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Comparison of indirect and direct quantification of esters of monochloropropanediol in vegetable oil

Mathieu Dubois[a,*], Adrienne Tarres[a], Till Goldmann[a], Anna Maria Empl[b], Alfred Donaubauer[b], Walburga Seefelder[a]

[a] Nestlé Research Centre, Nestec Ltd, Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland
[b] Nestlé Quality Assurance Center Weiding, Nestlé Deutschland AG, Frankfurt am Main, Germany

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ABSTRACT

The presence of fatty acid esters of monochloropropanediol (MEs) in food is a recent concern raised due to the carcinogenicity of their hydrolysable moieties 2- and 3-monochloropropanediol (2- and 3-MCPD). Several indirect methods for the quantification of MEs have been developed and are commonly in use until today, however significant discrepancies among analytical results obtained are challenging their reliability. The aim of the present study was therefore to test the trueness of an indirect method by comparing it to a newly developed direct method using palm oil and palm olein as examples. The indirect method was based on ester cleavage under acidic conditions, derivatization of the liberated 2- and 3-MCPD with heptafluorobutyryl imidazole and GC–MS determination. The direct method was comprised of two extraction procedures targeting 2-and 3-MCPD mono esters (co-extracting as well glycidyl esters) by the use of double solid phase extraction (SPE), and 2- and 3-MCPD di-esters by the use of silica gel column, respectively. Detection was carried out by liquid chromatography coupled to time of flight mass spectrometry (LC–ToF-MS). Accurate quantification of the intact compounds was assured by means of matrix matched standard addition on extracts. Analysis of 22 palm oil and 7 palm olein samples (2- plus 3-MCPD contamination ranged from 0.3 to 8.8 μg/g) by both methods revealed no significant bias. Both methods were therefore considered as comparable in terms of results; however the indirect method was shown to require less analytical standards, being less tedious and furthermore applicable to all type of different vegetable oils and hence recommended for routine application.

1. Introduction

The presence of fatty acid esters of 3-monochloro-1,2-propanediol together with 2-monochloro-1,3-propanediol (MCPD esters, MEs) has been reported in various types of food products and raw materials, especially in refined vegetable oils such as palm oil [1–8]. MCPD are known to be chemical food contaminants, detected first as a by-product of hydrolyzed vegetable protein by action of hydrochloric acid on residual lipids [9], and later as MCPD esters in vegetable oils where they are formed during the deodorization step in the refining process [3,6,10–14]. Due to their structural similarity to triglycerides, MCPD esters have been anticipated to be readily hydrolyzed during the gastro intestinal digestion through the action of intestinal lipases, triggering the hypothesis that MCPD esters may constitute an additional, previously unconsidered, source of MCPD human exposure (Statement of the Scientific Panel on Contaminants in the Food chain (CONTAM) on a request from the European Commission related to 3-MCPD esters; Question No EFSA-Q-2008-2258; 2008; http://www.efsa.europa.eu/fr/scdocs/doc/1048.pdf). 3-MCPD is known to have an in vivo carcinogenic and in vitro genotoxic activity [15], and 2-MCPD has been shown to cause severe toxic effects in striated muscles and hearts of rats [16]. Several indirect quantitative methods have been published focusing mainly on the determination of 3-MCPD esters [10,17,18]. They are based on the transesterification of the esters under acidic or alkaline conditions in order to release 3-MCPD, analysis of which awards more than 20 years of expertise. The liberated 3-MCPD is then usually transformed into a stable volatile derivative using mostly phenyl boronic acid (PBA) or heptafluorobutyryl imidazole (HFBI), which is then further characterized usually by the use of gas chromatography coupled to mass spectrometry (GC–MS). A detailed review of methods to determine 3-MCPD has been published by Baer et al. [19]. These types of methods do not provide any further information on the detailed structure of the individual esters which may be important bearing in mind that the individual 3-MCPD esters (mono- or di-) as well as the in parallel occurring 2-MCPD

* Corresponding author. Tel.: +41 21 785 8578; fax: +41 21 785 8553.
E-mail address: mathieu.dubois@rdls.nestle.com (M. Dubois).

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esters (mono- or di-) may show different toxic effects, following the principles of the human fat metabolism [16,20]. However, the full characterization of the health risks originating from each individual type of ester would require significant time and resources. Precautionary mitigation approaches are instead likely to become the best way to design health protective management measures, making the need of direct measurement of the individual compounds for monitoring purposes obsolete in this context.

Nevertheless the development of accurate direct analytical methods for MCPD esters may become relevant as no reference materials are available until today and since doubts were expressed on the precision and trueness of commonly applied indirect analytical methods, whose performances are thought to be compromised by the transesterification and derivatization step. Indeed Kuhlmann reported beginning of 2008 discrepancies in generated results depending on the transesterification conditions [8,21]. Amounts of 3-MCPD esters were found to be overestimated when an alkaline based cleavage of the esters was combined with the use of a derivatization solution containing inorganic chloride. These findings triggered the postulation of the presence of additional reactive compounds in refined oils which have been later identified as glycidyl esters (GEs) [21,22]. A proficiency test recently organized by the European Joint Research Center (JRC) in 2010 (http://www.irmm.jrc.be/interlaboratory_comparisons/3_MCPD/Documents/eur_24356_en_3-mpcd_esters_in_edible_oil.pdf) indicated furthermore the need to assess the trueness and accuracy of commonly applied indirect analytical methods.

In the absence of certified reference materials, the comparison of indirect and direct analytical methods may serve as an alternative for evaluating the trueness and precision of indirect analytical methods. Until today, such data are very limited and are referring mainly to the comparison of alkaline based methods as indirect methods and liquid chromatography–mass spectrometry (LC–MS) based methods as direct methods. First method comparison for MCPD esters had been carried out by ADM (Archer Daniels Midland Company; Research Division) [23]. The DGF Standard Method C-III 18 applied as to measure the total MCPD equivalents only (without differentiating MCPD- and glycidyl esters) was reported to give results that were constantly greater than the LC–ToF-MS (liquid chromatography couple to time of flight mass spectrometry) method measuring directly MCPD- and glycidyl esters. 2-MCPD esters had not been included in the analysis. J.D. Pinkston and P.J. Stoffolano from P&G presented recently the development of a direct analytical method for MCPD-esters applying a “dilute and shoot” approach using LC–MS/MS (liquid chromatography couple to tandem mass spectrometry) for measurement [24]. The method, taking not into consideration 2-MCPD esters had been compared to a modified version of the original “Weisshaar method” where sodium chloride is substituted by sodium sulfate (approach similar to DGF CVI 18 (10) [25]). Results of the 9 samples presented showed in general a good correlation but results have a slight bias and values of the P&G method were below (2 samples significantly) those obtained by the indirect analysis.

This publication describes for the first time the comparison of a newly developed direct method for the quantification of MEs taking into account the presence of 2-MCPD esters with an indirect method for 3- and 2-MCPD measurement based on acidic cleavage of the esters as developed by Divinova et al. [17]. The choice of MEs analytical standards for direct method was done in a pragmatic way to identify required standards using a simple theoretical approach, which has been afterwards confirmed by samples analysis. The direct method presented here used two separate extractions: one for MCPD mono-esters isolation (double SPE modified from [26–29]), and another one for MCPD di-esters (silica gel column). Sample extracts were then analyzed with a LC–ToF-MS detection system. Direct and indirect method comparison has been carried out on a set of 22 palm oil and 7 palm olein samples contaminated over a broad range. Results from the direct measurement of MCPD mono- and di-esters were correlated to results of the indirect measurement of 2- and 3-MCPD esters.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade LiChrosolv Water, 2-propanol, acetonitrile, n-hexane, tetrahydrofuran (THF), isooctane, diisopropyl ether, formic acid 98–100%, sulfuric acid 98%, sodium hydrogen carbonate, sodium chloride, sodium sulfate anhydrous and EXtrelut® NT 20 were from Merck (Darmstadt, Germany). Methanol LC/MS grade was from Fisher (Waltham, MA). HPLC grade acetone, cyclohexane and dichloromethane as well as ammonium formate were supplied by Sigma Aldrich (Fluka, Buchs, Switzerland). Ethyl-acetate was from Carlo Erba Reactifs SDS (Val de Reuil, France). Heptafluorobutyryl imidazole (HFBI) was from Thermo Scientific (Waltham, MA). Bakerbond 500 mg/3 mL silica cartridges, and Bakerbond SPE C18 2 g/6 mL were provided by Aventor (Phillipsburg, NJ). Silica gel high-purity grade, pore size 60 Å, 230–400 mesh was from Fluka (St Louis, MO). 3-MCPD was from Sigma Aldrich and 2-MCPD was from Santa Cruz Biotechnology (Santa Cruz, CA). Isotope-labeled d_5-3-MCPD was from CDN Isotopes (Augsburg, Germany). 1-Myristoyl-3-chloropropanediol, 1-lauroyl-3-chloropropanediol, 1-palmitoyl-3-chloropropanediol, 2-palmitoyl-3-chloropropanediol, 1-stearoyl-3-chloropropanediol, 1-linoleoyl-3-chloropropanediol, 1-linolenoyl-3-chloropropanediol, 1,2-bis-palmitoyl-3-chloropropanediol, 1-palmitoyl-2-stearoyl-3-chloropropanediol, 1,2-dilinoleoyl-3-chloropropanediol, 1-oleoyl-2-stearoyl-3-chloropropanediol, 1-linoleoyl-2-stearoyl-3-chloropropanediol, 1-oleoyl-2-linolenoyl-3-chloropropanediol, 1-oleoyl-2-linoleoyl-3-chloropropanediol, 1-palmitoyl-2-linoleoyl-3-chloropropanediol, 1,2-distearoyl-3-chloropropanediol, 1,3-distearoyl-2-chloropropanediol were from Toronto Research Scientific (Ontario, Canada). 1-Oleyl-3-chloropropanediol, 1,2-bis-oleoyl-3-chloropropanediol, 1-oleyl-2-palmitoyl-3-chloropropanediol were from Atlanchim Pharma (Nantes, France). Five isotopically labeled chemical standards have been custom synthesized also by Atlanchim Pharma (Nantes, France) and were: $^{13}C_4$-1-palmitoyl-3-chloropropanediol and $^{13}C_4$-1-oleyl-3-chloropropanediol labeled with three ^{13}C on glycidol or MCPD backbone and one ^{13}C on carboxyl group, $^{13}C_5$-1,2-bis-palmitoyl-3-chloropropanediol, $^{13}C_5$-1,2-bis-oleoyl-3-chloropropanediol and $^{13}C_5$-1-oleyl-2-palmitoyl-3-chloropropanediol labeled with three ^{13}C on MCPD backbone and one ^{13}C on each carboxyl group.

2.2. Standard solutions

Individual stock solutions of 2-MCPD, 3-MCPD and d_5-3-MCPD for indirect method were prepared in ethyl-acetate at a 1 mg/mL concentration. Two composite stock solutions containing 2- and 3-MCPD at a 10 μg/mL concentration for mix1, and labeled 3-MCPD at a 1 μg/mL concentration for mix1_IS were prepared in isooctane. Quantification of 2- and 3-MCPD was performed by external calibration curve, generated in isooctane by diluting mix1 and mix1_IS at concentrations of: 1000, 500, 200, 100, 50, 20 and 0 ng/mL, keeping internal standard in each calibrant solution at a constant concentration of 25 ng/mL. Calibration standard solutions (1 mL) were transferred to a set of seven 5-mL conical vials (reactive vial) for derivatization at the same time as oil sample extracts as

described in Section 2.4. A solution of 1-palmitoyl-2-stearoyl-3-MCPD at 50 μg/mL was prepared in tetrahydrofuran for recovery assessment in each batch of samples.

Individual stock solutions of MEs (isotopically labeled and unlabeled) for the direct method were prepared in dichloromethane at a 1 mg/mL concentration (min. weighed amount: 20 mg). An unlabeled 3-MCPD mono-ester composite stock solution (mix2) comprising 1-lauroyl-3-chloropropanediol, 1-myristoyl-3-chloropropanediol, 1-palmitoyl-3-chloropropanediol, 1-stearoyl-3-chloropropanediol, 1-oleoyl-3-chloropropanediol, 1-linoleoyl-3-chloropropanediol and 1-linolenoyl-3-chloropropanediol each at 50 μg/mL in acetone, was subsequently prepared from individual stock solutions. Similarly, an unlabeled 3-MCPD di-esters composite stock solution (mix3) was prepared comprising all the 3-MCPD di-esters mentioned in Section 2.1, except 1,2-distearoyl-3-chloropropanediol (total of 10 MCPD di-esters each at 50 μg/mL in acetone). Two other mix solutions also at 50 μg/mL in acetone were prepared for isotopically labeled MEs. Mix2_IS contained $^{13}C_4$-1-palmitoyl-3-chloropropanediol and $^{13}C_4$-1-oleyl-3-chloropropanediol, mix3_IS contained $^{13}C_5$-1,2-bis-palmitoyl-3-chloropropanediol, $^{13}C_5$-1,2-bis-oleoyl-3-chloropropanediol and $^{13}C_5$-1-oleyl-2-palmitoyl-3-chloropropanediol. All composited standard solutions were stored at +4 °C and allowed warming at room temperature before use. Quantification of mono and di-esters of MCPD was conducted independently by means of standard addition on sample extracts. Spiking solutions were prepared daily by diluting in acetone mix2 and mix2_IS to have MCPD mono-esters at 0.1 μg/mL (spike1) and 0.4 μg/mL (spike2). Similarly, mix3 and mix3_IS were diluted in acetone to have MCPD di-esters at 0.04 μg/mL (spike1′) and 0.16 μg/mL (spike 2′).

2.3. Samples

A total of 32 edible oil samples were considered for analysis by direct and indirect method, including palm oil (22), palm olein (7), palm kernel oil (1), coconut oil (1), and sunflower oil (1). For direct method development, canola oil (1), blended oil (1), safflower oil (1) and soy oil (1) were also considered. All samples were kept at +4 °C and protected from light in air-tight containers until analysis. Solid oil samples were melted in an oven at 60 °C (maximum 1 h, depending on the sample size) and thoroughly homogenized prior analysis.

2.4. Sample preparation for the analysis using the indirect method

Melted oil (0.5 g) was vigorously shaken into a mixture made of water (20 mL) and hexane (40 mL) for at least 1 min. After phase separation, the aqueous lower phase (containing free MCPD) was discarded. The upper organic phase (containing oil and bound MCPD) was dried over sodium sulfate and evaporated under a stream of nitrogen. A 125 mg aliquot was weighted into a glass beaker with screw cap, mixed with 25 μL of mix1_IS (corresponding to a 0.2 μg/g equivalent in sample concentration), 2.5 mL tetrahydrofuran and 1.8 mL of a methanolic sulfuric acid solution (1.8 mL sulfuric acid 98% in 100 mL methanol). After 16 h at 40 °C, 0.5 mL of a saturated $NaHCO_3$ solution was added to stop acidic hydrolysis, the tube was placed under a stream of nitrogen to evaporate organic solvent, and followed by addition of 16 mL of a saturated NaCl–Water:MeOH (9:1) solution and vigorously shaken. After phase separation, 15 mL of the aqueous phase was mixed with 20 g EXtrelut® NT 20 and quantitatively transferred into a 25-mm diameter column fitted with cotton wool and filled with 15 g of anhydrous sodium sulfate. MCPD were eluted with 250 mL diisopropyl ether into a 500 mL dried round bottomed flask. The eluate was evaporated to a small volume (ca. 1 mL) under vacuum (700 mbar at 40 °C), quantitatively transferred to a reactive-vial for evaporation under a gentle stream of nitrogen to approximately 0.1 mL, diluted with 5 mL ethyl acetate, further evaporated to 0.1 mL and filled up to 1 mL with isooctane. Derivatization of sample extracts and standards solution for calibration curve (1 mL of each concentration level, described in Section 2.2) was achieved by addition of 50 μL HFBI. After 30 min at 60 °C, excess reagent was removed by washing twice the organic phase with 2 mL of distilled water, the lower water phase being discarded with a Pasteur pipette. After phase separation, organic phase was transferred into GC vials for GC–MS analysis.

2.5. Extraction of MCPD mono-esters for the analysis using direct method

Extraction of MCPD mono-esters was performed on two Solid Phase Extraction cartridges (SPE), the first one being a C_{18} SPE and the second one a silica SPE. In a 5-mL flask, 2 g of melted oil was mixed with 20 μL of mix2_IS (corresponding to a 0.5 μg IS per gram of oil), and further dissolved in dichloromethane up to the 5-mL mark, 250 μL of this solution (equivalent to 40 mg oil) being loaded onto the first C_{18} SPE, equilibrated beforehand gravimetrically with 20 mL acetonitrile. The SPE cartridge was eluted with 25 mL acetonitrile. The eluate was then dried under a gentle stream of nitrogen and reconstituted with 500 μL of dichloromethane for an additional silica SPE cleanup. The first C_{18} SPE reconstituted extract was then quantitatively loaded onto a 500 mg silica cartridge conditioned first with 10 mL of dichloromethane. Elution was then performed with 25 mL of dichloromethane (2 mL of which being used for rinsing twice the initial vial for quantitative transfer). Extract was then dried under a stream of nitrogen and finally reconstituted in 500 μL of acetone, transferred to a first LC-vial and further diluted 5 times in acetone in a second one, the two extracts being analyzed by LC–ESI-ToF-MS.

2.6. Optimization of MCPD di-esters extraction for direct analysis

In the frame of method development for intact MCPD di-esters extraction from oil samples, a Mid Performance Liquid Chromatography system (MPLC, Isolera Biotage, Uppsala, Sweden) was used for optimization of eluting solvent composition. Briefly, 1 g palm oil dissolved in 10 mL hexane was spiked with 100 μL of mix3 (equivalent to 5 μg/g of oil). One mL was loaded on a Samplet (Dry load frit for 10 g SNAP cartridges, Biotage), which was then inserted into a silica cartridges (KP-Sil™ SNAP Cartridge, silica, 10 g, Biotage) pre-equilibrated with n-hexane. The sample was eluted at a flow rate of 10 mL/min and 12-mL fractions were collected, which were dried under a stream of nitrogen and reconstituted in 500 μL of acetone for LC–ESI-ToF-MS analysis. Different eluting solvents such as diethyl-ether, methyl tert-butyl ether, ethyl-acetate and dichloromethane were tested as linear gradient in hexane. Best parameters were then used for an isocratic and gravimetric elution from a silica gel column described in Section 2.7.

2.7. Extraction of MCPD di-esters for direct analysis

Extraction of MCPD di-esters was performed on a silica gel column, prepared by mixing 3 g of silica gel with 20 mL n-hexane in a glass beaker and then transferred into a chromatographic glass tube (400 mm length, 11 mm internal diameter) having a plug of cotton fiber placed just above the stopcock. In a 10-mL volumetric flask, 1 g of melted oil was mixed with 10 μL of mix3_IS, and further dissolved in n-hexane up to the 10-mL mark before transferring 400 μL (40 mg oil) into the silica gel column. The sample was let running through the column into a 100 mL round bottomed flask

and 65 mL dichloromethane:hexane (4:6, v:v) was subsequently loaded on the column for a gravimetric elution until flow stop. The eluate was evaporated to a small volume (ca. 1 mL) under vacuum (140 mbar at 40 °C), quantitatively transferred into a 7-mL amber tarred vial and then dried under a gentle stream of nitrogen. The residue was reconstituted in 500 μL of acetone, transferred to a first LC-vial and further diluted 5 times in a second one, the two extracts being analyzed by LC–ESI-ToF-MS.

2.8. GC–MS analysis

The analyses of 2- and 3-MCPD were performed using an Agilent HP 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to an Agilent HP 5975C series mass spectrometer (Agilent Technologies) and equipped with an HP 7683B autosampler (Agilent Technologies). A fused silica capillary column HP Ultra 2, 50 m × 0.32 mm i.d. and 0.17 μm film thickness from J&W Scientific (HP 19091B-015 Agilent Technologies). The flow rate of the helium carrier gas was 1 mL/min (constant flow). Two μL sample was injected in pulsed splitless mode (splitless time 1.2 min). The following oven temperature program was used: 60 °C hold for 1 min, to 84 °C at 4 °C/min (6 min), afterward to 280 °C at 25 °C/min (7.8 min), for a total cycle time of 25 min. The MS settings were as follow: source temperature, 230 °C; transfer line temperature, 280 °C. The MS system was operated in the electron ionization (EI) mode at 70 eV, focusing on the ions with a mass to charge (m/z) ratio of 291 (target) and m/z 453 (qualifiers) for 3-MCPD, m/z 291 for 2-MCPD and m/z 294 for d_5-3-MCPD. Dwell time was 35 μs. Typical retention time for 2- and 3-MCPD were 9.71 and 9.66 min, respectively.

2.9. LC–ESI-ToF-MS analysis

An Agilent 1290 Infinity UHPLC (Agilent Technologies, Santa Clara, CA) coupled to a 6540 Agilent Ultra High Definition Q-ToF MS analyzer (Agilent Technologies, Santa Clara, CA) was used to detect and quantify MEs directly. Chromatography columns used were an Acquity UPLC HSS T3 VanGuard column (5 mm × 2.1 mm i.d., 1.8 μm) (Waters, Milford, MA) coupled to an Acquity UPLC HSS T3 (50 mm × 2.1 mm i.d., 1.8 μm). Mobile phase A consisted of methanol/water (75/25, v/v) and mobile phase B consisted of 2-propanol both supplemented with 0.1% formic acid and 10 mM ammonium formate. A gradient program was applied at a 600 μL/min flow rate as follows: linear gradient from 0% B to 95% B from 0 to 12 min, then kept at 95% B for 3 min, and column was reconditioned from 12 to 15 min at 100% A. The column temperature was maintained at 60 °C and injection volume was 2 μL. Ionization and detection of analytes were performed with an electro spray ionization source (Jet Stream) operating in the positive ionization mode, using the following operating parameters: capillary voltage, 3500 V; nebulizer pressure, 50 psig; drying gas flow rate, 9 L/min; gas temperature, 350 °C; skimmer voltage, 60 V; octapole dc 1, 37.5 V; octapole rf, 250 V; fragmentor voltage (in-source CID fragmentation) was 150 V. The 2 GHz extended dynamic range was used, which allowed a mass resolution from 12,000 at m/z 200 to 25,000 at m/z 1500 with an acquisition range from 100 to 1600 m/z (2 scans/s). Accurate mass measurement was achieved thanks to an automated calibrant delivery system for mass spectra correction. A dual-nebulizer electro spray source introduces the outlet of the chromatography at the same time as the calibrant solution containing purine ($C_5H_4N_4$, m/z 121.050873) and HP-0921 (hexakis-(1H,1H,3H-tetrafluoropentoxy)-phosphazene, $C_{18}H_{18}O_6N_3P_3F_{24}$, m/z 922.009798). Identification of analytes was performed through both their exact mass measurement and their retention time (Table 1) using the "searching compounds by molecular formula" option in the Qualitative Mass Hunter software. The identification criteria were set at ±10 ppm for accurate mass tolerance and ±0.1 min for retention time tolerance. The window for extracted ion chromatogram generation was set at 10 ppm. Analysis and quantification of MCPD di-esters extracts was conducted independently from MCPD mono-esters. An injector program was used to perform standard addition on sample extracts. Five additional vials were placed in the autosampler, first vial containing acetone, second and third vial containing spike1 and spike2 for MCPD mono-esters, fourth and fifth containing spike1′ and spike2′ for MCPD di-esters. For each of the four sample extracts (MCPD mono- and di-esters, non diluted and diluted), three runs were performed as follows: the needle withdraw 1 μL of the extract and 1 μL of one of the three standard level (acetone, spike1 and spike2 for MCPD mono-esters;

Table 1
Chemical formula of GEs, MEs, and their respective retention time used for data treatment.

	Compound	Chemical formula	RT (min)	Monoisotopic mass (Da)
3-MCPD mono-esters	1-Myristoyl-3-chloropropanediol	$C_{17}H_{33}ClO_3$	2.37	320.2118
	1-Lauroyl-3-chloropropanediol	$C_{15}H_{29}ClO_3$	1.64	314.1335
	1-Palmitoyl-3-chloropropanediol	$C_{19}H_{37}ClO_3$	2.95	348.2431
	2-Palmitoyl-3-chloropropanediol	$C_{19}H_{37}ClO_3$	2.98	348.2431
	1-Stearoyl-3-chloropropanediol	$C_{21}H_{41}ClO_3$	3.86	376.2744
	1-Oleyl-3-chloropropanediol	$C_{21}H_{39}ClO_3$	3.32	374.2588
	1-Linoleoyl-3-chloropropanediol	$C_{21}H_{37}ClO_3$	2.87	372.2431
	1-Linolenoyl-3-chloropropanediol	$C_{21}H_{35}ClO_3$	2.48	370.2275
3-MCPD di-esters	1,2-Bis-palmitoyl-3-chloropropanediol	$C_{35}H_{67}ClO_4$	6.88	586.4728
	1-Palmitoyl-2-stearoyl-3-chloropropanediol	$C_{37}H_{71}ClO_4$	7.22	614.5004
	1,2-Dilinoleoyl-3-chloropropanediol	$C_{39}H_{67}ClO_4$	6.58	634.4728
	1-Oleoyl-2-stearoyl-3-chloropropanediol	$C_{39}H_{73}ClO_4$	7.28	640.5197
	1-Linoleoyl-2-stearoyl-3-chloropropanediol	$C_{39}H_{71}ClO_4$	7.03	638.5041
	1-Oleoyl-2-linolenoyl-3-chloropropanediol	$C_{39}H_{67}ClO_4$	6.58	634.4728
	1-Oleoyl-2-linoleoyl-3-chloropropanediol	$C_{39}H_{69}ClO_4$	6.81	636.4884
	1-Palmitoyl-2-linoleoyl-3-chloropropanediol	$C_{37}H_{67}ClO_4$	6.73	610.4728
	1,2-Bis-oleoyl-3-chloropropanediol	$C_{39}H_{71}ClO_4$	7.03	638.5041
	1-Oleyl-2-palmitoyl-3-chloropropanediol	$C_{37}H_{69}ClO_4$	6.95	612.4884
2-MCPD di-esters	1,2-Distearoyl-3-chloropropanediol	$C_{39}H_{75}ClO_4$	7.54	642.5354
	1,3-Distearoyl-2-chloropropanediol	$C_{39}H_{75}ClO_4$	7.60	642.5354
IS	$^{13}C_4$-1-palmitoyl-3-chloropropanediol	$^{13}C_4C_{15}H_{37}ClO_3$	2.99	352.2565
	$^{13}C_4$-1-oleyl-3-chloropropanediol	$^{13}C_4C_{17}H_{39}ClO_3$	3.19	378.2722
	$^{13}C_5$-1,2-bis-palmitoyl-3-chloropropanediol	$^{13}C_5C_{30}H_{67}ClO_4$	6.88	591.4896
	$^{13}C_5$-1,2-bis-oleoyl-3-chloropropanediol	$^{13}C_5C_{34}H_{71}ClO_4$	7.03	643.5209
	$^{13}C_5$-1-oleyl-2-palmitoyl-3-chloropropanediol	$^{13}C_5C_{32}H_{69}ClO_4$	6.95	617.5052

Table 2
Fatty acid distribution (weight percentage) in various oils (above 40% in bold). Some minor fatty acids were not included, leading to totals slightly lower than 100%.

	Caprylic acid	Capric acid	Lauric acid	Myristic acid	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Canola oil					4	2	**62**	22	10
Coconut oil	8	6	**47**	18	9	3	6	2	0.2
Corn oil					11	2	28	**58**	1
Cotton seed oil				1	22	3	19	**54**	1
Flaxseed oil					3	7	21	16	**53**
Grape seed oil					8	4	15	**73**	
Olive oil				0.1	13	3	**71**	10	1
Palm oil			0.2	1	**45**	4	**40**	10	0.4
Palm olein				1	37	4	**46**	11	
Palm kernel oil		4	**48**	16	8	3	15	2	
Safflower oil					7	2	13	**78**	0.2
Soybean oil					11	4	24	**54**	7

acetone, spike1′ and spike2′ for MCPD di-esters), mix in the syringe and inject (total of 12 injections per oil sample). In such conditions, standards added were equivalent to 0.5 μg/g and 2 μg/g of oil for non diluted extract, 2.5 μg/g and 10 μg/g for diluted extract.

2.10. Indirect and direct method comparison

To ensure results comparability, 2-MCPD and 3-MCPD from indirect analysis were summed up to give a total MEs amount expressed as MCPD. All the individual results for MEs (7 MCPD mono-esters and 10 MCPD di-esters) were expressed as MCPD equivalent: individual results were divided by the molecular weight of the corresponding ME and multiplied by the molecular weight of MCPD and finally summed up. The 32 samples were analyzed by both methods, but only palm oil and palm olein were used for result correlation as analytical standards were especially chosen to fit the analysis of these oils.

2.11. Calculation model for MCPD di-esters distribution in oils

Considering 3-MCPD, substituted in Sn1- and Sn2- with the 7 main fatty acids encountered in vegetable oil (laurate, myristate, palmitate, stearate, oleate, linoleate and linolenate fatty acids) as shown in Table 2, 49 different standards can be generated ($7^2 = 49$). For 2-MCPD, the same 7 possible fatty acids can be positioned in Sn1- and Sn3-, but symmetry of the molecule (Sn1- equivalent to Sn3-) leads to 28 different standards (number of multisets of 2 fatty acids from a set of 7 fatty acids: $\left(\binom{7}{2}\right) = \binom{7+2-1}{2} = 28$). To summarize, 77 MCPD di-esters could be found on a basis of 7 different fatty acids and this number increases to 126 if caprylic and capric fatty acids are added. Gathering all these analytical standards entails huge costs and efforts and was therefore considered as non realizable. Therefore a more pragmatic analysis of the situation has to be conducted in order to drastically reduce the number of required standards, while minimizing the impact on final results. First, most of the MCPD di-esters available today are esters of 3-MCPD, a fact which already reduces the choice of standards. Then, the theoretical abundance of each MCPD di-ester has been calculated for each type of oil, assuming a similar fatty acid profile in MCPD di-esters than in the oil. This approach is based on simple combinations, without taking into account natural preferences position of fatty acid on MCPD. Theoretical MCPD di-ester abundance has been calculated as follows:

$$\textit{Theoretical abundance}\,(\mathrm{MCPD}_{\mathrm{FA1;FA2}}) = \textit{Abundance}(\mathrm{FA1}) \times \textit{Abundance}(\mathrm{FA2}) \times k$$

with

- $\mathrm{MCPD}_{\mathrm{FA1;FA2}}$ being MCPD substituted with two fatty acid (FA1 and FA2)
- *Abundance* (FA1) being fatty acid abundance from Table 2
- $k = 1$ if (fatty acid 1) = (fatty acid 2)
- $k = 2$ if (fatty acid 1) $\neq$ (fatty acid 2)

Positional isomers can neither be resolved by the current liquid chromatography method, nor ToF-MS (same chemical formula, see Section 3.1), Sn1- and Sn-2 positions of 3-MCPD di-esters were not differentiated. The theoretical abundance of the two isomers for 3-MCPD were thus summed to give a global abundance, hence $k = 2$ in the previous calculation (above diagonal in example given in Table 3) and $k = 1$ when MCPD is substituted with the same fatty acid (diagonal in Table 3). Same considerations were taken for 2-MCPD di-esters.

3. Results and discussion

The indirect method for quantification of MEs reported here was derived from [3,17,30] for the hydrolysis of bound MCPD. The detection parameters were similar as reported under [31] and in the European standard EN 14573:2004 [32]. The method has been applied in a routine environment for several years, and thus was not modified in the frame of this study. Our main work focused on the development of a direct method for MEs and is therefore mainly discussed hereafter.

3.1. Choice of the analytical standards

Direct methods have the advantage to analyze MEs as such, without any chemical transformation, hence reducing the risk of sample preparation artifacts leading to over or underestimation. However, to ensure accurate quantification, adequate reference standards for MEs compounds have to be selected. Whereas indirect methods require only two standards (2- and 3-MCPD) plus ideally two IS (isotopically labeled 2- and 3-MCPD), the situation is more complex for MEs. MCPD mono-esters analytical standards were chosen in order to cover a broad range of oil, based on their fatty acid composition. Assumption was made that the relative abundances of these contaminants would follow the fatty acid composition of the individual types of oil (Table 2) as already shown for glycidyl esters (GEs) in a previous paper [33]. Sn1-3-MCPD mono-esters esterified with laurate, myristate, palmitate, stearate, oleate, linoleate and linolenate were included in the method. However, three isomers of MCPD mono-esters may exist for each fatty acid: one as the 2-MCPD mono-ester and two as the 3-MCPD mono-esters according to the two positions of ester (Sn1-3-MCPD and Sn2-3-MCPD mono-esters as shown in Fig. 1). Considering the seven fatty esters mentioned above, 21 different compounds have to be taken into account, whereas Sn1-3-MCPD mono-esters were the main isomers commercially available. It was however

Table 3
Theoretical distribution (percentage) of MCPD di-esters in palm oil and palm kernel oil, based on a similar distribution of fatty acid in oil and in MCPD di-esters (substituted with fatty acids in *Sn1-* and *Sn2-*). Abundances were calculated as described in calculation section. The most abundant MCPD di-esters to reach 95% coverage are in bold, whereas those commercially available are in the dotted line box.

Palm Oil		*Sn1-* Caprylic acid	Capric acid	Lauric acid	Myristic acid	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Sn2-	Caprylic acid									
	Capric acid									
	Lauric acid			*<0.1*	*<0.1*	*0.2*	*<0.1*	*0.2*	*<0.1*	*<0.1*
	Myristic acid				*<0.1*	*0.9*	*<0.1*	*0.8*	*0.2*	*<0.1*
	Palmitic acid					**20.3**	**3.6**	**36.0**	**9.0**	0.4
	Stearic acid						0.2	**3.2**	0.8	<0.1
	Oleic acid							**16.0**	**8.0**	0.3
	Linoleic acid								1.0	<0.1
	Linolenic acid									<0.1

Palm kernel Oil		*Sn1-* Caprylic acid	Capric acid	Lauric acid	Myristic acid	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Sn2-	Caprylic acid	*0.2*	*0.3*	***3.9***	***1.3***	***0.7***	*0.2*	***1.2***	*0.2*	*<0.1*
	Capric acid		*0.2*	***3.8***	***1.3***	*0.6*	*0.2*	***1.2***	*0.2*	*<0.1*
	Lauric acid			***23.0***	***15.4***	***7.7***	***2.9***	***14.4***	***1.9***	*<0.1*
	Myristic acid				***2.6***	***2.6***	***1.0***	***4.8***	*0.6*	*<0.1*
	Palmitic acid					0.6	0.5	**2.4**	0.3	<0.1
	Stearic acid						<0.1	**0.9**	0.1	<0.1
	Oleic acid							**2.3**	0.6	<0.1
	Linoleic acid								<0.1	<0.1
	Linolenic acid									<0.1

important to know if the three isomers could be differentiated with our analytical conditions. Thus the 2-palmitoyl-3-MCPD ester (the only Sn2 ester commercially available) and the 1-palmitoyl-2-MCPD ester (synthesized according to Haines et al. [23] but not purified) were compared to the 1-palmitoyl-3-MCPD in terms of retention time, mass profile and signal intensity. As shown in Fig. 2A, these three compounds cannot be resolved by our chromatography column/gradient used: 1-palmitoyl-3-MCPD ester elutes at 2.95 min, 1-palmitoyl-2-MCPD ester elutes at 2.98 min and 2-palmitoyl-3-MCPD ester elutes also at 2.98 min. The mass spectrometry response of Sn1- and Sn2- palmitoyl esters of 3-MCPD could only be compared (analytical standards), and response of Sn2- was 40% lower than Sn-1-palmitoyl-3-MCPD ester.

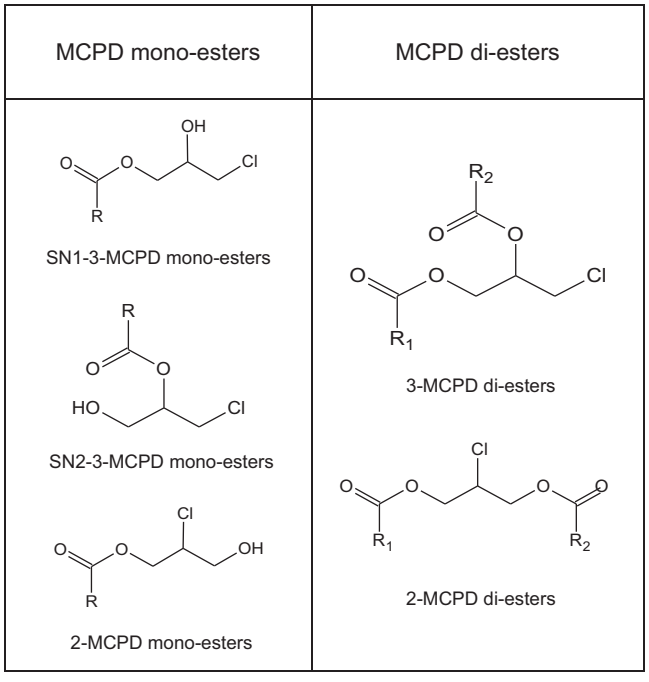

Fig. 1. Chemical structures of esters of monochloropropanols.

The diversity of MCPD mono-esters is a fact, but the situation is even more complex for MCPD di-esters. Similarly to MCPD mono-esters, the main challenge of direct determination of MCPD di-esters lies in the right choice and availability of the analytical standards. A pragmatic approach was chosen to drastically reduce the number of required analytical standards: the distribution of MCPD di-esters was calculated for each type of oil based on a simple model described in Section 2.11 as shown in Table 3 for palm oil and palm kernel oil. The most abundant MCPD di-ester allowing to reach at least 95% of total MCPD di-ester content were then selected for each type of oil. However, only few of them were commercially available (within the dotted-line box in Table 3). Due to this constraint, ten 3-MCPD di-esters detailed in Section 2.1 (except 1,2-distearoyl-3-chloropropanediol included later) have been included in the method. Reversely, MCPD di-ester coverage with the ten mentioned standards was calculated for various oils. A good coverage of MCPD di-esters in most of the oils and especially in palm oil was theoretically achieved (coverage above 95% for corn oil, cotton seed oil, grape seed oil, olive oil, palm oil, safflower oil and sunflower oil) but was not fit for palm kernel (8%) and coconut oil (4%) analysis. This simple model developed to predict MCPD di-esters relative abundance and to identify key analytical standards to be used for palm oil analysis was confirmed afterwards by analyzing 22 palm oil and 7

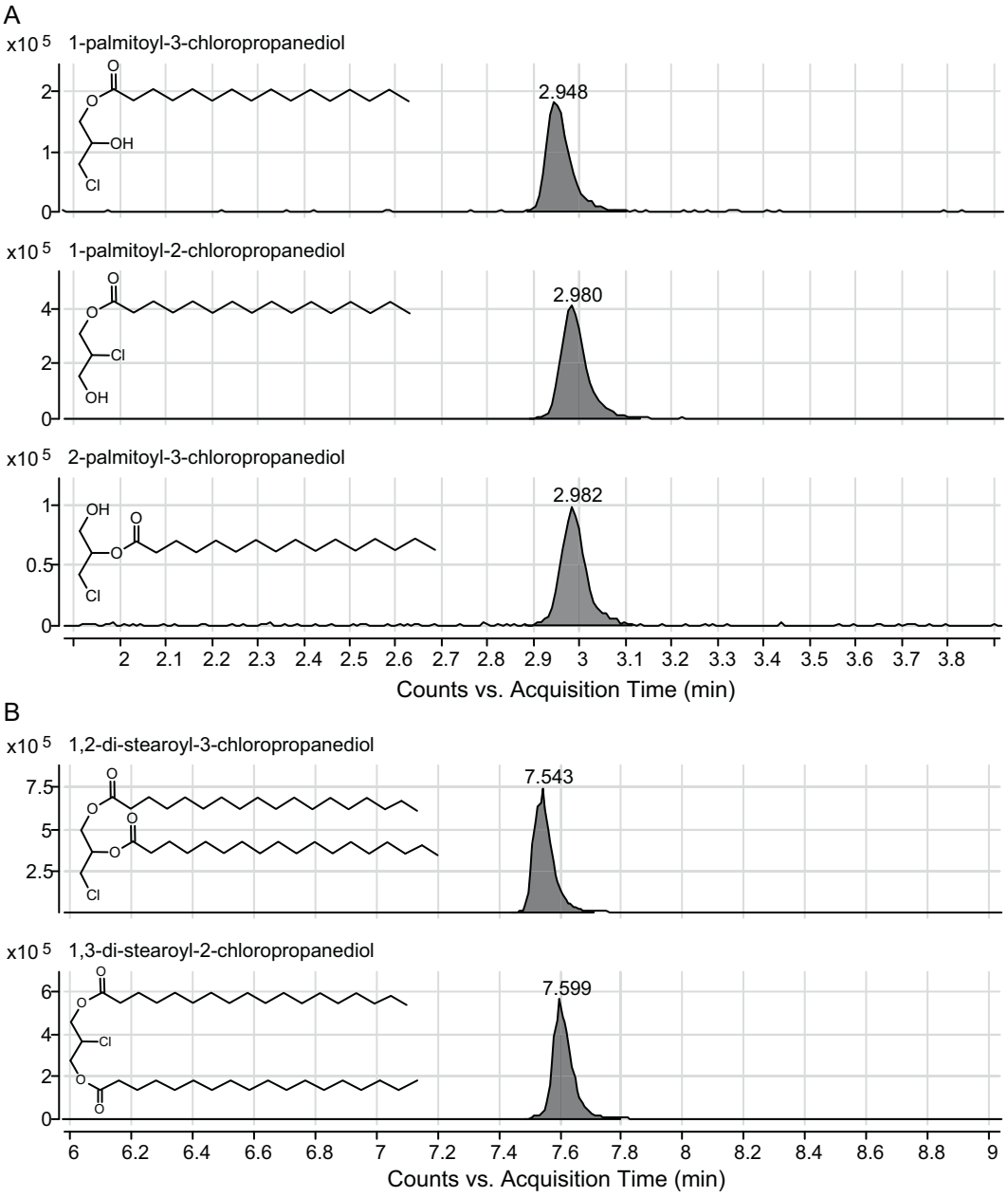

Fig. 2. Extracted ion chromatograms of: (A) 1-palmitoyl-3-MCPD (upper chromatogram), 1-palmitoyl-2-MCPD (middle chromatogram) and 2-palmitoyl-3-MCPD (lower chromatogram). These three isomers cannot be differentiated by liquid chromatography (same retention time) and by mass spectrometry (same chemical formula, same fragments). (B) Di-stearoyl-3-MCPD (upper chromatogram) and di-stearoyl-2-MCPD (lower chromatogram) can neither be differentiated by LC–ESI-ToF-MS.

palm olein samples. Only 19 over 22 palm oil samples had a MEs level high enough to perform a distribution evaluation (oleate-stearate-3-MCPD and bis-linoleate-3-MCPD were not detectable in the other two samples). Fig. 3 shows that the measured relative abundance of the ten different MCPD di-esters species analyzed in samples confirmed our theoretical approach proposed previously. This simple approach does not take into account natural preferential positions of fatty acids on glycerol as well as natural variability of fatty acid profile, which could explain the spread values and slight difference between median values of the 26 samples and calculated distribution. It should be noticed that, following this correlation between observed MCPD di-esters distribution in palm oil and calculated distribution, a reliable direct determination of MCPD di-esters in palm kernel oil and coconut oil seems today difficult to implement without custom synthesis of numerous analytical standards (bold number outside dotted-line box in Table 3). Furthermore it has been shown that palm oil, corn oil and coconut oil possess the highest potential for 3-MCPD formation [12]. Moreover, only one 2-MCPD di-esters (1,3-distearoyl-2-chloropropanediol) was commercially available. This standard was used as well as its corresponding 3-MCPD di-esters (1,2-distearoyl-3-chloropropanediol) in order to confirm extractability of 2-MCPD di-esters and to compare mass spectrometry response. Here again, LC–ESI-ToF-MS analysis could not differentiate di-stearoyl-3-MCPD and di-stearoyl-2-MCPD as shown in Fig. 2B. These two compounds had the same retention time and the same chemical formula, but a 30% lower response for the 2-MCPD ester was observed. It should be noticed that also some 3-MCPD di-esters own an identical chemical formula but are substituted with different fatty acids. Among the selected ten 3-MCPD di-esters analytical standards, this was the case of di-oleate-3-chloropropanediol with 1-stearoyl-2-linoleate-3-chloropropanediol ($C_{39}H_{71}ClO_4$), as well

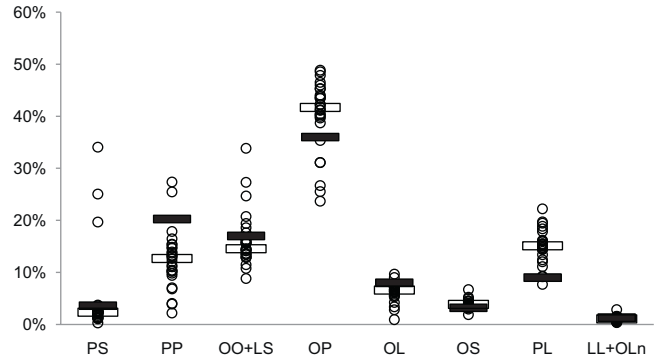

Fig. 3. MCPD di-esters distribution measured in 19 palm oil and 7 palm olein samples (○, as percentage of total MCPD di-esters weight). MCPD di-esters are abbreviated by their two fatty acids (P for palmitate, S for stearate, O for oleate, L for linoleate and Ln for linolenate). Median of measured values (▭) were comparable to theoretical distribution calculated for palm oil (▬).

as di-linoleate-3-chloropropanediol with 1-oleate-2-linolenate-3-chloropropanediol ($C_{39}H_{67}ClO_4$). These compounds could not be separated by our liquid chromatography conditions as they have identical retention time and cannot be differentiated by LC–ESI-ToF-MS. However, MS response observed between these isobaric compounds is identical, which allows a joint quantification. Combining previous observations and as an example, a LC–ESI-ToF-MS chromatographic peak attributed to di-oleate-3-chloropropanediol (combination of retention time and mass) gathers also signal from four other compounds: di-oleate-2-MCPD, 1-stearoyl-2-linoleate-3-MCPD, 1-stearoyl-3-linoleate-2-MCPD and 1-linoleate-2-stearoyl-3-MCPD.

3.2. Internal standards

To control the extraction process used for direct method, five isotopically labeled MEs standards were included into the present analytical approach, two as labeled 3-MCPD mono-esters and three as labeled 3-MCPD di-esters. To avoid any isotope contribution of the non labeled MEs to the labeled one, a shift in mass higher than 4 amu is highly recommended for MEs due to the presence of a chlorine atom within the molecule, that increases high mass isotopes abundance. IS used in the present method are labeled with three ^{13}C on the MCPD backbone and one ^{13}C on each carboxy group. A difference of 5 amu between non-labeled and labeled MCPD di-esters could lead to ambiguity between sodium adduct of non-labeled compound and ammonium adduct of labeled compound. However, the difference between these two ions is 100 ppm, which is above the identification criteria and the extraction window both set at 10 ppm for LC–ESI-ToF-MS data treatment, ensuring selectivity of detection.

3.3. Optimization of extraction methods

Each time an elution profile was determined, the main triglycerides (TAGs) and diglycerides (DAGs) observed for palm oil and coconut oil samples as summarized in Table 4 were analyzed in each elution fraction by LC–ESI-ToF-MS using their exact mass as identification criteria. The extracted ion chromatogram of the individual compounds was integrated and areas of compounds belonging to the same class were summed up to provide an elution profile of TAGs, DAGs, GEs and MEs. The gel permeation chromatography (GPC) extraction developed for GEs by Weißhaar and Perz in 2010 [22] and validated for a wide range of oil samples in another communication [33] was not applicable to MEs due to coelution with the main matrix components TAGs and DAGs as shown in Fig. 4.

Table 4
TAGs, DAGs and GEs molecular species detected in palm oil. Retention time and exact mass were used for detection by LC–ToF-MS.

ACN:DB[a]	Chemical formula	RT (min)	Monoisotopic mass (Da)
TAG			
38:0	$C_{41}H_{78}O_6$	7.10	666.5798
42:1	$C_{45}H_{84}O_6$	7.46	720.6268
48:0	$C_{51}H_{98}O_6$	8.15	806.7363
48:1	$C_{51}H_{96}O_6$	8.16	804.7207
48:2	$C_{51}H_{94}O_6$	8.02	802.7050
50:1	$C_{53}H_{100}O_6$	8.35	832.7520
50:2	$C_{53}H_{98}O_6$	8.21	830.7363
50:3	$C_{53}H_{96}O_6$	8.08	828.7207
52:1	$C_{55}H_{104}O_6$	8.55	860.7833
52:2	$C_{55}H_{102}O_6$	8.40	858.7676
52:3	$C_{55}H_{100}O_6$	8.27	856.7520
52:4	$C_{55}H_{98}O_6$	8.12	854.7363
52:5	$C_{55}H_{96}O_6$	8.00	852.7207
54:2	$C_{57}H_{106}O_6$	8.60	886.7989
54:3	$C_{57}H_{104}O_6$	8.46	884.7833
54:4	$C_{57}H_{102}O_6$	8.31	882.7676
54:5	$C_{57}H_{100}O_6$	8.17	880.7520
54:6	$C_{57}H_{98}O_6$	8.03	878.7363
DAG			
32:2	$C_{35}H_{64}O_5$	5.56	564.4754
34:1	$C_{37}H_{70}O_5$	6.20	594.5223
34:2	$C_{37}H_{68}O_5$	5.95	592.5067
36:1	$C_{39}H_{74}O_5$	6.56	622.5536
36:2	$C_{39}H_{72}O_5$	6.30	620.5380
36:3	$C_{39}H_{70}O_5$	6.05	618.5223
36:4	$C_{39}H_{68}O_5$	5.80	616.5067
38:1	$C_{41}H_{78}O_5$	6.90	650.5849
38:2	$C_{41}H_{76}O_5$	6.67	648.5693
Glycidyl-laurate	$C_{15}H_{28}O_3$	1.58	256.2040
Glycidyl-myristate	$C_{17}H_{32}O_3$	2.30	284.2351
Glycidyl-stearate	$C_{21}H_{40}O_3$	3.82	340.2977
Glycidyl-palmitate	$C_{19}H_{36}O_3$	3.07	312.2664
Glycidyl-oleate	$C_{21}H_{38}O_3$	3.27	338.2821
Glycidyl-linoleate	$C_{21}H_{36}O_3$	2.81	336.2664
Glycidyl-linolenate	$C_{21}H_{34}O_3$	2.42	334.2508

[a] Number of acyl group carbons: number of double bonds.

Finally, two different extractions were developed targeting MCPD mono-esters for the first one and MCPD di-esters for the second one.

3.3.1. Extraction of MCPD mono-esters

The present method is similar to the extraction approach proposed by [26], developed initially for GEs quantification. It has been modified in this study to include extraction of MCPD

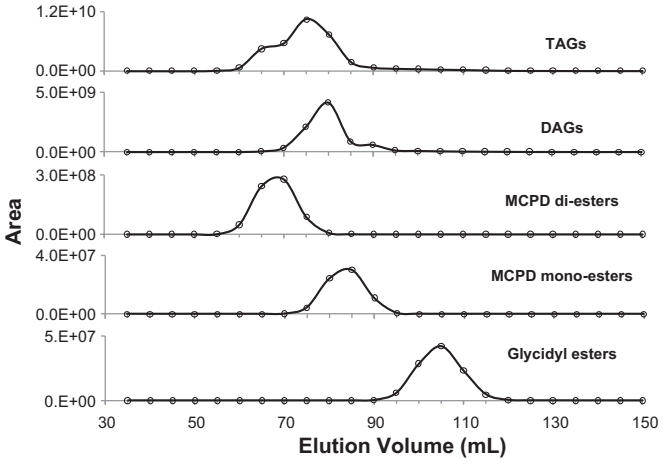

Fig. 4. GPC elution of a palm oil sample spiked at 5 μg/g with 7 MCPD mono-esters and 10 MCPD di-esters, obtained by collection of 5-mL fractions from 30 to 150 mL elution volume, analyzed by LC–ESI-ToF-MS.

Table 5
Recoveries obtained by the double SPE extraction method for GEs and MCPD mono-esters, and obtained by silica gel column extraction method for MCPD di-esters, on 6 palm oil samples spiked at 2 fortification levels (0.5 μg/g and 1 μg/g).

	Analyte	Absolute recovery ± RSD_{IR}	
		0.5 μg/g level	1 μg/g level
MCPD mono-esters	1-Myristoyl-3-chloropropanediol	61 ± 32	74 ± 41
	1-Lauroyl-3-chloropropanediol	89 ± 25	88 ± 30
	1-Palmitoyl-3-chloropropanediol	118 ± 27	101 ± 33
	1-Stearoyl-3-chloropropanediol	80 ± 13	97 ± 20
	1-Oleyl-3-chloropropanediol	150 ± 53	110 ± 31
	1-Linoleoyl-3-chloropropanediol	131 ± 37	116 ± 35
	1-Linolenoyl-3-chloropropanediol	113 ± 26	97 ± 24
MCPD di-esters	1,2-Bis-oleoyl-3-chloropropanediol	103 ± 7	108 ± 14
	1,2-Bis-palmitoyl-3-chloropropanediol	86 ± 29	127 ± 24
	1,2-Dilinoleoyl-3-chloropropanediol	56 ± 17	59 ± 16
	1-Oleoyl-2-linoleoyl-3-chloropropanediol	92 ± 16	86 ± 12
	1-Oleoyl-2-stearoyl-3-chloropropanediol	121 ± 10	117 ± 13
	1-Palmitoyl-2-linoleoyl-3-chloropropanediol	103 ± 9	101 ± 13
	1-Palmitoyl-2-oleoyl-3-chloropropanediol	124 ± 40	127 ± 29
	1-Palmitoyl-2-stearoyl-3-chloropropanediol	130 ± 8	117 ± 12
	1,2-Bis-oleoyl-3-chloropropanediol	103 ± 7	108 ± 14
	1,2-Bis-palmitoyl-3-chloropropanediol	86 ± 29	127 ± 24

mono-esters. This extraction was performed on two SPE (C_{18} and silica cartridge) cartridges. Two oils were considered for the method optimization: palm oil (the most relevant one for MEs and GEs content) and coconut oil (containing short myristic- and lauric-based TAGs which are eluting at lower volume and are likely to overlap with the elution of MCPD mono-esters). Acetonitrile and methanol were evaluated as eluting solvents for the C_{18} SPE. These solvents elute first monoglycerides (MAGs) and GEs, then MCPD mono-esters, followed by DAGs and finally TAGs. Acetonitrile (Fig. 5B) allowed a better separation of the MCPD mono-esters (and GEs) from DAGs and TAGs when compared to methanol (Fig. 5A), even if a higher elution volume was required (20 mL acetonitrile vs. 15 mL methanol). To further reduce the content of DAGs and MAGs, due to their coelution with MCPD mono-esters (even if present at low level, typically between 0 and 10% [34], DAGs and MAGs lead to significant matrix effects), the dried extract was reconstituted in 500 μL dichloromethane, and further cleaned on a silica SPE cartridge. Using dichloromethane as eluting solvent, TAGs eluted

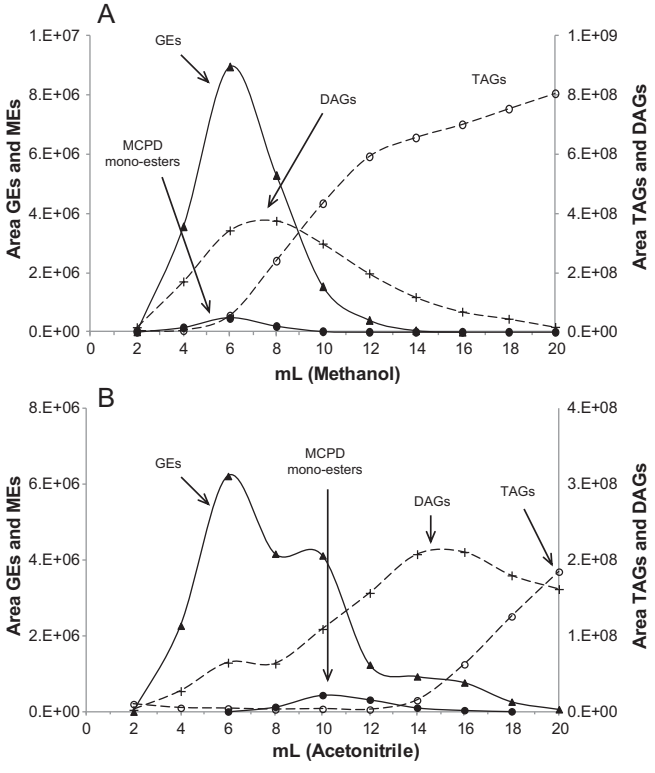

Fig. 5. Elution profile of GEs (▲) and MCPD mono-esters (●) spiked in Coconut Oil, as well as DAG (+) and TAG (○) elution profile. 2 g oil has been dissolved in 5 mL acetone and 250 μL was loaded on a 2 g C18 SPE cartridge. Eluting solvent was methanol (A) or acetonitrile (B).

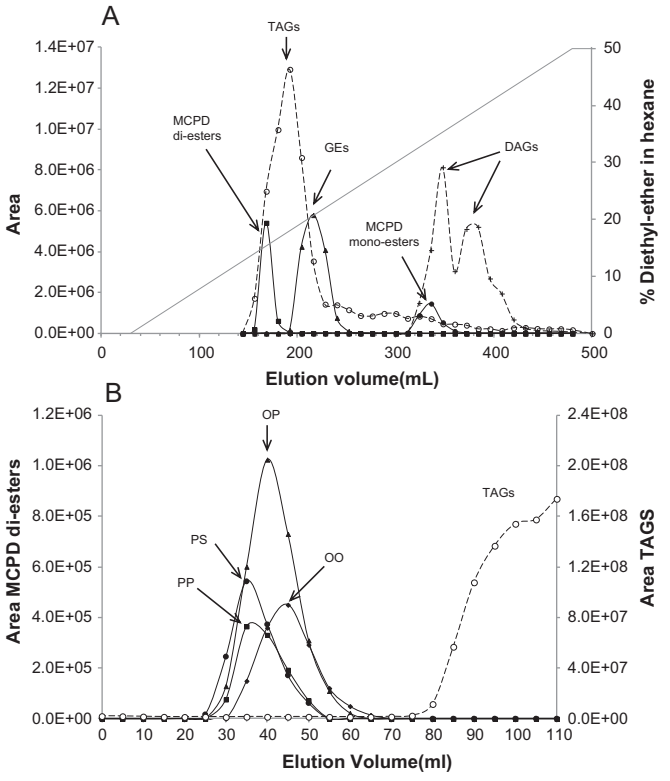

Fig. 6. (A) Elution of MCPD di-esters (■), GEs (▲) and, MCPD mono-esters (●) from 100 mg palm oil loaded on a 10 g KP-Sil™ SNAP Cartridge, with a linear gradient of diethyl-ether in hexane (grey line —). Area of TAGs (○) and DAGs (+) were divided by 50 for display convenience. Separation was improved (B) by use of isocratic elution (40% dichloromethane in hexane, 40 mg oil loaded on 3 g silica) to elute MCPD di-esters in the first 65 mL (●: 1-palmitoyl-2-stearoyl-3-chloropropanediol (PS); ■: 1,2-bis-palmitoyl-3-chloropropanediol (PP); ▲: 1-oleyl-2-palmitoyl-3-chloropropanediol (OP); ♦: 1,2-bis-oleoyl-3-chloropropanediol (OO)) and then TAGs (○).

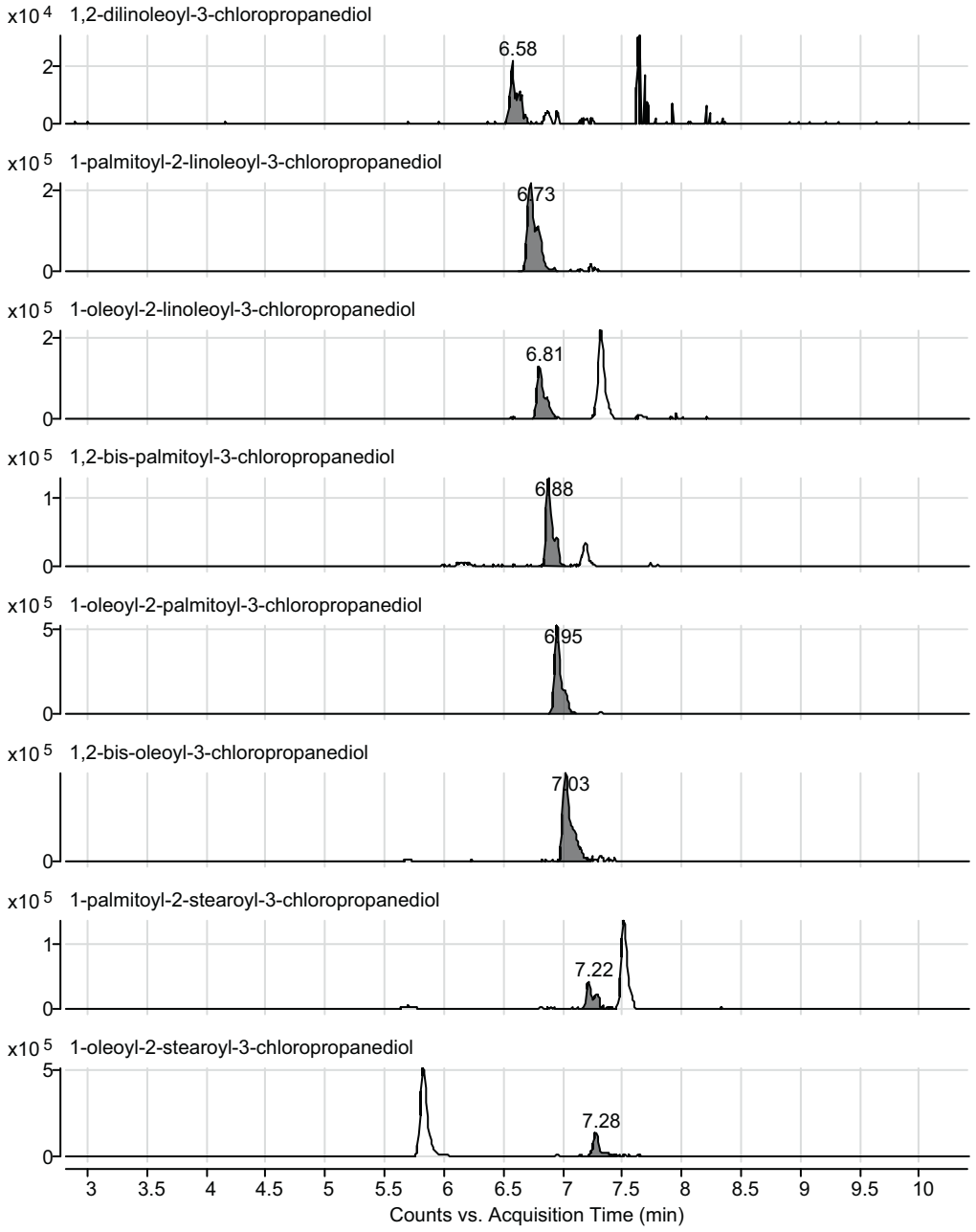

Fig. 7. Extracted ion chromatogram of MCPD di-esters in a palm olein sample after extraction on silica column and LC–ToF-MS analysis. Measured level were 0.1 μg/g di-linoleoyl-3-MCPD, 1.5 μg/g palmitoyl-linoleoyl-3-MCPD, 0.8 μg/g oleyl-linoleoyl-3-MCPD, 1.3 μg/g di-palmitoyl-3-MCPD, 5 μg/g palmitoyl-oleyl-3-MCPD, 1.8 μg/g di-oleyl-3-MCPD, 0.3 μg/g palmitoyl-stearoyl-3-MCPD and 0.6 μg/g oleyl-stearoyl-3-MCPD.

first, hence the importance of the first C_{18} SPE, followed by the elution of MCPD mono-esters (and GEs), then DAGs and finally MAGs. An elution volume of 25 mL dichloromethane allowed the efficient elution of MCPD mono-esters (10 mL for GEs), whereas DAGs and MAGs remained on the column. To assess recoveries, a low contaminated palm oil sample was spiked at two levels (0.5 μg/g and 1 μg/g of oil) and extracted in duplicate over three different days ($n = 6$). Recoveries obtained were between 61% and 151% for MCPD mono-esters as shown in Table 5. GEs recoveries by this approach were lower (between 44% and 87% for GEs, data not shown) than by the GPC extraction method, confirming the GPC extraction to remain the technique of choice for GEs determination in oils.

3.3.2. Extraction of MCPD di-esters

MCPD di-esters have physico-chemical properties close to those of TAGs, but an abundance ratio about 10^6, making their extraction challenging. The direct method was optimized with a Mid Performance Liquid Chromatography system, which offered the advantage of testing different size of silica cartridges as well as different particle size of silica (data not shown), in combination with different eluting solvent being applied to cartridges as gradient in hexane. A palm oil sample eluted with a diethyl-ether gradient in hexane is shown in Fig. 6A, illustrating the order of elution. MCPD di-esters and TAGs eluted very closely, but separation was enormously improved replacing diethyl-ether by dichloromethane and optimizing conditions for an isocratic elution

Table 6
MCPD esters measured by direct and indirect method in palm oil, palm olein, sunflower and palm kernel oil. Summed results (bold) are expressed as MCPD equivalent.

Samples	Comparison		Indirect method		Direct method															
	Sum MCPD[a] indirect method (μg/g)	Sum MCPD[b] direct method (μg/g)	MCPD (μg/g)		MCPD di-esters (μg/g)[c]									MCPD mono-esters (μg/g)[e]						
			3-MCPD	2-MCPD	MCPD eq.[d]	PS	PP	OO + LS	OP	OL	OS	PL	LL + OLn	MCPD eq.[f]	La	My	P	S	O	L	Ln
Palm oil 1	***1.22***	***0.82***	0.81	0.41	***0.70***	0.10	0.93	0.93	1.20	0.24	0.13	0.35	0.04	***0.12***	–	–	0.14	–	0.26	–	–
Palm oil 2	***1.86***	***1.55***	1.25	0.61	***1.50***	2.08	2.28	0.73	2.12	0.08	0.28	0.64	0.10	***0.05***	–	–	0.08	–	0.08	–	–
Palm oil 3	***2.54***	***2.81***	1.64	0.90	***2.16***	0.18	1.26	2.51	4.90	0.64	0.60	1.95	0.06	***0.65***	–	–	0.46	–	1.38	0.33	–
Palm oil 4	***3.90***	***3.53***	2.70	1.20	***3.34***	6.33	2.87	2.01	4.41	0.63	0.35	1.73	0.28	***0.19***	–	–	0.26	0.21	0.15	–	–
Palm oil 5	***7.90***	***8.85***	5.12	2.78	***8.39***	0.82	5.35	8.75	21.92	2.63	2.15	5.24	0.26	***0.45***	–	–	0.34	–	0.95	0.22	–
Palm oil 6	***3.56***	***3.02***	2.26	1.30	***2.95***	3.22	4.17	1.88	4.37	0.46	0.57	1.50	0.20	***0.07***	–	–	0.10	–	0.09	0.04	–
Palm oil 7	–	***0.12***	–	–	***0.10***	–	–	0.12	0.22	–	0.09	0.13	–	***0.02***	–	–	–	–	0.07	–	–
Palm oil 8	***5.88***	***5.23***	3.81	2.07	***4.87***	0.63	3.45	4.42	11.26	1.99	0.94	4.19	0.40	***0.36***	–	–	0.47	–	0.57	0.16	–
Palm oil 9	–	***0.14***	–	–	***0.11***	–	–	0.17	0.22	–	0.14	0.10	–	***0.02***	–	–	–	–	0.08	–	–
Palm oil 10	***1.55***	***1.35***	0.96	0.59	***1.17***	0.21	0.65	1.04	2.63	0.48	0.27	1.21	0.09	***0.18***	–	–	0.18	–	0.31	0.11	–
Palm oil 11	***1.00***	***1.34***	0.64	0.36	***1.10***	0.22	0.58	0.97	2.54	0.41	0.22	1.17	0.06	***0.24***	–	–	0.22	–	0.49	0.07	–
Palm oil 12	***2.37***	***1.96***	1.49	0.88	***1.85***	0.35	1.25	1.49	4.11	0.75	0.46	1.85	0.10	***0.11***	–	–	0.24	–	0.13	–	–
Palm oil 13	***0.27***	***0.11***	0.15	0.12	***0.11***	0.03	0.13	0.12	0.33	–	–	–	–	–	–	–	–	–	–	–	–
Palm oil 14	***6.29***	***6.15***	4.23	2.06	***5.50***	0.72	4.22	4.37	13.39	2.11	1.11	4.56	0.33	***0.64***	–	–	1.15	–	0.83	0.12	–
Palm oil 15	***4.87***	***3.85***	3.11	1.76	***3.47***	0.21	2.70	2.62	8.78	1.21	0.66	3.08	0.14	***0.38***	–	–	0.47	–	0.65	0.13	–
Palm oil 16	***1.74***	***1.53***	1.49	0.25	***1.11***	0.13	1.02	1.01	2.81	0.26	0.18	0.77	0.02	***0.42***	–	–	0.74	–	0.65	–	–
Palm oil 17	***7.02***	***6.84***	4.64	2.38	***4.98***	0.56	3.70	4.06	11.64	2.07	1.12	4.51	0.24	***1.86***	–	–	3.37	–	2.34	0.34	–
Palm oil 18	***5.55***	***5.39***	3.65	1.90	***4.20***	0.55	3.06	3.04	10.80	1.40	0.80	3.61	0.23	***1.19***	–	–	0.57	–	2.78	0.65	–
Palm oil 19	***7.27***	***6.51***	4.80	2.47	***4.97***	0.71	3.68	3.94	12.27	1.83	0.97	4.10	0.33	***1.54***	–	–	2.22	–	2.18	0.67	–
Palm oil 20	***4.59***	***4.35***	2.91	1.68	***4.17***	0.55	3.42	3.12	9.85	1.78	0.94	3.36	0.31	***0.18***	–	–	0.13	–	0.47	–	–
Palm oil 21	***8.84***	***7.75***	5.61	3.23	***7.04***	0.93	6.04	5.07	16.64	2.81	1.51	5.92	0.45	***0.70***	–	–	0.81	–	1.40	0.13	–
Palm oil 22	***5.55***	***4.80***	3.41	2.14	***4.80***	0.65	3.99	3.68	11.68	1.78	0.95	3.90	0.25	–	–	–	–	–	–	–	–
Palm olein 1	***6.63***	***6.59***	4.07	2.56	***5.45***	0.39	1.25	7.60	10.88	2.98	1.36	6.00	0.33	***1.14***	–	–	0.97	0.05	2.17	0.59	–
Palm olein 2	***3.69***	***3.02***	2.27	1.42	***2.91***	0.43	1.68	3.18	6.34	1.03	1.10	2.54	0.07	***0.11***	–	–	0.17	–	0.18	–	–
Palm olein 3	***6.63***	***6.23***	4.42	2.21	***6.13***	0.44	2.43	6.06	16.48	2.18	1.35	5.21	0.29	***0.10***	–	–	0.15	–	0.17	–	–
Palm olein 4	***3.26***	***2.85***	2.17	1.09	***2.59***	0.04	0.32	4.98	4.57	1.32	0.46	2.90	0.09	***0.26***	–	–	0.24	–	0.52	0.11	–
Palm olein 5	***1.88***	***1.51***	1.09	0.79	***1.46***	0.17	0.32	2.25	2.56	0.60	0.26	1.83	0.23	***0.05***	–	–	0.07	–	0.09	–	–
Palm olein 6	***5.87***	***6.79***	3.87	2.00	***6.18***	0.44	2.37	5.02	16.97	2.67	1.35	5.37	0.54	***0.61***	–	–	0.63	–	1.20	0.20	–
Palm olein 7	***2.39***	***2.19***	1.47	0.92	***2.02***	0.30	1.25	1.77	4.97	0.81	0.59	1.52	0.12	***0.17***	–	–	0.15	–	0.38	0.04	–
Sunflower oil	***4.52***	***4.97***	3.04	1.48	***4.33***	0.07	0.04	19.36	1.53	2.13	1.14	0.23	0.63	***0.63***	–	–	–	–	2.07	0.08	–
Palm kernel oil	***1.46***	***0.08***	0.97	0.49	***0.08***	0.05	0.15	0.04	0.19	0.03	–	0.02	–	–	–	–	–	–	–	–	–

[a] Sum of 2- and 3-MCPD obtained by indirect method.
[b] Sum of MCPD mono- and di-esters obtained by direct method expressed as MCPD equivalent.
[c] MCPD di-esters abbreviated by their fatty acid (P: palmitate; S: stearate; O: oleate; L: linoleate; Ln: linolenate).
[d] Sum of MCPD di-esters expressed as MCPD equivalent as described in Section 2.10.
[e] MCPD mono-esters abbreviated by their fatty acid (La: laurate; My: myristate; P: palmitate; S: stearate; O: oleate; L: linoleate; Ln: linolenate).
[f] Sum of MCPD mono-esters expressed as MCPD equivalent as described in Section 2.10.

(40% dichloromethane in hexane). Method was finally transferred on a chromatographic glass tube filled with silica gel and gravimetrical elution in order to increase sample preparation throughput (several samples could be extracted in parallel). As shown in Fig. 6B, MCPD di-esters elute in the first 65 mL window, separated baseline from TAGs. However, the critical parameter of this extraction procedure turned out to be the ratio between silica gel and matrix loaded: to keep extraction efficiency (ratio over 50) and to reduce solvent consumption (proportional to silica gel amount), a ratio of 75 in combination with 3 g silica gel conditioned with hexane was chosen. These conditions allowed an extraction of the MCPD di-esters from 40 mg oil with an elution volume of 65 mL (40% dichloromethane in hexane).

First recovery experiments were performed with the 4 MCPD di-esters analytical standards initially used (bis-palmitate-, bis-oleate-, palmitate-stearate- and palmitate-oleate-3-MCPD) in various types of oil including canola oil, coconut oil, blended oils, safflower oil, soy oil, sunflower oil, palm kernel oil and palm oil. These 8 oils were extracted before and after spiking at the 1 μg/g level for each MCPD di-ester. Quantification performed by means of standard addition on extracts led to an average absolute recovery of 103% (n = 32, median 100%, min 79%, max 137% and standard deviation 14%). Once analytical standards relevant for palm oil analysis were included in the method, further recovery experiments were performed in palm oil using a total of 10 MCPD di-esters. A low contaminated palm oil sample was spiked at two levels (0.5 μg/g and 1 μg/g) and extracted in duplicate over three different days (n = 6). Recoveries values are reported in Table 5, and were between 56% and 127%. Dilinoleoyl-3-MCPD was shown to be particularly lower (56 ± 17% at 0.5 μg/g and 59 ± 16% at 1 μg/g) than all the other MCPD di-esters recoveries which were above 86% at the two spiking levels. As an example, extracted chromatograms of the 8 MCPD di-esters in a palm olein sample obtained by LC–ToF-MS after extraction are shown in Fig. 7.

3.4. Direct method quantification approach

Since for most of the targeted analytes an internal standard was not available, matrix matched standard addition on extracts was considered as the best quantification approach. Hereby, addition of standards was carried out automatically using the LC-autosampler in order to ensure repeatability as described under Section 2.9 LC–ESI-ToF-MS analysis section. In addition, oil samples were always spiked with internal standards at a 0.5 μg/g level prior to extraction. The IS were quantified by standard addition similar to the non-labeled MEs, allowing evaluation of recoveries for each sample. For the 32 samples analyzed (22 samples of palm oil, 7 samples of palm olein, 1 sample of coconut oil, 1 sample of palm kernel and 1 sample of sunflower oil), the average of absolute recoveries for the three labeled MCPD di-esters (labeled bis-oleyl-, oleyl-palmitoyl- and bis-palmitoyl-3-MCPD) were 108 ± 14%, 92 ± 17% and 119 ± 45%, respectively. Due to matrix interference labeled bis-palmitoyl-3-MCPD was difficult to quantify resulting in a high variability of recoveries obtained for this analyte. Concerning the two labeled MCPD mono-esters (labeled 1-palmitoyl-3-MCPD and 1-oleyl-3-MCPD), average recoveries were 63% ± 31% and 76% ± 29% respectively. Limits of detection were estimated to 0.02 μg/g for MCPD di-esters and 0.05 μg/g for MCPD mono-esters.

3.5. Oil samples analysis

Results obtained by applying the indirect method (2- and 3-MCPD) to 22 palm oil (two were under LOQ at 0.1 μg/g for both 2- and 3-MCPD) and 7 palm olein samples were ranging between 0.3 and 8.8 μg/g for total MCPD (0.2–5.6 μg/g for 3-MCPD and 0.1–3.2 μg/g for 2-MCPD), as summarized in Table 6. Interestingly, contribution of 2-MCPD on total MCPD was stable at a 35% ± 5% value in average, which is far to be negligible. The same 29 samples analyzed by the newly developed direct method gave results between 0.1 and 8.8 μg/g for total MCPD, with MCPD di-esters between 0.1 and 8.4 μg/g, and up to 1.9 μg/g for MCPD mono-esters (expressed as MCPD equivalent). Individual results for each MCPD mono-ester and MCPD di-esters are given in Table 6. The major strength of the developed direct method is to allow characterization of samples in terms of MEs composition and especially MCPD di-esters distribution, as illustrated in Fig. 3 (results of palm oil 7, 9 and 13 in Table 6 were not included in this distribution evaluation as some MCPD di-esters were under limit of detection). Among the 29 studied samples, contribution of MCPD di-esters on total MEs was stable within samples at a 89% ± 8% value in average. Direct analysis by LC–ESI-ToF-MS allowed differentiating MCPD mono-esters from MCPD di-esters but not 2-MCPD esters from 3-MCPD-esters. In contrary, indirect analysis allows differentiating 2-MCPD from 3-MCPD but not mono- from di-esters as any separation had been performed prior to the acidic methanolysis. Combining results from the direct and indirect analysis lead to an interesting mapping of the MCPD esters present in the oil samples: 89% of the MEs detected in the 29 palm oil and palm olein samples were MCPD di-esters, 35% of which were esters of 2-MCPD. The distribution of the fatty acids esterified with MCPD followed the distribution pattern of the fatty acids in palm oil. The correlation between results is shown in Fig. 8. A slope of 0.972 and a constant bias of −0.13 μg/g indicate that the two methods provided very similar results. Results were also comparable for one sample of sunflower oil (4.5 μg/g when analyzed by the indirect method compared to 5 μg/g when analyzed by the direct method). As discussed in Section 3.1 and confirmed throughout our studies, the direct method with analytical standards as selected here was shown not to be suitable for coconut oil and palm kernel oil. Results are especially not correlated for the palm kernel oil. In the sample analyzed, 1.46 μg/g MCPD was detected by applying indirect method and 0.09 μg/g MCPD was detected applying the direct method. As a reminder, these types of oils are characterized by short chain fatty acids (capric, lauric and myristic fatty acids) that are not available as MCPD di-esters standards, and thus not quantified. This may lead to an underestimation of MCPD content by the direct method for palm kernel and coconut oil samples.

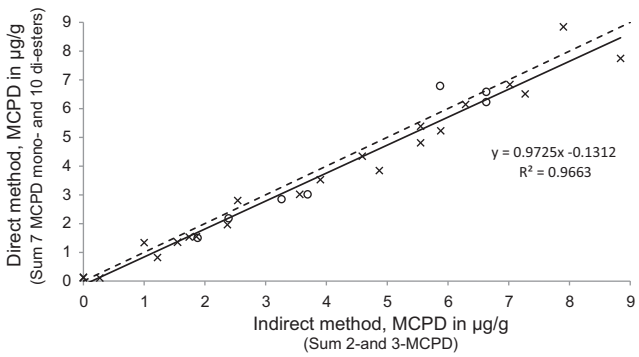

Fig. 8. Correlation plot of indirect method versus direct method for the determination of MEs in 22 palm oil samples (×) and 7 palm olein samples (○). For direct method, 2- and 3-MCPD results were summed. For indirect method results of 7 MCPD mono-esters and 10 MCPD di-esters were expressed as MCPD equivalent and summed.

4. Conclusion

A direct method for quantification of MEs has been developed, using two individual extraction steps to specifically and reliably isolate MCPD di-esters on one hand side (by silica gel extraction), and

to isolate MCPD mono-esters in the other hand side (by a double SPE) from the fat matrix. The main objective of this method development was to obtain a better understanding on the reliability of commonly applied indirect methods for the determination of MEs. Direct method has been mainly developed for palm oil and palm olein in the frame of method comparison, but preliminary results have shown that other types of edible oils (such as sunflower, canola, safflower and soy oil) can be targeted if adequate analytical standards are available. The comparison of the presented direct method with an indirect method (acidic methanolysis, HFBI derivatization and GC–MS), showed very similar results when analyzing 29 oil samples with both methods. However, the two methods differ in their applicability in routine analysis. The indirect approach requires a minimum of chemical standards (ideally 4 standards: 2- and 3-MCPD and their respective internal standards), is less cumbersome in sample preparation, and is applicable to all type of commodities compared to the direct approach. For a rapid determination of the total MCPD esters content in a broad type of oils, the indirect method as presented here (acidic methanolysis based) is therefore the method of choice for routine analysis.

Still the direct method may be considered as relevant for e.g. the preparation of reference materials or for toxicological studies related to MCPD-esters.

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