Comparison of Five Purification Methods for Chlorogenic Acids in Green Coffee Beans (*Coffea* sp.)

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Quantification of chlorogenic acid content in large populations of green coffee beans needs an accurate, fast, and unbiased purification method. Five different procedures of purification were compared. The first consisted of a successive use of different organic solvents, the second was based on a filtration through a C_{18} cartridge, the third used two combined reagents, and the remaining two methods (4 and 5) were a simplification of the third. One of the two simplest methods of purification (method 4) was also the fastest, the most accurate, and the least biased. Consequently, this method could be used routinely to quantify chlorogenic acids in green coffee beans.

Keywords: *Chlorogenic acids; Coffea sp.; purification procedures; HPLC*

INTRODUCTION

Quinyl esters of hydroxycinnamic acids, which are generally know as chlorogenic acids (CGA) in green coffee beans were first reported by Robiquet and Boutron in 1837 (Sondheimer, 1964). CGA are present in many *Coffea* species (Clifford, 1985a,b; Clifford et al., 1989; Rakotomalala et al., 1993; Anthony et al., 1993). In the two main cultivated species, *Coffea canephora* and *C. arabica*, CGA account for 7−10% and 5−8% of dry matter basis (dmb), respectively (Clifford, 1985b; Rakotomalala, 1992). CGA include three main groups which are caffeoylquinic acids (CQAs), feruloylquinic acids (FQAs), and dicaffeoylquinic acids (diCQAs) (Clifford et al., 1977). Each group contains three isomers on the basis of the number and identity of the acylating residues (Clifford, 1985a,b).

Quantitative analysis of CGA in large populations needs an accurate, nonbiased, fast, and cheap method. An accurate method allows reduction in sample size without a decrease in precision. A nonbiased method should give estimations centered on average on the true value.

The experimental procedure to estimate CGA contents includes three steps: (1) extraction, (2) purification, and (3) separation and quantification of the different isomers by HPLC. The extraction, separation, and quantification of the different isomers are well defined in terms of their optimal chemical reagents or solvents (Colonna, 1979; Clifford et al., 1985; Trugo, 1984; Van Der Stegen et al., 1980). By contrast, at least four different purification procedures are described in the literature, whereas a fifth variation is the standard technique used in Germany.

The first method consists of the successive use of different organic solvents (Fleuriet and Macheix, 1972). The second is a purification through a C_{18} cartridge (Sep-Pak ref 51910, Millipore Waters) (Bicchi et al., 1995). The third consists of a treatment by two specific reagents (Carrez Reagents) to precipitate colloidal material (Balyaya and Clifford, 1995). Removal of the rotary evaporation step leads to the next method (Trugo and Macrae, 1984), whereas a fifth method differs from the others by absence of cleaning with Carrez solutions. These five methods were compared, in terms of their precision and efficiency.

MATERIALS AND METHODS

Plant Material. Green coffee beans were supplied by the Agricultural Coffee Tree Station ORSTOM-IDEFOR (Man, Ivory Coast). In the first experiment, we used 1000 beans of the species *C. liberica* var. dewevrei (Bridson, 1994) from 10 trees. In the second experiment, 100 beans of the same species were used.

Extraction Procedure. The beans were divided into 100-bean lots to be crushed in a ball mill (Dangoumill) which was previously frozen by liquid nitrogen in order to minimize CGA degradation (Colonna, 1979). Crushing yielded a fine powder (15 min per lot). All powder was bulked again before being split into 1 g samples. Each sample was extracted in a 250 mL Erlenmeyer flask containing 100 mL of a methanol−water mixture (70/30) and 0.5% Na_2SO_3. The Erlenmeyer was shaken overnight at 4 °C in darkness on a stirring table at 125 rpm (Colonna, 1979). The organic extract was filtered through cotton to eliminate powder. All extracts were bulked again in order to obtain a homogeneous extract for comparison of purification methods.

Experimental Design. A first experiment involved the comparison of the four first methods. Five replicate purifications were done per method. The 20 samples were totally randomized before purification. A second experiment allowed the testing of the Carrez reagents step (comparison between methods 4 and 5). Four repetitions were performed per method.

Purification Procedures. The first step of methods 1−3 was elimination of methanol by rotary evaporation (Rotavapor) to obtain an aqueous extract.

Method 1 (Rakotomalala, 1992). To aqueous extracts (about 22 mL each) were added ammonium sulfate to a final concentration of 20 g/L (precipitation of proteins by an increase in ionic strength) and 4% phosphoric acid (acidification of the medium to make the CGA more soluble in ethyl acetate). Extracts were then treated three times with petroleum ether (+40;−60 °C) to remove lipids and pigments. Depigmented extracts were treated three times with $CHCl_3$ to eliminate caffeine and wax insoluble in petroleum ether. Residual solvent was then eliminated in a rotary evaporator Bioblock 94200 (Rotavapor). CGAs were extracted by four successive treatments with ethyl acetate. The four ethyl acetate phases were pooled and dried by anhydrous Na_2SO_4. After filtration (Whatman 1PS), ethyl acetate was evaporated (Rotavapor) and the residue was dissolved in 100 mL of methanol.

Method 2 (Bicchi et al., 1995). Aqueous extracts (about 30 mL) were filtered through a 0.2 μm Millipore filter, and 6 mL aliquots were loaded on a C_{18} Sep-Pak cartridge 51910 (Millipore Waters) previously conditioned with 5 mL of methanol and 3 mL of distilled H_2O of HPLC grade. The cartridge was

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eluted with 20 mL of methanol/water mixture (70/30), and the eluate was then directly submitted to HPLC−UV analysis.

Method 3 (Balyaya and Clifford, 1995). Carrez reagents consist of solutions I and II. Solution I was prepared by dissolving 21.9 g of crystallized zinc acetate and 3 mL of glacial acetic acid in 100 mL of distilled water. Solution II consisted of 10.6 g of potassium hexacyanoferrate (Fe^{2+}) in 100 mL of distilled water. Colloidal material present in aqueous extracts (about 30 mL) was precipitated by adding 1 mL of solution I, plus 1 mL of solution II, diluted to 50 mL with methanol−water mixture (70/30).

Method 4 (Trugo and Macrae, 1984). Methanol extracts (100 mL) were directly treated with Carrez reagents, without previous evaporation in a Rotavapor.

Method 5 (DIN Standard 10 767, 1992). Methanol extracts (100 mL) were directly analyzed by HPLC.

Analytical HPLC. Chromatography was carried out on a system consisting of two Waters Associates Model 510 pumping units, an automated sample injector (Waters 717 plus autosampler), a variable-wavelength UV detector (Waters 996 photodiode array detector), a C_{18} precolumn, and a 250 × 4 mm Merck Superspher 100 RP 18 column, 5 μm particle size. The elution program used two solvents, A and B. Solvent A was 2 mM phosphoric acid, pH 2.7, containing 5% methanol. Solvent B was methanol containing 5% of 2 mM phosphoric acid, pH 3.9. These two mobile phases were filtered (0.2 μm), degassed, and sonicated (Ney, 300 ultrasonik) before use. Sample or standard (10 μL) was analyzed at room temperature using the following elution program: A−B mixture (75/25) to pure solvent B in 45 min of linear gradient. Flow rate was 0.8 mL/min. UV detection was carried out at a wavelength of 325 nm, corresponding to the CGA maximum absorption.

Isomer Identification. CGA isomers (3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) were identified by comparing chromatograms (280 and 325 nm), retention times, and UV spectra to published results (Rakotomalala, 1992). Identification of the CQA isomers was confirmed by isomerization of 5-CQA standard (ref D11.080-9, Aldrich-Chimie). Isomerization was achieved by heating the standard in dilute ammonia solution at pH 8 for 30 min (Trugo et al., 1984).

Isomer Quantification. Quantification was achieved by peak area measurement and by comparison with a 5-CQA standard. A calibration curve ($C = 0.27896 \times$ peak area) was generated using three replicate points of 5-CQA at 25, 50, 75, 100, 125, and 150 mg/L. All amounts were referred to the same dilution factors, corresponding to the final volume of 100 mL, to obtain comparable results.

Statistical Analysis. Results were analyzed using Statistica software. The precision of the methods was compared by testing the homogeneity of their variance (Levene, 1960). In the first experiment, heterogeneity of variances led to compare efficiency of methods by the median test (Conover, 1971). In the second experiment, homogeneity of variances permitted the use of a one-way ANOVA. These tests were applied to the absolute amounts of each isomers, total CQA, total FQA, total diCQA, and total CGA.

RESULTS

The major problem encountered with the chromatographic resolution was the peak due to 4-CQA (Trugo and Macrae, 1984). The 4-CQA peak was located by isomerization of the 5-CQA standard, which yielded an equilibrium mixture of the 3-, 4-, and 5-isomers. The 4-CQA and 5-CQA isomer peaks overlapped (Figure 1), and so the common peak of these undifferentiated isomers was called the 5-&4-CQA peak. Eight peaks were then considered for the routine statistical analysis (Figure 2). The 5-&4-CQA peak was the main peak whatever the method, followed by the diCQA peaks. The FQA peaks were the lowest.

First Experiment. Differences between methods were recorded for each isomer (Table 1). For CQA isomers and total CQA, the best methods were 3 and 4, with a average gain of 153% by comparison with methods 1 and 2. For FQA isomers and total FQA, method 4 was significantly better than the three others. The gains were then 84%, 111%, and 146% by comparison with methods 3, 2, and 1, respectively. For diCQA isomers and total diCQA, methods 2 and 4 were similar and better than methods 1 and 3. The gain was then

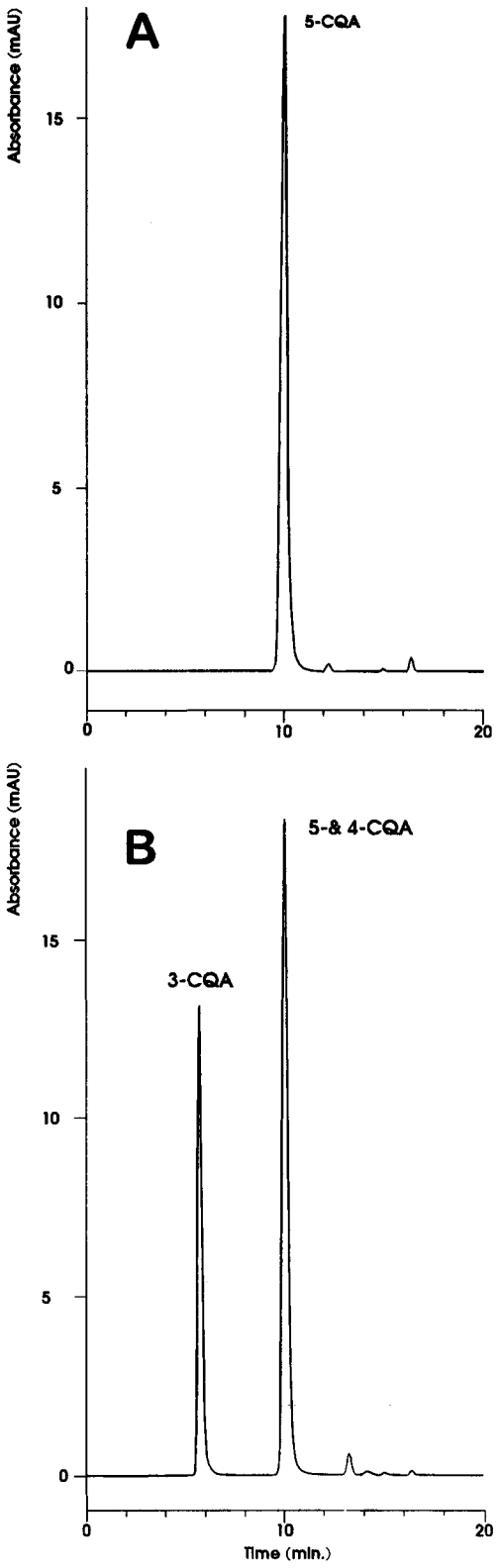

Figure 1. Chromatograms of (A) 5-CQA standard and (B) CQA isomers mixture obtained by isomerization of 5-CQA. Detection at 325 nm and chromatographic conditions are as described in the text

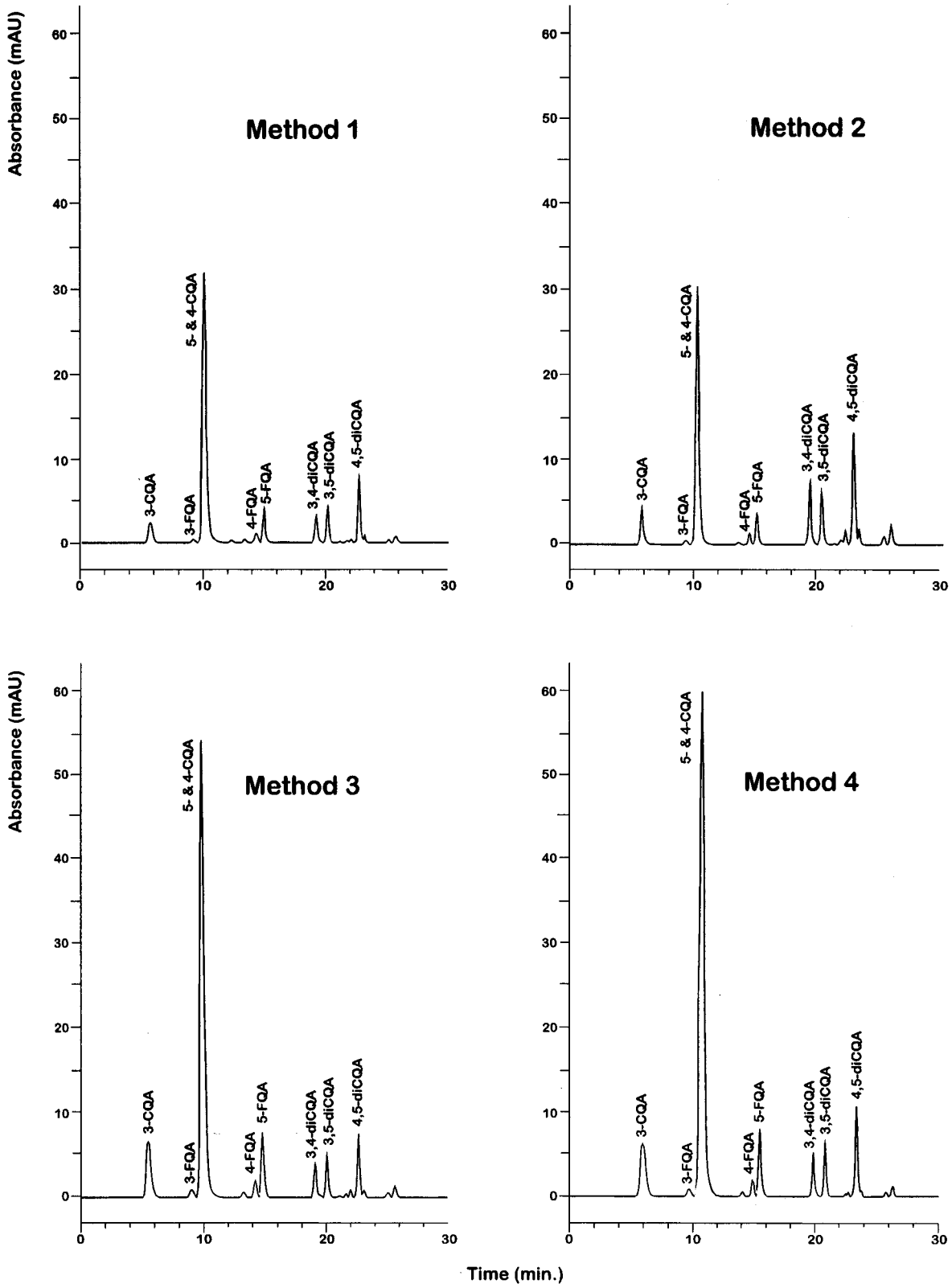

Figure 2. HPLC chromatograms of the four methods of purification of chlorogenic acids from green *C. liberica* var. dewevrei beans. Chromatographic conditions were as follows: solvent A, 5% methanol in 2 mM phosphoric acid; solvent B, 5% of 2 mM phosphoric acid in pure methanol; gradient A−B mixture (75/25) to pure B solvent in 45 min; column 250 × 4 mm Merck Superspher C_{18}; 10 μL injection; flow rate, 0.8 mL min^{-1}; detection at 325 nm.

53% and 108% by comparison with methods 3 and 1, respectively.

The lower the within-method variance, the better the absolute precision, which did not differ between the methods for CQA isomers (Table 2). By contrast, the precision of method 2 was clearly lower for FQA and diCQA isomers. The same respective conclusions were reached when isomers were regrouped in total CQA, total FQA, and total diCQA. The relative precision, measured by the coefficient of variation, was again lowest with method 2 (30−43%). Relative precision was mediocre with method 3 (8−22%) and acceptable with method 1 (2−8%). Method 4 was still the best method (1−7%), especially for FQA (Table 2).

Table 1. Median Contents (% dmb), Chi-Square Values (χ^2), and Associated Probabilities (*P*) for the Different Chlorogenic Acid Isomers (Results of 2 × 2 Comparisons Are Indexed)

	method 1	method 2	method 3	method 4	χ^2	*P*
3-CQA	0.2[a]	0.2[a]	0.7[b]	0.8[b]	19.0	0.000
5-&4-CQA	2.7[a]	2.1[a]	5.6[b]	6.5[b]	19.0	0.000
total CQA	2.9[a]	2.3[a]	6.2[b]	7.3[b]	19.0	0.000
3-FQA	0.03[a]	0.04[a]	0.04[a]	0.08[b]	10.1	0.017
4-FQA	0.07[a]	0.09[a]	0.09[a]	0.15[b]	10.1	0.017
5-FQA	0.22[a]	0.23[a]	0.27[b]	0.53[c]	10.1	0.017
total FQA	0.31[a]	0.36[ab]	0.41[b]	0.76[c]	10.1	0.017
3,4-diCQA	0.18[a]	0.43[c]	0.22[ab]	0.32[bc]	9.5	0.022
3,5-diCQA	0.19[a]	0.47[c]	0.26[b]	0.37[bc]	9.5	0.022
4,5-diCQA	0.39[a]	0.90[c]	0.56[b]	0.73[bc]	9.5	0.022
total diCQA	0.78[a]	1.80[c]	1.05[b]	1.43[bc]	9.5	0.022
total CGA	4.1[a]	4.6[a]	7.7[b]	9.5[c]	19.0	0.000

Table 2. Within-Method Variation: Standard Deviations and Coefficients of Variation (in Parentheses) Given for Each Method and Variable[a]

	method 1	method 2	method 3	method 4	*F*	*P*
3-CQA	0.016 (8%)	0.074 (31%)	0.070 (10%)	0.041 (5%)	1.83	>0.05
5-&4-CQA	0.143 (5%)	0.610 (37%)	0.441 (8%)	0.156 (3%)	2.63	>0.05
total CQA	0.156 (6%)	0.688 (31%)	0.506 (8%)	0.195 (3%)	2.51	>0.05
3-FQA	0.001 (6%)	0.015 (43%)	0.008 (18%)	0.001 (2%)	4.55	0.018
4-FQA	0.010 (5%)	0.039 (41%)	0.012 (13%)	0.001 (1%)	4.79	0.015
5-FQA	0.014 (6%)	0.105 (41%)	0.040 (14%)	0.006 (1%)	6.31	0.005
total FQA	0.018 (6%)	0.159 (41%)	0.061 (14%)	0.005 (1%)	6.05	0.006
3,4-diCQA	0.005 (3%)	0.115 (30%)	0.050 (22%)	0.022 (7%)	3.68	0.036
3,5-diCQA	0.008 (4%)	0.152 (35%)	0.049 (18%)	0.025 (7%)	3.81	0.032
4,5-diCQA	0.009 (2%)	0.250 (31%)	0.086 (15%)	0.034 (5%)	4.34	0.021
total diCQA	0.013 (3%)	0.519 (32%)	0.182 (17%)	0.078 (6%)	4.07	0.026
total CGA	0.182 (5%)	1.325 (31%)	0.733 (9%)	0.286 (3%)	2.66	>0.05

[a] *F* is the observed value and *P* the associated probability from comparison of variance (Levene test).

Table 3. Comparison of Methods 4 and 5 (Mean Contents (% dmb), *F* Values, and Associated Probabilities (*P*) Are Done for Each Chlorogenic Acid Isomers)

	method 4	method 5	*F*	*P*
3-CQA	0.59	0.50	15	0.008
5-&4-CQA	4.8	4.2	15	0.008
total CQA	5.4	4.7	15	0.008
3-FQA	0.054	0.049	5.46	0.058
4-FQA	0.054	0.049	5.46	0.058
5-FQA	0.37	0.35	8.87	0.025
total FQA	0.48	0.45	7.90	0.030
3,4-diCQA	0.26	0.19	99	0.000
3,5-diCQA	0.28	0.23	46	0.000
4,5-diCQA	0.56	0.43	55	0.000
total diCQA	1.1	0.8	63	0.000
total CGA	6.95	6.05	20	0.004

Second Experiment. For all isomers, estimations by method 4 were higher than by method 5, even if the test was not significant for 3- and 4-FQA (Table 3). For total CQA, total FQA, and total diCQA, the gains were of 15%, 6%, and 37%, respectively. The precision obtained did not differ between methods 4 and 5 for any isomers.

DISCUSSION

The method giving the higher contents was considered as being the least biased. Indeed, the true and unknown estimation would be obtained if, and only if, no loss occurs during the different steps of the analysis. This ideal case cannot be reached; however, we only expect a minimal loss (bias). Consequently, method 4 was the least biased for all chlorogenic acids, although methods 2 and 3 were similar to method 4 in the case of diCQA and CQA, respectively. Nevertheless, the quality of our choice supposes that the results we obtained with other methods were similar to literature values. Our results were comparable with published data on *C. liberica* var, dewevrei reported by Anthony et al. (1993) using method 3 and by Rakotomalala (1992) using method 1 (Table 4).

Table 4. Comparison of Our Data with Those of Anthony et al. (1993), Using Method 3 of Purification, and Those of Rakotomalala (1992), Using Method 1 (Data Are Expressed in % dmb)

	our results		literature results	
	method 1	method 3	method 1	method 3
total CQA	2.9	6.2	2.6	6.1−6.65
total diCQA	0.78	1.05	−	0.64−0.98
total FQA	0.31	0.41	0.28	0.38−0.61

Simplification of methods led to an improvment of results, except for method 5. Although the latter is a standard method to estimate CGA content, the absence of Carrez reagents decreased recovery in the case of coffee beans. As Carrez reagents precipitate polysaccharides, soluble proteins, and other colloidal materials, the latter observation suggests that some of these products could react with CGA during the HPLC phasis in relation with the acidification of medium (Trugo, 1984; Clifford, 1985a).

In conclusion, method 4 should be selected for routine analysis of CGA in green beans of *Coffea* species when repeatability, precision, rapidity, and cost are at a premium.

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