Simultaneous Determination of Trigonelline, Caffeine, Chlorogenic Acid and Their Related Compounds in Instant Coffee Samples by HPLC Using an Acidic Mobile Phase Containing Octanesulfonate

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In order to analyze trigonelline, caffeine, chlorogenic acid, and their related compounds simultaneously, an HPLC method using an InertSustain C18 column and a mobile phase containing octanesulfonate as an ion-pairing reagent under an acidic condition was developed. The optimum mobile phase conditions were determined to be 0.1% phosphoric acid, 4 mM octanesulfonate, and 15% methanol at 35°C. Using the proposed method, trigonelline, nicotinic acid, caffeine, theophylline, chlorogenic acid, and caffeic acid in ten instant coffee samples were analyzed. These analytes except for theophylline were detected in all samples. An increase in the caffeine content in instant coffee samples tended to decrease in both trigonelline and chlorogenic acid contents, and the trigonelline content was found to be correlated well with the chlorogenic acid content (R^2 = 0.887).

Keywords Instant coffee, trigonelline, caffeine, chlorogenic acid, HPLC

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Introduction

Coffee, a popular beverage all over the world, has characteristic taste and aroma. Typical compounds in coffee, such as caffeine, trigonelline and chlorogenic acid, are known to influence coffee flavor, contributing to the acidity and conferring astringency and bitterness.[1,2] These compounds are known to be biologically active as reviewed.[3–7] Caffeine, an alkaloid, stimulates the central nervous system, heart rate and respiration. Chlorogenic acid exhibits various biological properties including anti-bacterial, anti-oxidant and anti-carcinogenic activities, particularly hypoglycemic and hypolipidemic effects. Chlorogenic acids are a family of esters formed between caffeic acid and (–)-quinic acid. According to Clifford,[8] using the preferred IUPAC numbering,[9] chlorogenic acid is generally referred to as 5-caffeoylquinic acid, while it is still often called chlorogenic acid or 3-caffeoylquinic acid (pre-IUPAC numbering). Trigonelline, which is derived from the methylation of the nitrogen atom of nicotinic acid, has hypoglycemic, hypolipidemic, sedative, anti-migraine, anti-bacterial, anti-viral and anti-tumor effects, and potency to improve memory retention and inhibit platelet aggregation.

Two coffee tree species are distributed worldwide. *Coffea arabica* (Arabica) and *Coffea canephora* (commonly known as Robusta) are adapted to very different ecological environments, which may cause different taste and aroma. Arabica, with its lower bitterness and better flavor, is more appreciated by consumers, and its cost is higher than that of Robusta. Some chemicals in coffee such as sucrose and trigonelline, give appreciated flavor, while other chemicals such as chlorogenic acid and caffeine increase bitterness.[10,11] To evaluate the quality of coffee commodities from chemical constituents and their contents, sensitive, precise, and accurate analytical means are required for determining these chemicals.[12]

A number of investigations for the determination of components in coffee, including caffeine, chlorogenic acids, and/or trigonelline, have been conducted by high-performance liquid chromatography (HPLC) as reviewed.[12–14] Casal *et al.* reported that two coffee species, Arabica and Robusta, could be clearly distinguished by their trigonelline and caffeine contents,[15] but that neither trigonelline nor caffeine could be used to identify the geographical origin of the roasted coffee. Ky *et al.* reported higher trigonelline and sucrose contents in Arabica and higher chlorogenic acid and caffeine contents in Robusta.[10] The roasting process is also one of the important factors concerning the characteristic flavor and the final quality of coffee. The key

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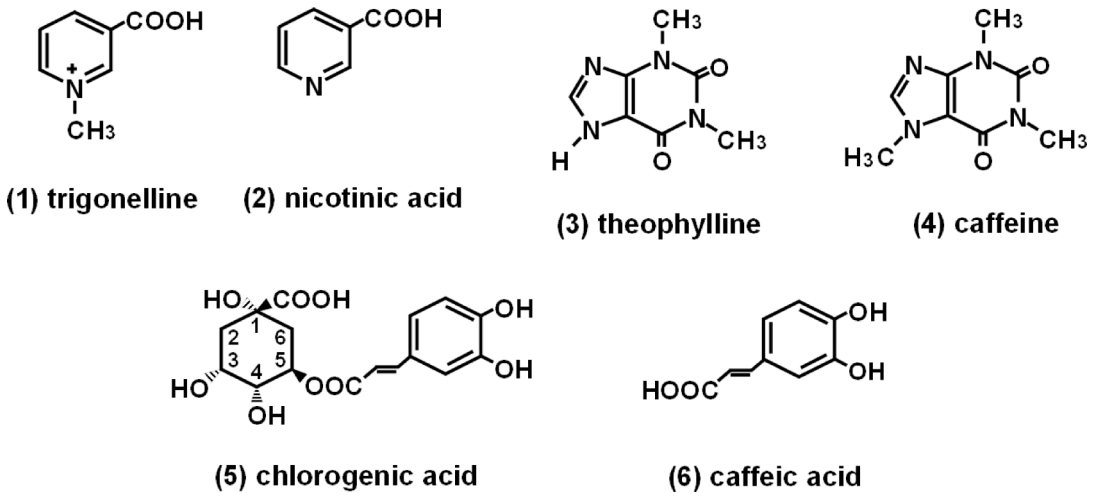

Fig. 1 Structural formulas of trigonelline, caffeine, chlorogenic acid, and their related compounds.

substances are subjected to chemical changes in this process.[16] For example, green coffee beans contain high levels of chlorogenic acids in their composition, while these levels were drastically reduced during roasting.[17] The loss of trigonelline was strongly dependent upon the degree of roasting and was associated with the formation of nicotinic acid, but caffeine levels were decreased only slightly.[18] Considerable stability of caffeine during roasting was also shown for both Arabica and Robusta samples by Bicho *et al*.[19]

The simultaneous determination of these compounds can be useful for the quality control of raw coffee beans and monitoring of coffee roasting conditions.[20] However, the three typical compounds (trigonelline, caffeine, and chlorogenic acid) in coffee have not been determined at the same time by one HPLC method. For example, trigonelline and caffeine were simultaneously analyzed, while chlorogenic aid was separately analyzed by another HPLC method.[10,19,21] Rodrigues and Bragagnolo used an HPLC method for the determination of caffeine and chlorogenic acids, and another HPLC method for determining trigonelline, nicotinic acid, 5-hydroxymethylfurfural, theobromine and theophylline.[22] De Maria *et al.* reported a simultaneous determination of trigonelline, caffeine, and total chlorogenic acid, in green coffee samples by high-performance gel filtration chromatography[23] other than reversed phase HPLC. The aim of the present study was to develop a method for the simultaneous analysis of trigonelline, caffeine, chlorogenic acid, and their related compounds (nicotinic acid, theophylline, and caffeic acid, as shown in Fig. 1) in instant coffee samples by a reversed phase HPLC using a mobile phase containing octanesulfonte as an ion-pairing agent under an acidic condition.

Experimental

Reagents and chemicals

Trigonelline hydrochloride, theophylline, chlorogenic acid hydrate, and sodium 1-octanesulfonate were obtained from Tokyo Kasei (Tokyo, Japan). Methanol was from Kanto (Tokyo, Japan). Nicotinic acid, caffeic acid, caffeine, and other chemicals (analytical grade) were obtained from Wako Pure Chemicals (Osaka, Japan).

HPLC apparatus and chromatographic conditions

The HPLC system consisted of a Jasco (Tokyo, Japan) Model PU-2080 pump, a Jasco Model UV-2075 detector, a Rheodyne (Cotati, CA) manual injector, a Shimadzu (Kyoto, Japan) Model CTO-10A column oven, and a Shimadzu Model DGU-14A degasser. An InertSustain C18 column (4.6 mm i.d. × 150 mm, GL Sciences, Tokyo, Japan) was used. A mobile phase consisted of 0.1% phosphoric acid, 4 mM octanesulfonate, and 15% methanol. Elution was carried out at a flow rate of 1.0 mL/min at 35°C. Analytes were detected at 220 nm. Data acquisition and processing were conducted with a Chromato-PRO (Runtime Instruments Co., Kanagawa, Japan).

Standard solution and sample preparation

Stock solutions (10 mM) of trigonelline, nicotinic acid, caffeine, theophylline, chlorogenic acid, and caffeic acid were separately prepared with purified water or 20% acetonitrile. Nine brands of instant coffee, one brand of decaffeinated instant coffee and one brand of regular coffee were purchased from a local market. Analytes in these instant coffee samples (0.0500 g) were extracted with ultrasonicating in 10 mL of 15% methanol at 43 kHz for 5 min at room temperature. Then, the mixture was centrifuged at approximately 1200*g* for 5 min and the supernatant was filtered with 0.45 μm filter and was analyzed by the HPLC method.

Preparation of 3-, 4-, and 5-caffeoylquinic acids

Commercial regular coffee (10 g) was added to 100 mL of hot purified water at 80°C. After stirring for one minute, the mixture was centrifuged at approximately 1200*g* for 5 min. The supernatant was filtered with 0.45 μm filter and was applied to HPLC described by Tfouni *et al.*[16] with minor corrections, using an InertSustain C18 column thermostated at 40°C. A mobile phase consisted of 0.1% phosphoric acid and 20% acetonitrile. Elution was carried out at a flow rate of 1.0 mL/min and analytes were detected at 320 nm. Three fractions, corresponding to the 3-, 4-, and 5-caffeoylquinic acids, were separately collected.

Results and Discussion

Factors affecting separation

Among the six analytes in Fig. 1, the purine related compounds, caffeine and theophylline, were retained by the ODS column and could be analyzed by reversed phase HPLC using neutral mobile phases. When the acidic mobile phase was used, chlorogenic and caffeic acids were easily analyzed by reversed phase HPLC,[16,22,24-26] because their protonated forms are neutral. However, trigonelline and nicotinic acid, which are cationic in an acidic medium and zwitterionic and anionic, respectively, in neutral or alkaline media, are difficult to be analyzed under any pH conditions. We examined the retention behaviours of trigonelline and nicotinic acid using HPLC with an InertSustain C18 column and a mobile phase containing 0.1% phosphoric acid and 15% methanol. As a result, trigonelline and nicotinic acid were detected at 1.6 and 2.1 min, respectively. This result were almost the same compared to previous reports,[10,19,21] showing that these zwitterionic compounds were difficult to be retained by an ODS column with an acidic mobile phase. The ionic nature of the analyte is suppressed by association with an ion-pair reagent of the opposite charge. The resulting uncharged ion-pair interacts with a non-polar stationary phase. HPLC with a mobile phase containing the ion-pair reagent (known as ion-pair chromatography) can be used for the simultaneous determination of both ionic and neutral compounds. Ion-pair chromatography is versatile and offers more possibilities for changing the stationary and/or mobile phase parameters.[27] Waksmundzka-Hajons[28] and Cecchi[29] have discussed the retention mechanism of ion-pair chromatography in detail. It was shown that the addition of C7 – C9 alkylsulfonates to a mobile phase at concentrations over the range of ~1 – 10 mM can provide the separation of compounds having amino groups.[30] Octanesulfonate, a C8 alkylsulfonate, as an ion-pair reagent has been widely used for the analysis of basic and zwitterionic componds in foods and other real samples.[31-35]

In order to analyze all analytes by using a reversed HPLC method, we added octanesulfonate to the acidic mobile phase as an ion-pairing reagent for the cationic analytes. The effect of the octanesulfonate concentration (2 – 8 mM) on the retention times of analytes was evaluated (Fig. 2). An increase in the octanesulfonate concentration brought about increases in the retention times of both trigonelline and nicotinic acid. However, the retention times of the other four analytes were gradually decreased with increasing the octanesulfonate concentration. These facts suggest that the hydrophobic interaction between neutral analytes and the C18 group on the column were weakened by adding octanesulfonate having a hydrophobic alkyl group, while ion-paring between the cationic analytes and the sulfonate group could promote the interaction between the analytes and the hydrophobic ODS column. The 4 mM octanesulfonate concentration was adopted for the simultaneous separation of all analytes considering the separation of the positional isomers of coffeoylquinic acid and other contaminants contained in the instant coffee samples (*vide infra*).

By increasing the methanol concentration (10 – 25%), the retention times of all the analytes in the acidic mobile phases decreased, where the degree of the effect of methanol pronouncedly depends on the analytes. The methanol concentration was fixed at 15% for the simultaneous separation of all the analyte considering a moderately short retention time and the separation of the positional isomers of coffeoylquinic acid and other contaminants contained in the instant coffee samples (*vide infra*). Therefore, the optimum mobile phase conditions were determined to be 0.1% phosphoric acid, 4 mM octanesulfonate, 15% methanol at 35°C.

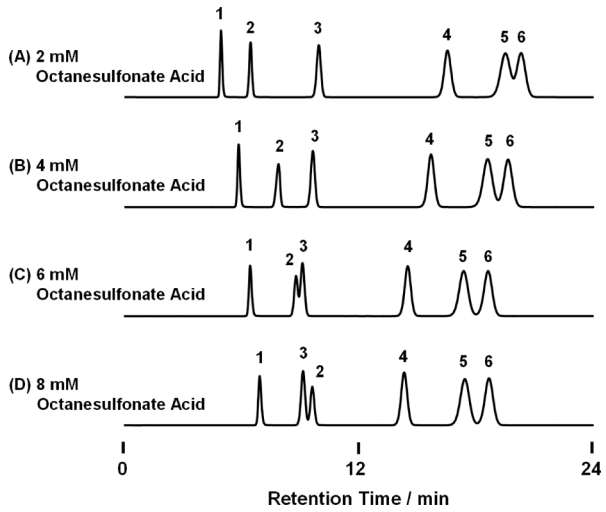

Fig. 2 Effect of the octanesulfonate concentration on the separation of six analytes. HPLC was done by using an InertSustain C18 column and mobile phases consisting of 0.1% phosphoric acid, 2 – 8 mM octanesulfonate, and 15% methanol. 1, trigonelline; 2, nicotinic acid; 3, theophylline; 4, caffeine; 5, chlorogenic acid; 6, caffeic acid.

Analysis of instant coffee samples

Six analytes were subjected to the HPLC method using the above optimum conditions. The detection limit, defined as a signal-to-noise ratio of 3, was 0.3 μM for all analytes. Linearity ($R^2 > 0.992$) was demonstrated in the range of 1 μM – 3 mM for the standard curves of all the analytes. Good reproducibilities of the peak areas (RSD < 0.6%) and the migration times (RSD < 0.13%) were obtained by five consecutive determinations at 1 mM for all six analytes. Recoveries were between 96.5 and 100.1%.

Roasting is a time-dependent process, whereby chemical changes are induced in the coffee beans.[17] Bicho *et al.* reported that the caffeine contents did not vary significantly with the roasting degree for the Arabica and Robusta samples, revealing a considerable stability during roasting.[19] However, the trigonelline contents in both coffees decreased significantly with the roasting intensity and the levels of chlorogenic acids remained higher in Robusta coffee beans, but decreased sharply with the roasting intensity. Rodrigues *et al.* reported that regular roasted ground coffee brews showed higher contents of chlorogenic acids, chlorogenic acid lactones, trigonelline, nicotinic acid, caffeine, and theobromine than regular soluble coffee brews.[22] In this work, trigonelline, nicotinic acid, theophylline, caffeine, chlorogenic acid, and caffeic acid in ten instant coffee samples were analyzed by using the proposed HPLC method (Table 1) and a representative chromatogram is shown in Fig. 3B. The amounts of trigonelline, caffeine, and chlorogenic acid in sample A* extracted with hot water were almost the same as those in sample A extracted with 15% methanol using ultrasonication at 43 kHz for 5 min. The amounts of nicotinic acid and caffeic acid in sample A were higher than those in sample A*. Thus, analytes in samples A – J were extracted with 15% methanol. The caffeine levels in samples A – I ranged from 28.8 to 35.0 mg/g, while caffeine level in decaffeinated sample J was understandably only

Table 1 The amounts of coffee components in instant coffee samples (mg g^{-1})

Instant coffee	Trigonelline	Nicotinic acid	Theophylline	Caffeine	Chlorogenic acid	Caffeic acid
A*	4.78 ± 0.01	0.0474 ± 0.0041	ND	33.8 ±0.1	4.24 ± 0.03	0.0567 ± 0.00376
A	4.68 ± 0.04	0.294 ± 0.001	ND	32.5 ± 0.1	4.39 ± 0.04	0.102 ± 0.001
B	4.72 ± 0.04	0.292 ± 0.001	ND	35.0 ± 0.1	4.52 ± 0.04	0.0879 ± 0.0001
C	3.02 ± 0.02	0.364 ± 0.001	ND	34.7 ± 0.2	2.65 ± 0.01	0.107 ± 0.003
D	6.71 ± 0.06	0.315 ± 0.005	ND	34.6 ± 0.2	10.6 ± 0.1	0.168 ± 0.003
E	6.94 ± 0.07	0.339 ± 0.007	ND	34.1 ± 0.2	8.14 ± 0.16	0.0489 ± 0.0017
F	7.06 ± 0.08	0.300 ± 0.001	ND	31.7 ± 0.6	9.45 ± 0.07	0.0734 ± 0.0013
G	7.59 ± 0.09	0.233 ± 0.004	ND	32.8 ± 0.3	9.10 ± 0.009	0.0644 ± 0.0039
H	7.73 ± 0.08	0.391 ± 0.001	ND	32.2 ± 0.3	9.81 ± 0.22	0.316 ± 0.011
I	9.76 ± 0.13	0.531 ± 0.022	ND	28.8 ± 0.3	11.6 ± 0.3	0.161 ± 0.006
J	6.67 ± 0.05	0.327 ± 0.005	ND	0.947 ± 0.010	9.43 ± 0.08	0.232 ± 0.013

Analytes in instant coffee samples were extracted with 15% methanol (A – J) or hot water (A*).

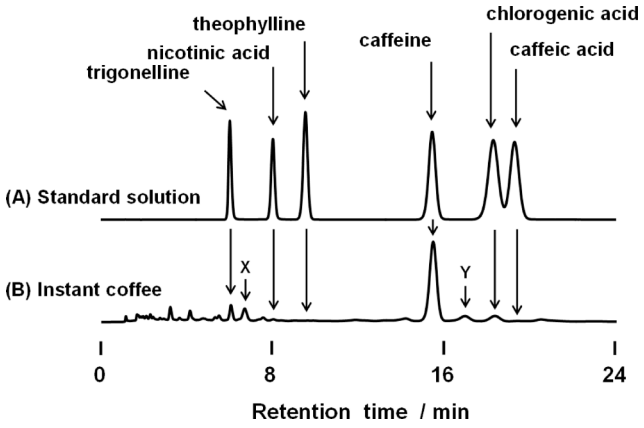

Fig. 3 Chromatograms of standard solution (A) and instant coffee sample (B). An instant coffee (0.0500 g) was dissolved by 15% methanol (10 mL). HPLC was done by using an InertSustain C18 column and mobile phases consisting of 0.1% phosphoric acid, 4 mM octanesulfonate, and 15% methanol. Analytes were detected at 220 nm.

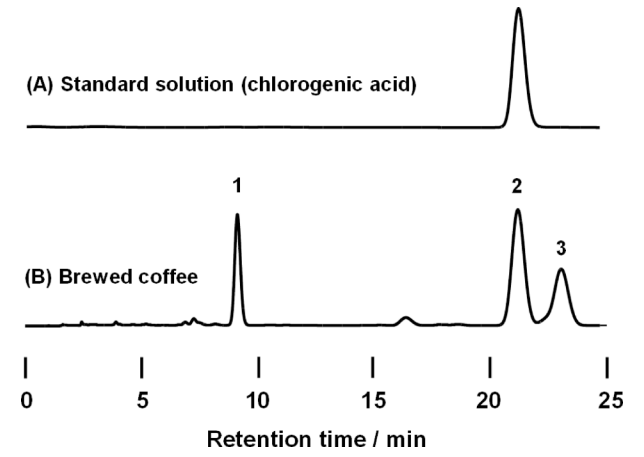

Fig. 4 Chromatograms of standard solution of chlorogenic acid (A) and brewed regular coffee (B). A regular coffee (10 g) was added to 100 mL of hot purified water (80°C). HPLC was done by using an InertSustain C18 column and mobile phases consisting of 0.1% phosphoric acid, 4 mM octanesulfonate, and 15% methanol. Analytes were detected at 320 nm. 1, 3-caffeoylquinic acid; 2, 5-caffeoylquinic acid; 3, 4-caffeoylquinic acid.

0.947 mg/g. It seems that those samples with higher concentrations of caffeine tend to have lower concentrations of trigonelline and chlorogenic acid. However, the trigonelline levels in samples A – I were found to be correlated well with the concentration of chlorogenic acid. The regression equation for the relationship between the amounts of trigonelline (x) and chlorogenic acid (y) was expressed as $y = 1.48x - 1.74$ with a correlation coefficient (R^2) of 0.887. Further studies are needed to clarify whether this correspondence resulted in coffee varieties, roasting processes and/or manufacturing process of instant coffee.

When instant coffee samples were analyzed by the proposed method with UV detection at 320 nm instead of at 220 nm, two peaks X and Y as well as a peak of chlorogenic acid, which were detected at 220 nm in Fig. 3B, were also detected at 320 nm. Chlorogenic acids are a family of esters formed between caffeic acid and (–)-quinic acid, which is referred to 5-caffeoylquinic acid. The three positional isomers of *mono*-caffeoylquinic acids, 3-, 4-, and 5-caffeoylquinic acid, were contained in regular coffee, where the isomer contents were increased in this order.[19,25] In order to assign the peaks X and Y in Fig. 3B, the three isomers of mono-caffeoylquinic acids were prepared by a similar method reported by Tfouni *et al.*,[16] as stated above. The chromatographic pattern in Fig. 4B was almost the same as that by Tfouni *et al.*,[16] and the three major peaks 1, 2, and 3 corresponded to 3-, 5-, and 4-caffeoylquinic acids, respectively. These three fractions were separately collected and each of those was applied to the proposed HPLC method. As a result, peaks X and Y were found to be peaks for 3- and 4-caffeoylquinic acids, respectively.

In conclusion, trigonelline, caffeine, chlorogenic acid, and their related compounds were simultaneously analyzed by the HPLC method using octanesulfonate as the ion-paring reagent under an acidic condition. These analytes in instant coffee samples could be analyzed using the proposed HPLC method. As a result, the amounts of trigonelline and chlorogenic acid in instant coffee samples were found to be correlated well to each other.

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