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Accurate quantitation of short-, medium-, and long-chain fatty acid methyl esters by split-injection capillary gas–liquid chromatography

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Abstract

Theoretical FID response factors were obtained with cold on-column injection for the major fatty acid methyl esters (FAMEs) occurring in bovine milk fat, except for methyl butyrate and methyl caproate. For these two substances the response factors were larger than expected by theory. Preliminary experiments showed that syringe handling is paramount for obtaining accurate results when a split injector was used for sampling. The solvent flush technique in combination with preheating of the needle and an additional dwell time after depressing the plunger proved to be best suited. Other operational parameters of the split injector were studied by two-level factorial designs. In the case of a FAME test mixture resembling the composition of bovine milk fat, temperature of the injector, septum penetration speed as well as the interaction of these two factors were found to govern the accuracy of the results. Using the same experimental set-up, all the factors tested (injector temperature, penetration speed, split liner design, and split vent flow) proved to be influential with a vegetable oil FAME test mixture. Nevertheless, the magnitude of these effects was so small that it seems justifiable to neglected them in routine testing of vegetable oil samples.

1. Introduction

It is widely accepted that gas–liquid chromatography (GLC) is the method of choice for the analysis of the fatty acid (FA) composition of fats and oils. In addition, the advent of inert and powerful fused-silica capillary columns fostered the attractiveness of this technique. Traditionally, lipid bound FAs are converted to methyl esters (FAMEs) by one of several possible basic procedures and the resulting esters separated by GLC. By theory, the area percentages of the recorded chromatogram should approximately represent the FA composition of the analysed fat. In some cases this assumption holds true, at least with modern instrumentation and simple FAME mixtures consisting of only 4 or 5 species with a similar number of carbon atoms. Accurate quantitation of mixtures of FAMEs whose boiling points differ to a greater extent represents a much more difficult task. Badings and De Jong [1] as well as Craske and Bannon [2] listed several factors which may adversely affect the accuracy of FAME analyses by GLC. Errors due to (i) inadequate sample preparation and (ii)

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faulty sample introduction seem to play the most prominent role.

The FA composition of milk fat (MF) from ruminant animals is not easily quantitated because it is made up of a unique mixture of components with low, medium and high boiling points. A base-catalysed transmethylation of the sample avoids losses of volatile and partly water-soluble short-chain FAMEs, hence ruling out a major source of error. As a consequence, failures in the GLC process per se or during calibration should account for inaccurate MF-FAME analyses. The necessity for special calibration runs results from the fact that the flame ionisation detector (FID) does not respond to the carbonyl carbon [3]. Therefore, the weight percentage of ionisable carbon varies with the chain length, thus giving rise to an unequal response for FAME with a different number of carbon atoms.

Sample introduction by split injection (SI) could aggravate this quantitation problem due to non-linear splitting. Several key factors involved in the process leading to sample discrimination have been identified so far [4]. Syringe handling techniques, temperature and design of the split injector seem to be most influential. In a series of articles the problems connected with the reliable GLC quantitation of FAMEs were addressed by Bannon, Craske and co-workers [2,5–9]. According to these studies, analytical parameters (ester preparation, FID linearity, injection technique, etc.) have to be optimised in such a way that only theoretical response factors (RFs), as proposed by Ackman and Sipos [3], have to be applied to convert FAME area percentages to mass percentages. By using packed-column technology they showed that this concept was also applicable to fats containing volatile, short-chain FAs like butyric acid in MF [5]. On the contrary, Badings and de Jong [1], working also with MF, reported empirical RFs, found by capillary-column GLC with cold on-column injection (OCI), which were close, but not identical with the theoretical factors.

A specially designed injector insert, high temperature of the injector (ca. 350°C), a high split ratio and rapid injection (preferably done by an automatic injector) were recommended for SI onto capillary columns to ensure high accuracy [2,7]. However, the appropriateness of these measures in case of FAME profiling of MF was not demonstrated. Although OCI injection is unequivocally regarded as the sampling technique best suited for quantitation, split injection is still very popular with lipid analysts. Therefore, we address, in this article, SI capillary GLC of FAMEs with large differences in chain length, with a focus on measures to minimise sample discrimination.

2. Experimental

2.1. Reagents

Reference methyl esters for gas chromatography (purity +99%) and solvents (AR grade) were obtained from Merck (Darmstadt, Germany). The AOCS Oil Reference Standard No. 1 was purchased from Sigma (Sigma-Aldrich, Vienna, Austria) and the CRM No. 162 (soya-maize oil blend) from the Community Bureau of Reference, Commission of the European Union, Brussels, Belgium. The Sep-Pak SiOH cartridge was from Waters Millipore (Millipore, Vienna, Austria).

2.2. Preparation of methyl esters

Methanolysis of the CRM No. 162 reference oil was done with KOH in methanol [10].

2.3. Gas–liquid chromatography

A Fisons/Carlo Erba MEGA 5300 gas chromatograph (Fisons Instruments, Vienna, Austria) equipped with both a cold on-column and a split/splitless injector was used. The FID signal was processed by a ChromJet integrator (Spectra-Physics, San Jose, CA, USA). FAMEs were separated with a 30 m × 0.32 mm I.D., 0.25 μm film thickness, J&W DB-Wax fused-silica capillary column (Fisons Instruments, Vienna, Austria). All experiments were run with the following oven temperature program: 40°C held for 4

min, 10°C/min to 140°C held there for 1 min, 5°C/min to 220°C held there for 10 min. Detector temperature was set at 250°C. Hydrogen was the carrier gas at 40 kPa head pressure (gas velocity: 45 cm/s determined at 40°C). Flow-rates of the FID fuel gases were: hydrogen 22 ml/min, nitrogen (make-up gas) 19 ml/min, air 290 ml/min. Secondary air cooling for OCI was turned on 2 min before an injection and shut off 5 s after injection. Sample introduction was done by means of a Hamilton (Bonaduz, Switzerland) 701N syringe (10-μl volume, 5-cm needle length, point style No. 2) for SI and a Hamilton 701SN syringe (5-μl volume, 7.5-cm needle length, point style No. 3) for OCI. Either an empty glass tube (79.5 × 4 mm I.D., Fisons Cat. No. 45300400) or a "laminar cup" splitter (79.5 × 5.5 mm I.D., Restek Cat. No. 20809, Bellefonte, PA, USA) were employed as splitter sleeves. Other splitter parameters are given in the text.

2.4. Needle handling techniques

The following needle handling techniques for SI were evaluated:

–(A) Filled needle injection: the needle of the syringe was cleaned with solvent and dried by applying vacuum. Sample was drawn up to the 1-μl mark, the syringe needle pushed through the septum and the plunger depressed immediately. Thereafter, the syringe was withdrawn rapidly.

–(B) Filled needle injection with post-injection dwell time: The injection was performed as described in (A) but the needle was left for an additional 2 s in the hot injector before being removed.

–(C) Hot needle injection: the syringe was cleaned as described in (A), sample was drawn up to the 1-μl mark and, thereafter, completely withdrawn into the barrel. The needle was pushed through the septum and, after a dwell time of 3 s, the plunger was depressed and the needle removed rapidly.

–(D) Solvent flush injection: first, the needle was filled with pure solvent. Second, the plunger was pulled back to the 1-μl mark to suck an air bubble into the syringe, followed by sample until the plunger reached the 2-μl mark. Finally, the sample was withdrawn from the needle into the barrel. Injection was performed as described under (C).

–(E) Solvent flush injection with post-injection dwell time: The injection was performed as described in (D) but the needle was left for an additional 2 s in the hot injector before being removed.

For OCI, the needle was filled with solvent followed by sample until the plunger reached the 1-μl mark. All the liquid was pulled back into the barrel before injection.

2.5. Error estimation

Analytical error was calculated in two ways. The first estimate was computed as proposed by Bannon et al. [5]: Total error (%) = Σabs($C_i - c_i$), where C_i was the known mass % of an individual FAME in the calibration mix, and c_i the mass % found by converting GLC area %. Only theoretical RFs were used for conversion.

The second estimate was calculated as: Discrimination (%) = Σabs($A_i - a_i$), where A_i was the area % of an individual FAME in the calibration mixture found by OCI, and a_i the area % found by SI.

2.6. Statistical analysis

Calculations were done using SAS/STAT Ver. 6.03 (SAS Institute, Cary, USA) procedures.

3. Results

3.1. On-column injection

Three calibration mixtures approximating the composition of bovine MF were prepared by gravimetry and analysed by GLC using OCI. Theoretical RFs and those obtained experimentally are listed in Table 1. C18:2 and C18:3 were omitted deliberately from the mixtures due to stability problems. This will not introduce a serious error in the analysis, since MF is not rich in polyunsaturated FAs. For all experimental

Table 1
Empirical response factors of fatty acid methyl esters as obtained by cold on-column GLC

FAME	Relative response factors					
	Theor.	Mix 1[a]	Mix 2	Mix 3	Mean	S.D.
C4:0	1.540	1.730	1.734	1.754	1.739	0.013
C6:0	1.308	1.430	1.427	1.398	1.418	0.018
C8:0	1.193	1.224	1.226	1.212	1.214	0.019
C10:0	1.123	1.132	1.141	1.145	1.139	0.007
C12:0	1.077	1.079	1.099	1.074	1.084	0.013
C14:0	1.044	1.066	1.047	1.046	1.053	0.011
C16:0	1.019	1.031	1.023	1.032	1.029	0.005
C18:0	1.000	1.000	1.000	1.000	–	–
C18:1	0.993	1.002	0.993	1.000	0.998	0.005

[a] Mean values of quadruplicate injections.

mixtures the found RFs compared favourably with the theoretical values, except for C4:0 and C6:0. These factors were by 0.199 and 0.110 larger than the theoretical RFs.

3.2. Split injection: needle techniques

In a preliminary trial, we compared six needle-handling techniques for sample introduction by SI (Fig. 1). The % total error and the % discrimination figures were employed as criteria to judge the efficacy of the different techniques. Mean values of replicated OCI ($n = 5$) of the calibration mixture were taken as a basis for % discrimination calculations.

The widely used filled needle technique (A in Fig. 1) produced the largest error of all methods tested. Both methods for estimating analytical error yielded equivalent results. The same technique, but with an additional dwell time of the syringe needle in the injection port after releasing the sample, improved accuracy considerably (B1 in Fig. 1). An even better error estimate was observed when the needle was pushed as rapidly as possible through the septum (B2 in Fig. 1) compared to variant B1, where needle insertion was done in a smooth, normal way. Interestingly, the two methods to calculate analytical error did not agree. Hot needle injection (C in Fig. 1) led to error estimates which were comparable to B1 and B2 when % total error was used as criterion but was less accurate when % discrimination was employed. Solvent flush in combination with a hot syringe needle (D in Fig. 1) was best suited as judged by % total error, while a SI method recommended by Ackman [11] gave results which were closest to those obtained by OCI (E in Fig. 1). Therefore, we decided to use

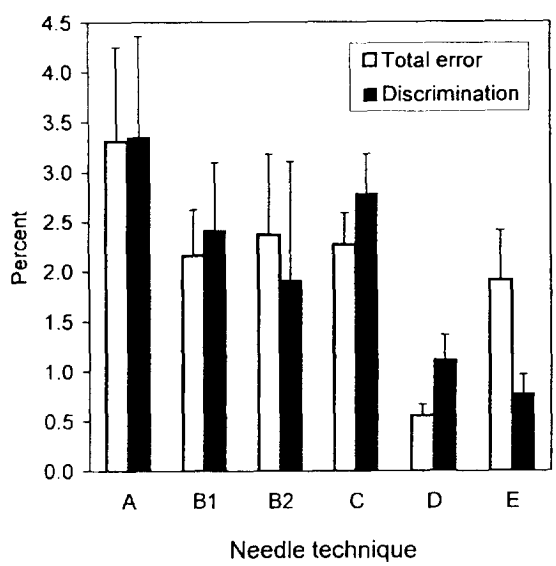

Fig. 1. Sample discrimination caused by different syringe needle handling techniques. (A) Filled needle. (B) Filled needle injection with post-injection dwell time; 1, normal septum penetration speed; 2, rapid septum penetration speed. (C) Hot needle injection. (D) Solvent flush injection. (E) Solvent flush injection with post-injection dwell time.

the latter technique in further experiments targeted at studying thoroughly those operating parameters which we expected to be of importance in SI of FAMEs.

3.3. Experimental design: milk fat

A two-level factorial design in the variables (a) temperature of the injector, (b) split-vent flow, (c) speed of needle penetration, and (d) injector sleeve design was set up to explore the experimental domain for an ester mixture simulating MF. The design matrix is given in Table 2. Injection technique E was termed injection with a "regular penetration speed (= 4 s)". The same technique but with a penetration speed as rapidly as manually possible (ca. 1 s) and immediate release of the sample was termed "rapid injection". The design was blocked in factor (d). Each run was performed in duplicate and the average non-corrected area percentages of the 9 individual FAMEs for the 16 (2^4) factor combinations were taken for principal component analysis (PCA). This enabled us to visualise the outcome of the experiment without employing any response variable (Fig. 2). For ANOVA computation, the % discrimination parameter was chosen as response.

A plot of the first two principal components, which accounted for 91.8% of the total variation, revealed that the velocity of needle insertion was most influential and led to a clearly visible grouping of the results (Fig. 2). At normal penetration speed the points were homogeneously dispersed around the result obtained by OCI, whereas rapid penetration produced two distinct sub-groups. Mean values for the factor levels, using % deviation as response variable, are shown in Fig. 3. ANOVA of the data confirmed the influence of penetration speed ($p = 0.0001$). Temperature of the injector proved also to be statistically significant ($p = 0.0001$), in contrast to split-vent flow and liner design ($p = 0.2798$ and $p = 0.2589$). Of the two-factor interactions involving significant variables, only the temperature × liner design ($p = 0.0092$) and the temperature × penetration speed ($p = 0.0001$) interaction terms were significant. At an injector temperature of 230°C, rapid septum penetration produced the largest deviation value observed, whereas at 330°C the difference due to penetration speed was negligible (Fig. 3). Moreover, the effect was variable for different FAMEs, depending on chain length. Penetration of the injector at 330°C at regular speed, although giving satisfactory figures for % discrimination, overvalued short-chain FAMEs to a considerable extent.

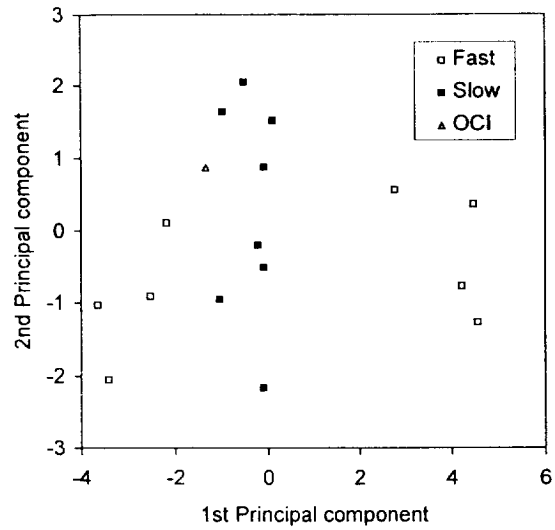

Fig. 2. Principal component plot of the milk fat experiment (fast, fast septum penetration; slow, slow septum penetration; OCI, on-column injection).

Table 2
Factor and factor levels used in the 2^4 experimental designs

Factor	Factor level	
	Low (−)	High (+)
Injector temperature	230°C	330°C
Split vent flow	50 ml/min	250 ml/min
Liner design	Regular	Laminar cup
Penetration speed	4 s[a]	1 s[b]

[a] Septum penetrated at regular speed and needle kept inside the hot injector for further 3 s before depressing the plunger.
[b] Septum penetrated as rapidly as possible and plunger depressed immediately thereafter.

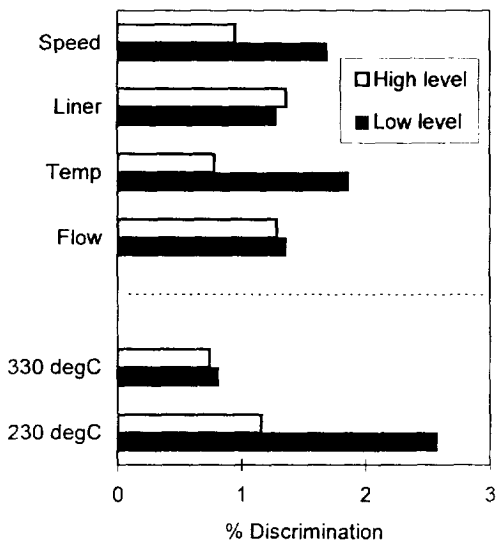

Fig. 3. Mean values at factor levels for the milk fat experiment.

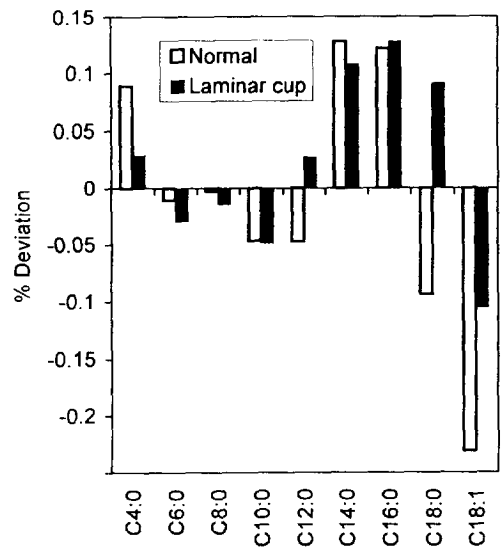

Fig. 4. Discrimination of individual FAMEs at an injector temperature of 280°C and normal septum penetration speed (peak areas obtained by cold on-column injection served as a basis for the evaluation of discrimination effects).

In a search for optimum conditions, the non-influential split vent flow was set to 50 ml/min, the septum was penetrated with a normal speed and the injector temperature set to 280°C (mid-point of the two factor levels). With these operating parameters both injector sleeves produced % deviation figures < 1 (Fig. 4).

3.4. Experimental design: vegetable oil

The same factorial design was applied to a FAME standard resembling the composition of an average seed oil (ca. 10% C16:0, 10% C18:0, 30% C18:1 and 50% C18:2). To ensure that the C18:2 preparation was peroxide free, a solution of the ester in *n*-hexane was passed through a Sep-Pak silica cartridge, and the non-oxygenated C18:2 eluted with 5% diethyl ether in *n*-hexane.

As the chain lengths of the FAMEs in the vegetable oil standard did not differ to a greater extent, no particular influence of the factors tested was expected. PCA of the data strengthened this assumption since data points did not cluster in specific groups (Fig. 5). However, ANOVA revealed that all the factors tested were significant at $p < 0.05$. Nevertheless, the % discrimination response variable remained $< 0.5\%$ for all factor combinations (Fig. 6). Therefore, the influence of the factors should be

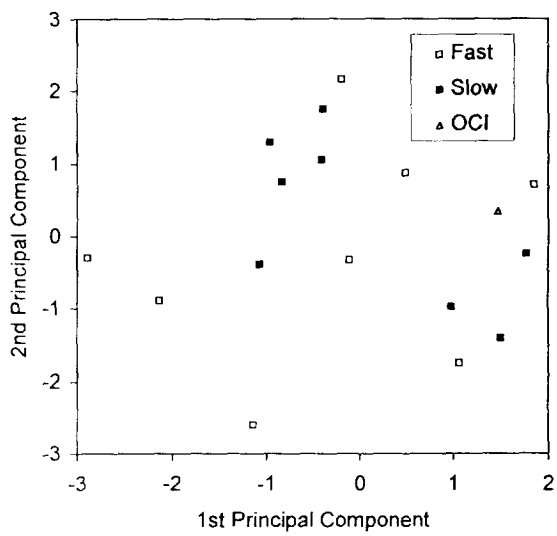

Fig. 5. Principal component plot of the vegetable oil experiment (fast, fast septum penetration; slow, slow septum penetration; OCI, on-column injection).

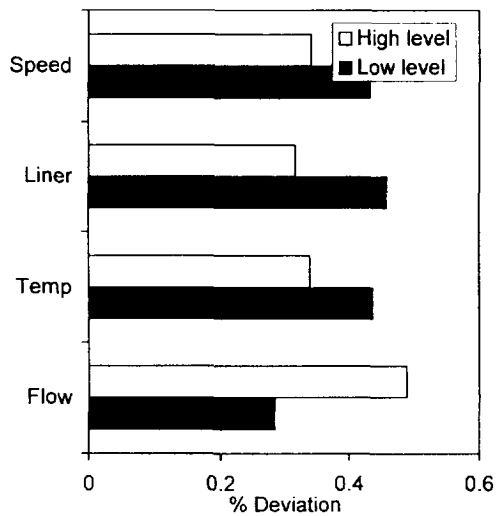

Fig. 6. Mean values at factor levels for the vegetable oil experiment.

only of minor importance in practical situations. This hypothesis was validated by analysis of commercially available standard mixtures (Oil Reference Standard AOCS No. 1 and BCR CRM 162) applying the same injection parameters as found in the MF optimisation trial. Only theoretical RFs were taken to convert % area to % weight. Excellent agreement between certified and experimentally determined values was observed (Table 3).

Table 3
Accuracy check by means of the AOCS reference mixture No. 1 and the BCR CRM 162

FAME	Certified value	Uncertainty	Found value ($n = 4$)
AOCS No. 1			
C16:0	6.0		6.02 ± 0.01
C18:0	3.0		2.92 ± 0.01
C18:1	35.0		34.92 ± 0.04
C18:2	50.0		50.25 ± 0.05
C18:3	3.0		2.98 ± 0.01
C20:0	3.0		2.92 ± 0.01
BCR CRM 162			
C16:0	10.65	0.17	10.69 ± 0.02
C18:0	2.87	0.07	2.92 ± 0.03
C18:1	24.14	0.28	24.21 ± 0.03
C18:2	56.66	0.54	56.59 ± 0.05
C18:3	4.48	0.21	4.74 ± 0.06

4. Discussion

4.1. Response factors of fatty acid methyl esters

Although serious shortcomings of classical SI have been reported, it is still the preferred sample introduction technique in most lipid laboratories [11]. In particular, whether SI allows the accurate quantitation of multi component mixtures is under discussion [12,13]. The most common remedy for this problem is to determine empirical correction factors by analysing a calibration mixture with known composition. Methyl esters of short-chain FAs are notoriously volatile. As a result, the accurate preparation of a calibration mixture resembling bovine MF is a delicate task. Using large amounts of FAME to lessen weighing errors is prohibitive due to the high price of pure standard substances. In addition, a one-pot preparation of the calibration mixture where nine individual standards are weighed in a suitable flask and made up to the mark with solvent, is time consuming. Thus, the risk that some of the volatile components evaporate gradually during handling is strongly increased. As a compromise we prepared 5–10 fold concentrated stock solutions of individual short and medium chain FAMEs (C4:0–C12:0) and added aliquots to the less-volatile long chain FAMEs, whose weight can be obtained with sufficient accuracy.

Remaining errors due to evaporation of volatile esters during mixture preparation were ruled out on two grounds. Firstly, three independently prepared FAME mixtures gave RFs whose relative S.D.s were well below 2% (Table 1). Furthermore, it is unlikely that mixtures flawed by evaporative losses, which is an uncontrolled process, will give such estimates of analytical precision. Secondly, a faulty mass taken for only one of the individual FAME during weighing will bias the percentages for all mixture components. This, in turn, will be reflected in deviations of all RFs from their theoretical values.

The validity of theoretical RFs, as originally proposed by Ackman and Sipos [3] and further advocated by an Australian research group [2,5–8], was confirmed, except for C4:0 and C6:0. Both behaved aberrantly in that their carbon deficiency was >1. This anomaly has even been recognised by Ackman and Sipos, who postulated in another publication [14] a carbon deficiency of 1.4 for C4:0 and C6:0, and 1.2 for C8:0. A re-calculation of RF (relative to C18:0) taking into account this deficiency, resulted in values of 1.7107, 1.4016 and 1.2232 for C4:0, C6:0 and C8:0, respectively. Experimentally found values (Table 1) were in close agreement with these factors. Shehata et al. [15] and Kemppinen and Kalo [16] also reported RFs value for C4:0 which were larger than the theoretical factors, viz. 1.62 and 1.64, although their factor for C6:0 agreed well (1.31) or was smaller (1.26). In a very carefully conducted study, employing OCI, RF values of 1.43, 1.18 and 1.09 were found for C4:0–C8:0 [1]. These RF values were intended to express the final result on a FA instead of a FAME basis. Conversion with appropriate factors leads to FAME-based figures of 1.579, 1.262, and 1.140, respectively [17].

Factors other than the relative proportion of "effective" carbon atoms in the molecule may thus relate to its FID response. A study aimed at predicting FID response by a quantitative structure–property relationship (QSPR) approach revealed that, besides the relative weight of "effective" carbon atoms, other features referred from quantum-chemical modelling of the molecules were involved [18].

Other possible sources of error, which could have been responsible for the deviation of our results from those found in the literature, such as irreversible adsorption, detector overload, or non-linearity of the detector, were ruled out, because adsorption is very unlikely to occur with fused-silica capillary columns and highly diluted samples were injected (ca. 100 ng total sample mass).

4.2. Discrimination caused by split injection

Processes taking place during sample introduction into a hot, vaporising injector are complex and may cause a serious alteration of the composition of that part of the sample actually reaching the column [13]. Numerous parameters are known to influence sample splitting [4]. Bannon et al. [7] identified three critical factors which governed accuracy of FAME analysis by SI to a large extent. Avoidance of the so-called needle discrimination by high injection speed, rapid vaporisation of the sample, ensured by high injector temperature, small sample volume and low sample concentration, and intensive mixing of the sample with carrier gas, facilitated by specially designed injector sleeves, were the key measures they recommended to achieve linear splitting. Our results confirmed the crucial importance of factors related to needle discrimination (syringe handling and injector temperature) but no indication about the significance of the other parameters mentioned were seen in the MF experiments. Needle handling exerted the most prominent influence on accuracy (Fig. 1). In agreement with the findings of Grob [13], filled needle techniques were least suited.

Interestingly, the solvent flush/hot needle technique (method D) produced results where total error, calculated according to the Bannon and Craske method, was as small as 0.56 ± 0.11 (3 different calibration mixtures). With this injection technique individual RFs did not differ by more than 0.038 from their theoretical values (largest difference found with C4:0: 1.578 vs. 1.540), but non-corrected area % did not agree with results obtained by OCI, a technique which is generally accepted to be non-discriminative [12,13,19]. Therefore, we believe that this particular type of sample introduction created such peculiar conditions that the resulting RFs were, misleadingly, close to the theoretical values put forward by Bannon et al. [5,7].

The factorial design with simulated MF showed clearly that neither split vent flow nor injector insert design was influential, while others [20–22] stressed the importance of high split ratios and/or special inlets to obtain linear sample splitting. In agreement with earlier reports [2,7,13], a high injector temperature was paramount for achieving high accuracy. On the other hand, the high temperature demanded rapid penetration of the septum to avoid needle

discrimination. Use of special autosamplers capable of performing injections in <0.2 s as recommended by Bannon et al. [7] is, therefore, mandatory for productivity since manual high speed injections, though possible, are prone to errors, and are likely to result in bent needles. A somewhat lower injector temperature (280°C) in combination with regular penetration speed, which promotes pre-heating of the needle, was an effective means to obviate this difficulty without loss of accuracy.

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