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APPLICATIONS OF REVERSED-PHASE CHROMATOGRAPHY AND NEPHELOMETRIC DETECTION TO ANALYSIS OF NON-POLAR MIXTURES AT MICROGRAM LEVEL

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SUMMARY

Non-aqueous reversed-phase chromatography with light-scattering detection is suitable for analysis of various non-polar mixtures. Microprecipitations are conveniently used in solute recovery for further investigations by auxillary chromatographic or spectral techniques. Described applications include determination of the triglyceride and fatty acid composition of vegetable oils, hydrocarbons in petroleum wax, and terpenoids in natural mixtures.

INTRODUCTION

Much recent progress in lipid chemistry is the result of extensive applications of chromatographic analytical and micropreparative techniques. However, separation and identification of the individual components of various non-polar mixtures still remains a non-trivial task. While many established chromatographic techniques in this area are confined to the "classical" mobile-phase/sorbent systems[1–3], high-performance liquid chromatography (HPLC) holds much promise for improvements[4].

Limited exploration of suitable HPLC column systems together with a lack of detection techniques have been major problems in extending this powerful method to mixtures of neutral lipids, essential oils, petroleum fractions, plant waxes, and other non-polar substances. During recent development of the light-scattering LC detector in our laboratory[5], we have shown that non-aqueous reversed-phase chromatography can be used with advantage to separate mixtures of cholesterol esters and triglycerides.

Light-scattering (nephelometric) detection and reversed-phase chromatography can complement each other[5], with no substantial compromises in either separation or detection conditions. While the non-polar solutes are separated on conventional

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(octadecylsilane) small-particle columns in organic solvents miscible with water (acetone, methanol, tetrahydrofuran, etc.), an aqueous buffer is added at the column end, thus forcing lipids out of solution and making them detectable by the light-scattering detector.

The design, operating conditions, and analytical performance of the light-scattering detector have already been described[5], while another recent publication[6] describes some applications of clinical and biomedical interest. The purpose of this communication is to demonstrate further applications of this general method to non-polar mixtures originated from diverse samples that may be of interest in different fields. Microgram amounts of various non-polar materials are shown to be effectively analyzed. Certain micropreparative aspects of this technique are also stressed, concerning the easy recovery of separated substances and feasibility of their further investigations by additional analytical techniques, such as mass spectrometry (MS).

EXPERIMENTAL AND RESULTS

Chromatographic system

A Waters Assoc. Model ALC/GPC 202 liquid chromatograph was used throughout the experiments. The light-scattering detector has been described earlier[5], including its modified version[6] with improved linearity. An additional high-pressure pump (Varian Model 8500) was used to introduce various amounts of aqueous solutions into a mixing tee between the column and the nephelometric detector. Two reversed-phase (octadecyl silica) columns used in this work were a 30 cm × 3.9 mm I.D., 10 μm μBondapak (Waters Assoc., Milford, Mass., U.S.A.) and a 25 cm × 4.6 mm I.D., 5 μm Zorbax ODS (Dupont, Wilmington, Del., U.S.A.) column.

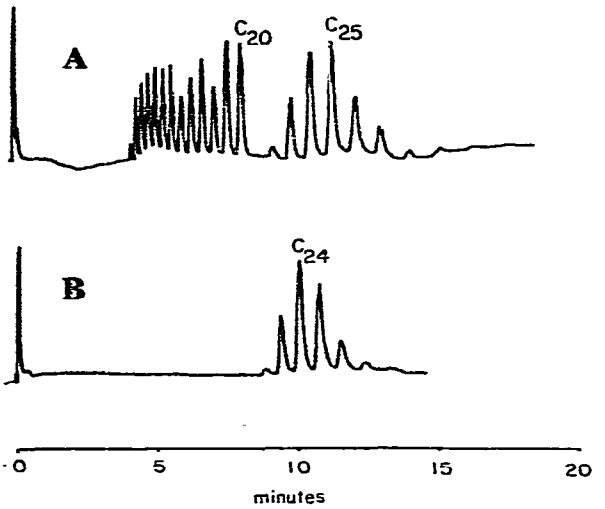


Fig. 1. (A) Chromatogram of standard hydrocarbon mixture (normal alkanes C_8–C_{30}, C_{21} omitted). Conditions: column flow-rate, 1.0 ml/min; mobile phase, methanol–tetrahydrofuran (2:1); precipitation agent concentration, 0.01 *F* ammonium sulfate; precipitation agent flow-rate, 2.0 ml/min; column, 5 μm Zorbax ODS. (B) Chromatogram of 50 μg petroleum wax sample. Conditions as in (A).

Sample preparation and chromatography

A hydrocarbon standard mixture and a sample of petroleum wax were dissolved in tetrahydrofuran and a 1-μl aliquot was chromatographed on the column, using a methanol–tetrahydrofuran (2:1) mixture as the mobile phase at 1.0 ml/min. The precipitation agent was 0.01 *F* ammonium sulfate, and its flow-rate was 2.0 ml/min. Chromatograms of these mixtures are shown in Fig. 1. Assignment of the carbon number to the individual homologs was made using standard hydrocarbons and the previously reported gas chromatographic (GC) data[7]. Comparison with the peak areas of standards indicate amounts of a few micrograms per component to be present in the analyzed mixtures.

Vegetable oils were diluted with tetrahydrofuran and 2-μl aliquots were injected onto the octadecyl silica column. Again, carbon-number separations for the individual triglyceride mixture components are indicated (Fig. 2). However, the overall chromatographic profiles of different vegetable oils are dependent upon the carbon number and the degree of unsaturation, both of which influence compound retention in a reversed-phase system.

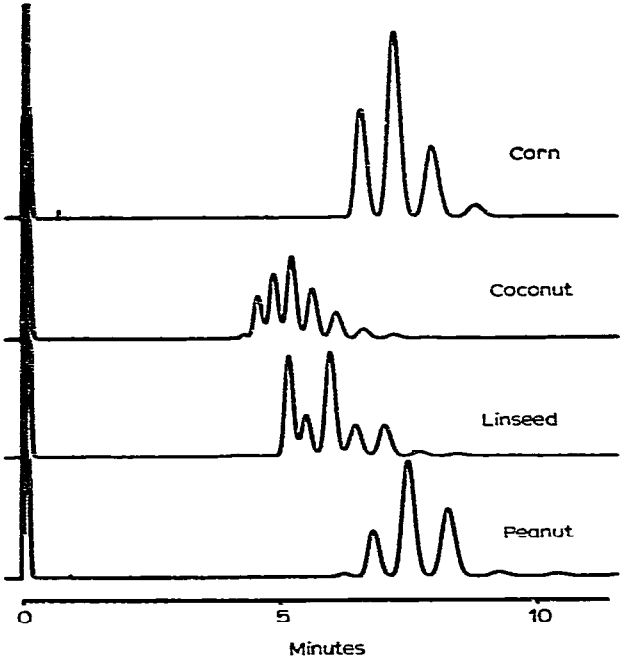


Fig. 2. Chromatograms of four vegetable oils (100 μg each). Conditions: column flow-rate, 1.0 ml/min; mobile phase, acetone–methanol (2:1); precipitation agent concentration, 0.025 *F* ammonium sulfate; precipitation agent flow-rate, 2.0 ml/min; column, 10 μm μBondapak.

One of the requirements for certain HPLC applications to complex natural mixtures with unknown constituents is an easy micropreparative technique, one which is compatible with further investigations by various structure elucidation techniques. Here, the precipitated solute is detected in a small-volume cell (approximately 10 μl) and can be easily recovered in a small vessel for further studies.

The microgram amounts of lipids collected after detection can be further investigated by MS, nuclear magnetic resonance spectrometry (NMR), GC–MS, etc., as shown in the following example of an analytical scheme used for the determination of fatty acid distribution within vegetable oil triglycerides.

A 200-μg aliquot of cottonseed oil was chromatographed on a 10-μm particle column, using an acetone–methanol (2:1) mixture as the mobile phase (Fig. 3A). Fraction 2 of this chromatogram was collected and rechromatographed on the same type of column, but with a different mobile phase (acetone–methanol (2:3), containing 0.4% $AgNO_3$). This phase system allows for the "argentation chromatography" and a subsequent resolution of isomers with different degree of unsaturation; fraction 2 of chromatogram A has now been resolved into three peaks (Fig. 3B). At this stage, an aliquot of any of the separated fractions can be subjected to MS to yield molecular weight information.

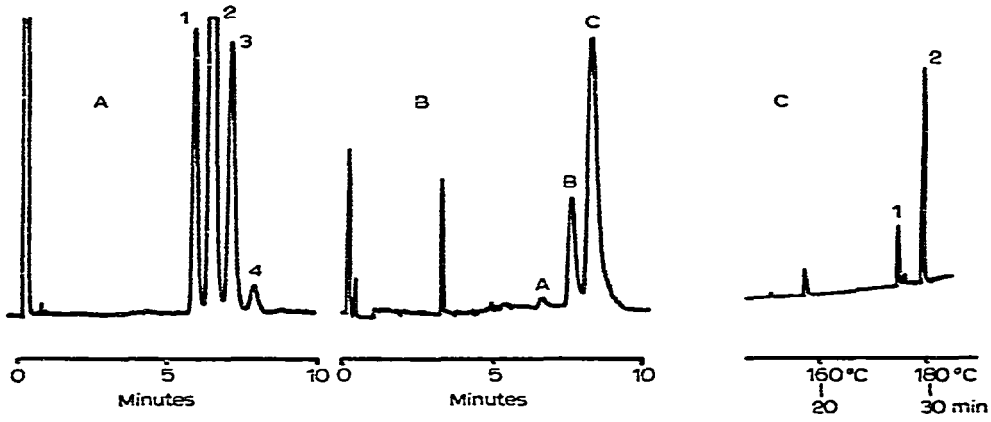


Fig. 3. Analysis scheme for the determination of fatty acid distribution within vegetable oil triglycerides. (A) Chromatogram (HPLC) of cottonseed oil. Conditions as in Fig. 1 except mobile phase, acetone–methanol (2:1); column, 10 μm μBondapak. (B) Chromatogram (HPLC) of fraction 2 of chromatogram A. Conditions as in Fig. 1 except mobile phase composition acetone–methanol (2:3), containing 0.4% $AgNO_3$; column, 10 μm μBondapak. (C) Gas chromatographic separation of the hydrolysis products of fraction B of chromatogram B, chromatographed as fatty acid methyl esters. Chromatographic conditions: 30 m × 0.2 mm I.D., OV-101 glass capillary column, temperature programmed from 120° at 2°/min. Peaks: 1 = methyl oleate; 2 = methyl linoleate.

Fraction B of chromatogram B was trapped and the material hydrolyzed with methanolic KOH. A subsequent derivatization[8] with methanol–HCl yielded methyl esters for GC analysis. A glass capillary column (30 m × 0.2 mm I.D.) coated with OV-101 methyl silicone fluid was attached to a Hewlett-Packard 5980A dodecapole mass spectrometer and an appropriate aliquot of the hydrolyzed sample was injected. Whereas a pertinent part of this chromatogram is shown in Fig. 3C (with flame ionization detection), the mass spectral data established that the two major peaks are methyl esters of oleic and linoleic acids.

Various samples of standard terpenes, long-chain alcohols, and sterols, as well as chromatography of waxy plant materials gave sufficient indication of a potential applicability of the light-scattering detector in the area of natural

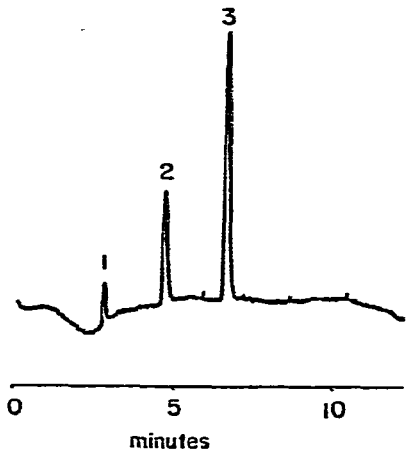
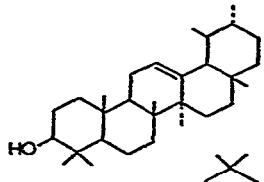
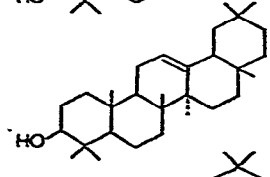
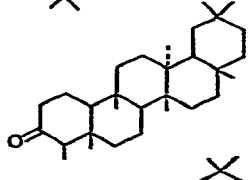
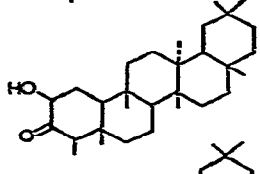
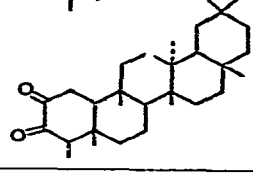


Fig. 4. Chromatogram of ethyl acetate extract of cork. Conditions as in Fig. 1. Peaks: 1 = friedelan-2,3-dione (tentative); 2 = cerin; 3 = friedelin.

TABLE I

STRUCTURES OF TERPENES

Compound	*Structure*
α-Amyrin	
β-Amyrin	
Friedelin	
Cerin	
Friedelan-2,3-dione	

products separation and analysis. Examples included an almost complete separation of α- and β-amyrin, in addition to the analysis of main triterpene components of the ethyl acetate extract[9] of cork, which is shown in Fig. 4. The individual peaks were collected and identified by MS as cerin, friedelin, and the third component tentatively identified as friedelan-2,3-dione. For structures of the mentioned terpenes, see Table I.

A simple procedure was also developed for the chromatographic analysis of solanesol, an important component of tobacco leaf[10]. A sample of commercial cigarette tobacco was extracted for 2 h with *n*-hexane in a Soxhlet extractor. The hexane extract was then partitioned with nitromethane[11] and the remaining hexane fraction concentrated for analysis. The resulting chromatogram is shown in Fig. 5. Whereas the first eluting large peak is a mixture of several components, the second large peak was tentatively identified by MS as solanesol from its molecular ion at *m/e* 630, in addition to the commonly observed[12] solanesenes (*m/e* 612), major thermal decomposition products of solanesol.

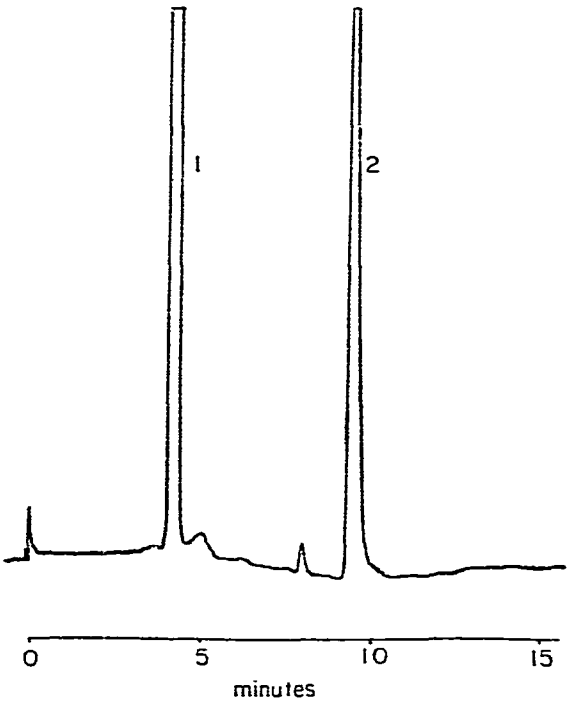


Fig. 5. Chromatogram of hexane extract of tobacco. Conditions as in Fig. 1 except mobile phase, acetone–methanol (2:1).

DISCUSSION

This communication provides further evidence that a simple light-scattering detector[5,6] is a valuable addition to the family of selective detectors for HPLC. In particular, its application is well illustrated here for the types of compounds that are not easily detectable by any other monitoring devices.

Although lipids with some degree of unsaturation can also be detected in the UV spectral region, light-scattering detection provides a more universal, regular and predictable response. As illustrated with the different vegetable oil samples (Fig. 2) their triglyceride composition can be more easily determined than through an alternative method of gas–liquid chromatography that must be carried out at inconveniently high temperatures[13].

Other non-polar mixtures, petroleum samples, waxes, etc., are also easily chromatographed in the described reversed-phase systems. Yet, no other detector is currently available for such compounds with microgram sensitivity. Although the profiles of hydrocarbons demonstrated in Fig. 1 can also be obtained through high-temperature capillary GC[7], the range of compounds can further be extended by a more non-polar mobile phase and/or gradient elution. The method may be suitable for a rapid determination of the hydrocarbon composition of petroleum waxes or distillation residues.

Micropreparative techniques are important to establish the chemical nature of the separated mixture components. While trapping and solvent removal are common in HPLC when further investigations are needed, the solute recovery after nephelometric detection is quantitative and very easy. Further sample manipulations are trivial in that the collected precipitate can be easily transferred into an NMR or MS probe, infrared cell, dissolved in a small amount of suitable solvent, etc., as needed. Some examples have been demonstrated in this work.

A good example of sample micro-manipulations is the determination of the fatty acid distribution in triglycerides shown in Fig. 3: a quantity of substance under one triglyceride peak from the sample of cottonseed oil was sufficient for further purification step by complexation chromatography, subsequent hydrolysis, methylation for a GC–MS run and quantitative fatty acid analysis. The results indicate that the particular analyzed triglyceride consists of one residue of oleic acid and two residues of linoleic acid.

Separations and micro-isolations of plant waxes and terpenoids are of considerable interest to natural products chemists. Chromatograms and spectral investigations of the samples shown here represent only a small fraction of many potential applications of the described system. The analysis of the ethyl acetate extract of cork (Fig. 4) and subsequent MS established the presence of major terpene components, isolated previously by classical approaches[9] as individual substances.

Solanesol, a terpenoid alcohol of tobacco leaf, has been known to be a major precursor of carcinogenic polycyclic aromatic hydrocarbons. Since a rapid and quantitative method is needed for its determination, gas–liquid chromatography has recently been advocated[10-12]. However, insufficient thermal stability, need for derivatization, as well as a limited specificity of the GC approach are obvious drawbacks. Also, the elution of trimethylsilylated solanesol necessitates a short (and inefficient) GC column together with very high temperature. On the other hand, our results with reversed-phase HPLC and the nephelometric detection suggest that a far more simple method can be devised for quantitation of solanesol in tobacco leaf extracts.

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