Journal of Chromatography, 247 (1982) 63–69
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,052

ANALYSIS OF FATTY ACID METHYL ESTERS WITH HIGH ACCURACY AND RELIABILITY

II. METHYLATION OF FATS AND OILS WITH BORON TRIFLUORIDE–METHANOL

CECIL D. BANNON*, JOHN D. CRASKE, NGO TRONG HAI, NEIL L. HARPER and KERRY L. O'ROURKE

Central Research Department, Unilever Australia Proprietary Limited, P.O. Box 9, Balmain, N.S.W. 2041 (Australia)

(Received May 26th, 1982)

SUMMARY

Procedures generally recognized as suitable for the preparation of fatty acid methyl esters using boron trifluoride–methanol have been shown to be inadequate when the analyte contains appreciable amounts of low-molecular-weight esters. A modified technique is described which maximizes extraction of the esters into iso-octane solution, and thereby improves quantitative accuracy and precision for esters down to and including methyl caproate.

INTRODUCTION

The quantitative methylation of fats and oils with boron trifluoride–methanol for gas–liquid chromatographic (GLC) analysis was first described by Metcalfe *et al.*[1] and is now the basis of a number of essentially identical internationally accepted procedures[2–5]. Even though the Metcalfe group described the quantitative analysis of a butterfat sample, including the determination of butyrate, the recognized methods state or imply that it is suitable for "long-chain fatty acids". The implication that it may not be suitable for short-chain esters has received mixed support in the literature. Kaufmann and Mankel[6] found that boron trifluoride–methanol consistently gave the best recovery of the short-chain esters for coconut oil compared with other reagents, while Sheppard and Iverson[7] noted that losses of the lower fatty acids could be avoided, but stressed the importance of salting-out with saturated brine. Vorbeck *et al.*[8], on the other hand, reported losses of the lower esters using boron trifluoride–methanol, as did McGinnis and Dugan[9], who found the use of sulphuric acid–methanol to be preferable. DeMan[10] also found losses of short-chain fatty esters when coconut oil was methylated with boron trifluoride–methanol and found a sealed tube method to be the only satisfactory procedure. Solomon *et al.*[11] found a dependence of the yield of esters on the sample size. Thus, for a mixture containing C_{12}–C_{18} chain

0021-9673/82/0000–0000/$02.75

lengths, the yield of esters was variable, with methyl laurate giving the poorest recovery, which was as low as 37.6% under the least favourable conditions. The high variability of analysis of the short chain esters is indicated in collaborative studies[12]. Using the American Oil Chemists' Society (AOCS)–International Union of Pure and Applied Chemistry (IUPAC) methodology for preparation of the esters, the coefficient of variation (C.V.) for the determined level of methyl caprylate in a coconut oil sample was variously found to be 18.3% (isothermal GLC), 12.6% (temperature-programmed GLC), 14.5% (correction factors) and 10.7% (no correction factors). In contrast, the C.V.s for methyl linolenate (which occurs at a similar concentration, *viz.*, 8%) in two samples of soybean oil were 6.2 and 6.0%, while the C.V.s for methyl palmitate (about 10.5%) in the same oils were only 2.9 and 3.5%.

The variability of our own results for the short-chain esters in coconut oil when using the AOCS method[2] led us to query the methodology for their preparation. We now show that, by strict adherence to the standard methods, there is a strong tendency to underestimate low-molecular-weight esters, because they tend not to transfer quantitatively from the aqueous into the hydrocarbon phase used for extraction. We also illustrate that a modified procedure gives much more precise and accurate results in the analysis of a primary standard mixture of triacylglycerols.

EXPERIMENTAL

Chemicals

All reagents were chemically pure. Isooctane (2,2,4-trimethylpentane) was of Baker Analyzed Reagent grade (J. T. Baker, Phillipsburgh, PA, U.S.A.). Heptane was of HPLC grade from Merck (Darmstadt, G.F.R.). A purity check by GLC under conditions similar to those used throughout the experiments showed that there were no peaks on the tail for either solvent.

Reference triacylglycerols were tricaproin, tricaprylin, tricaprin, trilaurin, trimyristin, tripalmitin and tristearin (puriss grade, Fluka, Buchs, Switzerland), and all were individually checked for purity by GLC after conversion into the methyl esters using the modified procedure described below. A primary standard mixture with a chain length distribution which simulated that of coconut oil was made from the reference triacylglycerols. The actual composition was calculated after applying corrections indicated by the purity checks using a procedure similar to that described previously[13]. Only minor corrections were required.

Methylation procedure

Premolten sample or oil (14 drops or approximately 150 mg) was transferred into a 50-ml volumetric flask fitted with a B14 ground-glass joint. The mixture was boiled under reflux for 3 min with 5 ml of 0.5 *M* methanolic potassium hydroxide solution, 5 ml of 14% methanolic boron trifluoride were added and the mixture was boiled under reflux for a further 3 min. The flask was removed from the heat source, 3 ml of isooctane and approximately 15 ml of saturated sodium chloride were added and the flask was stoppered and *shaken vigorously for 15 sec while tepid.* The liquid level was brought to the neck of the flask with more sodium chloride solution and the phases were allowed to separate. Approximately 2.5 μl of the upper layer were injected into the chromatograph.

The modified procedure was evaluated against the AOCS[2] and International Organization for Standardization (ISO)[5] methods by determining the means and standard deviations for each of the component esters of the primary triacylglycerol mixture and also by determining the grade[13,14] of analysis with respect to the known composition. Duplicate analyses of a soybean oil sample were also carried out by each of the three procedures.

Chromatography

GLC was carried out on a Varian Model 2700 chromatograph as described previously[13], except that the samples were injected manually. The detector was optimized for linearity as described previously[13]. Peak areas were measured using a Hewlett-Packard Model 3354 Laboratory Automation System, and sample composition was determined by normalizing the corrected peak areas after applying the theoretical response factors of Ackman and Sipos[15].

RESULTS AND DISCUSSION

The modified procedure which we have evaluated was developed from preliminary studies which indicated that the extraction step was highly critical for the recovery of the short chain esters. As not all of the results from these experiments were relevant to the essential modifications, and as the ultimate test was the performance of the final method for quantitative analyses, we do not report these experiments in detail here, but summarize the conclusions as follows. First, we found reflux times of 3 min to be desirable for both the saponification and methylation steps, and increases in these times had no further effect on the results. Second, various other salts were no more effective than sodium chloride for the salting-out step. Third, isooctane was preferred as the solvent phase as it was found invariably to be free of impurities which appear on the solvent peak tailing during GLC, in contrast to heptane, where only high-performance liquid chromatography grades were found to be acceptable. Fourth, extraction of the esters under tepid conditions (30–40°C) promoted rapid transfer of the esters into the organic phase, while cold conditions (0°C) inhibited transfer. Fifth, under tepid conditions, extraction was satisfactory after shaking vigorously for 15 sec and was not improved by shaking for 1 or 5 min. Finally, the use of a volumetric flask for the reaction facilitated both excellent contact of the phases during extraction and later recovery of the ester solution; it is of interest that Metcalfe *et al.*[1] used such a flask when the procedure was first described.

These experiments led to the selection of several of the particular details described in the modified procedure and also indicated why the various official procedures failed to extract short-chain esters quantitatively. Thus, all four procedures which have been cited differ only in small detail, but one such detail is the extraction step. All are identical in that, after refluxing with boron trifluoride–methanol, the required amount of heptane is added and the mixture boiled for 1 min. The AOCS method[2], which is described as being suitable for "long-chain fatty acids", simply requires that the flask is then topped up with saturated salt solution (without additional mixing). The IUPAC[4] and ISO[5] methods, both of which are stated to be suitable for fatty acids with six or more carbon atoms, and the Association of Official Analytical Chemists (AOAC)[3] method, for which a fatty acid chain length limit is not specified, all require a small portion of salt solution to be added at this stage. It is then

required to "swirl" or "rotate" the flask gently several times before floating the ester solution to the neck by adding more sodium chloride solution. The AOCS procedure thus has the mildest of the extraction steps, the ISO procedure is representative of a gentle extraction step, while the method described here includes a very vigorous extraction step. In our evaluation of the various procedures, the extraction steps were followed in the closest possible detail. The results of analyses of the triacylglycerol mixture are given in Table I for the AOCS procedure, in Table II for the ISO procedure and in Table III for the modified procedure. Statistical data and absolute grades of analysis are included in the tables, and the known composition of the triacylglycerol mixture is included in Table I.

TABLE I

ANALYSIS OF TRIACYLGLYCEROL MIXTURE USING AOCS METHYLATION PROCEDURE

Sample No.	*Composition by GLC analysis (%)*							*Grade of analysis (%)*
	Fatty acid methyl ester							
	6:0	*8:0*	*10:0*	*12:0*	*14:0*	*16:0*	*18:0*	
1	0.39	6.66	6.05	49.09	19.67	8.46	9.69	95.33
2	0.45	6.51	5.92	48.61	19.76	8.67	10.09	94.88
3	0.39	6.35	5.84	48.37	19.96	8.85	10.24	93.29
4	0.43	6.30	5.76	48.33	20.01	8.85	10.31	94.12
5	0.31	5.88	5.62	48.71	20.37	8.96	10.15	92.76
6	0.36	6.57	5.94	48.39	19.67	8.85	10.23	94.84
7	0.40	6.67	6.08	49.41	19.42	8.36	9.67	95.40
8	0.38	6.05	5.66	48.11	20.20	9.02	10.58	93.08
Mean	0.39	6.37	5.86	48.63	19.88	8.75	10.12	94.21
S.D.	0.044	0.28	0.17	0.43	0.31	0.24	0.31	1.05
C.V. (%)	11	4.4	2.9	0.88	1.6	2.7	3.1	
Δ*	−0.54	−1.93	−0.34	+0.90	+0.80	+0.57	+0.54	
Δ (%)	−58	−32	−6	+2	+4	+7	+6	
Known %	0.93	8.31	6.20	47.72	19.08	8.18	9.58	

* Difference between mean and known value.

The above results indicate the deficient recovery of short-chain esters in both the AOCS and ISO procedures. Predictably, the AOCS procedure was marginally less efficient than the ISO method, but both were well short of what we consider to be an acceptable standard (grade $\geq 99\%$). The progressive change in the relative difference between the mean for each ester and the known value (Δ %) from strongly negative to positive indicated that losses were appreciable for all esters up to and including methyl laurate and that only esters of longer chain length gave acceptable recovery. The modified procedure showed much improved results and reached an acceptable standard. Even here, however, there was still some deficiency in the recovery of methyl caproate, underlining the difficulty of obtaining quantitative extraction of this ester.

Analysis of a soybean oil sample confirmed that there was no significant dif-

TABLE II

ANALYSIS OF TRIACYLGLYCEROL MIXTURE USING ISO METHYLATION PROCEDURE

Sample No.	*Composition by GLC (%)*							*Grade of analysis (%)*
	Fatty acid methyl ester							
	6:0	*8:0*	*10:0*	*12:0*	*14:0*	*16:0*	*18:0*	
1	0.50	6.81	6.04	48.38	19.96	8.85	9.67	95.82
2	0.60	7.19	6.03	47.82	19.68	8.64	10.03	96.78
3	0.51	6.75	5.95	48.27	19.90	8.69	9.93	95.56
4	0.52	6.77	5.94	48.15	19.95	8.71	9.97	95.57
5	0.49	6.56	5.95	48.64	19.94	8.60	9.81	95.15
6	0.51	6.70	5.95	48.52	19.99	8.62	9.71	95.45
7	0.54	6.85	6.01	48.90	19.81	8.30	9.60	95.93
8	0.33	6.67	5.92	48.57	19.82	8.67	10.03	94.97
Mean	0.50	6.79	5.98	48.40	19.88	8.61	9.84	95.65
S.D.	0.076	0.19	0.047	0.33	0.10	0.13	0.17	0.55
C.V. (%)	15	2.3	0.79	0.68	0.50	1.5	1.7	
Δ	−0.43	−1.52	−0.22	+0.68	+0.80	+0.43	+0.26	
Δ (%)	−46	−18	−4	+1	+4	+5	+3	

TABLE III

ANALYSIS OF TRIACYLGLYCEROL MIXTURE USING MODIFIED METHYLATION PROCEDURE

Sample No.	*Composition by GLC (%)*							*Grade of analysis (%)*
	Fatty acid methyl ester							
	6:0	*8:0*	*10:0*	*12:0*	*14:0*	*16:0*	*18:0*	
1	0.67	8.12	6.33	47.79	19.11	8.29	9.69	99.11
2	0.76	8.10	6.28	47.70	19.16	8.34	9.67	99.20
3	0.71	8.06	6.32	47.90	19.14	8.30	9.57	99.05
4	0.71	8.10	6.29	47.89	19.13	8.33	9.54	99.07
5	0.67	8.13	6.32	47.84	19.13	8.27	9.64	99.13
6	0.69	8.10	6.31	47.89	19.12	8.31	9.58	99.11
7	0.72	8.19	6.34	47.88	19.04	8.33	9.50	99.10
8	0.63	8.30	6.36	47.91	18.98	8.28	9.54	99.10
Mean	0.69	8.14	6.32	47.85	19.10	8.30	9.59	99.10
S.D.	0.039	0.07	0.027	0.07	0.06	0.027	0.07	0.05
C.V. (%)	5.6	0.9	0.4	0.15	0.3	0.3	0.7	
Δ	−0.24	−0.17	+0.12	+0.13	+0.02	+0.12	+0.01	
Δ (%)	−25	−2	+2	+0.3	+0.1	+2	+0.1	

TABLE IV

DUPLICATE ANALYSES OF SOYBEAN OIL SAMPLE USING THE AOCS, ISO AND MODIFIED METHYLATION PROCEDURES

Fatty acid methyl ester	*Composition by GLC (%)*					
	AOCS		*ISO*		*Modified procedure*	
14:0	0.09	0.07	0.07	0.07	0.07	0.07
16:0	10.59	10.40	10.47	10.33	10.47	10.39
16:1	0.11	0.12	0.14	0.12	0.12	0.12
17:0	0.09	0.11	0.16	0.11	0.10	0.11
17:1	0.08	0.06	0.10	0.06	0.07	0.05
18:0	3.55	3.52	3.50	3.57	3.48	3.49
18:1	20.92	21.08	20.95	21.34	20.89	20.96
18:2	54.98	54.97	54.93	54.74	54.97	55.09
20:0	0.35	0.36	0.35	0.36	0.33	0.33
18:3	8.83	8.91	8.93	8.86	9.08	8.97
22:0	0.43	0.42	0.42	0.44	0.42	0.42

ference between the three procedures for the methylation of long chain fatty acids ($>C_{14}$). These results are given in Table IV.

CONCLUSION

The above studies indicate that the recognized procedures for the methylation of fats and oils with boron trifluoride–methanol, when followed literally, are not suitable for analytes containing significant amounts of fatty acids with chain lengths shorter than about C_{12}. This criticism is particularly relevant to the IUPAC[4] and ISO[5] procedures, which are specifically stated to be suitable for fatty acids with six or more carbon atoms. It is likely that the gentle extraction step in these procedures is meant to avoid emulsification. In our experience this is not a problem. In any event, the alternative, which is poor quantitative accuracy for the shorter chain acids, is unacceptable. It also follows that the procedure of boiling the reaction mixture with heptane for 1 min after methylation is not effective in transferring the shorter chain esters quantitatively into the organic layer; the modified procedure which we evaluated does not include this step. We believe that the many satisfactory results which appear in the literature for the analysis of coconut oil and similar materials may be influenced by two factors. First, it is likely that many analysts carry out the extraction with more vigorous action than that described in the methods. Second, the accuracy of the results may be improved by the application of arbitrary correction factors. In this respect, our results confirm that excellent quantitation may be obtained using only the theoretical response factors of Ackman and Sipos[15] provided the detector is optimized for linearity[13] and provided a truly representative extraction is achieved. While the recognized methods give satisfactory results for long chain fatty acids, it would appear to be a wise precaution to include a vigorous extraction step in the preparation of the methyl esters of all fats and oils.

ACKNOWLEDGEMENTS

This work was supported financially by the Australian Industrial Research and Development Incentives Board. Thanks are due to the Directors of Unilever for permission to publish this paper.

REFERENCES

1 L. D. Metcalfe, A. A. Schmitz and J. R. Pelka, *Anal. Chem.*, 38 (1966) 514.
2 *Official and Tentative Methods of the American Oil Chemists' Society*, American Oil Chemists' Society, Champaign, IL, 3rd ed., 1973 (revised to 1980), Method Ce 2-66.
3 *Official Methods of Analysis of the AOAC*, Association of Official Analytical Chemists, Washington, DC, 13th ed., 1980, sections 28.053–28.056.
4 *Standards Methods for the Analysis of Oils, Fats and Derivatives*, International Union of Pure and Applied Chemistry (IUPAC), Applied Chemistry Division, Commission on Oils, Fats and Derivatives, Part I (Sections 1 and 2), Pergamon Press, Oxford, 6th ed., 1979, Method 2.301, Section 3.
5 *International Standard*, International Organization for Standardization (ISO), Geneva, 1st ed., 1978, Ref. No. ISO-5509-1978 (E), Section 4.
6 H. P. Kaufmann and G. Mankel, *Fette-Seifen-Anstrichm.*, 65 (1963) 179.
7 A. J. Sheppard and J. L. Iverson, *J. Chromatogr. Sci.*, 13 (1975) 448.
8 M. L. Vorbeck, L. R. Mattick, F. A. Lee and C. S. Pederson, *Anal. Chem.*, 33 (1961) 1512.
9 G. W. McGinnis and L. R. Dugan, Jr., *J. Amer. Oil Chem. Soc.*, 42 (1965) 305.
10 J. M. DeMan, *Lab. Pract.*, 16 (1967) 150.
11 H. L. Solomon, W. D. Hubbard, A. R. Prosser and A. J. Sheppard, *J. Amer. Oil Chem. Soc.*, 51 (1974) 424.
12 D. Firestone and W. Horwitz, *J. Ass. Offic. Anal. Chem.*, 62 (1979) 709.
13 D. E. Albertyn, C. D. Bannon, J. D. Craske, N. T. Hai, K. L. O'Rourke and C. Szonyi, *J. Chromatogr.*, 247 (1982) 47.
14 S. F. Herb and V. G. Martin, *J. Amer. Oil Chem. Soc.*, 47 (1970) 415.
15 R. G. Ackman and J. C. Sipos, *J. Amer. Oil Chem. Soc.*, 41 (1964) 377.