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ARTICLES

Separation of Chlorogenic Acids and Caffeine on a Diasfer-110-C10CN Stationary Phase

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Abstract—It is shown that a transition from traditional C18 (I) stationary phases with non-polar endcapping to the C10CN (II) phase, containing terminal polar groups, leads to a change in selectivity comparable to an increase in the activity of residual silanol groups in phases I. The effect was detected in the separation of isomeric monocaffeoylquinic acids. Two versions of the gradient mode are proposed using a Diasfer-110-C10CN column and water–acetonitrile components of the mobile phase acidified with H_3PO_4 for the separation of chlorogenic acids and caffeine in green coffee extracts from different manufacturers. It was shown that the proposed chromatographic method can also be used to determine trigonelline, the retention of which significantly increased in replacing phase I with phase II. The proposed method was used to differentiate the fruits of two types of coffee—Arabica and Robusta. It was found that the total amount of chlorogenic acids and caffeine is higher in Robusta coffee extracts.

Keywords: reversed-phase HPLC, C18 phase, C10CN phase, selectivity, chlorogenic acids, caffeine, trigonelline, Arabica coffee, Robusta coffee

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In [1], it was shown that the change in the elution order of monocaffeoylquinic acids under reversed-phase chromatography conditions is determined by the activity of residual silanol groups. For this reason, differences in the selectivity of the separation of the pair of 4-caffeoylquinic (4CQA) and 5-caffeoylquinic (5CQA) acids are experimentally detected: the most frequently observed elution order t_R(3CQA) $\ll$ t_R(5CQA) < t_R(4CQA) [2–5] through coelution [6] can change to another t_R(3CQA) $\ll$ t_R(4CQA) < t_R(5CQA) [7, 8] with an increase in the activity of residual silanol groups [1]. However, in this case, it is of interest to study the selectivity of the separation of chlorogenic acids on the stationary phase Diasfer C10CN, the hydrophilic properties of the cyanide group in which can complement the lipophilic properties of the base of ten methylene groups in the stationary phase. To date, the number of publications on the study of the chromatographic behavior of substances on a stationary phase of this type is very limited [9–12].

The aim of this work was to compare the selectivity of the separation of trigonelline, caffeine and chlorogenic acids, which in the broad sense include three isomeric monocaffeoylquinic, three isomeric dicaffeoylquinic and 5-feruloylquinic acids, and to use the developed method for determining the composition of green coffee extracts and differentiate between the types of Arabica coffee (*Coffea arabica*) and the cheaper type, Robusta coffee (*Coffea canephora* var. *robusta*).

EXPERIMENTAL

To extract the ingredients of green coffee, coffee beans were ground in a household coffee grinder and the fraction passed through a 0.125 mm sieve was sifted for analysis. A sample of ground coffee weighing approximately 0.050 g was poured into 20 mL of a mixture of ethanol and water (1 : 1, by volume) and kept on an LSS 220 stirrer for 40 min. Then the mixture was centrifuged, the extract was decanted, and the solid residue was back extracted. It was experimentally established that more than 97% of the active ingredients were extracted in two successive extractions.

To calibrate the detector response, the following standards were used: chlorogenic acid (5CQA·0.5H_2O) (Aldrich, India) for the determination of all monocaffeoylquinic, dicaffeoylquinic acids and 5-feruloylquinic acid (when recalculated per mole, the peak area of dicaffeoylquinic acids was divided by two) and caffeine (China).

An Agilent 1260 Infinity chromatographic system with a diode array detector was used. To separate the components of green coffee extracts, various versions of mobile phases of the ethanol–0.2 vol % orthophosphoric acid–water system were used in the isocratic

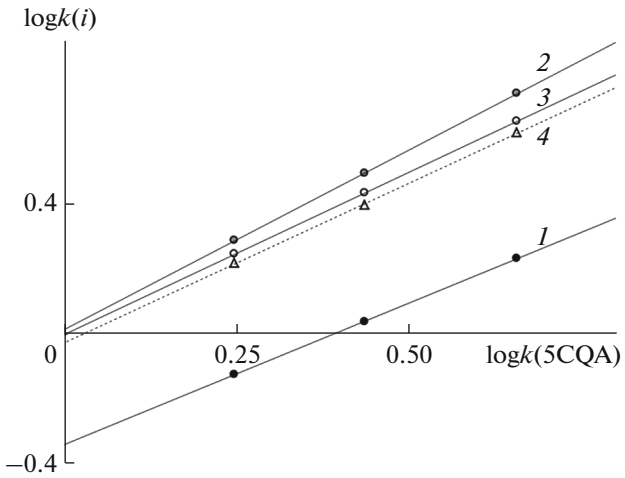

Fig. 1. Type II separation map of 3CQA (*1*), 4CQA (*2*), 5CQA (*3*), and caffeine (*4*) on the stationary phase of Kromasil 100-5C18 in the mobile phases of the ethanol–0.2 vol % orthophosphoric acid–water system at 30°C.

mode to compare the separation selectivity, and in gradient modes to determine the concentration of the active ingredients in the extract. A 150 × 4.0 Diasfer-110-C10CN, 5 μm column and a 100 × 4.6 Kromasil 100-5C18 column were used to compare the separation selectivity. In determining the active ingredients, only the first column was used in two versions of gradient elution with two components of the mobile phase: A—10 vol % ethanol, 0.2 vol % orthophosphoric acid in water, B—40 vol % ethanol, 0.2 vol % orthophosphoric acid in water in the gradient modes: (1) 0 min – 0% B; 3 min—0% B; 25 min—100% B; 26 min—0% B; 35 min—0% B; (2) 0 min—20% B; 3 min–20% B; 25 min—100% B; 26 min—20% B; 35 min—20% B.

Green coffee extracts cannot be introduced into the chromatograph due to the formation of artifacts—broadening and distortion of the shape of peaks of monocaffeoylquinic acids due to an excess in the elution capacity of the sample solvent compared to the starting eluent. It was experimentally determined that a two-fold dilution with distilled water was sufficient to exclude their formation, although a four-fold dilution was used to determine the active ingredients. After the dilution, the sample solutions were filtered through a Nylon filter with 0.45 μm pores (Millipore Millex HP, China) without additional purification.

The flow rate of the mobile phase was 0.8 mL/min at a column thermostat temperature of 30°C. A wavelength of 325 nm was used to detect all chlorogenic acids, and a second wavelength of 273 nm was used to determine caffeine. It was found that the calibration curves for chlorogenic acids and caffeine were linear without statistically significant intercepts (indicating systematic errors) in the concentration ranges from 0.01 to 0.06 mg/mL for chlorogenic acid and from 0.005 to 0.06 mg/mL for caffeine in the chromatographed sample.

RESULTS AND DISCUSSION

In this work, various compositions of the ethanol–0.2 vol % H_3PO_4–water system were used as mobile phases harmless to humans and the environment. To stabilize the charge state of chlorogenic acids, 0.2 vol % orthophosphoric acid was added to the mobile phases. In this case, the pH of the mobile phase was slightly higher than 2. This led, firstly, to the transfer of all acids to an almost completely unionized state [13]. Secondly, such a composition of the mobile phase does not go beyond the pH range 2–8 recommended for conventional "monomer" stationary phases [14].

The Kromasil 100-5C18 stationary phase was used as a reference phase. The chromatographic behavior of the main components of green coffee, including three isomeric monocaffeoylquinic acids, 5-caffeoylquinic (5CQA), 4-caffeoylquinic (4CQA), and 3-caffeoylquinic (3CQA) and caffeine, is shown on the type II separation map [15] (Fig. 1).

It follows from the presented data that, in the entire considered range of ethanol concentrations in the mobile phase (from 10 to 16 vol %), the elution order on the stationary phase of the Kromasil brand does not change:

$$\begin{aligned} t_R\,(3\text{CQA}) < t_R(\text{caffeine}) \\ < t_R\,(5\text{CQA}) < t_R\,(4\text{CQA}). \end{aligned} \quad (1)$$

In Fig. 1, trigonelline is absent, because its retention (probably due to ion exclusion) was lower than the retention of oxalic acid, used as a marker of the column's "dead" time.

A transition to the Diasfer C10CN stationary phase led to significant changes in the retention of the main components (Fig. 2), which is apparently due to the presence of a hydrophilic CN group at the end of the grafted alkyl radical. As expected, the selectivity of the separation of a pair of isomeric chlorogenic acids changed: the retention of 5CQA in all mobile phase compositions was higher than that of 4CQA:

$$t_R\,(3\text{CQA}) < t_R\,(4\text{CQA}) < t_R\,(5\text{CQA}). \quad (2)$$

In addition, the slope of the trend line for caffeine was less than that for 4CQA and 5CQA:

$$\log k\,(4\text{CQA}) = 0.947\log k\,(5\text{CQA}) - 0.140, \quad (3)$$

$$\log k\ (\text{caffeine}) = 0.737\log k\,(5\text{CQA}) + 0.020; \quad (4)$$

therefore, the elution position of caffeine changes with a change in the ethanol content of the mobile phase from the order given below at an ethanol content of more than 16 vol %:

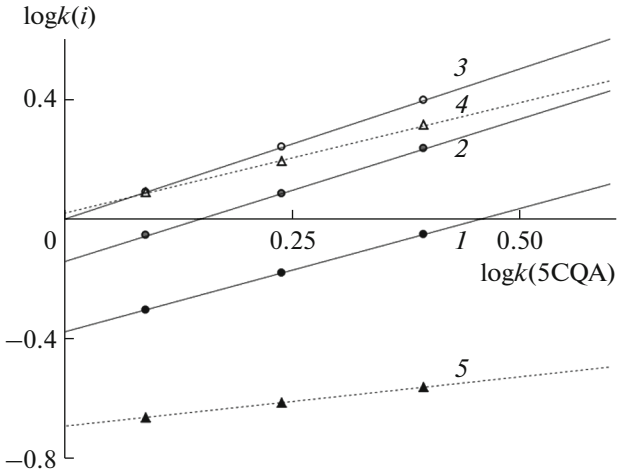

Fig. 2. Type II separation map of 3CQA (*1*), 4CQA (*2*), 5CQA (*3*), caffeine (*4*), and trigonelline (*5*) on the stationary phase of Diasfer 110-C10CN in the mobile phases of the ethanol–0.2 vol % orthophosphoric acid–water system at 30°C.

$$t_R(\text{3CQA}) < t_R(\text{4CQA}) < t_R(\text{5CQA}) < t_R(\text{caffeine}), \quad (5)$$

up to a series with one inversion at an ethanol content of less than 16 vol %:

$$t_R(\text{3CQA}) < t_R(\text{4CQA}) < t_R(\text{caffeine}) < t_R(\text{5CQA}). \quad (6)$$

In addition, for the Diasfer C10CN stationary phase, trigonelline has retention time greater than the "dead" time determined by the retention of oxalic acid.

A comparison of the peaks in the chromatograms (Fig. 3), recorded at two wavelengths (273 and 325 nm), shows that the Kromasil 100-5C18 column has higher efficiency despite its shorter length: more than 47000 tp/m for the Kromasil 100-5C18 phase versus about 28000 tp/m for the Diaspher C10CN phase. Despite this, in using the latter stationary phase, the complete (at the baseline level with $R_s > 1$) separation of the peaks of all sequentially eluted compounds is achieved.

According to [5], the main components of green coffee extract should include a peak of 5-feruloylquinic acid (5FQA) and three isomers of dicaffeoylquinic acids: 3,4-dicaffeoylquinic acid (3,4diCQA), 3,5diCQA, and 4,5diCQA [5]. However, the three isomers of dicaffeoylquinic acids are more lipophilic than monocaffeoylquinic acids (Fig. 4).

The vertical section *a* in Fig. 4, which determines the retention of all substances, shows that with a retention factor of 5CQA <1, the retention factor of 4,5diCQA is 16: i.e., the time required to record a chromatogram exceeds 30 min. In this regard, it is

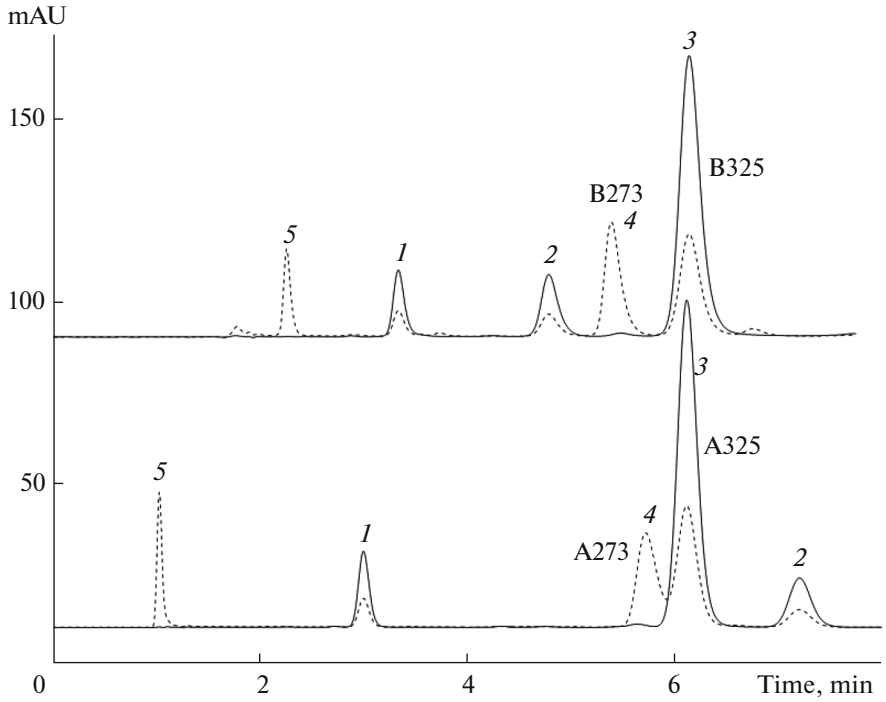

Fig. 3. Separation of 3CQA (*1*), 4CQA (*2*), 5CQA (*3*), caffeine (*4*) and trigonelline (*5*) on the stationary phase Kromasil 100-5C18: A325 and A273, recorded at wavelengths of 325 and 273 nm, respectively, and on the stationary phase Diasfer-110-C10CN: B325 and B273; mobile phase 10 vol % ethanol and 0.2 vol % orthophosphoric acid in water, 0.8 mL/min, 30°C.

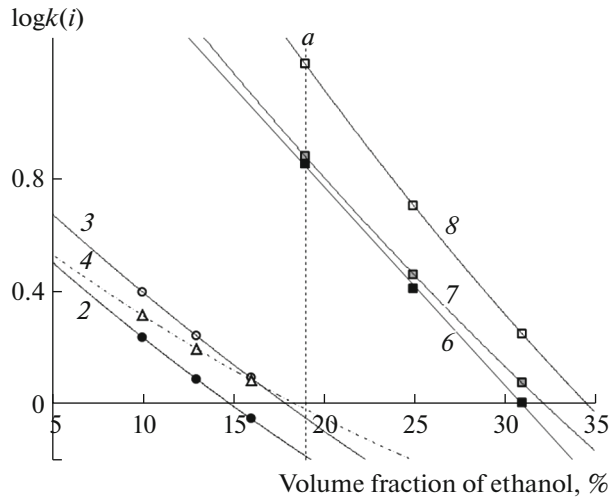

Fig. 4. Type I separation map of 4CQA (*2*), 5CQA (*3*), caffeine (*4*), 3,4diCQA (*6*), 3,5diCQA (*7*) and 4,5diCQA (*8*) on the stationary phase of Diasfer 110-C10CN in the mobile phases of the ethanol–0.2 vol % orthophosphoric acid–water system at 30°C.

more convenient to use gradient elution for the simultaneous determination of all chlorogenic (mono- and dicaffeoylquinic) acids and caffeine. In this paper, two versions of the gradient mode with different localization of caffeine in the series of chlorogenic acids are proposed (Fig. 5).

In both cases, however, it was not possible to achieve the complete separation of 3,4diCQA and 3,5diCQA, but this is not essential: recording chromatograms requires no more than 20 min in the case of the first type of gradient and 12 min for the second type of gradient. This made it possible to determine the concentration of active ingredients in the first extracts of green coffee, the results of which for the first extraction are presented in Table 1.

Judging from the results presented in Table 1, three isomers of monocaffeoylquinic acids are found in all samples of extracts, the main one being 5CQA. In the samples of Robusta coffee extracts, the content of this acid is slightly higher than in Arabica coffee varieties, but this difference is not great. A significantly more noticeable difference between the extracts is the concentration of 5-feruoylquinic acid—almost twice as high for the Robusta coffee (Tables 1, 2). Finally, the sum of concentrations of all chlorogenic acids for the Robusta coffee is significantly higher than for the Arabica coffee. Another indicator of the difference between the extracts of the two types of coffee is the higher concentration of caffeine in the Robusta coffee.

Let us note that the relatively high discrepancy between the concentrations of active components between parallel observations (more than 6 rel. %) may be due to the unevenness of the degree of grinding and the heterogeneity of the plant material in the samples taken for analysis. But this does not interfere with the differentiation of coffee by the type (Arabica or Robusta) according to the criteria proposed above.

At the same time, the Diasfer C10CN stationary phase again demonstrated the uniqueness of its sorption properties, due to which it was possible to change selec-

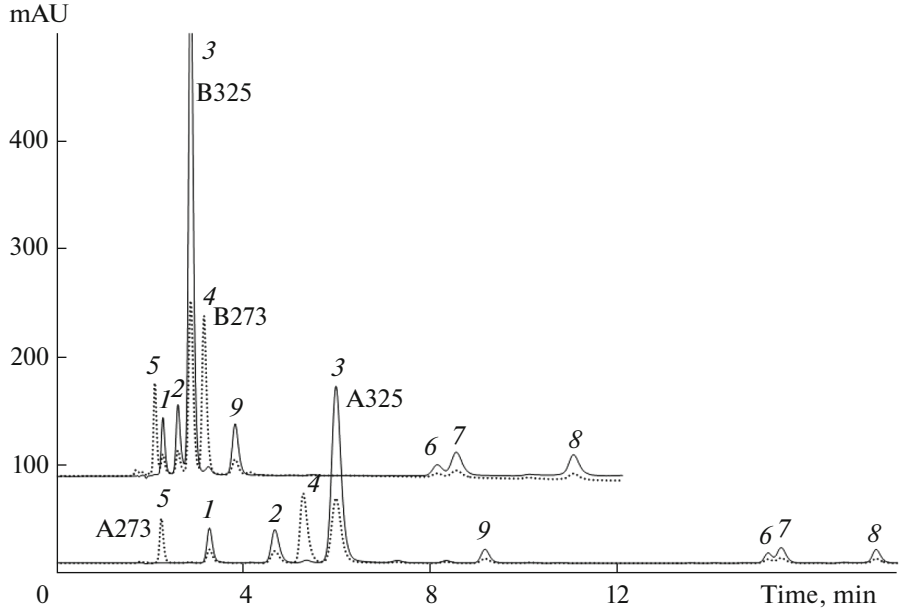

Fig. 5. Separation of the main components of green coffee extract: 3CQA (*1*), 4CQA (*2*), 5CQA (*3*), caffeine (*4*), trigonelline (*5*), 5FQA (*9*), 3,4diCQA (*6*), 3,5diCQA (*7*) and 4,5diCQA (*8*) on the stationary phase Diasfer 110-C10CN with gradient elution in modes 1 (A273 and A325) and 2 (B273 and B325); recording of chromatograms at 273 and 325 nm at 30°C.

Table 1. Concentration (mg/100 mL) of chlorogenic acids in the first green coffee extract (±6.5 rel. %, $n = 2$)

Manufacturer, variety	3CQA	4CQA	5CQA	5FQA	3,4diCQA	3,5diCQA	4,5diCQA
Brazil, Fernando	1.04	1.36	8.44	0.84	0.23	0.42	0.34
Colombia	0.82	1.14	9.31	0.88	0.22	0.54	0.38
Kenya	0.70	1.00	9.47	0.97	0.15	0.46	0.37
Ethiopia	0.53	0.74	8.51	0.74	0.19	0.62	0.47
Guatemala	0.56	0.78	7.03	0.75	0.01	0.03	0.02
Uganda	1.52	2.11	9.94	2.27	0.73	0.69	1.01
Peru	0.81	1.12	8.71	1.11	0.26	0.63	0.54
Ethiopia, Ranch	0.59	0.87	11.39	0.90	0.11	0.54	0.55
Vietnam, Kopi Luwak	0.95	1.41	10.09	1.03	0.36	0.58	0.63
(Arabica + Robusta) Verde	1.40	2.02	10.74	1.89	0.81	0.94	1.15
Robusta, Pachment	0.97	1.43	10.32	1.85	0.72	0.85	0.98
El Salvador, Pacamara	1.12	1.51	8.77	0.70	0.42	1.12	0.69

Table 2. Total concentration (mg/100 mL) of monocaffeoylquinic, dicaffeoylquinic and feruloylquinic acids and caffeine in the first green coffee extract (±6.5 rel %, $n = 2$)

Manufacturer, variety	Sum mono	5FQA	Sum di	Sum	Caffeine
Ethiopia	9.78	0.74	1.28	11.81	2.16
Guatemala	8.37	0.75	0.06	9.18	2.41
Kenya	11.17	0.97	0.98	13.12	2.54
Peru	10.64	1.11	1.43	13.17	2.55
Colombia	11.28	0.88	1.14	13.29	2.64
Ethiopia, Ranch	12.85	0.90	1.20	14.95	2.66
Brazil, Fernando	10.84	0.84	0.99	12.66	2.81
Vietnam, Kopi Luwak	12.44	1.03	1.56	15.04	2.89
El Salvador, Pacamara	11.40	0.70	2.23	14.33	3.12
(Arabica + Robusta) Verde	14.16	1.89	2.90	18.96	5.25
Uganda	13.57	2.27	2.43	18.28	5.25
Robusta, Pachment	12.72	1.85	2.55	17.12	5.89

tivity (in comparison with traditional C18 phases) for separating substances sensitive to polar interactions.

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CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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