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1 Off-line two-dimensional liquid chromatography separation for the quality control of saponins  
2 samples from *Quillaja Saponaria*

3

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10

11 **Running title:** off-line LC × LC for Quil-A quality control

12

13 **Non-standard abbreviations:**

14 1D LC: one-dimensional LC

15 <sup>1</sup>D: first dimension

16 <sup>2</sup>D: second dimension

17 RPLC: Reversed-phase liquid chromatography

18 HILIC: Hydrophilic interaction chromatography

19 QC: Quality control

20 LC x LC: comprehensive two-dimensional liquid chromatography

21

22 **Keywords**

23 Two-dimensional chromatography; Reversed-phase liquid chromatography; Hydrophilic  
24 interaction chromatography; Saponins; off-line optimization

25 **Abstract**

26 Quil-A is a purified extract of saponins with strong immunoadjuvant activity. While isolated  
27 molecules have been tested in clinical trials, Quil-A is mostly used as a totum of the Quillaja  
28 Saponaria bark extract. Quality control of the extract stability is usually based on the monitoring  
29 of specific saponins, whereas the comparison of samples with an initial chromatogram seems  
30 more appropriate. A reference fingerprint based on comprehensive two-dimensional liquid  
31 chromatography offers a rapid detection of non-conform samples. To fulfill quantity control  
32 constraints, off-line configuration was promoted. Hence, reversed-phase LC × reversed-phase LC  
33 and hydrophilic interaction chromatography × reversed-phase LC methods with single-  
34 quadrupole MS detection were kinetically optimized. The reversed-phase LC × reversed-phase LC  
35 method used a pH switch between dimensions to maximize orthogonality. Despite  
36 diagonalization, it led to a peak capacity of 831 in two hours. Moreover, the combination of  
37 hydrophilic interaction chromatography and reversed-phase LC offered a larger orthogonality but  
38 a lower, yet satisfactory peak capacity of 673. The advantages of both methods were illustrated  
39 on degraded samples, where the reversed-phase LC × reversed-phase LC contour plot highlighted  
40 the loss of fatty-acid chains, while the hydrophilic interaction chromatography × reversed-phase  
41 LC method attested enzymatic loss of sugar moieties.

42

## 44 1. Introduction

45 Plant extracts enter the composition of many cosmetic and pharmaceutical products and their  
46 bioactivity is most of the time the result of the combination of a large number of molecules [1].

47 The quality control (QC) of natural ingredients is very important at every stage of the production,  
48 from the incoming raw material to the stability of the plant material during storage. In general,  
49 the quantitative determination of selected markers is performed to ensure the quality of plant  
50 extracts. However, the lack of individual standards may reduce the number of monitored  
51 molecules. Moreover, the assay of such markers may not fully reflect the bioactivity of the totum.

52 As such, the European guidelines for herbal medicine recommend to consider the whole of the  
53 plant extract as active substance [2]. Metabolite profiling using highly resolutive techniques  
54 provides a better understanding of the chemical profile. Unfortunately, in an industrial context,  
55 these powerful tools are often reserved for research purposes and are not available in routine  
56 QC laboratories. Qualitative chromatographic profiling of the samples with a comparison to a  
57 validated reference material may help rejecting non-conform samples in a timely manner. As so,  
58 the European guidelines state that the stability of the plant material “should be demonstrated  
59 by means of appropriate fingerprint chromatograms and its content should remain comparable  
60 to the initial fingerprint” [2].

61 Saponins are a class of naturally occurring molecules presenting surface-active or detergent  
62 properties. Saponins are commercially used in many industrial contexts: as foaming agent and  
63 emulsifier in food and cosmetics [3], as a supplement in the agri-food industry [4] or by  
64 pharmaceutical companies as vaccines adjuvants since some specific saponins are able to  
65 modulate the immune response [5, 6]. The bark of the tree *Quillaja Saponaria Molina* represents

66 one of the main saponin source as it contains up to 10 % of saponins. *Quillaja* saponins present  
67 unique structural features with a triterpenic aglycone core, usually quillaic acid but also  
68 phytolaccinic acid or gypsogenin, substituted with two saccharide groups that are further  
69 extended by oligosaccharides and with different degrees of acylation (generic structure available  
70 in supplementary material, Figure S1). This results in a very significant molecular complexity.  
71 Hundreds *Quillaja* saponins were discerned so far, using a combination of NMR [7], two-  
72 dimensional liquid chromatography coupled with high resolution mass spectrometry (LC × LC –  
73 HRMS) [8] or LC – MS/MS [9]. In 2019, 60 structures were identified from *Quillaja Saponaria* bark  
74 extracts [6]. The commercial Quil-A® product is a highly purified extract of *Quillaja Saponaria*  
75 *Molina* bark that presents a strong immunoadjuvant activity. It is considered as a reference  
76 material for many industrial uses, especially for animal vaccines [10]. Because of the large  
77 number of structurally close molecules present in such sample, the fingerprint chromatogram  
78 used in quality control has to offer a maximal peak capacity that one-dimensional LC methods  
79 hardly provide. Off-line comprehensive LC × LC can be implemented in routine QC laboratories,  
80 thanks to the limited instrumental requirements. Off-line 2D separations can offer as much peak  
81 capacity as online LC × LC, provided the overall separation is over 2 hours [11]. Moreover, off-  
82 line transfer allows fraction solvent evaporation, hence limiting band broadening due to injection  
83 effects and the subsequent dilution. In LC × LC, the increase in peak capacity over 1D LC is directly  
84 linked to the orthogonality offered by the two selected dimensions. Several LC × LC methods have  
85 been developed for the separation of saponins. While online HILIC x HILIC separation carried in  
86 130 min has shown limited orthogonality [8], a RPLC × RPLC combination has been suggested, in  
87 which an improved orthogonality is provided thanks to a pH switch between dimensions [12].  
88 Unfortunately, this off-line method lasts 12 hours, which is not compatible with a QC routine. On

89 the other hand, the combination of HILIC and RPLC mechanisms has been suggested to separate  
90 saponins [13], but has never been attempted on *Quillaja Saponaria*.

91 In this paper, an off-line comprehensive two-dimensional liquid chromatography method was  
92 developed in respect with QC constraints. Two combinations, namely RPLC × RPLC with pH switch  
93 and HILIC × RPLC were optimized with a maximum chromatographic duration of 120 min, taking  
94 into account the instrumental limitations of the fraction collection system. These two methods  
95 were evaluated in terms of orthogonality, peak capacity and dilution factor. Finally, the utility of  
96 the initial chromatographic fingerprint of Quil-A sample was demonstrated for the control of  
97 hydrolytically and enzymatically degraded samples.

98

## 99 2. Material and methods

### 100 2.1. Chemicals

101 LC-MS grade acetonitrile (ACN), methanol (MeOH) and 2-butanol (2-BuOH; ≥ 99.5%), disodium  
102 phosphate (Na<sub>2</sub>HPO<sub>4</sub>) and citric acid were purchased from Sigma Aldrich (Isle d'Abeau, France).  
103 Formic acid (FA) (LC-MS grade) and ammonium acetate (AA) (analytical reagent grade) were  
104 obtained from Fisher scientific (Waltham, MA, USA). Sodium bicarbonate was from Laurylab  
105 (Brindas, France) and hydrochloric acid from Carlo Erba Reagents (Val-de-Reuil, France). Water  
106 was purified by an Elga purification system (Veolia, Paris, France). Mobile phases (all proportion  
107 expressed as v/v) were filtered through 0.2 µm PTFE membranes (Merck, Germany) before use.

### 108 2.2. Sample preparation

109 The dry sample Quil-A<sup>®</sup> (InvivoGen, San Diego, USA) was prepared in 100 % water at 50 mg/mL  
110 for RPLC × RPLC, and at 12.5 mg/mL in 75/25 2-butanol/water (v/v) for HILIC × RPLC. All samples  
111 were stored at 4°C until use.

112 Quil-A sample was hydrolyzed by two different processes to mimic potential degradation. On one  
113 hand, under mild basic conditions: 20 mg of NaHCO<sub>3</sub> were added to 300 mg of Quil-A in 50 mL of  
114 MeOH/water (50/50 v/v). The mixture was heated under reflux for 1h before neutralization with  
115 HCl and filtration on a 0.22 µm membrane. The sample was then evaporated to dryness and  
116 reconstituted in 100 % water at 50 mg/mL. On the other hand, Quil-A was enzymatically  
117 hydrolyzed using Rapidase® Revelation Aroma (La Littorale, Servian, France): 20 mg of enzyme  
118 preparation was added to 10 mg of Quil-A in 5 mL of citrate-phosphate buffer (Na<sub>2</sub>HPO<sub>4</sub> 0.2 M  
119 and citric acid 0.1 M) at pH 5. The mixture was left for 2 h on a plate shaker incubator (50°C; 1  
120 000 rpm). The sample was then evaporated to dryness and reconstituted in 1 mL of 75/25 2-  
121 butanol/water (v/v). Protein insoluble fraction was discarded by centrifugation.

## 122 2.3. Instrumentation and conditions

### 123 2.3.1. Instrumentation

124 One-dimensional LC as well as method developments and second dimension LC-UV-MS were  
125 performed on an Agilent (Santa Clara, CA, USA) UHPLC (1290 Infinity) ( $V_{dwell}$  0.20 mL) connected  
126 via a 1:1 split to a photodiode array detector set at 205 nm (external variance 13 µL<sup>2</sup>) and to an  
127 Agilent single quadrupole with ESI source in negative mode (external variance 26 µL<sup>2</sup>). The spray  
128 chamber was set with a drying gas flow of 12 L/min (N<sub>2</sub>), nebulizer pressure at 35 psig, drying gas  
129 temperature at 350°C and capillary voltage at 3 000 V. The fragmentor was at 70 V. The scan  
130 range was m/z 300 to 2 000. Compounds with m/z below 1 000 were considered as non-saponins  
131 Optimized <sup>1</sup>D separations for fractions collection were performed on an Acquity I-Class (Waters,  
132 Milford, MA, USA) ( $V_{dwell}$  0.11 mL; external variance 5.6 µL<sup>2</sup>) equipped with a fraction collector  
133 (Fraction Manager). Agilent equipments were controlled by OpenLab and Waters equipment by  
134 Empower. A homemade Matlab program (S. Heinisch and F. Rouviere) was used to generate  
135 contour plots.

136

### 137 2.3.2. 1D-reversed-phase LC separation

138 The one-dimensional RLPC analysis was conducted with a Waters BEH C18 column (100 mm x 2.1  
139 mm, 1.7  $\mu\text{m}$ ) using H<sub>2</sub>O with 0.1 % FA (A) and ACN with 0.1 % FA (B) and a gradient elution as  
140 follows : 0 min/15 % B, 117.1 min/42 % B, 120.0 min/42 % B, 120.5 min/95 % B, 121.8 min/95 %  
141 B, 123.0 min/15 % B, 126.9 min/15 % B. The normalized gradient slope was 0.3 %, the flow-rate  
142 0.2 mL/min, the injection volume 40  $\mu\text{L}$  and the column temperature 50°C.

### 143 2.3.3. LC $\times$ LC separations

144 For <sup>1</sup>D RPLC optimization, three columns were screened: Atlantis T3 (150 mm x 3 mm, 3  $\mu\text{m}$ ,  
145 Waters), Purospher STAR RP-18 endcapped (250 mm x 4 mm, 5  $\mu\text{m}$ , Merck) and Kinetex PFP F5  
146 (150 mm x 4.6 mm, 5  $\mu\text{m}$ , Phenomenex). The final <sup>1</sup>D RP separation was conducted on a Kinetex  
147 PFP F5 column. For <sup>1</sup>D HILIC analyses, three columns were screened: XBridge BEH Amide (150  
148 mm x 4.6 mm, 5  $\mu\text{m}$ , Waters), Polyhydroxyethyl A (200 mm x 4.6 mm, 5  $\mu\text{m}$ , PolyLC) and TSKgel  
149 amide (150 mm x 4.6 mm, 3  $\mu\text{m}$ , Tosoh). The chromatographic conditions used after optimization  
150 are summarized in Table 1.

151 The fractions collected from the first dimension were evaporated to dryness (30°C, 250 mbar for  
152 4 h then 40°C, 150 mbar for 4 h) and recovered in water in the same volume as collected.

153 The second dimension was conducted on a Waters Acquity BEH C18 column (50 mm x 2.1 mm,  
154 1.7  $\mu\text{m}$ ) using H<sub>2</sub>O (A) and ACN (B) with 0.1 % FA in both phases. Gradient conditions are indicated  
155 in Table 1.

## 156 2.4. Theory supporting off-line LC $\times$ LC optimization

157 Predicted peak capacities  $n_{th}$  were calculated based on the hypothesis of Linear Solvent Strength  
158 (LSS) theory [14], with the hypothesis of very large retention factors at initial composition :

159  $n_{th} = 2.3 S \Delta C_e \frac{1}{1+2.3sS} \frac{\sqrt{N_{col}}}{4} + 1$  (Eq. 1)

160 where  $\Delta C_e$  is defined as the range of composition at elution  $C_e$  between the first and last  
 161 saponins.  $S$  is the absolute value of the slope of the relationship between the logarithm of the  
 162 retention factor and the stronger solvent composition,  $s$  the normalized gradient slope (Eq. 2)  
 163 and  $N_{col}$  is the column efficiency. The normalized gradient slope  $s$  is given by:

164  $s = \Delta C \times \frac{t_0}{t_g}$  (Eq. 2)

165 Where  $t_0$  and  $t_g$  are respectively the column dead time and the gradient time and  $\Delta C$  the gradient  
 166 composition range.

167 In off-line two-dimensional separation, the overall chromatographic duration  $t_{total}$  is the sum of  
 168 the <sup>1</sup>D cycle time  ${}^1t_{total}$  and  $n_f$  times the <sup>2</sup>D cycle time  ${}^2t_{total}$ , where  $n_f$  is the number of fractions  
 169 transferred from the first dimension to the second dimension (Eq. 3).

170  $t_{total} = {}^1t_{total} + n_f \times {}^2t_{total} = ({}^1t_{dwell} + {}^1t_0 + {}^1t_g) + n_f \times ({}^2t_{dwell} + {}^2t_0 + {}^2t_g)$   
 171 (Eq. 3)

172 with  ${}^i t_0$ ,  ${}^i t_g$ ,  ${}^i t_{dwell}$  the column dead time, the gradient time and the system dwell time of the  
 173 dimension  $i$ , respectively.

174 Considering the <sup>1</sup>D flow-rate  ${}^1F$ , the <sup>1</sup>D gradient slope  ${}^1s$  and the sampling rate  $\tau$  as variables, for  
 175 each triplet ( ${}^1F$ ,  ${}^1s$ ,  $\tau$ ), it is hence possible to determine the <sup>1</sup>D cycle time  ${}^1t_{total}$  (Eq. 4) and the  
 176 number of fractions  $n_f$  (Eq. 5).

177  ${}^1t_{total} = \left( {}^1V_{dwell} + {}^1V_0 \left( 1 + \frac{{}^1\Delta C}{{}^1s} \right) \times {}^1F \right)$  (Eq. 4)

178 where  ${}^1V_{dwell}$  and  ${}^1V_0$  stand for the dwell volume and column void volume of the first dimension,  
 179 respectively.

180  $n_f = \frac{1}{6} \frac{{}^1t_g}{{}^1\sigma_{exp}} \times \tau$  (Eq. 5)

181 where  ${}^1\sigma_{exp}$  reflects the  ${}^1D$  experimental peak standard deviation. The external dispersion is  
 182 mainly due to the injection process. One of the advantages of off-line LC  $\times$  LC is that the injection  
 183 volume can be tuned in each dimension so that the ratio  $\beta = \frac{\sigma_{col}}{\sigma_{exp}}$  (Eq. 6) remains above a value  
 184 of 0.9, corresponding to a plate loss of less than 20 %.

185 Combining Eq. 5 with Eq. 6, the number of fractions can be expressed as followed:

$$186 \quad n_f = \frac{1}{6} \frac{{}^1t_g}{{}^1\sigma_{col}} \times \frac{{}^1\sigma_{col}}{{}^1\sigma_{exp}} \times \tau = \frac{1}{6} \frac{{}^1\Delta C}{{}^1s} \frac{\sqrt{{}^1N}}{(1+{}^1ke)} \times {}^1\beta \times \tau \quad (\text{Eq. 7})$$

187 By extension, the  ${}^2D$  cycle time  ${}^2t_{total}$  is deduced, hence the  ${}^2D$  gradient time  ${}^2t_g$  (Eq. 3).

188 Working at maximal flow rate in second dimension, the associated normalized gradient slope is  
 189 thus imposed.

190 The effective theoretical peak capacities in off-line LC  $\times$  LC can hence be predicted for each triplet  
 191 ( ${}^1F$ ,  ${}^1s$ ,  $\tau$ ). The calculation has to take into account corrections for the injection band broadening  
 192 effect in both dimensions as well as for undersampling and occupation space rate.

$$193 \quad n_{2D,predicted} = {}^1\beta \times {}^1n_{th} \times \alpha \times {}^2\beta \times {}^2n_{th} \times f_{coverage} \quad (\text{Eq. 8})$$

194  ${}^1n_{th}$  and  ${}^2n_{th}$  are the theoretical peak capacities (Eq. 2) in  ${}^1D$  and  ${}^2D$  respectively.  $\alpha$  is a correction  
 195 factor, taking into account under-sampling in  ${}^1D$  and calculated according to [15] (Eq. 9) and  
 196  $f_{coverage}$  is a correction factor which takes into account the occupation space rate of the 2D  
 197 separation. For prediction purposes, it was set at 0.5 for RPLC  $\times$  RPLC and 0.75 for HILIC  $\times$  RPLC.

$$198 \quad \alpha = \frac{1}{\sqrt{1+0.21\left(\frac{6}{\tau}\right)^2}} \quad (\text{Eq. 9})$$

199 Experimental peak capacities for each dimension in LC  $\times$  LC were measured according to:

$$200 \quad n_{exp} = 1 + \frac{t_n - t_1}{w_{4\sigma}} \quad (\text{Eq. 10})$$

201  $t_n$  and  $t_1$  are the retention times of the last and first eluted compound respectively, and  $w_{4\sigma}$  is the  
 202 average peak width at  $4\sigma$  over about 10 peaks.

203 The effective experimental peak capacities in LC × LC was estimated using (Eq. 11), where  $f_{coverage}$   
204 was measured on the UV contour-plot using Convex-Hull approach [16]. The external dispersion  
205 was already taken into account in the measurement of the experimental peak widths.

$$206 \quad n_{2D,effective} = f_{coverage} \times \alpha \times {}^1n_{exp} \times {}^2n_{exp} \quad (\text{Eq. 11})$$

207 The dilution factor  $D_f$  is defined as the quotient of the peak volume  $V_{exp}$  over the injected volume  
208  $V_{inj}$ . Since the column variance can be predicted according to chromatographic operating  
209 conditions, it is possible to predict the dilution factor:

$$210 \quad D_f = \frac{V_{exp}}{V_{inj}} = \frac{\sigma_{exp} \times \sqrt{2\pi}}{V_{inj}} = \frac{\sigma_{col} \times \sqrt{2\pi}}{\beta V_{inj}} \quad (\text{Eq. 12})$$

211

### 212 3. Results and discussion

213 The first objective of this study was to develop a reference chromatogram of Quil-A. The total  
214 chromatographic duration of 120 minutes was selected in order to generate sufficient peak  
215 capacity while limiting the instrumental occupancy rate in the QC laboratory, the latest being  
216 usually the bottleneck of routine analysis. This duration is compatible with the fact that the  
217 analysis of the stock plant material has to be performed in a regular basis, but on a limited  
218 number of samples. The duration of sample handling (evaporation) is not taken into account as  
219 it does not involve costly operations nor human supervision.

#### 220 3.1. One-dimensional separation

221 The separations of saponins reported in the literature are usually based on reversed phase  
222 mechanism using C18 stationary phases. A BEH C18 column was selected as suggested in  
223 literature [13]. The addition of a small quantity of formic acid increased the retention without  
224 inhibiting the MS signal. Figure 1 shows the resulting optimized one-dimensional RPLC analysis  
225 of Quil-A. Saponins peaks were identified in the extract as exhibiting simultaneously UV

226 absorbance at 205 nm and m/z over 1 000. The first saponin eluted at 12.5 min ( $C_e$  17 %) and the  
227 last one at 116 min ( $C_e$  41 %). A total of 122 peaks were detected but a cluster was present  
228 between 90 and 115 min with many co-eluting peaks including the saponins QS18 and QS21 that  
229 are commonly used as quality markers. The experimental peak capacity was found to be 260 (Eq.  
230 10) and the dilution factor 1.3 (Eq. 12). This example highlights the fact that the 1D-LC separation  
231 does not provide sufficient resolution power to analyze such a complex sample without high-  
232 resolution mass spectrometer.

233

### 234 3.2. Development of reversed-phase LC × reversed-phase LC and hydrophilic interaction 235 chromatography × reversed-phase LC

236 The second dimension was selected to provide high efficiency, speed and compatibility with both  
237 UV and MS detection. The RPLC method described previously was selected in the second  
238 dimension, with adjustment of the column length to 50 mm to reduce the <sup>2</sup>D gradient time. The  
239 selection of the first dimension mode was based on the search for the highest degree of  
240 orthogonality relative to the RPLC used in the second dimension.

#### 241 3.2.1. *Screening for the first dimension*

242 Saponins present an ionizable glucuronic acid group with a pKa of around 3.2 [17]. Using a basic  
243 mobile phase with 10 mM ammonium acetate (pH around 7) was suggested to induce a different  
244 selectivity compared to the second dimension [18]. Three different RP columns were screened  
245 using a generic gradient: Atlantis T3, Purospher STAR RP-18 endcapped and Kinetex PFP F5. The  
246 two C18 columns provided identical  $\Delta C_e$  of 20 %. The PFP stationary phase offered a larger  
247 elution range (44 %) with a different selectivity (Figure S2a). Saponins were found to elute  
248 between 37 % and 81 % MeOH. The injection process was optimized both in concentration and  
249 in volume (Figure S3). Thanks to the high solubility of saponins in water and to the on-column

250 focusing effect, it was possible to inject 87  $\mu\text{L}$  at 50 mg/mL, while limiting the loss of plates down  
251 to 20 % ( $\beta$  above 0.9 in Eq. 6).

252 For HILIC, a generic gradient was set up from 5 to 40 % of water + 10 mM ammonium acetate, to  
253 screen the three pre-selected columns: XBridge BEH Amide, Polyhydroxyethyl A and TSKgel  
254 amide. The elution window was found the same on the three columns, in the range of 15 % to 35  
255 % water. The TSKgel amide column was selected for the <sup>1</sup>D HILIC separation due to its higher  
256 kinetic performances (Figure S2b). Injection in HILIC is much more challenging as water is the  
257 strong eluent. At the HILIC initial composition (15 % water) the solubility was below 0.5 mg/mL,  
258 compromising detection. Using butanol instead of acetonitrile significantly improved Quil-A  
259 solubility [19], reaching 12.5 mg/mL in a 75/25 2-BuOH/water. This high-strength solvent allowed  
260 the injection of only 20  $\mu\text{L}$  but was found the best compromise between solubility and injection  
261 variance.

262 Repeatability is a critical parameter in quality control. For RPLC, an average standard deviation  
263 of 0.009 min was calculated on the retention times of five compounds ( $n = 5$ ), which is about 15  
264 times lower than the average base peak width (0.14 min). For HILIC, an average standard  
265 deviation of 0.002 min was calculated based on the retention times of ten compounds and an  
266 average base peak width of 0.21 min. Therefore, both methods ensured that compounds  
267 remained in the same fraction during repeated sampling. Worth mentioning, HILIC separation  
268 was repeatable with an equilibration volume of only 5 void volumes between analyses.

269 First dimension effective peak capacity was 99 for RPLC and 39 for HILIC (Eq. 10). The lower peak  
270 capacity in HILIC mode was due to the much lower elution composition range. However this low  
271 separation space may be counterbalance in LC  $\times$  LC by a large orthogonality related to the second  
272 dimension.

273

### 274 3.2.2. *Off-line LC × LC optimization*

275 The off-line chromatographic conditions were optimized for a 120-min duration, this overall time  
276 including the first dimension duration and the cumulative second dimension durations to analyze  
277 the  $n_f$  fractions (Eq. 3). It is to be noted that the dwell volume is twice the column volume in the  
278 second dimension so a repeatable equilibrium was easily reached [20] without additional  
279 equilibrium time.

280 To determine kinetically optimised conditions, analyte constants were determined. The viscosity  
281 was calculated using empirical equations derived by Cabooter et al. [21] and appropriate  
282 molecular diffusion references were from Li and Carr [22, 23] in 100 % water at 20 °C. Considering  
283 an average molecular weight of 1500, the diffusion coefficient of saponins was estimated  
284 according to Wilke-Chang equation [24] at  $2.76 \cdot 10^{-11} \text{ m}^2/\text{s}$  for <sup>1</sup>D RPLC (30 % - 85 % MeOH, 30  
285 °C),  $6.7 \cdot 10^{-11} \text{ m}^2/\text{s}$  for <sup>1</sup>D HILIC (40 °C, 65 % - 85 % ACN) and  $1.13 \cdot 10^{-12} \text{ m}^2/\text{s}$  for <sup>2</sup>D RPLC (15 % -  
286 65 % ACN, 50 °C). The LSS constants were derived from linear retention models for RPLC. For  
287 HILIC, linear plots of  $\log k$  vs. % water were considered in the very narrow composition of interest.  
288 LSS equations were applied on HILIC dimension [25], aware of the errors this model can generate  
289 on amide columns [26]. Of course, due to the diversity of the molecules engaged in this extract,  
290 all the constants are approximated as average. Nonetheless, the aim of the study was to find  
291 optimal conditions, as opposed to predict accurately the performances.

292 The variables for this optimization were the <sup>1</sup>D flow-rate and normalized gradient slope, as well  
293 as the sampling rate. The <sup>2</sup>D flow-rate was set to a maximal value of 1 mL/min, limited by the  
294 system pressure. The <sup>2</sup>D gradient time was dictated by the three variables through Eq. 3.

295 The plot of the dilution factor vs the predicted peak capacity (Figure 2) were illustrated for RPLC  
296 × RPLC (Figure 2a) and HILIC × RPLC (Figure 2b). Each open mark represents the results for a  
297 variable triplet (<sup>1</sup>F, <sup>1</sup>s,  $\tau$ ). The mark shape relates to the gradient slope <sup>1</sup>s, its color to the sampling

298 rate  $\tau$  and the various data points to the scanned <sup>1</sup>D flow-rates. Technical constraints due to the  
299 collector were also taken into account, namely the maximal number of fractions (96 vials) and  
300 the minimal sampling time required by the collecting needle (in our case 0.2 min). Conditions  
301 that meet those criteria are reported as full marks. Improvements on collector designs may allow  
302 access to better off-line 2D performances.

303  
304 As a general trend, the conditions leading to the largest peak capacities in RPLC  $\times$  RPLC were  
305 found for a low sampling rate and a short <sup>1</sup>D separation (around 10-15 min) as shown in Figure  
306 S4a. This trend is opposite to what is observed in online LC  $\times$  LC. For HILIC  $\times$  RPLC, the trend was  
307 similar but the gradient steepness rate was found to have more influence on the peak capacity  
308 than on RPLC  $\times$  RPLC, as clearly visible on Figure 2b and Figure S4b, with better results at low  
309 gradient slope. This is partly due to the poor kinetic performances of the selected HILIC column  
310 at large flow rates.

311 The best conditions providing both a large peak capacity and a low dilution factor are indicated  
312 with a black arrow. For RPLC  $\times$  RPLC, working with <sup>1</sup>F = 1.4 mL/min;  $s = 3\%$ ;  $\tau = 1$  generated a  
313 predicted peak capacity of about 750 alongside with an acceptable dilution factor around 0.3,  
314 whereas in HILIC  $\times$  RPLC, the best conditions were <sup>1</sup>F = 0.6 mL/min;  $\tau = 2$ ;  $s = 4\%$  and gave a  
315 predicted peak capacity of 600 and a dilution factor around 0.7. Table 1 summarizes these  
316 kinetically optimized conditions.

317

### 318 3.2.3. *Quil-A reference chromatograms*

319 The experimental optimized off-line RPLC  $\times$  RPLC (a) and HILIC  $\times$  RPLC (b) separations are shown  
320 as UV contour plots (Figure 3). Because of the necessary zoom to illustrate the presence of minor

321 compounds, major compounds spots may not be all distinguishable. Nonetheless, these UV  
322 contour plots are helpful in a QC environment to rapidly spot non-conform samples.

323 The observed occupation space was quite low in RPLC × RPLC. Diagonalized clusters were visible  
324 (Figure 3a). The ConvexHull method provided an occupation rate of 46 %. This measurement  
325 overestimated the occupation space since clusters were present. Unfortunately, the  
326 orthogonality is still difficult to apprehend when molecules are not evenly distributed over the  
327 2D separation space [27]. An effective peak capacity 831 was calculated, which is close to the  
328 predicted value of 748. Qiao et al. [28] obtained comparable results with a peak capacity of 524  
329 for the online RPLC × RPLC separation on triterpenoid saponins in licorice.

330 The HILIC × RPLC separation (Figure 3b) was characterized by a large occupation space rate (73  
331 %), which is close to the values found in the literature. For instance, Qiu et al. calculated a value  
332 of 79 % on *Panax ginseng* HILIC x LC separation [29]. The diversity of oligosaccharides (nature,  
333 number and positions) explains the better spread along the separation space using HILIC as first  
334 dimension. The peak capacity for this 2D system was 673, close to the predicted value (594) and  
335 slightly under the RPLC × RPLC result. The HILIC dimension resulted in a dilution factor of 4. But  
336 the evaporation of ACN-rich fractions and their recovery in 100 % water allowed on-column  
337 focusing in the second dimension, resulting in an overall dilution factor of 0.5 (Eq. 12).

338 Table 2 summarizes the performances of the three proposed methods. At first sight, it is clear  
339 that the performances of the 1D RPLC are well below those of LC × LC separations, in terms of  
340 peak capacity, dilution factor or signal-to-noise ratio for a selected target. Regarding the two LC  
341 × LC separations, the <sup>1</sup>D RPLC exhibited a higher peak capacity than <sup>1</sup>D HILIC, but the larger  
342 orthogonality of HILIC × RPLC led to a comparable effective peak capacity (831 vs 673).

343 From the sensitivity point of view, HILIC mode is unfavorable. The quantity of water-soluble  
344 samples that can be injected without inducing large band broadening is limited. In this

345 application, only 0.25 mg of saponin sample could be injected in HILIC, which is more than 17  
346 times lower than in RPLC. Despite on-column focusing in the second dimension, the HILIC × RPLC  
347 method provided a lower signal-to-noise ratio for QS18.

348

#### 349 *3.2.4. Application for degraded samples*

350 While RPLC × RPLC seems to offer better performances, the potential of each LC × LC  
351 configuration was highlighted through their application on degraded samples. Quil-A was  
352 chemically and enzymatically hydrolyzed in order to obtain two different altered samples.  
353 Contour plots are displayed as Figure 4 and the pattern of the original Quil-A sample was  
354 overlapped (in black dotted lines) to easily observe the differences.

355 The contour plot of the RPLC × RPLC analysis (Figure 4a) showed a general decrease of the  
356 retention along the main diagonal. This was in accordance with the assumption that the  
357 hydrolysis under mild basic conditions cleaved the ester bonds and cut the fatty-acid chain-  
358 arabinose carried by the sugar at the C-28 position arabinose (noted X on Figure S1). The overall  
359 hydrophobicity decreased, hence the retention in RPLC. MS data confirmed the disappearance  
360 of the two saponins QS18 and QS21 (noted 1 and 2, respectively in Figure 4). The corresponding  
361 degradation products, namely QS18-X and QS21-X ( $m/z=1674$  noted 1a and  $m/z=1512$  noted 2a,  
362 respectively) were identified in the degraded sample, exhibiting as expected, a lower retention  
363 in both dimensions. On the contrary, saponins located in the upper diagonal cluster (i.e. more  
364 retained in first dimension) do not have the fatty-acid chain-arabinose and remained consistent  
365 with the original fingerprint despite hydrolysis. Discrete spots with lower masses appeared,  
366 exhibiting poor retention in both RPLC dimensions. They were identified as sugar moieties  
367 resulting from the degradation.

368 For the illustration of enzymatic degradation, the commercial mixture “Rapidase Revelation  
369 Aroma” was selected as it shows, among others, an apiofuranosidase activity [30]. The loss of  
370 sugars led to a shift of compounds towards lower retention in HILIC and larger retention in RPLC,  
371 as expected (Figure 4b). QS18 and QS21 were degraded as they present an apiofuranose at R<sub>3</sub>  
372 position (Figure S1). MS also confirmed the presence of corresponding degradation products  
373 QS18-Api (identified as 1b on Figure 4b; m/z=2019) and QS21-Api (identified as 2b on the same  
374 figure; m/z=1857). Interestingly, the selected enzyme mixture did not degrade all carbohydrate  
375 moieties as illustrated by the still large coverage of the HILIC separation space.

#### 376 4. Conclusion

377 The objective of this study was to develop a separation method appropriate to an industrial  
378 quality control context, for the comparison of samples of *Quillaja Saponaria* to a reference  
379 extract. A 1D-RPLC separation was not providing enough separation power to be used without  
380 high resolution mass spectrometry detection, so LC × LC methods were investigated. RPLC × RPLC  
381 and HILIC × RPLC were optimized with a total separation time of 120 min and then compared in  
382 terms of peak capacity. The RPLC × RPLC separation was based on a pH and solvent change.  
383 However, only a limited space coverage of 46 % was reached. Nonetheless, due to high efficiency  
384 in both dimensions, an effective peak capacity of 831 was obtained. On the contrary, the HILIC ×  
385 RPLC led to a high space coverage of 73 % but the effective peak capacity was only 673 because  
386 of the limited peak capacity of the HILIC first dimension. Sensitivity wise, the dilution factor Df  
387 was below 1 for both configurations, thanks to the recovery of fractions in water between the  
388 two dimensions. Despite the best performances of RPLC × RPLC for Quil-A separation, both  
389 approaches are of interest for the quality control of saponins samples, as illustrated by the  
390 analysis of degraded samples. When RPLC × RPLC is ideal to spot modifications of the carbon

391 skeleton, HILIC × RPLC is more suitable to detect cleavages of sugar moieties. For both, a simple  
392 UV contour plot is sufficient to rapidly spot non-conform samples in a quality control  
393 environment, while being fully compatible with MS detection if further identification is required.

394  
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396 express their gratitude to Agilent Technologies for the gracious loan of the UHPLC 1290 Infinity.

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398 Conflict of interest statement

399 The authors declared they have no conflict of interest.

## 400 5. References

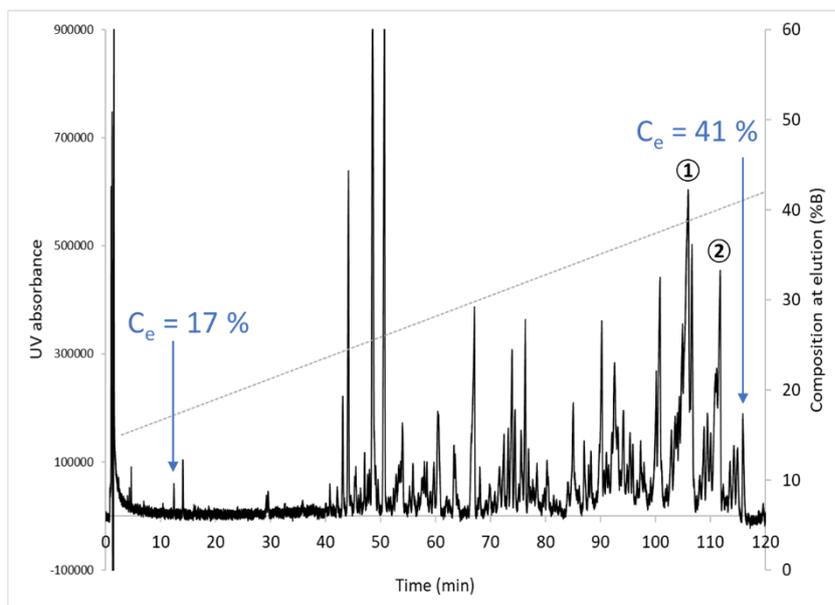
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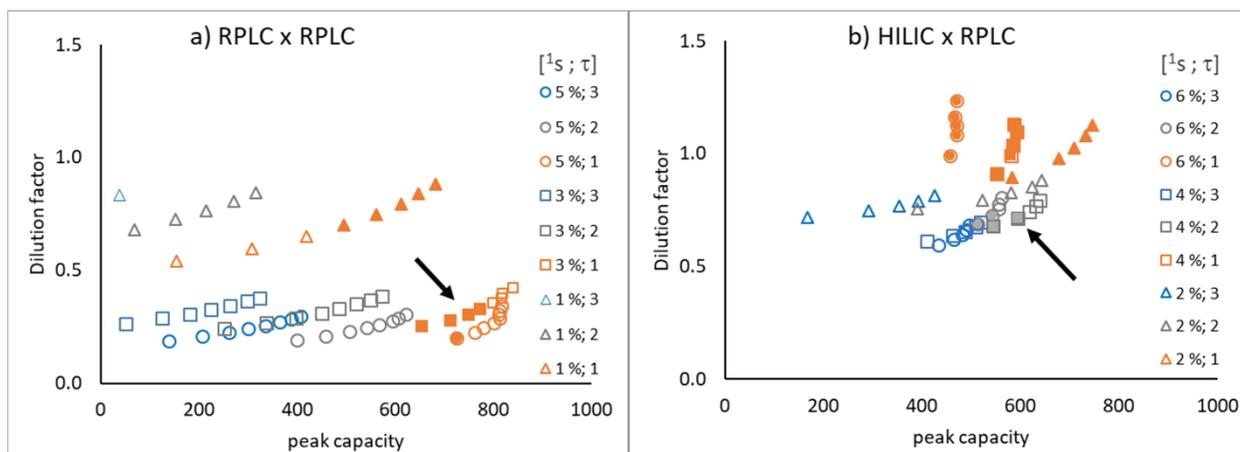
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487 **Figures**

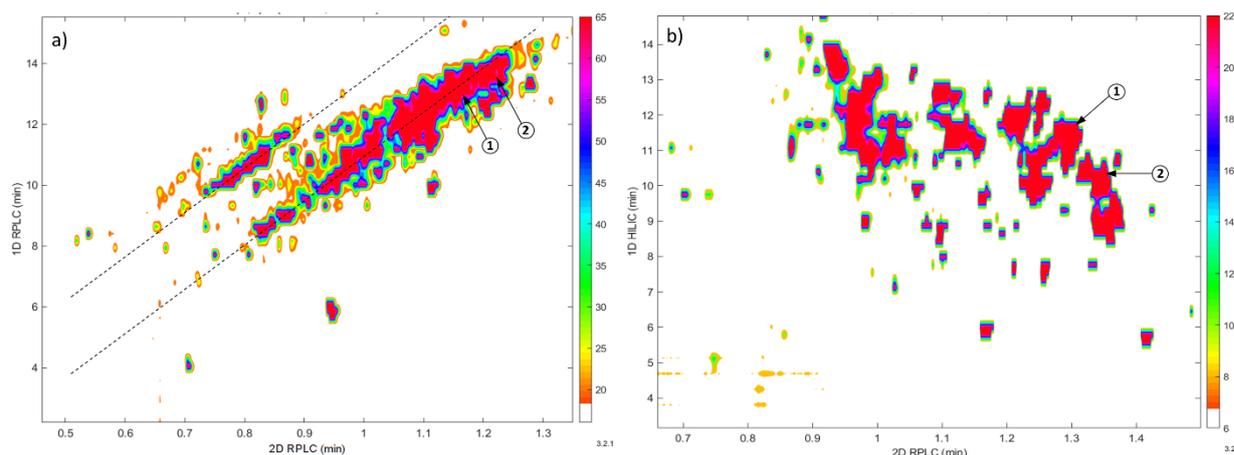


488 Figure 1. UV chromatogram of the 120-min RPLC separation of Quil-A. BEH C18 column (100  
489 mm x 2.1 mm, 1.7  $\mu$ m). Mobile phase: H<sub>2</sub>O (A) and ACN (B) with 0.1 % FA in both phases.  
490 Gradient 15 % to 42 % (B). Flow-rate 200  $\mu$ L/min. Injected volume 40  $\mu$ L (50 mg/mL in water).  
491 UV detection: 205 nm. Secondary axis represents the composition at elution (% B) along the  
492 analysis. Arrows point the first and the last saponin peak with the corresponding composition at  
493 elution and numbers (1) and (2) indicate saponins QS18 and QS21 respectively.

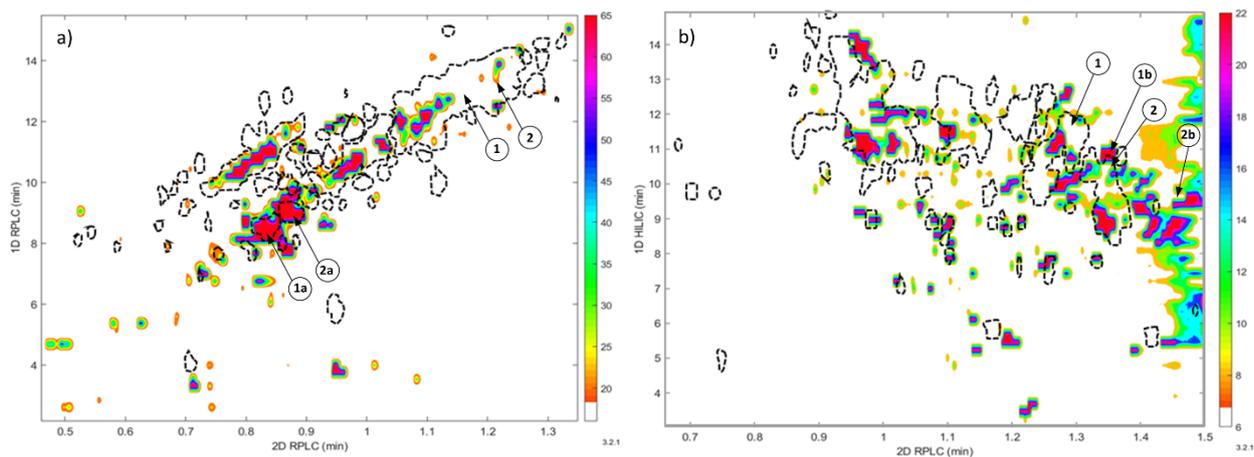
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 498 Figure 2. Plot of the predicted dilution factor vs predicted peak capacity for a chromatographic  
 499 duration of 120 min. a) RPLC  $\times$  RPLC and b) HILIC  $\times$  RPLC. Calculations were performed using  
 500 three selected variables:  $^1F$  [1- 3 mL/min] for RPLC  $\times$  RPLC and [0.2 – 1.2 mL/min] for HILIC  $\times$   
 501 RPLC ;  $^1s$  [1 %; 3 %; 5 %] for RPLC  $\times$  RPLC and [2 %; 4 %; 6 %] for HILIC  $\times$  RPLC; sampling rate  $\tau$  [1;  
 502 2; 3] for both methods. Open marks represent theoretical results and full marks represents  
 503 results that are compatible with technical constraints: number of transferred fractions below 96  
 504 and a sampling time over 0.2 min. The arrow indicates the selected operating conditions.



506  
 507 Figure 3. UV contour plots of off-line separation of Quil-A using a) RPLC  $\times$  RPLC and b) HILIC  $\times$   
 508 RPLC. Optimized conditions displayed in Table 1. Saponins QS18 (1) and QS21 (2) are indicated.



510

511 Figure 4. 2D-contour plots of off-line separations of degraded Quil-A. a) RPLC × RPLC of Quil-A

512 hydrolyzed; b) HILIC × RPLC of enzymatically hydrolyzed Quil-A. Original RPLC × RPLC and HILIC

513 × RPLC contour plot patterns of Quil-A overlapped (black dotted line). Similar conditions as

514 displayed in Table 1. UV signal at 205 nm. Numbers indicate saponins QS18 (1) and QS21 (2)

515 and corresponding degraded species a) QS18-X (1a) and QS21-X (2a) or b) QS18-Api (1b) and

516 QS21-Api (2b).

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## Supplementary material

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525 **Figure S1.** Structures of saponins from *Quillaja Saponaria*

526 **Figure S2.** UV chromatogram of <sup>1</sup>D separation of QuilA, a) RPLC Kinetex PFP F5 column.

527 **Figure S3.** Injection study in RPLC.

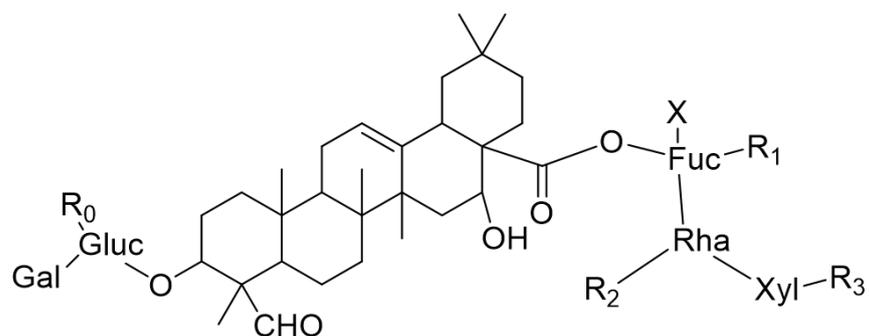
528 **Figure S4.** Plot of the predicted 2D peak capacity vs. the gradient time of the first dimension

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Generic structure



Some known saponins

Name	R <sub>0</sub>	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	X	Mw (g/mol)
<b>QS21</b>	Xyl	H	H	Api/Xyl	FA-Araf	1990.1
<b>QS18</b>	Xyl	H	Glc	Api	FA-Araf	2152.3

Structure of FA-Araf

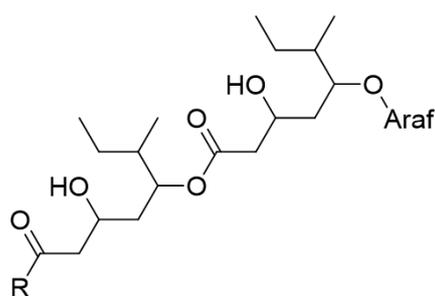


Figure S1. Structures of saponins from *Quillaja Saponaria*

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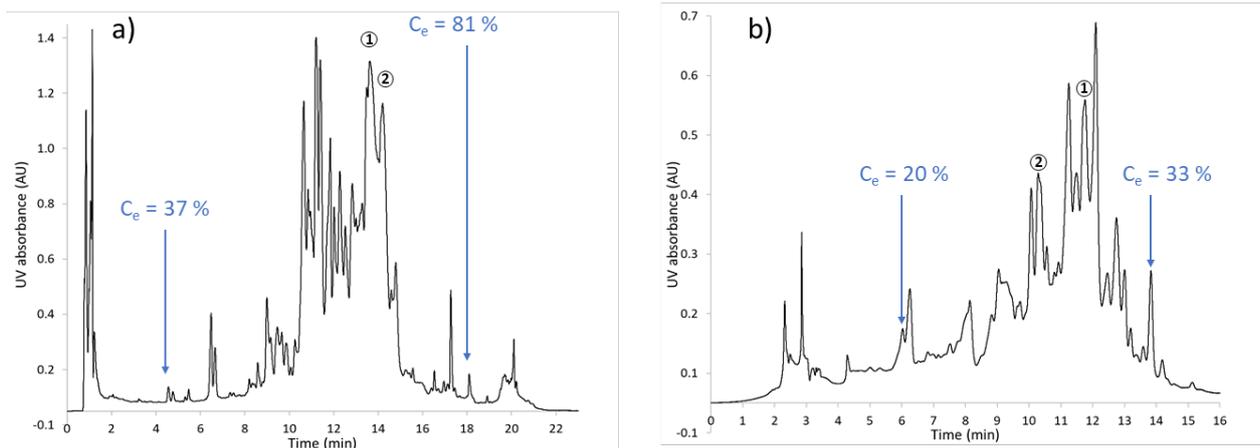


Figure S2. UV chromatogram of <sup>1</sup>D separation of QuilA, a) RPLC Kinetex PFP F5 column. Mobile phase: 10 mM AA in water (A) and in MeOH (B). Organic gradient started from 30 to 85 % (B) in 17.5 min at 1.4 mL/min. Injected volume of 50  $\mu$ L (50 mg/mL in water); b) HILIC TSKgel amide column. Mobile phase: ACN (A) and 10 mM ammonium acetate in water (B). Aqueous gradient started from 15 % to 35 % (B) in 12.5 min at 0.6 mL/min. Injected volume of 20  $\mu$ L (12.5 mg/mL in 2-BuOH/water75/25 v/v). UV detection at 205 nm. Arrows point the first and the last saponin peak with the corresponding composition at elution and numbers (1) and (2) indicate major saponins QS18 and QS21 respectively.

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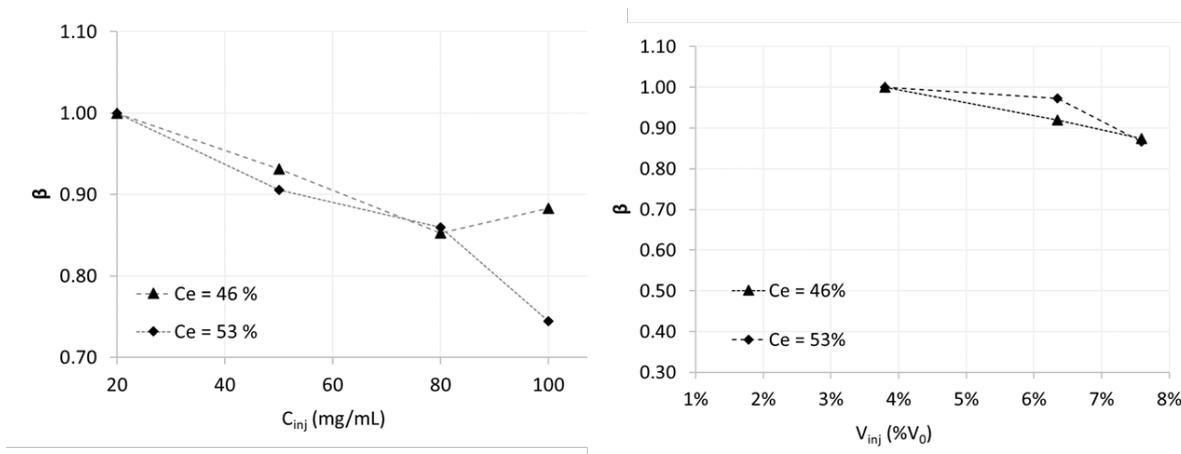


Figure S3. Injection study in RPLC. Plot of the fraction of remaining peak capacity  $\beta$  for two saponins, as a function of a) the concentration of Quil-A sample in water ( $V_{inj}$  10  $\mu$ L) and of b) the injection volumerelated to the column volume (50 mg/mL in water). Kinetex PFP F5 column. Mobile phase: 10 mM AA in water (A) and in MeOH (B). Gradient 30 % to 85 % (B) in 17.5 min. Flow-rate 1.4 mL/min.

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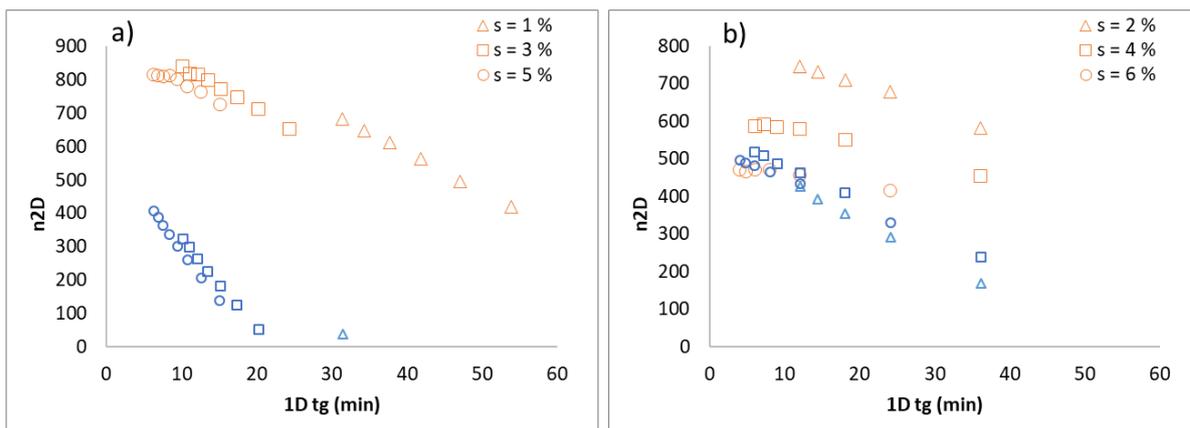


Figure S4. Plot of the predicted 2D peak capacity vs. the gradient time of the first dimension, for a chromatographic duration under 120 min. a) RPLC x RPLC and b) HILIC x RPLC. Calculations were performed using three variables :  $^1F$  [1- 3 mL/min] for RPLC x RPLC and [0.2 – 1.2 mL/min] for HILIC x RPLC;  $^1s$  (normalized gradient slope) [1 %; 3 %; 5 %] (marked as triangle, square and circle, respectively) for RPLC x RPLC and [2 %; 4 %; 6 %] for HILIC x RPLC; sampling rate  $\tau$  of 1 (orange) and 3 (blue) for both methods.

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