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A MICROLITER METHOD FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF LONG-CHAIN NON-ESTERIFIED FATTY ACIDS IN HUMAN SERUM OR PLASMA

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SUMMARY

Non-esterified fatty acids (NEFA) from C_{12} to C_{24} are assayed in human serum or plasma in a four-step procedure: extraction, volume reduction, methylation and gas chromatography. NEFA are extracted with chloroform—heptane—methanol from 50—100 μl of serum or plasma buffered with phosphate. After adding ethyl acetate the volume of the extract is reduced under partial reflux to 5—7 μl. Potassium carbonate, methyl iodide and a crown ether are added to the dry concentrate and the NEFA are selectively methylated with a yield of 100% by heating in a microrefluxer for 10 min. Gas chromatography is carried out with 1 μl of the reaction mixture on a packed column by temperature-programmed operation. Thirteen individual fatty acids are determined in sera of normal adults. The coefficients of variation for 24 determinations of a pooled serum were 2.7% for the total NEFA content and 3—10% for most of the individual NEFA.

INTRODUCTION

Long-chain (C_{12}—C_{24}) non-esterified fatty acids (NEFA) are present in human blood in physiologically varying concentrations [1]. Clinical interest follows from the fact that also certain pathological situations lead to changes in the NEFA pattern; for example, general hyperlipidacidemia in diabetes mellitus or the decrease of the linoleic acid:oleic acid ratio in stroke patients [2].

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Therefore assay of NEFA can be useful for diagnosis and in controlling the course of diseases as well as in the search for risk patients in preventive medicine.

A large number of methods for the quantitative determination of total as well as individual NEFA in serum/plasma have been published. The total NEFA content is usually assayed by titration [3—8] or colorimetrically after converting the fatty acids into their copper or cobalt soaps [9—14]. Recently an enzymatic method was described [15]. Individual NEFA are best determined by gas chromatography (GC) [16—22]. However, GC methods with sufficient sensitivity and precision are too laborious and therefore unsatisfactory for clinical use [16—18].

This paper presents a new micromethod for the GC assay of the individual NEFA in serum/plasma, which is based on the prechromatographic microliter techniques described recently [23]. It is as accurate as previous methods but is faster, easier in application and less expensive. In addition to this it is more sensitive so that NEFA such as lauric, myristoleic, linolenic, arachidonic or erucic acid, which are present in serum only in minor amounts, can also be determined.

EXPERIMENTAL

Materials and methods

All reagents and solvents which were of the highest available quality were used without further purification. Undecanoic, lauric, tridecanoic, myristic, myristoleic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachic, arachidonic, behenic, erucic, lignoceric acid and their methyl esters were from Sigma (Munich, G.F.R.). 2,6-Di-*tert.*-butyl-*p*-cresol and methyl iodide were purchased from Roth (Karlsruhe, G.F.R.). Potassium carbonate, iodine, potassium dihydrogen phosphate, disodium hydrogen phosphate, dibenzo-18-crown-6, ethyl acetate, propan-2-ol, chloroform, *n*-heptane, and methanol were from E. Merck (Darmstadt, G.F.R.).

For the extraction solution, one part of chloroform is mixed with one part of *n*-heptane and 2% methanol added. The buffer solution is 0.53 *M* potassium dihydrogen phosphate and 0.27 *M* disodium hydrogen phosphate. The methylation solution is a saturated solution of dibenzo-18-crown-6 in methyl iodide prepared at room temperature. The internal standard solution is 2.5 m*M* tridecanoic acid in propan-2-ol.

The calibration mixture consists of 0.2 m*M* each of lauric, tridecanoic, myristic, myristoleic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidonic, behenic, erucic and lignoceric acid methyl esters in ethyl acetate.

The microrefluxer [24] and boiling chips suitable for the microvessels were purchased from Forschungsinstitut Berghof (Tübingen, G.F.R.). The microvessels (Fig. 1) were prepared by a glass-blower according to specifications [25]. They were then cleaned adhering strictly to the given instructions [26, 27].

Thin-layer chromatography was performed on 40 mm × 80 mm silica-gel plates without fluorescence indicator (Merck) in a vapour phase saturated flat tank according to the method of Seiler and Knödgen [28]. The plate was

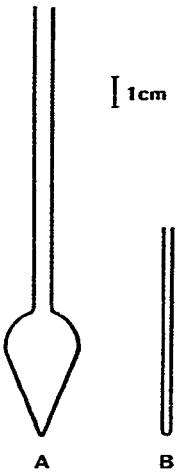

Fig. 1. Special equipment for the microliter technique. (A) Glass vessel of type A for volume reduction from 2000 to 50 μl. (B) Glass vessel of type B for volume reduction from 50 to 5 μl and for the methylation reaction.

developed in a solvent system consisting of *n*-hexane—diethyl ether—glacial acetic acid (90:10:1, v/v; Merck). After drying, the plate was placed in an iodine chamber for visualisation of the lipid and NEFA spots. For the identification of spots the following lipids were run in parallel: dipalmitoyl L-α-phosphatidyl choline, dimyristoyl L-α-phosphatidyl ethanolamine, glycerol tripalmitate, and cholesterol palmitate (Serva, Heidelberg, G.F.R.).

Standard procedure

Extraction. Serum or plasma (50—100 μl) is pipetted into a stoppered glass vessel of about 10 ml volume and 2 μl of the internal standard solution are added. After stirring for 1 min with a Vortex mixer followed by standing for 5 min, 150 μl of buffer solution and 1.25 ml of extraction solution are added. The mixture is vortexed for 2 min and after 15 min standing it is centrifuged at 800 *g* for another 15 min. Most of the upper aqueous layer is then removed to obtain direct access to the lower organic phase.

Concentration. One thousand microliters of the extract are transferred to a microvessel of type A (Fig. 1A) and 1000 μl of ethyl acetate are added. Heating the mixture in a water-bath at 95°C under partial reflux reduces the volume of the extract. Optimum conditions are reached when a condensation zone of some millimeters width can be seen 2—5 mm under the rim of the vessel [25]. This is achieved by varying the immersion depth of the vessel in the water-bath. When the condensation ring begins to drop, another 500 μl of ethyl acetate are added. Heating is continued until only a few microliters of liquid can still be seen in the vessel. Then the vessel is immediately chilled by ice. After cooling for 5 min about 50 μl of the extract have gathered at the bottom of the vessel. The solution is quantitatively transferred to a microvessel of type B (Fig. 1B) by means of a Pasteur pipette. Concentration is then performed as described above at 90°C until about 1 μl of liquid can be recognized. By cooling in the ice-bath a final volume of 5—7 μl of extract is obtained.

Methylation. A 3.5-μl volume of the concentrated extract is transferred to another type B vessel provided with a boiling chip and 3.5 μl of the methylation solution are added. In a controlled-humidity environment [24,29] about 0.5 mg of finely powdered potassium carbonate which had been dried in a drying pistol for 24 h at 250°C over phosphorus pentoxide is added with a special device [30]. The mixture is then refluxed under atmospheric pressure in a microrefluxer for 10 min. Up to twelve reactions can be carried out simultaneously. A sample of the extract can now be directly injected into the gas chromatograph. If the vessels are closed with suitable PTFE caps and kept at about 0°C no loss occurs during days of storage.

Gas chromatography. A Hewlett-Packard Model 5710A double-column gas chromatograph equipped with a flame ionization detector was used. One microliter of the sample was injected on one column while the other was conditioned by several injections of solvent at the same time. GC was carried out on 183 cm × 0.64 cm glass columns packed with 10% Silar 10 CP on Chromosorb W HP, 100—120 mesh (Hewlett-Packard, Frankfurt, G.F.R.), by temperature-programmed operation. The conditions were: oven temperature T_1 130°C, T_2 220°C, ΔT 4°C/min; injection port temperature 250°C; detector temperature 300°C; flow-rates were nitrogen 44 ml/min, hydrogen 60 ml/min, air 240 ml/min; range 10, attenuation 2.

Fatty acid concentrations were calculated electronically with a Hewlett-Packard Model 3380A integrator using the internal standard operation.

RESULTS AND DISCUSSION

Extraction

NEFA are extracted from serum or plasma by a modification of the method of Falholt et al. [14]. When the serum/plasma is buffered at pH 6.4 with potassium dihydrogen phosphate and disodium hydrogen phosphate, chloroform—*n*-heptane—methanol extracts the NEFA without significant co-extraction of other lipids. As shown by thin-layer chromatography, cholesterol esters, triglycerides and phospholipids were present only in trace amounts, whereas the extraction procedures of Dole [3] or of Folch et al. [31] lead to considerable amounts of these lipids in the extract (Fig. 2). As other lipids of serum/plasma can interfere with the analysis of NEFA the extracts obtained by the Dole or Folch procedures have to be purified by thin-layer chromatography [16—18,22]. This laborious step is omitted in our method.

Concentration

The extracts are concentrated to about 1/200 of their original volume under partial reflux in specially designed glass vessels (Fig. 1). Upon addition of ethyl acetate the solvent is rapidly removed in a water-bath at 90—95°C. At the same time the extract is dried azeotropically. The dryness of the concentrates was proved by cooling with acetone—CO_2. No turbidity could be observed, which indicated that the water content was lower than 0.1% [24]. It was demonstrated with serum extracts containing definite amounts of added undecanoic acid and with standard solutions of C_{12}—C_{24} fatty acids that no loss of material occurs with this concentration technique.

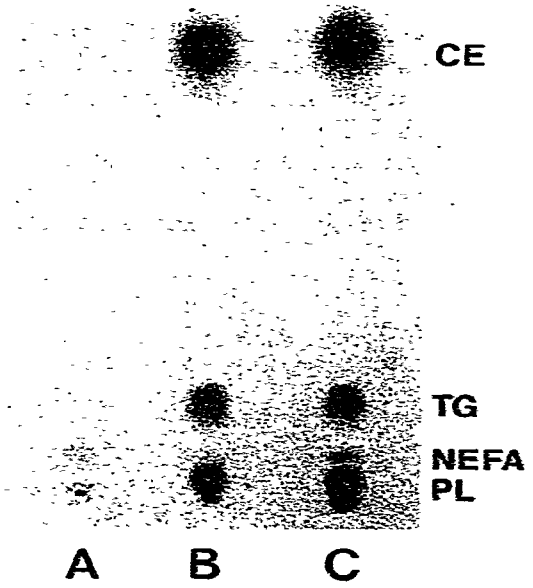

Fig. 2. Thin-layer chromatogram of concentrated serum extracts obtained by different procedures for the extraction of NEFA. (A) Extraction with chloroform—heptane—methanol modified after Falholt et al. [14]. (B) Extraction with propan-2-ol—heptane—HCl according to the method of Dole [3]. (C) Extraction with chloroform—methanol according to the method of Folch et al. [31]. Concentration of the extracts and thin-layer chromatography are described in Experimental. CE = cholesterol esters; TG = triglycerides; PL = phospholipids.

Methylation

The preparation of methyl esters from fatty acids by methyl iodide in the presence of potassium carbonate has already been described [19,32]. However, as the reaction had to be carried out under pressure for 1 h to achieve complete yield it did not seem suitable for a routine assay. The reaction conditions can be mitigated substantially by addition of a crown ether [33]. With dibenzo-18-crown-6, 100% yield of fatty acids from C_6 to C_{24} in the concentration range 5 μM (detection limit under the GC conditions used) to 10 mM is achieved within 10 min of refluxing at atmospheric pressure. The sample is then ready to be injected into the gas chromatograph. Other procedures commonly used to prepare the methyl esters, such as the boron trifluoride—methanol [17,18] or N,N-carbonyldiimidazole—methanol [16] methods, are much more laborious because additional steps of washing, extraction or volume reduction are necessary before chromatography.

The methylation step has also been tested on possible undesired reactions. Neither methylation of the carbon chain of unsaturated fatty acids nor transesterification of esterified fatty acids occurred even under prolonged refluxing [33]. Methylation by the methyl iodide—potassium carbonate method can be carried out in the presence of glyceryl esters in total lipid extracts of serum/plasma. Selective NEFA derivatization is also achieved by methylation with diazomethane [34] and trimethylsilylation [35].

Gas chromatography

With most of the serum or plasma NEFA assays, GC on packed columns is carried out isothermally because bleeding stationary phases such as DEGS or FFAP are used. Isothermal operation, however, means insufficient separation and unequal detection sensitivity for the long-chain NEFA spectrum in serum/plasma. These disadvantages can be avoided with temperature-programmed operation on the low-bleeding stationary phase Silar 10CP. As shown in Fig. 3, the peaks of fifteen equally concentrated fatty acid methyl esters of a standard mixture are clearly separated and equally high.

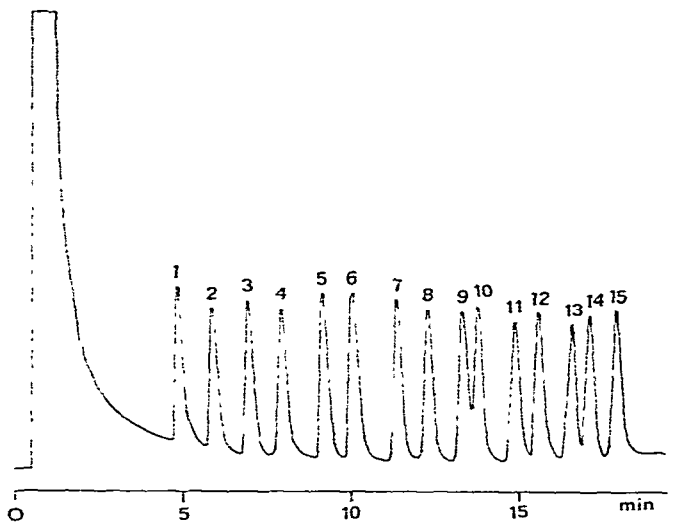

Fig. 3. Gas chromatogram of a standard mixture of the methyl esters of fifteen long-chain fatty acids with carbon chain length C_{12}—C_{24} obtained by temperature-programmed operation on packed Silar 10CP columns. The GC conditions are given in Experimental. Concentration of each fatty acid methyl ester was 0.2 m*M*. 1 = Lauric acid methyl ester; 2 = tridecanoic acid methyl ester; 3 = myristic acid methyl ester; 4 = myristoleic acid methyl ester; 5 = palmitic acid methyl ester; 6 = palmitoleic acid methyl ester; 7 = stearic acid methyl ester; 8 = oleic acid methyl ester; 9 = linoleic acid methyl ester; 10 = arachinic acid methyl ester; 11 = linolenic acid methyl ester; 12 = behenic acid methyl ester; 13 = erucic acid methyl ester; 14 = arachidonic acid methyl ester; 15 = lignoceric acid methyl ester.

In Fig. 4 a chromatogram of a pooled human serum is demonstrated. The corresponding blank chromatogram contains only one prominent peak which derives from an impurity of methyl iodide. This peak is clearly separated from the fatty acid peaks of the serum.

The sample injected into the gas chromatograph contains the methyl esters in about an eightfold concentration compared to the original concentration in the serum/plasma. Therefore, also the fatty acids lauric, myristoleic, linolenic, arachidonic and erucic acid, which are present only in minor amounts, can be precisely determined.

Autoxidation

A series of serum NEFA analyses has been performed in the presence of 0.05% of 2,6-di-*tert.*-butyl-*p*-cresol (BHT) in the unconcentrated extract and

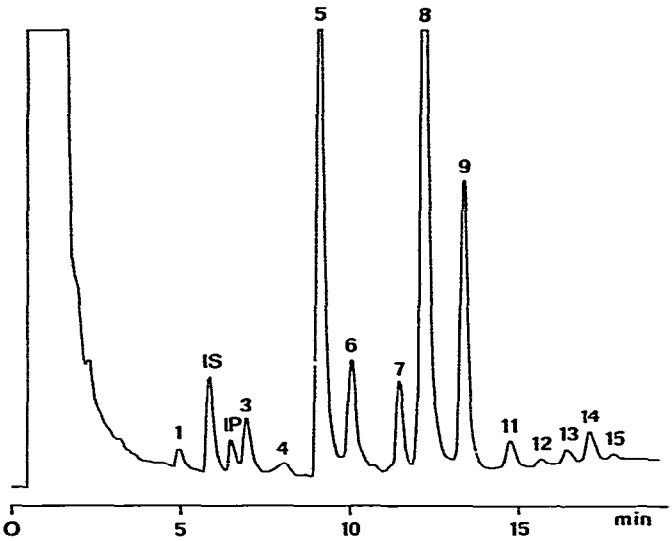

Fig. 4. Gas chromatogram of the NEFA methyl esters from a pooled human serum. The conditions are given in Experimental. IP = impurity from methyl iodide; ISTD = tridecanoic acid methyl ester (from added internal standard). For further designations see legend of Fig. 3.

compared to another series of analyses of the same serum without BHT. No significant difference in the amount of the fatty acids, for example of linolenic and arachidonic acid, was observed. With our technique impairment of the results of NEFA analyses by autoxidation of unsaturated fatty acids in the course of the assay can therefore be excluded [36].

Sample pretreatments

The influence of different pretreatments (coagulation, storage) of a human blood sample has been examined (Table I). NEFA assays were carried out with serum, heparin plasma and EDTA plasma of blood from the same collection. No difference in the NEFA content could be determined between serum and heparin plasma of blood kept for 30 min at room temperature before centrifuging. However, EDTA plasma values were significantly ($p < 0.01$) lower than those of serum. Also in heparin plasma of blood which was allowed to stand at room temperature for 30 min a somewhat higher NEFA content was found compared to heparin plasma of blood which was centrifuged immediately after collection. NEFA analyses of serum or plasma which had been kept at —70°C for several weeks did not differ from those of corresponding samples assayed immediately.

Evaluation of the method

The precision of the method has been evaluated by 24 NEFA assays of a pooled human serum. Mean values, standard deviations and coefficients of variation of thirteen individual long-chain NEFA and of the total fatty acid content are shown in Table II.

Sensitivity has been tested with aliquots of a pooled serum to which decreasing amounts of tridecanoic acid dissolved in propan-2-ol were added. Trideca-

TABLE I

THE EFFECT OF ANTICOAGULANTS AND DIFFERENT STORAGE CONDITIONS ON THE TOTAL* NEFA CONTENT OF A HUMAN BLOOD SAMPLE

The blood obtained by venipuncture from a healthy female adult was collected either in a serum-monovette® (Sarstedt, Nümbrecht, G.F.R.) or in syringes containing heparin (75 U/ml Thrombophob®, Nordmark, Hamburg, G.F.R.) or EDTA (Sarstedt). Centrifugation was carried out at 4°C for 10 min at 1300 *g*.

	Assay immediately after centrifugation (μM; mean ± S.D.; n = 8)	Assay after storage for four weeks at −70°C (μM; mean ± S.D.; n = 8)
Heparin plasma of blood centrifuged immediately after collection	524 ± 43	512 ± 35
Heparin plasma of blood kept 30 min at room temperature before centrifugation	553 ± 37	545 ± 30
EDTA plasma of blood centrifuged immediately after collection	461 ± 28	470 ± 18
EDTA plasma of blood kept 30 min at room temperature before centrifugation	467 ± 33	458 ± 25
Serum of blood kept 30 min at room temperature	550 ± 21	560 ± 19

*The sum of the individual NEFA determined by GC.

TABLE II

MEAN VALUES, STANDARD DEVIATIONS AND COEFFICIENTS OF VARIATION OF 24 DETERMINATIONS OF INDIVIDUAL AND TOTAL NEFA CONCENTRATIONS OF A POOLED HUMAN SERUM

NEFA		NEFA concentration (μM)		Coefficient of variation (%)
		Mean	S.D.	
$C_{12:0}$	lauric acid	8	0.9	11.3
$C_{14:0}$	myristic acid	22	1.8	8.2
$C_{14:1}$	myristoleic acid	8	0.8	10.0
$C_{16:0}$	palmitic acid	242	8.0	3.3
$C_{16:1}$	palmitoleic acid	53	2.6	4.9
$C_{18:0}$	stearic acid	37	3.6	9.7
$C_{18:1}$	oleic acid	287	9.4	3.3
$C_{18:2}$	linoleic acid	121	7.0	5.8
$C_{18:3}$	linolenic acid	12	1.3	10.8
$C_{20:4}$	arachidonic acid	16	1.7	10.6
$C_{22:0}$	behenic acid	1	—	—
$C_{22:1}$	erucic acid	5	0.7	14.0
$C_{24:0}$	lignoceric acid	1	—	—
Total		810	22.0	2.7

noic acid of 0.5 μM could still be clearly determined in a serum with a total NEFA content of about 0.5 mM.

For recovery studies distinct amounts of either tridecanoic, stearic or linoleic acid were added to samples of a serum. The mean recoveries of twelve experiments were 98% for tridecanoic acid, 100% for stearic acid and 97% for linoleic acid (Table III). Advantages of the method for its application as a clinical routine assay are due to the microliter scale. The amount of serum/plasma

TABLE III

RECOVERY EXPERIMENTS

Either tridecanoic, stearic or linoleic acid dissolved in propan-2-ol (2.5 mM) was added to aliquots of a pooled serum and assayed with the standard procedure. In experiments 1—3 oleic acid in the serum was chosen to serve as reference, otherwise the added tridecanoic acid was used as internal standard. The mean values of four assays for each experiment are given.

Exp.	Added fatty acid concentration	Assayed concentration (μM)			Recovery (%)
		Tridecanoic acid	Stearic acid	Linoleic acid	
1	25 μM tridecanoic acid	23	39	114	92
2	50 μM tridecanoic acid	52	40	125	104
3	100 μM tridecanoic acid	98	37	123	93
4	25 μM stearic acid	—	64	120	103
5	50 μM stearic acid	—	88	118	101
6	100 μM stearic acid	—	130	125	95
7	25 μM linoleic acid	—	35	140	96
8	50 μM linoleic acid	—	40	169	99
9	100 μM linoleic acid	—	38	214	97

necessary for the assay is reduced to 50—100 μl. As less solvents and reagents are used the interference from their impurities in the GC analysis is minimized. Furthermore, the costs for chemicals and glassware are lower. The prechromatographic part of the assay can be done with 10—12 samples in parallel and then requires about 3 h.

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REFERENCES

1 L. Hagenfeldt, Ark. Kemi, 29 (1968) 57.
2 S.-N. Lin and E.C. Horning, J. Chromatogr., 112 (1975) 465.
3 V.P. Dole, J. Clin. Invest., 35 (1956) 150.
4 V.P. Dole and H. Meinertz, J. Biol. Chem., 235 (1960) 2595.
5 D.L. Trout, E.H. Estes and S.J. Friedberg, J. Lipid Res., 1 (1960) 199.
6 T.F. Kelley, Anal. Chem., 8 (1965) 1078.
7 J. Keul, N. Linnet and E. Eschenbruch, Z. Klin. Chem. Klin. Biochem., 5 (1968) 394.
8 J.S. Braun, Z. Klin. Chem. Klin. Biochem., 9 (1971) 387.
9 W.G. Duncombe, Clin. Chim. Acta, 9 (1964) 122.
10 K. Itaya and M. Ui, J. Lipid Res., 6 (1965) 16.
11 M. Novák, J. Lipid Res., 6 (1965) 431.
12 S. Laurell and G. Tibbling, Clin. Chim. Acta, 16 (1967) 57.
13 E.J.M. Regouw, P.J.F.C. Cornelissen, R.A.P. Helder, J.B.F. Spijkers and Y.M.M. Weeber, Clin. Chim. Acta, 31 (1971) 187.
14 K. Falholt, B. Lund and W. Falholt, Clin. Chim. Acta, 46 (1973) 105.
15 S. Shimizu, K. Ihone, Y. Tani and A. Yamada, Anal. Biochem., 98 (1979) 341.
16 H. Ko and M.E. Royer, J. Chromatogr., 88 (1974) 253.
17 R.G. McDonald-Gibson and M. Young, Clin. Chim. Acta, 53 (1974) 117.
18 V. Rogiers, Clin. Chim. Acta, 78 (1977) 227.
19 A. Grünert, Z. Klin. Chem. Klin. Biochem., 13 (1975) 407.
20 D. Sampson and W.J. Hensley, Clin. Chim. Acta, 61 (1975) 1.
21 A. Wirth, J. Eckhard and H. Weicker, Clin. Chim. Acta, 71 (1976) 47.
22 M.L. Michailov, Biochem. Pharmacol., 23 (1974) 2625.
23 W. Dünges, Prächromatographische Mikromethoden, Hüthig, Heidelberg, 1979.
24 W. Dünges, Prächromatographische Mikromethoden, Hüthig, Heidelberg, 1979, p. 204.
25 W. Dünges, Prächromatographische Mikromethoden, Hüthig, Heidelberg, 1979, p. 33.
26 W. Dünges, K. Kiesel and K.E. Müller, in E. Reid (Editor), Blood Drugs and other Analytical Challenges, Ellis Horwood, Chichester, 1978, p. 294.
27 W. Dünges, Prächromatographische Mikromethoden, Hüthig, Heidelberg, 1979, p. 62.
28 N. Seiler and B. Knödgen, J. Chromatogr., 131 (1977) 109.
29 W. Dünges, K.E. Müller and M. Müller, in E. Reid (Editor), Blood Drugs and other Analytical Challenges, Ellis Horwood, Chichester, 1978, p. 257.
30 W. Dünges, Prächromatographische Mikromethoden, Hüthig, Heidelberg, 1979, p. 135.
31 J. Folch, M. Lees and G.H. Sloane-Stanley, J. Biol. Chem., 226 (1957) 497.
32 W. Dünges, Chromatographia, 6 (1973) 197.
33 W. Dünges, M. Höckel and W. Hinze, in preparation.
34 A. Kuksis, N. Kovacevic, D. Lau and M. Vranic, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 34 (1975) 2238.
35 A. Kuksis, J.J. Myher, L. Marai and K. Geher, Anal. Biochem., 70 (1976) 302.
36 A. Seher, in J. Schormüller (Editor), Handbuch der Lebensmittelchemie, Vol. II/2, Springer, Berlin, Heidelberg, New York, 1967, p. 952.