Limitations of Ambient Temperature Methods for the Methanolysis of Triacylglycerols in the Analysis of Fatty Acid Methyl Esters With High Accuracy and Reliability

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A comparison is presented of a method for the preparation of fatty acid methyl esters, involving hydroxide catalyzed transesterification at ambient temperature, with a second method employing methoxide catalyzed transesterification at reflux temperature. The first of these methods was specifically designed for the analysis of fats that contain very short chain length fatty acids (butyric and caproic acids), but it has been suggested that it might be suitable as a general method for the preparation of esters. It is now shown that the methoxide/reflux method gives more accurate results than does the hydroxide/ambient method when the samples to be analyzed contain high levels of long chain length fatty acids (e.g. stearic, palmitic, elaidic, oleic) and that it is quicker and at least as simple to carry out. The hydroxide/ambient method should be used only for its specific purpose and, when used, the procedure should be strictly followed and carefully standardized. Results obtained from fats that contain significant quantities of long chain length components should be viewed with suspicion.

In a recent publication(1), we demonstrated that the method of Christopherson and Glass(2) for the preparation of fatty acid methyl esters (FAME) from fats that contain very short chain length fatty acids cannot be relied upon to produce highly accurate results because, following the initial conversion of triacylglycerol (TAG) into FAME, a secondary reaction takes place in which FAMEs are saponified. As the shorter chain length FAMEs saponify faster than do those of longer chain length, the composition of the analyte changed significantly in as little as 15 min. The problem was resolved for the types of fats examined by neutralizing the alkaline catalyst after exactly six min reaction time. As stated in the paper, and since confirmed further in practice, such neutral analyte solutions are stable in composition providing precautions are taken to prevent evaporative losses of short chain length and autoxidative deterioration of unsaturated FAMEs.

It has been indicated to us (Y.F. Homans, Unilever Research Laboratory, Vlaardingen, The Netherlands, private communication) that some laboratories now use the hydroxide/ambient method as a general procedure for the preparation of FAME from all types of fats and oils. This is a matter of concern, as we found some indication in our work (1) that the conversion of saturated long chain length TAG into FAME might be significantly slower than that of the shorter chain length homologues.

In the present work, we have again confirmed the more rapid loss of short chain length FAME by saponification, more clearly demonstrated the slow conversion of long chain length TAG to FAME and, as a consequence, now recommend that the hydroxide/ambient method should not be considered as applicable to all types of fats. By contrast, the methoxide/reflux method of Bannon et al. (3) was shown to give more accurate and repeatable results when analyzing the primary standard selected. In addition, this method is quicker, at least as simple to perform and has been shown to perform reliably for a wide range of sample types containing fatty acids with chain lengths of eight or more carbon atoms.

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EXPERIMENTAL PROCEDURES

Apparatus. GLC was carried out on a Hewlett Packard model 5880 gas chromatograph fitted with a capillary inlet system and a flame ionization detector (FID). The column was 22 m x 0.25 mm i.d. fused silica coated with 0.2μDEGS (Chrompack, Middelburg, The Netherlands). The carrier gas was high purity hydrogen at an inlet pressure of 10 psi, which produced a column flow rate of ca. 0.5 ml/min. The split vent flow rate was ca. 100 ml/min, but adjusted occasionally as described previously (4) to optimize linearity of splitting. Septum purge flow rate ca. six ml/min. The total hydrogen flow rate to the detector was 30 ml/min, the make up gas was high purity nitrogen at a flow rate of 23 ml/min, and oil-free compressed laboratory air was supplied at a flow rate of ca. 240 ml/min. Injections were made by a Hewlett Packard model 7673A rapid automatic injector; the injector insert was a "double Jennings" design as recommended by Bannon et al. (4), operated at 375 C. Column oven temperature was 160 C and the detector temperature was 250 C. Peak areas were measured using a Hewlett-Packard model 3350A Laboratory Automation System.

Standard mixtures. A primary standard mixture of saturated FAME was prepared as previously described (1,5). This standard comprised the saturated even carbon number FAME from C8 to C18 inclusive in proportion similar to that of coconut oil and was used to ensure that the operating parameters of the chromatograph had been optimized. Actual composition was 8:0, 8.46%; 10:0, 7.29%; 12:0, 42.94%; 14:0, 20.18%; 16:0, 9.62%, and 18:0, 11.51%. As a measure that equipment was properly optimized, it was required that, on analysis, a grade of analysis of at least 99% be obtained. (Grade of Analysis $= 100 - \Sigma \mid C_i - C_i^1 \mid$,
where C_i = known concentration of ester component in mixture
and C_i^1 = amount of ester component determined.)

A primary standard mixture of TAG was prepared from individual saturated even carbon number TAG

TABLE 1

Analysis of Saturated Triacylglycerol Primary Standard

Methoxide/reflux methanolysis procedure

FAME	Known composition	Analysis number 1	2	3	4	5	6	7	8	9	10	Mean	SD (n-1)	CV (%)
		Composition by GLC (%)												
8:0	16.97	16.98	17.00	17.00	17.06	16.95	16.95	16.96	16.91	16.95	16.94	16.97	0.042	0.25
10:0	16.88	16.90	16.87	16.89	16.90	16.84	16.85	16.86	16.82	16.85	16.83	16.86	0.028	0.17
12:0	16.69	16.73	16.70	16.72	16.71	16.70	16.72	16.72	16.70	16.71	16.69	16.71	0.012	0.07
14.0	16.75	16.78	16.78	16.78	16.76	16.81	16.80	16.80	16.81	16.80	16.79	16.79	0.016	0.09
16:0	16.59	16.60	16.61	16.60	16.58	16.64	16.63	16.62	16.66	16.64	16.65	16.62	0.025	0.15
18:0	16.12	16.01	16.02	16.01	15.99	16.06	16.06	16.04	16.09	16.05	16.09	16.04	0.034	0.21
Total	100.00	100.00	99.98	100.00	100.00	100.00	100.01	100.00	99.99	100.00	99.99			
		Difference from known composition (%)												
8:0		+0.01	+0.03	+0.03	+0.09	-0.02	-0.02	-0.01	-0.06	-0.02	-0.03			
10:0		+0.02	-0.01	+0.01	+0.02	-0.04	-0.03	-0.02	-0.06	-0.03	-0.05			
12:0		+0.04	+0.01	+0.03	+0.02	+0.01	+0.03	+0.03	+0.01	+0.02	0.00			
14:0		+0.03	+0.03	+0.03	+0.01	+0.06	+0.05	+0.05	+0.06	+0.05	+0.04			
16:0		+0.01	+0.02	+0.01	-0.01	+0.05	+0.04	+0.03	+0.07	+0.05	+0.06			
18:0		-0.11	-0.10	-0.11	-0.13	-0.06	-0.06	-0.08	-0.03	-0.07	-0.03			
Grade of analysis (%)		99.78	99.80	99.78	99.72	99.76	99.77	99.78	99.71	99.76	99.79	99.77	0.029	0.03

Hydroxide ambient methanolysis procedure (temperature 25 C)

FAME	Known composition	Reaction time 2 min	4 min	6 min	8 min	10 min	15 min	30 min	1 hr	5 hr	24 hr
		Composition by GLC (%)									
8:0	16.97	17.76	17.65	17.77	17.37	17.34	17.25	17.10	17.09	16.86	15.95
10:0	16.88	17.16	17.23	17.39	17.02	17.00	16.97	16.91	16.93	16.88	16.63
12:0	16.69	16.73	16.82	17.06	16.73	16.72	16.72	16.72	16.74	16.78	16.84
14:0	16.75	16.58	16.65	16.96	16.69	16.68	16.72	16.76	16.78	16.85	17.08
16:0	16.59	16.25	16.25	16.46	16.43	16.46	16.49	16.57	16.57	16.64	16.98
18:0	16.12	15.53	15.40	14.36	15.77	15.80	15.84	15.94	15.90	15.99	16.52
Total	100.00	100.01	100.00	100.00	100.01	100.00	99.99	100.00	100.01	100.00	100.00
		Difference from known composition (%)									
8:0		+0.79	+0.68	+0.80	+0.40	+0.37	+0.28	+0.13	+0.12	-0.11	-1.02
10:0		+0.28	+0.35	+0.51	+0.14	+0.12	+0.09	+0.03	+0.05	0.00	-0.25
12:0		+0.04	+0.13	+0.37	+0.04	+0.03	+0.03	+0.03	+0.05	+0.09	+0.15
14:0		-0.17	-0.10	+0.21	-0.06	-0.07	-0.03	+0.01	+0.03	+0.10	+0.33
16:0		-0.34	-0.34	-0.13	-0.16	-0.13	-0.10	-0.02	-0.02	+0.05	+0.39
18:0		-0.59	-0.72	-1.76	-0.35	-0.32	-0.28	-0.18	-0.22	-0.13	+0.40
Grade of analysis (%)		97.79	97.68	96.22	98.85	98.96	99.19	99.60	99.51	99.52	97.46

from C8 to C18 inclusive, each ester being present in approximately equal amount. The exact fatty acid composition was determined by the technique previously described (1, 5). The composition of this standard is recorded in Table 1. Two other mixtures were prepared, similar in composition to this standard except that the tristearin content was replaced with trielaidin and triolein, respectively.

METHODS

The TAG primary standard and the mixtures containing unsaturated TAG were converted into FAME by each of the methods of Bannon et al., viz. the hydroxide/ambient method (1) and the methoxide/reflux method (3). In the case of the hydroxide/ambient method, variations of reaction time from that specified (6 min) were made, so that the effects of differential rates of saponification and transesterification could be studied. Reaction times (i.e. time of reaction in the presence of hydroxide catalyst before neutralization with acid) were 2, 4, 6, 8, 10, 15 and 30 min and 1, 5 and 24 hr. In the case of the methoxide/reflux method, 10 replicate methylations were

TABLE 2

Analysis of Triacylglycerol Standard Containing Methyl Elaidate

Methoxide/reflux methanolysis procedure

FAME	Analysis number												
	1	2	3	4	5	6	7	8	9	10	Mean	SD (n-1)	CV (%)
	Composition by GLC (%)												
8:0	16.34	16.34	16.35	16.28	16.36	16.31	16.33	16.33	16.32	16.36	16.33	0.024	0.15
10:0	16.31	16.31	16.32	16.30	16.32	16.25	16.31	16.30	16.31	16.32	16.31	0.021	0.13
12:0	20.67	20.67	20.67	20.68	20.67	20.63	20.67	20.67	20.68	20.66	20.67	0.014	0.07
14.0	15.63	15.63	15.62	15.65	15.62	15.64	15.63	15.64	15.63	15.63	15.63	0.009	0.06
16:0	15.41	15.42	15.41	15.43	15.41	15.47	15.42	15.43	15.42	15.41	15.42	0.018	0.12
tr18:1	15.64	15.63	15.64	15.66	15.62	15.70	15.64	15.64	15.64	15.62	15.64	0.023	0.15
Total	100.00	100.00	100.01	100.00	100.00	100.00	100.00	100.01	100.00	100.00			

Hydroxide ambient methanolysis procedure (temperature 25 C)

FAME	Reaction time									
	2 min	4 min	6 min	8 min	10 min	15 min	30 min	1 hr	5 hr	24 hr
	Composition by GLC (%)									
8:0	16.99	16.97	16.73	16.78	16.67	16.71	16.53	16.50	16.17	14.68
10:0	16.64	16.60	16.48	16.51	16.46	16.51	16.41	16.43	16.30	16.01
12:0	20.77	20.72	20.71	20.74	20.72	20.74	20.70	20.73	20.74	20.85
14:0	15.45	15.45	15.53	15.51	15.54	15.52	15.57	15.58	15.67	16.06
16:0	15.04	15.09	15.23	15.18	15.25	15.20	15.33	15.32	15.47	16.05
tr18:1	15.11	15.17	15.32	15.29	15.36	15.32	15.46	15.44	15.66	16.36
Total	100.00	100.00	100.00	100.01	100.00	100.00	100.00	100.00	100.01	100.01
	Difference from composition determined by methoxide/reflux method (%)									
8:0	+0.66	+0.64	+0.40	+0.45	+0.34	+0.38	+0.20	+0.17	-0.16	-1.65
10:0	+0.33	+0.29	+0.17	+0.20	+0.15	+0.20	+0.10	+0.12	-0.01	-0.30
12:0	+0.10	+0.05	+0.04	+0.07	+0.05	+0.07	+0.03	+0.06	+0.07	+0.18
14:0	-0.18	-0.18	-0.10	-0.12	-0.09	-0.11	-0.06	-0.05	+0.04	+0.43
16:0	-0.38	-0.33	-0.19	-0.24	-0.17	-0.22	-0.09	-0.10	+0.05	+0.63
tr18:1	-0.53	-0.47	-0.32	-0.35	-0.28	-0.32	-0.18	-0.20	+0.02	+0.72
Grade of analysis (%)	97.82	98.04	98.78	98.57	98.92	98.70	99.34	99.30	99.65	96.09

made so that a statistical analysis could be made. Samples obtained from the hydroxide/ambient and methoxide/reflux methods were analyzed alternately.

The FAME standard was analyzed at the beginning and end at regular intervals during the course of the analytical series as a check that the chromatograph was still operating optimally. On each occasion a grade of analysis in excess of 99% was obtained.

From the raw peak areas, a wt% composition of the sample was estimated by normalization to 100% after applying to each component theoretical FID relative response factors calculated using the method of Ackman and Sipos (6). These factors were: methyl caprylate, 1.1927; methyl caprate, 1.1233; methyl laurate, 1.0771; methyl myristrate, 1.0440; methyl palmitate, 1.0193; and methyl stearate, 1.000. Analyses were evaluated by determination of the grade of analysis and by an examination of the errors observed for individual FAMEs.

RESULTS AND DISCUSSION

Because the two methods under comparison have different strengths and limitations, considerable care had to be exercised in selecting the composition of the standard. The critical features of each method that required consideration were:

- *Methoxide/reflux method*
  - —Quantitative recovery of C8 and longer chain length FAME.
  - —Some loss of C6 due to adverse partition coefficient.
- *Hydroxide/ambient method*
  - —Quantitative recovery of C4 and C6.
  - —Rapid loss of C4 by way of saponification as a secondary reaction.
  - —Possible slow conversion of tristearin into methyl stearate.

It has already been shown (1) that the hydroxide/

TABLE 3

Analysis of Triacylglycerol Standard Containing Methyl Oleate

Methoxide/reflux methanolysis procedure

FAME	Analysis number												
	1	2	3	4	5	6	7	8	9	10	Mean	SD (n-1)	CV (%)
	Composition by GLC (%)												
8:0	16.53	16.71	16.66	16.62	16.38	16.61	16.66	16.67	16.60	16.74	16.62	0.102	0.62
10:0	16.60	16.65	16.68	16.66	16.50	16.60	16.67	16.64	16.65	16.68	16.63	0.054	0.33
12:0	16.65	16.62	16.67	16.68	16.62	16.62	16.66	16.63	16.66	16.64	16.65	0.022	0.13
14:0	17.26	17.20	17.23	17.24	17.29	17.24	17.22	17.21	17.24	17.20	17.23	0.028	0.16
16:0	16.94	16.86	16.86	16.87	17.06	16.92	16.87	16.88	16.90	16.85	16.90	0.063	0.37
c18:1	16.02	15.95	15.90	15.93	16.16	16.01	15.92	15.97	15.95	15.89	15.97	0.079	0.50
Total	100.00	99.99	100.00	100.00	100.01	100.00	100.00	100.00	100.00	100.00			

Hydroxide ambient methanolysis procedure (temperature 25 C)

FAME	Reaction time									
	2 min	4 min	6 min	8 min	10 min	15 min	30 min	1 hr	5 hr	24 hr
	Composition by GLC (%)									
8:0	18.43	17.49	18.00	17.55	17.67	17.27	16.93	16.83	16.56	14.81
10:0	17.34	17.03	17.19	17.03	17.03	16.96	16.83	16.82	16.73	16.23
12:0	16.68	16.72	16.66	16.66	16.65	16.72	16.74	16.74	16.73	16.92
14:0	16.69	16.98	16.79	16.93	16.90	17.04	17.15	17.19	17.25	17.83
16:0	15.96	16.42	16.19	16.42	16.36	16.52	16.69	16.74	16.87	17.68
c18:1	14.90	15.36	15.17	15.41	15.40	15.48	15.66	15.68	15.85	16.53
Total	100.00	100.00	100.00	100.00	100.01	99.99	100.00	100.00	99.99	100.00
	Difference from known composition (%)									
8:0	+1.81	+0.87	+1.38	+0.93	+1.05	+0.65	+0.31	+0.21	-0.06	-1.81
10:0	+0.71	+0.40	+0.56	+0.40	+0.40	+0.33	+0.20	+0.19	+0.10	-0.40
12:0	+0.03	+0.07	+0.01	+0.01	.00	+0.07	+0.09	+0.09	+0.08	+0.27
14:0	-0.54	-0.25	-0.44	-0.30	-0.33	-0.19	-0.08	-0.04	+0.02	+0.60
16:0	-0.94	-0.48	-0.71	-0.48	-0.54	-0.38	-0.21	-0.16	-0.03	+0.78
c18:1	-1.07	-0.61	-0.80	-0.56	-0.57	-0.49	-0.31	-0.29	-0.12	+0.56
Grade of analysis (%)	94.90	97.32	96.10	97.32	97.10	97.89	98.80	99.02	99.59	95.58

ambient method can give inaccurate results because of rapid saponification of very short chain length FAME. Accordingly, it was considered unnecessary to demonstrate this again by inclusion of FAMEs of chain length shorter than C8. On the other hand, the evidence that long chain length TAG and particularly tristearin might be slow to undergo transesterification was not so conclusive. Accordingly, a rather high level of tristearin and tripalmitin was included in the standard that contained saturated TAG. The effect observed could have been caused equally by long chain length FAME crystallizing from the reaction mixture and thus failing to react. For this reason, the elaidate and oleate mixtures were examined, as these would be progressively less prone to the problem of crystallization. In addition, it would be more common in practice for hard fats to contain trielaidin and triolein than tristearin.

The results obtained when the saturated TAG primary standard was analyzed are recorded in Table 1. Results for the elaidate mixture are collated in Table 2 and for the oleate mixture in Table 3.

In preliminary experiments, which were carried out before the experiments described herein, we observed that, when the hydroxide/ambient method was carried out at 20 C, there were occasions when some of the sample crystallized from the solvent. No cases of crystallization have been observed at 25 C, hence this temperature was selected for the hydroxide/ambient reaction, although it is recognized that this must be considered as about the upper limit of "ambient." It will be shown later that variation of the reaction temperature within the range 15-30 C had little influence on the relative rates of transesterification as a function of chain length.

From an examination of Table 1, it was concluded that the methoxide/reflux method consistently gave results of very high accuracy. By contrast, the hydrox-

ide/ambient method gave acceptable results only during the time span 15 min to 5 hr and never achieved quite the accuracy of the methoxide/reflux method. The reasons can be ascertained by an analysis of the errors of individual components. At all reaction times up to and including 5 hr the methyl stearate determined was less than the known figure, generally trending up to a more accurate figure. By contrast, the figures obtained for methyl caprylate were greater than theoretical at short reaction times, trending downward throughout the series to a figure that was less than theoretical at 24 hr (−1.02%). This can be interpreted as a summation of two factors, viz. differential rates of methanolysis of TAG and saponification of FAME of differing chain lengths. As the figures are normalized to 100%, a slow conversion of long chain length TAG must appear as an apparent excess content of those shorter chain length TAG that do convert more completely or more quickly into FAME. There is evidence of differential reaction rate throughout the range of chain lengths of this mixture. Thus for the 2-min sample, the errors from C8 to C18 trend from strongly positive through nearly correct to strongly negative.

Evidence of differential rates of saponification can be inferred from the significant loss of methyl caprylate after 24 hr (−1.02%). Further evidence of saponification was obtained by the appearance on this chromatogram of two broad trailing peaks, attributed to free caprylic and capric acids. Admittedly, a reaction time of 24 hr must be considered to be impractical. However, the experiment does corroborate the previous finding of rapid saponification of methyl butyrate (1), and indicates that to achieve maximum accuracy for samples of this type, it is necessary to react for at least 15 min, preferably 30 min to 5 hr, but no longer. Such reaction times would give poor results for samples that contain very short chain length FAME.

As it is not possible to calculate the composition of a mixture that contains unsaturated TAG with the same accuracy and by the same method used for those that are fully saturated (7), the compositions of the elaidate and oleate mixtures were estimated by repeated analysis using the methoxide/reflux method. The comparative performance of the hydroxide/ambient method was assessed by relating the figures obtained at different reaction times to the mean of those obtained by the methoxide/reflux method.

Essentially the same pattern of results was obtained. In the case of the elaidate mixture, grade of analysis was satisfactory (>99%) through the time span 30 min to 5 hr and was at a maximum at 5 hr. For the oleate mixture, the acceptable time span was 1 to 5 hr with the optimum at 5 hr. In each case, saponification of short chain length FAME was evident at 24 hr.

That the need to react for 5 hr is not a problem of poor solubility of long chain length FAME was also shown by reacting each of the mixtures for 6 min, at temperatures ranging from 15 to 30 C (Table 4). In no case was a good grade of analysis obtained. While there were variations of grade as the temperature changed, there was no significant trend for the grade to increase with temperature. In the case of the saturated primary standard reacted at 20 C, it was observed that crystallization did occur and this no doubt explains the very low grade. In none of the others was crystallization observed. It must be concluded that differential reaction rates of TAG of differing chain length is the major factor in adversely influencing the accuracy of results delivered by the hydroxide/ambient method.

TABLE 4.

Influence of Temperature on Conversion of TAG to FAME: Hydroxide/Ambient Procedure

Standard	Reaction temperature (C)			
	15	20	25	30
	Grade of analysis			
Saturated	92.90	77.56	96.22	96.80
Elaidate	97.49	97.67	98.78	96.18
Oleate	94.70	94.08	96.10	95.46

From the above it may be concluded that the hydroxide/ambient method is fragile and should be used only when it is essential to quantitate methyl butyrate and/or methyl caproate. Even when this is the case, it is important to follow the method exactly, to standardize carefully and to view with suspicion results obtained from fats that contain significant quantities of long chain length components. While we have investigated in this study only one specific ambient methanolysis method, it would appear probable that any similar ambient temperature method would suffer from the same limitations.

By contrast, the methoxide/reflux method gives very accurate results for a wide variety of fat types, is very simple to perform and is quick (2-3 min). It should not be used for quantitation of methyl butyrate and caproate, but apart from this limitation is a robust, practical method.

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