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Running title: Fast liquid chromatography determination of polyphenols in vegetables

Fast simultaneous determination of prominent polyphenols in vegetables and fruits by reversed phase liquid chromatography using a fused-core column

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Abstract

A reversed-phase high-performance liquid chromatography method with photodiode array detection has been developed enabling the joint determination of 17 prominent flavonoids and phenolic acids in vegetables and fruits. A multi-segmented gradient program using a fused-core column for the separation of several phenolic classes (phenolic acids and flavonoids) has been optimized. The influence of extraction conditions (sample freeze-drying, ultrasound extraction, solvent composition and extraction time) has been also optimized using response surface methodology with tomato samples as a model. Complete recoveries (76-108 %) were obtained for the phenolic compounds present in tomato. The developed method provided satisfactory repeatability in terms of peak area (RSD < 2.9 %) and retention time (RSD < 0.2 %) both for standards and real samples. Detection limits ranged between 3 and 44 μg kg^{-1} for the detected polyphenols. This method is recommended for routine analysis of large number of samples typical of production quality systems or plant breeding programs.

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1. Introduction

Regular consumption of fruit and vegetables has been associated with reduced risk of certain types of cancer, cardiovascular diseases and other functional declines associated with aging and modern lifestyle. It seems that phenolic compounds may play an important role in the antioxidant activity found in these products (Liu, 2003). Several epidemiological studies have shown a direct relationship between the intake of fruits, vegetables and their products, which are rich in polyphenols, and a protective effect against these diseases (Arts & Hollman, 2005; Petti & Scully, 2009).

Polyphenols are secondary plant metabolites widely distributed in plant tissues, being usually accumulated in fruit skins (Torres, Davies, Yañez, & Andrews, 2005). The polyphenol profile and concentration depend largely on the species considered. For instance, in tomato (S*olanum lycopersicum* L.), chlorogenic acid is the main phenolic acid, and the main flavonoids are rutin, naringenin and myricetin (Helmja, Vaher, Püssa, Raudsepp, & Kaljurand, 2008; Martínez-Valverde, Periago, Provan, & Chesson, 2002; Sakakibara, Honda, Nakagawa, Ashida, & Kanazawa, 2003). On the other hand, in bell pepper the main flavonoids and phenolic acids are quercetin and luteolin glycosides; onion accumulates quercetin and its glycosides; eggplant chlorogenic and ferulic acids; orange hesperidin and naringenin glycosides, etc. (Miean & Mohamed, 2001; Sakakibara et al., 2003).

Consumers are aware of the functional characteristics of agricultural food products, and more consumers choose foods considering their healthy characteristics. Thus, there is an increasing attention in the development of new antioxidant-rich varieties via breeding programs (Goldman, 2011). A great effort has already been done in the case of carotenoids, and right now phenolic compounds are receiving more attention. In order to develop breeding programs to achieve this target or to develop quality controls of food products, it is necessary the presence of rapid and inexpensive analytical procedures for the quantitation of the main flavonoid and phenolic acids present in each species.

59 Several analytical methods have been published for the determination of these compounds in
60 food samples. The most widely used are based on reversed-phase high-performance liquid
61 chromatography (RP-HPLC) coupled with UV–vis detection and/or mass (LC-MS) or tandem
62 mass spectrometry (LC-MS/MS) (Barros et al., 2012; De Paepe et al., 2013; Helmja et al., 2008);
63 however, the number of published studies dealing with LC-MS and LC-MS/MS techniques and
64 the possibility of access to these technologies for most laboratories are so far limited.
65 Most of the chromatographic procedures developed for the simultaneous measurement of
66 phenolic acids and flavonoids in foods require long analysis times (1 hour or more per sample)
67 (Merken & Beecher, 2000; Sakakibara et al., 2003), or they are focused on a single or a few
68 groups of phenolic structures (Mattila & Kumpulainen, 2002; Repollés, Herrero-Martínez, &
69 Ràfols, 2006). In addition, the most above-reported RP-HPLC methods did not have taken full
70 advantage of recent advances in LC instrumentation (Nováková & Vlčková, 2009). Indeed, it
71 could be beneficial to further improve chromatographic performance in terms of throughput
72 and/or resolution particularly when numerous complex food extracts have to be analysed. In
73 this context, several analytical strategies related to column technology have been developed in
74 HPLC, including the use of monolithic supports, packed columns with sub-2 µm particles
75 operating at ultra-high pressure (UHPLC) or with core-shell or fused-core particles. Advantages
76 of this latter technology are the ability to reach high peak efficiency (even at higher flow rates)
77 without the necessity to use instrumentation and consumables of higher costs required for
78 sub-2-µm particles (McCalley, 2010). However, only few works have used this core-shell
79 particle technology for the analysis of phenolic compounds, mostly focused in tea samples
80 (Rostagno et al., 2011).
81 Several extraction techniques have been also developed for the isolation of phenolic
82 compounds, namely ultrasound-assisted extraction (UAE) (Jerman, Trebše, & Vodopivec,
83 2010), supercritical fluid extraction (Adil, Cetin, Yener, & Bayındırlı, 2007), microwave-assisted
84 extraction (Li et al., 2012) and pressurized liquid extraction (Alonso-Salces et al., 2001). These

techniques reduce considerably the consumption of solvents, increase the speed of the extraction process and simplify it. Among these, ultrasound-assisted extraction is an inexpensive, simple and efficient alternative to conventional extraction techniques. Despite of the large number of investigations made, there is still a great interest in the development of analytical procedures for an easy, inexpensive and quick extraction and determination of phenolic acids and flavonoids in vegetable and fruit and samples.

In this work, a methodology to separate and quantify simultaneously the most representative phenolic compounds in several vegetables and fruits, using an UAE protocol followed by RP-HPLC analysis with diode array detection has been developed to cover this demand. For this purpose, gradient elution conditions were optimized to achieve a rapid separation of phenolic compounds of interest. Additionally, the extraction procedure was also optimized using a response surface methodology (RSM) to obtain the optimum extraction conditions of tomato polyphenols by considering the effects of freeze-drying, MeOH-H_2O proportion in the extraction solvent, extraction time and ultrasound-assisted extraction. The optimized method was carefully validated and applied to the quantitation of different vegetable and fruit matrices.

2. Material and methods

2.1. Chemicals and reagents

The standards of phenolic compounds: gallic acid, caffeic acid, p-coumaric acid, trans-ferulic acid, benzoic acid, chlorogenic acid, (+)-catechin, kaempferol, quercetin, myricetin, naringenin, genistein, luteolin, apigenin, rutin, naringin and hesperidin were purchased from Sigma-Aldrich (Syeinheim, Germany). Butylated hydroxytoluene (BHT), formic acid and HPLC-grade methanol (MeOH) were also supplied by Sigma. HPLC-grade acetonitrile (ACN) was purchased from Panreac (Castellar del Vallés, Spain). Water was purified on a Milli-Q water system (Millipore, Molsheim, France). Stock solutions of polyphenols were prepared in a methanol/water mixture (80:20, v/v) at 500 mg L^{-1}, except for apigenin and hesperidin, which was prepared in a mixture of methanol/acetonitrile (70:30, v/v). All stock solutions were stored at -20ºC until their use and protected from light. Prior to injection, working solutions (25 mg L^{-1}), were prepared daily by dilution of stock solutions with mobile phase, and filtered through a 0.2 µm pore diameter PTFE filter.

2.2. Instrumentation and conditions

A 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany), equipped with a quaternary pump, a degasser, a thermostatic autosampler, and a diode array detector (DAD), was used to separate the analytes. The analytical column was a fused-core Kinetex-XB C18 column (150 mm×4.6 mm i.d.; particle size, 2.6 µm) from Phenomenex (Torrance, USA). The column and guard column were thermostatically controlled at 35ºC, the flow rate was kept constant at 0.9 mL min^{-1} and the sample injection volume was 10 µL. The mobile phase solvents consisted of water, ACN and MeOH, each of them containing 0.1% (v/v) of formic acid. Detection and quantification was performed at 255 nm (for genistein and rutin), at 280 nm (for gallic and benzoic acids, catechin, naringin and hesperidin), at 290 nm (for naringenin), at 320 nm (for caffeic, p-coumaric, ferulic and chlorogenic acids) and at 365 nm (for kaempferol,

127 quercetin, myricetin, luteolin and apigenin). Each polyphenol UV-vis spectrum was also
128 recorded using a DAD detector for the identification of the studied compounds. Peak purity
129 was studied with the ChemStation Rev B.03.01 software (Agilent Technologies, Waldbronn,
130 Germany). In addition, samples were spiked in order to corroborate the peak identification.
131
132 *2.3. Plant material*
133 Fresh tomatoes from an experimental line ("Fortuna-C"), two highly consumed cultivars
134 ("Pera" and "Kumato®") and an accession of a wild species related to tomato (*Solanum*
135 *neorickii* D.M: Spooner, G.J. Anderson & R.K. Jansen, S.) were used. Standard cultivars
136 commonly available at local markets were used for onion, celery, grape, green pepper (Italian
137 type), red pepper (Lamuyo type), eggplant, muskmelon (Piel de sapo type), apple (cv. "Fuji")
138 and orange. Organic soy milk (13.2% peeled soy seeds blended in water) from a local
139 supermarket was also analysed. For each sample, the edible part was processed. When the
140 skin was included in the sample, it was previously washed with tap water. Sample was ground
141 in an 1100W blender until it was completely homogenized and stored at -80ºC until analysis.
142 When required a SilentCruher M homogenizer (Heidolph, Schwabach, Germany) was also used.
143 "Fortuna-C" and *S. neorickii* accession were provided by the Genebank of the Instituto
144 Universitario de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV, Spain).
145 Other fruits and vegetables were purchased in local markets. Samples were extracted using
146 the optimized extraction procedure.

147 In order to provide a supplementary quantification of free aglycones the extracts were also
148 hydrolyzed. For this purpose, a slight modification of the conditions reported by Hertog et al.
149 (1992) was adopted. HCl was added to the extracts at final concentration of 1.5M and
150 hydrolysis was performed at 90°C for 90 minutes. Each sample was analyzed twice. In order to
151 discard negative effects of hydrolysis conditions on flavonoid aglycones recoveries after
152 hydrolysis were studied in tomato, obtaining recovery values of 99% for quercetin and 76% for

153 naringenin. These values are similar to those reported in the original method (e.g. 98% for
154 quercetin) by Hertog et al. (1992).

155

156 *2.4. Extraction procedures*

157 The effect of several extraction conditions was examined using tomato as a reference matrix. A
158 two stage study was conducted. The first stage was aimed to select the factors and their
159 experimental range relevant for phenolic extraction. The second stage analyzed in depth only
160 the most relevant factors to determine the optimal extraction conditions. In the first stage,
161 factors analyzed included: MeOH:H_2O proportion of extraction solvent (mixture variable),
162 extraction time (numerical variable) from 10 to 180 minutes, ultrasound assisted extraction vs
163 standard solid-liquid extraction (categorical variable) and the use of fresh vs. freeze-dried
164 samples (categorical variable). In the case of MeOH:H_2O mixtures a range from 30% to 80%
165 MeOH was studied considering preliminary works. The response variables used were
166 chlorogenic, caffeic acid, p-coumaric acid, ferulic acid, rutin and naringenin, as they were the
167 polyphenols that could be quantified in the tomato variety selected for this assay and with the
168 optimized HPLC procedure, and had been described as main tomato polyphenols in previous
169 literature (Martínez-Valverde et al., 2002). A combined D-optimal design with experimental
170 point determination by point exchange method (Anderson & Withcomb, 2005) was obtained
171 and analyzed with Design Expert Software (Version 9.0, Stat-Ease, Inc., Minneapolis, USA). This
172 design used 44 runs in three blocks. A combined regression model (cubic for the mixture factor
173 and quadratic for the other factors) was adjusted and fittings to the data were checked with
174 ANOVA. In the second stage, the factors considered were the composition of the extraction
175 solvent and the extraction time (90 to 180 minutes), fixing the use of fresh samples and
176 ultrasound assisted extraction. A similar combined D-optimal design with 22 runs was used.
177 The optimum conditions of extraction were determined using a weighted desirability function
178 (targeted to find a compromise maximizing the extraction of each of the polyphenols analyzed)

179 in conjunction of variable sized simplex algorithm (Anderson & Withcomb, 2005). The
180 verification of the validity and adequacy of the predictive extraction model was checked with
181 the optimum conditions of extraction (three replicates) comparing predictions with observed
182 values using a two sided t-test (α= 0.05).

183 For extraction, approximately 1 g of fresh homogenized sample or 0.05g of freeze-dried
184 sample was weighted and 5 mL of MeOH/water (30 to 80% MeOH) containing 0.1% BHT (w/v)
185 was added. When appropriate, homogenized samples were freeze-dried using the freeze dryer
186 ALPHA 1–2LD plus (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz,
187 Germany) during 24 hours at 1.5 mbar. For ultrasound extraction, samples were immersed in
188 an ultrasonic bath Transsonic T470/H (Elma Electronics AG, Wetzikon, Switzerland) at a
189 frequency of 35 KHz, and room temperature during different extraction times. For
190 conventional solid-liquid extraction, samples were stirred using a swing agitator (Ovan,
191 Badalona, Spain). All extraction procedures were performed in absence of light to avoid the
192 oxidation of target compounds. The resulting extracts were centrifuged at 4000 rpm (2361 g)
193 at 4ºC during 5 minutes. The supernatants were filtered through a 0.2 μm pore size PTFE filter
194 prior to their analysis by HPLC. Orange extract was diluted 1/20 v/v.

195

196 *2.5. Method validation*

197 The method was validated using standards and tomato samples. Linear calibration curves with
198 a minimum of 6 levels, comprising concentrations between 0.1 to 20 mg L^{-1} were obtained. The
199 optimized procedure of extraction was used for the validation using tomato samples. The LODs
200 were calculated for the polyphenols present in tomato extracts as the lowest concentration
201 that provided a signal-to-noise ratio (S/N) equal to 3.

3. Results and discussion

3.1. Optimization of chromatographic conditions

Due to the wide range of polarity of phenolic compounds, a gradient elution system was developed. Simple linear gradients were first considered, by using ACN or MeOH as organic modifier. Fig. 1a shows the separation of polyphenols obtained under ACN-based mobile phase. The gradient elution conditions selected were as follows: a linear gradient started with 30% ACN and raised to 50% in 20 min followed an increase up to 100% ACN in 23 min. Under these gradient conditions, the analytes eluted in less than 10 min, but several overlapping peaks were obtained. In order to improve the resolution the eluotropic strength in initial mobile phase composition was reduced to 20% ACN (Fig. 1b). With these conditions, the early eluting compounds were reasonably well separated; however, the separation remained critical for luteolin/quercetin pair and also for naringenin, genistein and apigenin. Changes in final mobile phase composition (reduction in elution strength) or the use of lower gradient slopes did not offer a significant improvement in the resolution of these peaks and led to a noticeable increase in the analysis time (45-50 min) which did not offer any improvement compared to other procedures that required similar analysis times (Merken & Beecher, 2000; Sakakibara et al., 2003). In addition, different temperatures were tested (15, 25, 35 and 45ºC) to improve the separation. Although lower temperatures offered a better separation for quercetin and luteolin, the rest of problematic compounds were not resolved and lower temperatures led to an increase in the backpressure of the system. Thus, a temperature of 35ºC was selected as a compromise between the best resolution and a moderate pressure.

Alternatively, MeOH/water mixtures were employed as mobile phase. Fig. 1c shows the chromatogram obtained under the same gradient conditions as Fig. 1a by replacing ACN for MeOH as organic modifier. These new conditions were effective for separating a wide range of phenolics, with good resolution; however, the separation of quercetin/naringenin and luteolin/genistein pairs was not feasible.

228 In order to resolve the overlapping pairs of peaks and taking into account the different
229 selectivity offered by each eluent system, ternary mobile phases, composed by
230 MeOH/ACN/water mixtures, were investigated. In order to speed up the elution, multi-
231 segmented gradients were also tested.
232 A well-resolved chromatogram of phenolic compounds (including hesperidin, a flavonoid not
233 included in the previous attempts) obtained under the best elution conditions is shown in Fig.
234 1d. The analysis time for the 17 studied polyphenols was reduced to 20 minutes, which means
235 an improvement with respect to other authors (Merken & Beecher, 2000; Sakakibara et al.,
236 2003), who reported separation of similar compounds in 66 and 95 minutes respectively. Other
237 methods such as reported by Vallverdú-Queralt, Jáuregui, Di Lecce, Andrés-Lacueva, &
238 Lamuela-Raventós (2011) also employed 20 minutes for the analysis of only flavonoids in
239 tomato derivatives but, in this case, the equipment required (HPLC-ESI-QTOF) involved a
240 considerably higher investment, which is not usually available in common laboratories.
241 Final conditions included a multi-segmented gradient with a linear gradient starting with 30%
242 MeOH and 0% ACN and ending with 24% MeOH and 18% ACN at minute 12, a raise of MeOH
243 concentration up to 30% until minute 13, maintaining the obtained 18% ACN concentration
244 and finally, from minute 13 to minute 20, a decrease in MeOH and ACN concentrations down
245 to 20% and 10% respectively.

246 *3.2. Extraction optimization*

247 To optimize the extraction procedure, tomato samples were selected and the influences of
248 different variables on several phenolic compounds (chlorogenic, caffeic, p-coumaric and ferulic
249 acids and rutin and naringenin) were considered.

250 In a preliminary phase, an extensive study was performed to identify the most important
251 factors affecting extraction efficiency. Factors included in the design were extraction solvent
252 composition (MeOH:water, factors A and B respectively), extraction time (factor C), agitation
253 or ultrasound-assisted extraction (factor D) and sample preprocessing (fresh vs. freeze-dried
254 sample, factor E). The response surface models developed to explain the influence of these
255 factors on extraction efficiency were all significant ($p < 0.01$) and the adjusted determination
256 coefficients obtained (R^2adj) showed that, in general, an adequate explanation of the
257 extraction results was obtained (Table 2). The models showed that, generally, solvent
258 composition had a high influence in the extraction procedure. This was the main factor for all
259 the polyphenols, except for rutin. For this compound, the main factors were ultrasound vs.
260 agitation and sample pretreatment (fresh vs. freeze-drying). The factor extraction time was not
261 detected as a significant single factor, but it influenced extraction efficiency through
262 interactions with other factors, mainly solvent composition. The optimum conditions of
263 solvent composition and extraction time were compound dependent and required a specific
264 study in a more limited experimental region of response with better design point coverage.

265 Regarding the effect of ultrasound-assisted extraction vs. agitation and fresh vs. freeze-drying
266 samples, narigenin was considerably affected, while the rest of the compounds showed lower
267 levels of variation (the best MeOH:H_2O and time conditions can be observed in Table 2). For
268 naringenin extraction, the use of fresh sample and ultrasound-assisted extraction provided the
269 maximum extraction. Other conditions led to a considerably reduction in naringenin
270 extraction. The better efficiency of ultrasound-assisted extraction would be related to the

271 effect of ultrasound waves breaking the cells and releasing their contents (antioxidants among
272 others) of the vegetal matrix (Vinatoru et al., 1997). This effect would be compatible with the
273 fact that naringenin and its glycosides are mainly present in the peel of the fruit (Yamamoto et
274 al., 2004), being it more difficult to disrupt with standard homogenization and agitation.
275 Therefore, considering the importance of this specific compound in tomato samples, it was
276 necessary to fix these conditions to guarantee an efficient extraction. This reduction in the
277 number of variables enabled a more detailed analysis of the effect of solvent composition and
278 extraction time in a second phase.

279 In the second phase, solvent extraction composition was kept as in the previous phase and the
280 extraction time was studied between 90 and 180 minutes. The results obtained from the new
281 combined D-optimal design allowed to develop response surface models which were
282 significant ($p < 0.05$) and offered a better explanation of the extraction procedure than in the
283 previous phase (Fig. 2). The new models showed that factors A and B (solvent mixture) were
284 the main factors influencing extraction for all polyphenols present in tomato except for
285 naringenin. For this flavonoid, the only factor conditioning extraction was time, with better
286 results obtained with higher values. For chlorogenic, caffeic, p-coumaric and ferulic acids and
287 for rutin the models also showed complex interactions between solvent composition and
288 extraction time (Fig. 2). Consequently, the determination of common optimum combination
289 was extremely difficult, since the maximum of all these models were not coincident. To solve
290 this problem, a weighted desirability function targeted to maximize extraction for each
291 polyphenol was applied. Weights took into account the relative occurrence and concentrations
292 of each polyphenol in previous literature (Martínez-Valverde et al., 2002; Mattila &
293 Kumpulainen, 2002). High importance was assigned to chlorogenic acid, rutin and naringenin,
294 medium importance to caffeic acid and low importance to p-coumaric and ferulic acids.
295 Desirability results indicated that these best joint extraction would be obtained using a 48:52%
296 (MeOH: H2O) solvent mixture for 177 minutes (conditions represented with a vertical line for

297 each polyphenol in Fig. 2). A verification experiment was performed to check the reliability of
298 the model. No significant differences were detected between the predicted (mean ± 95%
299 confidence interval, mg kg^{-1}) vs. real contents (mean ± SD, mg kg^{-1}) for all the compounds:
300 chlorogenic (30.56 ± 3.02 vs 33.17 ± 0.28), caffeic (4.99 ± 0.73 vs 4.66 ± 0.05), p-coumaric
301 (1.97 ± 0.24 vs 1.75 ± 0.01), ferulic (3.00 ± 0.50 vs 2.71 ± 0.03), rutin (6.48 ± 0.72 vs 5.79 ± 0.04)
302 and naringenin (14.45 ± 6.83 vs 12.23 ± 0.99).

303

304 *3.3. Validation of HPLC method*

305 This method has been targeted to the analysis of different fruits and vegetables, but among
306 them tomato has a special importance considering the relevancy of this species at the global
307 level. It alone represents 5% of the total value of fresh vegetable market and more than 50% of
308 the processed vegetable market. In addition, tomato represents an 11% of the vegetable seed
309 market (Kapur, 2013). Therefore, validation was done using tomato samples as a
310 representative food matrix. Extraction conditions used were the optimum conditions obtained
311 in the previous section (non-freeze-dried samples, 48:52 % MeOH-H_2O during 177 minutes).
312 Linearity, precision, sensitivity, limits of detection (LOD) and recoveries of analytes of proposed
313 method were evaluated. Peak purity evaluated was higher than calculated threshold for all the
314 compounds. Only in certain cases with low concentrations peak purity could not be evaluated.
315 Excellent linear regression models, $r^2 > 0.999$, for all analytes, except for benzoic acid with $r^2 >$
316 0.998 (Table 1). Precision was calculated studying the intra- and inter-day repeatabilities
317 (%RSD) of retention times and peak areas for standards and spiked tomato extracts at 4 mg L^{-1}.
318 Intra-day (n=5) precision of peak areas ranged from <0.1 to 2.9% for standards and from 0.2 to
319 2.0% for spiked samples (Table 1). Inter-day (n=2) precision ranged from 0.2 to 5.4% for
320 standards and from 0.7 to 7.1% for spiked samples (Table 1). These %RSD values are lower or
321 similar than those reported by other authors who employed more sophisticated techniques

such as UHPLC-MS/MS (De Paepe et al., 2013). Intra-day (n=5) precision of retention times were lower than 0.2% in all cases and inter-day (n=2) precision ranged from 0.3% to 3.5% for standards and from 1.3% to 2.9% for spiked samples (Table 1), values similar to those results reported by other authors (De Paepe et al., 2013).

Limits of detection ranged from 3 to 44 μg kg^{-1} fresh weight (Table 1). These results are lower than those obtained by other authors, working with diode-array detection (Ribas-Agustí, Cáceres, Gratacós-Cubarsí, Sárraga, & Castellari, 2012) or more powerful techniques (De Paepe et al., 2013).

Recoveries obtained in tomato samples (average, n=5) ranged from 79% to 108% for the low fortification level (2 mg kg^{-1}) and from 76% to 102% for the high fortification level (20 mg kg^{-1}) (Table 1). The obtained recoveries were better or similar than those obtained by other authors who employed UHPLC-MS/MS (De Paepe et al., 2013; Sakakibara et al., 2003).

3.4. Quantitation studies and application to real samples

The applicability of the optimized RP-HLPC method was studied with the analysis of different fruit and vegetable matrices (Fig. 3). The food matrix affected retention times, but peaks could be identified by comparing absorption UV-vis spectra with those of the standards, by obtaining peak purity values higher than calculated thresholds and by spiking the sample extracts with the standards.

Tomato samples presented quantifiable levels of rutin, naringenin and of the caffeic, p-coumaric, ferulic and chlorogenic acids (Table 3 and Fig. 3a). Among them, chlorogenic acid showed the highest concentration (up to 25.5 mg kg^{-1}), followed by naringenin (up to 9.2 mg kg^{-1}). A strong genotypic effect was detected, with changes both in single and relative contents in the tomato varieties analyzed. In hydrolyzed samples (Fig. 3b), quantifiable levels of quercetin were detected, and the levels of caffeic and p-coumaric acids and narigenin

increased. Several authors contemplate the quantification of the aglycones (resulting from hydrolysis) avoiding the complex quantification of a large number of glycosides (Crozier, Lean, McDonald, & Black, 1997). A clear example of the possible benefits of the analysis of aglycones would be the case of naringenin, as it has been described, this compound in tomato is usually present as naringenin chalcone (Yamamoto et al., 2004). Considering genotypic differences, the polyphenol contents detected with this procedure were similar to those described by other authors (Martínez-Valverde et al., 2002).

The contents of caffeic, p-coumaric and chlorogenic acids and rutin found in the wild species *S. neorickii* were much higher (up to 12-fold) than those found in the cultivated species. These results highlight the interest of this species as a source of variation to develop breeding programs targeted to increase the level of flavonoids and phenolic acids in tomato.

The use of wild species, in this case related with tomato, to develop breeding programs targeted to increase flavonoid content has previously been successful. Willits et al. (2005) used the wild species *Solanum pennellii* Correl to restore the flavonoid pathway that appears to be suppressed in fruit flesh. Germplasm expressing chalcone isomerase in the flesh was used for this purpose, and hybrids of this species with tomato showed higher levels of quercetin diglycoside (Willits et al., 2005). It seems that the accession used in this work could also be a valuable source of variation for breeding programs, in this case targeted to rutin accumulation. In our case we cannot confirm if the high levels obtained could be due to high accumulation only in fruit peel or also in fruit flesh, although the levels obtained seem to suggest the second possibility.

The muskmelon sample (Fig. 3c) presented only quantifiable levels of rutin (1.3 mg kg^{-1}). This species does not outstand for high phenolic contents though it has not been extensively studied. Nevertheless, the presence of rutin in bitter melon leaves has been described (Zhang, Hettiarachchy, Horax, Chen, & Over, 2009). In eggplant the only compound detected was

372 chlorogenic acid, with levels (5.9 mg kg^{-1}) similar to those reported by other authors (Hanson
373 et al., 2006). In the orange sample (Fig. 3d) hesperidin and naringin were detected (Table 3)
374 and the concentrations found were similar or higher than those reported by other authors
375 (Plaza et al., 2011; Vanamala, Reddivari, Yoo, Pike, & Patil, 2006).

376 A different profile was obtained in the two varieties analyzed of pepper, red (Fig. 3e) and
377 green. Low quantities of chlorogenic acid and myricetin were detected in green pepper but not
378 in red pepper. Nevertheless, a high number of possible glycosides were detected. Recent
379 studies with Italian pepper in green stage and Lamuyo pepper in yellow stage have shown that
380 glycoside derivatives of flavonoids could be mainly detected in these varieties (Morales-Soto,
381 Gómez-Caravaca, García-Salas, Segura-Carretero, & Fernández-Gutiérrez, 2013). In fact, in our
382 case, the aglycones quercetin, naringenin and luteolin were detected after hydrolysis. In the
383 case of pepper, a strong dependence of genotype and ripening stage can be found (Howard &
384 Wildman, 2007). Thus, different concentration ranges can be found in each study. The
385 aglycone concentrations obtained here were lower than those reported by Sakakibara et al.
386 (2003), but similar to those reported by Bae et al. (2013).

387 In onion samples (Fig. 3f), only quercetin was detected, but in the hydrolyzed samples its
388 concentration increased considerably indicating the existence of glycosides. In this case, the
389 levels of this aglycone (31.1 mg kg^{-1}) were higher than those reported in other studies using
390 hydrolyzed samples (Patil, Pike, & Yoo, 1995). The main polyphenol present in the apple
391 samples (Fig. 3g) was rutin (6.1 mg kg^{-1}), with concentrations similar to those reported by other
392 authors in apple juice (Suárez-Vallés, Santamaría-Victorero, Mangas-Alonso, & Blanco-Gomis,
393 1994). Significant amounts of gallic acid (6.3 mg kg^{-1}), catechin (26.3 mg kg^{-1}) and rutin (4.4 mg
394 kg^{-1}) were also detected in grape samples (Fig. 3h). In addition the free aglycone quercetin (2.2
395 mg kg^{-1}) was detected in hydrolyzed samples. These results are in agreement with the data
396 reported by other authors (Pastrana-Bonilla, Akoh, Sellappan, & Krewer, 2003).

In the raw extracts of celery samples (Fig. 3i) only p-coumaric acid was detected. But in the hydrolyzed samples, amounts of apigenin and luteolin were detected, indicating the existence of glycosides. The concentrations obtained of these aglycones were lower than those reported by other authors (Sakakibara et al., 2003). Considering these differences, the analysis was repeated with new celery samples, but the concentrations obtained were similar. As the extraction procedure is quite similar to that reported by Miean & Mohamed (2001), it was considered that probably these differences could be associated with a varietal effect. Genistein and chlorogenic acid were detected in the soy milk samples (Fig. 3j). In the hydrolyzed extracts, the concentration of genistein (13.4 mg kg^{-1}) increased and was similar to that reported by other authors (Fukutake et al., 1996).

4. Conclusions

Polyphenol analysis has been extensively studied in plant materials due to the increasing importance of the functional value of these compounds. Despite the effort already made, it was still necessary to develop rapid and efficient methods compatible with the analysis of high number of samples with common equipment. The method developed in this work fulfills these requirements. It enables the joint quantification of 17 prominent phenolic acids and flavonoids in less than 18 minutes, with a good resolution. The repeatability, limit of detection and recovery in tomato samples even improve the results of previous methods. Furthermore, the reliability of the method has been proved in eleven species of fruits and vegetables. Its characteristics make it ideal for its application to common quality controls or to the development of breeding programs, where a large number of samples are analyzed and different polyphenol profiles are expected.

In addition, the extraction procedure of polyphenols has been optimized using tomato as a model. This evaluation has enabled the identification of the negative effects of the use of a

422 common procedure such as freeze-drying or agitation on the content of naringenin, one of the
423 most important polyphenols in tomato. The different response surfaces obtained for the
424 extraction of each polyphenol is due to a high level of interaction between factors, thus it is
425 necessary to identify a compromise that maximizes the extraction efficiency in a global
426 perspective.
427
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559 **Figure captions**

560

561 **Fig. 1.** Chromatograms of a standard mixture of phenolic compounds under different gradient
562 elution systems. Mixture A: 0.1% HCOOH in H_2O; mixture B: 0.1% HCOOH in ACN; mixture C:
563 0.1% HCOOH in MeOH. Gradient elution conditions (A:B:C in %): (a) From 70:30:0 to 50:50:0
564 (t=20min) and 0:100:0 (t=23min). (b) From 80:20:0 to 50:50:0 (t=20min) and 0:100:0
565 (t=23min). (c) From 70:0:30 to 50:0:50 (t=20min) and 0:0:100 (t=23min). (d) From 70:0:30
566 (t=0min) to 58:18:24 (t=12min) to 52:18:30 (t=13min) to 70:10:20 (t=20min) to 0:100:0
567 (t=23min). Peak identification: 1, gallic acid; 2, (+)-catechin; 3, chlorogenic acid; 4, caffeic acid;
568 5, p-coumaric acid; 6, ferulic acid; 7, benzoic acid; 8, rutin; 9, naringin; 10; hesperidin; 11,
569 myricetin; 12, quercetin; 13, luteolin; 14, naringenin; 15; genistein; 16, kaempferol; 17,
570 apigenin.

571

572 **Fig. 2:** Response surface plots showing the effect of solvent mixture (%MeOH and $\%H_2O$) and
573 extraction time (min) on the content (mg kg^{-1}) of main polyphenols found in tomato (at fixed
574 conditions: ultrasound-assisted extraction and fresh samples). The selected conditions with the
575 weighted desirability function to maximize global extraction are represented with a vertical
576 black solid line.

577

578 **Fig. 3:** Chromatograms of selected fruit and vegetable samples using the conditions specified
579 in Fig. 1d. Peak identification: 1, gallic acid; 2, (+)-catechin; 3, chlorogenic acid; 4, caffeic acid;
580 5, p-coumaric acid; 6, ferulic acid; 7, benzoic acid; 8, rutin; 9, naringin; 10; hesperidin; 11,
581 myricetin; 12, quercetin; 13, luteolin; 14, naringenin; 15; genistein; 16, kaempferol; 17,
582 apigenin.

Table(s)

Table 1. Repeatability of the method using standards and tomato samples and limit of detection and recovery analysis (at low and high fortification levels) in tomato samples

Compounds	Calibration equation y = ax + b			Intra-day repeatability[a] (n = 5) RSD (%)		Inter-day repeatability[a] (2 days) RSD (%)			Recovery (%), n = 5	
	a	b	r^2	t_R	Area	t_R	Area	LOD[c] (μg kg^{-1})	Low (2 mg kg^{-1})	High (20 mg kg^{-1})
Gallic acid	29.382	1.5286	1.0000	<0.1; n.d.[b]	2.6; n.d.	0.3; n.d.	5.4; n.d.	n.d.	n.d.	n.d.
Caffeic acid	54.240	-3.5350	1.0000	0.1; 0.1	0.4; 0.5	2.0; 1.9	2.0; 0.8	3	96 ± 3	95 ± 1
p-Coumaric acid	65.198	-1.1404	1.0000	0.1; 0.1	0.6; 2.0	2.6; 2.4	0.8; 0.7	15	79 ± 2	97 ± 2
Ferulic acid	57.203	1.2490	0.9999	0.1; 0.1	0.8; 0.2	2.4; 2.2	0.6; 1.5	15	99 ± 2	102 ± 0
Benzoic acid	3.328	0.5716	0.9985	0.1; n.d.	1.7; n.d.	1.8; n.d.	0.4; n.d.	n.d.	n.d.	n.d.
Chlorogenic acid	25.110	-4.6135	0.9995	0.1; 0.2	1.0; 0.8	1.8; 2.0	3.9; 7.1	10	108 ± 9	84 ± 2
Catechin	6.653	0.3788	1.0000	0.1; n.d.	2.0; n.d.	1.4; n.d.	1.8; n.d.	n.d.	n.d.	n.d.
Kaempferol	37.244	-2.4881	0.9992	0.2; 0.1	0.9; 1.9	3.0; 2.9	0.2; 1.3	15	95 ± 3	83 ± 2
Quercetin	29.628	-5.3835	0.9994	0.1; 0.1	1.4; 1.3	1.7; 1.6	0.2; 6.0	16	105 ± 1	92 ± 1
Myricetin	24.582	-8.2129	0.9993	0.1; 0.1	0.5; 1.0	2.4; 2.3	0.6; 2.3	44	107 ± 1	76 ± 1
Naringenin	39.703	-0.1397	0.9991	0.1; 0.1	1.2; 0.9	1.3; 1.3	2.8; 4.6	21	79 ± 4	90 ± 1
Genistein	139.260	6.9763	0.9996	0.1; n.d.	<0.1; n.d.	1.7; n.d.	2.7; n.d.	n.d.	n.d.	n.d.
Luteolin	24.195	-1.2115	0.9994	0.1; n.d.	2.9; n.d.	1.3; n.d.	1.3; n.d.	n.d.	n.d.	n.d.
Apigenin	30.035	2.0148	0.9994	0.2; n.d.	0.5; n.d.	3.5; n.d.	1.0; n.d.	n.d.	n.d.	n.d.
Rutin	16.788	1.5212	0.9999	0.1; 0.1	1.3; 1.9	2.6; 2.4	3.1; 2.0	10	90 ± 4	98 ± 2
Naringin	15.769	3.7647	0.9993	0.1; n.d.	0.5; n.d.	2.2; n.d.	5.0; n.d.	n.d.	n.d.	n.d.
Hesperidin	15.830	2.9013	0.9993	<0.1; n.d.	0.5; n.d.	0.3; n.d.	0.3; n.d.	n.d.	n.d.	n.d.

[a]RSD obtained with: standards; tomato extracts.

[b]n.d. = not determined since in tomato is not present.

[c]Detection limits obtained with tomato extracts.

Table(s)

Table 2: Results obtained in the initial stage of optimization of polyphenol extraction in tomato samples using RSM. Goodness of fit, significant factors identified**,** maximum predicted values and percentage extraction reduction referred to maximum (in parenthesis) are indicated.

		Chlorogenic	Caffeic	p-Coumaric	Ferulic	Rutin	Naringenin
Model R^2adjusted		0.62	0.53	0.82	0.54	0.68	0.90
Significant factors and interactions in the RSM models		[a] A, B, AC, ACD, ADE, AC^2,ABDE	A,B, BE	A,B,AD,AE,BC,BD,BE ADE,BCD,BCE, BCDE, AC^2D, AD^2E,BC^2D, BC^2E, AC^3, BC^3	A,B,AC,AD,ADE, AC^2D, AC^3	D, E, DE, C^2D	A,B,AC,AD,AE, BC,BD,BE,ACD, ADE
Best factor combination	MeOH:H_2O	56:44	30:70	39:61	80:20	Not dependent	30:70
	Time	175	136	164	180	10, 180	180
		Maximum response (mg kg^{-1}) and % reduction					
Utrasound	Fresh	35.24 (max.)	5.03 (-0.1%)	2.46 (-2.0%)	2.83 (-10.2%)	6.10 (-11.2%)	12.79 (max.)
Utrasound	Freeze-drying	31.94 (-9.3%)	5.04 (-0.1%)	2.51 (max.)	2.90 (-8.1%)	6.80 (-1.1%)	2.69 (-78.9%)
Agitation	Fresh	31.27 (-11.3%)	5.04 (-0.1%)	1.61 (-35.9%)	3.15 (max.)	6.80 (-1.0%)	7.92 (-38.1%)
Agitation	Freeze-drying	32.16 (-8.7%)	5.04 (max.)	1.91 (-24.0%)	3.08 (-2.2%)	6.87 (max.)	1.04 (-91.8%)

[a] Model factors: A = MeOH (%), B=H_2O (%), C= extraction time (min), D = Agitation/ultrasound assisted extraction, E = sample pretreatment (fresh/ freeze-drying)

Table(s)

Table 3. Mean contents (raw sample/hydrolyzed sample) of the phenolic compounds analyzed[a] (mean ± standard deviation in mg kg^{-1}) detected in several food matrices.

Sample	Gall	Caff	p-Cou	Fer	Benz	Chlor	Cat	Kaem	Quer	Myr	Naring	Gen	Lut	Apig	Rut	Nar	Hes
Tomato "Fortuna-C"	- [b] / -	5.31±0.06 /12.02±0.04	2.76±0.02 /8.46±0.02	2.09±0.03 /1.44±0.01	- / -	25.50±0.29 / -	- / -	- / -	- / 2.41±0.03	- / -	8.21±0.17 /4.75±0.05	- / -	- / -	- / -	6.08±0.06 / -	- / -	- / -
Tomato "pera"	- / -	1.40±0.01 /5.64±0.12	0.50±0.01 /3.17±0.01	1.12±0.01 /1.17±0.06	- / -	10.26±0.08 / -	- / -	- / -	- / 4.82±0.01	- / -	9.24±0.07 /13.76±0.14	- / -	- / -	- / -	6.52±0.12 / -	- / -	- / -
Tomato "Kumato®"	- / -	1.57±0.08 /6.00±0.00	0.65±0.01 /2.87±0.03	1.71±0.02 /1.31±0.02	- / -	7.15±0.27 / -	- / -	- / -	- / 4.51±0.08	- / -	4.38±0.10 /2.84±0.06	- / -	- / -	- / -	9.08±0.16 / -	- / -	- / -
S. neorickii	- / -	10.10±0.04 /103.05±1.01	22.01±0.31 /53.04±0.01	- / 10.60±0.04	- / -	38.16±0.96 / -	- / -	- / -	- / 28.88±0.14	4.2±0.08 /22.15±0.32	- / -	- / -	- / -	- / -	114.15±1.21 / -	- / -	- / -
Melon "piel de sapo"	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	1.28±0.03 / -	- / -	- / -
Eggplant	- / -	- / 1.61±0.03	- / -	- / -	- / -	5.93±0.09 / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
Orange	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / 31.27±0.05	- / -	- / -	- / -	- / -	81.79±0.09 /52.64±2.14	657.39±0.14 /166.65±1.11
Red pepper	- / -	1.01±0.01 / -	- / 3.02±0.01	1.91±0.02 /2.73±0.05	- / -	- / -	- / -	- / -	- / 6.94±0.05	- / -	- / 1.82±0.01	- / -	- / 4.85±0.01	- / -	0.61±0.03 / -	- / -	- / -
Green pepper	- / -	- / -	- / 1.43±0.04	0.90±0.01 / -	- / -	2.15±0.01 / -	- / -	- / -	- / 1.47±0.01	2.47±0.05 / -	- / -	- / -	- / 0.63±0.02	- / -	- / -	- / -	- / -
Onion	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	6.44±0.06 /31.05±0.19	2.38±0.02 / -	- / -	- / -	- / -	- / -	1.52±0.01 / -	- / -	- / -
Apple "Fuji"	- / -	0.80±0.01 /1.87±0.07	- / -	- / -	- / -	1.73±0.03 / -	- / -	- / -	- / 2.92±0.01	2.93±0.05 / -	- / -	- / -	- / -	- / -	6.12±0.16 / -	- / -	- / -
Grape	6.33±0.21 / -	- / -	- / 1.20±0.02	- / 0.45±0.03	- / -	- / -	26.33±0.62 / -	- / -	- / 2.20±0.01	- / -	- / -	- / -	- / -	- / -	4.40±0.04 / -	- / -	- / -
Celery	- / -	- / -	0.31±0.01 /0.66±0.0	- / 0.71±0.00	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / 1.30±0.01	- / 1.25±0.08	- / -	- / -	- / -
Soy milk	- / -	- / -	- / 1.81±0.01	- / 1.03±0.02	- / -	4.44±0.27 / -	- / -	- / -	- / -	- / -	- / -	0.15±0.03 /13.42±0.04	- / -	- / -	- / -	- / -	- / -

[a]Gallic acid (gall), caffeic acid (caff), p-coumaric acid (p-cou), ferulic acid (fer), benzoic acid (benz), chlorogenic acid (chlor), catechin (cat), kaempferol (kaem), quercetin (quer), myricetin (myr), naringenin (naring), genistein (gen), luteolin (lut), apigenin (apig), rutin (rut), naringin (nar) and hesperidin (hes)

[b] = not detected

Figure(s)

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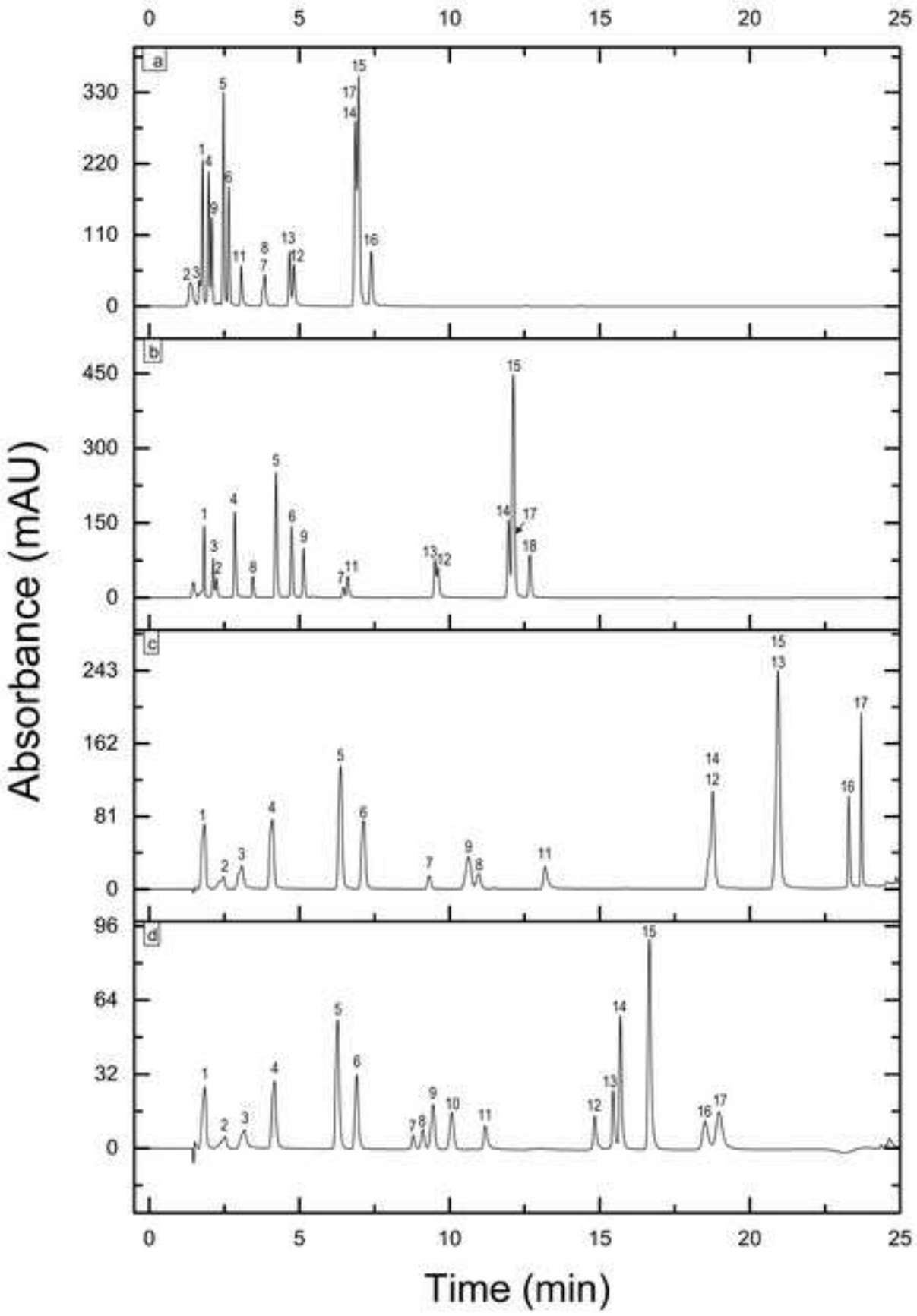
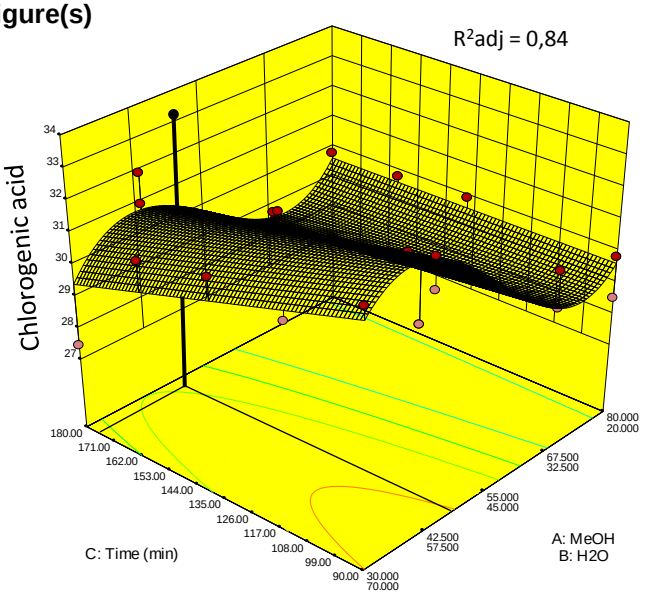
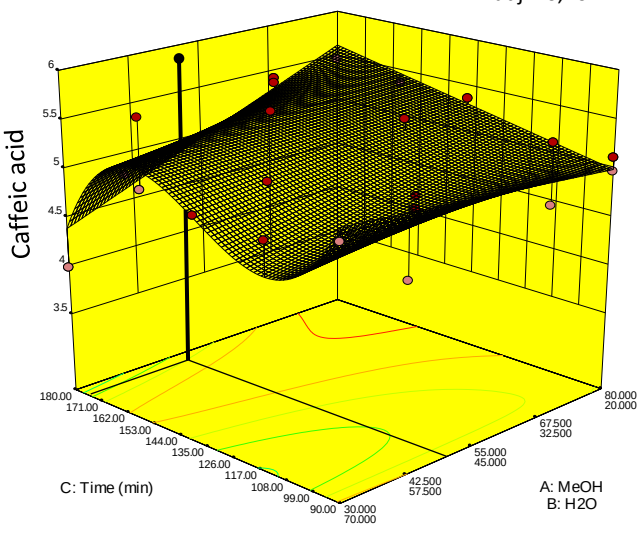
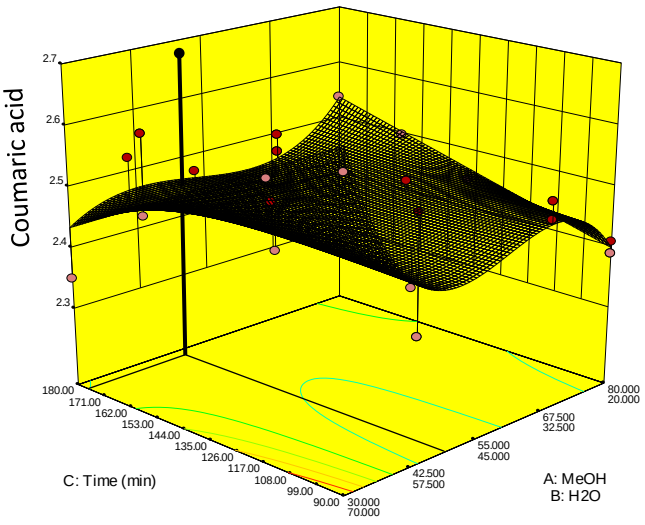
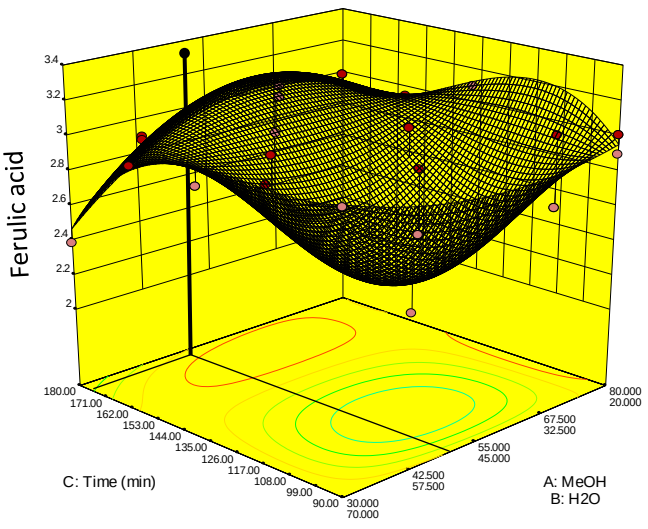


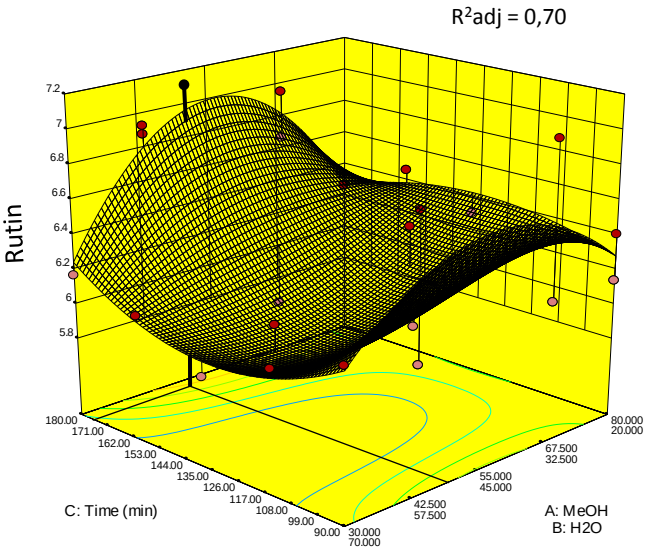
Figure(s)

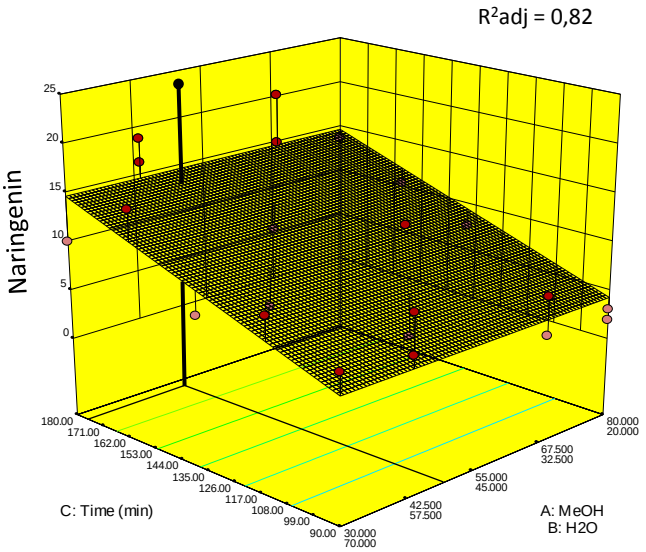

R²adj = 0,84
Chlorogenic acid
C: Time (min)
A: MeOH
B: H2O


R²adj = 0,70
Caffeic acid
C: Time (min)
A: MeOH
B: H2O


R²adj = 0,83
Coumaric acid
C: Time (min)
A: MeOH
B: H2O


R²adj = 0,72
Ferulic acid
C: Time (min)
A: MeOH
B: H2O


R²adj = 0,70
Rutin
C: Time (min)
A: MeOH
B: H2O


R²adj = 0,82
Naringenin
C: Time (min)
A: MeOH
B: H2O

Figure(s)

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