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POST-COLUMN EXTRACTION SYSTEM FOR USE WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple system for on-line sample extraction following reversed-phase high-performance liquid chromatographic (HPLC) analysis is presented. The system can be used to prepare samples for direct probe insertion mass spectrometry (MS), infrared analysis or other techniques. The extraction cell has also been used for continuous on-line HPLC–MS. The system is based on post-column chloroform extraction of the eluted compounds from polar mobile phases consisting of methanol or acetonitrile–0.1 *M* potassium dihydrogen orthophosphate buffer or water. The organic solvent is mixed with the column effluent using a commercially available post-column reaction device. The non-polar phase is separated by pressurized filtration through a Teflon membrane, which acts as a barrier to the aqueous portion of the eluent. The extracted solutes are detected using an ultraviolet spectrophotometer. A portion of the organic solvent that contains the eluted component can be collected easily, examined by other analytical techniques or, as will be shown, the solvent can be applied to a moving belt interface for introduction into a mass spectrometer.

The system has been used for the extraction and detection of four model compounds (dimethyl, diethyl, diisobutyl and diamyl phthalate esters). The linear range of detection by on-line mass spectrometry was shown to extend to at least 1.2 μg of each ester.

INTRODUCTION

Extraction systems for continuous flow phase separation have been used successfully in segmented and non-segmented flow injection analysis (FIA) and in post-column high-performance liquid chromatography (HPLC) with fluorimetric[1–5], and mass spectrometric (MS) detection[6,7]. These systems mostly rely on a "Tee"-type glass extractor, which is similar to that developed for air-segmented continuous flow

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analysis. Recently, Fossey and Cantwell[8] described a PTFE membrane separator for use in FIA.

This type of separator has several distinct advantages over the "Tee" device. First, the organic solvent must pass through the pores of the membrane so that a physical barrier exists to prevent the aqueous portion of the mobile phase from contaminating the extract. Second, the membrane acts as a filter and thereby prevents extraneous matter from the column or solvents from entering the detector. Third, with the proper design, the membrane can be used for a considerable period of time (several months) without any observable deterioration.

On-line extraction of HPLC effluent, under controlled conditions, can provide reproducible results with increased specificity and in addition reduce the sample preparation time when off-line analysis is required.

In this study, we have designed a PTFE membrane, organic-phase extractor for post-column use with reversed-phase HPLC. Using chloroform as the extraction solvent, the device has been used successfully with on-line spectrophotometric and mass spectrometric analyses and as a means of preparing samples for off-line post-column infrared or direct probe mass spectrometric analysis.

EXPERIMENTAL

Reagents

Dimethyl, diethyl, diisobutyl and diamyl phthalate esters were purchased from Chem Service (West Chester, PA, U.S.A.). A stock solution containing about 40 mg of each of the esters was prepared in 50 μl of acetonitrile. Dilutions of this solution were made as required. Acetonitrile and chloroform (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) were of HPLC grade. Potassium dihydrogen orthophosphate (KH_2PO_4) (Mallinckrodt, Paris, KY, U.S.A.) was of analytical-reagent grade.

High-performance liquid chromatography

For the on-line spectrophotometric studies and off-line infrared analysis, the mobile phase pump was a Spectra-Physics Model 8770 (Spectra-Physics, San Jose, CA, U.S.A.), and the sample injector was a Valco six-port loop injector (VICI, Houston, CA, U.S.A.), fitted with a 15-μl loop. A Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 1040A UV–visible detector was programmed to monitor the absorbance of the chloroform extract at 254 nm. A Digilab FTS-15C Fourier transform IR instrument was used to generate the infrared spectra.

For on-line HPLC–MS studies, a Spectra-Physics Model 3500B high-performance liquid chromatograph operated in the isocratic mode was used as the pumping system. The mass spectrometer was a Finnigan (San Jose, CA, U.S.A.) Model 4500 equipped with a moving-belt HPLC interface. A Schoeffel Model 770 HPLC detector was used in line to locate the separated bands.

Off-line MS studies were conducted on an LKB 9000 S mass spectrometer using direct probe insertion.

In all of the studies, either a Spectra-Physics RP-8 or a Hewlett-Packard C_8 reversed-phase column was used. Unless defined otherwise, the mobile phase consisted of acetonitrile–0.1 *M* KH_2PO_4 (1:1) at a flow-rate of 1.0 ml/min.

All tubing used with this equipment was 0.009 in. I.D. stainless steel, except the line from the extractor to the MS interface, which was 0.009 in. I.D. PTFE.

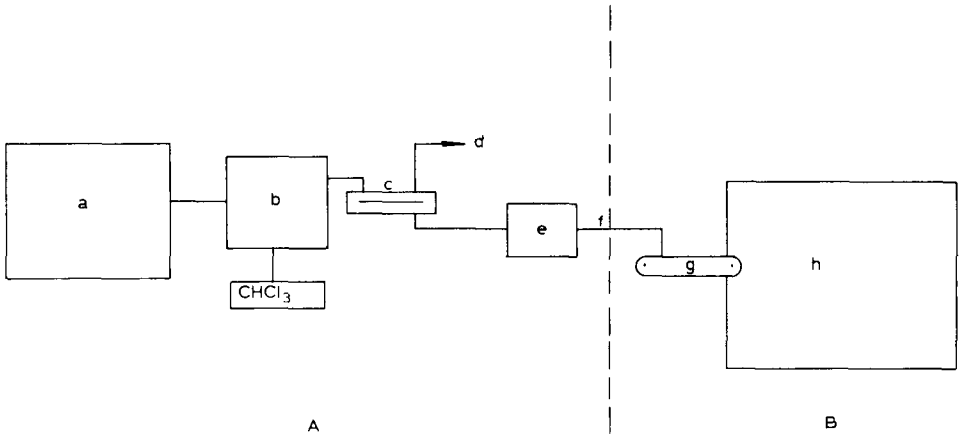

Fig. 1. (A) Basic system design for spectrophotometric detection and sample trapping. (a) High-performance liquid chromatograp; (b) post-column reactor system; (c) extractor; (d) waste line; (e) spectrophotometer); (f) collection point for off-line analysis. (B) Mass spectrometer interface. (g) Moving belt interface; (h) mass spectrometer.

Extraction system

The basic instrumentation used for most of these studies is shown in Fig. 1A. The effluent from the HPLC column is mixed with chloroform in a post-column reactor system (Model URS 051; Kratos Analytical Instruments, Ramsey, NJ, U.S.A.). This consists of two pumps and mixing chambers and a 2.0-ml delay coil that provides for mixing and allows time for the extraction to proceed.

Following the extraction step, the chloroform–mobile phase mixture moves into the extractor, which is shown diagrammatically in Fig. 2. This consists of two stainless-steel blocks, 1¼ in. wide and 2 in. long. Each of these blocks contains a channel that is 0.260 in. wide, 1.60 in. long and 0.003 in. deep. On the HPLC side of the separator, there are inlet and outlet holes at either end of the channel (0.02 in. I.D.). An outlet (0.02 in. I.D.) is located on the detector side of the device. A "race track" has been designed into the inlet side of the device that surrounds the channel. This is actually a ridge that is 0.002 in. high and is used to hold the PTFE membrane in place during the analysis and reduce the tendency of the membrane to collapse into the shallow channel. The total volume of each channel is approximately equal to three times the volume of a similar length of 0.009 in. tubing. The extractor used for these studies was manufactured by J. L. Behmer (Philadelphia, PA, U.S.A.).

A polyethylene-backed, 0.5-μm pore size, PTFE membrane (FHLP 047-00) (Millipore, Bedford, MA, U.S.A.) is folded over on itself (over the smooth side) and cut to fit the separator. The polyethylene mesh backing from another membrane is stripped and cut to fit into the inlet side channel. This aids in reducing the volume of the channel and also promotes separation of the organic–aqueous mixture. The membranes have been shown to last for up to 6 months before replacement is necessary.

A piece of stainless-steel tubing (0.09 in. I.D.) is attached to the waste outlet. Studies have shown that this length of tubing provides the necessary back-pressure

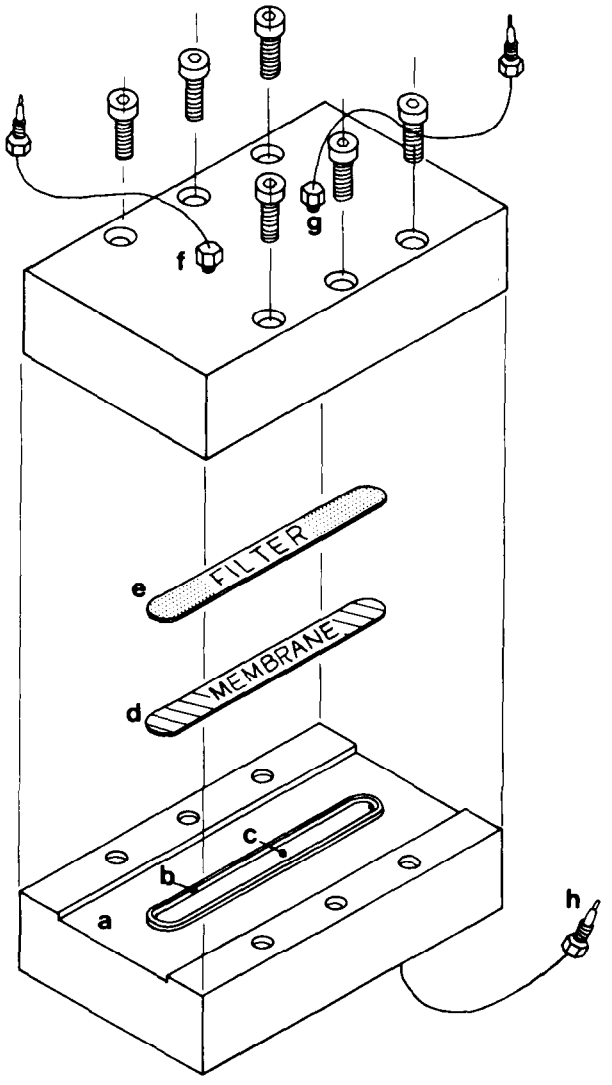

Fig. 2. Extractor. (a) Platform for membrane; (b) "race track"; (c) organic phase channel; (d) PTFE membrane; (e) polyethylene backing from PTFE membrane; (f) inlet of mobile phase and organic solvent mixture; (g) outlet to waste; (h) outlet to detector.

to promote filtration of the organic phase through the Teflon membrane. This tubing is used as a flow restrictor is the same way as auxiliary pumps or vacuum systems have been used in previously cited work.

RESULTS AND DISCUSSION

The characteristics and limitations of the extractor were studied using chloroform as the extracting solvent with several different reversed-phase systems con-

TABLE I

FLOW-RATE OF CHLOROFORM TO DETECTOR FOLLOWING SEPARATION AT EXTRACTOR

Mobile phase flow-rate: 1.0 ml/min.

Mobile phase components	*Proportions*	*Chloroform flow-rate (ml/min)*	
		1.0 ml/min	*0.5 ml/min*
Acetonitrile–0.1 *M* KH_2PO_4	0:100	0.36	0.20
	25: 75	0.33	0.10
	50: 50	0.38	0.09
	60: 40	0.36	0.10
Acetonitrile–water	0:100	0.38	0.07
	25: 75	0.36	0.08
	50: 50	0.37	0.08
	60: 40	0.37	0.07
Methanol–0.1 *M* KH_2PO_4	25:75	0.52	0.09
	50:50	0.44	0.09
	60:40	0.44	0.09
Methanol–water	25:75	0.42	0.09
	50:50	0.44	0.08
	60:40	0.40	0.08

sisting of various amounts of either methanol or acetonitrile combined with water and 0.1 *M* KH_2PO_4. All of these evaluations were conducted with an octylsilane column using the instrument configuration shown in Fig. 1A.

A primary concern for any on-line extraction system centers around the amount of organic phase that is delivered to the detector and that which is sent to waste.

The amount of organic waste that is separated from the extraction mixture when various mobile phase compositions were used was studied at two different flow-rates of chloroform. From the data presented in Table I, it is readily apparent that although the amount of organic phase that crosses the Teflon barrier is dependent on the basic composition of the mobile phase, the effluent velocity remains relatively constant as the methanol or the acetonitrile concentration is changed. Additionally, the data collected using chloroform at a flow-rate of 1.0 ml/min show a marked difference between methanol and acetonitrile. When these extractions were conducted in separating funnels, it was apparent that any emulsion formed broke down more rapidly with acetonitrile than with methanol. It appears that the increase in volume extracted is due to the rate of clarification of the sample. That is, the clarified organic phase passes through the pores of the membrane more easily than an emulsion.

Similarly, these studies show that when chloroform is used as the extracting solvent, the concentration of both acetonitrile and methanol cannot exceed 60% in either water or 0.1 *M* KH_2PO_4, as above this concentration a stable emulsion is formed that can permeate the PTFE membrane. This allows for the introduction of

the aqueous phase into the detector or collector. Early attempts to extend this limit beyond 60% by the simultaneous addition of chloroform and 10% sodium chloride appeared promising, but more work is required to verify this point.

As chloroform is able to extract acetonitrile from aqueous solutions, the total volume of the organic phase is increased when this extraction is performed. In this regard, samples that are generally too polar to be extracted into chloroform alone have been separated from reversed-phase systems of acetonitrile and 0.1 *M* KH_2PO_4 (25:75).

Classical linear gradient elution as well as hyperbolic and hypobolic gradient elution systems have been used successfully. When methanol was added to water or 0.1 *M* KH_2PO_4 at concentrations ranging from 10 to 50%, no perturbation of the baseline was observed at 254 nm. However, owing to the solubility of acetonitrile in chloroform, a measurable negative slope was observed that mirrored the gradient being studied. When a linear gradient was employed, this was easily compensated for by starting the recorder baseline at 40% of full scale.

The maximum flow-rate of the mobile phase that could be used for the chromatographic separation was determined using acetonitrile–0.1 *M* KH_2PO_4 (60:40). Chloroform was pumped into the extractor at flow-rates of 0.5 and 1.0 ml/min. These studies show that a combined flow-rate of 2.3 ml/min for the mobile phase and chloroform is apparently the limit for this system. Any attempt to increase the flow-rate of the mobile phase beyond 1.3 ml/min while pumping chloroform at 1.0 ml/min or beyond 1.8 ml/min with chloroform at 0.5 ml/min led to the introduction of the aqueous phase into the detector. This limit is thought to be due to the pressure exerted on the membrane by the mixed solvents and signifies a point at which the pore size of the membrane is widened and passage of the aqueous solvent is permitted.

The effect of increased column temperature on the extraction was studied using both methanol and acetonitrile with 0.1 *M* KH_2PO_4 (40:60) at 25, 30, 40 and 50°C. No perturbation of the baseline was observed over this temperature range when the organic solvent was monitored at 254 nm. During this study, the delay coil and extractor were left at room temperature (18°C).

The efficiency of the extractor for desalting was determined using mobile phase compositions of either acetonitrile or methanol with 0.1 *M* KH_2PO_4 (40:60). The mobile phase and chloroform were both pumped at a flow-rate of 1.0 ml/min. A 50-ml sample of the extract was collected after passing through the spectrophotometer and evaporated to dryness with a stream of dry nitrogen. In a similar manner, mobile phases were prepared using water in place of KH_2PO_4 and the extracts collected were to serve as blank reference solutions. Following evaporation, 5 ml of 70% perchloric acid were added to each flask to dissolve any residue present. These solutions were then analyzed using the vanadium phosphomolybdate procedure at 400 nm[9]. No phosphate ion in addition to that observed in the respective blanks was determined. This capability provides a means of rapidly preparing samples for analysis by techniques such as infrared and mass spectrometry, where contamination of the sample by inorganic ions could complicate the results.

To study the effect of the extractor on band broadening, reproducibility of analysis and applicability to off-line infrared and mass spectrometric analysis, as well as on-line mass spectrometry, dimethyl, diethyl, diisobutyl and diamyl phthalate esters were chosen as model compounds. These compounds are easily separated from

TABLE II

REPRODUCIBILITY STUDIES ON FOUR PHTHALATE ESTERS USING ON-LINE EXTRACTION WITH CHLOROFORM FOLLOWING SEPARATION BY ACETONE–0.1 *M* KH_2PO_4 (1:1)

Ester	*Relative standard deviation (%)*
Dimethyl phthalate ester	2.51
Diethyl phthalate ester	1.73
Diisobutyl phthalate ester	5.99
Diamyl phthalate ester	4.74

polar media with chloroform owing to their relative non-polar character. In addition, owing to their ubiquitous presence, they are often encountered as contaminants in samples following contact with plastics and therefore are well worth examination.

In all of the studies outlined below, a mobile phase consisting of acetonitrile–0.1 *M* KH_2PO_4 (1:1) was used with an octylsilane (C-8) HPLC column operated at ambient temperature at a flow-rate of 1.0 ml/min. Each of the esters was prepared at a concentration of between 80 and 120 ppm in acetonitrile. Chloroform was used to effect the extraction at a flow-rate of 0.5 or 1.0 ml/min as noted.

The effect of the extractor on band broadening was determined for the four phthalate esters with the chloroform flow-rate at 1.0 ml/min. A UV–visible detector was used to monitor the peaks at 254 nm. Unextracted samples were monitored by connecting the column effluent line directly to the spectrophotometer. The band width at half-height was compared for each of the esters and, on average, an increase of 12% was observed when the samples were extracted. This is apparently due to the effect of the mixing coil on the post-column reactor and the internal volume of the extractor. No attempt has yet been made to reduce this effect, but further studies are planned.

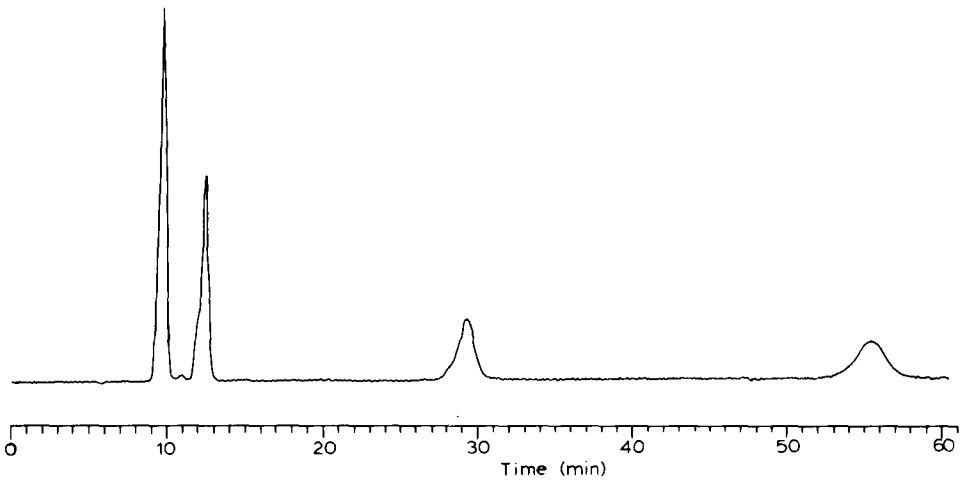

Fig. 3. Chromatogram recorded at 254 nm following on-line extraction with chloroform. The elution order is dimethyl, diethyl, diisobutyl and diamyl phthalate ester.

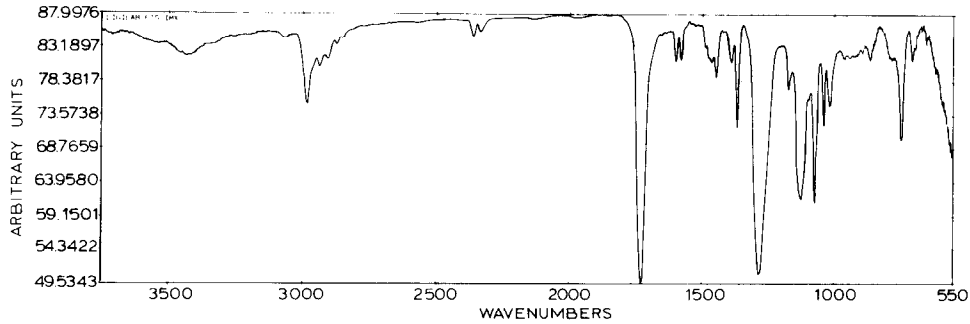

Fig. 4. Infrared spectra of dimethyl phthalate collected after separation by reversed-phase HPLC and on-line extraction into chloroform.

The reproducibility of the signal generated at 254 nm for each of the esters was studied using the conditions stated above with a chloroform flow-rate of 1.0 ml/min. The results obtained from ten 40-μl injections are shown in Table II for each of the esters. A representative chromatogram from this analysis is presented in Fig. 3. The apparent decrease in precision for the higher-molecular-weight esters is thought to be due to reduced peak intensity at the exaggerated retention times.

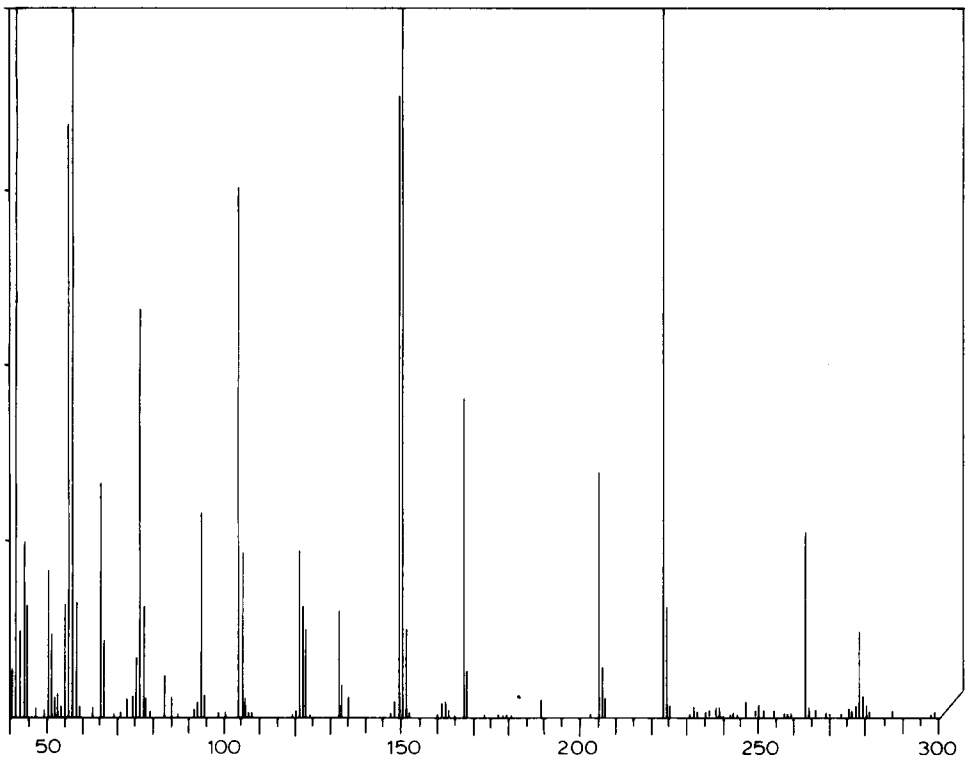

Fig. 5. Probe insertion electron-impact mass spectrum of isobutyl phthalate ester following separation by reversed-phase HPLC and on-line extraction with chloroform.

Following detection at 254 nm, the sample can easily be collected and further analyzed. To demonstrate this point, the diethyl phthalate peak was collected in an agate mortar from three successive injections of the sample used for the precision studies. The solvent was evaporated to dryness with a stream of dry nitrogen. A syringe containing 100 μl of chloroform was used to redissolve the sample and to apply the solution to a 1-cm potassium bromide pellet. Once the solvent had evaporated, the Fourier transform IR spectrum was recorded (Fig. 4). This is a very convenient means of preparing samples for infrared analysis, as there is little chance of losing any of the separated sample, and the sample workup time was reduced to about 4 min.

To determine the applicability of this procedure to off-line mass spectrometric analysis, a band corresponding to the isobutyl phthalate ester from a single injection was collected in a glass ampoule. The solvent was evaporated to about 100 μl using dry nitrogen. This was applied to the MS probe, evaporated to dryness and inserted into the instrument. The electron-impact mass spectrum obtained is shown in Fig. 5. Again, the sample was obtained following spectrophotometric detection in a form amenable to this type of analysis. No residue was observed on the probe following analysis, nor were any problems encountered in the operation of the instrument.

Finally, on-line HPLC–MS studies were conducted using a moving belt interface to the mass spectrometer. The equipment was arranged as shown in Fig. 1B. For this series of studies, the mobile phase composition was maintained as acetonitrile–0.1 *M* KH_2PO_4 (1:1). However, the flow-rate of the chloroform into the separator

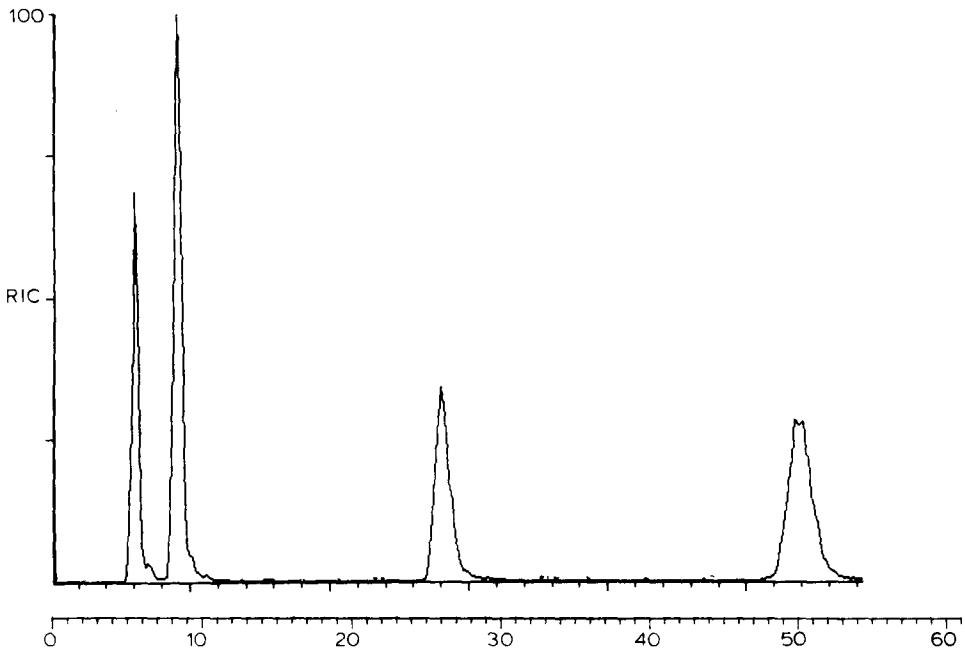

Fig. 6. Total ion chromatogram following separation by reversed-phase HPLC, on-line extraction with chloroform and introduction of the eluent into the mass spectrometer using a moving belt interface. The elution order is dimethyl, diethyl, diisobutyl and diamyl phthalate ester.

was reduced to 0.5 ml/min so that the belt would not be overloaded with solvent. The interface was operated with the external ovens on, and the front belt access door was removed to allow ventilation of the belt. The sample was applied to the belt through a stream splitter and finally through a glass capillary supplied with the instrument. At start-up, the organic solvent was directed away from the belt and the splitter was gradually adjusted until all of the solvent was deposited on the belt at a flow-rate of 0.09 ml/min.

As the total ion chromatogram in Fig. 6 shows, this method of sample application was successful. The total ion peak areas obtained by injecting various sample volumes representing from 1.2 to 120 μg produced a linear response for all four of the esters. An average correlation coefficient of 0.998 was obtained when the areas were compared with the sample mass injected.

Mass spectra were obtained for each of the eluted compounds. These were compared with spectra gathered for the neat phthalate esters using probe analysis. Each of the spectra show the presence of a parent ion (m/z = 194, 218, 278 and 306 for dimethyl, diethyl, diisobutyl and diamyl phthalate ester, respectively). The comparison also shows the presence of two major ions for each ester that can easily be accounted for by the loss of the alkyl group or the alkoxy functional group. For all of the spectra obtained using the HPLC interface, a base peak at m/z 149 was observed. The comparison clearly indicates that the molecules are not adversely affected by the chromatography or extraction procedure and that reliable data can be obtained using this procedure.

CONCLUSION

The extraction device presented offers a simple and easily implemented tool for the investigation of components separated from non-volatile, polar mobile phases when the compounds are amenable to extraction with chloroform. Samples of the model compounds were easily quantified and identified using this technique. This device has been shown to reduce dramatically the time required to prepare samples for further analysis following separation by HPLC.

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