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EVALUATION AND IMPROVEMENT OF TRANSESTERIFICATION METHODS OF TRIGLYCERIDES

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ABSTRACT

The causes responsible for the low reproducibility of the gas chromatographic method used to analyze fatty acid methyl esters, derived from the transesterification of triglycerides in n-hexane by means of methanol potassium hydroxide, were identified and eliminated.

Partition coefficients (Kd) of short-chain fatty acid methyl esters between n-hexane and methanol were determined. These results indicate that even under the best experimental conditions, a substantial portion of these compounds are transferred in the methanol phase and therefore are not analyzed. Accurate results, to within 2%, were obtained by rigorously controlling the volume of the reagents and introducing appropriate correction factors.

The accuracy of the results, reported as a percentage by weight, did not depend on the completeness of the transesterification reaction.

In the analysis of fatty acids as butyl esters, a measurable quantity of butyric ester was lost during the washing phase with water. By contract, butyric acid pentyl ester is insoluble in water. Therefore we propose an accurate method to analyze fatty acids as pentyl esters .

Oleic and elaidinic acids derived from triglyceride transesterification are easier to separate as methyl esters than as longer butyl or pentyl esters.

INTRODUCTION

The characterization of fatty material is carried out by a determination of both triglycerides and fatty acids. In particular, the determination of the composition of fatty acids clarifies the effect of some diets for animals[1]; the determination of elaidic acid reveals if fats have been treated with decolorizing earth [2].

The determination of butyric acid is of particular importance, for the detection of the quantity of butter mixed with vegetable fats[3]; butyric acid is present in butter, but not in vegetable fats. Generally, fatty acids are analyzed as esters using gas chromatography (GC). This is the most common procedure, because of its simplicity and speed, in the formation of esters via triglycerides transesterification. The method proposed by Christopherson and Glass[4], with slight modifications, allows one to obtain methyl, ethyl, propyl and butyl esters [5]. Unfortunately, according to this technique the analysis of short-chain fatty acids, in particular C4, as methyl esters , does not lead to satisfactory results[5]. The basic technique of Christopherson and Glass is based on the addition of a solution of NaOH or KOH in methanol to a solution of triglycerides in n-hexane and then by the GC analysis of the methyl esters contained in the hexane phase. The low reproducibility is due to both the volatility of methyl butyrate and its solubility in methanol[5]. In the same study Iverson concludes that the analysis of fatty acids as butyl esters confers more accurate results; butyl esters are less volatile and less

soluble in the aqueous phase. In our opinion, the volatility of short-chain methyl esters (C4-C6-C8) cannot be the cause of the low accuracy of the analytical results; to begin with, methyl butyrate has a boiling point above that of water, which is well known not to cause problems of volatility under ordinary lab conditions. In addition, after transesterification, methyl butyrate becomes diluted in n-hexane, so its vapor pressure becomes practically negligible due to Raoult's law. Experiments carried out in solutions of methyl butyrate in n-hexane (2 mg/ml) with n-undecane as the internal standard demonstrated that at a maximum of 25°C and for at least four hours, the concentration of the solution remains constant. As for solubility, short-chain methyl esters are soluble both in n-hexane as well as in methanol. Their solubility in methanol could be the cause of error only if, after the reaction, the esters are not given enough time to reach an equilibrium between the methanol and hexane phases. In any case, once equilibrium is reached, an appropriate correction factor can take into account the quantity of esters that dissolve in the methanol phase and therefore are not analysed. After transesterification of a hexane solution of butter, we looked for free fatty acids in the methanol phase using ion chromatography; the absence of these acids indicates slight saponification reactions that could contribute to making the method less reproducible. The scope of the present study is to identify the causes that reduce the accuracy of the results regarding short-chain fatty acid methyl esters and thus to propose more accurate procedures.

Samples of fat were analyzed as methyl esters and as esters of higher molecular weight; the results were compared, after standardization, as free fatty acids.

EXPERIMENTAL

Chemicals

n-hexane, analytical grade (Lab-Scan, Dublin, Ireland); methanol, analytical grade (Lab-Scan, Dublin, Ireland); butanol, analytical grade (Fluka Chemie, Switzerland); 1-pentanol, puriss. plus (Fluka Chemie, Switzerland)

Instruments

DANI gas chromatograph, model 8610-HT, equipped with autosampler, PTV injector and FID detector (DANI, Milan, Italy); HP electronic integrator, model 3394, (Hewlett-Packard, Palo Alto, California, U.S.A.); RTX-2330, 90% biscyanopropyl-10% cyanopropylphenyl polysiloxane capillary column, l=60 meters, i.d.=0.25 mm; f.t.=0.1 m (Restek Corporation, Bellefonte, U.S.A.)

Gas chromatographic conditions

Carrier gas: helium, flow rate: 1.5 ml/min.; oven 50 °C for 2 min., increasing 8° C/min. to a final temperature of 250 °C, for 7 min.; PTV injector: 50 °C for 10 sec., increasing 90 °C/min. to a final temperature of 270 °C, for 3 min.; split: 1:80; FID temperature: 270 °C

Trans-esterification reagents

The 2 M solutions of sodium butoxide (reagent A) in n-butanol and sodium pentoxide in n-pentanol (reagent B) were prepared following the method used by Zaugg [6] for the preparation of sodium ethoxide.

A 2 M solution of sodium hydroxide in methanol was prepared by dissolving 8.0 g sodium hydroxide in 100 ml anhydrous methanol (reagent C).

PROCEDURE

Determination of the partition coefficient (K) between n-hexane and methanol for methyl esters of short-chain fatty acids, without matrix

In a 20 ml volumetric flask weigh 100 mg each of methyl butanoate, methyl hexanoate and methyl octanoate, dissolve each with n-hexane saturated with methanol at 20°C (solution A contains a concentration of 5 mg/ ml of each ester).

In three 10 ml volumetric flasks, add 2.00, 4.00 and 6.00 ml of solution A and dilute them to the mark with n-hexane saturated with methanol; the solutions should have a concentration of 1, 2 and 3 mg/ml of each ester (L solutions).

The three solutions were analysed five times each by GC, and the average should have a concentration of 1, 2 and 3 mg/ml of each ester (L solutions).

The three solutions were analysed five times each by GC, and the average peak area (arbitrary unit) of the three esters for each of the three solutions was calculated as follows: in three centrifuge tubes, 5.00 ml of each of the three L solutions was placed and 5.00 ml of methanol saturated with n-hexane added. The tubes were stirred for two minutes and then centrifuged for two minutes at 2000 rpm. The hexane phase of each tube was analyzed five times by GC and the average peak areas for each of the esters was determined.

Calculation:

$$K = \frac{C}{D-C}$$

where :

C is the average of the five peak areas, for each ester, relative to the five analyses of the hexane L solutions, agitated with methanol;

D is the average of the five peak areas, for each ester, relative to the five analyses of the hexane L solutions, not agitated with methanol.

Determination of the partition coefficient (K) for the methyl esters in the presence of matrix

The three L solutions were analyzed five times by GC. In two 10ml volumetric flasks, weigh 250 mg and 350 mg of olive oil, add 2.00 ml and 4.00 ml, respectively, of solution A and dilute to the mark with n-hexane saturated with methanol (M solutions). In two centrifuge tubes, add 5.00 ml of each of the M solutions and 1.00 ml of transesterification reagent C. Agitate in a stirrer for two minutes and centrifuge at 2000 rpm for two minutes. Analyze by GC the n-hexane phase of the three M solutions five times.

Calculation:

$$K = \frac{C}{5*(D-C)}$$

where:

C is the average of the five peak areas, for each ester, relative to the five analyses of the M hexane solutions, after transesterification;

D is the average of the five peak areas, for each ester, relative to the five analyses of the L hexane solutions.

Determination of the partition coefficient (K) between n-hexane and water for butyl and pentyl esters

In three centrifuge tubes, weigh 25, 50 and 75 mg of anhydrous butter, respectively, and dissolve them in 5 ml of n-hexane. Add 1.00 ml of the transesterification reagent (reagent A for butyl esters and reagent B for pentyl esters), and stir for two minutes. Add one ml of HCl 2N solution, stir for two minutes and centrifuge at 2000 rpm for two minutes. Analyze the hexane phase five times by GC. Transfer 1.00 ml of hexane solution and 5.00 ml of water saturated with n-hexane to a centrifuge tube, agitate in a stirrer for two minutes and centrifuge for two minute at 2000 rpm; analyze the hexane phase five times by GC.

Calculation:

$$K = \frac{5*B}{A-B}$$

where:

A is the average of the five peak areas of the esters in the hexane phase before the agitation with water;

B is the average of the five peak areas of the esters in the hexane phase after agitation with water.

Procedure for the determination of fatty acids as methyl esters

500 mg of an anhydrous butter sample was placed in a centrifuge tube and dissolved in 5.00 ml of n-hexane; 1.00 ml of transesterification reagent C was added, stirred for two minutes, then centrifuged for two minutes at 2000 rpm. The hexane phase was analyzed by GC.

Procedure for the determination of fatty acids as pentyl esters

Weigh 50 mg of anhydrous butter sample in a centrifuge tube and dissolve it with one ml of n-hexane; add 200 ml of transesterification reagent B, and stir for two minutes. Then add 400 ml of aqueous solution of HCl 1N, stir for two minutes then centrifuge for two minutes at 2000 rpm. Analyze the hexane phase by GC.

Determination of correction factors for methyl esters

In a 10 ml volumetric flask, weigh 500 mg of an appropriate mixture of fatty acid methyl esters of a known concentration; dissolve in n-hexane and dilute to the mark. Transfer 5.00 ml of the solution to a centrifuge tube, add 1.00 ml of transesterification reagent C, agitate it in a stirrer for two minutes and centrifuge for two minutes at 2000 rpm. Analyze by GC the hexane phase five times and average the results. Gather the correction factors for every fatty acid, comparing the results deduced from the integrator report with the known composition of the mixture.

RESULTS AND DISCUSSION

Evaluation of the partition coefficient (K) of short chain fatty acid esters (C4-C8)

The partition coefficients (K) of methyl butanoate, methyl hexanoate and methyl octanoate are obtained by transesterification of synthetic solutions of

methyl esters, in the presence of olive oil, to check for any matrix effect. Two olive oil concentrations of about 25 and 50 mg/ml were used, similar to that employed in the analysis of butter; the n-hexane solution and the NaOH solution in methanol are presaturated with methanol and n-hexane, respectively, to avoid variations in volumes at the moment of contact between the two phases. The K values are reported in TABLE 1. In the same TABLE are reported the K values obtained solely by agitating simply the methyl esters in hexane solution with methanol.

The comparison of the two series of K values brings one to believe that at least in these intervals of concentration and under our experimental conditions, the matrix does not influence the K value. The K value relative to methyl butanoate (0.46) indicates that the equilibrium is predominately shifted toward the methanol phase. The K value relative to C6 (2.3) and the K value relative to C8 (6.0) indicate that the equilibria are instead shifted toward the hexane phase, in particular for C8. To avoid an excess drop in the concentration of methyl esters in the hexane phase after transesterification, it is best to operate with a relatively large volume of n-hexane in respect to the volume of transesterification reagent. Since we verified that 4 ml of NaOH 2N in methanol assures a complete transesterification of 1 mg of fat, in our experimental conditions a ratio of five to one (v/v), of n-hexane to methanol, should be employed. In any case, even under these experimental conditions, a significant amount of methyl butanoate, about 30%, is solubilized in methanol. A smaller amount of C6, about 8%, solubilizes in the methanol phase. Finally, the quantity of C8 which solubilizes in methanol is negligible.

Partition coefficient (K) of short-chain fatty acids as butyl and pentyl esters

The K partition coefficients between n-hexane and water for the butyl and pentyl esters of short-chain fatty acids were determined, since the methods for deriving these esters calls for washing with water [5].

Since short-chain fatty acid butyl esters are slightly soluble in water, in the measurement of K between n-hexane and water, in order to point out the

TABLE 1

Partition coefficient (K) between n-hexane and methanol for short-chain fatty acid methyl esters.

	K^*	K^1	K^2
Methyl butanoate	0.46	0.47	0.45
Methyl hexanoate	2.3	2.3	2.3
Methyl octanoate	6.0	6.1	6.1

K^*: Partition coefficient in the absence of olive oil
K^1: Partition coefficient in the presence of olive oil (25 mg/ml)
K^2: Partition coefficient in the presence of olive oil (50 mg/ml)

difference in concentration of the esters in the hexane phase before and after contact with water, a ratio of 1 to 5 between n-hexane and water was used. It was possible to determine the K values for butanoate and hexanoate butyl esters; it was not possible to determine the K for octanoate butyl ester, in as much as it is insoluble in water.

In TABLE 2 the relative values are reported. Taking into account the values found in the determination of fatty acids like butyl esters using the usual procedure [5], the washing of five ml of hexane phase with three 2-ml portions of water brings a loss of about 6 % of the butyl butanoate concentration; the losses of butyl hexanoate and butyl octanoate were negligible. Therefore this loss can be taken into account by using an appropriate correction factor. The K value of pentyl ester of butanoic acid between n-hexane and water was greater than 50; the loss of pentyl butanoate during the washing phase is negligible.

Precision and accuracy of the determination of the methyl esters

Considering the noteworthy quantity of short-chain methyl esters which dissolve in the methanol phase, to obtain precise results and it is necessary to

TABLE 2

Partition coefficient (K) between n-hexane and water for short-chain fatty acid butyl and pentyl esters in presence of butterfat.

	K
Butyl butanoate	27
Butyl hexanoate	51
Butyl octanoate	>>51

	K
Pentyl butanoate	51
Pentyl hexanoate	>>51
Pentyl octanoate	>>51

measure exactly the volumes of n-hexane and the transesterification reagent used. We also observed that since the presence of methanol in the hexane phase brings a momentary high concentration of C4, if phases are allowed to separate spontaneously after transesterification the gas chromatograms of the hexane phase show a notable quantity of methanol; the results for C4 are excessively high and are scarcely reproducible. If however, the system is allowed to rest for a longer period and then again analyze the hexane phase, the presence of methanol disappears and the result of C4 stabilizes at constant values. A vigorous centrifugation allows a rapid and complete separation of the phases; in these conditions the analytical results are immediately satisfyingly reproducible.

In TABLE 3 the values of the analyses of five different amounts of the same butter are reported, just as they appear on the integrator report. In the same TABLE the corrected values are reported, obtained by multiplying the first series

TABLE 3

Results of the analysis of five aliquots of the same butter sample as methyl esters and their adjusted value after using a correction factor (C.F.).

Fatty Acid (%)	Integrator Report (%)*	RSD(%)	Corrected Values	RSD (%)
Butyric	2.83	1.63	4.61	1.9
Caproic	1.85	1.42	2.63	1.5
Caprylic	0.91	1.46	1.33	1.1
Capric	2.03	1.35	2.74	1.0
Lauric	3.27	1.22	3.98	1.0
Mirystic	10.1	0.99	10.0	0.9
Palmitic	25.6	0.98	25.1	0.9
Stearic	10.5	0.96	10.1	05
Oleic	26.5	0.98	26.0	0.6
Linoleic	1.84	1.01	1.86	09
Linolenic	1.00	1.12	1.12	1.1

*: Average values obtained on the basis of five analyses

of values with the appropriate correction factor, as indicated in the PROCEDURE, which takes into account the effect of distribution and any other discriminatory effects caused by gas chromatographic injection. The standard deviation in percentage does not exceed 2 % in respect to the average even for the short-chain fatty acid esters.

Some researchers have reported that the reaction of transesterification that brings the formation of butyl esters presents a yield of 85% [4]; other researchers declare, instead, that the yield depends upon the ratio between the quantity of fat and the quantity of transesterification reagent and thus can be complete [5]. Experimentally we have observed that the use of 4 ml of 2N transesterification reagent per mg of triglycerides ensures a complete transesterification, while a reduced quantity of reagent does not ensure a quantitative yield. During this phase of research we observed that, fortunately, even if you use a reduced

quantity of transesterification reagent, the methyl, butyl and pentyl esters which form have the same composition, percentage-wise, as when the reaction is complete. And thus, it is important that the reaction be complete only if the determination of the fatty acids is made in absolute.

Standardization of the results

In the analyses conducted by GC the response of the FID detector is sent to an electronic integrator which supplies the evaluation and the printing of the results. In the case of the analyses of the fatty acids, the results generally are expressed as a percentage in weight. When the same mixture of triglycerides is analyzed in terms of fatty acids with methods that cause the formation of different esters, in order to compare the results it is necessary to standardize them; in scientific studies it is often not clear if this procedure is carried out.

In TABLE 4 a typical composition of free fatty acids derived from butter is reported; in the same TABLE are reported the percentages of methyl, butyl and pentyl esters that the free fatty acids assume after transformation to respective esters. As can be observed, passing from the free acids to the esters and the percentage in weight of the short-chain fatty acids increases and as a consequence the percentage decreases for the long chain ones. In particular, the percentage of butyric acid increases from 4 % as a free acid to 5.9 % as a pentyl ester, an increase of over 45%.

Analysis of the trans isomers

As mentioned in the INTRODUCTION, the analysis of fatty acids can be used as a control for the presence of trans isomers that form when the fats are subjected to certain processes, such as passing through decolorizing earth. In FIG. 1 the results of the chromatographic of the separation of oleic acid and elaidic acid are reported as methyl, butyl and pentyl esters, under the same gas chromotographic conditions. As can be observed, methyl esters present the best resolution (R) since they have a separation factor higher than the others.

TABLE 4

Typical composition by percentage of butterfat free fatty acids and their conversion to methyl, butyl and pentyl esters.

Fatty Acid	Composition (%)	Methyl Esters	Butyl Esters	Pentyl Esters
Butyric	4.02	4.63	5.66	5.90
Caproic	2.31	2.67	2.94	3.07
Caprylic	1.25	1.38	1.42	1.55
Capric	2.52	2.79	2.97	3.15
Lauric	3.67	3.95	4.01	4.28
Mirystic	10.1	10.0	9.90	9.81
Palmitic	28.0	27.8	27.2	27.0
Stearic	10.5	10.4	10.1	10.0
Oleic	25.0	24.7	24.0	23.9
Linoleic	2.03	1.87	1.66	1.52
Linolenic	1.02	1.00	0.95	0.93

*: Average values obtained on the basis of five determinations

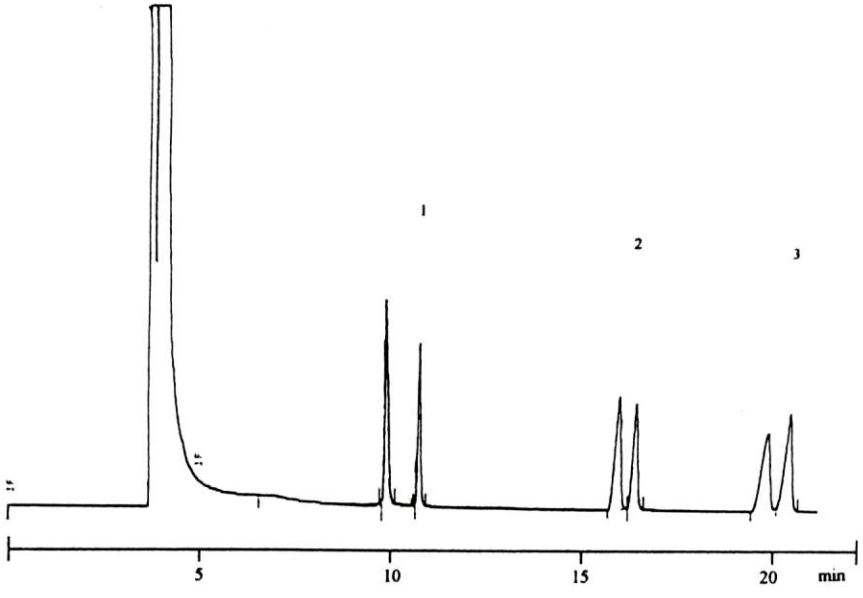

Figure 1. Separation of methyl (1), butyl (2) and pentyl (3) esters as oleic and elaidic trans isomers acid. The resolution is higher for methyl esters (R = 2.9) than for butyl esters (R = 1.1) and for pentyl esters (R = 1.0).

CONCLUSIONS

Triglyceride fatty acids can be accurately analyzed as methyl, butyl and pentyl esters. The K partition coefficient, obtained for short-chain fatty acids such as methyl esters, shows that a significant quantity of the ester dissolves in the methanol phase and thus is not determined. The partition coefficients, obtained for butyl esters, seem to conclude that only butanoic acid, during the phases of washing, is lost in a measurable quantity. And therefore, for methyl and butyl esters, accurate results can be obtained only by introducing appropriate correction factors . Pentyl esters are not soluble in water, and so, have no need for correction factors. Mixtures of fatty acids with trans isomers are more easily separated as methyl esters than as butyl or pentyl esters.

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