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Abstract: The solid phase extraction (SPE) method used by Banni et al. (2001) for fractionation of liver phospholipids (PLs) into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) was modified for improving its separation efficiency. A mixture of PL standars containing PC, PE, PS and PI was separated into individual classes by using aminopropyl minicolumns, following the method of Banni et al. (2001). Obtained eluted fractions were further analysed by thin-layer chromatography (TLC) and PL classes were identified. TLC evidenced the coelution of PC and PE, that of PE with PS and no elution of PI. The SPE method was subsequently modified in order to obtain a correct PLs fractionation into PC, PE, PS and PI. Main modifications consisted in increased solvent volumes for PC, PE and PS elution, and a different solvent mixture to allow PI elution. The effectiveness of the modified SPE method was checked by TLC, using both standards and muscle samples, showing a correct elution of PC, PE, PS and PI.

* Manuscript

1 TITLE

Improvement of a solid phase extraction method for separation of muscle phospholipid classes

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11 RUNNING TITLE

12 Separation of phospholipid classes by SPE

13 ABSTRACT

14 The solid phase extraction (SPE) method used by Banni et al. (2001) for
15 fractionation of liver phospholipids (PLs) into phosphatidylcholine (PC),
16 phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI)
17 was modified for improving its separation efficiency. A mixture of PL standars
18 containing PC, PE, PS and PI was separated into individual classes by using
19 aminopropyl minicolumns, following the method of Banni et al. (2001). Obtained eluted
20 fractions were further analysed by thin-layer chromatography (TLC) and PL classes
21 were identified. TLC evidenced the coelution of PC and PE, that of PE with PS and no
22 elution of PI. The SPE method was subsequently modified in order to obtain a correct
23 PLs fractionation into PC, PE, PS and PI. Main modifications consisted in increased
24 solvent volumes for PC, PE and PS elution, and a different solvent mixture to allow PI
25 elution. The effectiveness of the modified SPE method was checked by TLC, using both
26 standards and muscle samples, showing a correct elution of PC, PE, PS and PI.

27

28 KEYWORDS

29 SPE, phospholipid classes, muscle.

30

31 INTRODUCTION

32 The more representative PLs in mammal muscle are PC, PE, PS and PI (Olsson &
33 Salem, 1997). PC is usually the major PL in animal tissues, often amounting to almost 50%
34 of the total (Christie, 2005). PE is generally the second most abundant PL in animal, while
35 PS usually constitutes less than 10% of the total PLs (Christie, 2005) and the proportion of
36 PI is often lower than 4% in animal tissues (Genge, Licia, Wutier & Wutier, 2003). PC and
37 PE are neutral PLs, while PS is a weakly acidic one and PI is strongly acidic (Christie,
38 2005). Due to this feature the correct separation of PS and PI results difficult, and thus,
39 these two PLs are usually isolated together (Alasnier & Gandemer, 1998; Sánchez & Lutz,
40 1998; Tejeda, 1999). Fractionation of muscle PLs into major PL classes is becoming a
41 common analytical procedure for meat scientists because it allows the analysis of
42 individual PL classes and therefore, to obtain important information for each one (Kim &
43 Salem, 1990).

44 Separation of PL classes has conventionally been performed by TLC (Christie,
45 1982). High-performance liquid chromathography (HPLC) is getting more and more used
46 for this type of analysis due to its excellent quantification and resolution (Hemming &
47 Hawthorne, 2001). Solid phase extraction (SPE) is also a widespread technique for
48 separation of PL classes (Christie, 1982; Hemming & Hawthorne, 2001). This technique
49 has been widely used due to its easy and fast sample preparation.

50 Pietsch and Lorenz (1993) developed a SPE method for the separation of individual
51 PL classes (PC, PE, PS and PI) using single aminopropyl SPE cartridges and standards of
52 PLs. Latter on, Banni et al. (2001) separated liver PL classes according to the method
53 described by Pietsch and Lorenz (1993) with minor modifications. We have checked the
54 effectiveness of these methods using pure PL standards and coelutions of PC, PE and PS
55 and no elution of PI was found. Therefore, our objective was to optimize a SPE method for

56 separation of PL classes using standards of PLs and to subsequently check its effectiveness
57 using muscle samples.
58
59 MATERIALS AND METHODS
60 ***Materials***.
61 PL standars were obtained from Sigma Aldrich (St Louis, MO, CA): synthetic L-α-
62 phosphatidylcholine, L-α-phosphatidylethanolamine from *Escherichia coli*, 3-sn-
63 phosphatidyl-L-serine from bovine brain and L-α-phosphatidylinositol from bovine liver.
64 500 mg aminopropyl minicolumns were supplied from Varian (Harbor City, CA). A "Vac
65 Elut" apparatus from Varian (Harbor City, CA) was used for flusing the cartridges. TLC
66 plates of silica gel 60 G/UV_{254} (20x20 cm; 0.25 mm thickness) were from Macherey-Nagel
67 (Düren, Germany). All solvents used in this study were obtained from Scharlau
68 (Barcelona, Spain) or Panreac (Barcelona, Spain).
69 ***Preparation of biological samples***
70 Adult rats fed a mixed feed (AO4 from Panlab) were decapited and their *Longissimus*
71 *dorsi* muscle was dissected and stored at –80ºC until analysis. *Pectoralis major* muscle
72 from chicken was obtained in a local supermarket. Samples were ground using a
73 commercial grinder inmediately before fat extraction. Intramuscular total lipids were
74 extracted with chloroform:methanol (1:2; v/v) according to the method described by Bligh
75 & Dyer, 1959.
76 ***Fractionation of phospholipid classes by the Banni et al. (2001) method.***
77 Briefly, 50 µl of a mixture of PL standards (PC, PE, PS and PI) at 5 µg $µl^{-1}$ was added
78 to a 500 mg aminopropyl minicolum, which has been previously activated with 3 ml of
79 hexane. PC, PE, PS and PI were sequentially eluted with 20 ml of acetonitrile:n-propanol
80 (2:1; v/v), 7 ml of methanol, 7.4 ml of isopropanol:methanolic HCl 3N (4:1; v/v) and 10 ml

81 of methanol:methanolic HCl 3N (9:1; v/v), respectively. The vacuum was adjusted to
82 generate a flow of 1 ml min^{-1} through the minicolumn. Composition and volume of eluting
83 solvents in this SPE method is shown in Table 1.

84 ***Fractionation of phospholipid classes by the Banni et al. (2001) modified method.***

85 Briefly, 50 µl of a mixture of PL standards (PC, PE, PS and PI) at 5 µg μl^{-1} was added
86 to a 500 mg aminopropyl minicolum (Varian, Harbor City, USA), which has been
87 previously activated with 7.5 ml of hexane. PC, PE, PS and PI were sequentially eluted
88 with 30 ml of acetonitrile:n-propanol (2:1; v/v), 10 ml of methanol, 7.5 ml of
89 isopropanol:methanolic HCl 3N (4:1; v/v) and 17.5 ml of chloroform:methanol:HCl 37%
90 (200:100:1; v/v/v), respectively. Composition and volume of used eluted solvents in this
91 SPE method is shown in Table 1.

92 ***Thin layer chromatography.***

93 The efecctiveness of the studied SPE methods to separate PL classes was visually
94 checked following the TLC method described by Ramadan and Mörsel (2003). After
95 evaporation of solvents under nitrogen, each SPE eluted fraction was dissolved in 50 µl of
96 chloroform:methanol (2:1). TLC plates were divided into eigth lanes and were activated at
97 120ºC for 2 h inmmediately before use. 15 µl of each PL standard dissolved in
98 chloroform:methanol (2:1) at 5 µg μl^{-1} was loaded onto lanes 1 to 4 (PC, PE, PS and PI,
99 respectively). Sequentially eluted SPE fractions were loaded onto lanes 5 to 8. Plates were
100 developed with chloroform:methanol:25 % ammonia solution (65/25/4; v/v/v), air dried
101 and visualised by spraying the plates with molybdenum blue spray (1.3 % molybdenum
102 oxide in 4.2 M sulfuric acid) from Sigma (St Louis, USA). The R_f values of the eluted
103 fractions were compared with those of PL standars.

104 ***Isolation of phospholipids from biological samples.***

105 Lipid extracts from rat and chicken muscle were separated into lipid classes using
106 aminopropyl minicolumns (500 mg), following the method described by Ruiz, Antequera,
107 Andres, Petron and Muriel (2004). Briefly, minicolumns were activated with 7.5 ml of
108 hexane. 20 mg of total lipids dissolved in 150 µl of hexane:chloroform:methanol (95:3:2;
109 v/v/v) were loaded onto the column. Neutral lipids were eluted with 5 ml of chloroform
110 and free fatty acids with 5 ml of diethyl ether:acetic acid (98:2; v/v). In this way, PLs were
111 kept in the aminopropyl cartridge. Then individual PL classes (PC, PE, PS and PI) were
112 sequentially eluted using the improved Banni et al. (2001) method to check its
113 effectiveness. Thereafter, PL classes were identified using the previously described TLC
114 method.

115

116 RESULTS AND DISCUSSION

117 We have carried out an optimization of the SPE method of Banni et al. (2001) using a
118 mixture of PL standards (PC, PE, PS and PI). Examination of TLC plates of eluted
119 fractions obtained with the original SPE method for separating individual PL classes
120 (Figure 1) showed that the first eluted fraction (fraction 1) was only constituted by PC.
121 However, the second eluted fraction obtained (fraction 2) was constituted by both PE and
122 PC, as can be observed in lane-6 of the TLC plate (Figure 1). This indicates the coelution
123 of these two PLs. Similarly, lane 7 of the TLC plate showed two spots with R_f
124 corresponding to PS and PE, indicating the cross-contamination of these two PLs in the
125 third eluted fraction obtained (fraction 3). Finally, lane 8 of the TLC, in which the fourth
126 eluted fraction (fraction 4) was loaded, did not show any spot, indicating that the solvent
127 used for eluting the PI in the Banni et al. (2001) method (methanol:methanolic HCl 3N 9:1;
128 v/v) is not able to elute such PL class.

129 Due to the problems found in analysing PL classes by the Banni et al. (2001) method,
130 summarised in the co-elution of PE and PC, that of PS and PE and the retention of PI in the
131 aminopropyl minicolumn, several modifications were carried out in order to obtain a
132 correct PL classes fractionation.

133 Using the Banni et al. (2001) method, part of the PC was not eluted in the fraction 1,
134 and was thereafter eluted in the second fraction, which should contain only PE. For the
135 elution step, the objective is to elute the analytes in a volume as small as possible.
136 However, enough volume of solvent for eluting the whole amount of the analyte retained
137 in the column is needed. Thus, when analytes are coeluted, a higher volume of solvent
138 might be necessary. Taking this into consideration, we tried to correctly separate PC and
139 PE from the aminopropyl minicolumn increasing the volume of the eluting solvent. The
140 efficacy of increased volumes (22.5 ml, 25 ml, 27.5 ml, 30 ml) of the solvent used in the
141 Banni et al. (2001) method (acetonitrile:n-propanol 2:1; v/v) in eluting the whole PC were
142 checked. Finally, by using 30 ml of the proposed solvent mixture, no PC spot was
143 evidenced in lane 6 of the TLC plate (figure 2), corresponding to the second eluted fraction
144 of the modified SPE method, and only a spot corresponding to the PE was observed in
145 such fraction.

146 The third eluted fraction using the Banni et al. (2001) method showed a coelution of
147 PE and PS, indicating that the solvent (7 ml of methanol) used for PE elution (fraction 2)
148 was not able to elute the whole PE retained in the aminopropyl cartridge. Different
149 volumes of methanol (10 ml and 12.5 ml) were tested, and with both the PE spot was not
150 present in the third obtained fraction (that should only contain PS), indicating that the
151 lower volume (10ml) was enough for eluting the whole PE. The volume of
152 isopropanol:methanolic HCl 3N (4:1; v/v) used for eluting PS was adjusted to 7.5 ml for
153 practical purposes, with no incidence in the subsequent elution of PI.

154 Pietsch and Lorenz (1993) and Banni et al. (2001) were able to correctly elute PI.
155 However, using the same mixture of solvents (methanol:methanolic HCl 3N 9:1; v/v) as
156 these authors, PI was not present in the fourth fraction in our study. Difficulties in the
157 elution of acidic PLs such as the PI have also been reported by other authors (Kim &
158 Salem, 1990; Rizov & Doulis, 2000). In fact, Bateman and Jenkins (1997) reported only
159 39% of theoretical recovery of PLs when eluting with methanol, while the rest remained
160 attached to the column most likely due to their high acidity.

161 In order to extract PI from minicolumns, we tried first with increasing volumes of the
162 proposed mixture of solvents (methanol: methanolic HCl 3N 9:1; v/v) (12.5 ml, 15 ml,
163 17.5 ml and 20 ml), but none of them allowed the elution of PI (TLC plates not shown).

164 The cartridiges used in this study, NH_2-aminopropyl, are weak anion exchange
165 phases. Their primary retention mechanism is mainly based on the electrostatic attraction,
166 retaining negatively charged or anionic compounds. Secondary interaction depends on the
167 polarity of the compounds, those compounds showing higher polarity being more strongly
168 retained than less polar ones.These secondary interactions can cause incomplete elution.
169 Therefore, the more polar the functional groups contained in the PL are, the more strongly
170 the PL is adsorbed into the stationary phase (Christie, 1982). PI contains many hydroxyl
171 groups (Leningher, 1995), and this could lead to a strong retention into the aminopropyl
172 minicolumns Thus, both the acidity and the polarity of the eluting mixture of solvents
173 influence the correct extraction of the PL molecules from the stationary phase.

174 The acidity of the solvent used by Banni et al. (2001) might be insufficient to break
175 the interactions between PI and the stationary phase. The elution strategy usually consists
176 in incorporating an acid or a base in the eluting solution to convert the analytes to their
177 molecular, nonionic form (Fritz, 1999). In fact, Kim and Salem (1990), observed that the
178 elution of PI was achieved through adding phosphoric acid (5%) to a mixture of solvents,

179 and did not show elution of such acidic PL without this acid in the mixture of the mobile
180 phase. Previous investigations (Suzuki, E., Sano, A., Kuriki, T., & Miki, T., 1997) have
181 pointed out that the elution of acidic glycerophospholipids from the NH_2-phase cartridges
182 might be affected by the pH and salt concentration of the eluent. These authors tested a
183 wide range of pH and obtaining a correct elution of phosphatidylglicerol, PS and
184 cardiolipin, but PI was anyway retained in the cartridge. Enough volume of the HCl in
185 methanol solution must be used in order to neutralizes all the hydroxide ions on the ion
186 exchange SPE (Fritz, 1999). If insufficient HCl is used, the remaining active groups will
187 retain the PLs ions farther down the column. In fact, Pietsch and Lorenz (1993) found that
188 PI could be eluted from the aminopropyl matrix with methanol/methanolic HCl (9:1, v/v).

189 On the other hand, the polarity of the solvent used in the Banni et al. (2001) method
190 (methanol:methanolic HCl 3N 9:1; v/v), might also be inadequate for elution of PI from
191 the NH2-phase cartridge. In this sense, several researchers had found inadequate elution of
192 the acidic PLs with methanol (Pinkart, Devereux & Chapman, 1998; Kim & Salem, 1990;
193 Bateman & Jenkins., 1997). By using chloroform:methanol (1:6) followed by an eluting
194 solvent of 0.05M sodium acetate in chloroform:methanol (1:6), the average recovery of PC
195 and PE increased respect to the obtained using methanol (Pinkart et al., 1998).

196 Taking the previous discussion into consideration, we tried to correctly elute the PI
197 from the aminopropyl minicolumn with a more acid and less polar mixture of solvents than
198 that used in the Banni et al. (2001). Such a mixture was constituted by
199 chloroform:methanol:HCl 37% and it was tested for different proportions of solvents and
200 different volumes. The mixture of solvents in proportion (200:100:5) produced the
201 degradation of the stationary phase, which was colleted with the elutingnsolvents and with
202 elution products in the test tubes. This type of silica based phases have a stable pH range of
203 2 to 7.5. At pH levels above and below this range, the bonded phase can be hydrolyzed and

204 cleaved off the silica surface, or the silica itself can dissolve. The pH of the tested solution
205 was lower than 2, explaining the instability of the cartridge. TLC lanes where this eluted
206 fraction was loaded onto did not show any PL but a light blue stain was observed in the
207 place in which this fraction was loaded.It might be that the high acidity of the mixture of
208 solvents employed (chloroform:methanol:HCl 37%, 200:100:5) could hydrolyze the
209 inositol or the phosphate group from the phosphatidylinositol molecule. The hydrolyzed
210 products are different to PI, and thus, their R_f should be also different. The other possible
211 reason could be that the degraded stationary phase might interfere in the normal run of PI
212 in the TLC plates. Anyway, due to these results, the acidity of this mixture of solvents was
213 reduced by using less HCl. Thus, different volumnes of a mixture of
214 chloroform:methanol:HCl 37% (200:100:1) were tested. By using 10 and 15 ml of this
215 mixture PI was not eluted (TLC plates not shown). 20 ml of this mixture also produced the
216 degradation of the stationary phase, and TLC lanes in which this eluted fraction was loaded
217 onto showed a blue stain with a R_f lower than that of PI. Both the high volume and the low
218 pH of the mixture of solvents might lead to the stationary phase degradation, which could
219 again interfere with PI run in TLC plates.

220 A lower volume of chloroform:methanol:HCl 37% (200:100:1) (17.5 ml) was tested
221 and PI was correctly eluted. Figure 2 shows TLC plates in which fractions eluted using the
222 Banni et al. (2001) modified SPE method were loaded onto. As it can be observed, all PL
223 included in the mixture (PC, PE, PS and PI) were separately eluted in their respective
224 fractions. Validation of this SPE method was carried out using biological samples
225 (*Longissimus dorsi* from rat and *Pectoralis major* from chicken). The eluted fractions were
226 also analysed by TLC, showing that fraction 1, 2, 3 and 4 were constituted by PC, PE, PS
227 and PI, respectively (Figure 3).

228 It can be concluded that the modifications proposed for the Banni et al. (2001) SPE
229 method for separation of PL classes allow the correct elution of PC, PE, PS and PI using
230 both standards of PLs and muscle samples.

231 REFERENCES

232 Alasnier, C. & Gandemer, G. (1998). Fatty acid and aldehyde composition of individual
233 phospholipid classes of rabbit skeletal muscles is related to the metabolic type of the
234 fibres. *Meat Science,* 48, 225-235.

235 Banni, S., Carta, G., Angioni, E., Muru, E., Scanu, P., Melis, M.P., Bauman, D.E.,
236 Fischer, S.M., & Ip, C. (2001). Distribution of conjugated linoleic acid and
237 metabolites in different lipid fraction in the rat liver. *Journal of Lipid Research,*
238 42, 1056-1061.

239 Bateman, H.G. & Jenkins, T.C. (1997). Method for extraction and separation by solid
240 phase extraction of neutral lipid, free fatty acids, and polar lipids from mixed
241 microbial cultures. *Journal of Agricultural and Food Chemistry.,* 45, 132-134.

242 Bligh, E.G. & Dyer, E.J. (1959). A rapid method of total lipid extraction and
243 purification. *Canadian Journal of Biochemistry and Fisiology,* 37, 911.

244 Christie, W.W. (1982). Structural analysis of lipids by means of enzymatic hydrolysis.
245 In *Lipids analysis* (pp. 155-166). Oxford: Pergamon Press.

246 Christie, W.W. (2005). Lipid library (http://www.lipid.co.uk/infores/lipids.html).

247 Fritz, J.(1999). Advantages of SPE. In *Analitical Solid-Phase Extraction*. Iowa Satate:
248 Wiley.

249 Genge, B.R., Licia, N.Y., Wuthier, W. & Wutier, R.E. (2003). Separation and
250 quantification of chicken and bovine growth plate cartilage matrix vesicle lipids
251 by high-performance liquid chromatography using evaportive light scattering
252 detection. *Analytical Biochemistry,*. 332, 104-115.

253 Hemming, F.W & Hawthorne, J.N. (2001). Técnicas básicas. In *Análisis de Lípidos*, (pp.
254 117-131). Barcelona: Acribia.
255 Kim, H-Y. & Salem Jr, N. (1990). Separation of lipid classes by solid phase extraction.
256 *Journal of Lipid Research,* 31, 2285-2289.
257 Lehninger, A.L. (1995). Bioquímica. Las bases moleculares de la estructura y función
258 celular, (pp. 293-297). Barcelona: Omega.
259 Olsson, M.U. & Salem Jr, N. (1997). Molecular species analysis of phospholipids.
260 *Journal of Chromatography B*, 692, 245-256.
261 Pietsch, A. & Reinhard L.L. (1993). Rapid separation of the major phospholipid classes
262 on a single aminopropil cartridge. *Lipids,* 28, 945-947.
263 Pinkart, H.C., Devereux, R. & Chapman, P.J. (1998). Rapid separation of microbial
264 lipids using solid phase extraction columns. *Journal of Microbiological Methods,*
265 34, 9-15.
266 Ramadan, F.W. & Mörsel, J.T. (2003). Determination of the lipid classes and fatty acid
267 profile of niger (*Guizotia abyssinca* Cass.) seed oil. *Pyochemistry Analitica.,* 14,
268 366-370.
269 Rizov, I. & Doulis, A. (2001). Separation of plant membrane lipids by multiple solid-
270 phase extraction. *Journal of Chromatography a,* 922, 347-354.
271 Ruiz, J., Antequera, T., Andres, A.I., Petron, M.J. & Muriel, E.. (2004). Improvement of
272 a solid phase extraction method for analysis of lipid fractions in muscle foods.
273 *Analítica Chimica Acta,* 520, 201-205.
274 Sánchez, V. & Lutz, M. (1998). Fatty acid composition of microsomal phospholipids in
275 rats fed different oils and antioxidant vitamins supplement. *Journal of Nutritonal.*
276 *Biochemistry.,* 9, 155-163.

277 Suzuki, E., Sano, A., Kuriki, T., & Miki, T. (1997). Improved separation and
278 determination of phospholipids in animal tissues employing solid phase extraction.
279 *Biological and pharmaceutical bulletin,* 20, 299-303.
280 Tejeda, J.F. (1999). Estudio de la influencia de la raza y la alimentación sobre la
281 fracción lipídica intramuscular del cerdo Ibérico. Doctoral thesis. Departament of
282 Zootechny. Faculty of Veterinary. University of Extremadura.

283 Caption Figure 1.

284 Figure 1. TLC plate of eluted PL fractions from the mixture of PL obtained following
285 the Banni et al. (2001) method. Phosphatidylcholine (PC), phosphatidylethanolamine (PE),
286 phosphaticylserine (PS), phosphatidylinositol (PI).

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308 Caption Figure 2.

309 Figure 2. TLC plate of eluted PL fractions from the mixture of PL obtained following
310 the Banni et al. (2001) modified method. Phosphatidylcholine (PC),
311 phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI).

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333 Caption Figure 3.

334 Figure 3. TLC plate of eluted PL fractions from the *Longissimus dorsi* muscle of rats
335 obtained following the Banni et al. (2001) modified method. Phosphatidylcholine (PC),
336 phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI).

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358 Caption Table 1.

359 Table 1. Solvents, eluent proportions, eluent volumes and eluted phospholipids by

360 Banni et al. (2001) method and by Banni et al. (2001) modified method.

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Table(s)

1 Table 1.

		SOLVENT	PROPORTION	VOLUME	ELUTED PLs
Fraction 1	BM[a]	Acetonitrine:n-propanol	2:1	20 ml	PC
	MM[b]	Acetonitrine:n-propanol	2:1	30 ml	PC
Fraction 2	BM[a]	Methanol	Net	7 ml	PE + PC
	MM[b]	Methanol	Net	10 ml	PE
Fraction 3	BM[a]	Isopropanol:methanonolic HCl 3N	4:1	7.4 ml	PS + PE
	MM[b]	Isopropanol:methanonolic HCl 3N	4:1	7.5 ml	PS
Fraction 4	BM[a]	Methanol:methanolic HCl 3N	9:1	10 ml	-
	MM[b]	Chloroform:methanol:HCl 3N	(200:100:1)	17.5 ml	PI

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3 BM[a] = Banni et al. (2001) method.

4 MM[b] = Modified method.

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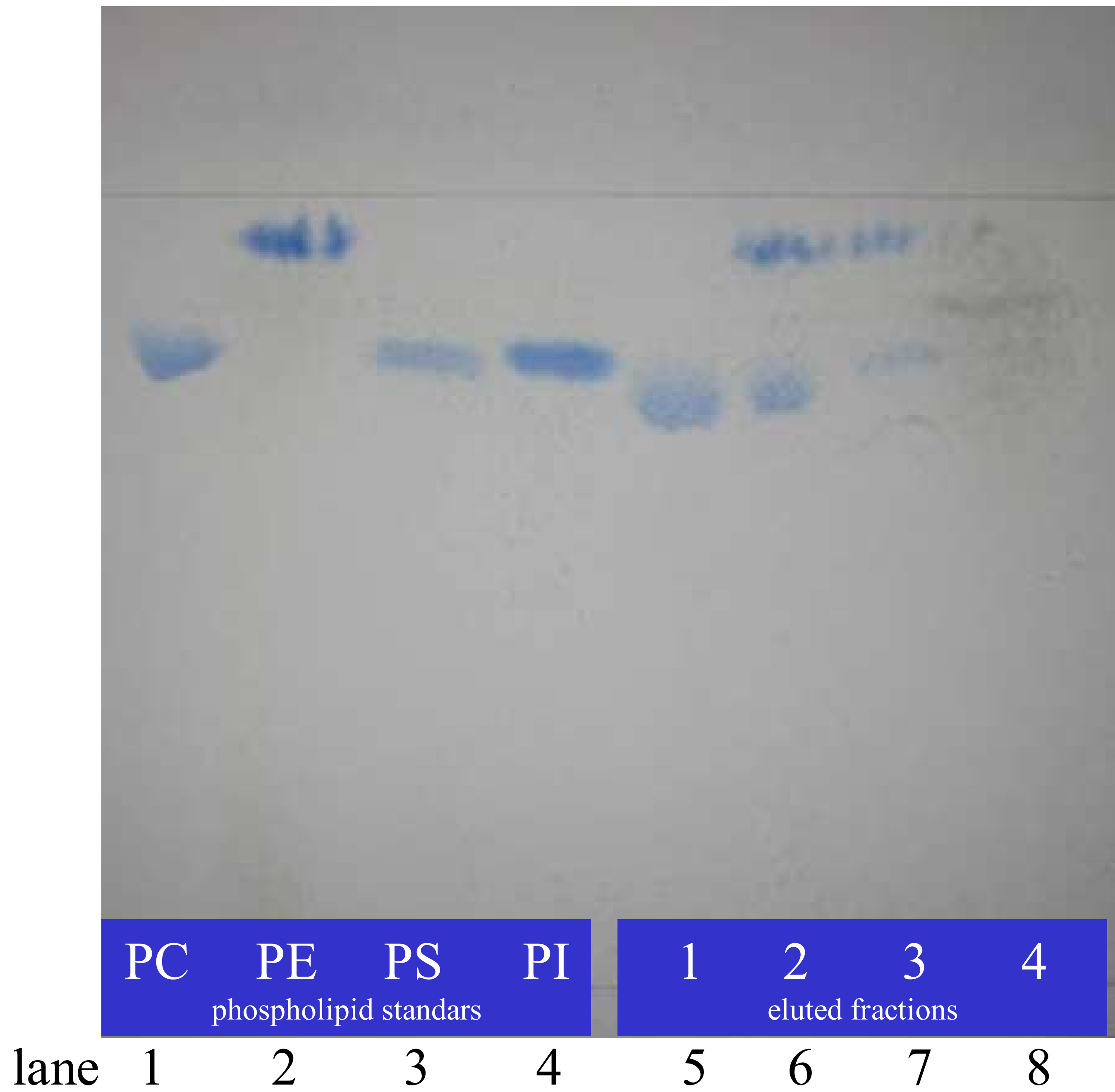
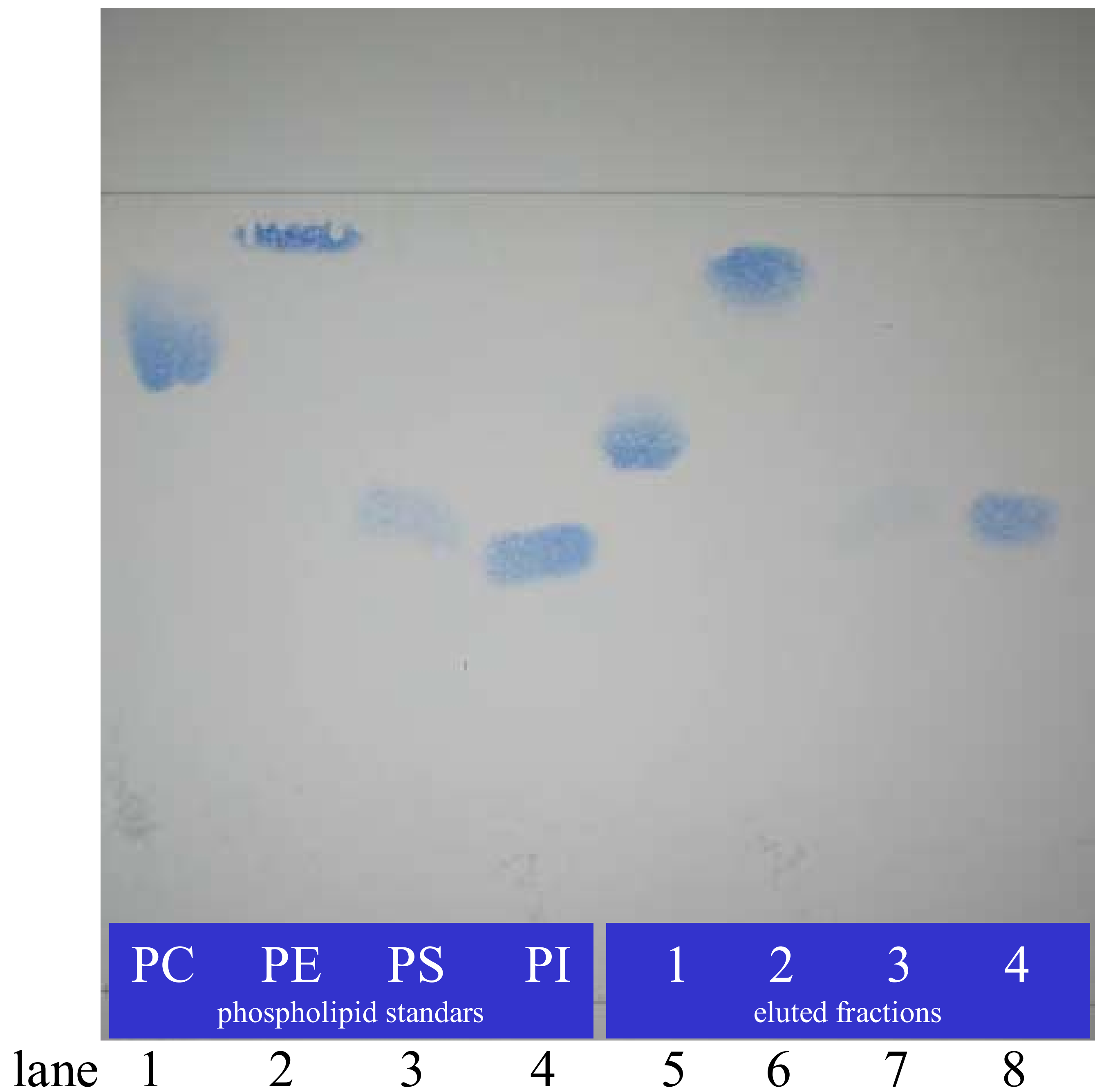
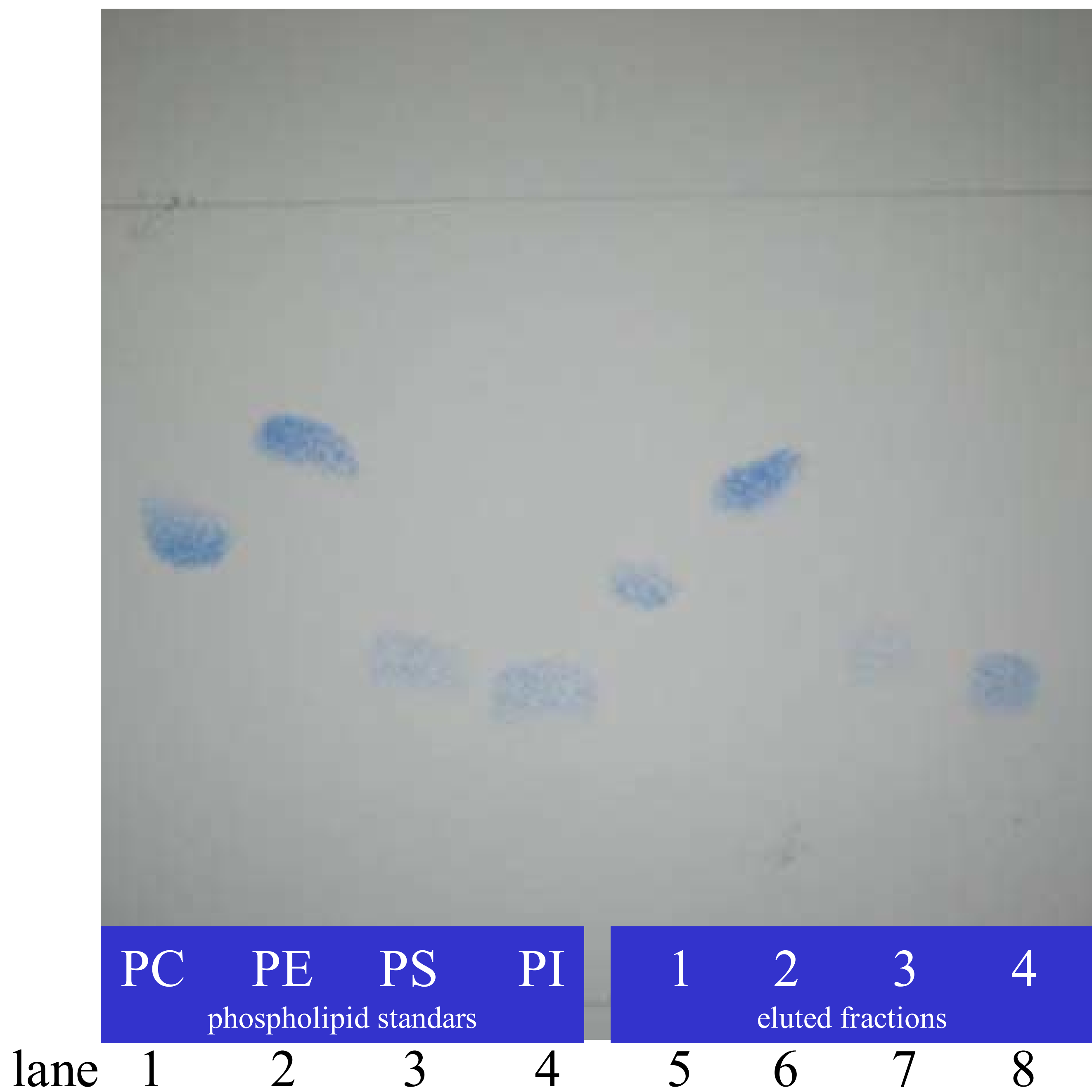
Figure(s)



Figure(s)



Figure(s)



* **Cover Letter**

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Improvement of a solid phase extraction method for separation of muscle phospholipid classes

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The present manuscript constitutes a further development of a previously established SPE method for separation of phospholipids classes. In this case, the method was optimized using phospholipids standards and checked using muscle phospholipids.