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CHROMATOGRAPHIC BEHAVIOUR OF AROMATIC COMPOUNDS ON NON-POLAR PHASES WITH ADSORBED QUATERNARY AMMONIUM SALTS

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(Received March 18th, 1981)

SUMMARY

Hexadecyltrimethylammonium (HDTMA) acetate and glycolate were strongly adsorbed from 1 m*M* solution on ODS-Hypersil (silica gel with octadecyl groups) and on Hitachi gel (porous styrene–divinylbenzene copolymer) in aqueous sodium acetate or glycolate solution. The presence of HDTMA favoured the sorption of aromatic carboxylate anions, while their retention was suppressed by increasing the concentration of the sodium salts. Under comparable conditions the presence of HDTMA had no significant effect on the sorption of the aromatic anions onto a commerical anion-exchange resin of the quaternary ammonium type (Dowex 1-X8), indicating that the ion pairing in the external solution was negligible. The results show that, in the presence of HDTMA, the non-ionic phases were converted into anion exchangers, and that ion exchange had a dominant effect on the retention of the benzoates and phenylacetates studied. Hydrophobic interactions contributed markedly to the sorption of the hydrophobic anions and phenols, while, for both types of solutes, hydrogen bonding between phenolic protons and the stationary phases was less important than with Dowex 1-X8.

INTRODUCTION

In a previous paper[1] the retention volumes were determined for phenols and aromatic carboxylate anions on a styrene–divinylbenzene anion exchanger containing quaternary ammonium groups (Dowex 1-X8), in solutions of sodium salts of simple aliphatic acids. Non-polar interactions of alkyl groups, and hydrogen bonding between phenolic hydroxyl protons and the aliphatic carboxylate anions in the resin, led to increased retention volumes of both ions and non-electrolytes, while intramolecular hydrogen bonding suppressed the hydrogen bonding with the resin. We here report on the sorption of the same solutes on non-polar stationary phases in aqueous sodium acetate and glycolate solutions containing hexadecyltrimethylammonium (HDTMA) ions. A porous copolymer, Hitachi gel 3011, with a chemical composition (styrene–divinylbenzene) similar to that of the matrix in the anion-exchange resin and a silica gel with covalently linked octadecyl groups (ODS-Hypersil) were used as column packings.

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Chromatography on non-polar phases in eluents containing ionic long-chain alkyl compounds has recently been reviewed[2,3]. Ion-pair chromatography and dynamic ion-exchange chromatography are among the names commonly used for related techniques. As shown below, ion-pair formation in the external solution is of little importance in the systems studied in the present work. Chromatography is inherently a dynamic process. We feel that the term "chromatography on sorbed ionic sites" would be better, and that the term "ion-pair chromatography" should be used only when ion pairing in the external solution is the predominant reason for the retention of the solutes.

EXPERIMENTAL

The jacketed stainless-steel columns (100 × 5 mm I.D.) were thermostatted by circulating water. ODS-Hypersil (5 μm; Shandon Southern, Runcorn, Great Britain) was packed according to the procedure recommended by Aslin[4]. The Hitachi gel (12 ± 5 μm; Hitachi, Tokyo, Japan) was kept in methanol. The solvent was displaced by water. The resin slurry was transferred to a reservoir attached to the column, and the column was packed by pumping distilled, boiled-out water through the system at 30 MPa.

Commercial hexadecyltrimethylammonium (HDTMA) chloride was converted into the acetate or glycolate by passing a 20% solution of it through a column packed with a polystyrene–divinylbenzene adsorbent, Amberlite XAD-2 (150 ± 30 μm; Rohm & Haas, Philadelphia, PA, U.S.A.). The column was then treated with ten bed volumes of 1 *M* sodium acetate (or glycolate) solution followed by five bed volumes of water. The acetate or glycolate form of HDTMA was eluted with five bed volumes of 92% (w/w) aqueous ethanol. Ethanol was removed by evaporation under reduced pressure and the sample dried under vacuum before weighing. The salts, which were free from chloride, were dissolved in water and stored as 10% (w/w) aqueous solutions.

The aromatic compounds were dissolved in distilled, boiled-out water and applied to the column, which was preconditioned with the eluent. Sparingly soluble acids were added as their sodium salts. To determine the retention volume, single compounds were chromatographed and the UV-absorbance was measured at 254 and 280 nm. The reported retention data were obtained with well separated mixtures of three to six compounds. The sample solutions (20 μl) were introduced through a sample loop. The nominal linear (empty tube) flow-rate was 5.1 cm min^{-1}.

Repeated runs under identical conditions showed that the retention volumes on both columns were reproducible within ± 1% for compounds eluted within less than 50 bed volumes. For compounds which appeared very late as broad peaks, the reproducibility was within ±2%. In the experiments referred to in Tables II and III, the loaded amounts were 0.20 μmol of each compound. Variations between 0.10 and 0.40 μmol had no significant effect on the retention volume.

The retention volumes are reported as $\bar{v}/X$, where $\bar{v}$ is the peak elution volume and X the bed volume. Some compression of the column packings occurred during prolonged use. This was disregarded when calculating the bed volume. The space at the top of the columns was filled with a thin layer of glass beads. The difference between ln $\bar{v}/X$ for a substituted compound, *e.g.*, methylphenol, and the parent com-

pound (phenol) in the same medium is denoted by Δ. The breakthrough curves of HDTMA were determined for solutions of sodium glycolate and sodium acetate. The curves were recorded with a refractive index detector, and the results checked by colorimetric tests based on addition of potassium permanganate at pH 3 and extraction with chloroform[5].

RESULTS AND DISCUSSION

Influence of the HDTMA concentration

Like the experiments with Dowex 1-X8 reported previously[1], those with the stationary phases reported here were made at pH 7, which means that the aromatic carboxylic acids are almost completely ionized, while the phenols can be considered as non-electrolytes. The comparison between the results obtained in these investigations is facilitated by the fact that the distribution coefficients on Dowex 1-X8 were only slightly affected when 1 m*M* HDTMA was present. For 4-hydroxyphenol the distribution coefficient decreased by 1 % compared to a blank without HDTMA, while an increase of 3 % was recorded for 4-hydroxy-3-methoxybenzoate and 1 % for 4-hydroxy-3,5-dimethoxyphenylacetate. Evidently, ion-pair formation in the eluent has no decisive influence on the sorption in this system. The experiments were made at 50°C, and, in agreement with results reported for other solutes, the distribution coefficients were higher than those at 60°C reported in the previous paper[1].

As shown in Fig. 1, the retention volumes for aromatic carboxylate anions in 0.5 *M* sodium acetate were much higher when HDTMA was present than in its absence. On ODS-Hypersil the highest values were obtained at a concentration of 0.1 mmol HDTMA per litre of eluent, while a slight, but significant, decrease was observed at the ten-fold concentration (*cf.*, ref. 6). For the Hitachi gel an asymptote was approached in 0.25 m*M* HDTMA solution. No further change was obtained by an increase to 1 m*M* HDTMA.

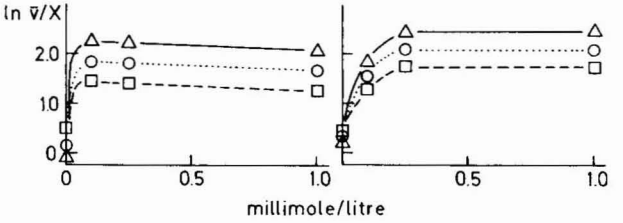

Fig. 1. Relationships between ln $\bar{v}/X$ and concentration of HDTMA in 0.5 *M* sodium acetate at pH 7 and 50°C on ODS-Hypersil (left) and Hitachi gel (right). △, 4-Hydroxybenzoate; ○, 4-hydroxy-3-methoxybenzoate; □, 4-hydroxy-3,5-dimethoxybenzoate.

In the absence of quaternary ammonium ions, 4-hydroxybenzoate was, on both stationary phases, held less strongly than the other anions studied. This was expected since this anion is more hydrophilic than the other anions. 4-Hydroxy-3-methoxybenzoate, with the phenolic proton linked to the vicinal methoxyl group by hydrogen bonding, is less hydrophilic. Accordingly, this anion exhibited a larger retention volume on the non-ionic stationary phases. More effective hydrogen bond-

ing, and shielding of the hydroxyl group by two adjacent methoxyl groups, explain the strong sorption of 4-hydroxy-3,5-dimethoxybenzoate.

The elution order was reversed when HDTMA was present in the eluent, and was the same as the order previously established for conventional anion-exchange resins in acetate and glycolate media. The results indicate that, in the presence of HDTMA, the stationary phases constitute anion exchangers[7,8] with an ability to bind the carboxylate ions as counter ions.

The effect of the HDTMA concentration was less dramatic for the non-ionized phenolic compounds than for the anions (Fig. 2). A large increase in the retention volume was observed for 4-hydroxyphenol, which is held by hydrogen bonding to acetate counter ions in anion exchangers[1]. The smallest effect of HDTMA, and at the same time the largest adsorption on both stationary phases in the presence and absence of quaternary ammonium ions, was observed for 2-methoxyphenol. Intramolecular hydrogen bonding, resulting in increased hydrophobic interactions and a decreased ability to enter into hydrogen bonding with the stationary phases, explains both the high retention volumes and the small influence of HDTMA. The larger retention volume of 4-hydroxy-3-methoxyphenol compared to 4-hydroxyphenol, in the absence of HDTMA, is also attributed to intramolecular hydrogen bonding leading to increased hydrophobic interactions. The reverse of this order, in the presence of HDTMA, is explained by a less effective hydrogen bonding to the stationary phase for 4-hydroxy-3-methoxyphenol compared with 4-hydroxyphenol.

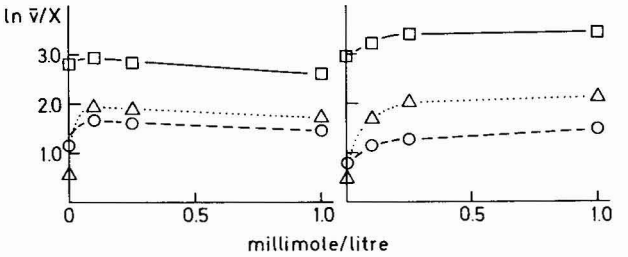

Fig. 2. Relationship between ln $\bar{v}/X$ and concentration of HDTMA in 0.5 *M* sodium acetate at pH 7 and 50°C on ODS-Hypersil (left) and Hitachi gel (right). □, 2-Methoxyphenol; △, 4-hydroxyphenol; ○, 4-hydroxy-3-methoxyphenol.

Adsorption of HDTMA

The adsorption of HDTMA, under the conditions used for the determinations of the retention data for the aromatic compounds, was calculated from the breakthrough curves recorded on the same columns after displacement of HDTMA. Gradient elution with methanol, 0–100%, was used for the displacement from the ODS-Hypersil column, while ethanol, 0–30% (w/w), was used for the Hitachi gel. The columns were then conditioned with sodium acetate (or glycolate) solution free from HDTMA. The breakthrough curves were recorded at influent compositions identical to the eluent compositions in most experiments with aromatic compounds.

Fig. 3 shows the breakthrough curves for ODS-Hypersil at an influent concentration of 1 m*M* HDTMA. The adsorption on both phases, calculated from the curves, is given in Table I, which shows that HDTMA was retained more strongly than any of the aromatic compounds studied in the present work. Very sharp breakthrough curves were obtained, indicating that the adsorption equilibrium was ap-

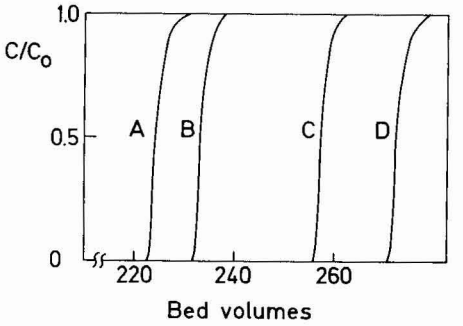

Fig. 3. Breakthrough curves for 1 m*M* HDTMA in acetate and glycolate solutions on ODS-Hypersil at pH 7 and 50°C. A, 0.3 *M* sodium glycolate; B, 0.5 *M* sodium glycolate; C, 0.3 *M* sodium acetate; D, 0.5 *M* sodium acetate.

proached rapidly. As can be seen, in 0.5 *M* sodium acetate the amount of HDTMA per ml of the bed volume was 272 times the amount present in 1 ml of the external solution. The adsorbed amount decreased only slightly when the eluent concentration was lowered by a factor of 10. Hence, the number of quaternary ammonium ions calculated per ml of the bed (which is equal to the ion-exchange capacity) was only slightly dependent on the concentration in the external solution. The capacity approaches that of conventional strongly basic anion-exchange resins of the styrene–divinylbenzene type having a low proportion of divinylbenzene. The adsorption increased slightly with increasing concentration of acetate or glycolate in the external solution. While the aromatic compounds (in the presence of HDTMA) were retained more strongly on the Hitachi gel than on ODS-Hypersil, the opposite was found for HDTMA in both acetate and glycolate solutions.

Influence of eluent concentration

Previously published reports on the effect of salt addition to eluents containing amines[9] or quaternary ammonium ions[10] show that electrolytes suppressed the retention of aromatic carboxylate anions. At high concentrations the effect became negligible[9]. For all aromatic carboxylate anions studied on ODS-Hypersil and the Hitachi gel in the present work (Tables II and III), the retention volume in aqueous solution

TABLE I

ADSORPTION OF HDTMA ON ODS-HYPERSIL AND HITACHI GEL FROM AQUEOUS SOLUTIONS CONTAINING 1 m*M* HDTMA

External solution	*Adsorbed amount (μmol per ml bed volume)*	
	ODS-Hypersil	*Hitachi gel*
Sodium acetate, 0.3 *M*	257	180
Sodium acetate, 0.5 *M*	272*	185
Sodium glycolate, 0.3 *M*	224	159
Sodium glycolate, 0.5 *M*	233	167

* In 0.1 m*M* HDTMA the adsorbed amount was 255 μmol ml^{-1}.

TABLE II

RETENTION DATA ON ODS-HYPERSIL FOR PHENOLS, BENZOATES AND PHENYLACETATES AT 50°C AND pH 7.0 IN ACETATE AND GLYCOLATE SOLUTIONS WITH ADDITIONS OF HDTMA (1 m*M*)

Parent compounds and substituents	*Acetate, 0.3 M*		*Acetate, 0.5 M*		*Glycolate, 0.3 M*		*Glycolate, 0.5 M*		*Acetate, 0.25 M**		*Acetate, 0.5 M**	
	$\bar{v}/X$	Δ	$\bar{v}/X$	Δ	$\bar{v}/X$	Δ	$\bar{v}/X$	Δ	$\bar{v}/X$	Δ	$\bar{v}/X$	Δ
Phenol	16.7		19.2		14.5		16.0		4.34		4.37	
4-Methyl	44.0	0.96	47.7	0.91	37.2	0.95	40.5	0.93	9.40	0.77	9.37	0.76
4-Hydroxy	4.9	−1.23	5.5	−1.25	3.6	−1.40	3.8	−1.44	1.37	−1.15	1.40	−1.14
4-Methoxy	13.3	−0.23	14.5	−0.28	11.4	−0.24	12.6	−0.24	3.08	−0.34	3.11	−0.34
2-Methoxy	12.5	−0.29	13.5	−0.35	12.0	−0.19	12.6	−0.24	3.24	−0.29	3.25	−0.30
4-Hydroxy-3-methoxy	3.8	−1.48	4.2	−1.51	3.1	−1.54	3.4	−1.56	1.21	−1.28	1.23	−1.27
Benzoate	31.1		22.5		46.1		35.3		8.82		5.34	
4-Methyl	84	0.98	64	1.04	119	0.95	96	1.00	21.6	0.89	12.7	0.86
4-Hydroxy	10.3	−1.11	7.9	−1.06	11.1	−1.42	8.4	−1.44	3.17	−1.02	2.03	−0.97
4-Methoxy	35.4	0.13	26.9	0.17	54	0.15	41.8	0.17	8.95	0.01	5.42	0.01
4-Hydroxy-3-methoxy	7.1	−1.48	5.3	−1.45	9.5	−1.57	7.2	−1.59	2.32	−1.34	1.60	−1.21
4-Hydroxy-3,5-dimethoxy	4.7	−1.90	3.5	−1.86	6.6	−1.95	5.0	−1.95	1.68	−1.66	1.23	−1.47
Phenylacetate	15.2		11.0		23.3		17.7		5.59		3.49	
4-Hydroxy	8.3	−0.61	5.9	−0.62	9.4	−0.91	7.0	−0.92	2.65	−0.75	1.82	−0.65
4-Methoxy	16.5	0.08	11.7	0.06	25.2	0.08	18.8	0.06	5.13	−0.09	3.27	−0.07
4-Hydroxy-3-methoxy	5.4	−1.03	3.9	−1.05	7.1	−1.20	5.3	−1.20	2.03	−1.01	1.44	−0.89
4-Hydroxy-3,5-dimethoxy	3.6	−1.44	2.8	−1.39	5.1	−1.51	3.9	−1.50	1.50	−1.32	1.11	−1.15

* Sodium acetate dissolved in 20% (w/w) methanol to a final concentration of 0.25 or 0.5 *M*.

with 1 m*M* HDTMA decreased markedly when the acetate or glycolate concentration was increased from 0.3 to 0.5 *M*. This is consistent with the observation that HDTMA cations adsorbed onto the stationary phases convert the latter to anion exchangers with acetate or glycolate counter ions. According to the law of mass action, the increased eluent concentration should in an idealized system lead to a decrease in the distribution coefficients for the aromatic anions by 40%.

The observed decrease in $\bar{v}/X$ was much less and varied between 20 and 32%. If it is assumed that the relative interstitial volume in the column was 0.4, the calculated decrease in the distribution coefficients would be 20–33%. The low values can only in part be ascribed to the increased ion-exchange capacity of the stationary phase resulting from the increased adsorption of HDTMA. On both stationary phases and in both media, 4-methylbenzoate exhibited the largest deviation from the calculated value. This shows that hydrophobic interactions exerted a great effect on the uptake of the aromatic carboxylate anions. Large deviations from ideality have previously been found in ion exchange of aromatic carboxylate ions on conventional ion-exchange resins[1]. As expected, an increased eluent concentration led to a large decrease in the retention volume also in the experiments with methanol present.

For all the phenolic compounds, the retention increased with increasing concentration of acetate or glycolate in the aqueous eluent. The effect was smaller than the salting out observed in experiments with an anion exchanger with a styrene–divinylbenzene matrix. It can be assumed that for this resin the salting-out parameter reflects the activity coefficients in the external solution. Hence, the smaller effect

TABLE III

RETENTION DATA ON HITACHI GEL FOR PHENOLS, BENZOATES AND PHENYLACETATES AT 50 C AND pH 7.0 IN ACETATE AND GLYCOLATE SOLUTIONS WITH ADDITIONS OF HDTMA (1 m*M*)

Parent compounds and substituents	*Acetate, 0.3 M*		*Acetate, 0.5 M*		*Glycolate, 0.3 M*		*Glycolate, 0.5 M*	
	$\bar{v}/X$	Δ	$\bar{v}/X$	Δ	$\bar{v}/X$	Δ	$\bar{v}/X$	Δ
Phenol	32.0		34.5		25.0		26.0	
4-Methyl	83	0.95	90	0.96	65	0.95	67	0.95
4-Hydroxy	7.9	−1.40	8.5	−1.40	5.1	−1.58	5.4	−1.57
4-Methoxy	27.5	−0.16	29.4	−0.16	20.8	−0.18	22.7	−0.14
2-Methoxy	28.8	−0.11	31.6	−0.09	25.7	0.03	26.9	0.03
4-Hydroxy-3-methoxy	3.9	−2.12	4.4	−2.06	4.7	−1.68	5.0	−1.66
Benzoate	46.5		32.8		52		37.9	
4-Methyl	122	0.96	88	0.99	129	0.91	99	0.96
4-Hydroxy	16.4	−1.04	11.6	−1.04	14.6	−1.27	10.4	−1.28
4-Methoxy	56	0.18	38.9	0.17	62	0.17	45.5	0.19
4-Hydroxy-3-methoxy	11.9	−1.36	8.2	−1.39	12.6	−1.42	9.2	−1.41
4-Hydroxy-3,5-dimethoxy	8.1	−1.74	5.6	−1.76	9.1	−1.74	6.7	−1.74
Phenylacetate	25.1		17.3		28.4		20.3	
4-Hydroxy	13.4	−0.63	9.5	−0.60	12.9	−0.79	9.2	−0.79
4-Methoxy	28.5	0.13	19.9	0.14	32.7	0.14	23.8	0.16
4-Hydroxy-3-methoxy	9.3	−0.99	6.4	−1.00	9.8	−1.07	7.1	−1.05
4-Hydroxy-3,5-dimethoxy	6.4	−1.37	4.4	−1.37	7.3	−1.36	5.5	−1.31

obtained in the systems studied in the present work indicates that the effect of the altered activity coefficients in the external solution on the distribution coefficients is suppressed by changes in the stationary phase.

In the experiments with 20% methanol in the eluent, an increase in the sodium acetate concentration from 0.25 to 0.5 *M*, had no significant influence on the retention volume of the phenolic compounds.

Experiments with the Hitachi gel in aqueous 1 m*M* HDTMA (pH 7) in the absence of sodium acetate or sodium glycolate showed that carboxylate ions were held very strongly by the resin. Hence, 4-hydroxy-3,5-dimethoxybenzoate, which exhibited very low retention volumes in the salt solutions, remained in the column after an elution volume corresponding to 120 column volumes. In contrast, the non-electrolytes appeared earlier in the eluate when no sodium salts were present. Compared to the retention volume in 0.3 *M* sodium acetate, the retention of 2-methoxyphenol was lowered by 22%. The corresponding values for 4-hydroxyphenol and 4-hydroxy-3-methoxyphenol were 61 and 59%, respectively. Hence, chromatography at a very low concentration of the eluting anion can be useful for separations of carboxylate anions from non-electrolytes.

Comparison between acetate and glycolate

Table I shows that HDTMA was held more strongly in sodium acetate than in sodium glycolate by both stationary phases studied in the present work. Hence, the ion-exchange capacity of the column packings will be higher in acetate medium, which will favour the uptake of the aromatic carboxylate ions. It can also be predicted that the sorption of the alkyl ammonium ion will depend on the activity coefficients of the counter ions. An analogous phenomenon is the adsorption of dyes onto cotton in aqueous solution. For dyes containing sulphonic acid groups the sorption is favoured by counter ions, which are held strongly by cation exchangers of the sulphonic acid type[11,12], *i.e.*, by counter ions with low activity coefficients in the gel phase. This suggests that glycolate ions present in the stationary phases exhibited larger activity coefficients than acetate ions. With regard to the activity coefficients of the competing counter ions, it can therefore be predicted that the aromatic carboxylate ions should be retained more strongly in glycolate solution than in acetate.

As shown in Table II, all aromatic carboxylate anions were held more strongly on ODS-Hypersil in glycolate than in acetate solution of the same concentration. The same was, with two exceptions, found also for the Hitachi gel. These results indicate that the direct effect of the activity coefficients of the eluent anion in the stationary phase on the ion-exchange equilibrium was more important than the indirect effect due to their influence on the sorption of the quaternary ammonium ion.

The exceptions obtained for the Hitachi gel were 4-hydroxyphenylacetate, with virtually the same retention in both eluents, and 4-hydroxybenzoate, which exhibited a slightly higher retention in acetate than in glycolate. It is noteworthy that for ODS-Hypersil the smallest differences between glycolate and acetate were observed for 4-hydroxy-substituted anions. Moreover, the most hydrophilic anions, *i.e.*, those with 4-hydroxy or 4-hydroxy-3-methoxy groups, were held more strongly by Dowex 1-X8 in acetate than in glycolate solution, while also with this resin the more hydrophobic anions exhibited higher distribution coefficients in glycolate[1]. The results indicate that the same interactions have an influence on the retention of the aromatic carbox-

ylate anions in these systems but that their relative importance differs from one stationary phase to another.

In the presence of HDTMA all phenols were held more strongly on ODS-Hypersil in acetate solution than in glycolate. The same holds true for Dowex 1-X8 studied previously[1], and with the exception of 4-hydroxy-3-methoxyphenol, also for the Hitachi gel. One factor which would favour the sorption in acetate medium is that the acetate counter ions will render the stationary phase more hydrophobic than glycolate ions. Hydrophobic interactions are of importance even for the sorption of the more hydrophilic aromatic compounds. The results indicate, however, that other interactions, such as hydrogen bonding of the solutes to the stationary phase, are also affected by the counter ions.

Influence of substituents on retention data

Tables II and III show that in aqueous media the substituent parameter Δ for a given compound was affected only slightly when the concentration of acetate or glycolate was increased from 0.3 to 0.5 *M*. This is true both for ODS-Hypersil and for the Hitachi gel. Similarly, the Δ values were hardly affected when the eluent was changed from 0.5 to 1.0 *M* sodium acetate on Dowex 1-X8[1]. Very similar Δ values were obtained for 4-methylphenol and 4-methylbenzoate in all media and on both stationary phases. In contrast, there were large differences between the Δ values for the three compounds containing a 4-hydroxyl substituent. For these compounds, less negative values were found in acetate than in glycolate. The results show that several types of interactions are of importance for the retention volume of the solutes.

In aqueous media, the sorption of ions and non-electrolytes on ion-exchange resins increases when hydrophobic substituents are introduced in the solutes. Accordingly, Tables II and III show that strongly positive Δ values were obtained for 4-methylphenol and 4-methylbenzoate on both stationary phases. The Δ values were larger than those reported for Dowex 1-X8 at 60°C in acetate and glycolate media[1]. Experiments at 50°C showed that the decrease in temperature from 60°C had no significant effect on the Δ values. Evidently, the change in free energy related to the transfer of a methyl substituent from the external solution to the stationary phase depends not only on the properties of the external solution but also on the stationary phase, and is larger (more negative) for ODS-Hypersil and Hitachi gel than for Dowex 1-X8. As expected, Δ was suppressed by the presence of 20% methanol in the eluent. In aqueous solutions the corresponding ethyl derivatives were held so strongly that it was impossible to determine their retention volumes with sufficient accuracy.

For anion-exchange resins with various carboxylate counter ions, the Δ values resulting from the introduction of a phenolic hydroxyl group in the 4-position were positive for phenol, benzoate and phenylacetate while, as can be seen from Tables II and III, these substituents gave rise to strongly negative Δ values both for ODS-Hypersil and for the Hitachi gel. For the anion exchanger the enhanced sorption is ascribed to hydrogen bonding between the phenolic hydroxyl proton and the acetate or glycolate counter ions present at high concentrations in the resin phase. For the systems studied in the present work, the positive contributions of hydrogen bonding to the distribution coefficients are less, due to a lower number of acetate or glycolate counter ions per unit volume of the column than in Dowex 1-X8. Possibly, the steric conditions are also more favourable for hydrogen bonding in the elastic ion-exchange

resin than in the more rigid phases used in the present work. Moreover, the negative contributions to Δ resulting from a decreased hydrophobicity of the solutes will be larger for ODS-Hypersil and the Hitachi gel. These factors together may explain why negative Δ values were obtained on ODS-Hypersil and the Hitachi gel.

For the species containing a methoxyl group in the *para* position, the hydrophobic interactions should be less than for the methylated compounds[13]. Moreover, 4-methoxyphenol is a weaker acid than phenol. Hence, the methoxyl group will decrease the hydrogen bonding of the phenolic proton to the stationary phase. This can explain the negative Δ values observed on both phases in both media (and also in the experiments with methanol present). In agreement with the experiments with Dowex 1-X8, positive Δ values were obtained for 4-methoxybenzoate and 4-methoxyphenylacetate.

The Δ values observed for 2-methoxyphenol on ODS-Hypersil in acetate and glycolate were negative and very close to those found on Dowex 1-X8, while the values found on the Hitachi gel were less negative. The results are consistent with the observation that intramolecular hydrogen bonding suppresses the hydrogen bonding of the phenolic proton to the stationary phase, but tends to increase the contribution of hydrophobic interactions to the retention[14].

4-Hydroxy-3-methoxyphenol exhibited lower retention volumes than 4-hydroxyphenol on ODS-Hypersil and Hitachi gel in both acetate and glycolate. Similarly, the 4-hydroxy-3-methoxy-substituted carboxylate ions were held less strongly than those with a 4-hydroxy substituent. Hydrogen bonding between the phenolic proton and the methoxyl group may have an influence in this direction. The introduction of a second methoxyl group, vicinal to the hydroxyl group, led to an additional decrease in the retention volume. The same effect was found on Dowex 1-X8, and was explained by effective intramolecular hydrogen bonding and the steric shielding of the phenolic hydroxyl group.

Both in 0.3 and 0.5 *M* solutions of the sodium salts, all aromatic solutes were held more strongly on the Hitachi gel than on ODS-Hypersil. The retention of all aromatic compounds, except 4-hydroxy-2-methoxyphenol, was previously determined in 0.5 *M* sodium acetate and glycolate solutions at 60°C on Dowex 1-X8. All benzoates and phenylacetates were, despite the higher temperature, held more strongly on the anion exchanger than on the Hitachi gel. The largest difference was observed for the ions containing a phenolic hydroxyl group. Likewise, 4-hydroxyphenol exhibited a much higher retention on the anion exchanger, while for 4-methoxyphenol the difference was small. The other phenolic compounds were held more strongly on the Hitachi gel. This holds true both in acetate and in glycolate media. The results confirm that hydrogen bonding between the resin and the phenolic proton in phenols, and the presence of carboxylate anions, give much larger contributions to the observed retention volumes on Dowex 1-X8 than on the stationary phases studied in the present work.

Finally, the chromatogram shown in Fig. 4 illustrates the usefulness of the applied technique for practical separations. As can be seen, six closely related aromatic carboxylic acids were separated within about 20 min by isocratic elution with 0.25 *M* sodium acetate, 1 m*M* HDTMA, in the presence of 3% (v/v) methanol. A decrease in the methanol concentration to 2% (v/v) led to a less good resolution. At 10% (v/v) methanol, the first two peaks overlapped seriously. In addition to organic

solvents, changes in pH, eluent concentration and of eluting anion offer great possibilities to optimize the conditions for separations of various acids and non-electrolytes. Under comparable conditions, a better resolution was obtained with ODS-Hypersil than with the Hitachi gel. On the other hand, the Hitachi gel is more stable and can be used over a wider pH range.

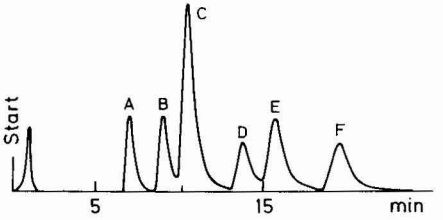

Fig. 4. Separation of benzoates and phenylacetates on ODS-Hypersil in 1 m*M* HDTMA and 0.25 *M* sodium acetate (3 % (v/v) methanol present) at pH 7 and 50°C. Wavelength, 280 nm. A = 4-Hydroxy-3,5-dimethoxy-phenylacetate (40 nmol); B = 4-hydroxy-3,5-dimethoxybenzoate (8 nmol); C = 4-hydroxy-3-methoxyphenylacetate (40 nmol); D = 4-hydroxy-3-methoxybenzoate (8 nmol); E = 4-hydroxyphenylacetate (40 nmol); F = 4-hydroxybenzoate (48 nmol).

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PARAMETERIZATION OF HYDROPHOBIC PROPERTIES OF AQUEOUS POLYMERIC BIPHASIC SYSTEMS AND WATER–ORGANIC SOLVENT SYSTEMS

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SUMMARY

Partition coefficients for a homologous series of dinitrophenylated amino acids with aliphatic side-chains have been determined in two aqueous polymeric Ficoll–dextran 70 and dextran 500–polyethylene glycol 6000 biphasic systems and in the systems formed by *n*-octanol and the aqueous phases of the above systems. The results afford an estimation of the free energy of transfer of a CH_2 group from one to the other phase of the systems examined. This parameter ($\Delta g_{tr}^{CH_2}$) was taken as a measure of the hydrophobic character of an aqueous phase with respect to *n*-octanol. It was shown that when the partition of a set of homologues in two biphasic systems is correlated according to the known equation $\ln K_i = a \ln K_0 + b$, where K_i and K_0 are the partition coefficients for a given solute in the *i*th system and in the system chosen for reference, respectively, the parameter a is related to the $\Delta g_{tr_i}^{CH_2}$ and $\Delta g_{tr_0}^{CH_2}$ by $a = \Delta g_{tr_i}^{CH_2} / \Delta g_{tr_0}^{CH_2}$.

This equation was used to determine the hydrophobic character of various organic solvents and that of the aqueous polymeric phases of the aqueous biphasic systems studied, and was found to be valid for comparison of the partition values determined in an aqueous polymeric biphasic system and in the water–*n*-octanol system. This seems to extend the possibilities of structure–activity relationships studies as the Ficoll–dextran aqueous biphasic system provides as promising a means for their study in biological chemistry as the water–*n*-octanol system in drug research.

INTRODUCTION

It has been shown[1–8] that the aqueous biphasic polymeric systems described by Albertsson[9] provide a means for estimating the relative hydrophobicities of biological solutes and particles. We developed[2–8] a new Ficoll–dextran biphasic system that has several advantages over the common dextran–polyethylene glycol system, particularly for analytical studies of both macromolecule and cell surface properties.

It should be noted, however, that the Ficoll–dextran system cannot be used for partitioning non-polar compounds because of their low solubility in water. The

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hydrophobic character of drugs is generally measured by partition coefficients determined using *n*-octanol–water as the distribution system[10–12]. Various solvent systems can be used for this purpose but measurements of the relative hydrophobicities of ionizable compounds always suffer from non-identity of the solute species in the two phases of a given water–organic solvent biphasic system[10–12]. Obviously the conventional biphasic systems containing an organic solvent cannot be applied to biopolymers because of the denaturing effects of organic solvents.

Therefore, it seems that the best way to formulate a general hydrophobicity scale for both biological macromolecules and low-molecular-weight polar and non-polar solutes is to establish an extra-thermodynamic relationship between the partition values determined in the above aqueous polymeric biphasic system and in the *n*-octanol–water system similar to those reported for various pairs of partition systems[10–13].

In this study we have attempted to establish such a relationship and to measure the hydrophobic character specific for the phases of two different aqueous polymeric biphasic systems relative to *n*-octanol.

EXPERIMENTAL

Materials

Ficoll-400 (lot 11069) and dextran T-500 (lot 9307, $\bar{M}_w = 484 \cdot 10^3$, $\bar{M}_n = 165.5 \cdot 10^3$) were obtained from Pharmacia (Uppsala, Sweden). Dextran 70 (lot 580870, $\bar{M}_w = 64.5 \cdot 10^3$, $\bar{M}_n = 22.7 \cdot 10^3$) was obtained under the trade-name Polyglucinum from Minmedprom (Moscow, U.S.S.R.). Polyethylene glycol 6000 was purchased from Serva (Heidelberg, G.F.R.).

Dinitrophenylated amino acids (DNP-glycine and DNP-L-alanine) were supplied by Serva. 2,4-Dinitrofluorobenzene was obtained from Calbiochem (Los Angeles, CA, U.S.A.). L-Norleucine and DL-norvaline were supplied by Reanal (Budapest, Hungary), and DL-2-amino-*n*-octanoic acid was purchased from BDH (Poole, Great Britain). The amino acids were dinitrophenylated as described in ref. 14. All dinitrophenylated amino acids were checked for purity by thin-layer chromatography, and their sodium salts were prepared by titration.

1-Octanol and other chemicals and salts were analytical-reagent grade materials and were used without further purification.

Methods

Buffered Ficoll–dextran 70 and Dextran 500–polyethylene glycol 6000 biphasic systems were prepared by weighing appropriate amounts of the stock polymer solutions as described previously[1]. The Ficoll–dextran biphasic systems contained 12.5% (w/w) Ficoll-400, 10.8% (w/w) dextran 70 and either 0.15 *M* sodium chloride in 0.01 *M* sodium phosphate buffer (pH 7.4) or 0.11 *M* sodium phosphate buffer (pH 7.4). The dextran–polyethylene glycol biphasic systems contained 7% (w/w) dextran 500 and 4.4% (w/w) polyethylene glycol 6000 (salt and buffer concentrations as indicated above).

The partition experiments were carried out as described elsewhere[2,5,6]. The phases of the Ficoll–dextran system were allowed to settle at 23°C for 24 h, then aliquots of both phases were pipetted from the system and each was used for the

solute concentration measurements and for subsequent partition experiments with *n*-octanol. The absorbance of each aliquot, appropriately diluted with water, was measured at 360 nm against a correspondingly diluted top or bottom phase blank.

The same partition technique was used with the dextran–polyethylene glycol systems except that the systems were centrifuged for 20 min at 1200 *g* to speed phase settling.

After settling of the aqueous biphasic systems, aliquots of the phases were mixed with equal volumes of *n*-octanol. The biphasic systems formed by a given aqueous polymeric phase with *n*-octanol were centrifuged for 5 min at 1200 *g*. The same technique was used with the buffer–*n*-octanol systems. The concentrations of a solute in both phases of the systems were measured as above, except those in the *n*-octanol phases, which were measured at 350 nm.

The partition coefficient, *K*, in the aqueous polymeric biphasic systems is defined as the ratio of the sample concentration in the Ficoll-rich (polyethylene glycol-rich) phase to that in the dextran 70-rich (dextran 500-rich) phase. The partition coefficient in the aqueous solution–*n*-octanol systems is defined as the ratio of the sample concentration in the *n*-octanol phase to that in the aqueous phase.

The partition coefficients were measured for each solute over approximately 10-fold concentration ranges and were found to be independent of the solute concentration in the aqueous polymeric biphasic systems and also in the aqueous solution–*n*-octanol systems.

The partition coefficient for each solute was determined as the mean of two measurements on three dilutions from each partition experiment carried out 2–4 times in a given biphasic system.

RESULTS

The approach used is based on the linear relationship between the logarithm of the partition coefficient and the number of CH_2 groups in the aliphatic chain of the compounds distributed in a given biphasic system[1,2,7]. Some of the relationships found are shown in Fig. 1a and b. These relationships can be described by the equation

$$\ln K = C + En \tag{1}$$

where n is the number of CH_2 groups in the amino acid aliphatic side-chain, C is the ln K value for DNP-glycine and E represents an average ln K increment per CH_2 group. It is clear that E is related to the free energy of transfer of a CH_2 group from one to the other phase of a given biphasic system ($\Delta g_{tr}^{CH_2}$) according to the equation $\Delta g_{tr}^{CH_2} = RTE$.

A least-squares treatment of the data obtained led to the C and E values listed in Table I, together with the corresponding $\Delta g_{tr}^{CH_2}$ values.

Linear relationships are known to exist between the logarithms of partition coefficients of substances determined separately in a pair of partition systems[10–13]. These relationships are described by the equation

$$\ln K_i = a \ln K_0 + b \tag{2}$$

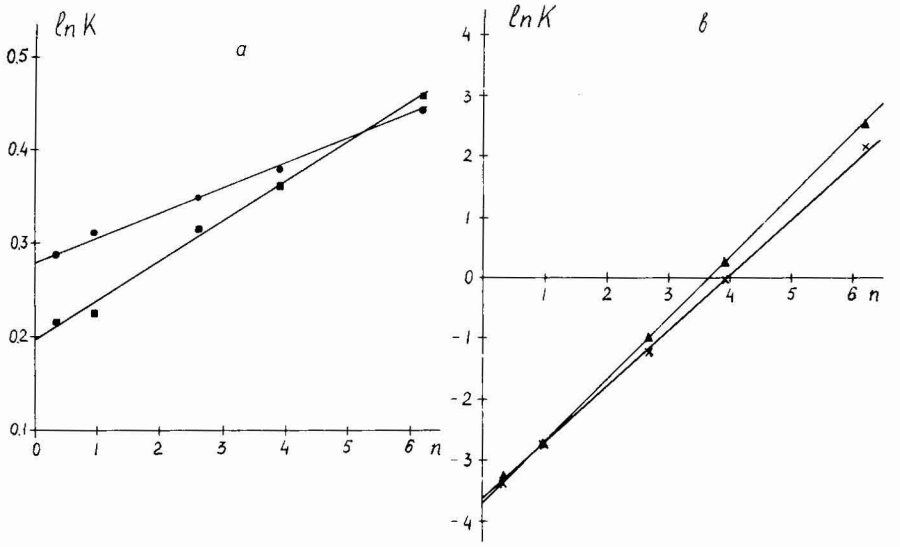

Fig. 1. Logarithm of the partition coefficient as a function of the aliphatic side-chain length (n) of the dinitrophenylated amino acids glycine, alanine, norvaline, norleucine and 2-amino-n-octanoic acid in the following biphasic systems: (a) 1, aqueous polymeric biphasic system containing 12.5% (w/w) Ficoll-400, 10.8% (w/w) dextran 70, 0.11 M sodium phosphate buffer (pH 7.4); 2, aqueous polymeric biphasic system including 7.0% (w/w) dextran 500, 4.4% (w/w) polyethylene glycol 6000, 0.11 M sodium phosphate buffer (pH 7.4); (b) 1, n-octanol–aqueous solution of 0.11 M sodium phosphate buffer (pH 7.4); 2, n-octanol–polyethylene glycol 6000-rich phase taken from the above aqueous polymeric system including 0.11 M sodium phosphate buffer (pH 7.4).

where K_i and K_0 are, respectively, the partition coefficients for a given solute in the ith phase system under study and in the system chosen for reference, and a and b are constants.

The values of a and b (which were calculated using the experimental K values with the corresponding buffer–n-octanol biphasic system as the reference) are given in Table II.

DISCUSSION

It has been suggested[1,15] that the free energy of the interface transfer of a CH_2 group specific for a given biphasic system ($\Delta g_{tr}^{CH_2}$) should be used as a measure of the difference in the relative hydrophobicities between the two phases of the system. When this criterion is used for comparison of different aqueous phases with a given organic phase it can be taken as a measure of the relative hydrophobicity of these phases. The same parameter can be applied for comparison of different organic solvents with water or with any salt solution. n-Octanol was chosen here as the reference organic solvent for the reasons given above. As the $\Delta g_{tr}^{CH_2}$ value specific for the water–n-octanol system is known[10–12], it is possible to calculate the $\Delta g_{tr}^{CH_2}$ values for the hypothetical polymer solution–water biphasic systems from the data given in Table I.

The relative hydrophobicities of the polymeric aqueous phases examined are shown in Fig. 2 and are described quantitatively (in terms of the $\Delta g_{tr}^{CH_2}$ values) in Table III. The hydrophobic character of different organic solvents was evaluated

TABLE I

CHARACTERISTICS OF THE PARTITION BEHAVIOUR OF SODIUM SALTS OF DNP-AMINO ACIDS WITH ALIPHATIC SIDE-CHAINS IN AQUEOUS POLYMERIC BIPHASIC SYSTEMS AND IN AQUEOUS PHASE–*n*-OCTANOL BIPHASIC SYSTEMS

Partition of a homologous series of solutes in a given phase system is described by the equation $\ln K = C + En$ (see text). Parameter E is related to the free energy of interface transfer of a CH_2 group ($\Delta g_{tr}^{CH_2}$) by $\Delta g_{tr}^{CH_2} = RTE$.

*Biphasic system**	*Salt composition***	C	E	$\Delta g_{tr}^{CH_2}$ *(cal/mole)*
n-Octanol–buffer	I	−3.686 ± 0.018	1.008 ± 0.005	598 ± 8
	II	−3.886 ± 0.024	1.008 ± 0.006	598 ± 8
Ficoll–dextran 70	I	0.281 ± 0.006	0.027 ± 0.002	16 ± 1
	II	0.099 ± 0.006	0.027 ± 0.002	16 ± 1
Polyethylene glycol–dextran 500	I	0.196 ± 0.011	0.042 ± 0.003	25 ± 2
	II	−0.073 ± 0.008	0.037 ± 0.002	22 ± 1
n-Octanol–dextran 70	I	−3.77 ± 0.11	1.014 ± 0.027	601 ± 16
	II	−3.95 ± 0.05	1.007 ± 0.013	597 ± 8
n-Octanol–Ficoll	I	−3.92 ± 0.09	0.979 ± 0.022	580 ± 13
	II	−4.03 ± 0.10	0.984 ± 0.025	584 ± 15
n-Octanol–dextran 500	I	−3.47 ± 0.02	0.963 ± 0.007	571 ± 4
	II	−3.57 ± 0.10	0.928 ± 0.030	550 ± 18
n-Octanol–polyethylene glycol	I	−3.63 ± 0.07	0.921 ± 0.021	546 ± 12
	II	−3.50 ± 0.12	0.890 ± 0.035	528 ± 21

* Polymer compositions of the aqueous biphasic systems are given in the text. For details of the preparation of the *n*-octanol–aqueous phase systems see text.

** Salt composition: I, 0.11 *M* sodium phosphate buffer (pH 7.4); II, 0.15 *M* sodium chloride in 0.01 *M* sodium phosphate buffer (pH 7.4).

TABLE II

FACTORS FOR CALCULATING THE PARTITION VALUES DETERMINED IN VARIOUS BIPHASIC SYSTEMS

The relationship between the partition values for a solute determined in two different biphasic systems is described by the equation $\ln K_i = a \ln K_0 + b$, where K_i and K_0 are the partition coefficients of a solute in the ith phase system under study and in the system chosen for reference, respectively, and a and b are constants. The system buffer–*n*-octanol was chosen for reference.

Biphasic system	*Salt composition**	a	b
Ficoll–dextran 70	I	0.027 ± 0.002	0.380 ± 0.02
	II	0.027 ± 0.002	0.204 ± 0.02
Polyethylene glycol–dextran 500	I	0.042 ± 0.003	0.351 ± 0.02
	II	0.037 ± 0.002	0.071 ± 0.02
n-Octanol–dextran 70	I	1.006 ± 0.025	−0.062 ± 0.11
	II	0.999 ± 0.013	−0.068 ± 0.06
n-Octanol–Ficoll	I	0.971 ± 0.020	−0.341 ± 0.09
	II	0.976 ± 0.023	−0.237 ± 0.10
n-Octanol–dextran 500	I	0.955 ± 0.008	0.050 ± 0.03
	II	0.921 ± 0.028	0.009 ± 0.10
n-Octanol–polyethylene glycol	I	0.914 ± 0.019	−0.261 ± 0.07
	II	0.883 ± 0.020	−0.069 ± 0.12

* Salt composition: I, 0.11 *M* sodium phosphate buffer (pH 7.4); II, 0.15 *M* sodium chloride in 0.01 *M* sodium phosphate buffer (pH 7.4).

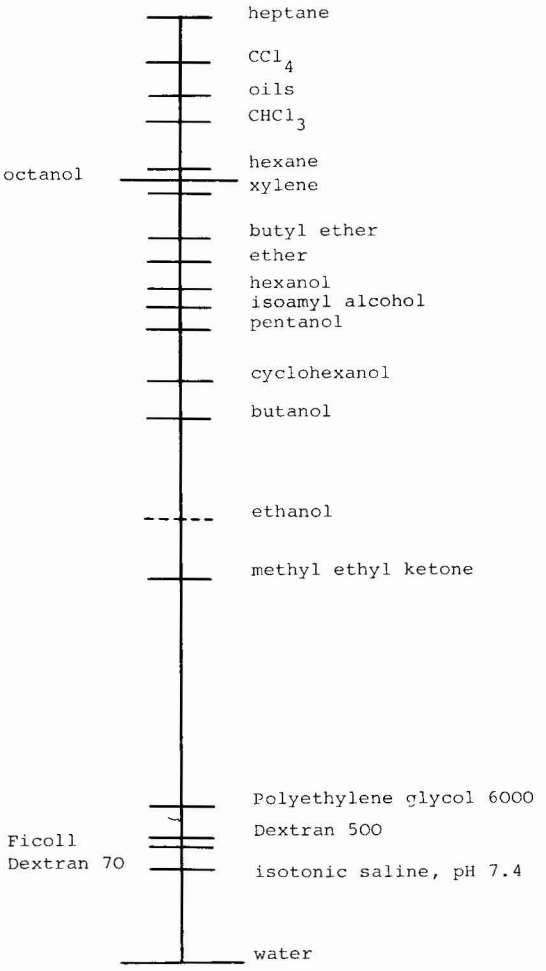

Fig. 2. Hydrophobicity scale for organic solvents and aqueous polymeric phases. Hydrophobicity is shown according to the $\Delta g_{tr}^{CH_2}$ values given in Table III. Polyethylene glycol 6000–polyethylene glycol 6000-rich phase, dextran 500–dextran 500-rich phase, Ficoll–Ficoll-rich phase and dextran 70–dextran 70-rich phase —all the phases from the corresponding aqueous polymeric biphasic systems containing 0.11 *M* sodium phosphate buffer (pH 7.4). Ethanol is scaled according to the extrapolated value of $\Delta g_{tr}^{CH_2}$ as indicated in the text.

from the literature data[10,12] as follows. It was shown earlier[8] that parameter a in eqn. 2 represents the difference in the relative hydrophobicities between the two phases of a given biphasic system and is related to the corresponding $\Delta g_{tr_i}^{CH_2}$ and $\Delta g_{tr_0}^{CH_2}$ by the equation

$$a_i = \Delta g_{tr_i}^{CH_2} / \Delta g_{tr_0}^{CH_2} \tag{3}$$

where the subscripts i and 0 denote the biphasic system under study and the reference, respectively.

All of the data given in Tables I and II are consistent with eqn. 3. Thus, the a_i

TABLE III

RELATIVE HYDROPHOBICITY OF ORGANIC SOLVENTS AND AQUEOUS SOLUTIONS EXPRESSED IN TERMS OF THE FREE ENERGY OF TRANSFER OF A CH_2 GROUP FROM WATER TO A GIVEN MEDIUM ($\Delta g_{tr}^{CH_2}$)

Solvent	$-\Delta g_{tr}^{CH_2}$ *(cal/mole)*	*Calculated from the data given in ref.*
n-Heptane	825 ± 10	Taken as reported in ref. 17
CCl_4	784 ± 8	16[a]
Oils	757 ± 16	10[b]
$CHCl_3$	731 ± 17	16[a]
n-Hexane	692 ± 60	19[c]
n-Octanol	683 ± 12	10, 12[b]
Oleyl alcohol	682 ± 43	10[b]
Xylene	672 ± 43	10[b]
Di-*n*-butyl ether	631 ± 34	16[a]
Diethyl ether	612 ± 74	12[b]
n-Hexanol	587 ± 24	16[a]
Isoamyl alcohol	573 ± 30	16[a]
Primary pentanols	552 ± 49	10[b]
Cyclohexanol	509 ± 62	10[b]
Primary butanols	476 ± 16	10[b]
Ethanol	388 ± 27	Extrapolated value from data in Fig. 3
Methyl ethyl ketone	337 ± 48	10[b]
Polyethylene glycol 6000[d]	155 ± 24	This work, Table I
Polyethylene glycol 6000[e]	137 ± 17	This work, Table I
Dextran 500[f]	133 ± 22	This work, Table I
Dextran 500[g]	112 ± 13	This work, Table I
Ficoll-400[h]	101 ± 18	This work, Table I
Dextran 70[i]	84 ± 22	This work, Table I
Isotonic saline (pH 7.4)[j]	85 ± 14	This work, Table I

[a] The $\Delta g_{tr}^{CH_2}$ values are calculated using the linear relationship between the logarithm of the partition coefficient and the number of CH_2 groups in the aliphatic side-chains of the amino acid derivatives studied in ref. 16.

[b] The $\Delta g_{tr}^{CH_2}$ values are calculated from the *a* values for the corresponding solvent regression equations reported in refs. 10 and 12 and the $\Delta g_{tr}^{CH_2}$ value of 683 cal/mole as reported for the *n*-octanol–water biphasic system[10–12].

[c] The $\Delta g_{tr}^{CH_2}$ value was calculated from the solubility data for amino acids reported in ref. 19.

[d] Polyethylene glycol-rich phase of the aqueous biphasic system polyethylene glycol 6000–dextran 500 in the presence of 0.15 *M* sodium chloride in 0.01 *M* sodium phosphate buffer (pH 7.4) (polymer composition is indicated in the text).

[e] As in [d] but in the presence of 0.11 *M* sodium phosphate buffer (pH 7.4).

[f] Dextran 500-rich phase of the system indicated in [d].

[g] As in [f] but in the presence of 0.11 *M* sodium phosphate buffer (pH 7.4).

[h] Ficoll-rich phase of the aqueous biphasic system Ficoll–dextran 70 in the presence of 0.11 *M* sodium phosphate buffer (pH 7.4) or in the presence of 0.15 *M* sodium chloride in 0.01 *M* sodium phosphate buffer (pH 7.4) (polymer composition is indicated in the text).

[i] Dextran 70-rich phase of the system indicated in [h].

[j] Isotonic saline containing either 0.11 *M* sodium phosphate buffer (pH 7.4) or 0.15 *M* sodium chloride in 0.01 *M* sodium phosphate buffer (pH 7.4).

values reported by Hansch[12] and Leo *et al.*[10] were treated according to eqn. 3 using the $\Delta g_{tr}^{CH_2}$ value of 683 ± 12 cal/mole for the *n*-octanol–water system[10-12]. The $\Delta g_{tr}^{CH_2}$ values for isoamyl alcohol–water, di-*n*-butyl ether–water, *n*-hexanol–water, chloroform–water and carbon tetrachloride–water systems were calculated from the experimental data reported by Nandi[16] using the approach applied in this study. The $\Delta g_{tr}^{CH_2}$ value for the *n*-heptane–water system was taken from ref. 17.

It should be noted that the $\Delta g_{tr}^{CH_2}$ values obtained for various *n*-alcohols appear to depend on the alcohol chain length, as shown in Fig. 3, except for the value for ethanol calculated from the data reported in ref. 18. However, the latter data are the only ones obtained not by the partition technique but by solubility measurements[18]. The extrapolated value of 388 ± 27 cal/mole seems to represent the hydrophobic character of ethanol much better than 589 ± 83 cal/mole estimated from the solubility data reported in ref. 18, as ethanol is the only alcohol under consideration that is completely miscible with water. Hence, the relative hydrophobicity of ethanol is shown in Fig. 2 according to the extrapolated value of $\Delta g_{tr}^{CH_2}$.

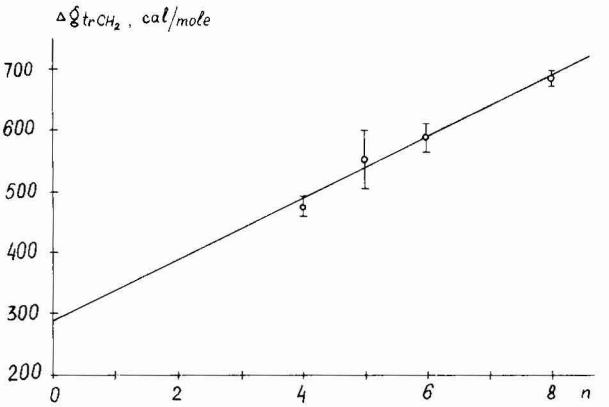

Fig. 3. $\Delta g_{tr}^{CH_2}$ values for *n*-alcohols as a function of the alcohol chain length (*n*).

The approach described above affords an evaluation of the relative hydrophobicities of aqueous polymer solutions, which appears to open up a promising line of research on polymer–solvent interactions. It is surprising that, to our knowledge, no use has been made of this approach previously.

It is necessary to emphasize that although $\Delta g_{tr}^{CH_2}$ appears to be completely satisfactory as a measure of the hydrophobic character of a given organic solvent or aqueous solution, it undoubtedly has some limitations. These limitations seem to be concerned with the different characters of the interactions between different moieties of a solute molecule and water or various organic solvents. A methylene group has a simple geometry and seems to be inert towards any aqueous or organic solvent. However, the affinity of polar, ionizable and many complex non-polar moieties for a solvent may vary widely. These variations are reflected by differences in the extent of solvation specific for different molecules in various solvents and they may affect the corresponding partition values. It seems reasonable to assume that the parameter *b* in

eqn. 2 represents the effects of the specific solute–solvent interactions on the solute partition coefficient. If this is so, b (not a, which is constant for a given pair of biphasic systems and does not depend on the chemical nature of the solute being partitioned) must be considered as a measure of the specific interactions of a given solute moiety with a particular solvent.

The above meaning of both parameters a and b in eqn. 2 seems to be consistent with the data reported in the literature[10–12]. A more comprehensive discussion of the a and b values reported for various water–organic solvent pairs[10–12], however, is beyond the scope of the present study and will be given in a subsequent publication.

The data given in Tables I and II allow one to compare the aqueous binary Ficoll–dextran and dextran–polyethylene glycol systems. The relative hydrophobicities of the two phases of the dextran–polyethylene glycol system appear to exceed those characteristic of the phases of the Ficoll–dextran system. It was shown[8], however, that the relative hydrophobicities of the phases of the Ficoll–dextran system depend particularly on the molecular weight of dextran and on the polymer composition of the system. Comparison of the data given in Table I for the dextran–polyethylene glycol system with those reported earlier for the same system with another polymer composition[1] indicates that the polymer composition of an aqueous polymeric biphasic system does affect its relative hydrophobicity. That seems to be in line with Albertson's view[9] with respect to the position of aqueous polymer solutions in a range of solvents with different hydrophobicities.

The data obtained in this study indicate that it is possible to correlate the partition values obtained in the aqueous polymeric biphasic system Ficoll–dextran with those determined in the water–*n*-octanol (or any water–organic solvent) system. The Ficoll–dextran biphasic system appears to provide a new means for studying the hydrophobic character of biological particles and any polar and ionizable solutes. This biphasic system can be considered as promising a tool for the study of structure–activity relations in biological chemistry as the water–*n*-octanol system was proved to be in drug research.

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STABILITY OF THE *o*-PHTHALALDEHYDE–HISTAMINE COMPLEX

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SUMMARY

Three methods of derivatization of histamine with *o*-phthalaldehyde (OPT) reagent were compared in order to confirm the stability of the fluorescent product. A reversed-phase high-performance liquid chromatographic system with a fluorescence detector was used. Immediately after derivatization, samples were injected at intervals of 10 min for a total of 60 min. The stability of the fluorescent complex varied according to the method of derivatization. For method 1 (histamine–OPT reaction at ambient temperature) the peak area after 10 min was only half that of its original value. For method 3 (acidification with hydrochloric acid of the end-product of derivatization) only a slight decrease occurred over the 60-min period; method 2 (extraction of the OPT complex with ethyl acetate) was the most stable.

INTRODUCTION

The derivatization of amines and amino acids with *o*-phthalaldehyde (OPT) has been the subject of numerous studies. The chemical reactions involved, however, are not fully understood[1].

In an attempt to quantify histamine in wine using a reversed-phase high-performance liquid chromatographic (HPLC) system, we observed that the histamine–OPT complex prepared according to Subden *et al.*[2] was unstable. A more thorough investigation, using three other methods of derivatization with OPT, was carried out in order to seek confirmation of the instability.

MATERIALS AND METHODS

Two isocratic mobile phases were used, one consisting of methanol–0.08 *M* acetic acid (52:48)[3] and the other acetonitrile–water (40:60) buffered to pH 7.0 with dipotassium hydrogen orthophosphate[2]. The solvents were filtered through a 0.45-μm filter and degassed by sonication just prior to use. A flow-rate of 1 ml/min was used throughout. OPT, mercaptoethanol and histamine were obtained from Sigma

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(St. Louis, MO, U.S.A.). All other chemicals met the ACS specifications. A stock solution of histamine in water (100 mg/l) was prepared, filtered through a 0.45-μm filter and stored at 4°C.

Liquid chromatography

The liquid chromatographic system consisted of a Waters Model 6000A solvent delivery device, a Waters U6K injector and a Waters Model 420 fluorescence detector (Waters Assoc., Milford, MA, U.S.A.). The detector was equipped with an F4T5BL lamp, a 340-nm excitation filter and a 440-nm emission filter; the gain was set at $\times 64$.

A Spectra-Physics Model SP4000 central processor and a Model SP4020 data interface (Spectra-Physics, Santa Clara, CA, U.S.A.) were used for integration and recording.

A μBondapak C_{18} column (Waters Assoc.) was used for reversed-phase HPLC. All analyses were performed in an air-conditioned room at 22°C.

Derivatization

Three methods of derivatization were investigated.

Method 1 (according to Mell et al.[3]. OPT reagent was prepared freshly each day by dissolving 160 mg of OPT in 3 ml of ethanol and 0.2 ml of 2-mercaptoethanol under a ventilated hood. This solution was added to 100 ml of 0.4 mol/l boric acid which was adjusted to pH 9.5 with potassium hydroxide.

For derivatization, 0.1 ml of stock histamine solution was treated with 1 ml of OPT reagent in a Teflon-lined screw-capped test-tube protected against light with aluminium foil and mixed for 1 min on a Vortex mixer. After incubation for 2 min at ambient temperature (20–22°C), 0.9 ml of doubly distilled water was added and mixed. The solution was then ready for injection. The injection volume of 20 μl corresponded to 0.1 μg of histamine. The time required from derivatization to injection was 5 min.

Method 2 (according to Davis et al.[4]*)*. OPT reagent was prepared by dissolving 0.5 g of boric acid in 19 ml of distilled water and titrating with potassium hydroxide solution (450 g/l) to pH 10.40 $\pm$ 0.02. After transferring the titrated solution to a dark-glass bottle with a Teflon-lined screw cap, 17.5 mg of OPT were weighed into a 5-ml beaker, dissolved in 200 μl of glass-distilled methanol and added to the borate solution together with 40 μl of 2-mercaptoethanol. The reagent was prepared fresh daily.

For derivatization, an aliquot of 0.5 ml of the histamine stock solution was diluted to approximately 10 ml, the pH was adjusted to 7.0 $\pm$ 0.2 with 0.5 mol/l potassium hydroxide solution and the volume was adjusted to 10 ml. A 1-ml volume of this solution was derivatized in a graduated conical centrifuge flask protected against light with aluminium foil by adding 0.5 ml of the OPT reagent. Two grams of sodium chloride were added to break any emulsion formed during two consecutive extractions with 2-ml portions of ethyl acetate. The sample was shaken on a Vortex mixer for 1 min during each extraction followed by sedimentation for 1 min (instead of centrifugation) to separate the phases. After extraction, the ethyl acetate phase was extracted twice with 2-ml portions of dibasic sodium phosphate buffer (50 mol/l, pH 10.0 $\pm$ 0.1), shaken for 1 min and allowed to sediment for 1 min. The ethyl acetate

was reduced to 1 ml under oxygen-free, dry nitrogen. The injection volume of 20 μl corresponded to 0.1 μg of histamine. The time required from derivatization to injection was 25 min.

Method 3 (according to Rice et al.[5]). OPT reagent was prepared by dissolving 10 mg of OPT in 1 ml of methanol.

For derivatization, an aliquot of 135 μl of the histamine stock solution was mixed with 1.86 ml of 0.1 *N* hydrochloric acid in a Teflon-lined screw-capped test-tube protected from light with aluminum foil. To this was added 0.4 ml of 1 *N* sodium hydroxide solution and the solution was mixed and left for 1 min. A 0.1-ml volume of the OPT reagent was added and the solution was mixed and left for 4 min, followed by the addition of 0.2 ml of 3 *N* hydrochloric acid. The injection volume of 20 μl corresponded to 0.1 μg of histamine. The time required from derivatization to injection was 7 min.

Procedure

The samples were injected immediately after the derivatization process (time zero), and thereafter at intervals of 10 min for a total of 60 min.

The study was conducted according to a 2 × 3 × 7 split–split plot design in two replications. The factors were, respectively, two mobile phases, three methods of derivatization and seven-fold injection.

RESULTS

The fluorescence of the histamine–OPT complex is represented by the area under the related peak (not shown). Immediately after derivatization (time zero) the

TABLE I

FLUORESCENCE RESPONSES IN TERMS OF OBSERVED AREAS UNDER THE HISTAMINE PEAK AS A FUNCTION OF TIME AFTER DERIVATIZATION (MEAN OF TWO REPLICATES)

Experimental conditions: reversed-phase HPLC system; 2 isocratic phases; 3 methods of derivatization; 7 injections per phase/method/replicate combination; 0.1 μg of histamine injected.

Time after derivatization (min)	*Observed areas under peaks* $\times 10^{-2}$					
	Methanol–acetic acid (52:48)			*Acetonitrile–water (40:60)*		
	Method 1	*Method 2*	*Method 3*	*Method 1*	*Method 2*	*Method 3*
0	1326 (3.12)*	1343	3666	2095 (3.31)*	1461	3434
10	625 (2.80)	1358	3575	1117 (3.04)	1442	3370
20	277 (2.44)	1330	3571	589 (2.76)	1393	3350
30	141 (2.14)	1385	3480	445 (2.64)	1415	3336
40	73 (1.87)	1405	3448	317 (2.50)	1492	3224
50	39 (1.58)	1372	3386	221 (2.34)	1527	3252
60	25 (1.40)	1408	3347	214 (2.33)	1450	3220
Standard error of slope, s_b	50.76 (0.009)	29.32	4.50	68.04 (0.014)	13.27	30.49
Coefficient of determination, r^2	(98.8)	1.1	91.8	(92.0)	3.2	10.3
R^2	73.42			76.1		

* Log_{10} (area).

observed areas for methods 1 and 2 for both mobile phases were about half those for method 3 (Table I).

The fluorescence of the histamine–OPT complex obtained by method 1 decreased markedly during standing; 10 min after derivatization it was half of its original value. Statistical analysis showed that this decrease followed a linear logarithmic curve (r^2 – 98.8 and 92.0 for mobile phases 1 and 2, respectively). Antilogarithms of this linear curve were used to draw the regression lines in Fig. 1 (method 1).

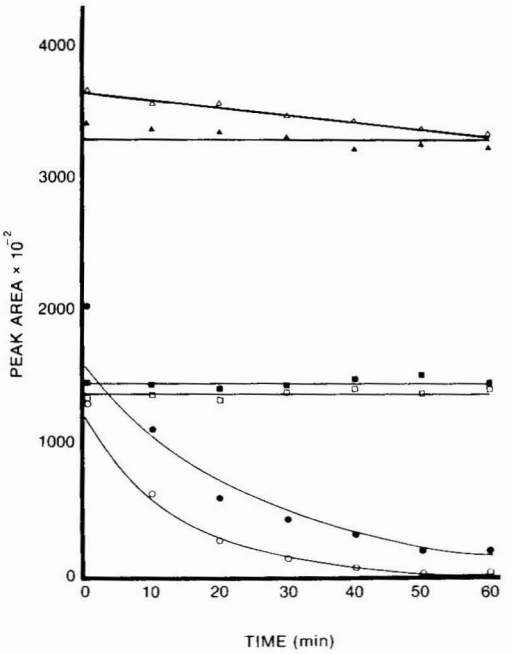

Fig. 1. Regression lines as a function of time of injection on the fluorescence responses of the histamine–OPT complex obtained by three methods of derivatization and using two HPLC isocratic phases (mean of two replicates). Open symbols: methanol–acetic acid. ○, Method 1, $\log_{10} \hat{y}^{\star} = 3.07 - 0.029x$; □, method 2, $\hat{y}^{\star\star} = 1339.2 + 1.07x$; △, method 3, $\hat{y} = 3652.0 - 5.2x$. Closed symbols: acetonitrile–water. ●, Method 1, $\log_{10} \hat{y}^{\star} = 3.20 - 0.016x$; ■, method 2, $\hat{y}^{\star\star} = 1428.9 + 0.84x$; ▲, method 3, $\hat{y}^{\star\star} = 3415.4 - 3.59x$.

Derivatization according to method 2 resulted in a stable complex; any variation in the area was independent of the duration between derivatization and injection (r^2 = 1.1 and 3.2).

A fairly stable omplex was obtained with method 3, although a decrease in fluorescence did occur upon standing when methanol–acetic acid was used as the mobile phase (r^2 = 91.8). With the other mobile phase (r^2 = 10.3), the apparent decrease was not statistically significant.

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* The plotted line was based on the antilog of $\log_{10} \hat{y}$.

** The slopes were not statistically significant, and therefore $\hat{y}$ was taken as $\bar{y}$.

DISCUSSION

Although the choice of a mobile phase is based on optimal resolution, the interrelation that may exist between the solvents and components under investigation should not be neglected. In this work, for two out of the three methods of derivatization methanol–acetic acid gave lower fluorescence values than acetonitrile–water. The acetate anion is reported to affect adversely the fluorescence of the OPT derivatives[1].

Of the three methods used, one had its fluorescence reduced by half 10 min after derivatization, while the other two were fairly stable. Adequate standardization of the procedure (duration of derivatization, interval between derivatization and measurement) is required for reproducibility and accuracy. The sensitivity of a method is also important when small amounts need to be detected and quantified (method 3 *versus* method 1, time zero, or method 2).

CONCLUSION

OPT is a useful reagent for the determination of histamine but careful choice and standardization of a method of derivatization are necessary in order to obtain reproducible data.

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COORDINATION POLYMERS AS ADSORBENTS AND STATIONARY PHASES IN GAS CHROMATOGRAPHY

VII. ALKALINE EARTH METAL DI-*n*-HEXYLPHOSPHINATES: SPECIFIC INTERACTIONS

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SUMMARY

It is shown that alkaline earth metal di-*n*-hexylphosphinates may interact specifically with unsaturated hydrocarbons. The dependence of logarithms of retention volumes on the boiling points of hydrocarbons is suggested for use in quantitative calculations of these interactions.

INTRODUCTION

In a previous paper[1], we described a method for the calculation of stability constants of π-complexes on the basis of gas–solid chromatographic data. It was stated there that a reference metal had to fulfil a number of conditions. In this work we demonstrate, using alkaline earth metal di-*n*-hexylphosphinates (DHPs) as an example, that main-group metals may interact specifically with unsaturated hydrocarbons and that these interactions depend on the kind of metal involved.

EXPERIMENTAL

The experimental methodology has been described previously[2,3]. Adsorbents were prepared to contain 10^{-4} mole of phosphinate per gram[4]. Stainless-steel columns (1 m × 4 mm I.D.) were used throughout. Infrared spectra were measured with a Perkin-Elmer 180 or 250 IR spectrometer. Calculations of heat of adsorption were carried out on an Odra 1204 computer. Retention volumes of specific interactions and the thermal effects of specific interactions were calculated by linear regression on a TI 59 programmable calculator.

RESULTS AND DISCUSSION

Retention times and volumes of 22 C_6 hydrocarbons were measured at four temperatures between 70 and 90°C. We have emphasized previously[4] that it is not

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possible to confirm the existence of specific interactions on the basis of either absolute or relative retention volumes. However, differences between the retention indices of various hydrocarbons on these adsorbents and their standard retention indices (SRI)[5] do suggest the existence of specific interactions.

Retention indices of these hydrocarbons on the phosphinate adsorbents studied and squalane, together with SRI values, are shown in Table I.

TABLE I

RETENTION INDICES OF C_6 HYDROCARBONS ON PHOSPHINATES AND SQUALANE

Compound	*Mg-DHP*	*Ca-DHP*	*Sr-DHP*	*Ba-DHP*	*Sq*[6]	*Sq*[7]	*SRI*
n-Hexane	600	600	600	600	600	600	600
1-Hexene	596.9	592.8	597.4	595.9	583.9	582.7	588
1-Hexyne	650.3	632.9	648.6	643.9	586.9		610
1,5-Hexadiene	592.8	585.9	592.6	590.4	565.7	563.7	577
2,4-Hexadiene	696.7	668.4	681.7	681.5	655.1		642
cis-2-Hexene	622.6	611.5	619.6	616.6	605.6	604.1	600
trans-2-Hexene	614.3	605.0	610.4	609.0	598.2	596.7	598
cis-3-Hexene	610.8	604.1	611.1	609.2	594.0	593.0	595
trans-3-Hexene	609.5	603.0	608.8	607.2	592.9	591.6	596
Cyclohexane	659.2	635.8	649.8	651.7	667.9	667.2	645
Cyclohexene	691.1	654.6	675.0	675.6	676.9		652
1,3-Cyclohexadiene	699.4	655.0	677.0	677.0			647
1,4-Cyclohexadiene	739.4	688.0	712.3	712.8	694.4		670
Benzene	713.8	661.2	695.9	685.9	644.6	641.8	643
3-Methylpentane	585.3	586.0	587.9	586.5	585.4	585.1	587.0
3-Methyl-1-pentene	567.1	567.7	571.2	568.8		553.0	563
cis-3-Methyl-2-pentene	618.0	608.9	618.1	615.4	604.6	603.4	607
trans-3-Methyl-2-pentene	627.0	617.0	625.1	624.0	614.4	612.9	598
Methylcyclopentane	624.7	609.7	619.0	620.0	632.1	631.0	612
2-Methyl-1-pentene	595.2	592.4	599.4	596.9	581.9	580.6	581
4-Methyl-1-pentene	565.1	568.5	571.4	569.5		550.4	562
trans-4-Methyl-2-pentene	579.1	578.8	582.6	580.6	563.0	561.6	575

Normal unsaturated hydrocarbons have higher retention indices on phosphinates than on squalene. These indices are also higher than the SRIs. The difference in retention indices between phosphinates and squalane and between phosphinates and SRI increases with the degree of unsaturation. These results indicate the existence of specific interactions between the double bonds of the solute and the alkaline earth metal of the phosphinates. There is a "cyclic effect" for cyclic hydrocarbons, because such compounds have higher retention indices than SRIs[5]. Copper and magnesium phosphinates when melted demonstrated a larger cyclic effect[8]. One may conclude that this effect is caused by the dispersive interactions of the phosphorus–alkyl radicals, so the rigidity of the structure of a solid phosphinate in contrast to a melted phosphinate may explain the smaller value of the effect. Alkene derivatives of 3-methylpentane have much higher retention indices than SRIs in the group, which

indicates apparent interactions of the double bonds of these hydrocarbons with the alkaline earth metals.

To make a quantitative evaluation of these interactions one must take into account the volatility of the hydrocarbons. There is a rectilinear dependence for a limited range of temperatures:

$$\log V_g^{pT} = a t_b + b$$

where V_g^{pT} = retention volume and t_b = hydrocarbon boiling point.

The linear relationship between retention data and boiling points of *n*-alkanes can serve as a reference line from which one can define a hypothetical retention volume, V_{g1}^{pT}, belonging to any *n*-hydrocarbon having a boiling point T_x. Knowing its real retention volume, V_{g2}^{pT}, one can calculate the difference that may indicate tne specific interaction contributions to the retention volume:

$$\Delta V_{\text{spec}}^{pT} = V_{g2}^{pT} - V_{g1}^{pT}$$

where $\Delta V_{\text{spec}}^{pT}$ = retention volume due to a specific interaction.

A similar procedure has been considered for evaluation of the retention mechanism of chlorinated methanes in hydrocarbon stationary phases[9]. Fig. 1 illustrates schematically the basis for the calculation of $\Delta V_{\text{spec}}^{pT}$.

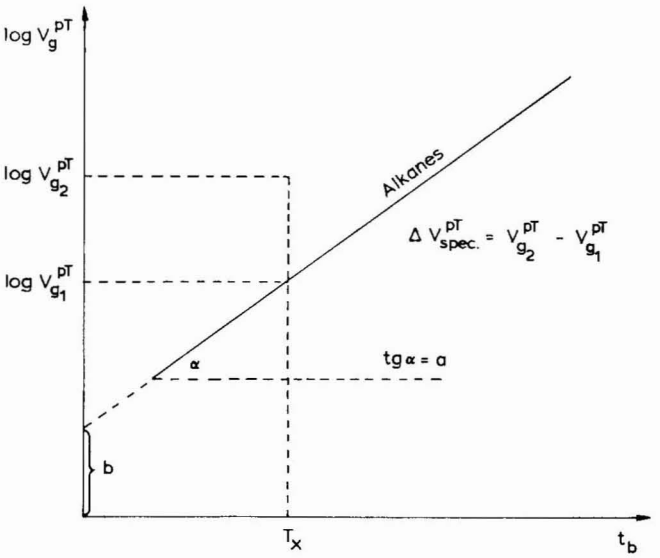

Fig. 1. Specific interactions of hydrocarbons with boiling point T_x.

Coefficients *a* and *b* and correlation coefficients (*r*) have been calculated for all of the columns. Values of *r* close to unity support the rectilinear relationship for each column used. The values of $\Delta V_{\text{spec}}^{pT}$ were calculated for unsaturated *n*-hydrocarbons and are presented in Table II.

TABLE II

VALUES OF RETENTION VOLUMES DUE TO SPECIFIC INTERACTIONS OF SOME C_6 HYDROCARBONS, V_{spec}^{pT} (ml/g)

Compound	t_b (°C)	*DHP-Mg*	*DHP-Ca*	*DHP-Sr*	*DHP-Ba*
1-Hexene	63.49	0.307	0.191	0.314	0.296
1-Hexene	71.33	1.566	0.899	1.523	1.370
cis-2-Hexene	68.84	0.648	0.248	0.530	0.451
trans-2-Hexene	67.87	0.441	0.113	0.287	0.265
cis-3-Hexene	66.44	0.475	0.262	0.481	0.451
trans-3-Hexene	67.08	0.355	0.136	0.319	0.293
1,5-Hexadiene	59.46	0.573	0.398	0.567	0.537
2,4-Hexadiene	80.00	3.013	1.480	2.139	2.220
a		$2.35 \cdot 10^{-2}$	$2.40 \cdot 10^{-2}$	$2.47 \cdot 10^{-2}$	$2.35 \cdot 10^{-2}$
b		$1.84 \cdot 10^{-3}$	$7.65 \cdot 10^{-2}$	$9.68 \cdot 10^{-2}$	$8.80 \cdot 10^{-2}$
r		0.9992	0.9992	0.9990	0.9992
r^2		0.998	0.998	0.998	0.998

The following conclusions can be drawn from these results: alkenes interact specifically with alkaline earth metals; the strength of those interactions depends on the degree of hydrocarbon unsaturation, alkynes and dienes interacting more strongly than alkenes; interactions of *cis*-isomers are stronger than those of *trans*-isomers; DHP-Mg shows the strongest and DHP-Ca the weakest interactions; and the specific interactions decrease with increasing ionic radius, excluding DHP-Ca.

Similar manipulations can be performed for cycloalkenes and unsaturated 3-methylpentane derivatives, but only if the reference line is based on cycloalkanes or 3-methylalkanes, respectively.

Specific free energy of interaction (ΔG_{spec})

The same dependence, $\log V_g^{pT} = f(t_b)$, can be used for the quantitative determination of thermal effects of specific interactions. The increase in retention volume together with the increase in the length of the *n*-alkane chain can be attributed to an increase in non-specific interactions[10]. Thus, the perpendicular (to the abscissa) section connecting the point (T_x, $\log V_{g2}^{pT}$) with the line set by *n*-alkanes can be used for the calculation of the free energy of specific interactions. If there are several sources of such interactions, then the total free energy, ΔG_a, will be

$$\Delta G_a = \sum \Delta G_{spec} + \Delta G_{non\text{-}spec}$$

where the sum contains all the possible sources of interactions different from non-specific. Based on the straight line set by *n*-alkanes:

$$\Delta G_{spec} = -RT \ln \left(\frac{V_{g2}^{pT}}{V_{g1}^{pT}} \right)$$

where:

V_{g2}^{pT} = real retention volume of an *n*-hydrocarbon having a boiling point equal to T_x.

V_{g1}^{pT} = hypothetical retention volume of the hydrocarbon (or retention volume of hypothetical *n*-alkane having the same boiling point T_x).
The hypothetical retention volume V_{g1}^{pT} shows which part of the total (real) retention volume V_{g2}^{pT} is caused by non-specific interactions. The values of ΔG_{spec} calculated in this way are shown in Table III.

TABLE III

VALUES OF $-\Delta G_{spec}$ OF SOME UNSATURATED HYDROCARBONS (kcal/mole)

Unsaturated hydrocarbon	*DHP-Mg*	*DHP-Ca*	*DHP-Sr*	*DHP-Ba*
1-Hexene	0.047	0.028	0.049	0.042
1-Hexyne	0.177	0.095	0.175	0.146
1,5-Hexadiene	0.092	0.059	0.093	0.080
2,4-Hexadiene	0.259	0.127	0.195	0.186
cis-2-Hexene	0.084	0.031	0.071	0.055
trans-2-Hexene	0.060	0.016	0.041	0.035
cis-3-Hexene	0.066	0.035	0.069	0.058
trans-3-Hexene	0.050	0.019	0.046	0.039

Heats of adsorption and specific heats of adsorption

Determination of thermal effects of adsorption is one of the commonest methods for the characterization of an adsorbent surface. The heats of adsorption of C_6 hydrocarbons on the packings discussed are presented in Table IV.

The heats of adsorption increase in the order DHP-Ba < DHP-Mg < DHP-Ca < DHP-Sr. It is more useful for our discussion to determine which part of the thermal effects is due to specific interactions of unsaturated hydrocarbons with phosphinates. The linear dependence of the heats of adsorption of alkanes on their boiling points has been known for a long time. Taking advantage of this relationship, we can calculate the heats of specific interactions, ΔH_{spec}. The results are presented in Table V.

The independence of ΔH_{spec} from temperature (in contrast to ΔV_{spec}^{pT}) makes this factor more valuable for the surface characterization of an adsorbent than the retention volume due to specific interactions. The regularities discovered before, *viz.*, dependence of the interaction on the degree of unsaturation of the hydrocarbon and stronger interactions of *cis*-isomers than those of *trans*-isomers, are fully confirmed by the results in Table V. However, no distinct dependence of these interactions on the ionic size of alkaline earth metals has been observed. To explain the this lack of dependence we shall consider the IR spectra of the phosphinates.

The spectra in the solid state and in solutions in the vibration range of the $-PO_2-$ group (900–1300 cm^{-1}) were measured for all of the phosphinates. The spectrum of DHP-Mg (potassium bromide pellet) shows three strong absorption bands at 1165, 1083 and 1023 cm^{-1}. The spectrum of the same compound dissolved in *n*-heptane demonstrates two strong absorption bands at 1160 and 1095 cm^{-1}, and two much weaker bands at 1006 and 976 cm^{-1}. DHP-Mg dissolved in carbon tetrachloride shows only two strong bands at 1163 and 1093 cm^{-1}. Taking into account literature data[11], one can conclude that the $-P=O$ group takes part in the coordi-

TABLE IV

HEATS OF ADSORPTION OF C_6 HYDROCARBONS ON ALKALINE EARTH METAL PHOSPHINATES (kcal/mole)

Compound	*DHP-Mg*	*DHP-Ca*	*DHP-Sr*	*DHP-Ba*
n-Pentane	5.81	6.37	6.64	5.13
n-Hexane	6.75	7.34	7.66	6.20
n-Heptane	7.67	8.41	7.86	7.27
n-Octane	8.64	9.49	10.17	8.32
1-Hexene	6.75	7.28	7.76	6.36
1-Hexyne	7.88	8.33	9.11	7.63
cis-2-Hexene	7.15	7.48	8.15	6.61
trans-2-Hexene	7.10	7.46	8.05	6.56
cis-3-Hexene	6.91	7.44	8.00	6.62
trans-3-Hexene	6.94	7.43	7.98	6.54
1,5-Hexadiene	7.08	7.32	7.85	6.52
2,4-Hexadiene	7.90	8.39	9.12	7.76
Cyclohexane	6.94	7.40	8.03	6.31
Cyclohexene	7.49	7.83	8.54	6.87
1,3-Cyclohexadiene	7.74	7.98	8.78	7.10
1,4-Cyclohexadiene	8.16	8.24	9.24	7.60
Benzene	7.85	8.14	9.28	7.45
Methylcyclopentane	6.61	7.20	7.66	6.15
3-Methylpentane	6.41	6.79	7.57	5.98
3-Methyl-1-pentene	6.17	6.96	7.48	5.95
cis-3-Methyl-2-pentene	6.73	7.41	8.25	6.59
trans-3-Methyl-2-pentene	6.85	5.89	8.22	6.76
2-Methyl-1-pentene	6.43	7.16	7.98	6.47
4-Methyl-1-pentene	5.92	6.79	7.43	6.04
trans-4-Methyl-2-pentene	6.35	7.03	7.65	6.31

TABLE V

SPECIFIC HEATS OF ADSORPTION OF UNSATURATED *n*-HYDROCARBONS ON ALKALINE EARTH METAL PHOSPHINATES (kcal/mole)

Compound	*DHP-Mg*	*DHP-Ca*	*DHP-Sr*	*DHP-Ba*
1-Hexene	0.12	0.03	0.16	0.30
1-Hexyne	1.01	0.81	1.21	1.29
cis-2-Hexene	0.35	0.04	0.34	0.36
trans-2-Hexene	0.33	0.06	0.28	0.34
cis-3-Hexene	0.19	0.09	0.29	0.45
trans-3-Hexene	0.20	0.06	0.24	0.35
1,5-Hexadiene	0.58	0.21	0.41	0.60
2,4-Hexadiene	0.75	0.57	0.88	1.11
a	$3.15 \cdot 10^{-2}$	$3.49 \cdot 10^{-2}$	$3.93 \cdot 10^{-2}$	$3.56 \cdot 10^{-2}$
b	4.63	5.04	5.10	3.80
r	0.9988	0.9979	0.9954	0.9993
r^2	0.998	0.996	0.991	0.999

nation of the magnesium atom. Three absorption bands (in the solid state) justify the existence of at least two kinds of such a coordination. The change in the phosphinate IR spectrum after dissolution of the compound in *n*-heptane indicates the flexibility of its structure and its susceptibility to external conditions. One may also assume that coordination bonds existing in the solid state are so weak that they might be of an intermolecular nature.

Carbon tetrachloride molecules that can coordinate with a metal atom probably take part in the coordination. A similar influence on phosphinate spectra were observed with $Mn[OP(C_8H_{17})_2O]_2$; the spectrum in the solid state (Nujol) demonstrated three bands at 1140, 1067 and 1015 cm^{-1}, whereas when dissolved in carbon tetrachloride it showed only two at 1135 and 1065 cm^{-1}.

The spectrum of DHP-Ca (potassium bromide pellet) shows several bands in the vibration range of the $-PO_2-$ group with similar intensities at 1168, 1138, 1025, 980 and 960 cm^{-1} and can prove the existence of several asymmetric kinds of coordination of calcium with the $-P=O$ group[11–13]. The spectrum of DHP-Ca dissolved in *n*-heptane consists of three dominating bands at: 1150, 1050 and 960 cm^{-1}. Dissolution of the phosphinate in carbon tetrachloride shifts these bands to 1165, 1033 and 955 cm^{-1}. Three absorption bands usually appear for octahedrally coordinated transition metal phosphinates, *e.g.*, for manganese, iron and cobalt di-*n*-octylphosphinates[11–13], so one may assume that the calcium atom is less accessible than magnesium in DHP-Mg. Hence DHP-Ca may be assumed to undergo weaker interactions with solutes under chromatographic conditions.

The spectra of DHP-Sr and DHP-Ba (potassium bromide pellets) are very much similar, the former having bands at 1143 and 1048 cm^{-1} and the latter at 1133 and 1034 cm^{-1}. In solution in the polar solvent dichloromethane (in non-polar solvents they are not soluble enough for a spectrum to be obtained) the bands are shifted to 1133 and 1040 cm^{-1} (1020 cm^{-1}) and 1130 and 1032 cm^{-1} (1020 cm^{-1}), respectively.

Dissolution of DHP-Sr and DHP-Ba in dichloromethane does not result in considerable differences in the IR spectra, although splitting of the symmetrical vibration band into a doublet is noticeable; this indicates that the structure of the dissolved compound differs from that of a phosphinate in the solid form. The splitting of both symmetrical and asymmetric vibration bands of the $-PO_2$ group in dichloromethane solution has already been observed for lead diphenylphosphinate[14].

The above discussion leads to the conclusions that interactions of alkaline earth metals with compounds that have π-bonds and heteroatoms are possible, calcium interactions will be weaker than those of magnesium and strontium and barium should have similar interactions.

CONCLUSIONS

Gas chromatography has been used to demonstrate the interactions of main-group metals with unsaturated hydrocarbons and their dependence on the degree of unsaturation and the structure of the solutes. The IR spectra of the alkali metal phosphinates have confirmed the possibility of the interactions and enabled the relatively weak specific interactions of DHP-Ca to be explained.

The linear dependence of logarithms of retention volumes on boiling points of

the sorbates has been used for the quantitative determination of specific interactions. The results presented in this and previous papers indicate that the relative retention volumes should not be used for this purpose even if one employs as a reference an alkane having the same number of carbon atoms as a compound able to interact.

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IDENTIFICATION OF GINSENOSIDES FROM *PANAX GINSENG* IN FRACTIONS OBTAINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY BY FIELD DESORPTION MASS SPECTROMETRY, MULTIPLE INTERNAL REFLECTION INFRARED SPECTROSCOPY AND THIN-LAYER CHROMATOGRAPHY*

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SUMMARY

The high-performance liquid chromatographic (HPLC) determination of the ginsenoside saponins from *Panax ginseng* C. A. Meyer (Araliaceae) was used to investigate the possibilities and limitations of peak identification with on-line and off-line methods. Thin-layer chromatography was used to separate the ginsenosides with a running distance of 6.5 cm, the detection limit being 0.2 μg. By means of multiple internal reflection infrared (IR) spectroscopy, IR spectra were obtained with 20 μg of ginsenoside. Field desorption (FD) mass spectrometry permitted not only the identification and determination of the molecular weights of underivatized ginsenosides, but also gave important information about the sequence of the sugar moities in the molecule. Less than 1 μg of ginsenoside is needed to produce an FD spectrum. The time required for the FD investigation of an HPLC fraction containing a ginsenoside is about 1 h, including sample preparation, FD measurements, data processing, output, evaluation and interpretation.

INTRODUCTION

We recently reported on the possibility of separating ginsenosides (triterpene saponins from *Panax ginseng* C.A. Meyer) by means of high-performance liquid chromatography (HPLC) and of determining them quantitatively in plant materials and in various galenical preparations[1,2]. An important aspect, which often is not considered when using HPLC, is the unambiguous and reliable identification of the eluted substances. The examination of the purity of a peak is particularly important if there are substances to be determined in complex mixtures, such as body fluids or

* Field desorption mass spectrometry of natural products, Part IX; for Part VIII, see ref. 12.

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plant extracts. Utilizing the HPLC determination of the ginsenosides, we report here the possibilities and limitations of peak identification using on-line methods such as ultraviolet (UV) spectroscopy and absorbance ratios and off-line methods, such as thin-layer chromatography (TLC), multiple internal reflection infrared (MIR-IR) spectroscopy and field desorption mass spectrometry (FD-MS). The structures of the ginsenosides investigated are shown in Fig. 1.

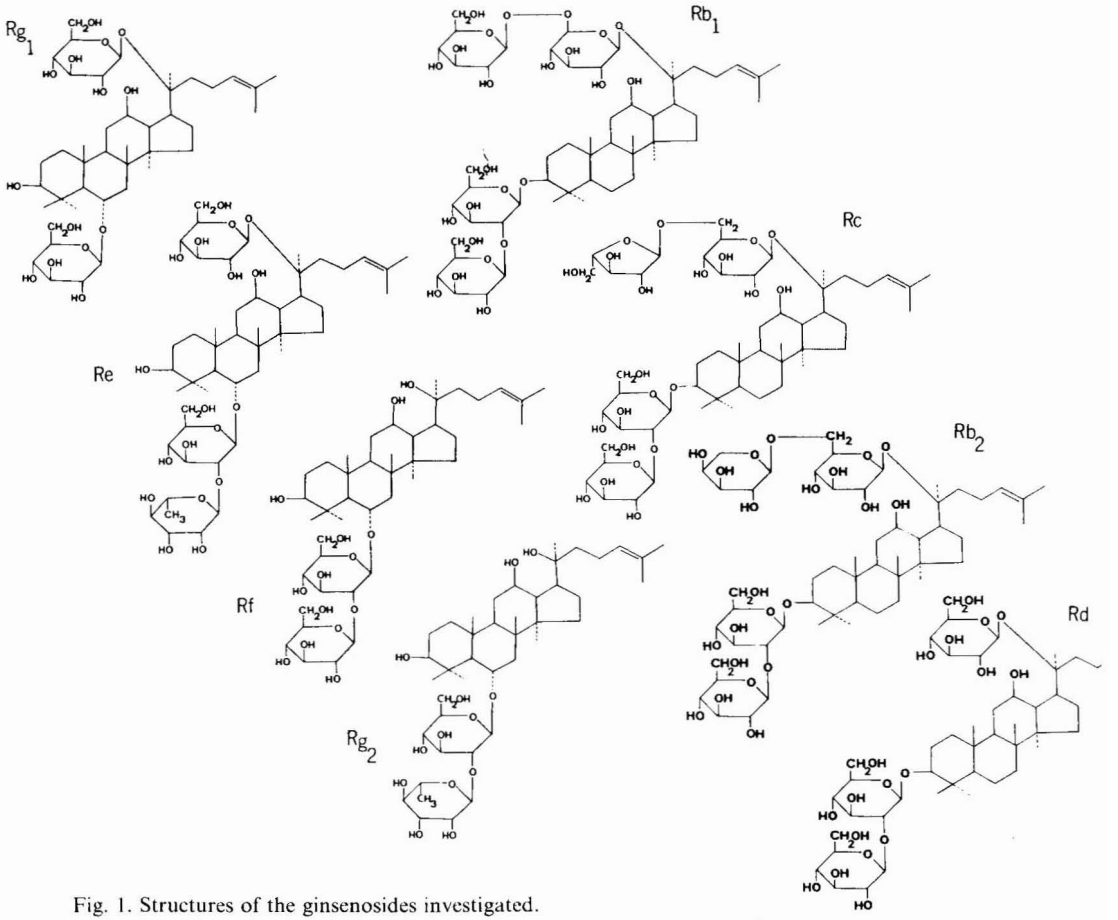

Fig. 1. Structures of the ginsenosides investigated.

EXPERIMENTAL

Apparatus

A Model 6000 A chromatographic pump, a Model U6K sample injection valve and a pre-packed μBondapack C_{18} column (30 cm $\times$ 3.9 mm I.D.) were obtained from Waters Assoc. (Milford, MA, U.S.A.), and an LC 75 spectrophotometric detector, Model 598 infrared spectrophotometer and micro MIR accessory from Perkin-Elmer (Norwalk, CT, U.S.A.). An Extrelut column and silica gel HPTLC plates (10 $\times$ 10 cm) were purchased from Merck (Darmstadt, G.F.R.) and a Linomat III from Camag (Muttenz, Switzerland). A W + W Series 1100 recorder was supplied by W +

W Electronic (Basle, Switzerland) and a MAT 731 mass spectrometer and Spectro-System 200 by Varian (Bremen, G.F.R.).

Materials

Methanol *n*-butanol and acetonitrile (Uvasol grade), chloroform, acetic anhydride, sulphuric acid and ethanol were Merck products. Perfluorokerosene and tris-(perfluorononyl)-*s*-triazine were obtained from J. T. Baker (Deventer, The Netherlands). Standardized ginseng extracts G115 and G115 S and ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1 and Rg_2 were supplied by Pharmaton (Lugano-Bioggio, Switzerland).

Sample preparation

A 1.0-g amount of ginseng extract was dissolved in 20.0 ml of water in a tared flask and 4.0 ml of this solution and 6.0 ml of water were added to an Extrelut column. After 15 min the column was eluted with 80.0 ml of water-saturated *n*-butanol. The eluate was evaporated on a Rotavapor and the residue dissolved in 2.0 ml of methanol. A 5–30-μl volume of this solution was injected into the HPLC system. The plastic material of new columns must be soaked in methanol for 1 h prior to the first use; before further use washing with common detergents is sufficient. The refilling material must be washed with methanol prior to use (for instance, six bags of refilling material with 1 l of methanol for 6 h in a Soxhlet apparatus, then dryed at 80°C). The column is packed with 11.3 g of refilling material. For further information, see ref. 1.

HPLC

The high-performance liquid chromatograph consisted of a constant-flow pump, a valve-type injector, a UV detector (203 nm, absorbance 0.08) and a strip-chart recorder (0.5 cm/min). The stainless-steel column was packed with reversed-phase material. The mobile phase was acetonitrile–water (29:71) at a flow-rate of 2 ml/min for ginsenosides Rb_1, Rb_2, Rc, Rd, Rf and Rg_2 and acetonitrile–water (18:82) at a flow-rate of 4 ml/min for ginsenosides Re and Rg_1. After each chromatographic run the column was washed with 30 ml of methanol at 4 ml/min. Room temperature (22°C) was used.

TLC

The fractions relating to the peaks of Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1 and Rg_2 were collected from the chromatograph in semi-micro flasks with conical bottoms, then the solvent was evaporated. The residue was dissolved in a few microlitres of methanol and the solution drawn off with a syringe. Pre-coated HPTLC plates were used for the chromatography, with Linomat III spray-on equipment. The sample volume was 10 μl, band length 5 mm, running speed 50 mm/μl, piston speed 10 sec/μl and nitrogen pressure 2 bar. Eluent A (chloroform–*n*-butanol–methanol–water, 20:40:15:20; lower phase) or eluent B (chloroform–methanol–water, 65:35:10; lower phase) can be used as the mobile phase. The eluent front was 6.5 cm for A and 7.5 cm for B. The plate was sprayed with 10 ml of acetic anhydride–sulphuric acid–ethanol (5:5:90) and heated at 105°C for 10 min. The ginsenosides were revealed with brown-violet colouring.

MIR-IR

A microprocessor-controlled IR spectrophotometer was used. The scan time was 8 min, abscissa expansion 56 cm, Slit medium (resolution at 1100 cm^{-1}, 3 cm^{-1}). A micro multiple internal reflection accessory, including a 45° KRS-5 micro crystal (thallium bromide–iodide), 10 mm long × 5 mm high × 0.5 mm thick, was used. To record the spectrum, 10–20 μl of the peak fractions that are to be investigated are placed with a syringe on the crystal and the solvent (methanol) is evaporated in air.

FD-MS

The FD spectra were produced on a commercially available double-focusing mass spectrometer equipped with a combined electron impact/field ionization/FD ion source. All spectra were recorded electrically with scan times between 4 and 8 sec/decade and at a mass resolution of better than 3000 (10% valley definition). Data acquisition and processing were performed using the data system. For mass calibration the electron impact mass spectra of perfluorokerosene and tris(perfluorononyl)-*s*-triazine were taken. The FD emitters used in all experiments were prepared by high-temperature activation of 10-μm diameter tungsten wires in a home-built multiple activation chamber. In general, emitters with an average length of 30 μm for the carbon microneedles were used and their ionization efficiency and adjustment were determined by means of m/z 58 of acetone in the field ionization mode. All spectra were produced at ion source potentials of +8 kV for the field anode and −3 kV for the slotted cathode plate, an ion source pressure of *ca.* 10^{-7} Torr and an ion source temperature between 50 and 60°C. The samples were desorbed by direct heating using the supplied emitter heating current (0–70 mA) and by controlling the emission of FD ions roughly by the total ion monitor. Methanol was used as solvent for all HPLC fractions and an estimated amount of *ca.* 1 μg was transferred to the emitter by the syringe technique. The time required for the FD investigation of an HPLC fraction containing a saponin was about 1 h, including sample preparation, FD measurements, data processing, output, evaluation and interpretation.

RESULTS AND DISCUSSION

On-line methods

In general, the identification and determination of the substances separated by chromatography can be performed on-line, that is at the same time as the chromatographic separation is achieved. Alternatively, an off-line investigation can be applied, involving collection of the effluent and consecutive examination of the chromatographically isolated fractions by physical or chemical methods. The on-line methods can be characterised as follows.

Retention time. Identical capacity factors of a reference substance and the substance to be determined in the test material is a unreliable peak identification method, as several substances can show identical retention times.

UV spectroscopy. If a UV spectrophotometer is used as a detector, it is possible to record the UV spectrum of substances in the stop-flow mode. In this method, the flow of the mobile phase is stopped when a substance that we wish to identify is in the absorption cell. One must work either with a double-beam spectrophotometer or a single-beam spectrophotometer with a background corrector. Although this peak

identification method has proved effective in certain instances[3,4], it is not suitable when there are two or more substances with identical UV spectra under the same peak. Owing to the C_{24}–C_{25} double bonding, all ginsenosides have identical UV spectra ($\lambda_{max.}$ 203 nm) and molar absorptivities (log ε = 4.66). Therefore, if two or more ginsenosides lie under the same peak, it would not be possible to distinguish them.

Absorbance ratios. Another technique for confirming the purity of compounds that appear within a single peak is to use the absorbance ratios at several UV wavelengths[5]. The flow of the mobile phase is stopped when a substance is in the absorption cell and the absorption at several wavelengths is measured. The ratios of these absorbances give an indication of the spectral properties of the compound in the cell. This technique is also useful for confirming the purity of compounds. It is not suitable when unseparated substances have the same UV spectra and identical molar absorptivities.

Off-line methods

For the off-line methods, which were preferred in this work, the following techniques were employed. After previous treatment the extracts were injected into the high-performance liquid chromatograph; Fig. 2 shows typical chromatograms. The steps in the possible off-line methods for peak identification are shown in Fig. 3. The fractions corresponding to peaks Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1 and Rg_2 are collected from the chromatograph in semi-micro flasks with conical bottoms (step 1). In step 2 the solvent (acetonitrile–water) is evaporated. The residue is then dissolved in a few microlitres of methanol and drawn off with a syringe (step 3).

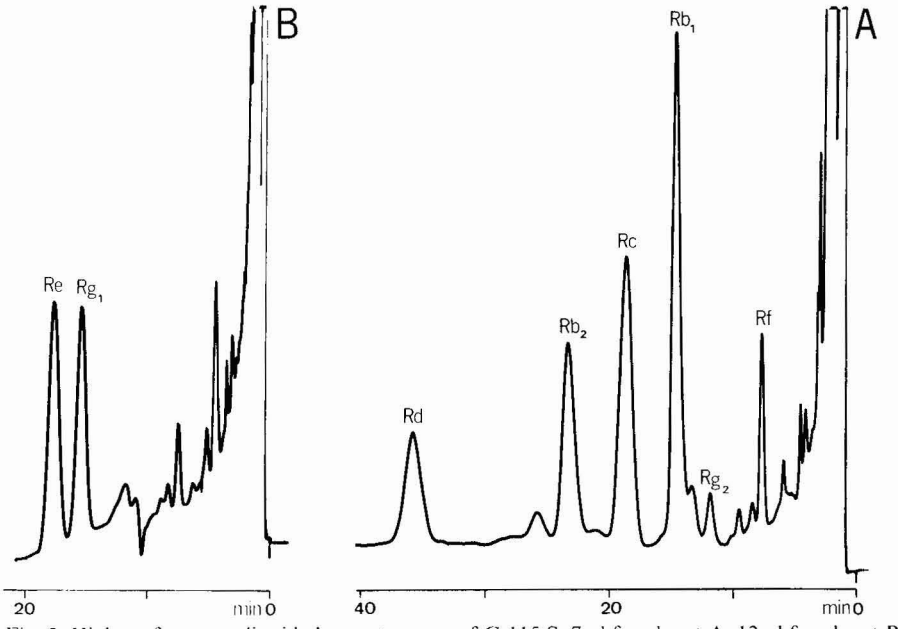

Fig. 2. High-performance liquid chromatograms of G 115 S, 7 μl for eluent A, 12 μl for eluent B. Eluent A: acetonitrile–water (29:71), 2 ml/min. Eluent B: acetonitrile–water (18:82), 4 ml/min. Rb_1, 13 μg; Rb_2, 8 μg; Rc, 11 μg; Rd, 4.4 μg; Re, 17 μg; Rf, 2 μg; Rg_1, 11 μg; Rg_2, 1 μg.

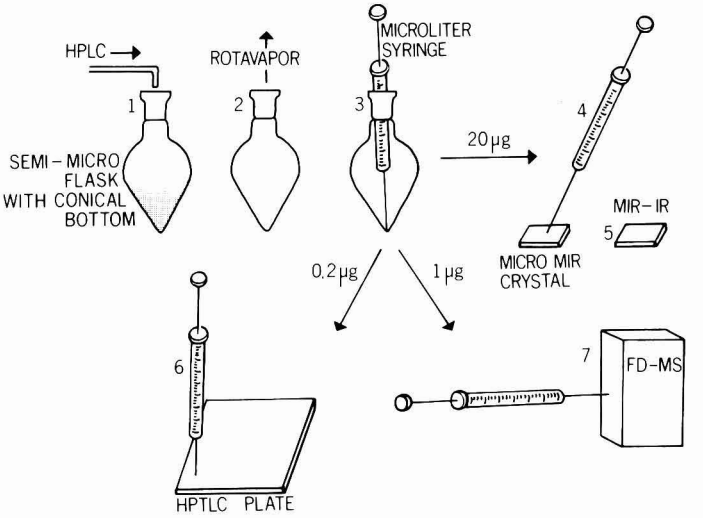

Fig. 3. Off-line methods for identifying substances separated by HPLC.

TLC. Chromatography with HPTLC (step 6) has considerable advantages over classical TLC[6]. For good resolution, it is important to have the minimal diffusion of the spot over as small an area as possible. This is achieved by using a small sample volume and a suitable non-polar solvent. Therefore, water has to be removed from the solvent (step 2).

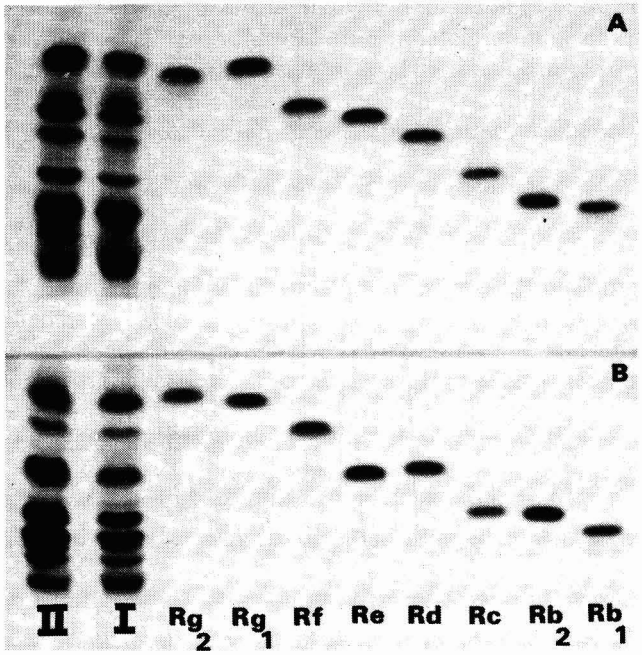

Fig. 4. Thin-layer chromatograms of G115 (I) (10 μl), G115 S (II) (10 μl) and of the peaks Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2 (10 μg each) after HPLC. For A and B, see Experimental.

It is of advantage to combine HPTLC plates with the Linomat spray-on technique, in which nitrogen is used as a carrier to spot the sample on the plate. The free selection of the sample volume between 1 and 100 μl, the accuracy of sample volume sprayed (better than 1%) and the freely selectable band length ensure the highest possible resolution within the shortest time. The results obtained with the HPLC spray-on technique are shown in Fig. 4. The ginsenosides can be separated with a running distance of 6.5 cm with eluent A within 60 min. To carry out an accurate peak identification, it is also possible to chromatograph with other eluents (for instance, B). Table I gives the R_F values obtained. This technique has a detection limit of 0.2 μg of ginsenoside.

TABLE I

RETENTION TIMES (R_F VALUES) OF THE GINSENOSIDES INVESTIGATED BY HPTLC

Ginsenoside	*Eluent A*	*Eluent B*
Rg_2	0.52	0.40
Rg_1	0.53	0.39
Rf	0.44	0.34
Re	0.42	0.25
Rd	0.37	0.26
Rc	0.28	0.23
Rb_2	0.22	0.23
Rb_1	0.21	0.14

MIR-IR spectroscopy. A useful application of MIR-IR spectroscopy is in the identification of peaks separated by HPLC (steps 4 and 5). Typical fraction sizes are 5–20 μg and, after evaporation of the solvent, they can easily be run as films cast on a micro MIR crystal. Total internal reflection occurs within a crystal of high refractive index, along the crystal–sample interface, when energy is passed through it at an angle of incidence greater than the critical angle. At each reflection energy penetrates the sample, which must be in optical contact with the crystal, and a small amount is absorbed at the characteristic absorption frequencies of the sample, producing an IR spectrum[5]. This technique allows the recording of IR spectra with very small amounts of substance. Spectra that we obtained with 20 μg each of the peak fraction corresponding to Rg_1 (A) and Re (B) are illustrated in Fig. 5. In these spectra the absorption bands at 1640 cm^{-1} (C=C) and at 3380 cm^{-1} (OH) are also characteristic for the other ginsenosides.

FD-MS. Gas chromatography–electron impact MS (GC–EI-MS) has been shown to be very useful for the identification and determination of the purity and structures of ginsenosides[7]. However, for the MS investigation volatile derivatives (peracetates or permethyl ethers) have to be produced. Moreover, the ginsenosides do not give molecular ions even when derivatized and even when the spectrum is taken at a low ionizing potential (20 eV)[8].

Previous investigations of natural products by FD-MS have demonstrated that the technique is suitable for the determination of the molecular weights of large, polar and underivatized glycosides. Steroid and triterpene saponins[9], physiologically active pennogenin and hederagenin glycosides[10], cytotoxic cardiac glycosides from *Lopho-*

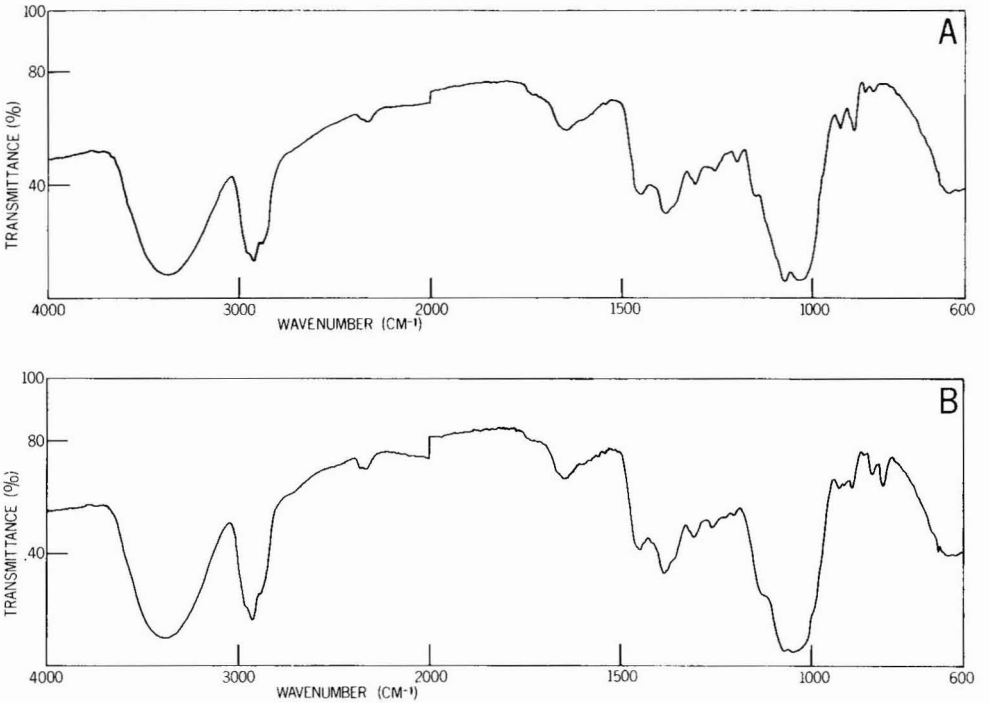

Fig. 5. MIR-IR spectra of the peaks Rg_1 (20 μg) (A) and Re (20 μg) (B) after HPLC.

petalum toxicum[11], lignan glycosides from the barks of *Ligstrum japonicum*[12] and some dammarane saponins[13] have been identified in extracts from plant materials. Moreover, the sequencing of underivatized oligoglycosides and the comparison of sequence-specific fragmentation with the mechanism of solvolysis in solution chemistry have been reported[14]. On the other hand, the utility of FD-MS for the identification of biocides[15–17] and drugs[18–20] in fractions of HPLC effluents, and thus the use of the FD spectrometer as a highly sensitive and specific "off-line" detector, have been amongst the most successful applications of the technique. These results prompted the present investigation to identify and characterize saponins from *Panax ginseng* extracts in HPLC fractions (step 7). The question was whether the FD method allows the rapid and reliable identification and, to some extent, the quality control of physiologically active natural products. In general, all of the ginsenosides investigated gave the $[M + Na]^+$ ion as base peak of the FD mass spectrum, and from this basic feature the molecular weight was easily derived. The compounds Rb_2, Re and Rg_1 had already been studied by FD[13] and the samples from the HPLC fractions gave similar results. For instance, Re gave the $[M + Na]^+$ ion at m/z 969 as base peak (6000 counts), a weak $[M + {}^{39}K]^+$ ion at m/z 985, the $[(M + Na) - H_2O]^+$ ion at m/z 951 (32% relative abundance), and a doubly charged $[M + 2Na]^{2+}$ ion at m/z 496 (15% relative abundance). All of these ions can be used to confirm the correct assignment of the molecular weight and, as will be shown below, are common in the FD spectra of the oligoglycosides. In addition, the loss of a terminal desoxyhexose, rhamnose, is indicated by the $[(M + Na) - 146]^+$ ion at m/z 823 (40% relative

abundance). Further, the loss of a terminal hexose, glucose, results in a weaker signal at m/z 807 for the $[(M + Na) - 162]^+$ ion (22% relative abundance). No other FD ions were detected when eight FD spectra were averaged at emitter heating currents of between 30 and 35 mA.

When six FD spectra of compound Rb_1 were taken between emitter heating currents of 35 and 40 mA the base peak was again the $[M + Na]^+$ ion at m/z 1131 (70,384 counts) and the $[M + 2Na]^{2+}$ ion at m/z 577 was of 12% relative abundance. The sequence-specific ions for the loss of one glucosyl unit, $[(M + Na) - 162]^+$, at m/z 969 and for the loss of the disaccharide consisting of two glucose units at m/z 807, $[(M + Na) - 324]^+$, were of minor importance under these experimental conditions (below 10% relative abundance). Also, compound Rg_1 gave an abundant ion at m/z 807 for $[M + Na]^+$, at m/z 823 for $[M + {}^{39}K]^+$ and m/z 415 for $[M + 2Na]^{2+}$. At the applied emitter heating current of 30–35 mA no sequence-specific fragmentation was induced.

In order to survey which thermally induced fragmentation can be generated and how these fragments can be explained, the ginsenosides Rf, Rd and Rc were investigated in that section of the desorption process which gave the most intense ion currents for structurally significant fragments.

The FD mass spectrum of Rf obtained in this manner is shown in Fig. 6. As mentioned above, the determination of the molecular weight is straightforward as the signal at m/z 823 produced by attachment of a sodium cation to the intact molecule (cationization) gives the base peak of the spectrum. Again, owing to small inorganic

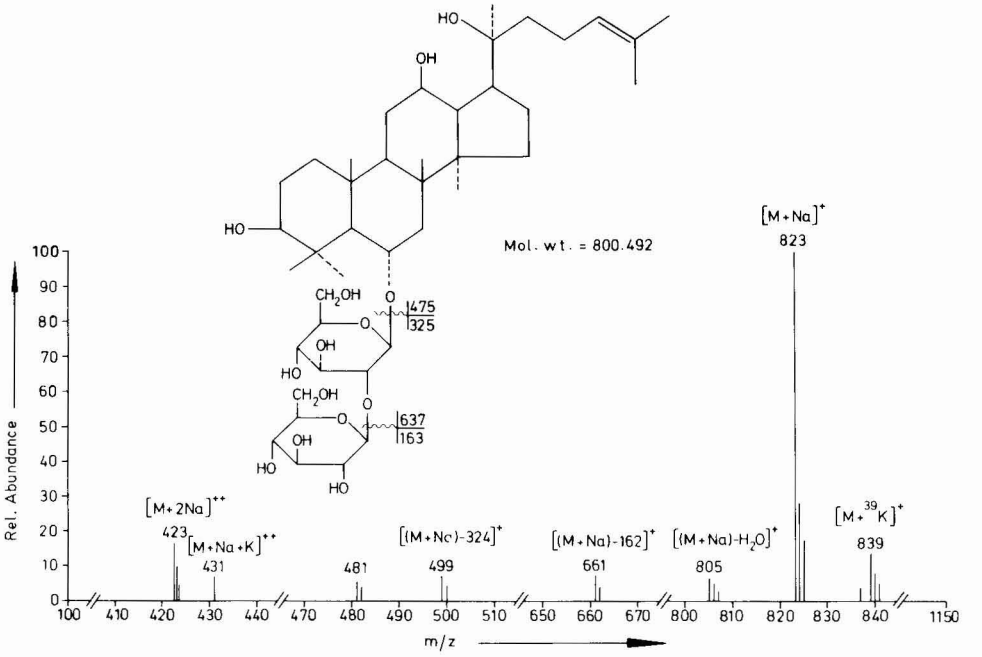

Fig. 6. FD mass spectrum of the HPLC fraction containing compound Rf. Seven mass spectra which were obtained between emitter heating currents of 29 and 32 mA were averaged by the data system. The base peak corresponds to 14,536 counts; the noise level is about 50 counts.

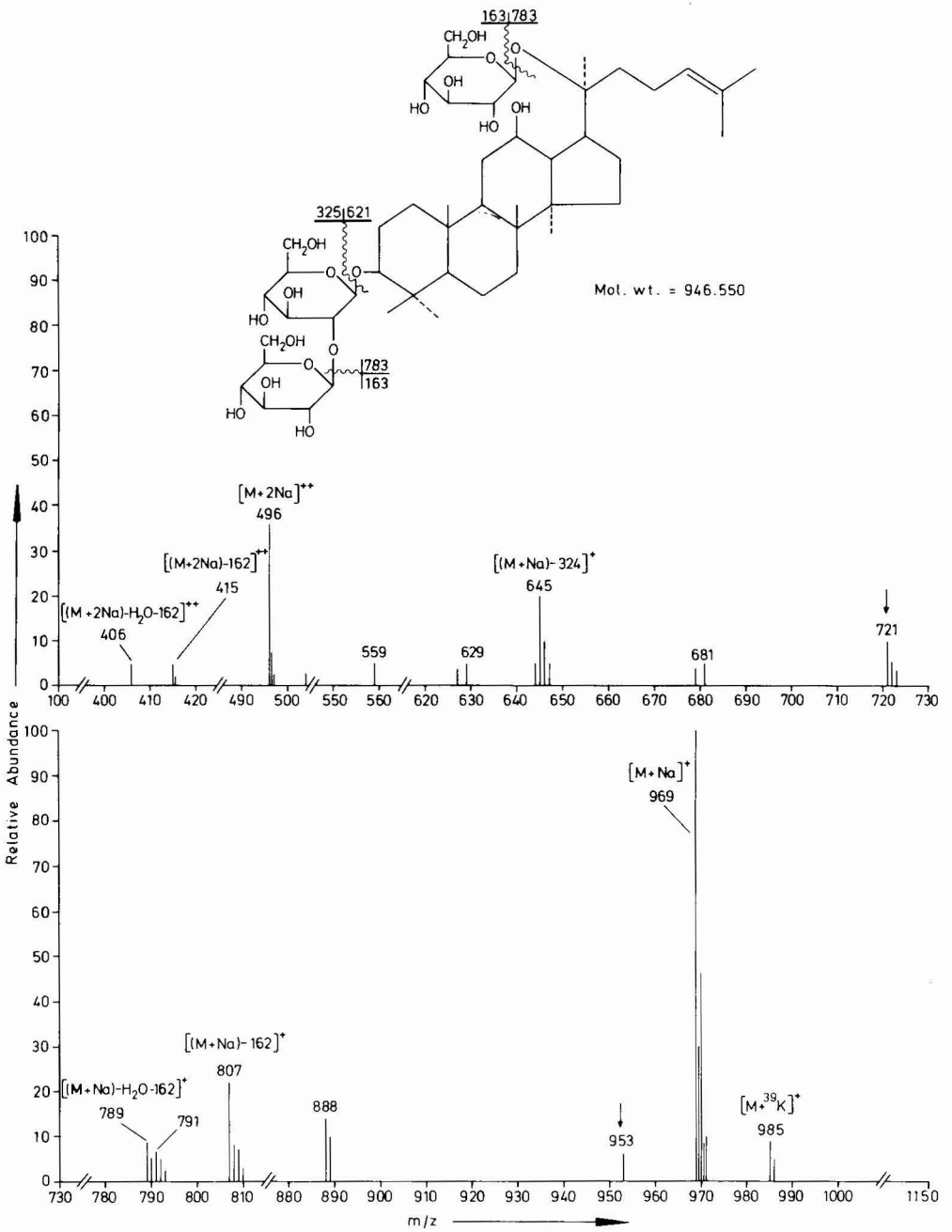

Fig. 7. FD mass spectrum of the HPLC fraction containing compound Rd. Eight mass spectra were averaged by the data system in a temperature range that corresponds to emitter heating currents between 30 and 40 mA. Whereas the noise level is comparable to that in Fig. 6, the base peak here represents 115,436 counts.

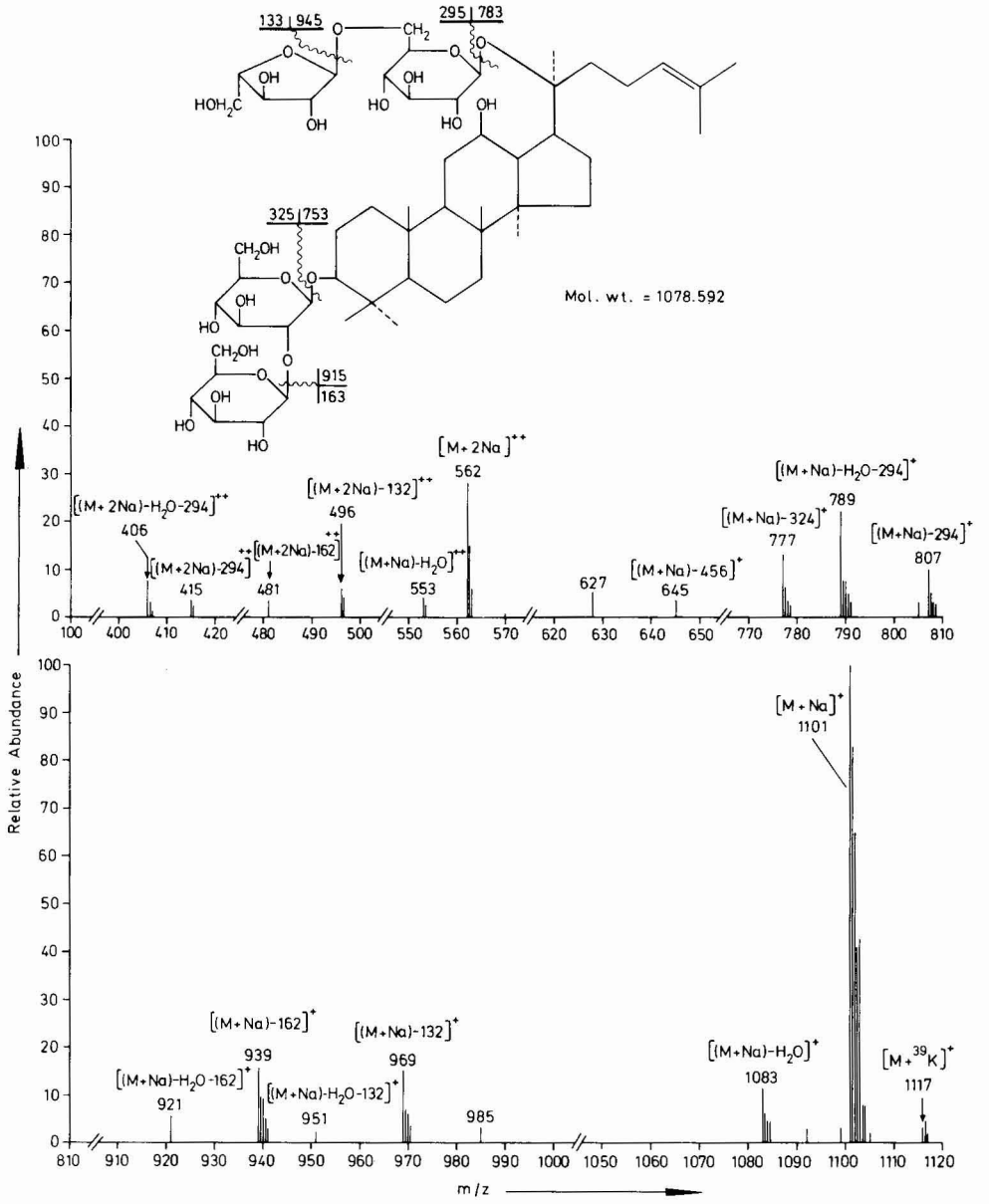

Fig. 8. FD mass spectrum of the HPLC fraction containing compound Rc. Within an emitter heating current in the range between 25 and 30 mA eight FD spectra were recorded and averaged. The base peak represents 57,776 counts; the background level (noise) is as in Figs. 6 and 7.

salt impurities, the $[M + {}^{39}K]^+$ ion at *m/z* 839 and its isotopic satellites are found. Loss of water from the molecule, probably from the aglycone, and cationization give the $[(M + Na) - H_2O]^+$ ion at *m/z* 805. An indication that the water loss is indeed from the aglycone and not the sugar moieties is derived from the signal at *m/z* 481, which can be explained as loss of water *after* cleavage of the disaccharide unit from

the glycosidic oxygen in position 6 of the aglycone. A general feature in FD-MS is the formation of doubly charged ions, and this is particularly valid for glycosides. This is clearly demonstrated by the FD ions at m/z 423, $[M + 2Na]^{2+}$, and m/z 431, $[M + Na + K]^{2+}$. The usefulness of these types of ion for additional confirmation of the molecular weight has been discussed previously in detail[21].

Cleavage of the terminal glucose from the molecule, proton transfer to the glycosidic oxygen and cationization are the processes that lead to the ion at m/z 661, explained as $[(M + Na) - 162]^+$. Similarly, the loss of both glucose units from position 6 of the aglycone occurs and, as the glycosidic oxygen is retained and proton transfer results in the hydroxyl function, an ion of comparable intensity is found at m/z 499 for $[(M + Na) - 324]^+$.

For compound Rd more fragmentation was found and, as shown in Fig. 7, the molecular weight of this ginsenoside is unambiguously determined by four series of FD ions. The $[M + {}^{39}K]^+$ ion at m/z 985, $[M + Na]^+$ at m/z 969, $[M + 2Na]^{2+}$ ion at m/z 496 and $[M + Na + {}^{39}K]^{2+}$ at m/z 504 all give information on the molecular weight. However, minor accompanying substances that could also be oligoglycosides cannot be excluded because the signals at m/z 953 and 721 are unlikely to be frag-

TABLE II

DETERMINATION OF THE MOLECULAR WEIGHTS AND ASSIGNMENT OF STRUCTURALLY SIGNIFICANT SIGNALS FOR CONFIRMATION OF THE SUGAR SEQUENCE IN THE HPLC FRACTION OF COMPOUND Rc BY FD-MS

*Type of ion**	*Relative abundance***	*Accurate mass*
$[M + K]^+$	+	1117.556
$[M + Na]^+$	+ + + + +	1101.582
$[(M + Na) - X]^+$	+	1083.573
$[(M + K) - Z]^+$	+	985.514
$[(M + Na) - Z]^+$	+	969.540
$[(M + Na) - X - Z]^+$	+	951.529
$[(M + Na) - Y]^+$	+	939.529
$[(M + Na) - X - Y]^+$	+	921.519
$[(M + Na) - Z - Y]^+$	+	807.487
$[(M + Na) - X - Z - Y]^+$	+ +	789.427
$[(M + Na) - (Y - Y)]^+$	+	777.477
$[(M + Na) - X - (Y - Y)]^+$	+	759.466
$[(M + Na) - (Y - Y) - Z]^+$ $[(M + Na) - (Z - Y) - Y]^+$	+	645.434
$[(M + Na) - X - (Y - Y) - Z]^+$ $[(M + Na) - X - (Z - Y) - Y]^+$	+	627.424
$[M + 2Na]^{2+}$	+ +	562.286
$[(M + 2Na) - X]^{2+}$	+	553.281
$[(M + 2Na) - Z]^{2+}$	+	496.265
$[(M + 2Na) - Y]^{2+}$	+	481.266
$[(M + 2Na) - Y - Z]^{2+}$	+	415.238
$[(M + 2Na) - X - Y - Z]^{2+}$	+	406.233

* X = water; Y = glucose; Z = arabinose.

** The relative abundances for electrical detection are given with five levels: relative abundance 0–20% = +; 20–40% = + +; 40–60% = + + +; 60–80% = + + + +; 80–100% = + + + + +.

ments of Rd. A possible explanation is that in this compound the hydroxyl function in position C_{12} is labile and was exchanged against hydrogen (*e.g.*, after elimination of water). The molecular weight of this new compound would be 930 and the corresponding $[M + Na]^+$ ion at *m/z* 953 is detected with low relative abundance. Loss of one glucose unit $[(M + Na) - 162]^+$ would explain the ion at *m/z* 791 and the loss to two hexose sugars would give the ion at *m/z* 629. In view of the pronounced signals for elimination of water from the aglycone of Rd, *viz.*, $[(M + Na) - 162 - H_2O]^+$ at *m/z* 789, $[(M + Na) - 324 - H_2O]^+$ at *m/z* 627 and $[(M + 2Na) - 162 - H_2O]^{2+}$ at *m/z* 406, elimination of water from position 12 is a very likely process. Owing to the two possibilities for the loss of a terminal glucose from the molecule and the abundant $[(M + Na) - 162]^+$ ion, a doubly charged ion for the loss of a sugar unit *and* water was observed for the first time at *m/z* 406. Very interesting is a novel type of ion for $[(M + Na) - 162]^{2+}$, a signal which represents a doubly charged sequence ion.

The FD mass spectrum of Rc is shown in Fig. 8 and can be regarded as an optimal FD spectrum as far as structurally important fragmentation is concerned. As all types of ions described above for the other ginsenosides are detected, the accurate masses of these ions and their interpretation are listed in Table II in order to facilitate the understanding of the information obtained for molecular weight and structure.

ACKNOWLEDGEMENTS

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RAPID HIGH-RESOLUTION SEPARATION OF OLIGOSACCHARIDES ON SILVER FORM CATION-EXCHANGE RESINS*

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SUMMARY

The effect of silver as a counter-ion for the aqueous separation of oligosaccharides on cation-exchange resins has been investigated. Silver form cation-exchange resins retain oligosaccharides to a greater extent than the calcium form of the same resins, resulting in a greater number of oligosaccharides being separated. By varying the amount of silver placed on the resin the amount of separation and column efficiency can be optimized. By using the optimum silver loading technique, the operating range, *i.e.*, the number of oligosaccharides separated, has been increased by a factor of two for 4, 5, 6 and 8% cross-linked resins.

INTRODUCTION

Corn-derived oligosaccharides have been separated on calcium form 4% cross-linked cation-exchange resins using water as the sole eluent[1–3]. When operated at low flow-rates the calcium form column separates the oligosaccharides in corn syrups up to a degree of polymerization (DP) of 8. The remaining higher-molecular-weight saccharides elute in the void volume of the column as a single peak at the beginning of the chromatogram. While the resolution of the separation is generally excellent for dextrose and maltose, the resolution of the higher corn-derived oligosaccharides deteriorates markedly after maltotriose and again even more markedly after maltohexaose. These sudden changes in resolution are probably due to the unique helical structure of α-1-4 linked corn-derived oligosaccharides since no corresponding effect has been observed for the separation of cellulose-derived oligosaccharides[4] or for those derived from inulin.

In addition to the resolution losses caused by the structure of corn-derived oligosaccharides, considerable losses in efficiency are also encountered with increases in flow-rate on calcium form columns. Efficiency losses on the order of 50% are not uncommon for a two-fold increase in flow-rate.

Due to these losses and to the rather high compressibility of 4% cross-linked

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resins, usable high-speed separation of corn-derived oligosaccharides is usually limited to DP 1 through DP 5 with analysis times of 15–20 min.

Since some of our earlier work[2] indicated that the number of oligosaccharides separated by a given resin could be doubled with the use of silver as the counter-ion, it was decided to study this system further to see if more mechanically stable and efficient resins could be used to improve the quality of the separation and further reduce the time of the analysis.

EXPERIMENTAL

Materials

The chromatographic resins Aminex A-7 (7–11 μm), Aminex A-5 (10–15 μm), Aminex 50W-X4 (10–15 μm), and an experimental 6% cross-linked, 10–15-μm resin were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). The HC-40 (10–15 μm) resin was obtained from Hamilton (Reno, NV, U.S.A.). The silver nitrate used for resin preparation was reagent grade from Mallinckrodt (St. Louis, MO, U.S.A.).

Chromatographic grade tubing (60 cm × 7.8 mm I.D.) was obtained from Waters Assoc. (Milford, MA, U.S.A.) and 30-cm columns were cut from this stock size. Chromatographic end fittings (Cat. No. 6-1Z2HBZ7-3) were obtained from Parker-Hannifin (Huntsville, AL 35802, U.S.A.).

Instrumentation

The automated liquid chromatographic system used in this study consisted of a Waters Model ALC201 equipped with a Model 401 differential refractometer and a 1000-lbs. solvent delivery system (Waters Assoc.), a System I computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.), an Omniscribe Model 5211-12 dual-pen recorder (Houston Instruments, Austin, TX, U.S.A.) and a custom-built autosampler/system controller of our own design. Details of this system have been described in a previous paper[2].

Resin preparation

Before conversion to the silver form the resin of choice is degassed using a 1:4 slurry of resin in deionized water under aspirator vacuum for 30 min. Following degassing, the resin is transferred to a 350-ml medium porosity sintered glass büchner funnel and the excess water is drawn through the resin bed. The resin is then washed with successive 200-ml portions of 0.5, 1.0, and 2.0 M HNO_3. Each wash is allowed to stand on the resin for 20 min before being drawn through the resin bed. Finally the resin is washed with three 200-ml washes of deionized water as in the previous step.

Since silver form resins perform more efficiently at less than 100% loading, conversions are carried out using equilibrium techniques. In order to convert resins to the desired silver level the following equations and equilibrium constants are used.

The standard equation for silver–hydrogen ion-exchange equilibria is

$$K = \frac{M_{H^+}}{M_{Ag^+}} \times \frac{\%\ \text{Ag resin}}{\%\ \text{H resin}} \tag{1}$$

where K is the equilibrium constant, M_{H^+} is the final molar concentration of H^+,

M_{Ag^+} is the final molar concentration of Ag^+, % Ag resin is the percentage of the resin in the silver form and % H resin is the percentage of the resin in the hydrogen form. For 8% cross-linked resins, the value of 5.84 has been reported[5] for K. For 6, 5 and 4% cross-linked resins the values of K were experimentally determined to be 5.3, 5.0 and 3.0, respectively. By rearranging eqn. 1 and inserting the desired values of silver and hydrogen loading, the final Ag^+ concentration can be calculated using eqn. 2.

$$M_{Ag^+}\,(\text{final}) = \frac{M_{H^+}}{K} \times \frac{\%\ \text{Ag resin}}{\%\ \text{H resin}} \tag{2}$$

Using eqn. 2 along with the amount and the capacity of the resin, the initial concentration of Ag^+ for a given conversion can be calculated using eqn. 3.

$$M_{Ag^+}\,(\text{initial}) = \left(\frac{M_{H^+}}{K} \times \frac{\%\ \text{Ag resin}}{\%\ \text{H resin}}\right) + \frac{\text{mequiv. of silver on resin}}{\text{volume of solution}} \tag{3}$$

The following example illustrates the use of eqn. 3 in performing an equilibrium conversion:

A 25-ml volume of Aminex A-7 (H^+) is to be converted to the 75% silver form. The capacity of this 8% cross-linked resin is 1.7 mequiv./ml resulting in a total capacity of 42.5 mequiv. for the 25-ml amount. Seventy-five percent of this value, *i.e.* 31.88 mequiv., is the amount of Ag^+ that will be placed on the resin and will also be the amount of H^+ that will be displaced into the solution. Using 500 ml as the final volume, the following list of values can be tabulated: mequiv. of silver on resin, 31.88; volume of solution, 500 ml; M_{H^+} (final), 31.88/500 = 0.0638; % Ag resin, 75; % H resin, 25; K, 5.84.

Inserting these values into eqn. 3 gives the following results:

$$M_{Ag^+}\,(\text{initial}) = \left(\frac{0.0638}{5.84} \times \frac{75}{25}\right) + \frac{31.88}{500} = 0.0966$$

To carry out the actual conversion, 25 ml of the resin of choice is slurried in 100 ml of deionized water and is transferred to a 500-ml erlenmeyer flask. The appropriate amount of silver nitrate is then added from a 0.4 M stock solution. The slurry is diluted to 500 ml with deionized water and a magnetic stir bar is added. From this point, the resin is protected from light with aluminum foil. Finally, the slurry is gently stirred for 30 min.

After conversion the resin slurry is transferred to a 350-ml medium porosity sintered glass büchner funnel, 250 ml at a time and the excess silver nitrate solution is drawn off with aspirator vacuum. The resin is then washed with three 200-ml portions of deionized water as in the initial step. All washings are collected and residual Ag^+ is determined by atomic absorption spectroscopy. From this determination the amount of silver retained by the resin can be checked.

Finally, the resin is reslurried in 200 ml of deionized water and is transferred into a light-tight container where it is stored until needed.

Column packing procedure

A 60-cm column is joined with a 30-cm column using a 3/8-in. tubing union and the assembly is clamped in a vertical position with the long column on the upper end. A 500-ml reservoir is attached to the upper end of the 60-cm column and a plugged 5-μm end fitting is attached to the lower 30-cm column. Next the assembly is filled to the bottom of the reservoir with degassed deionized water. The assembly is tapped during this step to insure that no air bubbles stick to the walls of the tubing. The prepared resin is reslurried in 200 ml of water and is transferred into the reservoir. The resin is then allowed to settle into the assembly for at least 4 h. After settling is complete the reservoir is removed and the upper end of the assembly is connected to an undamped Milton-Roy pump. Next the plug is removed from the lower end fitting and flow from the pump is initiated at a rate appropriate for the resin being packed. Pumping schedules for the various resins are listed in Table I. After packing is complete the pump is shut off and the upper column is removed from the assembly. The union is removed from the lower column and the resin from it is transferred into an end fitting. A small amount of water is added to the resin to settle it into the end fitting. The water is then removed from the resin by applying suction to the outlet of the fitting. The fitting so prepared is attached to the column and will be used as the inlet.

TABLE I

COLUMN PACKING PUMPING SCHEDULE

Time (min)	*Cross-linking (%)*					
	4 (10–15 μm)		*5–6 (10–15 μm)*		*8 (7–11 μm)*	
	Flow-rate (ml/min)	*Pressure (p.s.i.g.)*	*Flow-rate (ml/min)*	*Pressure (p.s.i.g.)*	*Flow-rate (ml/min)*	*Pressure (p.s.i.g.)*
0– 60	0.1	0– 80	0.5	0–300	0.5	0– 500
60–120	0.2	80–400*	1.0	300–600*	1.0	500–1000
120–180	–	–	–	–	1.5	1000–1500*

* Packing is stopped and the column is capped if pressure exceeds this value.

Sample preparation

Since salts, acids, soluble protein and particulate matter interfere with the quantitative analysis of corn syrups they must be removed prior to analysis. Simplified clean-up procedures for corn syrup samples have been described in a previous paper[2].

Chromatographic conditions

Unless otherwise specified the following conditions were used throughout. Solvent, deionized, degassed water maintained at 95°C; column dimensions, 30 cm × 7.8 mm I.D.; column temperature, 85°C; detector temperature, 45°C; detector attenuation, 8×; recorder sensitivity, 100 mV full scale; sample concentration, 2–5% dry solids basis; sample volume, 5–15 μl.

RESULTS AND DISCUSSION

Shown in Fig. 1 is the separation of a 42 D.E.* acid-hydrolyzed corn syrup on a commercially prepared 4% cross-linked resin (Ca^{2+}) run at various flow-rates. The chromatograms in the figure are typical for corn syrups separated on this support. As Fig. 1 illustrates, marked decreases in resolution occur after the DP 3, DP 6 and DP 9 regions. In addition to losses in resolution due to the structure of the corn-derived oligosaccharides, Fig. 1A–C also illustrates the loss in resolution due to increases in flow-rate. In terms of theoretical plates, the values based on the dextrose peak are 3611 for the 72-min separation, 2958 for the 36-min separation and 1738 for the 18-min separation.

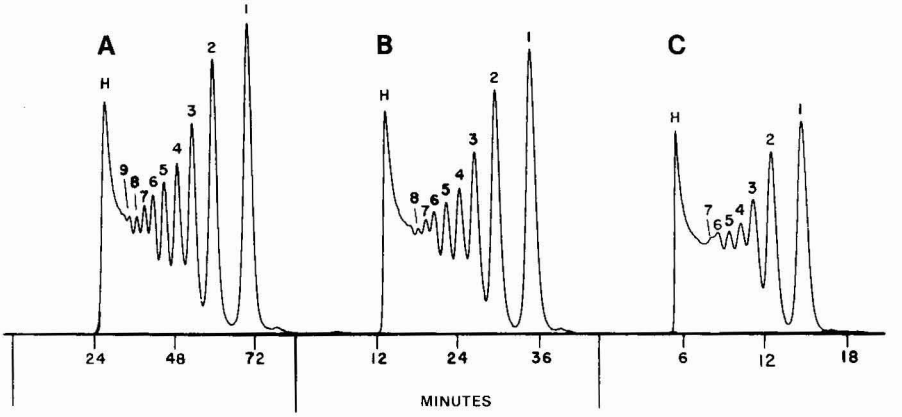

Fig. 1. Chromatograms of a 42 D.E. acid-hydrolyzed corn syrup at various flow-rates. Chromatographic conditions: column (30 cm × 7.8 mm I.D.) Aminex HPX-42 (Ca^{2+}), 4% cross-linked resin (20–30 μm). Flow-rate, (A) 0.15 ml/min; (B) 0.30 ml/min; (C) 0.60 ml/min. Numbers over peaks indicate DP; H indicates higher excluded oligosaccharides.

These problems, along with considerable lot-to-lot variation in the compressibility of 4% cross-linked resins, prompted the continued study of silver form resins.

Shown in Fig. 2 is the separation of a 42 D.E. acid-hydrolyzed corn syrup on a 4% cross-linked resin, Aminex 50W-X4 (Ag^+) (20–30 μm). This column, described in an earlier study[2] indicated that almost twice as many oligosaccharides could be separated on a 4% cross-linked resin in the silver form than could be separated on the same resin in the calcium form. However, the resolution of the oligosaccharides on these early silver form columns, while different in character, was in general no better than that obtained on the calcium form of the same resin.

Initially it was believed that these resins were being completely converted to the silver form. However, subsequent preparations of the same resin under slightly different conditions gave considerably different results in terms of the number of oligosaccharides separated and the resolution of the separation itself. These variations in the chromatographic behavior of early silver form resins finally lead to the discovery that they were only partially loaded with Ag^+, with the remainder of the resin being

* Throughout this article, D.E. (dextrose equivalent) is defined as a measure of reducing power using a modified Lane and Eynon procedure[6].

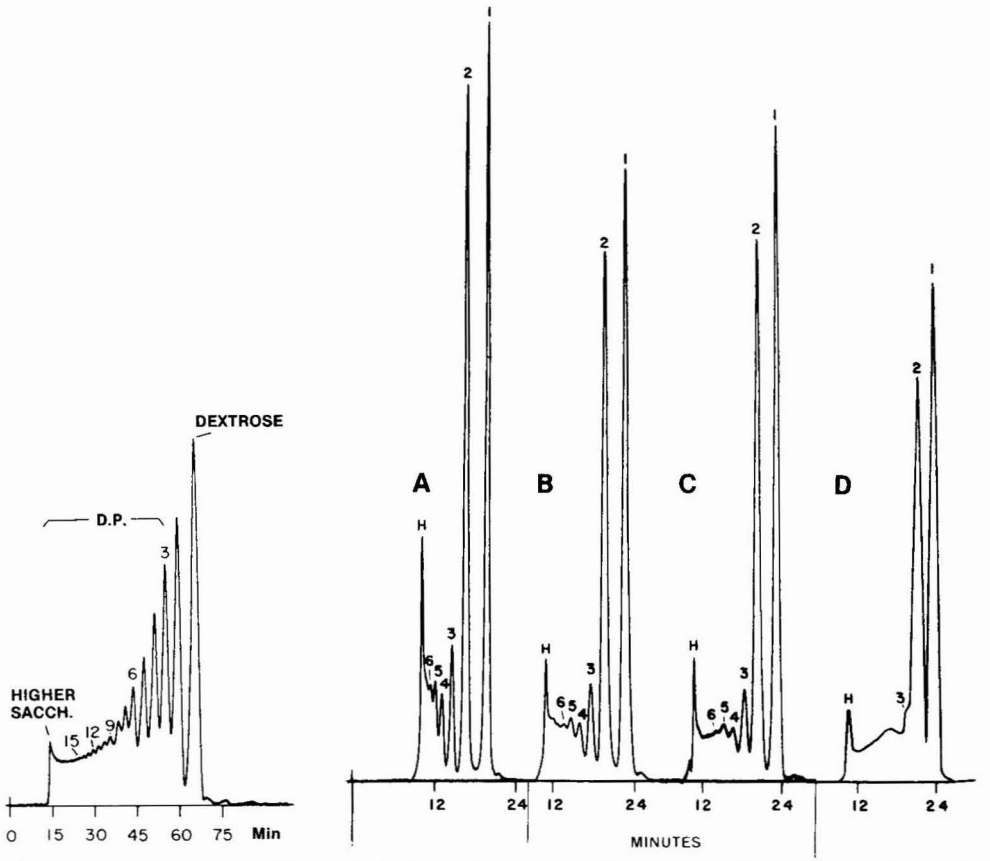

Fig. 2. Chromatogram of a 42 D.E. acid-hydrolyzed corn syrup. Chromatographic conditions: column (60 cm × 7.8 mm I.D.) Aminex 50W-X4 (Ag^+) 4% cross-linked resin (20–30 μm). Flow-rate, 0.3 ml/min. Numbers over peaks indicate DP.

Fig. 3. Effect of silver loading on resolution. Chromatograms of a 62 D.E. enzyme-hydrolyzed corn syrup chromatographed on columns with various amounts of silver loading. Chromatographic conditions: all columns (30 cm × 7.8 mm I.D.) Aminex Q-15S (19–25 μm) with (A) 70% silver form; (B) 85% silver form; (C) 95% silver form; (D) 100% silver form resin. Flow-rate, 0.4 ml/min. Numbers over peaks indicate DP; H indicates higher excluded oligosaccharides.

in the hydrogen form. As a result of this finding, a study was conducted to determine the effect of percentage silver loading on the separation of oligosaccharides.

Separation of oligosaccharides on 8% cross-linked resins

Shown in Fig. 3 are the chromatographic results of a study in which an 8% cross-linked resin, Aminex Q-15S (19–25 μm) was converted to various silver loading levels with silver nitrate. In the calcium form this resin separates a number of monosaccharides, maltose, and maltotriose from the higher oligosaccharides. However, we found the separation of the oligosaccharides on this resin could be extended beyond maltohexaose by increasing the silver loading level to about 80%. It was noted that while increased silver loading effectively increases the retention of the oligosaccha-

rides, it also, beyond a certain level, causes a marked reduction in the resolution of the separation. From this study it was determined more highly cross-linked resins could be used to separate oligosaccharides with more efficiency than the 4% calcium form resins, providing that the amount of silver on the resin was adjusted to an optimum level.

Shown in Fig. 4 are improved separations of a 62 D.E. enzyme-converted syrup on another 8% cross-linked resin, Aminex A-7 (7–11 μm) at the 75% silver loading level at various flow-rates. By using this high-performance resin converted to its optimum silver loading level of 75%, high resolution separation of the oligosaccharides can be obtained through DP 6 in 20 min. By increasing the flow-rate two-fold to 0.8 ml/min, the time of the analysis can be further reduced to 10 min. At this flow-rate the only peak to suffer a significant loss in resolution is the DP 6 peak. This excellent retention of resolution with increases in flow-rate is due to two additional beneficial features of silver form resins. First, silver form resins are more efficient, in terms of theoretical plates, than the same resin in the calcium form. Second, silver form resins retain their efficiency to a greater extent with increases in flow, than the

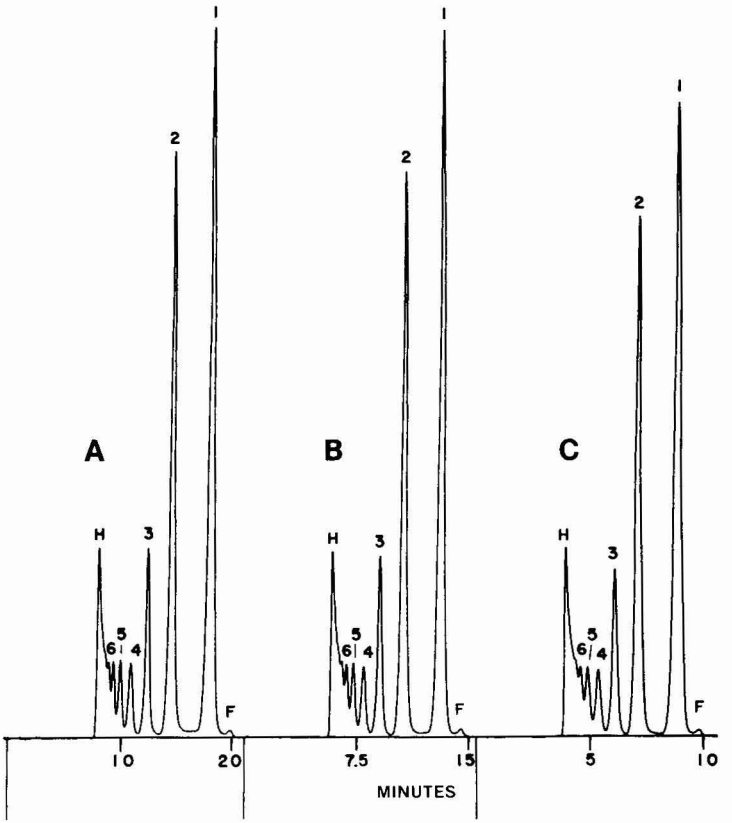

Fig. 4. Chromatograms of a 62 D.E. enzyme-converted corn syrup at various flow-rates. Chromatographic conditions: column (30 cm × 7.8 mm I.D.) Aminex A-7 (7–11 μm), 75% silver form. Flow-rate, (A) 0.40 ml/min; (B) 0.60 ml/min; (C) 0.80 ml/min. Numbers over peaks indicate DP; H indicates higher excluded oligosaccharides.

TABLE II

CAPACITY FACTORS (k') OF CORN-DERIVED OLIGOSACCHARIDES ON VARIOUS RESINS

Resin	*Counter-ion*	*Loading level (%)*	*DP*														
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
Aminex A-7	Ca^{2+}	100	0.63	0.33	0.21												
Aminex A-7	Ag^{+}	75	1.25	0.82	0.55	0.37	0.25	0.17	0.12								
HC-40	Ca^{2+}	100	1.18	0.82	0.61	0.46	0.35	0.26	0.21								
Aminex HPX-42	Ca^{2+}	100	1.65	1.25	1.01	0.85	0.70	0.56	0.47	0.39							
Aminex 6% X	Ag^{+}	86	1.54	1.19	0.94	0.75	0.58	0.45	0.36	0.28	0.21						
HC-40	Ag^{+}	71	2.09	1.69	1.40	1.16	0.94	0.77	0.64	0.52	0.42	0.34	0.28	0.22			
Aminex HPX-42	Ag^{+}	70	2.17	1.84	1.57	1.36	1.15	0.96	0.81	0.69	0.59	0.50	0.43	0.36	0.31	0.27	0.22

same resins in the calcium form. Fig. 5 illustrates the comparison of Aminex A-7 in both the silver and the calcium form *versus* flow-rate. At a flow-rate of 0.4 ml/min Aminex A-7 (Ag^+) is more than five times as efficient as Aminex A-7 (Ca^{2+}). Moreover, at an increased flow-rate of 0.8 ml/min Aminex A-7 (Ag^+) is more than ten times as efficient as Aminex A-7 (Ca^{2+}). These features are again due to the unique complex that silver forms with the oligosaccharides.

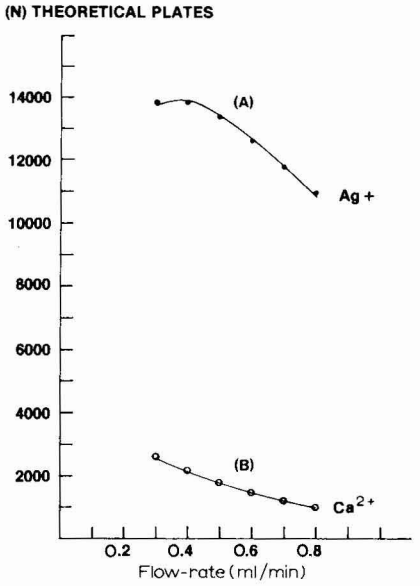

Fig. 5. Comparison of efficiency of Aminex A-7 (Ag^+) and Aminex A-7 (Ca^{2+}) *versus* flow-rate. Efficiency *versus* flow for (A) (30 cm × 7.8 mm I.D.) Aminex A-7 8% cross-linked resin column (7–11 μm), 75% silver form and (B) (30 cm × 7.8 mm I.D.) Aminex A-7 8% cross-linked resin column (7–11 μm), 100% calcium form. Theoretical plate calculations based on $N = 5.554\ (t_R/W_{1/2})^2$ for dextrose, where N = number of theoretical plates, t_R = retention time, and $W_{1/2}$ = peak width at half-height.

It has been reported by Goulding[7] that Ag^+ resins form strong monodentate complexes with most monosaccharides, while Ca^{2+} forms bi- and tridentate complexes with the same series of sugars depending on their structures. While the silver monodentate complex is less selective for monosaccharides, it is much stronger than the bidentate complex of calcium for the oligosaccharides. This stronger complex brings about the greater retention of all the oligosaccharides and allows more of them to be resolved from the truly excluded higher-molecular-weight sugars. Table II lists the capacity factors of the corn-derived oligosaccharides on various resins in both the silver and calcium form.

It was also reported by the same author[7] that Ag^+ resins have little or no ability to resolve α and β anomers of glucose, while Ca^{2+} resins at ambient temperature give partial separation of the same pair. While this feature of calcium form resins is less than desirable for most purposes, it can be suppressed by operating at elevated temperature. However, since Ag^+ resins show no tendency to separate α and β anomers of the glucose oligosaccharides, operation at elevated temperatures gives rise

to significantly narrower peaks than those obtained on the calcium form. This reduction in peak width along with increased retention accounts for the large differences in efficiency observed between the silver and calcium forms.

Separation of oligosaccharides on a 6% cross-linked resin

While additional oligosaccharides can be resolved from the higher excluded saccharides on 8% cross-linked resins by increasing the amount of silver on the resin, it generally results in some loss in resolution on the lower-molecular-weight end of the separation. An alternate method to increase the number of saccharides resolved is to use a resin with less cross-linking. Shown in Fig. 6 is a 42 D.E. acid-hydrolyzed syrup separated on a 6% cross-linked resin in the silver form at various flow-rates. The resin used in this portion of the study is currently an experimental 10–15-μm product with a capacity of 1.6 mequiv./ml. As illustrated in Fig. 6, this resin can separate the oligosaccharides through DP 9 when converted to 86% of its capacity with Ag^+. Also apparent is again the excellent retention of resolution with increased flow. This resin, which is only slightly less rigid than the 8% cross-linked Aminex resins, can be pumped at flow-rates of 0.8 ml/min in a 30 cm × 7.8 mm I.D. column and produce separations of the oligosaccharides in a corn syrup through DP 7 in less than 12 min. At this flow-rate the only significant loss in resolution occurs around the DP 8 region. However, in order to counteract this loss, a flow-rate of 0.6 ml/min is used and the samples are injected on an over-lap basis every 12 min with an autosampler of our own design.

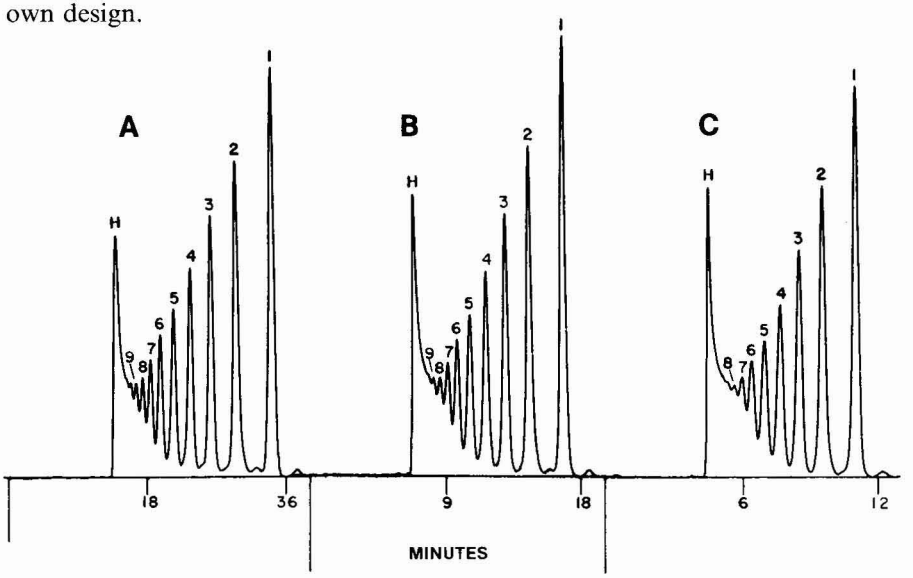

Fig. 6. Chromatograms of a 42 D.E. acid-hydrolyzed corn syrup at various flow-rates. Chromatographic conditions: column (30 cm × 7.8 mm I.D.) Aminex experimental 6% cross-linked resin (10–15 μm), 86% silver form. Flow-rate, (A) 0.25 ml/min; (B) 0.55 ml/min; (C) 0.80 ml/min. Numbers over peaks indicate DP; H indicates higher excluded oligosaccharides.

Fig. 7 illustrates the analysis of a series of different corn syrups using the over-lap injection technique. As illustrated in this figure, operation at a flow-rate of 0.6 ml/min offers the resolution of an 18-min analysis but with a sampling rate of one sample per 12 min, utilizing the relatively large void time of resin columns.

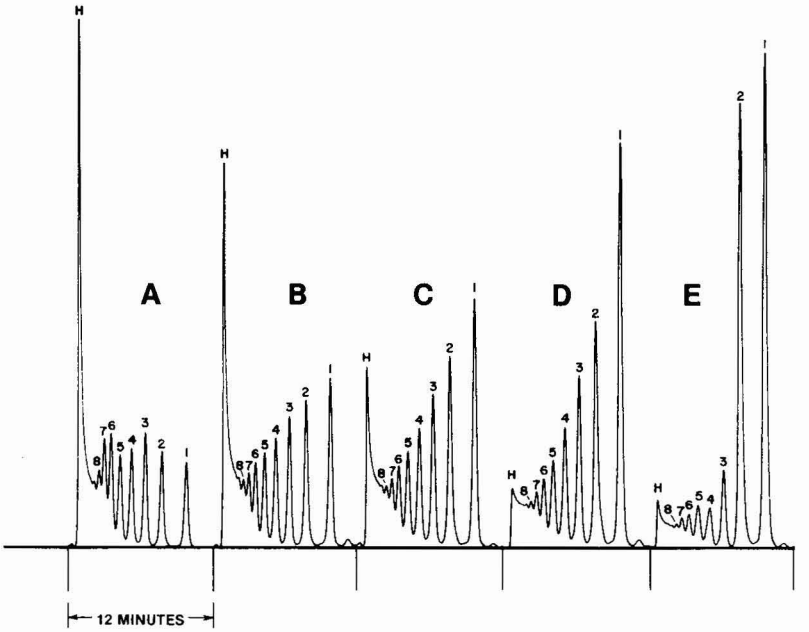

Fig. 7. Chromatograms of various corn syrups injected on an overlap basis. (A) a 28 D.E. enzyme-converted corn syrup; (B) a 35 D.E. acid-hydrolyzed corn syrup; (C) a 42 D.E. acid-hydrolyzed corn syrup; (D) a 53 D.E. acid-hydrolyzed corn syrup; (E) a 62 D.E. enzyme-converted corn syrup. Chromatographic conditions: column (30 cm × 7.8 mm I.D.) Aminex experimental 6% cross-linked resin (10–15 μm) 86% silver form. Flow-rate, 0.6 ml/min. Numbers over peaks indicate DP; H indicates higher excluded saccharides.

Although not originally intended for monosaccharide separations the high-performance silver form resins also give a satisfactory separation of fructose from dextrose. Shown in Fig. 8 is the separation of a series of saccharide standards with fructose included. While the amount of separation is not as large as that obtained on the calcium form, it is sufficient for the analysis of high fructose/regular corn syrup blends.

Separation of oligosaccharides on 4–5% cross-linked resins

While rapid separations through DP 8 are generally satisfactory for the routine characterization of corn syrups, occasionally a need arises that requires extended separations beyond this range. In an effort to fill this need, several 4–5% cross-linked resins were studied in the silver form, using the optimum loading technique.

Shown in Fig. 9 is the separation of a 42 D.E. acid-hydrolyzed corn syrup on a 5% cross-linked resin in the silver form at various flows. Although the manufacturers' original specification for this product stated that its degree of cross-linking was 4%, its mechanical strength along with its chromatographic behavior in both the silver and calcium forms indicate that it has an effective cross-linking of approximately 5%. As illustrated in Fig. 9A this resin can separate the oligosaccharides through DP 12 when converted to 71% of its capacity with Ag^+. Capable of withstanding flow-rates of over 0.6 ml/min it can produce separations of the oligosaccharides up through DP 10 when pumped at a rate of 0.7 ml/min as illustrated in Fig. 9C. However, since this

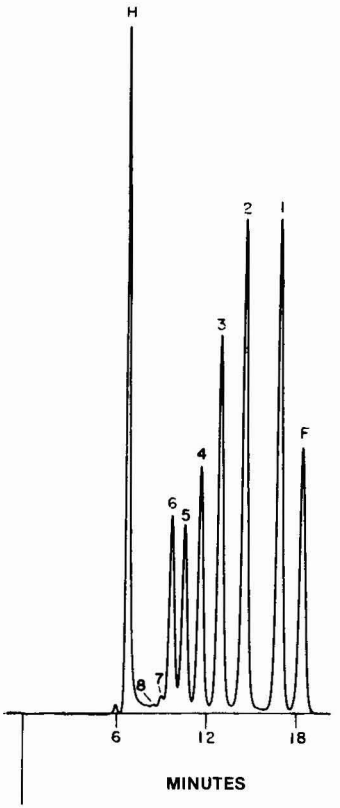

Fig. 8. Chromatogram of saccharide standards on (30 cm × 7.8 mm I.D.) Aminex experimental 6% cross-linked resin (10–15 μm) 86% silver form. Flow-rate, 0.55 ml/min. F = fructose; 1 = dextrose; 2 = maltose; 3 = maltotriose; 4 = maltotetraose; 5 = maltopentaose; 6 = maltohexaose; 7 = maltoheptaose; 8 = maltooctaose; H = dextran 10.

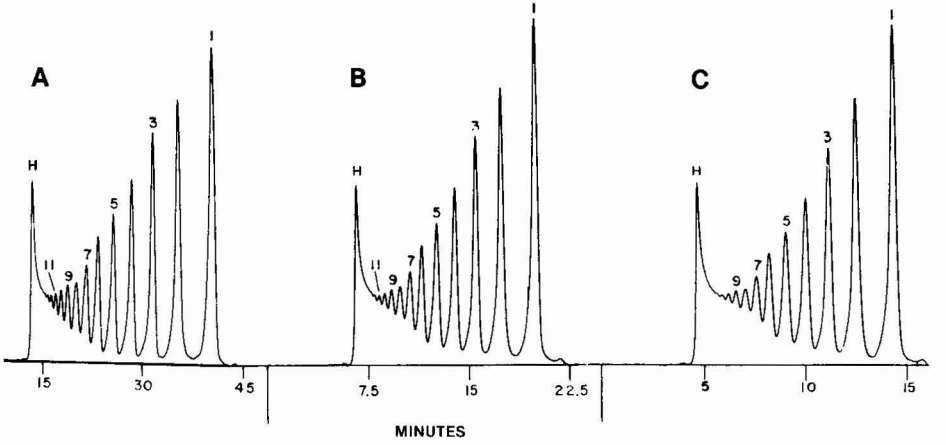

Fig. 9. Chromatograms of a 42 D.E. acid-hydrolyzed corn syrup at various flow-rates. Chromatographic conditions: column (30 cm × 7.8 mm I.D.) Hamilton HC-40 5% cross-linked resin (10–15 μm) 71% silver form. Flow-rate (A) 0.25 ml/min; (B) 0.55 ml/min; (C) 0.70 ml/min. Numbers over peaks indicate DP; H indicates higher excluded oligosaccharides.

resin loses considerable resolution at this rate, due to a slightly asymmetrical particle size distribution, it is generally operated at the 0.55 ml/min rate shown in Fig. 9B and is injected on an over-lap basis every 18 min.

The final resin studied in this series is another experimental product designated Aminex HPX-42 (10–15 μm). This product is a smaller particle size version of the 4% cross-linked resin shown in Fig. 1. Shown in Fig. 10 is the separation of a 62 D.E. enzyme-converted corn syrup on Aminex HPX-42 (10–15 μm) converted to 70% of its capacity with Ag^+. As illustrated, this high-performance resin can separate oligosaccharides through DP 15 in 60 min when operated at a flow-rate of 0.2 ml/min. Shorter analysis times are not practical with this resin since its small particle size gives rise to critically high backpressure at flow-rates above this value. While this column does not operate at the higher speeds of the other columns shown, it does illustrate that with optimum silver loading, high-resolution separation of corn-derived oligosaccharides can be made through DP 12 without marked losses in resolution after every half turn of the helix.

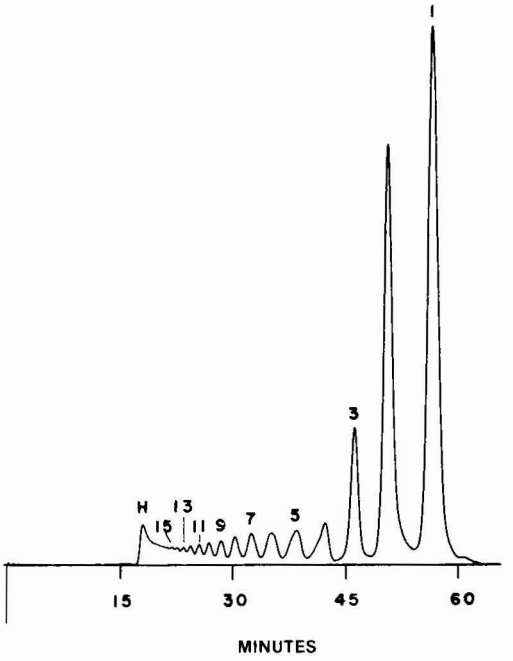

Fig. 10. Chromatogram of a 62 D.E. enzyme-hydrolyzed corn syrup. Chromatographic conditions: column (30 cm × 7.8 mm I.D.) Aminex 4% cross-linked resin (10–15 μm) 70% silver form. Flow-rate, 0.2 ml/min. Numbers over peaks indicate DP; H indicates higher excluded oligosaccharides.

CONCLUSION

We have found that the increased retention and resolution characteristics of silver form resins provide an analysis of corn derived oligosaccharides superior to that obtained on equivalent resins in the calcium form. While the aspect of partial silver loading is not yet clearly understood, it has provided a method to produce high-resolution separations of oligosaccharides on mechanically stable resins that in other

metallic forms resolve only mono-, di- and trisaccharides. Partial loading also appears to increase column life of silver form resins. Several columns from this study have been in operation for over 22 months and they show no indication of silver reduction.

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SEPARATION OF REDUCED DISACCHARIDES DERIVED FROM GLYCOSAMINOGLYCANS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for the analysis of reduced unsaturated disaccharides derived from enzymatic digestion followed by reduction with sodium borohydride of chondroitin sulfates, dermatan sulfate, heparan sulfate and heparin is described. This method is well suited for the HPLC analysis of glycosaminoglycans (GAGs) because the possibility of obtaining anomeric forms of unsaturated disaccharides is eliminated which provides a major advantage for quantitation. This procedure is more sensitive than existing HPLC methods for the determination of enzymatic degradation products from GAGs. In particular, the resolution of disaccharide products from heparan sulfate is improved after reduction. The applicability of this method for the determination of GAGs in biological samples is demonstrated.

INTRODUCTION

Glycosaminoglycans (GAGs) are members of a group of related heteropolysaccharides which can be degraded by specific enzymes to produce unsaturated disaccharides containing a reducing end. Two major groups of disaccharides have been reported: (1) disaccharides from chondroitin sulfate isomers after digestion with chondroitinase enzymes[1], (2) disaccharides from heparan sulfate and heparin after digestion with heparinase and/or heparitinase enzymes[2–4]. The assays of these disaccharide products provide useful tools for identification, structural analysis and quantification of GAGs[5,6]. High-performance liquid chromatography (HPLC) has been shown to be a successful technique for these assays. It is more sensitive, precise and faster than other chromatographic methods[7–11].

When HPLC is used for the quantitation of the unsaturated disaccharides from GAGs two peaks often have been observed for each disaccharide, which has been considered to be due to the presence of sugar anomeric forms[7,11]. Ototani *et al.*[10] also have assumed that anomers are present during the ion-pair reversed-phase HPLC of chondroitin sulfate disaccharides. These GAG disaccharides contain a reducing end which is the anomeric center in each molecule and therefore mixtures of α- and β-

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anomeric forms may be detected under appropriate chromatographic conditions. This phenomenon interferes with separations and has limited the quantification of these disaccharides.

In this report the effects of sodium borohydride reduction of sugar carbonyl to terminal hydroxymethyl groups are described. This approach does indeed eliminate anomeric forms and also increases the sensitivity and resolution in HPLC of enzymatic degradation products from GAG.

EXPERIMENTAL

Apparatus

The HPLC system employed in this work included a Model 6000A solvent delivery system, a U6K universal injector and a Model 440 UV detector (Waters Assoc., Milford, MA, U.S.A.). The recorder used was a Houston Instrument (Austin, TX, U.S.A.) OmniScribe A5211-5 dual-pen recorder. Peak heights, peak areas and retention times were measured by an on-line Model Supergrator-1 integrator (Columbia Scientific, Austin, TX, U.S.A.). A prepacked HPLC column, Partisil-10 PAC, 10 μm, 25 cm $\times$ 4.6 mm I.D. (Whatman, Clifton, NJ, U.S.A.) was employed.

Reagents and materials

Chondroitin 4-sulfate, the unsaturated disaccharides (ΔDi-OS, ΔDi-6S and ΔDi-4S)* from chondroitin sulfate isomers, chondroitinase ABC (E.C. 4.2.2.4) from *Proteus vulgaris* and chondroitinase AC (E.C. 4.2.2.5) from *Arthrobacter aurescens* were products of Seikagaku Kogyo, Tokyo, Japan and were purchased from Miles Labs. (Elkhart, IN, U.S.A.). Chondroitin 6-sulfate was purchased from Calbiochem (San Diego, CA, U.S.A.). The unsaturated disaccharides (ΔDi-HS_b-I, ΔDi-HS_b-II, ΔDi-HS_b-III and ΔDi-He_a-I)** from heparan sulfate and heparin were generous gifts from Dr. Alfred Linker (University of Utah, Salt Lake City, UT, U.S.A.). The urine sample from a patient with Maroteaux-Lamy disease was supplied by Dr. John T. Dulaney (University of Tennessee, Memphis, TN, U.S.A.).

Methanol and acetonitrile, HPLC grade, were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Sodium borohydride and sodium tetraborate were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Doubly distilled water passed through two mixed-bed ion-exchange cartridges was used in preparation of ammonium formate buffer for the HPLC mobile phase. The buffer was passed through a 0.45-μm filter prior to use.

Isolation of urinary GAGs and enzymatic procedure

The isolation of GAGs from 2 ml urine specimens and the enzymatic digestions

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* ΔDi-OS = a non-sulfated disaccharide from chondroitin; ΔDi-6S = a 6-sulfated disaccharide from chondroitin 6-sulfate; ΔDi-4S = a 4-sulfated disaccharide from chondroitin 4-sulfate. For detailed structures and preparations, see ref. 1.

** ΔDi-HS_b-I = a non-sulfated disaccharide from heparan sulfate; ΔDi-HS_b-II = a disaccharide from heparan sulfate containing one sulfate group on the acetylglucosamine unit; ΔDi-HS_b-III = a disaccharide from heparan sulfate containing N-sulfated glucosamine; ΔDi-He_a-I = a trisulfated disaccharide from heparin. For detailed structures and preparations, see refs. 2 and 4.

of standard chondroitin sulfates and urinary GAGs by chondroitinases were carried out as described previously[8,12].

Chemical reduction

Standard unsaturated disaccharides (200 μg), and enzymatic degradation product mixtures obtained from 200 μg standard chondroitin sulfates or from 1 ml Maroteaux-Lamy urine were individually dissolved in 200 μl of 0.1 *M* sodium borate buffer (pH 8.2). A 2.5-mg amount of sodium borohydride in 100 μl of 0.1 *M* sodium borate (pH 8.2) was added to each solution in several steps. The vortex mixing was done with a Super-Mixer (Matheson Scientific) after each addition. After shaking gently at room temperature for 3 h, the solutions were refrigerated for 3 h and then neutralized to pH 5.0 with 0.5 *M* acetic acid to destroy excess sodium borohydride. Each solution was dried under a stream of nitrogen and the residue, dissolved in 90% aqueous methanol, was applied to the HPLC column.

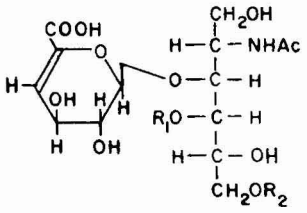
HPLC

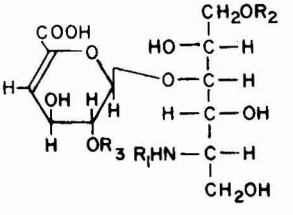
An aliquot from each reduced disaccharide product was injected and chromatographed at 1.5 ml/min with a ternary solvent system of acetonitrile–methanol–ammonium formate buffer on a Whatman Partisil-10 PAC column. The composition of the mobile phase was systematically varied in order to select the optimal conditions for separation.

The chromatography was carried out isocratically at room temperature. Details are given separately with each chromatogram.

RESULTS AND DISCUSSION

Sodium borohydride has been widely used for the derivatization of reducing sugars (neutral sugars[13,14], amino sugars[15,16] and sugar acids[17,18]) for gas chromatography. The reduction of the unsaturated disaccharides of GAGs using sodium borohydride was reported previously[4,19] and is assumed to reduce the carbonyl groups to give the structures shown in Fig. 1. Recently, in sequencing studies of





	R_1	R_2		R_1	R_2	R_3
1. r△Di—0S	H	H	4. r△Di-HS_b-I	Ac	H	H
2. r△Di—6S	H	SO_3H	5. r△Di-HS_b-II	Ac	SO_3H	H
3. r△Di—4S	SO_3H	H	6. r△Di-HS_b-III	SO_3H	H	H
			7. r△Di-He_a-I	SO_3H	SO_3H	SO_3H

Fig. 1. Structures of reduced disaccharides from GAGs.

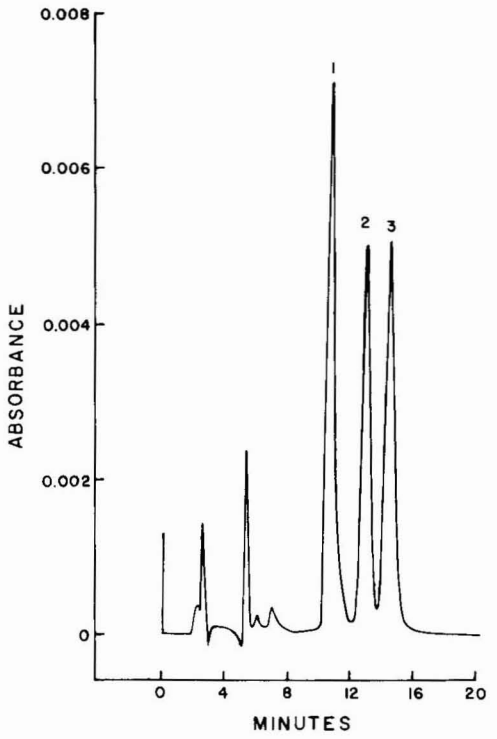


Fig. 2. HPLC of the reduced unsaturated disaccharides from chondroitin sulfate isomers. Peaks: 1 = rΔDi-OS; 2 = rΔDi-6S; 3 = rΔDi-4S. Column: Partisil-10 PAC, 10 μm, 25 cm $\times$ 4.6 mm I.D. Solvent system: acetonitrile–methanol–0.5 *M* ammonium formate, pH 6.0 (69:14:17). Flow-rate: 1.5 ml/min. Pressure: 600 p.s.i. UV detection at 254 nm, 0.01 a.u.f.s.

chondroitin sulfates, sodium borohydride has been used to form reduced oligosaccharides and unsaturated disaccharides which were analyzed by HPLC on a Partisil-10 SAX anion-exchange column[20]. Compounds 1–7 are the reduced disaccharides from ΔDi-OS, ΔDi-6S, ΔDi-4S, ΔDi-HS_b-I, ΔDi-HS_b-II, ΔDi-HS_b-III, and ΔDi-He_a-I, respectively. A separation of the three reduced chondroitin sulfate disaccharides rΔDi-OS, rΔDi-6S and rΔDi-4S is shown in Fig. 2. The resolution of these three reduced compounds is similar to that of non-reduced disaccharides[8]. However, the efficiency in the chromatography of the reduced compounds is much higher as indicated in Table I which shows the comparison of the number of theoretical plates of reduced and non-reduced disaccharide peaks under a variety of compositions of mobile phases. The effective baseline separations for reduced and non-reduced disaccharides were achieved by mobile phases 1 and 2, respectively. It is clearly indicated that the number of theoretical plates of reduced disaccharide peaks is increased from two- to seven-fold when compared with non-reduced disaccharides depending on acetonitrile–methanol ratio and the molarity and pH of aqueous ammonium formate in the mobile phase.

Fig. 3 illustrates the HPLC separation of reduced non-sulfated and monosulfated disaccharides from heparan sulfate. Much better resolution was achieved in

TABLE I

THE DEPENDENCE OF THE NUMBER OF THEORETICAL PLATES (N) FOR THE SEPARATION OF REDUCED AND OF NON-REDUCED CHONDROITIN SULFATE DISACCHARIDE PEAKS ON ACETONITRILE–METHANOL RATIOS AND pH AND MOLARITY OF AMMONIUM FORMATE IN THE MOBILE PHASE

N was calculated by half peak height method, using $N = 5.54 (t_R/W_{\frac{1}{2}})^2$ where t_R = retention time and $W_{\frac{1}{2}}$ = peak width at half peak height. Column: Partisil-10 PAC, 10 μm, 25 cm × 4.6 mm I.D. Flow-rate: 1.5 ml/min.

Disaccharide	*N*			
	Acetonitrile–methanol–ammonium formate (molarity, pH) ratio			
	(1) 69:14:17 (0.5 M, pH 6.0)	*(2) 59:24:17 (0.5 M, pH 6.0)*	*(3) 69:14:17 (0.5 M, pH 5.0)*	*(4) 69:14:17 (0.8 M, pH 6.0)*
ΔDi-OS	572	646	504	441
ΔDi-6S	871	870	604	680
ΔDi-4S	820	854	653	695
rΔDi-OS	2861	1766	2681	3215
rΔDi-6S	2428	1930	2458	2930
rΔDi-4S	2459	1920	2506	2833

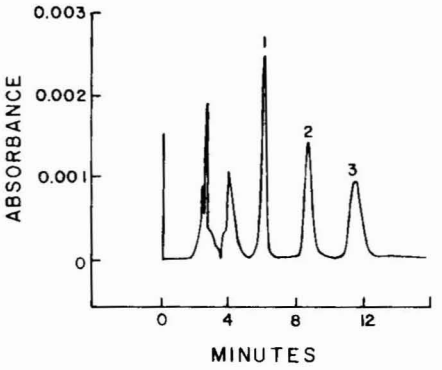


Fig. 3. HPLC of the reduced unsaturated disaccharides from heparan sulfate. Peaks: 1 = rΔDi-HS_b-I; 2 = rΔDi-HS_b-II; 3 = rΔDi-HS_b-III. Column: Partisil-10 PAC, 10 μm, 25 cm × 4.6 mm I.D. Solvent system: acetonitrile–methanol–0.5 M ammonium formate, pH 4.5 (60:20:20). Flow-rate: 1.5 ml/min. Pressure: 750 p.s.i. UV detection at 254 nm, 0.01 a.u.f.s.

comparison with HPLC of non-reduced compounds[11]. Each reduced compound formed from chondroitin sulfates as well as heparan sulfate gave a single and much sharper peak under all chromatographic conditions. This is an important advantage for quantitation and supports the conclusion that anomeric forms were eliminated.

The retention behavior of these reduced disaccharides, as chromatographed on the cyano-amino bonded stationary phase, showed a strong and sensitive dependence on the ratio of acetonitrile and methanol content in the eluent. Table II shows the capacity ratios (k') of these reduced disaccharides determined as a function of the

TABLE II

THE DEPENDENCE OF CAPACITY RATIOS (k') OF THE REDUCED DISACCHARIDES FROM GAGs ON THE ACETONITRILE AND METHANOL CONTENT OF THE MOBILE PHASE

Values given are mean k' values determined from five separate chromatograms. Column: Partisil-10 PAC, 10 μm, 25 cm × 4.6 mm I.D. Flow-rate: 1.5 ml/min. UV detection at 254 nm, 0.01 a.u.f.s.

Reduced disaccharide from chondroitin sulfates	k'					
	Ratio of acetonitrile–methanol–0.5 M ammonium formate (pH 6.0)					
	0:83:17	*19:64:17*	*39:44:17*	*59:24:17*	*69:14:17*	*74:9:17*
rΔDi-OS	0.94	1.01	1.31	2.23	3.40	5.96
rΔDi-6S	7.43	4.56	3.35	3.30	4.27	7.12
rΔDi-4S	7.26	4.47	3.51	3.74	4.90	8.35
Reduced disaccharide from heparan sulfate	*Ratio of acetonitrile–methanol–0.5 M ammonium formate (pH 4.5)*					
	0:80:20	*20:60:20*	*40:40:20*	*60:20:20*	*65:15:20*	*75:5:20*
rΔDi-HS_b-I	0.99	1.01	1.11	1.56	1.95	6.90
rΔDi-HS_b-II	6.78	4.09	3.05	2.73	3.06	8.54
rΔDi-HS_b-III	10.85	7.09	4.90	3.93	4.24	11.05

concentration of acetonitrile and methanol of the mobile phase, while keeping the content of aqueous ammonium formate constant. The k' values of reduced sulfated disaccharides from both chondroitin sulfates and heparan sulfate first decrease with increasing acetonitrile content and decreasing methanol content, pass through a minimum, and then increase sharply. The k' values of reduced non-sulfated disaccharides (rΔDi-OS and rΔDi-HS_b-I) increase slightly in the region in which k' values of reduced sulfated disaccharides decrease and then increase sharply as did the reduced sulfated disaccharides. A reversed retention order of rΔDi-6S and rΔDi-4S was observed when these two organic solvent contents were varied. The k' values of all reduced disaccharides decrease with increasing aqueous ammonium formate content. After using a column for a period of time with a given mobile phase, the retention times of all disaccharides may decrease due to loss of column efficiency. An adjustment of mobile phase composition was necessary in order to maintain the optimal conditions for separation. The difference in retention behaviors of these compounds from column to column can also be overcome in the same manner. The reduced trisulfated disaccharide (rΔDi-He_a-I) derived from heparin can be quantitated using an ion-pair reversed-phase method as described for HPLC of the non-reduced trisulfated disaccharide[11].

In order to evaluate the linearity and the sensitivity of this procedure, the following experiment was performed. Duplicate aliquots containing 200 μg of the unsaturated disaccharides were prepared. One set of these aliquots was reduced with sodium borohydride and the other set was used without chemical modification. Both sets, reduced and non-reduced disaccharides, were chromatographed separately under the conditions which gave effective separations for each disaccharide isomer group. The peak height of each disaccharide was plotted against the amount of

compound injected. Excellent linear relationships exist for both sets of disaccharides as shown in Fig. 4. The results also indicate that the detection sensitivity is increased by a factor of at least 2 by employing sodium borohydride reduction.

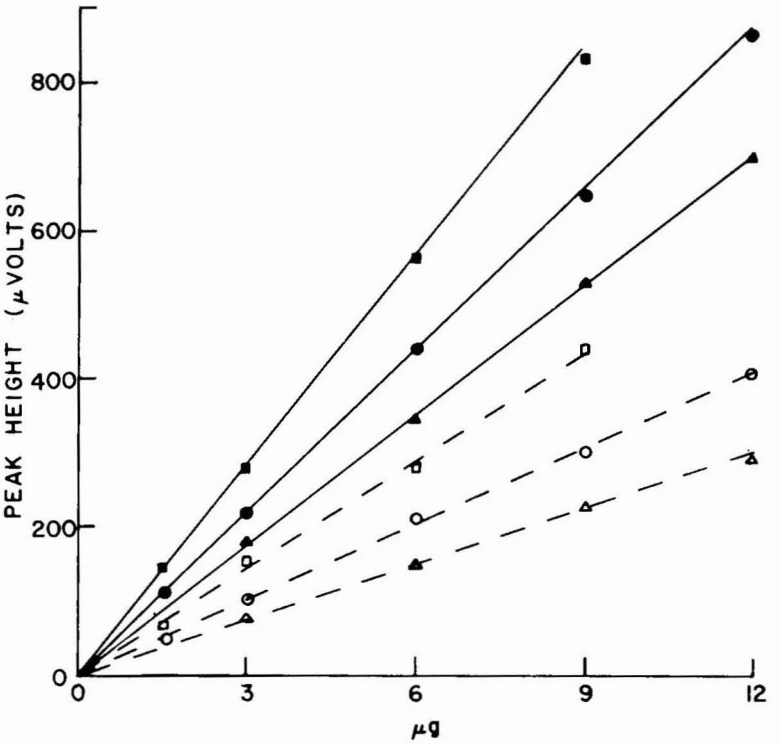


Fig. 4. The linearity of HPLC analysis for reduced disaccharides and the comparison of detection sensitivity between reduced products and original non-reduced compounds. Curves: ■, rΔDi-OS; ●, rΔDi-6S; ▲, rΔDi-4S; □, ΔDi-OS; ○, ΔDi-6S; △, ΔDi-4S.

The applicability of this reduction procedure to biological samples was demonstrated by the analysis of enzymatic degradation products of standard chondroitin sulfates and urinary GAGs from a patient with Maroteaux-Lamy disease (Fig. 5). The two top plots in Fig. 5 illustrate HPLC separations of reduced disaccharides obtained after the treatment of either chondroitin 4-sulfate (left) or chondroitin 6-sulfate (right) with chondroitinase ABC and borohydride reduction. As expected, rΔDi-4S and rΔDi-6S are the major products of chondroitin 4-sulfate and chondroitin 6-sulfate, respectively. The two bottom plots in Fig. 5 show separations of reduced disaccharides from Maroteaux-Lamy urinary GAGs after digestion with chondroitinase ABC (left) or AC (right) followed by borohydride reduction. The large difference in amounts of rΔDi-4S produced by the two different chondroitinases confirms the excretion of large amounts of dermatan sulfate[1], a characteristic of Maroteaux-Lamy disease[21]. Several additional impurity peaks appear in the chromatographs. However, they are eluted long before reduced disaccharide peaks and do not interfere with this quantification.

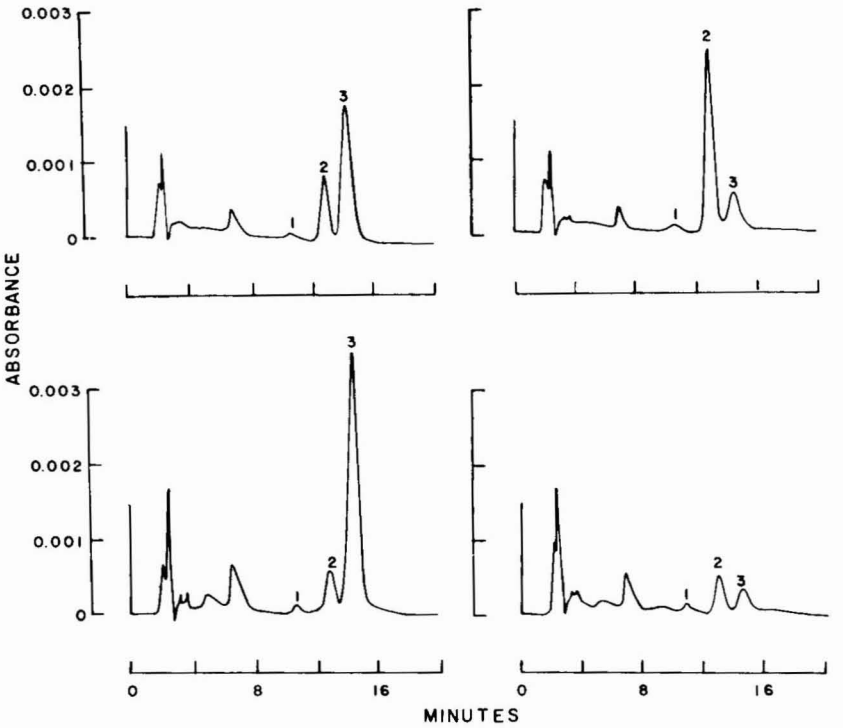


Fig. 5. HPLC of products of enzymatic degradation followed by sodium borohydride reduction of standard chondroitin sulfates and urinary GAGs: standard chondroitin 4-sulfate (top left) and chondroitin 6-sulfate (top right) degraded by chondroitinase ABC; GAGs from a Maroteaux-Lamy urine degraded by chondroitinase ABC (bottom left) and by chondroitinase AC (bottom right). Peaks: 1 = rΔDi-OS; 2 = rΔDi-6S; 3 = rΔDi-4S. The amount injected was 2.5% of the reaction products from 100 μg standard chondroitin sulfates or from GAG of 1 ml Maroteaux-Lamy urine. Chromatographic conditions as in Fig. 2.

In this laboratory the procedure described is a valuable addition to the existing methodology for the analyses of GAGs. It permits a facile analysis of lower levels of GAGs in biological samples when compared to previously described procedures involving enzymatic degradation.

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GAS CHROMATOGRAPHIC AND MASS SPECTROMETRIC STUDIES OF GINGER CONSTITUENTS

IDENTIFICATION OF GINGERDIONES AND NEW HEXAHYDROCURCUMIN ANALOGUES

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SUMMARY

The pungent principles of ginger (*Zingiber officinale* Roscoe), the gingerols and related compounds, were investigated by gas chromatography and gas chromatography–mass spectrometry using several chemical derivatives and deuterium labelling. Gingerdiones, postulated intermediates in the biosynthesis of the gingerols, were identified together with desmethylhexahydrocurcumin and the shogaol analogues of the hexahydrocurcumins.

INTRODUCTION

Previous studies on the composition of the pungent principles of ginger (*Zingiber officinale* Roscoe) have shown that the major constituents are the homologous gingerols (I) and the shogaols (II)[1–4]; the latter compounds are thought to be partly formed from the gingerols by decomposition[1,5,6]. Other related compounds present in smaller quantities include paradols (III), gingediols (IV), zingerone (V)[2,3,7], hexahydrocurcumin (VIa)[8] and the O-methyl ethers of many of these compounds[1,4]. Lower boiling extracts contain a range of terpenes and related compounds[9–12]. It has been proposed that the biosynthesis of the gingerols involves the condensation of dihydroferulic acid first with malonic acid and then with a short chain carboxylic acid such as hexanoic acid to give the intermediate gingerenedione (X, Fig. 1). This can either be reduced to the ketoalcohol (XI) and hydrogenated to gingerol (I), or hydrogenated to gingerdione (XII) and then reduced to gingerol[13–15]. Of these pathways, the first seems to be preferred as the reduction of gingerdione (XII) appears to be a relatively inefficient process[15]. However, neither gingerdione or the intermediate (XI) appears to have been identified in the ginger plant. In this paper, we describe the identification of three gingerdiones corresponding in chain lengths to the three major gingerols (Ia, $n = 4$, 6 and 8). Other new compounds identified were desmethylhexahydrocurcumin (VIb) and the shogaol equivalents (XIIIa, b) of both hexahydrocurcumin (VIa) and desmethylhexahydrocurcumin (VIb). These are probably produced

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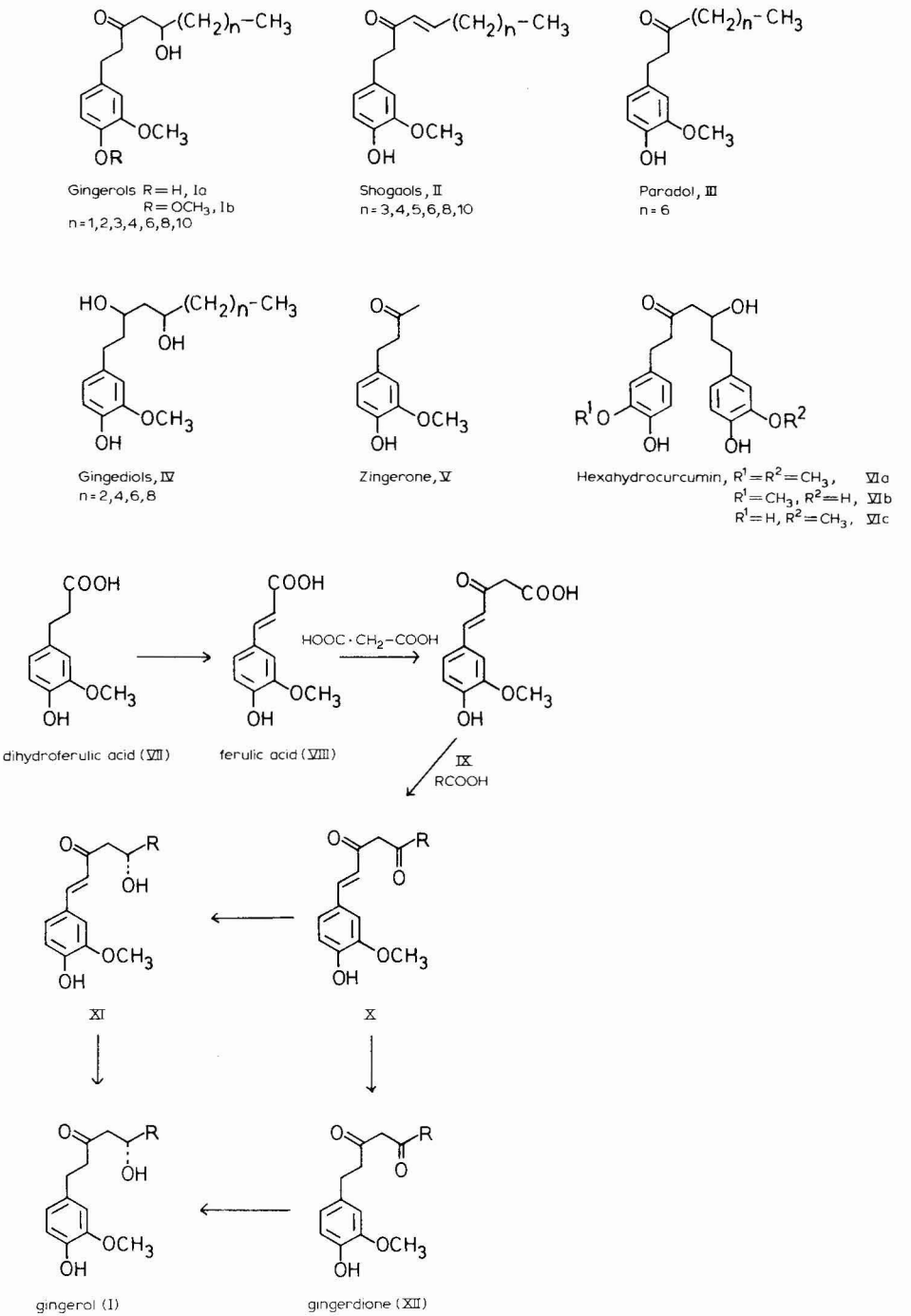
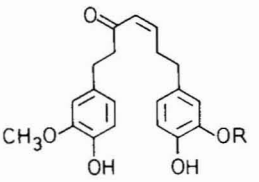
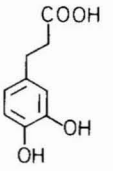


Fig. 1. Proposed scheme[14] for the biosynthesis of the gingerols and hexahydrocurcumins. Gingerols are produced when R is an aliphatic carboxylic acid such as hexanoic whereas the hexahydrocurcumins result from condensation with an aromatic acid such as dihydroferulic acid.

by the same biosynthetic pathway as the gingerols by condensation of the intermediate IX with either dihydroferulic or dihydrocaffeic acid (XIV).



R=CH_3, XIIIa
R=H, XIIIb



Dihydrocaffeic acid, XIV

EXPERIMENTAL

Preparation of samples

Root ginger from Jamaica was obtained locally. Gas chromatographic–mass spectrometric (GC–MS) studies on several samples showed similar profiles.

A 1.5-g amount of dry root was crushed and left to stand with ethyl acetate for 30 min. The solution was filtered and evaporated to dryness to yield 200 mg of oil. This was dissolved in 20 ml of ethyl acetate to give the stock solution. Aliquots of this were derivatized as described below.

Trimethylsilyl (TMS) derivatives. The sample (0.1 ml) was blown to dryness (nitrogen stream) and heated with a mixture of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 4 μl), trimethylchlorosilane (TMCS, 2 μl) and acetonitrile (4 μl) at 60°C for 10 min. Aliquots of the mixture were injected directly into the chromatograph.

[$^2H_9$]TMS derivatives[16]. The sample was heated with [$^2H_{18}$]bis(trimethylsilyl)-acetamide ([$^2H_{18}$]BSA, 5 μl) and acetonitrile (5 μl) for 10 min at 60°C.

Methyl ether–TMS derivatives. A 0.2-ml volume of the stock solution was blown to dryness and dissolved in dimethylformamide. Methyl iodide (10 μl) and potassium carbonate (*ca.* 50 mg) were added and the mixture was stood overnight at room temperature. The mixture was then diluted with water, the products were extracted with ethyl acetate, washed with water, dried ($MgSO_4$) and blown to dryness. The sample was then reacted with BSTFA as described above.

[$^2H_3$]Methyl ether–TMS derivatives. These were prepared as above using [$^2H_3$]-methyl iodide in place of the methyl iodide.

Methyloxime–TMS (MO-TMS) derivatives. The dry sample was dissolved in pyridine (20 μl) and heated for 1 h at 60°C with an excess of methoxyamine hydrochloride. The mixture was then blown to dryness and the TMS derivatives were prepared as described above.

Lithium aluminium hydride reduction. The sample was dissolved in ether (0.5 ml) and allowed to react with lithium aluminium deuteride (*ca.* 10 mg) for 1 h at room temperature. The products were extracted with ethyl acetate following destruction of the excess reagent by standard techniques. The solvent was removed from the dried ($MgSO_4$) solution with a nitrogen stream and the residue was trimethylsilylated as described above.

Deuterium exchange. The sample was dissolved in dioxane (0.5 ml), ^2H_2O (0.5 ml) and NaO^2H (trace) and allowed to stand at room temperature overnight. The

mixture was then diluted with ^2H_2O (2 ml) and acidified (H_2SO_4) and the products were extracted with ethyl acetate. The extract was washed with water, dried ($MgSO_4$) and converted into trimethylsilyl derivatives as described above.

Deuterium exchange, reduction. The deuterium exchange reaction was performed as above and the dried product was reduced with lithium aluminium deuteride as described under *Deuterium exchange*. The products were trimethylsilylated as described under *Trimethylsilyl (TMS) derivatives.*

Oxidation. The sample was dissolved in a mixture of dimethylsulphoxide (0.5 ml) and acetic anhydride[17] and left at room temperature overnight. Water was added and the products were extracted with ethyl acetate, washed with sodium bicarbonate solution, water and evaporated to dryness. The residue was hydrolysed in a mixture of methanol (1 ml) and dilute sodium hydroxide solution (0.1 ml, 0.1 *N*) for 1 h at 60°C, diluted with water and the products were extracted with ethyl acetate. This extract was washed with water, dried ($MgSO_4$) blown to dryness and converted into TMS derivatives as described above.

Gas–liquid chromatography

GC data were recorded with a Varian 2440 gas–liquid chromatograph fitted with dual flame-ionisation detectors and two 2 m × 2 mm glass columns packed with 3% SE-30 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.). Nitrogen at 30 ml/min was used as the carrier gas, the injector and detector temperatures were 300°C and the column oven was temperature programmed from 100 to 300°C at 4°C/min.

Gas chromatography–mass spectrometry

GC–MS data were recorded with a VG Micromass 12B mass spectrometer interfaced to a VG type 2040 data system and via a glass jet separator to a similar chromatographic system to that described above. Helium at 30 ml/min was used as

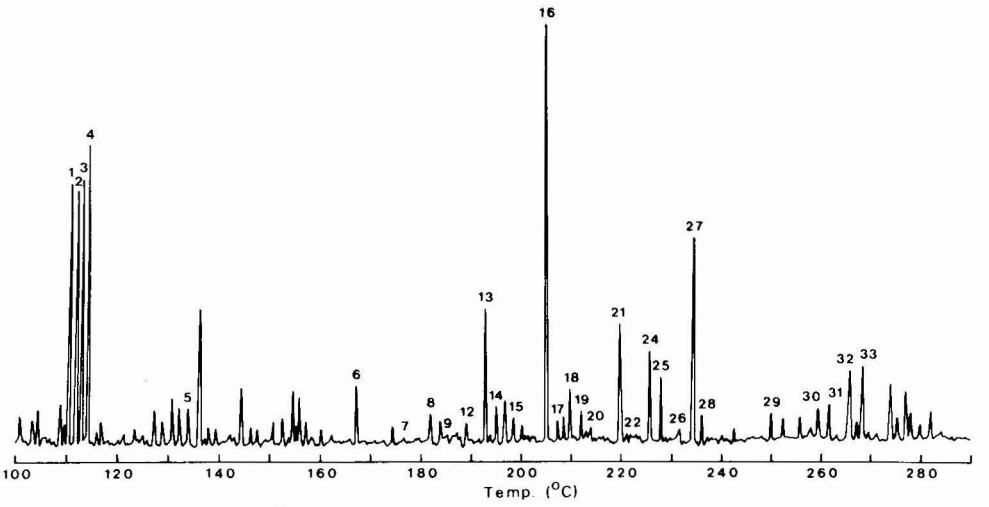


Fig. 2. Computer reprocessed[18] chromatogram of the ginger constituents separated on a 3% SE-30 packed column programmed from 100 to 300°C at 4°C/min. Peaks are identified in Tables I and II and in the text.

TABLE I

GC–MS DATA FOR THE TMS DERIVATIVES OF THE GINGEROLS AND RELATED COMPOUNDS

Compound type	*No.*	*n*	*Peak No.*	*MU (SE-30)*	M^{+}	*Base*	*Major ions*		
Gingerol	Ia	2	12	22.73	410*	209	145**	320***	222
					(31)		(50)	(17)	(20)
Gingerol	Ia	4	16	24.57	438	209	173**	348***	222
					(41)		(37)	(33)	(25)
Gingerol	Ia	6	21	26.39	446	209	201**	356***	222
					(44)		(31)	(29)	(27)
Gingerol	Ia	8	27	28.35	494	209	229**	404***	222
					(46)		(26)	(34)	(25)
Gingerol	Ia	10	29	30.30	522	209	257**	432***	222
					(24)		(13)	(23)	(20)
Methylgingerol	Ib	4	15	–	380	151	173**	290***	164
					(17)		(46)	(21)	(33)
Methylgingerol	Ib	6	20	–	408	151	201**	318***	164
					(14)		(40)	(20)	(38)
Methylgingerol	Ib	8	26	–	436	151	229**	346***	164
					(17)		(38)	(28)	(45)
Shogaol	IIa	2	7	–	320	209	223		
					(27)		(15)		
Shogaol	IIa	4	13	23.24	348	209	223	277	
					(53)		(15)	(15)	
Shogaol	IIa	6	18	25.26	376	209	277	223	
					(43)		(15)	(13)	
Shogaol	IIa	8	24	27.28	404	209	277	223	
					(51)		(17)	(13)	
Methylshogaol	IIb	4	10	–	290	151	165	219	
					(83)		(72)	(71)	
Methylshogaol	IIb	8	22	–	346	151	219	165	
					(51)		(58)	(32)	
Paradol	III	4	11	–	350	209	320	223	
					(56)		(23)	(22)	
Gingediol	IV	4	17	24.91	512	209	173**	332 §	422***
					(11)		(98)	(92)	(44)
Gingediol	IV	6	22	26.77	540	209	360 §	201**	450***
					(11)		(99)	(83)	(43)
Gingediol	IV	8	28	28.69	568	209	388 §	229**	478***
					(9)		(99)	(71)	(43)
Gingerdione	VII	4	14	23.62	364	209	179		
					(29)		(26)		
Gingerdione	VII	6	19	25.52	392	209	179		
					(30)		(24)		
Gingerdione	VII	8	25	27.55	420	209	179		
					(36)		(22)		

* m/z and (relative intensity).

** TMS-O^{+}=CH–$(CH_2)_n$–CH_3.

*** $[M - TMS\text{-}OH]^{+}$.

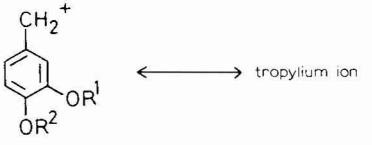
§ $[M - 2 \times TMS\text{-}OH]^{+}$.

the carrier gas. Operating conditions were: injector, separator and ion source temperatures, 300, 280 and 260°C, respectively; accelerating voltage, 2.5 kV; electron energy, 25 eV; trap current, 100 μA; scan, 3 sec/decade; exponential, down.

RESULTS AND DISCUSSION

Fig. 2 shows a computer reprocessed[18] total ion chromatogram of the ginger constituents separated as TMS derivatives on a 2-m SE-30 column. The compounds which were eluted in the low temperature region of the chromatogram were not studied in detail although a few identifications were made. Thus, peak 1 was identified as α-curcumene[19], peaks 2–4 were $C_{15}H_{24}$ sesquiterpenes, peak 5 was zingerone (V) and peaks 6, 8 and 9 were palmitic, oleic and stearic acids, respectively. Most of the other compounds in this region appeared to be hydroxylated derivatives of the sesquiterpenes (peaks 1–4).

Peaks 7, 10–29 were produced by the gingerols and related compounds (I–IV, VII), the compounds identified are listed in Table I together with their GC–MS characteristics. Identification was based on the GC–MS properties of the TMS, [$^2H_9$]TMS and MO–TMS derivatives. In addition the methylgingerols (Ib) and methylshogaols (IIb) were identified by comparing their GC–MS properties with synthetic samples prepared by methylation of the gingerols. The gingediols (IV) were identified similarly by reduction of the gingerols with lithium aluminium deuteride. The reduction yielded two diasteroisomers, epimeric about the reduced group, but only one of these was present in the ginger sample. Its stereochemistry was not determined. The compounds were located in the ginger fraction by plotting single ion chromatograms of characteristic ions; thus *m/z* 209 (a) was diagnostic of the compounds such as gingerol having one hydroxy and one methoxy substituent in the phenyl ring and *m/z* 151 (b) was characteristic of the methylgingerols and related compounds. Single ion chromatograms for the corresponding tropylium ions from other commonly occurring phenyl derivatives (*e.g.* methylenedioxy) failed to reveal any additional series of gingerols. Acetyl derivatives of the gingerols and gingediols recently found in samples of Japanese ginger[4] were not detected.



R^1=CH_3, R^3=TMS, *m/z* 209 (a)
R^1=R^2=CH_3, *m/z* 151 (b)
R^1=R^2=TMS, *m/z* 267 (c)

Further evidence that the shogaols are predominantly decomposition products of the gingerols was obtained from the experiments on labelling the α-hydrogens of the gingerols and shogaols with deuterium. GC–MS analysis of the products showed that most of the contribution to the shogaol peaks was by molecules which had incorporated 3 deuterium atoms. The shogaols should have incorporated 6 atoms as the result of double bond migration whereas the gingerols with 4 α-hydrogens incorporate 4 atoms. Thermal elimination of water from the gingerols, a 1:2 elimination, would remove one of these deuterium atoms (from C_4) to give a shogaol with

ABLE II

C–MS DATA FOR THE TMS DERIVATIVES OF THE HEXAHYDROCURCUMINS AND RELATE OMPOUNDS

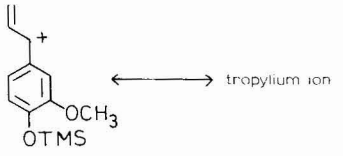
ompound	*No.*	*Peak No.*	*MU*	M^+	*Base*	*Major ions*		
exahydrocurcumin	VIa	32	32.55	590* (13)	209	500** (26)	179 (16)	235 (14)
esmethylhexahydrocurcumin	VIb	33	32.96	500 (10)	209	179 (17)		
	XIIIa	30	31.30	648 (21)	209	267 (47)	558** (37)	179 (31)
	XIIIb	31	31.86	558 (14)	209	267 (63)	179 (24)	

* *m/z* and (relative intensity).
** $[M - TMS\text{-}OH]^+$.

3 deuterium atoms. Thus, considerable dehydration took place after the completion of the exchange reaction, demonstrating the instability of the gingerols.

The compounds producing peaks 14, 19 and 25 were identified as gingerdiones (VII). They formed mono-TMS derivatives as shown by spectra of the $[^2H_9]$TMS derivatives, and the base peak in the spectrum of the TMS derivative at *m/z* 209 (a) showed that this hydroxy group was on the phenyl ring. Reaction with methoxyamine hydrochloride gave bis-oximes (2 peaks produced by *syn*- and *anti*-isomers) showing the presence of two ketone groups in the original molecules. Confirmation of the presence of these ketone groups in the 3 and 5 positions of the alkyl chain was obtained by oxidation of the gingerols with a mixture of acetic anhydride and dimethylsulphoxide[17]. The GC–MS characteristics of the products of oxidation (TMS derivatives) were identical to those of the diones in the ginger extracts. These compounds do not appear to have been reported before.

Peaks 30–33 were identified as hexahydrocurcumins and related compounds (Table II). Hexahydrocurcumin itself (VIa, peak 32) formed a tris–TMS derivative (molecular weight 590) and its mass spectrum (Fig. 3) showed an abundant loss of TMS-OH (*m/z* 500) and a base peak at *m/z* 209 (a). Ions at *m/z* 189, 193, 223, 249 and 251 were typical of the dihydroferulic acid residue and were also prominant in the spectra of the gingerols (Fig. 4). The ion at *m/z* 235 (d) which was absent from the spectra of the gingerols, came from the second dihydroferulic acid residue (C5–C7) by loss of TMS-OH (the site of the hydrogen loss was not determined) as shown by (a) the absence of exchangeable hydrogens and (b) the presence of the ion in the spectra of the TMS derivatives of the gingediols. Ion d is thus associated with the Ar–CH_2–CH_2–CHO-TMS- and not the Ar–CH_2–CH_2–CO-moiety.



d, *m/z* 235

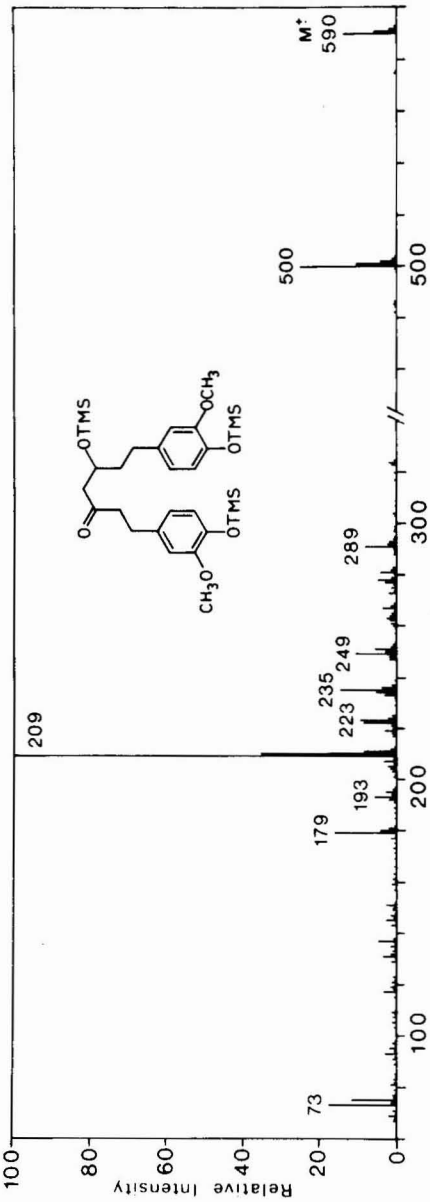


Fig. 3. 25 eV mass spectrum of the TMS derivative of hexahydrocurcumin.

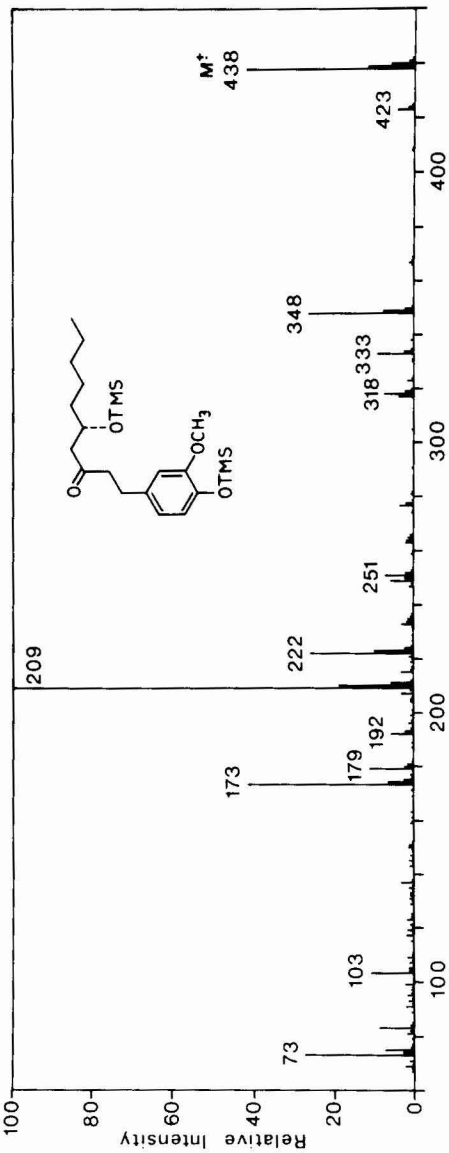


Fig. 4. 25 eV mass spectrum of the TMS derivative of [6]-gingerol (the name [6]-gingerol indicates the chain length of the acid condensed with intermediate IX).

The compound producing peak 33 formed a tetrakis-TMS derivative with a molecular weight of 648. Its mass spectrum was similar to that of the corresponding derivative of hexahydrocurcumin (VIa), but prominent ions at m/z 209 (a) and 267 (c) indicated that one of the methoxy groups had been replaced by a hydroxy group. Methylation with methyl iodide gave the same permethylhexahydrocurcumin from both hexahydrocurcumin and the compound producing peak 33. By the use of [$^2H_3$]-methyl iodide the two starting materials could be distinguished by their incorporation of two and three [$^2H_3$]methyl groups, respectively. Thus, peak 33 was produced by a desmethylhexahydrocurcumin. Two isomers (VIb and VIc) of this compound are possible but the mass spectrum of the TMS derivative showed that the compound was VIb. This was based on the shift of ion d (m/z 235) by 58 mass units. As this ion comes from the second aromatic residue (attached to C_7), then clearly this must contain the dihydroxy and not the hydroxy methoxy substituents.

A possible biosynthetic pathway for the production of these compounds can be proposed along the lines of the biosynthesis of the gingerols. Thus, condensation of dihydroferulic acid with the intermediate IX (Fig. 1) ultimately leads to hexahydrocurcumin (VIa) whereas condensation of IX with dihydrocaffeic acid (XIV) gives the desmethylhexahydrocurcumin (VIb).

Peaks 30 and 31 had GC–MS characteristics (Table II) fully consistent with their being produced by the hexahydrocurcumin equivalents to the shogaols (VIII). They formed bis-TMS derivatives and monomethyloximes. Results from the deuterium labelling experiments indicated that, like the shogaols, they were produced mainly by thermal dehydration of the hexahydrocurcumins.

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GAS–LIQUID CHROMATOGRAPHY OF *s*-TRIAZINES ON A SURFACE-BONDED SUPPORT IN SHORT COLUMNS

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SUMMARY

The use of surface-bonded PEG 20M Chromosorb P as the support, coated with Versamid 900 or PEG 20M stationary phases, in short columns allows the rapid isothermal gas–liquid chromatographic separation of *s*-triazine derivatives. The preparation of the support involved preliminary acid washing and subsequent polymer deactivation; a modified Soxhlet apparatus was used for acid washing, in which hot extraction acid vapours were used. The iron content of Chromosorb P (80–100 mesh) was reduced to 0.01–0.02% in 6 days.

Two columns were suggested: 3% Versamid 900 (0.45 m × 3 mm I.D.) for the separation of a mixture of methoxy-, chloro- and thiomethyl-*s*-triazines and 2% PEG 20M (0.35 m × 3 mm I.D.) for the separation of chloro-*s*-triazines alone. The analysis time on the former column was about 9 min and on the latter about 4 min.

INTRODUCTION

s-Triazine derivatives are widely used as herbicides, and in the last two decades a number of methods for their analysis have been developed[1–3], such as spectroscopy (UV–visible), gas–liquid chromatography (GLC) and paper and thin-layer chromatography. Although some of the first investigations were carried out by GLC[4–6] and the number of publications continued to grow up to 1970, by about 1976 the number of such papers had fallen to half that in 1970, probably owing to the time-consuming GLC analysis on conventional stationary phases in long columns[4]. The disadvantages of the other methods employed[3], *viz.*, insufficient separation possibilities of spectroscopy and the semi-quantitative results of paper and thin-layer chromatography, have directed the efforts of investigators during the last 2–3 years towards high-performance liquid chromatography (HPLC)[7] and back to GLC[8–14]. One of the main reasons is the suggested use of surface-bonded phases, allowing the solution of adsorption problems related to inefficient, poorly silanized commercially available supports[14–16].

In this paper we report the application on surface-bonded PEG 20M Chromosorb P as the support, coated with Versamid 900 or PEG 20M, for the separation of methoxy-, chloro- and thiomethyl-*s*-triazines using short columns and isothermal temperatures.

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EXPERIMENTAL

Preparation of surface-bonded packings

The surface-bonded support was prepared in accordance with the original idea suggested by Aue *et al.*[19,20] and the procedure described in a previous paper[17]. Acid washing of Chromosorb P (80–100 mesh) with 6 *N* hydrochloric acid was carried out in a modified Soxhlet apparatus of our own design (Fig. 1), in which hot extraction acid vapours were used; the acid-washing period was shortened to 6 days and the iron content was reduced to 0.01–0.02%. After coating the support with PEG 20M, heat conditioning to 280°C, removal of non-bonded PEG 20M by solvent extraction with methanol and drying, the surface-bonded support was ready. The support was divided into two parts: one was coated with Versamid 900 and the other with PEG 20M.

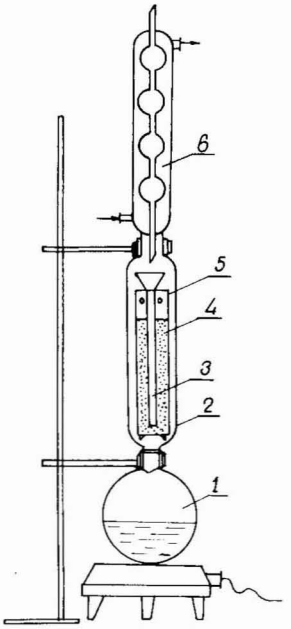


Fig. 1. Modified Soxhlet apparatus for acid washing of the support with the hot extraction acid vapours. 1, Flask (500 cm^3) with a ground-glass joint; 2, 340 mm × 450 mm I.D. tube with ground-glass joints at both ends; 3, 160 mm × 11 mm I.D. funnel with an elongated tube; 4, Chromosorb P (80–100 mesh); 5, 210 mm × 28 mm I.D. overflow cartridge with three openings for the extraction solution; 6, reflux condenser with ground-glass joint.

Gas–liquid chromatography

GLC measurements were carried out isothermally on a Carlo Erba Fractovap GI instrument equipped with a flame-ionization detector (FID) and an injector connected directly to the column (Rasoterm U-shaped 0.35 and 0.45 m glass columns of our design; the end fittings were straight without any additional glass or metal components for connection to the FID).

The glass columns were previously deactivated with dimethyldichlorosilane (DMCS) and then filled with the prepared packings. Versamid 900 was deposited on

the support from chloroform–*n*-butanol (1:1) and PEG 20M from dichloromethane. The working temperatures of the Versamid 900 and PEG 20M columns were 200 and 195°C, respectively. Oxygen-free argon was used as the carrier gas.

A Leeds and Northrup Spedomax W recorder was employed at a chart speed of 1l6 cm min^{-1}.

Chemicals

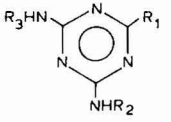
s-Triazines of analytical-reagent grade were employed. The Chromosorb P (80–100 mesh) support was supplied by Johns-Manville (Denver, CO, U.S.A.) and the Versamid 900 and PEG 20M liquid phases by Carlo Erba (Milan, Italy).

GLC measurements

The herbicides selected were polar, high-molecular-weight 1,3,5-triazines, substituted at the second, fourth and sixth positions (Table I). The methoxy-, chloro- and thiomethyl-*s*-triazine mixture (Fig. 2A) and the chloro-*s*-triazine mixture (Fig. 2B) were injected in the form of a dimethylformamide solution.

TABLE I

s-TRIAZINES USED



s-Triazine	*2-Substituent* (R_1)	*4-Substituent* (R_2)	*6-Substituent* (R_3)	*Molecular weight*
Prometone	OCH_3	iso-C_3H_7	iso-C_3H_7	225.3
Atratone	OCH_3	C_2H_5	iso-C_3H_7	211.0
Simetone	OCH_3	C_2H_5	C_2H_5	197.2
Propazine	Cl	iso-C_3H_7	iso-C_3H_7	229.7
Atrazine	Cl	C_2H_5	iso-C_3H_7	215.7
Simazine	Cl	C_2H_5	C_2H_5	201.5
Prometryne	SCH_3	iso-C_3H_7	iso-C_3H_7	241.3
Ametryne	SCH_3	C_2H_5	iso-C_3H_7	227.3
Simetryne	SCH_3	C_2H_5	C_2H_5	213.3

The retention times were measured by means of a stop watch and the peak widths by a micrometric magnifying glass ($\pm$0.1 mm) "Karl Zeiss"-Jena.

The reproducibility of GLC measurements such as resolution criterion ($R_{t,w}$), relative retention times (RRT) and asymmetry coefficient (K_a) was evaluated from the standard deviation (SD) and the relative standard deviation (RSD, %), obtained in 6 experiments with each mixture analyzed on the corresponding column (Table II).

RESULTS AND DISCUSSION

A large number of polar and non-polar stationary liquids have been suggested[4,8,11,13] for the GLC analysis of *s*-triazines, such as XE-60, Reoplex 400, PEG

TABLE II

STATISTICAL EVALUATION OF RELATIVE RETENTION TIME (*RRT*), $R_{t,w}$ AND K_a DATA

SD = standard deviation; RSD = relative standard deviation.

Column	*s-Triazine*	*RRT*	*SD*	*RSD*	$R_{t,w}$	*SD*	*RSD*	K_a		*RSD*
Versamid 900	Prometone	1.0	–	–	–	–	–	1.4	0.03	2.1
	Atratone	1.2	0.04	3.3	1.2	0.01	0.8	1.1	0.02	1.8
	Simetone	1.3	0.03	2.3	1.2	0.02	1.7	1.2	0.04	3.3
	Atrazine	1.6	0.05	3.1	2.8	0.12	4.2	1.3	0.02	1.5
	Prometryne	1.8	0.06	3.3	1.4	0.05	3.2	1.4	0.05	3.6
	Ametryne	2.1	0.07	3.5	1.5	0.02	1.6	1.3	0.04	3.1
	Simetryne	2.5	0.08	3.2	1.4	0.05	3.3	1.5	0.05	3.4
PEG 20M	Propazine	1.0	–	–	–	–	–	1.4	0.02	1.4
	Atrazine	1.3	0.03	2.3	1.6	0.05	3.1	1.2	0.04	3.3
	Simazine	1.7	0.02	1.2	1.7	0.03	1.8	1.3	0.04	3.0

20M, PEG A, OV-210, Versamid 900 and SE-30 with liquid loadings mainly below 5% (w/w), and also a combination of polar and non-polar liquids (SE-30 and Reoplex 400)[13]. Diatomaceous supports of the Chromosorb W type[4,11,13] treated or untreated with silanes have been employed. However, in most instances peak tailing and/or broadening was observed and there was a lack of reproducibility between different laboratories. A possible explanation is that separations on packed columns with liquid loadings below 5% depend on the support employed[21] because the separations are effected by a complex mechanism of retention. Recently these problems and those related to the use of poorly silanized supports in the analysis of polar compounds were overcome by the use of surface-bonded supports[8,20,21], which allowed an efficient low liquid loading of 1–3% (w/w)[17]. Some disadvantages associated with the use of Chromosorb P for the analysis of polar compounds[18], including pesticides[8], have been mentioned[8,18] in comparison with other diatomaceous supports such as Chromosorb W and G and Gas-Chrom P and Q. Nevertheless, in our investigation we used Chromosorb P.

Before we discuss the reliable results achieved by applying surface-bonded Chromosorb P as the support for the analysis of polar *s*-triazines, the reasons why this type of support was selected will be given.

First, as was mentioned above, the preparation of a surface-bonded support is time consuming (minimum 15 days). Therefore, it is better to use Chromosorb P, which has a higher mechanical resistance and is less fragile than Chromosorb W, with which the removal of fines at all stages is required.

Second, for additional deactivation of the support to achieve a fairly inactive clean surface, it is important to remove iron and other mineral impurities. As mentioned above, during acid washing (Fig. 1) the iron content is reduced to 0.01–0.02%. Moreover, Chromosorb P does not contain Fe_2O_3 as a complex as does Chromosorb W, and the iron is more readily extracted from the Chromosorb P surface.

Third, Chromosorb P is more suitable than Chromosorb W and G for modification and additional coating with polar liquids such as PEG 20M and Versamid 900. Moreover, according to Ottenstein[22], Chromosorb W supports have a larger pore size (about 9 μm), compared with about 2 μm for Chromosorb P. This difference could explain the variation in the column behaviour of the two types of supports: the former retains the liquid stationary phase in broad pools, whereas the latter does it in smaller pools, leading to less tailing with Chromosorb P.

As can be seen from Table II and Fig. 2, the surface-bonded Chromosorb P support employed in short columns provides good reproducibility of the relative retention times (relative standard deviation not over 3.5%), with good resolution. The resolution criterion ($R_{t,w}$) values for both columns are higher than 1.2 and the reproducibility of $R_{t,w}$ measured as the relative standard deviation is 0.8–4.2%. The values of the asymmetry coefficient (K_a) are close to unity with good reproducibility (relative standard deviation not over 3.6%).

When the relative standard deviation of the relative retention time was higher (*e.g.*, the relative standard deviation for atrazine was 4.2%), a check with the t_α criterion was made. The tabulated values of the t_α criterion (for a significance level of $\alpha = 0.05$ and five degrees of freedom) were higher than those of the calculated t_α criterion. Therefore, there was no systematic error in the relative retention data obtained.

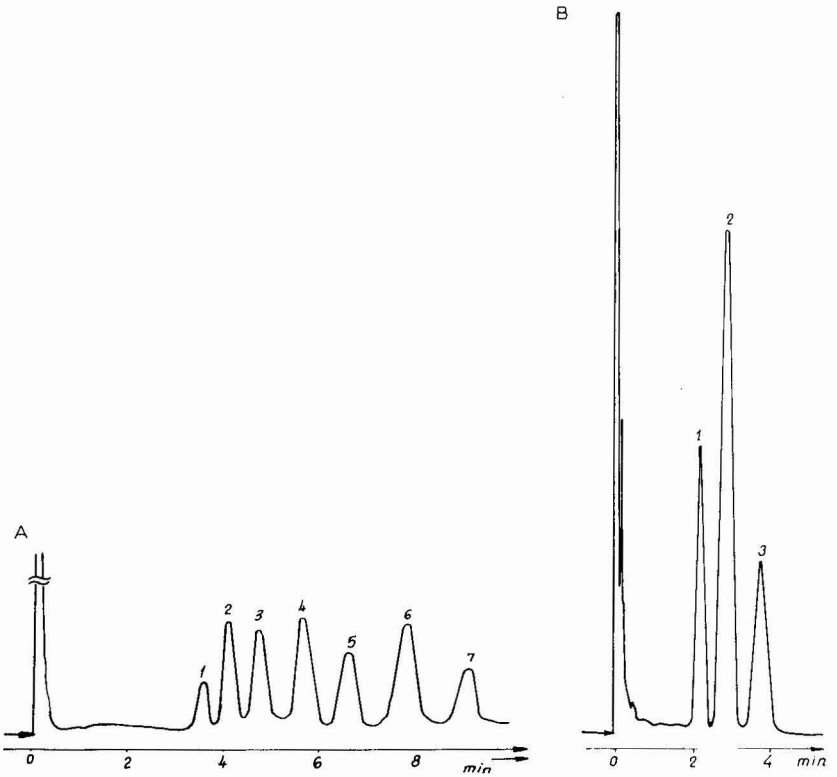


Fig. 2. Gas chromatograms of *s*-triazine mixtures. (A) Mixture of methoxy-, chloro- and thiomethyl-*s*-triazines: 1 = prometone; 2 = atratone; 3 = simetone; 4 = atrazine; 5 = prometryne; 6 = ametryne; 7 = simetryne. Operating conditions: column, 0.45 m × 3 mm I.D., packed with 3% Versamid 900 on Chromosorb P AW (80–100 mesh) (0.01% Fe), deactivated with a non-extractable layer of PEG 20M; argon flow-rate, 35 ml min^{-1}; column temperature, 200°C. (B) Mixture of chloro-*s*-triazines: 1 = propazine; 2 = atrazine; 3 = simazine. Operating conditions: column, 0.35 m × 3 mm I.D., packed with 2% PEG 20M on the support as in (A); argon flow-rate, 45 ml min^{-1}; column temperature, 195°C. Injection volume, 0.8 μl; attenuation, 16 × 10^2.

The time of analysis for the mixture of methoxy-, chloro- and thiomethyl-*s*-triazines on the Versamid column (Fig. 2A) is about 9 min. For the separation of the chloro-*s*-triazine series alone the most polar column PEG 20M (Fig. 2B) was found to be more suitable, with a shorter analysis time of about 4 min.

CONCLUSION

Surface-bonded supports in short columns have proved suitable for the GLC analysis of *s*-triazines, with an efficiency approaching that of HPLC[7,23,24].

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DETERMINATION OF TRACE ATMOSPHERIC ISOCYANATE CONCENTRATIONS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING 1-(2-PYRIDYL)PIPERAZINE REAGENT

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SUMMARY

A reversed-phase high-performance liquid chromatographic method is described for the determination of both aliphatic and aromatic isocyanates in air. The test atmosphere is drawn through a $2 \cdot 10^{-4}$ *M* solution of 1-(2-pyridyl)piperazine in toluene at a sampling rate of 1 l min^{-1} for 5–20 min. The sample solution is evaporated to dryness and the residue dissolved in 100 μl of acetonitrile. A 10-μl aliquot of the resulting solution is chromatographed using a 25 cm × 4.6 mm I.D. stainless-steel column packed with ODS-Hypersil 5 μm silica gel and eluted, isocratically, with acetonitrile–0.1 *M* ammonium acetate mobile phase at a flow-rate of 2.0 ml/min. The ammonium acetate solution is adjusted to pH 6.2 with acetic acid.

INTRODUCTION

Diisocyanates and their high-molecular-weight oligomers are extensively used in the production of polyurethane materials ranging from flexible foams to enamel wire coatings, so that a large number of workers are potentially at risk of isocyanate exposure. Adverse physiological effects of inhalation of isocyanate vapour or aerosol extend from mucous membrane irritation to respiratory sensitization[1,2]. The latter effect may possibly be due to combination reactions of isocyanate groups with body protein[3,4]. A progressive diminution in lung function has been reported to occur when workers are regularly exposed to sub-threshold limit value[5] isocyanate concentrations[6,7]. Although conflicting evidence has been put forward on this point[8,9], the current trend suggests that lower threshold limit values may be considered in the future[10]. Consequently, sensitive analytical methods for the determination of isocyanates and their oligomers are of increasing importance.

In order to achieve adequate sensitivity and resolution, the majority of recent analytical methods for the determination of isocyanates employ either high-perform-

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ance thin-layer chromatography (HPTLC) or high-performance liquid chromatography (HPLC). Previous colorimetric-based methods are generally incapable of resolving individual isocyanate component concentrations and are susceptible to interferences in many cases[11–18]. Although several HPLC methods have been reported for the determination of free isocyanates in their bulk prepolymers[19–22], the first generally applicable atmospheric isocyanate determination employed a double derivative thin-layer chromatographic technique[23]. The method, based on the reaction of isocyanates with N-4-nitrobenzyl-N-*n*-propylamine (nitro reagent) to form urea derivatives, has a lower detection limit of 80 $\mu g\ m^{-3}$, and an improved sampling technique[24] has recently increased its sensitivity to 2 $\mu g\ m^{-3}$ (for toluene diisocyanate, 4,4′-methylene bisphenyl isocyanate and 1,6-hexamethylene diisocyanate) for a 1-l air sample.

The determination of atmospheric isocyanates, using nitro reagent as the isocyanate-reactive entity, was later extended to gradient-elution HPLC as the method of analysis[25]. Further studies[26–28] indicate, however, that the presence of excess nitro reagent in the sample solution seriously reduces the life of the pellicular-silica column specified due to adsorption onto the column packing. As a result of such reagent adsorption the urea derivatives have a tendency to elute with sloping baselines, especially in the case of higher retention time peaks, causing a reduction in precision. The need to use gradient elution leading to significant column recovery times is a further limitation to be considered. Sangö[29] has recently examined the application of a bonded octadecylsilyl phase using isocratic elution with acetonitrile–water (75:25) to minimise the disadvantages associated with the nitro reagent. Nevertheless, decomposition of the nitro reagent, both during and after sampling, may occur in reducing or oxidizing atmospheres[25,26], possibly due to the presence of the aromatic nitro group in this secondary amine.

The reversed-phase HPLC method reported here is based on the reaction of isocyanates with 1-(2-pyridyl)piperazine to form stable urea derivatives. 1-(2-Pyridyl)piperazine has negligible steric hindrance at the –NH position and reaction with both aliphatic and aromatic isocyanates is rapid and exothermic. In addition, the substituted ureas formed possess significantly higher molar absorptivities in the ultraviolet region than those derived from the nitro reagent[30]. The method is capable of measuring 500 pg of methylene bisphenyl isocyanate, equivalent to 48 ppt (10^{12}) in a 10-l air sample.

EXPERIMENTAL

Chromatographic apparatus

The liquid chromatograph comprised an Altex Model 110A constant-flow reciprocating diaphragm pump, a Rheodyne Model 7120 syringe loading sample injector with a 10-μl loop and a Pye-Unicam LC–UV detector set at 254 nm. The column consisted of 25 cm × 4.6 mm I.D. Apollo stainless-steel tubing, slurry packed at 6000 p.s.i. with ODS-Hypersil (5 μm diameter mean particle size, Shandon Southern Products, Runcorn, Great Britain). The mobile phase was deaerated using helium and pumped at ambient temperature through the column at a flow-rate of 2.0 ml/min.

Mobile phase

Three mobile phases, consisting of acetonitrile in 0.1 *M* aqueous ammonium acetate solution were used in the isocratic mode. The ammonium acetate solution was adjusted to pH 6.2 with acetic acid. Mobile phase A (acetonitrile–ammonium acetate solution, 31:69) was used in the determination of 2,4- and 2,6-toluene diisocyanate (TDI), 1,6-hexamethylene diisocyanate (HMDI), phenyl isocyanate (PhI) and naphthalene diisocyanate (NDI) urea derivatives. Mobile phase B (acetonitrile–ammonium acetate solution, 37:63) was used in the determination of the isophorone diisocyanate (IPDI) urea derivatives, while mobile phase C (acetonitrile–ammonium acetate solution, 40:60) was used in the determination of the methylene bisphenyl isocyanate (MDI) and HMDI oligomer (Desmodur N; Bayer, Richmond-upon-Thames, Great Britain) urea derivatives.

Isocyanates

The following isocyanates were used: HMDI (Desmodur H, Bayer); IPDI (Veba-Chemie, Gelsenkirchvuer, G.F.R.); 4,4′-MDI (ICI, Macclesfield, Great Britain); 1,5-NDI (Desmodur 15, Bayer); PhI (Eastman-Kodak, Rochester, NY, U.S.A.); 2,4-TDI (Fluorochem, Glossop, Great Britain); 2,6-TDI (ICI, Blackley, Manchester, Great Britain) and HMDI oligomer (Desmodur N, Bayer).

Standard urea derivatives

A freshly prepared solution of pure, monomeric isocyanate (0.8 mmole) in 3 ml of dimethyl sulphoxide (DMSO), for example, is added to a stirred solution of 1-(2-pyridyl)piperazine (280 μl, 1.8 mmole) in DMSO (3 ml). The mixture is stirred for 15 min at 60°C and then 200 ml of distilled water is slowly added. A white precipitate forms which is filtered off and washed with distilled water. The white solid is dried at 50°C and recrystallised to produce approximately 500 mg of the urea derivative. The aromatic isocyanate urea derivatives are recrystallised from a mixture of toluene and DMSO, except for the phenyl isocyanate urea derivative which is recrystallised from a mixture of toluene and *n*-hexane. The aliphatic isocyanate urea derivatives are recrystallised from a mixture of water and dimethylformamide.

To prepare an HMDI oligomer urea derivative, it is necessary to use a three-fold molar excess of 1-(2-pyridyl)piperazine with dry acetonitrile as the solvent. The reaction mixture is stirred at 60°C for 30 min, after which it is set aside for several days, during which time the urea derivative forms as a gummy off-white deposit. Excess distilled water is then added at ambient temperature, the solution is decanted off, the residue is dried at 50°C and eventually solidifies to a white solid.

Alternatively, standard urea solutions can be produced for all the isocyanates and their oligomers by adding a suitable quantity of the isocyanate under investigation, dissolved in dichloromethane, to a $2 \cdot 10^{-2}$ *M* solution of 1-(2-pyridyl)piperazine in dichloromethane. The resulting solution is diluted to give standards in the required range.

Absorbing solution

A 320-μl volume (326 mg) of 1-(2-pyridyl)piperazine is pipetted into a 100-ml volumetric flask and made up to volume with toluene. A 5-ml aliquot of this solution is made up to 500 ml with toluene to give a $2 \cdot 10^{-4}$ *M* absorbing solution. This

solution should be stored in a dark glass-stoppered bottle. Experience indicates that the stored solution remains stable for several months.

Air sampling and sample preparation

A sample of the test atmosphere is drawn through 8 ml of the absorbing solution in a Greenburg–Smith type midget impinger at a flow-rate of 1 l min^{-1} for at least 5 min. Alternatively, for time-weighted-average sampling purposes, a lower flow-rate (50 ml min^{-1}) can be used. After sampling, the impinger and its contents are removed to an uncontaminated atmosphere and any liquid is expelled from the inlet tube with a blow-ball. Suitable amounts of the absorbing solution are successively transferred into a 2-ml micro-reaction vessel and evaporated to dryness with nitrogen. Finally, the impinger receiver is washed with 0.5 ml of toluene and the washings also transferred to the micro-reaction vessel. The residue, after evaporation, is redissolved in 100 μl of dry acetonitrile and a 10-μl aliquot is injected into the liquid chromatograph.

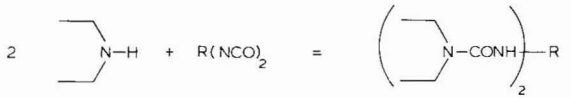
Determination of collection efficiency

A dynamic standard atmosphere apparatus[31], modified to include double dilution, was used to generate known concentrations of HMDI in a constant-flow stream of dried air. These atmospheres were sampled at 1 l min^{-1} through three midget impingers connected in series, each containing 8 ml of the absorbing solution. The absorbing solutions were treated as described above and the extent of breakthrough determined.

RESULTS AND DISCUSSION

Selection of derivatising reagent

The choice of 1-(2-pyridyl)piperazine as a suitable derivatising reagent for isocyanates was made after a systematic study of several related compounds with the required characteristics, that is (i) a secondary aliphatic amine group as part of a ring structure with negligible steric hindrance and (ii) an aromatic substituent. The one-step addition reaction of isocyanates with the –NH group eliminates the possibility of further reaction, as may occur with 1-naphthyl methylamine[32], which has recently been used in the HPLC analysis of isocyanates using fluorescence detection. The presence of an aromatic substituent on the derivatising molecule ensures that the urea derivatives will have sufficiently high molar absorptivities even when derived from aliphatic isocyanates, and the presence of the pyridyl group confers solubility on the urea derivatives in dilute acids, from which they may be recovered unchanged by alkali precipitation. Crystalline urea derivatives of the general formula



were prepared from 2,4-TDI and HMDI using the range of secondary aliphatic amines shown below. The molar absorptivities and wavelengths of maximum absorbance, using dioxan as reference solvent, are shown in Table I.

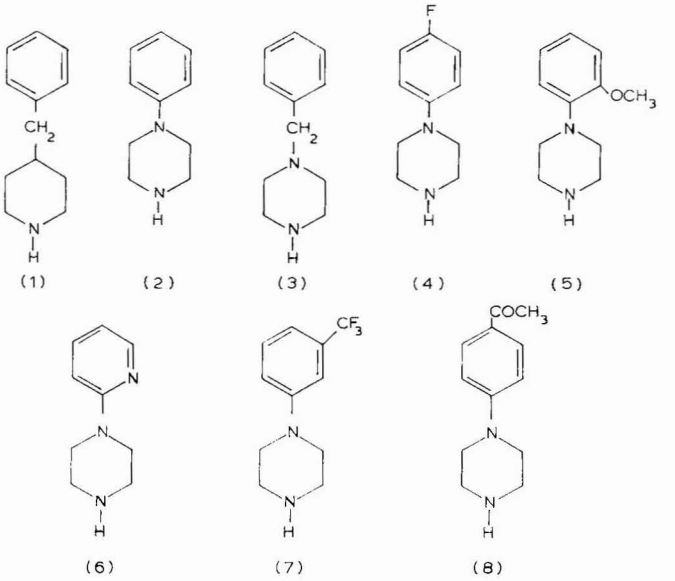


TABLE I

MOLAR ABSORPTIVITIES AND WAVELENGTHS OF MAXIMUM ABSORBANCE FOR ISOCYANATE UREA DERIVATIVES USING DIOXAN AS A REFERENCE SOLVENT

Reagent	*2,4-TDI*		*HMDI*	
	$\lambda_{max.}$ *(nm)*	ε $(l \cdot mol^{-1} \cdot cm^{-1})$	$\lambda_{max.}$ *(nm)*	ε $(l \cdot mol^{-1} \cdot cm^{-1})$
1 4-Benzylpiperidine	247	14 900	260	763
2 1-Phenylpiperazine	249	52 400	250	24 800
3 1-Benzylpiperazine	248	20 300	245	1 100
4 1-(4-Fluorophenyl)piperazine	250	49 500	249	17 300
5 1-(2-Methoxyphenyl)piperazine	247	42 100	255	20 000
6 1-(2-Pyridyl)piperazine	252	60 500	252	34 700
7 N-(α,α,α-Trifluoro-*m*-tolyl)-piperazine	255	51 800	258	26 300
8 4-Piperazinoacetophenone	314	45 900	317	35 900

It can be seen from Table I that 1-(2-pyridyl)piperazine-based urea derivatives are the most suitable for use in the determination of isocyanates by HPLC with UV detection. 1-(2-Pyridyl)piperazine also has high solubility in water, which makes the preparation of the urea standards easier, and the pyridyl ring enables visualisation of the urea derivatives, using HPTLC, with, for example, iodoplatinate reagent. Such a method has recently been developed[33] and provides the occupational hygienist with the facility to determine isocyanate in air concentrations on-site; HPLC analysis in the laboratory can be used to achieve greater precision and further qualitative confirmation when necessary.

Collection efficiency of sampling method

The efficiency of the sampling procedure was examined for a range of standard atmosphere concentrations using three impingers in series in the sampling train. HMDI was selected for this experiment because of its relatively low boiling point compared with those of the other aliphatic isocyanates studied. The results, shown in Table II, indicate that the collection efficiency of one impinger is greater than 90 % for HMDI levels as high as 1600 $\mu g\ m^{-3}$. Similar experiments with TDI, reported earlier[33], showed the collection efficiency of one impinger to be greater than 97 % for TDI levels up to ten times the current threshold limit value ($10 \times 140\ \mu g\ m^{-3}$).

TABLE II

EFFICIENCY OF THE SAMPLING PROCEDURE FOR THE COLLECTION OF HMDI IN $2 \cdot 10^{-4}$ *M* 1-(2-PYRIDYL)PIPERAZINE

Calculated concentration of atmosphere at 20° C⋆		*Mean concentration of isocyanate collected* ($\mu g\ m^{-3}$)			*Efficiency of trap 1* (%)
$\mu g\ m^{-3}$	*ppm*	*Trap 1*	*Trap 2*	*Trap 3*	
146	0.021	143	3	<2	98
170	0.024	150	20	<2	88
173	0.025	170	3	<2	98
173	0.025	170	3	<2	98
198	0.028	190	8	<2	96
233	0.033	221	8	4	95
242	0.035	237	5	<2	98
269	0.038	259	10	<2	96
271	0.039	255	16	<2	94
309	0.044	288	14	7	93
324	0.046	305	19	<2	94
425	0.061	389	36	<2	92
460	0.066	435	18	7	95
492	0.070	462	20	10	94
524	0.075	491	33	<2	94
579	0.083	507	72	<2	88
672	0.096	629	43	<2	94
685	0.098	626	48	11	91
722	0.103	646	60	16	89
761	0.109	667	60	34	88
783	0.112	653	82	48	83
823	0.118	748	48	27	91
979	0.140	938	41	<2	96
1122	0.160	1040	61	21	93
1366	0.195	1278	88	<2	94
1661	0.237	1530	131	<2	92

⋆ Assuming a collection efficiency of 100 % in a 3-impinger sampling system.

Chromatographic conditions

Previous workers have found that the presence of a basic reagent in the reaction mixture to be chromatographed presents certain problems when silica is used as the column packing[34]. The use of nitro reagent, for example, in normal-phase HPLC studies, was reported to produce significant peak tailing and progressive column

degradation, possibly because of the secondary amino group on the nitro reagent, which interacts with the silanol groups of the silica column to produce retardation of the reagent on the column[36]. The presence of excess reagent[27] requires the addition of a suitable reagent scrubber such as *p*-tolyl isocyanate[27] or acetic anhydride[35] to the reaction mixture. In order to overcome this problem several workers[29,37] have successfully investigated the use of reversed-phase HPLC using a bonded octadecylsilyl stationary phase with isocratic pH-buffered elution.

ODS-Hypersil was selected as the most suitable reversed-phase column packing in the present investigation. For all three mobile phases used in the separation of the various urea derivatives excess 1-(2-pyridyl)piperazine is eluted first, and since the elution mode is isocratic, the next analysis can, if required, be started immediately. The reagent as commercially supplied contains the disubstituted piperazine, 1,4-(2,2′-dipyridyl)piperazine, as an impurity. This can be removed by vacuum distillation if desired (boiling point of the reagent is 114°C at 3 Torr), but this was not found to be necessary in this study as the impurity did not interfere with the resolution of the urea derivative peaks.

Fig. 1 shows a chromatogram of the standard urea derivatives of a mixture of the two TDI isomers and PhI with unreacted reagent and the disubstituted piperazine impurity present. Fig. 2 shows a typical chromatogram of a polyurethane foam (TDI-oligomer based) hot-wire cutting process air sample. TDI monomer is a degradation

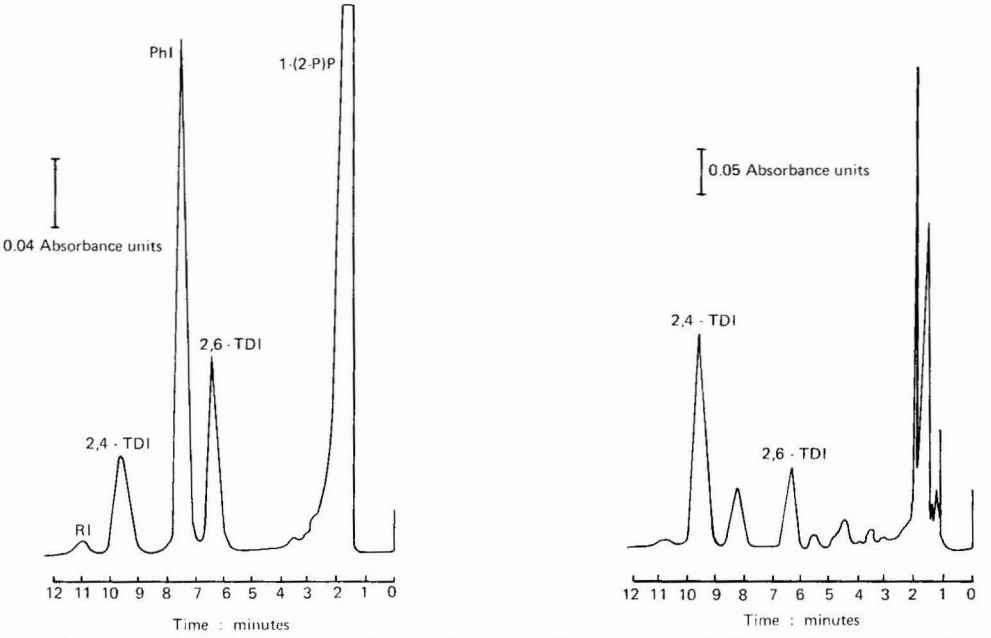
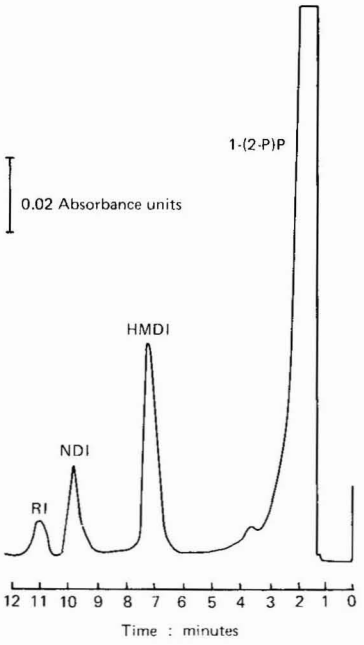


Fig. 1. Chromatogram of 1-(2-pyridyl)piperazine [1-(2-P)P] reagent, 1,4-(2,2′-dipyridyl)piperazine impurity and the PhI and TDI urea derivatives. Conditions: column, 250 × 4.6 mm I.D., ODS-Hypersil; mobile phase, acetonitrile 0.1 *M* aqueous ammonium acetate (31:69), the water phase adjusted to pH 6.2 with acetic acid; temperature, ambient; flow-rate, 2.0 ml/min; sample volume, 10 μl; detection, UV at 254 nm and 0.08 a.u.f.s.

Fig. 2. Chromatogram of a TDI in air sample taken above the hot-wire cutting of a flexible polyurethane foam based on a TDI oligomer. Conditions as in Fig. 1, except: sensitivity, 0.64 a.u.f.s.

product of such a process, and as can be seen from the chromatogram, is well resolved from the other breakdown products. Fig. 3 shows a chromatogram of the HMDI and NDI standard urea derivatives.

By increasing the concentration of acetonitrile in the mobile phase it is possible to elute the MDI and HMDI oligomer urea derivatives within 11 min as shown in Figs. 4 and 5, respectively indicating that the chromatographic system can be used in the analysis of the higher molecular weight isocyanate prepolymers frequently used by industry. The retention times and wavelengths of maximum absorbance for the urea derivatives studied are listed in Table III.



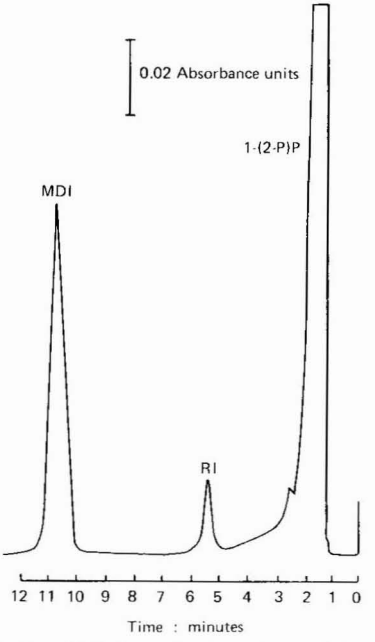


Fig. 3. Chromatogram of 1-(2-pyridyl)piperazine reagent, 1,4-(2,2′-dipyridyl)piperazine impurity and the HMDI and NDI urea derivatives. Conditions as in Fig. 1, except: sensitivity, 0.16 a.u.f.s.

Fig. 4. Chromatogram of the MDI urea derivative together with excess reagent and its impurity. Conditions as in Fig. 1, except: mobile phase, acetonitrile–0.1 *M* aqueous ammonium acetate (40:60), the water phase adjusted to pH 6.2 with acetic acid; sensitivity, 0.16 a.u.f.s.

It can be seen from Table III that the separation of isocyanate mixtures that commonly occur together in certain industrial atmospheres is easily achieved. For example, HMDI oligomer, commonly used in two-pack polyol-cure paint systems, generally contains approximately 0.7% of HMDI monomer. In many cases mixed isocyanates do not occur and it is relatively easy to modify the elution conditions to reduce the retention times; this will additionally give sharper peaks.

Calibration graphs, over the concentration 0–100 ng, were prepared for the isocyanate urea derivatives studied. A linear response was exhibited in each case between the concentration of the urea and its absorbance. A typical calibration graph, for the 2,6-TDI urea, is shown in Fig. 6. Detection limits, based on a signal-to-

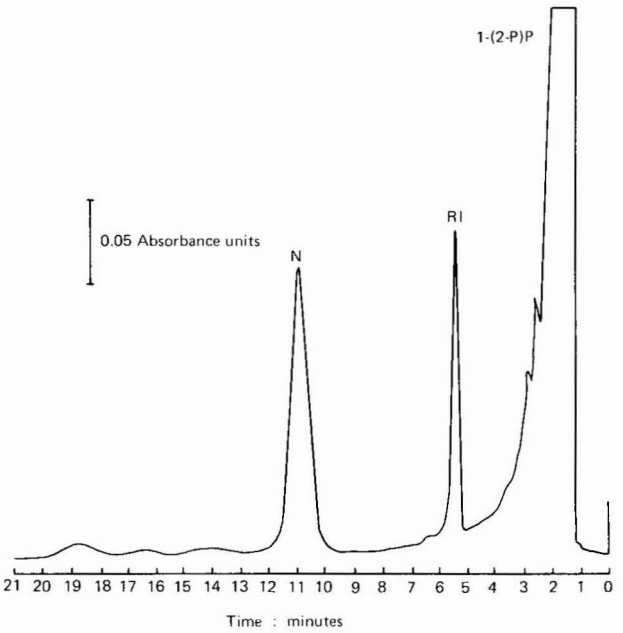


Fig. 5. Chromatogram of the HMDI oligomer (Desmodur N) urea derivative together with excess reagent and its impurity. Conditions as in Fig. 1, except: sensitivity, 0.32 a.u.f.s.

TABLE III

RETENTION TIME DATA AND WAVELENGTHS OF MAXIMUM ABSORBANCE FOR ISOCYANATE UREA DERIVATIVES
See text for description of mobile phases.

Compound	*Mobile phase*	*Retention time (min)*	$\lambda_{max.}$ *(nm)*
1-(2-Pyridyl)piperazine	A	1.3	255
2,6-TDI-urea	A	6.3	252
HMDI-urea	A	7.2	255
PhI-urea	A	7.3	251
2,4-TDI-urea	A	9.5	252
NDI-urea	A	9.7	253
1,4-(2,2-Dipyridyl)piperazine	A	10.9	–
1-(2-Pyridyl)piperazine	B	1.3	255
1,4-(2,2-Dipyridyl)piperazine	B	8.6	–
IPDI-urea	B	12.9 and 23.2	254
1-(2-Pyridyl)piperazine	C	1.3	255
1,4-(2,2-Dipyridyl)piperazine	C	5.5	–
MDI-urea	C	10.9	256
HMDI oligomer urea	C	11.0	253

noise ratio of 3 with the detector set at 0.005 a.u.f.s., are given in terms of free isocyanate in Table IV.

The lower limit may be decreased by taking a larger air sample, and the upper limit increased by using a more concentrated absorbing solution or smaller air sample.

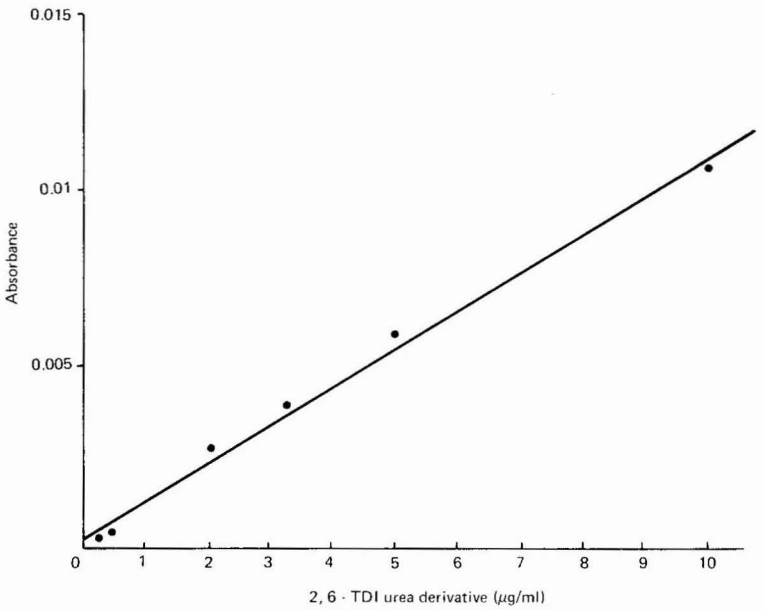


Fig. 6. Calibration curve for the 2,6-TDI urea derivative. Conditions as in Fig. 1.

TABLE IV

ISOCYANATE CONCENTRATION LIMITS

Concentration limits are based on a 10-litre air sample.

Isocyanate	*Lower limit ($\mu g\ m^{-3}$)*	*Upper limit ($mg\ m^{-3}$)*
2,4-TDI	0.5	17
2,6-TDI	0.5	17
MDI	0.5	25
PhI	0.5	21
HMDI	0.7	17
NDI	1.0	21
IPDI	1.5	22
HMDI oligomer	0.75	32

CONCLUSION

A reversed-phase HPLC method has been developed for the determination of atmospheric isocyanate concentrations using 1-(2-pyridyl)piperazine to form urea derivatives which exhibit very high molar absorptivities in the ultraviolet region. The sensitivity of the method is such that as little as 48 ppt of MDI or TDI may be determined in a 10-l air sample, equivalent to one four-hundreth of the current threshold limit values for these compounds[5]. This means that shorter sampling periods can be used giving rise to better hygiene control. In theory, the sensitivity of the method is sufficiently high to omit the concentration step prior to HPLC analysis, and to inject a 100-μl aliquot directly into the chromatograph. However, toluene is not suitable as an

injection solvent and there are problems associated with the use of acetonitrile as the sample absorbing solvent due to its toxic and volatile nature. In addition, we have evidence that the concentration step may be of importance in ensuring that urea formation goes to completion. It has also been shown that impurities in acetonitrile may react with isocyanates.

Unlike the nitro reagent and 9-(N-methylaminomethyl)anthracene, a reagent recently suggested for the HPLC-fluorescence determination of isocyanates[38], 1-(2-pyridyl)piperazine is a stable, high-boiling liquid (boiling point 283°C) requiring no preparation before use. In addition, HPTLC analysis[33] may be carried out on-site prior to HPLC analysis, using 1-(2-pyridyl)piperazine reagent, enabling the occupational hygienist to make an immediate assessment of the extent of any isocyanate hazard that may be present.

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Note

Fraction collector controller for collecting liquid chromatographic peaks

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In the course of our work on drug metabolism, it is often necessary to collect fractions from a liquid chromatographic column. This procedure is used frequently in analytical work when metabolites are collected for further analysis by methods such as high resolution mass spectrometry. Less frequently it is used in large-scale chromatography for the purification of drugs synthesized in the laboratory.

In many cases the peaks which one wishes to collect are not so well separated that conventional timed collections of fractions is satisfactory. Yet many laboratories have excellent fraction collectors which can be made to collect peaks when they are connected to an appropriate peak detecting device. In this communication a device which senses the start of a peak, arranges for peak collection, can accommodate a noisy baseline and corrects for sloping baselines, is described. The approach to the problem was to design a device which performed these functions and also had other useful features. It should be inexpensive, made of commonly available components and simple to construct. The design should be applicable to most detection systems and fraction collectors which are already in use or easily available. The fraction collector controller was built for an LKB fraction collector and a Houston Instruments strip chart recorder which are popular and representative of equipment in the field.

MATERIALS AND METHODS

Materials

The electronic components used are all readily available from electronic supply dealers and from electronic hobby houses. The recorder modifications were made in the machine shop.

Construction

The components were soldered on a printed circuit board which was made in the laboratory. In lieu of a custom-made printed circuit one can use a commercially available multiple-purpose printed circuit board and make the appropriate connections with "jumper" wires. The integrated circuits (IC) were mounted in IC sockets.

The design shown in the circuit diagram of Fig. 1 fulfils our requirements. Its function is more easily comprehended by following the functional block diagram (Fig. 2). The sensing unit consists of integrated circuit 1 (half of a 556) and a slider assembly on the recorder. The slider was a 12 mm long metal cylinder mounted on a

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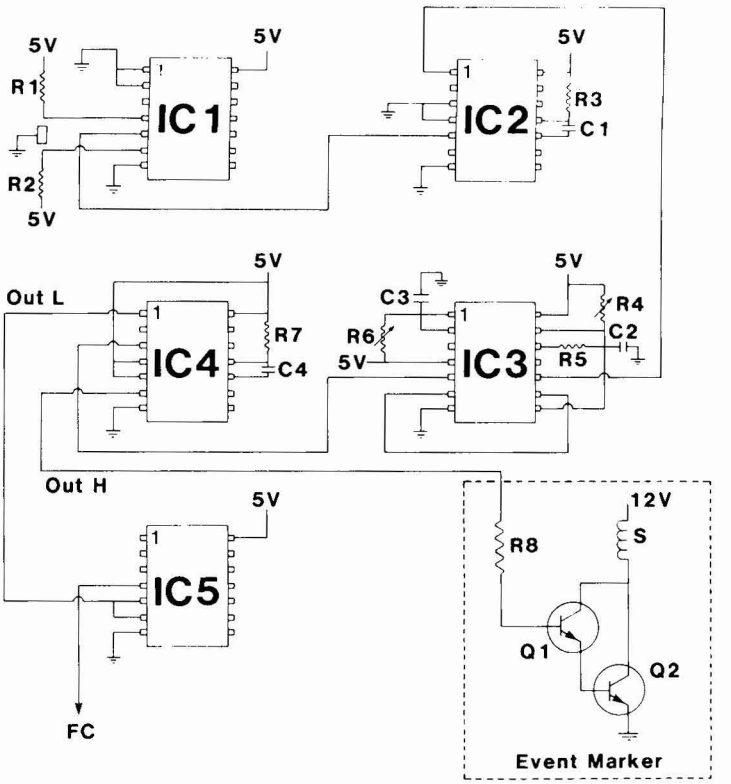

Fig. 1. Circuit diagram. The components were as follows: IC 1 and IC 3, 556 dual timer; IC 2 and IC 4, 74121; IC 5, 7402; Q1 and Q2, 2N2222 transistor; R1, R2 and R7, 10 kΩ; R3, 62 kΩ; R4 and R6, 1 mΩ variable; R5, 56 Ω; R8, 280 Ω; C1 and C4, 10 μF; C2, 330 μF; C3, 33 μF. Out L = output pulse low; Out H = output pulse high; S = event marker solenoid; FC = fraction collector.

Teflon® bushing which fits on the pen support rod of the recorder. An "L" shaped piece of metal (L-bar) was attached to the pen holder so that the slider was pulled or pushed to follow the movement of the pen. The slider was connected to electrical earth and made electrical contact with the rest of the circuit by touching an electrical contact on the L-bar when moving in one direction and on the pen holder when moving in the opposite direction. When contact with the L-bar is made a peak is detected and IC 2 (one-shot) is fired. IC 2 cannot be activated again until after the opposite electrical contact is made and IC 1 is reset by the pen moving downward during the declining phase of the peak. the first half of IC 3 contains a free running timer which fires at a preset frequency (every 5 min) unless it is interrupted. The output from IC 2 interrupts the 5-min timer, causing it to fire when a peak is detected. The output from the 5-min timer initiates a time delay (7 sec) which is contained in the second half of IC 3. This allows time for the column effluent to travel from the detector to the fraction collector. The output from the time delay fires a one-shot pulse (IC 4) which is used to drive the recorder event marker and to step the fraction collector. The circuit to this point is applicable to practically all fraction collectors and recorders. The time between fraction collector steps when no peaks appear can be

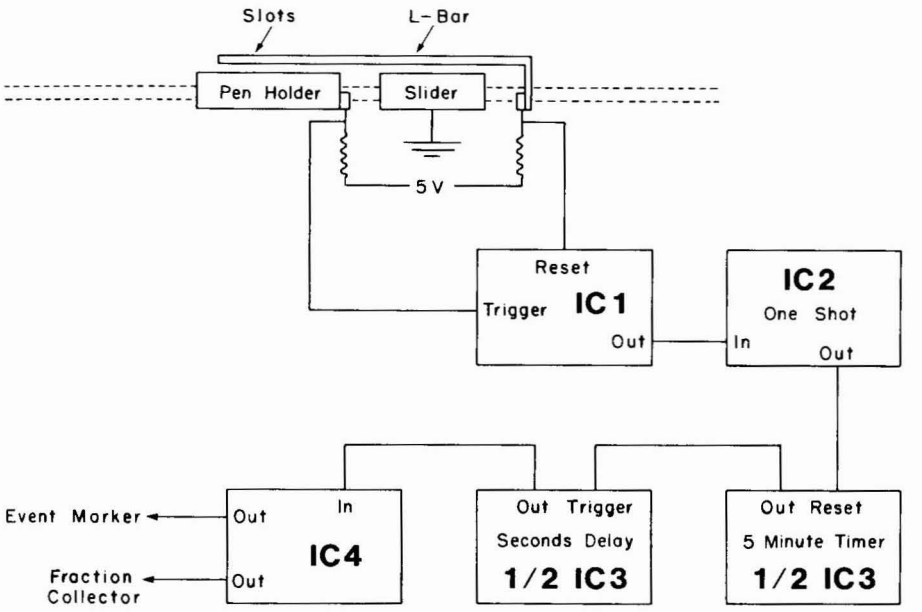

Fig. 2. Function block diagram.

varied by adjusting the resistance at R4. Similarly the time delay is adjusted at R5 and should be set for each chromatographic unit.

The output from IC 4 was not capable of directly driving either the fraction collector or the event marker. Consequently a pair of transistors (2N2222) was used for the event marker and another integrated circuit (7402) was used for the fraction collector. The input to the fraction collector was via the drop counter head; the input line was soldered to the wire which enters the fraction collector at pin 4. The fraction collector was set in the drop-count mode for one drop. This approach leaves the fraction collector essentially unchanged so that it may also be used in its conventional time- or drop-count modes.

To allow for use with noisy baselines the L-bar which pulls the slider was made with a slotted mount. This allows the operator to alter the amount of pen oscillation permitted without triggering the circuit. Note also that as long as the slider has not made both reset and trigger contacts the circuit does not sense a peak. This allows the baseline to drift in either direction without causing false triggering.

Before arriving at this final design, a purely electronic method was tried which would have obviated modifications to the recorder. In this approach the detector output was amplified and put through a first derivative circuit to detect changes in slope; this was abandoned for two reasons. Firstly it was difficult and time consuming to adjust for baseline noise, whereas L-bar adjustment (Fig. 2) is readily made and easy to see. Secondly, the solenoids in the event marker and the fraction collector always produced some electrical noise which was amplified by the derivatizing circuit to give false triggering.

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Note

Use of organic modifiers in the mobile phase for the reversed-phase high-performance liquid chromatographic separation of steroidal hormones

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(Received March 10th, 1981)

There have been several reports of the use of small percentages of various organic compounds in the mobile phases to modify chromatographic selectivity for different polar functional groups in reversed-phase high-performance liquid chromatography (HPLC)[1–7]. In the course of studies on the separation of various steroidal hormones we encountered several mixtures which we were unable to resolve completely by means of binary mixtures of acetonitrile and water or methanol and water on an octylsilane-bonded phase column. A systematic examination of several organic modifiers was initiated to improve the separations. As a result of these studies, several ethers were found to improve the separations significantly and an explanation for these effects was attempted.

EXPERIMENTAL

Reagents and materials

Estradiol-17α, estradiol-17β and estrone were purchased from Steraloids (Wilton, NH, U.S.A.). Testosterone, progesterone, estriol, mestranol, ethinylestradiol and norethindrone were products of Syntex (Palo Alto, CA, U.S.A.). Methanol, acetonitrile (UV grade), tetrahydrofuran (UV grade) and diethyl ether (distilled-in-glass grade), were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). 1,4-Dioxane, 1,2-dimethoxyethane, diisopropyl ether and cyclohexene oxide were purchased from Fisher (Pittsburgh, PA, U.S.A.), MCB (Cincinnati, OH, U.S.A.), Mallinckrodt (St. Louis, MO, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.), respectively.

Apparatus

All HPLC separations were performed with Waters Assoc. (Milford, MA, U.S.A.) equipment. The solvent delivery system consisted of a Model 6000A and two Model 45 pumping systems. Either a Model U6K universal injector or a Model 710B sample processor was used for the injection of samples. The detector was a Model 450 variable-wavelength UV monitor. The flow-rate and solvent composition were controlled by a Model 720 system controller. Peak heights, peak areas and retention times were measured by means of a Model 730 data module. The columns used were

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prepacked with 10-μm or 5-μm LiChrosorb RP-8, containing covalently bonded octylsilane functions (Rheodyne, Cotati, CA, U.S.A.).

High-performance liquid chromatography

Binary solvent mixtures of water–acetonitrile and water–methanol and ternary mixtures of these systems with ethers as modifiers were used as the mobile phases. Mobile phases were degassed in an ultrasonic bath for 30 min. The flow-rates used were 2 ml/min for the 10-μm RP-8 column and 1 ml/min for the 5-μm RP-8 column. Further details are given in the legends to the figures.

RESULTS AND DISCUSSION

Separation of a mixture of estrone, estradiol-17α and estradiol-17β by use of an acetonitrile–water system with or without diethyl ether as a modifier

The conditions employed for this comparison and the results obtained are summarized in Fig. 1. Addition of ether shortened the retention times, improved the resolution, and sharpened the peaks, thus increasing the sensitivity. Without ether, the separation of the epimeric estradiols was incomplete.

Effect of diethyl ether concentration on the elution volumes for estrone, estradiol-17α, estradiol-17β, testosterone and progesterone

The results of this study are summarized in Fig. 2. Elution volumes, expressed as a capacity ratio (k'), are plotted as a function of the volume percent of diethyl ether

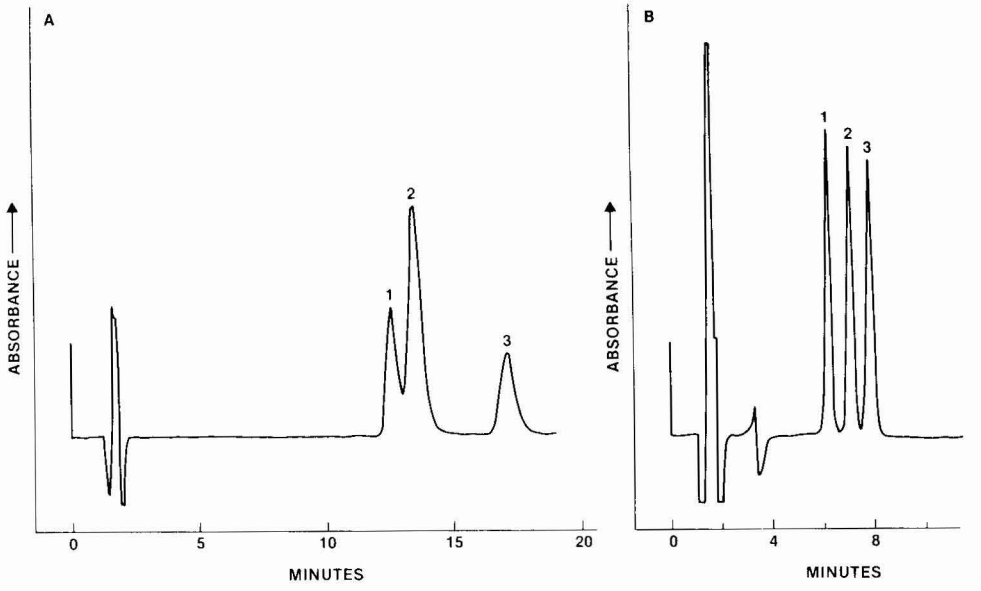

Fig. 1. HPLC of three estrogens with binary and ternary solvent systems. (A) Mobile phase: acetonitrile–water (35:65); (B) Mobile phase: (acetonitrile–water, 35:65)–diethyl ether (90:10, v/v). Column: LiChrosorb RP-8, 10 μm, 25 cm × 4.6 mm I.D.; flow-rate: 2.0 ml/min, pressure: 700 p.s.i.; UV detection at 280 nm, 0.04 a.u.f.s. Peaks: 1 = estradiol-17β; 2 = estradiol-17α; 3 = estrone.

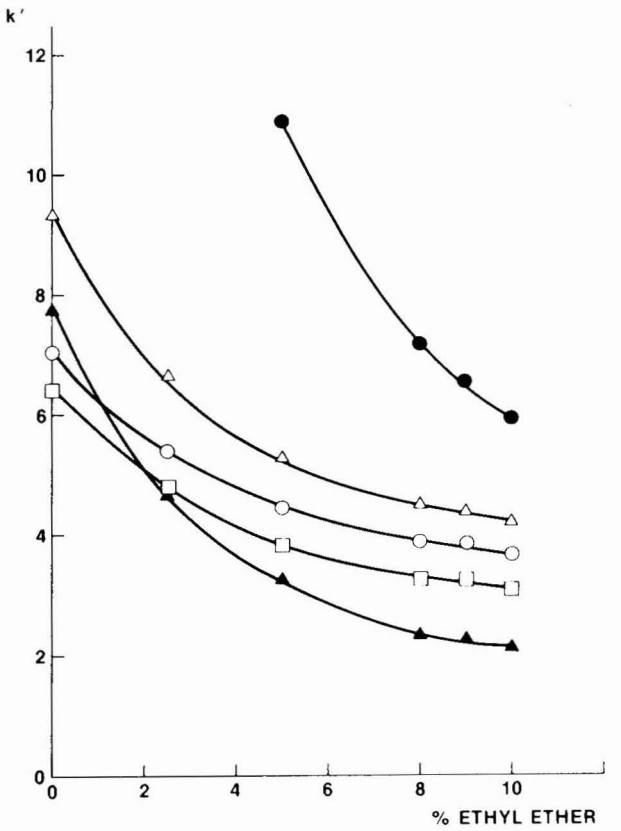

Fig. 2. k' Values of estrogens, testosterone and progesterone as a function of diethyl ether content of the mobile phase. Curves: ▲ = testosterone; □ = estradiol-17β; ○ = estradiol-17α; △ = estrone; ● = progesterone. Column: LiChrosorb RP-8, 10 μm, 25 cm × 4.6 mm I.D.; Solvent system: acetonitrile–water (35:65, v/v) plus diethyl ether.

in the mobile phase. In each case, lower values of k' were obtained when the concentration of diethyl ether was increased. However, the magnitude of the effect was different for different functional groups[1,2]. The retention times of α,β-unsaturated ketonic steroids were more drastically shortened.

Use of diethyl ether and tetrahydrofuran in the separation of ketonic from non-ketonic steroids

In Fig. 3 are plotted the k' values for 17α-ethinylestradiol, mestranol, norethindrone and progesterone as a function of the concentration of acetonitrile in water. Based on the closeness of the k' values for norethindrone and 17α-ethinylestradiol at all concentrations of acetonitrile, it is apparent that this binary system is unsuitable for the separation of these steroids. However, the addition of 10% (v/v) diethyl ether to the eluent containing 40% acetonitrile in water resulted in a lowering of the k' value for norethindrone in comparison with that obtained for 17α-ethinylestradiol and an improvement of the separation. Similarly, the addition of 10% (v/v)

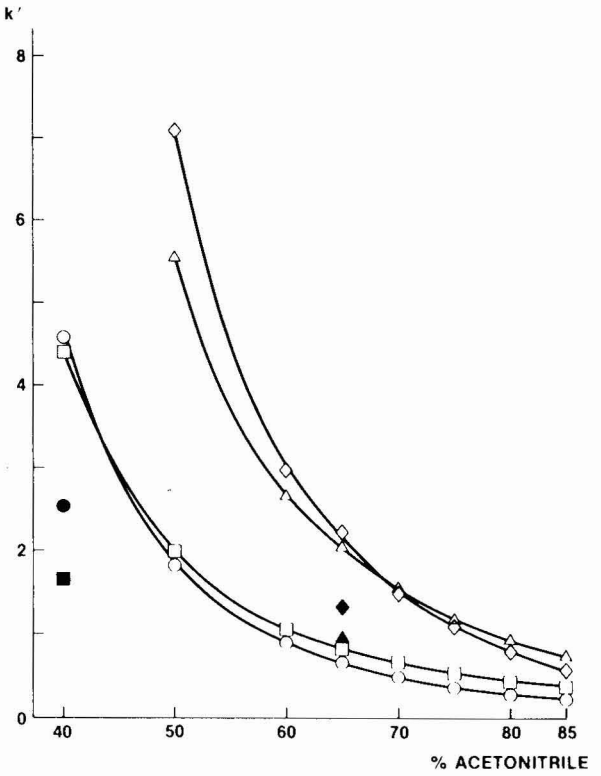

Fig. 3. k' Values of four steroids in binary and ternary solvent systems. Ethinylestradiol (○), norethindrone (□), mestranol (◇), and progesterone (△) were eluted with binary solvent systems of acetonitrile in water, as shown on the horizontal scale. Ethinylestradiol (●) and norethindrone (■) were eluted with a ternary solvent system of (acetonitrile–water, 40:60)-diethyl ether (90:10, v/v). Mestranol (◆) and progesterone (▲) were eluted with a ternary solvent system of (acetonitrile–water, 65:35)–tetrahydrofuran (90:10, v/v). Column: LiChrosorb RP-8, 5 μm, 25 cm × 4.6 mm I.D.

tetrahydrofuran to the eluent containing 65% acetonitrile provided a differential lowering of the k' values for mestranol and progesterone and allowed them to be separated. Although progesterone and mestranol could be separated from one another with a binary system at a lower concentration of acetonitrile, the presence of tetrahydrofuran reduced the elution volumes by at least a factor of 2 and shortened the time required for separation accordingly.

Selectivity effects for steroids of several organic modifiers with ether linkages

Since diethyl ether and tetrahydrofuran had significant effects on the retention behavior of various steroids and these modifiers both contain an ether linkage in their structures, the selectivity effects of other organic modifiers with ether linkages were investigated.

It is evident from earlier reports[8,9] and from our own results that the order of elution of the steroids used in this study depends on whether aqueous acetonitrile or methanol is used as the eluent in reversed-phase HPLC. For example, estradiol-17α

TABLE I

CAPACITY FACTORS (k') AND SELECTIVITY FACTORS (α) OF STEROIDS FOR DIFFERENT ORGANIC MODIFIERS WITH ETHER LINKAGES

	Steroids	*Water–methanol (45:55, v/v)*	*Ternary mixture of water–methanol (40.5:49.5, v/v) with 10 parts of*					*Water–methanol–cyclohexene oxide (44.1:53.9:2, v/v/v)*
			Diethyl ether	*Tetra-hydrofuran*	*Diisopropyl ether*	*1,2-Dimethoxy-ethane*	*1,4-Dioxane*	
Part A: k' values	Estrone	7.88	1.94	2.32	1.79	3.49	3.53	5.27
	Estradiol-17α	9.27	2.19	2.82	1.87	3.91	3.91	6.35
	Estradiol-17β	9.35	2.06	2.65	1.59	3.79	3.77	6.06
	Testosterone	11.3	1.59	2.09	0.56	4.12	4.53	5.47
	Progesterone	23.9	2.97	4.0	1.42	7.82	9.0	10.88
	Norethindrone (NET)	8.65	1.47	1.92	0.52	3.32	3.71	4.59
	17α-Ethinylestradiol (EE)	9.29	2.18	2.82	1.95	3.88	3.82	6.5
Part B: α values	α for NET and EE	1.07	1.48	1.47	3.75	1.17	1.03	1.42

and estradiol-17β are eluted before estrone by the eluent containing acetonitrile and after estrone by the eluent containing methanol. In addition, the order of elution of the two epimeric estradiols is reversed in these systems. However, when various modifiers containing ether linkages are added to both binary systems, the selectivity effect for polar functional groups is similar.

Table I shows the changes in elution volumes resulting from the addition of different ethers to aqueous methanol. Significant changes in elution order are observed. The elution volumes of all steroids are decreased by the use of an ether as a modifier. However, in systems containing diethyl ether, tetrahydrofuran, diisopropyl ether or cyclohexene oxide, the magnitude of the decreases in the elution volumes for steroids with a phenolic structure in the A ring (estrone, estradiol-17α, estradiol-17β and 17α-ethinylestradiol) is smaller than that for steroids containing an α,β-unsaturated ketone in the A ring (testosterone, progesterone and norethindrone). Similar results were obtained (Figs. 2 and 3) with the system containing acetonitrile. An example of polar functional group selectivity is shown in Part B of Table I. The values of the selectivity factor (α) for 17α-ethinylestradiol to norethindrone are significantly larger in the systems containing these monoether modifiers. These two steroids differ only in the A ring which is phenolic in the former and contains an unsaturated ketone in the latter. The role of organic modifiers on the retention mechanism in reversed-phase HPLC has previously been investigated by McCormick and Karger[10,11]. They indicated that polar group selectivities of solutes could in large part be rationalized on the basis of specific solute–modifier interactions in the stationary phase. The greater retardation of phenolic solutes may result from hydrogen bonding to the ethers which are partially extracted by the stationary phase. On a mole-percent basis in the mobile phase, diisopropyl ether, which is more hydrophobic than diethyl ether or tetrahydrofuran, provides the largest selectivity effect of the ethers. A large selectivity effect was also obtained for the relatively hydrophobic epoxide, cyclohexene oxide, which was tested at a concentration of only 2% (v/v) in the mobile phase. The modifiers containing two ether linkages, such as 1,2-dimethoxy ethane and 1,4-dioxane, do not show the differential effect for phenolic and ketonic steroids (Table I). The reduced hydrophobicity in these modifiers compared to monoethers could be responsible for the decreased effect, since it is reasonable that the concentration of these extracted modifiers in the non-polar stationary phase would be lower[10]. If this interpretation is correct, then a good modifier for mobile phases in reversed-phase HPLC must be a compound with a strong hydrophobic region to insure that it is adequately sorbed by the hydrophobic bonded phase, but it must also contain a polar region (such as an ether linkage), which can interact selectively (probably via hydrogen bonding) with certain compounds in the mixture to be separated.

Complete separation of a mixture of testosterone, estriol, estradiol-17β, estradiol-17α, estrone and progesterone with an eluent containing acetonitrile, water and diethyl ether

The conditions used and the separation obtained are shown in Fig. 4. In the absence of diethyl ether, the elution volume for progesterone was excessive and the separation of testosterone from estradiol-17α was marginal. The addition of diethyl ether to the binary mobile phase of water-acetonitrile changed the elution order of these steroids in a way that permitted rapid baseline separation of this complex mixture of steroids.

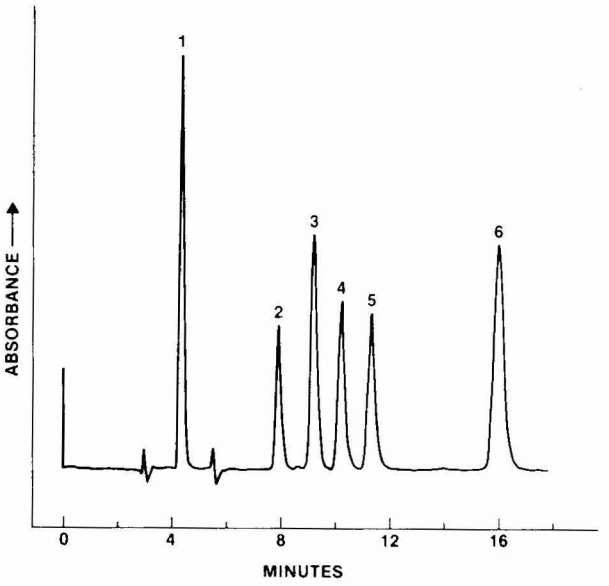

Fig. 4. HPLC separation of estrogens, testosterone and progesterone. Peaks: 1 = estriol; 2 = testosterone; 3 = estradiol-17β; 4 = estradiol-17α; 5 = estrone; 6 = progesterone. Column: LiChrosorb RP-8, 5 μm, 25 cm × 4.6 mm I.D.; Solvent system: (acetonitrile–water, 40:60)–diethyl ether (90:10, v/v); flow-rate: 1.0 ml/min; pressure: 2100 p.s.i.; UV detection at 270 nm, 0.04 a.u.f.s.

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Note

Detection of brominated and chlorinated organics by a gas chromatographic microcoulometric detector

Effect of pyrolysis tube conditions

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(Received April 8th, 1981)

The specific detection of halogenated organics is of considerable interest to environmental scientists. Low levels of organochlorine pesticides, polychlorinated biphenyls (PCBs), and polybrominated biphenyls (PBBs) entering the environment are important because of their refractory nature and potential for bioaccumulation. Low levels of some other halogenated compounds, such as haloforms, which are produced during the chlorine disinfection of water[1–3] must be monitored because they may be directly ingested by humans.

Gas chromatography (GC) with an electron-capture detector (ECD) is often used for the analysis of haloforms extracted from waters[4,5], as well as organochlorine pesticides, PCB, and PBB residues[6]. The ECD is used because of its sensitivity for halogenated compounds and its wide availability. The ECD has some disadvantages including: a lack of specificity; widely varying sensitivity with degree of chlorination, *e.g.* the ECD is 10^4 times more sensitive to $CHCl_3$ than to CH_3Cl (ref. 7); and sensitivity which varies with positional isomerism, *e.g.* different sensitivity for PCB isomers having the same degree of chlorination[8]. Microcoulometric detection does not have these disadvantages.

The microcoulometric detector responds to the weight of halogen present. Through oxidative pyrolysis the material is converted to titratable halides which are swept into the microcoulometric detector cell where the halides react with the silver ions present in the cell electrolyte. The efficiency of this pyrolysis process varies with compound, pyrolysis temperature, and furnace atmosphere. The coulometer produces a voltage proportional to the current required to replace the consumed silver ions. The detector has a selectivity of 10^6 for chlorine over carbon and of 10^4 for chlorine over sulfur, nitrogen and phosphorus[9]. Microcoulometric detection has been used to measure total organic halogen as a water quality parameter using a system with a sample boat for sample introduction[10].

In this paper the use of a commercially available microcoulometric detector specifically designed as a GC detector, the Envirotech (Santa Clara, CA, U.S.A.)

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Dohrmann DE-20, is discussed. Pyrolysis tube conditions were varied and the effect on the detection of certain halogenated organics including chloroform, bromoform, and halogenated benzenes was examined. At a pyrolysis tube temperature of 925°C, the analyses of four PBBs were compared.

EXPERIMENTAL

Solutions for microcoulometric detection

To avoid carbonaceous build-up in the pyrolysis tube, the solvent used to introduce the halogenated compounds into the GC system must be vented. Pentane was found to be a suitable solvent for the aliphatic halogens and halogenated benzenes studied. Hexane was utilized for the PBBs examined. Table I lists the columns and temperature programs utilized.

TABLE I

GC COLUMNS AND TEMPERATURE PROGRAMS UTILIZED FOR MICROCOULOMETRIC DETECTION

Compounds	*Retention time (min)*	*Column and temperature program*
Chloroform	4.8	4-ft. 10% Carbowax 20M column.
1,2-Dichloroethane	6.5	Isothermal at 75°C for 8 min,
Dibromethane	11.5	then 5°C/min to 130°C.
Bromoform	19.5	
Chlorobenzene	2.5	6-ft. 3%SE-30 column.
Bromobenzene	4.1	Isothermal at 68°C for 6 min,
1,2,4-Trichlorobenzene	11.4	then 10°C/min to 150°C.
1,2,4-Tribromobenzene	14.6	
2,4-Dibromobiphenyl	2.5	6-ft. 3% SE-30 column.
2,4′,5-Tribromobiphenyl	5.5	Isothermal at 220°C for 7 min,
2,2′,5,5′-Tetrabromobiphenyl	8.0	then 15°C/min to 290°C.
2,2′,4,4′,5,5′-Hexabromobiphenyl	15.4	

Three solutions for recovery studies with microcoulometric detection were prepared, each containing four structurally related compounds. Microliter quantities of $CHCl_3$, $CHBr_3$, $ClCH_2CH_2Cl$, and CH_2Br_2 were injected with a Hamilton 10-μl syringe into 10 ml of pentane. This stock solution was then diluted 1 to 50 to achieve the desired concentration for microcoulometric detection. The solution concentration was calculated from the density of the individual components. A solution containing three halogenated benzenes was prepared in the same manner; the fourth component, a tribromobenzene, was weighed on a microbalance. Four stock PBB standards (2,4-dibromo-, 2,4′,5-tribromo-, 2,2′,5,5′-tetrabromo-, and 2,2′,4,4′,5,5′-hexabromobiphenyl obtained from RFR, Hope, RI, U.S.A.) were prepared by weighing mg quantities and transferring to 100-ml flasks with hexane. The final PBB solution was prepared by mixing 2 ml each of the four PBB stock solutions.

Instrumental

An Envirotech DE-20 halogen specific microcoulometric GC detector was adapted to a Microtek MT-220 GC. The effluent from the GC column flows through a transfer line (wrapped in heating tape and kept at the maximum column temperature) to a pyrolysis tube in a furnace which is at 820°C or higher. As the GC effluent enters the pyrolysis tube it is joined by a 120 ml/min flow of oxygen. The halogenated species are converted to titratable halides and swept into the miniaturized microcoulometric cell.

A Texas Instruments strip chart recorder was used to record the voltage produced by the coulometer. A compensating polar planimeter (K & E) was used to measure the areas of the GC peaks for conversion to ng halide as follows:

$$\text{halide (ng)} = \frac{\text{GC peak area (coulombs)} \times 10^9 \times \text{atomic wt. of halide}}{96500 \text{ (faraday)}}$$

RESULTS AND DISCUSSION

Table II gives the results of triplicate injections of the solution containing $CHCl_3$, $ClCH_2CH_2Cl$, CH_2Br, and $CHBr_3$. Three pyrolysis tube temperatures, the lowest being the manufacturers recommended value of 820°C, were used. The % recoveries were determined as 100 × the ratio of the amount of halide represented by the GC peak area to the amount of halide equivalent to complete conversion of the organic halogen compound to titratable halides. For $CHCl_3$ and $ClCH_2CH_2Cl$ with oxygen pyrolysis gas the recovery could be increased by increasing the pyrolysis tube temperature. More complete pyrolysis to titratable halide probably results at the high

TABLE II

MICROCOULOMETRIC DETECTION OF $CHCl_3$, $ClCH_2CH_2Cl$, $CHBr_3$, CH_2Br_2 WITH OXYGEN OR CARBON DIOXIDE REACTANT

Reactant gas	*Pyrolysis tube temperature (°C)**	*Recovery (%) ± S.D. (n=3)*			
		*$CHCl_3$ (66 ng Cl^-)***	*$ClCH_2CH_2Cl$ (53 ng Cl^-)***	*$CHBr_3$ (137 ng Br^-)***	*CH_2Br_2 (138 ng Br^-)***
O_2	820	42 ± 1.5	41 ± 2.1	82 ± 2.6	77 ± 1.7
	920	47 ± 1.2	73 ± 1.5	55 ± 3.6	53 ± 4.2
	1020	47 ± 1.0	64 ± 2.5	39 ± 4.7	43 ± 2.1
CO_2	820	40 ± 2.0	45 ± 5.0	68 ± 3.8	79 ± 4.5
	920	48 ± 2.3	76 ± 1.5	82 ± 2.3	91 ± 2.3
	1020	66 ± 3.0	92 ± 3.5	81 ± 2.9	87 ± 0.6

* Deviation from specified temperatures ± 10°C.

** Amount of chloride or bromide equivalent to complete conversion of organic halogen compound to titratable halides.

temperatures. For $CHBr_3$ and CH_2Br_2 the opposite effect occurs. The recovery decreases with increased pyrolysis tube temperature (see Fig. 1). The brominated compounds may be converted to a nontitratable species such as bromate (BrO_3). Using carbon dioxide as the pyrolysis gas, the pyrolysis tube temperature could be increased without decreasing the recovery of brominated compounds. Comparable recoveries for the chlorinated compounds resulted with carbon dioxide as pyrolysis gas.

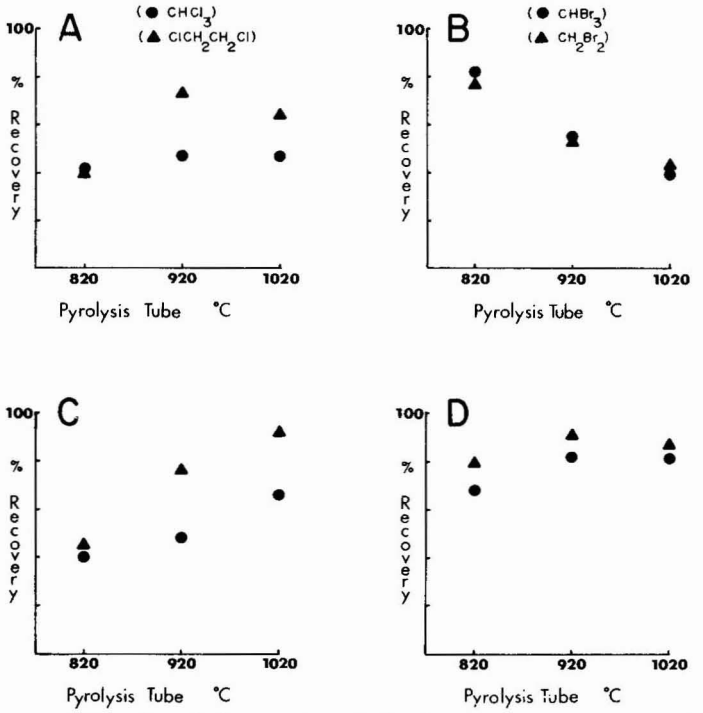

Fig. 1. Effect of pyrolysis gas and pyrolysis tube temperature on recovery. Chlorinated compounds A and C; ● $CHCl_3$, ▲ $ClCH_2CH_2Cl$. Brominated compounds B and D; ● $CHBr_3$, ▲ CH_2Br_2. A and B: Oxygen reactant gas; C and D: carbon dioxide reactant gas.

With oxygen as the pyrolysis gas, halogenated benzenes showed very poor recovery at 820°C (Table III). For both chlorinated and brominated benzenes, the recoveries increased with higher pyrolysis tube temperatures. The aromatic carbon–halogen bond is stronger than the aliphatic carbon–halogen bond. For example, the C–Br bond energy of bromobenzene is 71 kcal/mole; and the C–Br bond energy of ethyl bromide is 65 kcal/mole[11]. As a result, it would be expected that pyrolysis of the aromatic compounds require higher temperatures. Recovery of the bromobenzenes, as would be expected from the weaker C–halogen bond was better than recovery of the chlorobenzenes (C–Cl, 86 kcal/mole). Recoveries similar to the brominated benzenes were found (Table IV) for four PBB isomers (2–6 bromine atoms) using oxygen pyrolysis gas and a 925°C pyrolysis tube temperature.

Differences in thermal stability have been utilized to selectively detect chlorinated pesticides in the presence of PCBs with a Coulson electrolytic conductivity detector in the reductive mode[12,13]. It appears that the same sort of selective detection

TABLE III

MICROCOULOMETRIC DETECTION OF HALOGENATED BENZENES WITH OXYGEN REACTANT

*Pyrolysis tube temperature (°C)**	*Recovery (%) ± S.D. (n=3)*			
	*Chlorobenzene (87 ng Cl^-)***	*Bromobenzene (136 ng Br^-)***	*1,2,4-Trichlorobenzene (86 ng Cl^-)***	*1,2,4-Tribromobenzene (175 ng Br^-)***
820	< 5	< 5	< 5	< 5
870	14 ± 0.6	16 ± 1.2	3 ± 1.2	10 ± 2.5
920	28 ± 3.6	47 ± 5.7	18 ± 3.2	40 ± 2.9
1020	26 ± 2.1	50 ± 1.0	26 ± 1.7	47 ± 4.4

* Deviation from specified temperatures ± 10°C.

** Amount of chloride or bromide equivalent to complete conversion of organic halogen compound to titratable halides.

could be accomplished with the microcoulometric detector in the oxidative mode. The results with $CHBr_3$ and CH_2Br_2 point out, however, that if high pyrolysis tube temperatures are used to screen for all halogenated organics, there will be a tendency to discriminate against the aliphatic brominated organics. On the basis of this limited study, it is evident that to achieve maximum sensitivity by microcoulometric detection, the pyrolysis process must be optimized and for quantitation, standards must be used to determine the recovery for each compound.

TABLE IV

MICROCOULOMETRIC DETECTION OF PBBs WITH OXYGEN REACTANT

Pyrolysis tube temperature 925 ± 7°C.

Isomer	*Amount**	*Recovery of PBB (%) ± S.D. (n=3)*
2,4-Dibromobiphenyl	73.4 ng Br^-	43 ± 7.8
2,4′,5-Tribromobiphenyl	49.5 ng Br^-	34 ± 4.0
2,2′,5,5′-Tetrabromobiphenyl	69.8 ng Br^-	35 ± 6.8
2,2′,4,4′,5,5′-Hexabromobiphenyl	123.6 ng Br^-	44 ± 7.8

* Amount of bromide equivalent to complete conversion of the PBB to titratable bromide.

ACKNOWLEDGEMENT

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Note

Gas chromatographic determination of water in acetone

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In a study designed to develop a continuous process[1] for obtaining a cocoa butter-like fraction from beef tallow by acetone fractionation, the water content in the acetone was critical[2]. Therefore, monitoring the water content of acetone used in all stages of this experimental process was necessary. A quantitative method was required that was both rapid and reliable.

The standard Karl Fischer titration for determining water was not suitable because of ketonic interference. Even an alternate procedure of substituting freshly distilled pyridine for methanol as the solvent permitted only the approximate determination of small amounts of water in carbonyl compounds[3].

Other methods reported in the literature for determining water in liquids utilize gas chromatographic (GC) separation with thermal conductivity detection. Smith[4], probably one of the earliest researchers to publish GC data on the quantitative determination of water in an organic system, used three methods of quantitation, namely: internal normalization, calibrated standard curve, and an internal standard method. Bennet[5], in a further study of GC separation of aqueous organic solutions, measured peak height and interpreted the percent water from a corresponding calibration curve. However, because of possible changes in the column or detector, the calibration curve had to be reestablished periodically. Hogan *et al.*[6] used an internal standard technique employing methanol as the standard to eliminate the need for daily area calibrations. A shortcoming of this procedure was the necessity to measure the water content of the internal standard prior to addition to the samples.

MacDonald and Brady[7] combined the method of standard addition with gas chromatography to yield a technique that was superior to those previously available. However, when we employed this method in our study, we encountered difficulties. For example, water peaks were not reproducible because of moderate tailing, thus resulting in poor quantitation.

We modified MacDonald and Brady's method to permit analysis of a wider range of water contents (0.2–4.0%) in acetone. The changes include column packing, method of sample preparation, adjustment of sample size, and the use of a Chaney adaptor to insure injection of a constant volume of sample.

EXPERIMENTAL*

Apparatus

A Varian Aerograph chromatograph Model 1520 C with thermal conductivity detector (TCD) set at 200°C and 125 mA was used. The separating column was 6 ft. × 1/8 in. stainless steel packed with Chromosorb 102 (Johns-Manville), 80–100 mesh. Operating conditions were as follows: column, 150°C; injection port, 175°C; helium carrier gas flow-rate, 40 ml/min; sample size, 0.5 μl. An Infotronics Model CRS 100 integrator with automatic attenuation set at × 10 was used to integrate all peak areas.

Procedure

Sample preparation by the method of standard addition was as follows. Approximately 20.0 g of acetone was weighed into a tared 25-ml volumetric flask fitted with snap-cap closure. To this, varying weights of water (100 to 300 mg) were added with a weighing buret, and the solution was thoroughly mixed. In general, at least two separate weights of water were added to each acetone sample.

Initially, 0.5 μl of "neat" acetone was injected into the chromatograph with a 1.0-μl Hamilton syringe fitted with a Chaney adaptor. The retention time and area of the water peak were measured. Then, 0.5 μl of "addition" solution was injected, and the new area of the water peak was determined. Injections were repeated in this manner a minimum of four times, and the mean area value of the water peak was calculated. These values were used in calculating the weight of water in acetone.

Calculation

The weight percent of water in acetone was calculated by the following rearrangement of MacDonald and Brady's equation[7]:

$$M = \frac{(W_a \times A_a) \times (W_s - W_a)}{(W_s \times A_s) - (W_a \times A_a)}$$

where

M = weight of water in acetone
W_a = weight of acetone before standard addition
W_s = weight of acetone after standard addition
A_a = mean area of water peak before standard addition
A_s = mean area of water peak after standard addition

When M and W_a are known, the water content (%, w/w) = $(M/W_a) \times 100\%$

RESULTS AND DISCUSSION

Initially, Chromosorb 104 (an acrylonitrile–divinylbenzene polymer) was used as recommended by MacDonald and Brady. Moderate tailing of the water peak resulted in poor quantitation.

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Peak tailing is indicative of hydrogen bonding of water to the solid support of the column packing or to the column tubing itself. Tailing also may be caused by an overload of the thermal conductivity detector due to injection of excess sample volume. All three conditions will result in poor peak area reproducibility, thereby affecting the accuracy and precision of the method.

To minimize the adsorption effects, the stainless steel column was silanized before being packed. It has also been reported that inert porous polymer packings, such as the Chromosorb Century Series, have unique selectivities which depend on the functional groups chemically bound to the cross-linked polymer[8]. For example, Chromosorb 104 appears to be more suited for separating trace amounts of water (0.1 %) as demonstrated by MacDonald and Brady, whereas Chromosorb 102 (a styrene–divinylbenzene polymer) is more efficient for separating of higher concentrations of water (4.0 %) in acetone. For this reason, Chromosorb 102 was used in the present study. Because of the wide range (0.20–4.0 %) of water concentrations encountered it was observed that a 10-μl sample, as recommended by MacDonald and Brady, caused a TCD overload as manifested by a slow return of the pen to baseline. A study of the effect of sample volume indicated that a 0.5-μl aliquot gave a satisfactory separation of the two peaks (Fig. 1) and a consistent retention time for the water peak over the range of concentrations encountered. The method was further refined by the use of a 1.0-μl Hamilton syringe fitted with a Chaney adaptor and the technique of direct on-column injection of the sample.

These refinements permitted several grades of acetone as well as acetone cuts from various stages of the continuous extraction procedure to be analyzed. Table I includes quantitative data from three types of samples representative of all analyses made in this study.

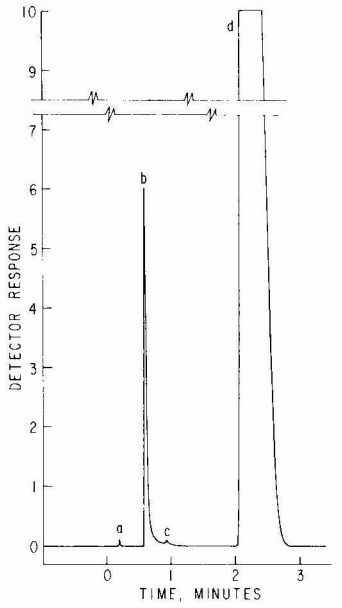

Fig. 1. Typical chromatogram showing the separation of a 2 % water in acetone solution. Peaks: a = air; b = water; c = impurity; d = acetone.

TABLE I

EXPERIMENTAL DATA AND ANALYTICAL RESULTS OF ACETONE ANALYSES

Sample	*Water added (g)*	*n*	*Area of water peaks (mV sec)*		*Water content (%)*	*C.V. (%)*
			Neat	*After addition*		
ACS-grade acetone	0.20707	5	4707	26,510	0.219	2.58
	0.20186	5	4707	25,136	0.226	0.94
Feed acetone	0.20109	5	12,883	15,716	4.19	2.01
	0.28471	4	12,883	17,316	3.92	1.20
Recycled acetone	0.18827	6	13,876	18,587	2.41	4.08
	0.10672	4	13,279	16,197	2.35	3.94

The first sample was the ACS- or reagent-grade acetone which had been used in the start-up of the continuous process for extraction of animal fats. It had an average mean area for the water peak of "neat" acetone of 4707 mV sec with an average standard deviation of 105 mV sec. After two separate additions of water to this acetone, the water peak areas averaged 26,510 and 25,136 mV sec with standard deviations between replicates of 583 and 195 mV sec, respectively. The water concentration of this sample as determined by the method of standard addition and calculated by the formula previously presented averaged 0.223%, having standard deviations of 0.006 and 0.002, respectively, for the two weights. The coefficient of variation (C.V.) averaged 1.75%.

The second sample, identified as feed acetone, also had been used in the experimental tallow fractionation pilot plant study. This sample came from the evaporator used to recover acetone from the fat for recycling. (The acetone must be dried before being recycled to the process.) A sample of this acetone was analyzed for water content. It had an average peak area of 12,883 mV sec for "neat" acetone with a standard deviation of 38 mV sec between replicates. Area counts of 15,716 and 17,316 mV sec were obtained after two separate additions of water to the feed acetone. The standard deviations of these data were 61 and 58 mV sec, and the calculated water content averaged 4.19 and 3.92%, respectively. The standard deviations of these data were 0.08 and 0.05, respectively, with an average C.V. of 1.60%.

The third sample shown is a recycled acetone, also from the tallow fractionation studies. This acetone, recovered from the evaporator, was partially dried by being passed through molecular sieves prior to GC analysis. The sample had averaged peak areas for "neat" acetone of 13,876 and 13,279 mV sec with standard deviations of 214 and 45 mV sec for 6 and 4 replicates, respectively. After two separate additions of water the area water peaks averaged 18,587 and 16,197 mV sec, respectively, with standard deviations of 195 and 120 mV sec. These data were used to calculate average water contents of 2.41 and 2.35% water with standard deviations of 0.1 and 0.09, equivalent to a C.V. of 3.99%.

This study has demonstrated the feasibility of using the method of standard addition for determining water in acetone up to a concentration of at least 4.0%.

Results of this work also show that concentrations of water as low as 0.2% can be determined accurately. Of the adsorbent systems tried, the Century Series Chromosorb 102 gave satisfactory separation of water and acetone and reproducible areas of the water peak. Finally, we found that overloading of the TCD could be avoided by limiting the sample volume to 0.5 μl, precisely measured each time with a Chaney adaptor.

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Note

High-performance liquid chromatography of maltosaccharides

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We have previously reported several improvements for fractionation and quantitative or qualitative determination of maltooligosaccharides using carbon column chromatography[1], paper densitometry[2], gas–liquid chromatography[3], gel filtration chromatography[4] and multiple descending paper chromatography[5]. These studies were closely related to our work on the fine structure of the starch molecule and on the mechanism of action of amylases. Recently, high-performance liquid chromatography (HPLC) has been recognized as a quick, convenient and accurate method of quantitative analysis of carbohydrates [6–10].

In this paper, we summarize our results on the quantitative analysis of maltosaccharides by HPLC. We also try to determine the range of error in the determination of higher maltooligosaccharides when maltose is used as a standard compound.

EXPERIMENTAL

Preparation of maltooligosaccharides

A 200-ml volume of 2% short-chain amylose (mean degree of polymerization, $\overline{D.P.} = 18$) was incubated with 5.5 I.U.* of bacterial liquefying α-amylase (Crystalline; Seikagu Kogyo, Tokyo, Japan) at 40°C for 30 min, then deionized on Amberlite MB-3 resin. The deionized hydrolyzate was lyophilized. Each pure maltooligosaccharide was prepared by macro paper chromatography. The concentrations of oligosaccharides were determined by the phenol–sulphuric acid method[11].

HPLC apparatus, column and operating conditions

A Trirotor HPLC apparatus (Nihon Bunko, Tokyo, Japan) was used with a Jascopak SN-01 column (25 cm × 4.6 mm, Nihon Bunko) and a 5-cm SN-01 guard column or a μBondapak carbohydrate column (30 cm × 4.0 mm, Waters Assoc.).

* One International Unit (I.U.) according to the International Commission on Enzymes is the amount of enzyme that will hydrolyze 1 μmol of glycosidic bonds in 1 min under optimal conditions.

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The elution of carbohydrate was detected with a differential refractometer (Shodex SE-11). The column was eluted with acetonitrile–deionized water (65:35 v/v) at room temperature. Samples of 10–30 μl were injected into the HPLC and eluted at a velocity of 2–3 ml/min. All the solvents and samples were degassed ultrasonically and filtered with a membrane filter (0.45 μm pore size).

RESULTS

Fractionation of maltooligosaccharides with Jascopak SN-01

The partial hydrolyzate of short-chain amylose and fractionated G_1, G_2, G_3 ··· G_7* were analyzed by HPLC with the Jascopak SN-01 column under the conditions described in Experimental.

Fig. 1 shows the elution profile of the mixture of maltooligosaccharides. The quantitative analysis of G_1 to G_8 is easily done within 20 min. Various amounts of pure G_1, G_2, G_4 and G_6 were injected into the HPLC in order to construct a calibration curve between detector response and the amount of maltooligosaccharides. The detector response, K, is defined as the slope of the calibration curve for each saccharide.

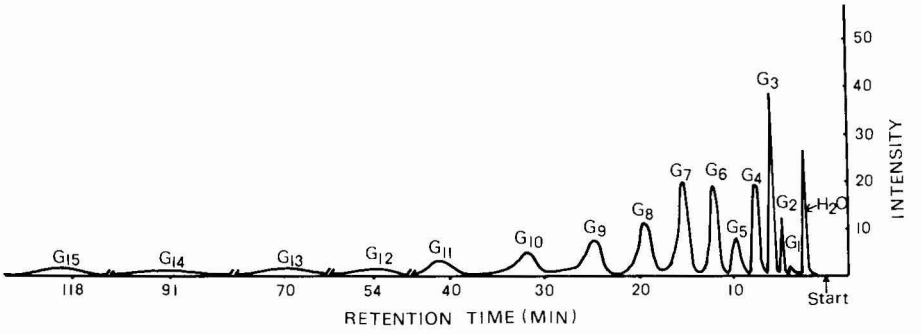

Fig. 1. Chromatogram of maltooligosaccharides mixture on Jascopak SN-01.

Fig. 2 shows the relation between peak height and the amount of saccharides. The slope of the curves gradually decreases with increasing molecular weight. The K value obtained from the G_1 curve is more than three times that from the G_6 curve. The values for G_1, G_2, G_4 and G_6 are 1.63, 1.35, 0.85 and 0.55 respectively.

Fig. 3 shows the relation between peak area, calculated as the area under a triangle, and the amount of saccharides. It is seen that the four curves are close to each other. The K values for G_1, G_2, G_4 and G_6 are 1.16, 1.23, 1.19 and 1.24.

Fig. 4 was obtained by plotting the peak weight (mg) against the amount of saccharides. Peak weight was measured by cutting and weighing the peak of each saccharide from a Xerox copy of the chromatogram. We found that the closest calibration curve was obtained when the peak weights were employed. The K values are 1.00 for G_1 and G_6, 0.99 for G_2 and G_4.

* Symbols G_1, G_2, G_3, ... etc. represent glucose, maltose, maltotriose, etc.

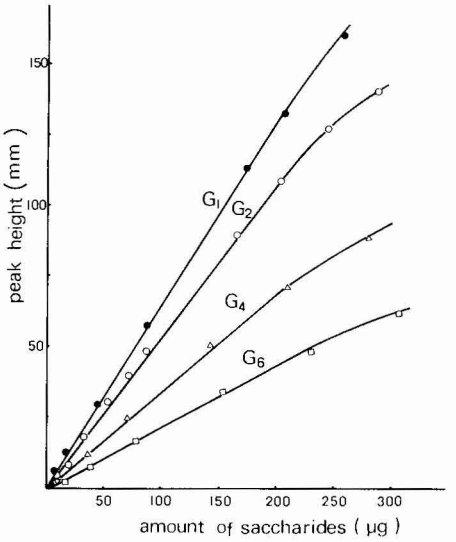

Fig. 2. Calibration curves obtained by plotting peak height against the amounts of each saccharide. Column: Jascopak SN-01.

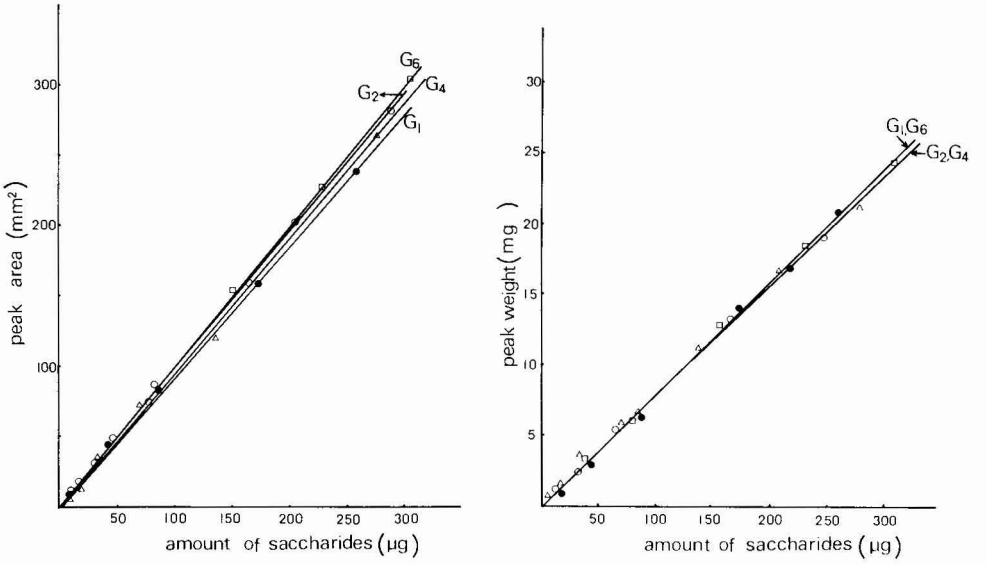

Fig. 3. Calibration curves obtained by plotting peak area against the amount of each saccharide. Column: Jascopak SN-01.

Fig. 4. Calibration curves obtained by plotting peak weight against the amount of each saccharide. Column: Jascopak SN-01.

Fractionation of maltooligosaccharides on μBondapak carbohydrate

The results of the fractionation of maltooligosaccharides on a μBondapak carbohydrate column are shown in Fig. 5. Glycerol was added as an internal standard. The column satisfactorily fractionated oligosaccharides up to G_6 or G_7, even

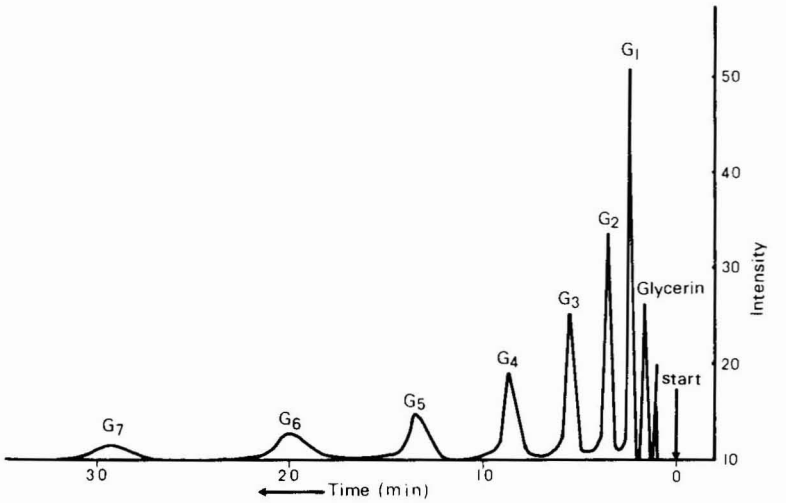

Fig. 5. Chromatogram of maltooligosaccharides mixture on μBondapak carbohydrate.

though the peaks were broad. It was difficult to analyze maltooligosaccharides higher than 10, which was easily done on the Jascopak SN-01 column. Fig. 6 shows the calibration curves for each oligosaccharide plotted as peak area against concentration. The K values calculated by various methods with and without internal standard are shown in Table I.

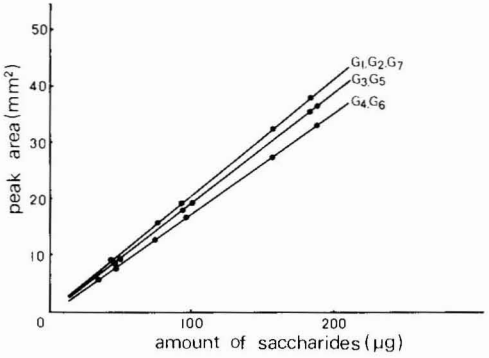

Fig. 6. Calibration curves obtained by plotting peak area against the amount of each saccharide. Column: μBondapak carbohydrate.

DISCUSSION

Because of the difficulty in obtaining commercially pure maltooligosaccharides as standard compounds for chromatography, one of the purposes of this study was to determine the range of error which might arise by using pure maltose, which is fairly readily obtainable, as a standard compound for the determination of higher malto-

TABLE I

DETECTOR RESPONSE VALUES (K) OF MALTOSACCHARIDES

Response values: H, based on peak height of maltooligosaccharide; A, based on peak area of maltooligosaccharide; H_I, based on peak height of internal standard; A_I, based on peak area of internal standard.

	H	A	H/H_I	A/A_I
G_1	0.18	0.21	0.91	1.36
G_2	0.14	0.21	0.59	1.11
G_3	0.11	0.20	0.52	1.17
G_4	0.06	0.18	0.27	1.05
G_5	0.05	0.20	0.23	1.13
G_6	0.03	0.17	0.14	1.01
G_7	0.03	0.21	0.15	1.37

oligosaccharides. Tables II and III show the correction factors for each maltooligosaccharide when G_2 is used as a standard. The correction factor (C.F.) was defined as:

$$\frac{K \text{ value of } G_2}{K \text{ value of maltooligosaccharide}}$$

As seen in Table II, the C.F. values of G_1, G_4 and G_6 are very close to 1 when the peak areas or peak weights are measured. This indicates that G_2 could be used as the standard compound for maltooligosaccharide determination with high accuracy because all the standard curves shown in Figs. 4 and 5 are very close to each other.

TABLE II

CORRECTION FACTORS OF EACH MALTOOLIGOSACCHARIDE WHEN G_2 IS USED AS STANDARD COMPOUND (JASCOPAK SN-01)

Correction factors were calculated as described under Discussion.

	Based on peak height	*Based on peak area*	*Based on peak weight*
G_1	0.83	1.06	0.99
G_4	1.59	1.03	1.00
G_6	2.45	0.99	0.99

TABLE III

CORRECTION FACTORS OF EACH MALTOOLIGOSACCHARIDE WHEN K VALUE OF G_2 IS USED (μBONDAPAK CARBOHYDRATE)

	H	A	H/H_I	A/A_I
G_3	1.27	1.05	1.13	0.94
G_4	2.33	1.17	2.19	1.06
G_5	2.80	1.05	2.57	0.98
G_6	4.67	1.24	4.21	1.10
G_7	4.67	1.00	3.93	0.81

Using the correction factors, the amount of each maltooligosaccharide is easily determined accurately from;

$$\mu g \text{ of maltooligosaccharide} = (\mu g \text{ as } G_2) \times \text{C.F.}$$

Correction factors obtained from the results on the μBondapak column were widely scattered when compared with the data obtained on Jascopak SN-01 (Fig. 3).

The experimental results described show that maltose can be employed as a standard saccharide for the determination of maltooligosaccharides with high accuracy, if the peak area or peak weight is measured. We have obtained satisfactory results with the Jascopak SN-01 column in terms of peak resolution and reproducibility. We also tested the SN-01 column packed in our laboratory and obtained exactly the same resolution of oligosaccharides. The use of the latter column reduced the cost of the column in HPLC to 5–10% of the commercially packed column.

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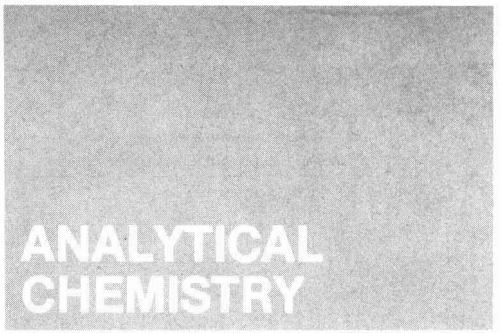

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Proceedings of the 6th International Symposium on Mass Spectrometry in Biochemistry and Medicine, Venice, 21 and 22 June, 1979

A. FRIGERIO *and* **M. McCAMISH.** *(Editors)*

Analytical Chemistry Symposia Series 4

This book, comprising 52 papers, provides a comprehensive insight into the latest research and developments in biochemistry, medicine, toxicology, drug research, forensic science, clinical chemistry and pollution.

Dec. 1980 564 pp.
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G. SVEHLA *(Editor)*

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ten by two co-workers of Fritz Feigl, the initiator of the technique. The second chapter comprises a brief history of analytical chemistry, providing a valuable and highly readable source of information on the early analytical work.

Aug. 1980 396 pp.
US $83.00/Dfl. 170.00
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J. GRIMSHAW, P. MORITZ *and* W. E. VAN DER LINDEN

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Nov. 1980 422 pp.
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Subscription Price: US $107.25/Dfl. 220.00
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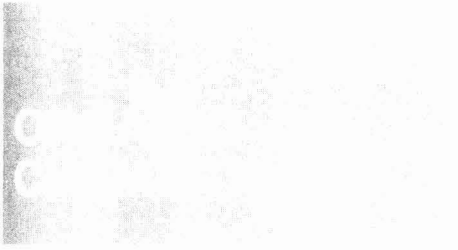

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E. BUNCEL *and* T. DURST *(Editors)*

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Studies in Organic Chemistry 5

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Aug. 1980 408 pp.
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Jan. 1980 522 pp.
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Their Chemistry, Biochemistry and Physiology

D. J. COSGROVE *(with contributing chapters by* **G. C. J. IRVING)**

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March. 1980 204 pp.
US $46.25/Dfl. 95.00
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May 1980 411 pp.
US $109.75/Dfl. 225.00
0-444-41895-4

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Including selected plenary lectures from the Fifth International Symposium on Organosilicon Chemistry, held in Karlsruhe, August 14-18, 1978

D. SEYFERTH *(Editor)*

Journal of Organometallic Chemistry Library 9

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Jan. 1980 440 pp.
US $105.00/Dfl. 215.00
0-444-41840-7

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D. SEYFERTH *and* **R. B. KING** *(Editors)*

Journal of Organometallic Chemistry Library 10

This volume brings the Annual Surveys for the year 1978 of the organometallic chemistry of the Group IV elements.

April. 1980 616 pp.
US $119.50/Dfl. 245.00
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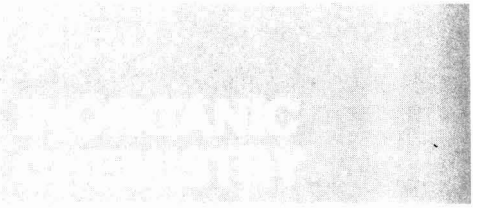

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May 1980 570 pp.
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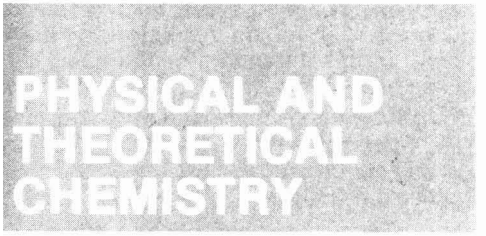

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July 1980 280 pp.
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STATISTICAL THERMODYNAMICS OF SIMPLE LIQUIDS AND THEIR MIXTURES

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This monograph outlines potential problems in the description of the state and the thermodynamic behaviour of fluids when using the radial distribution function. Necessary mathematical details are included with the more complicated operations described in the extensive appendices.

Jan. 1980 146 pp.
US $41.50/Dfl. 85.00
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"... is a compact and balanced account ... can be recommended to those who want a clear account of the authors' chosen field".

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GROWTH AND PROPERTIES OF METAL CLUSTERS

Applications to Catalysis and the Photographic Process

Proceedings of the 32nd International Meeting of the Société de Chimie Physique, Villeurbanne, France, September 24-28, 1979

J. BOURDON *(Editor)*

Comprising 53 papers, these proceedings offer a comprehensive and up-to-date account of recent progress on cluster build-up. Fundamental aspects such as nucleation, growth and electrocrystallization of clusters, their applications to photographic processes and catalysis were discussed as plenary lectures. Other communications reviewed for example, theoretical ad-

vances, the validity of the Hückel theory and advances resulting from increased detection sensitivity.

April. 1980 580 pp.
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Studies in Physical and Theoretical Chemistry 9

Two specific areas related to the physics of dielectrics, the gaseous and liquid states, are discussed in this book. The qualities characterizing a free molecule, such as polarizability and dipole moment are presented, as well as the effects which make it possible for those quantities to be determined. The chapters are: Static Electric Susceptibility of Gases and Liquids; Dielectrics in Alternating Fields; Anisotropy of the Polarizability and Molecular Structure; Non-Linear Dielectric Effects; Experimental Methods of Measuring Dielectric Susceptibility.

Sept. 1980 408 pp.
US $80.50/Dfl. 165.00
0-444-99766-0

SPECTROSCOPY IN CHEMISTRY AND PHYSICS: MODERN TRENDS

A Collection of the Invited Papers Presented at the 14th European Congress on Molecular Spectroscopy, Frankfurt/Main 3-7 September 1979

F. J. COMES, A. MÜLLER *and*
W. J. ORVILLE-THOMAS *(Editors)*

Studies in Physical and Theoretical Chemistry 8

Reprinted from the Journal of Molecular Structure, Vol. 59

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Proceedings of the International Symposium, Antwerp, Belgium, October 13-15, 1980

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This proceedings volume is devoted to the scientific aspects of deactivation of heterogeneous catalysts by poisoning, coking and solid state transformations. For each topic an attempt is made to understand the fundamental processes involved, to scientifically describe the practical consequences of the phenomena, and to show how this understanding of the basic processes can be related to the overall observed phenomena.

Oct. 1980 612 pp.
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A. E. MARTIN

Vibrational Spectra and Structure 8

J. R. DURIG *(Series Editor)*

This volume sets out to describe in a thorough but relatively uncomplicated manner the theoretical background of Fourier transform spectroscopy together with a number of associated instruments and techniques. The inclusion of a considerable amount of previously unpublished work and suggestions for processing interferogram data without need for a computer, complete the text.

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"Overall this is an excellent book for the spectroscopy specialist."
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This book is the first to present a systematic study of atoms in external fields. The

data required for such a study are presented through the tabulation of electric dipole polarizabilities, shielding potentials, probabilities of barrier penetration, electric dipole transition probabilities, and radial integrals of the B- through No-isoelectronic series.

Dec. 1980 568 pp.
US $119.50/Dfl. 245.00
ISBN 0-444-41936-5

WEAK INTERMOLECULAR INTERACTIONS IN CHEMISTRY AND BIOLOGY

P. HOBZA *and* **R. ZAHRADNIK**

Studies in Physical and Theoretical Chemistry 3

This monograph explains methods for calculation of weak interactions with special attention to the variation and perturbation methods and empirical and hybrid methods and their applications. The text is supplemented with numerous tables and figures.

Jan. 1980 354 pp.
US $48.75/Dfl. 100.00
0-444-99785-7

"... there is something in this book for everyone interested in the different aspects of the physico-chemical sciences, and it should find a place in every scientific library."
-Bioelectrochemistry and Bioenergetics

CATALYSIS BY ZEOLITES

Proceedings of an International Symposium organized by the Institut de Recherches sur la Catalyse - CNRS - Villeurbanne, and sponsored by the Centre National de la Recherche Scientifique, Ecully (Lyon), September 9-11, 1980

B. IMELIK, C. NACCACHE, Y. BEN TAARIT, J. C. VEDRINE, G. COUDURIER *and* **H. PRALIAUD** *(Editors)*

Studies in Surface Science and Catalysis 5

The invited papers in this volume comprise reviews of recent developments in catalysis by zeolites, including the conversion of hydrocarbons by acidic zeolites, the concepts of shape selectivity, the catalytic, properties of ions and metals trapped in solid matrices etc.

Sept. 1980 356 pp.
US $65.75/Dfl. 135.00
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ELECTRODES OF CONDUCTIVE METAL OXIDES: PART A

S. TRASATTI *(Editor)*

Studies in Physical and Theoretical Chemistry 11

This two-part work provides a general unifying introduction plus a state-of-the-art review of the physicochemical properties and electrochemical behaviour of conductive oxide electrodes. The present volume (Part A) discusses structure and thermodynamic properties from a multidisciplinary viewpoint. For the first time different groups of oxides are treated together in order to place emphasis on their similarities and differences.

Aug. 1980 382 pp.
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DESIGN OF INDUSTRIAL CATALYSTS

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Chemical Engineering Monographs 11

This book describes the design procedure by which catalysts may be selected for a particular reaction. It discusses the underlying theories and provides numerous examples of catalyst design. This is the first book on the subject and as such will be welcomed by chemical engineers and chemists in a wide variety of industries.

Aug. 1980 326 pp.
US $61.00/Dfl. 125.00
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Proceedings of the 4th International Meeting on Modern Ceramics Technologies, Saint-Vincent, Italy, 28-31 May 1979

P. VINCENZINI *(Editor)*

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This proceedings volume, comprising more than 100 papers, represents a complete overview of recent developments and new channels of research in the ceramics industry. Recent improvements in processing methods to reduce energy consumption to a minimum and new classes of ceramic material currently being introduced in various areas of energy production and conversion are discussed in depth in the text.

Mar. 1980 1312 pp.
US $192.75/Dfl. 395.00
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A Literature Source Book

J. WISNIAK *and* **A. TAMIR**

Physical Sciences Data 7

Providing an organised literature reference source for liquid-liquid equilibrium and for the equilibrium distribution of components between two immiscible liquids, this two-part work deals with data published between 1900 and early 1980. From 1500 references scanned (from Chemical Abstracts and allied publications in several fields), 8500 were found to contain pertinent material, thus providing an up-to-date guide for workers in fields such as chemistry, metallurgy, and chemical engineering.

Aug. 1980 1274 pp.
US $183.00/Dfl. 375.00
0-444-41909-8

STRUCTURE AND PROPERTIES OF AMORPHOUS POLYMERS

Proceedings of the Second Cleveland Symposium on Macromolecules, Cleveland, Ohio, 31 October - 2 November, 1978

A. G. WALTON *(Editor)*

Studies in Physical and Theoretical Chemistry 10

This symposium was organised so that leading researchers in the field of amorphous polymers could present reviews of structure/property relations for polymers in the amorphous or glassy state by thermal, mechanical and spectroscopic methodology. The understanding of design structure and properties of these polymers is of growing importance in industry and commerce, making this volume a valuable guide to research in the field.

July 1980 239 pp.
US $53.75/Dfl. 110.00
0-444-41905-5

ATMOSPHERIC POLLUTION 1980

Proceedings of the 14th International Colloquium, UNESCO Building, Paris, France, 5-8 May 1980

M. M. BENARIE *(Editor)*

Studies in Environmental Science 8

This book comprises a selection of 61 of the 81 papers presented at the bi-annual colloquium. The aim of this meeting was to offer specialists from the various subfields an opportunity to discuss the present state of research and development in the field.

April 1980 452 pp.
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H. SIEBERT, J. EICHBERGER, R. GRONYCH *and* **R. PETHIG**

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This book analyzes the relationship between environmental policy, environmental quality and international trade, and goes on to discuss how environmental policy

will affect the comparative advantage of a country, import and export flows, the terms of trade and the gains obtained from trade. The authors approach the subject from an analytical-theoretical angle with the results derived from analytical models interpreted verbally and diagrammatically.

May 1980 360 pp.
US $63.50/Dfl. 130.00
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FIELD WORKER EXPOSURE DURING PESTICIDE APPLICATION

Proceedings of the Fifth International Workshop of the Scientific Committee on Pesticides of the International Association on Occupational Health, The Hague, October 9-11, 1979

W. F. TORDOIR *and* **E. A. H. VAN HEEMSTRA-LEQUIN** *(Editors)*

Studies in Environmental Science 7

These proceedings comprise twenty-five invited papers in which international experts discuss the current state-of-the-art and related scientific developments in the field of occupational health related to the manufacture, formulation and application of pesticides.

April 1980 222 pp.
US $47.25/Dfl. 97.00
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GENERAL CHEMISTRY

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General Chemistry and Radiochemistry

P. BENEŠ *and* **V. MAJER,**

Topics in Inorganic and General Chemistry, Monograph 18

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March. 1980 312 pp.
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TITLES APPEARING EARLY 1981

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S. AMIEL *(Editor)*

Studies in Analytical Chemistry 3

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Jan. 1981 380 pp.
US $83.00/Dfl. 170.00
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SOLUBILITY IN INORGANIC TWO-COMPONENT SYSTEMS

M. BROUL, J. NYVLT *and* **O. SÖHNEL**

Physical Sciences Data 6

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Sept. 1980 576 pp.
US $90.25/Dfl. 185.00
ISBN 0-444-99763-6

IONIC HYDRATION IN CHEMISTRY AND BIOPHYSICS

B. E. CONWAY

Studies in Physical and Theoretical Chemistry 12

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Jan. 1981 794 pp.
US $131.75/Dfl. 270.00
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Feb. 1981 536 pp.
US $139.00/Dfl. 285.00
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Studies in Physical and Theoretical Chemistry 13

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THEOCHEM

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Editors:
W. J. ORVILLE-THOMAS, *Salford, U.K.*
I. G. CSIZMADIA, *Toronto, Canada*

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1981: Volumes 2-3 (in 8 issues)
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TrAC

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1981-82: Volume 1, issues in March, October, November and December 1981, and thereafter monthly January to December 1982.

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PUBLICATION SCHEDULE FOR 1981

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

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2 209/3 210/1	210/2 210/3 211/1	211/2 211/3 212/1 212/2	The publication schedule for further issues will be published later.				
Chromatographic Reviews							220/1							
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1	224/2					

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher)

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), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

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