Scientific Papers. Series D. Animal Science. Vol. LXV, No. 2, 2022
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

QUANTITATIVE AND SIMULTANEOUS GAS CHROMATOGRAPHIC DETERMINATION OF VARIOUS FORMS OF LONG-CHAIN FATTY ACIDS IN BIOLOGICAL MATERIAL

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Abstract

The literature describes non-quantitative gas chromatographic methods for determining the relative content of various forms of long chain fatty acids in the biological material under study. Our task was to improve the quantitative and simultaneous gas chromatographic method for determining the absolute content of various forms of long-chain fatty acids in the biological material under study (plant and animal tissues and liquids). To do this, the studied biological material, a simple and complex internal standard is treated with various extracting mixtures. Various forms of long chain fatty acids are isolated from extracted lipids and their methyl esters are chromatographed. The results of gas chromatographic studies are calibrated. The improved method for low error allows quantitatively and simultaneously in absolute units to determine the content of total long chain fatty acids, long chain fatty acids of common lipids, esterified, non-esterified and anionic long chain fatty acids in the studied biological material. Thus, using the improved gas-chromatographic method at a lower cost of reagents and time, quantitatively and simultaneously in the biological material under study in absolute units we determine the content of various forms of long chain fatty acids.

Key words*: accurate, biological material, definitions, gas chromatographic method, long chain fatty acids.*

INTRODUCTION

The literature describes gas chromatographic methods for determining the content of various forms of long-chain fatty acids in the biological material under study: total fatty acids (Brian & Gardner, 1991; Rivis & Danylic, 1995; Rivis et al., 1996a; Chen & Chuang, 2002), fatty acids of general lipids (Panagiotopoulou & Tsimidou, 2002), esterified (Ostermann et al., 2014; Liguon et al., 2015; Miwa, 2002; Weatherly et al., 2016), non-esterified (Miwa, 2002; Rivis & Danylic, 1997a) or anionic (Rivis et al., 1996b) fatty acids. Moreover, the existing methods are not quantitative (calibrated) (Ostermann et al., 2014; Liguon et al., 2015; Miwa, 2002) and give the results of studies in relative units (percentages) (Miwa, 2002; Rivis & Danylic, 1997b). It should be noted that in the literature there are limited data regarding quantitative and simultaneous gas chromatographic determination of the absolute content of different forms of individual long-chain fatty acids in biological material (Rivis et al., 1997a). Such gas chromatographic studies are necessary with the aim to obtain objective quantitative data and significant savings of reagents and time.

Based on the above, we have been set a task to improve the quantitative and simultaneous gas chromatographic method for determining the absolute content of total long-chain fatty acids, long-chain fatty acids of total lipids, esterified, non-esterified and anionic long-chain fatty acids in studied biological material (plant and animal tissues and liquids).

MATERIALS AND METHODS

For the analysis, 1.0-2.0 g or ml of plant tissues or liquids, 0.5-1.0 g or ml of tissues or liquids of the animal body, 0.08-1.00 g of microor-

ganisms were used. The amount of methyl ester of heptadecanoic acid (methylheptadecanoate), which is added to the sample as an internal standard, is (% of sample taken): to the liver of various species of animals, birds and fish - 0.39, to the mucous membrane of the wall of adult females - 0.145, to the mucous membrane of the small intestine of animals and birds - 0.066, to plasma or serum of animals, birds and fish - 0.027, to cow's milk - 0.645, to the rumen fluid of ruminants - 0.008, to insect tissues - 0.09, to ruminant rumen microorganisms - 0.116, to feces of livestock and poultry - 0.065 to natural fodder of ponds (zoobenthos, zooplankton, phytoplankton or higher aquatic vegetation) - 0.0095 to cereals and legumes or grains - 0.028.

Methylheptadecanoate is prepared as follows. The amount of heptadecanoate required for the tests introduced into a test tube (250 × 6 mm) with a ground. Then to the content of the test tube pour 8-10 drops of absolute methyl alcohol. Subsequently, 4-5 drops of acetyl chloride are poured into it. The latter is poured very carefully until a distinctive sound is heard from the test tubes. The tube is then quickly closed with a ground glass stopper and placed in a converted thermostat. In the latter, the upper, larger part of the test tube is cooled by flowing water from the water supply system for 1.5 hours and the lower, smaller part is in a water bath (65 C). After methylation is completed, the methanol from the test tube is evaporated in a vacuum cabinet at 45-50ºC. The obtained methylheptadecanoate is dissolved in a certain amount of hexane and used for research.

The amount of unesterified form of heptadecanoate, which is also added to the test sample as an internal standard, is (% of sample taken): 0.01 to the liver of different species of animals, birds and fish, 0.005 to the mucous membrane of the wall of mature females of animals, to the mucosa membrane of the small intestine of animals and poultry - 0.004, to plasma or serum of animals, poultry and fish - 0.003, to cow's milk - 0.005, to rumen fluid of ruminants - 0.004, to insect tissues - 0.01, to ruminant rumen microorganisms - 0.004, to feces of animals and birds - 0.005, to natural forage ponds (zoobenthos, zooplankton, phytoplankton or higher aquatic vegetation) - 0.0005, to cereals and legumes or cereals - 0.002.

The absolute content of total long-chain fatty acids, long-chain fatty acids of total lipids, esterified, non-esterified, and anionic long-chain fatty acids in the tissues of the honey bee (*Apis mellifera*) was determined quantitatively and simultaneously in three replicates. For this purpose, in the first 30 ml by volume, a vial of colored glass is placed 1,0 g of tissues of the abdomen and a simple internal standard (1.0 mg of heptadecanoate), and in the second - 1.0 g of tissues and a complex internal standard (mixture 0.95 mg methylheptadecanoate with 0.05 mg heptadecanoate). Next, 20 ml of chloroform-methanol-hydrochloric acid extraction mixture (200:100:1 by volume) poured into the contents of the first vial and 20 ml of chloroform-methanol extraction mixture (2:1 by volume) is added to the second vial. The vials are closed with plastic corks and placed on a shuttle apparatus, on which they will be shaken vigorously for 2 hours. After the extraction is completed, 7 ml of distilled water are poured into both vials. Subsequently, after delamination of the content of the bottles, the top water-methanol layers are selected and discarded by a water jet pump and a glass pipette with the spout is removed, and the lower (chloroform) pipette is transferred to a paper filter with a blue filter (180 × 13 mm). Moreover, the chloroform extract from the first bottle is filtered into one tube, and from the second into two tubes (in one of them only 1/10 of the total amount of chloroform extract is filtered). The chloroform of the above tubes is evaporated in a stream of dried nitrogen in a water bath or a vacuum cabinet (37-40ºC).

Then, 4 to 5 drops of 5% alcohol (methyl) sodium metal solution are added to the tube containing the first bottle extract and to the tube containing the smaller quantity of the extract from the second bottle. Test tubes are placed in a boiling water bath. After 30 seconds from the beginning of boiling 1.5 ml of distilled water are poured to the contained test tube`s content. Boiling continues until the smell of alcohol disappears (approximately 30 minutes). After boiling, the test tubes in a tripod are cooled in running water from the water supply system.

To convert the resulting saponification sodium salts of long-chain fatty acids to the free state, 5-7 drops of glacial acetic acid are added to the content of tubes. After that, free long-chain fatty acids are extracted with hexane (2 times in 1-2 ml). The hexane solution of free long-chain fatty acids is transferred automatically with the pipette into test tubes (250 × 6 mm) with a ground.

Hexane from the above tubes is evaporated in a stream of dried nitrogen in a water bath or a vacuum cabinet (37-40ºC), and 6-8 drops of absolute methyl alcohol and 2-3 drops of acetyl chloride are poured into their content. The tubes are quickly closed with a ground glass stopper and placed in a converted ultra thermostat, in which their upper, larger, part is cooled by running water from the water main, and the lower, smaller part is in a water bath (65ºC) for 1.5 hours.

After the methylation is completed, the methanol from the test tubes is evaporated in a vacuum cabinet at a temperature of 45-50ºC. Then, the methyl esters of the long-chain fatty acids are dissolved in test tubes in a few drops of hexane. Afterward, using a microsyringe, 1 ml of hexane methyl esters of long-chain fatty acids are alternately selected from each tube and injected into the evaporator of the gas-liquid chromatographic apparatus. The chromatograms yield fatty acid peaks according to the total amount of long-chain fatty acids (esterified, non-esterified, and anionic forms of long-chain fatty acids) and long-chain fatty acids of general lipids (esterified and non-esterified long-chain acids). The difference in the content of long-chain fatty acids between the two test tubes is formed by the anionic forms of long-chain fatty acids.

The lipids in the second tube, which had a larger amount of extract from the second vial, were dissolved in 2 ml of hexane. Next, 5-6 drops of 5% alcohol (methyl) sodium metal solution are added to the content of this tube. The tubes will then be shaken vigorously for 2 minutes. After a few minutes, it is necessary to stratify the content of the tube, using a glass pipette with a stretched spout, select the upper hexane layer and transfer it to a glass tube (50 × 5 mm) with a conical bottom. Hexane from this tube is evaporated in a stream of dried nitrogen in a water bath or a vacuum cabinet (37-40ºC) to several drops. Next, using a syringe, 1 ml of the hexane solution of pure methyl esters of long-chain fatty acids from this tube is introduced into the evaporator of a gas-liquid chromatographic apparatus. The chromatogram gives peaks of long-chain forms of fatty acids that have been esterified in phospholipids, esterified cholesterol (phytosterol), triacylglycerols, diacylglycerols and monoacylglycerols.

Next, add 2 ml of hexane to the content of the tube containing the larger extract from the second vial. After that, the test tube is shaken vigorously for 2 minutes. Subsequently, the upper hexane layer is removed and discarded after separation of the tube contents, with the aid of a water jet pump and a glass pipette with an extended nose. It is repeated another three times.

After that the contents of the above tubes is dissolved in 2 ml of hexane. Then we add 5-7 drops of glacial acetic acid to the test tube. Next, the test tube is shaken vigorously for 2 minutes. Subsequently, after separation of the content of the tube, using a glass pipette with a stretched spout, the upper hexane layer is removed and transferred to a tube (250 × 6 mm) with a ground. Hexane from this tube is evaporated in a stream of dried nitrogen in a water bath or a vacuum cabinet (37-40ºC). Then, 8-10 drops of absolute methyl alcohol are poured into the content of the tube with the ground. After that, 4-5 drops of acetyl chloride are poured into it. The test tube is then quickly closed with a ground glass stopper and placed in a converted thermostat. In the latter, the upper, larger portion of the test tube is cooled with running water from the water pipe for 1.5 hours, and the lower, smaller part is in a water bath (65ºC). After methylation is completed, the methanol from the test tube is evaporated in a vacuum cabinet at 45-50ºC. The methyl esters of the obtained long-chain fatty acids are dissolved in a few drops of hexane. Next, using a syringe, 1 ml of the hexane solution of pure methyl esters of long-chain fatty acids is introduced into the evaporatorof the gas-liquid chromatographic apparatus. On the chromatogram peaks of unesterified forms of long-chain fatty acids are obtained.

We determined among the total long-chain fatty acids, long-chain fatty acids of general

lipids, esterified, non-esterified and anionic forms of long-chain fatty acids in the tissues of the honey bee belly, quantitatively in absolute units, the contents of saturated fat with pair (caprylic − 8:0, capric − 10:0, lauric − 12:0, myristic − 14:0, palmitic − 16:0, stearic − 18:0, arachic − 20:0) and an odd (pentadecanoic − 15:0) number of carbon atoms in the chain, monounsaturated fatty acids of families ω − 7 (palmitooleic − 16:1) and ω − 9 (oleic − 18:1, eicosaenoic − 20:1) and polyunsaturated fatty acids of families ω − 3 (linolenic − 18:3, eicosapentaenoic − 20:5, docosatriene − 22:3, docosapentaenoic − 22:5, docosahexaenoic − 22:6) and ω − 6 (linoleic − 18:2, eicosadiene − 20:2, eicosatriene − 20:3, eicosatetraene-arachidone − 20:4, docosadiene − 22:2, docosatetraene − 22:4).

A gas-liquid chromatographic apparatus "Chrom-5" (Laboratorni pristroye, Praha), which has a stainless steel column with a length of 3700 mm and an internal diameter of 3 mm, was used for the study of fatty acid methyl esters. The column was filled with Chromaton-N-AW, 60-80 mesh grain, silaneised HMDS (hexamethyldisilysane), coated with polydiethylene glycoladipinate (fixed liquid phase) in an amount of 10% ("LACHEMA", Praha). The consumption of carrier gas, chemically pure and dried nitrogen (mobile phase) through the column at an inlet pressure of $1{,}5x10^5$ PA was about 65 ml/min. The combustion of the flames was provided with hydrogen (25 ml/min) and air (380 ml/min). The isothermal mode of operation of the packed column with the polar liquid phase was kept at the level of 196ºC, and of the evaporator and the detector − 245ºC. The detector was flame ionized. The record of the results of the analysis is differential. The identification of peaks on the chromatogram was also performed by the method of calculation of "Carbon atoms", well as using chemically pure, standard, hexane solutions of long-chain fatty acid methyl esters.

To obtain quantitative data, the results of gas chromatographic studies are calibrated. Moreover, the calibration of the results of gas chromatographic studies is carried out by the method of internal normalization. The norm (unit) is accepted as peak parameters of a simple (heptadecanoate) or complex (a mixture of heptadecanoate with methylheptadecanoate) internal standard. To this norm, the parameters of the peaks of the investigated fatty acids are equated.

For calibration of the results of gas chromatographic studies in test tubes (250 × 6 mm) we prepared mixtures of chemically pure heptadecanoic and investigated fatty acids in mass ratios of 1:1, 1:2, 1:4, 1:8, 8:1, 4:1 and 2:1. Next such an amount of absolute methyl alcohol is poured into these tubes which covers the fatty acids present in them. Then, acetyl chloride is poured into the content of the tubes. Immediately thereafter, the tubes are closed with ground glass stoppers and placed in a converted thermostat. In the latter, the upper, larger part of the test tubes is cooled with running water from the water tubes for 1.5 hours, and the lower, smaller one is in a water bath (65ºC). After completing of the methylation, the methanol from the tubes is evaporated in a vacuum cabinet at the temperature of 45-50ºC. Further, the methyl esters of the long-chain fatty acids under study are dissolved in a small amount of hexane. Then, using a microsyringe, 1 ml of a hexane solution of pure methyl esters of long-chain fatty acids from each tube is introduced into the evaporator of the gas-liquid chromatographic apparatus. On the chromatograms, the peaks of the long-chain fatty acids under study are obtained and their parameters are measured. Then the peak parameters of the heptadecanoic acid (internal standard) are equated with the peak parameters of the fatty acids under study. The results of the comparisons are converted to a ratio of 1:1. As a result of such calibration, numerical values are obtained, which are the correction coefficients for each fatty acid under study.

The absolute content of individual long-chain fatty acids in the tested biological material is determined by the following formula:

$$X, \text{ g/kg or l} = [(P \times K \times C) / Pst] \times 1000 / P$$

where:

X - quantitative content of the investigated long-chain fatty acid in absolute units, g/kg or l;

P - peak parameters of the investigated long-chain fatty acid, mm^2 or mm;

K - correction factor for the investigated long-chain fatty acid;

C - the amount of internal standard added (heptadecanoate), mg;
P_{st} - internal standard (heptadecanoate) peak parameters, mm^2 or mm;
1000 - conversion factor in kg or l;
P - a sample of the tested biological material, g or ml.
Based on the above formula for each chromatogram, first of all, it was determined the exact peak parameters of the studied long-chain fatty acids, mm^2 or mm:

$$P_1 = P \times K$$

Next, we determined the conversion factor in g/kg or l:

$$K_1 = 1000/P$$

Then, we calculated the amount of internal standard (heptadecanoate), which should be in kg or l of the biological material under study:

$$C_2, \text{ mg} = K_1 \times C$$

Then, we determined the conversion factor of the exact peak parameters of the studied individual long-chain fatty acids in absolute units of g/kg or l:

$$K_2 = C_2/P_{st}$$

Finally, the absolute content of individual long-chain fatty acids is calculated in the studied biological material:

$$X, \text{ g/kg or l} = K_2 \times P_1$$

The obtained digital material was processed by the method of variational statistics (Lopach et al., 2011). We calculated the arithmetic mean (M), and error of the arithmetic mean (± m). For the calculations a special computer program was used, Microsoft Exel for Windows XP.

RESULTS AND DISCUSSIONS

The results of studies of the absolute content of total long-chain fatty acids, long-chain fatty acids of total lipids, esterified, non-esterified and anionic long-chain fatty acids in the tissues of the honey bee are shown in the Table 1. The data of the Table 1 indicate that the error of the advanced gas chromatographic method of quantitative and simultaneous determination of the content of different forms of long-chain fatty acids in the studied biological material does not exceed 1%.
It should be noted that the absolute results of the research obtained by the advanced method can easily be translated into relative quantitative indicators. They can also be easily converted to molar units.

Table 1. The content of various forms of long-chain fatty acids in the tissues of the abdomen of the honey bee, g • 10^{-3}/kg wet weight, M ± m, n = 3

Long-chain fatty acids and their code	Forms of long-chain fatty acids				
	Esterified, non-esterified and anionic fatty acids in the total amount of fatty acids	Esterified and non-esterified fatty acids in the composition of total lipids	The esterified fatty acids	Non-esterified fatty acids	Anionic fatty acids
Caprylic, 8:0	67.2±0.53	64.3±0.53	58.3±0.51	6.1±0.05	3.0±0.02
Capric, 10:0	21.7±0.19	20.6±0.16	18.3±0.12	2.2±0.01	1.2±0.01
Lauric, 12:0	23.9±0.18	22.6±0.18	20.4±0.16	2.3±0.02	1.2±0.01
Myristic, 14:0	31.3±0.22	29.9±0.21	27.0±0.20	4.1±0.03	1.7±0.01
Pentadecanoic, 15:0	67.2±0.59	64.5±0.56	54.7±0.49	9.5±0.07	2.9±0.02
Palmitic, 16:0	1353.5±10.22	1293.3±9.97	1176.1±9.80	114.3±0.95	62.2±0.49
Palmitooleic, 16:1	94.0±0.77	89.3±0.69	81.8±0.68	7.8±0.06	4.4±0.04
Stearic, 18:0	1004.2±8.33	954.8±8.90	877.5±8.17	80.5±0.67	46.2±0.39
Oleic, 18:1	4026.0±32.83	3852.4±31.58	3534.1±29.92	311.2±2.76	188.6±1.12
Linoleic, 18:2	3139.2±26.38	2949.7±24.03	2705.6±22.54	250.5±2.04	184.5±1.34
Linolenic, 18:3	4217.9±36.04	4028.2±34.52	3716.4±29.78	301.4±2.57	198.7±1.55
Arachic, 20:0	125.4±1.00	118.6±0.90	109.7±0.90	9.3±0.07	6.2±0.05
Eicosaenoic, 20:1	311.3±2.19	296.4±2.16	271.5±2.14	23.8±0.19	15.5±0.11
Eicosadiene, 20:2	357.8±3.05	340.2±3.10	315.0±2.84	26.7±0.21	16.8±0.12
Eicosatriene, 20:3	197.1±1.54	179.9±1.37	165.1±1.35	14.2±0.10	17.5±0.14
Eicosatetraene-arachidone, 20:4	3370.8±26.98	3198.0±26.07	3006.3±26.02	202.1±1.78	162.5±1.27
Eicosapentaenoic, 20:5	2502.3±19.78	2395.4±18.95	2238.1±18.88	150.6±1.17	111.9±0.96

Docosadiene, 22:2	335.4±2.98	319.6±2.87	298.7±2.27	22.1±0.16	16.4±0.12
Docosatriene, 22:3	336.8±2.77	320.2±2.80	298.0±2.42	21.1±0.15	15.8±0.13
Docosatetraene, 22:4	387.3±3.21	369.4±3.12	348.8±3.05	21.2±0.16	18.8±0.15
Docosapentaenoic, 22:5	588.9±5.17	558.7±5.18	527.5±4.90	33.4±0.28	28.8±0.21
Docosahexaenoic, 22:6	668.4±6.22	641.3±5.96	601.6±5.17	37.1±0.30	30.5±0.24

CONCLUSIONS

The improved gas chromatographic method at lower costs of reagents and time allows quantitatively and simultaneously to determine the content of common long-chain fatty acids, long-chain fatty acids of common aliphatic alkaline chains acids in the studied biological material (vegetable and animal tissues and liquids) in absolute units. The error of the advanced gas chromatographic method of quantitative and simultaneous determination of different forms of long-chain fatty acids in the biological material under study does not exceed 1%.

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