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Determination of the carbon deficiency in the flame ionization detector response of long-chain fatty acid methyl esters and dicarboxylic acid dimethyl esters

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Abstract

Carbon deficiencies (CDs) of long-chain fatty acid methyl esters (FAMEs) and dicarboxylic acid dimethyl esters (DDMEs), which lead to decreased response in a flame ionization detection (FID) system, were determined by using full responding hydrocarbons (heptadecane, eicosane and alpha-cholestane) as references. For saturated FAMEs ranging from C_{12} to C_{22} and for DDMEs ranging from C_4 to C_{10}, CDs between 1.3 ± 0.12 and 1.7 ± 0.36 per ester group were recorded. All values were significantly ($P < 0.05$) greater than 1. Generally, response factors for gas-chromatographic analysis using FID have been calculated on the theory that the CD of FAMEs is 1 per ester group. However, this theory could not be confirmed experimentally for short-chain FAMEs of less than 8 carbons as CDs of around 1.5 were reported for C_4 and C_6 FAMEs. The study presented here contributes an approach to this problem by confirming the validity of response factors calculated from a CD of 1.5 per ester group as well as for long-chain FAMEs and DDMEs.

Keywords: Carbon deficiency; Response factors, theoretical; Fatty acid methyl esters

1. Introduction

Based on the observations of Ackman and Sipos [1], the flame ionization detection (FID) response depends on the relative (mass) amount of "active" carbon in the sample molecule. It is generally accepted today that the carboxyl carbon of the fatty acid (FA) does not contribute to the FID response and is therefore not active [1–3]. On the other hand, there is more uncertainty about the role of the methyl carbon, or more precisely the C_1 carbon of the alcohol portion of the ester. This uncertainty was expressed by Ackman and Sipos [1], but the authors concluded that there was no evidence that the alcohol portion of the ester does not give a full response. Based on the study of Ackman and Sipos, the generally accepted way of calculating theoretical response factors (TRFs) has been to determine the number of active carbons as the total number of carbon atoms minus 1 (C-1) per ester group. This concept has been confirmed experimentally for fatty acid methyl esters (FAMEs) containing more than 8 carbons in their FA chain [1], unsaturated FAMEs [2], ethyl, propyl and butyl esters [3], as well as for triglycerides [4]. However, this concept failed for short-chain FAMEs, as the carbon deficiency (CD) was found to be around 1.5 for the methyl esters (MEs) of butyric acid and caproic acid [5]. In theory, such deviations can take place when losses of the highly volatile short-chain FAMEs occur during standard preparation and therefore have to be compensated by higher correction factors. This explanation was highlighted by Bannon et al. [6], who proposed that for the short-chain FAMEs, only the TRF based on the C-1 concept should be used. However, this issue must be critically reviewed, since the data could not be confirmed empirically for butyric acid ME [7].

The formation of ions in a FID system seems to follow more complex mechanisms, which have been investigated by using quantitative structure–response relationships [8], artificial neuronal networks [9] or mass-spectrometric analysis of the ions formed in the combustion zone of the FID

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system [10]. However, a more practical approach is needed for the lipid analyst. Badings and De Jong [11] pointed out the problem that numerous parameters, mainly analytical errors, play an important role when one attempt to predict response factors. Because of this, Badings and De Jong recommended empirical response factors (ERFs), individually obtained for each laboratory and instrument, to be used for FAME analysis.

The evaluation of Ulberth et al. [3] could not confirm the C-1 concept for short-chain FAMEs, and thus substantiate the data previously reported by Ackman and Sipos [5]. The raw data of the response of FAMEs measured by Ulberth et al. [3] reveal the general problem encountered in the determination of carbon deficiencies: Whilst the CDs for butyric and caproic acid MEs are clearly in the region of C-1.5, it is difficult to assess the CDs for FAMEs longer than C_8. This is due to the fact that TRFs are normalized to $C_{18} = 1$ by convention, causing the TRF, obtained from different methods of calculation, to converge towards C_{18}ME. For FAMEs ranging from C_{10} to C_{18}, the analytical error in the study of Ulberth et al. [3] was in the same magnitude as the relative differences between the TRFs calculated with CDs of 1 and 1.5. On the other hand, the TRFs calculated as C-1.5 gave good agreement with the ERFs from C_6 to $C_{18:1}$ ($P > 0.05$) and only slight deviations from C_4 (1.6% versus 11.4% for C-1). These slight deviations are likely to be caused by analytical differences due to the volatility of C_4 FAME. However, the differences between ERFs and TRFs (C-1) in this study were significant ($P < 0.01$) for C_4, C_6, C_{10} and C_{16} FAMEs.

Based on the interpretation of the published data [1,3,5] and as a result of preliminary trials, the authors suggest a theory that the C_1 carbon of the alcohol portion of the ester also causes a reduced FID response. An approach to confirm this theory is presented in this study. It is based on the use of non-discriminative on-column injection and comparison of long-chain FAMEs with near-eluting hydrocarbons (HCs), which should permit a clearer view of the problem as well as a direct calculation of CDs. In addition, dicarboxylic acid dimethyl esters (DDMEs) were measured versus HCs. The elevated polarity of DDMEs compared to FAMEs results in a decreased volatility, thereby avoiding problems with regards to evaporative losses of low-molecular-weight compounds. Furthermore, the doubled number of heteroatoms produces a clearer CD, allowing a more accurate determination of ERFs.

2. Experimental

2.1. Reagents

Methyldodecanoate (C_{12}ME), methyltetradecanoate (C_{14}ME), methylhexadecanoate (C_{16}ME), methyloctadecanoate (C_{18}ME), methyleicosanoate (C_{20}ME), methyldocosanoate (C_{22}ME), dimethylsuccinate (C_4DME), dimethyladipate (C_6DME), dimethylsuberate (C_8DME) and dimethylsebacate (C_{10}DME) were obtained from Larodan (Malmö, Sweden). Heptadecane (C_{17}HC) and α-cholestane were purchased from Fluka (Buchs, Switzerland), and eicosane (C_{20}HC) was obtained from Supelco (Bellefonte, PA, USA). Standards were dissolved in hexane (HPLC grade), obtained from Merck (Darmstadt, Germany). The final concentration was 0.05 mg/ml per component for on-column injection and 1 mg/ml for split-injection.

2.2. GC–FID analysis

Analyses were made on a Carlo-Erba HRGC 5160 gas chromatograph equipped with an AS-550 on-column autosampler. Additional experiments were made with manual split-injection (split ratio 1:50) using a cup-split liner (Restek, Bellefonte, PA, USA; part number 20885). The determination of the response factors was performed on a RTX-225 capillary column (Restek, Bellefonte, PA, USA), which was 30 m long and had an I.D. of 0.25 mm and 0.25 μm d_f. A 40-cm methyl-deactivated retention gap (0.53 mm I.D.) was connected to the analytical column by using a fused silica tubing connector (both from Supelco, Bellefonte, PA, USA). Purity checks of the standards were performed on a HP-FFAP capillary column (Agilent Technologies, Palo Alto, CA, USA), which was 25 m × 0.32 mm I.D. and 0.52 μm d_f. Hydrogen, at flow rates of 6 ml/min (RTX-225) and 3.5 ml/min (HP-FFAP), was used as carrier gas. Injection volumes were 0.5 μl (on-column) and 1 μl (split). The oven temperature program for the on-column mode was 70 °C (2 min), followed by a 30 °C/min ramp to 240 °C. Secondary cooling was activated 2 min prior to injection and stopped immediately after injection. In the split mode, the oven temperature was kept at 140 °C for 2 min and then raised at a rate of 10 °C/min to 240 °C. The injector temperature was set at 280 °C and the detector was kept at 250 °C.

2.3. Response factors and carbon deficiencies

The relative response of a substance was calculated as the mass amount of active carbon relative to its total molecular mass. The response factors were calculated as the reciprocal value of the response, and were normalized to C_{17}HC = 1, and in some cases to C_{20}HC. The number of active carbons was considered to be the number of total carbon minus 1 per ester group for the conventional method (TRF1) and minus 1.5 for the theory to be tested (TRF1.5). Consequently, the CD of the HC was considered to be zero. Empirical CDs were obtained by back-calculation of the response factors.

2.4. Precision and accuracy

The results were calculated from eight (FAMEs) and five (DDMEs) independent analyses, respectively. For each replicate, a separate standard was prepared by accurately weighing ∼100 mg to nearest 0.1 mg of each component into a

100-ml volumetric flask. Purity checks were carried out for each component, and corrections for impurities were applied according to Albertyn et al. [7]. 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. Differences of empirical data versus theoretical values were obtained by calculating the $P = 0.05$ confidence interval by using Microsoft Excel software. The analytical error was calculated according to Bannon et al. [6] and was expressed as: Grade of analysis $= 100 - \sum(C_i - c_i)$, where C_i was the known mass% of an individual FAME in the calibration standard, and c_i the mass% measured after converting the peak areas using TRF1 or TRF1.5, respectively.

3. Results and discussion

As outlined in Table 1, the ERFs of FAMEs ranging from C_{12} to C_{22}, obtained by on-column injection and normalized to C_{17}HC = 1, were found to be higher ($P < 0.05$) than TRF1. On the other hand, there was good agreement of ERF versus TRF1.5, except for C_{20}ME. These results are also reflected by the CDs shown in Table 2. These data, however, have to be interpreted with greatest care, and all possible interferences that might lead to increased CDs have to be taken into consideration. However, even when calculating all possible statistical ranges, the results are clear, that the CD is higher than 1, thus indicating that the alcohol portion of the ester causes a reduced FID response as well. On the other hand, the statistical power of the results do not permit one to judge, whether the CD of FAME is exactly C-1.5 or somewhat lower, possibly in the range of 1.3–1.5. The grade of analysis for this experiment was 99.26 ± 0.10 for TRF1, and 99.54 ± 0.08 for TRF1.5. In an inter-laboratory trial, Craske [12] arbitrarily defined grades of 99.50+ as very good and 99.00–99.49 as good. Accordingly, both methods of calculation showed excellent performance, pointing to the fact that both kinds of TRF do not cause considerable deviations when calculating long-chain FAME. Although the measurement of "grade of analysis" is not sufficient to detect whether an error is systematic or random, it provides information on the absolute deviation of measured values from true values, and is therefore a useful tool for method development.

A measure for the reliability of the analysis is the ERF or CD determined for α-cholestane. As this cyclic HC elutes markedly behind C_{22}ME, it is most endangered by losses due to thermodegradation, and can provide an estimation of the maximum error caused by the chromatographic system. The results reported here show a slightly elevated ERF of α-cholestane, which was around 1% above the theoretical value. For a better understanding, these data are prepared graphically in Fig. 1. These results clarify the problem that even under optimized conditions, including

Table 1
Theoretical and empirical response factors of fatty acid methyl esters normalized to heptadecane[a]

	TRF		ERF	S.D.	Range ($P = 0.05$)	
	C-1	C-1.5			−	+
C_{17}HC	1.000	1.000	1.000	0.000	–	–
C_{12}ME	**1.263**	1.318	1.314	0.012	1.306	1.323
C_{14}ME	**1.224**	1.269	1.275	0.016	1.264	1.287
C_{16}ME	**1.195**	1.234	1.229	0.007	1.224	1.234
C_{18}ME	**1.172**	1.206	1.197	0.016	1.186	1.208
C_{20}ME	**1.154**	**1.184**	1.172	0.010	1.165	1.179
C_{22}ME	**1.140**	1.166	1.165	0.012	1.157	1.174
α-Cholestane	**0.976**	**0.976**	0.985	0.008	0.980	0.991

HC: hydrocarbon; ME: methyl ester; TRF: theoretical response factor; ERF: empirical response factor; C-1:carbon deficiency − 1; C-1.5: carbon deficiency − 1.5. S.D.: standard deviation. Range gives the $P = 0.05$ confidence interval of ERF. In boldface: values differ from ERF ($P < 0.05$).

[a] Sampling was performed by on-column injection.

Table 2
Carbon deficiencies of fatty acid methyl esters normalized to heptadecane[a]

	CD	S.D.	Range ($P = 0.05$)	
			−	+
C_{17}HC	0.000	0.000	–	–
C_{12}ME	1.474	0.101	1.404	1.544
C_{14}ME	1.537	0.198	1.400	1.675
C_{16}ME	1.418	0.116	1.337	1.500
C_{18}ME	1.365	0.234	1.204	1.527
C_{20}ME	1.316	0.131	1.225	1.407
C_{22}ME	1.500	0.194	1.365	1.634
α-Cholestane	0.265	0.220	0.112	0.417

HC: hydrocarbon; ME: methyl ester. CD: carbon deficiency; S.D.: standard deviation. Range gives the $P = 0.05$ confidence interval of CD.

[a] Sampling was performed by on-column injection.

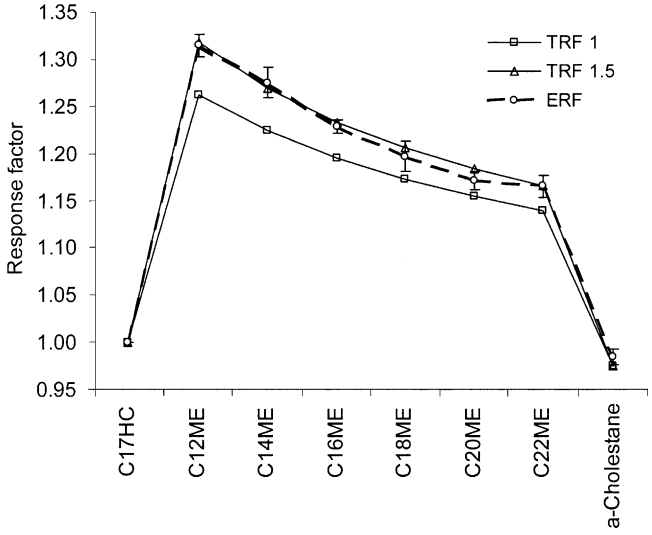

Fig. 1. Theoretical and empirical response factors of fatty acid methyl esters normalized to heptadecane.

Table 3
Carbon deficiencies of fatty acid methyl esters normalized to eicosane[a]

	CD	S.D.	Range ($P = 0.05$)	
			−	+
C_{20}HC	0.000	0.000	–	–
C_{18}ME	1.504	0.029	1.485	1.523
C_{20}ME	1.496	0.128	1.412	1.579
C_{22}ME	1.517	0.145	1.423	1.612
α-Cholestane	0.042	0.210	−0.094	0.179

HC: hydrocarbon; ME: methyl ester. CD: carbon deficiency; S.D.: standard deviation. Range gives the $P = 0.05$ confidence interval of CD.
[a] Sampling was performed by on-column injection.

non-discriminative on-column injection, it is practically impossible to obtain full recovery over a wide chromatographic range, such as from C_{17}HC to α-cholestane. Consequently, the CDs of the longer-chain FAMEs (C_{18}–C_{22}) were determined in an additional experiment using C_{20}HC as reference. This aliphatic HC elutes just ahead of C_{18}ME, and is expected to be the more reliable reference for these FAMEs. The results presented in Table 3 clearly support the C-1.5 concept, as even the α-cholestane reference does not deviate ($P > 0.05$) from the theoretical value. Furthermore, it can be concluded that such different HCs as alkanes and cholestane exhibit equal molar FID response.

The ERFs (Table 4) and CDs (Table 5) obtained by split-injection reveal the limitations of this injection technique. With increasing chain lengths, the CD increases constantly, indicating that losses due to discrimination inside the injector port had taken place. These results were obtained after careful optimization of the injection technique, which resulted in the decision to use a cup-split-injection liner, and to inject the sample by using the hot-needle, solvent-flush technique [13], combined with a post-injection dwell time, as recommended by Ackman [14]. The results of experiments using split-injection are presented primarily to illustrate one specific point. That is problems regarding discriminations during sampling can cause losses even within a narrow range of chain lengths. In this case, even C_{18}ME is affected by reduced recovery, which has to be compensated by higher RF. When following the usual convention, the TRF for C_{18}ME is normalized to 1, thereby affecting all other RFs. As a consequence, the other RFs would appear smaller than expected by theory. Consequently, a review of the data would suggest that ERFs are more likely to support

Table 4
Theoretical and empirical response factors of fatty acid methyl esters normalized to heptadecane[a]

	TRF		ERF	S.D.	Range ($P = 0.05$)	
	C-1	C-1.5			−	+
C_{17}HC	1.000	1.000	1.000	0.000		
C_{12}ME	**1.263**	**1.318**	1.299	0.015	1.287	1.310
C_{14}ME	**1.224**	**1.269**	1.257	0.007	1.252	1.263
C_{16}ME	**1.195**	1.234	1.233	0.007	1.227	1.239
C_{18}ME	**1.172**	1.206	1.213	0.009	1.206	1.220
C_{20}ME	**1.154**	**1.184**	1.201	0.013	1.191	1.212
C_{22}ME	**1.140**	**1.166**	1.212	0.015	1.200	1.224
α-Cholestane	**0.976**	**0.976**	1.026	0.028	1.004	1.048

HC: hydrocarbon; ME: methyl ester; TRF: theoretical response factor; ERF: empirical response factor; C-1: carbon deficiency − 1; C-1.5: carbon deficiency − 1.5. S.D.: standard deviation. Range gives the $P = 0.05$ confidence interval of ERF. In boldface: Values differ from ERF ($P < 0.05$).
[a] Sampling was performed by split-injection.

Table 5
Carbon deficiencies of fatty acid methyl esters normalized to heptadecane[a]

	CD	S.D.	Range ($P = 0.05$)	
			−	+
C_{17}HC	0.000	0.000	–	–
C_{12}ME	1.331	0.132	1.225	1.437
C_{14}ME	1.372	0.071	1.315	1.428
C_{16}ME	1.488	0.093	1.414	1.563
C_{18}ME	1.604	0.126	1.503	1.704
C_{20}ME	1.783	0.206	1.618	1.947
C_{22}ME	2.318	0.256	2.113	2.523
α-Cholestane	1.317	0.691	0.764	1.870

HC: hydrocarbon; ME: methyl ester. S.D.: standard deviation. Range gives the $P = 0.05$ confidence interval of CD.
[a] Sampling was performed by split-injection.

Table 6
Theoretical and empirical response factors of dicarboxylic acid dimethyl esters normalized to heptadecane[a]

	TRF		ERF	S.D.	Range ($P = 0.05$)	
	C-1	C-1.5			−	+
C_{17}HC	1.000	1.000	1.000	0.000		
C_4DME	**2.583**	3.444	3.425	0.147	3.296	3.553
C_6DME	**2.052**	**2.463**	2.279	0.099	2.192	2.366
C_8DME	**1.787**	2.043	2.065	0.041	2.029	2.101
C_{10}DME	**1.628**	1.809	1.880	0.160	1.740	2.020
C_{20}HC	0.999	0.999	1.006	0.097	0.992	1.020

HC: hydrocarbon; ME: methyl ester; TRF: theoretical response factor; ERF: empirical response factor; C-1: carbon deficiency –1; C-1.5: carbon deficiency –1.5. S.D.: standard deviation. Range gives the $P = 0.05$ confidence interval of ERF. In boldface: values differ from ERF ($P < 0.05$).
[a] Sampling was performed by on-column injection.

Table 7
Carbon deficiencies of dicarboxylic acid dimethyl esters normalized to heptadecane[a]

	CD	S.D.	Range ($P = 0.05$)	
			−	+
C_{17}HC	0.000	0.000	–	–
C_4DME	1.489	0.064	1.433	1.546
C_6DME	1.294	0.121	1.188	1.400
C_8DME	1.537	0.067	1.478	1.595
C_{10}DME	1.645	0.361	1.328	1.962
C_{20}HC	0.140	0.240	−0.132	0.412

HC: hydrocarbon; DME: dimethyl ester. CD: carbon deficiency; S.D.: standard deviation. Range gives the $P = 0.05$ confidence interval of CD.
[a] Sampling was performed by on-column injection.

Table 8
Theoretical response factors for fatty acid methyl esters normalized to C18

	TRF 1 (number of double bonds)	TRF 1.5 (number of double bonds)						
	0	0	1	2	3	4	5	6
C_4ME	1.54	1.71	–	–	–	–	–	–
C_6ME	1.31	1.39	–	–	–	–	–	–
C_8ME	1.19	1.24	–	–	–	–	–	–
C_{10}ME	1.12	1.15	1.14	–	–	–	–	–
C_{12}ME	1.08	1.09	1.08	–	–	–	–	–
C_{14}ME	1.04	1.05	1.04	–	–	–	–	–
C_{16}ME	1.02	1.02	1.02	1.01	–	–	–	–
C_{18}ME	1.00	1.00	0.99	0.99	0.98	0.97	–	–
C_{20}ME	0.98	0.98	0.98	0.97	0.96	0.96	0.95	–
C_{22}ME	0.97	0.97	0.96	0.96	0.95	0.94	0.94	0.93
C_{24}ME	0.96	0.95	0.95	–	–	–	–	–

TRF 1.5: theoretical response factors for saturated and unsaturated fatty acid methyl esters; TRF 1: theoretical response factors for saturated fatty acid methyl esters (added for comparison); ME: methyl ester.

the TRF1 theory. This is also reflected by the grades of analysis for the split experiments, which were lower for TRF1.5 (TRF1: 99.20 ± 0.21; TRF1.5: 98.80 ± 0.33). The lower performance of the split-injection can be accounted for due to the fact that, contrary to the validation exercise of Craske [12], the range of FAMEs analyzed was from C_{12} to C_{22}, whereas Craske evaluated C_8 to C_{18}. As can be observed in Table 5, the longer-chain FAMEs, especially C_{22}ME, contribute greatly to the higher analytical error.

Also, the ERFs obtained for DDMEs (Table 6) clearly support the TRF1.5 theory, as the measured CD was higher ($P < 0.05$) than 1 and did not differ ($P > 0.05$) from 1.5, except for C_6DME (Table 7). In this case, C_{20}HC was selected as reference, since α-cholestane elutes too far behind the slowest moving substance (C_{10}DME). The response of C_{20}HC was within the statistical range of theoretical response compared to C_{17}HC (TRF for C_{20}CH = 0.999, ERF = 1.006 ± 0,097). However, in this case, it must be mentioned that C_{20}HC elutes between C_6DME and C_8DME.

The data collected in this study confirm the evidence that the alcohol portion of the ester indeed causes a reduced FID response. The results point to the fact that the C_1 carbon of the alcohol gives only 1/2 response of a normal carbon. Consequently, the correct way of calculating TRFs should be based on the C-1.5 concept. This concept is also sufficient to explain the response behaviour of short-chain FAMEs, without stressing the theory that a different scission of the carbon–oxygen bond, or different inductive effects from the HC chain, may be responsible for an abnormal response. The CDs measured for the DDMEs also indicate that the nature of the functional group is responsible for the relative FID response rather than the size and shape of the HC chain. Furthermore, the good agreement of response for the two alkanes compared to α-cholestane points in the same direction. This conclusion supports and extends the observation of Ackman and Sipos [5], who suggested that iso-branched FAs produce the same molar FID response as the corresponding normal acids.

TRFs calculated for some common saturated and unsaturated FAMEs are presented (Table 8). This table also presents the saturated FAMEs in the TRF1 version, thereby permitting a comparison of both methods. All results are given with two decimals behind the comma, which is sufficient for most analytical purposes. Assuming that preparation of calibration standards is performed by weighing 100 mg of each standard component, and that the 0.1 mg digit of an analytical balance is no more reliable, any correction of <1% seems unnecessary. The TRFs in Table 8 are normalized to C_{18}ME, as this is the usual convention. This also seems useful as most major FAs of edible fats are in the C_{18} region. However, as chromatographic performance can even be reduced in this region, especially when using split-injection, normalization to C_{16}ME might be advantageous. The data presented in Table 8 point out that differences between the two methods of calculation are less than 1% for all long-chain FAMEs (C_{14}–C_{24}), but differ markedly for the shorter FAMEs (C_4ME 10%; C_6ME 5.7%; C_8ME 3.6%; C_{10}ME 2.3%; C_{12}ME 1.4%). There is no relative difference in the TRF of unsaturated FAME versus their saturated analoga between the two methods of calculation.

4. Conclusions

The theory reported here applies only minor corrections for long-chain FAMEs, compared to the established theory [1], but also confirms the data for short-chain FAMEs, which could not be explained by this theory. Therefore, the theory reported here provides a conclusive explanation of the FID response for all FAMEs and other esters. Only a consistent theory can be the basis of method optimization that really results in the elimination of systematic errors, and not the compensation of one error by another. With respect to these findings, optimization of chromatographic systems appear in a slightly different light, and should be re-evaluated, especially for split-injection systems and/or unsaturated FAMEs.

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