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GC Analysis of Fatty Acid Composition of the Oil Containing Short Chain Fatty Acids

Preparation of Propyl-esters with BF_3-1-propanol Reagent

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GC analysis of the fatty acid composition of the oils containing short chain fatty acids has not been performed satisfactorily because of the loss of short chain methyl esters during the ester preparation procedure. The loss was supposed to be due to volatility and water solubility of these esters. The authors have worked on the simple and quantitative procedure for the preparation of esters from the oils containing short chain acids. It was confirmed that the loss of short chain esters could be avoided by preparing propyl esters with BF_3-1-propanol reagent and washing the ester solution with 10.5% aqueous sodium chloride. Then the propyl esters prepared from the standard mixture containing from butyric to octadecanoic acids were analyzed by GC. The propyl esters were prepared by following procedure. Five tenths grams of the oil containing short chain fatty acids and 5 ml of 0.5 N sodium hydroxide in 1-propanol were introduced into an 100 ml flask. Then the mixture was refluxed about 10 min. Seven ml of 14% BF_3 1-propanol reagent was added through the condenser and refluxed for 10 min. Then 5 ml of heptane was added and boiled for 1 min. After cooling to room temperature 100 ml of 10.5% aqueous sodium chloride was added to the flask, and the flask was shaken for 1 min. The heptane layer was transfered into a test tube and the aqueous solution was reextracted by 5 ml of heptane. The heptane solutions were combined and used as the sample for GC. It was found that the response of propyl ester in FID increased as the carbon number of fatty acid increased, although the rate of increase was not so large as that of methyl ester. So GC analytical value (area percent) must be corrected using the correction factor obtained through the analysis of standard mixture. Moreover, the corrected analytical value should be converted to the fatty acid composition, since the ratio of propyl moiety in the propyl ester does not respresent the fatty acid composition. The propyl and methyl esters of fatty acids were prepared from milk fat and fatty acid compositions were analyzed by GC.

1. Foreword:

The GC analysis of the fatty acid methyl esters carried out in order to determine the fatty acid composition of samples(such as milk fat) which contain such short chain fatty acids as butyric acid, has been severely hampered by the loss of the methyl esters during the esterification procedure. This is due to the water solubility of these short chain fatty acids and their methyl esters.

Many reports[1)-7)] have been completed on the fatty acid composition analysis by GC, of samples containing short chain fatty acids.

The authors, found that the loss of butyric acid to the water layer can be avoided by conversion to its propyl esters during the esterification process. They further investigated the sensitivity of the response to FID of the propyl esters of the saturated odd numbered fatty acids from butyric acid to stearic acid and of oleic acid.

In addition, the boron trifluoride-methanol method is that presently used as the official method of the Oil Chemists Society of Japan and of the AOCS for the preparation of fatty acid methyl esters. Thus from the standpoint of speed and convenience etc. in the preparation of propyl esters, it seems desireable to use boron trifluoride as the catalyst. p.110(36

Consequently, the author et al ran tests on this method which uses boron trifluoride as catalyst to prepare propyl esters from milk fat for GC analysis.

2. The Experiment:

2.1. Experimental Materials and Equipment:

The reagents and equipment employed in the experiments were as follows:

Boron trifluoride-methanol reagent:

Wako Junyaku's 14% boron trifluoride-methanol reagent(hereafter referred to as BF_3-MeOH).

Boron trifluoride-1-propanol reagent:

Eastman's 14% boron trifluoride-1-propanol reagent(hereafter referred to as BF_3-PrOH).

Fatty Acids:

The degree of purity of the following fatty acids or their methyl esters was confirmed by GC and only those with 99% or greater purity were used: butyric acid, hexanoic acid, octanoic acid, decanoic acid, lauric acid, myristic acid, palmitic acid, stearic acid and oleic acid.

Equipment:

GC: Model 163 Hitachi gas chromatograph.

Integrator: Model E1A Shimazu chromatopak.

GC Analysis Conditions:

GC 分析条件

Column	25% DEGS/Shimalite (60~80 mesh) 3 mm×2 m
Detector	FID
Injection temp.	280℃
Detection temp.	280℃
Column temp.	50~230℃
Program rate	5℃/min
Sample size	1.0 μl

2.2 Selection of the Alcohols:

Some of the short chain fatty acids such as butyric acid are lost into the aqueous layer when washing is carried out during the methyl esterification process.

Therefore, esters of butyric acid were prepared using ethanol, 1-propanol and 1-butanol and tests were conducted to determine the proportion of these which are lost to the aqueous layer.

Each of the butyric acid esters were mixed in 1:1 and 1:5 proportions(wt/wt) with methyl myristate. 300mg of each mixture was placed in a round bottom flask with stop cock and dissolved in 5ml of heptane. GC analysis was carried out 5 times on any given sample solution and the ratio of the peak areas was obtained for the butyric acid esters and methyl myristate. Values for the dispersion and the means of the 5 analytical values for a given sample were obtained.

Next, 50ml of an aqueous saturated sodium chloride solution was added to the round bottom flasks containing the heptane solutions of these fatty acid ester mixtures and the flasks were then shaken vigorously for 1 minute. After being left to stand, GC was carried out 5 times on the upper heptane layer and the values for the dispersion and the mean were obtained as before.

A T-test was carried out to determine whether or not there was any significant difference between the mean of the analytical values before the addition of saturated aqueous sodium chloride solution and the mean of the analytical values after the addition and subsequent shaking.

As shown in Table 1, in the case of ethyl butyrate, a significant difference was observed(at 5% significance level) between the means of the analytical values before and after the addition of the aqueous sodium chloride solution but was not observed in the cases of propyl butyrate and butyl butyrate.

In other words, the loss due to the washing by the aqueous sodium chloride solution can be ignored if the butyric acid is converted to its propyl and butyl esters.

2.3. Correction of the Analytical Values and their Conversion to the Fatty Acid Composition:

2.3.1. Correction of the Analytical Values:

Figure 1 shows the relative weight responses*, to FID, of the propyl esters of the saturated odd number fatty acids from butyric acid to stearic acid and of oleic acid, when methyl myristate was obtained as the standard weight response. In addition, it also shows the relative weight response of the fatty acid methyl esters.

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Translator's note: The original literally translated would be "relative weight sensitivity" but was translated as "relative weight response" so as to coincide with the figures and tables.

The relative weight response was observed to increase with the carbon number up to decanoic acid in the case of the propyl esters, although this increase was not as marked as with the methyl esters and hence the need to correct the analytical values in cases where the sample contains short chain fatty acids was recognized.

Table 2 shows the correction factors obtained from the relative weight responses.

2.3.2. Conversion to the Fatty Acid Composition:

Ordinarily, the peak area percentages obtained from the GC analysis of methyl esters are considered as they are to be the fatty acid composition. In other words, the percentages of the methyl esters of each fatty acid are regarded as the percentages of each of the fatty acids respectively.

Table 3 shows the ratios of the molecular weights of the fatty acids to their respective propyl and methyl esters. In the case of the methyl esters, the percentages of the methyl esters of the fatty acids can be regarded as the percentages of the fatty acid since there is no big difference from C_8 to C_{18}. On the other hand, with C_{18} being 1.28 times C_4(0.8719/0.6817) in the case of the propyl esters, the adoption of the peak area percentages from the GC analysis of the propyl esters, as they are the fatty acid composition would lead to large errors, especially the shorter the fatty acid.

Therefore, in order to remove this error, the peak area percentages of the fatty acid propyl esters, obtained by GC analysis, were corrected using the correction coefficient shown in Table 2. The propyl fatty acid composition was then converted to the fatty acid composition by multiplying by the coefficient shown in Table 3.

2.4. Esterification using the BF_3-PrOH Reagent:

2.4.1. Testing for the Reaction Time:

Tests were carried out to determine the reaction time necessary for the completion of the esterification when using the BF_3-PrOH reagent.

7ml of 14% BF_3-PrOH reagent were added to each of 300 mg of stearic and oleic acids and heated refluxing was carried out. The reaction products were analysed by TLC at fixed intervals after the start of the reaction. The reaction was considered to have ended when no more spots of the unreacted fatty acids appeared. Figure 2 shows the experimental results for oleic acid.

Virtually identical results were obtained with the other fatty acids.

As is evident from Figure 2, 10 minutes was sufficient for the propyl esterification of oleic acid by the 14% BF_3-PrOH reagent.

2.4.2. Propyl Esterification Method of Fatty Acids using the 14% BF_3-PrOH Reagent:

According to the results of 2.2 and 2.4.1, the appropriate way of carrying out esterification using the BF_3-PrOH reagent is as follows:

"Approximately 300mg of fatty acids are weighed out, and placed in a 50 ml round bottom flask with stopcock. 7 ml of 14% BF_3-PrOH reagent are added and heated refluxing carried out for 10 minutes.Next 5ml of heptane are added and heated refluxing carried out for a further 1 minute after which the solution is left to cool to room temperature. 50ml of aqueous sodium chloride solution are added to the flask and shaken vigorously for 1 minute. After being left to stand, the upper heptane layer is collected and transferred to other stoppered containers. 5ml of heptane are again added to the lower layer sodium chloride saturated solution and the solution then vigorously shaken for 1 minute. The heptane phase is then collected after being left to stand and is then combined with the previous heptane solution to make the sample solution for GC purposes."

2.5. The Propyl Esterification of the Fatty Acid Mixtures:

Butyric, hexanoic, decanoic, palmitic and oleic acids were combined in equal proportions(wt/wt) and propyl esterification was then carried out on these mixtures according to the method explained in 2.4.2.

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A GC analysis was carried out 3 times on the heptane solutions of the ester mixtures which were obtained, and the mean of the peak area percentages of each component was obtained. The mean of the peak area percentages of each propyl ester was corrected using the correction coefficient shown in Table 2 and was then converted to the fatty acid percentage. The recovery rate of each fatty acid was obtained from the fatty acid composition which was obtained. These results are shown in Table 4.

In addition, in the interests of a comparison, the recovery rates obtained when GC analysis was carried out on the products of methyl esterification done according to the official method of the Oil Chemists Society of Japan, are shown in Table 5. These values are also the result of correcting the peak area percentages with the correction coefficient and then converting the values to the fatty acid composition(the same hereafter).

As shown in Tables 4 and 5, the recovery rates of such short chain fatty acids as butyric and hexanoic acids were clearly higher(virtually 100%) with the GC analysis done with the propyl esterification as compared with that done with the methyl esterification. Further, when the fact that the fluctuation coefficient between the recovery rates of the fatty acids with propyl esterification is 1% or less is taken into account, then the propyl esterification method, discussed in 2.4.2, clearly appears to be reliable.

2.6. The Propyl Esterification of Fatty Acid Mixtures that were Prepared so as to Approximate the Composition of Milk Fat:

Fatty acid mixtures were prepared so as to approximate the fatty acid composition

of milk fat. Propyl esterification was carried out 3 times according to the method discussed in 2.4.2 and GC analysis done 3 times on each of the esters. Table 6 shows the recovery rate of each of the fatty acids. In the interests of a comparison, methyl esterification was carried out on the respective mixtures according to the official method of the Oil Chemists Society of Japan,. GC analysis done and then as with the propyl esterification, the recovery rates were obtained.

2.7. The Preparation of Propyl Esters from Glycerides:

2.7.1. The Removal of Excess Propanol:

Saponification must be carried out prior to esterification when preparing methyl esters from triglycerides. According to the official method of the Oil Chemists Society of Japan, saponification is carried out using 0.5N sodium hydroxide-methanol solution and then the esterification is carried out. According to that method, the excess methanol is washed into the saturated aqueous sodium chloride solution used in the washing after esterification. If the preparation of propyl esters is done according to this official method, then the quantity of 1-propanol used would be greater than that used in the propyl esterification of fatty acids discussed in 2.4.2. Consequently, some 1-propanol would remain in the sample solutions for GC analysis, despite washing with the saturated aqueous sodium chloride solution. If these samples are used as such for the GC analysis, the peaks of 1-propanol would be very large and would hence affect the peaks of propyl butyrate.

Therefore, various concentrations of aqueous sodium chloride solution were tested with the objective of lowering the quantity of 1-propanol in the sample solutions to the extent that it would not hinder analysis.

8 aqueous sodium chloride solutions of different concentrations(6.6-26.4%) were prepared and the subsequent experiment was carried out using each of the different solution concentrations.

50ml of aqueous sodium chloride solution were added to a separating funnel to which 13 ml of 1-propanol had been added. The solution was then vigorously shaken for 1 minute after which it was left to settle. As a result, aqueous sodium chloride solutions of concentration 10.5% or less formed a uniform solution with the 1-propanol. However, in the case of solutions of higher concentration 2 distinct layers were formed.

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Accordingly, almost all of the 1-propanol would pass into the aqueous phase on washing with 10.5% aqueous sodium chloride solution.

No ester loss was observed from heptane solutions containing the propyl esters of short chain fatty acids, even when washed with a saturated aqueous sodium chloride solution. However, the subsequent experiment was carried out for when a 10.5% aqueous sodium chloride solution was used.

When 5ml of heptane were added to 100mg of a 1/1 mixture of propyl butyrate and methyl decanoate in a 100ml separating funnel, a uniform layer was the result. After doing a GC analysis 3 times on the heptane solution, 50ml of 10.5% aqueous sodium chloride solution was added and the mixture then shaken vigorously for 1 minute. GC analysis was then done again 3 times on the heptane layer after the solution was let stand to settle.

A statistical comparison was done of the GC analytical values for before and after the addition of the aqueous sodium chloride solution. No significant difference was observed between these values in these results.

According to the experiments to this point, it has been found that 13ml of 1-propanol forms a uniform layer with 50ml of 10.5% aqueous sodium chloride solution and that the propyl butyrate in the heptane does not pass into the aqueous sodium chloride solution.

However, 1-propanol was observed to intermix with the heptane layer in a system of co-existing propyl esters, heptane solutions, 1-propanol and 10.5% aqueous

sodium chloride solution. Therefore the following experiment was done to determine how much to increase the quantity of aqueous sodium chloride solution so that the intermixing of the 1-propanol into the heptane layer will be decreased enough so as not to interfere with the GC analysis. In addition, it was carried out to investigate whether or not in that case, the propyl esters of the short chain fatty acids pass into the aqueous sodium chloride solution.

A uniform layer was created as a result of adding 5ml of heptane to 100mg of a 1/1 mixture of propyl butyrate and methyl decanoate in a 100ml separating funnel. Following 3 GC analyses on this solution, 13ml of 1-propanol was added and the solution was shaken lightly. Then, 50ml of 10.5% aqueous sodium chloride solution was added and the solution then shaken vigorously for 1 minute. After settling, GC was again done 3 times and the analytical values of before and after the treatment with the aqueous sodium chloride solution were studied. The cases where 70 and 100ml of aqueous sodium chloride solution were added were also similarly studied.

In the cases where 50 and 70ml of aqueous sodium chloride were added, the 1-propanol peak was found to affect the propyl butyrate peak due to a considerable mixing of the 1-propanol into the heptane layer. In the case of a 100ml addition of aqueous sodium chloride solution, on the otherhand, no influence was observed on the propyl butyrate peak since there was little mixing of the 1-propanol in the heptane layer.

It thus appears evident from the aforementioned tests, that 100ml of 10.5% aqueous sodium chloride solution should be used in the washing of heptane layers containing large quantities of 1-propanol.

2.7.2. Propyl Esterification from Fats and Oils containing Butyric Acid:

According to the aforementioned experimental results, the appropriate way to

conduct the preparation of propyl esters from fats and oils containing butyric acid and using BF_3-PrOH is as follows:

"500 mg of fats and oils are placed in a 100ml pear-shaped flask with ground glass joint to which 6ml of 0.5N sodium hydroxide-1-propanol solution is then added. Heated refluxing is carried out until the oil droplets disappear and the flask contents become a uniform phase. On becoming uniform, 7ml of 14% BF_3-PrOH reagent is added and heated refluxing done for 10 minutes. Next 5ml of heptane are added and a further heated refluxing done for a minute after which the solution is left to cool to room temperature.

100ml of 10.5% aqueous sodium chloride solution is added to the flask; the flask is stoppered and then vigorously shaken for 1 minute. After being left to stand, the heptane phase is collected and transferred to other stoppered containers. 5ml of heptane is added to the aqueous sodium chloride solution and the solution is then vigorously shaken for 1 minute. After being left to stand, the heptane phase is collected and combined with the previous heptane solution. This latter solution is then considered as the sample solution for GC analysis."

2.8. The Fatty Acid Composition of Milk Fat:

Propyl esters were prepared from milk fat and GC analyses done according to the method outlined in 2.7.2. In the interests of a comparison, methyl esterification was carried out according to the official method of the Oil Chemists Society of Japan using the same milk fats and a GC analysis was then done.

The results are shown in Table 7.

As shown in Table 7, an anlysis was carried out using samples that were obtained by different esterification procedures. A difference in the composition of butanic acid was observed on comparing the results. Further, the scattering of the analytical values for each component between different samples obtained by the same esterification procedure was observed to be markedly larger in the case with methyl esters as compared to that of the propyl esters.

Hence, propyl esterification is believed to be the more reliable procedure for use in the GC analysis of the fatty acid composition of fats and oils containing short chain fatty acids.

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3. Discussion:

As a result of preparing and studying the esters of short chain fatty acids and various alcohols, it was found that the loss due to the water solubility of the methyl esters of short chain fatty acids can be avoided by converting them to their propyl esters.

The authors took the official method of the Oil Chemists Society of Japan which uses BF_3-MeOH as catalyst in preparing propyl esters from milk fat and altered it such that the alcohol used was 1-propanol, the reaction time was 10 minutes, the aqueous sodiun chloride solution used in washing was 10.5% and a volume of 100ml was used. Thus it was observed that propyl esters can be quickly, easily and quantitatively obtained.

On the other hand, the decrease in the peak area percentage of methyl butyrate against the percentage by wt of butyric acid in the fatty acid mixtures, has generally been attributed to the loss that occurs due to the washing in the esterification process and due to the volatility of methyl butyrate. The volatility loss can be prevented by carrying out the GC analysis immediately after esterification. On the other hand, a loss of methyl butyrate due to washing was observed by the authors with a wash of saturated aqueous sodium chloride solution. However, the cause of the decrease in the peak area percentage of methyl butyrate does not lie simply in the washing. As shown in Fig. 1, the relative weight response of methyl butyrate to FID is about 0.71 and hence this too is a major cause of the decrease in the peak area percentage. The relative weight response of propyl butyrate for which no washing loss was observed, was observed to be somewhat higher at about 0.79.

Since such a decrease is a problem related with FID, the correction coefficients have to be obtained anew and the corrections carried out again.

Generally, the peak area percentages of the methyl esters obtained by GC analysis following the methyl esterification of fatty acids, can be regarded as the fatty acid composition. When the peak area percentages of the propyl esters are similarly used as the fatty acid composition, a large error results. In order to avoid this, the peak area percentages of the fatty acid propyl esters must be converted to the area percentages of the fatty acids.

If attention is paid to the aforementioned points, then completely satisfying experimental results can be obtained by the method discussed in 2.4.2 for fatty acid mixtures containing butyric acid and by the method in 2.7.2 for fats and oils.

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APPENDIX

Table-1 Passage of butyric acid esters into aqueous sodium chloride layer.

Esters	Mixed ratio (C_4-ester : C_{14}-Me)	
	1 : 1	1 : 5
C_4-Et	×	×
C_4-Pr	○	○
C_4-Bu	○	○

× : Passage was observed.*
○ : Passage was not observed.*
* Decided by the T-test between the GC analytical values before and after shaking with aqueous sodium chloride.

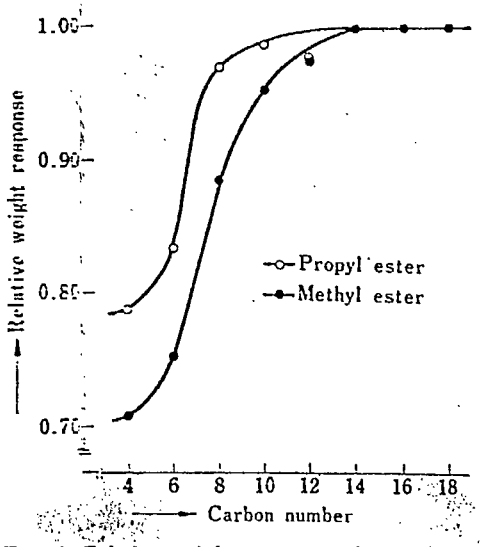

Fig.-1 Relative weight responses of methyl and propyl esters of fatty acids.

Table-2 Correction factors of methyl and propyl esters of fatty acids.

Fatty acid	Correction factors	
	Propyl ester	Methyl ester

Table-3 Molecular weight ratio of fatty acid to ester.

Fatty acid	Molecular weight ratio	
	Propyl ester	Methyl ester
C_4	0.6817	0.8653
C_6	0.7374	0.8939
C_8	0.7765	0.9125
C_{10}	0.8035	0.9255
C_{12}	0.8278	0.9362
C_{14}	0.8456	0.9426
C_{16}	0.8600	0.9485
C_{18}	0.8719	0.9533
$C_{18:1}$	0.8711	0.9530

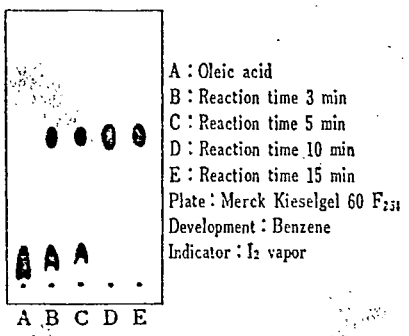

Fig.-2 TLC of reaction products of oleic acid with BF_3-1-propanol.

Table-4 Recoveries of short chain fatty acids from standard mixture-1* through the processes of esterification with BF_3-1-propanol.

Fatty acid	1	2	3	$\bar{X}$	CV(%)
	(%)	(%)	(%)	(%)	
C_4	99.7	99.3	98.1	99.0	0.84
C_6	102.4	102.4	102.9	102.6	0.28
C_8	101.7	99.9	100.9	100.8	0.89

*: Even mixture of fatty acids

Table-5 Recoveries of short chain fatty acids from standard mixture-1* through the processes of esterification with BF_3-methanol.

Fatty acid	1	2	3	$\bar{X}$	CV(%)
	(%)	(%)	(%)	(%)	
C_4	96.4	96.9	98.8	97.4	1.30
C_6	96.0	96.7	95.8	96.2	0.49
C_8	103.3	97.4	100.3	100.3	2.94

*: Even mixture of fatty acids

Table-7 Fatty acid composition of milk fat determined by GC analysis of methyl or propyl ester.

Fatty acid	Propyl ester (%)	Methyl ester (%)
C_4	3.61	2.98
C_6	2.19	2.20
C_8	1.01	1.02
C_{10}	2.53	2.49
C_{12}	3.29	3.24
C_{14}	11.08	11.13
C_{16}	32.17	32.94
C_{18}	11.20	11.82
$C_{18:1}$	27.58	27.04
Others	5.35	5.14

Table-6 Recoveries of fatty acids from standard mixture-2* through the processes of esterification.

Fatty acid	Mixed ratio (%)	Propyl ester		Methyl ester	
		Analytical value** (%)	Recovery (%)	Analytical value** (%)	Recovery (%)
C_4	5.46	5.72	104.8	3.66	67.0
C_6	2.36	2.09	88.6	1.84	78.0
C_{10}	3.03	2.66	87.8	2.62	86.5
C_{14}	31.41	34.11	108.6	33.57	106.9
C_{16}	32.35	32.43	100.2	35.48	109.7
$C_{18:1}$	25.38	22.98	90.5	23.04	90.8

*: Fatty acid mixture having the composition like that of milk fat.
**: Fatty acid composition calculated from GC analytical value.