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GLASS CAPILLARY GAS CHROMATOGRAPHY OF THE SERUM FATTY ACID FRACTION VIA AUTOMATIC INJECTION OF LIPID EXTRACTS

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SUMMARY

The combination of a consecutive double capillary column gas chromatographic system (Deans type) with a fully automatic injection device was developed to determine quantitatively the fatty acid fraction of serum extracts, without prior removal of the other lipids. A major difficulty arose through ghosting (memory), via reaction of methylating reagents with lipid deposits, mainly in the injection port. Use of diazomethane circumvented this, as the reagent was easily removed prior to analysis. The combination methyl iodide—potassium carbonate, convenient handling of which requires coinjection of methyl iodide, proved to be less than completely dependable. Dimethylformamide dimethylacetal is completely out of the question as a coinjectant. Lipid deposits in the injection port produced peak area drifts which could, however, be counteracted. The apparatus is capable of analyzing samples completely unattended for at least 20 hours (80 samples). It has been operated non-stop, day and night, for between 4 and 7 days with occasional reloading and servicing.

INTRODUCTION

In the course of studying energy substrate regulation in patients with selected brain lesions, it became necessary to determine the ("free") fatty acids (FA) in blood. Capillary columns were chosen for this task with the hope that at least some of their advantages would come to bear. The large number of samples made it desirable to devise a relatively simple work-up and use complete automation with septumless injection for the gas chromatographic (GC) analysis.

A great simplification of the sample preparation can be realized by sidestepping a chromatographic preseparation of FA and the other (mostly esterified) lipids which are extracted together [1]. It should be noted that phospholipids are not completely extracted by the method described here.

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Direct chromatography of the extract suggested the use of columns coupled in series — a Deans [2, 3] type system. This, it was presumed, should have the capability to cut out components which are not of interest. It was decided, therefore, to employ a proven [4, 5] capillary set-up with "valveless" switching of the columns. The combination of this sophisticated tool with a specially adapted septumless injector has apparently not previously been successfully applied. It took ca. 15,000 serum sample injections and some interspersed exploratory experiments before this undertaking could definitely be called a success. Improvements were made not so much in accuracy and precision but rather in the reduction of runaway values and in functional reliability. That is, the number of interdictions to correct deleterious developments could be curtailed drastically.

The experimentation was not limited to the apparatus itself but had to be extended to the chemistry, i.e. the methyl esterification. Thus, this report also relates the interesting findings made with several reagents, not only in regard to adaptation to the described procedure, but also in regard to FA chromatography in general. The findings regarding "ghosting" [6—8] should be especially of general interest. The terms "ghosting" and "memory" are used to describe the carry-over of substances form one injection to subsequent ones. Ghosting will, of course, be detected only when the composition of an injection is known and is sufficiently different from previous injections.

To demonstrate precision it was decided to use examples taken directly out of routine analyses (see Table I), although precision and accuracy are also demonstrated by the more usual idealized experiments (see Table III).

EXPERIMENTAL

Materials

The reference acids and esters were obtained from Nu-Check-Prep through Paesel GmbH (Frankfurt, G.F.R.). Reagents and analytical grade solvents were used as purchased. The diazomethane was generated from N-methyl-N'-nitro-N-nitrosoguanidine in an apparatus (Aldrich-Europe, Beerse, Belgium) as described in ref. 9. The control serum was Seronorm-Lipid from Nyegaard (Oslo, Norway) obtained through E. Merck (Darmstadt, G.F.R.). The capillary columns with poly(ethyleneoxy)glycol (Carbowax 20M) stationary phase were prepared by Gerstel (Mülheim/Ruhr, G.F.R.). This stationary phase was chosen for its stability, promising a conveniently long column life.

Instrumentation

The chromatograph is a Siemens L 402 with Deans type column switching. This is controlled by valves outside the oven, so that the term "valveless switching" has come into use. The automatic injection is done by a Siemens system that has been adapted from preparative chromatography (see Fig. 1). The peaks were integrated and concentrations calculated (internal reference) by the Spectra Physics System I electronic integrator.

Sample collection and preparation

Blood was collected, immediately stoppered, put on ice, and protected from direct light until it was centrifuged after standing a minimum of 30 min and a maximum of 360 min. The serum of each sample was frozen and stored at —18°C in several vials. For the analysis a vial was thawed and to each of two 200-μl portions were added 0.5 ml of buffer* (citrate—sodium hydroxide, pH 6, Merck 9437) and 2 ml of a chloroform—methanol (2:1, v/v) solution containing heptadecanoic acid as internal reference. The mixture was mechanically shaken for 15 min; the lower chloroform phase was carefully separated and the solvent evaporated in special pointed vials on a heating block and under a stream of nitrogen. Usually 24 samples were handled at a time. After this step the procedure was varied according to the methylating reagent.

Dimethylformamide dimethylacetal (DMFDMA) [11—16]. The extract residue from above was dissolved in 100 μl of hexane, ethyl acetate, or benzene, and 10—100 μl of DMFDMA (Serva, Heidelberg, G.F.R.) were added. The vials were sealed (Teflon-laminated septum and aluminium seal; Krebber, Keltern-Weiler, G.F.R.), and allowed to stand for various times at room temperature or up to 120 min at 90°C. The apparatus then removed 0.5 μl for the chromatography. Benzene, one of the solvents usually recommended, was scrutinized a little more closely. A solution of a known mixture of several acids and C_{17} methyl ester with 10 or 20 μl of DMFDMA in 100 μl of benzene was kept at room temperature for up to 20 min. The sealed vials were then opened and the contents shaken with 200 μl of water, extracted with 750 μl of hexane, and the solvent evaporated under a stream of nitrogen. The samples were ready for injection after the addition of 100 μl of hexane. That the acids were extracted by the hexane was then confirmed by adding 10 μl of DMFDMA and reinjecting.

Methyl iodide—potassium carbonate [17, 18]. The extract residue was dissolved in 100 μl of ethyl acetate—methyl iodide (50:50, v/v). A small spatula tip of solid granular K_2CO_3 and glass beads of 0.5 mm diameter were added, the latter to heighten the level of the solution. The vials were then sealed and heated at 90°C for 90 min. Again the solution was injected without further modification.

Diazomethane [9—19]. This reagent became attractive because of the possibility that it could easily be removed before the samples were injected. The adopted procedure was determined by trials using various reaction times, reagent amounts, and the presence or absence of methanol. Thus, 0.45 g of the guanidine and 1.5 ml of water were placed into the inner compartment and 6 ml of anhydrous diethyl ether were placed in the outer compartment. After placing on an ice-bath and tightly closing (a leak detection solution should be applied as a check), 1.8 ml of an aqueous 5 *N* sodium hydroxide solution was added through the septum within ca. 2 min. After standing for 30 min on the ice-bath, 100 μl of the cold ethereal diazomethane solution and 20 μl of

*Dole and Meinertz [10] suggest a pH of 2.5. However, there is less tendency toward lipid hydrolysis at pH 6 while differential extraction of FA is not seen either (Dr. W. Adoph, personal communication).

methanol were added to the extract residue and the mixture stoppered and allowed to stand at room temperature for a maximum of 20 min. The volatile components were then evaporated on a heating block (56°C) under a stream of nitrogen. Then 100 μl of hexane were added and the vials closed as above, being now ready for analysis.

A total of 48 samples can conveniently be handled in one batch. Ethanol was later substituted for methanol in order to avoid the possibility of a hidden, unwanted reaction [20, 21].

Tests to elucidate source of ghosting

In order to devise conditions to keep ghosting under control it became important to understand more about how this phenomenon comes about. This meant injecting relevant pure solvents and solutions (hexane, benzene, ethyl acetate, methanol, methyl iodide—ethyl acetate, DMFDMA—hexane) under conditions employed in the extract analyses. The external standard method was found to be the most convenient one for the quantitation of ghosting. To find the location of the ghosting some liquids were also injected after each of the following steps: (1) thorough cleaning of the injector by taking apart, scrubbing with solvents, soaking in sulphuric acid and reassembling, (2) repeating this routine for the injection port, and (3) changing the pre-column. Occasionally air was also injected.

Tests to check on possible reactions between coinjected substances

This simply consisted of an extensive series of injections, alternating most of the above solvents and solutions with corresponding mixtures of these and pooled serum extracts, C_{17} acid, FA C_{17} ester, tripalmitoylglycerol, cholesteryl palmitate or phosphatidylcholine (egg lecithin). Part of this was done both with dirty (used) and clean plumbing up to and including the pre-column. Concentrations of lipid solutions were chosen to resemble those in serum.

Description of the GC system

The set-up used is shown diagrammatically in Fig. 1. Basically the system performs two consecutive chromatographic separations. The pre-column should remove low- and high-boiling components via the outlet (cut) at the end of the pre-column and via flow reversal (backflush), respectively. The main column must then perform the desired separations. This can be done as a continuation of the separation that was started by the pre-column, or one can start from scratch by using a cold trap to gather completely or partially the desired components at the start of the main column.

The length of the pre-column is 10 m, that of the main column is 20 m. Pressure P_M determines the flow of the main column. Pressure P_A is so adjusted that the pressure at the end of the pre-column equals that of P_M (0.7 bar). Typical operational conditions and timing of flow switching are as follows. Nitrogen flow-rate of the main column is 2 ml/min (cold), that of the cut is 24 ml/min (greater than flow of pre-column) with the split closed. The split has a flow-rate of above 100 ml/sec with P_A on. Oven, injection port, and detector temperatures are 193°C, 270°C and 260°C, respectively, the oven being in the isothermal mode. The injection commences with the lowering and

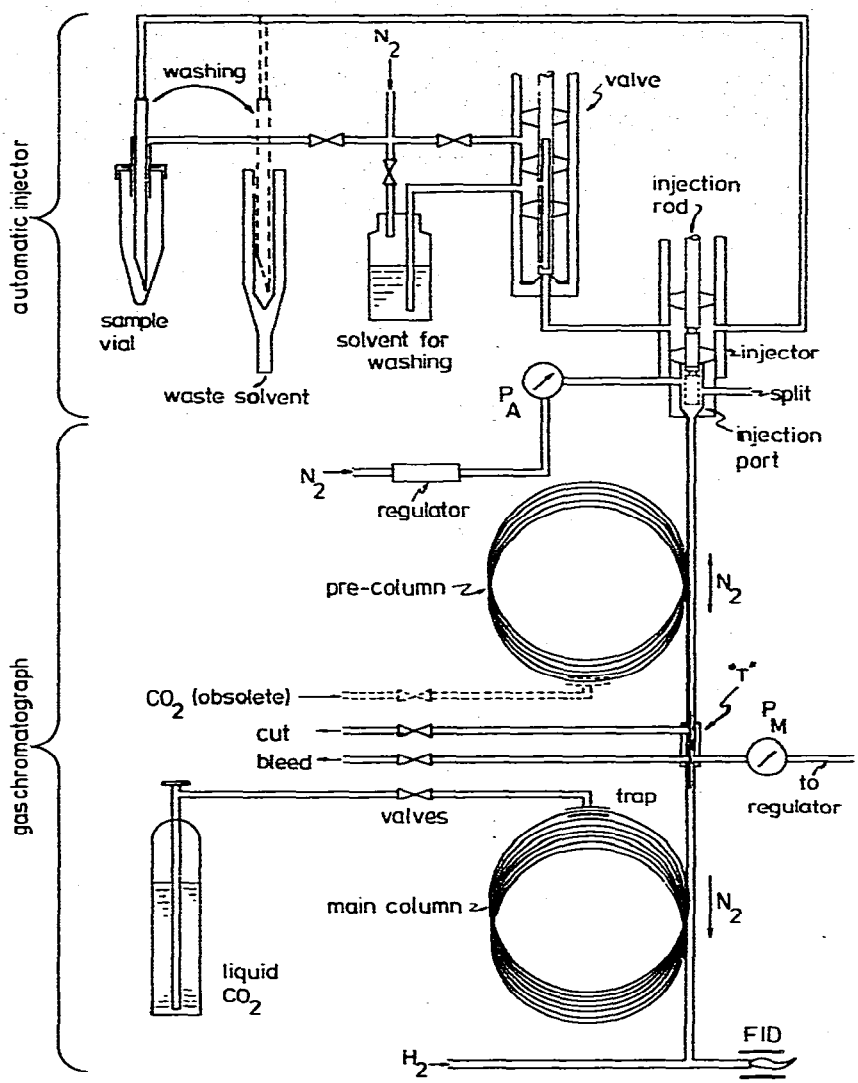

Fig. 1. Diagrammatic representation of the GC system.

electrical heating (0.6 sec, heating rate 200°C/sec) of the injection rod while P_A is on, the cut open, and the split closed. The split opens 25 sec later and after another 5 sec the injection rod is raised, washed, the liquid carbon dioxide trap turned on, and the cut closed. At this point the transfer of sample to the main column begins and most of the solvent has been removed through the cut. After another 95 sec the carbon dioxide is turned off and a further 65 sec later P_A is allowed to drop nearly to zero so that backflush of the pre-column takes place. The FA have now been passed on to the main column and are in the process of being separated. While the trap was on, it collected the more volatile acids to obtain a clean separation from solvent which reached the main column. The backflush position is kept until completion of the analysis (another 610 sec). Volatiles still on the pre-column and in the injection port

TABLE I

FATTY ACID CONCENTRATION IN TWO SERUM POOLS AND A CONTROL SERUM

Row 1–3: Values determined over a time span of about two years, each sample was frozen and thawed only once. Row 4: Values gathered over a period of about two months. Row 5: Commercial control serum, in use for about five months. Rows 1–5: These substrates were randomly interspersed through routine FA determinations. Row 6: The supplier's mean ± 2 S.D. as determined by the titration method.

Row No.	Substrate	Reagent	No. of samples	mmol/l · 10^3 (mean ± S.D.)					
				C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	Total
1	Pool 1	CH_3I–ethyl acetate*	12	166 ± 10	33 ± 5	58 ± 9	210 ± 18	67 ± 5	534 ± 32
2	Pool 1	diazomethane*	12	156 ± 16	27 ± 4	49 ± 5	198 ± 13	67 ± 5	497 ± 28
3	Pool 1	diazomethane**	27	161 ± 17	32 ± 4	52 ± 4	209 ± 11	72 ± 5	526 ± 33
4	Pool 2	diazomethane**	29	158 ± 12	29 ± 3	50 ± 5	211 ± 15	72 ± 6	520 ± 32
5	Control serum	diazomethane**	11						347 ± 18
6	Control serum								469 ± 88

*With trap on pre-column.
**With trap on main column.

should now pass out through the split. The early analyses were performed with a non-heatable injection rod which required the trap on the pre-column. This set-up was highly prone to trap malfunctions, because the amount of carbon dioxide had to be closely regulated in order to hold FA but not solvent. Furthermore, the solvent, benzene, could not be used as it was completely held back by the trap, negating the function of the cut. The present system is largely insensitive to fluctuations in the flow of liquid carbon dioxide.

The integrator is calibrated every tenth injection with a standard consisting of methyl esters of C_{14}, C_{16}, $C_{16:1}$, C_{18}, $C_{18:1}$, $C_{18:2}$, and C_{19} in hexane.

In anticipation of a possible future project, some trials including arachidonic acid ($C_{20:4}$) were conducted. Only the most pertinent readjustments of the chromatograph are mentioned here: (a) splitless evaporation time was lengthened to 55 sec, (b) a simple temperature program, starting with 160°C and ending with 198°C after the initial 2 min, was needed, and (c) the total analysis time was lengthened to 29 min.

RESULTS AND DISCUSSION

Methylations — general comments

There are sundry methods that can be used to methylate carboxylic acids for GC analysis. If esterified lipids are not removed prior to methylation it is safer to refrain from using reagents that are capable of transesterification. Careful determination of reaction parameters and strict adherence to them is an inconvenient prerequisite to their use [22]. Trimethylsilylation reagents are not known to react with esters [23]; nevertheless, they were not considered for the derivatization on the basis of anticipated difficulties in their removal prior to injection. Coinjection of these reagents is reported to cause reactions with the Carbowax 20M phase or with metal surfaces in the chromatograph [12—15]. The reagents dimethylsulfate [24] (maybe in conjunction with potassium carbonate) and N-methyl-2-halopyridinium iodide [25] were judged not to be an improvement over the methyl iodide technique and were not tested. Also not tried was chromatography of the acids without derivatization [26], simply because it was felt that the extensive literature on methyl ester analysis provided a broader base for our work.

Methylations — specifics

Methyl iodide. The two-phase system, which uses crystalline potassium carbonate as the base, has been extensively used in this laboratory while more attractive methods are being sought. Even though methyl iodide—potassium carbonate has yielded usable results (many thousands of samples were determined using it) its disadvantages stood in the way of complete satisfaction. A slight variation in physical dispostion can cause variations in reaction rate, so that a long, 90-min, reaction time had to be adopted in order to assure uniformly high yields. Worse than this was that the potassium carbonate sometimes clogged the plumbing of the automatic injection apparatus.

Homogeneous base catalysis with lutidine or collidine is not possible as these substances react readily with methyl iodide. A search for less nucleophilic, more hindered [27] bases was discontinued when the ghosting problems

mentioned subsequently were recognized. Coinjection of any bases should be expected to compound those problems.

Table I lists the means and standard deviations of values obtained for a pooled serum. The determinations were interspersed throughout analyses of patients' serum and include errors from the sample work-up, mechanical changes, and ghosting.

Dimethylformamide dimethylacetal. This highly promoted methylating agent [12—15] resisted all efforts to prepare anything more than possible traces of methyl ester until it was heated far above room temperature. The well-publicized procedures claiming FA dissolution as rate-limiting initially dissuaded us from considering temperature as a variable in improving yields until a search of the organic chemical literature [28] revealed the scope of this reaction. It was gratifying to learn, therefore, that a similar observation had already been reported [29]. This report presented evidence indicating that a carboxylic acid (indoprofen) was methylated in the injection port rather than after dissolution in DMFDMA—methanol. We can extend this observation to FA dissolved in the solvents benzene, ethyl acetate, and hexane. Furthermore, the absolute yield of reaction in the gas chromatograph appears to be highly sensitive to changes in unknown parameters. We obtained yields of between 5 and 87% at different times when DMFDMA (10 μl) was coinjected with the known acid—C_{17} ester mixture in the solvents hexane or ethyl acetate (100 μl).

As Table II shows, the reaction is relatively slow in benzene, even near 100°C and with a large excess of reagent. It must be reiterated that the older literature [28] allows the prediction of this outcome.

TABLE II

REACTION OF DMFDMA WITH FA

The substrate was an FA mixture and C_{17} methyl ester, all of known concentration.

DMFDMA (μl)	Temp. (°C)	Time (min)	mmol/l · 10^3					
			C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	Total
Calculated from weight			323	90	148	260	126	947
10	Ambient	12	5	?	4	4	?	15
Coinjection of above with 10 μl of DMFDMA			256	82	119	261	107	825
20	Ambient	20	6	2	4	10	4	26
10	100	60	302	77	153	255	121	908
10	100	120	311	80	156	270	136	953

Diazomethane. This is the reagent of choice for our apparatus as it should be for a host of applications. Its use has eliminated the ghosting due to in situ methylation. It is also quicker than the other reagents. About 2500 analyses have shown that conscientious work is necessary if the advantages are to be exploited. Thus, poor methylation yield will result if one does not make sure that the generator is gas-tight and that it is kept on ice after it is opened. Polymer precipitation, which can clog the system, is negligible during the short

reaction time possible with alcohol catalysis. The 20 min mentioned in the procedure are not necessary for the methylation but are allowed to make unharried work possible. Table III lists the results of some tests with this reagent, which reiterates the catalytic role of methanol [19]. Table I compares routine results with those obtained with methyl iodide.

Some of the many negative comments about diazomethane merit a digression at this point. A direct reaction with normal esters (triglycerides, etc.) is not known. If it occurred it would give Arndt—Eistert synthesis type products [30] which would not go undetected in GC. The catalysis by diazomethane of a reaction between a glycerol ester and methanol is so ineffective [20, 21] that one need not expect it here. Nevertheless, we substituted ethanol for methanol to be able to detect any conceivable and inadvertent catalysis of alcoholysis. We have not yet encountered any. Reaction with the double bonds is also not expected (nor evident; see Table III), as this reaction is known to be slow [31] under the circumstances that hold here.

TABLE III

REACTION OF DIAZOMETHANE WITH FA

The same FA mixture as in Table II was used. The diethyl ether—diazomethane solution used here was made by entraining the diazomethane from 0.45 g of the guanidine in 3 ml of diethyl ether. Reactions were carried out at room temperature.

Diazomethane—diethyl ether	Methanol (μl)	Time (min)	mmol/l · 10^3					
			C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	Total
Calcd.			323	90	148	260	126	947
100	0	10	132	37	64	117	59	409
100	0	10	145	42	66	126	63	442
50	0	10	142	41	61	112	60	416
50	0	10	not injected					
100	0	20	156	45	71	133	67	471
100	0	20	151	44	69	130	66	461
50	0	20	173	48	75	136	71	503
50	0	20	172	49	77	145	73	515
100	10	10	318	87	146	250	126	927
100	10	10	322	87	148	254	128	939
50	10	10	323	88	148	256	129	944
50	10	10	323	87	148	256	129	943
100	10	20	319	87	148	254	128	935
100	10	20	322	87	149	257	129	944
50	10	20	322	87	149	256	129	943
50	10	20	322	88	148	256	129	943

Ghosting

Fig. 2 gives examples of carry-over, which quickly reaches a plateau at tolerable levels that lasts for several days. This type of ghosting, obtained with inert solvent injections, usually resembles the previous samples; that is, it is a composite of previously injected samples. The error introduced by it is thus

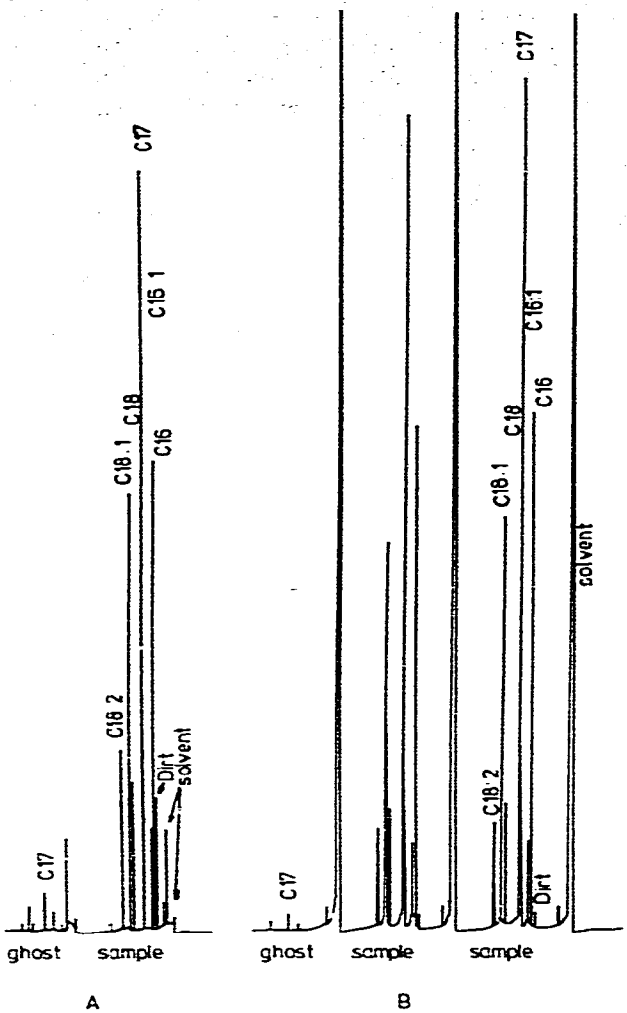

Fig. 2. Traces of chromatograms showing two examples of ghosts produced by injections of hexane. Trace A was obtained with a different gasket and injection rod than used for Trace B (heated rod used for B, trap on main column; cold rod for A, trap on the pre-column). Corrective measures are indicated when the level of ghosting shown in Trace A is reached (sample/ghost ca. 20). It should be noted that Figs. 2—4 and 7 are reductions of traced chromatograms in the same scale.

normally lower than appears from its size, because nearly proportional amounts are added to all the peaks, including the C_{17} internal standard. In short, the error is partially cancelled. It can become quite large when the Teflon gaskets sealing the injection rod (see Fig. 1) become worn. The lower gasket must be replaced after about two weeks and several tightenings. The upper gasket lasts for months. When hexane, benzene, or ethyl acetate is injected straight after the gaskets have been renewed or cleaned and after the injection rod is cleaned, methyl ester peaks are no longer detected. The ghosting obtained with inert solvents is thus clearly caused by retention of methyl esters at the contact point of gaskets and rod, an area which is not reached by washing solvent. This

phenomenon will be referred to subsequently as "physical ghosting". Successive injections of solvent diminish this deposit slowly.

Cleaning of the gaskets and rod does not prevent ghosting when methanol or especially DMFDMA (10 μl in 100 μl of hexane) is injected. Methyl iodide behaves only rarely in this fashion, but sometimes as strongly as DMFDMA. The absolute amounts vary greatly in all cases, but the reason for this could not be ascertained. The peaks so elicited usually have a relative composition which bears no resemblance to the previously analyzed samples (Fig. 3); subsequently,

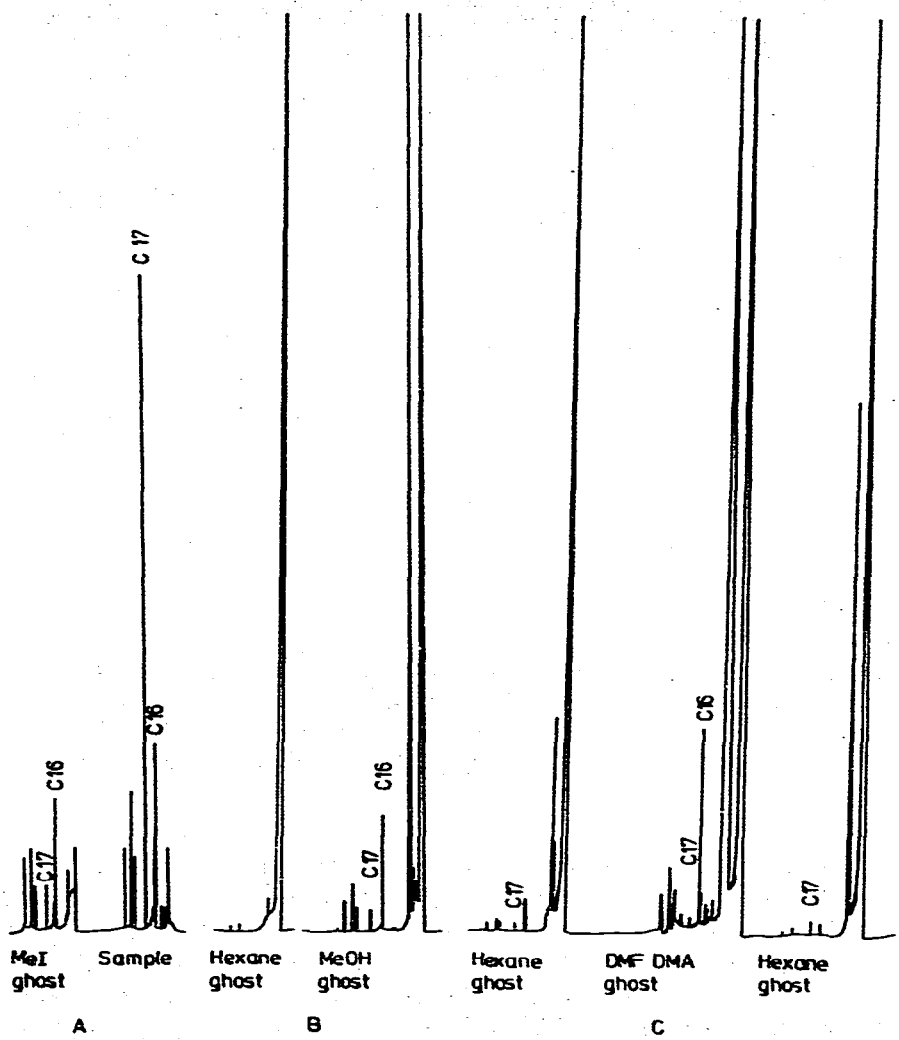

Fig. 3. Examples of ghosts elicited by injections of methyl iodide (MeI)—ethyl acetate (A), methanol (B), and DMFDMA (C). The trap was on the pre-column for A and on the main column for B and C. The trap position influences only the retention time and the solvent peak of the chromatogram. Water was inadvertently coinjected several samples before the ghost in A. This may or may not have had something to do with creating this "chemical ghost". The sample shown in A can be expected to include a similar ghost since the methyl iodide—K_2CO_3 method was still employed at that time (involving coinjection of methyl iodide).

these will be called "chemical ghosts". The most striking aspect about these is the small C_{17} peak. This pattern is also obtained with hexane (Figs. 3 and 4) if the reactive liquids were injected recently and if the gaskets were not cleaned. The "chemical ghosts" can be reduced considerably by cleaning the injection port, and all but traces disappear when the pre-column is renewed on top of it. This evidence can best be explained by invoking reactions with esterified lipid deposits.

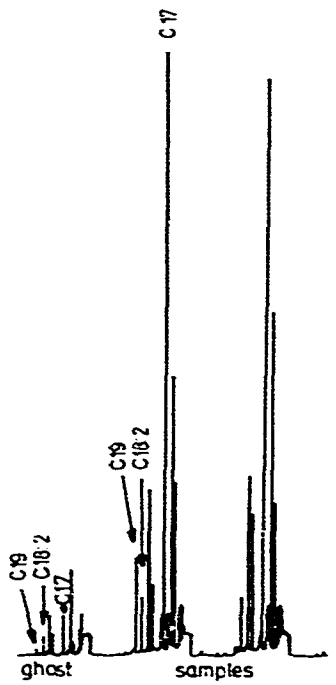

Fig. 4. Example of chemical-type ghosting obtained with hexane (compare Fig. 3C). The carry-over of C_{19} (was added as ester only to the previous sample, prior to methylation) displays normal "physical ghosting" behaviour. The C_{16} through $C_{18:2}$ ghost pattern points toward transesterification of extract deposits on the Teflon gasket, somewhere before the hexane injection.

To learn more about this transesterification, the above reagents were coinjected with an extract of pooled serum or, in the case of methanol and DMFDMA, also with known amounts of cholesteryl palmitate, tripalmitoylglycerol, and phosphatidylcholine. Coinjection with C_{17} acid and/or the known mixture of FA and C_{17} methyl ester was also carried out in order to determine how deposited acids may behave. Methyl iodide and methanol showed little or no reaction with coinjected acids, the maximum was about 3%. DMFDMA has been covered above. Methanol and DMFDMA showed higher ambivalence toward the coinjected esterified lipids than toward extract deposits. Whereas a reaction was always obtained when deposits were present, there were cases in which coinjected reagents and lipids did not give products. However, a certain consistency was evident in that the pair, phosphatidylcholine and DMFDMA, was the most reactive, while the combinations of methanol and cholesteryl

palmitate or the triglyceride gave the lowest conversion. The other possible combinations were of intermediate reactivity. All reactions have the potential to render serum FA determinations useless. As an example, the phosphatidylcholine—DMFDMA combination often yielded methyl ester peaks that amounted to 70% of a 0.5 mmol/l FA sample (external standard).

During these trials the fear arose that transesterification of the types

$$\text{Deposit—}CO_2R + R'CO_2CH_3 \rightarrow RCO_2CH_3$$

or

$$RCO_2H + R'CO_2CH_3 \rightarrow RCO_2CH_3 + R'CO_2H$$

may take place in the gas chromatograph. Both of these were ruled out by the lack of reaction when the FA C_{17} methyl ester mixture, or a C_{19} methyl ester and C_{17} acid were coinjected after deposits had formed.

Thin-layer chromatography [32] including spot staining [33] of a hexane and methanol wash of the injection port glass insert clearly indicated that the deposit contains cholesteryl esters, triacylglycerols, and phosphorus-containing substances. FA and methyl esters of FA were not seen. This was somewhat unexpected as the literature [34, 35] leads one to predict that all but the more polar compounds like phosphatidylcholines would be backflushed.

Drift and arachidonic acid

Two seconds for the splitless evaporation time, an injection port temperature of 180°C, and an injection rod heating time of 0.25 sec were sufficient to evaporate an unchanging proportion of FA methyl esters on to the pre-column when only standard was chromatographed, and when no extract deposition was present. Also, a complete cut of solvent and the determination of C_{14} was possible. The extended evaporation time as described in the Experimental section was instituted to reduce drastically a progressive, rapid decrease in especially the higher FA methyl ester peaks upon injection of serum extracts. This process is now so slow that it is usually limited to the C_{19} peak of the intermittent standards (see Fig. 5). Leaving the gas chromatograph on backflush overnight partly reverses the drift. Total restoration is achieved when the injection port glass insert is rid of the deposit, something that is done only rarely. When the attempt was made to determine $C_{20:4}$ methyl ester with a "dirty" injection port it was found that 25 sec of evaporation time was still not enough. When this splitless time was lengthened to 55 sec the C_{16} peak could no longer be separated from solvent, while the $C_{20:4}$ peak appeared. This was remedied by lowering the initial oven temperature so that only solvent moved rapidly through the pre-column. As a matter of fact, C_{14} may now be determined if desired. Results with a serum pool indicate little or no drift; however, at least twice as much sample must be injected so that the integrator can handle the broad $C_{20:4}$ peak. The pool used to adjust the machine was that of row 4 in Table I. After about 60 trial injections a further and consecutive injection of 14 pool extracts produced the following means and ranges (in parentheses): C_{16} = 155 (152—159), $C_{16:1}$ = 33 (32—33), C_{18} = 50 (49—50), $C_{18:1}$ = 207 (205—210), $C_{18:2}$ = 83 (82—84), $C_{20:4}$ = 8 (7—9), total = 535 (531—539). Though these results are pleasing, the analytical parameters will, most likely, still undergo developmental changes.

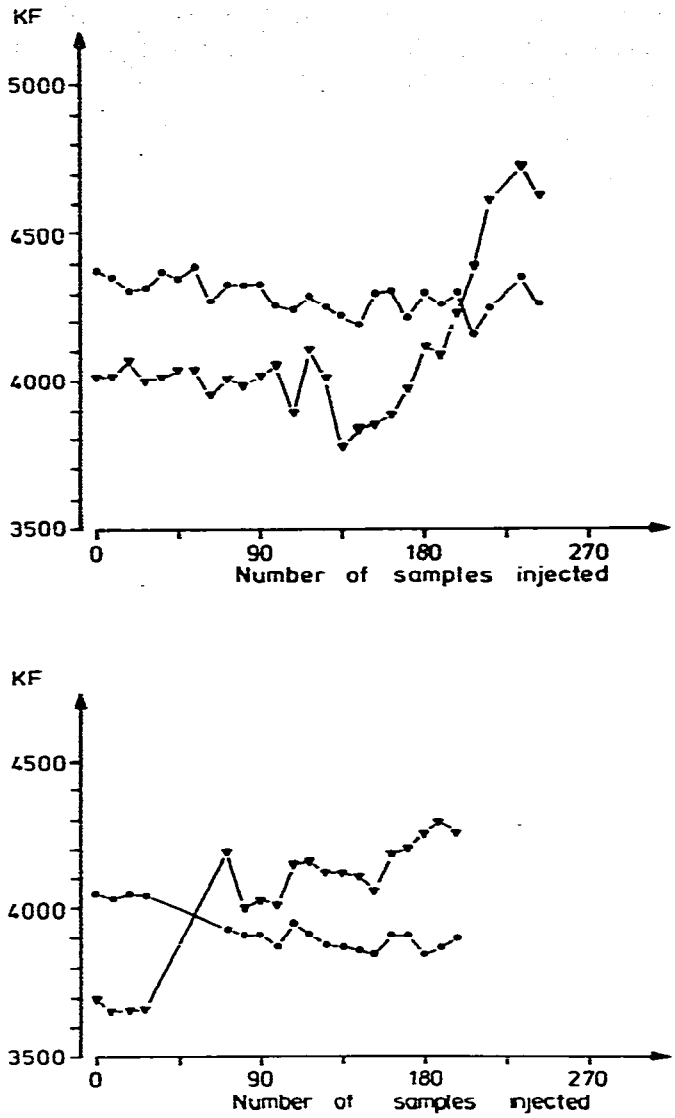

Fig. 5. Two recent series showing the drift of C_{19} standard caused by deposits in the injection port. The plotted KF, the integrator's correction—calculation factors, varies inversely with the peak area changes of the standard. The abscissa counts only the injections between standards (always 9). (▼), C_{19}; (●), $C_{18:2}$.

Incidentally, drifting of C_{19} was virtually absent with the carbon dioxide trap on the pre-column, which also required 55 sec of splitless evaporation time.

At this point it is quite obvious that "chemical ghosting" and the drift are primarily and solely, respectively, caused by the deposit in the injection port. The "chemical ghosting" forces one to conclude that the backflush does not function perfectly. On the other hand, "chemical ghosting" also reaches a plateau. Together with the fact that drifting is created only in the injection port, this shows that backflushing is definitely operative. One can well imagine that

frequent reconditioning of the column would be essential whenever lipid extracts are injected without backflushing.

Accuracy, precision and performance

Although the chromatograms in Fig. 6 were obtained using the carbon dioxide trap on the pre-column, so that the "T" deadspace intervened in the separation process, the resolution is close to a comparable single-column [36] chromatography. Later we switched to 20-m main columns and noted that the resulting decrease in resolution was less with the trap on the main column. We

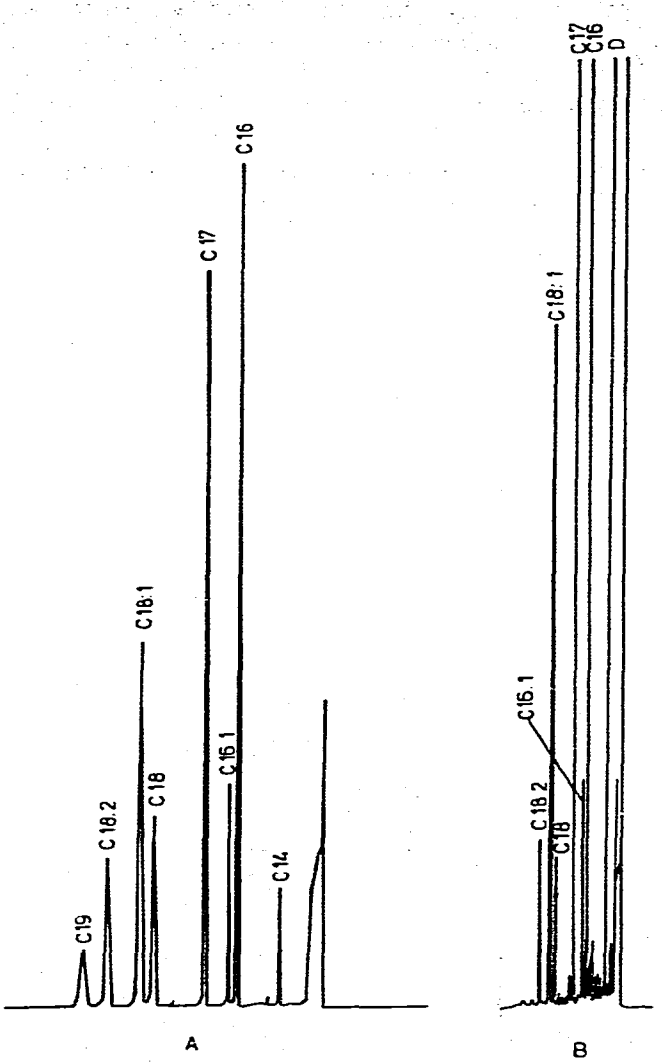

Fig. 6. Results obtained with a 50-m main column; flow was 2 ml/min, oven temperature was 195°C. (A) Methyl ester standard used to calibrate the integrator; chart speed was 5 mm/min. (B) Serum sample (methylated with methyl iodide) which contains extraneous peaks caused by the extraction of rubber in a partially Teflon-covered vial septum. The septa were later changed to completely Teflon-covered specimens. Chart speed here was 2 mm/min.

take this to be a demonstration that the consecutive-column technique can deliver ideal samples on to the analytical column, thus giving results at least equivalent to the most idealized single-column methods.

The reason for switching to the shorter columns was to decrease analysis time. The resulting lower resolution is more than adequate for the present purpose, all examples except those of Fig. 6 are obtained with 20-m columns. Thus, the accuracy and precision of even the $C_{16:1}$ quantitation in Table III can hardly be improved. Fig. 7 shows that even differing injection amounts have no discernible concentration shifts which are common to more strongly overlapping peaks. With the exception of C_{16} the concentrations differ in proportion to the calibration factor (KF) differences. Apparently, the typical capillary column peak sharpness limits such shifts. Now, the peak shape we obtained for arachidonic acid led to integrator performance reminiscent of past experience with packed columns.

It should be reiterated that we strived to take our examples for precision, etc., right out of routine analyses in order to present a more realistic picture.

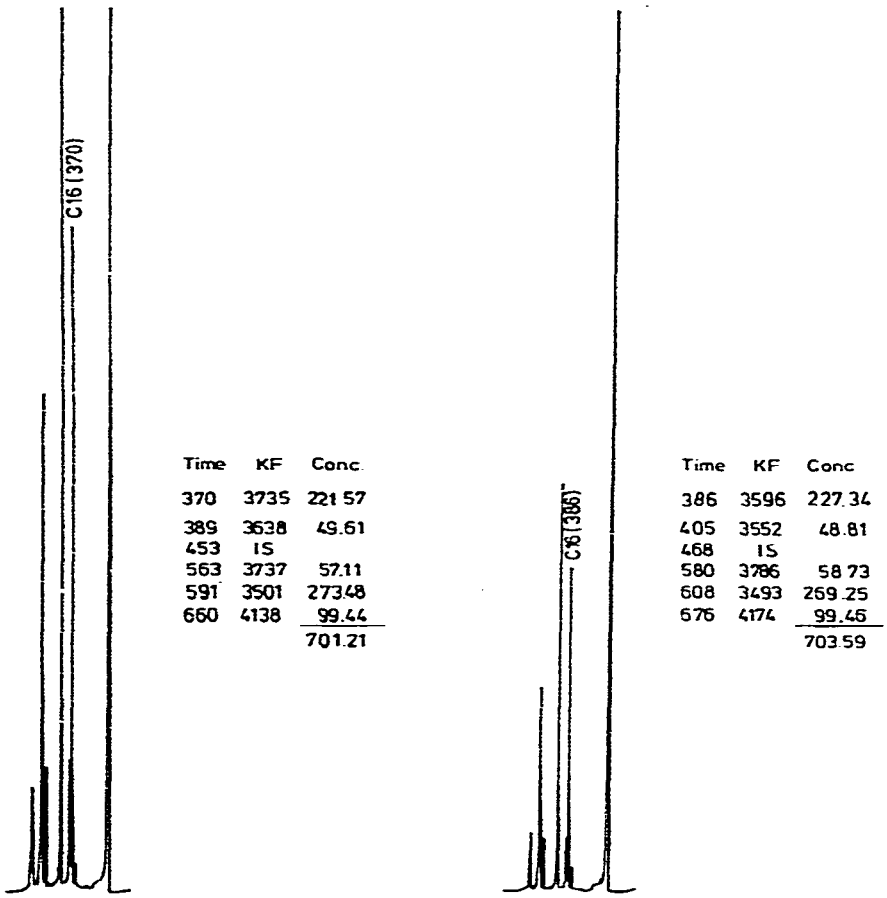

Fig. 7. One of the many examples showing that reproducibility is negligibly influenced by variation in sample concentration. The two chromatograms are separated by 17 injections.

The results of specific, and thus idealized, tests like that of Table III were included to demonstrate how the apparatus itself compares.

The KF values of Fig. 5 and 7 give an indication of how much ghosting and drifting (virtually limited to C_{19}) add to errors. Thus the largest variation in the KF of $C_{18:2}$ from one standard to the next (one point to the next, or every tenth injection) is 3.5% in Fig. 5. Most of the variations are about half that. Of course, one can choose to do worse if unwilling to readjust the Teflon gasket or change it after two to three weeks of operation to control "physical ghosting". When Table I is now compared to this it becomes obvious that most of the error is introduced by the work-up.

The retention time changes in Fig. 7 are about the maximum allowable, and are caused by weakening liquid carbon dioxide delivery to the trap. This increase of retention time signals the necessity to change the carbon dioxide. The retention times change less than 1% at other times. The practical consequence is that the integrator never confuses peaks.

A comparison with the titrimetric method of FA determination of a commercial control serum shows that we are in an accepted range with the entire work-up and chromatography. The difference between the two methods is about as expected [37] (see Table I, rows 5 and 6).

Although the manufacturer specifies that a chain of 200 samples can be placed on the sampling tray, it is probably preferable to expose only about 80 samples to room temperature and light at a time, as is done at present. The chain is then simply refilled daily without stopping the apparatus until samples, air, nitrogen carrier gas, or hydrogen run out. The washing solvent, Teflon gasket, integrator and chart paper, and the liquid carbon dioxide can be changed without stopping the analysis. In actual practice the machine is sometimes run continuously for about one week. Twenty hours without attention of any kind have proved to be quite practical. The injection and chromatography cycle stops after the last sample has been run through. It is possible, therefore, to let analyses terminate without being present.

After completion of the injection the unused part of the sample is returned to the vial. This allows many repetitions of a sample determination, if desired. The large excess of sample (0.5 μl out of 100 μl are used each time; that is, ca. 10^{-10} moles of FA per injection) is used to ensure filling of the injector.

CONCLUSION

This work suggests that frequent checks on the status of unwanted reactions are necessary whenever derivatizing reagents are coinjected with total lipid extracts. DMFDMA and methanol must be added to the list of substances that are out of question for coinjection, while the status of methyl iodide is unclear in this respect. Uncontrollable peak area drifts should be expected whenever non-chromatographic separations of FA and other serum components are used prior to extensive single-column GC runs. Although this GC represents a solution for a specific analytical request, we are certain that it can be adapted to overcome general problems [38] encountered in natural-product analysis with capillary columns. The key is simply the delivery of only the compounds of interest on to the analytical column.

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