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NON-AQUEOUS REVERSED-PHASE CHROMATOGRAPHY OF GLYCERIDES USING INFRARED DETECTION

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SUMMARY

Non-aqueous reversed-phase liquid chromatography has been found to provide an effective means of resolving complex mixtures of low-polarity substances present in glyceride-based oils.

The most selective elution conditions from a column containing Zorbax ODS chromatographic packing are obtained with mobile phases formed from methylene chloride or tetrahydrofuran and acetonitrile. Separation of the essentially UV-transparent glyceride-type solutes can be monitored by either differential refractive index or infrared detection. The latter method has the advantage that temperature control of the detector is unneccessary and that the wavelength of operation imparts a degree of specificity to the detection system. By using infrared detection, it has been possible to monitor separations carried out under gradient elution conditions.

INTRODUCTION

The analysis of glycerides and fatty acids derived from both the animal and vegetable kingdoms has been the subject of extensive studies over the last 25 years. The characterization of the amount and quality of glycerides in oils and fats has important commercial and biological significance as these substances are used in food, lubricant, soap and cooking oil manufacture.

Chromatography has played an important role in the characterization of fats and oils; indeed, the first gas chromatographic methods to be reported, by James and Martin in 1952[1,2], were concerned with the separation of fatty acid homologs.

Commercially, the analysis of fats has been approached from several different standpoints. Traditionally many industries rely upon non-specific methods of testing such as the softening characteristics[3], the degree of unsaturation, as determined by iodine absorption[4], or the total amount of hydrolysable material in a sample, usually referred to as the saponification value[5].

With the advent of chromatographic modes of analysis, it has proved possible to separate quantitatively the fatty acids that form the glycerides in a given oil sample. Thus it is possible to specify a sample according to its chemical constituents. The last mentioned type of analysis has been most frequently performed on the methyl esters of fatty acids. The methyl esters are obtained by initially hydrolysing the oil

sample and subsequently methylating the liberated fatty acid mixture. Although this approach produces solutes that can be conveniently studied by gas chromatographic methods, it suffers from the basic limitation that valuable information regarding the structure of a given glyceride can be lost during the hydrolytic procedure.

Triglycerides have been studied by gas–liquid chromatography[6], but the technique has not been widely practised. The principal limitations are the thermal instability of polyunsaturated compounds and the fact that excessively high column temperatures are required to elute glycerides that have fatty acids with carbon chains longer than about C_{12}.

Chromatographic methods for the analysis of fats in the liquid phase have also been widely reported and are the subject of a detailed review by Aitzetmuller[7]. As mono-, di- and triglycerides possess two, one and zero hydroxyl functional groups, respectively, the separation of these species by liquid chromatography should be relatively straightforward.

Liquid–solid chromatography using columns packed with silica have been the most widely studied for glyceride separations. Typical solvents used as mobile phases in this approach are 5% (v/v) diethyl ether in *n*-hexane, to elute the triglyceride components, 25% (v/v) diethyl ether in *n*-hexane to elute the diglycerides and 80% (v/v) diethyl ether in *n*-hexane to elute the more polar monoglycerides[8]. The elution behavior obtained under these conditions is based essentially on resolving the sample components into their respective classes. There is little, if any, resolution of homologs, *i.e.*, one triglyceride from another. The use of highly efficient chromatographic columns could possibly yield some degree of resolution within a given class, but the requirement for gradient elution for these essentially UV-transparent compounds creates a detection problem. Many workers have reported on the use of the so-called moving-wire flame-ionization detector for monitoring separations of glycerides. Unfortunately, in spite of many reported modifications, this type of detector lacks widespread popularity and, at present, such detectors are no longer manufactured commercially.

The separation of glycerides in natural oils by the size exclusion method has also been considered. This technique has experienced a considerable revival in the last 2 years with the introduction of highly efficient column packings of small particle diameter[9]. Unfortunately, even the best size exclusion systems are only able to resolve compounds that differ in molecular size by a factor of two. Typical glycerides such as tristearin (mol.wt. 891) and tripalmitin (mol.wt. 807) clearly are unlikely to be separable by such procedures.

Reversed-phase chromatography has been applied successfully to fatty acid phenacyl esters[9] and to the separation of polyunsaturated triglycerides[10]. The technique would, therefore, seem to possess promise for the separation of other glycerides according to hydrocarbon chain length. The above-mentioned examples, however, have physical characteristics that help the separation process considerably, *i.e.*, they are soluble in alcohols and are readily monitored by ultraviolet absorbance detectors. This situation does not apply to all glycerides: many are essentially insoluble in alcohol and are very poorly detected by ultraviolet absorbance methods. The lipophilic character of the sample components makes their solubility in aqueous–organic solvent mixtures exceedingly low; thus elution and detection can be a problem.

Recently, non-aqueous reversed-phase chromatography has been found to of-

fer considerable potential for the analysis of lipophilic samples such as aliphatic hydrocarbons, carotenoids and oil-soluble vitamins[11]. This paper is concerned with the applicability of this method to the practical problem of separating complex glyceride mixtures under mild conditions, *i.e.*, at room temperature without prior hydrolysis or derivatization of the sample. Of particular interest in the extent to which infrared absorbance detection can be used to monitor the separation of glyceride-type samples.

EXPERIMENTAL

Apparatus

All chromatographic studies were carried out on the following units, all obtained from DuPont, Instrument Products Division (Wilmington, Del., U.S.A.): Model 830 liquid chromatograph, Model 838 gradient elution accessory, Model 845 differential refractometer (cell volume 3 mm^3) and a DuPont infrared detector. The last unit had variable-wavelength capability over the range 2.5–14.5 μm. The optical pathlength of the sodium chloride flow cell was 0.23 mm and the internal volume was approximately 30 mm^3. Chromatograms were displayed on the chart of a Model 7130A potentiometric recorder (Hewlett-Packard, Avondale, Pa., U.S.A.).

Samples were introduced with a microsyringe of 50-μm^3 capacity (Type 705-SN, Hamilton, Reno, Nev., U.S.A.) into a Model 7120 septumless injector (Rheodyne, Berkeley, Calif., U.S.A.). The chromatographic columns (250 × 4.6 mm I.D.) were obtained pre-packed with Zorbax ODS from the supplier (DuPont). Zorbax ODS is a chromatographic packing based on 6-μm diameter porous silica microspheres having a bonded monolayer stationary phase of octadecylsilane.

All solvents were of distilled-in-glass quality obtained from Burdick & Jackson (Muskegon, Mich., U.S.A.).

Development of a non-aqueous reversed-phase system

The chromatographic column was maintained at 35° and the inlet pressure of the solvent delivery system was adjusted so as to maintain a mobile phase flow-rate of 1 cm^3/min. The retention behavior of glycerides with various compositions of the mobile phase was studied using a commercial cod liver oil sample as the test mixture. Mobile phases were prepared by mixing acetonitrile with methylene chloride or tetrahydrofuran in the appropriate proportions.

The course of a separation was monitored using the infrared detector operating at a wavelength of 5.75 μm, which corresponds to the absorption of infrared radiation by the carbonyl group present in the glyceryl esters and fatty acids. A limited investigation was also made into the feasibility of monitoring separations of glycerides using the infrared detector when the analysis is carried out under gradient elution conditions.

Elution behavior of vegetable oils

The most suitable mobile phase composition for the separation of components present in samples of corn oil and palm oil was investigated using the methylene chloride–acetonitrile system. Chromatograms obtained when monitoring the differential refractive index of the column effluent from these two oils were compared under otherwise identical operating conditions.

Elution behavior of mono- and diglycerides

Chromatographic conditions that were found to be applicable to the analysis of commercial oil samples were used to examine the elution behavior of pure and commercial samples of monostearoylglycerol. Similarly solutions containing 1,2- and 1,3-distearoylglycerol were analysed in order to establish the feasibility of resolving isomeric diglycerides by this procedure.

RESULTS

Development of a non-aqueous reversed-phase system

Rapid and complete elution of the components of cod liver oil was observed when either methylene chloride or tetrahydrofuran was used as the mobile phase. Conversely, no elution of the sample components from the Zorbax ODS column was observed when the mobile phase contained only acetonitrile. Systemmatic tests using 80%, 60%, 40% and 20% (v/v) of methylene chloride in acetonitrile as the mobile phase clearly indicated the dependence of elution speed on the concentration of methylene chloride. Fig. 1 illustrates the results obtained by reproducing the chromatograms obtained under these conditions.

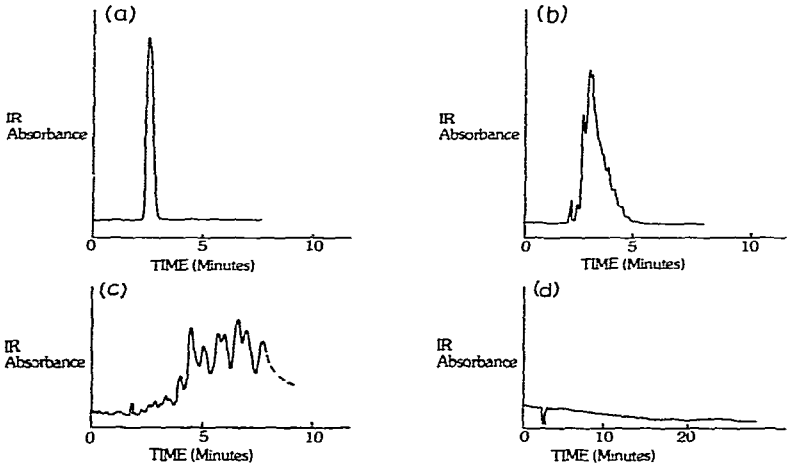

Fig. 1. Influence of methylene chloride concentration in acetonitrile mobile phase on the elution of cod liver oil components from Zorbax ODS. Operating conditions: column, 250 × 4.6 mm I.D.; temperature, 35°; mobile phase flow-rate, 1 cm^3/min; inlet pressure, *ca.* 53 bar; infrared detector operating at 5.75 μm. Concentration of methylene chloride in acetonitrile: (a) 80%; (b) 60%; (c) 40%; (d) 20%.

In a parallel study, shown in Fig. 2, tetrahydrofuran was shown to behave in an analogous manner to methylene chloride, *i.e.*, an increase in the concentration of this solvent in the mobile phase accelerated the elution of the sample components.

It was apparent that a mobile phase containing less than about 25% of tetrahydrofuran failed to elute the glycerides present in the oil sample, whereas mobile phases containing 50–75% of tetrahydrofuran would cause rapid elution. The overall difference in the infrared absorbance, measured at a wavelength of 5.75 μm, between

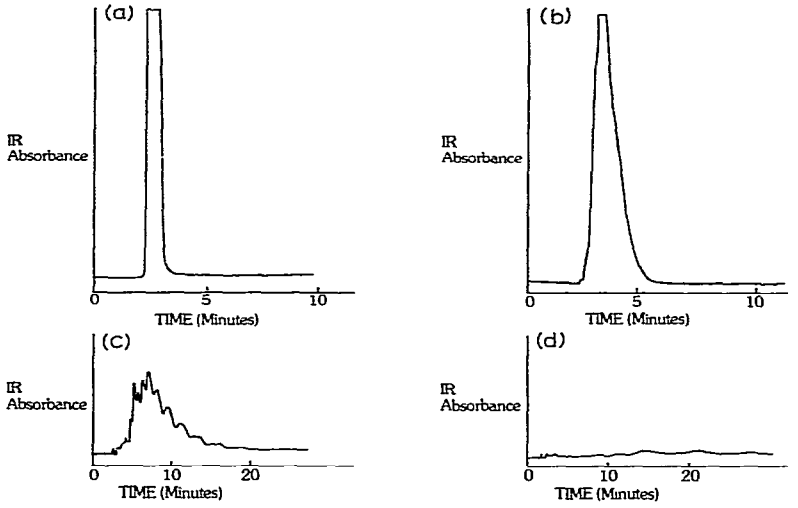

Fig. 2. Influence of tetrahydrofuran concentration in acetonitrile mobile phase on the elution of cod liver components from Zorbax ODS. Operating conditions: as for Fig. 1. Concentration of tetrahydrofuran in acetonitrile: (a) 80%; (b) 60%; (c) 40%; (d) 20%.

20% and 50% of tetrahydrofuran in acetonitrile was relatively small (of the order of 0.02 absorbance unit). This small difference in absorbance between two binary solvent mixtures, one of which is capable of retaining and the other of eluting the sample, suggested the possibility of using infrared detection to monitor separations carried out under gradient elution conditions.

A "blank" chromatographic analysis was carried out using a mobile phase composition which was changed from 20% to 60% of tetrahydrofuran in acetonitrile.

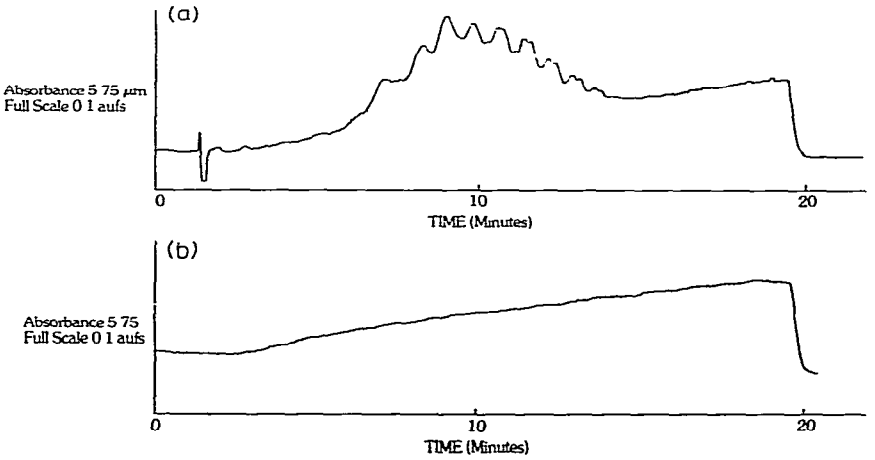

Fig. 3. Gradient elution separation of cod liver oil components monitored by infrared detection. Operating conditions: as for Fig. 1 except that a sensitivity of 0.1 absorbance unit equivalent to full scale of recorder chart was used. Concentration of tetrahydrofuran concentration in the mobile phase was increased linearly from 20% to 60% over a 20-min period.

Fig. 3b indicates the magnitude of the baseline shift observed when a linear concentration gradient was programmed and the infrared detector was operated at a sensitivity equivalent to 0.1 absorbance unit equal to full scale of the recorder chart. Fig. 3a illustrates, for comparison, a sample of cod liver oil analysed under the same conditions. The strong retention of the sample components in the early part of the chromatogram, followed by more rapid elution as the tetrahydrofuran concentration was increased, is apparent.

Elution behavior of glycerides in selected vegetable oils

Much of the development of the solvent mixtures used to form the mobile phase in this investigation was undertaken using cod liver oil as the test mixture. The elution characteristics of two vegetable oils, namely palm oil and corn (maize) oil, were also studied in order to ensure the versatility of the technique. A mobile phase containing 40% of methylene chloride in acetonitrile proved a useful isocratic mixture for the analysis of these oils. Figs. 4 and 5 compare, under identical operating conditions, the chromatograms obtained from 5 mg of palm oil and corn oil, respectively. A differential refractive index detector was used to monitor these separations, *i.e.*, the system was not responding selectively to carbonyl compounds. Independent tests, using the corn oil sample, demonstrated that the infrared and differential refractive index detector offered approximately equivalent sensitivities. Fig. 6, for example, is a chromatogram obtained using an infrared detector to monitor the separation of a 5-mg sample of corn oil. The operating conditions were similar to, but not identical with, those used for the separation monitored by refractometry shown in Fig. 5.

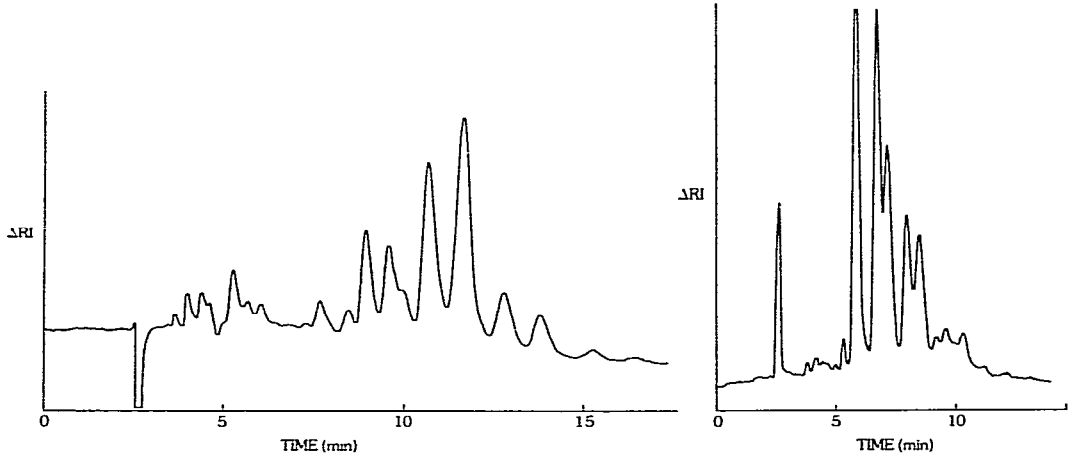

Fig. 4. Non-aqueous reversed-phase chromatography of palm oil. Operating conditions: as for Fig. 1 except that a differential refractive index detector operating at a sensitivity of $2 \cdot 10^{-5}$ refractive index units equivalent to full scale of recorder chart was used. Sample size: 5 mg. Mobile phase composition: 40% of methylene chloride in acetonitrile.

Fig. 5. Non-aqueous reversed-phase chromatography of corn (maize) oil. Operating conditions: as for Fig. 4.

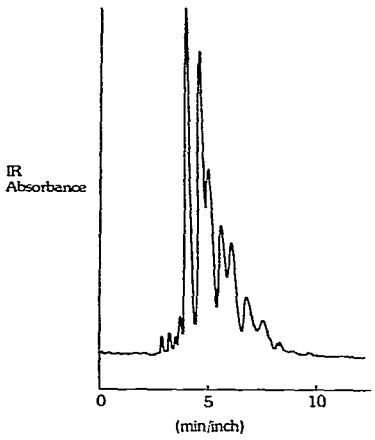

Fig. 6. Non-aqueous reversed-phase chromatography of corn oil monitored by infrared detection. Operating conditions: as for Fig. 4 except that an infrared detector was used (operating wavelength 5.75 μm). Mobile phase composition: 50% of methylene chloride in acetonitrile.

Elution of mono- and diglycerides

A sample of commercial quality monostearoylglycerol was examined using a mobile phase containing 40% of methylene chloride in acetonitrile. Under these conditions, a series of well resolved components were observed in the chromatogram generated from the output of the infrared detector operating at a wavelength of 5.75 μm. Injection of a solution prepared from reference-quality monostearoylglycerol yielded a single chromatographic peak, well resolved from the solvent front. These two chromatograms are shown in Figs. 7a and b.

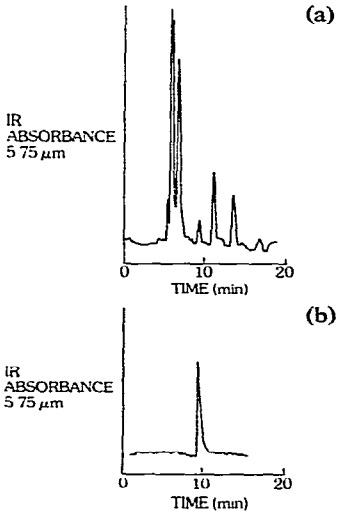

Fig. 7. Comparison of (a) commercial and (b) pure monostearoylglycerol by non-aqueous reversed-phase chromatography. Operating conditions: as for Fig. 4 except that an infrared detector was used, operating at a wavelength of 5.75 μm.

Small amounts of reference-quality 1,2- and 1,3-distearoylglycerol were dissolved individually in small portions of the mobile phase. Analysis of these solutions under identical conditions showed that the two isomers could be resolved under these non-aqueous reversed-phase conditions. The isomers were eluted with capacity factors (k') of 1.90 and 2.10 for the 1,2- and 1,3-distearoylglycerols, respectively. Fig. 8 shows a chromatogram of a synthetic mixture of the two distearoyl isomers and monostearoylglycerol. The early elution of monostearoylglycerol ($k' = 0.61$) is in keeping with the more polar nature of this component on this reversed-phase system.

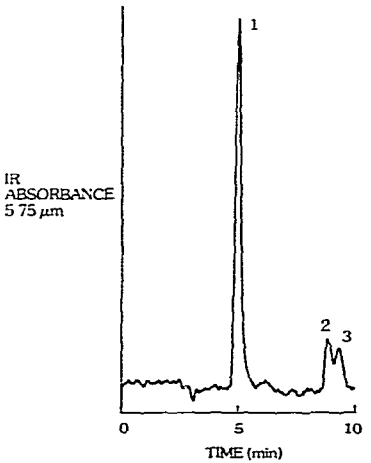

Fig. 8. Separation of distearoylglycerol isomers from monostearoylglycerol by non-aqueous reversed-phase chromatography. Operating conditions: as for Fig. 7. Peaks: 1 = monostearin ($k' = 0.61$); 2 = 1,2-distearin ($k' = 1.90$); 3 = 1,3-distearin ($k' = 2.10$).

DISCUSSION AND CONCLUSIONS

Earlier studies[11] showed that non-aqueous reversed-phase chromatography offers considerable potential for the analysis of hydrocarbons, carotenoids and oil-soluble vitamins. This investigation has shown that the glycerides, in common with the previously mentioned lipophilic substances, can be chromatographed successfully by the non-aqueous method. The principal advantages of this approach are summarized below.

All separations can be performed at room temperature or at a slightly elevated temperature, *e.g.*, 35–40°. Under these conditions, the stability of the compounds is not affected; this is particularly important when analysing polyunsaturated glycerides as they are easily degraded if subjected to the high temperatures necessary to separate them by gas–liquid chromatography.

The non-aqueous reversed-phase method utilizes mobile phases that are excellent solvents for lipophilic compounds, *viz.*, tetrahydrofuran and methylene chloride. With a high concentration of either of these solvents in the mobile phase, all sample components appear to be eluted rapidly from the columns and contamination of the chromatographic system is therefore avoided. Success at retaining lipophilic substances on the chromatographic packing, Zorbax ODS, when using these

excellent solvents mixed with acetonitrile is due essentially to the heavy coating, *ca.* 25% of octadecylsilane, chemically bonded to the particle surface. Several other commercial reversed-phase packings were examined in preliminary studies, but failed to maintain sufficient retention under these operating conditions, presumably because of an insufficient loading of stationary phase.

There are two practical consequences of the use of mobile phases that will readily dissolve the sample. Firstly, relatively concentrated sample solutions can be prepared, *e.g.*, 10–20% (v/v) of oil in a mobile phase is not uncommon. This high solubility permits the use of refractive index and infrared detection, which, while being most useful for monitoring these poor UV-absorbing species, lack the high sensitivity associated with ultraviolet absorbance and fluorescence techniques. In a similar way, it is possible to separate samples containing 10–20 mg of oil on analytical-scale columns, *i.e.*, those with internal diameters of 4.6 mm. Fraction collection of milligram amounts of eluting components is clearly feasible: these amounts are ideal for further investigation by complementary methods.

Infrared detection appears to be ideally suited to the analysis of glycerides. This detection principle has been employed in gas chromatography[12] and also in size exclusion chromatography[13], but little has been reported on its use for liquid column chromatography. The main advantages over refractometric methods are that a wavelength can be selected so as to impart a degree of specificity to the detector response and neither a differential column system nor temperature control of the detector is necessary.

The choice of solvents that can be used successfully with infrared detectors is inevitably much more restricted than with the differential refractive index system. It should be appreciated, however, that some of the best solvents for lipophilic samples are the most useful solvents for infrared spectrophotometry, *e.g.*, methylene chloride, chloroform and carbon disulfide. This work indicates that the transmission of light is sufficient at 5.75 μm to operate the detector at high sensitivity when working with acetonitrile, methylene chloride or tetrahydrofuran. The example of using infrared detection to monitor a separation achieved under gradient elution conditions must raise an interesting possibility for other applications. Although a baseline shift does occur, the magnitude of the shift is considered to be acceptable for practical day-to-day investigations.

The popular ultraviolet absorbance detectors are much less useful for the analysis of glyceride-based oils owing to the overall poor absorbance characteristics of the samples. Additionally, "total analysis" is complicated by the fact that poly-unsaturated solutes posses higher extinction coefficients than saturated solutes. The use of ultraviolet detectors operating at short wavelengths, *i.e.*, 190–210 nm, has been reported for the detection of essentially saturated acids and bile acids[14]. This approach is not satisfactory for glycerides as the solvents in which the samples are soluble do not transmit light below about 230 nm.

A combination of infrared detection and non-aqueous reversed-phase chromatography appears to give a system that is capable of separating milligram amounts of fish and vegetable oils as well as mono- and diglycerides. The system is versatile and easy to operate in that no temperature control of the detector or lengthy equilibration time is required. Under selected conditions, gradient elution chromatography can be performed satisfactorily. Clearly, more work is required in order to establish

the identities of the many components that appear in the chromatograms shown here. A detailed study with high-quality reference materials is planned in order to establish the overall potential of the method.

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EDITOR'S NOTE

Reversed-phase chromatography with non-aqueous solvents is not really a new field. Extensive work on paper or thin layers impregnated with liquid paraffin, silicone oil or lower boiling hydrocarbons and using solvents such as acetone–acetonitrile (8:2), glacial acetic acid or acetone–methanol (9:1) has been reported (for a review see F. B. Padley, *Chromatogr. Rev.*, 8 (1966) 208).

The best results were obtained using multiple development with the same solvent and using rather sensitive spot tests. At first sight these compare well with those described in this paper.

Editor of J. Chromatogr.