



**HAL**  
open science

# Development of an analytical method for chlorophyll pigments separation by reversed-phase supercritical fluid chromatography

Thibault Lefebvre, Arwa Talbi, Sirine Atwi-Ghaddar, Emilie Destandau, Eric Lesellier

## ► To cite this version:

Thibault Lefebvre, Arwa Talbi, Sirine Atwi-Ghaddar, Emilie Destandau, Eric Lesellier. Development of an analytical method for chlorophyll pigments separation by reversed-phase supercritical fluid chromatography. *Journal of Chromatography A*, 2020, 1612, pp.460643 -. 10.1016/j.chroma.2019.460643 . hal-03489536

**HAL Id: hal-03489536**

**<https://hal.science/hal-03489536v1>**

Submitted on 7 Mar 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 Development of an analytical method for Chlorophyll pigments separation  
2 by Reversed-phase Supercritical Fluid Chromatography

3

4 *Thibault Lefebvre, Arwa Talbi, Sirine Atwi-Ghaddar, Emilie Destandau, Eric*

5 *Lesellier\*, ICOA, CNRS UMR 7311, Université d'Orléans, rue de Chartres, BP 6759,*

6 *45067 Orléans Cedex 2*

7 *\*correspondence author: Eric Lesellier*

8 *Email address: [eric.lesellier@univ-orleans.fr](mailto:eric.lesellier@univ-orleans.fr)*

9

10

11

12

13

14 **Keywords**

15 Reverse phase supercritical fluid chromatography; retention behavior; phytylated  
16 chlorophyll derivatives; modifier percentage and nature; method development;  
17 backpressure modulation with flow rate gradient.

18

## 19 **Abstract**

20 Chlorophyll pigments give the green colour to plants, which is a quality attribute  
21 of food and vegetables. However, the chemical structure of native chlorophyll can  
22 change during varied processes (drying, freezing, extraction) applied to plants, which  
23 produce degradation compounds that could have a brown and unwanted colour.

24 Systematic experiments have been conducted in supercritical fluid  
25 chromatography with a C18 stationary phase to understand and model the  
26 chromatographic behaviour of the compounds with respect to the nature of the  
27 modifier (MeOH, ACN, and MeOH/ACN 50/50) and its percentage, from 10 to 100%.  
28 Specific retention changes were observed, which provide numerous analytical  
29 conditions to achieve compound separation. The chromatographic profile of the  
30 extract containing native chlorophyll *a*, *b* and numerous phytylated chlorophyll  
31 derivatives (pheophytin *a*, *a'*, *b*, *b'*; hydroxypheophytin *a*, *a'*, *b*, *b'*; pyropheophytin  
32 and lactone derivatives) is strongly impacted by the nature of the modifier and,  
33 because of the complexity of the extract, the optimal conditions obtained are unusual  
34 for supercritical fluid chromatography. An original method development using an  
35 optimization criterion was discussed for the analyses of samples, leading to a fast  
36 analytical method with a very low backpressure and a flow rate gradient, but a  
37 simplest and rapid method is also suggested for samples displaying fewer  
38 derivatives.

39

## 40 **1 Introduction**

41 Chlorophyll compounds are involved in photosynthetic chemical reaction of  
42 plants. They are divided into two main classes, the non-polar phytol esterified  
43 (chlorophylls *a* and *b*), and the non-esterified and polar ones (chlorophylls *c1*, *c2*, *c3*).  
44 The non-polar ones, chlorophyll *a* and chlorophyll *b*, are found in the leaf chloroplasts  
45 of higher plants, while the polar ones are found in marine phytoplankton. These  
46 pigments are responsible for the bright green colour of vegetables, or vegetable  
47 extracts, and their degradation can be damageable for the quality of the products.  
48 The chlorophyll *a* and *b* pigments are composed of a porphyrin-type skeleton,  
49 consisting of four pyrrole groups linked by methyne bridges (Figure 1). These  
50 porphyrin rings chelate a magnesium ion in the centre, and are esterified by a

51 terpenoid alcohol, called the phytyl chain, on carbon 17. A vinylic group is located on  
52 carbon 3, and a methyl ester on carbon 13<sup>2</sup>. The *a* and *b* forms differ by the nature of  
53 the substituent on carbon 7, which is either a methyl group for chlorophyll *a* or an  
54 aldehydic one for chlorophyll *b*.

55 Under normal maturing or postharvest storage, natural degradation of pigments  
56 can be observed in vegetables leading to changes in colour, which could depreciate  
57 the quality of the products [1,2]. During the ripening of olive fruit, the decrease in  
58 chlorophyll content associated to the carotenoid esterification is responsible for the  
59 colour evolution, from green to black [3]. For instance, an enzymatic reaction due to  
60 chlorophyllase induces the loss of the phytyl chain to give acidic forms of pigments  
61 called chlorophyllides (S1).

62 Other colour changes, related to chlorophyll structure modification, are reported  
63 during plant processes, such as dehydration [4-7] or freezing [8]. Most often, the  
64 increase in temperature during drying, the extraction with protic solvents or long  
65 storage all promote the demetallation of magnesium, leading to pheophytins *a* and *b*,  
66 or the epimerisation on carbon 13<sup>2</sup>, leading to *a'* and *b'* compounds, either for  
67 chlorophylls or pheophytins [9](S1). Additional decarbomethoxylation (on carbon 13<sup>2</sup>)  
68 can also be favoured by heating, leading to pyro-pheophytin from pheophytin [10].  
69 Besides, the presence of hydroxylated chlorophyll compounds is also reported, either  
70 related to the vegetable maturity [11] or to the conditions of the extraction process  
71 [12].

72 The chromatographic separation of these compounds is very useful to study the  
73 effects of the varied processes applied to vegetables: dry- or moist-heating, freeze-  
74 drying [13], rehydration, canning or extraction, to check the adulteration due to the  
75 addition of food colorant (copper complexes of chlorophyll) ([9] or the re-greening  
76 process in the presence of zinc [13]. Moreover, a chromatographic method can also  
77 follow the eventual degradation of chlorophyll during plant extraction processes when  
78 using modern extraction methods such as pressurised fluid extraction [14] or  
79 supercritical fluid extraction [15].

80 The presence of the phytyl chain, which contains 20 carbons, makes  
81 chlorophylls and some of their derivatives strongly non-polar (Log P>8).

82 One paper reported the separation of chlorophylls and chlorophyll derivatives  
83 with normal-phase liquid chromatography on a silica stationary phase with a dioxane-  
84 hexane elution gradient [16]. Thanks to the good quality of new generation-silica, the  
85 stabilization time between the analyses was lower than 10 min.

86 Apart from this example, most of the separations reported in the literature were  
87 achieved in non-aqueous reversed-phase liquid chromatography (NARP-LC), i.e.  
88 without any water in the mobile phase [6, 7, 11, 17], or with a constant small amount  
89 of water of 4 % [5, 10]. To observe both the derivatives that have lost their phytyl  
90 chain, meaning the more polar compounds (chlorophyllides and pheophorbide),  
91 water was used in the first part of the gradient. To achieve an optimal separation of  
92 these acidic derivatives, an ion-pairing agent was required. Ammonium acetate was  
93 selected in most cases [1, 4, 9, 13, 15, 18, 19], whereas tetrabutylammonium acetate  
94 [3, 12] or varied amines (pyridine, piperidine, pentylamine) were also added to the  
95 mobile phase [20,21,22].

96 Whatever the nature of the derivatives, elution gradients were largely used, with  
97 varied organic solvents: methanol, acetonitrile, acetone, methyl-t-butyl-ether,  
98 isopropanol, ethyl acetate or tetrahydrofuran. These organic solvents were used  
99 alone or in binary or ternary mixtures. The number of steps in the gradients generally  
100 varied from 4 to 8, including the stationary phase equilibria.

101 The use of C8-bonded phase was reported [19, 22] but most of the stationary  
102 phases employed were C18-bonded ones, with fully porous particles. For these C18  
103 phases, some studies reported retention changes related to the type of bonding,  
104 monomeric or polymeric [19, 20]. The use of polymeric C18 phases, for instance  
105 Vydac 201 TP 54, provided different separations for mono-vinyl and di-vinyl  
106 chlorophyll *c3*, or for chlorophyll *c1* and *c2*. These two pairs of compounds differ by  
107 the nature of substituents on carbon 8, either an ethylene or an ethane group. The  
108 specific behaviour of these polymeric C18-bonded phases for shape-constrained  
109 solutes was described elsewhere [23].

110 A C30 ligand was also selected for the separation of chlorophylls [5,14] for its  
111 ability to separate compounds with rigid well-defined molecular shape [23]. Recently,  
112 this C30 phase was used for an SFC x LC two-dimensional separation (in the second

113 LC dimension) [24], to be applied to the separation of both chlorophylls and  
114 carotenoid pigments of bell pepper (*Capsicum annuum* L.).

115 In order to improve the speed of chlorophyll compounds analysis, monolithic  
116 C18-bonded silica phases, which have a high porosity, have been used with a flow  
117 rate of 4 ml/min allowing the separation of the two chlorophylls *a* and *b* and the two  
118 pheophytins *a* and *b* in less than 5 min [19]. Recently, the use of core-shell particles  
119 was reported to achieve the separation of 24 pigments in a 28 min-analysis [6],  
120 including the epimer forms and the pyro-forms of chlorophyllide, pheophorbide,  
121 chlorophyll and pheophytin.

122 Reversed-phase supercritical fluid chromatography (RP-SFC), which can be  
123 achieved with a non-polar C18-bonded phase, is an interesting alternative for the  
124 separation of this type of non-polar compounds. With regards to the organic solvents  
125 used in NARP-LC, carbon dioxide has many advantages: ecological, economic and  
126 physico-chemical. Moreover, it can be mixed with many other solvent (often called  
127 modifier) to tune the polarity of the mobile phase, inducing large changes in retention  
128 and sometimes great improvement in the separation performances.

129 The first use of supercritical fluid chromatography (SFC) for the separation of  
130 chlorophyll pigments was reported twenty years ago. It was achieved with a modifier  
131 gradient of methanol from 6 to 25% on a C18-bonded phase [25], but no separations  
132 between hydroxylated and epimeric pheophytins were studied.

133 The goal of the present paper was to study the effects of a large composition  
134 range of varied modifiers (MeOH, ACN, MeOH/ACN) on the retention behaviour and  
135 on the separation of chlorophyll pigments, and to describe the analytical development  
136 of a separation method by RP-SFC based on an combined optimisation criterion.

137

## 138 **2 Material and methods**

139

### 140 *2.1 Instruments*

141 Chromatographic separations were carried out with the following instruments:  
142 the carbon dioxide pump was JASCO PU-2080-CO<sub>2</sub>, the modifier pump was JASCO  
143 PU-2080, the mixing unit was GILSON Dynamic mixer model 811, the column oven  
144 was a column thermostat JETSTREAM 2 PLUS (Sigma-Aldrich, l'Île d'Abeau,  
145 France), the back-pressure regulator was BP-2080 Plus. This device was equipped  
146 with a PDA detector JASCO MD40-10 (and an interface unit LC-NETII/ADC).  
147 Chromatograms were recorded using CHROMNAV software (Jasco, Lisses, France).  
148 The chromatographic column was an octadecylsiloxane-bonded superficially porous  
149 silica CORTECS C18+ (4.6 x 150 mm, 2.7 µm) provided by Waters (Dublin, Ireland).  
150 This column was selected for the type of particles (core-shell) providing high  
151 efficiencies, and for the positive charge surface modification. Retention factors (*k*)  
152 were calculated using the following equation.

$$153 \quad k = \frac{t_r - t_0}{t_0}$$

154 with retention times (*t<sub>r</sub>*) and dead time (*t<sub>0</sub>*). The dead time was measured at each  
155 analytical condition, because it varies in function of the compressibility of the carbon  
156 dioxide that can change following the inlet pressure of the column, which is related,  
157 at constant flow rates, to the mobile phase viscosity, i.e. to the modifier percentage  
158 [27,28].

159

160 Characterisation of an ivy extract was carried out with a Nexera UC SFC  
161 system coupled with a mass spectrometer, equipped with a dual ionization source  
162 and a simple-quadrupole analyser from SHIMADZU (Kyoto, Japan). The following  
163 parameters were used for the characterization: ionization in positive mode; interface  
164 voltage, 4.5 kV; interface temperature 350 °C; Nebulizing gas flow, 1.5 L/min; heat  
165 block, 200 °C. A make-up solvent was also used to enhance the ionization of the  
166 molecules: 0.100 ml/min of methanol with 20 mM ammonium formate were  
167 introduced after the column.

168

169 The plant dryer was a Memmert model 500. The sample was dried at 50°C for  
170 48 hours. Plant extraction was carried out with Ultrasonic bath BRANSON 3510. The  
171 centrifugation device was BR4i multifunction centrifuge (Thermo-electron, Nantes,  
172 France).

173 The microwave extractor was a Microsynth from Milestome (Soriso, Italy).  
174 Plant extraction was carried out in a 50 ml-reactor (QRS1550). Evaporation was  
175 made with a BUCHI rotary evaporator using the different modules (B-480, R-114, I-  
176 100, V-100 and F-308).

177

## 178 *2.2 Chemicals and plant material*

179 The solvents used for extraction and chromatographic purpose were methanol  
180 and ethanol, gradient grade (VWR International, Fontenay-sous-Bois, France), and  
181 acetonitrile, gradient grade (Carlo Erba, Val de Reuil, France). Carbon dioxide  
182 (>99.5%) was from AIR LIQUIDE (Fleury-les-Aubrais, France).

183 The plant used was wild ivy (*Hedera canariensis* Willd) collected in Orleans  
184 (France).

185

## 186 *2.3 Extraction methods*

187 Two extracts were used. The first one called in the next pages "*fresh extract*"  
188 was made using 1 g of the dried plant material and 15 mL of ethanol. The mixture  
189 was then sonicated for 60 min. Another extract called "*degraded extract*" was  
190 obtained with microwave assisted extraction with three heating cycles at 500 W for  
191 30 seconds, and cooldown to 25°C between two consecutive cycles. Both extracts  
192 were filtrated and centrifugated at 7000 rpm and at 20°C for 10 min. The *Fresh*  
193 *extract* was stored in the fridge in tainted bottle during analyses, whereas the  
194 *Degraded extract* was evaporated with a rotary evaporator. It was then solubilised in  
195 methanol for analysis.

196

## 197 *2.4 Analytical methods*



198 Different composition of mobile phase comprising carbon dioxide and methanol  
199 and/or acetonitrile were employed as further detailed in the results section. The flow  
200 rate was 1.5 mL/min, outlet pressure 10 MPa, oven temperature 25°C, and UV  
201 detection was monitored at 650 nm.

202

### 203 **3 Results and discussion**

204

#### 205 **3.1 Fresh extract**

206

207 A first extract was analysed. As shown in the following chromatogram (Fig.2),  
208 this extract mainly contains two compounds absorbing visible light at 650 nm. After  
209 characterisation using mass spectrometry and UV spectroscopy, chlorophyll *a* and *b*  
210 were identified. As expected, when using a C18-bonded stationary phase, chlorophyll  
211 *b* having an aldehyde function thus being slightly more polar is eluted before  
212 chlorophyll *a* bearing a methyl group. In addition, the relative peak heights are in  
213 accordance with other reported studies on plants, with chlorophyll *a* being more  
214 abundant than chlorophyll *b*.

215 In order to better understand the chromatographic behaviour of these two  
216 compounds and develop a separation method, different mobile phase compositions  
217 were tested. Then, for all these conditions, retention times of the two compounds  
218 were monitored at 650 nm.

219 From methanol 10% to 100%, retention factors were plotted for both  
220 chlorophylls (Figure 3). For all the studied conditions, both chlorophylls *a* and *b* were  
221 separated. Moreover, the two chlorophylls had similar retention behaviours. From 10  
222 to 40% methanol, there is a decrease of the retention factor. This decrease is  
223 explained by the increase of the polarity of the mobile phase which favours the  
224 solubility of compounds in the mobile phase. However, from 40% methanol, the  
225 polarity of the mobile phase continues to increase that decreases the solubility of the  
226 compounds. This phenomenon was already observed with other non-polar  
227 compounds (carotenoids [29, 30], UV absorbers [27] or triglycerides) with octadecyl-  
228 bonded silica stationary phase in SFC [31, 32].

## 229 3.2 Degraded extract 230

231 To develop a separation method that would also be suited to chlorophyll  
232 derivatives formed during varied processes applied to plants, a degraded extract was  
233 examined. As shown on figure 4, it contains more peaks than was observed in the  
234 fresh extract, and they are eluted later (between 4 and 12 minutes) than the native  
235 chlorophylls eluted in the first 4 minutes. Because the stationary phase is not polar,  
236 this increase of the retention time for these new compounds indicates that they are  
237 rather less polar than the chlorophylls. It means that these compounds are not  
238 chlorophyllides, which are obtained after the loss of the non-polar phytol chain. The  
239 molecular weight of the main compounds were obtained from SFC-MS analysis  
240 (Table 1). From these mass values and from the relative retention of the compounds,  
241 an identification attempt was done. With the MS parameters described previously,  
242  $[M+H]^+$  ions could be detected and correlated with expected degradation products. In  
243 addition, the ionisation parameters allowed to confirm the main compounds with  
244 fragments found in MS/MS methods [9, 13, 33]. However, due to the weak ionisation  
245 conditions and the use of a simple-quadrupole MS, the fragments observed in  
246 literature were detected with a much lower abundance.

247

248 The four compounds eluted first on figure 5 are hydroxy-pheophytin (OHPheo),  
249 whereas compounds 5 and 6 are pheophytin *a* and *b*, and compounds 7 and 8 are  
250 pheophytin *a'* and *b'*. In this work, the retention order of pheophytin compounds is  
251 reversed compared to the one observed in other publications in SFC or NARP-LC.  
252 Whereas the pheophytin *b* is generally less retained than pheophytin *a*, due the  
253 presence of the aldehydic group on pheophytin *b* with the C18 columns used in  
254 literature, our experiments show that the pheophytins with an aldehydic group (*b* and  
255 *b'*), were more retained than the ones with a methyl group (*a* and *a'*). This  
256 unexpected chromatographic behaviour can be explained by the positive charge  
257 surface modification of the selected C18-bonded phase, which favours the retention  
258 of compounds having an aldehydic group. However, this unexpected behaviour did  
259 not occur for the chlorophylls, indicating that this behaviour may be related to the  
260 presence of the magnesium group in the porphyrin ring.

261 Figure 5 shows that the chromatographic behaviour of these phytylated  
262 chlorophyll derivatives vs methanol percentage is similar to the one of chlorophylls.  
263 The separation behaviour of the two pairs of pheophytin (*a/b* and *a'/b'*) follows the  
264 same pattern, *i.e.* increasing separation when increasing the methanol percentage. It  
265 also appears very clearly on figure 6, showing the chromatograms obtained from 60  
266 to 75 % methanol in CO<sub>2</sub>. Using a temperature of 25°C and a back-pressure of 10  
267 MPa, we can consider that the method is not supercritical or subcritical fluid  
268 chromatography anymore, but rather non-aqueous enhanced-fluidity liquid  
269 chromatography (EFLC). When comparing the separation quality of the  
270 chromatograms plotted in figure 6, for the peaks that were eluted before pheophytins  
271 5 to 8 (see chromatogram at 70%), it appears that the best separation was achieved  
272 for 70% methanol. In this condition, the four hydroxy-pheophytins *a*, *b*, *a'* and *b'*  
273 (compounds 1, 2, 3 and 4) were well resolved, as well as one additional minor  
274 impurity (i) which co-elutes either with compound 2 or 3 for other methanol  
275 percentages. From its retention time with regards to pheophytin compounds, this  
276 impurity could be a 15-lactone form of pheophytin *a*, which is eluted before [9,12, 34],  
277 or a pyro form of hydroxy-pheophytin which is eluted after [6].

278 However, increasing the methanol percentage increases the fluid viscosity and  
279 the inlet pressure. Because we did not wish reach 40 MPa inlet pressure with regards  
280 to the pumping system, flow rate was reduced at the highest methanol proportions  
281 (superior to 50%), thus pressure drop was maintained stable (around 30 MPa).

282 Consequently, the study began, for 10% of modifier, at 3 mL/min, and for the  
283 optimal conditions (70% methanol added to CO<sub>2</sub>), the flow rate was reduced down to  
284 1.8 ml/min. To overcome this flow rate decrease, and to shorten the analysis  
285 duration, the back-pressure was decreased to the lowest possible value. At the  
286 optimal conditions, *i.e.* 70% methanol, it was possible to decrease the back-pressure  
287 value down to 4 MPa, without causing any phase separation between methanol and  
288 CO<sub>2</sub>. Supplementary information (Figure S2) shows the separation variation of the  
289 chlorophyll derivatives at this low back-pressure for varied flow rates, from 1.8 to 3  
290 mL/min. At 2 mL/min and 4 MPa, the chromatogram shows an almost identical  
291 separation quality, with a little smaller retention, with regards to the analysis achieved  
292 at 10 MPa and 1.8 mL/min. This retention decrease was not as significant as could  
293 be expected from an increase of 0.2 mL/min of the flow rate. However, as the back-

294 pressure was decreased from 10 to 4 MPa, the average pressure, i.e. the average  
295 density of the fluid was also lower, thereby reducing the elution strength of the mobile  
296 phase. At higher flow rates, from 2.2 to 3 mL/min maintaining constant back-pressure  
297 (4 MPa), the analysis duration was shortened. However, for most pairs of  
298 compounds, 1/2, 3/4, 5/6 and 7/8, a reduction of the resolution was observed. In  
299 general, the change in resolution is due either to separation factor modifications, i.e.  
300 to interactions variations between the compounds and the mobile phase, or to the  
301 efficiency, related to diffusion or mass transfer coefficients of the compounds. When  
302 modifying the flow rate, no change in the mobile phase composition occurs, except  
303 for compressible fluids, because of density changes. However, with 70% methanol  
304 added to CO<sub>2</sub>, no significant density variation should occur with flow rate increase.  
305 This observed behaviour of the regular decrease in resolution for the four pairs of  
306 compounds due to the flow rate increase from 2 to 3 mL/min can mostly be related to  
307 kinetic phenomena, and is in accordance with the one observed elsewhere for C18-  
308 bonded superficially porous particles [32]. From a practical point of view, this  
309 decrease in resolution was measured by the discrimination factor  $d_0$  [35] for the 3/4  
310 pair of compounds, which displayed the worst resolution. As can be seen in Figure  
311 7a, this criterion is well suited to examine the changes in separation quality without  
312 measuring peak width as it is required for the resolution factor. The discrimination  
313 factor can vary from zero for co-eluted peaks to one for fully separated peaks  
314 (meaning with a baseline resolution). As shown in figure 7b, for the 3/4 pair, this  
315 factor varied from 0.85 at 2 mL/min, to 0.4 at 3.2 mL/min. It underlines that the gain in  
316 analysis duration, from 10.5 min to 7 min (S2), was counterbalanced by the loss in  
317 separation quality.

318 In order to manage these opposite effects a combined criterion ( $C_c$ ) was  
319 calculated as the ratio between the discrimination factor and the analysis duration  
320 ( $t_a$ ). Figure 7b shows that this combined criterion first increased when flow rate  
321 increased from 2 to 2.6 mL/min, due to the greater effect of the analysis time  
322 reduction, then decreased when flow rate further increased from 2.6 to 3.2, due to  
323 the greater loss in separation quality. As a consequence, the value of 2.6 mL/min can  
324 be selected as a satisfying compromise between the two chromatographic changes.  
325 The chromatogram achieved at this flow rate and a back pressure of 4 MPa (fig. 8)  
326 displays the eight chlorophyll derivatives pheophytins and hydroxy-pheophytins, the

327 impurity (i) and at least seven unknown minor compounds (u) that also absorb visible  
328 light at 650 nm, indicating that they must all have a porphyrinic structure. The retention  
329 time of the last unknown compound is 11 minutes. Finally, to reduce the analysis  
330 duration, a flow rate gradient was applied from 2.6 to 3.2 mL/min (S3). The first  
331 gradient (S3a) was a step one at 5 minutes, allowing to maintain good resolution for  
332 hydroxy-pheophytins that were eluted before 5 minutes. The chromatographic profile  
333 was satisfactory both for hydroxy-pheophytins eluted at 2.6 mL/min and for  
334 pheophytins eluted at 3.2 ml/min. As expected, this gradient allowed to reduce the  
335 analysis duration because the last unknown compound was then eluted at 9.5  
336 minutes instead of 11. Three other gradient profiles (S3b,c,d) were attempted, by  
337 beginning earlier the increase in the flow rate (4.5 min, 4 min, 3.5 min) with a more or  
338 less smooth gradient. The use of a smoother slope could provide a more progressive  
339 change in average pressure in the column, i.e. a less dramatic variation of the fluid  
340 properties (density, viscosity, diffusion coefficients). However, no significant change  
341 of the chromatographic profile was noticed for these different gradient profiles,  
342 indicating once again that, with this high methanol proportion, the fluid behaves more  
343 as an incompressible liquid than a compressible supercritical fluid.

344 Additional studies were carried out replacing methanol by acetonitrile or by a  
345 50/50 mixture of acetonitrile and methanol. The proportions in carbon dioxide varied  
346 from 10 to 80%. In Figure 9, the chromatographic behaviour of pheophytin *a* and *b*  
347 are represented to exemplify the effects observed, because they are the main  
348 compounds produced by physico-chemical treatments on plants or plant extracts. As  
349 appears in figure 9, the chromatographic behaviour of the compounds followed the  
350 same pattern as the one observed when methanol alone was used as modifier. The  
351 percentage of acetonitrile providing minimum retention (25-30%) was lower than the  
352 one of methanol (40-50%). For the MeOH/ACN mixture, this percentage was  
353 intermediate (30-40%). Moreover, the separation factor ( $\alpha$ ) for these two pheophytins  
354 increased continuously with the modifier percentage (S4). For high modifier  
355 percentages, the use of acetonitrile or methanol/acetonitrile 50/50 favoured the  
356 separation of these two compounds. Figure 10 displays the chromatograms obtained  
357 with the three different modifiers at the percentages providing the best separation for  
358 the degradation compounds of chlorophylls. Unfortunately, one impurity is located at  
359 the beginning of the chromatogram when using methanol (see arrows). When using,

360 the two other modifiers (acetonitrile or methanol/acetonitrile mixture), this impurity is  
361 shifted later in the chromatogram and is co-eluted with some of the target  
362 degradation compounds. The presence of hydrophilic compounds eluted in the early  
363 part of the chromatogram when using C18-bonded phase was also reported  
364 elsewhere [14].

365 Finally, when studying the effects of the plants drying or storage, the extraction  
366 conditions with solvents or the conditions of evaporation of these solvents, in most  
367 cases, few degradation products from chlorophylls are observed, and the separation  
368 method can be simplified, by using only 20% of modifier (S5). The main chlorophyll  
369 derivatives observed are the pheophytins, but in comparison to the abundant native  
370 chlorophylls, their concentrations are very low. This chromatogram was obtained with  
371 only 20% methanol in carbon dioxide, which was sufficient to analyse samples which  
372 do not contain too many chlorophyll derivatives.

373

## 374 4 Conclusion

375

376 This study displays the chromatographic behaviour of chlorophylls and  
377 chlorophyll derivatives *versus* the nature and the percentage of modifier for different  
378 modifiers. The variations of retention factor on a C18-bonded phase is rather  
379 unusual, although it was observed before for carotenoid pigments, triglycerides or UV  
380 absorbers. This change is related to the non-uniform solubility variation of the non-  
381 polar compounds towards the mobile phase. These observations could also be used  
382 in the future to improve the modelling of extraction conditions by using supercritical  
383 fluids.

384 This paper also describes an original method development to achieve the  
385 separation of phytolated chlorophyll derivatives, varying the modifier nature and  
386 percentage, but also other parameters impacting the fluid density and viscosity. For  
387 instance, the modifier addition changes the interactions between the compounds and  
388 the mobile phase, as expected, but also impacts the fluid viscosity, that changes the  
389 inlet pressure. With regards to the pressure limit of the pumping system, this side-  
390 effect can be managed by reducing the back-pressure in the goal to increase the flow  
391 rate to reduce analysis duration. Besides, when using high modifier percentages, this

392 study also underlines that the changes in the gradient slope when modifier proportion  
393 is high, keeping constant the medium time between the initial and the final  
394 compositions, does not affect the chromatogram, because of the low compressibility  
395 of the mobile phase, which is rather in a liquid state.

396 Finally, the separation obtained at optimal conditions allows the separation of  
397 epimers of pheophytin and hydroxy-pheophitin, *a* and *b*, with a reduced analytical  
398 retention time compared to classical HPLC methods.

399

400 Acknowledgements: this work was supported by the Centre-Val de Loire region,  
401 which provided the funding of the “Khloros” APR-IR (2017).

402

403

404

## 405 References

406

407 [1] L. Almela, J.A. Fernandez-Lopez, M.J. Roca, High-performance liquid  
408 chromatographic screening of chlorophyll derivatives produced during fruit storage, J.  
409 Chromatogr. A 870 (2000) 483-489.

410 [2] M.H. Roiser, T. Muller, B. Krautler, Colorless chlorophyll catabolites in senescent  
411 florets of broccoli (*Brassica oleracea* var. *italica*), J. Agric. Food Chem. 63 (2015)  
412 1385-1392.

413 [3] B. Gandul-Rojas, M. R-L. Cepero, M.I. Minguéz-Mosquera, Chlorophyll and  
414 carotenoid patterns in olive fruits, *Olea europaea* Cv. Arbequina, J. Agric. Food  
415 Chem. 47(1999) 2207-2212.

416 [4] D. Borrmann, J. Castelhana de Andrade, U.M. Lanfer-Marquez, Chlorophyll  
417 degradation and formation of colorless chlorophyll derivatives during soybean  
418 (*Glycine max* L. Merrill) seed maturation, J. Agric. Food Chem. 57 (2009) 2030-2034.

- 419 [5] A. Kaiser, M. Brinkmann, R. Carle, D. Kammerer, Influence of thermal treatment  
420 on color, enzyme activities, and antioxidant capacity of innovative pastelike parsley  
421 products, *J. Agric. Food Chem.* 60 (2012) 3291-3301.
- 422 [6] J.L. Lafeuille, S. Lefebvre, J. Lebuhotel, Quantitation of chlorophylls and 22 of  
423 their colored degradation products in culinary aromatic herbs by HPLC-DAD-MS and  
424 correlation with color changes during the dehydration process, *J. Agric. Food Chem.*  
425 62 (2014) 1926-1935.
- 426 [7] T. H. Kao, C.J. Chen, B.H. Chen, An improved high performance liquid  
427 chromatography-photodiode array detection-atmospheric pressure chemical  
428 ionization-mass spectrometry method for determination of chlorophylls and their  
429 derivatives in freeze-dried and hot-air-dried *Rhiacanthus nasutus* (L.) Kurz, *Talanta*,  
430 86 (2011) 349-355.
- 431 [8] M. Eledenbos, L.P. Christensen, K. Grevsen, HPLC determination of chlorophyll  
432 and carotenoid pigments in processed green pea cultivar (*Pisum sativum* L.), *J.*  
433 *Agric. Food Chem.* 49 (2001) 4768-4774.
- 434 [9] A. Delpino-Rius, D. Cosovanu, J. Eras, F. Vilaro, M. Balcells, R. Canela-Garayoa,  
435 A fast and reliable ultrahigh-performance liquid chromatography method to assess  
436 the fate of chlorophylls in teas and processed vegetable foodstuff, *J. Chromatogr. A*  
437 1568 (2018) 69-79.
- 438 [10] X. Li, M. Woodman, S.C. Wang, High-performance liquid chromatography with  
439 fluorescence detection for the rapid analysis of pheophytins and pyropheophytins in  
440 virgin oil, *J. Sep. Sci.* 38 (2015) 2813-2818.
- 441 [11] S.C. Huang, C.F. Hung, W.B. Wu, B.H. Chen, Determination of chlorophylls and  
442 their derivatives in *Gynostemma pentaphyllum* Makino by liquid chromatography-  
443 mass spectrometry, *J. Pharm. Biomed. Anal.* 48 (2008) 105-112.
- 444 [12] M. Roca, L. Gallardo-Guerrero, M.I. Minguéz-Mosquera, B. Gandul-Rojas,  
445 Control of olive oil adulteration with copper-chlorophyll derivatives, *J. Agric. Food*  
446 *Chem.* 58 (2010) 51-56.
- 447 [13] A. Gauthier-Jaques, K. Bortlik, J. Hau, L.B. Fay, Improved method to track  
448 chlorophyll degradation, *J. Agric. Food Chem.* 49 (2001) 1117-1122.



- 449 [14] K.H. Cha, H.J. Lee, S.Y. Koo, D-G. Song, D-U. Lee, C-H. Pan, Optimization of  
450 pressurized liquid extraction of carotenoids and chlorophylls from *Chlorella vulgaris*,  
451 J. Agric. Food Chem. 58 (2010) 793-797
- 452 [15] J. A. Mendiola, F.R. Marin, S.F. Hernandez, B.O. Arredondo, F. J. Senorans, E.  
453 Ibanez, G. Reglero, Characterization via liquid chromatography coupled to diode  
454 array detector and tandem mass spectrometry of supercritical antioxidant extracts of  
455 *Spirulina platensis* microalga, J. Sep. Sci. 28 (2005) 1031-1038.
- 456 [16] R. Cruz, S. Casal, Direct analysis of vitamin A, vitamin, carotenoids, chlorophylls  
457 and free sterols in animal and vegetable fats in a single normal-phase liquid  
458 chromatographic run, J. Chromatogr. A 1565 (2018) 81-88.
- 459 [16] T. Bohn, T. Walczyk, Determination of chlorophyll in plant samples by liquid  
460 chromatography using zinc(phthalocyanin as an internal standard, J. Chromatogr. A  
461 1024 (2004) 123-128.
- 462 [17] Z. Kamffer, K.A. Bindon, A. Oberholster, Optimization of a method for the  
463 extraction and quantification of carotenoids and chlorophylls during ripening in grape  
464 berries (*Vitis vinifera* cv. Merlot), J. Agric. Food Chem. 58 (2010) 6578-6586.
- 465 [18] F. Rodriguez, M. Zapata, J.L. Garrido, High performance liquid chromatography  
466 separation of chlorophyll c form from marine phytoplankton on octadecyl bonded  
467 phases, Chromatographia 48 (1998) 677-680.
- 468 [19] J. L. Garrido, M. Zapata, Ion-pair reversed-phase high-performance liquid  
469 chromatography of algal chlorophylls, J. Chromatogr. A 738 (1996) 285-289.
- 470 [20] J.L. Garrido, F. Rodriguez, E. Campana, M. Zapata, Rapid separation of  
471 chlorophylls *a* and *b* and their demetallated and dephytylated derivatives using a  
472 monolithic silica C18 column and a pyridine-containing mobile phase, J. Chromatogr.  
473 A 995 (2003) 85-92.
- 474 [21] L. C. Sander, S. A. Wise, The influence of column temperature on selectivity in  
475 reversed-phase liquid chromatography for shape-constrained solutes, J. Sep. Sci. 24  
476 (2001) 910-920.

477 [22] Y. Susuki, Y. Shioi, Identification of chlorophylls and carotenoids in major teas by  
478 high-performance liquid chromatography with photodiode array detection, J. Agric.  
479 Food Chem. 51 (2003) 5307-5314.

480 [23] C. Emenhiser, L.C. Sander, S.J. Schwartz, Capability of a polymeric C30  
481 stationary phase to resolve cis-trans carotenoid isomers in reversed-phase liquid  
482 chromatography, J. Chromatogr. A 707 (1995) 205-216.

483 [24] I. Bonaccorsi, F. Cacciola, M. Utczas, V. Inferrera, D. Giuffrida, P. Donato, P.  
484 Dugo, L. Mondello, Characterization of the pigment fraction in sweet bell peppers  
485 (*Capsicum annuum* L.) harvested at green and overripe yellow and red stages by  
486 offline multidimensional convergence chromatography/liquid chromatography-mass  
487 spectrometry, J. Sep. Sci. 39 (2016) 3281-3291.

488 [25] S. Buskov, H.Sorensen, S. Sorensen, Separation of chlorophylls and their  
489 degradation products using packed column chromatography supercritical fluid  
490 chromatography (SFC), J. High Resolut. Chromatogr. 22 (1999) 339-342.

491 [26] E. Lesellier, C. West, The many faces of packed column supercritical fluid  
492 chromatography- A critical review, J. Chromatogr. A 1382 (2015) 2-46.

493 [27] K. Gurdale, E. Lesellier, A. Tchaplá, Study of the dead volume measurement in  
494 packed subcritical fluid chromatography with ODS columns and carbon dioxide-  
495 modifier mobile phases, J. Chromatogr. A 866 (2000) 241-251.

496 [28] E. Lesellier, A.M. Krustulovic, A. Tchaplá, Influence of the modifiers on the  
497 nature of the stationary phase and the separation of carotenes in sub-critical  
498 fluid chromatography, Chromatographia 36 (1993) 275-282.

499 [29] E. Lesellier, Overview of the retention in subcritical fluid chromatography  
500 with varied polarity stationary phases, J. Sep. Sci. 31 (2008) 1238-1251.

501 [30] E. Lesellier, A. Tchaplá, Retention behavior of triglycerides in octadecyl packed  
502 subcritical fluid chromatography with CO<sub>2</sub>/modifier mobile phases, Anal. Chem. 71  
503 (1999) 5372-5378.

504 [31] E. Lesellier, J. Bleton, A. Tchaplá, Use of relationships between retention  
505 behaviors and chemical structures in subcritical fluid chromatography with

506 CO<sub>2</sub>/modifier mixtures for the identification of triglycerides, *Anal. Chem.* 72 (2000)  
507 2573-2580.

508 [32] E. Lesellier, Efficiency in supercritical fluid chromatography with different  
509 superficially porous and fully porous particles ODS bonded phase, *J. Chromatogr. A*  
510 1228 (2012) 89-98.

511 [33] K. Chen, J.J. Rios, A. Pérez-Galvez, M. Roca, Development of an accurate and  
512 high-throughput methodology for structural comprehension of chlorophylls  
513 derivatives.(I) Phytylated derivatives, *J. Chromatogr. A* 1406 (2015) 99-108.

514 [34] R. Aparicio-Ruiz, M.I. Minguez-Mosquera, B. Gandul-Rojas, Thermal  
515 degradation kinetics of chlorophyll pigments in virgin oils. 1. Compounds of series *a*,  
516 *J. Agric. Food Chem.* 58 (2010) 6200-6208.

517 [35] M.Z. El Fallah, M. Martin, Influence of the peak height distribution on separation  
518 performances: discrimination factor and effective peak capacity, *Chromatographia* 24  
519 (1987) 115-122.

520

521 Figure captions.

522 Figure 1: Chemical structure of chlorophyll *a* and *b*.

523 Figure 2: Chromatogram of fresh Ivy extract in SFC. Column: Cortecs C18+; flow rate  
524 1.5 mL/min, T=25°C, P<sub>out</sub>= 10 MPa, mobile phase: CO<sub>2</sub>-MeOH (60/40, v/v) with (1)  
525 chlorophyll *b* and (2) chlorophyll *a*

526 Figure 3: Variation of log *k* vs the percentage of MeOH. Blue squares: chlorophyll *b*;  
527 red triangles: chlorophyll *a*.

528 Figure 4: Chromatogram of degraded Ivy extract in SFC. Mobile phase: CO<sub>2</sub>/MeOH  
529 90/10; other conditions as in figure 3. Identification of peaks in table 11 and figure 5.

530 Figure 5: Variation of log *k* vs the percentage of MeOH. 5= pheophytin *a*; 6/  
531 pheophytin *b*; 7/ pheophytin *a'*; 8/ pheophytin *b'*.

532 Figure 6: Chromatograms of chlorophyll derivatives at varied methanol percentages  
533 from 60 to 75%. P<sub>out</sub> = 10 MPa.

534 Figure 7: Variation of the discrimination factor *d*<sub>0</sub> and of the combined criterion (*C*<sub>c</sub>)  
535 for the pair of hydroxypheophytin 3 and 4 vs the flow rate with a back pressure of 4  
536 MPa.

537 Figure 8: Chromatogram of the degraded Ivy extract at 2.6 mL/min. P<sub>out</sub> = 4 MPa; T=  
538 25°C, mobile phase CO<sub>2</sub>/MeOH 30/70.

539 Figure 9: Variation of log *k* of pheophytin *a* (triangles) and *b* (squares) with different  
540 modifiers (MeOH, ACN, ACN/MeOH). Other analytical conditions as in figure 4.

541 Figure 10: Chromatograms of the degraded Ivy extract at the optimum of the  
542 separation for three modifiers. Lower chromatogram: methanol 70%; medium:  
543 methanol/acetonitrile (50/50) 50%; upper: acetonitrile 60%.

544 Figure S1: Structural modification of chlorophyll pigments

545 Figure S2: Chromatograms of chlorophyll derivatives at varied flow rates. P<sub>out</sub> = 4  
546 MPa for flow rates from 2 to 3 mL/min.

547 Figure S3: Chromatograms of the degraded Ivy extract for varied elution gradient  
548 profiles. Other analytical conditions are described in fig. 8.

549 Figure S4: Separation factor ( $\alpha$ ) for the pheophytin *a* and *b* vs the modifier  
550 percentage for three modifiers. Other analytical conditions as in figure 2.

551 Figure S5: Chromatogram of a fresh Ivy extract with 20% methanol as modifier.  
552  $T=25^{\circ}\text{C}$ ;  $P_{\text{out}}= 10 \text{ MPa}$ , Flow rate: 1.5 mL/min.

553

554

555

556

557

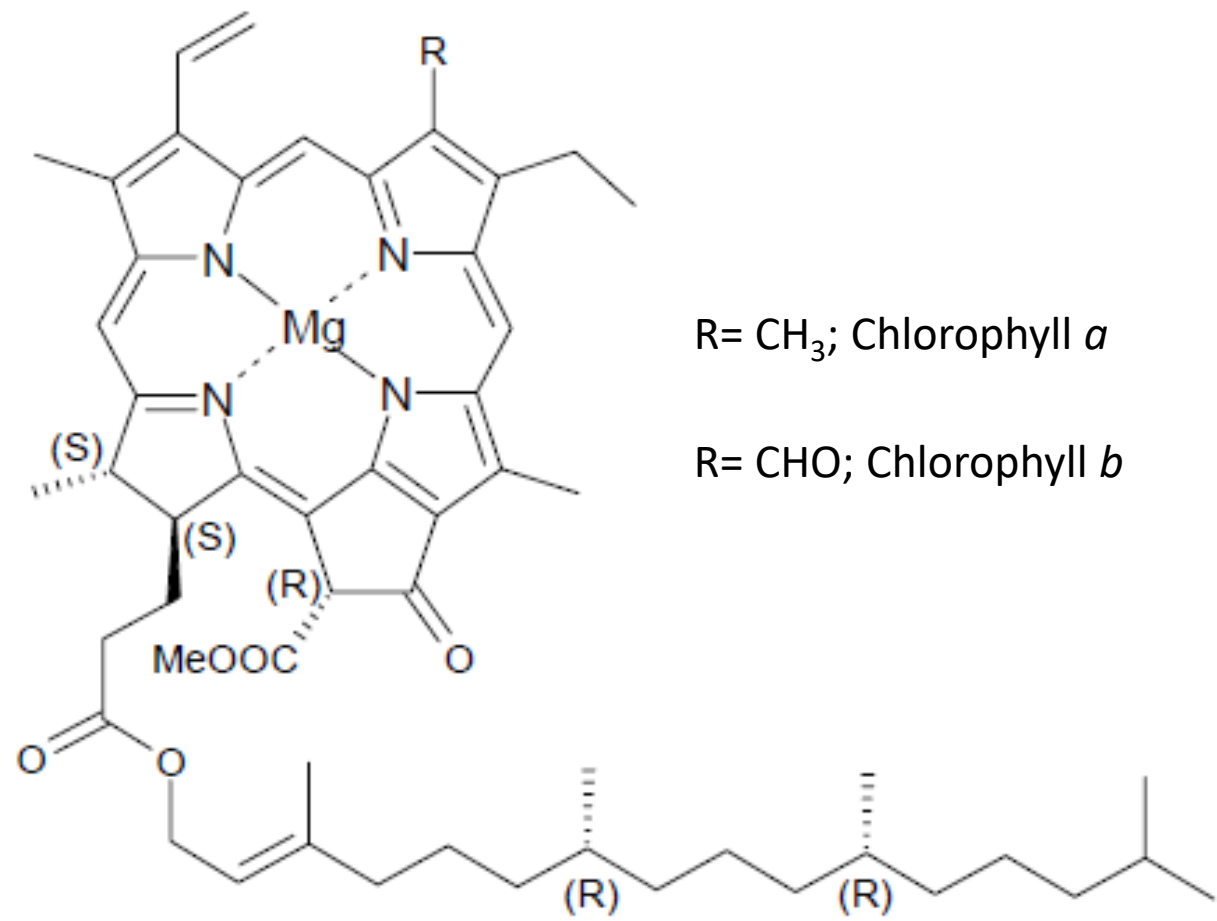


Figure 1

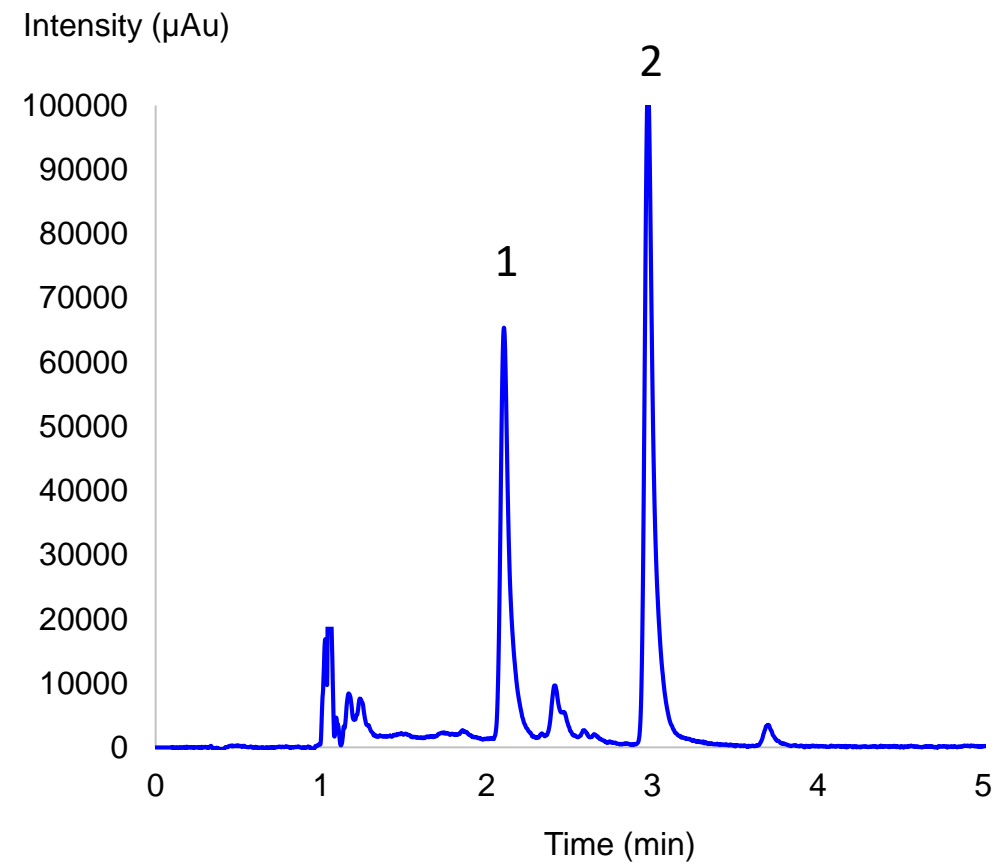


Figure 2

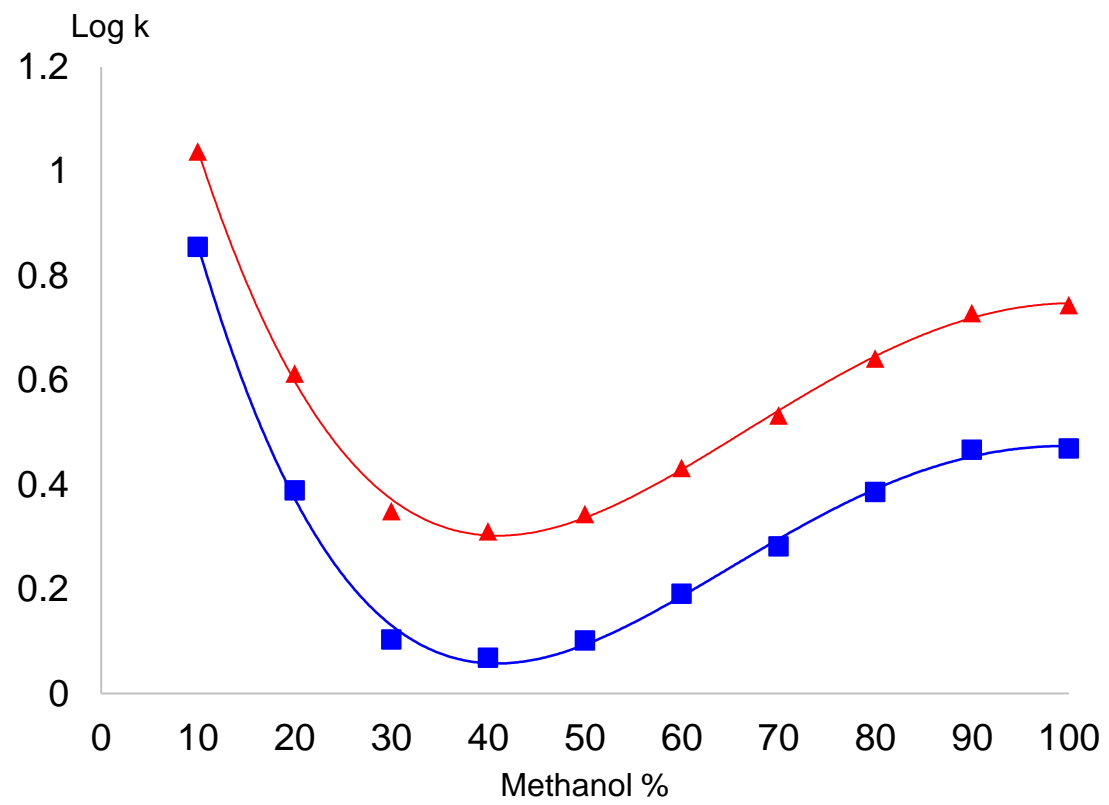


Figure 3



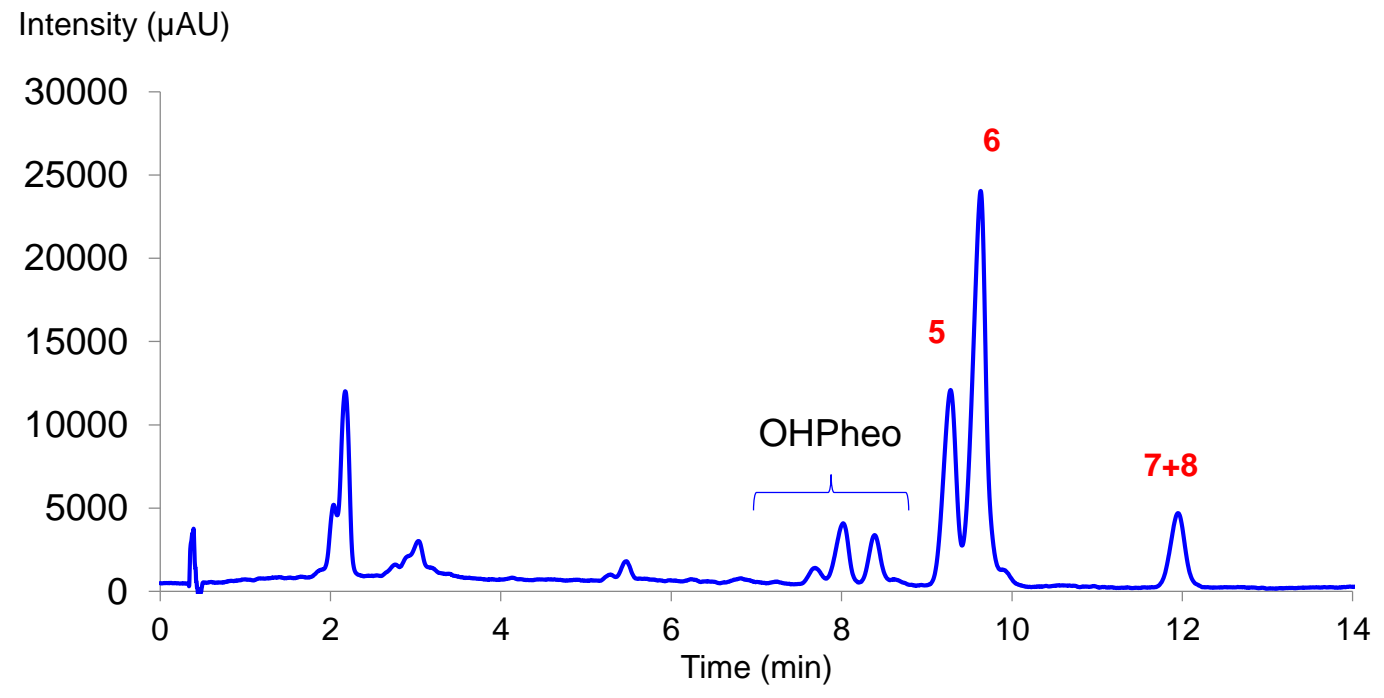


Figure 4

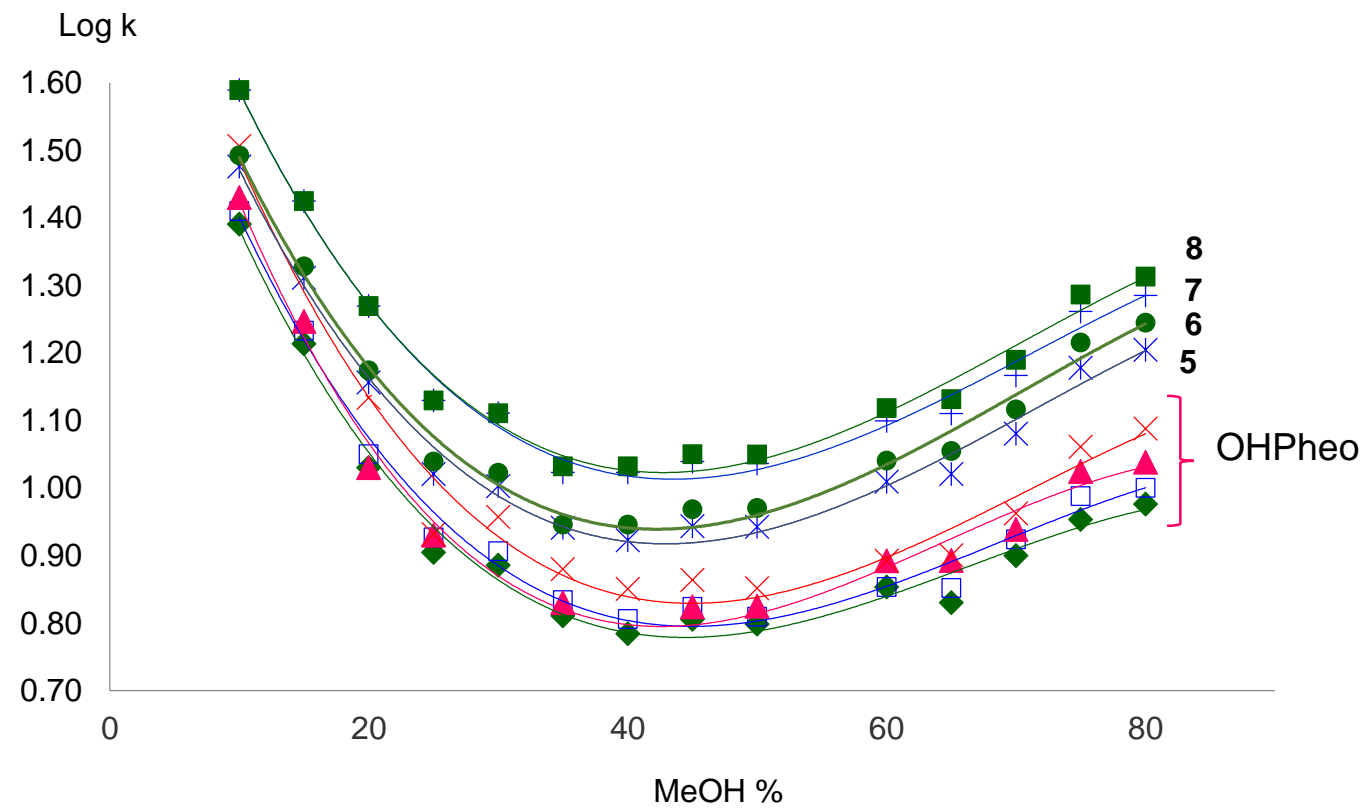


Figure 5

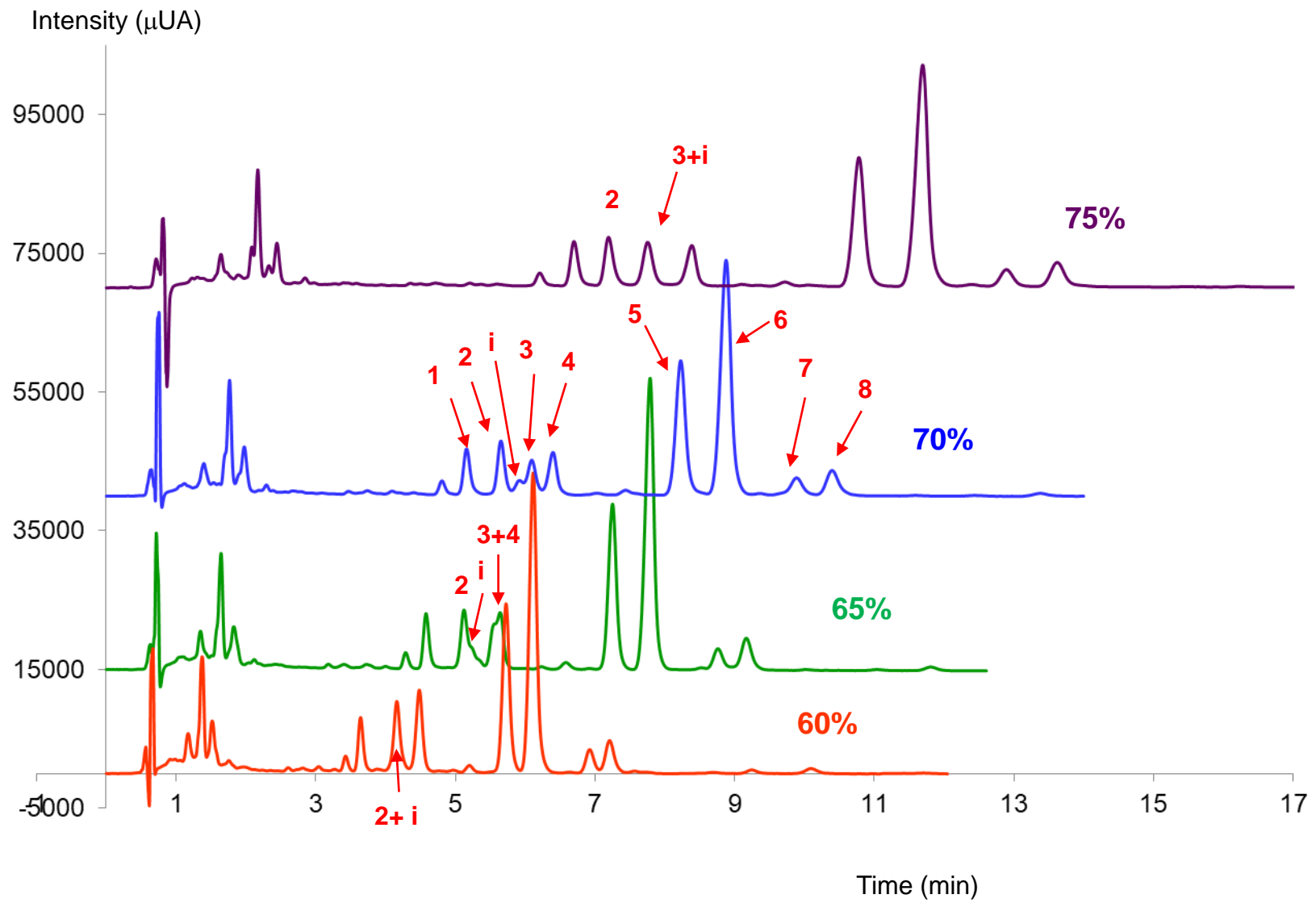


Figure 6

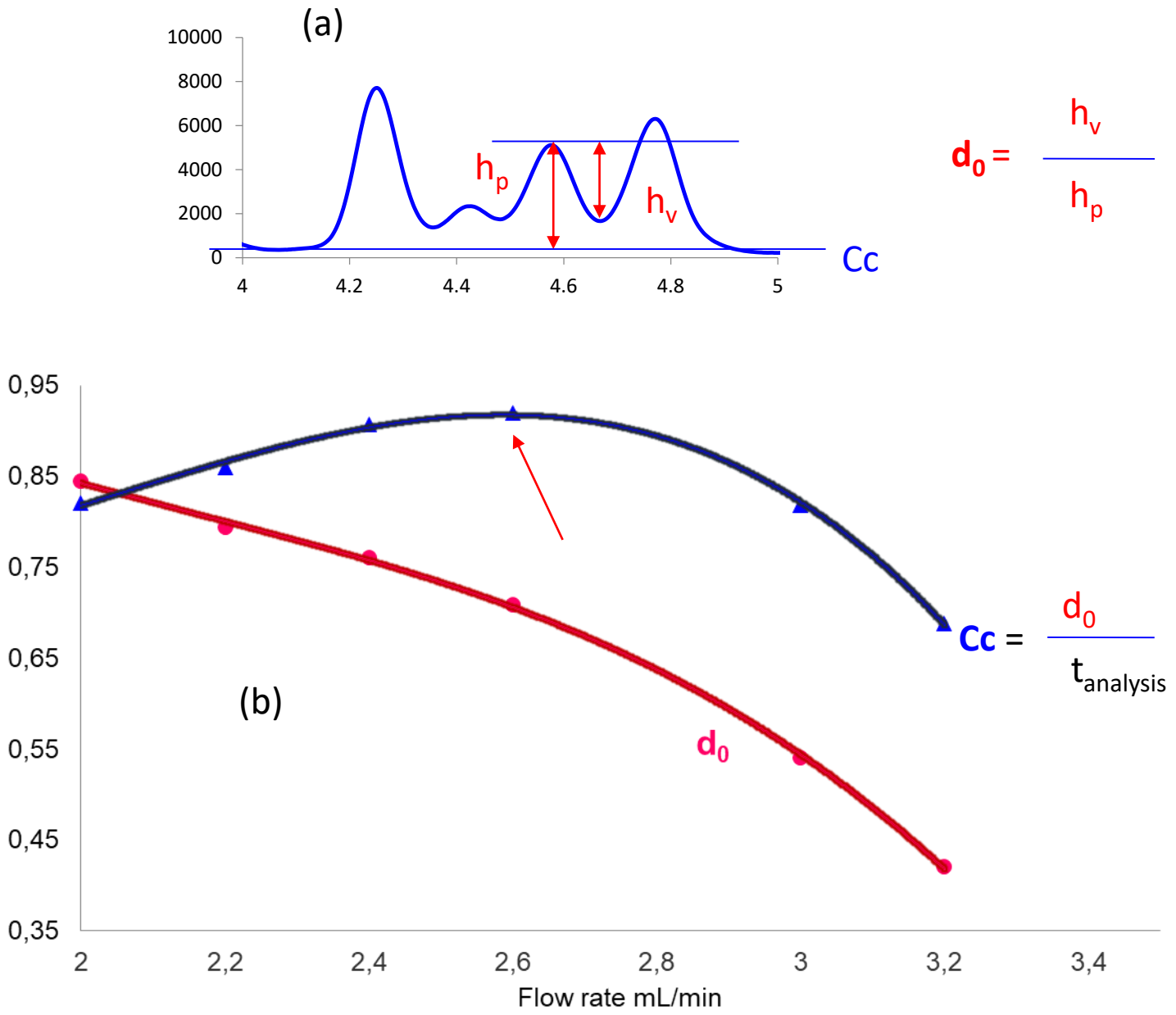


Figure 7

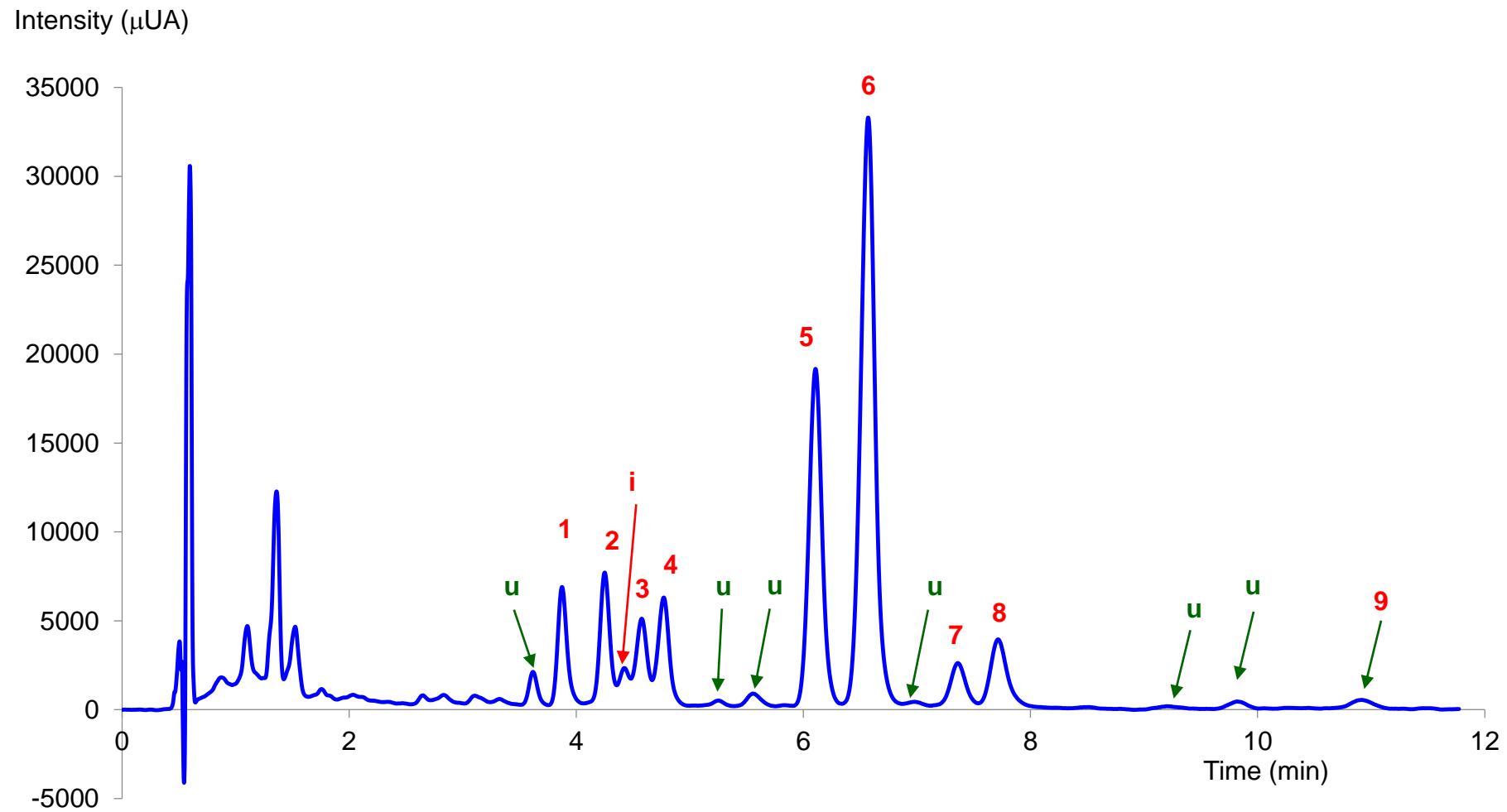


Figure 8

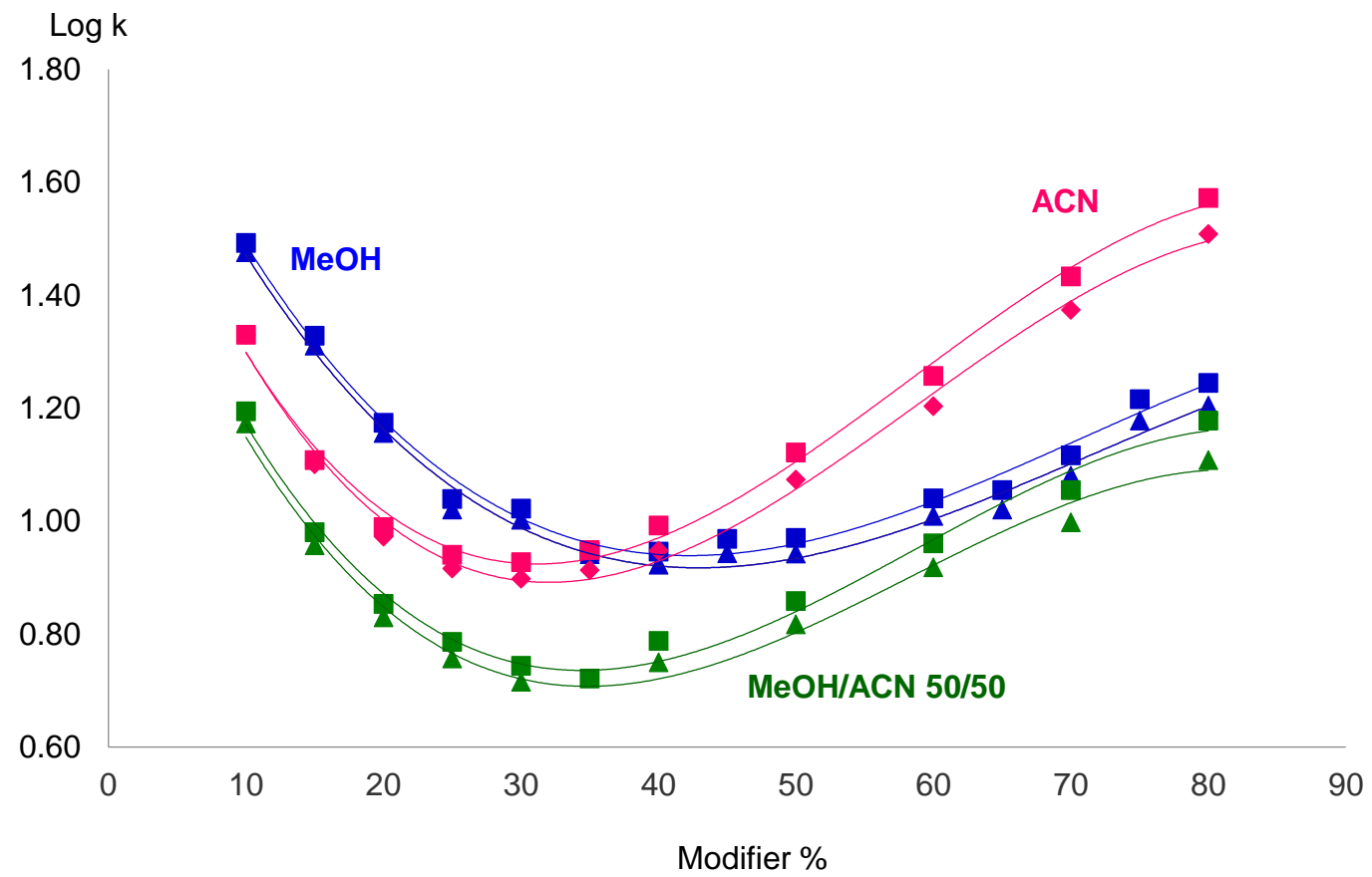


Figure 9

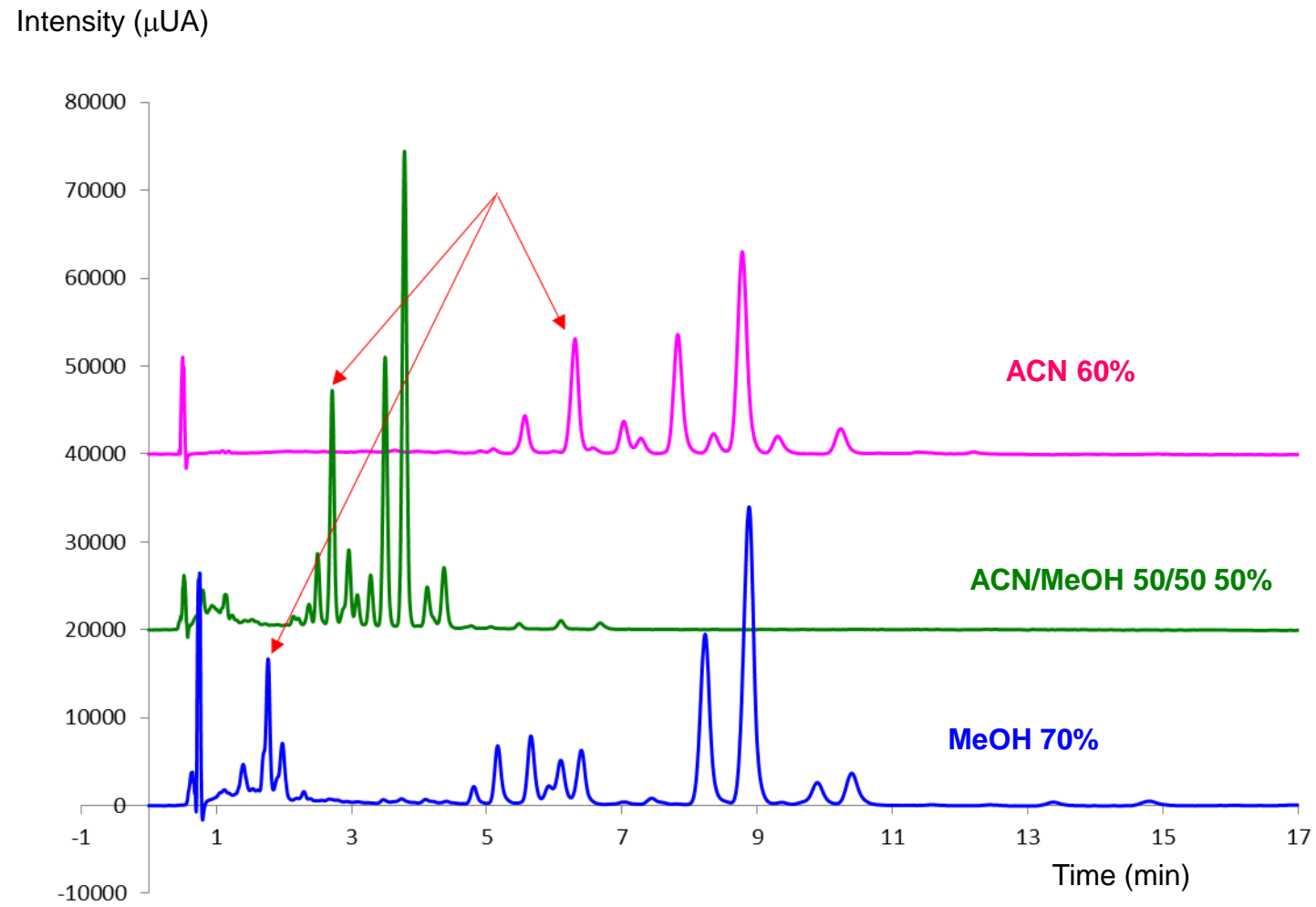


Figure 10

Peak	Identification	RT with method Fig 10 (min)	UV-Vis max, nm	[M+H] <sup>+</sup> m/z calculated	[M+H] <sup>+</sup> m/z detected	Fragments	ref
1	<i>13<sup>2</sup>-Hydroxypheophytine a</i>	3,88	666/407	888	887	869/610	[9]
2	<i>13<sup>2</sup>-Hydroxypheophytine a'</i>	4,26	664/408	888	887		
<i>i</i>	<i>15<sup>1</sup>-Hydroxy-lactone-pheophytine a</i>	4,45	668/400	904	904	565	[9]
3	<i>13<sup>2</sup>-Hydroxypheophytine b</i>	4,59	652/435	902	902	623/605	[9]
4	<i>13<sup>2</sup>-Hydroxypheophytine b'</i>	4,79	656/436	902	902		
5	<i>Pheophytine a</i>	6,11	665/408	872	872	593	[9]
6	<i>Pheophytine b</i>	6,57	665/408	886	886	607	[13]
7	<i>Pheophytine a'</i>	7,37	665/408	872	872	593	[9]
8	<i>Pheophytine b'</i>	7,73	665/408	886	886	607	[13]
9	Pyro-pheophytine a	10,95	665/408	814	814		[33]

Table 1: Identification of phytylated derivatives of chlorophylls by UV/visible and Mass-spectrometry (peak number refer to figure 10)