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CONTENTS

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(Continued overleaf)

Contents (continued)

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ACCURACY AND PRECISION IN THE DETERMINATION OF STOKES RADII AND MOLECULAR MASSES OF PROTEINS BY GEL FILTRATION CHROMATOGRAPHY

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SUMMARY

The accuracy and precision of the estimates of hydrodynamic parameters of globular proteins obtained by inverse regression from gel filtration chromatographic data are discussed. The usefulness of gel filtration chromatography as the basis for a rapid and reliable method for the determination of the Stokes radius and the molecular mass is considered. The discussion is supported by an analysis of the models already proposed in the literature, and is based on the precision of the estimates.

INTRODUCTION

Many analytical procedures have been used for the determination of the relative molecular mass (M_r) and the molecular size of proteins and nucleic acids. Whereas the most accurate of these techniques, *e.g.*, sedimentation velocity and sedimentation equilibrium measurements, viscosity and density determinations and light scattering (see refs. 1–5 and the references cited therein), require selected and expensive instrumentation, gel filtration is a very simple method. Through a mathematical approach, we have checked the quality of the results that can be obtained from gel filtration chromatographic data for globular proteins by using the various models already available.

Gel filtration chromatography can be considered as a transport phenomenon. Although the mechanism of separation of macromolecules by gel filtration is not completely understood[6] it is now well established that the behaviour of proteins in the gel matrix can be better related to their hydrodynamic radius (Stokes radius, R_S) than to their relative mass, M_r[7–9]. Considering the molecules of globular proteins as spheres with a defined hydrodynamic radius[10,11] is a simple assumption which can be very useful in the determination of molecular masses if it is combined with the determination of sedimentation coefficients. This combination is necessary because proteins are

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not truly spherical but have various shapes and extents of hydration, and therefore no unique relationship exists between R_S and M_r[12]. Therefore, it is not reasonable to assume that there exists a method for determining one parameter from the other, valid for any protein, and testing the mathematical models already proposed in the literature, involving any of these two parameters and the chromatographic variables, seems to be a reasonable step.

It is convenient to distinguish between accuracy and precision in the determination. The accuracy is related to bias, *i.e.*, the mean of the deviations from the real value, and the precision is concerned with the reproducibility of the determination[13]. It is possible for a given method to be accurate, *i.e.*, no systematic error is involved, but of low precision; conversely, the same erroneous value could be repeatedly obtained. It is not unusual to find in the literature different estimates of the molecular mass of the same protein even if the same method has been used; specimen purity and calibration technique can account for discrepancies on this magnitude in many instances[13].

In this paper, we discuss seven models that have been already introduced, in the light of real data for nine proteins used for calibration. These models are judged according to the accuracy and precision obtained when determining R_S or M_r by inverse regression from gel filtration chromatographic data.

EXPERIMENTAL

Proteins

The following proteins were used for calibration (see Table I): thyroglobulin (bovine thyroid), ferritin (horse spleen), catalase (bovine liver), and aldolase (rabbit muscle) (all from Pharmacia); albumin (bovine serum) (Serva); and ovalbumin (egg white), chymotrypsinogen A (bovine pancreas), myoglobin (whale muscle) and cytochrome *c* (horse heart) (all from Sigma).

TABLE I

MOLECULAR MASSES AND STOKES RADII OF NATIVE PROTEINS USED FOR CALIBRATION

Standards in aqueous solution obtained from sedimentation equilibrium.

Protein	*Molecular mass*	R_S *(nm)*
Thyroglobulin	670 000[a]	8.60[a]
Ferritin	440 000[b]	6.06[b]
Catalase	230 000[a]	5.23[a]
Aldolase	148 000[c]	4.60[d]
Albumin	67 000[e]	3.55[e]
Ovalbumin	43 500[e]	2.73[e]
Chymotrypsinogen A	23 000[e]	2.24[e]
Myoglobin	17 000[e]	2.08[e]
Cytochrome *c*	13 400[e]	1.65[e]

[a] Potschka[5].
[b] Frigon *et al.*[14].
[c] Righetti *et al.*[15].
[d] Hoorike *et al.*[6].
[e] Mantle[16].

Reagents

Blue Dextran 2000 was purchased from Pharmacia and potassium dichromate from Merck. Distilled water, further purified with a Millipore Milli-Q system, was used throughout.

Gel filtration chromatography

Gel filtration was carried out at 4°C on an Econo-column (Bio-Rad Labs.) of Sephacryl S-300 (Pharmacia) (111 × 1 cm I.D.) equilibrated with 50 m*M* Tris–HCl buffer (pH 8.2)–0.1 *M* NaCl. A 0.8-ml volume of each sample was applied, at a concentration of 3 mg/ml, with elution at a rate of 10 ml/h. The absorbance at 280 nm of the effluent was continuously recorded.

Chromatographic data were expressed in terms of the distribution coefficient, K_D or K_{av}, defined by the equations

$$K_D = \frac{V_e - V_0}{V_i}$$

and

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the elution volume of the protein under study, V_0 is the void volume (elution volume of Blue Dextran 2000, 1 mg/ml), and V_i is the internal volume (given by $V_i = V_t - V_m - V_0$, V_t being the total volume and V_m the matrix volume).

The calibration proteins were chromatographed 3–8 times.

Calibration of the column by Stokes radius

Several equations have been proposed in order to describe the relationship of the distribution coefficient of the protein with R_S. The following models have been considered in this paper.

Model I:

$$\text{erf}^{-1}(1 - K_D) = a + bR_S$$

was used by Horiike *et al.*[17], according to Ackers[18], who assumed that the effective radius of the pores follows a Gaussian distribution. This Gaussian distribution has also been considered by several workers in this context[6,12,19–23].

Model II:

$$K_D^{1/3} = a + bR_S$$

was proposed by Porath[24], and used later by Horiike *et al.*[17].

Model III:

$$(-\log K_{av})^{1/2} = a + bR_S$$

proposed by Laurent and Killander[25], and later by Siegel and Monty[26], is usually used when the measurements are obtained for a variety of proteins at the same gel concentration.

Model IV:

$$\frac{1000}{V_e} = a + bR_S$$

was proposed by Davis[23] as a simplified calibration procedure for gel filtration columns.

Correlation of distribution coefficient and molecular mass

Three models relating the distribution coefficient to the molecular mass have been considered.

Model V:

$$K_{av} = a + b \log M_r$$

has been used by several authors and is usually considered in studies of gel filtration[20].

Model VI:

$$\text{erf}^{-1}(1 - K_D) = a + bM_r^{1/3}$$

was developed by Fish[11].

The sigmoidal model VII:

$$K_{av} = \frac{1}{1 + (M_r/a)^b}$$

can be transformed into a linear model using the function logit $Y = \ln[Y/(1 - Y)]$[20]:

$$\text{logit } K_{av} = a + b \log M_r$$

In all the equations a and b are empirical constants for a given chromatographic system, and were estimated by a linear regression discussed below. R_S and M_r are assumed to be free from errors of determination and were therefore taken as control variables. The experimental variables were considered as response variables and written on the left-hand side of the equations.

RESULTS

Preliminary analysis

Let us consider the model

$$y_{ij} = a + bx_i + u_i + \varepsilon_{ij} \qquad 1 \leqslant i \leqslant 9; \qquad 1 \leqslant j \leqslant n_i \tag{1}$$

where x represents the control variable in any of the seven models introduced above and y is the response variable; n_i replications were made for the ith calibration protein (n_i varies between 3 and 8); y_{ij} is the value of y in the jth replication for the ith protein, and ε_{ij} is the error in the determination of y_{ij}, which is assumed to follow a Gaussian distribution, $N(0,\sigma_i)$; u_i represents the deviation of the ith protein from "ideal" behaviour, *i.e.*, the exact linear model. This deviation has been commented upon in the Introduction and including it in the model allows us to assume that ε_{ij} has a zero mean. Not much can be guessed, at present, about u_i for an individual protein and, more important, nothing can be known from the bare value x_i. Therefore, the best we can do is to make the simplest hypothesis concerning u_i, *i.e.*, that u_i follows a Gaussian distribution $N(0,\sigma)$ (σ independent of x). It is important to distinguish between both types of error, because omission of u_i leads to a model that does not pass the usual test of linearity. Nevertheless, this distinction makes eqn. 1 unmanageable, and a reduction must be made.

Taking means in eqn. 1:

$$\bar{y}_i = a + bx_i + u_i + \bar{\varepsilon}_i \qquad 1 \leqslant i \leqslant 9 \tag{2}$$

and now $\bar{\varepsilon}_i$ is $N(0,\sigma_i/\sqrt{n_i})$. An exploratory analysis of the values of $\sigma^2 + \sigma_i^2/n_i$, carried out by estimating the residual variance after fitting by the ordinary least-squares (OLS) method a linear model to the pairs $(x_i, \bar{y}_i)$, shows that the values σ_i^2/n_i, although different, are small in comparison with σ^2, and thus we are led to a model

$$\bar{y}_i = a + bx_i + w_i \qquad 1 \leqslant i \leqslant 9 \tag{3}$$

where the variance of w_i assumed to be constant, $\bar{y}_i$ can be taken as an estimate of the true value of y for the ith protein (not the expected value corresponding to $x = x_i$, unless the ith protein could be assumed to be "ideal").

Predictions from the models

To check the seven models, the following operations were performed. For each i we considered the sample obtained by omitting the pair $(x_i, \bar{y}_i)$ and fitted a linear model by the OLS method to this sample. Then the model obtained was used to calculate x_i by inverse regression, and this prediction was recorded. We thus obtained nine errors for each model. The predictions, together with the relative errors, in the form of percentages, are presented in Tables II–VIII. We can use now these errors in order to discuss the accuracy and precision of these methods for determining R_S and M_r. The purpose of omitting one pair $(x_i, \bar{y}_i)$ when fitting the models is to avoid the influence of the pair in the prediction of x_i when the prediction is made using a model obtained from a sample in which the pair itself was included (see ref. 27, Chapter 2, for an elementary discussion of this subject).

Let us look first at the models involving R_S (models I–IV). The estimates of R_S obtained by means of these models are negatively biased, the mean of errors being *ca.* -0.7 for all of them. Therefore, these models can be considered as reasonably and similarly accurate.

The precision of a method can be measured in different ways (variance, mean square error, median absolute deviation, etc.). Nevertheless, we are not interested here

TABLE II

VALUES OF THE CHROMATOGRAPHIC VARIABLE AND THE STOKES RADIUS IN MODEL I

Each predicted value of R_S was obtained by inverse regression from a linear model fitted to the eight other points. The percentage errors are referred to the true values of R_S.

$erf^{-1}(1 - K_d)$	R_S	*Predicted* R_S	*Percentage error*
1.2078	86.0	80.73	6.12
0.9437	60.6	64.28	6.07
0.7686	52.3	48.88	6.53
0.7257	46.0	45.92	0.17
0.6244	35.5	38.50	8.45
0.4871	27.3	27.60	1.09
0.4234	22.4	22.70	1.34
0.3778	20.7	18.74	9.46
0.3158	16.5	13.68	17.09

TABLE III

VALUES OF THE CHROMATOGRAPHIC VARIABLE AND THE STOKES RADIUS IN MODEL II

Details as in Table II.

$K_d^{1/3}$	R_S	*Predicted* R_S	*Percentage error*
0.4442	86.0	80.07	6.98
0.5667	60.6	64.80	6.93
0.6519	52.3	49.00	6.31
0.6730	46.0	45.92	0.17
0.7226	35.5	38.32	7.94
0.7888	27.3	27.35	1.83
0.8190	22.4	22.52	0.54
0.8401	20.7	18.73	9.52
0.8685	16.5	13.96	15.39

TABLE IV

VALUES OF THE CHROMATOGRAPHIC VARIABLE AND THE STOKES RADIUS IN MODEL III

Details as in Table II.

$(-\log K_{av})^{1/2}$	R_S	*Predicted* R_S	*Percentage error*
1.0166	86.0	78.76	8.42
0.8463	60.6	64.51	6.45
0.7306	52.3	49.65	5.07
0.7016	46.0	46.76	1.61
0.6322	35.5	39.39	10.96
0.5340	27.3	28.07	2.82
0.4862	22.4	22.67	1.21
0.4489	20.7	17.89	13.50
0.3997	16.5	11.80	28.40

TABLE V

VALUES OF THE CHROMATOGRAPHIC VARIABLE AND THE STOKES RADIUS IN MODEL IV

Details as in Table II.

$1000/V_e$	R_S	*Predicted* R_S	*Percentage error*
25.21	86.0	77.63	9.73
22.28	60.6	66.18	9.20
19.95	52.3	49.64	5.09
19.35	46.0	46.34	0.74
17.96	35.5	38.44	8.28
16.14	27.3	26.92	1.39
15.34	22.4	22.47	0.31
14.79	20.7	18.84	8.99
14.08	16.5	14.54	11.38

TABLE VI

VALUES OF THE CHROMATOGRAPHIC VARIABLE AND THE MOLECULAR MASS IN MODEL V

Each predicted value of M_r was obtained by calculating log M_r by inverse regression from a linear model fitted to the eight other points and transforming the resulting estimate into an estimate of M_r. The percentage errors are referred to the true values of M_r.

K_{av}	M_r	*Predicted* M_r	*Percentage error*
0.0927	670 000	872 048.30	30.16
0.1926	440 000	363 710.70	17.34
0.2928	230 000	189 132.62	17.77
0.3219	148 000	158 330.34	6.98
0.3985	67 000	94 474.86	41.01
0.5187	43 500	37 503.20	13.79
0.5803	23 000	25 380.36	10.35
0.6265	17 000	18 025.14	6.03
0.6921	13 400	10 275.22	23.32

TABLE VII

VALUES OF THE CHROMATOGRAPHIC VARIABLE AND THE MOLECULAR MASS IN MODEL VI

Each predicted value of M_r was obtained by calculating $M_r^{1/3}$ by inverse regression from a linear model fitted to the eight other points and transforming the resulting estimate into an estimate of M_r. The percentage errors are referred to the true values of M_r.

$erf^{-1}(1 - K_d)$	M_r	*Predicted* M_r	*Percentage error*
1.2078	670 000	959 386.11	43.19
0.9437	440 000	332 089.95	24.52
0.7686	230 000	196 804.47	14.43
0.7257	148 000	162 754.97	9.97
0.6244	67 000	102 509.69	53.00
0.4871	43 500	41 021.98	5.70
0.4234	23 000	25 862.72	12.45
0.3778	17 000	16 516.54	2.84
0.3158	13 400	7 246.70	45.92

TABLE VIII

VALUES OF THE CHROMATOGRAPHIC VARIABLE AND THE MOLECULAR MASS IN MODEL VII

Details as in Table VI.

Logit(K_{av})	M_r	*Predicted* M_r	*Percentage error*
−2.2828	670 000	1 850 502.70	176.19
−1.4359	440 000	313 949.36	28.65
−0.8829	230 000	152 276.88	33.79
−0.7452	148 000	127 496.24	13.85
−0.4119	67 000	79 888.69	19.24
0.0750	43 500	36 274.601	16.61
0.3232	23 000	26 574.56	15.54
0.5192	17 000	19 712.90	15.96
0.8119	13 400	12 267.75	8.45

in checking the quality of the models from a purely mathematical point of view, *i.e.*, the goodness of fit, but from a practical point of view, according to the precision of the estimates of R_S which could be obtained using them. In order to stress such an approach, the errors are presented as relative errors, and our discussion will be based on these. This presentation makes the result look worse than when the absolute error/length of interval ratio for the x variable is expressed, which would be the natural way for a linear model. Moreover, the correlations are high, above 0.99 for any of the nine fittings made for each model. However, the main interest here is the usefulness of the model for the determination of R_S, and the approach used here seems to be correct and easy to understand, and any one can draw his or her own conclusions from the results in Tables II–V.

For the models involving the molecular mass (models V–VII), the same analysis was performed. Nevertheless, M_r was transformed to linearize the models and, in spite of the high correlations (always above 0.975), the estimates of M_r show errors whose size is partly due to the change in dimension.

CONCLUSIONS

Gel filtration chromatography is considered to be a rapid and useful technique for the determination of the size and relative molecular masses of proteins[5,12]. Classical physico-chemical methods, such as sedimentation analysis, light scattering and electron microscopy, require very specific instrumentation[13], but gel filtration chromatography has the advantages of being relatively simple and of providing accurate results when the column has been calibrated properly.

The separation mechanism of gel filtration chromatography involves not only the molecular mass but also the shape of the molecules. Potschka[5] suggested that the universal calibration principle for gel filtration chromatography is the viscosity radius, *i.e.*, the molecular volume times a shape function which is defined by the intrinsic viscosity. Nonetheless, the reported differences between the Stokes radius based on the translational frictional coefficient, *i.e.*, calculated for native proteins from the diffusion coefficient with the Stokes–Einstein equation, and that based on the intrinsic

viscosity are usually not larger than 10%[28] or are indistinguishable[6,29]. We consider that the use of any Stokes radius for calibration in gel filtration chromatography could lead to good results.

We present here some conclusions from the results of the analysis made on seven models taken from the literature. The technical details have been given in the preceding section. The most obvious fact is that the use of the Stokes radius leads to better results, as could be expected considering what was previously known about the subject.

The models I–IV can be taken as acceptable for the determination of R_S, but some facts deserve attention. The errors obtained for proteins 1 and 9 must be considered, bearing in mind that they come from predictions corresponding to values of x falling outside the interval used in the determination of the parameters. It is interesting that, for all the models checked, the same proteins have either a low (aldolase) or a high (albumin) percentage error. This truly reflects the fact that some proteins behave anomalously with respect to the others. The basis for the difference is probably a greater deviation from a spherical shape (or, less likely, greater hydration) of some proteins. Without considering the error for cytochrome *c*, we do not find significant differences among the four models. Model IV has the advantage of using V_e directly, allowing an easier interpretation, but model I has a suggestive physical explanation, based on the assumption that the pore size of the matrix is Gaussian[18], as mentioned earlier. However, this model is limited as this assumption is not valid except for a particular Gaussian distriution of pore size centred at the origin, and Le Maire *et al.*[12] have shown that when the pore size distribution is calculated using an experimentally determined $K_D = f(R_S)$, the pore site is bimodal and therefore in no way Gaussian.

The importance of robustness in these analyses must be emphasized, because of the risk that the presence of a protein with very far from ideal behaviour could adversely affect the estimates of the parameters. We have already described the cautious approach followed in this work to the analysis of the size of the errors. Unless a deep knowledge of the proteins used for the calibration allows the experimenter to disregard such problems, we consider it advisable to use a robust regression technique in the calculation of the parameters of the model to be used for future determinations.

With respect to models V–VII, our results confirm that the use of M_r as a parameter for the description of the behaviour of the molecule inside the column is not adequate, as has been repeatly stated in the literature. However, if truly spherical proteins, hydrated to the same extent, are used, the errors can be minimized and a direct relationship between M_r and R_S can be achieved. In any event, the size of the errors obtained in this work does not allow us to consider these models as the basis for any precise method of determination of molecular masses of proteins. Nevertheless, they could be used to obtain an approximation of the relative magnitudes of the molecular masses of different proteins, *i.e.*, as a basis for comparative methods.

Finally, it is interesting to emphasize that a combination of R_S and sedimentation coefficient measurements to obtain M_r[26] leads to an error most generally smaller than that which results from a direct determination of M_r by gel chromatography.

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REFERENCES

1 H. Aburatani, T. Kodama, A. Ikai, H. Itakura, Y. Akanuma and F. Takatu, *J. Biochem. (Tokyo)*, 94 (1983) 1241–1245.
2 H. Hagamoto and K. Yagi, *J. Biochem. (Tokyo)*, 95 (1984) 1119–1130.
3 K. Takeuchi and K. Ishimura, *J. Biochem. (Tokyo)*, 97 (1985) 1695–1708.
4 Y. Morita, H. Iwamoto, S. Aibara, T. Kobayashi and E. Hasegawa, *J. Biochem. (Tokyo)*, 99 (1986) 761–770.
5 M. Potschka, *Anal. Biochem.*, 162 (1987) 47–64.
6 K. Horiike, H. Tojo, T. Yamano and M. Nozaki, *J. Biochem. (Tokyo)*, 93 (1983) 99–106.
7 W. W. Fish, J. A. Reynolds and C. Tanford, *J. Biol. Chem.*, 245 (1970) 5166–5168.
8 K. G. Mann and W. W. Fish, *Methods Enzymol.*, 26 (1972) 25–42.
9 M. le Maire, L. P. Aggerbeck, C. Monteilhet, J. P. Andersen and J. V. Møller, *Anal. Biochem.*, 154 (1986) 525–535.
10 C. Tanford, *Physical Chemistry of Macromolecules*, Wiley, New York, 1961, Ch. 6.
11 W. W. Fish, *Methods Membr. Biol.*, 4 (1975) 189–276.
12 M. le Maire, A. Ghazi, J. V. Møller and L. P. Aggerbeck, *Biochem. J.*, 243 (1987) 399–404.
13 A. J. Rowe, in *Techniques in Protein and Enzyme Biochemistry*, Elsevier/North-Holland, Amsterdam, 1978, B105a, pp. 1–31.
14 R. P. Frigon, J. K. Leypoldt, S. Uyeji and L. W. Henderson, *Anal. Chem.*, 55 (1983) 1349–1354.
15 P. G. Righetti, G. Tudor and K. Ek, *J. Chromatogr.*, 220 (1981) 115–194.
16 T. J. Mantle, in *Techniques in Protein and Enzyme Biochemistry*, Elsevier/North-Holland, Amsterdam, 1978, B105b, pp. 1–17.
17 K. Horiike, H. Tojo, M. Iwaki, T. Yamano and M. Nozaki, *Biochem. Int.*, 4 (1982) 477–483.
18 G. K. Ackers, *J. Biol. Chem.*, 242 (1967) 3237–3238.
19 G. K. Ackers, *Adv. Protein Chem.*, 24 (1970) 343–446.
20 D. Rodbard, in N. Catsimpoolas (Editor), *Methods of Protein Separation*, Vol. 2, Plenum Press, New York, 1976, pp. 145–218.
21 R. Vales, Jr. and G. K. Ackers, *Methods Enzymol.*, 61 (1979) 125–142.
22 R. S. Ehrlich, S. Hayman, N. Ramachandran and R. F. Colman, *J. Biol. Chem.*, 256 (1981) 10560–10564.
23 L. C. Davis, *J. Chromatogr. Sci.*, 21 (1983) 214–217.
24 J. Porath, *Pure Appl. Chem.*, 6 (1963) 233–244.
25 T. C. Laurent and J. Killander, *J. Chromatogr.*, 14 (1964) 317–330.
26 L. M. Siegel and K. J. Monty, *Biochim. Biophys. Acta*, 112 (1966) 346–362.
27 A. C. Atkinson, *Plots, Transformations and Regression*, Clarendon Press, Oxford, (1985).
28 C. Tanford, Y. Nozaki, J. A. Reynolds and S. Makino, *Biochemistry*, 13 (1984) 2369–2376.
29 R. E. Martenson, *J. Biol. Chem.*, 253 (1978) 8887–8893.

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CHROM. 21 429

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS ON N-METHYLPYRIDINIUM POLYMER COLUMNS

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SUMMARY

Two types of 4-methylpyridinium polymers (4VP–DVB–Me and 4VP–EG–Me, cross-linked with divinylbenzene and ethylene glycol dimethacrylate, respectively) were employed for the analysis of proteins in ion-exchange high-performance liquid chromatography. These polymers had different physical properties in the dry state, but showed similar retentions in size-exclusion chromatography using carbohydrate standards. Generally, the 4VP–EG–Me column was superior to the 4VP–DVB–Me column with regard to separation and recovery of proteins.

INTRODUCTION

Of the available techniques for the purification and analysis of proteins, high-performance liquid chromatography (HPLC) in various modes has been widely used. Ion-exchange chromatography has the advantage that the chromatography can be carried out in an aqueous buffer solution without an organic solvent. Therefore, it is possible to prevent the denaturation of proteins caused by organic solvents.

Among the column packings for the ion-exchange HPLC of proteins, organic polymer-based supports[1–4] have the advantages over surface-modified silica gels of chemical stability in alkaline solutions, higher sample loading capacities and a longer column life. In general, improvements in these matrices occur with hydophilic polymers such as poly(vinyl alcohol) gel[1–3] and glycol methacrylate gel[4].

In this work, two types of N-methylpyridinium polymers, cross-linked with either hydrophobic divinylbenzene (DVB) or hydophilic ethylene glycol dimethacrylate (EG), were applied to the ion-exchange HPLC of proteins, and the effect of cross-linkages on the retention behaviour was studied. Various anion-exchange polymers containing diethylaminoethyl (DEAE)/quaternary ammonium groups acting as the functional groups have been applied to the separation of proteins by HPLC However, polymers having quaternary pyridinium groups intended for protein analysis have not previously been studied.

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EXPERIMENTAL

Apparatus and materials

Chromatographic measurements were carried out with a Hitachi 655A-11 high-performance liquid chromatograph equipped with a Hitachi L-5000 gradient generator and a Hitachi L-4000 variable-wavelength UV monitor or a Hitachi L-3300 refractive index detector.

The specific surface area was measured with a Monosorb instrument (Yuasa Battery, Osaka, Japan) and the pore volume and pore diameter with a mercury porosimeter (Carlo Erba, Milan, Italy).

All the proteins employed were commercial products. Bovine serum albumin (BSA), soybean trypsin inhibitor (STI), chicken egg albumin (CEA), bovine milk β-lactoglobulin (β-LG), bovine milk β-lactoglobulin A (β-LGA), bovine milk β-lactoglobulin B (β-LGB), bovine γ-globulin (γ-G), bovine pancreatic α-chymotrypsin (α-CHT) and horse heart cytochrome *c* (CYC) were obtained from Sigma (St. Louis, MO, U.S.A.) and chicken egg lysozyme (LY) from Merck (Darmstadt, F.R.G.).

All other chemicals were of analytical reagent grade from Wako (Osaka, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan).

Preparation of column packings

Macroporous 4-vinylpyridine polymer cross-linked with DVB (4VP–DVB) was prepared as described[5] previously and macroporous 4-vinylpyridine polymer cross-linked with EG (4VP–EG) was prepared in the same manner. Both polymers (particle size 10–15 μm) were converted by methylation with methyl bromide into N-methylpyridinium polymers[6], which are abbreviated to 4VP–DVB–Me and 4VP–EG–Me, respectively.

Chromatography

The bromide forms of 4VP–DVB–Me and 4VP–EG–Me were packed into a stainless-steel column (25 cm × 4 mm I.D.) and conditioned with 0.05 *M* Tris–HCl buffer (pH 7.0) containing 0.5 *M* sodium chloride or 0.05 *M* phosphate buffer (pH 7.0) containing 0.5 *M* sodium chloride. These columns showed a pressure drop of 30–40 kg/cm^2 at a flow-rate of 0.5 ml/min. There was no problem with the use of high pressure (150 kg/cm^2).

Sample proteins were eluted with a 30-min linear gradient from 0 to 0.5 *M* sodium chloride in 0.05 *M* Tris–HCl buffer (pH 7) or 0.05 *M* phosphate buffer (pH 7) at a flow-rate of 0.5 ml/min. The chromatographic procedure was performed at room temperature, with detection at 280 nm. Unless specified otherwise, 60–80 μg of protein were injected onto the column.

The pore size of the polymers in the wet state was evaluated by measuring the relationship between the molecular weight and the elution time in size-exclusion chromatography (SEC) with standard samples of the dextrans T10, T40, T70 and T2000 (Pharmacia, Uppsala, Sweden) and the oligosaccharides xylose and lactose (Wako), maltotriose and maltoheptaose (Sigma). The amount of sample injected was 2–20μg. The hold-up volume of a column was measured with heavy water (^2H_2O). The retention behaviour of the carbohydrates was measured on the same column with 0.5 *M* sodium chloride solution as the eluent and by using the refractive index detector.

Recovery of proteins

The recovery of proteins during chromatographic operation was evaluated by injection of the proteins (0.2 mg) into the column equilibrated with 0.05 *M* Tris–HCl buffer (pH 7.0) and by eluting with 0.05 *M* Tris–HCl (pH 7.0) containing 0.5 *M* sodium chloride at a flow-rate of 0.5 ml/min. Spectrophotometric determination at 225 nm of the proteins in the column effluent, which was pooled for 20 min after protein injection, gave the recovery[2].

RESULTS AND DISCUSSION

Characterization of the polymer

Ion-exchange chromatography requires an electrostatic interaction between the solutes and the functional groups on the support surface. Therefore, it is generally desirable that the ion exchangers are macroporous in order to permit the penetration of proteins into the ion-exchange group.

It has been reported that the pore size of the support markedly affects the resolution and recovery of proteins in reversed-phase chromatography[7–9]. Similar results were obtained by Vanecek and Regnier[10,11] with ion-exchange HPLC systems. Table I shows the physical properties of the dried polymers used in this experiment. The characteristic surface properties of both polymers substantiate a porous structure. However, the most common pore radius of 4VP–DVB–Me is larger than that of 4VP–EG–Me. Fig. 1 shows molecular-weight calibration graphs obtained from the retention behaviour of carbohydrates. The exclusion limits of both polymers for the carbohydrates used were about 500 daltons.

Although 4VP–DVB–Me has a larger pore diameter than 4VP–EG–Me in the dry state, the exclusion limits of both polymers are similar and small. The pore volumes of the polymers in the wet state were determined by comparing the elution volume of an excluded molecule (T2000) with that of an included molecule (^2H_2O)[12]. The pore volumes (ml per ml of polymer) were 0.38 ml (4VP–DVB–Me) and 0.34 ml (4VP–EG–Me), *i.e.* nearly identical, indicating that the pore size distribution of both polymers in the wet state is different from that in the dry state. This may suggest that the proteins used scarcely penetrate into the pores and interact only on the limited surface of the polymers. Recently, non-porous polymer packings with covalently coupled functional groups on the surface (ion-exchange capacity 0.1–0.15 mequiv./ml) have been used for the separation of proteins in ion-exchange HPLC[12–14]. It was suggested that the interaction related to the retention of proteins occurs only on the

TABLE I

CHARACTERIZATION OF POLYMERS IN THE DRY STATE

Polymer	*Elemental analysis (%)*		*Specific surface area* (m^2/g)	*Pore volume* (*ml/g*)	*Most frequent pore radius* (*nm*)
	N	*Br*			
4VP–DVB–ME	4.62	27.90	23.90	0.287	27.5
4VP–EG–ME	3.87	22.28	9.49	0.077	8.7

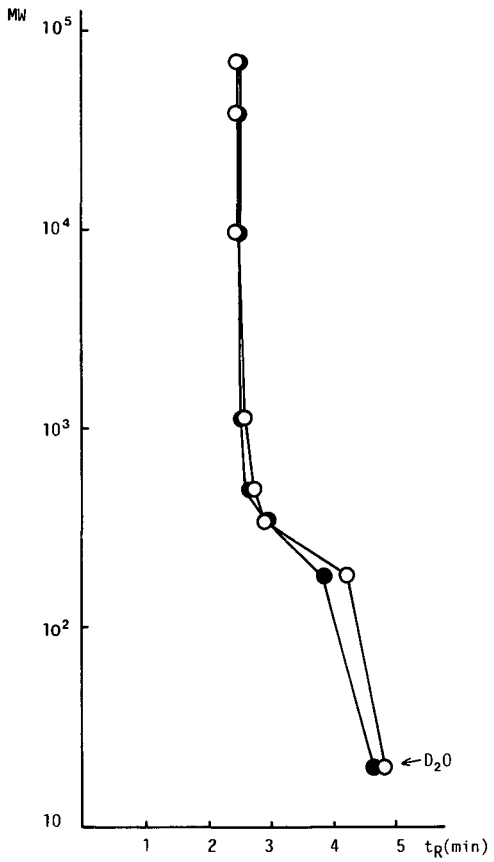

Fig. 1. Plots of molecular weights against retention time (t_R) for carbohydrates. ○, 4VP–DVB–Me; ●, 4VP–EG–Me. Column, 25 cm × 4 mm I.D.; eluent, 0.5 *M* NaCl; flow-rate, 0.5 ml/min; detection, refractive index. Samples: xylose (20 μg), lactose (20 μg), maltotriose (2 μg), maltoheptaose (2μg), dextrans T10 (2 μg), T40 (2 μg) and T70 (2 μg).

outer surface of the polymers. In the proposed pyridinium polymers, a similar behaviour in the retention of proteins is expected.

Anion-exchange HPLC of proteins

Table II shows the retention times of proteins obtained using a 30-min linear gradient from 0 to 0.5 *M* sodium chloride in Tris-HCl buffer (pH 7.0). The retention times of the proteins in both polymer columns are approximate. Proteins having lower isoelectric points (p*I*) than the pH of the mobile phase (pH 7) were retained on the columns, but those having p*I* > 7.0 were eluted in the void volume of the column. The retention times of the proteins varied with the pH of the buffer, as shown in Fig. 2. Considerable changes in the retention of proteins were observed at pH values near the p*I* valvues of the proteins. The major retention process of a protein having an overall negative charge may be governed by ionic interactions in the present system.

TABLE II

RETENTION TIMES (t_R) OF PROTEINS ON 4VP–DVB–ME (I) AND 4VP–EG–ME (II) COLUMNS IN TRIS–HCl BUFFER SYSTEM

The proteins were eluted using a 30-min linear gradient of sodium chloride from 0 to 0.5 *M* in 0.05 *M* Tris–HCl (pH 7) at a flow-rate of 0.5 ml/min. Sample loading, 60 μg. Column, 25 cm × 4 mm I.D.

Protein	*pI*	t_R *(min)*	
		I	*II*
BSA	4.7–4.9	15.82	17.2
		16.50	17.98
		16.89	18.40
β-LGA	5.2	21.4	23.34
β-LGB	5.1	19.12	21.32
CEA	4.6	15.63	17.1
STI	4.3	20.62	21.44
γ-G	8.0	2.42	2.40
LY	11.4	2.45	2.40
CYC	11.0	2.42	2.40
α-CHT	8.1	2.57	2.48

However, it is also necessary to take into account the effect of other interactions, such as hydrophobic and π–π interactions, on the retention of proteins.

The kind of buffer solution used markedly affected the retention of proteins as reported with other ion-exchange HPLC systems[15,16]. When gradient elution with sodium chloride in a phosphate buffer system was used, the protein peak became broad and poor peak resolution was observed compared with that in the Tris–HCl system.

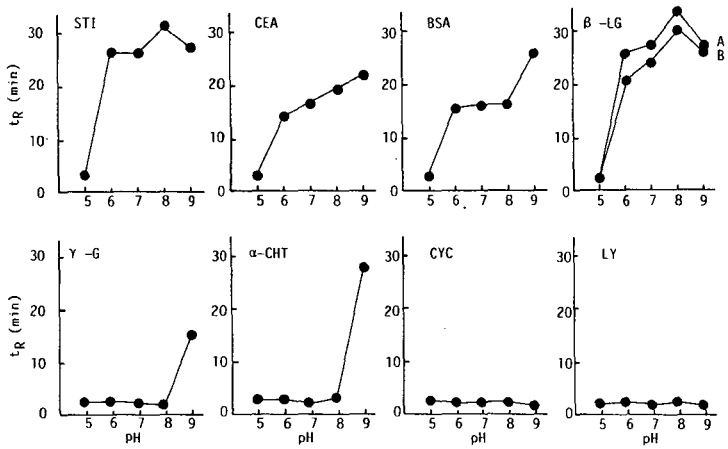

Fig. 2. Relationship between pH of mobile phase and retention time (t_R) on 4VP–DVB–Me column. Eluent, 30-min linear gradient of NaCl from 0 to 0.5 *M* in 0.05 *M* phosphate buffer (pH 5–9); flow-rate, 0.5 ml/min.

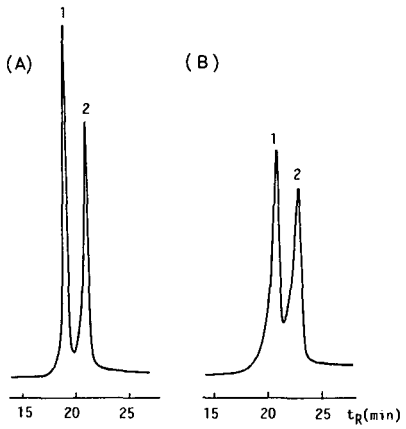

Fig. 3. Chromatograms of β-LG on (A) 4VP–DVB–Me and (B) 4VP–EG–Me columns. Peaks: 1 = β-LGB; 2 = β-LGA. Chromatographic conditions as in Table II. Sample loading, 80 μg.

The resolution of β-LGA and β-LGB in the Tris–HCl system is shown in Fig. 3. Good separation of these proteins was achieved on the 4VP–DVB–Me column. On the TSK Gel IEX-645 DEAE column, for which the exclusion limit of proteins is much higher than 10^6 (ref. 2), similar resolutions have been reported. Hence it is concluded that a polymer with a small pore size that excludes large molecules can also be applied to packing materials for protein analysis in ion-exchange chromatography.

The loading capacity of the 4VP–DVB–Me columns was examined with β-LG. Good resolution of β-LGA and β-LGB was obtained with up to 0.5 mg of sample but it decreased with increasing sample load (Fig. 4). In the investigations with the non-porous polymer packing, β-LG (60 μg) was loaded onto the non-porous MA7P column (10-μm particle diameter, 5 cm × 4.6 mm I.D.)[12] and a crude lipoxidase (100–

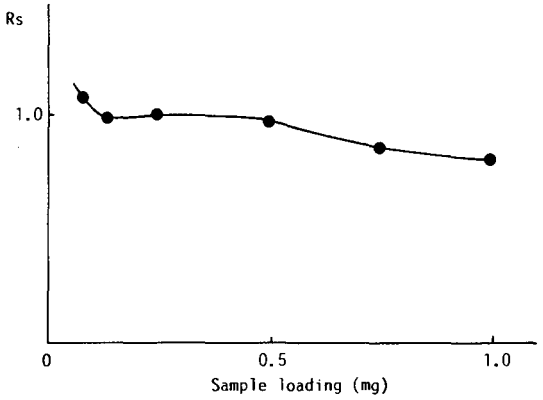

Fig. 4. Dependence of resolution (R_s) on sample load in the separation of β-LGA and β-LGB on the 4VP–DVB–Me column. (25 cm × 4 mm I.D.). Chromatographic conditions (except for sample loading) as in Table II.

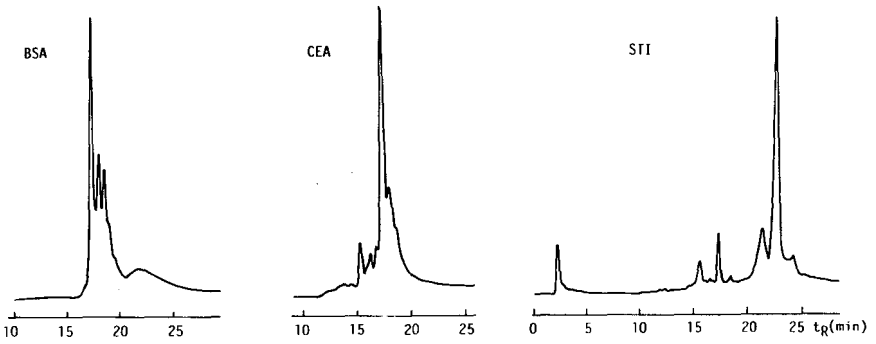

Fig. 5. Chromatograms of proteins on the 4VP–EG–Me column. Chromatographic conditions as in Table II.

200 μg) was applied to the TSKgel DEAE-NPR column (2.5-μm particle diameter, 3.5 cm × 4 mm I.D.)[13]. The loading capacity of the 4VP–DVB–Me column was higher than those of the non-porous polymer columns.

On the other hand, the chromatograms of other proteins show slightly better separations on the 4VP–EG–Me column than on the 4VP–DVB–Me column, as shown in Fig. 5. For BSA, multiple peaks were observed, owing to the intrinsic heterogeneity of BSA; serum albumin consists of several components, such as mercaptoalbumin, non-mercaptoalbumin and fatty acid binding albumin[17]. Identification of the peaks is now under investigation.

The recovery of proteins is shown in Table III. The recovery on the 4VP–EG–Me column was almost quantitative for all the proteins investigated, whereas that on the 4VP–DVB–Me column was poor. A hydrophobic interaction between the protein and the cross-linking part of the polymer may affect the non-specific adsorption of the protein.

In order to examine the stability of the column packings in alkaline solution, the columns were treated with 30 ml of dilute sodium hydroxide solution containing

TABLE III

RECOVERY OF PROTEINS FROM 4VP–DVB–ME (I) AND 4VP–EG–ME (II) COLUMNS

Protein	*Recovery (%)*[a]	
	I	*II*
BSA	85.9	101.2
β-LG	74.6	92.5
CEA	70.1	93.8
STI	63.6	89.3
γ-G	84.3	104.6
LY	97.8	103.1
CYC	95.1	102.2
α-CHT	95.9	100.9

[a] Recovery was obtained from absorbance at 225 nm as described under Experimental. Sample loading, 0.2 mg.

0.2 *M* potasium chloride (pH 12) at a flow-rate of 0.5 ml/min. The resolution of proteins on the columns did not change with this treatment. No significant deterioration of the column was observed even after continuous use for 3 months.

In conclusion, the porous 4VP polymers studied here are useful for protein analysis by anion-exchange HPLC, despite their low-molecular-weight exclusion limits. 4VP–EG–Me prepared by using a hydrophilic cross-linker is particularly promising for high protein recoveries.

REFERENCES

1 Y. Kato, K. Nakamura and T. Hashimoto, *J. Chromatogr.*, 294 (1984) 207.
2 Y. Kato, K. Nakamura and T. Hashimoto, *J. Chromatogr.*, 266 (1983) 385.
3 T. Kadoya, T. Isobe, Y. Amano, Y. Kato, K. Nakamura and T. Okuyama, *J. Liq. Chromatogr.*, 8 (1985) 635.
4 O. Mikes, *Int. J. Pept. Protein Res.*, 14 (1979) 393.
5 A. Sugii, N. Ogawa, K. Harada and I. Sato, *J. Chromatogr.*, 294 (1984) 185.
6 A. Sugii, K. Harada and N. Ogawa, *J. Chromatogr.*, 354 (1986) 211.
7 K. J. Wilson, E. van Wieringen, S. Klauser, M. W. Berchtold and G. J. Hughes, *J. Chromatogr.*, 237 (1982) 407.
8 R. V. Lewis, A. Fallon, S. Stein, K. D. Gibson and S. Udenfriend, *Anal. Biochem.*, 104 (1980) 153.
9 J. D. Pearson, W. C. Mahoney, M. A. Hermodson and F. E. Regnier, *J. Chromatogr.*, 207 (1981) 325.
10 G. Vanecek and F. E. Regnier, *Anal. Biochem.*, 109 (1980) 345.
11 G. Vanecek and F. E. Regnier, *Anal. Biochem.*, 121 (1982) 156.
12 J. K. Duncan, A. J. C. Chen and C. J. Siebert, *J. Chromatogr.*, 397 (1987) 3.
13 Y. Kato, T. Kitamura, A. Mitsui and T. Hashimoto, *J. Chromatogr.*, 398 (1987) 327.
14 D. J. Burke, J. K. Duncan, L. C. Dunn, L. Cummings, C. J. Siebert and G. S. Ott, *J., Chromatogr.*, 353 (1986) 425.
15 J. P. Mahieu, B. Sebille, M. Gosselet, M. C. Garel and Y. Beuzard, *J. Chromatogr.*, 359 (1986) 461.
16 K. M. Gooding and M. N. Schmuck, *J. Chromatogr.*, 266 (1983) 633.
17 T. Peters, Jr., in F. W. Putman (Editor), *The Plasma Proteins*, Vol. 1, Academic Press, New York, 1975, pp. 133–181.

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CHROM. 21 418

ISOLATION AND CONCENTRATION OF ORGANOPHOSPHORUS PESTICIDES FROM WATER USING A C_{18} REVERSED PHASE

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SUMMARY

A simple, rapid and effective method for the extraction and enrichment of organophosphorus pesticides based on the use of Sep-Pak C_{18} cartridges was studied as alternative method to those based on extraction with organic solvents. The influence of the elution solvent, pH, salinity and volume of water filtered was studied for ten organophosphorus pesticides. The pesticides were determined by gas chromatography with a BP-1 capillary column and a thermionic detector. Recoveries at the 100 and 200 ng/l spiking levels were greater than 85%, except for disulfoton.

INTRODUCTION

The occurrence of organophosphorus pesticides (OPs) in aquatic environments is mainly due to their increasing agricultural use. The analysis of water for organophosphorus pesticides by high-resolution gas chromatography with a thermionic detector is the most widely used procedure, and requires previous efficient extraction, concentration and clean-up procedures.

The use of partitioning, in which the OPs are extracted from one solvent into another[1–3], and liquid–solid chromatography using various adsorbents such as activated carbon[4], silica gel[5], synthetic polymers such as XAD-2[6,7], XAD-4[8,9] and Tenax[10] and commercially available cartridges packed with octadecylsilica by formation of ion-association complexes with tetraphenylarsonium cation[11] or by simple adsorption of the pesticides[12,13] have been developed for the isolation and concentration of OPs in water.

In this work we examined the performance of Sep-Pak C_{18} cartridges for the extraction and concentration of ten OPs from water. The effects of elution solvent, pH, salinity and volume of water filtered and a comparison with the Rodier[2] and the APHA methods[3] are reported.

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EXPERIMENTAL

Reagents

The OPs used were disulfoton, purity 97.1% (Bayer Hispania Comercial); ethion, purity 95% (Industrias Quimicas Argos); fonofos, purity 93.5% (Industrias Quimicas Serpiol); heptenophos, purity 93% (Industrias Quimicas Argos); malathion, purity 98% (Industrias Quimicas Serpiol); parathion-ethyl, purity 99.2% (Bayer Hispania Comercial); parathion-methyl, purity 80% (Bayer Hispania Comercial); phenthoate, purity 93% (Industrias Quimicas Serpiol); trithion, purity 90% (Industrias Quimicas Serpiol); and sumithion, purity 95% (Agrocros). Stock solutions of the OPs were prepared in ethyl acetate and diluted further with distilled water.

Sep-Pak C_{18} cartridges were obtained from Waters Assoc.

Dichloromethane, ethyl acetate, diethyl ether, *n*-hexane, methanol and light petroleum (b.p. 40–60°C) were glass distilled and free from interfering residues as tested by gas chromatography (concentration 100:1). Buffer solutions of pH 2–9 were prepared[14].

Apparatus

A Konik 2000-C gas chromatograph equipped with a splitless injector, an alkali flame ionization detector and a Spectra-Physics SP 4290 integrator was used. A 25 × 0.22 mm I.D. BP-1 0.25-μm fused-silica capillary column provided by Scientific Glass Engineering with helium as the carrier gas was used to separate the pesticides. The injector and detector temperatures were 260 and 280°C, respectively. Splitless injection at 45°C was employed, followed by a 0.6-min delay before heating the column to 140°C at 30°C/min. The column temperature was maintained at 140°C for 2 min, followed by further heating to 260°C at 5°C/min; the final temperature was maintained for 5 min.

Procedure

The cartridge was connected to a 1-l separating funnel with appropriate fittings. The cartridge was activated before use by passing 5 ml of methanol through it, followed by 10 ml of distilled water.

A volume of 1 l of water was spiked with a mixture containing 100–200 ng/l of each OP. This sample was eluted through the cartridge, by applying a vacuum by water aspiration, at a flow-rate of about 40–45 ml/min. The adsorbed residues were eluted with 5 ml of organic solvent. The organic layer was concentrated to 1 ml using a gentle stream of nitrogen. Samples of 2 μl were injected into the gas chromatograph.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram for the separation of the ten OPs after Sep-Pak extraction from water. This chromatogram, obtained using a nitrogen–phosphorus detector, did not contain extraneous peaks.

The recovery efficiency for evaluating the overall performance of the Sep-Pak C_{18} cartridge was tested by analysing fortified distilled water samples. In order to establish the optimum conditions for the extraction of the pesticides, the elution solvent, pH, salinity and volume of water filtered were varied.

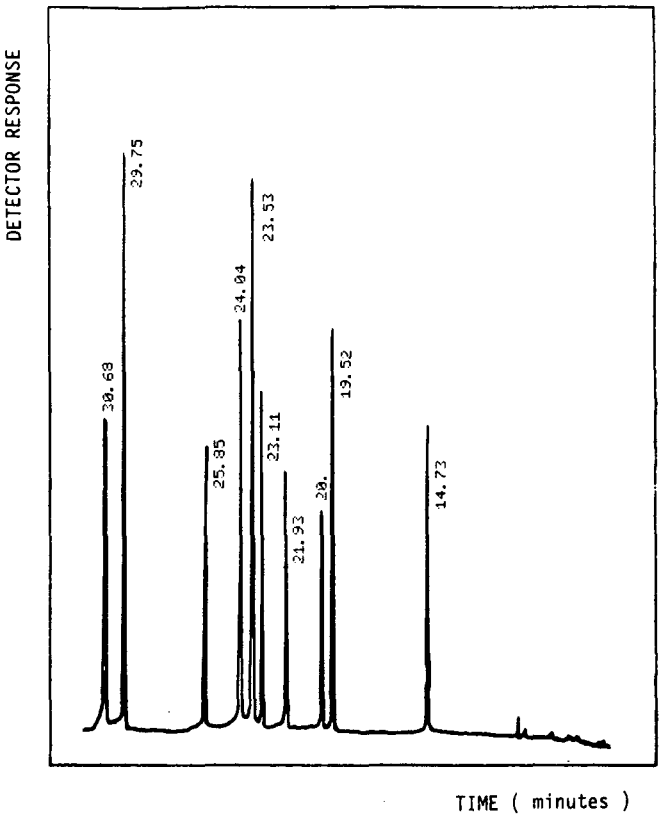

Fig. 1. Chromatogram for the separation of ten OPs. Volume injected, 2 μl. Amount of OPs 200–400 pg. OPs and retention times (indicated at the peaks): heptenophos, 14.73 min; fonofos, 19.52 min; disulfoton, 20.0 min; parathion-methyl, 21.93 min; malathion, 23.11 min; sumithion, 23.53 min; parathion-ethyl, 24.04 min; phenthoate, 25.85 min; ethion, 29.75 min; trithion, 30.68 min.

The recoveries reported are means of five analyses. The pesticide concentrations in water was maintained constant through all the tests (0.2 μg/l, except for disulfoton, ethion and fonofos, which were at 0.1 μg/l). The coefficients of variation ranged between 5 and 10%.

Table I gives the recoveries obtained using 1 ml of ethyl acetate, *n*-hexane and light petroleum as eluents for the ten OPs at pH 7. A study of the effect of pH on the recovery of 1 ml of ethyl acetate it showed (Table II) that pH values between 4.8 and 8 give the best recoveries.

When the assay was carried out in presence of 35 g/l of sodium chloride at pH 7 and eluting with 1 ml of ethyl acetate, the followed recoveries were obtained: heptenofos 77%, fonofos 79%, disulphoton 68%, parathion-methyl 89%, malathion 99%, sumithion 93%, parathion-ethyl 85%, phentoate 84%, ethion 73% and trithion 83%. Comparing these values with those in Tables I and II, a negligible effect of salinity, as in sea water, on the performance was observed.

For heptenofos, fonofos and ethion, the recovery was improved by increasing

TABLE I

PERFORMANCE OF Sep-Pak C_{18} CARTRIDGES

Pesticide	*Recovery (%)*		
	Ethyl acetate	*n-Hexane*	*Light petroleum*
Heptenophos	75	36	29
Fonofos	83	71	89
Disulfoton	76	58	46
Parathion-methyl	96	81	79
Malathion	101	85	76
Sumithion	98	20	24
Parathion-ethyl	93	85	82
Phenthoate	83	69	65
Ethion	79	94	86
Trithion	89	90	80

TABLE II

EFFECT OF pH ON ADSORBENT PERFORMANCE

Pesticide	*Recovery (%)*						
	pH 2.0	*pH 3.5*	*pH 4.8*	*pH 6.1*	*pH 7.0*	*pH 8.0*	*pH 9.0*
Heptenophos	59	63	68	74	75	73	61
Fonofos	48	71	72	79	83	85	52
Disulfoton	43	60	69	72	76	81	37
Parathion-methyl	66	71	89	93	96	77	64
Malathion	69	72	88	98	101	98	60
Sumithion	67	78	91	95	98	89	57
Parathion-ethyl	57	80	87	89	93	85	65
Phenthoate	53	68	77	80	83	84	36
Ethion	42	63	70	76	79	79	45
Trithion	53	75	75	84	89	92	52

TABLE III

EFFECT OF VOLUME OF FILTERED WATER ON THE RECOVERY AND DETECTION LIMITS OBTAINED

Pesticide	*Recovery (%)*		*Detection limit (pg)*
	1 l	*10 l*	
Heptenophos	86	88	52
Fonofos	95	69	20
Disulfoton	74	89	15
Parathion-methyl	98	91	39
Malathion	99	97	78
Sumithion	98	93	15
Parathion-ethyl	94	92	52
Phenthoate	89	93	31
Ethion	95	89	18
Trithion	89	94	52

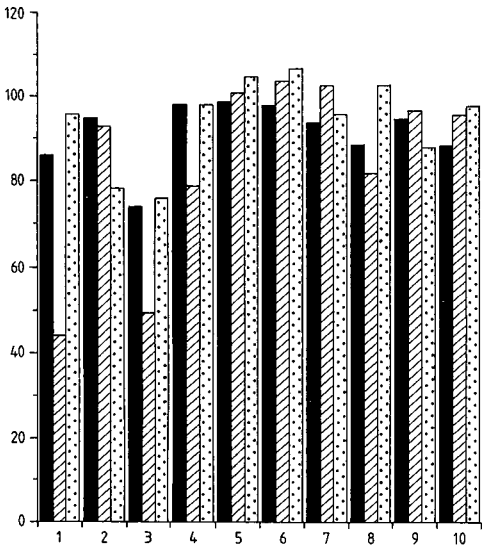

Fig. 2. Comparison of recoveries between the APHA[3] (hatched) and Rodier[2] (shaded) methods and the present method (black). 1 = Heptenophos; 2 = fonofos; 3 = disulfoton; 4 = parathion-methyl; 5 = malathion; 6 = sumithion; 7 = parathion-ethyl; 8 = phenthoate; 9 = ethion; 10 = trithion.

TABLE IV

RECOVERY OF ORGANOPHOSPHORUS PESTICIDES ADDED TO 1 l OF DIFFERENT WATERS

Pesticide	*Level of spike (μg/l)*	*Recovery (%)*		
		Tap water	*Lake water*	*Sea water*
Heptenophos	1	87	83	88
	0.1	72	65	77
Fonofos	1	94	78	96
	0.1	85	63	81
Disulfoton	1	95	72	93
	0.1	76	56	79
Parathion-methyl	1	108	90	104
	0.1	91	87	89
Malathion	1	103	92	99
	0.1	98	85	96
Sumithion	1	99	95	102
	0.1	94	78	100
Parathion-ethyl	1	95	92	97
	0.1	91	81	89
Phenthoate	1	89	75	91
	0.1	80	69	80
Ethion	1	93	91	90
	0.1	79	68	85
Trithion	1	100	79	101
	0.1	90	60	88

the volume of ethyl acetate, the best recovery being observed with 5 ml of eluent (Table III). Greater volumes of ethyl acetate did not improve the results.

Similar recoveries were obtained with the same amount of pesticide but increasing the volume of filtered water from 1 to 10 l, except for fonofos and disulphoton, which gave lower and higher recoveries, respectively, as shown in Table III. Table III also gives the detection limits[15] using a 10-l water sample. As a low volume of organic eluent is finally obtained (1 ml), a high preconcentration ratio is achieved (1:10 000), allowing a detection limit of lower than 0.4 ppt[a] in all instances.

The recoveries reported here are in good agreement with those obtained using the methods reported by Rodier[2] and APHA[3] (Fig. 2).

Table IV demonstrates the performance of the octadecyl-bonded porous silica when local environmental water samples were spiked with a mixture of the ten OPs. Fortified and non-fortified samples were run in parallel. Albufera lake waters were very muddy, requiring filtration through a 1-μm glass filter prior to elution through the Sep-Pak C_{18} cartridge, whereas the Mediterranean Sea and tap waters were eluted directly through the adsorbent.

This rapid and efficient method obviates the use of long sequential solvent extraction methods, thus substantiating the usefulness of Sep-Pak C_{18} cartridges for determining OPs in water.

ACKNOWLEDGEMENT

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REFERENCES

1 R. Mestres and Ch. Chevallier, *Ann. Falsif. Expert. Chim.*, 70 (1977) 101.
2 J. Rodier, *L'Analyse de l'Eau,* Dunod, Paris, 1984, pp. 414–416.
3 *Standard Methods for the Examination of Water and Wastewater, supplement to the 15th ed.*, American Public Health Association, Washington, DC, 1981, pp. 51–57.
4 V. Drevenkar, Z. Fröbe, B. Stengl and B. Tkalcevic, *Int. J. Environ. Anal. Chem.*, 22 (1985) 235.
5 E. M. Lores, J. C. Moore and P. Moody, *Chemosphere,* 16 (1987) 1065.
6 G. L. Lebel, D. T. Williams, G. Griffith and F. M. Benoit, *J. Assoc. Off. Anal. Chem.*, 62 (1979) 241.
7 J. J. Garrido and M. Monteoliva, *An. Edafol. Agrobiol.*, 40 (1981) 1781.
8 A. Verweij, M. A. Van Liempt-Van Houten and H. L. Boter, *Int. J. Environ. Anal. Chem.*, 21 (1985) 63.
9 J. E. Woodrow, M. S. Majewski and J. N. Seiber, *J. Environ. Sci. Health,* 21 (1986) 143.
10 A. Agostiano, M. Caselli and M. R. Provenzano, *Water Air Soil Pollut.*, 19 (1983) 309.
11 V. Drevenkar, Z. Fröbe, B. Stengl and B. Tkalcevic, *Mikrochim. Acta,* 1 (1985) 143.
12 J. J. Richard and G. A. Junk, *Mikrochim. Acta,* 1 (1986) 387.
13 G. A. Junk and J. J. Richard, *Anal. Chem.*, 60 (1988) 451.
14 J. Lurie, *Handbook of Analytical Chemistry,* Mir, Moscow, 1978.
15 J. E. Knoll, *J. Chromatogr. Sci.*, 23 (1985) 422.

[a] The American trillion (10^{12}) is meant.

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FLUORIMETRIC DETERMINATION OF VITAMIN K_3 (MENADIONE SODIUM BISULFITE) IN SYNTHETIC ANIMAL FEED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A POST-COLUMN ZINC REDUCER

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for analysis of vitamin K_3 (menadione), as the water-soluble sodium bisulfite salt, in synthetic animal feed is described. The menadione sodium bisulfite (MSB) is extracted with aqueous methanol, converted to oil-soluble menadione with sodium carbonate, and partitioned into *n*-pentane. After evaporation, the menadione is dissolved in methanol and analyzed by reversed-phase HPLC. The menadione is reduced on-line to its fluorescent 1,4-dihydroxy analogue by zinc reduction prior to fluorescence determination at 325 nm excitation wavelength and 425 nm emission wavelength.

The fluorescence response was linear in the range 1 to 100 ng of menadione injected. Recovery experiments were performed on synthetic animal feed spiked at 20 and 200 mg/kg levels of MSB. Average recoveries from feed were greater than 90% with an average relative standard deviation of 5.5%. Additional confirmation of menadione in actual feed extracts was performed using capillary gas chromatography–Fourier transform infrared spectroscopy (GC–FT-IR). The HPLC–fluorescence and GC–FT-IR methods had lower limits of detection of 20 μg/kg and 2 mg/kg, respectively.

INTRODUCTION

Vitamin K, a class of closely related naphthoquinones, is essential to all human and animal diets. It plays an important role in blood coagulation as a clotting factor and in bone mineralization processes[1]. Naturally occurring K vitamins, phylloquinone (K_1) and farnoquinone (K_2) are usually available to humans from the daily intake of leafy vegetables. However, at the National Center for Toxicological Research (NCTR), laboratory-raised rats and mice, must rely upon synthetic feeds that have been fortified with vitamin premixes for their vitamin K_3 or menadione intake. Menadione is an oil-soluble vitamin, which has exhibited extreme instability in most industrial feed matrices. Therefore, the more stable, water-soluble form, menadione sodium bisulfite (MSB) or some other menadione salt, is used to give a reliable source

of vitamin K_3 in synthetic feeds. Levels found in animal diets used at the NCTR range from approximately 2 to 20 mg/kg of K_3. A fast and dependable analytical method was necessary for determining the K_3 content, in the form of MSB, of synthetic feed prior to feeding laboratory animals at the NCTR.

Several methods are described in the literature to analyse for MSB in animal feed and vitamin premixes. Many of these methods have utilized gas chromatography with flame ionization detection (GC–FID) for detection of the pyrrolytic product of MSB[2–4]. In addition various direct spectrophotometric determinations[5–8] of vitamin K_3 have been reported. These methods lack the sensitivity needed to quantitate microgram amounts of MSB in synthetic feed matrices. Most of the available extraction methods for vitamin K_3 are similar to the official European Community (EC) colorimetric method[8] in which an aqueous alcohol solution extracts the MSB from the feed matrix. The MSB is then converted to the oil-soluble menadione form in the aqueous extract and solvent-partitioned into an organic solvent (*i.e.* 1,2-dichloroethane, *n*-hexane, chloroform, etc.). Recently, a number of high-performance liquid chromatographic (HPLC) methods for vitamin K have been published using ultraviolet (UV) spectrophotometric detection[9,1] and fluorescence detection[10,11]. These UV and fluorescence detection methods utilized reversed-phase HPLC analysis of menadione and other vitamin K compounds with subsequent post-column reaction. The fluorescence detection exhibited higher sensitivity and specificity than the UV methods. Speek *et al.*[11] reported the analysis of MSB in several different types of animal diets and premixes at levels ranging from 0.1 to 362 mg/kg with a minimum detection limit (MDL) of 0.02 mg/kg. However, their method is based upon an extremely complicated post-column reaction with sodium borohydride requiring a reaction coil, a secondary pumping system, a debubbler to remove incidental hydrogen bubbles, and a special debubbler fluorescence flow cell. In 1987, Haroon *et al.*[12] reported utilizing an 'on-line' zinc metal post-column reducer consisting of a 20 × 3.9 mm I.D. steel precolumn packed with high purity 200-mesh zinc particles, for reduction of phylloquinone (K_1) and its metabolite (phylloquinone-2,3-epoxide) in plasma samples with subsequent fluorometric detection of the generated hydroquinones.

The method reported here for analysis of MSB (K_3) in synthetic animal feed is a modification of several extraction methods[6,8,11] coupled with reversed-phase HPLC and an improved zinc post-column reducer and fluorescence detection. The method is simple, fast, and requires no complex derivatization reactions or specialized HPLC hardware. In addition, a confirmation procedure for the qualitative determination of menadione in synthetic feed is described using capillary gas chromatography–Fourier transform infrared spectroscopy (GC–FT-IR).

EXPERIMENTAL

Reagents

All reagents and organic solvents were analytical-reagent grade or comparable. The menadione sodium bisulfite standard (95% purity) was obtained from Sigma, St. Louis, MO, U.S.A. and the menadione standard from United States Biochemical, Cleveland, OH, U.S.A. Stock standard solutions of MSB and menadione were prepared by dissolving 50 mg in 50 ml of deionized water and methanol, respectively. The resulting 1-mg/ml MSB stock solution was diluted to 20 μg/ml and 200 μg/ml in

water–methanol (60:40, v/v) for use in HPLC analysis and MSB recovery experiments. The 1 mg/ml menadione stock solution was diluted with methanol or *n*-pentane to appropriate concentrations for HPLC–fluorescence linearity checks and GC–FT-IR assays, respectively. All standard solutions of MSB and menadione were stored in low actinic glassware at 4°C due to the long-term instability to light and ambient temperatures of these compounds in solution. The synthetic laboratory animal meals and vitamin premixes were analyzed as received. Pelletized feeds were ground using a Wiley Mill Model 4 (Arthur H. Thomas, Philadelphia, PA, U.S.A.) prior to analysis.

Extraction procedure

A 1.00-g sample of ground synthetic feed was extracted with 10 ml of water–methanol (60:40, v/v) in a 30-ml culture tube equipped with a PTFE-lined plastic cap by shaking for 30 min on a mechanical shaker. The sample was centrifuged at 1000 *g* for 10 min. A 5-ml aliquot of the clear supernatant was quantitatively transferred to a second 30-ml culture tube. After addition of 10 ml of 5% sodium carbonate diluted in deionized water and 10 ml of *n*-pentane, the tube was shaken by hand for 1 min and centrifuged at 1000 *g* for 1 min to separate the phases. The *n*-pentane (upper phase) was removed using a Pasteur pipette with care not to disturb the aqueous phase. Two additional 10-ml volumes of *n*-pentane was added to the aqueous and treated as before. The combined *n*-pentane extracts (30 ml total) were collected in a 100-ml round-bottom flask containing one borosilicate-glass boiling bead and evaporated to dryness under vacuum at room temperature maintained using a water bath. The residue was dissolved in 10 ml methanol. The finished extract containing 100 mg-equiv. of feed per ml of methanol was then ready for injection into the HPLC system.

High-performance liquid chromatographic system

A 10-μl sample volume was injected onto the 250 × 4.6 mm I.D. Supelcosil LC-18 (5-μm particle size) column using an Altex Model 210 HPLC injector connected to a Waters Model M-6000 pump. The column was eluted isocratically at ambient temperature with methanol–water (75:25, v/v) at a flow-rate of 0.9 ml/min. The effluent from the column was directed into a post-column zinc reducer consisting of a 20 mm × 2 mm I.D. stainless-steel Uptight guard precolumn with 2-μm frits (Upchurch Scientific, Oak Harbor, WA, U.S.A.) packed with powdered zinc (F&J Scientific, Monroe, CT, U.S.A.) sieved to 38-μm particle size or less. A 0.5-μm Uptight precolumn filter (Upchurch Scientific) was connected to the zinc reducer to remove any zinc particles from plugging the line to the detector. The 0.5-μm filter was then connected to the fluorescence flow-cell of a Shimadzu RF-535 fluorescence HPLC monitor operated at 325 nm excitation wavelength and 425 nm emission wavelength. Fluorescence response was recorded on a Spectra-Physics SP 4270 integrator.

Partitioning value determinations for menadione

An experiment was performed to determine the partitioning (*P*) values of menadione from alcoholic solutions into organic phases. Equal volumes (5 ml) of alcohol–water (40:60, v/v) solutions containing ethanol or methanol and 4 μg/ml menadione were shaken together with several organic solvents. The organic solvents studied in the experiment included *n*-hexane, *n*-pentane, chloroform, and methylene chloride.

After shaking for 1 min, the two phases were separated by centrifugation at 500 *g* for 1 min. The organic layers were analyzed by HPLC–fluorescence as described previously for menadione content. *P* values were calculated for each of the alcohol–water solutions and organic solvents by dividing the amount of menadione (μg/ml) present in the organic phase by the initial amount (4 μg/ml) in the aqueous phase.

Recovery experiment

Triplicate 1.00-g samples of ground synthetic animal feed in 30-ml culture tubes were spiked at levels of 20 and 200 mg/kg MSB. The spiked feed samples were extracted and analyzed by HPLC–fluorescence as previously described and compared to MSB standard solutions treated identically without feed present. Triplicate 1.00-g unspiked feed samples were analyzed for use in correcting the recovery data for inherent levels of MSB in the vitamin K-fortified feed.

GC–FT-IR procedure

For confirmation of the presence of menadione in an unspiked synthetic feed extract, a 10.0-g sample of synthetic feed was extracted using ten-fold increases in the extraction reagents described previously in a 180-ml culture tube. Two 20-ml aliquots of *n*-pentane were used to extract free menadione from the aqueous sodium carbonate solution. The *n*-pentane was transferred to a 100-ml round-bottom flask and reduced to approximately 1 ml with vacuum. This concentrate was quantitatively removed to a graduated 2-ml conical vial together with a 1-ml *n*-pentane rinse of the flask. The sample was evaporated to 0.2 ml volume under a gentle stream of nitrogen. A volume of 2 μl of this concentrate was injected into a Hewlett-Packard HP 5890 gas chromatograph equipped with a splitless capillary injector operated at 220°C. A 30 m × 0.32 mm I.D. fused-silica J&W DB-1701 capillary column was programmed from 80 °C initial temperature for 0.5 min up to 250°C at 16°C/min for 15 min final hold time. The GC column was interfaced to a Digilab FTS-40 FT-IR spectrometer using a Digilab GC/C 32 module containing a 10 cm × 1.0 mm I.D. gold-coated light pipe (250°C) and liquid nitrogen-cooled mercury–cadmium–telluride (MCT) detector. Spectral searches were provided by a Digilab 3200 data station with Search 32 software and a Hewlett-Packard HP 7550A graphics plotter for plotting out the infrared data.

RESULTS AND DISCUSSION

Extraction procedure for menadione sodium bisulfite in animal feed

The extraction procedure was derived from several excellent methods[6,8,11] for MSB analysis. A water–methanol (60:40, v/v) solution is used to solubilize the MSB water-soluble salt from the feed matrix. After centrifugation to precipitate the feed particles, a 50% aliquot of the supernatant is reacted with 5% aqueous sodium carbonate which destroys the bisulfite salt releasing the free menadione, 2-methyl-1,4-naphthoquinone. In contrast to the method of Speek *et al.*[11], *n*-pentane, rather than *n*-hexane, was used as the organic solvent for partitioning the free quinone from the aqueous alcolhol–carbonate solution. This was because *n*-pentane (*P* value = 0.83) performed as well as *n*-hexane (*P* value = 0.81) as seen in Table I and the lower boiling point of *n*-pentane which allowed for faster evaporation of extracts under

TABLE I

PARTITION VALUES FOR VITAMIN K_3 (MENADIONE) BETWEEN AQUEOUS ALCOHOLIC SOLUTIONS AND VARIOUS ORGANIC SOLVENTS

Partition values (*P* values) were determined by partitioning equal 5-ml volumes of 4 μg/ml aqueous alcohol solutions of menadione with four organic solvents and subsequent HPLC analysis of the organic phase.

Aqueous alcohol solution	*P value (1.00 = 100% extraction into organic phase)*			
	n-Pentane	*n-Hexane*	*Methylene chloride*	*Chloroform*
Water–methanol (60:40)	0.83 ± 0.02[a]	0.81 ± 0.07	1.00 ± 0.05	0.91 ± 0.05
Water–ethanol (60:40)	0.86 ± 0.05	0.85 ± 0.02	0.93 ± 0.02	0.91 ± 0.02

[a] *P* values are reported as the average of triplicate analyses (mean ± standard deviation).

vacuum at room temperature. A centrifugation step following the aqueous–organic partition has eliminated the filter paper drying step used on several methods[8,11,13]. After centrifugation, the *n*-pentane (upper) and aqueous (lower) layers are easily separated by using a Pasteur pipette to remove the menadione containing *n*-pentane. The organic extract is evaporated to dryness at reduced pressure as described elsewhere[8,11]. The residue is then dissolved in a known amout of methanol for menadione analysis by reversed-phase HPLC.

Post-column zinc reducer

Metals, such as zinc and cadmium, are well known for their ability to reduce quinones to their corresponding hydroquinones. However, only recently has a metal-filled column been developed for on-line reduction of a variety of menaquinones exhibiting vitamin K activity. Haroon *et al.*[12] used a zinc reducer to perform HPLC–fluorescence analysis of plasma samples for vitamin K_1 (phylloquinone) and K_1 epoxide. In their study, an acidic mobile phase (methanol– aqueous solution of 0.05 mol/l acetic acid, pH 3, 95:5) and one containing zinc ions (methanol–aqueous solution of 0.01 mol/l zinc chloride, 95:5) was necessary for reduction of vitamin K_1. In this study, vitamin K_3 has been shown to be reduced to its fluorescent analogue (2-methyl-1,4-dihydroxynapthalene) using methanol–water (75:25) as mobile phase coupled with an improved low volume zinc powder (< 38 μm particle size) reducer. The fluorescence response to menadione was linear in the range 1–100 ng of menadione injected. An acidic mobile phase (methanol–water, 75:25, pH 2.1 with acetic acid) was studied to detect any increase in fluorescence intensity for menadione (*i.e.* increase in reduction efficiency of the zinc metal). However, lowering the pH of the mobile phase resulted in an increase in pressure and finally plugging of the line to the detector. This may have been due to the depletion of zinc particles by acidic reaction and formation of hydrogen bubbles causing channeling and leakage of zinc particles around the frits. As shown in Fig. 1, the menadione was reduced to a fluorescent analogue in the presence of zinc metal as evidenced by the large fluorescence response with the zinc reducer in-line (chromatogram A) and without the reducer (chromatogram B). The small peak at 5.90 min in chromatogram A was due either to a small fluorescent impurity (possibly the dihydroxy reduction product) in the menadione standard or a reduction reaction of menadione with the stainless-steel tubing and column.

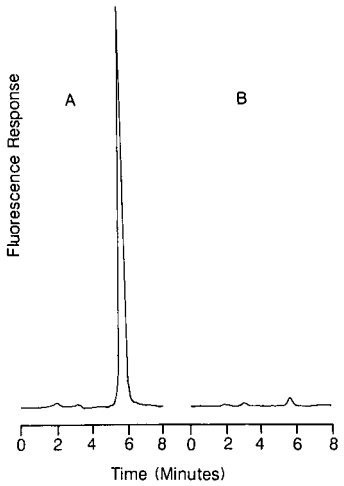

Fig. 1. HPLC–fluorescence chromatograms of 4-μg/ml menadione standard solutions (A) with and (B) without the post-column zinc reducer.

Recovery of MSB from synthetic animal feed

Triplicate samples of synthetic animal feed from a single lot were spiked at 20 and 200 mg/kg of MSB. Control animal feed samples containing only a vitamin premix were determined to contain 14.8 ± 0.9 mg/kg of MSB without correction for recovery (Table II). The percent recoveries of MSB at 20- and 200-mg/kg levels in feed of 91.2 ± 5.7 and 94.5 ± 4.4 were corrected for the control feed level. A typical HPLC–fluorescence assay of an MSB standard of 1 μg/ml in methanol (A), a control synthetic feed sample (B), and a 20-mg/kg synthetic feed spike (C) are shown in Fig. 2. The minimum detection limit for a 1-g synthetic feed sample was determined to be 20 μg/kg. Synthetic laboratory animal feed specifications for rat and mouse diets are usually 2–20 mg/kg of vitamin K_3.

TABLE II

RESULTS OF ANALYSIS OF SYNTHETIC ANIMAL FEED SPIKED WITH VITAMIN K_3 (AS MENADIONE SODIUM BISULFITE) USING HLPC–FLUORESCENCE

All results are mean ($\bar{x}$), standard deviation (S.D.), and relative standard deviation (R.S.D.) of triplicate 1.00-g synthetic feed samples from a single lot of feed.

Amount added (mg/kg)	*Amount found (mg/kg)*	*Amount recovered (%)*	
		$\bar{x}$ ± *S.D.*[a]	*R.S.D.*
Control	14.8 ± 0.9	–	–
20	33.0 ± 1.1	91.2 ± 5.7	6.3
200	203.8 ± 8.7	94.5 ± 4.4	4.7

[a] Recoveries are corrected for the menadione sodium bisulfite level found in the control feed samples.

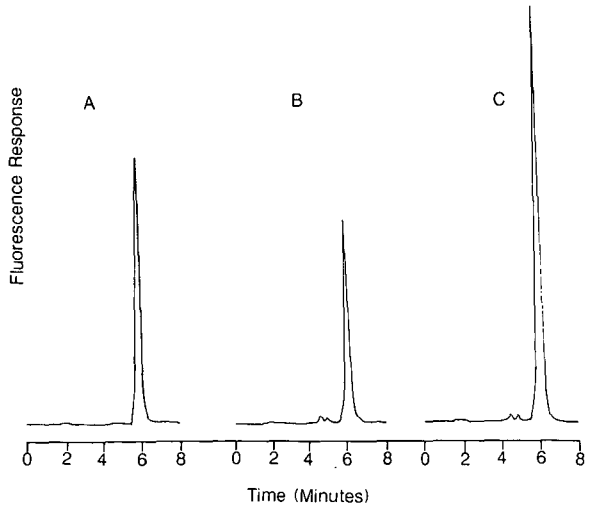

Fig. 2. Typical HPLC–fluorescence chromatograms of a (A) 1-μg/ml MSB standard, (B) 1.00-g control synthetic animal feed, and (C) 1.00-g synthetic animal feed sample spiked with MSB at the 20-ppm level.

Vitamin K_3 (MSB) analysis of synthetic animal feeds and vitamin premixes

Several synthetic feed lots and feed premixes were analyzed. The vitamin K_3 contents in mg/kg are reported in Table III. Vitamin K_3 in unspiked synthetic feed samples ranged from 1.90 to 20.7 mg/kg with premixes containing high levels (approx. 5%, w/w). As shown in Table III, vitamin-fortified meals and pellets stored for

TABLE III

RESULTS OBTAINED FROM HPLC–FLUORESCENCE ANALYSES FOR VITAMIN K_3 IN SYNTHETIC FEED AND VITAMIN PREMIXES

NA = Not available.

Type of sample analyzed	*Manufacturer*	*No. of days stored prior to sampling*	*Vitamin K_3 found (mg/kg)*
Meal, fortified[a]	A	2	9.67
Meal, fortified	A	280	1.90
Meal, fortified	B	2	20.7
Meal, fortified	B	125	14.8
Pellets, unfortified[b]	B	2	6.71
Pellets, unfortified	C	10	1.97
Pellets. fortified	C	16	5.89
Pellets, fortified	C	219	2.13
Vitamin feed premix[c]	D	NA	$55.4 \cdot 10^3$
Vitamin feed premix	D	NA	$57.3 \cdot 10^3$

[a] Meal or pellets contain vitamin premix.

[b] Pellets contains no vitamin premix.

[c] Vitamin premix, high in vitamin K_3 content and were diluted 1:5000 with methanol prior to HPLC analysis.

125–280 days were much lower in K_3 content than those stored 2–16 days indicating the instability of menadione, even as the more stable bisulfite salt, in synthetic animal feed stored for long periods. For determining if feed samples meet vitamin K_3 specifications, it will be advantageous for analyses to be performed as soon as possible after receiving the samples.

GC–FT-IR confirmation of menadione in synthetic feed

To confirm the presence of menadione in a synthetic feed extract, a 10.0-g sample of a typical synthetic animal feed was analyzed by capillary GC–FT-IR. Gram-Schmidt chromatograms are shown in Fig. 3 of a 0.5-mg/ml menadione standard (A) and a 10.0-g synthetic feed sample in 200 μl *n*-pentane (B). The minimum detection limit for menadione using the GC–FT-IR method was determined to be 2 mg/kg for a 2-μl splitless injection of the pentane–feed extract. Following a computer spectral-library search, an infrared spectral match was found for the animal feed peak at 6.48 min retention (IR trace A) with the 3250 compound EPALIB 8 library spectrum number 1343, menadione (IR trace B) as shown in Fig. 4.

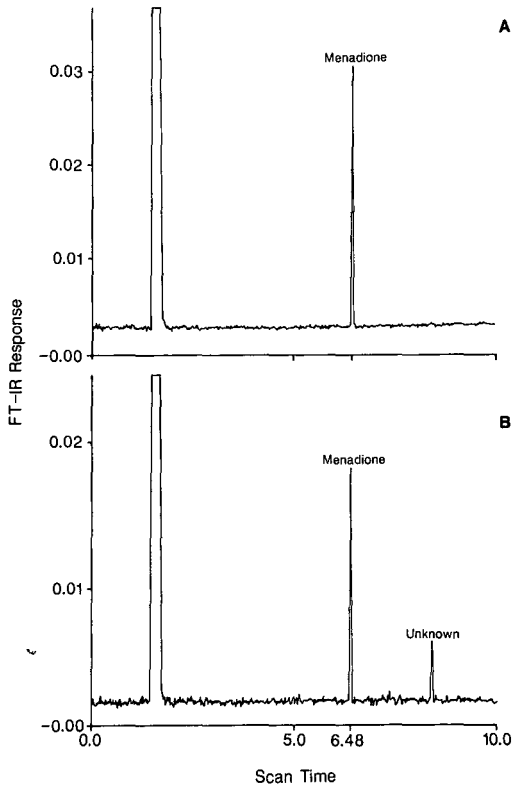

Fig. 3. DB-1701 capillary GC–FT-IR Gram-Schmidt chromatograms of 2-μl splitless injections of (A) a 0.5-mg/ml menadione standard in *n*-pentane and (B) a 10.0-g animal feed in 200 μl of *n*-pentane. Menadione is the peak at 6.48 min scan time.

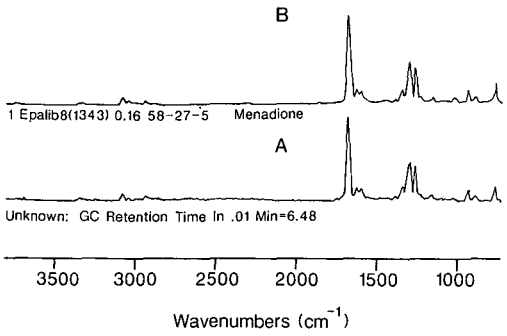

Fig. 4. Results of the FT-IR spectral-library search showing an FT-IR trace (A) of the 6.48 min peak for a 10.0-g synthetic animal feed sample and the FT-IR trace (B) for compound No. 1343, menadione, from the 3250 compound EPALIB 8 vapor phase library.

CONCLUSIONS

The improved zinc reducer reported here has been shown to be a very effective on-line method for reduction of menadione to its fluorescent analogue, 2-methyl-1,4-hydroxynaphthalene. Using this technique, a fast and sensitive HPLC method for analysis of vitamin K_3, as menadione sodium bisulfite, in synthetic animal feed has been described. The GC–FT-IR method has produced a good confirmatory analysis in addition to the specificity of the fluorescence method.

REFERENCES

1 V. Manz and R. Maurer, *Int. J. Vitam. Nutr. Res.*, 52 (1982) 248.
2 D. A. Libby and A. J. Sheppard, *J. Assoc. Off. Anal. Chem.*, 48 (1965) 973.
3 V.W. Winkler and J. M. Yoder, *J. Assoc. Off. Anal. Chem.*, 55 (1972) 1219.
4 V. W. Winkler, *J. Assoc. Off. Anal. Chem.*, 55 (1973) 1277.
5 S. S. M. Hassan, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 611.
6 R. Berrut, *Methods of Vitamin Assay: Vitamin K*, Wiley, New York, 4th ed., 1985, p. 295.
7 H. J. M. van Zijl and H. Geerling, *Chem. Weekbl.*, 55 (1959) 597.
8 *Off. J. Env. Commun.*, 17 (1979) 108/23, 22.4.74.
9 K. Ranfft and H. Ruckemann, *Z. Lebensm. Unters. Forsch.*, 167 (1978) 150.
10 K. Abe, O. Hiroshima, K. Ishibashi, M. Ohmae, K. Kawabe and G. Katsui, *Yakugaki Zasshi*, 99 (1979) 192.
11 A. J. Speek, J. Schrijver and W. H. P. Schreurs, *J. Chromatogr.*, 301 (1984) 441.
12 Y. Haroon, D. S. Bacon and J. A. Sadowski, *Biomed. Chromatogr.*, 2 (1987) 4.
13 *The United States Pharmacopeia*, Mack Printing, Easton, PA, 17th ed., 1965, p. 1082.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MIXTURES OF CATECHOL AND ITS REACTION PRODUCTS WITH GLYOXYLIC ACID

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SUMMARY

Complex mixtures of catechol and its α-hydroxyacetic acid derivatives formed by reaction with glyoxylic acid in aqueous alkaline media were analysed with high-performance liquid chromatography. A complete qualitative and quantitative analysis of the reaction was developed using a strong cation-exchange resin (SCX) and a reversed-phase *n*-octadecylsilane-modified silica (ODS) column combined with refractive index and fixed-wavelength UV detection. An almost identical elution pattern was observed for both columns, indicating a separation mechanism based on hydrophobic interactions. The ODS column appeared to be more selective than the SCX column towards the aromatic compounds.

INTRODUCTION

The reaction of catechol (1,2-dihydroxybenzene) with glyoxylic acid in aqueous alkaline media results in a complex mixture of α-hydroxyacetic acid derivates. The reaction scheme is shown in Fig. 1.

The main and most interesting reaction product is α,3,4-trihydroxybenzeneacetic acid (the *para* derivative), from which 3,4-dihydroxybenzaldehyde can be obtained by selective catalytic oxidation. The *para* derivative may also be a precursor for other α-substituted benzeneacetic acid derivatives (*e.g.*, D,L-3,4-dihydroxyphenylglycine) or 3,4-dihydroxybenzeneacetic acid. All such products are interesting starting materials for specialities and pharmaceutical products.

The development of a process for the preparation of the *para* derivative on a technical scale requires a complete kinetic description of the electrophilic substitution reactions of glyoxylic acid with catechol and its monosubstituted α-hydroxyacetic acid derivatives as shown in Fig. 1. A thorough investigation of the kinetics is possible only if an accurate method for the qualitative and a quantitative analysis of the reaction mixture is available.

Chromatographic techniques seem to be appropriate for the analysis of these complex reaction mixtures. Analysis for catechol is possible with gas–liquid chromatography (GLC)[1–3]. Nothing appears to have been reported previously about the

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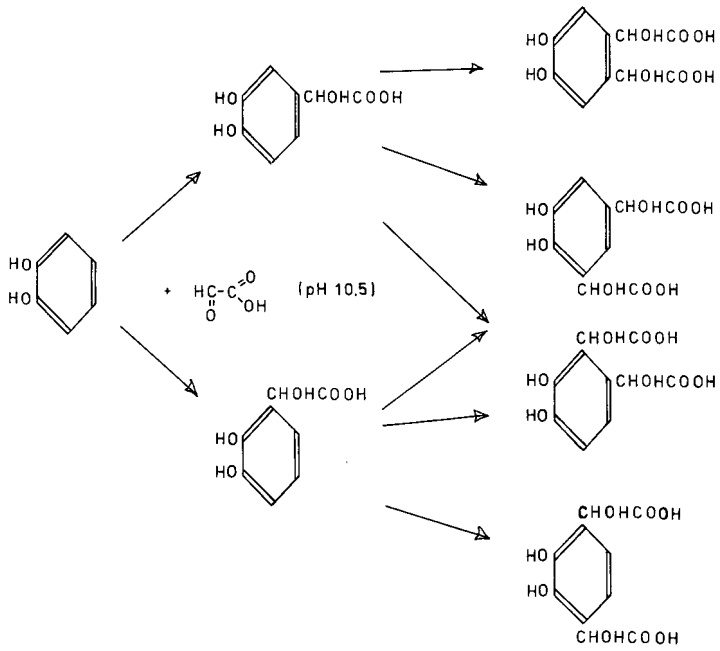

Fig. 1. Possible reactions in aqueous alkaline solutions of catechol and glyoxylic acid.

analysis of mixtures of catechol and its α-hydroxyacetic acid derivatives. For the separation of the α-hydroxyacetic acid derivatives of catechol by GLC, a time-consuming precolumn derivatization is necessary to increase their volatility and thermal stability. The application of liquid chromatography as separation technique for the components of the reaction mixtures of catechol and glyoxylic acid therefore seems an obvious choice. A procedure for the qualitative and quantitative analysis of reaction mixtures of glyoxylic acid and phenol or guaiacol (2-methoxyphenol) based on liquid chromatography has been described recently[4]. The chromatograms were obtained with a completely sulphonated copolymer of styrene and 1,4-divinylbenzene in the acidic form as the stationary phase and in an independent procedure with a reversed-phase system based on an *n*-octadecylsilane-modified silica phase[4]. For both columns the eluent was an aqueous $5 \cdot 10^{-3}$ mol/dm^3 sulphuric acid solution. It appeared useful to test the same method for the reaction mixtures of catechol and glyoxylic acid, which show a close resemblance to the less complicated reaction mixtures of glyoxylic acid and phenol or guaiacol. This paper describes the application of this slightly modified method to reaction mixtures of catechol and glyoxylic acid.

EXPERIMENTAL

Materials

Catechol and α,3,4-trihydroxybenzeneacetic acid were obtained from Janssen Chimica (Beerse, Belgium), phenol from Merck (Darmstadt, F.R.G.), glyoxylic acid (50 wt.-% in water) and sodium α,4-dihydroxybenzene acetate monohydrate from Andeno (Venlo, The Netherlands).

Apparatus

The chromatographic system, stationary phases, column dimensions and eluent composition were the same as described previously for the analysis of reaction mixtures of glyoxylic acid and phenol or guaiacol[4]. The following abbreviations for the column–detection combinations are used: SCX–RI, a completely sulphonated copolymer of styrene and divinylbenzene (8%) in the acidic form (a strong cation-exchange SCX column) as stationary phase combined with refractive index detection; SCX–UV, the same stationary phase as above combined with UV detection at 254 nm; ODS–RI, a stationary phase consisting of particles of *n*-octadecylsilane-modified silica (ODS column) combined with refractive index detection; and ODS–UV, the same ODS column as in the preceding system combined with UV detection.

Sample preparation

Samples for chromatography were taken from the solutions obtained during the reaction of catechol and glyoxylic acid. The reactions were carried out in aqueous solution at a constant pH of 10.50 and at constant temperatures between 25 and 50°C. The reactions were started by mixing equimolar amounts of the reactants. Before mixing, the pH of the solution of catechol and glyoxylic acid was adjusted to 10.50 with concentrated sodium hydroxide solution. The concentration of the reactants immediately after mixing was generally 0.25 mol/dm^3. The reaction was quenched immediately after sampling by mixing the sample with an equal volume of 1 mol/dm^3 hydrochloric acid. The resulting solution (pH $<$ 1) was introduced into the chromatographic system without further treatment.

RESULTS AND DISCUSSION

The elution volumes of catechol, phenol and guaiacol as a function of the column temperature were determined for the SCX and ODS columns. From these elution volumes the capacity factors, k', were calculated as described by Simpson[5]. The results are given in Fig. 2, which shows an identical influence of the column temperature on the capacity factors for the three aromatic compounds on both columns, suggesting an identical retention mechanism. Retention originates from hydrophobic interactions between the non-polar parts of the stationary phase and the aromatic ring of the eluted compounds[4]. The hydrophobic character increases in the order catechol $<$ phenol $<$ guaiacol.

The samples taken from the reaction mixtures of catechol and glyoxylic acid were analysed with the SCX and ODS columns at 30°C. Compared with the SCX column temperature of 85°C used in the analysis of reaction mixtures of glyoxylic acid and phenol or guaiacol[4], the temperature of the SCX column in this study is low. The choice of this low column temperature can be explained by a slightly but continuously decreasing stability of catechol on increasing the temperature of the SCX column above about 30°C. For the ODS column, no significant change in the molar response of catechol was observed for column temperatures between 20 and 60°C. Higher column temperatures were not investigated because of the limited thermal stability of the stationary phase.

Typical examples of chromatograms of samples taken from reaction mixtures of catechol and glyoxylic acid obtained with the SCX–RI and ODS–RI column–detec-

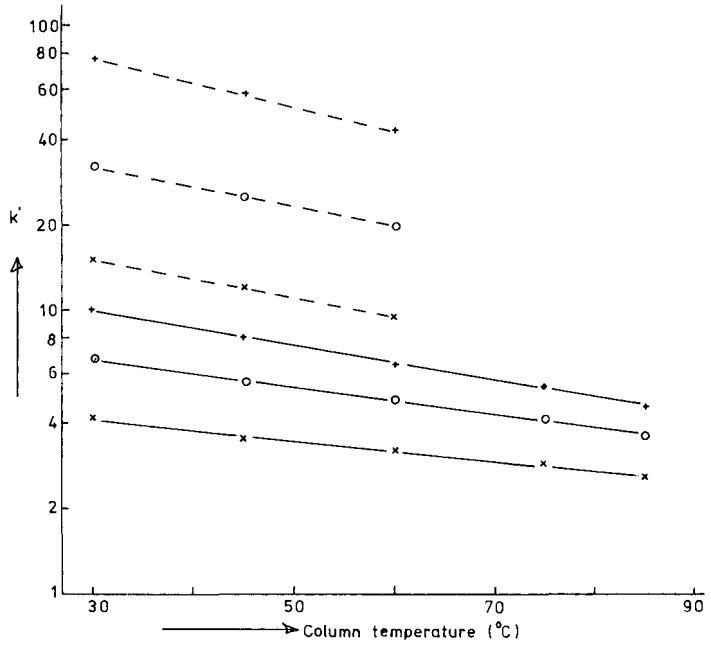

Fig. 2. Capacity factors (k') of (○) phenol, (+) guaiacol and (×) catechol as a function of column temperature for (solid lines) the SCX and (broken lines) the ODS column.

tion combinations are shown in the Figs. 3 and 4, respectively. The results show that quantification of catechol is possible with both combinations. The position of the elution zone of α,3,4-trihydroxybenzene acetic acid (the *para* derivative) in the chromatograms of the reaction samples was assigned by comparing the chromatograms with those obtained after injection of a solution of the pure compound. The elution volume of α,2,3-trihydroxybenzene acetic acid (the *ortho* derivative) on the ODS column is assumed to be significantly higher than that of the *para* derivative as a result of a more pronounced hydrophobic interaction of the *ortho* derivative with the non-polar stationary phase. For the SCX column co-elution of the *ortho* and *para* derivatives is observed. The difference in selectivity with respect to the *ortho* and *para* derivatives can be explained in terms of the hydrophobic properties of the stationary phases in the SCX and ODS columns[4]. As a result of their more polar character, the elution volumes of the doubly substituted derivatives of catechol are expected to be lower than those of the *ortho* and *para* derivatives for both columns. In the chromatograms of the reaction samples obtained with the ODS–RI system (Fig. 4), only two elution zones are observed between the injection peak and the peak of the *para* derivative. With the ODS–UV combination it was demonstrated that these elution zones can be assigned to aromatic compounds and therefore to doubly substituted derivatives of catechol.

Considering the structures of the four possible doubly substituted isomers, a different elution behaviour for each of these compounds may be expected on the ODS column. Probably only two isomers are formed in detectable amounts: α,α′,4,5-tetrahydroxybenzene-1,3-diacetic acid (the *ortho,para* derivative), from the *ortho* and the

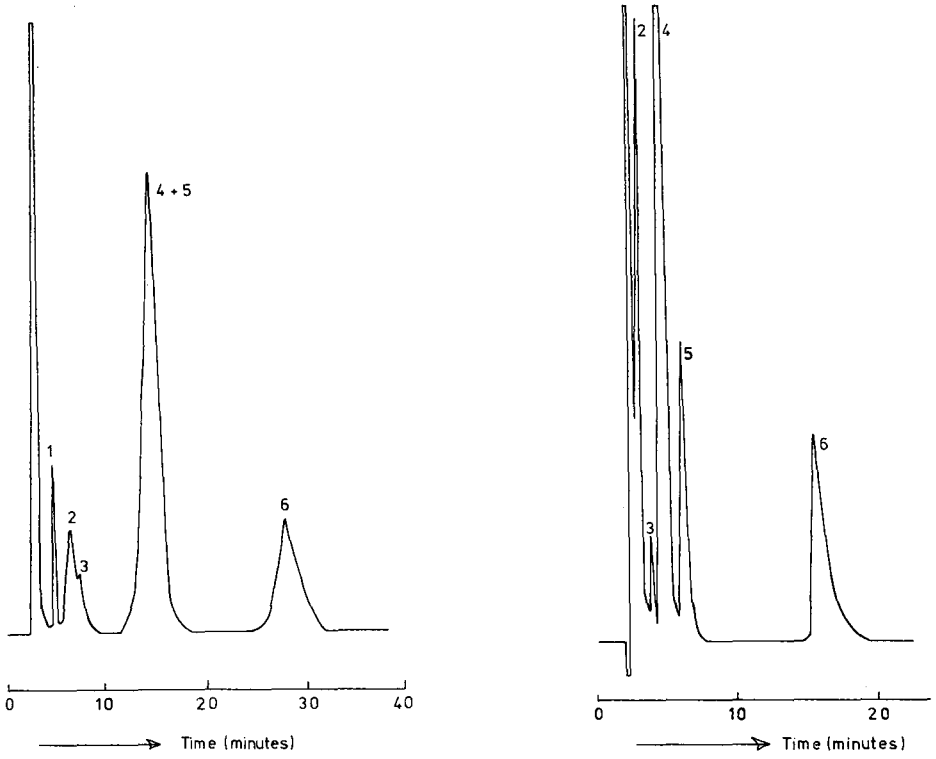

Fig. 3. Chromatogram of a sample from a reaction mixture of catechol and glyoxylic acid obtained with the SCX–RI combination. Analytical conditions as given under Experimental and in ref. 4. Column temperature 30°C; flow-rate of the eluent, 0.4 ml/min. Peaks: 1 = glyoxylic acid; 2 = α,α′,4,5-tetrahydroxybenzene-1,3-diacetic acid; 3 = α,α′,2,3-tetrahydroxybenzene-1,4-diacetic acid; 4 = α,3,4-trihydroxybenzeneacetic acid; 5 = α,2,3-trihydroxybenzeneacetic acid; 6 = catechol.

Fig. 4. Chromatogram of the same sample as in Fig. 3 obtained with the ODS–RI combination. Conditions as in Fig. 3, except flow-rate of the eluent, 0.8 ml/min. Peaks as in Fig. 3.

para derivatives, and α,α′,2,3-tetrahydroxybenzene-1,4-diacetic acid (the *ortho, ortho* derivative), from the *ortho* derivative. The formation of α,α′,3,4-tetrahydroxybenzene-1,2-diacetic acid from the *ortho* derivative and the formation of α,α′,4,5-tetrahydroxybenzene-1,2-diacetic acid from the *para* derivative are probably prevented by the steric hindrance caused by the α-hydroxyacetic acid group, already present in the singularly substituted derivative. As the hydrophobic properties of the *ortho,ortho* derivative are more pronounced than those of the *ortho,para* derivative, the elution volume of the *ortho,para* derivative on the ODS column will be significantly lower than that of the *ortho,ortho* derivative. For the SCX column, the elution peak of the *ortho,ortho* derivative appears as a shoulder to the rear part of the elution peak of the *ortho,para* derivative. This interpretation is supported by a chromatogram obtained with the SCX–UV combination. In this chromatogram, the same elution zones are observed as for the SCX–RI system, with the exception of the peak immediately following the injection peak in the SCX–RI system. This peak should therefore be

assigned to the elution of glyoxylic acid. Summarizing, it can be concluded that the elution volumes on the SCX column increase in the order glyoxylic acid < *ortho,para* < *ortho,ortho* < *para* ≈ *ortho* derivative < catechol. For the ODS column, on which only retention of the aromatic compounds can be expected, an almost identical sequence of the elution volumes is observed, *viz.*, *ortho,para* < *ortho,ortho* < *para* < *ortho* derivative < catechol.

The SCX–RI and ODS–RI combinations are both necessary for complete quantification of the reaction mixture. The ODS–RI combination has to be used for the quantification of the aromatic components in the reaction mixture, whereas the determination of the concentrations of catechol and glyoxylic acid is possible with the SCX–RI system.

The molar response of the *ortho* derivative and both doubly substituted derivatives of catechol, which are not available as pure compounds, can be calculated from the molar responses of catechol and the *para* derivative. For this calculation procedure, it is assumed that for a given number of α-hydroxyacetic acid groups present, the molar response for refractive index detection is independent of the position of the substituents in the aromatic ring. It is further assumed that for RI detection a linear relationship exists between the molar response and the number of α-hydroxyacetic acid groups present. These assumptions are supported by the values of the difference between the molar responses of α,3,4-trihydroxybenzeneacetic acid and catechol and the difference between the molar responses of α,4-dihydroxybenzeneacetic acid and phenol. The ratios of the measured values of the molar responses of these four compounds and the molar response of phenol are presented in Table I. These relative molar responses show that the difference between the molar responses of α,3,4-trihydroxybenzeneacetic acid and catechol is identical with that between the molar responses of α,4-dihydroxybenzeneacetic acid and phenol, within experimental error. This equality can lead to the conclusion that the change in the molar response after introduction of an α-hydroxyacetic acid group is independent of the other substituents in the ring.

These considerations support our assignment of the elution peaks in the chromatograms obtained with the SCX–RI (Fig. 3) and ODS–RI combinations (Fig. 4) to the doubly substituted derivatives α,α′,4,5-tetrahydroxybenzene-1,3-diacetic acid and α,α′,2,3-tetrahydroxybenzene-1,4-diacetic acid given above. From Fig. 4 (ODS–RI) and the assumed equality of the molar responses of both doubly substituted derivatives, it can be concluded that the concentration of the *ortho,para* derivative responsible for the first elution peak is much greater than that of the *ortho,ortho* derivative

TABLE I

RELATIVE MOLAR RESPONSES OF PHENOL, CATECHOL AND THEIR *PARA*-SUBSTITUTED α-HYDROXYACETIC ACID DERIVATIVES

Compound	*Relative molar response*
Phenol	1.00
α,4-Dihydroxybenzeneacetic acid	1.57
Catechol	1.16
α,3,4-Trihydroxybenzeneacetic acid	1.72

TABLE II

RELATIVE MOLAR RESPONSES OF CATECHOL, GLYOXYLIC ACID AND THEIR REACTION PRODUCTS FORMED IN AQUEOUS ALKALINE SOLUTIONS

Compound	*Relative molar response*
Catechol	1.00
Glyoxylic acid	0.42
α,3,4-Trihydroxybenzeneacetic acid	1.48
α,2,3-Trihydroxybenzeneacetic acid	1.48
α,α',4,5-Tetrahydroxybenzene-1,3-di-acetic acid	1.96
α,α',2,3-Tetrahydroxybenzene-1,4-di-acetic acid	1.96

assigned to the second elution zone on the ODS column. This difference in the amounts of the *ortho,para* and *ortho,ortho* derivatives is confirmed by the reaction scheme in Fig. 1 and by the ratio of the concentrations of the monosubstituted *para* and *ortho* derivatives, which is much higher than unity during the whole course of the reaction.

Table II shows the ratios of the molar responses of the components in the reaction mixtures of catechol and glyoxylic acid and the molar response of catechol for RI detection. With these values and the molar response of catechol, the concentra-

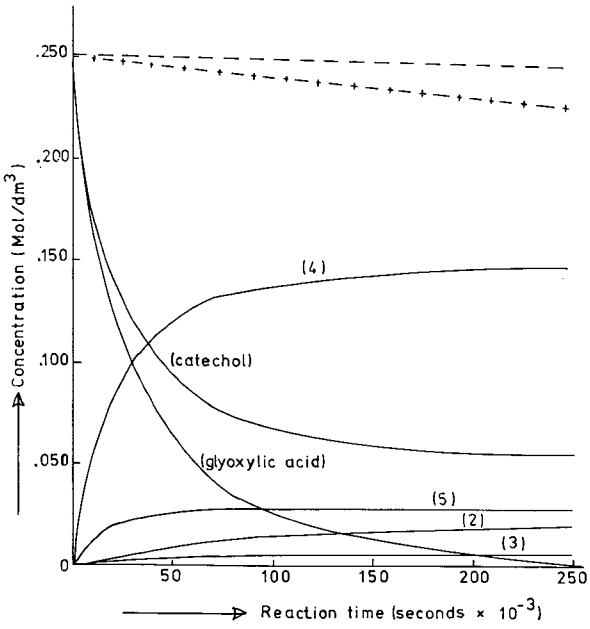

Fig. 5. Aromatic (− − − −) and glyoxylic acid equivalents (− + − + −), and the concentrations of the components of a reaction mixture of catechol and glyoxylic acid as a function of time. Solvent, water; temperature, 25°C; pH, 10.50. The numbers of the components correspond to those in Fig. 3.

tions of the various compounds in the reaction mixture can be calculated from the responses of the RI detector. Fig. 5 shows the concentrations of the various components in the reaction mixture as a function of time for a reaction at 25°C and a pH of 10.50, and indicates a complete mass balance for the aromatic compounds in the reaction mixture for a glyoxylic acid conversion between 0 and 1. The glyoxylic acid concentrations and the concentrations of the α-hydroxyacetic acid derivatives in the reaction mixture show that the mass balance for glyoxylic acid is not fully complete. For a complete conversion of glyoxylic acid, only about 90% of the glyoxylic acid is converted into α-hydroxyacetic acid derivatives. A few percent may be converted into glycolic and oxalic acid by the Cannizzaro reaction during the preparation of the starting solution. The nature of the other side-products formed from glyoxylic acid could not simply be determined. In the chromatograms obtained with the SCX–RI combination, no elution of eventual side-products of glyoxylic acid was observed.

CONCLUSIONS

Mixtures of catechol and its reaction products with glyoxylic acid can be characterized qualitatively by using high-performance liquid chromatography. The separation mechanism is based on hydrophobic interactions for both ODS and SCX columns.

The concentrations of the aromatic compounds can be determined with the ODS–RI combination and glyoxylic acid and catechol can be quantified using the SCX–RI combination. In the same way as for reaction mixtures of glyoxylic acid and phenol or guaiacol, it has been shown that reaction mixtures of catechol and glyoxylic acid can be completely quantified, even if not all aromatic reaction products are available in the pure form.

It is likely that the molar response using RI detection can be calculated from the contribution of the starting aromatic compounds and that of the α-hydroxyacetic groups attached to the ring. The contribution of each α-hydroxyacetic acid group is independent of the kind and number of substituents already present.

REFERENCES

1 I. O. O. Korhonen and J. Knuutinen, *Chromatographia*, 17 (1983) 154.
2 J. S. O'Grodnick, G. D. Dupré, B. J. Gulizia, S. H. Blake and R. A. Kuna, *J. Chromatogr. Sci.*, 21 (1983) 289.
3 H. Mayer, J. Nolte and B. Paschold, *Fresenius' Z. Anal. Chem.*, 315 (1983) 708.
4 L. A. Th. Verhaar, J. Meuldijk, W. P. Th. Groenland and K. van der Wiele, *J. Chromatogr.*, 437 (1988) 185.
5 C. F. Simpson, *Practical High Performance Liquid Chromatography*, Heyden, London, 1976, p. 7.

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CHROM. 21 477

Note

Simple modification to compensate for the baseline variation due to the introduction of a reduced-pressure gas sample into a gas chromatograph

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Gas chromatography (GC) is the most convenient method for the analysis of materials such as industrial gases. In most of commercially available gas chromatographs, a pneumatic mass flow controller is used to provide a constant flow of carrier gas. This flow controller has the disadvantage that it is slow to respond to variations in carrier gas pressure[1]. Gas analysis is also widely carried out using a gas chromatograph with a packed column and a conventional gas sampler. The gas sampler is used to introduce the gas sample, which is withdrawn under atmospheric pressure, into the gas chromatograph.

The pressure of the sample gas is not always equal to that of the carrier gas. Therefore, if a sample gas at close to or less than atmospheric pressure is introduced into a gas chromatograph, baseline variations may be found on the chromatogram[2]. This baseline variation, called a ghost peak, becomes progressively greater as the pressure difference between the sample and carrier gas pressures increases. Such a phenomenon is commonly observed on chromatograms obtained by the use of a gas chromatograph equipped with a packed column, a pneumatic mass flow controller and a flow-sensitive detector such as a thermal conductivity detector. In order to avoid such baseline variations, it is usually necessary to reduce further the volume of the sample loop, or to increase the pressure of the sample gas to that of the carrier gas by the use of a special valve[3,4].

In this paper, we report a new introduction system developed for gas samples at reduced pressures and to reduce the irregular baseline variations on gas chromatograms.

EXPERIMENTAL

The experiments were carried out using a Shimadzu GC-9A gas chromatograph equipped with a thermal conductivity detector and a computerized integrator (Shimadzu C-R3A). In this gas chromatograph, the carrier gas flows are controlled with pneumatic flow controllers. The sampling and injection system were modified as shown in Fig. 1. This system consists of an ordinary sampling system and a bypass line. The bypass line with a 10 p.s.i. relief valve (NUPRO SS-2C-10) (12) and a 5-ml

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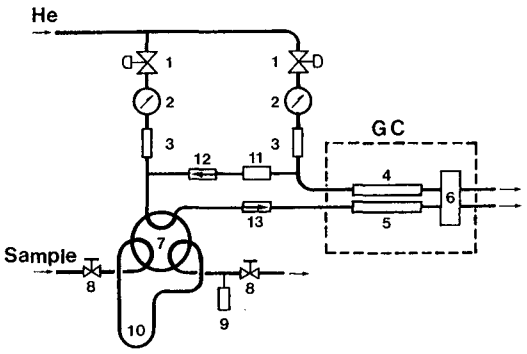

Fig. 1. Schematic diagram of apparatus. 1 = Flow controller; 2 = pressure gauge; 3 = flow meter; 4 = reference column; 5 = analytical column; 6 = detector; 7 = six-port air-actuated rotary valve (Valco Instruments, C-6P); 8 = diaphram stop valves (Nupro, SS/DLS4); 9 = pressure transducer (Trans Metrics, P-21BA); 10 = sample loop; 11 = surge tank (5 ml); 12 = 10 p.s.i. relief valve; 13 = 5 p.s.i. relief valve.

surge tank (11) was installed between the two carrier gas lines; one is for the analytical (chromatographic) column line and the other for the reference column line. The relief valve (12) can be operated to open the bypass line when the pressure difference between the inlet and outlet of the relief valve is over *ca.* 10 p.s.i.

Additional helium is consequently fed through the bypass line from the reference column line into the sample loop (10); the pressure in the sample loop can be close to and/or equalized to the pressure of the carrier gas. A 5 p.s.i. relief valve (NUPRO SS-2C-5) (13) is installed to prevent suction of the carrier gas into the analytical column during the pressurizing period. In this way, the sample gas can be pressurized quickly, and pressure variations of the carrier gas can be reduced.

The carrier gas helium was prepurified through an oxygen trap column (Applied Research, Oxytrap). A standard gas generator (STEC, SGGU-9000) was used to prepare gas mixtures ranging in concentration from 1 to several tens of ppm; and gas mixtures in cylinders were also used, if necessary. The operating parameters of the gas chromatograph are given in Table I.

TABLE I

OPERATING CONDITIONS OF THE GAS CHROMATOGRAPH

Parameter	*Component*	*Conditions*
Columns	Analytical	MS-5A, 60–80 mesh, 1.5 m × 3 mm I.D.
	Reference	MS-5A, 60–80 mesh, 1.5 m × 3 mm I.D.
Temperatures	Oven	50°C
	Injector	70°C
	Detector	70°C
Thermal conductivity detector		180 mA
Carrier gas (He)	Analytical column	50 ml/min at 1.8 kg/cm^2 G
	Reference column	70 ml/min at 1.9 kg/cm^2 G

RESULTS AND DISCUSSION

A series of experiments were conducted to assess and demonstrate the effects of this system for the reduction of baseline variations. The 10 p.s.i. relief valve was used in order to open and/or close the bypass line. This relief valve was chosen after considering the pressure drop in the sample loop; it is necessary that additional helium can be quickly fed into the sample loop without overpressurizing. Under the proposed conditions, even if the column length, oven temperature and carrier gas flow-rate were changed, the system could be succesfully operated without any modification of the system. As mentioned above, however, it is required that the carrier gas pressure in the reference column line be maintained slightly higher than that in the analytical column line; the pressure difference between the two carrier gas lines was set in the range 0–0.25 kg/cm^2. As shown in Fig. 2, when a gas sample that was taken into the sample loop (4.47 ml) under atmospheric pressure was introduced into the analytical column using the conventional system, the ghost peak overlapped other peaks of rapidly eluted gases such as hydrogen, oxygen and nitrogen. Consequently, it was difficult to determine their peak areas accurately with the tailing of the ghost peak, even with the use of a computerized integrator. On the other hand, such a large ghost peak did not appear on the chromatogram obtained with the present system, as shown in Fig. 2. The results indicate that the present modified system could be successfully compensate for the large baseline variations on the chromatogram.

The calibration graphs obtained with both systems are shown in Fig. 3. The graphs for methane did not show much difference between the two systems as this peak eluted separately from the ghost peak and there was little interference from the tailing of the latter. On the other hand, the argon peak, which eluted more rapidly, overlapped considerably with the tailing of the ghost peak when the conventional system was used. Therefore, the ghost peak caused a large interference in the measurement of the peak area of argon; the results showed that the calibration graph had a large deviation from the origin. However, the large ghost peak did not appear on the chromatogram obtained with the present system, as shown in Fig. 3. The repro-

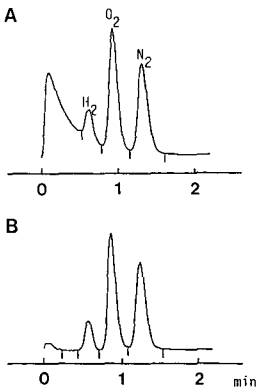

Fig. 2. Gas chromatograms of hydrogen, oxygen and nitrogen obtained with (A) the conventional and (B) the present systems. Sampling pressure: 744 mmHg. GC conditions as in Table I.

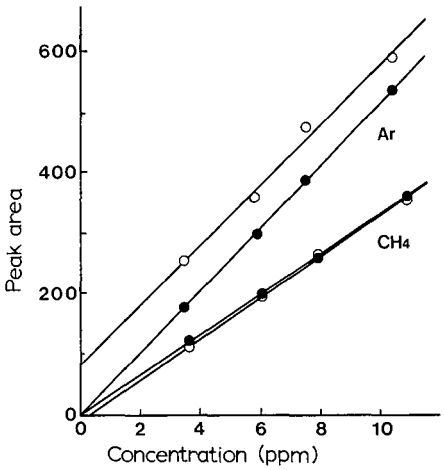

Fig. 3. Calibration graphs obtained with (○) the conventional and (●) the present systems. Sampling pressure: *ca.* 760 mmHg. GC conditions as in Table I.

ducibility (relative standard deviation, $n=8$) was 2.70% at a level of 4.7 ppm of oxygen in helium and was half of that with the conventional system.

The present system was found to be useful and reliable for the analysis of gas samples, taken under reduced pressure, with a gas chromatograph equipped with a packed column and a flow-sensitive detector such as a thermal conductivity detector.

REFERENCES

1 C. J. Cowper and A. J. DeRose, *The Analysis of Gases by Chromatography (Pergamon Series in Analytical Chemistry*, Vol. 7), Pergamon Press, Oxford, 1983.
2 S. G. Kulkarni, G. A. Rama, V. K. Manchada and P. R. Natarajan, *J. Chromatogr. Sci.* 23 (1985) 68.
3 T. J. Bruno, *J. Chromatogr. Sci.*, 23 (1985) 325.
4 H. Niehuis and M. Sevens, *J. Chromatogr.*, 94 (1974) 276.

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CHROM. 21 532

Note

Flavonoid aglycones identified by gas chromatography–mass spectrometry in bud exudate of *Populus balsamifera*

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Poplar bud exudate is a complex mixture which includes terpenoids, free and substituted benzoic and phenolic acids and their esters, and flavonoid aglycones[1–3]. The flavonoids of poplar bud exudate can provide a guide to species identification[4] and even clones which are difficult to distinguish morphologically (although known to be genetically different) can be correctly identified by analysis of their bud exudate[5].

We wish to apply analysis of bud exudate to the chemotaxonomy of the genus *Populus* and to use such analyses to determine the interrelationship of species and hybrids. During our preliminary analyses of poplar bud exudates by gas chromatography–mass spectrometry (GC–MS) we found a number of compounds which were previously unknown in poplars[1–3] and here report the flavonoids of *P. balsamifera* L. (or, *P. tacamahaca* Mill.) which are unusual in having dihydrochalcones as a major constituent.

Previous work on poplar bud exudate has identified a number of flavone and flavanone aglycones[1,4,6–8], but in *P. balsamifera* we find these to be minor components, the chalcones and dihydrochalcones predominating. We are aware of reports of several chalcones in *Populus*[1,4,9–11] but can find only a single dihydrochalcone, 2′,6′-dihydroxy-4′-methoxydihydrochalcone, reported[4,10,11].

We here identify five chalcones and six dihydrochalcones. Of these, three chalcones and five dihydrochalcones have not, we believe, been previously identified in *Populus* bud exudate, though most occur as aglycones or glycosides in other plant genera[12–14]. The technique of GC–MS has not been previously applied successfully to the resolution of such a complex mixture of chalcones and dihydrochalcones.

EXPERIMENTAL

Reagents and materials

Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was obtained from Sigma (Dorset, U.K.). Ethyl acetate was Mallinckrodt (St. Louis, CA, U.S.A.) Nanograde. Flavonoid standards were either purchased from Apin Chemicals (Abingdon, U.K.) or from Plantech U.K. (Reading, U.K.), or provided as a gift by Professor E. Wollenweber (Darmstadt, F.R.G.).

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Poplar bud exudate

Bud exudate was obtained from two specimens (No. 347 and No. 349) of *P. balsamifera* at Alice Holt Lodge, Forestry Commission, Farnham, U.K. No. 347 originated from Ontario, Canada and No. 349 from Oscoda County, MI, U.S.A. Both derived from material provided by Professor Scott Pauley.

Sample preparation and GC–MS

These were carried out as previously described[2].

RESULTS

Analysis by GC–MS allowed the separation (Fig. 1a) and identification of the compounds in bud exudate. These compounds included a number of terpenoids, substituted benzoic and phenolic acids and their esters, a series of chalcones and dihydrochalcones, flavones and flavanones (Fig. 1b; Tables I and II) which were identified as their trimethylsilyl (TMS) derivatives by comparison of their GC and MS characteristics with those of known reference standards. The chalcones chroma-

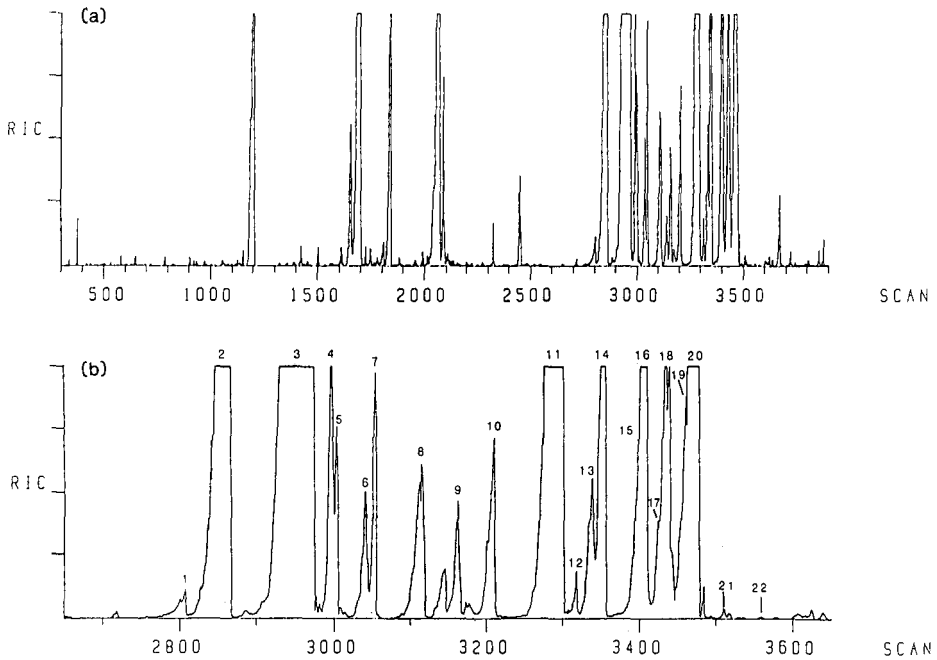


Fig. 1. (a) Reconstructed ion chromatogram (RIC) of bud exudate from *Populus balsamifera* No. 349, scans 300–3900 (MU 11–31). The peak at 1200 is *trans*-cinnamic acid mono-TMS, that at 2070 is *trans*-4-coumaric acid bis-TMS. Other peaks prior to 2650 scans are mostly terpenoids. (b) scans 2650–3650 (MU 22.6–29.4), which contain all the major flavonoid peaks listed in Table I. In addition to the flavonoids the following are identified: *trans*-cinnamyl-*trans*-cinnamate (1); benzyl-*trans*-4-coumarate mono-TMS (5); phenylethyl-*trans*-4-coumarate mono-TMS (8); *trans*-cinnamyl-*trans*-4-coumarate mono-TMS (20); and *trans*-4-coumaryl-*trans*-cinnamate mono-TMS? (18). We cannot confirm the identification of (18) as we do not have the trans-4-coumaryl alcohol for the required synthesis.

TABLE I

SUMMARY OF THE MAJOR CONSTITUENTS OF *P. BALSAMIFERA* BUD EXUDATE

	% Total ion current[a]	
	No. 347	*No. 349*
Substituted benzoic acids and their esters	< 0.1	< 0.1
Substituted phenylpropenoic[b] acids and their esters	8.9	15.9
Chalcones	3.7	3.3
Dihydrochalcones	63.3	49.3
Flavones	4.5	5.4
Flavanones	2.4	2.0
Terpenoids[c]	12.7	11.2

[a] The total ion current generated depends on the characteristics of the compound concerned and is not a true quantitation (see Greenaway *et al.*[1]).

[b] These are cinnamic acid together with coumaric acid and its esters. Other substituted phenylpropenoic acids (*e.g.* caffeic) and their esters are virtually absent.

[c] Whereas there are over 40 terpenoid peaks, more than 50% of the terpenoid content is represented by a single peak, which is probably bisabolol (see also ref. 15).

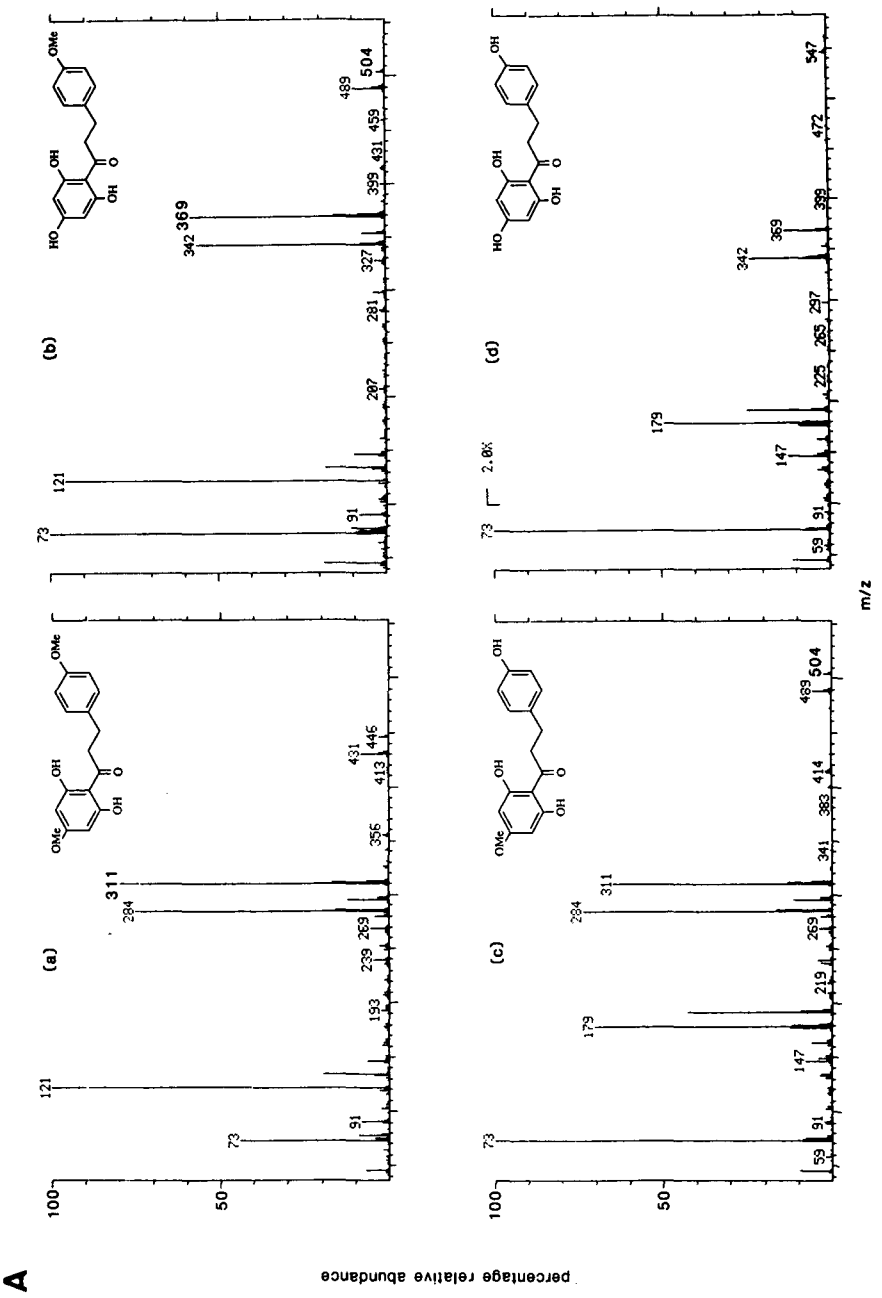
tographed about 0.5–1.0 methylene units (MU) after the corresponding dihydrochalcones (Fig. 1b; Table I).

The mass spectra of phloretin [synonyms: 2′,4′,6′,4-tetrahydroxydihydrochalcone, 2′,4′,6′-trihydroxy-3(4-hydroxyphenyl)propiophenone], peak 16 in Table I, 2′,4′,6′-trihydroxy-4-methoxydihydrochalcone (11)[a], 2′,6′,4-trihydroxy-4′-methoxydihydrochalcone (14) and 2′,6′-dihydroxy-4′,4-dimethoxydihydrochalcone (10) are shown as their TMS derivatives in Fig. 2A, and the spectra of 2′,6′-dihydroxy-4′-methoxychalcone (4) and 2′,4′,6′-trihydroxychalcone (7) are shown together with those of their corresponding dihydrochalcones, as their TMS derivatives, in Fig. 2B.

Whereas chalcones can form from their corresponding flavanones during preparation of TMS derivatives[1], we do not believe that this has happened here, as we do not find the chalcone corresponding to pinobanksin (9), which would be expected if conversion to chalcones occurred. Additionally we have no reason to believe that dihydrochalcones can form from chalcones during sample preparation or chromatography and note that these compounds chromatograph as TMS derivatives more successfully than do the flavones and flavanones (see ref. 1).

The buds of both specimens of *P. balsamifera* analysed here have a very similar flavonoid composition (Table I); we find the same pattern in bud exudate which we have analysed from other specimens of *P. balsamifera*.

[a] These numbers refer throughout to peak numbers in Table I and Fig. 1b.


A
percentage relative abundance
m/z
(a)
(b)
(c)
(d)
100
50
73
121
91
311
284
369
342
504
489
179
147
59
2.0%

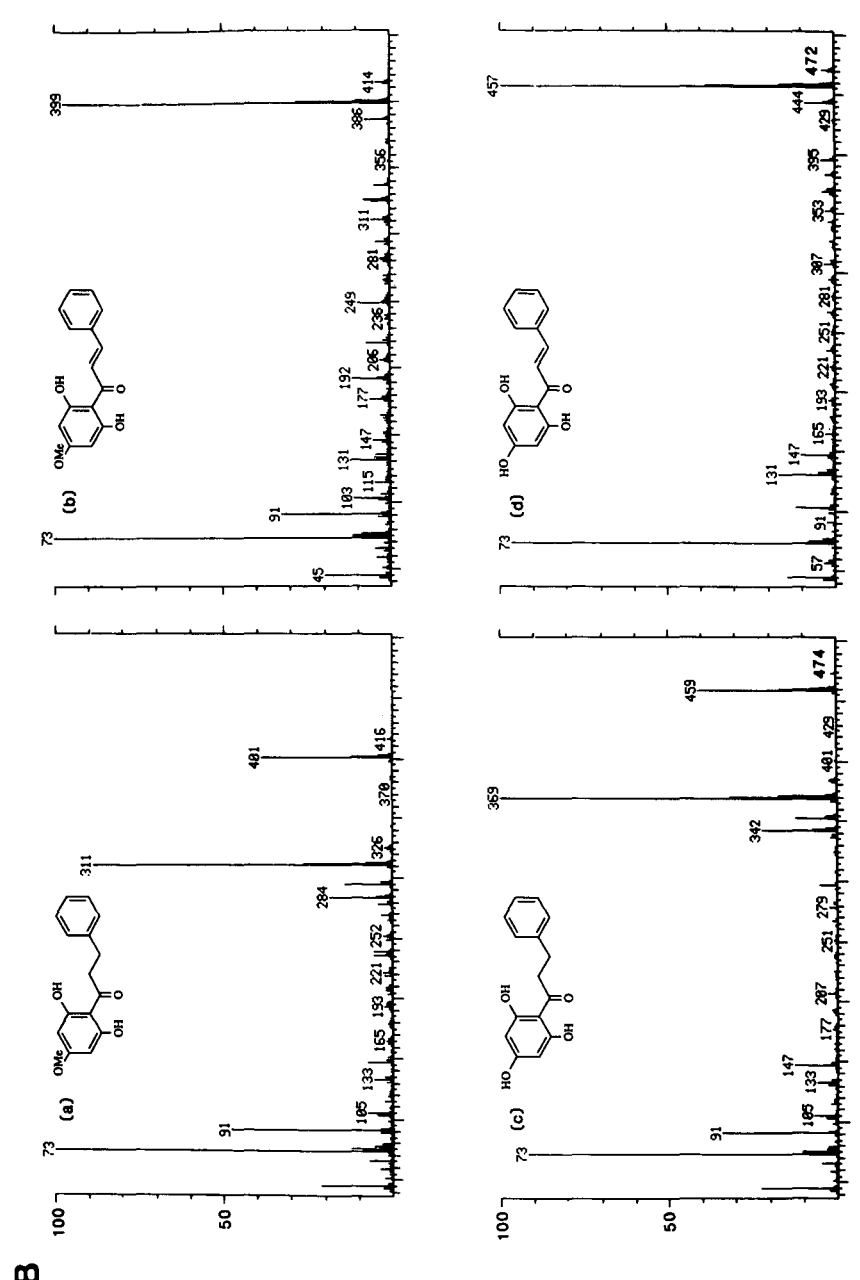


Fig. 2. (A) Mass spectra recorded at 70 eV of 2′,6′-dihydroxy-4′,4-dimethoxydihydrochalcone bis-TMS $[M]^+$ m/z = 446 (a); 2′,4′,6′-trihydroxy-4-methoxydihydrochalcone tris-TMS $[M]^+$ m/z = 504 (b); 2′,6′,4-trihydroxy-4′-methoxydihydrochalcone tris-TMS $[M]^+$ m/z = 504 (c); 2′,4′,6′,4-tetrahydroxydihydrochalcone (phloretin) tetra-TMS $[M]^+$ m/z = 562 (d). The spectrum of phloretin was recorded from a standard, as phloretin cochromatographs with 3,5,7-trihydroxyflavone tris-TMS in bud exudate. All other spectra are recorded from bud exudate. (B) Mass spectra recorded at 70 eV of 2′,6′-dihydroxy-4′-methoxydihydrochalcone bis-TMS $[M]^+$ m/z = 416 (a); 2′,6′,-dihydroxy-4′-methoxychalcone bis-TMS $[M]^+$ m/z = 414 (b); 2′,4′,6′-trihydroxydihydrochalcone tris-TMS $[M]^+$ m/z = 474 (c); 2′,4′,6′-trihydroxychalcone tris-TMS $[M]^+$ m/z = 472 (d).

TABLE II

FLAVONOIDS IDENTIFIED IN *P. BALSAMIFERA* BUD EXUDATE

Peak numbers correspond to those given in the chromatogram shown in Fig. 1b. GC retention times in methylene units (MU; defined by Dalgliesh *et al.*[16]) are given to two decimal places to indicate the elution sequence of peaks which chromatograph closely. Factors such as concentration of the compound concerned, together with the characteristics of a particular GC column are liable to affect the chromatography and for general purposes the MU figures are probably reliable to only a single decimal place.

Peak No.	*Compound*	*No. TMS*	*Retention Time (MU)*	*% Total ion-current*	
				No. 347	*No. 349*
2	2',6'-Dihydroxy-4'-methoxydihydrochalcone	2	23.78	7.8	7.6
3	2',4',6'-Trihydroxydihydrochalcone[a]	3	24.41	32.8	25.0
4	2',6'-Dihydroxy-4'-methoxychalcone	2	24.53	2.8	2.3
6	5,7-Dihydroxyflavanone	2	24.92	1.1	0.8
7	2',4',6'-Trihydroxychalcone	3	24.99	0.9	1.0
9	3,5,7-Trihydroxyflavanone[b]	3	25.77	0.5	0.6
10	2',6'-Dihydroxy-4',4-dimethoxydihydrochalcone[a]	2	26.10	2.5	1.2
11	2',4',6'-Trihydroxy-4-methoxydihydrochalcone[a]	3	26.65	15.4	12.3
12	3,5,7-Trihydroxyflavone[c]	2	26.83	0.1	0.1
13	5,7-Dihydroxyflavone	2	27.04	1.6	1.0
14	2',6',4-Trihydroxy-4'-methoxydihydrochalcone[a]	3	27.12	4.6	3.1
15	3,5,7-Trihydroxyflavone[c]	3	27.38	1.2	4.3
16	2',4',6',4-Tetrahydroxydihydrochalcone[a]	4	27.40	0.2	0.1
17	5,7-Dihydroxy-4'-methoxyflavanone[a]	2	27.52	0.8	0.6
19	2',4',6'-Trihydroxy-4-methoxychalcone[a]	3	27.77	<0.1	<0.1
21	2',6',4-Trihydroxy-4'-methoxychalcone[a]	3	28.10	<0.1	<0.1
22	2',4',6',4-Tetrahydroxychalcone[a]	4	28.54	<0.1	<0.1

[a] We are not aware of previous identifications of this compound in poplar bud exudates.

[b] We have previously found this compound in *Populus* X *euramericana* bud exudate (peak 71)[1], but could not identify it.

[c] 3,5,7-Trihydroxyflavone (galangin) was seen as both the bis-TMS and the tris-TMS derivatives.

DISCUSSION

We report here two series of chalcones and dihydrochalcones. Of these the methoxychalcones are presumably derived by simple methylation of other components: from 2',4',6',4-tetrahydroxychalcone [naringenin chalcone (22)] giving 2',4',6'-trihydroxy-4-methoxychalcone (19) and 2',6',4-trihydroxy-4'-methoxychalcone (21); and from 2',4',6'-trihydroxychalcone [pinocembrin chalcone (7)] giving 2',6'-dihydroxy-4'-methoxychalcone (4). The methoxydihydrochalcones could be derived either by methylation of phloretin or by reduction of the corresponding methoxychalcone. Thus, for example, either methylation of the 4-hydroxy group of phloretin [2',4',6',4-tetrahydroxydihydrochalcone (16)] or reduction of 2',4',6'-trihydroxy-4-methoxychalcone (19) would produce 2',4',6'-trihydroxy-4-methoxydihydrochalcone [4-O-methylphloretin (11)].

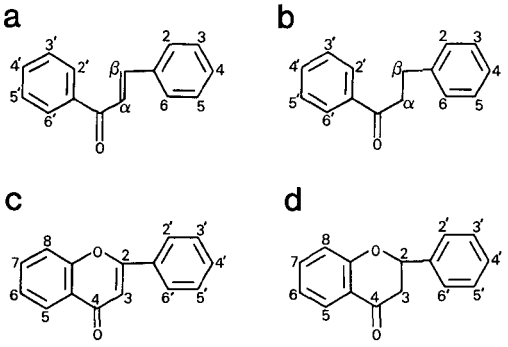


Fig. 3. Ring structure of chalcone (a), dihydrochalcone (b), flavone (c) and flavanone (d). The chalcone equivalent to a flavanone has a 2'-hydroxy substitution; thus 2',4',6'-trihydroxychalcone (7) is the chalcone corresponding to 5,7-dihydroxyflavanone (6).

We have previously analysed[1] the bud exudate of *Populus* X *euramericana* (Dode) Guinier, a hybrid between *P. deltoides* and *P. nigra*[17]. The bud exudates of *P.* X *euramericana* (Section *Aigeiros*) and *P. balsamifera* (Section *Tacamahaca*) are similar in that both contain similar classes of chemicals. Their percentage composition is however very different.

The content of terpenoids is high is *P. balsamifera* (> 10%, Table I) but low in *P.* X *euramericana* (< 0.1%)[1] and this may correlate with the strong and characteristic smell which *P. balsamifera* produces. Both species contain similar overall amounts of substituted benzoic and phenylpropenoic acids and their esters but their relative distributions are different. Thus *P.* X *euramericana* contains predominantly benzoic acid, together with caffeic acid and its esters[1,3], whereas in *P. balsamifera* these compounds are essentially lacking, cinnamic and coumaric acids and their esters predominating.

The major difference, however, is seen in the flavonoid composition. The dihydrochalcones, which form 50% or more of the total bud exudate of *P. balsamifera*, represent < 0.1% of *P.* X *euramericana* bud exudate[1]. This series of dihydrochalcones, which may be typical of *P. balsamifera* or may occur throughout the Section *Tacamahaca*, is reported here for the first time from poplars.

In *P. balsamifera* then we see high levels of dihydrochalcones. Chalcones will be converted to flavanones if chalcone isomerase is present and active. However, if this enzyme is absent or suppressed chalcones can accumulate and dihydrochalcones can be formed from them by reduction of the chalcone α,β-double bond (Fig. 3). In *P. balsamifera* we suggest that chalcone isomerase has low activity resulting in some accumulation of chalcones and the consequent production of the dihydrochalcones which we report here.

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REFERENCES

1 W. Greenaway, T. Scaysbrook and F. R. Whatley, *Proc. R. Soc. London, Ser. B*, 232 (1987) 249.
2 W. Greenaway, E. Wollenweber, T. Scaysbrook and F. R. Whatley, *J. Chromatogr.*, 448 (1988) 284.
3 W. Greenaway, E. Wollenweber, T. Scaysbrook and F. R. Whatley, *Z. Naturforsch. Biosci.*, 43C (1988) 795.
4 E. Wollenweber, *Biochem. Syst. Ecol.*, 3 (1975) 35.
5 W. Greenaway, J. Jobling and T. Scaysbrook, *Silvae Genet.*, 38 (1989) 28.
6 E. Wollenweber and K. Egger, *Phytochemistry*, 10 (1971) 225.
7 E. Nágy, V. Pápay, Gy. Litkei and Z. Dinya, in L. Farkas, M. Gabór and F. Kállay (Editors), *Flavonoids and Bioflavonoids 1985, Proceedings of the 7th Hungarian Bioflavonoid Symposium, Szeged, 16–18 May, 1985* (*Studies in Organic Chemistry*, Vol. 23), Elsevier, Amsterdam, 1986, p. 223.
8 V. Pápay, L. Tóth, M. Soltész, E. Nágy and Gy. Litkei, in L. Farkas, M. Gábor and F. Kallay (Editors), *Flavonoids and Bioflavonoids 1985, Proceedings of the 7th Hungarian Bioflavonoid Symposium, Szeged, 16–18 May, 1985* (*Studies in Organic Chemistry*, Vol. 23), Elsevier, Amsterdam, 1986, p. 233.
9 E. Wollenweber and W. Weber, *Z. Pflanzenphysiol.*, 69 (1973) 125.
10 A. Goris and H. Canal, *C.R. Acad. Sci.*, 121 (1935) 1435.
11 A. Goris and H. Canal, *C.R. Acad. Sci.*, 121 (1935) 1520.
12 J. B. Harborne, T. J. Mabry and H. Mabry (Editors), *The Flavonoids*, Chapman and Hall, London, 1975.
13 J. B. Harborne and T. J. Mabry (Editors), *The Flavonoids: Advances in Research*, Chapman and Hall, London, 1982.
14 J. B. Harborne (Editor), *The Flavonoids: Advances in Research since 1980*, Chapman and Hall, London, 1988.
14 B. R. Mattes, T. P. Clausen and P. B. Reichardt, *Phytochemistry*, 26 (1987) 1361,
16 C. E. Dalgliesh, E. C. Horning, M. G. Horning, K. L. Knox and K. Yarger, *Biochem. J.*, 101 (1966) 792.
17 M. Viart (Editor), *Poplars and Willows in Wood Production and Land Use*, Food Agricultural Organization of the United Nations, Rome, 1979, p. 34.

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Note

High-performance liquid chromatography of mercury and phenylmercury as the N-disubstituted dithiocarbamate complexes

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Phenylmercury (PhHg) salts are frequently used as antibacterial preservatives in pharmaceutical products such as eye drops[1] and an assay for PhHg in these products by high-performance liquid chromatography (HPLC) of the morpholinedithiocarbamate (MDTC) complex has been reported from these laboratories[2]. This assay can be succesfully applied to most pharmaceutical products. However, in recent studies on the stability of PhHg salts in the presence of sodium metabisulphite, which is sometimes included in products as an antioxidant, it has been found that the PhHg is degraded to inorganic mercury[3] and that the divalent $Hg(MDTC)_2$ co-elutes with the PhHgMDTC. Simple modification of the mobile phase failed to affect resolution of the two complexes and studies have been undertaken to investigate alternate derivatives to overcome this problem.

The quantitation of heavy metals by HPLC using a diverse range of N-disubstituted dithiocarbamate (DTC) complexing agents has been widely reported in recent years[4,5]. However, little has been published on the relationship between chemical structure of the DTC complexing agents and the chromatographic characteristics of the resultant complexes[6–8]. The separation and quantitation of mercury and organomercury compounds has received considerable attention, the usual complexing agent chosen being diethylaminedithiocarbamate (DEADTC)[9–13]. The resolution of metal ions of differing valency should be particularly amenable to modification of the complexing agent resulting in a differential change in polarity of the complexes. This paper reports the influence of changing the polarity of the DTC on the chromatographic characteristics of Hg^{2+} and $PhHg^{+}$ complexes.

EXPERIMENTAL

General reagents and chemicals

PhHg nitrate was obtained from BDH (Poole, U.K.) and the methanol and acetonitrile were HPLC-grade from Mallinckrodt (Melbourne, Australia). The dimethylamine, diethylamine, morpholine, pyrrolidine, piperidine and carbon disulphide were obtained from BDH or Ajax Chemicals (Sydney, Australia) and redistilled prior to use.

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Complexing reagents

The corresponding amine salts of the N-disubstituted DTC acids were prepared by the slow addition of carbon disulphide to a two-fold excess of the amine by previously reported methods[2] and stored under refrigeration. The complexing reagents were prepared by dissolving 50 mg of the salts in the minimum quantity of water (always less than 5 ml) and making to 100 ml with HPLC-grade methanol or acetonitrile as appropriate. The complexes were prepared by a 1:1 addition of these reagents to a $2 \cdot 10^{-4}$ *M* solution of either PhHg nitrate or Hg nitrate.

Chromatographic equipment and conditions

The liquid chromatograph consisted of a pump and variable-wavelength detector (LC-3, Pye-Unicam, Cambridge, U.K.), 20-μl loop injector (Rheodyne 7125, Cotati, CA, U.S.A.), integrating recorder (Hewlett-Packard 3380 A, Palo Alto, CA, U.S.A.) and a μBondapak C_{18} column (30 cm × 3.9 mm I.D., 10 μm particle size) (Waters Assoc. Sydney, Australia). All HPLC solvents incorporated $1 \cdot 10^{-4}$ *M* disodium EDTA and operated at a flow-rate of 1.5 ml min^{-1}. The monitoring wavelength was 258 nm.

Determination of capacity and separation factors

Capacity factors (k') were determined by duplicate injections of compounds and calculated by the formula: $k' = (t - t_o)/t_o$; and separation factors (α) by the formula: $\alpha = (t_2 - t_o)/(t_1 - t_o)$, where t is the retention time of the substance, t_1 the retention time of PhHg, t_2 the retention rime of Hg, and t_o the retention time of sodium nitrate, all determinations being made at a flow-rate of 1.5 ml min^{-1}.

RESULTS AND DISCUSSION

The capacity factors (k') for $PhHg^+$ and Hg^{2+} complexes derived from a diverse range of DTCs —dimethylamine (DMADTC), morpholine (MDTC), pyrrolidine (PYDTC), piperidine (PIDTC) and diethylamine (DEADTC)— have been determined for practical concentrations of 65–85% methanol in water and 55–80% acetonitrile in water as mobile phases. In some instances dilution of the sample with methanol based or acetonitrile based derivatising reagent resulted in the precipitation of the complexes from solution (consisting of approximately 50% acetonitrile or methanol in water) (Table I). For the sparingly soluble complexes the retention characteristics were determined using $2 \cdot 10^{-5}$ *M* $PhHg^+$ or Hg^{2+}. At the relatively high concentration of $PhHg^+$ used in eye drop formulations only PYDTC presented problems of solubility. Only DEADTC afforded soluble derivatives with both $PhHg^+$ and Hg^{2+} in both acetonitrile–water and methanol–water mobile phases. Numerous studies have been performed demonstrating the relationship between log k' derived from reversed-phase HPLC and calculated or experimentally derived log P values, where P is the octanol–water partition coefficient[14–16]. A similar relationship should exist for the complexes. Table IIA lists the correlation coefficients obtained from the linear plots of log k' and the log P of the amines from which the DTCs are derived, which serve as relative measures of the hydrophobicity of the resultant complexes. Correlation coefficients > 0.94 were obtained for all combinations of ion and solvent composition.

TABLE I

SOLUBILITY OF DITHIOCARBAMATE COMPLEXES FOLLOWING ADDITION OF REAGENT

Dithiocarbamate substituent	*Solvent*			
	Methanol		*Acetonitrile*	
	$PhHg^+$	*Hg^{2+}*	*$PhHg^+$*	*Hg^{2+}*
Dimethylamine	Soluble	Insoluble	Soluble	Insoluble
Morpholine	Soluble	Insoluble	Soluble	Insoluble
Pyrrolidine	Insoluble	Insoluble	Soluble	Insoluble
Diethylamine	Soluble	Soluble	Soluble	Soluble
Piperidine	Soluble	Insoluble	Soluble	Soluble

As the Hg^{2+} forms divalent complexes with DTCs, whereas $PhHg^+$ forms only monovalent complexes, the log k' for Hg^{2+} would be influenced to twice the extent of $PhHg^+$ by change in hydrophobicity of the side-chain groups. This is demonstrated in Table IIB where linear relationships are demonstrated between the two sets of data of slope close to 2.00 and with high correlation coefficients (> 0.99). As expected, the separation factors (α) are far more influenced by relative hydrophobicities of the DTC complexes than modification of solvent composition, due to the differing valency of

TABLE II

RELATIONSHIPS BETWEEN LOG CAPACITY FACTORS (k') AND LOG P FOR $PhHg^+$ AND Hg^{2+} COMPLEXES

Log P values used in calculations: morpholine, -1.08[17]; diethylamine, $+0.55$[18]; piperidine, $+0.85$[18]; dimethylamine, -0.49; pyrrolidine, $+0.32$. Dimethylamine and pyrrolidine values were calculated from the values of diethylamine and piperidine respectively, using the method of Rekker[18].

Percent modifier	*(A) Correlation coefficients of lines through log k' versus log P of corresponding amines*		*(B) Correlation coefficients and slopes of lines through log k' Hg^{2+} versus log k' $PhHg^+$*	
	$PhHg^+$	*Hg^{2+}*	*Slope*	*Correl. coeff.*
Methanol				
65	0.955	0.948	1.944	0.9969
70	0.966	0.967	1.945	0.9977
75	0.960	0.967	1.965	0.9970
80	0.965	0.971	1.972	0.9980
85	0.994	0.998	1.886	0.9975
Acetonitrile				
55	0.978	0.972	1.978	0.9992
60	0.969	0.971	1.975	0.9996
65	0.966	0.974	1.977	0.9983
70	0.961	0.973	2.046	0.9979
75	0.967	0.969	1.984	0.9989
80	0.967	0.974	1.896	0.9919

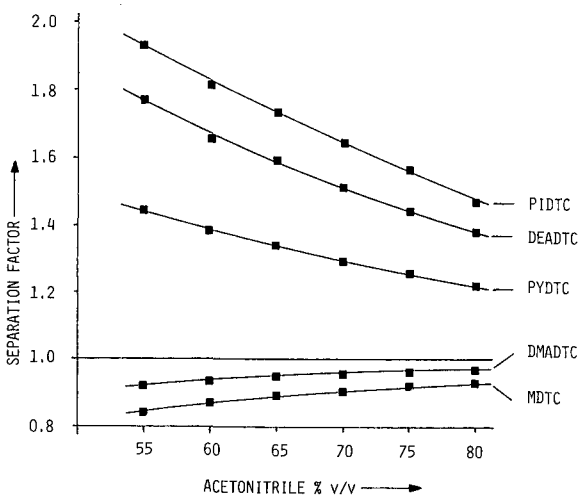

Fig. 1. Relationship between separation factor (α) and the acetonitrile content of the mobile phase and the nature of the dithiocarbamate complex. MDTC = morpholinedithiocarbamate; DMADTC = dimethylaminedithiocarbamate; PYDTC = pyrrolidinedithiocarbamate; DEADTC = diethylaminedithiocarbamate, and PIDTC = piperidinedithiocarbamate.

the ions (Figs. 1 and 2), the separation being improved by the use of more hydrophobic DTC complexing agents. For the quantitation of $PhHg^+$ in eye drops, any of the investigated DTC complexing agents would serve, provided no Hg^{2+} is present, with the exception of PYDTC, in which the complex is sparingly soluble in the derivatising solvent. For samples containing both $PhHg^+$ and Hg^{2+} the DEADTC would appear to be the best DTC agent due to its excellent separation and solubility of both com-

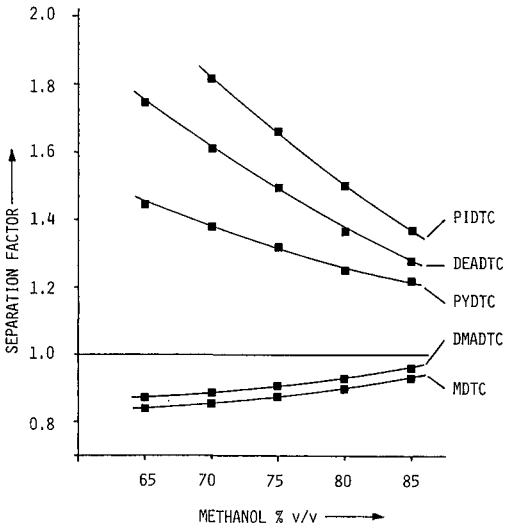

Fig. 2. Relationship between separation factor (α) and the methanol content of the mobile phase and the nature of the dithiocarbamate complex.

plexes in the mobile phases. The use of alternative DTC agents should enable separations of $PhHg^+$ and eye drop components to be achieved where the previously reported MDTC agent fails to resolve the $PhHg^+$ complex from other components in the formulation.

REFERENCES

1 *The Pharmaceutical Codex*, The Pharmaceutical Press, London, 11th ed., 1979.
2 J. E. Parkin, *J. Chromatogr.*, 407 (1987) 389.
3 J. E. Parkin, unpublished results.
4 J. W. O'Laughlin, *J. Liq. Chromatogr.*, 7 (1984) 127.
5 B. Steinbrech, *J. Liq. Chromatogr.*, 10 (1987) 1.
6 J. Lehotay, O. Liška, E. Brandšteterová and G. Guichon, *J. Chromatogr.*, 172 (1979) 379.
7 O. Liška, J. Lehotay, E. Brandšteterová, G. Guiochon and H. Colin, *J. Chromatogr.*, 172 (1979) 384.
8 O. Liška, G. Guiochon and H. Colin, *J. Chromatogr.*, 171 (1979) 145.
9 O. Evans and G. D. McKee, *Analyst (London)*, 113 (1988) 243.
10 W. Langseth, *J. Chromatogr.*, 438 (1988) 414.
11 S. Inoue, S. Hoshi and M. Mathubara, *Talanta*, 32 (1985) 44.
12 L. H. J. Lajunen, E. Eijarvi and P. Niemi, *Finn. Chem. Lett.*, 6 (1984) 146; *C.A.*, 102 (1985) 142433 (s).
13 S. Inoue, S. Hoshi and M. Sasaki, *Bunseki Kagaku*, 31 (1982) E243; *C.A.*, 97 (1982) 174195 (k).
14 C. F. Carney, *J. Liq. Chromatogr.*, 8 (1985) 2781.
15 R. Kaliszan, *J. Chromatogr. Sci.*, 22 (1984) 362.
16 M. D'Amboise and T. Hanai, *J. Liq. Chromatogr.*, 5 (1982) 229.
17 A. Leo, C. Hansch and D. Elkins, *Chem. Rev.*, 71 (1971) 525.
18 R. F. Rekker, *The Hydrophobic Fragmental Constant*, Elsevier, Amsterdam, 1977.

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CHROM. 21 476

Note

Liquid chromatographic separation and behaviour of some triazines on styrene–divinylbenzene columns

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Derivatives of *s*-triazines are widely employed in agriculture as effective herbicide components for weed control in food crops, and their determination is therefore important. A common method for determining triazines is gas chromatography, but it is not suitable for some of the higher molecular weight compounds and those with polar groups. High-performance liquid chromatography (HPLC) seems an attractive alternative as triazines absorb strongly in the UV region. Both reversed-phase[1–5] and normal-phase applications[6–8] have been reported.

In the last decade, styrene–divinylbenzene resins have been applied in HPLC and in previous papers[9–11] the preparation and characterization of polymeric columns were reported. The aim of this study was to investigate the elution behaviour of some *s*-triazines with different substituents at positions 2, 4 or 6 on PRP-1 and Porapak Q polymeric laboratory-prepared columns. The influence of the mobile phase pH on retention data and the relationships between capacity factors (k') and mobile phase composition (φ) were investigated using water–acetonitrile eluents. The results are discussed from the point of view of the application of such materials to the preconcentration and determination of *s*-triazines in water.

EXPERIMENTAL

The chromatographic measurements were carried out on a Spectra-Physics (San Jose, CA, U.S.A.) Model 8700 liquid chromatograph, equipped with a Uvidec-100 UV spectrophotometer (Jasco, Tokyo, Japan) set at 220 nm. This wavelength was determined experimentally to be the optimum for all triazines tested. Chromatograms were recorded on a Spectra-Physics SP 4270 integrator. Samples were introduced with a Rheodyne Model 7125 injector (Spectra-Physics), equipped with a 10-μl sampling loop. HPLC-quality acetonitrile (Rudi Pont, Eurobase, Milan, Italy) and deionized, distilled water were filtered through a Millipore 0.45-μm filter and degassed with a helium purge. The pH was adjusted with 0.01 *M* phosphoric acid and 0.01 *M* potassium dihydrogen- or sodium monohydrogenphosphate. The reported pH values are the pH of the solution before the addition of acetonitrile. Solvent mixtures are expressed as percent by volume.

The solutes (Table I) (Ciba-Geigy, Basle, Switzerland) were dissolved in

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methanol. Their concentrations were about 1 mg/l and allowed suitable responses from the detector without overloading the column. PRP-1 and Porapak Q columns (150 × 4.6 mm I.D.) were prepared as described previously[11]. Potassium nitrate was used as the unretained solute. All experiments were run in triplicate at a flow-rate of 1 ml/min.

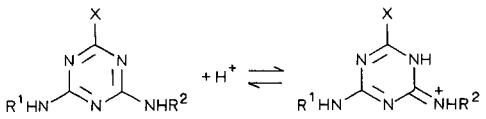
Enrichment experiments were carried out with a commercial PRP-1 cartridge containing about 100 mg of adsorbent. Similar Porapak Q cartridges were prepared in the laboratory. Before use, the traps were washed with 2 ml of acetonitrile and 2 ml of water. A vacuum was applied by a water pump and, after 250–1000 ml of aqueous sample had passed through, *s*-triazines were desorbed passing 1 ml of acetonitrile, which was collected in a glass vial.

The re-usability of the cartridge was investigated by making repeated pre-concentration runs and by restoring the adsorbents with 10 ml of acetonitrile.

RESULTS AND DISCUSSION

All *s*-triazines have similar structures and their physico-chemical properties are determined primarily by the substituent in the 2-position. Aminoalkyl groups in the 4- and 6-positions have a less marked effect. Some characteristics of the studied compounds are given in Table I.

s-Triazines are weak bases and they behave in aqueous solutions according to the equation

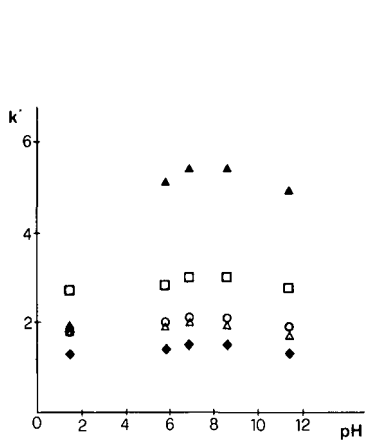


where X = Cl, OCH_3 or SCH_3 and R^1 and R^2 are alkyl groups. One would expect their retention to be affected by a change in the pH of the mobile phase. Consequently, the dependence of the capacity factors on mobile phase pH was evaluated in the pH range 2–11 and the results are shown in Figs. 1 and 2. The pK_a values of individual triazines indicate that the protonation of thiomethyl and methoxy derivatives occurs at pH < 4, while a pH < 2 is required for the protonation of chloro derivatives. Figs. 1 and

TABLE I

CHARACTERISTICS OF *s*-TRIAZINES STUDIES[6]

Compound	*Substituent*			pK_a	*MW*
	X	R^1	R^2		
Propazine	Cl	$CH(CH_3)_2$	$CH(CH_3)_2$	1.85	229.7
Atrazine	Cl	C_2H_5	$CH(CH_3)_2$	1.70	215.7
Simazine	Cl	C_2H_5	C_2H_5	1.90	201.5
Prometryne	SCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	4.05	241.3
Prometone	OCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	4.20	225.3



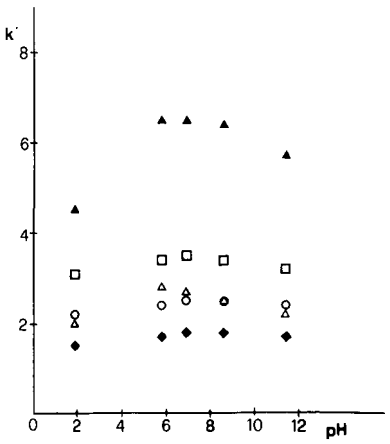


Fig. 1. Plot of k' *vs.* pH of the mobile phase on PRP-1 column. Mobile phase: acetonitrile–water (60:40). ◆ = Simazine; ○ = atrazine; □ = propazine; ▲ = prometrine; △ = prometone.

Fig. 2. Plot of k' *vs.* pH of the mobile phase on Porapak Q column. Mobile phase and symbols as in Fig. 1.

2 indicate that the retention of chlorotriazines is independent of the mobile phase pH, whereas the retention of prometone and prometryne increase with increase in pH up to about pH 7. The behaviour of the compounds studied is similar on the PRP-1 and Porapak Q phases, but the *s*-triazines are more retained on the latter phase. Further, the curvature for prometone is more pronounced on Porapak Q. A pH of 7 was selected as the optimum, as it gives both the highest retention and the best peak symmetry. In acidic mobile phases the peaks of more basic compounds exhibit noticeable tailing.

The relationships between log k' and acetonitrile volume fraction, φ, are shown in Figs. 3 and 4. Again, the retention behaviour of the compounds studied was the same on PRP-1 and Porapak Q phases. *s*-Triazines with the same substituent in positions 4 and 6 showed the retention order $SCH_3 > Cl > OCH_3$. Such a behaviour was observed by Jork and Roth[1] on a μBondapak C_{18} stationary phase with methanol–water as eluent, and Pacáková *et al.*[5] observed that methoxy compounds were more retained than chloro compounds on a Separon SI C_{18} phase. Further, methoxy derivatives could not be separated from chloro derivatives on either of these stationary phases. However, on PRP-1 and Porapak Q polymeric phases, such a preparation is possible at all acetonitrile concentration.

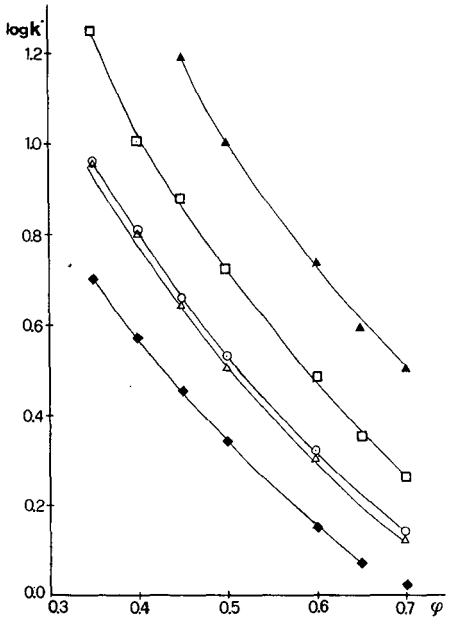
With *s*-triazines with the same substituent in position 2, the retention on polymeric phases increases with increasing number of methylene groups on the amino substituents.

Figs. 3 and 4 show non-linear relationships between log k' and φ. Regression analysis showed that all data points can be fitted to a quadratic expression[12]:

$$\log k' = A\varphi^2 + B\varphi + C$$

The regression parameters and correlation coefficients are given in Table II.

Extrapolation to 0% organic modifier concentration yielded the logarithm of



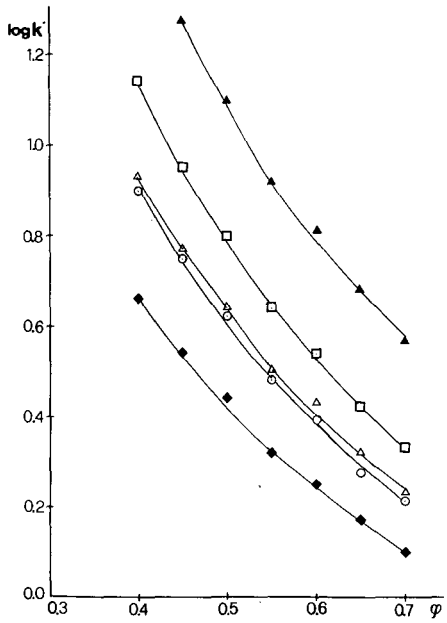


Fig. 3. Plot of log k' *vs.* φ on PRP-1 column. Mobile phase: acetonitrile–water (pH 7). Symbols as in Fig. 1.

Fig. 4. Plot of log k' *vs.* φ on Porapak Q column. Mobile phase and symbols as in Fig. 1.

the capacity factor in pure water (k'_w). The high k'_w values suggest that PRP-1 and Porapak Q materials could be effective in extracting *s*-triazines from water. In fact, herbicides are usually present at concentrations below the detection limit (detector sensitivity), so that a preconcentration step is necessary. The suitability of PRP-1 and Porapak Q for enrichment from aqueous solutions was evaluated by sampling distilled water containing *s*-triazines at concentrations below the detection limit (signal-to-noise ratio = 3), *i.e.*, about 0.5 ng of the tested substance in the volume injected (0.01–0.05 ppm).

TABLE II

REGRESSION PARAMETERS OF THE RELATIONSHIP BETWEEN LOG k' AND φ

Log $k' = A\varphi^2 + B\varphi + C$.

Compound	*PRP-1*				*Porapak Q*			
	A	*B*	*C*	r^a	*A*	*B*	*C*	r^a
Propazine	3.2	−6.2	3.03	0.999	3.7	−6.7	3.20	0.999
Atrazine	2.7	−5.2	2.46	0.999	2.8	−5.4	2.62	0.999
Simazine	2.4	−4.4	1.96	0.999	2.1	−4.2	2.00	0.998
Prometryne	3.7	−7.0	3.60	0.998	3.9	−7.3	3.70	0.997
Prometone	3.4	−6.0	2.65	0.997	2.8	−5.3	2.60	0.997

[a] r = Correlation coefficient.

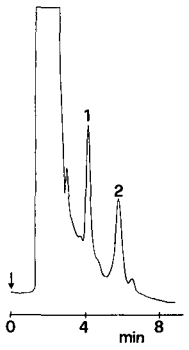


Fig. 5. Chromatogram obtained on sampling on a PRP-1 cartridge 200 ml of tap water containing 0.4 μg/l of atrazine (peak 1) and 0.3 μg/l of propazine (peak 2). Mobile phase: acetonitrile–water (60:40) (pH 7).

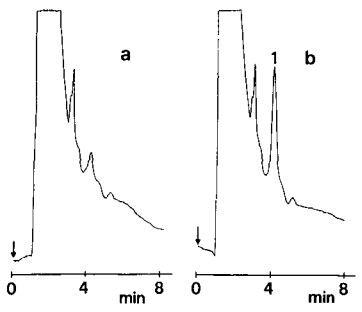


Fig. 6. Chromatograms obtained on sampling on a Porapak Q cartridge 100-ml aliquots of Po river water: (a) unspiked; (b) spiked with 0.8 μg/l of atrazine (peak 1). Mobile phase as in Fig. 5.

Recoveries of herbicides were evaluated by comparing the areas of the peaks obtained for the sample and for a standard solution. The recoveries were 90–100% for all the *s*-triazines and they did not vary with increase in water sample volumes (up to 1000 ml) or increase in herbicide concentration (up to 1 mg/ml). Further, it was observed that after four extractions the adsorbent capacity of polymeric traps had not decreased significantly.

Figs. 5 and 6 show typical chromatograms obtained by this procedure for tap water and Po river water samples, both unspiked and spiked with atrazine and/or propazine.

The results obtained show that polymeric columns could be usefully employed in the analysis of *s*-triazines. Further, such materials could also make it possible to concentrate aqueous solutions of herbicides at parts per 10^{12} levels in a simple manner.

REFERENCES

1 H. Jork and B. Roth, *J. Chromatogr.*, 144 (1977) 39.
2 A. Di Corcia, M. Marchetti and R. Samperi, *J. Chromatogr.*, 405 (1987) 357.
3 N. M. J. Vermeulen, Z. Apostolides, D. J. J. Potgieter, P. C. Nel and N. S. H. Smit, *J. Chromatogr.*, 240 (1982) 247.
4 M. Popl, Z. Voznakova, V. Tatar and J. Strnadova, *J. Chromatogr. Sci.*, 21 (1983) 39.
5 V. Pacáková, K. Štulík and M. Příhoda, *J. Chromatogr.*, 442 (1988) 147.
6 E. Smolkova and V. Pacakova, *Chromatographia*, 11 (1978) 698.
7 P. Dufek and V. Pacáková, *J. Chromatogr.*, 187 (1980) 341.
8 P. Dufek, V. Pacáková and E. Tesařová, *J. Chromatogr.*, 191 (1980) 115.
9 S. Coppi, G. Blo and A. Betti, *J. Chromatogr.*, 388 (1987) 135.
10 S. Coppi, A. Betti and S. Caldari, *J. Chromatogr.*, 395 (1987) 159.
11 S. Coppi, A. Betti, C. Bighi, G. P. Cartoni and F. Coccioli, *J. Chromatogr.*, 442 (1988) 97.
12 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 185 (1979) 179.

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CHROM. 21 471

Note

High-performance liquid chromatographic determination of the herbicide terbutylazine and its dealkylated metabolites in soil

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Terbutylazine (2-*tert*.-butylamino-4-chloro-6-ethylamino-1,3,5-triazine; GS 13529) is a chloro-*s*-triazinic herbicide of the homologous series to which the better known atrazine and simazine belong.

Degradation of *s*-triazines in soil may be either microbiological or physico-chemical; the main degradative mechanisms are photodecomposition, volatilization, hydroxylation and dealkylation[1]. The first two are negligible with terbutylazine, whereas the last two have great importance[2]. Within microbiological mechanisms of degradation, dealkylation seems to be the most significant; oxidative dealkylation has been proved to occur not only by microbial enzymatic systems[1] but also by free-radical reactions[3]. In this process three metabolites are formed: two monoalkyl derivatives and one completely dealkylated compound[4] (Fig. 1); two of these compounds

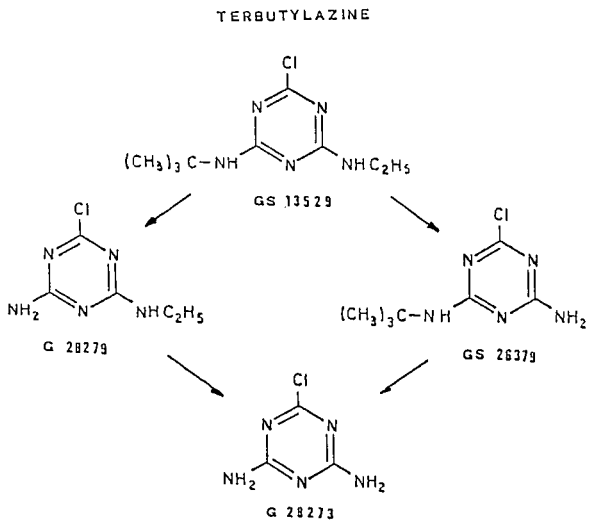

Fig. 1. Metabolic route of terbutylazine in soil.

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(G 28273 and G 28279) originate also from dealkylation of atrazine and simazine. Dealkylation of *s*-triazines has been observed not only in soil but also in higher plants[5,6].

Several methods have been described for the determination of terbutylazine, usually in the presence of other *s*-triazines, including gas chromatography[7–13] and high-performance liquid chromatography (HPLC)[11,13,14–16]; with regard to the study of metabolites, an HPLC method has been reported for the determination of hydroxy-*s*-triazines[17] but, to our knowledge, there is no reference to compounds formed by dealkylation of *s*-triazines.

In this paper, the HPLC separation of terbutylazine from its dealkylated metabolites and their determination in soil are described.

EXPERIMENTAL

Apparatus

A Varian (Palo Alto, CA, U.S.A.) Model 5020 liquid chromatograph was used, fitted with a variable-wavelength UV-100 UV–VIS detector and a Rheodyne injector (50-μl loop). The chromatograph was connected to a Hewlett-Packard 3390 A recorder–integrator.

Chromatography

Merck (Darmstadt, F.R.G.) Hibar RP-8, RP-18 and NH_2 (10 μm) columns (250 × 4.0 mm I.D.) were employed; the mobile phase was water–acetonitrile at various ratios and flow-rates (Table II). The analyses were performed at different wavelengths (Fig. 2), depending on the absorbance maxima previously determined for terbutylazine (221 nm) and its metabolic products G 28273 (205 nm), G 28279 (213 nm) and GS 26379 (213 nm) with a Varian Model DMS 90 UV–VIS spectrophotometer.

Chemicals and materials

Acetonitrile, chloroform, dichloromethane, diethyl ether and methanol were of HPLC grade (Carlo Erba, Milan, Italy); water was distilled twice and filtered through a Milli-Q apparatus (Millipore, Milan, Italy) before use.

Terbutylazine (GS 13529) and its metabolites GS 26379, G 28279 and G 28273 were obtained from Ciba Geigy (Milan, Italy).

TABLE I

PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE SOILS

Characteristic	*Soil*		
	A	*B*	*C*
Sand (%)	82.7	62.5	36.6
Silt (%)	9.0	9.3	27.4
Clay (%)	8.3	28.2	36.0
pH (in water)	6.46	7.67	8.37
Organic matter (%)	1.56	2.10	3.93

Three soils of different physical and chemical characteristics (Table I) were used to set up the extraction procedure.

Extraction procedure

Ten grams of air-dried, finely sieved (< 2 μm) soil were weighed in a 250-ml screw-capped flask, 50 ml of the extraction solvent (chloroform, dichloromethane, diethyl ether or methanol) were added and the mixture was agitated in a flash-shaker (Stuart Scientific) for 30 min. The soil was left to settle and the organic layer was filtered with a PTFE syringe-filter (diameter 25 mm, 0.45 μm) (Alltech, Milan, Italy); a 5-ml aliquot of the filtered extract was transferred into a 20-ml beaker, evaporated to dryness in a thermo-ventilated stove at 50–70°C (depending on the boiling point of the solvent used), the residue was taken up in 1 ml of mobile phase and the solution was injected for HPLC analysis.

RESULTS AND DISCUSSION

In order to separate terbutylazine and its dealkylated metabolites, both normal-phase (NH_2) and reversed-phase (RP-8 and RP-18) columns were employed (Table II). The latter two allowed a good separation of the four compounds with water–acetonitrile (50:50, v/v) as eluent. Under these conditions with the RP-8 column the metabolites were slightly more retained than by the RP-18 column, whereas terbutylazine was less retained. On increasing the water content in the mobile phase, the four compounds were more retained by the column, in the order terbutylazine > GS 26379 > G 28279 > G 28273.

With the normal-phase column (NH_2) and acetonitrile as eluent, the peak elution order was reversed, with the last peak (of the metabolite G 28273) being asym-

TABLE II

RETENTION TIMES OF TERBUTYLAZINE (IV) AND ITS DEALKYLATED METABOLITES (I–III) WITH DIFFERENT COLUMNS AND ELUENTS

Column	*Water–acetonitrile (v/v)*	*Flow-rate (ml/min)*	*Retention time (min)*			
			I	*II*	*III*	*IV*
RP-8	50:50	1.0	2.25	2.86	4.47	8.19
	60:40		2.39	3.30	6.75	16.20
	70:30		2.52	4.06	12.59	–
	80:20		3.09	6.30	–	–
	90:10		4.66	16.45	–	–
	95:5		7.00	–	–	–
RP-18	50:50	1.0	2.01	2.56	4.39	9.68
	60:40		2.07	2.90	6.64	19.58
	70:30		2.48	3.95	14.47	–
	80:20		2.96	5.82	–	–
	90:10		4.10	–	–	–
	95:5		6.85			
NH_2	0:100	0.5	13.83	7.73	6.35	5.40
	1:99		8.45	6.65	5.76	5.26

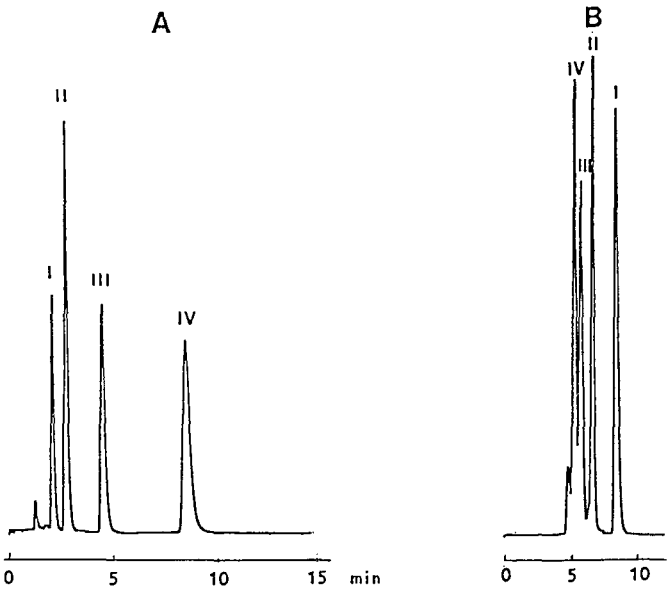

Fig. 2. Chromatography of terbutylazine (IV) and its dealkylated metabolites GS 26379 (III), G 28279 (II) and G 28273 (I). (A) On an RP-8 column: mobile phase, water–acetonitrile (50:50, v/v); flow-rate, 1 ml/min; detection, UV at 210 nm for 6.5 min, then at 220 nm. (B) On an NH_2 column: mobile phase, water–acetonitrile (1:99, v/v); flow-rate, 0.5 ml/min; detection, UV at 215 nm for 7.5 min, then at 205 nm.

metric and not sharp enough. Adding 1% of water to the mobile phase produced a sharp and symmetric peak (Fig. 2), with a considerable decrease in its retention time, whereas a moderate reduction in the retention times of the other three compounds was observed.

TABLE III

RECOVERIES OF TERBUTYLAZINE AND ITS DEALKYLATED METABOLITES WITH DIFFERENT SOLVENTS

Compound	*Fortification level (ppm)*	*Soil*	*Recovery ± R.S.D. (%)*[a]		
			Diethyl ether	*Methanol*	*Dichloro-methane*
G 28273	0.5	A	40.3 ± 1.1	71.6 ± 1.3	6.9 ± 2.4
		B	79.5 ± 3.3	80.8 ± 4.2	25.2 ± 3.6
		C	60.0 ± 1.2	72.8 ± 5.0	12.6 ± 7.1
G 28279	1.0	A	81.4 ± 1.0	80.6 ± 1.3	28.8 ± 5.1
		B	94.0 ± 6.8	84.7 ± 2.4	65.8 ± 2.8
		C	88.1 ± 1.0	77.7 ± 1.7	54.0 ± 3.5
GS 26379	1.0	A	91.1 ± 1.6	69.9 ± 3.6	54.7 ± 6.4
		B	93.8 ± 5.9	60.8 ± 0.1	67.8 ± 4.3
		C	90.7 ± 2.7	63.2 ± 1.4	76.3 ± 4.8
Terbutylazine	1.5	A	94.2 ± 3.0	64.0 ± 2.6	71.1 ± 5.1
		B	91.3 ± 6.1	52.6 ± 0.1	59.0 ± 7.1
		C	86.2 ± 1.7	51.7 ± 1.6	80.9 ± 2.0

[a] Mean values of duplicate analyses from three replicates.

Calibration graphs for each compound were constructed by plotting concentrations against peak areas; good linearities were achieved in the range 0–1.5 ppm, with correlation coefficients between 0.9991 and 0.9997. Under the optimum conditions the detection limit was 0.003 ppm for all compounds.

For recovery assays of terbutylazine and its dealkylkated metabolites three different soils that had never been treated with any pesticide, were used.

The blanks of the extraction solvents (chloroform, dichloromethane, diethyl ether and methanol) did not give any interfering peaks at the retention times of the compounds studied.

With chloroform a very low recovery (< 20%) was achieved for each compound in the three soils. Diethyl ether showed the highest extraction power and, with the exception of the most polar metabolite, G 28273, allowed very good recoveries from the three soils (Table III). In comparison with diethyl ether, methanol gave a poorer extraction of the less polar compounds (terbutylazine and GS 26379) but a better recovery of G 28273. Dichloromethane was less efficient than diethyl ether and methanol and did not allow satisfactory recoveries.

CONCLUSIONS

Considering the extraction percentages, it can be seen that there is no appreciable difference between methanol and diethyl ether as solvents, both giving good recoveries from the three different soils.

The simultaneous separation of terbutylazine and its dealkylated metabolites can be achieved with an RP-8 column and water–acetonitrile (50:50, v/v) as eluent when the concentration of the four compounds in the sample is greater than 0.1 ppm. For lower concentrations, when the presence of interfering compounds is more substantial, it is advisable, mainly for the determination of the metabolites G 28273 and G 28279, to increase the percentage of water in the mobile phase.

The separation on the NH_2 column can be used for the determination of terbutylazine and its metabolites and could be valuable as a confirmatory assay.

REFERENCES

1 L. S. Jordan, W. J. Farmer, J. R. Goodin and B. E. Day, *Residue Rev.*, 32 (1970) 267.
2 G. Rapparini, *I. Diserbanti*, L'Informatore Agrario, Verona, 1986.
3 J. R. Plimmer, P. C. Kearney and J. R. Rowlands, paper presented at the *156th American Chemical Society meeting, Atlantic City, NJ, 1968*, Abstract A47.
4 D. D. Kaufman and P. C. Kearney, *Residue Rev.*, 32 (1970) 235.
5 R. H. Shimabukuro, R. E. Kadunce and D. S. Frear, *J. Agric. Food Chem.*, 14 (1966) 392.
6 R. H. Shimabukuro, *J. Agric. Food Chem.*, 15 (1967) 557.
7 K. A. Ramsteiner, W. D. Hoermann and D. O. Eberle, *J. Assoc. Off. Anal. Chem.*, 57 (1974) 192.
8 R. Bailey, G. LeBel and J. F. Lawrence, *J. Chromatogr.*, 161 (1978) 251.
9 V. Pacáková and I. Němec, *J. Chromatogr.*, 148 (1978) 273.
10 V. Pacáková and H. Kozáková, *J. Chromatogr.*, 154 (1978) 251.
11 M. Popl, Z. Voznakova, V. Tatar and J. Strnadova, *J. Chromatogr. Sci.*, 21 (1983) 39.
12 V. Janda and K. Marha, *J. Chromatogr.*, 329 (1985) 186.
13 Y. Xu, W. Lorenz, G. Pfister, M. Bahadir and F. Korte, *Fresenius' Z. Anal. Chem.*, 325 (1986) 377.
14 P. Dufek and V. Pacáková, *J. Chromatogr.*, 187 (1980) 341.
15 D. J. Subac, *Chromatographia*, 14 (1981) 371.
16 C. E. Parker, C. A. Haney, D. J. Harvan and J. R. Hass, *J. Chromatogr.*, 242 (1982) 77.
17 K. A. Ramsteiner and W. D. Hoermann, *J. Agric. Food Chem.*, 27 (1979) 934.

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Note

Multiresidue method for fungicide residues in fruit and vegetables

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The efficiency and cost-effectiveness of multiresidue methods have prompted their application to a number of pesticides in food. Luke *et al.*[1] have developed a procedure involving acetone extraction followed by partitioning into hexane–dichloromethane which is capable of determining at least 112 compounds in a variety of foods. This method does not require clean-up other than partitioning and relies on gas–liquid chromatography (GLC) using specific detectors to avoid interference by co-extractives. Similarly, Martindale[2] developed a multiresidue method for 21 pesticides on potatoes which employed only a partitioning step for clean-up and a combination of six different chromatographic systems for determination. In a modification of the Luke method, Blaha and Jackson[3] used solvents of increasing polarity in the partitioning step, followed by gel permeation chromatography and GLC to determine 17 organophosphorous pesticides in 41 foods. Andersson and Ohlin[4] also employed the Luke extraction and gel permeation chromatography to determine 126 pesticides in several foods. Quantitative recoveries were obtained using GLC and selective thermionic and flame photometric detection. A different extraction procedure[5] involving the use of dichloromethane and methanol followed by water removal with Extrelut cartridges was used for some compounds such as carbendazim and thiophanate methyl, which were poorly recovered by acetone extraction.

These multiresidue methods involve the use of relatively large volumes of organic solvents in partitioning and clean-up steps and require associated glassware or automated apparatus in the case of gel permeation chromatography. Solid-phase extraction cartridges containing normal- or reversed-phase supports have become available commercially and offer the potential of simplifying the purification of the initial extract as well as reducing the amount of solvent consumed. Successful applications have been found in the determination of N-methyl carbamates in fruits and vegetables[6] and for a variety of pesticides in water[7]. The present study was conducted to examine the use of solid-phase extraction in a multiresidue method for fungicide residues in foods.

EXPERIMENTAL

Instrumentation

GLC was carried out on a Varian 3500 apparatus equipped with a ^{63}Ni elec-

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tron-capture detector, a Model 8035 autosampler, an on-column injector, a 30 m × 0.32 mm I.D. column coated with DB-5 to a film thickness of 0.25 μm, and a Model 650 data system. Helium carrier gas was supplied to the column at a linear velocity of 40 cm/s, and the nitrogen make-up gas flow-rate to the detector was 22 ml/min. The detector was maintained at 300°C. The initial injector temperature was 100°C and after a 0.5-min delay programmed at 100°C/min to 250°C. The initial column temperature was 100°C and after a 0.5-min delay programmed first at 50°C/min to 190°C and then, after 9 min at this temperature, at 30°C/min to 25°C where it was maintained for 5 min to elute high-boiling co-extractives.

High-performance liquid chromatographic (HPLC) separations were performed on a 30 cm × 3.9 mm I.D. column packed with 10-μm μBondapak C_{18} stationary phase. The mobile phase was delivered to the column isocratically at 1.0 ml/min with a Beckman 110B pump connected through a 100-μl injection loop and a Hamilton C_{18} guard column. The effluent was monitored by a Waters Model 440 absorbance detector with a 254-nm filter connected in series to a Schoeffel FS 970 fluorometer set at an excitation wavelength of 302 nm with a KV 370 emission filter.

Reagents

Solvents were purchased from Caledon Labs. (Georgetown, Canada) and were HPLC grade. Standards of the twelve respective fungicides were obtained from the repository of pesticide standards maintained in the Food Research Division and were at least 98% pure. Each compound was weighed accurately and dissolved in acetonitrile to give a stock solution containing approximately 2 mg/ml. A composite spiking solution containing 48 μg/ml was used to spike samples at the 5-ppm level and was prepared by adding 0.6 ml of each stock solution to a 25-ml volumetric flask and making to the mark with acetonitrile. A 1:10 dilution of this composite was used to prepare samples spiked at 0.5 ppm.

Solid-phase extraction was carried out using a cartridge containing 0.5 g of C_{18} stationary phase (Supelco, Bellefonte, PA, U.S.A.). The cartridge was washed before use by rinsing sequentially with 60 ml each of methanol, dichloromethane, again with methanol, and then 30 ml of water. This procedure removed substances which produced background peaks on the gas chromatogram. The extraction cartridges were eluted using a vacuum manifold supplied by the manufacturer and were fitted with detachable reservoirs to contain the sample and elution solvents.

The mobile phase for HPLC separations contained 60% methanol and 40% aqueous buffer prepared by adjusting 0.01 *M* H_3PO_4 to pH 7.0 with aqueous trimethylamine. The mobile phase was supplied to the column at a flow-rate of 1 ml/min.

Analytical procedure

The respective commodities were purchased at a local food store and 500-g samples homogenized in a Waring blender. Subsamples (10 g) were spiked with the composite fungicide standard in acetonitrile (1 ml) to give 0, 0.5 or 5.0 ppm of each compound, mixed, and extracted with acetone (35 ml) by blending on a Polytron homogenizer. The extract was filtered through Whatman No. 1 paper on a Buchner funnel using gentle vacuum and the filtrate transferred to a volumetric flask and brought to 50 ml with acetone. A 10-ml aliquot of extract was diluted to 50 ml with water and added to the reservoir of a pre-washed extraction cartridge.

After passage of the diluted sample extract through the cartridge, the adsorbent was washed with 10 ml of 40% methanol in water which was discarded and the fungicides were eluted with 5 ml of methanol. Care was taken during the washing and elution steps to prevent the column from going dry. For analysis by GLC, an aliquot of the eluate (4 ml) was transferred to a 15-ml centrifuge tube, diluted to 10 ml with water, and extracted with 5 ml of toluene. A 1:5 and 1:50 dilution of the toluene extract were placed in the autosampler rack for injection. Quantitation was carried out by reference to a linear calibration curve defined by the injection of a standard mixture of all compounds at five concentrations ranging from 20 to 80 ng/ml.

For determination by HPLC, a 0.5-ml aliquot of eluate was diluted to 2.5 ml with mobile phase and 100 μl were injected into the chromatograph. Biphenyl was determined by its absorbance at 254 nm and *o*-phenylphenol by flurorescence. Quantitation was accomplished by reference to a 40 ng/ml standard of each compound injected in duplicate with each set of six samples. Samples containing 5 ppm of fungicide were determined after dilution 1:25 with mobile phase.

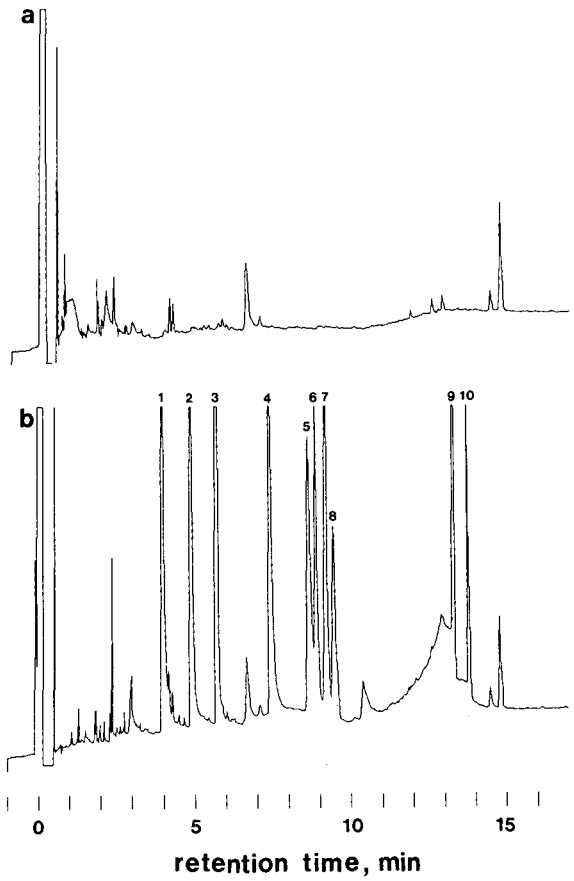

Fig. 1. GLC profiles of (a) unspiked apple and (b) apple spiked with 0.5 ppm of various fungicides. Peaks: 1 = dichloran; 2 = chlorothalonil; 3 = vinclozolin; 4 = triadimefon; 5 = anilazine; 6 = captan; 7 = folpet; 8 = procymidone; 9 = captafol; 10 = iprodione.

RESULTS AND DISCUSSION

Acetone was selected as the solvent for initial extraction because it has been shown[1,3,8] to be effective in the extraction of a large number of pesticides of diverse nature from a range of matrices with good recovery. Dilution of the extracts to a 20% acetone content was necessary to effect adsorption of all the fungicides onto the stationary phase. They were then recovered quantitatively from the cartridge by elution with methanol. Prewashing of the C_{18} cartridge before use with several column volumes of solvent was necessary to remove substances which interfered with the GLC and HPLC determinations. Contamination of both the packing material and the plastic container have been noted previously and the identity of several of these compounds reported[9].

GLC profiles of purified apple extract are shown in Fig. 1 and are typical of all commodities examined. The rise in baseline before the captafol peak (9) is due to decomposition on the GLC column and limited the quantitation limit of it to 1 ppm, since the peak area of captafol was linear with concentration injected above this level but decreased in a non-linear manner below it. Thus, the spiking levels for captafol were 1 and 10 ppm whereas the remainder of the fungicides were spiked at 0.5 and 5 ppm.

HPLC profiles from the same apple extract are given in Fig. 2. The fluorometric detector tracing is free of interferences while that of the UV detector indicates the presence of several UV-absorbing materials in the earlier part of the chromatogram. However, no interferences were observed in any commodities at the retention time of

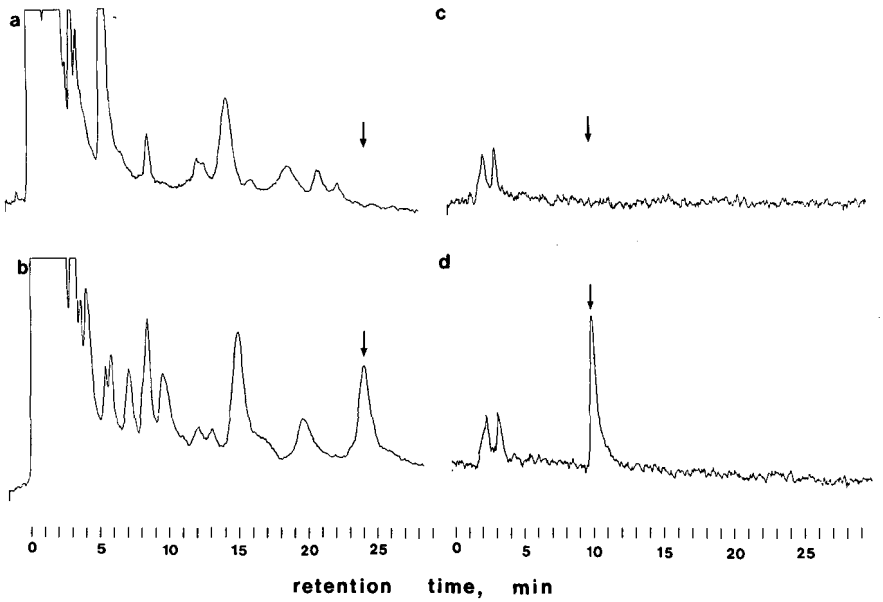

Fig. 2. HPLC profiles of unspiked apple (a and c) and of apple spiked with 0.5 ppm of biphenyl (b) and *o*-phenylphenol (d). Tracings a and b show absorbance at 254 nm while c and d record fluorescence emission. Retention time of biphenyl is 24 min and that of *o*-phenylphenol is 10.5.

TABLE I

RECOVERIES (%) OF FUNGICIDES ADDED TO FRUIT AND VEGETABLES AT 0.5 AND 5.0 ppm

Compound	*Grape*	*Apple*	*Tomato*	*Pear*	*Cucumber*	*Strawberry*	*Orange*	*Potato*
0.5 ppm spike								
Dichloran	83	87	93	94	100	85	81	76
Chlorothalonil	93	86	83	92	97	90	83	82
Vinclozolin	96	87	91	97	101	100	89	90
Triadimefon	96	84	98	94	98	102	91	94
Anilazine	97	82	93	89	102	102	91	101
Captan	92	85	88	103	91	119	76	88
Folpet	96	84	85	98	77	102	86	91
Procymidone	92	80	96	94	96	100	87	82
Captafol[a]	96	82	84	92	88	102	85	85
Iprodione	105	81	90	96	102	96	93	106
o-Phenylphenol	92	99	92	101	99	92	92	90
Biphenyl	91	88	80	83	97	82	82	90
5.0 ppm spike								
Dichloran	74	88	89	88	95	77	77	76
Chlorothalonil	82	84	87	89	97	85	90	89
Vinclozolin	83	85	88	88	99	88	89	88
Triadimefon	83	85	91	88	98	89	95	92
Anilazine	86	87	96	87	98	91	96	101
Captan	79	86	83	92	90	84	80	86
Folpet	83	85	85	90	91	87	90	90
Procymidone	81	85	90	86	97	88	88	84
Captafol[b]	83	84	84	86	95	88	91	88
Iprodione	85	84	92	92	106	87	100	97
o-Phenylphenol	96	102	91	104	101	95	95	99
Biphenyl	82	78	98	95	94	87	87	90

[a] 1.0 ppm.
[b] 10.0 ppm.

biphenyl. Although anilazine and dichloran were separated from the solvent front and are detectable by UV absorption, interfering co-extractives prevented their accurate quantitation, particularly at 0.5 ppm.

Satisfactory recoveries of the twelve fungicides were obtained from the eight commodities spiked in duplicate at 0.5 and 5 ppm as shown by the data in Table I. To obtain an indication of the repeatability of the procedure, apple was spiked at 0.5 ppm and analyzed in triplicate on two separate days. A mean coefficient of variation of 4.0% (range 2.3–8.0%) was obtained for recoveries of the twelve compounds.

The present data indicate that solid-phase extraction using a C_{18} cartridge is capable of effecting rapid isolation and clean-up of several fungicides from acetone extracts of fruit and vegetables while reducing the amount of organic solvent required for the clean-up phase of the analytical procedure. This reduction in solvent volume could be further improved if manufacturers provided materials free of platicizers and other monomeric materials which interfere in the determinative step. Although we did not examine the products of different suppliers, it has been shown[9] that some contain materials of a higher degree of purity and may be capable of providing

satisfactory blanks without extensive washing. The extension of this approach to other classes of pesticides and to compounds of greater polarity remains to be addressed in other studies.

REFERENCES

1 M. A. Luke, J. E. Freberg, G. M. Doose and H. T. Masumoto, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 1187.
2 R. W. Martindale, *Analyst (London)*, 113 (1988) 1229.
3 J. J. Blaha and P. J. Jackson, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 1095.
4 A. Andersson and B. Ohlin, *Var Foeda*, 38 (Suppl. 2) (1986) 79.
5 B. Ohlin, *Var Foeda*, 38 (Suppl. 2) (1986) 111.
6 A. de Kok, M. Hiemstra and C. P. Vrecker, *Chromatographia*, 24 (1987) 469.
7 R. Bagnati, E. Benfanati, E. Davoli and R. Fanelli, *Chemosphere*, 17 (1988) 59.
8 H. A. McLeod and R. A. Graham (Editors), *Analytical Methods for Pesticide Residues in Foods*, Canadian Government Publishing Centre, Supply and Services Canada, Ottawa, 1986.
9 G. A. Junk, M. J. Avery and J. J. Richard, *Anal. Chem.*, 60 (1988) 1347.

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Note

Immobilized metal-ion affinity chromatography of human growth hormone[a]

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(First received November 22nd, 1988; revised manuscript received January 19th, 1989)

Affinity chromatography on immobilized metal ions (IMAC) is a separation technique that takes advantage of the different capabilities of proteins of interacting with metal ions mainly through their exposed histidine residues. The metal ions are immobilized on suitable supports through a chelating ligand, usually iminodiacetate, which is covalently bound to the matrix. This technique was introduced by Porath *et al.*[1] in 1975 and since then it has been adopted for the purification of many proteins[2]. Recent advances with this method include the semipreparative purification of proteins from cell culture filtrates[3] and high-performance applications[4].

We have studied the behaviour of the recombinant human growth hormone (hGH) when subjected to IMAC and found this technique to be particularly useful for hGH purification. Further, by comparing our results with those observed for some model proteins[5], we have attempted to draw some conclusions about the number and location of histidine residues exposed on the molecule surface.

EXPERIMENTAL

Sepharose 6B (Pharmacia) was activated with epichlorohydrin and coupled with iminodiacetic acid according to Porath and Olin[6]. This iminodiacetate–agarose (IDA–agarose) was used for all IMAC experiments. Biosynthetic human growth hormone was cloned and purified in our laboratory from *B. subtilis* cells[7]. Metal chlorides ($CuCl_2$, $NiCl_2$, $CoCl_2$ and $ZnCl_2$) and imidazole were from E. Merck. Diethylpyrocarbonate (DEP) was purchased from Aldrich. All other chemicals were of analytical-reagent or reagent grade.

All chromatographic experiments were performed at 4°C in an 8-ml column (10 × 1 cm I.D.); a post-column of 3 ml of the same IDA–agarose was used to trap leaking metal ions. The column was loaded with the appropriate metal ion, using 50 ml of a 50 m*M* solution of the respective chlorides in water and making it recirculate for several hours, usually overnight. It was then equilibrated with 50 m*M* sodium

[a] Part of this work was presented as a poster at the *4th International Symposium on Separation Science and Biotechnology, Gargnano del Garda, Italy, August 31st–September 3rd, 1988.*

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phosphate (pH 7)–0.8 *M* sodium chloride (buffer A) and connected to the post-column, equilibrated with the same buffer. The high concentration of sodium chloride is used to prevent any ion-exchange adsorption of proteins. The flow-rate was maintained at 12 ml/h throughout. After loading the sample (1–1.5 ml of 0.5 mg/ml hGH), the column was washed with 30 ml of buffer A; elution was accomplished either with a pH gradient to pH 5 (50 m*M* acetate buffer–0.8 *M* sodium chloride) or with an imidazole concentration gradient from 0 to 30 m*M* in buffer A. With the IDA–Cu^{2+} column, the pH gradient went from 7 to 4 and then elution was continued with 1 *M* glycine adjusted to pH 8 with 1 *M* sodium hydroxide solution.

DEP modification of histidyl residues was performed according to Miles[8] in 20 m*M* phosphate buffer (pH 6) and followed in a Perkin-Elmer 551S UV–VIS spectrophotometer by recording difference spectra between 320 and 237 nm every fifth minute after the addition of DEP; the increase in absorbance at 242 nm corresponds to the amount of modified histidyl groups ($\varepsilon = 3200\ l\ mol^{-1}\ cm^{-1}$). Concentrations of 0.025 m*M* hGH and 0.25 m*M* DEP were used. Removal of the carbethoxy group from modified histidines was achieved by treatment with 20 m*M* hydroxylamine for 30 min.

RESULTS

hGH binding to different metal–agarose columns

We subjected biosynthetic hGH to IMAC using four different metal ions chelated to an IDA–agarose. According to what is considered to be a general rule, the extent of adsorption varies with the metal in use. These results are summarized in Fig. 1, in which the four chromatograms developed under the same conditions, except for the metal, are superimposed. The strongest retention is observed when the IMA

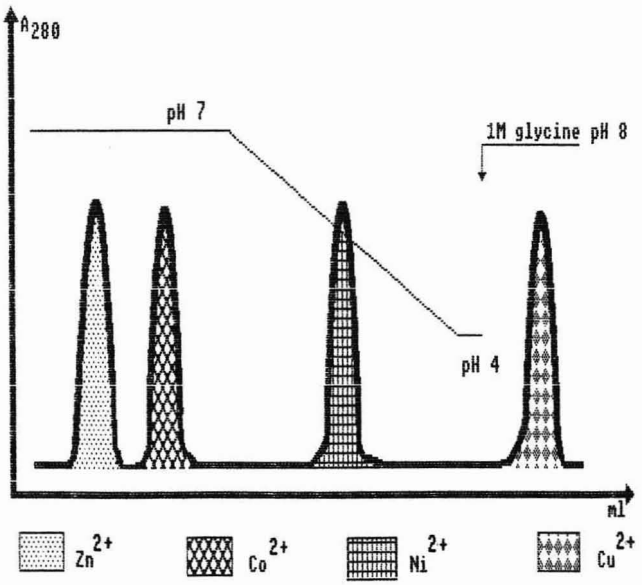

Fig. 1. Superimposition of IMA chromatograms of hGH using different metals.

column is loaded with Cu^{2+}, where the pH gradient does not elute the protein and a stronger displacer, such as 1 *M* glycine, is required. A weaker adsorption is exerted by Ni^{2+} ions and hGH can be eluted by decreasing the pH from 7 to about 5. Cobalt ions interact slightly with hGH molecules which are only retarded, but the technique may still be useful for isocratic enrichment of hGH samples. In contrast, zinc ions do not appear to have any effect and the protein is eluted in the void volume. Owing to this variety of possible behaviour, suitable conditions for IMAC purification of hGH can be chosen according to the kind of contaminants, but the most suitable adsorbent is probably IDA–Ni^{2+} gel. Fig. 2 shows the results of IMAC purification of a partially purified hGH, using an IDA–Ni^{2+} column eluted with a 2-h gradient from 0 to 30 m*M* imidazole in buffer A. Before IMAC, the crude cellular extract was purified only by ion-exchange chromatography on DEAE-cellulose. Therefore, a purity of over 90% is achieved in two steps, with no need for any sample preparation before IMAC except for the addition of sodium chloride up to 0.8 *M*.

DEP treatment

In view of the fact that in IMAC the interaction is believed to take place mainly through histidine residues, we prepared some modified hGH by attacking carbethoxy groups to the histidines. This strategy was followed by Al-Mashikhi and Nakai[9], who showed a decreased adsorption of carbethoxylated ovotransferrin on IMAC columns.

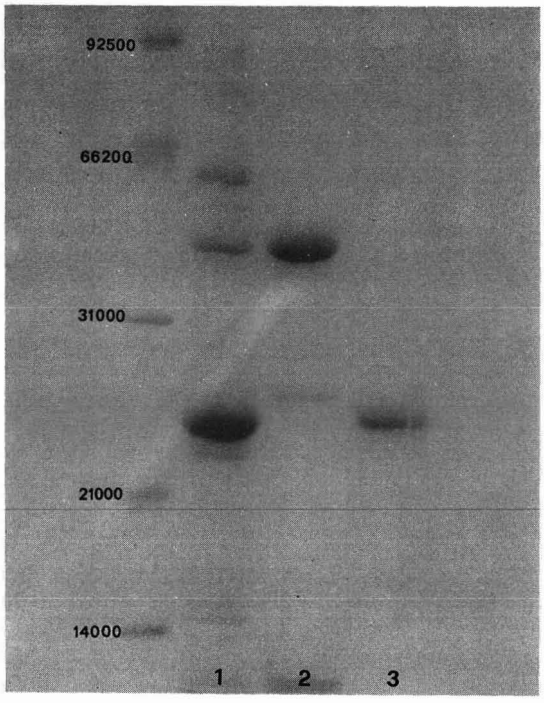

Fig. 2. Purification of hGH on IDA–Ni^{2+} gel. Lane 1 = partially purified hGH; lane 2 = unretained fraction; lane 3 = hGH eluted with 0–30 m*M* imidazole gradient. Numbers on vertical scale indicate molecular weights.

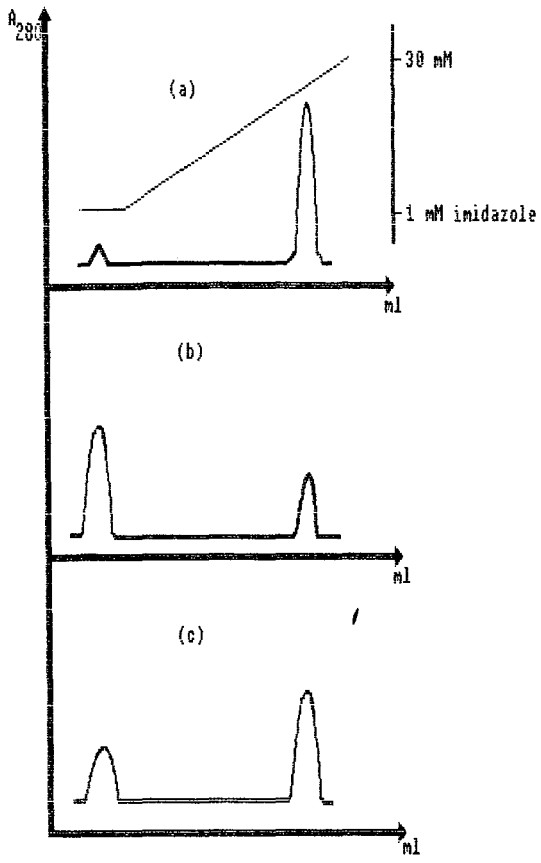

Fig. 3. IDA–Cu^{2+} chromatograms of modified hGH. (a) Unmodified hGH; (b) DEP-modified hGH; (c) DEP–hGH after removal of carbethoxy groups.

Diethyl pyrocarbonate (DEP) was ten times more concentrated than hGH, but we observed the modification of only about two of the three hGH histidines; this can be attributed to the poor accessibility of the third histidine. When subjected to IDA–Cu^{2+} chromatography, the amount of carbethoxylated hGH retained was only 30%, and it could be recovered with an imidazole concentration gradient. This residual adsorption was probably due to incomplete carbethoxylation of exposed histidines. Proper adsorption was almost fully restored by removing the carbethoxy groups by treatment with hydroxylamine (Fig. 3).

DISCUSSION

IMAC is a very flexible technique because the degree of retention can be modulated not only by changing external parameters such as pH, ionic strength or concentration of competitive molecules, but also by varying the intrinsic strength of the adsorbent, using different metal ions. Among the metals explored, nickel appears

to be the best candidate for hGH purification, owing to its moderate adsorption. hGH can be released from IMA–Cu^{2+} column by a decrease in pH or an imidazole concentration gradient. The results are satisfactory in terms of selectivity and degree of purity, as in this work where none of the traditional low-pressure chromatographic techniques (cation-exchange, hydrophobic interaction, size-exclusion chromatography) could achieve the same degree of purification (data not shown).

According to the proposed mechanism for IMAC interaction[5], the number of histidine residues and their location on the protein molecule should determine the extent of adsorption in this kind of chromatography, and other amino acids, such as tryptophan and cysteine, could play a secondary role. Our carbethoxylation studies of the histidine residues in hGH suggest that only two of the three histidines present in the molecule are exposed to the solvent and therefore readly accessible. The hydropathic profile[10] of the protein, which shows that His-151 is located within a highly hydrophobic region whereas His-18 and His-21 are present in a more hydrophilic environment, indicates that His-151 is the least reactive histidine. This prediction is consistent with the work of Fukushima *et al.*[11], who found that in bovine growth hormone, a protein which shares 66% homology with hGH, His-19 and His-21 are exposed to the solvent.

Predictions based on the study of model proteins suggest that protein retention by Zn^{2+} and Co^{2+} takes place when two proximal histidines located in an α-helical structure are present in the molecule[5]. Although proximal histidines are present in hGH (His-18 and His-21), the molecule does not bind to such sorbents. A plausible conclusion is that the two residues are in a conformational status that does not constitute a strong binding site for the metals.

It is interesting that a recently published three-dimensional model of hGH[12] predicts that His-18 and His-21 are not located in an α-helical structure. If this is so, the two histidines are not likely to behave as "proximal" in metal binding. On the other hand, analysis according to Chou and Fasman[13] does predict an α-helical conformation between residues 11 and 21 in hGH.

We tend to favour the prediction of the three-dimensional model on the basis of the consideration that α/α proteins such as hGH could not be particularly suitable for Chou and Fasman analysis. For example, this analysis would also predict about a 17% β-structure conformation in hGH, in contrast with the circular dichroism spectrum of hGH[14] which indicates only the presence of α-helices.

In conclusion, IMAC adsorption of hGH is consistent with the model of a protein containing two histidine residues that interact with the metals bound to the matrix in a non-cooperative fashion. The third histidine of the molecule (His-151) probably does not take part in the interaction, even with the strong IDA–Cu^{2+} gel for which a single exposed histidine would determine the binding.

To our knowledge, this is the first attempt to elucidate the topological distribution of histidine residues in hGH by means of IMAC and, as has already been envisaged by Sulkowski[5], this could be a major application of the technique in the future.

REFERENCES

1 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature (London)*, 258 (1975) 598.
2 B. Lonnerdal and C. L. Keen, *J. Appl. Biochem.*, 4 (1982) 203.

3 S. Krishnan, M. A. Vijayalakshmi and I. Geohel, *J. Chromatogr.*, 397 (1987) 339.
4 M. Belew, T. T. Yip, L. Andersson and R. Ehrnström, *Anal. Biochem.*, 164 (1987) 457.
5 E. Sulkowski, *Trends Biotechnol.*, 3 (1985) 1.
6 J. Porath and B. Olin, *Biochemistry*, 22 (1983) 1621.
7 E. Franchi, F. Maisano, S. Astrua Testori and G. Grandi, in preparation.
8 E. W. Miles, *Methods Enzymol.*, 43 (1977) 431.
9 S. A. Al-Mashikhi and S. Nakai, *Agric. Biol. Chem.*, 51 (1987) 2881.
10 J. Kyte and R. F. Doolitle, *J. Mol. Biol.*, 157 (1982) 105.
11 J. G. Fukushima, M. J. Biscoglio De Jimenez Bonino, O. Cascone and J. A. Santomè, *Int. J. Pept. Protein Res.*, 21 (1983) 451.
12 F. E. Cohen and I. D. Kuntz, *Proteins: Structure, Function and Genetics*, 2 (1987) 162.
13 P. J. Chou and G. D. Fasman, *Adv. Enzymol.*, 47 (1987) 45.
14 L. A. Holladay, R. G. Hammonds, Jr. and D. Puett, *Biochemistry*, 13 (1974) 1653.

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Note

High-performance liquid chromatographic determination of the free *o*-quinones of *trans*-caffeoyltartaric acid, 2-S-glutathionylcaffeoyltartaric acid and catechin in grape must

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The enzymatic oxidation of hydroxyphenolic compounds to quinones, catalysed by polyphenoloxidases, is a ubiquitous phenomenon[1,2], responsible for many biologically and technologically important reactions, such as melanin pigmentation[3–5], insect cuticle sclerotization[6,7] and fruit and vegetable browning during processing and storage[2,8,9]. Numerous studies have dealt with the enzymatic substrates and/or the condensation products, but the quinone intermediates have not received much attention. The purpose of this work was to develop a method to measure free quinones in grape musts in order to establish the kinetics of phenolic oxidation and subsequent quinone reactions.

Hydroxycinnamic acids and especially *trans*-caffeoyltartaric (caftaric) and *p*-coumaroyltartaric (coutaric) acids are the major phenolic compounds of white grape musts[10–12]. When no special care is taken to avoid oxidation during crushing and pressing, they are rapidly oxidized by the grape polyphenol oxidase (PPO) to caftaric acid *o*-quinone. The latter reacts readily with the available glutathione to form 2-S-glutathionylcaffeoyltartaric acid, known as Grape Reaction Product (GRP)[13–15]. This compound is not a substrate for the grape PPO[15] but can be oxidized by the excess of caftaric acid quinones[16]. Similar coupled oxidations have been demonstrated with a number of flavans[17], which are minor components of white grape juice but can be extracted in larger amounts when pomace contact takes place during the wine-making process[18–21]. The resulting quinones are rapidly involved in polycondensation reactions, leading to the formation of brown polymers[17].

The high-performance liquid chromatographic (HPLC) separation of grape and must phenolics has been extensively studied[22–26]. On the other hand, no method is available for measuring the free quinones, probably because of their instability. In particular, they are readily reduced back to the corresponding hydroquinones by sulphur dioxide and/or ascorbic acid added to prevent sample oxidation when assaying for grape must phenolics. However, the instantaneous concentration of *o*-quinones, and especially that of caftaric acid quinones, might be fairly high in oxidizing musts.

The ability of benzenesulphinic acid to react with *o*-quinones has been known for some time[27–29]. The method reported here involves benzenesulphinic acid deriva-

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tization followed by reversed-phase HPLC separation for the determination of caftaric acid, GRP and catechin-free *o*-quinones in grape musts.

EXPERIMENTAL

Chemicals

(+)-Catechin and sodium benzenesulphinate were purchased from Fluka (Buchs, Switzerland), ascorbic acid and hydrogen peroxide (35% solution) from Merck (Darmstadt, F.R.G.) and horseradish peroxidase (E.C. 1.11.1.7) from Sigma (St. Louis, MO, U.S.A.). Crude grape PPO extract was prepared from grape juice as described previously[14].

Caftaric acid was extracted from grape juice following the procedure of Singleton *et al.*[12] and 2-S-glutathionylcaftaric acid prepared by aerating 2 m*M* caftaric acid and 10 m*M* reduced glutathione in the presence of 6 g/l crude grape PPO extract in 2.5 g/l aqueous potassium hydrogentartrate (pH 3.65). Both were purified by preparative HPLC. The preparative HPLC system was a Jobin-Yvon (Longjumeau, France) system, consisting of a Modulprep compression module, a Modulprep hydraulic module, a Modulprep pump, a manual injection system, an ISA-SM 25 UV detector set at 280 nm and a Linseis recorder. The column was an axial compression column (500 × 22 mm I.D.), filled with LiChrosorb RP-18 stationary phase (Merck, 15–25-μm packing). Isocratic elution was performed using 10% methanol in 3% acetic acid solution at a flow-rate of 20 ml/min.

Caftaric acid and catechin sulphones were synthesized by incubating 2 m*M* caftaric acid and 2 m*M* catechin, respectively, with 20 m*M* sodium benzene sulphinate and 10 g/l crude grape PPO extract in 2.5 g/l aqueous potassium hydrogentartrate. The GRP *o*-quinones were obtained by peroxidase oxidation of 2 m*M* GRP in the presence of a stoichiometric amount of hydrogen peroxide. After a few minutes, a red-brown colour characteristic of GRP quinones[16] developed and the sulphones were produced by addition of 20 m*M* sodium benzenesulphinate. Sulphur dioxide (0.5%) was added 30 s after the sodium benzenesulphinate to reduce the hydrogen peroxide remaining and inhibit peroxidase. The three sulphones were purified by preparative HPLC as described above but using 30% methanol in 3% acetic acid as the eluent.

Sample preparation

Model solutions were prepared by incubating the substrate(s) in the presence of crude grape PPO extract in 2.5 g/l aqueous potassium hydrogentartrate at 30°C and with air agitation on a magnetic stirrer. Oxidized must samples were obtained at the INRA experimental winery at Pech Rouge by crushing and pressing white grapes with regular winery equipment, unless specified otherwise.

Sodium benzenesulphinate crystals were added to all samples (approximately 2.5 mg/ml) and the mixture was stirred for 10–15 s, stabilized by addition of sulphur dioxide (0.2%) and filtered through 0.45-μm membrane filters prior to injection (injection volume 20 μl) on to the HPLC column.

Instrumentation

The HPLC apparatus was a Millipore-Waters (Milford, MA, U.S.A.) system including a 710B autoinjector, a 720 system controller and two M510 pumps

connected to a Spectromonitor 3100 (Milton Roy) variable-wavelength detector set at 280 nm and to an Enica 21 integrator (Delsi, France). The column was reversed-phase Spherisorb ODS-2 (5-μm packing) (250 × 4 mm I.D.) protected with a guard column of the same material (Knauer, F.R.G.). The elution conditions were as follows: flow-rate, 1 ml/min; solvent A, 2.5% acetic acid; solvent B, acetonitrile–solvent A (80:20, v/v); elution starting with 5% B, isocratic for 4 min, and continuing with a linear gradient from 5 to 20% B in 16 min and from 20 to 80% B in 10 min, followed by washing and reconditioning of the column.

The UV spectra were recorded from 250 to 400 nm using a Millipore-Waters photodiode-array detector under the same chromatographic conditions.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms for a combined standard of caftaric acid, GRP, catechin and their benzene sulphones and for an oxidizing white grape must sample. Each quinone gave a single derivative, as expected from the results of Piretti *et al.* [29] on catechin and Pierpoint [28] on caffeic and chlorogenic acids.

The mean retention times (± S.D.) for a series of twelve injections were 28.47 ± 0.04, 29.42 ± 0.05 and 30.84 ± 0.03 min for caftaric acid, GRP and catechin sulphones, respectively. It was checked that the quinone derivatives detected in grape

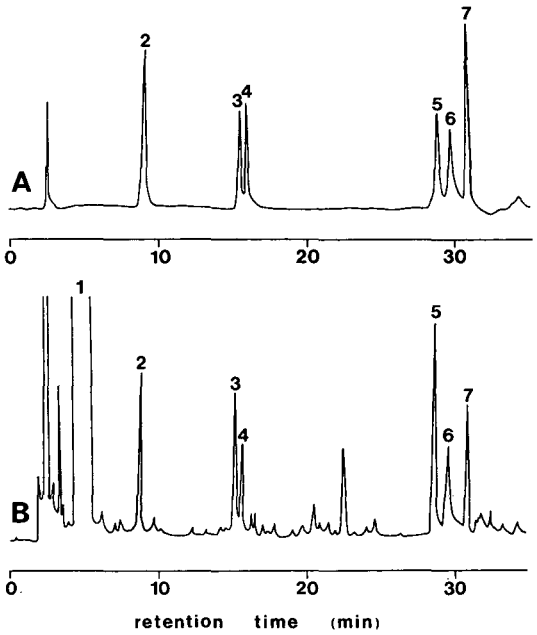

Fig. 1. HPLC traces of reduced (hydroquinones) and oxidized (*o*-quinones) grape juice phenolics in (A) 0.05 m*M* standard solution and (B) 0.1 m*M* catechin-treated Sauvignon must oxidized for 10 min. Peaks: (1) benzenesulphinic acid; (2) *trans*-caffeoyltartaric acid; (3) 2-S-glutathionylcaffeoyltartaric acid; (4) catechin; (5) *trans*-caffeoyltartaric acid *o*-quinone, (6) 2-S-glutathionylcaffeoyltartaric acid *o*-quinone; (7) catechin *o*-quinone.

musts were identical with the standards and did not coelute with other grape must components by co-injection of must samples with quinone derivative standards and comparison of the retention times and of the UV spectra recorded using a diode-array detector.

Known dilutions of each compound in water and in a Chardonnay wine must prepared at the INRA experimental winery were used to determine the response factors (concentration/unit peak area) at 280 and 313 nm. The calibration graphs for all compounds were linear over a concentration range of 0–2 m*M* (0–40 nmol injected). The coefficients of variation over the range 0.01–2m*M* were 1.46, 4.8 and 3.9% ($n=8$) for caftaric acid, GRP and catechin quinones, respectively. The detection limits for caftaric acid, GRP and catechin benzene sulphones were 10, 20 and 13 ng, respectively, in grape must.

The derivatization rates were studied on a solution containing 1 m*M* caftaric acid, 0.5 m*M* GRP and 0.5 m*M* catechin incubated for 10 min with 1 g/l PPO. Derivatization appeared to be very rapid for all three compounds, as immediate discoloration of the samples was observed following addition of benzenesulphinate and no further increase in the amount of catechin and GRP sulphones was obtained when benzenesulphinate was allowed to react longer with the quinones before addition of sulphur dioxide (Fig. 2). In addition, the amount of caftaric acid quinone derivative increased slightly, indicating that the enzymatic oxidation but not the coupled oxidations continues in the presence of excess of benzenesulphinate until sulphur dioxide is added. It was therefore concluded that the optimum delay between benzenesulphinic acid and sulphur dioxide addition was 5–10 s.

The quinone derivatives were stable over a period of several weeks when the samples to which sulphur dioxide had been added were kept in the dark at 4°C.

Reproducibility studies were performed on model solutions containing caftaric acid and GRP or caftaric acid and catechin (each 0.2 m*M*) with the crude grape PPO extract and on a white must prepared by crushing Sauvignon grapes under vacuum, adding 0.1 m*M* catechin and oxidizing by stirring in air at 30°C. Samples were taken in

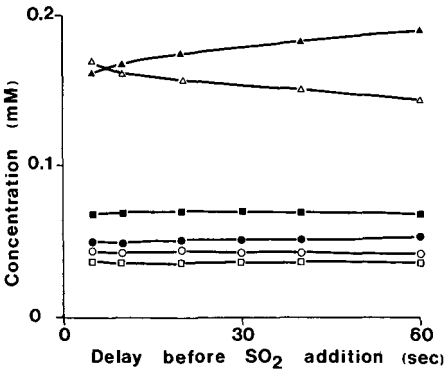

Fig. 2. Influence of the delay between sodium benzenesulphinate and sulphur dioxide addition on the concentration of hydroquinones (open symbols) and *o*-quinones (full symbols) in samples taken after 10-min oxidation from a solution containing initially (△,▲) 1 m*M* caffeoyltartaric acid, (□,■) 0.5 m*M* 2-S-glutathionylcaffeoyltartaric acid, (○,●) 0.5 m*M* catechin and 1 mg/ml crude grape polyphenol oxidase extract.

five replicates from each solution after oxidation for 10 min. The coefficients of variation for the determination of quinones in the model solution and grape must were 2.4, 3 and 2.3% for caftaric acid, GRP and catechin, respectively, in model solutions, and 2.4, 4 and 2.5%, respectively, in Sauvignon must.

The method described is simple, fast and of adequate sensitivity for the measurement of free caftaric acid, GRP and catechin *o*-quinones in oxidizing grape must. Further work on its application to the determination of other *o*-quinones potentially present in grape musts, in particular epicatechin and procyanidin *o*-quinones, is in progress. One of the advantages of the method is that it is suitable for the simultaneous determination of hydroquinones and *o*-quinones. It should also be adaptable to other phenolic substances such as DOPA and its derivatives and, consequently, offer a very useful approach to the study of phenolic oxidation and condensation mechanisms in various research fields.

REFERENCES

1 A. M. Mayer and E. Harel, *Phytochemistry*, 18 (1979) 193.
2 M. Sugumaran, H. Dali, V. Semensi and B. Hennigan, *J. Biol. Chem.*, 262 (1987) 10546.
3 H. S. Raper, *Biochem. J.*, 21 (1927) 89.
4 V. J. Hearing and T. M. Ekel, *Biochem. J.*, 157 (1976) 549.
5 G. Prota, *J. Invest. Dermatol.*, 75 (1980) 122.
6 H. Lipke, M. Sugumaran and W. Henzel, *Adv. Insect Physiol.*, 17 (1983) 1.
7 K. J. Kramer and T. L. Hopkins, *Arch. Insect. Biochem. Physiol.*, 6 (1987) 279.
8 A. G. Mathew and H. A. B. Parpia, *Adv. Food Res.*, (1971) 75.
9 L. Vamos-Vigyazo, *CRC Crit. Rev. Food Sci. Nutr.*, (1981) 49.
10 T. E. Myers and V. L. Singleton, *Am. J. Enol. Vitic.*, 30 (1979) 98.
11 C. W. Nagel, J. D. Baranowski, L. W. Wulf and J. R. Powers, *Am. J. Enol. Vitic.*, 30 (1979) 198.
12 V. L. Singleton, C. F. Timberlake and A. G. H. Lea, *J. Sci. Food Agric.*, 29 (1978) 403.
13 V. L. Singleton, J. Zaya, E. Trousdale and M. Salgues, *Vitis*, 23 (1984) 113.
14 V. L. Singleton, M. Salgues, J. Zaya and E. Trousdale, *Am. J. Enol. Vitic.*, 36 (1986) 50.
15 V. F. Cheynier, E. K. Trousdale, V. L. Singleton, M. J. Salgues and R. Wylde, *J. Agric. Food Chem.*, 34 (1986) 217.
16 V. F. Cheynier and M. W. Van Hulst, *J. Agric. Food Chem.*, 36 (1988) 10.
17 V. Cheynier, C. Osse and J. Rigaud, *J. Food Sci.*, 53 (1988) 1732.
18 C. Cantarelli and C. Peri, *Am. J. Enol. Vitic.*, 15 (1964) 146.
19 J. Oszmianski, F. M. Romeyer, J. C. Sapis and J. J. Macheix, *Am. J. Enol. Vitic.*, 37 (1986) 7.
20 D. Ramey, A. Bertrand, C. S. Ough, V. L. Singleton and E. Sanders, *Am. J. Enol. Vitic.*, 37 (1986) 99.
21 V. L. Singleton, J. Zaya and E. Trousdale, *Am. J. Enol. Vitic.*, 31 (1980) 14.
22 V. Cheynier and J. Rigaud, *Am. J. Enol. Vitic.*, 37 (1986) 248.
23 A. W. Jaworski and C. Y. Lee, *J. Agric. Food Chem.*, 35 (1987) 257.
24 S. M. Lunte, *J. Chromatogr.*, 384 (1987) 371.
25 V. L. Singleton and E. Trousdale, *Am. J. Enol. Vitic.*, 34 (1983) 27.
26 L. W. Wulf and C. W. Nagel, *Am. J. Enol. Vitic.*, 29 (1978) 42.
27 W. S. Pierpoint, *Biochem. J.*, 98 (1966) 567.
28 W. S. Pierpoint, *Biochem. J.*, 112 (1969) 619.
29 M. V. Piretti, G. P. Serrazanetti and G. Paglione, *Ann. Chim. (Rome)*, 67 (1977) 395.

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Note

Determination of the principal anionic components in wines and soft drinks, by ion interaction reversed-phase high-performance liquid chromatography

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Previous work[1,2] in this laboratory has shown the applicability of ion interaction reversed-phase high-performance chromatography in separations of anionic species. Salicylates of some aliphatic amines were used as ion interaction reagents. Retention and resolution were shown[2] to depend on the alkyl chain length of the ion interaction reagent, the flow-rate, the stationary phase and the method of detection, which leads to the well known[3–8] versatility of the technique.

The aim of this work was to optimize the experimental conditions for the identification, separation and determination of anionic species, both inorganic and carboxylic. The method was applied to the analysis of the major anionic components of some Italian wines and soft drinks.

EXPERIMENTAL

Apparatus

Analyses were performed using a Varian LC 5000 chromatograph, equipped with a Vista 401 data system and a UV-100 spectrophotometric detector. Alternatively, a Wescan 213A conductimetric detector was employed; it was interfaced to the Vista 401 data system with a 1 V exit. For pH measurements a Metrohm 654 pH-meter was used.

Merck Hibar LiChrosorb RP-18, 10 μm (250 × 4 mm I.D.), Merck Hibar LiChrospher RP-18, 10 μm (250 × 4 mm I.D.) and Waters Bondapak C_{18}, 10 μm (150 × 3.9 mm I.D.) columns, equipped with a Merck Hibar LiChrocart LiChrosorb RP-18 guard column (25 × 4 mm I.D.), were used.

Chemicals

Analytical-reagent grade chemicals and ultra-pure water from a Millipore Milli-Q system were used. Heptylamine was from Aldrich and octylamine from Fluka. Salicylic acid and all other reagents were obtained from Carlo Erba.

The solutions of the eluents were prepared by dissolving a weighed amount of the amine in ultra-pure water and adjusting the pH to 6.2 ± 0.4 by addition of salicylic acid. Taking into account the acid formation constants of amines, the com-

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TABLE I

RETENTION TIMES OF SOME ANIONS USING DIFFERENT ION INTERACTION REAGENTS, STATIONARY PHASES AND FLOW-RATES

Estimates of standard deviations are based on at least four determinations.

Anion	*Retention time (min)*					
	0.005 M heptylamine salicylate		*0.005 M octylamine salicylate*			
	Merck LiChrosorb RP-18 (10 μm), 0.5 ml/min	*Waters Bondapak C_{18} (10 μm), 0.5 ml/min*	*Merck LiChrosorb RP-18 (10 μm), 3.0 ml/min*	*Waters Bondapak C_{18} (10 μm), 1.9 ml/min*	*Merck LiChrospher RP-18 (10 μm)*	
					2.0 ml/min	*2.5 ml/min*
Chloride	6.4 ± 0.2	5.2 ± 0.2				
Carbonate	7.1 ± 0.2	5.0 ± 0.2			3.0 ± 0.3	2.1 ± 0.3
Acetate	7.2 ± 0.2	5.2 ± 0.2	1.7 ± 0.3	2.0 ± 0.2	3.0 ± 0.3	
Nitrate	7.1 ± 0.2	5.5 ± 0.2				
Orthophosphate	9.1 ± 0.2	6.2 ± 0.3				
Glycolate	7.4 ± 0.3			2.2 ± 0.3		
Gluconate				2.2 ± 0.3		
Ascorbate	7.7 ± 0.3	5.7 ± 0.2		2.2 ± 0.3		
Lactate	7.9 ± 0.2	5.2 ± 0.3	2.0 ± 0.3	2.2 ± 0.3		
Butyrate			4.3 ± 0.3			
Succinate	12.6 ± 0.2	8.6 ± 0.2	9.3 ± 0.3	9.2 ± 0.3	17.2 ± 0.3	
Malate	13.5 ± 0.4	11.5 ± 0.4	10.4 ± 0.4	10.1 ± 0.3	19.8 ± 0.3	
Glutarate	15.0 ± 0.4	11.9 ± 0.4		9.7 ± 0.3		
Malonate	15.0 ± 0.4	11.9 ± 0.4		10.0 ± 0.4	21.6 ± 0.4	17.4 ± 0.4
Tartrate	15.5 ± 0.5	12.2 ± 0.5	12.7 ± 0.5	12.3 ± 0.5	24.9 ± 0.5	
Maleate	17.8 ± 0.5	12.7 ± 0.4	11.7 ± 0.4	11.2 ± 0.5	22.6 ± 0.5	
Adipate				11.5 ± 0.4	20.9 ± 0.5	17.0 ± 0.4
Citrate	34.0 ± 2.0	15.6 ± 0.5				
System peak	67.0 ± 2.0	62.0 ± 2.0	15.0 ± 0.6	20.4 ± 0.6	24.0 ± 1.0	

position of the eluents so prepared is not exactly stoichiometric. For simplicity, however, they will be referred to henceforth as amine salicylates.

Chromatography

In order to condition the chromatographic system, eluent was allowed to flow through the column until a stable baseline was obtained, generally requiring about 1 h. Eluent solution was prepared freshly every third day.

The reproducibility of the retention times and sensitivity was very good for sequential analyses, but it was poorer for different eluent preparations and column conditionings. The sensitivity and, consequently, the accuracy and precision of determined followed a similar trend. Retention times and quantitation data were evaluated for different preparations.

Between use, the columns were regenerated by passage of water–methanol (1:1); no particular deterioration of the column was observed with respect to its use in other chromatographic techniques.

The samples of wines and beverages to be injected were prepared by filtration through a Nucleopore Syrfil 0.45-μm filter and diluting, when necessary, in ultra-pure water.

RESULTS AND DISCUSSION

Preliminary measurements showed that, in the separation of carboxylic acids, 10-μm C_{18} stationary phases are more suitable than the 5-μm type as the former allow a better separation and generally lower retention times. In this study, different 10-μm stationary phases (with spherical or irregular particles) were compared.

Salicylates of heptylamine and octylamine were used as ion interaction reagents. The relatively low ionic equivalent conductivity and the high molar absorptivity in the UV region ($\varepsilon = 308 \pm 2$ l mol^{-1} cm^{-1} at 254 nm) of salicylate ions allow both conductimetric and spectrophotometric, direct and indirect, detection. The results obtained for a series of anionic species are given in Table I.

The behaviour of the ion interaction reagent and its elution flow agree with previous conclusions[2] that the shorter is the alkyl chain length, the shorter are the retention times and, as the flow-rate increases, the retention decreases.

Further, interactions between the eluate and modified stationary phase depend on the alkyl chain length and modify the elution order (compare, for example, the retention times of tartaric and maleic acids obtained with the two eluents).

Fig. 1 shows the separation of a mixture of acetic, ascorbic, lactic, succinic, malic, glutaric, tartaric and maleic acids under optimized experimental conditions. Owing to the molar absorptivities at 254 nm of ascorbate [$\varepsilon_{254\ nm} = (8.72 \pm 0.04) \cdot 10^3$ l mol^{-1} cm^{-1}] and maleate [$\varepsilon_{254\ nm} = (7.2 \pm 0.2) \cdot 10^2$ l mol^{-1} cm^{-1}], these anions give rise to positive peaks. Hence they can be easily identified and separated from UV-transparent anions, in spite of the poor separation factors. Taking into account the molar absorptivity of salicylate ions at this wavelength, UV-transparent anions appear as negative peaks.

The use of octylamine salicylate, characterized by lower retention, permits the separation of the series of the anions listed in Table I, whereas the use of heptylamine allows the separation of the components with lower retention times to be improved.

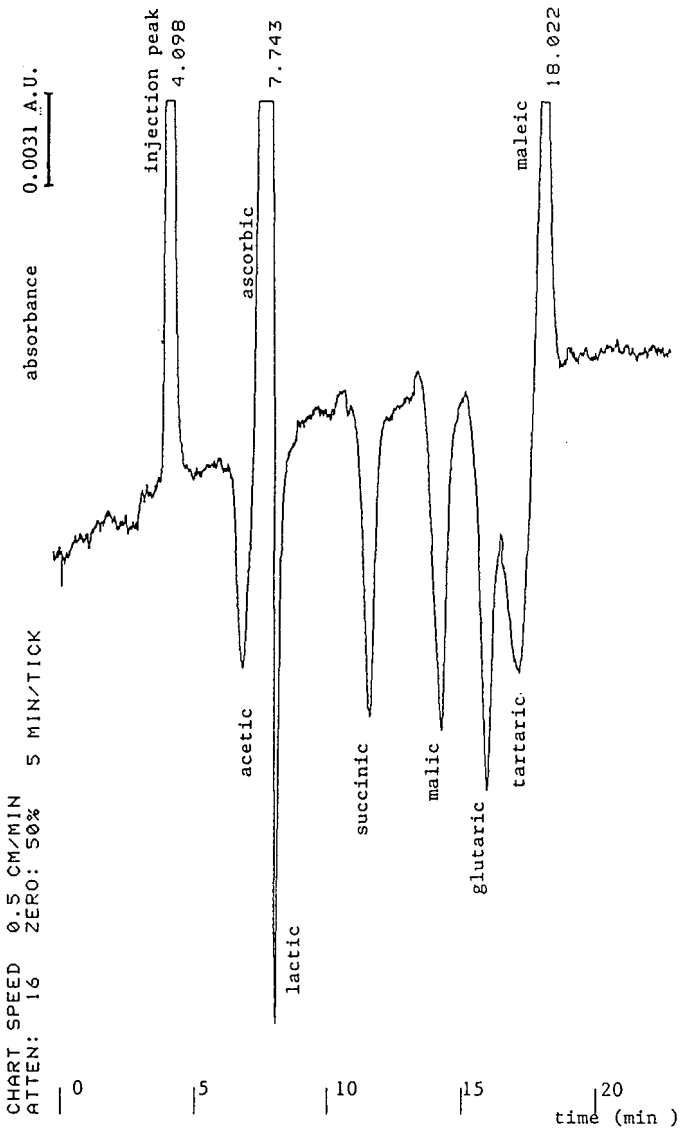

Fig. 1. Separation of a mixture of acetic, ascorbic, lactic, succinic, malic, glutaric, tartaric and maleic acids (50.0 ppm each). Injection volume, 100 μl; column, Merck LiChrosorb RP-18, 10 μm; ion interaction reagent, 0.0050 *M* heptylamine salicylate; flow-rate, 0.5 ml/min; spectrophotometric detection (254 nm).

These observations were applied to the analysis of some Italian wines and soft drinks. The qualitative and quantitative determination of the anionic species is of interest with regard to organoleptic properties and quality control.

Red wines (Barbera delle Langhe 1987, Barbaresco DOCG 1984, Grignolino 1987, Chianti DOCG 1987), and white wines (Pinot Grigio 1987 and boxed Tavernello Trebbiano) were considered. Fig. 2 shows, as an example, the chromatograms for Barbera wine when using (a) 0.0050 *M* octylamine salicylate as the eluent at a flow-

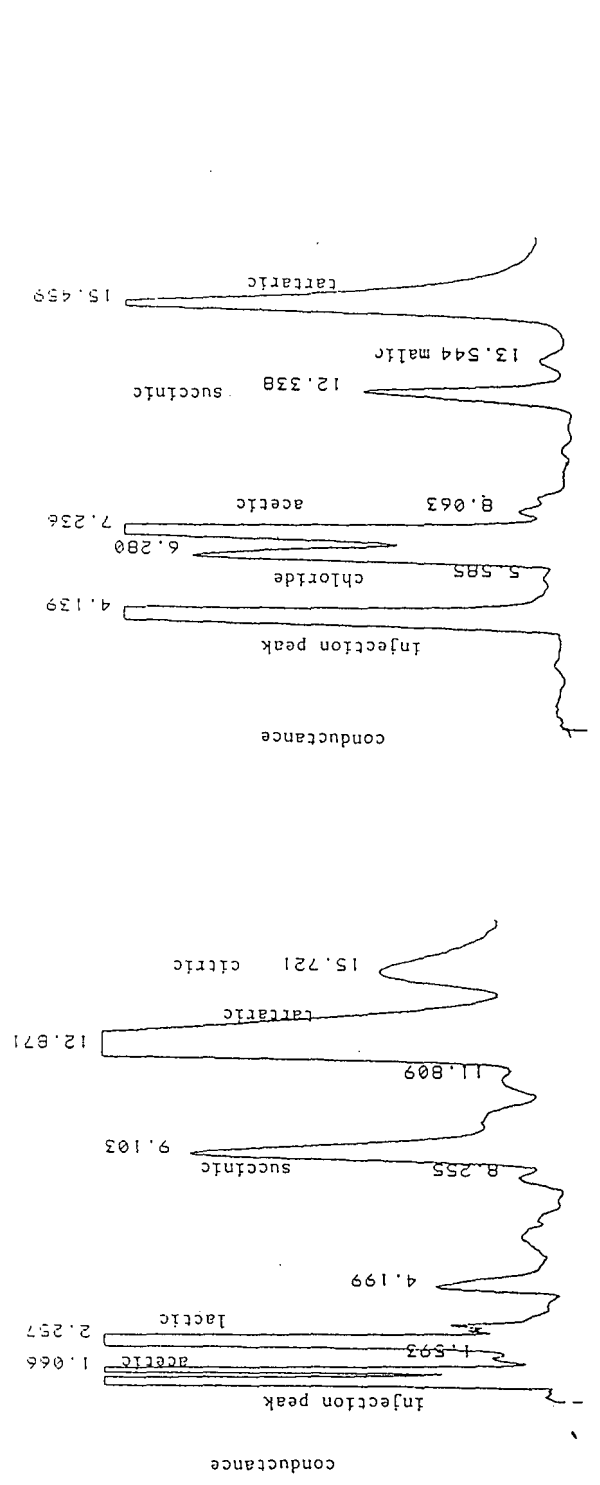

Fig. 2. Analysis of Barbera delle Langhe 1987 wine. Injection volume, 100 μl; column, Waters Bondapak C_{18}, 10 μm; conductimetric detection. (a) Ion interaction reagent, 0.0050 *M* octylamine salicylate; flow-rate, 1.9 ml/min; sample dilution, 1:10 (v/v). (b) Ion interaction reagent, 0.0050 *M* heptylamine salicylate; flow-rate, 0.5 ml/min; sample dilution, 1:50 (v/v).

rate of 1.9 ml/min and, for comparison, (b) 0.0050 *M* heptylamine salicylate at a flow-rate of 0.5 ml/min. The sample was diluted 1:10 (v/v) with ultrapure water when using octylamine salicylate and 1:50 (v/v) when using heptylamine salicylate. The sensitivity of the method for the analysis of the of major organic acids in wines is satisfactory.

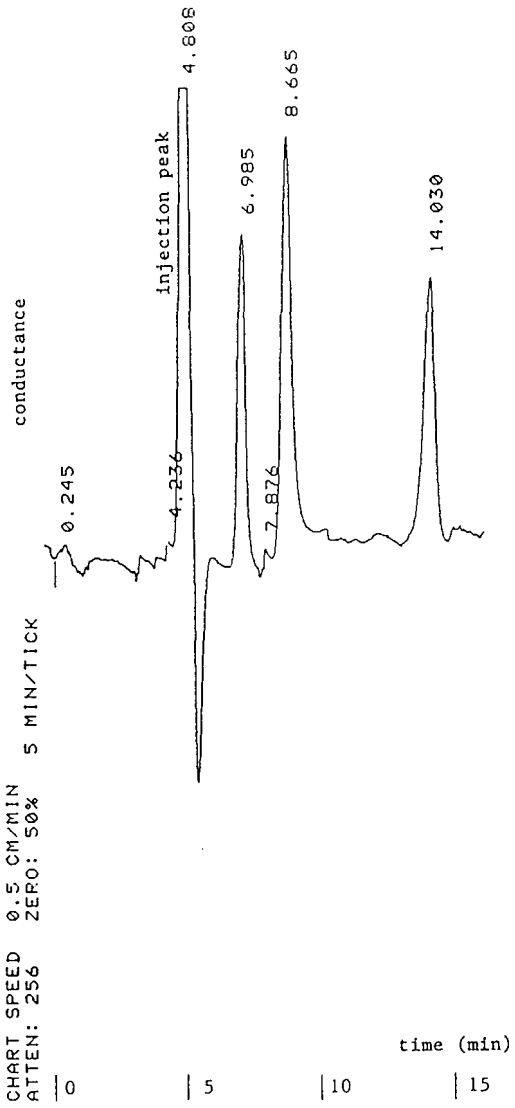

Fig. 3. Chromatogram recorded for a cola drink, diluted 1:15 (v/v). Column, Merck LiChrosorb RP-18, 10 μm; ion interaction reagent, 0.0050 *M* heptylamine salicylate; flow-rate, 0.7 ml/min; injection volume, 100 μl; conductimetric detection. Peaks: 6.985 min = chloride; 8.665 min = orthophosphate; 14.030 min = tartrate.

TABLE II

DETERMINATION OF MAJOR CARBOXYLIC ACIDS IN ITALIAN WINES

Estimates of standard deviations are based on at least three determinations.

Wine	*Acid (g/l)*			
	Succinic	*Malic*	*Tartaric*	*Citric*
Grignolino 1987	0.85 ± 0.08	0.11 ± 0.02	1.93 ± 0.19	0.23 ± 0.07
Barbera delle Langhe 1987	0.60 ± 0.07	traces	2.20 ± 0.20	0.08 ± 0.03
Chianti DOCG 1987	0.79 ± 0.07	0.27 ± 0.04	2.41 ± 0.24	0.09 ± 0.03
Barbaresco DOCG 1984	0.69 ± 0.06	0.59 ± 0.06	2.13 ± 0.11	0.22 ± 0.03
Pinot Grigio 1987	0.40 ± 0.05	1.41 ± 0.14	1.34 ± 0.13	0.06 ± 0.02
Tavernello Trebbiano	0.70 ± 0.07	0.63 ± 0.06	2.42 ± 0.24	0.11 ± 0.02

As mentioned above, the use of heptylamine permits a better separation of the less retained components. As inorganic anions are characterized under these conditions by lower retentions, separations of mixtures of organic and inorganic anions are therefore made possible by the use of this reagent. A typical example (flow-rate 0.7 ml/min, LiChrosorb RP-18 column and conductimetric detector) is the analysis of anions in commercial soft drinks, namely a cola beverage, a fizzy drink and a dietetic drink, previously degassed and diluted 1:15 (v/v). Fig. 3 shows, as an example, a chromatogram of a cola drink.

Preliminary measurements performed on wines and drinks to which known amounts of the investigated acids had been added permitted any matrix effect on the determination of the anions to be excluded. Plots of peak area *versus* standard concentration showed good linearity. For all the wines the concentrations of succinic, malic, tartaric and citric acids were determined (Table II). Table III reports quantitative data for chlorides, orthophosphates, tartrates and citrates in soft drinks. The results for the cola drink agree reasonably well with literature data[9] with respect to the pH of the drink. However, our results show that not only orthophosphoric acid but also citric acid contribute to the acidity.

The advantage of the proposed technique is that it requires no pretreatment or derivatization of the sample, except for a filtration and, sometimes, dilution.

If chromatograms of the sample and of standard solutions are recorded sequentially for the same eluent preparation, a precision below 2% is obtained in quantitative analysis.

TABLE III

DETERMINATION OF ANIONIC SPECIES IN SOFT DRINKS

Estimates of standard deviations are based on at least three determinations.

Soft drink	*Anion (g/l)*			
	Chloride	*Orthophosphate*	*Tartrate*	*Citrate*
Dietetic drink	0.28 ± 0.04	0.12 ± 0.03	–	3.0 ± 0.2
Fizzy drink	0.05 ± 0.02	–	0.17 ± 0.03	1.5 ± 0.2
Cola drink	0.03 ± 0.02	0.27 ± 0.03	0.24 ± 0.04	–

The sensitivity depends on the operating conditions, as can be seen by comparing the chromatograms for Barbera wine obtained using octylamine and heptylamine salicylate (Fig. 2). The chromatograms show similar peak areas, notwithstanding the different dilutions of the samples [1:10 (v/v) for octylamine salicylate and 1:50 (v/v) for heptylamine salicylate]. The interpretation of this difference in sensitivity, related to the alkyl chain length, requires further investigation. It can be concluded that the sensitivity of the proposed method is more than sufficient for the analysis of anionic species in wines and beverages. The precision is comparable to that obtained by other methods that require derivatization reaction[10,11]. The lack of a need for pretreatment is a major advantage, especially in food control analysis.

ACKNOWLEDGEMENT

This work was supported by the Italian National Council of Researches (CNR, grant No. 88.00075.03).

REFERENCES

1 M. C. Gennaro, *J. Liq. Chromatogr.*, 10 (1987) 3347.
2 M. C. Gennaro, *J. Chromatogr.*, 449 (1988) 103.
3 B. B. Wheals, *J. Chromatogr.*, 262 (1983) 61.
4 W. E. Barber and P. W. Carr, *J. Chromatogr.*, 316 (1984) 211.
5 M. Dreux, M. Lafosse, P. Agbo-Hazoumé, B. Chaabane-Doumandji, M. Gibert and Y. Lévi, *J. Chromatogr.*, 354 (1986) 119.
6 B. A. Bidlingmeyer, C. T. Santasania and F. V. Warren, Jr., *Anal. Chem.*, 59 (1987) 1843.
7 M. Lookabaugh, I. S. Krull and W. R. LaCourse, *J. Chromatogr.*, 387 (1987) 301.
8 B. A. Bidlingmeyer, *J. Chromatogr. Sci.*, 18 (1980) 525.
9 J. Murphy, *J. Chem. Educ.*, 60 (1983) 420.
10 E. Mentasti, M. C. Gennaro, C. Sarzanini, C. Baiocchi and M. Savigliano, *J. Chromatogr.*, 322 (1985) 177.
11 F. Caccamo, G. Carfagnini, A. Di Corcia and R. Samperi, *J. Chromatogr.*, 362 (1986) 47.

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CHROM. 21 456

Note

Determination of rubidium, sodium, calcium and thiamine in a pharmaceutical preparation by capillary isotachophoresis

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(Received February 27th, 1989)

Rubidium and sodium iodide in association with calcium and vitamins such as thiamine and vitamin C are used in Italy in the treatment of cataracts, iritis etc.[1]. Several methods have been used to determine alkali and alkaline-earth metals and thiamine, including atomic emission spectrometry, ion-selective electrodes, high-performance liquid chromatography and isotachophoresis[2–6]. The method recommended by the Italian Pharmacopoeia for sodium and calcium determination is titrimetry[7].

The composition of the pharmaceutical formulation is particularly appropriate for capillary isotachophoretic analysis, and this method was therefore applied to the determination of rubidium, sodium, calcium and thiamine in eye-drop samples.

As rubidium has a relatively high mobility, which is similar to that of the common leading cations used, we considered the possibility of modifying its effective mobility. For the separation of alkaline-earth metals by capillary isotachophoresis, complex-forming equilibria [*e.g.*, with SO_4^{2-}, α-hydroxyisobutyric acid (HIBA) and 1,2-cyclohexanediamine-N,N,N′,N′-tetraacetate (CyDTA)] have been used[8–10]. In order to modify the mobility of alkali metal ions, generally inclusion complex formation with non-ionic substances (*e.g.*, 18-crown-6) is used[11–13].

In this work, leading electrolyte containing 30 m*M* 18-crown-6 was used for the rapid determination of rubidium, sodium, calcium and thiamine in an eye-drop formulation.

EXPERIMENTAL

Apparatus

The analysis was carried out by using a LKB (Bromma, Sweden) 2127 Tachophor apparatus equipped with a conductivity detector. Also used were a PTFE capillary tube (240 mm × 0.5 mm I.D.) and a laboratory-made conductivity detector cell[14]. The resistance and its derivative were recorded with an LKB 2210 line recorder at a chart speed of 50 mm/min. The current used was 200 μA, changed to 25 μA during detection.

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Chemicals

Ammonia solution and acetic acid were purchased from Carlo Erba (Milan, Italy), 2-pyridinecarboxylic acid (2-picolinic acid), 18-crown-6, Triton X-100 from Fluka (Buchs, Switzerland) and thiamine hydrochloride from Sigma (St. Louis, MO, U.S.A.).

The sample composition was 0.800 g of rubidium iodide, 0.500 g of calcium formate, 1.200 g of sodium iodide, 0.225 g of sodium ascorbate and 0.250 g of thiamine hydrochloride in 100 ml of solution; boric acid, sodium tetraborate and lactose were also present as excipients. The sample and the standards analysed were kindly supplied by Dipartimento di Studi Farmaceutici, Università la Sapienza (Rome, Italy).

Electrolytes

A 10 m*M* solution of ammonium picolinate (pH 5.4) containing 30 m*M* 18-crown-6 and 0.4% (w/v) Triton X-100 was used as the leading electrolyte and 5 m*M* acetic acid as the terminating electrolyte.

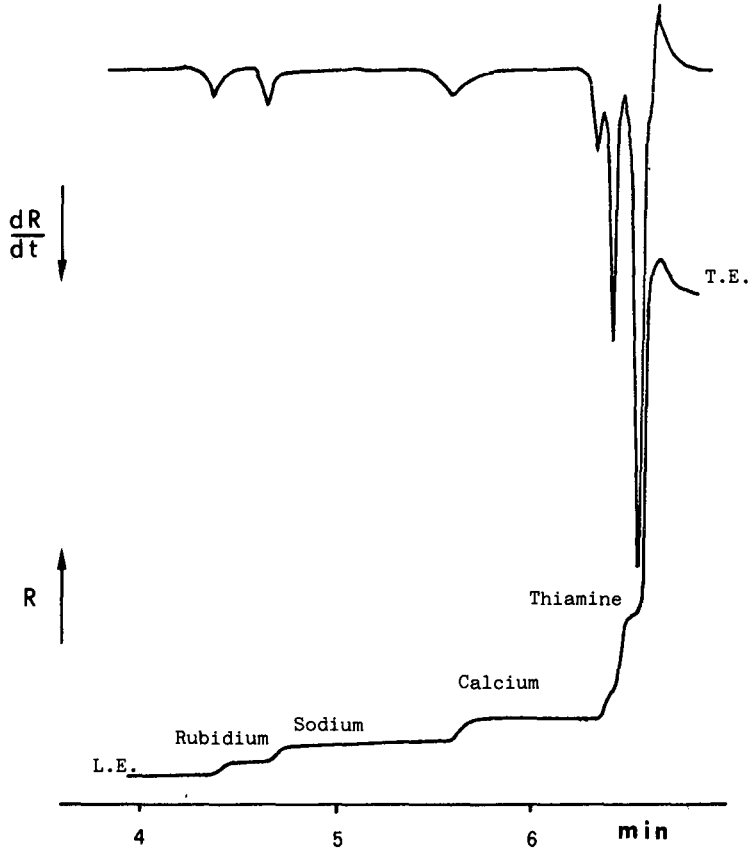

Fig. 1. Isotachopherogram for the separation of rubidium, sodium, calcium and thiamine in an eye-drop sample diluted 100-fold; 8 μl injected. Leading electrolyte: 10 m*M* ammonium picolinate (pH 5.4) containing 30 m*M* 18-crown-6 and 0.4% (w/v) Triton X-100. Terminating electrolyte: 5 m*M* acetic acid.

RESULTS AND DISCUSSION

In order to find optimal conditions for the separation of rubidium, sodium, calcium and thiamine, several electrolyte systems were tested. H^+ was used as a terminator, ammonium was the leading ion and, to ensure very low mobility for H^+, picolinate anion was the leading counter anion[15]. With this electrolyte system it was possible to obtain good resolution between sodium, calcium and thiamine. Rubidium gave a mixed zone with ammonium.

In isotachophoresis, if mixed zones are obtained it is necessary to alter the original electrolyte system in order to change the effective mobilities, usually by appropriate selection of the pH of the leading electrolyte and the use of complex formation[16]. In order to reduce the mobility of rubidium, inclusion complexation was used and 18-crown-6 was added to the leading electrolyte.

Different concentrations of the inclusion complexing agent were added to the leading electrolyte and the relative step heights were observed. The effective mobilities of calcium and thiamine were not reduced, and rubidium was separated from ammonium at a concentration of 18-crown-6 of 25 m*M*.

The optimum concentration of 18-crown-6 was found to be 30 m*M*. A standard solution containing rubidium, sodium, calcium and thiamine at concentrations similar to those in the diluted sample was used for the calibration graphs.

Fig. 1 shows an isotachopherogram of a sample of the pharmaceutical preparation analysed. The linearity of the calibration graphs was tested in the ranges $(7.2–38) \cdot 10^{-10}$, $(8–80) \cdot 10^{-10}$, $(3.8–38) \cdot 10^{-10}$, and $(2–10) \cdot 10^{-10}$ mole for rubidium, sodium, calcium and thiamine, respectively. The correlation coefficients and the relative standard deviations (eight determinations) were 0.9979 and 1.3%, 0.9989 and 0.7%, 0.9984 and 0.8%, and 0.9927 and 2.1% for rubidium, sodium, calcium and thiamine, respectively.

Complete resolution of a standard mixture is possible if the sodium and calcium amounts are not greater than $8 \cdot 10^{-9}$ and $3.8 \cdot 10^{-9}$ mole, respectively.

The results obtained show that capillary isotachophoresis is readily applicable to the determination of inorganic and organic ions in pharmaceutical formulations (no preliminary operations are required), rapid (less than 7 min) and sensitive, with good reproducibility. The method can be used for routine drug monitoring.

REFERENCES

1 *Repertorio Farmaceutico Italiano*, Cedof Editor, Milan, 2nd ed., 1988.
2 K. Kimura, M. Matsuo and T. Shono, *Chem., Lett.*, (1988) 615.
3 M. Suzuki and K. Ohta, *Anal. Chem.*, 57 (1985) 26.
4 M. Miyazaki, K. Hayakawa and S.-G. Choi, *J. Chromatogr.*, 323 (1985) 443.
5 F. L. Lam and A. Lowande, *J. Pharm. Biomed. Anal.*, 6 (1988) 87.
6 R. Roben and K. Rubach, in C. J. Holloway (Editor), *Analytical and Preparative Isotachophoresis*, Walter de Gruyter, Berlin, New York, 1984, p. 109.
7 *Farmacopea Ufficiale Italiana*, Vol. I, Istituto Poligrafico dello Stato, Rome, 9th ed., 1986, p. 118.
8 E. Schumaker, W. Thormann and D. Arn, in F. M. Everaerts (Editor), *Analytical Isotachophoresis*, Elsevier, Amsterdam, 1981, p. 33.
9 F. M. Everaerts, Th. P. E. M. Verheggen, J. C. Reijenga, G. V. A. Aben, P. Gebauer and P. Boček, *J. Chromatogr.*, 320 (1985) 263.
10 I. Nukatsuka, M. Taga and H. Yoshida, *Bull. Chem. Soc. Jpn.*, 54 (1981) 2629.

11 F. S. Stover, *J. Chromatogr.*, 298 (1984) 203.
12 M. Tazaki, M. Takagi and K. Ueno, *Chem. Lett.*, 5 (1982) 639.
13 A. A. G. Lemmens, F. M. Everaerts, J. W. Venema and H. D. Jonker, *J. Chromatogr.*, 439 (1988) 423.
14 S. Fanali and M. Sinibaldi, *J. Chromatogr.*, 442 (1988) 371.
15 V. Jokl, J. Pospichalova and M. Polasek, *Electrophoresis*, 7 (1986) 433.
16 P. Boček, M. Deml, P. Gebauer and V. Dolnik, in B. J. Radola (Editor), *Analytical Isotachophoresis (Isotachophoresis Library)*, Verlag Chemie, Weinheim, 1988.

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CHROM. 21 459

Letter to the Editor

Dot immunobinding assay of high-performance liquid chromatographic fractions on poly(vinylidene difluoride) membranes

Sir,

The presence of immunoreactive peptides in tissue extracts can be demonstrated by radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) or dot immunobinding assay (DIA). The purification of the immunoreactive peptides is facilitated by the use of high-performance liquid chromatographic (HPLC) techniques. We routinely use DIA in HPLC procedures to identify the peptides because of its great simplicity in comparison with RIA or ELISA. However, many HPLC buffers contain high concentrations of organic solvents which damage the nitrocellulose paper, the most widely used solid support for DIA[1]. We therefore tried a new hydrophobic membrane, made of poly(vinylidene difluoride) (PVDF)[2], as an alternative solid support. We have now made use of the caudodorsal cell hormone (CDCH, MW 4500), a neuropeptide which regulates egg-laying in the mollusc *Lymnaea stagnalis*, to demonstrate the usefulness of the PVDF membrane as a solid support for DIA in HPLC.

An acid extract of cerebral commissures (the neurohaemal storage site of CDCH) was size-fractionated on three high-performance gel permeation columns connected in series (one I_{300} and two I_{125} protein columns from Waters Assoc.) using 7.0 m*M* trifluoroacetic acid (TFA)–acetonitrile (70:30) as running buffer. Fractions of 1 ml were collected and 2 μl per fraction were directly dot blotted on to a PVDF membrane and immunostained using a monoclonal anti-CDCH antibody[3] as described by Batteiger *et al.*[4]. The second antibody was rabbit against mouse peroxidase

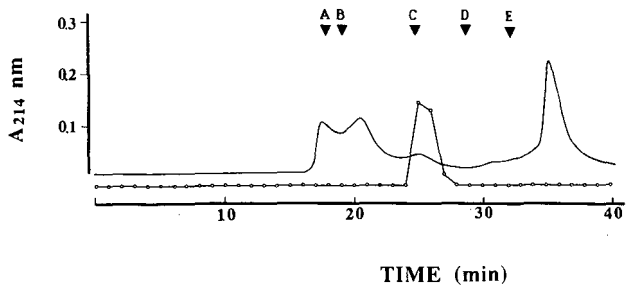

Fig. 1. High-performance gel permeation separation of cerebral commissure extracts. A 2-μl volume of each fraction was dot blotted on to a PVDF membrane, and the absorbance at 214 nm (solid line) and coloured peak areas (open circles) were measured. The molecular weight markers were (A) ovalbumin (43 kDa), (B) myoglobulin (17.8 kDa), (C) synthetic CDCH (4.5 kDa), (D) Glu–Pro–Arg–Leu–Arg–Phe–His–Asp–Val (1.2 kDa) and (E) Arg–Leu–Arg–Ala–Ser (0.6 kDa).

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conjugated antibody from Dako. The assay was developed in diaminobenzidine solution (2 mg per 10 ml of phosphate-buffered saline) containing hydrogen peroxide (2 μl of 30% H_2O_2 per 10 ml of phosphate-buffered saline), which produced coloured spots. After the membrane had been placed in 100% methanol, it was scanned with a densitometer. The coloured spots were then quantified by cutting and weighing. The results are presented in Fig. 1.

We have also used other HPLC buffers for peptide purification, including triethylamine–formate, pyridine–acetate and heptafluorobutyric acid as counter ions in reversed-phase HPLC, and in all instances specific immunostained fractions were demonstrated. We are not aware of any HPLC buffer that is not compatible with the PVDF membrane. This unique property makes the PVDF membrane an ideal solid support for DIA of proteins and peptides separated by HPLC.

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1 R. Hawkes, E. Niday and J. Gordon, *Anal. Biochem.*, 119 (1982) 142–147.
2 M. F. Pluskal, M. B. Prizekop, M. R. Kavonian, C. Vecoli and D. A. Hicks, *BioTechniques*, 4 (1986) 272–283.
3 J. van Minnen, Ch. van de Haar, A. K. Raap and E. Vreugdenhil, *Cell Tissue Res.*, 251 (1988) 477–484.
4 B. Batteiger, V. Newhall and R. B. Jones, *J. Immunol. Methods*, 55 (1982) 297–307.

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Author Index

Errata

J. Chromatogr., 465 (1989) 87–93
p. 92, Table I, first column, "μmol/l" should read "μmol/24-h".

J. Chromatogr., 467 (1989) 332–335
p. 333, 8th line of the *Validation* section, "concentration range 0.025 to 0.075 mg/ml" should read "0.05 to 0.15 mg/ml".
p. 335, ref. 1, the volume number is 227.

Vol. 472, No. 2

journal of chromatography news section

NEW BOOKS

Modern supercritical fluid chromatography, edited by C.M. White, Hüthig, Heidelberg, 1988, 239 pp., price DM 98.00, ISBN 3-7785-1569-1.

Advances in chromatography, Vol. 28, edited by J.C. Giddings, E. Grushka and P.R. Brown, Marcel Dekker, New York, 1988, 392 pp., price US$ 99.75 (U.S.A. and Canada), US$ 119.50 (rest of world), ISBN 0-8247-7878-2.

Analysis of carbohydrates by GLC and MS, edited by C.J. Biermann and G.D. McGinnis, CRC Press, Boca Raton, FL, 1988, 304 pp., price *ca.* US$ 145.00 (U.S.A.), *ca.* US$ 170.00 (rest of world), ISBN 0-8493-6851-0.

Ligand exchange chromatography, by V.A. Davankov, J.D. Navratil and H.F. Walton, CRC Press, London, 1988, 244 pp., ISBN 0-8493-6775-1.

Ion chromatography applications, by R.E. Smith, CRC Press, London, 1988, 192 pp., price £ 73.00, ISBN 0-8493-4967-2.

Experimental design: A chemometric approach, (*Data Handling in Science and Technology*, Vol. 3) by S.N. Deming and S.L. Morgan, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1988, XIV+286 pp., price (paperback) Dfl. 125.00, US$ 65.75, ISBN 0-444-43032-6.

Physical adsorption on heterogeneous solids, by M. Jaroniec and R. Madey, Elsevier, Amsterdam, New York, 1988, XIV + 353 pp., price US$ 131.50, Dfl. 250.00, ISBN 0-444-87117-9.

Analytical aspects of drug testing, edited by D.G. Deutsch, Wiley, New York, Chichester, 1989, *ca.* 320 pp., price *ca.* US$ 86.25, ISBN 0-471-85309-7.

Chemistry for the protection of the environment 1987 (*Proc. 6th Int. Conf., Torino, 15–18 Sept. 1987*), edited by L. Pawlowski, E. Mentasi, C. Sarzanini and W.J. Lacy, Elsevier, Amsterdam, New York, 1988, X + 412 pp, price US$ 168.50, Dfl. 320.00, ISBN 0-444-87130-6.

A guide to materials characterization and chemical analysis, edited by J.P. Sibilia, VCH, Weinheim, 1988, X + 318 pp., price DM 75.00.

Organic luminescent materials, by B.M. Krasovitskii and B.M. Bolotin, VCH, Weinheim, 1988, XI + 340 pp., price DM 198.00, ISBN 3-527-26880-4.

Biological monitoring for pesticide exposure: measurement, estimation and risk reduction (*ACS Symposium Series No. 382*), edited by R.G.M. Wang, American Chemical Society, Washington, DC, 1988, XI + 490 pp., price US$ 69.95 (U.S.A. and Canada), US$ 83.95 (rest of world), ISBN 0-8412-1559-6.

ANNOUNCEMENTS OF MEETINGS

ANATECH '90, 2nd INTERNATIONAL SYMPOSIUM ON APPLICATIONS OF ANALYTICAL TECHNIQUES TO INDUSTRIAL PROCESS CONTROL, NOORDWIJKERHOUT, THE NETHERLANDS, APRIL 3–5, 1990

The 2nd International Symposium on Applications of Analytical Techniques to Industrial Process Control will be held at the Leeuwenhorst Congress Centre in Noordwijkerhout, The Netherlands, from April 3 to 5, 1990.

Like its predecessor this second symposium is aimed at an interdisciplinary audience of analytical chemists with an academic or industrial background, and those involved in process analytical chemistry and process control.

Speakers will focus on recent developments in analytical techniques and applications in process control. Topics will include: sampling systems for on-line analysis of difficult to handle material *(K. Carr-Brion, U.K.)*; statistical aspects of process control/process analysis *(K. Doerffel, D.D.R.)*; teaching of process analytical chemistry *(J. Inzcedy, Hungary)*; flow injection analysis in process control and optimization *(E.D. Yalvac, U.S.A.)*; progress and perspectives in on-line process gas and liquid chromatography *(C.L. Guillemin, France)*; state-of-the-art in NIR process hardware *(D.E. Honigs, U.S.A.)*; trends and perspectives in process analyzers from the instrumentation point of view *(H. van den Hauten, The Netherlands)*. Particular attention will be paid to sampling problems, sample preparation, in-line and on-line measurements and remote sensing. The scientific programme will comprise invited as well as submitted papers (oral and posters). Time will also be reserved for roundtable discussion sessions on selected topics. The official language of the symposium will be English.

In conjuction with the symposium, an exhibition of instruments within the scope of the symposium will be held. Companies interested in participating in the exhibition should direct their enquiries to the conference secretariat.

Participants wishing to present a paper should submit an abstract, in English, of about 250 words before October 15, 1989 to the address given below. Papers presented at the symposium will be refereed for publication in a special issue of *Analytica Chimica Acta*. A copy of this issue will be sent to all participants as part of the conference package.

Fur further details, contact: Professor Willem E. van der Linden, Laboratory for Chemical Analysis-CT, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands. Tel.: (53) 892629; telex 44200; fax (53) 356024.

2nd INTERNATIONAL SYMPOSIUM ON APPLIED MASS SPECTROMETRY IN THE HEALTH SCIENCES, BARCELONA, SPAIN, APRIL 17–20, 1990

The 2nd International Symposium on Applied Mass Spectrometry in the Health Sciences will be held at the Palau de Congressos in Barcelona, Spain, from April 17–20, 1990.

The scientific programme will consist of invited lectures, group discussions and poster presentations covering the whole field of applied mass spectrometry in the health sciences.Topics will include: new developments in mass spectrometric instrumentation and novel techniques (CZE–MS, high mass methods, FTICR, ion mobility MS, etc.); recent trends and horizons in combined chromatographic and mass spectrometric techniques (LC–MS, SFC–MS nad MS–MS); applications in: clinical, metabolic and biochemical studies, molecular biology and biotechnology, respiratory gas analysis, environmental and food chemistry, toxicology and doping control, and drug assay methods and pharmacology; fundamental studies and fragmentation mechanisms of biomolecules.

All those intending to participate are invited to submit papers to be included in the scientific programme and Proceedings. Contributed papers will be refereed. Final date for submission of abstracts will be 15th November, 1989.

For further details, contact: Professor Emilio Gelpí, Symposium Secretariat, 2nd International Symposium on Applied Mass Spectrometry in the Health Sciences, Palau de Congressos, Dept. Convencions, Av. Reina Ma. Cristina s/n, 08004 Barcelona, Spain. Tel.: (343) 423 31 01, Ext. 8208-8213; telex. 53117; telefax 426 28 45.

20th INTERNATIONAL ROLAND W. FREI MEMORIAL SYMPOSIUM ON ENVIRONMENTAL ANALYTICAL CHEMISTRY, STRASBOURG, FRANCE, APRIL 17–20, 1990

The 20th International Roland W. Frei Memorial Symposium on Environmental Analytical Chemistry, organized by the International Association of Environmental Analytical Chemistry and the University of Strasbourg, will be held in Strasbourg, France, April 17–20 1990. Unfortunately the First President of the Association. Professor Dr. Roland W. Frei passed away January 29, 1989, much too early. He saw ahead to make the Strasbourg Symposium an anniversary event, and had still so many scientific plans in the field of environmental analytical chemistry. In his spirit scientists from many countries will thus again present the state of the knowledge about trace analysis related to environmental problems.

Besides the traditional broad range programme on analytical topics in water, air and soil pollution, special feature topics will be discussed. The scientific programme consists of lectures, poster presentations, and three round table discussions. The Ordinary Assembly of the International Association of Environmental Analytical Chemistry will also take place. This list of speakers and poster presentators is not yet complete. It is planned that at the most 26 oral presentations of 25 minutes each (including discussion) will take place. Additional contributions are welcome. Additional participants who want to present an oral lecture should contact Professor M.F.F. Leroy or Dr. E. Merian before November 1, 1989.

All Abstracts (up to 200 words) must arrive as one page offset original February 1, 1990, at the latest at Dr. Merians address. Speakers and poster presentators should bring their final manuscript to Strasbourg to be reviewed and to be published in the *International Journal of Environmental Analytical Chemistry* and in the *Journal of Toxicological and Environmental Chemistry* (Gordon and Breach Science Publishers). Proceedings will be composed by the editors and will be included in the full registration fee. Posters may still be submitted until February 1, 1990. The poster boards will be 80 cm wide and 110 cm high.

A Technical Exhibition of international suppliers of analytical and laboratory equipment will be located on the same floor as the lecture hall and poster/coffe area.

The chairman of the symposium is: Professor Dr. Maurice J.F. Leroy, Laboratory of Mineral Chemistry, Ecole Européenne des Hautes Études des Industries Chimiques, 1 rue B. Pascal, P.p. 296/R8, F-67008 Strasbourg cédex, France, Tel.: (33) 88-619-576; telex: ULP 870-260F; fax: EHICS 88 61 78 52. The secretary of the scientific programme is: Dr. Ernest Merian, Im Kirsgarten 22, Ch-4106 Therwil, Switzerland, Tel.: (41) 61-732950. For registration contact (before February 1, 1990): Mrs. Marianne Frei-Häusler, P.O. Box 46, CH-2123 Allschwil, Switzerland, Tel.: (41) 61-632789.

4th WORKSHOP ON THE CHEMISTRY AND ANALYSIS OF ENVIRONMENTAL HYDROCARBONS, STRASBOURG, FRANCE, APRIL 19–21, 1990

The 4th Workshop on the Chemistry and Analysis of Environmental Hydrocarbons, organized by the International Association of Environmental Analytical Chemistry and the University Louis Pasteur, will follow the 20th International Roland W. Frei Memorial Symposium on Environmental Analytical Chemistry in Strasbourg, France.

The following major topics will be covered: source and transport of hydrocarbons (or their functionalized counterparts); biochemical and photochemical transformations; pollution indicators; analytical methods; and case studies. Also the Workshop will consist of about 12 lectures (of 25 minutes, including discussion), poster presentations, and discussions. Additional contributions are welcome (additional participants who want to present an oral presentation should contact Professor P. Albrecht or Dr. E. Merian before November 1, 1989).

All abstracts (up to 200 words) must arrive as one page offset original February 1, 1990 at the latest at Dr. Merians address. Speakers and poster presentators should bring their final manuscript to Strasbourg to be reviewed and published in the *International Journal of Environmental Analytical Chemistry* and in the *Journal of Toxicological and Environmental Chemistry* (Gordon and Breach Science Publishers). Proceedings will be composed by the editors and will be included in the full registration fee. The poster boards will be 80 cm wide and 110 cm high.

The chairmen of the workshop are: Professor Dr. Joan Albaiges, C.I.D.-C.S.I.C., Girona Salgado 18–26, E-08034 Barcelona, Spain, tel.: (343) 2040-600, and Professor Dr. Pierre Albrecht, University Louis Pasteur, Blaise Pascal 1, F-67008 Strasbourg, France, tel.: (33) 88-416841; Co-chairman: Dr. Ernest Merian, Im Kirsgarten 22, CH-4106 Therwil, Switzerland, tel.: (41) 61-732950. For registration contact: Mrs. Marianne Frei-Häusler, P.O. Box 46, CH-4123 Allschwil, Switzerland, tel.: (41) 61-632789.

4th SYMPOSIUM ON THE ANALYSIS OF STEROIDS, PÉCS, HUNGARY, APRIL 24–26, 1990

The 4th Symposium on the Analysis of Steroids will be held in Pécs, one of the most beautiful historical cities in Hungary, from April 24–26, 1990.

The topics and scope of the symposium are: (1) determination of steroids in biological samples: clinical steroid analysis; analytical methodology for studying the biosynthesis and metabolism of steroids as well as the pharmacokinetic study of stereoid drugs; determination of steroids in samples of plant and animal origin; and (2) industrial and pharmaceutical steroid analysis: methods for the purity testing of steroids; analysis of pharmaceutical dosage forms including stability assays; analysis of the intermediates of steroid syntheses; structure elucidation of steroids. The scope of the symposium covers discussion of methodological problems as well as practical applications drawn from the fields of all the main steroid groups.

The language for presentation and discussion of all papers will be English.

Plenary lectures by acknowledged international authorities on the field of steroid analysis will highlight new developments. Contributed papers are invited for oral presentation (time: 15 min, including discussion) or poster presentation. Abstracts (up to 200 words) must be submitted not later than September 15, 1989. Please indicate in the covering letter if you wish to present your paper as a lecture or as a poster. The material will appear in the Symposium Book of Abstracts, which will be distributed to all registered participants. Authors will be informed of the acceptance of their submitted papers and the form of presentation (lecture or poster) by November 15, 1989.

All correspondence concerning the 4th Symposium on the Analysis of Steroids should be addressed to the Chairman of the Organizing Committee: Professor S. Görög, c/o Chemical Works of Gedeon Richter Ltd., P.O. Box 27, H-1475 Budapest, Hungary. Tel.: (361) 574566, telex: 22-5067 RICHT H, fax: (361) 473973.

HPLC '90, 14th INTERNATIONAL SYMPOSIUM ON COLUMN LIQUID CHROMATOGRAPHY, BOSTON, MA, U.S.A., MAY 20–25, 1990

The 14th International Symposium on Column Liquid Chromatography will be held at the Boston Park Plaza Hotel, Boston, MA, U.S.A. on May 20–25, 1990. Boston was also the site of the 4th CLC Symposium, HPLC '79.

The symposium, which alternates between the U.S.A. and Europe, is the scientific meeting for the presentation of the most recent advances in the rapidly growing fields of liquid chromatography and related separation methods. Various sessions will address advances in separation methods, mechanisms, selective and sensitive detection, sample preparation, automation, derivatization, preparative liquid chromatography, biopolymer separation, chiral recognition, capillary and conventional electrophoresis, supercritical-fluid chromatography, field flow fractionation, and related techniques.

The scientific programme will include presentations on the latest HPLC developments and related techniques. Expert scientists will be invited to present lectures on topics of high interest. A special emphasis for HPLC '90 will be on bioanalytical chemistry, including the separation and detection of biopolymers, pharmaceuticals, bioorganics, and related biologically active materials. The topics will be covered in several formats: general and invited lectures devoted to specific topics; poster sessions for interaction among the presenters and their audience; informal discussion sessions covering new and developing techniques; and an exhibition of the latest instrumentation will be organized.

The Organizing Committee welcomes your contributions of recent research results. Please submit a 150-word abstract of your proposed presentation to the Symposium Manager before October 1, 1989.

The social programme will include a reception and scheduled sightseeing tours of Boston and its environs.

For additional information contact: Ms. Shirley E. Schlessinger, Symposium Manager, HPLC '90, 400 East Randolph Drive, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011.

3rd EUROPEAN MEETING ON BIO-CHROMATOGRAPHY AND MOLECULAR AFFINITY, DIJON, FRANCE, MAY 22–25, 1990

The 3rd European Meeting of "Groupe Francais de Bio-Chromatographie" will be held at the Congress Center in Dijon, France, May 22–25, 1990.

The scientific programme will consist of invited lectures, posters and oral presentations. The main topics are:

— Biochromatography: supports and derivatization; ion-exchange chromatography; hydrophobic interactions; gel filtration.
— Molecular recognition and affinity chromatography: interactions with solubilized ligands; interactions with immobilized ligands; biospecific affinity; immuno-affinity.
— Pseudo-specific affinity: dye ligand affinity; immobilized metal affinity; amino acid affinity.
— Protein conformation and chromatographic behaviour.

Details regarding submission of papers will be given in the second announcement. The deadline for submission is December 31, 1989.

Manufacturers, distributors and suppliers of products and service to the specific field of biochromatography will find this meeting an opportunity to display products and services. an estimated 300 scientific specialists, researchers, teachers and students from all countries will attend the meeting.

Please address all correspondence concerning the congress to: Groupe Francais de Bio-Chromatographie, Unité d'Immuno-Allergie, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France. Tel.: (1) 45 68 80 00; telex: 250 609 F; fax: (1) 43 06 98 35.

3rd WORKSHOP ON CHEMISTRY AND FATE OF MODERN PESTICIDES, BILTHOVEN, THE NETHERLANDS, SEPTEMBER 4–6, 1991

The first Workshop on Chemistry and Fate of Organophosphorus Compounds was held in Amsterdam (June, 1986) and a second one in Barcelona (November, 1987). The third Workshop in this series will be entitled 3rd Workshop on Chemistry and Fate of Modern Pesticides. The previous topic, organophosphorus compounds, will be extended with nitrogen- and sulphur-containing pesticides.

Environmental chemists, analytical chemists, toxicologists and biologists will discuss in an interdisciplinary way relevant scientific problems. Emphasis will be placed on environmental and analytical chemistry (sample preparation, GC, LC, GC–MS, LC–MS, SFC–MS), ecotoxicology and environmental modelling. The proceedings are planned in the *International Journal of Environmental Analytical Chemistry*. The symposium language is English, no simultaneous translation will be given.

For further details contact: Pesticides Workshop Office, Dr. P. van Zoonen, RIVM, P.O. Box 1, 3720 BA, Bilthoven, The Netherlands.

COURSE

SHORT COURSE ON DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLOW INJECTION ANALYSIS, CORDOBA, SPAIN, SEPTEMBER 18–19, 1989

This Short Course, to be held at the Department of Analytical Chemistry, Science Faculty of the University of Cordoba, Spain, will precede the International Symposium on HPLC and FIA, September 20–22, 1989, at the same location.

The course is intended to introduce participants to recent approaches to detection in continuous analytical flow systems (liquid chromatography and flow injection analysis). This will include both spectroscopic (atomic and molecular) and electrochemical (potentiometric and amperometric) detectors as well as hyphenated techniques. Instrument demonstrations and applications will be offered by instrument manufacturers: Hewlett-Packard for HPLC and Scharlau Science for FIA.

The registration fee is: SFr. 550 (SFr. 500 for attendants to the symposium). Detailed information on the course is made available upon registration (deadline August 15, 1989).

The contact address for registration is: Workshop Office IAEAC, Mrs. M. Frei-Häusler, P.O. Box 46, CH-4123 Allschwil, Switzerland. Tel.: (41) 61-632789 and 632950.

DATABASE

EVENTLINE, A NEW EVENTS DATABASE

Eventline, recently launched by Elsevier, is a new database of conferences, conventions, symposia, trade fairs, exhibitions, and sporting events. Eventline will be a 'one stop' source of information on all types of events taking place globally, from high level scientific conferences to major sporting events. None of this information will be made available in a printed format, other than Eventline's so called 'on demand' service. This means that the data supplied to a customer comes directly from the database in the form of a printout. Current information sources offer primarily either printed directories or subscriptions to periodicals which inevitably means that they can soon be out of date and provide information which is surplus to requirements. Eventline is designed to be a moderately priced, easy to use, 'one stop' information service on all types of events. The basic idea is that you specify what sort of search you want done, and that you only receive information on those events which meet the criteria you have specified. Eventline currently holds more than 13 000 events which are due to be held between now and the year 1999. This number is being added to daily in order to make the database even more complete. A Full brochure on Eventline, which includes a tariff of fees, is available from any of the offices below: in the U.S.A. and Canada: Eventline, P.O. Box 57101, Philadelphia, PA 19111, U.S.A., Tel.: (215) 572 7424, Fax: (215) 572 7393; in Asia/Pacific (excl. Japan): Eventline, 67 Wyndham St, 8th Floor, Hong Kong, Tel.: 852 5 222536, Telex: 011-75909; in Europe: Eventline, P.O. Box 521, 1000 AN Amsterdam, The Netherlands, Tel.: 31 20 5862751, Fax: 31 20 5862850; and in Japan: Elsevier, 3-28-1 Yushima, Bunkyo-ku, Tokyo, Tel.: 81 3 836 0810, Fax: 81 3 836 0204.

CALENDAR OF FORTHCOMING EVENTS

July 17–18, 1989 Ithaca, NY, U.S.A.	**Short Course on LC—MS, SFC–MS, and CZE–MS** Contact: Department of Conference Services, LC–MS, Box 3, Robert Purcell Union, Cornell University, Ithaca, NY 14853, U.S.A.
July 19–21, 1989 Ithaca, NY, U.S.A.	**6th (Montreux) Symposium on Liquid Chromatography–Mass Spectrometry (LC–MS, SFC–MS, CZE–MS, MS–MS)** Contact: Department of Conference Services, LC–MS, Box 3, Robert Purcell Union, Cornell University, Ithaca, NY 14853, U.S.A. (Further details published in Vol. 467, No. 2.)
July 30–Aug. 4, 1989 Denver, CO, U.S.A.	**3rd International Symposium on Supercritical Fluid Chromatography** Contact: B. Wenclawiak, Physikalisches Institut der Universität Münster, Wilhelm-Klemm Strasse 10, D-4400 Münster, F.R.G.

July 30–August 5, 1989 Cambridge, U.K.	**SAC 89, International Conference on Analytical Chemistry** Contact: SAC 89, Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, U.K. Tel.: (01) 437-8656. (Further details published in Vol. 407.)
July 31–Aug. 4, 1989 New Hampton, NH, U.S.A.	**Gordon Research Conference on Statistics in Chemistry and Chemical Engineering** Contact: Dr. A.M. Cruickshank, Gordon Research Conferences, University of Rhode Island, Kingston, RI 02881, U.S.A. Tel.: (401) 783-4011.
Aug. 2–7, 1989 Lund, Sweden	**32nd IUPAC Congress** Contact: IUPAC, c/o Stockholm Convention Bureau, P.O. Box 6911, S-102 39 Stockholm, Sweden. Tel.: (46) 8230990, telex: 11556, FAX: 46 8 34 84 41. (Further details published in Vol. 450, No. 3.)
Aug. 21–25, 1989 Leipzig, G.D.R.	**7th Danube Symposium on Chromatography** Contact: 7th Danube Symposium on Chromatography, Karl-Marx-Universität Leipzig, Sektion Chemie, Talstrasse 35, Leipzig, G.D.R. (Further details published in Vol. 411.)
Aug. 21–25, 1989 Amsterdam, The Netherlands	**5th International Conference on Particle Induced X-Ray Emission and its Analytical Applications** Contact: 5th PIXE Conference, Department of Physics and Astronomy, Free University, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5486224. (Further details published in Vol. 445, No. 1.)
Aug. 28–Sept. 1, 1989 Wiesbaden, F.R.G.	**11th International Symposium on Microchemical Techniques** Contact: Gesellschaft Deutscher Chemiker, Abt. Tagungen, P.O. Box 900440, D-6000 Frankfurt/Main 90, F.R.G. Tel.: (069) 79 17-366/360, telex: 4170497 gdch d. (Further details published in Vol. 456, No. 2.)
Sept. 1–3, 1989 Leiden, The Netherlands	**2nd International Symposium on Disposition and Delivery of Peptide Drugs** Contact: Dr. J. Verhoef, Center for Bio-Pharmaceutical Sciences, P.O. Box 9502, 2300 RA Leiden, The Netherlands.
Sept. 4–8, 1989 Colymbari, Crete Greece	**Pesticides and Alternatives, International Conference on Innovative Chemical and Biological Approaches to Pest Control** Contact: Professor John Casida, Department of Entomological Sciences, University of California, Berkeley, CA 94720, U.S.A. Tel.: (415) 642-5424.
Sept. 5–8, 1989 Guildford, U.K.	**8th International Bioanalytical Forum: Analysis for Drugs and Metabolites, Including Anti-infective Agents** Contact: Dr. E. Reid, Guildford Academic Associates, 72 The Chase, Guildford, GU2 5UL, U.K. Tel.: (0483) 65324. (Further details published in Vol. 464, No. 2.)

Sept. 10–15, 1989 Antwerp, Belgium	**International Symposium on Gas Separation Technology** Contact: Dr. R. Dewolfs, University of Antwerp, Department of Chemistry, Universiteitsplein 1, B-2610 Antwerp-Wilrijk, Belgium. Tel.: (32) 3-828 25 28, ext. 204 or 215; telex: 336 46 UIA B; telefax: (32) 3-827 08 74. (Further details published in Vol. 438, No. 2.)
Sept. 12–15, 1989 Guildford, U.K.	**2nd International Symposium on Chiral Separations** Contact: The Chromatographic Society, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, U.K. Tel.: (0602) 418248, ext. 2187; Fax: (0602) 484266; telex: 377534.
Sept. 18–19, 1989 Cordoba, Spain	**Short Course on Detection in High-Performance Liquid Chromatography and Flow Injection Analysis** Contact: Colegio Mayor La Asuncion, Avda. Menendez Pidal s/n, 14004 Cordoba, Spain. Tel.: (9) 57-297144.
Sept. 18–22, 1989 Edmonton, Canada	**Annual Conference of the Canadian Society of Forensic Science** Contact: Canadian Society of Forensic Science, Suite 215, 2660 Southvale Cresent, Ottawa, Ontario, Canada K1B 4W6. Tel.: (613) 731-2096
Sept. 19–21, 1989 Birmingham, U.K.	**5th BOC Priestly Conference** Contact: Dr. B.D. Crittenden, School of Chemical Engineering, Claverton Down, Bath BA2 7AY, U.K. Tel.: (0225) 826826, telex: 449097.
Sept. 19–22, 1989 Antwerp, Belgium	**International Symposium on the Analysis of Nucleoside, Nucleotide and Oligonucleotide Compounds** Contact: Dr. E.L. Esmans or Mr. J. Schrooten, University of Antwerp (R.U.C.A.), Laboratory for Organic Chemistry, Groenenborgerlaan 171, B-2020 Antwerp, Belgium. Tel.: (03) 2180233 or (03) 2180496, telex: 33362 rucabi, Fax: (03) 2180217. (Further details published in Vol. 448, No. 3.)
Sept. 20–22, 1989 Cordoba, Spain	**Symposium on Detection in Flow Injection Analysis and High-Performance Liquid Chromatography** Contact: Workshop Office IAEAC, Ms. M. Frei-Häusler, Postfach 46, CH-4123 Allschwil 2, Switzerland. (Further details published in Vol. 448, No. 3.)
Sept. 24–29, 1989 New York, NY, U.S.A.	**28th Eastern Analytical Symposium** Contact: David S. Klein, 642 Cranbury Cross Road, North Brunswick, NJ 08902, U.S.A.
Sept. 25–28, 1989 St. Louis, MO, U.S.A.	**103rd AOAC International Meeting and Exposition** Contact: Margaret Ridgell, AOAC, 1111th North 19th Street, Suite 210, Arlington, VA 22209, U.S.A. Tel.: (703) 522-3032. (Further details published in Vol. 467, No. 2.)
Sept. 27–29, 1989 Paris, France	**Euro Food Chem V, 5th European Conference on Food Chemistry** Contact: G.A.M.S., 88 Boulevard Malesherbes, 75008 Paris, France. Tel.: (161) 45639304.

Oct. 1–4, 1989 Hamilton, Canada	**2nd International Conference on Separation Science and Technology** Contact: V. Lakshmanan, Ontario Research Foundation, Mississauga, Ontario, L5K 1B6 Canada.
Oct. 1–5, 1989 Toronto, Canada	**3rd International Congress on Chemical Sensors** Contact: Dr. M. Thompson, Department of Chemistry, University of Toronto, 80 St. George Street, Ontario, M5S 1A1 Canada.
Oct. 1–6, 1989 Chicago, IL, U.S.A.	**FACSS XVI, 16th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies** Contact: Robert Michel, FACSS XVI Program Chair, Department of Chemistry, University of Connecticut, U-60, Storrs, CT 06268, U.S.A. Tel.: (203) 486-3143. (Further details published in Vol. 467, No. 2.)
Oct. 17–20, 1989 Tokyo, Japan	**10th International Symposium on Chromatography, CIS '89** Contact: Tadao Hoshino, Pharmaceutical Institute, School of Medicine, Keio University, 35-Shinanomachi, Shinjuku-ku, Tokyo 160, Japan. (Further details published in Vol. 456, No. 2.)
Oct. 22–27, 1989 Knoxville, TN, U.S.A.	**6th Symposium on Separation Science and Technology for Energy Applications** Contact: Dr. J.T. Bell, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6268, U.S.A. Tel.: (615) 574-4934 or 574-6795. (Further details published in Vol. 456, No. 2.)
Oct. 29–Nov. 3, 1989 Rehovot, Israel	**8th International Symposium on Affinity Chromatography and Biological Recognition** Contact: E.A. Bayer and F. Kohen, AC&BR Secretariat, The Weizmann Institute, Institute of Science, Rehovot 76100, Israel. (Further details published in Vol. 448, No. 3.)
Nov. 6–8, 1989 Philadelphia, PA, U.S.A.	**9th International Symposium on High-Performance Liquid Chromatographic Separation of Proteins, Peptides and Polynucleotides** Contact: Janet E. Cunningham, Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772. (Further details published in Vol. 462.)
Nov. 15, 1989 London, U.K.	**Applications of Evolved Gas Chromatography** Contact: Dr. C.J. Keattch, Honorary Secretary of the Thermal Methods Group, Industrial and Laboratory Services, P.O. Box 9, Lyme Regis, Dorset DT7 3BT, U.K. Tel:: (02974) 2472.
Feb. 4–8, 1990 Bendigo, Australia	**18th Australian Polymer Symposium** Contact: Dr. J.D. Wells, Bendigo College of Advanced Education, Bendigo, Victoria 3550, Australia.
April 3–5, 1990 Noordwijkerhout, The Netherlands	**ANATECH '90, 2nd International Symposium on Applications of Analytical Chemical Techniques to Industrial Process Control** Contact: Professor Dr. Willem E. van der Linden, Laboratory for Chemical Analysis-CT, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

April 17–20, 1990 Barcelona, Spain	**2nd International Symposium on Applied Mass Spectrometry in the Health Sciences** Contact: Professor Emilio Gelpí, Palau de Congressos, Dept. de Convensions, Av. Reina Ma. Cristina s/n, 08004 Barcelona, Spain.
April 8–11, 1990 Ghent, Belgium	**7th International Symposium on Preparative Chromatography** Contact: Professor M. Verzele, RUG-LOS, Krijgslaan 281 (S4), B-9000 Ghent, Belgium. Tel.: (091) 225715; fax: (091) 228321.
April 17–20, 1990 Strasbourg, France	**20th International Roland W. Frei Memorial Symposium on Environmental Analytical Chemistry** Contact: Mrs. Marianne Frei-Häusler, P.O. Box 46, CH-2123 Allschwil, Switzerland. Tel.: (41) 61–632789.
April 19–21, 1990 Strasbourg, France	**4th Workshop on the Chemistry and Analysis of Environmental Hydrocarbons** Contact: Mrs. Marianne Frei-Häusler, P.O. Box 46, CH-2123 Allschwil, Switzerland. Tel.: (41) 61-632789.
April 24–26, 1990 Pécs, Hungary	**4th Symposium on the Analysis of Steroids** Contact: Professor S. Görög, c/o Chemical Works of Gedeon Richter Ltd., P.O. Box 27, H-1475 Budapest, Hungary. Tel.: (361) 574566, telex: 22–5067 RICH H, fax: (361) 473973.
May 20–25, 1990 Boston, MA, U.S.A.	**HPLC '90, 14th International Symposium on Column Liquid Chromatography** Contact: Ms. Shirley, E. Schlessinger, Symposium Manager HPLC '90, 400 East Randolph Drive, Chicago, IL 60601, U.S.A. Tel.: (312) 527 2011.
May 22–25, 1990 Dijon, France	**3rd European Meeting on Bio-Chromatography and Molecular Affinity** Contact: J.-P. Dandeu, Groupe Français de Bio-Chromatographie, Institut Pasteur, Unité d'Immuno-Allergie, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France. Tel.: (1) 45688000. (Further details published in Vol. 456, No. 2.)
July 9–11, 1990 Wrexham, U.K.	**Ion-Ex 90, International Conference and Industrial Exhibition on Industrial, Analytical and Preparative Applications of Ion-Exchange Processes** Contact: Ion-Ex 90, Conference Secretariat, Faculty of Research and Innovation, The North East Wales Institute, Connah's Quay, Deeside, Clwyd CH5 4BR, U.K. Tel. (0244) 817531, ext. 276 or 234, telex: 61629 NEWI G, fax: (0244) 822002. (Further details published in Vol. 464, No. 2.)
Aug. 26–31, 1990 Vienna, Austria	**Euroanalysis VII, 7th European Conference on Analytical Chemistry** Contact: Professor Dr. M. Grasserbauer, c/o Interconvention, Austria Center Vienna, A-1450 Vienna, Austria. Tel.: (43) 222-2369/647; telex: 111803 icos a, Fax: (43) 222-2369/648. (Further details published in Vol. 445, No. 1.)

Aug. 26–31, 1990 Prague, Czechoslovakia	**10th International Congress on Chemical Engineering, Chemical Equipment Design and Automation** Contact: Congress CHISA '90, P.O. Box 857, 111 21 Prague 1, Czechoslovakia. Telex: 121114 chp c.
Sept. 23–28, 1990 Amsterdam, The Netherlands	**18th International Symposium on Chromatography** Contact: 18th International Symposium on Chromatography, RAI Organisatie Bureau Amsterdam bv, Europaplein 12, 1078 GZ Amsterdam, The Netherlands. Tel.: (31-20) 549 1212; telex: 13499 raico nl; Fax: (31-20) 464469. (Further details published in Vol. 464, No. 2.)
Oct. 19–23, 1990 Adelaide, Australia	**27th Meeting of the International Association of Forensic Toxicologists** Contact: V.J. McLinden, Chemistry Center (WA), 125 Hay Street, Perth, Western Australia 6000, Australia. (Further details published in Vol. 467, No. 2.)
Oct. 28–31, 1990 San Francisco, CA, U.S.A.	**ANABIOTEC '90, 3rd International Symposium on Analytical Methods in Biotechnology** Contact: Shirley Schlessinger, ANABIOTEC '90, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A. (Further details published in Vol. 448, No. 3.)
March 4–7, 1991 Les Diablerets, Switzerland	**4th Hans Wolfgang Nürnberg Memorial Workshop on Toxic Metal Compounds (Interrelation Between Chemistry and Biology)** Contact: Dr. Ernest Merian, Im Kirsgarten 22, CH-4106 Therwil, Switzerland.
Sept. 4–6, 1991 Bilthoven, The Netherlands	**3rd Workshop on Chemistry and Fate of Modern Pesticides** Contact: Pesticides Workshop Office Dr. P. van Zoonen, RIVM, P.O. Box 1, 3720 Bilthoven, The Netherlands.

PUBLICATION SCHEDULE FOR 1989

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	J	A	S	
Journal of Chromatography	461 462 463/1	463/2 464/1	464/2 465/1 465/2	466 467/1 467/2	468 469 470/1 470/2	471 472/1 472/2 473/1	473/2 474/1 474/2 475	476 477/1 477/2		The publication schedule for further issues will be published later
Bibliography Section		486/1		486/2		486/3		486/4		
Biomedical Applications	487/1	487/2	488/1 488/2	489/1 489/2	490/1 490/2	491/1	491/2	492 493/1	493/2	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 445, 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.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Notes and Letters to the Editor are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press" (journal should be specified), "submitted for publication" (journal should be specified), "in preparation" or "personal communication".

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