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Rapid and Green Separation of Mono- and Diesters of Monochloropropanediols by Ultrahigh Performance Supercritical Fluid Chromatography–Mass Spectrometry Using Neat Carbon Dioxide as a Mobile Phase

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ABSTRACT: This study demonstrates the effect of column selectivity and density of supercritical carbon dioxide (SC-CO_2) on the separation of monochloropropanediol (MCPD) esters, known as food toxicants, using SC-CO_2 without addition of cosolvent in ultrahigh performance supercritical fluid chromatography–mass spectrometry (UHPSFC-MS). This study shows that over 20 2-monochloropropanediol (2-MCPD) and 3-monochloropropanediol (3-MCPD) mono- and diesters are separated on a 2-picolylamine column in less than 12 min. The presence and position of a hydroxyl group in the structure, the number of unsaturated bonds, and the acyl chain length play a significant role in the separation of MCPD esters. The flow rate, backpressure, and column oven temperature, which affect the density of the mobile phase, were shown to have a substantial impact on retention, efficiency, and selectivity. The developed method was successfully applied for the determination of MCPD esters in refined oils and showed a close to excellent green analysis score using the Analytical Eco-Scale.

KEYWORDS: *chloropropanol, mass spectrometry, method development, 2-MCPD esters, 3-MCPD esters, orthogonal column screening, SFC, UPC²*

INTRODUCTION

Edible oils and fats are industrially refined in order to remove undesired components, which may influence the quality of a final food product. This process however causes side reactions resulting in the formation of heat-induced toxicants such as 3-monochloropropane-1,2-diol (3-MCPD) and 2-monochloropropane-1,3-diol (2-MCPD) fatty acid esters.[1] The concern related to the presence of these contaminants in fats and fat-containing foods is because of the possible release of free chlorinated propanols from their esterified form during digestion.[2] Toxicological research on metabolism of free 3-MCPD proved its contribution to nephrotoxicity and antifertility.[3] For these reasons, the experts of International Agency on Cancer Research classified 3-MCPD as a non-genotoxic threshold carcinogen (category 2B).[4] In addition, experts of the European Food Safety Agency (EFSA) released recently an opinion on the risks for human health related to the presence of MCPD in foods and the Panel on Contaminants in Food Chain lowered the tolerable daily intake (TDI) of 3-MCPD from 2.0 μg/kg body weight per day to 0.8 μg per kg body weight per day.[5] There is still limited data on the behavior of 2-MCPD esters in the gastrointestinal tract. Also, the toxicological potential of 2-MCPD cannot be clearly stated due to the limited, mostly unpublished data.[6] According to the EFSA experts, the difference in the localization of the chlorine within the molecule structure makes it unlikely that 2-MCPD exhibits a similar metabolic pattern to 3-MCPD although the structure of both compounds is analogous.[5] According to the recent *in vitro* studies, 2-MCPD was not cytotoxic in contrast to the 3-MCPD esters, which displayed some cytotoxic potential.[7]

The hydrophobic properties and similar structure of MCPD esters (Figure 1) make their separation a challenging task. Furthermore, they occur at low concentration in foods, as part of the lipid fraction of the food sample.[8] To address such challenges, several analytical procedures dedicated to the determination of 3-MCPD and 2-MCPD fatty acid esters in food samples have been developed.[9] These methods offer two kinds of analytical approaches: direct (determination of intact MCPD esters) and indirect (measurement of the total MCPD ester content converted into free forms).[10,11]

Indirect methodologies are characterized by extensive sample preparation involving hydrolysis and transesterification (when MCPD esters are released into the free diols), sample cleanup, and derivatization of the hydroxyl groups present in the diol form.[12] Obtained derivatives are determined by GC-MS. Moreover, harsh chemical treatment applied in these protocols is likely to cause mutual interconversion/transformation reactions affecting the trueness of the analysis.[13] Several indirect methods of MCPD ester determination in oily/fatty

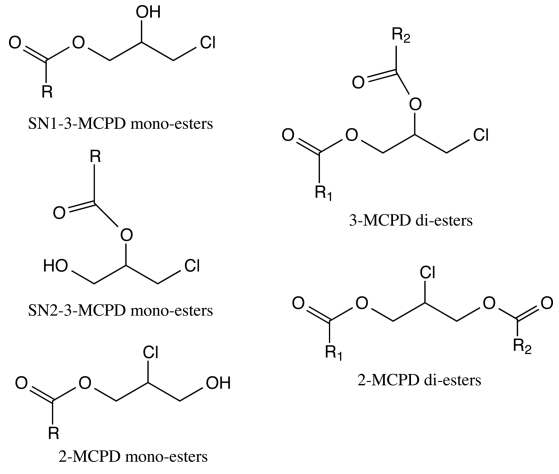

Figure 1. Chemical structures of mono- and difatty acid esters of MCPDs.

food samples have been adopted by American Oil Chemists' Society as official methods.[14,15] Although such an approach is simple in the case of chromatographic analysis (two peaks of MCPD derivatives to be identified), the method does not provide any information regarding the chemical structure of intact MCPD esters that may play a significant role in toxicological studies.[3]

Direct methods on the other hand allow for differentiation between various species of 2-MCPD and 3-MCPD mono- and diesters. Direct approaches do not require any chemical conversion of the analytes as needed in indirect procedures, inherently leading to less risk of undesired side reactions.[16] There are several direct protocols utilizing reversed-phase chromatography available in the literature, however they all have limitations in terms of low sensitivity, inadequate selectivity, severe matrix effects, and utilization of significant amounts of organic solvents.[17,18] One of the major issues is the limited separation selectivity for structurally similar MCPD esters, including isomers, thereby limiting the determination of many of the intact ester species in lipid samples. Furthermore, to the best of our knowledge, there is no one method that enables determination of both 3-MCPD mono- and diesters and 2-MCPD mono- and diesters in a single run. The most advanced direct method, which successfully targets these analytes, is based on separate sample preparation procedures for monoesters and diesters.[19,20] Each procedure involves two-step solid phase extraction (SPE) for sample cleanup. The authors focused on the major development in efficient removal

Table 1. Names and Abbreviations of the MCPD Esters Used in This Study[a]

					m/z of characteristic ions		
					precursor ion		
analyte	name	abbreviation		std mix	$[M + NH_4]^+$	$[M + Na]^+$	product ion, $[M - RCO_2]^+$
1	1-lauroyl-2-oleoyl-3-chloropropanediol	1-La-2-Ol-3M	C12:0 C18:1 3M	1, 2	574.457		357.253
2	1-lauroyl-2-linoleoyl-3-chloropropanediol	1-La-2-Li-3M	C12:0 C18:2 3M	1, 2	572.443		355.239
3	1,3-dipalmitoyl-2-chloropropanediol	1,3-diPa-2M	C16:0 C16:0 2M	1, 2	604.504		331.238
4	1,2-dipalmitoyl-3-chloropropanediol	1,2-diPa-3M	C16:0 C16:0 3M	1	604.503		331.237
5	1-palmitoyl-2-stearoyl-3-chloropropanediol	1-Pa-2-St-3M	C16:0 C18:0 3M	1, 3	632.530		359.266
6	1-palmitoyl-2-oleoyl-3-chloropropanediol	1-Pa-2-Ol-3M	C16:0 C18:1 3M	1, 3	630.515		357.251
7	1-palmitoyl-2-linoleoyl-3-chloropropanediol	1-Pa-2-Li-3M	C16:0 C18:2 3M	1, 2	628.501		355.236
8	1,3-distearoyl-2-chloropropanediol	1,3-diSt-2M	C18:0 C18:0 2M	1, 3	660.558		359.265
9	1,2-distearoyl-3-chloropropanediol	1,2-diSt-3M	C18:0 C18:0 3M	1, 2	660.557		359.264
10	1-oleoyl-2-stearoyl-3-chloropropanediol	1-Ol-2-St-3M	C18:1 C18:0 3M	1, 2	658.541		359.263
11	1,2-dioleoyl-3-chloropropanediol	1,2-diOl-3M	C18:1 C18:1 3M	1, 2	656.527		357.249
12	1-linoleoyl-2-stearoyl-3-chloropropanediol	1-Li-2-St-3M	C18:2 C18:0 3M	1, 3	656.526		359.264
13	1-oleoyl-2-linoleoyl-3-chloropropanediol	1-Ol-2-Li-3M	C18:1 C18:2 3M	1, 2	654.510		355.233
14	1,2-dilinoleoyl-3-chloropropanediol	1,2-diLi-3M	C18:2 C18:2 3M	1, 2	652.498		355.235
15	1-oleoyl-2-linolenoyl-3-chloropropanediol	1-Ol-2-Ln-3M	C18:1 C18:3 3M	1, 2	652.497		353.219
16	1-palmitoyl-3-chloropropanediol	1-Pa-3M	C16:0 3M	1, 3		371.238	
17	1-linoleoyl-3-linolenoyl-2-chloropropanediol	1-Li-3-Ln-2M	C18:2 C18:3 2M	1, 2	650.482		355.232
18	1-linoleoyl-2-linolenoyl-3-chloropropanediol	1-Li-2-Ln-3M	C18:2 C18:3 3M	1, 3	650.482		353.219
19	1-heptadecanoyl-3-chloropropanediol	1-Hep-3M	C17:0 3M	1, 2		385.252	
20	1,2-dilinolenoyl-3-chloropropanediol	1,2-diLn-3M	C18:3 C18:3 3M	1, 2	648.465		353.218
21	1-stearoyl-3-chloropropanediol	1-St-3M	C18:0 3M	1, 3		399.265	
22	1-palmitoyl-2-chloropropanediol	1-Pa-2M	C16:0 2M	1, 3		371.238	
23	1-oleoyl-3-chloropropanediol	1-Ol-3M	C18:1 3M	1, 2		397.251	
24	1-linoleoyl-3-chloropropanediol	1-Li-3M	C18:2 3M	1, 2		395.236	
25	1-linolenoyl-3-chloropropanediol	1-Ln-3M	C18:3 3M	1, 2		393.222	
26	1-oleoyl-2-chloropropanediol	1-Ol-2M	C18:1 2M	1, 3		397.251	
I.S. 1	1,2-dipalmitoyl-3-chloropropanediol-5d	1-Pa-3M-5d	C16:0 3M 5d				
I.S. 2	1-palmitoyl-3-chloropropanediol-5d	1,2-diPa-3M-5d	C16:0 C16:0 3M 5d				

[a]Standard mixture 1 was used for column screening, and standard mixtures 2 and 3 were used in repeatability and recovery studies, respectively. Experimental *m/z* values of the most abundant characteristic ions and adduct types of the MCPD esters obtained by UHPSFC-MS.

of possible interferents in order to enhance the chromatographic separation. Although the chromatographic run lasted over 30 min, there were still some peaks that coeluted.[19,20]

Recently, a supercritical fluid chromatography (SFC) method has been established for the analysis of 3-MCPD esters (but not for 2-MCPD esters) without the need for sample purification or derivatization.[21] In that study, methanol and isopropanol, up to 30%, were utilized as cosolvents together with a cyano column, and the main objective of the study was to enhance the sensitivity rather than the selectivity.

We demonstrate here a rapid method allowing for the first time the separation of intact 2-MCPD and 3-MCPD mono- and diesters without the need for a separate sample cleanup procedure or any chemical conversion. In comparison to published direct methods, our new method offers higher selectivity and less usage of organic solvents. The exclusion of sample cleanup did not result in the deterioration of method sensitivity in comparison to the available literature protocols involving double SPE sample preparation procedures. Several MCPD diesters, differing from each other only in the structure (position of hydroxyl group or fatty acid chains), are difficult to differentiate by UHPSFC-MS alone. In these cases MS/MS was beneficial. The analytes were separated in less than 12 min. It should be emphasized that, in our method, organic solvent is utilized only at the stage of oil sample dilution before injection. The fact that we use neat SC-CO_2 as a mobile phase in modern UHPSFC contributes significantly to the novelty of this study. The mobile phase density (at varying temperature and pressure), the mobile phase flow rate, the type of injection solvent, the injection volume, and the effect of ionization method in MS on the chromatographic resolution and detection of MCPD esters were explored. Finally, a green analysis score has been estimated for our new "dilute and shoot" UHPSFC-MS method and then compared to one of the direct protocols available in the literature.

MATERIALS AND METHODS

Materials. Analytical standards of all investigated MCPD esters presented in Table 1 were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Methanol of LC-MS grade, *n*-hexane, and acetonitrile were purchased from Scharlau (Sentmenat, Spain), ethyl acetate was purchased from Fisher Scientific (Loughborough, Leciestershire, U.K.), dichloromethane, acetone, and 2-propanol were purchased from VWR International (Fontenay-sous-Bois, France), ethanol (99.7%) was purchased from Solveco (Rosenberg, Sweden), and ammonium formate, formic acid, and heptane were purchased from Sigma-Aldrich (St. Louis, MO). Refined palm oil was purchased from Sigma-Aldrich. Corn oil, rapeseed oil, and sunflower oil were purchased from a local supermarket.

Equipment. The separation was performed using an Aquity Ultra Performance Convergence Chromatography (UPC^2) system coupled to a XEVO-G2 Q-TOF (quadrupole time of flight) mass spectrometer (Waters, Milford, MA). The outlet pressure (backpressure) was set by the automatic back pressure regulator (ABPR). A pre-ABPR split approach (Aquity UPC^2 splitter) was used to interface the UHPSFC system to the MS, and the approximate split ratio was estimated to 1:100.[22] The columns used were 100 mm × 3 mm i.d., 1.7 μm, Torus 2-picolylamine (2-PIC), Torus diethylamine (DEA), Torus high density diol (Diol), Torus 1-aminoanthracene (1-AA), Aquity UPC^2 CSH fluoro-phenyl (CSH-FP), Aquity UPC^2 BEH (BEH) and 100 mm × 3 mm i.d., 1.8 μm, Aquity UPC^2 HSS C_{18} SB (HSS-C_{18}). All columns were purchased from Waters. Data collection and system control were performed using Waters MassLynx 4.1 equipped with application manager TargetLynx.

Method Development. Standard solution 1 containing all 26 MCPD esters (Table 1) prepared at 1 μg/mL in ethyl acetate was used for method development. Standard solution 2 containing 16 MCPD esters (Table 1) prepared at 20 μg/mL in heptane was used to determine repeatability and intermediate precision of the developed UHPSFC-MS method. Standard solution 3 containing nine MCPD esters (Table 1) prepared at 20 μg/mL in heptane was used to determine the recovery of the developed UHPSFC-MS method.

The chromatographic conditions for column screening were as follows: mobile phase was composed of neat SC-CO_2 (no cosolvent), flow rate was 2 mL/min, column oven temperature was 40 °C, backpressure was 140 bar, and the injection volume was 1 μL of standard solution 1. The effects of column oven temperature (35, 40, 45, and 50 °C), backpressure (110, 140, 170, and 200 bar), and the flow rate (1.0, 1.5, and 2.0 mL/min) on retention factors and kinetic performance were investigated using the 2-PIC column, injecting 1 μL of standard solution 1. Temperatures and pressures were studied at 1 mL/min; the effect of flow rate was studied at 50 °C and 110 bar.

The effects of the type of injection solvent and the injection volume were investigated by injecting 1−10 μL (with an increment of 1 μL) of 1-La-2-Ol-3M, **1**, 1,2-diPa-3M, **4**, 1-Pa-3M, **16**, 1-Li-3-Ln-2M, **17**, and 1-Li-3M, **24**, at a concentration of 1 μg/mL in methanol, ethanol, 2-propanol, ethyl acetate, heptane, dichloromethane, acetonitrile, and acetone respectively, on the 2-PIC column and neat SC-CO_2 at 1 mL/min and 50 °C. The backpressure gradient was as follows: 0−3 min, 110−110 bar; 3−11 min, 110−160 bar; 11−13 min, 160−160 bar; 13−14 min, 160−110 bar; and 14−15 min, 110−110 bar.

UHPSFC-MS Analysis of MCPD Esters. The best UHPSFC-MS conditions for separating MCPD esters in this study were as follows: injection volume, 5 μL; column, 100 mm × 3 mm i.d., 1.7 μm Aquity UPC^2 2-PIC column (Waters); column oven temperature, 50 °C; mobile phase, neat SC-CO_2. The backpressure gradient was as follows: 0−3 min, 110−110 bar; 3−11 min, 110−160 bar; 11−13 min, 160−160 bar; 13−14 min, 160−110 bar; and 14−15 min, 110−110 bar.

The data acquisition was done in positive ion electrospray ionization (ESI$^+$) mode monitoring the sodium adducts by MS for monoesters while the diesters were detected by MS/MS under the following conditions: data acquisition range was m/z 50−800; capillary voltage, 3.5 kV; cone voltage, 35 V; source temperature, 120 °C; desolvation temperature, 300 °C; collision energy of 20 eV for diesters; cone gas flow rate 50 L/h; and desolvation gas flow rate 600 L/h. Ammonium formate in methanol, 0.2% (w/v), was used as a makeup solvent, 0.5 mL/min, to enhance the ionization process for MCPD esters.

Method Validation. Determination of the limits of detection (LODs), limits of quantitation (LOQs), and intra-assay and interassay precision were done according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Q2 (R1).[23] Calibration curves were obtained by plotting the ratio of the response area of base peak extracted ion chromatogram to that of the corresponding internal standard (0.5 μg/mL of each 1,2-diPa-3M-5d and 1-Pa-3M-5d) against the concentration of MCPD esters. LODs and LOQs were calculated based on the manually measured signal-to-noise ratios (S/N) in the chromatograms, i.e., LOD, 3S/N, and LOQ, 10S/N.

Repeatability and intermediate precision, which are reported as relative standard deviation (RSD %) of peak area of standards, were calculated according to the following equation:

$$\text{RSD \%} = (\text{standard deviation}/\text{mean}) \times 100$$

Repeatability was determined by analyzing a mixture of MCPD esters, standard solution 2, at four different concentrations (50, 500, 1000, and 2000 ng/mL) of each standard in triplicate on the same day, whereas the intermediate precision was determined by analyzing analogous samples on three consecutive days.

The recovery tests were performed by spiking 0.5 g of three edible oils, corn oil, rapeseed oil, and sunflower oil, with a known amount of standard, standard solution 3. The preparation of samples was performed as described below. The recovery of each standard was evaluated by comparing the concentration of three replicates of low, medium, and high concentrations (50, 500, and 2000 ng/mL) spiked into the edible oils before and after the analysis of standard MCPD

esters using the developed UHPSFC-MS method using the following equation:

$$\text{recovery \%} = \frac{\text{actual concn of std determined by UHPSFC-MS}}{\text{theor concn of std added to the oil sample}} \times 100$$

Preparation of Samples. Prior to the UHPSFC-MS analysis, 0.5 g of each oil sample was accurately weighed into a screw-capped glass tube. Internal standards (I.S.) solution (10 μL, 0.5 mg/mL of 1-Pa-3M-5d and 1,2-diPa-3M-5d in ethyl acetate) and 10 mL of heptane were added into the sample. The mixture was then vortexed until the oil sample completely dissolved in heptane. The final concentration of each I.S. in oil samples was 0.5 μg/mL, and oil samples were analyzed in triplicate (n = 3) using the developed UHPSFC-MS method.

RESULTS AND DISCUSSION

Tuning of MS Parameters. The signal intensity of ESI and APCI, both in positive mode, were assessed by direct infusion analysis of pure analytical standards dissolved in methanol and compared to SFC separation of the standard mixture. Direct infusion analysis resulted in a significant difference between the investigated ionization techniques. APCI generated higher response, especially in the case of monoesters. Regarding the diesters, both ionization techniques exhibited comparable performance. In spectra obtained after ESI, the signal of the highest intensity corresponded to the molecular ions in the form of sodium (monoesters) or ammonium (diesters) adducts. After stronger ionization of the same compound by APCI, in-source fragmentation took place, which resulted in high intensity of ions corresponding to fragments after fatty acid residue cleavage.

Obtained spectra of diesters after APCI ionization allowed for distinguishing heteroesters of 3-MCPD, cleavage of two different fatty acid residues at the sn-1/sn-2 and sn-1/sn-3 positions, which are of the same molecular mass, without the need of fragmentation by MS/MS. After in-source fragmentation, the fragments after fatty acid moiety disconnection from outer (sn-1 and sn-3) positions generate signals of higher intensity than those after disconnection from the sn-2 position.

In contrast, after combining ESI or APCI with UHPSFC, the signal intensity of UHPSFC-APCI/MS was significantly lower than that of UHPSFC-ESI/MS. The configuration used for the UHPSFC-MS equipment was with the MS connected via a T-junction and a narrow capillary between the column and the ABPR, to allow for minimal zone spreading. As a result, only a small mass fraction of the eluent reaches the MS, which is detrimental for the APCI since this is known to be a mass-dependent ionization source.[24] Therefore, in this case ESI was selected as the ionization technique for further research. Our observations confirm those discussed previously.[25] Characteristic ions of highest intensity generated by ESI are for monoesters, sodium adducts $[M + Na]^+$; and for diesters, ammonium adducts $[M + NH_4]^+$ as precursor ions and ions after residue cleavage of acyl group from sn-1 position $[M - RCO_2]^+$ as product ions, Table 1.The best ionization conditions within the studied range of ESI in positive mode were found to be 3.5 kV and 35 V for capillary cone voltage and sampling cone voltage, respectively. Desolvation gas flow and desolvation temperature turned out to have only minor impact on the ionization performance. For the transitions in Table 1 optimal collision energies were 18−22 eV for diesters, thus 20 eV was selected.

The effect of modifier in the makeup fluid on type of ion produced and its intensity was not studied, thus the MS parameters could be further improved.

Column Selection. MCPD esters are hydrophobic compounds, and they are divided into two groups according to the position of the chlorine atom in their molecules, i.e., 2-MCPD and 3-MCPD (Figure 1). MCPD monoesters are more polar than MCPD diesters due to the presence of a hydroxyl group instead of a fatty acid ester. Some of these compounds have a similar structure; e.g., they differ only in the position of chlorine, such as the MCPD monoester 1-Ol-2M and 1-Ol-3M; or the position of the MCPD diesters 1,3-diPa-2M and 1,2-diPa-3M. This structural similarity and their weak ability for strong polar interactions make the separation of MCPD esters difficult.

Due to the low polarity of MCPD esters neat SC-CO_2 without addition of polar cosolvent is required in order to ensure retention. A benefit worth mentioning of neat SC-CO_2 as eluent is that the selectivity of the chromatographic system mainly is governed by the stationary phase.

Understanding the column interaction chemistry and separation mechanisms in UHPSFC in general is still limited to a relatively small number of studies.[26] In this study, seven stationary phases packed with sub 2 μm particles, 2-PIC, 1-AA, BEH, CSH-FP, HSS-C_{18} SB, DEA, and Diol, were tested for the MCPD ester separation using fixed initial chromatographic conditions.

Retention factors (k-values) of all 26 compounds are presented on stationary phases normally used for reversed phase applications (Figure 2A) and on polar stationary phases (Figure 2B). When comparing the stationary phases, it is observed in Figures 2A and 2B that the k-values differ significantly (except for 1-AA) for MCPD monoesters and MCPD diesters, respectively, depending on the chain length and the degree of unsaturation of the fatty acid groups. For instance, a comparison of hydrophobic columns in Figure 2A shows that MCPD monoesters, especially the ones containing unsaturated fatty acids, are best retained on the CSH-FP column but least retained on the 1-AA column. MCPD diesters on the other hand are least retained on the CSH-FP column. Both the CSH-FP and the 1-AA columns, but not the HSS-C_{18}, where retention factors decrease with the degree of unsaturation, show an increased trend in k-values with the degree of unsaturation due to $\pi-\pi$ interactions. The polar columns all exhibited an increased trend in k-value with increased chain length and degree of unsaturation (Figure 2B).

The position of the chlorine group (2M and 3M isomers labeled within dotted boxes) (Figure 2) does not seem to give an effect on the k-values, especially of the 2M and 3M diester isomers, with some few exceptions. The k-values of 2M and 3M monoesters differ in most cases on the polar columns while a few of the diester isomers show different k-values on the HSS-C_{18} and the 1-AA columns.

On the polar columns, all MCPD diesters were eluted before the MCPD monoesters. This is due to the presence of a hydroxyl group in the MCPD monoesters that provides hydrogen-bonding interaction with polar sites on the stationary phases. The same phenomenon was observed on the CSH-FP, but not on the 1-AA and HSS-C_{18} SB columns. However, BEH, DEA, and Diol columns showed poor selectivity for separating MCPD diesters as compared to the 2-PIC column.

Low numbers of α-values were found for all columns, which further supports the statement of a challenging separation. Comparing polar columns, the BEH provided higher k-values and slightly better selectivity in terms of fewer coeluting peaks, but the zone broadening was quite severe. The Diol and DEA

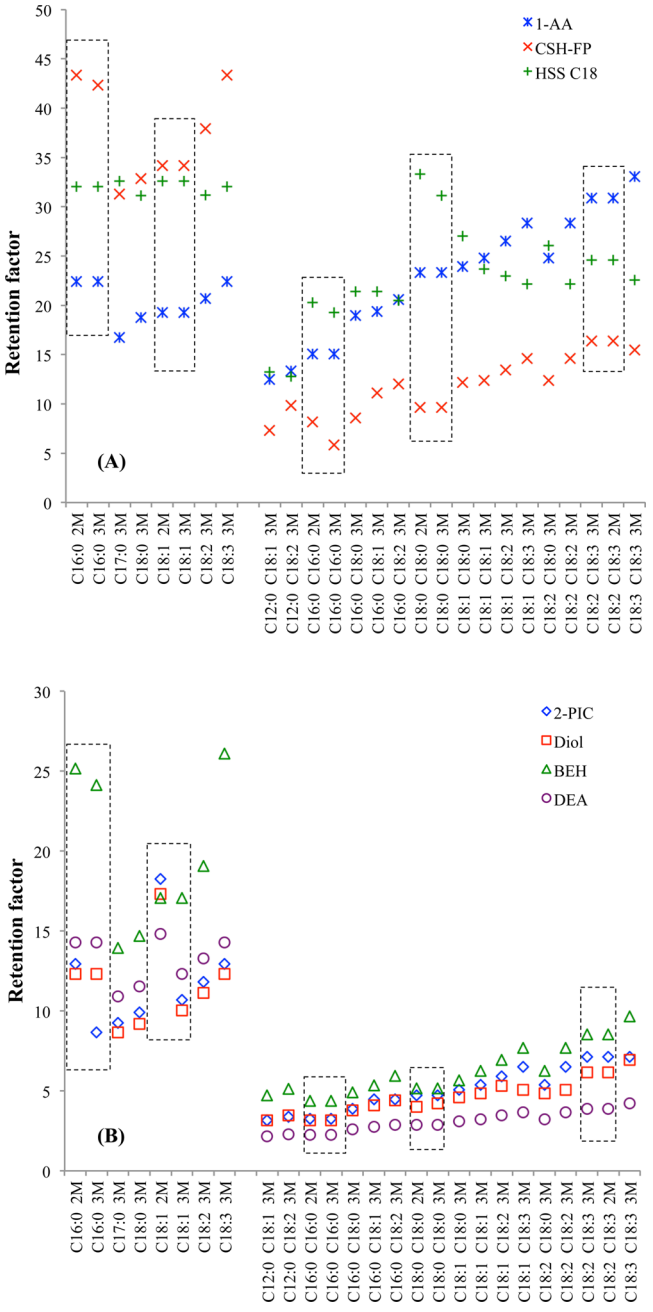

Figure 2. Retention factors (k) of MCPD esters on seven columns of different interaction chemistry, including (A) apolar and (B) polar columns. Monoesters on the left- and diesters on the right-hand side of the figure. 2M and 3M isomers (position of chlorine) are labeled with dotted boxes.

columns delivered lower k-values and α-values of the MCPD diesters as when compared to the 2-PIC column. Figure 3 shows the cumulative number of peak pairs with α-values above a certain value (x-axis). Thus, Figure 3 reveals that the 2-PIC column gives the overall best performance in terms of column selectivity. Furthermore, the 2-PIC column was one of the best performing in terms of zone broadening (Tables S1 and S2).

In summary, combining the performance with respect to retention, selectivity, and kinetic performance, the 2-PIC was selected for further method development for separating MCPD esters.

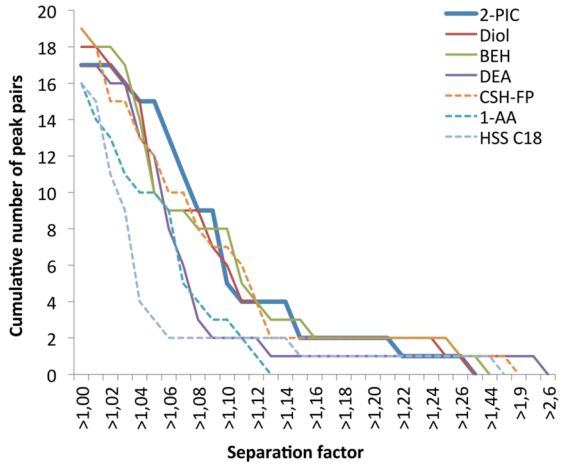

Figure 3. Cumulative number of peak pairs with separation factors above a certain value. Apolar columns are displayed with dashed lines and polar columns with solid lines; a bold line highlights the performance of the 2-PIC column that was chosen for further method development.

Effect of Flow Rate, Backpressure, and Column Oven Temperature. It is a known fact that the inlet pressure increases with flow rate, leading to an increase in the mobile phase density and viscosity of neat SC-CO_2 due to its compressibility. This change in density directly affects the elution strength and the k-values.[25,27,28]

Figure 4A illustrates the influence of flow rate (1.0, 1.5, and 2.0 mL/min) on the k-values of MCPD esters on the 2-PIC column at 50 °C and 110 bar. The k-values were drastically decreased with increasing flow rate due to higher density and thus the elution strength. The effects of density on retention factors and viscosity on diffusivity affect both longitudinal diffusion and mass transfer resistance, which explains the negative effect on the peak shapes (Table S3 and Figure S4).[28]

Density and viscosity can also be increased by means of increased backpressure or reduced temperature.[23,26,27] The summarized effects on the 2-PIC column at 1 mL/min of obtained k-values at the highest and the lowest backpressure (110−200 bar) and column oven temperature (35−50 °C) respectively are presented in Figure 4B. It is obvious that the retention factors increase with increasing column oven temperature from 35 to 50 °C due to decreased density and that the temperature effect is more significant at the lowest backpressure, 110 bar, which can be explained by the higher compressibility of neat SC-CO_2 at low backpressures. Moreover, at lowest backpressure, increasing the column oven temperature from 35 to 50 °C led to a loss in efficiency most likely due to the radial temperature gradient associated with expansion and cooling the mobile phase.[29,30] As expected, k-values decrease with increasing backpressure at different column oven temperatures due to an increased density of the mobile phase. Moreover, a minor change in retention factors was observed when the column oven temperature was increased from 35 to 50 °C at the highest backpressure since that also makes the mobile phase less compressible (Figure S4 and Table S5).

Furthermore, at the highest backpressure, efficiency was only slightly changed with increasing column oven temperature due to the relatively lower compressibility of the mobile phase. In summary, the best results in terms of selectivity were observed at 110 bar and at oven temperatures of 50 °C.

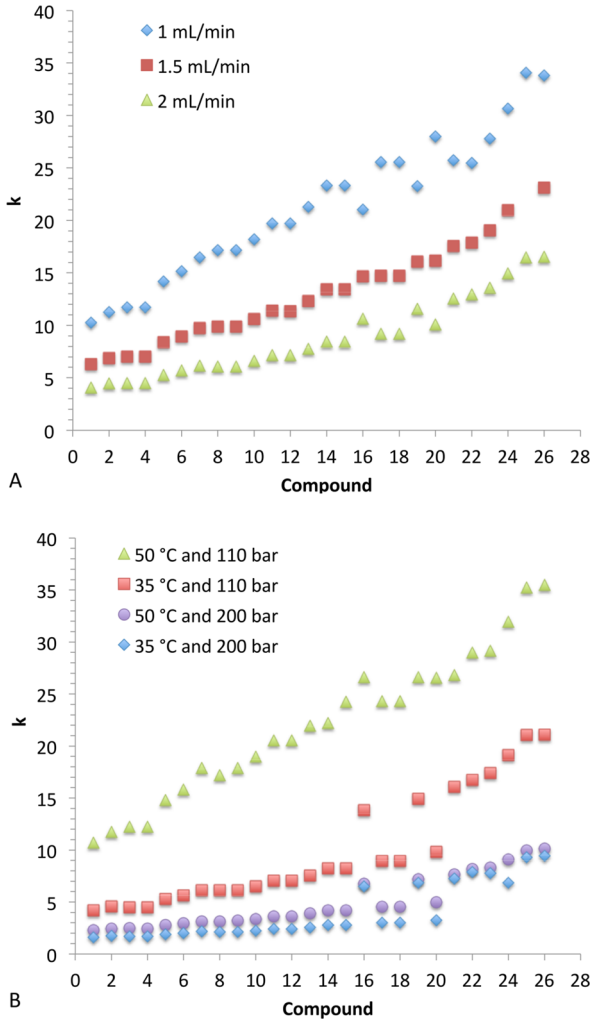

Figure 4. Effect of (A) flow rate and (B) column oven temperature and backpressure on the retention factors. UHPSFC conditions: mobile phase neat SC-CO_2; column, 2-PIC; column oven temperature, 50 °C; backpressure, 110 bar (A); flow rate, 1 mL/min (B). Analyte identification is as in Table 1.

Backpressure Isobaric vs Gradient Program. In order to control the zone spreading without losing selectivity, a backpressure gradient program was applied. Figure 5 shows the effect of a backpressure gradient program (0−3 min, 110 bar; 3−15 min, 200 bar; 15−20 min, 200 bar; 20−21 min, 200−110 bar; and 21−22 min, 110 bar) as compared to a backpressure isobaric program (110 bar) at 50 °C and 1 mL/min on the 2-PIC column. With the backpressure gradient program, narrower peaks and shorter separation time were observed due to a gradual increase in viscosity and elution strength of the mobile phase.

The peak widths and resolutions of all MCPD esters were improved with the backpressure gradient program. A final tuning of the backpressure gradient program resulted in the chromatogram in Figure 6, which illustrates the separation of 26 MCPD esters (Figures S6 and S7, Table S8).

Elution Order of MCPD Esters. As mentioned, the separation mechanism of MCPD esters on the selected 2-PIC column is based on the presence or absence of a hydroxyl group, i.e., MCPD monoester or MCPD diester, and the length of the fatty acid chains as well as the number of double bonds. The retention time increased linearly with the number of double bonds and the carbon number due to $\pi-\pi$ interactions occurring between the pyridyl functional groups of the stationary phase and the double bonds of the analytes (Figure S9A,B).

2-MCPD and 3-MCPD monoesters that have the same chemical structure, i.e., 1-Pa-2M/1-Pa-3M, **16**/**21**, and 1-Ol-2M/1-Ol-3M, **23**/**26**, were also separated under the selected UHPSFC conditions. The separation was based on the position of hydroxyl group in the chemical structure of MCPD monoesters. 1-Pa-2M and 1-Ol-2M with hydroxyl group in position 1, i.e., a primary alcohol, were retained more than 1-Pa-3M and 1-Ol-3M, which are secondary alcohols. Primary alcohols are more acidic than secondary alcohols and, thereby, form stronger hydrogen-bonding interaction with the polar sites on the 2-PIC stationary phase.

Isomers of some MCPD diesters, i.e., 1,3-diPa-2M/1,2-diPa-3M, **3**/**4**, 1,3-diSt-2M/1,2-diSt-3M, **8**/**9**, and 1-Li-3Ln-2M/1-Li-2-Ln-3M, **17**/**18**, were not separated. Also 1,3-diOl-3M/1-Li-2 St-3M, **11**/**12**, and 1,2-diLi-3M/1-Ol-2Ln-3M, **14**/**15**, were coeluted. The two compounds in each pair have the same chemical formulas, the only difference between them being the double bond position in the acyl group. Although 1-Ln-3M, **25**, and 1-Ol-2M, **26**, have a hydroxyl group in a different position

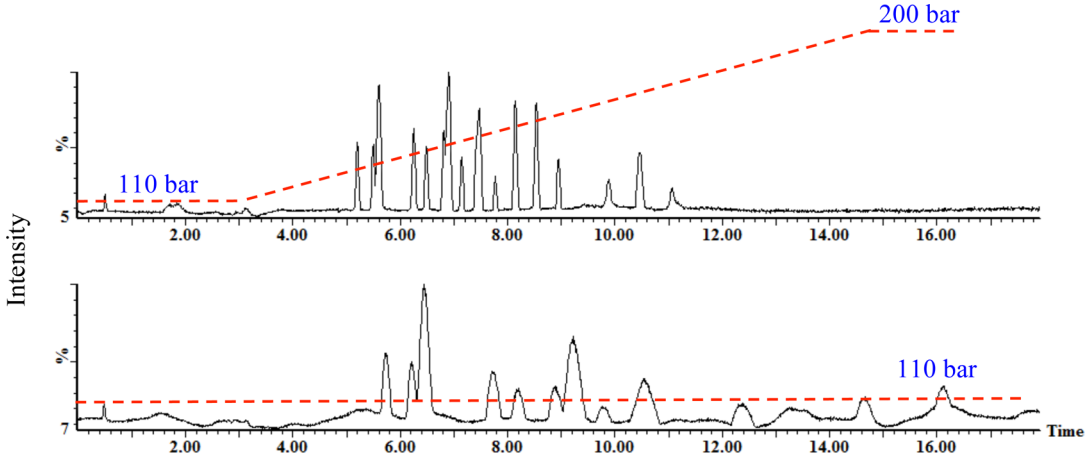

Figure 5. Effect of backpressure gradient on the separation of 2-MCPD and 3-MCPD compounds on the 2-PIC column using UHPSFC-MS. Flow rate, 1 mL/min, and column oven temperature, 50 °C.

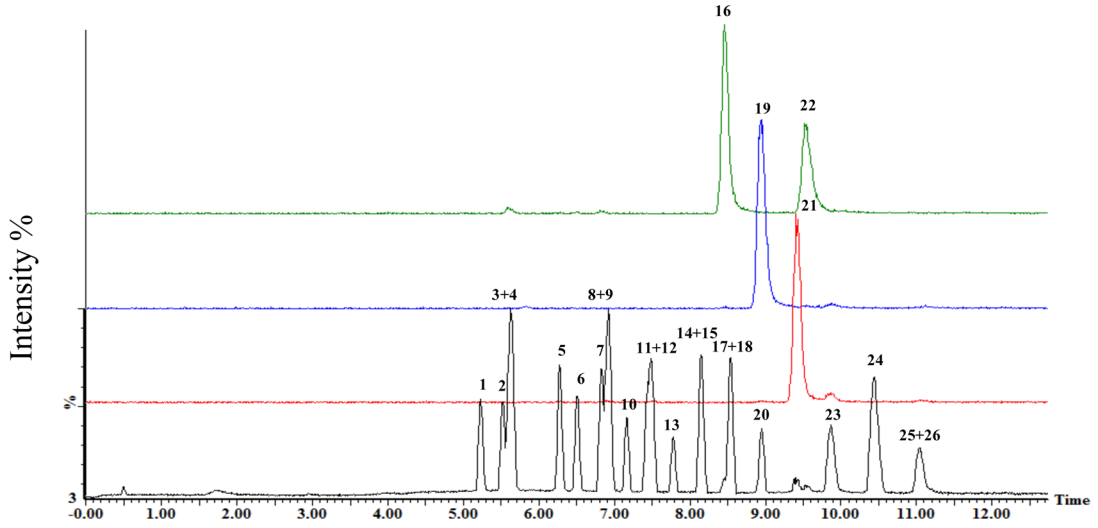

Figure 6. UHPSFC-MS chromatogram for separating MCPD esters on the 2-PIC column. The improved UHPSFC separation conditions are as follows: flow rate, 1 mL/min; column oven temperature, 50 °C. Backpressure gradient program: 0−3 min, 110−110 bar; 3−11 min, 110−160 bar; 11−13 min, 160−160 bar; 13−14 min, 160−110 bar; and 14−15 min, 110−110 bar. Analyte identification is as in Table 1.

as well as the same acyl chain length in their molecules, these two compounds were not resolved due to 1-Ln-3M having two double bonds more than 1-Ol-2M, resulting in increasing the retention time of 1-Ln-3M.

The last three pairs, i.e., **11**/**12**, **14**/**15**, and **25**/**26**, could be distinguished by utilizing UHPSFC-MS/MS instead of UHPSFC-MS, as they produce different product ions.

Effect of Injection Solvent and Injection Volume. The effects of injection solvent and injection volume on the chromatographic performance of MCPD esters using a 2-PIC column were also investigated. A standard mixture (1 μL) containing five MCPD esters (1-La-2-Ol-3M, 1,2-diPa-3M, 1-Pa-3M, 1-Li-3-Ln-2M, and 1-Li-3M) diluted in nine different solvents were injected onto the 2-PIC column. No change in the retention time, elution order, or peak shape was observed when 1 μL was injected using all nine different solvents. Increasing the injection volume from 1 to 10 μL has substantial impact on the retention time and peak shape especially when polar protic solvents such as methanol or ethanol were used as injection solvents. This is due to the adsorption of molecules of alcoholic solvents to hydroxyl and silanol groups on the 2-PIC column.[31,32] The results also showed that larger injection volumes for ethyl acetate, acetonitrile, acetone, and dichloromethane did not affect the retention times of the studied compounds. However, a distortion of peaks was noticed and more so at larger injection volumes (Figure S10A,B).

The best results were obtained using nonpolar solvents such as heptane and *n*-hexane, which provided better peak shapes even at a very high injection volume (10 μL). However, peak broadening increased with the injection volume when using heptane or *n*-hexane as injection solvents. In further studies, 5 μL was used for quantitative analysis of MCPD esters with heptane as injection solvent (Figures S11 and S12).

Validation of the UHPSFC-MS Method. To show its potential to be used for quantitative analysis of MCPD esters in some oil samples, the investigated UHPSFC-MS method was partially validated. Calibration curves were found to be linear over the range 20−2000 ng/mL for MCPD esters (Table 2). The coefficient of determination (R^2) of 0.995 and above was observed for all of the studied compounds. LOD was determined to be 0.5−7.5 ng/mL, whereas the LOQ was found to be 1.3−17.5 ng/mL (Table 2).

Table 2. Validation of the UHPSFC-MS Method for the Analysis of MCPD Ester Standard Solutions

compd	dynamic range (ng/mL)	slope	R^2	LOD (ng/mL)	LOQ (ng/mL)
1	20−2000	11.56	0.9992	2.5	7.5
2	20−2000	7.23	0.9996	5.0	15.0
3	20−2000	7.12	0.9985	0.5	1.3
4	20−2000	16.31	0.9992	0.5	1.3
5	20−2000	20.38	0.9995	1.0	2.5
6	20−2000	14.17	0.9989	5.0	15.0
7	20−2000	9.56	0.9991	5.0	15.0
8	20−2000	15.35	0.9991	1.0	2.5
9	20−2000	25.64	0.9996	1.0	2.5
10	20−2000	43.93	0.9993	2.5	7.5
11	20−2000	26.00	0.9992	2.5	7.5
12	20−2000	18.45	0.9994	1.3	5.0
13	20−2000	6.92	0.9995	2.5	7.5
14	20−2000	11.76	0.9993	2.5	7.5
15	20−2000	3.66	0.9995	2.5	7.5
16	20−2000	1.55	0.9995	1.0	2.5
17	20−2000	9.56	0.9958	5.0	15.0
18	20−2000	4.07	0.9993	2.5	7.5
19	20−2000	1.65	0.9990	7.5	17.5
20	20−2000	3.93	0.9999	7.5	17.5
21	20−2000	1.92	0.9995	5.0	15.0
22	20−2000	1.21	0.9991	1.0	2.5
23	20−2000	2.46	0.9998	7.5	17.5
24	20−2000	4.40	0.9994	7.5	17.5
25	20−2000	2.64	0.9994	7.5	17.5
26	20−2000	2.50	0.9985	7.5	17.5

Repeatability and intermediate precision were determined for 16 MCPD esters (standard solution 2), in order to ensure the precision of the UHPSFC-MS method for the quantitation of MCPD esters. The RSD % values of peak area for both repeatability and intermediate precision were found to be less than 4.8% and 10.6%, respectively, which indicates that the

repeatability of the developed UHPSFC-MS method is acceptable (Table S13).

Recoveries were obtained by spiking known amounts of 9 MCPD esters (standard solution 3), 50, 500, and 2000 ng/mL for each compound, in three different edible oils, corn oil, rapeseed oil, and sunflower oil. The recovery range of all tested compounds was 68.8−112.8%, which indicates the potential of the investigated UHPSFC-MS method for the determination of MCPD esters in edible oils (Table S14).

Determination of MCPD Esters in Edible Oils and Refined Palm Oil. The partially validated UHPSFC-MS method was further applied for the determination of MCPD esters in some edible oils as well as in refined palm oil. Seven MCPD esters were quantitated in refined palm oil at concentrations ranging from 0.29 to 2.74 mg/kg, and only two MCPD esters were quantitated in rapeseed oil at concentrations ranging between 0.30 and 5.35 mg/kg, whereas no MCPD esters were found in either corn oil nor in sunflower oil (Table S15, Figures S16 and S17).

Obtained results indicate the strong relation between MCPD ester species present in the oil and its fatty acid composition. According to Codex Alimentarius, palm oil consists mainly of palmitic and oleic acid and, as a result, MCPD esters present in this kind of oil in highest concentrations are 1,2-dipalmitoyl-3-MCPD, 1-lauroyl-2-oleoyl-3-MCPD, and 1-palmitoyl-2-oleoyl-3-MCPD. The same situation may be observed in the case of rapeseed oil, where the high concentrations of 1-oleoyl-2-linoleoyl-3-MCPD and 1,2-dioleoyl-3-MCPD are related to fatty acid composition of rapeseed oil consisting mainly of oleic and linoleic fatty acids. However, these results are only semiquantitative since no full validation using matching deuteriated internal standards for each analyte was performed, nor were matrix effects studied. Quantitative analysis can be further improved by utilizing a triple-quadrupole MS instead of a Q-TOF/MS.

Assessment of Method Greenness. The evaluation of greenness of methodology described in this paper and the comparative one (SPE-LC-MS/MS based protocol) was carried out with the use of the so-called Analytical Eco-Scale.[33] This comprehensive semiquantitative tool is based on assigning penalty points to parameters of the analytical protocol, which are not in accordance with ideal green analysis principles. Penalty points for reagents are estimated on the basis of the amount of each reagent utilized and its hazard (number and character of pictograms). The final assessment of the greenness of the methodology is based on the following calculation:

$$\text{Analytical Eco-Scale score:} \quad 100 - \text{total penalty points}$$

which means that the higher the score, the greener the procedure.

For the greenness evaluation, our methodology has been compared with one of the direct methods available in the literature, which allows for the information regarding 3-MCPD and 2-MCPD diester and 3-MCPD monoester content to be obtained. The sample preparation processes for diesters and monoesters are separated, and both involve SPE. Hence they consume relatively large amounts of organic solvent, not to mention further liquid chromatographic analysis. In the evaluation of penalty points, only the sample preparation processes before chromatographic analysis and the actual chromatographic separation have been taken into account, since standard solution preparation and spiking are analogous in both cases and may be omitted.

It is evident that the methodology described in this study with the use of SC-CO_2 known as a green solvent is much more environmentally friendly than the comparative one. The value of Analytical Eco-Scale score equal to 24 (<50) represents inadequate green analysis, whereas the value of 69 (>50) represents green analysis, very close to excellent green analysis (>75) (Table 3).

Table 3. Eco-Scale Penalty Points (PPs) for MCPD Ester Determination by One of the Methodologies Available in the Literature and the Developed "Dilute and Shoot" UHPSFC-MS Methodology

	penalty points	
assessed parameters (e.g., reagents, instruments)	SPE-LC-MS/MS[19,20]	dilute and shoot UHPSFC-MS
diethyl ether	4	0
methyl *t*-butyl ether	4	0
methanol	12	6
silica SPE cartridge	1	0
hexane	16	0
ethyl acetate	8	0
dichloromethane	1	0
acetonitrile	8	0
isopropyl alcohol	8	0
formic acid	6	0
heptane	0	16
CO_2	0	2
LC-MS/SFC-MS (energy)	2	2
occupational hazard	0	0
waste	5	5
total penalty points	76	31
Analytical Eco-Scale total score	24	69

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Notes

The authors declare no competing financial interest.

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