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1 Determination of the collision cross sections of cardiolipins and phospholipids from  
2 *Pseudomonas aeruginosa* by traveling wave ion mobility spectrometry-mass spectrometry  
3 using a novel correction strategy

4

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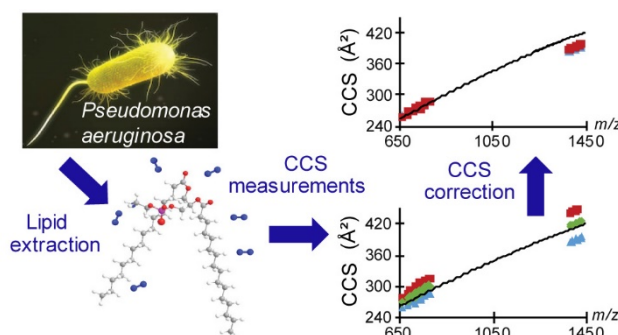
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22 **ABSTRACT**

23

24 Collision Cross Section (CCS) values are descriptors of the 3D structure of ions which  
25 can be determined by ion mobility spectrometry (IMS). Currently, most lipidomic studies  
26 involving CCS values determination concerns eukaryote samples (*e.g.* human, bovine) and to  
27 a lower extent prokaryote samples (*e.g.* bacteria). Here, we report CCS values obtained from  
28 Traveling Wave Ion Mobility spectrometry ( $^{TW}CCS_{N_2}$ ) measurements from the bacterial  
29 membrane of *Pseudomonas aeruginosa* - a bacterium ranked as priority 1 for the R&D of  
30 new antibiotics by the World Health Organization. In order to cover the lack of reference  
31 compounds which could cover the  $m/z$  and CCS range of the membrane lipids of *P.*  
32 *aeruginosa*, three calibrants (polyalanine, dextran and phospholipids) were used for the  
33  $^{TW}CCS_{N_2}$  calibration. A shift from the published lipids CCS values was systematically observed  
34 ( $\Delta CCS\%$  up to 9%), thus we proposed a CCS correction strategy. This correction strategy  
35 allowed to reduce the shift ( $\Delta CCS\%$ ) between our measurements and published values to  
36 less than 2%. This correction was then applied to determine the CCS values of *Pseudomonas*  
37 *aeruginosa* lipids which have not been published yet. As a result, 32  $^{TW}CCS_{N_2}$  values for  $[M+H]^+$   
38 ions and 24  $^{TW}CCS_{N_2}$  values for  $[M-H]^-$  ions were obtained for four classes of phospholipids  
39 (phosphatidylethanolamines (PE), phosphatidylcholines (PC), phosphatidylglycerols (PG) and  
40 diphosphatidylglycerols —known as cardiolipins (CL)).

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## 44 INTRODUCTION

45 *Pseudomonas aeruginosa* is a bacterium known to have intrinsic resistances to  
46 antibiotics and easily acquire genes encoding resistance to new drugs [1]. This bacterium is  
47 responsible of 18% of the bacteria-induced nosocomial pneumonia [2] which, combined with  
48 its virulence, dramatically increases the death rate of immunocompromised patients. As a  
49 result, *P. aeruginosa* is one of the deadliest bacteria in hospitals, thus ranked by the World  
50 Health Organization (WHO) as priority number one for the R&D of new antibiotics [3].

51 Due to the numerous resistance mechanisms of *P. aeruginosa* against antibiotics  
52 targeting proteins or enzymes [4], new antibiotic strategies are required. One of the current  
53 new drug strategies involves membrane cell destabilization or destruction leading to the  
54 bacteria lysis [5]. Thus, it is necessary to know the composition and 3D structure of the  
55 membrane cell lipids to develop such antibiotics. However, while the proteome of *P.*  
56 *aeruginosa* has been extensively studied [6–8], only few data have been reported on the  
57 mem- brane cell lipidome of *P. aeruginosa* [9, 10].

58 Here, we focused on the membrane lipids of *P. aeruginosa* and especially on the  
59 identification of the different compounds by ion mobility spectrometry coupled to mass  
60 spectrometry (IMS-MS). IMS is a post-ionization separation technique in which analyte ions  
61 travel through an inert gas (generally N<sub>2</sub> or He) in a cell under the influence of an electric  
62 field. For equal charge state, the most compact ions will have the smallest drift times since  
63 they are less slowed down by the buffer gas than the largest ions. Consequently, the IMS  
64 separation depends on charge, size and shape [11, 12]. Moreover, drift time ( $t_D$ ) can be  
65 converted to the experimental collision cross section (CCS) of the ion within the buffer gas.  
66 CCS values are closely related to the 3D structure of each ion. These experimental CCS can  
67 be compared with calculated CCS obtained from theoretical structures using computational  
68 methods (*i.e.* trajectory meth- od (TM), projection approximation (PA) and exact hard sphere  
69 scattering (EHSS) methods [13]) or CCS predicted by using a machine learning approach [14]  
70 as CCS are predictable values [15].

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72 For CCS determination, in most cases, and in particular for non-uniform field  
73 instruments such as traveling wave ion mobility spectrometry (TWIMS), the use of  
74 calibration is required with compounds of known CCS [16]. As an example, polyalanine, one  
75 of the most common calibrant for TWIMS, has been used for lipid CCS determination [17–  
76 19]. However, caution must be taken when choosing calibrants since shifts with the  
77 literature can be observed when using calibrants not chemically close to the sample [13].  
78 Indeed, Hines *et al.* [20] confirmed that using calibrants structurally close to targets reduced  
79 the error in calibrated CCS values obtained with TWIMS. Thus, PCs are recommended for  
80 phospholipid calibration in positive mode and PEs for phospholipid calibration in negative  
81 mode [21]. Using definite calibrants, CCS values are extremely repeatable (RSD < 0.5%) [22],  
82 reproducible in intra-laboratory studies (RSD < 2%) [23] and reproducible in inter-laboratory  
83 studies (RSD < 5% using different ion mobility techniques, RSD < 2% using drift-tube) [19].  
84 Consequently, CCS values can be used as an additional descriptor, together with  $m/z$  and  
85 retention time, to increase the confidence in automatic identification [19, 24]. For such  
86 reasons, the number of biological sample studies using IMS as a separation and  
87 characterization tool has been constantly increasing over the last decade [15, 17–20, 24–30].

88 Herein, we report the identification of the cell membrane phospholipids of *P.*  
89 *aeruginosa* strain PAK (PAK) and the experimental CCS values of their positive and negative  
90 ions. The data were acquired on a TWIMS, with nitrogen as drift gas, yielding  $^{TW}CCS_{N_2}$  values  
91 based on conventional notations [23]. To ensure our  $^{TW}CCS_{N_2}$  measurements, we used three  
92 approaches with different calibrating substances: (i) polyalanine [19] and (ii) lipid calibrants  
93 [20] which have been previously used for phospholipids, (iii) dextran which is a calibrant that  
94 can cover the required  $m/z$  and CCS ranges of PAK lipids, from the short fatty acyl chain  
95 phospholipids to the cardiolipins. We show here that the use of polyalanine or dextran  
96 requires a post-calibration  $^{TW}CCS_{N_2}$  correction using commercial phospholipid standards. This  
97 correction strategy was validated by comparing our corrected  $^{TW}CCS_{N_2}$  values with  $^{DT}CCS_{N_2}$   
98 values of the literature. Then, the correction was applied to all the  $^{TW}CCS_{N_2}$  values of *P.*  
99 *aeruginosa* lipids. It is the first time to our knowledge that experimental  $^{TW}CCS_{N_2}$  values of  
100 both protonated and deprotonated ions of diphosphatidylglycerols—commonly called  
101 cardiolipins (CL)—are reported.

## 102 MATERIAL AND METHODS

### 103 *Chemicals and reagents*

104 Milli-Q grade water was obtained from an 18 M $\Omega$  Milli-Q (EMD Millipore  
105 Corporation, Molsheim, France). Methanol, chloroform, acetonitrile and isopropanol were  
106 purchased from Fischer Scientific (UK), ammonium formate from Sigma-Aldrich (Saint  
107 Quentin Fallavier, France) and formic acid from Merck (Germany). Except for chloroform  
108 which was HPLC grade, all solvents and buffers used were LC-MS grade. Polyalanine and  
109 dextran, used as IMS calibration substances, were purchased from Sigma-Aldrich (Saint  
110 Quentin Fallavier, France). Phospholipid standards for the IMS calibration were purchased  
111 from Avanti Polar Lipids (AL, USA): PE 6:0/6:0, PE 10:0/10:0, PE 15:0/15:0, PE 17:0/17:0, PC  
112 17:0/17:0, PC 20:0/20:0, PC 24:0/24:0. Leucine enkephalin was purchased from Sigma-  
113 Aldrich (Saint Quentin Fallavier, France).

114

### 115 *Biological sample preparation*

116 A *P. aeruginosa* strain PAK (PAK) was pre-cultured in a 50-mL centrifugal tube  
117 containing 20  $\mu$ L of bacterial stock solution (Muller-Hinton Broth (MHB)/glycerol, 70/30) and  
118 20 mL of MHB. The flask was incubated overnight at 37 °C on a rotary shaker (140 rpm).  
119 After incubation, the pre-culture was used to inoculate 330 mL of MHB at a final  
120 concentration of 10<sup>4</sup> colony-forming units (CFU)/mL in a 1 L Erlenmeyer flask. The culture  
121 was incubated on a rotary shaker (140 rpm) at 37 °C for 7 h. Then, the culture was  
122 centrifuged (2600 $\times$ g, 10 min, 20 °C). The obtained pellet was washed in 10 mL of 100 mM  
123 Tris pH 8.0. Ten milliliters of phosphate-buffered saline (PBS) and 3 mg of alginate lyase were  
124 added at room temperature during 1 h for the lysis of alginates, produced by *P. aeruginosa*  
125 PAK strain, which can be deleterious for mass spectrometric analysis. A pellet was obtained  
126 by centrifugation (5000 $\times$ g, 10 min, 20 °C) and washed in 100 mM Tris pH 8.0. The pellet was  
127 resuspended in 6 mL of 100 mM Tris pH 8.0 and was subjected to sonication (cycles of 30 s  
128 on/10 s off for 2 min). The suspension was then centrifuged (5200 $\times$ g, 20 min, 20 °C). The  
129 supernatant, containing the inner and outer membranes, was ultra-centrifuged (70,000 $\times$ g, 1  
130 h, 4 °C), then the resulting pellet was stocked at -20 °C.

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131 Finally, the lipid extraction was carried out according to an adapted Bligh and Dyer  
132 protocol [31]. After waiting for the pellet to warm up to room temperature, 500  $\mu\text{L}$  of Milli-Q  
133 water was added to dissolve the pellet and then vortex mixed for 5 min. 1.2 mL of methanol  
134 was added and then vortex mixed for 15 min. To degrade and eliminate proteolipids, the  
135 mixture was centrifuged ( $4000\times g$ , 10 min,  $22\text{ }^{\circ}\text{C}$ ), then the liquid phase was recovered. After  
136 adding 0.6 mL of chloroform, the solution was vortex mixed for 15 min and sonicated for 15  
137 min. 0.6 mL of chloroform was added and then vortex mixed for another 15 min. 0.62 mL of  
138 a 1 M ammonium acetate was added and vortex mixed for 15 min. Finally, the mixture was  
139 left to rest for 30 min in order to allow the three phase separation (aqueous phase, protein  
140 interphase and organic phase). One milliliter of the organic phase was recovered and 0.94  
141 mL of chloroform was added to the aqueous phase for a second liquid-liquid extraction.  
142 After stirring for 15 min, the mixture was left to rest again for 30 min. The aqueous phase  
143 was removed and organic phases were mixed and evaporated under argon. The organic  
144 phase was separated in aliquots and evaporated under argon. Lipid extracts were conserved  
145 under argon at  $-20\text{ }^{\circ}\text{C}$ . To ascertain the quantity of lipids obtained, one aliquot of the lipid  
146 extract was diluted in a chloroform: methanol (4/1, v/v) solution. Then, the lipid extract  
147 concentration was checked by measuring the surface pressure of Langmuir monolayers.

148

#### 149 *Sample and calibrant mix solution preparation*

150 The lipids extracted from the membrane of PAK were reconstituted in  
151 chloroform/methanol (2/1, v/v) to obtain a concentration of  $1\text{ }\mu\text{g}/\mu\text{L}$  which was estimated as  
152 previously mentioned. Five microliters of this solution was then diluted in  $20\text{ }\mu\text{L}$  of  
153 isopropanol/acetonitrile/water (4/3/1, v/v) for analysis. The dextran calibrant solution was  
154 prepared from a stock solution (1 mg/mL in water) diluted to  $10^{-5}\text{ mol/L}$  in water/  
155 acetonitrile (1/1, v/v). The polyalanine calibrant solution was prepared from a stock solution  
156 (1 mg/mL in water/acetonitrile (1/1, v/v)) diluted to  $10^{-5}\text{ mol/L}$  in water/acetonitrile/acetic  
157 acid (49.5/49.5/1, v/v). Commercial PEs and PCs used as IMS standards were individually  
158 dissolved at  $10^{-4}\text{ mol/L}$  in chloroform/methanol (2/1, v/v), then mixed and diluted in  
159 isopropanol/acetonitrile/water (4/3/1, v/v) to obtain a lipid calibrant solution at  $10^{-5}\text{ mol/L}$ .

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160 *Instrumentation*

161 The LC-IMS-MS experiments were performed using an UHPLC system (Dionex  
162 Ultimate 3000 RSLC, Thermo Scientific, San Jose, CA, USA) coupled to an IMS Q-TOF mass  
163 spectrometer (SYNAPT G2 HDMS, Waters MS Technologies, Manchester, UK)  
164 equipped with an electrospray interface, a traveling wave ion mobility (TWIMS) cell.  
165 Data treatment was performed with MassLynx 4.1, DriftScope 2.8 and UNIFI 1.8.2.169  
166 software (Waters, Manchester, UK).

167 The column used was a 1.0 × 100 mm, Acquity UPLC CSH C18 1.7 μm column (Waters,  
168 Manchester, UK) equipped with a 0.2 μm prefilter. The sample tray and column oven  
169 temperatures were set at 4 °C and 50 °C, respectively. The injection volume was set at 1 μL.  
170 A flow rate of 100 μL/ min was used. Mobile phase A consisted of acetonitrile/ aqueous 10  
171 mM ammonium formate (60/40) + 0.1 % formic acid and mobile phase B of  
172 isopropanol/acetonitrile/aqueous 10 mM ammonium formate (88/8/4) + 0.1 % formic acid.  
173 The gradient elution was the following: 0–3 min, 6–62% B; 3–8 min, 62–70% B; 8–13 min,  
174 70% B; 13–15 min, 70–89% B; 15–20 min, 89% B; 20–22 min, 89–100% B; 22–26 min,  
175 100% B; 26–27 min, 100–6% B; 27–30 min, 6% B.

176 LC-IMS-MS data were acquired in triplicate in positive and negative ion modes. The  
177 source parameters were in positive mode: capillary, extraction cone and cone voltages set at  
178 3 kV, 6 V and 50 V, respectively; the cone gas and desolvation gas flows were 40 L/h and 600  
179 L/h, respectively; and the desolvation temperature was 400 °C. For negative ion mode, the  
180 capillary, extraction cone and cone voltage were – 2.4 kV, 5 V and 45 V, respectively; the cone  
181 gas and desolvation gas flows were 60 L/h and 900 L/h, respectively; and the desolvation  
182 temperature was 500 °C.

183 The IMS parameters were helium cell flow 180 mL/min, traveling wave height 40 V, IMS cell  
184 nitrogen flow 70 mL/min (3.01 mbar) and traveling wave velocity 600 m/s.

185 Mass spectra were recorded over a  $m/z$  50–2000 range in resolution mode (resolution 20 000  
186 FWHM). External  $m/z$  calibration was performed with a sodium formate solution.



187 Lockmass correction was performed by infusing leucine enkephalin (2 ng/ $\mu$ L, 3  $\mu$ L/min) via the  
188 LockSpray™ interface (reference scan frequency, lock spray capillary and cone voltage of 10 s,  
189 3.5 kV and 40 V, respectively, in positive mode and 10 s, 2.5 kV and 30 V, respectively, in  
190 negative mode).

191 MS/MS experiments were performed in both positive and negative ionization mode.  
192 Precursor ions were selected in the quadrupole, at a resolution of 1  $m/z$  unit, using retention  
193 time windows in the acquisition method. Several acquisitions were performed to obtain the  
194 MS/MS spectra for all co-eluting lipids. The collisional activation was performed in the trap cell  
195 using argon as target gas ( $6.10^{-3}$  mbar) and a collision energy of 35 eV. MS/MS spectra were  
196 processed using MassLynx 4.1 (Waters).

197 For calibrants, raw data were first opened with DriftScope 2.8 (Waters) in order to  
198 only select the singly charged ions on the 2D map (drift times plotted against  $m/z$ ). Ion  
199 mobility spectra were then exported and processed with MassLynx 4.1 (Waters). For PAK  
200 samples, raw data were processed with UNIFI 1.8.2.169 (Waters). PAK lipids were first  
201 manually identified in order to create a home-made library from retention time ( $t_R$ ) and  $m/z$ ,  
202 for each of the lipids described in this publication. This library was then used to  
203 automatically obtain the lipids  $t_D$ .

204

#### 205 *Collision cross section measurements*

206 Due to the use of an inhomogeneous electric field in the TWIMS cell, an external CCS  
207 calibration is required. For this purpose, we used singly charged ions of three published  
208 calibrants: dextran oligomers [32, 33], polyalanine oligomers [34] and phospholipids (PEs  
209 and PCs) [20]. Thus, these calibrant solutions were individually infused for 2 min in positive  
210 and negative modes. Ion mobility spectra were fitted with a Gauss function [35] using  
211 OriginPro 2016 9.3.226 (OriginLab Corporation) to obtain the drift time of each calibrant  
212 ion. Using charge- and mass-independent  $^{DT}CCS_{N_2}$  values ( $CCS'$ ) and drift times ( $t_D$ ), calibration  
213 curves  $\ln CCS' = f(\ln t_D)$ , for singly charged positive and negative ions, were obtained in  
214 nitrogen according to the procedure of Smith [16]. LC-IMS-MS data of the PAK sample were

30

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215 processed with the UNIFI software (Waters) in order to automatically obtain drift time  
216 values.

217 Drift time values were collected for retention time variation lower than 0.3 min, mass error  
218 lower than 5 ppm and relative intensity higher than 0.1%.

219 Then,  $^{TW}CCS_{N_2}$  were determined using the calibration curve established previously. Finally, the  
220  $^{TW}CCS_{N_2}$  values were corrected using the CCS correction strategy that will be developed in the  
221 results section of this paper. This correction involves plotting  $^{DT}CCS_{N_2}$  against  $^{TW}CCS_{N_2}$ , for the  
222 phospholipid CCS standards, using calculation spreadsheets (see Electronic Supplementary  
223 Material (ESM) ESM\_3).

224

## 225 **RESULTS AND DISCUSSION**

### 226 *Lipid profiling*

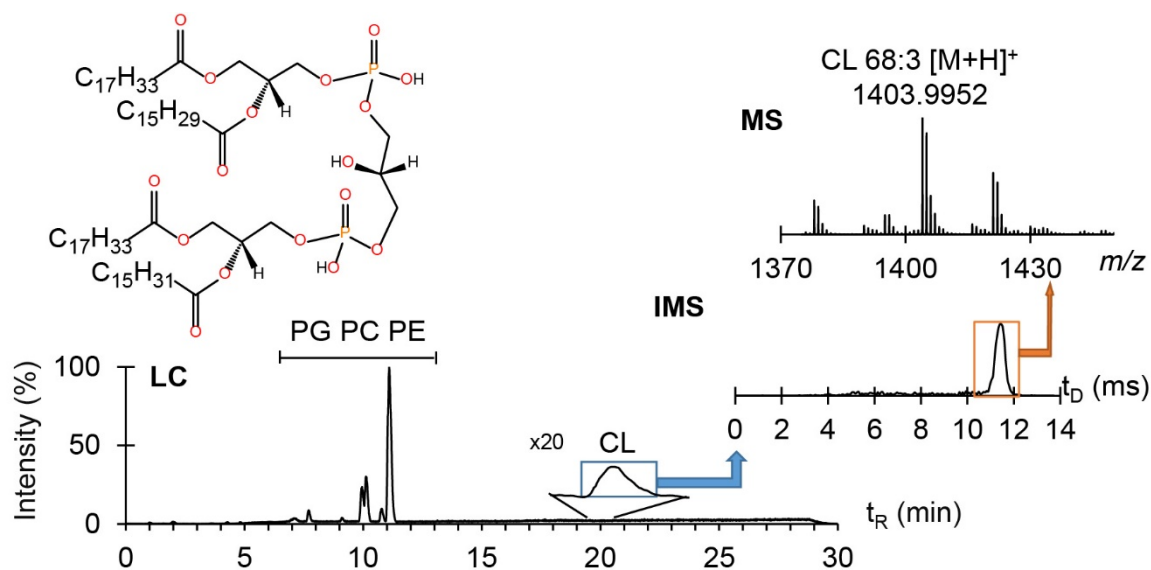
227 The LC-IMS-MS analysis of PAK membrane extract is reported in Fig. 1. The LC  
228 dimension allows the separation of the different classes of lipids, *i.e.* phosphatidylcholines  
229 (PC), phosphatidylethanolamines (PE), phosphatidylglycerols (PG) and cardiolipins (CL). Using  
230 our chromatographic conditions, the following separation could be achieved: PC, PE and PG  
231 classes (two fatty acid chains on phospholipids) from 8.5 to 12 min and CL class (four fatty  
232 acid chains on phospholipids) from 18.8 to 20.5 min. Some co-elution was still observed for  
233 isomers of phospholipids exhibiting different combinations of fatty acyl chains (*e.g.* PG  
234 16:1/16:0 and PG 18:1\_14:0, see ESM\_1 Fig. S1) and for phospholipids consisted of fatty acyl  
235 chains showing two methylene and one more double bond (noted as DBE for double bond  
236 equivalent) than other phospholipids of the same class (*e.g.* PE 30:0, PE 32:1 and PE 34:2).  
237 Lipid identification was achieved thanks to the orthogonal MS dimension, more precisely  
238 from MS/MS data and accurate mass measurements.

239 Thus, 41 lipids were clearly identified in the membrane of PAK (see ESM\_1: Tables S1,  
240 S2, and S3): 28 phospholipids (8 PGs, 13 PEs and 7 PCs) and 13 CLs. Please note that with our  
241 analytical conditions (*i.e.* buffered mobile phases), the cardiolipins were detected as singly

242 charged species but not as doubly charged species—as previously described with other  
 243 analytical conditions [36]. The lipid annotation is based on the nomenclature of Liebisch *et*  
 244 *al.* [37].

245 To our knowledge, this study is the first one on the membrane lipids of the strain PAK —  
 246 other studies were performed on the strains PAO1 and PA14 [9, 10]. No relevant differences  
 247 between the membrane lipids of the strains PAK, PAO1 and PA14 were observed.

248



249

250 **Fig. 1** Base Peak Chromatogram (BPC), extracted ion mobility spectrum and mass spectrum  
 251 from LC-IMS-MS analysis of PAK membrane extract. The enlarged retention time zone  
 252 corresponds to cardiolipins. The ion mobility spectrum was extracted for CL 68:3 (*m/z*  
 253 1403.9952). The mass spectrum is obtained by integrating the ion mobility spectra from 10.5  
 254 ms to 12.5 ms. A possible structure for *m/z* 1403.9952 is given on the left.

255

256 <sup>TW</sup>CCS<sub>N<sub>2</sub></sub> determination, comparison with published CCS values and post-calibration correction

257 Most of the CCS reported in the literature for phospholipids (PL) have been obtained  
 258 using either a drift-tube IMS instrument [15, 20, 38, 39] or a TWIMS cell calibrated with  
 259 polyalanine ions [17–19]. In the latter case, Hines *et al.* [20, 25] had previously

260 demonstrated that the  $^{TW}CCS_{N_2}$  determination of PL requires TWIMS cell calibration with PL  
261 calibrants. However, obtaining the most representative  $^{TW}CCS_{N_2}$  values would require to  
262 have lipid calibration curves for each chemical class present in the sample (PC, PE, PG or CL)  
263 and for each number of double bonds on the fatty acyl chains (0, 1 or 2).

264 Because of the lack of such calibrants, we chose to use a single calibrant to determine the  
265 CCS values of all the PAK membrane lipids. For this reason, we choose the dextran calibrant  
266 which covers the required  $m/z$  and CCS ranges, in particular for CLs.

267 Please note that an extrapolated polyalanine calibration was considered as well, but  
268 for clarity, the following methodology will be described for the dextran calibration first. The  
269 calibration curves obtained with dextran (in both positive and negative modes) were then  
270 used for our PAK sample (see ESM\_2). Thus, the  $^{TW}CCS_{N_2}$  of  $[M+H]^+$  were measured for PEs,  
271 PCs and CLs in positive ion mode while the  $^{TW}CCS_{N_2}$  of  $[M-H]^-$  were measured for PEs, PGs  
272 and CLs in negative ion mode. Triplicate of  $^{TW}CCS_{N_2}$  measurements were carried out for each  
273 sample (in positive and negative ion mode) and gave repeatable CCS values ( $0.1\% \leq \%RSD \leq$   
274  $0.4\%$ ,  $n = 3$ ). However, when we compared our measured  $^{TW}CCS_{N_2}$  values with those  
275 published (5 PEs and 6 PCs in positive mode, 6 PEs and 4 PGs in negative mode), shifts in CCS  
276 were systematically observed ( $\Delta CCS \geq 2\%$ ) (ESM\_1: Tables S4 and S5). This is also illustrated  
277 in Fig. 2a: the experimental  $^{TW}CCS_{N_2}$  values obtained after dextran calibration in positive ion  
278 mode (red squares) are significantly shifted towards higher CCS values compared with the  
279 lipid CCS values from the literature [20] (extrapolated black line).

280 In order to avoid this issue, we propose here a CCS correction strategy to reduce the  
281 shift due to the dextran calibration. The CCS correction strategy was carried out, using the  
282 IMS lipid calibrants (PEs and PCs, see ESM\_2), by plotting correlation curves between their  
283 experimental  $^{TW}CCS_{N_2}$  calibrated with dextran and their published  $^{DT}CCS_{N_2}$  [20]. In both  
284 positive and negative ion mode, a linear regression was obtained with high coefficient of  
285 determination ( $R^2 \geq 0.999$ , ESM\_3). This post-calibration correction was then applied to the  
286  $^{TW}CCS_{N_2}$  values of lipids present in PAK strain (red squares of Fig. 2b). Please note that  
287 previous work had shown that the fatty acyl chains of phospholipids extracted from *P.*  
288 *aeruginosa* can be different from the ones of eukaryotes —especially odd-chains which can

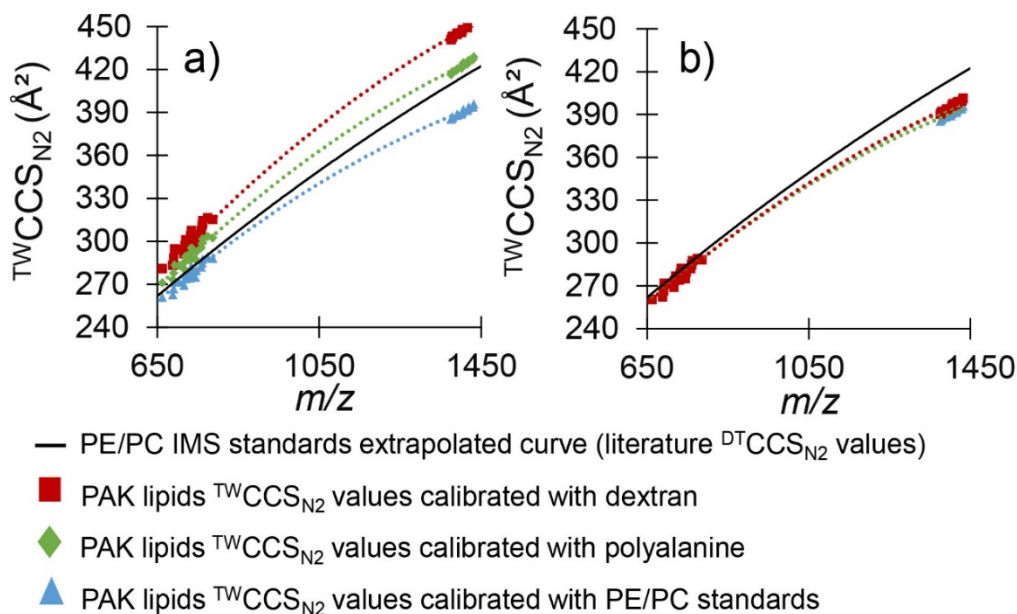
289 be cyclopropylated or methylated [9, 40]. However, considering the resolving power of  
290 TWIMS, linear, cyclopropylated or methylated fatty acyl chain isomers would not be  
291 separated. Hence, we could use easily available phospholipid standards rather than bacterial  
292 phospholipids for the correction.

293 In parallel, as proof of concept for our correction strategy, we also determined  $^{TW}CCS_{N_2}$   
294 for PAK lipids using polyalanine or lipid calibration curves (see ESM\_2). These calibration curves  
295 were extrapolated to higher CCS values in order to exceed those of CLs.

296 The experimental  $^{TW}CCS_{N_2}$  for PAK lipids, using these extrapolated calibration curves, are  
297 reported in Fig. 2a as green (polyalanine cal) and blue (PL cal) points. One can notice that the  
298 CCS range of our  $^{TW}CCS_{N_2}$  obtained with polyalanine calibration (ESM\_1: Table S2) are  
299 consistent with the cardiolipins  $^{TW}CCS_{N_2}$  of  $[M+2K]^{2+}$  and  $[M-2H]^{2-}$  from *Escherichia coli*  
300 recently published by Hines *et al.* (CCS range of 380–425 Å<sup>2</sup>) [25]. The correction strategy,  
301 previously described for the  $^{TW}CCS_{N_2}$  calibrated with dextran, was also applied for  $^{TW}CCS_{N_2}$   
302 values calibrated with polyalanine (green points of Fig. 2b).

303 Figure 2b shows that the correction strategy applied for  $^{TW}CCS_{N_2}$  values calibrated by  
304 either dextran or polyalanine, and the  $^{TW}CCS_{N_2}$  calibration —without correction— with PL, lead  
305 to convergent experimental  $^{TW}CCS_{N_2}$  values. Moreover, for CLs, these convergent  $^{TW}CCS_{N_2}$  values  
306 are smaller than the extrapolated PE/PC  $^{DT}CCS_{N_2}$  black line. This result suggests that  
307 cardiolipins are more compact (smaller  $^{TW}CCS_{N_2}$  value) than any PE or PC which would have a  
308 similar m/z value (extrapolated black line). This might be explained by the fact that  
309 cardiolipins are biosynthesized from a phosphatidylglycerol and a phosphatidic acid [41] which  
310 have smaller CCS values than PEs or PCs [15]. The details of all the  $^{TW}CCS_{N_2}$  values we measured  
311 are reported in the supplementary information (ESM\_1: Tables S1 to S3):  $^{TW}CCS_{N_2}$  values using  
312 the three types of calibration (dextran (Table S1), polyalanine (Table S2) and lipids (Table S3))  
313 and the two corrections (dextran corrected with lipids (Table S1) and polyalanine corrected  
314 with lipids (Table S2)).

315



316

317 **Fig. 2**  $^{TW}CCS_{N_2}$  plotted against  $m/z$  for positive ions of PAK lipids. (a) represents  $^{TW}CCS_{N_2}$  of PAK  
 318 lipids before correction of the  $^{TW}CCS_{N_2}$  values. (b) represents  $^{TW}CCS_{N_2}$  of PAK lipids after the  
 319 correction. In both (a) and (b), the black line represents the lipid IMS standards extrapolated  
 320 curve ( $^{DT}CCS_{N_2}$  values from Hines *et al.* [20]).  $^{TW}CCS_{N_2}$  of PAK lipids are represented by red  
 321 squares when calibrated with dextran, green squares when calibrated with polyalanine and  
 322 blue squares when calibrated with lipid standards.

323

324 In order to validate our correction strategy (calibration with dextran followed by a  
 325 post-calibration correction with lipids, as mentioned above), the corrected  $^{TW}CCS_{N_2}$  values  
 326 were compared with the published ones. Note that several phospholipids (PEs, PGs and PCs)  
 327 present in our sample had published CCS values (17 in positive mode and 16 in negative  
 328 mode). On the other hand, CCS values of protonated and deprotonated prokaryote  
 329 cardiolipins had never been reported. Considering that published CCS values from different  
 330 ion mobility techniques have some disparities ( $0.04\% \leq \Delta CCS\% \leq 3.84\%$ , ESM\_1: Tables S6  
 331 and S7), our  $^{TW}CCS_{N_2}$  are consistent with the CCS values from the literature, as reported in  
 332 Table S5. One can note that the CCS disparities in the literature could be due to differences  
 333 in lipid structures (*e.g.* double bond location and geometry cis/trans). This structural  
 334 heterogeneity would give rise to small, but measurable, CCS difference [38].

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335 In addition, one can observe that the correction strategy allows a shift reduction to less than  
 336 2% between our corrected  $^{TW}CCS_{N_2}$  and those reported by Zhou *et al.* [15] and Hines *et al.*  
 337 [20], as shown in Table 1. Since such deviation is acceptable for inter-laboratory  
 338 reproducibility, we can highlight the validation of the post-calibration correction strategy.  
 339 Furthermore, the  $^{TW}CCS_{N_2}$  values of phospholipids extracted from PAK membrane are similar  
 340 to the CCS values of lipid standards from the literature; therefore, we can suppose that the  
 341 phospholipids in PAK have similar tridimensional structures in gas phase than the  
 342 phospholipids used by Zhou *et al.* and Hines *et al.* (synthetic standards) [15, 20]. Besides, a  
 343 similar decrease of the shift between our experimental  $^{TW}CCS_{N_2}$  values and those of the  
 344 literature was observed for the experimental CCS values obtained by polyalanine calibration  
 345 and post-calibration lipid correction, as illustrated in Fig. 2b. All comparisons with the  
 346 literature are given in ESM\_1 Tables S4 and S5.

347

**Table 1.** Comparison of our experimental  $^{TW}CCS_{N_2}$ , from PAK, with the published values. a and b correspond to relative shifts between our CCS and  $^{DT}CCS_{N_2}$  from a: Zhou *et al.* (2017) [15] and b: Hines *et al.* (2016) [20], respectively.

Positive ion mode [M+H] <sup>+</sup>						
Lipid	Calibration using dextran			After calibration correction		
	$^{TW}CCS_{N_2}$ (Å <sup>2</sup> )	$\Delta CCS^a$ (%)	$\Delta CCS^b$ (%)	$^{TW}CCS_{N_2}$ (Å <sup>2</sup> )	$\Delta CCS^a$ (%)	$\Delta CCS^b$ (%)
PC 32:0	307.6	8.0	8.9	282.0	-1.0	-0.2
PE 32:0	294.6	7.8	8.5	271.4	-0.7	0.0
PE 34:0	301.0	7.5	8.5	276.6	-1.2	-0.3
PE 34:1	297.1	7.4	8.7	273.4	-1.2	0.0
Negative ion mode [M-H] <sup>-</sup>						
Lipid	Calibration using dextran			After calibration correction		
	$^{TW}CCS_{N_2}$ (Å <sup>2</sup> )	$\Delta CCS^a$ (%)	$\Delta CCS^b$ (%)	$^{TW}CCS_{N_2}$ (Å <sup>2</sup> )	$\Delta CCS^a$ (%)	$\Delta CCS^b$ (%)
PE 32:0	274.4	5.6	5.8	257.1	-1.1	-0.9
PE 34:0	281.4	5.7	6.0	263.4	-1.1	-0.8
PE 34:1	279.5	5.5	5.7	261.6	-1.3	-1.1

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349 *CCS correction application*

350 Once the post-calibration correction was validated, this methodology was then  
 351 extended to all the lipids of PAK. Table 2 reports the  $^{TW}CCS_{N_2}$  values of lipids in PAK extracts  
 352 which constitute new findings, in particular for CLs. Indeed, the  $^{TW}CCS_{N_2}$  of  $[M+H]^+$  and  $[M-H]$   
 353  $^-$  are determined for the first time for PAK cardiolipins (Table 2, ESM\_1: Tables S1 to S3). As  
 354 previously mentioned, the doubly charged cardiolipins were not detected since the  
 355 ionization of the singly charged species was favored by our analytical conditions. In the case  
 356 of phospholipids, we could verify that our corrected experimental CCS were consistent with  
 357 those of phospholipids described in literature (ESM\_1: Tables S4 and S5).

358 In total, 56  $^{TW}CCS_{N_2}$  values were measured and corrected, including 32 for positive  
 359 ions and 24 for negative ions. Using the dextran calibration, the  $^{TW}CCS_{N_2}$  range was from 303  
 360  $\text{\AA}^2$  to 455  $\text{\AA}^2$  in positive mode and from 266  $\text{\AA}^2$  to 418  $\text{\AA}^2$  in negative mode, whereas when  
 361 the dextran calibration was corrected, the  $^{TW}CCS_{N_2}$  range was from 278  $\text{\AA}^2$  to 401  $\text{\AA}^2$  in  
 362 positive mode and from 249  $\text{\AA}^2$  to 387  $\text{\AA}^2$  in negative mode. More precisely for cardiolipins,  
 363 the  $^{TW}CCS_{N_2}$  range was from 441  $\text{\AA}^2$  to 455  $\text{\AA}^2$  in positive mode and from 411  $\text{\AA}^2$  to 418  $\text{\AA}^2$  in  
 364 negative mode, whereas when the dextran calibration was corrected, the  $^{TW}CCS_{N_2}$  range was  
 365 from 390  $\text{\AA}^2$  to 401  $\text{\AA}^2$  in positive mode and from 380  $\text{\AA}^2$  to 387  $\text{\AA}^2$  in negative mode.  
 366 Moreover, for cardiolipins in negative mode, the  $^{TW}CCS_{N_2}$  values were consistent with the first  
 367 two  $^{DT}CCS_{N_2}$  of CL (CL 72:6 and CL 72:7) described from bovine heart by Groessl *et al.* [38].

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**Table 2.**  $^{TW}CCS_{N_2}$  values, with dextran calibration and lipid correction, for phospholipids  
 extracted from the membrane of PAK.

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Origin	Lipid	$^{TW}CCS_{N_2}$ ( $\text{\AA}^2$ )	$^{TW}CCS_{N_2}$ ( $\text{\AA}^2$ )
		for $[M+H]^+$	for $[M-H]^-$
Phospholipids extracted from the membrane of PAK	PE 30:1	260.2	249.7
	PE 33:1	271.0	258.7
	PE 35:1	276.0*	264.9
	PG 30:0	nd	258.3
	PG 35:2	nd	269.7
	CL 66:3	390.1	380.4
	CL 66:2	391.2	381.6
	CL 66:1	391.9	nd
	CL 67:3	392.8	nd
	CL 67:2	393.7	nd
	CL 68:4	394.0	nd
	CL 68:3	395.7	nd
	CL 68:2	397.4	386.9
	CL 69:4	396.7	nd
	CL 69:3	398.3	nd
	CL 69:2	399.0	nd
CL 70:4	399.4	nd	
CL 70:3	401.2	nd	

These values are new to the literature, apart from the CCS noted with \*. nd: not detected.

374

### 375 *PAK lipid $^{TW}CCS_{N_2}$ vs $m/z$ correlation*

376 Fig. 3 illustrates the correlation between the corrected  $^{TW}CCS_{N_2}$  values and  $m/z$  values  
 377 in negative mode: for all lipids in Fig. 3a and for PEs and PGs in Fig. 3b. As shown in Fig. 3a, a  
 378 polynomial fit for  $^{TW}CCS_{N_2}$  plotted against  $m/z$  in negative mode ( $> 0.999$ ) was observed. A  
 379 polynomial fit ( $> 0.99$ ) was also observed for positive values (ESM\_1: Fig. S9). By plotting  
 380  $^{TW}CCS_{N_2}$  against  $m/z$  for each chemical family, we can observe that PGs, PEs and CLs present  
 381 similar slopes.

382 In negative ion mode, deprotonated molecules of PEs and PGs present close  $^{TW}CCS_{N_2}$  values  
 383 for a same  $m/z$  range (Fig. 3b), also for PEs and PGs constituted of the same acyl chains. The

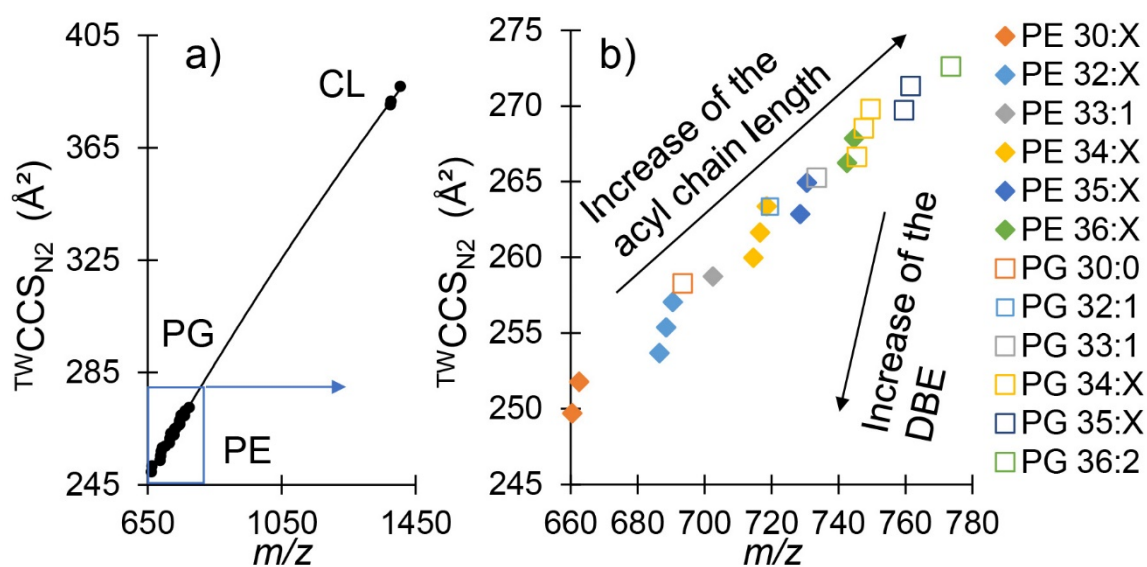
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384  $^{TW}CCS_{N_2}$  of PGs are slightly higher than the ones of the PEs (e.g.  $^{TW}CCS_{N_2}(PG\ 34:1) = 268.5\ \text{\AA}^2 >$   
 385  $^{TW}CCS_{N_2}(PE\ 34:1) = 261.6\ \text{\AA}^2$ ). A similar trend was observed for PEs and PCs, in positive mode,  
 386 with PC's  $^{TW}CCS_{N_2}$  being slightly higher than the ones of the PEs (ESM\_1: Fig. S9).

387 Moreover, as shown in Fig. 3b, we can make several observations. First, as previously  
 388 described by Jackson *et al.* [28], the CCS linearly decreases when the number of double-  
 389 bonds increases in the fatty acyl chains for definite chain lengths (e.g. C32:0, C32:1 and  
 390 C32:2). Secondly, the slope observed from this linear pattern was identical for every  
 391 chemical classes, leading to a symmetrical figure, as described by Leaptrot *et al* [39]. Thirdly,  
 392 for positive ions (ESM\_1: Fig. S9), CCS values of phospholipids with odd number of carbon  
 393 atoms in the fatty acyl chains and with 0, 1 or 2 DBE were closer than the CCS values of  
 394 phospholipids with even number of carbon atoms. All of these observations could be  
 395 explained by the folding of the fatty acyl chain with the increase of the DBE, as previously  
 396 described [15,28,42].



397

398 **Fig. 3**  $^{TW}CCS_{N_2}$  values plotted against  $m/z$  for negative ions. 3a represents values for every  
 399 class of phospholipids, whereas 3b only represents values for PEs and PGs. 3b was inspired  
 400 from the Fig. 4 from Zhou *et al* (2017) [15]. PGs are represented by squares, PEs by  
 401 diamonds. X refers to the number of double-bonds on the fatty acid moiety of the  
 402 phospholipid which can be 0, 1 or 2 for PEs and PGs.

## 403 **CONCLUSION**

404 In this study, new knowledge about the membrane phospholipids of *P. aeruginosa* was  
405 obtained. In particular, new prokaryote lipid CCS values were determined. We worked on a  
406 methodology to allow the measurement of <sup>TW</sup>CCS<sub>N2</sub> within high *m/z* and CCS ranges. Our  
407 work resulted in a CCS correction strategy. After applying the correction, we showed that the  
408 CCS deviation observed between the CCS values of the literature and the ones measured  
409 with the dextran or polyalanine calibration could be lessened. We measured 32 <sup>TW</sup>CCS<sub>N2</sub>  
410 values in positive ionization mode and 24 in negative mode, with a good repeatability (RSD ≤  
411 0.4%). In particular, we measured 18 new <sup>TW</sup>CCS<sub>N2</sub> values for cardiolipins, a unique class of  
412 phospholipids whose CCS values were lacking in the literature for protonated and  
413 deprotonated molecules. Such values can be used to complete existing CCS databases.  
414 Moreover, regarding antibiotic research against *P. aeruginosa*, cardiolipins are of high  
415 interest as a recent study described new antibiotic therapies targeting cardiolipins [43].

416

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420 support.

421

## 422 **COMPLIANCE WITH ETHICAL STANDARDS**

## 423 **CONFLICT OF INTEREST**

424 The authors declare that they have no conflict of interest.

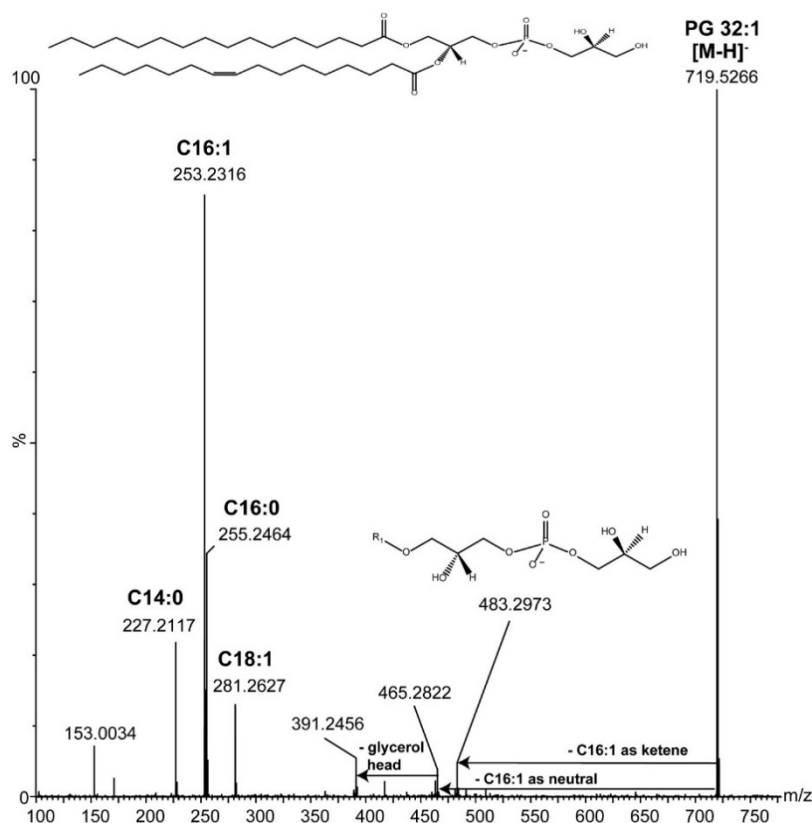
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- 537

538 **Supplementary Information S1 : MS/MS spectrum and <sup>13</sup>C<sub>CS</sub><sub>N2</sub> measurements.**

539

540 **Fig S1.** MS/MS Spectra of PG 32:1. A possible structure of the most abundant isomer is given  
 541 (cis or trans isomerism cannot be determined with this experiment). The ion of  $m/z$   
 542 153.0034 corresponds to the Glycerol-3-phosphate ion with loss of  $H_2O$ . The  $[M-H]^-$  of the  
 543 fatty acyl chains show the presence the isomers PG 16:0\_16:1 and PG 14:0\_18:1. Knowing  
 544 that the loss of *sn*-2 is favoured, since ions of  $m/z$  483.2973, 465.2822, 391.2456 and  
 545 253.2316 are the most abundant, we can conclude that for PG 16:0/16:1, the most abundant  
 546 isomer, the 16:0 is on position *sn*-1 and the 16:1 is on position *sn*-2.

547 Structural elucidation was performed according to Hsu F-F, Turk J (2009) Electrospray  
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**Table S2** <sup>TW</sup>CCS<sub>N2</sub> values calibrated with **dextran** for the membrane lipids of PAK

membrane lipids of PAK	Formula	Positive ions					Negative ions				
		ion	m/z	<sup>TW</sup> CCS <sub>N2</sub> without correction	<sup>TW</sup> CCS <sub>N2</sub> %RSD	corrected <sup>TW</sup> CCS <sub>N2</sub> (Å <sup>2</sup> )	ion	m/z	<sup>TW</sup> CCS <sub>N2</sub> without correction	<sup>TW</sup> CCS <sub>N2</sub> %RSD	corrected <sup>TW</sup> CCS <sub>N2</sub> (Å <sup>2</sup> )
PC 32:1	C <sub>40</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	732.553	303.6	0.1	278.7					
PC 32:0	C <sub>40</sub> H <sub>80</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	734.569	307.6	0.1	282.0					
PC 34:2	C <sub>42</sub> H <sub>82</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	758.569	307.5	0.1	281.9					
PC 34:1	C <sub>42</sub> H <sub>82</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	760.585	311.0	0.1	284.7					
PC 34:0	C <sub>42</sub> H <sub>84</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	762.600	314.2	0.4	287.3					
PC 35:1	C <sub>43</sub> H <sub>84</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	774.600	316.3	0.1	289.0					
PC 36:2	C <sub>42</sub> H <sub>82</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	786.600	315.1	0.1	288.1					
PE 30:1	C <sub>35</sub> H <sub>68</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	662.475	280.8	0.1	260.2	[M-H] <sup>-</sup>	660.461	266.3	0.1	249.7
PE 30:0	C <sub>35</sub> H <sub>70</sub> NO <sub>8</sub> P						[M-H] <sup>-</sup>	662.476	268.6	0.1	251.8
PE 32:2	C <sub>37</sub> H <sub>70</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	688.491	283.2	0.1	262.2	[M-H] <sup>-</sup>	686.476	270.7	0.2	253.7
PE 32:1	C <sub>37</sub> H <sub>72</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	690.506	288.3	0.1	266.3	[M-H] <sup>-</sup>	688.492	272.6	0.1	255.4
PE 32:0	C <sub>37</sub> H <sub>74</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	692.522	294.6	0.0	271.4	[M-H] <sup>-</sup>	690.507	274.4	0.1	257.1
PE 33:1	C <sub>38</sub> H <sub>74</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	704.522	294.1	0.1	271.0	[M-H] <sup>-</sup>	702.507	276.3	0.1	258.7
PE 34:2	C <sub>39</sub> H <sub>73</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	716.522	291.4	0.1	268.8	[M-H] <sup>-</sup>	714.507	277.7	0.1	260.0
PE 34:1	C <sub>37</sub> H <sub>76</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	718.538	297.1	0.1	273.4	[M-H] <sup>-</sup>	716.523	279.5	0.0	261.6
PE 34:0	C <sub>39</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	720.553	301.0	0.1	276.6	[M-H] <sup>-</sup>	718.539	281.4	0.1	263.4
PE 35:2	C <sub>40</sub> H <sub>76</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	730.538	297.8	0.1	274.0	[M-H] <sup>-</sup>	728.523	280.9	0.1	262.9
PE 35:1	C <sub>40</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	732.553	300.3	0.1	276.0	[M-H] <sup>-</sup>	730.539	283.2	0.0	264.9
PE 36:2	C <sub>41</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	744.553	298.8	0.1	274.8	[M-H] <sup>-</sup>	742.539	284.6	0.1	266.2
PE 36:1	C <sub>41</sub> H <sub>80</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	746.569	304.4	0.1	279.3	[M-H] <sup>-</sup>	744.554	286.4	0.1	267.9



membrane lipids of PAK	Formula	Positive ions					Negative ions				
		ion	m/z	<sup>TW</sup> CCS <sub>N2</sub> without correction	<sup>TW</sup> CCS <sub>N2</sub> %RSD	corrected <sup>TW</sup> CCS <sub>N2</sub> (Å <sup>2</sup> )	ion	m/z	<sup>TW</sup> CCS <sub>N2</sub> without correction	<sup>TW</sup> CCS <sub>N2</sub> %RSD	corrected <sup>TW</sup> CCS <sub>N2</sub> (Å <sup>2</sup> )
PG 30:0	C <sub>36</sub> H <sub>71</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	693.4712	275.8	0.1	258.3
PG 32:1	C <sub>38</sub> H <sub>71</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	719.4869	281.4	0.1	263.4
PG 33:1	C <sub>39</sub> H <sub>75</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	733.5025	283.5	0.1	265.3
PG 34:2	C <sub>40</sub> H <sub>75</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	745.5025	285.1	0.1	266.6
PG 34:1	C <sub>40</sub> H <sub>77</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	747.5182	287.1	0.1	268.5
PG 35:2	C <sub>40</sub> H <sub>75</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	759.5182	288.5	0.1	269.7
PG 35:1	C <sub>40</sub> H <sub>77</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	761.5338	290.2	0.1	271.3
PG 36:2	C <sub>42</sub> H <sub>79</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	773.5338	291.7	0.1	272.6
CL 66:3	C <sub>75</sub> H <sub>140</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1375.963	441.0	0.2	390.1	[M-H] <sup>-</sup>	1373.949	411.2	0.3	380.4
CL 66:2	C <sub>75</sub> H <sub>142</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1377.979	442.3	0.0	391.2	[M-H] <sup>-</sup>	1375.965	412.5	0.4	381.6
CL 66:1	C <sub>75</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1379.995	443.2	0.1	391.9					
CL 67:3	C <sub>76</sub> H <sub>142</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1389.979	444.4	0.2	392.8					
CL 67:2	C <sub>76</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1391.995	445.4	0.2	393.7					
CL 68:4	C <sub>77</sub> H <sub>142</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1401.979	445.8	0.2	394.0					
CL 68:3	C <sub>77</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1403.995	447.9	0.1	395.7					
CL 68:2	C <sub>77</sub> H <sub>146</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1406.010	450.1	0.2	397.4	[M-H] <sup>-</sup>	1403.996	418.4	0.3	386.9
CL 69:4	C <sub>78</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1415.995	449.2	0.2	396.7					
CL 69:3	C <sub>78</sub> H <sub>146</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1418.010	451.1	0.2	398.3					
CL 69:2	C <sub>78</sub> H <sub>148</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1420.026	452.0	0.1	399.0					
CL 70:4	C <sub>79</sub> H <sub>146</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1430.010	452.4	0.1	399.4					
CL 70:3	C <sub>79</sub> H <sub>148</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1432.026	454.7	0.1	401.2					

**Table S3** <sup>TW</sup>CCS<sub>N2</sub> values calibrated with **polyalanine** for the membrane lipids of PAK

membrane lipids of PAK	Formula	Positive ions					Negative ions				
		ion	m/z	<sup>TW</sup> CCS <sub>N2</sub> without correction	<sup>TW</sup> CCS <sub>N2</sub> %RSD	corrected <sup>TW</sup> CCS <sub>N2</sub> (Å <sup>2</sup> )	ion	m/z	<sup>TW</sup> CCS <sub>N2</sub> without correction	<sup>TW</sup> CCS <sub>N2</sub> %RSD	corrected <sup>TW</sup> CCS <sub>N2</sub> (Å <sup>2</sup> )
PC 32:1	C <sub>40</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	732.5538	291.9	0.1	278.9					
PC 32:0	C <sub>40</sub> H <sub>80</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	734.5694	295.5	0.1	282.1					
PC 34:2	C <sub>42</sub> H <sub>82</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	758.5694	295.4	0.1	282.0					
PC 34:1	C <sub>42</sub> H <sub>82</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	760.5851	298.6	0.1	284.8					
PC 34:0	C <sub>42</sub> H <sub>84</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	762.6007	301.6	0.4	287.4					
PC 35:1	C <sub>43</sub> H <sub>84</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	774.6007	303.5	0.1	289.1					
PC 36:2	C <sub>42</sub> H <sub>82</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	786.6007	302.4	0.1	288.2					
PE 30:1	C <sub>35</sub> H <sub>68</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	662.4755	270.9	0.1	260.4	[M-H] <sup>-</sup>	660.4610	259.2	0.1	249.7
PE 30:0	C <sub>35</sub> H <sub>70</sub> NO <sub>8</sub> P						[M-H] <sup>-</sup>	662.4766	261.6	0.1	251.8
PE 32:2	C <sub>37</sub> H <sub>70</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	688.4912	273.1	0.1	262.4	[M-H] <sup>-</sup>	686.4766	263.8	0.2	253.7
PE 32:1	C <sub>37</sub> H <sub>72</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	690.5068	277.8	0.1	266.5	[M-H] <sup>-</sup>	688.4923	265.7	0.1	255.4
PE 32:0	C <sub>37</sub> H <sub>74</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	692.5225	283.6	0.0	271.6	[M-H] <sup>-</sup>	690.5079	267.6	0.1	257.1
PE 33:1	C <sub>38</sub> H <sub>74</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	704.5225	283.2	0.1	271.2	[M-H] <sup>-</sup>	702.5079	269.5	0.1	258.8
PE 34:2	C <sub>39</sub> H <sub>73</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	716.5225	280.6	0.1	269.0	[M-H] <sup>-</sup>	714.5079	270.9	0.1	260.0
PE 34:1	C <sub>37</sub> H <sub>76</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	718.5381	285.9	0.1	273.6	[M-H] <sup>-</sup>	716.5236	272.8	0.0	261.7
PE 34:0	C <sub>39</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	720.5538	289.5	0.1	276.8	[M-H] <sup>-</sup>	718.5392	274.8	0.1	263.4
PE 35:2	C <sub>40</sub> H <sub>76</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	730.5381	286.5	0.1	274.2	[M-H] <sup>-</sup>	728.5236	274.2	0.1	262.9
PE 35:1	C <sub>40</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	732.5538	288.8	0.1	276.2	[M-H] <sup>-</sup>	730.5392	276.6	0.0	265.0
PE 36:2	C <sub>41</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	744.5538	287.4	0.1	274.9	[M-H] <sup>-</sup>	742.5392	278.0	0.2	266.3
PE 36:1	C <sub>41</sub> H <sub>80</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	746.5694	292.5	0.1	279.5	[M-H] <sup>-</sup>	744.5549	279.9	0.1	267.9

membrane lipids of PAK	Formula	Positive ions					Negative ions				
		ion	<i>m/z</i>	<sup>TW</sup> CCS <sub>N2</sub> without correction	<sup>TW</sup> CCS <sub>N2</sub> %RSD	corrected <sup>TW</sup> CCS <sub>N2</sub> (Å <sup>2</sup> )	ion	<i>m/z</i>	<sup>TW</sup> CCS <sub>N2</sub> without correction	<sup>TW</sup> CCS <sub>N2</sub> %RSD	corrected <sup>TW</sup> CCS <sub>N2</sub> (Å <sup>2</sup> )
PG 30:0	C <sub>36</sub> H <sub>71</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	693.4712	269.0	0.1	258.3
PG 32:1	C <sub>38</sub> H <sub>71</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	719.4869	274.8	0.1	263.4
PG 33:1	C <sub>39</sub> H <sub>75</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	733.5025	276.9	0.1	265.3
PG 34:2	C <sub>40</sub> H <sub>75</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	745.5025	278.5	0.1	266.7
PG 34:1	C <sub>40</sub> H <sub>77</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	747.5182	280.6	0.1	268.6
PG 35:2	C <sub>40</sub> H <sub>75</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	759.5182	282.0	0.1	269.8
PG 35:1	C <sub>40</sub> H <sub>77</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	761.5338	283.8	0.1	271.4
PG 36:2	C <sub>42</sub> H <sub>79</sub> O <sub>10</sub> P						[M-H] <sup>-</sup>	773.5338	285.3	0.1	272.7
CL 66:3	C <sub>75</sub> H <sub>140</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1375.9639	416.5	0.2	388.9	[M-H] <sup>-</sup>	1373.9493	409.5	0.3	382.4
CL 66:2	C <sub>75</sub> H <sub>142</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1377.9795	417.7	0.0	389.9	[M-H] <sup>-</sup>	1375.9650	410.9	0.4	383.6
CL 66:1	C <sub>75</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1379.9952	418.6	0.1	390.6					
CL 67:3	C <sub>76</sub> H <sub>142</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1389.9795	419.6	0.2	391.5					
CL 67:2	C <sub>76</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1391.9952	420.6	0.1	392.4					
CL 68:4	C <sub>77</sub> H <sub>142</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1401.9795	420.9	0.2	392.7					
CL 68:3	C <sub>77</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1403.9952	422.8	0.1	394.4					
CL 68:2	C <sub>77</sub> H <sub>146</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1406.0108	424.7	0.2	396.1	[M-H] <sup>-</sup>	1403.9963	417.0	0.3	389.1
CL 69:4	C <sub>78</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1415.9952	423.9	0.2	395.4					
CL 69:3	C <sub>78</sub> H <sub>146</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1418.0108	425.6	0.2	396.9					
CL 69:2	C <sub>78</sub> H <sub>148</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1420.0265	426.5	0.1	397.6					
CL 70:4	C <sub>79</sub> H <sub>146</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1430.0108	426.8	0.1	398.0					
CL 70:3	C <sub>79</sub> H <sub>148</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1432.0265	428.8	0.1	399.7					

**Table S4** <sup>TW</sup>CCS<sub>N2</sub> values calibrated with **phospholipids** for the membrane lipids of PAK

membrane lipids of PAK	Formula	Positive ions				Negative ions			
		ion	m/z	<sup>TW</sup> CCS <sub>N2</sub> without correction (Å <sup>2</sup> )	<sup>TW</sup> CCS <sub>N2</sub> %RSD	ion	m/z	<sup>TW</sup> CCS <sub>N2</sub> without correction	<sup>TW</sup> CCS <sub>N2</sub> %RSD
PC 32:1	C <sub>40</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	732.5538	279.0	0.1				
PC 32:0	C <sub>40</sub> H <sub>80</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	734.5694	282.2	0.1				
PC 34:2	C <sub>42</sub> H <sub>82</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	758.5694	282.1	0.1				
PC 34:1	C <sub>42</sub> H <sub>82</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	760.5851	284.9	0.1				
PC 34:0	C <sub>42</sub> H <sub>84</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	762.6007	287.4	0.3				
PC 35:1	C <sub>43</sub> H <sub>84</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	774.6007	289.1	0.1				
PC 36:2	C <sub>42</sub> H <sub>82</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	786.6007	288.2	0.1				
PE 30:1	C <sub>35</sub> H <sub>68</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	662.4755	260.8	0.1	[M-H] <sup>-</sup>	660.4610	253.9	0.1
PE 30:0	C <sub>35</sub> H <sub>70</sub> NO <sub>8</sub> P					[M-H] <sup>-</sup>	662.4766	256.0	0.1
PE 32:2	C <sub>37</sub> H <sub>70</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	688.4912	262.7	0.1	[M-H] <sup>-</sup>	686.4766	257.9	0.2
PE 32:1	C <sub>37</sub> H <sub>72</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	690.5068	266.8	0.1	[M-H] <sup>-</sup>	688.4923	259.5	0.1
PE 32:0	C <sub>37</sub> H <sub>74</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	692.5225	271.8	0.0	[M-H] <sup>-</sup>	690.5079	261.2	0.1
PE 33:1	C <sub>38</sub> H <sub>74</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	704.5225	271.5	0.1	[M-H] <sup>-</sup>	702.5079	262.9	0.1
PE 34:2	C <sub>39</sub> H <sub>73</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	716.5225	269.2	0.1	[M-H] <sup>-</sup>	714.5079	264.1	0.1
PE 34:1	C <sub>37</sub> H <sub>76</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	718.5381	273.8	0.1	[M-H] <sup>-</sup>	716.5236	265.7	0.0
PE 34:0	C <sub>39</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	720.5538	277.0	0.1	[M-H] <sup>-</sup>	718.5392	267.4	0.1
PE 35:2	C <sub>40</sub> H <sub>76</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	730.5381	274.4	0.1	[M-H] <sup>-</sup>	728.5236	266.9	0.1
PE 35:1	C <sub>40</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	732.5538	276.4	0.1	[M-H] <sup>-</sup>	730.5392	269.0	0.0
PE 36:2	C <sub>41</sub> H <sub>78</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	744.5538	275.1	0.1	[M-H] <sup>-</sup>	742.5392	270.2	0.1
PE 36:1	C <sub>41</sub> H <sub>80</sub> NO <sub>8</sub> P	[M+H] <sup>+</sup>	746.5694	279.6	0.1	[M-H] <sup>-</sup>	744.5549	271.8	0.1

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membrane lipids of PAK	Formula	Positive ions				Negative ions			
		ion	<i>m/z</i>	<sup>TW</sup> CCS <sub>N2</sub> without correction (Å <sup>2</sup> )	<sup>TW</sup> CCS <sub>N2</sub> %RSD	corrected <sup>TW</sup> CCS <sub>N2</sub> (Å <sup>2</sup> )	ion	<i>m/z</i>	<sup>TW</sup> CCS <sub>N2</sub> without correction (Å <sup>2</sup> )
PG 30:0	C <sub>36</sub> H <sub>71</sub> O <sub>10</sub> P					[M-H] <sup>-</sup>	693.4712	262.4	0.1
PG 32:1	C <sub>38</sub> H <sub>71</sub> O <sub>10</sub> P					[M-H] <sup>-</sup>	719.4869	267.4	0.1
PG 33:1	C <sub>39</sub> H <sub>75</sub> O <sub>10</sub> P					[M-H] <sup>-</sup>	733.5025	269.3	0.1
PG 34:2	C <sub>40</sub> H <sub>75</sub> O <sub>10</sub> P					[M-H] <sup>-</sup>	745.5025	270.6	0.1
PG 34:1	C <sub>40</sub> H <sub>77</sub> O <sub>10</sub> P					[M-H] <sup>-</sup>	747.5182	272.5	0.1
PG 35:2	C <sub>40</sub> H <sub>75</sub> O <sub>10</sub> P					[M-H] <sup>-</sup>	759.5182	273.7	0.1
PG 35:1	C <sub>40</sub> H <sub>77</sub> O <sub>10</sub> P					[M-H] <sup>-</sup>	761.5338	275.3	0.1
PG 36:2	C <sub>42</sub> H <sub>79</sub> O <sub>10</sub> P					[M-H] <sup>-</sup>	773.5338	276.5	0.1
CL 66:3	C <sub>75</sub> H <sub>140</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1375.963	385.4	0.2	[M-H] <sup>-</sup>	1373.9493	381.4	0.3
CL 66:2	C <sub>75</sub> H <sub>142</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1377.979	386.4	0.0	[M-H] <sup>-</sup>	1375.9650	382.6	0.4
CL 66:1	C <sub>75</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1379.995	387.1	0.1				
CL 67:3	C <sub>76</sub> H <sub>142</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1389.979	387.9	0.2				
CL 67:2	C <sub>76</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1391.995	388.8	0.1				
CL 68:4	C <sub>77</sub> H <sub>142</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1401.979	389.0	0.2				
CL 68:3	C <sub>77</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1403.995	390.6	0.1				
CL 68:2	C <sub>77</sub> H <sub>146</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1406.010	392.2	0.1	[M-H] <sup>-</sup>	1403.9963	387.7	0.3
CL 69:4	C <sub>78</sub> H <sub>144</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1415.995	391.6	0.1				
CL 69:3	C <sub>78</sub> H <sub>146</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1418.010	393.0	0.2				
CL 69:2	C <sub>78</sub> H <sub>148</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1420.026	393.7	0.1				
CL 70:4	C <sub>79</sub> H <sub>146</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1430.010	394.0	0.1				
CL 70:3	C <sub>79</sub> H <sub>148</sub> O <sub>17</sub> P <sub>2</sub>	[M+H] <sup>+</sup>	1432.026	395.8	0.1				

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**Table S5** Comparison between the experimental <sup>TW</sup>CCS<sub>N<sub>2</sub></sub> values for the phospholipids of PAK and CCS values of the literature for [M+H]<sup>+</sup>.  
1: dextran calibration, 2: dextran calibration and lipid correction, 3: polyalanine calibration, 4: polyalanine calibration and lipid correction, 5 phospholipids calibration

Lipid	Leaptrot 2019 - drift tube						Zhou 2017 - drift tube						Hines 2016 - drift tube						Paglia 2015 - TWIMS polyalanine calibration					
	CCS [M+H] <sup>+</sup> (Å <sup>2</sup> )	% Δ 1	% Δ 2	% Δ 3	% Δ 4	% Δ 5	CCS [M+H] <sup>+</sup> (Å <sup>2</sup> )	% Δ 1	% Δ 2	% Δ 3	% Δ 4	% Δ 5	CCS [M+H] <sup>+</sup> (Å <sup>2</sup> )	% Δ 1	% Δ 2	% Δ 3	% Δ 4	% Δ 5	CCS [M+H] <sup>+</sup> (Å <sup>2</sup> )	% Δ 1	% Δ 2	% Δ 3	% Δ 4	% Δ 5
PC 32:0	278.4	10.5	1.3	6.0	1.3	1.4	283.4	8.5	-0.5	4.1	-0.5	-0.4	282.5	8.9	-0.2	4.4	-0.1	-0.1	291.0	5.7	-3.1	1.4	-3.1	-3.0
PC 32:1	276.5	9.8	0.8	5.6	0.9	0.9	282.6	7.4	-1.4	3.3	-1.3	-1.3							287.0	5.8	-2.9	1.7	-2.8	-2.8
PC 34:0							289.7	8.5	-0.8	4.1	-0.8	-0.8							297.0	5.8	-3.3	1.5	-3.2	-3.2
PC 34:1	282.0	10.3	1.0	5.9	1.0	1.0	287.1	8.3	-0.8	4.0	-0.8	-0.8							295.0	5.4	-3.5	1.2	-3.5	-3.4
PC 34:2	279.5	10.0	0.9	5.7	1.9	1.9	285.2	7.8	-1.2	3.6	-0.1	-0.1							293.0	4.9	-3.8	0.8	-2.8	-2.8
PC 35:1	287.1	10.2	0.7	5.7	0.7	0.7	289.2	9.4	-0.1	4.9	0.0	0.0												
PC 36:2	288.6	9.2	-0.2	4.8	-0.1	-0.1	291.3	8.2	-1.1	3.8	-1.1	-1.1							301.0	4.7	-4.3	0.5	-4.3	-4.3
PE 32:0							271.4	8.5	0.0	4.5	0.1	0.1	271.5	8.5	0.0	4.5	0.0	0.1						
PE 32:1							267.2	7.9	-0.3	4.0	-0.3	-0.2												
PE 32:2													263.8	7.4	-0.6	3.5	-0.5	-0.4						
PE 34:0							277.5	8.5	-0.3	4.3	-0.3	-0.2	277.4	8.5	-0.3	4.4	-0.2	-0.1						
PE 34:1	270.1	10.0	1.2	5.9	1.3	1.4	274.7	8.2	-0.5	4.1	-0.4	-0.3	273.4	8.7	0.0	4.6	0.1	0.1	272.0	9.2	0.5	5.1	0.6	0.7
PE 34:2	268.5	8.5	0.1	4.5	0.2	0.3	272.7	6.8	-1.4	2.9	-1.4	-1.3												
PE 35:1							276.6	8.6	-0.2	4.4	-0.1	-0.1												
PE 35:2							275.0	8.3	-0.4	4.2	-0.3	-0.2												
PE 36:1	277.0	9.9	0.8	5.6	0.9	0.9	281.3	8.2	-0.7	4.0	-0.7	-0.6												
PE 36:2	275.1	8.6	-0.1	4.5	-0.1	0.0	279.3	7.0	-1.6	2.9	-1.6	-1.5	277.9	7.5	-1.1	3.4	-1.1	-1.0	279.0	7.1	-1.5	3.0	-1.5	-1.4

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**Table S6** Comparison between the experimental <sup>TW</sup>CCS<sub>N<sub>2</sub></sub> values for the phospholipids of PAK and CCS values of the literature for [M-H]<sup>-</sup>.

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1: dextran calibration, 2: dextran calibration and lipid correction, 3: polyalanine calibration, 4: polyalanine calibration and lipid correction, 5 phospholipids calibration

Lipid	Leaptrot 2019 - drift tube					Zhou 2017 - drift tube					Hines 2016 - drift tube					Paglia 2015 - TWIMS polyalanine calibration								
	CCS [M-H] <sup>-</sup> (Å <sup>2</sup> )	% Δ 1	% Δ 2	% Δ 3	% Δ 4	% Δ 5	CCS [M-H] <sup>-</sup> (Å <sup>2</sup> )	% Δ 1	% Δ 2	% Δ 3	% Δ 4	% Δ 5	CCS [M-H] <sup>-</sup> (Å <sup>2</sup> )	% Δ 1	% Δ 2	% Δ 3	% Δ 4	% Δ 5	CCS [M-H] <sup>-</sup> (Å <sup>2</sup> )	% Δ 1	% Δ 2	% Δ 3	% Δ 4	% Δ 5
PE(30:0)													253.3	6.0	-1.4	2.3	-1.4	0.2						
PE(32:1)							256.6	6.2	-0.5	3.5	-0.5	1.1												
PE(32:0)													259.4	5.8	-0.9	3.2	-0.9	0.7						
PE(32:2)													256.3	5.6	-1.0	2.9	-1.0	0.6						
PE(34:0)													265.5	6.0	-0.8	3.5	-0.8	0.7	274	2.7	-3.9	0.3	-3.9	-2.4
PE(34:1)	266.9	4.7	-2.0	2.2	-2.0	-0.5	263.1	6.2	-0.6	3.7	-0.5	1.0	264.5	5.7	-1.1	3.1	-1.1	0.5						
PE(34:2)	265.6	4.6	-2.1	2.0	-2.1	-0.6																		
PE(36:1)	273.6	4.7	-2.1	2.3	-2.1	-0.7																		
PE(36:2)	272.1	4.6	-2.2	2.2	-2.1	-0.7	268.5	6.0	-0.9	3.5	-0.8	0.6	270.7	5.1	-1.7	2.7	-1.6	-0.2						
PG(32:1)							263.3	6.9	0.0	4.4	0.0	1.6												
PG(33:1)							266.4	6.4	-0.4	4.0	-0.4	1.1												
PG(34:0)																			283	2.0	-4.7	-0.3	-4.6	-3.3
PG(34:1)							270.2	6.3	-0.6	3.8	-0.6	0.9							281	2.2	-4.4	-0.1	-4.4	-3.0
PG(34:2)							268.7	6.1	-0.8	3.6	-0.7	0.7							279	2.2	-4.4	-0.2	-4.4	-3.0
PG(35:1)							272.7	6.4	-0.5	4.1	-0.5	0.9												
PG(36:2)																			289	0.9	-5.7	-1.3	-5.6	-4.3

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**Table S7:**  $\Delta$ CCS (%) comparison between CCS values of the literature for  $[M+H]^+$ 

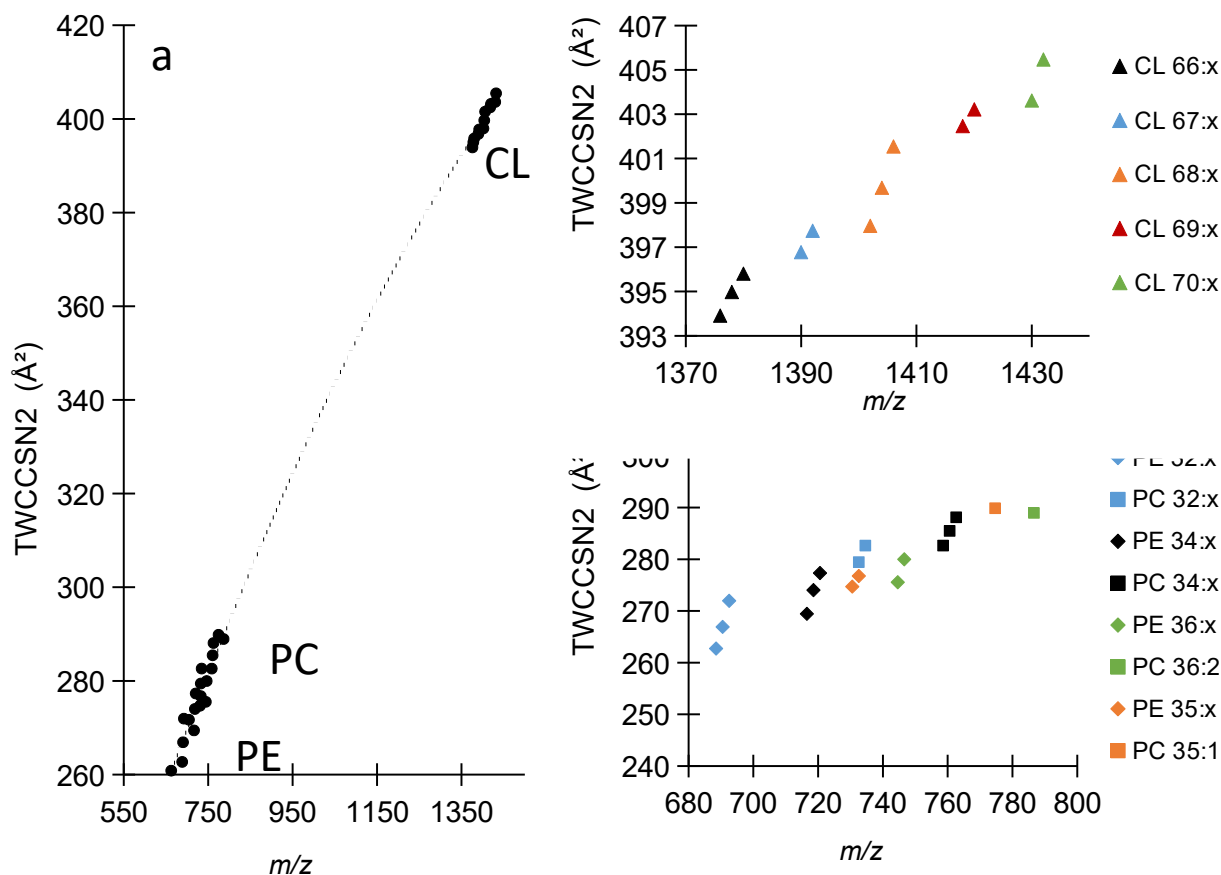
Lipid	Leaptrot / Zhou	Leaptrot / Hines	Zhou / Hines	Zhou / Paglia	Hines / Paglia
PC(32:0)	-1.8	-1.5	0.3	-2.6	-2.9
PC(32:1)	-2.2			-1.5	
PC(34:0)				-2.5	
PC(34:1)	-1.8			-2.7	
PC(34:2)	-2.0			-2.7	
PC(36:2)	-0.9			-3.2	
PE(32:0)			0.0		
PE(34:0)			0.0		
PE(34:1)	-1.7	-1.2	0.5	1.0	0.5
PE(36:2)	-1.5	-1.0	0.5	0.1	-0.4

**Table S8:**  $\Delta$ CCS (%) comparison between CCS values of the literature for  $[M-H]^-$ 

Lipid	$\Delta$ Comparison between the literature (%)			
	Leaptrot / Zhou	Leaptrot / Hines	Zhou / Hines	Zhou / Paglia
PE(34:1)	1.4	0.9	-0.5	
PE(36:2)	1.3	0.5	-0.8	
PG(34:1)				-3.8
PG(34:2)				-3.7

556 The following figure describes the  $^{TW}CCS_{N_2}$  vs  $m/z$  correlation

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558 **Figure S9:**  $^{TW}CCS_{N_2}$  values plotted against  $m/z$  for positive ions. (a) represents values for every  
 559 class of phospholipids, whereas (b) only represents values for CLs and (c) only represents  
 560 values for PCs and PEs. CLs are represented by triangles, PCs are represented by squares, PEs  
 561 by diamonds. x refers to the number of double-bonds on the fatty acid moiety of the  
 562 phospholipid which can be equal to 0, 1, 2 (for PCs and PEs) or up to 3, 4 (for CLs).

## 563 S2 Dextran, polyalanine and phospholipids CCS Calibration

		M(N <sub>2</sub> )				28.0134							
		Glc <sub>n</sub>	m/z	z	m	CCS (N <sub>2</sub> ) in Å <sup>2</sup>	1/μ	Ω'(N <sub>2</sub> ) litt	td	ln(Ω'(N <sub>2</sub> ))	ln(td)		
Positive Dextran Calibration	2	[M+Na] <sup>+</sup>	365.11	1	365.11	179.54	0.04	915.77	2.28	6.82	0.82		
	3	[M+Na] <sup>+</sup>	527.16	1	527.16	215.00	0.04	1108.88	3.21	7.01	1.17		
	4	[M+Na] <sup>+</sup>	689.21	1	689.21	243.36	0.04	1262.66	4.01	7.14	1.39		
	5	[M+Na] <sup>+</sup>	851.26	1	851.26	273.81	0.04	1425.94	4.89	7.26	1.59		
	6	[M+Na] <sup>+</sup>	1013.32	1	1013.32	302.06	0.04	1577.08	5.75	7.36	1.75	x	0.590
	7	[M+Na] <sup>+</sup>	1175.37	1	1175.37	330.94	0.04	1731.10	6.74	7.46	1.91	lnA	6.328
	8	[M+Na] <sup>+</sup>	1337.42	1	1337.42	355.80	0.04	1863.77	7.68	7.53	2.04	A	560.026
	9	[M+Na] <sup>+</sup>	1499.48	1	1499.48	377.70	0.04	1980.68	8.53	7.59	2.14	r <sup>2</sup>	0.9998

		M(N <sub>2</sub> )				28.0134							
		Sequenz	m/z	z	m	CCS (N <sub>2</sub> ) in Å <sup>2</sup>	1/μ	Ω'(N <sub>2</sub> ) litt	td	ln(Ω'(N <sub>2</sub> ))	ln(td)		
Positive Polyalanine Calibration	A4		303.208	1	303.2	166.00	0.04	840.62	2.06	6.73	0.72		
	A5		374.208	1	374.2	181.00	0.04	924.03	2.42	6.83	0.88		
	A6		445.208	1	445.2	195.00	0.04	1001.08	2.76	6.91	1.02		
	A7		516.308	1	516.3	211.00	0.04	1087.66	3.17	6.99	1.15		
	A8		587.308	1	587.3	228.00	0.04	1178.96	3.67	7.07	1.30	x	0.563
	A9		658.308	1	658.3	243.00	0.04	1259.62	4.12	7.14	1.42	lnA	6.336
	A10		729.408	1	729.4	256.00	0.04	1329.66	4.54	7.19	1.51	A	564.400
	A11		800.408	1	800.4	271.00	0.04	1409.88	5.01	7.25	1.61	r <sup>2</sup>	0.9991
	A12		871.508	1	871.5	282.00	0.04	1469.14	5.50	7.29	1.70		
	A13		942.508	1	942.5	294.00	0.04	1533.45	5.98	7.34	1.79		
	A14		1013.508	1	1013.5	306.00	0.04	1597.66	6.45	7.38	1.86		

		M(N <sub>2</sub> )				28.0134							
		Lipide	m/z	z	m	CCS (N <sub>2</sub> ) in Å <sup>2</sup>	1/μ	Ω'(N <sub>2</sub> ) litt	td	ln(Ω'(N <sub>2</sub> ))	ln(td)		
Positive Lipids Calibration	PE 6:0/6:0		412.2095	1	412.2095	202.1	0.04	1035.08	2.94	6.94	1.08		
	PE 10:0/10:0		524.3347	1	524.3347	233	0.04	1201.54	4.06	7.09	1.40		
	PE 15:0/15:0		664.4912	1	664.4912	265	0.04	1373.92	5.30	7.23	1.67		
	PE 17:0/17:0		720.5538	1	720.5538	277.4	0.04	1440.48	5.76	7.27	1.75	x	0.512
	PC 20:0/20:0		846.6946	1	846.6946	306.4	0.04	1595.52	6.96	7.37	1.94	lnA	6.380
	PC 24:0/24:0		958.8198	1	958.8198	330.9	0.04	1726.34	7.98	7.45	2.08	A	590.169
													r <sup>2</sup>

		M(N <sub>2</sub> )				28.0134							
Glc <sub>n</sub>		m/z	z	m	CCS (N <sub>2</sub> ) in Å <sup>2</sup>	1/μ	Ω'(N <sub>2</sub> ) litt	td	ln( Ω'(N <sub>2</sub> ))	ln(td)			
Negative Dextran Calibration	2	[M-H] <sup>-</sup>	341.11	1	341.11	174.56	0.04	888.17	2.02	6.79	0.70		
	3	[M-H] <sup>-</sup>	503.16	1	503.16	202.34	0.04	1042.30	3.03	6.95	1.11		
	4	[M-H] <sup>-</sup>	665.21	1	665.21	233.96	0.04	1213.00	3.92	7.10	1.37		
	5	[M-H] <sup>-</sup>	827.27	1	827.27	265.39	0.04	1381.45	4.86	7.23	1.58		
	6	[M-H] <sup>-</sup>	989.32	1	989.32	296.73	0.04	1548.76	5.85	7.35	1.77	x	0.546
	7	[M-H] <sup>-</sup>	1151.37	1	1151.37	319.21	0.04	1669.34	6.71	7.42	1.90	lnA	6.374
	8	[M-H] <sup>-</sup>	1313.43	1	1313.43	343.55	0.04	1799.25	7.71	7.50	2.04	A	586.226
	9	[M-H] <sup>-</sup>	1475.48	1	1475.48	365.21	0.04	1914.89	8.64	7.56	2.16	r <sup>2</sup>	0.9953

		M(N <sub>2</sub> )				28.0134							
Sequenz		m/z	z	m	CCS (N <sub>2</sub> ) in Å <sup>2</sup>	1/μ	Ω'(N <sub>2</sub> ) litt	td	ln( Ω'(N <sub>2</sub> ))	ln(td)			
Negative Polyalanine Calibration	A4		301.151	1	301.2	165.00	0.04	835.32	2.09	6.73	0.74		
	A5		372.188	1	372.2	179.00	0.04	913.65	2.48	6.82	0.91		
	A6		443.225	1	443.2	195.00	0.04	1000.94	2.86	6.91	1.05		
	A7		514.262	1	514.3	209.00	0.04	1077.24	3.28	6.98	1.19	x	0.576
	A8		585.300	1	585.3	223.00	0.04	1153.02	3.67	7.05	1.30	lnA	6.301
	A9		656.337	1	656.3	238.00	0.04	1233.63	4.12	7.12	1.42	A	545.101
	A10		727.374	1	727.4	253.00	0.04	1314.01	4.58	7.18	1.52	r <sup>2</sup>	0.9998
	A11		798.411	1	798.4	267.00	0.04	1389.01	5.06	7.24	1.62		
	A12		869.448	1	869.4	279.00	0.04	1453.45	5.50	7.28	1.70		
	A13		940.485	1	940.5	294.00	0.04	1533.40	6.04	7.34	1.80		
	A14		1011.522	1	1011.5	308.00	0.04	1608.06	6.58	7.38	1.88		

		M(N <sub>2</sub> )				28.0134							
Lipide		m/z	z	m	CCS (N <sub>2</sub> ) in Å <sup>2</sup>	1/μ	Ω'(N <sub>2</sub> ) litt	td	ln( Ω'(N <sub>2</sub> ))	ln(td)			
Negative Lipids Calibration	PE 6:0/6:0		410.1949	1	410.1949	199.3	0.04	1020.58	3.03	6.93	1.10		
	PE 10:0/10:0		522.3201	1	522.3201	223.2	0.04	1150.89	3.85	7.05	1.34		
	PE 15:0/15:0		662.4766	1	662.4766	253.3	0.04	1313.18	4.92	7.18	1.59	x	0.517
	PE 17:0/17:0		718.5392	1	718.5392	265.5	0.04	1378.61	5.39	7.23	1.68	lnA	6.359
	PC 20:0/20:0		904.7012	1	904.7012	305.1	0.04	1590.39	7.15	7.37	1.96	A	577.820
	PC 24:0/24:0		1016.8264	1	1016.8264	331	0.04					r <sup>2</sup>	0.9998

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[M+CH<sub>3</sub>COO<sup>-</sup>]

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566 **S3 Dextran and polyalanine correction curves**

Positive values

Measured with dextran calibration

Lipids	m/z	z	td	td'	CCS' (N2)	<sup>TW</sup> CCS <sub>N2</sub>	<sup>DT</sup> CCS <sub>N2</sub> lit.	Ω %Δ
PE 6:0/6:0	412.210	1	2.94	2.91	1058.4	206.7	202.1	2.3
PE 10:0/10:0	524.335	1	4.06	4.02	1280.7	248.4	233.0	-6.6
PE 15:0/15:0	664.491	1	5.30	5.26	1498.7	289.1	265.0	-9.1
PE 17:0/17:0	720.554	1	5.76	5.72	1574.0	303.1	277.4	-9.3
PC 20:0/20:0	846.695	1	6.96	6.91	1759.8	337.9	306.4	-10.3
PC 24:0/24:0	958.820	1	7.98	7.93	1907.4	365.6	330.9	-10.5

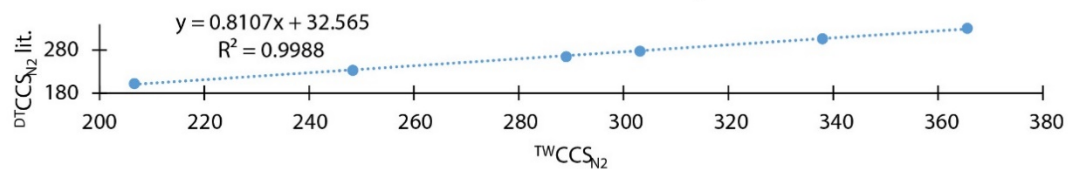
Equation A: lipid standards  
positive correction

a 0.8107

b 32.5654

r<sup>2</sup> 0.9988

correction curve for dextran calibration in positive mode



Negative values

Measured with dextran calibration

Lipids	m/z	z	td	td'	CCS' (N2)	<sup>TW</sup> CCS <sub>N2</sub>	<sup>DT</sup> CCS <sub>N2</sub> lit.	Ω %Δ
PE 6:0/6:0	410.195	1	3.03	3.00	1075.0	209.9	199.3	5.3
PE 10:0/10:0	522.320	1	3.85	3.81	1225.3	237.6	223.2	-6.5
PE 15:0/15:0	662.477	1	4.92	4.88	1400.8	270.2	253.3	-6.7
PE 17:0/17:0	718.539	1	5.39	5.35	1472.4	283.6	265.5	-6.8
PC 20:0/20:0	904.701	1						
PC 24:0/24:0	1016.826	1						

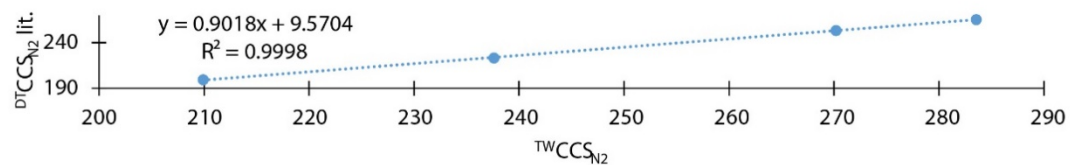
Equation B: lipid standards  
negative correction

a 0.9018

b 9.5704

r<sup>2</sup> 0.9998

correction curve for dextran calibration in negative mode

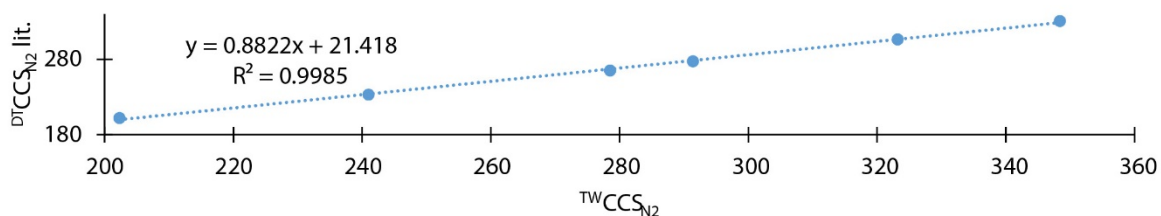


Positive values

Measured with polyalanine calibration

Lipids	m/z	z	td	td'	CCS' (N <sub>2</sub> )	<sup>TW</sup> CCS <sub>N<sub>2</sub></sub>	<sup>DT</sup> CCS <sub>N<sub>2</sub></sub> lit.	Ω %Δ
PE 6:0/6:0	412.210	1	2.94	2.91	1036.1	202.3	202.1	2.3
PE 10:0/10:0	524.335	1	4.06	4.02	1242.9	241.0	233.0	-6.6
PE 15:0/15:0	664.491	1	5.30	5.26	1444.0	278.5	265.0	-9.1
PE 17:0/17:0	720.554	1	5.76	5.72	1513.2	291.4	277.4	-9.3
PC 20:0/20:0	846.695	1	6.96	6.91	1683.1	323.2	306.4	-10.3
PC 24:0/24:0	958.820	1	7.98	7.93	1817.5	348.4	330.9	-10.5

correction curve for polyalanine calibration in positive mode



Negative values

Measured with polyalanine calibration

Lipids	m/z	z	td	td'	CCS' (N <sub>2</sub> )	<sup>TW</sup> CCS <sub>N<sub>2</sub></sub>	<sup>DT</sup> CCS <sub>N<sub>2</sub></sub> lit.	Ω %Δ
PE 6:0/6:0	410.195	1	3.03	3.00	1033.2	201.8	199.3	5.3
PE 10:0/10:0	522.320	1	3.85	3.81	1185.5	229.9	223.2	-6.5
PE 15:0/15:0	662.477	1	4.92	4.88	1364.7	263.2	253.3	-6.7
PE 17:0/17:0	718.539	1	5.39	5.35	1438.1	276.9	265.5	-6.8
PC 20:0/20:0	904.701	1						
PC 24:0/24:0	1016.826	1						

correction curve for polyalanine calibration in negative mode

