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COUPLED COLUMN CHROMATOGRAPHY EMPLOYING EXCLUSION AND A REVERSED PHASE

A POTENTIAL GENERAL APPROACH TO SEQUENTIAL ANALYSIS

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SUMMARY

The application of on-line, coupled column chromatography (CCC) using exclusion chromatography on microparticles as the preliminary separation technique and reversed-phase chromatography as the secondary technique is described. The potential universality of the CCC approach is illustrated by applications in three areas: additives in compounded rubber, the pesticide malathion in vegetable matter and limonin in grapefruit peel. The advantages and limitations of the coupling technique are discussed. The use of a double-beam variable-wavelength spectrophotometric detector set at 215 nm as a "universal" detector for exclusion chromatography with unstabilized tetrahydrofuran as the mobile phase is considered.

INTRODUCTION

Coupled column chromatography (CCC) is a powerful technique for separating multi-component samples in high-performance liquid chromatography (HPLC)[1–3]. In the CCC approach, fractions from one column are transferred selectively to one or more secondary columns for further separation. The columns may be of the same type (*e.g.*, silica) but with different phase ratios[2,4] or they can be of different types such as silica gel and bonded nitrile[5,6].

In principle, the CCC technique can be off-line or on-line. Off-line CCC is carried out by collection of solutes at the detector exit of the first column and re-injection onto the second column. On-line CCC is achieved through coupling to a second column by means of a switching valve. This valve either traps a defined volume of collected sample, usually in a loop, and directs it on to the second column, or it can divert the mobile phase containing the desired solute from the first to the second column for a defined period of time. Obviously, on-line techniques are preferred but, as will be pointed out here, they are not always feasible.

In most instances in CCC, the mobile phase remains the same and additional selectivity or retention is achieved by changing the stationary phase. In this work, two

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modes of HPLC were used: high-performance exclusion chromatography (HPEC) and bonded reversed-phase chromatography (RPC). The first column was an exclusion column or columns whereas the second column was packed with a chemically bonded reversed-phase microparticulate packing. The mobile phase used in the first column was different from that in the second. In previous investigations[7,8] these two modes were combined but large-particle polystyrene–divinylbenzene (PS–DVB) exclusion packings were used, which gave broad elution profiles that did not permit direct coupling. The microparticulate exclusion packings of particle size 10 μm employed here gave much narrower peaks and consequently these peaks could be injected directly on to a reversed-phase column without adverse effects. As will be pointed out, this coupled column approach may prove to be a potential general approach for tackling complex samples. Three applications in diverse areas were chosen to illustrate this potential of the CCC technique.

EXPERIMENTAL

Instrumentation

Fig. 1 is a schematic diagram of the coupled column system employed. The HPEC system consisted of a Model 8510 syringe pump (Varian) equipped with a six-port sampling valve (Valco) for sample injection. The exclusion columns used were MicroPak TSK 10-μm PS–DVB gels (Varian) with various pore sizes. The actual set used varied with each application but in general the effective molecular weight range was 15,000–50 (based on polystyrene standards). Technical data for the columns employed are given in Table I.

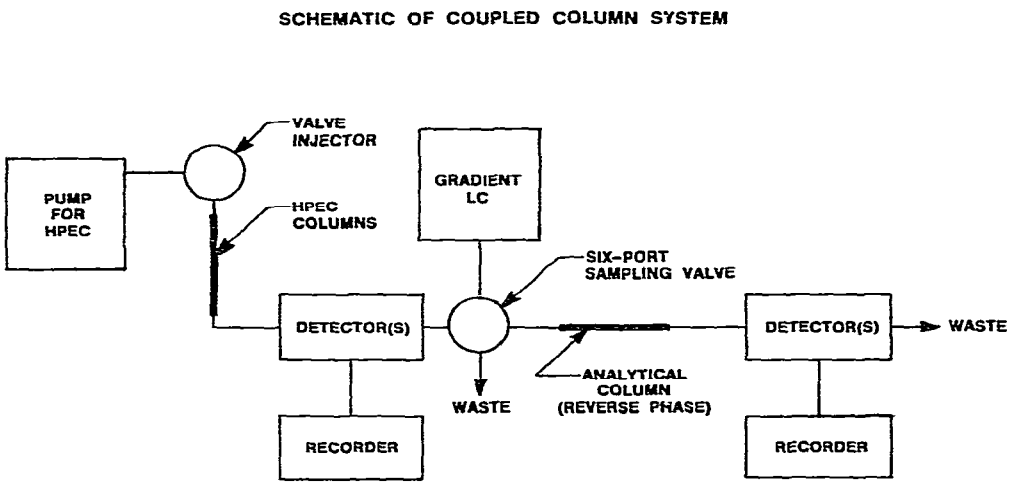


Fig. 1. Schematic diagram of coupled column system.

In the HPEC step, the solvent employed was tetrahydrofuran (THF). As one of the aims of the study was to devise a universal system for multicomponent samples, a variable-wavelength detector (Varichrom, Varian) was set at a wavelength of 215 nm. At this wavelength, many substances that possess a polar functional group show some

TABLE I

PROPERTIES OF HPEC COLUMNS

Packing	*Average pore size (Å)*	*Approx. exclusion limit**	*Molecular weight operating range**
MicroPak 1000H	40	10^3	50–800
MicroPak 2000H	250	10^4	100–8000
MicroPak 3000H	1500	$6 \cdot 10^4$	100–60,000

* Based on polystyrene standards.

absorbance and other compounds that show moderate UV absorbance at longer wavelengths display enhanced sensitivity at low UV wavelengths[9,10]. Because, at 215 nm, THF shows some UV absorbance, the detector was equipped with a flowing reference cell to provide compensation. The system was arranged so that the pump effluent entered the reference cell, sample valve, column and finally the analytical cell in that order. The pressure on the HPEC columns (and consequently on the reference cell) never exceeded 100 atm and therefore cell leakage was not a problem.

For direct coupling experiments, the outlet from the variable wavelength detector was routed to the sample inlet loop of another six-port valve already attached to a gradient chromatograph (Varian Model 8520) operating in the reversed-phase mode. The column employed was a MicroPak-MCH (monolayer octadecylsilane phase). At the appropriate time the six-port valve was actuated and the contents of the loop filled with the desired fraction from the HPEC column were injected into the reversed-phase system. The mobile phase employed in this instance was usually water–acetonitrile (ACN). The effluent from the reversed-phase column was monitored with a variable-wavelength detector and/or fluorescence detector (Fluorichrom, Varian).

For off-line CCC, the fractions from the HPEC column were collected manually in microvolume tapered vials at the exit of the UV detector. If concentration was required, the THF was removed by evaporation in a stream of dry nitrogen. The sample could then be dissolved in another solvent and injected into the reversed-phase system.

Solvents and chemicals

The THF and ACN were UV-grade, distilled-in-glass materials obtained from Burdick and Jackson (Muskegon, Mich., U.S.A.). The water was distilled in an all-glass system.

The compounded rubber samples and the standards of the various additives were kindly supplied by the Goodyear Tire and Rubber Co. (Akron, Ohio, U.S.A.). Wingstay 100 (a mixed diarylphenylenediamine), Wingstay 300 (an alkylarylphenylenediamine), Plioflex (a copolymer of styrene and butadiene), and Chemigum (a copolymer of acrylonitrile and butadiene) are registered trade names of Goodyear Tire and Rubber Co.

The malathion, tomato plants and grapefruit were obtained locally.

Procedures

Preparation of rubber samples. The compounded uncured stocks were dissolved in THF at the 2% (w/v) level. The insoluble carbon black was removed by filtration

and centrifugation prior to injection into the HPEC system. The standard samples of individual additives were dissolved in THF at the appropriate concentration and then chromatographed. In all instances, the direct coupled column system was used and 10-μl aliquots of the various HPEC peaks were injected into the reversed-phase system.

Malathion on tomato plants. The tomato plants were treated with a commercial 50% malathion in xylene formulation at the recommended level of two tablespoons per gallon of water and allowed to stand overnight. Tomato plant fronds were then removed and macerated in a blender. A 5-g portion of the sample was extracted with methylene chloride, the extract was evaporated to dryness and the residue dissolved in THF. The solution was then ready for injection into the HPEC system. A second sample of tomato fronds was taken from the same plants after 1 week and treated in the same manner. The initial sample was completely amenable to the coupled column system. The 1-week sample required manual collection of the malathion-containing fraction, concentration by evaporation and subsequent separation by reversed-phase chromatography. The reasons for this concentration step are discussed later. Fronds from unsprayed tomato plants were carried through the entire procedure to act as a control.

Limonin in grapefruit peel. The peel from a grapefruit of average size was cut into small pieces, which were combined in a flask and extracted with hot methylene chloride for 1 h. Methylene chloride was chosen in order to minimize the amount of water extracted. The extract was taken to dryness and the oily residue re-dissolved in THF. This solution was then subjected to the direct HPEC–RPC coupled column approach. For the RPC system, the variable-wavelength detector was set at 207 nm with ACN–water as the mobile phase. It was determined that this wavelength was a maximum for limonin.

APPLICATIONS OF CCC

Analysis of compounded rubber stock

Rubber stocks contain a large number of additives, the purpose of which is to improve the physical properties of the final cured product. Typical stocks contain carbon black, process oil, antioxidants, accelerators and sulfur. The types and amounts of these materials present are traditionally determined by extraction with an appropriate solvent and subsequent analysis by other techniques. Combining HPEC with the RPC technique provides a possible direct route to the separation and determination of many of these additives.

The two rubber stocks selected for study were a butadiene–acrylonitrile copolymer (Chemigum N-615) and a styrene–butadiene copolymer (Plioflex 1502). Table II lists the compounding recipes for the two samples. Initially, the elution behavior of the various "pure" constituents for the HPEC and RPC systems was studied. With the exception of elemental sulfur, the compounds studied in HPEC eluted as expected with an increase in retention volume with decreasing molecular weight (or more correctly, size). Sulfur existing as S_8 (molecular weight 256) had an elution volume much greater than that of any other constituent including benzene used to establish the total permeation volume (V_t). These studies confirm the similar observations made by Cassidy[11], who reported a rapid specific determination of elemental sulfur in water. Our studies of a number of sulfur-containing compounds suggest that abnor-

mally long retention (*i.e.*, longer than predicted from molecular weight *versus* elution volume calibration graphs) is a general phenomenon. Work is in progress to study this observation in more detail.

TABLE II

COMPOSITION OF COMPOUNDED RUBBER STOCKS

Component	*Parts added*	
	Chemigum N-615	*Plioflex 1502*
Polymer	100.0	100.0
HAF carbon black	50.0	50.0
Aromatic process oil	—	5.0
Dibutyl phthalate	5.0	—
Stearic acid	1.0	1.0
Wingstay 100*	—	1.0
Wingstay 300**	1.0	—
Zinc oxide	5.0	5.0
MBTS	1.0	1.0
TMTD	0.3	0.3
Sulfur	1.5	1.5
Total	164.8	164.8

* Mixed diarylphenylenediamine.
** Alkylarylphenylenediamine.

The chromatogram of Chemigum achieved on the HPEC system employing 50-cm MicroPak 3000H, 50-cm 2000H and 80-cm 1000H columns is illustrated in Fig. 2A. Using these high-resolution columns, the compounds were sometimes sufficiently resolved to make a tentative identification based on molecular size. However, more conclusive identification was accomplished by directing a 10-μl fraction of each HPEC peak sampled at its apex into the RPC system through the injection valve. An ACN–water gradient was employed and retention times were matched with standards run in the same manner on the CCC system. Two of the HPEC fractions, labelled fractions 1 and 2, run on the RPC system which matched known standards are shown in Figs. 2B and 2C. The matching of two retention volumes for each component using the combination of HPEC and RPC allows increased confidence in accurate identifications. Direct injection of the rubber solution into the RPC system was not feasible as the extracted polymer was found to be precipitated on the column in the aqueous environment.

As can be seen in Table II and Fig. 3A, Plioflex 1502 is a more complex sample than Chemigum in that it contains a process oil having a wide molecular weight range of aromatic compounds, which tend to interfere with the HPEC chromatogram of other components. In Fig. 3, the broad envelope eluting between approximately 30 and 50 min is caused by the process oil. Superimposed on this envelope are other components giving more defined peaks. Fractions were further separated by RPC using the CCC system and two were identified. One was identified as Wingstay 100 (Fig. 3B), which gave its characteristic five-peak pattern relating to various diarylphenylenediamine isomers. The second fraction gave two compounds, as shown

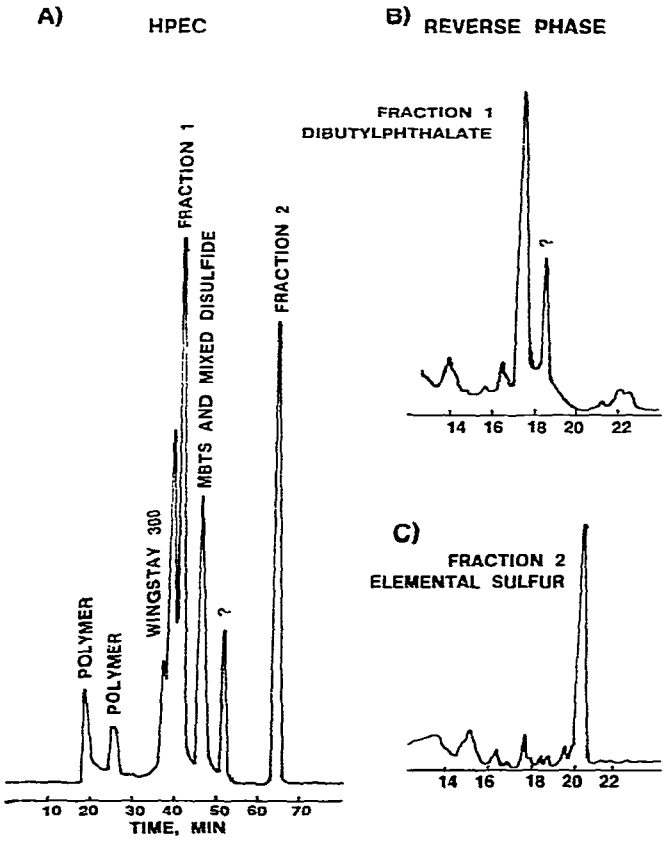


Fig. 2. Coupled column separation of compounded Chemigum. (A) HPEC: columns, 50 cm 3000H 50 cm 2000H, and 80 cm of 1000H MicroPak TSK (8 mm I.D.); THF at 1 ml/min; detection at 215 nm; 1.0 a.u.f.s.; 200 μl injected. (B) RPC: 25 cm × 2.2 mm MicroPak MCH; flow-rate, 0.5 ml/min; injection volume, 10 μl; gradient, acetonitrile–water (20:80, v/v) to 100% acetonitrile at 3% acetonitrile/min; detection at 254 nm; 0.05 a.u.f.s.

in Fig. 3C. The second peak of fraction 2 was found to be 2,2′-thiobis(benzothiazole) MBTS). There was no evidence of a peak for tetramethyl-thiuramdisulfide (TMTD) in any other fraction; however, it was known to have been in the formulation of Plioflex. This led us to attempt to confirm the identity of an unknown peak in fraction 2.

From previous knowledge[12], we knew that disulfides may undergo interchange in solution. An equimolar mixture of MBTS and TMTD was prepared in THF and injected into the CCC system. Fig. 3A indicates the presence of two peaks, each of which was directed separately on to the RPC system. Fraction 1 (Fig. 4B) gave three peaks, one of which was confirmed to be MBTS. Fraction 2 (Fig. 4C) gave one peak which was TMTD, confirmed by running a standard. As depicted in Fig. 4D, the equimolar mixture, when injected directly into the RPC system, revealed the presence of three peaks, the retention times of two of which matched MBTS and TMTD. The third peak, which matched the retention time of the largest unknown peak in Fig. 4B, was suspected to be a mixed disulfide. Variation of the molar ratio of MBTS and

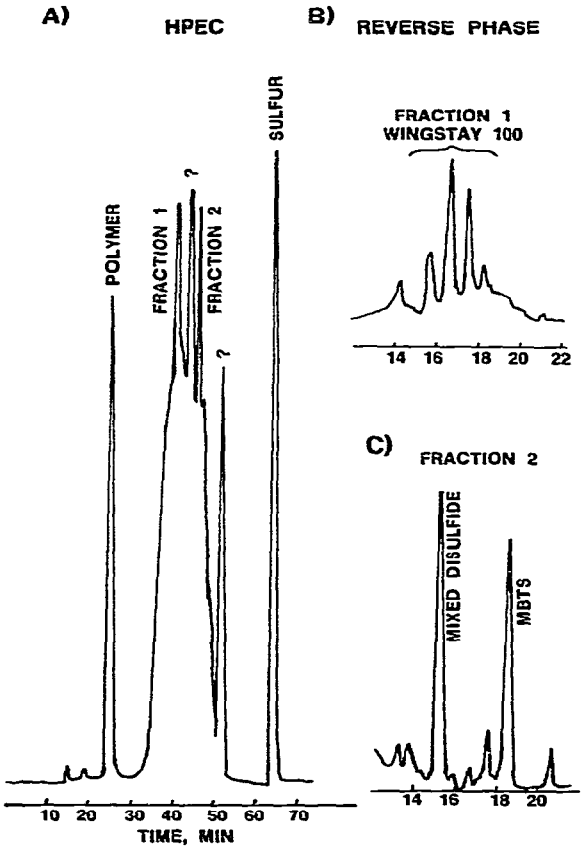
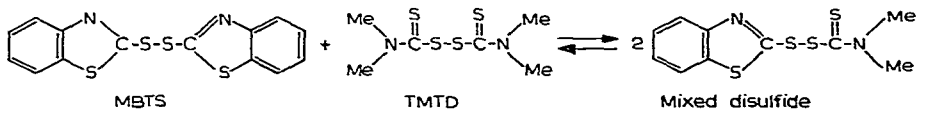


Fig. 3. Coupled column separation of compounded Plioflex by (A) HPEC and (B) RPC. Conditions as in Fig. 2.

TMTD in THF solution showed that the product was derived from TMTD and MBTS and its formation was limited by the component present in the least amount. The suspected interchange is illustrated in eqn. 1:



Because in the formulation the molar ratio of MBTS to TMTD is approximately 2.4, one would expect to find little, if any, TMTD but would expect to find the mixed disulfide, which is confirmed in Fig. 3C.

To summarize, if a compounded rubber contains no process oil, it may be possible to quantitate the additives directly from the HPEC separation using peak heights or peak areas. In addition, elemental sulfur can be determined quickly and accurately using HPEC. When process oil is also present, CCC is necessary. The HPEC–RPC combination has been shown to be most useful.

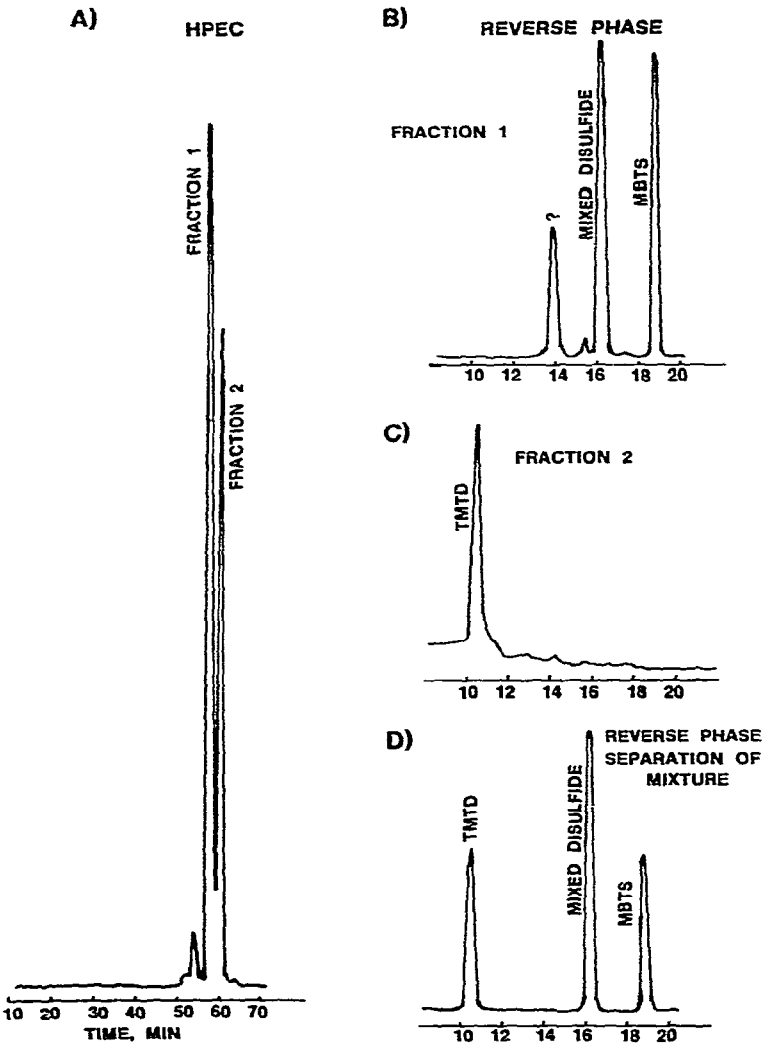


Fig. 4. Coupled column separation of disulfide mixture by (A) HPEC and (B–D) RPC. Conditions as in Fig. 2.

Malathion on tomato plants

The exclusion chromatographic clean-up technique has been applied to the removal of chlorinated pesticides and other contaminants from such diverse samples as grains[8], fish[13] and animal and plant extracts[14], using large-particle exclusion columns of 200–400 mesh. The fraction containing the pesticides was generally well diluted and required extensive concentration. The use of microparticulate exclusion columns results in much narrower fractions and these are more amenable to coupled column work.

To investigate the potential of the CCC technique in pesticide clean-up, tomato plants were sprayed with malathion and allowed to dry overnight. Fronds were col-

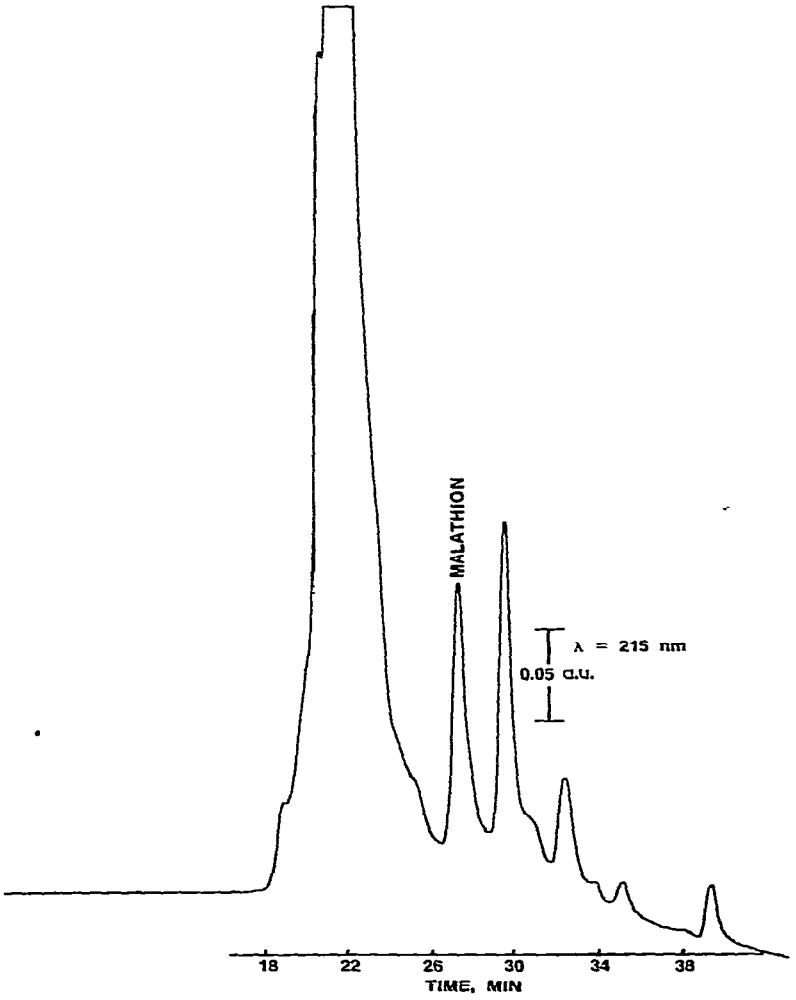


Fig. 5. HPEC separation of tomato plant extract. Sample isolated 16 h after treatment with malathion; 20-μl injection; conditions, columns, 50 cm 2000H, 80 cm 1000H, MicroPak TSK (8 mm I.D.); flow-rate, 1 ml/min; eluent, THF; UV detection at 215 nm and 0.5 a.u.f.s.; 200-μl injection.

lected the following day (16 h) and 1 week later in order to assess if the technique could follow the known degradation of malathion in the atmosphere[15].

Fig. 5 depicts the HPEC chromatogram that resulted from the injection of the green-colored extract. A sufficient amount of malathion remained on the plant to give strong absorbance at 215 nm. The blank chromatogram showed only a minor peak in the malathion elution region. From the peak height of a standard malathion sample run on the HPEC system, the concentration on the tomato plant was calculated to be about 200 ppm.

The CCC approach was used to confirm the presence of malathion in the collected fraction. As shown in Fig. 6, the malathion fraction gave a clean, well resolved

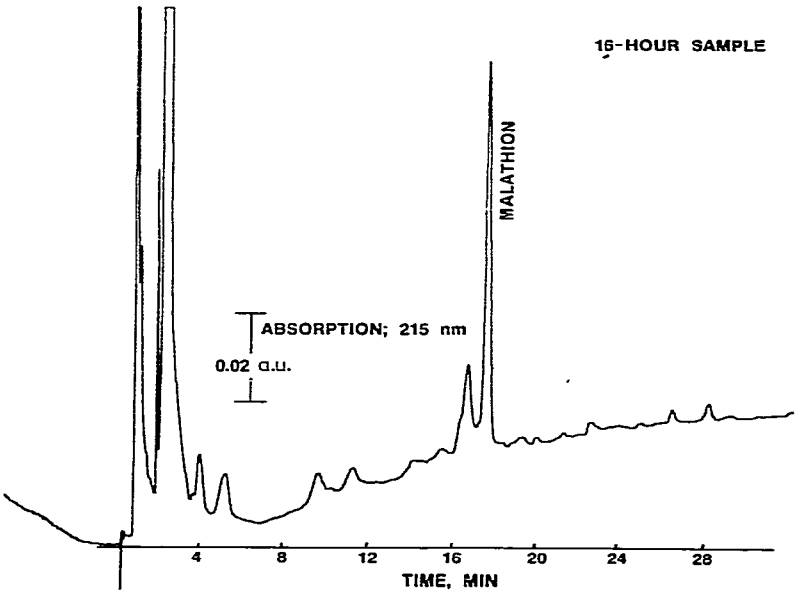


Fig. 6. RPC separation of suspected malathion fraction isolated by HPEC. Conditions, extractant, THF, 10-μl injection; column, 25 cm × 2.2 mm MicroPak MCH; flow-rate, 0.5 ml/min; gradient, acetonitrile–water (10:90, v/v) to 100% acetonitrile at 3% acetonitrile/min. UV detection at 215 nm and 0.2 a.u.f.s.; fluorescence, excitation at 254 nm, emission at > 370 nm; 5 mV full scale.

peak with a retention time matching that of a malathion standard on the RPC column.

Fig. 7 illustrates the chromatogram obtained for the 7-day sample carried through the work-up procedure. In the malathion region, no perceptible difference was observed in comparison with the blank. Using the CCC system, a 50-μl portion of this fraction was diverted into the RPC column but no malathion could be perceived. For this reason, a 1-ml fraction of the THF eluent was collected (at the region depicted in Fig. 7), evaporated to dryness and re-dissolved in 50 μl of acetonitrile. This solution was subjected to RPC analysis and the resulting chromatogram is presented in Fig. 8. A higher sensitivity was required and many more substances are observed in the concentrated fraction in comparison with the chromatogram in Fig. 6. Clearly, a well resolved peak for malathion was present in the chromatogram. From the height of the malathion peak one can calculate that roughly 1 ppm of malathion was present in the tomato plant even after exposure to the atmosphere for 1 week. Such a degradation rate (*i.e.*, from 16 h to 7 days) is consistent with other studies of malathion breakdown[15].

Other chromatographic techniques such as GC[16] or adsorption chromatography[17] could have been used for the secondary separation after the cleanup by exclusion chromatography. Separation and quantitation by GC using the flame photometric phosphorus —and sulfur— specific detectors would be preferred for trace levels (*i.e.*, parts per trillion*) of malathion. Adsorption chromatography would not

* The American trillion (10^{12}) is meant.

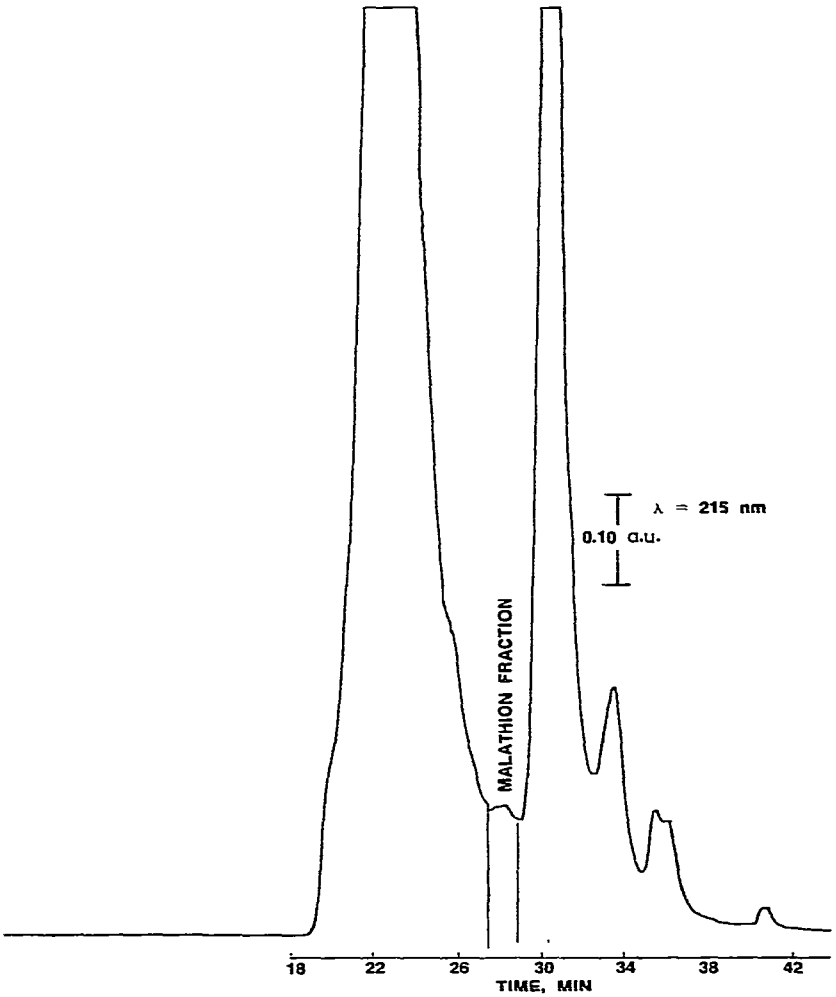


Fig. 7. HPEC separation of tomato plant extract. Sample isolated 7 days after treatment with malathion. Conditions as in Fig. 5 except 200-μl injection.

have proved advantageous over RPC since the injected tetrahydrofuran from the exclusion step would have deactivated a silica gel column requiring extensive regeneration after each run.

Limonin in grapefruit peels

Grapefruit oil is a major by-product of the citrus-processing industry and is used as a flavoring material, not only for citrus products but also for a wide range of foodstuffs throughout the world. Limonin, the structure of which is depicted in Fig. 9, is the bitter principle found in lemon and grapefruit peel. Thus, the limonin content of grapefruit oils can have an effect on quality. Also, the quality of grapefruit juice made by commercial cold-pressing can be affected. Therefore, it is of interest to isolate and measure the amount of limonin in grapefruit peel.

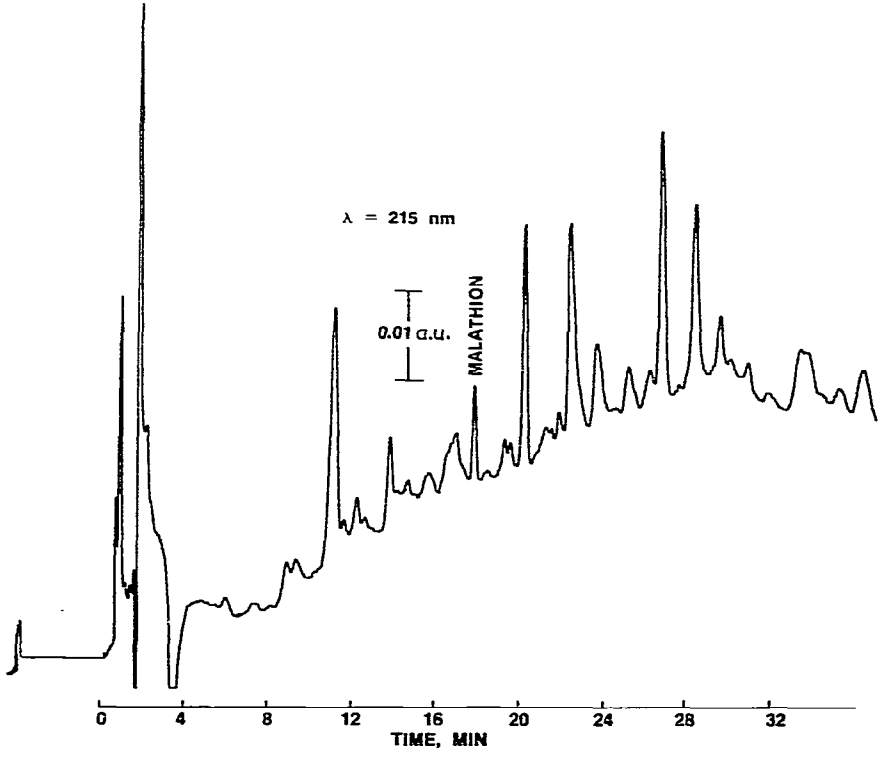


Fig. 8. RPC separation of suspected malathion fraction isolated from 7-day sample. HPEC fraction concentrated 20 times before injection; 5-μl injection; other conditions as in Fig. 6.

A complex natural product such as grapefruit peel obviously contains many extractable substances. CCC offered a simple system for the clean-up and separation of the components of grapefruit peel extracts. First, the deep yellow THF solution was injected into the HPEC system, resulting in the chromatogram presented in Fig. 9A. The molecular weight fraction which was known to coincide with a known limonin standard was sampled at its apex and injected by means of the sample valve on to the RPC column. A peak was found to elute at the retention time of a pure limonin standard. To confirm the identity of this peak, a fraction was collected manually, chromatographed on the RPC system (Fig. 9B), then, after spiking the collected fraction with a known amount of limonin, was re-run on the RPC system. As illustrated in Fig. 9C, the suspected limonin peak was enhanced. Of course, this observation does not necessarily confirm the presence of limonin. However, in a separate identical experiment, a detection wavelength of $\lambda = 285$ nm was used. This wavelength corresponds to a second maxima of limonin ($\varepsilon \sim 30$). The height of the peak corresponding to limonin was compared to that of Figure 9B ($\lambda = 207$, $\varepsilon \approx 7000$). The expected peak height ratio was observed giving further credence to the belief that this peak was limonin. The CCC approach thus offers a direct method for the determination of limonin in grapefruit peel.

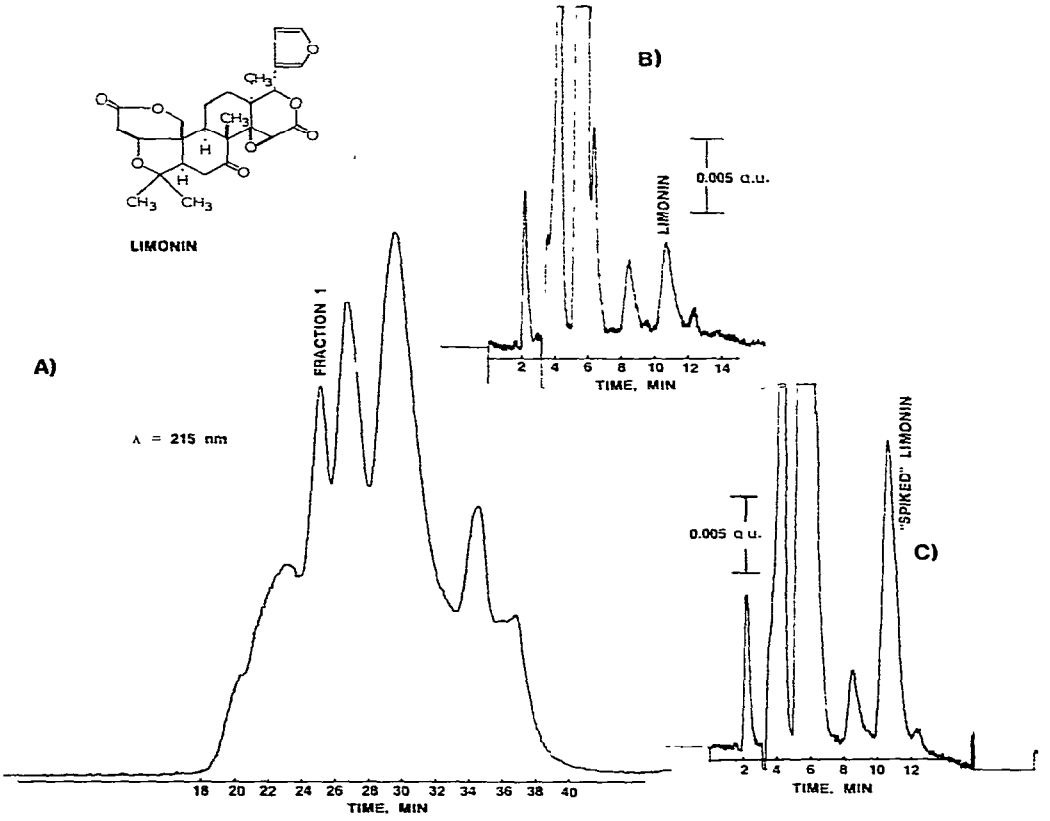


Fig. 9. (A) Isolation of limonin in grapefruit by HPEC of total extract; conditions as in Fig. 5. RPC of suspected limonin fraction: (B) sample; (C) "spiked" with limonin; conditions, acetonitrile–water (15:85, v/v); flow-rate, 0.5 ml/min; column, 25 cm × 2.2 mm MicroPak MCH; detection, 207 nm, 0.05 a.u.f.s.

DISCUSSION

The technique of CCC has proved to be highly effective when size separation is the first mode chosen. The HPEC technique provides information about the molecular weight range of the samples, usually without regard to chemical functionality. In addition, all sample components are eluted in one column volume. With microparticulate gel columns, a sufficient degree of separation may occur on the HPEC column alone to permit quantitation. Also, the peaks are often fairly sharp, making direct coupling more feasible as collection and concentration are not required. The exclusion technique has the advantage that the sample capacity is higher than in other liquid chromatography (LC) modes[8]. Thus, a large sample can be fractionated and the individual fractions still contain an appreciable amount of material for detection during the secondary chromatographic step. The solvent most often used, THF, is compatible with both aqueous and many organic solvent systems which might be used for the second LC mode.

On the other hand, the RPC technique in its various forms —regular partition

(adsorption), ion suppression, ion-pair partition— is the most widely used technique in HPLC. An estimated 50% of the work reported in current LC literature used this mode. This popularity is undoubtedly due to the fact that: (1) many non-ionic, ionic and ionizable samples can be separated using a single column and mobile phase, with or without added salts; (2) the bonded-phase columns are relatively stable, reproducible and easily regenerated after gradient elution; (3) the predominant mobile phase, water, is inexpensive and plentiful; (4) the most frequently used organic modifier, methanol, can be obtained at a reasonable price in most places in the world; and (5) the order of elution is often predictable based on the solubilities of the sample components in the mobile phase.

As has been demonstrated here, fairly complex separation problems in diverse areas can be handled by CCC provided that certain conditions are met. Firstly, the compound of interest must be eluted from the HPEC column in a sufficient concentration to allow on-line coupling. If the sample is too dilute, then a large volume of THF mobile phase must be injected on to the reversed-phase column in order to detect separated components. A large injection volume may cause a partial movement (*i.e.*, spreading out) of some sample components. Of course, the amount of THF that can be tolerated is dependent upon the solvent composition of the RPC system. For high percentage of water, only 2–5% of the column void volume can be injected without detrimental effect. However, for high percentages of organic modifier much larger volumes (10–20%) of the void volume can be injected. In RPC, THF is considered to be a strong solvent in an aqueous environment. Thus, resolution is sometimes destroyed by these large-volume injections.

In many instances where the sample is too dilute for direct coupling, off-line technique must be used. Samples can be collected at the HPEC detector exit and concentrated by driving off the THF in a stream of dry nitrogen. The collected fraction can be taken to dryness, then re-dissolved in a small volume of a solvent that is more compatible with the RPC system. The off-line approach proved more feasible for the 1 ppm sample of malathion in the plant extract, or when larger fractions of HPEC effluent were injected. Compared with gas chromatography, off-line techniques in LC are carried out easily owing to the simplicity of sample collection of liquid effluent.

When on-line CCC techniques are used, each of several sample components can be diverted from the HPEC column during the course of the coupling experiments. Thus, the flow was stopped on the HPEC column while each eluted fraction was separated by RPC. No detrimental band broadening was observed on subsequent fractions as they are eluted from the HPEC column once the flow was resumed. This was undoubtedly true as longitudinal solute diffusion is very slow in packed columns.

The use of a double-beam variable-wavelength detector at 215 nm proved very fruitful. Although not reported here, on several occasions the wavelength was set at longer values and indeed fewer peaks were detected. As long as a reference cell was used the baseline remained very stable, despite the fact that THF, with a UV cut-off at 210 nm, was used as the HPEC mobile phase. Attempts to recycle the spent THF, however, met with less success. Apparently, during use, the THF, which was unstabilized, forms compounds, possibly peroxides, which give rise to strong UV absorbance at 215 nm. If this THF was used to fill the pump, a large negative baseline offset was observed when this solvent reached the reference cell of the variable-wave-

length detector. No such problem was observed at 254 nm. Means of purifying the spent THF are under investigation.

CONCLUSIONS

The technique of CCC, using HPEC + RPC, provides a powerful combination for approaching a "universal" LC separation system. With the advent of microparticulate aqueous compatible gels and silica-based packings[19], this approach can be further extended to ionic, ionizable or other polar materials.

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