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RESEARCH ARTICLE

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Evaluation of a quick one-step sample preparation method for the determination of the isotopic fingerprint of rapeseed (*Brassica napus*): Investigation of the influence of the use of 2,2-dimethoxypropane on compound-specific stable carbon and hydrogen isotope analyses by gas chromatography combustion/pyrolysis isotope ratio mass spectrometry

Lili Xia[1] | Kaori Sakaguchi-Söder[1] | Daniel Stanojkovski[2] | Liselotte Schebek[1]

[1]Institute IWAR, Technische Universität Darmstadt, Franziska-Braun-Straße 7, Darmstadt, Hessen, DE, 64287, Germany

[2]Jager Biotech GmbH, An der Ziegelei 57, Groß-Zimmern, DE, 64846, Germany

Correspondence
L. Xia, Institute IWAR, Technische Universität Darmstadt, Franziska-Braun-Straße 7, Darmstadt, Hessen, DE 64287, Germany.
Email: l.xia@iwar.tu-darmstadt.de

Rationale: Gas chromatographic analyses for vegetable oils require transesterification, which generally involves multiple steps, mainly to generate fatty acid methyl esters (FAMEs). A quick method based on acid-catalyzed transesterification using 2,2-dimethoxypropane (DMP) enables the conversion in one step, in a single reactor. For compound-specific stable carbon and hydrogen isotope analyses (C- and H-CSIA) of individual fatty acids (FAs) in oil, the verification of this one-step method has not yet been reported.

Methods: In this study, we evaluated the feasibility of the one-step method for C- and H-CSIA of individual FAMEs in rapeseed samples. The focus was on the investigation of the influence of methanol, which was produced from the reactions of DMP with glycerol and water during transesterification, on the accuracy of isotope composition of FAMEs, consequently of the FAs. The reproducibility of the one-step method was assessed by the measurement of the FAMEs from rapeseed and rapeseed oil. For the C- and H-CSIA of individual FAMEs, a gas chromatography combustion/pyrolysis isotope ratio mass spectrometry system was used.

Results: Our results showed that no significant differences arise in the carbon and hydrogen isotope compositions of the selected main FAMEs produced with and without DMP except for the H-CSIA value of C18:3. The reproducibility of the one-step method for rapeseed was in the range of ±0.1 mUr to ± 0.3 mUr for C-CSIA and ±1 mUr to ±3 mUr for H-CSIA of the main FAMEs.

Conclusions: DMP improves the transesterification efficiency without influencing the accuracy of the C- and H-CSIA of FAMEs. The performance of the one-step method for rapeseed samples for the determination of C- and H-CSIA values of FAMEs is satisfactory. Thus, the applicability of the one-step method for isotopic fingerprint analyses of FAs in oilseeds is reported for the first time.

1 | INTRODUCTION

The combination of analytical techniques and chemometrics has been successfully applied to investigate agricultural commodities for the protection of high-quality origin varieties against adulteration and fraud.[1–3] For edible vegetable oils (e.g. olive oil, sesame oil, palm oil, pumpkin oil), chromatographic techniques with stable isotope analysis are often used to determine isotope compositions, i.e. "fingerprints," of individual oil components.[4–9] For instance, Spangenberg et al. showed that the ratio of the stable carbon isotope composition ($\delta^{13}C$ values) of two fatty acids (FAs), namely oleic acid (C18:1) and palmitic acid (C16:0), could indicate the adulteration of olive oil with other vegetable oils.[8] Potočnik et al. verified the botanical and geographical origin of pumpkin oil based on the composition of individual FAs and the stable carbon isotope composition of selected FAs, i.e. C16:0, stearic acid (C18:0), C18:1 and linoleic acid (C18:2).[6] Ehtesham et al. showed that the stable hydrogen isotope composition ($\delta^{2}H$ values) and $\delta^{13}C$ values of four FAs, i.e. butyric acid (C4:0), myristic acid (C14:0), C16:0 and C18:1, can be applied as biomarkers to trace the regional origin of milk powders.[10]

To determine the isotope composition of FAs in vegetable oil, triacylglycerols (TAGs), the main components of oils, and/or free fatty acids (FFAs) are chemically converted to fatty acid methyl esters (FAMEs) in the presence of methanol and certain catalysts.[4–9] The stable isotope compositions of FAMEs are then determined using gas chromatography combustion/pyrolysis isotope ratio mass spectrometry (GC-C/Py-IRMS).[7,11,12] Due to the addition of the CH_3 group of methanol to FAs when producing FAMEs, the $\delta^{13}C$ and $\delta^{2}H$ values of FAMEs differ from the corresponding values of FAs in oil. Thus, the isotope composition of a certain FA (e.g. C16:1) is calculated based on the isotope composition of its corresponding FAME (e.g. C16:1 FAME) and the methanol employed, using mass balance correction equations.[4–9,12]

Potentially, kinetic isotope effects could cause additional differences in isotope compositions between reactant FAs and product FAMEs.[11] Whereas for carbon no kinetic isotope effects that change the isotope composition are expected in the reaction using excess methanol with catalyst,[13] hydrogen isotopes can undergo fractionation due to a secondary kinetic isotope effect. However, this effect was determined to be small when boron trifluoride in methanol was used to carry out the methylation reaction.[14] In addition, isotope fractionation of hydrogen may be caused by organic $^{1}H/^{2}H$ exchange in C-H bonds in FAs if oil is transesterified under acidic and hot conditions, although isotope fractionation due to hydrogen exchange was found to be trivial during that reaction.[15]

GC-C/Py-IRMS is a powerful technique for precisely determining slight differences in the abundances of isotopes (i.e. C, H, O and N) in various compounds.[16] For compound-specific stable carbon analysis (C-CSIA), individual target compounds are first separated in a GC column then transferred to an online-connected combustion chamber. In the combustion chamber, each target compound is converted to CO_2 and the CO_2 gas is analyzed in the IRMS system where the abundances of the masses 44 ($^{12}C^{16}O_2$), 45 ($^{12}C^{16}O^{17}O$ and $^{13}C^{16}O_2$) and 46 ($^{12}C^{16}O^{18}O$, $^{12}C^{17}O_2$ and $^{13}C^{16}O^{17}O$) of the CO_2 are determined.[12] For compound-specific stable hydrogen isotope analysis (H-CSIA), target compounds separated in the GC column are transferred to a high-temperature conversion system and pyrolytically converted to H_2 gas.[16] The abundances of the masses 2 ($^{1}H_2$) and 3 ($^{1}H^{2}H$) of the H_2 gas are determined in the IRMS system. The $\delta^{13}C$ and $\delta^{2}H$ values are determined from the carbon stable isotope ratio $^{13}C/^{12}C$ and hydrogen stable isotope ratio $^{2}H/^{1}H$, respectively, of each compound in a sample and of corresponding reference standards.

To implement C-CSIA and H-CSIA for a vegetable oil, the oil should be first extracted from the oilseed. Depending on the form of samples, extraction of oil may require multiple steps. For instance, to extract oil from maize and 16 nonmaize seeds, nuts or kernels, Woodbury et al. first milled the samples and placed them in petroleum ether for a total of 6 h. The remaining solvent in the extracted oil was then removed under vacuum at 60°C.[9] Jeon et al. collected sesame oil by centrifuging the roasted and pressed seeds for 10 min. The extracted sesame oil was then completely dried under nitrogen flushing.[17]

The extracted oil is converted to FAMEs for GC analysis to determine the FA profile of the oil or for stable isotopic analysis of individual FAs. For authentication studies, FAMEs are normally produced using a boron trifluoride (BF_3) catalyst as described in DIN EN ISO 12966-2.[7,9,18] This DIN method also consists of multiple steps, i.e. saponification, transmethylation/methylation, purification and extraction of FAMEs prior to the instrumental analysis.[19] Briefly, TAGs are first transmethylated in the presence of methanolic sodium hydroxide to form FAMEs. All the FFAs present are converted to sodium soaps in an alkaline-catalyzed step. The soaps are then converted into methyl esters by reaction with a BF_3–methanol complex. Isooctane and saturated sodium chloride solution are subsequently added to separate the FAMEs into the upper isooctane phase. After the removal of trace amount of water by the addition of anhydrous sodium sulfate, the FAMEs are ready for GC analysis.

The involvement of multiple steps and the use of BF_3 are known disadvantages of the DIN method when analyzing a large number of samples for chemometrics evaluation. Labor-intensive multistep sample preparation is not only unsuitable for processing a high number of samples but also may lead to certain sample loss and increase the chances of contamination. BF_3 is a toxic substance and the methanolic BF_3 reagent is highly reactive, and thus not suitable for long-term storage.[19,20]

To overcome these disadvantages of the DIN method, Garcés and Mancha developed a unique and quick one-step sample preparation method.[21] This one-step method enables lipid extraction from seeds, transesterification, methylation and extraction of FAMEs simultaneously in a single vial within a couple of hours with the aid of 2,2-dimethoxypropane (DMP), toluene and *n*-heptane as components of a transmethylation mixture. DMP enables the transesterification and methylation reactions to be performed simultaneously. A transesterification product, glycerol, reacts with DMP to produce isopropylidine glycerol and methanol.[22] Removal of glycerol

accelerates the transesterification reaction and the methanol produced via the reaction will be fed as a reactant for the transmethylation/methylation processes. In addition, DMP reacts with water generated from the methylation process to produce acetone and methanol. Water hinders the transmethylation reaction; thus, the removal of water from the system helps the methylation process to proceed.[23] The FAMEs produced in these two simultaneous reactions can then be directly extracted in the *n*-heptane available in the same reaction tube.

The applicability of the quick one-step method of Garcés and Mancha to determining the lipid content and the FA profile of various oilseeds was verified. However, its verification for the determination of the isotope composition of individual FAs has not yet been reported.

In the study reported here, we evaluated the feasibility of the one-step method of Garcés and Mancha for the C- and H-CSIA of rapeseed (*Brassica napus*) with the following two objectives: (1) determining the influence of the aggregate states of samples on the reproducibility of C- and H-CSIA and (2) determining the influence of DMP, more specifically the influence of the reaction intermediate methanol due to the use of DMP, on the accuracy of the C-and H-CSIA of FAMEs, and consequently of FAs.

The isotope composition of FAs necessary for the authentication study of vegetable oils should be calculated from the isotope composition of the FAMEs as well as the methanol consumed in the reaction processes. The isotope composition of the initially added methanol in the transmethylation mixture can be determined, whereas that of the reaction intermediate methanol formed by the use of DMP cannot. If our study shows that the influence of the reaction intermediate methanol on C- and H-CSIA of FAMEs can be quantified or is negligible, the isotope composition of FAs can be determined using the quick one-step method with DMP, as long as the isotope composition of the initially added methanol is known.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

All the solvents and reagents were of analytical grade or higher purity. For the production of transmethylation mixtures, methanol, toluene, DMP and sulfuric acid (H_2SO_4) were used. Heptane was also added to each transmethylation mixture for the purpose of extracting the FAMEs produced in the reaction.

Supelco® nonadecanoic acid methyl ester (C19:0 FAME; Merck, Darmstadt, Germany) was used as an internal standard to determine the recovery rate of FAMEs in the *n*-heptane phase and the FAME yield. Supelco® 37 component FAME Mix and ROTICHROM® FO 7 FAMEs mixture (Carl Roth, Karlsruhe, Germany), which consists of 11 FAMEs, were applied for the quantification of individual FAMEs. ROTICHROM® oleic acid methyl ester (C18:1 FAME) was applied as in-house reference material for quality control of the isotope measurements. The stable isotopic reference materials USGS 70 and USGS 71 were purchased from the USGS Reston Stable Isotope Laboratory (Reston, VA, USA). The icosanoic acid methyl esters (C20:0) in these reference materials were used for the normalization of stable carbon ($\delta^{13}C$) and hydrogen ($\delta^{2}H$) measurement of FAMEs.

2.2 | Samples

A bottle of rapeseed oil was purchased from a shop in Dottenfelderhof (Germany), and a rapeseed sample cultivated in Hesse (Germany) was provided by the Landesbetrieb Landwirtschaft Hesse. For each experiment, 20 mg of rapeseed oil or 50 mg of rapeseed was used for the one-step extraction. Each experiment was done in triplicate. For the rapeseed samples, 10 g of rapeseed was ground with a coffee grinder for 1 min to obtain a fine and homogeneous powder.

2.3 | One-step sample preparation method of Garcés and Mancha

With a transmethylation mixture of methanol, toluene, DMP, sulfuric acid and *n*-heptane (39:20:5:2:34 vol%), Garcés and Mancha achieved complete transmethylation of seeds at 80°C in a reaction time of 120 min in one reaction tube.[21] The transmethylation and methylation reactions taking place in the conversion of TAGs to FAMEs and two more reactions occurring in the presence of DMP are displayed in Figure 1.[22,24]

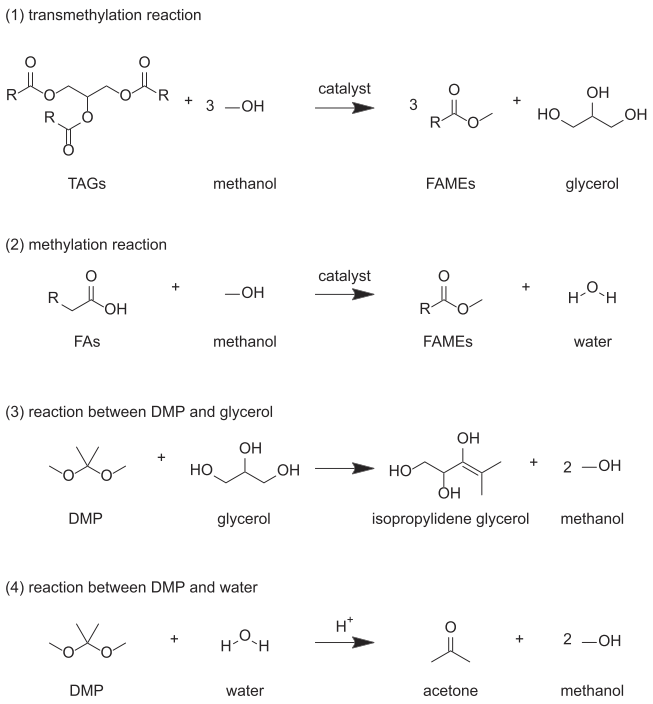

FIGURE 1 Four major reactions occurring in the one-step method

2.4 | Preparation of transmethylation mixtures and production of FAMEs

For this study, three transmethylation mixtures, TM+D, TM-D and TX-D, were prepared. The composition of these transmethylation mixtures is summarized in Table 1.

The volume ratio of methanol, toluene, DMP, sulfuric acid and *n*-heptane for these mixtures was as follows: TM+D (39:20:5:2:34), TM-D (44:20:0:2:34) and TX-D (56:14:0:1:29).

The TM+D mixture containing 5 vol% of DMP was proven as the best mixture for the highest transmethylation efficiency by Garcés and Mancha.[21] TM-D was formulated without DMP by replacing DMP in the TM+D mixture with the same volume of methanol.

TX-D was also formulated without DMP in the same way as TM-D. The volume of methanol used for TX-D and TM-D was, respectively, 3.9 mL (0.096 mol) and 2.2 mL (0.054 mol), but the volume of the other reagents in the transmethylation mixture stayed the same. Each transmethylation mixture was first prepared without *n*-heptane and stored in a refrigerator.

From a homogenized and ground batch of a rapeseed sample, nine aliquots were taken. Each aliquot contained 50 mg of ground rapeseeds, which is roughly equivalent to 20 mg of rapeseed oil. For each transmethylation mixture, three aliquots were handled under the same conditions. An amount of 20 mg of rapeseed oil or 50 mg of ground rapeseed was placed in a 20 mL crimp-top glass vial (reactor) where a certain volume of TM+D, TM-D or TX-D was added. A volume of 1.7 mL of *n*-heptane was then added to the reactors containing TM+D and TM-D shortly before the reaction started, whereas a volume of 2 mL of *n*-heptane was added to the reactors with TX-D when the reaction was complete.

Each reactor was closed with a Teflon-lined crimp cap, strongly shaken by hand and placed in a water bath at 80°C for 120 min. The reactor was then cooled to room temperature and shaken again. The FAMEs produced in the reaction were extracted in the upper layer mainly consisting of toluene and *n*-heptane. The upper layer was taken and diluted eight times with *n*-heptane. The original or diluted aliquot was analyzed directly for its FA profile using GC with flame ionization detection (FID), and for the δ^2H and $\delta^{13}C$ values of individual FAMEs using GC-C/Py-IRMS. The rapeseed samples were prepared under every condition in triplicate and isotope measurement was performed three times per sample.

2.5 | Determination of FA profile

For the FA profile analysis, an Agilent 7890B gas chromatograph with FID (Agilent, Santa Clara, CA, USA) was employed. The GC instrument was equipped with a TG-WAX column (30 m × 0.32 mm, 0.5 μm; Thermo Fisher Scientific, Dreieich, Germany). Helium was used as the carrier gas at a flow rate of 1.5 mL/min. A PAL autosampler (CTC Analytics, Zwingen, Switzerland) was used to inject 1 μL of the sample into the GC inlet heated at 250°C with a split ratio of 1:10. The GC oven temperature program was started at 160°C for 1 min, heated to 190°C at 20°C/min and further to 220°C at 7°C/min where it was held for 29 min. Each sample was analyzed twice. The FAs in rapeseed were identified using GC-FID. For the determination of the FA profile, the samples and the ROTICHROM® FO 7 FAME mixture were analyzed ing GC-FID. In accordance with DIN EN ISO 12966-4, the weight percentage of the individual FAME (wi) was calculated using the following equation:

$$wi = Fi \times Ai / \Sigma(Fi \times Ai) \quad (1)$$

where Ai is the chromatographic area of the FAME. The correction factor Fi was determined by the ROTICHROM® FO 7 FAME mixture using the equation:

$$Fi = mi \times \Sigma A / Ai \times \Sigma m \quad (2)$$

where $mi/\Sigma m$ is the known mass percentage of the FAME.

2.6 | Compound-specific stable carbon and hydrogen isotope analyses

For C- and H-CSIA of individual FAMEs, a Trace 1310 gas chromatograph with FID coupled to a Delta V advantage isotope ratio mass spectrometer with an IsoLink II combustion/pyrolysis interface under a continuous helium flow regulated by a Universal ConFlo IV Interface (GC-C/Py-IRMS system; all supplied by Thermo Fisher Scientific, Bremen, Germany) was employed. The chromatographic separation of the FAMEs was performed with the same column as and similar oven temperature program to those used for FA profile analysis. About one-tenth of the GC effluent

TABLE 1 Composition of transmethylation mixtures and *n*-heptane for every 20 mg of oil or 50 mg of seed

		Volume (mL)			Amount (mmol)		
	Reagent	TM+D	TM-D	TX-D	TM+D	TM-D	TX-D
Transmethylation mixture	Methanol	1.95	2.2	3.9	48.2	54.4	96.4
	Toluene	1	1	1	9.4	9.4	9.4
	DMP	0.25	0	0	2	0	0
	Sulfuric acid	0.1	0.1	0.1	1.9	1.9	1.9
n-Heptane		1.7	1.7	2	11.6	11.6	13.7
Sum		5	5	7	73.1	77.3	121.3

was directed to the FID system and the remaining 90% into the combustion reactor interface. The targeted FAMEs from the GC effluent transferred to the IsoLink II were oxidized to CO_2 in a capillary combustion reactor at 1000°C for carbon isotope analysis. The capillary combustion reactor was made of a nickel tube into which copper, nickel and platinum wires are inserted. Moisture generated in the combustion process was removed by a tubular Nafion membrane. The CO_2 flow was directed to the IRMS system and the peak signal intensities of the three major ions of *m/z* 44, 45 and 46 were monitored using three Faraday cup detectors.

For hydrogen isotope analysis, the targeted FAMEs from the GC effluent were directed to a high-temperature conversion reactor and pyrolyzed to H_2 gas at 1420°C. The ceramic high-temperature conversion reactor tube was conditioned by injecting a mixture of alkanes to generate a glassy carbon surface before the first measurement. The peak signal intensities of *m/z* 2 and 3 of the H_2 gas were monitored by two additional Faraday cup detectors. The H_3^+ factor for correction of the H_3^+ contribution to the *m/z* 3 signal was determined daily before the sample measurement. It was shown to be lower than 10 ppm/nA, as suggested by the manufacturer.

The peak signal intensities of *m/z* 44, 45 and 46 of CO_2 and of *m/z* 2 and 3 of H_2 generated from individual FAMEs were integrated automatically with a background subtraction using Isodat 3.0 software (Thermo Fisher Scientific), and the data were exported for further correction.

2.7 | $\delta^{13}C$ and $\delta^{2}H$ normalization

The stable isotope compositions of carbon and hydrogen are reported in delta notation (δ) as the per mille deviation of the isotope composition relative to reference materials:

$$\delta = \left(R_{sample} - R_{RM}\right)/R_{RM} \quad (3)$$

where R_{sample} and R_{RM} are the ratio of the heavy to light isotopes of sample and reference materials, respectively. The unit of δ is mUr (milliurey) instead of per mille based on the IUPAC guidelines for SI units.[25]

The measured $\delta^{13}C$ and $\delta^{2}H$ values of samples were calculated from the isotope ratio of samples relative to that of working gases CO_2 and H_2, respectively, in our laboratory. They were converted and reported on an international isotope reference scale (VPDB for carbon: Vienna Pee Dee Belemnite; VSMOW for hydrogen: Vienna Standard Mean Ocean Water) using a two-point normalization method. The reference materials, USGS 70 and USGS 71, were dissolved in *n*-heptane and used for the two-point normalization. The true $\delta^{13}C$ values of C20:0 FAME in USGS 70 and 71 are, respectively, −30.53 ± 0.04 and −10.50 ± 0.03 mUr, expressed relative to VPDB on a scale normalized by assigning a value of +1.95 mUr to NBS 19 calcium carbonate and −46.6 mUr to LSVEC lithium carbonate. The true $\delta^{2}H$ values of C20:0 FAME in USGS 70 and 71 are, respectively, −183.9 ± 1.4 and −4.9 ± 1.0 mUr, reported relative to VSMOW on a scale normalized such that the $\delta^{2}H$ value of SLAP (Standard Light Antarctic Precipitation) is −428 mUr.[25]

Linear regression Equation 4 of the measured and true $\delta^{13}C$ and $\delta^{2}H$ values was established using USGS 70 and 71 in each analysis sequence. The true $\delta^{13}C$ and $\delta^{2}H$ values (δ^{T}_{spl}) of each unknown sample were normalized according to Equation 5[26]:

$$\text{Slope} = \frac{\delta^{T}_{spl} - \delta^{T}_{RM2}}{\delta^{M}_{spl} - \delta^{M}_{RM2}} = \frac{\delta^{T}_{RM1} - \delta^{T}_{RM2}}{\delta^{M}_{RM1} - \delta^{M}_{RM2}} \quad (4)$$

$$\delta^{T}_{spl} = \frac{\delta^{T}_{RM1} - \delta^{T}_{RM2}}{\delta^{M}_{RM1} - \delta^{M}_{RM2}} \times \left(\delta^{M}_{spl} - \delta^{M}_{RM2}\right) + \delta^{T}_{RM2} \quad (5)$$

where δ^{T}_{spl}, δ^{T}_{RM1} and δ^{T}_{RM2} denote the true isotope compositions of samples, USGS 70 and USGS 71, relative to international reference materials; and δ^{M}_{spl}, δ^{M}_{std1} and δ^{M}_{std2} represent the measured isotope compositions of the sample, USGS 70 and USGS 71, relative to the working gas.

2.8 | Quality assurance and quality control

To control the day-to-day performance of the GC-C/Py-IRMS system and the data normalization, the in-house reference material C18:1 FAME was used. The carbon and hydrogen isotope compositions of C18:1 FAME were measured after every 10–12 measurements and normalized using the USGS 70 and USGS 71 reference materials. The system stability was controlled by checking the deviation from the mean carbon and hydrogen isotope composition of the in-house reference material −28.4 and −165.1 mUr, respectively, which were determined from 15 measurements. The analytical run was accepted when the deviations fell in the range of ±0.6 mUr for $\delta^{13}C$ values and ±3.3 mUr for $\delta^{2}H$ values.

3 | RESULTS AND DISCUSSION

3.1 | Influence of DMP on transmethylation efficiency of the one-step method

To investigate the influence of DMP on the transmethylation efficiency, first, the FA profile and the absolute mass of FAMEs were determined using three transmethylation mixtures: TM+D (with DMP), TM-D and TX-D. The TM+D transmethylation mixture contained 5 vol% of DMP and 39 vol% of methanol, whereas the TM-D and TX-D mixtures were prepared without DMP, with 44 and 56 vol% of methanol, respectively.

The FA profile (mol%) was calculated by the mass percentage and the respective molecular weight of the individual FAs. The results are summarized in Table 2. The mean and standard deviation (SD) of each FA in the Table are determined from the mol% of the FA of three mixtures for comparison purposes. Details are presented in Table S1 (supporting information).

The main FA components of rapeseed oil are C18:1 (61.8 ± 0.6%), C18:2 (18.1 ± 0.4%), C18:3 (9.8 ± 0.5%) and C16:0 (5.5 ± 0.1%). The FA composition remains unchanged regardless of the transmethylation mixtures, with or without DMP. Furthermore, the absolute mass of each of the four main FAs was determined by the addition of a known amount of an internal standard C19:0 FAME in each reactor. The results are presented in Figure 2 and Table S2 (supporting information). For all major FAMEs, the absolute mass per 100 g of rapeseed determined using TM+D was greater than that using TM-D and TX-D.

Based on the results presented in Table S2 (supporting information), the yield of the FAMEs of TM-D and TX-D was calculated to be 86% and 91%, respectively, relative to the FAME yield of TM+D. The results showed that the use of DMP has a clear advantage in the transmethylation efficiency of TAGs to FAMEs. The yield increased from 86% to 100% by replacing 5 vol% methanol in TM-D with 5 vol% DMP of TM+D. This result shows consistency with the result by Garcés and Mancha that the presence of DMP can significantly improve the transmethylation efficiency of oil, if the proportions of methanol and H_2SO_4 and oil in the mixtures are kept the same. Another remark is that an increase of methanol from 44 vol % (TM-D) to 56 vol% (TX-D) contributed to an increase of the FAME yield of only 5%.

As presented in Figure 1, stoichiometrically, 1 mol of TAGs requires 3 mol of methanol, for the production of 1 mol of glycerol and 3 mol of FAMEs. In reality, however, a large excess of methanol ensures a high transmethylation reaction rate. Zheng et al. recommended a molar ratio of methanol to oil of 245:1,with acid catalyst, to achieve the maximum FAME yield from frying oil.[27] The yield of FAME was reduced by 27% when the molar ratio of methanol to oil was 106:1.[27]

The TAGs are composed of FAs esterified on a glycerol backbone.[28] As presented in the FA profile in Table 2, the average molar mass of the TAGs in rapeseed oil is estimated to be 881 g/mol (see Table S3, supporting information). Thus, 50 mg of rapeseed, which contains 20 mg of rapeseed oil, is equivalent to 0.02265 mmol. Based on this value, the molar ratios of methanol to oil in the transmethylation mixtures were calculated and are presented in Table 3. The methanol-to-oil molar ratios in TM+D, TM-D and TX-D are 2128:1, 2401:1 and 4256:1, respectively, indicating that the amount of methanol for the reaction is sufficient with or without DMP. Thus, the increase of the yield of TM+D is probably not due to the increase of methanol produced by the use of DMP.

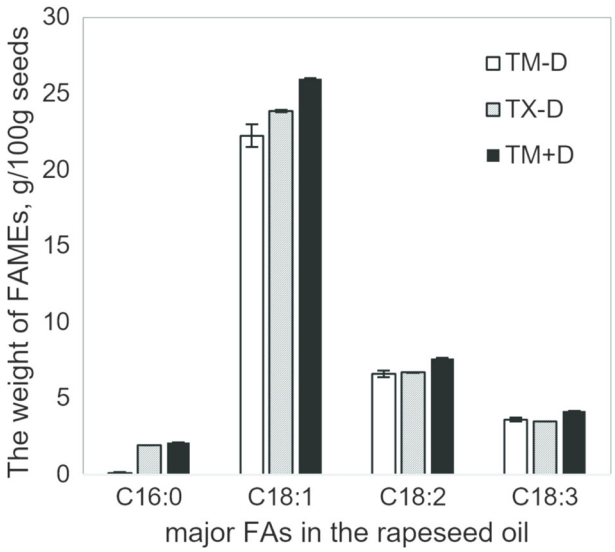

FIGURE 2 Weight of four major FAMEs (C16:0, C18:1, C18:2 and C18:3) in 100 g of rapeseed sample prepared using three transmethylation mixtures

TABLE 3 Molar ratio of each component to oil and FAME yield in transmethylation mixtures

	Molar ratio		
	TM+D	TM-D	TX-D
Rapeseed oil	1	1	1
Methanol	2128	2401	4256
Toluene	415	415	415
DMP	90	0	0
Sulfuric acid	82	82	82
FAME yield (%)	100	86	91

We assume that the main effects of DMP in the transmethylation reaction are (1) consumption of a reaction product (glycerol) and (2) removal of water from the system. The addition of DMP to the system ensures complete conversion of lipid by consuming glycerol, a reaction product of the transmethylation reaction (see Figure 1). From

TABLE 2 FA profile of a rapeseed sample determined using three transmethylation mixtures

	Fatty acid composition (mol%)												
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0	C22:1	C24:0	C24:1
TM+D	0.1	5.5	0.4	1.7	61.6	18.2	10.0	0.5	1.2	0.3	0.3	0.1	0.1
TM-D	0.1	5.6	0.4	1.7	61.4	18.4	10.1	0.5	1.2	0.3	0.3	0.1	0.1
TX-D	0.1	5.5	0.3	1.8	62.5	17.7	9.3	0.6	1.3	0.3	0.4	0.1	0.2
Mean	0.1	5.5	0.3	1.7	61.8	18.1	9.8	0.5	1.2	0.3	0.3	0.1	0.1
SD	0.02	0.06	0.05	0.09	0.61	0.36	0.47	0.05	0.04	0.03	0.04	0.03	0.03

20 mg of rapeseed oil (=0.02265 mmol), 0.02265 mmol of glycerol is expected to be produced. The amount of DMP (2 mmol) used is in excess for the reaction between DMP and glycerol. If the entire amount of glycerol reacts with DMP, 0.0453 mmol of additional methanol is produced, which will increase the methanol-to-oil molar ratio from 2128:1 to 2130:1. Since the increase of the methanol-to-oil molar ratio from 2401:1 (TM-D) to 4256:1 (TX-D) contributed to the increase of the yield only by 6%, the additional methanol generated by the reaction of DMP and glycerol in TM+D might not have a significant influence on the increase of yield.

Furthermore, the ability of DMP to react with water promotes the transmethylation reaction (see Figure 1). The presence of water in the system may result in the oil hydrolysis reaction, which suppresses the transmethylation reaction. Commonly, rapeseed contains 7–10% water and 0.5–1.8% FFAs under optimal storage conditions.[28] The methylation reaction of FFAs also produces water (see Figure 1). In addition, a very small quantity of water is present in solvents. For instance, the methanol product that we used contains about 0.02% of water. By increasing the methanol amount from 48.2 mmol (TM+D) to 54.4 mmol (TM-D) to 96.4 mmol (TX-D), the amount of water associated with the methanol product also increased from 0.017 to 0.019 to 0.034 mmol, respectively. Generally, the increase of methanol in the system will contribute to the increase of the FAME yield, although at the same time it may inhibit the production of FAMEs to some extent due to the increase of water associated with a methanol product if DMP is not used.[20,21] This can be an explanation for the transmethylation efficiency of TX-D stagnating at 91%, despite the addition of a large amount of methanol.

3.2 | Influence of DMP on $\delta^{13}C$ and $\delta^{2}H$ values of FAMEs using the one-step method

Prior to the investigation of the influence of DMP on the $\delta^{13}C$ and $\delta^{2}H$ values of the FAMEs produced in the different transmethylation mixtures applied for the quick one-step sample preparation, we investigated the precision of GC-C/Py-IRMS by analyzing two FAME reference materials and the influence of the aggregate states of samples on the reproducibility of the H- and C-CSIA of FAMEs.

3.2.1 | Precision of GC-C/Py-IRMS

Two in-house FAME reference materials, namely oleic acid methyl ester (C18:1) and nonadecanoic acid methyl ester (C19:0), were analyzed nine times (n = 9) using GC-C/Py-IRMS at the Institute of Applied Geosciences (IAG), Technical University Darmstadt, Germany and the mean and standard deviation were determined. The standard deviation (1σ) of the $\delta^{13}C$ and $\delta^{2}H$ values of the reference materials determined from the repeat measurements was considered as the precision of the C- and H-CSIA achieved by this GC-C/Py-IRMS system. The results are presented in Table 4. For both FAMEs, a precision of ±0.1 and ±1.7 mUr was achieved for C-CSIA and H-CSIA, respectively. These values showed the good stability of the instrument for the C- and H-CSIA of FAMEs.

3.2.2 | Influence of aggregate states of samples on reproducibility of H- and C-CSIA of FAMEs

One advantage of this one-step method is to save the step of extracting fluid oil from seeds, which may result in a certain degree of isotopic fractionation.[14] On the other hand, compared with fluid oil, the solid seeds are coarse and inhomogeneous, and this may influence the reproducibility in transmethylation and methylation reactions. An inconsistent and incomplete conversion of TAGs to FAs to FAMEs leads to a poor reproducibility, i.e. precision, of the isotope analysis. The FAMEs were prepared from rapeseed oil and ground rapeseed under the same conditions using the TM+D transmethylation mixture. Three reactors were prepared from three aliquots of each sample.

The ranges of the standard deviation of the carbon and hydrogen compositions of the four major FAMEs of oil and rapeseed samples are summarized in Table 5. Since the origin of the fluid oil and the rapeseed samples are different, the mean values differ (see Figure S1, supporting information).

The precisions of the $\delta^{2}H$ values of different FAMEs prepared from the fluid oil and solid seed samples are in the range of ±0.9 to ±2.4 mUr and ±0.7 to ±1.8 mUr, respectively. The results show that the precision of H-CSIA of the four FAMEs produced from the fluid oil sample was slightly outside the precision of the GC-C/Py-IRMS instrument (1σ = ±1.7 mUr) whereas the precision of the H-CSIA of the solid rapeseed sample fell within the precision of the instrument.

TABLE 5 Comparison of reproducibility of $\delta^{13}C$ and $\delta^{2}H$ values of four FAMEs of oil and seed

Aggregate state	SD (±1σ) of $\delta^{13}C$ values, n = 3 (mUr)	SD (±1σ) of $\delta^{2}H$ values, n = 3 (mUr)
Rapeseed oil	±0.06 to ±0.1	±0.9 to ±2.4
Rapeseed	±0.1 to ±0.3	±0.7 to ±1.8

TABLE 4 Precision of C- and H-CSIA by analyzing two in-house reference materials using GC-C/Py-IRMS

	$\delta^{13}C$ (mUr)			$\delta^{2}H$ (mUr)		
In-house reference material	n	Mean	1σ	n	Mean	1σ
C18:1 FAME	9	−28.8	±0.1	9	−165.1	±1.7
C19:0 FAME	9	−28.7	±0.1	9	−244.3	±1.7

Regarding the C-CSIA measurements, the standard deviations of the $\delta^{13}C$ values of the four FAMEs from the fluid oil sample and the solid seeds are in the range of $\leq \pm 0.1$ mUr and ±0.1 to ±0.3 mUr, respectively. Unlike for H-CSIA, the precision of the C-CSIA of the fluid oil sample fell in the range of the precision of the instrument (1σ = ±0.1 mUr) but that of the solid seed sample was somewhat worse.

The precision of the $\delta^{13}C$ values of different FAMEs is similar to that published by Richter et al. (±0.05 to ±0.4 mUr), who prepared FAMEs from rapeseed oil using the DIN Norm method (saponification and methylation with BF_3).[7]

As a summary, the precisions of both C- and H-CSIA of the selected FAMEs prepared from fluid oil and from solid seeds are similar. The results indicate that there is no difference in the reproducibility of the conversion process of FAs to FAMEs by the one-step method due to the aggregate state. Furthermore, the precisions of C- and H-CSIA of these FAMEs produced from solid rapeseed samples by the one-step method and from fluid rapeseed oil by the DIN method are in the same range, indicating that the degree of consistency and completion of FAME production by the one-step method using the TM+D transmethylation mixture under the selected conditions and by the DIN method are comparable.

3.2.3 | Influence of DMP on $\delta^{13}C$ and $\delta^{2}H$ values of FAMEs

During the formation of FAME, 1 mol of $-CH_3$ from methanol will be added to 1 mol of FA. The isotope composition of FAMEs could be influenced by that of the methanol used for the production of FAMEs.

If the entire 2.0 mmol of DMP added to TM+D is consumed in a reaction with glycerol as well as with water, theoretically 4.1 mmol of methanol will be produced intermediately. Thus, the molar ratio of the reaction intermediate methanol to the originally added methanol is about 1:12. Since the $\delta^{13}C$ and $\delta^{2}H$ values of the reaction intermediate methanol are not measurable, the influence of DMP on the isotope composition of FAMEs has to be evaluated from the carbon and hydrogen isotope compositions of the individual FAMEs produced with and without DMP.

We selected the TM+D and TX-D mixtures to produce FAMEs from the same batch of rapeseed sample. One of the reasons of this selection was that the reactions in both TM+D and TX-D reactors must have reached equilibrium, as proved by Garcés and Mancha under comparable conditions.[21] Another reason was that the FAME yields of TM+D (100%; see Table 3) and of TX-D (91%; see Table 3) are comparable. Panetta and Jahren showed that FAME yields influence the $\delta^{13}C$ values, if the reactions had not reached equilibrium.[29] From their results, however, the difference in $\delta^{13}C$ values between the FAME yield of 90% and 100% was less than 0.1 mUr.[29]

Figure 3 shows the isotope compositions of the four major FAMEs produced using TM+D (with DMP) and TX-D (without DMP). A T-test was carried out to compare the carbon and hydrogen isotope composition of individual FAMEs between these two reactors. As presented in Tables S4 and S5 (supporting information), statistically, there is no significant difference in isotope compositions between TX-D and TM+D, except for the $\delta^{2}H$ value of C18:3. The difference in hydrogen isotope composition of C18:3 is 2.6 mUr, somewhat greater than the reproducibility of the seed sample preparation (see Table 5), but similar to that of the oil sample preparation.

The transmethylation reaction is a complicated process with regard to bond formation between the carboxyl carbon and the incoming methoxide oxygen.[14] In simpler terms, every mole of FAME generated is composed of 1 mol of $-CH_3$ from methanol and 1 mol of FA. According to Paolini et al., the relationship of the $\delta^{2}H$ values of the FAs and the FAMEs can be estimated using the following mass balance equation[12]:

$$(H_n + 3) \times \delta^2 H_{FAME} = H_n \times \delta^2 H_{FA} + 3 \times \delta^2 H_{MeOH} \quad (6)$$

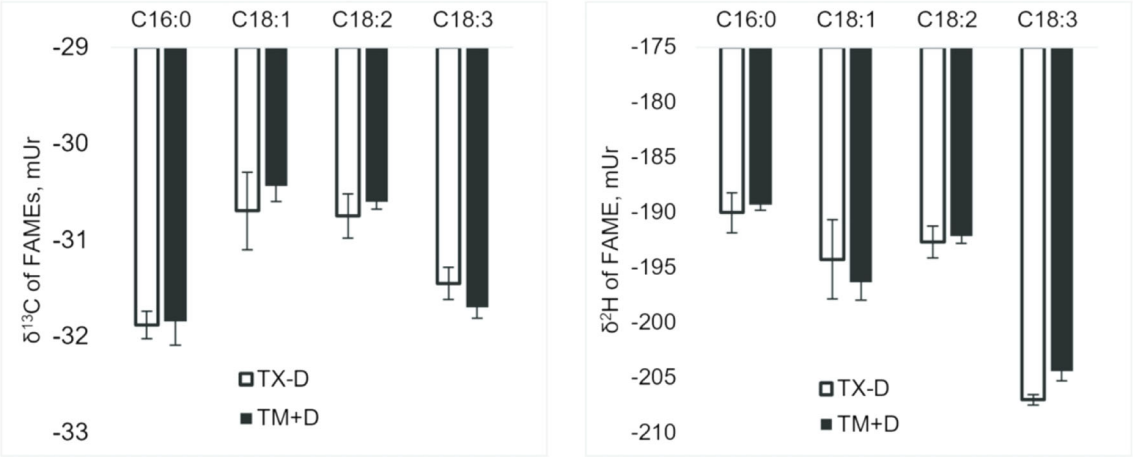

FIGURE 3 The $\delta^{13}C$ (left) and $\delta^{2}H$ (right) values of C16:0, C18:1, C18:2 and C18:3 FAMEs in rapeseed using TX-D and TM+D transmethylation mixtures

where H_n is the number of H atoms of the FA, and δ^2H_{FAME}, δ^2H_{FA} and δ^2H_{MeOH} are the hydrogen isotope compositions of the FAME, the FA and the methyl group of methanol, respectively.

Assuming that the C18:3 FAME from TX-D is generated from the initially added methanol whereas that from TM+D is generated from the mixture of the initially added and the reaction intermediate methanol, the difference in δ^2H values ($\Delta\delta^2H$) of methanol (δ^2H_{MeOH}) between TM+D and TX-D is 28 mUr, derived from the measured $\Delta\delta^2H$ of FAME 18:3 of 2.6 mUr and using Equation 6. Since the $\Delta\delta^2H$ values of both initially added and reaction intermediate methanol should also cause the difference in δ^2H values of C16:0, C18:1 and C18:2 FAMEs, based on this assumption the calculated $\Delta\delta^2H$ values of these FAMEs should fall into the range 2.3 to 2.4 mUr (see Table S6, supporting information). However, our result showed that there was no significant difference in the measured δ^2H values of C16:0, C18:1 and C18:2 FAMEs between TM+D and TX-D. Thus, a quantifiable influence of the reaction intermediate methanol produced by DMP on the $\delta^{13}C$ and δ^2H values of FAMEs was not observed in our study. Thus, the isotope composition of FAs can be determined as long as the isotope composition of the initially added methanol is known.

3.3 | Estimation of $\delta^{13}C_{FAME}$ and δ^2H_{FAME} values from $\delta^{13}C_{H3CO\text{-}DMP}$ and $\delta^2H_{H3CO\text{-}DMP}$ values with the assumptions

The influence of DMP on the isotope composition of FAMEs could also be estimated if reasonable assumptions are made for the $\delta^{13}C$ and δ^2H values of the H_3CO- group in the DMP structure ($\delta^{13}C_{H3CO\text{-}DMP}$, $\delta^2H_{H3CO\text{-}DMP}$). This H_3CO- group in DMP will be the source of the H_3CO- group in the reaction intermediate methanol, which will be added to generate the FAME during methylation (see Figure 1).

DMP is generally produced by the reaction of methanol and acetone under acidic conditions, so the H_3CO- group in DMP should be from the reactant methanol.[30] According to the literature, the $\delta^{13}C$ value and the bulk δ^2H value of methanol from different sources vary between −25 and −46 mUr and between −18 and −227 mUr, respectively.[12,31–33] As the $\delta^{13}C$ and δ^2H values of methanol used for the production of our DMP are unknown, we made assumptions. Our assumptions are that (1) the $\delta^{13}C$ and the δ^2H values of the H_3CO- group in DMP are similar to those of methanol found in the literature and (2) all the DMP used in the TM+D mixture was transformed to produce the reaction intermediate methanol, which was used to form FAMEs.

For the estimation of $\delta^{13}C_{FAME}$ and δ^2H_{FAME} values in FAMEs generated using the TM+D mixture, the $\delta^{13}C$ and the δ^2H values of the H_3CO- group in DMP are assumed to be in the range −80 to −20 mUr and −300 to −20 mUr, respectively, considering that isotope fractionations may occur during the production of DMP from methanol (e.g. separation of the reactants and products in distillation and in complete reaction of methanol). The $\delta^{13}C$ and the bulk δ^2H values of the methanol originally used in the transmethylation reagent (TX-D and TM+D) were −38.9 ± 0.08 and −169.5 ± 2.8 mUr, measured by GC-C/Py-IRMS, based on the method given by Ai et al., although the δ^2H value of H_3CO- group in methanol is in fact needed.[32] In the TM+D mixture, the ratio of the originally added methanol to the reaction intermediate methanol due to the DMP is 12:1, if all the DMP is transformed into methanol.

Taking these assumptions into account, the $\delta^{13}C$ and δ^2H values of the methanol potentially available in TM+D during the methylation process were estimated to be −37.4 to −42.2 mUr and −157.5 to −179.9 mUr, respectively (see Table S7, supporting information). Based on these assumptions, the $\delta^{13}C$ and δ^2H values of FAMEs were determined (see Tables S8 and S9, supporting information). The absolute differences between the measured and estimated isotope compositions of the individual FAMEs were 0.08 to 0.4 mUr for $\delta^{13}C$ values and 1.2 to 3.5 mUr for δ^2H values. This shows that our assumption is reasonable.

Furthermore, the maximum absolute differences between estimated isotope compositions of the individual FAMEs with and without DMP were determined to be approximately 0.2 mUr for $\delta^{13}C$ values and 1 mUr for δ^2H values. This outcome indicates that the influence of DMP on isotope measurement is smaller than the analytical precision, which was consistent with our experimental results. In other words, the difference, i.e. the influence of DMP on C- and H-CSIA of FAMEs, cannot be detected unless the analytical precision is markedly improved.

4 | CONCLUSIONS

This study has demonstrated that the quick one-step sample preparation method of Garcés and Mancha is feasible not only for FA profile analysis and the estimation of lipid content but also for C- and H-CSIA for individual FAMEs of both solid rapeseed and fluid rapeseed oil. The reproducibility of the one-step method for the determination of the isotope composition of rapeseed was within ±0.3 mUr for $\delta^{13}C$ values and ± 3 mUr for δ^2H values. Our results showed no significant influence on the $\delta^{13}C$ and δ^2H values of three of four major FAMEs by the addition of 5 vol% DMP to the transmethylation mixture. The performance of the one-step method for rapeseed samples for the determination of the $\delta^{13}C$ and δ^2H values of FAMEs is satisfactory. The quick one-step method, therefore, can be implemented to deal with a large number of oilseed samples for the investigation of the isotopic fingerprints of their FAs in a much shorter time, fostering the application of chemometrics for the purpose of the authentication of a variety of oilseeds.

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ORCID

Lili Xia https://orcid.org/0000-0002-3571-2847
Kaori Sakaguchi-Söder https://orcid.org/0000-0001-6927-661X

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