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# Analysis of complex mixtures of polyglycerol fatty esters using liquid chromatography and high-resolution mass spectrometry: Retention, structural and relative composition study

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1 **Analysis of complex mixtures of polyglycerol fatty esters using liquid chromatography**  
2 **and high-resolution mass spectrometry: retention, structural and relative composition**  
3 **study.**

4  
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13  
14 **Abstract**

15 Polyglycerol esters (PGEs), produced by esterification of fatty acids on polyglycerols, were  
16 analysed by High Resolution Mass Spectrometry (HRMS), HPLC-MS and U-HPLC-MS. A  
17 structural study of PGEs in 4 samples synthesised by the Gattefossé company was carried out  
18 using an elemental analysis of HRMS spectra and modelling of all probable isomers and cyclic  
19 structures. The results were used to construct a structural database of all species present in the  
20 4 samples. After an assessment of the selectivity of 5 reversed phase columns: Aeris Widepore  
21 XB-C8, 3.6 $\mu$ m, 2.1x150 mm (Phenomenex), Acquity CSH C18 1.7 $\mu$ m 2.1x50mm, Acquity  
22 CSH Phenyl-Hexyl 1.7 $\mu$ m 2.1x50 mm, Acquity CSH Fluoro-Phenyl 1.7 $\mu$ m 2.1x50mm (Waters  
23 Co.) and Kinetex F5 1.7 $\mu$ m 2.1x100mm (Phenomenex), HPLC-MS and U-HPLC-MS analyses  
24 were performed on an Aeris Widepore XB-C8 (Phenomenex) column (HPLC) and Acquity  
25 CSH Fluoro-Phenyl (Waters) column (U-HPLC) with aqueous formic acid /acetonitrile in

26 gradient mode. The separation was optimised with 10 minutes (HPLC) and 5 minutes (U-  
27 HPLC) of gradient. The detection, performed on a QDA detector (Waters), produced extracted  
28 ion chromatograms (XICs) based on all adducts identified in the HRMS analysis. HPLC and  
29 U-HPLC analyses showed the different mono- and di-ester species and provided relative  
30 quantification of all identified constituents. The combined analyses of the HRMS, HPLC-MS  
31 and U-HPLC-MS results were used to compare the different PGE batches and quantify the  
32 molecular constituents according to their relative abundance, for these complex mixtures. With  
33 HPLC and U-HPLC analyses, using 2 different gradient times and 2 different selectivity  
34 columns, and comparing the retention factors and log P of the different species, it was possible  
35 to link structural identification and relative quantification of all PGEs identified in the samples.

36

### 37 **Highlights**

- 38 • Batch comparisons and quantification of constituents of complex formulations
- 39 • Structural database construction with HRMS spectra and modelling of all probable  
40 PGEs
- 41 • HRMS and (U)-HPLC-MS can link structural identification to relative PGEs  
42 quantification

43

### 44 **Keywords**

45 HPLC; HRMS; Formulation; Complex matrices; Polyglycerol esters; Green surfactant.

46

47

## 48 **1. Introduction**

49 Polyglycerol is a viscous, non-toxic, water-soluble polyether with a high hydrophilic character  
50 and excellent biocompatibility. Polyglycerol esters (PGEs), obtained via esterification of fatty

51 acids on polyglycerols, are odourless, biodegradable non-ionic surfactants. They are mainly  
52 used in cosmetics, the food industry and pharmaceuticals and they come from renewable  
53 resources [1-3]. These products have excellent skin tolerance, better thermal stability than other  
54 conventional non-ionic surfactants, and moderate antimicrobial activity. Furthermore in  
55 addition to their surface-active properties, the lipophilic part, consisting of a fatty acid,  
56 combined with a hydrophilic part based on glycerol, gives them emollient and moisturizing  
57 properties [4]. PGEs are considered as green surfactants with promising physicochemical  
58 properties.

59 Polyglycerol can be synthesised from glycidol [5] but for safety concern glycerol is preferred.  
60 Glycerol is a safe, renewable material generated as a by-product during the biodiesel process  
61 [6]. The polymerisation of glycerol is ideally conducted at a high temperature in the presence  
62 of metal hydroxide [7,8] or more selective catalysts [9,10]. As previously mentioned,  
63 polyglycerol esters are produced from the esterification of fatty acids, derived from  
64 triglycerides of vegetable oils, and polyglycerols. Accordingly, polyglycerol esters are made of  
65 a complex mixture of compounds depending on the degree of glycerol polymerisation, on the  
66 polyglycerol branching isomers and on the number and type of fatty acids that have reacted per  
67 molecule of surfactant. It is important to determine the composition of these complex mixtures  
68 both qualitatively and quantitatively. Indeed, the properties of PGEs are connected to their  
69 composition [11]. Various chromatographic techniques are available for analysing PGEs: thin-  
70 layer [12], GC [13], RP-HPLC [14] and SFC [15]. Specific methods have also been developed  
71 by combining GC and HPLC analyses [16] or using stationary phases of porous graphitic carbon  
72 stationary phases [17]. In this study, the complexity of 4 mixtures of PGEs was analysed by  
73 direct infusion in a Q-ToF HRMS system. With these structural data, PGEs retention were  
74 studied on five stationary phases (one HPLC and four U-HPLC) in reversed mode, in order to  
75 optimise methods adapted to the total range of PGE structures. By integrating molecular

76 modelling data, we connected the PGEs' retention factors to specific mono-, di- and tri-esters.  
77 These identified structures were then quantified in relative quantity in the 4 mixtures.

78

## 79 **2. Materials and methods**

### 80 2.1 Synthesis

#### 81 2.1.1 Chemicals

82 Products used for synthesis: polyglycerol (Spiga spa, Genova, Italy), stearic acid 98%  
83 (Mosselman) and technical oleic acid (Berg & Schmidt).

84

#### 85 2.1.2 Procedure for PG distillation

86 Prior to the synthesis, diglycerol was removed by means of short path molecular distillation  
87 (UIC GmbH) to increase the content in polyglycerols with a higher degree of polymerisation.

88 The short path distillation unit (UIC GmbH) was equipped with temperature regulators (Julabo  
89 ME4, HT30M1) and connected to a cold trap filled with liquid nitrogen. The distillation  
90 conditions were the following: feeding the polyglycerol-6 into the distillation unit at 80°C and  
91 300mL/h, pressure 0.05 mbar, heating mantle set at 200°C and internal condenser at 90°C.

92

### 93 2.2 Analytical methods

#### 94 2.2.1 Chemicals

95 HPLC-grade acetonitrile (ACN), methanol (MeOH) and formic acid (HCOOH) were obtained  
96 from Sigma-Aldrich Co. (St. Quentin Fallavier, France). Water (H<sub>2</sub>O) was purified using a  
97 milli-Q purification system (Millipore, St. Quentin Yvelines, France).

#### 98 2.2.2 HRMS

99 Solutions (1 mg.mL<sup>-1</sup>) of samples S1, S2, S3 and S4 were obtained by dissolution in MeOH  
100 with 0.1% HCOOH, sonication at 20°C and filtration through 0.2 µm nylon filters (Waters

101 Corporation, Milford, MA, USA). The samples were injected (5  $\mu\text{L}$ ) without using a column  
102 on a Xevo G 2 QToF (Waters) system, with elution water 0.1% HCOOH/ACN (90%:10% v:v)  
103 and flow 0.5  $\text{mL}\cdot\text{min}^{-1}$ , capillary voltage ESI+ (3kV), fragmentor potential (30 V), nitrogen ESI  
104 (800 $\text{L}\cdot\text{H}^{-1}$ ), nitrogen cone (20 $\text{L}\cdot\text{H}^{-1}$ ) and source temperature (110°C). Masslynx was used to  
105 process the results. After summation of 1 min of acquisition, all spectra were analysed and  
106 compounds identified by comparison with the theoretical mass with a maximum deviation of 3  
107 ppm.

108 The deviation is calculated as shown below:

$$109 \Delta\text{ppm} = (| \text{theoretical mass} - \text{experimental mass} |) / \text{theoretical mass} \times 1\,000\,000$$

110

### 111 2.2.3 HPLC-MS and U-HPLC-MS

112 HPLC and U-HPLC analyses were performed on an Acquity<sup>®</sup> UPLC H-Class system composed  
113 of a quaternary solvent manager, a sample FTN manager and an Acquity<sup>®</sup> QDA detector  
114 (Waters Corporation, Milford, MA, USA). Analyses were monitored by Empower 3 software  
115 (Waters Co.). Eluents were filtered through 47 mm, 0.45  $\mu\text{m}$  nylon membrane discs (Waters).  
116 HPLC separations were carried out on Aeris Widepore XB-C8, 3.6 $\mu\text{m}$ , 2.1x150 mm  
117 (Phenomenex, Torrance, CA, USA) and U-HPLC separations were carried out on 4 columns:  
118 Acquity CSH C18 1.7 $\mu\text{m}$  2.1x50mm, Acquity CSH Phenyl-Hexyl 1.7 $\mu\text{m}$  2.1x50 mm, Acquity  
119 CSH Fluoro-Phenyl 1.7 $\mu\text{m}$  2.1x50mm from Waters Co and Kinetex F5 1.7 $\mu\text{m}$  2.1x100mm  
120 (Phenomenex).

121 For HPLC analysis, a gradient mode was used with all time conditions : (% vol.)  $t_{0\text{min}}$ : 40%  
122 H<sub>2</sub>O, 50% ACN, 10% ACN + 1% HCOOH;  $t_{10\text{min}}$ : 90% ACN, 10% ACN + 1% HCOOH; Flow  
123 1  $\text{mL}\cdot\text{min}^{-1}$ ; the run time was 10 minutes and the column temperature was 25°C. For U-HPLC  
124 analysis, all-time conditions were (% vol.)  $t_{0\text{min}}$ : 50% H<sub>2</sub>O, 40% ACN, 10% ACN + 1%  
125 HCOOH;  $t_{5\text{min}}$ : 90% ACN, 10% ACN + 1% HCOOH; Flow 0.5  $\text{mL}\cdot\text{min}^{-1}$ ; the run time was 5

126 minutes and the column temperature was 35°C. MS analyses were performed with an  
127 electrospray ionisation source (ESI) in positive mode with a sampling frequency of 8.8 Hz. The  
128 capillary and fragmentor potentials were set respectively to 0.8 kV and 15 V. The QDA analyser  
129 worked in full scan mode, and the mass range was set at  $m/z$  145–1250. To identify and analyse  
130 each PGE, full-scan survey spectra were used to generate extracted ion chromatograms (XICs)  
131 using the  $[M+Na]^+$  or  $[M+H]^+$  mass value adjusted at 2 decimals, from theoretical or  
132 experimental masses (Table S1 to S4 in supplementary data). For the semi-quantitative analysis  
133 of each PGE, the peaks obtained for each XIC were integrated and the relative abundance was  
134 calculated by the ratio of each PGE's peak area on the sum of the integrated peaks areas.

135

### 136 2.3 Molecular modelling

137 All PGE structures were optimised using semi-empirical AM1 quantum mechanical  
138 calculations in the MOPAC application, using Chem 3D Pro version 14.0 software  
139 (CambridgeSoft) with an RMS gradient of 0.100 to minimise energy. The optimised structures  
140 were used to calculate the octanol/water partition coefficient ( $\log P$ ) and Connolly molecular  
141 area (CMA) with Chem 3D Pro 14.0 software (CambridgeSoft).

142

143

## 144 **3. Results and discussion**

### 145 3.1. Modelling of structures

146 Prior to the experiments, modelling was performed for all the structures that could possibly be  
147 present. This comprised free polyglycerols (PG), with a degree ranging from 1 to 12, and  
148 including possible loss of water molecules, whether because of intrinsic cyclic structures or  
149 dehydration processes in the mass spectrometer. Then the same kind of work was done for fatty  
150 esters, combining every single free PG with up to 12 fatty chains. Since the analysis of PGEs

151 mixtures by HRMS, HPLC and U-HPLC-MS did not resolve all probable isomers and branched  
152 PGs, modelling was limited to linear PGs with 1, 2 or 3 chains of esterified fatty acids in  
153 terminal positions for mono and di-esters. For tri-esters models, the 3<sup>rd</sup> fatty chain is positioned  
154 on the central OH function of the PGs. For PGs and PGEs with water molecule loss, the models  
155 incorporate 1 or 2 water loss.

156

157 The molecules will be named as follows:

- 158 - PG<sub>n</sub> = free polyglycerol with a polymerisation degree n
- 159 - PG[x] = PG with a loss of x water molecules (maximum 2)
- 160 - PG<sub>n</sub>+mC<sub>x</sub> = PGE with PG<sub>n</sub> and m linear chains of x carbon atoms, for a set of four  
161 possible fatty acids (C16, C18, C18:1 and C18:2)
- 162 - PG<sub>n</sub>+mC<sub>x</sub>/pC<sub>y</sub> = PGE with PG<sub>n</sub> and m linear chains of x carbon atoms and p linear  
163 chains of y carbon atoms, for a set of four possible fatty acids (C16, C18, C18:1 and  
164 C18:2)
- 165 - [M+H]<sup>+</sup> = ionic structure of a PGE with a hydrogen adduct
- 166 - [M+Na]<sup>+</sup> = ionic structure of a PGE with a sodium adduct

167

168 For the study, we had samples of the mixtures of the free PGs used in the esterification reaction,  
169 one centred around PG3 and two around PG6 (either distilled or not).

170 We had 4 samples of PGEs obtained with different fatty acid mixtures, whose initial reactive  
171 compositions are given in Table 1 below.

172

Sample	Free PGs	Fatty acids
S1	Distilled PG6	C18 (98.8%)
S2	Distilled PG6	C18:1 (79.4%)



		C18:2 (12.4%)
		C16 (4.8%)
		C18 (1.9%)
S3	Non distilled PG6	C18 (50%)
		C16 (50%)
S4	PG3	C18:1 (79.4%)
		C18:2 (12.4%)
		C16 (4.8%)
		C18 (1.9%)

---

173 Table 1. Samples S1 to S4 initial reactive composition.

174

175 Then, for every possible molecule, the molecular formula was determined, and the exact mass  
 176 at the fourth decimal level was calculated for both hydrogen and sodium adducts (which may  
 177 form during the synthesis process or in the ESI source).

178 Possible molecules are modelled and three-dimensional structures minimised, for calculation  
 179 of the physicochemical parameters log P and Connolly molecular area. These physicochemical  
 180 parameters will provide information in the structural study on possible formulae, linking the  
 181 HRMS and experimental retention data obtained using the two chromatographic methods.

### 182 3.2 HRMS analyses

183 Once this preliminary work was done, all 4 samples were analysed using high resolution mass  
 184 spectrometry in order to identify all the molecules present in each sample. Figure 1 shows the  
 185 ESI+ TOF mass spectrum obtained with sample S3. In all acidified and dissolved methanol  
 186 samples,  $[M+H]^+$  and  $[M+Na]^+$  adducts were detected in ESI positive mode. This can be  
 187 explained by the ability of the polyglycerol polar-head group to behave as a relatively good  
 188 chelators for sodium ions, depending on the PG structure. The analysis of these complex HRMS

189 spectra consisted of elemental analysis of the PGEs corresponding to the molecular formulae  
190 of the modelled PGEs for signals greater than 10% of the relative intensity.

191 For S1, (Table S1, supplementary data) obtained from distilled PG6 and C18 fatty acid, esters  
192 with a polar head comprising between 3 and 8 glycerol units combined with one or two fatty  
193 chains were mainly detected, as expected as this is consistent with the starting materials used.  
194 In this sample, 27 PGEs were found with a relative intensity above 10% for 143 modelled,  
195 corresponding to 19% identification score.

196 For S2, (Table S2, supplementary data) which resulted from the reaction between distilled PG6  
197 and a mixture of fatty acids mainly composed of oleic acid, here again esters with a polar head  
198 comprising between 3 and 8 glycerol units esterified by one to up to 3 fatty chains were  
199 detected. For compounds that have two or more chains, one cannot distinguish between a  
200 combination of 2 C18:1 and 1 C18 + 1 C18:2, for they have the exact same formulas and  
201 therefore theoretical mass. Nonetheless, as there is nearly 80% of oleic acid and only less than  
202 2% stearic acid in the starting mixture of fatty acids used to prepare S2, it is statistically most  
203 likely that it is the combination of 2 C18:1 fatty chains. In this sample, 33 PGEs were found  
204 with a relative intensity above 10% for 327 modelled, corresponding to 10% identification  
205 score.

206  
207 S3 (Table S3, supplementary data) was obtained from the reaction between non distilled PG6  
208 and a 1:1 mixture of palmitic and stearic acid. As expected, many more compounds can be  
209 detected here as diglycerol and other fairly volatile compounds (cyclic ones) were not removed  
210 by the distillation step, as in S1 and S2. PG2 to PG7 combined with up to 3 chains can therefore  
211 be detected in S3. In sample S3, 59 PGEs were found with a relative intensity above 10% for  
212 150 modelled, corresponding to 39% identification score.

213 Finally, S4 (Table S4, supplementary data was obtained by the combination of PG3 with the  
 214 same fatty acid mixture as for S2, mainly composed of oleic acid. As expected, PG2 to PG5  
 215 bearing up to 3 chains can be found in the mixture, with the same uncertainty for compounds  
 216 having 2 or more chains, whether 2C18:1 or 1C18 + 1C18:2. Same conclusion applies here. .  
 217 In sample S4, 32 PGEs were found with a relative intensity above 10% for 327 modelled,  
 218 corresponding to 10% identification score.

219  
 220 In every sample mixture, non-reacted free PGs can be detected, depending on the starting free  
 221 PGs mixture, along with the esters.

222 In all mixtures, no PG with a degree exceeding 12 was found, even at very low level.

223 No structure with more than 2 water loss was detected.

224 Table 2 summarises the rough composition of each sample.

225

Sample	Free PGs	PG esters
S1	PG3 to PG8	PG3 to PG8 with up to 2 C18 chains
S2	PG3 to PG6	PG3 to PG7 with up to 3 chains / mixed grafting (C16/C18/C18:1/C18:2)
S3	PG2 to PG8	PG2 to PG7 with up to 3 chains / mixed grafting (C16/C18)
S4	PG3 to PG4	PG3 to PG5 with up to 3 chains / mixed grafting (C16/C18/C18:1/C18:2)

226 Table 2. Samples 1 to 4 composition.

227

### 228 3.3 HPLC-MS

229 In order to check that the analytical methods ensure the separation and identification of the  
 230 various classes of PGEs, we chose test compounds, for which the probable developed formulas

231 are given in Figure 2 and optimised structures after AM1 minimisation are given in Figure S1  
232 (supplementary data). The esterification reaction involves the terminal hydroxyl of each PG, as  
233 they are more reactive than the other OH groups [18]. The terminal positions are therefore  
234 chosen in all models to represent the monoesters and di-esters. The test compounds were  
235 selected to simplify method development and to verify that they could be separated on the  
236 columns used in this study.

237 These 4 test compounds were identified by HRMS in sample S3 (table S4, supplementary data).  
238 We chose esterified PGEs from PG4, 2 monoesters with a single C16 and a single C18 chain  
239 and 2 di-esters with 2 C16 and 2C18 chains. Because the exact structures and positions of the  
240 fatty acids are unknown, the formulae given in Figure 2 are only probable structures, in  
241 reference to the more reactive terminal hydroxyl groups. We used these four test compounds  
242 because they show strong variations in their hydrophobic (log P) and steric (Connolly molecular  
243 area) values (Table 3) and enable us to assess the selectivity of the stationary phase and the  
244 conditions of gradient on these model PGEs with varied steric and hydrophobicity footprints.  
245 These hydrophobic and steric parameters are used for modelling the reversed-phase retention  
246 mechanisms [19] and are therefore relevant indicators of the structural variability of  
247 interactions.

248

Compound	Compound code	log P	Connolly Molecular Area (Å <sup>2</sup> )
PG4[1]+1C16	A	4.8	617.9
PG4[1]+1C18	B	5.9	654.9
PG4+2C16	C	12.1	932.7
PG4[1]+2C18	D	15.2	993.0

249 Table 3. Log P and Connolly molecular area calculated for PG4[1]+1C16, PG4[1]+1C18,  
250 PG4+2C16, PG4[1]+2C18.

251

252 The XIC chromatogram of sample S3 (Figure 3) shows the separation of the 4 PGEs from the  
253 least hydrophobic (A: log P 4.8) to the most hydrophobic (D: log P 15.2) in less than 10 minutes,  
254 with a simple aqueous gradient using ACN as an organic modifier. This XIC chromatogram  
255 was obtained by summation of m/z: 557.36, 563.41, 813.60 and 851.65. Each separated  
256 chromatograms obtained with the 4 m/z values is given in Figure S2 (supplementary data).

257 Wide-pore core-shell columns showed good efficiency and peak capacity for the separation of  
258 macromolecules [20]. The Aeris™ WIDEPORE XB-C8 3.6 µm silica particles, surrounded by  
259 a 0.2 µm porous layer, showed good selectivity in the separation of the 4 test PGEs. The semi-  
260 quantitative analysis was performed from XIC chromatograms for all PGEs theoretically  
261 present in each sample. Tables 4 to 6 present the results for samples S1 to S4 for relative  
262 abundances above 1%.

263 For S1 (Table S5, supplementary data), the major species were PG4, PG5 and PG6 esters  
264 containing two C18 chains, accounting for 7.3 to 8.1% (relative abundance) of the mixture.  
265 There was also 5.9% PG3+2 C18. Di-esters accounted for 35.7% of the S1 sample. The  
266 majority-identified monoesters were PG3 and PG4 with one C18 chain, at 6.5 and 4.6%  
267 respectively. Other classes of monoesters were found with different PGs with loss of a water  
268 molecule: PG3[1], PG4[1], PG5[1] and PG6[1] associated with one C18 chain. Monoesters  
269 accounted for 42.5% of the S1 sample. The remaining 21.8% were made up of 70 free PGs,  
270 mono- and di-ester minority species, all identified with this analytical method. All free PGs were  
271 found at retention times between 0.20 and 0.35 minutes. Monoesters are found between 0.59  
272 and 1.96 min and di-esters separated between 7.3 and 9.3 min. PG8, PG6, PG5 and PG3 esters  
273 with one C18 chain are found with a loss of one water molecule with higher retention times.

274 For PG8[1], PG6[1], PG5[1] and PG3[1] esters with one C18 chain, this water loss occurred  
275 prior to separation during the esterification reaction. For PG7[1], PG4[1], and PG3[1] ] esters  
276 with one C18 chain, This water loss can also occur in the ESI source and the water-loss PGEs  
277 are then identified at the same retention time as linear PGEs.

278 For S2 (Table S6, supplementary data), a very complex mixture obtained from distilled PG6,  
279 the analysis showed that the major species were PG3, PG4, PG5 and PG6 esters bearing two  
280 alkyl chains (C18, C16, C18:1, C18:2), accounting for 13.5 to 6.9% (relative abundance) of the  
281 mixture. The simple quadrupole system resolution does not discriminate isobaric compounds.  
282 The PGE identified with an experimental mass of 791.78 can be attributed to either  
283 PG3+1C18+1C18:2, PG3+2C18:1 or PG4+2C16. As the C18:1 fatty acid accounts for nearly  
284 80% of the fatty acid mixture used for the preparation of sample 2, we can only suggest that  
285 PG3+2C18:1 may be the major compound having this mass. Di-esters account for 50.1% of  
286 sample S2. The majority-identified monoesters were PG3, PG4 and PG4[1] with one C18:1  
287 chain, at 4.6, 4.1 and 3.0% respectively. Monoesters account for 26.9% of the S2 sample. The  
288 remaining 23% made up of 70 free PGs, mono and di-ester minority species. Most of the  
289 monoesters and di-esters with one or two identified water loss are found at the same retention  
290 time as linear PGEs, this mechanism is linked to the ESI source.

291 For S3 (Table S7, supplementary data), a complex mixture of non-distilled PG6 with C18 and  
292 C16, the analysis showed that the major species were PG4 esters containing one C18 and one  
293 C16 alkyl chains and PG3[1] monoester with one C18 chain, accounting for 6.6 and 6.1%  
294 (relative abundance) of the mixture, respectively. Di-esters account for 46.9 % of the S3 sample.  
295 The majority-identified monoesters comprised all the PGs from PG2 to PG8[1] with one C18  
296 or one C16 chain. Monoesters accounted for 25.7% of sample S3. The remaining 27.4% were  
297 made up of 65 free PGs, mono and di-ester minority species. PG3, PG4, and PG5 esters with

298 one C16 or one C18 chain are found with a loss of one water molecule with higher retention  
299 times. This water loss occurred prior to separation during the esterification reaction.

300

301 The S4 sample analysis (Table S8, supplementary data) showed results close to S2. The major  
302 species were PG3, PG4 and PG5 di-esters containing C18, C18:1 and C18:2 alkyl chains,  
303 accounting for 19.3 and 11.5% (relative abundance) of the mixture. Di-esters account for 55.9%  
304 of the S4 sample. The majority-identified monoesters comprised PG3 and PG4 with one C18:1  
305 chain. Monoesters accounted for 21.2% of the S4 sample. The remaining 27.4% were made up  
306 of 60 free PGs, mono and di-ester minority species. As observed for sample S2, most of the  
307 monoesters and di-esters with one or two identified water loss are found in S4 at the same  
308 retention time as linear PGEs, this mechanism is linked to the ESI source.

309

310

### 311 3.4 U-HPLC-MS analyses

312 The first work was to select the suitable column for separating the PGEs according to the chosen  
313 gradient. We followed the 4 typical PGEs (Figure 2), by injecting sample S3 for the 4 study  
314 columns with a XIC chromatogram extraction for each compound. With the Acquity CSH C18  
315 column, only PG4[1]+1C16 and PG4[1]+1C18 were identified. With the CSH Phenyl-Hexyl  
316 and Kinetex F5 columns, the 2 test monoesters PG4[1]+1C16, PG4[1]+1C18 and one di-ester  
317 PG4+2C16 were identified.

318 The XIC chromatogram of sample S3 (Figure 4) shows the separation of the 4 test PGEs on the  
319 Acquity fluoro-phenyl column in less than 5 minutes, with a simple aqueous gradient using  
320 ACN as an organic modifier. The column selectivity of the Acquity fluoro-phenyl column was  
321 adapted to the separation of the test PGEs in a wide range of log Ps (from 4.8 to 15.2) and  
322 Connolly molecular areas (from 617 to 993), (Table 2). The Acquity fluoro-phenyl column was

323 selected for all identification and semi-quantitative analysis of the PGEs samples, with the U-  
324 HPLC method. These analytical conditions gave an equivalent resolution compared to the  
325 HPLC method (Figure 3) while dividing the analysis time by 2. This XIC chromatogram was  
326 obtained by summation of m/z: 557.36, 563.41, 813.60 and 851.65. Each separated  
327 chromatograms obtained with the 4 m/z values is given in Figure S3 (supplementary data).

328  
329 The semi-quantitative analysis was performed from XIC chromatograms for all PGEs  
330 theoretically present in each sample. Tables S9 to S12 summarise the results for samples S1 to  
331 S4 for relative abundances above 1%. For sample S1 (Table S9, supplementary data), the U-  
332 HPLC method provided identification of 37 compounds with more than 1% relative abundance,  
333 which is 24 additional compounds compared to the HPLC method. It is possible with the U-  
334 HPLC to identify 6 free PGs (PG3 to PG7) at an rt close to 0.31 min, accounting for between  
335 1.0 and 1.5% of all PGEs and PGs detected. The major species were PG3 and PG4 esters  
336 containing two C18 chains, accounting for 6.1 and 5.81% (relative abundance) of the mixture.  
337 All the most abundant di-esters identified by HPLC, from PG3 to PG6, detected and quantified  
338 with similar yields. With U-HPLC, di-esters accounted for 36% (35.7% with HPLC),  
339 monoesters accounted for 37.1% (42.5% with HPLC) and free PGs were quantified at 6.9%  
340 (ND with HPLC) of the S1 sample. The remaining 20% are made up of free PGs, mono and  
341 di-ester minority species. For sample S2 (Table S10, supplementary data), the U-HPLC method  
342 provided identification of 33 compounds with more than 1% of relative abundance, which is 7  
343 additional compounds compared to the HPLC method. With the U-HPLC analysis, the majority  
344 identified species were PG3, PG4, PG5 and PG6 esters containing two alkyl chains (C18, C16,  
345 C18:1, C18:2), accounting for between 7.9 and 3.3% (relative abundance) of the mixture. All  
346 the most abundant di-esters identified by HPLC, from PG3 to PG6, were detected and quantified  
347 with similar yields except for the compound with an experimental mass of 791.71 attributed to



348 [PG3+1C18+1C18:2+Na]<sup>+</sup> or [PG3+2C18:1+Na]<sup>+</sup> or [PG4+2C16+H]<sup>+</sup> identified at a relative  
349 abundance of 7.9% with the U-HPLC method and 13.5% with the HPLC method. Di-esters  
350 represent 35.8% (50.1% with HPLC), and monoesters 26.9% (25.6% with HPLC). For S3  
351 (Table S11, supplementary data), a complex mixture of non-distilled PG6 with C18 and C16,  
352 the analysis showed that the major species were PG3[1] monoester containing one C18 alkyl  
353 chains and the PG4[1] di-ester with one C18 and one C16 chain, accounting for 8% (6.1% with  
354 HPLC) and 2.9% (6.6% with HPLC) of the mixture, respectively. Di-esters accounted for  
355 33.9% and monoesters for 29.5% of sample S3. One free PG was also identified at 1.1%  
356 (PG4[1]). The S4 analysis (Table S12, supplementary data) showed results close to S2 and to  
357 the HPLC analysis. The major species were PG3, PG4 and PG5 di-esters containing C16, C18,  
358 C18:1 and C18:2 alkyl chains, accounting for 10.1% (19.3% with HPLC) 5.6% (11.5% with  
359 HPLC) and 5.2% (5.6% with HPLC) of the mixture. Di-esters accounted for 40.4% of the S4  
360 sample. The majority-identified monoesters comprised PG3 and PG2 and PG4 with one C18:1  
361 chain. Monoesters accounted for 28.8% of sample S4.

362 Isobaric ions can be detected with both HPLC and U-HPLC methods. The adduct m/z 749.80  
363 can be detected in sample S2, S3 and S4 with the U-HPLC-MS method. This signal can be  
364 attributed to 2 different PGEs: PG3[1]+1C18+1C16, with a calculated log P of 20.9 and a  
365 Connolly molecular area 876.3 Å<sup>2</sup> or PG6+1C18:1, with a calculated log P of 2.8 and a  
366 Connolly molecular area 811.7 Å<sup>2</sup>. For samples S2 and S4, the analysis of the low retention  
367 time of the corresponding PGE (0.7 min) was compatible with PG6+1C18:1, with lower values  
368 for the calculated log P and Connolly molecular area (Table S10 and Table S12, supplementary  
369 data). For sample S3, the retention time of this PGE was higher (3.4 min) and compatible with  
370 a di-ester, PG3[1]+1C18+1C16, with a high value for log P and the Connolly molecular area  
371 (Table S11, supplementary data). The analysis done with the HPLC-MS method detected m/z  
372 749.68 in sample S2 and S4, with low retention time values (0.6 min, Table S6 and Table S8),

373 compatible with the monoester PG6+1C18:1, as analysed with the U-HPLC-MS method. This  
374 signal was not detected in the S3 sample. The comparison of the retention time and the  
375 molecular calculated parameters log P and Connolly molecular area, can be used to discriminate  
376 probable PGEs structure for isobaric ions.

377 HRMS results confirm the interpretation given with the comparison of the retention time with  
378 the calculated values for log P and the Connolly molecular area. In sample S2, the elemental  
379 analysis confirmed the probable formulae of PG6+1C18:1 (Table S2, supplementary data), and  
380 for S3 the identification of the di-ester PG3[1]+1C18+1C16 (Table S3, supplementary data).

381 The development of the two HPLC-MS and U-HPLC-MS methods is complementary in  
382 analysing these complex matrices. Some di-esters of the S3 sample, such as  
383 PG3[1]+1C18+1C16, are identified by the U-HPLC MS method (m/z: 749.80, Table S11,  
384 supplementary data) and not detected by the HPLC-MS method. These very hydrophobic  
385 diesters (log P: 20.9) have a high steric effect with the stationary phase (Connolly molecular  
386 area 876.3 Å<sup>2</sup>) and are therefore not correctly eluted by the HPLC-MS method, whereas this  
387 PGE is identified with HRMS (Table S3, m/z: 749.5917, supplementary data). The simple  
388 HRMS analysis of the samples, without chromatographic separation, cannot identify some of  
389 the less apolar monoesters, such as PG6+1C18:1. This PGE was identified well in sample S2  
390 using HRMS (Table S2, m/z: 749.4655) and was also identified with the HPLC-MS and U-  
391 HPLC-MS methods, with a relative abundance ranging from 1.3% to 1.4% (Table S6 and S10,  
392 m/z 749.68 and 749.59, supplementary data). PG6+1C18:1 was not identified with HRMS for  
393 sample S4 (Table S4, supplementary data), whereas it was found using HPLC-MS and U-  
394 HPLC-MS, with a relative abundance of 1.3% to 1.4% with the two methods, for the same  
395 sample (Table S8 and S12, m/z: 749.69 and m/z 749.62, supplementary data). It appears that  
396 the development of the two chromatographic methods brings complementary resolution to  
397 HRMS for the study of these complex matrices.

398 Both the HPLC and U-HPLC methods can discriminate cyclic PG, generated by ESI reaction  
399 or during the polymerisation process, which are found in various PGE structures. For sample 1,  
400 PG4+2C18 was the most abundant PGE, identified at an rt of 8.30 min with HPLC (Table S5).  
401 An intramolecular condensation was found with the identification of PG4[1] + 2C18 at an rt of  
402 9.33 min, related to an increase in hydrophobic and steric interactions with the stationary phase.  
403 The PG3 + 1C18 monoester was identified at an rt of 1.32, and the corresponding cyclic PG3[1]  
404 + 1 C18 was found at the same rt of 1.32, related to ESI-induced dehydration and at rt 1.96,  
405 also related to an increase in hydrophobic and steric interactions with the stationary phase  
406 (Table S5). The same analysis can be achieved for all samples, for a precise characterisation of  
407 these specific classes of cyclic PGEs, in accordance with the previous work of Chaimbault et  
408 al. [21] and Crowther *et al.* [22]. The detailed PGEs analysis with one or two water loss is  
409 identical to the analysis performed with the U-HPLC method.

410

#### 411 **4. Conclusions**

412 The study of the composition of polyglycerol ester mixtures remains a challenge, even after  
413 decades of studies, as they display numerous structures that have very close features.  
414 Thanks to the modelling of all possible PG and PGE structures up to 12 PG units and High  
415 Resolution Mass Spectrometry (HRMS), we were able to identify the compounds present in the  
416 4 different samples provided by the Gattefossé company, and to construct a structural database  
417 with HRMS data. HPLC-MS and U-HPLC-MS analyses using a QDA detector (Waters),  
418 enabled us to perform PGE separations of PGEs in 10 minutes (HPLC) and 5 minutes (U-  
419 HPLC) gradients. The detection produced extracted ion chromatograms (XICs), based on all  
420 the possible adducts identified in the HRMS analysis. Using 2 different gradient times and  
421 comparing the retention factors and the log P of the different species, we were able to  
422 distinguish between different possible isobaric ions and therefore to make a reliable relative

423 quantification of all the PGEs identified in order to compare different synthesised samples with  
424 each other.

425

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429

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