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JOURNAL OF CHROMATOGRAPHY A

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

SYMPOSIUM VOLUMES

ELSEVIER

JOURNAL OF CHROMATOGRAPHY A

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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SYMPOSIUM ISSUE

6TH SYMPOSIUM ON HANDLING OF ENVIRONMENTAL AND BIOLOGICAL SAMPLES IN CHROMATOGRAPHY

Guildford (UK), July 19–21, 1993

Guest Editors

D. BARCELÓ
(Barcelona, Spain)

D. STEVENSON
(Guildford, UK)

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Foreword

The *6th International Symposium on Handling of Environmental and Biological Samples in Chromatography* took place at the University of Surrey, Guildford, UK on July 19–21, 1993. It was attended by close to 100 participants and there were twenty oral and twenty poster presentations. This series of meetings was initiated by the late Professor Roland W. Frei under the auspices of the International Association of Environmental Analytical Chemistry and was run in collaboration with the Chromatographic Society.

Growing public concern about the risk to human health caused by exposure to chemicals means that more environmental and biological analysis is required. Although there have been many important advances with instrumentation, columns, etc., in the field of environmental and biological analysis, sample preparation is often the rate-limiting step and the area most prone to errors. Analytical scientists will thus require methodology that is both reliable (fit-for-purpose) and economic. Advances in sample preparation for chromatography thus have a pivotal role to play. The conference thus focused on new methodologies as well as the need to simplify, modify and automate existing procedures.

There were discussion sessions during the conference, but the exchange of ideas and information was greatly facilitated by the informal discussions taking place during coffee breaks, lunch sessions and in the evenings. The benefits of a short campus-based meeting with all facilities, including accommodation, on one site was commented on by many delegates. The help and ideas from the Scientific Committee is gratefully acknowledged and in particular the support of Dr. I. Wilson, Dr. D. Barceló, Dr. K. Zech and Mrs. M. Frei for the running of the symposium was much appreciated. This issue of the *Journal of Chromatography A* contains a selection of the papers presented at the symposium. I hope that it is informative and useful to all those with an interest in sample preparation. The Editor, staff and referees of the *Journal of Chromatography A* have produced the issue to their continuing high standard. The next symposium in this series will be held in Lund, Sweden in May 1995 under the Chairmanship of Professor J.Å. Jönsson, my best wishes to him and his team for another successful conference.

Guildford (UK) Derek Stevenson

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Review

On-line sample treatment for or via column liquid chromatography

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Abstract

It is generally recognized that sample treatment often is the bottleneck in trace-level organic analysis. As far as column liquid chromatography (LC) is concerned, the design, and commercialization, of on-line and fully automated precolumn/analytical column LC systems with diode-array UV or mass spectrometric detection is a distinct step towards solving the existing problems. Small precolumns that can be packed with sorbents of divergent selectivity, and also on-line (electro)dialysis modules, have been shown to perform well in many environmental and biomedical applications. In addition, it is noteworthy that LC or rather, LC-type, trace enrichment and clean-up on such precolumns are increasingly being used as an on-line treatment step for aqueous samples prior to capillary gas chromatographic analysis.

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1. Introduction

In modern trace-level organic analysis, chromatographic techniques play a predominant role. They are used to create an efficient separation of the analytes of interest in complex environmental, biological, food(stuff) or pharmaceutical samples, prior to the actual measurement, *i.e.*, the detection step. Unfortunately, however, even the combined force of an efficient separation and a sophisticated mode of detection does not always create sufficient selectivity and/or sensitivity for the final goal to be reached: quantification and/or identification of sample constituents typically present at low-ppm to low-ppt (10^{-5}–10^{-11} g/g) levels. In such instances, special attention has to be devoted to sample pretreatment (for trace enrichment and clean-up) and pre- or post-separation derivatization or conversion of the analytes (for improved detection selectivity and/or sensitivity). In recent years, column liquid chromatography (LC), and especially reversed-phase LC with aqueous–organic eluents, has achieved widespread accept-

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ance and popularity. No doubt this is at least partly due to the increasing attention being given to polar compounds, irrespective of whether these are drugs, pesticides, industrial chemicals or their breakdown products. Selectivity and sensitivity enhancement in LC by means of (on-line) postcolumn reaction detection has been the subject of many reviews and books [1–3] and will not be considered here. Rather, attention will be devoted to sample treatment for LC, and emphasis will be given to the use of on-line techniques. These are becoming increasingly important in all those situations where (i) large series of samples have to be analysed routinely, making rapid analysis, (semi-)automation and unattended operation aspects of major concern, and (ii) sensitive trace-level determination requires the analysis of total samples or sample extracts rather than aliquots, under conditions in which analyte losses, due to, *e.g.*, evaporation or irreversible sorption to the vessel walls, and contamination, caused by the solvents or reagents used, laboratory air and/or sample manipulation in general, must be rigorously minimized.

In a majority of such cases, on-line sample treatment for LC is carried out by means of a so-called precolumn technique. Most attention will therefore be devoted to this alternative and, especially, to the role played by the nature of the precolumn packing material. In recent years, techniques such as on-line dialysis and electrodialysis have also received attention and they will be discussed accordingly. On-line precolumn derivatization is another topic of current interest, partly because pre- and postcolumn strategies aimed at enhancing detection sensitivity and/or selectivity can be readily compared. Conventional column-switching LC–LC procedures, which, in essence, are fairly straightforward heart-cutting techniques, will not be considered. The reader interested in state-of-the-art applications should consult refs. 4 and 5, which present several elegant studies on the rapid and automated determination of, *e.g.*, bentazone, isoproturon and ethylenethiourea in ground- and rain water (0.2–1 μg/l level). Finally, some attention will be devoted to on-line LC–GC (capillary gas chromatography), a topic of much current interest in which LC is used for either preseparation or trace enrichment, while the highly efficient separation–detection is performed in the GC part of the set-up.

2. On-line precolumn/analytical column LC

In an on-line precolumn/analytical column LC procedure, four main steps can be discerned (see Fig. 1): (i) loading of the sample (typically an aqueous sample or aqueous extract), which results in trace enrichment of the analytes of interest, *i.e.*, in increased sensitivity; (ii) flushing of the precolumn to wash out potentially interfering sample constituents, which ensures improved selectivity; (iii) desorption of the analytes from the precolumn, a step which should be rapid and efficient in order to ensure that the starting zone on the top of the analytical column will be sufficiently small; and (iv) reconditioning of the precolumn, which is preferred with expensive precolumns and/or when reconditioning is rapid, or exchange of the (disposable) precolumn cartridge which is recommended for all other situations.

Currently, there is an increasing tendency to use relatively small precolumns which typically have dimensions 2–10 mm × 4.6–2 mm I.D. Packed precolumns are commercially available from several manufacturers, but manual slurry or dry packing of a precolumn does not present any

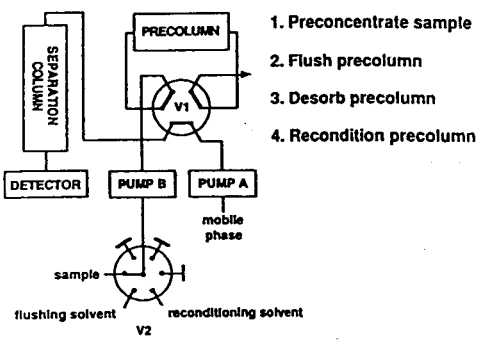

Fig. 1. Set-up of a precolumn sample pretreatment system for LC. V1 = High-pressure switching valve; V2 = low-pressure solvent-selection valve.

real problems and, in addition to rapid exchange, allows the easy screening of new packing materials. Recently, as an alternative to precolumns, so-called Empore membrane extraction discs have been introduced [6,7] that contain a suitable stationary phase (C_{18}-bonded silica, ion exchanger or copolymer; typically about 90%, w/w) enmeshed in a polytetrafluoroethylene network (10%, w/w). The successful use of small 4.6 mm diameter discs for trace enrichment coupled on-line with LC has been reported [7].

In most published procedures for on-line precolumn/analytical column LC, C_{18}- or C_8-bonded silicas are used as the precolumn packing material. A good illustration of the general usefulness of these materials is presented in Table 1 for a number of chlorophenols [8]. Obviously, even compounds as hydrophilic as dichlorophenols will have breakthrough volumes of *ca.* 10 ml on a small precolumn, whereas for a really hydrophobic compound such as pentachlorophenol the breakthrough volume is over 300 ml. Although the latter figure is, of course, more impressive, one should realize that even a 10-ml sample loading represents a 100-fold improvement in analyte detectability over a conventional 100-μl loop injection!

Alkyl-bonded silicas are very effective for trace enrichment, as is well illustrated by the data summarized in Table 2, which deal with a prolonged study on the determination of urapidil and two of its metabolites in serum and urine [9]. However, their general drawback is that together with the analytes of interest, many potentially interfering sample constituents will also be retained. Hence the selectivity will be increased to only a minor extent, if at all. In such instances, enhanced selectivity will have to be provided in the detection step. An example is given in Fig. 2, which shows the trace-level determination of several highly chlorinated phenols in tap water at

Table 1
Breakthrough volumes of selected chlorophenols on precolumns packed with alkyl-bonded silica or polymer materials

Packing material	Breakthrough volume of chlorophenol (ml)				
	Mono-	Di-	Tri-	Tetra-	Penta-
LiChrosorb RP-18	0	12	58	180	320
Hypersil C-18	0	10	35	150	340
Polymer PRP_1	30	200			

2 mm × 4.6 mm I.D. precolumn; sample, water of pH 3.

Table 2
On-line precolumn/analytical column LC of urapidil and its metabolites M_1 and M_2

Parameter	Result[a]
Recovery	95–110%
Accuracy/precision	10% or better
Detection limit:	
ECD	5 ng/ml in serum
UV	50 ng/ml in urine
Precolumn:	
Lifetime	15–20-ml of sample
New column	Every 10 samples

[a] Based on *ca.* 15 000 analyses carried out over a 2-year period; one metabolite stabilized as a result of sorption on precolumn packing material.

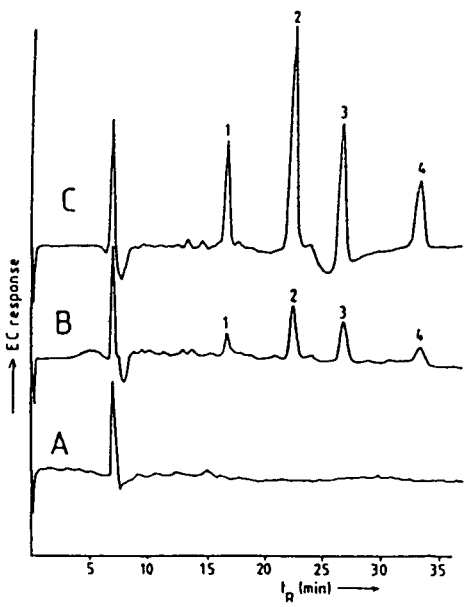

Fig. 2. LC with electron-capture detection of 40 ml of tap water after trace enrichment. (A) Non-spiked, (B) spiked with 14–45 ng/l and (C) spiked with 60–180 ng/l of (1) 2,3,6-tri-, (2) penta-, (3) 2,3,5-tri- and (4) 2,3,4-trichlorophenol. Normal-phase LC on silica with hexane–toluene–glacial acetic acid (79:20:1) as eluent.

the 10–100 ng/l level using trace enrichment combined with normal-phase LC and electron-capture (!) detection [10]. An alternative means of improving selectivity is to use postcolumn reaction detection. In one such study [11,12], N-methylcarbamates were preconcentrated on C_{18}-bonded silica and separated by conventional reversed-phase LC, the LC effluent then being led through a solid-phase reactor containing an anion-exchange resin which was kept at 100°C. The carbamates decomposed and methylamine was formed, which reacted with *o*-phthalaldehyde in a second open-capillary-type reactor. The highly fluorescent reaction product was then monitored directly, because *o*-phthalaldehyde itself does not fluoresce. As a continuation of this work, the determination of twenty parent N-methylcarbamates and twelve major degradation products in surface water was carried out, using 50 ml of water and low-carbon C_{18}-bonded (C_{18}/OH) silica for trace enrichment [13]. Although the published procedure is not fully on-line, the experimental results are highly rewarding, with detection limits of, typically 20–30 ng/l (see Fig. 3).

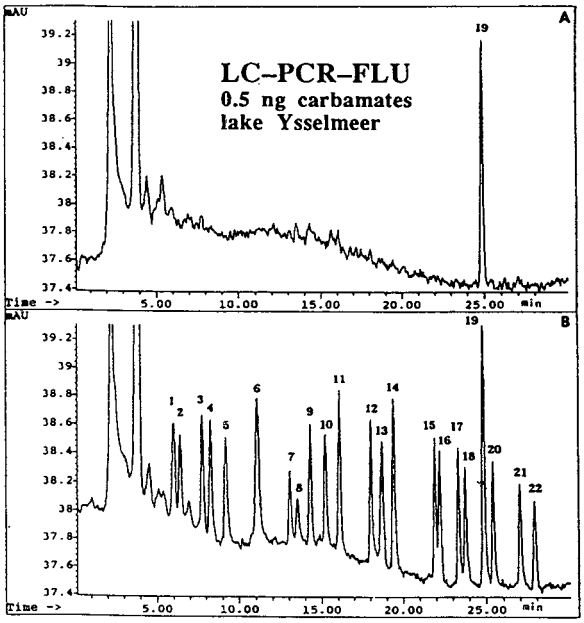

Fig. 3. LC with post-column reaction detection and fluorescence monitoring of lake IJsselmeer surface water samples after trace enrichment of 50 ml of (A) a blank water sample and (B) a water sample fortified with eleven N-methylcarbamates and ten degradation products at the 0.1 μg/l level on 500-mg C_{18}/OH cartridges. Landrin (peak 19; 1.0 ng injected) was used as an internal standard. In the case of 100% recovery, the injected amount (in 100 μl) of all other carbamates is 0.5 ng.

Obviously, if precolumns have to be used in order to also enhance selectivity, one alternative is to use a series of precolumns containing different packing materials or a selective, *e.g.*, a metal-loaded or an antibody-loaded, precolumn. Two well known examples of the former approach deal with monitoring the effluent from a wastewater treatment plant (C_{18}-bonded silica/polymer/ion exchanger precolumns) [14] and with the determination of carbohydrates in fermentation broths and spent sulphite liquor [15]. The set-up of the latter system involves two off-line and two on-line precolumns and, after the LC separation, either UV absorbance monitoring or postcolumn reaction detection using an immobilized enzyme reactor and electrochemical detection. In some instances, clean-up was sufficiently dramatic to allow UV detection of sugars at 195 nm (!).

Metal-loaded packing materials can easily be prepared by flushing an (inexpensive) thiol- or 8-hydroxyquinoline-containing poly(methyl methacrylate) polymer with an excess of an aqueous solution of a suitable metal salt, *e.g.*, silver nitrate. Ag(I)-loaded precolumns have been used to preconcentrate pyrimidine nucleobases such as uracil and thymine, structurally related compounds such as the drugs 5-fluorouracil and AZT and the pesticide bromacil [16]. The precolumn set-up used for the trace-level determination of AZT in plasma is shown in Fig. 4. Initially, the AZT-containing sample was loaded directly on the metal-loaded precolumn (sample pH = 5). Desorption with a very small (60 μl) volume of 0.1 *M* perchloric acid effected the rapid and quantitative desorption of the analyte from the precolumn and its transfer to a conventional reversed-phase LC system. Even after prolonged use, the repeated injection of the plug of strong acid did not cause a noticeable deterioration in the performance of the analytical column. However, the precolumn pro-

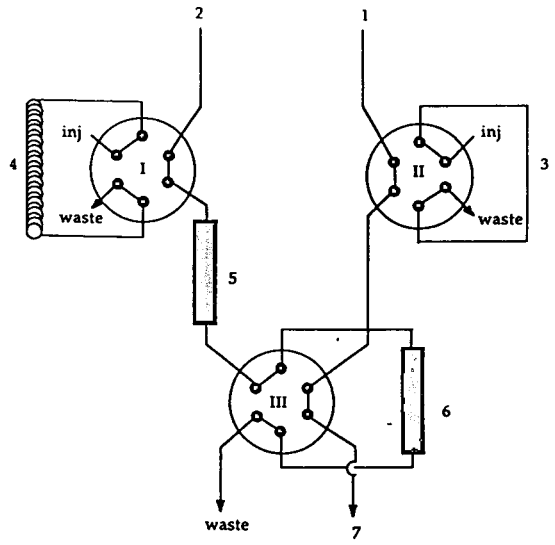

Fig. 4. Scheme of the analytical system used for the determination of AZT in biological samples. 1 = LC pump; 2 = preconcentration pump; 3 = injection loop (perchloric acid); 4 = sample injection loop; 5 = polymer-based clean-up precolumn; 6 = Ag(I)–thiol column; 7 = to LC column and UV detector.

cedure did not remove all endogenous compounds having the –NH(=CO) structure responsible for retention. For that reason, a PLRP-S copolymer precolumn was inserted (Fig. 4; No. 5) to effect additional clean-up. Under these conditions, AZT could be determined in plasma down to a concentration of 10^{-8} *M* using ordinary UV detection at 269 nm.

Interesting studies on metal-loaded precolumns are also being performed by Boos *et al.* [17]. They have synthesized a tailor-made Cu(II)–phthalocyanine-modified porous glass precolumn packing material and have set up a fully automated precolumn/analytical column LC procedure for the low-level detection of free and conjugated 1-hydroxypyrene in urine (detection limit 0.01 pmol).

The use of immobilized antibodies for selective on-line sample treatment in LC has been reported for, *e.g.*, the anabolic steroid β-19-nortestosterone and its main metabolite, α-19-nortestosterone, in calf urine and meat [18]. Other applications include the determination of β-trenbolone, chloramphenicol, clenbuterol and aflatoxin M_1 [19]. After sorption of the analyte(s) of interest from, typically, a biological fluid or milk on to a properly pretreated immunoaffinity precolumn, on-line desorption is carried out either selectively, *i.e.*, by using an essentially aqueous solution containing a so-called displacer (norgestrel in the case of nortestosterone), or non-selectively with a methanol–water or acetonitrile–water mixture containing a high proportion of modifier. In such instances, additional water has to be pumped in between the immunoaffinity precolumn outlet and the inlet of a second precolumn packed with an alkyl-bonded silica to allow refocusing of the analyte-containing zone on the latter precolumn (see Fig. 5). In most studies published so far, detection limits are well below the 1 μg/l level. Some of the immunoaffinity precolumns have been used for over 100 runs. Two relevant applications [19,20] are shown in Figs. 6 and 7.

Under certain conditions, the above line of reasoning regarding selectivity does not provide the proper answer. Within the framework of the Rhine Basin Program, an international partnership of several Dutch, German and Swiss universities, governmental institutes and instrument companies [21], a fully automated precolumn–LC–diode-array UV system has been designed for monitoring polar pesticides in river Rhine water and also water from other Euro-

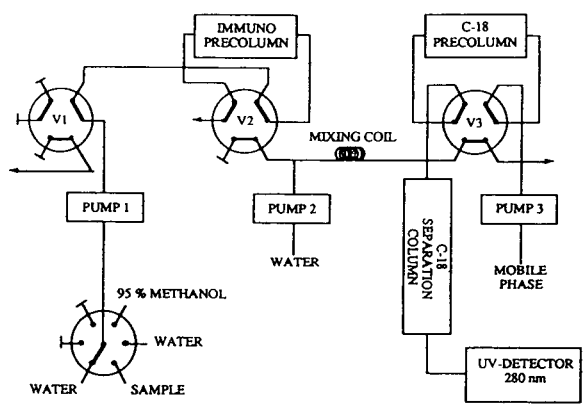

Fig. 5. Typical set-up of an on-line immuno precolumn/analytical column LC system used for non-selective desorption.

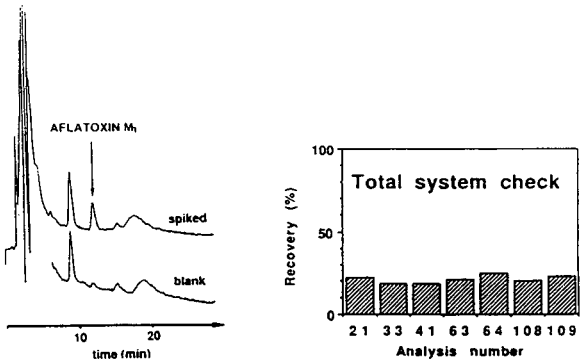

Fig. 6. LC with fluorescence detection of a crude milk sample spiked with 50 ng/l of aflatoxin M_1, and of the corresponding blank. Hollow-fibre dialysis–immunoaffinity preconcentration was performed with donor and acceptor volumes of 25 ml each. Total system check is also shown.

pean rivers (monitoring level 0.5–3 μg/l; at present, 50–80 pesticides are included). In such a case, the presence of even a single pollutant at or near the alert (1 μg/l) or alarm (3 μg/l) level is, and should be, the exception rather than the rule. In other words, instead of trying to improve selectivity by combining several types of pre-columns or by using selective stationary phases, one now must try to trap whatever (unknown) pollutant is present in the river water sample on a single phase, obviously the most hydrophobic one available, *i.e.*, on a polymer packing material [22,23]. As an example, Fig. 8 shows the identification of an extremely low level of atrazine in river Rhine water. The completely automated SAMOS (System for Automated Monitoring of Organic compounds in Surface water) LC monitoring system, which combines a Prospekt (Spark Holland, Emmen, Netherlands) sample treatment module and an HP 1090 (Hewlett-Packard, Waldbronn, Germany) LC separation–diode-array detection unit, is commercially available through the latter company.

The same SAMOS approach has also been found very useful in work using on-line pre-column/analytical column LC combined with thermospray mass spectrometric (TSP-MS) detection [24]. Using, for example, 50-ml river Rhine water samples, all fifteen phenylurea herbicides tested could be detected at the 0.1

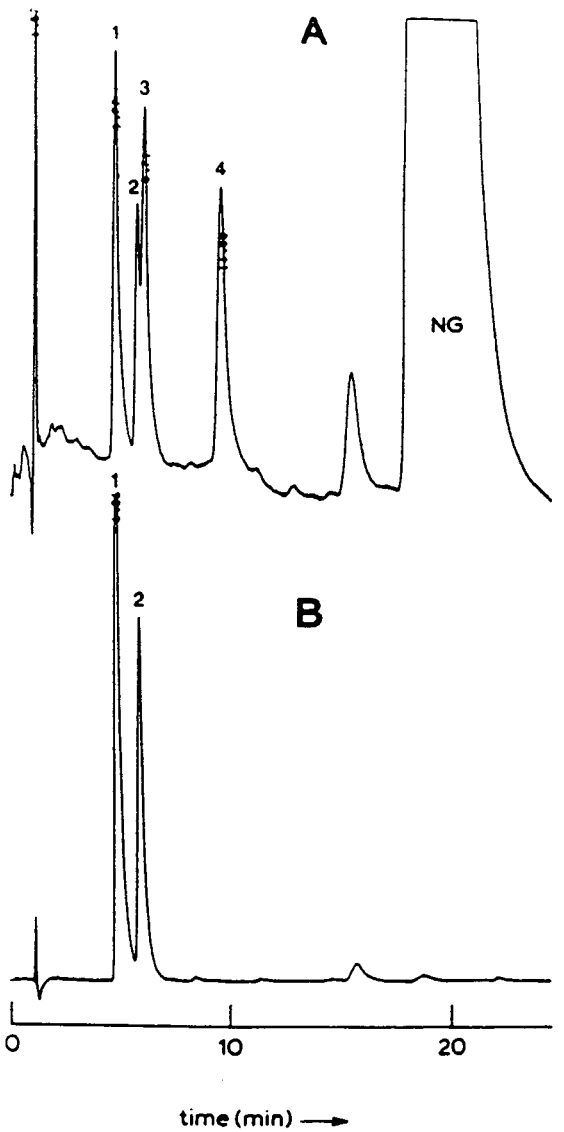

Fig. 7. LC of a mixture of (1) 17-β-trenbolone (1 μg/l), (2) 17-α-trenbolone (2 μg/l), (3) β-19-nortestosterone (0.2 μg/l) and (4) α-19-nortestosterone (0.3 μg/l); 53 ml of this mixture were loaded on to the immuno precolumn. (A) UV detection at 247 nm (0.002 AUFS); (B) UV detection at 340 nm (0.008 AUFS).

μg/l level (selected-ion monitoring). A relevant example of real analysis is given in Fig. 9.

Finally, one should mention the so-called restricted access materials such as internal-surface reversed phases, shielded hydrophobic

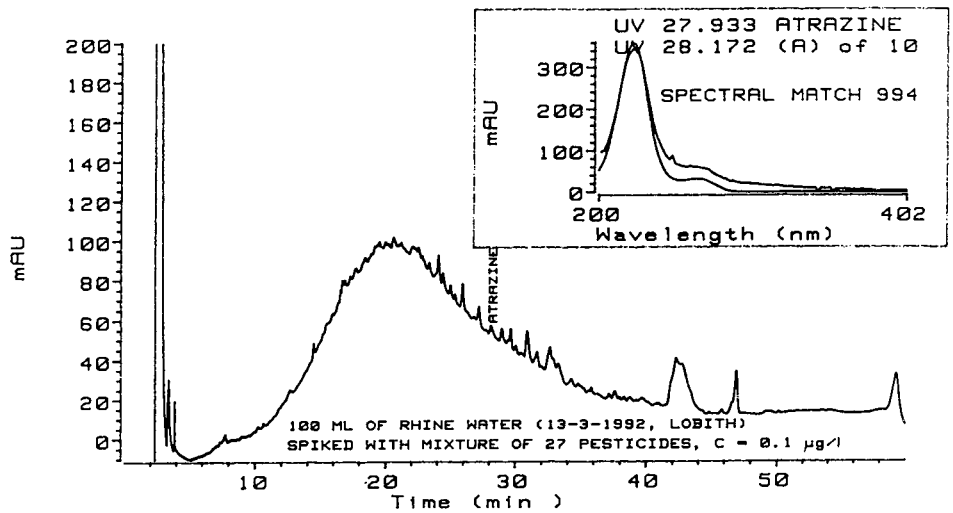

Fig. 8. Identification of a 0.1 μg/l spike of atrazine in 100 ml of river Rhine water using trace enrichment on a polymer precolumn coupled on-line with reversed-phase LC–diode-array UV detection (SAMOS LC system).

phases, semi-permeable surfaces and dual-zone materials [25,26]. The unique feature of these packings is that they prevent the access of matrix components such as proteins (which are allowed to interact only with hydrophilic, non-adsorptive layers on the outer packing surfaces), whilst selectively retaining small molecules such as drugs and their metabolites (which can penetrate

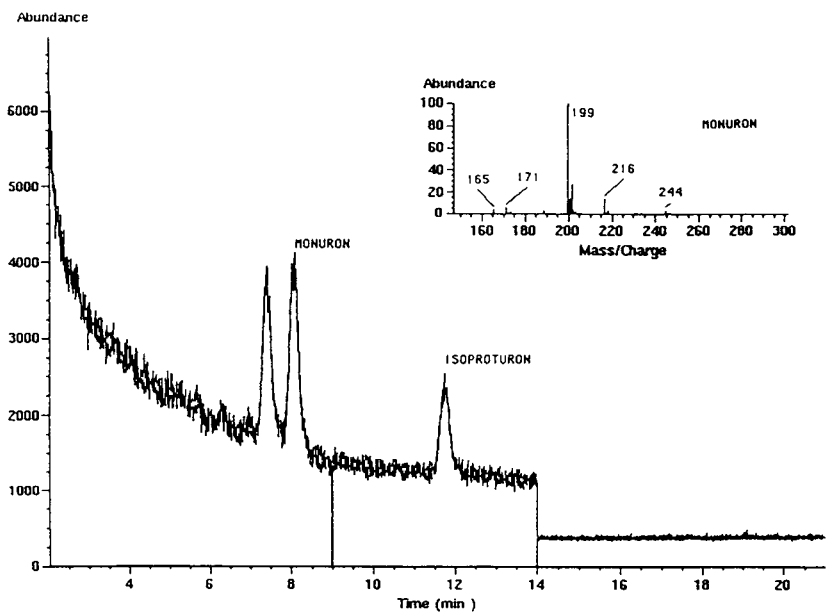

Fig. 9. On-line precolumn/analytical column LC–thermospray MS of 50 ml of river Rhine water using C_{18} membrane extraction discs and a linear methanol–0.1 *M* ammonium acetate gradient. MS monitoring: 0–10 min, *m/z* 199 and 201; 10–14 min, *m/z* 207; 14–21 min, *m/z* 275 (for detection of neburon).

the packings and gain full access to partitioning phases). Although the restricted access materials have not yet gained widespread acceptance in practice, a number of interesting single- and coupled-column mode applications have been reported, and there is no doubt that the possibility to carry out, *e.g.*, drug determinations by direct serum injection will stimulate further activity in this area.

3. Alternative on-line sample treatment techniques

In recent years, the use of dialysis as an on-line sample treatment technique for the removal of macromolecules prior to LC has received much interest; an extensive review is available [27]. A dialysis module consists of two Perspex blocks with the dialysis membrane (molecular mass cut-off typically 10 000–15 000) in between to separate the donor (sample) phase from the acceptor phase. Fig. 10 shows the general set-up of a system. It is important to realize that depending on the aim and the boundary conditions of the analysis (high recovery, rapid analysis, large or limited sample volume), a different donor–acceptor mode of operation should be selected from among the four combinations generally recommended, *viz.*, (i) stagnant–stagnant, (ii) stagnant–flowing, (iii) pulsed–flowing and (iv) flowing–flowing.

Because dialysis is based on molecular diffusion occurring as a result of the concentration gradient across the cell of the analyte of interest, it is obvious that the in itself simple stagnant–stagnant mode will be time consuming and will never effect more than a 50% recovery. In practice, therefore, a flowing acceptor stream is almost invariably selected. Dialysis now proceeds more rapidly and the recovery will be higher. Unfortunately, however, dilution is an unavoidable consequence. It is therefore necessary to insert a precolumn in the system (*cf.*, Fig. 10) to reconcentrate the analytes, while avoiding the use of too large volumes of acceptor solvent which may cause breakthrough. If relatively large sample volumes are available as with, for example, milk, the process can be further accelerated by adding the sample as a number of pulses (thus restoring the initial, high concentration gradient) or by using a continuously flowing donor (sample) stream. Relevant examples include the determination of sulphonamides [28] and nitrofurans [29] in (aqueous extracts of) eggs, meat and milk and of oxytetracycline [30] (Fig. 11) in salmon plasma and whole blood. Hollow-fibre dialysis has been shown to be

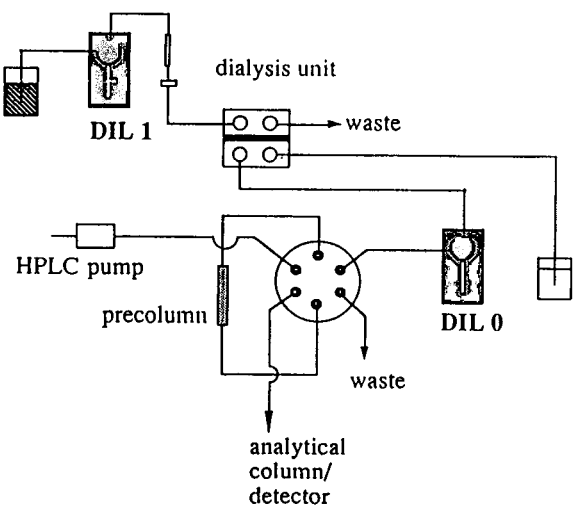

Fig. 10. General set-up for dialysis combined on-line with LC.

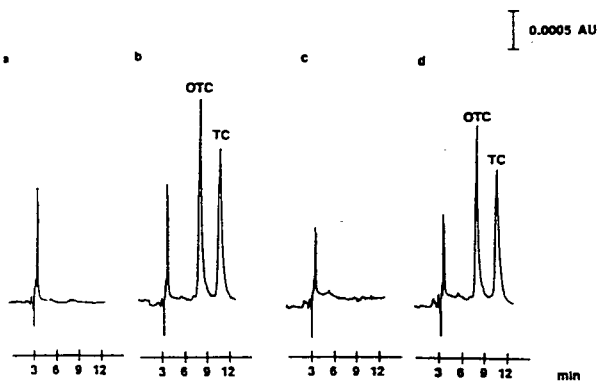

Fig. 11. Reversed-phase LC–UV detection after dialysis of (a) drug-free salmon plasma, (b) salmon plasma spiked with 1 μg/ml of oxytetracycline (OTC), (c) drug-free salmon whole blood and (d) salmon whole blood spiked with 1 μg/ml of OTC. Internal standard: tetracycline (TC).

highly rewarding in the determination of trace amounts of aflatoxins in milk by immunoaffinity precolumn/analytical column LC [20].

Recently, first results have been published [31] concerning electrodialytic sample treatment, where 5–10 V are applied across the dialysis cell (now containing electrode compartments and ion-exchange membranes next to the ordinary cut-off membrane). This promotes the transfer of charged analytes from the donor to the acceptor phase by means of electromigration. In such a case, a slowly flowing donor stream and a stagnant acceptor stream are used, and the dilution effect on the acceptor side, which invariably occurs with conventional dialysis, is absent. Rather, selective enrichment (up to 10–20-fold) of the charged analytes is achieved, as has been shown for, *e.g.*, the determination of several aromatic sulphonic acids in river Rhine water and of paraquat and diquat in groundwater (Fig. 12) [32].

A quick survey of the literature shows that, whereas postcolumn reaction detection is invariably carried out on-line, precolumn labelling or conversion of analytes is usually done off-line. In recent years, however, on-line precolumn derivatization has attracted some attention. Most studies have dealt with the low-level determination of primary and/or secondary amino acids in a variety of samples, using reagents such as 9-fluorenyl(m)ethyl chloroformate or *o*-phthalaldehyde [33–35]. Modern autosamplers or autosampler-related devices are used to carry out the required reactions under such (geometrical) conditions that on-line coupling with the LC part of the system is possible.

When utilizing *o*-phthalaldehyde as reagent, the addition of a mercapto reductant containing a chiral centre allows the separation of D- and L-amino acids, as their diastereomers, on a conventional alkyl-bonded silica column [36]. A similar result can be obtained by using pure enantiomeric (+)-9-fluorenylethyl chloroformate as the reagent (see Fig. 13) [35]. A different type of application [37] deals with the separation of nitrated polyaromatics, *e.g.*, nitrated pyrenes, as their amino analogues. Reduction is carried out on a precolumn filled with a mixture of small zinc particles and glass beads. The (chemiluminescence) detection limits are between 0.1 and

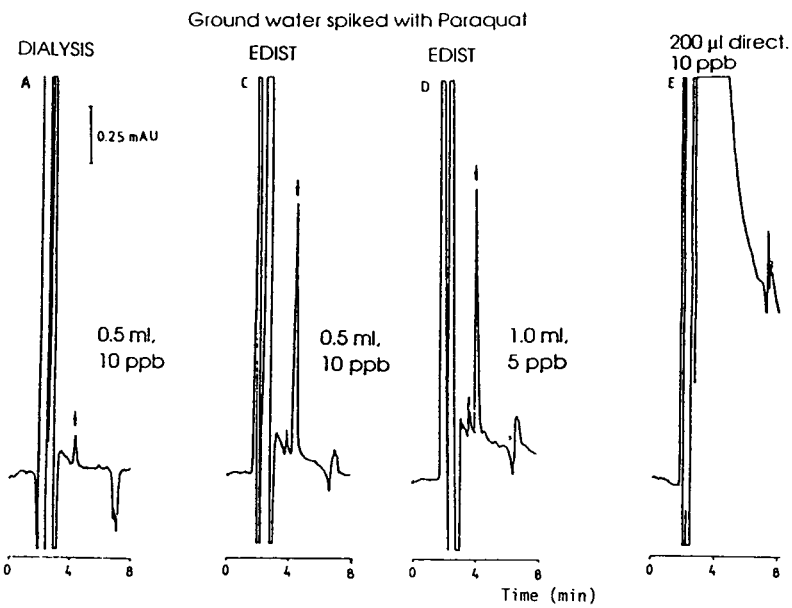

Fig. 12. LC–UV detection of ground water samples spiked with 5–10 µg/l of paraquat (see arrow). Pretreatment by means of dialysis and electrodialysis (EDIST) is compared with direct injection.

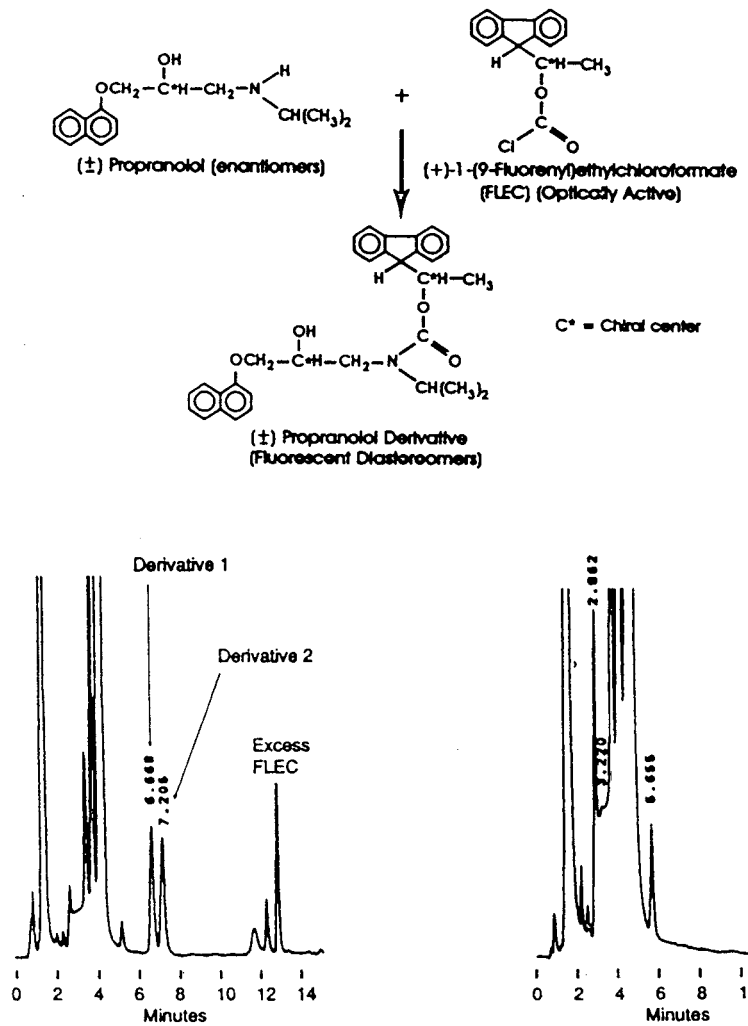

Fig. 13. Determination of (±)-propranolol using on-line precolumn derivatization using an optically active reagent and LC with fluorescence detection. Left chromatogram; FLEC-(±) propranolol, 200 pmol. Right chromatogram: FLEC-buffer (blank). Fluorescence detection: excitation wavelength 265 nm, emission wavelength 345 nm. Mobile phase: acetate–acetonitrile (30:70).

10 pg. It is interesting to add that the reduction can also be carried out successfully in the post-column (Fig. 14). The differences in retention times observed for the precolumn compared with the postcolumn procedure are, of course, due to the fact that the compounds that are actually separated are different, *i.e.*, amino and nitro analogues, respectively. Phase-transfer catalysis has also been used for on-line precolumn analyte derivatization. In one study, solutes such as ethinylestradiol and 4-monochlorophenol were determined in urine samples [38]. Dansylation in an aqueous–organic two-phase system was followed by on-line LC separation and fluorescence detection. In another study [39], a micellar system was used for the rapid derivatization of the drug valproic acid and free fatty acids in plasma using substituted coumarins and acridines as reagents. Under suitable conditions [Arkopal N130 as non-ionic surfactant, tetrakis(decyl)ammonium bromide as transfer catalyst and 9-bromomethylacridine as reagent; 50°C], the reac-

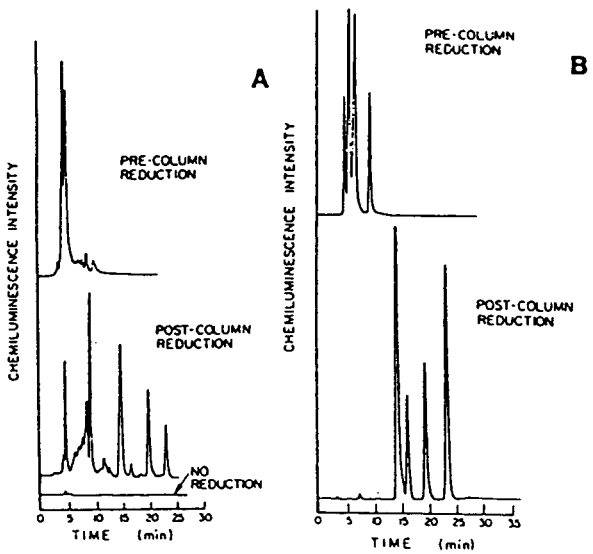

Fig. 14. LC with chemiluminescence detection of (A) a carbon black extract and (B) a nitrated pyrene sample. The nitrated polynuclear aromatics are detected as their amino analogues after on-line pre- or postcolumn reduction with powdered zinc contained in a small reactor.

tion is completed within about 10 min, and on-line coupling to an LC system presents no serious problems. In recent years, the use of off-line and (automated) on-line solid-phase derivatization has repeatedly been reported by Krull and co-workers [40–42], and achiral and chiral derivatizations of both nucleophilic analytes and amino acids have been studied. An interesting recent example is the direct determination of 1-adamantanamine in plasma and urine using a solid-phase reagent containing a covalently bound activated ester of 9-fluoreneacetate. Surprisingly, the technique has not yet gained much acceptance.

4. On-line LC–GC

Another approach to sample treatment that has been shown to be rewarding is the on-line coupling of LC and capillary GC. Admittedly, in an LC–GC system the role of LC is reduced to that of providing sample treatment (either heart-cutting or trace enrichment plus clean-up; see below) while GC provides the real separation. However, the on-line coupling of LC with, in principle, the full range of universal and selective GC detectors makes LC–GC too good a combination to be missed in an overview on pretreatment of (often aqueous) samples. A discussion of the various LC–GC interfaces currently available is inappropriate in the present context. For this aspect, the reader should consult, *e.g.*, refs. 43 and 44. Here, some interesting recent applications will be briefly discussed.

In one (heart-cutting) study [45], olive oil (diluted with hexane) was injected into a normal-phase LC system; pentane containing 2% of methyl *tert.*-butyl ether was used as eluent. The (300 μl) fraction containing the polycyclic aromatic hydrocarbons was transferred on-line via a 3-m retention gap to a GC–MS system and analysed. A relevant result is shown in Fig. 15. Another example [46] deals with the determination of the herbicide fenpropimorph in cereals.

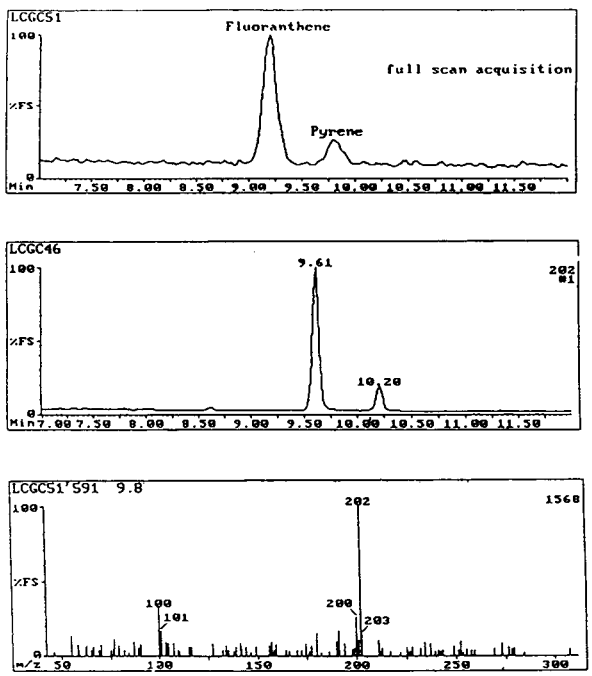

Fig. 15. On-line LC–capillary GC with MS detection of polycyclic aromatics in an olive oil sample (low μg/l level). Part of the final gas chromatogram is shown in the full-scan acquisition and the selected-ion monitoring (SIM) (m/z 202) mode; the mass spectrum of pyrene is also shown.

After reversed-phase LC, the appropriate heart-cut was mixed on-line with an organic solvent. A segmented stream resulted, the analyte of interest being extracted into the organic phase, which was separated from the aqueous phase in a non-membrane-type phase separator, collected in a loop and analysed by GC with thermionic detection (NPD).

In the literature, by far the most attention has been devoted to the heart-cut type of application. We have, however, mainly studied the trace-enrichment alternative, with emphasis on application to aqueous samples. In that case, the main concern is to remove most, if not all, of the water before the analyte-containing zone or plug enters the GC part of the system. A set-up that can be used to that end is shown in Fig. 16. An aqueous sample is loaded on the same type of small precolumn as discussed above for LC operation. Next, ethyl acetate with a flow-rate of, typically, *ca.* 50 μl/min is led through the precolumn. The organic solvent serves the double purpose of pushing any water remaining in the precolumn or the capillaries out to waste and desorbing the trapped analytes of interest, transferring them to the retention gap. Traces of water that dissolve in the ethyl acetate (*ca.* 3%, w/w) will be completely removed during partially concurrent solvent evaporation in the retention gap when ethyl acetate, which forms an azeotrope with water containing *ca.* 8% (w/w) of the latter solvent, is evaporated prior to the actual GC run. Good results have been obtained with polar analytes in surface water [47]. In another instance, three norsteroids were determined at the 0.5–5 ng/ml level in 5 ml of urine [48].

Situations have also been encountered, however, in which the presence of traces of water in the retention gap caused severe peak distortion and even loss of peaks in GC analysis. As an alternative, we therefore introduced the use of Empore membrane extraction discs for trace enrichment in LC–GC. As regards loading of these discs with analytes from aqueous samples, the situation is of course identical with that in LC discussed above. The main advantage of using extraction discs in LC–GC is that on-line drying with a stream of nitrogen takes only 10–15 min at room temperature. Work on a series of organophosphorus pesticides showed that, under these conditions, analyte loss was negligible even at the sub-nanogram level. As an example, on-line membrane extraction–GC–nitrogen–phos-

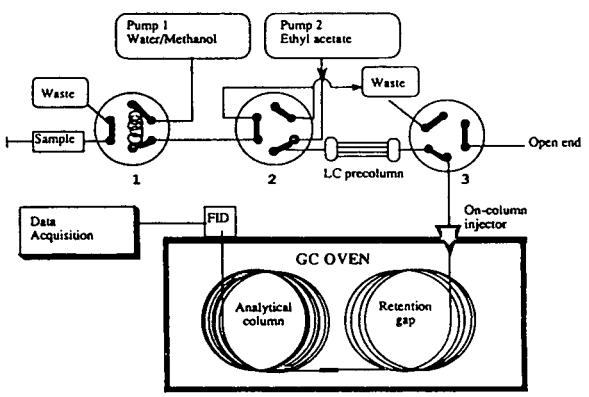

Fig. 16. Scheme of an LC–GC system involving LC-type (aqueous samples) trace enrichment and sample clean-up and on-line GC analysis (SAMOS GC system). The desorption step is shown.

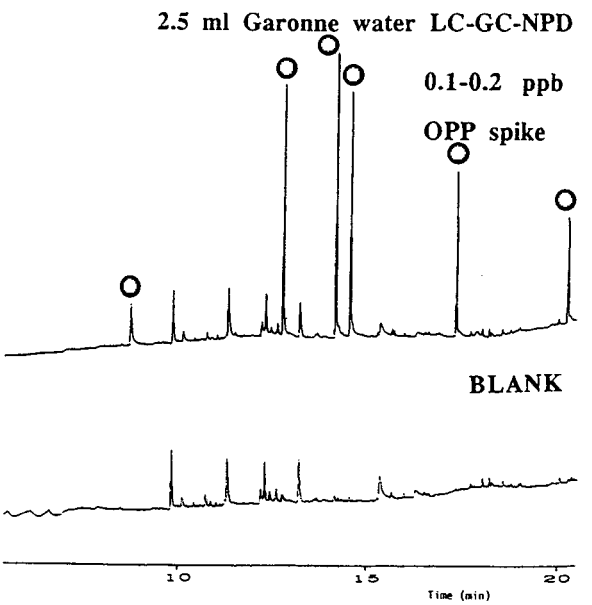

Fig. 17. LC–GC–NPD of (bottom) 2.5 ml of Garonne water and (top) 2.5 ml of Garonne water spiked with six organophosphorus pesticides. The 0.1–0.2 ng/ml spikes (see black spots) are (with increasing retention time) mevinfos, diazinon, fenitrothion, fenthion, triazofos and coumafos.

phorus detection (NPD) analyses of blank and spiked surface water are shown in Fig. 17. The six pesticides were added as 0.1–0.2 ng/ml spikes and trace enrichment was carried out using 2.5 ml of water. The cartridge holder held three 0.5-mm thick, 4.6-mm diameter polymer-loaded extraction discs, which could be re-used at least ten times [49]. Obviously, this meanwhile automated SAMOS GC approach is a promising one to meet the current directives for the determination of trace levels of pesticides in surface and drinking waters. The small volumes of sample required are an additional benefit. Very recently, similar studies have been carried out for LC–GC–MS using both full-scan acquisition and single-ion recording [50]. An interesting result obtained for a 10-ml surface water sample is shown in Fig. 18.

5. Conclusions

Sample treatment is often the bottleneck in modern organic trace-level analysis. Most procedures are still off-line in nature and they tend to be laborious, time consuming and prone to error. As there is an increasing demand for the routine monitoring of ever larger numbers of samples, the development of on-line sample treatment procedures is critically important: sen-

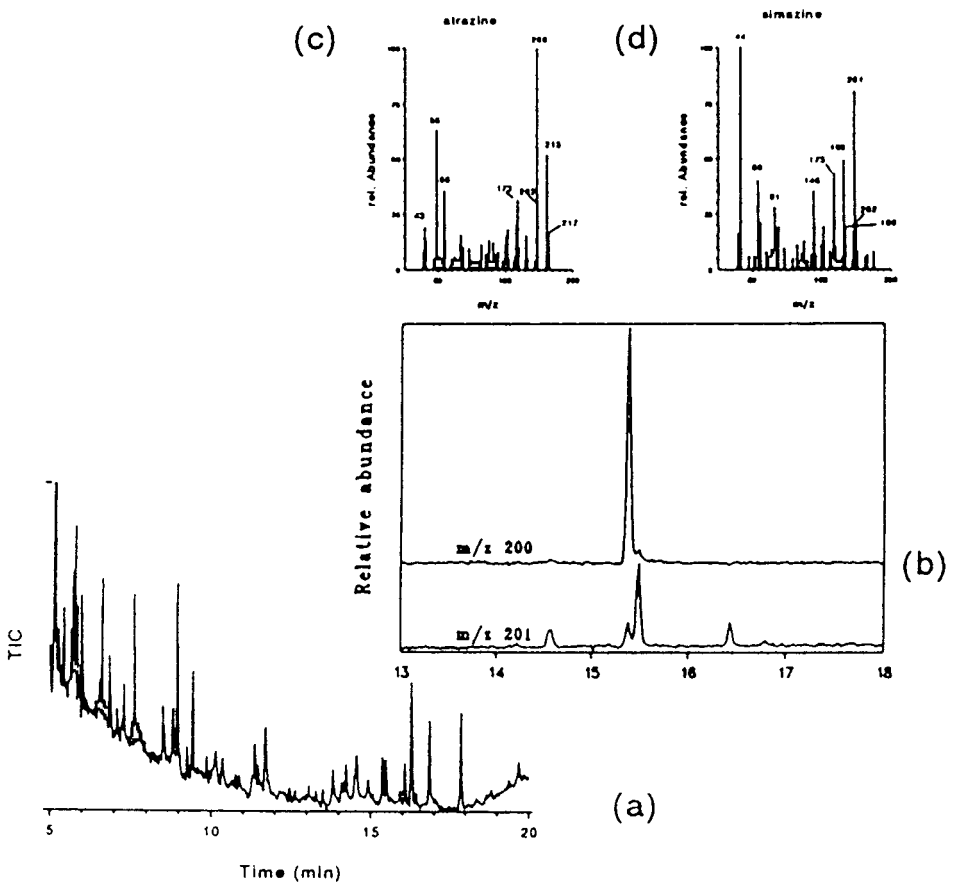

Fig. 18. On-line LC–GC–MS of 10 ml of river Meuse water with full-scan acquisition over the m/z 35–385 range. (a) Total ion current. (b) Reconstructed ion traces for atrazine (m/z 200) and simazine (m/z 201). Mass spectra recorded for atrazine (c) and simazine (d) peaks are also shown.

sitivity increases, losses are prevented and automation becomes readily accessible, as is demonstrated by the development of the SAMOS LC and SAMOS GC systems, and of automated systems for on-line precolumn derivatization.

Today, both disposable and re-usable precolumns filled with a variety of polymers, alkyl-bonded silicas or selective stationary phases are routinely used for on-line precolumn/analytical column LC. Many biomedical and environmental applications have been published and the robustness of such systems is highly satisfactory. Several alternative solutions are increasingly attracting attention. Amongst these, dialysis, with its efficient removal of high-molecular-mass material, already takes a prominent place. The on-line coupling of (reversed-phase) LC or, more correctly, solid-phase extraction of aqueous samples and capillary GC still poses a number of problems. However, the separation and detection power of such a system is sufficiently rewarding to justify the research activities required to solve these problems.

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JOURNAL OF CHROMATOGRAPHY A

Potential of receptor–ligand interactions for sample handling in liquid and gas chromatography

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Abstract

The use of receptor proteins for biospecific sample handling in liquid and gas chromatography is described. As a model system the uterine estrogen receptor was chosen for the isolation of 17β-estradiol and its synthetic agonist diethylstilbestrol. Biochemical characteristics relevant for the use of receptors in sample handling such as the kinetics of receptor–ligand binding, reproducibility and capacity are examined by means of an estrogen radioreceptor assay. Different techniques for the non-covalent immobilization of the estrogen receptor were investigated. Both protamine-coated glass fibre filters and silica particles which bind the receptor via electrostatic interactions have been used for this purpose. The use of the estrogen receptor in the isolation of 17β-estradiol and diethylstilbestrol prior to GC–MS analysis is demonstrated and discussed.

1. Introduction

The control on the use of illegal growth promotors in animal production has become an important issue in the European Community in recent years. This resulted in an increased demand for analytical methods which are able to identify and quantitate not only known drugs but also newly synthesized agonists. Sample pretreatment methods based on immunoaffinity interactions have been applied for this purpose; however, they fail in isolating all agonists, which are structurally different. The only common property of these compounds is their biological activity. Moreover, antibody–antigen interactions do not reflect biological activity. On the other hand, binding of ligands to receptors is a measure for biological activity. We therefore investigated the use of receptors in sample handling which due to the group selectivity should allow the isolation of a broader range of compounds.

Currently the application of receptors in the determination of *e.g.* hormonal anabolic compounds such as 17β-estradiol (17βES) and diethylstilbestrol (DES) is restricted to radioreceptor assays (RRAs) mainly [1]. Following C_{18} preconcentration, reversed-phase LC separation and fraction collection, the amount of estrogenic anabolic compounds is determined using an estrogen RRA. This method is used for routine screening of urine samples of cattle for the presence of growth promotors prior to GC–MS determination. While the RRA is a multi-residue method with high sensitivity it is rather laborious and requires long incubation times.

The use of receptors in sample handling techniques has been described by Banner *et al.* [2] for the determination of progesterone and several of its metabolites. The sample handling procedure included an incubation step with the

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progesterone receptor ligand-binding domain, expressed as a protein A fusion protein in *Escherichia coli*. The covalent immobilization of the estrogen receptor (ER) was investigated by Ikeda *et al.* [3] to study association and dissociation phenomena. They reported a significant change in kinetics for the immobilized ER compared to the ER in solution.

In this paper we describe the study to the application of receptor–ligand interactions for biospecific sample handling. The ER was used to demonstrate the potential of the use of receptor–ligand interactions in the isolation of 17βES and DES prior to GC–MS determination. Special attention is paid to important biochemical parameters such as binding capacity, kinetics of association and dissociation and receptor stability. Different possibilities to immobilize the ER on silica or glass fibre filters were investigated. Ligands bound to the receptors were recovered by extraction with ethyl acetate at low pH and identified and quantitated by GC–MS.

2. Experimental

2.1. Apparatus

A B. Braun (Melsungen, Germany) Potter S homogenizer, a Waring (New Hartferd, CT, USA) blender, a DuPont (Den Bosch, Netherlands) Sorvall RC-5B centrifuge and a Beckman (Berkeley, CA, USA) L8-50M/E ultracentrifuge were used for the isolation of the ER from calf uterus. A Jouan (Saint Nazaire, France) GR 2000 SX cooled centrifuge was used in the protamine precipitation RRA. An LKB (Turku, Finland) Wallac 1214 RackBeta "Excel" liquid scintillation counter and Packard (Meriden, USA) Pony Vial 6000292 counting vials were used for radioactivity measurements. A Millipore (Bedford, MA, USA) 1225 sampling manifold filtering device equipped with Whatman (Maidstone, UK) glass fibre filters was used in the filter experiments. A HBI Vortex evaporator (Saddle Brook, NJ, USA) was used for the evaporation of ethyl acetate. A Varian (Sunnyvale, CA, USA) 3400 gas chromatograph equipped with a column (30 m $\times$ 0.25 mm I.D.) coated with a DB-5 stationary phase (0.20 μm film thickness) was used for separation of the ligands. A Finnigan (Bremen, Germany) MAT 900 mass spectrometer was used for detection.

2.2. Chemicals

All chemicals used were of analytical grade. All aqueous solutions were prepared using water purified with a Millipore Milli-Q system. Sodium molybdate, aprotinine, protamine sulphate, ovalbumin, 17βES, DES, ethylenediaminetetraacetic acid (EDTA) and dithiotreitol were purchased from Sigma (St. Louis, MO, USA). Bacitracin was obtained from Serva (Heidelberg, Germany). Tris(hydromethyl)aminomethane hydrochloride (Tris) was obtained from Aldrich (Steinheim, Germany) and silica 60 (9–10 μm particle size) was obtained from Merck (Darmstadt, Germany). Hydrochloric acid, methanol, isooctane and ethyl acetate were obtained from J.T. Baker (Deventer, Netherlands). N,O,-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Pierce (Rockford, IL, USA). Polychlorobiphenyl-138 (PCB-138) was obtained from Schmidt (Amsterdam, Netherlands). Tritium-labelled 17βES ([^{3}H]17βES) was obtained from DuPont (Dreiech, Germany). Emulsifier-SAFE scintillation liquid was obtained from Packard (Meriden, USA). [16,16,17-$^2H_3$]17βES (99.5 atom% ^{2}H) was obtained from MSD (Montreal, Canada). [$^2H_6$]DES (No. 91M3507) was kindly donated by the Laboratory of Residue Analysis (RIVM, Bilthoven, Netherlands).

2.3. Reagents

Dilution buffer consisted of 15 mmol/l dithiotreitol and 15 mmol/l EDTA in 10 mmol/l Tris · HCl buffer of pH 7.5. Homogenization buffer consisted of 0.25 mol/l sucrose, 25 mmol/l sodium molybdate, 0.1 mmol/l bacitracin, aprotinin [80 kallikrein inhibitor units (KIU) per ml] and 10% glycerol in dilution buffer. Assay buffer consisted of dilution buffer containing

0.1% (w/v) ovalbumin. Precipitation buffer consisted of 15 mmol/l dithiotreitol, 1.5 mmol/l EDTA and 0.1% (w/v) protamine sulphate in 10 mmol/l Tris · HCl buffer, pH 8.0. Coating buffer consisted of 15 mmol/l dithiotreitol, 1.5 mmol/l EDTA and 0.3% (w/v) protamine sulphate in 10 mmol/l Tris · HCl buffer, pH 8.0. A 17βES tracer solution contained [2,4,6,7-$^3H_4$]17βES (specific activity 111.6 Ci/mmol) in methanol to a concentration of 0.4 ng/ml. A 17βES standard solution contained 17βES in methanol to a concentration of 1 μg/ml.

2.4. Procedures

Biochemical characterization

Isolation of the ER. The cytosolic ER was isolated from immature calf uteri as described earlier by Ingerowsky and Stan in 1978 [4]. All procedures were performed at 4°C according to the same method used by Arts *et al.* [1]. Freshly dissected uterine tissue was chopped. The tissue was homogenized 1:1 with homogenization buffer in a Waring blender. The total volume was doubled with homogenization buffer and pottered in a Potter S. The homogenate was centrifuged at 1500 *g* and 4°C for 15 min, and the supernatant at 300 000 *g* and 4°C for 50 min subsequently. The supernatant which contained the ER was frozen and stored at −80°C in aliquots of 3.5 ml.

Radioreceptor assay. An estrogen RRA has been developed which is based on the ability of protamine (a cationic peptide with a molecular mass ranging from 5000 to 10 000) to form a precipitate with the ER [5,6]. After incubation (2 h at 20°C) of the receptor with radioligand in a 4-ml counting vial the receptor-bound fraction and unoccupied receptors were precipitated by addition of an equal amount of precipitation buffer, followed by 30 s of vortexing. After centrifugation at 3500 *g* and 4°C for 15 min the supernatant was decanted and the precipitate was washed three times with 3 ml of water cooled on ice. Finally, the receptor–ligand complex was broken by addition of 400 μl of an aqueous sodium hydroxide solution (0.4 mol/l). All assays were performed in triplicate unless stated otherwise. Scintillation liquid was added and radioactivity was counted after vortexing of the vial contents for 30 s. Non-specific binding (NSB) was determined in an identical way, however in the presence of an additional 100-fold excess of non-radioactive 17βES.

Determination of receptor capacity. Aliquots of 400 μl of an eight times diluted receptor solution were incubated during 2 h at 20°C with increasing amounts of [^{3}H]17βES, ranging from 0 to 200 pg. For the determination of the NSB a 100-fold excess of non-radioactive 17βES was added to each vial. The incubates were treated as described in the previous section. The capacity of the receptor solution was derived from saturation plots according to Michaelis–Menten. Day-to-day variation of capacity was examined by performing the saturation experiments on three different days with the same batch of receptor solution.

Kinetics of receptor–ligand association and dissociation. The association rate of receptor–ligand complex formation was examined ($n = 4$) by incubating 6 ml of a ten times diluted receptor solution at 20°C with 250 pg [^{3}H]17βES in a polypropylene tube. At certain time intervals 200-μl samples were taken from each tube to determine the amount of [^{3}H]17βES specifically bound per milligram protein. For the separation of receptor-bound ligand from free ligand, each sample was sucked through a protamine-coated glass fibre filter onto which the receptor–ligand complex was adsorbed [7,8].

The dissociation rate of the receptor–ligand complex was investigated ($n = 3$) by means of comparison of specific binding (SB) and NSB of [^{3}H]17βES to the vials between the described protamine precipitation assay (20 min) and an extended protamine precipitation assay (50 min). Aliquots of 200 μl of a 4-ml incubation mixture consisting of 200 pg of [^{3}H]17βES dissolved in a four times diluted cytosol solution were incubated for 2 h at 20°C to establish total binding. NSB to receptor solution constituents as well as

to the vials was determined as mentioned previously. In the extended assay receptor–ligand–protamine precipitate derived after centrifugation and washing (see section *Radioreceptor assay*) was vortexed with 2 ml of dilution buffer and centrifuged again at 3500 *g* and 4°C for 15 min. The precipitate was then washed for the second time and centrifuged an additional 7 min at 3500 *g* and 4°C. Finally, 400 μl of a 0.4 mol/l sodium hydroxide solution in water were added to dissolve the precipitate and break the receptor–ligand complex. Radioactivity was measured subsequently.

Trapping of the ER after incubation

Glass fibre filters. Protamine-coated glass fibre filters were prepared by soaking the filters in coating buffer for 1 h. The filters were placed in a Millipore filtering device and washed with a total volume of 9 ml of dilution buffer before use. A 4-ml volume of a four times diluted cytosol solution was incubated with 240 pg of [^{3}H]17β-ES. The estrogen receptor–ligand complex was then trapped on the protamine-coated filters. NSB to receptor solution constituents was determined in the same way, but in the presence of a 100-fold excess of non-radioactive 17β-ES. NSB of the radioligand to the filters was examined by applying an aliquot of 200 μl of an incubation mixture consisting of 4 ml assay buffer and 240 pg of [^{3}H]17β-ES onto each of the filters. After application of the samples onto the filters a reduced pressure of 50 mbar was used to suck the sample through the filter which was then washed three times with 3 ml of dilution buffer. Next the filters were dried at a 200 mbar reduced pressure and transferred to counting vials. Finally, 4 ml of scintillation liquid was added and 2 h afterwards radioactivity was counted.

Silica 60. Silica 60 (9–10 μm particle size) was coated with protamine by gently shaking 1 g of silica in 10 ml of coating buffer overnight. After centrifugation at 2000 *g* during 10 min the supernatant was decanted. The pellet was washed twice with 6 ml of dilution buffer and vortexed for 1 min. Different amounts of slurries of both protamine-coated and plain silica were mixed with 200 μl of an incubation mixture consisting of 5 ml of an eight times diluted receptor solution which was previously incubated with 270 pg [^{3}H]17β-ES. After vortexing for 30 s and a 5 min incubation time the mixture was transferred onto a single uncoated GF/C filter which was placed in the filtering device and washed and dried according to the procedure as described for the glass fibre filters.

Application of the ER in sample handling followed by GC–MS

A 4-ml volume of a four times diluted receptor solution was used to isolate amounts of 1 ng and 250 pg, respectively, of 17β-ES, DES and a 1:1 mixture containing both ligands ($n = 3$). Incubation took place for 2 h at 20°C under gentle shaking. Isolation of the receptor–ligand complex from other matrix constituents was performed with the previously described filtering technique using two protamine-coated GF/C filters. The filters were transferred into glass tubes and the ligands were extracted with 5 ml ethyl acetate after breaking of the receptor–ligand complex with 1 ml of a 0.1 mol/l hydrochloric acid solution. Finally, a 4-ml volume of the organic phase was evaporated to dryness in a vacuum Vortex and 500 μl of methanol were added to dissolve the residue, of which an aliquot of 400 μl was evaporated and derivatized with 100 μl of BSTFA–TMCS at 60°C during 1 h. After evaporation of the derivatization mixture, the residue was dissolved in 25 μl isooctane in which 1 ng/μl PCB-138 was dissolved (external standard). Finally, 1 ng of both [$^2H_6$]DES and [$^2H_3$]17β-ES were added as internal standards. Aliquots of 2 μl of the final mixture were injected onto the gas chromatograph at a carrier (helium) gas flow of 25 cm/s, split flow of 20 ml/min, split time of 1 min, injector temperature of 250°C and interface temperature of 260°C. The following temperature programme has been used: 175°C (1 min), 175–200°C (10°C/min), 200–260 (2.5°C/min) and 260°C (14 min). High-resolution electron impact (70 eV) MS detection, as described earlier [9], was carried out in the

positive ion mode, at a source temperature of 225°C. The signal at different m/z ratios was determined by selected ion monitoring (SIM).

3. Results and discussion

3.1. Principle of biospecific sample handling

Receptor–ligand complex is trapped on a protamine-coated surface. The sample handling technique consists of an incubation step in which the sample is allowed to react with the receptor. Subsequently, both receptor and receptor–ligand complex are trapped on a protamine-coated surface. After removal of free ligand and non-binding matrix components the receptor–ligand complex is dissociated at low pH, the analyte extracted with ethyl acetate and determined by GC–MS.

3.2. Biochemical characterization

The receptor concentration, kinetics of association and dissociation of the receptor–ligand complex and temperature dependency of complex formation were determined in order to evaluate their influence on sample capacity, speed of preconcentration and stability of the receptor during sample handling.

Determination of receptor capacity

Mean values for the equilibrium dissociation constant (K_d) of the 17βES–receptor complex and the total concentration of specific binding sites ($[R]_T$) and standard deviations were obtained by means of saturation experiments at three different days. The capacity of the eight times diluted receptor solution yielded 536 ± 80 pmol/l, meaning that 1 ml of undiluted receptor solution contains 4.3 pmol of specific binding sites. The K_d yielded 172 ± 3 pmol/l. From the K_d, which reflects the ligand concentration at which 50% of the total binding sites are occupied, the binding affinity K_a can be derived according to the relation $K_a = 1/K_d$. The average binding affinity of the receptor for 17βES at 20°C yields $5.8 \cdot 10^9$ l/mol. Day-to-day variation of 2% for K_d demonstrates the high reproducibility of receptor–ligand complex formation. In comparison to immobilized antibody supports the capacity using 1 ml receptor solution is approximately 20 times lower. Batch-to-batch variation in K_d and $[R]_T$ can occur. This is mainly determined by the quality of the tissue and by the conditions during isolation (temperature and time needed for different isolation steps).

Kinetics of receptor–ligand association and dissociation

Fig. 1 shows the relation of SB *vs.* incubation time displayed as a typical association curve. From the association curve the association rate constant (k_{+1}) and the dissociation rate constant (k_{-1}) of the complexation reaction of the ligand and the receptor in equilibrium can be calculated [10]. This yields $k_{+1} = 532 \cdot 10^6$ $(\text{mol/l})^{-1}$ min^{-1}.

Association, as can be seen in Fig. 1, is completed within 30 min under the described experimental conditions. This, however, does not have to be a limitation for the application of receptor–ligand interactions in a dynamic system.

Data on the experimental estimation of dissociation of the receptor–ligand complex are shown in Table I. No significant difference in SB between the conventional protamine precipitation assay of 20 min and the extended assay of 50 min is observed, implying that dissociation does not occur within 50 min. NSB of [^{3}H]17βES to

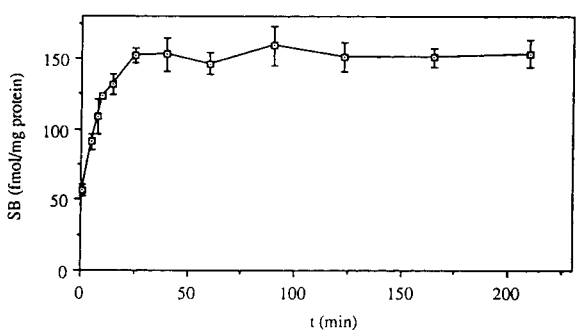

Fig. 1. Graphical representation of specific binding (SB), expressed as fmol [^{3}H]17βES bound per mg protein *vs.* time (t) as a typical association curve ($n = 4$).

Table I
Different values of relative binding (%B/T) and non-specific binding (NSB) of the radioligand to the vials, expressed as percentage of specific binding (SB), for the 20-min protamine precipitation (PP20) assay and for the extended protamine precipitation assay (PP50) of 50 min ($n = 3$)

	%B/T	NSB (%SB)
PP20	80	4.9
PP50	84	1.2

the vials is even lower in the 50 min assay. The k_{-1} can not be determined by substitution of the K_d and the association rate constant in the equation $k_{-1} = K_d k_{+1}$, since both values were determined by different methods.

3.3. Trapping of the ER after incubation

To facilitate the recovery of bound ligand after incubation the estrogen receptor–ligand complex was trapped on a protamine-coated surface. Protamine-coated glass fibre filters or silica were investigated for this purpose. Table II summarizes the properties of several filters with respect to their capability of binding receptor–ligand complex in proportion to the protamine precipitation method. For this purpose the trapping efficiency (%B/T), *i.e.* the percentage of the total amount of [^{3}H]17βES specifically bound to the receptor, was determined for each filter as well as for the protamine precipitation method. It can be concluded that protamine-coated GF/C filters containing two filter layers are most efficiently trapping the receptor–ligand complex. The %B/T equals the value of that of the protamine precipitation method, whereas NSB is even slightly lower.

Attempts to immobilize the ER to protamine-coated filters prior to incubation resulted in recoveries of only 10%. The low recovery can probably be attributed to the low surface density of active binding sites on the filter.

Alternatively to the tested glass fibre filters the trapping properties of protamine-coated silica were investigated. Table III shows the trapping efficiencies of protamine-coated silica and protamine precipitation experiments. Trapping efficiency of protamine-coated silica (P-Si50) and the protamine precipitation method (PP) are approximately the same. Moreover, 50 mg of silica is sufficient for complete recovery of the receptor–ligand complex, since no significant difference in trapping efficiency for the different amounts of silica is observed. The difference in %B/T between coated and plain silica is more clearly than for the filters.

Data shown in Tables II and III exhibit similar

Table II
Different values of relative binding (%B/T) and non-specific binding (NSB) of the radioligand to the vials/filters, expressed as percentage of specific binding (SB) for the different filters

	%B/T	NSB (%SB)
PP	79	5.5
1 × GF/C	20	1.5
2 × GF/C	46	1.5
1 × P-GF/C	45	1.2
2 × P-GF/C	73	1.5
P-GF/B	57	1.3

PP = Protamine precipitation assay; 1 × GF/C = uncoated GF/C filter; 2 × GF/C = two uncoated GF/C filters; 1 × P-GF/C = protamine-coated GF/C filter; 2 × P-GF/C = two protamine-coated GF/C filters; 1 × P-GF/B = protamine-coated GF/B filter ($n = 3$).

Table III
Different values of relative binding (%B/T) and non-specific binding (NSB) of the radioligand to the vials/silica material (NSB), expressed as percentage of specific binding (SB) for the different amounts of silica, either coated or uncoated with protamine

	%B/T	NSB (%SB)
PP	69	7.4
Si50	37	4.5
Si150	36	5.5
P-Si50	65	3.0
P-Si150	59	4.1

PP = Protamine precipitation assay; Si50 = 50 mg of uncoated silica; Si150 = 150 mg of uncoated silica; P-Si50 = 50 mg of protamine-coated silica; P-Si150 = 150 mg of protamine-coated silica ($n = 3$).

trapping properties for both protamine-coated glass fibre filters and silica. In terms of automation silica is preferred to glass fibre filters since silica can be incorporated more easily into conventional solid-phase extraction techniques.

3.4. Application of the ER in sample handling followed by GC–MS

Fig. 2 shows mass chromatograms at different m/z ratios obtained after isolation of a 1:1 mixture of 1 ng of 17βES and DES with the ER solution and subsequent derivatization of the extract. By means of SIM at m/z 412 and 413 the isotope ratio of DES-TMS (peaks 1 and 2, respectively) could be determined. SIM at m/z 416 and 417 yielded the isotope ratio of the 17βES-TMS derivative (peaks 3 and 4, respectively). SIM at m/z 418 shows the derivatized internal standard [$^2H_6$]DES-TMS (peak 5), whereas at m/z 419 the ^{13}C isotope of [$^2H_6$]DES-TMS (peak 6) as well as the derivative of the other internal standard [$^2H_3$]17βES-TMS (peak 7) are represented. Overall recoveries (including derivatization for GC) are 59 and 68% for 1 ng and 250 pg of 17βES, respectively and 5 and 15% for 1 ng and 250 pg of DES, respectively. Incubation of the ER solution with 1 ng of both ligands reveals recoveries of 47 and 7%, respectively. Since an endogenous amount of 17βES is present in the receptor solution (120 pg/ml), recoveries for 17βES are higher than theoretically possible. For the same reason recoveries for DES after isolation of DES only are low, due to competition with endogenous 17βES.

These experiments demonstrate that receptor–ligand interactions can be implemented in sample handling techniques for steroids in the absence of interfering matrix components. In experiments not described calf urine has been pretreated on a XAD-2 precolumn. The column extract was added to a protamine precipitation assay and the amount of receptor–ligand complex was determined and compared to that obtained with a protamine precipitation assay in the absence of the extract. This experiment showed that the presence of the calf urine extract in a binding assay reduced the maximal binding with approximately 60%. Determination of steroids in calf urine pretreated on a XAD-2 precolumn only is hindered by the presence of high concentrations of competing compounds, such as phytohormones and lignanes. The biological activity of these compounds which occur in many forage plants has been described extensively [11–14]. Banner *et al.* [2] also reported an extensive sample pretreatment prior to the isolation of the ligands with the receptor ligand-binding domain, necessary to obtain optimal conditions for receptor–ligand complex formation.

4. Conclusions

The receptor–ligand complex can efficiently be trapped after incubation on either protamine-coated glass fibre filters or silica. The experiments with the protamine-coated filters show that adsorption of the complex to the coated filters occurs within a few seconds. Moreover, washing of the filters after adsorption of the complex, which reduces NSB, did not lead to a decrease in recovery of the steroids investigated. These experiments reveal a strong interaction between the ER and the protamine coating.

It is demonstrated that receptors can be applied to the isolation of ligands from clean sample extracts in sample handling on basis of biological recognition. However, the low recovery of DES in the presence of 17βES is the result of competition between the two ligands in equilibrium for a limited number of receptor binding sites. An excess of receptor binding sites will eliminate competition. Furthermore, immobilization of a high amount of ER onto a solid support for the use in a dynamic sample handling system will probably contribute to an increase in the rate of association. Affinity constants are high enough to ensure immediate association. However, dissociation of the receptor–ligand complex in a dynamic system should be slow in order to obtain retention of the ligands on the affinity column within the time interval needed for sample handling.

Thus, to obtain an operational screening meth-

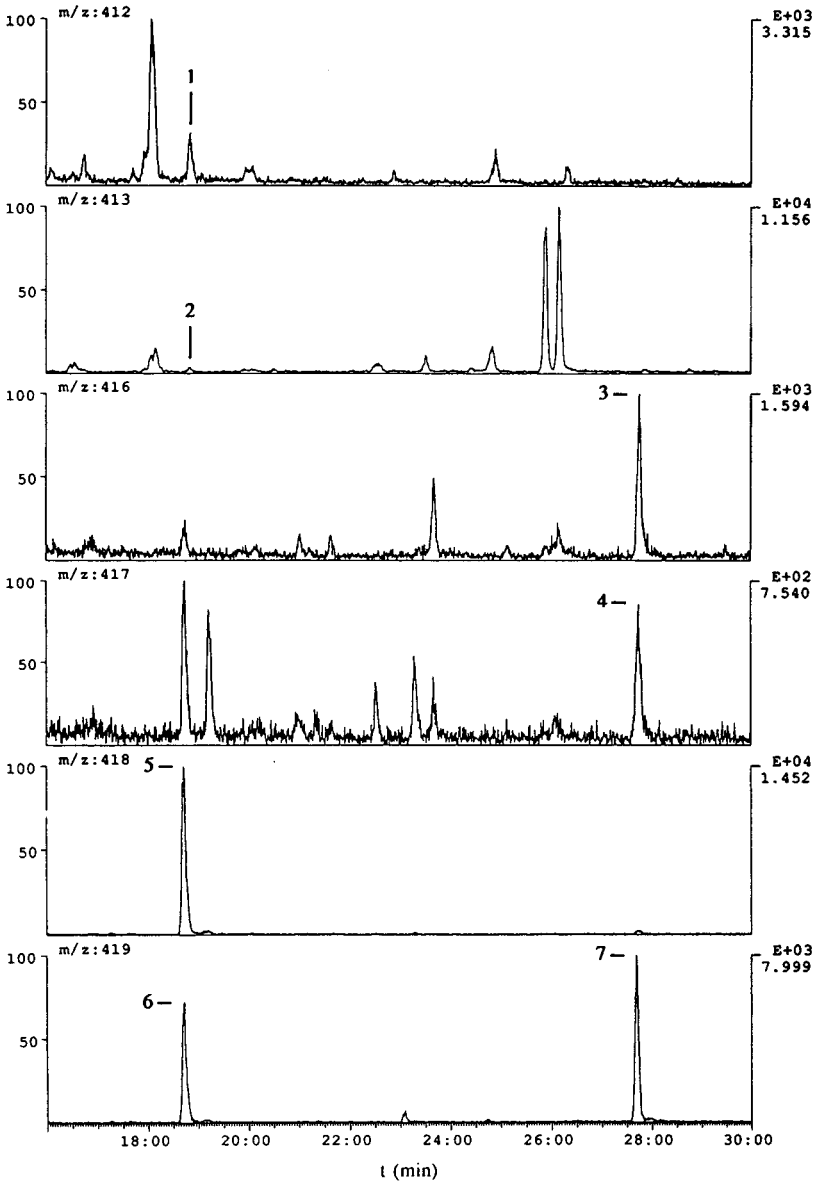

Fig. 2. GC–MS chromatograms by SIM at different m/z ratios of a 1:1 mixture of 17βES and DES (1 ng each) obtained after performing the procedure as described under *Application of the ER in sample handling followed by GC–MS*. Relevant peaks: 1 = DES-TMS; 2 = ^{13}C-DES-TMS; 3 = 17βES-TMS; 4 = ^{13}C-17βES-TMS; 5 = [$^{2}H_6$]DES-TMS; 6 = [$^{2}H_6$]^{13}C-DES-TMS; 7 = [$^{2}H_3$]17βES-TMS.

od for estrogenic compounds a high receptor capacity is required to eliminate competition. This eventually leads to higher recoveries which makes the technique more suitable to be combined with LC. Recombinant DNA techniques should be employed to obtain high amounts of

ER. The development of a screening method for estrogenic compounds in calf urine is under investigation.

5. Acknowledgements

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On-line sample handling of water-soluble organic pollutants in aqueous samples using porous graphitic carbon

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Abstract

An on-line technique coupling preconcentration via a precolumn packed with porous graphitic carbon (PGC) and liquid chromatography with a PGC analytical column was investigated and found to be a very simple and efficient system for the trace-level determination of some very polar and water-soluble organic pollutants (characterized by logarithms of their water–octanol partition coefficients below 1) from environmental waters. As these analytes are much more retained by PGC than they are by C_{18} silica, preconcentration on a PGC precolumn cannot be coupled on-line with the widely used and more efficient C_{18} silica analytical columns, but with a PGC analytical column. Applications are presented for the trace-level determination of some organic compounds included in the EEC environmental priority pollutant list such as 2-chloro-4-aminophenol, chloroanilines, aminophenols and cyanuric acid. The influence of the sample matrix was investigated with drinking and river water samples.

1. Introduction

The determination of trace amounts of organic compounds in environmental aqueous samples requires a preconcentration step before the chromatographic analysis. Polar and water-soluble compounds cannot be determined at trace levels in these media because no simple method exists for their extraction from water samples. These compounds are characterized by weak hydrophobicity, as measured by values of the logarithms of their water–octanol partition coefficients (log P_{oct}) lower than 1, or they are hydrophilic with log $P_{oct} < 0$.

Trace enrichment is still often carried out by means of liquid–liquid extractions in many environmental procedures; nevertheless, recoveries are low for polar analytes and this technique cannot be applied to hydrophilic analytes which are more soluble in water than in usual organic solvents. Solid-phase extraction (SPE) techniques have grown in interest as an alternative to the laborious and time-consuming liquid–liquid extractions. SPE can be considered as a simple chromatographic process and retention of analytes by the sorbent occurs provided they are not eluted by the sample water. Consequently, reversed-phase sorbents and ion exchangers are convenient sorbents for the extraction of organics from water. The key parameter in SPE is the sample volume that can be handled without any breakthrough. In a first approximation, the breakthrough volume can be calculated from the capacity factor of analytes in water, k'_w. In general, C_{18} silicas are convenient sorbents for the trace determination of apolar compounds

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(*i.e.*, log $P_{oct} > 3$). Nevertheless, the capacity factors of moderately polar analytes (*i.e.*, log $P_{oct} = 1–3$) are too low with C_{18} silica to allow the handling of a sufficiently large volume. The retention of organic compounds in water is about 25–40 times higher with apolar copolymers such as PRP-1 or PLRP-S, so that these sorbents are more convenient for the preconcentration of moderately polar compounds [1–3]. As an example, the breakthrough volume of aniline (log $P_{oct} = 0.9$) was measured as 180 ml using a large precolumn (9 cm × 0.46 cm I.D.) packed with the PRP-1 copolymer [4]. This sorbent cannot be used for on-line preconcentration of more polar compounds, because small-sized precolumns are required.

Previous studies have shown that the retention of some polar compounds in water can be very high using porous graphitic carbon (PGC), available recently as a stationary phase for LC [3]. The capacity factors in water of 1,3-dihydroxybenzene and 1,3,5-trihydroxybenzene were measured as 21 and 3, respectively, with PRP-1 and as 331 and 1050, respectively, with PGC [5]. PGC shows a highly ordered crystalline structure with large bands of delocalized electrons, so that the retention mechanism is a mixture of hydrophobic and electronic interactions and is very different from that observed with C_{18} silicas and PRP-1 copolymers [5–9]. PGC is a reversed-phase sorbent and it is observed that the retention of compounds decreases when the organic content of the mobile phase increases. However, for some planar molecules such as benzene derivatives, the retention increases with increasing number of polar substituents on PGC whereas it decreases with the other two reversed-phase sorbents.

The aim of this work was to investigate the potential of PGC for both extracting and determining some water-soluble compounds in aqueous samples. An on-line approach coupling SPE and LC was studied. In contrast to off-line procedures, the advantages are that (i) there is no risk of loss and contamination as there is no sample manipulation between preconcentration and analysis, (ii) more quantitative results are expected and (iii) the entire sample is transferred and analysed, which allows the handling of a smaller sample volume. As many polar analytes are slightly volatile or are partly degraded when heated, on-line procedures are often more convenient for such analytes.

2. Experimental

2.1. Apparatus

A Model 5060 liquid chromatograph equipped with a UV 200 variable-wavelength spectrophotometer (Varian, Palo Alto, CA, USA) was used for direct injections and precolumn elution. On-line percolation of samples was performed using a Varian Model 2010 pump. Precolumn and analytical column switching were connected with two Rheodyne (Berkeley, CA, USA) valves. Quantitative measurements of peak areas were provided by a CR 3A integrator–computer (Shimadzu, Kyoto, Japan).

2.2. Stationary phases and columns

A C_{18} analytical column (25 cm × 0.46 cm I.D.) prepacked with Whatman Spherisorb ODS-2 (Nagel, Düren, Germany) and a column prepacked with Hypercarb porous graphitic carbon (10 cm × 0.46 cm I.D.) (Shandon, Runcorn, UK) were used. Preconcentrations were made through experimental stainless-steel precolumns (1 cm × 0.46 cm I.D.) prepacked with 10–15-μm Hypercarb PGC (Shandon). A 1 cm × 0.2 cm I.D. column available from Chrompack (Middelburg, Netherlands) was laboratory packed with C_{18} silica RP18 from Merck (Darmstadt, Germany) and used in series with the PGC precolumn. Off-line cartridges were packed with experimental 40–60-μm PGC material provided by Shandon.

2.3. Chemicals

HPLC-grade acetonitrile was obtained from Rathburn (Walkerburn, UK) and methanol from Prolabo (Paris, France). LC-grade water was prepared by purifying demineralized water in a

Milli-Q filtration system (Millipore, Bedford, MA, USA). Other chemicals were obtained from Prolabo, Merck or Fluka (Buchs, Switzerland). Stock standard solutions of selected solutes were prepared by weighing and dissolution in methanol or in water. The final standard solutions did not contain more than 0.5% of methanol.

3. Results and discussion

3.1. On-line methodology using a PGC precolumn

On-line coupling of SPE to LC is easily performed in any laboratory and automatic devices are now commercially available. In its simplest form, the extraction precolumn is placed in the sample-loop position of a six-port switching valve. After conditioning, sample application and cleaning via a low-cost pump, the precolumn is coupled to an analytical column by switching the valve to the inject position. The extracted compounds are then eluted directly from the precolumn to the analytical column by a suitable mobile phase which also permits the chromatographic separation of trapped compounds. The LC system is often run in the reversed-phase mode with a C_{18} analytical column and acetonitrile or a methanol–water gradient because the residual water does not have to be removed before desorption.

Band broadening

The size of the precolumn is an important parameter in the coupling because the profile of concentrated species transferred from the precolumn to the analytical column should ideally be as narrow as possible at the beginning of the separation. Consequently, the precolumn dimensions should be as small as possible and adapted to those of the analytical column [10,11]. For a classical analytical column of 15 cm × 0.46 cm I.D., the length of the precolumn should be a maximum of 1 cm and the diameter smaller than 0.46 cm. On another hand, if solutes are slightly retained by the sorbent, it is necessary to increase as much as possible the amount of sorbent and therefore the precolumn dimensions, in order to obtain breakthrough volumes as high as possible. For this reason, precolumns of 1 cm × 0.46 cm I.D. prepacked with 10–15-μm PGC were selected.

Chromatograms obtained by direct loop injection (20 μl) of a benzene solution on to a 10-cm long analytical column prepacked with 7-μm PGC (Hypercarb) and by preconcentrating a 5-ml water sample spiked with 20 μl of the same benzene solution are represented in Fig. 1a and b. The band broadening was assessed by calculating the number of plates from chromatograms corresponding to three replicate direct injections and on-line preconcentrations (Table 1). First, it can be seen that the plate number in the PGC column is low, around 1000, whereas the test carried out with xylenol having a similar k', but in a mobile phase containing 95% of methanol, gave around 3000 plates for the same column. This decrease is due to the high proportion of water (56%) in the mobile phase. When comparing efficiencies obtained by direct injection and via the precolumn, a decrease in the plate number of about 20% is observed and the peak-height ratio is 0.45. A similar decrease was observed when desorbing the precolumn in the opposite way to the percolation (backflush desorption). One reason is that the dimensions of the precolumns are too large in comparison with those of the analytical column, but no analytical column longer than 10 cm is available. The same precolumn was coupled to a 25-cm long analytical column packed with C_{18} silica. The mobile phase was adjusted in order to give the same retention time of benzene (Fig. 1c) and the experiments described above were carried out (Fig. 1d). Comparison of the efficiencies reported in Table I shows that no band broadening is obtained for benzene when the analytical column is longer. Despite equal efficiencies, the peak heights are different and the ratio between direct injection and on-line preconcentration of the same amount of benzene is 0.68. This is explained by the calibration of the loop which is specified to an average accuracy of 20%. Calibration of the loop is a delicate operation and was not performed in this study.

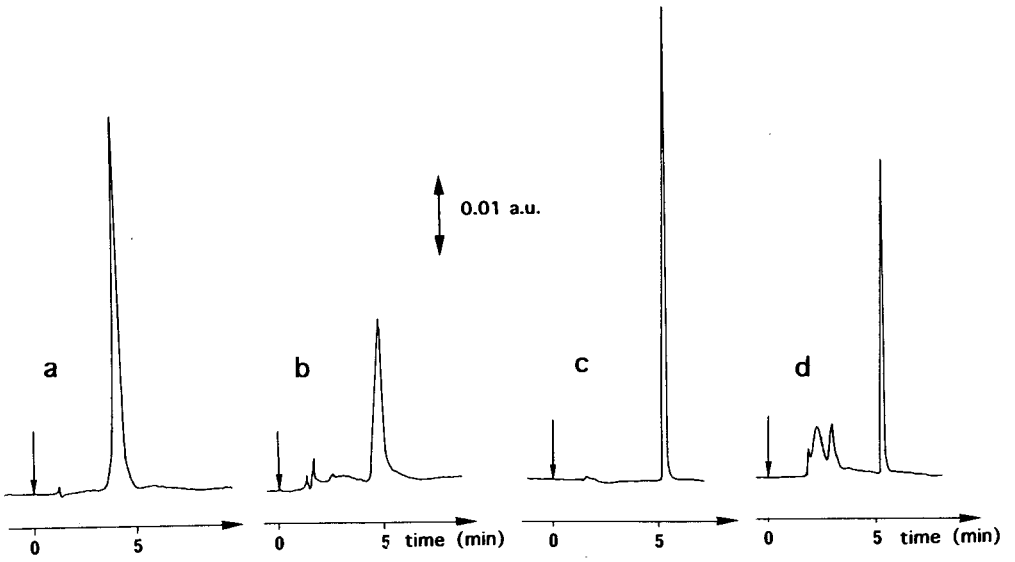

Fig. 1. Chromatograms obtained by direct injection on to PGC and C_{18} columns and by on-line preconcentration with a PGC precolumn. (a) Direct injection of 20 μl of a 10 mg/l benzene solution on to a 10 cm × 0.46 cm I.D. Hypercarb column. Mobile phase, methanol–water (44:56, v/v); flow-rate, 1 ml/min; UV detection at 254 nm. (b) On-line preconcentration of a 5-ml water sample spiked with 20 μl of the 10 mg/l benzene solution. Precolumn, 1 cm × 0.46 cm I.D. packed with PGC; elution in the same column and under the same experimental conditions as in (a). (c) Direct injection of 20 μl of a 10 mg/l benzene solution on to a 25 cm × 046 cm I.D. C_{18} Spherisorb ODS column. Mobile phase, acetonitrile–water (64:36, v/v); flow-rate, 1 ml/min. (d) On-line preconcentration of a 5-ml water sample spiked with 20 μl of the 10 mg/l benzene solution. Precolumn, 1 cm × 0.46 cm I.D. packed with PGC; elution in the same column and under the same experimental conditions as in (c).

Sorbent compatibility

Band broadening can also occur when the analyte is more retained by the sorbent of the precolumn than it is by that of the analytical column [1]. Nevertheless, many examples have been presented using a PRP-1 or PLRP-S precolumn and a C_{18} analytical column. The potential of PGC is in the extraction of compounds that cannot be extracted by C_{18} silica because of insufficient retention. Separation of polar analytes on C_{18} silica is usually achieved with water or water-rich mobile phases which are unable to desorb analytes that are more retained by the PGC precolumn. This is illustrated in Fig. 2 for the trace determination of polar aniline derivates included in the EEC priority pollutant list. On C_{18} silica, the more polar 2-chloro-4-aminophenol ($\log P_{oct} = 1.16$) is eluted before the monochloroanilines ($\log P_{oct} = 1.8$) and separation is achieved with a mobile phase containing 33% of acetonitrile (Fig. 2a). When the same mixture is analysed on-line via the PGC precolumn (Fig. 2b), a large band broadening occurs for 2-chloro-4-aminophenol. A similar separation including also a chloromethylaniline and a dichloroaniline was performed on the PGC col-

Table 1
Peak heights (h) and column efficiencies (plate number, N) measured with (a) direct injections of benzene and (b) on-line preconcentration with the PGC precolumn (mean values of three experiments)

Material	h(a)	h(b)	h(b)/h(a)	N(a)	N(b)
PGC	9.1 ± 0.2	4.1 ± 0.3	0.45	1260 ± 60	960 ± 50
C_{18} silica	11.4 ± 0.2	7.8 ± 0.3	0.68	10900 ± 400	11100 ± 600

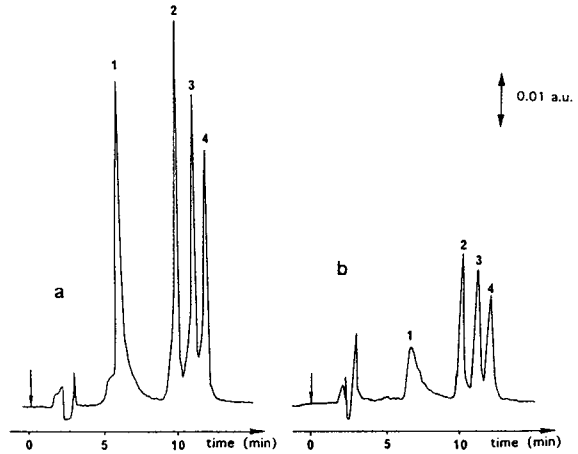

Fig. 2. Chromatograms obtained by direct injection and on-line preconcentration using a C_{18} analytical column. (a) Direct injection of 20 μl of an aniline derivative solution at 10 mg/l on to a 25 cm × 0.46 cm I.D. C_{18} Spherisorb ODS column. Mobile phase, 33% of acetonitrile and 67% of a 0.05 *M* sodium acetate–acetic acid solution at pH 4.6; flow-rate, 1 ml/min; UV detection at 240 nm, 0.1 AUFS Solutes: 1 = 2-chloro-4-aminophenol; 2 = 2-chloroaniline; 3 = 3-chloroaniline; 4 = 4-chloroaniline. (b) On-line preconcentration of a 10-ml water sample spiked with 20 μg/l of each compound. Precolumn, 1 cm × 0.46 cm I.D. packed with PGC; on-line elution into the C_{18} column under the same experimental conditions.

umn (Fig. 3a) and the more polar 2-chloro-4-aminophenol is eluted after the monochloroanilines. The mobile phase allowing the separation of the first compounds contains 68% of methanol. Fig. 3b illustrates clearly that when the sorbents in the precolumn and the analytical column are the same, the band broadening is decreased. Comparison between Fig. 2 and 3 indicates also that the difference in efficiencies between PGC and C_{18} silica is not as large for the aniline derivatives as it was for benzene, because in that example the mobile phase used with PGC contained a higher content of organic solvent than that with C_{18} silica.

It is concluded that for more polar analytes with log $P_{oct} < 1$, the direct coupling of a PGC precolumn and a C_{18} analytical column is impossible unless water is added to the mobile phase after the desorption of the precolumn via another pump. However, coupling with a PGC analytical column is easy.

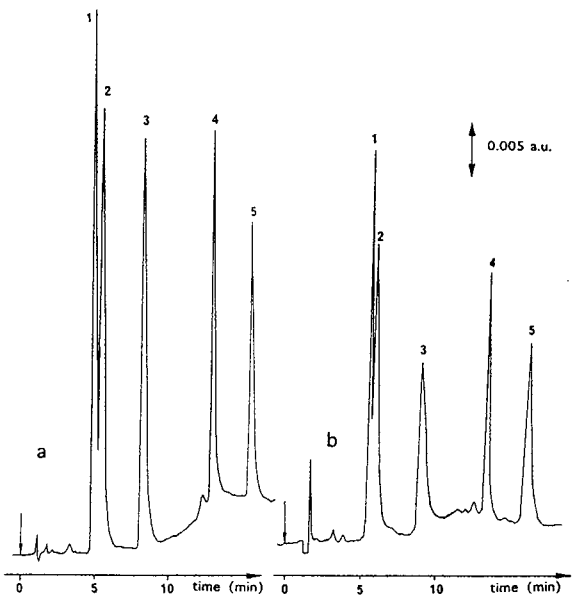

Fig. 3. Chromatograms obtained by direct injection and on-line preconcentration using a Hypercarb analytical column. (a) Direct injection of 20 μl of a solution containing 2-chloro-4-aminophenol of 64 mg/l and other aniline derivatives at 20 mg/l on to a 10 cm × 0.46 cm I.D. Hypercarb column. Mobile phase, methanol gradient with 0.05 *M* sodium acetate–acetic acid solution at pH 4.6, 68% of methanol from 0 to 6 min and up to 91% at 9 min; flow-rate, 1 ml/min; UV detection at 240 nm, 0.05 AUFS. Solutes: 1 = 4-chloroaniline; 2 = 2-chloroaniline; 3 = 2-chloro-4-aminophenol; 4 = 5-chloro-2-methylaniline; 5 = 2,3-dichloroaniline. (b) On-line preconcentration of a 10-ml water sample spiked with 128 μg/l of 2-chloro-4-aminophenol and 45 μg/l of the other compounds. Precolumn: 1 cm × 0.46 cm I.D. packed with PGC; on-line elution into the Hypercarb column under the same experimental conditions.

Precolumn stability

The same precolumn was re-used about 30–50 times with drinking waters and was just washed with pure acetonitrile or methanol after each run. With surface waters, the precolumn was washed with water–tetrahydrofuran (50:50) containing 0.1% of perchloric acid after each run and was changed about after ten runs.

3.2. Applications of water-soluble analytes

Aminophenols

These products are important industrial chemicals and also degradation products of aniline and

Table 2
Water–octanol partition constants and retention in water of aminophenols on PRP-1 and PGC

Solute	Log P_{oct}	Log k'_w	
		PRP-1	PGC
2-Aminophenol	0.52	1.7	1.6
3-Aminophenol	0.17	1.3	1.7
4-Aminophenol	0.04	1.1	2.1

some pesticides. Their extraction from water is difficult owing to their high polarity, as shown in Table 2. A method has been described which involved the preparation of acetate derivatives before extraction with methylene chloride and a second derivatization with trifluoroacetic anhydride before GC analysis and electron-capture detection [12]. 4-Aminophenol is not retained on C_{18} silica and only slightly on PRP-1, but its retention on PGC is ten times higher. We especially studied the on-line determination of 3- and 4-aminophenol because 2-aminophenol is easily oxidized and some drastic experimental conditions have to be applied in order to avoid degradation of this isomer [13]. The analytical separation was obtained with a methanol gradient containing initially 30% of methanol and is shown in Fig. 4a. As the UV maxima are different for the two compounds, a change in the UV wavelength was made at 7.5 min. The retention order of the two isomers follows the decreasing polarity order indicated by the log P_{oct} values in Table 2. Sample volumes of 10 and 20 ml containing the same amount of analytes as the direct injection were analysed on-line (Fig. 4b and c). The peak height of 4-aminophenol is constant for 10 and 20 ml, whereas that of 3-aminophenol decreases between 10 and 20 ml, indicating that breakthrough occurred between

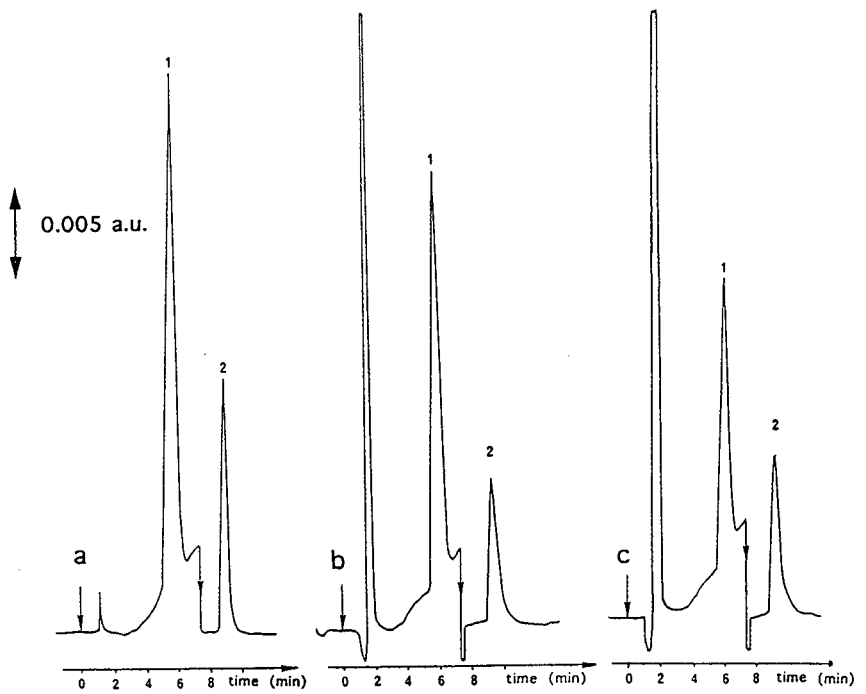

Fig. 4. On-line determination of aminophenols. (a) Direct loop injection of 600 ng of 3-aminophenol (solute 1) and 4-aminophenol (solute 2) in to the 10 cm × 0.46 cm I.D. Hypercarb column; (b) on-line analysis of a 10-ml sample spiked with 60 μg/l and (c) on-line analysis of a 20-ml sample spiked with 30 μg/l. Precolumn, 1 cm × 0.46 cm I.D. packed with PGC; analytical column, 10 cm × 0.46 cm I.D. Hypercarb; mobile phase, methanol gradient with a 0.05 *M* sodium phosphate solution at pH 7, 30 to 50% of methanol from 0 to 5 min; UV detection at 220 nm from 0 to 7.5 min and at 235 nm at 7.5 min (autozero at 7.8 min).

10 and 20 ml for the 3-isomer but not for the 4-isomer. The detection limit for these two compounds was measured as 2 μg/l in a 20-ml sample and can easily be lowered to the μg/l level using this simple on-line system with electrochemical detection, as a factor 500 has been reported between the electrochemical and UV responses [14].

Cyanuric acid

Another example is the trace-level determination of cyanuric acid (2,4,6-trihydroxy-1,3,5-triazine). Cyanuric acid is the ultimate hydroxylated degradation product of some widely used triazine herbicides. Chlorinated derivatives of cyanuric acid are also used in swimming pools as disinfectants and are transformed into cyanuric acid, so that the residual cyanuric acid has to be controlled in pool waters. HPLC methods have been described for the characterization of this analyte with detection limits of the order of mg/l, but no preconcentration method has been reported [15–17]. The logarithm of the water–octanol partition constant was calculated relative to that atrazine (2.7), and was found to be −0.2, thus indicating a high polarity and solubility in water [18]. This analyte is very slightly retained by C_{18} silica in water ($\log k'_w = -0.3$) and not at all by PRP-1, whereas it is highly retained by PGC. Log k'_w was extrapolated from the linear relationship between log k' and the methanol content of the mobile phase to be 2.5 ± 0.2. This result is equivalent to that obtained for 1,3,5-trihydroxybenzene [5] and is another example of a water-soluble analyte that cannot be extracted from aqueous media by conventional sorbents or by liquid–liquid extraction.

Fig. 5a and b represent the direct injection of 20 μl of swimming-pool water on to the PGC analytical column and a C_{18} analytical column and the cyanuric acid concentration was measured to be about 4 mg/l. The difference in retention can be assessed by the difference in the mobile phase composition, pure water with the C_{18} column and 30% methanol for the PGC column. The effect of pH was studied on the PGC column and the retention did not vary between pH 3 and 8, despite the pK_a value of

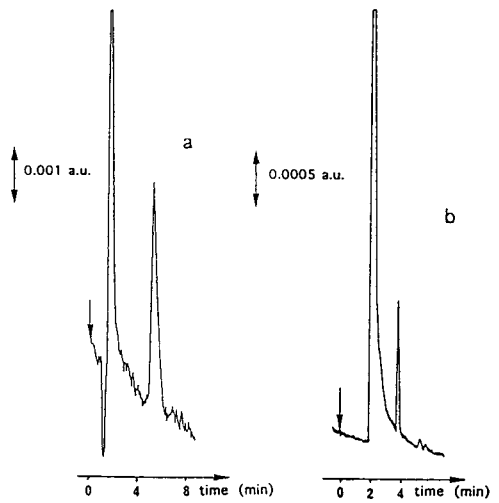

Fig. 5. Determination of cyanuric acid in a swimming pool water sample by direct injection of a 20-μl aliquot. (a) Analytical column, 10 cm × 0.46 cm I.D. Hypercarb; mobile phase, 30% of methanol with 70% of a 0.05 *M* sodium phosphate solution at pH 7, flow-rate, 1 ml/min UV detection at 220 nm, 0.01 AUFS. (b) Analytical column, 25 cm × 0.46 cm I.D. C_{18} Spherisorb ODS; mobile phase, water adjusted to pH 3 with perchloric acid; UV detection at 205 nm, 0.005 AUFS.

6.9. A 0.005 *M* sodium phosphate solution of pH 7 was selected as the mobile phase because it provided a sharper peak. As the UV spectrum of cyanuric acid does not show any absorbance above 220 nm, this compound cannot be identified in unknown samples using only diode-array UV detection and the retention times using the two different columns can serve as confirmation.

A lower detection level is obtained using the on-line preconcentration set-up. From the log k'_w found by extrapolation of the log k'–methanol content relationship, the breakthrough volume, V_b, was calculated to be between 25 and 70 ml. The V_b value was measured more accurately to be 50 ml by percolating increasing volumes of samples containing the same amount of cyanuric acid and by measuring the peak height on the chromatograms obtained by on-line elution as reported in Table 3 [2].

Fig. 6 represents the chromatograms obtained by on-line analysis of 50 ml of LC-grade drinking water and river water samples spiked with 3 μg/l

Table 3
Variation of peak heights (in cm) with the sample volume analysed on-line and corresponding recoveries (mean values of two experiments) according to ref. 2

Parameter	Sample volume (ml)							
	10	20	30	50	60	70	80	100
Concentration (μg/l)	85.6	42.8	28.5	17.1	14.3	12.2	10.7	8.6
Peak height	8.6	8.4	8.7	8.4	6.3	3.6	2.6	2.1
Recovery (%)	100	98	101	98	73	41	33	24

of cyanuric acid. The peak heights are equivalent for the different waters. An autozero has to be set at 3 min for river water owing to the larger amounts of interferences in these waters. A concentration of 3 μg/l is close to the detection limit in each water. If a lower detection level is required, the only means is to increase the amount of carbon in order to increase the breakthrough volume and an off-line procedure using a cartridge has to be applied. A cartridge was packed with 500 mg of 40–60-μm PGC sorbent and after conditioning with methanol and water a sample of 250 ml of drinking water spiked with 5 μg/l of cyanuric acid were percolated through it. Elution was carried out with 20 ml of methanol with subsequent evaporation to dryness. As cyanuric acid is more soluble in water than it is in methanol, injection of the extract reconstituted in 500 μl of methanol gave a bad peak shape, whereas reconstitution in 500 μl of water gave a correct peak. A mean recovery of 96% was measured with three replicate experiments by direct injection of 20 μl of the extracts corresponding to samples spiked at the 5 μg/l level. A volume of 500 μl was necessary to achieve total dissolution of the extract and 100 μl is the largest volume that can be directly injected into a 10-cm column. Therefore, it is obvious that the same detection limits are obtained using the on-line procedure with handling of a 50-ml sample volume and using off-line preconcentration with a 250-ml sample volume, as the injection of a 100-μl aliquot corresponds to a 50-ml fraction of the 250 ml of off-line preconcentrated solution. This problem was overcome by on-line analysing nearly the totality of the extract.

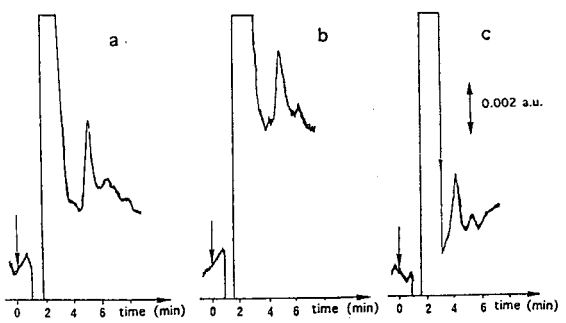

Fig. 6. On-line analysis of 50 ml of (a) LC-grade water, (b) drinking water and (c) river Seine water samples each spiked with 3 μg/l of cyanuric acid. Precolumn packed with PGC and other conditions as in Fig. 5a; UV detection at 220 nm, 0.02 AUFS.

After off-line preconcentration the dry extract was reconstituted in 30 ml of LC-grade water and 25 ml were analysed on-line, thus allowing the analysis of 83% of the extract from the 250 ml of sample concentrated in the cartridge. Using this coupling of an off-line extraction with on-line analysis, the chromatogram in Fig. 7 was obtained for the analysis of 250 ml of drinking water spiked with 5 μg/l of cyanuric acid. The UV attenuation is 2.5 times higher and the peak height much larger than in Fig. 6. The detection limit was 0.2 μg/l under these experimental conditions and can be easily lowered because the sample volume can be increased to 500 ml without breakthrough on a 500-mg cartridge and also because the extracts coming from two cartridges can be mixed together before on-line analysis. The on-line analysis of this extract was

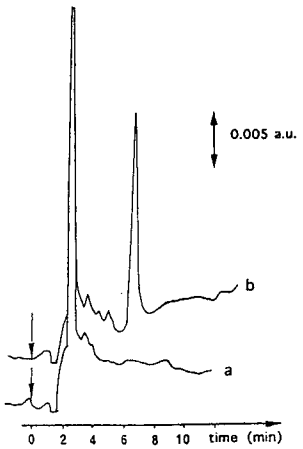

Fig. 7. On-line analysis of an off-line drinking water extract from 250 ml of drinking water, (a) non-spiked and (b) spiked with 5 μg/l of cyanuric acid. Dissolution of the dry extract in 30 ml of water and on-line analysis of a 25-ml aliquot (83% of the extract); other conditions as in Fig. 6; UV detection at 220 nm, 0.05 AUFS.

possible only because this analyte is water soluble.

4. Conclusions

The trace determination of some very polar and water-soluble organic pollutants in environmental waters requires specific strategies as illustrated in this study. On-line sample handling coupling the preconcentration of compounds using a 1-cm long precolumn packed with porous graphitic carbon and liquid chromatographic separation using a PGC analytical column is a very simple and efficient system for their trace-level determination, although smaller precolumns and more efficient analytical columns should be required. The potential of PGC as an extraction sorbent for water-soluble analytes will allow the study of the monitoring and behaviour of numerous compounds and degradation products in the environment.

5. Acknowledgements

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JOURNAL OF CHROMATOGRAPHY A

Characterisation of C_{18}-bonded silicas for solid-phase extraction by solid-state NMR spectroscopy

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Abstract

^{29}Si and ^{13}C cross-polarisation magic-angle-spinning (CP-MAS) NMR spectroscopy have been used to characterise a total of seven C_{18}-bonded silicas used for solid-phase extraction. Wide variations were observed in the extraction properties of these silicas for basic analytes such as the drug propranolol. These differences depended upon the degree of carbon loading of the phases and whether or not endcapping had been performed. The different properties of the phases appear to correlate well with the differences observed using CP-MAS NMR. This demonstrates the utility of such techniques as a means of further understanding the underlying mechanisms responsible for the observed extraction properties of the phases.

1. Introduction

The use of silica based materials for sample preparation in the form of solid-phase (or liquid–solid) extraction (SPE, LSE) is now well established, particularly in the fields of biomedical and environmental analysis. With the widespread and increasing use of these products it has become clear that there can be wide variations in extraction properties for nominally similar packings (*e.g.* C_{18}) obtained from different manufacturers. Further, batch-to-batch variation has also been noted for materials produced by the same manufacturer. Whilst this situation is by no means unique, and indeed such differences have been widely discussed for liquid chromatography packings (where they have also been exploited as a useful source of chromatographic selectivity) it nevertheless merits study as a means of improving our understanding of the extraction behaviour of SPE phases. For some time we have been interested in the extraction of basic drugs (particularly β-blockers), where it is suspected that a mixed mode of extraction onto C_{18}-bonded silica (involving an ion-exchange interaction with residual silanols) occurs [1–4]. Recently we have investigated the SPE of a range of β-blockers onto a number of C_{18}-bonded silicas produced by the same manufacturer but with different degrees of carbon loading and endcapping [5]. Clear differences were apparent in the extraction behaviour of these phases under the conditions employed, with extraction efficiency decreasing with increasing carbon loading/endcapping. Such results provided a further indication of the likely importance of residual silanols in explaining the SPE of the analytes. Accordingly we have undertaken a further study in which the phases have been investigated using solid-state NMR. This

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approach has previously been found to be of value in the study of the bonded phases used for HPLC [5–11]. Here, ^{29}Si and ^{13}C cross-polarisation magic-angle-spinning (CP-MAS) NMR spectroscopy have been utilised to characterise the various bonded silicas in order to attempt to better understand the mechanisms responsible for the SPE results obtained with these materials.

2. Experimental

2.1. Cartridges

Three types of C_{18}-bonded cartridges, without endcapping, were used. These corresponded to the ODS [5% nominal carbon loading (CL)], ODS1 (14%CL), and the ODS2 (16% CL). Four types of C_{18}-bonded and endcapped cartridges were studied corresponding to the ODS3 (10.5% CL), ODS4 (14% CL), ODS5 (18% CL) and the ODS6 (22% CL). The cartridges were obtained from Whatman (Maidstone, UK). Elemental analysis gave values of 3.8, 11.6, 16.9, 10.7, 13.6, 20.4 and 21.5% for the carbon loadings of the ODS, ODS1, ODS2, ODS3, ODS4, ODS5 and ODS6 phases, respectively.

2.2. Extraction procedure

The extraction procedure used for [^{14}C]propranolol has been described in detail elsewhere [5]. Briefly, aqueous samples (0.2 *M* sodium acetate buffer, pH 5) were applied to columns, preconditioned by washing with methanol. Following sample application the columns were washed with water and then acetonitrile. The retained material was then eluted from the cartridges with methanol–triethylamine acetate (0.1 *M*, pH 7) (80:20, v/v). Radioactivity in column eluates was determined by scintillation counting.

2.3. CP-MAS NMR spectroscopy

Details of the solid-state NMR spectroscopy are as follows: NMR measurements were performed on a Bruker MSL 200 spectrometer on samples of the various C_{18}-bonded silicas (2–300 mg) in double bearing rotors of zirconium oxide. Magic-angle spinning was carried out at a rate of 3500 Hz. The spectra were recorded using a flip-back pulse sequence with pulse lengths of 5–7 μs and a repetition rate of 1 s. For each spectrum 2000 scans were accumulated. ^{29}Si CP-MAS NMR spectra were recorded with a contact time of 5 ms. For ^{13}C CP-MAS NMR spectra contact times of 3 ms were used. All NMR spectra were externally referenced to liquid tetramethylsilane.

3. Results and discussion

Typical results for the extraction of propranolol on the SPE materials studied here are shown in Fig. 1 [5]. Samples were applied at pH 5 to ensure the ionisation of the analyte and thus promote ionic interactions with the silanols, followed by an acetonitrile wash (to elute compounds retained only by reversed-phase partition). Under these conditions the higher the carbon loading and degree of endcapping the lower the extraction efficiency and the more easily the compound was eluted from the column at the wash step. Similar results were obtained for a number of other β-blockers [5]. As we have discussed elsewhere [1–3,5], such results are most easily explained by postulating an ionic interaction with residual silanols on the silica surface. Increasing the degree of coverage with ODS groups coupled with endcapping would therefore be expected to affect such interactions by both decreasing the number of available silanols and also reducing access to them as a result of steric effects. The investigation of these modified silicas using CP-MAS NMR might therefore be expected to reveal differences between the various SPE phases. Previously we have successfully used such techniques to characterise bonded phases for HPLC, [7–9,11–12] and the resonances for the various groups present on the surface of the silica have been assigned (see Fig. 2). The ^{29}Si CP-MAS NMR spectra for each of these phases are shown in Figs. 3 (unend-

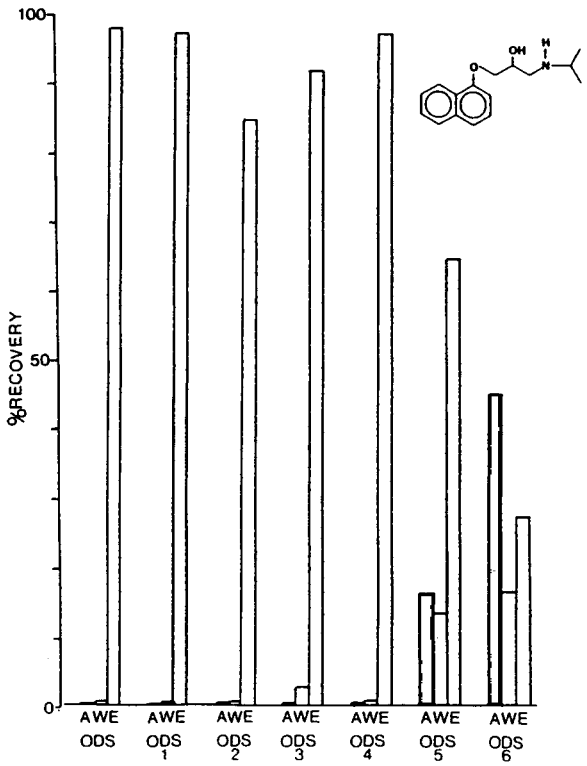

Fig. 1. Typical results for the solid-phase extraction of [^{14}C]propranolol from aqueous buffer on to the various C_{18}-bonded silicas showing the proportion of the applied radioactivity recovered at each stage of the extraction. A = Application step; W = wash step; E = elution step. Each value is the mean of two determinations.

capped) and 4 (endcapped) respectively. The spectra show three groups of peaks. The first group, from −90 to −110 ppm, result from the surface silicon atoms present on the silica gel. The resolution of the peaks is sufficient to distinguish between the silanediols [−90 ppm, Si(OH)2, Q_2], silanols [−100 ppm, Si(OH), Q_3] and siloxanes (SiOSi, −110 ppm, Q_4). The second group of peaks, covering the range −48 to −66 ppm indicate that the silica gel has been modified by reaction with a trifunctional alkylsilyl group. The exact chemical shift of the signal being dependent on the degree of cross-linking. Thus monodentate bonded and non-cross-linked silicons appear at −48 ppm (T_1), bidentate at −56 ppm (T_2) and fully cross-linked

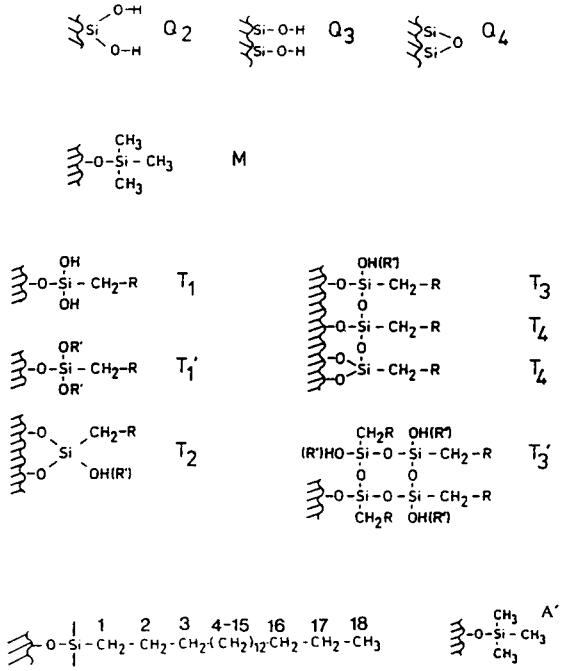

Fig. 2. The various types of groups likely to be found on the surface of C_{18}-bonded silicas. The lettering (Q_2 etc.) is used in the subsequent CP-MAS NMR spectra.

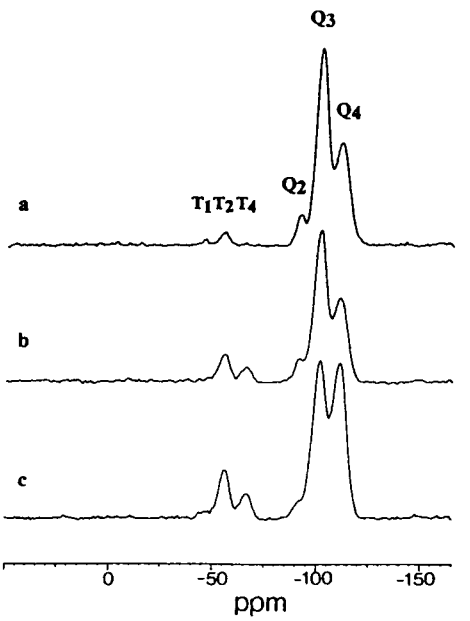

Fig. 3. ^{29}Si CP-MAS NMR spectra of the unendcapped C_{18}-bonded silicas. (a) ODS; (b) ODS1; (c) ODS2. See Fig. 2 for key to signal assignments.

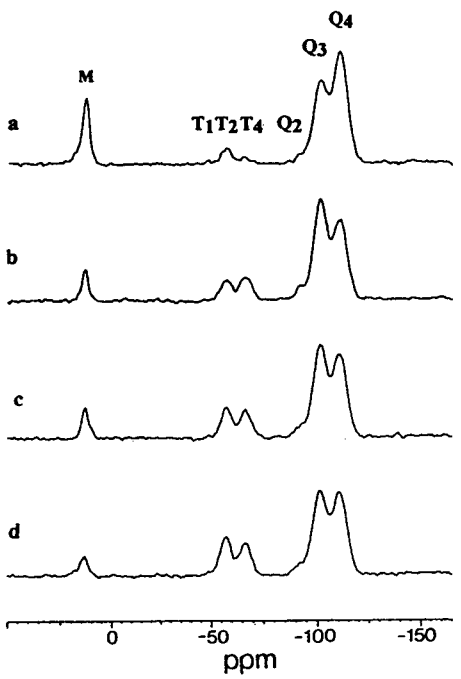

Fig. 4. ^{29}Si CP-MAS NMR spectra of the endcapped C_{18}-bonded silicas. (a) ODS3; (b) ODS4; (c) ODS5; (d) ODS6.

species at −66 ppm (T_4). The endcapped silicas show a further signal at +13 ppm corresponding to the so-called M group of a monofunctional alkylsilyl, in this case that of the trimethylsilyl (TMS) group used for endcapping.

Given that the spectra for all of the various phases were recorded and processed under the same conditions the intensities of the peaks of the M and T groups can be compared. In the case of the unendcapped phases the comparison is simplified by the fact that only ODS (T) groups are present, and therefore a higher carbon loading causes an increase in the intensity of the T-group family of peaks. Clearly, in the case of those silica gels with a low carbon loading, such as the ODS material (nominal 5% carbon loading), and in the absence of endcapping, a considerable number of residual silanols remain available (Fig. 3a) for interaction with a basic analyte such as propranolol. As the spectra show (Fig. 3a–c), with increasing carbon loading (*e.g.* ODS1 and ODS2 with a nominal loading of 14 and 16% carbon, respectively) the proportion of these silanols was steadily reduced. However, without endcapping large numbers are still present. Where the silica gel has been endcapped the number of TMS groups (M groups) must also be considered. However, the M and T groups have similar dynamic behaviours and thus their ratio can readily be determined. For the ODS3 phase some 70% of all the groups bonded to the silica are in fact M groups. For the remaining endcapped silicas (ODS4, 5 and 6) the ratio of TMS to ODS groups ranges from 20 to 30%.

Comparison of the intensities of the Q_2, Q_3 and Q_4 peaks in the ^{29}Si spectra allows an assessment of the numbers of residual silanols to be made. Thus as a first approximation it can be seen that, as the carbon loading increases, the intensity of the Q_2 and Q_3 groups decreases. The intensity of the Q_4 peak (−110 ppm), which corresponds to the Si–O–Si of the siloxanes should remain unchanged, as these groups take no part in bonding with ODS or TMS groups. Nevertheless the intensity of the Q_4 peak does fall as the carbon loading is increased. This is a consequence of the CP-MAS NMR experiment in which signal intensity is proportional to the number of ^{1}H nuclei in the immediate vicinity of the silicon atoms. Modification of the silica, causing a reduction in the number of silanols, therefore results in a decrease in the intensity of the siloxane signals. In general the results obtained are consistant with these expectations. The ODS2 phase does, however, provide an exception to this trend, giving the highest Q_4 peak of all the phases. Furthermore the intensity of the Q_3 peak for the silanols is also higher in this material than in the ODS1 phase, despite the ODS2 material having a higher carbon loading. It is not easy to rationalise this behaviour. It is possible that some other magnetisation mechanism(s) are operating (*e.g.* through space from the ODS chain to the surface silicon atoms) or some other dynamical behaviour of the silica gel is responsible. Alternatively some further treatment of the silica may have been undertaken (*e.g.* heating or acid treatment), or another type/batch of silica may have been used for the manufacture of the ODS2 material. The reduc-

tion in the number of silanols present on the surface of the ODS3 material, an endcapped material which has a carbon loading of 10.5% is however, quite marked. As noted above the amount of TMS relative to ODS on this material is quite high. At the higher carbon loadings obtained with the ODS4 (14%), ODS5 (16%) and ODS6 (22%) the endcapping with TMS appears to have been less efficient and more silanols are detectable on these phases than on the ODS3 silica. This perhaps results from steric factors, due to the higher ODS coverage, preventing the endcapping reagent from gaining access to the silanols.

The ^{13}C CP-MAS NMR spectra reveal a range of peaks between 0 and +50 ppm (Figs. 5 and 6). Many of the carbon atoms of the ODS chains have similar chemical shifts and are not resolved in these spectra. For the ODS chains the signal assignments are as follows: C-1 12 ppm, C-18, 14 ppm; C-2 and 17, 22.5 ppm, C-4 to C-15, 30 ppm and C-3 and C-16, 32 ppm. The endcapped phases also show resonances for the methyl carbons of the TMS groups (C-A′) which appear

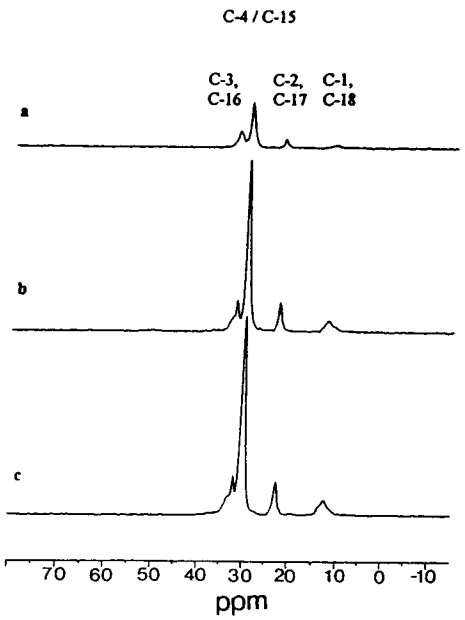

Fig. 5. ^{13}C CP-MAS NMR spectra of the unendcapped silicas. (a) ODS; (b) ODS1; (c) ODS2. See Fig. 2 for key to signal assignments.

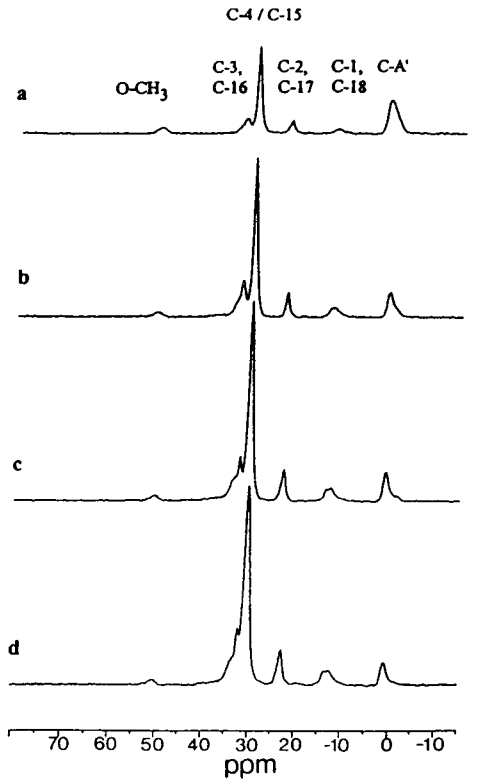

Fig. 6. ^{13}C CP-MAS NMR spectra of the endcapped silicas. (a) ODS3; (b) ODS4; (c) ODS5; (d) ODS6. See Fig. 2 for key to signal assignments, O–CH3 is residual methanol.

at +1 ppm. A further peak at +50 ppm results from the presence of adsorbed methanol. The ^{13}C spectra show a good agreement between carbon loading and peak intensity. With the higher carbon loading the integral of peaks for the ODS chain increases, and the peak intensity of the C-A′ groups also increases in proportion to the degree of endcapping. Thus, the ^{29}Si and ^{13}C CP-MAS NMR results show clear differences between the seven C_{18}-bonded phases examined, and differences in the SPE properties of these phases for β-blockers have also been demonstrated [5]. The question to be addressed therefore is, can the differences in extraction properties be reconciled with the NMR spectra of the phases?. Clearly the presence of silanols on the surface of these phases is consistent with a role in an ionic extraction mechanism, and the reduced number available on the more highly

loaded and endcapped phases is also consistent with the extraction characteristics of these phases. However, this is clearly not the complete explanation as significant numbers of silanols are still present even at the very highest carbon loadings. Indeed it is interesting to note that the material which had the fewest residual silanols was the ODS3 and not the ODS6 material. However, despite the lower number of silanols present reasonably efficient extractions were obtained on ODS3 but not on the ODS6 phase. This result is explicable not merely in terms of numbers of silanols but also access to them. In fact close examination of the data do show a trend for the extractions of propranolol and a number of other β-blockers to be less good on the ODS3 material than on either ODS2 or ODS4 silicas, with small but significant loss of material from the ODS3 column at the wash step [5]. The relatively low loading of C_{18} groups on the ODS3 phase probably means that, whilst the overall number of silanol groups is lower than on the other endcapped phases, steric factors do not prevent the ready interaction of the analyte with such silanols as remain. In contrast it seems likely that on the ODS6 phase the access of the analytes to the silanols present on the silica surface might be significantly reduced, both as a result of steric factors and the need for the analyte, which will be positively charged under the conditions used, to penetrate the highly lipophilic C_{18} layer.

4. Conclusions

Solid-state CP-MAS NMR spectroscopy revealed clear differences between a number of different silicas used for SPE. These differences appear to relate to the observed extraction behaviour of the phases suggesting that CP-MAS NMR may be a useful tool for the investigation of these types of phenomena. Given these results it would seem that it would be highly desirable for such information to the generated by the manufacturers of SPE (and indeed bonded phases for chromatography) as part of the quality control process. In this way batch-to-batch variations might become more easily predicted.

Further experiments to explore the utility of CP-MAS NMR in the study of SPE are continuing.

5. Acknowledgements

We thank Brigitte Schindler for help in obtaining the CP-MAS NMR spectra. Useful discussion with Dr. B. Law and Professor J.K. Nicholson are gratefully acknowledged.

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Automated system for the trace analysis of organic compounds with supported liquid membranes for sample enrichment

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Abstract

Supported liquid membranes, mounted in flow systems, can be used for selective and efficient extraction and enrichment of various types of analytes. The basic principle is a continuous extraction from an aqueous phase into an organic solvent, immobilized in a porous hydrophobic membrane, followed by a continuous back-extraction to a stagnant aqueous acceptor phase on the other side of the membrane. The entire acceptor phase is then used for further analysis.

The ASTED (Gilson) automated sample preparation system was modified by exchanging the dialysis unit for a supported liquid membrane unit. This permits a direct connection to a liquid chromatographic column with minimal sample losses. The instrument can be loaded with up to 60 samples, which are automatically processed before the final chromatographic analysis. With this equipment, the same sample can pass the membrane extractor several times (while the acceptor phase remains stagnant) to give a higher recovery. When one sample is chromatographed the next one is enriched simultaneously.

The technique was evaluated for the extraction of a basic drug and its metabolite from water solutions with the purpose to be applied to blood plasma samples. The recovery depends on the time used for the enrichment. With enrichment times similar to the chromatographic run, or *ca.* 15 min, the recovery was *ca.* 35%.

1. Introduction

Automation of a complete analysis is desirable when a large number of samples regularly must be handled, as often is the case in *e.g.* bioanalysis. Automation of a trace analysis procedure often leads to reduced costs, better reproducibility and a reduced risk of contamination. When biological samples are handled, the risk of being infected is also smaller with a closed, automated system.

Classical workup techniques, such as liquid–liquid extraction and column fractionation [1,2] are difficult to automate. The most successful approach has been to use flow systems as Autoanalyzers (Technicon) and systems based on flow injection analysis (FIA) techniques for relatively simple applications [3,4].

Solid phase extraction (SPE) [5] is a relatively new technique, which in many cases is very efficient for sample cleanup, especially for lipophilic compounds, but also in this case, automation is not straightforward. A few commercial robotic systems, notably the ASPEC (Gilson, Villiers-le-Bel, France), Millilab (Millipore, Bedford, MA, USA) and Benchmate (Zymark,

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Hopkinton, MA, USA), are available for SPE and related sample workup operations in combination with HPLC [6]. The combination of automated on-line sample workup with GC is not common.

A novel approach for selective extraction and enrichment is the supported liquid membrane (SLM) technique [7–9]. This technique has been applied to various classes of compounds, including amines [10,11], acids [12–14] and metals [8]. It can handle complex biological matrices as urine [10], blood plasma [11] and faeces [13] and be connected on-line to gas [10,11] or liquid [12,14] chromatographic equipment. A notable feature is that chromatograms obtained from blank samples containing these matrices are very similar to chromatograms from distilled water blanks, showing a very efficient cleanup. The SLM principle is superficially similar to dialysis and these techniques share the efficient rejection of macromolecular matrix components. However, in dialysis [3,9], low molecular components nonselectively pass the membrane and are diluted in the acceptor liquid (and, with the ASTED, later concentrated on a SPE cartridge). The SLM technique gives a selective enrichment in a small volume.

Here we present a novel technical solution for automated analysis of ionizable substances. It is based on the SLM technology for sample workup with the membrane unit incorporated into a modified ASTED (Gilson) equipment, directly connected to a HPLC system. The object is to process small sample volumes (<1 ml) and to obtain a final extract with a volume compatible with a direct injection into a HPLC. Due to low concentrations, a high transfer rate through the membrane is needed, and the time for the enrichment should be in the same range of time as the chromatographic run. Three basic compounds, the drug Amperozide and two related compounds (Kabi-Pharmacia AB, Lund, Sweden), were used as model substances. Amperozide is an amine (see under Chemicals) with a pK_a of 6.8.

In a forthcoming publication, the application of this methodology to blood plasma samples will be presented.

2. Experimental

2.1. Apparatus

An ASTED (Automated Sequential Trace Enrichment of Dialysates) instrument (Gilson Medical Electronics, Villiers-le-Bel, France) was modified in the following way: the original dialysis membrane holder was exchanged for a custom made holder for the supported liquid membrane, shown in Fig. 1. This consisted of two $10 \times 25 \times 70$ mm PVDF (polyvinyliden fluoride) blocks with identical machined grooves having the dimensions $0.1 \times 2.5 \times 40$ mm, forming channels with a nominal volume of 10 μl each. The membrane (6×46 mm) was placed between the blocks, which were clamped together with six bolts. To prevent stoppage in the acceptor channel, especially when high donor flow rates were used, a thin polyethene spacer was placed between the acceptor block and the membrane. Low-volume connections for standard Altex type fittings were provided in the blocks.

The original dialysis membrane holder in the ASTED instrument was replaced with the liquid membrane unit. The original SPE cartridge, connected to the injection valve, was replaced with a 30 μl loop of stainless steel tubing. The parts of the ASTED equipment needed for this application can also be purchased separately

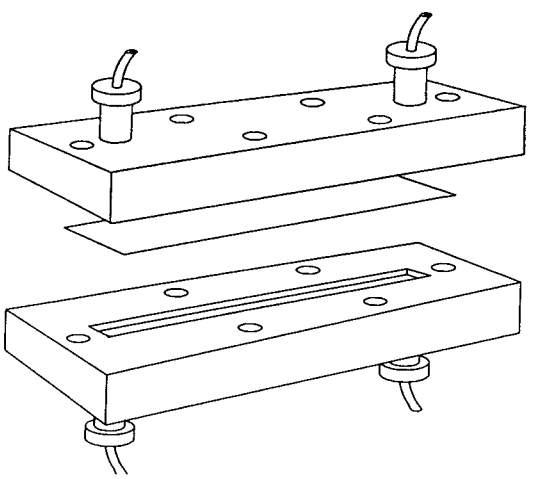


Fig. 1. Membrane unit.

(Model 231 sample handling unit and an extra syringe pump). The hardware related to the dialysis membrane as well as the special ASTED software is not needed.

The porous PTFE membrane used (Model TE35, 0.2 μm, Schleicher and Schuell, Dassel, Germany), was cut to fit in the holder and soaked for 15 min in the selected solvent. After mounting, a washing sequence was run to ensure that excess solvent was washed away from the membrane surfaces.

The equipment for the analysis consisted of a HPLC pump (Model 2150, LKB, Bromma, Sweden), a column (150 mm × 4.6 mm I.D., packed with 5 μm Nucleosil C_{18}, Hichrom), a variable-wavelength UV detector (Spectroflow, Kratos, Ramsey, NJ, USA) and a strip-chart recorder (Model 2210, LKB, Bromma, Sweden). The mobile phase flow-rate was usually 0.65 ml/min and the UV detector was set at 265 nm. Baseline separation of the compounds studied was obtained in about 15 min.

2.2. Operation

The operation is described with reference to Fig. 2. Sample from one of the vials (1), usually 800 μl, is sucked into the needle (2) by the syringe pump (3) after mixing with basic donor buffer (4). The needle is then moved to the injection port (5) and the sample is slowly pressed into the donor channel (6) of the membrane unit, where the unprotonated amines (drug compounds) are extracted into the membrane and subsequently re-extracted into the acceptor (7), where they are protonated and irreversibly trapped in a stagnant acidic buffer. The sample passes into a coil (8), large enough to accommodate the entire sample. The sample may then be sucked back through the donor channel, passing the membrane again and into a second coil (9). These steps can easily be repeated, permitting extractions of the same sample. After the extraction is completed, the donor channel is washed with sample-free donor solution. This step can remove uncharged compounds which may have been transferred into the acceptor phase together with the amines [8]. Finally, the acceptor volume, containing the extracted amines, is transferred by means of the second syringe (10) into the loop injector valve (11) and injected into the HPLC column (12), which is connected to the pump (13). Before a new sample cycle is started, both the donor and the acceptor channels are rinsed with donor (4) and acceptor (14) buffers, respectively.

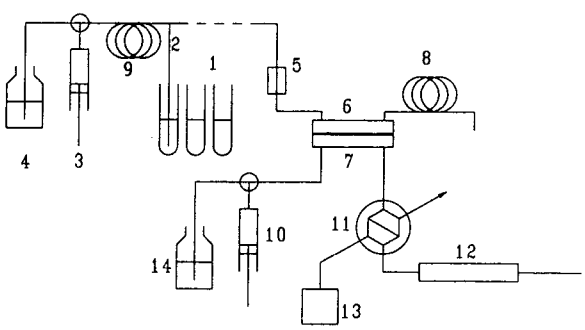


Fig. 2. Experimental setup. For details see the text.

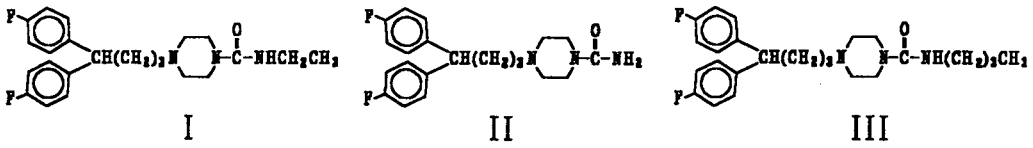
The sequence of operation of the equipment was programmed into the ASTED microprocessor. The complete program can be obtained from the corresponding author of this paper.

With the ASTED, up to 60 samples can be processed, and five different reagents can be automatically added to the samples.

2.3. Chemicals

Amperozide, 4-[4,4-bis(4-fluorophenyl)butyl]-N-ethyl-1-piperazinecarboxamide (**I**), a main metabolite, 4 - [4,4 - bis(4 - fluorophenyl)butyl] - 1-piperazinecarboxamide (**II**), and an analogue compound intended for use as an internal standard, 4-[4,4-bis(4-fluorophenyl)butyl]-N-butyl-1-piperazinecarboxamide (**III**), were obtained as hydrochlorides from Kabi-Pharmacia. Their purity was >99%. Stock solutions were prepared in water (200 μg/ml) and were stable for at least several months when kept in a refrigerator.

The donor solution contained 12.5 m*M* EDTA and NaOH to the desired pH, usually 9.0. To suppress adsorption, 3.8 m*M* $(NH_4)_2SO_4$ was added in most cases. The acceptor solution



I II III

contained 0.5 m*M* $(NH_4)_2SO_4$ and H_2SO_4 to give a pH of usually 2.3.

The membrane was impregnated by soaking for 15 min in either *di*-hexyl ether (Sigma, St. Louis, MO, USA) or in a solution of 5% (w/w) of TOPO, trioctyl phosphine oxide (Fluka, Buchs, Switzerland), in *di*-hexyl ether.

The mobile phase for the liquid chromatography was ammonium phosphate buffer (pH 8.2) in methanol (E. Merck, Darmstadt, Germany, HPLC grade) (55:155, v/v). To prevent bubble formation, the mobile phase was degassed with helium. All water was purified using a Milli-Q unit (Millipore, Bedford, MA, USA) and all chemicals were of analysis grade or better.

3. Results and discussion

3.1. Chromatograms

Fig. 3a shows a chromatogram of compounds **I–III**, enriched with the liquid membrane technique, after optimization of various parameters

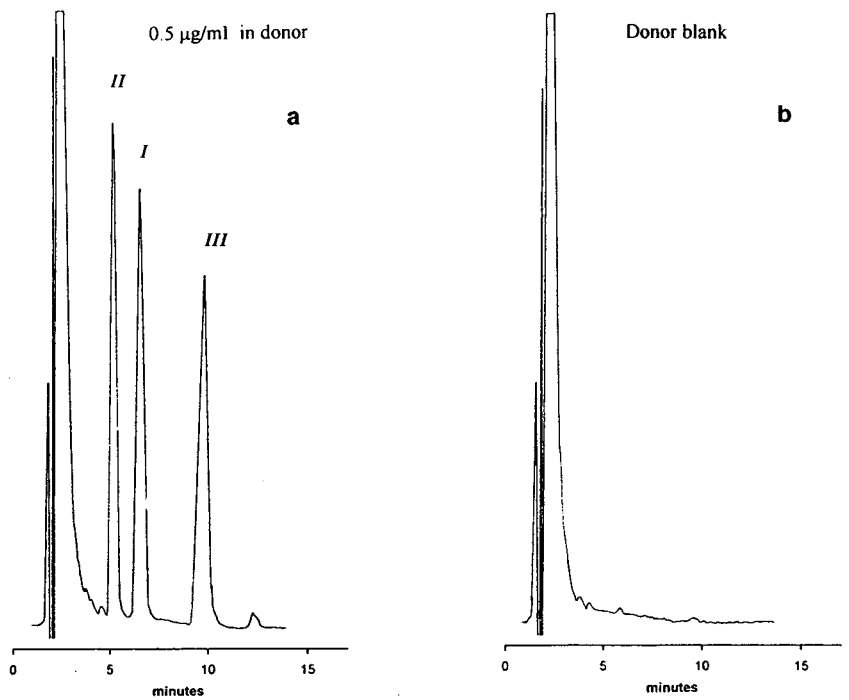


Fig. 3. (a) Chromatogram of compounds **I** (Amperozide), **II** (its metabolite) and **III**; 0.5 µg/ml each in donor buffer after enrichment as described in the text. (b) Chromatogram after enrichment of pure donor buffer.

as discussed below. Fig. 3b shows the corresponding blank.

The peaks appearing in the beginning of the chromatogram are solvent peaks, caused by the HPLC system and not by the liquid membrane procedure, as they turn up also in a direct injection on the column.

3.2. Optimization of dimensions and flow-rates

Strategies for the optimization of the recovery (expressed as extraction efficiency) are reviewed in ref. 15. In the present case, the basic requirements were:

(1) it must be possible to analyze small sample volumes (<1 ml), as required by future bioanalytical applications; (2) the extract volume should be limited to *ca.* 10 μl, since an on-line connection with complete sample transfer to HPLC is desired; (3) the sample workup should be finished within the time of the chromatographic run, which means that the analysis time is determined by the chromatographic separation; and (4) the extraction should be as selective as possible, leading to cleaner blanks and lower detection limits.

The first two conditions necessitate the use of a small extraction unit, with a channel volume of *ca.* 10 μl. As the membrane area should be as large as possible [15], this calls for shallow channels. The minimum depth that can be machined and is practical to use is *ca.* 0.1 mm, which leads to the dimensions stated above. The limited sample volume demands a high extraction efficiency in order to meet the requirement of a low detection limit. This is contrary to applications of the supported liquid membrane technique to environmental samples such as river water, where a low detection limit may be achieved by incomplete extraction of large sample volumes [7,8,15–17].

The efficiency of liquid membrane extraction depends on a number of experimental parameters [7,8,15], such as flow-rate, dimensions of the membrane unit, chemical composition of the phases, kinetic and thermodynamic properties, etc.:

$$E = 1 - \exp\left[\frac{3D_D}{\pi h_D K k_M} \ln\left(1 + K k_M \sqrt{\frac{2\pi h_D}{3D_D \phi}}\right) - \sqrt{\frac{6D_D}{\pi h_D \phi}}\right] \quad (1)$$

Here, E is the extraction efficiency (number of moles collected in the acceptor phase divided by number of moles originally in the extracted sample), K is the distribution coefficient of the analyte between the organic membrane phase and the donor phase, k_M is the mass transfer coefficient in the membrane phase, D_D is the diffusion coefficient of the analyte in the donor phase, ϕ is equal to $F_D/(L \cdot w)$. F_D is the volume flow-rate of the donor phase and L, w, and h_D are the length, width and depth of the donor channel, respectively.

The extraction efficiency increases when F_D is decreased, *i.e.*, when the enrichment time of the analyte increases, other parameters being the same. With the ASTED instrument, the lowest flow-rate that can be obtained is 180 μl/min, leading to a maximum enrichment time of only *ca.* 5.5 min for a 1-ml sample, which for the polar analytes considered is insufficient to give high recoveries. However, the construction of the instrument permits the sample to pass by the membrane several times, in a "push–pull" mode. Thereby the total enrichment time can easily be extended up to *e.g.* 15–20 min, significantly increasing the efficiency without exceeding the time for a reasonable HPLC analysis cycle.

The extraction efficiency after n passes, E_n, is given by [15]:

$$E_n = 1 - (1 - E_1)^n \quad (2)$$

A plot of $\log(1 - E_n)$ *vs.* n should thus be a straight line passing through the origin. As is seen in Fig. 4, the agreement with eqn. 2 is better for higher than for lower flow-rates. This is probably due to the fact that the broadening of the sample plug is larger at low flow-rates, leading to some losses during the subsequent passages. The volume set for the syringe ("3" in Fig. 2) in the push–pull operation was the same as that of the sample aliquot.

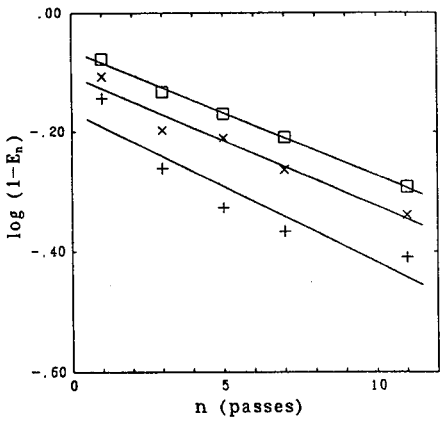


Fig. 4. Plots of $\log(1 - E_n)$ of Amperozide *vs.* the number of passes (n) through the extraction unit at different donor flow-rates. Donor pH = 9.0, acceptor pH = 2.3, membrane liquid was 5% TOPO in di-*n*-hexyl ether. + = 0.18 ml/min; × = 0.36 ml/min; □ = 0.75 ml/min.

In Fig. 5, the extraction efficiency of Amperozide (**I**) is plotted *vs.* total enrichment time at three different donor flow-rates (F_D). This experiment suggests that the extraction efficiency of Amperozide, at a constant enrichment time, increases only slightly with the flow-rate. Theoretically, this implies that the mass transfer in this system is mainly limited by the mass transfer in the membrane [8,15]. It appears to be somewhat better to use a higher flow-rate and compensate for the decreased enrichment time with an increased number of passages.

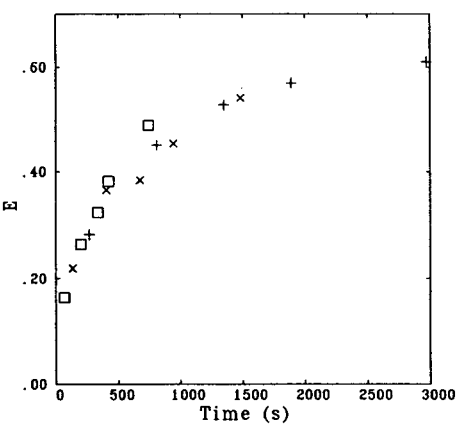


Fig. 5. Extraction efficiency (E) of Amperozide (**I**) *vs.* enrichment time at three different donor flow-rates. Conditions and symbols as in Fig. 4.

The results show that reasonable extraction efficiencies (in the order of 50%) can be obtained even for polar substances within *ca.* 15 min. With an acceptor volume of *ca.* 10 μl and a sample volume of *ca.* 1 ml, a considerable enrichment (*ca.* 50 times) of the analyte is achieved.

3.3. Composition of the membrane liquid

When the mass transfer in the membrane phase is controlling the overall mass transfer, it is important that the distribution coefficient K for the analyte compound is as large as possible. For polar compounds, as the ones investigated, a polar liquid in the membrane is desirable. Since polar liquids tend to be at least somewhat soluble in water, a compromise between membrane stability and efficiency must be made.

In Table 1, experimental extraction efficiencies are given for the three model compounds with different membrane liquids under otherwise identical conditions.

Obviously, there is a prominent influence of the nature of the membrane solvent on the recovery. The best pure solvent is di-*n*-hexyl ether. With the addition of 5% trioctyl phos-

Table 1
Extraction efficiencies for compounds **I**, **II** and **III** with different membrane solvents

Solvent	Compound		
	I	**II**	**III**
n-Undecane	0.19	0.025	0.13
1-Undecanol	0.14	0.14	0.08
1-Undecanal	0.05	0.06	0.04
2-Undecanone	0.27	0.24	0.16
6-Undecanone	0.28	0.25	0.16
Di-*n*-hexyl ether	0.30	0.13	0.20
Di-*n*-hexyl ether + 5% TOPO	0.31	0.20	0.20
n-Undecane + 50% di-*n*-hexyl ether	0.22	0.07	0.13

F_D = 0.18 ml/min, n = 1, other conditions as in Fig. 4.

phine oxide (TOPO), the extraction efficiency for the most polar compound, the metabolite (**II**), is increased, and this combination was selected as the optimal one for the following experiments.

With this membrane liquid, the membrane lasted for *ca.* 80 samples.

3.4. Optimization of the donor pH

In order to examine the influence of pH of the donor phase on the extraction efficiency, it was varied between 5.0 and 12.0 by changing the sodium hydroxide concentration. As the pK_a of Amperozide is 6.8, any pH < 9 (*ca.* two pH units over pK_a), will lead to an incomplete deprotonation [15] and thus to a decreased extraction efficiency. At pH > 9, an increase in the sodium hydroxide concentration may increase the partition coefficient by salting out, thereby increasing the mass transfer in the membrane (*cf.* eqn. 1). Simultaneously, the viscosity of the donor solution will increase, decreasing the mass transfer in the donor phase. As the mass transfer in the membrane limits the overall mass transfer, the extraction efficiency is expected to increase slightly with increasing pH, also when pH > 9. As seen in Fig. 6, this is approximately the case for compounds **I** and **II**, while the pH dependence is less for the extraction of compound **III**.

The addition of ammonia is advantageous for suppression of adsorption (see memory effects). Therefore, the dependence of E on donor phase pH was investigated with the addition of ammonia as 3.8 mM ammonium sulphate. The results in Fig. 7 show that at pH > 9, a markedly decreased extraction efficiency is observed. This is probably due to extraction of significant amounts of NH_3, increasing the pH in the acceptor phase close to the membrane surface and preventing complete protonation of the amines.

To examine the influence of TOPO on the shape of the curve of E *vs.* pH, similar experiments were performed with only *di*-hexyl ether as the membrane liquid (and with ammonia included in the donor phase). In this case, the extraction efficiency of compound **II**, which is the most polar of the compounds, is diminished as the polarity of the membrane liquid is decreased (in agreement with Table 1). The shape of the curves is, however, more or less the same.

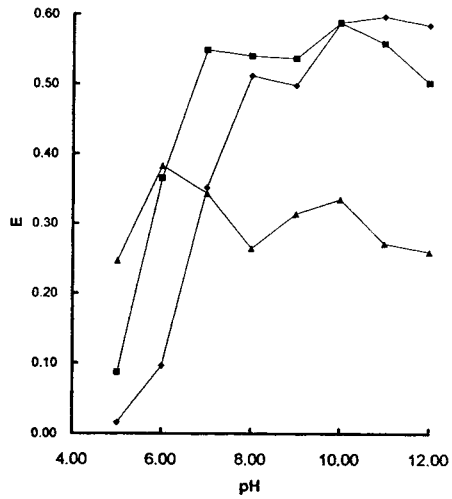


Fig. 6. Extraction efficiency for compounds **I** (□), **II** (◆) and **III** (▲) *vs.* donor pH. Donor: NaOH (different concentrations), EDTA (12.5 mM). Membrane liquid was 5% TOPO in di-hexyl ether. F_D = 0.18 ml/min, n = 3. Acceptor pH = 2.3.

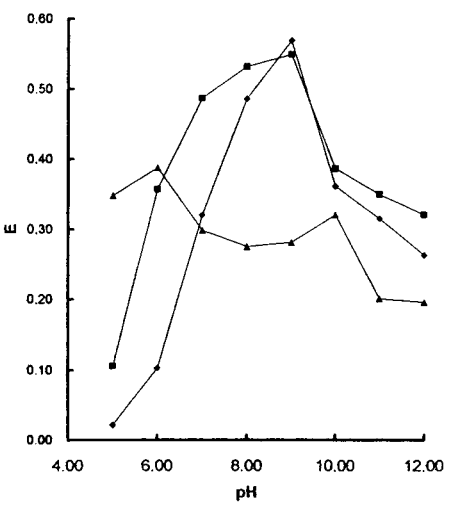


Fig. 7. Extraction efficiency *vs.* donor pH, as in Fig. 6, except that the donor phase additionally contains 3.8 mM ammonium sulphate.

3.5. Optimization of the acceptor pH

The pH of the acceptor phase was varied between 1.5 and 3.1. As can be seen in Fig. 8, all three compounds showed a maximum in extraction efficiency around pH 2.3. From earlier experiments [7] and theory [15], the recovery was expected to be independent of the acceptor pH as long as this is sufficiently low to ensure immediate protonation of the analytes. The same experiment was performed without TOPO. In this case, all three substances showed a similar maximum, only at a slightly higher pH (2.5).

From these comparisons, it seems that TOPO enhances the extraction of polar substances, but doesn't significantly influence the pH dependence of the extraction process.

3.6. Quantification

Calibration curves, based on peak heights, were made for all three compounds (Table 2). Seven aqueous samples with concentrations in the range 0–1000 ng/ml were processed in triplicate using a donor flow-rate of 0.18 ml/min, and one passage by the membrane, giving an enrichment time of *ca.* 5.5 min. All curves showed good linearity and the intercepts did not differ significantly from zero at a 95% confidence level. The mean recoveries were 35%, 34% and 20% for compounds **I**, **II** and **III**, respectively.

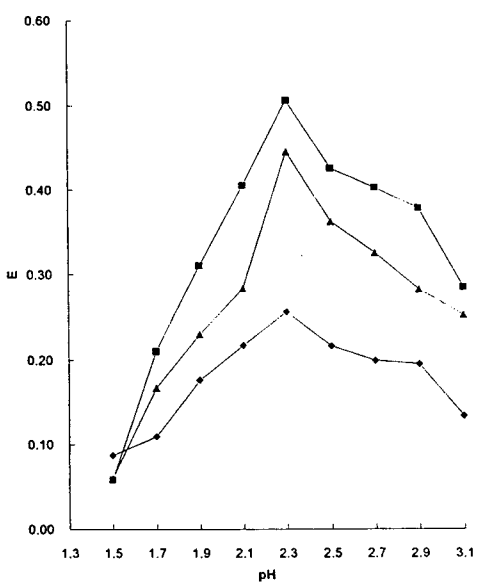


Fig. 8. Extraction efficiency *vs.* acceptor pH. Membrane liquid was 5% TOPO in di-*n*-hexyl ether. Donor pH = 9.0, other conditions were as in Fig. 6.

Table 2
Calibration curve parameters for the model compounds

Compound	Slope[a]	Intercept[a]	*r*
I	0.108 ± 0.004	0.05 ± 1.59	0.9996
II	0.121 ± 0.001	−0.22 ± 0.46	0.9999
III	0.098 ± 0.005	−1.45 ± 1.98	0.9992

[a] Arbitrary units; 95% confidence intervals.

3.7. Repeatability

The overall repeatability (relative standard deviation) was *ca.* 2% based on three analyses of a 0.5 μg/ml solution. Fifteen consecutive analyses of a 1 μg/ml solution were also made (see Memory effects, below). The peak heights of compound **III** showed a slight increase, which gave rise to a R.S.D. of 3.4%, but the other substances still showed a R.S.D. of *ca.* 2%. The first injection gave markedly smaller peak heights and is not included in the reproducibility calculations.

3.8. Detection limit

The detection limit with UV detection after one passage is about 20 ng/ml for all three substances. This corresponds to peak heights twice the baseline noise. For bioanalysis, much lower detection and determination limits are needed, which can be achieved with electrochemical detection [18].

3.9. Memory effects

Amines are in general apt to be adsorbed on surfaces of both polymers, glass and metals. One way to minimize this tendency is to keep the amines in ionized form. To decrease the ad-

sorption in the enrichment process when the amines are unprotonated, *ca.* 200 μg/ml of ammonia as ammonium sulphate (3.8 m*M*) was added to both the donor and acceptor phases as a competing adsorbate.

To evaluate the adsorption in the HPLC step, the substances were first injected directly on the column, without the ASTED equipment. All three substances exhibited no memory effects at all in a subsequent blank injection, except for compound **III**. For this substance, less than 1% of the first peak showed up in the subsequent blank. The experiment was performed with both a 0.5 and a 1.5 μg/ml solution.

To prevent adsorption in the sample vials waiting for analysis, the solutions were kept in glass vials at a pH around 4 and each sample was automatically mixed with the alkaline donor solution prior to the enrichment (see Operation). It was found that the recovery decreased with increasing number of mixing cycles, probably due to adsorption in the needle and the mixing coil ("9" in Fig. 2). The highest recovery was obtained when the donor solution was added and manually mixed with the sample before loading into the sample rack. This would, however, lead to the samples being stored in alkalized form in the sample rack, waiting for analysis during different periods of time.

This was evaluated with 15 identical samples which were injected consecutively. The substances (1 μg/ml) were dissolved in donor buffer (pH 9) and kept in glass vials during the 4-h experiment. There was a pronounced decrease of the peak heights during the experiment, for compound **I** 11%, for compound **II** 7% and for compound **III** 20%. When the substances were kept in a solution at pH 4 instead, and automatically mixed with pH 9 donor buffer immediately prior to the extraction, no decrease in peak height was observed. The experiment shows that it is important to keep the amines ionized as long as possible and that the mixing with alkali should be made immediately before each analysis, and with one mixing cycle.

With earlier set-ups [11] the alkalization was made in a flow system immediately before the membrane. It is a disadvantage with the ASTED equipment that the samples must be alkalized already in the vials so the amines are transferred through the needle and tubing in neutral form. The resulting memory effects can be controlled by washing as described below, but the overall recovery will be adversely affected. The standard materials in the equipment is stainless steel for the needle, EPF for the coiled tubing (9), PTFE for other tubing and PVDF for the membrane unit. Some experiments have been made with other materials, but without striking improvements.

The memory effects in the tubing and the membrane unit are dependent on the extent of washing. With the ASTED equipment three different washing procedures can be used: "wash", washing of the donor channel, "rinse", washing of the needle, and "regeneration", washing of both the donor and the acceptor channel simultaneously. By changing the different washing times, it was found that washing the acceptor channel had greater influence on the memory effects than washing the donor channel. With the different washing volumes chosen, wash: 1 ml, rinse: 2 ml and regeneration: 3 ml, the memory effects for compounds **I**, **II** and **III** were 2%, 0% and 10%, respectively.

3.10. Discussion

Compared to the previously developed methods for membrane enrichment, the approach presented here has two main advantages; a large number of samples, up to 60, can be automatically processed and the recovery of polar analytes, which normally is low, can be improved without seriously extending the analysis time by passing the same sample plug by the membrane several times.

The technique is especially interesting for bioanalysis of plasma samples. Often only small sample volumes are available (less than 1 ml) and the concentrations of the analytes are often so low that efficient enrichment is needed. The membrane workup results in a considerable enrichment and, additionally, a solution free from macromolecules, which is favourable for the final HPLC separation.

The extent of enrichment is determined by the ratio between the sample volume and the acceptor volume and by the analyte recovery. Here we have shown that with the procedure used, the recovery can be increased by extending the residence time. If this residence time exceeds the time needed for the final HPLC step, the time for the total analysis will be prolonged, otherwise, one sample can be extracted while the previous one is chromatographed. The choice may be to pay in analysis time to obtain sufficient recovery.

In this work we have used an UV detector. With the effective sample clean up in the enrichment step, an electrochemical detection approach seems promising. The model compounds, as is usually the case in bioanalysis, are electrochemically active [18], which makes the approach especially interesting in this area. Work along these lines is in progress.

Here we have shown how the system can be used for basic compounds. With small changes in the system set-up, *i.e.* by changing the composition of the donor and acceptor buffers and of the membrane liquid [8], other substances, such as organic acids or permanent ions, can be processed.

4. Acknowledgements

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JOURNAL OF CHROMATOGRAPHY A

Determination of pesticides in environmental waters by automated on-line trace-enrichment and liquid chromatography

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Abstract

Automated on-line trace-enrichment, liquid chromatographic analysis and UV diode-array detection (DAD) were investigated for the determination of pesticides at the 0.1 μg/l level in drinking and surface waters. The selection of the preconcentration parameters (sorbent, sample volume, reproducibility) is discussed. Conditions are specified for quantification methods such as using calibration graphs constructed with spiked samples, standard addition methods and a calculation method included in the DAD software depending on the sample nature. Detection limits of 0.1 μg/l were obtained using 150 ml of river waters without any clean-up.

1. Introduction

Liquid chromatography (LC) has been shown to be an effective technique for the determination of pesticides and organic pollutants in aqueous media [1–3]. Its increasing availability is easily explained by its suitability for analysing simultaneously thermolabile and non-volatile organic compounds over a wide range of polarity without any derivatization such as is required in gas chromatographic (GC) analysis. Other reasons are its easy on-line coupling with the enrichment step using solid-phase extraction on precolumns [2,4–8] and the development of sensitive UV diode-array detection (DAD). Trace enrichment and LC analysis with DAD can be automated and this methodology has been applied for the automated monitoring of a broad range of pesticides and pollutants in drinking and surface waters [9]. On-line methodology coupling solid-phase extraction to LC separation is easily performed in any laboratory. In its simplest form, a precolumn is placed in the sample-loop position of a six-port switching valve. After conditioning, sample application and cleaning via a low-cost pump, the precolumn is coupled to an analytical column by switching the valve into the inject position. The extracted compounds are then eluted directly from the precolumn to the analytical column by a suitable mobile phase which permits the separation of the trapped compounds. The sequence described above can be totally automated using commercially available programmable systems, *e.g.*, the Prospekt module. As there is no sample manipulation between preconcentration and analysis, no loss or contamination risk can occur and one can expect more accurate quantitative results. Another advantage is that the whole species are analysed, allowing the handling of a smaller volume in comparison with off-line procedures where only an aliquot is usually analysed. One constraint of on-line techniques is the need to avoid a decrease in the analytical column ef-

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ficiency when coupling the precolumn. This is obtained by selecting a precolumn of small dimensions, typically 1 cm × 0.2 cm I.D., packed with LC-grade stationary phases. Therefore, when low detection levels are required, one has to select a stationary phase giving a high retention of analytes in water in order to handle a sufficiently large volume without breakthrough. A comparison of reversed-phase extraction sorbents has shown that the retention volumes of many organic compounds were about 25 times higher with apolar copolymers (PRP-1 or PLRP-S) than with octadecylsilicas [10].

Most of the studies using on-line trace enrichment, LC separation and DAD have discussed the identification of co-eluting analytes with similar UV spectra or with weak UV absorption. Limits of determination in LC-grade waters were shown to be lower than 0.1 μg/l, but determinations at this level in drinking waters were difficult [9]. In river water, the limits of detection are generally above 1 μg/l, although a multi-residue analysis of pesticides in river water at the 0.3 μg/l level using an on-line solid-phase disk extraction has been reported [11].

The aim of this study was not to determine in one run as many pesticides as possible, but to investigate the quantitative aspects encountered in trace-level determinations carried out using automated on-line trace enrichment and LC analysis with DAD. The effects of the sample matrix and optimization of the preconcentration parameters are discussed. Detection limits that can be obtained for drinking and river waters in multi-residue analysis and for triazine and phenylurea groups are presented.

2. Experimental

2.1. Apparatus

LC analyses were performed with a Varian LC System Workstation including a Varian Star 9010 solvent-delivery system and a Model 9065 Polychrom diode-array detector. The analytical column was connected to a Valco valve (VICI, Houston, TX, USA). Trace enrichment was performed on disposable cartridges using the Prospekt (Spark Holland, Emmen, Netherlands), which is an automated programmable sample preparation unit allowing direct elution to the LC column. Conditioning of the cartridges and sampling were performed via a solvent-delivery unit (SDU) (Spark Holland).

2.2. Stationary phases and columns

The analytical column was 25 cm × 4.6 mm I.D. prepacked with 5-μm Varian ODS-TSK 80TM octadecylsilica (Toyo Soda Manufacturing). Samples were preconcentrated on 10 mm × 2 mm I.D. cartridges prepacked with styrene–divinylbenzene copolymer (15–25 μm PLRP-S; Polymer Laboratories, Church Stretton, UK) and the octadecylsilica (Baker, Deventer, Netherlands).

2.3. Chemicals

HPLC-grade acetonitrile was kindly given by J.T. Baker. Methanol was purchased from Prolabo (Paris, France). LC-quality water was prepared by purifying demineralized water in a Milli-Q filtration system (Millipore, Bedford, MA, USA). Other chemicals were obtained from Prolabo, Merck or Fluka.

The various pesticides were supplied by Riedel-de Haën (Seelze, Germany) or Promochem (Wesel, Germany). Stock standard solutions of selected solutes were prepared by weighing and dissolving them in methanol.

Three standard solutions were prepared, one containing ten representatives of the main classes of pesticides (simazine, atrazine, methabenzthiazuron, isoproturon, linuron, carbaryl, propanil, fenamiphos, fenitrothion and parathion), another containing most of the triazines used in France and some degradation products (deisopropylatrazine, deethylatrazine, hydroxyatrazine, hexazinone, simazine, cyanazine, simetryne, atrazine, prometon, sebutylazine, propazine and terbutylazine) and the third containing phenylurea herbicides (fenuron, methoxuron, monuron, methabenzthiazuron, chlortoluron, fluometuron, monolinuron, isoproturon,

diuron, difenoxuron, buturon, linuron, chloroxuron, chlorbromuron, diflubenzuron and neburon). These standard solutions were stored at 4°C and were used for the preparation of dilute working standard solutions and for spiking water samples. No change in the chromatogram of the standard solutions was observed during the 3 months of the study. The final spiked samples did not contain more than 0.5% of methanol.

2.4. Procedure

Automation of on-line trace enrichment was performed using the Propekt system equipped with a disposable cartridge unit. This system contains an SDU for conditioning and washing the trace-enrichment cartridges and percolating samples.

The procedure was as follows: (1) washing the cartridges with 10 ml of acetonitrile; (2) conditioning them with 10 ml of methanol and then 10 ml of LC-grade water; (3) percolation of samples; and (4) desorption from the cartridge to the analytical column by an acetonitrile gradient with phosphate buffer (pH 7). Three different gradients were used for the separation of triazines, phenylureas and multi-residue pesticides and are described in the figure captions.

3. Results and discussion

3.1. Analytical LC separation

The pesticides used are some of the most commonly applied in the European Community. Three standard solutions were prepared, one containing pesticides from different classes such as triazines, phenyureas, carbamates, organophosphorus and propionanilides and the other two containing twelve triazines and sixteen phenylureas, respectively. As each solution contained pesticides with a wide range of polarity, the analytical separation was carried out by reversed-phase chromatography using a C_{18} analytical column and an acetonitrile gradient with phosphate buffer (pH 7). The chromatographic conditions are reported in Fig. 2 for the multiresidue separation and in Fig. 6 for the separation of phenylurea and triazine herbicides.

3.2. Preconcentration parameters

Choice of sorbent

Once the analytical separation is obtained by direct injection of the standard solution onto the analytical column, it is possible to determine the detection limit for each compound and then to calculate the sample volume that has to be percolated without breakthrough in order to detect the analyte of interest at the required concentration. As an example, quantitative analysis at the 0.1 μg/l level necessitates at least a minimum limit of detection of 0.05 μg/l and, if the detection limit measured by direct injection is 5 ng, a sample volume of 100 ml has to be handled. Therefore, the main parameter of the preconcentration procedure is the choice of a sorbent that gives for this analyte a convenient breakthrough volume (V_b). The choice of the sorbent and the knowledge of the V_b values of the analytes are more important when using on-line preconcentration, in contrast to off-line preconcentration where it is possible to increase the breakthrough volume by increasing the amount of sorbent in the cartridge.

Breakthrough volumes

The breakthrough volume can be measured on a "breakthrough curve" obtained by percolating a spiked solution through the precolumn and recording the UV signal of the effluent [2,12]. This method is very time consuming and the direct UV recording requires a solution spiked at the 0.1 mg/l level. An experimental method for determining both breakthrough volumes and recoveries has been described and is easily performed with the on-line set-up [12,13]. A small volume spiked with a trace concentration (μg/l level) of all the analytes is percolated through the precolumn and the chromatogram corresponding to the on-line elution is recorded and peak areas are measured. This first volume is chosen so that breakthrough does not occur for any solute that can be verified approximately by

direct loop injection of the same amount. The sample volume is then increased and the concentration decreased in order to have a constant amount of analytes in the percolated samples. Provided that breakthrough does not occur for any analyte, the amounts concentrated remain constant and peaks areas measured on chromatograms obtained with on-line elution are constant. The breakthrough volume of an analyte is calculated when the peak area begins to decrease and the corresponding recovery can be also calculated by dividing the peak area obtained for the sample volume by the constant peak area obtained for sample volumes before breakthrough. One advantage of this method is that these values can be obtained from three or four on-line preconcentrations for all the analytes and under experimental conditions that correspond to those used for real analysis (trace level and several analytes together).

This method was applied and breakthrough volumes were compared using cartridges packed with C_{18} silica and PLRP-S. The results are reported in Fig. 1a and b. On C_{18} precolumns, breakthrough occurs rapidly for a volume between 40 and 60 ml for the four more polar compounds of the multi-residue standard solution whereas on PLRP-S precolumns V_b is between 300 and 400 ml for simazine and methabenzthiazuron and above 500 ml for atrazine. The differences in V_b values between C_{18} silica and the apolar copolymer are consistent with retention measurements [10]. Nevertheless, the V_b values measured here are higher than some published values obtained using the same cartridges [7,9]. For example, values of 50 and 90 ml were obtained for simazine and atrazine on PLRP-S cartridges. Nevertheless, the same workers have also measured V_b values of 180 ml for simazine and >400 ml for atrazine with a 10 mm × 2 mm I.D. precolumn laboratory packed with PLRP-S [8]. We have also obtained similar results for these two compounds on a similar precolumn packed with the PRP-1 copolymer [12]. As the V_b values in ref. 9 were measured by recording the breakthrough curves, the lower values obtained are likely to be due to the high concentrations of the spiked solutions and overloading of the precolumn.

It has also been reported that the breakthrough volume was the same when measured in LC-grade water, drinking water and surface waters [8]. The more polar the analytes, the lower are the V_b values on both C_{18} silica and apolar copolymers. Many modern pesticides and degradation products are more polar than simazine; Liska *et al.* [7] reported a method allowing the screening of about 50 "polar" pesticides, which are nowadays applied and which were classified in order of increasing retention times. In a first approximation, this order reflects the polarity order and simazine is

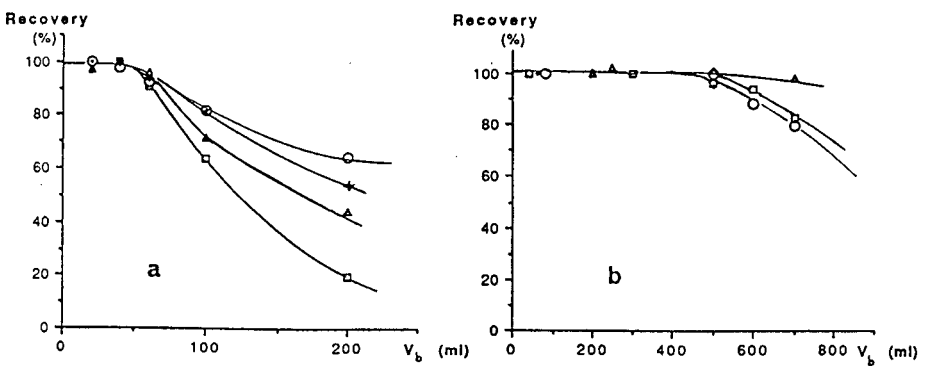

Fig. 1. Experimental variations of recovery with percolated Milli-Q-purified water samples having a constant amount (about 100 ng) of pesticides. Precolumn: (a) C_{18}; (b) PLRP-S. Analytical column, Varian ODS (25 × 0.46 cm I.D.); flow-rate, 1 ml/min; mobile phase, acetonitrile gradient with 0.05 *M* phosphate buffer at pH 7, gradient 30% acetonitrile from 0 to 38 min, 30 to 45% from 38 to 44 min, 45 to 47% from 44 to 52.5 min, 47 to 100% from 52.5 to 70 min; detection at 220 nm.

the 23rd compound. Therefore, it is advisable to use PLRP-S cartridges for trace-level determinations of pesticides in water. On a C_{18} precolumn, the sample volume that can be handled without any breakthrough for many moderately polar pesticides is lower than 50–100 ml.

Reproducibility

One advantage of automation in on-line preconcentration is that more reproducible results are expected, provided that the precolumns are packed with the same amount of sorbent and with the same efficiency. The repeatability of peak areas and heights obtained by direct loop injections into the analytical column was first studied. Table 1 (top) reports results from five 10-μl injections of a 2.5 mg/l solution containing four pesticides. The relative standard deviation (R.S.D.) is between 3–7% and 3–5% when measuring peak areas and peak heights, respectively. The reproducibility between cartridges was measured by preconcentrating 50 ml of LC-grade water spiked with 0.5 μg/l of the same pesticides. Five experiments were carried out using a new precolumn in each run. As can be seen in Table 1 (bottom), the R.S.D. is around 10% for measurements of both peak areas and peak heights. These results indicate that the precolumns were packed under reproducible conditions.

When percolating 50 ml of the spiked solution, breakthrough does not occur for each compound and therefore recoveries of 100% are expected. As the spiked solutions contained 25 ng of each compound, the peak areas obtained by preconcentration should be equal to those obtained by direct injection, as the amount injected directly is also 25 ng of each analyte. The average ratio between peak areas obtained by preconcentration and direct injection is 76%; it is 78% when using peak heights. If a decrease in efficiency was to be observed owing to the coupling of the precolumn, the ratio calculated from peak heights should be different from that calculated from peak areas. The difference observed is due to the volume of the injection loop, which is specified to an average accuracy of 20%. Calibration of a 20-μl loop is a delicate operation and is not necessary. When using on-line techniques, quantitative analyses should not be carried out by comparison with direct injections. Once the sample volume has been selected, calibration graphs should be constructed with spiked solutions under the same experimental conditions as selected for the analyses of unknown samples.

Flow-rate

Flow-rates of 2 and 5 ml/min for the preconcentration step were studied. The peak areas

Table 1
Test of reproducibility

Method	Compound	Peak area		Peak height	
		Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)
Direct injection[a]	Simazine	31 473 ± 866	2.7	1740 ± 45	2.6
	Cyanazine	19 558 ± 686	3.5	1182 ± 59	5.0
	Atrazine	22 460 ± 1570	7.0	1964 ± 99	5.0
	Londax	4059 ± 210	5.2	448 ± 18	4.0
Preconcentration[b]	Simazine	23 414 ± 2071	8.8	1256 ± 121	9.6
	Cyanazine	14 983 ± 1580	10.5	984 ± 111	11.3
	Atrazine	17 000 ± 1929	11.3	1520 ± 152	10.0
	Londax	3057 ± 245	8.0	349 ± 30	8.6

[a] Data obtained from five direct injections of 10 μl of a 2.5 mg/l solution of each analyte (amount injected 25 ng) in methanol.
[b] Data obtained from five preconcentrations of 50 ml of Milli-Q-purified water spiked with 0.5 μg/l of each compound (amount injected 25 ng) using different PLRP-S precolumns.

and heights of the different pesticides were measured under the same experimental conditions as those used for the results in Table 1 (bottom). Reproducibility was observed within a similar R.S.D. of 10%. For experiments allowing the measurement of breakthrough volume or calibration with LC-grade waters, the same precolumn was often reused after washing with pure acetonitrile and a flow-rate of 2 ml/min was applied. For natural water such as river water containing many interferences that cannot be eliminated easily by a washing step, a new precolumn was used in each run and the percolation was carried out at a flow-rate of 5 ml/min.

3.3. Determination in different matrices

It was recommended that calibration graphs should be obtained in the same conditions as for real analyses of unknown samples. Nevertheless, it is interesting to assess whether a calibration graph that was obtained with spiked LC-grade waters could be used for drinking or river waters. Some quantification methods are also included in the software of diode-array detectors and calculations are often made from data from spiked LC-grade solutions.

Matrix interference peaks

PLRP-S is a non-selective sorbent and many other compounds from the matrix of natural samples are preconcentrated and can be eluted together with the analytes of interest. Interferences depend on the nature of the water. They have an effect on both detection limits and quantification [9]. No clean-up can be applied such as by washing the precolumn after the percolation, because the more polar analytes would be eluted.

Different waters, LC-grade water, drinking water and Seine river water, were spiked with the standard multi-residue solution at a concentration of 0.3 μg/l and 150 ml of each sample were analysed on-line. The chromatograms obtained are represented in Fig. 2. The blank (Fig. 2a) indicates the baseline obtained at 220 nm with the gradient applied. LC-grade water (Fig. 2b) contains some impurities at the end of the chromatogram. In drinking water (Fig. 2c), interferences are visible at the beginning of the chromatogram by a broad peak spread over 8 min and several other peaks that do not correspond to the standard solution. In Seine water (Fig. 2d), the interfering peak at the beginning

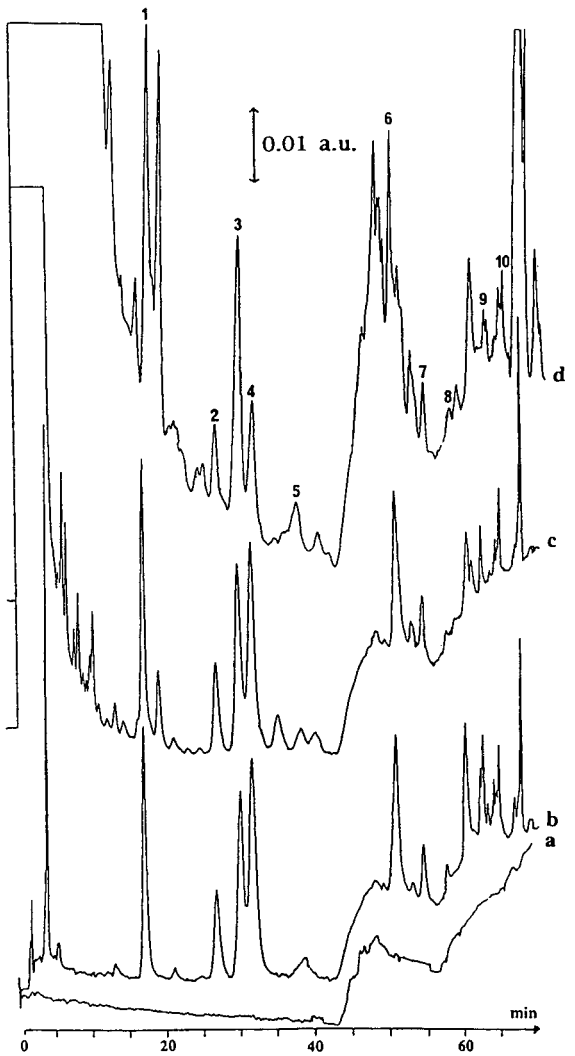

Fig. 2. On-line analysis of 150 ml of different water samples spiked with 0.3 μg/l of (1) simazine, (2) methabenzthiazuron, (3) atrazine, (4) carbaryl, (5) isoproturon, (6) propanil, (7) linuron, (8) fenamiphos, (9) fenitrothion and (10) parathion. Precolumn, PLRP-S; other experimental conditions as in Fig. 1. (a) Blank gradient; (b) Milli-Q-purified water; (c) drinking water; (d) surface water from the Seine (June 28th, 1993).

of the chromatogram is much broader than in drinking water and is spread over 15 min, and there are also other interfering peaks from around 40 min until the end of the gradient. The non-spiked sample of surface water is represented in Fig. 5.

First, with this gradient applied, a peak will not be detected in river water if its retention time is lower than 15 min. Peaks 1–4,6 and 7 are easily visible at this wavelength even in river water, in spite of the low concentration. It is important to note that within an R.S.D. of 10%, the peak heights are close in LC-grade water, drinking water and river water. Calibration graphs for these compounds should be similar in these three different matrices. Peak 3 is higher in river water owing to the presence of atrazine in the raw sample.

Effect of the gradient applied

Determination of a compound in natural samples is more difficult if a matrix interference peak is co-eluted. The shape of the matrix peaks depends on the nature of the sample and also of the gradient which is applied. It can appear as a broad peak at the beginning of the sample, and this corresponds in general to a gradient with an initial content of acetonitrile higher than 20–30%, as shown in Fig. 3a. With the same water and preconcentration parameters, but with a gradient containing only 5% of acetonitrile at the beginning, the matrix interfering peak has a different shape and appears in the middle of the chromatogram, as shown in Fig. 3b. This was also observed by Slobodnik *et al.* [9], with a gradient from 10% to 100% acetonitrile in 55 min. Low-level detection means that the interfering peak in river water is not too large and is well situated. This is often incompatible with the simultaneous determination of a large number of compounds spread over a large polarity range, which requires a gradient from pure water to pure acetonitrile or methanol. As an example, the retention time of simazine is 17.7 min with the gradient in Fig. 3a and 22.2 min with the gradient in Fig. 3b. If a limit of detection in river

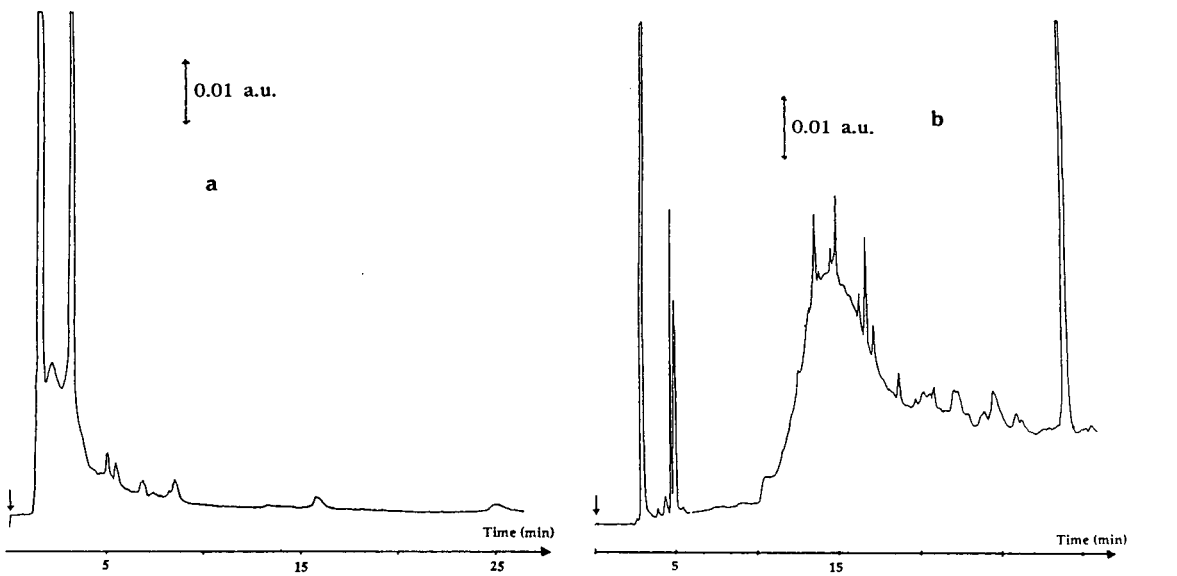

Fig. 3. On-line analysis of 150 ml of drinking water with two different acetonitrile gradients for the elution of the precolumn. (a) Acetonitrile gradient with a 0.05 *M* sodium phosphate solution (pH 7) at a flow-rate of 1 ml/min: 30% acetonitrile from 0 to 38 min, 30 to 45% from 38 to 44 min, 45 to 47% from 44 to 52.5 min, 47 to 100% from 52.5 to 70 min. (b) Acetonitrile gradient with a 0.001 *M* solution of perchloric acid at a flow-rate of 1 ml/min: 5 to 10% acetonitrile from 0 to 5 min, 10 to 35% from 5 to 8.5 min, 35% from 8.5 to 18 min, 35 to 75% from 18 to 30 min. Precolumn, PLRP-S; analytical column, Varian ODS (25 × 0.46 cm I.D.); detection at 220 nm.

waters below 0.5 μg/l is required, a compromise has to be found between the gradient shape and the polarity range of compounds detected.

Calibration graphs

When carrying out multi-residue analyses, the chromatograms are often presented with the detector set at 220 or 230 nm, because most compounds absorb at these wavelengths [3]. Nevertheless, when very low levels are required, it is advisable to draw the calibration graphs at wavelengths adapted to the compounds and to the matrix interferences. This is illustrated in Fig. 4 by chromatograms obtained at three wavelengths from the on-line analysis of 150 ml of drinking water spiked with 0.3 μg/l of the multi-residue standard solution. Whereas peaks 1–4 are well detected at 220 nm, peaks 5–10 are not. Peak 5 (isoproturon) is better detected at 239 nm (not represented), peaks 6–8 at 249 nm and peaks 9 and 10 (fenitrothion and parathion) at 268 nm. Although peak 10 is higher at 220 nm, its determination at 268 nm is certainly easier and more accurate because it can also be seen that the background of interfering materials detected after 40 min is higher at 220 nm than at 249 or 268 nm.

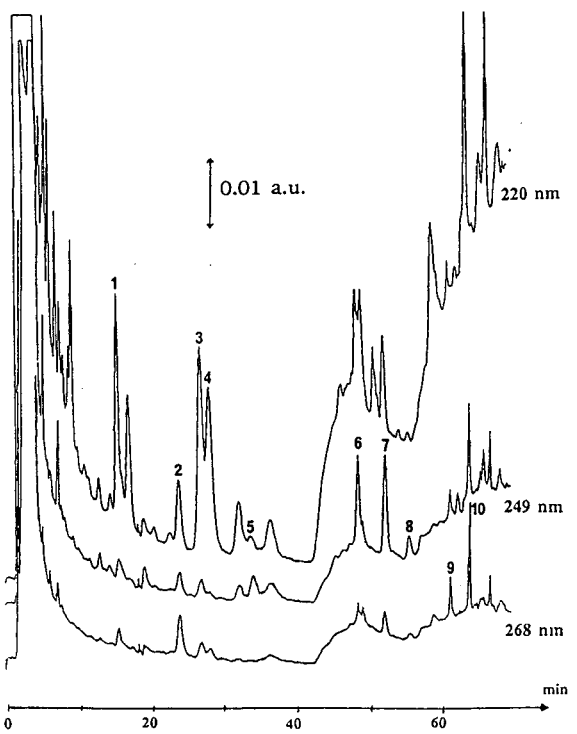

Fig. 4. Chromatograms corresponding to the on-line elution of 150 ml of drinking water at different wavelengths; drinking water spiked with 0.3 μg/l of ten pesticides as in Fig. 2. Precolumn, PLRP-S; analytical column, Varian ODS (25 × 0.46 cm I.D.); flow-rate, 1 ml/min; acetonitrile gradient as described in Fig. 1.

Calibration plots for the ten solutes were drawn for spiked LC-grade and drinking water in the trace-level range of 0.1–1.5 μg/l and using the same experimental conditions with a sample volume of 150 ml. Non-spiked drinking water was also analysed in order to be sure that no peak was obtained at the retention times of the analytes. Calibration equations are reported in Table 2. The correlation coefficients are all satisfactory ($R^2 > 0.99$) except for fenamiphos in LC-grade water. The important point is that calibration equations are not very different when constructed from LC-grade water or drinking water. That means that correct quantitative results can be obtained by on-line preconcentration of a non-spiked drinking water sample, using the calibration equation obtained with spiked LC-grade solution. In river waters, matrix interferences are higher and there are often many peaks for the non-spiked samples, so that it is difficult to obtain calibration graphs in the same range from 0.1 to 1.5 μg/l.

Determination using standard addition method and DAD software

In river water, the standard addition method is expected to be more accurate because of the high background of interfering compounds on the baseline. A non-spiked 150-ml sample of Seine river water was analysed on-line and the chromatogram obtained at 220 nm is presented in Fig. 5. Peaks corresponding to the retention times of simazine and atrazine were obtained. DAD identified atrazine, but not simazine, as can be seen by the match of the UV spectra. Aliquots of the same water samples (150 ml) were spiked with 15, 45 and 75 ng of each compound of the multi-residue standard solution. The slope calculated as concentration is

Table 2
Calibration data and detection wavelengths for multi-residue analysis (in the range 0.1–1.5 μg/l) in LC-grade water and drinking water

Compound	Detection wavelength (nm)	LC-grade water			Drinking water		
		N^a	Calibration equation[b]	R^2	N^a	Calibration equation[b]	R^2
Simazine	220	6	$y = 2058 + 1.12 \cdot 10^5 x$	0.999	7	$y = -2017 + 1.48 \cdot 10^5 x$	0.994
Methabenzthiazuron	220	6	$y = 790 + 6.26 \cdot 10^4 x$	0.997	7	$y = 164 + 7.65 \cdot 10^4 x$	0.998
Atrazine	220	6	$y = 1485 + 9.63 \cdot 10^4 x$	0.992	7	$y = 4880 + 1.16 \cdot 10^5 x$	0.998
Carbaryl	220	6	$y = 1290 + 1.92 \cdot 10^5 x$	0.998	7	$y = 3768 + 2.09 \cdot 10^5 x$	0.996
Isoproturon	239	6	$y = -5425 + 6.14 \cdot 10^4 x$	0.998	7	$y = -309 + 5.96 \cdot 10^4 x$	0.995
Linuron	249	6	$y = -1596 + 5.63 \cdot 10^4 x$	0.992	7	$y = -1352 + 5.73 \cdot 10^4 x$	0.981
Propanil	249	6	$y = -281 + 5.59 \cdot 10^4 x$	0.993	6	$y = -4382 + 6.19 \cdot 10^4 x$	0.998
Fenamiphos	249	6	$y = -426 + 1.67 \cdot 10^4 x$	0.960	4	$y = -0.14 + 7.47x$	0.994
Fenitrothion	268	6	$y = -1106 + 1.63 \cdot 10^{-4} x$	0.995	6	$y = -751 + 1.80 \cdot 10^4 x$	0.985
Parathion	268	6	$y = 69 + 1.89 \cdot 10^4 x$	0.995	6	$y = -85 + 2.09 \cdot 10^4 x$	0.997

Sample volume: 150 ml. Data from peak areas, except for fenaminphos where peak heights were used for the calibration in drinking water.
[a] Number of experimental points.
[b] y = area; x = concentration (ppb).

close to that obtained for calibration with LC-grade or drinking water. Nevertheless, it must be pointed out that the baseline around the atrazine peak is correct and does not contain too much interfering material. The calibration graph with LC-grade water gives a concentration of 0.30 μg/l in the raw sample. Standard addition calculations give a concentration of 0.28 μg/l.

Another quantification method was used which is included in the Polyview software and named MultiComponent Analysis (MCA). This spectrophotometric-based method allows the identification and determination of several analytes in an unknown sample by comparison with standard solutions. Pesticides are characterized by their retention time, their spectrum and the amount injected, and these data are stored in a library. In our experiment, the data were introduced from a chromatogram corresponding to the on-line analysis of a 150-ml spiked LC-grade water sample, and not by direct injection. The report of the MCA method gives the spectra from the library and the spectrum recorded on the peak of the unknown solution (Fig. 5). The result of quantification via the MCA software is 0.26 μg/l. Taking into account an average R.S.D. which can be estimated to be at least 15%, the values given by the three methods are consistent. The MCA calculation is, of course, the fastest and has also the advantage of indicating rapidly by means of the spectrum if there is a co-eluting analyte in the peak. No conclusion can be drawn in that case. This is clearly shown for the identification of simazine. It is visible in the chromatogram that there is a co-eluting peak. A standard addition made the first peak increase with good linearity. We tried to eliminate the second part of the peak for the spectrum recording, but no confirmation was obtained. Confirmation of the compound has to be carried out with a second on-line analysis in which the nature of the analytical column is changed to a cyano-bonded type, as shown by Di Corcia and Marchetti [3]. The coupling of on-line trace enrichment and LC coupled to mass spectrometry is now being developed and has been applied to the trace-level determination of phenylureas [14].

3.4. Detection limits

In drinking waters

From Fig. 4, representing the handling of 150 ml of drinking water spiked at the 0.3 μg/l level, it is obvious that detection limits depend on the UV properties of analytes, but are lower than 0.1

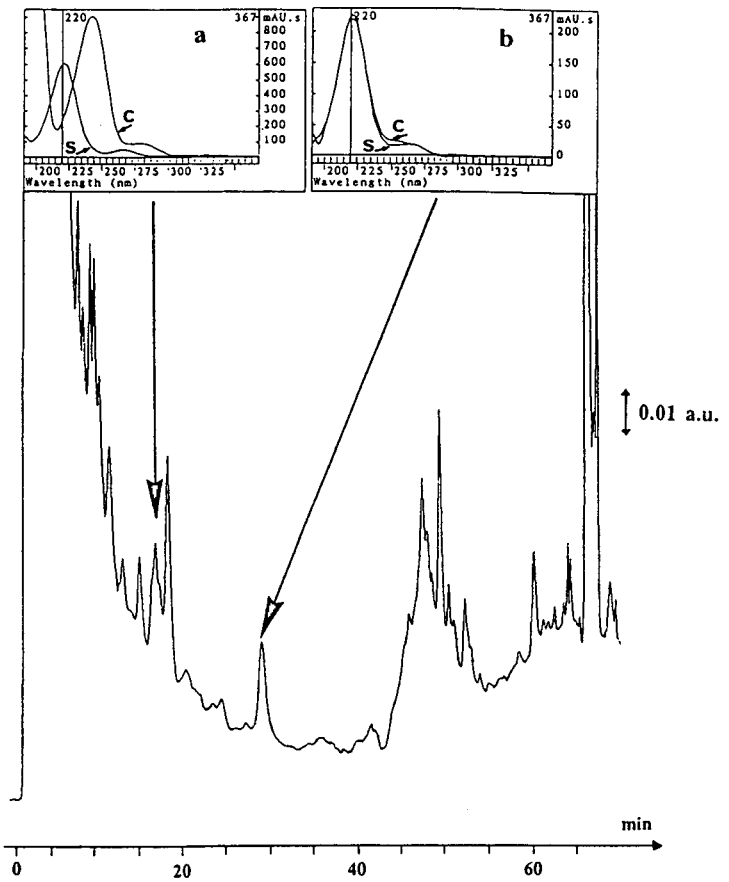

Fig. 5. On-line analysis at 150 ml of Seine river water sample (non-spiked) and quantification methods; experimental conditions as in Fig. 2. Comparison of standard spectra and unknown peak spectra for (a) simazine and (b) atrazine. S refers to the standard and C to the peak of the unknown compound.

μg/l for most of the compounds. This level is required for the control of drinking water in European countries and the handling of 150 ml is sufficient. Now, if quantification is required, a lower detection limit has to be obtained and this is easily done with drinking water by increasing the sample volume. A sample volume of 300 ml was tried, as each compound having a retention time higher than that of simazine or methoxuron has a breakthrough volume higher than 300 ml. Fig. 6 shows the on-line analyses of 300 ml of drinking water spiked with (a) triazines and some of their degradation products or (b) phenylurea herbicides. In each group of herbicides, a wide range of polarity is covered from the first to the last eluted peaks. These chromatograms show that the detection limits are from 5 to 30 ng/l, depending on the analytes. Similar detection limits have been obtained in various ground waters.

In surface water

The Seine river in Paris can be taken as providing convenient samples for studying detection limits in surface water. Fig. 2, which compared drinking and river waters spiked with 0.3 μg/l of the multi-residue solution, showed that the detection limits for some analytes should be of the same order than those obtained in drinking waters. Fig. 7a and b represent the on-line

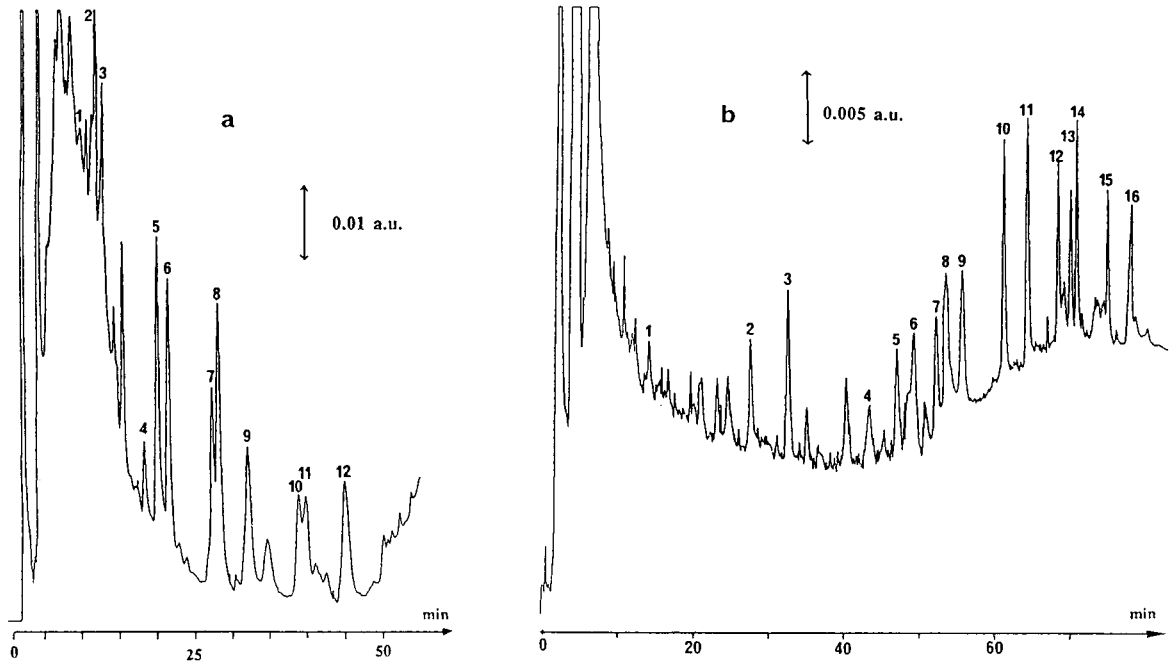

Fig. 6. Preconcentration on PLRP-S of 300 ml of drinking water spiked at 0.1 μg/l with (a) triazines and (b) phenylureas. Triazines: 1 = deisopropylatrazine; (2) = hydroxyatrazine; 3 = deethylatrazine; 4 = hexazinone; 5 = simazine; 6 = cyanazine; 7 = simetryne; 8 = atrazine; 9 = prometon; 10 = sebutylazine; 11 = propazine; 13 = terbutylazine. Analytical column, Varian ODS (25 × 0.46 cm I.D.); flow-rate, 1 ml/min; acetonitrile gradient with phosphate buffer at pH 7, gradient 15 to 30% acetonitrile from 0 to 9 min, 30 to 34% from 9 to 16 min, 34 to 40% from 16 to 45 min and 40 to 60% from 45 to 55 min; detection at 220 nm; zero offset, −30%; attenuation, 16. Phenylureas: 1 = fenuron; 2 = methoxuron; 3 = monuron; 4 = methabenzthiazuron; 5 = chlortoluron; 6 = fluometuron; 7 = monolinuron; 8 = isoproturon; 9 = diuron; 10 = difenoxuron; 11 = buturon; 12 = linuron; 13 = chloroxuron; 14 = chlorbromuron; 15 = diflubenzuron; 16 = neburon. Acetonitrile gradient with phosphate buffer at pH 7: 20 to 35% acetonitrile from 0 to 52 min, 35 to 70% from 52 to 77 min; detection at 249 nm; zero offset, 5%; attenuation, 8.

analysis of 150 ml of river waters spiked with 0.1 μg/l of the triazine mixture and of the phenylurea mixture, respectively. The degradation products of atrazine are not visible owing to the broad interfering peak due to the low attenuation of the detector and peak 5 is high owing to the presence of atrazine in the raw sample. The sample analysed in Fig. 7b contained 1.2 μg/l of diuron, which was identified and measured by the MCA method and the standard addition method. Other compounds were not present in the raw samples and the two chromatograms in Fig. 7 show that detection limits of 0.1 μg/l can be reached for river waters without any clean-up. This excellent result is due to (i) the efficient coupling of the precolumn with the analytical column provided by the Prospekt device, (ii) the low sample volume which lowers the matrix interferences and (iii) the shape of the analytical gradient which can decrease the interfering peaks. The sample volume was increased to 300 ml, but it was impossible to obtain a chromatogram at the same attenuation of detection, which had to be increased by a factor of 2. The result is that the same information was obtained with a higher interfering peak.

The results described above imply that the detection limits depend on the interfering peak shape and that a compromise should be found between low-level detection in river waters and the number of analytes and polarity range analysed. As the sample volume is low, analyses are automated and not too time consuming, so that

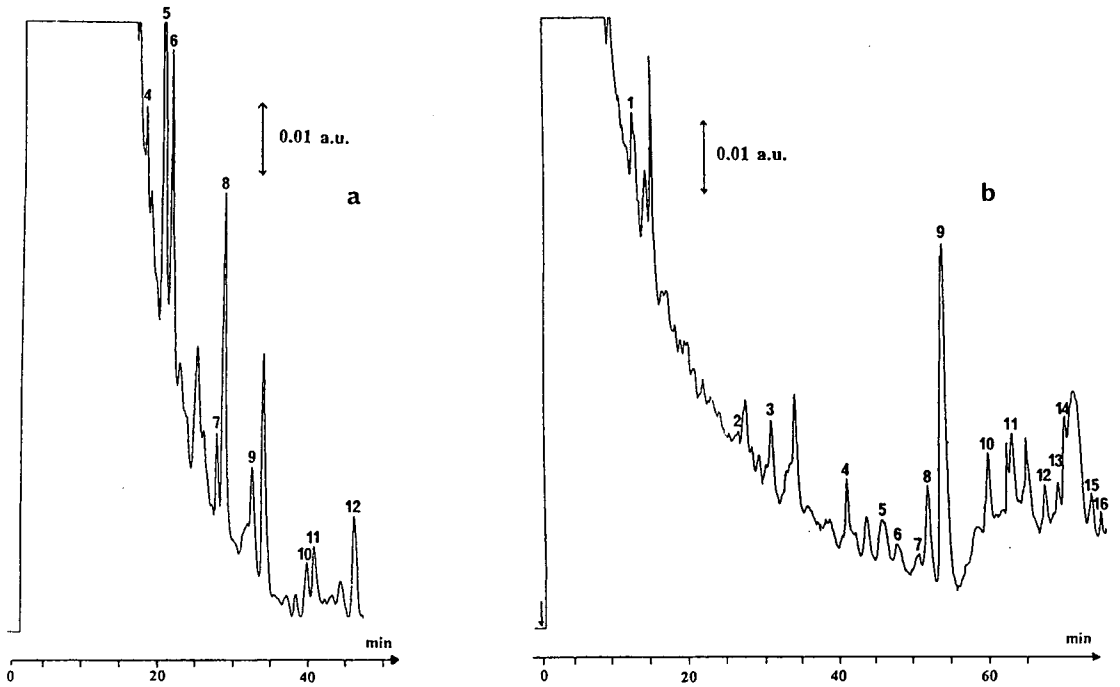

Fig. 7. Preconcentration on PLRP-S of 150 ml of surface water (Seine river) spiked at 0.1 μg/l with (a) triazines and (b) phenylureas. Experimental conditions and peaks as in Fig. 6. Triazines: detection at 220 nm; attenuation, 16; zero offset, −55%. Phenylureas: detection at 244 nm; attenuation, 16; zero offset, −15%.

it is alway possible to divide the polarity range into two groups and to perform two analyses.

4. Conclusions

On-line trace enrichment and LC analysis provide a powerful tool for the analysis of aqueous environmental samples. We have shown that, provided a good choice of the preconcentration parameters and a good selection of the analytical conditions are made, low-level determinations can be performed for many pesticides, even in highly contaminated surface waters and without any clean-up. Determinations below the 1 μg/l level can be achieved within an average R.S.D. of 15%. The weakest point is still the identification of compounds which cannot be confirmed only by a retention time and a UV spectrum for all the compounds. The use of a confirmation column is recommended. This problem could be solved by the development of efficient interfacing with mass spectrometry.

5. Acknowledgements

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On-line and off-line sample preparation of acidic herbicides and bentazone transformation products in estuarine waters

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Abstract

The isolation and trace-enrichment of the acidic herbicides benazolin, bentazone, (2,4-dichlorophenoxy)acetic acid (2,4-D), (4-chloro-2-methylphenoxy)acetic acid (MCPA), 2-(4-chloro-2-methylphenoxy)propionic acid (MCPP) and the transformation products (TPs) of bentazone 6- and 8-hydroxy in estuarine waters is described. The pesticides were spiked at 0.3 μg/l and 1–3 μg/l (for the TPs) in estuarine waters and were preconcentrated using solid-phase extraction (SPE) methods using C_{18} and styrene divinylbenzene (SDB) Empore extraction disks of 47 mm and 4.6 mm diameter in the off-line and on-line modes, respectively, followed by liquid chromatography–diode array (LC–DAD), fluorescence (FD) and/or thermospray mass spectrometric (TSP-MS) detection.

In the off-line mode 5 liters of water were preconcentrated and recoveries of 40–76% were obtained except for the TPs that were not recovered at all. Blanks were investigated within a pH range of 0.7–2, thus recommending the use of SDB over C_{18} Empore disks. When on-line SPE was used only 150 ml of river water were needed, with a limit of detection (LOD) varying between 5–100 ng/l and higher recoveries than in the off-line mode, followed by LC–DAD in both cases.

When on-line SPE with Empore disks was used for the determination of the acidic herbicides in real estuarine waters followed by LC–FD, only bentazone showed similar detection levels as in DAD whereas MCPA and MCPP exhibited quenching interferences from the water matrix.

Confirmation of the acidic herbicides in estuarine waters was accomplished by on-line SPE–LC–TSP-MS using selected-ion monitoring (SIM) and negative-ion mode thus showing similar LOD to those achieved with on-line SPE–LC–DAD.

Illustrative examples of the trace level determination of the acidic herbicides bentazone and MCPA in real estuarine water samples of the Ebro delta (Tarragona, Spain) are shown.

1. Introduction

The acidic herbicides are of interest within the European Community (EC) countries. In a recent report [1] it was shown that the acidic herbicides benazolin, bentazone, (2,4-dichlorophenoxy)acetic acid (2,4-D), (4-chloro-2-methylphenoxy)acetic acid (MCPA), 2-(4-chloro-2-methylphenoxy)propionic acid (MCPP) and TCA are being used in Europe in amounts over 500 tons per year. Since most of them are considered transient and probable leachers through the soil and into the ground-water, they may pose problems for the ground-water resources. In addition to the parent pesticides, in the same report [1] it was recommended to monitor their toxic transformation products (TPs). Due to their current use they have been identified in environmental water samples. Bentazone, for instance, is being applied as rice

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herbicide and it has been determined together with 8-hydroxybentazone in estuarine water samples from the Ebro delta (Tarragona, Spain) when using our current monitoring programmes, either based on liquid–liquid extraction (LLE) [2] or on-line solid-phase extraction [3] (SPE) followed by liquid chromatography with diode array (LC–DAD) and mass spectrometric (TSP-MS) detection.

The official and most current methods for the determination of acidic herbicides in water samples are based on the adjustment of the waters to basic pH followed by LLE with diethyl ether or dichloromethane, their conversion to methyl esters by diazomethane or as pentafluorobenzyl derivatives and gas chromatography–electron capture detection (GC–ECD) [4]. Recently, the use of single or double Empore disks at pH from 1–7 was described as a previous step replacing the tedious LLE and using the same determination protocol as the official methods of analysis [5].

To avoid the derivatisation step, the use of either LLE at acidic pH < 2 and LC with DAD and FD has been proposed [2,6]. Another alternative is to replace LLE by SPE techniques with Carbopack cartridges, that permit high flow-rates and high water volumes of 100 ml/min and 2 l, respectively. In this way a LOD of 0.1 μg/l can be obtained when using LC–UV detection [7].

On-line SPE coupled to LC has been recently reported for the determination of various acidic herbicides [3,8–10]. Such SPE systems use either PLRP-S, C_{18} with large volume injection or anion exchange. One of the difficulties encountered with the analysis of acidic herbicides in real river water samples was the interference of humic substances that could not be totally removed even when working at different pH values [3,8].

One of the key parameters in environmental analysis is the confirmation of the trace organics tentatively identified by the UV spectra of the LC–DAD. In this respect, MS is the ideal technique that avoids false positive determinations. Thermospray LC–MS in the negative-ion mode of operation has been recommended for the identification of acidic herbicides [11–14]. In addition, the use of chloroacetonitrile as eluent additive in TSP-MS has proved to be useful for obtaining additional structural information via chloride attachment to chlorinated phenoxy acids [11,13].

The aim of this work was to develop and compare various LC methods for the determination of acidic herbicides and bentazone TPs in estuarine waters at levels of 0.1 μg/l. The approach has consisted in the use of (i) an off-line method with Empore extraction disks of C_{18} and SDB by preconcentrating 5 liters of water at acidic pH of 0.7–2; (ii) an on-line SPE method using Empore disks followed by LC–DAD, LC–FD and LC–TSP-MS which involved the preconcentration of 150 ml and (iii) the systems developed for the determination of the different herbicides in real estuarine waters of the Ebro delta (Tarragona, Spain).

2. Experimental

2.1. Chemicals

HPLC grade water and acetonitrile gradient grade LiChrosolv from Merck (Darmstadt, Germany) were passed through a 0.45 μm filter before use. Ammonium formate, formic acid, sulphuric acid and trifluoroacetic acid were also purchased from Merck. All of these chemicals were of analytical reagent grade. Tripropylamine was obtained from Fluka (Buchs, Switzerland). Tripropylammonium formate was prepared by mixing amounts of tripropylamine (98%) and formic acid. Benazolin, 8-hydroxybentazone, 6-hydroxybentazone, bentazone, 2,4-D, MCPA and MCPP were purchased from Promochem (Wesel, Germany). Stock standard solutions (1000 μg/ml) of the herbicides were prepared in acetonitrile. The stock solutions were diluted and mixed in HPLC grade water containing formic acid (pH = 3) before LC analysis.

2.2. Chromatographic conditions

The eluent was delivered by two Knauer 64 high-pressure pumps (Bad Homburg, Germany)

coupled to a 1000S Applied Biosystems diode array detector (Foster City, CA, USA) or a Model LC 240 Fluorescence detector from Perkin-Elmer (Beaconsfield, UK). Two LiChrocart cartridge columns 60 RP-8 and 60 RP-8 select B (25 cm × 4.6 mm I.D.) packed with 4-μm Supersphere from Merck were used. Gradient elution was performed from an eluent containing 10% of A [acetonitrile–water (90:10)] and 90% of B [water–formic acid pH = 2.9] to 35% of A–65% of B in 30 min; from 35% of A–65% of B up to 45% of A–55% of B in 20 min. From 45% of A–55% of B up to 100% of A in 2 min and back to the initial conditions in 3 min. Post-run time was 10 min at a flow-rate of 0.9 ml/min.

Quantification of the compounds was done by external calibration and peak area measurements. The calibration graphs were constructed for compounds showing recoveries higher than 70%. The concentration range studied ran from 0.02 to 2 μg/l using preconcentration volumes of 100–150 ml and UV detection at 200 nm.

2.3. Mass spectrometric analysis

A Hewlett-Packard (Palo Alto, CA, USA) Model 5988A Thermospray LC–MS quadrupole mass spectrometer and a Hewlett-Packard Model 35741B instrument for data acquisition and processing were employed. The Thermospray temperature parameters were programmed from 100°C to 90°C and from 180°C to 170°C for the stem and tip, respectively. Source temperature was set at 220°C when using ammonium formate as mobile phase additive and at 250°C when using tripropylammonium formate as mobile phase additive. In all experiments the filament-on mode was used in which conventional negative-ion chemical ionization can be carried out. The chromatograms were recorded under time scheduled selected-ion monitoring (SIM) conditions as shown in Table 1.

Table 1
Time scheduled selected ion monitoring (SIM) conditions under TSP-MS with NI detection

Time in min	Ions monitored
0 to 35	242, 288, 255, 239, 283
38 to 45	239, 199, 245, 219, 265
45 to 50	213, 259

2.4. Sample pretreatment

Estuarine water samples from Ebro Delta (Tarragona, Spain) of 150 ml were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA) and were then acidified at pH = 1–1.5 in case of C_{18} disks and at pH = 1 in case of SDB disks with sulphuric acid. Samples were spiked with the different pesticides giving final concentrations in the range of 0.3–3 μg/l. In the off-line method a Baker Empore Extraction System filtration apparatus, which was a gift from J.T. Baker (J.T. Baker, Deventer, Netherlands) was used. The general extraction procedure using 4–5 liters of water samples was described elsewhere [15]. In the on-line method a MUST column switching device from Spark Holland (Emmen, Netherlands) was used. Sample preconcentration was carried out with an SSI Model 300 LC pump from Scientific Systems Inc., (State College, PA, USA). The precolumn consisted of a stainless steel membrane disks holder constructed in the workshop of the Free University of Amsterdam (Netherlands) where 10 × 4.6 mm I.D. C_{18} or SDB Empore extraction disks were placed, as described elsewhere [3]. Extraction disks manufactured under the trade mark Empore were a gift of J.T. Baker (Deventer, Netherlands).

3. Results and discussion

3.1. Analytical LC separation

It has been shown that the use of a C_{18} bonded silica column with an acidic mobile phase, *e.g.*, perchloric acid at pH = 2, allows a good separation of the acidic herbicides [10]. When the acidic mixture contains the TPs, then the separation between closely related compounds becomes critical. This is the case of 2,4-D and

MCPA or 6-hydroxybentazone and 8-hydroxybentazone. To achieve a complete separation of all these compounds a high-resolution end-capped LC column of 25 cm length and 4-μm particle size becomes necessary exhibiting a better resolution for the two TPs of bentazone with a reduction in the peak tailing.

The influence of the pH on the separation was also studied. Due to the ionization suppression, the separation of the acidic herbicides increased with a pH decrease with an optimal separation at pH = 2.82. This pH value was selected as a compromise that prevented a decrease of the column life. When a polymeric column Zorbax SB phenyl of 250 × 4.6 mm I.D. 5-μm particle size was used, a lack of separation efficiency was noticed even thought its use was possible over a pH range 1–13. Two acid modifiers were investigated: trifluoroacetic acid and formic acid. The former allowed a good background line at wavelengths lower than 220 nm and the full separation of 6-hydroxybentazone and the 8-hydroxybentazone, but 2,4-D and MCPA were not resolved (figure not shown). The latter one, used at the same pH but with formic acid, permitted a really good resolution between 2,4-D and MCPA but 6-hydroxybentazone and benazolin coeluted.

When performing on-line precolumn technology, a good separation is necessary because otherwise the extra band broadening caused by the coupling may lead to an overlapping of peaks when analyzing real samples [10]. Therefore, we have selected formic acid as modifier for further analysis, even thought benazolin and 6-hydroxybentazone coelute. This coelution was not so important for us, as compared to 2,4-D and MCPA (these two compounds are also much more in use in the area of study). With respect to the UV detection a wavelength of 200 nm was used as the result of a compromise between selectivity and sensitivity.

In addition, it was difficult to find a commercially available 6-hydroxybentazone standard of high purity. In ethylacetate solvent, that was commercially available, three peaks with UV detection appeared. These peaks did not come from a degradation process on the analytical column but from impurities present in the analytical standard.

3.2. Preconcentration studies with LC–DAD

Off-line SPE

The aim of the off-line preconcentration procedure was to compare two kinds of Empore disk materials: C_{18} and SDB. Previous studies have shown that, in general, PRP-1 or PLRP-S were more suitable than C_{18} silica sorbent for the extraction of relative polar compounds from water [8,10].

It is known that in reversed-phase chromatography the trapping capacity of an ionisable analyte depends on its ionisation state. The retention of a compound is higher in its neutral than in its ionic form. Since the pK_a values of phenoxy acids is around 2.5 [10] a pH value lower than the pK_a is needed to achieve good recovery values.

In our experiments a pH = 1 in the water sample was established in order to prevent recovery losses. This creates a problem since C_{18} bonded silica material is no more stable at pH = 1, especially when preconcentrating large water volumes like 5 liters, which require 150 min of preconcentration time leading to decomposition of the sorbent and high blank interferences (figure not shown). So, when using C_{18} disks the pH was kept at 1.5 to avoid these decomposition problems. In contrast, when using SDB disks even at pH 0.7 the blanks are quite clear. The main advantage of SDB Empore disks was the possibility of using them over the pH range 0.7–13 without any decomposition of the sorbent material in the off-line method. Table 2 shows the recoveries and LODs when using such water volumes. The recoveries varied from 40–76%, bentazone being the only compound that shows recovery values over 70% in both sorbents. In general, there is a surplus of the breakthrough volumes for most of the compounds with no recovery at all for TCA and the two TPs of bentazone. The somewhat higher recoveries values for SDB as compared to C_{18} can be explained by the structural molecular properties

Table 2
Average % recovery (Av) and relative standard deviation (R.S.D.) of pesticides in estuarine waters using off-line SPE with SDB or C_{18} Empore extraction disks of 47 mm diameter

Compound	UV (nm) absorption	SDB[a]		C_{18}[b]		LOD g/l
		Av	R.S.D.	Av	R.S.D.	
Benazolin	220	45	12	39	15	1
Bentazone	220	76	7	72	6	0.05
MCPA	200	65	9	53	11	0.2
2,4-D	200	59	11	44	9	0.2
MCPP	200	70	8	58	12	0.1

Water volume preconcentrated: 5 l at a flow-rate of 30 ml/min. Spiking level: 0.25 μg/l. Analysis: LC–DAD. 6-hydroxybentazone, 8-hydroxybentazone and TCA were not recovered.
[a] Water pH = 0.7.
[b] Water pH = 1.5.

involving $\pi-\pi$ interactions between polymeric matrix and organic compounds with free electrons.

On-line SPE

When using on-line SPE only 150 ml of water were needed for the preconcentration studies. The contact time between the mobile phase and the sorbent is shorter and we did not see any alteration of the C_{18} sorbent at pH = 1. Table 3 gives the recoveries and the coefficient of variation of each compound spiked at 0.3 μg/l in a estuarine water after preconcentrating a volume of 150 ml followed by LC–DAD.

As noticed in Table 3, the on-line SPE method using SDB Empore disks allowed only a slight improvement of the recovery values as compared to C_{18} Empore disks due to some decrease in the interferences of the early eluting peaks. Fig. 1A and 1B show typical chromatograms obtained with the on-line method after preconcentrating 150 ml of Ebro river surface water spiked at 0.3 μg/l using C_{18} and SDB disks, respectively. The use of a wavelength of 200 nm permitted the detection of most of the compounds. In the case of bentazone a better quantitation is obtained by the use of $\lambda = 215$ nm (Fig. 1C). Benazolin and 8-hydroxybentazone exhibited low recoveries

Table 3
Average % (Av) recovery and relative standard deviation (R.S.D.) of pesticides in surface water using on-line SPE with 10 SDB or 10 C_{18} Empore extraction disks of 4.6 mm diameter

Compound	UV (nm) absorption	SDB		C_{18}		LOD μg/l
		Av	R.S.D.	Av	R.S.D.	
Bentazone	220	95	5	98	3	0.01
Benazolin	220	65	10	58	8	0.3
MCPA	200	80	5	78	6	0.05
2,4-D	200	82	6	74	8	0.05
MCPP	200	85	3	81	7	0.05
8-Hydroxybentazone	220	33	20	30	22	0.7

Surface water (pH = 1) volume preconcentrated: 150 ml at a flow-rate of 3 ml/min ($n = 6$ for each pesticide). Spiking level: TCA: 500 μg/l; 6-hydroxybentazone: 1.5 μg/l; 8-hydroxybentazone: 3 μg/l; others: 0.3 μg/l. Analysis: LC–DAD. 6-Hydroxybentazone and TCA could not be recovered.

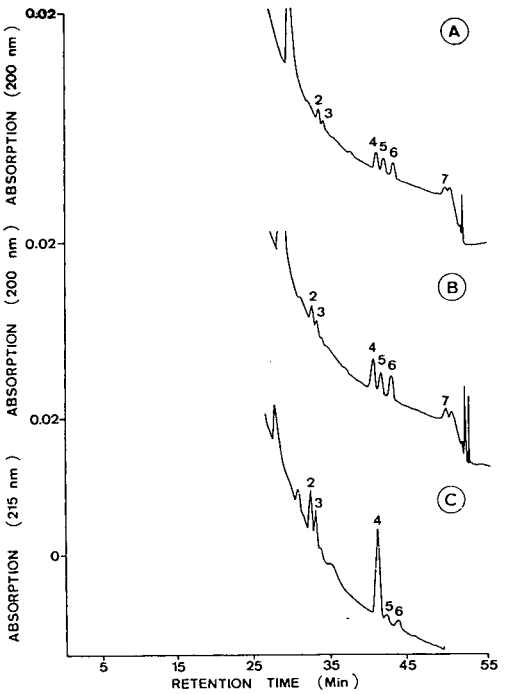

Fig. 1. LC–Diode array chromatograms at 200 nm obtained after on-line preconcentration of 150 ml of Ebro river water sample spiked at 0.3 μg/l: (A) on 10 C_{18} Empore extraction disks; (B) on 10 SDB Empore extraction disks; (C) same analysis as (B) but recorded at 215 nm. Peaks: (1) benazolin, (3) 8-hydroxybentazone, (4) bentazone, (5) 2,4-D, (6) MCPA, (7) MCPP. LC conditions: see Experimental.

due to matrix interferences present in the estuarine waters.

Similar explanations as discussed in the off-line method can be given for the somewhat higher recoveries obtained for SDB as regards to C_{18} Empore disks (see Table 3). 6-Hydroxybentazone and TCA were not recovered at all due to matrix interferences and poor detection, respectively. The present study expands the application of the use and comparison of SDB and C_{18} Empore disks. In previous studies from our group [3] we observed that C_{18} was superior to SDB Empore disks for a variety of carbamate and triazine pesticides.

Because of evident difficulties (due to degradation) encountered in previous studies when analysing carbamates in river water samples immediately after sampling [3], it was decided to perform an analyte stability study during 20 days in water. All the 8 compounds were spiked into river water, stored at 4°C in the dark and kept at pH = 4.8 with ammonium formate–formic acid buffer that avoids biological degradation. All the 8 compounds remained stable.

3.3. Preconcentration studies with LC–FD

Schüssler [6] used fluorescence detection after LLE of MCPA, MCPP and bentazone from water sample. Fig. 2A shows a typical LC–FD of the pesticide standard mixture. 2,4-D, which has a closely related structure, does not fluoresce naturally. It is known that with halogen substituents on the ring fluorescence can be reduced or even eliminated [16]. 6-Hydroxybentazone

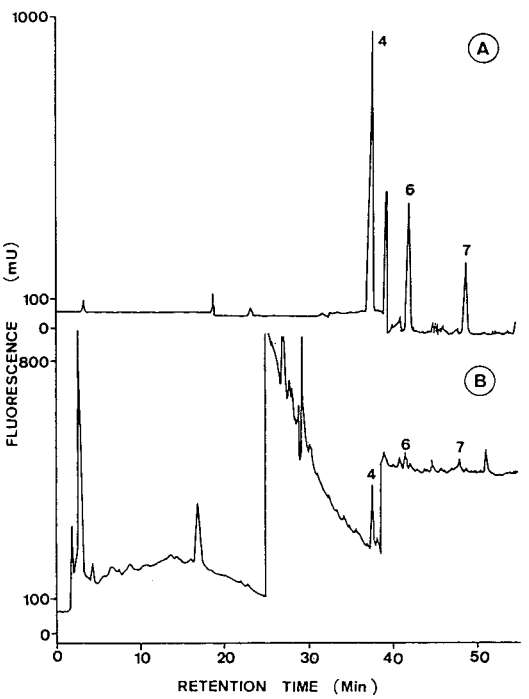

Fig. 2. (A) LC–FD chromatogram of the pesticide containing 10 mg/l (injection 20 μl) each of (4) Bentazone (Ex: 340 nm, Em: 430 nm), (6) MCPA (Ex: 280 nm, Em: 310 nm), (7) MCPP (Ex: 280 nm, Em: 310 nm). (B) LC–FD chromatogram obtained after on-line preconcentration on Empore extraction disks of SDB 150 ml of Ebro river water sample spiked at 0.3 μg/l. Other experimental conditions as in Fig. 1.

and 8-hydroxybentazone exhibited moderate fluorescence in a mobile phase water–acetonitrile (50:50, v/v) with neutral media [17]. The fluorescent intensity is strongly influenced by the pH and the type of the mobile phase. When using an acidic mobile phase, the chromatographic separation of 6-hydroxybentazone and 8-hydroxybentazone showed very low fluorescence even when injecting high amounts of the compounds, the LOD being 200 ng and 250 ng, respectively. At this LOD it is impossible to use the FD for trace level monitoring in water.

For bentazone, MCPA and MCPP the LOD were 0.5 ng, 1 ng and 1.5 ng, respectively. It was necessary to preconcentrate a volume of 100–150 ml in the on-line SPE method to reach the LOD of 0.1 μg/l, in a similar way to the use of LC–DAD. Average recoveries and LOD using SDB and C_{18} Empore extraction disks are indicated in Table 4. Fig. 2B shows a LC–FD chromatogram obtained after preconcentrating 150 ml of estuarine water. We can see that the recoveries for MCPA and MCPP are low. This is attributed to humic substances, considered to be polydisperse hydrophobic polymers of average molecular mass 1000–10 000, that contain structures as metoxy aromatics, phenols and aryl ketones [3]. Due to their aromatic structure, humic substances including humic acids and fulvic acids could be directly responsible for the quenching phenomenon explaining the low recovery values and the high coefficient of variation obtained in the case of MCPA and MCPP. Besides, the interference level depends strongly on the excitation and emission wavelength values. Actually, only bentazone was monitored in estuarine waters using LC–FD. This was demonstrated by performing the recovery studies using HPLC water instead of estuarine water with recoveries in the range of 85–95% for the three acidic herbicides, demonstrating the negative effect of the natural humic materials on the recoveries. Bentazone, with a less acidic structure as compared to the chlorinated phenoxy acids, showed good recoveries using estuarine waters.

3.4. Thermospray LC–MS

In order to confirm the compounds of interest in the HPLC chromatogram and avoid false positives, an on-line SPE–LC–TSP-MS method has been developed in our laboratory for a variety of pesticides [18]. In this paper we have optimized such a method for the acidic herbicides using NI mode detection, as recommended [11–14]. The major ions, relative abundances and LOD obtained under SPE–LC–TSP-NI MS are shown in Table 5.

In general, two mechanisms of ion formation were observed: proton abstraction and anion attachment. When ammonium formate–formic acid buffer (pH = 2.9) was used as additive different base peaks were obtained depending on the compound: for phenoxy acids and benazolin $[M + HCOO]^-$ ion, following expectations [11,12], whereas the $[M - H]^-$ ion for bentazone and 8-hydroxybentazone [19] and the $[M - H - OH + HCOO]^-$ ion for 6-hydroxybentazone. The use of tripropylammonium for-

Table 4
Average % recovery (Av) and relative standard deviation (R.S.D.) of pesticides in estuarine water (pH = 1) using on-line SPE with 10 SDB or 10 C_{18} Empore disks of 4.6 mm

Compound	Wavelength (nm)		SDB		C_{18}		LOD (μg/l)
	Ex	Em	Av	R.S.D.	Av	R.S.D.	
Bentazone	340	430	85	8	86	7	0.01
MCPA	280	310	22	30	18	24	1
MCPP	280	310	31	19	25	15	1

Water volume: 150 ml, spiking level 0.3 μg/l. Detection by fluorescence. (When HPLC water was used instead of estuarine water recoveries were of 85–95% for all three compounds at the same spiking level.)

Table 5
Main ions, relative abundance and LOD (when preconcentrating 100 ml of estuarine water) for acidic herbicides under on-line SPE–LC–TSP-MS under NI mode of operation

M_w	Compound and ions (m/z)	Carrier stream				LOD μg/l
		1	2	3	4	
240	Bentazone					0.02
	239 $[M-H]^-$	100	100	100	100	
200	MCPA					0.1
	199 $[M-H]^-$	12	18	17	16	
	245 $[M+HCOO]^-$	100	100	100	100	
	235 $[M+Cl]^-$		18			
214	MCPP					0.1
	213 $[M-H]^-$	10	10	23	16	
	259 $[M+HCOO]^-$	100	100	100	100	
	249 $[M+Cl]^-$		10			
221	2,4-D					0.1
	219 $[M-H]^-$	12	8	30	100	
	265 $[M+HCOO]^-$	100	100	100	7	
	256 $[M+Cl]^-$		8			
243	Benazolin					2
	242 $[M-H]^-$	25	30	30	30	
	288 $[M+HCOO]^-$	100	100	100	100	
256	8-Hydroxybentazone					2
	255 $[M-H]^-$	100	100	100	100	
256	6-Hydroxybentazone					4
	239 $[M-OH]^-$	23	20	5	10	
	255 $[M-H]^-$	23	20	15	10	
	283 $[M-H-OH+HCOO]^-$	100	100	100	100	

Carrier stream: (1) water–acetonitrile (65:35, v/v) with a buffer of 50 m*M* ammonium formate–formic acid, pH = 2.9; (2) water–acetonitrile + 2% chloroacetonitrile (65:35, v/v) with a buffer of 50 m*M* ammonium formate–formic acid, pH = 2.9; (3) water–acetonitrile (65:35, v/v) with a buffer of 50 m*M* tripropylammonium formate–formic acid, pH = 2.9; (4) water–acetonitrile (65:35, v/v) with a buffer of 50 m*M* tripropylammonium formate buffer, pH = 7.2.

mate (pH = 2.9) was also investigated. Since tripropylamine has a higher basicity than ammonia [20] an increased deprotonation was expected. This hypothesis was confirmed in a basic mobile phase (pH = 7.2) and was important in the case of 2,4-D, that showed $[M-H]^-$ ion as base peak instead of $[M+HCOO]^-$ (see Table 5). When formic acid was added in greater amounts to lower the pH (pH = 2.9) the $[M+HCOO]^-$ ion remained as base peak. This shows once more that TSP is a solution-dependent ionization technique being affected by the pH of the mobile phase [21]. With this mobile phase a two-fold, three-fold and one order of magnitude increase in signal intensity was found for MCPP, benazolin and bentazone, respectively. In addition, it showed slightly better sensitivity for the rest of the compounds and therefore it was

selected for performing the on-line SPE–LC–MS analysis.

The addition of 2% of chloroacetonitrile to the mobile phase was also investigated in order to investigate the additional structural information obtained, as reported [13]. Although no change was observed in the base peak the abundance of the $[M + HCOO]^-$ adduct ion decreased as compared with the absence of chloroacetonitrile. Chloride attachment leads to $[M + Cl]^-$ ion formation with relative abundances of at best 18%. Although the addition of chloroacetonitrile to the LC eluent allowed complementary molecular mass information to be obtained, unfortunately a two-fold decrease in the signal intensity was noticed. Consequently, it was not used for performing further studies with the on-line system since the major analytical requirements of the present work is to achieve a low detection limit.

The chromatograms were recorded under time-scheduled selected-ion monitoring (SIM) conditions, each compound being identified by two main ions. The time scheduled SIM is reported in the experimental section. Fig. 3 shows a typical on-line SPE–LC–TPS-MS separation in the NI mode of the pesticides mixture obtained after preconcentrating 100 ml of Ebro river water spiked at 0.6 μg/l. The monitoring of benazolin and the two bentazone TPs was impossible because of the lack of sensitivity under TSP-MS detection, and requires further investigation by other techniques, *e.g.*, electrospray. The optimization of the stem temperature showed that no thermal decomposition of the analytes occurred in the interface. The LOD obtained with the proposed method are indicated in Table 5 being somewhat higher than with LC–DAD.

In NI mode TCA gives one main ion corresponding to $[2M - 2Cl + HCOO]^-$ using water + formic acid pH = 2.82–methanol (65:35, v/v) as mobile phase when 1 μg was injected. In a mobile phase of acetonitrile–water, the relative abundance of this ion is even lower. Due to the difficulties in its response and low molecular mass, 162, this compound can not be determined at the low μg/l level required in the present method.

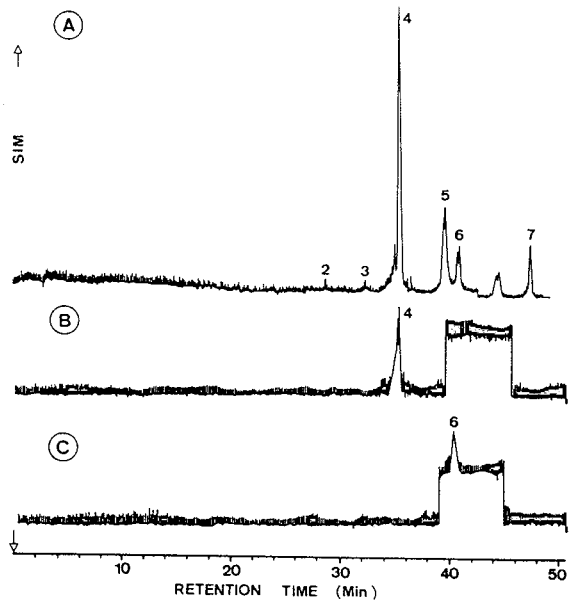

Fig. 3. (A) On-Line SPE using 10 SDB Empore extraction disks followed by LC–TSP-MS of 100 ml of Ebro river water sample spiked at 0.6 μg/l in NI mode under SIM conditions (for details, see Experimental). Gradient elution as in Fig. 1 but the mobile phase contained as additive tripropylammonium–formic acid buffer pH = 2.9. (B) and (C) TSP-MS detection under NI and SIM detection after preconcentrating 100 ml of the same non-spiked estuarine Ebro delta water that permitted confirmation of the presence of: (B) (4) bentazone (0.12 μg/l) (stem, tip and source temperatures: 95°C, 180°C and 250°C, respectively); (C) (6) MCPA (0.18 μg/l) (stem, tip and source temperatures: 70°C, 160°C and 200°C, respectively).

3.5. Confirmation of environmental levels

The current developed methods were applied to the determination of acidic herbicides in the Ebro delta estuarine waters. A monitoring programme that measures the contamination level from acidic herbicides is continuously performed. The Ebro river delta is a typical rice cultivation area where bentazone and MCPA are applied in large amounts during spring and summer [2]. An example of the on-Line SPE

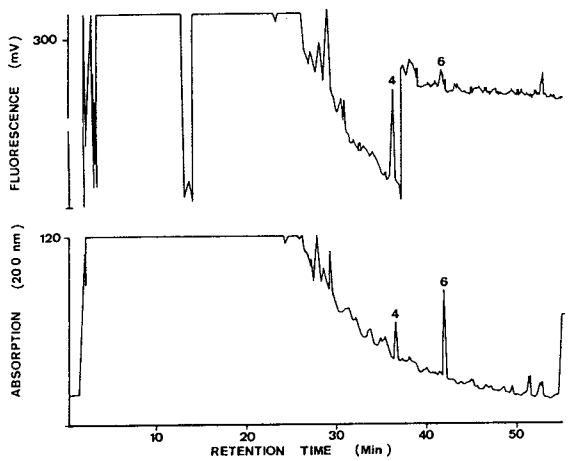

Fig. 4. LC chromatogram obtained after on-line preconcentration on SDB Empore extraction disks of a non-spiked estuarine Ebro Delta water sample using: (A) FD, (preconcentration volume 150 ml) (B) DAD at 200 nm, (preconcentration volume 150 ml) that permitted confirmation of: (4) bentazone at 0.12 μg/l and (6) MCPA at 0.18 μg/l.

with LC–DAD and LC–FD traces are shown in Fig. 4A and 4B. Bentazone (peak no. 4) was clearly identified in both detectors, whereas the presence of MCPA was somewhat suspect and FD, due to the quenching effects mentioned before, could not be used for an unequivocal identification. The use of on-line SPE–LC–TSP-MS of the same sample is shown in the chromatograms of Fig. 3B and 3C. Due to the low level of MCPA, and lower signal under TSP-MS as regards to bentazone, it was necessary to perform the analysis of the sample twice. In the first case we monitored the $[M-H]^-$ ion of bentazone at m/z 239 (Fig. 3B) whereas in the second case we monitored the $[M-H]^-$ and $[M+HCOO]^-$ ions of MCPA, at m/z values of 199 and 245, respectively. In the latter case the experimental conditions were set up in order to increase the MCPA signal intensity (stem, tip, and source temperatures: 70°C, 160°C and 200°C, respectively). Under these conditions, the signal intensity of bentazone decreased by a factor of 10. In this way the unequivocal determination of bentazone and MCPA at 0.12 μg/l and 0.18 μg/l levels, respectively, was feasible in estuarine Ebro river waters samples collected during June of 1993.

4. Conclusions

An off-line SPE system based on the use of C_{18} or SDB Empore disks was evaluated for the determination of various acidic herbicides spiked at 0.25 μg/l level using 5 liters of estuarine waters. The method permitted only the determination of bentazone and MCPP at 0.1 μg/l level when using SDB Empore disks, whereas the recoveries for C_{18} Empore disks were below 60%, with the exception of bentazone. The off-line method demonstrated that with a pH close to 1 the use of SDB disks is superior to C_{18} disks since better blanks and recoveries are achieved.

The on-line SPE method using both types of material and only 150 ml of estuarine water spiked at levels of 0.3–1.5 μg/l permitted recoveries greater than 80% for all the pesticides, with the exception of benazolin and the two TPs of bentazone. This was attributed to interferences in the LC–DAD traces caused by the humic substances present in the real estuarine waters. On-line SPE–LC with FD permitted a complementary detection to LC–DAD for bentazone in real estuarine water samples, whereas for MCPA and MCPP quenching effects were noticed. This behaviour presents a novelty in the analysis of real estuarine waters containing bentazone and it is a useful confirmation method.

The use of on-line SPE–LC–TSP-MS with NI and time-scheduled SIM permitted confirmation of most of the studied pesticides at LOD below or equal to 0.1 μg/l. Exceptions were benazolin, 6-hydroxybentazone and 8-hydroxybentazone with LOD of 2–4 μg/l.

It is our plan to investigate in the near future other adsorbent materials, *e.g.*, carbon type, together with other MS detection systems, *e.g.*, electrospray, to improve the LOD of certain compounds, such as benazolin, TCA and bentazone TPs.

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JOURNAL OF CHROMATOGRAPHY A

Application of on-line solid-phase extraction followed by liquid chromatography–thermospray mass spectrometry to the determination of pesticides in environmental waters

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Abstract

A multi-residue method for the trace-level determination of 34 pesticides and various transformation products was developed by using on-line solid-phase extraction and either ten 4.6-mm Empore extraction discs containing C_{18} or a conventional precolumn, packed with PRP-1 copolymer, followed by liquid chromatography–thermospray mass spectrometry with time-scheduled selected-ion monitoring. Two main ions (usually $[M + H]^+$ and $[M + NH_4]^+$ or $[M + CH_3CN]^+$) were used for each pesticide in the positive-ion operational mode, while $[M - H]^-$ and $[M + HCOO]^-$ ions were used in the negative-ion mode. Losses of CH_3NCO were also monitored for many of the carbamate pesticides. The proposed method requires 100 ml of sample for a limit of detection of 0.01–0.4 μg/l, depending on the particular compound and the operational mode. Calibration graphs were constructed by preconcentrating 100 ml of an estuarine water sample, spiked with the pesticide mixture at various concentration levels, varying from 0.025 to 1.2 μg/l. Good linearity was observed for sixteen of the analytes studied, the relative standard deviation ($n = 5$) being 4–13%. Some examples of the trace-level determination of various pesticides in European river and ground water samples are given.

1. Introduction

The on-line combination of LC and MS is the most powerful tool available for confirmation of the presence of pesticides in water matrices with no false-positive determinations. Of the different LC–MS systems, the thermospray (TSP) method has been widely used for water analysis, usually following off-line liquid–liquid extraction (LLE) and/or solid-phase extraction (SPE) [1]. One of the assets of LC–MS systems is their suitability for determining a wide variety of pesticides, which makes them appealing for multi-residue analysis. In this respect, LC–TSP-MS has permitted the determination of 23 and 19 pesticides in water [1] and fruits and vegetables [2], respectively. LC–particle beam (PB)–MS has also been evaluated for the characterization of pesticides in ground waters included in the National Pesticide Survey (NPS), where it proved to be sensitive enough for only 43 of the 126 polar pesticides on the NPS list [3]. Recent studies on the performance of TSP and PB in the determination of pesticides have shown TSP to excel

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over PB with acidic herbicides {*e.g.*, (2,4-dichlorophenoxy)acetic acid (2,4-D) and (4-chloro-2-methylphenoxy)acetic acid (MCPA) [4]} and carbamates (*e.g.*, carbaryl and carbofuran [5]); the limit of detection is 10–50 times lower with TSP than that with PB, the former method also featuring a wider linear dynamic range.

In previous work, our group has characterized a wide variety of pesticides including chlorinated phenoxy acids [6], carbamates and triazines [7] and various pesticide transformation products (TPs) [8] by LC–TSP-MS. Other workers [9] have also accomplished the preconcentration of phenylurea herbicides from water samples by using on-line SPE–LC–TSP-MS and positive-ion (PI) mode detection. However, results were only obtained in the PI mode, quantification aspects were lacking and no examples of on-line preconcentration of a broad range of pesticides (only a few TPs were addressed) were reported, with also few applications to real environmental samples. In two previous papers, we reported on the optimization of two on-line systems based on Empore SPE discs and a PRP-1 precolumn for the preconcentration of 30 pesticides and various transformation products included on the NPS list of the US Environmental Protection Agency (EPA) and the European Community [10,11] and for the characterization of phenylurea and triazine herbicides [12]. As a rule, quantification was performed by UV spectrophotometry at 220 nm or by using a postcolumn reaction with fluorescence derivatization (for carbamate pesticides).

Based on previous studies, the purpose of this work was to apply on-line SPE–LC–MS by using two different precolumn sorbents (Empore C_{18} discs and PRP-1) and two detection modes (PI and NI) for the determination of pesticides bearing various chemical groups and selected TPs at concentrations of 0.01–0.5 μg/l in real environmental water samples.

2. Experimental

2.1. Chemicals

HPLC-grade water, acetonitrile (gradient-grade LiChrosolv) and methanol (Merck Darmstadt, Germany) were passed through a 0.45-μm filter before use. Ammonium formate and formic acid were also purchased from Merck. Aldicarb sulphoxide, butocarboxim sulphoxide, aldicarb sulphone, oxamyl, methomyl, deisopropylatrazine, 3-hydroxy-7-phenolcarbofuran, deethylatrazine, 3-hydroxycarbofuran, methiocarb sulphoxide, methiocarb sulphone, methiocarb, 3-ketocarbofuran, 3-ketocarbofuranphenol, 1-naphthol, carbofuran, butocarboxim, aldicarb, bentazone, symazine, baygon, carbaryl, chlortoluron, MCPA, atrazine, isoproturon, propanil, molinate, alachlor, metolachlor, diuron, propazine, terbuthylazine, linuron, propoxur, neburon and metoxuron were purchased from Promochem (Wesel, Germany).

2.2. Chromatographic conditions

The eluent was delivered by two Model 510 high-pressure pumps coupled to a Model 680 automated gradient controller (Waters Chromatography Division, Millipore, Bedford, MA, USA) and a Model 7125 injection valve furnished with a 20-μl loop (Rheodyne, Cotati, CA, USA). The general scheme of the system used for carrying out the on-line preconcentration of pesticides from water samples was similar to that described elsewhere [10–12]. However, in this work we used thermospray mass spectrometry instead of conventional diode-array and/or fluorescence detection. The general scheme of the method used is shown in Fig. 1.

After the membrane discs has been placed in the disc holder, this holder was fitted in a MUST column-switching device (Spark Holland, Emmen, Netherlands) and connected to an SSI Model 300 LC pump (Scientific Systems, State College, PA, USA) which delivered the water samples containing the pesticides. The discs were first conditioned by flushing 10 ml of methanol and then 10 ml of HPLC-grade water at 1 ml/min. Water volumes of 100 ml spiked with pesticides and TPs at concentrations of 0.2 μg/l were preconcentrated on ten membrane extraction discs of 4.6 mm diameter at a flow-rate of 5 ml/min. Following the preconcentration step, the MUST valve was switched and the com-

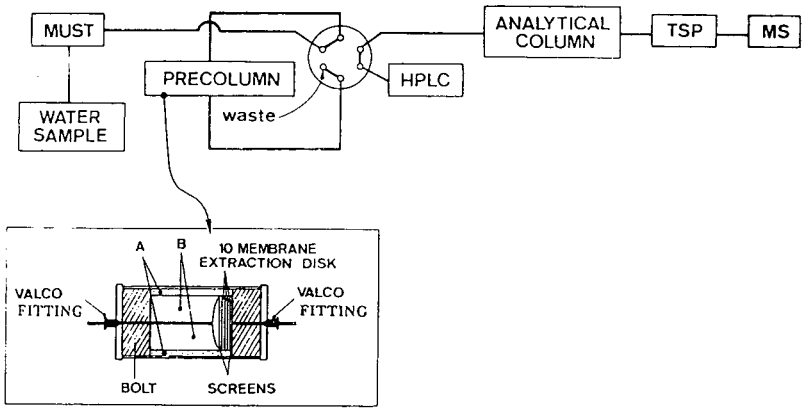

Fig. 1. General scheme of the system used for the preconcentration and determination of pesticides in water samples. A = Cylinder to adjust diameter; B = cylinder to adjust length of holder.

ponents were desorbed and separated in an analytical column. Instead of the discs, a conventional precolumn was also used and the system operated under conditions similar to those with the discs.

Two multi-residue analyses were carried out. The first involved a 250 × 4.6 mm I.D. analytical column (Interchim, Paris, France) packed with ODS-2 silica of 5-μm particle size. Preconcentration was carried out through a precolumn of 20 × 3 mm I.D. packed with PRP-1 copolymer (Brownlee Columns, Applied Biosystems, San Jose, CA, USA) of 10-μm particle size at a flow-rate of 4 ml/min. Gradient elution was accomplished in 35 min from an eluent containing 80% of solvent A (water + 0.05 *M* ammonium formate) and 20% of solvent B (acetonitrile) to 80% A–20% B at a flow-rate of 1 ml/min. The second analysis used a precolumn consisting of ten Empore C_{18} extraction discs, 4.6 mm in diameter, and preconcentrating the water at a flow-rate of 3 ml/min. The analytical column was a LiChroCART cartridge column (250 × 4.6 mm I.D.) packed with 4-μm Supersphere 60 RP-8 from Merck. Gradient elution was performed from an eluent containing 5% of solvent A [acetonitrile–methanol–water (40:40:20) + 0.0075 *M* ammonium formate)] and 95% of solvent B [acetonitrile–water (10:90) + 0.05 *M* ammonium formate–formic acid buffer (pH 3)] to 20% A–80% B (15 min), from 20% A–80% B to 30% A–70% B (20 min) and from 30% A–70% B to 55% A–45% B (20 min). The isocratic mode was used for 10 min, followed by gradient elution from 55% A–45% B to 100% of A (10 min), then the isocratic mode for 5 min and back to the initial conditions in 5 min. The post-run time was 10 min (flow-rate 0.85 ml/min).

2.3. Mass spectrometric analysis

A Hewlett-Packard (Palo Alto, CA, USA) Model 5988A thermospray LC–MS quadrupole mass spectrometer and a Hewlett-Packard Model 35741B instrument for data acquisition and processing were employed. The thermospray temperatures used varied from 90 to 80°C (stem) and from 190 to 180°C (tip) at the beginning and end of the gradient. The ion source temperature was set at 240°C. The filament-on mode was used in all experiments, with conventional positive- and negative-ion chemical ionization. Chromatograms were recorded under time-scheduled selected-ion monitoring (SIM) conditions as shown in Table 1.

2.4. Quantitative analysis

The linearity and reproducibility of the response of the on-line system used was examined. Calibration graphs were constructed by injecting estuarine water samples spiked at five different concentration levels encompassing the range of interest; they were linear over the range 0.025–1.2 μg/l (see Table 2). Sixteen compounds

Table 1
Time-scheduled SIM conditions, with preconcentration through a PRP-1 precolumn and through Empore C_{18} discs

Preconcentration	Time (min)	Compounds (m/z monitored) Positive-ion mode	
PRP-1	From 0 to 12	Deisopropylatrazine (174, 215) Deethylatrazine (188, 229)	
	From 12 to 18	Simazine (202, 243) Metoxuron (229, 270)	
	From 18 to 23	Chlortoluron (213) Isoproturon (207, 248) Atrazine (216, 257)	
	From 23 to 27	Propazine (230, 271) Terbuthylazine (230, 271)	
	From 27 to 35	Neburon (275, 316)	
		Compounds (m/z monitored)	
		Positive-ion mode	Negative-ion mode
Empore	From 0 to 12	Butocarboxim sulphoxide (207, 224) Aldicarb sulphoxide (207, 224)	
	From 12 to 15.5	Aldicarb sulphone (223, 240) Oxamyl (220, 237)	(267)
	From 15.5 to 23	Methomyl (163, 180) Deisopropylatrazine (174, 215) 3-Hydroxycarbofuranphenol	(225)
	From 23 to 35	3-Hydroxycarbofuran (238, 255) Methiocarb sulphoxide (185, 226) Deethylatrazine (188, 229) Methiocarb sulphone (218, 259)	(183, 229) (199, 245)
	From 35 to 50	3-Ketocarbofuranphenol Butocarboxim (191, 208) Aldicarb (191, 208) 3-Ketocarbofuran	(178, 223) (178, 235)
	From 50 to 59.5	Simazine (202, 243) Propoxur (210, 227) Carbofuran (222, 239) Bentazone	(239)
	From 59.5 to 72	Carbaryl (219, 260) Chlortoluron (213, 254) MCPA 1-Naphthol Atrazine (216, 257) Isoproturon (207, 248)	(199, 245) (143, 189)
	From 72 to 77	Propanil Methiocarb (226, 243) Molinate (188, 213)	(217, 262)
	From 77 to 80	Alachlor (238, 270, 287) Metolachlor (284)	

Table 2
Calibration data for selected pesticides (spiked at 0.025, 0.1, 0.4, 0.8 and 1.2 μg/l) after preconcentration of 100 ml of estuarine water

Analyte[a]	Calibration equation	R^2	R.S.D. (%)[b]	LOD (μg/l)
Aldicarb sulphone	$y = 69.2x + 2.56$	0.962	7	0.1
Methomyl	$y = 1.43x + 1.26$	0.975	10	0.05
Deisopropylatrazine	$y = 15.9x + 1.43$	0.915	9	0.02
3-Hydroxycarbofuran	$y = 8.31x + 1.67$	0.995	7	0.1
Deethylatrazine	$y = 14.1x + 1.18$	0.990	8	0.02
Butocarboxim	$y = 9.21x + 1.20$	0.977	10	0.05
Aldicarb	$y = 13.5x + 1.76$	0.922	11	0.02
Simazine	$y = 18.7x + 1.45$	0.995	8	0.01
Propuxor	$y = 24.8x + 1.62$	0.931	13	0.02
Carbofuran	$y = 152.4x + 7.51$	0.992	12	0.02
Carbaryl	$y = 192.6x + 3.12$	0.931	11	0.02
Chlortoluron	$y = 122.5x + 9.69$	0.922	13	0.01
Atrazine	$y = 23.1x + 1.32$	0.977	6	0.02
Isoproturon	$y = 27.2x + 1.58$	0.961	4	0.01
Methiocarb	$y = 143.6x - 5.68$	0.938	11	0.05
Molinate	$y = 51.8x + 1.86$	0.918	6	0.01

Calibration was performed by plotting peak area (y) *versus* amount injected on to the on-line precolumn system (x, μg/l) using positive-ion mode time-scheduled SIM.

[a] Aldicarb sulphoxide, butocarboxim sulphoxide and oxamyl could not be properly determined as their LODs were *ca.* 0.4 μg/l. Alachlor and metholachlor were not measured owing to difficult quantification (large variation in the tip temperature of the TSP interface) when working at 100% of organic modifier in the LC eluent.

[b] Relative standard deviation ($n = 5$) at 0.4 μg/l.

showed a good linearity range in the positive-ion mode and with time-scheduled SIM. Aldicarb sulphoxide, butocarboxim sulphoxide and oxamyl could not be measured owing to a poor limit of detection (LOD), close to the concentration range studied. Other compounds such as 3-hydroxycarbofuran and 3-hydroxy-7-phenolcarbofuran could not be measured owing to the lack of detection. Methiocarb sulphone and 1-naphthol showed instability in water during the preconcentration step. Alachlor and metolachlor could not be properly measured owing to their elution at 100% of organic modifier, making it difficult to keep the tip temperature of the thermospray interface stable and consequently high standard deviations were observed with no linearity in the quantification values. Other compounds showed good sensitivity only in the NI mode, such as 3-hydroxycarbofuranphenol, 3-ketocarbofuranphenol, 3-ketocarbofuran, bentazone, MCPA and propanil.

The dynamic range provided by TSP was consistent with reported values whether the PI (carbamate pesticides) [5] or the NI mode (chlorinated phenoxy acid herbicides) [4] was used. However, none of these previous studies used an on-line preconcentration system as described here.

The LODs in Table 2 were calculated by using a signal-to-noise ratio of 3–4 and assuming that 1 cm was the minimum peak height that could be measured with reasonable confidence. This corresponds, in the Hewlett-Packard TSP mass spectrum, to *ca.* 500 arbitrary units (the noise level is *ca.* 150 units). Table 2 gives the values for the compounds measured using the PI mode. The LODs using the NI mode varied from 0.02 to 0.4 μg/l depending on the compound studied,

the worst values being obtained for the TPs of carbofuran and methiocarb.

3. Results and discussion

3.1. Mass spectral information

As ion formation and fragmentation patterns in thermospray LC–MS may be strongly influenced by various operational parameters (*e.g.*, the nature and composition of the mobile phase, the source and vaporization temperature), each group of pesticides should be studied in detail prior to tackling trace-level determinations in surface waters. The major ions and their relative abundances obtained by TSP-MS in the PI and NI modes for a selection of eight analytes are given in Table 3. Because complete separation of all the pesticides studied entailed the use of gradient elution (see Fig. 2), the major ions for each compound were obtained at three different eluent compositions, depending on the retention time in the liquid chromatogram.

Each compound gave two main ions corresponding to $[M + H]^+$ and $[M + NH_4]^+$ in the PI mode. Ammonium formate was chosen as an eluent additive instead of ammonium acetate because it enhanced the chromatographic resolution at pH 3 and also adduct formation as a result of its slightly higher gas-phase acidity [6,13]. However, the sensitivity was similar in both instances and $[M + NH_4]^+$ appeared to be the base peak for the whole group of carbamate pesticides except carbofuran, which had $[M + H]^+$ as its base peak. This behaviour meets the expectations for carbamates [5,7,14,15] except for carbofuran, the ions of which did not match previous findings published by our group showing $[M + H]^+$ as the base peak [7]. This difference in the relative abundances of the ions may be ascribed to the presence of formic acid in the mobile phase; hence, depending on the compounds and experimental conditions used, TSP is a solution-dependent ionization technique, being affected by the pH of the mobile phase. Chlorotriazine and phenylurea pesticides usually generate $[M + H]^+$ as their base peak, thus meeting the expectations for compounds that exhibit a higher proton affinity that does ammonia [9,16–18]. Fragmentation was only observed in a few isolated instances. Methiocarb sulphoxide and methiocarb sulphone showed a loss corresponding to CH_3NCO, and the herbicide alachlor exhibited a fragment at m/z 238 resulting from the loss of the CH_3OH moiety [1].

The NI mode was also assessed as it generally involves more fragmentation processes, so it provides complementary structural information to that offered by the PI mode; however, few compounds provide adequate sensitivity. Formate adducts (propanil, MCPA, aldicarb sulphone, 3-hydroxy-7-phenolcarbofuran) and/or deprotonated molecular ions (bentazone, 1-naphthol) were observed as base peaks. Electron capture was observed to occur for 3-ketocarbofuranphenol and 3-ketocarbofuran, leading to $[M]^{-\cdot}$ as the base peak; on the other hand, the N-methylcarbamates methiocarb sulphoxide, methiocarb sulphone and 3-ketocarbofuran showed fragmentation, with the loss of a CH_3NCO moiety. The relative abundances of the ions from the four metabolites of carbofuran were low owing to thermal decomposition in the probe. When electrospray (EPS)-MS experiments were performed they exhibited $[M + Na]^+$ and $[2M + Na]^+$ as their base peak and main adduct ion, respectively [19]. Monitoring them by ESP may be easier, but our current ESP system is not yet operational with the proposed multi-residue method.

3.2. Water analysis

Efficient monitoring of surface water samples requires LODs close to 0.1 μg/l, which is the maximum allowable concentration established by the European Community in its Directive on the Quality of Water Intended for Human Consumption [20]. LC–TSP-MS provides LODs of 10 μg/l (0.2 ng if a 20-μl loop is used) by SIM, so obtaining a three orders of magnitude lower LOD entails the use of a preconcentration step. The advantages of SPE over conventional LLE are well documented [21]; also, the use of SPE coupled on-line to LC with UV and diode-array

Table 3
Main ions and their relative abundances (%) for eight compounds using TSP-MS in the PI and NI modes and filament-on conditions

Peak No.	M_r	Compound and ions	Eluent	PI mode	NI mode
7	180	3-Hydroxycarbofuranphenol	1	n.d.[a]	
	225	$[M + HCOO]^-$			100
8	237	3-Hydroxycarbofuran	1		n.d.
	238	$[M + H]^+$		12	
	255	$[M + NH_4]^+$		100	
9	241	Methiocarb sulphoxide	1		
	185	$[M + H - CH_3NCO]^+$		100	
	226	$[M - CH_3NCO + H + CH_3CN]^+$		55	
	183	$[M - H - CH_3NCO]^-$			20
	229	$[M - CH_3NCO + HCOO]^-$			100
11	257	Methiocarb sulphone	1		
	218	$[M - CH_3NCO + NH_4]^+$		100	
	259	$[M - CH_3NCO + NH_4 + CH_3CN]^+$		20	
	199	$[M - H - CH_3NCO]^-$			45
	245	$[M - CH_3NCO + HCOO]^-$			100
12	178	3-Ketocarbofuranphenol	2	n.d.	
	178	$[M]^{-\cdot}$			100
	233	$[M + HCOO]^-$			5
15	235	3-Ketocarbofuran	2	n.d.	
	178	$[M - CH_3NCO]^{-\cdot}$			60
	235	$[M]^{-\cdot}$			100
18	221	Carbofuran	2		n.d.
	222	$[M + H]^+$		100	
	239	$[M + NH_4]^+$		40	
29	269	Alachlor	3		n.d.
	238	$[M + H - CH_3OH]^+$		50	
	270	$[M + H]^+$		100	
	287	$[M + NH_4]^+$		50	

The relative abundances indicated were obtained on injecting 100 ng of each individual compound (after preconcentrating 100 ml of a solution of 1 μg/l of each analyte) and using the following eluent compositions: (1) water–acetonitrile–methanol (80:10:10) with ammonium formate–formic acid buffer (pH 3); (2) water–acetonitrile–methanol (50:25:25) with ammonium formate–formic acid buffer (pH 3); (3) water–acetonitrile–methanol (20:40:40) with ammonium formate–formic acid buffer (pH 3).
[a] Not detected.

detection has been reported [22–24]. Recently, Empore C_{18} discs were used with similar detection systems to accomplish the highly efficient trace enrichment of both polar and non-polar analytes from surface water samples [10,11,25]. In addition to C_{18} SPE, PRP-1 copolymer has been used as a sorbent for the preconcentration of relatively polar pesticides [12].

Both SPE sorbents, Empore C_{18} discs and PRP-1, were employed in a multi-residue approach to the determination of 34 pesticides and various transformation products in water samples. In order to compare both sorbents, the average recoveries for four compounds are given in the Table 4; they range from 20% for compounds with low breakthrough volumes (*e.g.*, deisopropylatrazine) to 92% for compounds with high breakthrough volumes (*e.g.*, atrazine).

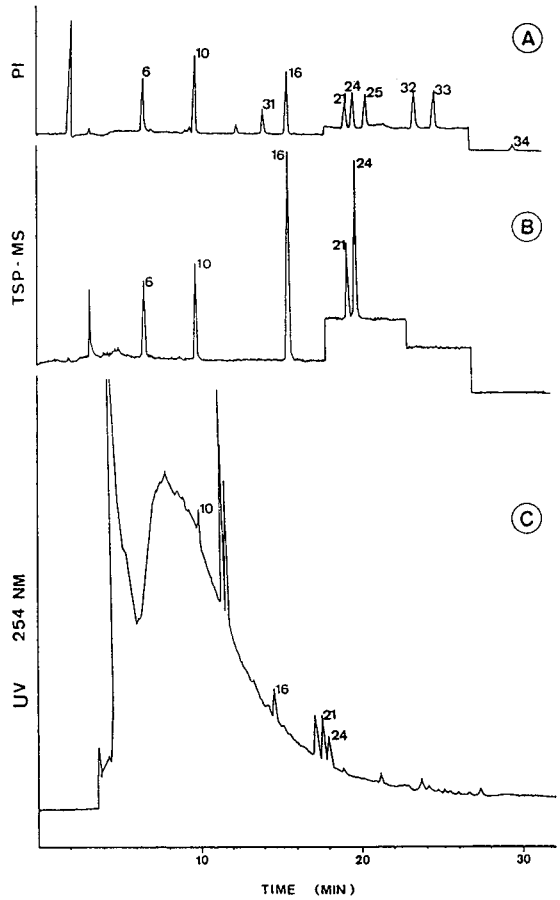

Fig. 2. (A) On-line SPE using PRP-1 sorbent followed by LC–TSP-MS of a river water sample spiked with 0.2 μg/l each of (6) deisopropylatrazine, (10) deethylatrazine, (31) metoxuron, (16) simazine (21) chlortoluron, (24) atrazine, (25) isoproturon, (32) propazine, (33) terbuthylazine and (34) neburon obtained with a preconcentration volume of 50 ml in the PI mode under SIM conditions (for details, see Table 1). The analytical LC column (250 × 4.6 mm I.D.) was packed with ODS-2 silica. Gradient elution programme: from 80% of A (water–0.05 *M* ammonium formate) and 20% of B (acetonitrile) to 80% of A–20% of B in 35 min and back to the initial conditions 10 min post-run. Flow-rate: 1 ml/min. (B) On-line SPE using PRP-1 sorbent followed by LC–TSP-MS of 50 ml of non-spiked River Dropt water obtained in the PI mode under SIM conditions (for details, see Table 1). The PI mode confirmed (6) deisopropylatrazine, (10) deethylatrazine, (16) simazine, (21) chlortoluron and (24) atrazine. Other experimental conditions as in (A). (C) On-line SPE using PRP-1 sorbent with LC–DAD at 254 nm of 200 ml of the same River Dropt water as in (B). Other experimental conditions as in (A) and (B) except that eluent B contained potassium acetate–0.1 *M* acetic acid (pH 4.7) instead of 0.05 *M* ammonium formate.

These two on-line methods usually require the use of 50–100 or 150–200 ml of water as the preconcentration volume when MS or UV detection, respectively, is employed [10,11,24]. Using a lower preconcentration water volume an additional advantage is gained as the analysis time is decreased (*ca.*, 30 min) and recoveries are improved. Typical results for the analysis of 50 ml of spiked (0.2 μg/l) water from the River Dropt (France) and 100 ml of spiked (0.3 μg/l) water from the River Ebro (Spain) are shown in Figs. 2A and 3A, respectively. PRP-1 (Fig. 2A) and Empore C_{18} discs (Fig. 3A) were used as on-line solid-phase sorbents, followed by LC–TSP-MS in the PI mode under time-scheduled SIM conditions. By using this detection mode, the sensitivity was increased by one order of magnitude relative to the traditional SIM detection mode. The two m/z values selected for each compound are given in table 1. Only the base peak was selected for monitoring at trace-level concentrations when the relative abundance of the second ion was very low (5–10%). The time-scheduled SIM monitoring is shown in Table 1. Fig. 3B shows an on-line LC–TSP-MS trace obtained in the NI mode under time-scheduled SIM conditions by preconcentrating 150 ml of spiked (0.3 μg/l) water from the River Ebro. Despite the well known lower sensitivity of the NI relative to the PI mode for most of the pesticides studied [2,7], the LODs for pesticides possessing an electrophilic moiety (acidic and phenolic compounds) were lower in the NI than the PI mode, consistent with previous results [4,6]. Two of the pesticide TPs studied, methiocarb sulphone and 1-naphthol, could not be determined by this method owing to instability in water during the preconcentration step and to the latter not being trapped on the C_{18} sorbent at an acidic pH (pH 3) [11]. Monitoring 3-hydroxycarbofuran and 3-hydroxy-7-phenolcarbofuran was impossible because of the lack of sensitivity of MS detection, which was ascribed to the thermal decomposition of the analytes in the TSP probe during analysis.

Fig. 2B and C illustrate the analysis of a non-spiked real river water sample using on-line SPE and PRP-1 copolymer followed by MS

Table 4
Breakthrough volumes (V_B) and average recovery of pesticides using on-line SPE with ten Empore C_{18} discs or a PRP-1 precolumn

Compounds	Empore C_{18} discs			PRP-1 precolumn		
	V_B (ml)[a]	Recovery (%)[b]		V_B (ml)[a]	Recovery (%)[b]	
		A	B		A	B
Deisopropylatrazine	8	17	60	14	22	65
Deethylatrazine	70	55	90	64	51	90
Simazine	>150	87	95	>160	90	94
Atrazine	>150	92	97	>320	92	100

[a] Breakthrough volumes were calculated as described in refs. 10 and 12.
[b] Average recoveries were calculated by preconcentrating (A) 150 or (B) 50 ml of water at 2 ml/min with a spiking level of 0.3 μg/l.

detection in the PI mode under SIM conditions and or diode-array detection (DAD) at 254 nm, respectively. In the LC–DAD spectrum, the matrix peak appears at the beginning of the chromatogram (20–30 min) and can vary according to the water type and gradient elution performed. Low recoveries were obtained for the first few compounds eluted (deethylatrazine, deisopropylatrazine) when 150–200 ml of water were used, and are attributed to fulvic and humic substances present in the river water. In Fig. 2B it can be observed that the use of MS detection under SIM conditions removed all kinds of interfering peaks and permitted the determination of deisopropylatrazine in real water samples with a volume of only 50 ml, giving a better recovery than using 150 ml of preconcentrated water (see Table 4).

Although all the dirty water samples were injected directly into the source of the MS apparatus, no decrease in sensitivity was observed during our analyses.

The LODs obtained with the method are given in Table 2. We noted an increase in the sensitivity for all the compounds studied with respect to LC–DAD, ranging from a factor of 200 for aldicarb sulphoxide to a factor of 10 for atrazine when comparing the results at the same preconcentration volume (150 ml). The performances of the two types of preconcentration procedures were similar for the common compounds studied, as illustrated in Table 4; however the recoveries were better when using smaller volumes as a result of the lower breakthrough volumes of the chlorotriazine transformation products. It should be noted that the Empore C_{18} discs were replaced after five analyses in order to avoid band broadening; the PRP-1 precolumn can be used much longer (*ca.* twenty analyses).

3.3. Confirmation of environmental levels

Our groups are currently working on the trace-level determination of pesticides in water samples from three different areas. The River Dropt area (south-western France) is mainly used for cereals and triazine and phenylurea herbicides are applied extensively. The Ebro delta (Tarragona, Spain) is a typical rice cultivation area, where propanil, molinate and bentazone are used [26]. The third region is Almeria (southern Spain), where carbamate insecticides are used for green and fruit crops. Fig. 2B shows a chromatogram obtained in the PI mode under SIM conditions following preconcentration of 50 ml of water from the River Dropt which allows the unequivocal determination of deisopropylatrazine, deethylatrazine, simazine, atrazine and chlortoluron at 0.2, 0.2, 0.3, 0.3 and 0.25 μg/l, respectively. Fig. 4A shows an LC–TSP-MS trace obtained in the PI mode under SIM con-

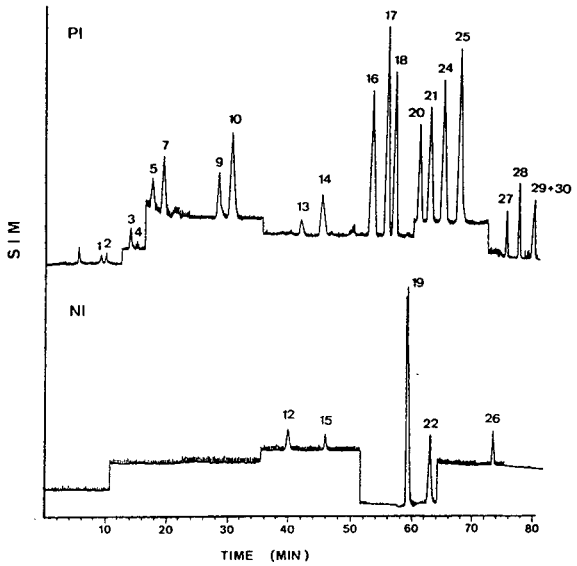

Fig. 3. On-line SPE with C_{18} Empore discs followed by LC–TSP-MS of a River Ebro water sample spiked with 0.3 μg/l each of (1) butocarboxim sulphoxide, (2) aldicarb sulphoxide, (3) aldicarb sulphone, (4) oxamyl, (5) methoxyl, (6) 3-hydroxy-7-phenolcarbofuran, (7) deisopropylatrazine, (8) 3-hydroxycarbofuran, (9) methiocarb sulphoxide, (10) deethylatrazine, (11) methiocarb sulphone, (12) 3-ketocarbofuran, (13) butocarboxim, (14) aldicarb, (15) 3-ketocarbofuran, (16) simazine, (17) baygon, (18) carbofuran, (19) bentazone, (20) carbaryl, (21) chlortoluron, (22) MCPA, (23) 1-naphthol, (24) atrazine, (25) isoproturon, (26) propanil, (27) methiocarb, (28) molinate and (29 + 30) alachlor + metolachlor, obtained after preconcentrating 100 and 150 ml of sample in the PI and NI modes, respectively, and under SIM conditions (for time-scheduled SIM conditions, see Table 1). Precolumn packed with ten Empore C_{18} extraction discs and LiChroCART cartridge column (25 cm × 4.6 mm I.D.) packed with 4-μm Supersphere 60 RP-8. Gradient elution programme: from 5% of A [acetonitrile–methanol–water + 0.075 *M* amonium formate (40:40:20)] and 95% of B [acetonitrile–0.05 *M* ammonium formate–formic acid buffer (pH 3) (10:90)] to 20% A–80% B in 15 min, from 20% A–80% B to 30% A–70% B in 20 min, from 30% A–70%–B to 55% A–45% B in 20 min, isocratic for 10 min, from 55% A–45% B to 100% A in 10 min, isocratic for 10 min and back to initial conditions 10 min post-run. Flow-rate: 0.85 ml/min.

ditions following preconcentration of 100 ml of water from Almeria where simazine, carbofuran and methiocarb were detected at 0.03, 0.02 and 0.05 μg/l, respectively. Fig. 4B shows an LC–TSP-MS trace of Ebro river water obtained

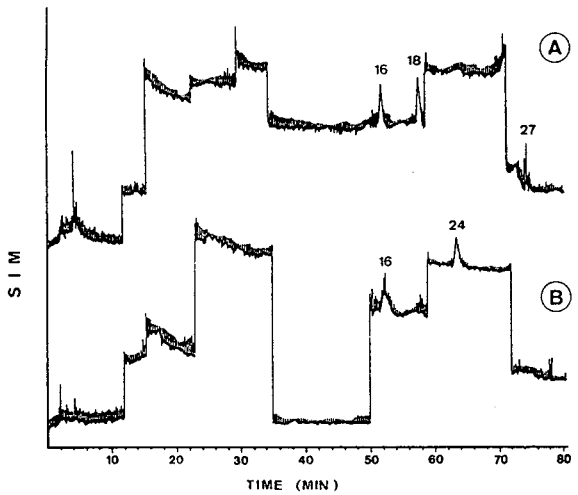

Fig. 4. (A) On-line SPE using Empore C_{18} extraction discs followed by LC–TSP-MS in the PI mode under time-scheduled SIM conditions (see Table 1) of 100 ml of Almeria ground-water. Compounds present were (16) simazine, (18) carbofuran and (27) methiocarb at concentrations of 0.03, 0.02 and 0.05 μg/l, respectively. For other experimental conditions, see Experimental. (B) On-line SPE using Empore C_{18} extraction discs followed by LC–TSP-MS in the PI mode under time-scheduled SIM conditions (see Table 1) of 100 ml of River Ebro water samples. Compounds detected at 0.01 μg/l were (16) simazine and (24) atrazine.

under the same conditions. The presence of simazine and atrazine at 0.01 μg/l was confirmed. The herbicide concentrations in the last sample are ten times lower than those usually expected [26] because the samples were collected during the winter, when herbicides are used sparingly.

4. Conclusions

The proposed on-line SPE–LC–TSP-MS method uses preconcentration of only 50–100-ml volumes for the determination of pesticides in water samples at concentrations of 0.02–0.4 μg/l. It is worth emphasizing that early-eluting compounds (*e.g.*, deisopropylatrazine and deethylatrazine), which usually cannot be determined by DAD with the same on-line system owing to low breakthrough volumes and/or interferences from humic substances in the water matrix, were

unequivocally identified by on-line SPE LC–TSP-MS using time-scheduled SIM. A good linearity range was obtained for sixteen compounds on preconcentrating 100 ml of estuarine water samples spiked at 0.025–1.2 μg/l. When determination was performed at the 0.4 μg/l level, the relative standard deviation for the different pesticides varied from 4 to 13%, which is acceptable for the on-line SPE–LC–TSP-MS technique used. A better detection limit was also obtained with on-line SPE–LC–TSP-MS than with DAD as only 50–100 ml of water were required compared with 150–200 ml in order to obtain a similar LOD.

PRP-1 and Empore C_{18} discs provide similar results for the breakthrough volumes of various triazine herbicides. The proposed method permits the unequivocal determination of deisopropylatrazine and deethylatrazine at concentrations as low as 0.15 and 0.2 μg/l, respectively, in river water samples, which was impossible with our current monitoring system based on on-line coupled SPE–LC–DAD. This is particularly important for deisopropylatrazine, which is a ubiquotus water pollutant and frequently escapes detection owing to interferences and/or extraction problems. Removal of fulvic and humic interferents is the greatest advantage of this technique over DAD.

5. Acknowledgements

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JOURNAL OF CHROMATOGRAPHY A

Supercritical fluid extraction of organochlorine pesticides from an aqueous matrix

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Abstract

Supercritical fluid extraction (SFE) conditions were optimised for the removal of organochlorine pesticides (OCPs) from water. OCPs were collected and extracted from solid-phase extraction disks (Empore) and also directly from a water sample using a modified extraction cell. High recoveries (>90%) were obtained for two of the three OCPs with Empore disks. Despite the good solubility of OCPs in pure CO_2, the analyte recoveries decreased when they were extracted directly from water. Three different flow-rates were used in the direct SFE with no apparent change in recovery, indicating that extraction was diffusion-controlled. The effect of increasing the ionic strength of the aqueous sample on analyte recovery was investigated.

1. Introduction

There has been increasing interest in supercritical fluid extraction (SFE) as a sample preparation technique in analytical chemistry. SFE has increasingly replaced more conventional extraction methods, such as Soxhlet and solvent extraction with organic solvents [1–3]. Supercritical fluids possess physical properties that are intermediate between those of liquids and gases. These unique properties lead to increased diffusion rates and low viscosity which ensure rapid extraction of analytes is possible. Solvent strength is related to supercritical fluid density which can be altered by changing the pressure and temperature of the fluid. This enables selective extractions to be performed [4].

Uncertainty about the long-term environmental effects of organochlorine pesticides (OCPs) has led to voluntary or compulsory control of their use in most countries [5]. However, because of their highly persistent nature and known mammalian toxicity residue analysis is still continued. All of the OCPs studied (lindane, dieldrin and aldrin) are found on the Department of the Environment's "Red-List" [6] of dangerous substances in water.

The majority of literature published on the supercritical fluid extraction of pesticides is concerned with removal from solid matrices with little or no water content [7–9]. There are several problems associated with extraction of analytes from aqueous solution. For direct extraction, the nature of the sample necessitates the use of an extraction cell different in design to the conventional "flow-through" type to retain

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the sample in the cell. However the main problem is the relatively high solubility of water in supercritical carbon dioxide, approximately 0.3% [10]. This can cause restrictor plugging by ice during the supercritical fluid adiabatic expansion and carry over of water into the collection solvent and ultimately into the chromatographic detection system.

Initial studies on SFE from water matrices used "closed loop stripping", where the supercritical fluid was recirculated by a pump back into the water sample. After equilibration a sample is taken via an injection loop and analysed by supercritical fluid chromatography (SFC) [11,12]. Another method incorporates a sandwich type phase separator to remove the aqueous phase from the supercritical carbon dioxide before analysis by on-line SFC [13].

The recent introduction of solid-phase extraction (SPE) disks (Empore) has led to direct extraction of trace organics from aqueous samples. SPE allows more rapid extraction than is possible using conventional methods. The disks comprise a PTFE membrane impregnated with C_{18} bonded silica. They are used in a conventional filtration apparatus where the sample is filtered under vacuum [14]. The large surface area of the disk ensures the high flow-rates used do not cause break-through of sample. Preconditioning of the disk prior to use is necessary to activate the sorbent sites [15]. Since the eluting solvent is carbon dioxide only methanol and water are used for preconditioning. The disks were used to isolate the OCPs from the water matrix prior to SFE. After filtration of sample, the disks are dried, loosely rolled and placed in a conventional 10-ml extraction cell for SFE with carbon dioxide.

The cell used for direct extraction of OCPs from the aqueous phase is shown in Fig. 1 [16]. The cell is made from stainless steel and has an internal volume of 50 cm^3. The inlet tube has a conventional HPLC solvent filter attached to it to aid mixing of the sample with the supercritical carbon dioxide. The 'head-space' configuration of the cell allows the supercritical fluid to pass through the aqueous sample before exiting via an outlet frit into the restrictor to be collected.

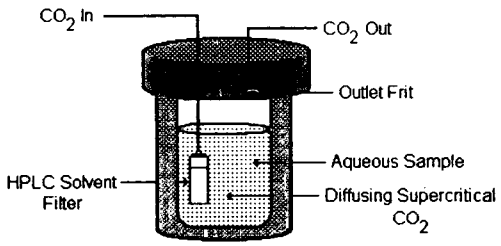

Fig. 1. Schematic diagram of the "headspace" extraction cell.

1.1. Mechanism of pesticide extraction from water

The hydrides of small non-metallic elements are gaseous at room temperature. Water is the sole exception and its existence in condensed phases is due to the strength of the O-H···O hydrogen bonds and to the fact that each water molecule can form four such bonds acting as both a hydrogen donor and receptor. As a consequence, the structure adopted by ice is a tetrahedral one with unfilled space left within the crystal. Pure water has a similar tetrahedral arrangement [17].

Hydrophobic substances are defined as those that are readily soluble in many non-polar solvents, but only sparingly soluble in water. In fact the attraction of non-polar groups for each other plays only a minor role in the hydrophobic effect. The hydrophobic effect primarily arises from the strong attractive forces between water molecules, which being isotropically arranged, must be disrupted or distorted when any solute is dissolved in water. If the molecule is ionic or contains polar groups, it can form strong bonds to water molecules, which more than compensate for the disruption or distortion of the bonds existing in pure water; and ionic or polar substances will tend to be soluble in water. No such compensation occurs with non-polar groups and their solution in water is accordingly resisted.

When a hydrophobic pesticide is introduced in the water matrix there is a disruption in the ordering of the structure. The removal of the molecule is entropically favoured and it will be

partitioned at the supercritical CO_2–water interface. Extraction from a solid matrix tends to be initially equilibrium controlled (due to interaction of the supercritical fluid with the analyte on the surface of the matrix) followed by diffusion (kinetic controlled) through the matrix as the extraction proceeds. However, extraction from water differs from this since water is sparingly soluble in supercritical CO_2. This implies that extraction will only be kinetically controlled as the supercritical CO_2 diffuses through the aqueous matrix.

Addition of salt to the water sample causes an increase in the ionic strength of the solution which in turn increases the solvent–solvent interactions. The relative hydrophobicity of the pesticides are enhanced and the molecules tend to aggregate together to reduce the disruption to the water structure. These larger pesticides structures can then be more easily removed from the aqueous matrix by the diffusing supercritical CO_2. Once the concentration of pesticide falls below the "aggregation point" the effect of ionic strength on the recovery will be reduced. The addition of salt to the aqueous sample should allow pesticide molecules to be more efficiently removed in the earlier stages of extraction until the "aggregation point" is reached. No additional effect on recovery will be noted once the pesticide concentration is below the "aggregation point".

2. Experimental

2.1. Reagents

Lindane, aldrin, dieldrin (purities 99.5%) and hexabromobenzene (99.2%) were obtained from Promochem (St. Albans, UK). Methanol, hexane (HPLC grade) and acetone (AnalaR) were obtained from various sources. Carbon dioxide (SFC grade) was supplied from Air Products (Sunderland, UK). Celite, 60–80 mesh, (used as an inert matrix for initial optimisation experiments) was purchased from BDH (Merck, Poole, UK). Solid-phase extraction disks (Empore) and Bond Elut extraction cartridges were both obtained from Phase Separations (Clwyd, UK).

2.2. Apparatus

The optimisation and SPE were performed on a Carlo Erba SFE 30 system (Carlo Erba, Milan, Italy) using a 150-ml syringe pump (SFC 300, Fig. 2). All SFE was performed in off-line mode through a heated metal restrictor maintained at 150°C with the analytes being collected in a suitable solvent (hexane). Static and dynamic extraction were achieved by means of an air actuated pneumatic valve. All extractions were performed at constant pressure (between 15 and 45 MPa) and temperature (between 40 and 150°C). Optimisation experiments were carried out using a 1.67-ml Keystone extraction cell (Mettler-Toledo, Halstead, Essex, UK). The size of the solid-phase extraction disks necessitated the use of a larger extraction cell. A 10-ml cell supplied by Jasco (Mettler-Toledo) was used for these extractions.

Due to the large size (50 ml) of the 'headspace' extraction cell used for direct analysis of water samples a Jasco SFE system was used. The Jasco SFE system has been described in detail elsewhere [16] The collection system was modified to ensure effective trapping of the pesticides which could be lost due to aerosol formation during the violent depressurisation of the CO_2. The modified system uses a 25 cm^3 glass vial, containing 6–7 ml of solvent. A PTFE-coated rubber septum cap is pierced by the 1/16 in. (1 in. = 2.54 cm) stainless-steel tubing from the

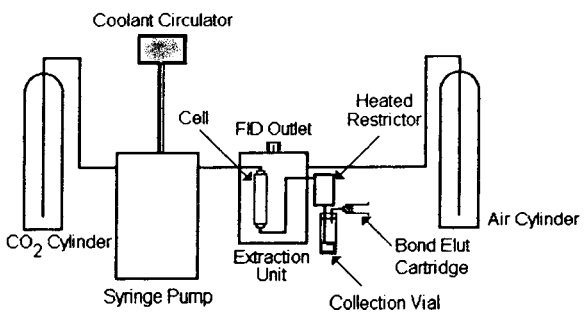

Fig. 2. Schematic diagram of the Carlo Erba SFE apparatus.

back-pressure regulator (BPR) and by a syringe needle to which a Bond Elut C_{18} cartridge is attached. The needle allows the depressurized CO_2 to escape while the cartridge traps any analyte that may be carried with the aerosol and can be subsequently back flushed with a small amount of collection solvent into the vial.

Analysis of the extracts was by a Perkin Elmer 8420 gas chromatograph (Buckinghamshire, UK) with electron-capture detection (ECD) and split injection (50:1 ratio). A 0.5-μl volume was injected onto a 12 m × 0.25 mm I.D. BP-5 fused-silica column (SGE, Ringwood, Australia). The oven was maintained at 240°C throughout the analysis with the injector and detector temperatures 250 and 350°C, respectively. Nitrogen was used as both carrier and make-up gas. Hexabromobenzene was used as an internal standard. A typical chromatogram is shown in Fig. 3.

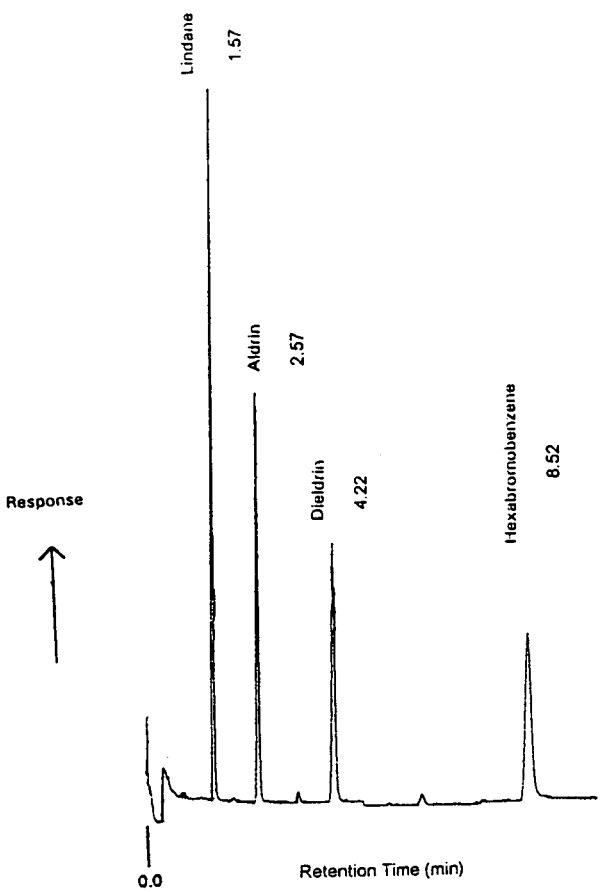

Fig. 3. A typical GC–ECD chromatogram.

2.3. Procedure

Optimisation

Pressure and temperature variables were initially optimised by extracting the OCPs from an inert matrix (Celite). A known amount of pesticide was spiked onto 0.2 g of Celite and the solvent allowed to evaporate. A simple experimental design approach was used to combine various pressure/temperature combinations. Extraction time was not used as a variable because the fixed restrictor on the Carlo Erba SFE dictates that the fluid flow-rate is not constant. The amount of CO_2 passing through the extraction cell was kept constant and therefore extraction time varied during each experiment. The optimum conditions for extracting the three OCPs from Celite were found to be 300 atm (1 atm = $1.01 \cdot 10^5$ Pa) and 50°C (density 0.85 g ml^{-1}).

Solid-phase extraction

These conditions were then used to extract the OCPs that had been trapped on a solid-phase extraction disk. A sample consisting of 200 ml of distilled water to which 10 μg of each OCP had been added was used for the extractions. The sample was then pre-treated prior to filtration by adding 5 ml of methanol and adjusting the pH to 2 with concentrated hydrochloric acid [15].

The disks were first pre-treated by activating with 10 ml of methanol for 3 min and then passing through air for 1 min. A further 5 ml of methanol was added and allowed to soak for another 3 min followed by 10 ml of distilled water and the sample which was filtered in approximately 5 min (care must be taken not to allow the disk to become dry during this state). The disk was then air dried for 10 min, placed in an oven (45°C, 20 min) for further drying and then rolled and placed in the 10-ml extraction cell. A 30-min static extraction was then carried out under the optimum conditions followed by a

dynamic extraction where two lots of 30 ml of CO_2 were passed through the cell. The extracts were then combined and analysed as before.

Direct extraction from water

Direct analysis of a water sample containing OCPs was evaluated using the "headspace" extraction cell (Fig. 1). The extraction conditions used in the SFE of the solid-phase extraction disks were used to extract lindane, aldrin and dieldrin (10 μg each in acetone) from 45 ml of distilled water. The effect of flow-rate on recovery was investigated by extracting at 0.7, 1.0 and 1.5 ml min^{-1}. Extraction times were varied from 15 min to 2 h and the extracts analysed by GC–ECD.

The effect of adding sodium chloride (8 g) to the sample (45 ml) prior to extraction was investigated. Salt is commonly used in solvent extraction [18] as it increases the ionic strength of the solution. This increases the relative hydrophobicity of the non-polar OCPs and therefore will aid their removal from the matrix. The amount of salt used in the experiment corresponds to the ratio used in the EPA method [18].

3. Results and discussion

In the initial stages of developing a SFE method the supercritical fluid composition, pressure and temperature must all be considered. This can be achieved by extracting the analytes from an inert matrix such as Celite to prevent matrix effects. This can also be useful in determining the trapping efficiency of the collection device. Preliminary studies on such spiking experiments indicated that the conventional collection devices on both the Jasco and Carlo Erba instruments were unsatisfactory at trapping the extracted analytes. This led to the development of the modified collection vessel now used incorporating a Bond Elut cartridge (Fig. 2).

3.1. Optimisation of SFE conditions for OCPs

The optimum conditions for extraction of OCPs by pure CO_2 were determined by dynamically extracting from Celite at various pressure/temperature combinations using a simple factorial design. It must be noted that the extraction conditions may have to be altered when the OCPs are extracted from a real matrix.

3.2. SPE–SFE of aqueous samples

The optimum conditions for SFE of OCPs from Celite were found to be a dynamic extraction at 300 atm and 50°C. These conditions were used in extracting OCPs which had been previously trapped on a SPE disk. However, a 30-min static extraction, at the same pressure and temperature, was performed prior to the dynamic extraction to successfully remove all of the trapped OCPs from the disks. The recoveries of the OCPs are shown in Table 1.

It is seen that quantitative recoveries are possible for aldrin and dieldrin using a combined SPE–SFE method. These results are comparable to extraction of OCPs from spiked sand [8]. The recoveries for lindane are lower than expected although it is not possible to determine whether this is due to poor retention on the SPE disk or to the actual SFE of the disks. The R.S.D. values on four extractions are high; however this

Table 1
Combined solid-phase extraction–supercritical fluid extraction of organochlorine pesticides from an aqueous sample

Extraction number	% Recovery (10 μg)		
	Lindane	Aldrin	Dieldrin
1	65.3	103.0	80.0
2	78.8	89.9	96.6
3	88.5	96.8	99.6
4	75.5	104.4	90.2
Average	77.0	98.5	91.6
% R.S.D.	12.4	6.7	9.5

is an accumulated error generated over three separate stages *i.e.* SPE, SFE and GC.

3.3. Direct analysis of aqueous samples

SFE of OCPs from an aqueous sample was carried out directly, at different flow-rates of supercritical CO_2 using the "headspace" extraction cell (Fig. 1). The same conditions were used as in the combined SPE–SFE experiment. The effect of increasing the ionic strength of the solution by adding sodium chloride to the sample was also investigated. The percentage recoveries of the extractions at various extraction times were determined. The results are shown in Figs. 4 and 5. As expected the recovery increases with increased extraction time as more CO_2 is allowed to pass through the cell. However it would not be practical to extend extraction time beyond 2h because of the amount of water carry over observed at long extraction times. The number of cell volumes swept for the three flow-rates studied (0.7, 1.0, 1.5 ml min^{-1}) and at a typical extraction time of 60 min are 0.9, 1.3 and 1.9, respectively. It can be seen from the graphs that within experimental error (see below) flow-rate has little effect on the recoveries of OCPs. Although the cell volumes swept is more than double, at flow-rates between 0.7 and 1.5 ml min^{-1}, the recoveries do not show a marked increase. The curves obtained increase rapidly and then gradually plateau indicating that the extraction is kinetically controlled [19]. Diffusion from the aqueous matrix or slow desorption kinetics limiting the rapid extraction of analytes.

Eventhough the effect of "salting out" is well known and is frequently used to assist extraction [18] the results reported in Fig. 5 indicate that salt has no significant effect on the recovery of the analytes. This may be due to the much increased water carry over observed when salt is added to the extraction cell (even at short extraction times) causing problems in detecting the OCPs by GC–ECD. It therefore appears impractical to use salt to assist the extraction of hydrophobic molecules by supercritical CO_2.

Fig. 6 shows the percentage recoveries for all the OCPs with respect to flow-rate and salt addition and the variation in data obtained. It is concluded that all the results reported fall within an extraction "envelope" and that this represents the actual limits of the method to extract OCPs directly from water. The deviation in results observed at longer extraction times may be due to the increased amount of water carry over observed which becomes more noticeable at higher flow-rates (1.5 ml min^{-1}). This observation combined with a removal of the majority of collection solvent by the violent depressurisation of CO_2 from the BPR may cause an increased error in the overall analytical procedure.

A repeatability study ($n = 5$) was undertaken on a 15-min extraction at 300 atm, 50°C and 1 ml min^{-1}. The % R.S.D. for lindane, aldrin and dieldrin was found to be 6.7, 7.3 and 8.2%, respectively. These recoveries compare favourably with those reported for the combined SPE–SFE method.

The overall recoveries obtained for direct extraction from water are in the order of 20% lower than the extractions involving prior trapping of the analytes onto C_{18} solid-phase extraction disks using the combined SPE–SFE method. The lower recoveries of OCPs obtained by direct extraction may be indicative of poor diffusion of the supercritical CO_2 through the aqueous matrix. However, the inclusion of a modified cell with HPLC solvent filter to increase the diffusion of the supercritical CO_2 should allow extraction from within the bulk aqueous sample and not just the CO_2–water interface [16]. The lower recoveries of OCPs obtained may however be acceptable in qualitative analysis particularly as no preconcentration step is required.

4. Conclusions

The extraction of OCPs from an aqueous matrix has been achieved by two different methods. The techniques are simple to undertake and are faster than conventional solvent extraction. Solid-phase extraction disks are shown to be efficient at trapping OCPs and give quantitative results with supercritical CO_2. This leads to the

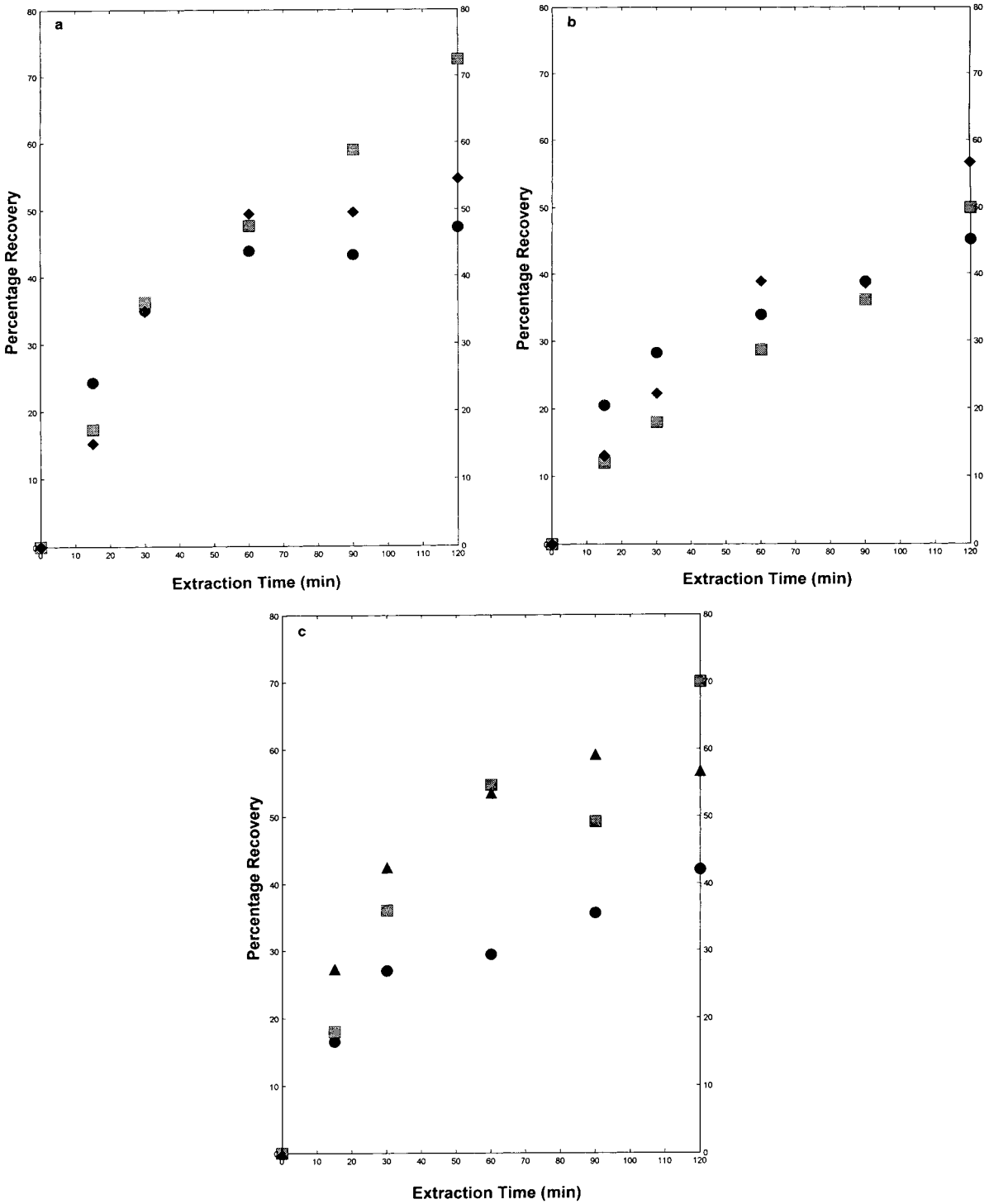

Fig. 4. Percentage recovery *versus* extraction time. (a) Lindane, (b) aldrin, (c) dieldrin. Flow-rates: ● = 0.7; ■ = 1.0; ◆ (a and b) = 1.5, ▲ (c) = 1.5 ml/min.

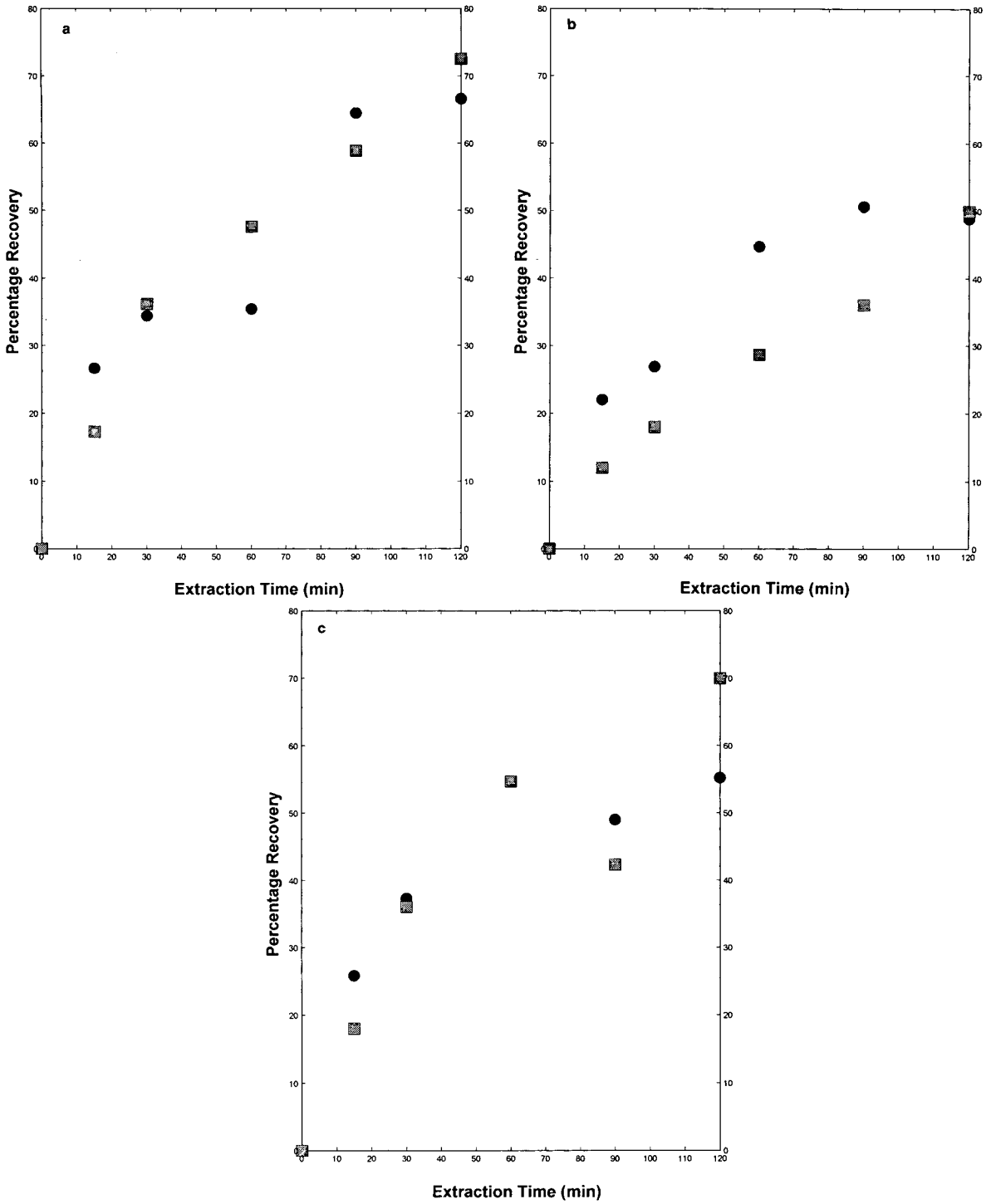

Fig. 5. Percentage recovery *versus* extraction time. Effect of salt for (a) lindane at 1.0 ml/min, (b) aldrin at 1.0 ml/min, (c) dieldrin at 1.0 ml/min. ● = With salt; ■ = without salt.

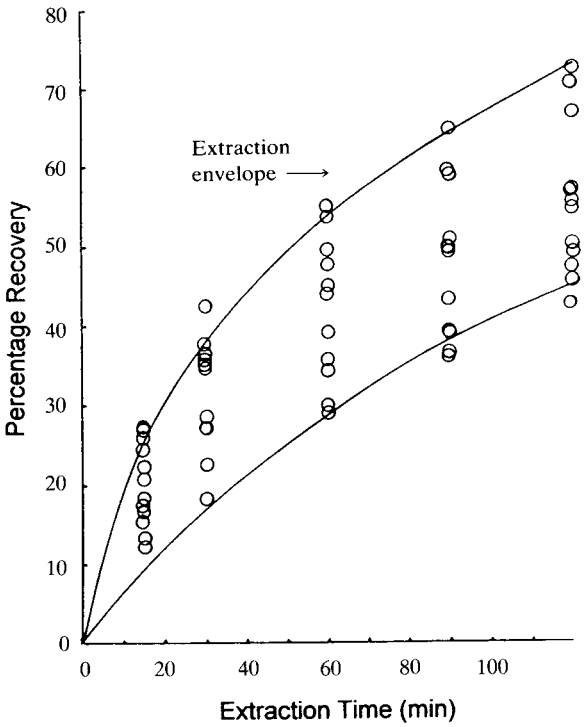

Fig. 6. A plot of percentage recovery data for aldrin, dieldrin, lindane at the three flow-rates studied plus the data when salt was added (1.0 ml/min only).

possibility of selectively extracting pesticides from the disks by using SFE rather than conventional solvents. Direct extraction from water was not affected by the addition of salt to the matrix although this did have an adverse effect on detection at longer extraction times. Flow-rate appears to have little effect on the recoveries of OCPs direct from water indicating that the process is kinetically limited by diffusion through the aqueous matrix. The method can potentially be used for trace analysis of pesticides in waste waters without the need for a preconcentration step which is usually required in solvent extraction.

Acknowledgement

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Qualitative and quantitative carbohydrate analysis of fermentation substrates and broths by liquid chromatographic techniques

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Abstract

Quantitative and qualitative aspects of the column liquid chromatographic analysis of carbohydrates in complex fermentation media are considered. Two fermentations of lignocellulose hydrolysates with *Saccharomyces cerevisiae* for the production of fuel ethanol were followed. In one spent sulphite liquor and in another an enzymatic hydrolysate of *Salix caprea* was used as the fermentation substrate. Two of the most commonly used chromatographic set-ups, one ligand-exchange and one ion-exchange system with refractive index and pulsed amperometric detection, respectively, were used to determine the carbohydrates. Some interfering compounds were eliminated by solid-phase extraction prior to sample introduction into the separation system. However, incomplete clean-up of the samples before chromatographic separation resulted in co-elution of matrix compounds with the sugars, introducing quantitative and qualitative errors in the evaluation of the sugar content. In fact, only in 30% of the samples analysed did the results between the two methods agree within 5%. The carbohydrate content of the fermentation samples as given by the two chromatographic methods is presented. Liquid chromatography coupled with thermospray mass spectrometry in both positive- and negative-ion modes was used for the characterization of the molecular ions of glucose, xylose, galactose, arabinose, mannose and well known interfering compounds such as phenolics and related aromatic compounds, and applied to biotechnological fermentation samples for qualitative analysis. Diode-array UV spectrophotometry was used as a complementary detection technique in order to identify unequivocally carbohydrates present in these fermentation media and trace interfering phenolic compounds.

1. Introduction

Since the oil crisis in the mid 1970s, alternative energy sources have been of great importance. For instance, ethanol produced in bioprocesses has been considered as a renewable energy source suitable for usage as a liquid fuel or fuel additive. In North America ethanol is blended with gasoline, and in Brazil ethanol has been used as a fuel for several years [1]. In Sweden, fuel ethanol produced from lignocellulosic material has been suggested as a possible energy source, and it has been successfully employed in 32 buses in Stockholm [2]. These buses have a positive effect on the environment and a further 300–400 ethanol-fuelled buses are planned for Stockholm. As fuel ethanol is a bulk product, the manufacturing process needs to be efficient

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to be cost effective in comparison with gasoline. Factors such as environmental benefits and political decisions will be of great importance to the extent of application of fuel ethanol in the future [1,3].

Fuel ethanol can be produced from different sources of lignocellulose, *e.g.*, agricultural residues, forestry products or municipal waste material [4–7]. The lignocellulosic raw material consists of cellulose, hemicellulose and lignin with variable distributions. The cellulose and hemicellulose fractions can be degraded to a fermentable hydrolysate from which ethanol can be produced. Depending on the raw material and the pretreatment, the lignocellulose hydrolysates will contain different and variable amounts of mono-, di- and oligosaccharides [5]. During the pretreatment not only monosaccharides but also acetic acid, furfural and extractives are released. These substances have an inhibitory effect on the fermenting microorganisms. Other substances with inhibitory action, such as sugar and lignin degradation products, are produced during the pretreatment to variable extents [8–13]. The fermentation process produces not only ethanol but also by-products, *e.g.*, acetic acid, glycerol, succinic acid and xylitol [14]. To ferment the hydrolysates successfully requires a microorganism that can ferment all sugars present to ethanol and also withstand the inhibitors present. Most attention has been given to how the pentose sugars can be fermented to ethanol. Several possible fermentation alternatives have been suggested, *e.g.*, recombinant *Escherichia coli*, *Pichia stipitis*, *Pachysolen tannophilus*, *Candida shehatae* and recombinant *Saccharomyces cerevisiae* [15–17]. Altogether this means that the fermentation substrate has a complex composition which changes throughout the process, and accurate analytical techniques are of utmost importance in developing and optimizing the fermentation process.

The production of fuel ethanol is an example of an industrial fermentation process where a highly complex substrate is used for which analytical techniques have been developed to only a limited extent. In this study we chose to work with two lignocellulose hydrolysates, spent sulphite liquor (SSL) and an enzymatic hydrolysate of steam-pretreated *Salix caprea* (EH), the compositions of which are completely different regarding sugar content, inhibitors and the matrix as a whole.

For the analysis of these highly complex fermentation substrates and broths, containing a broad range of lignin breakdown products, intermediates and products, there is a need to find a reliable methodology for accurate qualitative and quantitative analyses for monosaccharides present in these samples. Sample-handling techniques such as solid-phase extraction prior to separation for selective enhancement in chromatographic analysis are commonly utilized. Liquid chromatography (LC) in combination with mass spectrometry (MS) is another powerful method in biotechnological applications. Thermospray (TSP) LC–MS is an appropriate technique for quantification, as demonstrated in the determination of carbamates and chlorinated phenoxy acids, for which positive- and negative-ion detection modes were used [18,19]. TSP as an interfacing system has been found appropriate for quantification purposes at a level of 100 ppb, whereas the absolute detection limit is around a few nanograms under full-scan conditions and below 1 ng in the selected-ion monitoring (SIM) mode. Of the three commercial interfaces available, TSP, electrospray (ES) and particle beam (PB), only TSP and ES were adequate for quantitative purposes. PB showed statistically significant differences in quantification and was inefficient in transporting the analytes into the ion source. The linear dynamic range is 10–1000 ng/ml and the use of SIM is recommended for quantification as the relative standard deviation is lowered to 19%. The advantage of using TSP is that it can be used in both positive-ion (PI) and negative-ion (NI) modes [18,19]. Here, we report on the investigation of qualitative and quantitative evaluations of carbohydrates present in fermentation substrates and broths by using two of the most common LC methods utilized for sugar determination. Mass spectrometry and diode-array UV spectrophotometry are complementary techniques used in the characterization of these processes.

2. Experimental

2.1. Chemicals

High-performance liquid chromatographic (HPLC) grade water, methanol and acetonitrile from Merck (Darmstadt, Germany) were passed through a sterile filter (0.45 μm) (Waters Chromatography Division, Millipore, Bedford, MA, USA) before use. Ammonium formate was supplied by Fluka (Buchs, Switzerland). All carbohydrates, L-(+)arabinose, cellobiose, D-(+)galactose, D-(+)glucose, D-(+)mannose and D-(+)xylose, were of Sigma grade (Sigma, St. Louis, MI, USA) and all phenolic standards were of analytical-reagent grade from Merck. Baker's yeast, *Saccharomyces cerevisiae*, was obtained from Jästbolaget (Rotebro, Sweden). Nutrients added were yeast extract (Difco, Detroit, MI, USA), and analytical-reagent grade ammonium hydrogenphosphate, magnesium sulphate heptahydrate and sodium phosphate (all from Merck). The two fermentation substrates, spent sulphite liquor (SSL) and the enzymatic hydrolysate (EH) produced from fast-growing willow (*Salix caprea*), were kindly supplied by MoDo AB (Örnsköldsvik, Sweden) and the Department of Chemical Engineering I, Lund Institute of Technology, University of Lund (Lund, Sweden), respectively. All other standards were of analytical-reagent grade and purchased from Merck or Sigma. All solutions were prepared by dissolving the substances in water obtained from a Millipore (Bedford, MA, USA) Milli-Q water-purification system or HPLC-grade water.

2.2. Instrumentation

Two chromatographic systems for the determination of sugars were used. The first contained a high-pressure LC pump (Waters Model 600 programmable solvent-delivery module) equipped with a Model 7045 six-port injection valve with a 20-μl loop (Rheodyne, Cotati, CA, USA), a Model 2142 refractive index (RI) detector (LKB, Bromma, Sweden) and a Model 2210 chart recorder (LKB). The analytical column (300 × 7.8 mm I.D.) was a ligand-exchange column in the Pb^{2+} form (Aminex HPX-87P; Bio-Rad Labs., Richmond, CA, USA) heated to 85°C in a chromatographic oven (Waters column heater module, controlled by a Waters temperature-control module). Milli-Q-purified water was used as the mobile phase, pumped at a flow-rate of 0.6 ml/min. This system is referred to as LEC–RI below.

The second chromatographic system consisted of a high-pressure LC pump, an injection valve with a 25-μl loop, a guard column (CarboPac guard 25 × 3 mm I.D.), an analytical column (CarboPac PA1, 250 × 4 mm I.D.) and a pulsed amperometric detection (PAD) system, all from Dionex (Sunnyvale, CA, USA). The mobile phase was 10 m*M* NaOH, except where stated otherwise, and was pumped at a flow-rate of 1.0 ml/min. The separations were carried out at room temperature. This set-up is referred to as AEC–PAD.

Gel permeation chromatography (GPC) was performed on an LC system consisting of a Model 400 high-pressure pump (Applied Biosystems, Foster City, CA, USA), a Model 6020 injector (Rheodyne) and a Vari-Chrom UV–visible spectrophotometric detector (Varian, Sunnyvale, CA, USA). Analyses of spectra were performed on a Chrom-A-Scope diode-array UV detector (Barspec, Rehovot, Israel). The analytical columns used were a Bio-Beads SX-12 (Bio-Rad Labs.) stainless-steel column (450 × 10 mm I.D.) with dichloromethane as the mobile phase and a Phenogel (Phenomenex; Remuko, Palos Verdes, CA, USA) column (40 × 4.6 mm I.D.) with tetrahydrofuran as the mobile phase.

Reversed-phase separations were carried out on a LiChroCART cartridge column (12.4 cm × 4.0 mm I.D.) packed with 5-μm LiChrospher 100 RP-18 (Merck) using methanol–50 m*M* acetate buffer (pH 4.2) (20:80) as the mobile phase, followed by a step gradient to a 90% methanol content of the mobile phase run after each separation, using a Chrom-A-Scope rapid-scanning UV detector. The scanning was performed between 190 and 380 nm.

The LC–MS system for qualitative evaluation of sugars consisted of a Model 510 high-pressure pump (Waters Chromatography Division). Injection was carried out using a Model 7125 six-port injection valve with a 20-μl loop (Rheodyne). In flow-injection experiments, a reversed-phase eluent of methanol–water (50:50) containing 0.05 *M* ammonium formate was used at a flow-rate of 1 ml/min. All mobile phases were degassed in either an ultrasonic bath or by helium gas.

A Hewlett-Packard (Palo Alto, CA, USA) Model 5988A thermospray quadrupole mass spectrometer and a Hewlett-Packard Model 59970C instrument for data acquisition and processing were employed. The temperatures of the TSP were 100, 188, and 270°C for the steam, vapour and ion source, respectively. In all experiments the filament was on. Full-scan conditions were used in most of the experiments, with scanning from m/z 92 to 500 and from m/z 138 to 400 in the PI and NI modes, respectively.

2.3. Fermentation substrate

For fermentation, two different substrates were used; spent sulphite liquor (SSL) and enzymatic hydrolysate (EH) of steam-pretreated *Salix caprea*. The substrates were supplemented with 2.5 g/l of yeast extract, 0.25 g/l of $(NH)_4HPO_4$ and 0.025 g/l of $MgSO_4 \cdot 7H_2O$ and buffered with 0.1 *M* sodium phosphate to a final pH of 5.5.

2.4. Fermentation conditions

For fermentation, a 25-ml beaker sealed with a rubber stopper and supplied with a cannula for carbon dioxide removal was used. The beaker was inoculated with compressed baker's yeast (2 g) and either SSL or EH was added to give a final volume of 25 ml. The beaker was slowly stirred at 30°C. Samples (100 μl) were taken after 0, 1, 3 and 24 h of fermentation and the pH was adjusted to 5.5 at each sampling.

2.5. Sample clean-up and fractionation of the fermentation samples

All samples were diluted 50-fold and filtered through a 0.45-μm membrane filter (Schleicher & Schüll, Dassel, Germany) to eliminate particulate contaminants. In the purification by solid-phase extraction (SPE), a diluted sample (1.0 ml) was stirred in a beaker for 5 min with a mixture of polymeric anion- (20 mg) and cation-exchange (10 mg) support (Amberlite; BDH, Poole, UK).

The SSL and EH samples were centrifuged after dilution in a Wifug bench-top centrifuge at 5700 rpm for 1 min, prior to filtration and prior to SPE treatment. Fractionation of SSL and EH samples was performed on the ligand-exchange LC system with the Aminex HPX-87P column and fractions corresponding to each eluting peak (glucose, xylose, galactose, arabinose–ethanol and mannose) were collected.

3. Results and discussion

3.1. Composition of biotechnological samples

The nature of these fermentation samples and substrates is highly complex and the knowledge of their compositions is limited. SSL is a waste material from the pulping industry having a high salt content and produced under harsh conditions in a recycling system. In SSL the sugars solubilized are mainly those from the hemicellulose fraction as the cellulose fraction is utilized in the pulping. The EH was prepared in a two-stage steam-pretreatment process (acidic conditions) combined with enzymatic hydrolysis [20,21]. In the EH, both the cellulose and the hemicellulose can be hydrolysed and thus oligo-, di- and monosaccharides from both fractions are expected to be present. Moreover, SSL is produced from softwood whereas EH is produced from hardwood, and therefore the compositions of the hemicellulose fractions differ [15]. Depending on the conditions of hydrolysis, pentoses and hexoses emanating from the hydrolysed polysac-

charides can easily be converted into different degradation products, *e.g.*, furfural and 5-hydroxymethylfurfural, which not only decrease the amount of usable carbon source for the yeast but also are toxic and may inhibit the fermentation process. In enzymatic hydrolysis the conditions are mild, resulting in a more controlled composition of the product, subsequently used as a substrate. The enzymatic hydrolysis step is specific in contrast to the chemical treatment, which yields a broader range of matrix components. SSL is obtained from an industrial recycling system where interferents can be accumulated. Both substrates will consist of poly-, oligo- and monomers of lignin and various oxidation and breakdown products thereof. Humic substances and browning compounds have also been found in these substrates. Humics are dark-brown, amorphous, colloidal compounds. The major constituents are humic acids, aromatic polymers with a high molecular mass to which a high number of functional groups are attached. Many of the components in the matrix not only interfere in the analysis but also have an inhibitory action on the fermenting microorganism. The fermentation process itself causes a complexity in sample composition as cell mass and nutrients are added to the substrate and different products are produced. A further complication is that the ethanol produced during the process will increase the solubility of more hydrophobic substances in the broth, such as various substituted phenols.

3.2. LC separation systems for carbohydrates

A series of complementary investigations were performed to characterize these highly complex fermentation samples. The analytical procedures used are shown in Fig. 1. These were applied to both the fermentation substrate and the broth, and also to the fractions collected from the LEC–RI system. The crude samples and fractions were independently investigated with four different methods, *i.e.*, UV spectrophotometry, TSP-MS, GPC and reversed-phase chromatography. Two well established LC separation systems for carbohydrate analysis were used for

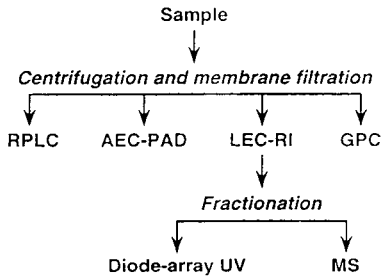

Fig. 1. Illustration of analytical techniques used for the characterization of biotechnological samples. RP-LC = reversed-phase liquid chromatographic system; AEC–PAD = anion-exchange chromatographic system with pulsed amperometric detection; LEC–RI = ligand-exchange chromatographic system with refractive index detection; GPC = gel permeation chromatography; MS = mass spectrometry.

quantitative and qualitative evaluation of these fermentation substrates and broths. In both systems the stationary phase has a poly(styrene–divinylbenzene) matrix, differently derivatized in the two types. One system makes use of ligand exchange as the basis for separation, with Milli-Q-purified water at neutral pH as the mobile phase, while the other utilizes anion exchange with aqueous sodium hydroxide solution of pH 12–13 as the mobile phase. The ligand-exchange system is coupled to a refractive index detector (LEC–RI) and the anion-exchange system to a pulsed amperometric detection unit (AEC–PAD). Polarimetry is the classical way of determining sugar levels and is still used in many industries, but as LC has been introduced for quality control, RI has become the most popular detection method for sugar analysis. This is due mainly to the simplicity of operation of these units. Their lack of selectivity and sensitivity, however, limits their usefulness in analyses of samples with some degree of complexity. This has been shown in several industrial and biological applications [22,23].

The LC systems were investigated with respect to linear response characteristics, repeatability, reproducibility and sensitivity, with five different monosaccharide standards, glucose, xylose, galactose, arabinose and mannose (Table 1). In the concentration range investigated (0.25–5 mM), the LEC–RI system was found to respond

Table 1
Data for the two separation and detection methods

Parameter	LEC–RI	AEC–PAD
Calibration graph concentration range	0.25–5 m*M*	2–30 μM
Linearity:		
Glucose	$y = 21.9x + 4.6$ ($R = 0.9943$)	$y = 2.1x + 0.86$ ($R = 0.9989$)
Xylose	$y = 20.2x + 4.2$ ($R = 0.9944$)	$y = 2.0x + 0.43$ ($R = 0.9990$)
Galactose	$y = 19.6x + 4.3$ ($R = 0.9942$)	$y = 2.0x + 1.5$ ($R = 0.9992$)
Arabinose	$y = 18.0x + 3.9$ ($R = 0.9931$)	$y = 2.3x + 0.77$ ($R = 0.9994$)
Mannose	$y = 10.2x + 2.4$ ($R = 0.9940$)	Not determined
Limit of detection (LOD)[a]	0.1 m*M* (mannose 0.15–0.2 m*M*)	0.1–1 μM (mannose 5–20 μM)
Repeatability:		
Retention time	0.34%[b] ($n = 14$)	0.1–3%[c] ($n = 4$–5)
Peak height	<1%[d] ($n = 7$)	5–6% ($n = 4$)
Reproducibility[c]	*ca.* 1%	Not determined

[a] For AEC–PAD, this depends on the condition of the PAD detector. LOD is defined as 2–3 times the noise level.
[b] Five calibration solutions and nine real samples.
[c] 0.1% = four identical samples; 3% = five calibration solutions.
[d] Earlier investigations [25].

linearly up to about 2–3 m*M* with regression coefficients (R) very close to 1.0. Including the response obtained for a 5 m*M* concentration in the linear regression calculations lowered the R values to about 0.994, indicating a small deviation from linearity. With the AEC–PAD system, the calibration graph ranged between 2 and 30 μM. High R values (0.9989–0.9994) were obtained over the entire interval, indicating good linearity. No response for mannose, however, appeared until a concentration of 20 μM was injected and subsequently this sugar was not used for the calibration graph. The limit of detection (LOD) is normally low for sugars in this system 0.1–1.0 μM (Table 1). Our experience is that the performance of the PAD unit determines the LOD. Poisoning of the electrode will eventually occur after repetitive injections of these complex samples, although the surface of the electrode is cleaned after each measurement [24,25] (see below). We found that after running the system for 24 h, a drastic increase in response was obtained by flushing the detector cell with 0.2 *M* NaOH for 1 h. A lowering of the detection limits by a factor between 5 and 10 was feasible with this cleaning step, indicating that matrix compounds causing electrode fouling were eliminated from the electrode surface. The LOD value for all sugars except mannose was therefore found to be about 1 μM for an "old" cell surface and about 0.1 μM for a "fresh" one. As mentioned above, the response for mannose is lower, giving LODs of between 5 and 20 μM depending on the condition of the detector. In the LEC–RI system, the limits of detection were *ca.* 0.1 m*M* except for mannose, which was about twice as high. The lowering of the response is due to the high k' value of mannose and its lower RI factor, which is about half that of the other sugars.

The AEC–PAD system thus offers an LOD two to three orders of magnitude lower than that of the LEC–RI system. Sensitivity, however, is not a critical parameter in these applications during most of the process time, owing to the relatively large amounts of the analytes. Baseline separation of the five sugars is obtained with the AEC–PAD system, in contrast to the LEC–RI system, in which the peaks of arabinose and mannose partly overlap. The improved separation is due to the formation of the enolate form of the sugars at this high pH. As anions, they are more easily separated compared with the separation system based on ligand exchange, where

the sugars are uncharged. In the ligand-exchange mode, the forces of interaction are obviously much weaker, resulting in lower resolution. On the other hand, the repeatability and reproducibility of the LEC–RI system are better, regarding both retention time and peak height (Table 1). In fact, the repeatability of retention times of the AEC–PAD system can be very poor. We found that the electrochemical detector was sensitive to changes in pH resulting from dissolution of carbon dioxide into the NaOH-based mobile phase, which also changes its ionic strength and thus affects the chromatographic separation.

A difference between the samples analysed by the two systems was the utilization of an SPE step prior to injection into the LEC–RI system. A mixed anion- and cation-exchange SPE support was used in order to exclude Cl^- ions and cations such as metal ions present in the samples. The presence of these ions will otherwise ruin the performance of the analytical column. In addition, we have found in other studies that these polymeric SPE supports eliminate interfering matrix compounds to a varying extent, aromatic lignin breakdown products being removed very efficiently [26,27]. Typical recovery values for the five sugars were found to be 88–94%. This clean-up step should also be taken into consideration in evaluating the stability of the two systems. The better stability found for the LEC–RI system could well be due to the less complex samples handled by the analytical column and the detector, while all matrix components were introduced into the AEC–PAD system.

The amounts of each sugar in the two hydrolysates and the corresponding chromatograms are shown in Table 2 and Fig. 2. As is evident from Table 2, some results obtained with the two methods for the contamination of the sugars agree very well whereas others differ. In only 30% of the cases (12 out of 40) did the results of the two methods agree within 5%. There are several reasons for these differences. First, with the LEC–RI system, it is sometimes difficult to determine small amounts owing to the elution of interfering matrix compounds (see Fig. 2A). The samples run using the AEC–PAD system had to be more diluted so as to fall within the linear concentration range. Any interfering compounds were then equally diluted. Indeed, these chromatograms appear very clean (see Fig. 2C), resembling those obtained when running standard carbohydrate solutions, and are accordingly easier to quantify. The chromatograms (Fig. 2C and D) also reflect the superior discrimination of the amperometric detector where the chosen operating potential seems to be optimum with respect to the overwhelming number of matrix

Table 2
Determination of sugars (m*M*) in fermentations of SSL and EH using ligand-exchange chromatography with refractive index detection and anion-exchange chromatography with pulsed amperometric detection

Method	Sugar	SSL 0	SSL 1	SSL 3	SSL 24	EH 0	EH 1	EH 3	EH 24
LEC–RI	Glucose	28	9.0	0	0	390	160	4.4	0
	Xylose	66	66	54	44	54	30	48	26
	Galactose	29	32	26	21	19	14	4.2	3.0
	Arabinose	10	14	10	8.5	12	0	0	0
	Mannose	120	89	0	0	11	0	0	0
AEC–PAD	Glucose	25	6.7	2.6	0	357	165	4.0	0
	Xylose	63	66	52	36	55	30	47	26
	Galactose	32	31	26	17	6.8	2.8	6.0	2.4
	Arabinose	14	15	12	8.7	4.9	4.0	3.7	2.1
	Mannose	85	48	5.8	2.2	11[a]	0	0	0

SSL = spent sulphite liquor; EH = enzyme hydrolysate; 0, 1, 3 and 24 = time (h) after start of fermentation.
[a] This value was determined in a separate analysis which does not correspond to the chromatogram shown in Fig. 1D.

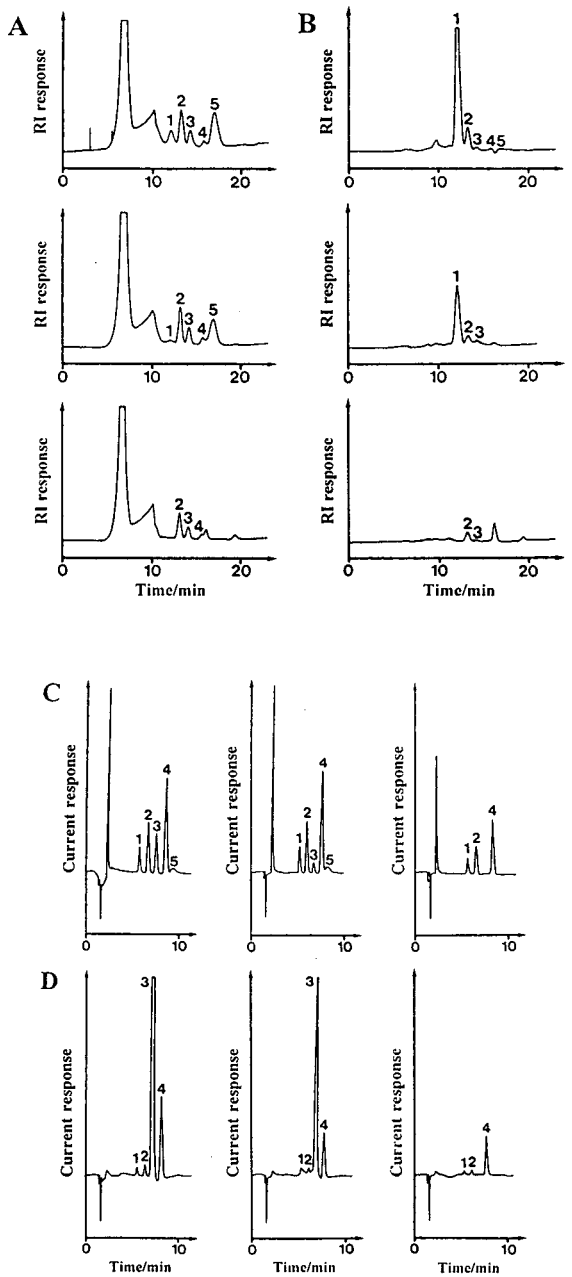

Fig. 2. Chromatograms of samples taken after 0, 1 and 24 h of fermentation in SSL and EH using two different separation and detection principles. (A) LEC–RI of SSL; (B) LEC–RI of EH; (C) AEC-PAD of SSL; (D) AEC–PAD of EH. Peaks (LEC–RI): 1 = glucose; 2 = xylose; 3 = galactose; 4 = arabinose; 5 = mannose. Peaks (AEC–PAD): 1 = arabinose; 2 = galactose; 3 = glucose; 4 = xylose; 5 = mannose.

compounds in these crude samples. Second, as the PAD is more sensitive with lower LOD values, the determination of smaller amounts can be made more accurately.

The sugar initially having the highest concentration in SSL is mannose, followed by xylose, about equal amounts of glucose, galactose, and arabinose (see Fig. 2A and C). In EH, glucose is the totally dominant sugar and only small amounts of the others are traceable (Fig. 2B and D). When *Saccharomyces cerevisiae* is used in fermentation, glucose and mannose are the first two fermentable carbohydrates to be metabolized. The two methods give very similar values for the concentration of the virtually non-fermentable pentose xylose (see Table 2), whereas the largest discrepancies between the methods are found for mannose in the SSL fermentation. A reliable comparison cannot be made for arabinose owing to the lower sensitivity of the ligand-exchange system. Galactose and arabinose seem also to vary at the beginning of the process when EH is fermented. It was found in another study that, when the ligand-exchange system was used, unidentified peaks appeared in the chromatogram during the process [22]. In addition, some of the sugar peaks did not decrease in the order expected during the production of ethanol [27].

The variation in the evaluated concentrations can be explained partly by the different separation mechanisms of the two systems, although the largest difference lies in the detection methods. RI detection measures the difference in refractive index between the solutes eluting through the flow cell and the eluent present in the reference cell. Compounds eluting through the cell with either a higher or a lower refraction index than that in the reference cell will result in a positive or a negative peak. However, if several analytes pass simultaneously, an additive multi-component signal will be obtained. This is the case if the resolution is incomplete in the chromatographic separation. The corresponding peak will be positive if the sum of the RI factors of the solutes in the unresolved peak is value higher than that in the reference cell and negative if the sum is lower, and a flat baseline is

obtained if the sum is zero or close to zero. The last case has been found to occur in certain industrial applications [22]. The RI detector is thus much affected by the presence of interfering substances, to which the PAD does not necessarily respond.

Oxidation of sugars at electrode surfaces often suffers from electrode fouling due to electrochemical formation of products that adsorb on the electrode surface [24]. Electrochemical detection principles such as PAD, pre- and post-column derivatization systems with various chemical reagents and enzymes as catalytic reagents have been intensively studied in order to circumvent the irreversible fouling effects of the electrode [22,25].

3.3. Characterization by gel permeation and reversed-phase chromatography

Samples were further characterized by GPC and reversed-phase chromatography. GPC in combination with diode-array detection provided complementary information on the distribution of molecular size and shape of the components present in the samples withdrawn from the process 0, 1, 3 and 24 h after the initiation of the fermentation process. Fingerprint spectra can be obtained for the specific compounds present in each substrate and broth [28,29], and also for each resulting chromatographic peak. These data provide information about changes in matrix composition and possible indications of the presence of substituted phenolics and structurally related molecules, as breakdown of lignin oligo- and polymers can give products of these types.

Typical chromatograms obtained from two of the samples using this technique are shown in Fig. 3, which indicates the distribution of predominantly mono-, di- and oligomeric breakdown products of lignin present in the broth. It was found that the composition of these lignin breakdown products differed widely depending on the time the sample was taken during the fermentation. The GPC column used in this instance (SX-12) has an upper molecular mass exclusion limit of 400, which means that the compounds eluting after the front peaks are

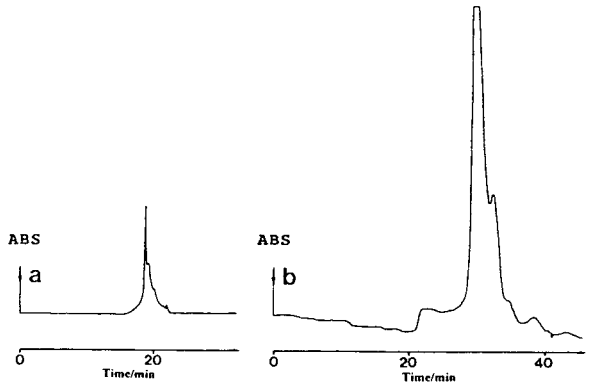

Fig. 3. Gel permeation chromatographic separations of (A) fermented SSL diluted 1000-fold on a Phenogel separation column using tetrahydrofuran as the mobile phase and (B) fermented SSL diluted 1000-fold on an SX-12 separation column using dichloromethane as the mobile phase. Flow-rate, 1 ml/min; injection volume, 20 μl; UV detection 280 nm, 0.1 AUFS.

predominantly of molecular mass below 400. The front will contain lignin breakdown products with a molecular mass higher than the exclusion limit of the column, whereas the last peak will typically contain, among all other fermentation products and intermediates, lignin monomers, mono- and disaccharides, small oligosaccharides, furan aldehydes and the alcohol produced. In the chromatogram shown in Fig. 3B, where the molecular mass exclusion limit of the column used (Phenogel) is 1000, the front contains all excluded compounds with molecular mass above 1000. The rest of the sample is seen as eluting peaks with a poorer resolution. These results clearly show the presence of compounds having a range of different molecular masses in the samples, despite pretreatment by filtration, centrifugation and SPE.

Isocratic reversed-phase separations were utilized to separate and determine furan aldehydes. The level of furfural and 5-hydroxymethylfurfural can be correlated with the efficiency of the hydrolysis steps, as these two aldehydes are products originating from hexoses and pentoses. Low levels of 5-hydroxymethylfurfural present in the substrates will reflect optimum hydrolysis conditions with high hexose levels. This was of particular interest as the low molecular masses of

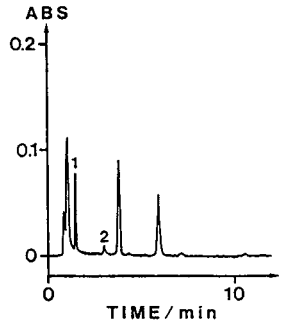

Fig. 4. Reversed-phase separation from SSL (t = 3 h) diluted 200-fold on a LiChrospher 100 RP-18 analytical column. Mobile phase, methanol–50 mM acetate buffer (pH 4.2) (20:80); flow-rate, 1 ml/min; injection volume, 20 μl; UV detection at 280 nm, 0.05 AUFS. Peaks: 1 = 5-hydroxymethylfurfural (40 μM); 2 = furfural (0.6 μM).

the two aldehydes did not permit analysis with TSP-MS (see below). Fig. 4 illustrates a reversed-phase separation in which both furfural and 5-hydroxymethylfurfural can be identified. The mobile phase was chosen to contain smaller amounts of organic additives such as methanol [methanol–10 mM acetate buffer (pH 4.2) (20:80)] in this particular case in order to achieve a good resolution. A step gradient to a 90% methanol content of the mobile phase was run after each separation (data not sown) to clean the column from strongly bound hydrophobic lignin breakdown products. The column was equilibrated with the mobile phase (10 ml) before the next injection was made.

3.4. *Fractionation from the ligand-exchange system*

UV spectra of the fractions obtained by LEC–RI showed that they contained not only sugars but also other compounds. This is clearly seen for both the hexoses and the pentoses by comparison of the spectra from a pure glucose solution (Fig. 5A), the glucose and xylose fractions from SSL (Fig. 5B and C) and arabinose and mannose from EH (Fig. 5D and E). The UV spectra from pure hexose and pentose solutions appear almost identical. As is clearly seen, there is a marked difference when comparing the pure standard solution with the fractionated samples.

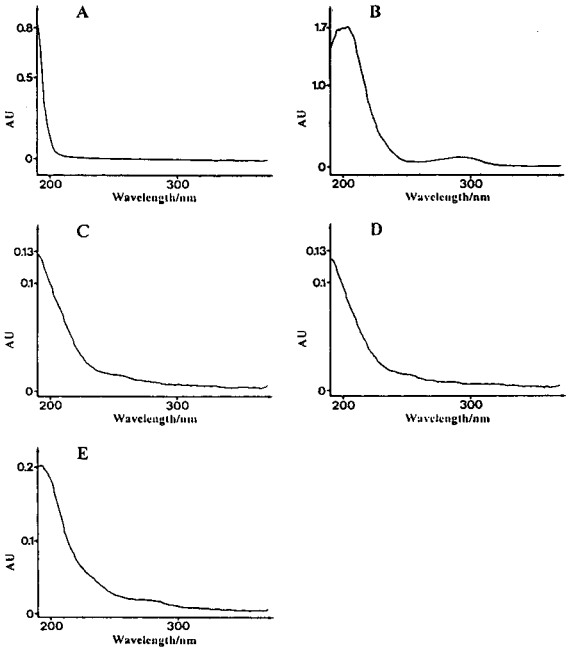

Fig. 5. Diode-array UV spectra of (A) a pure glucose standard, (B) a fractionated glucose peak present in SSL, (C) a fractionated xylose peak present in SSL, (D) a fractionated arabinose peak present in EH and (E) a fractionated mannose peak present in EH.

In Fig. 5B and E there are similar peak maxima around 220 and 280 nm similar to those in the spectra of many of the phenolic and related aromatic compounds. In fact, the spectra obtained from these fractionated chromatographic eluates resemble those of some of the fermentation broths previously investigated after liquid–liquid extraction [28].

3.5. *LC–MS*

Sugars are a class of compounds difficult to handle by MS. They are not amenable to study directly by GC–MS and chemical derivatization is needed. In order to characterize the sugars directly, either chemical ionization mass spectrometry (CI-MS) (using methane) [30] or different combinations of LC–MS [31–33] have been suggested. Advantages claimed in the use of LC–MS for sugars include ease of sample preparation, speed of analysis, similar sensitivity and

good reproducibility. In CI-MS the $[MH]^+$ ion was usually present together with a pattern of fragmentation that permitted the deduction of the sugar structures [30]. When LC–MS was used with a moving belt interface, xylose, fructose, glucose and sucrose being characterized by ammonia CI-MS, the $[M + NH_4 - H_2O]^+$ and $[M + NH_4]^+$ ions were major peaks in the spectrum [31]. It should be noticed that with LC–TSP-MS, the $[M + NH_4]^+$ ion was the base peak for glucose [32]. However, when the TSP probe temperature is increased above 230°C, a slight increase in the relative abundance of the dehydrated $[M + NH_4 - H_2O]^+$ ion is noticed (usually it gives a 10% relative abundance). Although most of the experiments performed under LC–MS conditions were carried out in the PI mode, one example reported in the literature indicated that LC–TSP-MS in the NI mode was feasible for glucose, thus giving $[M - H]^-$ as the base peak [33], with a sensitivity similar to that of GC–MS.

The objective of our work was to use LC–TSP-MS for the characterization of the different sugars in the PI and NI modes. From the results with the sugars shown in Table 3, it can be seen that all the compounds behave similarly in the PI and NI modes of detection, and that degradation does not occur. The $[M + NH_4]^+$ ion is always the base peak in the PI mode, as expected for

Table 3
Important fragments, relative abundances (RA) and arbitrary units (AU) given in an absolute scale of units in mass spectrum observed in LC–TSP-MS in positive-(PI) and negative-ion (NI) modes and filament-on mode of operation for sugars

Molecular mass	Compounds and ions (m/z and tentative identification)	RA		AU
		PI	NI	Base peaks
180	Glucose			
	180 $[M + NH_4 - H_2O]^+$	10		
	198 $[M + NH_4]^+$	100		150
	179 $[M - H]^-$		20	
	225 $[M + HCOO]^-$		100	40
150	Xylose			
	150 $[M + NH_4 - H_2O]^+$	10		
	168 $[M + NH_4]^+$	100		60
	149 $[M - H]^-$		1	
	195 $[M + HCOO]^-$		100	15
150	Arabinose			
	150 $[M + NH_4 - H_2O]^+$	25		
	168 $[M + NH_4]^+$	100		20
	149 $[M - H]^-$		1	
	195 $[M + HCOO]^-$		100	15
180	Galactose			
	180 $[M + NH_4 - H_2O]^+$	25		
	198 $[M + NH_4]^+$	100		2
	179 $[M - H]^-$		5	
	225 $[M + HCOO]^-$		100	4
180	Mannose			
	180 $[M + NH_4 - H_2O]^+$	15		
	198 $[M + NH_4]^+$	100		12
	179 $[M - H]^-$		5	
	225 $[M + HCOO]^-$		100	4

Amount injected: 1 μg.

TSP. In contrast, when LC–MS was used with a moving belt interface or the sugar solution was spotted directly on the belt, the ratio between the $[M + NH_4 - H_2O]^+$ and $[M + NH_4]^+$ ion abundances changed. Generally, with direct spotting the relative abundance of the $[M + NH_4]^+$ ion increased whereas that of $[M + NH_4 - H_2O]^+$ decreased, suggesting that during moving belt LC–MS some thermal degradation occurred [31]. In our experiments, the abundance of $[M + NH_4]^+$, similar to that reported by other workers using TSP conditions [32], was also similar to that reported with direct spotting, indicating that LC–TSP-MS offers a better approach than the moving belt as it avoids thermal degradation. One reason for the abundance of degradation under TSP conditions in this study is that TSP temperatures below 230°C were employed, as recommended by other workers [32].

With TSP-MS in the NI mode, the common base peaks correspond to the adduct ion with the eluent additive formate, and as the second peak the proton abstraction peak ($[M - H]^-$ ion). In this respect these data differ from the TSP-MS of glucose in the NI mode in that we obtain as the base peak the adduct with formate, whereas Reid *et al.* [33] did not report adduct formation with their additive, ammonium acetate. This may be explained in this work by the use of a different source; the instrument used usually gives much more adduct formation under NI TSP-MS conditions, as reported [34]. The different eluent additive, formate in our experiments, forms adducts more readily than acetate [33]. The formation of both ions in TSP-MS in the NI mode indicates an intermediate behaviour and shows that adduct formation is always more important than proton abstraction, as has been noted for various phenoxyacetic acid herbicides [35]. By calculating the relative abundance (RA) values (see arbitrary units) as shown in Table 3 (the percentage of each ion in the spectrum), comparison of the spectra is feasible. It is possible to overcome fluctuations and experimental variations in the instrumental performance by use of these calculated values. It is readily seen from Table 3 that glucose and xylose gave signal responses at least one order of magnitude better than those given by galactose and mannose. This implies that the LODs for glucose and xylose will be lower than those for galactose and mannose.

The relative abundances of the important ions obtained from different phenolics in TSP-MS can be found, as indicated in Table 4. The fragmentation pattern for the different phenolic compounds under NI TSP-MS conditions follows expectations [34,36] and gives, in general the $[M - H]^-$ and $[M + HCOO]^-$ ions as the main peaks. In some instances $[M - H]^-$ is not observed for a series of compounds, *e.g.*, 4-hydroxybenzoic acid, 2,4-dihydroxybenzaldehyde and benzoic acid (see Table 4); this is due to the fact that the scan range started at m/z 138, so that it was impossible to detect their proton abstraction peak for a compound with a lower molecular mass. It is interesting to note that when an ethoxy group is introduced, as in 3-ethoxy-4-hydroxybenzaldehyde, the compound gives a response under PI conditions, which indicates the enhancement of the proton affinity of this compound by the addition of an ethoxy group to the molecule. With hydroxycoumarin, the behaviour is reversed; when a hydroxy group is introduced, the gas-phase basicity increases and therefore the hydroxy compound gives a response in the NI mode, whereas coumarin does not. Benzoic acid derivatives can only stabilize the negative charge by electron delocalization in the aromatic ring and so give good responses in TSP-MS in the NI mode.

To distinguish between sugars, the use of MS–MS will be needed, as has been demonstrated for triazine herbicides [37]. The identification of *m*-coumaric acid was also feasible in an SSL fraction (from flow-injection chromatograms; see Fig. 6). All the individual peaks correspond to different injections and each peak at m/z values of 163 and 209 also matches the total ion chromatogram. The use of the NI mode permitted SIM using the two main ions in the isolated fraction. This technique was found to be useful for identification in these complex samples, as this mode of operation enhances the signal in the total current trace. This is illustrated in Fig. 6 (peaks between 12 and 17 min) by the definite identification of the presence of this compound

Table 4
Important fragments, relative abundances (RA) and arbitrary units (AU) given in an absolute scale of units in mass spectrum observed in LC–TSP-MS in positive-(PI) and negative-ion (NI) modes and filament-on mode of operation for substituted phenols

Molecular mass	Compounds and ions (m/z and tentative identification)	RA		AU
		PI	NI	Base peaks
138	4-Hydroxybenzoic acid			
	183 $[M + HCOO]^-$	n.d.[a]	100	3
154	3,4-Dihydroxybenzoic acid			
	153 $[M - H]^-$	n.d.	100	1
	199 $[M + HCOO]^-$	n.d.	100	1
154	3,5-Dihydroxybenzoic acid			
	153 $[M - H]^-$		10	
	199 $[M + HCOO]^-$	n.d.	100	1
166	3-Ethoxy-4-hydroxybenzaldehyde			
	167 $[M + H]^+$	100		3
	184 $[M + NH_4]^+$	30		
	165 $[M - H]^-$		100	5
	211 $[M + HCOO]^-$		60	
138	2,4-Dihydroxybenzaldehyde			
	183 $[M + HCOO]^-$	n.d.	100	5
138	3,4-Dihydroxybenzaldehyde			
	183 $[M + HCOO]^-$	n.d.	100	11
122	Benzoic acid			
	167 $[M + HCOO]^-$	n.d.	100	6
154	2,5-Dimethoxyphenol			
	155 $[M + H]^+$	100		4
	199 $[M + HCOO]^-$		100	1
126	5-Hydroxymethylfurfural			
	144 $[M + NH_4]^+$	100	n.d.	100
148	Cinnamic acid			
	147 $[M - H]^-$	n.d.	10	
	193 $[M + HCOO]^-$	n.d.	100	3
146	Coumarin			
	147 $[M + H]^+$	5	1	164
	164 $[M + NH_4]^+$	100		
162	4-Hydroxycourmarin			
	163 $[M + H]^+$	30	5	180
	180 $[M + NH_4]^+$	100		
	161 $[M - H]^-$		100	3
	207 $[M + HCOO]^-$	25	1	
162	7-Hydroxycoumarin			
	163 $[M + H]^+$	7		
	180 $[M + NH_4]^+$	100		140
	161 $[M - H]^-$		100	8
	207 $[M + HCOO]^-$		90	
164	*m*-Coumaric acid			
	163 $[M - H]^-$	n.d.	3	
	209 $[M + HCOO]^-$	n.d.	100	5

Amount injected: 1 μg.
[a] n.d. = Not detected. Other phenolics not reported in this table gave no signal under the experimental conditions used.

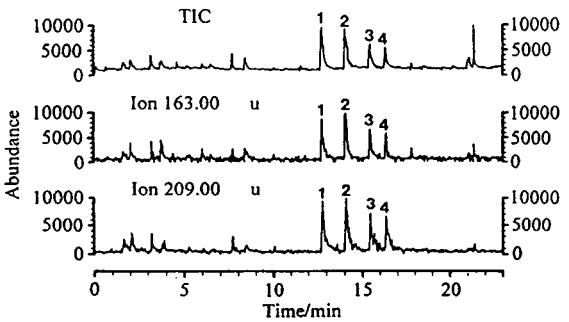

Fig. 6. Total ion current and selected-ion chromatograms of a ligand-exchange chromatographic fraction of SSL under NI TSP-MS conditions. Peaks 1 and 2 are from SSL 1 h and peaks 3 and 4 are from SSL 3 h. Ions monitored correspond to $[M-H]^-$ and $[M+HCOO]^-$ with m/z values of 163 and 209, respectively. Carrier stream, methanol–water (50:50) containing 0.05 *M* formate at 1 ml/min.

in the sample. Although only *m*-coumaric acid could be identified in these samples there are many other peaks with various masses. These ions are seen after background correction and in many instances have high molecular masses, above 2000. Hence it cannot be qualitatively proved that these compounds are breakdown products of lignin, with aromatic structures as shown in earlier investigations [28]. Studies of the use of LC–TSP-MS for identification and quantification purposes with phenolics in these types of complex samples are continuing. In this context, the use of new interfacing systems such as electrospray [38] has been demonstrated to be useful for the determination of phenolics in waste streams, in many instances in combination with MS–MS. The combination of TSP and ES may also be very powerful in this field.

4. Conclusions

This study has clearly shown the problems encountered in the determination of sugars in fermentation substrates and broths. These problems are alleviated using lignocellulose hydrolysates as a model substrate, utilizing the most commonly used LC techniques. Today, many processes are operated with subsequent analysis of these or similar carbohydrates; however, very little information is available regarding their qualitative and quantitative validation.

For the proper utilization of these lignocellulose hydrolysates it is essential that a true picture of the distribution of the various sugars is known, in addition to their individual concentrations. Different hydrolysis methods or small changes in the parameters of the process may cause alterations in the sugar yield, which may in turn cause changes in the chemical environment with which the microorganism has to cope in the fermenter. This is of special importance when new strains of microorganisms are tested as candidates for possible utilization in biotechnological processes or in understanding the metabolic pathways of different (*e.g.*, recombinant) species. These applications using a combined analytical methodology show advantages for the characterization of carbohydrates and phenolic compounds possibly present in fermentation substrates and broths interfering with the chromatographic evaluation.

It is also envisaged that ES and TSP will be used as ionization methods in conjunction with MS for quantitative purposes in the analysis of carbohydrates in complex samples. Both interfaces will be compared in the PI mode for sugars and in the NI mode for phenolics. This approach will be useful for scientists working in the biotechnological field for identifying and determining compounds present in the complex fermentation processes. The technique will also be complementary to current analytical methods used in the biotechnological field.

There are methods of avoiding interferences from matrix components in these samples, such as the introduction of small precolumns in a coupled column system in order to eliminate interfering compounds on-line in a chromatographic system. The combination of a coupled-column clean-up step may be combined with selective detection utilizing enzyme-based detection systems, specially developed for these types of fermentation samples. LC sensors based on chemical recognition as opposed to those based on physical recognition is currently under study due to the lack of such detection units. Several papers have reported the use of biological recog-

nition in sensor technology. Immobilized enzyme reactors are currently marketed by a few companies for use in pre- or postcolumn derivatization of substrates inherently difficult to monitor selectively. Enzyme electrodes have been studied mainly for the selective determination of a single substrate and have therefore found only few applications in LC. However, most enzymes are specific to a group rather than a single substrate and there are many enzymes with a broad selectivity pattern. Therefore, in this context, with recent knowledge of enzyme stabilization and organic-phase enzymology, it is expected that biological recognition will in the future have a great impact in sensor technology for LC applications.

5. Acknowledgements

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JOURNAL OF CHROMATOGRAPHY A

Screening method for the determination of volatiles in biomedical samples by means of an off-line closed-loop trapping system and high-resolution gas chromatography–ion trap detection

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Abstract

A method is described for the analysis of volatile organic compounds in biological matrices (faeces and urine). The technique is based on off-line preconcentration by means of a closed-loop trapping system followed by high-resolution gas chromatography–ion trap detection (HRGC–ITD) for separation and identification of the compounds. The technique has been validated for pattern recognition in faecal and urine samples from healthy volunteers. It is considered a very promising tool in metabolic research.

1. Introduction

For many years, volatile substances are an important object of research in medicine. Organic volatiles are studied in different human matrices such as skin [1], saliva, tissue homogenates, cerebrospinal fluid, serum [2], breath [3,4], flatus [5,6], faeces [7–9] and urine [10]. In gastroenterology volatile organic compounds (VOCs) are related to health and diseases in the gastrointestinal tract [11–14].

In this study VOCs are especially related to fermentation products released by the bacterial degradation process in the human colon. Because the colon is difficultly accessible for *in vivo* studies, we used an *in vitro* faecal incubation system. These *in vitro* studies have been applied successfully for the study of colonic fermentation [15–18].

The first aim of this study was to develop a technique useful for determination of the volatile fermentation products in biomedical samples such as faeces and urine. The second aim was to demonstrate different patterns of fermentation products, formed when different substrates were added to an *in vitro* faecal incubation system. The substrates used were a carbohydrate, a protein and a lipid suspension.

2. Experimental

2.1. Closed-loop trapping apparatus

The device described in this paper is a slightly modified one, basically described by Grob and co-workers [19–21]. This technique has successfully been applied for different complex matrices in environmental studies [22–24], but at our

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knowledge has never been evaluated in biomedical samples.

The closed-loop trapping system is modified by adding a water-condensing system, a flow meter and a gas bulb as shown in Fig. 1 (numbers between parentheses refer to the figure).

Pure nitrogen (99.9995%) (Air Products, Vilvoorde, Belgium) (1) is entering the system by a 4-way valve (Whitey, Highland Heights, OH, USA) (2) with a stainless-steel inner housing. Switching the valve opens or closes the loop. When it is closed, the nitrogen gas is continuously flowing through the loop. When the valve is open, the system is continuously flushed by pure nitrogen. For a very clean background, a charcoal filter between the nitrogen gas bulb and the 4-way valve is recommended.

The gas enters the sample container (3) vertically and leaves through an elbow. This vessel is made of glass and is easily connectable with finger tight Cajon ultra-torr couplings (Macedonia, OH, USA). The sample vessel is dipped in a thermostated water bath (4) at 37°C.

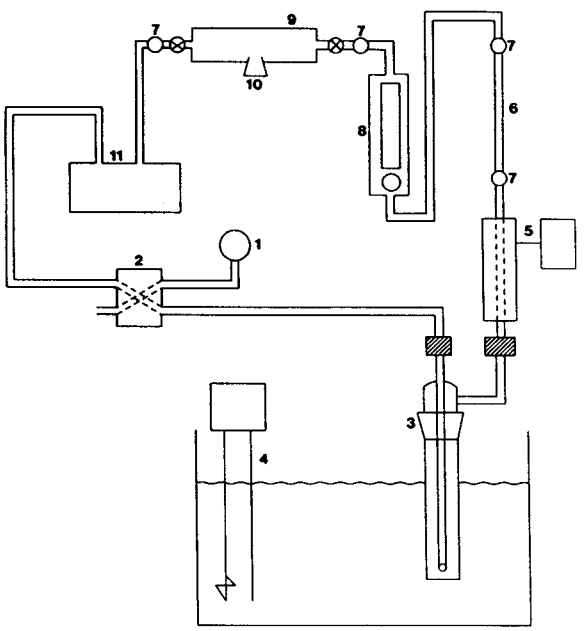

Fig. 1. The closed-loop trapping system: (1) nitrogen supply; (2) 4-way valve; (3) sample container; (4) thermostatic water bath; (5) water condensing system; (6) adsorption trap; (7) Rotulex joints; (8) flow meter; (9) gas bulb; (10) septum; (11) pump system.

Just before the adsorption trap, the glass tube is passed through a heating block (5) to minimize possible condensation of water from the gas leaving the sample container. The temperature of the heated block is kept at 60°C above the temperature of the sample container.

The adsorption trap (6) is filled with 160 mg Tenax GR (Chrompack, Middelburg, Netherlands) and is connected in the system by Rotulex knee-joints (7), providing an excellent tightness and a quick exchange. The traps are preconditioned overnight in an oven at 240°C with a gentle stream of nitrogen (99.9995%) (SoVirel, France). They are used the same day. An aluminium foil is kept around to prevent Tenax from degradation by light.

The gas bulb (Alltech, Laarne, Belgium) (9) has a volume of 125 ml and is modified with Rotulex knee-joints at the ends for quick exchange. Through the septum (10) of the flask it is possible to add internal standards or to withdraw samples.

A PTFE membrane pump (KNF Neuberger, Freiburg, Germany) (11) and a flow meter (GEO/National, Hasbrouck Heights, NJ, USA) (8) provide the flow through the system. The pump has a stainless-steel diaphragm coated with PTFE. This diaphragm is easily accessible for cleaning or exchange. The outlet of the pump is connected to the 4-way valve. The flow through the system is about 250 ml/min. All tubing is made of 1/8 in. (1 in. = 2.54 cm) stainless steel, except for the connection between the sample container and the adsorption trap, which is made of glass.

2.2. Desorption–separation–detection system

To release the VOCs from the adsorption trap, a modified thermodesorption cold trap (TCT) injector (Chrompack) is used [25,26]. Thermodesorption and cryofocussing were carried out in the following manner: holding the desorption trap at 200°C for 5 min with a helium (99.9999%) (Air Products) flow of 20 ml/min (sufficient for the desorption of the VOCs). Meanwhile the cryotrap, 0.32-mm uncoated deactivated fused silica, was held at − 120°C.

Breakthrough was non-existent under these conditions.

The VOCs concentrated in this manner in the cryotrap were released into the analytical column by heating the cryotrap to 200°C for another 20 min.

Separation and identification of the compounds were performed on a HRGC–ITD system. The gas chromatograph was a Vega 6000 instrument (Carlo Erba, Milan, Italy) equipped with a cryostat (cryo 620) with carbon dioxide, a split–splitless injector and a TCT injector. The analytical column was a 25 m × 0.32 mm CP-Sil 5 CB, film thickness 1.2 μm (Chrompack). Helium (99.9999%) at 70 kPa inlet pressure was the carrier gas. Oven temperature program: 30°C (5 min); 5°C/min; 200°C (6 min). The analytical column was coupled to the ion trap detector, model ITD 700 (Finnigan, San Jose, CA, USA) by means of an open split interface. The transfer line was held at 220°C.

Detection was carried out both in electron impact (EI) and chemical ionization (CI) mode. In the EI mode, multiple ion monitoring was used from m/z 34–39, m/z 41–43 and m/z 45–300. Automatic Gain Control was "on". Scans were averaged every second, as the result of 5 μscans. In the CI mode, isobutane 99.95% (Air Products) was used as reaction gas with a maximum ionization time of 800 μs and a maximum reaction time of 800 ms.

2.3. Reagents and standard solutions

Tenax GR was purchased from Chrompack. $HNa_2PO_4 \cdot 12H_2O$ and $H_2NaPO_4 \cdot 2H_2O$ were purchased from Fluka (Buchs, Switzerland). Pure water was purchased from the Central Bureau for Nuclear Measurements (CBNM) (Geel, Belgium). Sterile pyrogene-free water was obtained from Baxter (Lessines, Belgium). The internal standard mixture containing bromochloromethane, 1,4-dichlorobutane and 1-chloro-2-bromopropane, each 20 mg/ml methanol, was purchased from Supelco (Bellefonte, PA, USA). Hexane p.a. and acetone p.a. came from Merck (Darmstadt, Germany).

For closed-loop trapping performance control, a standard solution of acetone is made by diluting 100 ml acetone in 100 ml pure water. For urine and faecal analysis 5 μl internal standard mix is diluted in 20 ml pure water. An aliquot of 5 μl of this dilution is used for standardisation, so an amount of 25 ng for each standard on the analytical column is achieved. The standard was freshly made every day and kept at 4°C in the dark.

The buffer solution contained 2.9 g $Na_2HPO_4 \cdot 12H_2O$ and 0.7 g $NaH_2PO_4 \cdot 2H_2O$ per litre (pH 7). This solution was freshly made every day with pyrogene-free water.

The substrates added to the incubation medium were glucose, casein and Intralipid. Glucose was purchased from Cerestar (Vilvoorde, Belgium), casein from the Melkindustrie (Veghel, Netherlands) and Intralipid from Kabi Pharmacia (Stockholm, Sweden).

2.4. Sample preparation

Faecal samples

Total fresh faecal samples were diluted to obtain a faecal suspension of 1 part faeces per 124 parts of buffer solution (w/w). Dilutions were made by means of a Kenwood-mixer and kept anaerobic by pouring 30 ml of liquid nitrogen into the mixing beaker before sample addition. A 5-ml aliquot of the faecal suspension was transferred to the sample container, flushed with dry nitrogen and firmly closed by means of a clamp and two Swagelok fittings with PTFE seals at the small glass ends .

The sample container was placed in the incubator (Polylab, Antwerpen, Belgium) at 37°C for 24 h before it was plugged into the closed-loop trapping system.

For fermentation pattern recognition with different substrates 10 mg of each substrate was added to the diluted 5 ml faecal sample.

Urine samples

From a morning mid-stream urine, a 2-ml sample was transferred to the sample container and 3 ml of pyrogene-free water was added. No incubation of urine samples was performed prior to the closed-loop trapping procedure.

2.5. Methods

For determination of the reproducibility of the closed-loop trapping system, 5 ml acetone standard was added to the sample container. No adsorption trap was used in the closed loop. After 15 min of closed-loop running, the stopcocks on both ends of the gas bulb were closed simultaneously and 0.2 μl hexane was added to the flask through the septum as a calibration standard. After 15 min of equilibration, 100 μl gas was withdrawn from the flask and was injected into the splitless injector of the GC.

For determination of the faecal and the urine samples, an adsorption trap was added to the closed-loop system just before plugging in the sample container. The loop was closed by means of the 4-way valve, 5 μl of internal standard solution was added through the septum of the gas bulb and the sample container was immersed into the water bath at 37°C. After 15 min of closed-loop trapping, the adsorption trap was removed and closed on both ends with glass cocks and Rotulex clamps until analysis was carried out on the same day. Internal standard could not be added to faecal suspensions before incubation since some methanogenic bacteria may break down halogenated hydrocarbons [27]. Between each sample run a wash-out period of 30 min was performed with an empty adsorption trap and an open loop, to make sure that all trace organics left the system. The contribution of the VOCs from the buffer solution was determined by handling 5 ml buffer in the same way as the faecal samples. Blank procedure for handling urine samples was done by determination of VOCs in 3 ml water.

3. Results

3.1. Quality control of the system

To determine the purity of the whole system, an empty sample container is plugged into the closed-loop system. Internal standard is added and the procedure is run for 15 min at 37°C. A chromatogram of the system purity is given in Fig. 2. Some methylated benzene peaks are present due to the Tenax of the adsorption trap.

The reproducibility of the closed-loop system was determined by computing the ratios of the peak areas of the specific m/z from acetone and hexane (Table 1) on nine different tests. The mean of the ratios was 1.13 with an R.S.D. of 4%.

The reproducibility of the whole system (closed-loop system and HRGC–ITD) was determined by means of the three internal stan-

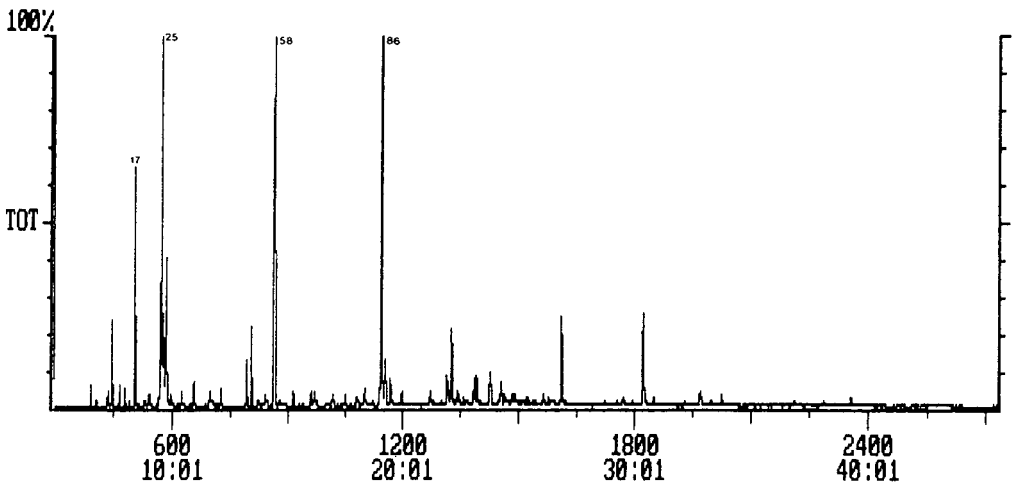

Fig. 2. Total ion chromatogram of the system purity. x-Axis: scan number and time (min:s). Peak numbers indicate compound No., listed in Table 2.

Table 1
Specific m/z values used for the calculation of the quality control of the closed-loop system

Product	Specific m/z used for calculation
Acetone	43
Hexane	57
Bromochloromethane	49
1,4-Dichlorobutane	41
1-Chloro-2-bromopropane	55

dards (I.S.) added to 5 ml pure water. The mean of the ratio of I.S. **3**/I.S. **1** was 4.46 with an R.S.D. of 5.8%. The mean of the ratio of I.S. **3**/I.S. **2** was 1.50 with an R.S.D. of 6.2%.

3.2. Volatile compounds in faeces and urine

For the determination of the volatile fermentation products, five healthy volunteers were studied. During a normal western European diet period, samples were collected and assayed as

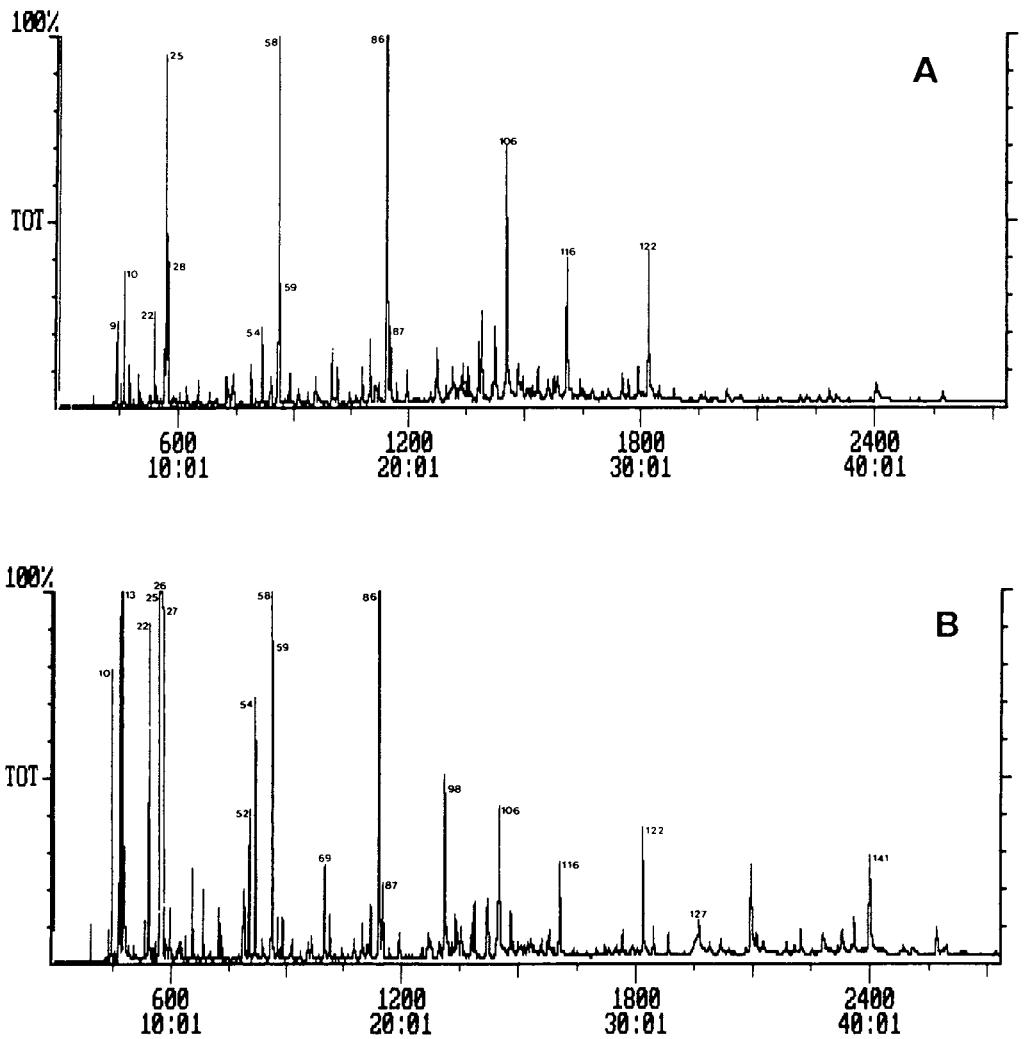

Fig. 3. Total ion chromatogram of the VOCs: (A) in the buffer solution; (B) of a typical faecal sample. Labelling of axes and peaks as in Fig. 2.

described above. Fig. 3A and B shows chromatograms of the background peaks resulting from both the system and the buffer solution and from a typical normal faecal sample.

Fig. 4A and B shows chromatograms of the background peaks from both the system and the pure water and from a typical normal urine sample.

Compounds were identified on the basis of purity and fitted matches between the EI mass spectrum generated by the ion trap detector and the EI spectrum in the computer-based NBS library (Environmental Protection Agency, National Institute of Health, Washington, DC, USA). CI was used to determine molecular masses of the different compounds. Peaks with a signal-to-noise ratio less than 10 in total ion content were not taken into account. Some products remained unknown due to difficulties in spectrum interpretation.

The peak area of the specific m/z value of all peaks was divided by the peak area of the specific m/z value of internal standard **2**, 1-chloro-2-bromopropane, to give a relative index (RI) for each compound. After subtraction of the RI of the background compounds from the

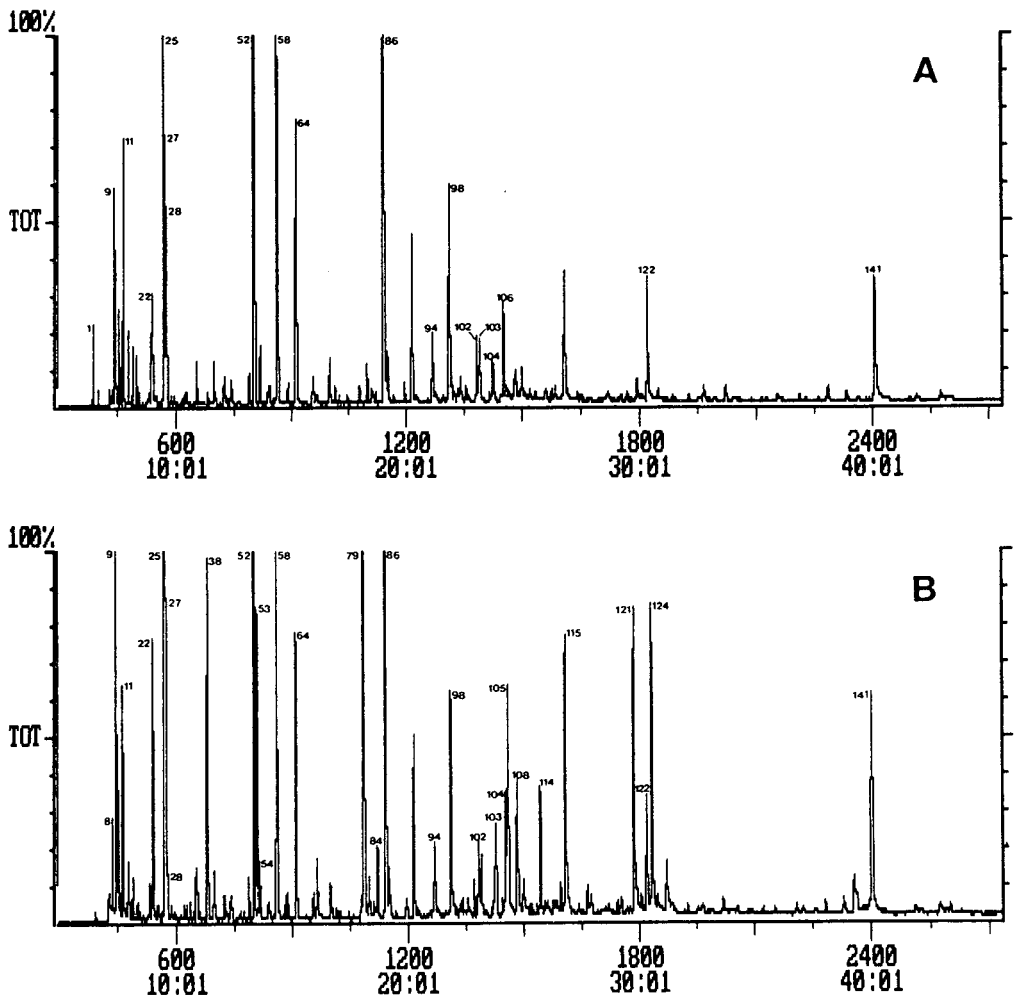

Fig. 4. Total ion chromatogram of the VOCs: (A) in the pure water; (B) of a typical normal urine sample. Labelling of axes and peaks as in Fig. 2.

Table 2
List of volatile compounds present in faeces and/or urine (F/U) and their *m/z* values, used for RI-computing

No.	Compound	F/U	*m/z*	No.	Compound	F/U	*m/z*
1. S-compounds				*3. Alcohols*			
1	Sulphur dioxide	FU	64	7	?4-Pentene-2-ol	FU	45
2	Hydrogen sulfide	FU	34	24	3-Methyl-2-butanol	FU	45
5	Methanethiol	FU	47	26	4-Methyl-2-pentanol	FU	45
6	Thioglycolic acid	FU	47				
11	Methylthiirane	FU	45	*4. Phenyl compounds*			
13	Methyl sulfide	FU	62	37	Benzene	FU	78
15	Carbondisulfide	FU	76	59	Toluene	FU	91
17	Dimethylsulphoxide	FU	79	77	Phenyl compound	FU	91
39	Methylthiocyanate	F	73	80	1,2-Dimethylbenzene	FU	91
40	2-Methyl-2-propane thiol	FU	41	87	Phenyl compound	FU	91
41	Thioacetic acid	FU	43	94	Benzaldehyde	FU	105
52	Dimethyl disulfide	FU	94	100	Phenol	FU	94
60	Methanesulphonylchloride	FU	79	103	Phenyl compound	FU	105
72	3-Pentanethiol	FU	43	105	3-Carene	FU	93
81	2-Phenyl-4-(phenylmethylene)-5(4H)-thiazolone	FU	77	108	Limonene	FU	67
				112	4-Methylphenol	FU	107
85	1,3-Propanedithiol	U	61	127	Indole	F	117
90	Methylpropyl disulfide	F	80	131	Skatole	F	130
91	2-Methyl-2-[(1-methylethyl)-thio]propane	FU	41	141	Phenol compound	F	205
				142	Diphenylamine	FU	169
98	Dimethyl trisulfide	FU	45				
110	4-Methylthiobutanenitrile	U	61	*5. Alkanes/alkenes*			
113	1,1-Methylenebis(thio)bis-ethane	U	75	8	?1-Propene	FU	41
				9	2-Methylpropane	FU	43
117	Methyl(methylthio)methyl-disulfide	F	61	12	1,3-Pentadiene	FU	67
				16	Pentane	FU	41
123	Dimethyl tetrasulfide	FU	45	29	Methylpropene	FU	41
				36	1-Butene	FU	41
2. Acids and esters				65	3-Octene	FU	41
30	Methylacetate	FU	43	97	Methyl-1,3-cyclopentadiene	FU	79
32	Acetic anhydride	FU	43				
35	2-(2-Propenyloxy)-ethanol	FU	41	*6. Ketones/aldehydes*			
43	Propanoic acid	FU	45	20	?4-Pentene-2-one	F	43
56	2-Methylpropanoic acid	FU	41	23	2-Ethylbutanal	U	43
63	Butanoic acid	FU	60	34	3-Methyl-2-butanone	FU	43
71	Pentanoic acid	FU	60	38	3,3-Dimethyl-2-hexanone	FU	43
73	2-Methylhexanoic acid	FU	74	51	2-Butenal	FU	41
74	Ester	F	57	54	3-Methyl-2-pentanone	FU	43
75	Ester	FU	43	62	2-Hexanone	FU	43
82	Hexanoic acid	FU	60	69	3-Methyl-3-pentene-2-one	FU	55
96	Ester	FU	41	79	4-Heptanone	FU	43
99	Heptanoic acid	FU	60	84	2-Heptanone	FU	43
111	Ester	FU	43	101	6-Methyl-5-heptene-2-one	FU	43
118	Ester	U	41	115	2-Nonanone	FU	43
119	Ester	F	43	129	Undecanone	FU	43
121	Ester	FU	73				
126	Ester	F	41	*7. Halogenated compounds*			
132	Ester	F	104	3	Acetylchloride	FU	43
134	Ester	F	104	28	Trichloromethane	FU	83
138	Ester	F	104	31	Fluoro compound	FU	51
139	Ester	F	104	44	Fluoro compound	FU	51

(*Continued on p. 340*)

Table 2 (*continued*)

No.	Compound	F/U	*m/z*	No.	Compound	F/U	*m/z*
50	Bromohexane	FU	43	78	2,3-Dihydropyrane	FU	41
55	Fluoro compound	FU	51	83	Unknown	FU	39
66	Fluoro compound	FU	51	88	Unknown	FU	41
67	2-Bromo-2-methylpentane	FU	41	92	Unknown	F	43
68	Tetrachloroethene	FU	35	93	Unknown	F	57
76	Fluoro compound	FU	51	95	β-Pinene	FU	93
89	Fluoro compound	FU	51	102	Unknown	FU	41
				104	Unknown	FU	281
8. Miscellaneous/unknown				106	Unknown	FU	57
4	Unknown	FU	43	107	Unknown	F	67
10	Diisopropyl ether	FU	45	109	Unknown	FU	41
14	Unknown	FU	59	114	Unknown	FU	93
18	1,2-Dimethylhydrazine	FU	42	116	Unknown	FU	41
19	Unknown	FU	43	120	Unknown	U	45
21	Unknown	FU	51	122	?Tetradecyloxirane	FU	41
22	Unknown	FU	43	124	Unknown	FU	41
27	Unknown	U	43	125	Unknown	U	39
33	Unknown	FU	41	128	Unknown	F	148
42	Unknown	U	43	130	Unknown	FU	43
45	2-Methyl-1-nitropropane	FU	41	133	Unknown	F	161
46	Hydroxylamino compound	FU	41	135	?O-Decylhydroxylamine	FU	41
47	Unknown	FU	39	136	Unknown	FU	43
48	Furanone compound	FU	41	137	?Caryophyllene	F	41
49	Pyridine	FU	52	140	Unknown	FU	43
53	1H-Pyrrole	FU	67				
57	Furanone compound	FU	41	*9. Internal standards*			
61	1,3-Epoxy-4-methylpentane	FU	43	25	Bromochloromethane (I.S. **1**)		49
64	Unknown	FU	41	58	1-Chloro-2-bromopropane (I.S. **2**)		41
70	Unknown	FU	207	86	1,4-Dichlorobutane (I.S. **3**)		55

Compounds were identified on the basis of purity and fitted matches between the electron impact mass spectrum generated by the ion trap detector and the electron impact spectrum in the computer-based NBS library. Additional information on molecular mass was given by the chemical ionization mode.

RI of the sample compounds, 142 compounds (internal standards included) remained. These compounds are listed in Table 2. An attempt is made to classify these compounds in nine categories following their chemical nature. Additional characterization is done by appearance of each compound in faeces and/or urine, and by specific *m/z* value used for RI computation. This table is the result of all compounds found in 5 healthy volunteers.

3.3. Quality control of the sample handling

The reproducibility of the analytical procedure was examined from homogenization of the faecal samples to detection and quantitation of the volatile compounds by mass spectrometry. For this purpose, three faecal samples from the same fresh faecal aliquot have been taken. Peak areas at specific *m/z* values (Table 2) were determined and R.S.D. values of each compound have been calculated. Out of 90 products found in this faecal sample, 58 had an R.S.D. $< 10\%$, 18 had an R.S.D. between 10 and 20% and 14 products had an R.S.D. $> 20\%$. Of the 14 compounds in the last series, 7 had a signal-to-noise ratio < 10 in specific *m/z* mode, 2 were overloaded and 5 showed bad chromatographic resolution.

The reproducibility of handling urine samples was examined in the same way. Three urine samples from the same urine stock sample were examined. Out of 81 products found in the urine

sample, 69 had an R.S.D. < 10%, 8 had an R.S.D. between 10 and 20%, and 4 had an R.S.D. > 20%. From the last series, 2 had a signal-to-noise ratio <10 in specific m/z mode and 2 were overloaded.

3.4. Fermentation patterns of different substrates

To determine the influence of substrates on the formation of volatile compounds by fermentation processes, different substrates were added to the faecal samples.

Figs. 5, 6 and 7 show a faecal sample incubated with glucose, casein and Intralipid, respectively.

Upon careful examination of chromatographic patterns of Figs. 5–7, different patterns in formation of VOCs are obtained, which are summarized in Table 3. Upon adding glucose to the incubation system, short-chain fatty acids (SCFA) are predominant. They are quantitatively the principal metabolites of carbohydrate fermentation. No primary alcohols could be detected due to limitations in the mass selection of the ITD. A faecal sample incubated with casein shows a different pattern, as mainly the sulphur compounds are present in relatively high concentrations. Indole and skatole could only be demonstrated in case Intralipid was added to the incubation medium.

4. Discussion

In this study, a closed-loop trapping system has been developed to study the formation of volatile compounds in faecal media and urine. Different methods of trapping and concentrating trace substances have been described. A short survey of preconcentration methods in capillary GC has been published by Roeraade [28], and trapping on solid adsorbents has been reviewed by Núñez *et al.* [29].

A closed-loop system for trapping VOCs, hitherto only used for environmental studies [22,23,24], has been applied to demonstrate VOCs in faecal and urine samples. At our knowledge, this is the first time that this system has been applied for the study of the formation of volatile bacterial fermentation products in the human colon. The rationale of this study is that in the last decade there is a growing interest in bacterial fermentation products as causing agents for colonic cell epithelium damage and systemic diseases. Indeed, the colon is an open system with nutrients continuously flowing into the

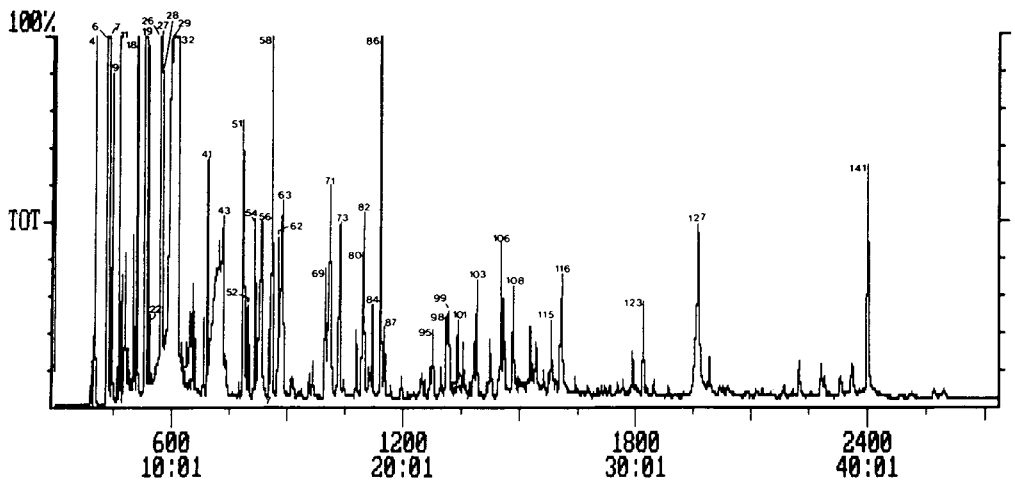

Fig. 5. Total ion chromatogram of the VOCs of a faecal sample incubated *in vitro* with glucose. Labelling of axes and peaks as in Fig. 2.

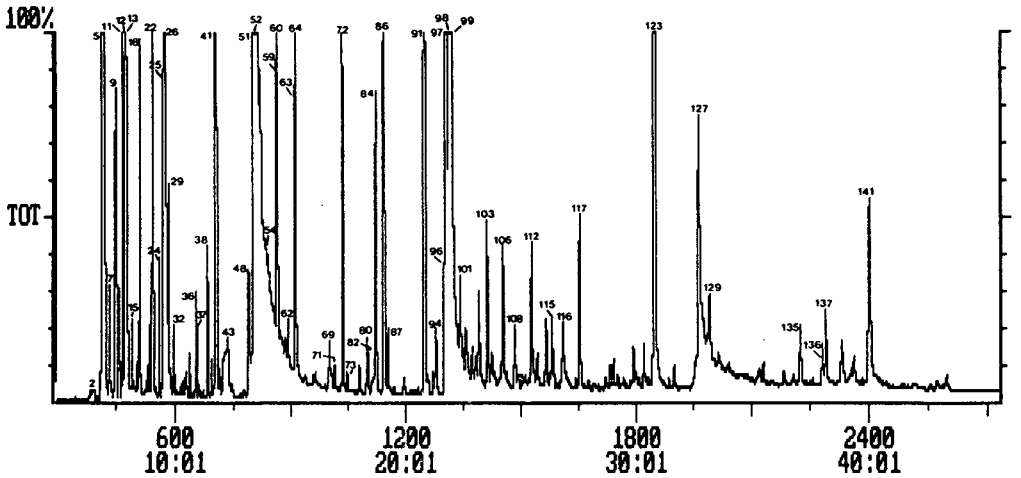

Fig. 6. Total ion chromatogram of the VOCs of a faecal sample incubated *in vitro* with casein. Labelling of axes and peaks as in Fig. 2.

caecum, and where the end products are excreted as faeces. This 1.5 meter anaerobic system contains about 90 g of total bacterial mass [9], representing more than 400 different bacterial species. They degrade the luminal content of the colon to a wide variety of metabolites. Some of them are beneficial for the host [30], others are known to be toxic [31,32] or even potentially carcinogenic [33,34]. The control of fermentation is thus of critical importance to man. An excellent overview of the degradative processes occurring in the colon is given by Macfarlane and Cummings [8,35].

To understand the profile of fermentation products in normal individuals, methods to analyse short-chain fatty acids [36] and bacterial fatty acids composition [37] have been described. The present study on analysis of VOCs is to be considered as an additional tool to gain insight in the fermentation processes.

The authors are well aware of the fact that these analyses show some fundamental short-

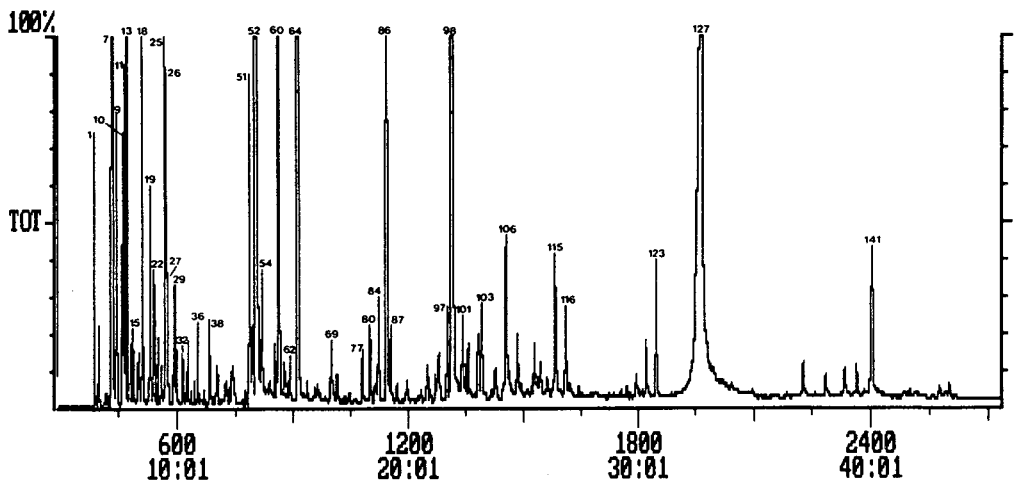

Fig. 7. Total ion chromatogram of the VOCs of a faecal sample incubated *in vitro* with Intralipid. Labelling of axes and peaks as in Fig. 2.

Table 3
The most important volatile compounds of the *in vitro* substrate study with their RI-values

No.	Blank	Glucose	Casein	Intralipid	Product
2	0	0	26	2	Hydrogen sulfide
5	0	0	15 198	8	Methanethiol
6	0	9801	0	0	Thioglycolic acid
7	22	4426	427	0	4-Pentene-2-ol
13	1210	54	8718	980	Dimethyl sulfide
26	394	2313	3505	304	4-Methyl-2-pentanol
32	0	8121	660	190	Acetic acid anhydride
35	0	102	4	27	2-(2-Propenyloxy)ethanol
41	0	614	9008	66	Thioacetic acid
43	0	1714	384	68	Propanoic acid
52	123	80	50938	1644	Dimethyldisulfide
56	0	524	0	0	2-Methylpropanoic acid
60	0	0	184	7	Methanesulphonylchloride
63	0	630	20	0	Butanoic acid
71	0	537	10	0	Pentanoic acid
72	0	0	760	0	3-Pentanethiol
73	0	333	3	0	2-Methylhexanoic acid
82	0	360	24	0	Hexanoic acid
91	6	36	1875	58	2-Methyl-2-[(1-methylethyl)thio]propane
98	83	98	4674	554	Dimethyl trisulfide
99	0	28	534	0	Heptanoic acid
100	8	8	50	27	Phenol
112	95	94	291	80	4-Methylphenol
117	0	0	555	6	Methyl(methylthio)methyldisulfide
123	4	5	4087	197	Dimethyl tetrasulfide
127	44	616	1136	2364	Indole
131	0	0	0	5	Skatole
142	2	3	11	7	Diphenylamine

comings because they have been done on faeces, which is an end product. But sampling "*in situ*" is not possible. In the past, this indirect approach, together with faecal incubation studies, has been proved to give reliable results on colonic fermentation processes [15–18]. Moreover, authors agree that inter-individual changes, due to different diets and different bowel habits, may influence analysis outcome.

Therefore, maximal precaution has been given to the described method to minimize errors in analytical data. As the described system, *i.e.* closed-loop trapping thermodesorption–gas chromatography–ion trap detection, is very sensitive for external contamination, each step has been controlled carefully. As an example, the contamination of water, originating from the Central Bureau of Nuclear Measurements, is given. Although this water has been chosen out of six other water samples and considered the purest, there are still volatile contaminants present. Additionally highest attention in interpreting mass spectra has been paid, and, although CI mass spectra have been used to complete EI data, it was not possible to identify all compounds. Due to the mass spectrometric overload at lower mass range, multiple ion monitoring could only be performed within specific mass ranges. By doing this, all primary alcohols (specific m/z 30 and 31) have been excluded from analysis. Compounds with signal-to-noise ratios less than 10 also have been excluded (in an arbitrary way) from data interpretation. This does certainly not mean that they would not exert any physiological significance, as trace quantities also may have great impact on colonic cell functions.

In this study, quantitative analysis of com-

pounds has not been done, due to the quasi impossible task to study response factors of each compound *versus* internal standards. These internal standards however are of great importance to control the stability of the system and to support the reliability of the qualitative data. Due to the sometimes poor chromatographic resolution in total ion mode, all compounds were examined with their specific m/z value. The calculation with relative indices gives only a first approximation on quantitative interpretation of data. There is a large difference between the contribution of the specific m/z values of each compound to the total mass of each compound. Attention should also be paid to the fact that equimolar quantities of different compounds give different responses in the detector.

Out of 90 compounds in faeces, 58 had an R.S.D. value of less than 10%; 14 products had an R.S.D. value of more than 20%. The latter was due, either to very low signal-to-noise ratios (≤ 10) or to product overload, which may cause misinterpretation of mass spectra. This study is the first attempt to gain insight in formation of volatile compounds upon bacterial fermentation in the colon. Some firm statements may already be made: formation of short-chain fatty acids upon carbohydrate presence. They are rapidly absorbed from the human large bowel, so their concentration in a blank faecal sample is relatively low [8]. Another statement is the formation of sulphur-containing compounds upon protein (casein) incubation. These compounds are intermediary or end products of the breakdown of cysteine and methionine, two amino acids present in casein. Phenol and *p*-cresol are end products of the fermentation of tyrosine, also present in casein [38]. The formation of acids has already been confirmed in other "*in vivo*" studies, whereas ongoing "*in vivo*" investigations on supplementary protein intake do confirm the faecal incubation studies, described in this study [39]. Addition of lipid (as Intralipid) shows only minor differences in the VOCs chromatogram. It seems reasonable to assume that the appearance of indole and skatole might be due to the protein content [40] of the Intralipid solution, rather than to the lipid content itself.

This study aims to contribute substantially to better knowledge of metabolite formation due to bacterial anaerobic metabolism. Together with other chemical compounds, VOCs may contribute in metabolic pattern recognition. This may lead to better understanding of pathophysiological processes occurring in the colon, and may also influence the etiology of diseases, related to the systemic system.

In conclusion the combination of an off-line preconcentration by means of a closed-loop trapping system followed by GC–ITD is a reliable method for screening volatiles in faecal and urine samples on a qualitative base.

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