TITLE: Assessment of Total (Free and Bound) Phenolic Compounds in Spent Coffee Extracts

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ABSTRACT

Spent coffee is the main by-product of the brewing process and a potential source of bioactive compounds, mainly phenolic acids easily extracted with water. Free and bound caffeoylquinic (3-CQA, 4-CQA, 5-CQA), dicaffeoylquinic (3,4-diCQA, 3,5-diCQA, 4,5-diCQA), caffeic, ferulic, *p*-coumaric, sinapic and 4-hydroxybenzoic acids were measured by HPLC, after applying three treatments (alkaline, acid, saline) to spent coffee extracts. Around 2-fold high content of total phenolics has been estimated in comparison to free compounds. Phenolic compounds with one or more caffeic acid molecules were approximately 54% linked to macromolecules like melanoidins, mainly by non-covalent interactions (up to 81% of bound phenolic compounds). The rest of the quantitated phenolic acids were mainly attached to other structures by covalent bonds (62-97% of total bound compounds). Alkaline hydrolysis and saline treatment were suitable to estimate total bound and ionically bound phenolic acids, respectively, whereas acid hydrolysis is an inadequate method to quantitate coffee phenolic acids.

KEYWORDS: Coffee; by-products, phenolics, hydrolysis, melanoidins.

INTRODUCTION

Coffee is one of the most consumed beverages in the world, and the richest source of phenolic compounds in the daily diet.[1,2] Chlorogenic acids (CGAs) are the major phenolic components of coffee brews, mainly caffeoylquinic acids (CQAs), feruloylquinic acids (FQAs), *p*-coumaroylquinic acids (*p*CoQAs) and di-caffeoylquinic acids (diCQAs), as well as caffeoylquinic acid lactones (CQLs) and feruloylquinic acid lactones (FQLs) generated during the roasting process.[3,4] Spent coffee is the main by-product of coffee brewing process and it also has substantial amounts of phenolic acids.[5,6] In fact, spent coffee extracts with high antioxidant activity have been obtained and proposed to be added as a food ingredient to enhance food health properties.[7,8] However, their phenolic composition remains partially unknown, because the studies have been focused on the identification and quantitation of the free phenolic acids. Phenolic compounds are also found in the food matrix attached to other structures such as proteins, polysaccharides, etc. by hydrogen, covalent, ionic bonds and other interactions.[9,10] Free and bound bioactive compounds are bioavailable after their release from food matrices by gastrointestinal enzymatic action or further microbiota activity.[11,12] Consequently, they might contribute to health related properties associated with the consumption of coffee or eventually spent coffee extracts added to other foods.

Some authors have reported that hydroxycinnamic acids play an important role in the melanoidins formation during roasting process, and consequently certain amount of these phenolic compounds remain linked to the coffee melanoidins structure.[13,14] Several techniques have been applied to break covalent bonds. For example, alkaline pressure-hydrolysis was one of the first methodologies used to detect compounds attached to the high molecular weight fraction of coffee extracts.[15] Saponification or alkaline hydrolysis is frequently used to release covalently bound phenolic compounds.

63 Previous studies have detected caffeic and ferulic acids after applying this method to
64 coffee brew,[16] and also chlorogenic acids in high molecular weight coffee melanoidins
65 fractions.[17-21] Recently, phenol and benzoic acid derivatives and chlorogenic acids have
66 been found after alkaline fusion.[18,21] Acid conditions have also been used on cereals,
67 fruits, vegetables and beverages to release phenolic compounds covalent linked to other
68 structures.[22,23]
69 Non-covalent interactions have been less studied. Barbeau and Kinsella [24] reported that
70 a high ionic strength medium with NaCl decreased the bindings of chlorogenic acids to
71 protein fractions. Another study showed higher concentrations of phenolic acids after
72 the addition of NaCl, confirming that NaCl breaks the ionic bindings between phenolic
73 compounds and proteins.[25] Also some authors used high ionic strength solutions to
74 break non covalent bonds between melanoidins and low molecular weight compounds,
75 such as phenolics.[26,27]
76 The knowledge of the total content of phenolic compounds (free and bound) in spent
77 coffee extracts is crucial for their potential use as functional ingredients by the food
78 industry. Until now, neither of the techniques previously described have been applied to
79 spent coffee extracts. Therefore, three of the most common hydrolytic procedures were
80 applied to spent coffee extracts which have proven genoprotective, antimutagenic and
81 antimicrobial activity,[7,8] and also to coffee brew as a reference point. Thus, the main
82 aim of the work was to measure free and bound compounds for the assessment of the
83 total phenolic compounds content of spent coffee extracts, and to determine the most
84 accurate method for this purpose.
85

MATERIALS AND METHODS

Chemicals and Reagents. Methanol HPLC grade, sodium chloride, ethylenediaminetetraacetic acid (EDTA), ascorbic acid, sodium hydroxide, hydrochloric acid were obtained from Panreac (Barcelona, Spain). Pure reference standards of caffeic acid, ferulic acid, *p*-coumaric acid, 4-hydroxybenzoic acid, sinapic acid and 5-caffeoylquinic acid were purchased from Sigma-Aldrich (Steinheim, Germany) and 3,4-, 3,5-, and 4,5-dicaffeoylquinic acids from Phytolab (Vestenbergsgreuth, Germany).

Coffee brew preparation. Roasted coffee from Guatemala (*Coffea arabica*, 3.03% water content, L* = 24.69 ± 0.74, roasted at 219 °C for ca 15 min) was provided by a local factory. The lightness value (L*) indicates the coffee roasting degree, and it was analyzed by means of a tristimulus colorimeter (Chromameter-2 CR-200, Minolta, Osaka, Japan) using the D65 illuminant and CIE 1931 standard observer. The instrument was standardized against a white tile before sample measurements. Ground roasted coffee was spread out in an l cm Petri plate, and the L* value was measured in triplicate on the CIELab scale.

Roasted coffee beans were ground to a powder in a Moulinex coffee grinder (model Super Junior “s”, Paris, France) for 20 s immediately before sample preparation. Filter coffee brew was prepared from 36 g of ground roasted coffee for a volume of 600 mL, using a filter coffee machine (model Avantis 70 Aroma plus, Ufesa, Spain). Extraction took approx. 6 min at 90 °C. Extraction as the percentage of total solids with respect to ground roasted coffee was 23.3%.

Spent coffee extract. Spent coffee extracts were prepared according to the method described by Bravo et al.[6] Briefly, first, spent coffee was defatted with petroleum ether (1:11, w/v) for 3 h at 60 ºC in a Soxhlet extraction system (Extraction Unit B-811 Standard BUCHI, Flawil, 127 Switzerland). Then, 24 g of spent coffee were extracted

111 with a volume of 400 mL of water using a filter coffeemaker (model AVANTIS 70
112 Inox, Ufesa, Spain). Extraction took approximately 6 min at 90 ºC. Extraction as the
113 percentage of total solids with respect to ground spent coffee was 11%.
114 Both coffee brew and spent coffee extract were lyophilized using a Cryodos Telstar
115 (Terrassa, Spain).
116 **Alkaline hydrolysis.** The procedure was performed according to Nardini et al.[16], with
117 some modifications. A volume of 5 mL of spent coffee extract (0.05 g) or coffee brew
118 (0.08 g) was added to a 5 mL of 2 M NaOH solution containing 1% (w/w) ascorbic acid
119 and 10 mM ethylenediaminetetraacetic acid (EDTA). The mixture was incubated for
120 30min at 30ºC.
121 **Acid hydrolysis.** The hydrolytic method was applied according to Alves et al.[22], with
122 some modifications. An aliquot (20 mL) of coffee brew (0.32 g) or spent coffee extract
123 (0.2 g) were hydrolyzed by adding 20 mL of methanol, 4 mL of concentrated HCl (10.2
124 M) and 600 µL of antioxidant solution (1% BHT and 1% ascorbic acid). The mixtures
125 were heated under reflux at 75 °C for 150 min. After the hydrolysis, samples were
126 neutralized with 10 M NaOH.
127 **Saline treatment.** Ionically bound phenolic compounds were obtained according to the
128 method described by Delgado-Andrade and Morales.[14] Briefly, NaCl was added to an
129 aliquot (50 mL) of coffee brew (0.8 g) or spent coffee extract (0.5 g) to have a 2 M
130 concentration. Then, samples were maintained at 4 ºC overnight.
131 After each treatment, samples were acidified to pH 3 with concentrated HCl, then were
132 centrifuged and the supernatant was stored at 4 ºC for further analysis.
133 **Chlorogenic acids analysis.** Extraction of chlorogenic acids was carried out according
134 to Bicchi et al.[28] The compounds were analyzed by HPLC following the method
135 described by Farah et al.[4] with some modifications.[5] HPLC analysis was achieved with

136 an analytical HPLC unit model 1100 (Agilent Technologies, Palo Alto, CA, USA)
137 equipped with a binary pump and an automated sample injector. A reversed-phase
138 Poroshell 120 C-18 (2.7 μm particle size, 250 x 4.6 mm) column was used at 25 °C.
139 Samples were properly diluted and the sample injection volume was 100 μL. The
140 chromatographic separation was performed using a gradient of methanol (solvent A)
141 and Milli-Q water acidulated with phosphoric acid (pH 3.0, solvent B) at a constant
142 flow of 0.8 mL/min. Elution was initiated at 10% A and maintained for 5 min, the
143 percentage of solvent A was increased to 20% in 10 min and maintained for 10 min,
144 then increased to 50% in 20 min and maintained for 3 min, and finally increased to 80%
145 for 15 min. Detection was accomplished with a diode-array detector (DAD), and
146 chromatograms were recorded at 325 nm. Identification of 5-CQA and diCQAs was
147 performed by comparing the retention time and the photodiode array spectra with those
148 of their reference standards compounds. 3-CQA and 4-CQA were identified by the
149 isomerization of 5-CQA standard. Quantitation of 5-caffeoylquinic acid (5-CQA) was
150 made by comparing the peak areas with those of the standards. Quantitation of the other
151 CGAs was performed using the area of 5-CQA standard combined with their respective
152 molar extinction coefficients as reported by Trugo and Macrae [29] and Farah et al.[4]
153 **Other Phenolic acids.** The extraction of hydroxycinnamic acids and benzoic acid
154 derivative was carried out according to Alvarez-Vidaurre et al.[30] The HPLC analysis
155 was performed following the method described by Nardini et al.[16], with modifications.
156 HPLC analysis was achieved with an analytical HPLC unit model 1200 (Agilent
157 Technologies, Palo Alto, CA, USA) equipped with a binary pump and an automated
158 sample injector. The sample injection volume was 50 μL. Chromatographic separation
159 was performed at 25 ºC using a reversed-phase Gemini NX (5 μm particle size, 250 x
160 4.6 mm) column (Phenomenex, USA) and a mobile phase consisting methanol (solvent

161 A) and Milli-Q water acidulated with phosphoric acid (pH 2.5, solvent B). The flow rate
162 was 1 mL/min. Elution was initiated at 15% A and maintained for 20 min, the
163 percentage of solvent A was increased to 20% in 10 min, to 45% in 5 min, to 55% in 10
164 min, then maintained for 10 min, and finally increased to 80% for 15 min. Detection
165 was accomplished with a diode-array detector at 325 nm for caffeic acid, ferulic acid, *p*-
166 coumaric acid and sinapic acid, and at 260 nm for 4-hydroxybenzoic acid. Identification
167 of phenolic acids was performed by comparing the retention time and the photodiode
168 array spectra with those of their reference compounds. Calibration curves of standard
169 were used to quantitate. Coefficients of linearity for the calibration curves were
170 typically R^2>0.99.
171 **Statistical analysis.** Each parameter was analyzed in triplicate. Results are shown as
172 means ± standard deviations. A Student's *t*-test was applied to determine differences of
173 phenolic compounds between non-treated samples and each treatment. All statistical
174 analyses were performed using the SPSS v.15.0 software package.

175 **RESULTS AND DISCUSSION**

176 Spent coffee is the by-product generated after a brewing process, and might be
177 considered a valuable source of easily extracted phenolic compounds by the food
178 industry. However, identification and quantitation of the total (free and bound) phenolic
179 compounds should be a previous step before further applications. Therefore, spent
180 coffee extracts were submitted to three treatments (alkaline, acid and saline). Phenolic
181 compounds were analyzed by HPLC-DAD and compared to those in their respective
182 coffee brew. Figures 1 and 2 showed the chromatograms of phenolic compounds of
183 coffee brews before and after each treatment. Similar chromatograms were obtained for
184 spent coffee extract because samples were properly diluted before injection in HPLC to
185 have areas ranged within calibration curves.

First, free chlorogenic acids (CGAs), the most abundant phenolic compounds in coffee, were identified and quantitated in non-treated samples. Figure 1C shows the CGAs peaks, which were identified as 3-caffeoylquinic acid (peak 3), 4-caffeoylquinic acid (peak 2), 5-caffeoylquinic acid (peak 1), 3,4-dicaffeoylquinic acid (peak 4), 3,5-dicaffeoylquinic acid (peak 5) and 4,5-di caffeoylquinic acid (peak 6), as compared with the standards mixtures (figure 1A and B). Spent coffee extract showed less content of free CQAs than the coffee brew, whereas the concentration of free diCQAs was 1.8 fold higher in the by-products (Table 1). Furthermore, the content of other phenolic compounds, such as hydroxycinnamic acids and benzoic acid derivative, was also measured by HPLC and chromatograms are shown in Figure 2. The results (Table 1) showed low amounts of caffeic acid (peak 8), ferulic acid (peak 9), *p*-coumaric acid (peak 10), sinapic acid (peak 11), as well as 4-hydroxybenzoic acid (peak 12) in both spent coffee extract and coffee brew (Figure 2B).

Alkaline hydrolysis

Alkaline hydrolysis or saponification is applied to release compounds bound to polymers by covalent interactions.[10] Spent coffee extract and coffee brew treated with alkaline solutions showed differences in the chromatographic phenolic acids profile compared with non-treated samples. The chromatogram (Figure 1D) showed the disappearance of the major CGAs being detected only one large peak (8). Even though the elution time of peak 8 was quite similar to 4-CQA (2), the spectral data confirmed that it was caffeic acid. Some authors have reported that phenolic compounds are susceptible to oxidation at pH 8 and higher, leading to degradation into their corresponding molecules derivatives.[31,9] Quantitation of the caffeic acid was carried out with the second chromatographic method (Figure 2C). It was also observed that ferulic acid, *p*-coumaric acid, sinapic acid and 4-hydroxybenzoic acid peaks remained after the

211 hydrolysis. Moreover, the numerical data showed a significant increase ($p<0.01$) in the
212 amounts of hydroxycinnamic acids in samples treated with alkaline conditions in
213 comparison with non-treated spent coffee extract and coffee brew (Table 1). These
214 results agree with previous studies, where high amounts of caffeic and ferulic acids
215 were found in coffee brew or in the high molecular weight melanoidins fraction after
216 applying alkaline hydrolysis.[16,17]
217 Chlorogenic acids are an ester formed between a quinic acid molecule and one or more
218 hydroxycinnamic acids molecules. Alkaline medium can break not only the covalent
219 bonds between melanoidins and phenolic compounds, but also CGAs internal bonds.
220 Our findings suggest that the high concentration of caffeic, ferulic and coumaric acids
221 found in hydrolyzed samples were partially due to the cleavage of the ester linkages in
222 free CGAs. In fact, an additional experiment showed that all 5-CQA from coffee brew
223 and spiked standard (300 and 500 ppm 5-CQA) has been hydrolyzed into caffeic acid,
224 but only 50% of the expected caffeic acid was quantitated (Figure 3). Taking into
225 account this lost, our results indicate that the amount of caffeic acid obtained after
226 alkaline hydrolysis was approximately 58% and 35% higher than the expected in spent
227 coffee extract and coffee brew, respectively. This fact could be partially explained by
228 the presence of other chemical compounds with caffeic acid in their structure. A
229 complete free phenolics profile of Arabica spent coffee extract and coffee brew
230 (unpublished data) showed that CQLs were 18% of the total CQAs and diCQAs, as well
231 additional 8% caffeoylquinic isomers were found. Then, the caffeic acid derived from
232 overall free chlorogenic acids was deducted from the total caffeic acid, which included
233 the obtained after alkaline hydrolysis and the estimated due to losses. Thus, the results
234 suggest that around 47% (spent coffee extract) and 19% (coffee brew) of the caffeic
235 acid found after the alkaline hydrolysis could come from CGAs or caffeic acids attached

to other structures. In the case of coffee brew, this percentage of bound phenolic compounds is in the range of 1 to 29% proposed by Perrone et al.[19] depending on the roasting degree. The amount of bound phenolics in coffee might also be influenced by coffee variety because it has been reported that darker roasted Robusta coffees showed higher losses of chlorogenic acids, but an increase in bound compounds.[19,32]

Similarly, the increment of ferulic and coumaric acids concentration could be explained by the release of hydroxycinnamic acids from feruloylquinic and coumaroylquinic acids presents in coffee brew and spent coffee extract. Unlike the caffeic acid, the other hydroxycinnamic acids do not suffer losses by the alkaline treatment. Moreover, the proportion of FQAs and FQLs in the samples was close to 9% of the total content of free CGAs (unpublished data). Thus, the percentage of extra ferulic acid found in hydrolyzed samples was similar, which indicate that minor amount of this compound was released from melanoidins and other macromolecules. Even though the concentrations of sinapic and 4-hydroxybenzoic acids were lower than caffeic, ferulic and coumaric acids, 2-3 fold significant increases ($p<0.001$) were found in comparison to the values in the non-treated samples. There is scarce literature about the presence of sinapic acid in coffee. Up to our knowledge, sinapic acid linked to quinic, caffeoylquinic and feruloylquinic acids has only been reported in Robusta green coffee.[33] Consequently, part of these chlorogenic acids could remain attached to melanoidins during roasting process. Finally, the increase of 4-hydroxybenzoic acid agrees with Nunes and Coimbra,[18] who found benzoic acid and derivatives as 4-hydroxybenzoic acid attached to HMW melanoidins fraction of roasted coffee after applying alkaline fusion.

Acid hydrolysis

Acid hydrolysis is also proposed to release bound compounds. The chemical based is similar to alkaline hydrolysis, which is the rupture of covalent bonds using in this case a strong acid (HCl). Figure 1E shows a chromatogram of a hydrolyzed coffee brew, where four small peaks has been observed. Two peaks were identified as 5-CQA and caffeine (peaks 1 and 7). The peak 8 eluted at the same time as 4-CQA, but it has been identified as caffeic acid by the spectrum data. The last peak (ca 35 min) was the antioxidant used in the assay. Similarly, antioxidants used were detected in Figure 2 (ca 71 and 77 min), as well as the caffeine (ca. 26 min). In spent coffee, caffeoylquinic and dicaffeoylquinic acids were completely lost after acid hydrolysis. Also, ferulic, coumaric, sinapic and 4-hydroxybenzoic acids totally disappeared in both spent coffee extracts and coffee brews after acid treatment. Furthermore, minor changes in caffeic acid concentration were found in comparison to non-treated samples (1.50 μmol/ g of spent coffee extract and 2.01 μmol/ g of coffee brew). A previous study reported that this technique could be used to release and to quantitate phenolic compounds like isoflavones from other coffee components.[22] However, our results strongly suggest that phenolic acids, such as CGAs and hydroxycinnamic acids are very susceptible to acid hydrolysis. This in agreement with the study of Mattila et al.[23], which found that phenolic compounds are affected by extreme pH conditions, but oxidation processes are more likely to occur in acid pH. Consequently, acid hydrolysis is an inadequate technique to release the main bound coffee phenolic compounds (phenolic acids), but it can be applied to evaluate others like isoflavones.

Saline treatment

This method has been used to release phenolic compounds ionically bound to proteins or melanoidins, using 2 M NaCl. Barbeau and Kinsella [24] reported that chlorogenic acid carboxyl group is predominately ionized at neutral pH. Therefore, the increase of ionic

strength tends to neutralize charge interaction between dissociated carboxylic groups of chlorogenic acid and positively charge side chain groups, like the amide carbonyls of the peptide bonds in proteins and melanoidins leading to the release of ionically bound phenolics. The chromatograms of CGAs (Figure 1F) and hydroxycinnamic acids (Figure 2E) after saline treatment show similar peaks profiles than coffee samples without treatment. However, the results (Table 1) showed that samples treated with 2 M NaCl had higher concentrations of caffeoylquinic acids, with highly significant differences ($p<0.001$). The amount of diCQAs was also higher ($p<0.01$) in comparison to non-treated samples, even though no statistical differences were detected for 3,5 diCQA in spent coffee extracts. The extra amounts of CQAs and diCQAs found after the saline treatment mean those compounds ionically attached to other structures. Thus, it should be highlighted that free and ionically bound CQAs were found in similar amounts in spent coffee extracts, whereas clearly lower amounts of bound CQAs (33.23 μmol/ g) were in coffee brew. The addition of a caffeic acid moiety in the case of diCQAs increases the hydroxyl groups that can ionically interact with melanoidins explaining the higher amount of bound diCQAs in coffee brews in comparison to spent coffee extracts. This is in agreement with previous works, which showed that a second extraction of ground coffee to obtain spent coffee extracts using a filter coffeemaker favors the extraction of bound CQAs, mainly diCQAs, probably due to the turbulences which facilitate contact of grounds and water.[5,34]

Regarding to hydroxycinnamic acids, coumaric acid showed the largest increase, with values threefold higher than in non-treated samples. Sinapic acid and 4-hydrobenzoic acid raised their concentrations from 1.2 to 1.5 folds. However, caffeic acid and ferulic acid showed slight variations in the final content, with no significant differences ($p>0.05$). Some authors did not find phenolic acids increases after applying high ionic

310 strength treatment to coffee brew HMW melanoidins fraction,[18,19] whereas others found
311 caffeoylquinic acids ionically bound to the HMW melanoidins core contributing to high
312 antioxidant capacity of coffee.[14] Our data strongly suggest that the extra phenolic
313 compounds found after the ionic treatment have been released from the melanoidins
314 core or other medium and low molecular weight melanoidins or Maillard reaction
315 products. In fact, our results support the theory of Bekedam et al.[20] that chlorogenic
316 acids are also incorporated into the melanoidins through nonester linkages.

317 **Total phenolic compounds**

318 Free and estimated bound and total phenolic acids of spent coffee extracts are
319 summarized in Table 2. Spent coffee extracts had around 2-fold higher content of total
320 phenolics than those measured directly (without hydrolysis or saline treatment) showing
321 an underestimation of phenolic acids. Phenolic compounds with one or more caffeic
322 acid molecules were approximately 54% linked to macromolecules like melanoidins,
323 mainly by non-covalent interactions (up to 81% of bound phenolic compounds). The
324 rest of the quantitated phenolic acids were mainly attached to other structures by
325 covalent bonds (62-97% of total bound compounds).

326 In conclusion, spent coffee extract is a rich source of phenolic acids with a high
327 percentage of compounds linked to macromolecules like melanoidins or other Maillard
328 reaction products, mainly by non-covalent interactions. In contrast, coffee brew only
329 had around 20% of attached phenolics. Moreover, caffeoylquinic acids are the most
330 abundant and represent 70% of the total CGAs. Regarding to the treatments, it could be
331 said that two of the three methodologies provide accurate information about spent
332 coffee extract and coffee brew bound phenolic compounds. The alkaline hydrolysis is a
333 suitable method to know total phenolic compounds both free and bound. However, it
334 cannot be used to directly quantitate total parent compounds, due to the susceptibility of

coffee component internal linkage and oxidative losses at high pH conditions. On the other hand, saline treatment allowed to quantitate ionically bound phenolic compounds. Thus, both methods increased the knowledge about the total content of phenolic compounds (free + bound) in spent coffee. All the reported data must be taken into account for the characterization of this by-product in order to be used as a potential food ingredient by the food industry. Spent coffee phenolic compounds (free and bound) may add beneficial health properties to food, specifically in the prevention of oxidative stress related diseases, such as cancer, cardiovascular and neurodegenerative diseases.

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Figure caption

Figure 1. Chromatograms of (A) Standards mixture, (B) Standards mixture (CQA Isomers), (C) Non-treated coffee brew, (D) Alkaline hydrolyzed coffee brew, (E) Acid hydrolyzed coffee brew, (D) Saline treated coffee brew. Peaks (1) 5-CQA; (2) 4-CQA; (3) 3-CQA; (4) 3,4-diCQA; (5) 3,5- diCQA; (6) 4,5-diCQA; (7) caffeine; (8) caffeic acid.

Figure 2. Chromatograms of (A) Standards mixture, (B) Non-treated coffee brew, (C) Alkaline hydrolyzed coffee brew, (D) Acid hydrolyzed coffee brew, (E) Saline treated coffee brew. Peaks (8) caffeic acid; (9) p-coumaric acid; (10) ferulic acid; (11) sinapic acid; (12) 4-Hydroxybenzoic acid.

Figure 3. Chromatograms of (A) Non-treated coffee brew spiked with 5-CQA standard, (B) Alkaline hydrolyzed coffee brew spiked with 5-CQA standard. Peaks (1) 5-CQA; (8) caffeic acid.

Table 1. Free and total phenolic acid content of coffee brew and spent coffee extract. Data are expressed as µmol/ g of lyophilized coffee brew or spent coffee extract.

	Non-treated	Alkaline hydrolysis	Acid hydrolysis	Saline treatment
3-CQA				
Coffee brew	43.52±0.87	nd	nd	50.89±0.55***
Spent coffee	27.36±0.62	nd	nd	55.71±0.31***
4-CQA				
Coffee brew	49.27±2.32	nd	nd	57.43±1.01**
Spent coffee	35.88±0.88	nd	nd	62.92±0.56***
5-CQA				
Coffee brew	78.40±1.09	nd	24.61±0.34***	96.10±2.40***
Spent coffee	53.59±2.15	nd	nd	112.87±1.28***
Total CQA				
Coffee brew	171.19	nd	24.61	204.42
Spent coffee	116.83	nd	nd	231.51
3,4-diCQA				
Coffee brew	0.83±0.03	nd	nd	1.14±0.07**
Spent coffee	1.39±0.12	nd	nd	1.65±0.00ns
3,5-diCQA				
Coffee brew	0.57±0.02	nd	nd	0.79±0.50**
Spent coffee	0.99±0.07	nd	nd	1.07±0.00ns
4,5-diCQA				
Coffee brew	0.90±0.05	nd	nd	1.27±0.09**
Spent coffee	1.70±0.13	nd	nd	1.88±0.00ns
Total diCQA				
Coffee brew	2.30	nd	nd	3.20
Spent coffee	4.08	nd	nd	4.60
Total CQA+diCQA				
Coffee brew	173.49	nd	24.61	207.62
Spent coffee	120.91	nd	nd	236.11
Caffeic acid				
Coffee brew	1.40±0.04	136.53±7.44***	2.01±0.08**	2.24±0.10***
Spent coffee	2.00±0.00	156.27±15.53***	1.50±0.01ns	2.51±0.09**
Ferulic acid				
Coffee brew	0.09±0.01	13.66±1.49***	0.39±0.01***	0.11±0.03ns
Spent coffee	0.17±0.01	17.49±1.18***	nd	0.19±0.03ns
p-Coumaric acid				
Coffee brew	0.19±0.02	2.73±0.25***	nd	0.50±0.00***
Spent coffee	0.24±0.02	2.60±0.14***	nd	0.67±0.06***
Sinapic acid				
Coffee brew	0.07±0.00	0.16±0.02**	nd	0.10±0.00***
Spent coffee	0.12±0.01	0.34±0.04**	nd	0.15±0.00ns
4-Hydroxybenzoic acid				
Coffee brew	0.16±0.03	0.62±0.04***	nd	0.22±0.00*
Spent coffee	0.17±0.02	0.46±0.02***	nd	0.26±0.05ns

All values are shown as means ± SD (n=3). nd, not detected. In each row, asterisk indicates different significance ns $P < 0.05$, * $P > 0.05$, ** $P > 0.01$, *** $P > 0.001$ from non-treated sample.

Table 2. Free and estimated bound phenolic acids content of spent coffee extract. Data are expressed as µmol per g of lyophilized spent coffee extract.

	Free	Bound	Ionically bound	Total
Total CQA	116.83	141.41	114.68	258.24
Total diCQA	4.08	4.94	0.52	9.02
Caffeic acid	2.00	2.42	0.51	4.42
Ferulic acid	0.17	6.44	0.02	6.61
p-Coumaric acid	0.24	2.36	0.43	2.60
Sinapic acid	0.12	0.22	0.03	0.34
4-Hydroxybenzoic acid	0.17	0.29	0.09	0.46

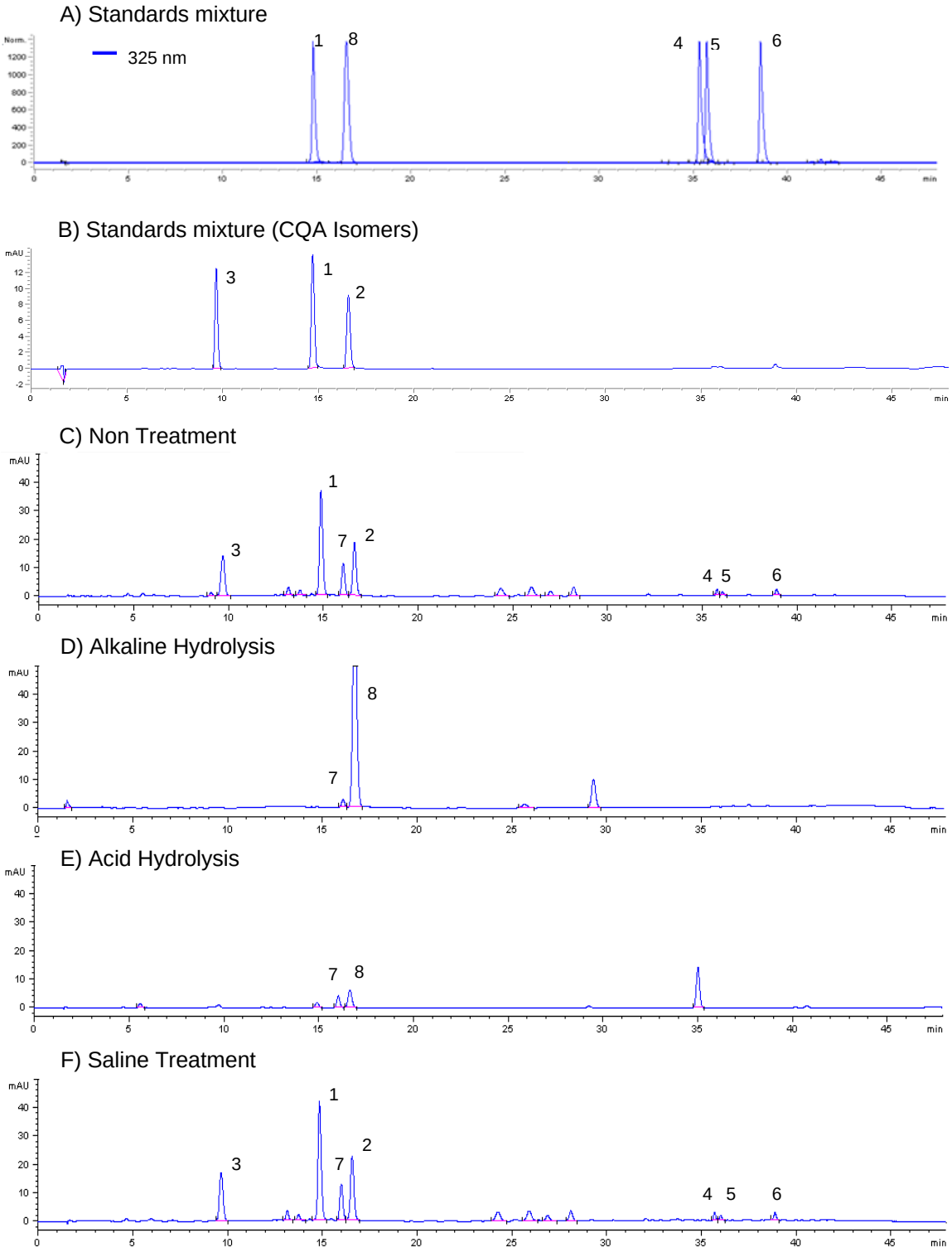
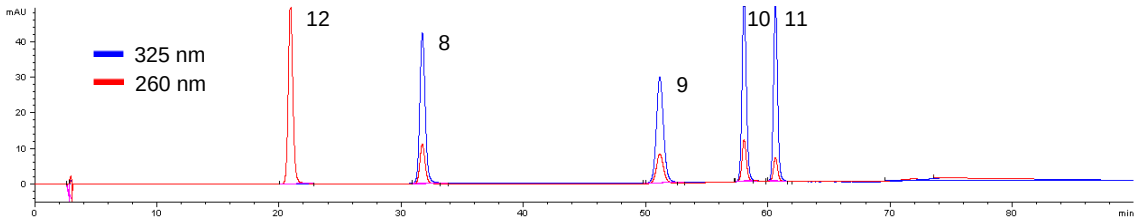
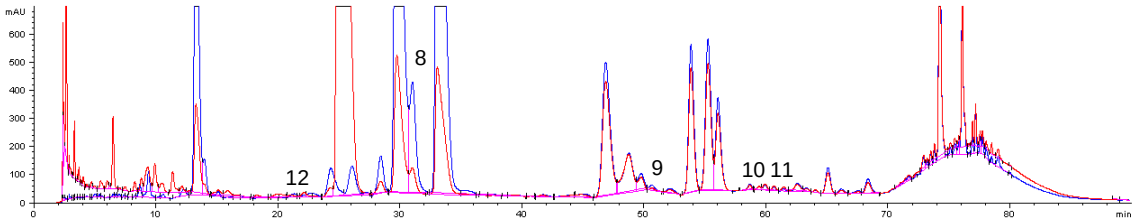

A) Standards mixture
325 nm
1
8
4
5
6
B) Standards mixture (CQA Isomers)
3
1
2
C) Non Treatment
1
3
7
2
4 5
6
D) Alkaline Hydrolysis
8
7
E) Acid Hydrolysis
7 8
F) Saline Treatment
1
3
7
2
4 5
6
min

Figure 1.

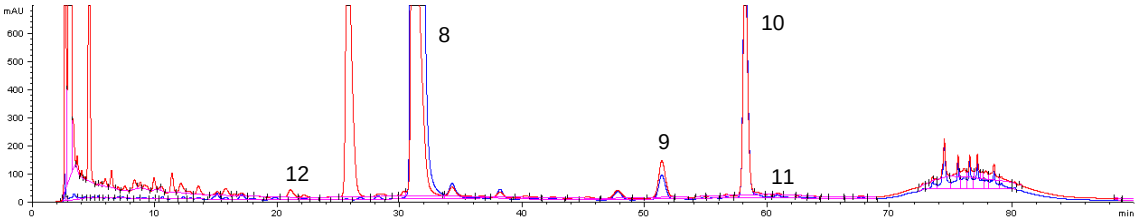
A) Standards mixture



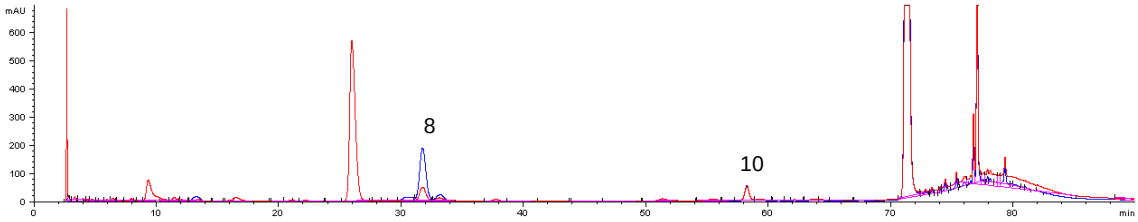
B) Non Treatment



C) Alkaline Hydrolysis



D) Acid Hydrolysis



E) Saline Treatment

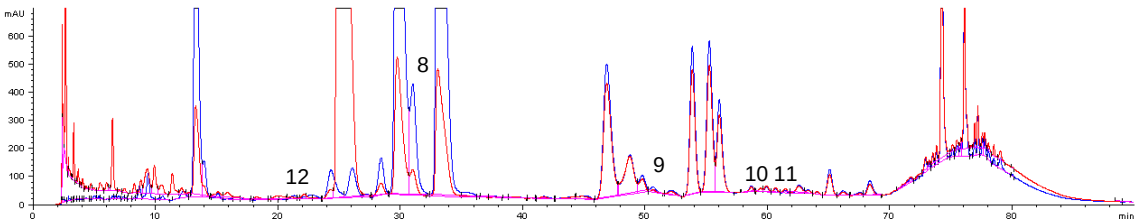


Figure 2.

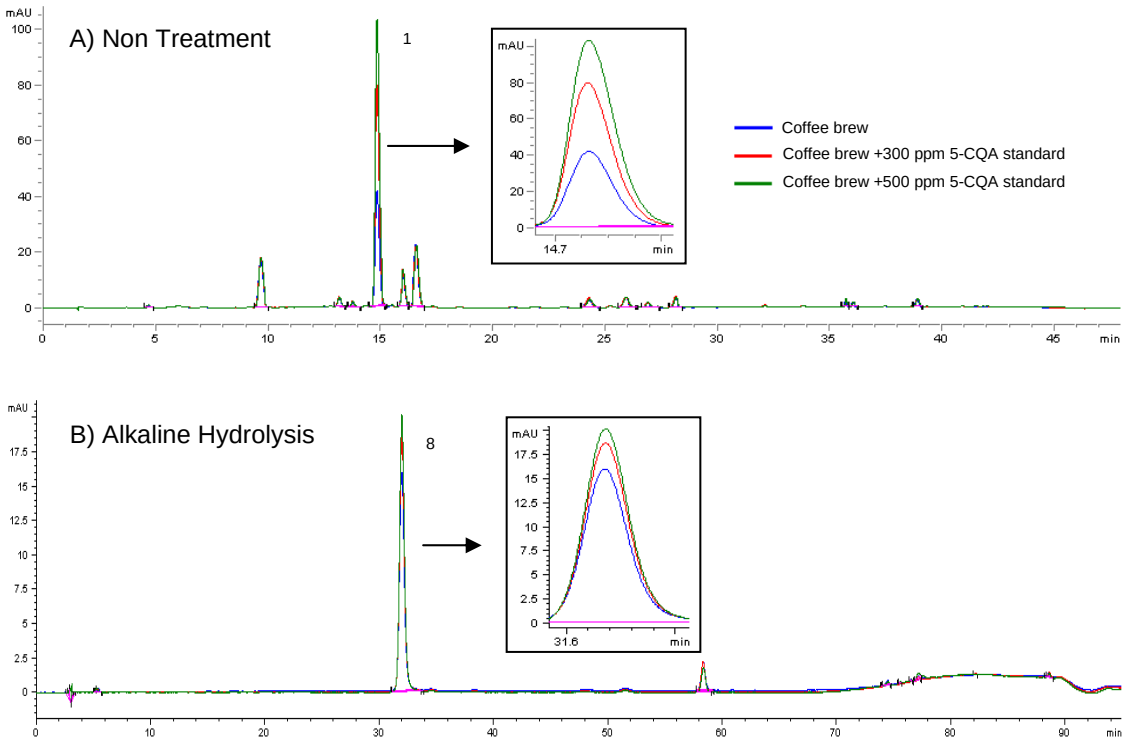
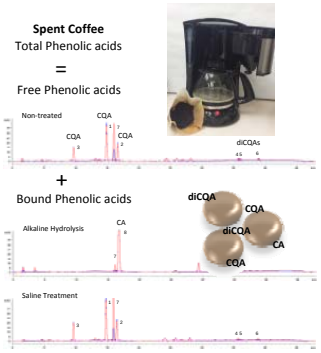

A) Non Treatment
1
mAU
min
14.7
Coffee brew
Coffee brew +300 ppm 5-CQA standard
Coffee brew +500 ppm 5-CQA standard
B) Alkaline Hydrolysis
8
31.6

Figure 3.

TOC GRAPHIC


Spent Coffee
Total Phenolic acids
=
Free Phenolic acids
Non-treated
CQA
CQA
CQA
diCQAs
+
Bound Phenolic acids
Alkaline Hydrolysis
CA
Saline Treatment
diCQA
CQA
diCQA
CA
CQA