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Two-dimensional separation of fatty acids by thin-layer chromatography on urea and silver nitrate silica gel plates

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Abstract

Semipreparative separation of fatty acid methyl esters (FAMEs) using two-dimensional TLC is described. The part of layer was impregnated with urea (first dimension of development) and second part with $AgNO_3$ (second dimension). The FAMEs were separated according the structure of the chain (branched) and also by number of their double bonds.

Keywords: Fatty acid methyl esters

1. Introduction

Many methods have been used for the preparation of fatty acids according to their individual structural characteristics. The chromatography methods: gas chromatography (GLC), thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC), belong to those that are the most elaborate of all; their current frequency of use goes in the opposite order to that mentioned above. Nowadays, GLC is used very rarely and the emphasis is on HPLC. TLC can compete with HPLC owing to its modest financial requirements.

In addition to these techniques, non-chromatographic techniques have also been in use to isolate fatty acids, e.g., crystallization, either at low temperatures or in the form of cluster compounds. Urea is usually employed in these methods. The compounds are separated according to the spatial arrangement of their chain. Use of urea for impregnation of the stationary phase–silica gel is very rare [1]. TLC on urea–silica gel plates has so far been published only once [2].

In contrast, impregnation of silica gel with silver salts and subsequent separation according to the number and configuration of double bonds is routinely used [3]. To our knowledge, the combination of the two types of impregnation has not yet been described even though plates with preconcentration zones are commercially available.

The present paper is focused upon semipreparation of methyl esters of fatty acids (FAMEs) using two-dimensional TLC. One part of the layer on the plate was impregnated with urea (first dimension of development), the other part with $AgNO_3$ (second dimension). The FAME mixture can then be separated according to both the structure of the chain (branched) and the number of double bonds.

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2. Experimental

2.1. Reagents

All solvents used were HPLC grade. Silica gel TLC grade and urea (p.a.) were obtained from Sigma-Aldrich (Prague, Czech Republic). Fatty acids (see Table 1) were also obtained from Sigma. A mixture of FAMEs (see Table 2) was prepared by mixing roughly equal amounts of FAMEs from *Streptomyces avermitilis* [4] and cod liver oil (Czechoslovak pharmacopeia No. 4), transesterified by a mixture of MeONa in MeOH [5].

2.2. Slurry preparation

The slurry was prepared by mixing silica gel [2] with a 10% urea solution or a 10% solution of $AgNO_3$.

A 0.5-mm layer of silica gel with urea (20 × 5 cm) was put on a glass plate that was dried in a KOH-containing desiccator overnight, and the free surface (20 × 15 cm) was overlayered with silica gel with $AgNO_3$. The plate was dried again in a similar way and activated for 1 h at 105°C. After cooling the plates were immediately transferred into a desiccator for storage.

2.3. TLC and GC–MS separation

The lower, right-hand corner of the plate was loaded with an amount of 250–500 μg of a sample and the plate was developed with butyl acetate. After the chromatographic run was finished, the plate was dried until no solvent smell remained, turned around by 90° and developed with a mixture of hexane–diethyl ether–methanol (90:10:1), where the polar solvent had to be present to disrupt the urea complex. After desiccation the plate was sprayed with a 0.1% ethanol solution of 2′,7′-dichlorofluorescein and the corresponding spots marked under UV light. The silica gel was scraped and extracted with a mixture of hexane–diethyl ether (1:1) and after evaporation, the FAMEs were converted into oxazolines.

Oxazolines were prepared using a modified method of Yu et al. [6] and determined by means of GC–MS. For identification of oxazolines a GC–MS Shimadzu QP-1000 (Shimadzu, Kyoto, Japan) with a 60 m × 0.32 mm I.D. (0.2 μm) Supelcowax fused-silica capillary column (Supelco, Gland, Switzerland), split/splitless injector and He as a carrier gas were used. Oven temperature was programmed from 150 to 250°C at a rate of 5°C/min. Ionization energy was 70 eV; electron multiplier voltage was 2.5 kV. The identification of separate peaks was made using total mass spectra (from *m/z* 50 to *m/z* 500) according to the method used in previous papers [6,7].

In the previous work [2], the author had big problems with the impregnation of the TLC layer. We did not meet with similar problems, perhaps because the other modifier, ammonium sulfate, was not used.

Table 1
Standard mixture of fatty acid methyl esters

FAME	Total (μg)	Fractions (in percentage of total)							
		n[a]	i-ai[a]	m-br[b]	1 × C=C	2 × C=C	3 × C=C	4 × C=C	5+6 × C=C
18:0	13.5	95.3	4.6	0.1					
i-18:0	10.1	4.5	89.2	5.8					
phytanic	11.9	0.2	5.7	94.1					
18:1w9	13.8				99.9	0.1			
18:2w6	11.5				0.1	99.8	0.1		
18:3w3	14.6					0.1	99.8	0.1	
20:4w6	11.7						0.1	98.5	1.4
20:5w3	12.9							1.4	98.6

Analyses were repeated five times, the R.S.D. was ±1%.
[a] n = normal chain; i = isoacid; ai = anteisoacid.
[b] m-br = multi-branched acid.

Table 2
Fatty acid composition from total sample individual fractions

Fatty acid[a]	Fractions								
	Total	*n*[b]	i-ai[b]	m-br[c]	1 × C=C	2 × C=C	3 × C=C	4 × C=C	5+6 × C=C
12:0	0.15	0.13	0.02	0	0	0	0	0	0
13:0	0.10	0.09	0.01	0	0	0	0	0	0
i-14:0	0.14	0.01	0.11	0.02	0	0	0	0	0
14:0	4.18	3.61	0.56	0.01	0	0	0	0	0
br-13:0	0.09	0	0.01	0.08	0	0	0	0	0
i-15:0	0.75	0.08	0.56	0.11	0	0	0	0	0
ai-15:0	1.47	0.16	1.09	0.22	0	0	0	0	0
15:0	0.58	0.49	0.09	0	0	0	0	0	0
9-15:1	0.92	0	0	0	0.92	0	0	0	0
i-16:0	1.12	0.13	0.83	0.16	0	0	0	0	0
br-15:0	0.28	0.01	0.03	0.24	0	0	0	0	0
16:0	12.39	10.39	1.98	0.02	0	0	0	0	0
7-16:1	0.56	0	0	0	0.56	0	0	0	0
9-16:1	8.71	0	0	0	8.70	0.01	0	0	0
11-16:1	0.34	0	0	0	0.34	0	0	0	0
6,9-16:2	0.15	0	0	0	0	0.15	0	0	0
9,12-16:2	0.21	0	0	0	0	0.21	0	0	0
i-17:0	0.47	0.05	0.35	0.07	0	0	0	0	0
ai-17:0	0.51	0.06	0.38	0.07	0	0	0	0	0
br-16:0	0.25	0.01	0.02	0.22	0	0	0	0	0
17:0	0.18	0.15	0.03	0	0	0	0	0	0
4,7,10-16:3	0.12	0	0	0	0	0	0.12	0	0
6,9,12-16:3	0.34	0	0	0	0	0	0.34	0	0
9-17:1	0.42	0	0	0	0.42	0	0	0	0
4,7,10,13-16:4	0.08	0	0	0	0	0	0	0.08	0
6,9,12,15-16:4	0.26	0	0	0	0	0	0	0.26	0
i-18:0	0.01	0	0.01	0	0	0	0	0	0
18:0	3.15	2.64	0.50	0.01	0	0	0	0	0
9-18:1	15.24	0	0	0	15.22	0.02	0	0	0
11-18:1	3.12	0	0	0	3.09	0	0	0	0
13-18:1	1.42	0	0	0	1.42	0	0	0	0
9,12-18:2	3.17	0	0	0	0	3.17	0	0	0
19:0	0.09	0.07	0.02	0	0	0	0	0	0
6,9,12,-18:3	0.35	0	0	0	0	0	0.35	0	0
10-19:1	0.03	0	0	0	0.03	0	0	0	0
9,12,15-18:3	1.45	0	0	0	0	0	1.45	0	0
6,9,12,15-18:4	1.47	0	0	0	0	0	0	1.45	0.02
20:0	0.24	0.21	0.03	0	0	0	0	0	0
9-20:1	1.96	0	0	0	1.96	0	0	0	0
11-20:1	4.83	0	0	0	4.82	0.01	0	0	0
13-20:1	0.42	0	0	0	0.42	0	0	0	0
11,14-20:2	0.53	0	0	0	0	0.53	0	0	0
21:0	0.07	0.06	0.01	0	0	0	0	0	0
8,11,14-20:3	0.18	0	0	0	0	0	0.18	0	0
5,8,11,14-20:4	0.83	0	0	0	0	0	0	0.82	0.01
11,14,17-20:3	0.12	0	0	0	0	0	0.12	0	0
8,11,14,17-20:4	0.47	0	0	0	0	0	0	0.46	0.01
5,8,11,14,17-20:5	7.50	0	0	0	0	0	0	0.11	7.39

Table 2 continued.

Fatty acid[a]	Fractions								
	Total	n[b]	i-ai[b]	m-br[c]	1×C=C	2×C=C	3×C=C	4×C=C	5+6×C=C
22:0	0.19	0.16	0.03	0	0	0	0	0	0
9-22:1	2.11	0	0	0	2.11	0	0	0	0
11-22:1	4.21	0	0	0	4.21	0	0	0	0
13-22:1	2.47	0	0	0	2.47	0	0	0	0
4,7,10,13,16-21:5	0.18	0	0	0	0	0	0	0	0.18
7,10,13,16-22:4	0.15	0	0	0	0	0	0	0.15	0
4,7,10,13,16-22:5	0.68	0	0	0	0	0	0	0.01	0.67
7,10,13,16,19-22:5	2.14	0	0	0	0	0	0	0.03	2.11
24:0	0.17	0.14	0.03	0	0	0	0	0	0
15-24:1	0.37	0	0	0	0.37	0	0	0	0
4,7,10,13,16,19-22:6	5.91	0	0	0	0	0	0	0.08	5.83

[a] First number, number of carbon atoms in the chain; second number, number of double bonds; number(s) before the hyphen, position(s) of double bond(s).

[b] n = normal chain; i = isoacid; ai = anteisoacid.

[c] m-br = multi-branched acid.

3. Results and discussion

Table 1 shows the results of the separation of pure standards, a model mixture commercially available. As seen in Fig. 1, excellent separation of the individual lipid classes was obtained. During development in the first dimension on a layer of silica gel impregnated with urea, saturated FAMEs were separated to obtain the unsubstituted, monosubstituted and polysubstituted esters. After the plate was dried and developed in the second dimension, the FAMEs differing in the number of double bonds were separated.

The separation of the individual fractions is shown in Fig. 1 and the results are summarized in Table 1. The two-dimensional chromatography enabled us to separate all eight FAMEs. The separation of branched FAMEs was incomplete only when silica gel–urea was used. In spite of that, however, this method yielded fractions enriched with the corresponding structural type up to 90% of the total. This amount by far exceeded the yields obtained after crystallization in the presence of urea when, in contrast, polyenoic acids were contaminated even with branched FAMEs. In order to separate them, another separation method had to be used.

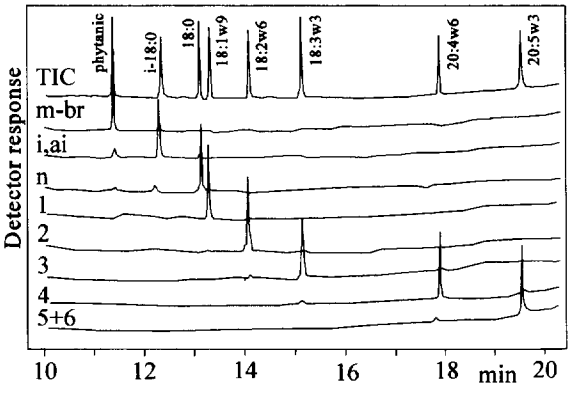

Fig. 1. GC–MS of total FAME standards and single fractions after TLC; for conditions see Section 2.3.

Using Ag–TLC, five fractions are usually obtained in separations of fatty acid methyl esters with 0–4 double bonds [8]. It is reportedly possible to separate methyl esters with zero to six double bonds on an Ag–TLC plate with a double development, provided that the relative humidity is maintained below 50% [9]. By contrast most workers have preferred to separate methyl esters with zero to three double bonds on one plate, and those with four to six double bonds on another. With minor difficulties it is possible to obtain separation from zero to four double bonds, but separation of methyl esters with five and six double bonds on the same plate is impossible. In all cases of simple development, the analyst must choose between two problems that need to be solved. Firstly separation is not obtained for methyl esters with zero and one double bonds (all others are separated) [10], or secondly, the mixture is

separated except for methyl esters with five and six double bonds, and it is this case.

Many compounds can produce inclusion compounds but the most important in analytical chemistry are urea and cyclodextrins. To our great surprise only one paper on the use of urea in TLC was published [2]. A literature search of Chemical Abstracts (1983–1994) has shown that only thirteen papers using chromatography of fatty acid derivatives in the presence of cyclodextrin were published but there is not any paper which describes the application of cyclodextrins for separation of fatty acids according to their chemical structure, using TLC. The paper of Mack and Hauck [11] describes the chiral separation of hydroxy acids. In contrast, many hundreds of applications are described in gas or liquid chromatography.

The above methods can be successfully used for separations of small amounts of complex mixtures into the individual fractions, which was verified with a more complex mixture.

The above-mentioned method was employed to separate a natural FAME mixture (Table 2). The mixture was obtained from cod liver oil, known for the complex nature of the fatty acids present. Up to about sixty identified FAMEs have been described to be present in this material [12].

In our case, to reach an even higher complexity of the analyzed mixture (especially with respect to the monobranched acids present), an addition of FAMEs from *Streptomyces avermitilis* was used. Streptomycetes are well known for their production of iso acids (i) and anteiso acids (ai) [13]. The mixture created by mixing the two completely different FAME types exhibited an unusual complexity, showing the presence of almost sixty FAMEs.

The results from Table 2 show a successful application of the above-mentioned method. The chromatography of methyl esters by urea showed that separation between normal, monobranched (iso or anteiso) and polybranched compounds was excellent. The iso-16:0 was obtained in all three fractions, but in the parent fraction the amount was five times higher than in the other fractions (straight chain and/or polybranched). Also the longer homolog, e.g., ai-17:0 was obtained in all three fractions but with a different amount. The normal-chain (n) fraction as well as the multi-branched acid (m-br) fraction contained ca. one sixth but a principal amount was in the i-ai fraction.

On the other hand the separation with Ag–TLC was excellent. The separation of oleic acid (as methyl ester) from the other acids, i.e. saturated or dienoic acids, showed that only 0.13% of this acid was obtained from the dienoic fraction and none from the saturated fraction. The separation of the final fractions, i.e. fractions with four, five or six double bonds, was also good. The methyl ester of 4,7,10,13,16,19-22:6 acid was divided in the ratio $\approx$1:100 between fraction 4 (4 $\times$ double bond) and fraction 5+6 (5 and/or 6 double bonds).

The main contribution of the above-mentioned method is the possibility of semipreparation of the individual fractions of fatty acids, even when the initial amount of the compound is in the range of only micrograms. This situation occurs frequently, especially when FAMEs from not very common animals or plants are being analyzed. It was the reason why we sought to develop the method and use it to analyze such sources of FAMEs.

The only limitation of urea–TLC involves the chain length of the FAMEs used. Crystallographic data suggest that the C_8 and shorter acids do not form inclusion compounds [14]. The limitation for short-chain acids is not important since the shortest fatty acid chains in most natural mixtures contain 12–14 carbon atoms. A more serious limitation is the possibility of poor separation of esters longer than C_{18}. These FAMEs, reportedly, do not form adducts with urea. As shown in the paper of Tiffany [2] and also in our work, those long-chain FAMEs can be separated without problems.

References

[1] R.J. Hamilton and S. Hamilton, Lipid Analysis, A Practical Approach, IRL Press, Oxford, 1992.
[2] J.M. Tiffany, J. Chromatogr., 243 (1982) 329.
[3] M. Kates, Techniques of Lipidology, Elsevier, Amsterdam, 1991.
[4] T. Řezanka, H. Míková and M. Jurková, FEMS Microbiol. Lett., 96 (1992) 1.
[5] W.W. Christie, Lipid Analysis, Pergamon Press Oxford, 1982.
[6] Q.T. Yu, B.N. Liu, J.Y. Zhang and Z.H. Huang, Lipids, 23 (1988) 804.

[7] T. Řezanka, I.V. Zlatkin, I. Viden, O.I. Slabova and D.I. Nikitin, J. Chromatogr., 558 (1991) 215.
[8] W.W. Christie, Gas Chromatography and Lipids, The Oily Press, Ayr, Scotland, 1989.
[9] P.A. Dudley and R.E. Anderson, Lipids, 10 (1975) 113.
[10] R. Wilson and J.R. Sargent, J. Chromatogr., 623 (1992) 403.
[11] M. Mack and H.E. Hauck, Chromatographia, 26 (1988) 197.
[12] R.G. Ackman, J.C. Sipos and P.M. Jangaard, Lipids, 2 (1967) 251.
[13] A. Ballio and S. Barcellona, Ann. Inst. Past. Microbiol., 114 (1968) 121.
[14] E.V. Truter, J. Chem. Soc., (1951) 2416.