Automated glass capillary gas–liquid chromatography of fatty acid methyl esters with reference to *cis* and *trans* isomers

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Summary The availability of an excellent separation method for fatty acid methyl esters, including separation of *cis* and *trans* isomers and of isomers that differ only in the position of double bonds, has become more and more important. The present glass capillary chromatography system combines high separation power with high precision and easy handling. Moreover, the system is completely automated and therefore provides a time saving method. As compared to a conventional packed column, the glass capillary column provides about one hundred fold more theoretical plates (227,000), as well as narrower peaks, thus giving rise to less error when integrating with electronic integrators. The reproducibility for relative retention time is better with the capillary column (0.26%) and reproducibility of the weight percent values is at least similar to that of the packed column (1.53%). When handling only small sample amounts the capillary provides better values because of its low capacity. This powerful system should open up new possibilities in the field of fatty acid investigation.

Supplementary key words positional isomers • automated gas–liquid chromatography • electronic integration

Chromatography of fatty acid methyl esters on packed columns is a well established method (1–3). Liquid phases such as DEGS, DEGA, EGSS-X, or, in the last two years, Silar 5 C and 10 C (4), and, most recently, Apolar 10 C (5) (which apparently is the same as the silar phases) are most commonly used. Despite their general applicability in fatty acid research, gas–liquid chromatography systems using packed columns have the distinct disadvantage of comparatively low separation power which leads to poor separation of *cis* and *trans* isomers and, in some cases, of isomers that differonly in the position of double bonds (positional isomers). Although this might be a question of mere theoretical importance, some recent studies of the role of essential fatty acids in atherosclerosis demonstrate the need for a gas–liquid chromatography system that provides a reliable separation of *cis* and *trans* isomers (6–9).

In addition, an optimal separation of the positional isomers is required because essential fatty acid activity, which is defined as biological activity in standardized growth tests in rats, varies with the position of double bonds in fatty acids with the same carbon number, e.g., the γ-linolenic acid with

Abbreviations: GLC, gas–liquid chromatography; DEGS, diethylene glycol succinate; DEGA, diethylene glycol adipate; EGSS-X, ethylene glycol succinate–methylsilicone copolymer; TLC, thin-layer chromatography.

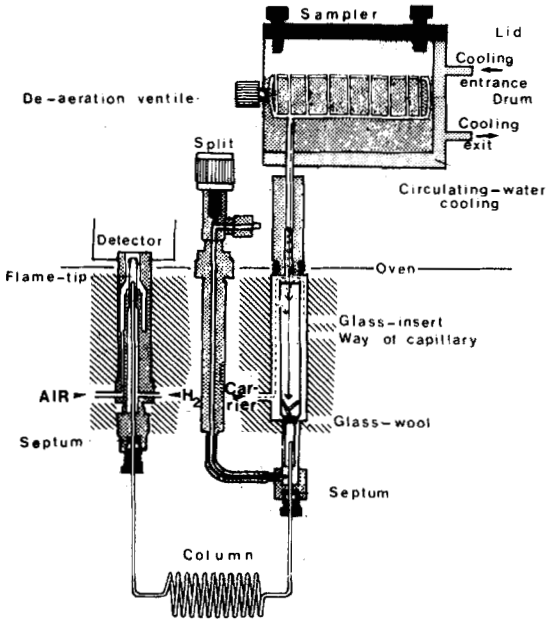

Fig. 1. Automatic solid sampler, injection port, and part of detection system of the Fractovap 2301 AC (Carlo Erba S.p.A., Milan, Italy).

double bonds in the 6,9, and 12 positions has a ten-fold higher activity than α-linolenic acid with double bonds in the 9,12, and 15 positions. From the studies of Thomasson (10) it is well known, that high essential fatty acid activity is associated with acids having both n-6 and n-9 double bonds[1] as in γ-linolenic acid.

A packed column, even with the new apolar phases, cannot separate positional isomers to a satisfactory degree. Capillary columns have been used for the separation of fatty acid methyl esters since 1959. One of the first applications for the separation of *cis* and *trans* isomers was carried out by Lipsky, Lovelock, and Landowne (11). Further studies of the resolution of isomeric fatty acids have been presented by Ackman (12), Scholfield and Dutton (13), and Hooper and Ackman (14). Many investigations have been done with stainless steel capillary columns but glass capillary columns can be used with a somewhat better efficiency or with smaller samples.

By employing a glass capillary column we could approximately double the number of resolved peaks as compared to a packed column coated, e.g., with DEGS. The present work compares the packed column to the new capillary column and shows the effectiveness of a glass capillary column in routine quantitative analysis.

Materials and methods. Lipids were extracted from the plasma of healthy donors by the method of Folch, Lees, and Sloane Stanley (15). 4-Methyl-2,6-di-*tert*-butylphenol (BHT) was added as antioxidant (16). Phospholipids, monoglycerides, 1,2 diglycerides, 1,3 diglycerides, cholesterol, free fatty acids, triglycerides, and cholesterol esters were separated by thin-layer chromatography, employing a double-development procedure first with diethylether–benzene–acetic acid–methanol 45:55:1:1 (v/v) and then with diethylether–hexane–acetic acid 20:90:1 (v/v), using precoated silica gel plates (Merck AG, Darmstadt, West Germany).[2] The lipids were eluted from the silica gel and methylated by the method of Morrison and Smith (17). A fatty acid methyl ester mixture from Applied Science Lab. Inc., State College, Pa., was used as a reference standard.

The gas chromatograph used was a Fractovap 2301 AC (Carlo Erba S.p.A., Milan, Italy) equipped with a flame ionization detector and linear temperature programmer. The injection port system was as described by Grob and Grob (18). It is an all-glass system, which has the advantage that the sample never comes in contact with any material other than glass (**Fig. 1**). The other end of the capillary protrudes into the flame tip, and thus has the advantage of an almost complete absence of dead volume.

The separating conditions were as follows. Carrier gas was purified hydrogen (99.999%) at a flow rate of 4 ml/min at room temperature. Fuel gases were synthetic air (99.99%) and hydrogen at flow rates of 150 ml/min and 12 ml/min, respectively. Synthetic air is a mixture of 80% highly purified nitrogen and 20% highly purified oxygen (Linde, Thalfingen, FRG). Nitrogen as make up gas was added to optimize the flame ionization detection. The injection port temperature was kept at 250°C. Injection of the samples was performed at 40°C column temperature. The oven was then heated to 95°C within one minute and then to 198°C at a rate of 1°C/min. The upper temperature was kept constant for 7 min before cooling down. The attenuation was two times to the integrator and another four times to the recorder. Total analysis time was 110 min.

Such a long routine analysis needs automatic injection and integration in order to achieve optimal utilization of the chromatography system. The automatic injection was performed with a solid sampler system (Carlo Erba) (Fig. 1). For this purpose, an aliquot of the sample was sucked into a container-capillary, which was coated with GE SE-30, a silicone liquid phase. Before coating with a 0.5% solution of GE SE-30 in chloroform (w/v), the container-capillary had to be treated with a 4% solution of trimethylchlorosilane in chloroform (v/v) in order to obtain an even film of liquid on the inner column wall, because chemical modification of the surface decreases the contact angle between the liquid and the surface. In a cooled system (3°C) the solvent was evaporated from the container-capillary under nitrogen, so that only the sample material without solvent was on the inside walls of the container-capillary. This method cannot be employed for low boiling substances or gases, and in our case the sampler had to be cooled down to 3°C. The containers were then brought into the automatic sampler, which was sealed off until all 22 analyses were performed. The air was completely blown out of the sampler by carrier gas and, after 10 min of

[1] The number following "n-" designates the position of the first double bond counted from the terminal methyl group, numbers in front of the name of the acid designate the positions of double bonds counted from the carboxyl end of the acid.

[2] Jaeger, H., H. U. Klör, and H. Ditschuneit. Unpublished results.

stabilization, the first analysis was started. The others followed completely automatically, controlled either by time or by the end of the temperature program. In most cases, the sample sequence was programmed so that the next s mple fell into the injection port as soon as the temperature program had reached its starting temperature of 40°C. This "cold trapping" (19) (an injection temperature that is at least 30–40°C lower than the separating temperature) is necessary to obtain small, well separated peaks. The solid sampler described here has the advantages of high reproducibility, injection of the entire sample, the absence of a solvent peak, a complete lack of septum bleed, low cost, and high stability.

Our integrator was a System I Computing Integrator (Spectra Physics, Darmstadt, West Germany) with a storage memory of 160 peaks and an additional calculation accessory that enabled the integrator to calculate the mean correction factors (response factors) automatically out of three or more calibration runs with a standard mixture. Furthermore, the percentage by weight values were calculated from the corrected peak areas. The fatty acid peaks were identified by relative retention time (RRT) and the specific correction factors. They were transferred by the integrator to a teletype (Teletype Corp., Skokie, Ill., USA), which punched the values into a tape in ASCII-Code, a 7 bit code, which can be read through an interface by almost any large computer. The computer calculated the statistical data from the analyses and compared them to other data. Relative retention times and correction factors change with time and the peaks cannot be identified by the computer if they are designated only by these values. Therefore for long-term investigations the peaks have to be identified by the computer by their carbon numbers, number of double bonds, and position of first double bond counted from the methyl end of the fatty acid. This form of description of a fatty acid originates from Klenk (20).

The column used was a 50 m glass capillary, coated with free fatty acid phase, a modified polyether which is stable up to 200°C (Laboratory for Gas Chromatography, H. and G. Jaeggi, Trogen, Switzerland). About 40 capillary columns of the same phase and quality as the one described here are in use now. The technique of coating has reached such a high standard that only one column in 15 is unsatisfactory. To improve the separation, coating with Silar 10 C was tried but the laboratory has not yet succeeded in making a stable column.

The identification of fatty acid methyl esters in our analyses was performed by comparison with the relative retention times of standard fatty acid methyl esters and was controlled on a second column. The percentage by weight values were calculated by application of the normalization method using correction factors. These ranged from 965 to 992 for saturated fatty acid methyl esters, and from 1010 to 1100 for unsaturated and branched fatty acids. Correction factors changed by about 10% after one year of use of the column, and the column to column variation was similar. The variation from day to day was about 2%.

Results. **Fig. 2** shows a chromatogram of a standard mixture of fatty acid methyl esters and **Fig. 3** shows a chromatogram of fatty acids of normal human plasma phospholipids.

One can see from Fig. 2 that the important separations of

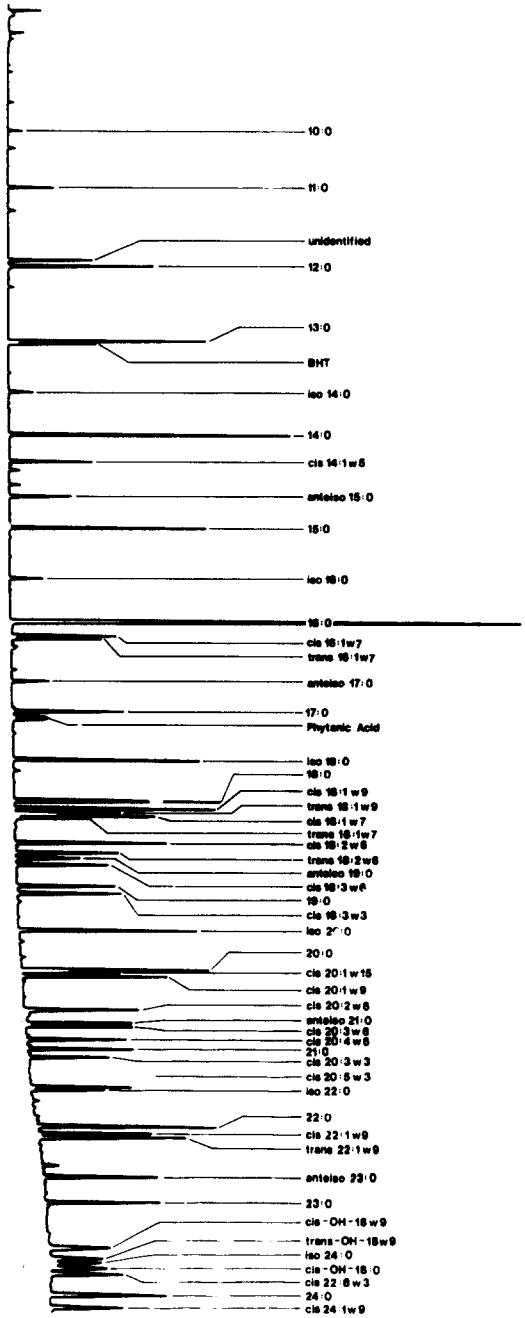

Fig. 2. Chromatogram of a standard mixture of fatty acid methyl esters.

cis- from *trans*-linoleic acid (18:2 n-6), of α- from γ-linolenic acid (*cis* 18:3 n-3, *cis* 18:3 n-6), of 8,11,14- from 11,14,17-eicosatrienoic acid (*cis* 20:3 n-6, *cis* 20:3 n-3), and of 5- from 11-eicosenoic acid (*cis* 20:1 n-15, *cis* 20:1 n-9) are all good baseline separations. The 18:1 peak from the packed column, which in human serum lipids consists mainly of oleic acid, was separated into four peaks: oleic (*cis* 18:1 n-9), elaidic (*trans* 18:1 n-9), *cis*-vaccenic (*cis* 18:1 n-7), and *trans*-vaccenic (*trans* 18:1 n-7). Furthermore, it is even possible to separate the 18:1 n-6 acids, but we did not have available an appropriate standard for definite identification.

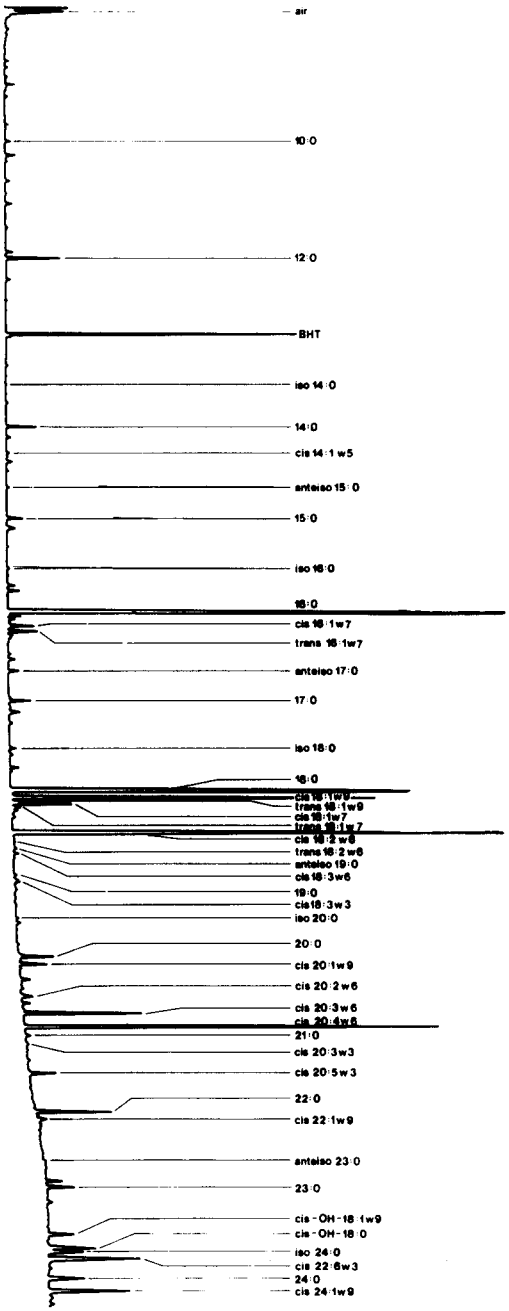

Fig. 3. Chromatogram of fatty acid methyl esters of normal plasma phospholipids.

In addition, we have seen a separation of mixed isomers (*cis/trans* and *trans/cis*) of linoleic acid on our column but could not get them pure enough to include them in our standard mixture. We have not yet chromatographed fatty acid aldehydes or dimethylacetals, but we are convinced that it is possible to achieve a good separation of these compounds as well. The separation of other *cis* and *trans* isomers, e.g., those of *cis*- and *trans*-palmitoleic acid (16:1 n-7), *cis*- and *trans*-13-docosenoic acid (erucic-, brassidic acid — 22:1 n-9), and *cis*- and *trans*-ricinoleic acid (OH — 18:1 n-9), were all either baseline or 4,6σ separations (i.e. where peaks did not overlap more than 1–2% of their peak area value) thus providing a precision of about ± 0.3% with electronic integrators. The iso- and anteisoacids were all separated from the *n*-acids by baseline separation.

It is further to be seen from Fig. 2 that the separations of elaidic (*trans* 18:1 n-9) from *cis*-vaccenic acid (*cis* 18:1 n-7) and of *n*-eicosenoic (arachic — 20:0) from *cis*-5-eicosenoic acid (*cis* 20:1 n-15) were not good enough for integration, but only trace amounts (less than 0.1%) of elaidic and *cis*-5-eicosenoic acid were found in our samples. For special purposes when these two components show higher values, it is possible to calculate the error of the integrator and correct the values.

To further characterize the properties of our chromatographic system, we have tested the reproducibility over a 24 hr period. Ten different amounts of the same fatty acid methyl ester mixture were chromatographed. The reproducibility expressed as standard deviation (σ) in percent of the mean value for each peak out of 10 consequent analyses (variation) was better than 0.26% for relative retention times and better than 1.53% for the percentage by weight values.

The main properties of the capillary column as compared to those of a packed column are given in **Table 1.** The formulas for the determination are described by Kaiser (21). It is seen that the capillary is much longer than a conventional packed column. Therefore, most investigators prefer hydrogen as carrier gas because of its low viscosity (about one half of all other carrier gases). Hydrogen as carrier gas does not cause double bond reduction or isomerization. This was investigated with a quantitative standard fatty acid methyl mixture. It was also shown by Neuner-Jehle and Etzweiler (22). With its small inner diameter, the capillary has a much higher column efficiency than the packed column. The capacity (loadability) was much higher for the packed column, because this value depends on the cube of the inner diameter (21), but the reproducibility was better with the capillary.

Discussion. The main problem with conventional packed columns is the limitation of theoretical plate number to about 5000, which represents the lowest value for capillary columns. In principle, a packed column has about the same number of theoretical plates per meter of column length as a capillary column, but for technical reasons it is not possible to operate a conventional packed column of similar length.

In order to separate two close peaks, e.g., *cis* and *trans* 16:1 n-7, on a packed column, it is possible to change the polarity of the column by using another liquid phase. However, this leads to a poorer separation of two other neighboring peaks such as 18:0 and *cis* 18:1 n-9 (5). To overcome this problem it is possible to perform a pre-separation on TLC into saturated-, mono-, di-, and polyunsaturated compounds and to perform gas–liquid chromatography of these groups on different columns or with different temperature programs (23). However, this procedure is laborious and time-consuming, and includes many potential errors.

All these difficulties can be overcome by using a glass capillary chromatographic system because all compounds, including *cis* and *trans* isomers, can be separated in a single run. With regard to electronic integration, capillary chromatog-

TABLE 1. Properties of a capillary column in comparison to those of a packed column

	Capillary	Packed
Carrier gas	hydrogen	nitrogen
Liquid phase	FFAP	DEGS
Length (m)	50.0	2.0
Inner diameter (mm)	0.33	2.0
Flow rate of carrier gas (ml/min)	4.0	25.0
Number of theoretical plates (n)	227,052	6,000
n per meter column length	4,541	3,000
Separation number (n_{sep})	47.0	7.0
n_{sep} per meter column length	0.9	3.5
Gas hold up time t_m (mm)[a]	52.6	23.3
Capacity ratio k (for C 10:0)	16.0	9.2
Capacity (g) (for C 10:0)	5.3×10^{-6}	7.1×10^{-4}
Reproducibility of relative retention time[b]	0.26	2.14
Reproducibility of percentage by weight[b]	1.53	3.17

[a] Measured at a recorder chart speed of 80 in./hr.

[b] As maximal percent deviation from mean value.

All calculations are means from three measurements, except the calculations for reproducibility (n = 10). Separation number is defined as the number of separated peaks within one carbon number range. Gas hold up time is the retention time of an inert gas that is not retained by the liquid phase. Capacity ratio is the ratio of adjusted retention time to gas hold up time. Capacity is defined as amount of sample that decreases the column efficiency to 90% of maximal value.

raphy provides narrow peaks even at the end of the chromatogram and gives better results with fewer integration errors.

Another advantage, at least in some cases, is that smaller amounts of sample material are needed for capillary GLC. In other situations, however, where high capacity is needed, this might be a disadvantage.

Nevertheless there are chromatographic problems that cannot be solved by capillary GLC, which is a purely analytical technique. GLC on a preparative scale or of trace amounts together with very large amounts cannot be carried out with capillary columns.

Since the beginning, capillary GLC has found a wide field of application such as the analysis of tobacco smoke, diesel oil, pesticides, steroids, drugs, etc (24). Most recently we have used this chromatographic system for the determination of cholesterol and have found a precision better than 0.8% in the range of 3–50 ng sample material.[3]

In recent years many investigators have hesitated to use capillary GLC because the technique was thought to be too expensive and too difficult to handle. The presumed low reproducibility and precision in quantitative analyses was another argument for not using the capillary system. From our experience, the use of capillary systems is different from that of GLC systems with packed columns, and it takes a little longer to establish a method with capillary GLC. However the extraordinarily high separation power should pursuade most investigators to use the capillary system. Furthermore, low reproducibility and low precision can no longer be an argument against the glass capillary column.

We wish to thank Mr. G. Blos from the German Division of Carlo Erba, S.p.A., Milan, Italy, for his invaluable technical assistance. We also thank Mrs. A. Jaeger for her most helpful drawing and typing.

Manuscript received 22 August 1975; accepted 11 December 1975.

[3] Jaeger, H., H. U. Klör, and H. Ditschuneit. Manuscript in preparation.

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