Analysis of Fatty Acid Methyl Esters With High Accuracy and Reliability. V. Validation of Theoretical Relative Response Factors of Unsaturated Esters in the Flame Ionization Detector

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Because unsaturated fatty acid methyl esters (FAME) are subject to autoxidation, it is virtually impossible to obtain and maintain high purity standards. Accordingly, it is not possible to determine flame ionization detector response factors by the usual technique of analyzing standard mixtures of known composition. In an alternative approach, the response factors of methyl oleate, methyl linoleate, methyl linolenate, methyl arachidonate and methyl 4,7,10,13,16,19-docosahexaenoate relative to methyl stearate were estimated by determining the peak areas before and after quantitative hydrogenation in the presence of an internal standard. The estimates showed excellent agreement in all cases with the theoretical factors predicted by Ackman and Sipos and thus constitute an independent and unambiguous proof that the theoretical factors are highly accurate for all olefinic unsaturated FAME. Whereas it is common practice to determine an empirically derived correction factor for each FAME by analyzing standard mixtures of known composition, the thesis is now proposed that, for both saturated and olefinic unsaturated FAME, the proper approach to accurate analysis requires that peak areas be corrected using the theoretical response factors as the only correction factors. If the correct result cannot be obtained when analyzing a primary standard of saturated FAME, it is an indication of faulty technique or equipment, and the only acceptable resolution of the problem is to locate and correct the fault(s).

In recent studies (1,2) we have shown that the theoretical factors first proposed by Ackman and Sipos (3) in 1964 for the relative response of fatty acid methyl esters (FAME) in the flame ionization detector (FID) are highly accurate for all saturated, straight-chain esters from methyl stearate down to and including methyl butyrate. These factors were stated (3) to be proportional to the weight percent in the molecule of "active" carbon atoms, which includes all carbon atoms except that of the carbonyl group. Ackman and Sipos proposed that olefinic carbon atoms give a full active carbon atom response and that corrections for unsaturated esters should, therefore, be made on the same basis as those for saturated esters. To support this thesis, the authors showed that slightly improved estimates of the iodine value (IV) could be made from the gas liquid chromatographic (GLC) analysis when the theoretical factors were applied. While these results supported the need to apply theoretical response factors in the case of unsaturated FAME, it cannot be claimed that this constituted a proof of the accuracy of the theoretical factors.

In 1970, Shehata et al. (4) compared the observed factors for a number of FAME, including some unsaturated esters, with the theoretical factors and reported good agreement. However, when the observed factors are expressed relative to methyl stearate, the following estimates for the unsaturated FAME are obtained: 16:1, 1.20 (theoretical 1.012); 18:1, 1.03 (0.993); 18:2, 1.12 (0.986); 18:3, 1.07 (0.980). These figures actually show poor agreement between the observed and theoretical factors. Such data do not disprove the accuracy of the factors, because the discrepancies could be explained alternatively on the grounds that significant systematic errors were present. No further studies aimed specifically at investigating the accuracy of the theoretical factors for unsaturated esters have been reported.

Despite the lack of accurate supportive data for the relative ponse factors of unsaturated FAMEs, there are plausible theoretical grounds for expecting that the Ackman and Sipos factors may be highly accurate. These factors would be expected if olefinic carbon atoms have an exactly equal per carbon response in the FID compared to saturated carbon atoms, and a degradation mechanism such as the hydrogen cracking model proposed by Blades (5) might readily account for this. On the other hand, estimates of the exact response of olefinic carbon atoms have varied and are represented by the data of Sternberg et al. (6), who found an effective carbon number of 0.95 for olefinic carbon atoms: of Blades (7), who found values of 1.02 for ethylene and 1.00 for both propylene and butylene, and of Nicholson (8), who found a value of 0.99 for ethylene. Values other than 1.00 would result in deviations from the Ackman and Sipos factors.

Relative response factors normally are determined directly by the analysis of a mixture of the analyte of interest and the reference compound. In the case of unsaturated FAMEs, however, it is virtually impossible to obtain and maintain high purity standards for reasons of autoxidation. If the purity of the unsaturated ester is not known, an accurate estimate of the relative response factor cannot be made. In the present study we have endeavored to overcome this problem by adding an internal standard to the unsaturated ester and converting the latter into a saturated ester of known relative response factor by quantitative hydrogenation. The relative response factor of the unsaturated ester may then be determined by relating the peak areas before and after hydrogenation using the internal standard, while allowing for the stoichiometry of hydrogenation.

Because the effect of a double bond on the relative response factor is small, e.g. methyl oleate has a theoretical response of 0.9932 relative to methyl stearate, the accuracy with which measurements need to be made is very high. Hydrogenation must be quantitative, and the formation of by-products, such as alicyclic or aromatic FAME monomers (9), must be avoided. Detectable impurities which coincide with the internal standard, the unsaturated ester or the hydrogenation product in the chromatograms must be taken into account. Other impurities, including undetected and undetectable impurities in the unsaturated ester, may be ignored: it is in this respect that the hydrogenation method has a crucial advantage over the direct determination of relative response factors. Finally, care must be taken to eliminate all systematic errors of analysis from the chromatographic system.

[1]Part IV is Reference 2.

The hydrogenation method may be expressed in general terms according to equation [1].

$$A'_x R_x = A_x R_x + \Sigma c_i A_i R_i \quad [1]$$

where A'_x = area of a given saturated FAME x resulting from hydrogenation
R_x = response factor of the same FAME x
A_x = area of the same FAME x before hydrogenation (if present as an impurity)
A_i = area of any unsaturated FAME i which, when hydrogenated, is converted into saturated FAME x
R_i = response factor of FAME i
c_i = stoichiometry factor for the conversion of FAME i into FAME x as a result of hydrogenation

The various values of A in equation [1] must be determined before and after hydrogenation under identical analytical conditions, hence, an appropriate correction is made to the value of A'_X using the internal standard as indicated in equation [2].

$$A'_x = A'_x \text{ (observed)} \cdot \frac{A_s}{A'_s} \quad [2]$$

where A'_x (observed) = apparent area of x after hydrogenation
A_s = area of internal standard before hydrogenation
A'_s = area of internal standard after hydrogenation

In the present study we have applied the hydrogenation technique to estimate the response factors of the FAMEs 18:1, 18:2, 18:3, 20:4 and 22:6 relative to 18:0.

EXPERIMENTAL PROCEDURES

Isooctane (2,2,4-trimethylpentane) and diethyl ether were both Pronalys analytical reagent grade (May and Baker, West Footscray, Victoria, Australia). The hydrogenation catalyst was 10% palladium on active charcoal (E. Merck, Darmstadt, G.F.R.), and silica was 200-325 mesh CC-7 (Mallinckrodt, New York, New York). High purity hydrogen was supplied from a Mark V Elhygen hydrogen generator (Milton Roy, Riviera Beach, Florida). Methyl esters of the purest available grades were methyl margarate, methyl oleate, methyl linoleate, methyl linolenate, methyl arachidonate, methyl 4,7,10,13,16,19-docosahexaenoate (all Sigma, St. Louis, Missouri), methyl stearate, methyl arachidate and methyl behenate (all E. Merck, Darmstadt, G.F.R.). Purity checks were carried out on the esters both before and after hydrogenation under the experimental conditions used for the main experiments. The saturated esters were also examined for the presence of free fatty acids as described previously (2). The model compound used to investigate the possible formation of aromatic FAME monomers during hydrogenation was methyl 9(2'-propylphenyl)-nonanoate.

APPARATUS

GLC was carried out on a Hewlett-Packard model 5790 gas chromatograph fitted with a capillary inlet system and an FID. The column was 10 m x 0.25 mm ID fused silica coated with 0.2μ of DEGS (Chrompack, Middleburg, The Netherlands). The carrier gas was high purity hydrogen which had an inlet pressure of 3.5 psi. The split vent flow-rate was 170-210 ml/min and the septum purge flow-rate ca. 3 ml/min. The total hydrogen flow-rate to the detector was 30 ml/min, the make-up gas was high purity nitrogen and had a flow-rate of 30 ml/min, and oil-free compressed laboratory air was supplied at a flow-rate of ca. 400 ml/min. The injector temperature was 245-250 C, and the detector temperature was 250 C. The column oven temperature was 170 C. Peak areas were measured using a Hewlett-Packard model 3354 Laboratory Automation System.

PROCEDURE

Hydrogenations. Hydrogenations were carried out by shaking a mixture of the internal standard (ca. 100 mg) with the unsaturated ester (ca. 150 mg) in isooctane (4 ml) under high purity hydrogen in the presence of catalyst (50 mg) for 20 min at room temperature. Diethyl ether (10 drops) was added to the mixture, which was filtered through silica (50 mg) before GLC analysis.

Determination of relative response factors of unsaturated esters. The determination of the relative response factor of a given unsaturated ester was carried out in two steps. In the first step, the quantitative accuracy of the total experimental procedure was established using a primary standard mixture consisting of the selected internal standard and the saturated ester of the same chain-length as the unsaturated ester being studied. This mixture was subjected to the hydrogenation procedure and GLC analysis and was required to be analyzed with an accuracy of ± 0.1% for each of the two components before the unsaturated ester of interest was studied. It was further required that this accuracy be achieved using as the only correction factors the theoretical relative response factors of Ackman and Sipos, which were 1.0091 for methyl margarate, 1.000 for methyl stearate, 0.9846 for methyl arachidate and 0.9720 for methyl behenate. If a standard did not come up to the required accuracy, adjustments to the chromatographic system were made until the stated accuracy was achieved. In the present study, small adjustments to the injector temperature and/or split vent flow-rate were all that were required to obtain satisfactory results. For each of the three C18 unsaturated esters, this saturated primary standard consisted of a mixture of methyl margarate (internal standard) and methyl stearate. For 20:4, the primary standard consisted of methyl stearate (internal standard) and methyl arachidate, while for 22:6 the primary standard consisted of methyl arachidate (internal standard) and methyl behenate.

In the second step, the relative response factor of a given unsaturated ester was determined immediately after the system had been standardized satisfactorily as above. A mixture of the internal standard and unsaturated ester in isooctane was prepared for hydrogenation as described above and analyzed in duplicate by GLC. The mixture was hydrogenated immediately and again analyzed in duplicate by GLC. Raw areas were corrected, when necessary, for impurities from one or both of the two major components which coincided with any peak of interest. Thus, the raw area of the internal standard was corrected pro rata for impurities in the unsaturated ester which coincided with it, and vice versa, using data derived from the purity checks on the individual esters. Corresponding corrections were made to the results of analyses after hydrogenation using data derived from the internal standard and the unsaturated ester after they had been hydrogenated individually. The corrected areas were normalized with respect to an arbitrary internal standard raw

area of 500,000. The mean results for a given pair of duplicate analyses were used to calculate the required relative response factor. In the case of the three C18 esters, each was found to contain small, but significant, amounts of one or both of the other two and also of 18:0. The corrected and normalized areas for the three esters were thus used to construct three linear equations according to equation [1] which were then solved to give the estimates of the relative response factors. This was not possible in the case of 20:4 and 22:6, and minor approximations were made as discussed later in order to deal with small, but significant, amounts of impurities which were found in both cases. Each of two operators carried out duplicate hydrogenations on each of the unsaturated esters so that four estimates of the relative response factor were made.

The stoichiometry factors, which correct for the change in molecular weight during hydrogenation, for the conversion of the various unsaturated esters into the corresponding saturated esters, were as follows: 18:1, 1.00680; 18:2, 1.01369; 18:3, 1.02068; 20:4, 1.02532 and 22:6, 1.03531. In the cases of 20:4 and 22:6, the initial estimates of the response factors were relative to 20:0 and 22:0, respectively. These were converted to response factors relative to 18:0 by multiplying by the appropriate theoretical factors, which were 0.9846 for 20:0 and 0.9720 for 22:0.

RESULTS AND DISCUSSION

Table IA summarizes the results of the purity checks on the individual C18 FAMEs before and after hydrogenation, and Table IB summarizes similar data for the C20 and C22 FAMEs. Only those figures are detailed which were relevant to the corrections required for the primary standard mixtures of saturated FAMEs and for the results of the hydrogenation studies with the unsaturated esters.

Only minor corrections were needed to calculate the exact compositions of the primary standard mixtures. The unsaturated esters all showed small, but significant, amounts of impurities, both before and after hydrogenation, which were taken into account later when determining the required relative response factors.

ANALYSIS OF PRIMARY STANDARDS

Typical results of analyses of the three primary standard mixtures are given in Table II.

An incidental conclusion from the results in Table II is that the accuracy of the theoretical relative response factors for methyl arachidate and methyl behenate, which have not been previously investigated, was verified.

TABLE IA

Purity Check on Reference C18 Fatty Acid Methyl Esters

Fatty acid methyl ester	Composition by GLC analysis (%)									
	Reference ester									
	Methyl margarate		Methyl stearate		Methyl oleate		Methyl linoleate		Methyl linolenate	
	A[a]	B[b]	A	B	A	B	A	B	A	B
17:0	99.51	99.56	0.33	0.33						
18:0			99.32	99.43	0.22	99.72		99.97		99.55
18:1			0.08		99.24		0.20		0.01	
18:2	0.06		0.02		0.37		99.61		0.53	
18:3							0.11		98.82	
18:2-con					0.12		0.03			
18:U						0.21				0.12
18:3-con									0.56	
Fatty acid			0.05							
Irrelevant peaks	0.43	0.44	0.20	0.24	0.05	0.07	0.05	0.03	0.08	0.33

[a]A, before hydrogenation. [b]B, after hydrogenation.

TABLE IB

Purity Check on Reference C20 and C22 Fatty Acid Methyl Esters

Fatty acid methyl ester	Composition by GLC analysis (%)							
	Reference ester							
	Methyl arachidate		Methyl arachidonate		Methyl behenate		Methyl 4,7,10,13,16,19 – docosahexaenoate	
	A	B	A	B	A	B	A	B
20:0	99.05	99.05		99.85				
20:2			0.81					
20:4			98.74					
20:5			0.12					
22:0	0.38	0.38			99.83	99.83		99.40
22:4							0.51	
22:5							0.09	
22:6							98.87	
Fatty acid								
Irrelevant peaks	0.57	0.57	0.33	0.15	0.17	0.17	0.53	0.60

TABLE II

Analysis of Primary Standard Mixtures

Fatty acid methyl ester	Composition (%)								
	Mixture 1			Mixture 2			Mixture 3		
	Observed		Known	Observed		Known	Observed		Known
17:0	36.86	36.78	36.85						
18:0	63.14	63.22	63.15	41.34	41.42	41.36			
20:0				58.66	58.58	58.64	34.44	34.53	34.48
22:0							65.56	65.47	65.52

INVESTIGATION OF POSSIBLE HYDROGENATION BY-PRODUCTS

No peaks corresponding to possible aromatic FAME monomers were detected in the appropriate regions of the chromatograms. Trace peaks which possibly could have been alicyclic FAME monomers were detected in certain of the hydrogenation products, especially that of methyl linolenate. It was more likely, however, that such peaks were incompletely hydrogenated monoenes, and minor corrections were made as discussed below to allow for their presence. Even smaller peaks corresponding to probable unhydrogenated monoenes were seen in the methyl oleate and methyl linoleate samples: corrections for these also were readily made. No such peaks were seen in the hydrogenation products of methyl arachidonate and methyl 4,7,10,13,16,19-docosahexaenoate. It was concluded that significant amounts of cyclic fatty acids had not formed during the hydrogenations.

DETERMINATION OF RESPONSE FACTORS OF UNSATURATED ESTERS

C18 esters. The corrected raw areas for all three C18 unsaturated esters before and after hydrogenation are summarized in Table III. The figures have been normalized with respect to an arbitrary raw area of 500,000 for the internal standard (methyl margarate).

The methyl oleate sample contained small, but significant, amounts of 18:0 and 18:2, which were included as such in the appropriate equation. Also present were two small peaks which had retention times corresponding to conjugated isomers of 18:2. That these compounds were unsaturated was indicated by their disappearance after hydrogenation. As conjugated dienes could reasonably be expected to be present in the sample as a result of autoxidation, the peaks were assigned this identity and their areas summed with the 18:2 peak in the appropriate equation. The hydrogenated samples contained traces of a peak denoted in Table III as "18:U." The concentration of this peak varied considerably, suggesting that it probably was incompletely hydrogenated monoene. This identity was assumed and the area added to that of the 18:0 peak after the appropriate stoichiometric conversion.

The methyl linoleate sample was similarly found to contain small amounts of impurities which included 18:1, 18:3 and suspected conjugated dienes. The data were treated as for the methyl oleate. The hydrogenated sample contained traces of a peak which was again designated as "18:U" for reasons similar to those given above. The area was thus added to that of the 18:0 peak after an appropriate stoichiometric conversion.

TABLE III

Corrected Mean Raw Areas For C18 Unsaturated FAME Before and After Hydrogenation Normalized With Respect to an Internal Standard Raw Area of 500,000

Fatty acid methyl ester	Corrected and normalized raw area											
	18:1				18:2				18:3			
	Operator 1		Operator 2		Operator 1		Operator 2		Operator 1		Operator 2	
	A[a]	B[b]	A	B	A	B	A	B	A	B	A	B
18:0	1,894	824,523	1,852	782,638		676,188		739,216		678,362		782,162
18:1	821,035		775,056		1,367		1,486		81		60	
18:2	2,981		2,805		673,295		737,419		3,604		4,191	
18:3					756		824		674,846		777,044	
18:2-con	980		904		1,054		806					
18:3-con									2,125		2,874	
18:U		1,439		58		176		157		1,098		1,286
	Duplicate Hydrogenations											
18:0	1,891	766,616	1,630	751,302	444	755,277	2,658	826,790	336	660,332	226	809,598
18:1	758,856		749,211		1,330		2,199					
18:2	2,768		2,613		752,706		819,714		1,530		1,880	
18:3					713		817		654,980		803,338	
18:2-con	934		485									
18:3-con									1,151		1,500	
18:U		3,393		3,877						690		640

[a]A, before hydrogenation. [b]B, after hydrogenation.

The methyl linolenate sample contained traces of 18:2 and two peaks with retention times corresponding to conjugated trienes whch disappeared after hydrogenation. This last pair is summed in Table III as "18:3-con," and the area was added to that of the 18:3 peak for calculation purposes. The hydrogenated sample showed a peak corresponding to "18:U" which was reasonably reproducible throughout the results and for those in Table IA. This compound thus could have been a by-product of hydrogenation such as an alicyclic FAME monomer, or it simply may have been the hydrogenation product of a minor impurity in the methyl linolenate. The area was added to that of the 18:0 peak after an appropriate stoichiometric conversion assuming the compound to be a C18 ester containing one double bond equivalent.

The salient feature of the above results was that the vast bulk of the unsaturated ester was converted in each case into methyl stearate during hydrogenation. While it was essential to include the areas of the minor peaks in the calculations, their actual identities were of little significance in the circumstances. If, for example, a peak designated "18:1" was actually 18:2, then an error would be introduced due to the differing response factors of the two. However, as all the peaks of uncertain identity were trace components, the effect of any such error was calculated to be negligible.

The data in Table III were used to construct sets of three linear equations for each of the two operators and two hydrogenations according to equation [1]. This provided four estimates of the required response factors. The resulting estimates of the response factors, together with those obtained below for 20:4 and 22:6, are given later in Table VI.

Methyl arachidonate. The corrected raw areas for methyl arachidonate before and after hydrogenation are summarized in Table IV. The figures have been normalized with respect to an internal standard (methyl stearate) raw area of 500,000.

Two minor impurities with retention times corresponding to 20:2 and 20:5 were detected in the methyl arachidonate sample. For calculation purposes, the areas of both peaks were added to that of the 20:4 peak. This necessarily introduced an error into the estimates of the relative response factor for 20:4, but it was calculated that the magnitude of this error was very small. The hydrogenated sample showed no evidence of hydrogenation by-products.

The above data afforded estimates of the response factor of 20:4 relative to 20:0, which then were converted into estimates relative to 18:0. The final estimates are included in Table VI.

Methyl 4,7,10,13,16,19-docosahexaenoate. The corrected raw areas for methyl 4,7,10,13,16,19-docosahexaenoate before and after hydrogenation are summarized in Table V. The

TABLE IV

Corrected Raw Areas For Methyl Arachidonate Before and After Hydrogenation Normalized With Respect to an Internal Standard Raw Area of 500,000

Fatty acid methyl ester	Corrected and normalized raw area of 20:4			
	Operator 1		Operator 2	
	A[a]	B[b]	A	B
20:0		824,385		732,131
20:2	6,696		5,934	
20:4	821,270		727,326	
20:5	1,146		1,066	
	Duplicate Hydrogenations			
20:0		777,233		758,058
20:2	6,340		6,094	
20:4	772,464		745,736	
20:5	537		992	

[a]A, before hydrogenation. [b]B, after hydrogenation.

TABLE V

Corrected Raw Areas for Methyl 4,7,10,13,16,19-Docosahexaenoate Before and After Hydrogenation Normalized With Respect to an Internal Standard Raw Area of 500,000

Fatty acid methyl ester	Corrected and normalized raw area of 22:6			
	Operator 1		Operator 2	
	A[a]	B[b]	A	B
22:0		684,680		841,081
22:4	3,338		3,919	
22:5	583		714	
22:6	679,868		832,810	
	Duplicate Hydrogenations			
22:0		726,672		747,084
22:4	1,190		1,212	
22:5			150	
22:6	726,123		743,118	

[a]A, before hydrogenation. [b]B, after hydrogenation.

TABLE VI

Estimates, Means and Standard Deviations of Response Factors of Unsaturated Esters Relative to Methyl Stearate

Fatty acid methyl ester	Relative response factor								
	Operator 1		Operator 2		Mean	Standard Deviation	Theoretical		
							Effective 1.00	Carbon 0.99	Number 0.95
18:1	0.992	1.000	0.996	0.995	0.996	0.003	0.993	0.994	0.999
18:2	0.986	0.987	0.985	0.988	0.986	0.001	0.986	0.989	0.998
18:3	0.978	0.984	0.979	0.984	0.981	0.003	0.980	0.983	0.996
20:4	0.958	0.955	0.957	0.967	0.959	0.005	0.960	0.964	0.980
22:6	0.940	0.938	0.943	0.942	0.941	0.002	0.939	0.944	0.965

figures have been normalized with respect to an internal standard (methyl behenate) raw area of 500,000.

The methyl 4,7,10,13,16,19-docosahexaenoate sample contained small amounts of impurities which corresponded in retention times to 22:4 and 22:5, and their areas were added to that of the 22:6 peak. The error resulting from this approximation was again calculated to be very small. The hydrogenated sample showed no evidence of hydrogenation by-products.

The above data afforded estimates of the response factor of 22:6 relative to 22:0, which were then converted into estimates relative to 18:0. The final estimates are included in Table VI, together with the estimates of all the other response factors. Included in Table VI are the predicted factors for effective carbon numbers for olefinic carbon atoms of 1.00, which are the Ackman and Sipos factors, as well as those obtained using effective carbon numbers of 0.95(6) and 0.99(8).

The estimates of the relative response factors showed the best agreement with the Ackman and Sipos factors in the cases of 18:2, 18:3, 20:4 and 22:6 and acceptable agreement in the case of 18:1. We attribute this poorer agreement in the case of 18:1 to experimental error resulting from the formidable demands on accuracy required by the experiment. It is argued that the overwhelming weight of evidence of the other four esters militates against a possible deviation from the Ackman and Sipos theoretical for 18:1. The results thus indicate that these factors are highly accurate for the esters studied and, in turn, it is reasonable to extend this conclusion to all other olefinic unsaturated FAME, which allows the response in the FID of any such ester to be calculated accurately. We thus extend to all the common saturated and unsaturated FAME our thesis that the proper approach to accurate fatty acid analysis requires that peak areas should be corrected using the Ackman and Sipos theoretical factors as the only correction factors, and that all other sources of systematic errors should be eliminated.

The results also illustrate that the corrections accordingly required are not insignificant, e.g., an error of about 6% relative to 18:0 is incurred in the case of 22:6 if no correction is applied, with often is the practice.

With regard to the hydrogenation technique itself as a means of determining relative response factors, we already have stated that, in principle, an advantage of the technique is that it is possible to work with esters of unknown purity. In practice, we have sometimes found that esters known to be severely autoxidized may lead to inconsistent results and that it is advisable to work with good quality esters. This does not detract from the stated advantage, as it is not possible to estimate the absolute purity with certainty of even the best quality unsaturated esters obtainable.

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