Accred Qual Assur (2015) 20:411–419
DOI 10.1007/s00769-015-1144-1

PRACTITIONER'S REPORT

# Validation of a GC–IDMS method for the metrologically traceable quantification of selected FAMEs in biodiesel

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Received: 21 January 2015 / Accepted: 27 May 2015 / Published online: 21 June 2015
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**Abstract** Current methods for the analysis of fatty acid methyl esters (FAMEs) in rapeseed oil-based biodiesel refer to operationally defined measurands, which is a practical solution for routine analysis. In this paper, a new method for the SI-traceable quantification of selected FAMEs in biodiesel and its validation are described. This method has the potential to be a reference method for applications requiring structurally defined measurands and traceability to the SI as it allows direct comparisons to well-characterised calibrants through the use of isotopically labelled analogues of the analytes as well as establishing a full uncertainty budget. The method is based on gas chromatography–isotope dilution mass spectrometry. Its performance is demonstrated through its implementation and validation in two independent laboratories and is shown to provide reliable and traceable results for selected FAMEs in biodiesel test samples.

**Keywords** Biofuels · Biodiesel · GC–IDMS · Traceability · Fatty acid methyl ester

## Introduction

The European Union sets goals to reach a market share of energy from renewable sources in the transport sector of 10 % in 2020 [1]. Biofuels have an essential role to achieve this target, especially biodiesel, which is used as admixture to conventional diesel as well as full replacement of conventional diesel.

Biodiesel is a fuel that is produced from biological sources, principally vegetable oils, but also, to a lesser extent, from animal fats, microalgae oil or recycled restaurant grease. A wide variety of vegetable oils can be converted into biodiesel. Four oil crops are the most employed: rapeseed, soybean, palm and sunflower, with rapeseed being most frequently used [2].

The long-term operation of conventional diesel engines with vegetable oils can lead to gumming, injector coking and ring sticking [3]. Currently, transesterification is most frequently used to obtain a fuel compatible with the specifications of the diesel engines [2]. Transesterification, also called alcoholysis, consists of transforming triglycerides into fatty acid alkyl esters in the presence of an alcohol and a catalyst, such as alkali or acid, with glycerol as a by-product. Ethanol and methanol are the alcohols most frequently utilised, especially the latter because of its low cost [2]. By using methanol in this process, the final product comprises a mixture of fatty acid methyl esters (FAMEs).

European legislation [4] sets specifications for FAME biodiesel used as admixture to conventional diesels and refers to the European Standard EN 14214 [5] for further specifications of the FAMEs. This standard specifies limit values for the total FAME content as well as for the linolenic acid methyl ester content and refers to a standard method (EN 14103:2011 [6]) for their quantification, i.e., EN 14214 refers to operationally defined measurands. The standard method uses capillary gas chromatography (GC) on polar stationary phases, applying internal calibration with nonadecanoic acid methyl ester (C19:0) and detection via flame ionisation (FID) for the quantification of the

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FAMEs. The standard method is suited to routine applications, as it does not require derivatisation, and compounds are identified on the basis of identical retention times of a standard and the peak corresponding to the compound of interest in the sample. A limitation of this method, with regard to its metrological traceability, is the use of C19:0 as the single internal calibrant. None of the FAMEs is quantified versus a calibrant containing the same FAMEs. Further, as for many standardised methods, application of the standardised method is assumed to give "true" results, i.e., the performance characteristics listed in the standard method are limited to repeatability and reproducibility, trueness is not considered. While this approach is very practical and widely used in standardised methods for routine applications, for particular purposes, requiring structurally defined measurands and well-described traceability to the SI, a different approach may be needed. The "White Paper on Internationally Compatible Biofuel Standards" recommended the "development of internationally accepted reference methods […] that underpin assessment of product quality and help facilitate trade" [7]. This issue was tackled in a project of the European Metrology Research Programme (EMRP), called "ENG09: Metrology for Biofuels". As part of the project, new methods were developed to enable the provision of metrologically traceable reference values for selected FAMEs in biodiesel, being aware that these may differ from the operationally defined measurements required for the application of EN 14214. In particular, a method using gas chromatography combined with isotope dilution mass spectrometry (IDMS) was developed, using isotopically labelled FAMEs synthesised specifically for this project. IDMS, if applied properly to homogeneous liquid samples, has the potential of providing metrologically traceable reference values with well-characterised uncertainties. This technique has also been widely used in procedures applied for the certification of reference materials or in other applications requiring high accuracy [8, 9]. To the best of our knowledge, the validation of a GC–IDMS method for the quantification of FAMEs in biodiesel has not been reported in literature yet.

In this paper, the description and full in-house validation of the GC–IDMS method for the analysis of selected FAMEs in biodiesel is presented. The method has been implemented in two independent laboratories (further denoted as laboratory A and laboratory B), employing slightly different procedures. The results of the in-house method validations in the two laboratories are shown, demonstrating the possibility to establish a full traceability chain for the method, including an assessment of trueness of the results. This is further supported by a comparison of the results obtained by the two laboratories.

## Experimental section

### Reagents

#### *Native FAMEs*

The native FAMEs considered in this study are palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acid methyl esters, as these are the most abundant FAMEs present in biodiesel. Laboratory A purchased the native FAMEs from Alfa Aesar (Ward Hill. MA, USA), whereas laboratory B obtained them from Sigma-Aldrich (St. Louis, MO, USA).

#### *Labelled FAMEs*

For the purpose of this article, the isotopically labelled FAMEs are referred as C16:0*, C18:0*, C18:1*, C18:2* and C18:3*. Labelled FAMEs were synthesised by TÜBİTAK UME (National Metrology Institute, Gebze, Turkey) according to the following procedure: 25 mg of each fatty acid standard was dissolved in 0.5 mL of toluene; then 1 mL of a solution of methanol-$d_4$ in $H_2SO_4$ (1:100 volume ratio) was added, and the mixture was stirred overnight at 50 °C. Next, 2 mL of aqueous sodium chloride (5 %) was added, and then the mixture was extracted twice with 5 mL of *n*-hexane. After washing the organic layer with 2 mL of $NaHCO_3$ aqueous solution (2 %) and drying over $Na_2SO_4$, the solvent was evaporated at reduced pressure. The residue was a deuterated fatty acid methyl ester.

Toluene, methanol-$d_4$, $H_2SO_4$, sodium chloride and *n*-hexane SupraSolv grade were obtained from Merck (Darmstadt, Germany). $NaHCO_3$ and $Na_2SO_4$ were purchased from Sigma-Aldrich (St. Louis, MO, USA).

#### *Purity assessment*

The purity assessment of native and synthesised labelled FAMEs was done by quantitative nuclear magnetic resonance (qNMR). Benzoic acid NIST-350b (NIST, Gaithersburg, MD, USA) was used as internal standard (IS), and chloroform-$d_6$ (Sigma-Aldrich, St. Louis, MO, USA) was used as solvent. In a glass vial, 40–50 mg of each native and labelled FAME and 25–30 mg of benzoic acid were accurately weighed. Then, about 2.8–3.2 mL of chloroform-$d_6$ was added to the vial and shaken till total dissolution. Finally, about 0.7 mL of the solution was transferred into an NMR tube. Purity of the samples was assessed in triplicate.

All NMR measurements were carried out on a Varian 600 spectrometer (Palo Alto, CA, USA) operating at

599.90 MHz. The probe used was a Varian's One NMR. All NMR spectra were processed with the software Mestrenova 8.1.0. The spectral width was 9615.4 Hz, the flip angle 90°, and the acquisition time 3.4 s. An appropriate window function was applied prior to Fourier transformation in order to enhance the spectral resolution (exponential multiplication with a line broadening factor of 0.30 Hz). The samples were measured at 25 °C.

Individual purities and their uncertainties were assessed for all native and isotopically labelled FAMEs. All purity values were larger than 98 % (mass fraction) with uncertainties ranging between 0.2 % and 0.3 %.

*Samples and certified references materials*

The final determination of FAMEs was carried out on a rapeseed-based biodiesel test material equivalent to the material used for the Certified Reference Material ERM®-EF001 (available from JRC-IRMM, Geel, Belgium [10]) which was distributed during the so-called proof-of-concept exercise of the EMRP ENG09 project. During method validation, a rapeseed-based biodiesel material produced in the BIOREMA project was used [11, 12]. For trueness assessment, two certified reference materials (CRMs) were used from the National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA: NIST SRM 2773 (animal-based biodiesel) and NIST SRM 2377 (mixture of FAMEs).

## Quantification of FAMEs

*Calibration solutions*

A series of calibration solutions (8 for laboratory A and 6 for laboratory B) were prepared resulting in the following concentration ranges for native FAMEs: 0.63–7.5 µg/g for C16:0, 0.13–1.5 µg/g for C18:0, 6.3–75 µg/g for C18:1, 3.1–38 µg/g for C18:2 and 1–12 µg/g for C18:3. To each calibration solution, the same amount of isotopically labelled FAMEs was added. The mass fraction of labelled FAMEs was chosen to be in the lowest third of the calibration range of each of the native compounds. Laboratory A added every single labelled FAME to the calibration solutions, whereas laboratory B excluded C18:3* from them due to problems with its stability. Instead, laboratory B quantified C18:3 using C18:2* as internal standard. This approach was accepted after ensuring its validity by quantifying C18:3 in the NIST SRM 2377 CRM.

All calibration solutions were prepared gravimetrically using as solvent kerosene (laboratory A) and toluene (laboratory B).

*Sample preparation*

Prior to preparation, all biodiesel samples were adjusted to room temperature. Laboratory A prepared a solution of biodiesel with a final concentration of 13.25 mg/g in *n*-hexane. Laboratory B diluted the biodiesel in toluene to reach a final concentration of 7 mg/g. Aliquots of these solutions were spiked with the isotopically labelled FAMEs to reach a final concentration in the samples identical to the ones achieved in the calibration solutions. Samples were prepared and analysed in triplicate on each day of the validation study.

The CRMs used to assess the trueness of the developed methods were prepared in the same way as the biodiesel samples by taking the amount of material that comprises a final concentration of FAMEs between the limits of the calibration ranges and spiking it with labelled FAMEs. The CRMs were analysed together with the biodiesel samples and the calibration solutions.

All solutions were prepared gravimetrically.

*GC–IDMS analysis*

Laboratory A performed all the analysis on a TSQ Quantum XLS GC–MS/MS instrument (Thermo Scientific, Waltham, MA, USA) equipped with a TRACE TR-BIO-DIESEL column (30 m × 0.25 mm ID × 0.25 µm film thickness, Thermo Scientific).

The equipment used by laboratory B was an Agilent 6890N gas chromatograph coupled to an Agilent 5975 Series GC/MSD (Agilent, Santa Clara, CA, USA). The column employed for the separation of FAMEs was a SP 2560 (100 m × 0.25 mm ID, 0.20 µm film thickness, SUPELCO, Bellefonte, PA, USA).

The analyses of the samples were carried out under the conditions shown in Table 1.

*Quantification principle*

The calibration data were used for the calculation of the relative response factors ($R$) according to Eq. (1):

$$R = \frac{A_{\mathrm{FAME,cal}} \cdot m_{\mathrm{labFAME,cal}}}{A_{\mathrm{labFAME,cal}} \cdot m_{\mathrm{FAME,cal}}} \quad (1)$$

where $R$ is the relative response factor, $A_{\mathrm{FAME,cal}}$ is the peak area of the specific FAME in the calibration solution, $m_{\mathrm{FAME,cal}}$ is the mass of the individual FAME in the calibration solution, $A_{\mathrm{labFAME,cal}}$ is the peak area of the labelled FAME, and $m_{\mathrm{labFAME,cal}}$ is the mass of labelled FAME in the calibration solution.

The mass fraction of each FAME in a test sample was calculated according to this Eq. (2)

**Table 1** GC–IDMS conditions used for the analysis of target FAMEs

| | Laboratory A | Laboratory B |
|---|---|---|
| *GC conditions* | | |
| Injection technique | Split (split–splitless, ratio 10:1) | Split (split–splitless, ratio 10:1) |
| Injection volume | 1.0 µL | 2.5 µL |
| Injector temperature | 250 °C | 250 °C |
| Carrier gas | Helium (99.9999 % purity) | Helium (99.9999 % purity) |
| Flow | Constant flow mode at 1.0 mL/min | Constant flow mode at 1.3 mL/min |
| Capillary column | TRACE TR-BIODIESEL (thermo scientific), 30 m × 0.25 mm ID × 0.25 µm film thickness | SP 2560 (SUPELCO), 100 m × 0.25 mm ID × 0.20 µm film thickness |
| Oven temperature programme | 120 °C (0.50 min), 30 °C/min to 220 °C (1 min), 10 °C/min to 240 °C (5 min) | 140 °C (3 min), 4 °C/min to 176 °C (22 min), 5 °C/min to 240 °C (2 min) |
| *MS conditions* | | |
| Transfer line | 250 °C | 230 °C |
| Ion source temperature | 200 °C | 150 °C |
| Ionisation mode | Electron impact (EI) at 70 eV | Electron impact (EI) at 70 eV |
| Acquisition mode (SIM mode) | C16:0: *m/z* 227.30, 241.85; C16:0*: *m/z* 230.40, 244.39<br>C18:0: *m/z* 255.37; C18:0*: *m/z* 244.24, 258.46<br>C18:1: *m/z* 141.15, 169.47, 213.50, 253.42; C18:1*: *m/z* 143.95, 175.26, 216.87, 256.63<br>C18:2: *m/z* 178.34, 262.63; C18:2*: *m/z* 150.27, 164.00<br>C18:3: *m/z* 93.51, 163.56; C18:3*: *m/z* 135.97, 150.76 | Diagnostic ions:<br>*m/z* 74, 77: native and labelled FAMEs (McLafferty rearrangement)<br>Quantifying ions:<br>C16:0: *m/z* 270.1; C16:0*: *m/z* 273.2<br>C18:0: *m/z* 298.1; C18:0*: *m/z* 301.2<br>C18:1: *m/z* 296.1; C18:1*: *m/z* 299.2<br>C18:2: *m/z* 294.1; C18:2*: *m/z* 312.3<br>C18:3: *m/z* 292.1 |

$$w = \frac{A_{\mathrm{FAME}} \cdot m_{\mathrm{labFAME}} \cdot m_{\mathrm{bdsol}}}{A_{\mathrm{labFAME}} \cdot R \cdot m_{\mathrm{aliquot}} \cdot m_{\mathrm{bd}}} \quad (2)$$

where $w$ is the mass fraction of a specific FAME in a biodiesel sample, $A_{\mathrm{FAME}}$ is the peak area of the FAME in the sample, $m_{\mathrm{labFAME}}$ is the mass of the labelled FAME added to the sample, $m_{\mathrm{bdsol}}$ is the mass of the biodiesel solution (biodiesel + toluene), $A_{\mathrm{labFAME}}$ is the peak area of the labelled FAME in the sample, $m_{\mathrm{aliquot}}$ is the mass of the biodiesel solution spiked and injected, and $m_{\mathrm{bd}}$ is the mass of the biodiesel taken to prepare the biodiesel solution.

*GC–FID approach*

The fatty acid composition of biodiesel was determined by capillary gas chromatography with flame ionisation detection (GC–FID) using analytical conditions identical to those prescribed in EN 14103 [6] in combination with a different quantification approach using experimentally determined relative response factors for the target FAMEs. Therefore, five in-house prepared FAME mix solutions were analysed using experimental conditions identical to those used for the test sample. The response factors for the different FAMEs were calculated relative to the internal standard, i.e., nonadecanoic acid methyl ester (C19:0). Additionally, the trueness of results for the individual FAMEs of interest, i.e., C16:0, C18:0, C18:1, C18:2 and C18:3 was assessed by the analysis of SRM 2377. The chromatographic separation carried out on a polar column (SP 2560 Supelco Bellefonte, PA, USA; 100 m × 0.25 mm ID × 0.20 µm) was done using helium as carrier gas (1.3 mL/min constant flow) and the following temperature programme: hold for 3 min at 140 °C then heat to 176 °C at 4 °C/min and hold for 22 min. Heat to 240 °C at 4 °C/min and hold for 15 min. The temperature of the FID and the split flow injector was set at 250 °C.

## Results and discussion

### Method validation

Method validation is one of the measures universally recognised as a necessary part of a comprehensive system of quality assurance in analytical chemistry. The in-house

validation procedure carried out at both laboratories was accomplished on the basis of the EURACHEM [13] and IUPAC [14] guidelines. As far as they are relevant, the following parameters of the analytical procedures were examined: linearity, limit of detection (LOD), limit of quantification (LOQ), precision and trueness for data on the most abundant FAMEs (C16:0, C18:0, C18:1, C18:2 and C18:3) in biodiesel. The validation study was carried out on five different working days.

As the FAMEs considered for validation constitute 95 % or more of the total mass of a biodiesel sample, most of the validation was carried out using 'synthetic' samples, i.e., mixtures of pure compounds or solutions of individual FAMEs. No additional matrix effects are expected when using 'real' samples.

*Linearity, working range and response factors*

In laboratory B, three replicates of every calibration solution were analysed on each of the 5 days of the validation study. The calibration curves were established considering all data obtained by plotting the peak area ratios (FAME/labelled FAME) versus mass fraction ratios (FAME/labelled FAME). The ions shown in Table 1 were selected to establish the ratio of the areas. The correlation coefficients obtained from the linear regression of the calibration curves for the target FAMEs were larger than 0.99, indicating a strong relationship between the variables within the entire mass fraction range.

**Table 2** Mass fractions $w$ at the limit of detection (LOD) and quantification (LOQ) of target FAMEs, estimated from the sample blank value plus 3 times and 10 times the standard deviation, respectively

| FAME | $w_{LOD}$ (mg/g) | $w_{LOQ}$ (mg/g) |
|---|---|---|
| C16:0 | 0.0090 | 0.030 |
| C18:0 | 0.0042 | 0.014 |
| C18:1 | 0.010 | 0.033 |
| C18:2 | 0.028 | 0.092 |
| C18:3 | 0.027 | 0.089 |

Visual inspection of the corresponding residual plots revealed a random pattern for each FAME compound, being consistent with an adequate straight-line model without any trend in the spread of residuals with concentration.

Since the calibration curves for all target analytes were linear over the studied mass fraction range, the average relative response factor $R$ (Eq. 1) obtained for all calibration points was used covering the whole calibration range. The values of $R$ obtained for any of the selected FAMEs on the five validation days did not show a relative standard deviation larger than 2 % for both methods.

*Limit of detection and limit of quantification*

The LOD and LOQ were estimated to be significantly below the observed FAME mass fractions, as the selected FAMEs represent the main components in biodiesel. Nevertheless, laboratory A determined LOD and LOQ and results are shown in Table 2.

*Repeatability and intermediate precision*

In laboratory A, repeatability and intermediate precision of the method were assessed by analysing kerosene spiked with C16:0 (0.60 mg/g), C18:0 (0.26 mg/g), C18:1 (0.13 mg/g), C18:2 (0.14 mg/g) and C18:3 (0.66 mg/g). Six replicates of the sample were prepared and analysed on each of three validation days, using six injections each. One-way ANOVA was applied to the results and yielded repeatability as "within-group" standard deviation, and day-to-day variation (intermediate precision) as "between-group" standard deviation. The results are given as relative standard deviation (Table 3).

In order to calculate the repeatability and intermediate precision in laboratory B, three independent replicates of the biodiesel sample were prepared and analysed on five different working days. Two injections were done for each replicate, and the mean value was calculated to be used for the final calculations. One-way ANOVA was applied to analyse the data similar to laboratory A. The obtained results are shown in Table 4.

**Table 3** Recoveries of individual FAMEs and related relative standard deviations (RSD) of repeatability (laboratory A)

| FAME | Recovery (%) | RSD of recovery (%) | RSD of repeatability of peak area (%) | RSD of repeatability of retention time (%) | RSD of intermediate precision (%) |
|---|---|---|---|---|---|
| C16:0 | 99.3 | 0.8 | 0.4 | 0.06 | 0.036 |
| C18:0 | 99.0 | 0.5 | 0.4 | 0.07 | 0.032 |
| C18:1 | 101.5 | 0.5 | 0.9 | 0.05 | 0.15 |
| C18:2 | 100.7 | 0.5 | 0.6 | 0.07 | 0.4 |
| C18:3 | 99.4 | 0.2 | 1.0 | 0.07 | 0.4 |

*Trueness*

For the assessment of the trueness, laboratory A used an animal-based biodiesel CRM (NIST 2773), whereas laboratory B used a CRM consisting of a mixture of FAMEs (NIST 2377). Two replicates were prepared and injected together with the biodiesel samples and calibration solutions on each of the 5 days of the validation study. The mean value obtained from the measurement of the analysed replicates was used for the final trueness assessment. Results from these measurements are shown in Table 5.

The trueness assessment was carried out according to ERM Application Note 1 [15], where the comparison of the measurement results with the certified values is described in detail. No significant differences between the measurement results and the certified values for the analytical procedures used by laboratory A and laboratory B were found.

*Robustness*

It was considered that the assessment of the robustness was not applicable to these analytical procedures since all temperatures and processes were well controlled when using the GC–MS. All steps of the analytical procedure were automated, and the different parameters were strictly controlled. The proper functioning of the GC–MS was regularly checked during maintenance and operational qualification. Any potential fluctuation (within the range admitted by the operational qualification test of the instrument) from the instrument settings is covered by the repeatability and intermediate precision of the method. Therefore, the assessment of robustness for the related parameters is not required.

Regarding sample preparation, it consists basically of weighing biodiesel and diluting it with organic solvent, and consequently there are no parameters for which robustness could be assessed.

**Table 4** Precision data obtained in validation study for FAMEs of interest in biodiesel (laboratory B)

| FAME | RSD of repeatability (%) | RSD of intermediate precision (%) |
|---|---|---|
| C16:0 | 0.163 | 0.439 |
| C18:0 | 0.218 | 0.797 |
| C18:1 | 0.014 | 0.12 |
| C18:2 | 0.078 | 0.16 |
| C18:3 | 0.159 | 0.591 |

**Table 5** Measured and certified mass fractions for SRM 2773 (laboratory A) and SRM 2377 (laboratory B); all data in mg/g; $U$ denotes expanded uncertainty at $k = 2$

| FAME | Measured value | Uncertainty $U$ of measured value | Certified value | Uncertainty $U$ of certified value |
|---|---|---|---|---|
| *Laboratory A—SRM 2773* | | | | |
| C16:0 | 190 | 7 | 184 | 6 |
| C18:0 | 91 | 4 | 87.8 | 4.2 |
| C18:1 | 340 | 15 | 343 | 8 |
| C18:2 | 225 | 16 | 226 | 5 |
| C18:3 | 25.3 | 1.4 | 25 | 1.0 |
| *Laboratory B—SRM 2377* | | | | |
| C16:0 | 7.5 | 0.4 | 7.38 | 0.32 |
| C18:0 | 7.71 | 0.18 | 7.68 | 0.12 |
| C18:1 | 6.8 | 0.4 | 7.01 | 0.31 |
| C18:2 | 7.54 | 0.17 | 7.33 | 0.14 |
| C18:3 | 4.5 | 0.4 | 4.26 | 0.26 |

## Uncertainty estimation

The estimation of the final uncertainty for the mass fraction of any of the target FAMEs was made up from different contributions, according to the Guide to the Expression of Uncertainty in Measurements [14], using a bottom-up approach. The expanded uncertainty is calculated taking into account the different contributions according to the Eq. (3):

$$U = k \cdot \sqrt{u_{\mathrm{cal}}^2 + u_{\mathrm{r}}^2 + u_{\mathrm{day}}^2 + u_{\mathrm{t}}^2} \tag{3}$$

where $U$ is the expanded combined relative uncertainty, $k$ is the coverage factor corresponding to a confidence level of approximately 95 % ($k = 2$), $u_{\mathrm{cal}}$ is the relative uncertainty contribution of the calibration, $u_{\mathrm{r}}$ is the relative uncertainty due to repeatability (15 replicates in total), $u_{\mathrm{day}}$ is the relative uncertainty due to intermediate precision (five measurement days), and $u_{\mathrm{t}}$ is the relative uncertainty due to trueness.

*Uncertainty of calibration*

The uncertainty of calibration includes contributions arising from purity of the calibrant, gravimetric preparations and as well as the determinations of the relative response factor $R$. It contributes to the final measurement uncertainty, which has been estimated during the method validation. The largest uncertainty contribution from the gravimetric preparation was coming from the preparation of the lowest concentration level for both methods. As a conservative approach, it was decided in all cases to use the highest uncertainty contribution among all gravimetric preparations of the calibration solutions as contribution to the final uncertainty of the calibration. This contribution was still lower than 0.05 % in all cases, resulting in an uncertainty of calibration lower than 0.35 % for all the FAMEs of interest for both methods.

*Uncertainty of repeatability and intermediate precision*

One-way ANOVA was applied to the measurement results. The uncertainty contribution related to the repeatability ($u_r$) was estimated as $s_r/\sqrt{n}$ with $s_r$ being the repeatability standard deviation from the validation study, and $n$ the number of replicates performed for the particular measurement in question.

The intermediate precision expresses the precision where at least one of the conditions for repeatability is not met. The experiment consisted in the analysis of three independent replicates of the same sample on five different days, employing the same methodology and the same operator. Applying one-way ANOVA to the obtained results enables estimating the uncertainty due to the intermediate precision ($u_{day}$) as $s_{day}/\sqrt{p}$ with $s_{day}$ being the relative day-to-day variation from the validation study and $p$ being the number of days over which the measurements were spread.

The obtained results showed an uncertainty due to repeatability ranging from 0.014 % (C16:0) to 0.22 % (C18:0), whereas the uncertainty contribution related to the intermediate precision varied from 0.12 % (C 16:0) to 0.8 % (C18:0).

*Uncertainty of trueness*

Equation (4) was used to calculate the uncertainty of trueness:

$$u_t = \sqrt{\frac{s_t^2}{n_t} + \frac{\sum u_{mat}^2}{n_{mat}^2}} \quad (4)$$

where $s_t$ and $n_t$ are the relative standard deviation and the number of replicates of the trueness experiment of the validation study, respectively, $u_{mat}$ is the relative uncertainty of the certified values of the reference material used, and $n_{mat}$ is the number of materials used for the trueness estimation.

The estimated relative uncertainty contributions for the individual FAMEs were ranging from 0.9 (C18:0) to 3.4 % (C18:3).

*Expanded uncertainty (U) and analysis of biodiesel*

For both methods, the expanded combined uncertainty was calculated using Eq. 3, taking into account the different contributions described above. As can be seen from Fig. 1 for laboratory B, the main contribution to the final uncertainty budget is due to the trueness assessment for each of the studied FAMEs except for C18:0, where the intermediate precision is of similar magnitude.

The developed methods were applied to the analysis of a rapeseed biodiesel test material distributed in the frame of the EMRP project. The quantification of the selected FAMEs together with the associated uncertainty of the measurements is shown in Table 6. This table also includes the results obtained by analysing this test sample using the GC–FID method to quantify individual FAMEs. The results obtained with the new GC–IDMS methods are in agreement with those obtained by GC–FID within the uncertainties stated.

*Traceability of the results*

The traceability of the measured values obtained using the described GC–IDMS methods is established on the one hand by measuring structurally well-defined individual FAMEs, which is ensured by the mass spectrometric

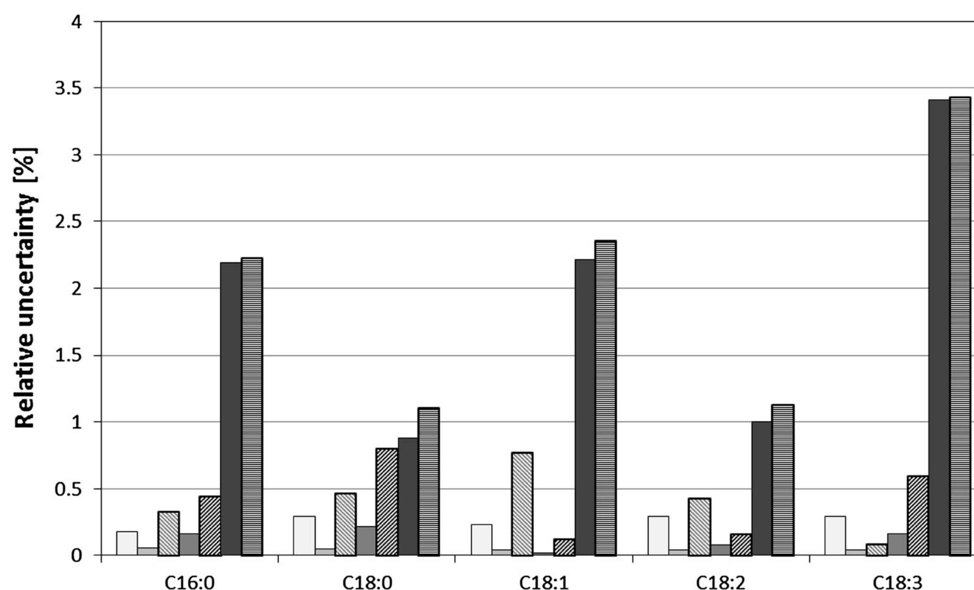


**Fig. 1** Individual uncertainty components together with the combined uncertainty of target FAME mass fractions as obtained in validation study (laboratory B), expressed as relative uncertainties. Components of the uncertainty of calibration due to □ standard purity, ▒ standard preparation and ▧ relative response factors; uncertainties of ■ repeatability, ▨ intermediate precision, ■ trueness and ▤ combined uncertainty

**Table 6** Comparison of results for individual FAMEs in biodiesel test material obtained with presented methods and GC–FID approach

| Laboratory | A | | B | | | |
|---|---|---|---|---|---|---|
| Method | GC–IDMS | | GC–IDMS | | GC–FID | |
| FAME | Mass fraction (mg/g) | $U$ ($k = 2$) (mg/g) | Mass fraction (mg/g) | $U$ ($k = 2$) (mg/g) | Mass fraction (mg/g) | $U$ ($k = 2$) (mg/g) |
| C16:0 | 46.3 | 1.9 | 44.5 | 1.9 | 44.0 | 2.1 |
| C18:0 | 15.7 | 0.7 | 15.9 | 0.4 | 16.1 | 0.6 |
| C18:1 | 600 | 26 | 593 | 28 | 596 | 26 |
| C18:2 | 214 | 11 | 207 | 5 | 199 | 7 |
| C18:3 | 85 | 5 | 87 | 6 | 86 | 6 |

detection employed. On the other hand, the methods allow calibration with calibrants containing the very same FAMEs, for which their mass fractions can be established in a metrologically sound way, through purity assessment and gravimetric preparations. Further steps in the calibration involve isotopically labelled analogues of the same FAMEs, thus minimising potential bias due to mismatches between internal standards and measurands. All steps in the measurement can be fully described and uncertainty contributions have been assigned and quantified.

## Conclusions

It has been shown in this work that it is possible to set up GC–IDMS-based procedures for selected FAMEs in biodiesel that are able to provide metrologically traceable results with a reliable uncertainty estimate. In case of the sample investigated here, results obtained are also comparable to those obtained by a more routinely applicable GC–FID method within their respective uncertainties. The reported GC–IDMS results have uncertainties similar to the GC–FID method. This can be mainly attributed to the fact that the uncertainty budgets are dominated by the uncertainty of trueness, which mainly depends on the uncertainty of the certified values of the CRMs employed.

As isotopically labelled FAMEs are generally more expensive than other substances commonly used as internal standards in GC–FID, the procedures described here are not suitable in routine measurements, but GC–IDMS of FAMEs opens the possibility to provide metrologically traceable reference values for special applications, such as resolving dispute between laboratories, further refinement of routine (standardised) methods or development of CRMs.

**Acknowledgments** The research leading to these results has received funding from the European Union on the basis of Decision No. 912/2009/EC, in the frame of the EMRP Project ENG09 'Metrology for Biofuels'.

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