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ASSAY OF LIPIDS IN DOG MYOCARDIUM USING CAPILLARY GAS CHROMATOGRAPHY AND DERIVATIZATION WITH BORON TRIFLUORIDE AND METHANOL

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SUMMARY

The fatty acids of three lipid classes (free fatty acids, triglycerides, and cholesteryl esters) from dog heart were analysed by gas chromatography. Samples of the left ventricle were homogenized and total lipids were extracted. After separation by thin-layer chromatography, the bands of the lipid classes studied were scraped off, transmethylated according to the boron trifluoride—methanol procedure, and the fatty acid methyl esters were extracted and analysed. The problems related to the quantitation of fatty acids were investigated, namely transmethylation procedure, thin-layer chromatography, and gas chromatographic conditions. Fatty acid methyl esters were separated on capillary columns coated in the laboratory with SP 2340 stationary phase. The high performance of the separation ensured the reliability and the precision of the analysis.

INTRODUCTION

In the study of the myocardial metabolism of fatty acids, three points need to be taken into consideration. First, their quantitative determination with

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a maximum of precision. Secondly, a qualitative profile with a maximum of information about isomers and minor components. Thirdly, a reasonable duration of analysis.

The quantitative determination of fatty acids requires control of each of the steps involved in the preparation procedure, which includes homogenization, extraction, derivatization and gas chromatographic (GC) analysis. In a recent paper, Badings and De Jong [1] discussed the factors which may affect the precision of the quantitative analysis, with special attention to the *trans*-esterification step and to instrumentation-related factors. Similar studies have previously been made [2—5].

The homogenization and extraction procedures have also been shown to be of a primary importance, especially in the determination of myocardial free fatty acids (FFA) [6—8], the main problem being the risk of autolysis increasing the FFA concentration.

The aim of this report was the quantitation of the fatty acids of three lipid classes of dog heart: FFA, triglycerides (TG), and cholesteryl esters (CE). For this purpose, the individual relative response factors (RRF) of ten fatty acids (14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:4, 22:1)* were determined. The influence of thin-layer chromatography (TLC) on the RRF was studied and the rate of conversion of bound fatty acids was checked by means of recovery experiments.

The profile of fatty acid methyl esters (FAME) was given by a highly polar liquid phase, SP 2340. The high resolution of this phase [9] allowed the separation of isomers of unsaturated fatty acids, and recent progress in the preparation of capillary columns [10—12] made possible their preparation in the laboratory. The reliability of the separation was fundamental for the identification and the quantitation of the fatty acids of the lipid classes studied.

MATERIALS AND METHODS

Standards and reagents

Analytical-grade solvents and isooctane (spectrosol grade) were purchased from SDS (Peypin, France). Dichloromethane was redistilled before use, and the purity of *n*-heptane was checked by GC in conditions similar to those used throughout the experiments.

Silica gel G60 and boron trifluoride 20% in methanol were purchased from E. Merck (Darmstadt, F.R.G.). 2,6-Di-*tert.*-butyl-*p*-cresol (BHT) was provided by Riedel de Haën (Seelze, Hannover, F.R.G.).

Standard fatty acids, fatty acid methyl esters, triacylglycerols and cholesteryl esters were all purchased from Sigma (St. Louis, MO, U.S.A.)

*Abbreviations used in the text, figures and tables: 14:0 = myristic acid; 14:1c,ω5 = myristoleic acid; 14:1tr,ω5 = myristelaidic acid; 15:0 = pentadecanoic acid; 16:0 = palmitic acid; 16:1c,ω7 = palmitoleic acid; 16:1tr,ω7 = palmitelaidic acid; 17:0 = margaric acid; 18:0 = stearic acid; 18:1c,ω9 = oleic acid; 18:1tr,ω9 = elaidic acid; 18:1c,ω7 = vaccenic (*cis*) acid; 18:2c,ω6 = linoleic aicd; 18:3c,ω3 = linolenic acid; 20:0 = arachidic acid; 20:1c,ω9 = *cis*-11-eicosenoic acid; 20:2c,ω6 = *cis,cis*-11,14-eicosadienoic acid; 20:3c,ω6 = dihomo-γ-linolenic acid; 20:4c,ω6 = arachidonic acid; 20:3c,ω3 = *cis,cis,cis*-11,14,17-eicosatrienoic acid; 22:0 = behenic acid; 22:1c,ω9 = erucic acid.

Gas chromatography

Conditions. Analyses were carried out on a Packard Model 427 chromatograph equipped with a moving needle injector and a flame-ionization detector which was connected with a Sefram recorder and a Delsi integrator. Capillary columns (60 m × 0.30 mm) were coated with SP 2340; dried hydrogen was used as the carrier gas at a flow-rate of 2.5 ml/min. Fuel gases were synthetic air (a mixture of nitrogen and oxygen, 80:20) and hydrogen at flow-rates of 300 and 30 ml/min, respectively. The injection port temperature was 220°C, the detector temperature 250°C, and oven temperature 130°C, isothermal for 3 min then programmed at 1°C/min up to 170°C.

Preparation of capillary columns. Capillaries drawn from soda lime glass tubes were prepared as described by Sisfontes et al. [11] by double etching with gaseous hydrochloric acid, deactivation with Carbowax 20 M [13], and static coating with a solution containing 0.15% (w/v) SP 2340 in dichloromethane. The free end was sealed with sodium silicate [14, 12], and the solvent was evaporated after 24 h under vacuum at 25°C at a rate of 1.5 cm/min. Capillary columns were conditioned for 1 h at 120°C, then the temperature was raised to 220°C and maintained overnight.

Sample preparation

Samples of the left ventricular myocardium were taken from anaesthetized dogs, after a left thoracotomy, using the drill biopsy technique [15]. The tissue samples were homogenized as described by Van der Vusse and coworkers [6, 7, 16], and the lipids were extracted by a mixture of chloroform—methanol (2:1) containing the internal standards (heptadecanoic acid, triheptadecanoin, and cholesteryl heptadecanoate) and 0.01% BHT. The mixture was shaken for 15 sec, filtered, and a biphasic system was produced by dilution of the extract with 1 ml each of chloroform and 0.73% sodium chloride solution [17, 18]. The aqueous phase was separated by centrifugation and the organic phase was evaporated under a stream of nitrogen without any heating. The residue was dissolved in 150 μl of chloroform and spotted on thin-layer plates (20 × 10 cm) coated with a 0.2-mm layer of silica gel G 60 and predeveloped with diethyl ether to remove contaminants. The lipids were separated by the double-development procedure described by Jaeger et al. [19]. The FFA, TG, and CE bands were located by viewing under UV light and by reference to a lipid standard mixture run in parallel and sprayed with 0.1% (w/v) Rhodamine B. These bands were scraped off and *trans*-methylated according to the procedure of Morrison and Smith [20].

Special precautions. All glassware used in the experiments was washed with alkaline detergent, sulphochromic acid (12 h), and rinsed with hot water, deionized water, and distilled water. The glassware was then washed successively with the organic solvents ethanol, acetone, and chloroform.

Identification and quantitation of FAME

Peaks of FAME were identified by comparing their relative retention times with those of standards (Fig. 1). Methyl esters of 14:1tr,ω5 and 20:3,ω6 were identified by comparison with profiles reported by authors using the same stationary phase [9, 21, 22].

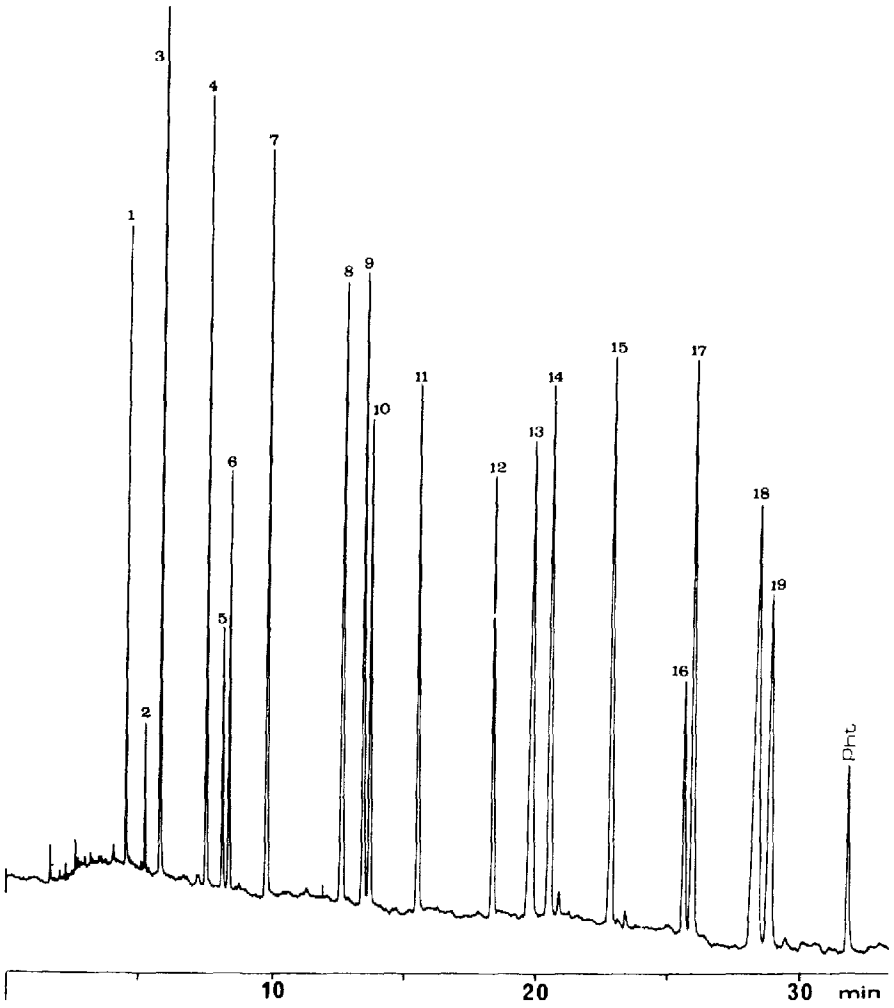

Fig. 1. Gas chromatogram of a standard mixture of FAME obtained on a glass capillary column (50 m) coated with SP 2340. Conditions are given in Materials and methods. The amounts injected ranged between 2 (14:1c,ω5) and 35 (22:0) ng. Peaks: 1 = 14:0; 2 = 14:1c,ω5; 3 = 15:0; 4 = 16:0; 5 = 16:1tr,ω7; 6 = 16:1c,ω7; 7 = 17:0; 8 = 18:0; 9 = 18:1c,ω9; 10 = 18:1c,ω7; 11 = 18:2; 12 = 18:3; 13 = 20:0; 14 = 20:1; 15 = 20:2; 16 = 20:4; 17 = 20:3,ω3; 18 = 22:0; 19 = 22:1; Pht = phthalate.

The concentrations of fatty acids (FA) were calculated using the internal standard method as follows:

$$\text{FA concentration (nmol/g wet weight)} = \frac{\text{Peak area of FA}}{\text{Peak area of 17:0}} \times \text{Amount of}$$

$$17{:}0\ (\text{nmol}) \times \text{RRF} \times \frac{1000}{\text{Sample weight (mg)}}$$

The RRF were determined, using a calibration mixture, from the peak

area ratios relative to 17:0. Ten fatty acids were studied (14:0, 16:0, 16:1c,ω7, 18:0, 18:1c,ω9, 18:2, 18:3, 20:0, 20:4, 22:1) in calibration mixtures containing 90, 135, 180, and 225 nmol of each fatty acid, the amount of 17:0 remaining constant at 180 nmol.

$$\text{RRF} = \frac{\text{Amount of FA/Amount of 17:0}}{\text{Peak area of FA/Peak area of 17:0}}$$

Values are presented as mean ± S.D. Differences between groups were calculated by analysis of variance.

RESULTS AND DISCUSSION

Determination of relative reponse factors

The results presented here were calculated over a period of six months and using four calibration mixtures. Table I shows that differences exist between the RRF of fatty acids methylated with BF_3—methanol: for the same chain length, the response of the saturated homologue was the highest and decreased with the increase in the number of double bonds.

To investigate whether the RRF were modified by TLC, as reported by Emilsson and Gudbjarnason [17], aliquots of the calibration mixtures were spotted on thin-layer plates and developed as described previously prior to methylation. Dienoic and polyenoic fatty acids (Table I) showed significantly higher RRF values ($P < 0.001$) after TLC, indicating a significantly lower relative response. The RRF value of 22:1 also increased ($P < 0.01$), but those of 16:0 and 16:1 remained unchanged. No correlation has been found between the number of double bonds and the changes in the RRF values.

Myristic acid represents a special case; it was included in the calibration mixtures, but has not been mentioned in Table I because its RRF value was systematically around 4.4 (4.432 ± 2.322, n = 21, values calculated after

TABLE I

RELATIVE RESPONSE FACTORS OBTAINED AFTER BF_3—METHANOL METHYLATION WITH AND WITHOUT PREVIOUS THIN-LAYER CHROMATOGRAPHY

Fatty acid	BF_3—methanol			TLC + BF_3—methanol			Statistical result* (p)
	RRF	S.D.	n**	RRF	S.D.	n	
16:0	1.132	0.212	37	1.172	0.163	26	NS
16:1c,ω7	2.164	0.612	35	2.059	0.768	29	NS
18:0	0.756	0.130	38	0.869	0.067	32	<0.001
18:1c,ω9	0.799	0.199	36	0.962	0.101	30	<0.001
18:2	1.070	0.182	39	1.384	0.299	29	<0.001
18:3	1.522	0.450	33	2.699	1.423	26	<0.001
20:0	0.755	0.131	32	0.800	0.175	31	NS
20:4	1.589	0.517	37	2.365	1.212	29	<0.001
22:1	0.901	0.188	32	1.096	0.347	25	<0.01

*NS = not significant.
**n = Number of determinations.

TLC + BF_3—methanol). The cause of this particularly low response is a loss in the injector: the methyl myristate was sufficiently volatile to be partially vaporized within 30—60 sec, the interval between deposition of the sample on the injector glass needle and injection into the capillary column.

TABLE II

RECOVERY EXPERIMENTS

Standards were added to the powdered tissue in the amounts indicated prior to extraction. Each result is the mean of three assays.

Lipid class	Added component	Amount added (nmol)	Recovery (%)	S.D.
FFA	18:0	3	96.83	15.95
	18:2	3	87.52	7.32
	20:4	3	95.17	13.66
CE	Cholesteryl myristate	5	115.87	17.68
	Cholesteryl stearate	5	95.72	8.40
TG	Tristearin	6	114.34	9.40
	Trilinolein	6	111.46	31.28

TABLE III

QUANTITATIVE PROFILE OF MYOCARDIAL FREE, TRIGLYCERIDE- AND CHOLESTERYL-ESTERIFIED FATTY ACIDS

Results are given in nmol/g wet weight as mean ± S.D. (n = 5).

	FFA	TG	CE
14:0	11.8 ± 9.6	316.9 ± 96.7	50.2 ± 18.0
14:1tr,ω5	3.3 ± 1.9	32.1 ± 18.2	24.2 ± 11.2
14:1c,ω5	5.7 ± 2.3	52.8 ± 26.7	8.9 ± 1.3
15:0	15.8 ± 8.7	81.3 ± 27.6	20.9 ± 2.5
16:0	55.6 ± 30.9	1039.2 ± 248.4	81.5 ± 29.1
16:1tr,ω7	8.1 ± 5.6	67.7 ± 27.2	40.2 ± 14.6
16:1c,ω7	4.4 ± 2.4	529.1 ± 190.0	17.1 ± 9.5
18:0	24.2 ± 4.4	295.7 ± 58.6	22.3 ± 8.9
18:1c,ω9	22.5 ± 9.4	2104.9 ± 514.5	71.0 ± 31.2
18:1c,ω7	1.4 ± 0.6	154.1 ± 37.6	7.5 ± 3.7
18:2	6.6 ± 3.1	845.8 ± 270.9	177.1 ± 73.5
18:3	1.0 ± 0.6*	67.5 ± 29.3	2.9 ± 1.4
20:0	1.9 ± 0.7	10.3 ± 4.8	1.8 ± 0.8
20:1	1.3 ± 0.4**	27.6 ± 13.4	4.7 ± 2.0
20:2,ω6	ND***	8.1 ± 4.1	1.9 ± 1.0**
20:3,ω6	3.5 ± 1.4	20.1 ± 13.8	6.5 ± 2.6
20:4	4.2 ± 2.3	51.8 ± 25.0	86.1 ± 44.0
20:3,ω3	ND	ND	ND
22:0	1.1 ± 0.5**	4.5 ± 3.8	2.8 ± 0.8
22:1	ND	ND	1.7 ± 0.6**

*Detected in two samples.
**Detected in three samples.
***ND = Not detected.

From these data, we may conclude that the methylation procedure, as well as the other possible manipulations (TLC), have a direct influence on the quantitative response of fatty acids. As this response is related to that of the internal standard, which is in our case a saturated fatty acid, it seems likely that the unsaturated fatty acids show a quite different behaviour. We agree with the conclusion expressed recently by Badings and De Jong [1]: the RRF must be calculated from standards that have been subjected to the complete procedure that is undergone by the analysed sample. Calculation of the RRF on the basis of standard FAME is useful to test the linearity of the detector response and the repeatability of the apparatus and that of the manipulator, but may give rise to an erroneous indication of the effective recovery of each fatty acid after laboratory methylation.

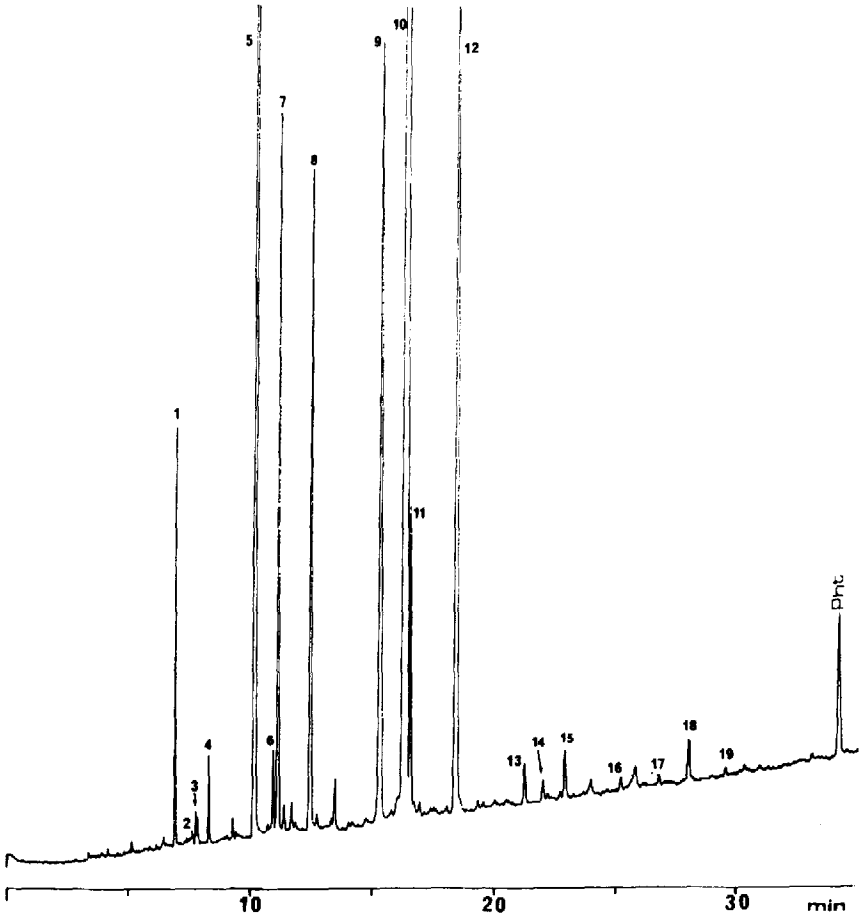

Fig. 2. Typical profile of FAME obtained from myocardial TG and separated on a glass capillary column (50 m) coated with SP 2340. To 141.5 mg of homogenized tissue were added 40 nmol of triheptadecanoin and 6 nmol each of tristearin and trilinolein for recovery tests. The residue of BF_3-methylated fatty acids was dissolved in 200 μl of *n*-heptane and 0.8 μl was injected into the gas chromatograph. GC conditions are given in Materials and methods. Peaks: 1 = 14:0; 2 = 14:1tr,ω5; 3 = 14:1c,ω5; 4 = 15:0; 5 = 16:0; 6 = 16:1tr,ω7, 7 = 16:1c,ω7; 8 = 17:0; 9 = 18:0; 10 ; 18:1c,ω9; 11 = 18:1c,ω7; 12 = 18:2; 13 = 18:3; 14 = 20:0; 15 = 20:1; 16 = 20:2; 17 = 20:3,ω6; 18 = 20:4; 19 = 22:0; Pht = phthalate.

Recovery experiments

These experiments were performed to test the rate of conversion of bound fatty acids and the rate of methylation of FFA. Standards of FFA, CE, and TG were added to homogenized biopsy tissues prior to extraction. The results are given in Table II; each is the mean of three experiments.

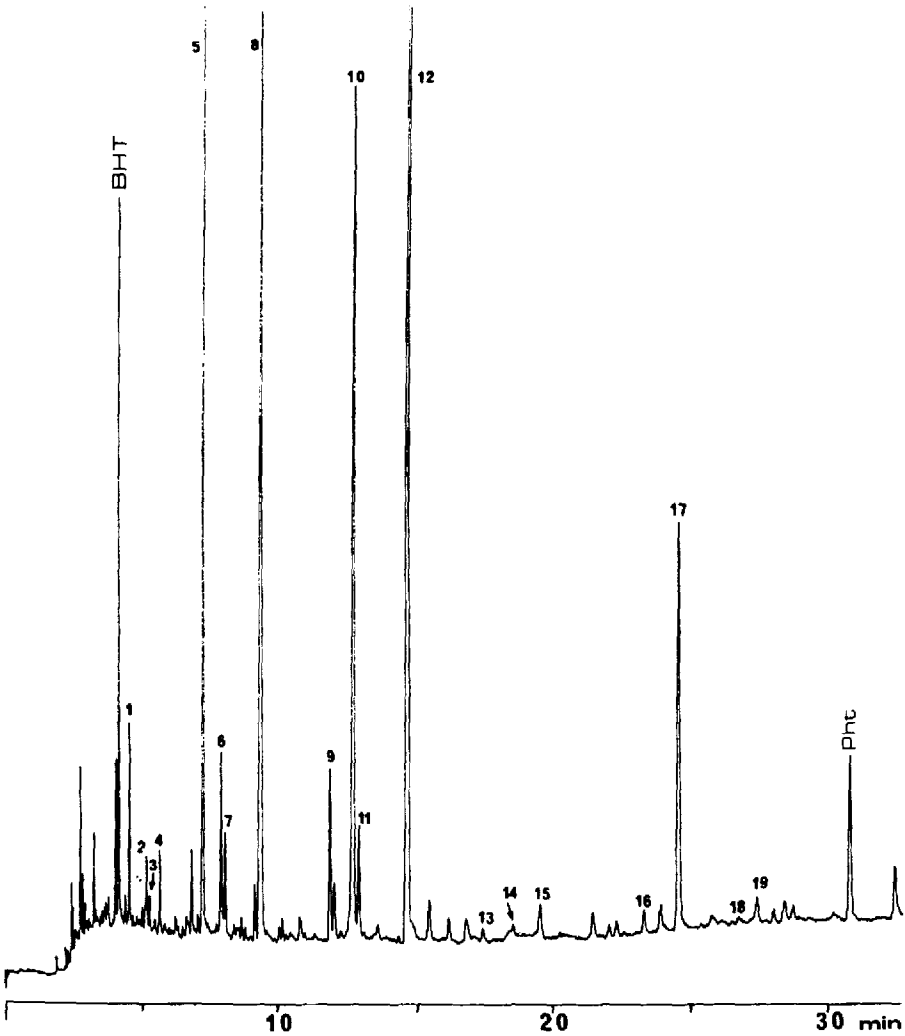

Fig. 3. Typical profile of FAME obtained from myocardial CE and separated on a glass capillary column (50 m) coated with SP 2340. To 241.9 mg of homogenized tissue were added 20 nmol of cholesteryl heptadecanoate. The residue of BF_3-methylated fatty acids was dissolved in 100 µl of *n*-heptane and 1.2 µl were injected into the gas chromatograph. GC conditions are given in Materials and methods. Peaks: 1 = 14:0; 2 = 14:1tr,ω5; 3 = 14:1c,ω5; 4 = 15:0; 5 = 16:0; 6 = 16:1tr,ω7; 7 = 16:1c,ω7; 8 = 17:0; 9 = 18:0; 10 = 18:1c,ω9; 11 = 18:1c,ω7; 12 = 18:2; 13 = 18:3; 14 = 20:0; 15 = 20:1; 16 = 20:3,ω6; 17 = 20:4; 18 = 22:0; 19 = 22:1; Pht = phthalate; BHT = 2,6-di-*tert.*-butyl-*p*-cresol.

Quantitative profile of myocardial free, triglyceride-, and cholesteryl-esterified fatty acids

The results of five experiments are given in Table III. For each experiment, three samples of the left ventricular wall were separately homogenized and treated, and the FAME derived from each extract were chromatographed twice. The RRF values used in the calculation of fatty acid concentrations are those listed in Table I (TLC + BF_3—methanol (Figs. 2—4).

Interference of BHT on the FAME profile

BHT was added in small proportion (0.01%) to prevent autoxidation during the analytical procedure. After TLC, BHT was detected just below the CE band, and it was generally included with this fraction when the gel was scraped off. For this reason, a second TLC was described to remove it [7]. After BF_3—methanol reaction, BHT might interfere with the FAME at two sites. The first is the BHT peak, which might overlap completely the peak of methyl myristate. The second is a derivative of BHT produced by permethyla-

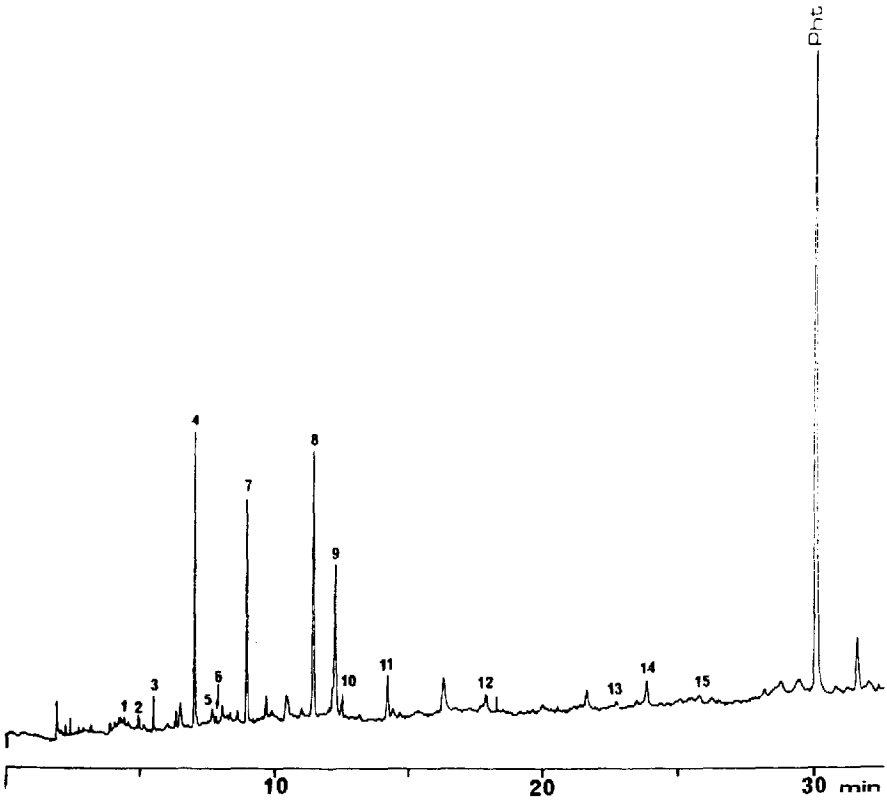

Fig. 4. Typical profile of FAME obtained from myocardial FFA and separated on a glass capillary column (50 m) coated with SP 2340. To 279.3 mg of homogenized tissue were added 4.5 nmol of heptadecanoic acid. The residue of BF_3-methylated fatty acids was dissolved in 30 μl of *n*-heptane and 6 μl were injected into the gas chromatograph. GC conditions are given in Materials and methods. Peaks: 1 ; 14:0; 2 = 14:1c,ω5; 3 = 15:0; 4 = 16:0; 5 = 16:1tr,ω7; 6 = 16:1c,ω7; 7 = 17:0; 8 = 18:0; 9 = 18:1c,ω9; 10 = 18:1c,ω7; 11 = 18:2; 12 = 20:0; 13 = 20:3,ω6; 14 = 20:4; 15 = 22:0; Pht = phthalate.

tion during the BF_3—methanol reaction. The BHT derivative was described by Heckers et al. [9] as having a retention time identical to that of 16:1c,ω7.

In our case, the columns were sufficiently resolutive to separate distinctly the peaks of BHT and methyl myristate (Fig. 3). Moreover, the BHT was partly lost in the injector. As the peak of the BHT derivative, it appeared at half the distance between those of 16:1c,ω7 and 17:0, so that BHT does not interfere in the analysis.

In conclusion, this study may be considered as an approach to the resolution of the analytical problem of the quantitation of fatty acids, and a basis for the study of their myocardial metabolism.

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