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1 Supercritical Fluid Chromatography development of a predictive analytical tool
2 to selectively extract bioactive compounds by Supercritical Fluid Extraction and
3 Pressurised Liquid Extraction

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8 Abstract

9

10 Selective extraction is a great concern in the field of natural products. The
11 interest is to apply specific conditions favouring the solubility of targeted secondary
12 metabolites and avoiding the simultaneous extraction of unwanted ones. Different
13 ways exist to reach selective extractions with suited conditions. These conditions can
14 be determined from experimental studies through experimental design, but a full
15 experimental design takes time, energy, and uses plant samples. Prediction from
16 varied solubility models can also be applied allowing a better understanding of the
17 final selected conditions and eventually less experiments.

18 The aim of this work was to develop and use a chromatographic model to
19 determine optimal extraction conditions without the need for numerous extraction
20 experiments. This model would be applied on the selective extraction of the desired
21 antioxidant compounds in rosemary leaves (rosmarinic and carnosic acids) vs
22 chlorophyll pigments to limit the green colour in extracts. This model was achieved
23 with Supercritical Fluid Chromatography (SFC) and then applied to Supercritical Fluid
24 Extraction (SFE) and Pressurized Liquid Extraction (PLE) assays.

25 SFC models predicted low solubility of chlorophylls for low (5%) and high
26 (100%) percentage of solvent in carbon dioxide. Also, low solubility was predicted
27 with acetonitrile solvent compared to methanol or ethanol. This was confirmed with
28 different extractions performed using SFE with different percentages of solvent (5,
29 30, and 70%) and with the three solvents used in the SFC models (acetonitrile,
30 methanol and ethanol). Also extractions using PLE were carried out using the same

31 neat solvents in order to confirm the SFC models obtained for 100% of solvent.
32 Globally, extractions validated the SFC models. Only some differences were
33 observed between ethanol and methanol showing the complexity of plant extraction
34 due to matrix effect. For all these extracts, the content of carnosic acid and
35 rosmarinic acid was also monitored and selective extraction conditions of bioactive
36 compounds could be determined.

37

38 1 Introduction

39

40 The need for selectivity during plant extraction has different reasons. One of
41 them, maybe the simplest and most logical one, is to enrich the final extract in
42 particular bioactive metabolites, without the need for additional purification steps by
43 preparative chromatography or counter-current chromatography. Numerous articles
44 were published about enrichment of extracts. For example, it has been studied in
45 order to extract more bioactive compounds such as hydroxytyrosol from olive waste
46 [1], or chlorophyll pigments extraction in order to valorise and use them as dye [2].
47 On the contrary, another interest can be the necessity to avoid particular compounds.
48 Some compounds considered as interferences can be toxic compounds coming from
49 the plant or from the soil or the water used during the plant growth [3–6].

50 Among many extraction approaches, supercritical fluid extraction with carbon
51 dioxide as extracting fluid has shown interesting selectivity properties, which result
52 from the carbon dioxide itself, and from the possibility to extract sequentially different
53 family of compounds when applying a dynamic and continuous method [7,8]. When
54 the fluid is in the supercritical state, different parameters such as pressure,
55 temperature and the addition of co-solvents can modify carbon dioxide behavior. This
56 change of parameters can then be used to selectively extract the target compounds,
57 due to their varied solubilities depending on the fluid properties.

58 The classical approach to determine extraction conditions is based on the
59 selection of some parameters and their variation in a defined range. All the
60 extractions are carried out, to determine the best. A more efficient way to obtain
61 optimal conditions with a minimum of experiments exists with design of experiments
62 (DoE).

63 Different parameters are screened simultaneously then, based on a pre-defined
64 response (concentration of target analytes, yield...), optimum conditions are
65 determined [9–11]. This strategy also helps understanding the influence of the
66 different parameters tested and their interactions. Even if a DoE reduces the number
67 of experiments, it still remains long. Applied to plant extraction, the number of
68 extracts increases exponentially with the number of parameters to be tested.

69 The aim of this study was to develop a model which would allow identifying the
70 best extraction conditions without the need for a DoE with numerous extractions. This
71 work was focused on chlorophyll pigments. On the one hand, there is an interest for
72 the extraction of chlorophyll pigments to use these compounds as natural dyes. On
73 another hand, there is also an interest for not extracting chlorophyll pigments. For
74 instance, for cosmetic raw materials, plant extracts must contain the lowest possible
75 amount of chlorophyll pigments (with the highest possible amount of bioactive
76 compounds), to obtain white final products. Also, chlorophyll compounds are very
77 sensitive and transform themselves into degradation products such as pheophytin
78 which change the color from green to gray [12] inducing possible colour change of
79 the final products. Rosemary (*Rosmarinus officinalis*) is a well known plant widely
80 used in cosmetic or food applications. Rosemary extracts can be used as
81 conservative agents for example for milk and dairy products [13]. The aerial part of
82 this plant from the *Lamiaceae* family, contains chlorophyll but also interesting
83 bioactive molecules such as carnosic or rosmarinic acids known for their antioxidant
84 or their antiproliferative properties [14,15].

85 In this paper, the behavior of chlorophyll compounds is first studied by
86 Supercritical Fluid Chromatography and Liquid Chromatography with pure organic
87 solvents as mobile phases. The retention observed in function of the different
88 conditions is then correlated to real extraction processes, and then to SFE or
89 pressurized liquid extraction (PLE). Finally, the different conditions favoring the
90 extraction or non-extraction of chlorophyll will also be studied regarding the extraction
91 of the main bioactive compounds of rosemary. Thus the behavior of carnosic acid
92 and rosmarinic acid will be also investigated in this work because they have different
93 polarities ($\text{Log } P_{\text{Carnosic acid}}=5.14$, $\text{Log } P_{\text{Rosmarinic acid}}=1.82$) [16].

94

95 2 Material and methods

96

97 2.1 Solvents and chemicals

98

99 For all this study, the solvents used were methanol, ethanol and 2-
100 methyltetrahydrofuran, gradient grade (VWR International, Fontenay-sous-Bois,
101 France), and acetonitrile, gradient grade (Carlo Erba, Val-de-Reuil, France).
102 Propylene carbonate used was for synthesis (Merck KGaA, Darmstadt, Germany).
103 Carbon dioxide (>99.5%) was from Air Liquide (Fleury-les-Aubrais, France). Different
104 additives were tested: water was supplied by an Elga Purelab Flex station from
105 Veolia (Antony, France), and citric acid was obtained from Sigma Aldrich (Saint-
106 Quentin Fallavier, France). In order to develop the analytical method, different
107 standards were used. Carnosic and rosmarinic acid standards were supplied by
108 Extrasynthese (Genay, France). Chlorophyll *a* and *b* were obtained from ivy extracts
109 (protocol described in extraction method part). The dispersant was diatomaceous
110 earth (DE) from Fisher Scientific (Illkirch- France), general purpose grade.

111

112 2.2 Analytical SFC method development

113

114 The chromatographic system used for the retention model or the quantification
115 of extract was a Jasco instrument (see details in [12]).

116 During the analytical method development in order to analyse rosemary
117 extracts, different columns were tested: HSS C18 (5 μm , 4.6 x 150 mm), Torus DEA
118 (1.7 μm , 3.0 x 100 mm) (diethylamine group), Torus 2-Pic (1.7 μm 3.0 x 100 mm)
119 (picolylamine group), Torus DIOL (1.7 μm 3.0 x 100 mm), all from Waters (Saint
120 Quentin en Yvelines). Two elution gradients were used. "Gradient 1": 5% of methanol
121 during 1 min, then from 5 to 50% until 15 min. The following conditions with "Gradient
122 1" were used for the initial column screening: flow rate: 1.0 mL/min, column oven
123 temperature: 35°C, injection volume: 5 μL . The columns packed with small diameter
124 particles and the elution gradient up to 50% methanol both induce a high pressure
125 drop along the column. As a result, the back-pressure was set at 10 MPa, to avoid
126 reaching the upper pressure limit of the pumping system (40 MPa).

127 Once the stationary phase was selected, and depending on the retention times
128 observed for the target compounds, the gradient was optimized and shortened into
129 “Gradient 2”: 5% methanol during 1 min, then increase to 50% in 4 min and maintain
130 at 50% during 4 min, to finally come back to initial condition during 3 min at 5%.

131 The final SFC method developed used coupled columns: HSS C18 coupled
132 with Torus DIOL.

133

134 2.3 Plants and extraction methods

135

136 Two plants were used in this study. Ivy leaves (*Hedera canariensis Willd*) used
137 for the production of chlorophyll-rich extract were collected in Orleans (France).
138 Rosemary leaves (*Rosmarinus officinalis*) for the study of selective extraction of
139 carnosic acid, rosmarinic acid and chlorophyll *a* were provided by one of our
140 industrial partners, Alban Muller (Fontenay-sur-Eure, France) specialised in plant
141 extraction.

142 Ivy was harvested and then dried using a Memmert model 500 dryer, at 50°C
143 for 24 h. Then the dried leaves were grinded using Polymix PX-MFC 90 D Kinematica
144 from Grosseron (Coueron, France).

145 Plant extraction for “standard” extracts used in the two first parts was carried out
146 with Ultrasonic bath Branson 3510. Ivy and Rosemary extracts were prepared from 1
147 g of plant material and 15 mL ethanol. The mixture was then sonicated for 60 min.
148 The extracts were centrifuged at 7000 rpm at 20°C for 10 min using BR4i
149 Multifunction Centrifuge (Thermo-electron, Nantes, France). The extracts were used
150 for no longer than one week, in order to maintain low amounts of chlorophyll
151 degradation products.

152 A Waters MV-10 ASFE was used for supercritical fluid extraction. In addition, a
153 UV detector was added between the oven and back-pressure regulator (BPR)
154 modules in order to monitor extraction kinetics. The detector was a Gilson 151 UV /
155 VIS Multiwavelength Detector (GILSON, Villiers-le-Bel, France). The 5-mL extraction
156 cell was prepared using 1 g of plant material mixed with 1 g of dispersant. Cotton
157 served to fill the cell completely and was placed at the top and bottom of the cell.

158 Extraction parameters for all rosemary extracts were using the percentage of modifier
159 with a total flow of 3.0 mL/min for 30 min at 25°C, 10 MPa. For 5% modifier, a make-
160 up solvent (ethanol) was used at 0.15 mL/min introduced before the BPR to avoid
161 compound precipitation in the transfer capillary. Final volume obtained was 24 mL
162 when using 5% modifier (plus make-up solvent) and 23 mL when using 30% modifier.

163 Pressurised liquid extraction (PLE) was performed with a Dionex ASE 150 from
164 Thermo (Thermo, Villebon sur Yvette, France): 1 g of plant material mixed with 1 g of
165 dispersant (DE) were extracted in a 22-mL extraction cell.

166 All extractions were carried out at ambient temperature (Temperature regulation
167 off), 10 MPa, and 3 cycles of 10 min static extraction with 60% flush. Final volume
168 was 32 mL.

169 Different fluid compositions were selected from varied points of retention values
170 and were applied in extraction by supercritical fluid, with ACN, MeOH, EtOH at 5, 30,
171 70% in CO₂ (SFE), and 100% liquid (PLE). All extractions of Rosemary leaves were
172 carried out three times in order to evaluate repeatability. The latter is then expressed
173 as standard deviation on the different figures.

174 Peak areas were measured with the SFC method developed in section 3.1.3.
175 The results are expressed in terms of the area of the peak of chlorophyll *a*
176 normalized by taking into account the final collected volume.

177 The volumes collected at the end of each extraction being different, it was
178 necessary to find a way to be able to compare these areas with each other. A simple
179 way is to evaporate the sample dissolve it again to a specific volume. With the mass
180 of dry extract and the volume, it is possible to use the concentration. However, since
181 chlorophylls are very sensitive to evaporation (possible degradation), another way
182 was implemented. The final volume of each extract was measured, and then
183 normalization by volumes was carried out. For example, if a normalized volume of 15
184 mL is selected, the area of an extract of 60 mL is then multiplied by 60 (volume of the
185 extract) and divided by 15 (reference volume).

186

187

188 2.4 Retention Model

189

190 To establish the model $\log k = f(\text{solvent percentage})$, a HSS C18 column (5 μm ,
191 4.6 x 150 mm) was used (Waters, Guyancourt, France). The flow rate was 1.5
192 mL/min, the outlet pressure set at 10 MPa, the oven temperature at 25°C, and UV
193 detection monitored at 650 nm.

194 Retention factors (k) were calculated using the following equation.

195
$$k = \frac{t_r - t_0}{t_0}$$

196 with retention times (t_r) and dead time (t_0). The dead time was measured at
197 each analytical condition, because it could vary with the compressibility of carbon
198 dioxide.

199 The latter can change depending on the inlet pressure of the column, which is
200 related, at constant flow rate, to the mobile phase viscosity, i.e. to the modifier (co-
201 solvent) percentage and nature [17,18].

202 An Ivy extract was used to examine the retention variation of chlorophyll *a* and
203 *b*. The characterisation of this extract was carried out with a Nexera UC On-line SFE-
204 SFC System with Mass-spectrometer in a previous study [12].

205

206 2.5 Correlation with Hansen solubility model

207

208 In order to correlate our retention model with Hansen solubility parameters,
209 other experiments were done with an HPLC instrument LC20-Series (two LC-20AD
210 pumps; autosampler SIL-20AC; degassing unit DGU-20A; Column oven CTO-20AC;
211 Diode array detector SPD-M20A) from Shimadzu (Kyoto, Japan) with the same flow
212 rate, temperature and detection conditions as the SFC experiments.

213 XLSTAT 2018.1.1 software (Addinsoft, Paris, France) was used for data
214 analysis.

215

216

217

218 3 Results and discussion

219

220 In this study, two different analytical SFC methods were used. Chlorophyll
221 retention to serve for extraction models was monitored using conditions summarized
222 in section 2.2. For this purpose, an isocratic SFC method using a C18 stationary
223 phase allowed the analysis of chlorophylls from ivy extracts. However, for other
224 compounds with different polarities, this method was limited. Another method was
225 needed to analyse chlorophylls together with carnosic and rosmarinic acid in
226 rosemary extracts (details in section 2.4). The following part presents the
227 development of this second method.

228

229 3.1 Analytical method development for quantification

230

231 The objective was to develop an analytical method to separate the phenolic
232 acids of interest in rosemary: carnosic and rosmarinic acid, as well as chlorophylls *a*
233 and *b*. During the development of an analytical method, many parameters influence
234 the separation quality. The use of carbon dioxide in sub- or supercritical conditions
235 makes the development of such a method even more complex. However, even if this
236 can be seen as an additional difficulty, it also allows us to have more parameters to
237 play on in order to separate the compounds and thus refine the method [19]. A diode
238 array UV-visible detector was used for selective detection, and different wavelengths
239 were selected depending on the absorbance of the compounds analysed: carnosic
240 acid: 221 nm, rosmarinic acid: 332 nm, chlorophylls: 650 nm.

241

242

243 One of the major parameters influencing SFC separations is the stationary
244 phase. In order to find the most suitable, a so-called "initial" analysis method was
245 used (described in section 2.2). Unlike RPLC or NPLC, chromatography in
246 supercritical phase allows a wide choice of columns to be used, in particular with
247 different polarity. Different columns were selected (section 2.2).

248 From the first column screening, the polar Torus DEA column was rejected
249 because chlorophylls eluted in the dead volume. On another hand, the Torus 2-PIC
250 column was unable to retain the two acidic compounds (rosmarinic and carnosic
251 acids), which may be surprising for a basic ligand. As expected, the HSS C18 column
252 was not able to retain rosmarinic acid but provided a satisfactory separation between
253 carnosic acid and chlorophylls. Finally, the Torus DIOL column was selected because
254 all compounds were sufficiently retained. In order to save time, the method was
255 shortened by programming the elution with Gradient 2 (described in section 2.2). It
256 was then possible to analyse all target compounds within 10 minutes.

257

258 Despite this change in the mobile phase gradient, the chromatogram obtained
259 with the Torus DIOL column showed broad peak shape for rosmarinic acid. The
260 mobile phase composition was varied to improve peak efficiency. Different additives
261 such as water or acid were added to the modifier in order to improve the peak shape
262 of rosmarinic acid. Finally, citric acid was selected, a polyacid which was reported in
263 other SFC methods [20].

264 The first noticeable thing is the change in retention time between the different
265 modifiers (Figure S1a). Rosmarinic acid is eluted faster using methanol with water
266 than methanol alone, and even faster using methanol with acidified water. The
267 addition of 5% of water in the modifier slightly decreases the retention of rosmarinic
268 acid.

269 Adding citric acid to water induced both a greater decrease in retention time of
270 the acidic compound and a strong peak width decrease. The modification of the
271 ionization state of rosmarinic acid (from anionic to neutral), and by consequence, its
272 change in solubility into the mobile phase can explain this change of retention.
273 Besides, as the column ligands carry hydroxyl groups, the addition of an acid in the
274 mobile phase could also change the apparent chemical nature of stationary phase
275 [21–23]. Citric acid may adsorb onto the polar groups, which will affect the nature of
276 the interactions between the analyte and the stationary phase as in HILIC mode [24–
277 26]. The adsorption of citric acid may repulse other acidic compounds from the
278 stationary phase and thus, reduce interactions between analytes and stationary
279 phase.

280 Despite the best improvement of peak shape of rosmarinic acid obtained with
281 citric acid, the use of this acid leads to visible degradation of the chlorophylls which
282 are therefore no longer detected (Figure S1b). Chlorophyll compounds are sensitive
283 to pH changes and degrade into other forms such as pheophytin (magnesium-free
284 chlorophyll).

285 Consequently, as water also refined the rosmarinic acid peak, the modifier
286 methanol /water 95/5 v/v was selected for the rest of the development. Thanks to
287 previous optimization steps, a method allowing the analysis of carnosic, rosmarinic
288 acid and chlorophyll *a* and *b* in less than 10 minutes was developed on the Torus
289 DIOL stationary phase. Figure S2 shows chromatograms obtained with the new
290 optimized conditions.

291 However, chlorophylls also absorb at 221 nm, the wavelength which is used to
292 study carnosic acid. Because it elutes close to the chlorophylls, the peak integration
293 could be biased. For this purpose, another approach with coupled columns was
294 developed.

295

296 From the previous column screening, the HSS C18 column seemed to be
297 suitable for carnosic acid and chlorophyll separation. However, a reversal of elution
298 order between chlorophyll *a* and *b* occurred, compared to the one obtained with the
299 Torus DIOL stationary phase. Chlorophyll *b* being more polar than chlorophyll *a*, it
300 was more retained on the polar diol stationary phase and less retained on the non-
301 polar C18 stationary phase. Whatever this drawback, the HSS C18 column was
302 coupled with the Torus DIOL column in order to combine the different effects.
303 Besides, due to the low viscosity of the mobile phase used in SFC, the column
304 coupling does not induce a great pressure drop increase, around 3 MPa more.

305 However, when coupling column with an elution gradient, placing the most
306 retentive column first is not advised, because the compounds leave the first column
307 and enter the second one with a high modifier amount. Therefore, the most retained
308 analytes in the first column will have very few interactions with the stationary phase of
309 the second column.

310 For these reasons, the first column was HSS C18 and the second was Torus
311 DIOL. As expected, this coupling made it possible to increase the difference in

312 retention times between chlorophylls and carnosic acid of about one minute (fig. 1).
313 Moreover, the separation of carnosic acid from impurities was enhanced. The
314 chlorophylls were well separated, with chlorophyll *b* eluting first, showing for these
315 two pigments the predominance of HSS C18 phase on their selectivity. To conclude,
316 the coupled columns are used with the elution conditions developed previously in
317 order to quantify carnosic acid, rosmarinic acid and chlorophylls *a* and *b* from extracts
318 obtained with SFE or PFE.

319

320 3.2 Solubility predictive model

321

322 To achieve a solubility predictive model, we monitored the retention of
323 chlorophyll *a* in different analytical SFC and LC conditions. Plotting the logarithm of
324 the retention factor according to the nature and the percentage of modifier is usually
325 used to improve the understanding of the retention of one compound compared to
326 another in order to develop an analytical method [12,27,28]. Another interest, related
327 to extraction field, is to study the solubility of an analyte in a fluid that is either a
328 mobile phase or an extraction phase. Consequently, specific extraction conditions,
329 extrapolated from the retention curves, may be applied to extraction techniques such
330 as supercritical fluid extraction (SFE) for CO₂/modifier eluents, or pressurized fluid
331 extraction (PFE) for neat solvent eluents.

332 Chlorophyll retention curves were obtained in SFC and LC, on a C18-bonded
333 phase. Chlorophylls have high log *P* values (superior to 8) [29]. Thus a non-polar C18
334 stationary phase is able to provide some retention for such compounds. In addition,
335 only limited changes in the stationary phase properties due to adsorption of the
336 mobile phase components are expected. On the opposite, a polar stationary phase
337 would not provide satisfactory retention of the non-polar chlorophylls, and the strong
338 mobile phase adsorption on the polar stationary phase surface when changing the
339 modifier percentage would induce some retention modification that would not be
340 solely related to changes in the analyte solubility.

341 Three solvents were selected in this study: acetonitrile, ethanol, and methanol.
342 Acetonitrile and methanol are quite different. Their log *P* may look similar (log
343 $P_{\text{acetonitrile}}=-0.34$ [30] Log $P_{\text{methanol}}=-0.77$ [31]), but acetonitrile is a non-protic solvent,

344 which can interact with the analytes through dipole-dipole interactions, whereas
345 methanol is protic and interacts through hydrogen bonding interactions, which leads
346 to different chromatographic behaviours. Ethanol, parent of methanol, is quite close
347 in terms of its polarity ($\text{Log } P_{\text{ethanol}} = -0.31$ [32]) but has an additional methylene group.
348 In the field of plant extraction, this solvent is widely used due to its polarity, its origin
349 (from agricultural resources), its biodegradability, its low toxicity, its moderate cost,
350 and favourable regulations allowing its use in regulated fields such as cosmetics.

351 An ivy extract used as chlorophyll standard (preparation method described in
352 section 2.3) was therefore injected on the HSS C18 column in SFC and LC with
353 different percentages of solvent in CO_2 , ranging from 5 to 100%. The other
354 parameters such as temperature, flow-rate and back-pressure are described in
355 section 2.2. The results are presented in Figure 2. Only the plots for chlorophyll *a* are
356 presented because the plots for chlorophyll *b* had a very similar shape [12]. For
357 acetonitrile, between 5 and 20%, retention was too high and was thus not measured.

358 The first noticeable point is the curve shape. Conventionally in RP-HPLC, when
359 using C18-bonded stationary phase, the retention is expressed with the logarithm of
360 the retention factor in order to have a straight line with a negative slope as a function
361 of the percentage of organic solvent. In Figure 3, two areas are identifiable. The first
362 one is a decrease in retention until about 40% for acetonitrile and methanol, and 50%
363 for ethanol. Because carbon dioxide is considered as a nonpolar solvent similar to
364 pentane, this decrease was fairly predictable. The more modifier there is, the more
365 the polarity of the mobile phase increases, approaching the polarity of chlorophyll *a*,
366 which favours the analyte solubility in the mobile phase. However, beyond these
367 percentages, the retention increases showing that the polarity of the mobile phase
368 continues to increase above the polarity of the analyte. This behavior was described
369 before with carotenoids [33] and triacylglycerols [19] on C18 stationary phase in SFC.

370 The shape of this curve also has other consequences. It shows that it is
371 possible to have equal $\log k$ values at different percentages of modifier. Identical $\log k$
372 k indicates conditions of iso-solubility. For example, similar $\log k$ values are
373 measured for the methanol curve around 20-30% and 50-60%. A similar trend is
374 observed with acetonitrile. For ethanol, we observe equal $\log k$ values between 40-
375 50% and 80%. These conditions applied to extraction could then yield similar results
376 taking into account this property of iso-solubility.

377 From these curves, it is possible to identify different zones which may be useful
378 in the extraction or non-extraction of chlorophyll, on the basis of the compound
379 solubility expected from the retention models. At low percentage of modifier,
380 chlorophyll *a* has a high retention which means low solubility in the mobile phase.
381 For example, it is the case with CO₂/EtOH 95/5 v/v. This fluid composition can
382 therefore be used in SFE in order to obtain extracts with low chlorophyll content.

383 Conversely, the minimum retention represents a high solubility in the mobile
384 phase. Lowest retention was obtained between 30-50% MeOH or 50-80% EtOH.
385 Similar compositions (i.e 30% and 70% modifier) can be applied in SFE in order to
386 obtain chlorophyll-rich extracts. The retention curve obtained with acetonitrile is
387 interpretable in the same manner, but because this curve is located higher than the
388 methanol and ethanol ones, it indicates lower solubility of chlorophylls in these
389 CO₂/acetonitrile mobile phases.

390 A final area is observed with 100% modifier, thus in near-HPLC conditions. The
391 only difference is the back-pressure of 10 MPa applied to the SFC system. As a
392 result, this specific point in the curve can be related to pressurised liquid extraction
393 (PLE).

394 We can see that at 100% modifier, acetonitrile is not a suited solvent for the
395 extraction of chlorophylls, whereas pure methanol is more efficient, and pure ethanol
396 should solubilise chlorophylls well.

397 In order to validate the expected compound solubility from this chromatographic
398 model, different extractions were done with these three solvents. But first, we
399 attempted to correlate this retention models with the Hansen solubility prediction
400 model.

401

402 3.3 Correlation with HANSEN solubility prediction model

403

404 Varied solubility models exist in order to theoretically determine if compounds
405 are soluble in a given environment, based on chemical interactions. From these
406 varied models [34], the Hansen solubility parameters (HSP), derived from the
407 Hildebrand solubility parameters, are widely used in plant extraction [35]. This model

408 uses three different parameters to describe the different interactions that can take
409 place between the compounds and the extraction solvent: δD for dispersion (van der
410 Waals forces), δP for polarity (related to dipole moment) and δH for hydrogen
411 bonding. Based on these parameters it is possible to organize all chemicals into a 3-
412 dimensions space (δP ; δD ; δH). Mixtures of liquid solvents can also have their own
413 HSP. It is simply calculated using the HSP values of the different components, and
414 then pondered according to composition. Equation (1) was used to calculate HSP
415 values for different solvent compositions:

$$416 \quad \delta_{ab} = \frac{(\delta_a * \%a + \delta_b * \%b)}{\%tot} \quad (1)$$

417 With δ_{ab} the HSP of the mixture, δ_a and δ_b the HSP values of solvent a and
418 solvent b respectively, $\%a$ and $\%b$ the percentages of solvent a and solvent b.

419 From these parameters, solubility can be determined by calculating a distance
420 (R_a) between a solvent (neat or mixed) and the compound to solubilize (here
421 chlorophyll a). This distance is calculated using equation (2):

$$422 \quad R_a = \sqrt{4(\delta D_2 - \delta D_1)^2 + (\delta P_2 - \delta P_1)^2 + (\delta H_2 - \delta H_1)^2} \quad (2)$$

423 With δD , δP and δH the HSP for component number 1 (solvent) and 2 (analyte).
424 Considering that the lowest distance should reflect the highest compatibility between
425 the solvent and analyte, and *vice versa*, it is then possible to predict the compound
426 solubility.

427 In this study, we carried out a statistical study in order to correlate the solubility
428 parameter of solvents (for the three solvents previously used as modifier in SFC
429 model ACN, MeOH and EtOH and their mixtures at different compositions) to the log
430 k values of chlorophyll a measured in SFC and LC conditions. Moreover, to improve
431 the relevance of this study, two more solvents (2-methyltetrahydrofuran and
432 propylene carbonate) were added in the study. These solvents, recognized as green
433 solvents, were chosen to extend the HSP range of tested solvents (i.e. lower δP with
434 2-methyltetrahydrofuran and higher δD with propylene carbonate). By including these
435 solvents, the population of three Hansen parameters is well scattered, improving the
436 applicability domain of the model. The values of log $k_{\text{chlorophyll a}}$ for different solvent
437 compositions were measured by HPLC and retention curves are presented in Figure
438 3. For the three solvents previously studied (MeOH, EtOH and ACN), the retention

439 with pure solvent is close to the ones obtained with 100% modifier in SFC. The
440 curves for the mixtures between these solvents evolve quite linearly. For the two
441 other solvents (Propylene carbonate - PC and 2-methyltetrahydrofuran – MeTHF),
442 mixtures with PC show high retention with high PC content. It is even impossible to
443 elute chlorophyll *a* with PC percentage above 80%. For MeTHF, the contrary is
444 observed as this solvent yields the lowest retention of all the solvents tested.

445 In addition, different mixtures of methanol and acetonitrile (5/95; 10/90; 20/80
446 and 50/50) as modifier were used in SFC to extend HSP range.

447 Population distribution is plotted in supplementary figures S3 and S4
448 (respectively for liquid-liquid and liquid-CO₂ mixtures) with δP in function of δH . δD
449 was not plotted because this parameter was less dispersed than the others, varying
450 only between 15.1 and 19.1, while δP varied between 4.8 and 18, and δH varied
451 between 4.6 and 22.3. Due to very high retention, some curves are not complete,
452 mainly when using propylene carbonate (PC). All the HSP for the different
453 composition are summarized in supplementary data. Data of calculated solubility
454 parameters for liquid mixtures are summarized in Tables S5-S11 and for liquid-CO₂
455 mixtures in Tables S12-S18.

456 A multilinear regression was computed using δP , δD , δH as independent
457 variables and $\log k$ values as dependent variable.

458 In the first place, only data using liquid mixtures are studied.

459 The regression on liquid mixtures yielded equation (3) with $R^2=0.905$:

$$460 \quad \log k \text{ chlorophyll } a = -4.02 + 0.13 * \delta D + 0.17 * \delta P + 0.03 * \delta H \quad (3)$$

461 The high regression coefficient demonstrates that $\log k$ values obtained
462 chromatographically can be correlated with theoretical solubility parameters, in this
463 case, HSP. This result first shows that the calculation of the solubility parameters of
464 liquid mixtures in proportion of the volume fraction seems relevant, and secondly, that
465 the retention of chlorophyll *a* should be predictable from the HSP values. In our point
466 of view, this validates the use of chromatographic retention to study the solubility
467 changes of this compound in liquids. A linear regression between experimental and
468 calculated values of $\log k$ is represented in supplementary material Figure S19.

469 Besides, from the coefficient values of the three solubility parameters, it is
470 difficult to deeply understand which interactions, dispersion, dipole-dipole or
471 hydrogen bonding play a preponderant role in the retention changes, i.e. in the
472 solubility changes. However, the dispersion and dipole-dipole interactions, which
473 have the highest coefficient values in Eq. (3), seem mainly involved in the retention
474 changes of chlorophyll *a*.

475 Based on the chromatographic retention model in Eq. (3), solvents with high δP
476 and δD would induce a poor solubility of chlorophyll *a*. It is the case for PC ($\delta P = 18$,
477 $\delta D = 20$) which shows poor solubility, so high retention in chromatographic model.

478 The contrary is observed with MeTHF ($\delta P = 4.8$; $\delta D = 16.8$): low retention
479 indicating high solubility.

480 The same data processing has been applied on CO₂-solvent data (log *k* values
481 measured in SFC conditions). However, these parameters depend on the fluid
482 density, they can change in function of pressure and temperature [36]. Due to the
483 complexity of the determination of its HSP, CO₂ HSP were selected for the closest
484 condition possible (40°C and 100 bar) [37].

485 Figure 4 shows the change in the R² regression coefficient related to the
486 calculation of multilinear regression starting with the data of neat liquids (Eq. (3), on
487 the right of the figure) and including progressively the data obtained with increasing
488 CO₂ percentage in the mobile phase, up to 95% CO₂ (5% of modifier, on the left of
489 the figure). For instance, it means that to obtain the R² value for the point 90% of
490 liquid, we added to the numerous values of retention obtained for all the pure liquids
491 studied (data of Figure 3), the retention values of the mobile phases composed by
492 the mixtures 90/10 solvent/carbon dioxide. Then, each following point of fig. 4 is
493 obtained by adding to the previous retention data at each CO₂/modifier percentage,
494 the retention values at the studied mixtures. Figure 4 shows that reasonably good
495 correlation coefficients are obtained for modifier percentage above 30% (R² > 0.8).
496 However, the correlation coefficient decreases dramatically for the lowest amounts of
497 modifier, from 30 to 5%

498 This result highlights the difficulty to calculate the HSP values for mixtures with
499 lower amounts of modifier. This difficulty is either related to the higher compressibility
500 of such fluids, or/and to the solubility changes in this area, which may be due to the

501 formation of solvent clusters around the analyte. It was shown in supercritical fluid
502 chromatography, that with low modifier percentages in carbon dioxide, a solvating
503 sphere of modifier can surround the compounds. Thus in the immediate vicinity of the
504 analyte (called the cybotactic region), the concentration of modifier is higher than in
505 the bulk of the fluid [19]. Obviously, this must have a strong effect on the real
506 solubility of the compounds, but this local environment is not easy to predict and
507 describe with macromolecular data, such as the HSP, or any other type or polarity
508 parameters.

509 Figure 5 shows the changes in the coefficient values for the three computed
510 Hansen parameters when the proportions of CO₂ and solvents are varied. All these
511 coefficients decrease when adding carbon dioxide to the modifier.

512 This was expected from the respective values of the HSP parameters of
513 acetonitrile, methanol, ethanol and carbon dioxide. The dispersion coefficient δD
514 decreases rapidly upon addition of CO₂-solvent data, as the decrease is observed
515 from 80% of liquid downwards. The other two are rather constant up to 40 % of liquid
516 and decrease only when the proportion of solvent is further reduced. This confirms
517 the apparent changes in the compound solubility model below 40% of liquid mixed to
518 carbon dioxide.

519 For the lowest modifier percentages, i.e. the highest carbon dioxide ones, the
520 main Hansen parameter acting on retention/solubility is the dipole-dipole parameter.
521 However, we have to keep in mind the very low regression coefficients obtained for
522 these mixtures, suggesting limited interpretability of these models, which moderates
523 this statement.

524 Nevertheless, almost of these results indicate reasonably good correlation
525 between the chromatographic retention (measured from reverse phase supercritical
526 and liquid chromatography) and the calculated HSP values either for liquid mixtures,
527 or for rich liquid/carbon dioxide ones. It is then possible to predict solubility by
528 choosing the right HSP: high δP and δD values should yield poor solubility, and *vice*
529 *versa*.

530 Further developments could be done in the future, to back-calculate the
531 accurate HSP values for low liquid content in carbon dioxide, based on retention
532 values, and including more analytes with varied chemical structures.

533 4 Extraction

534

535

536 First, we can notice the small amount of chlorophyll *a* extracted with acetonitrile,
537 compared to methanol and ethanol extractions (fig. 6). According to the retention
538 model (fig. 3), this solvent (mixed to carbon dioxide or used as pure liquid) yielded
539 high chromatographic retention of chlorophyll *a*, and this is related to the low amount
540 of chlorophyll extracted, whether in SFE or in PLE.

541 Between SFE points at 5, 30, and 70%, the worst extraction conditions are
542 obtained at 5% modifier in CO₂, regardless of the modifier used. These experimental
543 values of extracted amounts are in accordance with log *k* curves in Figure 2. Indeed,
544 log *k* at 5% was the highest retention point for each solvent (no measurement was
545 possible with acetonitrile due to very high retention of chlorophylls). Conversely, SFE
546 experiments using 30% and 70% modifier extracted a higher chlorophyll content for
547 all modifiers. However, between these two points, the content extracted at 70%
548 modifier is higher than the chlorophyll content extracted at 30% modifier for ACN,
549 MeOH and slightly higher for EtOH. These results do not follow the retention model
550 for acetonitrile and methanol (Figure 2) since the measured chromatographic
551 retention at 70% modifier was higher than the one at 30%. Differences at
552 intermediate points could come from a lack of accuracy of the model. This may be
553 because the model uses pure analyte molecules and then cannot predict all the
554 intraparticle diffusional resistances (for the dried plant particles), which may occur
555 into organelles (especially chloroplast) of plant cells. It could be some accessibility
556 phenomena for chlorophyll compounds stored in chloroplast and the use of 70% of
557 modifier would still be better to reach and solubilise them [38].

558 Besides, all PLE extracts all show lower content of extracted chlorophyll
559 compared to SFE at both 30% and 70% for all three solvents. In that case, and
560 whatever the intraparticle diffusivity, these results are in accordance with the
561 retention model.

562 However, a difference is observed between the retention model and extraction
563 content for methanol and ethanol. For percentages above 20% of modifier, log k_{ethanol}
564 was lower than log k_{methanol} . This result, when related to the compound solubility,

565 would mean that chlorophyll content would be lower for extractions with methanol
566 than ethanol at 30, 70 and 100% of modifier but the contrary was observed.
567 However, the retention model does not take into account the matrix effect, i.e. the
568 ability to the fluid to penetrate the matrix. Figure 7 shows the inlet pressure changes
569 from the chromatographic measurements. For acetonitrile and methanol, the increase
570 in pressure is regular and almost linear whereas for ethanol, inlet pressure strongly
571 increases to reach very high values. Obviously, because the inlet pressure is related
572 to the fluid viscosity, it shows the dramatic increase of viscosity with the addition of
573 ethanol, and consequently, the decrease of both the diffusivity of the fluid, meaning a
574 probable limitation of the fluid penetration in the dried plant, and of the diffusion
575 coefficient of analytes, that also could limit the recovery of compounds when viscous
576 solvent is mixed to carbon dioxide. Such behaviour, a decrease in the diffusion
577 coefficient reducing the penetration capacity of the solvent and consequently the
578 diminution in the extraction yield have been reported for chlorophyll in microalga
579 [39,40].

580 However, these diffusivity changes related to the viscosity variations do not
581 explain the increased extraction recovery of chlorophylls between 30 and 70% of
582 modifier that are observed whatever the modifier, acetonitrile, methanol or ethanol.
583 An additional phenomenon should take place, which is neither related to the modifier
584 nature, nor to the viscosity of the extraction solvent.

585 To conclude, the extraction experiments allowed us to correlate them with
586 predicted conditions obtained by chromatographic technique. Nevertheless, even if
587 most of the extraction results could be correlated to chromatographic observations,
588 this study demonstrated the complexity of plant extraction and all the matrix effects
589 that could impact the extraction. It is especially the case for conditions with low
590 retention meaning high extraction capacity.

591 After focusing on model validation for chlorophyll extraction, the study was
592 focused on the extraction of bioactive compounds of rosemary, in function of different
593 conditions tested previously. The results are shown in Figure 8. For any of the
594 selective conditions for chlorophyll extraction (70% of modifier) or non-extraction (5%
595 of modifier), carnolic acid was extracted. For the most selective SFE conditions, for
596 instance low percentage of any modifier or with acetonitrile, the amounts of extracted
597 carnolic acid were lower with regards to extraction conditions using higher modifier

598 contents and/or alcohol modifiers. With SFE, extraction of carnosic acid was favoured
599 by the use of ethanol compared to acetonitrile and methanol. However, in PLE
600 extraction, the amount of extracted carnosic acid was similar with the three solvents.
601 For rosmarinic acid, which is more polar compared to carnosic acid, the extraction
602 selectivity was much higher. With the lowest modifier percentage up to 30%, no or
603 small amounts of this compound were extracted. High methanol or ethanol
604 proportions were required to achieve satisfactory extraction of rosmarinic acid.
605 Whatever the ethanol amount in SFE and the extraction method (SFE and PLE), the
606 amounts of rosmarinic acid extracted with ethanol remained lower than with
607 methanol, which is in accordance with the higher polarity of methanol. Finally, the
608 yield obtained for both acids from PLE remained below the best yields obtained from
609 SFE. In that case, one can claim that it could be due to the difference of the
610 extraction mode between PLE and SFE, as the first one was done in static
611 conditions, whereas the second one was done in dynamic conditions.

612 To conclude, it is possible to extract selectively carnosic acid without chlorophyll
613 with SFE using low modifier percentage or acetonitrile. However, due to the high
614 polarity of rosmarinic acid, its extraction requires the use of high modifier percentage
615 and polar solvent, also yielding the extraction of chlorophylls.

616

617 5. Conclusion

618 During this study, a SFC method was developed to predict extraction behaviour
619 of chlorophyll *a*, and to easily find selective extraction conditions between pigments
620 and bioactive compounds, rosmarinic and carnosic acids. This approach can be used
621 either for liquid extraction by PLE with neat organic solvent or by SFE with mixtures
622 of carbon dioxide and solvent in supercritical state.

623 Besides, correlation between the Hansen solubility parameters and the
624 retention for chlorophyll *a* was studied. Rather satisfactory correlations were reported
625 when using pure liquids or mixed CO₂/liquid mobile phases with modifier percentages
626 higher than 30%. For the lower modifier contents, the use of the HSP model seems
627 inadequate to describe the retention values, either due to erroneous calculation of
628 the HSP values based on the volume fractions, or to inhomogeneity of such mobile
629 phases, with significant clustering of the modifier around the analyte.

630 This retention model can be helpful to determine the fluid compositions allowing
631 to reach selective extractions. For instance, the solubility of chlorophyll *a* was
632 strongly reduced with acetonitrile, whether employed as neat solvent in PLE or mixed
633 to CO₂ in different proportions in SFE. Moreover, low modifier (5%) in carbon dioxide
634 reduced chlorophyll solubility with regards to intermediate values of modifier (30%).
635 However, we also observed, based on triplicate extractions with real samples, that
636 some matrix effects can induce different results to the ones predicted from the
637 chromatographic model, maybe due to different diffusivity of the solvents in the plant
638 matrix. Nonetheless, selective conditions to extract carnosic acid without chlorophyll
639 were identified thanks to this method (using 5% acetonitrile as modifier). However, it
640 was harder to obtain selective conditions for rosmarinic acid due to its higher polarity.
641 In order to extract it, higher modifier percentages or more polar solvents are required,
642 which are also extracting significant amounts of chlorophyll. In addition, it was
643 possible to extract most of the chlorophyll using 30% of ethanol as modifier in CO₂,
644 showing that SFE can be a good alternative for more ecological extraction requiring
645 low amounts of green solvents. The latter (CO₂ and ethanol) are compatible with
646 cosmetic regulations.

647

648 Finally, once different conditions are identified that can extract selectively the
649 studied compounds, one of the solutions could be to use these different conditions
650 successively on the same plant sample. Supercritical fluid extraction techniques use
651 a dynamic extraction system which means that extraction conditions can be changed
652 at any time during the extraction process. This will be investigated in the future.
653 However, this study also shown that the modelling from the chromatographic
654 behaviour, could not take into account the various matrix effects, i.e. the fluid
655 diffusivity into the complex samples, and the presence of other compounds in the
656 plant.

657

658

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660

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663

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804

805 Figure captions

806 Figure 1. SFC chromatograms of carnosic acid, rosmarinic acid and
807 chlorophylls with final gradient conditions (gradient 2) and the coupled column
808 system (HSS C18 and Torus DIOL).

809 Figure 2. Logarithm of retention factor of chlorophyll *a* vs modifier percentage.
810 Column: HSS C18, Flow rate: 1.5 mL/min; T = 25°C; P = 10 MPa.

811 Figure 3. Logarithm of retention factor of chlorophyll *a* vs different percentage of
812 solvent B for different mixtures noted A – B with the solvents identified on the figure.
813 Column: HSS C18, Flow rate: 1.5 mL/min; T = 25°C.

814 Figure 4. Variation of the regression coefficient R^2 from cumulative retention
815 values of chlorophyll *a* measured either with pure mobile phases (100%) or added
816 with values from mixed CO₂/liquid mobile phases.

817 Figure 5. Variations of the (a,b,c) HSP coefficients from the computed
818 multilinear regression $\log k = f(a\delta P + b\delta D + c\delta H)$. Each point was computed from
819 cumulative retention values of chlorophyll *a* measured with pure liquid mobile phases
820 (100%) or progressively added with values from mixed CO₂/liquid mobile phases (see
821 text for details).

822 Figure 6. Chlorophyll *a* content in rosemary extracts vs the extraction conditions
823 both for SFE (left side) and PFE (right side), with different solvent compositions of
824 extracting solvent.

825 Figure 7. Variation of the inlet pressure vs the modifier percentage in SFC
826 system with the HSS C18 column.

827 Figure 8: Peak areas of Carnosic acid (solid color) measured in SFC at 221 nm
828 and rosmarinic acid (white dots) measured at 332 nm in function of extraction
829 conditions for SFE (left side) and PFE (right side).

830

831

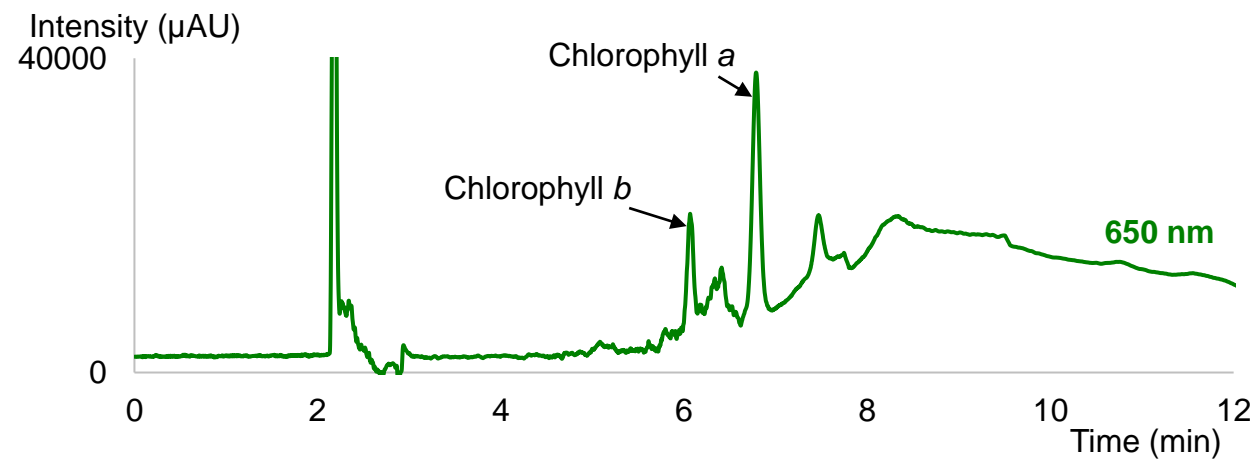
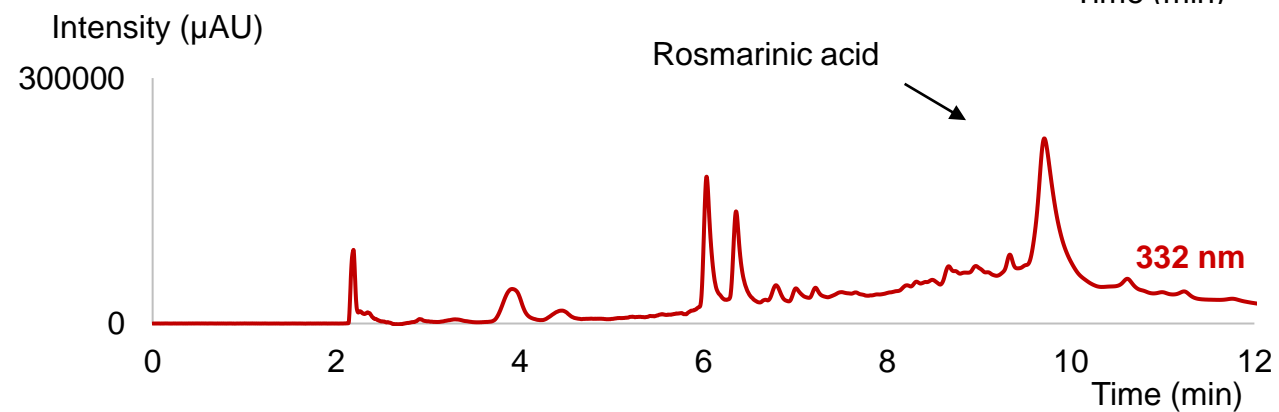
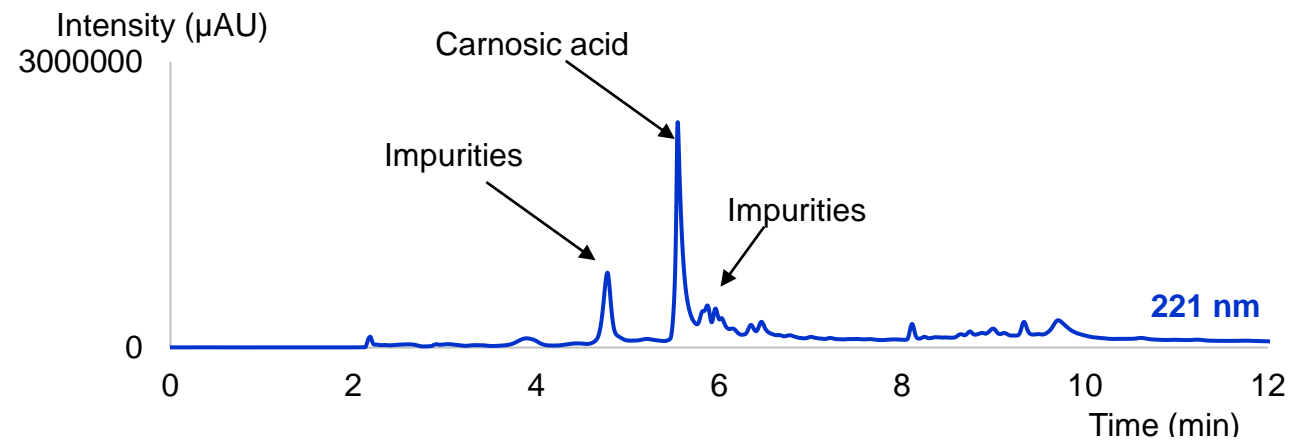


Figure 1

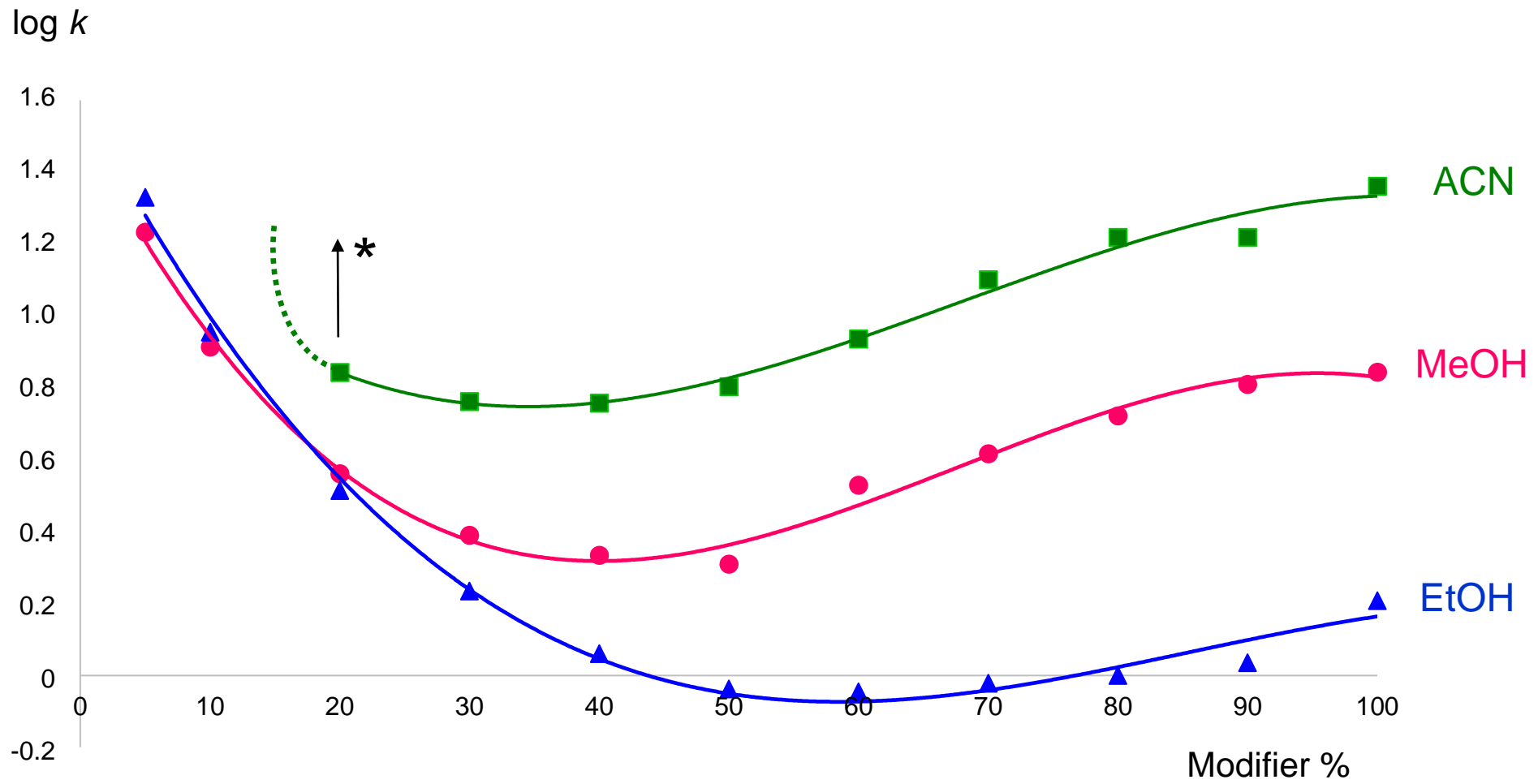


Figure 2

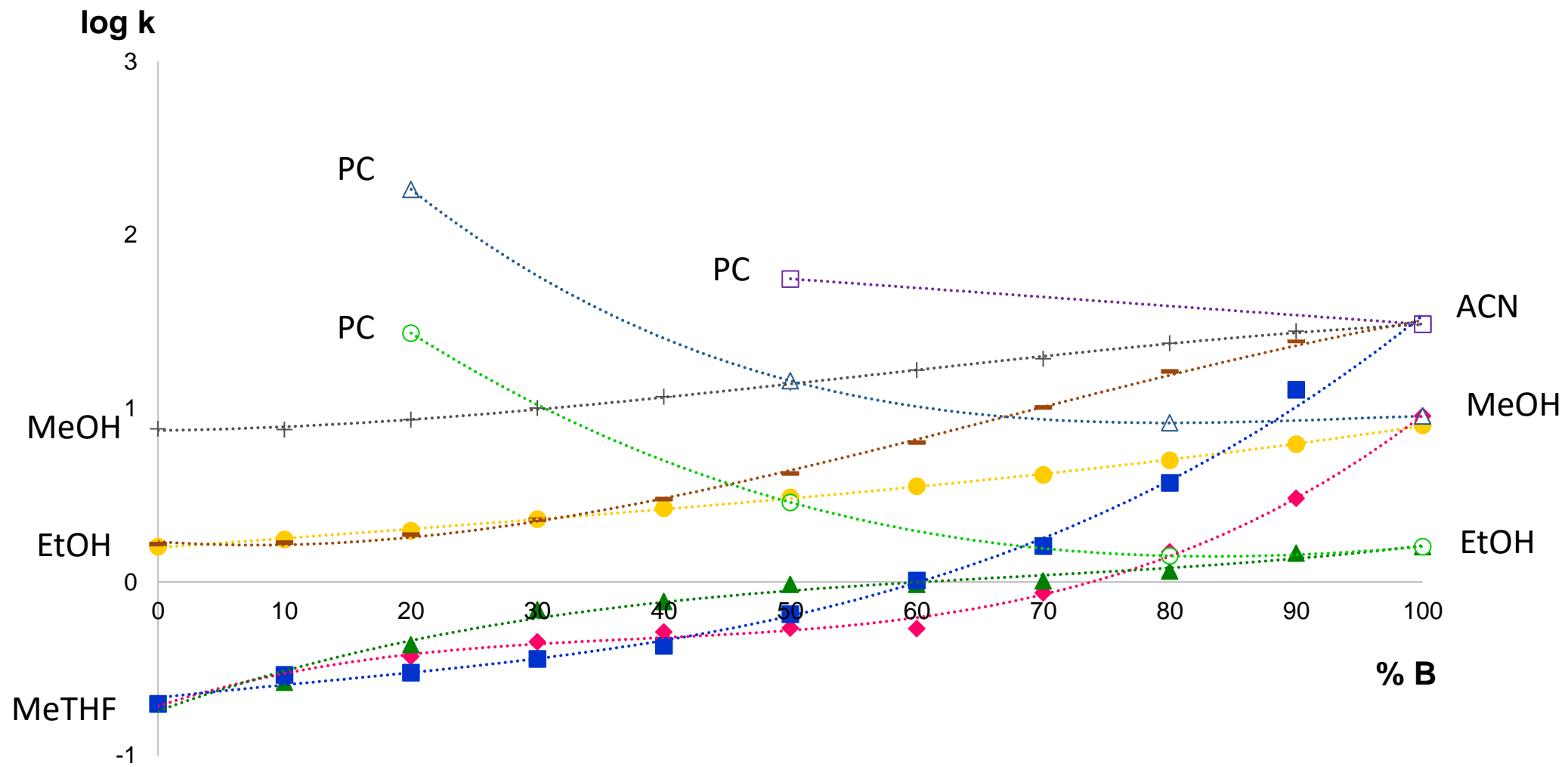


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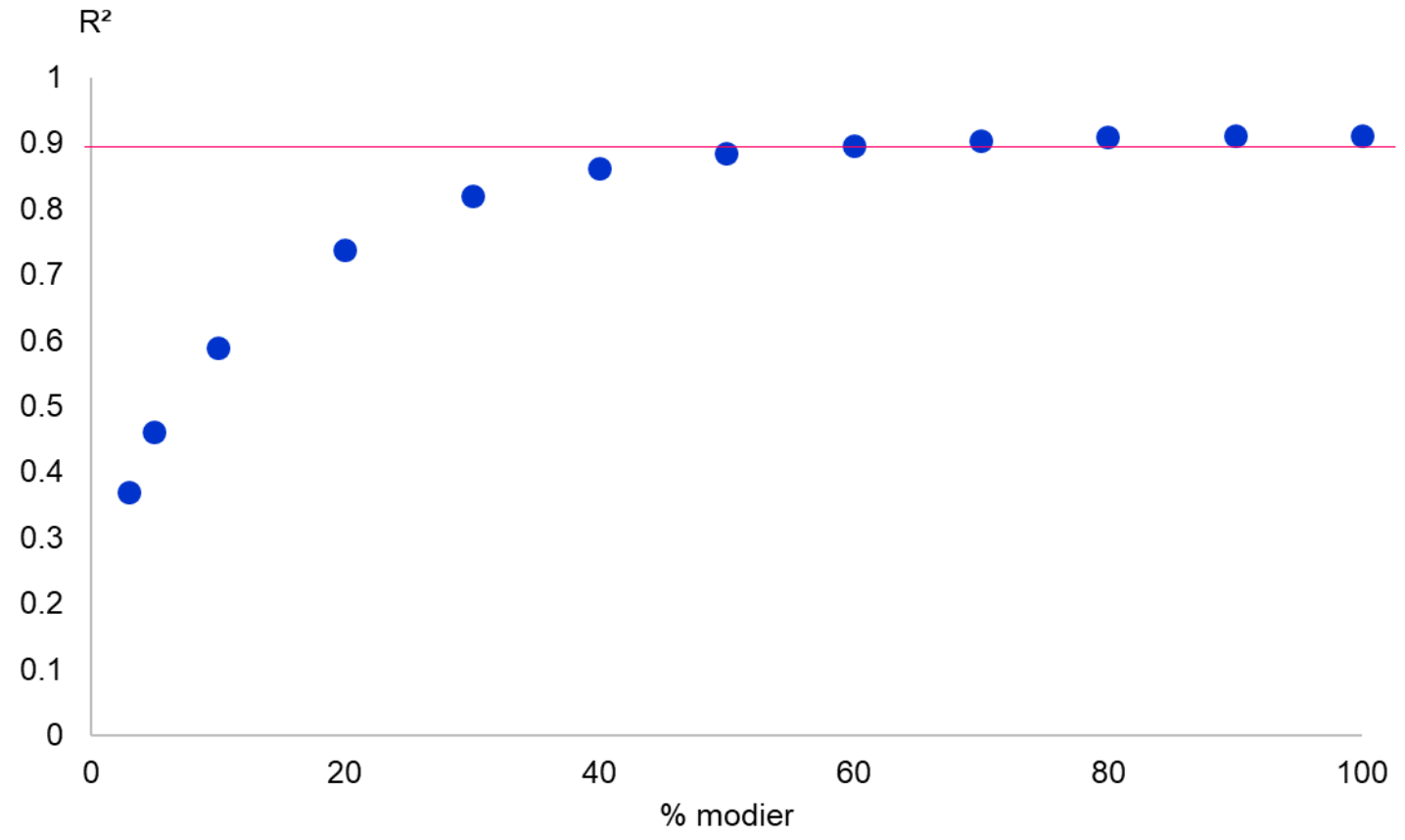


Figure 4

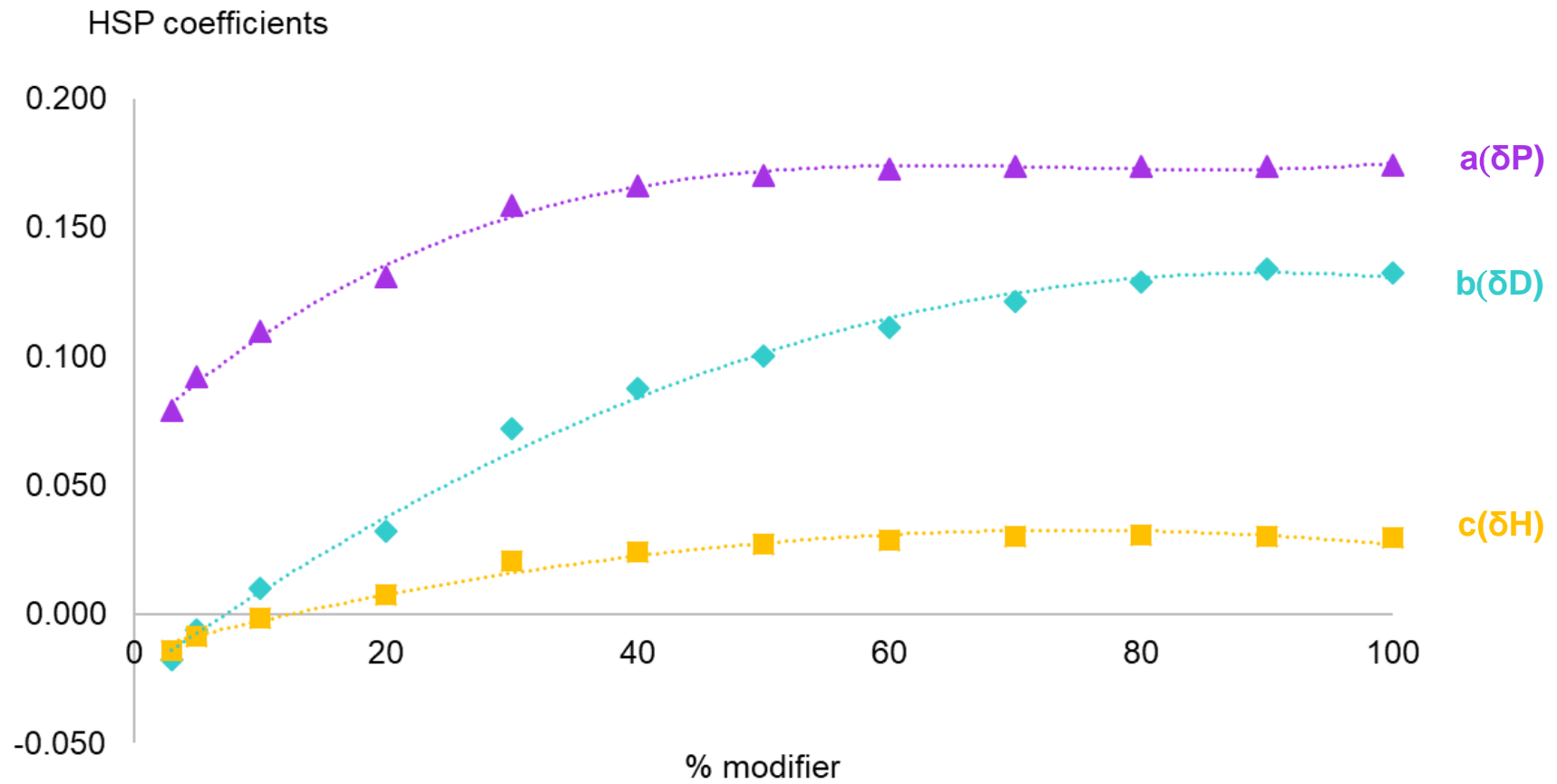


Figure 5

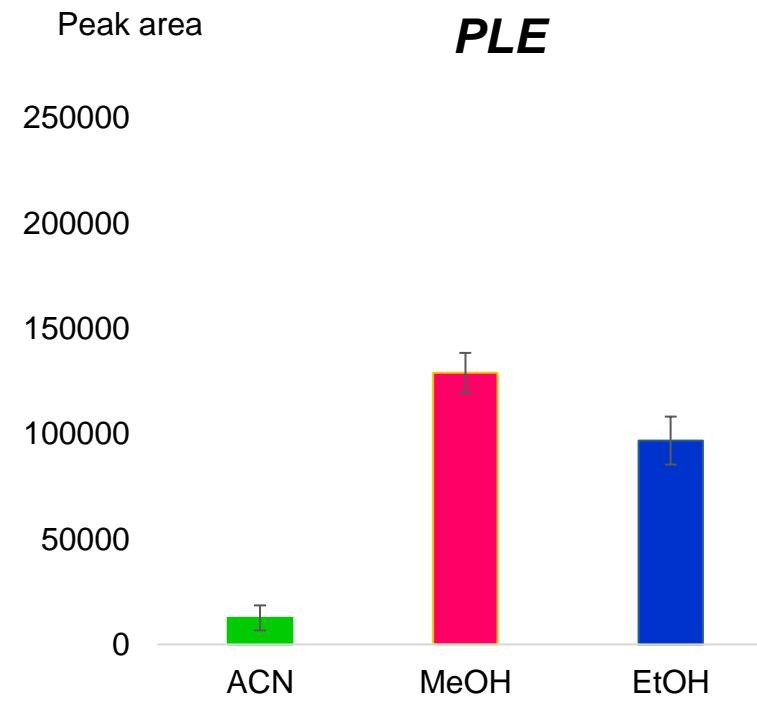
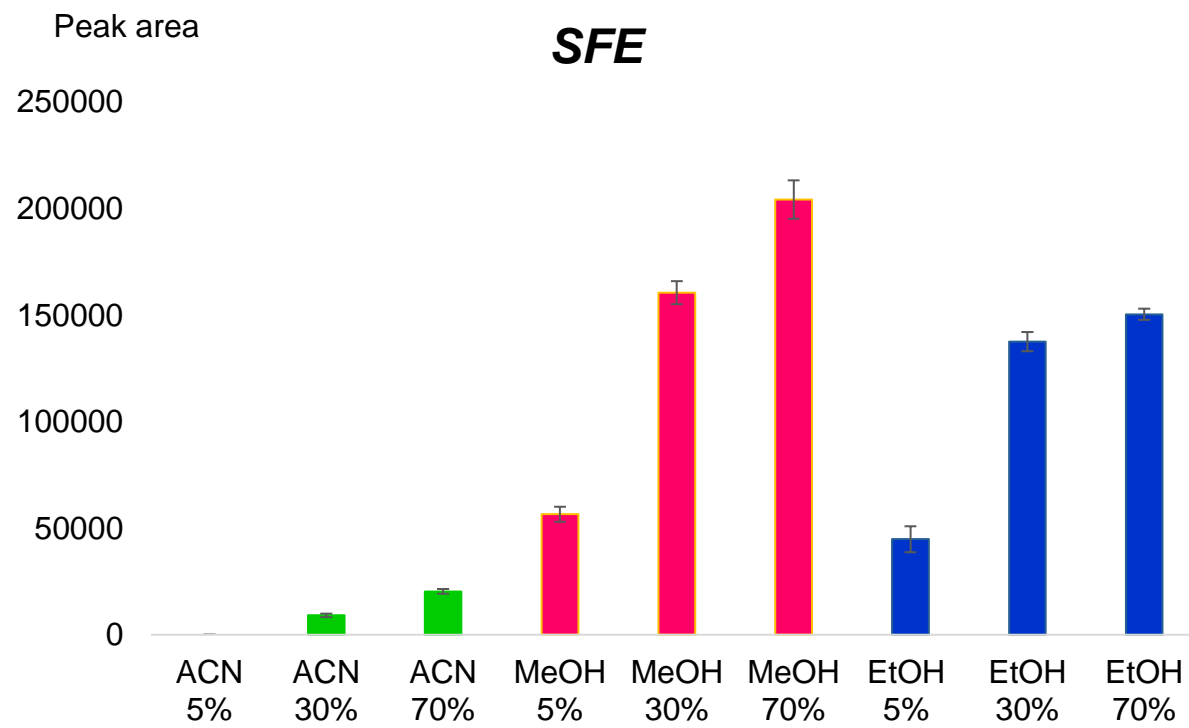


Figure 6

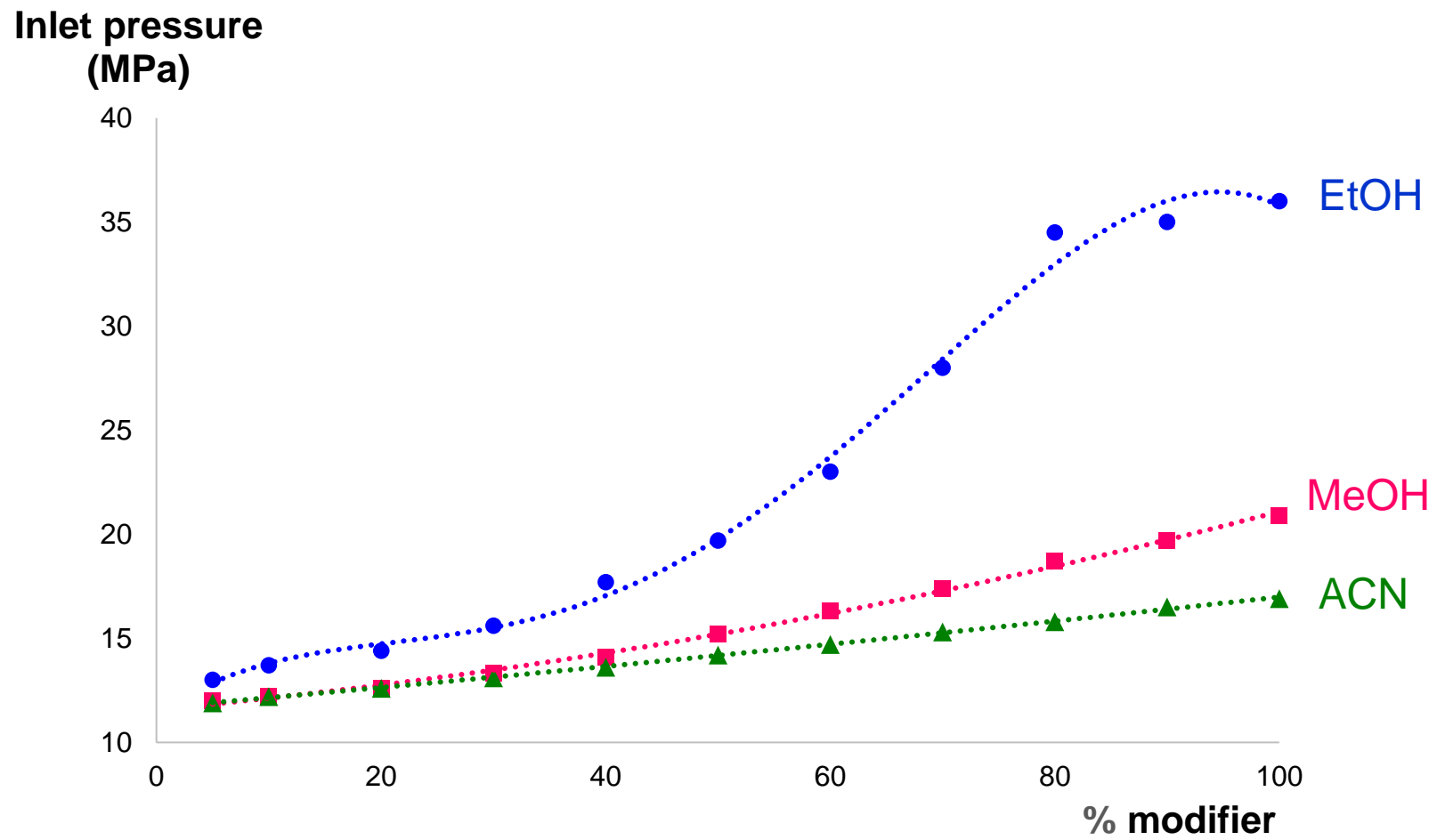


Figure 7

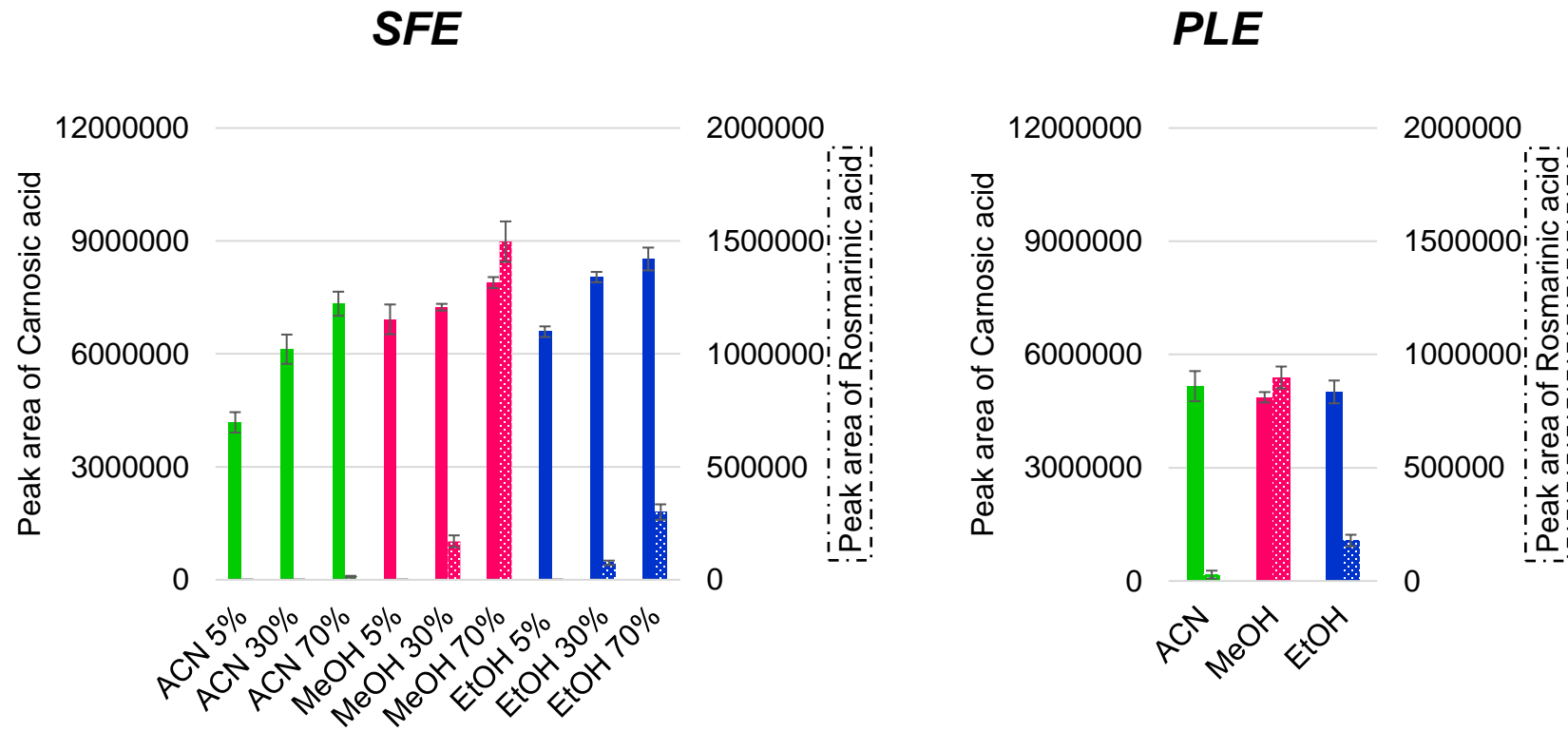


Figure 8