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AUTOMATED ON-LINE MULTI-DIMENSIONAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUES FOR THE CLEAN-UP AND ANALYSIS OF WATER-SOLUBLE SAMPLES*

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SUMMARY

The application of an automated on-line multi-dimensional liquid–liquid chromatographic technique for the clean-up and analysis of water-soluble samples was investigated. The use of microparticulate aqueous-compatible steric exclusion columns as the primary separation step coupled to either reversed-phase, normal-phase or ion-exchange columns as the secondary step allowed the direct injection of complex samples without prior clean-up. The entire operation was automatically controlled by a microprocessor-based liquid chromatograph with time-programmable events which allowed precise switching of high-pressure pneumatically operated valves. Both heart-cutting and on-column concentration methods were used. The heart-cutting technique had the advantage of selectivity but lacked sensitivity; more successful was the on-column concentration technique, which, by the concentration of the solute from a larger volume of exclusion column effluent on to the secondary column, gave better sensitivity. The technique was applied to the analysis of theophylline and caffeine in biological fluids, catecholamines in urine, vitamins in a protein food supplement and sugars in molasses and candy bars.

INTRODUCTION

Multi-dimensional chromatography (also known as coupled column chromatography or column switching) refers to the technique in which fractions from one column are selectively transferred to one or more secondary columns for further

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separation. Off-line multi-dimensional liquid chromatographic (LC) techniques have been in use for some time. In particular, the use of gel permeation chromatography (GPC) for the clean-up of complex sample matrices using organic solvents followed by either an LC step or a gas chromatographic (GC) separation of the collected fractions is widespread (for an example, see ref. 1). Such off-line techniques are often time consuming, inconvenient and difficult to quantitate and/or reproduce. Obviously, on-line techniques are to be preferred.

The on-line combination of high-performance GPC and reversed-phase chromatography (RPC) employing two chromatographic pumps has been reported earlier[2]. In the earlier approach, complex organic soluble samples were first fractionated on a microparticulate cross-linked polystyrene exclusion column using tetrahydrofuran (THF) as the mobile phase and, using a heart-cutting approach, these fractions were directed through a six-port sampling valve on to a reversed-phase chromatographic column using water–acetonitrile as the mobile phase. Two difficulties were encountered with this approach: (1) water-soluble samples could not be handled and (2) because THF is a powerful modifier in reversed-phase chromatography, only a small volume could be injected from the GPC column on to the reversed-phase column. Large injection volumes of THF would cause partial migration of injected fractions down the reversed-phase column, thereby limiting resolution. However, too small volumes would limit the sensitivity of the technique.

With the recent developments in aqueous-compatible, rigid microparticulate (10-μm) exclusion (gel filtration) columns[3], it was of interest to explore the feasibility of handling complex aqueous samples by multi-dimensional chromatography. Erni and Frei[4] reported the successful coupling of aqueous-exclusion and reversed-phase columns but used large porous glass particles as the exclusion media. Broad elution profiles from low-efficiency primary columns limit the ultimate resolution and sensitivity of the multi-dimensional technique. In this work we utilized 10-μm high-performance microparticulate aqueous-exclusion columns coupled on-line with reversed-phase, ion-exchange and normal bonded phase chromatographic columns. Such a combination allows the on-line clean-up of a wide range of aqueous samples, ranging from physiological fluids to water-soluble industrial polymers. This combination technique also provides the capability of easily concentrating samples on to a secondary column.

The combination of aqueous-exclusion chromatography (EC) and RPC is ideal from the standpoint that the solvents used in both techniques are compatible. The predominant aqueous mobile phase used in EC is, fortunately, a "weak" mobile phase in RPC. Such solvent compatibility allows, in addition to heart cutting, the technique of on-column concentration to be carried out. In this mode of operation, the flow of column 1 is directed for a finite period of time on to column 2; in effect, it directs a portion of the chromatogram from column 1 on to column 2. By the use of automatic valving, the entire operation can be carried out unattended.

In our earlier report[2], two or three pumps were employed. With the advent of modern single-pump ternary gradient chromatographs, this less expensive alternative appeared to be more attractive. Therefore, one aim of this work was also to investigate the use of a microprocessor-controlled single pump chromatograph in performing all required tasks.

EXPERIMENTAL

Instrumentation and columns

Fig. 1 is a schematic diagram of the multi-dimensional system employed. The chromatograph is a Varian Model 5060, which has a single pump with three-solvent capability. It was equipped with an automatic six-port sampling valve (Valco) for injection of the sample on to the exclusion column. In addition, up to three other automatic six-port, two-position valves (Valco) were employed. All valves could be controlled by time-programmable external events (powered contact closures) from the chromatograph. They could be automatically switched either off or on at predetermined times by single keyboard programming.

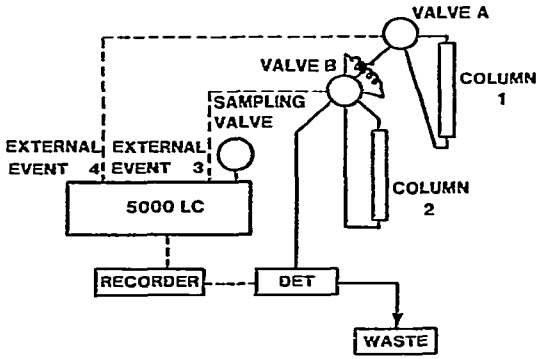

Fig. 1. System configuration for automated on-line column-switching techniques using Varian Model 5000 LC external events. Dashed lines represent electrical connections.

The aqueous compatible microparticulate (10-μm) exclusion columns were 30 cm $\times$ 7.5 mm I.D. MicroPak TSK Type SW or TSK Type PW columns. The Type SW columns are recommended for biopolymers and are packed with rigid particles of silica gel especially chemically deactivated to avoid adsorption of proteins. The Type PW columns are packed with microparticulate polyether gels which contain hydrophilic hydroxyl surface groups. They are recommended for simple saccharides, polysaccharides and water-soluble industrial polymers, oligomers and additives. Column 2 (30 cm $\times$ 4 mm I.D.) was one of three: (1) a MicroPak-MCH-10 reversed-phase column which contains 10-μm particles with a monolayer of bonded octadecylsilane; (2) an Aminex HPX-87 (polystyrene–divinylbenzene) cation-exchange resin in the Ca^{2+} form, recommended for carbohydrates; or (3) a MicroPak-NH_2-10 column, which possesses surface aminopropyl groups. All of these columns are available from Varian (Palo Alto, CA, U.S.A.).

A Varian UV-50 variable-wavelength detector, a Varian Fluorichrom filter fluorimeter or a Bioanalytical Systems (W. Lafayette, IN, U.S.A.) LC-2 electrochemical detector with a glassy carbon electrode were employed.

Valving configurations

By appropriate configurations of the switching valves, a number of flow options were available. Fig. 2 depicts the normal configuration when two switching valves

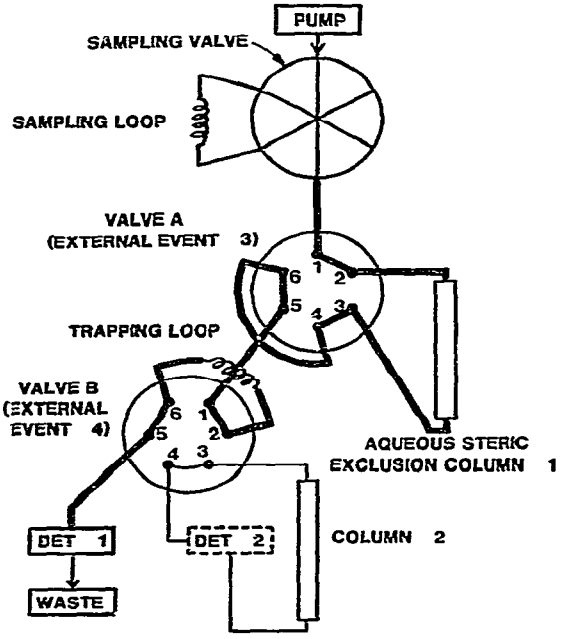

Fig. 2. HPLC solvent flow diagram for automated on-line multi-dimensional chromatography. Heavy lines represent solvent flow path for the normal configuration.

were employed. Such a configuration allows both heart cutting or on-column concentration to be carried out with no plumbing change. By using external events 3 and 4 from the Model 5060, valve A or valve B can be switched at different times in order to obtain different flow paths. The heavy lines represent the flow paths for the normal configuration at time zero. For heart cutting, when the solute of interest enters the trapping loop, having been eluted from column 1, both valves are switched and the trapped solute (the volume of which is determined by the loop volume) is directed on to column 2. In this switched configuration, using a single-pump chromatograph, some mobile phase used for the exclusion separation that resides in the chromatograph hydraulics and connecting lines is routed through column 2. In the usual case, the passage of this volume of mobile phase, which at most represents 4 ml in the Model 5060, is not detrimental since water or buffered water is a weak solvent for RPC. By switching valve B back to its normal state after all of the sample has been directed on to column 2, most of this solvent goes to waste. Then chromatography on column 2 can be carried out by again switching valve B. Note that column 1 is isolated and other fractions could, in turn, be chromatographed on column 2 when the first separation has been completed.

In the on-column sample concentration configuration, valve B is switched from the normal configuration in Fig. 2 when one desires to concentrate a portion of the chromatogram. The total time selected is dependent on the volume desired to be injected. When the volume is diverted from column 1 to column 2, then valve A is placed in its switched position and now chromatography proceeds on column 2.

In cases where the solvent used in column 1 remains in the lines and is,

therefore, partially pumped through column 2 during its chromatography step is detrimental to the column or the separation, a second valving configuration employing three valves can be used. Although not shown, an optional valve is placed between valves A and B. Its purpose is to act as a holding loop during heart cutting so that the solvent in the lines can be directed to waste. Once all lines have been flushed, then the sample is directed to column 2 and chromatography begins.

Solvents and chemicals

Acetonitrile and methanol were distilled-in-glass materials obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Water was purified by reverse osmosis and deionization. Buffer salts were of reagent grade.

Samples of molasses, candy bar and protein supplement were obtained locally. Sugars and vitamins used as standards were of reagent grade. Samples of reagent-grade DOPA (3,4-dihydroxyphenylalanine) standard were obtained from Aldrich (Milwaukee, WI, U.S.A.). Urine samples for DOPA analysis were obtained from the University of California at San Francisco, Department of Dermatology.

Preparation of samples

Samples of molasses and candy bar were either suspended or shaken with water for several minutes, then centrifuged, and the supernatant was filtered through an 0.45-μm membrane filter prior to injection. Urine and plasma were filtered through a membrane filter. Standard samples were dissolved in water or buffer at the appropriate concentration and then chromatographed.

Chromatography

The basic chromatographic procedures were carried out in a similar manner. The filtered sample was injected, either manually or with an AutoSampler, on to the exclusion column. The Model 5060 pump allows use of one, two or three solvents and either isocratic or gradient operation. Blending is accomplished by high-speed solvent proportioning valves on the low-pressure side of the pump. These solvent compositions can be time programmed. Thus, the EC solvent used in column 1 can be different from that used in column 2. For example, solvent A for EC can be water, and while EC is proceeding solvent A only can be pumping. When the second separation step commences on column 2, solvents B (aqueous buffer) and C (acetonitrile or methanol) can be programmed to begin flow and solvent A stopped. Thus a single pump can serve the purpose of a two- or three-pump system.

At the time heart cutting or on-column concentration is to occur, the chromatograph is simultaneously programmed to actuate the correct valves, thereby directing the desired trapped volume or portion of chromatogram as well as the desired solvent or solvent combination.

RESULTS

Caffeine and theophylline in biological fluids

Theophylline (1,3-dimethylxanthine) is used in the treatment of bronchial asthma and has been applied as a CNS stimulant and diuretic. High-performance liquid chromatographic (HPLC) analyses of theophylline and a common interferent,

caffeine, in plasma and urine have been given considerable attention recently. These analyses, however, rely on sample pre-treatment, such as extractions or pre-concentrations[5–7]. The following application allows the direct injection of biological fluids without pre-treatment.

The analysis of caffeine and theophylline in biological fluids was carried out using both the heart-cutting and on-column concentration methods. In both instances, the first column was a MicroPak TSK 2000SW exclusion column (50 cm × 7.5 mm I.D.) and the second column was a MicroPak MCH-10 reversed-phase column. The effluent from both columns was monitored at 272 nm. Exclusion chromatography was performed using water as the mobile phase at 1 ml/min. Caffeine and theophylline were co-eluted from the aqueous exclusion column at 18.8 ml under these conditions.

In the heart-cutting method used for the analysis of caffeine, 50 μl of raw urine were injected on to the exclusion column. At the elution volume of the caffeine peak from the exclusion column (18.8 ml corresponding to the peak apex), the solute was trapped by means of a 50-μl trapping loop. Solvent flow was switched to the reversed-phase column by appropriate valve actuation and, after an equilibration period of 4 min at a mobile phase flow-rate of 4 ml/min, the trapped solute was injected on to the reversed-phase column. RPC analysis of caffeine was performed isocratically using a mobile phase containing 20 m*M* tetramethylammonium chloride, 10 m*M* potassium dihydrogen orthophosphate and 24% acetonitrile at a flow-rate of 1 ml/min. Fig. 3A depicts the analysis of a caffeine standard using this technique. The elution volume of caffeine is slightly above 4 ml under these conditions. The heart-cutting method yielded a minimum detectable concentration of $5 \cdot 10^{-3}$ mg/ml of caffeine with 4 decades of linearity. Fig. 3B shows the analysis of a urine sample containing approximately $6 \cdot 10^{-3}$ mg/ml of caffeine. Although the heart-cutting method allowed high selectivity owing to the ability to trap small solute volumes, in this instance it was insufficiently sensitive to detect theophylline at therapeutic levels.

In the on-column concentration method for blood plasma and urine analysis, a 2.8-ml volume, corresponding to the elution of caffeine and theophylline from the exclusion column, was concentrated on to the head of the reversed-phase column. Caffeine and theophylline were then analyzed using gradient elution, as shown in Fig. 4A and B. Using this approach, a minimum detectable concentration of approximately $5 \cdot 10^{-4}$ mg/ml was obtained for both caffeine and theophylline, with linearity to 1 mg/ml. This can be compared with values in the literature[7] of $2 \cdot 10^{-5}$ mg/ml. It should be noted that although the method lacks the absolute sensitivity of previously reported analyses, it accomplishes automatic sample handling of complex matrices and yields detection limits within therapeutic ranges. Although not as selective as the heart-cutting method (necessitating gradient elution), the on-column method yielded substantially better sensitivity.

Analysis of vitamins in protein food supplement

Increasingly, many diverse food products are being fortified with vitamins to enhance their nutritional value. One such food product, a protein supplement, was selected for analysis of several B vitamins at levels ranging from 0.001% to 0.04% by weight in the protein supplement.

Direct injection of the protein supplement on to a reversed-phase column for vitamin analysis would result in irreversible adsorption of the protein content of the

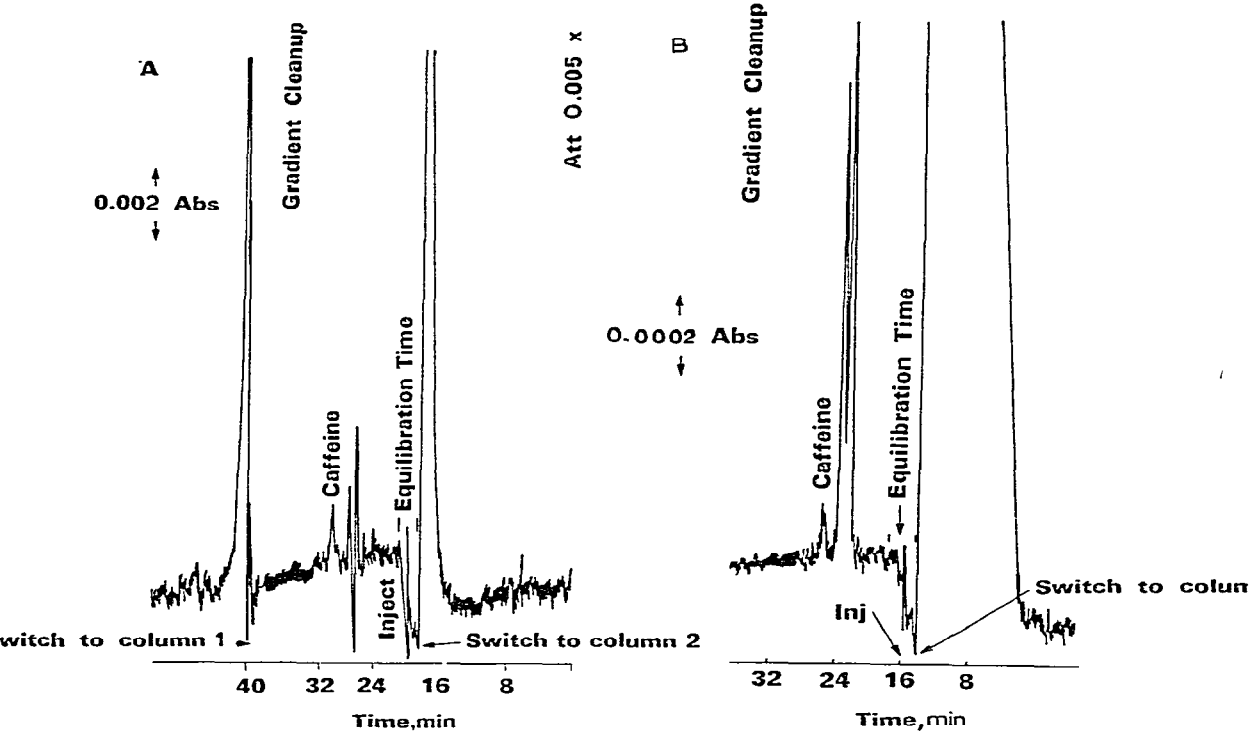

Fig. 3. Heart-cutting technique for separation of caffeine in raw urine. (A) Caffeine standard; (B) raw urine sample. Conditions: EC column, MicroPak TSK 2000SW (50 cm × 7.5 mm I.D.); flow-rate, water at 1.0 ml/min; detection at 272 nm; 50 μl injected. RPC column: MicroPak MCH-10 (30 cm × 4 mm I.D.); flow-rate, 1 ml/min 20 m*M* tetramethylammonium chloride, 10 m*M* KH_2PO_4 and 24% acetonitrile; 50 μl injected from trapping loop; detection at 272 nm; 0.002 a.u.f.s.

supplement to the column packing as well as possible matrix interferences. Therefore, an on-column concentration technique was used for sample clean-up in addition to concentration of low levels of the vitamins, accomplished by utilizing the high sample capacity of the exclusion columns.

An on-column concentration technique employing a MicroPak TSK 2000SW aqueous-exclusion column and a MicroPak MCH-10 reversed-phase column was used for the analysis of vitamins in the protein supplement. The chromatogram of the aqueous-exclusion analysis of the protein supplement is shown in Fig. 5A. A 1.3-ml volume of the eluent, corresponding to the elution interval of the vitamins from the exclusion chromatogram, was concentrated on to the head of the reversed-phase column by appropriate switching valve actuation. Fig. 5B depicts the analysis of niacin, pyridoxine, thiamine and riboflavin vitamin standards by reversed-phase gradient elution using 1-heptanesulfonic acid (HSA) as an ion-pairing reagent.

The vitamin analysis of the protein supplement with on-column concentration is shown in Fig. 5C. A 10–15-mg sample load was injected on to the aqueous-exclusion column and the solute eluting from 8.2 to 9.5 ml (1.3 ml volume) was concentrated on to the reversedphase column. Gradient elution of the vitamins was then initiated.

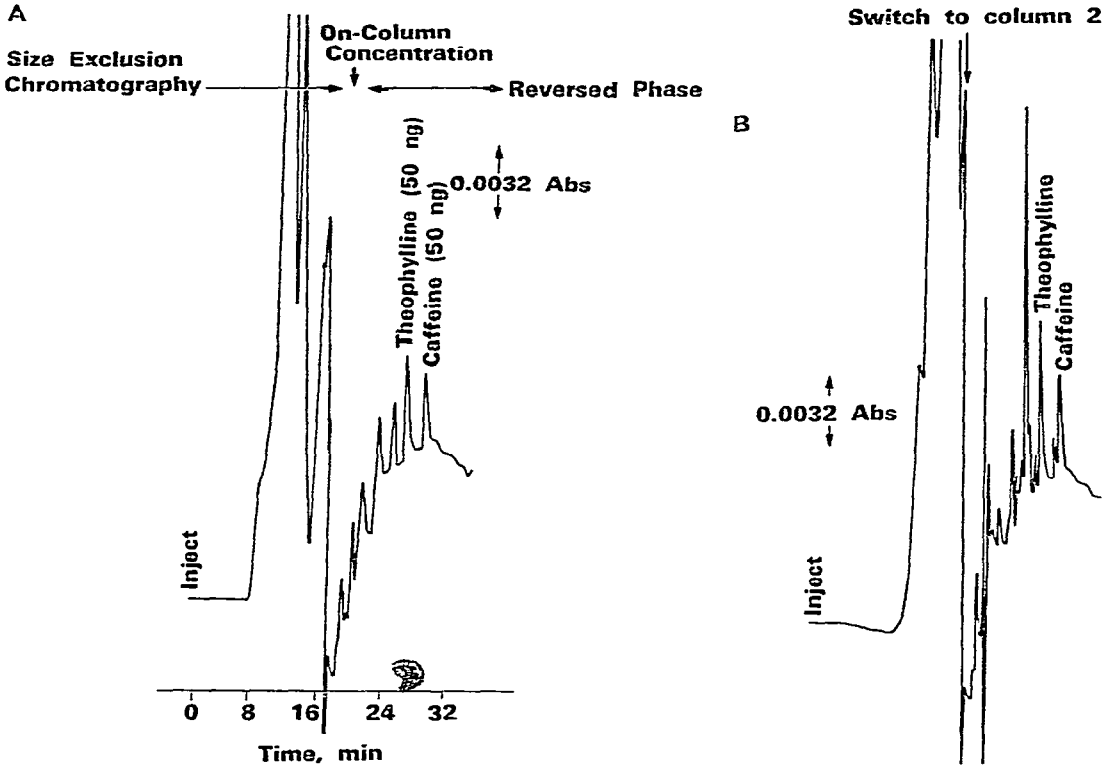

Fig. 4. On-column concentration technique for separation of caffeine and theophylline in biological fluids. (A) Blood plasma; (B) raw urine. EC conditions as in Fig. 3 except injection volume (10 μl). RPC conditions: column, MicroPak MCH-10 (30 cm × 4 mm I.D.); flow-rate, 1 ml/min; volume concentrated from EC column, 2.8 ml; gradient, water with 0.01 *M* KH_2PO_4 (pH 4)–acetonitrile, 0 to 12% acetonitrile at 6%/min, then held for 10 min; detection at 272 nm; 0.032 a.u.f.s.

The chromatographic conditions used were the same as those in Fig. 5A for exclusion analysis and Fig. 5B for reversed-phase analysis. Approximately 0.02% of niacin, 0.003% of pyridoxine, 0.009% of riboflavin and 0.003% of thiamine were found in the food supplement. The analysis of these B vitamins by HPLC has been favorably compared to chemical and microbiological analyses of these vitamins in foodstuff matrices[8,9]. On-column concentration for the analysis of vitamins in food matrices not only offers the advantage of speed by reducing the sample preparation time, but also extends the technique to trace levels by effectively increasing the detection sensitivity through the use of higher sample loadings on the EC column.

Analysis of DOPA in urine

The analysis of DOPA and other catecholamines by HPLC has received considerable attention owing to the advent of the highly sensitive and selective electrochemical detector. One of the major limitations of the currently available electrochemical detectors is their susceptibility to electrode poisoning, which is particularly pronounced with complex biological samples. This factor has required that usual

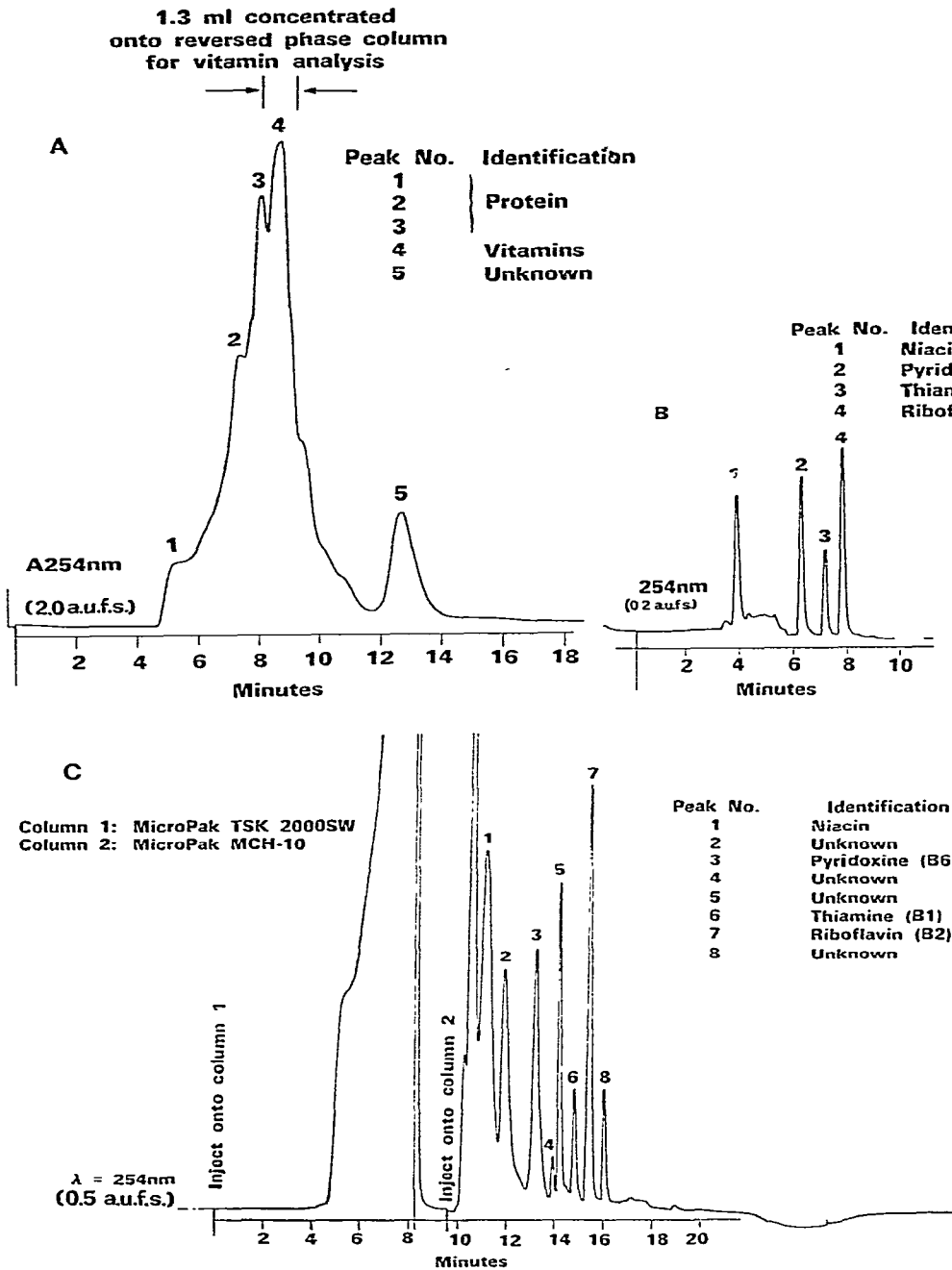

Fig. 5. On-column concentration technique for separation of B vitamins in food protein supplement: (A) EC analysis of supplement. Column, MicroPak TSK 2000SW (30 cm × 7.5 mm I.D.); methanol–water (1:9) with 0.1 *M* KH_2PO_4 and 0.01 *M* 1-heptanesulfonic acid (HSA) at 1.2 ml/min; detection at 254 nm; 2.0 a.u.f.s.; injection volume, 100 μl. (B) RPC analysis of vitamin standards. Column, MicroPak MCH-10 (30 cm × 4 mm I.D.); flow-rate, 2 ml/min; injection volume, 10 μl; gradient, methanol–water (10:90), to 80% methanol with 0.01 *M* KH_2PO_4 and 0.01 *M* HSA at 15% methanol/min; detection at 254 nm; 0.1 a.u.f.s. (C) On-column concentration techniques for vitamin analysis in supplement; conditions as in (A) and (B) except volume concentrated from exclusion chromatogram for injection on to RPC column (1.3 ml).

HPLC techniques include extensive sample pre-treatment procedures[10,11]. The use of two-dimensional on-line HPLC, as described below, eliminates sample pre-treatment by allowing only the portion of the sample that includes the compound of interest to pass through the electrochemical detector.

The analysis of DOPA in urine was carried out by a heart-cutting method. The exclusion column used was a MicroPak TSK 2000SW with water as the mobile phase. The second column was a MicroPak MCH-10 reversed-phase column using 20 m*M* camphorsulfonic acid, 100 m*M* sodium dihydrogen orthophosphate and 0.1 m*M* NaEDTA for the isocratic elution of DOPA. DOPA eluted at 8.9 ml on the exclusion column and a 100-μl volume was trapped at this point. The trapped solute was then injected on to the reversed-phase column and eluted at 15.2 ml. The exclusion chroma-

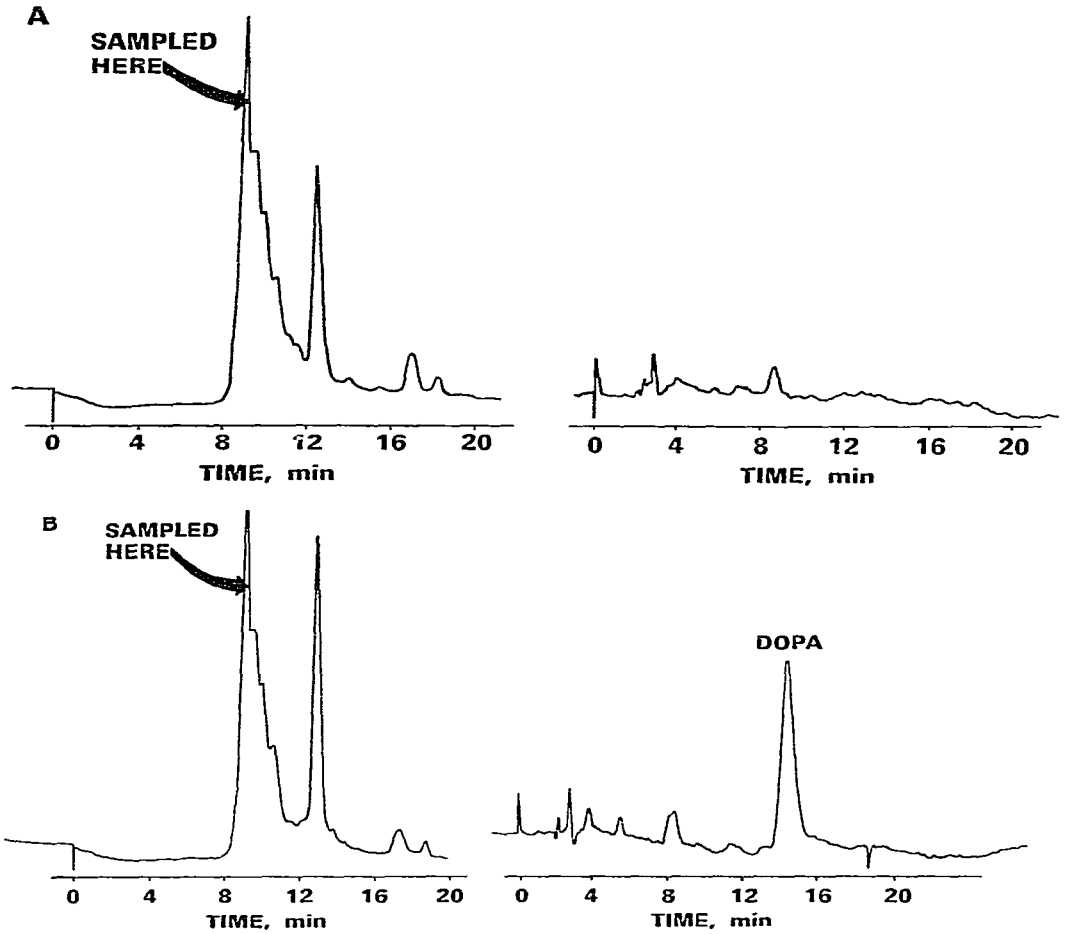

Fig. 6. Heart-cutting technique for the analysis of DOPA in urine. (A) Normal urine; (B) abnormal urine. EC analysis (left): column, MicroPak TSK 2000SW (30 cm × 7.5 mm I.D.); water at 1.0 ml/min; injection volume, 10 μl raw urine; detection at 230 nm; 2.0 a.u.f.s. RPC analysis (right): column, MicroPak MCH-10 (30 cm × 4 mm I.D.); water with 20 m*M* camphorsulfonic acid, 100 m*M* NaH_2PO_4 and 0.1 m*M* NaEDTA at 1 ml/min; detection, electrochemical detector with glassy carbon electrode operated at 0.720 mV *vs.* Ag–AgCl; attenuation, 2 nA/V; sample, 100 μl.

tography was monitored by UV at 230 nm and the reversed-phase effluent was monitored using an electrochemical detector operated at 720 mV *vs.* a silver–silver chloride electrode. Fig. 6A shows the analysis of normal urine by this method and Fig. 6B the analysis of urine from a melanoma patient with elevated DOPA levels. Note that by using this approach not only is the reversed-phase separation simplified considerably, but also the electrochemical detector is protected from poisoning by the raw urine sample.

The purpose of this application was to illustrate the possibility of on-line sample clean-up. Quantitative aspects are under investigation.

Analysis of sugars in molasses and candy formulations

The amounts and types of sugars present in food formulations has become a subject of great concern to both food processors and consumers. LC techniques employing both aminopropyl bonded phase columns for normal-phase chromatography and ion-exchange resins in the Ca^{2+} form have been used successfully for the analysis of sugars in various foodstuffs.

An on-column concentration technique was employed for the analysis of sugars in a candy bar formulation using a TSK Type PW aqueous-exclusion column and an Aminex HPX-87 cation-exchange column. The results for sucrose, glucose and fructose standards on these columns are shown in Fig. 7A.

The chromatogram of the EC analysis of the candy bar formulation is shown in Fig. 7B. A 1.1-ml portion of the chromatogram, encompassing the elution interval of the sugar standards, was concentrated on to the ion-exchange column for subsequent separation. The higher molecular weight components seen as peak 1 in this chromatogram are passed to waste to prevent contamination of the analytical ion-exchange column.

The results of the on-column concentration technique are shown in Fig. 7C. A sample of the candy formulation dissolved in water was injected on to the EC column and the solute eluting from 5.4 to 6.5 ml was concentrated on to the ion-exchange column by activation of switching valves. Analysis of the sugars was then begun on the HPX-87 column. The chromatographic conditions for analysis on both columns were the same as those for the sugar standards in Fig. 7A. Approximately 975 μg of sucrose and 75 μg of glucose were detected in the sample, representing 0.008% and 0.1% by weight, respectively, in the formulation.

The heart-cutting technique used for the analysis of sugars in molasses using normal-phase chromatography represents a special, non-ideal case in that the problem of non-compatible solvents for columns 1 and 2 greatly restricts the volume of solvent from the EC column (1) that can be passed over the normal-phase column (2). For this analysis, an EC column was employed with water as a mobile phase. A MicroPak-NH_2 column was used for sugar analysis. For such a normal-phase column water is a strong solvent. To circumvent this complication, a third switching valve was installed between the two valves shown in Fig. 2 such that, by appropriate actuation, the analytical columns and trapping loop (50-μl volume) can be isolated from the solvent flow path while the dead volume of the chromatographic pumping system is flushed directly to waste.

Fig. 8A depicts the EC analysis of molasses using a TSK 2000PW column. Note that the higher molecular weight polysaccharides eluting in peak 1 are flushed to waste

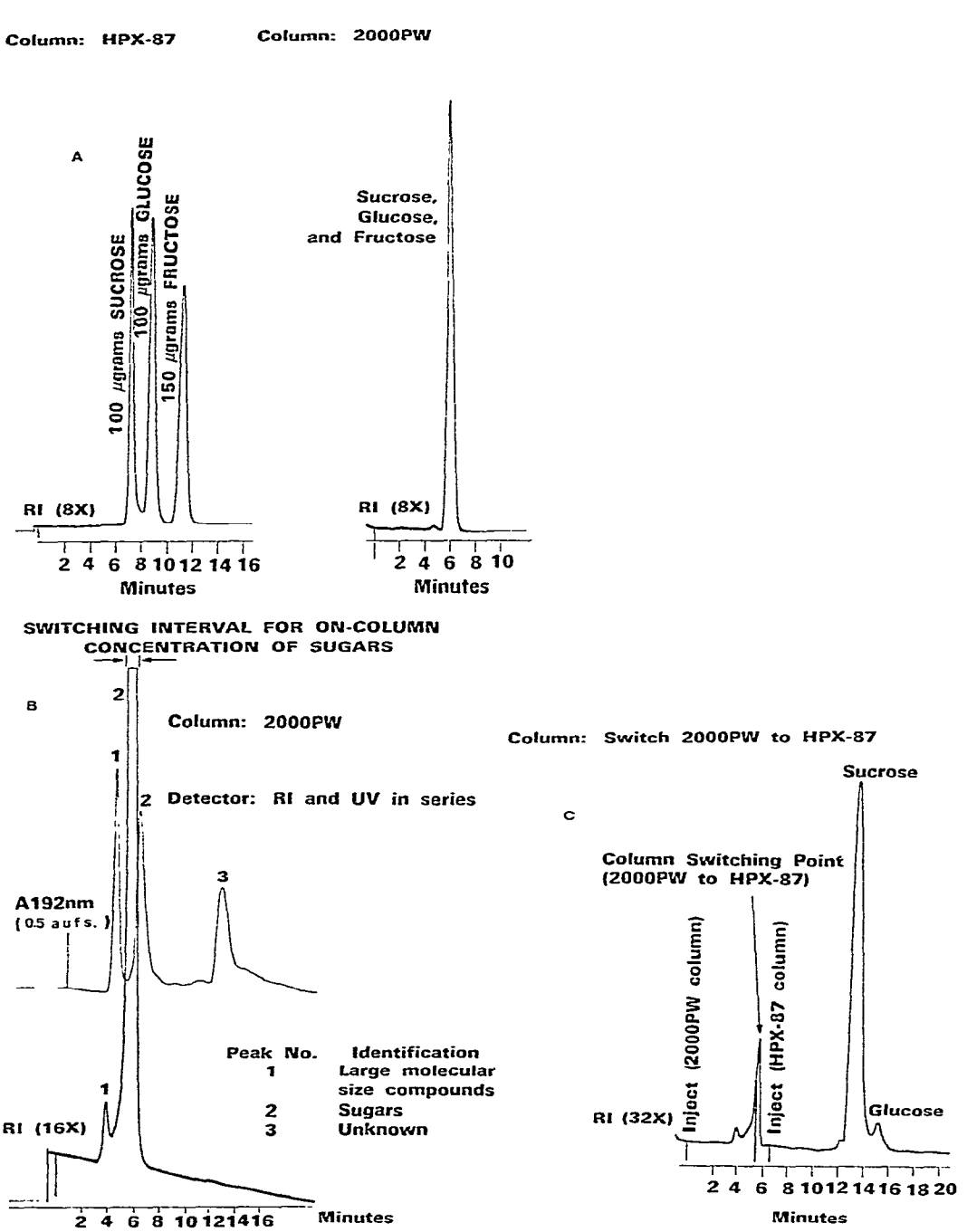

Fig. 7. On-column concentration method for sugar analysis in a candy bar formulation. (A) Sugar standards; (B) exclusion chromatography of candy bar formulation; (C) on-column concentration method for sugars in candy bar formulation. EC analysis: column, MicroPak TSK 2000PW (30 cm × 7.5 mm I.D.); water at 1.5 ml/min; injection volume, 10 μl; concentration volume, 1.1 ml; detection, RI; attenuation, 8×; UV, 192 nm, 0.5 a.u.f.s. Cation-exchange chromatography: column HPX-87; temperature, 85°C; water at 0.7 ml/min; detection as above.

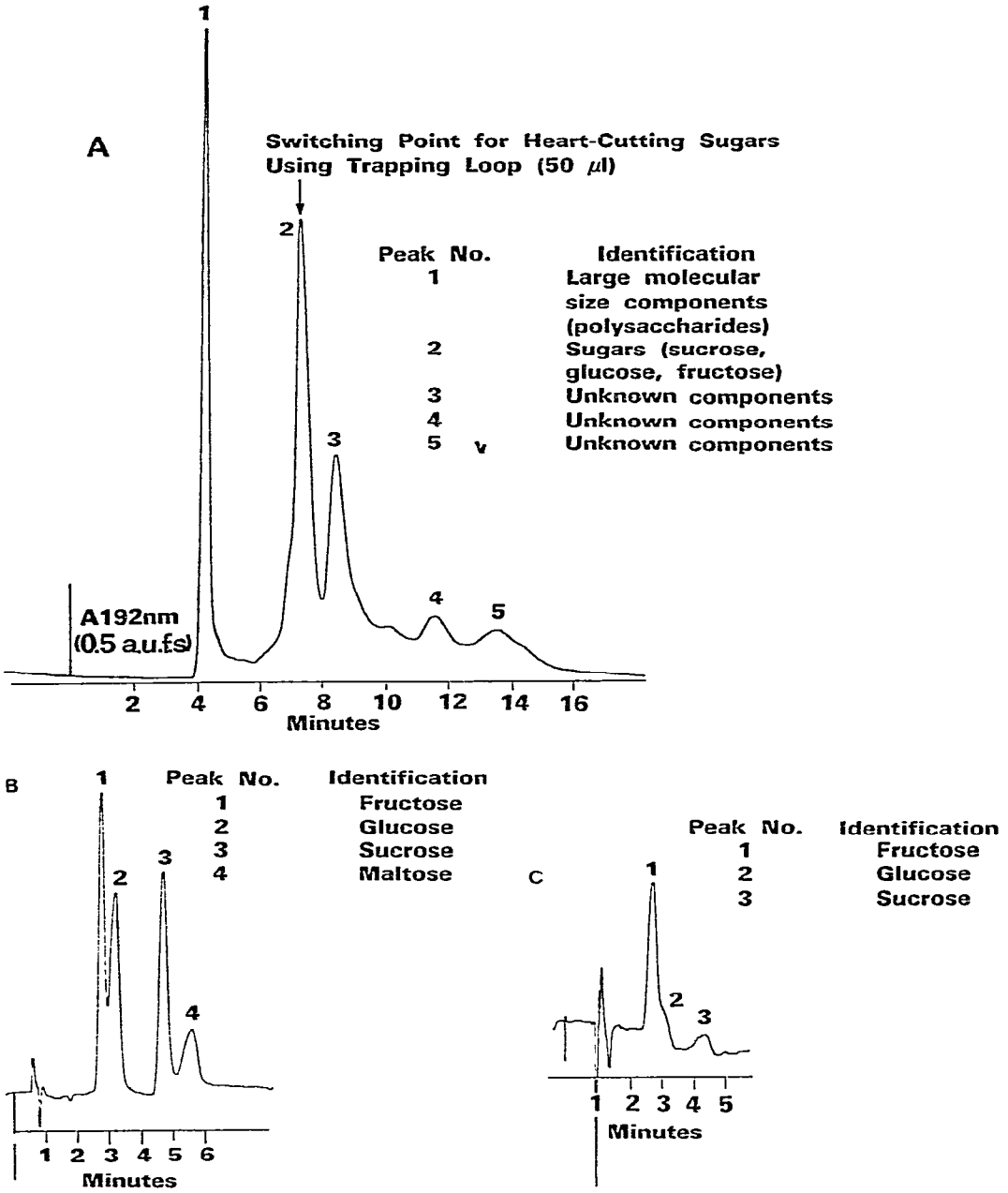

Fig. 8. Heart-cutting method for sugars in molasses. (A) Exclusion chromatography of molasses; (B) normal-phase chromatography of sugar standards; (C) normal-phase chromatography of molasses heart cut. Exclusion chromatography: column, MicroPak TSK 2000PW (30 cm × 7.5 mm I.D.); 0.1% acetic acid in water at 1.2 ml/min; detection, UV, 192 nm, 0.5 a.u.f.s.; injection volume, 100 µl. Normal-phase chromatography: column, MicroPak NH_2-10 (30 cm × 4 mm I.D.), acetonitrile–water (65:35) at 2.8 ml/min; detection, UV, 192 nm, 0.2 a.u.f.s. (B), 0.1 a.u.f.s. (C), trapping loop volume, 50 µl.

to prevent contamination of the analytical column for sugar analysis. At an elution volume of 7.8 ml, the sugars were trapped by means of a 50-μl trapping loop. The trapping loop was then isolated and solvent flow directed to waste by appropriate valve actuation. After flushing the chromatographic pumping system, switching valves were again actuated to direct the trapped sugar solutes onto a MicroPak-NH_2-10 normal-phase column for isocratic analysis of the sugars. Fig. 8B shows the analysis of several sugar standards on the normal-phase column. A chromatogram of the trapped sugar solutes from a molasses sample is shown in Fig. 8C. In this manner, sucrose, fructose and glucose can be analyzed in the molasses sample. Levels of 15% of fructose and 5% of sucrose were found in the molasses.

Methods for the HPLC analysis of sugars in molasses have been well documented using aminopropyl bonded phase columns[12], but lengthy sample preparation and column clean-up procedures are often required. It should also be noted that, although an additional valve is utilized in the heart-cutting scheme involving non-compatible solvents, through judicious choice of solvent flow schemes a third powered contact closure for actuation of this additional valve is not required and the third valve can be actuated from one of the contact closures already employed.

DISCUSSION

For aqueous samples, the technique of multidimensional LC has proved to be useful when aqueous-exclusion chromatography is the primary mode chosen. The LC technique separates on the basis of size so that the high-molecular-weight components in complex mixtures that frequently contaminate regular HPLC columns by being irreversibly adsorbed can be effectively eliminated by directing them to waste while the lower molecular weight components can be further separated on the second column, as was illustrated for the molasses sample.

A further advantage of EC is that, provided the appropriate pore size is selected, low-molecular-weight components frequently elute as a single peak that can be totally or partially directed to column 2 by on-column concentration or by heart cutting. EC also has a higher sample capacity than the other LC modes and thus a large sample can be fractionated and the individual fractions still contain an appreciable amount of material for detection during the chromatographic step. This was amply illustrated in the protein supplement example, where 10–15 mg of sample were injected and trace amounts of vitamins were clearly observed in the reversed-phase ion-pair separation.

For the secondary aqueous-compatible chromatographic step, the modes of RPC or ion-exchange chromatography appear to have the most promise. Reversed-phase chromatography is particularly attractive as it can be used for a wide variety of ionic, ionizable and non-ionic compounds. If water or buffer is used for the EC step, use of RPC is attractive in that water is a weak solvent in this mode. If for some reason, such as for elimination of hydrophobic interactions in the EC column, an organic solvent must be added to the mobile phase, care should be exercised when directing large sample volumes on to the RPC column. A large sample volume of a moderately strong solvent may cause a partial movement (*i.e.*, spreading out) of some sample components. The technique of using column 2 as a holding column or by use of a third valve as a holding loop will prove useful in such instances. After the sample is in holding, the

solvent lines can be flushed of the strong solvent to waste. After flushing, the chromatography on column 2 can be carried out under more typical elution conditions.

Analysis of molasses using an aqueous EC column as the primary column and a normal-phase NH_2 column for sugar analysis illustrates an example of a non-compatible solvent situation, water being the strong solvent for normal-phase columns. Through the use of a third switching valve to isolate the two columns and the trapping valve from the solvent flow, systems involving otherwise incompatible chromatographic modes can be effectively coupled provided that trapping loop volumes are kept small ($<$100–200 μl). Further work to evaluate such column-switching schemes more fully is currently under way.

As demonstrated with the urine and plasma samples, an exciting possibility is the direct injection of an untreated biological fluid on to an EC column. The columns show little interaction with the sample matrix and under normal circumstances all sample components elute in one column volume. Samples merely have to be filtered to remove particulate matter. With the use of sensitive electrochemical and fluorescence detectors, trace levels of endogenous compounds and drugs and drug metabolites can be determined.

CONCLUSION

The technique of multi-dimensional LC combining aqueous-exclusion with reversed-phase or ion-exclusion chromatography provides a powerful combination for the approach of a "universal" LC separation system for aqueous samples. Such a system can be used to analyze components in complex sample matrices with minimal sample pre-treatment. For maximal selectivity, a heart-cutting technique shows advantages over an on-column concentration technique, while the on-column concentration approach yields greater sensitivity for LC analyses.

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