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Quantification of long chain polyunsaturated fatty acids by gas chromatography Evaluation of factors affecting accuracy

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Abstract

The accurate and reproducible analysis of long-chain polyunsaturated fatty acids (PUFA) is of growing importance. Especially for labeling purposes, clear guidelines are needed in order to achieve optimum accuracy. Since calibration standards cannot be used for method validation due to the instability of PUFAs, there is no direct way to check for the absence of systematic errors. In this study the sources of error that weaken the accuracy were evaluated using theoretical considerations and calibration standards with corrected composition. It was demonstrated that the key role for optimum accuracy lies in the optimization of the split injection system. Even when following the instructions outlined in the official methods of the American Oil Chemist's Society (AOCS), systematic errors of more than 7% can arise. Clear guidelines regarding system calibration and selection of appropriate internal standards (IS) can improve precision and accuracy significantly.

Keywords: Gas chromatography; Optimization; Fatty acid methyl esters; Omega-3 fatty acids; Accuracy; Precision

1. Introduction

In most cases, research on polyunsaturated fatty acids (PUFA) is performed by comparative studies, in which different dietary groups are compared. The absolute accuracy in such studies is not the ultimate priority. However, a lack of accuracy cannot be accepted when analyzing functional foods enriched with long-chain omega-3 PUFA (*n*-3 LC-PUFA), at least when levels are declared on the product label. The main problem for the analysis of *n*-3 LC-PUFA is that PUFAs are rather unstable, so that calibration cannot be performed by using quantitative standards. This makes the use of correction factors a question of faith. Whereas some analysts use empirical response factors (ERF), others trust in theoretical response factors (TRF), but many do not use correction factors at all.

The official methods of the Association of Analytical Chemists (AOAC) [1] and the American Oil Chemists's Society (AOCS) [2] provide clear guidelines for accurate quantification of *n*-3 LC-PUFAs in marine oils. Both methods stipulate transmethylation by sodium hydroxide/boron trifluoride [3,4]. Furthermore, the use of C23:0 (methyl tricosanoate) as internal standard (IS) and wax-type capillary columns are mandatory. Both methods [1,2] describe the application of TRFs as proposed by Ackman and Sipos [5] and Bannon et al. [6]. However, the AOCS method does not strictly demand the application of these factors by indicating that they only "should" be used for optimum accuracy. Split injection at 250 °C and a split rate of 1:50 is recommended as the preferred injection technique in both methods. Nevertheless, the description of the procedure leaves the possibility to apply other injection techniques, such as on-column injection (OCI). It has been proposed that the injection technique, especially in vaporizing injectors, is the main source of error in quantitative GC [7].

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The validity of the concept of TRFs by Ackman and Sipos [5] has been confirmed for unsaturated fatty acids by Bannon et al. [6]. Applying this concept is the only practicable way to ensure accurate instrument set-up without using unsaturated calibration standards. However, the study of Bannon et al [6] was performed by using split injection. According to Grob, discrimination effects in vaporizing injection systems can be minimized extensively, but cannot be eliminated completely [8]. Discrimination effects inside the injector are mainly caused by different volatilities of analytes. Therefore, it is likely to assume that suboptimal performance of the injection system could affect long-chain saturated fatty acids (internal standards) to a different extent as PUFAs.

The aim of this study was (I) to reevaluate the concept of TRFs for unsaturated fatty acids by using non-discriminative OCI; (II) estimate to what extent the variables allowed in the official AOAC and AOCS methods affect the accuracy of these methods and (III) to suggest solutions that help to reduce the bandwidth of possible errors.

2. Experimental

2.1. Materials

Methyl esters of tetradecanoic acid (C14:0), hexadecanoic acid (C16:0), octadecanoic acid (C18:0), eicosanoic acid (C20:0), docosanoic acid (C22:0), tetracosanoic acid (C24:0), linoleic acid (C18:2n6), arachidonic acid (C20:4n6), docosahexaenoic acid (C22:6n3), heptadecanoic acid (C17:0), nonadecanoic acid (C19:0), heneicosanoic acid (C21:0) and tricosanoic acid (C23:0) were obtained from Larodan™ (Malmö, Sweden). Standards were dissolved in hexane (HPLC grade, containing 100 ppm of *t*-butylhydroxytoluene), obtained from Merck™ (Darmstadt, Germany). The final concentration of the methyl ester standards was 0.05 mg/ml per component for OCI and 1 mg/ml for split injection. Refined Seal Blubber Oil was obtained from Atlantis Marine Inc. (St. John's, NL, Canada) and was transmethylated with sodium methoxide according to Bannon et al. [9].

2.2. GC–FID analysis

In order to check for robustness, different GC systems were used in this study. Analyses were made on two Carlo Erba HRGC 5000 series gas chromatographs (Carlo Erba, Milan, Italy), one equipped with manual OCI and one with manual split injection, and one Shimadzu 9A (Shimadzu Europe Ltd., Duisburg, Germany) with manual split injection. In OCI mode, the analytical column was connected to a methyl-deactivated retention gap (0.53 mm I.D., 40 cm length), which was conducted through a 20 cm isolation mantle in order to ensure secondary cooling over the entire flooding zone [10]. Split injection was performed at 250 °C into an empty glass liner (Restek, Bellefonte, PA, USA) with an I.D. of 4 mm at a split ratio of 1:50. Injection was performed as follows: 0.8 μl of solvent ahead of 0.2 μl air and 1.0 μl sample were retracted completely into the glass-body of the syringe. The syringe was left inside the injector for 5 s followed by a rapid injection (hot-needle technique). The needle was kept in the injector for another 5 s, as recommended by Ackman [11]. In order to check for robustness, variations to this technique were applied, which are outlined in Section 3.

Two different types of capillary columns were used in this study: (1) Supelcowax 10 (Supelco, Bellefonte, PA, USA), 10 m, 0.10 mm I.D., 0.1 μm d.f.; (2) RTX 225 (Restek, Bellefonte, PA, USA), 30 m, 0.25 mm I.D., 0.25 μm d.f. Hydrogen, at inlet-pressures of 2.3 bar (column 1) and 1.0 bar (column 2) were employed. Injection volumes were 0.5 μl (OCI) and 1 μl (split). The oven temperature programs were: 170 °C (1 min), followed by a 10 °C/min ramp to 240 °C (column 1) and 170 °C (2 min), 3 °C/min to 220 °C (column 2). In the OCI mode secondary cooling was activated 3 min prior to injection and stopped immediately after injection.

Purity checks were carried out for each component, and corrections for impurities were applied according to Albertyn et al. [12]. In brief, the total amount of minor peaks found in the GC profile of a single standard was subtracted from the total amount of this standard substance in the calibration mixture. If one minor peak coincided in the chromatogram with another of the standard components, the weight contribution of this component was increased accordingly. TRFs were calculated as described previously [13].

ERFs of C18:2n6, C20:4n6 and C22:6n3 were measured in a mixed standard containing C17:0, C19:0 and C23:0 as reference. Two milliliters of this standard were dried under nitrogen, re-diluted in tetrahydrofuran and hydrogenated in a stream of hydrogen after the addition of ca 20 mg PtO_2 (Adam's catalyst). Hydrogen was taken from the split outlet of the GC at a rate of 20 ml/min and bubbled through the sample using a Pasteur pipette. During reaction, which was performed for 2 h, the flask was cooled under reflux in order to avoid evaporative losses of the solvent. Following the reaction the sample was filtered through a syringe filter (3 μm pore size), evaporated to dryness and re-dissolved in hexane. The hydrogenated sample was analyzed on the OCI–GC system and results were used to correct the weigh-in of the unsaturated fatty acid methyl esters (FAME). The reaction was considered complete as no remaining unsaturated components were detected in the chromatograms. ERFs were measured in relation to these corrected weights using the OCI–GC. Factors were normalized to C18:0 = 1 for general comparison and to C23:0 when using C23:0 as IS.

3. Results and discussion

The reassessment of the TRFs for unsaturated FAMEs using an OCI–GC system confirmed the data reported by Bannon et al. [6] (Table 1). It could therefore be concluded that the OCI system produced accurate results for PUFAs. Table 2

Table 1
Theoretical and empirical response factors of different unsaturated FAMEs for correction of flame ionization detector response (normalized to C18:0 = 1)

	TRF[a]	TRF[b]	ERF (OCI)[c]	ERF[d]
C18:2	0.986	0.986	0.990 ± 0.002	0.986 ± 0.001
C20:4	0.960	0.958	0.960 ± 0.011	0.959 ± 0.005
C22:6	0.939	0.934	0.935 ± 0.012	0.941 ± 0.002

[a] Theoretical response factors, calculated according to Ackman and Sipos [5].
[b] TRFs calculated according to Schreiner and Hulan [13].
[c] Empirical response factors, sample injected on-column ($n = 5$).
[d] Data from Bannon et al. [6].

presents the relative error expressed as percent discrimination versus the theoretical content of C22:6n3 obtained in four different single injections. ERFs of the calibration runs, upon which the data have been calculated, are also shown. Not surprisingly, the OCI system (Table 2, A) produced the most accurate result with only −0.01% discrimination when compared to C23:0 IS and still less than 1% (−0.69%) when compared to C17:0 IS. Injections B–D in Table 2 represent split injections of different quality (B: optimized, C: average, D: poor). Among the split injections, only injection "B" meets the requirements outlined in AOCS method Ce 1b-89 [2], that TRFs and ERFs should not deviate by more than 5% from each other. Consequently, the relative error is well below this 5% limit (3.49%), whereas injections C + D result in discriminations of more than 5%. However, when the analytical issue is to measure *n*-3 LC-PUFAs only (versus C23:0 IS), one could conclude that system calibration would only be needed in this chromatographic range. As seen in Table 2, also injection "C" would be acceptable in this instance, but the error for C22:6n3 would exceed 7%. It must be concluded, that system calibration should always be performed in a broad

Table 2
Relative error caused by injector discrimination of docosahexaenoic acid (C22:6n3) measured vs. two different internal standards

	A	B	C	D	TRF
ERF calibration[a]					
C14:0	1.113	1.059	**0.907**	**0.645**	1.096
C16:0	1.066	1.035	**0.873**	**0.605**	1.065
C18:0	1.038	1.017	**0.890**	**0.635**	1.041
C20:0	1.013	1.002	**0.944**	**0.678**	1.022
C22:0	1.004	0.987	0.995	**0.748**	1.006
C24:0	0.996	1.007	1.011	0.956	0.994
Discrimination (%)[b]					
C22:6n3 vs. C23:0	−0.01	3.49	7.38	13.87	
C22:6n3 vs. C17:0	−0.69	−2.51	−10.32	−22.61	

[a] ERF: empirical response factors obtained in the calibration run. Data obtained from four different single injections. A: on-column injection; B: split injection 250 °C, empty needle 5 s, quick injection, 5 s dwell time; C: same as B, but only 2 s dwell time; D: injector temperature 150 °C, full needle injection, TRF: theoretical response factor; all response factors are normalized to C23:0 = 1. Numbers in boldface differ from the TRF by more than 5%.
[b] Discrimination calculated on the basis of the calibration data presented in the respective column.

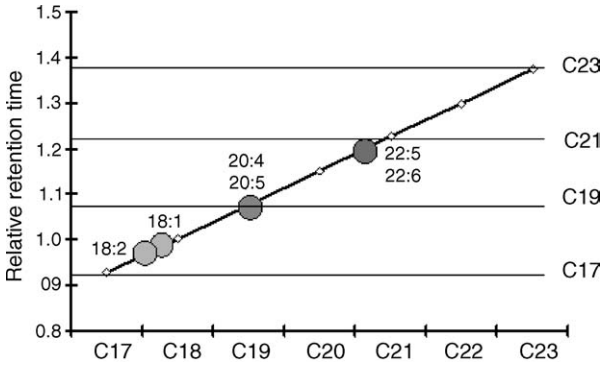

Fig. 1. Relative retention times of fatty acid methyl esters on a non-polar (methyl-phase) column.

chromatographic range (e.g. from C14:0 to C24:0) in order to meet the quality requirements of AOCS Ce 1b-89 [2], and that this demand should be outlined in that method.

Furthermore, it can be observed in Table 2 that comparison of C22:6n3 with C23.0 IS always results in positive error when using split injection, whereas comparison with C17:0 IS produces negative error. This can be explained by decreasing volatility in the order C17:0 > C22:6n3 > C23:0. According to Grob [8], discrimination in vaporizing injectors can be largely minimized by improving injection technique (syringe handling), but full elimination of discrimination can hardly be achieved. Consequently, C22:6n3 will always be overestimated when compared to the less-volatile C23:0, and underestimated versus the more volatile C17:0. Although this error should not exceed 5% in optimized systems, it must be regarded as systematic error and is therefore a lack of accuracy. It should be noted that injection C in Table 2 was performed similarly to injection B with only minor changes in syringe handling, which can be regarded as "case to case" variation. Consequently, selection of IS should be targeted towards similar volatility of components.

Data on volatility of FAMEs from PUFAs at chromatographic conditions (approximate pressure in the injector port) have not been described in the literature, but can be estimated by the elution order from unpolar (methyl-phase) columns. A plot of relative retention times of saturated and unsaturated fatty acids on such a column (HP 101, Agilent Technologies Inc., Palo Alto, CA, USA) using a linear temperature program is presented in Fig. 1. This plot shows co-elution of C20:4n6 and C20:5n3 with C19:0 and elution of C22:5n3 and C22:6n3 slightly ahead of C21:0 but far before C23:0. Therefore, it can be assumed that, from a quantitative standpoint, C21:0 would be the best choice for unsaturated C22 FAMEs and C19:0 would be the optimum for unsaturated C20 FAMEs. Furthermore, C17:0 would be the standard of choice for all unsaturated C18 FAMEs including conjugated linoleic acids (CLA), as it has been recommended by Kramer et al. [14], who discussed this problem from the standpoint of optimizing separation.

The data in Table 3 confirm this assumption by showing minimum discrimination for C18:2n6 versus C17:0, C20:4n6

Table 3
Systematic errors of polyunsaturated fatty acid methyl esters in comparison to different internal standards (discrimination vs. on-column injection (%))

	, Internal standard			
	C17:0	C19:0	C21:0	C23:0
System A				
C18:2n6	−**1.16** ± 0.612[a,*]	2.77 ± 1.479[b,*]	7.67 ± 2.965[c]	9.42 ± 4.136[c]
C20:4n6	−3.57 ± 1.923[a]	**0.24** ± 0.221[b,*]	5.01 ± 1.509[c]	6.70 ± 2.962[c]
C22:6n3	−7.56 ± 2.874[a]	−3.93 ± 1.418[b]	**0.63** ± 0.316[c,*]	2.24 ± 1.576[c,*]
System B				
C18:2n6	−**0.43** ± 0.222[a,*]	2.01 ± 0.860[a,*]	6.52 ± 1.750[b]	8.56 ± 2.452[b]
C20:4n6	−2.03 ± 0.941[a]	**0.37** ± 0.193[b,*]	4.81 ± 0.748[c]	6.81 ± 1.589[d]
C22:6n3	−5.93 ± 1.522[a]	−3.63 ± 0.870[b]	**0.62** ± 0.165[c,*]	2.54 ± 0.892[d]
System C				
C18:2n6	−**0.89** ± 0.856[a,*]	5.50 ± 3.114[ab,*]	12.28 ± 6.517[bc]	18.12 ± 9.016[c]
C20:4n6	−3.76 ± 2.958[a,*]	**2.38** ± 0.885[ab,*]	8.91 ± 4.043[bc]	14.54 ± 6.426[c]
C22:6n3	−7.19 ± 4.000[a]	−**1.30** ± 0.909[b,*]	4.96 ± 2.770[c,*]	10.36 ± 4.882[c]

Results are means of quadruplicate analyses ± standard deviation. System A: SUPELCOWAX 10, 0.1 mm I.D.; system B: RTX225, 0.25 mm I.D.—both optimized injections (refer to Section 2); system C: As system B but injected at 150 °C (not optimized). Values in a row with no common superscripts (a–c) differ ($P < 0.05$); values in boldface indicate minimum discrimination in the respective row.
* Values do not differ from 0 ($P > 0.05$).

versus C19:0 and C22:6n3 versus C21:0. The not-optimized injection (at 150 °C—injection C) had even better results for C22:6n3 versus C19:0. When compared to the appropriate standard, all results were within the required limit of 5% error. With optimized injection (samples A and B in Table 3), the relative error was not exceeding 1% except in one case (C18:2 versus C17:0 in sample A). The error for C22:6n3 was around 2.5% when compared to C23:0, but only 0.63% when compared to C21:0. It can be assumed that this trend was entirely caused by discrimination effects in the injector port, as retention times and elution orders were quite different in the two chromatographic systems employed (Table 4). Furthermore, it can be observed in Table 3 that choosing IS with similar volatility will produce higher precision (=smaller standard deviation). Although it is usually possible to measure C22:6n3 within the 5% limit of error, problems can arise for C20:5n3 (EPA). Following the data presented in Fig. 1, it must be concluded that C20:5n3 will behave similar to C20:4n6. When comparing C20:5n3 with C23:0, systematic errors of more than 5% must me expected (Table 3).

Table 4
Elution order of fatty acids in the two chromatographic systems used in this study

	Supelcowax 10[a]		RTX 225	
	RT[b]	RRT	RT	RRT
Standard				
C17:0	4.04	0.83	7.16	0.80
C19:0	5.77	1.18	11.02	1.23
C21:0	7.53	1.54	15.42	1.72
C23:0	9.39	1.92	20.05	2.23
Unsaturated fatty acid				
C18:2n6	5.47	1.12	9.92	1.10
C20:4n6	7.63	1.56	14.81	1.65
C22:6n3	10.69	2.19	21.04	2.34

[a] Supelcowax 10 (Supelco, Bellefonte, PA; 10 m length, 0.1 mm I.D.); RTX 220 (Restek, Bellefonte, PA; 25 m length, 0.25 mm I.D.).
[b] RT: retention time (min), RRT: relative retention time (C18:0 = 1).

The reason why C23:0 is normally considered as the standard of choice for comparison with *n*-3 LC-PUFAs is because it usually elutes without interference with other components. A potential overlap in wax-columns can occur between C23:0 and C21:5n3, a fatty acid that is found in some marine oils [15]. Base-line separation of these fatty acids is a prerequisite for accurate *n*-3 LC-PUFA analysis and is a mandatory task in the method optimization for the AOAC and AOCS methods [1,2]. The more polar cyanopropyl phases, such as CP-Sil 88 (Chrompack, Middelburg, The Netherlands), may co-elute C20:4n6 and C23:0, whereas the less polar RTX 225, which was used in this study, elutes C23:0 in the range of C22:4n6 and C22:5n6. However, all these problems can be solved by fine-tuning instrument parameters, such as temperature program or flow rate. C19:0 and C21:0 are often avoided because they elute in the most crowded region of the chromatogram. Nevertheless, it is possible to guide these peaks into empty places in most cases. Systems employing wax-columns can be optimized in that way that C21:0 elutes between C20:3n6 and C20:4n6 and C19:0 between C18:3n6 and C18:3n3. If an overlap cannot be avoided, it is still possible to inject the sample once without IS and to correct for the overlapping substances. This procedure is also needed when there is a natural content of the IS in the sample. In most animal fats, especially in marine oils, rather high amounts of C17:0 are common.

Table 5 presents the content of C20:5n3, C22:5n3 and C22:6n3 in a sample of natural marine oil (Seal Blubber Oil) measured versus different IS. This table shows in a pronounced way that choosing the appropriate standard results in much better repeatability. The standard deviations of C22:6n3

Table 5
Long-chain omega-3 fatty acids in natural marine oil measured vs. different internal standards (mg/g, oil ± standard deviation)

Internal standard	C20:5n3 (EPA)	C22:5n3 (DPA)	C22:6n3 (DHA)
C17:0	63.28 ± 0.783	33.00 ± 0.580	68.78 ± 1.168
C19:0	65.82 ± 0.146	34.33 ± 0.390	71.55 ± 0.800
C21:0	66.57 ± 0.698	34.71 ± 0.078	72.36 ± 0.091
C23:0	67.84 ± 1.474	35.37 ± 0.377	73.73 ± 0.826

Values are means of quadruplicate analyses.

and C22:5n3 versus C21:0 and of C20:5n3 versus C19:0 are considerably smaller compared to the other standards. It can be concluded that the variability between consecutive injections is mainly influenced by differences in volatility. This must be regarded as the main reason why results obtained by split injection show higher standard deviations as results from OCI systems [8].

4. Conclusions

Choosing C23:0 IS, as outlined in official methods for marine oils [1,2], can lead to systematic overestimation of *n*-3 LC-PUFAs. Selecting the appropriate IS (C19:0 for unsaturated C20 FAMEs and C21.0 for unsaturated C22 FAMEs) does improve both, accuracy and precision for the analysis of PUFAs. This will result in relative standard deviations for consecutive injections of less than 0.25% for *n*-3 LC-PUFAs. When TRFs are applied, systematic errors will usually be beyond 1%, whereas errors of more than 5% must be accepted with C23:0 as IS especially for EPA, even when the injection system is optimized. A GC system operated in this way would be a better basis for further method optimization regarding transmethylation, lipid extraction or sample preparation.

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