〈Review〉

Recent Developments in High-performance Liquid Chromatography of Lipids

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Abstract

The possibilities for HPLC analysis of lipids have been revolutionised by the availability of evaporative light-scattering detectors, with which the response is independent of the nature of the mobile phase and does not depend on the presence of specific chromophores in the lipids. It was thus possible to develop an HPLC procedure, involving ternary gradient elution, for separating all the lipid classes in animal tissues in a single step. Although reversed-phase HPLC has been widely used for the analysis of molecular species of lipids, silver ion chromatography can be a valuable alternative. For example, a stable silver ion column for HPLC was developed which permitted resolution of molecular species of triacylglycerols, even from such complex samples as fish oils, again with light-scattering detection and gradient elution. The capacity for HPLC resolution of diastereomeric diacyl-*sn*-glycerol derivatives, prepared from triacylglycerols, has lead to a new simple method for stereospecific analysis of the latter.

Introduction

Lipid analysts were slow in realising the potential of high-performance liquid chromatography (HPLC), largely because of the lack of a sensitive universal detector. There is no HPLC detector with the simplicity, ease of operation and linearity of the flame-ionisation detector used in gas chromatography (GC), but a useful, proven and versatile instrument is available, *i.e.* the evaporative light-scattering or "mass" detector. With this instrument, the mobile phase emerging from the end of the HPLC column is evaporated in a stream of air in a heated chamber; the solute does not evaporate, but is nebulized and is carried in the form of minute droplets through a light beam, which is reflected and refracted. The amount of scattered light is measured by a photomultiplier tube and bears a relationship to the concentration of material eluted. The first commercial detector, based on this principle, was one from Applied Chromatography Systems (A.C.S) Ltd (Macclesfield, Cheshire, U.K.), but French (Cunow S.A., Cergy St Christophe; Sedere, Vitry-sur-Seine) and American (Varex Corp., Burtonville, MD) instruments now afford greater sensitivity. Applications of such detectors in lipid

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analysis have recently been reviewed [1].

There are no special wavelength requirements for the light source, and it is simply a projector lamp in some of the commercial instruments. Such detectors can be considered to be universal in their applicability, in that they will respond to any lipid that does not evaporate before passing through the light beam. Even under gradient elution conditions, the instruments give excellent results, and they are simple and rugged in use. In more recent models, the sensitivity is higher than that of a refractive index detector, but the light-scattering detector is not affected by changes in the mobile phase or small variations in ambient temperature or in the flowrate of the mobile phase. Even with sharp changes in solvent composition, there is no disturbance of the base-line and little drift during long periods of use. Most organic solvents, including acetone, toluene and chloroform, for example, can be used in the mobile phase, and these can contain up to 20% water and even small amounts of ionic species. While the minimum limit of detection is to some extent dependent on the nature of the mobile phase, it is about 0.1μg in the best commercial instruments.

The detector is admittedly destructive in that the sample is lost in the current of air, but it is possible to insert a stream splitter between the end of the HPLC column and the detector to divert much of the sample for collection. When it is used in this way, the detector is an excellent research tool, because samples can be collected easily for analysis by other methods, *e.g.* for the determination of fatty acid compositions by GC (with an internal standard if necessary). An additional advantage in research applications is that rapid adjustments to the chromatographic conditions are easy to make, since changing the solvent has virtually no effect on the base line, and this is often necessary during the development of new methods. The availability of evaporative light-scattering detectors has given lipid analysts much greater opportunities to use a wide range of mobile phases, often in complex gradients, to improve the selectivity of particular separations.

Separation of Lipid Classes

An important objective for lipid analysts is to separate all the main lipid classes, differing widely in polarity, from tissues in a single chromatographic run. It is not too difficult with thin-layer chromatography(TLC) but has been more of a challenge for HPLC. With the aid of a light-scattering detector and a ternary solvent delivery system, this was accomplished on a short column(5×100mm) packed with Spherisorb™ sillca gel(3μ particles)[2]. The choice of solvents for the mobile phase was constrained only by the need for sufficient volatility for evaporation in the detector under conditions that did not cause the solute to evaporate, and by the necessity to avoid inorganictions, which could not be volatilised. A ternary-gradient elution system with eight programmed steps was devised, starting in essence with isooctane or hexane to separate the lipids of low polarity and ending with a mobile phase containing water to elute the phospholipids. To mediate the transfer from one exfreme to the other, a solvent of medium polarity was required and isopropanol-chloroform(4:1, v/v) gave satisfactory results as illustrated in Fig. 1. After each analytical run, a gradient was generated in the reverse direction to remove most of the water bound to the silica gel, and to re-establish the activity of the column before the next analysis. A high flowrate(2ml/min) aided the separation and appeared to compensate for the absence of strong acid or inorganic ions, which others have found necessary for resolution of phospholipid classes. Subsequently, I demonstra-

ted that better resolution of minor acidic components could be obtained by adding small amounts of organic ionic species to the aqueous component of the eluent [3]. 0.5 to 1mM Serine buffered to pH 7.5 with triethylamine gave the best results. Although the response changed a little and some re-calibration was necessary, such ionic species at this concentration had no effect on the base-line of the detector. Also, the useful working life of the column was greatly extended by this simple step.

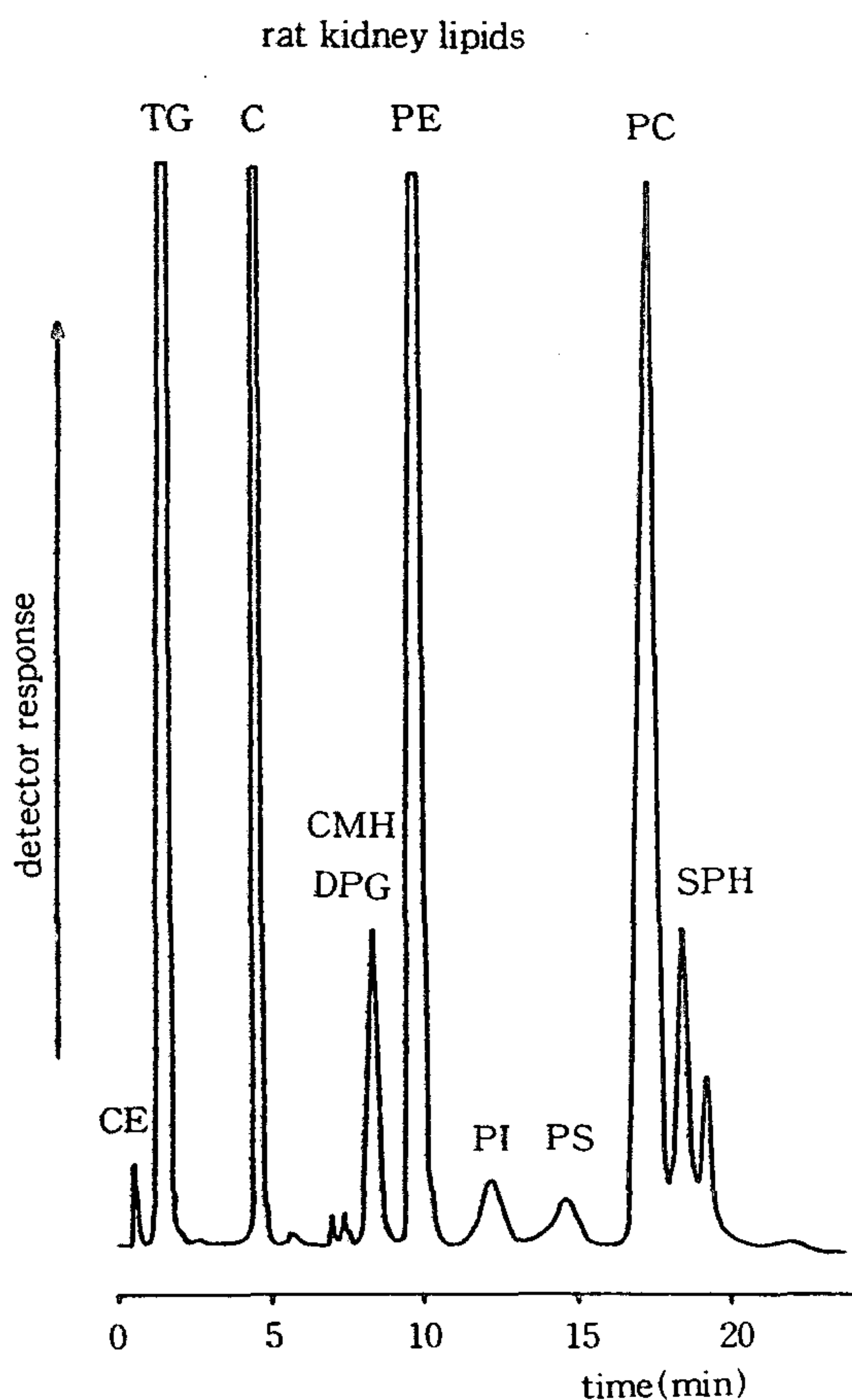

Fig. 1. Separation of lipids from rat kidney by HPLC on silica gel with a complex gradient and evaporative light-scattering detection [2, 3]. Abbreviations : CE, cholsterol esters : TG, triacylglycerols : C, cholesterol : CMH, ceramide monohexoside : DPG, diphosphatidylglycerol : PE, phosphatidylethanolamine : PI, phosphatidylinositol, PS, phosphatidylserine, PC, phosphatidylcholine : SPH, sphingomyelin.

In spite of the abrupt changes in solvent composition at various times, no disturbance of the base-line was apparent when animal tissue lipids were analysed, and all the main simple lipid(cholesterol esters, triacylglycerols and cholesterol) and phospholipid classes were separated from each other in only 20 minutes. After a further 10 minutes of elution to regenerate the column, the next sample could be injected onto the column.

The nature of the detector response in general has been studied in some detail [4~6], and it is known that it depends both on the nature of the solvent and of the solute. When a light-scattering detector is used in quantitative analysis of lipids, therefore, it is necessary to determine the optimum conditons for the desired separations first and then carry out a calibration with standards similar in nature to the samples to be analysed. The operating parameters of the detector, such as the air pressure, evaporator temperature and attenuation, must also be optimised and fixed. For most lipids, the response of the ACS detector was approximately linear in the range 50 to 200μg, but it tended to fall off rapidly below 10 μg; newer models of detector would be expected to exhibit much better sensitivity and linearity. The linearity problem occurs because light is scattered much less by small droplets as their diameters near the wavelength of light; with improved detector design, the problem might be reduced but not eliminated. The accuracy and precision of evaporative light-scattering detectors in quantitative analysis can also be improved by adding an internal standard. For example, phosphatidyldimethylethanolamine was used in lipid class analyses [7].

Methodology for the separation of simple lipids and phospholipids or of phospholipids alone, and adapted from the above or similar to it, has now

been described from a number of laboratories confirming its robustness [8~15]. Moreau has published an interesting comparison of the use of evaporative light-scattering and transport-flame ionisation detection in the analysis of plant lipid classes separated by a procedure adapted from the above [16]. The former was clearly superior.

Sliver Ion High-perfomance Liquid Chromatography

Many early attempts to adapt silver ion chromatography to HPLC met with limited success only. For example, an HPLC grade of silica gel was impregnated with silver nitrate and then packed into a columns, both the grade of silica gel and the method of impregnation being important [15, 17, 18]. Gradient elution with transport-flame ionisation detection was employed to obtain resolution and direct quantification of confectionery fats into molecular fractions. Unfortunately, a major problem was that silver ions eluted continuously in the mobile phase. While a precolumn of silver nitrate extended the life of the column and maintained the resolution, the fractions collected were contaminated with silver ions.

The alternative approach adopted by the author was to load a silica-based ion-exchange medium(chemically-bonded sulphonic acid groups) with silver ions in an HPLC column *in situ* by injection *via* the Rheodyne valve, while pumping water through it, before the aqueous phase was replaced with organic solvents [19]. Although only 50 to 80mg of silver nitrate was used, this was quite sufficient for the purpose. When this column was employed in the analysis of molecular species of triacylglycerols, the simplest elution scheme was a gradient of acetone into dichloroethane-dichloromethane [20]. This served for fats with small proportions of linoleic acid, such as sheep adipose tissue or bovine milk fat, but it gave excellent resolution of the principal components with zero to three double bonds in total in the fatty acyl chains. In addition, it was possible to separate fractions with *trans*- *from those with* *cis*- monoenoic residues.

In this and related studies, fractions were collected via a stream-splitter, and they were identified and quantified by adding methyl nonadecanoate as an internal standard and transmethylating for GLC analysis of the fatty acid components(If calibration is carried out with suitable standards, the response of the mass detector can be used directly). No silver salts were detectable in the fractions.

Most triacylglycerol samples contain a higher proportion of linoleic acid, and resolution into molecular species was then accomplished with a ternary gradient system simply by introducing acetonitrile into acetone after the first fractions were eluted. One dienoic acyl residue was retained 2.5 times as strongly as a monoene, and one triene(18:3(n−3)) was retained by the same amount as two dienoic residues in a molecule, so there was some overlap of dienoic and trienoic species when α-linolenic acid was present in samples. In contrast, molecular species of triacylglycerols containing γ-linolenic acid were retained a little less strongly and a distinctive resolution of evening primrose oil was attained, for example [21]. Triacylglycerols as highly unsaturated as linseed oil [20] and fish oils [20, 22, 23] have been fractionated successfully. With the former, the most abundant single fraction was trilinolenin, and a simple progression of fractions with increasing numbers of double bonds were eluted until this species was reached. Triacylglycerols from fish were fractionated by silver ion HPLC by extending the polarity range of the gradient, as shown for Baltic herring oil in Fig. 2 [22, 23]. When the more saturated molecules

eluted(Fractions 1 to 8), resolution was excellent and it was surprising to find appreciable amounts of trisaturated and disaturated-monoene species. Base-line resolution could no longer be sustained when molecules containing trienoic and more highly-unsaturated fatty acids began to elute (Fractions 9 to 19), because the wide range of positional isomers caused components to overlap. Valuable separations of species containing two saturated and/or monoenoic fatty acids and one polyenoic fatty acid were achieved, nevertheless. The final group of peaks(Fractions 20 to 26) contained 11 to 15 double bonds per molecule.

The order of elution of triacylglycerol species is easily understood with silver ion systems because only one property of the molecules is involved, *i.e.* degree of unsaturation. On the other hand, reversed-phase chromatography effects separation both by chain-length and degree of unsaturation, each double bond reducing the effective chain-length by the equivalent of about two methylene groups. Excellent resolution is possible, but chromatograms of unknown samples especially are rarely easy to interpret.

The two techniques used in sequence make a much more powerful tool. Thus, fish oils with reversed-phase HPLC gave immensely complex chromatograms and identification of individual components was not possible [23]. When fractions from silver ion HPLC were collected and then subjected to reversed-phase HPLC, separation was then in effect by chain-length alone and the peaks were identifiable intuitively in a broad sense. When each fraction was examined in turn in this way, much more information was obtained from the sample in comparison to when either technique was used on its own. However, each fraction from the reversed-phase column still contained a large number of distinct molecular species, and HPLC in combination with mass

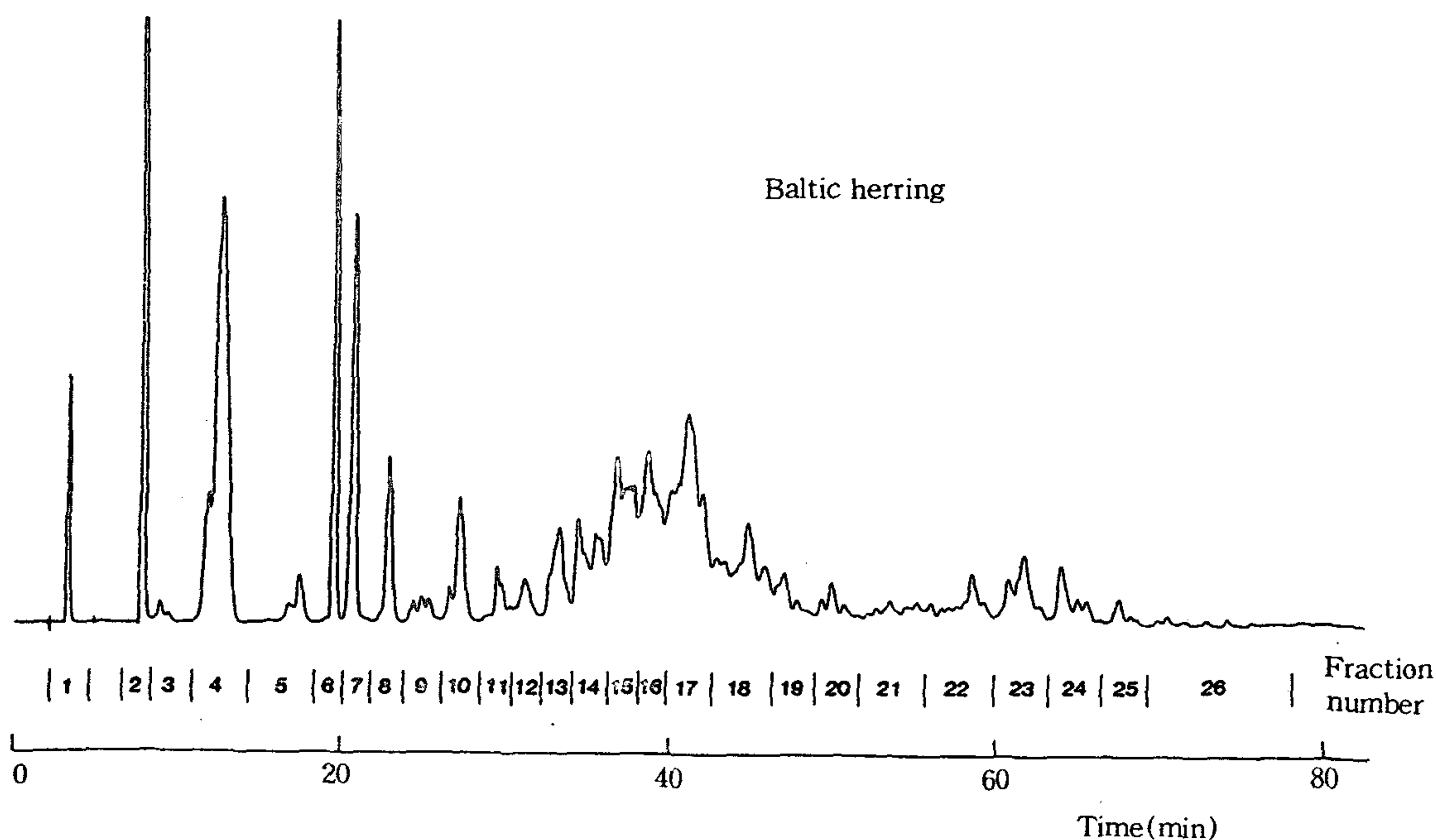

Fig. 2. Separation of molecular species of triacylglycerols from a fish oil, *i. e.* the Baltic herring, by silver ion HPLC with light-scattering detection [22]. A ternary gradient elution system, from dichloromethane-dichloroethane (1 : 1) to acetone to acetonitrile was employed.

spectrometry would be essential if individual components are to be identified.

This concerted approach was also of value in a related study of the seed oil of the meadowfoam plant(*Limnanthes alba*), which is unusual in that the triacylglycerols contain mainly C_{20} and C_{22} fatty acids with double bonds in positions 5 and 13, i.e. 5*c*−20 : 1, 5*c*−22 : 1, 13*c*−22 : 1 and 5*c* 13*c*−22 : 2, in addition to small amounts of fatty acids of the more normal kind. They were resolved by HPLC in the silver ion and reversed-phase modes, and by the two techniques used in a complementary fashion [24]. With silver ion HPLC, a distinctive resolution was achieved in which fractions differing only in the positiion of one double bond in a single monoenoic fatty acyl group were separated, the order of elution being 11−20 : 1, 5−20 : 1, 13−22 : 1, 5−18 : 1 and 9−18 : 1. Reversed-phase HPLC also gave fractions containing single positional isomers(11−20 : 1<5−20 : 1<13−22 : 1<5−22 : 1), but the pattern was less easy to ascertain as fractions containing 22 : 2 tended to overlap with those containing 20 : 1. When the techniques were used in sequence, many more fractions were obtained that when either technique was used on its own.

The silver ion HPLC procedure has some potential for the separation of positional and configurational isomers of fatty acids, and for quantitative determination of *trans*-unsaturation. To this end, phenacyl derivatives of fatty acids were prepared so that UV detection with accurate quantification was possible [25]. Dichloroethane-dichloromethane(1 : 1, v/v), sometimes with a little acetonitrile added, was used as the mobile phase to effect excellent resolution of positional and configurational isomers of mono−, di− and trienoic fatty acids. The distance of the double bond from the carboxyl group was found to be more important than the terminal region of the molecule in governing the separation of positional isomers [26]. As it was relatively easy to control the chromatographic parameters in silver ion HPLC in comparison with analogous TLC procedures, studies of the mechanism of the interaction between silver ions and unsaturated fatty acids were possible. It appears that this form of silver ion HPLC involves simultaneous interactions between one silver ion and either two double bonds, or one double bond and one other electron-rich moiety such as the ester group.

Resolution of Chiral Diacylglycerols as a Means of Stereospecific Analysis of Triacyl-*sn*-glycerols

One of the important stereochemical problem in lipid analysis is the resolution of the enantiomeric 1, 2− and 2, 3− diacyl-*sn*-glycerols, partly because of their importance as biosynthetic precursors of triacyl-*sn*-glycerols and partly as a step towards the stereospecific analysis of the latter(see below) [27]. One approach to the resolution of such diacyl-*sn*-glycerols has been to convert them to diastereomeric urethane derivatives such as the (*S*)−(+)−1−(1−naphthyl)ethyl urethanes, by reaction with isocyanates prepared from the enantiomericallypure amine. These diastereomers can be resolved by HPLC on columns of silica gel, and they are readily detected as they emerge from HPLC columns by their pronounced UV absorbance at 280 nm. The isocyanate reagent is available commercially. Reaction occurs rapidly with alcohols in the presence of a basic catalyst in toluene solution with no detectable racemization.

Diastereomeric naphthylethyl urethane derivatives were first used for resolution of chiral glycerol compounds by means of HPLC by Michelsen *et al.* [28], but the practical value of this work was limited as partial resolution only of

diastereomeric derivatives prepared from diacyl-*sn*-glycerols could be achieved under the conditions essayed. However, the desired separation was effected by Laakso and Christie [29], with a mobile phase of hexane with a small amount of isopropanol(or *n*–propanol) as modifier. (*S*)–(+)–1–(1–Naphty)ehthyl isocyanate derivatives of diacyl-*sn*-glycerols eluted in the order 1, 3–, followed by 1, 2–and then 2, 3–isomers. In addition, molecular species of single-acid diacyl-*sn*-glycerols derivatives were well resolved in the order $18:1<18:0<18:2<16:0$, *i. e.* neither that expected for normal–nor reversed-phase partition chromatography. When the (*R*)–(–)–form of the derivatizing agent was used, the order of elution changed so that 2, 3–emerged before 1, 2–isomers. It is should be noted that for the common range of fatty acyl groups studied, 1, 2–dipalmitoyl–and 2, 3–dioleoyl-*sn*-glycerol urethanes were just separable when the (*S*)–form of the derivatives was employed, but these were furthest apart with the (*R*)–form. If the enantiomeric forms of the derivatives were to be utilised in conjunction with HPLC–mass spectrometry, there is considerable potential for detailed analyses of complex mixtures.

This type of separation served as the basis for a novel procedure for stereospecific analysis of triacyl-*sn*-glycerols from natural oils and fats, *i. e.* for determining the compositions of fatty acids esterified to each of positions *sn*–1, *sn*–2 and *sn*–3 [29, 30]. Triacyl-*sn*-glycerols were subjected to partial hydrolysis with a Grignard reagent and the products were converted to the (*S*)–(+)–1–(1–naphthyl)ethyl urethane derivatives. The diacylglycerol derivatives were isolated by a solid-phase extraction procedure and were resolved into the diastereomeric forms by HPLC on silica gel. From high-precision GC analyses of the fatty acids of the intact triacylglycerols and of the 1, 2–and 2, 3–diacyl-*sn*-glycerol derivatives, the compositions of positions *sn*-3 and *sn*-1 respectively could be calculated; that of position *sn*-2 was obtained by difference. Previously, this type of analysis could only be accomplished by time-consuming procedures involving a difficult synthetic step, stereospecific lipase hydrolyses and more complex separation procedures (reviewed elsewhere [31, 32]). Comparable approaches to the problem, but using chiral phase HPLC columns, are under investigation in the laboratory of Takagi in Japan [33, 34].

Stereospecific analysis in conjunction with silver nitrate chromatography is an extremely powerful technique for revealing the fine structure of natural oils and fats. For example, stereospecific analysis of olive oil triacyl-*sn*-glycerols showed that the compositions of positions *sn*–1 and *sn*–3 were apparently identical [35], but similar analyses of fractions obtained by silver ion HPLC revealed marked asymmetry in different molecular species.

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