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DETERMINATION OF FATTY ACIDS AS PHENACYL ESTERS IN RAT ADIPOSE TISSUE AND BLOOD VESSEL WALLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Twenty-two biologically relevant (6:0–22:6) saturated, monounsaturated and polyunsaturated fatty acids were separated by reversed-phase high-performance liquid chromatography after derivatization with phenacyl bromide. An optimal resolution of the critical combinations linolenic–myristic, docosahexaenoic–palmitoleic–arachidonic and palmitic–oleic acids and *cis* and *trans* isomers of octadecenoic (n9) and octadecadienoic (n9, 12) acids was achieved by continuous gradient elution with methanol–acetonitrile–water. Elution of mixtures of 6:0–22:1 fatty acids was completed within 80 min at a flow-rate of 1 ml/min. By the use of UV detection at 242 nm the detection limits for short- and long-chain fatty acids were found to be about 0.8 and 12 ng per injection, respectively. Linearity was tested up to 100 ng. The method was applied to the determination of fatty acids in rat adipose tissue and blood vessel walls of animals fed hydrogenated fat diets. The results are comparable to those obtained by gas chromatography and surpass the latter for the resolution of oleic and elaidic acids.

INTRODUCTION

Adipose tissue is the main source of blood free fatty acids, which represent the most actively metabolized pool of lipid classes. The fatty acid pattern of adipose tissue is closely related to the fatty acid composition of dietary lipids and so may reflect not only a pathological situation but also the feeding history of the animal. The latter is particularly important in nutritional experiments and in establishing adequate biological models.

Although gas chromatographic (GC) methods are traditionally used for fatty acid analyses[1], they are accompanied by some disadvantages, particularly with re-

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spect to heat-labile[2] or short-chain fatty acids[3], and moreover the separation of *cis–trans* isomers is possible only with capillary GC columns.

In order to overcome some of these shortcomings, a number of high-performance liquid chromatographic (HPLC) methods have been introduced. These methods usually offer good resolution of biologically important fatty acids[4,5], but the detection of underivatized fatty acids is neither sensitive nor selective because these compounds generally do not contain suitable chromophores. Absorption of underivatized fatty acids near 200 nm cannot be recommended because it is adversely affected by the properties of and frequent impurities in organic solvents, which is specifically undesirable in gradient elution.

In order to increase the sensitivity and selectivity of detection, a number of UV-absorbing or fluorescent derivatives have been prepared. Phenacyl[3,6–12], *p*-nitrobenzyl[13], *p*-bromophenacyl[14–16,25], 2-naphthacyl[3,17,18], *m*-methoxyphenacyl[19], *p*-phenacylazophenacyl[20], naphthylamine[21,22], pentafluorobenzyl[23], 5-dimethylamino-1-naphtalenesulphonylethanolamine[24], 9-diazomethylanthracene[26,27], 2-nitrophenylhydrazide[28,29], isopropylidine hydrazide[30], 4-bromomethyl-7-acetoxycoumarin[31], 9,10-diaminophenanthrene[32], 9-aminophenanthrene[22] and 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone[33] derivatives are typical examples.

Phenacyl and substituted phenacyl esters are the most commonly used compounds. They are easily prepared in quantitative yields and are stable, and so permit the determination of fatty acids even at nanogram levels[3,9].

Separations are usually carried out on reversed-phase columns using isocratic or gradient elution with methanol, acetonitrile and water. The order of elution is governed by the length of the fatty acid carbon chain and the number of double bonds in it[3]. The retention time increases with increasing chain length and decreasing number of double bonds[34]. These opposing tendencies lead to the occurrence of several pairs of fatty acids that are difficult to separate[7], and the separation of which may be considered a criterion of the resolution efficiency of an analytical procedure. Examples of such critical pairs are linolenic (18:3) and myristic (14:0), palmitoleic (16:1) and arachidonic (20:4) and palmitic (16:0) and oleic (18:1) fatty acids[15,34] although difficulties with the separation of some other fatty acid pairs may also be expected. In addition, the retention of phenacyl derivatives of fatty acids on the column is affected by geometric and double-bond positional isomerism[3,10]. The *trans* isomers are generally eluted after the corresponding *cis* isomers. Positioning of the double bond in the proximity of the carboxy group of an acid usually leads to a decrease in retention compared with an isomer of a fatty acid with the double bond shifted in the direction of the methyl end of the carbon chain[11]. The resolution of various adjacent peaks of fatty acid phenacyl esters on reversed-phase columns is further significantly affected by the column temperature and the proportions of methanol and acetonitrile in the mobile phase.

In this work we compared various separation conditions in order to achieve an optimal resolution of biologically important fatty acids and applied the method to the determination of the fatty acid composition of rat adipose tissue and lipid components of blood vessel walls.

EXPERIMENTAL

Materials

Fatty acid standards were purchased from the following sources: caproic (6:0), caprylic (8:0), capric (10:0) and lauric (12:0) acids from Sigma (St. Louis, MO, U.S.A.), myristic (14:0), palmitic (16:0) and stearic (18:0) acids from Calbiochem (San Diego, CA, U.S.A.) and myristoleic (*cis* 14:1 n9), pentadecanoic (15:0), margaric (17:0), oleic (*cis* 18:1 n9), elaidic (*trans* 18:1 n9), linoleic (*cis,cis* 18:2 n9, 12), linoelaidic (*trans,trans* 18:2 n9,12), linolenic (all-*cis* 18:3 n9,12,15), eicosenoic (*cis* 20:1 n11), eicosadienoic (*cis,cis* 20:2 n11,14), eicosatrienoic (all-*cis* 20:3 n11,14,17), arachidonic (all-*cis* 20:4 n5,8,11,14), erucic (*cis* 22:1 n13), docosahexaenoic (all-*cis* 22:6 n4,7,10,13,16,19) acids from Serva (Heidelberg, F.R.G.). Methyl esters of these fatty acids were obtained from Supelco (Bellefonte, PA, U.S.A.). All standards were of analytical-reagent grade.

Water (silica-glass distilled) was further filtered through a Millipore 0.5-μm filter (Millipore, Bedford, MA, U.S.A.). Acetonitrile (HPLC grade) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and methanol (HPLC grade) from Merck (Darmstadt, F.R.G.), and were used without further purification. α-Bromoacetophenone was purchased from Fluka (Hauppage, NY, U.S.A.) or prepared in the laboratory by bromination of acetophenone and recrystallization from 96% ethanol (m.p. = 50°C). Triethylamine, acetone, chloroform and diethyl ether (analytical-reagent grade), purchased from Lachema (Brno, Czechoslovakia), were redistilled under reduced air pressure before the use.

Preparation of phenacyl esters of fatty acids

Fat from adipose tissue was extracted by the method of Folch *et al.*[35] and saponified with 25% (w/v) potassium hydroxide in 96% ethanol. The saponification was carried out in tightly closed 125 × 20 mm I.D. PTFE-lined screw-capped culture tubes (Corning Glass Works, Corning, NY, U.S.A.) by boiling the mixture in a water-bath for 1 h. For saponification of 0.05 g of fat, 1 ml of potassium hydroxide solution was used. After cooling and acidification to pH 2 with 3 *M* hydrochloric acid, free fatty acids were extracted with *n*-hexane–diethyl ether (1:1). The extraction procedure was repeated twice using 2 ml of the solvent mixture each time.

Standards of fatty acids or fatty acids released from the saponified fat (10–100 μg) were brought to dryness under a stream of dry nitrogen and phenacyl esters were then prepared by the method described by Wood and Lee[3], Except that the reaction mixture of fatty acids, phenacyl bromide (25 μl of a 10 mg/ml solution in acetone) and ethylamine (25 μl of a 10 mg/ml solution in acetone) was heated in a boiling water-bath for only 5 min. The excess of phenacyl bromide was reacted with acetic acid (40 μl of a 2 mg/ml solution in acetone) and, after evaporation of solvents under a stream of dry nitrogen at 40°C, the derivatization products were reconstituted in methanol.

HPLC analyses

Analyses of phenacyl esters of fatty acids were carried out on a Spectra-Physics SP 8100 high-performance liquid chromatograph, equipped with an SP 8400 UV–VIS variable-wavelength detector and an SP 4100 computing integrator (Spectra-

TABLE I

ELUTION CONDITIONS USED FOR SEPARATION OF FATTY ACID PHENACYL DERIVATIVES

*Elution mode**	*Mobile phase components present*			*Concentration gradient, CH_3OH–CH_3CN–H_2O (%, v/v) (time in minutes in parentheses)***
	CH_3OH	CH_3CN	H_2O	
A	×		×	83:17(0–35),90:10(70),100:0(90)
B		×	×	70:30(0–40), 90:10(60),100:0(80)
C	×	×	×	40:40.5:19.5(0),80.5:0:19.5(30),90:0:10(70),100:0:0(90)
D	×	×	×	40.5:41:18.5(0), 81.5:0:18.5(28–30),90:0:10(70),100:0:0(90)
E	×	×	×	40:40.5:19.5(0),81.5:0:18.5(25–27),90:0:10(70),100:0:0(90)
F	×	×	×	40:40.5:19.5(0),81.5:0:18.5(30),90:0:10(70),100:0:0(90)
G	×	×	×	40:40.5:19.5(0),81.5:0:18.5(28–30),90:0:10(70),100:0:0(90)

* Column, 250 × 4 mm I.D., Separon SGX C_{18}, 5 μm; guard column, 50 × 4 mm I.D., Separon SGX C_{18}, 7 μm; temperature, 40°C; flow-rate, 1.0 ml/min.

** Time to reach the composition cited using a linear gradient.

Physics, Santa Clara, CA, U.S.A.). Separations were performed on 250 × 4 mm I.D. columns packed with 5-μm Separon SGX C_{18} octadecyl-bonded spherical silica and coupled with an octadecyl 7-μm guard column (50 × 4 mm I.D.) (Tessek, Prague, Czechoslovakia).

Samples of derivatized fatty acids dissolved in methanol (1–10 μg/ml of each) were injected through a 10-μl sample loop. All solvents were degassed under vacuum and then maintained flushed with helium (99.996%) (Messer, Griesheim, Austria). The column temperature was maintained at 40°C and the eluted phenacyls were detected at 242 nm.

Elution was performed using a concentration gradient of a methanol–acetonitrile–water ternary mixture. The initial proportions of the components at the beginning of the run was 40:40.5:18.5 (v/v). The concentration of acetonitrile was then decreased linearly so that it reached 0% at 25 min while its concentration in the mobile phase was replaced with methanol at the same gradient rate. Elution was completed with a linear gradient of the methanol–water mixture so that the mobile phase usually contained 90% of methanol at 60–70 min and was 100% methanol at 90 min. The elution of phenacyl esters of 6:0–22:1 fatty acids was completed within 80 min at a flow-rate of 1 ml/min. For the detailed composition of mobile phase, see Table I, elution mode E.

GC analyses

Analyses of methyl esters of fatty acids[36] were performed on a Carlo Erba 2351 Fractovap gas chromatograph with a flame ionization detector (Carlo Erba, Milan, Italy) connected with a HP 3380 A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The column (2 m × 2 mm I.D.) was packed with 10% DEGS-PS on Aeropak 30 (100–120 mesh) (Supelco). The column temperature was maintained at 190°C, the injector temperature was 225°C and the detector temperature was 190°C.

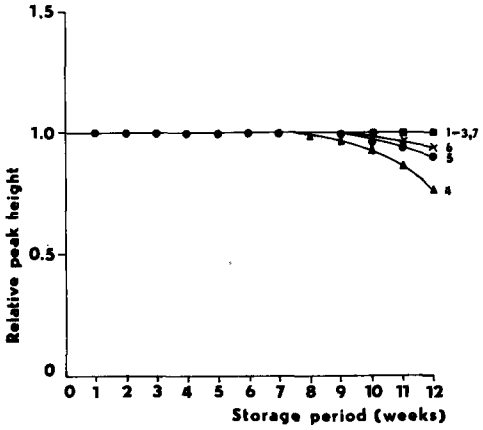

Fig. 1. Effect of storage period on relative peak heights of phenacyl esters of fatty acids. 1 = 12:0; 2 = 16:1; 3 = 18:0; 4 = 22:6; 5 = 20:4; 6 = 18:3; 7 = 18:2*cc*. Samples dissolved in methanol and stored closed in glass vials at 4°C, protected from daylight.

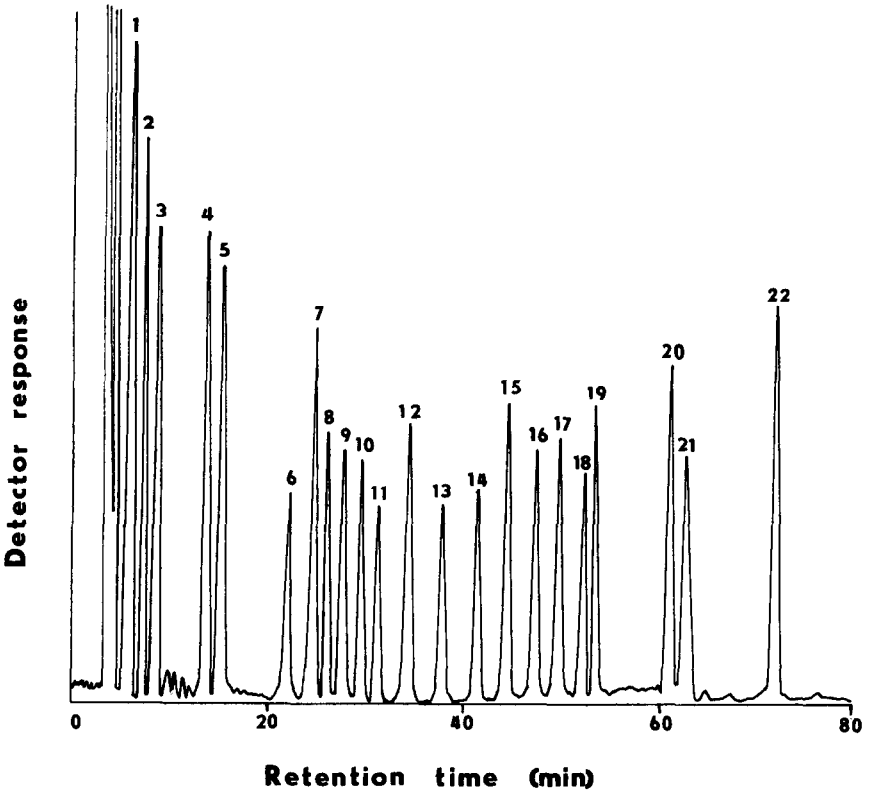

Fig. 2. HPLC of a standard mixture of fatty acid phenacyl esters (40 ng of each). Elution conditions as in Table I, elution mode E. Peaks: 1 = 6:0; 2 = 8:0; 3 = 10:0; 4 = 12:0; 5 = 14:1; 6 = 18:3; 7 = 14:0; 8 = 22:6; 9 = 16:1; 10 = 20:4; 11 = 18:2*cc*; 12 = 15:0; 13 = 18:2*tt*; 14 = 20:3; 15 = 16:0; 16 = 18:1*c*; 17 = 18:1*t*; 18 = 20:2; 19 = 17:0; 20 = 18:0; 21 = 20:1; 22 = 22:1.

TABLE II

EFFECTS OF ELUTION MODE ON RETENTION TIMES (t_R) AND RESOLUTIONS (R_S) OF SOME CRITICAL PAIRS OF FATTY ACID

Elution conditions are described in Table I. Resolution (R_S) calculated as $R_S = 2t_R/(W_1 + W_2)$, where W_1 and W_2 are the widths at the base of adjacent peaks. Individual R_S values refer to the neighbouring fatty acids. t_R = Retention time in minutes.

Elution mode	t_R *18:3*	R_S	t_R *14:0*	R_S	t_R *22:6*	R_S	t_R *16:1*	R_S	t_R *20:4*	t_R *16:0*	R_S	t_R *18:1*
A	25.3	0	25.3	2.1	28.0	1.9	30.1	1.9	32.6	42.7	2.2	44.7
B	19.8	3.4	24.6	1.5	26.8	1.2	28.9	0	29.9	41.3	2.0	44.5
C	31.3	2.4	34.2	1.9	36.4	1.6	39.1	0.5	39.8	59.4	2.2	62.5
D	21.3	2.6	25.8	0.7	26.1	1.7	29.3	1.8	31.6	49.4	2.6	53.3
E	26.5	2.5	29.4	1.5	33.9	1.4	34.5	1.9	37.5	51.4	2.5	54.0
F	26.3	2.6	29.5	2.0	33.0	1.7	34.4	1.9	36.9	53.4	2.4	56.6
G	26.7	2.6	29.4	1.9	31.7	0.9	33.0	1.9	35.6	52.8	2.4	56.4

RESULTS

Fatty acid derivatization

Investigation of the effect of reaction time on the formation of fatty acid phenacyl esters showed that the reaction proceeded at high speed and with reproducible quantitative yields. The detector response reached its maximum value within 1 min of boiling for all of the fatty acids studied. Prolonged boiling, for up to 30 min, did not produce any additional peaks in the chromatogram or lead to an increase or decrease in the detector response.

The phenacyl derivatives of fatty acids showed good stability, as demonstrated in Fig. 1. No decreases in the HPLC peak heights were observed over a storage period of up to 8 weeks for samples dissolved in methanol and stored in closed culture tubes at 4°C and with exclusion of daylight. After that period a slight decrease in absorbance was observed with the phenacyl esters of 22:6, 20:4 and 18:3 fatty acids; however, no decrease beyond 20% relative was observed even after 12 weeks.

Resolution

A typical chromatogram of a mixture of phenacyl esters of fatty acid standards is presented in Fig. 2. The chromatographic conditions were as described under *HPLC analyses.*

Elution with a methanol–water mobile phase only failed to resolve linolenic (18:3) and myristic (14:0) acids. An acetonitrile–water mobile phase could not resolve

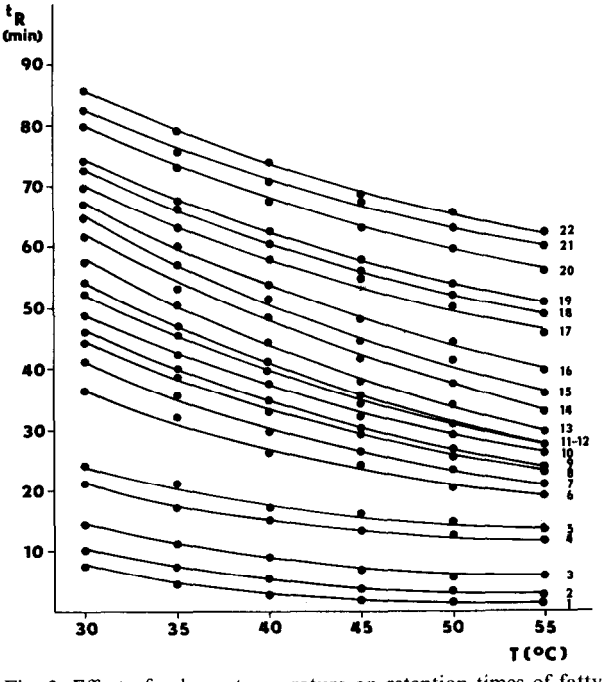

Fig. 3. Effect of column temperature on retention times of fatty acid phenacyl esters. Fatty acids as in Fig. 2. Elution mode E (Table I).

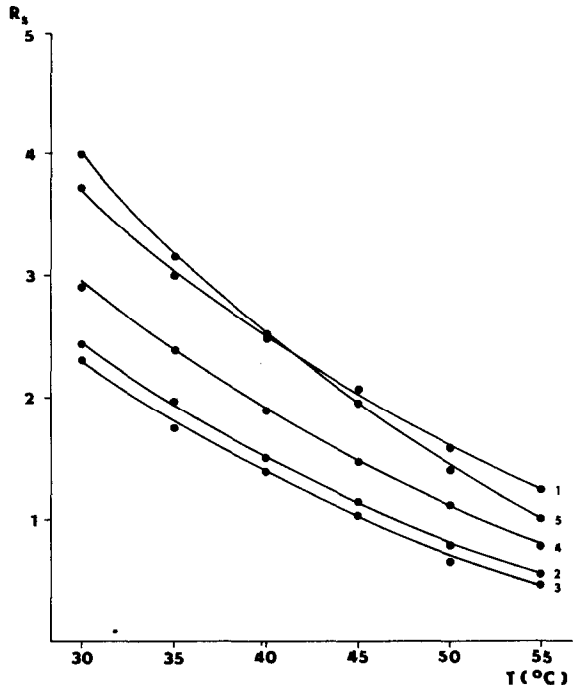

Fig. 4. Effect of column temperature on the resolution of critical pairs of fatty acid phenacyl esters. Elution mode E (Table I). Critical pairs: 1 = 18:3/14:0; 2 = 14:0/22:6; 3 = 22:6/16:1; 4 = 16:1/20:4; 5 = 16:0/18:1*c*.

adjacent peaks of palmitooleic (16:1) and arachidonic (20:4) acids. The retention times and resolutions of some important critical pairs of fatty acids under various elution conditions are summarized in Table II.

The effects of column temperature on the retention of some biologically important fatty acid phenacyl esters and on the resolution of their adjacent chromatographic pairs are depicted in Figs. 3 and 4. The optimal column temperature selected from these data was 40°C.

Quantitation

Calibration graphs were constructed from the chromatograms obtained by injecting 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, 60.0, 80.0 and 100.0 ng of each fatty acid in a standard mixture. The ratios of the peak areas or heights were plotted against the corresponding concentrations of the fatty acid (ng per 10 μl) and gave linear relationships over the concentration range studied. Under defined elution conditions both the measured parameters (peak heights and peak areas) gave the same results and therefore in routine analyses the peak heights were used. The linear regression equations for 22 biologically important mammalian fatty acids are presented in Table III. Identical linear equations were obtained for free fatty acid standards with those for methyl ester standards that were saponified prior to derivatization.

The detection limits for these fatty acids differ substantially depending on their

TABLE III

PARAMETERS OF THE CALIBRATION GRAPH FOR FATTY ACID PHENACYL ESTERS DETERMINED BY HPLC

Fatty acid	*Abbreviation**	$x = ah + b$**		r***
		a	b	
Caproic	6:0	0.0025	−0.0039	0.999
Caprylic	8:0	0.0031	−0.0046	0.999
Capric	10:0	0.0038	+0.0065	0.999
Lauric	12:0	0.0037	+0.0142	0.999
Myristic	14:0	0.0078	−0.0074	0.994
Myristoleic	14:1	0.0063	+0.0106	0.998
Pentadecanoic	15:0	0.0112	−0.0021	0.996
Palmitic	16:0	0.0094	−0.0368	0.985
Palmitoleic	16:1	0.0124	−0.0053	0.998
Heptadecanoic	17:0	0.0094	+0.0448	0.973
Stearic	18:0	0.0084	+0.0547	0.981
Oleic	18:1*c*	0.0106	+0.0607	0.987
Elaidic	18:1*t*	0.0104	+0.0558	0.986
Linoleic	18:2*cc*	0.0147	+0.0121	0.998
Linoelaidic	18:2*tt*	0.0145	+0.0111	0.997
Linolenic	18:3	0.0146	−0.0084	0.998
Eicosenoic	20:1*c*	0.0126	+0.0125	0.978
Eicosadienoic	20:2	0.0123	+0.0717	0.964
Eicosatrienoic	20:3	0.0137	+0.0147	0.996
Arachidonic	20:4	0.0122	−0.0174	0.998
Erucic	22:1	0.0073	+0.0418	0.993
Docosahexaenoic	22:6	0.0115	−0.0158	0.999

* c = *cis* isomer; t = *trans* isomer.

** x = Concentration of a fatty acid in ng per 10 μl; h = peak height in mm. Parameters a and b calculated for the range 1–100 ng of a fatty acid in an injection volume of 10 μl. Discrete measured concentrations were 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, 60.0, 80.0 and 100.0 ng per 10 μl ($n=5$).

*** r = Correlation coefficient.

molecular mass. Assuming a signal-to-noise ratio of 3, the limit of detection for short-chain (up to C_{14}) fatty acids was about 0.8 ng and for fatty acids with carbon chains longer than C_{20} it was about 1.2 ng per injection. The smallest amount of fat analysed was 5 μg.

Analytical recoveries calculated for each fatty acid from chromatograms of adipose tissue samples with and without added standard fatty acid mixture ranged from 92 to 106% with coefficients of variation in the range 1.5–6.3% ($n=5$) (Table IV).

Reproducibilities of fatty acid determinations by the proposed HPLC procedure, calculated from repeated analyses of identical adipose tissue fat samples, are presented in Table V. These results show that a given amount of each of these fatty acids can be determined accurately with a coefficient of variation of 2.1–8.2%.

The method was applied to the determination of the fatty acid composition of adipose tissue and blood vessel walls of rats fed various dietary fats (Figs. 5–8). Fatty acids were identified on the basis of the retention times of the components of the

TABLE IV

ANALYTICAL RECOVERY FOR THE DETERMINATION OF FATTY ACIDS ADDED TO RAT ADIPOSE TISSUE

Fatty acid	*Amount added (μg)**	*Increase determined (μg) (n = 5)*	*Recovery (%)*	*Coefficient of variation (%)*
12:0	5.0	5.30 ± 0.49	106.0	2.92
14:0	5.0	5.16 ± 0.30	103.2	3.81
14:1	2.0	1.98 ± 0.23	99.0	5.81
16:0	10.0	10.28 ± 0.67	102.8	1.52
16:1	15.0	14.84 ± 0.43	98.9	1.90
18:0	5.0	5.04 ± 0.36	100.8	2.14
18:1*c*	15.0	15.20 ± 0.67	101.3	2.41
18:1*t*	10.0	10.32 ± 0.81	103.2	2.85
18:2*cc*	5.0	4.76 ± 0.48	95.2	4.08
18:2*tt*	2.0	1.85 ± 0.50	92.5	4.03
18:3	5.0	4.74 ± 0.26	94.8	5.49
20:1*c*	2.0	1.86 ± 0.18	93.0	4.68
20:2	2.0	1.84 ± 0.29	92.0	3.76
20:4	2.0	1.86 ± 0.21	93.0	6.29
22:6	2.0	1.92 ± 0.16	96.0	2.89

* Amount of each fatty acid added to 100 μg of extracted adipose tissue fat before the saponification step.

TABLE V

REPRODUCIBILITY OF DETERMINATION OF FATTY ACIDS AS PHENACYL ESTERS IN ADIPOSE TISSUE BY HPLC

Fatty acid	*Mean* (μg)*	*Standard deviation (μg)*	*Coefficient of variation (%)*
12:0	0.28	0.08	3.00
14:0	2.78	0.23	8.20
14:1	0.30	0.10	3.33
16:0	24.54	0.55	2.24
16:1	12.30	0.55	4.47
18:0	3.18	0.28	3.81
18:1*c*	31.18	0.64	2.05
18:1*t*	15.36	0.67	4.36
18:2*cc*	2.08	0.43	2.67
18:2*tt*	2.24	0.11	4.91
18:3	1.18	0.18	5.25
20:1	0.14	0.06	3.29
20:2	0.14	0.06	3.29
20:4	0.28	0.08	3.57
22:6	0.40	0.10	2.50

* Expressed as mean amount in 100 μg of adipose tissue fat ($n = 5$).

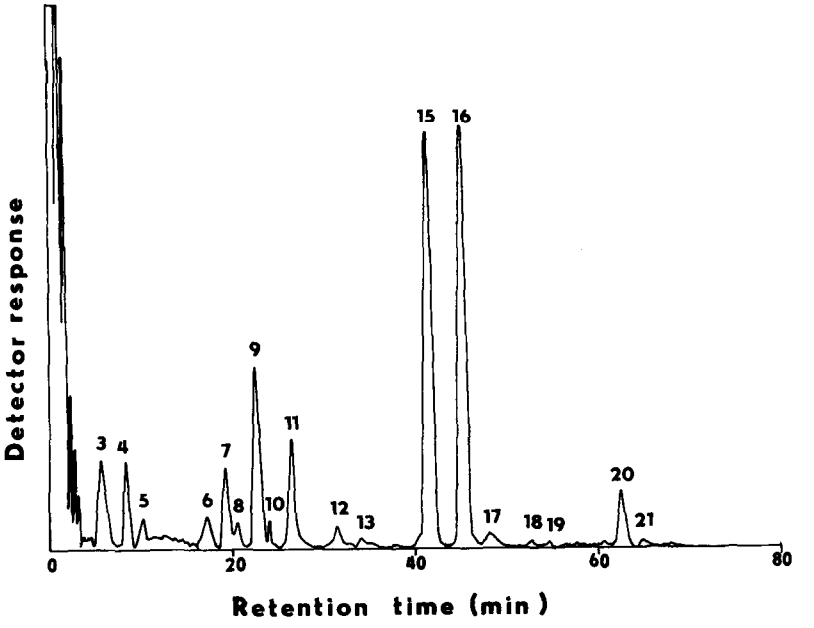

Fig. 5. HPLC of fatty acid phenacyl esters of adipose tissue of a laboratory rat fed diet A (cereal based). 100 μg of fat per 1 ml of methanol; injection volume, 10 μl; elution mode, E (Table I); absorbance scale, 0.04 a.u.

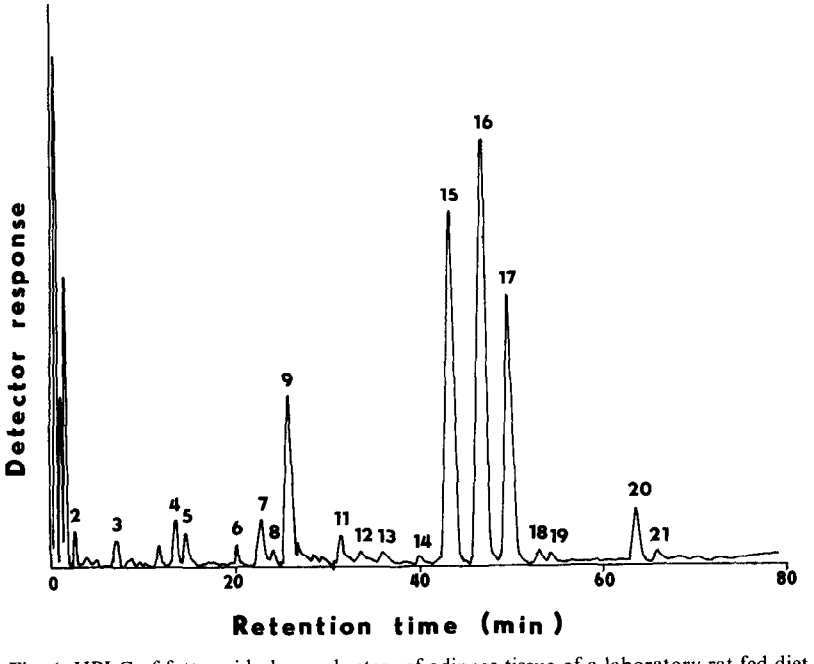

Fig. 6. HPLC of fatty acid phenacyl esters of adipose tissue of a laboratory rat fed diet B (hydrogenated vegetable oil). Conditions as in Fig. 5.

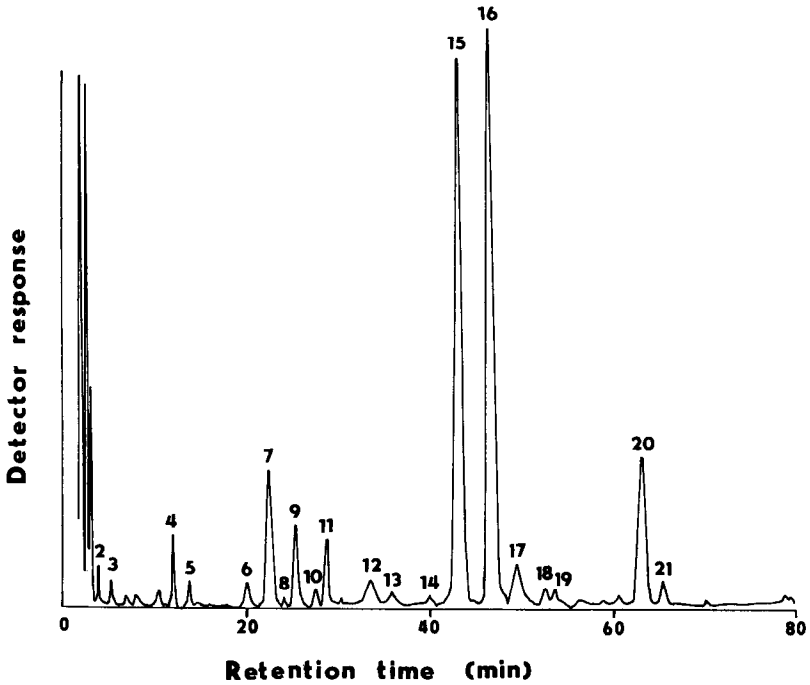

Fig. 7. HPLC of fatty acid phenacyl esters of blood vessel walls of a laboratory rat fed diet A. Conditions as in Fig. 5.

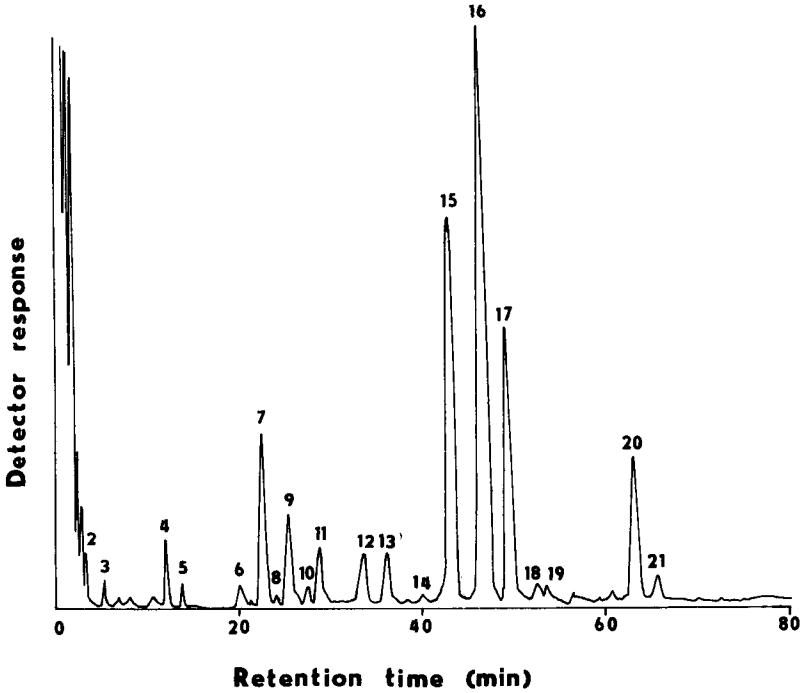

Fig. 8. HPLC of fatty acid phenacyl esters of blood vessel walls of a laboratory rat fed diet B. Conditions as in Fig. 5.

TABLE VI

COMPARISON OF HPLC AND GC RESULTS FOR DETERMINATION OF FATTY ACIDS IN ADIPOSE TISSUE FAT OF LABORATORY RATS FED DIFFERENT DIETARY FATS

Fatty acid	*Concentration (%, w/v)**			
	LC		GC	
	*A***	*B***	*A***	*B***
12:0	1.1	0.2	0.9	0.2
14:0	4.7	2.8	5.0	2.2
14:1	0.9	0.3	0.9	0.4
16:0	31.2	24.8	34.8	25.8
16:1	13.8	12.1	12.2	12.8
18:0	3.5	3.0	3.7	2.4
18:1*c*	32.6	31.5	32.9	48.3
18:1*t*	1.1	15.7	–	–
18:2*cc*	5.7	2.1	4.7	2.2
18:2*tt*	0.1	2.2	–	2.1
18:3	2.0	1.2	2.0	1.6
20:1*c*	0.3	0.1	0.3	0.2
20:2	0.1	0.1	0.2	0.1
20:4	0.3	0.3	0.1	0.1
22:6	1.1	0.4	–	–

* Expressed as means of three analyses.

** A and B represent adipose tissue fat from animals fed a control diet DOS (cereal-based) and a hydrogenated fat diet, respectively.

standard fatty acid mixture. Adipose tissue fatty acids were also examined by a GC method as described under Experimental and the results of the two methods are compared in Table VI. The observed differences were slight except for 18:1 *trans* isomers, which were not resolved from the corresponding *cis* isomers by the GC method but were well resolved by the HPLC method.

DISCUSSION

Phenacyl esters of fatty acids have often been used in analytical chemistry. Recently they proved to be useful derivatives for the HPLC determination of long-chain fatty acids, both saturated and unsaturated[3,6,7]. They can be prepared in various ways, differing mainly in the temperature and reaction time or in the presence or absence of various catalysts, for example 18-crown-6[6]. The most suitable for its simplicity and speed seems the procedure described by Wood and Lee[3], which does not require a catalyst. We tested the minimum time necessary for the derivatization and found that the reaction was completed within 1 min. Further boiling did not increase or decrease the detector response. For convenience we used a 5-min reaction time.

Quenching the excess of the derivatization reagent with acetic acid is important for the successful application of this procedure[3]. The phenacyl esters prepared in this way exhibited very good stability; when properly stored they did not change for

about 2 monts. The lowest stability was observed with phenacyl esters of polyunsaturated acids such as linolenic (18:3), arachidonic (20:4) and docosahexaenoic (22:6) acids. However, the decrease in the concentration of these acids observed after 3 months still did not exceed 20%.

The mobile phases usually used for the HPLC separation of fatty acid derivatives are acetonitrile–water[2,3,7,9–11,24,27,29], methanol–water[5,6,12,14,19,23,28,30,33] and acetonitrile–methanol–water[15,28,31,34]. Elution with acetonitrile–water is not efficient enough to separate the phenacyl derivatives of palmitoleic (16:1) and arachidonic (20:4) acids (Tables I and II), as also demonstrated elsewhere[3,15,24]. Partial separation could be achieved with a decreased ratio of acetonitrile to water in the mobile phase, as reported by Borch[7], but this is inconvenient owing to the substantial prolongation of the elution time (up to 4 h at a flow-rate of 2 ml/min) and the specific column requirements (90 × 0.64 cm I.D.).

Elution with methanol–water, on the other hand, cannot resolve adjacent peaks of linolenic (18:3) and myristic (14:0) acids (Tables I and II), as also reported elsewhere[12,15,34]. Korte *et al.*[12] in addition could not resolve the phenacyl esters of oleic and elaidic acids (*cis* and *trans* isomers of 18:1 n9 acids), stearic (18:0) and *cis*-11-eicosenoic (20:1) acids and all-*cis*-4,7,10,13,16,19-docosahexaenoic (22:6) and palmitooleic (16:1) acids.

These results demonstrate the importance of the carbon chain length and the number of double bonds with respect to the solubility of phenacyl derivatives in these two solvents. Whereas in acetonitrile the number of double bonds seems to be more important for the solubility, in methanol the chain length seems more important. Utilization of the different properties of these two solvents offers some possibility for the separation of fatty acids whose differences in chain length and degree of unsaturation may make them difficult to separate with the use of either acetonitrile or methanol alone. Isocratic elution with any mixture of these solvents cannot gain from all the potential advantages of the system. As can be seen in Tables I and II, acetonitrile improves the resolution of fatty acids eluted at the beginning of the run (18:3 and 14:0), but significantly reduces the resolution of fatty acids eluted later (16:1 and 20:4 and 16:0 and 18:1). For these reasons, a ternary concentration gradient elution with acetonitrile, methanol and water seems to be the best way to optimize the separation of the phenacyl derivatives of mammalian fatty acids by reversed-phase HPLC.

In our method, the mobile phase contained acetonitrile only at the beginning of the run and the elution was then completed with an increasing concentration gradient of methanol in water (Table I, elution mode E, Fig. 1). The resolution achieved with this gradient elution and the time required seem to be better for a wider range of fatty acid phenacyl esters than reported previously[3,7,12,14,15]. No previous paper has described the separation of 22:6 acid from palmitooleic (16:1) and myristic (14:0) acids. The resolution achieved under the chromatographic conditions suggested here is higher than 1.

The method is very suitable for the quantitation of fatty acids. The calibration graphs for both short- and long-chain fatty acids are linear over the investigated concentration range of 1.0–100.0 ng per injection. The calibration data presented in Table III demonstrate good precision and linearity.

The detection limits, close to those of fluorescence labelling methods of 0.5–2

fmol[33] or 5–1000 pmol[31], and the good analytical reporducibility and recovery make the method suitable for a wide range of applications.

In conclusion, the method reported here demonstrates the increasing capacity of HPLC methodology to solve problems related to fatty acid analysis. This method is more efficient in the resolution of *cis* and *trans* conformational isomers of fatty acids than the usually used GC methods with packed columns, and there are no problems with derivatization of short-chain fatty acids or heat-labile polyunsaturates. Another advantage over GC methods is that the separated fatty acids are not destroyed during their detection, which enables further analyses to be performed.

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