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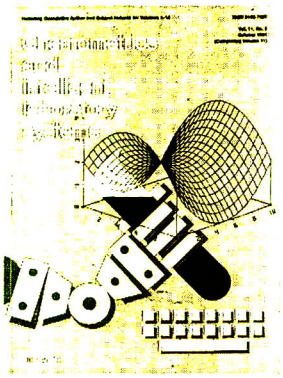

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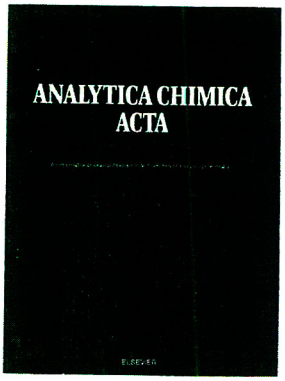

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On-line *versus* off-line solid-phase extraction in the determination of organic contaminants in water

Advantages and limitations

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ABSTRACT

Solid-phase extraction (SPE) has become and important and frequently employed method for the preconcentration of organic pollutants from water samples. From the methodological standpoint, two basic approaches can be recognized, on-line and off-line, each of which has its advantages and limitations. The original off-line modification is simple and highly flexible. Therefore, it is often used in analytical research and in quick testing methods. On the other hand, the possibility of automation and the high sample throughput of on-line SPE are the major reasons for its growing use in routine target analyses and in analytical methods for continuous monitoring of water quality. While the growing interest in automation in laboratory practice leads to promising perspectives for on-line techniques, the relatively unlimited flexibility of the classical off-line SPE makes it always a suitable procedure to be used for trace enrichment purposes in diverse analytical applications. This paper gives an overview of the basic principles and possibilities of both off-line and on-line SPE approaches and provides a brief survey of their benefits and limitations.

INTRODUCTION

Low concentrations (ng/l–μg/l) of toxic non-volatile organic compounds in water samples and the complexity of environmental matrices require the application of a suitable sample handling procedure prior to any assessment of water pollution via chromatographic techniques. The role of such a sample-handling step is to enrich all analytes of interest and to purify the original sample matrix by removing as much of the interfering components as possible. The present state of analytical chemistry can be characterized as a situation where highly efficient separation and detection systems are usually coupled with laborious and time-consuming sample handling procedures which limit the sample throughput and, often, also the overall performance of the method [1]. Thus, at present, sample handling is being considered to be the weakest aspect of environmental chromatographic analysis. This situation implies the need for the development of novel approaches to sample handling or, at least, for the thorough improvement of the effectiveness of current methods.

The techniques used most frequently for the preparation of aqueous samples prior to chromatographic analysis are liquid–liquid extraction (LLE) and solid-phase extraction (SPE). The greatest advantages of LLE are its simplicity and numerous practical verifications and performance evaluations. SPE, developed intensively in the last 20 years, has become a powerful alternative technique owing to its simplicity, flexibility and high sample throughput. Comparisons of LLE and SPE can be found in the literature

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[2–4] and experimental comparative tests performed by various workers [5,6] have shown a prevalence of SPE over LLE in many applications, especially when more polar compounds were to be preconcentrated.

The basic principle of SPE is the transfer of analytes from the aqueous phase to the active sites of the adjacent solid phase. This transfer is stimulated by the selection of appropriate operational conditions in the system of three major components, water (liquid phase)–sorbent–analyte. After the replacement of the water by a suitable liquid phase in this system, the analyte can be desorbed from the sorbent and further analysed. Usually, the SPE process is carried out in the column and is often referred to as low-performance liquid chromatography. Owing to the use of two extreme mobile phases, other synonyms such as digital chromatography, on–off or stop–go chromatography are also in use [7].

Many factors influence the efficiency of the SPE process, but the two most important are capacity and retention. An insufficient capacity of the sorbent surface can cause its overloading and, consequently, earlier breakthrough of analytes. However, this situation is considered not to be very likely owing to low concentrations of organic compounds in treated water samples and the relatively high sorption capacities of applied sorbents [8]. The more critical factor is the retention of analytes, which should be maximum in the water–sorbent–analyte system and minimum in the eluent–sorbent–analyte system. The existence of these two contradictory demands on the strength of the sorbent–analyte interactions leads to the necessity to make a compromise during the selection of working conditions for sorption and desorption so as to obtain an optimum preconcentration.

The retention of an analyte in the SPE column during sample application can be expressed via its breakthrough curve, *i.e.*, the dependence of the ratio of the effluent concentration (C) to influent analyte concentration (C_0) on the volume of the aqueous sample percolated through the SPE column (Fig. 1). In principle, the breakthrough curve has the integral shape of a common Gaussian curve. The retention time of an analyte (the maximum of the Gaussian peak) corresponds to the inflection point of the breakthrough curve. The breakthrough volume can be defined as the volume after passage of which a certain level of breakthrough of an analyte occurs. In practical applications, the breakthrough level, defined as the ratio of the outlet to inlet analyte concentration or as the fraction of the total mass of an analyte which has passed

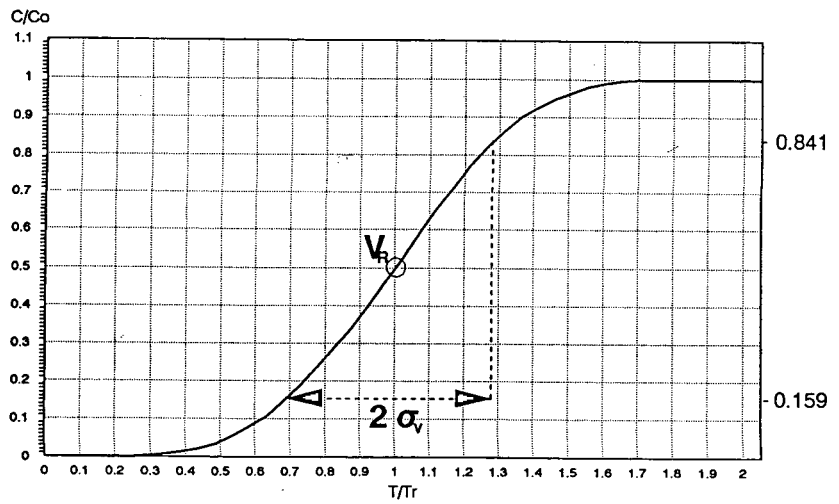

Fig. 1. Breakthrough curve. C is the solute concentration in the effluent, C_0 is the solute concentration in the sample (*i.e.*, in the influent), T is time, T_R is the retention time of the solute, V_R is the retention volume of the solute and σ_V is the elution band broadening.

out of the column, is set to 1–10% (*i.e.*, C/C_0 = 0.01–0.1; Fig. 1).

Having information of the shape of the breakthrough curve, the optimum volume of the preconcentrated water sample can be set so that the maximum recovery will be obtained ($V < V_B$) or the maximum amount of an analyte will be preconcentrated ($V > V_R + 3\sigma_V$; see Fig. 1), where V is the volume of the sample passed through the column, V_B is the breakthrough volume of the analyte and σ_V is the dispersion. If more analytes are to be preconcentrated on the same SPE column simultaneously, the selection of the sample volume has to be a compromise between losses of less retained components and low enrichment factors for highly retained compounds. The other solution of this problem is to use more SPE columns connected in series. The breakthrough curve can be measured experimentally either using direct methods based on the on-line (off-line) detection of the analyte in the SPE column effluent or by indirect methods, *i.e.*, evaluation of the effect of the volume of the sample on the recovery of the preconcentration.

The retention of an analyte in the SPE column during the desorption process can be demonstrated by the elution curve, *i.e.*, by the concentration profile of an analyte in the SPE column effluent (Fig. 2). From the shape of the elution curve the optimum volume of the solvent required for the quantitative desorption of the analyte can be obtained. In on-line applications the measurement of the elution curve is of minor importance as adequate information is provided by the character of additional band broadening in the analytical column.

The most often used sorbent materials in SPE applications are chemically bonded silicas, polymers and carbons. They bind analytes primarily due to hydrophobic interactions, but the secondary interactions can play a significant role in some instances, *e.g.*, ionic interactions of residual silanol groups on bonded silicas with positively charged analytes. For trapping of some groups of analytes, ion-exchange or ligand-exchange processes can also be applied using silica- and polymer-based ion exchangers or metal-loaded sorbents.

With respect to the system approach two

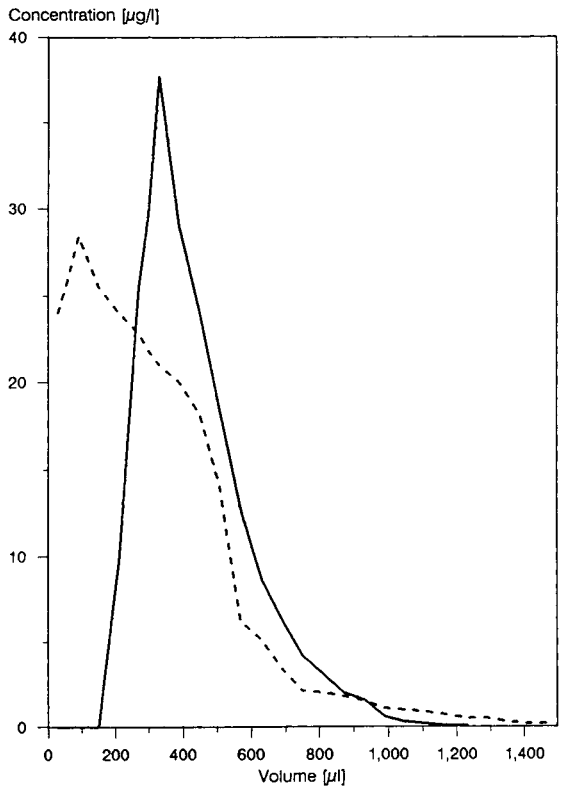

Fig. 2. Elution curves measured for (solid line) cyclohexanone and (dashed line) aniline on a C_{18} SPE column with an internal volume of 1 ml. The eluting solvent was methanol.

modes of SPE can be distinguished: off-line and on-line. In the on-line configuration the SPE column output is connected directly to the analytical column, so that elution and separation of analytes are performed in one step. In the off-line configuration, the elution and separation of analytes are two separate steps. The chemistry and general principles are the same for both of these variants, but the differences in their methodology are sometimes the reason for certain drawbacks or advantages of one of these approaches in a particular application. Nowadays, the opinions of analytical chemists on the usefulness of either of these two approaches are widely polarized.

The objective of this paper is to compare on-line and off-line SPE so as the advantages and drawbacks of these two approaches can be highlighted.

OFF-LINE APPROACH

Principles

In off-line SPE the analyte is accumulated from the water sample by a convenient SPE column and subsequently it is eluted with a suitable solvent. The eluate from the column is collected in a vial. Analytes in the eluate can be further concentrated by partial solvent evaporation, and finally, an aliquot of the eluate is injected into the chromatographic system. The volume of the water sample applied usually ranges from several tens of millilitres to tens of litres. In early SPE applications, the sorbent material was laboratory packed into a column made of stainless steel, glass or polymer with an internal volume of 1–100 ml. The introduction of commercially available disposable cartridges and suitable devices for forcing the water sample through these cartridges reduced the use of laboratory-packed columns substantially.

There is a wide choice of disposable cartridges packed with chemically bonded silicas. Cartridges with graphitized carbon black and ion exchangers are also available from several suppliers. The particle size of the sorbent packed in the SPE column is usually 40–60 μm. This diameter is sufficient to ensure an effective preconcentration and to avoid problems with the back-pressure due to suspended solids in the water sample.

To desorb trapped analytes, various organic solvents (*e.g.*, methanol, acetonitrile, ethyl acetate, diethyl ether) or their mixtures are used. To improve the efficiency of the desorption, the organic solvents can be modified by addition of an acid, base or buffer solution. An aliquot of the eluate is then analysed using one or more separation and detection systems. If needed, the analysis of the eluate can be repeated.

A typical sequence of SPE includes the following steps: activation of the sorbent (wetting with a suitable solvent), conditioning (replacing of the activation solvent by the aqueous phase), percolation of the water sample, clean-up (removal of interfering components), drying of the sorbent bed, elution of accumulated analytes and regeneration of the sorbent (usually not recommended for disposable cartridges because of memory effects [9] and/or hysteresis effects [10]).

As mentioned above, breakthrough and elution characteristics can be employed for optimization of the off-line SPE procedure in order to select suitable volumes of water sample and eluting solvent. Accordingly, the maximum theoretical preconcentration factor (F), given as the ratio of the breakthrough volume (V_B) and the width of the elution curve (W), $F = V_B/W$, was suggested [11]. This factor expresses the maximum possible preconcentration of an analyte in a particular analyte–sorbent–solvent (water/eluent) system while the water sample volume is minimal and the recovery is 100%. The practical significance of this factor is reduced when more analytes are to be preconcentrated simultaneously, but it can be used as a system parameter characterizing the efficiency of a given solid phase to preconcentrate a certain group of analytes.

Empirical approaches to optimization of the SPE procedure can be also found in the literature [12].

Advantages

The major advantages of the off-line approach are its operational flexibility and the simplicity of the equipment required. With certain simplifications, a syringe and an SPE cartridge are sufficient tools for a rapid trace enrichment. In practical applications, to avoid possible sample contamination from the sample-delivery system, a pressure difference (positive or negative, *i.e.*, compressed gas or vacuum) is used as a driving force. There are several types of a simple vacuum manifold available for the percolation of water samples. Such a device usually permits the processing of several samples simultaneously. Another way to force the water through the cartridge is to use a high pressure in the sample reservoir connected to the column inlet. This configuration is preferred in automated SPE systems.

The operational flexibility of the SPE means that there is a wide range available for setting of the operational conditions. The analyst can optimize the amount of sorbent, the type and vol-

ume of eluting solvent, the number of clean-up steps and their working conditions and the scheme of the eluent fractionation. Moreover, one can select a convenient separation and detection technique and the appropriate scale of the SPE procedure (ranging from small micro-columns to large fractionation columns).

One of the large-scale applications of off-line SPE often used in environmental analysis is its incorporation into methods dedicated to the investigation of the occurrence of organic contaminants in waters. For such qualitative broad range analysis, tens to thousands of litres of water sample were forced through a large laboratory-made column and the eluate obtained was further concentrated by evaporation to achieve a high preconcentration factor [13,14]. Such procedures were not sufficiently quantitative, but they provided extensive information on the character of water pollution. From the standpoint of scale, the opposite to large-scale SPE is solid-phase microextraction [15], a rapid, solventless and portable method employing a narrow fibre coated with a film of a suitable phase with thickness ranging from 15 to 150 μm.

To simplify the original complex sample matrix, a single operation to remove undesirable interferences or a suitable combination of several clean-up steps can be performed. Ionized analytes trapped on the solid phase can be separated from other compounds by flushing the column with alkalinized or acidified water. Subsequently, polar compounds can be eluted with appropriate mixture of water and an organic modifier. Finally, remaining non-polar compounds are eluted with pure organic solvent or a mixture of several solvents. Eluates obtained by these procedures are much simpler than the original sample and the subsequent analytical separation can be performed more easily. In principle, the analogous strategy can be used for the fractionation of sorbed analytes. The only substantial difference between clean-up and fractionation is that in clean-up there is usually only one fraction of interest and other fractions are discarded whereas in the fractionation procedure all fractions of the eluate obtained are further analysed. Generally, incorporation of clean-up and/or fractionation steps into the SPE procedure prolongs the analysis time and increases labour requirements, but the resulting information is usually worth this effort. The information gain provided by fractionation can be increased when several separation and/or detection techniques are used simultaneously.

Valls *et al.* [16] combined LLE and SPE for the accumulation of ionic and non-ionic organic contaminants from urban wastewaters and coastal sea waters. After sorption, the organic extracts were further fractionated by column chromatography and fractions were analysed by high-resolution GC–MS using different ionization techniques. This procedure allowed the identification of 290 anthropogenic contaminants in the different aquatic compartments. Asafu-Adjaye *et al.* [17] separated kepone from other pesticides by flushing the loaded SPE column with hexane, which eluted DDT, DDE and HCB. Kepone and its metabolites were subsequently eluted with a mixture of hexane and diethyl ether. Wells *et al.* [7] discussed SPE for the selective fractionation of wastewater effluents.

To optimize the recovery of a particular analyte sorbed on a given sorbent, a wide range of eluting solvents or their mixtures can be employed. Bacaloni *et al.* [18] tested nine eluents to desorb organochlorinate pesticides (OCPs) from graphitized carbon black. They found hexane–diethyl ether (50:50) to be the most efficient desorbing medium for OCPs. The recoveries for other groups of compounds showed that, using this eluent, OCPs could be completely separated from polycyclic aromatic hydrocarbons and, to a certain extent, from polychlorinated biphenyls. Combination of more SPE columns and the use of several eluting solvents can also increase the efficiency of the SPE procedure. DiCorcia *et al.* [19] extracted phenoxyacetic acid herbicides from water using a miniaturized cartridge containing graphitized carbon black (GCB) at the top and a silica-based strong anion exchanger at the bottom. After the percolation of the water sample through this cartridge the anion exchanger was activated by sodium acetate solution. Sorbed analytes were then transferred from the GCB to the anion exchanger using methyl-

ene chloride–methanol basified with sodium hydroxide. After washing, herbicides were desorbed from the anion exchanger with water–methanol containing trifluoroacetic acid and potassium chloride. A similar procedure with a cation exchanger was applied to chloroanilines [20]. The detection limits in both instances were in the ng/l range.

The great amount of work required in off-line SPE is, on the other hand, compensated for by the possibility of using various separation and/or detection techniques simultaneously, with only negligible technical limitations. Aliquots of an eluate can be injected into GC and/or HPLC systems and also transferred to other separation systems (*e.g.*, isotachophoresis, planar chromatography). The range of available GC detectors enables high sensitivities to be achieved (*e.g.*, an electron-capture or nitrogen–phosphorus detector) [21–27] and also considerable qualitative information (using MS) [28,29].

Screening methods for monitoring pesticides and other water pollutants have been at the focus of environmental analytical chemistry for a long time. Many of the screening procedures developed recently prefer the use of SPE for handling water samples. Off-line screening requires simple instrumentation and enables low detection limits to be achieved owing to the possibility of partial evaporation of the eluate from the SPE cartridge. Di Corcia and Marchetti [30] presented an off-line approach to monitoring a large group of pesticides in ground and river waters. The method incorporated fractionation of the analytes into basic, neutral and acidic compounds, based on two different interaction mechanisms on graphitized carbon black. Processing of large volumes of water (0.5–2 l) and evaporation of the eluates led to detection limits lower than 0.1 μg/l for most pesticides (Fig. 3).

The ease of manipulation with disposable SPE media has been further enhanced. Recently, membrane extraction discs have been introduced as an attractive kind of SPE material. These membranes consist of a fibrillated PTFE matrix in which sorbents such as bonded silicas, polymers or ion exchangers are enmeshed. Owing to the internal structure of the discs, high flow-rates of water samples can be achieved and the decrease in recovery due to channelling is avoided. The easy manipulation with discs makes them suitable for rapid testing methods. Hagen *et al.* [31] used discs containing C_{18} and C_8 bonded silica for the enrichment of phthalates and pesticides and Kraut-Vass and Thoma [32] preconcentrated pesticides and other pollutants. Owing to the obvious benefits of their use, discs are becoming preferred media for drinking water test methods as defined by US Environmental Protection Agency [33].

Limitations

The flexibility of the off-line approach is offset by the labour required for the overall SPE procedure. This is usually acceptable in the case of an occasional broad-range analytical survey, but it may become an obstruction when complex continuous monitoring or any similar water quality control programme with a large number of samples and high sampling frequency is to be carried out. Off-line procedures require a lot of manual work that can be automated only by means of robotic systems, at considerable financial cost.

In general, to achieve an exact determination, all measurements of the volume should be as exact as possible. In off-line SPE problems can sometimes arise due to handling of relatively small volumes of several tens of microlitres (*e.g.*, after partial evaporation of the eluate solvent after desorption). During this operation the loss of several microlitres of the eluate solvent can lead to a considerable error in the final result. Moreover, the poor reproducibility of such manual operations (especially between different operators) can also adversely affect the precision of the procedure. Injection of relatively small aliquots of the eluate increases the detection limit of the analytical procedure. This is not critical for non-polar compounds for which the handicap of aliquot injection is compensated for by the large volumes of water sample processed and by evaporation of a substantial part of the eluate solvent, but it becomes a serious problem in the case of polar and/or more volatile compounds which have low breakthrough volumes and high losses during evaporation.

The use of disposable cartridges for handling

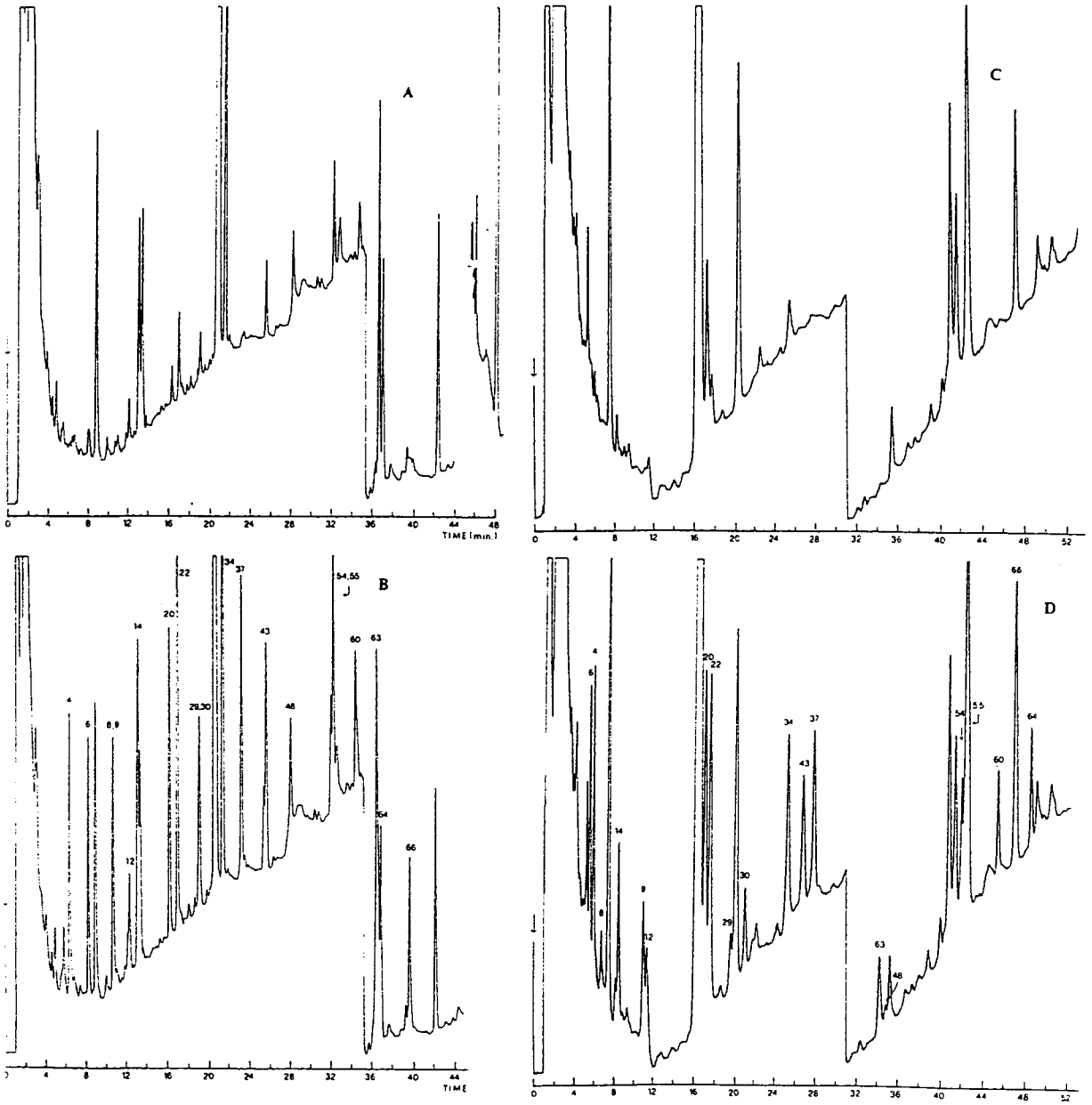

Fig. 3. Chromatograms obtained from 1.5 l of a well-water sample. Extract of unspiked sample chromatographed on the (A) C_{18} DB column and (C) the cyano column. Extract of the spiked sample chromatographed on the (B) C_{18} DB column and (D) the cyano column. The spiking level was 100 ng/l of each pesticide [except for chloridazon (peak 4; 50 ng/l) and carbaryl (peak 22; 25 ng/l)]. The extract volume injected on to the cyano column was 20 μl; UV DAD detection, attenuation 0.005 AUFS. The extract volume injected on to the C_{18} DB column was 40 μl; UV DAD detection, attenuation 0.01 AUFS. Peaks: 4 = chloridazon; 6 = mevinphos II; 8 = aldicarb; 9 = metoxuron; 12 = monuron; 14 = metribuzin; 20 = chlortoluron; 22 = carbaryl; 29 = metobromuron; 30 = paraoxon; 34 = propanil; 37 = linuron; 43 = prpyzmide; 48 = malathion; 54 = fenthion; 55 = parathion-ethyl; 60 = phoxim; 63 = butylate; 64 = metoxychlor; 66 = pendimethalin. From ref. 30 (© American Chemical Society).

water samples reduces the consumption of organic solvents when compared with LLE, but the costs of the sorbent material and solvents necessary for off-line SPE are still higher than those for on-line precolumn techniques.

Nowadays, there a broad range of bonded silica disposable cartridges are commercially available, but there is only limited access to cartridges packed with other kinds of sorbents such as polymers or carbons, which are suitable for trapping more polar compounds. Laboratory packing of the disposable cartridges can be a solution to this problem, but it increases the labour needed for the SPE procedure.

Drying of the wet cartridge prior to elution of sorbed non-polar analytes is usually applied to ensure quantitative collection of the eluate and the exact evaluation of its volume. Such operation is not difficult with this type of compound, but the application of a drying step in the SPE of some more polar compounds can adversely affect their recovery [11]. This problem can be solved by fractionation of the eluent, but it further increases the time and labour needed for the sample-handling procedure.

ON-LINE APPROACH

Principles

The chemical principle of the on-line approach is the same as that for the off-line version. The major difference is the direct transfer of sorbed analytes from the SPE column (precolumn, concentrator column) to the analytical column after changing the position of a switching valve. Hence this approach is also called precolumn switching or precolumn technology. Conventional or microbore HPLC columns usually serve as the analytical column, but GC capillaries have also been applied for this purpose. The principles and technical aspects of the on-line approach, especially for HPLC analyses, have been thoroughly described [8,34–37] and many applications have been published in environmental and biomedical analysis.

A typical simple on-line system is shown in Fig. 4. It consists of two circuits connected together by a switching valve. An aqueous sample is introduced into the precolumn by the

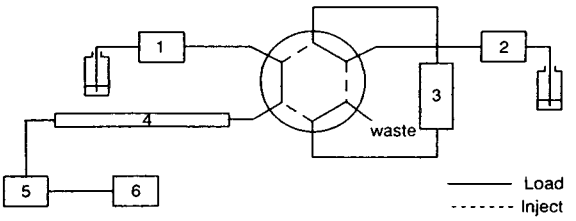

Fig. 4. Basic on-line precolumn set-up. 1 = Mobile phase pump; 2 = sample pump; 3 = precolumn; 4 = analytical column; 5 = detector; 6 = recorder/computer.

pump in the low-pressure (preconcentration) circuit and subsequently the precolumn is switched to the high-pressure circuit where the analytes are eluted by the mobile phase directly into the analytical column. The sample volume applied usually ranges from 10 to 200 ml, depending on the analytes and on the total organic load of the water sample. The internal volume of the precolumn ranges from several tens to several hundreds of microlitres. The optimum volume depends on the volume and the plate number of the analytical column and on the value of the capacity factor (k') of the analyte to be preconcentrated [38,39]. In general, the precolumn volume should be small compared with the volume of the analytical column and have a similar or smaller diameter [8]. These factors are important in minimizing the additional band broadening in the analytical column.

Theoretically, the sorbent material in the precolumn should be identical with the packing material in the analytical column. If two different sorbents are used, the retention of the analyte in the precolumn should be lower than that in the analytical column [8,37]. There is no problem in complying with this rule during the preconcentration of non-polar compounds. However, it becomes more critical in the trace enrichment of polar compounds, when a sorbent having stronger interactions with these analytes than a conventional C_{18} material should be used in the precolumn. If the precolumn containing more hydrophobic material is connected to a C_{18} analytical column, the danger of additional band broadening is obvious. However, investigations of the peak shape deterioration and some on-line applications published recently [40–44] showed that a styrene–divinylbenzene spherical copoly-

mer could retain many polar compounds sufficiently and that its connection to a C_{18} analytical column produced negligible or only low additional band broadening.

The particle size of the precolumn material should be the same as that used in the analytical column, but it is possible to use particles of a larger diameter when processing water samples with a high concentration of suspended solids to prevent problems with high back-pressure.

Advantages

The on-line approach minimizes the operator labour required for the sample preparation and his or her contact with the sample. This eliminates losses which can occur in off-line SPE during handling of the eluate (collection and evaporation, volume measurement) and during drying of the SPE column. The elimination of losses and the introduction of the whole amount of the preconcentrated analyte into the separation–detection unit improve both the sensitivity and the reproducibility of the analysis.

The regulating unit of an on-line system is the switching valve. It can be operated manually, but the whole preconcentration process can be automated using microprocessors for valve(s) control. The automation reduces the labour and time required for the analysis and allows easier control of multi-step SPE procedures. Consequently, this results in a high sample throughput. The basic on-line system (Fig. 4) can be converted into a more sophisticated set-up by incorporation of additional valves, precolumns and/or LC pumps. Such systems usually employ two or more precolumns with different sorbents connected in series (Fig. 5). Subra *et al.* [43] preconcentrated organic pollutants using C_{18} and polymeric precolumns in series connected to a C_{18} analytical column. Non-polar compounds were extracted by the first precolumn whereas the moderately and relatively polar compounds were recovered from both precolumns. The ratio of amounts preconcentrated on C_{18} and polymer (PRP-1) precolumns and variations of this ratio with the sample volume served as useful information for the identification of solutes (Fig. 6). Nielen *et al.* [45] used for the preconcentration and fractionation of organic pollutants in

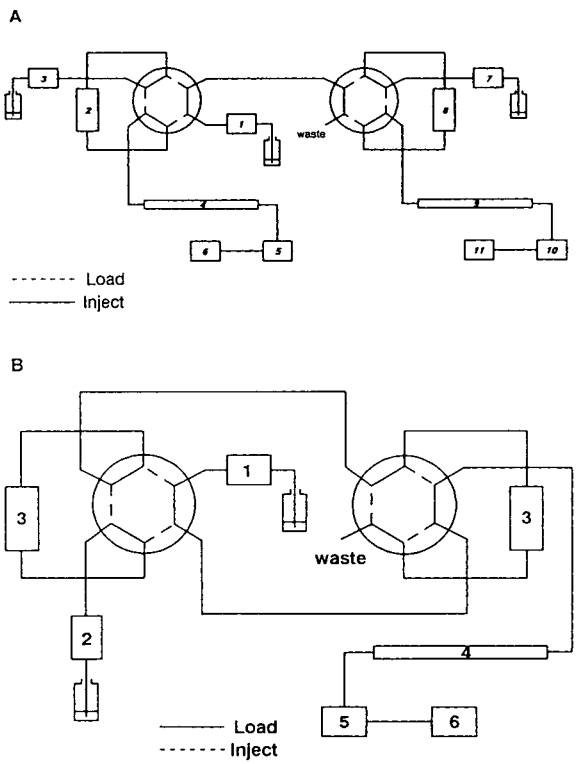

Fig. 5. Two precolumn on-line systems with (A) two or (B) one analytical column. The system components are as follows: (A) 1 = sample pump; 2, 8 = precolumns; 3, 7 = mobile phase pumps; 4, 9 = analytical columns; 5, 10 = detectors; 6, 11 = recorders/computers; (B) 1 = mobile phase pump; 2 = sample pump; 3 = precolumns; 4 = analytical column; 5 = detector; 6 = recorder/computer.

industrial effluents three precolumns in series (C_{18}, PRP-1 and cation exchanger) and eluted all precolumns separately. Brouwer *et al.* [46] connected two polymer (PLRP-S) precolumns in series and the outlet of each precolumn was directed on-line to a separate PLRP-S analytical column. While the first precolumn was operated in the reversed-phase mode, the trace enrichment in the second precolumn was based on an ion-pairing mechanism. This allowed the preconcentration of acidic and basic compounds within one analysis. The same group developed a similar system with one HPLC analytical column [47]. In this system they used as precolumns specially designed holders packed with membrane extraction discs. The advantage of this type of precolumn packing was that its amount

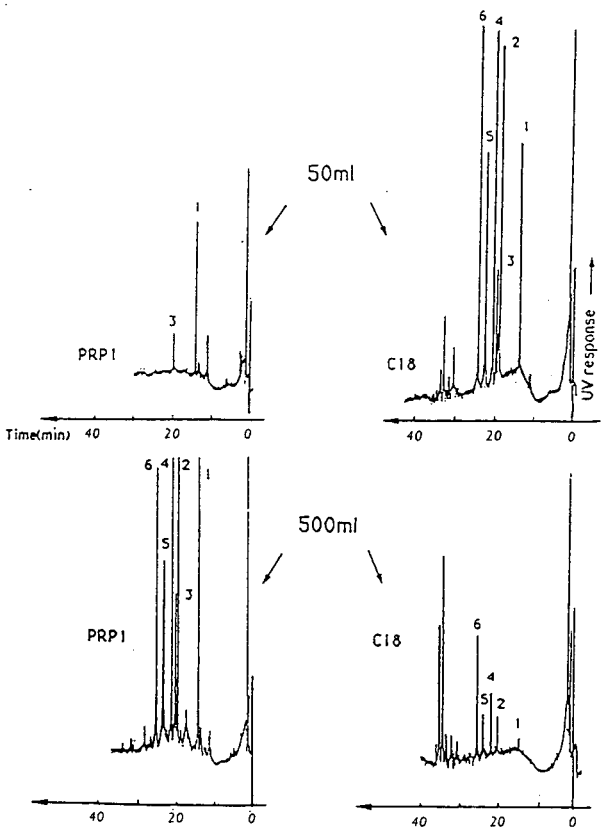

Fig. 6. On-line preconcentration of two samples of different volumes (50 and 500 ml) of a standard solution of phenylureas. The amount of the compounds is the same in the two samples (different concentrations). Solutes: 1 = metoxuron (0.83 μg); 2 = monolinuron (0.51 μg); 3 = buturon (0.83 μg); 4 = chlortoluron (0.89 μg); 5 = diuron (1.09 μg); 6 = linuron (1.18 μg). Preconcentration through two precolumns (10 × 2.1 mm I.D.) in series packed with RP-18 silica (10 μm) at a flow-rate of 3 ml/min; elution to the analytical column (150 × 4.6 mm I.D.) packed with ODS-2 silica (5 μm) at a flow-rate of 1.5 ml/min; mobile phase, acetonitrile gradient with a solution of 0.1 *M* potassium acetate–acetic acid (pH 6) and acetonitrile from 15% acetonitrile from time 0 to 5 min, 20% at 8 min, 50% at 25 min and 80% at 35 min; UV detection at 278 nm, sensitivity 0.01 AUFS. From ref. 43 (© Gordon and Breach).

was easily adjustable in accordance with the needs of a particular application.

The advantage of the high sample throughput can be demonstrated by an automated on-line system such as that developed by Ramsteiner [48]. This system allowed the selection of the sample volume to be preconcentrated and unattended processing of up to 32 water samples. He found this method to be advantageous from the viewpoints of sensitivity, rapid sample handling and costs when large monitoring programmes were to be performed and he demonstrated its superiority over previously used labour-intensive off-line techniques.

The need for continuous monitoring of water quality initiated the development of rapid screening on-line SPE–HPLC methods with UV diode-array detection. The use of on-line systems for this purpose allowed rapid access to information on water quality and a relatively high frequency of sampling. Reupert *et al.* [49] applied C_{18} materials for on-line preconcentration. They compared different C_{18} reversed phases with respect to their retention capacity. Their method was optimized for about 40 individual substances, mostly triazines and phenylureas.

The use of C_{18}, however, does not provide satisfactory results when compounds with higher polarity are to be preconcentrated. In this case the use of polymeric material in the precolumn increases the breakthrough volumes and, despite the fact that the k' values of analytes on the polymer are higher than those on C_{18}, its contribution to additional band broadening on a C_{18} analytical column is still acceptable. Such a combination of sorbents (*i.e.*, polymer in the precolumn and C_{18} in the analytical column) was tested in analyses for more than 50 compounds in aqueous matrices and for most of these compounds the results obtained were satisfactory [41]. The PLRP-S–C_{18} SPE–HPLC system with UV diode-array detection developed in that work was able to detect a wide range of water pollutants at the low- to sub-μg/l concentration level in less than 100 min (Fig. 7). After automation and optimization of all steps, this system was demonstrated to be a powerful tool for the rapid screening of surface water quality and its efficiency had already been recognized by its practical use in a surface water quality monitoring station [50].

The on-line coupling of SPE to GC is more complicated than that to HPLC owing to problems with the liquid phase–gas phase interface. However, interest in on-line LC–GC coupling is continuously increasing, as can be seen from the number of LC–GC applications in the review

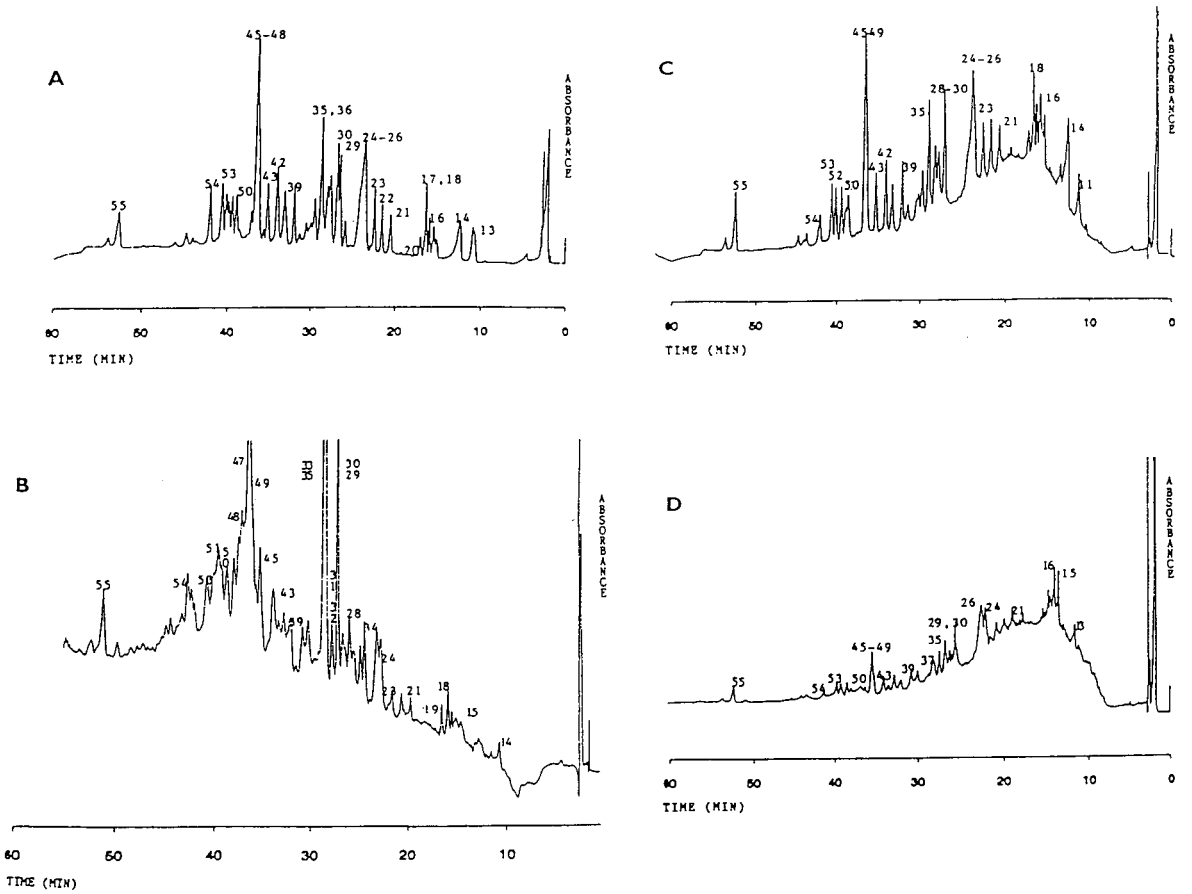

Fig. 7. Chromatograms of (A) HPLC-grade water spiked with the test compounds at concentrations of 2.5–5 μg/l, (B) HPLC-grade water spiked with the same compounds at a 25 times lower concentration, (C) non-buffered river Rhine water spiked with the test compounds at concentrations of 2.5–5 μg/l and (D) non-buffered river Rhine water spiked with the test compounds at a five times lower concentration. Detector scale 0.1 AUFS. Test compounds: 1 = diquat; 2 = paraquat; 3 = maleic hydrazide; 4 = ethylenethiourea; 5 = propylenethiourea; 6 = metham sodium; 7 = aniline; 8 = methyl isothiocyanate; 9 = aldicarb sulphone; 10 = benzenesulphonamide; 11 = asulam; 12 = oxamyl; 13 = fenaminosulf; 14 = carbendazim; 15 = metamitron; 16 = isocarbamide; 17 = 2,6-dimethylaniline; 18 = chloridazon; 19 = dimethoate; 20 = dicamba; 21 = aldicarb; 22 = bromacil; 23 = simazine; 24 = 2-chloroaniline; 25 = 2-nitrophenol; 26 = benzothiazole; 27 = bentazon; 28 = atrazine; 29 = 2,6-dichlorophenol; 30 = bromoxynil; 31 = thiram; 32 = diuron; 33 = triclopyr; 34 = monolinuron; 35 = DNOC; 36 = propachlor; 37 = dichlorprop; 38 = mecoprop; 39 = warfarin; 40 = metazachlor; 41 = linuron; 42 = 3,3-dichlorobenzidine; 43 = sethoxydim; 44 = coumafuryl; 45 = 2,4,5-trichloroaniline; 46 = captan; 47 = alachlor; 48 = metolachlor; 49 = barban; 50 = alloxydim sodium; 51 = dinoterb; 52 = dinoseb; 53 = pentachlorophenol; 54 = phoxim; 55 = permethrin. From ref. 41 (© Gordon and Breach).

paper by Davies *et al.* [51]. SPE–GC, which is also referred to as trace enrichment LC–GC, has been used for the determination of pesticides in water [52]. For that purpose a six-port switching valve was modified to incorporate an internal microcolumn packed with C_8-bonded silica. Pesticides were adsorbed on the precolumn from an aqueous sample, the precolumn was then dried by helium purging and vacuum and the analytes were eluted with hexane directly to a GC column using a retention gap. In the SPE–GC system developed by Van der Hoff *et al.* [53], the SPE precolumn served for the clean-up of the LLE extract prior to GC analysis. This approach of on-line clean-up of LLE extracts was developed by the same group also for HPLC analyses of environmental pollutants [54–57]. They used precolumn switching in combination with off-line

LLE and obtained a considerable selectivity enhancement. The main advantage of this approach, in which the on-line coupled precolumn was used only for clean-up purposes, was the removal of the large matrix peak from the chromatogram (this peak is a frequent interference in on-line SPE–HPLC systems). Even though the off-line extraction required a time-consuming manual operation, owing to the application of column switching a considerable gain in sample throughput was achieved.

The application of a sophisticated clean-up procedure is not the only possibility for achieving high selectivity. The alternative way is to use a highly selective solid phase, *e.g.*, ion exchangers, metal-loaded sorbents or a phase with immobilized enzymes. The procedures utilizing these materials are complicated and require a skilled operator, but their efficiency and suitability for special target analyses has been demonstrated by many workers [8,37,58].

Limitations

Loading of the whole amount of an analyte from the water sample on to the precolumn is usually associated with the delivery of a large number of other matrix components, unless a thorough clean-up is performed. Such a clean-up operation, however, prolongs the analytical procedure and makes it more complicated. It must be also pointed out that the addition of any further operation to the basic preconcentration cycle increases the probability of losses of analytes. Moreover, from the technical and financial standpoints, the addition of further steps to the procedure requires the incorporation of additional pumps, valves and other equipment.

Manipulation with sophisticated on-line systems also requires adequately trained technical personnel. Even though the fully automated systems can be operated, with certain simplifications, by pressing the "start" button only, troubleshooting of any minor malfunctions appearing in such a system will certainly require a skilled operator.

In on-line applications, prior to forcing the water sample through the precolumn, this sample is usually filtered to remove the suspended solid particles in order to prevent clogging of the precolumn. As a consequence, part of the analyte adsorbed on these particles cannot be trapped by the solid phase in the precolumn, and it is therefore lost for further analysis.

Using an on-line sample-handling procedure for polar compounds, it is possible to reach a relatively high preconcentration factor owing to the transfer of the whole mass of an analyte from the processed sample on to the precolumn. On-line preconcentration of polar compounds enables detection limits at low-ppb to high-ppt concentration levels to be achieved much more easily and faster than when the off-line approach is used. However, to reach the low-ng/l level, the volume of water sample to be processed has to be so large that the operation of small on-line precolumns is often problematic owing to interferences, clogging, time needed for sorption, etc. Hence for such applications many analysts prefer the use of larger off-line cartridges, which allow the processing of large volumes and which can sustain higher flow-rates. The increased volume of the eluate from a large SPE column is then reduced by partial evaporation.

Major problems can also occur during the on-line preconcentration of non-polar compounds. The very low detection limits required for these compounds generate similar difficulties with the processing of large sample volumes to those mentioned above. Moreover, the strongly hydrophobic character of such analytes causes their strong adsorption within the whole preconcentration system, which leads to so-called memory effects with the risk of obtaining false-positive results. The elimination of memory effects through extensive flushing is not always efficient. It is clear that off-line procedures that utilize disposable cartridges are more protected against memory effects.

The low flexibility in setting the desorption conditions in on-line configurations is another problem which has to be coped with when one tries to obtain an elution profile in the precolumn that is as narrow as possible and optimum separation in the analytical column. The composition of the mobile phase in an on-line SPE–HPLC system has to be primarily adjusted in accordance with the requirements for a good separation in the analytical column, hence the best solution for improving the shape of the desorption curve (*i.e.*, minimization of additional

band broadening) is focusing via an appropriate gradient profile of the mobile phase in LC.

CONCLUSIONS

Even though the development of environmental analytical chemistry towards rapid and efficient methods has led to an increase in interest in automated on-line sample-handling procedures, the high versatility and simplicity of off-line SPE often makes this approach a method of choice for a particular application. Considering the character of the reviewed applications it can be assumed that the off-line procedures are to be preferred when a complex analytical survey on water quality, requiring complicated fractionations and/or the use of several separation and detection techniques, is to be performed. The off-line approach is also often advantageous when a simple, inexpensive target method is required which can be executed in any common laboratory and also under field conditions.

In the future, the major part of routine off-line target methods will probably be gradually replaced by automated techniques. However, because of their great flexibility and simplicity, the off-line procedures will always be a valuable tool primarily in the area of analytical research and in diverse on-site applications.

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Influence of the chromatographic capacity factor (log k') as an index of lipophilicity in the antibacterial activity of a series of 6-fluoroquinolones

Relationship between physico-chemical and structural properties and their hydrophobicity☆

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ABSTRACT

The aim of this study was to establish the influence of lipophilicity on the antibacterial activity (log $1/MIC_{50}$) of 22 fluoroquinolones and to assess the influence of their electronic, steric and topological properties on their hydrophobicity. The lipophilicity of the compounds, expressed as the chromatographic capacity factor (log k'), was determined by ion-pair reversed-phase HPLC. On the basis of the mathematical models developed, an attempt was made to confirm the mechanism of interaction of the quinolones with DNA-gyrase proposed previously.

INTRODUCTION

Fluoroquinolones are a family of antibacterial agents extensively used in both human and veterinary clinics. They are bactericides and act by inhibiting bacterial DNA-gyrase [1].

In 1962, Lesher *et al.* [2] isolated nalidixic acid (Fig. 1) as a by-product in the synthesis of chloroquine, and 2 years later it was introduced into general practice for the treatment of urinary infections [3]. Later, structural modifications were made to the basic skeleton of nalidixic acid and new derivatives were synthesized, *e.g.*, ox-

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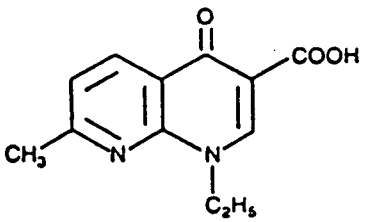


Fig. 1. Nalidixic acid.

olinic acid, piperamic acid and piromidic acid. All these quinolinecarboxylic acids constitute the first-generation quinolone series.

In 1973, Gerster synthesized flumequine [4]. For the first time the C-6 position was functionalized with a fluorine atom, thus constituting the first synthesis of a fluoroquinolone. Its antibacterial activity surpassed that of the first-generation quinolones, and this molecule formed a bridge between the first and third generations. Flumequine was included in the second generation of quinolones.

In 1980, Koga [5] synthesized norfloxacin, which was the first fluoroquinolone of the third generation. Two structural modifications were performed in this synthesis which are fundamental for the antibacterial activity of these drugs: the C-6 position was functionalized with a fluorine atom and the C-7 position was functionalized with an aliphatic cyclic amine (piperazine N-alkylpiperazine, etc.). It was soon realized that these compounds were much more active *in vitro* and that they showed a broader range of antibacterial activity. In contrast to quinolones of the first generation, the fluoroquinolones showed a systemic action *in vivo*. From the pharmacokinetic point of view, their absorption is excellent when taken orally and their metabolization is slight [6–11].

Since then, many pharmaceutical companies began to synthesize new fluoroquinolonic derivatives, carrying out functional and isosteric substitutions in the basic skeleton of 3-quinolinecarboxylic acid. Some of these derivatives are now commercially available in human and veterinary clinics, *e.g.*, norfloxacin, ciprofloxacin and enrofloxacin, and others are at an advanced clinical stage.

The aim of this work was to study the influence of the lipophilicity of these compounds, expressed as the chromatographic capacity factor (log k') (determined by ion-pair reversed phase HPLC) on their antibacterial activity (log $1/MIC_{50}$) by means of the establishment of the best simple linear relationship.

Budvári-Bárány and Szász [12] have correlated log k' with the logarithm of the partition coefficient at pH 7.40 for a series of fluoroquinolones. From the results they concluded that it is possible to use log k' as a descriptor of lipophilicity in quantitative structure–activity relationship (QSAR) studies for this series.

Numerous papers have described the use of reversed-phase HPLC to determine quinolones using ultraviolet and fluorescence detectors. The same authors Budvári-Bárány and Szász [12] indicated the need to use the ion-pair technique, determining the differences between cetrimide and sodium hexanesulphonate.

We also aimed to study the influence of electronic, steric and topological descriptors on hydrophobicity by means of a multiple linear relationship between the representative parameter of lipophilicity (log k') and the electronic, steric and topological parameters studied.

The electronic descriptors used are normally the net charges associated with each atom of a molecule, dipolar moment and HOMO and LUMO molecular orbits. Molecular surface area and volume are the steric descriptors most frequently used. Of the topological descriptors, the most usual is the molecular connectivity index of order i $^{i}\chi$, first introduced by Kier and Hall [13]. These indices allow the parameterization of a determinate chemical structure as a function of its chains, rings and branches. We thus aimed to confirm the mechanism of interaction of the quinolones with bacterial DNA-gyrase proposed by Shen and Mitsher [14].

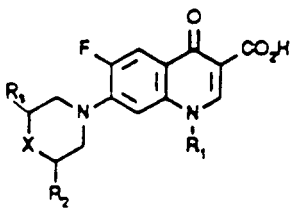
EXPERIMENTAL

Quinolones

Following the method described by Koga [5], 22 fluoroquinolones were synthesized in our laboratory. All intermediates used in the synthesis were of synthesis quality. All products were properly identified and analysed by IR and

TABLE I

CHEMICAL STRUCTURE OF THE 22 FLUOROQUINOLONES ASSAYED



Quinolone	R_1[a]	R_2	R_3	X
1	cC_3H_5	H	H	NH
2	cC_3H_5	H	H	NCH_3
3	cC_3H_5	H	H	NC_2H_5
4	cC_3H_5	H	H	CH_2
5	cC_3H_5	H	H	O
6	cC_3H_5	H	H	HCH_2CH_2OH
7	C_2H_5	H	H	NH
8	C_2H_5	H	H	NCH_3
9	C_2H_5	H	H	NC_2H_5
10	C_2H_5	H	H	O
11	C_2H_5	H	H	CH_2
12	cC_2H_5	CH_3	H	NH
13	C_2H_5	CH_3	H	NH
14	cC_3H_5	H	H	$CHCH_3$
15	cC_3H_5	CH_3	H	CH_2
16	cC_3H_5	H	CH_3	CH_2
17	cC_3H_5	H	H	S
18	C_2H_5	H	H	$CHCH_3$
19	C_2H_5	CH_3	H	CH_2
20	C_2H_5	H	CH_3	CH_2
21	C_2H_5	H	H	S
22	C_2H_5	H	H	NC_6H_5

[a] c = Cyclo.

NMR spectroscopy. Their structures are shown in Table I.

Materials

For the determination of MIC_{50}, Müller–Hinton agar was supplied by DIFCO (Detroit, MI, USA). HPLC-grade methanol was supplied by Merck (Darmstadt, Germany), analytical-reagent grade phosphoric acid 85% by Probus (Barcelona, Spain), HPLC-grade sodium heptanesulphonate by Scharlau (Barcelona, Spain) and analytical-reagent grade sodium hydroxide, methanol, potassium dihydrogenphosphate and potassium dichromate by Jansen Chimica (Geel, Belgium).

The analytical column (300 × 2 mm I.D.), filled with 10-μm C_{18}-functionalized silica particles, was μBondapak C_{18}, supplied by Waters (Milford, MA, USA).

Equipment

A Model PU 9700 IR spectrophotometer (Pye Unicam, Cambridge, UK) and a Model AW 80 NMR spectrometer (Bruker, Karlsruhe, Germany) were used for the identification of molecular structures. Petri plates for the MIC_{50} assay were grown in an oven (Selecta, Barcelona, Spain). Theoretical descriptors were calculated using a PC/AT computer (Olivetti, Ivrea, Italy), equipped with a math coprocessor. The HPLC equipment consisted of two Model 110B pumps, a Model 406 analogue interface, an injector with a 20-μl loop and a Model 167 variable-wavelength UV detector (Beckman Instruments, San Ramon, CA, USA). The chromatographic apparatus was controlled and the analogue signals were analysed using System Gold software (Beckman Instruments) installed in a PC/AT computer (Syswest, Barcelona, Spain).

Microorganisms and assay of antibacterial activity (MIC_{50})

Log $1/MIC_{50}$ was used as a descriptive parameter of antibacterial activity, where MIC_{50} is the minimum concentration that inhibits 50% of the bacterial strains analysed. We used 100 strains of *Escherichia coli* (kindly supplied by the Department of Microbiology at the Faculty of Medicine, Universitat Autònoma de Barcelona, Spain) to determined the value of MIC_{50}, and the assay was validated using the following typified strains: *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 2592, *Streptococcus faecalis* ATCC 29212 and *Staphylococcus aereus* ATCC 219213.

Antibacterial activity was determined using the method of serial dilutions in agar, following the specifications of the National Committee for Clinical Laboratory Standards [15]. The inoculum was applied to Müller–Hinton agar plates containing serial quantities of quinolone, using a

Steers replicator [16]. The plates were incubated at 37°C for 20 h and inspected immediately.

Chromatography and calculation of lipophilicity

The chromatographic capacity factor (log k') obtained by reversed-phase HPLC was used as an index of lipophilicity. It is defined as $k' = (t_r - t_0)/t_0$, where t_0 is the retention time of a control substance, which theoretically is not retained in the column, and t_r is the retention time of the analyte substance.

The isocratic mobile phase was composed of methanol–water (1:1), 25.5 m*M* potassium dihydrogenphosphate and 1 m*M* sodium heptanesulphonate, with the pH adjusted to 2.75 with 85% phosphoric acid. The flow-rate was 1 ml/min and the injection volume 20 μl. Absorption was monitored at 270 nm.

Approximately 0.3 m*M* of each quinolone was dissolved in 10 ml of 0.1 *M* NaOH, 10 ml of methanol were added and the volume was made up to 100 ml with water. A 1-ml aliquot was taken and diluted to 50 ml with water. Ten injections were made under the analytical conditions stated, with the aim of obtaining a mean value. In order to obtain the value of t_0, a 0.3 m*M* solution of potassium dichromate was used as a control. The deviation of the results was no greater than 8% for either t_r or t_0.

Calculation of electronic descriptors

The net charges associated with each atom of the molecules, the values of the dipolar moment and the energies of the HOMO and LUMO molecular orbits were calculated using the AM1 semi-empirical method [17], once the molecular geometry had been optimized. These calculations were performed on an IBM 3090-170 computer in the Physico-Chemical Section of the Faculty of Chemistry at Tarragona (Spain).

Calculation of steric descriptors

Molecular surface area and volume were calculated with the MOLSV program [18] in a version for PC-compatible computer, adapted by K.J. Tupper at the University of Indiana from the program developed by G.H. Smith. The program was run on a PC/AT computer. The cartesian coordinates introduced in the program corresponded to those structures with optimized geometry, based on the data obtained from calculations with the AM1 semi-empirical method.

Calculation of topological descriptors

The molecular connectivity indices of order i $^i\chi$ were calculated with the INDICES program, and the molecular connectivity indices of valency of order $^i\chi^v$ were calculated with the CONIND program, both running on a PC/AT-compatible computer.

RESULTS

A representative chromatogram, obtained from quinolone **12**, is shown in Fig. 2.

In order to study the influence of lipophilicity on the antibacterial activity of fluoroquinolones, a simple linear correlation was performed, in which antibacterial activity represented by the logarithm of $1/MIC_{50}$ was considered as the dependent variable and lipophilicity, as represented by log k', was considered as the independent variable. The calculation was performed using the linear regression program LREGR.

The values of log $1/MIC_{50}$ *vs.* the values of log k' are shown in Fig. 3. The linear correlation which best fits the experimental results corresponded to the following mathematical expression:

$$\log \frac{1}{MIC_{50}} = -0.8161\,(\text{S.D.} = \pm 0.1074) \cdot \log k' + 0.3700\,(\text{S.D.} = \pm 0.0900)$$

$$n = 22\,; \quad r = -0.8616\,; \quad F \text{ ratio} = 0.000\%$$

The theoretical and experimental values of log $1/MIC_{50}$ for the series of quinolones studied are shown in Table II, together with the residual values.

In order to study the influence of electronic, steric and topological descriptors on the lipophilicity of fluoroquinolones, a multiple linear correlation was performed in which lipophilicity represented by log k' was considered as the dependant variable and the following were considered as independent variables: the net charges

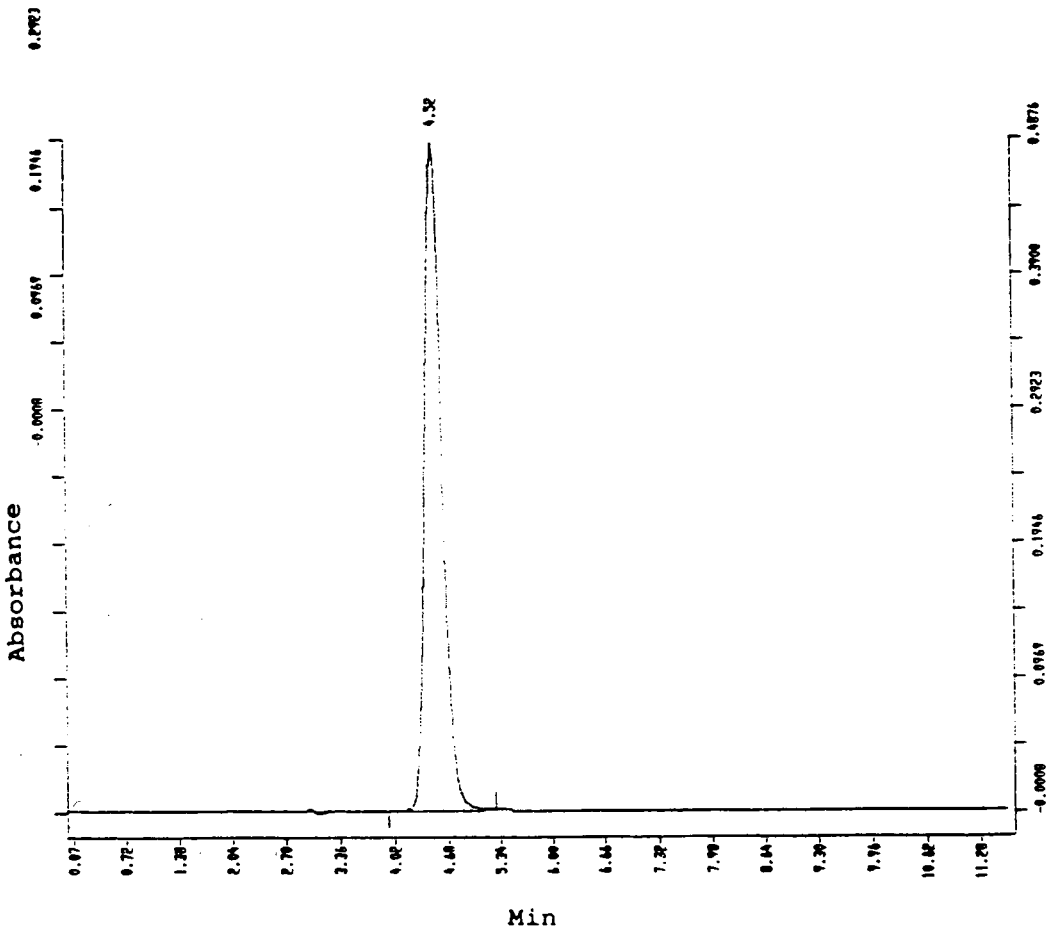


Fig. 2. Representative chromatogram of quinolone **12**.

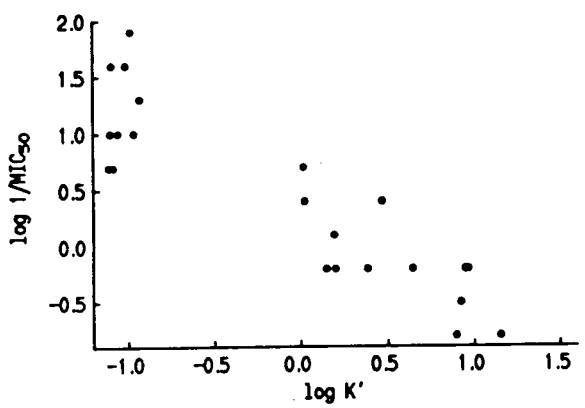


Fig. 3. Value of log $1/MIC_{50}$ *vs.* the value of log k'.

associated with the atom (QX_i), where X_i is the atom situated at position i, the dipolar moment (DM), the HOMO and LUMO molecular orbits, the molecular surface area (MS) and volume (MV) and the valence and non-valence molecular connectivity indices and their difference ($^{i}\chi^{v}$, $^{i}\chi$, DIF $^{i}\chi$).

Of all the electronic, steric and topological descriptors estimated for all the quinolones assayed, the values of those descriptors which, on the basis of the matrix correlation, are linearly independent are MV, QN_1, QC_7, QN_{16}, QX_{24}, DM and DIF $^{3}\chi_c$.

With the aim of obtaining this multiple linear correlation, the calculation was performed using the stepwise linear regression analysis program

TABLE II

THEORETICAL AND EXPERIMENTAL VALUES OF LOG $1/MIC_{50}$ FOR THE SERIES OF QUINOLONES STUDIED

Quinolone	Log $1/MIC_{50}$		Difference (%)[a]
	Experimental	Theoretical	
1	1.6020	1.2396	23
2	1.6020	1.1730	27
3	1.3010	1.1042	15
4	0.0969	0.1694	−75*
5	0.6980	0.3158	55
6	0.6980	1.2323	−77*
7	1.0000	1.2089	−21
8	1.0000	1.1336	−13
9	0.6980	1.2549	−80*
10	0.3979	0.3109	22
11	−0.2040	−0.2021	−1
12	1.9030	1.1486	40
13	1.0000	1.2465	−25
14	−0.2040	−0.4557	−123*
15	−0.2040	−0.4694	−130*
16	−0.2040	0.1638	20
17	0.3979	−0.0563	114*
18	−0.5050	−0.4337	14
19	−0.8060	−0.4112	49
20	−0.2040	0.2072	202*
21	−0.2040	0.0150	107*
22	−0.8060	−0.6140	24

[a] For explanation of the values marked with asteriks, see Discussion.

TABLE III

DESCRIPTORS CORRESPONDING TO THE MATHEMATICAL EXPRESSION RESULTING FROM THE MULTIPLE LINEAR CORRELATION

QX_{24} = net charge associated with the heteroatom X situated at position 4 of the heterocyclic aliphatic ring which functionalizes position C-7; MD = dipolar moment; DIF $^3\chi_c$ = difference between the molecular connectivity index of valency and non-valency of order 3 cluster.

Quinolone	QX_{24}	DM	DIF $^3\chi_c$
1	−0.2895	10.769	0.354
2	−0.2635	9.715	0.452
3	−0.2633	9.543	0.434
4	−0.1605	9.775	0.388
5	−0.2526	9.020	0.388
6	−0.2408	7.901	0.434
7	−0.2927	10.721	0.355
8	−0.2271	10.786	0.420
9	−0.2536	10.877	0.401
10	−0.2572	9.170	0.355
11	−0.1591	9.782	0.355
12	−0.2866	10.769	0.548
13	−0.2950	10.328	0.439
14	−0.0933	9.785	0.387
15	−0.1492	9.770	0.387
16	−0.1523	10.043	0.435
17	−0.0318	9.037	0.463
18	−0.0990	8.543	0.355
19	−0.1488	10.286	0.355
20	−0.1510	9.942	0.467
21	−0.0477	9.498	0.355
22	−0.2240	5.810	0.375

SLREG (part of Labsware computational package; Compudrug, Budapest, Hungary). The multiple linear correlation which best fits the experimental data was that corresponding to the following expression:

$$\log k' = 6.8448\ (\text{S.D.} = \pm 1.3184) \cdot QX_{24} - 0.2249\ (\text{S.D.} = \pm 0.0933) \cdot DM - 4.0366\ (\text{S.D.} = \pm 2.1135) \cdot \text{DIF}\ ^3\chi_c + 5.0421$$

$$n = 22; \quad r = 0.8543; \quad F \text{ ratio} = 0.002\%$$

where QX_{24} is the negative net charge associated with the heteroatom X situated at position 4 of the heterocyclic aliphatic ring which functionalizes position C-7, DM is the dipolar moment and DIF $^3\chi_c$ is the difference between the molecular connectivity index of valency and non-valency of order 3 cluster (Table III).

Table IV shows the theoretical and experimental values of log k' for the quinolone series studied, together with the residual values.

DISCUSSION

The antibacterial activity of the fluoroquinolones is related to lipophilicity, although differences are noted in some members of the series (marked with asterisks in Table II). Under the analytical conditions used there may be some kind of interaction of these compounds with the

TABLE IV

THEORETICAL AND EXPERIMENTAL VALUES OF LOG k' FOR THE SERIES OF QUINOLONES SERIES STUDIED TOGETHER WITH THE RESIDUAL VALUES

Quinolone	Log k'		Difference (%)[a]
	Experimental	Theoretical	
1	−1.0936	−0.7830	28
2	−1.0132	−0.8108	101
3	−0.9303	−0.6836	26
4	0.1972	0.2770	−40
5	0.0206	−0.2608	1366
6	−1.0847	−0.1340	88
7	−1.0565	−0.8015	24
8	−0.9657	−0.6217	36
9	−1.1120	−0.7560	32
10	0.0265	−0.1724	750
11	0.6453	0.4444	31
12	−0.9838	1.6905	272
13	−1.1019	−1.1319	−3
14	0.9512	0.7981	12
15	0.9677	0.3243	66
16	0.2040	0.0536	74
17	0.4695	1.0796	−130
18	0.9247	1.1918	−29
19	0.8975	0.4085	54
20	0.1516	−0.0665	144
21	0.3871	1.3780	256
22	1.1530	0.7640	34

residual silanol groups on the column, which might explain these differences.

According to Shen and Mitsher [14], the fluoroquinolones have three domains of action (Fig. 4): domain of hydrogen bridges with the bases of DNA; domain of hydrophobic interactions between N-alkyl groups and quinolones joined to each of the two strands of DNA; and domain of interaction with DNA-gyrase. The heterocycle that functionalizes position C-7 belongs to the last domain. An increase in the net negative charge associated with the heteroatom X_{24} produces a decrease in the lipophilicity of the fluoroquinolones and consistently an increase in the antibacterial activity. This increase in net negative charge follows the order, $S < C < O < N$. On the other hand, the

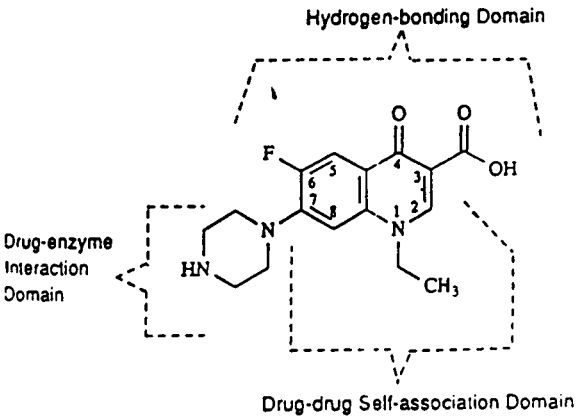


Fig. 4. Domains of action of the fluoroquinolones [14].

increase in antibacterial activity follows the order $C < S < O < N$. The increase in the value of the dipolar moment (DM) of the fluoroquinolones leads to a decrease in lipophilicity and also a consequent rise in antibacterial activity.

It thus appears that an increase in net negative charge associated with the heteroatom X_{24} favours the interaction of fluoroquinolones with DNA-gyrase, in such a way that the increase in the density of the negative charge of the heteroatom X_{24} would stabilize the interaction with DNA-gyrase.

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Elution behaviour of styrene–acrylonitrile copolymers in high-performance liquid chromatography with mixtures of chloroform and *n*-hexane as mobile phases

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ABSTRACT

With isocratic elution of styrene–acrylonitrile copolymers from an ODS silica column using chloroform–*n*-hexane mixtures as mobile phases, the elution profiles of the copolymers with an acrylonitrile content from 15 to 33% showed four different patterns, depending on the compositions of the copolymers and the mobile phases: (i) elution with a retention volume corresponding to the interstitial volume of the column; (ii) elution with a retention volume corresponding to the sum of the interstitial volume and the pore volume of the packing materials in the column; (iii) elution with both retention volumes; and (iv) precipitation on the column. A transition period was found during the process in which the copolymer which dissolved in a good solvent precipitated when the latter became a poor solvent on adding a non-solvent, and this was defined as the pre-precipitation state. Each molecule was in a condensed form in this state. In order to separate the copolymers according to composition, gradient elution from 100% *n*-hexane to 100% chloroform, in 30 min was performed, and the copolymers were separated in order of increasing the acrylonitrile content. No molecular mass dependence of the separation was observed.

INTRODUCTION

Synthetic random copolymers, in general, show a molecular mass distribution and a chemical composition distribution, and the determination of both distributions is important for the characterization of the copolymers. Several attempts have been made to determine chemical composition distribution by high-performance liquid chromatography (HPLC). For example, poly(styrene–methyl acrylate) [1], poly(styrene–methyl methacrylate) [2–4], poly(styrene–acrylonitrile) [5] and poly(styrene–butadiene) [6] random copolymers have been separated according to their composition by HPLC.

Styrene–acrylonitrile random copolymers, P(S–AN), are of technical importance and the determination of their chemical heterogeneity is of great interest. High-performance precipitation liquid chromatography (HPPLC), developed by Glöckner *et al.* [5], was first applied to the separation of P(S–AN) copolymers according to composition. HPPLC uses a mixture of a good solvent and a non-solvent for the copolymer concerned and the initial mobile phase consists of a non-solvent (isooctane in Glöckner *et al.*'s work) and, first, the copolymer which is dissolved in a good solvent and is injected into the chromatographic system is precipitated on top of the column. By applying gradient elution with a good solvent (tetrahydrofuran in their work), the copolymer is redissolved and is eluted according to its solubility (and composition).

The separation of P(S–AN) copolymers by HPPLC was found to be dependent on the stationary phase and the composition of the mobile phase [7]. By applying normal-phase

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gradient elution from *n*-heptane to dichloromethane with RP-C_{18} or polystyrene gel columns, the copolymers were separated in order of increasing acrylonitrile content, but the peaks were broad. Separation according to composition was also possible by normal-phase gradient elution from *n*-heptane–dichloromethane (20:80) to dichloromethane–2.75% methanol using 500-Å pore-size silica gel and the peaks were almost symmetrical.

In this work, the elution behaviour of P(S–AN) in HPPLC with mixtures of chloroform and *n*-hexane as mobile phases was evaluated. The copolymer was dissolved in chloroform in all instances. With isocratic elution with *n*-hexane–chloroform, the transition from elution from a column to precipitation on the column via the pre-precipitation state was clearly observed. Separation according to composition was possible by gradient elution from *n*-hexane to chloroform.

EXPERIMENTAL

The apparatus used was a Jasco (Tokyo, Japan) Trirotar-VI high-performance liquid chromatograph with a Uvidec-100 VI ultraviolet absorption detector operated at 260 or 270 nm. The packing material was ODS silica (Develosil) (Nomura Chemical, Aichi, Japan) packed in a column of 250 mm × 4.6 mm I.D. The number of theoretical plates was 2400, obtained by injecting 0.1 ml of a 0.1% solution of benzene in chloroform. The column was thermostated at 25°C in a Model AO-30C column oven (Showa Denko, Tokyo, Japan).

The samples used were styrene–acrylonitrile copolymers prepared by suspension polymerization and supplied by Mitsubishi Monsanto (Yokkaichi, Japan). The acrylonitrile contents of these copolymers were measured by nitrogen determination and were as follows: P(S–AN)-15, 14.8% (w/w); P(S–AN)-20, 19.6% (w/w); P(S–AN)-27, 26.2% (w/w); and P(S–AN)-33, 32.3% (w/w). These samples were dissolved in chloroform at a concentration of 0.05 or 0.1%. Chloroform is a good solvent for the copolymers and can dissolve them completely. The injection volume into the column was 0.1 ml, which was a reasonable size in this experiment: no difference was observed in the elution behaviour between sample sizes of 0.1 and 0.01 ml, the peak being difficult to recognize in the latter instance. Polystyrenes (PS) having narrow molecular mass distributions (Pressure Chemical, Pittsburgh, PA, USA) were used for the measurement of the molecular mass dependence of the HPPLC separation.

The mobile phases were chloroform, *n*-hexane and their mixtures. Chloroform contained 1% of ethanol as a stabilizer and henceforth chloroform where mentioned contains 1% of ethanol unless specified otherwise. Elution was performed by isocratic or gradient elution. The flow-rate was 0.5 ml/min and the pressure drop of the column system was between 21 and 23 kg/cm^2 during elution.

RESULTS AND DISCUSSION

Isocratic elution

Samples were dissolved in chloroform, which was a good solvent for the copolymers. The sample solutions were introduced into the LC system where good or poor eluents for the sample copolymers were used as the mobile phases. When good eluents for the copolymers were used, they eluted at V_0 (= 2.0 ml) [the exclusion limit in size-exclusion chromatography (SEC)] and when poor eluents were used, they eluted at $V_0 + V_i$ (= 3.0 ml) (V_i = pore volume) or precipitated on the top of the column.

When chloroform was used as the mobile phase, all the copolymers eluted at a retention volume V_R = 2.0 ml. With up to 20% of *n*-hexane in chloroform, the copolymers eluted at V_R = 2.0 ml. The elution behaviour of the copolymers obtained with increasing *n*-hexane content in the mobile phase is discussed below. All the measurement were monitored by UV detection at 260 nm.

P(S–AN)-33. When the composition of the mobile phase was chloroform–*n*-hexane (70:30, v/v), the intensity of the peak at V_R = 2.0 ml decreased and a small peak was observed at V_R = 3.0 ml. At the composition chloroform–*n*-hexane (60:40, v/v), the peak at V_R = 2.0 ml disappeared and only the second peak at V_R =

3.0 ml was observed. On increasing the *n*-hexane content to 60% or 70%, the peak intensity at $V_R = 3.0$ ml became significantly larger. When *n*-hexane alone was used as the mobile phase, the second peak at $V_R = 3.0$ ml disappeared and only a small peak due to the solvent used for the sample solution was observed. All the copolymer precipitated on the column. The results are shown in Fig. 1. The small peak at $V_R = 3.0$ ml in Fig. 1a is not a sample peak but that due to the solvent used for sample solution.

P(S–AN)-27. With up to 30% of *n*-hexane in the mobile phase, all the copolymer eluted at $V_R = 2.0$ ml. With chloroform–*n*-hexane (60:40, v/v), the intensity of the peak decreased and a second peak appeared at $V_R = 3.0$ ml. At the composition chloroform–*n*-hexane (50:50, v/v), the first peak disappeared and only the second peak was observed. With chloroform–*n*-hexane (30:70, v/v), the intensity of the second peak increased significantly. When *n*-hexane alone was used as the mobile phase, the second peak decreased and most of the copolymer precipitated on the column. The results are shown in Fig. 2.

P(S–AN)-20. With up to 40% of *n*-hexane in the mobile phase, all the copolymer eluted at $V_R = 2.0$ ml. When the composition was chloroform–*n*-hexane (50:50, v/v), the intensity of the peak decreased and a second small peak appeared at $V_R = 3.0$ ml. At chloroform–*n*-hexane (40:60, v/v), only the second peak was observed. The intensity of the second peak increased significantly with *n*-hexane alone as the mobile phase. The results are shown in Fig. 3.

P(S–AN)-15. With up to 50% of *n*-hexane in the mobile phase, all the copolymer appeared at $V_R = 2.0$ ml. With chloroform–*n*-hexane (40:60, v/v), the peak became small and a second peak appeared at $V_R = 3.0$ ml. With chloroform–*n*-hexane (30:70, v/v), the first peak disappeared and only the second peak was observed. When *n*-hexane alone was used as the mobile phase, the intensity of the second peak increased significantly as with P(S–AN)-20. The results are shown in Fig. 4.

The retention volume of 2.0 ml in this system corresponds to the volume at the exclusion limit ($= V_0$) in SEC and the solutes elute from the column through the interstices among the packing particles without entering the pores of the

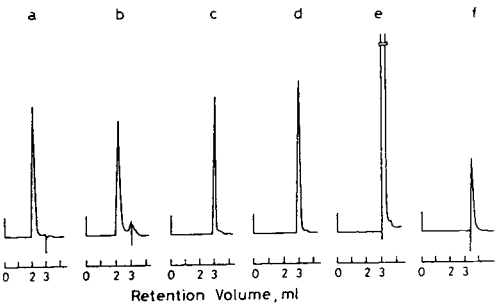

Fig. 2. Elution profiles of P(S–AN)-27 copolymer. Composition of chloroform–*n*-hexane mobile phase: (a) 70:30; (b) 60:40; (c) 50:50; (d) 40:60; (e) 30:70; (f) 0:100 (v/v). Other conditions as in Fig. 1.

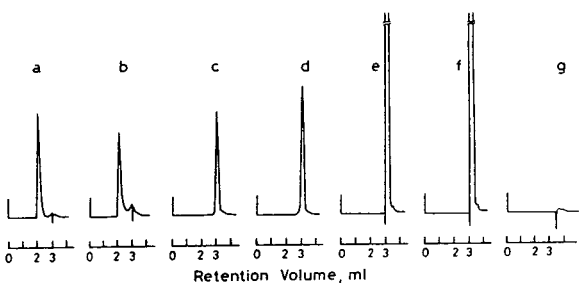

Fig. 1. Elution profiles of P(S–AN)-33 copolymer. Sample concentration, 0.05%; UV detection at 260 nm, 0.64 AUFS. Composition of chloroform–*n*-hexane mobile phase: (a) 80:20; (b) 70:30; (c) 60:40; (d) 50:50; (e) 40:60; (f) 30:70; (g) 0:100 (v/v).

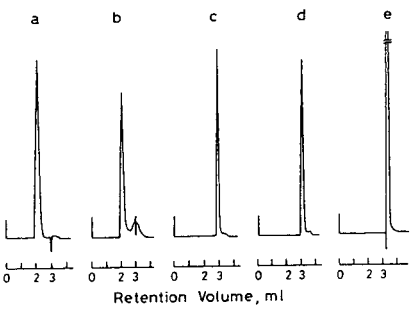

Fig. 3. Elution profiles of P(S–AN)-20 copolymer. Composition of chloroform–*n*-hexane mobile phase: (a) 60:40; (b) 50:50; (c) 40:60; (d) 30:70; (e) 0:100 (v/v). Other conditions as in Fig. 1.

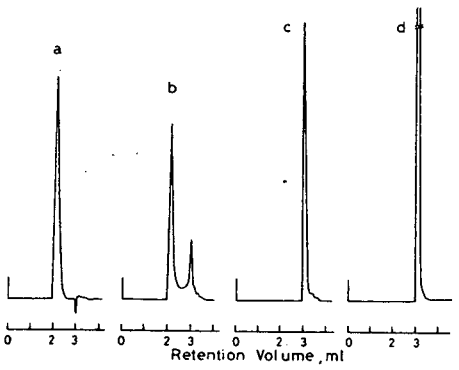

Fig. 4. Elution profiles of P(S–AN)-15 copolymer. Composition of chloroform–*n*-hexane mobile phase: (a) 50:50; (b) 40:60; (c) 30:70; (d) 0:100 (v/v).

particles. ODS silica packing materials prepared with silica gel having an average pore of 100 Å decrease the apparent average pore diameter to 30–40 Å and polymers having molecular masses over 1000 are totally excluded from the pores when they are eluted with a good solvent as the mobile phase [8]. The retention volume of 3.0 ml in this system corresponds to the sum of the interstitial volume, the pore volume of the packing materials in the column and the dead volume of tubing between the injection valve and the UV detector ($= V_0 + V_i$). Small molecules are capable of entering deeply into the pores and elute at this retention volume.

Mobile phases having a low content of *n*-hexane in chloroform are still good solvents for P(S–AN) copolymers and the copolymers will elute at $V_R = 2.0$ ml with these mobile phases. Mobile phases having a high content of *n*-hexane are poor solvents for the copolymers and with increasing *n*-hexane content in the mobile phase the copolymers precipitate on the column, depending on their composition. A transition period (the pre-precipitation state) may exist during the process in which the copolymer that dissolved in a good solvent precipitated as the solvent became a poor solvent on adding a non-solvent to it. Each molecule was in a condensed form during this period. Therefore, the size of the copolymer became very small in this period and the copolymer eluted at $V_R = 3.0$ ml as in Fig. 1c and d, Fig. 2c and d, Fig. 3c and d and Fig. 4c. These phenomena were different from those in HPPLC.

n-Hexane is a non-solvent for P(S–AN) copolymers and when it is used alone as the mobile phase, the copolymers should precipitate on the column according to the principle of HPPLC, as in Fig. 1g. On the other hand, a small peak at $V_R = 3.0$ ml is observed in Fig. 2f and the copolymers eluted from the column even with *n*-hexane as the mobile phase as in Figs. 3 and 4. The reason is considered to be as follows. As the copolymers were dissolved in chloroform as sample solutions, the copolymer molecules were surrounded tightly with chloroform molecules and the contact between *n*-hexane molecules and the copolymer molecules was interrupted for a while. Therefore, the copolymer eluted from the column without precipitating immediately, even though the mobile phase was *n*-hexane. This process depends, of course, on the composition of the copolymers and P(S–AN)-33 copolymer precipitated immediately in the *n*-hexane mobile phase.

A significant increase in the peak intensity with increasing *n*-hexane content in the mobile phase was observed, as in Fig. 1e and f, for example. The phenomena occurred just before the precipitation of the copolymers on the column. The reason is assumed to be that the transparency of the effluent containing the copolymer became poorer owing to the increase in turbidity, resulting in a decrease in the UV transmittance. At this moment some of the copolymer precipitated on the column (see *Gradient elution*, below). The peak areas at $V_R = 3.0$ ml were not constant, because the elution at this point was in a transition state and was influenced by the experimental conditions.

Turbidity measurement

The solubility of the copolymers decreases with increasing contents of acrylonitrile in the copolymers and of *n*-hexane in the mobile phase. The copolymers eluted at $V_R = 2.0$ ml at first, shifted to $V_R = 3.0$ ml with increasing *n*-hexane content in the mobile phase and were then retained, which is considered to be due to the transition of the state of the copolymers in the mobile phase. The copolymers are solvated with

a good solvent such as chloroform or chloroform with a small proportion of *n*-hexane, shrink in a poor solvent such as chloroform containing large amounts of *n*-hexane, and then are precipitated on the column in the non-solvent, *n*-hexane.

The solubility of the copolymers can be determined by turbidity measurement. A volume of 10 ml of a 0.1% copolymer solution in chloroform placed in a flask and was thermostated at 25°C in a water-bath and then visually titrated with *n*-hexane. For turbidity titration, the spectrometric method is preferable, but in order to understand the observations in this work, the visual technique may be sufficient. The volume of *n*-hexane when turbidity was first observed in the solution was taken as the cloud point. The volume of *n*-hexane required was as follows: P(S–AN)-15, 17.0 ml; P(S–AN)-20, 13.3 ml; P(S–AN)-27, 9.0 ml; and P(S–AN)-33, 6.0 ml. The *n*-hexane content in the solution required to precipitate the copolymers was plotted against the composition of the copolymers and is shown in Fig. 5 (closed circles).

The open circles in Fig. 5 indicate the *n*-hexane contents in the mobile phase at which the second peak at $V_R = 3.0$ ml appeared (bottom) and at which the first peak at $V_R = 2.0$ ml disappeared (top). Each cloud point is located between two open circles. The top open circles are close to the cloud points, indicating that the copolymers eluted from the column as if they were small molecules and they did not precipitate on the column at their cloud points. These results are different from those obtained by Glöckner and Van den Berg [9]. Mobile phases having compositions below the bottom open circles correspond to good solvents for the copolymer. The region between the bottom open circles and somewhat above the top open circles corresponds to the transition period (the pre-precipitation state) of the specified copolymer.

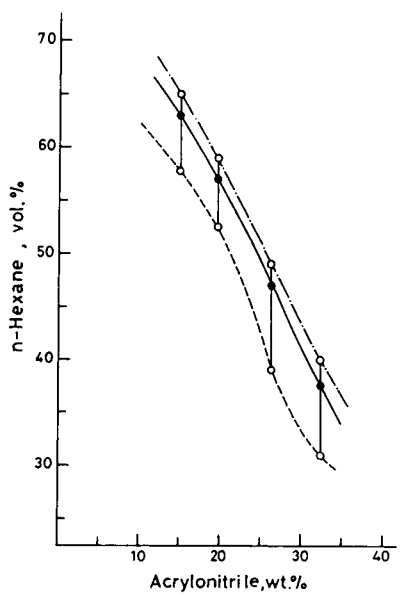

Fig. 5. Cloud point and elution characteristics plotted as *n*-hexane content in the mobile phase *versus* acrylonitrile content in the copolymer. ● = Cloud point; ○–○ = the range in which both peaks appeared at $V_R = 2.0$ ml and $V_R = 3.0$ ml, respectively.

Gradient elution

The elution profiles of P(S–AN) copolymers obtained by isocratic elution were grouped into the following four patterns: elution at $V_R = 2.0$ ml, elution at $V_R = 2.0$ and 3.0 ml, elution at $V_R = 3.0$ and precipitation on the column. Elution between $V_R = 2.0$ and 3.0 ml or after $V_R = 3.0$ ml was not observed. Therefore, in order to separate the copolymers according to composition, gradient elution should be performed with *n*-hexane–chloroform, the concentration of the latter increasing linearly.

Linear gradient elution was performed with a gradient from 100% *n*-hexane to 100% chloroform in 30 min, and then the elution was continued with chloroform for a further 15 min. In order to minimize the baseline drift during the elution, the wavelength of the UV detector was changed to 270 nm. A sample volume of 0.1 ml was sufficient to detect peaks and no difference in the elution profiles was observed between sample volumes of 0.1 and 0.01 ml.

The results are shown in Fig. 6. Elution was in the order of increasing acrylonitrile content in the copolymers and there was a linear relationship between retention volume and acrylonitrile content. After the gradient elution, the mobile phase was changed to *n*-hexane, the flow of which into the column was continued for 70 min. By this treatment, it was possible to minimize the peak eluted at $V_R = 3.0$ ml except for P(S–

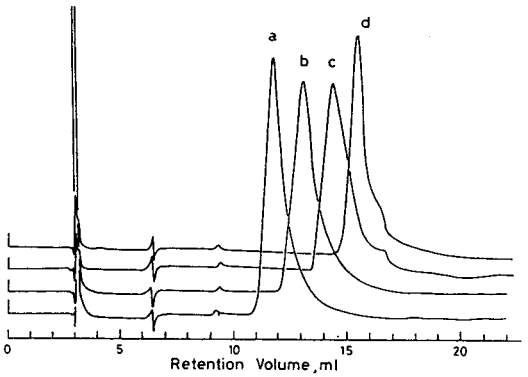

Fig. 6. Chromatograms of P(S–AN) copolymers obtained by gradient elution. Sample solution, 0.1%; volume injected, 0.1 ml; UV detection at 270 nm, 0.08 AUFS. Peaks: (a) P(S–AN)-15; (b) P(S–AN)-20; (c) P(S–AN)-27; (d) P(S–AN)-33.

AN)-15, which still had a large peak there. Although P(S–AN)-15 copolymer had a large peak at $V_R = 3.0$ ml, part of the copolymer was also eluted at the appropriate retention volume by gradient elution, and thus the assumptions that the extremely large peak at $V_R = 3.0$ ml was due to the decrease in the transmittance of UV radiation caused by turbidity of the sample effluent and that part of the copolymer precipitated on the top of the column at this moment seem to be correct. The peak at $V_R = 3.0$ ml except for P(S–AN)-15 and that appeared at $V_R = 6.5$ ml are those due to the sample solvent.

In order to check the molecular mass dependence of the separation by gradient elution, polystyrene (PS) standards having a narrow molecular mass distribution were separated in the same manner as in Fig. 6 and the results are shown in Fig. 7. PS having $M_r > 10^5$ eluted at $V_R = 10.0$ ml, PS having $M_r = 2200$ eluted at $V_R = 6.5$ ml and PS having $M_r = 20\,400$ eluted after $V_R = 6.5$ ml as a broad peak. All the polymers had a large peak at $V_R = 3.0$ ml as with the P(S–AN) copolymers.

These results were obtained with PS samples and therefore similar experiments with P(S–AN) copolymer samples are required in order to estimate the molecular mass dependence. No data have appeared in the literature to indicate that the molecular mass dependence of elution

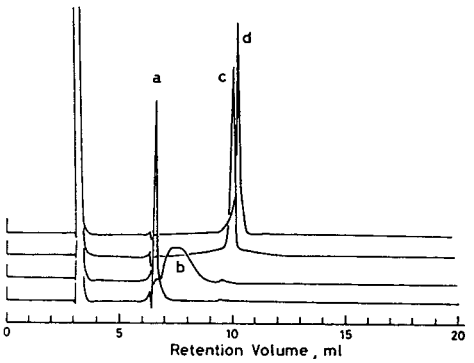

Fig. 7. Chromatograms of polystyrenes obtained by gradient elution. Sample solution, 0.05%; volume injected, 0.1 ml; UV detection at 270 nm, 0.16 AUFS. Peaks: PS of M_r (a) 2200, (b) 20 400, (c) 97 200 and (d) 411 000.

can or cannot be transferred from homopolymers to copolymers. If it could be, then it would be possible to state that no molecular mass dependence of the separation by gradient elution was observed for polymers and copolymers of high molecular mass, *e.g.*, $>10^5$. P(S–AN) copolymers with a 4% acrylonitrile content eluted at 10.3 ml (data not shown). From these results and Fig. 6, it could also be possible to state that the P(S–AN) copolymers examined in this work eluted according to their composition and no molecular mass dependence was observed.

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Solution properties of polyelectrolytes

VIII[☆]. A comparative study of the elution behaviour on two organic-based packings[☆☆]

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ABSTRACT

Aqueous size-exclusion chromatography was used to analyse the elution behaviour of several standard ionic polymers, including poly(L-glutamic acid), sodium poly(styrene sulphonate) and poly(acrylic acid), different in nature and chain flexibility, as a function of the pH and ionic strength of the eluent. Two organic-based hydrophilic packings, Spherogel TSK PW4000 and Ultrahydrogel 250, were tested in order to select the optimal conditions of macromolecular separation, and the results obtained for each column were compared. A set of calibration graphs for the above polyions as a function of eluent pH and ionic strength were obtained and compared with those obtained for uncharged standards (dextran and polyethylene oxide, PEO) under the same experimental conditions. The divergence between both charged and uncharged plots served to interpret the separation mechanisms for the polyions, other than pure size exclusion. Deviations from ideal elution behaviour have been attributed to ion-exclusion and hydrophobic effects, as a consequence of the repulsive or attractive interactions between the ionizable groups of the polyelectrolyte and the residual surface charge of the support.

INTRODUCTION

In the last decade, size-exclusion chromatography (SEC) has become firmly implanted in the areas of biomedical and life sciences, mainly as a result of the development of high-performance and selective hydrophilic backing columns, allowing the separation and identification of synthetic and natural water-soluble polymers as well as macromolecular assemblies, such as viral particles and liposomes [1–5].

Hydrophilic packings can be classified into two categories, inorganic (mostly silica based) and organic (formed by a three-dimensional polymer network with strategically inserted water-compatible organic functional groups). These functional groups, such as hydroxyl, carboxyl, etc., are dissociated or not depending on the pH of the mobile phase. In some cases, specific interactions between functional groups and certain atoms or ionic groups can distort the macromolecular separation expected from a pure size-exclusion mechanism. In the particular case of elution of a polyelectrolyte through organic- or silica-based packings, specific solute–matrix interactions, such as hydrogen bonding, ion exchange, hydrophobic and ion-exclusion effects, have been thoroughly studied from both experimental and theoretical points of view [6,7].

In this context, we should mention a series of

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papers published by Dubin and co-workers [8–10] on a model based on the reduction in the pore volume accessible to polyions, which predicts ion-exclusion effects. Another model calculates the free energy of partitioning of anionic polyelectrolytes into cylindrical pores of like charge. A theoretical contribution based on the Poisson–Boltzmann equation has recently been reported by Hoagland [11], which examines the interaction of a rod-like polyelectrolyte with nearby surfaces of like charge. Recently, our group [12,13] has evaluated the elution behaviour of sodium poly(styrene sulphonate) (PSS) and proposed an empirical correlation accounting for deviations of this polyion when compared with standard non-ionic polymers.

In this paper, a qualitative analysis of the elution behaviour of PSS, poly(L-glutamic acid) (PGA) and poly(acrylic acid) (PAA) polyions, at different mobile phase compositions and through two different packings, is presented. The studied packings, organic-based ones, were Spherogel TSK PW4000 and Ultrahydrogel 250 (UHG-250). The first has been widely used in the separation of both synthetic and biological macromolecules, however the second has scarcely been tested. Deviations in universal calibration curves, expressed as log $M[\eta]$ *vs.* elution volume, with respect to the uncharged polymer, have been considered in order to evaluate the contribution of non-exclusion effects to the total separation mechanism.

EXPERIMENTAL

Chemical and reagents

Dextran samples purchased from Pharmacia (Uppsala, Sweden) with nominal molecular masses of 10 000, 17 700, 40 000, 66 900, 83 300, 170 000, 500 000 and 2 000 000 g mol^{-1} were used as standards for uncharged polymers. The chromatographic low-molecular-mass range was covered by poly(ethylene oxide) (PEO) standards with molecular masses of 2000 and 4000 g mol^{-1}, from Fluka (Darmstadt, Germany). Studied polyelectrolytes were samples of PGA from Sigma (St. Louis, MO, USA), PSS from Pressure (Pittsburgh, PA, USA) and PAA from Aldrich (Milwaukee, WI, USA) and are listed in Table I. All samples show polydispersities lower than 1.1.

TABLE I

NOMINAL MOLECULAR MASSES AND INTRINSIC VISCOSITIES IN PURE WATER AT 25°C OF STUDIED POLYELECTROLYTES

PGA = Poly(L-glutamic acid); PSS = sodium poly(styrene sulphonate); and PAA = poly(acrylic acid)

Samples	M (g mol^{-1})	$[\eta]$ (ml g^{-1})
PGA-1	13 600	181
PGA-2	43 000	985
PGA-3	77 800	2220
PSS-1	1 600	87
PSS-2	16 000	611
PSS-3	31 000	856
PSS-4	88 000	2740
PSS-5	177 000	6080
PAA-1	5 000	44.5
PAA-2	90 000	1430
PAA-3	250 000	5420

Solvents used for viscometric measurements and as eluents in SEC were buffers made up from sodium dihydrogenphosphate and sodium monohydrogenphosphate for pH 7.0 and from sodium acetate and acetic acid for pH 5.0. Desired ionic strengths were adjusted from 0.005 to 0.20 *M*. Reagents used in the preparation of buffers were analytical grade from Merck (Darmstadt, Germany), and the conductivity of the HPLC water used (Merck) was tested daily.

Viscosities

Intrinsic viscosity values, $[\eta]$, for uncharged polymers in pure water at 25.0 ± 0.1°C were evaluated through the viscometric equations $[\eta] = 97.8 \cdot 10^{-3} M^{0.5}$ ml g^{-1} for dextrans [14] and $[\eta] = 2.0 + 0.016 M^{0.76}$ ml g^{-1} for PEO [15], where M = molecular mass. The effects of ionic strength and pH on the viscosity of those non-ionic polymers were ignored [8,12,13].

Viscosity measurements of polyelectrolyte samples at 25.0 ± 0.1°C and pH 5.0 were performed with an automatic Ubbelohde-type AVS 440 capillary viscometer from Schott Geräte (Hofheim, Germany). Original solutions were equilibrated at the working temperature for several hours prior to introduction into the

viscometer. At least five dilutions were obtained by adding the appropriate aliquots of solvent. Kinetic energy corrections were taken into account for the evaluation of $[\eta]$, which was determined by extrapolation to infinite dilution of Füoss plots [16], namely η_{red}^{-1} *vs.* $c^{1/2}$.

Chromatography

The LC equipment consisted of an M-45 solvent-delivery system, a U6K universal injector and an R-401 refractive index detector from Waters (Milford, MA, USA). An Ultrahydrogel 250 (UHG-250) column (30 × 0.78 cm I.D.) packed with hydroxylated poly(methacrylate)-based gel of 250 Å nominal pore size from Waters and a Spherogel TSK PW4000 column (30 × 0.75 cm I.D.) packed with hydroxylated polyether copolymer of 500 Å nominal pore diameter from Beckman Instruments (Galway, Ireland) were used. The chromatograms were recorded by a Yokogawa Electric Works dual-channel recorder (Tokyo, Japan). The exclusion volumes, V_0, and the total column volumes, V_T, were 5.48 and 10.46 ml, respectively, for the UHG-250 column and 5.15 and 10.40 ml, respectively, for the TSK one, as determined with blue dextran ($M = 2\,000\,000$ g mol^{-1}) and ^2H_2O, respectively.

Buffers used as eluents were degassed and filtered through regenerated cellulose 0.45-μm pore diameter filters from Micro Filtration Systems (Dublin, CA, USA). All chromatographic experiments were conducted at room temperature and the column was equilibrated overnight prior to starting any experiment. Chromatograms of polyelectrolytes were obtained at a flow-rate of 1.0 ml min^{-1} by injection of 100 μl of 0.1% (w/v) solute solutions, prepared using the corresponding mobile phase as solvent. Elution volumes of uncharged standards were obtained by extrapolation to zero concentration of peak elution volumes obtained for at least three different injected concentrations. Obtained values were independent of pH and of ionic strength, at least in the range studied here.

RESULTS AND DISCUSSION

The approach most commonly followed to analyse secondary effects in aqueous SEC of polyelectrolytes compares the calibration graphs obtained under the same experimental conditions both for the polyion under study and for some uncharged polymer used as a reference. In this context, some controversy arises about the physical magnitude that best defines the hydrodynamic volume of polyions. Thus, whereas several authors [17–19] believe that elution volumes, V_e, in aqueous SEC of polyelectrolytes are determined by the hydrodynamic volume, $M[\eta]$, only at ionic strengths that are sufficiently high to allow electric double-layer effects to be considered negligible, Potschka [20] shows that any macromolecule, regardless of its shape (solid sphere, expanded coil or more or less flexible rod), elutes at any condition according to a universal parameter, namely the viscosity radius, R_η, directly related to $M[\eta]$. Therefore, in what follows the product $M[\eta]_I$ will be used as the representative parameter of polyelectrolyte size at any external salt concentration, I.

Whereas pH causes negligible changes or no changes at all in the intrinsic viscosity of flexible polyelectrolytes, as experimentally ascertained for the polymers studied here and in agreement with previous reports [8,12], added salts do indeed cause large changes [21]. It is generally admitted that [22,23]:

$$[\eta]_I = [\eta]_\infty + SI^{-1/2} \quad (1)$$

where $[\eta]_I$ and $[\eta]_\infty$ stand for intrinsic viscosities at the ionic strength I and at $I \rightarrow \infty$, respectively, and S is related to the stiffness of the macromolecule according to Odijk [24]. In what follows units for $[\eta]$ and I will be ml g^{-1} and mol l^{-1}, respectively.

Eqn. 1 plots are shown in Fig. 1 for the three polyelectrolytes under study. As seen in the figure good linear correlations hold in the range of ionic strength so far studied (0.005–0.20 M), allowing evaluation of $[\eta]_\infty$ and S values through least-square fits. As seen, the higher the molar mass of the polyanion the larger the slope values of Fig. 1, as expected from the greater contribution to $[\eta]_I$ of the electrostatic persistence length, L_e, of the macromolecular chains. Moreover, good linear correlations of $\log[\eta]_I$ and $\log[\eta]_\infty$ *vs.* $\log M$ (not shown) allowed us to obtain Mark–

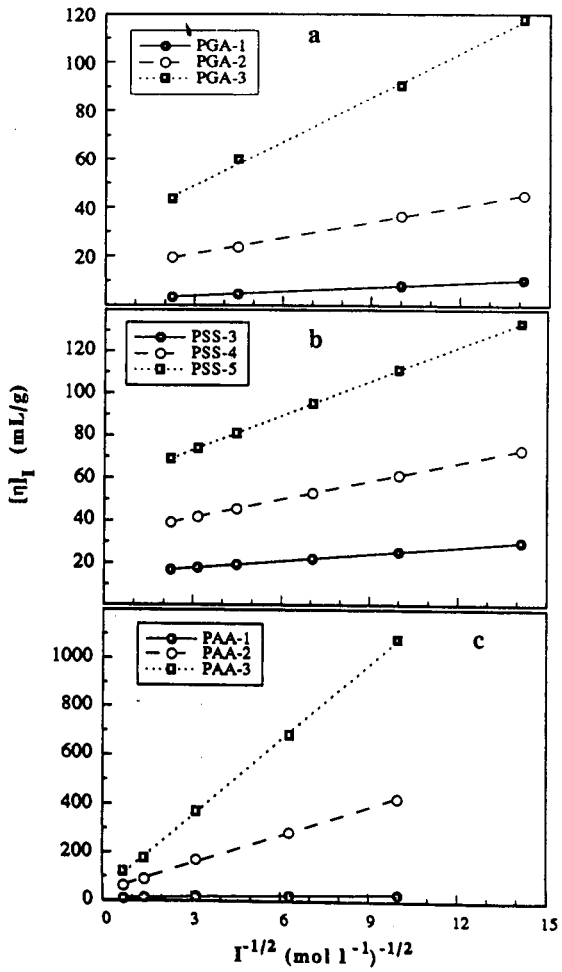

Fig. 1. Intrinsic viscosities of poly(L-glutamic acid), PGA (a); sodium poly(styrene sulphonate), PSS (b) and poly(acrylic acid), PAA (c) in acetate buffer (pH 5.0) as a function of the inverse square root of ionic strength.

Houwink constants K and a ($[\eta] = KM^a$) at the diverse I values studied.

Chromatograms of PGA-1, PSS-2 and PAA-1, as examples, on TSK PW4000 and UHG-250 columns in pure water (curves a) and in phosphate buffer (pH 7.0) at various ionic strengths (curves b–g) are shown in Fig. 2. In all cases the peaks are Gaussian and become broader with increasing ionic strength. In contrast to the SEC behaviour of dextran or any uncharged polymer, where the effects of I on elution volumes are negligible, it can be observed in Fig. 2 that the retention volumes of all polyanions significantly

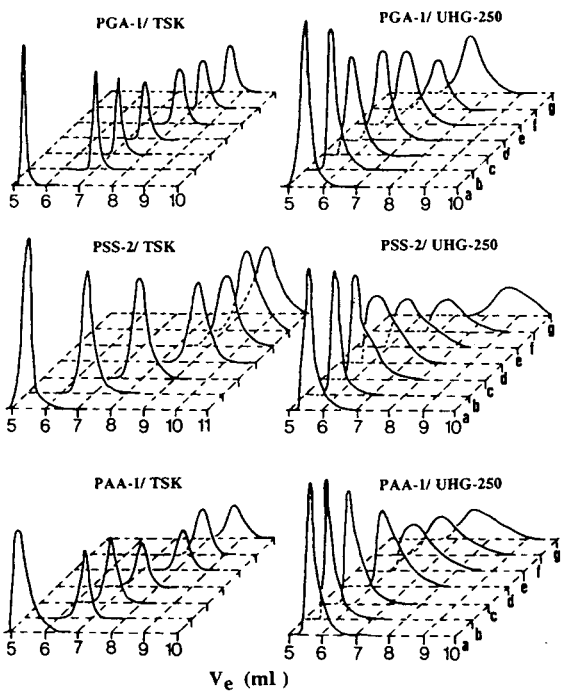

Fig. 2. Comparison of the elution profiles of poly(L-glutamic acid) (PGA-1), sodium poly(styrene sulphonate) (PSS-2) and poly(acrylic acid) (PAA-1) on TSK PW4000 and on UHG-250 columns at pH 7.0 and various ionic strengths: (a) pure water; (b) 0.005 M; (c) 0.01 M; (d) 0.02 M; (e) 0.05 M; (f) 0.10 M; and (g) 0.02 M.

increase with increasing ionic strength of the mobile phase. Both TSK PW4000 [25,26] and UHG-250 [4,13,27] are hydroxylated polymer-based gels containing residual carboxyl groups. At low ionic strength and pH > pK_a(–COOH), these groups become dissociated and the gels will then exhibit negative residual charges on their surfaces. Consequently, the elution volumes of polyanions shift towards lower values, denoting polymer–gel electrostatic repulsion. This effect can be suppressed at high ionic strength, but not always [9]. At pH 7.0 all ionic groups of both packing and polyelectrolyte sample should be negatively ionized. Therefore, in pure water (chromatograms a) or in dilute buffer solution at $I = 0.005$ M (chromatograms b), solutes cannot enter into pores because of electrostatic repulsions and elute near the exclusion volume, V_0, and their peak shapes are sharp regardless of their molar masses. With increasing eluent ionic

strength, the screening of charges implies a decrease of electrostatic repulsion and, as a result, solute elution profiles become broader according to the increasing amount of pore volume accessible to the polyelectrolyte. Note that the TSK PW column has wider pores than the UHG-250 column, so solutes can enter into a larger pore volume, as shown by the higher elution volumes at the same ionic strength on TSK PW than on UHG-250 (compare profiles b for the same sample in both columns). As a consequence, at moderate I values the predominant separation mechanism in TSK PW gel seems to be size exclusion, whereas in UHG-250, at the same I values, it is ion exclusion.

This complex behaviour is better illustrated through universal calibration plots. Thus, log $M[\eta]_I$ *vs.* V_e graphs are depicted in Fig. 3 for PGA and dextran on TSK PW4000 gel at different ionic strengths and at pH 5.0 (acetate buffer, Fig. 3a) and at pH 7.0 (phosphate buffer, Fig. 3b). As mentioned above, elution curves of PGA

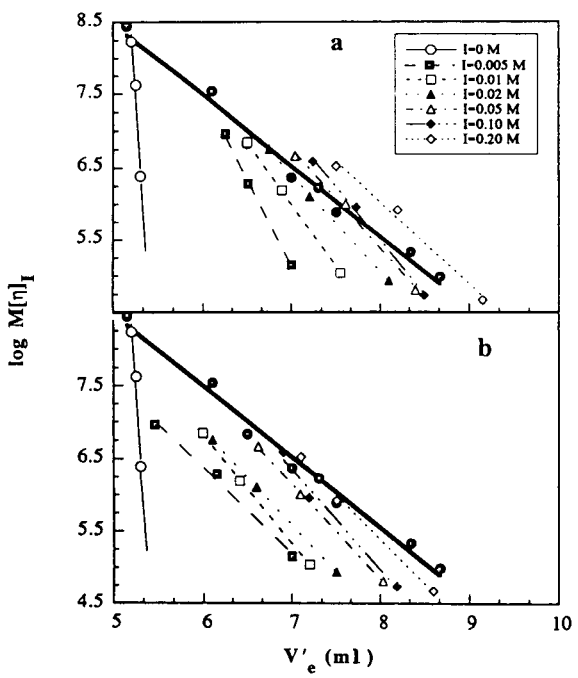

Fig. 3. Universal calibration plots for dextran (**O**) in pure water and poly(L-glutamic acid) (PGA) on a TSK PW4000 column at different ionic strengths and pH values: (a) pH 5.0 and (b) pH 7.0.

are far from the reference one at low ionic strength, approach the dextran curve with increasing saline content and cross it at pH 5.0 and high ionic strengths ($I = 0.10$ and 0.20 M). Although the influence of pH is less pronounced than that of ionic strength, dextran yields a single curve at any pH value while PGA plots deviate most from the reference curve at high pH values. This result is a consequence of the variation in the surface charge density provoked by the dissociation of carboxyl groups of both the PGA polymer and the support. At the lowest pH and highest I values, the elution volumes of polyelectrolyte (Fig. 3a) shift towards values higher than would be expected from ideal behaviour, probably because of the appearance of salt-induced matrix–solute hydrophobic interactions. In this regard, Mori [28] has reported hydrophobic retention of PSS on different supports at relatively high ionic strengths.

Because this general trend is also followed by the other studied polyanions (PSS and PAA) on both TSK PW and UHG-250 columns, a detailed analysis on the above deviations deserves to be undertaken. Since hydrodynamic volumes of solutes are not affected by pH, as mentioned above, changes in elution volumes with pH taking place at fixed ionic strength can be attributed to repulsive ionic effects between solute and gel surfaces. Effectively, PGA and matrix contain carboxyl groups, with pK_as in both cases about 4.25 and, whereas at pH 5.0 the degree of dissociation is about 50%, at pH 7.0 all functional groups are completely dissociated, greatly increasing the electrostatic repulsion. Of course, pH values lower than pK_a would diminish electrostatic effects but could also cause precipitation of polyanion and instability of supports. Note that for chromatographic purposes it is important to distinguish between the pH of eluent and the pH of the injected PGA solution. Whereas the former is constant, the latter depends on polymer concentration, this dependence being more pronounced as ionic strength decreases and molar mass increases [12]. In order to minimize this drawback all polyelectrolyte samples were injected at a constant concentration, namely 0.1% (1 g l^{-1}, dilute solution). In spite of this, changes in the pH of

the actual injected sample with respect to the pH of eluent could affect the extent of repulsive polymer–substrate interactions, and these contributions, though small, could add to the general trend followed by calibrations with pH.

Fig. 4 depicts calibration curves for PGA, PSS and PAA on both TSK PW4000 (Fig. 4a–c) and UHG-250 (Fig. 4d–f) columns at pH 5.0 and different ionic strengths. As can be seen, different sets of elution curves depending on I are obtained for each polyion/gel system. Comparison of Fig. 4a–c should illustrate the different elution behaviour of polyelectrolytes on the same TSK PW4000 column and eluents. Thus, the predominant mechanism for the PGA/TSK system, at low and moderate I values, is ion exclusion; ideal SEC is obtained at $I = 0.10$ M, and at higher I values adsorptions or attractive polymer–gel interactions are observed. It must be kept in mind, as commented above, that pH 5.0 is the pH value most suitable for elution because about 50% of ionizable groups of both polyanion (PGA) and gel remain protonated. Regarding the PSS/TSK system (Fig. 4b), at $I < 0.01$ M electrostatic repulsion effects predominate and pure SEC is achieved at $I = 0.01$ M. At higher I values the calibration curves shift towards higher elution volumes than those of the reference one, and they become vertical at the highest studied I. In fact, under these conditions elution volumes seem to be independent of solute molar mass, and they reach the total volume of column whatever the polyelectrolyte molar mass. In the system PAA/TSK (Fig. 4c), size exclusion is the predominant separation mechanism in the range $0.01 \leq I \leq 0.05$ M. Under these conditions repulsion and adsorption secondary effects would appear to cancel each

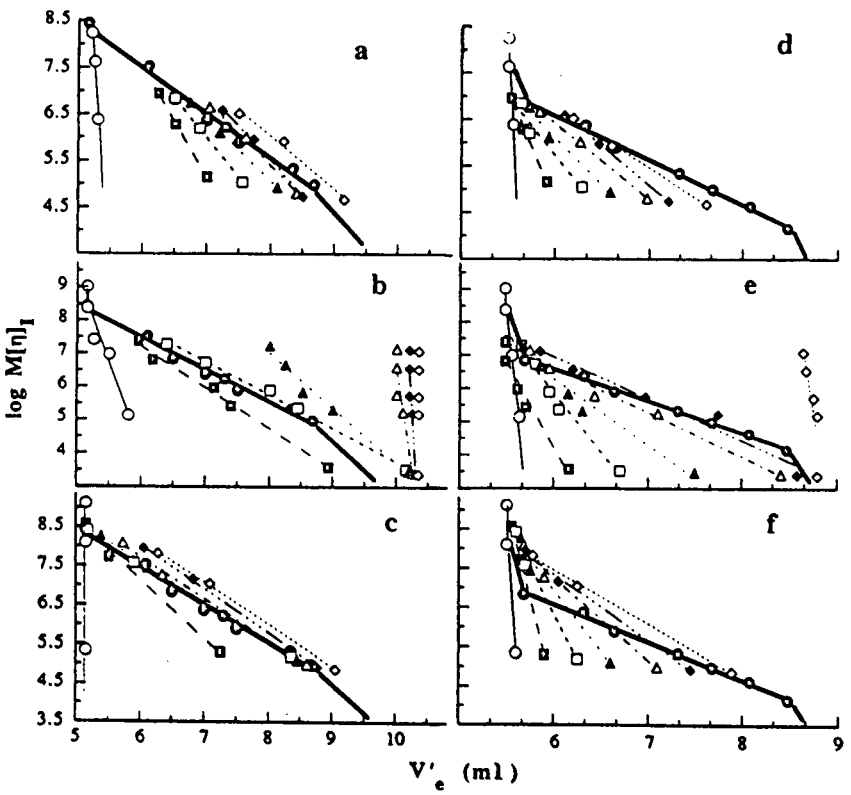

Fig. 4. Universal calibration plots for dextran (O) in pure water and poly(L-glutamic acid) (PGA) (a), sodium poly(styrene sulphonate) (PSS) (b) and poly(acrylic acid) (PAA) (c) on a TSK PW4000 column. Parts d, e and f refer to calibration of the same polyions on a UHG-250 column. In all cases acetate buffer solution at pH 5.0 has been used. The meaning of the symbols is the same as in Fig. 3.

other out. At higher I values, adsorption of polymer by gel takes place. In summary, under fixed I and pH conditions, elution volumes in TSK columns are in the order PGA < PAA < PSS. This order is in agreement with the non-polar character of solutes, that is with their expected hydrophobic interactions. In fact, in this gel hydrophobic interactions seem to play a more important role than electrostatic ones, since if electrostatic interactions were predominant the order of elution volumes would be the inverse of that above [electrostatic repulsions should be in the order PSS > PAA = PGA as expected from their pK_as, namely pK_a(PSS) < pK_a(PAA) = pK_a(PGA)].

Calibrations in UHG-250 (Fig. 4d–f) seem to follow similar trends to the above ones. In this gel, however, electrostatic effects seem to be the most important secondary effects, since, except for PSS at very high ionic strength ($I = 0.20$ M), adsorption effects seem to be negligible. This behaviour sounds reasonable, because on the one hand UHG-250 gel displays less apolar zones than TSK gel and, on the other, it has a higher percentage of ionizable groups per area unit, that is a larger surface charge density. Ideal SEC, of course, can only be obtained at higher I values in this gel. It is worth remarking that the usual order in elution volumes PGA < PAA < PSS is not obeyed by PAA samples with the largest molecular masses, which cross the reference calibration curve. This anomalous behaviour at the highest molecular masses can be explained by assuming additional specific polymer–gel interactions, via hydrogen bonds between the carbonyl ester group of methacrylate, the monomer base of UHG gel and the –COOH lateral groups of PAA (or PGA). These cooperative additional adsorptions, the larger the higher the molecular mass of solute, are in agreement with the increase in alcohol preferential sorption with polymer molecular mass reported by Horta and Katime [29] for poly(methyl methacrylate)–benzene–butanol systems, in which specific interactions between polymer and alcohol take place, and are also in line with the studies of Molyneaux and Vekavakayanondha [30] on specific interactions between phenols and poly(vinyl pyrrolidone). In these systems, a decrease in phenol–polymer interactions with decreasing polymer molar mass is also observed, as ascertained by the proportion of active sites on the polymeric chain occupied by the phenol.

From the complex behaviour mentioned above, it is deduced that the chemical nature of the polyelectrolyte is the main factor governing its elution behaviour, since secondary effects depend on the one hand on the ionizable group density (pK_a) and, on the other, on the extension of non-polar zones. Likewise, from the comparison of elution behaviours of a given polyanion on diverse columns, the following consequences can be pointed out:

(a) Regarding the electrostatic repulsion effects, modern gels based on cross-linked hydrophilic polymers seem to be more convenient for aqueous size-exclusion chromatography studies than the traditional silica-based ones.

(b) For the two gels studied here, the antagonistic secondary effects under the usual working conditions seem to be better equilibrated in UHG-250 than in TSK PW4000. In the latter electrostatic repulsions are weaker but undesirable adsorptions are present, at least for the solutes studied here.

Finally, it must be pointed out that the best experimental conditions (pH and I of mobile phase) in SEC of synthetic or biological polyelectrolytes depend on the solute/support system under study. Each specific problem demands an optimum selection of column and a rigorous search for the most suitable eluent.

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Dependence of retention of ionic solutes on the composition of the mobile phase electrolytes in partition chromatography

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ABSTRACT

On the basis of the model for partition chromatography of ionic solutes in the presence of background electrolytes, theoretical equations are developed to describe the retention dependence of ionic solutes on the composition of electrolytes in the mobile phase. The theoretical equations were tested by calculating the expected capacity factors of various inorganic ions in the presence of two or three background electrolytes using experimental values obtained in a single electrolyte system. The predictions from the equations agreed well with experimental retention data on hydrophilic polymer packings for both binary (acetone–water) and single solvent (water) systems.

INTRODUCTION

In any mode of liquid chromatography, it is essential to use an electrolyte solution as the mobile phase for elution of ionic solutes. In both partition chromatography and size-exclusion chromatography, an electrolyte is added to the mobile phase in order to eliminate ion exclusion and ion-exchange adsorption effects due to the low concentration of some fixed charges on the column packings.

The presence of a background of eluting electrolyte allows an analyte ion to move independently of the counter ion which is a constituent of the analyte salt injected. However, the presence of background electrolyte does not imply that the partitioning of an analyte ion is independent of its counter ion. The retention of the analyte ion depends strongly on the type of counter ion and co-ion in the mobile phase [1–10].

Shibukawa and Ohta [9] presented a simple model for partition chromatography of ionic solutes in the presence of background electrolyte and showed that the effect of various types of electrolytes on the retention of inorganic ions on non-ionic porous polymers, when water is used as the mobile phase solvent, can well be interpreted by the proposed model. This ion partition model was also used successfully to account for the effect of type of the background ions in binary solvent systems [10].

In this study, the ion partition model was used to describe the effect of the composition of uni–univalent electrolytes in the mobile phase on the retention of inorganic ions. A set of equations was derived to predict the capacity factors for cations and anions. Predictions from the theory

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were compared with experimental data obtained on hydrophilic porous polymers with acetone–water or water alone and it is shown that the equations can be used to predict the retention of analyte ions.

THEORETICAL

Let us consider the equilibrium distribution of an analyte cation, C^{p+}, between the mobile and stationary phases in the presence of N background univalent anions, $X_1^-, \ldots, X_N^-$ and a common cation, Y^+, based on the ion partition model [9]. The basic assumption of the ion partition model is that the association of an analyte ion with counter ions is negligible in both the mobile and stationary phases [9].

If the number of ionic groups in the stationary phase is so small that their electrostatic effect on the retention of the analyte ion is suppressed by the background electrolyte, the distribution equilibria of Y^+ and X_i^- $(i = 1, \ldots, N)$ are expressed as

$$Y^+(m) + X_i^-(m) \rightleftharpoons Y^+(s) + X_i^-(s) \quad (i = 1, \ldots, N) \quad (1)$$

where m and s denote the mobile and stationary phase, respectively. The distribution of C^{p+} can be expressed by either a set of N equilibria given by eqn. 2 or one "ion-exchange" equilibrium represented by eqn. 3 [9]:

$$C^{p+}(m) + pX_i^-(m) \rightleftharpoons C^{p+}(s) + pX_i^-(s) \quad (i = 1, \ldots, N) \quad (2)$$

$$C^{p+}(m) + pY^+(s) \rightleftharpoons C^{p+}(s) + pY^+(m) \quad (3)$$

If the ionic activity coefficient can be regarded as being approximately constant, we may use concentrations in place of activities to write the equilibrium constants. The molar equilibrium constants for the equilibria 1, 2 and 3 are given by eqns. 4, 5 and 6, respectively:

$$K_{YX_i} = \frac{[Y^+]_s[X_i^-]_s}{[Y^+]_m[X_i^-]_m} \quad (i = 1, \ldots, N) \quad (4)$$

$$K_{CX_i} = \frac{[C^{p+}]_s[X_i^-]_s^p}{[C^{p+}]_m[X_i^-]_m^p} \quad (i = 1, \ldots, N) \quad (5)$$

$$K_{C/Y} = \frac{[C^{p+}]_s[Y^+]_m^p}{[C^{p+}]_m[Y^+]_s^p} \quad (6)$$

The ionic activity coefficient in dilute solution (≤ 0.1 M) depends only on the total ionic strength of the solution and is independent of the type and concentration of the coexisting ions [11]. It is not difficult to maintain the ionic strength of the mobile phase constant, whereas that of the stationary phase depends on the distribution constant of the background electrolyte. However, it is reasonable to assume that the ionic activity coefficient in both the mobile and stationary phases is approximately unchanged if there is no extreme difference in the distribution constant of the background electrolyte; the constancy of the ionic activity coefficient was discussed in a previous paper [4].

It should be noted that K_{YX_i}, K_{CX_i} and $K_{C/Y}$ are not independent of one another [9]. The distribution of C^{p+} can be described by using either a combination of eqns. 4 and 5 or a combination of eqns. 5 and 6.

In most cases, the analyte ion is present in both the mobile and stationary phases at concentrations much lower than those of other background ions. Hence we have the equations of charge balance for the mobile phase and the stationary phase as follows, respectively:

$$[Y^+]_m = [X_1^-]_m + [X_2^-]_m + \cdots + [X_N^-]_m \quad (7)$$

$$[Y^+]_s = [X_1^-]_s + [X_2^-]_s + \cdots + [X_N^-]_s \quad (8)$$

From eqns. 4, 7 and 8, we obtain

$$\frac{[Y^+]_s}{[Y^+]_m} = \left(\frac{\sum K_{YX_i}[X_i^-]_m}{\sum [X_i^-]_m} \right)^{1/2} \quad (9)$$

The capacity factor, $k_C'^{YX_i}$, of the analyte ion, C^{p+}, when eluted with a single electrolyte, YX_i, is given by [9]

$$k_C'^{YX_i} = \phi \cdot \frac{[C^{p+}]_s}{[C^{p+}]_m} = \phi K_{C/Y} K_{YX_i}^{p/2} \quad (10)$$

where ϕ is the phase ratio. Combining eqn. 10

with eqns. 6 and 9 gives the following equation for the capacity factor of C^{p+} in the mixed background ion system:

$$k_C'^{Y(X_1+\cdots+X_N)} = \phi K_{C/Y} \frac{[Y^+]_s^p}{[Y^+]_m^p} = \left\{ \frac{\sum (k_C'^{YX_i})^{2/p} [X_i^-]_m}{\sum [X_i^-]_m} \right\}^{p/2} \quad (11)$$

The equation for the capacity factor of an anion, A^{p-}, is derived in a similar manner to that described for that of C^{p+}. The capacity factor, $k_A'^{YX_i}$ of A^{p-} is expressed as

$$k_A'^{YX_i} = \phi \cdot \frac{[A^{p-}]_s}{[A^{p-}]_m} = \phi \cdot \frac{K_{YA}}{K_{YX}^{p/2}} \quad (12)$$

where

$$K_{YA} = \frac{[Y^+]_s^p [A^{p-}]_s}{[Y^+]_m^p [A^{p-}]_m} \quad (13)$$

By combining eqns. 9, 12 and 13, we obtain the following equation, which represents the dependence of the capacity factor of A^{p-} on the composition of the background co-ions:

$$k_A'^{Y(X_1+\cdots+X_N)} = \phi K_{YA} \cdot \frac{[Y^+]_m^p}{[Y^+]_s^p} = \left\{ \frac{\sum [X_i^-]_m}{\sum \frac{[X_i^-]_m}{(k_A'^{YX_i})^{2/p}}} \right\}^{p/2} \quad (14)$$

In order to calculate the capacity factor of an analyte ion in a mixed background electrolyte system, $k_C'^{Y(X_1+\cdots+X_N)}$ or $k_A'^{Y(X_1+\cdots+X_N)}$, according to eqn. 11 or 14, one must obtain the capacity factors in all of the individual electrolyte solutions which constitute the mixed electrolytes, namely $k_C'^{YX_1}, \ldots, k_C'^{YX_N}$ or $k_A'^{YX_1}, \ldots, k_A'^{YX_N}$. However, it is not necessary to determine all of them experimentally. The capacity factor of the analyte ion, C^{p+}, in the electrolyte system YX_i can be calculated from the following equation by using the capacity factors of C^{p+}, X_i^- and X_j^- determined in the electrolyte system YX_j [9]:

$$k_C'^{YX_i} = k_C'^{YX_j} \left(\frac{k_{X_i}'^{YX_j}}{k_{X_j}'^{YX_j}} \right)^{p/2} \quad (15)$$

The capacity factor of the anionic analyte, A^{p-}, is calculated as

$$k_A'^{YX_i} = k_A'^{YX_j} \left(\frac{k_{X_j}'^{YX_j}}{k_{X_i}'^{YX_j}} \right)^{p/2} \quad (16)$$

EXPERIMENTAL

Materials

All chemicals used in this study were of analytical-reagent grade unless indicated otherwise.

HPLC-grade acetone was obtained from Kanto (Tokyo, Japan). Water purified through a Millipore Milli-Q water purification system was used throughout.

The column packings used were a cross-linked dextran gel, Sephadex G-25 (10–40 μm), purchased from Pharmacia (Uppsala, Sweden), and a cross-linked polyacrylamide gel, Bio-Gel P-2 (200–400 mesh), from Bio-Rad Labs. (Richmond, CA, USA). These hydrophilic polymer gels were washed with water, ethanol and acetone in that order and then dried at 90°C before use.

Chromatographic conditions

The pump was a Kyowa Seimitsu (Tokyo, Japan) Model KHP-010 solvent-delivery system. The eluent reservoir was a commercially available glass syringe with a 200-ml capacity [12]. Inorganic anions were detected with a Kyowa Seimitsu Model KLC-800 UV–visible variable-wavelength absorption detector or a Tosoh (Tokyo, Japan) Model CM-8 conductivity detector. A Perkin-Elmer (Norwalk, CT, USA) Model 3100 atomic absorption spectrometer was used for the detection of alkali and alkaline earth metal ions in a similar manner to that described in the literature [13].

The column packings were allowed to swell for 24 h in a large excess of the eluent solvent to be used, the solvent being decanted several times. The swollen gels were slurry packed into a 300 × 4 mm I.D. stainless-steel column. The column

was thermostated at 25.0 ± 0.1°C through a column jacket, using a Yamato (Tokyo, Japan) Model BH-71 constant-temperature circulator.

Eluents were water or acetone–water (70:30, v/v) containing sodium salts. Sample solutions were prepared by dissolving salts of the analyte ions in solutions whose compositions were the same as those of the eluents used.

Portions of 10 μl of the sample solutions (1–5 m*M*) were injected into the column with a Kyowa Seimitsu Model KHP-UI-130A injection valve. Elutions were carried out at a constant flow-rate of *ca.* 0.8 ml/min. Exact values of the volumetric flow-rate were measured using a burette designed to prevent vaporization of the solvent.

The mobile phase volume was determined according to the method proposed by Shibukawa and Ohta [14].

RESULTS AND DISCUSSION

Sephadex G-25/acetone–water system

In order to test eqns. 11 and 14, Sephadex G-25 equilibrated with acetone–water (70:30, v/v) was chosen as a partition chromatographic system, since Sephadex G-25 preferentially takes up water when it is brought into contact with a mixture of water and acetone and then forms a typical normal-phase partition system [10].

Capacity factors of inorganic ions were determined by elution with acetone–water (70:30, v/v) containing NaCl, $NaNO_3$ or $NaClO_4$ or two or three of them. The ionic strength of the eluents was kept constant at 0.01 *M*; ion exclusion and ion-exchange effects of fixed ionic groups of the matrix of Sephadex G-25 are almost negligible in these media [15].

Fig. 1 shows the capacity factor *vs.* background electrolyte composition data for the $NaNO_3$–$NaClO_4$ system. The solid lines represent the k' values calculated from eqn. 11 (for cations) or eqn. 14 (for anions) using only the experimental k' data obtained in $NaNO_3$ solution. The k' values of the analyte ions in the $NaClO_4$ system were calculated from eqn. 15 or 16 using the k' values of the analyte ions, NO_3^- and ClO_4^-, determined in the $NaNO_3$ system. For example, the capacity factor of K^+ in the

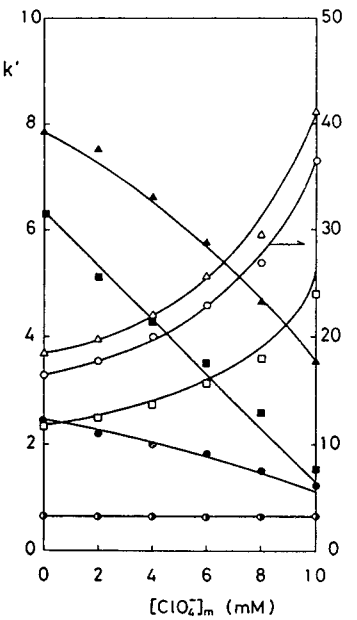

Fig. 1. Capacity factors of inorganic ions on Sephadex G-25 as a function of concentration of ClO_4^- in acetone–water (70:30, v/v) containing $NaNO_3$ and $NaClO_4$. The solid lines were calculated for the cations and anions according to eqns. 11 and 14, respectively. $[Na^+]_m = [NO_3^-]_m + [ClO_4^-]_m = 10$ m*M*. ● = Li^+; ▲ = K^+; ■ = Mg^{2+}; ○ = Cl^-; △ = I^-; □ = SCN^-; ◑ = nitrobenzene.

$NaClO_4$ system, $k'^{NaClO_4}_K$, is obtained by substituting $k'^{NaNO_3}_K = 7.87$, $k'^{NaNO_3}_{ClO_4} = 1.06$ and $k'^{NaNO_3}_{NO_3} = 5.31$ into eqn. 15 as follows:

$$k'^{NaClO_4}_K = 7.87 \cdot \left(\frac{1.06}{5.31}\right)^{1/2} = 3.52 \qquad (17)$$

The calculated k' values in $NaClO_4$ and NaCl systems obtained with eqn. 15 or 16 are listed in Table I together with the experimental values.

The capacity factor of NO_3^- in the $NaNO_3$ system was assumed to be the same as that of the system peak observed when $NaNO_3$ was injected. This assumption is reasonable if ion exclusion and ion-exchange effects exerted by the fixed ionic groups of the polymer matrix are negligibly small [16]. The details of the elution behaviour of system peaks in mobile phase systems containing more than one electrolyte will be reported in a subsequent publication.

It is seen from Fig. 1 that the calculated k' values are in remarkably good agreement with

TABLE I

CALCULATED AND EXPERIMENTAL CAPACITY FACTORS OF INORGANIC IONS ON SEPHADEX G-25 WITH ACETONE–WATER (70:30, v/v)

Background electrolyte	Value	Analyte ion							
		Li^+	K^+	Mg^{2+}	ClO_4^-	SCN^-	I^-	NO_3^-	Cl^-
$NaNO_3$	Exptl.	2.44	7.87	6.29	1.06	2.36	3.69	5.31[a]	16.56
$NaClO_4$	Exptl.	1.23	3.55	1.67	2.15[a]	4.80	8.22	11.83	36.48
	Calcd.	1.09	3.52	1.26	2.37	5.28	8.26	11.88	37.06
NaCl	Exptl.	4.59	15.45	17.80	0.83	1.62	2.54	3.88	12.02[a]
	Calcd.	4.31	13.89	19.61	0.60	1.34	2.09	3.01	9.38

[a] System peak.

the experimental values for both cations and anions. The k' value of nitrobenzene, which is a non-ionic solute, is independent of the composition of the mobile phase anions.

In Fig. 2, the experimental and theoretically predicted k' values are compared for NaCl–$NaNO_3$–$NaClO_4$ ternary mixtures, where concentrations of Na^+ and NO_3^- are kept constant at 10 and 2 mM, respectively. Again, theoretical k' values were calculated from eqn. 11 or 14 using only the experimental k' data obtained in $NaNO_3$ solution. Fig. 2 shows that there is fairly good agreement between the experimental and predicted k' values. The larger deviation between the predicted and experimental points for Mg^{2+} may be attributed to the possible ion association of Mg^{2+} with Cl^-.

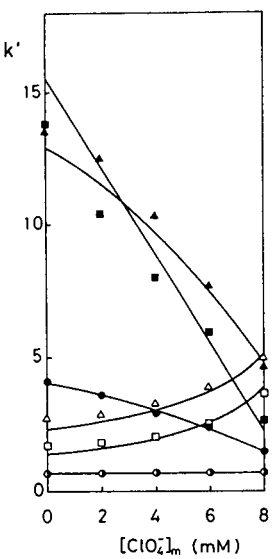

Fig. 2. Capacity factors of inorganic ions on Sephadex G-25 as a function of concentration of ClO_4^- in acetone–water (70:30, v/v) containing $NaNO_3$, NaCl and $NaClO_4$. $[NO_3^-]_m = 2$ mM; $[Na^+]_m = [NO_3^-]_m + [Cl^-]_m + [ClO_4^-]_m = 10$ mM. ○ = Li^+; ▲ = K^+; ■ = Mg^{2+}; △ = I^-; □ = SCN^-; ◑ = nitrobenzene. For other details, see Fig. 1.

The results shown in Figs. 1 and 2 indicate not only the validity of the theoretical approach in this paper but also the practical usefulness of the proposed equations for calculating capacity factors of ionic solutes from a very limited amount of experimental data.

Sephadex G-25/water and Bio-Gel P-2/water systems

As a second test of the validity of eqns. 11 and 14, the retention data of inorganic ions on Sephadex G-25 and Bio-Gel P-2 obtained when eluted with aqueous solutions of NaCl and $NaClO_4$ were used.

It is well known that water molecules sorbed in hydrophilic polymer gels generally exhibit physical properties distinct from those of ordinary free water by the interaction with polar groups and/or the hydrophobic part of the polymer matrices [17–21]. The retention of a solute compound on a water-swollen non-ionic polymer gel can be regarded as being governed by the partitioning of the compound between the external bulk water and the water in polymer

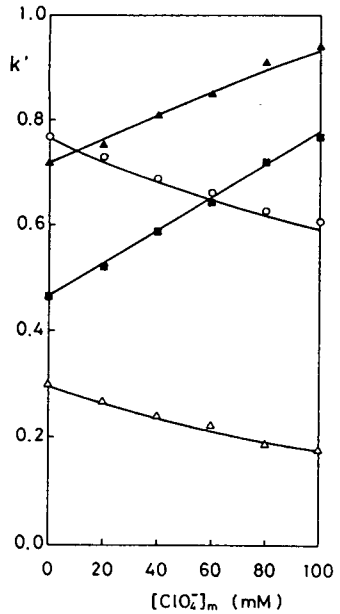

Fig. 3. Capacity factors of inorganic ions on Sephadex G-25 as a function of concentration of ClO_4^- in aqueous solution containing NaCl and $NaClO_4$. $[Na^+]_m = [Cl^-]_m + [ClO_4^-]_m$ = 100 m*M*. ▲ = K^+; □ = Mg^{2+}; ○ = NO_3^-; △ = SO_4^{2-}. For other details, see Fig. 1.

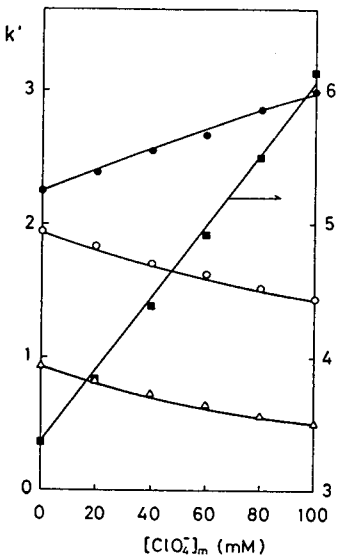

Fig. 4. Capacity factors of inorganic ions on Bio-Gel P-2 as a function of concentration of ClO_4^- in aqueous solution containing NaCl and $NaClO_4$. $[Na^+]_m = [Cl^-]_m + [ClO_4^-]_m$ = 100 m*M*. ○ = Li^+; □ = Mg^{2+}; ○ = NO_3^-; △ = SO_4^{2-}. For other details, see Fig. 1.

gels, which has properties different from those of ordinary bulk water [14,21].

Figs. 3 and 4 show the capacity factors of some inorganic ions on Sephadex G-25 and Bio-Gel P-2 as a function of the concentration of ClO_4^- in the mobile phase containing NaCl and $NaClO_4$; the total concentration of Cl^- and ClO_4^-, that is, the concentration of Na^+, was kept constant at 0.1 *M*. The theoretical k' values (solid lines) were calculated from eqn. 11 or 14 using the experimental k' data obtained in the NaCl eluent system; k'^{NaCl}_{Cl} was assumed to be the same as k' of the system peak observed when NaCl was injected into the column equilibrated with 0.1 *M* NaCl aqueous solution. As can be seen from Figs. 3 and 4, the calculated k' values are in good agreement with the experimental values. It should be noted that only divalent cations show the linear relationship between k' and the concentration of ClO_4^-, as expected from eqn. 11.

The results described above support the generality and practical applicability of the ion partition model to both single solvent systems and binary solvent systems. Although this work was carried out only on hydrophilic polymer gels, the argument presented here can be applied to the results obtained with other non-ionic packings as the model does not depend on a specific retention mechanism.

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Ion chromatographic determination of carboxylic acids in air with on-line liquid membrane pretreatment

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ABSTRACT

A method for the determination of low-molecular-mass carboxylic acids in air is reported. The method is based on impinger sampling in sodium hydroxide, selective enrichment across a liquid membrane and determination by ion-exclusion chromatography. The membrane enrichment is carried out in an automated continuous-flow system coupled to the chromatographic column. By impregnation of the membrane with 10% tri-*n*-octylphosphine oxide in di-*n*-hexyl ether, extraction efficiencies in the range 30–100% for C_1–C_4 carboxylic acids were achieved. Formic and acetic acid were measured in the range 17–300 nmol/m^3 and some other carboxylic acids were detected in air samples taken in southern Sweden.

INTRODUCTION

The occurrence of various carboxylic acids in the atmosphere, measured in the gas phase, in precipitates or in particles, has been reported in many papers. Relatively low concentrations (2–100 nmol/m^3) of formic and acetic acid have been found over oceans [1,2]. Model experiments and field investigations have shown that photochemical oxidation of hydrocarbons is an important source of gaseous organic acids in non-polluted areas [2]. Jacob and Wofsy [3] proposed isoprene emitted from biomass as a significant source of pyruvic, formic and methacrylic acid. This theory was supported by several studies carried out in the Amazon forest by Andreae and co-workers [4,5]. Direct emission of formic and acetic acid from vegetation has also been measured [6]. Anthropogenic sources such as motor exhausts [7,8] are reported to be the main origin of organic acids in urban areas. The average emission of monocarboxylic acids from motor vehicles in southern California basin was estimated to 15 000–20 000 kg/day, and gaseous formic acid and acetic acid in the range 50–800 nmol/m^3 were found in this area [9].

The contribution from organic acids to the total acidity in the atmosphere is important to air quality issues but has not been sufficiently studied. Grosjean [10] measured organic and inorganic acids (as Cl^- and NO_3^-) in ambient southern California air and found that 73.5% (mole basis) of the total gas-phase acids consisted of formic and acetic acid.

Long-path IR measurements are commonly used for various gaseous molecules including formic and acetic acid [11]. It is a rapid method that gives instantaneous results, but the sensitivity is lower than that of chromatographic techniques.

A number of sampling methods for gaseous and particle-bound formic and acetic acid have been compared in a comprehensive study [12]. It was concluded that only the mist chamber [13] and NaOH-coated denuders were free from

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significant interferences. In another recent study, various other collection devices, such as alkaline filters and solvent traps, have been found to give considerable interferences due to reactions with other compounds present in air (*e.g.*, aldehydes), while microimpingers were free from these interferences [14]. Thus, absorption in an alkaline solution appears to be the safest principle for air sampling of acids. The mist chamber is a recently developed device for wet absorption of gaseous components and it has been successfully used for the collection of formic and acetic acid [4,5,15]. The advantage is that this collector over a conventional impinger is that it allows a higher air flow-rate (up to 8 l/min), but a drawback is that it is not commercially available.

For the determination of acetic and formic acid in air, further preconcentration of the absorbing solution is usually not necessary, but the determination of other acids present in very low concentrations would require some treatment prior to the determination step. A range of organic acids (C_1–C_7) have been measured in rain and fog samples by GC after concentrating the sample using a rotary evaporator [16]. For analysis of antarctic ice an anion exchanger was used as a precolumn in order to improve the detection limits for formic, acetic, propanoic and butanoic acid [17].

An efficient technique for the selective enrichment of various classes of compounds using supported liquid membranes has been developed at our laboratory [18,19]. With this technique, the required enrichment of acids in aqueous absorbing solutions can be made in a closed automated system, with on-line connection to chromatographic instrumentation. Interfering compounds (non-acidic, particulate, etc.) are simultaneously rejected.

Carboxylic acids are most often determined by ion-exchange or ion-exclusion chromatography [5,7,8,17]. Generally, lower detection limits can be achieved with open-tubular column GC after derivatization of carboxylic acids to *p*-bromophenacyl esters [16,20]. This method in combination with mass spectrometry has been used for the determination of nineteen dicarboxylic acids in ambient air [21]. However, the difficulty of automating the derivatization and extraction procedures, the introduction of additional operations and chemicals and the long analysis times are disadvantages of the described GC methods compared with LC.

In this paper, we present an automated method for the determination of carboxylic acids at low concentrations, involving impinger sampling, liquid membrane enrichment and ion-exclusion chromatography. With this combination, a convenient and selective technique is obtained for the determination of carboxylic acids in air with few interferences and sufficient sensitivity.

EXPERIMENTAL

Equipment

Analyses were performed on a Dionex 4000i ion chromatograph connected on-line to a liquid membrane enrichment flow system. An HPICE-AS1 ion-exclusion column with 2 m*M* HCl as eluent was used. The recommended eluent, 1 m*M* octanesulphonic acid, was also tested. The eluent and NaOH solution (for the flow system) were kept in helium-pressurized bottles in the Dionex eluent degas module. An anion micro membrane suppressor (AMMS-ICE) with 5 m*M* tetrabutylammonium hydroxide as regenerant was used. The regenerant flow control valve (Dionex) was replaced with a three-way slider valve (Model 5301; Reodyne, Cotati, CA, USA) for on–off regulation of the regenerant solution. The chromatograms were collected and handled with a personal computer (Victor V386A; Victor Technologies, Stockholm, Sweden), using a JCL6000 chromatography data system (Jones Chromatography, Hengoed, UK), which also controlled the time sequence for the operation of the valves in the flow system.

The flow system is shown in Fig. 1. The three pneumatic valves in the Dionex chromatography module (injection valve, column-switching valve and regenerant flow control valve) were used for the flow system, which was constructed inside the chromatography module. A Minipuls 3 peristaltic pump (Gilson, Villers-le-Bel, France) with PVC pump tubes (Elkay, Shrewsbury, MA, USA) was used. The confluences, where the channels meet at a 60° angle, were made of PTFE. Reagents were mixed in a knotted tube

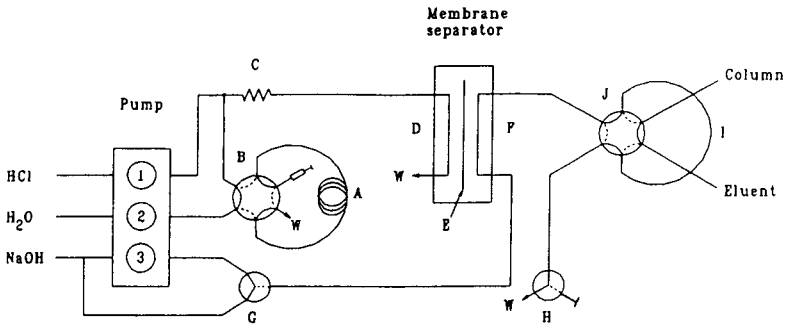

Fig. 1. Flow system for enrichment of organic acids. A = Sample loop; B and J = injection valves; C = knitted tube reactor; D = donor channel; E = liquid membrane; F = acceptor channel; G and H = valves; I = injection loop.

reactor (0.5 mm I.D., six knots). PTFE tubing, flange-free fittings (Alltech, Deerfield, IL, USA) and Dionex standard fittings were used to connect the various parts of the flow system.

The membrane used was a porous PTFE membrane (TE 35 membrane filter, Schleicher & Schüll, Dassel, Germany) with a pore size of 0.2 μm and cut to 36×77 mm^2. The membrane was held between two blocks of PTFE (outer dimensions $85 \times 55 \times 15$ mm^3), with meander channels facing each other (Fig. 2). Each channel was 0.1 mm deep, 2.5 mm wide and 750 mm long, giving a calculated volume of 188 μl. The PTFE blocks were tightened together with eight screws (see Fig. 2).

The solutions for impregnating the membrane were prepared by adding 10 ml of di-*n*-hexyl ether to the desired amount of tri-*n*-octylphosphine oxide and sonicating it for *ca.* 1 h. The membrane was immersed in the organic liquid in a petri dish for a few minutes (the time is not critical). After mounting the membrane, excess of solvent was washed out from the separator by pumping acceptor and donor solutions through the channels for about 30 min. A membrane prepared in this way can be used for several hundred enrichments.

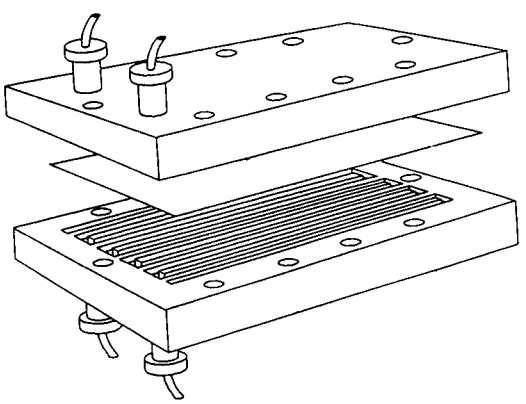

Fig. 2. Membrane separator unit.

Operation of the liquid membrane enrichment system

Referring to Fig. 1, the operation can be described as follows (for a detailed description of the liquid membrane extraction technique, see refs. 18 and 19). The sample is introduced through a 3.0-ml loop (A) into the aqueous carrier stream (channel 2, 0.17 ml/min) using valve B. The pH is decreased to *ca.* 1.4 by mixing with 1 *M* HCl (channel 1, 0.02 ml/min) in a knitted tube reactor (C). From the donor side of the membrane separator (D) the organic acids are extracted into the liquid membrane (E). On the other side of the membrane, the acids are trapped in a stagnant NaOH solution (acceptor phase, F). After the whole sample has passed the separator, valves G and H are switched. The enriched sample plug (*ca.* 200 μl) is then pushed by the acceptor solution into a 300-μl injection loop (I) and injected into the ion-exclusion separation column by switching valve J. A typical enrichment time is 20 min, during which time a previously extracted sample is chromatographed. Between the enrichment cycles the sample loop and the separator are washed with the donor and acceptor solutions, respectively, for 3 min to reduce memory effects. Only very

small losses (due to adsorption) of the free acids were observed during the transport from the membrane unit to the injection loop.

Determination of extraction efficiency

The enrichment efficiency for each acid was calculated in the following way. Calibration graphs for direct injections (without enrichment) were constructed in the range 10–320 μM. A 20-μM solution was enriched as described above and the corresponding concentration after enrichment was evaluated using the calibration graph. The extraction efficiency, E, is defined by $E = n_A/n_D$, where n_A is the number of moles of analyte collected in the acceptor phase and n_D is the number of moles pumped into the donor phase. Here n_A is given by the evaluated concentration after enrichment times the volume of the injection loop (300 μl) and n_D by the concentration before enrichment (20 μM) times the sample loop volume (3.0 ml).

Air sampling

Midget impingers with glass joints were used for collection of the air samples. The 20 mM NaOH absorption solution was prepared freshly every day and also analysed as a blank. The impingers were wrapped in aluminum foil to avoid photochemical reactions of the acids during sampling in daylight. Two parallel samples were collected simultaneously in most instances. The pumps were portable programmable air sampling pumps (Model 224-30, SKC) and 480 or 960 l were collected with a flow-rate of 2 ml/min. All connections were made of glass except that a PVC tube used between the outlet of the impinger and the pump.

The sampling recovery was determined by bubbling nitrogen through three impingers connected in series, the first containing a 20 mM solution of seven carboxylic acids (pH < 2) and the second and third containing 20 mM NaOH, followed by analysis of the contents of the second and third impingers [22]. Sources of contamination were identified by soaking plastic caps, screw-caps, tubing and filters in the sampling solution for 2 h. The samples were stored in a refrigerator and analysed within 2 days.

Chemicals

Formic, acetic, oxalic, succinic and hydrochloric acid and chloroform were obtained from Merck (Darmstadt, Germany), propanoic acid from Aldrich Chemie (Steinheim, Germany), butanoic acid and lactic acid from BDH (Poole, UK), malic acid, sodium pyruvate and di-*n*-hexyl ether from Sigma (St. Louis, MO, USA), glycolic acid (70% in water) from Kebo (Stockholm, Sweden), sodium hydroxide from EKA (Bohus, Sweden), tri-*n*-octylphosphine oxide and tetrabutylammonium hydroxide (1.5 M) from Fluka (Buchs, Switzerland) and octanesulphonic acid (0.1 M) from Fisons Scientific (Loughborough, UK). Analytical-reagent grade chemicals were used when available. Water was purified with a Milli-Q/RO-4 unit (Millipore).

Stock standard solutions of the acids (20 mM) were prepared in water; 0.5 ml of $CHCl_3$ (Merck) per 100 ml was added as a biocide. The stock standard solutions were stored in a refrigerator, and fresh working standard solutions were prepared every day. To minimize the carbonate content of the NaOH solution, it was prepared by dissolving prewashed NaOH pellets in water. The concentration was determined by titration with H_2SO_4. The solution was then kept in a Dionex eluent bottle under helium pressure.

RESULTS AND DISCUSSION

Membrane extraction efficiency

Choice of membrane solvent. The first experiments were carried out with a membrane impregnated with pure di-*n*-hexyl ether (DHE). The extraction coefficient was reasonably high for propanoic and butanoic acid but low for formic and acetic acid, and for the more polar acids (pyruvic, malic and lactic) no extraction was obtained. The extraction efficiency and the precision were substantially improved incorporating tri-*n*-octylphosphine oxide (TOPO) in the membrane solvent. Table I shows the differences in extraction efficiency and relative standard deviation for a 20 μM solution of seven acids in DHE without and with 10% of TOPO.

TOPO is a reagent that has been used for facilitating liquid membrane extraction of carboxylic acids [23]. It acts as a carrier molecule,

TABLE I

EXTRACTION EFFICIENCY AND PRECISION WITH 10% TOPO IN DHE IN THE MEMBRANE

Acid	Extraction efficiency (%)		R.S.D. (%)[a]	
	DHE	10% TOPO in DHE	DHE	10% TOPO in DHE
Pyruvic	–	69	–	3.1
Malic	–	29	–	5.8
Lactic	–	34	–	2.1
Formic	5.1	77	41	2.2
Acetic	16	63	8.2	6.0
Propanoic	46	99	9.6	7.0
Butanoic	81	93	4.8	2.6

[a] Relative standard deviation ($n = 4$).

which by hydrogen bonding to the acid forms an apolar species, more soluble in the membrane liquid than the free acid. In general, the extraction efficiency for the investigated acids increases with increased TOPO content in the membrane. The effect was more pronounced for the more polar hydroxy acids [24]. Extraction of HCl became disturbing at TOPO concentrations over 10%, as the chloride peak increased markedly with increasing TOPO concentration. The optimum membrane solvent was considered to be 10% TOPO in DHE.

Choice of acceptor solution. One of the most important parameters for controlling the enrichment across the membrane is pH. The pH of the stagnant acceptor solution is critical, as a high pH is essential in order to obtain enrichment of the acids. The initial pH of the acceptor solution (20 m*M* NaOH) before enrichment starts is *ca.* 12, but as the organic acids and hydrochloric acid accumulate in the acceptor phase, the pH decreases. The critical contribution was found to originate from HCl, especially at high TOPO concentrations. Extraction of carbonic acid generated from CO_2 also contributes to this pH drop. The CO_3^{2-} peak increased with increasing NaOH concentration in the acceptor, but the pH of the acceptor stabilized at *ca.* 8, owing to buffering. Different concentrations (10–100 m*M*) of NaOH in the acceptor solution were compared for extraction of six carboxylic acids (20 μM) with 5% TOPO in the membrane. The extraction efficiency was similar with 10 and 20 m*M* NaOH, but decreased markedly with 50 and 100 m*M* NaOH as the acceptor [24]. The reason for this is not fully understood but the phenomenon will be further investigated.

As an alternative to NaOH solution, various buffer systems could be considered, but the anions of a buffer are very likely to interfere with the analyte ions in the chromatogram. A borate and a phosphate buffer were tried but rejected owing to the large interfering peaks that arose. A tris(hydroxymethyl)aminomethane (Tris) buffer has been tried with promising results [24].

Air sampling

As the sampling procedure is a common source of errors, various experiments were performed in order to investigate suspected artifacts. Keene *et al.* [12] concluded that there are significant systematic and episodic artifacts among many currently developed measurement systems for formic and acetic acid.

Impingers were chosen for the sampling owing to their simplicity and high efficiency. Dilute NaOH was chosen as the absorption solution as water was found to be less efficient. The pH of the NaOH solution decreased (owing to collection of CO_2) from 12 to 9.4 in both the first and second impinger, regardless of the air volume sampled (480 or 960 l). The sampling recovery in NaOH was found to be >95% for the seven acids tested. The same recovery was achieved with a buffer solution (20 m*M* $NaHCO_3$ + NaOH) at pH 9.5, indicating that the pH drop of the NaOH solution during sampling is not a problem.

A particulate filter was not used as we found no significant difference between parallel samples with and without a filter. It has been shown in a study carried out in Virginia, USA, that ≥98% of atmospheric acetic and formic acid occur in gaseous form [15]. Significant contamination from various materials such as PVC and silicone-rubber tubing, polypropene caps, PTFE-faced screwcaps and polycarbonate filters was revealed when soaked in NaOH. Therefore, all

sample containers and connections used were made of glass or PTFE except between the outlet of the impinger and the pump. The risk of contamination of the sampling solution by vapour in the indoor air was observed by leaving an impinger with sampling solution exposed to the air in the laboratory via the inlet and outlet for 2 days. Small amounts of formic and acetic acid were found in the solution when analysed. The sampling solution was always prepared fresh in a separate room to circumvent contamination from sources in the laboratory. Similar problems with contamination from plastics and indoor air have been reported by others [25].

Addition of chloroform to the samples in order to prevent microbiological activity in the samples has been recommended by several workers [1,9]. Chloroform has also been added to the sampling solution before impinger collection [14]. However, it is known (and observed by us) that chloroform can be photochemically converted into formic acid. As the samples were analysed within 2 days after sampling, no chloroform was used for the samples.

Suppression of carbonate

The high level of carbon dioxide in air caused a large tailing carbonate peak, interfering with propanoic acid in the chromatogram. It was possible to separate the peaks with a weaker eluent (30 μM HCl, pH 4.5), but then the other acids were not sufficiently resolved.

An attempt was made to remove the carbonate with a gas membrane (an "empty" PTFE membrane) by letting the carbon dioxide diffuse from the acidified sample through the membrane into an acceptor phase consisting of 10 m*M* NaOH. The method could have been incorporated on-line in the flow system, but the loss of carboxylic acids was 10–30% and this method was rejected.

A more successful technique was to bubble nitrogen through the air samples after decreasing the pH to 2–3 by adding 1 *M* HCl. The carbonate peak disappeared from the chromatogram after bubbling for only 1 min. In order to check the stability of the carboxylic acids, a standard solution of 200 μM was treated with nitrogen for 0.5–10 min. The solutions were injected directly into the column (without membrane enrichment). In Fig. 3 it can be seen that there is no loss of acids, even with longer bubbling times. However, after membrane enrichment of any sample or blank, there was still a small carbonate peak originating from CO_2 dissolved in the acceptor solution and the carrier.

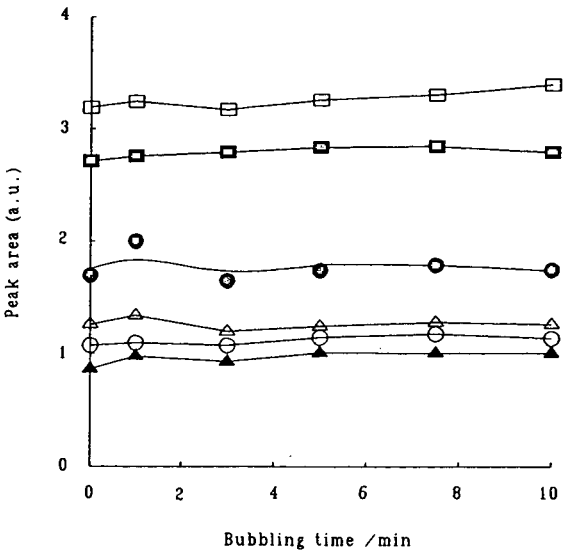

Fig. 3. Stability of a 200 μM solution of organic acids in 1 m*M* HCl after bubbling with nitrogen for 0–10 min. ○ = Lactic; ● = formic; □ = malic; ■ = pyruvic; △ = acetic; ▲ = butanoic acid.

Chromatography

In the first experiments, 1 m*M* octanesulphonic acid containing 2% of 2-propanol was used as the eluent, according to the recommendation of Dionex. The separation of seven carboxylic acids (pyruvic, malic, lactic, formic, acetic, propanoic and butanoic acid) was satisfactory but some acids occasionally eluted as negative peaks. With 1 m*M* HCl the separation of these acids was equally good and no peaks were negative. In order to improve the separation of pyruvic acid from the chloride peak (*i.e.*, the front peak), the eluent concentration was increased to 2 m*M*. The drawback was a slightly deteriorated resolution between lactic and formic acid. Other acids that unfortunately co-eluted in this system were glycolic, lactic and succinic acid. Fig. 4 shows chromatograms of a standard solution of

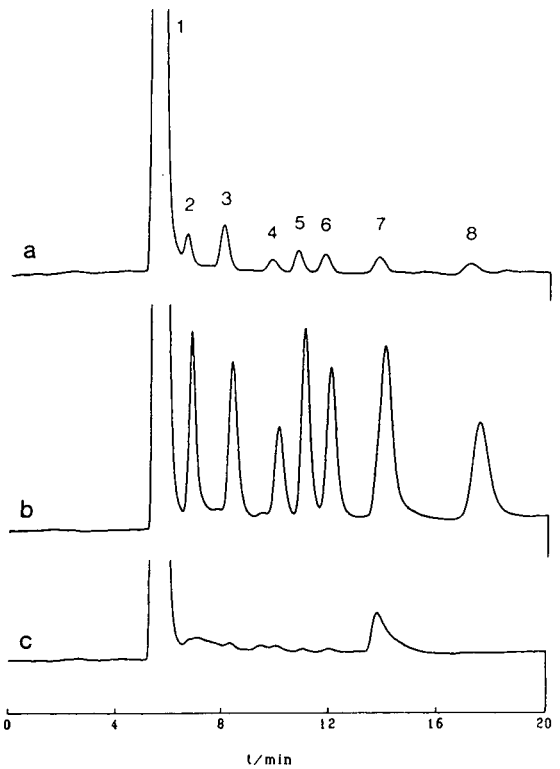

Fig. 4. Chromatograms of organic acids: (a) 24 μM standard solution in 1 mM HCl without enrichment; (b) after membrane enrichment; (c) blank (water). Peaks: 1 = Cl^-; 2 = pyruvic; 3 = malic; 4 = lactic; 5 = formic; 6 = acetic; 7 = propanoic (and CO_3^{2-}); 8 = butanoic acid.

seven organic acids (24 μM) injected with and without membrane enrichment and an enriched blank (water). This blank was injected directly after the enrichment of the standard solution showing also the overall memory effects. The blank peaks corresponded to 2–6% of the peaks of the standard solution. The contribution of carbonate to the propanoic acid peak in the chromatogram of the enriched solution (Fig. 4b) is revealed in the blank chromatogram (Fig. 4c). The interference from carbonate in the chromatogram of the directly injected solution (Fig. 4a) is negligible.

Quantification

Calibration graphs for the seven carboxylic acids after membrane enrichment were measured in the range 0.1–24 μM (Table II). The graphs

TABLE II

CALIBRATION GRAPHS AND PRECISION FOR VARIOUS ACIDS

Acid	Concentration range (μM)	Slope[a]	Intercept[a]	r
Pyruvic	1–24	10 ± 0.6	0.3 ± 8	0.9994
Malic	0.5–24	9 ± 0.5	−6 ± 6	0.9992
Lactic	0.5–24	7 ± 2	−0.9 ± 24	0.9911
Formic	0.1–24	12 ± 1	−3 ± 18	0.9966
Acetic	0.1–24	12 ± 0.6	−2 ± 7	0.9990
Propanoic[b]	1–24	15 ± 2	129 ± 30	0.9965
Butanoic	0.5–24	15 ± 0.4	−5 ± 4	0.9999

[a] Arbitrary units, 95% confidence interval.
[b] Including carbonate peak.

were linear with insignificant intercepts (95% confidence intervals) for all acids except propanoic acid, which has an intercept corresponding to the carbonate peak. The precision (Table I) is not significantly worse than for the other acids. The determination of pyruvic acid is limited by the front peak.

The detection limit with membrane enrichment of a 3-ml sample was 100 nM for formic and acetic acid and 500 nM for pyruvic, malic, lactic and butanoic acid. For propanoic acid the interfering carbonate peak resulted in a detection (and quantification) limit of *ca.* 6 μM, which is about the same as for direct injection of a carbonate-free solution without enrichment. The limit of quantification determined from the calibration graph was in the range 1–3 μM for all acids in Table II except propanoic acid. This corresponds to 20–60 nmol/m^3 in air with sampling of 480 l of air and enrichment of 3 ml. The detection limit can be lowered at the cost of time by prolonging the sampling time or by enriching larger volumes of the absorbing solution.

Measurement of acids in air

To demonstrate the applicability of the described technique to real air samples, some samples were collected at various sites in southern Sweden, giving the results presented in Table III. This was not intended as a detailed investigation of the occurrence of organic acids in air. Formic and acetic acid were detected in all

TABLE III

CARBOXYLIC ACIDS IN AMBIENT AIR IN SOUTHERN SWEDEN

Site	Date (May 1993)	Time	T (°C)[e]	Wind direction[f]	Concentration (nmol m^{-3})		
					Formic acid	Acetic acid	Propanoic acid[g]
Lund[a]	14	11.15	23	S	28	17	nd
Lund[a]	17	14.18	16	SE	26	18	nd
Lund[a]	18	11.15	18	E	48	20	nd
Lund[a]	18	13.21	15	E	35	19	tr
Lund[a]	18–19	22.02	10	E	43	25	tr
Lund[b]	17	20.24	12	E	44	36	tr
Sandhammaren[c]	20	13.17	20	E	300	140	tr
Vallby[d]	21	00.08	13	E	140	150	tr
Vallby[d]	21	11.15	22	E	170	120	tr

[a] *ca.* 3 km from city centre.
[b] City centre.
[c] Rural, 50 m from the sea.
[d] Rural, 10 km from the sea.
[e] Mean temperature.
[f] Predominant wind direction.
[g] nd = Not detected; tr = traces.

samples in the range 17–300 nmol/m^3. An interesting observation is that the concentrations were considerably higher in rural areas with intense plant growth [2] than in the city of Lund. The increased level of acetic acid in the city centre compared with the other city location may be attributed to the denser traffic (including ethanol-fuelled buses) in the centre.

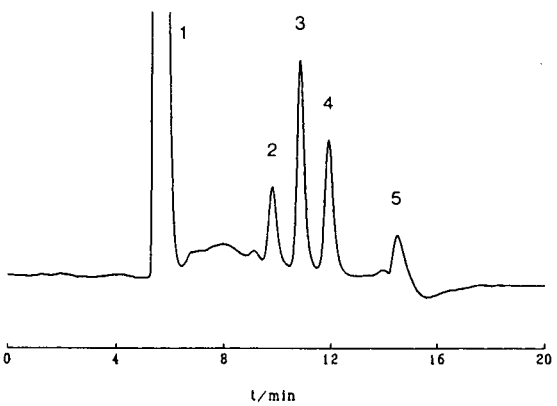

Fig. 5. Chromatogram of an air sample taken on May 21st in Vallby, S.E. Sweden. A 480-l volume of air was sampled with an impinger and 3 ml were enriched and analysed as described in the text. Peaks: 1 = Cl^-; 2 = unknown; 3 = formic acid; 4 = acetic acid; 5 = propionic acid and CO_3^{2-}.

In some samples traces of propanoic acid were found. In Fig. 5, a chromatogram is shown of an air sample taken at a rural site in the south east of Sweden. The unidentified peak could be lactic, succinic or glycolic acid or a mixture of them. Unfortunately, these acids are difficult to separate by ion-exclusion chromatography. Succinic acid up to 2.4 nmol/m^3 has been measured in Los Angeles with a GC method [21]. The concentrations of formic and acetic acid in two samples taken simultaneously with parallel impingers were the same (within 4%).

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Determination of phenols at the ng/l level in drinking and river waters by liquid chromatography with UV and electrochemical detection ☆

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ABSTRACT

Solid–liquid extraction of samples and liquid chromatography with UV and electrochemical detection with laboratory-made microcolumns were applied to the separation and identification of phenols and substituted phenols in waters. The compounds mainly studied were the eleven phenols considered as priority pollutants by the US Environmental Protection Agency. Chromatographic separation was carried out with several water–methanol isocratic mobile phases; use of the autoincrement mode of the electrochemical detector allowed the compounds in the samples to be confirmed. The chromatographic system worked automatically. The detection limits obtained with prior concentration of the samples were 40–600 ng/l, depending of the phenol. Electrochemical detection was used for the determination of phenols in river and drinking waters; phenols at the ng/l level was detected

INTRODUCTION

The determination of phenols in drinking and river waters is of great importance now that the MAC (Maximum Admissible Concentration) in the EEC countries for phenols in drinking water is 0.5 μg/l, excluding those natural phenols which do not react with chlorine [1]. The official methods [1] recommended for their determination are based on the measurement of an index of phenols using spectrophotometric methods based on 4-aminoantipyrine or 2-nitrophenol [2,3]. These methods are subject to interferences and only with difficulty can phenolic compounds be detected at the 0.5 μg/l level [4,5] demanded at present.

Chlorophenols and nitrophenols, which are used in industry and agriculture and as wood preservatives, etc., can be present in raw waters as a result of spillages or accidents. Because of this, in the 1970s the US Environmental Protection Agency (EPA) [6] created a list of the eleven most important phenol contaminants as priority pollutants. Chlorophenols can be formed during water chlorination [7], causing problems of taste and odour in waters at very low concentrations, near the μg/l level.

There are numerous standard methods for determining and for confirming the presence of

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phenols [2,3,5,8]. The most recent [8] are based on their concentration by liquid–liquid extraction, derivatization and measurement by gas chromatography with electron-capture detection. This is the official method of the EPA [9] and gives limits of detection between 0.58 to 2.2 μg/l for the eleven priority pollutant phenols.

Methods of liquid chromatography (LC) with UV detection [5,10–12] have also been used, together with LC with UV detection and derivatization "on-line" [13]. In this way, LC has been used with electrochemical detection (ED) [14–16], achieving excellent limits of detection with easy sample preparation.

In this work, the determination of a wide range of phenols, nitrophenols chlorophenols, methylphenols, hydroxyphenols and other phenols, mainly those considered to be priority pollutants by the EPA, was studied. LC–ED was applied, employing an amperometric detector which uses the electronic treatment technique to correct for the electrodeposition, as proposed by Wang and Lin [17,18]. This detector was studied by Gretzfeld-Hüsgen and Schuster [16] in the determination of certain phenols, but here all the phenols listed by the EPA were considered. ED and UV detection were compared in order to check the improvement in sensitivity and limits of detection for the former method.

To confirm the presence of phenols, a method similar to that proposed by Shoup and Mayer [14] and by Hennion *et al.* [15] was used. In this method the amperometric detector was equipped with two working electrodes to which different potentials were applied. The detector produced two signals and their ratio was used to confirm the compounds. In this work we used the detector function defined as the "autoincrement mode". This allowed us to obtain automatically the chromatograms of phenols and signal ratios at different potentials using only one working electrode.

To improve the limits of detection of phenolic compounds, solid-phase extraction with reversed-phase C_{18} microcolumns, prepared in the laboratory, was used. The proposed technique was applied to determine phenols in drinking and river waters, giving limits of detection lower than those of the EPA standard method [9]. With prior concentration, the limits of detection were 1.2–15 pg (40–600 ng/l), depending on the phenol.

EXPERIMENTAL

Equipment

The system of gradients, pump, furnace, automatic injector/autosampler and programmable UV detector were from a Series HP 1050 from Hewlett-Packard (Waldbron, Germany). The electrochemical amperometric detector was an HP 1049 A (Hewlett-Packard) connected in series to the output of the UV detector. All the modules of the configuration were connected by interfaces, and the whole system in turn was controlled by an HP QS/20 87 personal computer (Hewlett-Packard) with outputs to a screen and printer. The interface to connect the start–stop orders and to control the signals input from the two detectors was an HP 35900 C (Hewlett-Packard). The data analysis, results and system control were executed using the chromatographic software Chemstation HP 3365 Series II (Hewlett-Packard), which is controlled by Windows 3.0 (Microsoft).

The stability of the HP 1049 A electrochemical detector was studied by Gretzfeld-Hüsgen and Schuster [16] for tetrachlorophenol and pentachlorophenol, giving an R.S.D. lower than 2.5% for 200 injections, based on both area counts and peak heights.

Chromatographic separation and detection

The separation of the compounds was achieved using a 5-μm LiChrospher 100 RP-18 precolumn (4 × 4 mm I.D.) connected to a 5-μm Spherisorb ODS-2 column (250 × 4 mm I.D.), both supplied by Hewlett-Packard. The temperature of the column was kept at 40°C and the flow-rate of the eluents was 0.8 ml/min. The volume of sample injected by automatic injection was 25 μl in all instances (blanks, standards and samples).

The wavelength of the UV detector was set at 280 nm, except for pentachlorophenol, which was determined at 300 nm by programming the detector before its elution. The electrochemical detector worked in the amperometric mode with

a glassy carbon electrode at a potential of 1000 mV between the working and the reference electrodes. A solid-state AG/AgCl reference electrode was used, so the eluents used with the electrochemical detector contained KCl (0.05 g/l). This detector has a pretreatment function to clean the working electrode automatically, which was used every fifteen injections, applying in a cyclical form (two cycles) alternate potentials of −800 and +1300 mV during 500 ms. The working electrode was polished in the conventional way every 60 injections.

Mobile phases

The water–methanol gradients used for UV detection were prepared with HPLC-grade water acidified with 28 μl/l of 98% H_2SO_4 (pH 3), to which were added different proportions of methanol. The mobile phases used with the UV detector were as follows: gradient A, 0 min 35% of methanol, 10 min 40% of methanol, 25 min 80% of methanol; and gradient B, 0 min 40% of methanol, 20 min 80% of methanol. Gradient A was used for the general separation of 21 phenols and gradient B to separate the eleven EPA priority pollutant phenols.

The isocratic mobile phases of water–methanol used with the electrochemical detector were as follows: eluent A, 25% of methanol, eluent B, 50% of methanol and eluent C, 75% of methanol, with HPLC water acidified with 28 μl/l of 98% H_2SO_4 (pH 3) that contained KCl (0.05 g/l) and KNO_3 (2 g/l). Eluents B and C were used to screen for the eleven phenols being studied, and the eluent A exclusively for the determination of phenols in the real samples. The background currents registered for these three eluents with the working electrode in the correct state were 40–80 nA. The retention times obtained with the gradient and isocratic conditions used are given in Table I.

Sample treatment and solid-phase extraction

Samples were placed in glass bottles (1 l) with a PTFE-covered stopper and kept in the dark at 4°C. Volumes of 100 ml of these samples were

TABLE I

RETENTION TIMES (min) OF DIFFERENT PHENOLS UNDER THE ELUTION CONDITIONS USED

Phenol	UV detection		ED detector		
	Gradient A	Gradient B	Isocratic A	Isocratic B	Isocratic C
Hydroquinone	3.24				
Resorcinol	3.79				
Pyrocatechol	4.59				
Phenol	7.08	6.21	10.50	4.77	
4-Nitrophenol	9.53	8.20	16.80	5.60	
2,4-Dinitrophenol	11.33	9.65		6.50	
p-Cresol	11.80				
o-Cresol	12.10				
2-Chlorophenol	12.98	11.40		6.98	
2-Nitrophenol	14.56	13.20		8.25	
4-Chlorophenol	15.81				
2,6-Dimethylphenol	17.37				
2,4-Dimethylphenol	18.23	17.16		10.21	
2,6-Dichlorophenol	18.92				
2-Methyl-4,6-dinitrophenol	19.84	18.46		12.18	
4-Chloro-3-methylphenol	20.61	19.61		12.97	
2,4-Dichlorophenol	21.93	20.25		15.83	
2,4,6-Trimethylphenol	21.93				
2,4,6-Trichlorophenol	25.75	23.00		30.75	6.23
2,4,5-Trichlorophenol	26.14				
Pentachlorophenol	32.12	28.51			13.10

treated with 0.3 ml of 10% Na_2SO_3 solution to eliminate the free chlorine and with 100 μl of 65% HNO_3 (pH 2). The treated samples were then filtered through a 0.45-μm membrane filter from Schleicheir & Schüll (Dassell, Germany). Glass microcolumns (100 × 8 mm I.D.) filled with 500 mg of phase Bakerbond C_{18} type 7025-00 (J.T. Baker, Deventer, Netherlands) were used to concentrate phenols. The microcolumns were previously conditioned with 10 ml of acetonitrile, 10 ml of methanol and 100 ml of water (pH 2, adjusted with HNO_3). An aliquot of 25 ml of treated sample was taken and passed through the column, which was then washed with 2 ml of 0.01 *M* HNO_3 and the phenols were eluted with 1 ml of methanol. This methanolic solution was placed in 2-ml vials and injected into the chromatograph by the automatic injector/autosampler. The passage of the solvents and samples through the extraction equipment for microcolumns (J.T. Baker, type 70180) was accomplished under vacuum (250–500 mmHg); 1 mmHg = 133.322 Pa). Microcolumns were prepared and conditioned in the laboratory and before being used the column blanks were obtained with 1-ml samples of methanol, which were run to obtain the corresponding chromatograms.

Reagents and standards

The standards used were: 4-nitrophenol, 2,4-dinitrophenol, 2-nitrophenol, 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, 2,4,6-trichlorophenol, *p*-cresol, *o*-cresol, 2,4-dimethylphenol, 2,6-dimethylphenol, resorcinol, hydroquinone, pyrocatechol, phenol, 4-chloro-3-methylphenol and 2-methyl-4,6-dinitrophenol from Merck (Darmstadt, Germany), 2,4,5-trichlorophenol and 2,4,6-trimethylphenol from Fluka (Buchs, Switzerland) and pentachlorophenol from Janssen (Geel, Belgium). Stock standard solutions of 1000 mg/l were prepared in water–methanol (1:1) and kept in the dark at 4°C. Dilutions of the stock standard solutions were made with HPLC water immediately before use. All of the general reagents used, 98% H_2SO_4 and 65% HNO_3 (Merck), KCl, KNO_3 and Na_2SO_3 (Probus) were of analytical-reagent grade. The water, methanol and acetonitrile (Merck) used to prepare the eluents were of HPLC grade. The eluents were filtered using 0.45-μm membrane filters (Schleicher & Schüll) and were degassed with helium.

RESULTS AND DISCUSSION

Comparison between UV and electrochemical detection

The initial study was carried out to compare the detection of phenols with UV and electrochemical detectors. As water–methanol gradients allow UV detection the separation of the compounds in systems A and B described above was studied first. Fig. 1a shows the chromatogram of the 21 phenols studied (250 ng of each compound) with gradient A. Under these conditions 2,4-dichlorophenol and 2,4,6-trimethylphenol (retention time $t_R = 21.92$ min) did not separate, and it was possible partially to separate *p*-cresol and *o*-cresol ($t_R = 11.79$ and 12.02 min, respectively). This chromatogram was obtained using a wavelength of 280 nm, changing to 300 nm at 28 min, before pentachlorophenol was eluted. The wavelength change did not affect the baseline. Fig. 1b shows the chromatogram obtained for the eleven EPA priority phenols (250 ng of each phenol) with gradient B, measured at 280 nm and changed to 300 nm at 25 min (before the elution of pentachlorophenol). With this gradient all eleven phenols were well resolved in less than 30 min.

With an electrochemical detector it is not possible to work with gradients because the flow stability can be affected, causing baseline drift and random noise. Also, not all the solvents used in LC can be used, as some of them attack certain internal parts of the detector. Initially, we used the solvent mixture used by Gretzfeld-Hüsgen and Schuster [16] [water–methanol (40:60) at pH 3], modified to give eluents A, B and C described above. Eluant B (50% methanol) was used as screening for the nine more polar phenols. Under these conditions, 2,4,6-trichlorophenol and pentachlorophenol had a high t_R of 45 min. This meant that we had to use a solvent containing more methanol, *i.e.*, eluent C (75% methanol), with which we obtained the separation of the most non-polar phenols is less than 15 min. Eluent A (25% methanol) was used exclusively in the determination of phenol in real

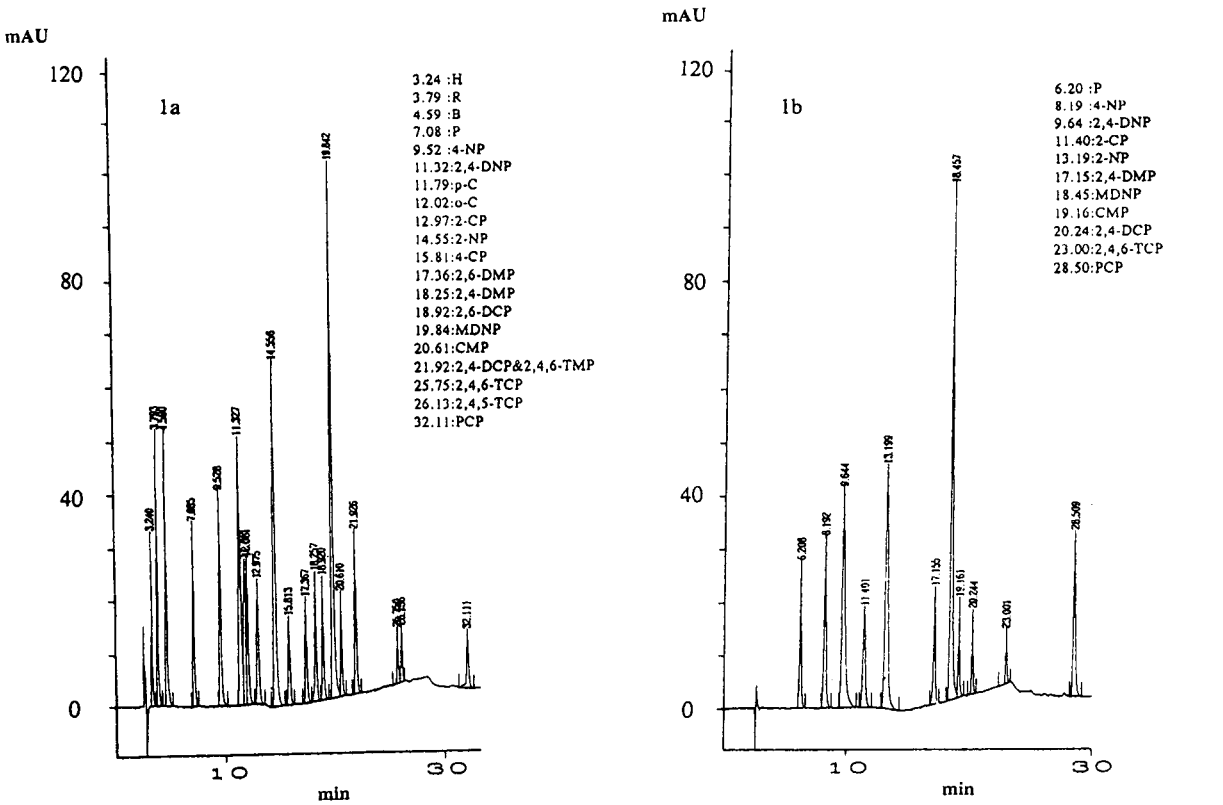

Fig. 1. Chromatograms obtained with UV detection: (a) separation of the 21 phenols and (b) separation of the EPA priority pollutant phenols (10 mg/l of each). H = hydroquinone; R = resorcinol; B = pyrocatechol; P = phenol; 4-NP = 4-nitrophenol; 2,4-DNP = 2,4-dinitrophenol; p-C = *p*-cresol; o-C = *o*-cresol; 2-CP = 2-chlorophenol; 2-NP = 2-nitrophenol; 4-CP = 4-chlorophenol; 2,6-DMP = 2,6-dimethylphenol; 2,4-DMP = 2,4-dimethylphenol; 2,6-DCP = 2,6-dichlorophenol; MDNP = 2-methyl-4,6-dinitrophenol; CMP = 4-chloro-3-methylphenol; 2,4-DCP = 2,4-dichlorophenol; 2,4,6-TMP = 2,4,6-trimethylphenol; 2,4,6-TCP = 2,4,6-trichlorophenol; 2,4,5-TCP = 2,4,5-trichlorophenol; PCP = pentachlorophenol.

samples because the matrix components of the sample caused a large peak at the beginning of the chromatogram and masked the results. With eluent B, the t_R of phenol was 4.77 whereas using eluent A phenol appeared at 10.50 min, far enough from the initial zone of the chromatogram to avoid the interferences mentioned. In Fig. 2a and b the sequential chromatograms of the eleven phenols (625 pg of each) are shown, with isocratic mobile phases B and C at a potential of 1000 mV. In these chromatograms all the peaks are measured in less than 35 min (analysis of both injections).

Table II gives the detection limits (DL) for the UV and electrochemical methods and the sensitivity ratios of the two methods, illustrating the sensitivity gain obtained by using the electrochemical detector. The DLs were obtained from calibration graphs constructed for each phenol and were calculated as the analyte concentration than caused a signal three times the standard error. Calibration graphs were also used to calculate the sensitivity expressed as area counts/concentration (μg/l) (in Table II only sensitivity ratios between the detectors are shown). The calibration graphs obtained had correlation coefficients >0.99 and were achieved with five points in the concentration range 2000–10 000 μg/l for the UV detector and 1–20 μg/l for the electrochemical detector. From Table II it can be observed that the sensitivity of electrochemical detection was more than 100 times better than that of UV detection for all the phenols except the nitrophenols, with gains of 566- and 728-fold

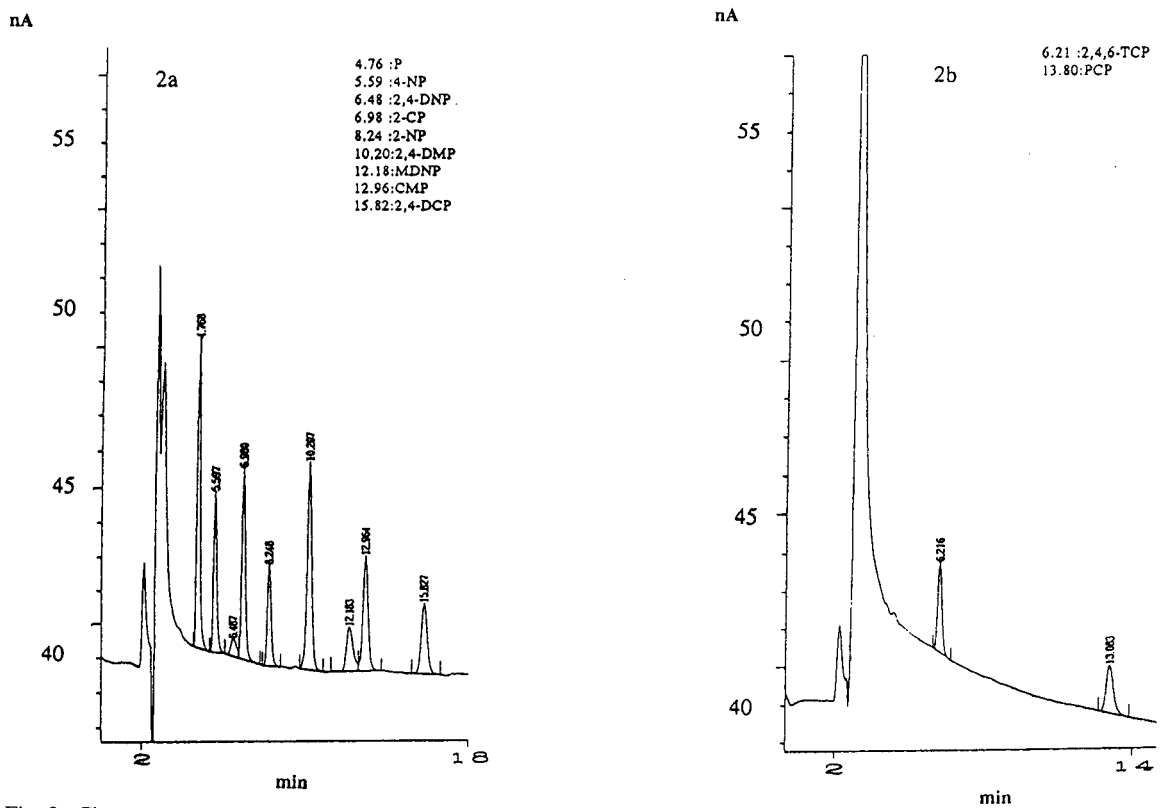

Fig. 2. Chromatograms of the EPA priority pollutant phenols (25 μg/l of each) obtained with ED: (a) eluent B and (b) eluent C. P = phenol; 4-NP = 4-nitrophenol; 2,4-DNP = 2,4-dinitrophenol; 2-CP = 2-chlorophenol; 2-NP = 2-nitrophenol; 2,4-DMP = 2,4-dimethylphenol; MDNP = 2-methyl-4,6-dinitrophenol; CMP = 4-chloro-3-methylphenol; 2,4-DPC = 2,4-dichlorophenol; 2,4,6-TCP = 2,4,6-trichlorophenol; PCP = pentachlorophenol.

TABLE II

DETECTION LIMITS AND RATIOS OF SENSITIVITY BETWEEN ELECTROCHEMICAL AND UV DETECTION

Phenol	Detection limit (μg/l)		Sensitivity ratios (ED/UV)
	UV detection	ED	
Phenol	22	0.9	566
4-Nitrophenol	341	1.5	47
2,4-Dinitrophenol	266	4.3	4
2-Chlorophenol	454	1.7	390
2-Nitrophenol	531	1.3	75
2,4-Dimethylphenol	240	0.9	403
2-Methyl-4,6-dinitrophenol	187	5.2	21
4-Chloro-3-methylphenol	292	1.6	633
2,4-Dichlorophenol	326	1.9	179
2,4,6-Trichlorophenol	263	4.2	728
Pentachlorophenol	809	6.1	419

for phenol and 2,4,6-trichlorophenol, respectively. For nitrophenols the sensitivity ratios are much lower, especially for 2,4-dinitrophenol (ratio only 4). This may be due to the fact that the working potential used (1 V) was not optimum for determining nitrophenols, as it is known that 1.2 V is to be preferred.

Concentration by solid–liquid extraction

The concentration technique studied was solid–liquid extraction of the sample with microcolumns containing 500 mg of C_{18} reversed-phase material. Aliquots of 25 ml of standard solutions of different initial concentrations were subjected to solid–liquid extraction. Standard solutions of 10 μg/l–1 mg/l were prepared for the UV detector, while for the electrochemical detector the starting concentration ranged from 0.5 to 1 μg/l; greater sensitivity and lower DLs were achieved in the latter separation mode, as mentioned above. In all instances the final volume was 1 ml of methanol solution in a concentration factor of 25.

Table III presents the recovery results for standard solutions of 50 μg/l of each phenol with UV detection and 0.5 μg/l of each phenol with electrochemical detection. Table III also gives the detection limits obtained for both methods. With the UV detector phenol concentrations between 4 and 85 μg/l could be detected whereas with the electrochemical detector concentrations between 0.04 and 0.59 μg/l could be detected, depending of the type of phenol. For the electrochemical detector the R.S.D. for the nine recovery experiments was of the order of 10%. With the UV detector an insufficient number of experiments (three) to allow the calculation of a reliable R.S.D. were carried out, owing

TABLE III

RECOVERY OF PHENOLS BY SOLID–LIQUID CONCENTRATION

Initial concentration of phenols: 50 μg/l for UV detector and 0.5 μg/l for electrochemical detector

Phenol	UV detection		ED		
	Recovery (%)	Detection limit (μg/l)	Recovery (%)	R.S.D. (%) ($n = 9$)	Detection limit (μg/l)
Hydroquinone	ND[a]	–	–	–	–
Resorcinol	ND	–	–	–	–
Pyrocatechol	18	85	–	–	–
Phenol	19	5	80	8	0.05
4-Nitrophenol	72	19	62	10	0.10
2,4-Dinitrophenol	95	11	65	11	0.27
p-Cresol	93	4	–	–	–
o-Cresol	79	14	–	–	–
2-Chlorophenol	90	20	102	5	0.07
2-Nitrophenol	103	21	102	7	0.05
4-Chlorophenol	91	13	–	–	–
2,6-Dimethylphenol	88	13	–	–	–
2,4-Dimethylphenol	98	10	93	7	0.04
2,6-Dichlorophenol	97	15	–	–	–
2-Methyl-4,6-dinitrophenol	91	8	64	8	0.33
4-Chloro-3-methylphenol	93	13	88	8	0.07
2,4-Dichlorophenol	102	13	116	6	0.07
2,4,6-Trimethylphenol	102	13	–	–	–
2,4,6-Trichlorophenol	78	14	81	5	0.21
2,4,5-Trichlorophenol	91	8	–	–	–
Pentachlorophenol	48	67	41	9	0.59

[a] ND = not detected.

to a lack of sensitivity in our application. Recovery data with UV detection were only used for comparison with electrochemical data.

Determination of phenols in samples of river and drinking water

Fig. 3 shows two examples of chromatograms for river and drinking waters. Fig. 3a for a concentrated sample of river water; Fig. 3a1 is for the concentrated sample alone and Fig. 3a2 is for the same sample spiked with 0.5 μg/l of phenol. Fig. 3b is for a concentrated sample of drinking water; Fig. 3b1 is for the sample alone and Fig. 3b2 is for the sample spiked with 0.5 μg/l of phenol. In both instances the peak at $t_R = 10.43$ min was identified as phenol; its concentration was calculated to be 250 and 70 ng/l in river and drinking water, respectively (using the standard addition method). These chromatograms were achieved using eluent A; with eluents B and C no electrochemically active substances attributable to the phenols studied were detected.

Na_2SO_3 was added to samples of drinking water to eliminate free chlorine, which could interfere in the determination using the standard addition method, because when the standard of phenols is added to samples of chlorinated water the chlorine can react with the phenols added and produce chlorophenols. The effect of sulphite addition was studied and no interference was observed in the analyses of samples extracted with microcolumns. Only direct injection of samples treated with sulphite showed a distorted area at the beginning of the chromatogram.

Confirmation of the peaks with electrochemical detection

The general working potential for the electrochemical detector was 1000 mV, although to confirm the phenolic compounds a feature of the detector called the "autoincrement mode" was used, in which a sample was injected repeatedly and automatically at different potentials, so obtaining chromatograms at several working voltages. The initial and final potentials, the voltage increments and the allowed baseline drift were fixed before the equipment made the injection. Here the conditions were initial potential

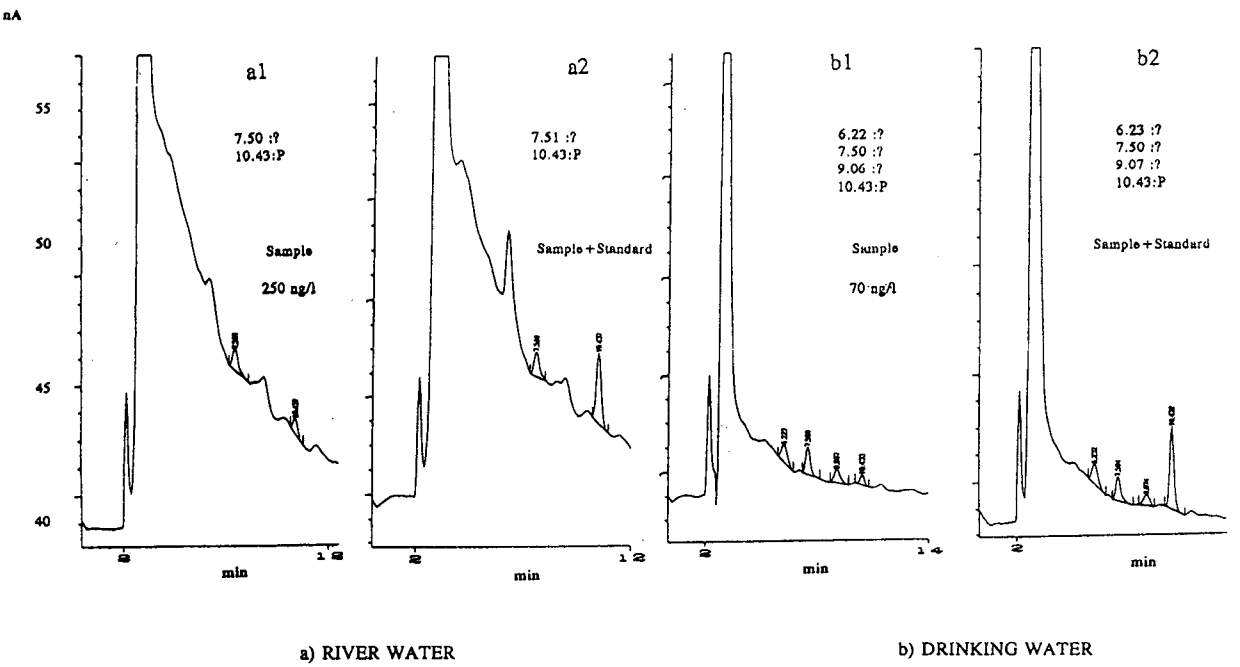

Fig. 3. Chromatograms of concentrated samples of (a) river and (b) drinking waters (eluent A). P = phenol.

TABLE IV

ELECTROCHEMICAL RATIOS OF THE AREAS AT DIFFERENT VOLTAGES

Phenol	Ratio of voltages					
	0.8/0.9	0.8/1.0	0.8/1.1	0.9/1.0	0.9/1.1	1.0/1.1
Phenol	0.45	0.40	0.40	0.90	0.90	1.00
4-Nitrophenol	0.00	0.00	0.00	0.00	0.00	0.17
2,4-Dinitrophenol	0.00	0.00	0.00	0.00	0.00	0.18
2-Chlorophenol	0.60	0.55	0.50	0.90	0.80	0.90
2-Nitrophenol	0.00	0.00	0.00	0.00	0.00	0.55
2,4-Dimethylphenol	0.85	0.85	0.85	1.00	1.00	1.00
2-Methyl-4,6-dinitrophenol	0.00	0.00	0.00	0.00	0.00	0.18
4-Chloro-3-methylphenol	0.75	0.65	0.50	0.85	0.70	0.80
2,4-Dichlorophenol	0.75	0.70	0.60	0.95	0.85	0.90
2,4,6-Trichlorophenol	0.80	0.80	0.80	1.00	1.00	1.00
Pentachlorophenol	0.30	0.35	0.40	1.00	1.20	1.20

800 mV, final potential 1100 mV, increments of 100 mV and allowed baseline drift 1 nA/min.

Table IV gives the electrochemical ratios found for standard solutions of 25 μg/l of each phenol. As can be seen, the nitrophenols did not respond to 800 or 900 mV in the range of concentrations studied. The most interesting ratios were 0.8/0.9, 0.8/1.0, 0.8/1.1, except for nitrophenols, for which the best ratio was 1.0/1.1. A similar confirmation technique had been used via ED with two working electrodes each at a different potential. In this way, for example, Shoup and Mayer [14] used potentials of 850 and 900 mV *versus* Ag/AgCl/3 *M* NaCl.

CONCLUSIONS

Using LC coupled to a UV detector, concentrations of phenols down to *ca.* 10 μg/l can be detected employing the concentration technique studied, whereas an electrochemical detector can reach levels below 500 ng/l, which is actually the MAC in drinking waters in EEC countries. Further, with ED phenols present in samples can be confirmed using the "autoincrement mode" and the retention times obtained with different eluents.

The electrochemical detector used in this study has a pretreatment function that allows the cleaning of the working electrode surface electrochemically, before or between analyses, so avoiding premature fouling of the electrode surface. This feature improves the stability of the response with this type of detector.

LC–ED has the advantage over the EPA standard method [9] that it is possible to obtain lower DLs in shorter analysis times because derivatization of the phenols is not required.

We consider that LC–ED could replace the standard official methods using 4-aminoantipyrine and 2-nitrophenol, which do not allow the determination of the specified levels (0.5 μg/l) and which are much less able to distinguish the types of phenols present in the samples.

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CHROM. 25 127

High-performance liquid chromatographic analysis of polyphenolic compounds predominating in sherry musts☆

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ABSTRACT

A chromatographic procedure has been developed for the analysis of polyphenolic compounds in sherry musts. The procedure allows for the use of internal standards to control the different steps of the analysis. A C_{18} reversed-phase column of 25 cm length and 4 mm diameter charged with 5 μm particle size filling is used in connection with a gradient in stages consisting of a 2% acetic acid solution in methanol. The choice of the gradient has been previously worked out as applied to a mixture of 22 polyphenolic compounds. In order to ensure the stability of the must samples they are mixed with 10% dimethylformamide and 2% ascorbic acid. The internal standard is then added to the samples, which are subsequently submitted to a continuous rotary extraction process for 3 h using diethyl ether.

INTRODUCTION

The importance of polyphenolic compounds in enology is well established [1]. Particularly in connection with sherry wines, such compounds are related to undesirable phenomena, such as the typical "browning", that occurs as a result of the ageing processes of these wines [2].

A high proportion of such compounds arise directly from the grapes so that the process of wine making, greatly influences the final polyphenolic content [3]. As a result it is of considerable interest to study these compounds in must samples taken from vintages as well as to follow their evolution during the wine-making process.

For reasons directly related to the complexity of the samples, in the analysis of these compounds it is mandatory to carry out a preliminary extraction of polyphenolic compounds, which are then determined by HPLC [4,5]. This stage of sample preparation is based mainly on liquid–liquid extraction techniques [6,7], solid-phase extraction [8–10], gel permeation chromatography (GPC) [11,12] or even chromatography on an open column [13]. In all cases considerable sample manipulation occurs, leading to the occurrence of a number of errors. Therefore, it is of particular interest to be able to control the quality of analyses, which can be achieved provided one or more internal standards can be added to the sample at the start of the analytical process. On the other hand, the great number of polyphenolic species that are dealt with finally by HPLC imposes the need to select a suitable separation process allowing for the inclusion of the internal standards that will appear in the final chromatogram.

The present work is concerned with the possibility of using internal standards with a view to controlling the overall analytical process of poly-

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phenols in must samples, carrying out the final quantification by HPLC. For this purpose a previously selected gradient is applied as worked out in connection with 22 polyphenolic species, allowing for the inclusion of internal standards in the must samples to be analysed.

EXPERIMENTAL

Reagents and standards

The standard compounds used were obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany) and Eastman Kodak (Rochester, NY, USA). Methanol of HPLC-gradient grade and all other chemicals used (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany). Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Preparation of the must samples

The samples of the investigated musts were obtained directly from the winery of "Bodegas Osborne & Cia" in different stages of processing: pressing, cleaning by sedimentation and fermentation.

Extraction of the samples was carried out under the cautionary conditions described elsewhere [14] in order to guarantee their stability throughout the different stages of the analysis: manual filtration with cotton cloth followed by the direct addition of 10% dimethylformamide (DMF) and 2% ascorbic acid and immediate freezing until the moment of analysis.

Extraction of polyphenolic compounds from the must

The extraction process was carried out by means of a continuous extraction rotary device, as designed by the authors [15], by adhering to the following procedure. A 1-ml volume of a 150 mg/l solution of 2,5-dihydroxybenzaldehyde (Fluka) was added to 100 ml of must followed by the addition of 1000 ml of water, thereafter saturating the obtained solution with NaCl. The solution was extracted with 80 ml of diethyl ether using a Mascré extractor for 3 h at a rate of 0.8 turns/min. The organic solvent was dried for 1 h with anhydrous sodium sulphate followed by evaporation in a rotavapor to attain a final volume of 5 ml.

TABLE I

GRADIENT ELUTION PROGRAMME

Time (min)	0	25	45
Solvent B (%)	0	15	50

Equipment

A Waters (Millipore, Milford, MA, USA) liquid chromatograph with Model 510 and M45 pumps, a Model M680 gradient programmer, a Model U6K injector and a Model M991 diode-array detector were used. Separation was carried out using a LiChrospher C_{18} steel cartridge, 250 × 4 mm I.D. and 5 μm particle size filling (Merck).

Chromatographic conditions

The chromatographic conditions adopted were as follows: flow-rate, 1 ml/min; detection, UV absorption at 210–390 nm; volume injected, 20 μl; and mobile phase, methanol–acetic acid–water (10:2:88, v/v) as solvent A and methanol–acetic acid–water (90:2:8, v/v) as solvent B with a gradient programme in two steps (Table I).

RESULTS AND DISCUSSION

As a rule, one of the most important stages of the analytical procedure used by the authors in the past for the chromatographic separation of polyphenolic species by HPLC was carried out using a 30-cm-long C_{18} column of irregular silica and 10 μm particle size [16]. Because of the great complexity of the sample, such a procedure precludes the use of internal standards that cannot be quantified reliably. Fig. 1a shows a chromatogram obtained from a must sample under the specified conditions, which clearly demonstrates the impossibility of including any internal standard.

In view of such difficulties a search was carried out for a reversed-phase column of more theoretical plates coupled with an elution gradient that would afford the inclusion of some internal standard. As a result five reversed-phase

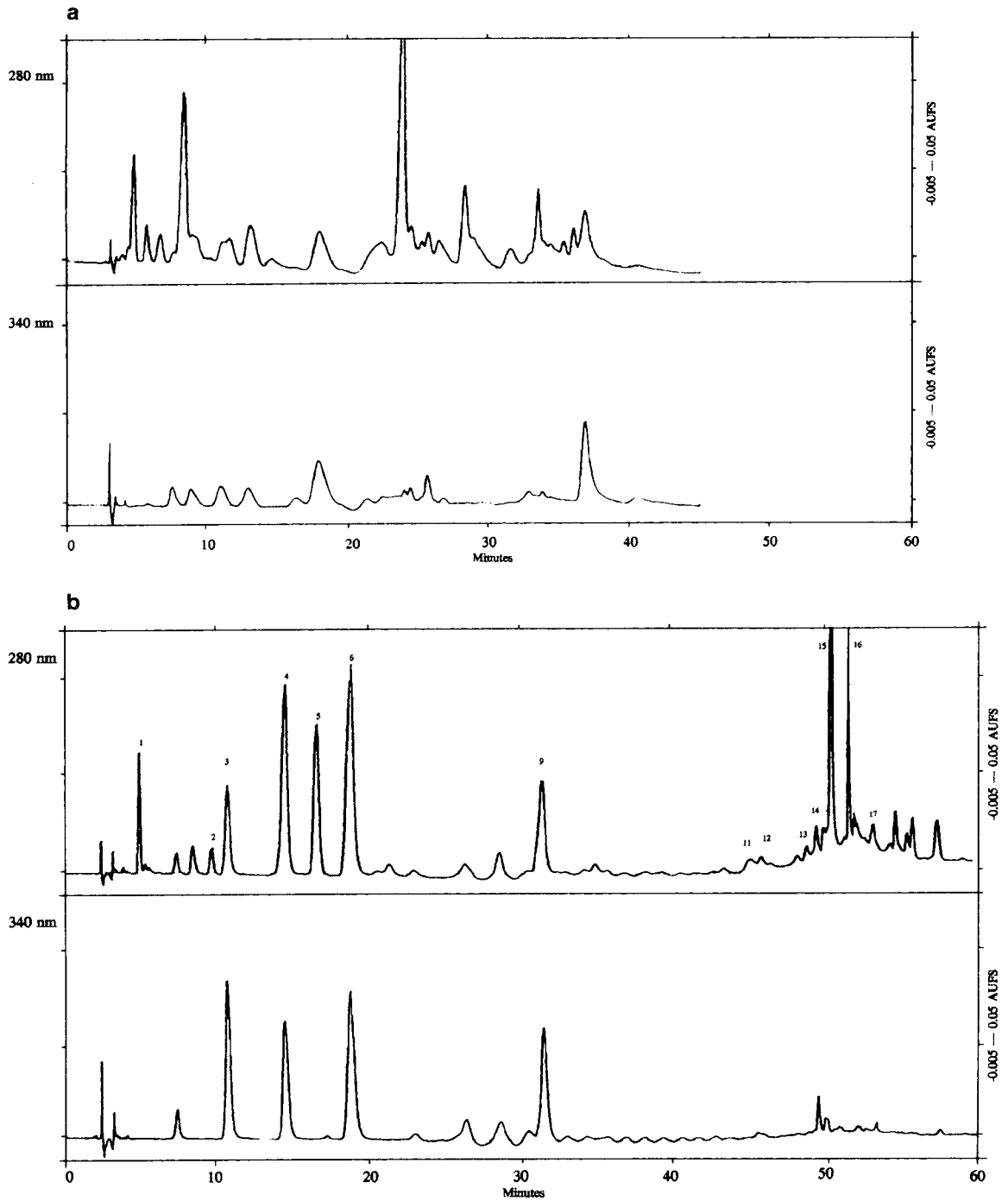

Fig. 1. Chromatograms obtained from the must sample (280 and 340 nm). The substances are identified in Fig. 2. (a) Chromatographic conditions: C_{18} column, 10 μm particle size, 30 cm length and the gradient described in ref. 16. (b) Chromatografic conditions: C_{18} steel cartridge, 5 μm particle size, 250 × 4 I.D. mm and the gradient described in the chromatographic procedure.

columns of different lengths and particle sizes were compared, determining the optimum elution gradient for each one [17]. Finally, a LiChrospher C_{18} steel cartridge, 250 × 4 mm I.D. with 5 μm particle size filling (Merck), was selected. Using this column in connection with the gradient as described before, the chromatogram reproduced in Fig. 1b was obtained for the same must sample.

The selected chromatographic conditions allow a better peak resolution. In addition, the void central zones, which can be easily appreciated in the chromatogram, hint at the possibility of including some polyphenolic substance (internal standard) that does not overlap with other compounds present in the must sample or at least able to be quantified reliably. Of course, such an internal polyphenolic standard must be absent from the analysed must samples.

Selection of internal standards

As a result of the preliminary investigations carried out by the authors, three compounds were chosen as potential internal standards: two phenolic acids (β-resorcilic acid; 2,6-dimethoxybenzoic acid) and one phenolic aldehyde (2,5-dihydroxybenzaldehyde). These compounds were added to a given sample of must and resulted in the chromatograms reproduced in Fig. 2, which allows the conclusion that two of the three tested compounds can be used as internal standards (2,6-dimethoxybenzoic acid, quantified at 280 nm, and 2,5-dihydroxybenzaldehyde, quantified at 340 nm).

Bearing in mind that the composition of the must samples in terms of polyphenolic species may vary widely, depending on the stage of the wine-making procedure, the chosen polyphenolic substances (internal standards) should be equally useful for any type of must sample arising during the wine-making procedures.

Application to different samples of must in the wine-making procedure

Taking into account the considerations described above, the selected standards were in-

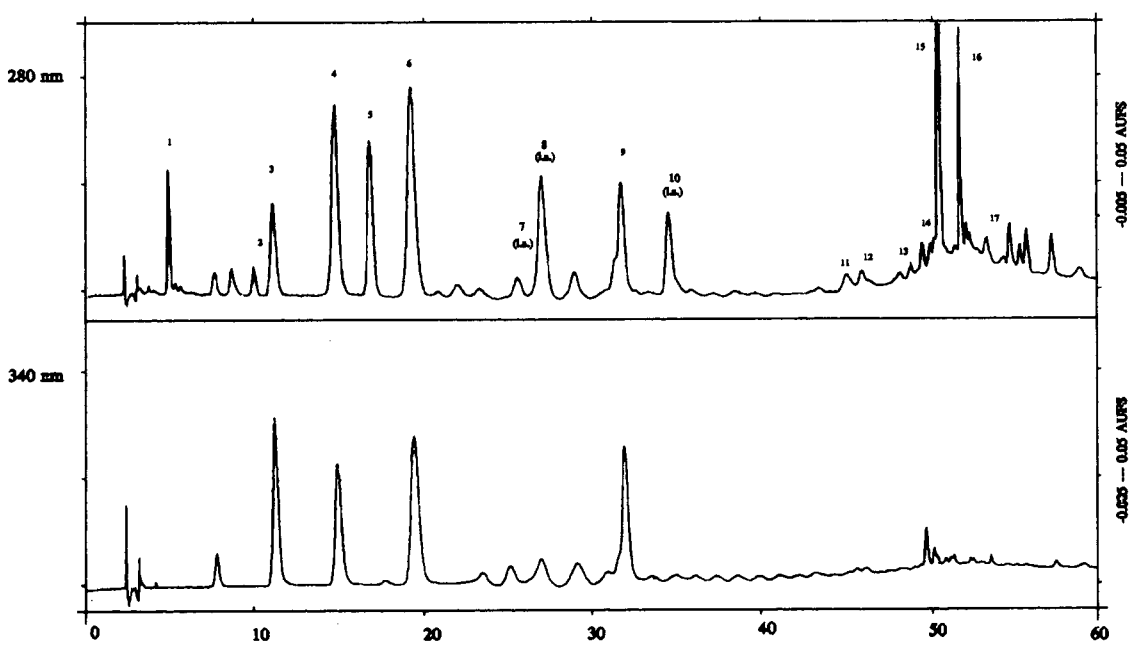

Fig. 2. Chromatograms (280 nm and 340 nm) obtained from must sample with addition of three internal standards. Peaks: 1 = gallic acid; 2 = protocatequialdehyde; 3 = *trans*-caffeoyltartaric acid; 4 = feruoyl tartrate; 5 = catechin + unknown; 6 = *p*-coumaroyl tartrate; 7 = 2,5-dihydroxybenzaldehyde (I.S.); 8 = β-resorcylic acid (I.S.); 9 = caffeic acid; 10 = 2,6-dimethoxybenzoic acid (I.S.); 11 = veratraldehyde; 12 = *p*-coumaric acid; 13 = 2,4-dimethoxybenzoic acid; 14 = ferulic acid; 15 = anisaldehyde; 16 = veratric acid; 17 = 3,4,5-trimethoxycinamic acid.

vestigated using a number of samples taken from a must deposit during different stages or processes of wine-making. The chromatograms obtained are reproduced in Fig. 3, and show different profiles depending on the wine-making process considered. The chromatograms of fermented musts (Fig. 3b) show a number of new peaks appearing in the region of the internal standard added (2,6-dimethoxybenzoic acid), which in some cases result in peak overlapping. Despite this, 2,5-dihydroxybenzaldehyde can be used as an internal standard to follow the wine-making process.

Once it was established that this compound can be used as an internal chromatographic standard, a study was carried out to determine its behaviour from the beginning, through sample preparation up to its final quantification by HPLC. With this aim a series of must samples were submitted to the extraction procedure and then added to a known concentration of the internal standard selected. The resulting chromatograms indicate that recovery of the standard was 98.9%, with a relative standard deviation of 1.98%. As a result the suitability of such a substance as a means of controlling the quality of the overall analytical procedures was confirmed.

CONCLUSIONS

Using a C_{18} steel cartridge (250 × 4 mm I.D.) and 5 μm particle size filling and the chromatographic conditions specified in the present paper, it is possible to use 2,5-dihydroxybenzaldehyde as an internal standard, quantified at 340 nm, for the analysis of phenolic compounds in the sherry

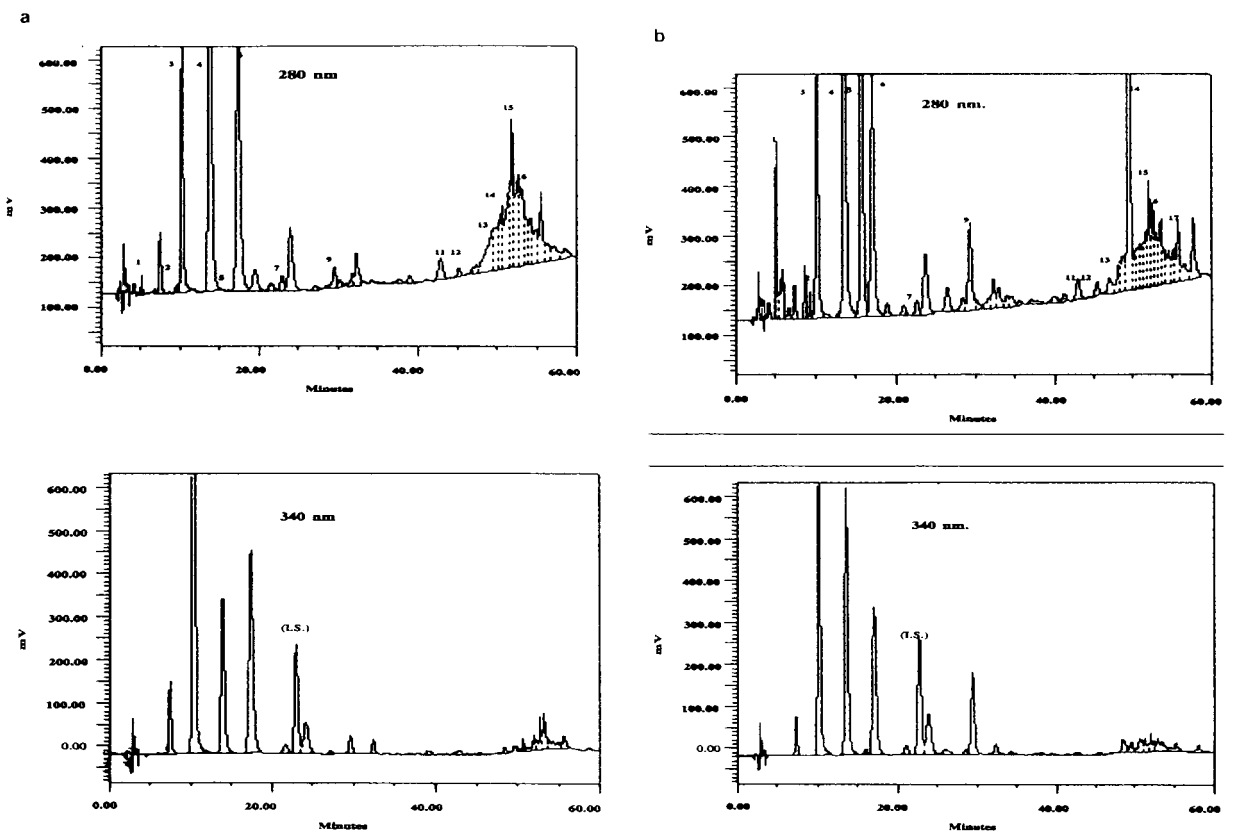

Fig. 3. Chromatograms (280 nm and 340 nm) obtained from different samples of must in the wine-making procedure, using 2,5-dihydroxybenzaldehyde as internal standard. (a) Must from the pressing stage. The substances are identified in Fig. 2. (b) Fermented must.

musts. In addition, this compound can be used to control the analytical steps preceding the HPLC analysis because the extent and relative standard deviation of its recovery are satisfactory. Such a possibility allows the efficiency of one of the stages of sample preparation to be checked, for instance the extraction step, which is a process highly related to sample manipulation.

In summary, we conclude that the proposed methodology can be used in connection with studies related to the control and evolution of the wine-making processes of sherry musts.

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Fast screening method for diuretics, probenecid and other compounds of doping interest☆

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ABSTRACT

A method to detect the presence of diuretics, probenecid and other agents of doping interest (morazone, mesocarb, caffeine) in urine was developed. The extraction procedure was optimized to obtain adequate recoveries of the compounds under study. The best results were achieved using alkaline extraction (pH 9.5) with ethyl acetate and the salting-out effect (sodium chloride). The extracts were analysed by HPLC with diode-array detection, using a high-speed reversed-phase column and a mobile phase containing acetonitrile and 0.1 *M* ammonium acetate (adjusted to pH 3), with gradient elution. Under these conditions, the time of analysis per sample was 12 min. Extraction recoveries, detection limits and results of the reproducibility assays are presented. The method allows the detection of the compounds in urine during at least 24 h after a therapeutic dose of the drug. Quantitative results obtained from excretion studies are also presented.

INTRODUCTION

Diuretics and probenecid are included in the list of compounds banned in sport by the Medical Commission of the International Olympic Committee since 1988. Hence, screening procedures to detect their presence in urine are required.

Although some diuretics are extensively metabolized (*e.g.*, spironolactone, excreted in urine as canrenone and other metabolic products [1,2]), most of them are excreted unchanged in urine to variable extents [3–11]. Therefore, procedures to screen for diuretics and probenecid in human urine can be designed to detect the suspected parent compounds.

The group of diuretics includes compounds with wide differences in molecular structures and physico-chemical properties. Basic (potassium-sparing diuretics, such as amiloride and triamterene), neutral (aldosterone antagonists, such as canrenone and spironolactone), weakly acidic (carbonic anhydrase inhibitors, such as acetazolamide and diclofenamide; thiazides and related agents, such as chlorthalidone) and strongly acidic compounds (loop diuretics, such as furosemide, bumetanide, piretanide and etacrynic acid) are to be considered, and these compounds cover a wide range of liposolubilities. Consequently, it is difficult to develop a common screening procedure for all these substances. Further, the presence of polar functional groups makes their determination by gas chromatography difficult without prior derivatization, which in most instances is a time-consuming step.

Most of the procedures described in the literature are high-performance liquid chromatographic (HPLC)-based methods. Tisdall *et al.* [12] and Fullinfaw *et al.* [13] reported HPLC methods to

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screen only for acidic diuretics. Other workers [14–16] described HPLC screening methods using two extraction procedures (in acidic and basic conditions) to obtain adequate recoveries for all the compounds. Nevertheless, Cooper *et al.* [15] and Tsai *et al.* [16] reported poor detection limits that would make the detection of some of the compounds difficult after a normal ingestion of the drug. Park *et al.* [17] described a unified HPLC method to screen for diuretics using solid-phase or liquid–liquid extraction procedures.

In this work, the extraction procedure and HPLC separation were optimized for fourteen diuretics and probenecid in order to afford a unified and rapid methodology. The method can also be used to screen for other diuretics and compounds belonging to other groups of banned substances (*e.g.*, stimulants such as mesocarb and morazone) and to determine caffeine.

EXPERIMENTAL

Chemicals and reagents

The compounds studied were supplied by the following pharmaceutical manufacturers: acetazolamide (Laboratorios Wassermann, Barcelona, Spain); amiloride, etacrynic acid and probenecid (Merck, Sharp & Dohme España, Alcalá de Henares, Madrid, Spain); furosemide and piretanide (Hoechst Ibérica, Barcelona, Spain); bendroflumethiazide (Laboratorios Davur, Madrid, Spain); triamterene (Laboratorios Jorba, Madrid, Spain); spironolactone and canrenone (Searle Ibérica, Madrid, Spain); bumetanide (Boehringer Ingelheim, Barcelona, Spain); chlorthalidone and hydrochlorothiazide (Laboratorios Ciba-Geigy, Barcelona, Spain); diclofenamide (Laboratorios Frumtost-Zyma, Barcelona, Spain); and benzthiazide (A.H. Robins, Richmond, VA USA). 7-Propyltheophylline, used as internal standard (I.S.), was synthesized from theophylline (Sigma Química, Alcobendas, Madrid, Spain) and propyl iodide (Merck, Darmstadt, Germany) in alkaline medium.

Water used in the HPLC mobile phase was of Milli-Q grade (Millipore Ibérica, Barcelona, Spain). Methanol, acetonitrile and ethyl acetate were of HPLC grade. Diethyl ether was of analytical-reagent grade and distilled before use. Other reagents were of analytical-reagent grade. Ammonium chloride buffer was prepared by dissolution of 28 g of ammonium chloride in 100 ml of deionized water and adjusting the pH to 9.5 with concentrated ammonium solution.

Standard solutions

Stock solutions were prepared by dissolving the compounds in methanol (1 mg/ml). Triamterene stock solution was prepared with methanol and concentrated hydrochloric acid (1:1, v/v). Working solutions were prepared by 1:10 dilution of the stock solutions with methanol (100 μg/ml). All solutions were stored in the dark at 4°C.

Extraction procedures

Four basic and two acidic procedures were tested (Table I).

Basic extractions

Procedure B1. A 5-ml urine sample was pipetted into a centrifuge tube and 50 μl of the I.S. working solution were added. The urine was made alkaline with 200 μl of ammonium chloride buffer (pH 9.5) and extracted with 6 ml of diethyl ether. After mixing (rocking at 40 movements/min for 20 min) and centrifugation (5 min at 800 *g*), the organic layer was separated and taken to dryness under a stream of nitrogen at 40°C.

Procedure B2. This was identical with B1 but ethyl acetate was used as organic solvent for extraction.

Procedure B3. This was identical with B2, but sodium chloride (2 g) was added before the organic solvent to promote the salting-out effect.

Procedure B4. This was identical with B3, but the alkalinized and salted urines were extracted twice with 6 ml of ethyl acetate. After mixing and centrifugation the combined organic layers were taken to dryness under a stream of nitrogen at 40°C.

TABLE I

EXTRACTION PROCEDURES TESTED

Parameter	Procedure					
	B1	B2	B3	B4	A1	A2
Urine volume (ml)	5	5	5	5	5	5
Extraction pH	pH 9.5	pH 9.5	pH 9.5	pH 9.5	pH 2.5	pH 2.5
Salt	No	No	NaCl (2 g)	NaCl (2 g)	No	No
Extraction solvent (ml)	Diethyl ether (6)	Ethyl acetate (6)	Ethyl acetate (6)	Ethyl acetate (2 × 6)	Diethyl ether (6)	Ethyl acetate (6)

Acidic extractions

Procedure A1. A 5-ml urine sample was pipetted into a centrifuge tube, acidified (pH 2.5) with 100 μl of a 40% phosphoric acid solution and extracted with 6 ml of distilled diethyl ether. After mixing (rocking at 40 movements/min for 20 min) and centrifugation (5 min at 800 *g*), the organic layer was separated and taken to dryness under a stream of nitrogen at 40°C.

Procedure A2. This was identical with A1, but ethyl acetate was used as organic solvent.

In all instances the residues were reconstituted with 200 μl of a mixture of deionized water–acetonitrile (85:15, v/v) and aliquots of 20 μl were analysed by HPLC.

High-performance liquid chromatography

Chromatographic analysis was carried out using a Series II 1090L liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode-array detector. The instrument was linked to an HP 9000/300 workstation (Hewlett-Packard).

The column was Ultrasphere ODS with 3-μm particle size (7.5 × 0.46 cm I.D.) (Beckman, San Ramon, CA, USA).

The mobile phase was a mixture of 0.1 *M* ammonium acetate solution (adjusted to pH 3 with concentrated phosphoric acid) and acetonitrile with gradient elution. The acetonitrile content (initially 10%) was increased to 15% in 2 min, to 45% in 3 min, to 60% in 3 min, maintained there for 1 min, decreased to the initial conditions in 1 min and stabilized for 2 min before the next injection. The flow-rate was 1 ml/min. The aqueous component of the mobile phase was filtered through a 0.45-μm filter (Reactivos Scharlau, Barcelona, Spain) before use.

The detector was set to monitor the signals at 240, 270, 290, 300, 318 and 350 nm. In addition, the full ultraviolet spectrum between 200 and 400 nm of the detected peaks was stored in the data system and plotted at the end of each run.

Recovery studies

Water samples spiked with 2 μg/ml of the compounds (for acetazolamide, 5 μg/ml) were subjected to the extraction procedures. The extraction recoveries were calculated by comparison of the peak area of each compound obtained from these extracted samples with that of a solution in deionized water–acetonitrile (85:15, v/v) at a concentration equivalent to 100% recovery.

Extraction recoveries were studied statistically using a one-way ANOVA test. Differences between procedures were considered significant when $p < 0.01$.

Excretion studies

Excretion studies involving drug administration and urine collections were performed with healthy male volunteers under the authorization of the Hospital del Mar Ethical Committee (Barcelona, Spain) and the Spanish Ministry of Health (assay No. 88/135). Compounds were administered as single therapeutic doses and urines were collected for a period up to 24 h.

Volunteers were under medical supervision throughout the study.

RESULTS

The extraction recoveries obtained for some of the compounds under study using the different procedures are compared in Fig. 1.

Under acidic conditions (procedures A), amiloride (basic compound) is not extracted. Hydrochlorothiazide and acetazolamide (weakly acidic compounds) are best recovered using ethyl acetate (procedure A2). In contrast, diethyl ether (procedure A1) gives higher yields for chlortalidone, furosemide and bumetanide. The extraction of the other compounds is not significantly affected by the solvent used at acidic pH.

Under basic conditions (procedures B), ethyl acetate (procedure B2) gives significantly higher recoveries than diethyl ether (procedure B1). The salting-out effect (procedure B3) also helps in enhancing the extraction yields for most of the compounds. The use of two consecutive extractions (procedure B4) leads to an increase in the recoveries, which is especially important for strongly acidic compounds. It is worth noting that weakly acidic compounds are better extracted under basic conditions (procedure B), except acetazolamide. The extraction recoveries for the compounds under study obtained using procedure B4, their detection limits and the intra- and inter-day reproducibilities are listed in Table II.

A chromatogram of a methanolic solution of some diuretics and probenecid is presented in Fig. 2. The separation of the compounds is achieved in less than 8.5 min. Additionally to the compounds used to optimize the methodology, retention times of other diuretics and other compounds of doping interest are listed in Table III. Although some of the compounds elute at similar retention times, all of them are easily distinguishable by their UV spectra [18]. Relative standard deviations (R.S.D.s) of the relative retention time (RRT) calculated using fresh mobile phase preparations during 8 days range from 0.24 to 1.94%.

With regard to excretion studies, all the com-

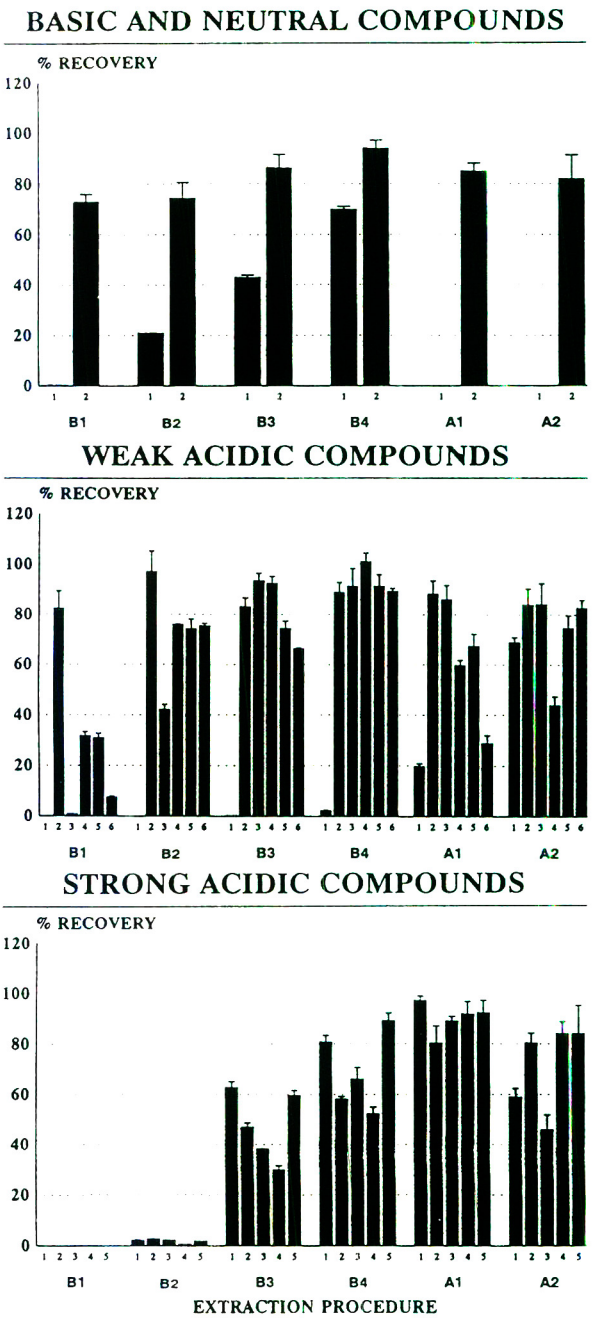

Fig. 1. Comparison of extraction recoveries obtained for some diuretics and probenecid. Extraction procedures are described under Experimental. Top: 1 = amiloride (basic); 2 = canrenone (neutral). Middle: 1 = acetazolamide; 2 = bendroflumethiazide; 3 = benzthiazide; 4 = chlorthalidone; 5 = diclofenamide; 6 = hydrochlorothiazide. Bottom: 1 = bumetanide; 2 = etacrynic acid; 3 = furosemide; 4 = piretanide; 5 = probenecid.

TABLE II

RESULTS OBTAINED USING PROCEDURE B4

Extraction recoveries, detection limits at different wavelengths and R.S.D.s obtained in intra- and inter-day reproducibility assays at the indicated concentrations.

Compound	Recovery		Detection limit		Reproducibility ($n = 8$)		
	Mean ± S.D. (%)	n	nm	μg/ml	Concentration (μg/ml)	R.S.D. (%)	
						Intraday	Interday
Basic compounds							
Amiloride	70.1 ± 1.07	4	350	0.02	1	3.7	6.7
Triamterene	67.9 ± 5.98	3	350	0.01	1	8.2	17.1
Caffeine	88.9 ± 5.27	3	270	NS[a]	3	1.6	5.9
7-Propyltheophylline (I.S.)	86.8 ± 7.05	4	270	NS	1	2.6	7.9
Neutral compounds							
Canrenone	94.0 ± 8.07	4	270	0.1	NS	–	–
Spironolactone	92.9 ± 9.66	4	240	0.1	NS	–	–
Weakly acidic compounds							
Acetazolamide	2.3 ± 0.30	4	270	2	10	4.5	13.1
Bendroflumethiazide	89.1 ± 3.81	3	270	0.05	NS	–	–
Benzthiazide	91.5 ± 7.03	4	270	0.1	0.5	8.0	8.7
Chlorthalidone	101.4 ± 3.35	4	270	0.2	1	3.2	5.8
Diclofenamide	91.6 ± 4.55	4	290	0.2	1	2.9	5.5
Hydrochlorothiazide	89.6 ± 1.11	4	270, 318	0.1	1	2.7	7.4
Strongly acidic compounds							
Bumetanide	80.8 ± 2.62	4	270, 350	0.02	0.5	2.2	6.0
Etacrynic acid	58.3 ± 0.96	3	270	0.2	NS	–	–
Furosemide	66.2 ± 4.52	4	270, 350	0.05	0.5	2.7	6.3
Piretanide	52.5 ± 2.57	4	270, 350	0.1	0.5	2.6	5.7
Probenecid	89.3 ± 3.05	4	270	0.1	0.5	3.2	5.7

[a] NS = Not studied.

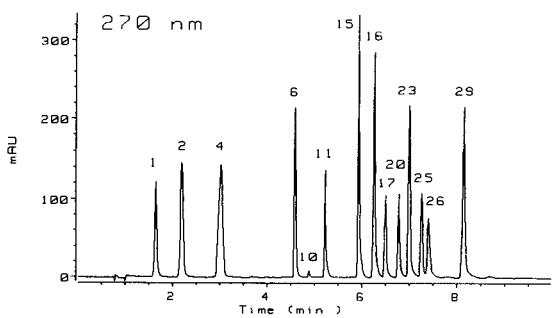

Fig. 2. Chromatogram obtained at 270 nm after analysis of a methanolic solution of some diuretics and probenecid. Peaks 1 = amiloride; 2 = acetazolamide; 4 = hydrochlorothiazide; 6 = 7-propyltheophylline (I.S.); 10 = diclofenamide; 11 = torasemide; 15 = furosemide; 16 = buthiazide; 17 = benzthiazide; 20 = piretanide; 23 = bendroflumethiazide; 25 = bumetanide; 26 = probenecid; 29 = canrenone.

pounds used to optimize the methodology were detected in urines collected from 0 to 24 h after intake of a single therapeutic dose. Quantitative results for urines from some excretion studies are listed in Table IV. Calibration equations for the determination of the compounds had correlation coefficients higher than 0.999.

Caffeine was well resolved from interfering compounds and showed a linear response in the range 2–20 μg/ml, following the equation $y = 1.054x - 0.012$ ($r = 0.9992$), where y is the peak-area ratio between caffeine and the I.S. and x is the caffeine concentration in μg/ml.

Examples of chromatograms corresponding to blank, spiked and true positive urine samples obtained either from excretion studies or from

TABLE III

ABSOLUTE AND RELATIVE (RRT) RETENTION TIMES OF THE COMPOUNDS STUDIED AND WAVELENGTHS USED TO MONITOR THEM

Compound	Retention time (min)	RRT	Wavelength (nm)
Amiloride	1.64	0.362	270, 350
Acetazolamide	2.19	0.483	270
Caffeine	2.76	0.610	270
Hydrochlorothiazide	3.01	0.664	270, 318
Triamterene	3.67	0.810	270, 350
7-Propyltheophylline (I.S.)	4.53	1	270
Morazone metabolite 1	4.54	1.002	270
Chlorthalidone	4.72	1.041	270
Morazone	4.76	1.050	270
Diclofenamide	4.85	1.070	270, 290
Torasemide	5.17	1.141	270, 290
Clopamide	5.20	1.147	270, 240
Morazone metabolite 2	5.45	1.203	270
Mesocarb metabolite	5.49	1.211	270, 350
Furosemide	5.88	1.298	270, 350
Buthiazide	6.23	1.375	270, 318
Benzthiazide	6.47	1.428	270
Cyclothiazide 1	6.60	1.457	270, 318
Cyclothiazide 2	6.70	1.479	270, 318
Piretanide	6.71	1.481	270, 350
Polythiazide	6.91	1.525	270, 318
Etacrynic acid	6.92	1.527	270
Bendroflumethiazide	6.97	1.538	270
Xipamide	7.11	1.569	270, 300
Bumetanide	7.18	1.584	270, 350
Probenecid	7.31	1.613	270, 240
Mesocarb	7.77	1.715	270, 350
Spironolactone	7.79	1.719	240
Canrenone	8.08	1.783	270

sport competitors (true doping positive cases) are shown in Figs. 3–9.

DISCUSSION

Owing to the different physico-chemical properties (acidbase behaviour, liposolubility) of the target compounds, it is difficult to develop single extraction procedures and chromatographic conditions to obtain adequate selectivity and sensitivity for all the compounds under study.

The extraction at acidic pH allows the recovery of acidic and neutral compounds but not of the basic diuretics. However, optimized basic extraction allows the recovery of all kind of compounds studied. For this reason, extraction in alkaline conditions with salting-out seems to be suitable for screening purposes to detect diuretics in urine. The basic extracts were much cleaner than the acidic extracts so an increase in signal-to-noise ratio is consequently obtained.

Ethyl acetate, a more polar solvent than diethyl ether, gives the best extraction recoveries for most of the compounds at alkaline pH. Weakly acidic compounds, except acetazolamide, are well extracted in the basic ethyl acetate extract owing to their high pK_a values [15–18]. The recovery of the strongly acidic compounds and acetazolamide in the basic extract is mainly achieved due to the salting-out

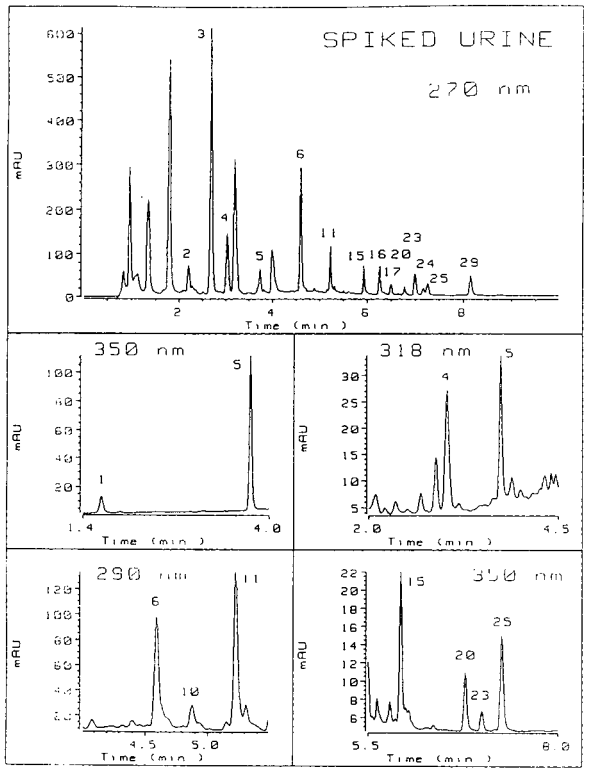

Fig. 3. Chromatograms at different wavelengths from a extract of a spiked urine containing various compounds: 1 = amiloride (0.1 μg/ml); 2 = acetazolamide (5 μg/ml); 3 = caffeine; 4 = hydrochlorothiazide (0.5 μg/ml); 5 = triamterene (0.2 μg/ml); 6 = I.S. (1 μg/ml); 10 = diclofenamide (0.5 μg/ml); 11 = torasemide (0.5 μg/ml); 15 = furosemide (0.2 μg/ml); 16 = buthiazide (0.2 μg/ml); 17 = benzthiazide (0.2 μg/ml); 20 = piretanide (0.2 μg/ml); 23 = bendroflumethiazide (0.2 μg/ml); 24 = xipamide (0.2 μg/ml); 25 = bumetanide (0.2 μg/ml); 29 = canrenone (0.2 μg/ml).

effect (comparison of procedures B3 and B2). Procedure B4 is the method of choice for screening purposes because it appears to be the best compromise. In general terms, procedure B4 allows higher yields than other methods previously described [15–17]. Only acetazolamide has a low recovery but it can be easily detected owing to the high concentrations appearing in urine after a single therapeutic dose (Table IV).

The use of gradient elution and an ammonium buffer is needed to obtain adequate retention times and good chromatographic behaviour for

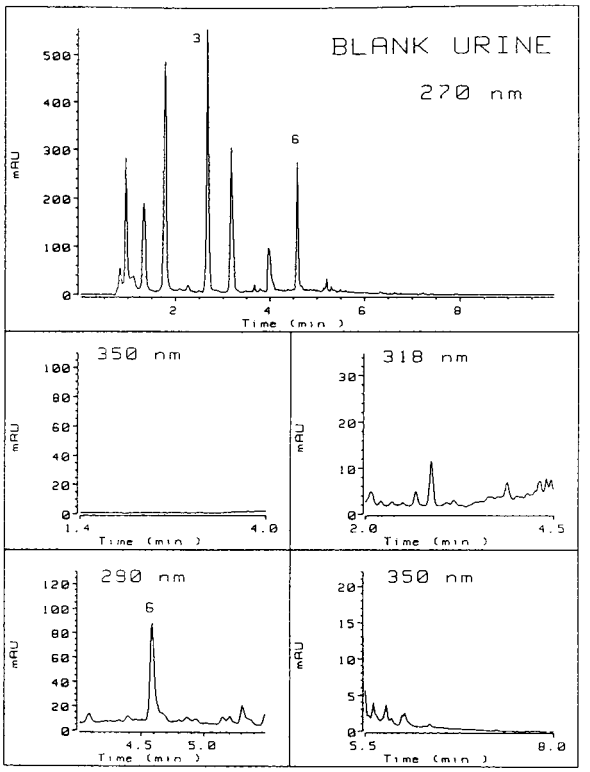

Fig. 4. Chromatogram at 270 nm and other selected wavelengths of a blank urine sample extracted using procedure B4. Peaks: 3 = caffeine; 6 = 7-propyltheophylline (I.S.).

all of the compounds, as reported previously [18]. The additional decrease in the retention times achieved with the 3-μm particle size column (Table III) results in a substantial decrease in the analysis time per sample as com-

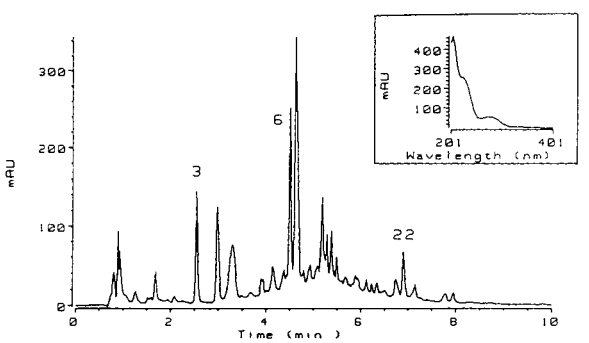

Fig. 5. Chromatogram at 270 nm of a urine sample from an excretion study of etacrynic acid and UV spectrum of the peak of etacrynic acid. Peaks: 3 = caffeine; 6 = I.S.; 22 = etacrynic acid.

TABLE IV

RESULTS OF EXCRETION STUDIES

Concentrations of the unchanged compounds detected in urines after administration of single therapeutic doses of the drugs to healthy volunteers.

Compound	Administered dose (mg)	Mean concentration in urine (μg/ml)		
		0–8 h	8–24 h	0–24 h
Amiloride	5	1.45	0.54	NS[b]
Triamterene	100	0.97	0.15	NS
Acetazolamide	250	185.56	20.83	NS
Diclofenamide	50	9.02	4.22	NS
Chlorthalidone	25	4.08	3.36	NS
	25	1.18	1.09	NS
Hydrochlorothiazide	25	10.77	2.81	NS
	50	23.02	7.87	NS
Bumetanide	1	0.13	0.15	NS
	1	0.21	0.06	NS
Etacrynic acid	50	1.57	0.61	NS
	50	3.56	0.19	NS
	50	2.91	0.33	NS
Furosemide	40[a]	5.49	0.82	NS
Piretanide	6	1.10	0.10	NS
Probenecid	250	NS	NS	26.83
	250	NS	NS	23.93

[a] Administered as furosemide xanthynol.
[b] NS = not studied.

pared with other HPLC methods described previously [15–17]. The good inter-day reproducibility of RRT and the acquisition of the full UV spectra allow the easy identification of the peaks detected.

Good reproducibility is obtained, as the intra- and inter-day R.S.D.s are in general lower than 10%. Acetazolamide was poorly recovered under the proposed conditions, thus increasing the analytical variations. For accurate quantitative or confirmatory studies, a neutral extraction using diethyl ether (recovery $21.5 \pm 0.99\%$, $n = 3$) is preferred.

The detection limits obtained using the proposed method are better than those described previously [12–17], except for acetazolamide. Although 270 nm was chosen as a compromise wavelength for most of the compounds [18], the use of specific wavelengths improves the detection, especially for those appearing at short retention times (Table III).

Additionally to the compounds used in the optimization of the screening procedure, the method has proved to be of great value for the detection of other diuretics and very polar or thermally unstable compounds, such as mor-

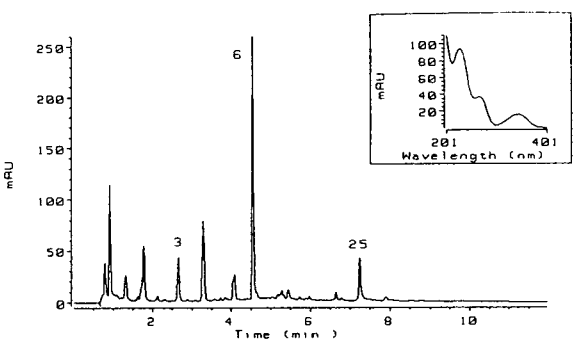

Fig. 6. Chromatogram at 270 nm of a urine sample from an excretion study of bumetanide and UV spectrum of the peak of bumetanide. Peaks: 3 = caffeine; 6 = I.S.; 25 = bumetanide.

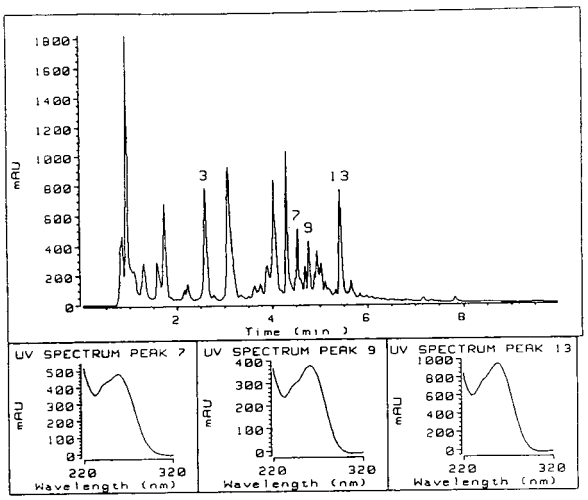

Fig. 7. Chromatogram at 270 nm of a urine sample from an excretion study of morazone and UV spectra of the peaks detected. Peaks: 3 = caffeine; 7 = morazone metabolite 1; 9 = unchanged morazone; 13 = morazone metabolite 2.

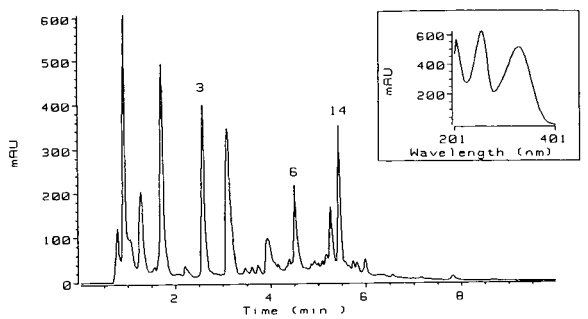

Fig. 8. Chromatogram at 270 nm of a urine sample from an excretion study of mesocarb and UV spectrum of the peak of the conjugated mesocarb metabolite. Peaks: 3 = caffeine; 6 = I.S.; 14 = conjugated mesocarb metabolite.

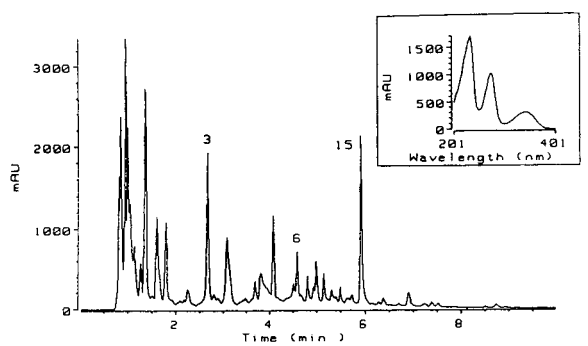

Fig. 9. Chromatogram at 270 nm of a true positive furosemide urine sample and UV spectrum of the peak of furosemide. Peaks: 3 = caffeine; 6 = I.S.; 15 = furosemide.

azone (Fig. 7) and conjugated metabolites of mesocarb (Fig. 8) [19,20].

The method developed is able to detect the ingestion of the compounds under study at least during 24 h after intake. It has been applied routinely to screen for the misuse of these compounds in the Barcelona antidoping laboratory and subjected to open and blind controls with total reliability of the results. An adaptation of this method was used at the Barcelona Olympic Games.

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Design and use of a thermal conductivity detector at reduced pressure for temperature-programmed capillary gas chromatography

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ABSTRACT

Based on a systematic study of the response characteristics of a thermal conductivity detector (TCD) at reduced pressure, a newly designed TCD was constructed and evaluated for use in temperature-programmed capillary gas chromatography. The effective cell volume is as low as 1.3 μl (physical volume 100 μl), and there is no dead corner along the sensing path. Capillary columns of I.D. $\geq$0.25 mm can couple directly with the TCD without a make-up gas. The detector response time is less than 0.1 s, the linear dynamic range is about four orders of magnitude and the detection limit is about 10 ppm (v/v) for octadecane. In the split injection mode, a simple configuration of the capillary inlet system offers a constant carrier gas flow-rate through the column with temperature-programmed operation. Practical examples are given that demonstrate the applicability of this detector and the system.

INTRODUCTION

The advantages of capillary columns over packed columns in the analysis of volatile compounds has led to renewed interest in the thermal conductivity detector (TCD). Although the TCD is well defined [1,2] and is among the most commonly used detectors in packed column gas chromatography, there is still much work to be done in order to apply it in capillary GC. The basic requirements are: (i) reducing the effective cell volume (V_e) of TCD to a few microlitres while maintaining its sensitivity and linear dynamic range; (ii) keeping the baseline of the detector output stable during a temperature-programmed analysis; (iii) maintaining the carrier gas flow constant during temperature programming (TP). This is essential for concentration-sensitive detectors in quantitative analysis.

It is known that the effective cell volume can be reduced substantially when a TCD is operated at low pressure, *i.e.*, $V_e = V_0 P/P_0$, where V_0 is the physical volume of the cell and P and P_0 are the cell pressure and atmospheric pressure, respectively [3,4].

In a previous study on the response characteristics of a TCD at reduced pressure, we found that an operating pressure limit (P_{min}) exists for a TCD [5]. When the operating pressure P is lower than P_{min}, both the absolute response and the relative response of the detector are pressure dependent, and the noise level increases exponentially as the pressure decreases. At $P > P_{min}$, the detector response is stable and insensitive to pressure, and the relative response factors measured at normal pressure can be used directly at lower pressures. An analytical equation was deduced in that study showing the relationship between P_{min} and the cell radius R, cell body temperature T and the molecule cross-section ϕ of carrier gas used, *i.e.*, $P_{min}(\text{Pa}) = 3.41 \cdot 10^{-6} T/$

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($R\phi^2$). These findings prompted us to design and construct a TCD [6] that has superior performance to the conventional TCD.

The intention of this study was to design and evaluate a new TCD, operated at reduced pressure, for use with capillary columns. The detector should meet the above requirements (i) and (ii), and should be as sturdy as a conventional TCD. Further, requirements (iii) has to be fulfilled in order to gain wider applicability.

EXPERIMENTAL

A ShangFen 1102 gas chromatograph (Shanghai Analytical Instrumentation, Shanghai, China) was used. The original TCD was replaced with the new designed detector as shown in Fig. 1. There are two heating blocks in the detector body, one surrounding the TCD cell and the other placed between the TCD cell and the oven. The second one is used for preheating the gases that flow through the detector. This measure ensured that the baseline of the TCD output was stable during the temperature programming of the oven. The capillary inlet system was modified (Fig. 2), a short piece of empty fused-silica tubing being used as the split restrictor, and was placed in the same oven as the analytical column. The splitting ratio was adjusted by changing the length of the restrictor. This configuration, together with the constant-flow controllers originally mounted on the chromatograph, provides a constant gas flow through both the analytical side and the reference side in temperature-programmed analysis. It also keeps the splitting ratio constant during temperature

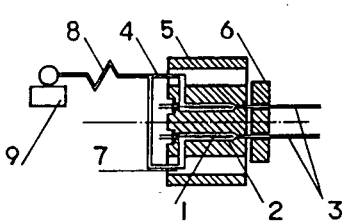

Fig. 1. Schematic diagram of detector system operating at reduced pressure [7]. 1 = Filament; 2 = TCD cell; 3 = inlet; 4 = outlet; 5 = heating block 1; 6 = heating block 2; 7 = heat-insulating material; 8 = restrictor; 9 = vacuum pump.

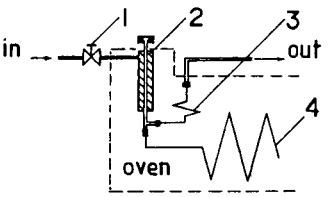

Fig. 2. Schematic diagram of split injection system allowing constant flow through both the analytical column and the reference column with temperature programming. 1 = Constant flow controller; 2 = split injector; 3 = short piece of empty capillary tubing; 4 = analytical column. The reference side is the same as above.

programming. The splitting ratio was about 1:50 in all experiments.

The fused-silica capillary columns used were (A) 50 m × 0.25 mm I.D. OV-101; (B) 25 m × 0.32 mm I.D. cross-linked PEG-20M and (C) 25 m × 0.25 mm I.D. cross-linked OV-1.

High-purity hydrogen (minimum concentration 99.999%) was used as the carrier gas.

RESULTS AND DISCUSSION

Design considerations for the TCD cell

The physical volume of the detector cell was 100 μl. It was designed to operate at pressures in the range 140–540 Pa for use with capillary columns. It can also be used at normal pressure with packed columns. To reduce the flow disturbances both at the gas inlet and outlet region within the cell, we took the following measures: the inlet gas flow was along the axis of the cell, and hence the filament; the hole of the outlet had the same diameter as the cell; and the position of the hole was at the end part of the filament support. Hence there was no dead corner and no abrupt change of gas flow along the sensing path. The noise level of the detector was only one quarter of that of the conventional type [5]. The minimum detectable concentration with this detector was 10 ppm for n-C_{16} and n-C_{18} at a TCD body temperature of 200°C and a bridge current of 120 mA, as was shown in Fig. 3.

The pressure P of the TCD cell has to be kept at $P \geq 1.2P_{min}$. To meet this criterion, a restrictor was placed between the outlet of the cell and the vacuum line. In principle, P should be as low

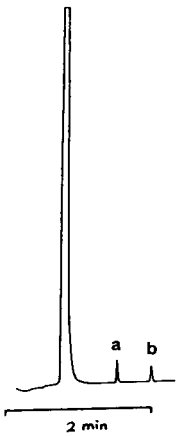

Fig. 3. Test for sensitivity of the detector. Sample: (a) n-C_{16} and (b) n-C_{18} in octane, 30 ppm (v/v) for each. TCD, 200°C, 120 mA; column, 30 m × 0.32 mm I.D. cross-linked SE-54 at 140°C.

as possible in order to reduce V_e, and the restrictor should be adjustable, such as with a vacuum needle valve, to control the cell pressure when using a column with a different diameter or on changing the flow-rate. A pressure gauge is also needed to indicate the cell pressure [5]. This measure will increase the complexity for both instrumentation and operation. We simplified the pressure control by using only one fixed restrictor, which just met $P = 1.2P_{min}$ at a column I.D. of 0.25 mm. When larger diameter columns are used, P and hence V_e will increase. However, the maximum permissible detector volume $V_{d,max}$ is proportional to $(\text{I.D.})^n$, where $n = 2–3$ [8], so the increase in V_e is slower than that of $V_{d,max}$, *i.e.*, $V_e \leq V_{d,max}$ for columns with I.D. ≥ 0.25 mm.

Baseline drift during TP

As the two filaments of the TCD were not identical, two factors can influence the baseline drift in TP operation: the change in inlet gas temperature caused by the oven temperature programming, and the change in carrier gas flow-rate because of the temperature dependence of the viscosity of gases. The heating block 2 in Fig. 1 was used to compensate for the first factor, and the constant flow control system was used not only for quantitative analysis but also for stabilizing the baseline. Fig. 4 demonstrates the temperature-programmed analysis of a naphtha sample using this detector. The drift of the baseline is very small, in contrast to the constant inlet pressure mode, where the baseline shows a *ca.* 1 mV drift under the same TP conditions. The first two peaks in Fig. 4 are air and water peaks (the identities of the two peaks were established by GC–MS), which are undetectable with flame ionization detection (FID). The column inlet pressure showed a >80% increase when the column temperature raised from 50 to 140°C.

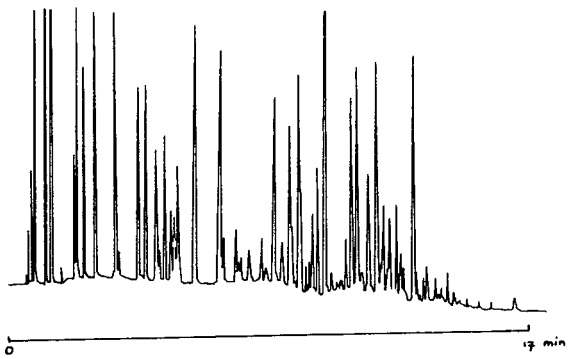

Fig. 4. Chromatogram of naphtha on column A. Initial temperature 50°C, maintained for 2 min, then programmed to 140°C at 2°C/min. TCD temperature, 200°C.

Accuracy of quantitative analysis with TP

For concentration-sensitive detectors, there is an additional requirement for flow control in TP operation. Normally it is very difficult to maintain a constant flow control to within *ca.* 1 ml/min, so we used the strategy of keeping the splitting ratio constant and controlling the total mass flow (shown in Fig. 2). The influence of this control on the accuracy of quantitative analysis was examined with a series of n-alkane mixtures analysed using both isothermal and TP operation. The results of the area percentage reports from the integrator and their elution temperatures (T_r) calculated from their elution times are given in Table I. The differences in the results of the isothermal and TP runs are caused mainly by the change in carrier gas flow-rate, which depends on the quality of the constant mass flow controller. The error was within 1% in the test, but when constant inlet pressure control is used the error can exceed 50% in the same TP operation.

TABLE I

PERCENTAGE PEAK AREAS (A) AND THE ELUTION TEMPERATURES (T_r) OF n-ALKANES MEASURED UNDER ISOTHERMAL (150°C) AND TP CONDITIONS (80°C FOR 2 min, THEN PROGRAMMED TO 160°C AT 8°C/min)

n-Alkane	A		T_r (°C)
	Isothermal	TP	
n-C_{10}	16.3	16.2	123.1
n-C_{11}	12.5	12.3	136.7
n-C_{12}	15.3	15.1	150.2
n-C_{13}	33.0	33.2	160.0
n-C_{14}	22.9	23.2	160.0

Examples of application

The chromatogram shown in Fig. 4 is for a real sample analysis. Because of the universal response characteristics of the TCD, the water content in naphtha was also determined in a single run.

The analysis of esterified evening primrose oil was carried out under isothermal conditions, and the resulting chromatogram is shown in Fig. 5. The same sample was also analysed under the same column conditions but using FID (chromatogram not shown), and the peak area of γ-linolenic acid was 6.5% lower than that obtained with the TCD because of the relatively low response factor of this compound in FID. The TCD response factors for molecules having similar cross-sections are the same, which benefits quantitative analysis, particularly when pure standard samples are not available.

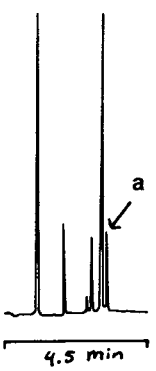

Fig. 5. Analysis of evening primrose oil on column B at 210°C. TCD temperature, 250°C. a = γ-linolenic acid.

Fig. 6. Chromatogram of C_4 mixture used as lighter fuel. Column C at 50°C.

Fig. 6 shows the rapid analysis of a gas mixture containing air, isobutane, n-butane and butene. This experiment was used to test the response speed of the detector. Note the time scale in the chromatogram.

The last example examined was a synthetic essential oil. It was originally analysed with FID and GC–MS. The components in the sample contained O, N and rings, but the exact positions of some functional groups were not known and several standards were unavailable in our laboratory. The relative response factors were set to unity for all of the components, based on the consideration that the molecular mass of all the components exceeded 240. This sample was then analysed with the TCD (Fig. 7). Unexpectedly,

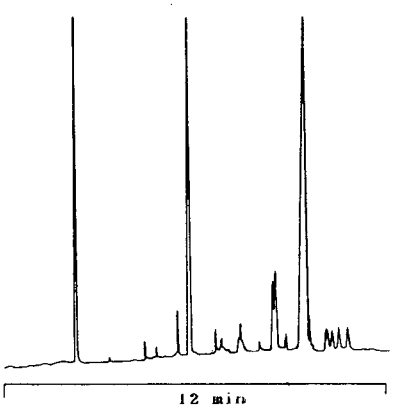

Fig. 7. Quantitative analysis of synthetic essential oil on column A with TCD detection. Column temperature, 200°C; detector temperature, 220°C; bridge current, 150 mA.

the quantitative results were different from the former data. We believe the accuracy of the latter analysis was better for this sample, for reasons explained above for the second example.

More important applications of this detector system are the quantitative analysis of natural gas and other gas mixtures containing inorganic gases. This aspect is under study.

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Headspace analysis with large sample volumes

Influence of sampling device volume, analyte concentration and sample matrix

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ABSTRACT

In three series of experiments the factors influencing headspace gas chromatography (HSGC) with large sample volumes were investigated. The main factors studied were sampling device volume and interactions between the substances and between the volatiles and the sample matrix. It could be shown that variation in any of these factors causes a dramatic change in the resulting headspace composition. The results of these experiments are compared, and the reasons for the different behaviour of the substances are discussed. It is demonstrated that the mechanisms of interaction are complicated and difficult to estimate, especially in complex samples such as food flavours.

INTRODUCTION

Headspace gas chromatography (HSGC) has been shown to be a mostly objective analytical method for investigating food flavours. It has therefore become a widely used and important tool for aroma analysts. Originally developed by Machata [1] for the measurement of blood alcohol concentration, this method has undergone many modifications and improvements for the analysis of volatile food components, including flavour compounds, in the last 20 years.

A dramatic increase in sensitivity after injection of large headspace volumes can be obtained when the volatiles are simultaneously concentrated by cryofocusing directly on the gas chromatographic column or on a deactivated precolumn [2]. The applicability of this method for the analysis of complex food flavours has been demonstrated by several examples [3–9]. When evaluating the results of HSGC, influencing factors such as interaction of the volatile compounds with the sample matrix and the vapour pressure of the substances at a given temperature must be considered. These compound-specific properties can be expressed by the activity coefficient, γ, which depends on the partial pressure of the substance according to Henry's law [10]:

$$p_i' = x_i \gamma p_i^0$$

where p_i' is the partial pressure of compound i, x_i is the mole fraction of i, p_i^0 is the saturated vapour pressure of compound i at a given temperature.

The aim of this work was to determine to what extent the final headspace composition of a model solution is influenced by interaction of compounds with the sample matrix and with each other. The test compounds were chosen for

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TABLE I

COMPOSITION OF THE INVESTIGATED TEST SOLUTION

No.	Substance	Boiling point (°C) [11,12]	Amount (mg/l)
1	2-Methylpropanal	64.2–64.6	199
2	3-Methylbutanal	92.5	102
3	2-Methylbutanal	92–93	137
4	Butanol	117.2	177
5	2,5-Dimethylfuran	93–94	193
6	1-Methylpyrrole	114–115	138
7	Dimethyldisulphide	109.7	147
8	3-Methylthiophene	115.4	201
9	Butylbutyrate	166.6	119
10	3-Methylpyridine	144.1	283
11	2,4-Dimethylthiazole	144–145	105
12	2,6-Dimethylpyrazine	155.6	129
13	2-Acetylpyrazine		114
14	Phenylacetaldehyde	193–194	188
15	Limonene	177–178	126
16	β-Caryophyllene	258–259	124

their differences in chemical structure (different substance classes), volatility and polarity, representing the complexity of food flavours.

MATERIALS AND METHODS

Sample preparation

Large headspace volumes were collected using a gas-tight syringe with a deactivated fused-silica needle according to the sampling procedure described by Wittkowski *et al.* [4]. The headspace sampling device (a 14-ml vial or a 250-ml or 1000-ml Erlenmeyer flask) was filled with 1 ml of the test solution in diethyl ether (for details of the chosen compounds and their concentrations, see Table I). This system was spiked with one of the substances in a great excess or with one of the test matrices listed in Table II. Afterwards the headspace sampling unit was connected to the flask and the sample was stirred for 20 min and finally maintained at exactly 20°C for 1 h.

Headspace injection

A 1-ml aliquot of the vapour phase was withdrawn from the thermostated headspace apparatus using a gas-tight syringe with a fused-silica needle and injected "on-column" with an injection speed of 0.5 ml/min. During the injection, the first loop of the deactivated precolumn was cooled with liquid nitrogen.

Gas chromatography

The gas chromatographic conditions were as follows: gas chromatograph, Carlo Erba HRGC 5160 Mega Series with an MFC 500 programming unit and an EL 480 electrometer; integrator, Shimadzu CR3-A; column, J&W DB-1, 60 m × 0.323 mm × 1 μm, connected to a 2-m deactivated precolumn; carrier gas, helium, average linear velocity 30 cm/s; temperature pro-

TABLE II

LIST OF INVESTIGATED MODEL MATRICES

Sampling device volume (ml)	Investigated model matrices
250	3 ml of glycerine
250	3 ml of paraffin
250	3 ml of water
250	1.4 g of gelatine + 10 ml of water
1000	3 ml of glycerine
1000	3 ml of paraffin
1000	3 ml of water
1000	1.6 g of gelatine + 10 ml of water

gramme, 30°C for 5 min, 30–40°C at 1°C/min, 40°C for 1 min, 40–260°C at 3°C/min, 260°C for 60 min; injector, on-column, room temperature; detector; flame ionization detector at a temperature of 280°C.

RESULTS AND DISCUSSION

To examine the interactions between the substances in headspace analysis we chose a model solution of test compounds dissolved in diethyl ether. It was necessary always to add the same amount of each component to the headspace vessel. Otherwise, without adding solvent, a similar dosing of the substances was not practicable. In the first series of experiments the volume of the headspace sampling device was varied. As depicted in Fig. 1 there were great differences in the resulting headspace chromatograms. Some of the higher-boiling substances could only be detected in larger amounts when sampling was carried out in the 1000-ml Erlenmeyer flask. For instance, phenylacetaldehyde and β-caryophyllene were detectable in the 14-ml vial only as trace components.

The peak areas shown graphically in Fig. 2 confirm these qualitative results. As shown, the peak areas of the higher-boiling substances were greatly increased when the 1000-ml Erlenmeyer flask was used, whereas the decrease in the low-boiling compounds was only small. This fact indicates the usefulness of increasing the sampling device volume for the investigation of substances with low volatility or decreasing the sampling device volume for the separation of high-boiling substances for the fast routine analysis of highly volatile compounds. Of course, diethyl ether as a low-boiling solvent would have some effect on the composition of the headspace compared with experiments without solvent. However, because it is present in large excess over the test solutes this effect will be the same in every experiment and can therefore be neglected.

On the basis of these results the 1000-ml configuration was preferred as the sampling device for further studies of headspace composition. In a second series of experiments the strength of interactions between the tested components was examined. For this the test solution

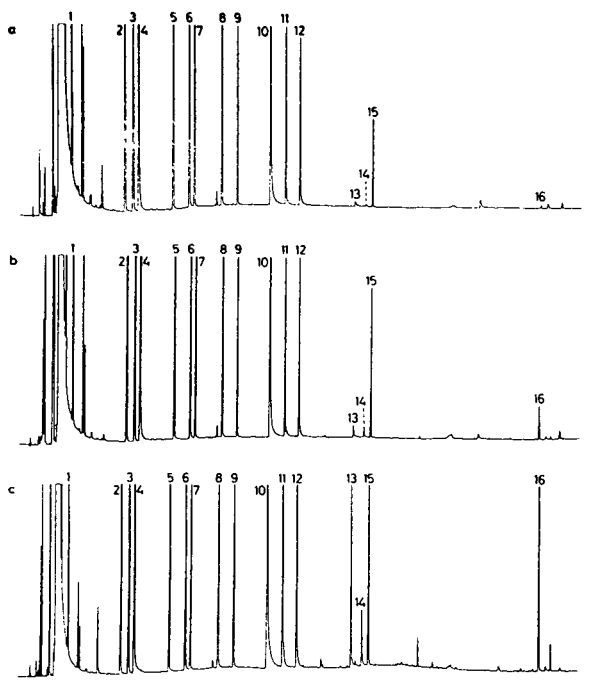

Fig. 1. Headspace gas chromatograms of a test solution after sampling from different sampling devices: (a) 14-ml vial; (b) 250-ml Erlenmeyer flask; (c) 1000-ml Erlenmeyer flask. (The peak numbers refer to compounds listed in Table I.)

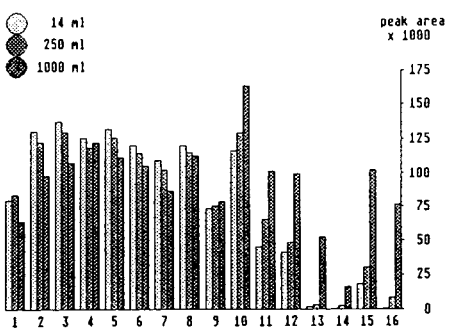

Fig. 2. Comparison of peak areas of reference compounds after HSGC with different sampling device volumes. For compound numbers, see Table I.

was spiked with an excess of one of the model compounds and the effect on the peak areas of the other substances was observed. The HSGC procedure was the same as described above.

Fig. 3 shows as an example, headspace gas chromatograms obtained after addition of 2-methylpropanal or 2,4-dimethylthiazole and, for comparison, the chromatogram of the original test solution headspace. These results are shown graphically in Fig. 4. In both cases all peak areas decreased drastically, but especially those of the higher-boiling substances. In the case of the addition of large amounts of 2-methylpropanal, some of the compounds (3-methylpyridine, 2-acetylpyrazine and β-caryophyllene) could no longer be detected.

The results of all substance addition experiments are summarized in Table III. As expected, there are some interactions between the compounds in the headspace above a sample. But it is surprising how strong these effects can be. On the one hand, a displacement of substances by others primarily takes place in the low-volatile fraction of the compounds investigated. These components are listed at the bottom of Table III. Several substances could only be detected as traces, or, if a greater excess of the additional compound (No. 1b, 2-methylpropanal; No. 3, 2-methylbutanal) was added, they disappeared. On the other hand, these interactions can also influence the headspace concentrations of higher volatile polar substances, such as butanol, which is displaced much more than other low-boiling compounds.

The addition of a low-volatile compound (*e.g.* 2-acetylpyrazine) influences the headspace composition only a little, as is shown in Table III, No. 6. The recoveries of all tested compounds were higher than 90%, except for butylbutyrate and phenylacetaldehyde. The recovery of the former was only a little less (79%), but the recovery of the latter was drastically reduced (6%).

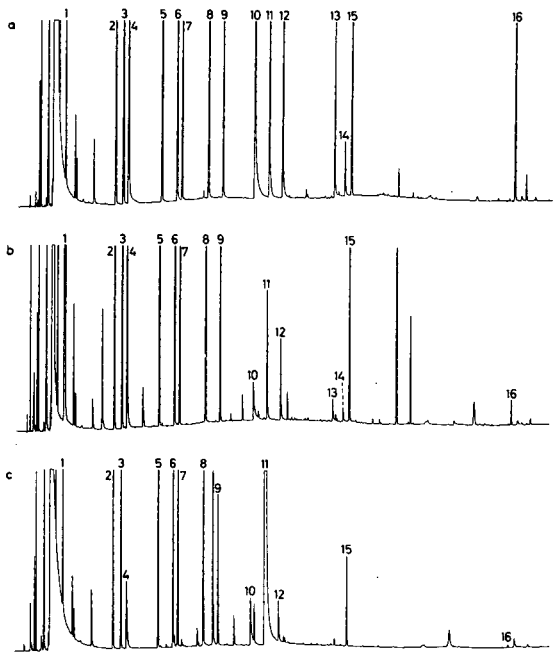

Fig. 3. Headspace gas chromatograms of the test solution before (a) and after addition of 2-methylpropanal (1) (b) or 2,4-dimethylthiazole [11] (c).

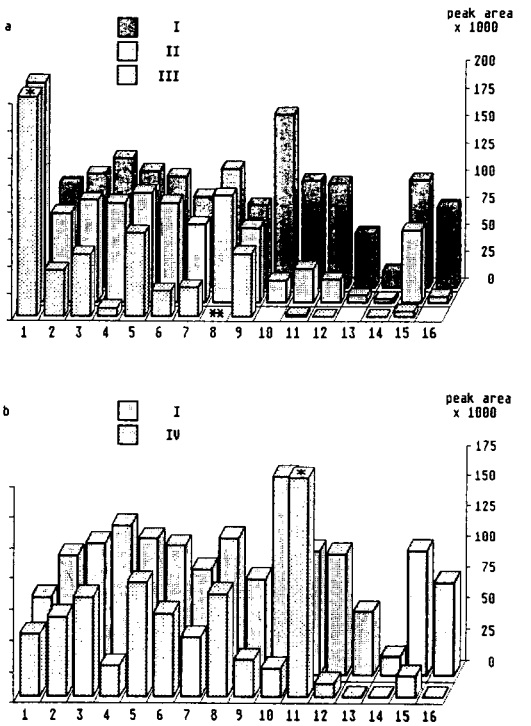

Fig. 4. Peak areas of test compounds. (a) After addition of 2-methylpropanal: I = original test solution; II = small excess; III = large excess. (b) After addition of 2,4-dimethylthiazole: I = original test solution; II = excess. * = Bars are not depicted at full height. ** = Peak overlapping with an impurity of 2-methylpropanal. (For compound numbers, see Table I.)

TABLE III

RELATIVE PEAK AREAS OF THE TEST COMPOUNDS IN DIFFERENT SUBSTANCE ADDITION EXPERIMENTS

Sampling device: 1000-ml Erlenmeyer flask. Std = Test solution; Ex = excess of the added compound; 1a/1b = small/great excess of 2-methylpropanal; 2 = excess of 3-methylbutanal; 3 = excess of 2-methylbutanal; 4 = excess of 3-methylpyridine; 5 = excess of 2,4-dimethylthiazole; 6 = excess of 2-acetylpyrazine.

Test compound	Std	1a	1b	2	3	4	5	6
2-Methylpropanal	100	Ex	Ex	68	46	85	81	90
3-Methylbutanal	100	87	46	Ex	29	61	67	96
2-Methylbutanal	100	90	55	67	Ex	72	76	95
Butanol	100	77	7	69	12	22	12	101
2,5-Dimethylfuran	100	93	71	78	42	82	84	93
1-Methylpyrrole	100	90	24	73	25	46	65	95
Dimethyldisulphide	100	86	33	60	18	47	57	100
3-Methylthiophene	100	91	[a]	79	29	68	76	96
Butylbutyrate	100	91	76	61	10	59	40	79
3-Methylpyridine	100	13	–	48	3	Ex	15	90
2,4-Dimethylthiazole	100	31	3	52	4	123	Ex	89
2,6-Dimethylpyrazine	100	22	1	47	4	13	12	102
2-Acetylpyrazine	100	14	–	24	–	2	2	Ex
Phenylacetaldehyde	100	22	7	11	–	6	6	6
Limonene	100	68	6	72	3	13	18	94
β-Caryophyllene	100	8	–	40	1	1	1	89

[a] Peak overlapping with an impurity of 2-methylpropanal.

These results indicate that there are strong interactions between the substances in the headspace over a complex sample. Even small changes in the sample composition can cause drastic changes in the resulting headspace composition, but the effects vary depending on the amounts and characters of the investigated substances. Therefore general rules cannot be given for the interactions of different compounds in the gas phase above complex samples because many different and unknown factors influence its composition.

We did not investigate the behaviour of individual test compounds. These data would only be of theoretical interest because they have no analytical relevance to the interpretation of the behaviour of complex mixtures. In such systems there are strong interactions between the compounds in the gas phase, as is shown in the substance addition experiments. The individual data are therefore not useful tools for interpreting the results.

Another important factor in HSGC is the sample matrix, which can also influence the headspace composition [13]. To measure the degree of these interactions, a third series of experiments was undertaken, in which the sample matrix was varied. This was done by adding one of the test matrices (water, glycerol, paraffin or gelatine). After equilibration the sample was treated as described above.

The relative peak areas of two subseries of experiments (250- or 1000-ml Erlenmeyer flask) are summarized in Table IV. Here the peak areas in the matrix investigations are related to those of the original standard solution. To aid interpretation, the results of the 1000-ml experiments are additionally depicted as a bar graph in Fig. 5. As shown, all test matrices greatly influence the headspace composition. This effect cannot be explained by an incomplete equilibration due to the short equilibration time of 1 h, since longer times (2 h) did not affect the results.

The results of the matrix variation experiments can be summarized as follows. Adding a model matrix to the test solution generally causes a

TABLE IV

RELATIVE PEAK AREAS OF THE TEST COMPOUNDS IN DIFFERENT MATRIX ADDITION EXPERIMENTS

Std = Test solution; Gly = glycerine; Par = paraffin; Wat = water; Ge = gelatine + 10 ml of water (1 = 1.4 g; 2 = 1.6 g).

Test compound	250-ml flask					1000-ml flask				
	Std	Par	Gly	Wat	Ge1	Std	Par	Gly	Wat	Ge2
2-Methylpropanal	100	56	66	60	35	100	80	71	81	43
3-Methylbutanal	100	29	69	69	28	100	46	84	77	73
2-Methylbutanal	100	33	76	74	44	100	50	87	80	78
Butanol	100	31	13	8	2	100	55	18	10	5
2,5-Dimethylfuran	100	28	83	86	80	100	42	89	86	94
1-Methylpyrrole	100	20	71	74	30	100	31	81	77	70
Dimethyldisulphide	100	12	76	87	72	100	19	87	91	94
3-Methylthiophene	100	14	86	96	89	100	22	89	88	97
Butylbutyrate	100	10	99	97	63	100	13	90	80	85
3-Methylpyridine	100	4	1	2	–	100	2	1	3	–
2,4-Dimethylthiazole	100	5	9	14	4	100	3	9	14	7
2,6-Dimethylpyrazine	100	5	3	2	–	100	2	2	1	–
2-Acetylpyrazine	100	–	–	3	–	100	–	–	2	–
Phenylacetaldehyde	100	–	–	–	–	100	–	–	6	–
Limonene	100	–	289	318	317	100	1	89	86	95
β-Caryophyllene	100	–	157	326	592	100	1	91	75	56

decrease in the peak areas of the reference compounds. Exceptions are limonene and β-caryophyllene, which were enriched after addition of water, glycerol or a gelatine solution in the 250-ml configuration. One reason for this behaviour could be that highly volatile substances, which would cause a displacement of these terpenes if no matrix were added, strongly interact with these test matrices. Therefore these substances no longer have any displacement power. This effect is additionally increased by only weak interactions of the non-polar terpenes with the more polar model matrices.

Looking at Table IV it is obvious that it can be divided into three parts. The substances at the top were only a little influenced by matrix addition, whereas the compounds in the middle show extreme interactions. These components were not detected or were detectable only in small concentrations in the test solution headspace. In contrast, the substances listed in the lower part of the table were only slightly influenced (or even enriched in the 250-ml configuration) in most matrix addition experiments.

A comparison between the tested matrices shows that the effect of displacement is stronger in the case of paraffin addition than in other cases. This effect is probably based on a better solubility of the test compounds in this matrix than in the others, as is shown by the extreme behaviour of the non-polar terpenes. These substances were hardly detectable in the paraffin addition experiments. On the contrary, butanol, a polar molecule, is less influenced by paraffin.

For the same reasons as in the substance addition experiments, no investigation was carried out in which the compounds were tested individually with one of the test matrices because these results cannot be extrapolated to the analysis of complex mixtures.

As in the case of substance addition, the matrix experiments show that it is not possible to predict the interactions between components in headspace analysis of a complex system.

CONCLUSIONS

The results of the experiments carried out show that there are strong interactions between substances in the headspace and between the

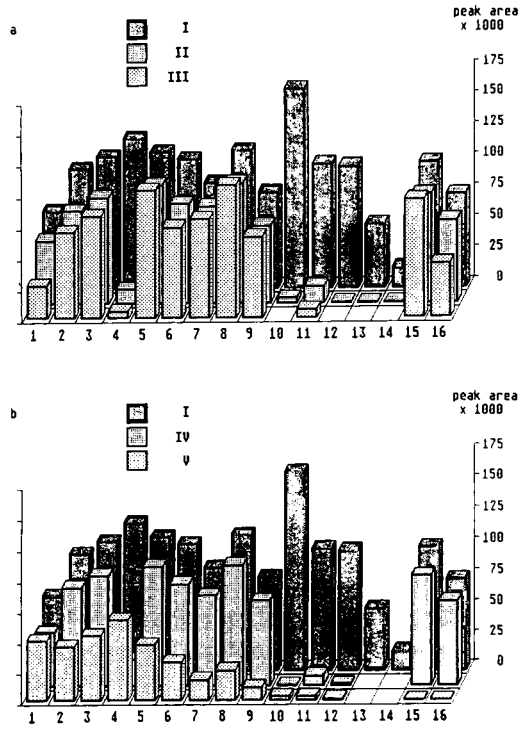

Fig. 5. Peak areas of test compounds before (I) and after addition of model matrices (sampling device: 1000-ml Erlenmeyer flask): (a) addition of water (II) or gelatine solution (III); (b) addition of glycerine (IV) or paraffin (V). (For compound numbers, see Table I.)

volatiles and the sample matrix. These factors and the sampling device volume influence the final headspace composition. Other influencing factors, such as the volatility and polarity of the analytes, their solubility in the sample matrix, etc., are also difficult to estimate, especially in HSGC with large sample volumes of complex samples. Nevertheless, HSGC is a suitable and easy method of investigating food flavours.

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Matrix effects and solute discrimination when injecting dirty samples in capillary columns

Comparative study between classical split and splitless injections☆

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ABSTRACT

Vaporization inside the injector chamber is a very complex process. The presence of non-volatile material may change the volatilization rate of a large number of species. In particular, splitting is far from ideal, as significant differences were observed in the splitting of clean and dirty samples and no discrimination-free chromatographic conditions could be found. The pattern of discrimination was strongly dependent on the glass liner geometry. When the non-volatile content of the sample was increased and packed inserts were used, smaller amounts of the most volatile compounds entered the column, whereas the least volatile compounds seemed not to be affected. The opposite effect was found when using empty injector inserts. Splitless injection was less affected by the presence of non-volatile components in the sample when the distance between the needle and the column entrance was large enough. Incomplete evaporation only occurred with very dirty samples, and this effect was avoided by increasing the injector temperature.

INTRODUCTION

Although current chromatographic research is focused mainly on the development of on-column injectors [1,2] and coupling between HPLC and high-resolution GC systems [3,4], large numbers of samples of very different types are still being analysed using classical injection techniques, *i.e.*, split and splitless injection. One reason is that most extracts are obtained via direct extraction from the original sample and accordingly these extracts are dirty samples that contain a certain amount of non-volatile material that may damage the chromatographic column on entry. On-column injection of dirty samples is now possible [5,6], but the precolumn has to be replaced after a short period of time, which means interrupting normal work and causing additional problems such as recalibration of the system. Classical splitless injection is still used because of the ability to inject dirty samples [7].

Split injection is also still used, not only to analyse concentrated mixtures but also for the analysis of dilute samples containing very volatile compounds. These types of samples are sometimes dirty and difficult to analyse via splitless injection owing to the impossibility of achieving good recondensation effects without cryofocus-

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ing system. This type of sample is very common in food and environmental research.

Heavy material in the sample may cause two main effects: it may change the vaporization rate of the sample and it may even cause incomplete evaporation. Non-volatile material is not vaporized and tends to remain in droplets [8]. These droplets may retain certain solutes from the sample which are released with some delay compared with a clean sample, or they may even be completely retained [8,9]. In split injection, the presence of long-lived droplets may drastically change the pressure wave generated by the almost instantaneous vaporization of a clean sample [10]; this is known to influence the true splitting ratio or, even worse, it may cause a large part of the sample to be lost directly through the split outlet. In splitless injection, the most intense effect is thought to be incomplete evaporation.

The main aim of this work was to evaluate the real ability of both vaporization techniques to analyse dirty samples in the field of flavour research. It is still common to extract volatile compounds with a low-boiling solvent in order to avoid thermal degradation of the solutes, concentrate the extract and analyse it using split injection. However, some microextractive techniques, which allow the concentration step to be eliminated, have been developed [11–14] and some of them allow the extract to be injected via splitless injection.

In this study, "dirtiness" was obtained with a dearomatized wine extract. The solutes considered belonged to different flavour groups, namely fatty acid esters and fusel alcohols.

EXPERIMENTAL

Dichloromethane and pentane (HPLC grade) were obtained from Carlo Erba (Milan, Italy), 1,1,2-trichlorotrifluoroethane (HPLC grade) from Aldrich (Milwaukee, WI, USA) and 2-propanol (for residue analysis) from Merck (Darmstadt, Germany).

The solutes used were obtained from Alltech (West Chester, PA, USA), and were of quantitative quality. Calibration solutions were prepared for each solvent. The solutions injected contained 40 mg l^{-1} of volatiles with split and 4 mg l^{-1} with splitless injection. The injection linearity was tested by injecting calibrated solutions between 20 and 200 mg l^{-1} with split and between 1 and 50 mg l^{-1} with splitless injection.

"Dirtiness" was obtained by continuous extraction of 5 l of a previously distilled red wine with 500 ml of dichloromethane. Subsequently this extract was concentrated by solvent evaporation at 45°C to a final volume of 1 ml. Dirty extracts were obtained by adding different volumes of this extract to clean solutions.

The chromatograph was an HP 5890 Series II (Hewlett-Packard) fitted with split/splitless and on-column injectors. In order to avoid discrimination in the syringe an HP 7673 automatic injector was used. Split injections were performed by the cold needle method. Splitless injections were performed by the hot needle method with a preheating time of 5 s. A 10-μl syringe and an injection volume of 1 μl were used.

A Supelcowax 10 column (60 m $\times$ 0.32 mm I.D.) with a film thickness of 0.50 μm was used. The column temperature was initially held at 40°C for 3 min, then programmed at 3°C min^{-1} to 180°C. Flame ionization detection was used. The carrier gas was hydrogen. The purge flow-rate was 3 ml min^{-1}. Two different injection temperatures were considered, 250 and 350°C.

For split injection, the carrier gas flow-rate was 1 ml min^{-1}, the split flow-rate was 40 ml min^{-1} and the pre-set splitting ratio was 1:40. Injections were carried out using two different glass liners: a Jennings cup type packed with Chromosorb and an empty cylindrical type. The distance between the column entrance and the tip of the syringe was 4.26 cm.

For splitless injection, the carrier gas flow-rate was 2.5 ml min^{-1}, the split flow-rate was 26 ml min^{-1} and the splitless time was 3 min. An empty cylindrical liner was used. The distance between the column entrance and the tip of the syringe was 4.26 cm unless specified otherwise.

Chromatographic signals were registered with an NEC computer using Maxima 820 from Waters Software. To obtain the true splitting ratio and the mass transfer efficiency, chromatographic peak areas from the different experiments

were compared with those obtained from on-column injection of clean samples.

RESULTS

Split injection

Clean samples. The results are given in Table I. Slight deviations from the preset split ratio were observed while working with the two different inserts, even with clean solutions. However, the linearity and precision of injection were very good in both instances. With the empty cylindrical insert the splitting ratio was not the same for all the compounds, being slightly lower for the least volatile substances. In contrast, with the Jennings cup packed insert, the splitting ratio was constant for all the compounds tested.

Dirty samples. When non-volatile material is added to the solutions, the splitting ratio changes and the pattern of that change is strongly dependent on the geometry of the system. When a packed insert is used, the splitting ratio tends to decrease, but this effect is more pronounced for the most volatile solutes, as can be clearly seen in Fig. 1 and Table II. Thus, when the sample contains non-volatile material, smaller amounts of the most volatile compounds and about the same amount of the least volatile compounds enter the column. However, if an empty insert is used, the splitting ratio tends to increase, and in this instance the least volatile compounds are most subject to this effect. The results are shown in Fig. 2. Surprisingly, the linearity and precision of injection were not affected by the presence of non-volatile material, as can be seen in Table II. Almost the same results were obtained using pentane as the solvent, showing that the solvent does not exert a significative influence.

From an analytical point of view, these effects make quantification erroneous, even using an internal standard. In Figs. 3 and 4, the relative error in the quantification was obtained on the basis of a calibration performed with 1-hexanol as internal standard. To avoid such errors, at least two different internal standards should be used or a standard addition calibration should be

TABLE I

TRUE SPLITTING RATIO IN THE INJECTION OF CLEAN AND DIRTY SAMPLES

Compound	True splitting ratio[a]											
	Clean samples				Dirty samples (in dichloromethane)							
	Dichloromethane		Pentane		E.I.	P.I.	Change (%)[b]		R.S.D. (%)[c]		r^{d}	
	E.I.	P.I.	E.I.	P.I.			E.I.	P.I.	E.I.	P.I.	E.I.	P.I.
1-Propanol	29.9	28.2	32.9	31.4	28.2	38.9	−5.6	39.0	3.1	2.8	0.9991	0.9992
Ethyl butyrate	28.8	27.9	31.7	30.8	27.3	38.3	−5.3	36.7	2.1	3.3	0.9996	0.9989
1-Butanol	29.1	27.3	32.6	30.7	26.7	38.4	−8.2	40.8	1.6	2.2	0.9998	0.9995
1-Pentanol	32.2	30.1	34.5	32.3	27.0	37.7	−16.0	25.1	1.9	1.7	0.9992	0.9996
Ethyl hexanoate	34.7	30.9	35.8	33.5	29.7	39.9	−14.4	22.6	3.2	2.9	0.9990	0.9990
1-Hexanol	35.7	28.7	36.9	32.0	28.1	38.0	−21.3	32.4	2.2	2.5	0.9997	0.9999
Ethyl octanoate	34.4	28.4	37.1	31.8	28.5	35.6	−17.2	25.1	1.7	1.2	0.9993	0.9994
Ethyl decanoate	37.2	31.6	38.9	32.4	29.1	37.5	−21.8	18.5	2.5	1.8	0.9992	0.9999
Phenylethyl acetate	33.9	32.1	36.0	32.1	28.3	27.3	−16.5	−15.0	1.9	2.4	0.9989	0.9991
Ethyl laurate	35.6	29.1	37.8	30.6	29.0	31.8	−18.5	9.3	2.6	3.1	0.9994	0.9995
2-Phenylethanol	34.0	28.7	36.3	30.5	30.0	30.2	−11.8	5.2	2.1	1.8	0.9997	0.9997

[a] Results are averages of five injections. E.I. = Empty insert; P.I. = packed insert.
[b] Relative increment of the true splitting ratio when the sample contains 28% of the dirty extract.
[c] Average of six injections.
[d] Linear regression coefficient.

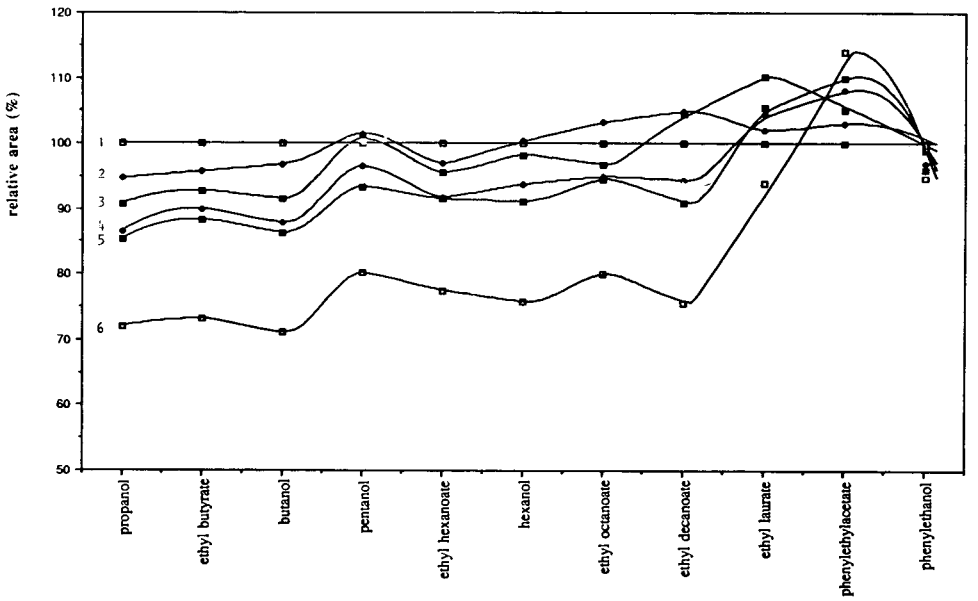

Fig. 1. Effect of non-volatile material. Relative areas of the chromatographic peaks for clean and dirty solutions. Split injection; packed insert; injector temperature, 250°C. Individual points are connected with lines to show the differences, but there is no physical measuring to these lines. For the nature of the contaminants, see Experimental. Percentages of the contaminants are: 1 = clean sample; 2 = with 2%; 3 = with 4%; 4 = with 8%; 5 = with 14%; 6 = with 28%.

performed. It is important to point out that the deviation of the splitting ratio seems to depend on the boiling point of the solute more than on its chemical characteristics, as can be seen in Figs. 5 and 6.

Temperature effect. With both kinds of inserts

TABLE II

ANALYTICAL CHARACTERISTICS OF THE SPLIT INJECTION OF DICHLOROMETHANE DIRTY EXTRACTS

Compound	True splitting ratio (dirty samples)		Change (%)[a]		R.S.D. (%)[b]		r[c]	
	E.I.	P.I.	E.I.	P.I.	E.I.	P.I.	E.I.	P.I.
1-Propanol	28.2	38.9	−5.6	39.0	3.1	2.8	0.9991	0.9992
Ethyl butyrate	27.3	38.3	−5.3	36.7	2.1	3.3	0.9996	0.9989
1-Butanol	26.7	38.4	−8.2	40.8	1.6	2.2	0.9998	0.9995
1-Pentanol	27.0	37.7	−16.0	25.1	1.9	1.7	0.9992	0.9996
Ethyl hexanoate	29.7	39.9	−14.4	22.6	3.2	2.9	0.9990	0.9990
1-Hexanol	28.1	38.0	−21.3	32.4	2.2	2.5	0.9997	0.9999
Ethyl octanoate	28.5	35.6	−17.2	25.1	1.7	1.2	0.9993	0.9994
Ethyl decanoate	29.1	37.5	−21.8	18.5	2.5	1.8	0.9992	0.9999
Phenylethyl acetate	28.3	27.3	−16.5	−15.0	1.9	2.4	0.9989	0.9991
Ethyl laurate	29.0	31.8	−18.5	9.3	2.6	3.1	0.9994	0.9995
2-Phenylethanol	30.0	30.2	−11.8	5.2	2.1	1.8	0.9997	0.9997

[a] Relative increment of the true splitting ratio when the sample contains 28% of the dirty extract.
[b] Average of six injections.
[c] Linear regression coefficient.

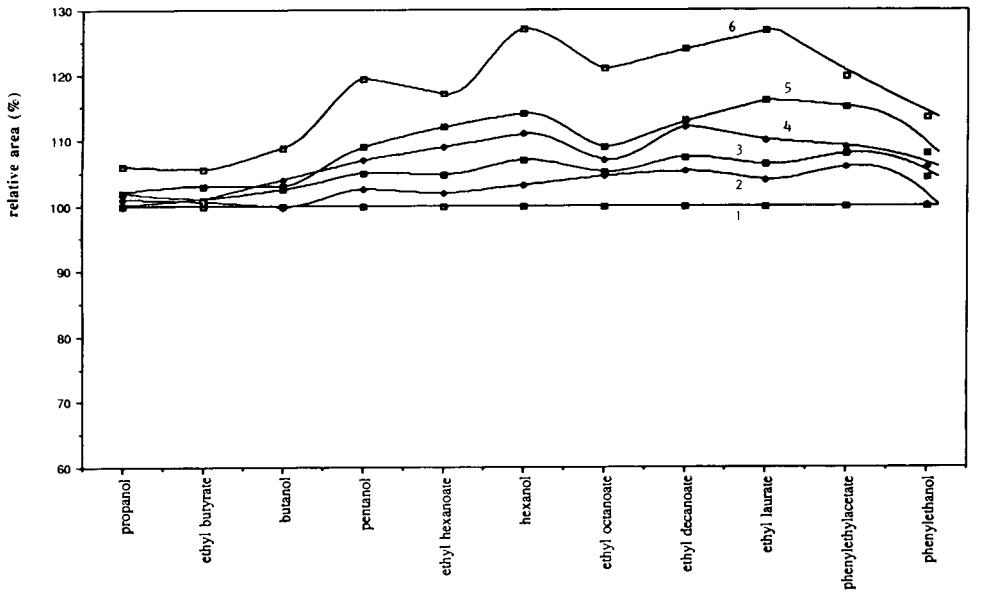

Fig. 2. Effect of non-volatile material. Relative areas of the chromatographic peaks for clean and dirty solutions. Split injection; empty insert; injector temperature, 250°C. Individual points are connected with lines to show the differences, but there is no physical measuring to these lines. For the nature of the contaminants, see Experimental. Percentages of the contaminants are: 1 = clean sample; 2 = with 2%; 3 = with 4%; 4 = with 8%; 5 = with 14%; 6 = with 28%.

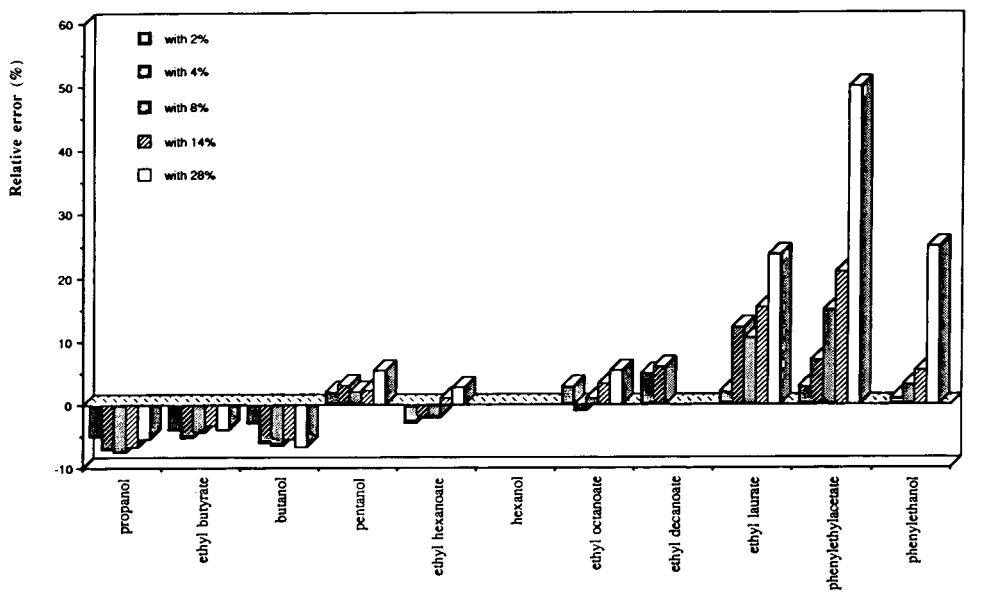

Fig. 3. Effect of non-volatile material on quantification. Relative errors in the quantification of dirty solutions with calibration based on a clean sample and with the use of an internal standard (1-hexanol). Split injection; packed insert; injector temperature, 250°C.

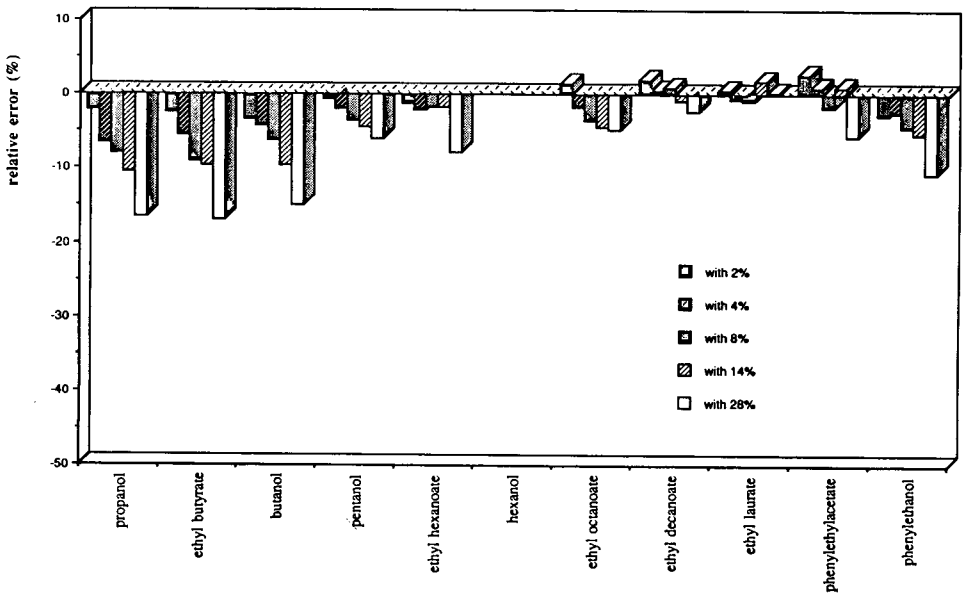

Fig. 4. Effect of non-volatile material on quantification. Relative errors in the quantification of dirty solutions with calibration based on a clean sample and with the use of an internal standard (1-hexanol). Split injection; empty insert; injector temperature, 250°C.

the temperature decreases slightly, but it does not avoid the effects of the addition of non-volatile material. Figs. 7 and 8 show the effect of an increase of 100°C in the injector temperature.

Splitless injection

Splitless injection was tested with different solvents: 2-propanol (b.p. 82°C), 1,1,2-trichlorotrifluoroethane (b.p. 56°C) and dichloromethane (b.p. 44°C). The solvent behaviour was similar in all instances, working with both clean and dirty solutions. In all instances, a very good mass transfer was achieved and only with a large amount of added non-volatile material were the least volatile solutes transferred with poor efficiency. In Fig. 9, results for the injection of 2-propanol extracts are presented. The results obtained with the other solvents were similar but slightly better. Increasing the injector temperature led to suppression of the deviations.

DISCUSSION

Vaporization of the sample in the injector depends not only on the thermodynamic properties of the solvent such as surface tension, boiling point and heat of evaporation, but also on other factors. The system geometry and injection methods also have a significant effect [15–17]. The amount of solute introduced into the column depends not only on the chosen splitting ratio, but also on the real amount of vaporized solute, on the actual splitting ratio at the moment the solute reaches the split point and on the concentration of vaporized solute at the column entrance. In other words, the extent and rate of vaporization will determine the process. Vaporization should become more difficult as surface tension increases and as non-volatile substances are introduced in the sample, preventing nebulization of the sample near the needle exit. Consequently, if incomplete evaporation occurs, smaller amounts of solutes will enter with samples in dichloromethane as solvent than with samples in pentane, and with dirty than with clean samples. However, this was not the case under the present conditions, where evaporation of the samples seemed to follow the first scenario described by Grob and De Martin [16], *i.e.*, flash evaporation, rather than the second one, *i.e.*, incomplete evaporation due to sample liquid not being nebulized. This may be

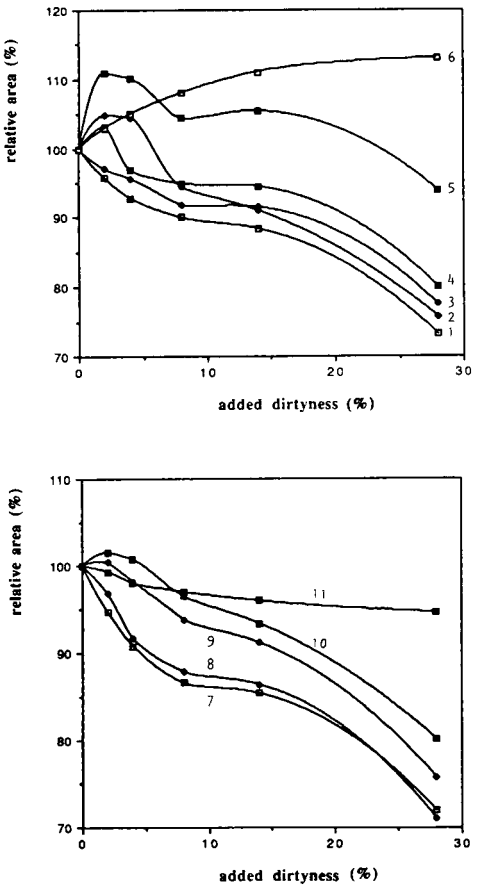

Fig. 5. Effect of non-volatile material. Behaviour of the different solutes. Split injection; packed insert; injector temperature, 250°C. 1 = Ethyl butyrate; 2 = ethyl decanoate; 3 = ethyl hexanoate; 4 = ethyl octanoate; 5 = ethyl laurate; 6 = phenylethyl acetate; 7 = propanol; 8 = butanol; 9 = hexanol; 10 = pentanol; 11 = phenylethanol.

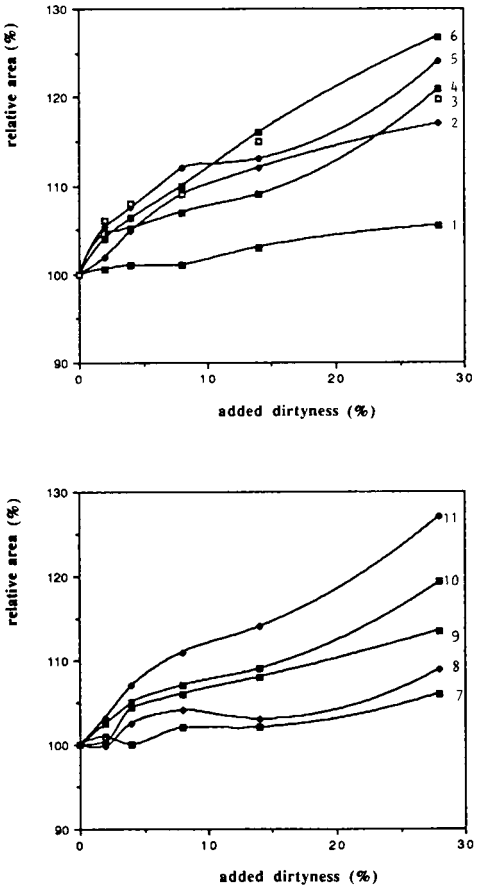

Fig. 6. Effect of non-volatile material. Behaviour of the different solutes. Split injection; empty insert; injector temperature, 250°C. 1 = Ethyl butyrate; 2 = ethyl hexanoate; 3 = phenylethyl acetate; 4 = ethyl octanoate; 5 = ethyl decanoate; 6 = ethyl laurate; 7 = 1-propanol; 8 = 1-butanol; 9 = 1-pentanol; 10 = phenylethanol; 11 = 1-hexanol.

due to the fact that the rapid autosampler injection (<0.02 s) could generate a mechanical spray effect, making vaporization easier. Another, although less probable, cause might be that the liquid droplets from the ejected sample reach the injector bottom and, instead of being lost through the split outlet, bounce and return to the vaporization chamber [17].

Whatever the cause, the fact is that with the injection of dichloromethane samples there is a slightly larger entry of solutes than with pentane samples, probably owing to the larger increase in volume caused by the vaporization of 1 μl of dichloromethane (450 μl) than of pentane (300 μl) and to the more intense pressure wave generated in the former instance.

Some differences are observed in the injection of clean samples, depending on the insert geometry. Better results are obtained with the Jennings cup packed insert which seems to be discrimination free. When injecting dirty samples the resulting effects also depend strongly on the geometry of the insert.

With an empty insert, where incomplete evaporation is more likely, an increase in non-volatile material not only does not reduce the

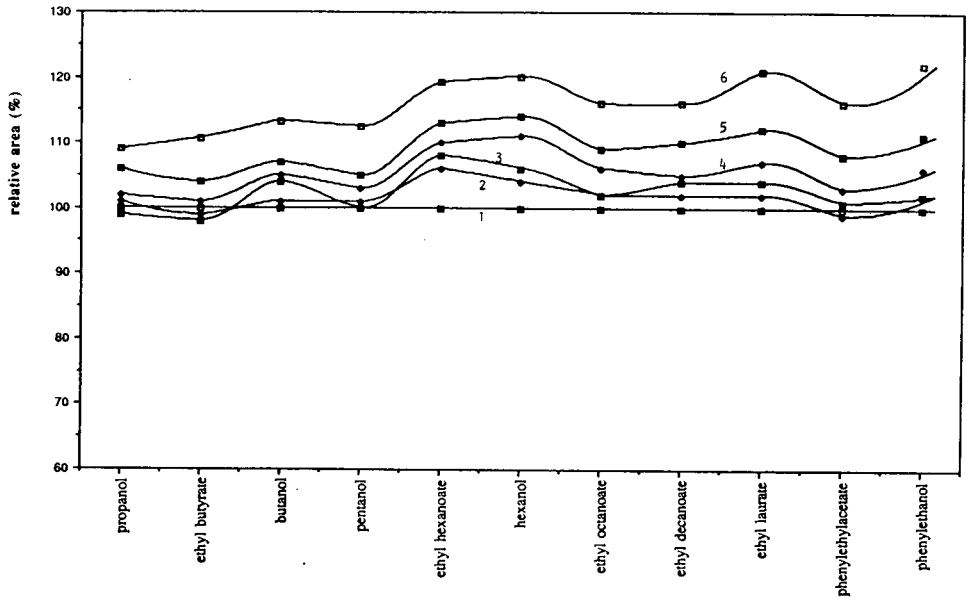

Fig. 7. Effect of increasing the injector temperature. Conditions and presentation as in Fig. 2; injector temperature, 350°C. 1 = Clean sample; 2 = with 2%; 3 = with 4%; 4 = with 8%; 5 = with 14%; 6 = with 28%.

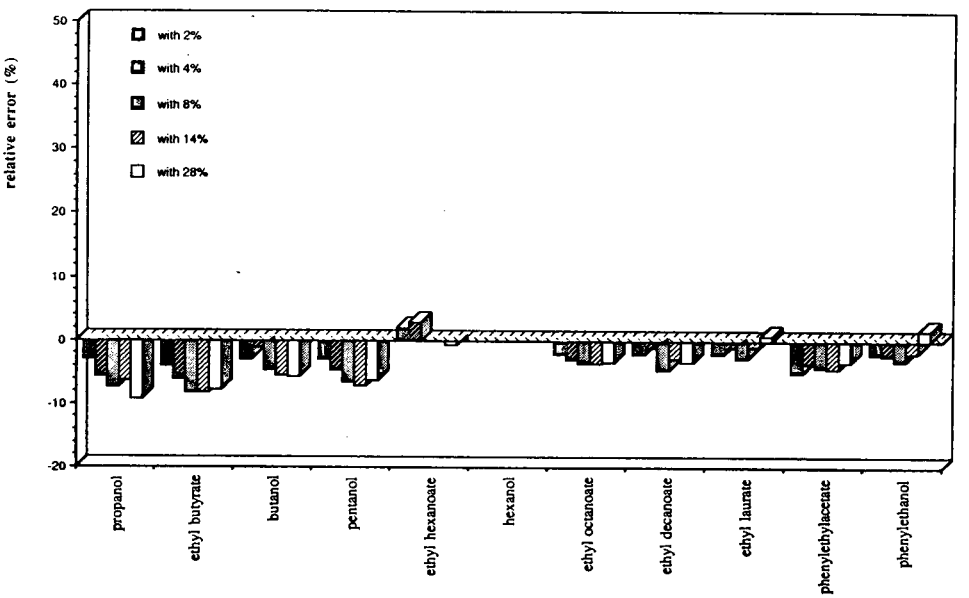

Fig. 8. Effect of increasing the injector temperature. Conditions as in Fig. 4; injector temperature, 350°C.

splitting ratio but also increases the ratio of the less volatile solutes introduced into the column. This could be due to a delay in vaporization caused by the presence of non-volatile substances, thus allowing the drops expelled by the syringe to travel further. For this reason, these droplets can transport the less volatile compounds faster inside the glass insert and they can

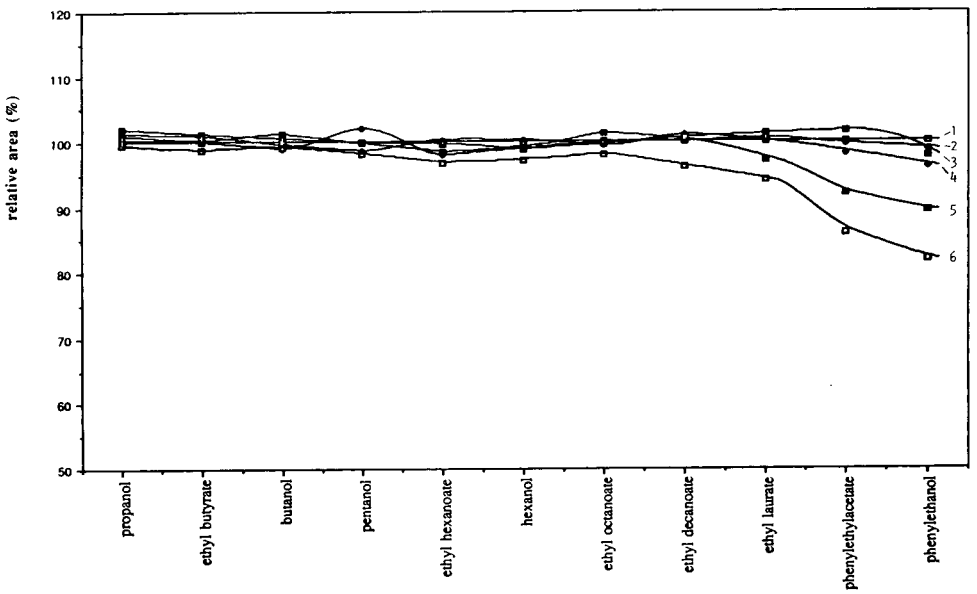

Fig. 9. Effect of non-volatile material. Relative areas of the chromatographic peaks in the dirty solutions compared with clean solutions. Splitless injection; 2-propanol as solvent; injector temperature, 250°C. Presentation as in Figs. 1 and 2. 1 = Clean sample; 2 = with 2%; 3 = with 4%; 4 = with 8%; 5 = with 14%; 6 = with 28%.

form a more concentrated cloud of vapour near the column entrance than in the injection of a clean sample. As a result, a stronger recondensation effect is produced, which is shown by a higher splitting ratio.

This means that droplets are not trapped by the layer of dirtiness covering the internal surfaces of the glass liner, otherwise the least volatile material would enter in a lower proportion. It is thought that droplets rebounce off the wall owing to their boiling material, which acts as a vapour cushion covering them [18]. In this instance, the effect of increasing the injector temperature is small because the vaporization time decreases slowly, according to the concept of Leidenfrost temperature introduced by Wang *et al.* [18]. The fact that the most volatile solutes do not seem to be affected in this kind of insert by the addition of non-volatile material may be due to the compensation of the decrease in the pressure wave by accelerated transport of the solutes in the micelles.

When the level of non-volatile compounds is increased in a Jennings cup packed injector, the entry of the most volatile solutes is reduced, whereas the least volatile solutes are hardly affected. It is thought that vaporization of the most volatile solutes is considerably delayed with respect to a clean injection, whereas this delay is not as significant with the least volatile solutes.

This seems to imply that even in the injection of clean sample there is a difference in the vaporization times between the most volatile solutes, which would vaporize faster, and the least volatile, which would vaporize more slowly. As a result, the delay caused by the presence of non-volatile compounds has a more significant effect on the most volatile compounds. This delay can be manifested in smaller pressure wave and recondensation effects. Accordingly, the most volatile solutes enter to a lesser extent than they would do with a clean injection. Again, the effect of increasing the injector temperature was minimal.

Splitless injection

Under the chosen conditions, the mass transfer efficiency is very high, although a solvent effect could not be achieved. A decrease in mass transfer efficiency should now be due to incomplete evaporation. The flow-rate of the carrier gas inside the liner is slow, and micelles could be

trapped by the layer of dirtiness covering the inner surface of the liner, thus following the third scenario described by Grob and De Martin [16], *i.e.*, liquid splashing on the insert wall, particularly in the case of 2-propanol. The solvent in this instance plays a secondary role because the vaporization rate is lowered for all the solvents in the same proportion, depending more on the amount of non-volatile material contained by the sample than on the boiling point of the solvent. Although above the Leidenfrost temperature the increase in heat transfer with increasing temperature is very small, as the vaporization time is larger compared with that of flash evaporation the effect of increasing the injector temperature is highly significant.

CONCLUSIONS

The vaporization process inside the injector is very complex and strongly dependent on the non-volatile content of the sample. Split injection is strongly affected by this and the true splitting ratio can be significantly altered, following different patterns according to the geometry of the system. In general, with an empty insert, solutes travel faster to the column entrance when the sample is dirty, and those solutes which are not greatly affected by the pressure wave enter in larger amounts in dirty than in clean samples. In contrast, the only effect observed with a packed insert is that the amount of the least volatile solutes entering the column is lower owing to the decrease in the pressure wave. An increase in temperature reduces these effects only slightly. It should be noted that under the conditions used no incomplete vaporization was observed, perhaps owing to the intense nebulization caused by rapid autosampler injection. Splitless injection appears to be less affected by the presence of non-volatile material. Thus, better results can be achieved by using solvents with a good recondensation effect than with the most volatile solvents. From this point of view, microextractions show several advantages over classical extractions for the routine determination of thermally degradable compounds.

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Capillary gas chromatographic separations of a multi-component mixture of polyalcohol compounds

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ABSTRACT

Capillary gas chromatographic separation conditions were established for a 31-component mixture of polyhydroxy compounds containing two tetritols, three pentitols, six hexitols, seven monoanhydropentitols, eight monoanhydrohexitols, three dianhydrohexitols, pentaerythritol and myoinositol. Mixtures of per-O-acetyl or per-O-trimethylsilyl derivatives of these compounds were separated on fused-silica columns using highly polar DB-23 and non-polar HP-5 stationary phases, respectively. Relative retention times for each compound with respect to pentaerythritol and myoinositol and methylene units were determined.

INTRODUCTION

Polyhydroxy compounds are widespread in the plant kingdom [1,2] and there is great interest in their detection and identification. High-resolution gas chromatography (HRGC) offers the possibility of the rapid detection of these compounds or their derivatives in mixtures from natural sources or resulting from chemical transformations of polyols.

The recent literature covering applications of GC to these compounds can be divided into three groups. The first is concerned with improvement of GC separation methods for alditol, anhydroalditol and cyclitol derivatives [3–19]. The second involves papers dealing with the detection and determination of these compounds occurring in nature, *e.g.*, in biological fluids [20,21], human cerebrospinal fluid [22], the blood serum [23], urine of patients with uraemia [24], human urine (6-deoxyallitol and 6-deoxyglucitol) [25], eyeballs of animals and healthy humans [26], eyeballs of patients with cataract [27], animal cell plasma [28], cow milk [29], clover and peanuts [30], seeds and fodder (methylinositol) [31], raw food [32], foodstuffs [33] and plasticizers [34]. The third relates to the GC separation of enantiomers on columns with chiral stationary phases [35–39].

Our previous studies concerning the separation and identification of anhydroalditol mixtures formed in cyclization–dehydration reactions of particular pentitols [40–44] and hexitols [45,46] were performed using capillary GC (cGC) with SE-52, OV-275, Carbowax 20M and 20MTPA stationary phases and helium or hydrogen as the carrier gas. Based on these results, we were able to separate more complicated mixtures.

In this paper we present the results of the separation of per-O-acetyl and per-O-trimethylsilyl derivatives of a 31-component mixture of polyols by cGC.

EXPERIMENTAL

Gas chromatographic conditions

The 31-component mixture of polyhydroxy compounds was separated on two columns: as per-O-acetyl derivatives on a DB-23 fused-silica

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column (60 m × 0.258 mm I.D.) and as per-O-trimethylsilyl derivatives on an HP-5 fused-silica column (50 m × 0.2 mm I.D.).

Both columns were separately installed inside a Carlo Erba Vega 6180 gas chromatograph. The gas chromatograph was equipped with a cold on-column injector, a flame ionization detector and an integrator (CE Instruments DP 700). Hydrogen was used as the carrier gas at a flow-rate of 2 ml/min measured at room temperature. The temperature programme for the HP-5 column was from 115 to 180°C at 3°C/min and from 180 to 250°C at 4°C/min, and that for the DB-23 column was from 80 to 150°C at 3°C/min, from 150 to 200°C at 4°C/min and from 200 to 240°C at 5°C/min with a final hold at 240°C for 15 min.

Reactants

The following compounds were used: allitol, obtained by reduction of D-allose (Aldrich) with $NaBH_4$; D-altritol and galactitol, obtained by reduction of D-tagatose (Sigma) with $NaBH_4$; 1,4-anhydro-D-arabinitol [47]; 1,5-anhydro-D-arabinitol [47]; 1,4-anhydro-D,L-galactitol [47];

TABLE I

RETENTION CHARACTERISTICS OF PER-O-ACETYLATED POLYOLS ON DB-23

GC peak	Per-O-acetyl derivative of	Retention time (min)	Relative retention time[a]		Methylene units[b]
			RRT^1	RRT^2	
1	1,5-Anhydroxylitol	33.07	0.811	0.667	24.75
2	1,5-Anhydroribitol	34.13	0.837	0.682	25.29
3	1,5-Anhydroarabinitol	34.39	0.844	0.687	25.42
4	1,4;3,6-Dianhydroglucitol	34.60	0.849	0.692	25.53
5	Erythritol + 1,4-anhydroarabinitol	34.87	0.856	0.697	25.63
6	1,4-Anhydroxylitol	35.06	0.860	0.701	25.74
7	1,4;3,6-Dianhydromannitol	35.24	0.864	0.705	25.85
8	1,4-Anhydroribitol	36.03	0.884	0.720	26.26
9	Threitol	36.29	0.890	0.726	26.43
10	1,5;3,6-Dianhydroglucitol	36.54	0.896	0.731	26.57
11	1,4-Anhydrolyxitol	36.68	0.900	0.733	26.66
12	Penthaerythritol	40.77	1.000	0.815	29.22
13	Ribitol	41.67	1.022	0.833	29.65
14	Arabinitol	42.02	1.031	0.840	29.76
15	1,4-Anhydroglucitol	42.32	1.038	0.846	29.86
16	1,5-Anhydroglucitol	42.91	1.052	0.858	30.29
17	1,4-Anhydrogalactitol	43.18	1.059	0.863	30.43
18	1,4-Anhydromannitol + 1,5-anhydrogalactitol	43.34	1.063	0.866	30.50
19	1,5-Anhydromannitol	43.49	1.067	0.869	30.57
20	Xylitol + 2,5-anhydroglucitol	43.84	1.076	0.876	30.74
21	1,6-Anhydroiditol	45.28	1.111	0.905	32.26
22	Allitol	46.35	1.137	0.927	32.58
23	Mannitol	47.16	1.157	0.943	32.84
24	Altritol	47.35	1.161	0.947	32.87
25	Galactitol	48.03	1.178	0.960	34.07
26	Glucitol	49.05	1.203	0.981	34.38
27	Myoinositol	50.02	1.227	1.000	34.68
28	Iditol	50.89	1.248	1.017	34.97

[a] RRT^1 relative to pentaerythritol, RRT^2 relative to myoinositol.
[b] Measured relative to C_{24}–C_{35} *n*-alkanes from 80°C at 3°C/min.

1,5-anhydro-D-galactitol [47]; 1,4-anhydro-D-glucitol [47]; 1,5-anhydro-D-glucitol [47]; 2,5-anhydro-D-glucitol [47]; 1,6-anhydro-L-iditol [47]; 1,4-anhydro-D,L-xylitol [44]; 1,5-anhydroxylitol [44]; 1,4-anhydro-D-lyxitol [44]; 1,4-anhydro-D-mannitol [47]; 1,5-anhydro-D-mannitol [47]; 1,4-anhydro-D,L-ribitol [44]; 1,5-anhydroribitol [44]; D-arabinitol (Sigma); 1,4;3,6-dianhydro-D-glucitol [47]; 1,5;3,6-dianhydro-D-glucitol [47]; 1,4;3,6-dianhydro-D-mannitol [47]; erythritol (Aldrich); galactitol (Sigma); D-glucitol (Sigma); L-iditol [48]; xylitol (Sigma); D-mannitol (Sigma); myoinositol (Sigma); pentaerythritol (Sigma); ribitol (Sigma); D,L-threitol (Aldrich); bis(trimethylsilyl)acetamide (BSTFA) (Aldrich); acetic anhydride (Polskie Odczynniki Chemiczne, Gliwice, Poland); sodium acetate (Polskie Odczynniki Chemiczne); C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, C_{28}, C_{32}, C_{36} *n*-alkanes kit (Alltech); C_9, C_{11}, C_{13}, C_{15}, C_{17}, C_{19}, C_{21}, C_{23}, C_{25} *n*-alkanes kit (Alltech); *n*-hexacosane (Alltech); *n*-triacontane (Alltech); and tetratriacontane (Alltech).

Solvents

Chloroform, pyridine and hexane were obtained from Aldrich.

Apparatus and columns

A VEGA 6180 gas chromatograph was obtained from Fisons Instruments (Vienna, Austria), a DB-23 capillary column from J&W Scientific (Folsom, CA, USA) and an HP-5 capillary column from Hewlett-Packard (Geneva, Switzerland).

Preparation of standard mixture

About 2–5 mg of each of the 31 reactants were dissolved in 10 ml of water and 20 μl of this mixture were placed in a Reacti-vial. Water was then removed under a stream of nitrogen and 0.5

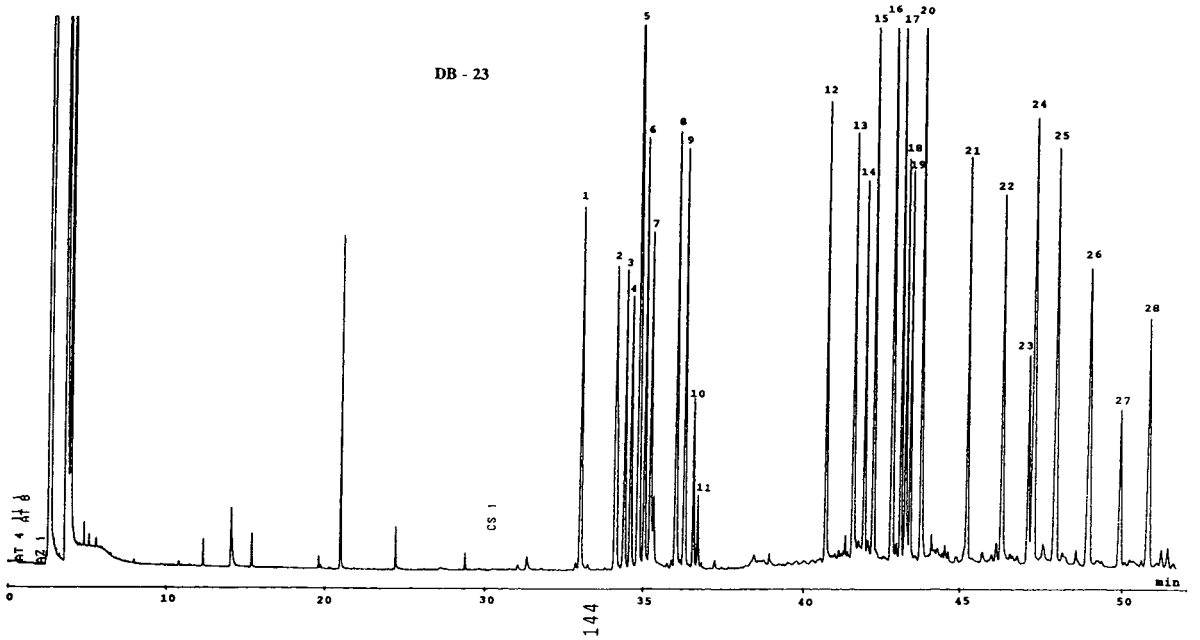

Fig. 1. Gas chromatogram of per-O-acetyl derivatives of the 31-component mixture separated on DB-23. Peaks: 1 = 1,5-anhydroxylitol; 2 = 1,5-anhydroribitol; 3 = 1,5-anhydroarabinitol; 4 = 1,4;3,6-dianhydroglucitol; 5 = erythritol + 1,4-anhydroarabinitol; 6 = 1,4-anhydroxylitol; 7 = 1,4;3,6-dianhydromannitol; 8 = 1,4-anhydroribitol; 9 = threitol; 10 = 1,5;3,6-dianhydroglucitol; 11 = 1,4-anhydrolyxitol; 12 = penthaerythritol; 13 = ribitol; 14 = arabinitol; 15 = 1,4-anhydroglucitol; 16 = 1,5-anhydroglucitol; 17 = 1,4-anhydrogalactitol; 18 = 1,4-anhydromannitol + 1,5-anhydrogalactitol; 19 = 1,5-anhydromannitol; 20 = xylitol + 2,5-anhydroglucitol; 21 = 1,6-anhydroiditol; 22 = allitol; 23 = mannitol; 24 = altritol; 25 = galactitol; 26 = glucitol; 27 = myoinositol; 28 = iditol.

ml of freshly distilled acetic anhydride and 10 mg of anhydrous sodium acetate were added. The mixture was heated at 100°C for 1 h, then the volatile components were removed under reduced pressure and the residue was dissolved in 100 μl of chloroform.

Another 20 μl portion of the same mixture, after removing water under a stream of nitrogen, was O-trimethylsilylated with 100 μl of BSTFA in 100 μl of pyridine at 100°C for 15 min.

RESULTS AND DISCUSSION

The separation with the optimum temperature programme and with the optimum carrier gas flow-rate of the per-O-acetylated 31-component mixture of polyol compounds on a fused-silica cGC column with the stationary phase DB-23 (Table I) gave 28 well separated peaks (Fig. 1). Erythritol and 1,4-anhydroarabinitol (peak 5), 1,4-anhydromannitol and 1,5-anhydrogalactitol (peak 18) and xylitol and 2,5-anhydroglucitol (peak 20) were not separated. It is noteworthy that a separately prepared mixture of per-O-acetyl-1,4-anhydro-D-mannitol and -1,5-anhydro-D-galactitol could be separated on a glass column coated, according to Grob and Grob's procedure [49], with a mixed Carbowax 20 MTPA–SP 2340 (2:1, w/w) stationary phase. The remaining components were separated to the baseline and could be quantitatively determined.

The elution order of per-O-acetylated compounds (Fig. 1, Table I), in particular polyol groups, is as follows:

(a) alditols: erythritol (5), threitol (9), ribitol (13), arabinitol (14), xylitol (20), allitol (22), mannitol (23), altritol (24), galactitol (25), glucitol (26) and iditol (28);

(b) anhydropentitols: 1,5-anhydroxylitol (1), -ribitol (2) and -arabinitol (3), 1,4-anhydro-arabinitol (5), -xylitol (6), -ribitol (8) and -lyxitol (9); the elution order of fully acetylated

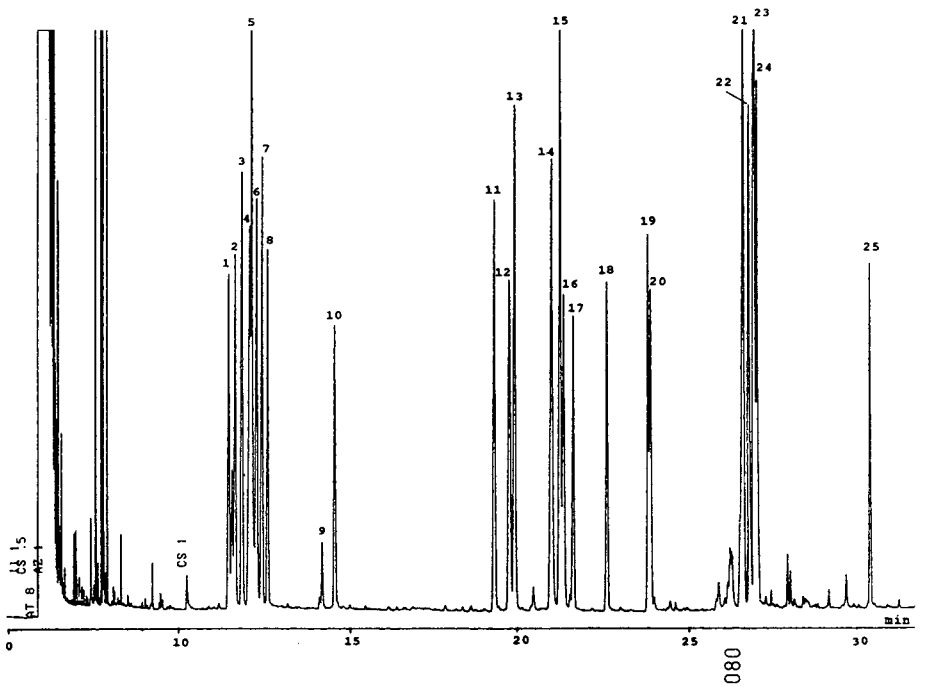

Fig. 2. Gas chromatogram of per-O-trimethylsilyl derivatives of the 31-component mixture separated on HP-5. Peaks: 1 = 1,5-anhydroarabinitol; 2 = 1,5;3,6-dianhydroglucitol + 1,4;3,6-dianhydroglucitol; 3 = 1,4-anhydroarabinitol + threitol; 4 = pentaerythritol + erythritol; 5 = 1,4;3,6-dianhydromannitol; 6 = 1,5-anhydroribitol; 7 = 1,4-anhydroribitol; 8 = 1,4-anhydroxylitol; 9 = 1,4-anhydrolyxitol; 10 = 1,5-anhydroxylitol; 11 = xylitol; 12 = arabinitol; 13 = ribitol; 14 = 2,5-anhydroglucitol + 1,4-anhydrogalactitol; 15 = 1,4-anhydroglucitol; 16 = 1,5-anhydromannitol; 17 = 1,6-anhydroiditol; 18 = 1,5-anhydrogalactitol; 19 = 1,4-anhydromannitol; 20 = 1,5-anhydroglucitol; 21 = mannitol; 22 = glucitol; 23 = allitol + galactitol + iditol; 24 = altritol; 25 = myoinositol.

1,4-anhydropentitols is in accordance with the values of their free energy differences [42];

(c) anhydroglucitols: 1,4- (peak 15), 1,5- (peak 16) and 2,5- (peak 20);

(d) anhydrogalactitols: 1,4- (peak 17) and 1,5- (peak 18);

(e) anhydromannitols: 1,4- (peak 18) and 1,5- (peak 19). Thus, among these isomeric per-O-acetyl-anhydropentitols, 1,5-anhydropentitols elute before 1,4-anhydropentitols. Further, per-O-acetyl-1,4-anhydrohexitols isomers elute before the 1,5-anhydro isomers.

The use of a fused-silica cGC column with HP-5 stationary phase for the separation of the per-O-trimethylsilylated mixture of the same polyols compounds gave only 25 peaks (Fig. 2, Table II). In this case five sets of compounds could not be separated: 1,4;3,6- and 1,5;3,6-dianhydroglucitol (peak 2), 1,4-anhydroarabinitol and threitol (peak 3), erythritol and pentaerythritol (peak 4), 2,5-anhydroglucitol and 1,4-anhydrogalactitol (peak 14) and allitol, galactitol and iditol (peak 23). Thus, only twelve peaks were separated to the base line (7–13, 18, 21, 22

TABLE II

RETENTION CHARACTERISTICS OF PER-O-TRIMETHYLSILYLATED POLYOLS ON HP-5

GC peak	Per-O-trimethylsilyl derivative of	Retention time (min)	Relative retention time[a]		Methylene units[b]
			RRT^1	RRT^2	
1	1,5-Anhydroarabinitol	11.60	0.380	0.950	15.17
2	1,5;3,6-Dianhydroglucitol + 1,4;3,6-dianhydroglucitol	11.79	0.386	0.966	15.23
3	1,4-Anhydroarabinitol + threitol	11.98	0.392	0.981	15.29
4	Pentaerythritol + erythritol	12.21	0.400	1.000	15.35
5	1,4;3,6-Dianhydromannitol	12.27	0.402	1.005	15.37
6	1,5-Anhydroribitol	12.40	0.406	1.016	15.42
7	1,4-Anhydroribitol	12.55	0.411	1.028	15.46
8	1,4-Anhydroxylitol	12.71	0.416	1.041	15.51
9	1,4-Anhydrolyxitol	14.27	0.467	1.169	16.00
10	1,5-Anhydroxylitol	14.66	0.480	1.201	16.11
11	Xylitol	19.43	0.636	1.591	17.48
12	Arabinitol	19.88	0.651	1.628	17.60
13	Ribitol	20.04	0.656	1.641	17.66
14	2,5-Anhydroglucitol + 1,4-anhydrogalactitol	21.09	0.691	1.727	17.97
15	1,4-Anhydroglucitol	21.36	0.699	1.749	18.00
16	1,5-Anhydromannitol	21.45	0.702	1.757	18.04
17	1,6-Anhydroiditol	21.76	0.713	1.782	18.13
18	1,5-Anhydrogalactitol	22.71	0.744	1.860	18.42
19	1,4-Anhydromannitol	23.91	0.783	1.958	18.78
20	1,5-Anhydroglucitol	23.99	0.786	1.965	18.81
21	Mannitol	26.73	0.875	2.189	19.75
22	Glucitol	26.92	0.881	2.205	19.82
23	Allitol + galactitol + iditol	27.07	0.886	2.217	19.88
24	Altritol	27.14	0.889	2.223	19.93
25	Myoinositol	30.54	1.000	2.501	21.29

[a] RRT^1 relative to myoinositol, RRT^2 relative to pentaerythritol.

[b] Measured relative to C_{15}–C_{22} *n*-alkanes from 115°C at 3°C/min.

and 25) and only these could be quantitatively analysed.

The elution order of per-O-trimethylsilylated compounds (Fig. 2, Table II), in particular polyol groups, is as follows:

(a) alditols: threitol (3), erythritol (4), xylitol (11), arabinitol (12), ribitol (13), mannitol (21), glucitol (22) and altritol (24);

(b) anhydropentitols: 1,5-anhydroarabinitol (1), 1,4-anhydroarabinitol (3), 1,5-anhydroribitol (6), 1,4-anhydroribitol (7), 1,4-anhydroxylitol (8), 1,4-anhydrolyxitol (9) and 1,5-anhydroxylitol (10);

(c) anhydroglucitols: 2,5- (peak 14), 1,4- (peak 15) and 1,5- (peak 20);

(d) anhydrogalactitols: 1,4- (peak 14) and 1,5- (peak 18);

(e) anhydromannitols: 1,5- (peak 16) and 1,4- (peak 19).

The identities of all peaks in both instances were determined by the standard cGC co-injection method.

The relative retention times with respect to pentaerythritol and myoinositol and the number of methylene units were determined in the usual manner for both types of derivatives and are given in Tables I and II.

On the basis of this and previous work, we can conclude that the cGC separations of the per-O-acetyl derivatives of polyols give better results than for their O-trimethylsilyl derivatives. Hence these derivatives can be used for the determination of these compounds in biological fluids [50].

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Improvements in the separation of polychlorinated biphenyl congeners by high-resolution gas chromatography

Application to the analysis of two mineral oils and powdered milk ☆

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ABSTRACT

A comparative study of the separation of seven polychlorinated biphenyl (PCB) congeners (IUPAC Nos. 28, 52, 101, 118, 138, 153 and 180) and other important PCBs (31, 44, 61, 105, 128, 149, 156, 163, 170 and 186), selected on the basis of their toxicity and levels found in environmental and food samples, was performed using DB-5 and CP-Sil 8 CB (5% phenyl–95% methyl polysiloxane), DB-1701 (14% cyanopropylphenyl–86% methyl polysiloxane) and DB-17 (50% phenyl–50% methyl polysiloxane) fused-silica capillary columns in gas chromatography with electron-capture detection. The combination of DB-5 or CP-Sil 8 CB and DB-17 capillary columns allowed the separation and determination of PCB congeners 118–149, 138–163 and 153–105–132. Some of these congeners were determined in candidate reference materials, two waste mineral oils of different concentration levels and a milk powder, as part of an intercalibration exercise. The recoveries obtained for thirteen selected PCBs were >90% in the three different samples. The concentrations of PCB congeners determined in the samples ranged between 0.086 and 2.054 mg kg^{-1}, 0.758 and 66.720 mg kg^{-1} and 0.208 and 14.00 μg kg^{-1} for oil of low level, oil of high level and milk powder, respectively. The methods were validated by participation in several round-robin exercises and the results obtained were in good agreement (R.S.D. = 20–40%) for all participating laboratories (between 11 and 19).

INTRODUCTION

Residue levels of polychlorinated biphenyls (PCBs) have been found in aquatic biota, both continental and marine, as a result of their widespread distribution, environmental persistence and high lipophilicity [1–3]. Among the 209 possible PCB congeners, only around 150 have been reported in the total environment. Seven of them (IUPAC Nos. 28, 52, 101, 118, 138, 153 and 180) were selected as the most relevant because of their distribution throughout chromatograms, coverage of the chlorination range,

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use in technical mixtures, relative ease of analytical determination and proved toxicity. Consequently, they are commonly mentioned in environmental and food regulations [4].

Following the initial selection of these seven PCBs, considerable efforts have been made in the past to detect potential interferences and/or co-elutions of these congeners by capillary gas chromatography (GC). Initially a problem was encountered in the determination of PCB 138 [5] that later was attributed to the presence of PCB 163 [6]. The complete separation of the two congeners was possible only by using a polar SP-2330 (biscyanopropylphenyl polysiloxane) capillary GC column [6]. The co-elution problems between these two congeners, PCB 138 and 163, were found during a certification exercise in which most of the laboratories used DB-5-type columns and the congeners were unresolved. Hence it was impossible to certify the PCB 138 congener, and consequently, certification was indicated as the total amount of PCB 138 + 163 [4]. Recently, the separation of the two congeners was achieved using a polar FFAP (polyethylene glycol terephthalic acid ester) column [7].

When using the most common stationary phases, which usually contain 95% dimethyl–5% phenyl polysiloxane (SE-54, CP-Sil 8, DB-5), other co-elution problems have also been reported, *e.g.*, for PCBs 101–84–90, 123–149, 171–156–202 and 153–105 [8,9]. Such a co-elution problem can be solved either using multidimensional GC, with a non-polar column in combination with a more polar column by using the heart-cutting technique [8], a dual-column system (DB-5 and DB-1701) operated in parallel with a glass T-split [10] or using other columns of different polarity, *e.g.*, HP-5, CP-Sil 19 and CP-Sil 88. It is clear that many co-elution problems with PCBs have not been solved and it is necessary to find an appropriate column(s) that will solve specific problems [7,11].

Therefore, to cope with the requirements of routine measurements of PCBs in food and environmental samples there is a need for the development of analytical methods for the confirmation of the different groups of PCBs, thus avoiding false-positive determination. Most of the PCBs can be easily separated using DB-5 or CP-Sil 8 CB apolar capillary columns. However, the complete separation of the whole group of PCBs is not possible even using these stationary phases in a 30- or 50-m column.

The purpose of this work was to carry out a comparative study of the separation of the seven common PCB congeners (28, 52, 101, 118, 138, 153 and 180) and a few other PCBs (31, 44, 61, 105, 128, 149, 156, 163, 170 and 187) selected on the basis of their proved toxicity and/or levels found in environmental and food samples [12], using DB-5 (60-m), CP-Sil 8 CB (50-m), DB-1701 (60-m) and DB-17 (30-m) capillary columns. These four columns were applied to the determination of some of the above PCB congeners in samples of two waste mineral oils and a milk powder, as a part of an intercalibration exercise of the BCR (Community Bureau of Reference) of the Commission of the European Communities (CEC). The results of the two laboratories were in good agreement in a round-robin exercise with the most probable values from different laboratories (total 22) with a relative standard deviation (R.S.D.) between 20 and 40%. By using the columns reported in this paper, an accurate assessment of the environmental levels of the different PCB congeners can be made, thus improving the quality assurance in the final analytical determination.

EXPERIMENTAL

Chemicals

The solvents *n*-hexane, acetone and dichloromethane of residue analysis grade were supplied by Merck (Darmstadt, Germany). The purity of the solvents was determined by concentration of a 150-ml volume to 0.5 ml and analysis by high-resolution GC with electron-capture detection (ECD). Isooctane of pesticide grade was obtained from Carlo Erba (Milan, Italy). Florisil (600–100 mesh), sulphuric acid (95%, G.R. grade) and anhydrous sodium sulphate for residue analysis were purchased form Merck. Florisil cartridges with 1 g of adsorbent were obtained from Analytichem (Varian, Palo Alto, CA, USA; purchased through Scharlau, Barcelona, Spain). Individual analytical-reagent grade PCBs were supplied from Promochem (Wesel, Germany) and 2,4-dichlorobenzylhexyl

ether (DCBE-C_6) and 2,4-dichlorobenzylhexadecyl ether (DCBE-C_{16}) (Dr. D. Wells, Aberdeen, U.K.) and 1,2,3,4-tetrachloronaphthalene (TCN) were used as internal standards. All glass materials were cleaned with AP-13 Extran alkaline soap (Merck) for 24 h, dried overnight at 180°C and rinsed with high-purity solvents immediately prior to use.

Sample treatment

Five replicates of 0.5 g of waste mineral oil of high and low PCB concentration levels were accurately weighed into a 25-ml volumetric flask and diluted to volume with *n*-hexane. An aliquot of diluted sample was removed for clean-up and analysis.

For the milk powder, five replicates of 5 g were placed in a mortar and then 15 ml of water, 12 g of Florisil (activated at 675°C for 2 h) and 130 g of anhydrous sodium sulphate were added. The mixture was mixed to yield a dry powder. This mixture was transfered into an extraction column (2 cm I.D.) previously filled with a small plug of glass-wool and eluted with 100 ml of *n*-hexane–acetone (2:1, v/v). The extract was concentrated to *ca.* 3 ml in a rotary evaporator.

Clean-up procedure

Laboratory 1. The oil extract was cleaned up by extensive shaking with 3 × 3 ml of sulphuric acid for about 3 min and, when a good phase separation had been obtained, the upper organic layer was removed and the extract was transfered into a Florisil cartridge that had previously been cleaned and activated with 20 ml of *n*-hexane. The PCB congeners were eluted with 10 ml of *n*-hexane. The organic fraction was evaporated and the final volume was adjusted to 0.5 ml, then being ready for injection into the GC–ECD system.

Laboratory 2. The clean-up column (1 cm I.D.) was prepared using 8 g of Florisil activated at 675°C for at least 2h, and stored at 150°C before use. At the two ends of the column an anhydrous sodium sulphate layer *ca.* 2 cm high was applied. Subsequently, the column was eluted with 50 ml of *n*-hexane to clean the absorbent. The oil extract (1 ml) was transfered quantitatively to a Florisil column and was eluted with 50 ml of *n*-hexane at a flow-rate of 1 ml min^{-1}.

A 1-ml volume of the hexane–acetone extract obtained for the milk sample as described previously was mixed with 5 ml of 95% sulphuric acid and after 3 h the organic phase was removed and quantitatively transferred to a Florisil column. The clean-up procedure for the milk was the same as that used for the waste mineral oil extracts. The overall procedures for the different samples are outlined in Fig. 1.

Chromatographic conditions

Purified extracts were analysed on a Hewlett-Packard (Palo Alto, CA, USA) Model 5890 capillary gas chromatograph with an HP 7673A autosampler and equipped with a ^{63}Ni electron-capture detector (laboratory 1) and on a Carlo Erba (Milan, Italy) 5300 Mega Series chromatograph equipped with a ^{63}Ni electron-capture detector (laboratory 2). Fused-silica capillary

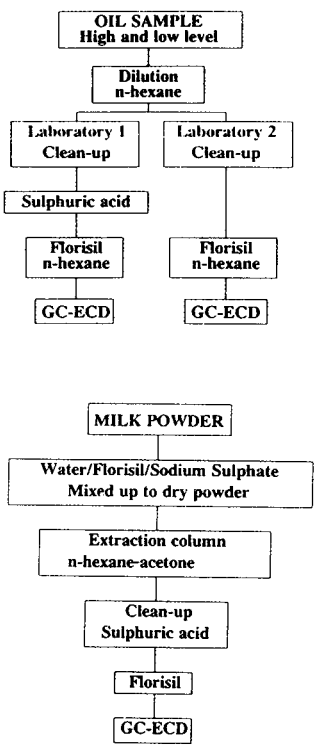

Fig. 1. Summary of procedures used for the separation and determination of PCBs in high- and low-level mineral oils and powdered milk. Solvent ratio in extraction column: *n*-hexane–acetone (2:1, v/v).

columns of DB-5 (J&W Scientific, Folsom, CA, USA) and CP-Sil 8 CB (Chrompack, Middelburg, Netherlands), both containing 5% phenyl–95% methyl polysiloxane, DB-1701 (J&W Scientific), containing 14% cyanopropylphenyl–86% methyl polysiloxane, and DB-17 (J&W Scientific), containing 50% phenyl–50% methyl polysiloxane, were used. The characteristics of the columns and the chromatographic conditions are given in Table I. The chromatographic data were recorded on an HP 59970C GC workstation data system and a Merck–Hitachi (Darmstadt, Germany) Model D-2000 integrator in laboratories 1 and 2, respectively.

The linearity of the ECD response for each congener was determined by plotting calibration graphs of peak height/mass injected *versus* mass injected. The linear range for the PCB congeners was between 10 and 300 $\mu g\ l^{-1}$. The reproducibility of the peak heights calculated from ten replicate analyses of a standard mixture of the PCB congeners was between 4.1 and 6.7%. Quantitative analysis was carried out using external calibration with an internal standard. Standard solutions containing 20–300 pg μl^{-1} in isooctane of the mixed PCB congeners and the internal standard were prepared and injected with the automatic sampler into the GC–ECD system.

Recovery

The recoveries of the PCBs were calculated by spiking the samples by careful mixing of standard solutions. Different amounts of each congener were used in order to obtain concentrations

TABLE I

EXPERIMENTAL CHROMATOGRAPHIC CONDITIONS

Parameter	Column					
	DB-5[a]		CPSil-8[c]	DB-1701[a]	DB-17[c]	DB-17[a]
	A[b]	B[b]				
Injector splitless	Yes	Yes	Yes	Yes	Yes	Yes
Splitter time (min)	50	60	35	60	35	60
Injection volume (μl)	1	1	2	1	2	1
Injector temperature (°C)	270	270	270	270	270	270
Carrier gas	He	He	He	He	He	He
Linear carrier gas velocity (cm^{-1} s)	25	28	26	26	25	27
Make-up gas	N_2	N_2	N_2	N_2	N_2	N_2
Make-up gas flow-rate (ml min^{-1})	35	31	60	32	58	32
Column length (m)	60	60	50	60	30	30
Column I.D. (mm)	0.25	0.25	0.25	0.25	0.25	0.25
Film thickness (μm)	0.25	0.25	0.25	0.25	0.25	0.25
Initial oven temperature (°C)	90	90	80	90	80	90
Initial isothermal period (min)	3	3	2	3	2	2
Initial programming rate (°C min^{-1})	20	2.5	6	3	6	20
Second isothermal temperature (°C)	150	–	–	–	–	150
Second isothermal period (min)	1	–	–	–	–	1
Second programming rate (°C min^{-1})	3	–	–	–	–	3
Final isothermal temperature (°C)	280	290	280	280	290	280
Final isothermal period (min)	20	30	15	30	10	30
Detection	ECD	ECD	ECD	ECD	ECD	ECD
Detector temperature (°C)	310	330	310	330	310	330

[a] Laboratory 2.

[b] (A) Conditions for low-level oil; (B) conditions for high-level oil.

[c] Laboratory 1.

TABLE II

RECOVERIES OF PCB CONGENERS IN MINERAL OIL AND MILK POWDER SAMPLES

PCB	Recovery (%)					
	Low-level oil		High-level oil		Milk powder	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
28	80.1	6.5	91.6	7.2	98.2	6.2
52	81.4	6.9	96.7	8.1	95.7	5.1
101	90.0	8.1	103.0	9.4	98.6	5.2
105	–	–	90.8	7.6	97.3	5.7
118	97.6	7.3	99.3	4.8	99.2	4.2
128	–	–	97.9	4.9	93.7	6.9
138	92.3	5.7	100.2	5.3	96.4	6.3
149	–	–	92.5	5.6	97.2	5.1
153	87.7	6.5	94.6	4.2	98.7	4.0
156	–	–	83.8	7.5	92.1	5.8
163	–	–	85.7	5.6	98.7	6.4
180	105.1	5.1	97.2	4.6	97.9	6.3
170	–	–	93.4	6.7	95.4	5.9

around 50, 100, 150 and 200% ($n = 3$) of the actual concentration in the real samples.

The efficiency was evaluated by studying the recovery of PCBs from samples of spiked oils and milk. The analytical recoveries observed for the thirteen congeners from the two oil samples and milk powder were calculated as described under Experimental and were >90% for all the compounds studied in the three different samples (see Table II).

RESULTS AND DISCUSSION

Chromatographic separation

Data in the literature shown that not all the PCBs can be separated in the columns usually used. The pairs 28–31, 118–149, 138–163 and 153–105 showed inadequate separation with a DB-5-type column and hence there is a need to use other GC columns. The columns studied in this work were DB-5, DB-1701, DB-17 and CP-Sil 8 CB. The retentions time of the PCB congeners 28, 31, 44, 52, 61, 101, 105, 118, 128, 138, 149, 153, 156, 163, 170, 180 and 186, relative to PCB 153, obtained using these columns are given in Table III. These PCB congeners are recommended by the BCR to be determined in milk powder and mineral oil, and are representative of the level of chlorination. Chromatograms of the mineral oils and milk powder using the DB-5 column are shown in Fig. 2 and a chromatogram of high-level mineral oil on the DB-17 column is shown in Fig. 3. It should be noted that all the studies reported in this paper on the separation of the different PCB congeners were performed with real samples of oils and milk.

Chlorobiphenyls 31 and 28. This pair of congeners can be separated using columns of CP-Sil 8 CB or DB-5 of sufficient length, 50 m or more, as can be seen in Table III. Adequate resolution, with a 75% of separation, between the two congeners can be obtained using helium as carrier gas. This separation of PCBs 28 and 31 is better than that using more polar stationary phases such as DB-1701 and DB-17. These results are in agreement with the literature data, showing that it is possible to separate these congeners on a routine basis by using DB-5-type columns of 50 m × 0.22 mm I.D. and a film thickness of 0.2 μm or more [13]. Narrow-bore columns of 0.15 mm I.D. give a much improved

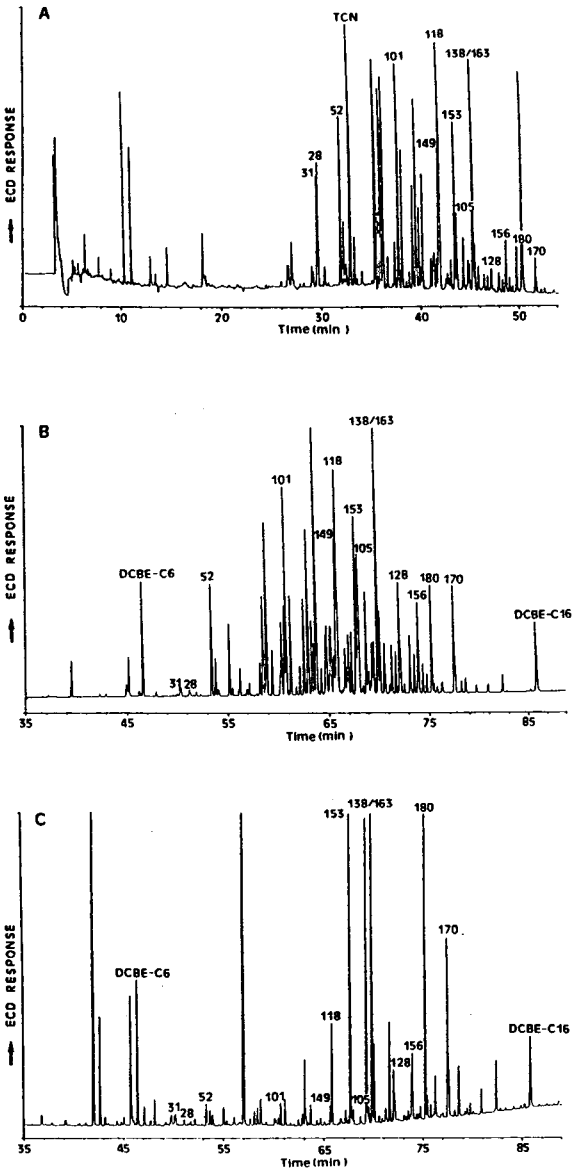

Fig. 2. GC–ECD using a DB-5 column (60 m × 0.25 mm I.D., 0.25 μm film thickness): (A) low-level waste mineral oil (TCN as internal standard); (B) high-level waste mineral oil; (C) powdered milk (DCBE-C_6 and DCBE-C_{16} as internal standards). The chromatographic conditions are given in Table I.

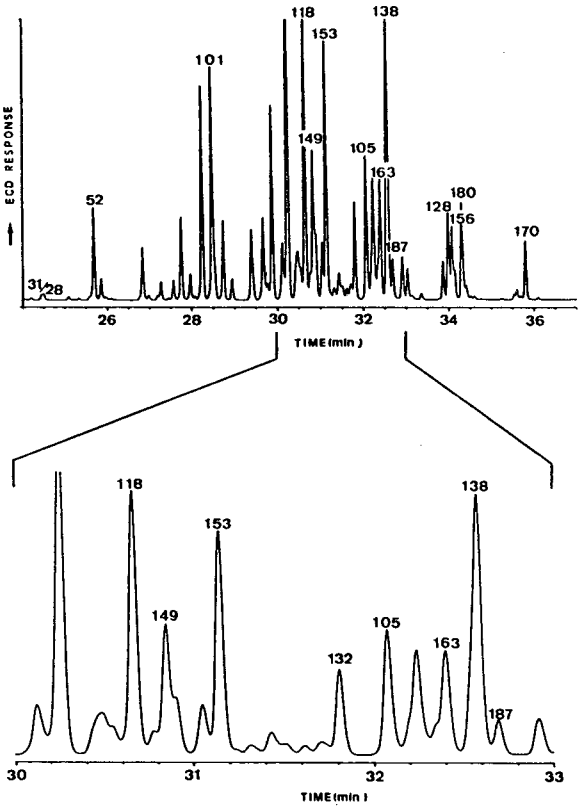

Fig. 3. GC–ECD using a DB-17 column (30 m × 0.25 mm I.D., 0.25 μm film thickness) for extract of high-level waste mineral oil. The chromatographic conditions are given in Table I.

separation [14] so these congeners can then be resolved even with a 25-m column, but the increased pressure required to maintain the optimum linear velocity requires close attention to the possibility of leaks at the front end of the column.

Chlorobiphenyls 118 and 149. It is difficult to force a baseline separation of the pair of PCBs 118 and 149 on a DB-5-type column, as can be seen in Fig. 2, but their separation is excellent on the two polar columns, DB-1701 and DB-17 (see Fig. 3), and the use of polar columns allows the baseline separation of this two congeners. Other workers have obtained similar separations using other polar stationary phases, such as CP-Sil 19 CB (7% phenyl–85% methyl–7% cyanopropyl–1% vinyl polysiloxane) [8,15]. The separation of the other problematic pairs of congeners with this last column is similar to that with the DB-1701 and poorer than that with the DB-17 column.

Chlorobiphenyls 138 and 163. This pair of

TABLE III

RETENTION TIMES AND RELATIVE RETENTION TIMES OF PCB CONGENERS

PCB	Column							
	CPSil-8		DB-5[a]		DB-1701		DB-17[b]	
	t_R (min)	RRT[c]	t_R (min)	RRT[c]	t_R (min)	RRT[c]	t_R (min)	RRT[c]
31	29.84	0.801	49.93	0.741	51.26	0.778	25.98	0.663
28	29.91	0.802	50.04	0.743	51.31	0.779	25.98	0.663
52	31.15	0.836	53.08	0.788	53.88	0.818	28.21	0.720
44	31.94	0.857	54.94	0.815	56.15	0.853	30.52	0.779
61	33.31	0.894	57.86	0.859	58.75	0.892	32.82	0.838
101	34.30	0.920	60.44	0.897	60.01	0.911	33.74	0.861
149	36.46	0.925	65.40	0.971	64.37	0.977	38.46	0.982
118	35.52	0.980	65.53	0.958	64.86	0.985	38.10	0.973
153	37.27	1.000	67.37	1.000	65.86	1.000	39.17	1.000
105	37.48	1.006	67.65	1.004	67.26	1.021	41.02	1.047
138	38.30	1.028	69.56	1.033	68.34	1.038	42.09	1.075
163	38.30	1.028	69.56	1.033	68.34	1.038	41.73	1.065
187	38.91	1.044	70.98	1.054	69.16	1.050	44.87	1.146
128	39.36	1.056	71.70	1.064	71.06	1.079	45.01	1.149
156	40.26	1.080	73.57	1.092	72.78	1.105	45.17	1.153
180	40.89	1.097	74.89	1.112	73.50	1.116	45.68	1.166
170	42.20	1.132	77.16	1.145	76.93	1.168	48.77	1.245

[a] Obtained with DB-5 (B) (laboratory 2) conditions (see Table I).
[b] Obtained with DB-17 (laboratory 1) conditions (see Table I).
[c] RRT = Retention time relative to PCB 153.

congeners is well separated using the most polar stationary phase studied, DB-17, and have similar retention times on CP-Sil 8 CB, DB-5 and DB-1701 phases. Most earlier workers considered that the PCB 163 was unlikely to exist to any extent in technical mixtures [16], but Larsen and Reigo [6] synthesized this compound and confirmed its presence in a large number of environmental matrices. The separation of PCBs 163 and 138 is important because they have relatively high toxicity. Until now, this separation was only possible with the very polar SP-2330 (biscyanopropylphenyl polysiloxane) [6], which shows interferences for the congeners 101, 153 and 180, or with the FFAP (polyethylene glycol terephthalic acid ester) that gives very long retention times for the PCBs 169, 170 and 206 [7]. One drawback with the DB-17 phase is the loss of resolution between the congeners 128 and 156, but these two compounds are not among the seven legislated for in EEC countries [4,17,18]. In addition, the DB-17 column is only 25 m long and the analysis time is shorter than with the FFAP column (see Fig. 3).

Chlorobiphenyls 153 and 105. It is possible to separate PCB 153 from 105 on a CP-Sil 8 CB column (see Table III), but it is more difficult to resolve PCB 132 from 105, which elutes immediately after the PCB 153. When using CP-Sil 19 and CP-Sil 88 columns a good separation between PCB congeners 153, 105 and 132 was obtained [11], although other problems occurred, *e.g.*, 105 now co-eluted with 141 and 176, and 132 with 179 (CP-Sil 19), and 105 co-eluted with 129 (CB-Sil 88). The separation of these congeners by multi-dimensional GC using a smectic phase as second column has been described [15], but this requires the use of more expensive equipment. A much simpler approach would be to use a 25-m DB-17 column, which

TABLE IV

RESULTS FOR PCB CONGENERS IN LOW-LEVEL WASTE MINERAL OIL ($n = 5$)

PCB	Concentration (mg kg^{-1})						
	Laboratory 1		Laboratory 2		Interlaboratory		
	Mean	S.D.	Mean	S.D.	No. of results	Mean	S.D.
28	0.615	0.075	N.D.[a]	N.D.	19	0.601	0.175
52	1.441	0.096	1.308	0.209	18	1.202	0.204
101	2.054	0.186	1.722	0.134	19	1.739	0.363
118	1.578	0.215	1.644	0.083	18	1.908	0.423
138 + 163	1.140	0.127	1.668	0.061	19	1.406	0.208
153	0.623	0.062	0.738	0.072	19	0.887	0.144
180	0.094	0.010	0.086	0.006	19	0.179	0.046

[a] ND = Not determined.

separate the PCBs congeners 153, 105 and 132 (see Fig. 3).

Validation of results

In order to validate the performance of the analytical methods used, we have participated in three interlaboratory round-robin exercises organized by the BCR. Tables IV, V and VI show the results obtained for low- and high-level waste mineral oils and milk powder, respectively. Our results are compared with the mean values obtained in several European laboratories. From the results for the low-level mineral oil, several comments can be made. First, PCBs 138 and 163 were quantified together because most of the laboratories involved in the round-robin test

TABLE V

RESULTS FOR PCB CONGENERS IN HIGH-LEVEL WASTE MINERAL OIL ($n = 5$)

PCB	Concentration (mg kg^{-1})						
	Laboratory 1		Laboratory 2		Interlaboratory		
	Mean	S.D.	Mean	S.D.	No. of results	Mean	S.D.
28	1.760[a]	0.055	0.758	0.026	13	0.769	0.116
52	32.640	2.535	29.076	1.593	21	31.523	3.936
101	66.720	3.255	56.526	2.612	22	60.662	7.358
105	16.160	0.241	17.254	0.745	18	18.581	2.699
118	42.660	0.483	39.918	3.877	21	47.756	6.630
128	16.420	0.973	12.178	0.666	19	11.998	2.288
138	58.240	0.695	42.526	5.049	22	53.226	7.938
149	22.280	1.217	30.986	0.934	14	34.308	3.090
153	38.260	2.390	32.738	4.451	21	39.547	4.911
156	4.540	0.385	9.137	0.320	16	7.033	0.825
163	12.30	0.500	10.508	0.303	11	11.505	4.706
180	10.020	0.882	11.256	0.419	20	10.525	0.831
170	7.320	0.606	11.326	0.466	19	6.970	1.462

[a] Sum of congeners 28 and 31.

TABLE VI

RESULTS FOR PCB CONGENERS IN DRIED MILK POWDER ($n = 5$)

PCB	Concentration ($\mu g\ kg^{-1}$)				
	Laboratory 2		Interlaboratory		
	Mean	S.D.	No. of results	Mean	S.D.
28	0.208	0.015	12	0.442	0.204
52	0.722	0.074	15	1.089	0.349
101	0.431	0.049	11	0.599	0.300
105	0.289	0.017	15	0.428	0.193
118	2.70	0.23	15	3.29	0.58
128	1.68	0.25	15	1.42	0.23
138	14.00	1.55	15	15.25	1.89
149	0.401	0.057	13	0.468	0.169
153	17.01	1.53	15	18.95	1.75
156	1.19	0.15	17	1.67	0.57
163	1.90	0.09	8	2.36	1.29
180	9.00	0.88	17	11.09	2.34
170	4.20	0.40	17	5.76	1.76

were unable to separate this mixture because they used DB-5 columns. The second point is that most of our results agreed with the mean values obtained in the different laboratories, with the exception of PCBs 153 and 180. In both of our laboratories these values were much lower than the mean values. We attribute this difference to the use of relatively "older" standard solutions, so a slight evaporation of this solution might have taken place, thus concentrating to some extent the more volatile PCBs. This would lead to lower values for PCBs 153 and 180 in comparison with other PCBs when using the external standard method. Another effect to consider was the recoveries of these congeners achieved using these standard solutions, which will affect the attainment of correct final values.

The problem of the determination of these two congeners was overcome when analysing the high-level waste mineral oil (see Table V). Here fresh solutions were prepared for the external standard determination (laboratory 1) and at the same time an internal standard (laboratory 2) was used for determination. A problem was noticed in the separation of PCBs 28 and 31 in laboratory 1, and this was attributed to the ageing of the GC column used. In Table V the problems encountered with the complete separation of PCBs 138 and 163 can still be noticed, as only eleven laboratories were able to give an acceptable mean value for such congeners. For analysis of milk powder only laboratory 2 was involved, as laboratory 1 only participated with the environmental samples. The first point to consider is the problem of determining PCB 163 in the different laboratories, for the same reasons as mentioned above. The second point to consider in the analysis of milk powder is the higher standard deviation obtained for the mean interlaboratory values compared with waste mineral oil. This could be attributed to the difficulties in performing the determination of PCB congeners in milk powder, which was also observed in the method of calculating recoveries. The extraction of fat from milk was a critical step in the analytical procedure because the PCB congeners are absorbed in the globules of fat and their reproducible extraction was time consuming and laborious.

CONCLUSIONS

The use of GC columns of different polarity was tested for the complete separation of thirteen PCB congeners found in waste mineral oils of low and high concentration levels and in milk

powder. The separation of congeners 118–149, 138–163 and 105–153 was achieved using a DB-17 capillary column much more effectively than using the conventional DB-5-type columns.

The performance of the different analytical methodologies was validated by participation in three interlaboratory exercises, showing that the different extraction and clean-up methods and also the chromatographic separation were adequate for such analyses. The proposed methodology can be easily implemented in routine determinations of PCBs in the two matrices described.

ACKNOWLEDGEMENT

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Clean-up and confirmation procedures for gas chromatographic determination of pesticide residues in contaminated waters. Part I☆

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ABSTRACT

The effects of sulphuric acid, potassium hydroxide and chromic acid on eleven organochlorine and ten organophosphorus pesticides were investigated. The treatments destroy some pesticides totally or partially, leave others unaltered and have a clean-up effect. These reactions can be used to confirm the presence of an identified pesticide. The clean-up extracts in the environmental samples facilitate the identification of the organochlorine and organophosphorus pesticides and their quantitative analysis. The results obtained for contaminated surface waters show the usefulness of these methods for multi-residue capillary gas chromatography without the need for other additional separative chromatographic steps to avoid interferences or the use of a highly sensitive mass spectrometer for confirmation.

INTRODUCTION

The correct identification of a particular pesticide residue in environmental samples is of major importance. The determination of pesticides in complex matrices presents difficulties because the biocides themselves, their metabolites, some contaminants and artifacts of synthesis often have similar physico-chemical properties and identical retention times when gas chromatography (GC) is used [1–4]. Therefore, false-positive results can be obtained. Several methods, such as chemical clean-up, sweep co-distillation and gel permeation, liquid–liquid partition and adsorption chromatography have been employed to remove co-extractants and minimize these difficulties in environmental contaminant analysis [5–10].

The efficacy of sulphuric acid clean-up has been studied and applied to organochlorine residue analyses of water samples [11], food commodities [12] and other samples [13–15]. Alkaline treatment of sample extracts provides the clean-up of co-extracted materials and dehydrochlorination to the corresponding alkenes of various organochlorine pesticides [16,17]. The same effect is achieved with a precolumn of sodium or potassium hydroxide on-line with the GC system [18–20]. Chromic acid has been used to remove interferences [21] and to distinguish polychlorinated biphenyls from DDT and its analogues [22–24].

The need for a rapid and inexpensive clean-up technique for routine multi-residue pesticide analysis of contaminated waters has led to the use of chemical treatments (concentrated sulphuric acid, ethanolic potassium hydroxide and chromium trioxide) of samples for organochlorine pesticide residue determination followed by GC with electron-capture detection (ECD) [25–27]. However, there are no refer-

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ences to their utilization in organophosphorus pesticide residue determination, with the exception of a recent report by Wan [28] in which the efficacy of sulphuric acid clean-up for the determination of four organophosphorus insecticides, such as malathion, fenitrothion, dimethoate and quinalphos, was evaluated in tea.

The purpose of this work was to determine the effect of different chemical treatments on organochlorine and organophosphorus pesticides in order to be able to use them in routine purification and confirmation in multi-residue analysis when ECD and alkaline flame ionization detection [nitrogen–phosphorus detection (NPD)] in capillary GC are simultaneously used. The clean-up procedures including treatments with sulphuric acid, potassium hydroxide and chromic acid were tested on extracts of surface water samples obtained by solid-phase extraction using octadecylsilica.

EXPERIMENTAL

Reagents

The organochlorine pesticides used were *o,p'*-DDE, *o,p'*-DDT, dieldrin, α-endosulfan, β-endosulfan, endosulfan sulphate, α-HCH, β-HCH, δ-HCH, isodrin and mirex and the organophosphorus pesticides used were disulfoton, ethion, fonofos, heptenophos, malathion, parathion-ethyl, parathion-methyl, phenthoate, sumithion and trithion. All of them, with purities between 95 and 99%, were purchased from Promochem (Wesel, Germany). Stock solutions were prepared in ethyl acetate and preserved at 4°C.

Ethyl acetate, *n*-hexane, ethanol and methanol (J.T. Baker, Phillipsburg, NJ, USA) were glass-distilled and free from interfering residues (as tested by GC following concentration by a factor of 100). Preparative octadecylsilica, 55–105 μm, was obtained from Waters–Millipore (Milford, MA, USA). Sulphuric acid, sp. gr. 1.84, and potassium hydroxide solution (2*M* in ethanol) were purchased from Merck (Darmstadt, Germany). To prepare chromic acid solution, 5 g of chromium(VI) oxide (Merck) were dissolved in 3 ml of water and 60 ml of glacial acetic acid (Merck) were added.

Apparatus

GC was performed with a Konik 2000-C gas chromatograph (Sant Cugat de Vallés, Barcelona, Spain) equipped with a splitless injector, a ^{63}Ni high-temperature electron-capture detector, an alkaline flame ionization detector and a Spectra-Physics SP 4290 integrator. Two fused-silica capillary columns were required; the working column was a BP-5 0.25-μm bonded-phase (5% phenyl–methylsiloxane) column (25 m × 0.22 mm I.D.), provided by Scientific Glass Engineering (Ringwood, Australia), and the second column, used for confirmatory purposes, was a DB-17 0.25-μm bonded-phase (50% phenyl–methylsiloxane) column (30 m × 0.24 mm I.D.), provided by J & W Scientific (Folsom, CA, USA). Helium was used as the carrier gas at a flow-rate of 2.8 ml min^{-1}. The injector temperature was set at 285°C and both detector temperatures (ECD and NPD) were set at 300°C. Splitless injection was performed with the column oven at 50°C. The column temperature was maintained at 50°C for 0.8 min, increased at 30°C min^{-1} to 140°C, which was maintained for 2 min, then at 5°C min^{-1} to 280°C, the final temperature being held for 12 min.

Extraction procedure

Water (1 l) was poured into a separating funnel connected by means of a microcolumn (100 mm × 9 mm I.D.) with a No. 3 (coarse) frit containing 0.5 g of octadecylsilica.

The octadecylsilica was conditioned before use by adding methanol (10 ml) followed by distilled water (10 ml). The water samples were forced through the solid phase with aid of a vacuum in order to obtain a flow-rate of about 30–40 ml min^{-1}. The material adsorbed was eluted with 5 ml of ethyl acetate and 5 ml of *n*-hexane. The organic layer was concentrated to 0.6 ml using a gentle stream of nitrogen. Samples of 3 μl were injected into the gas chromatograph.

Differences between the retention times of compounds on the non-polar BP-5 capillary column and semipolar DB-17 capillary column enable the identities of the studied pesticides to be confirmed and the products of pesticide reactions after chemical treatments to be discriminated.

Aliquots of 0.2 ml of this final hexane solution were used to identify the peaks with sulphuric acid, potassium hydroxide and chromic acid, according to the treatment procedures.

Acid treatment procedure

An aliquot of 0.2 ml of organic extract was introduced into a 5-ml centrifuge tube and 0.2 ml of concentrated sulphuric acid was added. The tube was stoppered and shaken vigorously for 5 min, 2 ml of distilled water were added and the mixture was allowed to stand until the two layers had separated. The organic layer was decanted with a Pasteur pipette and then diluted to 0.2 ml with *n*-hexane.

Alkali treatment procedure

An aliquot of 0.2 ml of organic extract was mixed with 0.2 ml of potassium hydroxide solution. The mixture was shaken vigorously for 2 min in an automatic vibrator and left to stand for 15 min at room temperature. Distilled water (3 ml) was added and the two layers were separated. The organic layer was decanted with a Pasteur pipette and diluted to 0.2 ml with *n*-hexane.

Oxidative treatment procedure

An aliquot of 0.2 ml of organic extract was evaporated to dryness in a tube, 5 ml of chromic acid solution was added to the residue and the mixture was placed in a water-bath at 75–80°C for 15 min. It was then removed and cooled. A 20-ml volume of distilled water and 20 ml of *n*-hexane were added and the mixture was shaken for 15 s. The *n*-hexane layer was retained and washed with water until the organic layer became colourless. The *n*-hexane portion was then decanted and concentrated to 0.2 ml.

In order to obtain the recoveries of the three chemical treatments (acid, alkali and oxidative), the chromatograms of treated samples were compared with those of the pesticides not subjected to the different treatments.

RESULTS AND DISCUSSION

In previous studies [29–32], the efficiency of octadecylsilica solid-phase extraction for these and other pesticides from tap, surface and sea waters was studied.

Figs. 1 and 2 show the chromatograms of standard mixtures of organochlorine and organophosphorus pesticides before and after chemical treatment, respectively. The results obtained with chemical treatments in terms of

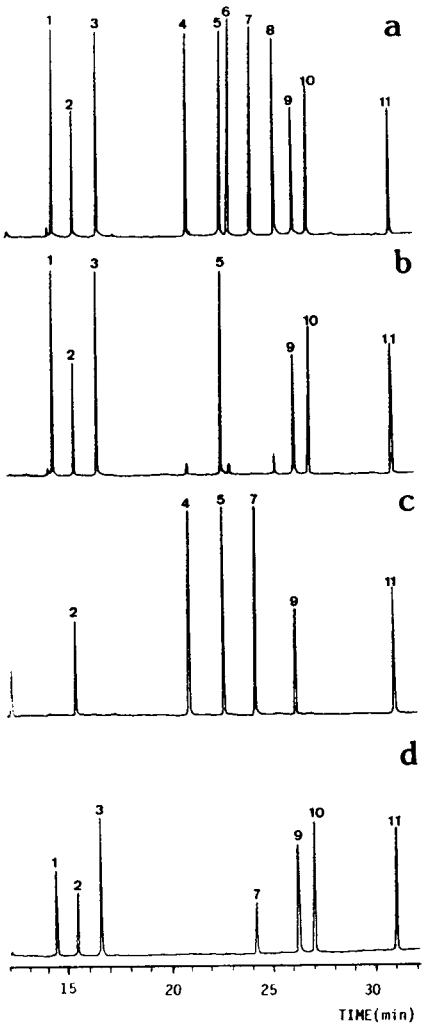

Fig. 1. Chromatogram of working organochlorine pesticide solution obtained with the BP-5 capillary column and ECD: (a) without any treatment; (b) after acid treatment; (c) after alkali treatment; (d) after oxidative treatment. 1 = α-HCH; 2 = β-HCH; 3 = δ-HCH; 4 = isodrin; 5 = *o,p'*-DDE; 6 = α-endosulfan; 7 = dieldrin; 8 = β-endosulfan; 9 = *o,p'*-DDT; 10 = endosulfan sulphate; 11 = mirex. For GC conditions, see Experimental.

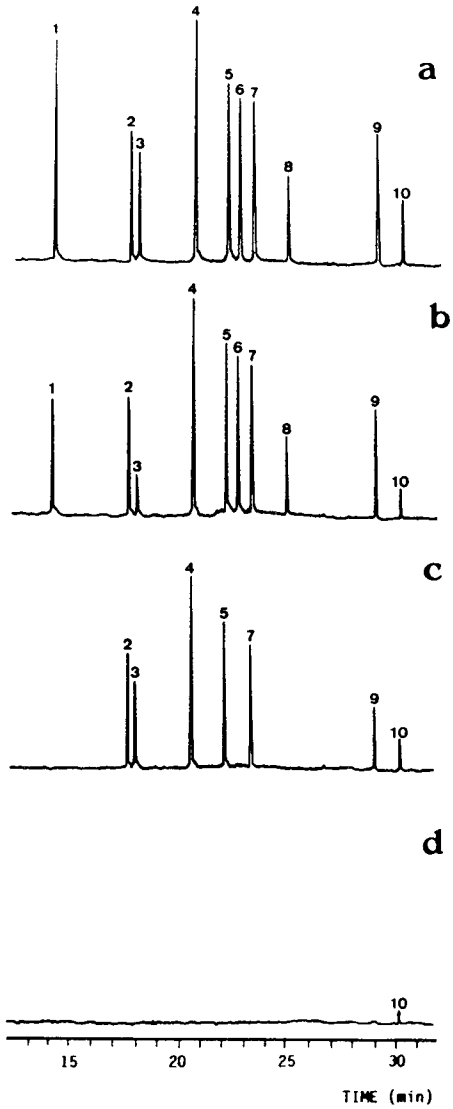

Fig. 2. Chromatogram of working organochlorine pesticide solution obtained with the BP-5 capillary column and NPD: (a) without any treatment; (b) after acid treatment; (c) after alkali treatment; (d) after oxidative treatment. 1 = Heptenophos; 2 = fonofos; 3 = disulfoton; 4 = parathion-methyl; 5 = malathion; 6 = sumithion; 7 = parathion-ethyl; 8 = phenthoate; 9 = ethion; 10 = trithion. For GC conditions, see Experimental.

either the decrease or disappearance of the chromatographic signal of the pesticides or the appearance of reaction products help to confirm and quantify the pesticides.

After the chemical treatments, the results obtained by GC using ECD for organochlorine and NPD for the organophosphorus pesticides are given in Table I. They are particularly valuable for monitoring residues in environmental waters. Table I shows the average recoveries and relative standard deviations (R.S.D.s) (n = 6) obtained when the standard aqueous solutions of organochlorine and organophosphorus pesticides were subjected to the previously described treatment procedures with sulphuric acid, potassium hydroxide and chromic acid.

According to Table I, the degradation of the studied pesticides after sulphuric acid treatment can be separated into three categories:

(I) no reaction occurred (less than 25% destroyed): o,p'-DDE, o,p'-DDT, endosulfan sulphate, α-HCH, β-HCH, δ-HCH, mirex, ethion, fonofos, malathion, parathion-ethyl, parathion-methyl, phenthoate, sumithion and trithion;

(II) partial reaction (25–75% destroyed): disulfoton, heptenophos and trithion;

(III) extensive reaction (more than 75% destroyed): dieldrin, α-endosulfan, β-endosulfan and isodrin.

For the ethanolic potassium hydroxide treatment the pesticides can also be divided into the same three categories:

(I) no reaction occurred (less than 25% destroyed): o,p'-DDE, o,p'-DDT, dieldrin, β-HCH, isodrin, mirex, disulfoton, fonofos, malathion, parathion-ethyl and parathion-methyl;

(II) partial reaction (25–75% destroyed): ethion and trithion;

(III) extensive reaction (more than 75% destroyed): α-endosulfan, β-endosulfan, endosulfan sulphate, α-HCH, δ-HCH, heptenophos, phenthoate and sumithion.

Finally, the pesticides after chromic acid treatment can also be divided into the same three categories:

(I) no reaction occurred (less than 25% destroyed): o,p'-DDT, endosulfan sulphate and mirex;

(II) partial reaction (25–57% destroyed): dieldrin, α-HCH, β-HCH and δ-HCH;

(III) extensive reaction (more than 75% destroyed): o,p'-DDE, which produces a new

TABLE I

RECOVERIES OF ORGANOCHLORINE AND ORGANOPHOSPHORUS PESTICIDES AFTER CHEMICAL TREATMENTS

Pesticides	Working solution ($\mu g l^{-1}$)	Recovery [% ± R.S.D. ($n = 6$)]			Detection limit ($ng l^{-1}$)
		H_2SO_4	KOH	Cr(VI)	
Organochlorine compounds					
o,p'-DDE	0.25	95 ± 3	98 ± 1	0[a]	3.6
o,p'-DDT	0.25	88 ± 9	78 ± 8	83 ± 11	3.5
Dieldrin	0.30	0	97 ± 5	27 ± 6	2.1
α-Endosulfan	0.25	4	0	0	3.5
β-Endosulfan	0.25	10	0	0	3.7
Endosulfan sulfate	0.25	106 ± 7	0	89 ± 9	3.9
α-HCH	0.20	91 ± 3	0	42 ± 12	1.9
β-HCH	0.20	86 ± 4	86 ± 7	55 ± 10	2.1
δ-HCH	0.20	92 ± 9	0	48 ± 15	1.9
Isodrin	0.30	5	98 ± 3	0	2.5
Mirex	0.40	101 ± 6	97 ± 8	91 ± 2	6.7
Organophosphorus compounds					
Disulfoton	0.50	29 ± 11	78 ± 8	0	3.0
Ethion	0.50	83 ± 12	51 ± 9	4	3.6
Fonofos	0.50	94 ± 4	90 ± 5	0	4.0
Heptenophos	1.00	53 ± 2	0	0	10.4
Malathion	1.20	97 ± 2	79 ± 9	0	15.6
Parathion-ethyl	1.00	97 ± 3	79 ± 7	0	10.4
Parathion-methyl	1.00	97 ± 4	83 ± 11	0	7.8
Phenthoate	1.00	94 ± 4	0	0	6.2
Sumithion	0.50	93 ± 6	0	0	3.0
Trithion	1.00	47 ± 15	52 ± 10	17 ± 2	10.4

[a] Degradation product dichlorobenzophenone.

degradation peak, α-endosulfan, β-endosulfan, isodrin and all organophosphorus pesticides; *o,p'*-DDE is converted into *o,p'*-dichlorobenzophenone, but is not seen because of the chromatographic conditions employed.

Generally, our results agree with the few studies found in the literature [18,21,26,27], except for *o,p'*-DDT [18,27] and β-HCH [18] with the alkali treatment. In order to clarify this discrepancy, the alkali treatments described in refs. 18 and 27 were carried out at 100°C for a longer time.

On the other hand, in the alkali treatment previously described, if 5 *M* potassium hydroxide solution is employed instead of 2 *M* potassium hydroxide, *o,p'*-DDT and β-HCH are destroyed.

Other chemical treatments reported in the literature are different and their results are not comparable.

The oxidative treatment is the most destructive of the three treatments studied, followed by the alkali treatment. The acid treatment is the least destructive. Consequently, the three treatments described in this paper can be used to confirm the peak of pesticides, taking into account the different behaviours observed for each of them and the fact that they are rapid, simple and applicable to many samples.

Application to water analysis

The content of organochlorine and organophosphorus pesticides in surface water samples was determined by using octadecyl-bonded

porous silica glass microcolumn extraction, followed by chemical treatment with sulphuric acid, potassium hydroxide and chromic acid for confirmation of peaks. The treatment procedures remove the interferences by destroying some of the organic matter contained in contaminated waters. Therefore, Florisil or alumina column adsorption chromatography was found to be unnecessary.

Fig. 3 shows the ECD and NPD chromatograms corresponding to the analysis of a surface water sample collected from a predominantly agricultural area in which pesticide residues are fairly common.

The results of the analysis of twenty surface water are given in Table II. The pesticides were identified by using the retention times obtained with two different polarity capillary columns. The concentration of pesticides was calculated before any chemical treatment, by comparing the peak areas with those of standards injected under identical conditions. The confirmation is

TABLE II

PESTICIDES PRESENT IN SURFACE WATER SAMPLES FROM THE VALENCIA AREA

Sample No.	Pesticide possible	Concentration (ng/l)	Chemical treatment[a]			Confirmation
			Acid	Alkali	Oxidative	
1	Ethion	10	+	+	−	Positive
	Sumithion	8	+	+	−	Positive
2	Fonofos	9	+	+	−	Positive
3	β-Endosulfan	55	+	−	−	Negative
4	Parathion-ethyl	34	+	+	−	Positive
	Trithion	49	+	+	−	Positive
5	Parathion-ethyl	25	+	+	−	Positive
6	Parathion-ethyl	39	+	+	−	Positive
	Ethion	10	+	+	−	Positive
7	Parathion-ethyl	35	+	+	−	Positive
8	α-Endosulfan	24	−	−	−	Positive
	β-Endosulfan	18	−	−	−	Positive
	Parathion-ethyl	176	+	+	−	Positive
	Sumithion	30	+	−	−	Positive
9	*o,p'*-DDE	10	+	+	−	Positive
10	α-Endosulfan	352	−	−	−	Positive
	β-Endosulfan	112	−	−	−	Positive
	Disulfoton	103	+	+	−	Positive
	Parathion-ethyl	125	+	+	−	Positive
11	Parathion-ethyl	21	+	+	−	Positive
	Trithion	30	+	+	−	Positive
12	Disulfoton	57	+	+	−	Positive
	Ethion	21	+	+	−	Positive
	Trithion	23	+	+	−	Positive
13	Ethion	6	+	+	−	Positive
	Parathion-methyl	84	+	+	−	Positive
14	Trithion	585	+	+	−	Positive
15	Trithion	26	+	+	−	Positive
16	Ethion	7	+	+	−	Positive
17	Sumithion	6	+	−	−	Positive
18	Parathion-methyl	81	+	+	−	Positive
19	Malathion	16	+	+	−	Positive
20	Sumithion	23	+	−	−	Positive

[a] + = Unaltered; − = destroyed.

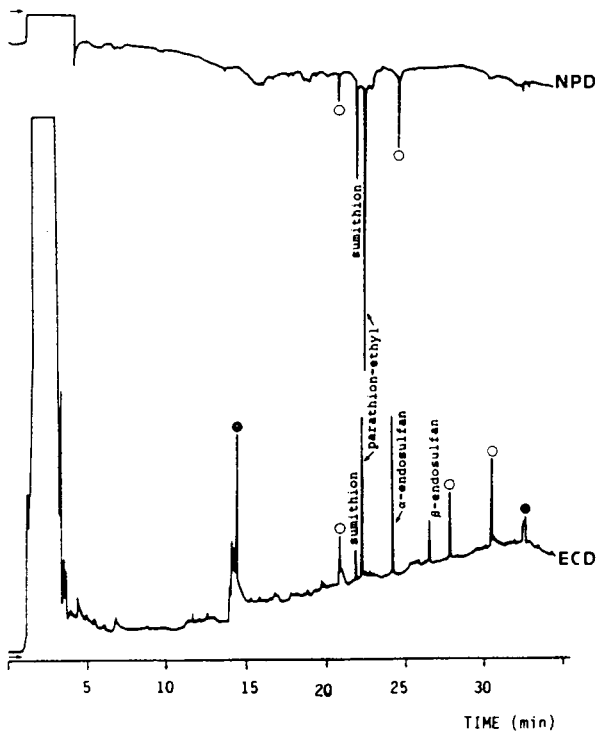

Fig. 3. Chromatogram corresponding to water sample No. 8 from the Valencia area (see Table II), obtained with parallel (ECD, NPD) detection using the BP-5 capillary column prior to any chemical treatment. ● = Unknown peak also in blank; ○ = unknown peak destroyed in all treatments. The peak identified as sumithion was destroyed in the oxidative and alkali treatments. The peak identified as parathion-ethyl was destroyed in the oxidative treatment. The peaks identified as α-endosulfan and β-endosulfan were destroyed in all treatments. See text for operating conditions.

positive when the results obtained by comparing the three chemical treatments are the same as those obtained with standards (Table I), and negative when at least one treatment differs from the standard.

As seen in Table II, the organochlorine pesticides *o,p'*-DDE, α-endosulfan and β-endosulfan were confirmed in surface water after the treatments, except for one sample in which the β-endosulfan gave a false-positive result.

The results obtained for all of the organophosphorus pesticides in the surface water samples analysed were confirmed by chemical treatments. The presence of organophosphorus compounds as the most important pesticides is due to the fact that the water samples were collected in an intensive agricultural area where non-persistent organophosphorus pesticides are predominantly used.

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Gas chromatographic analysis of organophosphorus pesticides of horticultural concern ☆

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ABSTRACT

An optimized GC method for the determination of 25 organophosphorus pesticides (OPs) currently applied to horticultural crops was developed. Four additional compounds were evaluated for their suitability as internal standards. The linearity, reproducibility and recoveries of the method are discussed. The reliability of the method for routine analysis of fruit and vegetable samples is demonstrated. Registration of the analytical results for a control sample in quality control charts demonstrated the performance of this method. The limits of detection are in the range 5–20 μg/kg. The sample throughput is *ca.* 68 per 24 h. Results are also presented for extracts of four real samples fortified with the target OPs. The method was evaluated and the results were confirmed by GC–MS.

INTRODUCTION

During the last decade the number of pesticides, including isomers and breakdown products, applied to fruits and vegetables and for which analytical methods are available has increased to about 180 [1]. Many official and commercial laboratories regularly examine these food products to determine compliance with maximum residue limits (MRLs) [2]. Such analyses also yield information on the effect of pesticide degradation processes on the horticultural crop and on the average amount of pesticide ingested by the consumer.

The importance of revising and improving multi-residue analytical methods periodically is readily apparent. The demand for efficient monitoring programmes requires that a set of multi-residue methods and measurement regimes be designed by taking into account the need for greater efficiency and lower costs and the requirements of consumer safety. Design considerations must also include recent technological advances such as macro open-tubular columns and new stationary phases [3–5].

Gas chromatography (GC) with phosphorus flame photometric detection (FPD-P) has frequently been the instrumental technique of choice for the analysis of volatile organophosphorus pesticides for reasons of selectivity, sensitivity and reproducibility. However, optimum GC temperatures and confirmation methods are necessary [6,7]. Interferences in analytical determinations may occur when FPD-P is used in the phosphorus mode and sulphur is present [8], when some thermally labile OPs, such as tri-

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chlorfon [9–11], are present or when bleeding of the liquid phase occurs [12]. On the other hand, experience gained during interlaboratory evaluations [13–15] of methodologies for this class of compounds indicates the need for analysts to be familiar with the OPs currently used on specific crops in each region.

In this study, a rapid, sensitive and accurate chromatographic procedure for the determination of 25 OPs currently applied to horticultural crops grown in greenhouses in southeastern Spain was developed. The work described proceeded in the following stages: (a) optimization of the GC conditions taking into account the factors mentioned below which gave the best chromatographic resolution and the shortest time for analysis; (b) confirmation by GC–MS; (c) study and selection of internal standards; (d) study of the behaviour of working and internal standards; (e) determination of the linearity, reproducibility and recoveries of the method; and (f) evaluation of the performance of the method on control samples and on different real samples fortified with selected target compounds. A list of the compounds included in this study together with total turnover values from three European countries and determinations of many of these compounds in residue amounts ≥20% of MRLs from one European country are given in Table I.

EXPERIMENTAL

Chemicals

Pesticide-grade ethyl acetate, acetone, light petroleum, anhydrous sodium sulphate (12–60 mesh) and triphenyl phosphate were obtained from Merck (Darmstadt, Germany). The pesticide standards (Pestanal quality) listed in Table I were obtained from Riedel-de Haën (Seelze, Germany). Stock standard solutions were prepared by dissolving 10.0–15.0 mg of each purity-certified pesticide in 100 ml of light petroleum or acetone (methamidophos) to give 100.0–150.0 mg/l solutions. A working standard mixed solution of 1.0–1.5 mg/l was prepared by transferring 1 ml of each stock standard solution into a 100-ml volumetric flask and diluting to volume with light petroleum.

Chromatographic analysis

GC–FPD. A Perkin-Elmer (Beaconsfield, UK) Model 8600 gas chromatograph equipped with a flame photometric detector was used with an SPB 1701 fused-silica (0.5 μm) capillary column (30 m × 0.53 mm I.D.) coated with cyanopropylphenylmethylsiloxane (Supelco, Bellefonte, PA, USA). The carrier gas was helium at a flow-rate of 10 ml/min. The temperatures of the injector and detector were maintained at 220 and 300°C, respectively. The conditions used for GC were optimized. The injection volume was 3 μl.

GC–MS. A Hewlett-Packard (Palo Alto, CA, USA) Model 5995 instrument with a Model 59970 data system were used for GC–MS in the electron impact (EI) mode. The same fused-silica column as described above was used. The sample was introduced directly into the ion source. The carrier gas was helium. The other chromatographic conditions were identical with those described for GC–FPD analysis. EI spectra were obtained at 70 eV.

Optimization

Response function. The selection of an objective function (*OF*) based on a penalty function (*p*-) was realized by applying criteria given by Deming [16]. This function is defined as

$$OF = n + \sum R_{i,j} + p$$

where

$$p = 0 \qquad \text{for } y < y_t$$

$$p = b(y - y_t) \qquad \text{for } y_t < y < y_t + 5$$

$$p = -\infty \qquad \text{for } y > y_t + 5$$

n is the number of peaks detected, $R_{i,j}$ is the resolution between adjacent peaks i and j ($R_{i,j}$ is limited to a maximum value of 1.5 to avoid $\Sigma R_{i,j}$ being determined largely by the largest values of $R_{i,j}$), y is the retention time of the last peak considered and y_t is the target retention time.

Only the four worst resolved pairs of peaks were considered in the present calculations. The target retention time was fixed at $y_t = 20$ min as

TABLE I

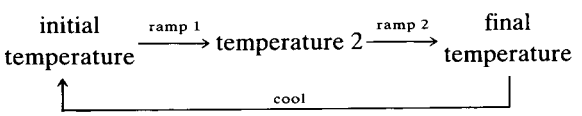
ORGANOPHOSPHORUS COMPOUNDS STUDIED, TOTAL TURNOVER OF SELECTED PESTICIDES IN THREE EUROPEAN COUNTRIES IN 1989 AND RESIDUES OF ORGANOPHOSPHATES FOUND AT ≥20% OF THE MRL IN FRUITS AND VEGETABLES IN 1991

No.	Compound	Total turnover (tonnes)[a]			Found at ≥20% of MRLs[b]
		Greece	Sweden	Denmark	
1	Dichlorvos	25.6	0.1	4.9	×
2	Methamidophos	317			×
3	Mevinphos		0.4		
4	Acephate	21	0.9	1.3	×
5	Naled				
6	Omethoate	29			×
7	Etrimphos		0.3		
8	Monocrotophos	118			×
9	Dimethoate	106	9.5	88.8	
10	Chlorpyriphos methyl				×
11	Pirimiphos methyl	20			×
12	Chlorpyriphos				×
13	Parathion methyl	25			×
14	Malathion	42.5	3.2	11.0	×
15	Fenitrothion	5.3	0.5	4.4	×
16	Quinalphos	4.3			
17	Chlorfenvinphos		0.7	1.3	×
18	Mecarbam	42			×
19	Metidathion	230			
20	Fenamiphos	42			
21	Carbophenothion				×
22	Triazophos	27.7			×
23	Pyridafenthion	1.2			
24	Phosalone	30.9			
25	Pyrazophos	7.1			

[a] Ref. 28.
[b] In fruits imported from Spain to Sweden; × = compound found. Ref. 1.

the maximum acceptable retention time of the last peak.

Selection variables. The next step was to define the variable space or search region. Relying on literature data [17] and previous experience, we selected the following temperature programming cycle:



Five preliminary runs (Table II) were made in order to establish the variables and ranges to be optimized and the survey limits of each of them.

A final temperature of 260°C was chosen to avoid column bleeding. A rate of 30°C/min was chosen for ramp 1. Thus a three-variable space (temperature 1, temperature 2 and ramp 2) with ranges 90–110°C, 180–240°C and 2–10°C/min, respectively, was used in the optimization procedure.

OPSP optimization. Over this domain an automated sequential method, optimization by search point (OPSP) as described previously [18], was used to determine the optimum GC conditions to accomplish the separation of the OPs [highest criterion value (*OF*)] within the desired time limits. A series of chromatograms were generated for which the experimental conditions were

TABLE II

PRELIMINARY RUNS

$OF_{max} = 14$.

Temperature 1 (°C)	Ramp 1 (°C/min)	Temperature 2 (°C)	Ramp 2 (°C/min)	Temperature 3 (°C)	*OF* value
100	10	210	2	260	0
100	30	210	2	260	10.02
100	10	210	10	260	8.76
100	30	210	10	260	9.72
120	20	230	5	260	9.43

set as indicated by the search algorithm. The optimum conditions were known after 23 experimental chromatographic runs had been performed. These optimum conditions temperature programme was an initial temperature of 110°C (held for 1 min), increased to 226°C at 30°C/min and then to 260°C at 4°C/min.

Sample preparation

Different fruit and vegetable samples were collected from greenhouses in the vicinity of Almería, Spain (where the most of the OP compounds mentioned in Table I are currently used) and extracted in our laboratory according to the following procedure.

The sample was macerated in a food chopper, 50 g of chopped sample were weighed into a high-speed blender and 40 g of anhydrous sodium sulphate were added. After thorough mixing, 100 ml of ethyl acetate were added and the mixture was blended for 5 min. The supernatant liquid was filtered with suction through a filter-paper and a layer of 20 g of anhydrous sodium sulphate. The extraction and filtration were repeated once. The filter was rinsed with 50 ml of ethyl acetate and the combined extracts were evaporated on a vacuum rotary evaporator using a 40–60°C water-bath. The volume of the residue solution was reduced to exactly 10 ml [19].

Recovery assays

Fresh green pepper samples, which had not been treated with the pesticides studied, were fortified with 0.20–0.30 mg/kg of each pesticide as follows. A 10-ml volume of the working standard mixed solution described above was added to 50 g of chopped sample in a high-speed blender jar. After evaporation of the light petroleum with a stream of air, the sample was homogenized for 2 min. After 30 min, the sample was again homogenized for 1 min and immediately analysed by application of the previously described method. The recovery assays were replicated three times.

RESULTS AND DISCUSSION

GC separation

The optimum temperature programme (see Experimental) resulted in the retention behaviour indicated in Table III. An example of a gas chromatogram of the mixed OP compounds is shown in Fig. 1A. Two critical pairs of compounds, chlorpyriphos–methylparathion and quinalfos–chlorfenvinos, remained partially overlapping, but the second pair could be well resolved using the detector in the sulphur mode (Fig. 1B). The total run time was 16 min plus 5 min for equilibration at the initial temperature. Hence the analysis of *ca.* 68 samples in 24 h is feasible.

GC–MS confirmation

Solutions containing all the target compounds were analysed by GC–MS in the EI mode with a scan range from m/z 30 to 600 under full-scan conditions. The main fragments obtained and their relative abundances are shown in Table IV. These data are in good agreement with the

TABLE III

RELATIVE RETENTION TIMES (RRT) AND THEIR RELATIVE STANDARD DEVIATIONS (R.S.D.s) AND AVERAGE RECOVERIES AND THEIR R.S.D.s OF TEST COMPOUNDS IN GREEN PEPPER SAMPLES USING GC–FPD-P

Fortification level 0.20–0.30 mg/kg ($n = 10$). Chromatographic conditions and sample preparation are described in the text.

No.	Compound	RRT[a]	R.S.D. (%)	Average recovery (%)	R.S.D. (%)
1	Dichlorvos	0.540	0.17	83.0	6.9
2	Methamidophos	0.602	0.17	68.4	33.9
3	Mevinphos	0.665	0.24	79.2	8.2
4	Acephate	0.737	0.26	69.1	30.8
5	Naled	0.793	0.28	70.2	8.4
6	Omethoate	0.835	0.27	65.1	21.2
7	Etrimphos	0.868	0.30	108.1	6.6
8	Monocrotophos	0.904	0.29	69.3	23.8
9	Dimethoate	0.925	0.30	78.8	12.7
10	Chlorpyriphos methyl	0.934	0.31	94.3	6.3
11	Pirimiphos methyl	0.965	0.31	96.2	6.8
12	Chlorpyriphos	1.000	0.32	98.2	4.9
13	Parathion methyl	1.006	0.39	96.9	7.9
14	Malathion	1.029	0.23	105.2	3.4
15	Fenitrothion	1.043	0.32	94.4	4.2
16	Quinalphos	1.120	0.35	93.7	10.6
17	Chlorfenvinphos	1.125	0.33	90.5	15.6
18	Mecarbam	1.152	0.34	95.7	7.5
19	Metidathion	1.217	0.34	94.5	7.9
20	Fenamiphos	1.268	0.37	99.9	11.6
21	Carbophenothion	1.425	0.36	96.0	3.7
22	Triazophos	1.496	0.35	100.2	8.2
23	Pyridafenthion	1.756	0.32	102.1	5.9
24	Phosalone	1.928	0.32	99.3	6.5
25	Pyrazophos	2.001	0.35	96.5	3.7

[a] Absolute retention time of chlorpyriphos = 7.698 min.

different diagnostic ions reported for these organophosphorus pesticides [20–26].

Selection of internal standard

Four compounds were considered for use as internal standards. Three of them, chlormephos, fenthion and demeton, are OP compounds but are not used on horticultural crops in this area, and triphenyl phosphate has been proposed in EPA Method 507 and National Pesticide Survey Method 1 [27]. Selection was based on both retention time and detector response. Chlormephos met all the criteria for an internal standard (retention time 5.05 min; detector response 2.5 times more than that for chlorpyrifos; recovery = 88%) and is therefore, recommended as the internal standard. Of the other three compounds investigated two (fenthion and demeton) co-eluted with the target compounds. Triphenyl-phosphate eluted at the end of the desired retention time range.

Evaluation of working and internal standards

An important criterion by which to assess the quality of the analytical results is the accuracy of determination. For this purpose we evaluated the stability, detector response and random deviations of the working and internal standard by quality control charts (QCC). The QCC for two representative OPs, methamidofos (high polarity) and chlorpyrifos (low polarity), and the internal standard chlormephos showed no results

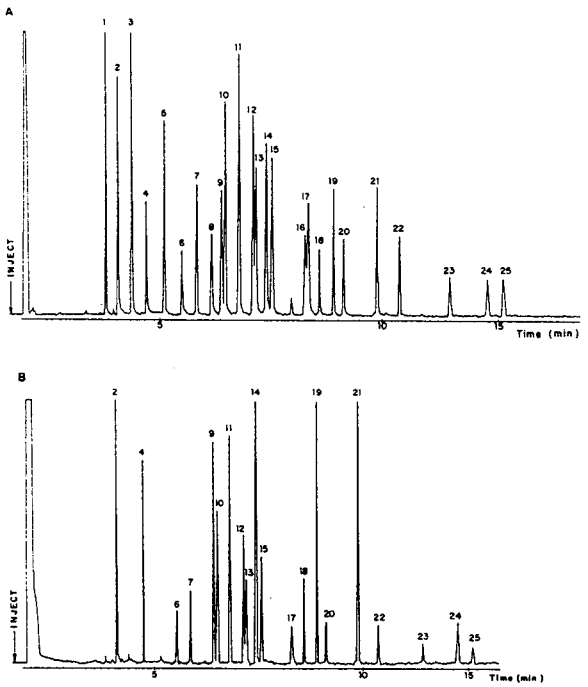


Fig. 1. Gas chromatogram of the working standard solution containing the 25 organophosphorus pesticides (concentrations 1.0–1.5 mg/l). (A) FPD in phosphorus mode; (B) FPD in sulphur mode. Numbers above the peaks correspond to compound numbers in Table I.

outside the limiting lines (three times the standard deviation) during at least 15 days if the standard solutions (see Experimental) were kept at 4°C for all compounds except methamidofos, for which it is recommended that a daily check is made.

Linearity, recoveries and repeatability

The linear dynamic range of the detector response was checked for all the target compounds in the working standard solution and appeared to be 0.15–50 ng absolutely amount injected (on-column) and the correlation coefficients were higher than 0.991 in all instances. This is also the practical working range. Detection limits based on a signal-to-noise ratio of 3:1 were on average 50 pg absolute amount injected.

The retention times and recoveries of the different OPs were tested by fortifying ten fresh pepper samples, using the procedure described above (Table III). The repeatability of retention time was satisfactory in all instances. The recoveries were in the range 68–108% with a relative standard deviation (R.S.D.) of less than 30% in all instances except methamidophos (33.90%) and acephate (30.83%), which is considered acceptable.

Control sample

As a quality assurance measure, a control sample was repeatedly analysed over a 2-month period. Green pepper was chosen as the relevant matrix, and it was shown that residues of target compounds and internal standard were stable for a long time if the sample is kept in a freezer at −18°C. The analytical data for the fortified control sample were registered on a control chart according to Shewhart. The results for chlorpyriphos are shown in Fig. 2 and confirm the overall reliability of optimized method in routine analysis.

Method performance with cucumber, beans and grapes

The proposed screening method was assessed for the analysis of cucumber, beans and grapes, collected from a greenhouse in Almería (Spain) in order to observe the effect of the matrix on the recoveries, separation and interfering peaks. The homogenized samples were fortified, using the procedure described above, with the 25 target compounds in the range 0.20–0.30 mg/kg and analyses were carried out in duplicate (in this test green pepper samples were also included). All compounds were identified correctly

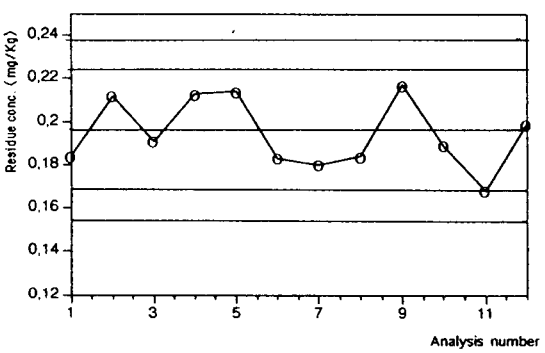


Fig. 2. Shewhart control charts for the fortified control sample for chlorpyrifos. The analytical results were registered over a 2-month period.

TABLE IV

MAIN IONS AND RELATIVE ABUNDANCES IN EI MASS SPECTRA OF THE ORGANOPHOSPHORUS PESTICIDES

No.	Compound	Molecular mass	Main ions, m/z (relative abundance, %)					
			I	II	III	IV	V	VI
1	Dichlorvos	220	79 (20)		109 (100)		185 (18)	220 (4)
2	Methamidophos	141	79 (14)	94 (100)				141 (39)
3	Mevinphos	224			109 (19)	127 (100)		224 (4)
4	Acephate	183		94 (68)		125 (14)	136 (100)	183 (4)
5	Naled	380			109 (100)			301 (5)
6	Omethoate	213	79 (48)		110 (100)	125 (17)		213 (4)
7	Etrimphos	292	79 (71)		109 (43)	125 (100)		292 (36)
8	Monocrotophos	223			109 (13)	127 (100)		223 (4)
9	Dimethoate	367				125 (90)	182 (100)	367 (9)
10	Chlorpyriphos methyl	321	79 (35)		109 (25)	125 (100)		286 (60)
11	Pirimiphos methyl	305	79 (40)		109 (44)	125 (100)		305 (57)
12	Chlorpyriphos	349		97 (47)			197 (100)	314 (47)
13	Parathion methyl	263	79 (30)		109 (100)	125 (83)		263 (35)
14	Malathion	330		93 (87)		125 (100)	173 (75)	330 (3)
15	Fenitrothion	277	79 (26)		109 (79)	125 (100)		277 (45)
16	Quinalphos	298		97 (39)			146 (100)	298 (17)
17	Chlorfenvinphos	358	81 (100)		109 (62)		267 (45)	323 (44)
18	Mecarbam	329		97 (82)		125 (49)	131 (100)	329 (12)
19	Metidathion	302		93 (21)		125 (18)	145 (100)	302 (3)
20	Fenamiphos	303			109 (14)		154 (100)	303 (69)
21	Carbophenothion	342		97 (66)		125 (49)	157 (100)	342 (15)
22	Triazophos	313		97 (55)			161 (100)	313 (7)
23	Pyridafenthion	340		97 (100)			188 (52)	340 (44)
24	Phosalone	367				121 (56)	182 (100)	367 (9)
25	Pyrazophos	373		97 (30)			221 (100)	373 (11)

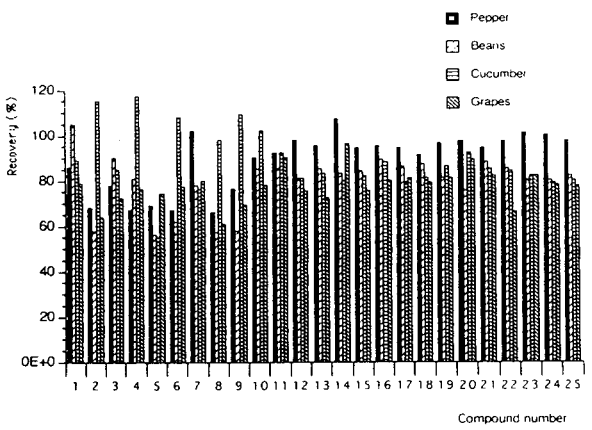


Fig. 3. Average recoveries (%) from duplicate determinations of the 25 OP compounds as a function of the matrix. Fortification level 0.20–0.30 mg/kg.

and the average recovery data were, as expected, dependent on the matrix, but nevertheless acceptable (Fig. 3). These values were in the range 65–111% in all instances, except for methamidophos, acephate, naled, omethoate and dimethoate, with values in the range 58–119%.

CONCLUSIONS

An optimized chromatographic method covering 25 organophosphorus pesticides in fruit and vegetable samples has been developed. The sample throughput was increased considerably (*ca.* 68 per 24 h) with the optimized method. The minimum detectable amount is about 50 pg (signal-to-noise ratio = 3) for each pesticide,

which means that residue levels in real crop samples down to 10 μg/kg can be detected.

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Field sampling and analysis of volatile reduced sulphur compounds in air, water and wet sediments by cryogenic trapping and gas chromatography☆

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ABSTRACT

A method for the simultaneous analysis of volatile reduced sulphur species such as hydrogen sulphide, carbonyl sulphide, methanethiol, dimethyl sulphide, carbon disulphide and dimethyl disulphide in air, water and wet sediments is described. In the atmosphere these compounds are preconcentrated by pumping air through a U-shaped cryogenic trap cooled with liquid argon. In waters and sediment pore waters they are concentrated by nitrogen purging and cryo-trap condensation. The trap is then connected on-line to a field-portable gas chromatograph provided with a secondary cryofocusing trap and a flame photometric detector. Detection limits less than or equal to 10 pg S/l (air), 1 ng S/l (water) and 10 pg S/g (wet sediment) are achieved for individual compounds. The variation in the quantitative results for water analyses is less than 7%, with the only exception being methanethiol (20%). Examples of application to air, water and wet sediment samples from a stratified karstic lake and a coastal saltern pond are shown.

INTRODUCTION

In the last decade, the number of analytical developments for the determination of volatile reduced sulphur compounds (VSCs) has increased considerably. The methods described are devoted to the study of different natural systems and involve a preconcentration step generally followed by gas chromatographic separation on either capillary [1,2] or packed [3,4] columns. Poor recoveries were obtained with preconcentration by adsorption onto solid surfaces [1,5,6], except when the adsorbent columns were kept at low temperatures [4]. Cryogenic trapping affords better results, being the technique of choice in several studies of air and water samples [2,3]. However, only some of these studies concern the whole set of VSCs (*e.g.* refs. 1, 3 and 5), many of them being only devoted to the analysis of one or two species (*e.g.* ref. 7). Likewise, very few approaches have addressed the determination of VSCs in air, water and sediment pore water [8].

The difficulties in the overall analysis of the VSC mixtures arise from the different properties of their components, such as volatility, polarity and reactivity. Furthermore, the concentrations of the individual species may range over spans as large as 10^{-3}–10^{5} ppt (w/w), involving diverse interference problems between major and minor constituents. The differences in sample matrix represent an additional difficulty which tends to restrict the range of application of the methods and requires dedicated handling procedures. However, these analytical problems are faced with the need for the study of the distribution

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and behaviour of all VSCs in the principal environmental compartments, namely air, water and sediments, for a better understanding of the mechanisms involved in the biogeochemical cycling of sulphur [9].

In order to contribute to a comprehensive description of VSC occurrence in the marine and continental environments we present here an analytical method for the determination of hydrogen sulphide (H_2S), carbonyl sulphide (COS), methanethiol (MeSH), dimethyl sulphide (DMS), carbon disulphide (CS_2) and dimethyl disulphide (DMDS) in air, water and sediment pore water samples, including procedures adapted for field studies. These analyses are performed by cryogenic preconcentration, separation by packed column gas chromatography (GC) and flame photometric detection (FPD). Sampling efficiencies, sensitivity and precision of the measurements are indicated. The procedures have been tested in natural systems as different as a stratified karstic lake and a shallow hypersaline pond. Characteristic GC profiles corresponding to air, water and sediment pore water collected in these environments are described.

EXPERIMENTAL

Cryogenic enrichment samplers

The traps for cryogenic preconcentration were U-shaped borosilicate glass tubes (20 cm × 10 mm O.D. × 6 mm I.D.). The lower portion of the tubes was packed with quartz wool to increase the condensation surface. The inner tube surfaces and quartz wool were silanized periodically. The traps were conditioned before sampling by nitrogen flushing under vacuum at 60–80°C. Series of five U-tubes were connected to two six-way PTFE rotating valves (Rheodyne, Cotati, CA, USA) by means of 0.16-cm PTFE tubing. Each loop was selected by setting both the inlet and the outlet valves to the required position. One position was short-circuited, allowing the loops to be closed without interrupting the gas flow. Further details on this cryogenic system are described elsewhere [10].

Field sampling and preconcentration

Air. Prior to sampling the U-shaped tubes were immersed in liquid argon. Ambient air samples were collected by connecting the cryogenic trap to a portable pump (Grinyo Rotamix, L'Hospitalet de Llobregat, Catalonia, Spain) and switching the six-way PTFE valves to select the appropriate loop. Air volumes of 1–10 l were drawn through the traps at flow-rates of 150–300 ml/min. After collection, the valves were switched either back to the closed position or to the next loop position for further sampling. The loaded traps were kept closed and under liquid argon until chromatographic analysis, which was performed within the following 2 h.

Water. Water samples were collected in dark, silanized glass bottles that were completely filled and tightly sealed to minimize headspace and air entrance. The samples were stored at 4°C in the dark and analysed within 1–8 h after sampling. The VSCs were determined in 1–50 ml aliquots, which were transferred to a bubbling flask, through a PTFE-coated septum, and purged with a nitrogen stream supplied through a fritted glass diffuser. The stripped volatile compounds were collected in a cryogenic trap as described for the air samples. The bubbling flask was periodically silanized. In the case of small subsample volumes, these were brought to 50 ml by dilution with degassed Milli-Q water. In waters with a high content of suspended particles, the subsamples were filtered through GF/F (Whatman, Maidstone, UK) prior to injection into the flask.

Sediment pore water. Small sediment cores were collected with 10 cm × 2 cm I.D. polyethylene cylinders and frozen immediately under liquid argon. In the laboratory, the cores were carefully sliced in millimetre layers. Each slice was then immersed in a 25-ml spinning tube filled to the brim with degassed water medium that contained a buffered Milli-Q water solution having similar alkalinity and salinity to the values measured in the overlying water. The tube was plugged with a PTFE-coated septum held with a holed screw cap. Homogenization was achieved by shaking for 3 min. Then, the samples were spun down at 4100 rpm for 10 min (2800 *g*). After centrifugation the supernatant solution was

taken by suction with a syringe while nitrogen was simultaneously introduced for restoration of the removed pressure. These water solutions were analysed as described above for water samples.

Blanks. Control blanks were periodically analysed. Volumes of 50 ml of Milli-Q water, previously degassed by flushing with nitrogen, were purged and analysed as an actual sample. Normally, no sulphur compounds were observed above the detection limit. Only small amounts of COS and CS_2 were detected in some cases, when the quality of the purging nitrogen was not adequate.

Gas chromatography

A portable gas chromatograph developed by W. Haunold, University of Frankfurt [10], was used for the *in situ* analysis of volatile reduced sulphur compounds. This instrument was equipped with an FPD (Perkin-Elmer, Norwalk, CT, USA) and a Hewlett-Packard Model 3393A integrator. The optimal detector gas flow-rates were 95 and 170 ml/min for hydrogen (99.999% quality) and synthetic air, respectively. The carrier gas was 99.999% quality nitrogen, additionally purified by passage through molecular sieves, Oxysorb and cryogenic traps. The sulphur components were separated on a 1.4 m × 0.32 cm O.D. Teflon (FEP) column packed with 63–200 μm Carbopack BHT 100 (Supelco, Bellefonte, PA, USA). Column heating and cooling were performed with Peltier elements. The column was conditioned overnight at 100°C with carrier gas at a flow-rate of 20 ml/min. Almost baseline separation of H_2S, COS, CH_3SH, DMS, CS_2 and DMDS was achieved using a temperature programme from −5°C up to 100°C at 30°C/min, with an initial 0.5-min delay and 8 min hold time at the end. Carrier flow-rate was 19 ml/min.

Cryofocusing and injection

A small second glass loop (25 cm × 1.5 mm I.D.) was located between the injection valve of the gas chromatograph and the column. This secondary loop has been demonstrated to be useful for cryofocusing and separation of the VSC from co-trapped water [2]. Before analysis, the sampling trap was connected to the GC injection valves and the lines were purged with nitrogen for 2–3 min. For injection, the GC valve was switched to the injection position, the valves of the selected sample loop were open and the liquid argon was replaced quickly by hot water (60–70°C). Then, the desorbed gases were brought by the carrier gas into the cryofocusing trap, which was immersed in liquid argon. After 1.5 min, the sample cold trap valves were switched back to the closed position so that the carrier gas flowed directly to the cryofocusing trap without going through the sample loop. This desorption time allowed a complete transfer of the volatile components with a minimal load of water vapour [3]. The VSCs were finally introduced into the GC column by heating the secondary loop with a hot water bath. The GC temperature programme was started at this point.

Calibration

Calibration was performed using certified permeation tubes containing H_2S, COS, CH_3SH, DMS, CS_2 and DMDS (Vici Metronics, Santa Clara, CA, USA). The gaseous standards were obtained in a permeation apparatus, keeping the tubes in glass vessels at a constant temperature of 30.0 ± 0.1°C and under continuous nitrogen flow. Variable volumes of the outcoming nitrogen stream were taken with gas-tight syringes and injected through a septum into a PTFE line connected to the cryofocusing trap of the gas chromatograph.

RESULTS AND DISCUSSION

Cryogenic trapping

Safe sample volumes. Experiments to establish the optimal conditions for cryogenic preconcentration were carried out. The permeation devices were used to generate VSC mixtures with concentrations representative of those found in sulphur-rich atmospheres. These mixtures were produced in a 25-l dilution chamber flowed with nitrogen. The outlet of the chamber was connected to two traps in serial arrangement. The

trapping efficiency was tested twice by collecting volumes of about 1 and 5 l of the outcoming nitrogen at 300 and 340 ml/min, respectively. In both cases no VSC traces were detected in the second trap for a total S of 10 ng. Nevertheless, in order to ensure a complete trapping, flow-rates lower than 300 ml/min were used in the regular field analyses.

Water interferences. Purge stream clogging in the cryo-trap is a common problem in VSC water analyses and results from the freezing of the water vapour carried by the stripping gas. Significant amounts of water are also trapped in the analysis of air samples with a high humidity content. Several methods have been developed to remove water vapour from the sample stream before reaching the cryo-trap, including absorption with hygroscopic salts [6,7] and Nafion driers (ref. 11 and references therein). Both systems have been successfully applied to the determination of DMS in humid air and water samples [6,7,12]. However, their suitability for the whole set of VSCs is still controversial because at low concentrations losses of one or more components are usually observed [1,3,4,11].

Driers were therefore not considered in the development of the analytical procedures of the present study. Clogging was avoided by optimization of both the stripping flow-rate and the depth at which the glass loop was immersed in liquid argon. At a water-saturated nitrogen flow-rate of 150 ml/min, no clogging occurred after 20 min of sampling. Higher flow-rates resulted in ice blocking of the cold trap after 10–15 min. On the other hand, the sample loop was immersed in liquid argon up to one half of its height. In this way, ice was accumulated in the upper portion of the wide loop and not in the narrower, preceding PTFE tube.

Another problem associated with the presence of water in the samples is the gradual deactivation of the chromatographic column after repeated VSC analysis. In the present study water entrance into the GC was minimized by means of the controlled desorption and cryofocusing method described in the Experimental section. The column was, however, periodically reconditioned by heating to 100°C for 1 h.

Stripping efficiency

The stripping efficiency of the purge system for water and sediment pore water samples was determined. Owing to the difficulty in preparing reliable standard solutions with gas components, representative real samples containing all the VSCs of interest were used as test solutions. Volumes of 50 ml of sample were sequentially purged for three periods of 10 min. The stripping gas flow was 150 ml/min. The components released were collected in three different traps corresponding to each period. Recoveries of 100% for most of the components were achieved within 20 min. Only in one case were traces of H_2S detected after 30 min.

Sensitivity and precision of the method

The detection limits of the chromatographic system, defined as signal-to-noise ratios of 2 for the different compounds of interest, are displayed in Table I. The values are similar to those reported previously for methods based on FPD [3,5,7]. The precision of the chromatographic method was determined by repeated injection of standards (four replicates) at the whole range of the response curve. The resulting mean standard deviations and the variation ranges are shown in Table I. Minimal standard deviations were obtained within the 0.5–100 ng S range. As expected, the highest variations were observed near the detection limit. The precision of the entire water sample procedure was determined by analysis of five replicates of a representative sample. Standard deviations are also included in Table I.

Identification and quantitation

Peak identifications were performed by retention time comparison between the GC traces of samples and standard mixtures. The quantitative composition of the reference mixtures was set by adequate operation of the permeation device. These mixtures were repeatedly injected between samples. Calibration curves for each VSC of interest were obtained prior to sample analysis.

The non-linearity of the FPD signal is illus-

TABLE I

DETECTION LIMITS AND PRECISION IN THE ANALYSIS OF AIR, WATER AND SEDIMENT PORE WATER

n.d. = Not determined.

	Detection limits				Precision (% of standard deviation)		
	Absolute (pg S)	5 l air sample (pg S/l)	50 ml water sample (ng S/l)	5 g wet sediment sample (pg S/g)	Gaseous standard mixtures		Water sample replicates
					Mean	Range	
H_2S	40	8	0.8	8	7	1–18	7
COS	25	5	0.5	5	7	0.1–13	5
MeSH	50	10	1.0	10	7	1–15	20
DMS	25	5	0.5	5	6	1–17	6
CS_2	15	3	0.3	3	6	1–16	2
DMDS	10	2	0.2	2	7	4–13	n.d.

trated in Fig. 1, where peak areas *versus* injected masses of sulphur are represented for COS and DMS. The chemiluminescence is generally proportional to $[S]^n$, where S is the amount of sulphur reaching the detector and n ideally equals 2 [13]. Accordingly, linearized plots of log (area) *vs.* log[S] have commonly been used for quantitation (Fig. 1). These plots flatten in the upper portion of the linearized response, which is probably due to analyte autoquenching when large amounts of a sulphur species reach the flame [13]. Obviously, quantitation was only performed within the linearity range.

Application to environmental samples

Field measurements were conducted in a variety of natural environments. Representative chromatograms of VSC in air and water column (from the surface to the deep layer) of a karstic monomictic lake (Cisó, Catalonia, Spain) are shown in Fig. 2. The highest concentrations were found in the anoxic sulphide-rich hypolimnion (3 m depth), where the predominant organic VSCs were MeSH (40 μg S/l) and COS (15 μg S/l) occurring together with very high H_2S amounts generated by sulphate reduction in the bottom part of the lake. Large amounts of CS_2 (34 μg S/l) were occasionally observed. These concentrations decreased sharply in the metalimnion (1.6 m), where MeSH and COS occurred in the 50 ng S/l level. In the oxic epilimnion (0.5 m), the only VSC observed, in addition to H_2S, was COS, which was found at concentration levels of 18 ng S/l. Consistently, COS was the only organic VSC detected in the air over the lake, at a concentration of 0.8 ng S/l, which is one-half of the mean value in the lower troposphere [14]. In brief, in the stratified Ciso Lake system VSC concentrations in the hypolimnion are extremely high, but an elevated bacterial sulphur consumption seems to occur in the metalimnion, considerably decreasing the VSC output to the atmosphere.

One representative gas chromatogram of a water sample from a shallow coastal salt marsh is displayed in Fig. 3A. The VSC composition was clearly dominated by DMS (124 ng S/l). This concentration is in good agreement with values reported for marine coastal areas [15] but somewhat lower than levels found in other salt ponds [16].

The vertical VSC distribution in microbial mats from several solar saltern ponds has also been studied. Enhanced VSC emission due to the osmotic shock of the living organisms was prevented by sediment suspension in water solutions of alkalinity and salinity values similar to

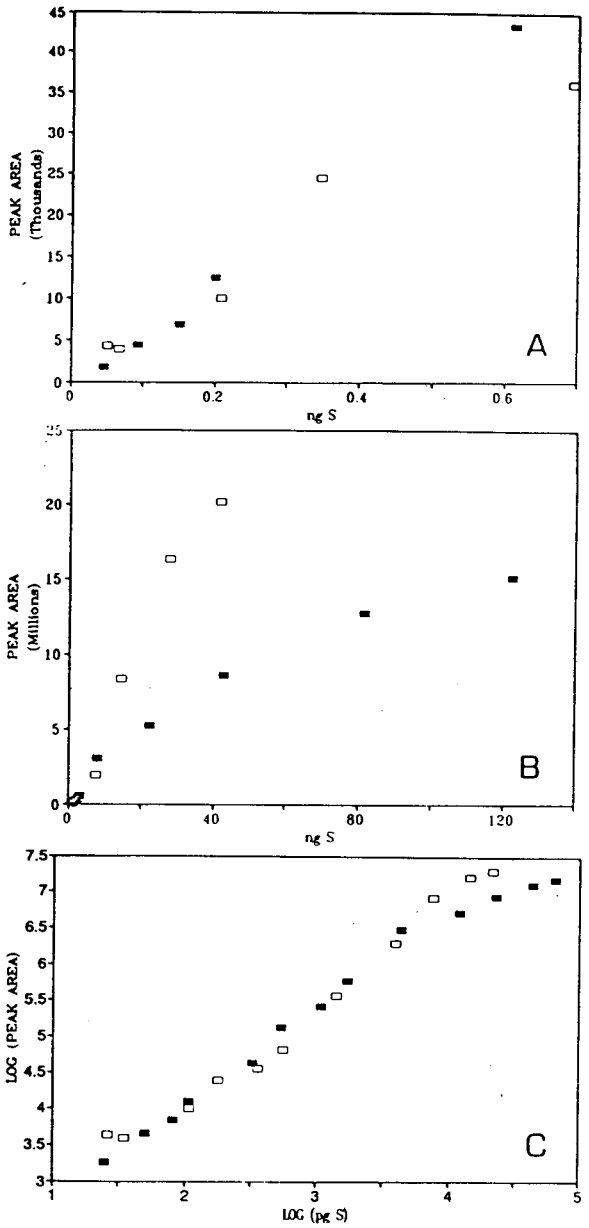

Fig. 1. Characteristic non-linear (A and B) and linearized (C) plots of FPD response *vs.* injected amount of sulphur as (■) COS and (□) DMS. (A) Peak area *vs.* weight of S in the 0.05–0.7 ng range. (B) Peak area *vs.* weight of S in the 1–120 ng range. (C) Log(peak area) *vs.* log(pgS) in the 0.05–120 ng range.

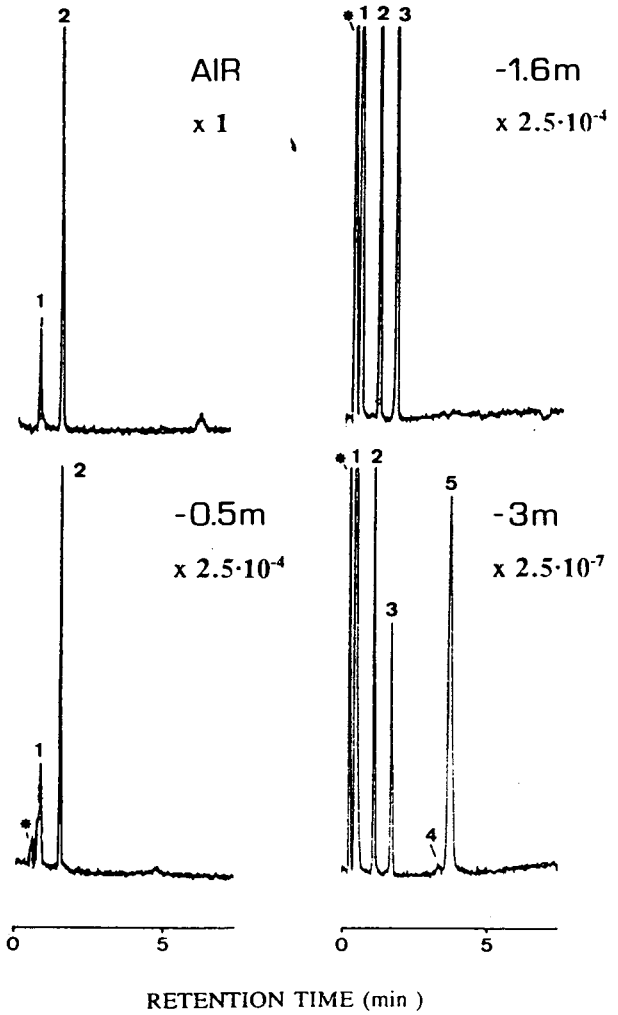

Fig. 2. Gas chromatograms of volatile sulphur compounds in the water column (0.5, 1.6 and 3 m depth) and adjacent air layer of Lake Ciso. Sample dilution is indicated as multiplying factors referred to the air sample. Peak assignments as follows: 1 = H_2S; 2 = COS; 3 = MeSH; 4 = DMS; 5 = CS_2; * = CO_2.

those in the overlying waters. The GC trace resulting from the 3–6 mm depth layer of a cyanobacterial mat essentially composed of *Aphanotece halophytica* is shown in Fig. 3B. This benthic community exhibits a VSC composition very distinct from that observed in the overlying water. In particular, the DMS content is very low, which may be due to either a poor capacity to produce the precursor, dimethylsulphonium propionate, or a strong DMS consumption within the mat system [17]. The difference between the water column and the underlying

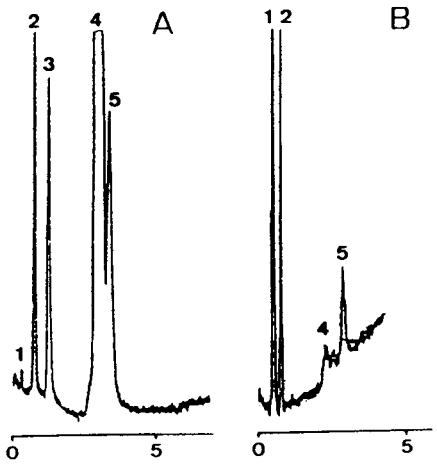

Fig. 3. Gas chromatograms of water (A) and sediment pore water (B) samples from an hypersaline pond containing an *Aphanotece halophytica* mat. Peak assignments as in Fig. 2.

sedimentary benthic community prompts the need for measurements in the diverse compartments of the same natural environment and the development of suitable technology to achieve this goal.

CONCLUSIONS

The methodology presented here enables the collection and simultaneous analysis of trace amounts of H_2S, COS, MeSH, DMS, CS_2 and DMDS in diverse types of environmental samples. Reliable determinations of VSCs in air, waters, and sediment pore waters are achieved with procedures derived from minor modifications of a basic analytical method. The use of cryogenic enrichment traps followed by gas chromatography in a portable apparatus enables the analysis close to the field site, thus minimizing storage times. The VSCs are efficiently separated from bulk co-trapped water by means of cryofocusing in a second cold trap, avoiding possible losses associated with the use of drying devices. This technique has been successfully applied to field studies of VSCs in salt ponds and stratified freshwater lakes.

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Micellar electrokinetic capillary chromatography theory based on conventional chromatography

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ABSTRACT

From the definitions of retention time (t_R) and resolution (R_s) in conventional chromatography, two fundamental equations for the retention behaviour and resolution of neutral solutes are derived and proved to be valid in all cases of micellar electrokinetic capillary chromatography (MECC). Two parameters, phase velocity ratio (P_r) and column availability (A_{co}), are introduced to reveal clearly the relationships and differences between MECC and conventional chromatography. The t_R and R_s values may be either positive or negative in MECC. A negative t_R indicates that the solute migrates toward the positive electrode and a positive t_R toward the negative electrode. $R_s > 0$ means that the solute with a smaller value of the capacity factor (k') in the pair of solutes reaches the detector first, while $R_s < 0$ means that the elution order is the opposite. MECC can be classified into eight cases depending on the values of P_r for convenience of discussion. So far, MECC was usually performed in case IV and the resolution was poorer than that in conventional chromatography for given values of theoretical plate number, selectivity and k'. However, a better resolution can be obtained in cases II, VI and VIII when $P_r < (1 - k')/2$. Cases VI, VIII and II are preferable to case IV for high resolution and should be more frequently employed in the future.

INTRODUCTION

Since micellar electrokinetic capillary chromatography (MECC) was first introduced to extend the power of capillary electrophoresis to the separation of neutral solutes by Terabe *et al.* in 1984 [1], it has exhibited great potential as an effective liquid separation technique [2,3]. With the rapid increase in applications, the basic theory to summarize and predict experimental results is desired, and is also necessary for this new technique to be widely accepted. In a theoretical approach, the distribution mechanism and two fundamental equations to describe the retention behaviour and resolution of neutral solutes have been put forward by Terabe and co-workers [1,4]:

$$t_R = \frac{1 + k'}{1 + (t_0/t_{mc})k'} \cdot t_0 \quad (1)$$

$$R_s = \frac{N^{1/2}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k'_2}{1 + k'_2} \cdot \frac{1 - t_0/t_{mc}}{1 + (t_0/t_{mc})k'_1} \quad (2)$$

where t_R is the retention time of a solute, k' is the capacity factor (moles of the solute in the micellar phase/moles the aqueous phase), t_0 and t_{mc} are the retention times of the aqueous and micellar phases, respectively, R_s is the resolution

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of a pair of solutes, subscripts 1 and 2 refer to the two solutes with $k'_1 < k'_2$, α is the selectivity (k'_2/k'_1) and N is the theoretical plate number.

Based on the eqns. 1 and 2, the retention behaviour and resolution in MECC has been widely investigated [4–9]. However, because eqns. 1 and 2 were derived under the assumption $0 < t_0/t_{mc} < 1$ [1,4], most discussions have been restricted within these narrow limits. In fact, eqns. 1 and 2 can be applied for all values of t_0/t_{mc} from $-\infty$ to ∞, which will be expounded in this paper. It can be seen from eqns. 1 and 2 that t_0/t_{mc} can affect the separation more significantly than k'. In some MECC analyses, a negative t_0/t_{mc} was demonstrated to be preferable to positive values for high resolution [10–13]. However, the theoretical explanation of the retention and resolution in terms of t_0/t_{mc} was inadequate. Gareil [8] developed three sets of retention and resolution equations for the three cases of MECC classified according to the value of t_0/t_{mc} from $-\infty$ to ∞. However, there was a lack of mathematical evidence and continuity [8]. It still remains ambiguous over what range of values t_0/t_{mc} should be employed for higher resolution.

In this paper, eqns. 1 and 2 are mathematically derived from the definitions of t_R and R_s in conventional chromatography without any assumptions. To reveal clearly the relationships and differences between MECC and conventional chromatography, the parameters phase velocity ratio, column availability and virtual column length are introduced, and then concise forms of eqns. 1 and 2 are presented. The retention behaviour and resolution in all cases of MECC are systematically discussed in comparison with those in conventional chromatography, and some conditions for MECC are suggested for improving the resolution.

As the separation is also based on the distribution between two phases, MECC naturally belongs to chromatographic systems according to the definition of chromatography [14]. The fundamental difference between MECC and conventional chromatography is only that both of the phases in MECC are moving. Hence the theory of MECC can be deduced on the basis of conventional chromatography.

THEORY AND DISCUSSION

Retention behaviour

One of the two phases in MECC is the bulk aqueous solution, migrating at a velocity strictly determined by the electroosmotic flow. The other phase is the micelles, migrating at a velocity (V_{mc}) determined by the sum of the electroosmotic velocity (V_{eo}) of the bulk solution and the electrophoretic velocity of the micelles (V_{ep}):

$$V_{mc} = V_{eo} + V_{ep} \tag{3}$$

The velocity is positive when the migration is towards the negative electrode and negative when it is towards the positive electrode [4,5,10]. In this instance, V_{eo}, V_{mc} and V_{ep} may be either positive or negative.

The definition of retention time in MECC is identical with that in conventional chromatography, that is, the time when just half amount of a solute has been eluted from the column or migrated away from the detector. At this moment the following equation is true:

$$V_{R,aq}C_{aq} + V_{R,mc}C_{mc} = V_{c,aq}C_{aq} + V_{c,mc}C_{mc} \tag{4}$$

where $V_{c,aq}$ and $V_{c,mc}$ are the volumes of the aqueous and micellar phases in the column, respectively, $V_{R,aq}$ and $V_{R,mc}$ are the retention volumes of the aqueous and micellar phases, respectively, and C_{aq} and C_{mc} are the equilibrium concentrations of the solute in the aqueous and micellar phases, respectively. The right-hand side of eqn. 4 represents the amount of the solute still remaining in the column and the left-hand side represents that having been eluted from the column. In contrast with conventional chromatography, the left-hand side of eqn. 4 has one more term, because the two phases both flow out the column. Eqn. 4 can be rewritten as

$$\frac{V_{R,aq}}{V_{c,aq}} + \frac{V_{R,mc}}{V_{c,aq}} \cdot \frac{C_{mc}}{C_{aq}} = 1 + \frac{V_{c,mc}}{V_{c,aq}} \cdot \frac{C_{mc}}{C_{aq}} \tag{5}$$

It is evident that $V_{R,aq}/V_{c,aq} = t_R/t_0$ and the right-hand of eqn. 5 is equal to $1 + k'$, as in conventional chromatography. The second term on the left-hand side can be expressed as

$$\frac{V_{R,mc}}{V_{c,aq}} \cdot \frac{C_{mc}}{C_{aq}} = \frac{t_R F_{mc}}{V_{c,aq}} \cdot \frac{C_{mc}}{C_{aq}}$$

$$= \frac{t_R}{V_{c,aq}} \cdot \frac{V_{c,mc}}{t_{mc}} \cdot \frac{C_{mc}}{C_{aq}} = \frac{t_R}{t_{mc}} \cdot k' \qquad (6)$$

where F_{mc} is the volume flow velocity of the micellar phase. By combination with eqn. 5, the fundamental retention equation is derived:

$$t_R = t_0(k' + 1) \cdot \frac{P_r}{(k' + P_r)} \qquad (7)$$

where P_r is the phase velocity ratio and defined as

$$P_r = \frac{V_{eo}}{V_{mc}} = \frac{t_{mc}}{t_0} \qquad (8)$$

It is worth noting that there are no assumptions in the above derivation and eqn. 7 is valid in all cases of MECC. Eqn. 7 is equivalent to eqn. 1 but more concise by using P_r instead of t_{mc}/t_0. The phase velocity ratio (P_r) highlights the relationships and difference in retention behaviour between MECC and conventional chromatography. P_r is the decisive parameter that determines the retention characteristics of MECC. Hence MECC can be classified into eight cases according to the value of P_r for convenience of discussion, as shown in Table I. This classification is made so that the cases of MECC change in turn with the continuously changing experimental conditions.

It can be seen from eqn. 7 that the retention time may be negative in MECC. A negative t_R has been accepted in the literature [5,10,12,13], although it is unnatural in conventional chromatography. A negative t_R indicates that the solute migrates towards the positive electrode and a positive t_R towards the negative electrode [5]. The migration direction of a solute can be seen straightforward from the signs of t_R and the polarity of power supply does not have to be specified. The absolute value of t_R indicates only the time that a solute takes to elute out of the column, if the power polarity is suitable. Hence adding signs to t_R in MECC is not only rational in theory but also meaningful in practice.

Almost all the discussions on t_R and R_s have been in terms of capacity factor [4–9]. In fact, the effect of the phase velocity ratio is more significant. Hence plots of t_R *versus* P_r are shown in Fig. 1, according to eqn. 7. The outline of the retention behaviour of MECC can be seen from

TABLE I

CHARACTERISTICS OF THE RETENTION BEHAVIOUR AND COLUMN AVAILABILITY (A_{co}) IN MECC

Subscripts 1 and 2 refer to a pair of solutes with $k'_1 < k'_2$. MECC is classified into eight cases depending on the value of the phase velocity ratio (P_r).

Case	P_r	Retention behaviour			A_{co}
		Elution order ($t_0 > 0$)	Elution order ($t_0 < 0$)	Absolute value of t_R	
I	0	$0 < t_{mc} < t_{R,2} < t_{R,1} < t_0$	$t_0 < t_{R,1} < t_{R,2} < t_{mc} < 0$	$\lvert t_R \rvert < \lvert t_0 \rvert$	$-1/k'_1$
II	0–1	$0 < t_{mc} < t_{R,2} < t_{R,1} < t_0$	$t_0 < t_{R,1} < t_{R,2} < t_{mc} < 0$	$\lvert t_R \rvert < \lvert t_0 \rvert$	$-1/k'_1$–0
III	1	$t_{mc} = t_{R,2} = t_{R,1} = t_0$	$t_0 = t_{R,1} = t_{R,2} = t_{mc}$	$\lvert t_R \rvert = \lvert t_0 \rvert$	0
IV	1–∞	$0 < t_0 < t_{R,1} < t_{R,2} < t_{mc}$	$t_{mc} < t_{R,2} < t_{R,1} < t_0 < 0$	$\lvert t_R \rvert < \lvert t_0 \rvert(1 + k')$	0–1
V	$-\infty$,∞	$0 < t_0 < t_{R,1} < t_{R,2} < \infty$	$-\infty < t_{R,2} < t_{R,1} < t_0 < 0$	$\lvert t_R \rvert = \lvert t_0 \rvert(1 + k')$	1
VI	$-\infty$ to $-k'_2$	$0 < t_0 < t_{R,1} < t_{R,2} < \infty$	$-\infty < t_{R,2} < t_{R,1} < t_0 < 0$	$\lvert t_R \rvert > \lvert t_0 \rvert(1 + k')$	1–∞
VII	$-k'_2$ to $-k'_1$	$t_{R,2} < 0 < t_{R,1}$	$t_{R,1} < 0 < t_{R,2}$		
VIII	$-k'_1$–0	$-\infty < t_{R,1} < t_{R,2} < t_{mc} < 0$	$0 < t_{mc} < t_{R,2} < t_{R,1} < \infty$	$\lvert t_R \rvert > \lvert t_0 \rvert(1 + k')$ when $P_r < -k'/2$	$-\infty$–0 $A_{co} < -1$ when $P_r < (1 - k'_1)/2$

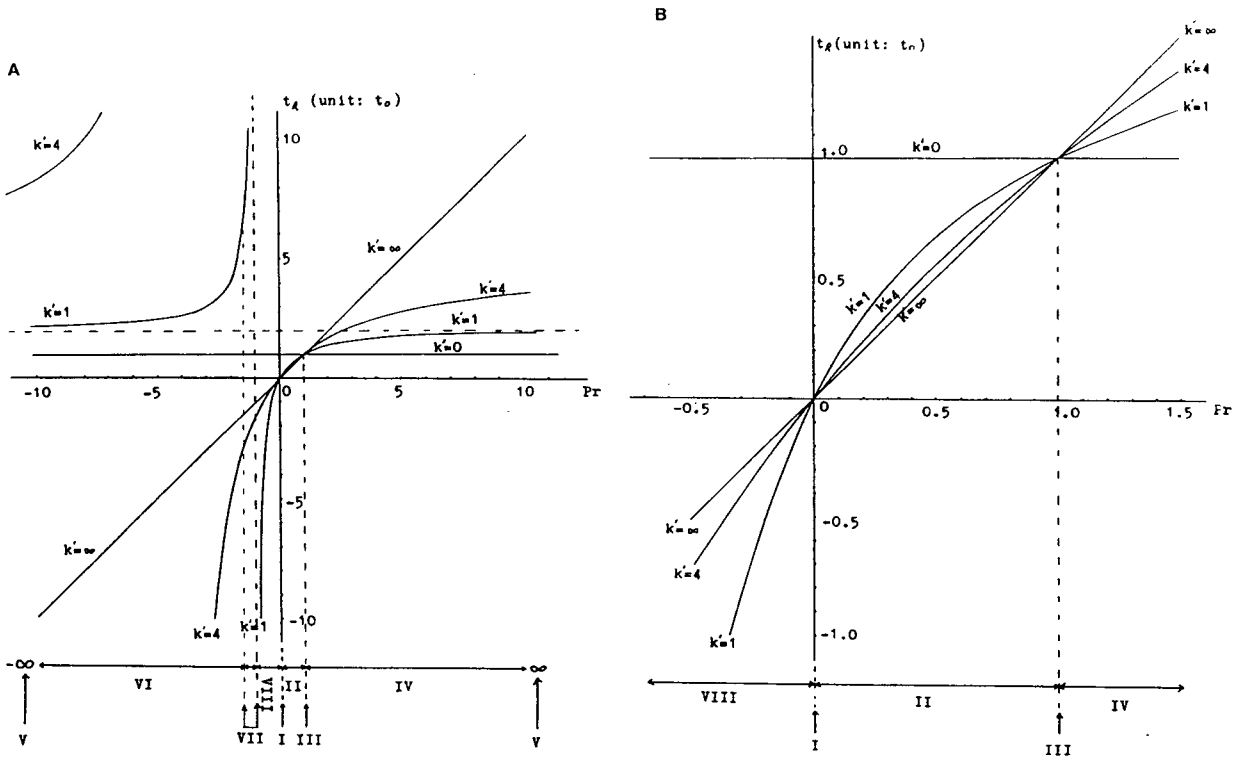

Fig. 1. Dependence of retention time on phase velocity ratio (P_r). The section for case II in (A) is expanded tenfold in (B). The values of the capacity factor are given on each line. The classifications of cases VI–VIII refer to a pair of solutes with $k'_1 = 1$ and $k'_2 = 1.5$. The units on the ordinate are relative to the retention time of the aqueous phase (t_0).

Fig. 1 and the details in each instance are discussed below. Some retention characteristics of the eight cases are summarized in Table I.

Case I: $P_r = 0$. In this case, the aqueous phase is immobile in contrast with the too fast moving micellar phase (see eqn. 8). As $P_r = 0$ and t_0 approaches infinity, the retention equation should be rewritten as $t_R = t_{mc}(1 + k')/k'$ by substituting $P_r t_0 = t_{mc}$ and $P_r = 0$ into eqn. 7. Because the unit of t_R is set as t_0 in Fig. 1, all the lines pass through the origin and the details of the retention behaviour cannot be seen.

Case II: $0 < P_r < 1$. It can be seen from Fig. 1B that the solutes with larger values of k' move faster than those with smaller values of k', thus having shorter retention times in this case, similarly to case I. The elution order in cases I and II seemingly contradicts that in conventional chromatography, but actually it does not. In fact, the micellar phase should be considered as the mobile phase and the aqueous phase as the stationary phase in cases I and II, because the micellar phase moves faster than the aqueous phase ($|V_{mc}| = |V_{eo}|/P_r > |V_{eo}|$). With this consideration, the dependence of t_R on k' would be similar to that in conventional chromatography if the capacity factor was defined as $V_{c,aq}C_{aq}/V_{c,mc}C_{mc}$.

Case III: $P_r = 1$. All the solutes migrate at the same velocity and there is no separation in this case, because the velocity of the micellar phase is equal to that of the aqueous phase and there is no relative movement of the two phases. Substitution of $P_r = 1$ into eqn. 7 yields $t_R = t_0$.

Case IV: $1 < P_r < \infty$. This case is the most frequently encountered in MECC analyses. The retention behaviour in this case has been widely investigated [4–9], which can also be seen from Fig. 1 and Table I.

Case V: $P_r = -\infty$ and ∞. The micellar phase

(pseudo-stationary phase) really becomes stationary in this case, as $V_{mc} = 0$. Hence the situation in this case is identical with that in conventional chromatography. Eqn. 7 turns into the well known equation in conventional chromatography by substituting $P_r = \infty$ into eqn. 7.

Case VI: $-\infty < P_r < -k'_2$. The micellar phase moves in the direction opposite to the aqueous phase in this and the following cases ($P_r < 0$), whereas in cases I–V the two phases move in the same direction ($P_r > 0$). It should be noted that the boundary conditions in this and the following cases are dependent on the capacity factors of the solutes. The discussion here refers to a pair of solutes with $k'_1 < k'_2$. Those solutes with $k' > -P_r$ cannot be detected in this case and their situations belong to case VIII, because they migrate in the opposite direction to the electroosmotic flow. By substituting the inequality $-\infty < P_r < -k'$ in eqn. 7, we obtain $|t_R| > |t_0|(1 + k')$, whereas in case IV $|t_R| < |t_0|(1 + k')$. This means that the retention time in this case is longer than that in case IV.

Case VII: $-k'_2 \leq P_r \leq -k'_1$. By consideration of the range of P_r in eqn. 7, one obtains that $t_{R,1}$ is of the same sign as t_0 but $t_{R,2}$ is not. The pair of solutes do not migrate in the same direction. When $P_r = -k'_2$ or $P_r = -k'_1$, $t_{R,2}$ or $t_{R,1}$ approaches infinity. Hence this case is not acceptable for common separations.

Case VIII: $-k'_1 < P_r < 0$. By substituting the inequality $-k' < P_r < 0$ in eqn. 7, we obtain that t_R has the opposite sign to t_0. In this case, the migration of the solute forced by the electroosmotic flow cannot compensate for the opposite migration of the solute induced by the oppositely moving micellar phase. As a result, the solute migrates in the opposite direction to the electroosmotic flow. The detection end must be set opposite to the electroosmotic flow in this case, in contrast to the other cases. It can be performed simply by reversing the polarity of the power supply. The elution order in this case is similar to that in cases I and II, but contrary to that in the other cases and conventional chromatography, as Fig. 1 shows. In cases I, II and VIII the micellar phase will arrive first at the detector, so those solutes which are inclined to be solubilized by the micellar phase (larger value of k') will elute early. Those solutes with $k' < -P_r$ migrate in the same direction as the electroosmotic flow and will not be detected in this case; their situations belong to case VI.

As an example, the elution order at pH < 3.5 was the reverse of that at pH >6 in the literature [5,11], where the situation at the lower pH corresponded to case VIII and that at the higher pH to case IV. Another example is the reported separation of amines [12], where P_r was −2.5 according to our calculation. Thus, Dns-methylamine ($k' = 0.82$) and Dns-methyl-[$^2H_3$]amine ($k' = 0.81$) migrated in the direction of the electroosmotic flow and were detected at the negative end, and the situation belonged to case VI ($P_r < -k'$), whereas the situation for Dns-hexylamine ($k' = 71.3$) and Dns-octylamine ($k' = 279$) belonged to case VIII ($P_r > -k'$) and the power polarity had to be reversed in order to detect them.

Resolution

The definition of the resolution (R_s) is identical with that in conventional chromatography [15]:

$$R_s = \frac{t_{R,2} - t_{R,1}}{2(\sigma_2 + \sigma_1)} \tag{9}$$

where subscripts 1 and 2 refer to the pair of solutes with $k'_1 < k'_2$ and σ is the standard deviation of the peak. By substituting eqn. 7 into eqn. 9 and using the same assumptions as in conventional chromatography [15], we obtain the approximate resolution equation of MECC:

$$R_s = \frac{N^{1/2}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k'_2}{1 + k'_2} \cdot A_{co} \tag{10}$$

where A_{co} is the column availability, given by

$$A_{co} = \frac{P_r - 1}{P_r + k'_1} \tag{11}$$

Eqn. 10 is equivalent to eqn. 2 and valid in all cases of MECC. The column availability (A_{co}) is an important parameter that determines the characteristics of R_s in MECC, and its physical meaning will be discussed in the next section. Plots of A_{co} *versus* P_r are shown in Fig. 2 and

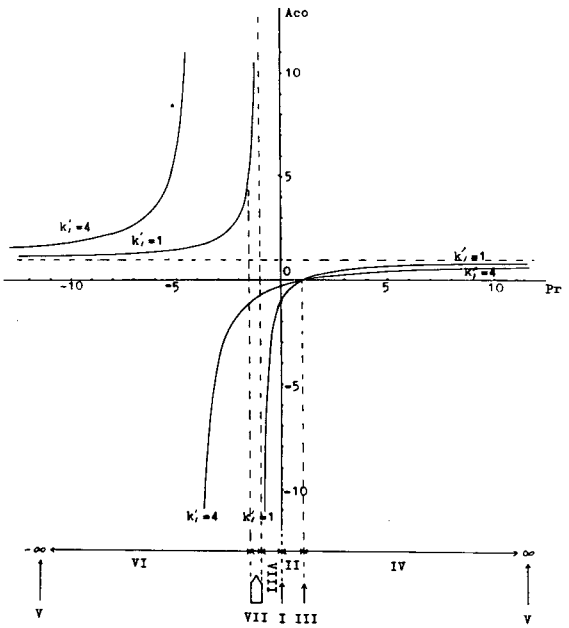

Fig. 2. Dependence of column availability (A_{co}) on phase velocity ratio (P_r). The values of the capacity factor are given on each line. Classifications of cases VI–VIII as in Fig. 1.

some characteristics of A_{co} in the eight cases are summarized in Table I. A_{co} indicates the relationships and differences in resolution between MECC and conventional chromatography, just as P_r does in retention behaviour.

In case I, the aqueous phase is stationary in contrast to the too fast moving micellar phase. The resolution is similar to that in conventional chromatography when the aqueous phase is considered as the stationary phase and the micellar phase as the mobile phase. Eqn. 10 would return to the style in conventional chromatography if the capacity factor was defined as $V_{c,aq}C_{aq}/V_{c,mc}C_{mc}$.

Substitution of $P_r = 1$ into eqn. 11 yields $A_{co} = 0$, and therefore there is no separation in case III. Case V corresponds to conventional chromatography, substitution of $P_r = \infty$ into eqn. 11 yielding $A_{co} = 1$. Case VII is useless for general separations because the pair of solutes will not elute at the same end.

MECC has usually been performed in case IV, where $0 < A_{co} < 1$, as Fig. 2 shows. The resolution resulting from the high plate number is partly offset by the column availability. Hence the resolution did not seem as good as expected when the plate number was as high as 40 000 [1]. Fortunately, better resolution can be obtained in cases VI, VIII and II.

By considering the range of P_r in case VI in eqn. 11, we have $A_{co} > 1$. This means that the resolution in case VI is always better than that in conventional chromatography for the given values of N, α and k', which is the opposite of that in case IV. The improvement in resolution in case VI has been demonstrated experimentally in the literature [10,12]. The deuterated and non-deuterated compounds could be separated in case VI, whereas no separation was observed in the normal mode, case IV [12].

As Fig. 2 shows, R_s is negative in cases I, II and VIII. A negative R_s is unnatural in conventional chromatography, but is valid in MECC. A negative R_s means that the solute with the larger values of k' reaches the detector earlier than the other solute ($|t_{R,2}| < |t_{R,1}|$), whereas a positive R_s means $|t_{R,2}| > |t_{R,1}|$. The signs of R_s clearly indicate the elution order of the pair of solutes, while the absolute value of R_s indicates how good a separation is. It can also be seen from the definition of R_s (eqn. 9) that it will be negative when $t_{R,1} > t_{R,2}$.

To have a better resolution than conventional chromatography, A_{co} should be smaller than -1 in cases II and VIII. By substituting $A_{co} < -1$ into eqn. 11, we have

$$P_r < \frac{1 - k'_1}{2} \tag{12}$$

The P_r range in case VI ($P_r < -k'_2$) is naturally sufficient for the demand of inequality 12, where $A_{co} > 1$. The values of P_r in case IV ($1 < P_r < \infty$) do not meet the requirement of inequality 12, thus $A_{co} < 1$. Hence inequality 12 is the necessary and sufficient condition to obtain a better resolution than in conventional chromatography for a given value of N, α and k'. The resolution in MECC can be greatly enhanced by adjusting the experimental parameters to meet inequality 12, which may be carried out by control of V_{eo} [10,16–18], V_{ep} and k' [4,19–21].

The enhancement of resolution in case VIII has also been demonstrated in the separation of racemic dansylated amino acids [13]. At pH 3 the enantiomers of Dns-DL-methionine ($k' = 1.13$ and 1.15) were completely separated, where the P_r was -0.69 and the situation belonged to case VIII. In contrast, no separation was observed at pH 7 (case IV).

Column availability

As the micellar phase is moving in the MECC procedure, the movement of a solute can be divided into two components. One is the migration of the solute relative to the micellar phase at a velocity V'_s, which is ascribed to the distribution of the solute between the micellar and aqueous phases. The other is that the solute moves with the micellar phase at a velocity V_{mc}, which does not contribute to the separation. We call the former component the effective movement and the latter the ineffective movement. The total migration velocity of the solute (V_s) is the sum of V'_s and V_{mc}:

$$V_s = \frac{L}{t_R} = V'_s + V_{mc} \tag{13}$$

where L is the apparent column length from the injection end to the detection point, sometimes called the effective length [13]. Substitution of eqns. 3 and 7 into eqn. 13 yields

$$V'_s = \frac{-V_{ep}}{1 + k'} \tag{14}$$

We define the virtual column length (L') as the distance that a solute has migrated relative to the micellar phase within the time t_R, or the difference between the distance the micellar phase has moved and the apparent column length:

$$L' = V'_s t_R = L - V_{mc} t_R \tag{15}$$

By substituting eqns. 7 and 14 into eqn. 15 and using eqn. 11, we obtain

$$L' = L A_{co} \tag{16}$$

The virtual column length corresponds to the actual distribution length a solute undergoes. In essence, the physical meaning of L' is identical with that of column length in conventional chromatography. However, L' may be either positive or negative, because the solute may migrate in two ways. A negative L' indicates that the solute moves more slowly than the micellar phase in the same direction and a positive L' that it moves faster.

Eqn. 16 can be rewritten as $A_{co} = L'/L$, so that column availability essentially represents the ratio of the distribution length to the apparent column length. $|A_{co}| < 1$ means that the actual distribution length that is useful for separation is shorter than the apparent column length, whereas $|A_{co}| > 1$ means that the actual distribution length is longer than the apparent column length, hence the resolution can be enhanced. $A_{co} = 0$ means that there is no distribution and therefore no separation.

CONCLUSIONS

The fundamental retention and resolution equations (eqns. 7 and 10) are concise with the introduction of phase velocity ratio and column availability, which highlight the relationships and differences between MECC and conventional chromatography. As the micellar phase may move either in the direction of or opposite to the aqueous phase, t_R and R_s may be either positive or negative. MECC can be classified into eight cases for discussion. Case I, III, V and VII are the mathematical limits, which may not be approached in practical analysis but relate the actual modes in an overall understanding of the separation behaviour. Case IV is the most common mode in the literature but the resolution is not as good as expected. A better resolution can be obtained by adjusting the experimental parameters to meet $P_r < (1 - k'_1)/2$ in cases VI, VIII and II. It should be noted that all the solutes may not elute at the same end in cases VI and VIII.

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Effects of organic mobile phase modifiers on elution and separation of β-blockers in micellar electrokinetic capillary chromatography

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ABSTRACT

A study was made of the effect of organic modifiers (acetone, acetonitrile, ethanol, ethylene glycol, methanol and 2-propanol) in phosphate buffer (0.08 *M*) containing 15 m*M* cetyltrimethylammonium bromide as surfactant on the elution and separation of eleven common β-adrenergic blocking agents. The amount of the modifier was varied from 0.1 to 10.0% (v/v). At maximum addition, the organic solvents increased the viscosity of the buffer solution as follows: acetone 16%, acetonitrile 9%, ethanol 26%, ethylene glycol 27%, methanol 20% and 2-propanol 29%. In contrast to the migration time of the other β-blockers, that of labetalol was not increased by the addition of organic solvent to the buffer solution. Rather, labetalol eluted more quickly with increase in the amount of modifier, and thereby effected changes in the elution order of the β-blockers. The addition of modifiers also affected the resolution, and the best resolution values were achieved with the following amounts of organic solvent in MEKC buffer: acetone 0.1%, acetonitrile 0.1–0.5%, ethanol 5.0–7.5%, ethylene glycol 1.0–2.5%, methanol 5.0% and 2-propanol 1.0–2.5% (v/v). No significant relationship was found between the elution order and separation and the structure of the β-blockers in micellar electrokinetic capillary chromatography with an organic modifier in buffer solutions.

INTRODUCTION

Micellar electrokinetic capillary chromatography (MEKC) is a high-resolution separation method that has been in use since 1984 [1,2]. Many types of molecules are amenable to MEKC, including phenols [1,2], amino acids [3], nucleosides and oligonucleotides [4], nucleic acids [5], chiral substances [6,7] and pharmaceuticals [8–14]. In studying the effects of organic modifiers on separation in MEKC we have been using β-adrenergic blocking agents (β-blockers) as model compounds. These are a therapeutically important group of drugs, chemically derived from the adrenergic agonist iso-

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prenaline [15]. β-Blockers are widely used in the treatment of angina pectoris and cardiac arrhythmias and more recently have been administered for the control of blood pressure and in ophthalmic disease (glaucoma). β-Blockers have also been used to improve athletic performance in cases where sympathetic activity causes the heart to race [16]. The structures of the β-blockers studied are shown in Fig. 1.

MEKC, which is a capillary zone electrophoretic (CZE) technique employing a surfactant above its critical micelle concentration (CMC), permits the separation of neutral and of uncharged molecules in an electroosmotically driven system [3,4]. The separation of neutral solutes is based on their differential partition between the electroosmotically pumped aqueous phase and the hydrophobic interior of the charged "pseudo-stationary" phase, or the micelles, which owing to electrophoretic effects are moving more slowly than the mobile phase. Charged compounds are distributed between the micellar and aqueous phases and simultaneously separated according to their electrophoretic mobilities.

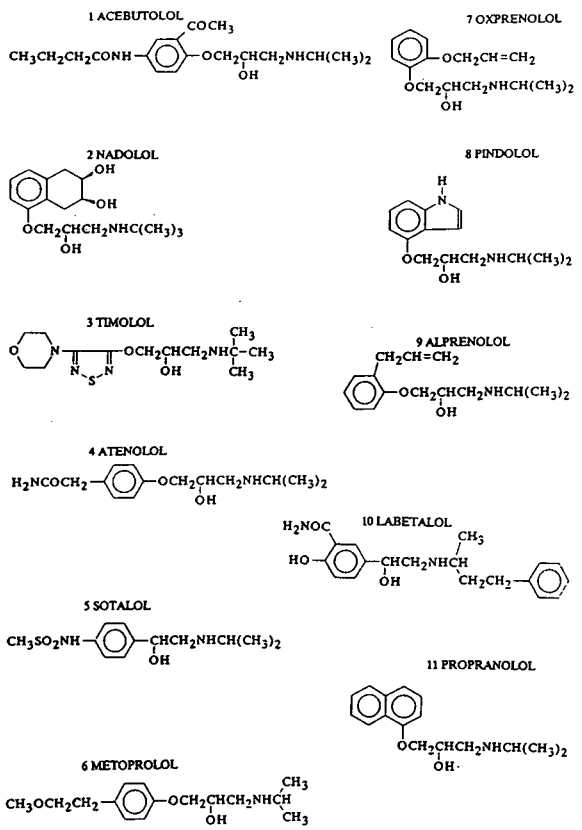

Fig. 1. Structures of β-blockers.

A surfactant exists in the form of micelles when its concentration in solution exceeds the CMC. The CMC and the aggregation number of micelles depend on physico-chemical parameters such as temperature, ionic strength and added electrolytes in a surfactant–water system [17]. Organic modifiers have been found to have several effects in MEKC. Even small concentrations of an organic solvent added to an aqueous micellar system may change the micelle formation and shape by decreasing the CMC [18]. Organic modifiers may also change the size of micelles and the electroosmotic flow (EOF) [19]. As the addition of an organic modifier to the MEKC electrolyte improves the wetting of the capillary wall, the silica surface is modified, with resulting changes in zeta-potential, and consequently in EOF [20]. Organic solvents also cause variations in EOF by changing the viscosity of the buffer solution [19]. Finally, modifiers affect the mass-transfer rates in and out of the micelles [20].

Molecular connectivity indices, introduced by Randic [21] and further developed by Kier and Hall [22], are numerical values describing the molecular structure, and are used to explain quantitatively the behaviour of an analyte in a chromatographic system. These indices have been applied to separations in both HPLC [23] and MEKC [24].

In this work, six organic modifiers (acetone, acetonitrile, ethanol, ethylene glycol, methanol and 2-propanol) of different selectivity [25], potentially useful in MEKC buffer solutions, were studied for their effects on the elution and separation of eleven β-blockers. Of interest were their effects on the corrected migration times ($t_r' = t_r - t_0$) of the β-blockers and on the electroosmotic breakthrough time (t_0). Resolution (R_s) values for each peak pair were calculated by the half-width method. In addition, an attempt was made to explain the migration behaviour of the β-blockers through reference to their structures as described by molecular and molecular connectivity indices.

EXPERIMENTAL

Apparatus

MEKC was performed in 580 × 0.050 mm I.D. fused-silica capillary tubes (Polymicro Technologies, White Associates, Pittsburgh, PA, USA), where the distance from the injector to the detector (L_d) was 500 mm. A Waters Quanta 4000 capillary electrophoresis system (Millipore, Waters Chromatography Division, Milford, MA, USA) was employed. The detection wavelength was 214 nm. All experiments were carried out at ambient temperature. Samples were injected hydrostatically for 15 s and the running voltage was −20 kV. The data (peak height) were collected with an HP 3392A integrator (Hewlett-Packard, Avondale, PA, USA).

The pH of the buffer solution was adjusted to 6.8 with a Jenway (Felsted, UK) Model 3030 pH meter connected to a Jenway electrode containing 4 *M* KCl in saturated AgCl. The electrode system was calibrated with potassium hydrogenphthalate (0.05 *M*, pH 4.00) and sodium tetraborate (0.01 *M*, pH 9.81) solutions.

The viscosities of the buffer solutions were measured with a Model 0.003 SIL viscometer (Gallenkamp, London, UK) at 20, 25, 30, 35 and 40°C. The kinematic viscosities in 10^{-2} m^2 s^{-1} were calculated according to the equation $V = 0.00282t - (7.1/t)$, where t is the measured time in seconds. The amounts of organic solvents added to buffer solutions in the viscosity studies were 0.1, 1.0, 2.5, 5.0 and 10.0% (v/v).

The Molconn X 1.0 program (Lowell H. Hall, Hall Associates Consulting, Eastern Nazarene College, Quincy, MA, USA) was used to calculate the molecular and molecular connectivity indices of the β-blockers up to ten order indices. The Stat View II 1.03 (Abacus Concepts, Berkeley, CA, USA) and Systat 5.1 (Systat, Evanston, IL, USA) procedures were used as the statistical programs. All of these programs were run on a MacIntosh IIsi computer.

Materials

The β-blockers studied were acebutolol hydrochloride, alprenolol hydrochloride, atenolol, labetalol hydrochloride, (±)-metoprolol (+)-tartrate, nadolol, oxprenolol hydrochloride, pindolol, (*S*)-(−)-propranolol hydrochloride, sotalol hydrochloride and timolol maleate, all from Sigma (St. Louis, MO, USA). The reagents used included sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dihydrate, N-cetyl-N,N,N-trimethylammonium bromide (CTAB), acetone, acetonitrile, ethylene glycol and methanol from Merck (Darmstadt, Germany). Ethanol was obtained from Alko (Helsinki, Finland) and 2-propanol from Rathburn Chemicals (Walkerburn, UK). Other reagents were of analytical-reagent grade. A Water-I system from Gelman Sciences (Ann Arbor, MI, USA) was used for ion-exchange of distilled water. All the micellar buffer solutions were filtered through 0.45-μm membrane filters (Millipore, Molsheim, France).

MEKC buffer was prepared from sodium dihydrogenphosphate and disodium hydrogenphosphate solutions containing CTAB so that, after addition of organic modifier, the concentrations were 0.08 *M* for phosphate and 15 m*M* for CTAB. The pH was adjusted to 6.8. The organic modifiers were added to achieve concentrations of 0.1, 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0% (v/v). All solutions were degassed before use. Before each injection, the separation capillary was purged for 2 min with the buffer solution.

RESULTS AND DISCUSSION

The magnitude of the EOF is determined according to the equation

$$\mu_{eo} = (L_d L_t / t_0) V \quad (1)$$

where μ_{eo} is electroosmotic mobility, L_d distance from injector to detector, L_t total capillary length, t_0 migration time of the electroosmotic flow marker and V applied voltage [26]. In this study EOF can be monitored with t_0 values, as the other factors are constant as is evident from eqn. 1; t_0 values were measured with methanol.

The repeatability of the migration of the β-blockers was confirmed by measuring corrected migration times (t_r') and t_0 values as six replicates at concentrations of organic modifier of 0.1, 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0% (v/v). The results for buffer solution when 10.0% (v/v) of acetone was added as modifier are not available because the separation conditions were too unstable for reliable analysis. The relative standard deviation

(R.S.D.) of the t_r' values of β-blockers varied from 0.5 to 9.9% ($n = 6$), and the R.S.D. of t_0 values varied from 0 to 4.3% ($n = 6$). The measurements can be regarded as reliable. However, the day-to-day repeatability was not satisfactory, perhaps because of the lack of temperature control in the apparatus, or because of the precipitation that occurs at the electrodes when phosphate-based buffers are used in MEKC. The changes in migration of β-blockers due to the organic modifiers were therefore measured on the same day.

The consequent changes of EOF, as measured by t_0 values, are shown in Table I. EOF showed an inverse correlation with the viscosity of the organic modifiers, which increased in the order acetonitrile, acetone, methanol, ethanol, ethylene glycol and 2-propanol (Table II). The addition of organic modifier increased the viscosity of the buffer solution. When the amount of acetonitrile was increased from 0.1 to 10.0% (v/v) the viscosity increased by 9.0%. Likewise, the other modifiers increased the viscosity of the buffer solution: acetone 16.0%, ethanol 25.9%, ethylene glycol 26.7%, methanol 19.6% and 2-propanol 28.9% (Table III). It is worth noting that acetonitrile changed the viscosity of the buffer solution much less than did the other organic solvents, and also effected the smallest changes in the migration times of the β-blockers (Fig. 2). The changes in EOF and in the elution behaviour of the β-blockers are probably due to the changes in interactions between the silica wall and the buffer solutions containing different organic modifiers.

TABLE I

THE ELECTROOSMOTIC BREAKTHROUGH TIMES (t_0) IN BUFFER SOLUTION MODIFIED WITH SIX ORGANIC SOLVENTS

The volumes of organic modifier are 0.1% and 10.0% (v/v).

Organic solvent	EOF (min)	
	0.1%	10.0%
Acetone	5.9	6.7
Acetonitrile	5.8	5.7
Ethanol	5.3	6.8
Ethylene glycol	5.8	8.7
Methanol	5.4	7.1
2-Propanol	5.8	8.3

TABLE II

KINEMATIC VISCOSITIES (10^{-2} m^2 s^{-1}) OF THE PHOSPHATE BUFFER SOLUTION AND PURE ORGANIC MODIFIERS AT TEMPERATURES FROM 20 TO 40°C

Solvent	20°C	25°C	30°C	35°C	40°C
Buffer	1.12	0.91	0.81	0.73	0.64
Acetone	0.41	0.36	0.33	0.31	0.29
Acetonitrile	0.40	0.39	0.37	0.35	0.34
Ethanol	1.85	1.36	1.26	1.07	1.09
Ethyl glycol	2.23	2.09	1.82	1.64	1.47
Methanol	0.97	0.66	0.62	0.59	0.54
2-Propanol	2.89	2.38	2.12	1.89	1.64

Except for acetonitrile and labetalol, when the amount of organic modifier was increased from 0.1 to 10.0% (v/v) the migration times (t_r') of the β-blockers increased (Fig. 2). With acetonitrile the migration times decreased or did not change. For labetalol the t_r' values were reduced in all modifiers. Acetonitrile decreased the t_r' of labetalol by 3.3 min, 2-propanol reduced it by 1.5 min and the other solvents reduced it by a few seconds (Fig. 2).

The migration window (difference between the first and last compounds) was decreased by 30% when the addition of acetonitrile was increased from 0.1 to 10.0% (v/v), but it was widened by at least 30% when the other organic solvents were added in increased amounts. The best separations of the β-blockers achieved with the following amounts of organic modifiers: 2-propanol 2.5%, ethylene glycol 1.0% and methanol 5.0% (v/v).

As the migration of labetalol was less affected than that of the other analytes, the elution order of labetalol relative to the other β-blockers changed markedly, but as the amount of modifier increased it co-eluted successively with alprenolol, pindolol, oxprenolol, metoprolol, sotalol, atenolol, timolol and nadolol (Fig. 2). Timolol and atenolol eluted together when the volume of methanol was below 2.5% (v/v) and when acetonitrile was the buffer modifier. Ox-

TABLE III

KINEMATIC VISCOSITIES OF THE PHOSPHATE BUFFER SOLUTION SUPPLEMENTED WITH ORGANIC MODIFIERS AT TEMPERATURES FROM 20 TO 40°C

Temperature (°C)	Kinematic viscosity (10^{-2} m^2 s^{-1})											
	Acetone						Ethylene glycol					
	0.1%	1.0%	2.5%	5.0%	10.0%	Δ (%)	0.1%	1.0%	2.5%	5.0%	10.0%	Δ (%)
20	1.04	1.07	1.10	1.16	1.27	18.1	1.04	1.10	1.15	1.22	1.46	28.8
25	0.93	0.93	0.96	1.05	1.11	16.2	0.92	0.94	0.99	1.08	1.26	27.0
30	0.86	0.88	0.90	0.92	1.04	17.3	0.80	0.83	0.88	0.94	1.11	27.9
35	0.73	0.74	0.77	0.80	0.86	15.1	0.73	0.75	0.79	0.85	0.99	26.3
40	0.65	0.65	0.67	0.71	0.75	13.3	0.66	0.67	0.70	0.75	0.86	23.3
	Acetonitrile						Methanol					
	0.1%	1.0%	2.5%	5.0%	10.0%	Δ (%)	0.1%	1.0%	2.5%	5.0%	10.0%	Δ (%)
20	1.04	1.06	1.08	1.11	1.20	13.3	1.04	1.06	1.10	1.18	1.32	21.2
25	0.93	0.93	0.96	1.00	1.02	8.8	0.92	0.95	0.98	1.04	1.14	19.3
30	0.82	0.83	0.85	0.88	0.91	9.9	0.81	0.82	0.87	0.91	0.99	18.2
35	0.75	0.74	0.75	0.77	0.80	6.2	0.72	0.76	0.77	0.80	0.89	19.1
40	0.66	0.66	0.67	0.69	0.71	7.0	0.63	0.65	0.69	0.71	0.79	20.3
	Ethanol						2-Propanol					
	0.1%	1.0%	2.5%	5.0%	10.0%	Δ (%)	0.1%	1.0%	2.5%	5.0%	10.0%	Δ (%)
20	1.03	1.08	1.12	1.22	1.45	29.0	1.04	1.12	1.17	1.30	1.57	33.8
25	0.93	0.95	0.99	1.08	1.25	25.6	0.93	0.96	1.01	1.10	1.32	29.5
30	0.80	0.85	0.88	0.94	1.09	26.6	0.81	0.85	0.87	0.95	1.13	28.3
35	0.73	0.75	0.77	0.84	0.98	25.5	0.72	0.74	0.79	0.85	0.99	27.3
40	0.65	0.66	0.69	0.75	0.84	22.6	0.64	0.68	0.69	0.75	0.86	25.6

prenolol and pindolol co-eluted when acetonitrile or ethanol was the organic solvent, and also when the volume of ethylene glycol exceeded 5.0% (v/v). Atenolol and sotalol could not be separated from each other in acetone-modified buffers, and they also eluted together when the volume of ethylene glycol exceeded 5.0% (v/v). With 10.0% (v/v) of 2-propanol, atenolol and sotalol co-eluted, as also did oxprenolol and pindolol. In MEKC buffer without an organic modifier labetalol eluted as the tenth compound between alprenolol and propranolol (Fig. 3).

Compounds were considered to elute together if the R_s value between them was less than 0.4. As can be seen in Fig. 4, when acetone or acetonitrile was used, no good separation was obtained for all eleven β-blockers. With the other organic solvents as buffer modifier the best R_s values were achieved in the following ranges: ethanol 5.0–7.5, ethylene glycol 1.0–2.5, methanol 5.0 and 2-propanol 1.0–2.5% (v/v). The separation of the β-blockers can therefore be improved through the addition of a suitable amount of these modifiers to the buffer solution [*e.g.*, 2-propanol 2.5% (v/v)] (Fig. 3).

In order to explain the overall migration behaviour of the solutes in terms of their structures, the molecular and molecular connectivity

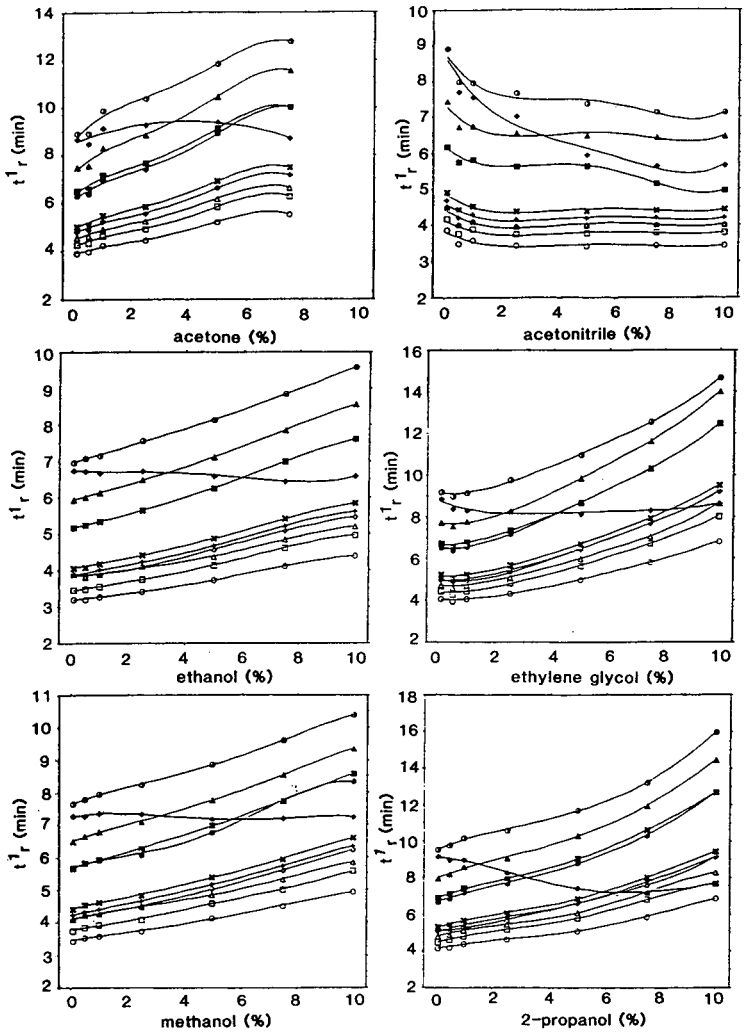

Fig. 2. Migration behaviour of eleven β-blockers in buffer solutions modified with six organic solvents. ○ = 1; □ = 2; △ = 3; ◇ = 4; + = 5; × = 6; ● = 7; ■ = 8; ▲ = 9; ◆ = 10; ◑ = 11 (for compound numbers, see Fig. 1).

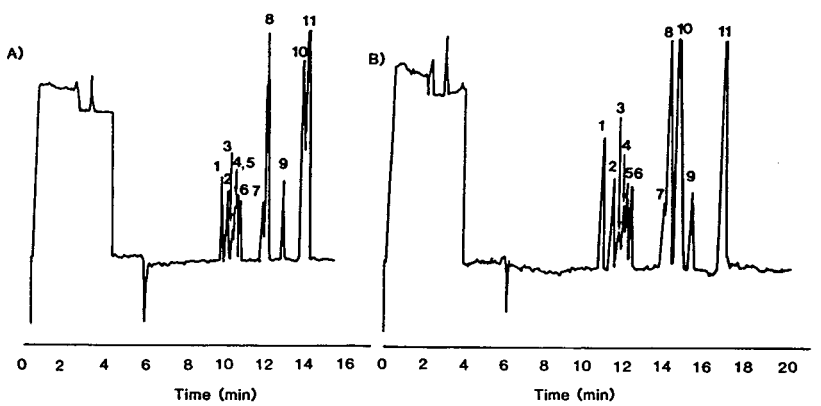

Fig. 3. Electropherograms of eleven β-blockers (A) in phosphate buffer and (B) in 2.5% (v/v) 2-propanol-modified buffer. Separation conditions are given under Experimental and compound numbers in Fig. 1.

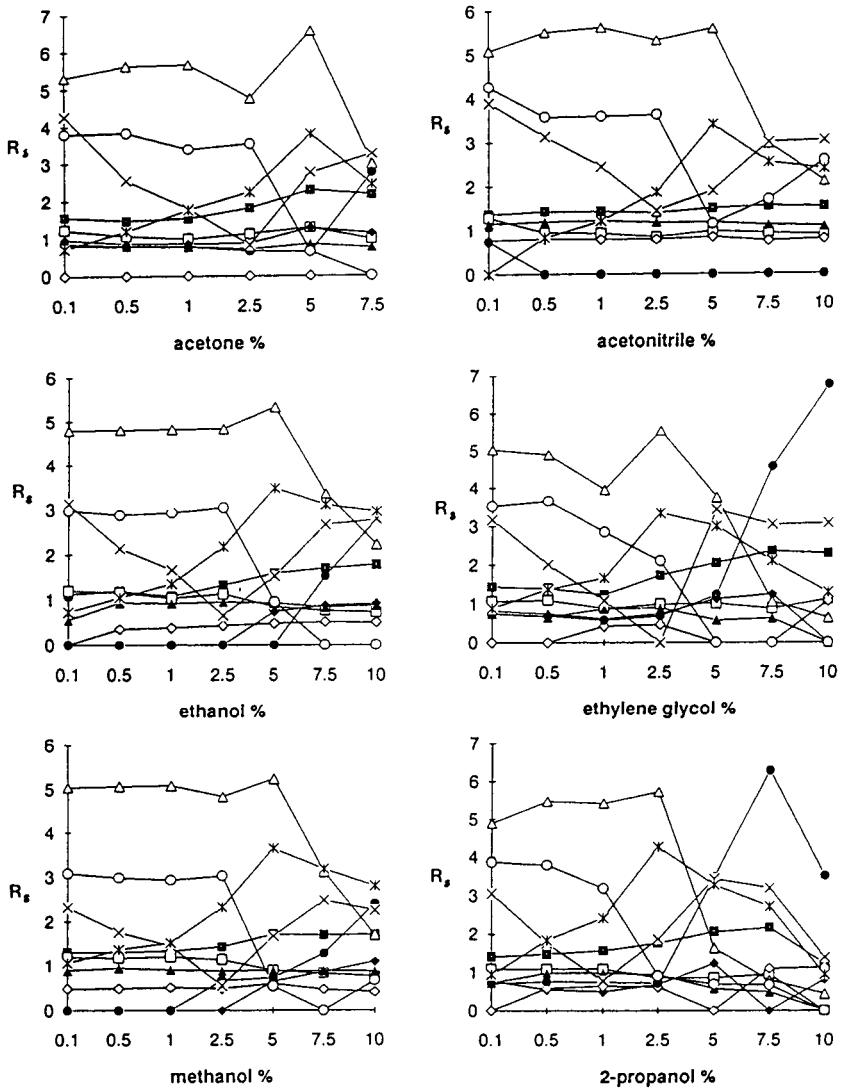

Fig. 4. Plotted resolution values for the β-blockers in different organic-modified buffers. For the identification of R_s values, see elution order from Fig. 2 and compound numbers from Fig. 1. Rx is the resolution value between the compounds x and $x + 1$. The analysis conditions are reported under Experimental. ■ = R1; □ = R2; ◆ = R3; ◇ = R4; ▲ = R5; △ = R6; ● = R7; ○ = R8; × = R9; * = R10.

indices of the β-blockers were related to migration, as in our earlier study [24]. The migration changes were described as migration differences between concentrations of 0.1 and 10.0% (v/v): $\Delta t'_r = t'_{r\ org\ 10.0\%} - t'_{r\ org\ 0.1\%}$. The structural descriptors of the β-blockers did not correlate with the migration changes under the conditions studied. Only 2-propanol produced changes in migration that could be related to structure. The atom indices for atoms 1 and 2 showed some correlation, *i.e.*, $r = 0.92$ and 0.91, respectively. Fig. 5 shows the atom numbering for the carbon skeleton of β-blockers.

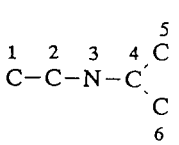

Fig. 5. Atom numbering of the skeleton common to all the β-blockers studied.

Because the molecular indices did not give satisfactory results, a non-numerical approach was used to explain the correlation between the migration and the structures of β-blockers. The exceptional behaviour of labetalol is evidently due to its carboxyl amide and hydroxy groups (Fig. 1). Normally, in dilute acidic or basic solutions, the carboxyl amide group hydrolyses to the corresponding acid, but with labetalol, the carboxyl amide and hydroxy groups form a six-membered ring via either intramolecular or intermolecular hydrogen bonding, so that competition for the ring-forming reaction occurs. These reactions are not possible for the other β-blockers studied as they do not contain carboxyl amide groups (Fig. 1). Formation of the six-membered ring decreases the interactions between labetalol and slowly eluting micelles, and probably explains why unlike the t_r' values of the other β-blockers, that of labetalol was not increased when the amount of organic modifier in MEKC buffers was increased.

CONCLUSIONS

The organic modifiers affected the separation of β-blockers by MEKC in several ways. Except for acetonitrile they lengthened the migration times and widened the migration window. In suitable amounts they improved the resolution values. 2-Propanol seems to be the best choice for the organic modifier in the separation of these eleven β-blockers by MEKC. The most likely explanation for the changes effected by the modifiers is a change in the viscosity of the buffer and consequently in the EOF. The organic modifiers studied increased the viscosity of the buffer solution in the order acetonitrile < acetone < methanol < ethanol < ethylene glycol < 2-propanol, and the magnitude of EOF decreased in approximately the same order. When using organic solvents as buffer modifiers in MEKC, one must also be aware of the exceptions in the migration behaviour. With fewer β-blockers the use of organic modifiers may not be necessary, as adequate separations can sometimes be achieved without them.

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Isotachophoretic desorption of anionic compounds from solid-phase adsorbents

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ABSTRACT

The isotachophoretic (ITP) desorption of anionic compounds adsorbed on solid-phase extraction (SPE) columns is described. Octadecyl (C_{18})-modified silica and styrene–divinylbenzene (ST–DVB) packings were used as the SPE adsorbents. The influence of methanol on the ITP desorption of the test compounds was also examined. The ITP desorption of amaranth red as the test compound from both the ODS and ST–DVB adsorbents showed good results. In contrast, the ITP desorption of bromophenol blue resulted in a discontinuous desorption and no sharp zones were obtained. However, the addition of methanol to both the leading and terminating electrolytes during the ITP desorption in combination with a counter flow improved the desorption of bromophenol blue significantly. The ITP desorption of benzoic acid from the ST–DVB phase showed no reproducible results, whereas its desorption yields from the ODS phase was significantly better.

INTRODUCTION

The application of high-performance liquid chromatography (HPLC) or capillary zone electrophoresis to the determination of compounds in complex matrices or at low concentration levels often necessitates a sample pretreatment step. As demonstrated previously, both zone electrophoresis and isotachophoresis (ITP) can be used as on-line sample pretreatment steps for ionic compounds prior to HPLC analysis [1–3].

Another interesting application of electrophoresis is solid-phase extraction (SPE), followed by the desorption of the compounds by ITP. This technique, which can be termed isotachophoretic desorption, was first described by Kašička and Prusík [4–7]. They used ITP to desorb some proteins from an affinity adsorbent. Other groups have reported the use of zone electrophoresis as the desorption step after SPE (*e.g.*, [8–18]). In principle, the electrophoretic desorption of solutes adsorbed on SPE materials may contribute to a more selective desorption of the target compounds in smaller sample volumes.

In this paper, the preliminary results of the

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ITP desorption of some anionic compounds from C_{18}-modified silica and styrene–divinylbenzene as SPE adsorbents are presented. To correlate the ITP desorption data with the adsorption capacities of the test compounds on both SPE adsorbents, breakthrough curves were also determined by HPLC experiments. Bromophenol blue, benzoic acid and amaranth red were used as test compounds. Because of the limited detection sensitivity of ITP equipment, the experiments were performed in the 10^{-5} mol/l range. The results of the breakthrough and desorption experiments are compared and discussed.

EXPERIMENTAL

Breakthrough volumes

The breakthrough volumes of the test solutes on the investigated SPE phases were measured by frontal analysis. The retention volume of a specific test solute minus 2.33 times the standard deviation was taken as the breakthrough volume [19]. A schematic diagram of the LC equipment for the determination of breakthrough volumes is presented in Fig. 1. This equipment consisted of a Model 100A pump (Beckman Instruments, Palo Alto, CA, USA), a Model PU4225 UV detector (Unicam, Cambridge, UK) and a Valco N-60 HPLC injection valve (VICI-AG Valco Europe, Schenkon, Switzerland). The 10 × 2 mm I.D. dry-packed Kel-F column was positioned in a C270 stainless-steel high pressure column cartridge holder (Upchurch Scientific, Oak Harbor, WA, USA). UV detection was performed at 225 nm. The detector output was recorded with a BD41 potentiometric recorder (Kipp & Zonen, Delft, Netherlands) and a DC268 computer (Tulip, 's-Hertogenbosch, Netherlands) equipped with a laboratory-made Multilab-TS interface. Data handling was performed with Caesar software (B-Wise Software, Geleen, Netherlands). To measure the breakthrough volumes the column was conditioned with methanol. After washing the column with 0.25 ml of acidified demineralized water (pH 3.0), the equipment was flushed with the sample solution. By switching the six-way valve, adsorption of a compound could be measured. The flow-rate through the column was 0.5 ml/min. The samples and the demineralized water for the washing steps were acidified to pH 3.0 by adding 1 *M* hydrochloric acid.

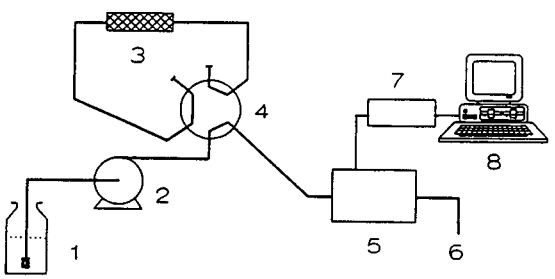

Fig. 1. Schematic diagram of the equipment for the determination of breakthrough volumes. 1 = Eluent; 2 = HPLC pump; 3 = SPE column; 4 = six-way valve; 5 = UV detector; 6 = waste; 7 = interface; 8 = computer.

Isotachophoresis

The ITP equipment for the desorption experiments consisted of a laboratory-constructed ITP apparatus [20] equipped with an in-line laboratory-made SPE column, two three-way Hamilton (Bonaduz, Switzerland) valves and a laboratory-made a.c. conductivity detector. Fig. 2 shows schematic diagrams of the ITP desorption equipment with and without the membrane pump. The I.D. of the 13-cm long PTFE capillary was 1.0 mm (Alltech, Laarne, Belgium) and the applied electric current during the ITP desorption was 100 μA. Kel-F columns of 7 × 2 mm

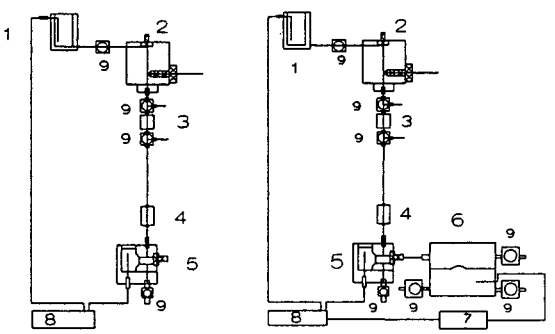

Fig. 2. Schematic diagrams of the ITP desorption equipment, (right) with and (left) without the membrane pump. 1 = Terminating electrolyte compartment; 2 = injection block; 3 = SPE column; 4 = conductivity detector; 5 = leading electrode compartment; 6 = membrane pump; 7 = interface; 8 = high-voltage power supply; 9 = Hamilton valve.

I.D. and 7 × 1 mm I.D. were used for the ITP desorption experiments. The 2-μm A113 polyethylene frits and the Tefzel C283 low-pressure column cartridge holder were from Upchurch Scientific. A number of ITP desorption experiments were also performed by applying a counter-flow membrane pump to increase the ITP desorption time. This pump was controlled by a Brandenburg (Thornton Heath, UK) 807R power supply, which also delivered the constant driving electric current for the ITP desorption experiment. The output of the conductivity detector to monitor the ITP desorption process was recorded and processed with the same data-handling equipment as described under *Breakthrough volumes*.

The leading electrolyte was 0.01 *M* HCl (pH 6.0). The counter ion was histidine and either 0.2% (w/w) hydroxyethylcellulose or 0.05% (w/w) poly(vinyl alcohol) was added to suppress the electroosmotic flow. The terminating electrolyte was a 0.005 *M* morpholinoethanesulphonic acid (MES) solution (pH 6.4) with histidine as the counter ion. If a counter-flow was applied, the terminating electrolyte was 0.00645 *M* MES solution (pH 6.43) with histidine as the counter ion. Conditioning and washing of the column were performed as described under the section *Breakthrough volumes*. After the column had been loaded with the sample, the column was washed with 0.25 ml of water (pH 3.0). Next, the ITP capillaries were filled with the leading and the terminating electrolytes and the desorption was started by applying the high-voltage power supply across the ITP equipment.

Chemicals

Benzoic acid ($pK_a = 4.2$), bromophenol blue ($pK_a = 4.0$), amaranth red, L-histidine and hydrochloric acid were from Merck (Darmstadt, Germany), HPLC-grade methanol from FSA Laboratory Supplies (Loughborough, UK), poly(vinyl alcohol) (Mowiol, $M_r = 44\,000$) from Hoechst (Frankfurt, Germany), morpholinoethanesulphonic acid from Sigma (St. Louis, MO, USA) and hydroxyethylcellulose from Polysciences (Warrington, UK). Water was demineralized with a Milli-Q water purification system (Waters–Millipore, Milford, MA, USA). The 8-μm styrene–divinylbenzene (ST–DVB) copolymer was obtained from Polymer Labs. (Zeist, Netherlands) and the 10-μm C_{18}-modified silica (Zorbax ODS) from Rockland Technologies (New Port, DE, USA).

RESULTS AND DISCUSSION

Breakthrough volume experiments

The capacities of the stationary phases for the test solutes were determined under defined conditions in order to establish what concentration could be loaded on to the SPE columns to perform the subsequent ITP desorption experiments. The results of the measurements of the breakthrough volumes of the test solutes are summarized in Table I. Large differences in the

TABLE I

BREAKTHROUGH VOLUMES OF THE TEST COMPOUNDS ON THE TWO ADSORBENTS

Kel-F column (10 × 2 mm I.D.). All samples were acidified to pH 3.0 by the addition of hydrochloric acid. Flow-rate, 0.5 ml/min.

Experiment	Compound	Concentration (*M*)	Breakthrough volume (ml)	
			ODS	ST–DVB
1	Benzoic acid	$1 \cdot 10^{-6}$	0.8	10.4
2	Benzoic acid	$1 \cdot 10^{-5}$	0.7	7.2
3	Benzoic acid	$1 \cdot 10^{-4}$	0.7	5.7
4	Bromophenol blue	$1 \cdot 10^{-5}$	114	34
5	Amaranth red	$1 \cdot 10^{-5}$	24	9

capacities of the two SPE packing materials for bromophenol blue were obtained. These and similar differences for the other two compounds can be understood from the large differences in the interaction forces between these solutes and the SPE adsorbents, resulting in significant differences in their chromatographic behaviour.

Isotachophoretic desorption experiments

To study the efficiency of the ITP desorption of amaranth red and bromophenol blue from the SPE adsorbents the effluents, from these adsorbents were monitored visually. First 1 ml of a solution containing $1 \cdot 10^{-5}$ *M* of either amaranth red or bromophenol blue was adsorbed on the C_{18}-modified silica or the ST–DVB adsorbent using the 7×2 mm I.D. column as the SPE cartridge. Amaranth red was desorbed by ITP from both the SPE adsorbents as a narrow red zone showing sharp zone boundaries. In addition, after each experiment the remaining SPE packing was extracted with methanol in order to determine whether the ITP desorption process was completed. In the extracts no amaranth red could be observed visually, indicating that the desorption of this compound had been performed efficiently. In contrast, the desorption of bromophenol blue resulted in a non-continuous desorption process with diffuse zones. To investigate whether a local change in the electric field, by the connection of the applied 2 mm I.D. column to a 1 mm I.D. ITP capillary, might disturb the electrodesorption process, the experiments were repeated using the 7×1 mm I.D. column. Also in these experiments the ITP desorption of bromophenol blue resulted in broad diffuse zones.

To establish whether the electroosmotic flow (EOF) could be responsible for the observed irregular desorption patterns of bromophenol blue, 0.05% (w/w) of Mowiol was added to the washing water to suppress the EOF. Also in these experiments no improvement of the desorption of bromophenol blue was obtained.

Generally, ITP processes are considered to be completed when the situation indicated as the steady state has been achieved. To obtain this steady state in relatively short ITP capillaries, a counter flow, opposite to the migration direction of the target compounds, can be applied. ITP desorption experiments of 1 ml of $1 \cdot 10^{-5}$ *M* bromophenol blue solutions applying a counter-flow were performed on both SPE packings in the 7×1 mm I.D. column. In these experiments a significant improvement of the desorption of bromophenol blue from both packings was obtained. These observations indicate that the ITP desorption of this compound can be improved by using longer ITP capillaries.

Next, the effects of the addition of 10% (v/v) of methanol to both the leading and terminating electrolytes on the desorption efficiency of bromophenol blue were investigated. In these experiments also a counter flow was applied during a short period of time. Again a significant improvement of the desorption of bromophenol blue was obtained. We assume that this might be explained by the decreased capacity of the stationary phase for this compound.

It is concluded that the ITP desorption of amaranth red from both SPE adsorbents could easily and completely be performed and resulted in sharp narrow zones. In contrast, the ITP desorption of bromophenol blue proved to be much more complicated and did not result in complete desorption from the SPE adsorbents. Reversed-phase liquid chromatographic stationary phases, which were used as the SPE adsorbents here, may show strongly different behaviour towards these compounds, especially under the aqueous conditions applied here. Therefore, we assume that the large differences in the ITP desorption of amaranth red and bromophenol blue may be explained by their different interaction forces towards the adsorbents.

In the above studies, the isotachophoretic desorption was monitored visually. Applying a conductivity detector in ITP analysis, electropherograms are obtained in which the conductivity signal is plotted on the ordinate as a function of time. In ITP the relative step height of the detector signal provides qualitative information about a solute while the length of a zone provides quantitative information.

To investigate the ITP desorption process further, experiments with benzoic acid as the test compound and applying a conductivity detector

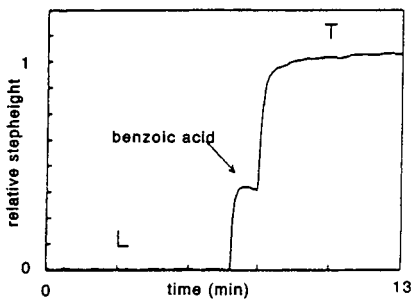

Fig. 3. Isotachopherogram of the electrodesorption of benzoic acid ($4 \cdot 10^{-8}$ mol) from the ST–DVB stationary phase (experiment 4, Table II); L = leading electrolyte, T = terminating electrolyte.

were carried out. The step height of benzoic acid in the applied operational system was 0.43. As an example, Fig. 3 shows an isotachopherogram of benzoic acid. Because the 10×2 mm I.D. column was too large to be fit into the cartridge holder and no evidence was found that a reduced I.D. (using the 7×1 mm I.D. column) improved the ITP desorption process, these electrodesorption experiments were carried out in 7×2 mm I.D. column, packed with either the C_{18}-modified silica or the ST–DVB adsorbent. The calibration graph of the amount of benzoic acid (A, in moles) *versus* the resulting ITP zone length (l, in seconds), determined by repeated direct injections of benzoic acid solutions in the injection compartment of the ITP equipment, was $l = 2.06 \cdot 10^{9} A + 9.2$, with a regression coefficient $r = 0.994$.

The SPE columns were loaded with aqueous solutions of benzoic acid of concentration $2 \cdot 10^{-5}$ or $1 \cdot 10^{-4}$ M. The loading volumes ranged from 0.25 to 2.0 ml of standard solution. As can be seen in Table I, in most instances the amount of benzoic acid loaded on the SPE columns did not exceed the maximum capacity of these columns. The results of the ITP desorption of benzoic acid from both SPE adsorbents are summarized in Table II. From these results, it can be concluded that the recoveries and the repeatabilities of the desorption of benzoic acid from the adsorbents are limited in most instances. The reasons for these observations are not yet clear. Further studies will be focused on the elucidation of the ITP desorption process and especially the role of the nature of the SPE adsorbent.

CONCLUSIONS

Preliminary results on the desorption of amaranth red, bromophenol blue and benzoic acid by ITP from two different SPE adsorbents have been presented. From the data, it can be concluded that for amaranth red and benzoic acid the ITP desorption from both adsorbents resulted in narrow zones with sharp boundaries. However, the recoveries and the repeatability of the ITP desorption process with benzoic acid were poor. The ITP desorption of bromophenol blue showed poor results on both SPE adsorbents. However, a significant improvement of the desorption efficiency could be obtained by the application of a counter flow and the addition of 10% (v/v) of methanol to both ITP

TABLE II

RECOVERY EXPERIMENTS OF BENZOIC ACID BY ITP DESORPTION FROM THE ODS AND THE ST–DVB PHASES AS SPE ADSORBENTS IN THE 7×2 mm I.D. COLUMN

Experiment	Concentration (M)	Adsorbed volume (ml)	Adsorbed amount (mol)	Recovery (%)[a]	
				ODS	ST–DVB
1	$2 \cdot 10^{-5}$	0.25	$5 \cdot 10^{-9}$	90, 110	
2	$2 \cdot 10^{-5}$	0.5	$1 \cdot 10^{-8}$	103, 93	78
3	$2 \cdot 10^{-5}$	1.0	$2 \cdot 10^{-8}$	47, 21	62
4	$2 \cdot 10^{-5}$	2.0	$4 \cdot 10^{-8}$		56
5	$1 \cdot 10^{-4}$	0.5	$5 \cdot 10^{-8}$		84, 34, 66
6	$1 \cdot 10^{-4}$	1.0	$1 \cdot 10^{-7}$		62, 37, 39

[a] ODS-phase: experiments 1–3 are duplicate measurements; ST-DVB-phase: experiments 5 and 6 are triplicate measurements.

electrolytes. Finally, it can be concluded that the ITP desorption of ionic substances from SPE adsorbents may have prospects as a sample treatment technique, although a more extensive study of the background of this technique is necessary.

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Short Communication

Separation and quantitation of nitrobenzenes and their reduction products nitroanilines and phenylenediamines by reversed-phase high-performance liquid chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method for the separation and quantitation of a mixture consisting of nitrobenzene, dinitrobenzene isomers, 1,3,5-trinitrobenzene and their reduction products: aniline, nitroanilines and phenylenediamines has been developed. The method is sensitive and highly reproducible. The mixture is resolved on a Zorbax C_8 column with 0.1% triethylamine and methanol as the mobile phase. The detection limits for individual chemicals at 254 nm are in the range of 25–50 ng.

INTRODUCTION

A number of nitroaromatic compounds have been shown to form as by-products during the manufacture of the explosive 2,4,6-trinitrotoluene (TNT) [1–3]. Among these by-products, dinitrobenzene isomers (*o*-, *m*-, *p*-DNB), 1,3,5-trinitrobenzene (TNB) and 3,5-dinitroaniline (*m*-DNAN) are frequently detected in waste water discharged from TNT production facilities and

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also at open-burn and open-detonation grounds and neighboring areas [1–3]. In addition, the formation of TNB and DNB in the environment by photochemical conversion of discharged TNT and 2,4-dinitrotoluene (2,4-DNT) has also been reported [3]. TNB, DNAN and DNB are mutagenic to *Salmonella typhimurium* [4,5] and were shown to be toxic to various animal species and microorganisms [3]. The DNB isomers are potential inducers of methemoglobinemia in experimental animals and in humans [6–10]. One isomer, *m*-DNB, causes splenomegaly and testicular atrophy in rats [11,12]. Several DNB isomers and TNB form covalent adducts with rat blood proteins [13,14] and with soft tissue DNA [14]. Reduction of the nitro group of DNB isomers and TNB to the corresponding nitroanilines by isolated hepatocytes [15] and by cell free extracts from *Veillonella alkalescence*, *Nocardia* species or phenol adapted bacteria from sewage sludge has been reported [16–18].

In recent years, several liquid chromatographic or gas chromatographic, mass spectrometric methods have been reported for the separation and quantitation of nitroaromatic compounds and their reduction products either obtained commercially or extracted from contaminated soil/water [19–24]. Several metabolites of nitrobenzene (NB) [25] and the DNB isomers [15] were resolved on reversed-phase high-performance liquid chromatography (HPLC). However, a single chromatographic method for the resolution of a mixture containing NB, DNB isomers, TNB, and the reduction products such as nitroanilines and phenylenediamines (*o*-, *m*-, *p*-isomers), is currently not available. In this communication we describe a reversed-phase HPLC method suitable for the resolution and quantitation of such mixtures.

EXPERIMENTAL

Chemicals

NB, *o*-DNB, *m*-DNB, *p*-DNB, aniline (AN), 2-nitroaniline (*o*-NAN), 3-nitroaniline (*m*-NAN), 4-nitroaniline (*p*-NAN), 1,2-phenylenediamine (*o*-PD), 1,3-phenylenediamine (*m*-PD), 1,4-phenylenediamine (*p*-PD), *m*-DNAN and 4-nitroacetanilide (4-NACAL) were obtained (97–99% purity) from Aldrich (Milwaukee, WI, USA). TNB was supplied 99% pure by the US Army Biomedical Research and Development Laboratory, Fort Detrick, Frederick, MD, USA. Methanol (HPLC grade) was obtained from Burdick and Jackson (Muskegon, MI, USA) and triethylamine (sequanal grade) was supplied by Pierce (Rockford, IL, USA) Glass-distilled water was prepared in our laboratory.

HPLC standards

Stock solutions of the individual chemicals were prepared in methanol (100 μg/ml). All stock solutions except *p*-PD (prepared as needed) were stable for at least 2 weeks at −20°C. A working standard mixture (0.5 μg/ml) was prepared daily by mixing 50 μl from each stock solution into a separate vial which was brought up to a final volume of 10 ml.

High-performance liquid chromatography

Millipore/Waters (Milford, MA, USA) HPLC equipped with a 600E solvent controller, a 484 tunable absorbance detector, a Berthold HPLC radioactivity monitor (LB 506 C-1) and interfaced with an Epson Equity III computer was used. Integration and peak quantitation were achieved by using Berthold HPLC Program version 1.43. The effluent was monitored at 254 nm (0.1 AU full scale) and separations were achieved on a Zorbax C_8 column (5 μm particle size, 25 cm × 9.4 mm) connected behind a cartridge guard column (1.25 cm × 4 mm).

Mobile phase and gradient conditions: gradient I. Solvent A: 0.1% triethylamine in water (pH 6.5–6.8); solvent B: methanol. Flow-rate was 3 ml/min and the gradient curve was set at G-5. A–B (90:10) was used as initial condition. The methanol concentration was increased from 10 to 30% B in 10 min, then to 50% B in 50 min and 50% B to 100% B in 5 min. After 5 min at 100% B, the gradient was reversed to initial conditions in 10 min and equilibrated for an additional 10 min before the next sample was injected. Solvents A and B were degassed every day by filtering through 0.22-μm membrane filters. During operation the solvents were sparged with helium gas (60 ml/min).

Mobile phase and gradient conditions: gradient II. This gradient was developed for the separation and quantitation of 4-NACAL from a mixture. A–B (80:20) was used as initial condition. The methanol concentration was linearly (G-6) increased from 20% to 50% in 15 min then to 65% in 25 min, and finally to 100% in 10 min. The column was washed for an additional 5 min and brought back to initial conditions (20% B) in 10 min by reverse gradient and was equilibrated for an additional 10 min at initial conditions before the next sample was injected. The flow-rate was 3 ml/min.

RESULTS AND DISCUSSION

In our early attempts with methanol–water gradients as a mobile phase, phenylenediamines were eluted as broad peaks with significant trailing and with elevated base line. However, the introduction of 0.1% triethylamine (pH 6.5–6.8) in water (solvent A) dramatically improved both the resolution (baseline separation) and the sharpness of all three phenylenediamine peaks. Further increasing the triethylamine concentration to 0.2% did not result in any improvement in the peak resolution or sharpness. Hence, the triethylamine concentration in water was maintained at 0.1% throughout (solvent A). For the separation and quantitation of nitrobenzenes and their possible reduction products on HPLC, several methanol–0.1% triethylamine gradients were employed. Two of the gradients that separated the mixture most effectively (gradients I and II) are described. A mixture, consisting of 13 components (nitrobenzenes, nitroanilines and phenylenediamines), was resolved using gradient I and is shown in Fig. 1. 4-NACAL, a potential metabolite of 1,3-DNB [26] did not resolve from TNB on gradient I. Therefore, gradient II was developed to resolve 4-NACAL from the mixture and is shown in Fig. 2. In gradient II, *o*-NAN co-eluted with *p*-DNB and *o*-DNB co-eluted with *m*-DNB. Therefore, *o*-NAN and *o*-DNB were not included in the mixture. Individual peak identification and their retention times for figures one and two are presented in Table I.

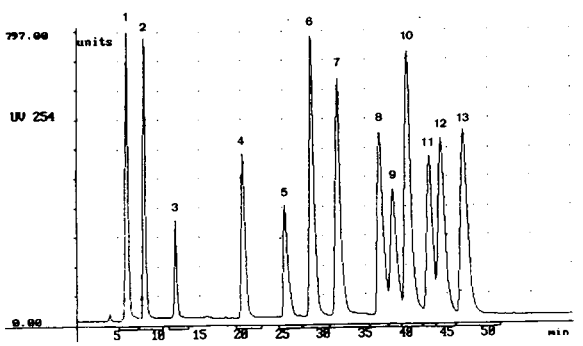

Fig. 1. Separation of a mixture containing nitrobenzenes and their reduction products on Zorbax C_8 column (25 cm × 9.4 mm). Injection volume: 100 μl, each peak represents 5 μg. For other details see Experimental section.

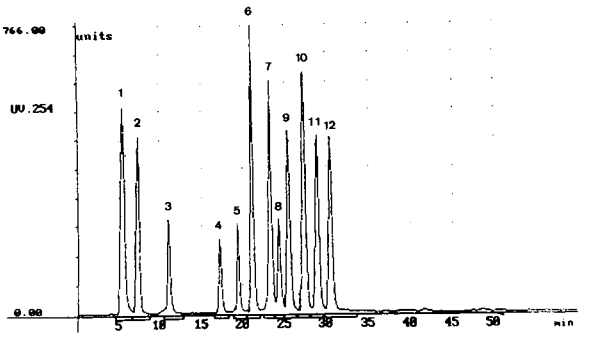

Fig. 2. Separation of a mixture containing nitrobenzenes and reduction products on Zorbax C_8 column (25 cm × 9.5 mm). Injection volume: 100 μl, each peak represents 5 μg. For other details see Experimental section.

Linear regression analysis. Regression analysis was carried out on each peak area against concentration and the correlation co-efficient was between 0.997 and 0.999 over the concentration range of 25–500 ng.

CONCLUSIONS

The method described here allowed a complete resolution and rapid determination of nitrobenzenes and their reduction products and the detection limit was 25–50 ng per chemical. The standard deviation (±S.D.) values presented from six different HPLC runs suggest that the retention times of individual peaks in Figs. 1 and 2 are highly reproducible. This method may be

TABLE I

PEAK IDENTIFICATION AND THE RETENTION TIMES OF CHROMATOGRAMS FROM FIGS. 1 AND 2

Gradients 1 and 2

Peak No.	Compound	Retention times (min:s)[a]	
		Gradient 1	Gradient 2
1	*p*-PD	6:42 ± 0:20	5:25 ± 0:11
2	*m*-PD	9:00 ± 0:24	7:15 ± 0:10
3	*o*-PD	12:54 ± 0:24	11:05 ± 0:05
4	AN	21:00 ± 0:18	17:00 ± 0:15
5	*p*-NAN	26:00 ± 0:18	19:18 ± 0:12
6	*m*-NAN	29:00 ± 0:18	21:06 ± 0:24
7	TNB	32:12 ± 0:18	23:12 ± 0:18
8	*p*-DNB/4-NACAL[b]	37:12 ± 0:12	24:30 ± 0:09
9	*o*-NAN/*p*-DNB[b]	38:37 ± 0:12	25:30 ± 0:19
10	*m*-DNB	40:27 ± 0:12	27:14 ± 0:12
11	*o*-DNB/*m*-DNAN[b]	43:00 ± 0:12	28:46 ± 0:07
12	*m*-DNAN/NB[b]	44:17 ± 0:15	30:22 ± 0:09
13	*NB*	47:06 ± 0:12	

[a] Mean ± S.D. from six HPLC runs.
[b] For gradient 2 (Fig. 2) only.

used to screen and determine the environmental fate of nitrobenzenes that were generated during munition manufacture and discharged into surface waters and soils. It can also be employed for the isolation, identification and quantitation of mammalian and plant metabolites of nitrobenzenes.

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DISCLAIMER

This document has been reviewed in accordance with US Environmental Protection Agency (EPA) and US Army policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. The findings in this report are not to be construed as official US EPA/US Army position unless so designated by other authorized documents.

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Short Communication

Solid-phase extraction of soluble proteins in grape musts☆

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ABSTRACT

Samples of soluble proteins were prepared from grape must by dialysis and solid-phase extraction (SPE) for analysis by reversed-phase HPLC. SPE yielded very good recovery rates and saved time as compared with other methods, *e.g.* concentration and purification of proteins from complex matrices such as grape musts.

INTRODUCTION

Considerable improvements have been achieved in solid-phase extraction (SPE) of late. SPE has been used on its own and in combination with conventional methods (centrifugation, filtration, distillation, precipitation, dialysis, etc.) to clean and concentrate samples of many different types for purification and instrumental analysis. Dialysis is a highly suitable method for preparing soluble proteins from grape musts for analysis by electrophoresis or by HPLC. It is simple to carry out, does not denature the proteins, and allows a number of samples to be prepared concurrently, thereby avoiding distortion caused by interference by high- and low-molecular-mass compounds [1,2]. However, the resulting dialysates must be concentrated to adapt protein levels to the sensitivity of the detectors commonly employed.

Concentration by eliminating solvent in a rotary vacuum evaporator (RVE) or by ultrafiltration is slow, and moreover requires pretreatment of samples.

Lyophilization and reversed dialysis do enable several samples to be treated concurrently but do not yield good results in subsequent analysis of the soluble fraction [3].

SPE affords several advantages when concentrating dialysates of this type: it is fast, simple and capable of treating several samples at the same time. In addition, extracts so treated can also be purified by separating out the high-molecular-mass species that are not removed by dialysis itself (clean-up).

Several authors have employed SPE to concentrate or separate proteins from complex matrices, although most such studies have dealt with only one or a small number of proteins [4,5].

The present study compared dialysis and SPE

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in preparing soluble proteins from grape must samples for RP-HPLC analysis.

MATERIALS AND METHODS

Samples

Grapes of the Airén variety collected in Tomelloso (La Mancha, Spain) were used.

Reagents

Deionized water was used in all solutions. Trifluoracetic acid (TFA) was from Fluka (Buchs, Switzerland). Acetonitrile, methanol (HPLC grade) and orthophosphoric acid (analytical grade) were from Panreac (Barcelona, Spain).

Sample preparation

Musts were obtained by lightly pressing the grapes, followed by filtration through a 0.4-μm nylon membrane.

Proteins were separated by dialysis of 150 ml of must against tap water in a Medicell 2 dialysis membrane for 18 h.

Concentration of dialysates

Two procedures were employed to concentrate the dialysates:

(a) An amount of 75 ml of dialysate was concentrated to 2 ml in an RVE. Water was eliminated at low pressure inside the RVE at a temperature of 30°C in order to avoid denaturation and precipitation of the proteins. The concentrate was filtered through a nylon membrane with a pore size of 0.2 μm.

(b) An extraction column (300-mg Extract-Clean RP-18, Alltech, IL, USA) was preconditioned for SPE by first passing 5 ml of methanol followed by 5 ml of distilled water through the column at a flow-rate of 2 ml/min at 20 mmHg (1 mmHg = 133.322 Pa). An 75-ml volume of dialysate was then passed through the column at a flow-rate of 2 ml/min. The bed, undried, was washed first with 2 ml of distilled water and then with 2 ml of a mixture of distilled water and 85% H_3PO_4 (50:50, v/v) at a flow-rate of 1 ml/min using syringe aspiration. Protein elution was carried out by syringe aspiration with 2 ml of methanol at a flow-rate of 2 ml/min.

RP-HPLC analysis

In line with the method previously described by us [2], the linear gradient of acetonitrile in 0.1% TFA–water ranged from 20% to 80% over 45 min. The flow-rate was 1 ml/min.

A 300-Å Nucleosil C_4 150 × 4.6 mm column (Análisis Vínicos, Tomelloso, Spain) was employed to perform the separations.

The injection volume of concentrated extract was 20 μl, and the detector wavelength was 220 nm.

RESULTS AND DISCUSSION

Fig. 1 shows the analysis of RP-HPLC results for the different fractions of Airén grape must obtained by SPE. Chromatogram d, for the methanolic elution fraction, displays considerable reduction of the polyphenolic front as compared with the results obtained by concentrating the dialysate in an RVE [2,6]. This was due to separation of the front compounds into different fractions; some of these compounds were not retained but were carried along with

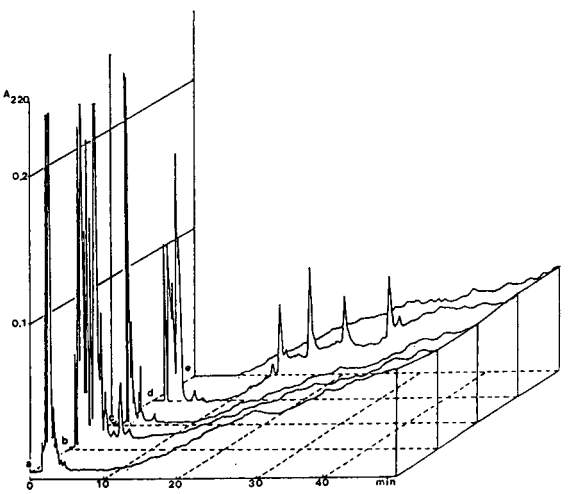

Fig. 1. Chromatogram showing the results of application of RP-HPLC to the different protein fractions of Airén grape must dialysed and concentrated by SPE. (a) Exclusion fraction of the dialysate concentrated 35 times using an RVE. (b) Fraction obtained by washing with distilled water. (c) Fraction obtained by washing with a water–H_3PO_4 mixture (50:50, v/v). (d) Methanolic elution fraction. (e) Methanolic post-elution fraction.

the dialysate, while other compounds eluted during washing (chromatograms b and c).

The reversed-phase SPE columns also acted as precolumns, thereby increasing the working life of the analytical column and enhancing analysis quality.

Elution carried out with 2 ml of methanol, a solvent with high eleutropic power, yielded sufficiently good results for recovery of all the separated proteins (chromatogram d). The appearance of the resulting peaks and the baseline was similar to that obtained for analyses of dialysate concentrated in an RVE [2,6].

Use of the eluting methanolic and acetonitrile fractions in the analyses did not improve recovery. Chromatograms for these analyses were nearly flat (chromatogram e).

The 300-mg extraction columns sufficed to retain the entire protein fraction (2–3 mg). No remaining proteins were detected either in the exclusion fraction of the dialysate afterwards concentrated in an RVE (chromatogram a) or in the washed fractions prior to elution with methanol (chromatograms b and c).

Table I sets out the quantitative data (mean values and relative standard deviations for three replications) for the RP-HPLC analysis and recovery of the soluble proteins from SPE as percentages of the values for the analysis of the RVE-concentrated extract (taken as 100% recovery).

TABLE I

MEAN PERCENTAGE PROTEIN RECOVERY VALUES FOR AIRÉN GRAPE MUST CONCENTRATED BY SPE AND ANALYSED BY RP-HPLC

Magnitude	Protein No.						
	1	2	3	4	5	6	7
t_r(min)	15.1	18.2	21.0	25.0	25.8	27.3	28.2
P_i(%)[a]	20.5	33.3	19.0	1.0	–[b]	19.0	7.1
R.S.D.(%)	6.5	5.0	4.1	7.1		5.5	5.4
R_i(%)[c]	155.3	100.9	87.9	35.0	–	95.2	107.5

[a] Mean percentage value for each protein and R.S.D. calculated for three replications using samples prepared by SPE.

[b] Minor protein (0.9%) determined in the sample concentrated using an RVE [3].

[c] Recovery values for each protein concentrated by SPE as a percentage of the results for samples concentrated using an RVE (100% recovery):

$$R_i(\%) = \frac{\text{Area}_i(\text{SPE})}{\text{Area}_i(\text{RVE})} \times 100$$

On the whole, the differences between the two methods were slight, although higher recoveries were achieved with SPE, which yielded a weighted mean of $R = 108.2\%$[1].

The most significant differences were recorded for the higher recovery (R) of protein 1 by SPE (155.3%) and the lower recoveries of minor proteins 4 and 5, once again as percentages in respect of the results obtained for concentration using an RVE.

$$^1R = \frac{\Sigma P_i \times R_i(\%)}{100}$$

Reproducibility of the SPE-based method was very good (relative standard deviations ranged from 4 to 7%).

In terms of performance, SPE enabled individual samples to be prepared in 30 min, a saving of one-third of the amount of time required when an RVE was used to concentrate the dialysates. Moreover, the method presented the further advantage of being able to process 24 samples simultaneously using commercially available systems (vacuum manifold, Alltech).

SPE makes it possible to automate the entire procedure by incorporating programmable equipment and an automatic injector and thus adapt it for on-line applications. This is an extremely important advantage, because it enables the method to be employed routinely in soluble protein analysis and in producing reliable determinations of the origin of grape musts [2,7] by reducing analysis time and automating sample processing.

CONCLUSIONS

Very good recovery rates were achieved when SPE was utilized to concentrate the soluble proteins in grape musts. Methanol proved to be suitable solvent for elution of the proteins for subsequent analysis by RP-HPLC.

SPE achieved good purification of protein extracts while reducing the time needed for

sample processing, thereby facilitating the application of this method.

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Short Communication

Supercritical fluid extraction of organotins from biological samples and speciation by liquid chromatography and inductively coupled plasma mass spectrometry

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ABSTRACT

Supercritical fluid extraction is used to extract tributyltin and triphenyltin from biological samples. The extraction conditions with carbon dioxide as supercritical fluid (methanol modifier used) are optimized for the organotins from fish tissue certified reference material. The total extraction time is found to be approximately 15 min. The recovery studies at the optimal conditions shows a recovery of 44% for tributyltin and 23% for triphenyltin. The reproducibilities for both the compounds extracted are within 2% R.S.D. The optimum conditions obtained are also used to extract tributyltin and triphenyltin from tuna fish obtained from a local grocery store.

INTRODUCTION

Extractions of trace organic analytes is usually performed by liquid solvents or with a Soxhlet apparatus. These extractions are generally time-consuming and the most error-prone step of an analytical procedure. Recent publications [1–5] have demonstrated the potential of using supercritical fluid extraction (SFE) as an alternative to the time-consuming, less efficient, and less quantitative conventional extraction procedures. Supercritical fluids have several characteristics that make them useful for the rapid and quantitative extraction and recovery of analytes. Properties of supercritical fluids that are attractive from an extraction viewpoint include: diffusion coefficients, density, and viscosity. Diffusion coefficients of supercritical fluids (between 10^{-4} and 10^{-3} cm^2 s^{-1}) are considerably greater than those of liquids (less than 10^{-5} cm^2 s^{-1}) which leads to more efficient and rapid extractions from a variety of sample matrices [6]. The low viscosity and the absence of surface tension in supercritical fluids facilitate pumping and fluid flow in the extraction process. Solvent strength is a function of the density of a supercritical fluid. Densities of supercritical fluids are closer to those of liquids enabling the greater interactions on a molecular level necessary for the solubilization process. With supercritical fluids, densities can be easily controlled by adjusting temperature

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and pressure, and thereby enhancing the solvating power which facilitates extraction of a host of analytes of varying polarity and molecular size [6]. Temperature or pressure changes, near the critical point of the supercritical fluid, can change solute solubilities [6] by as much as a factor of 100, or even 1000. Their lower viscosities and higher solute–fluid binary diffusion coefficients improve mass transfer from solid or liquid matrices, thus decreasing the overall extraction time. The use of modifiers can also increase the solvent strengths of the supercritical fluid for the extraction of polar and high-molecular-mass solutes. Moreover, the use of fluids that have low critical temperatures, such as, CO_2, N_2O and SF_6, allow extractions under thermally mild conditions, thereby protecting thermally labile analytes. The use of a non-toxic supercritical fluid as an extraction solvent offers many advantages over conventional liquid organic solvents in terms of solvent disposal and long term exposure of laboratory personnel to the extracting medium. Supercritical fluids thus provide a greater flexibility in optimizing the extraction conditions for a specific solute or a class of solutes from a complex matrix by changing the extraction pressure and/or temperature.

The analytes of interest are usually extracted by the supercritical fluid and are subsequently analyzed by an appropriate on-line or off-line analytical method. There are also several reports where SFE is directly interfaced with analytical chromatography. There are a number of examples of on-line SFE coupled with supercritical fluid chromatography [7–12] and gas chromatography [13–15] which include several environmental, pharmaceutical and industrial applications. With SFE directly coupled to a chromatographic technique, there is no sample handling required between the extraction and the chromatography stage, and the extraction effluents can be quantitatively and reproducibly transferred for the analysis. On-line SFE requires the SFE parameters, analyte trapping conditions, and the chromatographic separation conditions be understood before a successful analysis can be done. Also, once the on-line analysis is completed, the extract is no longer available for further evaluation using different techniques. Since, supercritical fluids undergo expansive (*i.e.* Joule–Thomson effect) cooling during decompression, even volatile components can be quantitatively and effectively collected off-line in various common solvents. The principal advantage of off-line SFE is that it does not require previous knowledge of the operating conditions and also allows the extract to be analyzed by any appropriate technique, even it is not readily interfaced to SFE. Organotin compounds have numerous applications [16–19], for example, mono and diorganotins are used to stabilize poly(vinyl chloride) (PVC) polymers. Triorganotins are used as biocides, catalysts, wood preservatives, fire retardants and reducing agents, as well as in the pharmaceutical, ceramic and glass industries. Tin compounds have also been used in the production of cans for food storage. These organotin compounds are of high environmental and toxicological concern, since they are released into the environment. A large fraction of the total organotin compounds used as biocides and algicides in antifouling paints have directly entered into the aquatic environment. Tributyltin and triphenyltin have been used as antifouling paints for fish nets and ship hulls. Pollution by triphenyltin is a more serious problem than pollution by tributyltin, since it accumulates in the lipophilic tissues in the fish.

This short communication describes the extraction of tributyltin (TBT) and triphenyltin (TPT) compounds from a fish tissue certified reference material and tuna fish that was obtained from a local grocery store. The extracts obtained were speciated by liquid chromatography with inductively coupled plasma mass spectrometry (LC–ICP-MS).

EXPERIMENTAL

Biological samples

Fish tissue (certified reference material No. 11) was obtained from National Institute for Environmental Studies, Japan Environmental Agency. The tuna fish was from canned tuna obtained at a local grocery store. Tuna was dried in the oven at 90°C and homogenized before the extraction procedure.

Reagents

SFE/SFC-grade carbon dioxide (with the helium head space option) was obtained from Air products (Middletown, OH, USA). Optima grade methanol (Fisher Scientific) was used for extractions. HPLC grade methanol (Fisher Scientific), deionized distilled water (Barnstead, 18 MΩ), reagent grade glacial acetic acid (Fisher Scientific) and certified ACS ammonium acetate (Fisher Scientific) were used to prepare the mobile phase.

SFE instrumentation

The SFE instrument is the Isco model 260D (Lincoln, NE, USA) supercritical fluid extractor. It consists of two pumps: one for the primary fluid, SFE/SFC-grade carbon dioxide and the other for the modifier, optima grade methanol. The temperature and pressure were adjusted to the desired value. The system was operated by first placing the sample into the extraction cell and warming the sample for about 5 min before the extraction process. The extraction procedure was started by letting the extraction cell pressurized with the supercritical fluid. During this process which is known as the static extraction, there is no outflow of the fluid. Static extraction was carried on for three minutes and the valve was then moved to the extract position to facilitate dynamic extraction. During dynamic extractions, the fluid continuously flows through the cell and the extracts were directly collected by placing the extraction cell outlet restrictor in 5 g of methanol. For all the extractions the fluid flow was maintained around 0.65 ml/min. The extracts obtained were injected directly into the chromatographic system.

LC–ICP-MS operating conditions

The extracts were analyzed by employing ion-pair reversed-phase LC–ICP-MS, following the procedure described previously [20]. The HPLC system consisted of a Model 300DX (Dionex, Sunnyvale, CA, USA) A Plasma Quad PQS (Fisons, VG Elemental, Winsford, Cheshire, UK) ICP-MS was used. The PRP-1 column was used with a guard column obtained from Anspec (Ann Arbor, MI, USA). Mobile phase of pH 6 containing methanol–water–acetate (94:5:1) (*i.e.* 0.046 *M* acetic acid and 0.012 *M* of ammonium acetate) was used for the analysis. Sodium pentane sulfonate (0.004 *M*) was used as the ion-pairing agent. The flow-rate of the mobile phase was 1 ml/min. About 2–3% of oxygen was added to the argon nebulizer gas to prevent carbon accumulation on the sampler and skimmer. A forward power of 1500 W was used with an argon coolant flow of 14 l/min, auxiliary flow of 1 l/min, and nebulizer gas flow of 0.8 l/min. The spray chamber was cooled to about −18°C, to enhance solvent condensation. The tin major isotope at m/z 120 (32.37% abundance) was monitored.

RESULTS AND DISCUSSION

Initial studies were performed to find the optimal conditions for the extraction of TBT and TPT in fish tissue. The parameters that were optimized include: extraction time, extraction temperature, extraction pressure and the amount of methanol content which is used as the modifier.

Optimization of the extraction time

The optimum extraction time depends on the pressure, temperature and the flow-rate of the fluid through the extraction cell. For unknown samples, the extraction time can be found by conducting successive extractions. Fig. 1 shows the chromatograms obtained for four successive extractions that were done with 0.14 g of fish tissue at 80°C and 6000 p.s.i. (1 p.s.i. = 6894.76 Pa), with a flow-rate of 0.65 ml/min. The first chromatogram represents a 3-min static extraction, followed by a 12-min dynamic extraction to yield a 15-min total extraction time. The rest of the chromatograms are from the dynamic extractions. These chromatograms indicate that a large amount of the soluble components can be extracted in first 15 min. This suggests that optimization of other parameters involved, might lead to a successful completion of extraction within a 15-min time period. Further optimization was performed by extracting for 20 min (3-min static extraction followed by a 17-min

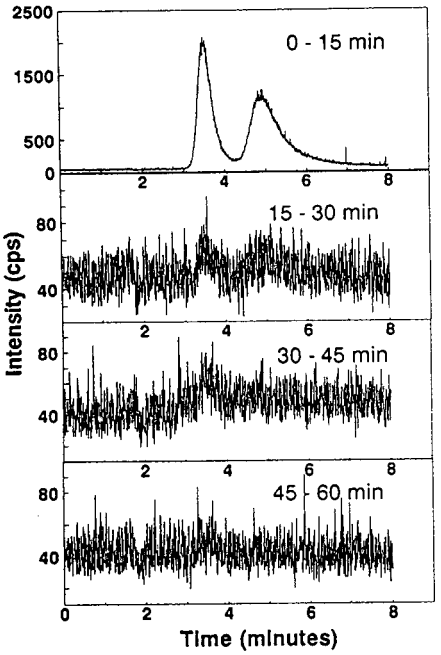

Fig. 1. Chromatograms of tin compounds from 0.14 g fish tissue extract demonstrating the optimization of the extraction time. The intensity scale should be noted.

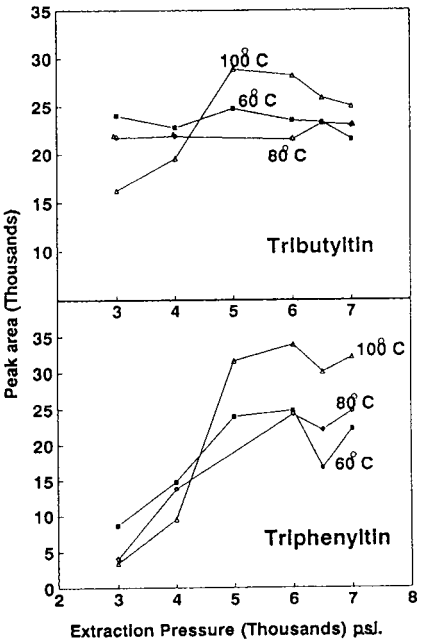

Fig. 2. Optimization of the extraction temperature and pressure for TBT and TPT from 0.14 g of fish tissue (supercritical fluid = CO_2, flow-rate = 0.65 ml/min; static extraction time = 3 min; dynamic extraction time = 17 min; total extraction time = 20 min).

dynamic extraction) at each set of extraction conditions.

Optimization of extraction temperature and pressure

The extractions were performed at temperatures 60, 80 and 100°C. At each temperature, the pressure was varied from 3000 to 7000 p.s.i. These studies were all done without any modifier present in the supercritical fluid carbon dioxide and the flow-rate of the fluid was again maintained at 0.65 ml/min. The effect of temperature and pressure on extraction is shown in Fig. 2. These results indicate that higher amounts of both TBT and TPT were extracted at a temperature of 100°C, when the pressure is higher than 4000 p.s.i. The optimum extraction pressure was found to be at 6000 p.s.i. at all temperatures for TPT, whereas for TBT, the optimum pressure was found to be between 6000 and 6500 p.s.i. Further extractions were done with extraction pressure of 6000 p.s.i. at a temperature of 100°C.

Optimization of the amount of modifier

The use of a binary phase system offers greater flexibility, since the modifier identity and concentration can be easily altered, thereby allowing the adjustment of supercritical fluid for extraction conditions. The modifier used in these extractions was methanol. The addition of methanol to CO_2 increases the solvent polarity, and enhancing the extraction of polar components (TBT and TPT). The effect of the amount of methanol content on extraction is shown in Fig. 3. These observations indicate an increase in the extraction of both TBT and TPT with the increase in the percentage of the modifier used.

Thus, the optimum conditions for TBT and TPT extractions using supercritical carbon dioxide with methanol were found to be CO_2 with

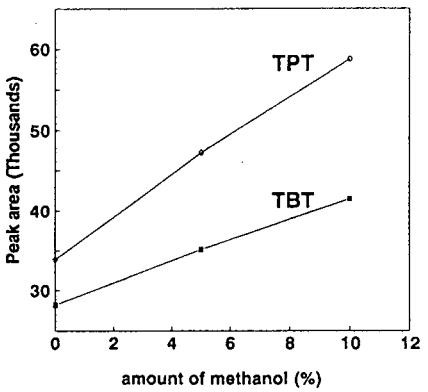

Fig. 3. Effect of the amount of methanol for extractions of TBT and TPT from 0.14 g fish tissue (extraction temperature = 100°C; extraction pressure = 6000 p.s.i.; flow-rate = 0.65 ml/min; static extraction time = 3 min; dynamic extraction time = 17 min; total extraction time = 20 min).

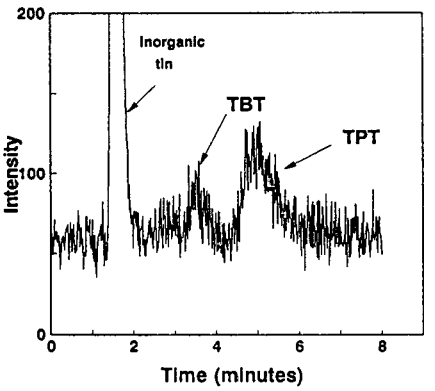

Fig. 4. Chromatogram of tin compounds from the tuna fish extract.

10% methanol, at a temperature of 100°C and a pressure of 6000 p.s.i.

Recovery and reproducibility

The recovery study of these compounds at optimal conditions shows a recovery of 44% (±1.4) for TBT and 23% (±0.9) for TPT, when 0.14 g of fish tissue was extracted. The precision of the extraction method was evaluated by three replicate sample analysis. The recovery study was also done with differing amount of sample sizes using small (0.5 ml and 6.9 mm I.D.), medium (2.5 ml and 7.6 mm I.D.) and large (10 ml and 15.1 mm I.D.) extraction cells. The results are shown in Table I. These results suggest that by keeping the extraction cell small with respect to the sample volume, the degree of sample contact with the extraction fluid increases which is reflected by greater recoveries.

SFE was also employed for extracting organotins from tuna fish. An appreciable amount of inorganic tin was found to be present in the tuna fish extract, which could result from the soldered can that is used to store the tuna. The chromatogram obtained from the tuna fish SFE is shown in Fig. 4. The amount of TBT and TPT were estimated to be about 1.7 ng/g (±2.2) and

TABLE I

RECOVERY OF TBT AND TPT FROM FISH TISSUE (CERTIFIED REFERENCE MATERIAL) BY SUPERCRITICAL FLUID EXTRACTION

Extraction cell	Amount of sample	Recovery of TBT (%) (mean ± S.D., $n = 3$)	Recovery of TPT (%) (mean ± S.D., $n = 3$)
Small (0.5 ml and 6.9 mm I.D.)	0.14 g	44 ± 1.5	23 ± 0.9
Medium (2.5 ml and 7.6 mm I.D.)	0.6 g	39 ± 2.3	22 ± 2.8
Large (10 ml and 15.1 mm I.D.)	3.0 g	31 ± 4.4	17 ± 1.8

3.4 ng/g (±1.6) respectively in 0.2 g of dried tuna fish.

CONCLUSIONS

The potential of using SFE for extracting organotins from fish tissue and tuna fish was demonstrated. It was observed that the total extraction time can be reduced between 15 to 20 min. The amount of sample used was reduced to 0.14 g as compared to 2.5 to 5 g of sample with the solvent extraction method [20]. The reproducibility of the recoveries with SFE were within 2%. However, the low recoveries obtained by SFE suggests procedural modifications, which might include addition of a complexing agent and/or investigating other supercritical fluid/modifier combinations.

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Short Communication

Orthoformates as reagents for derivatization of aminoalkanephosphonic acids for characterization by gas chromatography–mass spectrometry☆

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ABSTRACT

Studies of the derivatization of aminoalkanephosphonic acids by trialkyl orthoformates are reported. Gas chromatographic retention data and low-resolution electron impact mass spectra for the derivatives are presented.

INTRODUCTION

Functionalized alkanephosphonic acids constitute technologically important class of phosphoroorganic compounds [1,2]. Many phosphonic acids, *e.g.*, aminophosphonic acids, also exhibit biological activity and are therefore of pharmacological interest [3,4]. For these reasons their analysis, mostly based on chromatographic methods, is a continuing problem in the analytical chemistry of organophosphorus compounds [5–7].

However, nonvolatile phosphonic acids are only suitable for liquid chromatographic techniques [6,8–11] and their study by gas-phase methods necessitates prior derivatization to volatile compounds, usually diester-based derivatives [5]. Such derivatization, occurring smoothly for several acids derived from phosphorus with a variety of reagents (*e.g.*, silylating agents [9–23], benzyl- and benzacyl-based bromides [23,24], diazoalkanes [25–31]) became more difficult when applied to alkanephosphonic acids bearing an amino function. Thus silylation, widely used in the derivatization of organophosphorous

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acids, when applied to aminoalkanephosphonic acids does not give satisfactory results owing to the lability of the silylamino functions [11,20,32]. Also, the conversion of alkanephosphonic acids by means of diazoalkanes, which leads to diester derivatives [25,30,31], cannot be recommended for the direct esterification of aminophosphonic acids owing to a non-selective N-alkylation of the amino group [33,34].

Therefore, the derivatization procedures of aminoalkanephosphonic acids include the protection stage of the amino function (in the form of a Schiff base [20], isothiocyanate [20] or amide [20,29,35]) in addition to the esterification of the phosphonic moiety. O,O-Diethyl 1-aminoalkanephosphonates were also found to be suitable for gas chromatographic (GC) and gas chromatographic–mass spectrometric (GC–MS) characterization [36].

Esters of phosphonic and polyphosphonic acids [37], N-trifluoroacetyl derivatives of aminoalkanephosphonic acids [35] and free 1-aminoalkanephosphonic acids (synthetic application) [38] have also been obtained using trialkyl orthoformates. In the last application, the formation of the corresponding 1-(N-formylamino)alkanephosphonate diesters has been reported [38]. Recently we have reported on the scope of the derivatization of functionalized alkanephosphonic acids by means of orthoformates [39].

In this paper, we present our findings on the application of orthoformates to the derivatization of various aminoalkanephosphonic acids.

EXPERIMENTAL

Materials

Trimethyl and triethyl orthoformates, trifluoroacetic acid and tridecane were purchased form Aldrich (Milwaukee, WI, USA). Triisopropyl orthoformate was prepared according to ref. 40 and aminoalkanephosphonic acids according to refs. 41–43. All compounds applied were of the purity previously reported.

Preparation of derivatives

The reaction was carried out in Wheaton 1-ml microproduct V-vials equipped with a spin vane, placed in a thermostated oil-bath. The samples of aminoalkanephosphonic acids (0.1–5 mg) were suspended in a mixture of trifluoroacetic acid (0.075 ml) and orthoformate (0.30 ml) and the suspensions were stirred at 120°C (at 100°C for trimethyl orthoformate) for 2 h. Aliquots of these reactions mixtures were diluted with acetonitrile (0.2 ml) and analysed.

Gas chromatography

Gas chromatographic analyses were performed with a Chrom 5 gas chromatograph equipped with a flame ionization detector and a CI 100 integrator (Laboratórní Přístoje, Prague, Czech Republic). The columns were 2.8 m × 4 mm I.D. glass columns packed with 5% OV-17 on acid-washed and silanized Chromosorb W AW DMCS (80–100 mesh). Helium was used as the carrier gas and the flow-rate was adjusted to 30 ml min^{-1} at 200°C. The injector and the detector temperatures were maintained at 250°C.

Mass spectrometry

An LKB 2091 GCMS instrument was used to record the low-resolution mass spectra of the derivatives. Sample introduction was via the chromatographic column inlet, a 2.7 m × 2 mm I.D. glass coil packed with 3% OV-17 on 80–100-mesh Waraport. The initial column temperature was 50°C, programmed at 10°C min^{-1} to 250°C. Electron ionization mass spectra were recorded at an electron energy of 70 eV, ion source temperature 250°C and accelerating voltage 3.5 kV.

A Finnigan MAT 95 mass spectrometer was used for the GC–MS analysis of multi-component mixtures of derivatives. Sample introduction was via a Varian 3400 gas chromatograph equipped with a 25 m × 0.22 mm I.D. BP-5 capillary column (SGE). The column temperature was 100°C for 2 min, then programmed at 10°C min^{-1} to 250°C. The injector temperature was maintained at 200°C and the transfer line temperature was 250°C. The column was directly introduced to the ion source of the mass spectrometer. Mass spectra were recorded at an electron energy of 70 eV.

^{31}P NMR

^{31}P NMR spectra were recorded on a Bruker AC 200 spectrometer operating at 81.01 MHz.

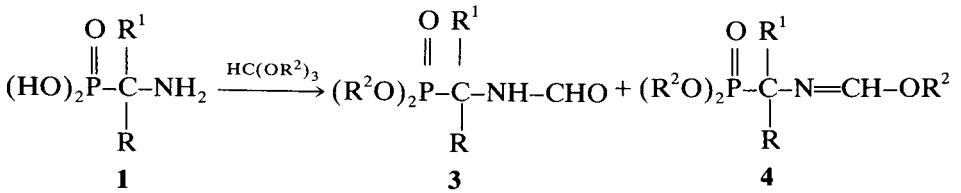
RESULTS AND DISCUSSION

Derivatization

The esterification of aminoalkanephosphonic acids (**1**) by means of trialkyl orthoformate afforded a mixture of diester derivatives (**3** and **4**), according to eqn. 1 [the formation of the diester derivative **2**, with a free amino function, was found to occur only in trace amounts, during esterification of 1-amino-1-methylethanephosphonic acid (**1h**)].

The structural assignment of derivatives **3** and **4** was accomplished on the basis of the chromatographic and mass spectral analysis of compounds **3**, **3A** and **4** obtained during esterification of 1-aminoethanephosphonic acid [Ala(P); **1a**] and 1-(N-methylamino)ethanephosphonic acid [Me-Ala(P); **1Aa**] by means of triethyl and trimethyl orthoformate, respectively [39].

The structure of aminoalkanephosphonic acids and the acidic catalysis exert a substantial influence on the course of derivatization and the



(1)

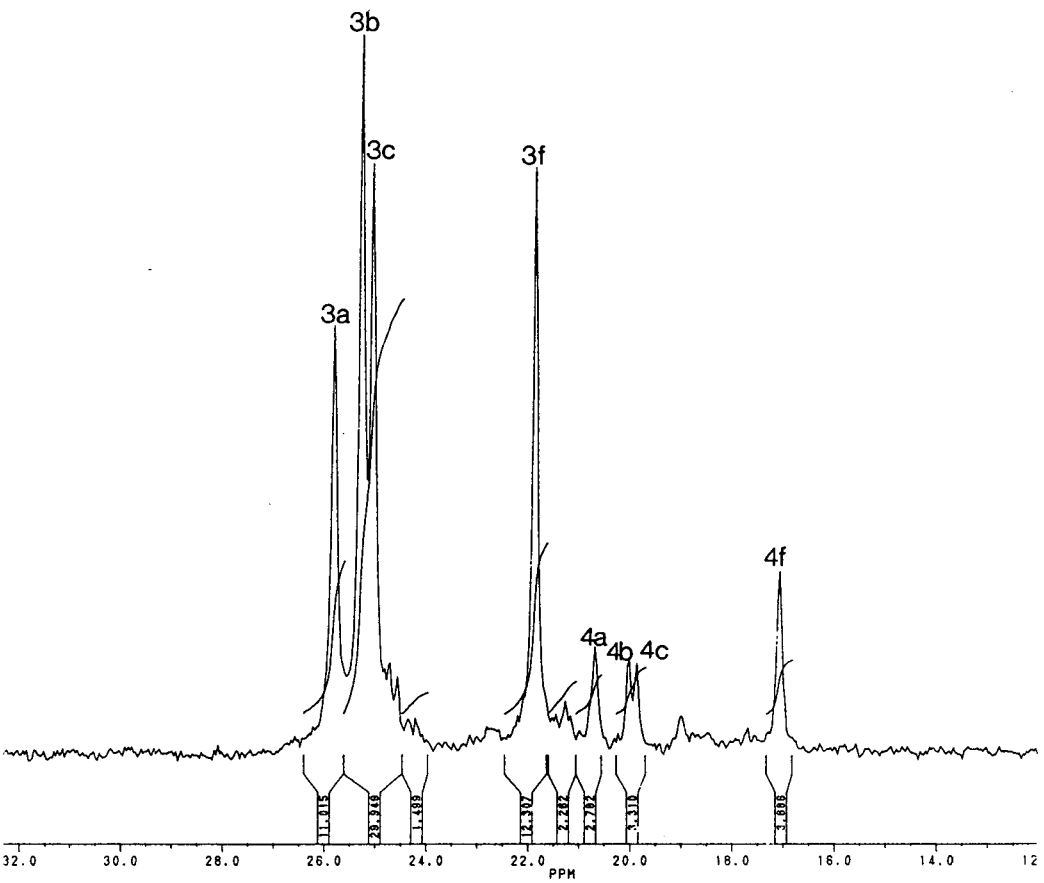


Fig. 1. ^{31}P NMR spectrum of the derivatization products of the mixture of aminophosphonic acids **1a**, **1b**, **1c** and **1f** obtained by means of triethyl orthoformate. Conditions as described under Experimental.

reaction rate [39]. Thus, the optimum reaction conditions were obtained in the reaction system consisting of triethyl orthoformate, trifluoroacetic acid and aminoalkanephosphonic acid in amounts of 0.30 ml, 0.075 ml and 1 mg, respectively. In this rection system the amino acids **1** were found to form homogeneous solutions in which at 120°C the derivatizations were usually completed in 1 h. The results on the reaction course of various types of aminophosphonic acids with trialkyl orthoformates will be presented elsewhere.

The optimization of the reaction course was carried out using ^{31}P NMR monitoring. These investigations revealed the complete conversion of aminoalkanephosphonic acids to mixtures of derivatives **3** and **4** with only small amounts of unidentified by-products. The ^{31}P NMR spectrum of the derivatization products of a mixture of amino acids **1a**, **1b**, **1c** and **1f** for the reaction run under standard conditions is presented in Fig. 1. A comparison of this and other representative procedures for the derivatization of aminoalkanephosphonic acids is given in Table I. It is worth noting that in earlier reports [11,29,35] the application of ^{31}P NMR spectroscopic investigations for optimization and confirmation of the quantitative course of the derivatization procedure was omitted.

*Chromatographic properties of derivatives **3** and **4***

Two sets of peaks of varying height were obtained on OV-17 for all the compounds studied. Analysis by mass spectrometry revealed that the first compounds eluted are formino-alkoxy derivatives **4** and the second are N-formyl derivatives **3**. These derivatives could be stored for several weeks at 0°C without extensive decomposition and were found to be suitable for characterization by means of GC. Both types of compounds give reproducible retention data. The results for their relative retention volumes (*vs.* tridecane) as a function of temperature (OV-17) are given in Fig. 2. The separation of derivatives **3** and **4** obtained from derivatization of the mixture of aminoalkanephosphonic acids **1a**, **1b**, **1c** and **1f** is presented on the Fig. 3.

TABLE I

COMPARISON WITH REPRESENTATIVE METHODS OF AMINOALKANEPHOSPHONIC ACIDS DERIVATIZATION

Conversion to derivative	Reagent[a]	Time (h)	Temperature (°C)	Range (mg)	Ref.
(I) O,O-Bis(trimethylsilyl)	(Ms. pr.)	≤8		1	11
1-(N-acetylamino)alkane-	(1) BSTFA–TMSC	≤4	80		
phosphonate	(2) Py–Ac_2O	0.5	80		
	(3) BSTFA–TMSC	0.2	80		
(II) O,O-Bis(trimethylsilyl)	(Ms. pr.)	≤8		1	11
1-(2-propenimine)-	(1) BSTFA–TMSC	≤4	80		
alkanephosphonate	(2) Acetone	0.5	80		
	(3) BSTFA–TMSC	0.2	80		
(III) O,O-Bis(trimethylsilyl)	(Ms. pr.)	16–18		1	11
1-isothiocyanato-alkanephosphonate	(1) BSTFA–TMSC	≤4	80		
	(2) CS_2	ca. 12	20		
(IV) O,O-Dibutyl	(Ms. pr.)	*ca.* 3		10	29
1-(N-trifluoroacetyl-amino)alkane-	(1) TFA–TFAA	1	40	0.25–0.5	
phosphonate	(2) Diazobutane	0.2	20		
(V) O,O-diethyl	(Ms. pr)	*ca.* 10		10	35
1-(N-trifluoroacetyl-amino)alkane	(1) TFA–TFAA	1	20		
phosphonate	(2) $HC(OEt)_3$	8	r.t.[b]		
(VI) This work	TFA–$HC(OEt)_3$	2	120		

[a] Ac = acetyl; Ac_2O = acetic anhydride; BSTFA = bis(trimethylsilyl)trifluoroacetamide; TFA = trifluoroacetic acid; TFAA = trifluoroacetic anhydride; TMSC = trimethylchlorosilane; Py = pyridine; Ms. pr. = multi-stage procedure.

[b] Reflux temperature.

TABLE II

REDUCED MASS SPECTRA OF DERIVATIVES OF 3

No.	Derivative of 3		Molecular formula (mass)	Mass spectrometry: m/z (relative intensity, %)											
	$R(R^1 \neq H)$	$O\text{-}R^2$		M + 1	M	Others									
3a	Me	O-Et	$C_7H_{16}NPO_4$	210	209	192	181	166	155	138	111	82	72	44	
			209.9	(1.2)	(3.3)	(1.3)	(2.5)	(4.5)	(17)	(54)	(60)	(50)	(50)	(100)	
3ba	Et	O-Me	$C_6H_{14}NPO_4$	196	195	180	166	152	138	137	127	111	110	86	58
			195.2	(0.6)	(4.3)	(0.4)	(3.6)	(2.3)	(3.1)	(2.1)	(13)	(12)	(28)	(100)	(63)
3b	Et	O-Et	$C_8H_{18}NPO_4$	224	223	208	180	178	166	138	86				
			223.3	(1.6)	(8.9)	(0.7)	(3.2)	(4.1)	(3.9)	(2.3)	(100)				
3ca	Bu	O-Me	$C_8H_{18}NPO_4$	224	223	194	181	180	167	166	138	114	110	86	
			223.2	(0.5)	(1.0)	(1.2)	(2.4)	(1.7)	(2.7)	(2.6)	(2.6)	(100)	(16)	(16)	
3c	Bu	O-Et	$C_{10}H_{22}NPO_4$	252	251	222	209	195	166	155	138	114	86		
			251.4	(2.8)	(2.4)	(3.3)	(6.8)	(4.9)	(3.3)	(14)	(50)	(100)	(51)		
3d	$EtSCH_2$	O-Et	$C_9H_{20}NPO_4S$	270	269	240	226	224	212	209	208	191	139	138	131
			269.3	(3.5)	(8.0)	(1.0)	(2.3)	(31)	(1.2)	(5.3)	(6.4)	(15)	(8.9)	(51)	(100)
3e	$EtSCH_2CH_2$	O-Et	$C_{10}H_{22}NPO_4S$	284	283	254	223	208	195	180	167	139	124	75	
			283.3	(2.4)	(13)	(3.7)	(5.0)	(20)	(100)	(7.5)	(16)	(25)	(12)	(38)	
3f	Ph	O-Et	$C_{12}H_{18}NPO_4$	272	271	243	242	226	197	162	155	134	138	106	
			271.2	(1.3)	(7.0)	(0.9)	(1.3)	(0.8)	(0.4)	(5.6)	(5.9)	(100)	(1.8)	(33)	
3g	$PhCH_2$	O-Et	$C_{13}H_{20}NPO_4$	286	285	242	241	240	194	166	148	147	139	138	
			285.2	(2.5)	(4.9)	(1.5)	(3.1)	(8.1)	(10)	(16)	(16)	(100)	(2.1)	(26)	
3h	Me (Me)	O-Et	$C_8H_{18}NPO_4$	224	223	205	194	180	155	138	111	86	82	58	
			223.3	(3.1)	(0.6)	(1.6)	(0.9)	(4.1)	(6.8)	(13)	(11)	(100)	(11)	(10)	

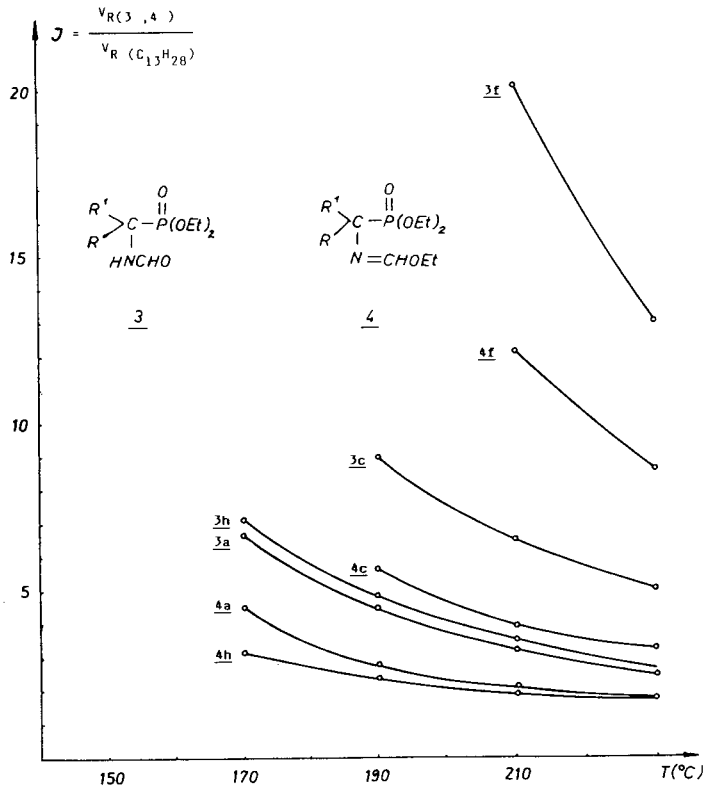


Fig. 2. Relationship between relative retention volume (*J vs.* tridecane) and temperature for the N-formyl (**3**) and N-forminoethoxy (**4**) derivatives.

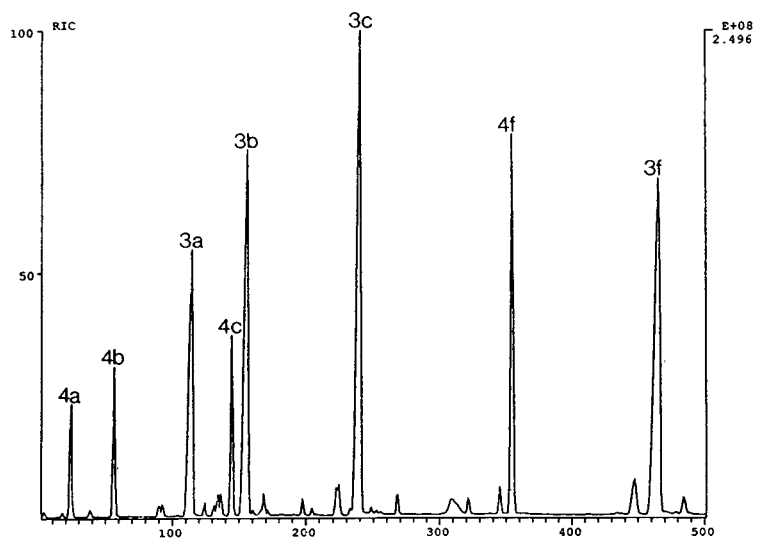


Fig. 3. GC–MS of the derivatization products of the mixture of aminophosphonic acids **1a**, **1b**, **1c** and **1f** obtained by means of triethyl orthoformate. Conditions as described under Experimental. The ^{31}P NMR spectrum of the derivatization products of this reaction mixture is presented in Fig. 1.

TABLE III

REDUCED MASS SPECTRA OF DERIVATIVES OF **4**

No.	Derivative of **4**		Molecular formula (mass)	Mass spectrometry: m/z (relative intensity, %)											
	$R(R^1 \neq H)$	$O\text{-}R^2$		M + 1	M	M − 1	Others								
4a	Me	O-Et	$C_9H_{20}NPO_4$	238	237	208	191	180	166	152	138	100	72	44	
			137.3	(0.5)	(0.8)	(5.9)	(0.5)	(2.7)	(4.2)	(6.9)	(1.8)	(100)	(37)	(33)	
4ba	Et	O-Me	$C_7H_{16}NPO_4$	210	209	194	180	166	152	151	149	137	109	100	
			209.3	(0.1)	(0.4)	(3.6)	(1.4)	(0.9)	(1.6)	(1.4)	(4.3)	(3.9)	(7.1)	(100)	
4b	Et	O-Et	$C_{10}H_{22}NPO_4$	252	251	250	222	194	180	166	138	123	114	86	
			251.4	(0.5)	(0.3)	(0.1)	(3.7)	(1.9)	(1.7)	(3.9)	(2.3)	(1.2)	(100)	(25)	
4ca	Bu	O-Me	$C_9H_{20}NPO_4$	238	237	236	222	180	179	128	109	99	93	72	
			237.2	(−)	(0.2)	(0.3)	(1.0)	(1.1)	(1.0)	(100)	(4.4)	(3.3)	(8.1)	(20)	
4c	Bu	O-Et	$C_{12}H_{26}NPO_4$	280	279	278	264	250	222	208	194	166	165	142	114
			279.4	(0.5)	(0.2)	(0.2)	(0.3)	(3.5)	(3.1)	(0.7)	(5.0)	(2.5)	(3.4)	(100)	(16)
4d	$EtSCH_2$	O-Et	$C_{11}H_{24}NPO_4S$	298	297	268	251	237	225	224	166	160	139	138	100
			297.4	(2.7)	(5.1)	(1.3)	(3.0)	(12)	(2.5)	(4.4)	(11)	(13)	(3.4)	(15)	(100)
4e	$EtSCH_2CH_2$	O-Et	$C_{12}H_{26}NPO_4S$	312	311	310	283	282	236	223	194	166	152	114	75
			311.4	(2.0)	(6.2)	(0.7)	(6.8)	(19)	(26)	(46)	(19)	(21)	(30)	(52)	(100)
4f	Ph	O-Et	$C_{14}H_{22}NPO_4$	300	299	298	227	199	187	162	134	117	106	91	79
			299.3	(0.2)	(0.4)	(0.3)	(0.2)	(0.4)	(0.4)	(100)	(13)	(1.1)	(14)	(4.3)	(8)
4g	$PhCH_2$	O-Et	$C_{15}H_{24}NPO_4$	314	313	312	284	268	256	240	222	176	175	146	138
			313.3	(3.0)	(6.0)	(8.2)	(5.2)	(1.5)	(1.9)	(2.5)	(38)	(100)	(35)	(40)	(31)
4h	Me (Me)	O-Et	$C_{10}H_{22}NPO_4$	252	251	236	222	194	180	151	138	114	86	58	
			251.4	(0.1)	(0.2)	(0.2)	(0.4)	(0.2)	(0.5)	(0.5)	(0.6)	(100)	(16)	(18)	

Mass spectral properties of derivatives 3 and 4

N-Formylaminoalkanephosphonates (3). The partial spectra of N-formyl derivatives **3** are summarized in Table II. The molecular ions $[M]^{+\cdot}$ (and $[M+1]^+$) of these derivatives were observed in low abundance. Charge localization on the nitrogen atom produced ions $[M-(R^2O)_2P(O)]^+$ ($[M-109]^+$ or $[M-137]^+$ for the O-methyl or O-ethyl derivatives, respectively) and $[M-R]^+$ ions (R is the alkyl chain at C-1) resulting from α-cleavage. The ions $[M-(R^1O)_2P(O)]^+$ or $[RCH=NH_2]^+$ represent the base peaks for majority of the N-formyl derivatives **3**. For derivatives **3** with R < Et the latter ions were dominant. This tendency turned in favour of $[M-(R^1O)_2P(O)]^+$ for higher homologues of **3** (R > Et), especially for the O-methyl esters (**3ba** and **3ca**). All compounds **3** exhibited in distinct abundance (1.7–5.1%, except for **3f**) the ions $[M-43]^+$, formed presumably by elimination of a molecule of isocyanic acid from the molecular ions of **3**.

The mass spectra of the sulphur-containing derivatives **3d** and **3e** differ from those of the other compounds **3**. They contained molecular ions $[M]^{+\cdot}$ and $[M+1]^+$ in relatively higher abundance. The base peaks were $[M-R+1]^+$ for **3e**, formed by elimination a molecule of olefin from the molecular ion, and $[M-(EtO)_2P(O)H]^+$ for **3d**. The ions $[M-(EtO)_2P(O)]^+$ and $[RCH=NH_2]^+$ (for **3e**) were of relatively low abundance (1.4% and 5.4%, respectively).

The ions at *m/z* 138, 137, 110 and 82 were characteristic of the presence of the diethylphosphonate system (or the ion at *m/z* 109 and related ions for O-methyl esters **3**) and had the mechanism of formation reported previously [44].

Forminoalkoxyalkanephosphonates (4). The partial spectra of derivatives **4** are summarized in Table III. Molecular ions of these derivatives were observed in low abundance and the latter decreased slightly with increasing molecular mass (0.8% in **4a** to 0.2% in **4c**). The presence of a sulphur atom in the molecule of compounds **4** caused a substantial increase in the molecular ions (5.7% in **4d** and 6.2% in **4e**).

Fragmentation was found to be predominantly directed by charge localization on the nitrogen atom. α-Cleavage, resulting in the loss of the O,O-diethyl phosphonate moiety, $[M-137]^+$, produced the base peaks most of the forminoethoxyphosphonates **4**, with the exception for sulphur-containing derivatives (13% for **4d** and 21% for **4e**). Further cleavage of the C-1–C-2 bond in compounds **4** produced the ions $[M-R]^+$, *m/z* = 180 for O-methyl and *m/z* = 222 for O-ethyl derivatives, respectively. The third α-cleavage (C-1–H) resulted in the formation of ions $[M-1]^+$, which were observed in low abundance in the spectra of **4b**, **4ca**, **4c**, **4e** and **4f** (0.1–0.3%), and in distinct abundance in the spectrum of **4g** (12%). The ion at *m/z* 223 in **4d** and **4e** was produced by the migration of an aliphatic hydrogen to the phosphonate moiety and the loss of the alkyl chain by cleavage at C-1–C-2, respectively. Its higher abundance in the spectrum of **4e** implied its formation via a McLafferty rearrangement from the molecular ion. The ions at $[M-58]^+$ and $[M-72]^+$ (for O-methyl and O-ethyl derivatives) resulted from the α-cleavage of the imine moiety from the molecular ions. These ions were accompanied by $[M-57]^+$ and $[M-71]^+$ ions derived from the migration of aliphatic hydrogen from the alkoxyimine function to the phosphonate moiety and subsequent cleavage of the C-1–N bond in compounds **4**.

CONCLUSIONS

A general derivatization procedure for aminoalkanephosphonic acids by means of triethyl orthoformate has been developed. ^{31}P NMR spectroscopic investigations confirmed the complete conversion of 1-aminoalkanephosphonic acids into volatile diester derivatives in 2 h, which is faster than in earlier procedures. The ester products of this reaction, N-formyl derivatives **3** and forminoalkoxy derivatives **4**, are satisfactory for characterization by both GC and combined GC–MS. The mass spectra of derivatives **3** and **4** were structurally informative. Ions characteristic of the phosphonate system were present in the mass spectra of all derivatives **3** and **4**, permitting rapid identification of these compounds.

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Short Communication

Trifluoroacetylation of muricholic acids and hyocholic acids

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ABSTRACT

Trifluoroacetylation of α-muricholic acid and α- and β-hyocholic acids is incomplete under routine conditions (at room temperature with trifluoroacetic anhydride for 30 min), whereas β-muricholic acid reacts completely. Complete trifluoroacetylation of these bile acids was achieved by reaction at room temperature for 16–24 h. Trifluoroacetylation of α-muricholic acid at room temperature for 48 h or at a higher temperature (50°C) for 0.5–1.0 h led to another unknown peak.

INTRODUCTION[a]

Methyl ester trimethylsilyl ethers of bile acids are popular derivatives for their determination by gas–liquid chromatography. However, it is well known that the derivatives tend to make a flame ionization detector dirty in a short period.

Trifluoroacetylation of a hydroxyl group is also frequently adopted in the analysis of bile acids by gas–liquid chromatography [2–7]. Usually, an excess amount of trifluoroacetic anhydride is added to bile acid methyl esters and left for 15–30 min or more at room temperature [4]. Most of the bile acids are quantitatively trifluoroacetylated by this procedures, but α-muricholic acid and α- and β-hyocholic acids are not, as described by Sjövall [8] and Mott *et al.* [9].

We describe here a method for the trifluoroacetylation of these bile acids.

EXPERIMENTAL

The α- and β-muricholic acids and β-hyocholic acid were kindly supplied by Dr. Isao

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[a] Nomenclature for bile acids was based on a recent proposal by Hofmann *et al.* [1]. In this paper the following trivial names are used: α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid; β-hyocholic acid, 3α,6α,7β-trihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; 7-epicholic acid, 3α,7β,12α-trihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-hydroxy-5β-cholanoic acid.

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Horibe (Shionogi Research Laboratories, Osaka, Japan). α-Hyocholic acid was purchased from Calbiochem-Behring (San Diego, CA, USA). Trifluoroacetic anhydride was obtained from Wako (Osaka, Japan).

Bile acids were dissolved in small amounts of methanol and methylated with freshly prepared diazomethane. The reaction mixture was evaporated to dryness under a stream of nitrogen and reduced pressure. Then, 0.3 ml of trifluoroacetic anhydride was added to the bile acid methyl esters. The test-tubes containing the reaction mixtures were capped and left for various periods (0.5–48 h) at room temperature, 0°C or 50°C. The reaction mixture was evaporated to dryness under a stream of nitrogen and reduced pressure.

The bile acid derivatives were dissolved in carbon disulphide–methyl ethyl ketone (1:1, v/v) and analysed by gas–liquid chromatography using a Hewlett-Packard (Avondale, PA, USA) HP 5890A gas chromatograph equipped with a hydrogen flame ionization detector. A 15 m × 0.25 mm I.D. capillary column coated with DB-17 (J & W Scientific, Folsom, CA, USA) was used. The operating conditions were as follows: oven temperature, increased from 200°C (held for 1 min) to 280°C at 7.5°C/min; detector temperature, 300°C; injection port temperature, 280°C; carrier gas, helium at 25 cm/s; splitting ratio, 1:50; data processor, HP 3393A; automatic sampler, HP 7673A.

RESULTS

Fig. 1 shows the chromatograms of methyl α-muricholate (Me-αMC) trifluoroacetylated for 0.5 h and 16 h at room temperature, Me-αMC trifluoroacetylated for 0.5 h gave two major peaks (5.48 and 10.27 min) and one minor peak (10.90 min), while the bile acid trifluoroacetylated for 16 h gave only one peak at 5.48 min.

Table I shows the effects of reaction temperature and reaction time. When Me-αMC was trifluoroacetylated for a shorter duration or at a lower temperature, peaks with longer retention times (10.27 and 10.90 min) appeared, but they disappeared when the trifluoroacetylation reaction was performed for 16–25 h at room temperature. However, when the reaction was performed for 48 h at room temperature or 0.5–1 h at 50°C, another minor peak appeared with a retention time of 5.80 min.

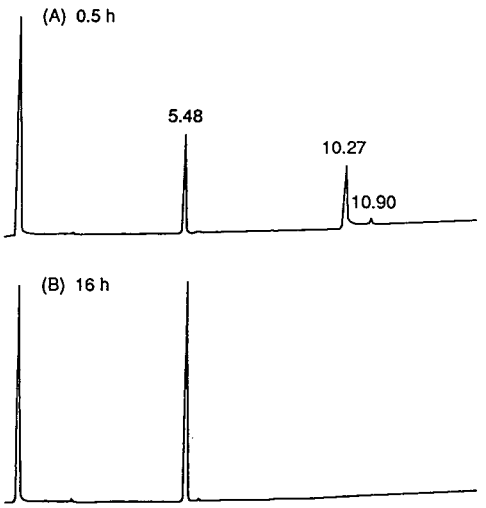

Fig. 1. Gas–liquid chromatograms of methyl α-muricholate after trifluoroacetylation with trifluoroacetic anhydride at room temperature for (A) 0.5 h and (B) 16 h. Numbers at peaks indicate retention times in min.

GC–MS analysis revealed that the peaks with retention times of 5.48, 10.27 and 10.90 min

TABLE I

EFFECTS OF REACTION TEMPERATURE AND REACTION TIME ON TRIFLUOROACETYLATION OF METHYL α-MURICHOLIC ACID

Results given are extent of trifluoroacetylation (%).

Temperature (°C)	Reaction time (h)	Retention time (min)			
		5.48	5.80	10.27	10.90
0	2	23.7	nd[a]	64.3	11.9
Room temp.	0.5	39.7	nd	49.5	10.7
Room temp.	1	58.3	nd	36.5	5.1
Room temp.	16	100.0	nd	nd	nd
Room temp.	24	100.0	nd	nd	nd
Room temp.	48	98.5	1.5	nd	nd
50	0.5	93.8	1.5	4.8	nd
50	1	98.3	1.7	nd	nd

[a] nd = Not detectable.

were tri-, di- and monotrifluoroacetylated Me-αMC, respectively. The peak at 5.80 min could not be identified.

Table II shows the effect of the reaction time on trifluoroacetylation of Me-αMC, Me-βMC, methyl α-hyocholate (Me-αHC) and methyl β-hyocholate (Me-βHC). In this experiment, Me-αMC gave only two peaks after reaction for 0.5 h at room temperature, but it gave one peak (5.48 min) after reaction for 16 h. Me-βMC showed one peak after reaction for both 0.5 and 16 h. Me-αHC gave three peaks and Me-βHC two peaks after a 0.5-h reaction, but both of them gave one peak after a 16-h reaction.

Table II shows the results of the analysis of these bile acids. When 0.1 μg of each bile acid was applied to the column, the peak area was *ca.* 20 000 (arbitrary units) for each bile acid, as shown by Jones *et al.* [10], after reaction for 16 h, suggesting that the reaction had proceeded quantitatively under this condition. In contrast, the peak areas of Me-αMC, Me-αHC and Me-βHC after a 0.5-h reaction were much lower than those after a 16-h reaction, even when the peak areas at 0.5 h were combined for each bile acid.

Although the experiments were carried out at one time in each instance, the data obtained were reasonable among the compared group, hence the data in Tables I and II are thought to be reliable.

TABLE II

PEAK AREAS OF α- AND β-MURICHOLIC ACIDS AND α- AND β-HYOCHOLIC ACID AFTER TRIFLUOROACETYLATION FOR 0.5 AND 16 h AT ROOM TEMPERATURE

Results are peak areas (in arbitrary units) corresponding to 0.1 μg of each bile acid, and in parentheses are the percentages of the values after a 16-h reaction.

Compound[a]	Peak retention times (min)	Reaction time (h)	
		0.5	16
Me-αMC	5.48	9290 (45.6%)	20 356
	10.27	4180 (20.5%)	nd[b]
Me-βMC	7.07	20 472 (105.1%)	19 485
Me-αHC	7.30	4948 (25.2%)	19 671
	9.81	2791 (14.2%)	nd
	11.20	5016 (25.5%)	nd
Me-βHC	7.44	3198 (16.6%)	19 232
	9.83	827 (4.3%)	nd

[a] MCA = Muricholic acid; HCA = hyocholic acid.
[b] nd = Not detectable.

DISCUSSION

The trifluoroacetylation of a hydroxyl group with trifluoroacetic anhydride is considered to proceed quantitatively within 30 min at room temperature [4], and this is true for most of the bile acids such as cholic, 7-epicholic, deoxycholic, chenodeoxycholic, ursodeoxycholic, hyodeoxycholic and lithocholic acids. However, the bile acids possessing hydroxyl groups at the C-3, C-6 and C-7 positions were found to require reaction times of over 16 h at room temperature to complete the reaction quantitatively. Me-αMC, Me-αHC and Me-βHC are the bile acids requiring a longer reaction time, while Me-βMC and methyl cholate and chenodeoxycholate are easily trifluoroacetylated. Therefore, the hydroxyl group at the 6α- or 7α(β)-position is considered to inhibit trifluoroacetylation, probably owing to steric hindrance by the previously formed trifluoroacetyl group at the 6α- or 7α(β)-position.

Mott *et al.* [9] reported the complete trifluoroacetylation of α-HCA at 80°C for 30 min but not at 35°C for 20 min. In our case, when α-MCA was treated with trifluoroacetic anhydride, trifluoroacetylation at 50°C for 30 min or at room temperature for 48 h led to another unknown peak, although it was minor (less than 2%). Therefore, the trifluoroacetylation of bile acids, including α-MCA, with trifluoroacetic anhydride should be performed at room temperature for 16–24 h.

These findings can be explained as follows. We assumed that the rate-determining step of these reactions is not the second trifluoroacetylation but the third. Further, the second trifluoroacetylation of trihydroxy bile acids would occur at the 6-position because 3,6-ditrifluoroacetates are sterically more stable than the corresponding 3,7-ditrifluoroacetates. On trifluoroacetylation, the direction of the lone pair orbitals of the

oxygen atom in the hydroxyl group on the bile acids is closely involved in the reactivity as the reagent attacks the lone pair of electrons in these orbitals. In ditrifluoroacetylates of bile acids, except for that of α-MCA, the carbonyl oxygen in the trifluoroacetyl group introduced would form a hydrogen bond with the proton in the adjacent hydroxyl group, which is the third reaction centre, so that the direction of the lone pair orbitals of the oxygen atom in this group is defined.

Molecular orbital calculation by the PM3 method in MOPAC (version 5.00) (QCPE No. 455) and molecular modelling suggest that the direction of the lone pair orbitals in the ditrifluoroacetylate of β-MCA is the most favourable to the reaction, *i.e.*, no steric hindrance for the approach of the reagent, that of α-HCA is sterically unfavourable as the lone pair orbitals comes under the B-ring, and that of β-HCA is sterically most unfavourable at the orbitals are directed to the C-15 atom so that it is difficult for the reagent to approach the reaction centre. On the other hand, with α-MCA, such hydrogen bonds cannot be formed because the 6- and 7-substituents are in opposite directions. Therefore, the reactivity on trifluoroacetylation of the 3,6,7-trihydroxy bile acids increases in order β-MCA, α-MCA, α-HCA and β-HCA.

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Book Review

Process gas chromatography—Fundamentals and applications, by R. Annino and R. Villalobos, Instrument Society of America, Research Triangle Park, NC, 1992, XI + 456 pp., ISBN 1-55617-272-9, price US$ 76.00 (members), US$ 95.00 (non-members).

In this book world-famous experts describe, in sixteen chapters, their own diverse experience of this specialized topic.

Three groups of chapters can be identified: The first three chapters (Overview of gas chromatography, Selectivity of stationary phases, and The rate theory and chromatographic efficiency) present the theoretical background of gas chromatography in a consistent and well balanced form with relevant examples of calculations illustrating the basic relations that must be understood by any beginner in process gas chromatography.

The second group of eight chapters (Process gas chromatographic hardware, Sample-handling and conditioning systems, Valves for sample injection and column switching, Detectors, Data acquisition and reduction, and Quantitative analysis) deals with all the technical aspects necessary for optimal performance of process gas chromatography.

The third group includes five chapters (I/O devices and communication, Diagnostics and alarms, Maintenance, A computer-aided approach to the gas chromatographic optimization problem, and Specialized methods of analysis) covering problems and examples of real applications and plant control using process gas chromatography.

All chapters include questions to test the reader's knowledge. The last chapter includes selected references, and answers to exercises, both arranged by individual chapter.

At present, only one book by G. Guiochon and Cl.L. Guillemin, *Quantitative gas chromatography for laboratory analyses and on-line process control* (Elsevier, Amsterdam, 1988) covers this topic, and that in only one chapter. Therefore, this book written by established experts is a very good contribution to the gas chromatography literature. On-line gas chromatographic analysis for process monitoring and control involves many more problems than GC in laboratories. The preparation period alone of any process control project needs about one-third of the resources spent on the project. Process gas chromatography is much more demanding in terms of sampling procedure, nature and complexity of samples ot be analyzed, long-term life and stability of columns, problems of baseline drift and noise, and possible signal artifacts, which may cause false alarms and thus waste large amounts of plant resources.

It is necessary to congratulate the authors not only on high factual content but also on a highly didactic level of presentation. The book is a good source of information not only for technicians preparing the gas chromatographic working conditions and selecting the right instrumentation but also for managers with decision-making responsibility. Many of the examples and exercises involving real calculations will be useful to those working in technical universities. I highly recommend this book for all who may be interested.

Brno (Czech Republic) Jaroslav Janák

Author Index

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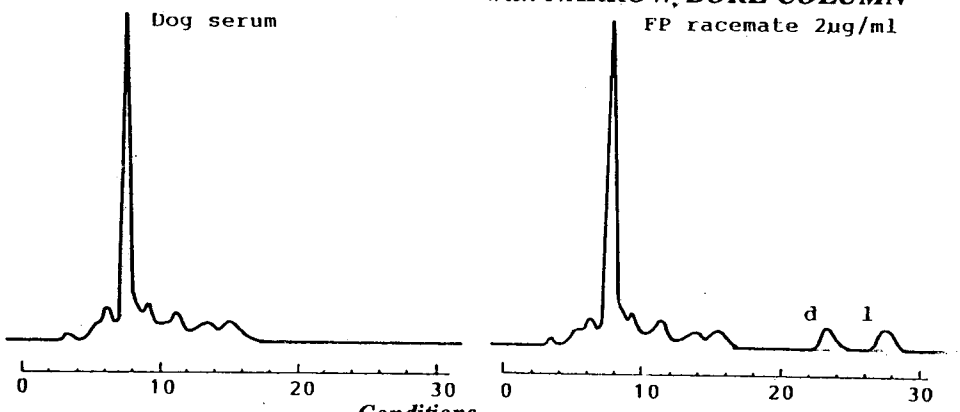

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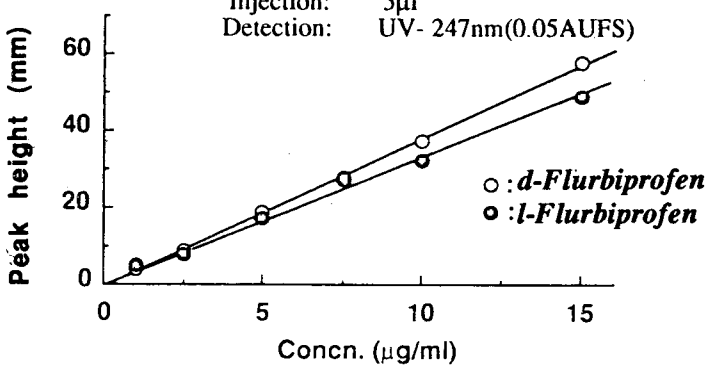

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