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A rapid and sensitive method for characterization and quantification of polyglycerol esters by Supercritical Fluid Chromatography coupled to High-Resolution Mass Spectrometry (SFC-HRMS)

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Abstract

Polyglycerol esters of fatty acids (PGEs), a very complex mixture of various isomers, are widely used as green surfactants in different industrial fields such as in cosmetic, pharmaceutical and food industries. However, no study related to the purification and the absolute quantification of these compounds has been described yet. In this study, we developed a rapid and efficient method for characterization and quantification of PGEs using Supercritical Fluid CO₂ coupled to High-Resolution Mass Spectrometry (SFC-HRMS). The SFC conditions were first considered including the stationary phase, the nature of mobile phase, the column temperature, the back-pressure regulator. The MS parameters (drying-gas temperature, capillary voltage, nozzle voltage, fragmentor voltage) were then investigated to get the best sensitivity for the PGE analysis. The MS/MS-based structural characterization of targeted PGE, triglycerol mono-oleate (PG3+1C18:1), was established and is helpful to study complex mixtures of PGEs with numerous isobaric PGEs. PG3+1C18:1 was then purified at lab scale and used as standard for quantification. This enabled to develop a rapid quantification method for PG3+1C18:1 within 12 min with good linearity ($R^2 = 0.9997$) as well as sensitivity (picogram level). The validated method was then successfully applied to quantify PG3+1C18:1 in commercial products in order to evaluate their composition.

Keywords

polyglycerol esters; surfactant; supercritical fluid chromatography; high resolution tandem mass spectrometry; quantification

Introduction

Polyglycerol esters (PGEs, Fig. 1) are a class of non-ionic emulsifiers which are extensively used in different industries including cosmetics, pharmaceuticals and food industries. Their interesting features such as broad range of polarity or hydrophilic-lipophilic balance number (HLB) values make them a highly valuable emulsifier [1]. Further promising physical-chemical properties are also found in this class of compounds as odorless, biodegradable, good thermal stability and moderate antimicrobial activity. These properties lead to widespread applications of PGEs in the industry [1-3].

PGEs are obtained by the synthesis from glycerol and fatty acid through 2 steps. The first one is the polymerization of glycerol, which generally come from the biodiesel process as a by-product [4] or fatty acid production, resulting in a complex mixture of polyglycerol oligomers [1, 5]. These oligomers could be linear, branched or cyclic. This mixture is then esterified in the second step with one or several fatty acids giving PGEs. When polyglycerol oligomers are available, PGEs could be directly formed by mixing them with fatty acids. The final product is very complex with various compounds different in the degree of polymerization, the branching type of oligomers, the number and the length of fatty acids [6]. Therefore, a method for the PGE qualification and/or quantification would be necessary to optimize the synthesis process. Furthermore, as the composition of a mixture designs its properties, it is worth to characterize as well as quantify the PGE mixture.

However, the similar physical-chemical properties of PGEs as well as their non-UV-absorbing behavior make difficult to purify and analyze these compounds. Furthermore, some PGEs are exhibiting chiral carbons leading to numerous stereoisomers in mixture [7]. Thus, no absolute quantitative method for PGEs has been described yet in the literature. Some studies about PGE analysis methods have been published by using thin-layer chromatography (TLC) [8], gas chromatography (GC) [9], high-performance liquid chromatography HPLC [6, 7, 10], GC combined to LC [11], supercritical fluid chromatography (SFC) [12] with mass spectrometry (MS) [6, 7, 9-11], evaporative light scattering detection (ELSD) [7], or flame ionization detector (FID) [12]. Recently, a (U)-HPLC-MS method for relative PGE quantification has been developed but without taking into account the difference in ionization yields of PGEs depending on their structures [6].

In recent years, SFC in analytical chemistry has attracted much attention thanks to its numerous advantages such as short run time analysis, high efficiency and low organic solvent consumption [13-15]. The addition of a polar solvent in the mobile phase with/without additive at low concentration allows analyzing compounds with wide polarity range. Furthermore, SFC has been shown to be performant in chiral separation [16, 17]. Thus, it would be interesting to use SFC to separate PGE mixtures.

Herein, we present a method using SFC coupled to high-resolution mass spectrometry (SFC-HRMS) to analyze PGEs and to establish the MS/MS-based structural characterization of this class of compounds. After a column screening performed on nine different stationary phases, the SFC conditions (nature of co-solvent, column temperature, back-pressure regulator (BPR) and MS parameters (drying gas temperature, capillary voltage, nebulizer voltage, fragmentor voltage) were optimized. A PGE purification method using HPLC was also introduced allowing to purify triglycerol mono-oleate PG3+1C18:1. This enabled for the first time to develop an absolute quantitative method for this particular ester by SFC-HRMS.

Materials and methods

General experimental procedures

1D and 2D NMR spectra were recorded in MeOH-D₄ on Bruker Avance 500 MHz spectrometers. Chemical shifts were referenced using residual solvent (δ_{H} 3.31 ppm and δ_{C} 49.15 ppm).

Synthesis

The following procedure was applied to synthesize polyglyceryl-3 esters PG3MO: Starting materials including 3.15 g of oleic acid (99%, Larodan, USA), 5.12 g of commercial polyglycerol-3 (Spiga spa, Italy) and 0.08 g of sodium hydroxide (30%, Alfa Aesar, Germany) were charged into a 25 mL pyrex tube equipped with a PTFE pipe designed for water elimination. The resulting mixture was stirred at 220 °C under a nitrogen flow (1 L min⁻¹). After 7 h of stirring, the mixture was decanted at 80 °C for 3 h affording PG3MO.

Samples PG3DO1 and PG3DO2 are two different batches of commercial polyglyceryl-3 dioleate supplied by Gattefossé (Plurol® oleique CC 497).

Chemicals

Carbon dioxide (CO₂, purity \geq 99.7%) was purchased from Air Liquide (Grigny, France). Methanol (MeOH, LC-MS grade) and acetonitrile (ACN, ultra-gradient HPLC grade) were purchased from J.T. Baker (Center Valley, PA, USA). ACN (HPLC grade), dichloromethane (CH₂Cl₂, stabilized with amylene) and ethanol absolute anhydrous (EtOH, HPLC-Isocratic grade) were purchased from Carlo Erba (Val de Reuil, France). Ammonium acetate (AcONH₄, LC-MS grade) was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Water was purified on a Millipore direct-Q apparatus (MilliporeMerck, Alsace, France). Three products were provided by the Gattefossé company: PG3MO, PG3DO1 and PG3DO2. Product PG3DO2 was used to study the optimal SFC-HRMS and SFC-HRMS/MS conditions at a concentration of 1 mg mL⁻¹ in MeOH/CH₂Cl₂ 1:1 v/v. Product PG3MO was used for the purification of PG3+1C18:1. Solutions of PG3+1C18:1 used in method validation section were prepared from a stock solution (0.1 mg mL⁻¹ in MeOH) and stored at -20 °C.

SFC

Analyses were performed on a 1260 Infinity Analytical SFC system (Agilent Technologies, Waldbronn, Germany) consisting of an Aurora module and an “LC-like” system. This system was equipped with a thermostated autosampler (kept at 5 °C) with a 5 μ L injection (full loop mode with overfill factor of 3) and two thermostated column compartments that can contain up to eight columns.

Nine columns were selected for the screening of the stationary phase. Three columns were purchased from Waters (Guyancourt, France): Torus Acquity UPC² 1-aminoanthracene (1-AA) (50 mm \times 2.1 mm, 1.7 μ m), TorusAcquity UPC² diethylamine-bonded silica (DEA) (50 mm \times 2.1 mm, 1.7 μ m) and Acquity UPC² BEH 2-ethylpyridine (2-EP) (100 mm \times 2.1 mm, 1.7 μ m). The Hypercarb™ (100 mm \times 2.1 mm, 5 μ m) was purchased from Thermo Fisher Scientific (Courtaboeuf, France). Four columns were purchased from Agilent Technologies (Massy, France): ZORBAX Eclipse Plus C18 - Rapid Resolution HT (C18) (150 mm \times 2.1 mm, 1.8 μ m), Pursuit 3 pentafluorophenyl (PFP) (150 mm \times 2 mm, 3 μ m), Pursuit XRs Diphenyl (DP) (250 \times 2 mm, 3 μ m) and Zorbax RRHD SB-CN (CN) (100 mm \times 2.1 mm, 1.8 μ m). The Amine Luna NH₂ (NH₂) (150 mm \times 2 mm, 3 μ m) was purchased from Phenomenex (Le Pecq, France). The DEA column was selected for the quantification step.

The mobile phase was composed of CO₂ (A) and co-solvent (B). For the screening of stationary phase, the mixture of MeOH/EtOH 1:1 v/v with 20 mM of AcONH₄ was employed as co-solvent. The flow rate was fixed at 1 mL min⁻¹. Run time of 16 min was programed as follows: 0-10 min (1-30% B), 10-12 min (30%

B), 12-13 min (30-1% B), 13-16 min (1% B). The column temperature was 50 °C. The BPR was set at 130 bar and 60 °C. The make-up solvent consisted of MeOH/EtOH 1:1 v/v with 20 mM of AcONH₄ and its flow rate was set at 0.2 mL min⁻¹.

To get the optimum SFC separation, the gradient, the nature of co-solvent together with different values of column oven temperature (40, 50 and 60 °C) and BPR pressure (130, 140 and 150 bar) were examined.

SFC-HRMS analysis

The SFC system was coupled to a quadrupole time-of-flight (QTOF) mass spectrometer 6540 Agilent (Agilent Technologies, Massy, France) equipped with an electrospray ionization ESI dual JetStream. After the UV detector, a make-up solvent was added into the mobile phase to improve the solubility of analytes. This solvent was pumped by a 1260 Infinity isocratic pump (Agilent Technologies, Massy, France) with a flow rate of 0.2 mL min⁻¹. Before the ion source entrance, a Caloratherm (Sandra Selerity Technologies, Kortrijk, Belgium) fixed at 60 °C was used to avoid the freezing of the transfer line.

MS parameters were set as follows: drying gas at 10 L min⁻¹, nebulizer at 50 psi, sheath gas temperature at 350 °C, sheath gas flow at 11 L min⁻¹, nozzle voltage at 1000 V, skimmer at 45 V, Oct 1 RF Vpp at 750 V. Other parameters including fragmentor voltage, drying-gas temperature, capillary voltage (Vcap) and nozzle voltage were further investigated to get the optimal values. Experiments were carried out in positive ionization mode. Full-scan mode from *m/z* 50 to *m/z* 1700 at 2 GHz was selected resulting in a mass resolution of 26 000 at *m/z* 922. Calibration solution containing two internal reference masses (purine, C₅H₄N₄, *m/z* 121.0509, and HP-921 [hexakis-(1*H*,1*H*,3*H*-tetrafluoro pentoxy)phosphazene], C₁₈H₁₈O₆N₃P₃F₂₄, *m/z* 922.0098) purchased from Agilent (Agilent Technologies, Massy, France) was injected routinely in order to get mass accuracy below 5 ppm. MassHunter Workstation software (B.08.00) was used to analyze the recorded data. For each ion of interest, the extracted ion chromatogram (EIC) was traced by selecting the theoretical *m/z* value with a selection window of 20 ppm.

SFC-HRMS quantification

Optimal SFC and MS parameters were set for the PG3+1C18:1 quantification method. Run time was reduced to 12 min including column stability time. The DEA column temperature was 50 °C. The injection volume was fixed at 5 µL. The mobile phase was composed of CO₂ (A) and MeOH (B) with a flow rate of 1.2 mL min⁻¹. The gradient was: 0-7 min (6-15% B), 7-9 min (15% B), 9-9.1 min (15-6% B), 9.1-12 min (6% B). The BPR was set at 140 bar and 60 °C. The make-up solvent was MeOH with a flow rate of 0.2 mL min⁻¹. MS parameters were set as follows: drying gas at 10 L min⁻¹, nebulizer at 50 psi, sheath gas temperature at 350 °C, sheath gas flow at 11 L min⁻¹, Vcap 3500 V, nozzle voltage at 1000 V, fragmentor 200 V, skimmer at 45 V, Oct 1 RF Vpp at 750 V.

SFC-HRMS/MS analysis

SFC conditions and MS scans were set as indicated above. The fragmentor voltage and the drying-gas temperature were studied to optimize the MS/MS analyses. The mass range of precursor and fragment ion was set between *m/z* 100 and *m/z* 1200. Selected parent ions (*m/z* 505.3735, 579.4103, 653.4471) were fragmented at 10 eV of collision energy with a mass isolation window of 1.3 amu.

SFC purification of PG3+1C18:1

Semi-preparative SFC was carried out on an Investigator SFC System (Waters) equipped with a diode array detector (DAD) and an ELSD. The diphenyl column (250 mm x 4.6 mm, 5 µm) purchased from Agilent Technologies (Massy, France) was employed. The DAD was set at 210 and 254 nm. Product PG3MO (100 mg mL⁻¹ in MeOH/CH₂Cl₂ 1:1 v/v) was subjected to SFC with an injection volume of 60 µL. The column temperature was set at 40 °C. The BPR was at 130 bar. The mobile phase consisted of CO₂ (A) and MeOH/EtOH 1:1 v/v (B) with a flow rate of 4 mL min⁻¹. The gradient was: 0-15 min (8% B), 15-18 min

(8-30% B), 18-23 min (30-30% B), 23-25 min (30-8% B), 25-29 min (8% B). The make-up solvent was MeOH/EtOH 1:1 v/v with a flow rate of 3 mL min⁻¹. Six fractions (SFC-F1 to 6) were collected and dried under nitrogen at ambient temperature.

HPLC purification of PG3+1C18:1

HPLC purification was performed on a Gilson system equipped with a 322 pumping device, a GX-271 fraction collector, a 171 DAD and a prepELSIII electrospray nebulizer detector. The DAD was set at 210, 220, 254, 272 and 310 nm. A Luna C₁₈ column (250 mm x 21.1 mm, 5µm, Phenomenex) was employed. The mobile phase consisted of H₂O (A) and ACN (B) with a flow rate of 21 mL min⁻¹. The gradient was: 0-15 min (85% B), 15-15.5 min (85-100% B), 15.5-23 min (100% B), 23-24 min (100-85% B), 24-30 min (85% B). Product PG3MO (50 mg mL⁻¹ in ACN/H₂O 1:1 v/v) was subjected to HPLC purification with an injection volume of 400 µL affording pure PG3+1C18:1 (5.8 mg).

Method validation

The selectivity of the method was studied by adding the mixture of polyglycerol and oleic acid used for the synthesis to the standard solution.

Linear least squares regression was calculated based on the peak area of the analyte at six different levels (0.5-20.0 ng mL⁻¹) in triplicate experiments. The slope, the y-intercept of the calibration curve as well as the coefficient of determination (R^2) were calculated.

The limits of detection (LOD) and quantification (LOQ) were defined as the concentration with a signal-to-noise ratio (S/N) of 3 and 10, respectively [18].

To study intra- and inter-day precision, six calibration levels were injected in triplicate in a single run and in three different days. The relative standard deviation (RSD), the back-calculated concentration as well as the accuracy were determined.

Application of the validated method

The quantification of PG3+1C18:1 in three products was performed using the validated method, based on the established external calibration curve.

Results and discussion

Optimization of SFC separation

First, SFC parameters including the chemistry of the stationary phases, the mobile phase composition and the column temperature were studied in order to separate and detect a large variety of PGEs. Table S1 in supplementary data displays 50 PGEs potentially expected in the commercial mixture. These molecules could be mono-, di-, tri-, tetra-, penta-esters of oleic acid and polyglycerols with a polymerization degree ranging from 2 to 6, and their analogs with one loss of water molecule. The esters of glycerol and polyglycerol having degrees of polymerization higher than 6 were not detected. The mass range acquisition was set at m/z 100-1700 so the detection was limited up to penta-esters of polyglyceryl-3 esters. Targeted compound in this study is PG3+1C18:1, which is major in the commercial product.

To study the influence of the stationary phases on the separation, the PG3DO2 solution was analyzed on nine different columns using a generic gradient (Fig. 2). When using the Hypercarb column, no PGE was detected. Surprisingly, a previous study had showed that the Hypercarb column allowed to efficiently separate PGEs diastereoisomer by HPLC [7]. This difference could be explained by the difference in the mobile phase condition, liquid *versus* supercritical, which could influence the affinity between the analyte and the stationary phase. The PGEs were mainly co-eluted on the PFP, CN and C18 columns. They were better separated on the other columns, *i.e.* 2-EP, 1-AA, DP, NH₂ and DEA.

Among 50 hypothetical PGEs in Table S1, 40 PGEs were detected (PGEs annotated in black bold, Table S1, supplementary data). The DEA enabled the best separation of all PGEs within 8 min so it was selected for the following optimization steps. The higher the degree of polymerization of polyglycerol, the more molecules were retained on column (Fig. 3-a). On the contrary, molecules bearing more esterified oleic acid were eluted earlier (Fig. 3-b). The PGEs having one water loss were eluted before their analogs without water loss (Fig. 3-c) suggesting that the loss of one water molecule was a product of an intramolecular condensation induced during the synthesis procedure. This could be an intrinsic cyclic or a dehydration phenomenon.

Furthermore, the DP column enabled to separate PGEs in class while the DEA separated them in class and in species (Fig. S1, supplementary data). It would thus be interesting to use these two columns to isolate PGEs in a successive procedure: the first step to separate PGEs in class by using the DP column, the fractions of interest would be further purified on the DEA column to access pure species.

The mobile phase composition as well as its flow rate were then studied. As mentioned above, the molecules of interest in this study were isomers of PG3+1C18:1. Thus all improvement steps to reach the best separation and detection were focused on these molecules. When using the generic SFC method, different isomers of PG3+1C18:1 were co-eluted (Fig. S2, supplementary data). Furthermore, we observed that all PGEs in the PG3DO2 were eluted between 4 and 15% of co-solvent. The gradient was then modified by decreasing the gradient slope through a gradient of 6 - 15% of co-solvent during 15 min. This led to a better separation of the 3 detected PG3+1C18:1 isomers. The flow rate of the mobile phase was slightly increased from 1 to 1.2 mL min⁻¹ to decrease retention times and improve peak shapes. The nature of co-solvent was also examined. As the presence of AcONH₄ in co-solvent was not favorable for the purification procedure, a study on the nature of co-solvent was proceeded by using a mixture of MeOH/EtOH 1:1 v/v, pure MeOH or pure EtOH. As expected, MeOH enabled the PGEs to elute earlier than the mixture of MeOH/EtOH 1:1 v/v and pure EtOH. We observed that the chromatographic peaks and the sensitivity were improved when using MeOH. Data (Fig. S2, supplementary data) also showed that AcONH₄ did not demonstrate significant improvement of separation and detection of PGEs by SFC-HRMS. MeOH was thus selected as co-solvent.

It has been demonstrated that the column temperature affects the state and the density of the mobile phase, resulting in significant influence on the SFC analyses including changes in retention time, peak shape and method sensitivity [19]. Optimization of the oven temperature was then studied at three different temperatures, *i.e.* 40, 50 and 60 °C. The results showed that setting oven temperature at 50 and 60 °C enabled a better sensitivity for PG3+1C18:1 (Fig. 4-a). This phenomenon has been observed in a previous study [19]. It should be related to the supercritical state of the mobile phase at high temperature, which resulted in better detection of the analytes by ESI-HRMS. The column temperature was thus kept at 50 °C to avoid potential column or compound degradation. The BPR was optimized with three pressure levels including 130, 140 and 150 bar. BPR at 140 bar offered a slight improvement in sensitivity of PG3+1C18:1 (Fig. 43-b) and was consequently chosen for further developments.

Optimization of MS parameters

Different MS parameter settings were optimized to get the best sensitivity for the PGE analysis. First, the drying-gas temperature was studied at three temperatures (250, 300 and 350 °C). The results presented in Fig. 5 show that the PGE peaks got the highest intensity at 350 °C. As a result, this value was kept as optimum. Other compound-dependent parameters were also examined including the V_{cap}, the fragmentor voltage, the nozzle voltage. The results showed that the V_{cap} did not present significant influence on the PGE analysis. A value of 3500 V was selected for this parameter. For the fragmentor voltage, the more important this value, the higher intensity of the PGEs. This parameter was thus kept at 200 V. In contrast

to the previous parameters, the nozzle voltage did not show a linear influence on the intensity of the PGE signals. At the highest tested value, *i.e.* 2000 V, intensities of PGE signals were smaller compared to intensities at 1000 V. That could be due to an in-source fragmentation of PGEs at high nozzle voltage. Nozzle voltage at 1000 V enabled the best sensitivity of the PGEs so it was kept as optimum.

SFC-HRMS/MS characterization of PGEs

The SFC-HRMS/MS-based characterization of PGEs, especially PG3+1C18:1, was then explored. The PGEs had three molecular ion forms: the minor form is the protonated molecule, the second one is the ammonium cationized species and the major one is the sodium cationized species. Since the protonated species is fragmented at the lowest energy and contains more characteristic fragments than the two others, it was used for the structural characterization study.

First, the SFC-HRMS/MS method was set using the optimum parameters described previously with the collision energy at 0 eV. We observed that all three isomers of PG3+1C18:1 were fragmented (Fig. S3, supplementary data) due to residual internal energies of the precursor ions. Our goal was then to minimize this activation process by reducing the fragmentor voltage. The results showed that the fragmentor voltage at 80, 100 and 120 V induced the same proportion of PG3+1C18:1 fragmentation even if the in-source fragmentation was less pronounced when decreasing this parameter value (Fig. S4, supplementary data). Therefore the fragmentor voltage was set at 120 V for HRMS/MS characterization study..

The collision energy was then increased to 10 eV leading to extended fragmentation of each isomer with similar fingerprints (Fig. 6 and S5). However, their relative intensity was different from one compound to the other. Losses of one, two and three glycerol moieties were observed. The fragments corresponding to free mono-, di-, tri-glycerol together with oleic acid were also present. The consecutive losses of water molecule were detected for each ion. When using our SFC-HRMS/MS conditions to analyze other PGEs in the mixture, such as PG4+1C18:1 and PG5+1C18:1, the same fragmentation pathway was observed (Fig. S6 and S7, supplementary data) indicating the efficiency of the method for PGE structural characterization. As the PGE mixtures are complex and sometimes they could contain many isobaric molecules, such as PG3+C18+C18:2 and PG3+2C18:1, the determination of each PGE type presents a challenge. By using our method, the MS/MS-based fragmentation will allow to characterize each PGE type and distinguish the isobaric PGEs.

Purification and structure elucidation of PG3+1C18:1

As previously indicated, the two columns DEA and DP showed interest in PG3+1C18:1 purification by SFC. Product PG3MO rich in PG3+1C18:1 was first fractionated by SFC on the DP column affording 6 fractions. SFC-HRMS analyses of these fractions showed an abundant intensity of free triglycerol (PG3), tetraglycerol (PG4) and dehydrated tetraglycerol (PG4(1)) in all fractions. Fraction SFC-2 was almost composed of these molecules and a very low quantity of PG3+1C18:1 (Fig. S8, supplementary data). By comparing SFC-HRMS profiles of PG3MO and corresponding SFC purified fraction, an important decrease of PG3+1C18:1 peak intensity was observed whereas the quantity of free polyglycerols was significantly increased. We supposed that a hydrolysis of PGEs occurred during CO₂ decompression and methanolysis/ethanolysis reaction with residual solvents [20].

The purification of PG3+1C18:1 from PG3MO on reversed-phase HPLC was then investigated. After one step of purification, a fraction containing PG3+1C18:1 (5.8 mg) was isolated as a colorless emulsion. The purity of this fraction was first verified by SFC-HRMS (Fig. S9, supplementary data). The MS spectrum of the peak eluted between 3-4 min displayed a major ion peak at m/z 527.3543, for which the molecular formula C₂₇H₅₂O₈ was deduced ([M+Na]⁺, Δ +3.22 ppm). According to Table S1 (supplementary data), this ion can only be attributed to PG3+1C18:1. A peak shoulder was detected suggesting that this fraction

contained two PG3+1C18:1 isomers named 1 (major) and 2 (minor). The percentage of isomer 2 calculated based on the peak area was estimated to 13%. In order to confirm the identification of PG3+1C18:1, 1D and 2D NMR experiments were performed (Fig. S10 to S15, supplementary data). Oleic acid and mixture of polyglycerol used for the synthesis were also analyzed by ^1H and ^{13}C NMR in the same condition. The comparison of ^1H and ^{13}C NMR spectra of these compounds allowed to identify proton and carbon signals belonging to the triglycerol head and to the fatty chain moiety. On ^1H NMR spectrum (Fig. S10, supplementary data), the total of proton integration at δ_{H} 2.35 ppm (1.61) and δ_{H} 2.17 ppm (0.43) was of 2.04 suggesting that these protons corresponded to two isomers of PG3+1C18:1 as detected by SFC-HRMS. Because of the weak 2D NMR intensity of minor isomer 2, only the structure of major isomer 1 of PG3+1C18:1 was established. All observed HSQC, HMBC, COSY and NOESY correlations confirmed the distribution of proton and carbon. HMBC spectrum showed the correlation between carbon of a carbonyl group (δ_{C} 175.6 ppm) and 2 protons of a methylene group (δ_{H} 4.06-4.17 ppm, δ_{C} 66.7 ppm) suggesting that the ester function was at position 1'. This result is in good agreement with the hypothesis that the esterification was preferred at the terminal hydroxyl group of PG3 [21].

Quantification

The quantification of PG3+1C18:1 was performed on the basis of the peak area of the pseudo-molecular ion $[\text{M}+\text{Na}]^+$. When comparing the peak area of PG3+1C18:1 in the standard solution and in the solution added with polyglycerol and oleic acid, no significant difference was observed indicating no significant matrix effect. Fig. S16 (supplementary data) displays the linear regression, the slope, the y-intercept and the determination coefficient (R^2) for PG3+1C18:1. The linearity was calculated over three orders of magnitude of concentration resulting in excellent linearity with the R^2 value of 0.9997. The RSD were in the range 4.1-13.6% at 6 different levels of concentration. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.250 and 0.625 pg injected, respectively. For intra-day study performed at six concentrations, the precisions were between 4.1% and 13.6%, and the accuracies were between 89.2% and 115.3%. Concerning inter-day data, the precisions were in the range 1.1% - 6.7%, and the accuracies were between 89.2% and 115.3% (Table S2, supplementary data). These results show that our method exhibits good repeatability.

The method was applied to quantify PG3+1C18:1 in three products PG3MO, PG3DO1 and PG3DO2 leading to content of 8.1, 7.4 and 7.4 % of PG3+1C18:1, respectively.

Conclusions

A rapid and efficient method for characterization of PGEs using SFC-HRMS/MS was established after optimizing SFC and MS parameters. After a single HPLC purification step, pure PG3+1C18:1 was obtained allowing to confirm its structure by HRMS and NMR analyses. For the first time, a method of quantification for PG3+1C18:1 was validated showing a good linearity ($R^2 = 0.9997$) as well as sensitivity (pg level). This method was then applied to quantify PG3+1C18:1 in three commercial samples allowing to better characterization of their fine composition. Using the same methodology, other PGE standards could be isolated for a more complete quantification of constituents of commercial PGE mixtures by SFC-HRMS.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure captions

Fig. 1. Chemical structure of PGEs ($R^1 = R^2 = R^3 = R^4 = H$ or acyl group).

Fig. 2. Base peak chromatograms of PG3DO2 solution analyzed by SFC-HRMS with 9 different stationary phases: Hypercarb, PFP (pentafluorophenyl-bonded silica), CN (cyano-bonded silica), C18 (octadecyl carbon-bonded silica), 2-EP (2-ethylpyridine-bonded silica), 1-AA (1-aminoanthracene-bonded silica), DP (diphenyl-bonded silica), NH₂ (amino-bonded silica) and DEA (diethylamine-bonded silica). Analysis conditions: The mobile phase was composed of CO₂ (A) and MeOH/EtOH 1:1 v/v with 20 mM of AcONH₄ (B) at 1 mL min⁻¹ flow rate. The gradient was: 0-10 min (1-30% B), 10-12 min (30% B), 12-13 min (30-1% B), 13-16 min (1% B). The temperature of column was 50 °C. The BPR was set at 130 bar and 60 °C. The make-up solvent was MeOH/EtOH 1:1 v/v with 20 mM of AcONH₄ at 0.2 mL min⁻¹ flow rate.

Fig. 3. EIC of a) monoesters of polyglycerol having n (n=2-6) degree of polymerization and oleic acid (number above each peak corresponds to n value); b) esters of triglycerol (n=3) and m oleic acid (m= 1-5) (number above each peak corresponds to m value); c) PG3+1C18:1 and its analog with one water loss PG3(1)+C18:1. Analysis conditions: The DEA column was set at 50 °C. The mobile phase was composed of CO₂ (A) and MeOH/EtOH 1:1 v/v with 20 mM of AcONH₄ (B) at 1 mL min⁻¹ flow rate. The gradient was: 0-10 min (1-30% B), 10-12 min (30% B), 12-13 min (30-1% B), 13-16 min (1% B). The BPR was set at 130 bar and 60 °C. The make-up solvent was MeOH/EtOH 1:1 v/v with 20 mM of AcONH₄ at 0.2 mL min⁻¹ flow rate.

Fig. 4. Influence of the oven temperature (a) and the BPR (b) on the signal of PG3+1C18:1.

Fig. 5. Influence of the drying-gas temperature (a), the Vcap (b), the fragmentor voltage (c) and the nozzle voltage (d) on the signal of PG3+1C18:1.

Fig.6. HRMS/MS spectra (10 eV of collision energy) of the most intense PG3+1C18:1 isomer with MS/MS fragmentation pattern suggested for a linear PG3+1C18:1 with the fatty chain positioned in terminal position of PG3 (G = glycerol, FA = fatty acid, PG(2)1 = diglycerol with one water loss, PG3(1) = triglycerol with one water loss).

Table

Table 1. ^1H and ^{13}C NMR data for PG3+1C18:1.

Position	PG3+1C18:1		
	Type	δ_{C} (ppm)	δ_{H} (ppm, J en Hz)
1	C	175.6	
2	CH ₂	35.1	2.33-2.37 (m)
3	CH ₂	26.2	1.57-1.66 (m)
4	CH ₂	30.4	1.25-1.39 (m)
5	CH ₂	30.3	1.25-1.39 (m)
6	CH ₂	30.5	1.25-1.39 (m)
7	CH ₂	31.0	1.25-1.39 (m)
8	CH ₂	28.3	2.00-2.07 (m)
9	CH	131.1	5.32-5.38 (m)
10	CH	131.0	5.32-5.38 (m)
11	CH ₂	28.3	2.00-2.07 (m)
12	CH ₂	30.9	1.25-1.39 (m)
13	CH ₂	30.8	1.25-1.39 (m)
14	CH ₂	30.6	1.25-1.39 (m)
15	CH ₂	30.3	1.25-1.39 (m)
16	CH ₂	33.2	1.25-1.39 (m)
17	CH ₂	23.9	1.25-1.39 (m)
18	CH ₃	14.6	0.90 (t, 6.9)
1'	CH ₂	66.7	4.06-4.17 (m)
2'	CH	69.8	3.92-3.97 (m)
3'	CH ₂	73.8	3.49-3.58 (m)
4'	CH ₂	74.1	3.49-3.58 (m)
5'	CH	70.9	3.87-3.92 (m)
6'	CH ₂	74.0	3.49-3.58 (m)
7'	CH ₂	72.8	3.56-3.64 (m)
8'	CH	72.4	3.73-3.79 (m)
9'	CH ₂	64.5	3.50-3.59 (m)