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NON-AQUEOUS REVERSED-PHASE LIQUID CHROMATOGRAPHY

A NEGLECTED APPROACH TO THE ANALYSIS OF LOW POLARITY SAMPLES

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SUMMARY

It has been found that many hydrophobic substances such as hydrocarbons and glyceride oils may not completely elute from a reversed-phase liquid chromatographic column when using semi-aqueous mobile phases containing methanol or acetonitrile. Addition of lower-polarity solvents such as methylene chloride or tetrahydrofuran to the carrier causes rapid elution of these non-polar substances. By using a highly retentive reversed-phase packing, Zorbax ODS, it has been demonstrated that non-aqueous mobile phases provide an effective way of separating water-insoluble, non-polar substances. The enhanced solubility of compounds in these non-aqueous solvents greatly facilitates the detection of compounds which have poor UV absorbance/fluorescence characteristics.

INTRODUCTION

Over the last few years, the so-called reversed-phase method of separating chemical species has enjoyed ever-increasing popularity. One of the most significant of the early developments in this field was the work described by Locke[1]. In 1968, he reported retention data for hydrocarbon samples on a chromatographic system comprising squalane as stationary phase and pure acetonitrile as the carrier liquid. Schmit *et al.*[2] in 1971 described the chromatographic behavior of a packing which possessed a chemically bonded hydrophobic stationary phase. This chromatographic packing, known as Permaphase ODS, was the first of many products of different manufacture which shared a similar octadecylsilane-type of bonded stationary phase. Chromatographic packings of the type described have been widely used for the separation of organic chemical mixtures.

Almost invariably the mobile phase used in reversed-phase work is prepared from a water–organic solvent mixture where the concentration of the organic component determines the rate of elution of a given compound from the chromatographic

column. Organic solvents such as methanol and acetonitrile have been widely used in admixtures with water to affect the separation of samples which are generally soluble in organic, but insoluble in aqueous solvents. In recent years application of the reversed-phase method has been extended by the use of solvents such as tetrahydrofuran and acetic acid[3] and dioxane[4] in aqueous admixtures. The scope of the reversed-phase method has also been broadened to include samples which are quite ionic in character; this is achieved by adding a complementary organic counter-ion to the mobile phase. This last-mentioned approach may be considered as a form of ion-pair chromatography, similar in principle to the work developed by Eksborg *et al.*[5], or alternatively, as a dynamically loaded ion-exchange system.

At the other end of the polarity scale from ionic species, lie many compounds which are very hydrophobic in character, for example, hydrocarbons, fats, vegetable and mineral oils. These substances, by their very nature, are insoluble in water and generally have negligible solubility in solvents such as methanol and acetonitrile. There have been many reports in the literature of the liquid chromatographic separation of low-polarity substances using hydrophobic chromatographic packings. Much of this literature relates to the use of porous polymer beads, especially of the styrene–divinylbenzene type, as the chromatographic support. Generally, the studies described in these reports fall into two separate approaches. Pokorny *et al.*[6] have utilized gel-permeation chromatography to affect the separation of low-molecular-weight solutes: tetrahydrofuran is the most commonly used carrier and sample components elute in order of decreasing molecular size. Conversely, studies such as those by Lawrence[7], Chu and Pietrzyk[8] and Janák *et al.*[9] have relied on semi-aqueous mobile phases to enhance solute–support interactions. Unfortunately, this approach suffers from two limitations. Firstly, the solubility of low polarity compounds in semi-aqueous solvents is generally very low. Secondly, porous polymer beads of the styrene–divinylbenzene type normally used have poor mass transfer characteristics which results in broad eluting peaks.

In the present study, the behavior of a range of low-polarity compounds has been examined on an efficient, modern reversed-phase chromatographic packing which is known to offer good mass transfer characteristics. The principal objective has been to assess the analytical utility of columns containing reversed-phase chromatographic packings when the mobile phase is prepared from solvents other than water, *i.e.*, solvents in which low-polarity compounds have an appreciable solubility. Very little has been reported in the literature relating to elution chromatography performed in this manner since the work of Martinů and Janák[10]. These authors described the separation of several simple aromatic compounds on Porapak T, a commercially available porous polymer type of chromatographic support, with hexane as the carrier solvent.

The objective throughout this study has been to demonstrate the feasibility of working with non-aqueous solvents in reversed-phase chromatography.

EXPERIMENTAL

Apparatus

Various high-performance liquid chromatographs were used during the course of this study. The principal components comprised the following units: (1) Model 830

liquid chromatograph, equipped with a thermostatically controlled column compartment, septumless injector and 254-nm ultraviolet absorbance detector (cell volume 8 mm^3, maximum operating sensitivity of 0.01 a.u.f.s.); (2) Model 833 flow controller; (3) Model 837 spectrophotometer (cell volume 8 mm^3, wavelength range 195 to 650 nm); (4) infrared detector (cell volume 30 mm^3, optical pathlength 0.23 mm); (5) twin pen strip chart recorder (1 mV span, Hewlett-Packard Model 7130A). All of these units are available from Du Pont Instruments.

Chromatographic columns

All columns were fabricated from precision stainless-steel tubing 250 × 4.6 mm I.D. End fittings were conventional Swagelok reducing units, bored out to eliminate dead volume in the column end fittings. The chromatographic packing material used throughout this study was Zorbax ODS (Du Pont, Wilmington, Del., U.S.A.). This material has a linear aliphatic octadecyl hydrocarbon monomolecular layer chemically bonded to 6-μm diameter porous silica microspheres. The packing was held in the column with Hastalloy frits. The porosity of the inlet and outlet frits was 5 μm and 2 μm, respectively.

Solvents

Liquid chromatography grade solvents were used throughout this work. Acetonitrile, methanol, methylene chloride and tetrahydrofuran were purchased from either Rathburn Chemicals (Walkerburn, Great Britain) or Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.).

Samples

Most of the compounds used in this study were obtained from other departments within the Du Pont Company. Exceptions are the triglycerides and aliphatic hydrocarbons which were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.) and Chem Service (West Chester, Pa., U.S.A.). The sample of cod liver oil was supplied by an undisclosed donor, but was identical with commercially available products.

Methodology

Studies concentrated on solutes which were known from previous experiments to be retained relative to the "solvent front" on Zorbax ODS chromatographic packing when either pure methanol or pure acetonitrile was used as the mobile phase. In these cases, the influence of a less polar solvent added to the mobile phase was studied. Solvents that were favored in this study were methylene chloride and tetrahydrofuran as these offer excellent solubilizing power for non-polar samples such as fats and oils.

The influence of mobile phase composition on sample retention was investigated by introducing dilute solutions of the samples into the chromatograph and noting the elution behavior at each of several fixed compositions of mobile phase. Typical tests were performed with 10%, 20%, 40%, 60% and 80% of the less polar solvent added to the acetonitrile. Samples were dissolved in either the mobile phase or methylene chloride at the 1% (w/v) level, when using an ultraviolet detector or at the 10% (w/v) level, when using refractometric or infrared detectors. Injection volumes ranged from 10 to 50 mm^3 depending on the operating conditions.

Several different chemical sample mixtures were studied which were considered to be representative of low-polarity compounds. The chromatograms obtained are described in the next section.

RESULTS AND DISCUSSION

Influence of mobile phase composition

Saturated triglycerides can be considered as typical samples which have low solubility in semi-aqueous solvents and tend to be retained strongly on reversed-phase chromatographic packings. The test mixture was selected to represent liquid solutes which mix readily with the mobile phase and solid solutes which possess different solubility characteristics in the mobile phase solvents, *i.e.*, tricaprylin, trilaurin and tripalmitin. Fig. 1 shows the influence on retention of the addition of tetrahydrofuran to an acetonitrile mobile phase. The chromatograms clearly demonstrate that the retention of these glyceride samples is decreased by increasing the proportion of tetrahydrofuran in acetonitrile through the range 10–60% (v/v). Tetrahydrofuran concentrations greater than 60% cause the glycerides to elute close to the solvent front. Progressively decreasing the concentration of this solvent, particularly below 20% by volume tetrahydrofuran, leads to distorted peaks with a characteristic leading edge. This is believed to be due to the low solubility of these solutes in the mobile phase.

Fig. 2 shows a similar examination of a group of hydrocarbons, specifically: decane, decene, dodecane, and dodecene. In this study, methylene chloride is used as the modifying solvent and the infrared detector is operated at 3.4 μm, the wavelength

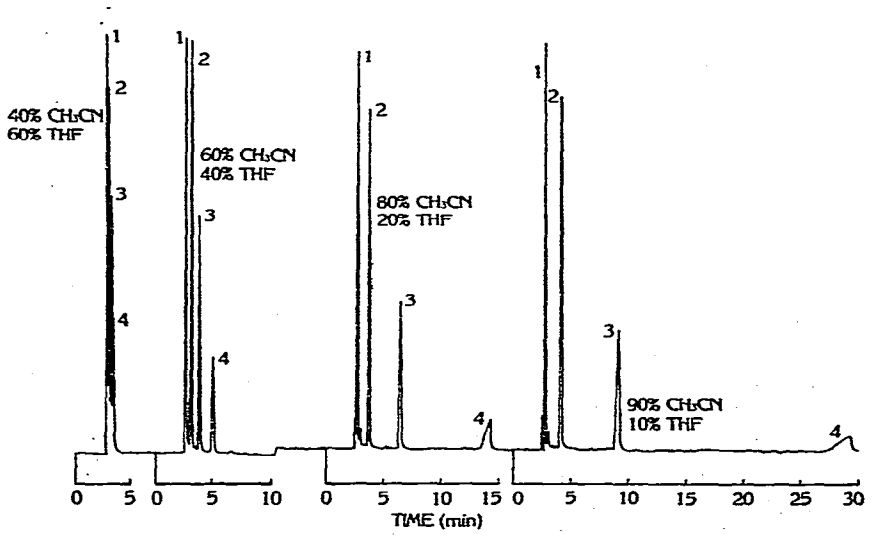

Fig. 1. Influence of mobile phase composition on elution of saturated triglycerides. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 mm × 4.6 mm I.D.; mobile phase: primary, acetonitrile, secondary: tetrahydrofuran; flow-rate: 0.6 ml/min; pressure: 21 bar; temperature: 40°; detector: infrared, 5.75 μm. Peak identity: (1) tributyrin; (2) tricaprylin; (3) trilaurin; (4) tripalmitin.

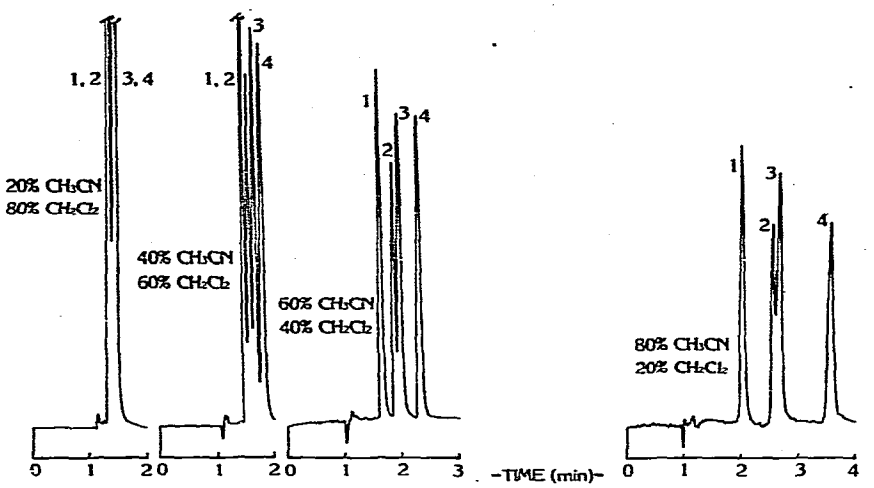

Fig. 2. Influence of mobile phase composition on elution of aliphatic hydrocarbons from Zorbax ODS. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 × 4.6 mm I.D.; mobile phase: primary, acetonitrile, secondary, methylene chloride; proportions: see figure; flow-rate: 1 ml/min; temperature: 24°; detector: infrared, 3.4 μm. Peak identity: (1) decene; (2) dodecene; (3) decane; (4) dodecane.

corresponding to methylene absorption rather than 5.75 μm (carbonyl absorption). Addition of 20–40% (v/v) of methylene chloride caused the sample components to elute within k' 1 to 10, the generally accepted practical working range of capacity factors. Any further increase in the methylene chloride concentration leads to rapid elution of all components, although even under these conditions a certain degree of resolution is obtained between the sample components.

Application of the method to the separation of hydrocarbons

Aliphatic compounds. Illustration of the potential separating power of this non-aqueous method is made by a high-speed separation of various aliphatic hydrocarbon mixtures.

Fig. 3 illustrates the isocratic separation of a number of saturated hydrocarbons by liquid chromatography. This chromatogram clearly indicates the principle of rapid liquid-phase separations of samples which would normally be studied by gas chromatography. Unsaturated hydrocarbons have been found to behave in a similar manner to alkanes. Fig. 4, for instance demonstrates the separation of homologs of linear alkenes of importance in the petroleum industry; again, high-speed, high-resolution separations are achieved.

An example with important significance to many industrial processes is shown in Fig. 5 where hydrocarbons with the same carbon number are resolved according to their chemical class: aromatic, olefinic, aliphatic. The figure illustrates the rapid separation of butylbenzene and decene from decane.

Aromatic hydrocarbons. Compounds which are aromatic in character can be readily detected by ultraviolet absorbance measurements. In this situation it is possible to change the composition of the mobile phase, *i.e.*, use gradient elution, to

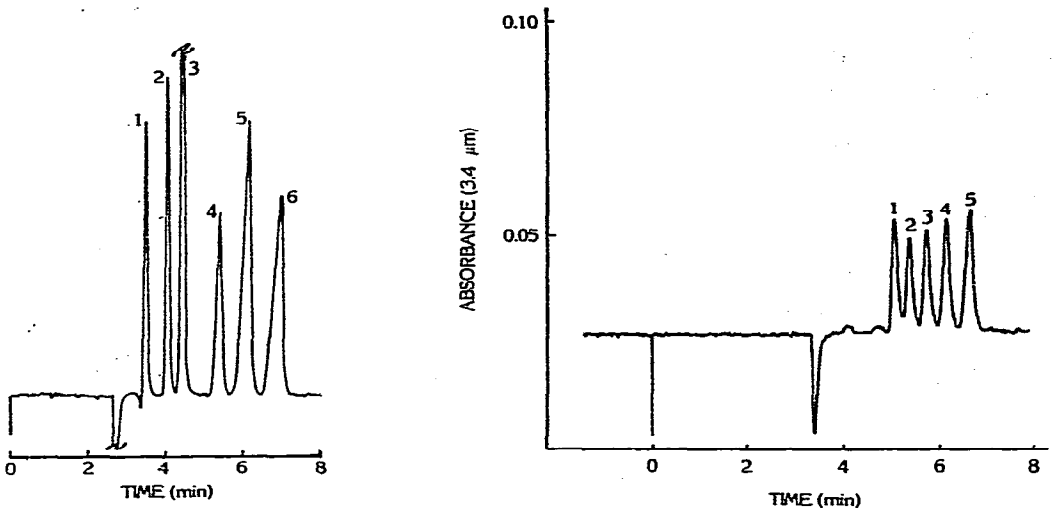

Fig. 3. Separation of saturated hydrocarbons using non-aqueous reversed-phase chromatography. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 × 4.6 mm I.D.; mobile phase: methylene chloride–acetonitrile (1:4); flow-rate: 1.0 ml/min; pressure: 20.5 bar; temperature: 40°; detector: IR, 3.4 μm, 0.1 a.u.f.s. Peak identity: (1) hexane; (2) decane; (3) dodecane; (4) hexadecane; (5) octadecane; (6) eicosane.

Fig. 4. Separation of alkene homologs by non-aqueous reversed-phase chromatography. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 mm × 4.6 mm; mobile phase: tetrahydrofuran–acetonitrile (1:9); flow-rate: 0.75 ml/min; temperature: 27°; detector: IR (3.4 μm). Peak identity: (1) *n*-decene-1; (2) *n*-undecene-1; (3) *n*-dodecene-1; (4) *n*-tridecene-1; (5) *n*-tetradecene-1.

increase progressively the speed of elution of the sample constituents. The effect of an acetonitrile to methylene chloride gradient on the elution of components in a synthetic mixture of aromatic hydrocarbons is illustrated in Fig. 6. In this example the methylene chloride concentration was increased from zero to about 30% (v/v) during the analysis. The order of elution is comparable to that achieved with semi-aqueous mobile phases, however, under the latter conditions, solutes such as coronene and decacyclene are retained very strongly on the column unless the column is operated at a temperature above 70°.

Aromatic polymers. In a manner analogous to the separation of polyaromatics, it is possible to fractionate some low-molecular-weight polymers by the non-aqueous method. Application to the separation of oligomers in a commercial sample of polystyrene, molecular weight 800, is illustrated in Fig. 7. The order of elution is in increasing molecular size, opposite to that observed in size-exclusion chromatography. The selectivity in this non-aqueous reversed-phase method is much greater than with separations based simply on steric considerations.

Application of the method to oil-soluble vitamins

Carotenoids. A sample of commercial β-carotene was examined using a gradient elution system from acetonitrile to methylene chloride. Fig. 8 illustrates the results obtained when the separation was monitored at a wavelength of 497 nm. The

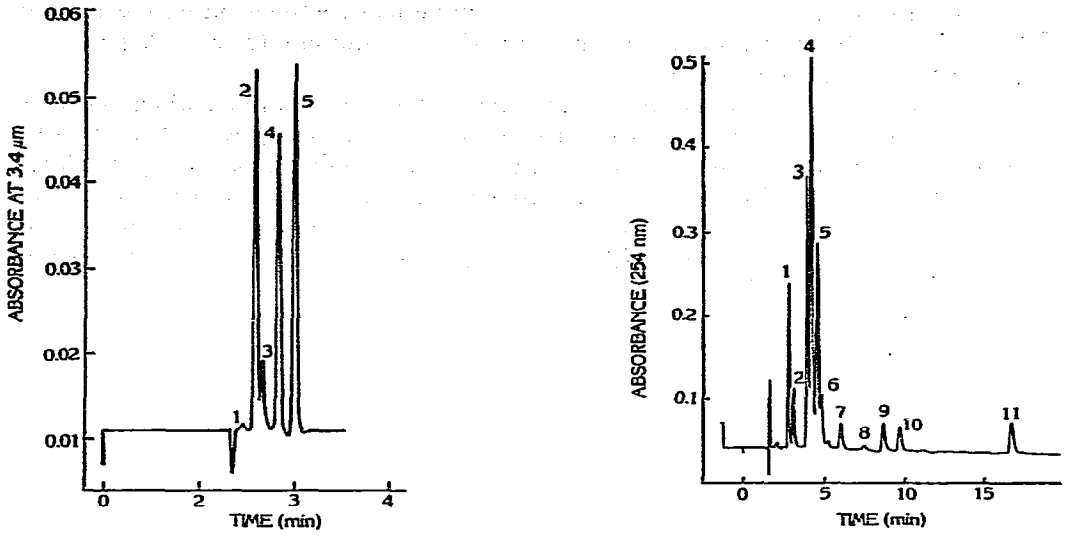

Fig. 5. Separation of hydrocarbons with same carbon number: aromatic, olefinic, aliphatic. Operating conditions: see Fig. 3. Peak identity: (1) impurity from butylbenzene; (2) butylbenzene; (3) impurity from decene; (4) decene; (5) decane.

Fig. 6. Separation of polynuclear aromatic hydrocarbons by non-aqueous reversed-phase chromatography. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 × 4.6 mm I.D.; mobile phase: primary, acetonitrile, secondary, methylene chloride; program: linear 0–30% methylene chloride in 15 min; flow-rate: 2 ml/min; temperature: 24°; detector: UV (254 nm). Peak identity: (1) fluoranthene; (2) pyrene; (3) benzo[*mno*]fluoranthene; (4) benzo[*e*]pyrene; (5) benzo[*a*]pyrene; (6) 3,4-benzotetraphene; (7) benzo[*ghi*]perylene; (8) benzo-[*rst*]pentaphene; (9) coronene; (10) benzo[*a*]naphtho[8,1,2-*c*,*d*,*e*]naphthacene; (11) decacyclene.

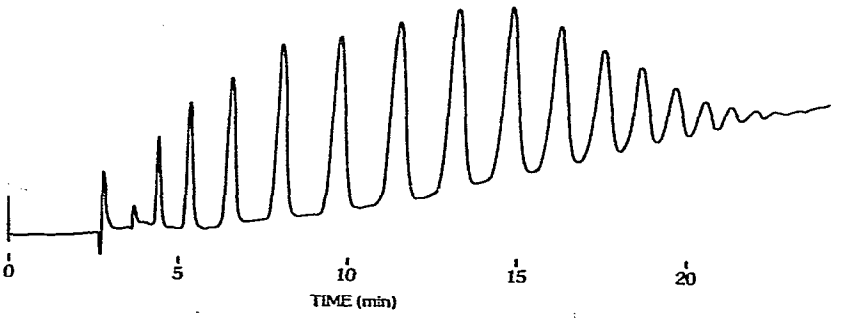

Fig. 7. Separation of polystyrene 800 oligomers by non-aqueous reversed-phase. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 15 cm × 4.6 mm I.D.; mobile phase: primary, methanol, secondary: tetrahydrofuran; program: linear (1.4%/min); flow-rate: 1.5 ml/min; temperature: ambient; detector: UV (254 nm).

upper trace represents the detector output signal amplified by a factor of ten over the lower trace. Minor components can be readily detected in the β-carotene. These trace components are almost certainly related to carotenoids since the operating wavelength selected to monitor the separation, 497 nm, would be insensitive to most non-carotenoid solutes likely to coexist in this sample.

Vitamin D_3 in fish liver oil. Samples of commercial cod liver oil were examined to establish the feasibility of direct determination of vitamin D_3 and A palmitate without any sample pre-treatment.

Fig. 9 contains reproductions of the chromatograms obtained when cod liver oil samples containing 85 and 285 I.U. of vitamin D_3 were examined by the reversed-phase method. The methylene chloride portion of the mobile phase greatly increased the solubility of the oil. This fact was particularly important as, in order to detect the low levels of vitamin, it was necessary to inject a sample prepared by mixing the cod liver oil with an equal volume of methylene chloride, *i.e.*, a 50% (v/v) sample concentration.

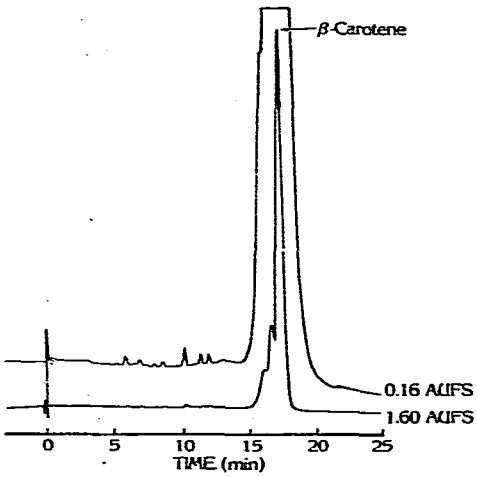

Fig. 8. Separation of minor components in β-carotene using non-aqueous reversed-phase chromatography. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 × 4.6 mm I.D.; mobile phase: primary, acetonitrile, secondary: methylene chloride; program: linear addition of methylene chloride at 2%/min; flow-rate: 1 ml/min; temperature: 24°; detector: visible absorbance, 497 nm.

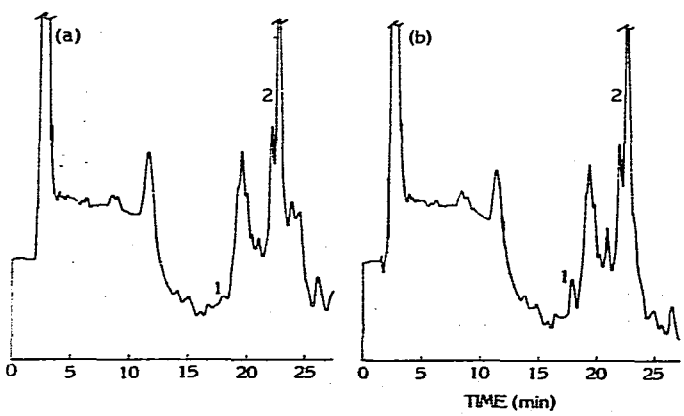

Fig. 9. Detection of vitamin D_3 in cod liver oil using non-aqueous reversed-phase chromatography. (a) Native cod liver oil, (b) Native cod liver oil, approx. 85 I.U. D_3/g. spiked to give 285 I.U. D_3/g. Operating conditions: see Fig. 8, except detector: UV, 254 nm. Peak identity: (1) vitamin D_3; (2) vitamin A palmitate.

CONCLUSIONS

The analytical utility of performing reversed-phase separations on modern, highly efficient bonded packings with non-aqueous mobile phase has been demonstrated. In this configuration, a mechanism of separation comparable to the earlier use of paraffin oil-impregnated cellulose layers can be envisaged. However, the use of chemically bonded chromatographic packings considerably increases the reproducibility and stability of the separation system.

Chromatographic systems using a totally organic mobile phase offer several attractions for the analysis of hydrophobic samples relative to semi-aqueous systems.

Firstly, many non-polar substances are insoluble in water and only sparingly soluble in methanol and acetonitrile, the most popular organic solvents used in reversed-phase work. Tetrahydrofuran and especially chlorinated solvents are exceedingly good solvents for hydrophobic substances; thus sample solubility is not a problem. In some instances a concentration approaching 20% (w/v) of the sample in the mobile phase is possible. Under these circumstances no precipitation of the sample on the column is observed. The high solubility of the sample in the carrier liquid is beneficial in that relatively large samples may be analyzed, aiding detection when using the less sensitive differential refractive index and infrared monitors. A high sample load also improves the limits of detection of minor components and makes semi-préparative chromatography more attractive.

The types of samples studied in the present work, namely hydrocarbons and fat-soluble vitamins, have been analyzed previously to some extent using semi-aqueous mobile phases. The observations in this work suggest that in many instances incomplete elution of the sample would have occurred unless the separations were carried out at elevated temperatures. It is evident, in retrospect, that the incomplete elution of sample components when using methanol or acetonitrile has probably contributed significantly to the steady deterioration of some chromatographic columns in use. Clearly, even if the component of interest is eluted from the column, as in the case of the analysis of vitamins A and D, the matrix, which is invariably a glyceride based oil, is still retained. Any retained constituent, if not flushed from the system routinely, will result in premature failure of the column. In spite of the relatively large samples frequently injected, *e.g.*, approximately 10 mg, during this study, none of the four columns used showed any signs of deterioration. The columns were in use intermittently over a period of eight months.

The analysis of hydrocarbons by this reversed-phase method is particularly interesting in that by adjusting the sample concentration and choosing the appropriate detector it is possible to monitor different hydrocarbon types. A fluorescence detector would observe only the heavier polynuclear aromatic substances. An ultraviolet detector with variable wavelength control could be used to monitor selectively various polyunsaturated and aromatic constitutents. An overall qualitative picture of the components present in the sample is probably the best obtained by using a refractive index or an infrared absorbance detector. It is clearly recognized that several of the solutes selected to demonstrate the non-aqueous approach to reversed-phase chromatography can be analyzed either by gas–liquid chromatography and/or more conventional reversed-phase methods. This use of well characterized samples should clearly indicate the type of results obtained, *i.e.*, a separation of homologs rather

than a class or type separation. Samples with a given carbon content, as shown in Fig. 5, are resolved, suggesting that compounds belonging to different hydrocarbon types do not elute at exactly the same time. Other experimental tests, not detailed in this report, suggest that higher-molecular-weight alkanes, *e.g.*, the kerosine range, can also be fractionated to some extent by using a mobile phase containing an increased concentration of methylene chloride.

Evidence to substantiate a reversed-phase mechanism can be drawn from the observation that a solvent of lower polarity, *e.g.*, methylene chloride, will accelerate elution of components whereas a more polar solvent, *e.g.*, acetonitrile holds the sample components on the column. If adsorption on non-chemically bonded sites of the support contributed to the separation mechanism, the opposite effect of solvent polarity would be expected.

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