Flame-Ionization Detector Response to Methyl, Ethyl, Propyl, and Butyl Esters of Fatty Acids

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ABSTRACT: The concept of theoretical response factors is not directly applicable to methyl esters of short-chain fatty acids (FA), since their carbon deficiency is larger than expected from theory. Substituting the methyl group by an ethyl, propyl, or butyl group improved the flame-ionization efficiency of fatty acid esters gradually, up to the point where the empirical response factors of the butyl esters were identical within experimental error to the theoretical values. Butyl esters of FA have a uniform flame-ionization detection (FID) response irrespective of the number of carbon atoms contained in the FA. They exhibit a carbon deficiency of 1.0, i.e. the carbonyl carbon atom does not respond, as expected from theory. Compared to methyl esters, which have a carbon deficiency of 1.4–1.5 for short-chain FA, use of butyl esters has the advantage that a precalculation of the FID response enables the analyst to judge whether the analytical system employed works properly and the data produced are accurate and reliable. Both acid (BF_3 or H_2SO_4)- and alkali (butoxide)-catalyzed butyl ester preparation were equally effective, giving the analyst a choice of methods so that different analytical needs can be addressed efficiently. Computing response factors and comparing the theoretically expected values with those obtained experimentally gives the experimenter an indication whether the analytical system employed for FA profiling (transesterification plus the subsequent gas–liquid chromatographic separation and quantitation by FID) works properly. This setup is particularly useful for an accurate analysis of the FA profile of milk fat.

Paper no. J8919 in *JAOCS 76,* 263–266 (February 1999).

KEY WORDS: Fatty acid esters, flame-ionization detector, gas–liquid chromatography, response factors.

Fatty acid (FA) profiling of edible fats and oils by gas–liquid chromatography (GLC) is an analytical procedure widely applied in the oils and fats industry. One of the major problems associated with this technique is discrimination of substances differing to a larger extent in molecular weight during sample introduction (1). Cold on-column injection (OCI) eliminates discrimination in the injector (2). Factors contributing to the fact that an FA profile expressed as area-% is mostly not equal to weight-% are: (i) discrimination effects occurring during sample introduction, (ii) irreversible adsorption of analytes in the chromatographic system, (iii) thermal instability of analytes, and (iv) uneven detector response towards different compounds (3–5). Bannon, Craske and co-workers (3,4,6–8) investigated thoroughly the accuracy of fatty acid methyl ester (FAME) analyses and concluded that by using an optimized analytical setup only theoretical response factors (TRF), calculated according to Ackman and Sipos (5), are necessary to convert FAME area-% to weight-%. The concept proved to be valid for fats with FA containing four or more carbon atoms. Milk fat (MF) is the only natural fat containing substantial amounts of short-chain FA. This unique feature is often used to determine either the presence of MF in a fat mixture or to quantitate its content in the mixture (9–11). To attain high accuracy and precision in case of FA profiling of MF is a delicate task. Not only do the mentioned chromatographic factors exercise influence on the final outcome but the preparation of calibration solutions is also a critical point due to the pronounced volatility of short-chain FAME. GLC analysis of calibration mixtures containing methyl esters of $C_{4:0}$ to $C_{18:0}$ plus $C_{18:1}$, and injected by the nondiscriminative OCI technique, underpinned the validity of the TRF concept, with the exception of $C_{4:0}$ and $C_{6:0}$ (12). Empirical response factors (ERF) for these short-chain FAME were higher than predicted. Since it is not easy to completely rule out losses of volatile $C_{4:0}$ and $C_{6:0}$ during the preparation of calibration solutions, in the present study we used involatile triacylglycerols (TAG) and converted them to methyl, ethyl, propyl and butyl esters, as the FA esters of higher alcohols are not as volatile as FAME. Furthermore we were interested to find out whether the alcohol moiety might influence the FID response, particularly for esters of short-chain FA. The experimentally obtained ERF are contrasted with the TRF values, and reported in this article.

MATERIALS AND METHODS

Materials. Solvents, alcohols, metallic sodium, concentrated H_2SO_4, anhydrous Na_2SO_4, NaOH pellets, and $NaHSO_4{\cdot}H_2O$

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were of Analytical Reagent grade and purchased from Merck (Darmstadt, Germany). The pre-coated Kieselgel 60 thin-layer chromatography (TLC) plates were also from Merck. Tributyrin, tricaproin, tricaprylin, tricaprin, trilaurin, trimyristin, tripalmitin, tristearin, and triolein were of 99% purity (Sigma, St. Louis, MO). BF_3 in *n*-butanol (10 %, w/w) was obtained from Supelco (Bellefonte, PA). Other esterification reagents were prepared by dissolving an appropriate amount of metallic sodium in alcohol to get 2 mol/L solutions of sodium methoxide in methanol, sodium ethoxide in ethanol, sodium propoxide in propanol, and sodium butoxide in butanol.

Standard solutions. Three individual TAG calibration solutions matching the FA composition of MF were prepared by using an analytical balance, and dissolved in cyclohexane by slightly warming in a water bath. The solutions contained between 25 and 50 mg total TAG/mL.

Derivatization. Base-catalyzed transesterification was performed at room temperature by reacting 5 mL of fat solutions (containing 10 mg total TAG/mL cyclohexane) in a tightly capped test tube with 50 µL alkoxide solution (13). The tubes were vigorously vortexed for 1 min after addition of the transesterification reagent and left to stand for another 5 (methoxide and ethoxide), 10 (propoxide), or 15 min (butoxide) before termination of the reaction by the addition of 0.2 g $NaHSO_4{\cdot}H_2O$. The ester solution was centrifuged, the supernatant diluted 1:10 with methyl *tert*-butyl ether, and used for GLC.

For acid-catalyzed ester preparation, the BF_3-methanol prescribed in the AOCS Official Method Ce 2-66 (14) was substituted by BF_3-butanol. To 1 mL fat solution (25 mg TAG/mL cyclohexane) in a screw-capped test tube, 1.5 mL 0.5 mol/L NaOH in butanol was added and heated for 4 min in a boiling water bath; then 2 mL BF_3-butanol was added and the solution was heated for a further 3 min. To the cooled solution, 1 mL cyclohexane and 5 mL distilled water were added, shaken, centrifuged, and the aqueous phase discarded. The organic phase was re-washed 5 × with 5 mL portions of distilled water to remove butanol and finally dried with anhydrous Na_2SO_4. The clear supernatant was diluted 1:10 with methyl *tert*-butyl ether and used for GLC.

Alternatively to the AOCS Official Method Ce 2-66, 2% (vol/vol) H_2SO_4 in butanol was also used a catalyst. To 1 mL fat solution (25 mg total TAG/mL cyclohexane) in a screw-capped test tube, 2 mL 2% H_2SO_4 was added and heated in a boiling water bath for 2 h. The butyl esters were recovered as described above for the modified AOCS Ce 2-66 method.

TLC. The purity of TAG as well as the completeness of the various transesterification reactions was monitored by spotting individual TAG dissolved in cyclohexane or the ester solutions on a silica gel TLC plate and development with *n*-hexane/diethyl ether/acetic acid (90:10:1, vol/vol/vol). Spots were made visible by spraying with 10% (vol/vol) H_2SO_4 in methanol and charring at 110°C.

GLC. Esters of FA were separated by means of a 30 m × 0.32 mm i.d. DB-Wax column, 0.25 µm film thickness (J&W Scientific, Folsom, CA) operated in a Fisons 5300 GC (Fisons Instruments, Milan, Italy). Samples (0.5 µl) were injected on-column at an oven temperature of 40°C. After 4 min the temperature was increased at 10°C/min to 220°C. Hydrogen at 25 kPa was the carrier gas. The FID was maintained at 250°C. The PC 1000 chromatographic software (Thermo Separation, San Jose, CA) was used for peak processing and area estimation.

Calculation of response factors. TRF of alkyl esters of FA were calculated as described by Ackman and Sipos (5), and ERF by the relationship [weight-%]/[area-%]. For comparison purposes all factors were related to $C_{18:0}$.

RESULTS AND DISCUSSION

To calculate FID response factors of FA esters according to the concept presented by Ackman and Sipos (5), and to compare the computed values with those obtained experimentally, is an attractive feature. If ERF differ to a greater extent from TRF, the analyst should check possible sources of error (proper functioning of the GLC system, purity of the standards, derivative formation, adsorption, decomposition, or discrimination of analytes during GLC), and the second step should be an optimization of the whole analytical system so that only TRF have to be employed to convert area-% to weight-% of FA esters (4). A prerequisite for this optimization strategy is an accurate knowledge of figures for TRF. Ackman and Sipos (5) demonstrated that the carbonyl C-atom does not contribute in general to the FID response of FAME, and that methyl esters of short-chain FA in particular have a carbon atom deficiency >1 (1.5 for $C_{3:0}$, 1.4 for $C_{4:0}$ and $C_{6:0}$, and 1.2 for $C_{8:0}$) (15). Substituting the methyl ester group in methyl propionate for a propyl group changed the carbon atom deficiency from 1.5 to 1.4.

ERF of TAG test mixtures derivatized by alkali to form methyl (FAME), ethyl (FAEE), propyl (FAPE), and butyl esters (FABE) are summarized in Table 1. Other factors, apart from the flame-ionization efficiency of an organic compound, which could influence the magnitude of ERF were ruled out on the following grounds. The purity of the TAG was checked by TLC, and no extraneous materials found by the charring technique were used. The completeness of the transesterification reactions was also monitored by TLC and found to be spontaneous with methyl and ethyl esters and somewhat slower with propoxide and butoxide. Consequently the reaction time was increased to 10 min for propyl ester formation and 15 min for butyl esters. Discrimination effects during sample introduction can be ruled out since OCI was used, and distortion of early eluting peaks—a problem encountered when flooding polar capillary column with an apolar solvent—was circumvented by using a polar solvent (methyl *tert*-butyl ether) as described previously (16).

Irrespective of the alcohol moiety, ERF of esters of FA with >10 carbon atoms matched within experimental error the TRF. For FAME of $C_{4:0}$, $C_{6:0}$, $C_{8:0}$, and $C_{10:0}$, the ERF and TRF differed markedly, indicative of a carbon deficiency >1. These differences diminished toward 1 as the chainlength of the alcohol moiety increased (Fig. 1). Butyl esters of short-

TABLE 1
Response Factors of Fatty Acid Esters[a]

	Methyl ester		Ethyl ester		Propyl ester		Butyl ester	
Fatty acid	ERF[b]	TRF	ERF	TRF	ERF	TRF	ERF	TRF
$C_{4:0}$	1.770 ± 0.033	1.540	1.613 ± 0.035	1.412	1.444 ± 0.024	1.329	1.336 ± 0.039	1.270
$C_{6:0}$	1.392 ± 0.037	1.308	1.393 ± 0.027	1.253	1.308 ± 0.020	1.211	1.239 ± 0.028	1.180
$C_{8:0}$	1.318 ± 0.026	1.193	1.276 ± 0.019	1.164	1.223 ± 0.018	1.141	1.174 ± 0.025	1.123
$C_{10:0}$	1.195 ± 0.015	1.123	1.166 ± 0.015	1.107	1.130 ± 0.010	1.094	1.082 ± 0.014	1.083
$C_{12:0}$	1.117 ± 0.012	1.077	1.080 ± 0.023	1.068	1.055 ± 0.011	1.060	1.019 ± 0.018	1.054
$C_{14:0}$	1.023 ± 0.012	1.044	0.999 ± 0.024	1.039	0.997 ± 0.009	1.035	0.995 ± 0.013	1.032
$C_{16:0}$	0.992 ± 0.005	1.019	0.990 ± 0.013	1.017	1.001 ± 0.005	1.016	0.999 ± 0.009	1.014
$C_{18:0}$	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
$C_{18:1}$	0.987 ± 0.004	0.993	0.990 ± 0.004	0.994	0.994 ± 0.004	0.994	0.996 ± 0.009	0.994

[a] Three individual triacylglycerol standard solutions were transesterified in quintuplicate; mean values ± standard deviation are reported.
[b] ERF, empirical response factor; TRF, theoretical response factor.

chain acids showed an almost ideal behavior; their ERF were virtually identical with the TRF. Therefore, combustion efficiency of FA esters obviously increases with increasing carbon number of the ester group. This is probably due to the fact that a different scission of the ester linkage may take place, giving a hydrocarbon residue, which would give the full carbon atom response, instead of an alcohol which has a carbon deficiency of 0.5 (17). Katritzky *et al.* (18), in modeling a quantitative structure-response factor relationship, found, besides the relative weight of effective carbon in a substance, some quantum-chemical descriptors (minimal total bond order of a carbon atom and total molecular one-center electron-electron repulsion) as being also of importance in model building, and this might come into effect with different esters of short-chain FA.

In addition to their ideal FID response behavior, butyl esters have the obvious advantage that butyl butyrate is not too volatile and not water-soluble, thus limiting losses due to vaporization and washing steps during ester preparation. Therefore, butyl esters have already been proposed as an alternative to methyl ester for MF analysis (19,20). Iverson and Sheppard (20) used BF_3-catalyzed formation of butyl esters of MF and claimed that ERF did not differ from unity. Esterification of one of the TAG calibration solutions by BF_3-butanol and H_2SO_4-butanol did not substantiate the findings of the latter authors (Table 2), who used packed-column GLC and also a hot injector, a setting which does not guarantee a nondiscriminative sample introduction. Nevertheless, the experiment demonstrated that acid-catalyzed transesterification of a TAG solution resembling the FA composition of MF is equivalent to the alkali-catalyzed reaction as judged by the close agreement of ERF with their TRF, although an extensive washing process was involved in the former procedures. The smaller differences between the ERF and TRF of butyl butyrate in the acid-catalyzed procedures, as compared to the alkali transesterification, may result from a secondary reaction following the initial transesterification reaction, in which the already formed esters start to saponify in the alkaline reaction mixture (8). As the short-chain esters saponify faster than the long-chain saturated esters, this fact might be responsible for the somewhat higher ERF found for butyl butyrate in the butoxide-catalyzed reaction (Table 2). However, when applied to a real MF, the butyl ester profile was in close agreement with the conventional methyl ester method (Table 3). Results in Table 3 are expressed as g FA/100 g of total FA to allow a direct comparison, and they agreed within experimental error.

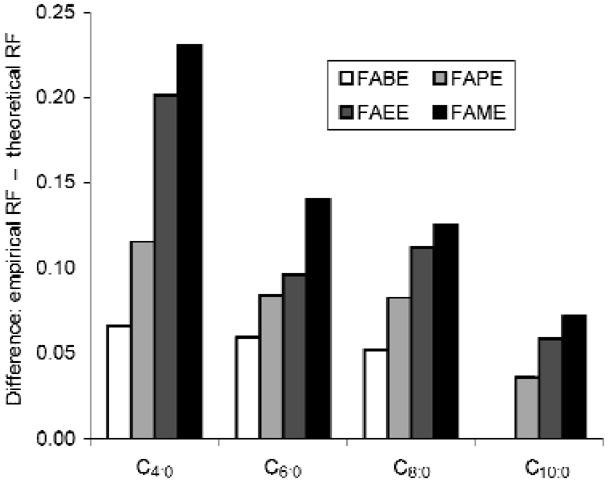

FIG. 1. Approximation of the theoretical response factors (RF) of various esters of short-chain fatty acids by the empirical RF. FABE, fatty acid butyl ester; FAPE, fatty acid propyl ester; FAEE, fatty acid ethyl ester; FAME, fatty acid methyl ester.

TABLE 2
Response Factors of Butyl Esters of Fatty Acid Esters[a]

	TRF[b]	Butoxide	H_2SO_4	BF_3
$C_{4:0}$	1.270	1.359 ± 0.062	1.274 ± 0.024	1.231 ± 0.008
$C_{6:0}$	1.180	1.258 ± 0.046	1.255 ± 0.014	1.222 ± 0.004
$C_{8:0}$	1.123	1.178 ± 0.042	1.174 ± 0.008	1.160 ± 0.005
$C_{10:0}$	1.083	1.097 ± 0.036	1.105 ± 0.003	1.095 ± 0.006
$C_{12:0}$	1.054	1.044 ± 0.026	1.062 ± 0.012	1.045 ± 0.016
$C_{14:0}$	1.032	1.011 ± 0.020	1.019 ± 0.010	1.008 ± 0.005
$C_{16:0}$	1.014	1.010 ± 0.011	1.011 ± 0.006	1.007 ± 0.001
$C_{18:0}$	1.000	1.000	1.000	1.000
$C_{18:1}$	0.994	1.008 ± 0.006	1.011 ± 0.002	1.040 ± 0.005

[a] Mean values ± standard deviation of three separate esterification reactions of one triacylglycerol standard solution are reported.
[b] See Table 1 for abbreviations.

TABLE 3
Fatty Acid Profile of an Anhydrous Milk Fat[a]

Fatty acid	Methyl ester	Butyl ester
$C_{4:0}$	3.86	3.88
$C_{6:0}$	2.17	2.28
$C_{8:0}$	1.33	1.34
$C_{10:0}$	2.90	2.92
$C_{12:0}$	3.39	3.40
$C_{14:0}$	11.10	11.32
$C_{14:1}$(n-9)	0.95	1.06
$C_{15:0}$	1.35	1.40
$C_{16:0}$	29.84	29.69
$C_{16:1}$(n-7)	1.71	1.72
$C_{18:0}$	9.00	9.05
$C_{18:1}$[b]	22.39	22.24
$C_{18:2}$(n-6)	1.92	1.82
$C_{18:2}$ conj.	1.14	1.10

[a] Mean value of duplicate determinations, results expressed as g fatty acid/100 g of total fatty acids.
[b] Sum of $C_{18:1}$(n-9) plus $C_{18:1}$(n-7).

We prefer to use the butoxide method for the routine preparation of butyl esters of MF, since it is less time-consuming and washing steps are not necessary. However, the acid-catalyzed methods would be better suited in cases where an MF with an increased content of free FA, e.g., a partially lipolyzed MF isolated from mold cheese, has to be analyzed.

ACKNOWLEDGMENT

Presented at the 2nd Meeting of the European Section of AOCS, October 1-4, 1998, Cagliari, Italy

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[Received June 23, 1998; accepted November 9, 1998]