Based upon our findings and the previous reports[1–5], a hypothesis was formed regarding the nature of this effect. It was thought that something in the injection valve could be acting as a site for the adsorption of the drug.

In most operations, the injection valve is overfilled several times[6] to ensure that the sample concentration in the valve is representative of the sample solution. If some component of the valve could adsorb material, then the mass of analyte in the valve would be larger than the product of the volume of the valve loop and the concentration of the sample solution. We hypothesized that the analyte may be pre-concentrated in the injection valve during the overfill process when low-solvent-strength preparations are analyzed.

The ability of the valve to adsorb a solute material would depend upon the relative free energies of adsorption and solution for the solute in the sample solvent. At low solvent strengths, the free energy of the adsorbed state might be favored over the solution state due to a lack of solubility. If the solvent strength of the sample preparation was increased, the free energies might again favor the solution state and the solute would not adsorb to the surface(s).

Confirmation of this hypothesis could explain some of the previously observed discrepancies. In the work of Perlman and Kirschbaum[2], each of the solutes exhibited the largest response in aqueous preparations as the model above suggests. Berridge[3]

did not find this phenomenon when using a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 1090 A HPLC system. Although it is not clear which injection system was used on this instrument, if the autosampler provided by the manufacturer was used, the sample is drawn by a syringe into a stainless-steel capillary of sufficient length that the sample solution does not contact the injection valve body. As there is no contact with the valve and no overfill, adsorption on potentially active sites, such as the rotor, would not be possible. (*Note:* The sample solution, bracketed by mobile phase, is passed through the injection valve at the time of injection. Pre-concentration of analyte on the rotor is not possible in this arrangement.)

If Berridge[3] used the Hewlett-Packard 1090 manual injection valve option (*i.e.*, overfilled loop and rotary injection valve), it is possible that he could not reproduce the observations of Perlman and Kirschbaum[2] due to batch-to-batch variation in the rotor material or due to the past history of this material. Chan and Yeung[4] were not able to reproduce the effect and pointed out several valid criticisms of the earlier work. Again, the past history or exact surface chemistry of the rotor may have been significantly different from that of Perlman and Kirschbaum's[2] system.

Inman *et al.*[1] also show findings consistent with this model in several areas. A relationship was found in which increases in acetate concentration could reduce the magnitude of the inaccuracy with vinblastine. This is consistent with displacement of the drug from an adsorbing surface by a competitive mechanism. The shape of the response curves for vancomycin hydrochloride and vinblastine are consistent with the model suggested above. The curves for each preparation of sample in the lowest concentration regions diverge from the origin and then become parallel. These regions may correspond to partial and complete saturation of the available adsorption sites. The variability between instruments suggests that there may be a difference in the valve surfaces both between and within manufacturers.

Several experiments were performed to test the hypothesis described above. The isocratic assay system in our examination used either a 250 × 4.6 mm I.D. Zorbax RX® column (Mac-Mod Analytical, Chadds Ford, PA, U.S.A.) which provided a capacity factor of approximately 7 with a minimum of 15 000 theoretical plates or a

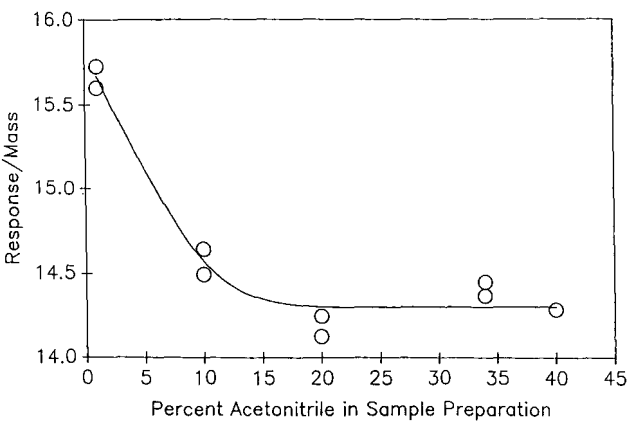

Fig. 1. Area response (arbitrary units) per mass of analyte injected as a function of percentage of acetonitrile in sample preparation. A Rheodyne injection valve was used with a 20-μl loop using a 200-μl overfill volume.

150 × 4.6 mm I.D. Zorbax C_8 column (Mac-Mod Analytical). The mobile phase was made of 35% aqueous acetonitrile and an amine modifier. The model compound in this work contains imine, amide and oxadiazole functionalities. It has a molecular weight of 335, is highly aromatic, is only slightly soluble in water and has no ionizable functions. The assay will be described in detail in a future report.

The instrumentation employed included a Perkin-Elmer (Norwalk, CT, U.S.A.) ISS-100 or a Varian Assoc. (Sunnyvale, CA, U.S.A.) 9090 autosampler each with a Rheodyne (Cotati, CA, U.S.A.) sample injection valve with a 20-μl loop and LDC UV monitor D (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) or Waters-Millipore (Milford, MA, U.S.A.) 441 line source detectors at 254 or 308 nm. In one study, a Hewlett-Packard 1090 M with factory-supplied autosampler and diode array detector was used. Data were collected using a Hewlett-Packard 3392 integrator, transferred to a VAX computer (Digital Electronics, Maynard, MA, U.S.A.) and integrated using software developed in-house.

Five solutions of analyte at approximately 6 μg/ml were prepared in 1, 10, 20, 34 and 40% acetonitrile. These solutions were injected using a Rheodyne valve with a 20-μl loop using a 200-μl overfill. The resulting chromatographic response as a function of acetonitrile concentration is shown in Fig. 1. The samples prepared with the lowest eluent strength (*i.e.* 1 and 10%) had the largest area response per mass of analyte. At levels of 20% acetonitrile and above the response was essentially flat. Although peak-height data showed the same effect, some effect of sample preparation solvent composition upon peak height was noted in other work and reflected in the efficiency of the chromatography, so peak-height data are not reported further in these studies.

When these same samples were injected using a Hewlett-Packard 1090 M system with the syringe-based autosampler, each of the five samples provided essentially equivalent responses. Using the diode array detector on the Hewlett-Packard 1090, the spectra of the peaks eluted from these solutions were indistinguishable from each other as would be expected and as was suggested by Chan and Yeung[4]. This experiment supports the suggestion that there is something unique in the injection valve-based system and that solvent composition is an important parameter.

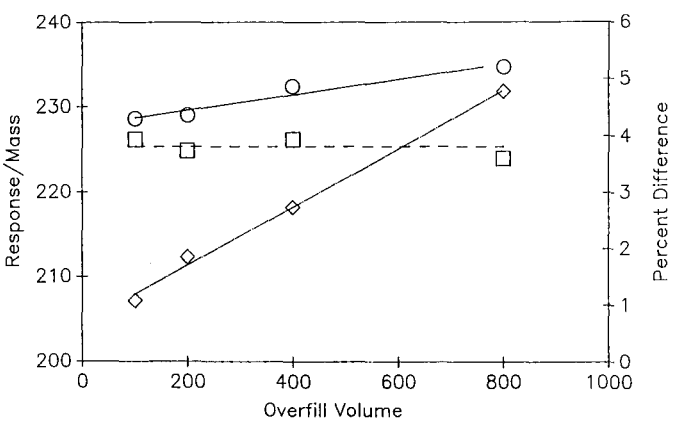

Fig. 2. Area response (arbitrary units) per mass of analyte as a function of overfill volume drawn through a 20-μl loop using samples prepared in 1% (○) and 34% (□) acetonitrile. The % difference curve (◇) shows the potential bias in the assay at each overfill volume.

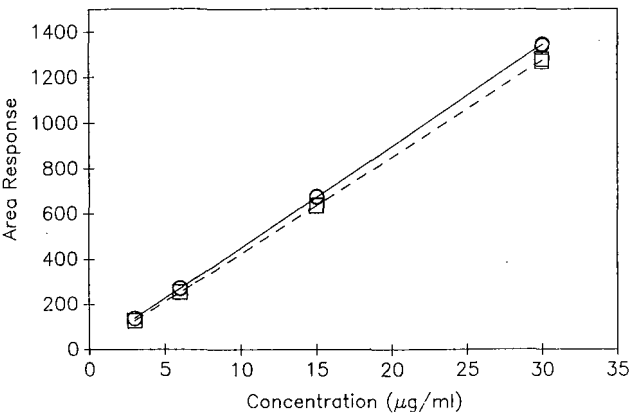

Fig. 3. Area response (arbitrary units) calibration curve for analyte in 1% (○) and 34% (□) acetonitrile sample preparations injected from a 20-μl loop with a 400-μl overfill. The slopes of these curves are statistically different.

A second experiment examined the impact of loop overfill on the proposed adsorption phenomenon. A Varian 9090 autosampler using a Rheodyne injector valve was programmed to utilize overfill volumes of 100, 200, 400 and 800 μl for the 20-μl loop. Samples at approximately 6 μg/ml were prepared in 1 and 34% acetonitrile and were analyzed with the results shown in Fig. 2. The response of these samples in 34% acetonitrile is virtually flat across the range of overfill volumes while the response of samples in 1% acetonitrile shows the tendency to increase with overfill volume. The percentage difference (or potential assay bias) between these responses is also plotted in Fig. 2. Again, these results support the hypothesis of analyte adsorption at low mobile phase strengths. No saturation of the surface(s) is observed at these concentration levels.

The effect of concentration was examined using samples at approximately 3, 6, 15 and 30 μg/ml in both 1 and 34% acetonitrile. These samples were injected using the Rheodyne valve with a 400-μl overfill volume. The response is plotted against concentration in Fig. 3 and demonstrates that there is a tendency for the 1% solution to produce a higher response than the corresponding 34% acetonitrile-based samples. Once again, no fixed amount of offset in response was observed. This suggests that the adsorption site(s) have not been saturated at these concentrations. Higher-concentration samples were not prepared due to concerns regarding the solubility of the compound in 1% acetonitrile.

These results confirm the hypothesis that the interactions and inaccuracies observed in this and other work[1–5] are due to adsorption of the analyte on surfaces of the injection system. The phenomenon only manifests itself when the eluting power of the sample solvent is below that needed to desorb the analyte from the adsorbing surface. We believe that the problem(s) noted are due to adsorption on the rotor surfaces because of the lack of effect in the Hewlett-Packard 1090 systems which have only stainless-steel contact surfaces. The variability of this phenomenon both within and between brands of autosamplers suggests that the rotor materials may be responsible.

In drug release testing and in biological or environmental analysis, the assay of aqueous samples using non-matrix matched standards is not uncommon. The poten-

tial bias could over-estimate the potency of a dosage, the drug level in a patient or the concentration of a contaminant. It is not unreasonable to expect that this phenomenon could occur in many systems, but may not be significant depending upon the difference between the potential of the injection system to adsorb the analyte and the analytical range of interest.

The compounds for which this effect have been observed seem to cover a wide range of physical and chemical properties[1–5]. The effect has been observed for large and small molecules, those with poor to excellent aqueous solubility, a wide variety of functionalities and a range of pK_a values. It is not clear that any *a priori* estimate of the tendency to adsorb may be made based on simple molecular characteristics.

It is also not unreasonable to expect that some components of mixtures may be pre-concentrated more than others resulting in biases in both relative and absolute levels. This would be expected to have the greatest impact in some assays for relative levels of impurities. The use of internal standards may also result in biases due to differential adsorption.

Fortunately, this phenomenon is relatively easily identified and corrected. By varying the amount of overfill in the injection system or by comparison of standards prepared at relevant solvent compositions, the potential for these problems can be readily found. Lack of apparent analyte adsorption in one system does not assure the lack of adsorption in all systems due to lot-to-lot variation in the rotor material as well as past history of each rotor. In our studies, the same samples resulted in a bias of 8–10% in one system while a bias of 3–5% was present in another even though both systems used the same brand of sample injection valve.

If solute adsorption on the valve is found to be significant, the analyst can avoid injector overfill provided only most assay precision is needed (*i.e.*, typically $\leqslant 0.5\%$ relative standard deviation for overfill *vs.* $\geqslant 1.0\%$ relative standard deviation for syringe-based non-overfill injectors). Matrix matching may not solve the problem if both samples and standards are prepared at low solvent strengths: the calibration curves may be non-linear if the adsorption saturation point is in the analytical range of interest. Standard addition techniques could fail for the same reasons. The problem might be reduced through the use of the new rotor materials which are commercially available. The best solution is probably to raise the solvent composition of the samples to increase eluting power during the overfill operation.

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CHROM. 22 053

Letter to the Editor

Fast separation of dihydro ergot alkaloids on an octylsilica column

Sir,

For the separation of the three components [dihydroergocornine (I), dihydroergocristine (III), dihydro-($\alpha+\beta$)-ergocryptine (II, IV), silica-based hydrocarbon stationary phase (octylsilica, octadecylsilica) and methanol[1] and acetonitrile[2–5] containing aqueous buffer (pH ≈ 7) as eluents are used. As buffers, phosphate[1], ammonium carbonate[2–5], triethanolamine–citric acid or triethanolamine–sodium acetate mixtures[5] are used. A reasonable separation of the four components, including the α- and β-isomers of dihydroergocryptine, was achieved only in alkaline eluents containing diethylamine[5] or triethylamine[6–8]. The developed method suggests the use of tetrabutylammonium hydroxide (TBAOH) rather than toxic and environmentally objectionable di- or triethylamine.

The analytical column (150 × 4.0 mm I.D.) was packed with 5-μm octylsilica (BST SI-100-S5 C_8; Bioseparation Technologies, Budapest, Hungary). In order to avoid rapid degradation of the stationary phase in analytical column, a short protecting column (30 × 4.0 mm I.D.) filled with octylsilica was inserted between the separation column and the injector and a silica column (100 × 4.0 mm I.D.) (10-μm LiChrosorb SI-100; Merck, Darmstadt, F.R.G.) was fitted between the pump and the injector. Before starting the measurements the analytical column was washed with acetonitrile–5 m*M* aqueous TBAOH (47:53) until the pH of the effluent reached the alkaline range (50–60 ml).

After the measurements, the column was washed to neutral with acetonitrile–water (47:53). Under these conditions, the analytical column could be used for 3 months (approximately 350–400 injections) without deterioration.

The separation of the four dihydro ergot alkaloids was studied as a function of eluent pH. At a constant acetonitrile concentration (47%), the concentration of the TBAOH was varied between 1 and 10 m*M* (pH 11.8–13.4). As Fig. 1 shows, the capacity factor (k') of the alkaloids decreases rapidly at pH > 12, and the elution sequence of dihydroergocristine (III) and dihydro-β-ergocryptine (IV) also changed. At pH > 12.4 there was a significant increase in selectivity. Without affecting the selectivity, the retention of the alkaloids changed when the acetonitrile concentation was varied in the range 40–55% at a constant (5 m*M*) TBAOH concentration.

With an eluent containing 45% acetonitrile, complete baseline separation is possible (Fig. 2) in 8 min. Although this separation time is twice that achieved by

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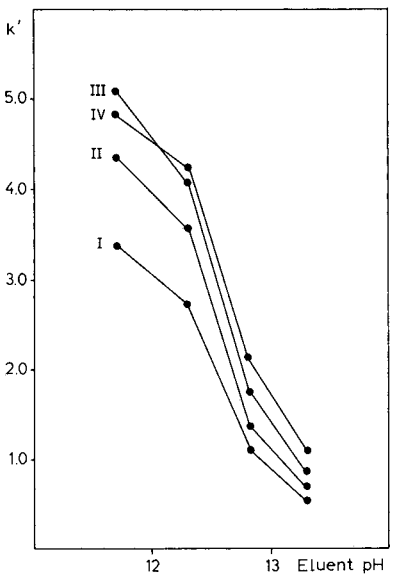

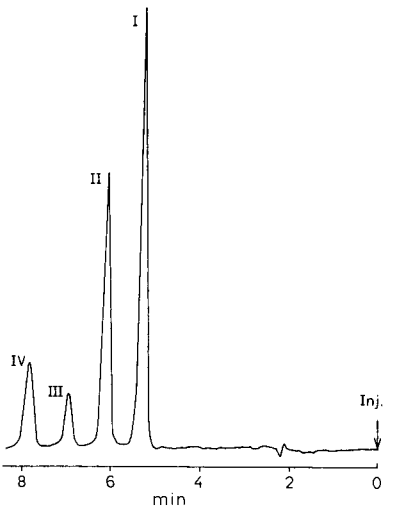

Fig. 1. Change in the capacity factor of the dihydro ergot alkaloids as a function of the eluent pH. Stationary phase, octylsilica; mobile phase, acetonitrile–10^{-2}–10^{-3} *M* aqueous TBAOH (47:53). Samples: I = dihydroergocornine; II = dihydro-α-ergocryptine; III = dihydroergocristine; IV = dihydro-β-ergocryptine.

Fig. 2. Chromatograms of the dihydro ergot alkaloids. Mobile phase; acetonitrile–$5 \cdot 10^{-3}$ *M* aqueous TBAOH (45:55); flow-rate, 0.8 ml/min; chart speed, 1.5 cm/min; injected sample, 1.56 μg of dihydro ergot alkaloids in 10 μl; λ = 280 nm, 0.2 a.u.f.s. Compounds as in Fig. 1.

high-performance liquid chromatography (HPLC)[8] at a flow-rate of 2.2 ml/min, it is half that obtained with the usual LC methods[6].

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ELISABETH PAPP

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CHROM. 22 079

Letter to the Editor

Journal of MicroColumn Separations

Sir,

Your recent review of the new *Journal of MicroColumn Separations*[1] came as a rather abrupt surprise to me and to many others who have since voiced their opinions concerning the review. While I am a firm supporter of the practice of close scrutiny, evaluation, and fair criticism of scientific reports and publications, I also believe that this practice should be undertaken according to acceptable prescribed rules of objectivity and fairness. In the case of this review, these rules were blatantly violated, offending me personally and the scientific publishing community in a broader sense. Several of the more important points of concern are as follows.

(1) While there is a precedent for journals to publish reviews of other journals, extreme care must be taken to ensure objectivity and impartiality, especially when the journal under review is a competing journal. It is unfortunate that for your review of *The Journal of MicroColumn Separations*, (a) the review was based only on the first issue and (b) the review was unsigned. In most cases, journals are not considered for review until they have been published for a full year. Only after such a period of time can proper perspective be given on matters such as public acceptance, timeliness of publication, scope of coverage and consistency of quality. Your review, based on one issue only, is critical through implication of what *might* be (*e.g.*, Are there to many journals? Will there be sufficient material? Will it have broad enough distribution? Will it provide good service?). Furthermore, reputable reviews are usually solicited from impartial, but knowledgeable, reviewers who are willing to sign their names and back their opinions.

(2) Whether or not the *Journal of Chromatography* or anyone else is happy about the situation, readers *are* supporting the creation of more specialized journals. In the field of chromatography alone, *Preparative Chromatography, Chirality, Journal of Planar Chromatography* and *The Journal of MicroColumn Separations* are among the new, specific journals that have recently appeared. Scientists specializing in particular areas of chromatography research often feel that their work is "buried" in more general journals and, therefore, is not being read by their colleagues to the degree desired. Furthermore, readers often must wade through voluminous amounts of material to find the important "kernels" of interest to them. In short, pertinent information is more efficiently and less expensively disseminated through specialized journals.

(3) The comments in the journal review concerning the level of rigor built into the review process for papers submitted to *The Journal of MicroColumn Separations*, and the comments made using one paper as an example, were very unprofessional. As

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a participant in the review process for many journals, I can safely claim that no journal adheres to a more careful and rigorous review process than does *The Journal of MicroColumn Separations*.

In conclusion, I feel that your review was inappropriately handled, and that you have violated the most important objective of the scientific publishing industry —that of setting in print correct information. *The Journal of MicroColumn Separations* has been quite successful in finding its public and is growing at a healthy rate. Despite your criticism of the title, we elected to focus the journal on the separation sciences that utilize microcolumns —an area in which the boundaries between the different techniques become less distinct. Every effort is being made to continuously improve the scientific quality of the journal and to respond to the needs and desires of the modern analytical community.

Chemistry Department, Brigham Young University, Provo, UT 84602 (U.S.A.) M. L. LEE

1 *J. Chromatogr.*, 472 (1989) 346.

(Received September 8th, 1989)

Reply

The Editors accept as reasonable the two main complaints in the accompanying letter: unsigned reviews and a review of one issue of a new journal. It was decided in June 1989 that book reviews accepted after that date must be signed. Future requests from publishers for reviews of a single issue of a new journal will be viewed with reserve.

Editors, Journal of Chromatography

Request for manuscripts

Y. Ito, W.D. Conway, M. Knight and Y.-W. Lee will edit a special, thematic issue of the *Journal of Chromatography*, entitled **"Counter-current Chromatography"**. Both reviews and research articles will be included.

Topics such as the following will be covered:

- theoretical aspects
- instruments
- solvent systems
- applications
- any other topics relating to counter-current chromatography.

Potential authors of reviews should contact Yoichiro Ito, National Institutes of Health, Bldg. 10, Rm. 7N 322, Bethesda, MD 20892, U.S.A. (Tel. 301-496-3237 or -2557, Fax: 301-402-0013).

The deadline for receipt of submissions is **May 1, 1990**. Manuscripts submitted after this date can still be published in the Journal, but then there is no guarantee that an accepted article will appear in this special thematic issue. **Five** copies of the manuscript should be submitted to Y. Ito. All manuscripts will be reviewed and acceptance will be based on the usual criteria for publishing in the *Journal of Chromatography*.

Request for manuscripts

R. Majors, F. Regnier and K. Unger will edit a special, thematic issue of the *Journal of Chromatography* entitled **"LC Column Packings"**. Both reviews and research articles will be included.

Topics such as the following will be covered:

- organic packings
- inorganic packings
- non-porous particles
- macroporous particles
- restricted access media
- functionalized membranes
- solid-phase extraction materials
- commercially available packings
- physical-chemical characterization
- relative performance of packings
- packing procedures and hardware
- column care.

Only minor coverage of topics such as affinity chromatography and chiral separations is planned since these will be the topics of other thematic issues.

Potential authors of reviews should contact Roger Giese, Editor, prior to any submission. Address: 110 Mugar Building, Northeastern University, Boston, MA 02115, U.S.A.; tel.: (617) 437-3227; fax: (617) 437-2855.

The deadline for receipt of submissions is **June 1, 1990**. Manuscripts submitted after this date can still be published in the Journal, but then there is no guarantee that an accepted article will appear in this special, thematic issue. **Five** copies of the manuscript should be submitted to R. Giese. All manuscripts will be reviewed and acceptance will be based on the usual criteria for publishing in the *Journal of Chromatography*.

PUBLICATION SCHEDULE FOR 1990

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	
Journal of Chromatography	498/1 498/2 499	500 502/1	502/2 503/1 503/2 504/1	504/2 505/1	505/2 506 507	The publication schedule for further issues will be published later
Cumulative Indexes, Vols. 451–500		501				
Bibliography Section		524/1		524/2		
Biomedical Applications	525/1	525/2	526/1	526/2 527/1	527/2	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 478, pp. 453–456. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Notes, Review articles and Letters to the Editor. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed six printed pages. Letters to the Editor can comment on (parts of) previously published articles, or they can report minor technical improvements of previously published procedures; they should preferably not exceed two printed pages. For review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

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Bunitrolol	3.08	Hydroxyzine	2.15	Tolperisone	3.91
Chloperastin	2.85	Ibuprofen	1.33	Trihexyphenidyl	5.16
Chlormezanone	6.48	Ketoprofen	1.99	Trimipramine	3.69
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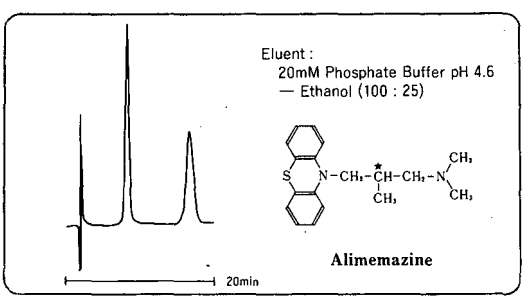

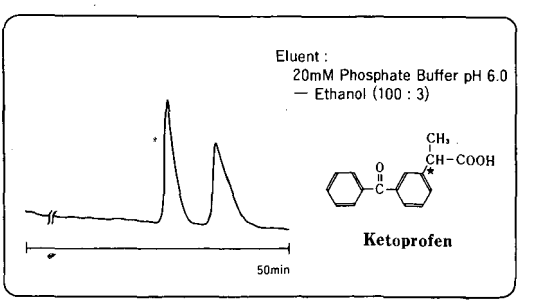

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