This paper must be cited as:

López-Froilán, R., Ramírez-Moreno, E., Podio, N. S., Pérez-Rodríguez, M. L., Cámara, M., Baroni, M. V., Wunderlin D.A & Sánchez-Mata, M. C. (2016). In vitro assessment of potential intestinal absorption of some phenolic families and carboxylic acids from commercial instant coffee samples. Food & Function, 7(6), 2706-2711.
DOI: doi.org/10.1039/c6fo00315j

Original Research Article

In vitro assessment of potential intestinal absorption of some phenolic families and carboxylic acids from commercial instant coffee samples

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Abstract

Coffee is one of the most consumed beverages in the world, being a source of bioactive compunds as well as flavors. Hydroxycinnamic acids, flavonols, and carboxylic acids have been studied in samples of instant coffee commercialized in Spain, The studies about contents of food components should be complemented with either *in vitro* or *in vivo* bioaccessibility studies to know the amount of food components effectively available for functions in the human body. In this sense, a widely used in vitro model has been applied to assess the potential intestinal absorption of phenolic compounds and organic acids. Contents of hydroxycinnamic acids and flavonols were higher in instant regular coffee samples than in decaffeinated ones. Bioaccesible phenolic compounds in most analyzed samples account to 20-25 % of hydroxycinnamic acids and 17-26 % of flavonols. This could mean that a great part of them can remain in the gut, acting as potential *in situ* antioxidants. Quinic, acetic, pyroglutamic, citric and fumaric acids were identified in commercial instant coffee samples. Succininc acid was found in the coffee blend containing chicory. All carboxylic acids showed a very high bioaccesibility. Particularly, acetic acid and quinic acid were found in higher contents in the samples treated with the *in vitro* simulation of gastrointestinal process, compared to the original ones, which can be explained by their cleavage from chlorogenic acid during digestion. This is considered as a positive effect, since quinic acid is considered as an antioxidant inducer.

Keywords: hydroxycinnamic acids, flavonols, carboxylic acids, organic acids, instant coffee, bioaccessibility.

Introduction

Coffee is one of the most widely consumed beverages in the world, but the types of coffee beverages and the modality of consumption are strictly associated with social habits and cultures in each country[1]. Commercial production of coffee mainly exploits the seeds of *Coffea arabica* L. (Arabica coffees), which represent approximately 70% of the world market, while *Coffea canephora* Pierre ex A. Froehner (Robusta coffees) is less used[2]. However, for the production of instant coffee, Robusta coffee is preferred for economic reasons and because the extraction yields of soluble solids in the manufacturing process is superior to Arabica coffee[3,4]. Instant coffee is produced by extracting the ground and roasted coffee in hot water under pressure. The coffee infusion is concentrated and either freeze-dried or spray-dried to a granular product, which readily dissolves in water; this is an important point related to consumer preferences. Sometimes decaffeinated coffee is produced by the extraction of caffeine from coffee beans (using organic solvents such as methylene chloride or supercritical CO_2).[5] The different characteristics of ground-roasted coffee as well as the different processes applied, make differences in the final soluble product.

In recent years, there has been an increasing interest in the possible positive implications of coffee consumption for human health[6]. Coffee is a rich source of bioactive phytochemicals, including methylxanthines, amino acids, phenolic compounds. The primary methylxantine in coffee is caffeine, which is well known for its stimulatory and metabolic effects. Phenolic and polyphenolic constituents of coffee have attracted recent attention due, in part, to their natural abundance and reported biological activities including antioxidant activities[7]. The most common phenolic

compounds in coffee are phenolic acids, mainly caffeic acid, a type of trans-cinnamic acid, and its derivative, chlorogenic acid [8]. Chlorogenic acids (CAs) are a family of esters formed between certain trans-cinnamic acids such as caffeic, ferulic, and *p*-coumaric, and quinic acid [9,10]. They are known, not only for their contribution to the final acidity, astringency, and bitterness of the coffee brew, but also for their potential antioxidant properties [11].

Another group of compounds with nutritional importance in coffee is low molecular weight organic acids, which contribute to both taste and flavor of foods, as most of them are volatile [12]. Some carboxylic acids may play a role in the physiology of the human body: citric, malic and lactic acids are considered available organic acids that provide energy [13], since they take part in colonocytes metabolism. In addition, quinic acid has been characterized as a pro-metabolite that leads to induction of efficacious levels of nicotinamide and tryptophan as antioxidants in the body, and thus it has been considered an antioxidant inducer [14].

To achieve a complete knowledge of the significance of nutrients and bioactive compounds in the diet, not only their content but also other factors in which bioavailability is a key point should be studied. The concept of bioavailability of a food component is related to the fraction of the component that can be used for physiological functions. Bioaccessibility is defined as the amount of a food constituent present in the gut due to its release from the solid food matrix, and which may be able to pass through the intestinal barrier, being therefore potentially bioavailable [15].

Estimating the bioaccessibility of food components should ideally be determined by *in vivo* studies, taking into account the whole range of nutritional, physiological and ecological factors that influence absorption. However, *in vitro* methods offer an appealing alternative to human and animal studies. They can be simple, rapid, and may

predict relative bioaccessibility by the determination of both the amount of food component released from the food matrix by the action of the digestive enzymes, and the non-digestible fraction that could reach the colon. This type of protocol has been used to estimate the intestinal accessibility of phenolic compounds and antioxidant capacity in different food sources [15].

Many researchers have studied coffee beans composition as well as the composition of roasted coffee, but intestinal accessibility studies are not frequent, and still less is known about instant products. For that reason, in this study, hydroxycinnamic acids and flavonols (as two of the main types of phenolic families present in coffee), as well as carboxylic acids, have been studied in different samples of instant coffee commercialized in Spain, together with their potential intestinal accessibility after a simulated *in vitro* digestion process.

Material and Methods

Instant coffees of commercial importance were purchased in several markets in the city of Tres Cantos, Madrid, Spain. Eleven different types of instant coffee were selected: 6 different brands of regular instant coffee (identified as 1R to 6R), 3 different brands of decaffeinated coffee (7D to 9D), and two blends of instant coffee with other ingredients. One of them was a blend of 65% roasted coffee and 35% green coffee (10GR); and the other contained 38% coffee, 60% chicory and 2% magnesium sulphate (11CC).

Samples preparation

Coffee infusions were prepared according to manufacturer instructions: 3 g of sample coffee were dissolved in 200 mL of deionized water to achieve a complete extraction at 75°C (however, for sample 11CC, 6 g were used for the coffee infusion). The infusion

was cooled to room temperature, filtered by gravity, divided into aliquots for later analysis, and stored at - 20°C until analysis.

Simulation of *in vitro* gastrointestinal digestion

The potential intestinal absorption was estimated using an *in vitro* digestion followed by a dialysis method[16], based on the method previously described by Miller et al.[17] Twenty -five millilitres of the coffee infusion was adjusted to pH 2.0 (6 M HCl) and incubated with 120 μL of pepsin solution (40 mg pepsin -Sigma Aldrich P-7000- per mL 0.1 M HCl) at 37° C for 2 h. Then, 1.5 mL pancreatin-bile solution (5 mg pancreatin enzyme -P-1750 Sigma Aldrich- plus 25 mg porcine bile-B-8631 Sigma Aldrich- per mL 0.1 M $NaHCO_3$) was added, and digestion products were placed in a dialysis bag (Medicell 7000/2, width 34 mm, 7000MW cut off) in a sodium bicarbonate solution (Merck) (pH 7.5) for 3 h. Then, aliquots of dialyzed materials were taken for later analysis.

The *in vitro* intestinal absorption of studied compounds was calculated according to the equation proposed by Rodríguez-Roque et al.[18]

$$Bioaccesibility\ (\%) = \frac{[compound\ dyalized]}{[compound\ no\ digested]} * 100$$

Analysis of phenolic families

For the analysis of hydroxycinnamic acid and flavonol phenolic families, all coffee infusions, before and after the *in vitro* simulation of digestion, were diluted 1 in 10 mL, based on the methodology developed by Bonoli et al.[19] For hydroxycinnamic acids analysis, 200 μL of diluted infusion + 300 μL of deionized water (or 500 μL of dialyzed material) were added with 4 mL of MeOH; then stirred and absorbance measured at 320 nm for hydroxicinnamic acids analysis, or at 350 nm for flavonols analysis (Lambda EZ 210 UV-visible spectrophotometer, Perkin Elmer, Massachusetts, USA). Ferulic acid

and quercetin (Sigma–Aldrich) were used as standards to build a linear calibration curve, in the range of 0 – 17 mg/L and 0 – 34 mg/L for hydroxycinnamic acids and flavonols, respectively. Final results were expressed as g per 100 g of sample.

Analysis of carboxylic acid by HPLC

The analysis of carboxylic acids was performed in the coffee infusions (diluted 1 in 10) and the digested coffee infusions, adjusted to pH 2.6 with sulphuric acid 9 M, and filtered through paper and PVDF filters (0.45 μm). The injection volume was 20 μL for coffee infusions and 80 μL for digested coffee infusions. The analytical equipment used was a liquid chromatographer (Micron Analıtica, Madrid, Spain), equipped with an isocratic pump (model PU-II), a 717 plus autosampler automatic injector (Waters), a Sphereclone ODS (2) 250 X 4.60, 5 μm Phenomenex column, a UV–visible detector (Thermo Separation Spectra Series UV100), and software Biocrom 2000 3.0. The mobile phase was 1.8 mMH_2SO_4 (pH = 2.6) at 0.4 mL/min flow rate, and UV detection was performed at 215 nm.[20] Standards of L-pyroglutamic acid, D-(−)-quinic acid, citric acid monohydrate, fumaric acid, succinic acid and acetic acid were purchased in Sigma Aldrich, and used to identify the compounds by their retention time (Fig. 1), as well as to build linear calibration curves.

Statistical analysis

All analyses were carried out in triplicate. Data were analyzed using SPSS (SPSS version 22.0 for windows) software. Analysis of variance (ANOVA) and Duncan's multiple range method were used to compare any significant difference between samples. Values were expressed as mean ± standard deviation. Differences were considered significant at $p < 0.05$.

Results and Discussion

Phenolic compounds

Results of phenolic families in the analyzed samples are shown in Table 1, expressed as contents of phenolic compounds extracted in infusions per 100 g of instant coffee powder.

As expected, coffee blend 10GR presented the highest content of both hydroxycinnamic acids and flavonols, since this sample contains 35% of green (non-roasted) coffee, and many phenolic compounds are known to decrease due to the roasting process[21]. The lowest content of hydroxycinnamic acids and flavonols was found in sample 11CC, where coffee had been partially substituted by chicory (only 38% coffee). The inclusion of roasted chicory root in coffee blends is a practice that has been traditionally made to get low caffeine levels in the infusion. Chicory roots (contrarily to chicory leaves) has been reported as a poor phenolic compounds source (presenting around 0.5 g of phenolic compounds per 100 of dried product, according to Jurgonski et al.[22]),. For that reason, the mixture of chicory with coffee, would result in less phenolic compounds compared to regular or decaffeinated coffee blends, as shown in this study.

Average values for all samples of regular coffee were 7.01 g/100 g of hydroxycinnamic acids and 6.88 g/100 g of flavonols; while for decaffeinated coffee were 6.25 g/100 g and 6.38 g/100 g, respectively. These differences are slight but statistically significant (p<0.05), and could be due to a partial extraction of these compounds in the decaffeination process of coffee. These results are in agreement with other values reported by Ramalakshmi et al.[23] and Mullen et al. [24]

The results of both families of phenolic compounds in the samples after the *in vitro* simulation of digestion process are also listed in Table 1. A similar trend was found in digested samples of coffee compared to initial coffee infusions; higher initial phenolic compounds content is linked to more phenolic compounds dialyzed, with a constant tendence in the percent of *in vitro* absorption among all analyzed samples: 20 – 25% for hydroxycinnamic acids, and 17 – 26% for flavonols, with the exception of 2R coffee, showing higher bioaccessibility for both families (29 and 33%, respectively).

According to the results obtained in this study, after ingestion of instant coffee infusion, 74 – 83% of phenolic compounds would probably remain unabsorbed in the gut, acting as *in situ* antioxidants. The role of non-absorbable phenolic compounds in the maintenance of intestinal health status has been well established by Halliwell et al.[25] These authors stated that the concentration of many plant-derived antioxidants, including phenolic compounds, may be much higher in the lumen of the GI tract than are ever achieved in plasma or other body tissues, allowing an antioxidant action in the GI tract. In the case of the studied samples, and with the limitations of *in vitro* assays, it could be estimated that the ingestion of about 100 mL of coffee infusion, obtained from about 1.5 g of instant coffee, would lead to the presence of about 80 mg of hydroxycinnamic acids and the same amount of flavonols in the lumen of the GI tract. These families may be able to locally act like antioxidants in the gut; approximately 20 mg of hydroxycinnamic acids or flavonols would be bioaccesible. These values would be slightly higher for regular coffee than for decaffeinated coffee, and even higher for coffee blends with green coffee, and lower for blends with chicory.

Organic acids

The following acids were identified in the different instant coffees: quinic, acetic, pyroglutamic, citric, fumaric and succinic acids as shown in Fig. 1, for 1R and 11CC samples. The amounts in the different samples analyzed are presented in Table 2 (coffee infusions) and Table 3 (digested coffee infusions).

In all samples, acetic, pyroglutamic and quinic acids were the major acids, with the exception of 11CC (coffee-chicory blend), where succinic acid was detected in higher amount than the other acids, coming probably from chicory. The presence of quinic acid is expected as a part of the molecule of CAs, and roasting promotes its release from CAs molecules by hydrolysis of the ester bond[26]. Heat is also expected to promote the formation of pyroglutamic acid from glutamate[27], while acetic acid has been reported to increase as a result of the processing of instant coffees, probably released from the decomposition of sugars and other organic compounds cleavage[26].

When comparing the obtained results with the literature, some differences were found. Alcazar et al.[28] and Rogers et al.[29] reported citric and malic acids as the major carboxylic acids in green coffee beans. Acid contents differ according to the kind of coffee bean, and they change easily depending on the mode of processing, roasting, and extraction.[30] Pyroglutamic acid contents here presented are similar to those obtained by Galli and Barbas [12] in freeze-dried Colombian coffee.

Although wide variations among the different samples analyzed were found ($p< 0.05$), the statistical study applied showed no statistically significant differences due to the types of coffee studied (regular *vs*. decaffeinated *vs.* coffee blends), with the exception of pyroglutamic acid contents in coffee blends, which were significantly lower than those in regular and decaffeinated coffee. In the case of 10GR sample, the presence of

other materials different from roasted coffee (green coffee or chicory) could explain this fact.

Table 3 presents the amount of each carboxylic acid found in digested samples, expressed per 100 g of initial instant coffee. In general, the average intestinal accessibility of all carboxylic acids is greater than the accessibility of the phenolic compound families. *In vitro* intestinal accessibility was 29% for quinic acid, and over 68% for acetic acid, with respect to the original instant coffee. Higher amounts of acetic and quinic acids were found in some of the digested samples compared to the initial coffee infusions. The increase of acetic acid in 7D sample can be due to its release from the hydrolysis of other acids such as citric acid (which always appears in a very low amount in digested samples, compared to the coffee infusions), and this fact, together with the small size of the molecule, would facilitate the absorption of acetic acid.

The increase of quinic acid in digested samples 4R, 6R, 8D and 9D with respect to content in instant coffee can be due to the hydrolysis from CAs during *in vitro* digestion, releasing quinic acid. This fact may be relevant since quinic acid has been considered as an antioxidant inducer through the metabolism of nicotinamide and tryptophan [14], and thus its higher content in the digested samples could be beneficial.

Fumaric acid presented a value of 30 – 50% *in vitro* intestinal accessibility, while for pyroglutamic acid it was 45 – 77%. As previously pointed out by Galli and Barbas [12], pyroglutamate is known to have a number of remarkable cognitive enhancing effects, which could also be related to the properties of coffee traditionally associated with caffeine. The high content of highly bioaccessible pyroglutamic acid found in this study also supports this hypothesis. On the other hand, citric acid was not found in several digested coffees, while in the chicory-coffee blend, the succinic acid showed an *in vitro* intestinal accessibility of 43%.

Conclusions

Contents of hydroxycinnamic acids and flavonols were higher in instant coffee blends containing green coffee than in roasted coffee samples; they were also higher in regular than in decaffeinated instant coffee. Bioaccesible phenolic compounds in most analyzed samples account to 20-25% of hydroxycinnamic acids and 17-26% of flavonols; the rest could remain in the gut acting as potential *in situ* antioxidants.

Quinic, acetic, pyroglutamic, citric and fumaric acids were identified in all the commercial instant coffee samples analyzed. Succininc acid was detected in the coffee blend containing chicory. Wide variations on the carboxylic acid contents were found among the different samples analyzed, although differences were not due to the type of coffee (regular *vs*. decaffeinated *vs.* coffee blends), except for pyroglutamic acid contents in coffee blends, which were lower than those in regular and decaffeinated coffee. Higher bioaccessibility is generally suggested for carboxylic acids than for phenolic compounds. Acetic and quinic acids may be released from citric acid during digestion, which could be relevant, since quinic acid may act as an antioxidant inducer.

In vitro or *in vivo* bioaccessibility studies should be encouraged to know the amount of food components effectively available for functions in the human body.

Acknowledgements

This work has been possible thanks to a scholarship granted by UCM - UNC (Madrid, España – Córdoba, Argentina) to Natalia Podio, and by PROMEP, UAEH-107 to Esther Ramírez Moreno, as well as to the funding of ALIMNOVA research group (UCM-GR35/10A).

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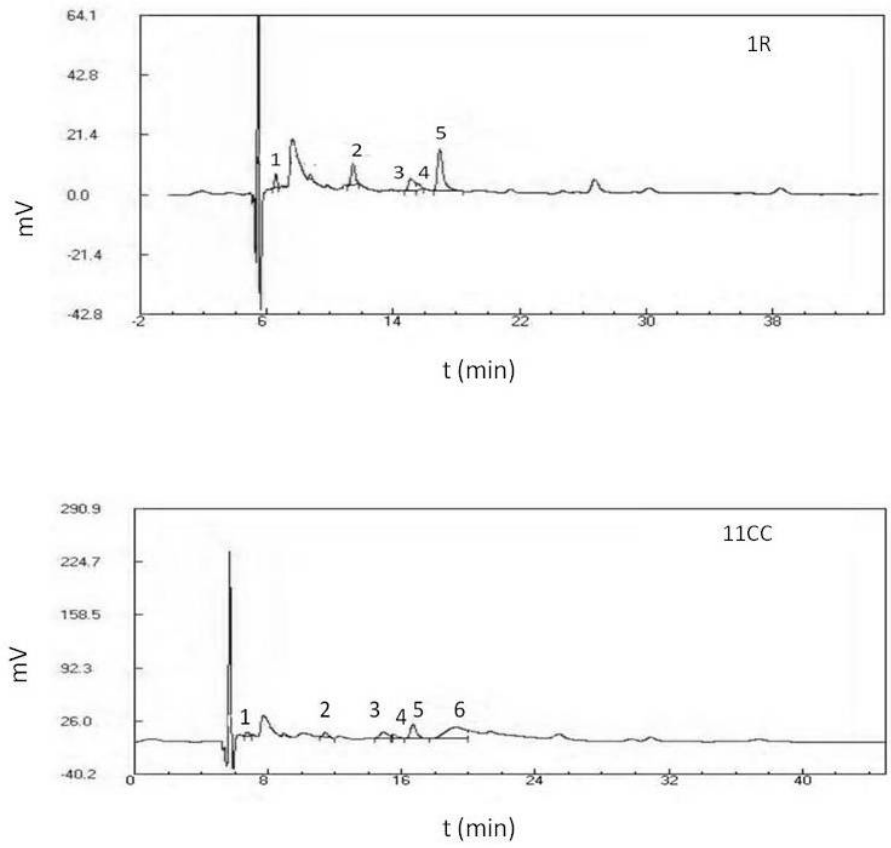

Figure 1: Chromatographic profiles of coffees 1R and 11CC. (peaks 1. Quinic acid. 2. Acetic acid. 3. Pyroglutamic acid. 4. Citric acid. 5. Fumaric acid. 6. Succinic acid)

Table 1. Phenolic families extracted in non-digested and digested instant coffees infusions, expressed per 100 g of instant coffee powder

Samples	Hydroxycinnamic acids (g ferulic acid equivalents/100 g)		Flavonols (g quercetin equivalents/100 g)	
	Coffee infusion	Digested coffee infusion	Coffee infusion	Digested coffee infusion
Regular Coffees				
1R	6.56 ± 0.07a	1.45 ± 0.07a	6.66 ± 0.07ab	1.38 ± 0.06b
2R	7.46 ± 0.02d	2.17 ± 0.01e	7.10 ± 0.03c	2.32 ± 0.06e
3R	6.68 ± 0.14a	1.39 ± 0.06a	6.53 ± 0.16a	1.22 ± 0.05a
4R	7.30 ± 0.03c	1.84 ± 0.01d	7.13 ± 0.06d	1.76 ± 0.07d
5R	7.11 ± 0.13b	1.76 ± 0.04c	6.77 ± 0.15b	1.78 ± 0.06d
6R	7.09 ± 0.13b	1.66 ± 0.05b	7.20 ± 0.13c	1.67 ± 0.06c
Average ± SD	7.01 ± 0.33	1.68 ±0.25	6.88 ±0.28	1.65 ± 0.33
Decaffeinated Coffees				
7D	6.43 ± 0.04b	1.35 ± 0.04b	6.59 ± 0.04c	1.25 ± 0.03b
8D	6.07 ± 0.10a	1.24 ± 0.05a	6.21 ± 0.11a	1.14 ± 0.05a
9D	6.34 ± 0.12b	1.60 ± 0.04c	6.42± 0.13b	1.58 ± 0.03c
Average ± SD	6.25 ±0.18	1.41 ± 0.18	6.38 ± 0.18	1.34 ± 0.21
Coffee blends				
10GR	8.66 ± 0.11b	2.04 ± 0.09b	8.31 ± 0.12b	2.22 ± 0.09b
11CC	3.73 ± 0.05a	0.79 ± 0.02a	3.85 ± 0.05a	0.67 ± 0.02a

Values are expressed as mean value ± standard deviation (SD) of three replicate experiments (n-1). Different letters in the same column means statistical differences ($p < 0.05$) within each type of coffee (regular coffee, decaffeinated coffee and coffee blends).

Table 2. Carboxylic acids extracted in infusions of instant coffees, expressed as mg/100 g of instant coffee powder

Sample	Quinic acid	Acetic acid	Pyroglutamic acid	Citric acid	Fumaric acid	Succinic acid
Regular coffees						
1R	3479 ± 89b	5938± 478cd	3962 ± 319b	729 ± 51a	58.9 ± 1.7a	N.D
2R	2451 ± 326b	6242 ± 221d	2701 ± 157a	880 ± 68ab	55.5 ± 3.2a	N.D
3R	5092 ± 145d	4732 ± 173a	4617 ± 163b	1707 ± 161d	72.3 ± 6.7b	N.D
4R	1782 ± 63a	4356 ± 458a	4382 ± 139b	784 ± 128a	53.4 ± 7.1a	N.D
5R	4076 ± 509c	4884 ± 250ab	6050 ± 491c	1362 ± 95c	72.3 ± 8.0b	N.D
6R	1555 ± 10a	5427 ± 268bc	4100 ± 145b	1111 ± 93bc	79.30 ± 2.5b	N.D
Average ± SD	3072 ±1317	5263 ± 737	4302 ± 1034	1096 ± 365	65.7 ± 10.7	N.D.
Decaffeinated coffees						
7D	3618 ± 539c	3878 ± 78a	4956 ± 142a	941 ± 58a	47.4 ± 2.3a	N.D
8D	2723 ± 387b	4626 ± 380b	4513 ± 449a	674 ± 71a	55.2 ± 5.1b	N.D
9D	1622 ± 97a	4859 ± 413b	4623 ± 437a	1903 ± 134b	75.6 ± 4.2c	N.D
Average ± SD	4455 ± 506	2650 ± 924	4651 ± 398	1172 ± 563	59.4 ± 13.2	N.D.
Coffee blends						
10GR	2666 ± 125a	4423 ± 155b	2543 ± 280a	1677 ± 7b	55 ± 8.9b	N.D
11CC	1155 ± 144a	1626 ± 34a	2374 ± 206a	594 ± 35a	25.0 ± 0.6a	3294 ± 331a

Values are expressed as mean value ± standard deviation of three replicate experiments (n-1). Different letters in the same column means statistical differences ($p < 0.05$) within each type of coffee (regular coffee, decaffeinated coffee and coffee blends). N.D Non-detected (limit of detection = 0.1 mg/100 g)

Table 3. Carboxylic acids extracted in digested infusions of instant coffees, expressed as mg/100g of instant coffee powder

Sample	Quinic acid	Acetic acid	Pyroglutamic acid	Citric acid	Fumaric acid	Succinic acid
Regular coffees						
1R	1857 ± 210a	4496 ± 202b	2628 ± 295bc	416 ± 46a	25.4 ± 1.2a	N.D
2R	1813 ± 155a	4544 ± 731b	1978 ± 33a	651 ± 25b	23.9 ± 0.7a	N.D
3R	1474 ± 137a	3664 ± 198a	2343 ± 160ab	1483 ± 101d	28.2 ± 1.8a	N.D
4R	2409 ± 335b	3740 ± 367a	3056 ± 291d	N.D	26.3 ± 0.4a	N.D
5R	1444 ± 60a	3597 ± 335a	2698 ± 167c	N.D	21.8 ± 2.3ab	N.D
6R	2275 ± 239ab	5091 ± 465b	2360 ± 209abc	856 ± 124c	34.7 ± 1.3b	N.D
Average ± SD	1878 ± 410	4189 ± 651	2510 ± 383	567 ± 533	27.2 ± 4.3	N.D.
Decaffeinated coffees						
7D	2803 ± 575ab	4901 ± 758b	2703 ± 367a	665 ± 78a	29.2 ± 1.2a	N.D
8D	4228 ± 644b	4265 ± 181ab	2663 ± 348ab	N.D	25.4 ± 1.5ab	N.D
9D	2673 ± 150a	3694 ± 92a	3694 ± 92b	914 ± 74.	46.9 ± 5.2b	N.D
Average ± SD	3066 ± 732	4287 ± 592	3099 ± 475	526 ± 412	34.9 ± 9.4	N.D.
Coffee blends						
10GR	2435 ± 119b	3646 ± 47a	1773 ± 176a	764 ± 80b	24.3 ± 1.8b	N.D
11CC	911 ± 121a	2410 ± 365 b	1188 ± 190b	351 ± 22a	5.7 ± 0.4a	1405.9 ±10.2a

Values are expressed as mean value ± standard deviation of three replicate experiments (n-1). Different letters in the same column means statistical differences ($p < 0.05$) within each type of coffee (regular coffee, decaffeinated coffee and coffee blends). N.D Non-detected (limit of detection = 0.1 mg/100g)