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CAPILLARY ZONE ELECTROPHORESIS. APPLICATION TO
WINE CHARACTERIZATION BY USING CHEMOMETRICS.**

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**DETERMINATION OF POLYPHENOLS IN SPANISH WINES BY
CAPILLARY ZONE ELECTROPHORESIS. APPLICATION TO WINE
CHARACTERIZATION BY USING CHEMOMETRICS.**

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35

36 **Abstract**

37

38 A capillary zone electrophoresis method for the simultaneous determination of twenty
39 polyphenols in wine was developed. The separation was performed using fused-silica
40 capillaries of 75 μm I.D. and a 30 mM sodium tetraborate buffer solution at pH 9.2
41 with 5% isopropanol as a background electrolyte. A capillary voltage of +25 kV with
42 pressure-assisted (3.5 kPa) separation from min 18 was applied, thus, achieving a total
43 analysis time lower than 20 min. Instrumental quality parameters such as limits of
44 detection (LOD values between 0.3 and 2.6 mg/L), linearity ($r^2 > 0.990$), and run-to-run
45 and day-to-day precisions (RSD values lower than 6.5% and 15.7%, respectively) were
46 established. Three different calibration procedures were evaluated for polyphenol
47 quantitation in wines: external calibration using standards prepared in Milli-Q water,
48 standard addition, and pseudo-matrix matched calibration using wine as a matrix. For a
49 95% confidence level, no statistical differences were observed, in general, between the
50 three calibration methods (p -values between 0.11 and 0.84), while for some specific
51 polyphenols, such as cinnamic acid, syringic acid and gallic acid, results were not
52 comparable when external calibration used. CZE method using pseudo-matrix matched
53 calibration was then proposed and applied to the analysis of polyphenols in 49 Spanish
54 wines, showing satisfactory results and a wide compositional variation between wines.
55 Electrophoretic profiles and other compositional data (e.g., peak areas of selected peaks)
56 were considered as fingerprints of wines to be used for characterization and
57 classification purposes. The corresponding data were analyzed by PCA in order to
58 extract information on the most significant features contributing to wine discrimination
59 according to their origins. Results showed that a reasonable distribution of wines
60 depending on the elaboration areas was found, being tirosol, gallic, protocatechuic, p-
61 coumaric and caffeic acids some representative discriminant compounds.

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66 **Keywords:** Polyphenols, phenolic acids, wines, capillary zone electrophoresis, PCA

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69 **INTRODUCTION**

70

71 Moderate consumption of wine has been associated with reduced risk of
72 cardiovascular diseases and cancer, as well as with several beneficial effects on the
73 human immune system and cognitive functions (1). Health-promoting properties such as
74 anti-oxidant, anti-bacterial, anti-inflammatory, anti-allergic and anti-thrombotic
75 activities have been related with the presence of polyphenols (2). Other phenolic
76 compounds, such as phenolic acids, catechins and some flavonoids play an important
77 role in wine quality, contributing in flavor and color properties, especially on red wines
78 (3,4). Thus, the determination of polyphenols in wines, using reliable methods, for
79 quality control and assessment of wines because of their effects on health and taste of
80 these products is considered at the moment a priority.

81 High performance liquid chromatography (HPLC) has been the technique of
82 choice for the quantification of phenolic compounds in wine using either UV absorption
83 spectroscopy (5-12) or mass spectrometry (LC-MS) (13,14). Other analytical techniques
84 such as gas chromatography coupled to mass spectrometry (15), polycyclic sensors
85 (16,17) or cyclic voltammetry (18) have also been recently reported for the analysis of
86 these compounds.

87 Lately, the utilization of capillary electrophoresis (CE) has increased as an
88 alternative to LC because of his high efficiency, rapid analysis and low reagent
89 consumption. The application of CE to the determination of phenolic compounds in
90 beverages (19) and foods (20,21), including wine, has been reviewed. A specific
91 revision of methods for quantifying resveratrol in wine is also given elsewhere (22). For
92 instance, capillary zone electrophoresis (CZE) methods using phosphate or borate-based
93 electrolytes has been described for the quantitative analysis of phenolic acids (23-28),
94 resveratrol (26,29), flavonols (26,30), catechins (27-30), and different flavonoids
95 (24,31). Other CE techniques, such as micellar electrokinetic chromatography (MEKC)
96 with sodium dodecyl sulfate (SDS) have also been applied to the determination of
97 phenolic acids (32,33) and flavonoids (32-34). However, from the point of view of wine
98 analysis, no more than 10 common polyphenols are usually quantified in many of these
99 works. Some of these CE studies focused solely on the determination of the phytoalexin
100 resveratrol (35-37). Detections often rely on UV spectroscopy using diode array devices,
101 but other techniques such as voltammetry (29), or CE coupled to mass spectrometry
102 (CE-MS) (14) have also been employed.

103 Obtaining reliable quantitative data for the quantification of polyphenols in wine
104 using capillary electrophoresis is still necessary. For instance, some comparisons
105 between the quantitative performance of HPLC and CE methods have been carried out.
106 In some studies, no significant qualitative and quantitative differences in the results
107 were obtained by the two techniques (28). In other cases, small differences were
108 reported (30,31). For this reason, different calibration procedures must be evaluated for
109 polyphenol quantitation in wine samples by CE.

110 The characterization and classification of wines can be tacked from
111 compositional profiles as a source of analytical information. Families of natural wine
112 components such as low molecular organic acids, alcohols, esters, polyphenols, amino
113 acids, biogenic amines and inorganic species have been found to be efficient descriptors
114 of some climatic, agricultural and oenological features. Hence, such compositional data
115 can be treated by chemometric methods such as principal component analysis (PCA)
116 and partial least square regression (PLS) and discriminant analysis (DA) for
117 classification, quantification and authentication purposes (38).

118 This work was aimed at developing and evaluating a CZE method for the
119 simultaneous determination of 20 polyphenols in wine, without any sample treatment.
120 Quality parameters, such as limits of detection (LODs), limits of quantitation (LOQs),
121 linearity, and run-to-run and day-to-day precisions were established by using two
122 different CE instruments. Three calibration procedures (external calibration, standard
123 addition and pseudo-matrix matched calibration) were also evaluated and compared for
124 the analysis of polyphenols in wine samples. The proposed CZE method was applied to
125 the quantification of polyphenols in various Spanish wines. Contents of representative
126 compounds were exploited as potential descriptors of geographical region of wines.
127 Graphs of the wine distribution obtained by using PCA showed significant clustering as
128 a function of origin.

129

130 **MATERIALS AND METHODS**

131

132 **Reagents and solutions**

133

134 Syringic acid, *p*-coumaric acid, homovanillic acid, protocatechuic acid,
135 resveratrol, fisetin, (-)-epicatechin, quercitrin hydrate, and 4-hydroxybenzoic acid
136 standards of analytical grade were obtained from Sigma-Aldrich (Steinheim, Germany).

137 2-(4-hydroxyphenyl)ethanol (Tyrosol), *trans*-cinnamic acid, gallic acid, veratric acid,
138 homogentistic acid, caffeic acid, sinapic acid, ferulic acid, vanillin, and (+)-catechin
139 were purchased from Fluka (Steinheim, Germany), and quercetin dihydrate was from
140 Riedel-de Haën (Seelze, Germany).

141 HPLC-gradient grade methanol and isopropanol were obtained from Merck
142 (Darmstadt, Germany), and sodium tetraborate was purchased from Sigma-Aldrich.

143 Stock standard solutions of all polyphenols (~1000 mg/L) were prepared in
144 methanol. Intermediate working solutions were prepared weekly from these stock
145 standard solutions by appropriate dilution with water. All stock solutions were stored at
146 4 °C for not more than 1 month. Background electrolyte (BGE) was prepared daily by
147 dilution of a 100 mM sodium tetraborate solution, and adding the appropriate amount of
148 isopropanol. BGE solutions were filtered through 0.45 µm nylon filters (Whatman,
149 Clifton, NJ, USA).

150 Water was purified using an Elix 3 coupled to a Milli-Q system (Millipore,
151 Bedford, MA, USA) and filtered through a 0.22 µm nylon filter integrated into the
152 Milli-Q system.

153

154 **Apparatus**

155

156 The experiments were performed on a Beckman P/ACE MDQ capillary
157 electrophoresis system (Fullerton, CA, USA) equipped with a diode array detection
158 system. The electrophoretic separation was carried out using uncoated fused silica
159 capillaries (Beckman) with a total length of 60 cm (effective length 50 cm) x 75 µm I.D.
160 The background electrolyte (BGE) consisted of 30 mM sodium tetraborate buffer
161 solution (pH 9.2) containing 5% (v/v) isopropanol. Capillary temperature was held at 25
162 °C. The BGE was filtered through a 0.45 µm membrane filter, and degassed by
163 sonication before use. Samples were loaded by pressure-assisted hydrodynamic
164 injection (10 s, 3.5 kPa). The electrophoretic separation of polyphenols was performed
165 by applying a capillary voltage of +25 kV. Pressure-assisted separation (3.5 kPa) from
166 minute 18 was used. Direct UV absorption detection was carried out from 190 nm to
167 310 nm (sample quantitation was performed at 280 nm). This CE instrument was
168 controlled using a Beckman 32 Karat software version 5.0. Peak integration was
169 performed valley-to-valley taking into account the baseline shift showed in the
170 electropherograms.

171 To study the method performance, a Beckman P/ACE 5500 CE System
172 (Beckman) was also used. With this instrument, a fused silica capillary with a total
173 length of 57 cm (effective length 50 cm) x 75 μm I.D. was used. This CE instrument
174 was controlled using a Beckman P/ACE station software version 1.2. All other
175 acquisition conditions were equal to those of MDQ CE instrument.

176

177 **Capillary conditioning**

178

179 New capillaries were pretreated with 0.1 M hydrochloric acid for 60 min, water
180 for 60 min, 0.1 M sodium hydroxide for 60 min, and finally they were washed with
181 water for 60 min. At the beginning of each working session, the capillary was rinsed
182 with sodium hydroxide for 30 min, water for 30 min, and with the BGE for 60 min. The
183 capillary was rinsed with BGE for 5 min between runs. At the end of each session, the
184 capillary was stored after rinsing with water.

185

186 **Data Analysis**

187

188 MATLAB (Version 6.5) was used for calculations. PCA was from the PLS-
189 Toolbox (39). A detailed description of this method is given elsewhere (40).

190 The plot of scores showing the distribution of the samples on the principal
191 components (PCs) may reveal patterns that may be correlated to sample characteristics,
192 in this case sample origin. The study of the distribution of variables (loadings' plot)
193 provided information dealing with their correlations and possible relationships with
194 wine properties. Additionally, the simultaneous study of the scores and loadings (bi-
195 plot) was used to explore the relationships between samples and variables.

196

197 **Samples**

198

199 A total of 102 red wines were purchased from a supermarket in Barcelona, Spain.
200 These wines were chosen in two batches: (i) one of 49 wines chosen to get a variety of
201 wines produced in several regions of Spain to establish the CZE method, and (ii)
202 another of 53 wines chosen from three selected Spanish regions (Catalunya, La Rioja
203 and Castilla – La Mancha) to study wine characterization according to their region of
204 origin. All wines were analyzed from freshly opened bottles; determinations were

205 always done in less than 48 hours to preserve polyphenol content. Samples were directly
206 injected into the CE system after a filtration step using 0.45 μm nylon filters (the first 1-
207 2 mL of filtrate were rejected). No further sample treatment was performed. The
208 analytes were identified by comparison of the migration times with those of aqueous
209 standards as well as those obtained by spiking the wines with standards.

210

211 **RESULTS AND DISCUSSION**

212

213 **Optimization of the separation**

214

215 As it has been mentioned in the introduction, most of the works dealing with
216 analysis of polyphenols in wines by CE have been focused on a few compounds (the
217 most abundant ones). However, for wine characterization and better understanding of
218 health-promoting properties, it can be interesting to study the presence of other
219 polyphenols although they may not occur at relatively high concentrations. For this
220 reason, in this study a CZE method was developed for the simultaneous separation and
221 determination of 20 polyphenols in red wines. Borate-based buffers were chosen as
222 BGE for the electrophoretic separation as they provided pH values around 9.2, making
223 them suitable for the separation of this family of compounds in positive polarity mode.
224 However, the addition of organic solvents is mandatory to improve the electrophoretic
225 separation. In this work, a solution of sodium tetraborate containing isopropanol as
226 organic modifier was selected as BGE separation. The optimization of the percentage of
227 organic solvent and electrolyte concentration in the running buffer relied on
228 experimental design. A standard mixture containing the 20 polyphenolic compounds
229 under study was prepared to evaluate the performance of the separation. In this case, a
230 2-factor grid design was defined. Concentrations of isopropanol and borate buffer were
231 assayed at 5 levels (from 1 to 5%, in steps of 1%) and 3 levels (10, 20 and 30 mM),
232 respectively. As a result, a total of 5 x 3 experiments were carried out. The criterion for
233 finding the optimal experimental conditions was based on obtaining the best separation,
234 in terms of number of resolved peaks (N_{peaks}) and resolution (R_s), in the minimum run
235 time (t_{run}). Figure 1 shows the response surfaces obtained for each of the objectives
236 considered. In the case of N_{peaks} , the maximum was achieved at 5% isopropanol and 30
237 mM borate buffer. For R_s of *p*-coumaric and quercetin peaks, two maxima were found
238 which corresponded to 5% isopropanol and, 10 mM and 30 mM borate. For t_{run} , which

239 was estimated from the migration time of the last peak of the electropherogram (2,3-
240 dihydroxybenzoic acid), the faster runs were obtained at 1% isopropanol and 10 mM
241 borate.

242 In order to reach a suitable compromise among these 3 objectives, a combined
243 desirability response was defined as follows: $D = (d_{\text{peaks}} \times d_{\text{res}} \times d_{\text{time}})^{1/3}$, being d_{peaks} , d_{res}
244 and d_{time} the normalized (desirability) contributions of N_{peaks} , R_s and t_{run} , respectively.
245 Experimental values of N_{peaks} , R_s and t_{run} were used to estimate the corresponding
246 individual desirabilities according to the following transformations: (i) $d_{\text{peaks}} = 0$ for
247 $N_{\text{peaks}} \leq 10$, $d_{\text{peaks}} = 1$ for $N_{\text{peaks}} = 20$, and $0 < d_{\text{peaks}} < 1$ for $10 < N_{\text{peaks}} < 20$; (ii) $d_{\text{res}} = 0$
248 for $R_s \leq 0.7$, $d_{\text{res}} = 1$ for $R_s \geq 1.5$, and $0 < d_{\text{res}} < 1$ for $0.7 < R_s < 1.5$; (iii) $d_{\text{time}} = 0$ for
249 $t_{\text{run}} \geq 45$ min, $d_{\text{time}} = 1$ for ≤ 10 min R_s , and $0 < d_{\text{time}} < 1$ for $45 < t_{\text{run}} < 10$, depicts the
250 overall desirability D . The maximum values of this surface were attained at 5%
251 isopropanol and 30 mM borate buffer so these experimental conditions were selected as
252 optimal. Under these conditions, analytes were separated in about 40 min by applying
253 +25 kV. An increase in capillary voltage was not useful to reduce analysis time because
254 the electrophoretic separation worsened significantly. However, as the last migrating
255 polyphenols 4-hydroxybenzoic acid, caffeic acid, gallic acid and 3,4-dihydroxybenzoic
256 acid (peaks 17 to 20, respectively) presented a high separation, an over-imposed
257 pressure of 3.5 kPa was applied at min 18 to reduce the analysis time. Separation was
258 then accomplished in less than 25 min. Figure 2 shows the electropherogram of a 30
259 mg/L standard of all polyphenols obtained under optimal conditions: 30 mM tetraborate
260 buffer with 5% isopropanol as BGE, separation at +25 kV, and pressure assisted
261 separation (3.5 kPa) from min 18. Although some pairs of compounds were not baseline
262 separated (pairs 3/4, 8/9 and 15/16 with resolutions of 0.7, 0.8 and 1.0, respectively), the
263 separation can be considered acceptable as a compromise between resolution and
264 analysis time. Hydrodynamic injection time (2 to 25 s) was also studied in order to
265 increase sensitivity. An injection time of 10 s (3.5 kPa) was selected as an optimal
266 compromise between peak signal and resolution.

267

268 Instrumental quality parameters

269

270 Instrumental quality parameters of the proposed CZE method under optimal
271 conditions were evaluated using two CE instruments. Figures of merit are given in

272 Table 1. LODs, based on a signal-to-noise ratio of 3:1, were calculated using standard
273 solutions at low concentration levels (in the range 0.3-2.6 mg/L). The values obtained
274 are similar to those reported in the literature with CE methods when using UV-detection
275 (26,33). LOQs, based on a signal-to-noise ratio of 10:1, between 1.0 and 8.5 mg/L were
276 obtained. Calibration curves based on peak area at concentrations between 1 and 100
277 mg/L (higher concentrations for some compounds) were established. Good linearity was
278 observed for all compounds with correlation coefficients (r^2) higher than 0.990.

279 Run-to-run and day-to-day precisions for compound quantification, at a
280 concentration level of 30 mg/L (using standard solutions), were calculated by external
281 calibration for the two CE instruments (P/ACE MDQ and P/ACE 5500). In order to
282 obtain the run-to-run precision, five replicate determinations were carried out. Similarly,
283 day-to-day precision was calculated by performing 15 replicate determinations on three
284 non-consecutive days (five replicates each day). To better validate the proposed method,
285 precision was evaluated using two different CE instruments. The RSDs obtained for
286 run-to-run and day-to-day precisions were similar using both CE instruments (in the
287 range 0.6-6.5% and 6.7-15.7%, respectively). These results showed that the proposed
288 method was satisfactory in terms of precision for the quantitative analysis of
289 polyphenols and phenolic acids. Run-to-run precision was also evaluated using pseudo-
290 matrix matched calibration by performing five replicate determinations of a wine
291 sample matrix spiked at two concentration levels (10 and 30 mg/L). RSD values in the
292 range 5.7-11.2% and 3.4-8.9% for concentration levels of 10 mg/L and 30 mg/L
293 respectively were obtained. Pseudo-matrix matched calibration showed better precision
294 as expected because it allows the correction of the baseline shift observed in the wine
295 electropherograms. Finally, Table 1 also shows that good run-to-run and day-to-day
296 precisions of migration times were also obtained, with RSD values lower than 3.4%.

297

298 **Analysis of polyphenols in Spanish wines**

299

300 In order to evaluate the applicability of the proposed method to the
301 determination of twenty polyphenols and phenolic acids in real samples, 49 commercial
302 Spanish wines were analyzed. No sample treatment was applied and the wines were
303 only filtered through 0.45 μm nylon membranes before injection. Figure 3a shows, as an
304 example, the electropherogram obtained for the analysis of a wine sample at three
305 different acquisition wavelengths. As can be seen, electrophoretic profiles of standards

306 are much simpler than those of the wines due to the components of the sample matrix.
307 For this reason, prior to analyze all wine samples, three different quantitation methods
308 were evaluated: (i) external calibration using standards prepared in water, (ii) standard
309 addition, and (iii) pseudo-matrix matched external calibration (using a wine sample as
310 matrix). These three calibration methods were applied to the analysis of five selected
311 wines.

312 First, wine samples were analyzed using standard addition in order to establish
313 the concentration of polyphenols in each sample. All the analyses were performed by
314 triplicate, and the results are given in Table 2. Compound identification was based on
315 the concordance of retention time and UV absorption spectrum with those of the
316 standards. The same samples were then analyzed by external calibration using standards
317 prepared in Milli-Q water, and by pseudo-matrix matched calibration. As no wine free
318 of polyphenols can be found, for pseudo-matrix matched calibration two wines with low
319 concentration of polyphenols were used as sample matrices to prepare all the other
320 standards to be used in the calibration, and concentration of each standard was then
321 calculated taking into account the basal level in the native wine. These analyses were
322 also performed by triplicate with each quantitation method and the results are also given
323 in Table 2. In all cases pseudo-matrix matched calibration provided similar results to the
324 standard addition calibration. External calibration using standards prepared in Milli-Q
325 water seems to give also similar results, or slightly different, than those observed with
326 standard addition. Nevertheless, in order to see if there is any statistical difference
327 between these results, a statistical paired-sample comparison analysis was performed
328 with the results obtained either using external calibration or pseudo-matrix matched
329 calibration procedures with those established by standard addition. For a 95%
330 confidence level, the results achieved with the three calibration procedures were not
331 significantly different, with p -values (Table 2) higher than 0.05 (probability at the
332 confidence level) in all cases. However, it must be mentioned that for some compounds
333 (such as *t*-cinnamic, syringic acid, and gallic acid) in some wines, statistical differences
334 between external calibration and standard addition were observed. In consequence, the
335 optimized CZE method, using pseudo-matrix matched calibration with standards
336 prepared in wine matrix, can be proposed as an economic and rapid method for the
337 analysis of polyphenols in wine samples, providing a good idea of polyphenol
338 concentration levels for wine characterization.

339 Table 3 shows the concentration levels of polyphenols found in 12 of the 49
340 commercial Spanish wines analyzed, and the concentration range observed for each
341 polyphenol, as well as the average concentration and the standard deviation, are also
342 included. As shown in the table, a wide compositional variation was observed. Five
343 polyphenols were found in all the analyzed samples: 2-(4-hydroxyphenyl)ethanol,
344 resveratrol, quercitrin, caffeic acid and gallic acid. Coumaric acid, veratric acid,
345 cinnamic acid, syringic acid, quercetin and 3,4-dihydroxybenzoic acid were also found
346 in almost all wines analyzed. Gallic acid was usually found at relatively high
347 concentrations, with values ranging from 9 to 209 mg/L. 2-(4-hydroxyphenyl)ethanol
348 was also found at relatively high concentrations in most of the samples (from 33 to 145
349 mg/L). The other polyphenols found in the analyzed samples presented, in general,
350 concentration levels ranging from LOD to ~50 mg/L, although in some wines high
351 concentration levels were observed for some specific polyphenols such as homovanillic
352 acid in wines 22 and 23 (155 and 181 mg/L, respectively), epicatechin in wine 49 (154
353 mg/L), or catechin in wines 22 and 24 (66 and 70 mg/L, respectively). Only two of the
354 twenty polyphenols analyzed (sinapic acid and homogentisic acid) were not detected in
355 any sample. It should be pointed out that polyphenol levels found in this work for red
356 wines are, in general, in agreement with those described in the literature for this kind of
357 samples (25,33). The wide compositional variation and number of polyphenols found in
358 the analyzed wines show that the determination of a high number of polyphenols is
359 necessary for a better wine characterization.

360

361 **Principal Component Analysis**

362 The developed CZE-UV method was also evaluated in order to see if either the
363 electrophoretic profile or the polyphenol profile was useful for wine characterization in
364 relation to the region of origin. For this purpose, a batch of 53 Spanish wines from three
365 different regions (Catalunya, La Rioja and Castilla – La Mancha) were analyzed with
366 the proposed CZE-UV (average concentrations for each polyphenol compound are
367 presented in Table 4) and the results were treated by PCA.

368 Raw electrophoretic profiles were firstly evaluated as a source of analytical
369 information for building characterization models. Since electropherograms showed
370 certain degree of variability in the migration time of components the extraction of solid
371 conclusions was hindered. This drawback was solved by peak alignment of
372 electropherograms at each recorded wavelength using Correlation Optimized Warping

373 (COW) written for MATLAB. Owing to the complexity of the electrophoretic profiles,
374 COW was inefficient to deal with peak shifting in the whole time range so the
375 correction was performed on three different time window subsets as follows: 0 to 11
376 min, 11 to 19 min and 19 to 25 min. After COW application, electropherograms at each
377 wavelength were reconstituted and the resulting data sets were analyzed by PCA.
378 Exploratory results showed the predominance of Catalunya and Rioja wines in some
379 parts of the plot of scores although some of the samples appeared in the wrong positions.
380 Regarding Castilla - La Mancha region, samples lay in an intermediate zone and mixed
381 with the other classes.

382 Since the presence of irrelevant data in the set under study may hinder the
383 extraction of reliable conclusion regarding to origin, next step was focused on the
384 selection of discriminat features. In this case, peak areas of the most descriptive peaks
385 were taken as analytical data to be treated by PCA. In particular, the data set consisted
386 of 15 peak areas of known and unknown compounds extracted as follows: 2 peaks at
387 280 nm, 6 peaks at 310 nm and 7 peaks at 370 nm (see Fig. 3a). PCA results showed
388 that PC1 was mainly focused on the description of the peak intensities and variance
389 dealing with geographical characteristics was not retained. Information of the origin of
390 wines was captured by PC2 and PC3. The scatter plot of scores of PC2 versus PC3 (Fig.
391 3b) suggested that wines from Catalunya were located on the right part while Rioja
392 wines appeared on the top and central-left side. Castilla - La Mancha wines were mainly
393 on the left side and they seemed to be less distinguishable from the other classes. The
394 distribution of variables with respect to PC2 and PC3 showed that samples with higher
395 contents of compounds S1, S3, S4 and S6 were typical of Catalunya. Species S9, S14
396 and S15 were quite characteristic of Rioja, and compounds S5, S11 and S12 were more
397 abundant in Castilla - La Mancha wines. Some of these peaks have not been identified
398 yet. For the known components, tirosol, gallic acid were more characteristic of
399 Catalunya, p-coumaric and caffeic acids were encountered at higher levels in Rioja
400 samples and protocatechuic was more specific of Castilla - La Mancha wines.

401

402 The results obtained in this study show that the developed CZE method, using
403 pseudo-matrix matched calibration with standards prepared in wine matrix, can be
404 proposed as a rapid and economic method for the determination of polyphenols in wine
405 samples. The method was applied to analyze these compounds in 49 commercial
406 Spanish wines from different regions. Eighteen of the twenty polyphenols studied were

407 detected and, in most of the samples, quantified, being gallic acid and 2-(4-
408 hydroxyphenyl)ethanol the compounds found at higher concentrations. The peak areas
409 of the most abundant compounds (some of them identified by comparison with
410 standards and some of them unknown) resulted in an excellent source of information to
411 carry out the wine characterization. Results from PCA proved that such compositional
412 data allowed wines to be clustered according to their origins. Besides, the most
413 discriminant analytes representative of each geographical area were identified.

414

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416

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545 **Figure captions**

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547 Figure 1. Simultaneous optimization of isopropanol percentage and borate buffer
548 concentration from a 5×3 grid design. (a) Number of peaks separated; (b) Resolution
549 between *p*-coumaric and quercetin peaks; (c) Run time; (d) Overall desirability.

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551 Figure 2. Electrophoretic separation of an aqueous standard mixture of 20 polyphenols.
552 BGE: 30 mM tetraborate buffer with 5% isopropanol. Capillary voltage: +25 kV,
553 pressure assisted separation (3.5 kPa) from minute 18. Acquisition wavelength: 280 nm.
554 Peak identification: see Table 1.

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556 Figure 3. (a) Electropherograms of a wines sample recorded at 280, 310 and 370 nm. (b)
557 PCA results (score and loading plots) using selected peak areas as analytical data.

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Table 1. Instrumental quality parameters

N°	Compound	LOD (mg/L)	LOQ (mg/L)	Working range (mg/L)	Linearity	run-to-run precision (% RSD, n=5)				day-to-day precision (% RSD, n=3x5)			
						migration time		Concentration ^a		migration time		Concentration ^a	
						MDQ CE	5500 CE	MDQ CE	5500 CE	MDQ CE	5500 CE	MDQ CE	5500 CE
1	2-(4-Hydroxyphenyl)ethanol	0.5	1.7	2-200	>0.990	0.1	0.3	2.1	4.5	0.8	1.6	9.5	11.0
2	Resveratrol	1.6	5.1	5-200	>0.992	0.3	0.1	0.6	1.7	1.2	1.3	6.7	6.7
3	(-)-Epicatechin	2.4	8.0	8-100	>0.990	0.6	0.5	1.2	2.3	0.6	0.8	8.5	9.2
4	(+)-Catechin	2.5	8.1	8-100	>0.996	0.3	0.4	1.5	2.8	0.7	1.2	7.8	8.9
5	Veratric acid	0.3	1.0	1-100	>0.997	0.2	0.4	2.9	4.2	0.8	1.8	12.3	11.5
6	Homovanillic acid	0.3	1.1	1-200	>0.998	0.3	0.2	1.4	2.1	0.6	1.9	11.4	10.7
7	Vanillin	0.7	2.4	2-100	>0.999	0.1	0.3	2.3	6.5	1.9	2.2	10.1	10.3
8	t-Cinnamic acid	0.4	1.4	1-100	>0.998	0.3	0.4	3.1	2.1	0.7	2.2	15.7	13.7
9	Sinapic acid	0.9	3.1	3-100	>0.996	0.2	0.3	2.9	1.5	0.5	2.3	11.6	10.6
10	Quercitrin	0.9	2.8	3-100	>0.990	0.3	0.3	2.5	1.3	0.4	2.5	14.6	11.8
11	Homogentistic acid	0.9	2.8	3-100	>0.998	0.4	0.4	3.9	2.1	0.7	2.7	13.8	10.5
12	Syringic acid	0.6	1.9	2-100	>0.996	0.2	0.6	4.4	2.8	1.5	3.4	11.6	11.3
13	Ferulic acid	0.5	1.8	2-100	>0.998	0.2	0.1	3.6	1.8	1.5	2.3	13.3	13.9
14	Fisetin	0.7	2.2	2-100	>0.999	0.6	0.1	2.7	5.8	0.9	1.0	14.8	10.9
15	p-Coumaric acid	0.7	2.3	2-100	>0.999	0.04	0.1	1.6	3.4	1.4	0.8	14.1	12.7
16	Quercetin	2.6	8.5	8-100	>0.998	0.2	0.2	1.4	2.4	0.7	1.0	10.2	10.6
17	4-Hydroxybenzoic acid	0.4	1.4	1-100	>0.999	0.1	0.1	1.9	2.5	1.3	0.8	9.9	9.8
18	Caffeic acid	0.5	1.7	2-100	>0.998	0.2	0.2	2.8	4.7	2.1	0.9	11.7	10.6
19	Gallic acid	2.1	6.9	7-250	>0.998	0.2	0.1	2.5	4.0	2.1	0.9	12.7	11.1
20	3,4-Dihydroxybenzoic acid	0.6	2.1	2-100	>0.998	0.2	0.2	5.0	4.2	2.2	1.8	10.7	11.6

^a Concentration: 30 mg/L. Quantitation performed by external calibration.

Table 2: Comparison of calibration procedures for polyphenol quantitation in Spanish wines by the proposed CZE method.

N°	Compound	Wine 1			Wine 2			Wine 3			Wine 4			Wine 5		
		EC	SA	pMM	EC	SA	pMM	EC	SA	pMM	EC	SA	pMM	EC	SA	pMM
1	2-(4-Hydroxyphenyl)ethanol	60.2±5.0	56.3±2.0	58.9±4.0	89.7±6.5	75.9±4.3	80.3±6.1	115.1±14.9	98.71±9.9	109.0±9.9	85.1±6.3	86.3±4.3	84.1±2.9	~LOD	~LOD	~LOD
2	Resveratrol	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD
3	(-)-Epicatechin	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD
4	(+)-Catechin	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD
5	Veratric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	Homovanillic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	Vanillin	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	n.d.	n.d.	n.d.	~LOD	~LOD	~LOD	n.d.	n.d.	n.d.
8	t-Cinnamic acid	5.2±0.1	1.0±0.1	1.2±0.1	5.1±0.2	1.5±0.3	2.1±0.3	6.5±0.1	2.5±0.2	2.0±0.2	~LOD	~LOD	~LOD	6.1±0.4	2.3±0.5	3.4±0.5
9	Sinapic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	Quercitrin	20.5±0.4	23.6±1.3	20.4±0.7	12.9±0.1	10.6±0.1	11.9±0.1	34.5±2.5	25.6±0.8	30.2±0.9	21.7±1.7	28.5±5.6	27.0±2.4	15.9±2.0	20.7±3.0	19.0±2.3
11	Homogentistic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	Syringic acid	7.6±0.8	3.2±1.0	4.0±1.0	~LOD	~LOD	~LOD	4.7±0.4	3.1±0.4	2.2±0.4	12.7±2.0	8.0±4.7	9.1±5.0	19.2±0.9	9.6±1.6	10.2±1.3
13	Ferulic acid	~LOD	~LOD	~LOD	16.3±1.9	6.8±0.7	7.5±0.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	~LOD	~LOD	~LOD
14	Fisetin	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	p-Coumaric acid	n.d.	n.d.	n.d.	4.8±0.7	3.3±1.5	6.7±1.5	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	2.5±0.3	5.1±0.8	4.9±0.7
16	Quercetin	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	n.d.	n.d.	n.d.	11.8±0.8	5.1±0.5	3.9±0.5
17	4-hydroxybenzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18	Caffeic acid	~LOD	~LOD	~LOD	9.0±1.2	8.0±1.9	8.9±1.7	2.2±0.3	3.8±1.0	3.9±0.9	5.3±0.8	6.6±1.2	6.6±1.1	8.4±0.7	9.1±0.7	12.7±0.5
19	Gallic acid	43.1±1.9	82.3±19.5	91.0±4.6	71.0±4.2	51.6±4.5	63.1±5.2	100.7±5.4	87.6±1.2	80.5±1.0	45.5±3.6	77.0±23.7	58.4±4.3	35.2±4.2	43.5±16.7	43.8±5.0
20	3,4-Dihydroxybenzoic acid	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD	n.d.	n.d.	n.d.	~LOD	~LOD	~LOD	~LOD	~LOD	~LOD
	<i>p</i> .values ^a	0.48	-	0.46	0.09	-	0.11	0.12	-	0.70	0.35	-	0.38	0.84	-	0.61

All concentrations are in mg/L. Quantitations performed by triplicate (n=3), results expressed as: Concentration mean of samples analyzed ± standard deviation

EC: External calibration; SA: Standard addition; pMM: pseudo-matrix matched calibration

n.d.: not detected

^a for a 95% confidence level

Table 3: Polyphenol concentration levels (mg/L) in Spanish wines obtained by the proposed CZE method.

Nº	Compound	Wine 6	Wine 10	Wine 16	Wine 18	Wine 20	Wine 25	Wine 36	Wine 38	Wine 40	Wine 45	Wine 47	Wine 49	Concentration range	average±st.dev.
1	2-(4-Hydroxyphenyl)etanol	99.4±9.7	125.8±17.5	59.7±2.0	63.4±0.8	77.23±2.1	45.8±1.2	61.1±0.5	70.0±2.6	68.1±1.4	54.2±8.0	145.9±4.9	65.2±2.5	0.3 - 145.9	71.34±22.59
2	Resveratrol	9.4±0.6	~LOD	22.5±0.2	~LOD	23.9±0.1	24.1±0.1	25.6±0.05	25.6±0.2	28.0±0.1	21.2±0.2	20.9±0.01	20.7±0.1	0.8 - 28	18.00±9.78
3	Epicatechin	~LOD	~LOD	16.9±1.7	n.d.	12.9±2.3	13.9±2.0	52.6±17.6	50.3±4.8	15.4±1.2	n.d.	5.1±0.1	2.2±0.04	1.2 - 154.1	24.34±31.93
4	Catechin	~LOD	~LOD	0.7±0.1	n.d.	0.8±0.05	0.6±0.05	n.d.	4.0±0.6	n.d.	11.2±1.1	n.d.	1.2±0.5	0.6 - 86.5	7.99±19.68
5	Veratric acid	7.14±0.8	10.7±1.3	35.6±0.1	9.5±0.0	39.9±6.0	31.0±2.9	19.3±1.6	n.d.	23.4±0.7	2.0±0.05	6.9±0.1	19.6±2.3	2 - 40.6	17.49±11.26
6	Homovanillic acid	n.d.	n.d.	n.d.	n.d.	n.d.	21.6±3.3	n.d.	n.d.	3.6±0.4	n.d.	n.d.	n.d.	2.24 - 181	49.17±68.27
7	Vanillin	n.d.	n.d.	n.d.	n.d.	n.d.	11.0±1.1	16.6±2.1	17.6±0.8	5.8±0.4	n.d.	6.8±0.8	n.d.	0.35 - 21.1	8.39±6.48
8	t-Cinnamic acid	n.d.	2.1±0.1	10.3±0.1	n.d.	n.d.	7.7±0.1	12.6±1.0	12.9±0.4	2.1±0.1	4.3±0.4	6.1±0.1	6.2±0.02	0.2 - 19.7	5.98±4.21
9	Sinapic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
10	Quercitrin	13.0±2.6	3.6±0.5	7.0±1.4	3.5±0.6	13.8±1.3	12.8±0.1	4.9±0.9	11.9±1.0	24.1±0.7	2.6±0.2	8.3±0.9	7.5±0.3	1.4 - 31.9	12.90±7.77
11	Homogentisic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
12	Syringic acid	~LOD	3.9±0.5	5.7±0.2	7.9±0.4	n.d.	3.4±0.5	6.9±0.3	9.3±0.07	1.7±0.3	3.6±0.4	4.6±0.7	5.3±0.4	0.3 - 15.5	4.94±3.19
13	Ferulic acid	~LOD	~LOD	n.d.	10.0±0.4	9.9±0.02	9.7±0.05	12.7±0.6	11.6±0.6	7.5±0.2	n.d.	5.4±0.7	n.d.	0.25 - 15.5	8.69±4.04
14	Fisetin	6.1±0.7	10.6±1.6	n.d.	15.0±0.3	n.d.	n.d.	18.5±1.3	17.4±0.5	7.5±1.2	11.6±2.0	n.d.	n.d.	0.35 - 18.5	10.40±5.17
15	p-Coumaric acid	1.35±0.01	7.9±1.2	16.1±0.6	19.0±0.5	11.7±0.5	8.6±0.5	15.0±0.3	14.4±0.02	6.4±0.3	2.3±0.4	9.1±0.6	11.0±0.3	0.35 - 19	9.37±5.42
16	Quercetin	~LOD	~LOD	30.7±0.1	32.6±1.2	8.5±0.06	30.4±1.0	34.2±0.6	33.9±0.02	33.3±0.9	28.6±0.4	1.6±0.2	1.7±0.3	0.3 - 34.7	18.88±14.36
17	4-hydroxybenzoic acid	~LOD	~LOD	n.d.	n.d.	n.d.	4.9±0.1	n.d.	8.2±0.2	n.d.	n.d.	n.d.	n.d.	0.2 - 13.2	3.92±4.70
18	Caffeic acid	1.9±0.2	4.3±0.1	8.3±0.1	11.1±0.3	9.1±0.02	8.4±0.1	13.9±0.3	12.6±0.3	3.9±0.2	4.5±0.3	2.4±0.3	4.3±0.01	0.25 - 15.6	7.55±3.92
19	Galic acid	57.9±8.1	103.5±3.5	46.7±2.5	59.8±0.9	69.8±2.5	16.3±0.6	35.8±0.3	53.7±2.0	54.6±0.7	111.8±15.7	50.9±7.2	9.1±5.5	9.1 - 209.2	55.40±30.49
20	3,4-Dihydroxybenzoic acid	4.0±0.6	~LOD	9.4±0.9	n.d.	8.8±0.0	6.7±0.4	14.0±0.3	21.45±0.9	13.1±0.7	2.3±0.3	2.9±0.03	4.6±0.1	0.3 - 21.45	7.04±5.12

All concentrations are in mg/L. Quantitations performed by triplicate (n=3), results expressed as: Mean of samples analyzed ± standard deviation

Table 4: Polyphenol concentration levels (mg/L) in the three analyzed regions.

Nº	Compound	Catalunya	La Rioja	Castilla-La Mancha
1	2-(4-Hydroxyphenyl)etanol	77.7±12.7	62.4±15.7	77.9±14.7
2	Resveratrol	22.7±4.4	23.9±1.6	13.1±7.0
3	Epicatechin	58.0±22.7	21.3±14.7	n.d.
4	Catechin	5.7±3.9	2.2±2.6	n.d.
5	Veratric acid	9.1±6.4	23.3±11.6	16.8±10.2
6	Homovanillic acid	n.d.	13.4±8.1	n.d.
7	Vanillin	11.1±5.6	11.9±4.9	n.d.
8	t-Cinnamic acid	7.2±3.1	8.1±3.7	4.9±1.2
9	Sinapic acid	n.d.	n.d.	n.d.
10	Quercitrin	12.7±6.6	12.7±9.1	11.3±7.6
11	Homogentisic acid	n.d.	n.d.	n.d.
12	Syringic acid	6.0±2.5	5.6±2.0	7.2±5.4
13	Ferulic acid	9.2±2.9	11.0±2.6	7.7±5.9
14	Fisetin	13.7±3.8	15.2±2.2	7.9±2.0
15	p-Coumaric acid	7.5±4.5	13.9±3.7	7.5±6.1
16	Quercetin	31.2±1.8	31.5±1.6	15.2±12.4
17	4-hydroxybenzoic acid	10.7±3.5	6.3±4.3	n.d.
18	Caffeic acid	7.9±3.0	9.0±3.0	6.2±3.8
19	Gallic acid	51.4±26.4	49.4±21.4	42.5±17.1
20	3,4-Dihydroxybenzoic acid	3.0±2.0	3.5±2.3	12.6±3.7

Results expressed as: Mean of samples analyzed ± standard deviation.

Figure 1

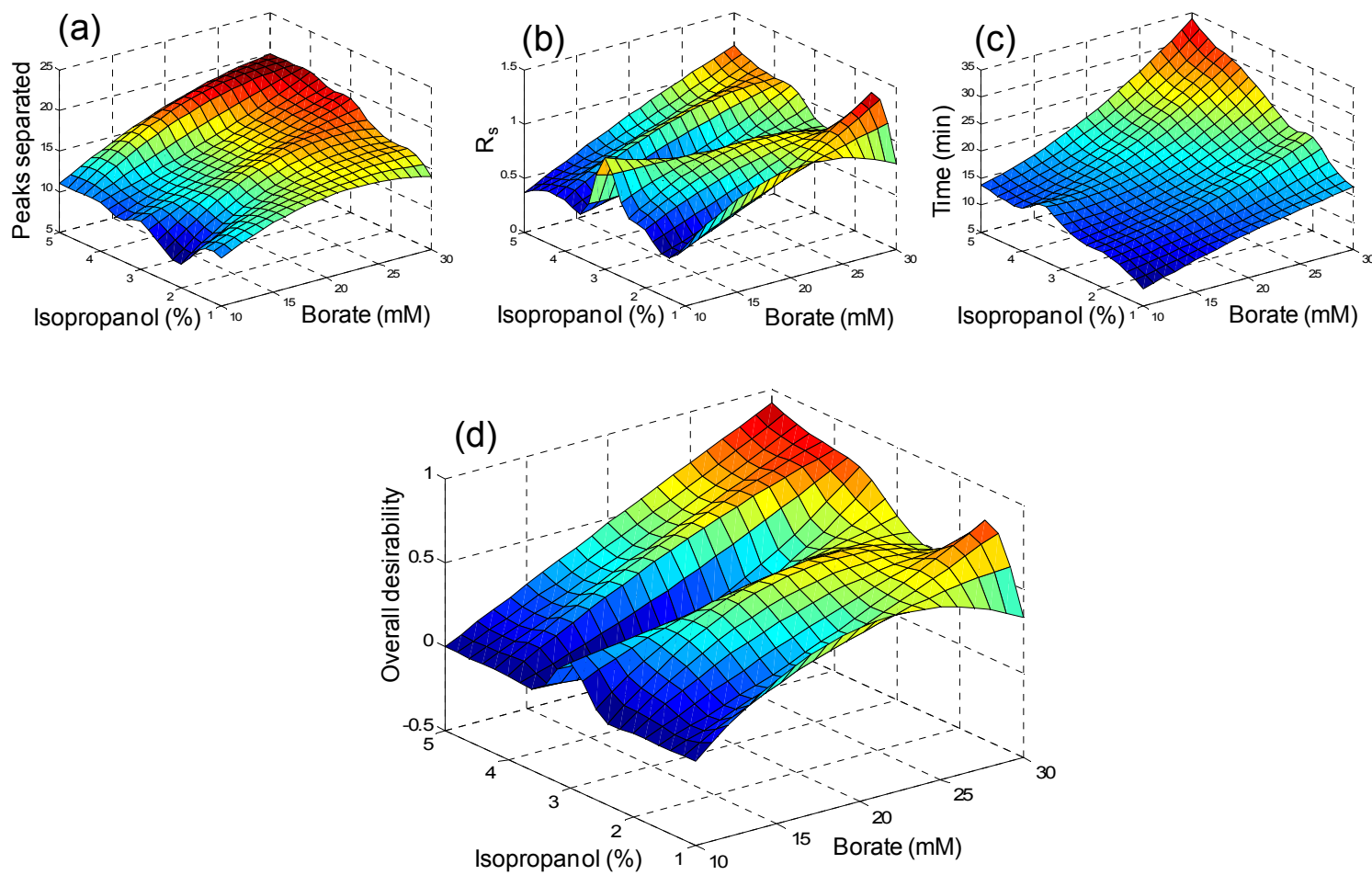


Figure 2

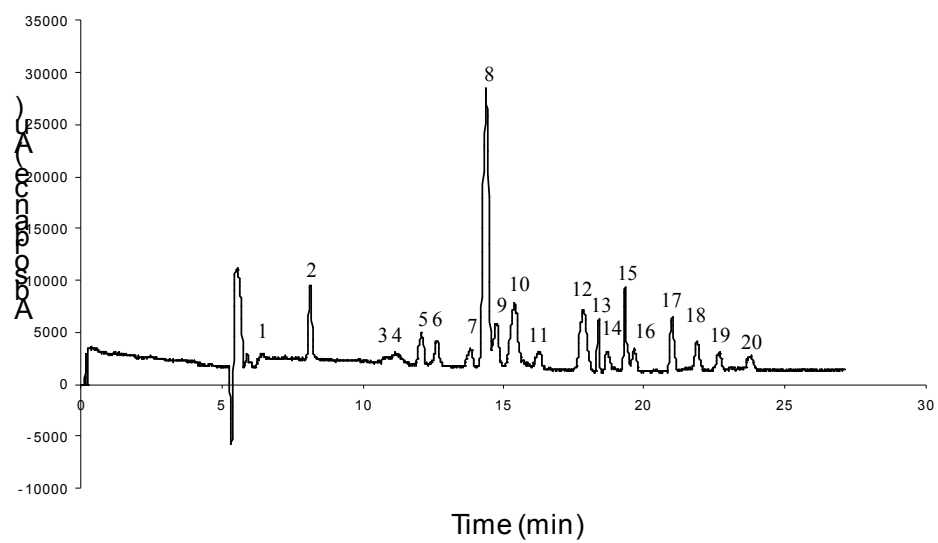
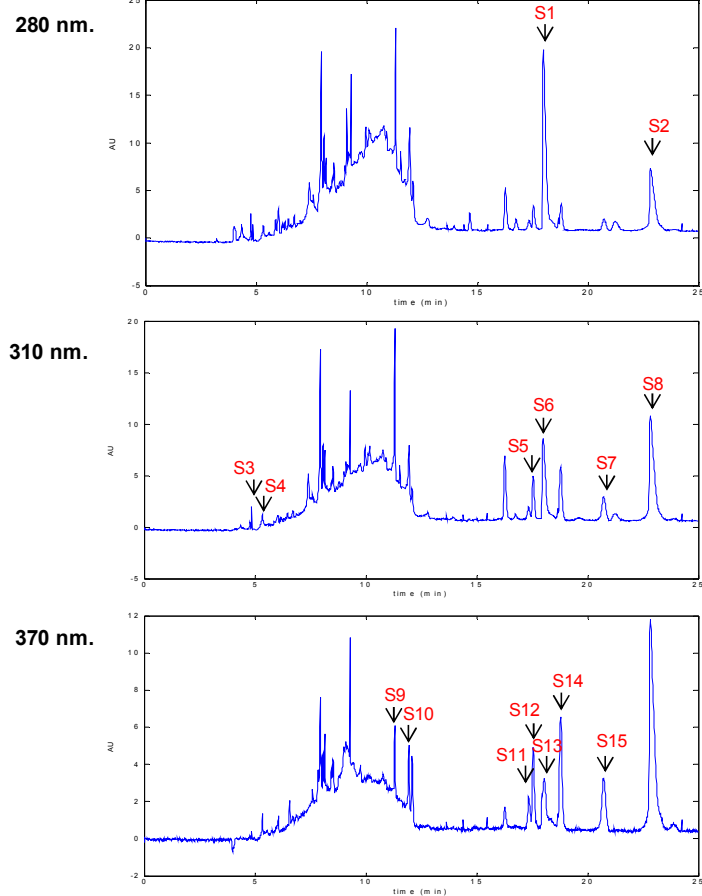
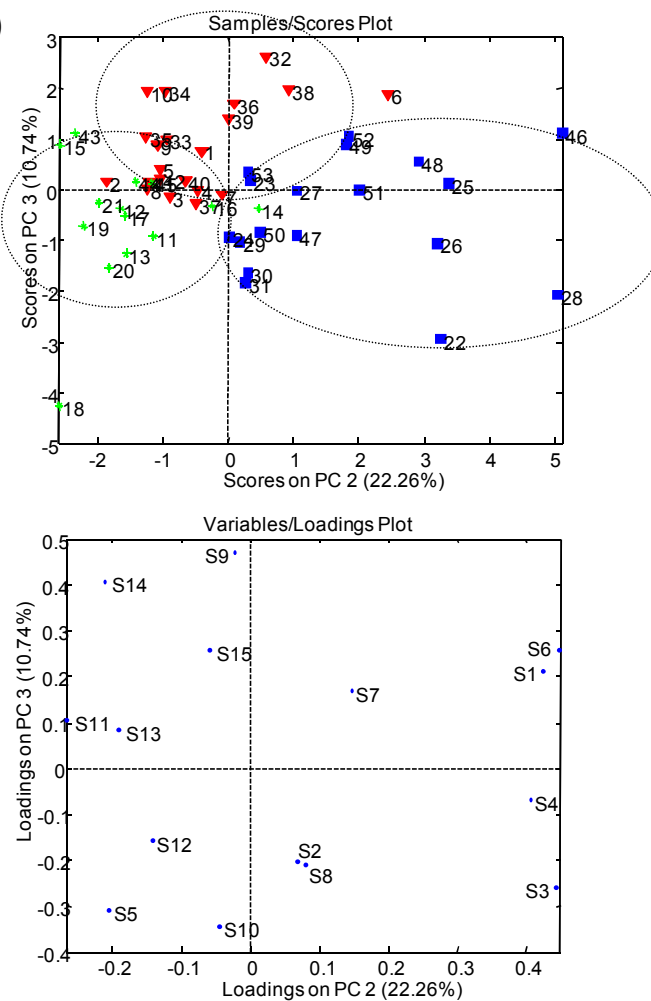


Figure 3

(a)



(b)



TOC Figure

