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Optimization of normal phase chromatographic conditions for lipid analysis and comparison of associated detection techniques

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18 **Abstract**

19 One important challenge in lipid class analysis is to develop a method suitable or, at least adaptable, for a
20 vast diversity of samples. In the current study, an improved normal-phase liquid chromatography (NPLC)
21 method allowed analyzing the lipid classes present in mammalian, vegetable as well as microorganism
22 (yeast and bacteria) lipid samples. The method effectively separated 30 lipid classes or subclasses with a
23 special focus on medium polarity lipids. The separation was carried out with bare silica stationary phase and
24 was coupled to evaporative light scattering detection (ELSD), charged aerosol detection (Corona-CAD[®])
25 and mass spectrometry. Solutions are provided to circumvent technical issues (such as pumping solvents of
26 low viscosity, solvent purity, rinsing step). The influence of mobile phase composition and addition of ionic
27 modifiers on the chromatographic behavior of particular lipid classes is documented. A comparison between
28 ELSD and Corona-CAD[®] confirmed the interest of this later detector for samples with a wide range of
29 concentration of different lipids. Three common atmospheric pressure ionization interfaces were used for
30 coupling the NPLC separation to a LTQ Velos Pro[®] mass spectrometer. The comparison of the
31 chromatographic profiles showed that atmospheric pressure chemical ionization (APCI) and atmospheric
32 pressure photoionization (APPI) are both suitable to detect the different lipid classes whereas APPI allows a
33 better sensitivity for lipids at low-concentration.

34

35 **Keywords:**

36 Lipids

37 Normal-phase liquid chromatography

38 Evaporative Light Scattering Detector

39 Charged Aerosol Detector

40 Atmospheric pressure photoionization

41 LC/MS

42

1. Introduction

Lipidomics has emerged with the growing progress in mass spectrometry and bioinformatics. This is a complex field of activity since more than 40 000 unique lipid structures are listed in the LIPIDMAPS database nowadays [1,2]. Analytical techniques for lipidomics represent a very dynamic and motivating area as the lipid composition of animal or vegetal tissue is influenced by external factors (such as metabolic state or diet) and as research methods have still to be developed. New technologies and/or new developments in analytical sciences are now addressing lipid analysis to promote alternative and original separation techniques coupled with mass spectrometry. An important review about LC/MS based lipid analysis was published by Cajka & Fiehn [3] in 2014. They analyzed 185 original research papers and covered the technical approaches involved in lipidomics. The scope ranges from sample preparation to data treatment and encompasses mass spectrometry and separation techniques.

Several interesting findings can be extracted from this article. First, the diversity of the biological samples addressed by these 185 studies: plasma and serum represent 39% of the samples, animal tissue 23% and cells 22%, only 3% are plant tissues and other matrix represent 13%. Second, when LC/MS is used, reversed-phase liquid chromatography (RPLC), normal phase liquid chromatography (NPLC) and hydrophilic interaction chromatography (HILIC) are the most important techniques and correspond respectively to 71, 19 and 8% of the studies. It is worthy to note that RPLC and HILIC take advantage of improved sub-2- μm or fused core 2.6-2.8 μm particle size stationary phases whereas NPLC methods use classical 3-5 μm . In the vast majority of cases, narrow bore columns (2.1 mm I.D.) are used whatever is the retention mechanism. RPLC and HILIC benefit from the high water content and water miscible solvents used as mobile phase that facilitate the coupling with MS. HILIC is presented as an alternative to NPLC with a better reproducibility and a better compatibility with MS. Finally, this review pointed out the dominant position of electrospray ionization mass spectrometry (ESI) in lipidomic studies, most of time used in positive mode. At the opposite, atmospheric pressure chemical ionization (APCI) appears to be limited to nonpolar lipids (mainly triacylglycerols).

With regard to separation techniques, the recent trends in lipidomic analysis can be illustrated by four impressive studies issued by Holčapek's group. Two of them concern alternative retention mechanisms (namely HILIC) or separation techniques i.e. supercritical fluid chromatography (SFC) and their coupling to MS. The two remaining studies take advantage of multidimensional techniques such as LCxLC or LCxIon Mobility to deal with sample complexity.

In a recent work (2016) [4], a considerable effort was made to optimize the HILIC separation of acidic phospholipids. This separation appears to be also suitable for medium polarity lipids (such as ceramides)

76 but, in case of a total lipid extract, both NPLC and HILIC must be employed to accommodate the sample
77 complexity and polarity [5]. The most nonpolar lipids cannot be retained in HILIC conditions.
78 SFC offers both efficiency and analysis speed when used with modern sub 2 μ m particles. A high-throughput
79 method by UHPSFC coupled with electrospray ionization mass spectrometry (ESI-MS) with a
80 chromatography column packed with silica particles of less than 2 μ m (2015) [6] was proposed. 24 classes
81 of lipids were separated in 6 min and 436 lipid species were identified in a porcine brain extract. The CO₂
82 co-solvents used were only water and methanol.

83 When the lipid sample is extremely complex, one-dimensional analysis becomes insufficient. The second
84 trend is to enhance the discriminating power of the chromatographic system by adding a supplementary
85 separation step before the mass spectrometer entry. Two-dimensional liquid chromatography-electrospray
86 ionization mass spectrometry (2D LCxLC ESI-MS) was developed for the comprehensive and simultaneous
87 separation of classes and species of lipids (2015) [7]. The authors used RPLC with a C18 column in the first
88 dimension (150 min), and HILIC with a silica column in the second dimension (1 min). Finally, in a study
89 dealing with the complex analysis of the regioisomers of triacylglycerol by differential mobility ESI-MS
90 (2016) [8], the authors describe how ionic mobility permits the separation of compounds which are
91 chromatographically co-eluted.

92 All these recent studies take advantage of recent sub 2 μ m or fused core particles that allow fast and efficient
93 separations. This trend is only scarcely represented when considering normal phase separations. In 2011,
94 McLaren [9], adapted the separation of total lipids to a Fused-Core particle column using UHPLC. He used
95 an Halo HILIC column with 2.7 μ m particle, but carried out the separation in normal-phase mode with
96 evaporative light scattering detection (ELSD). The separation of the main lipids found in plasma, liver and
97 heart was realized in 10 min. The method was developed with only 7 different lipid standards; however it is
98 advisable from the chromatogram that more lipid classes could be separated using this method. The solvent
99 program appears to be very similar to the one proposed by Christie [10] for mammalian lipid class analysis .

100 Thus, despite of its typically longer analysis time, NPLC remains attractive for the analysis of a total lipid
101 extract and the objective of a complete separation of lipid classes. Method development is more complex in
102 NPLC than in RPLC. The mobile phases are usually made of solvent mixtures to ensure miscibility and to
103 encompass a wide range of polarity. The main advantage of NPLC is the elution of lipids according to their
104 polar moieties or “head group” which also define their family or “lipid class”. Even complex lipid mixtures
105 lead to easily interpretable chromatograms where molecular species of the same class co-elute within a
106 single peak. Mass spectrometry is the usual way to access the molecular species identification and relative
107 distribution.

108 In most studies, the chromatographic technique is developed to resolve the lipid classes present in a sample
109 or a group of samples. For example some method are designed to address animal tissues (heart, liver, brain,
110 kidney...) but only few studies are assessed with samples of various origin (such as animal and vegetal).

111 In chronological order, Graeve's method [11], initially devoted to zooplankton analysis was further adapted
112 by Gerits [12] for wheat lipids analysis during bread making. Graeve's method [11] is particularly effective
113 for the analysis of nonpolar lipids. At the opposite, Gerits' solvent conditions offer a better separation for
114 lipids of intermediate polarity such as glycolipids. Both these methods were derived from the work of
115 Homan and Anderson [13] which studied lipids from animal tissues. Homan and Anderson, themselves,
116 adapted the pioneering work of Christie issued in 1985 [10]. By comparing all these publications, one can
117 easily notice the changes operated by these authors and the subsequent tailoring of the separation to assess
118 the complexity of specific samples. However, solvent programs are complex and it is difficult and almost
119 impossible to synthesize all these experimental conditions to arrive at a compromise gathering the strength
120 of both methods.

121 In this publication, our objective was to develop an almost universal method for the overall evaluation of the
122 lipid classes present in natural samples regardless of their origin. Further, as NPLC is rather complex to
123 develop due to subtle interactions between analytes and mobile and stationary phases, we tried to exemplify
124 the effect of changes in the solvent program on the lipid class separation. Ref [9] shows that even in NPLC,
125 the solvent program can be adapted from conventional LC to UHPLC and, we can expect the solvent
126 conditions we propose should be transposable as well.

127 As already pointed out, ESI is the most represented ionization interface in lipidomic studies, presumably
128 because RPLC and HILIC represent nearly 80% of the chromatographic separations and are known to be
129 compatible with this interface. Mobile phase compatibility with atmospheric pressure ionization (API)
130 sources depends on the API source and the solvent [14]. We investigated coupling our separation with ESI
131 and APCI but also atmospheric pressure photoionization (APPI) in order to select the most appropriate
132 interface. Finally, and because ELSD and corona charged aerosol detection (Corona-CAD[®]) are useful to
133 monitor the separation in parallel with MS, a short comparison between those two detectors is provided.

135 **2. Experimental**

136 2.1. Chemicals

137 Isooctane, ethyl acetate, acetone (all HPLC grade), acetic acid (AA) and trimethylamine (TEA) were all
138 purchased from Sigma-Aldrich. Chloroform and water (all HPLC grade) were obtained from VWR
139 (Fontenay-sous-Bois, France). Isopropanol (IPA) (ULC/MS grade) was from Biosolve (Dieuze, France).

140 Isooctane was purified prior analysis by pumping the solvent through a semi preparative column packed
141 with Lichroprep Si 60 5-20 μ m silica (Merck KGaA, Darmstadt, Germany).

142 2.2. Standards

143 Phosphatidylglycerols (PG), phosphatidylethanolamines (PE), phosphatidic acids (PA),
144 phosphatidylcholines (PC), sphingomyelins (SM), lysophosphatidylcholines (LPC) from egg yolk,
145 phosphatidylinositols (PI) from bovine liver, phosphatidylserines (PS) from bovine brain, Cardiolipins (CL)
146 from bovine heart, squalene (SQ), cholesteryl palmitic acid (CE(16:0)), methyl-nonadecanoic acid (FAME),
147 tristearic acid triglyceride (TG(18:0/18:0/18:0)), cholesterol (Chol), diacylglycerols (DG(18:2/0:0/18:2) and
148 DG(16:0/16:0/0:0)), stearic acid (FA(18:0)), monoacylglycerol (MG(18:0/0:0/0:0)) were purchased from
149 Sigma-Aldrich (St. Quentin Fallavier, France). Monogalactosyldiglycerols (MGDG) and
150 digalactosyldiglycerols (DGDG) (vegetable origin) was from Avanti Polar (Coger, Paris, France). Wheat
151 Glucosylceramides (GlcCer) were purchased from Extrasynthese (Genay, France). Acylated sterol
152 glycosides (ASG (18:2-Glc-Sitosterol)) came from Larodan (Limhamn, Sweden) Ceramides: CerII
153 (Cer(d18:1/18:0)), CerIIIb (Cer(t18:0/18:1)), CerVI (Cer(t18:0/18:0(2OH))), were purchased from Degussa
154 (Hanau, Germany) and CerV (Cer(d18:1/18:0(2OH))) from Matreya (State College, PA USA). Stock
155 solutions (5 g/L) of each standard compound were prepared in chloroform.

156 Table 1 presents detailed information about the lipid species composition of standards.

157 The lipid nomenclature follows the LIPID MAPS system and the shorthand notation for lipid structures [15].
158 When no abbreviation can be found for a specific lipid class or subclass in the LIPID MAPS nomenclature,
159 commonly used abbreviation was employed (squalene (SQ), cholesterol (Chol), fatty acids methyl esters
160 (FAME)). A list of abbreviations is proposed in table S-1.

161 2.3. Samples

162 Total lipid extracts from heart, brain and liver (all from bovine origin), *Escherichia coli*, yeast
163 (*Saccharomyces cerevisiae*) and soybean were purchased from Avanti Polar (Coger, Paris, France).

164 Lipowheat® oil is the food grade ingredient from ROBERTET Health & Beauty (Formerly HITEX, Vannes,
165 France). This sample comes from the ethanolic extraction of bakery wheat gluten.

166 Each sample is prepared in chloroform at the concentration of 5 g/L for analysis and storage.

167 2.3.1. Lipid test mixtures

168 Test mixture 1 is composed by 22 lipid standards: SQ, CE, FAME, TG, Chol, DG 1-3, DG 1-2, FA, MG,
169 ASG, MGDG, GlcCer, DGDG, PG, PE, PA, PI, CL, PS, PC, SM and LPC at an individual concentration of
170 0.5 g/L.

171 Test mixture 2 is a mixture of wheat oil (Lipowheat® oil) and soya lecithin supplemented with Chol in
172 (9:9:0.6, v/v) proportion with a total lipid concentration of 10 g/L.

173 Ceramides test mixture 3 is made of CerII, CerIIIb, CerV and CerVI sub-classes at an individual
174 concentration of 0.5 g/L.

175 Test mixture 1, 2 and 3 were prepared by mixing the appropriate stock solutions. After mixing all standards,
176 the solution is evaporated under a stream of nitrogen and dissolved in mobile phase A: chloroform (4:1 v/v)

177 2.4. Apparatus

178 Separation of lipids was performed with an Inertsil Si 5 μ m (150 mm \times 2.1 mm I.D.) column (GL Sciences
179 Inc., Tokyo, Japan) thermostated at 40°C. The HPLC instrumentation consisted of an Agilent system, 1050
180 injector and a 1260 pump (Agilent Technologies, Santa Clara, CA, USA). An overpressure of 0.2 bar was
181 applied to the mobile phase A reservoir. The 0.2 bar overpressure is set by a pressure regulator and the
182 nitrogen line connected to the solvent bottle via a solvent pressurization kit obtained from Thermo Fischer
183 Scientific. This kit consists in an adapted bottle cap and all necessary connections.

184 Two universal detectors, ELSD (Eurosep, Cergy, France) and Corona CAD® systems (both from ESA,
185 Chelmsford, MA, USA) are compared; the signal was acquired with a Chromeleon data station (Thermo
186 Fisher Scientific, Villebon-sur-Yvette, France). ELSD settings: nebulizer temperature 35°C, drift tube 45°C,
187 photomultiplier 600 and air pressure 1.5 bars. Corona-CAD® settings: range 500pA, filter none and air
188 pressure 35 psi.

189 MS analyses were performed with a LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific). The MS² and MS³
190 spectra were obtained in data dependent acquisition (DDA) mode. The LTQ-Orbitrap Velos Pro mass
191 spectrometer was equipped with an H-ESI II probe / and a combined APCI/APPI ion source.

192 2.5. Chromatographic method

193 The mobile phases and the solvent program are presented in table 2. The flow rate was set at 0.8 mL.min⁻¹.
194 The injected volume was 2 μ L.

195 2.6. MS method

196 H-ESI II probe: Spray voltage was set at 3.3 kV. Heater temperature of the probe was set at 300°C. Sheath
197 gas, auxiliary gas, and sweep gas flow rates were set at 40, 20, and 0 (arbitrary unit) respectively. Capillary
198 temperature was set at 350°C and S-lens RF level at 60%. Analysis was performed in negative and positive
199 ion mode.

200 APCI/APPI ion source: Corona needle voltage in APCI mode was set at 6 kV. Vaporizer temperature of the
201 probe was set at 400°C. Sheath gas, auxiliary gas, and sweep gas flow rates were set at 40, 10, and 0
202 (arbitrary unit) respectively. Capillary temperature was set at 325°C and S-lens RF level at 60%. Analyses
203 were performed in negative and positive ion mode.

205 **3. Results and Discussion**

206 There are two ways to understand the different NPLC methods developed for lipid class analysis and their
207 progress over time: either by the sample origin or by the stationary phase and solvent program.

208 Table 3, summarize the successive evolutions of Christie's method by the authors quoted in the introductory
209 part of the manuscript. Chloroform, a CMR (carcinogenic, mutagenic, or toxic for reproduction) solvent was
210 replaced by dichloromethane, itself substituted by the less toxic and peek-tubing compatible ethyl acetate.

211 IPA was replaced by acetone to improve the sterols (ST)/DG chromatographic selectivity. AA and TEA
212 were added at the end of the gradient program to enhance phospholipids (PL) analysis. In the same way, the
213 addition of 0.2% (v/v) of ethyl acetate in isooctane appeared to improve the separation of early eluting
214 lipids. Other modifications/improvements were made to help instrument performances. IPA was used as a
215 rinsing solvent, prior the equilibration with isooctane and to avoid pumping failure with this low viscosity
216 solvent that can be encountered with some pumping devices.

217
218 The mobile phases used in Graeve's [11] and Gerits' [12] methods differ by the presence of 0.2% (v/v) of
219 ethyl acetate in isooctane, the ionic modifiers concentration and the addition of IPA as rinsing solvent (table
220 3). In the present study, we intended to appreciate and exemplify the impact of these differences on the
221 chromatographic separation of lipid classes. The main goal being to achieve the highest degree of separation
222 allowing the characterization of biological lipid extracts from various origins (animal, vegetal, yeast,
223 bacteria). For this purpose, test mixture 1 composed of 22 lipid standards and test mixture 2 were
224 systematically chromatographed. Ceramides are biologically relevant lipids which have not been considered
225 in refs [11,12]. We used test mixture 3, to appreciate the impact of the changes in chromatographic
226 conditions on the retention of several compounds of this class.

227 The different methods listed in table 3 use ELSD as detection principle. In this study, we compared the
228 chromatographic profiles achieved by both ELSD and Corona-CAD[®]. Those two quasi-universal detectors
229 allow quantitation but are unable to provide any structural information. As this later aspect is mandatory in
230 lipidomic profiling of biological extracts, we assessed the MS response when coupling our separation
231 through three ionization sources, namely ESI, APCI and APPI.

232 Finally, 7 total lipid extracts from different origins (3 animal, 2 vegetal, 1 from yeast and 1 from bacteria)
233 were analyzed with the method presenting the best resolution between the lipid classes. In order to
234 appreciate the versatility of the method we briefly compared the results obtained with each extract to
235 information from the literature.

236 3.1. Mobile phase composition

237 3.1.1. Mobile phase A

238 Isooctane purification

239 In an attempt to reduce the impurity level, isooctane was flowed through a semi-preparative column packed
240 with Lichrospher Si-60, 5-20 μm bare silica prior its use in mobile phase [16–18].

241 Eluent pressurization

242 Gerits introduced the IPA rinsing to prevent pressure fluctuation encountered at the beginning and at the end
243 of the solvent program. From our experience, such phenomenon is more or less pronounced depending on
244 the equipment used and is encountered with highly compressible and low viscosity solvents such as alkanes.
245 A slight pressurization (0.2 bar) of the solvent reservoir minimize the pumping difficulties encountered with
246 alkanes.

247 Ethyl acetate

248 Graeve's method [11] allows the best separation of the most nonpolar lipids (SQ, SE (sterol ester), WE (wax
249 ester), FAME, DAGE (diacylglycerol ethers) and TG). Mobile phase A composition is isooctane:ethyl
250 acetate (99.8:0.2, v/v). Ethyl acetate was preferred to tetrahydrofuran (THF) initially proposed by Christie or
251 Homan since it is compatible with peek tubing [19]. The lower polarity and the selectivity change induced
252 by this solvent allow an improved separation of SQ, SE and WE.

253 Gerits [12] uses pure isooctane. Unfortunately, the most nonpolar lipids cannot be seen on its
254 chromatograms that start at 5 min after the injection. In order to appreciate the exact role of the 0.2% (v/v)
255 ethyl acetate addition in mobile phase A, test mixtures 1 and 2 were analyzed with a pure or modified phase
256 A (figure 1). Adding 0.2% (v/v) ethyl acetate only influences the selectivity between lipid classes eluted
257 during the first 5 minutes of the chromatogram. Both SE and FAME are less retained and show a broader
258 peak. Additionally, the resolution between the dead volume and SQ decreases. The chromatograms from
259 natural samples mixture show an improved separation of nonpolar compounds eluted during the 5 first
260 minutes. Co-elutions with solvent impurities are also limited.

261 To summarize, the addition of 0.2% (v/v) ethyl acetate enhances the discrimination of nonpolar lipids.
262 Alternatively, when SQ is a compound of interest, only pure isooctane used as initial mobile phase allows its
263 retention.

264 3.1.2. Mobile phase B

265 The mobile phase B, as optimized by Gerits for the analysis of medium polarity lipids (MG, MGDG and
266 DGDG), contains 20 fold more AA than the mobile phase proposed by Graeve [11]. In order to highlight the
267 effect of AA on lipid classes retention, three levels were compared and presented in figure 2: 0.02%
268 (Graeve), 0.15 % (v/v) (this work) and 0.4% (Gerits).

269 The retention of FA decreases with increasing concentration in AA in the mobile phase. This is probably due
270 to the competition between AA and FA for the adsorption onto the stationary phase. With 0.02% AA (v/v),
271 the FA peak co-elutes with a system peak and at 0.4% (v/v) FA and DG 1,3 co-elute. Among the tested
272 concentrations, 0.15% AA (v/v) offers the best compromise for the elution of FA and their resolution with
273 adjacent peaks.

274 In addition, AA concentration in mobile phase B has a noticeable impact on the second part of the
275 chromatogram. 0.15% AA (v/v) is also an optimum concentration for the elution of PL at the end of the
276 chromatogram as advisable from chromatographic profile of test mixture 1 in figure 2. Alternatively, using
277 0.4% AA (v/v) is interesting to improve the chromatographic profile of lipids eluting between DGDG and
278 PL.

279 We decided to favor FA retention and PL separation and thus, to use the intermediate 0.15% AA (v/v)
280 concentration in the solvent program.

281 3.1.3. Mobile phase C

282 Homan [13] first introduced a mobile phase C composed by IPA and water (85:15, v/v) with an equimolar
283 amount of AA and ethanolamine (EA) at 7.5 mM. The same C mobile phase is used by Graeve [11]. The
284 experimental conditions of Gerits [12] are close to those of Homan, except EA is replaced by TEA. The
285 mobile phases proposed by Homan [6] don't incorporate any ionic modifier. In order to understand the
286 influence of the ion pair concentration and volume ratio in mobile phase C, a separation of test mixtures 1
287 and 2 with A: 0.05% (v/v) AA and TEA, B: 7.5 mM AA and TEA (0.043% (v/v) AA and 0.104% (v/v)
288 TEA) C: neither AA nor TEA is presented in figure 3.

289 The AA/TEA relative amounts do not affect the lipids eluted at the beginning of the chromatogram with the
290 notable exception of FA. When absent or in equimolar ratio, FA retention is unaffected.

291 When the same volume of AA and TEA e.g. 0.05% (v/v) is added, the molar excess of AA represents the

292 addition of 0.025% (v/v) AA in the mobile phase. Although being present in the C mobile phase and despite
293 of the rinsing phase D, the AA amount influences the FA retention at the beginning of the chromatogram. In
294 these conditions, the AA amount in phase C is responsible for the co-elution of FA and Chol.
295 The relative amounts of AA and TEA influence polar lipid (PL) retention and selectivity. The best
296 separation is obtained with an equimolar amount of AA/TEA in phase C.

297 3.1.4. Mobile phase D

298 Graeve's method [11] doesn't make use of a particular rinsing solvent at the end of the solvent program.
299 After the phase C step, the solvent program goes back to the B phase and then re-equilibration is performed
300 using the A phase. The low viscosity of isooctane together with the high permeability of monolithic silica,
301 allow doubling the flow rate during the equilibration step. Gerits [12] introduced IPA as rinsing solvent and
302 indicated that this rinsing step improves the subsequent pumping of isooctane. As already underlined, this
303 step influences the first part of the chromatogram.

304 In this study, a rinsing step was introduced with ethyl acetate, an intermediate polarity solvent, in order to
305 facilitate equilibration while favoring the retention of nonpolar lipids at the beginning of the chromatogram.
306 As indicated in table 2, the rinsing step consists in a 50:50 mixture between mobile phase A and D. The
307 chromatograms of test mixtures 1 and 2 obtained with 3 rinsing conditions are presented in figure 4. When
308 no rinsing is performed (Fig 4.B), mobile phase D composition is identical to mobile phase A. In figure 4.A
309 and 4.C, IPA and ethyl acetate are respectively used.

310 As seen in Figure 4.A the use of IPA as rinsing solvent is not compatible with a proper retention of neutral
311 lipids at the beginning of the chromatogram. Indeed, we can hypothesize that the IPA adsorption energy is
312 so high that this solvent cannot be readily driven out the silanols of the silica by isooctane.

313 Repeated experiments have shown that the ethyl acetate rinsing step (Fig 4.C) gives a superior result
314 compared to an increase in equilibration time performed with mobile phase A (Fig 4 A). In the first part of
315 the chromatogram, SQ, CE, FAME and TG are more retained. The retention time of FA is slightly increased
316 but the influence of the ethyl acetate rinsing step is especially noticeable on peak geometry with FA eluting
317 as a sharp and intense peak.

318 It is also important to note that the rinsing step can have an influence on the most polar lipids of the
319 chromatogram. The PL profile in figure 4.A using IPA is noticeably different than the profiles obtained with
320 mobile phase A (Fig 4.B) and ethyl acetate (Fig 4.C).

321 Solvent consumption is directly impacting analysis cost and solvent rejection being an environmental and
322 safety issue, it is worthy to note that the method we propose use about 40% less solvent than refs [10-11].

323 The total solvent volume of our method is 42 mL, 32 mL for the solvent program plus 10 mL for rinsing and
324 equilibration whereas it is 68 mL when using the methods described in refs [10-11].

325 Medium polarity lipids (Ceramides) The aforementioned publications do not relate to ceramides. This lipid
326 class is frequently investigated in lipidomic studies, especially in skin research where a vast diversity is
327 encountered [23–26]. We considered important to evaluate the elution window of the ceramide subclasses
328 and to assess the possible co-elution with other lipid classes. Cer elute in the area of medium polarity lipids
329 with retention times intermediate between MG and MGDG (figure 6). ASG co-elutes with the first peak of
330 CerVI. CerV elutes as a double peak. CerV, a synthetic ceramide, is a racemic mixture of two epimers N-
331 (R,S)-alpha-Hydroxyoctadecanoyl-D-erythro-sphingosine. These two peaks presumably correspond to the
332 epimers. Bare silica had already proven to separate epimers, as for example sterols [27]. No information is
333 available about the stereochemistry of CerVI, but, as for CerV, its elution as two distinct peaks may be due
334 to the occurrence of two epimeric forms. Unlike in CerV and CerVI, carbon at position 2 of the fatty acyl
335 chain of CerII is not a chiral center. The double peak encountered with this compound is presumably due to
336 the Z and E configuration of the double bond of the fatty base moiety. To facilitate understanding,
337 drawings of the chemical structures are presented in figure S-1. The MS spectra of the 3 couples of peaks are
338 commented further in the document (ESI, APCI and APPI comparison).

340 3.2. ELSD / Corona-CAD[®] comparison

341 ELSD has gained a wide acceptance in lipid class analysis since the pioneering work of Robinson and
342 Macrae [20] at the beginning of the '80s. Indeed, ELSD is a nebulization-based detector that is compatible
343 with gradient elution and allows detecting any solute less volatile than the mobile phase. The Corona-CAD[®],
344 introduced in 2004 [21], shares the same operating principle except aerosol charging is used instead of the
345 light diffusion exploited in the ELSD.

346 Figure 5 presents the chromatograms of test mixtures 1 & 2 at 0.5 g/L (2 μ L injected) using both detectors.
347 Corona-CAD[®] appears to be more sensitive to the baseline drift phenomenon occurring from the gradient
348 program. The chromatogram of lipid standard at equal concentration (test mixture 1) shows more consistent
349 peak heights with Corona-CAD[®] than with ELSD. This can be explained by the typical response functions
350 of both detectors [22]. With ELSD, when Rayleigh scattering is the dominant phenomenon, the sensitivity
351 increases with increasing amount of solutes. From our experience, this is the case when narrow bore column
352 and reduced flow rates are used. Consequently, the peak intensities of minor compounds appear to be less
353 than their actual quantity. At the opposite, major compounds benefit from the increased sensitivity and their
354 peak intensities dominate the chromatogram. The response function of the Corona-CAD[®] is more consistent
355 among injected quantities and, at least, the sensitivity decreases for high solute amounts. Therefore, with the

356 Corona-CAD[®], the chromatographic profile is more likely to reflect the relative amounts of solutes involved
357 in complex samples such as mixture 2.

358 3.3. ESI, APCI and APPI comparison

359 Although combined positive-negative ionization mode could be achieved by the mass spectrometer, distinct
360 positive and negative profiles were recorded with the three ion sources to highlight the different behavior
361 of some lipid classes (figure 7).

362 ESI does not give any signal at the beginning of the chromatogram in either positive or negative ionization
363 mode. Only polar lipids are detected but with an unfavorable background noise. Coupling the present NPLC
364 method with ESI cannot be recommended.

365 The chromatograms acquired with either APCI or APPI showed similar profiles. In positive ion mode, peak
366 intensities of ionized lipid classes appeared to be similar. On the contrary, APPI delivered a 4 fold more
367 important signal than APCI in negative mode. APCI is much more readily available in mass spectrometry
368 platforms than APPI and can conveniently be used with this method. However, APPI will be preferred if
369 available. For example, the Cer class is detected with a ≈ 20 fold more intense response in negative APPI
370 detection compared to positive APPI (figure 8). This finding corroborates the fact that APPI appears to be
371 particularly interesting for lipid analysis [29], well suited to NPLC solvent conditions [30] and offering a
372 high sensitivity for lipid analysis [31].

373 The three API sources were compared by Imbert et al. [14] by coupling the Graeve's method [11] to mass
374 spectrometry for the analysis of *Leishmania donovani* lipids. In this previous study from our group, the
375 number of lipids classes was twice as less. In addition, when comparing chromatographic profiles, the
376 separation appears to be poorer than in the present study. It is worthy to note that mobile phases contain AA
377 and TEA in proportions that are different from the present study and this presumably influence ionization. In
378 addition, Imbert and al. employed an acetone post-column addition as dopant for APPI ionization. In the
379 present study, such addition did not improve ionization. The presence of 0.2% (v/v) ethyl acetate in the
380 initial mobile phase presumably plays this role. The effect of eluent composition on the ionization efficiency
381 of ESI, APCI and APPI in LC-MS was studied in [32].

382 Figure 9 contains a selection of MS spectra obtained for 4 standards that illustrate characteristic ions
383 obtained with the 3 API sources. Supplementary figure S-2 presents the LC/MS full scan spectra of all
384 standards considered in this study in either positive or negative ionization mode with ESI, APCI and APPI
385 interfaces.

386 3.3.1. CerIIIb : Cer(t18 :0/18 :1)

387 The three API sources produced similar ions for CerIIIb. The full scan spectra of other Cer standards
388 presented similar ionization patterns.

389 *In negative mode* [33,34], the most abundant ion was $[M-H]^-$ whereas the AA adduct $[M-H+CH_3COOH]^-$
390 was also observed. With ESI, the AA adduct represented 80% relatively to the $[M-H]^-$ but only 20% when
391 APCI and APPI were used. The total ion count (TIC) was low with ESI ($2.6E4$) whereas it was more
392 important with APCI and APPI. APPI provided a 2 fold more intense signal than APCI.

393 *In positive mode*, the most abundant ion was $[M+H]^+$ and was accompanied by the $[M+H-H_2O]^+$ that
394 correspond to the loss of a water molecule. This in source fragmentation was more pronounced with APPI
395 and less important with ESI. The TIC remained low in ESI ($6.3E4$) and more important with APCI and
396 APPI. At the opposite of the negative mode, the TIC was 2 fold more important with APCI than APPI.

397 The MS spectra obtained in full scan, MS^2 and MS^3 for each couple of peaks encountered with CerV and
398 CerVI were identical (figure S-3). The MS spectra of the two peaks of CerII showed the same ions but with
399 different relative intensities. This supposes quasi-identical structures and reinforces the hypothesis of the
400 epimeric forms separation of CerV and CerVI and the stereoisomerism of CerII.

401 3.3.2. ASG(18:2-Glc-Sitosterol)

402 *In negative mode*, the base peak was $[M-H+CH_3COOH]^-$ with the 3 ionization sources. Here also, APPI
403 provided a 3 fold more intense signal than APCI, whereas ESI exhibited a low TIC ($1.2E4$).

404 *In positive mode* [35], the ASG ions could not be retrieved from the ESI LC/MS profile. The most abundant
405 ion corresponds to $[M-FA-sugar+H+H_2O]^+$ and was 3 fold more intense with APPI compared to APCI.

406 3.3.3. PA from egg yolk, PA(16:0_18:1) as most abundant specie.

407 The adducts and fragmentation patterns observed with PA were also encountered with other PL (PG, PE, PI
408 and PS) [36,37].

409 *In negative mode*, the base peak was $[M-H]^-$ with all API sources. The TIC was comparable between ESI
410 and APCI (about $1E7$) and 4 fold more abundant with APPI.

411 *In positive mode*, the observed ions with ESI were different from the two other sources. TEA adducts were
412 observed with ESI as $[M+TEA+H]^+$ et $[M+2TEA+H]^+$. With both APCI and APPI, the major ion
413 corresponded to the loss of the polar head of the PL $[M+H-polar\ head]^+$ and a $[M+Na]^+$ of lesser intensity
414 was also observed.

415 3.3.4. PC from egg yolk, PC(16:0_18:1) as most abundant specie

416 The adducts and fragmentation patterns observed with PC were also encountered with other PL (SM and
417 LPC) [36,37].

418 *In negative mode*, ESI spectra were different from the two others and exhibited a predominant AA adduct.
419 With APCI and APPI, a radical ion $[M^\circ]^-$ was observed together with a $[M-CH_3]^-$ that correspond to the loss
420 of a methyl group. The signal intensity was comparable between ESI and APCI (about 3E6) and 5 fold more
421 intense with APPI.

422 In positive mode, the major ion was $[M+H]^+$ with ESI and no fragment ion was observable. With APCI and
423 APPI, the major ion was a $[M+H\text{-polar Head}]^+$ and $[M+H]^+$ was also observed. The signal intensity was
424 comparable between the 3 API sources.

425 It is important to note that, whereas APCI and APPI induce more fragments than ESI, the structural
426 information is preserved by combining both positive and negative mode of ionization.

427 3.3.5. The particular case of TG

428 Tristearin or TG(18:0/18:0/18:0) was arbitrary chosen to represent the TG class. The LC/MS tristearin
429 spectra obtained with APCI and APPI in positive mode showed an unique $[M+H\text{-FA}]^+$ ion whereas in TG
430 spectra from lipid samples, the $[M+H\text{-FA}]^+$ coexisted with the $[M+H]^+$. TG from natural oils are often
431 characterized using the $[M+H]^+$ ion [17,18]. In most publications, saturated TG are not reported. Saturated
432 TG are indeed only present in very low amount in natural oils. A systematic study of TG fragmentation
433 patterns using LC/APCI-MS shows that for saturated TG the $[M+H]^+$ ion is not observed [40]. For
434 TG(18:1/18:1/18:1) or TG(16:1/16:1/16:1)) the $[M+H]^+$ is weak and accounts for only 7% of the base peak
435 $[M+H\text{-FA}]^+$. The $[M+H]^+$ ion becomes the major ion of the spectrum when the degree of unsaturation
436 increases.

437 To summarize, APPI appeared to be the best-suited interface for coupling the present NPLC lipid classes
438 separation to mass spectrometry. A simplified list of ions encountered in positive and negative ion mode
439 APPI is presented in table 4. This table was built to facilitate the identification of lipid classes in different
440 sample types. In most cases, the lipid classes correspond to analytical standard. In some instances, when the
441 standards are not readily available, the reported ions correspond to lipid structures identified in natural
442 samples. For each lipid class or subclasses, the ions observed for a representative specie are reported
443 together with its molecular mass, retention time and adduct(s) observed. By working with the two ionization
444 modes, all the lipid classes studied herein could be detected. It is easily possible to trace back to the lipid
445 specie parent structure despite of the in source fragmentation.

446 3.4. Chromatographic profiles of 7 total lipid extracts from different samples

447 All total lipid extracts available from Avanti Polar were studied: 3 from animal, 2 from plants and one from
448 yeast and bacterium (figure 10).

449 3.4.1. Heart / Liver / Brain

450 Homan [13] studied the three animal tissues, Christie [10] and McLaren [9] studied the heart and liver, and
451 Holčapek's group [6] the brain. The three animal samples exhibit similar lipid profiles. TG, Chol, FA are
452 found in variable quantities according to the animal tissue considered. PE and PC are the major
453 phospholipids. Heart shows a higher amount of CL whereas brain contains high quantities of cerebrosides
454 (HexCer and HexCer(OH)) that are not present in other tissues. The lipid profiles obtained in the present
455 study are similar to those shown by Christie [10] or Homan [13]. The method proposed in herein presents a
456 better retention of CE and allows quantifying them if needed. In McLaren's study, the aim was to analyze
457 the major lipids with the shortest possible analysis time. Consequently, the chromatogram duration is only
458 10 minutes but low concentration lipids are not observable in the chromatograms of real samples. This is
459 possibly a consequence of the reduced amount of sample injected. As the method was developed with only 7
460 different lipid standards, it is difficult to appreciate the real ability of this method to separate a complex total
461 lipid extract. In contrast, the UHPSFC-MS profile of pork brain presented by [6], shows a very detailed
462 separation. All the lipids we evidenced in our profiles (except SQ) were found and additional lipids in low
463 amount were identified.

464 UHPSFC-MS appears to be a very promising technique for total lipid extract analysis together by its high
465 efficiency and high throughput. It is also worthy to note that this technique only uses supercritical CO₂ with
466 methanol and water as co-solvent or make-up fluid and is much more environment friendly than usual NP-
467 HPLC methods. At the present time, this equipment is scarcely present in the laboratories but these uses
468 should grow for both high throughput and extensive analysis.

469 3.4.2. Soybean / Wheat

470 The two vegetal samples soya and wheat are also the most complex samples involved in this study in term of
471 lipid composition. They are particularly rich in lipid of intermediate polarity typical from vegetal such as
472 ASG, SG, GlcCer and DGDG. Soya lecithin and wheat were already extensively studied in our laboratory
473 while developing separations with the poly(vinyl)alcohol stationary phase [41] and ASG, SG, GlcCER,
474 DGDG, PI, PE and PC were identified.

475 Soya lecithin is particularly rich in PL and most studies focus on these classes. An example of extensive
476 characterization using phosphorus 31 nuclear magnetic resonance can be found in [42] and the lipid classes
477 reported are PC, PE, PI, PA, PG, N-acylphosphatidylethanolamines (NAPE), LPC, diphosphatidylglycerol
478 (DPG), PS and lysophosphatidic acids (LPA) (by increasing order of magnitude in the sample). The

479 chromatographic profiles in figure 10 show that, whereas the main classes are identified, our method only
480 fails at detecting the quantitatively minor lipid classes. Those minor classes (LPC, DPG, PS and LPA) are
481 less than 0.8% mol/100 mol of phosphorylated lipids.

482 Concerning wheat lipids, the entirety of the lipid classes involved in Gerits' publication [12] are resolved in
483 our conditions. In addition, 6 extra lipids are identified (SQ, SE, ST, ASG, SG and GlcCer) and 4 unknown
484 peaks are resolved from the lipid classes. Compared to previously published solvent programs, the present
485 chromatographic conditions allow an appropriate retention of the nonpolar lipid classes (SQ and SE)
486 together with an improved separation in the intermediate polarity lipids area which allows to detect ST,
487 ASG, SG and GlcCer while preserving the resolution of PL classes.

488 3.4.3. Yeast (*Saccharomyces cerevisiae*)

489 *Saccharomyces cerevisiae* is often used as a model organism and there is abundant information concerning
490 its metabolism. The lipid profile of yeast (*Saccharomyces cerevisiae*) was not studied by the authors we
491 compared with and we referred to other bibliographical sources to ascertain the level of information provided
492 by our method. The yeast lipidome was investigated by shotgun mass spectrometry in ref [43] with a special
493 attention paid to lyso forms of the PL classes. Ref [44] also examines yeast lipidome and presents consistent
494 results (only Cer are found in addition to the lipids of Ref [43]). The main lipid classes highlighted in these
495 articles were TG, DG, Cer and ST and the phospholipids PA, PS, PE, PC, PI, PG, CL together with their
496 lyso forms. Inositol phosphoceramide (IPC) derivatives (IPC, Mannosylated-IPC and Man(IP)₂C) were
497 found in notable but also variable amount in *S. cerevisiae* wildtype stains.

498 Our method allowed identifying the main lipid classes (except PS and the lyso-forms, which was not in
499 noticeable quantity in our sample) but not the IPC derivatives. This result compared favorably with the
500 NPLC/Corona-CAD[®] of ref [45]. SQ, coenzyme Q6 and PG are detected in our chromatographic profile and
501 are not reported in [45].

502 Neither our method nor [45] allow to detect IPC derivatives. Except in the 12-14 min range no unidentified
503 peak could be detected and this elution window does not correspond to the IPC derivatives that, from our
504 experience [46], should elute near the PL elution windows. We unsuccessfully performed an in depth
505 examination of the APPI profiles searching for the quasi-molecular and possible adduct ions of the main
506 species of IPC and its derivatives. IPC derivatives are cell messengers and appear to increase dramatically as
507 a response to a stress or a change in the culture media (temperature for [44], AA for [47]). Their levels are
508 possibly too low to be detected in our sample. These compounds are also ionized very differently according
509 to the ionic modifiers added to the mobile phase both with respect to the adducts observed and their
510 intensities [36,41]. A detailed investigation of IPC derivatives detection under our mobile phase composition
511 conditions would be necessary but is out of the scope of this work.

3.4.4. Bacteria (*Escherichia coli*)

As for yeast, no NPLC of the *E. coli* lipids was found and the characteristic composition of these bacteria had to be retrieved from the literature. The typical composition as mentioned by the lipid standard supplier Avanti Polar is rather simple and *E. coli* extract contains 57.5% PE, 15.1% PG, 9.8 CL and 17.6% of non-identified lipids. Coenzyme Q8 [49] is not listed but is also present and signaled as being obtained from *E. coli* extract by the same supplier. Lipid A which is involved in the toxicity of Gram negative bacteria is also present in *E. coli* but wasn't detected in our experimental conditions as the m/z of its molecular ion is beyond the mass range of the full scan detection (m/z 1700). This lipid composition was confirmed by different publications using mass spectrometry [50–52] and our separation appeared to highlight the main component of the *E. coli* extract.

4. Conclusions

Most of NPLC methods for lipid classes analysis were developed to separate lipids issued from animal samples. Fewer methods were developed to assess the higher complexity of vegetal samples. Some plant lipids such as ASG, MGDG, SG and DGDG present an intermediate polarity and increase the sample complexity in this region of the chromatogram.

The method we propose compares favorably with those developed for animal tissues [9–11,13] and presents an improved separation for vegetal samples [12]. This method, developed using lipid classes of intermediate polarity is also interesting to separate ceramides.

NPLC method development is generally considered as difficult and requiring a specific skill. We decided to add information about the different issues solved during this development. Particularly the use of nonpolar, low viscosity solvent such as isooctane and the use of a pressurized solvent tank to circumvent the pumping difficulties encountered. In addition, the presence of impurities in isooctane led us to re-purify this solvent using a semi-preparative silica column. We also focused on the rinsing step and the importance of using ethyl acetate to both eliminate the C mobile phase from the stationary phase and favor the re-equilibration.

NPLC solvent programs frequently incorporate complex mobile phases. In this study, we documented the importance of adding 0.2% (v/v) ethyl acetate in the initial mobile phase. Also, the importance of the relative amounts of AA and TEA in the mobile phase on the retention of some specific lipid classes such as FA in the first part of the chromatogram but also the PL classes at the end and the compromise to be found to ensure a correct selectivity.

542 The comparison of the detection systems that are both heavily solvent dependent reinforce the interest of the
543 Corona-CAD[®] thanks to its increased sensitivity at low concentration compared to the commonly used
544 ELSD. When comparing the API interfaces under NPLC conditions, ESI did not succeed at detecting the
545 whole lipids in the chromatogram as signal extinction occurs at the beginning of the chromatogram. We
546 have shown that for lipids such as PL, in positive mode APCI and APPI present more in source
547 fragmentation compared with ESI. In negative mode, the in source fragmentation is comparable with the
548 three API sources. By using both positive and negative mode, structural information is visible in full scan
549 spectra. Coupling with APCI provided satisfactory results but APPI is generally more sensitive and does not
550 require any dopant make-up in our chromatographic conditions.

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- 695

Table 1. Information about the lipid species composition of standards.

SQ-squalene, CE-cholesteryl esters, FAME-fatty acids methylesters, TG-triacylglycerols, Chol-cholesterol, DG-diacylglycerols, FA-fatty acids, MG-monoacylglycerols, Ceramides (CerII (Cer(d18:1/18:0)), CerIIIb (Cer(t18:0/18:1)), CerV (Cer(d18:1/18:0(2OH)) and CerVI (Cer(t18:0/18:0(2OH))))), ASG-acylated steryl glycosides, MGDG-monogalactosyldiglycerols, SG-steryl glycosides, GlcCer-glucosylceramides, DGDG-digalactosyldiglycerols, NAPE-N-Acylphosphatidylethanolamines, PG-phosphatidylglycerols, PE-phosphatidylethanolamines, PA-phosphatidic acids, PI-phosphatidylinositols, CL-cardiolipins, PS-phosphatidylserines, PC-phosphatidylcholines, SM-sphingomyelins, LPC-lysophosphatidylcholines.

A

Monomolecular lipids standards							
Lipid Class	Isoprenoids	ST	FA	TG	DG	MG	Cer
Subclasses (spectes)	SQ	CE(16:0)	FAME(methyl- nonadecanoic acids)	TG (18:0/18:0/18:0)	DG (18:2/0/0/18:2)	MG (18:0/0/0/0:0)	Cer(d18 :1/18 :0)
		Chol	FA(18:0)		DG (16:0/16:0/0:0)		Cer(t18 :0/18 :1)
		ASG (18:2-Glc- Sitosterol)					Cer(d18 :1/18 :0(2OH))
							Cer(t18:0/18 :0(2OH))

B

Composition of natural PL standards (typical composition provided by Avanti Polar)									
Lipid Class	PG	PE	PA	PI	CL	PS	PC	SM	LPC
FA%/origin	egg yolk	egg yolk	egg yolk	bovine	bovine	bovine	egg yolk	egg yolk	egg yolk
	chicken	chicken	chicken	liver	heart	brain	chicken	chicken	chicken
16:0	32.9	17.3	34.2				32.7	86	69
18:0	12.2	24.2	11.5	46		42	12.3	6	24.6
18:1	30.2	18.1	31.5	8	5	30	32		3.4
18:2	18.7	14	18.5	6	90		17.1		0.3
20:3				13					
20:4	3.5	16	2.7	17		2	2.7		
22:6		4.2				11			

C

Natural lipids standards (typical composition provided by Avanti Polar)			
Lipid Class	DGDG	MGDG	Neutral glycosphingolipids
FA\origin	plant	plant	wheat
18:3-18:3	44.5	14.1	GlcCer(d18:2/16:0(OH))
18:2-18:3	10.7	3.2	GlcCer(d18:1/16:0(OH))
16:3-18:3	21.3	66.8	GlcCer(d18:1/20:0(OH))
16:3-18:2	7	12.9	
16:1-18:3	6.9	3	
16:0-18:3	9.7		

D

Other subclasses of lipids found in samples				
Lipid Class	Quinones and hydroquinones	ST	Neutral glycosphingolipids	PE
origin	brain	Wheat	brain	Wheat
Subclasses	coenzyme Q10	SG	HexCer HexCer(OH)	NAPE

Table 2. Quaternary gradient mobile phase composition. A, isooctane:ethyl acetate (99.8:0.2, v/v); B, acetone:ethyl acetate (2:1, v/v) containing 0.15% acetic acid (v/v); C, 2-propanol:water (85:15, v/v) containing 0.043% acetic acid (v/v) and 0.104% trimethylamine (v/v); D, ethyl acetate.

Time (min)	Percent solvent				Flow-rate (mL/min)
	A	B	C	D	
0	100	0	0	0	0.8
1.5	100	0	0	0	0.8
1.6	97	3	0	0	0.8
9	94	6	0	0	0.8
11	70	30	0	0	0.8
14	45	55	0	0	0.8
15	45	55	0	0	0.8
16	40	55	5	0	0.8
20	35	55	10	0	0.8
20.1	33	50	17	0	0.8
25	38	45	17	0	0.8
25.1	48	35	17	0	0.8
30	53	30	17	0	0.8
40	40	0	60	0	0.8
40.1	0	100	0	0	0.8
42	0	100	0	0	0.8
42.1	50	0	0	50	0.8
45	50	0	0	50	0.8
47	100	0	0	0	0.8
53	100	0	0	0	0.8

Table 3. Changes and evolution of the lipid class analysis method from Christie to this work. Abbreviation: acetic acid (AA), 2-propanol (IPA), ethanolamine (EA), trimethylamine (TEA), tetrahydrofuran (THF). The full name of lipids can be retrieved in table S-1. SQ-squalene, SE-sterol ester, CE-cholesteryl esters, FAME-fatty acids methylesters, DGE- diacylglycerol ethers, TG-triacylglycerols, Chol-cholesterol, DG-diacylglycerols, FA-fatty acids, FAlc- fatty acids alcohol, MG-monoacylglycerols, ceramides (CerII (Cer(d18:1/18:0)), CerIIIb (Cer(t18:0/18:1)), CerV (Cer(d18:1/18:0(2OH)) and CerVI (Cer(t18:0/18:0(2OH)))), ASG-acylated steryl glycosides, MGDG-monogalactosyldiglycerols, SG-steryl glycosides, GlcCer-glucosylceramides, HexCER- hexosylceramide, DGDG-digalactosyldiglycerols, NAPE-N-Acylphosphatidylethanolamines, PG-phosphatidylglycerols, DPG- diphosphatidylglycerol, PE-phosphatidylethanolamines, PA-phosphatidic acids, PI-phosphatidylinositols, CL-cardiolipins, PS-phosphatidylserines, PC-phosphatidylcholines, SM-sphingomyelins, LPC-lysophosphatidylcholines.

Author (year)	Christie [10] (1985)	Homan [13] (1998)	Graeve [11] (2009)	Gerits [12] (2013)	McLaren [9] (2011)	Present study (2017)
Column	Spherisorb 3µm Si 100 X 5 mm	Spherisorb 5µm Si 100 X 4.6 mm	Chromolith Si 100 X 4.6 mm	Chromolith Si 100 X 4.6 mm	Halo HILIC 2.7 µm 50 X 2.1 mm	Inertsil 5µm Si 150 X 2.1 mm
Temperature		45°C	40°C	40°C		40°C
Analysis time	30 min	30 min	35 min	35 min	10.5 min	53 min
Flow rate	2 ml/min	1.6 - 2 ml/min	1.4 – 3 ml/min	1.4 – 3 ml/min	1.2 ml/min	0.8 ml/ min
injected Volume	5 µl	10 µl	2 – 60 µl	1 – 50 µl	2 µl	2 µl
injection Solvent	Chloroform:isooctane (1:1, v/v)	Isooctane:THF (9:1, v/v)	Isooctane:ethyl acetate (9:1, v/v) or Dichloromethane:methanol (2:1, v/v)	Isooctane	Isooctane:THF:methanol (9:1:1, v/v/v)	Isooctane:chloroform (4:1, v/v)
Detector	ELSD	ELSD	ELSD	ELSD	ELSD	ELSD / CAD / ESI / APCI / APPI
Solvent A	Isooctane:THF (99:1, v/v)	Isooctane:THF (99:1, v/v)	Isooctane:ethyl acetate (99.8:0.2, v/v)	Isooctane	Isooctane:THF (99:1, v/v)	Isooctane:ethyl acetate (99.8:0.2, v/v) pressurized
Solvent B	IPA:chloroform (4:1, v/v)	Acetone:dichloromethane (2:1, v/v)	Acetone:ethyl acetate (2:1, v/v) + AA (0.02% (v/v))	Acetone:ethyl acetate (2:1, v/v) + 70 mM AA	Acetone:dichloromethane (4:1, v/v)	Acetone:ethyl acetate (2:1, v/v) + 35 mM AA
Solvent C	IPA:water (1:1, v/v)	IPA:water (85:15, v/v) + 7.5 mM AA + 7.5mM EA	IPA:water (85:15, v/v) + 7.5 mM AA + 7.5mM EA	IPA:water (85:15, v/v) + 7.5 mM AA + 7.5mM TEA	IPA:chloroform (80:20, v/v)	IPA:water (85:15, v/v) + 7.5 mM AA + 7.5mM TEA
Solvent D				IPA	IPA:water (1:1, v/v)	Ethyl acetate
Samples investigated	heart, erythrocytes, plasma	heart, liver, brain, Kidney	marine zooplankton	wheat	plasma, liver, heart	heart, liver, brain, yeast, <i>E. coli</i> , soy, wheat
Standards used to optimize the analytical method	CE, TG, Chol, DG, FA, DPG, PE, PI, PS, PC, SM, LPC	CE, TG, Chol, DG, FA, MG, HexCer, HexCer(OH), CL, PE, PI, PS, PC, SM, LPC	SQ, SE, WE, FAME, DGE, TG, FAlc, ST, DG, FA, HexCer, CL, PE, PI, PS, PC, LPC	TG, Chol, DG, FA, MG, MGDG, DGDG, NAPE, PE, PA, PG, NALPE, PI, PC, LPC	CE, TG, FA, Chol, DG, PE, PC	SQ, CE, FAME, TG, Chol, DG, FA, MG, CerII, CerIII, CerV, CerVI, ASG, MGDG, GlcCer, DGDG, PG, PE, PA, PI, CL, PS, SM, LPC
Lipids classes and subclasses identified in samples	CE, TG, Chol, DG, FA, DPG, PE, PI, PS, PC, SM, LPC	CE, TG, Chol, HexCer, HexCer(OH), CL, PE, PI, PS, PC, SM	Sq, WE, DGE, TG, FAlc, ST, FA, HexCer, CL, PE, PI, PS, PC, LPC	TG, Chol, DG, FA, MG, MGDG, DGDG, NAPE, PE, NALPE, PI, PC, LPC	CE, TG, FA, Chol, PE, PC, CL	SQ, SE, Coenzyme, TG, ST, Chol, DG, FA, MG, Cer, ASG, MGDG, SG, GlcCer, HexCer, HerCer(OH), DGDG, NAPE, PG, PE, PA, PI, CL, PS, PC, SM, LPC

Table 4. APPI negative and positive mode: characteristics ions of some lipid species encountered in lipid standards and samples (see text for details). A retention time noted x-y indicates a broad peak and x;y indicates a dual peak. Ions between brackets have an intensity of less than $5 \cdot 10^6$ counts. For PL, the FA indication is not positional.

SQ-squalene, CE-cholesteryl esters, FAME-fatty acids methylesters, TG-triacylglycerols, Chol-cholesterol, DG-diacylglycerols, FA-fatty acids, MG-monoacylglycerols, Ceramides (CerII (Cer(d18:1/18:0)), CerIIIb (Cer(t18:0/18:1)), CerV (Cer(d18:1/18:0(2OH)) and CerVI (Cer(t18:0/18:0(2OH)))), ASG-acylated steryl glycosides, MGDG-monogalactosyldiglycerols, SG-steryl glycosides, GlcCer-glucosylceramides, DGDG-digalactosyldiglycerols, NAPE-N-Acylphosphatidylethanolamines, PG-phosphatidylglycerols, PE-phosphatidylethanolamines, PA-phosphatidic acids, PI-phosphatidylinositols, CL-cardiolipins, PS-phosphatidylserines, PC-phosphatidylcholines, SM-sphingomyelins, LPC-lysophosphatidylcholines.

Lipid species	Rt. (min)	Molecular mass (g/mol)	APPI -					APPI +						
			[M-H] ⁻	[M-CH ₃] ⁻	[M ^o] ⁻	[M-H+CH ₃ COOH] ⁻	[FA-H] ⁻	[M+H] ⁺	[M + Na] ⁺	[M+H-FA] ⁺	[M+H-H ₂ O] ⁺	[M-FA-sugar+H+H ₂ O] ⁺	[Aglycone+H-H ₂ O] ⁺	[Aglycone+H] ⁺
SQ	0.7	410.4	(409.4)					411.4						
CE(16:0)	1.7-2.8	624.6					(255.2)					369.4		
FAME(methyl-nonadecanoic acids)	2.5	312.3					(297.3)	(313.3)						
TG(18:0/18:0/18:0)	4.6	890.8	(889.8)				283.3					607.6		
Coenzyme Q10	4.6	862.7	(861.7)					(863.7)						
Chol	7.4	386.4											(369.4)	
DG(18:2/0:0/18:2)	9.3	616.5	(615.5)			(675.5)	279.2	617.5					599.5	
DG(16:0/16:0/0:0)	10.4	568.5				(627.5)	255.2						551.5	
FA(18:0)	11.4	284.3	283.3				283.3							
Cer II : Cer(d18:1/18 :0)	13.4; 13.7	567.6	566.6				626.6	568.7					550.7	
Cer IIIb : Cer(t18:0/18 :1)	14.2	583.6	582.5				642.6	584.6					566.6	
Cer V : Cer(d18:1/18 :0(2OH))	14.0; 14.9	581.5	580.6				(640.5)						564.6	
Cer VI : Cer(t18:0/18 :0(2OH))	14.5; 15.8	599.5	598.5				(658.5)	600.6					582.5	
MG(18:0/0:0/0:0)	13	358.3					(417.3)	(283.3)	(359.3)				(341.3)	
ASG(18:2-Glc-Sitosterol)	14.5	838.7					897.7	279.2						
MGDG(18:3/16:3)	17	746.5	745.5				805.5	249.2 / 277.2		769.5				397.3
SG(Sitosteryl glucoside)	17.6	576.4					(635.5)							585.5
GlcCer(d18:2/20:0(OH))	19.5	771.6	770.6						(794.6)					(397.3)
HexCer	19	809.7	808.8											(610.6)
HexCer(OH)	19.5	827.7	826.8										792.8	
DGDG(18:3/18:3)	21.8	936.6	935.6				995.6	(277.2)		(959.6)				
NAPE(18:2-16:0-N18:2)	24.4	977.7	(976.7)					(255.2)/(297.3)		(1000.7)				(613.4)
PG(16:0_18:1)	28.7	748.5	747.5					(255.2)/(281.2)		771.5				
PE(18:2_18:0)	29.5	743.5	742.5					(279.2)/(283.3)		(766.5)			[M+H-172] ⁺ = 577.5	
PA(16:0_18:1)	30.7	674.5	673.5					(255.2)/(281.2)		(697.5)			[M+H-141] ⁺ = (603.5)	
PI(18:0_20:4)	33	886.6	885.5					(283.3)/(303.2)		(909.5)			[M+H- 98] ⁺ = 577.5	
CL([18:2/18:2],[18:2/18:2])	34	1449.0	1448.0					279.2		1472.0			[M+H-260] ⁺ = 627.6	
PS(18:0_18:1)	34.6	789.6	788.5					(280.2)/(283.3)		812.5				599.6
PC(16:0_18:1)	35.3	759.6						(255.2)/(281.2)	(760.6)				[M+H-185] ⁺ = 605.7	
SM(d18:1/16:0)	36.4	702.6		744.6	759.6				(703.6)				[M+H-183] ⁺ = 577.5	
LPC(0 :0/18 :0)	38.9	523.4	(508.4)	(523.4)				283.3		(524.4)			[M+H-183] ⁺ = (520.5)	
LPC(16:0/0 :0)	39.6	495.3	(480.3)	(495.3)				(255.2)		(496.3)				

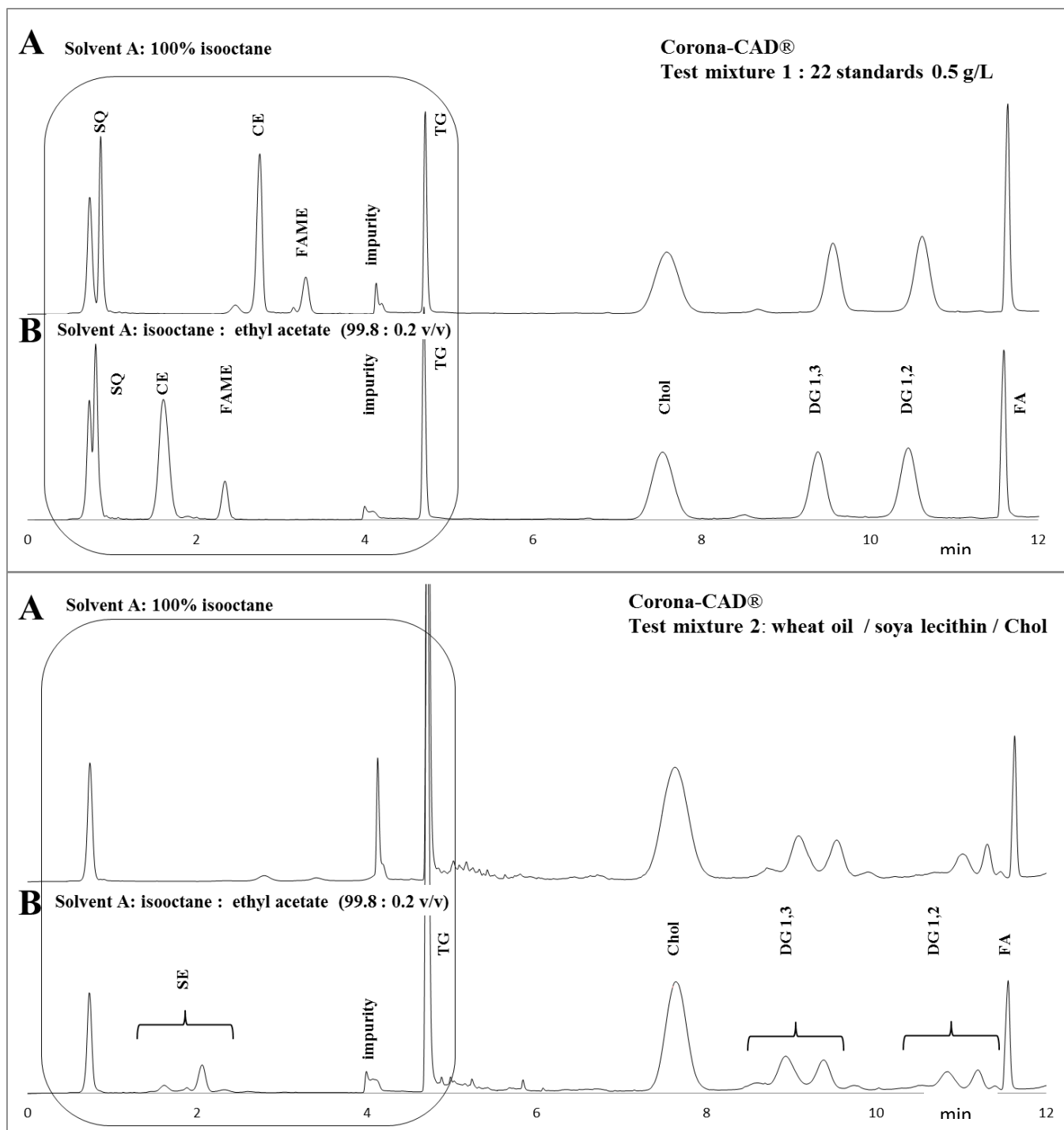


Figure 1. Influence of the initial mobile phase composition on the first part of the chromatogram (0~12 min) for test mixtures 1 (top) and 2 (bottom). (A) 100% isooctane (B) isooctane:ethyl acetate (99.8:0.2, v/v). The other mobile phases of the solvent program are as indicated in table 2. SQ-squalene, SE-sterol ester, CE-cholesteryl esters, FAME-fatty acids methylesters, TG-triacylglycerols, ST-sterols, Chol-cholesterol, DG-diacylglycerols, FA-fatty acids. Detector: Corona-CAD®.

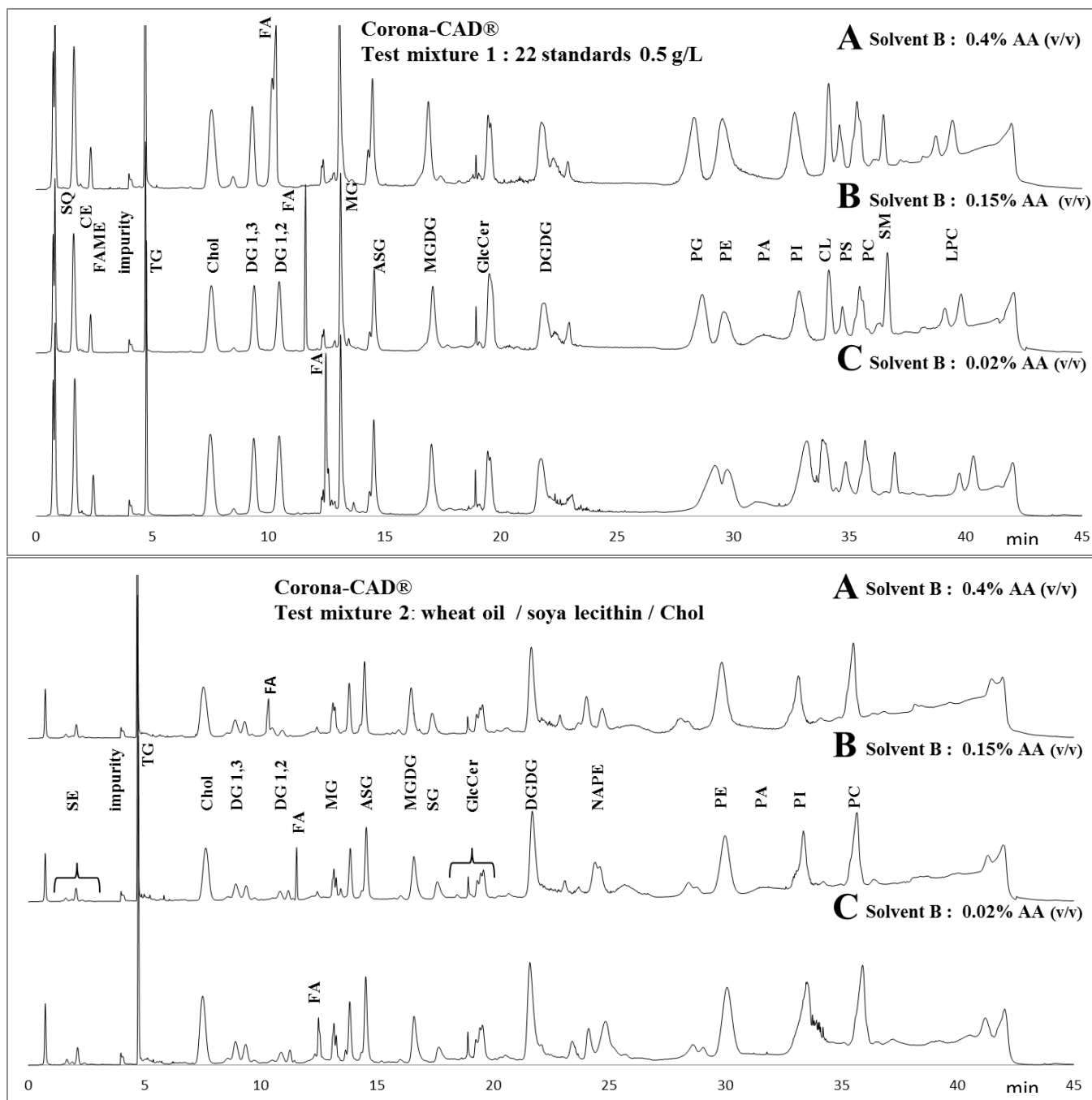


Figure 2. Influence of the amount of acetic acid in the B phase of the solvent program on the chromatogram of test mixtures 1 (top) and 2 (bottom). (A) 0.4 %, (B) 0.15 % (C) 0.02 % acetic acid (v/v) in acetone:ethyl acetate (2: 1, v/v). The other mobile phases of the solvent program are as indicated in table 2. SQ-squalene, SE-sterol ester, CE-cholesteryl esters, FAME-fatty acids methylesters, TG-triacylglycerols, Chol-cholesterol, DG-diacylglycerols, FA-fatty acids, MG-monoacylglycerols, ASG-acylated steryl glycosides, MGDG-monogalactosyldiglycerols, SG-steryl glycosides, GlcCer-glucosylceramides, DGDG-digalactosyldiglycerols, NAPE-N-Acylphosphatidylethanolamines, PG-

phosphatidylglycerols, PE-phosphatidylethanolamines, PA-phosphatidic acids, PI-phosphatidylinositols, CL-cardiolipins, PS-phosphatidylserines, PC-phosphatidylcholines, SM-sphingomyelins, LPC-lysophosphatidylcholines. Detector: Corona-CAD®.

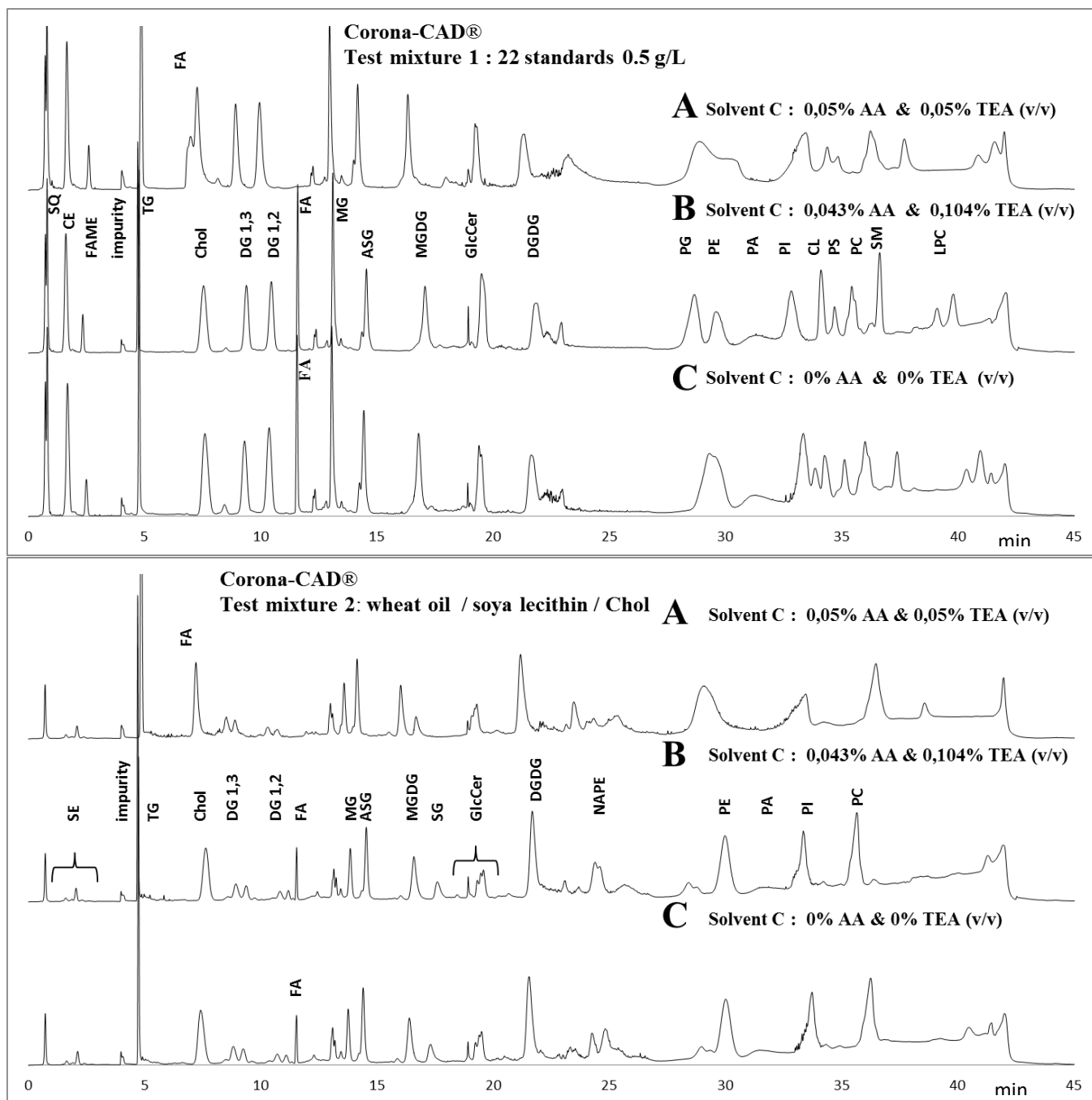


Figure 3. Influence of the amounts of acetic acid and trimethylamine in the C phase of the solvent program on the chromatogram of test mixtures 1 (top) and 2 (bottom). (A) 0.05% AA & TEA (v/v), (B) 7.5mM AA & TEA (0.043% AA & 0.104% TEA) and (C) without AA or TEA. The other mobile phases of the solvent program are as indicated in table 2. SQ-squalene, SE-sterol ester, CE-cholesteryl esters, FAME-fatty acids methylesters, TG-triacylglycerols, Chol-cholesterol, DG-diacylglycerols, FA-fatty acids, MG-monoacylglycerols, ASG-acylated steryl glycosides, MGDG-monogalactosyldiglycerols, SG-steryl glycosides, GlcCer-glucosylceramides, DGDG-digalactosyldiglycerols, NAPE-N-

Acylphosphatidylethanolamines, PG-phosphatidylglycerols, PE-phosphatidylethanolamines, PA-phosphatidic acids, PI-phosphatidylinositols, CL-cardiolipins, PS-phosphatidylserines, PC-phosphatidylcholines, SM-sphingomyelins, LPC-lysophosphatidylcholines. Detector: Corona-CAD®.

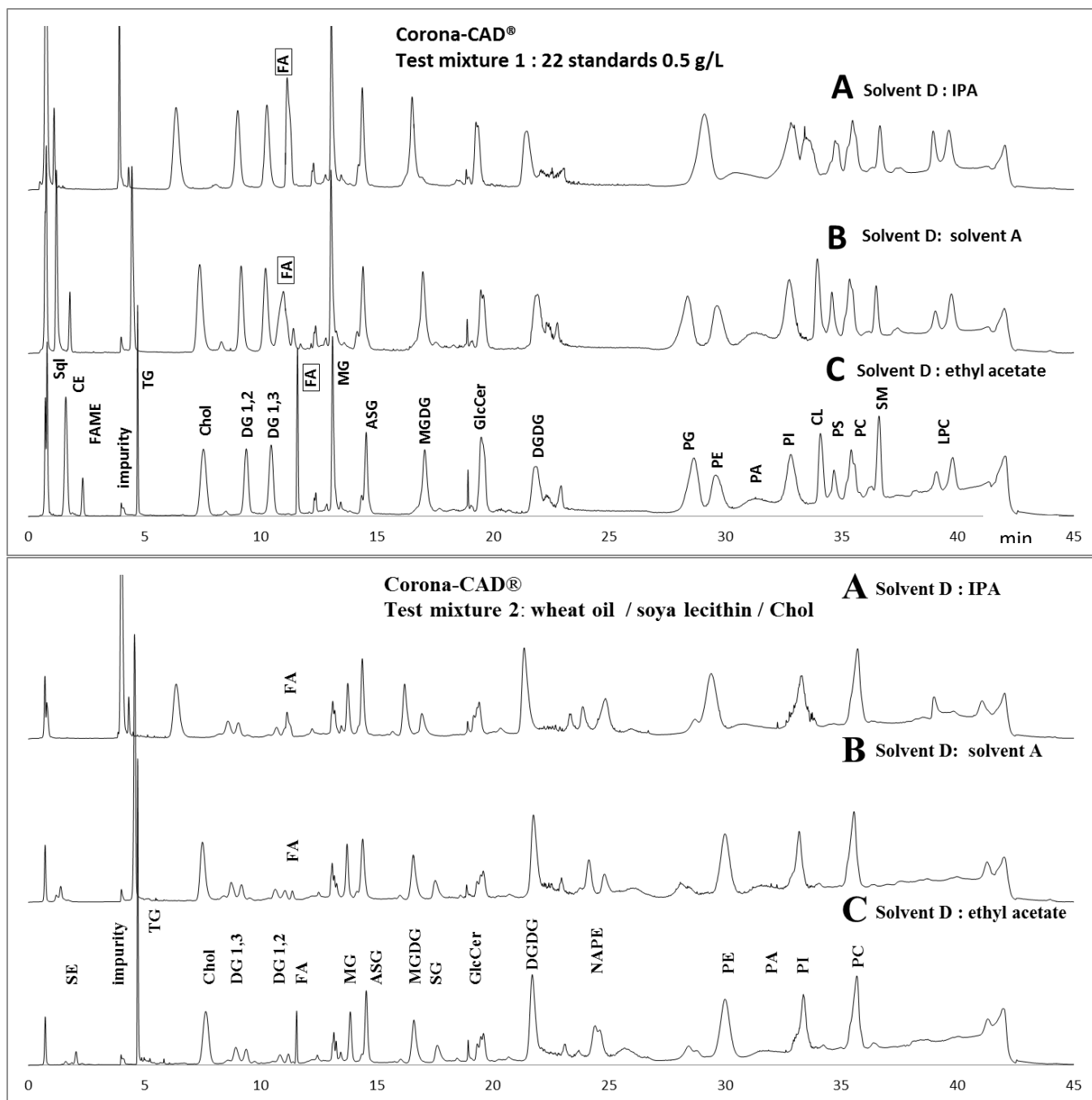


Figure 4. Influence of the rinsing step (mobile phase D) on the chromatogram of test mixture 1 (top) and 2 (bottom): (A) isopropanol (B) isoctane: ethyl acetate (99.8: 0.2, v/v) (C) ethyl acetate (C). The other mobiles phases of the solvent program are as indicated in table 2. SQ-squalene, SE-sterol ester, CE-cholesteryl esters, FAME-fatty acids methylesters, TG-triacylglycerols, Chol-cholesterol, DG-diacylglycerols, FA-fatty acids, MG-monoacylglycerols, ASG-acylated steryl glycosides, MGDG-monogalactosyldiglycerols, SG-steryl glycosides, GlcCer-glucosylceramides, DGDG-digalactosyldiglycerols, NAPE-N-

Acylphosphatidylethanolamines, PG-phosphatidylglycerols, PE-phosphatidylethanolamines, PA-phosphatidic acids, PI-phosphatidylinositols, CL-cardiolipins, PS-phosphatidylserines, PC-phosphatidylcholines, SM-sphingomyelins, LPC-lysophosphatidylcholines. Detector: Corona-CAD®.

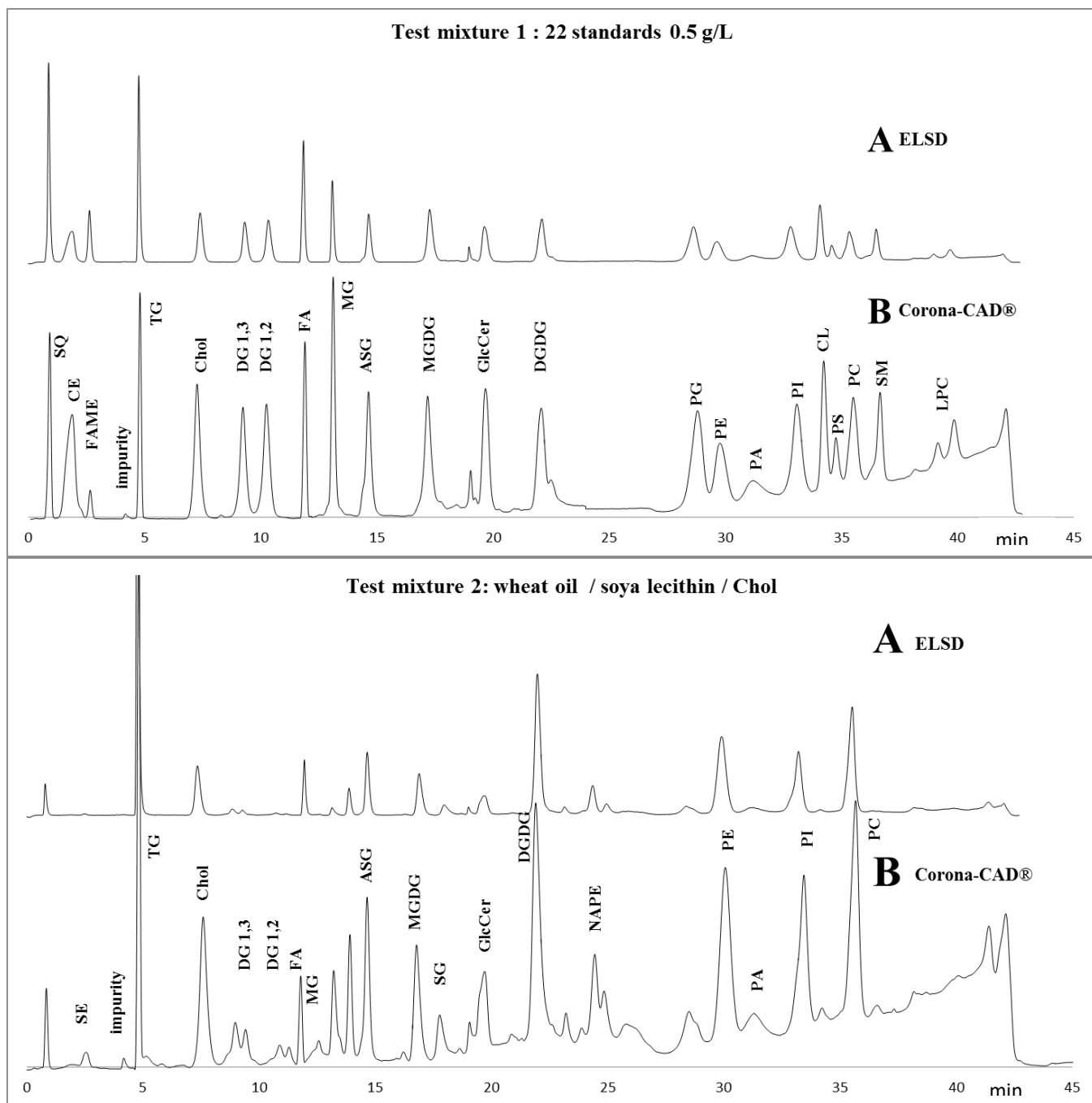


Figure 5. Chromatographic profiles of test mixtures 1 (top) and 2 (bottom) obtained with (A) ELSD and (B) Corona-CAD® detection. SQ-squalene, SE-sterol ester, CE-cholesteryl esters, FAME-fatty acids methylesters, TG-triacylglycerols, Chol-cholesterol, DG-diacylglycerols, FA-fatty acids, MG-monoacylglycerols, ASG-acylated steryl glycosides, MGDG-monogalactosyldiglycerols, SG-steryl glycosides, GlcCer-glucosylceramides, DGDG-digalactosyldiglycerols, NAPE-N-Acylphosphatidylethanolamines, PG-phosphatidylglycerols, PE-phosphatidylethanolamines, PA-phosphatidic acids, PI-

phosphatidylinositols, CL-cardiolipins, PS-phosphatidylserines, PC-phosphatidylcholines, SM-sphingomyelins, LPC-lysophosphatidylcholines. Detector: Corona-CAD®.

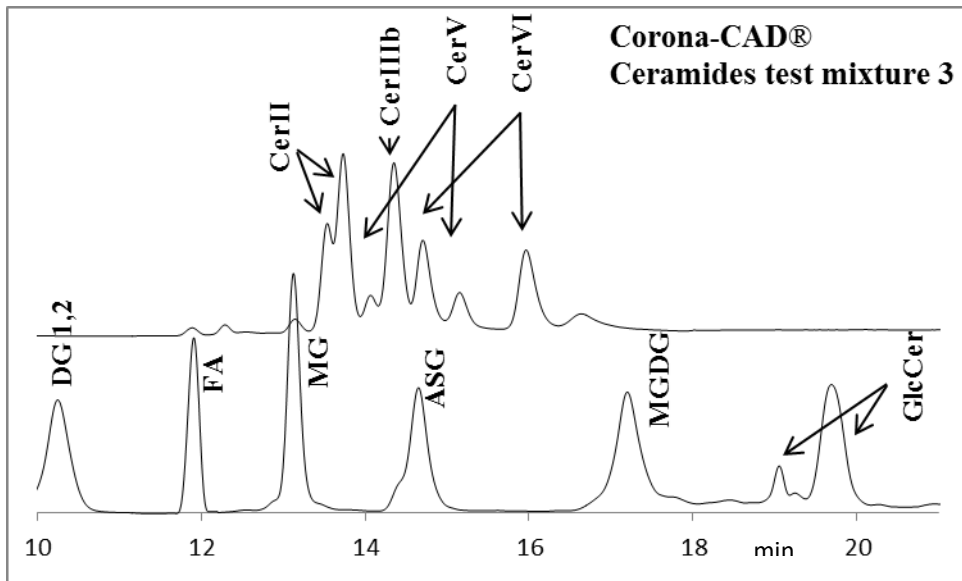


Figure 6. Superimposition of chromatographic profiles of ceramides subclasses (test mixture 3) and chromatographic profile of standards (test mixture 1) obtained with Corona-CAD®.

Ceramides (CerII (Cer(d18:1/18:0)), CerIIIb (Cer(t18:0/18:1)), CerV (Cer(d18:1/18:0(2OH))) and CerVI (Cer(t18:0/18:0(2OH))))), DG-diacylglycerols, FA-fatty acids, MG-monoacylglycerols, ASG-acylated steryl glycosides, MGDG-monogalactosyldiglycerols, SG-steryl glycosides, GlcCer-glucosylceramides. Detector: Corona-CAD®.

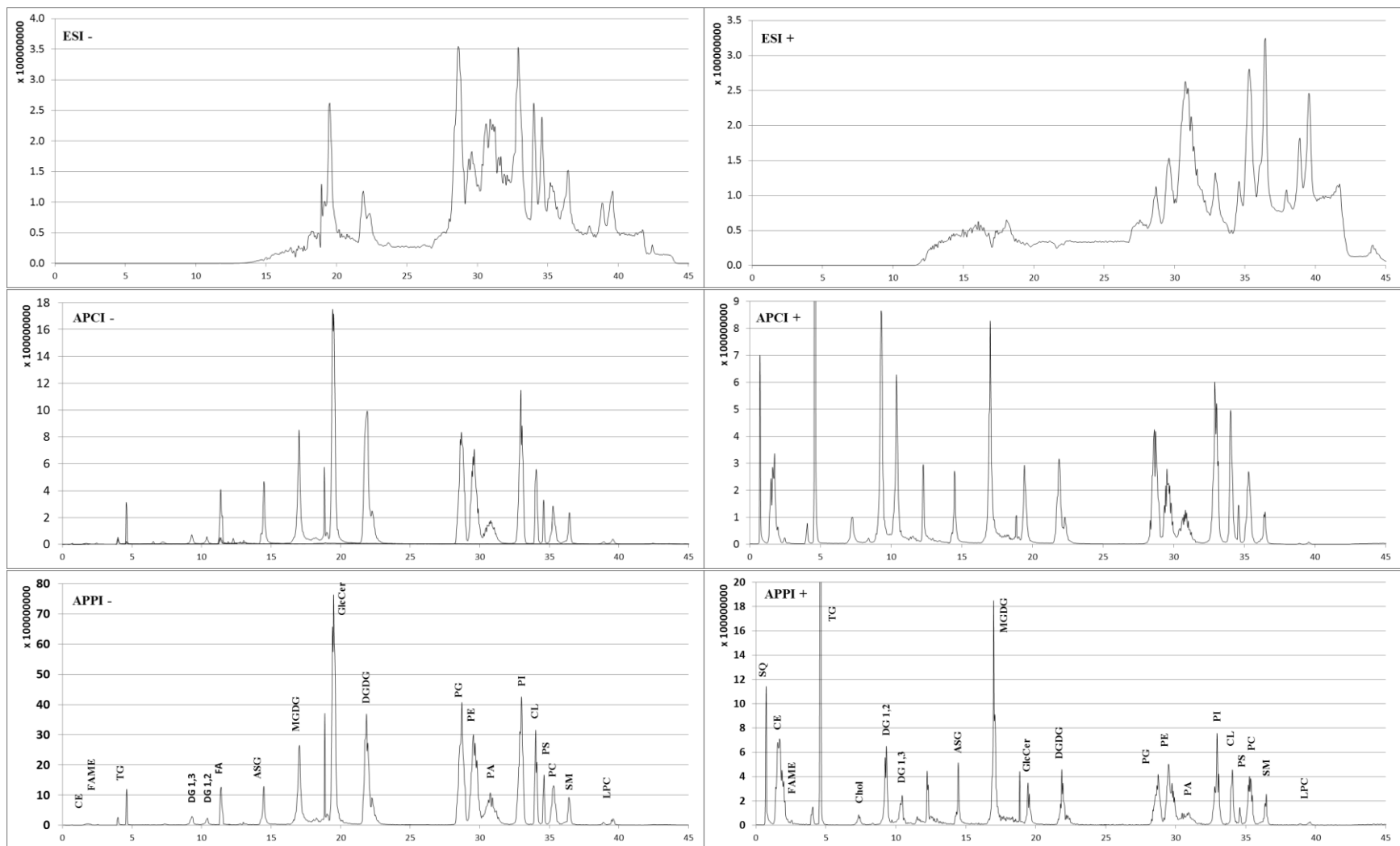


Figure 7. LC/MS chromatographic profile of mixture 1, using three different ion sources: ESI (top), APCI (middle) and APPI (down), in negative ion mode (left) and positive ion mode (right).

SQ-squalene, CE-cholesteryl esters, FAME-fatty acids methylesters, TG-triacylglycerols, Chol-cholesterol, DG-diacylglycerols, FA-fatty acids, ASG-acylated sterol glycosides, MGDG-monogalactosyldiglycerols, GlcCer-glucosylceramides, DGDG-digalactosyldiglycerols, PG-phosphatidylglycerols, PE-phosphatidylethanolamines, PA-phosphatidic acids, PI-phosphatidylinositols, CL-cardiolipins, PS-phosphatidylserines, PC-phosphatidylcholines, SM-sphingomyelins, LPC-lysophosphatidylcholines.

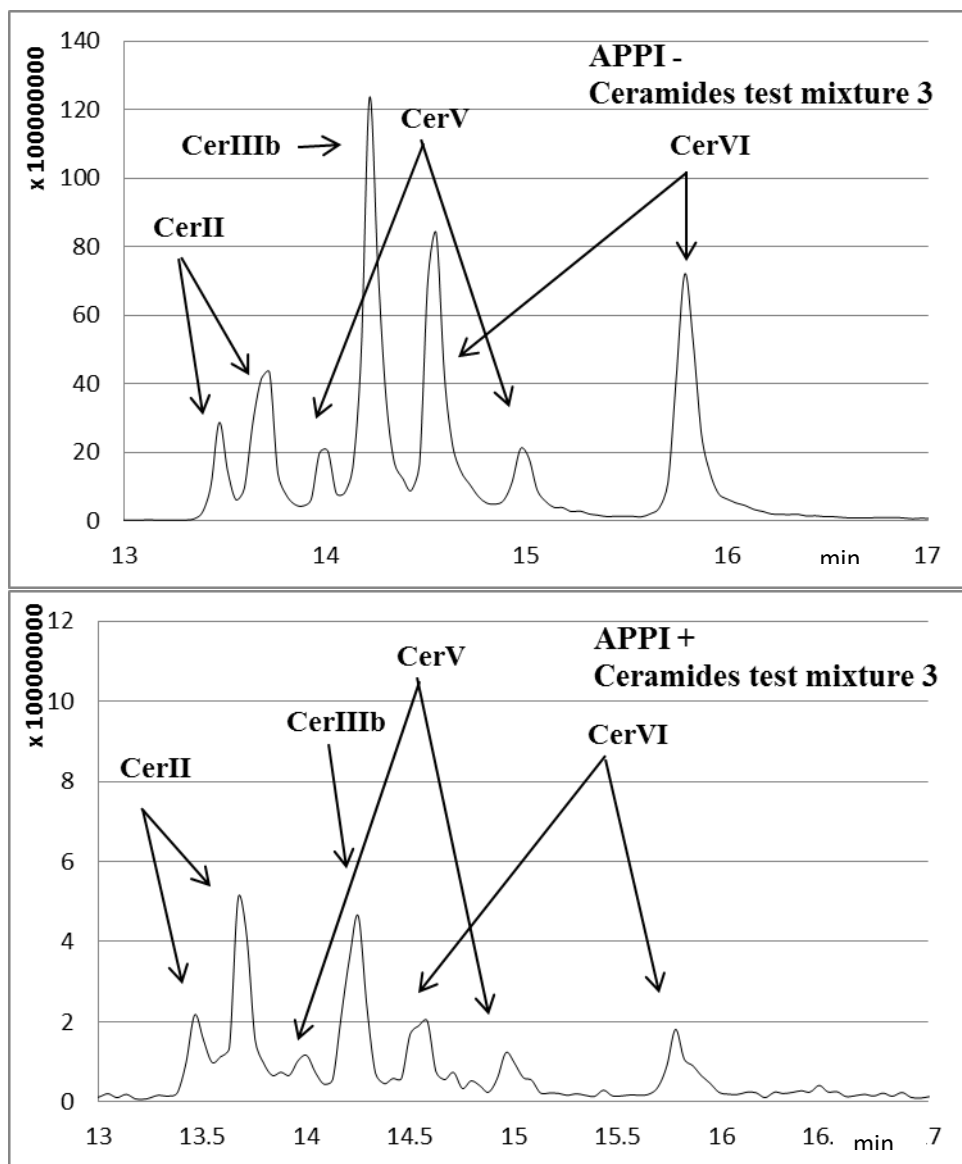


Figure 8. LC/MS chromatographic profile of test mixture 3, using APPI, in negative ion mode (top) and positive ion mode (down).

Ceramides (CerII (Cer(d18:1/18:0)), CerIIIb (Cer(t18:0/18:1)), CerV (Cer(d18:1/18:0(2OH))) and CerVI (Cer(t18:0/18:0(2OH))))).

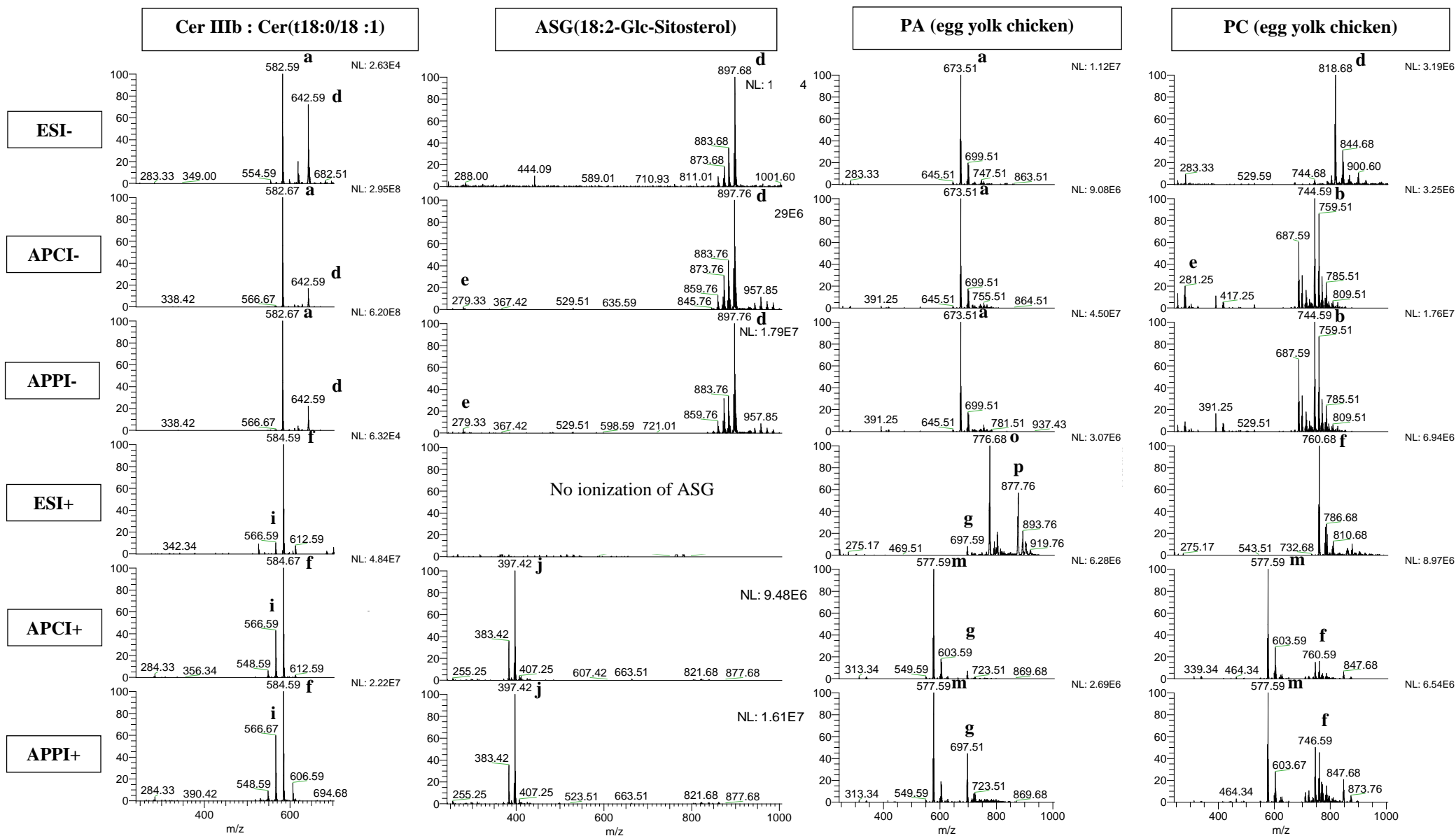


Figure 9. LC/MS full scan spectra obtained with the 3 API sources in positive and negative mode.

a : [M-H]⁻, b : [M-CH₃]⁻, c : [M^o]⁻, d : [M-H+CH₃COOH]⁻, e : [FA-H]⁻, f : [M+H]⁺, g : [M + Na]⁺, h : [M+H-FA]⁺, i : [M+H-H₂O]⁺, j : [M-FA-sugar+H+H₂O]⁺, k : [Aglycone+H-H₂O]⁺, l : [Aglycone+H]⁺, m : [M+H-polar Head]⁺, n : Diglyceride like ion, o : [M+H+TEA]⁺ and p : [M+H+2TEA]⁺.

CerIIIb (Cer(t18:0/18:1)), ASG-acylated sterol glycosides, PA-phosphatidic acids, PC-phosphatidylcholines.

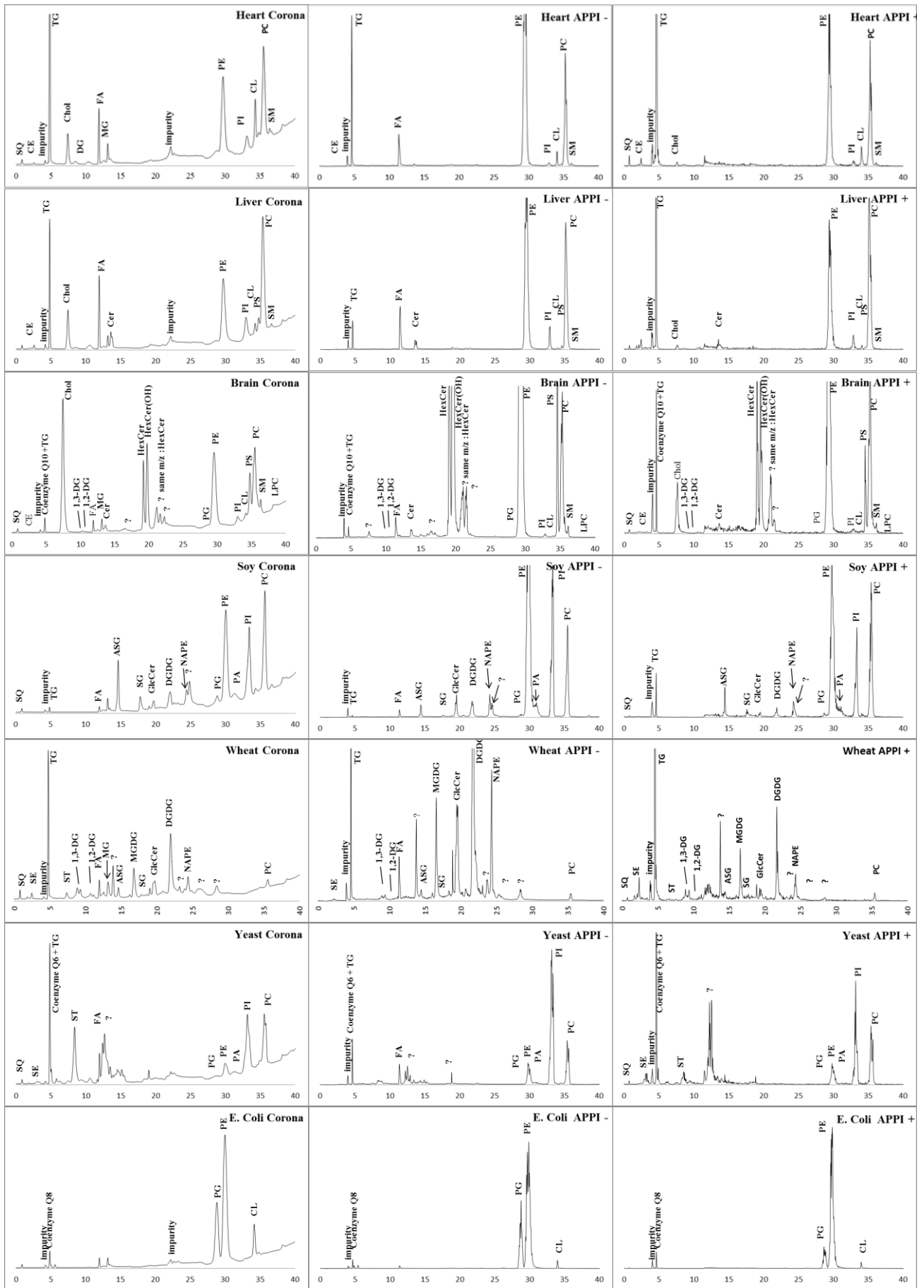


Figure 10. Chromatograms of 7 total lipid extracts, obtained by LC- Corona-CAD® (left), LC-APPI negative ion mode (middle) and LC-APPI positive ion mode (right). SQ-squalene, SE-sterol ester, CE-cholesteryl esters, TG-triacylglycerols, ST-sterols, Chol-cholesterol, DG-diacylglycerols, FA-fatty acids, MG-monoacylglycerols, ASG-acylated steryl glycosides, MGDG-monogalactosyldiglycerols, SG-steryl glycosides, GlcCer-glucosylceramides, HexCer-hexosylceramide, DGDG-digalactosyldiglycerols, NAPE-N-Acylphosphatidylethanolamines, PG-phosphatidylglycerols, PE-phosphatidylethanolamines, PA-phosphatidic acids, PI-phosphatidylinositols, CL-cardiolipins, PS-phosphatidylserines, PC-phosphatidylcholines, SM-sphingomyelins, LPC-lysophosphatidylcholines.