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Determination of phenolic acids in coffee by micellar electrokinetic chromatography

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Abstract

A seven-minute micellar electrokinetic chromatography analytical procedure capable of resolving the five principal phenolic acids in coffee, including the isomers of 5-caffeoylquinic acid (5-CQA), 4-CQA and 3-CQA, caffeic acid and ferulic acid is reported. The electrophoretic conditions consisted of an SDS (70 mM)-phosphate (17.6 mM)-methanol (5% v/v) buffer system, pH 2.5, 22.1 °C, −17 kV and detection at 325 nm. The Joule effect and the possible interactions between the buffer components and temperature with respect to peak quality, resolution and selectivity were assessed in the concentration range of 25–900 μg/mL. Performance evaluation of the system used a 3^{3-7} factorial design at the 95% confidence level. The lowest correlation coefficient for linearity was 0.99888 for the 4-CQA. Limits of detection and quantification were 0.98 and 4 μg/mL. The method was tested with both green and roasted coffee beans using four systems of extraction.

Keywords: HPCE; Capillary electrophoresis; MEKC; Phenolic acids; Chlorogenic acid; Ferulic acid; Caffeic acid

1. Introduction

Chlorogenic acid (or 5-caffeoylquinic acid, CQA) is a hydroxycinnamic acid that can function both as a dietary neurosignalling compound at adenosine receptors and as an antioxidant (Williams, Spencer, & Rice-Evans, 2004). CQA occurs in coffee together with two major positional isomers, 3-CQA and 4-CQA, whose physiological or technological importance have not been studied. Upon thermal treatment or storage CQAs can undergo hydrolysis, followed by lactone closing of their products (Farah, de Paulis, Trugo, & Martin, 2005).

A considerable number of phenolic compounds have been identified in roasted coffee, many of which are derived from or related to chlorogenic acid. From the standpoint of the sensorial properties the chlorogenic acids are also among the more than 29 compounds responsible for the characteristic flavor of roasted coffee (Sarrazin, Lequére, Gretsch, & Liardon, 2000). They may contribute, however, with variable degrees of bitterness, astringency and staleness upon aging (Menezes, 1994).

Recent epidemiologic studies associate coffee consumption to protective benefits to the central nervous system. Coffee solutions, particularly of the cultivar Robusta (*Coffea canephora*), exhibit notable free radical quenching ability as evidenced by the inhibitory activity of deoxyribose degradation in tissue culture assays and cell survival indices (Daglia et al., 2004).

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is also one of the most abundant phenolic acids in plants, reaching concentrations of up to 5% in corn kernels (Rosazza, Huang, Dostal, Volm, & Russeau, 1995), largely bound to cell wall polysaccharides. The cross-linked ferulic acid can then be released by the intestinal microbiota for

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general utilization by the body. Besides the antioxidant properties, anti-thrombotic, anti-inflammatory and anti-bacterial activities have been attributed to ferulic acid (Ou & Kwok, 2004).

The analytical determination of phenolic acids has been classically done by HPLC (Trugo & Macrae, 1984) or by further improved methods that may include the determination of 3,4-, 3,5- and 4,5-di-CQA (Negishi, Negishi, Yamaguchi, & Sugahara, 2004). Although capillary electrophoretic methodologies have been recently described using direct (Polasek, Petriska, Pospisilova, & Jahodar, 2006), indirect (Jiang, He, Zhao, & Hu, 2004) and electrochemical (Chen, Zhang, Xue, Chen, & Hu, 2004) detection, in addition to micellar electrokinetic chromatography – MEKC (Guan, Wu, Lin, & Ye, 2006), no electrophoretic or MEKC method for CQA has been reported that includes both the 3- and 4-isomers of CQA, plus caffeic and ferulic acids.

MEKC was first described by Terabe, Otsuka, Ichikawa, Tsuchiya, and Ando (1984) as a modification of the eletrophoretic separation of low-molecular weight aromatic phenols and nitro compounds by the introduction of a surfactant such as SDS to an aqueous buffer at a concentration above a critical level (Critical Micellar Concentration for SDS $= 8.1 \times 10^{-3}$ M), thus enabling the formation of a stable micellar phase that functions as a pseudo-stationary phase and partitions the analyte with the aqueous phase, while migrating towards the anode with a relatively large mobility.

The objective of this work was therefore to develop a capillary electrophoretic method capable of resolving the CQA from its two positional isomers and evaluate the influence of the main factors determining the efficiency and total analysis time of coffee extracts with respect to the CQA isomers, plus the ferulic and caffeic acids, using micellar electrokinetic chromatography.

2. Experimental

2.1. Standards and coffee samples

The chlorogenic acid (5-CQA) standard was purchased from MP Biomedicals, Inc., USA (cat no. 150618). Ferulic (F-3500) and caffeic (C 0625) acids were from Sigma, St. Louis, MO, USA.

Since neither the 3-CQA nor the 4-CQA isomers were commercially available as standards, it was necessary to prepare (Trugo & Macrae, 1984) and isolate the isomers by HPLC, following an appropriate procedure (Negishi et al., 2004) and then, to inject the isolated, corresponding fractions into the capillary electrograph (CE). For this, the isomers were collected from a preparative HPLC column (Waters X-Terra MS C_{18} 5 μm; 19 × 50 mm; Milford, MA, USA), which used the mobile phase and gradient system employed by Negishi et al. (2004). Identification of the peaks was based on previous identification of the isomers using MS and NMR spectroscopy as reported by Negishi et al. (2004).

Green coffee beans (*Coffea* sp.) were kindly supplied by Bernadete Silvarolla, from the Agronomic Institute of Campinas, Campinas, SP, Brazil.

2.2. Reagents and equipment

The running phosphate buffer was prepared from a 50 mM stock solution made with reagent grade phosphoric acid (85%, Merck), HPLC/UV grade methanol (J.T. Baker) and a 500 mM stock solution of sodium dodecyl sulfate (electrophoresis grade SDS, Sigma Chemical Co.), to give 17.6 mM phosphate, 5% methanol and 70 mM SDS as final concentrations and pH 2.50. Lots of 50 mL of buffer were routinely prepared by mixing 2.5 mL of a 200 mM monosodium phosphate (Merck) solution, 7.0 mL of the SDS stock solution, plus 2.5 mL methanol, and the pH later adjusted under a pH-meter with about 7.6 mL of a 50 mM phosphoric acid solution. The mixture was brought to volume with deionized water. The capillary electrophoresis was performed on a Hewlett–Packard 3D Capillary Electrophoresis system (Waldbronn, Germany), equipped with a DAD detector. The phenolic acids were detected at 325 nm. The molar absorptivities of the 3- and 4-isomers were taken from Trugo and Macrae (1984) in order to construct individual calibration curves for all the CQAs. For the caffeic and ferulic acids, standard curves were also determined for each analyte (concentration ranges used are given in Table 2). Data were collected using the HP 3D Chemstation software from Hewlett–Packard (Wilmington, DE, USA). The separations were carried out on a fused-silica capillary (75 μm ID) with effective (l) and total length (L) of 22.5 cm and 30 cm, respectively. Before the first run, capillary conditioning was accomplished by running 5 min of deionized and water, 30 min of 1 M NaOH, followed by 10 min deionized water, all filtered through a 0.47 μm membrane. For in-between samples, we selected the post-conditioning routine, consisting of 1 min of water, 2 min of 0.1 M NaOH, plus 1 min of water and 3 min of buffer. Injection was done hydrodynamically for 3 s at 25 mbar. Measurements of pH were made with a DM-20 pH-meter from Digimed (São Paulo, SP, Brazil), using a combined glass electrode.

2.3. Sample preparation

Green coffee beans (1.5 g/100 mL) were ground to pass a 40 mesh screen and ready-to-drink coffee (25 g L^{-1}) were samples to be extracted for 30 min in a mixture of methanol and deionized water (4:6) and later filtered through a 0.47 μm membrane. Injection samples were prepared by making a 1:1 dilution of the above extract in the 4:6 methanol:water solution.

3. Results and discussion

Joule tests were performed in order to select current–voltage ranges that would offer no significant heating

distortions. These values exhibited a linear relationship ($R^2 = 0.994$) between −2.5 and −20 kV. From these data, −17 kV was chosen as the running voltage.

For the experimental design, the methanol and SDS concentrations and temperature were selected among nine independent variables to be tested by the Minitab14 software for being, in that order, the most influential of all. The dependent variable was the migration time of the 5-caffeoylquinic acid. When selecting the injection characteristics, the number of theoretical plates, symmetry and width of the peaks were tested. From the various conditions of injection tested we found that those meeting the highest degree of consistency were 25 mbar for 3 s. Migration time repeatability showed standard deviations from the means for five different days of (in minutes) 0.01 for FA and 5-CQA, 0.005 for CA, 0.002 for 4-CQA and 0.001 for 3-CQA. Area repeatability is shown in Table 2. Linearity coefficients obtained were between 0.99888 (4-CQA) and 0.99997 (CA), along the concentration interval from 25 to 900 μg/mL. The equilibrium reached inside the capillary did not exhibit electroosmotic flow, thus separating the analytes by partition in the pseudo-chromatographic microenvironment established between the buffer and the micelle–methanol system. The ranges of variation and their corresponding codes are shown in the caption of Fig. 1.

With regard to the relationship time-optimized selectivity, the instrument found a migration time of >7 min for the 3-CQA. Since the methanol concentration was noticed to strongly influence this relationship, an effort was made to lower the level of methanol in the extraction system for real samples in order to lower the analysis time to a value below 7 min without diminishing the selectivity. Attempts were made to extract the chlorogenic acids with 40%, 50%, 60% and 80% (v/v) aqueous methanolic solutions, but concentrations above 50% hampered micelle migration.

Since the CQA has an alkaline pK (~8.5; Sauerwald, Schwenk, Polster, & Bengsch, 1998), it was early understood that an acid pH would be essential for the separation of the isomers from the main form, the 5-CQA. Because of the acid medium (pH 2.5) the molecular characteristics of the structures had a significant weight as selectivity factors. Molecular models (illustration not shown) suggest that the 3-isomer can interact more strongly with the system, most likely because of the closer clustering of the electronegative hydroxyl groups, as compared to the other isomers.

It was also interesting to note that when the polarity was inverted, as the positive pole was next to the detection end, the micelles played a double role in the electrophoretic system; while the analytes migrated toward the positive pole by partition, the negatively charged micelles also migrated to the same pole (detector side), displaying an EOF as in classical capillary electrophoresis.

At the beginning of the tests, unexplained current breaks occurred after a couple of trials. Upon examination of the capillaries it was evident that the outside of the capillary had been covered with conducting material. Since this was probably due to an outflow of the SDS medium, the problem was solved by placing small insulating pieces of latex gloves, wrapping a short stretch of the capillary projections out of the cassette. Between runs, the capillary was regenerated by applying a pressure of 1000 mbar for the recommended time for each solution (H_2O, 1.5 min; 0.1 M NaOH, 1.5 min; H_2O, 2 min; buffer, 2 min).

Although it was possible to operate at higher voltages than −17 kV with little effect on the total time of analysis, the operating voltage of −17 kV (~120 μA) was arbitrarily selected for the tests supported by the absence of distortions due to any Joule effect. A preliminary evaluation of the individual effects of the three most important variables on the total time of migration by the Minitab14 indicated that SDS +1; MeOH −1 and temperature −1 (see caption

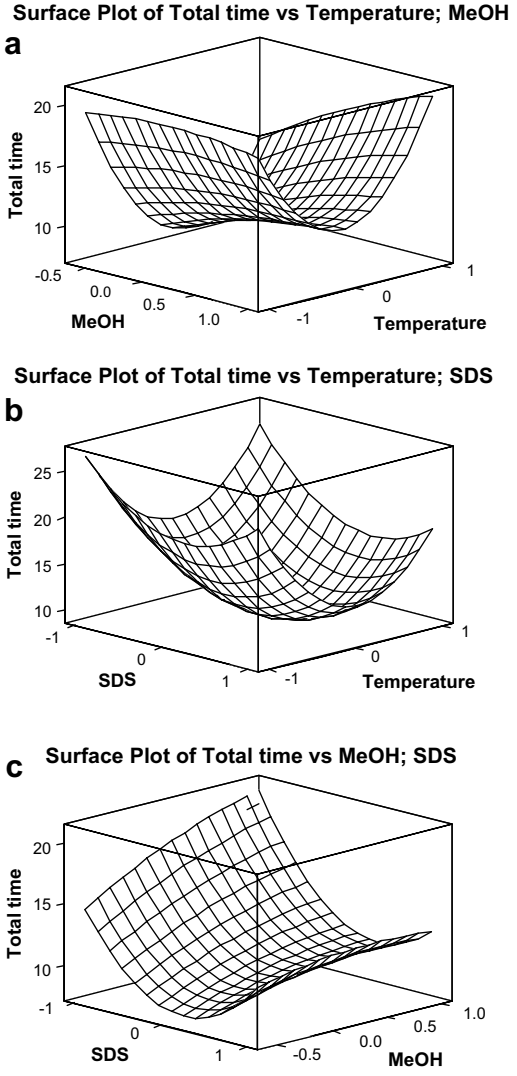

Fig. 1. Surface response of total analysis time to the independent variables: temperature, methanol and SDS concentrations. Code −1 (50 mM SDS, 2.5% methanol, 20.00 °C). Code −0.5946 (58 mM SDS, 3.5% methanol, 20.81 °C). Code 0 (70 mM SDS, 5.0% methanol, 22.00 °C). Code +0.5946 (82 mM SDS, 6.5% methanol, 23.19 °C). Code +1 (90 mM SDS, 7,5% methanol, 24.00 °C).

to Fig. 1) would each give the lowest migration time. Hence a 3^{3-7} factorial planning was carried out and the resulting surfaces are graphically depicted in Fig. 1.

For the preparation of the sample extracts, protein precipitating agents, metal ions or other clarification agents, were avoided in order to minimize the noise, thus improving the labeling of minor the components CA and FA. Characterization of the general electropherogram quality can be found in Table 1. The accuracy of the method can be found in the last column of Table 1.

In order to assess the performance of the method in real samples, green coffee bean filtered extracts and filtered roasted coffee drinks were injected and the results are shown in Fig. 2 and Table 2. The high quality of the

Table 1
Parameters of electropherogram, calibration curve quality for pure compounds at three concentration levels and accuracy evaluation of the method (recoveries) tested in coffee beans ($n = 3$)

Analyte	Migration time (min)	Slope	Intercept	R^2	RSD (%)	Peak symmetry	Theoretical plates	Concentration range (μg/mL)	Recovery (%)
Ferulic acid	2.265	918.22	−8.08E−01	0.99977	6.21	1.60	255,009	25–225	99.20
5-CQA	2.976	645.24	−7.12E−01	0.99996	2.31	1.53	187,740	100–900	93.64
Caffeic acid	3.392	2383.81	−1.74E−01	0.99997	1.82	1.73	160,553	25–225	95.23
4-CQA	4.224	654.47	−7.68	0.99888	3.38	1.03	158,460	99–890	95.09
3-CQA	6.100	598.75	−8.49	0.99906	1.64	0.88	100,228	99–889	94.68

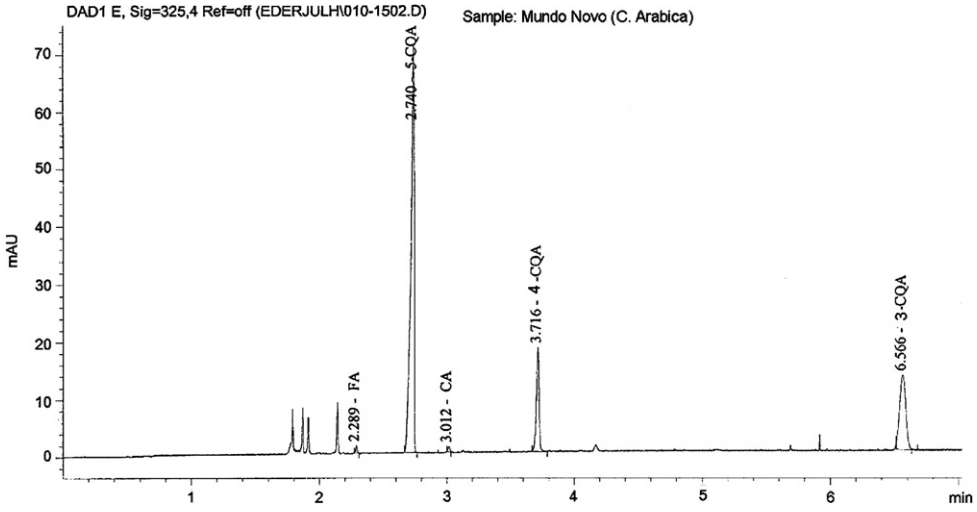

Fig. 2. Green coffee sample *Mundo Novo* IAC-501-5. Electropherogram shows four initial unidentified peaks, ferulic acid (peak 5), 5-CQA (peak 6), caffeic acid (peak 7), 4-CQA (peak 8) and 3-CQA (peak 9). Electrolyte: 10 mM phosphate + 70 mM SDS + 5% MeOH, pH: 2.50. Chemstation parameters: −17 kV; 22 °C. Capillary: 22.5 cm (effective) × 75 μm, Injection: 25 mbar/3 s.

Table 2
Simple phenolic acid contents of twelve Brazilian cultivars of *C. canephora* ($n = 3$)

Coffee cultivar	Phenolic acid contents (mg/100 g)				
	3-CQA	4-CQA	5-CQA	FA	CA
Mundo Novo IAC 376-4	516.8 ± 9	475.0 ± 7	1898.4 ± 9	15.0 ± 1	4.1 ± 0.1
Mundo Novo IAC 388-17-1	479.6 ± 21	486.1 ± 18	2027 ± 105	19.6 ± 1	5.2 ± 0.2
Mundo Novo IAC 501-5	595.4 ± 2	573.0 ± 3	2340.3 ± 8	20.9 ± 1	4.9 ± 0.3
Mundo Novo IAC 515-11	545.5 ± 12	537.8 ± 6	2237.2 ± 36	17.8 ± 0.1	4.9 ± 0.4
Acaiá	788.1 ± 2	735.8 ± 3	3040.2 ± 14	20.8 ± 0.2	4.7 ± 0.4
Icatu Vermelho IAC 2945	752.4 ± 4	728.1 ± 10	3579.5 ± 46	28.5 ± 1	10.2 ± 1
Icatu Amarelo IAC 2944	427.5 ± 21	416.2 ± 18	1677 ± 77	19.0 ± 1	5.1 ± 0.1
Catuaí Vermelho IAC 81	374.5 ± 1	369.7 ± 1	1339.8 ± 3	15.6 ± 1	6.1 ± 0.1
Catuaí Vermelho IAC 144	439.6 ± 1	434.2 ± 1	1604.3 ± 3	15.2 ± 0	5.6 ± 0.1
Catuaí Amarelo IAC 62	391.4 ± 20	391.5 ± 16	1534.1 ± 76	16.0 ± 1	4.3 ± 0.4
Obatã IAC 1669-20	522.8 ± 18	529.6 ± 24	2208 ± 119	21.4 ± 2	5.6 ± 0.1
Tupi IAC 1669-33	592.3 ± 38	555.2 ± 33	2227 ± 152	25.0 ± 1	11.6 ± 1

electropherogram and the actual values of the simple phenolic acid components of Brazilian coffees attest to the usefulness of the method.

4. Conclusions

A simple capillary electrophoretic system has been developed and evaluated for the rapid, simultaneous analytical determination of caffeoylquinic (chlorogenic) acid, caffeic and ferulic acid, giving particular attention to the determination of the two common positional isomers of CQA that previous methods did note include. The method, which utilizes phosphate buffer, methanol, straight silica capillaries and SDS above a critical concentration, is considered as a technique of micellar electrokinetic chromatography (MEKC) and can be used to monitor the presence of all the three CQA isomers in natural or processed vegetables as well as possible changes due to metabolism.

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