Determination of Biologically Active Substances in Roasted Coffees Using a Diode-Array HPLC System

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We studied the simultaneous quantitative analysis of biologically active substances, such as nicotinic acid, trigonelline, caffeine, qunolinic acid and tannic acid and pyrogallic acid, in several roasted coffees by an HPLC/diode-array system with a home-made sol-gel and ODS-2 columns. A simple method for simultaneous quantitative analysis of biologically active substances in the coffee brew became feasible by an HPLC/diode-array system with a sol-gel column at a single wavelength of 210 nm. The most efficient condition of the R_s value was above 1.05 when two sol-gel columns were connected. In addition, the elution behavior of nicotinic acid in brew extracted from commercially available coffee beans suggests the thermal decomposition process during roasting, and indicated the maximum value for full city roasted coffee.

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Introduction

The taste and aroma of coffee brew have been widely investigated in regard to improving the brain's recognition ability by coffee-aroma, which stimulates the bloodstream of the cerebrum.[1-3] The biological action of caffeine, which is one of coffee-aroma, is widely utilized in clinical medicine at present.[1,3] Recently, it has been reported that biologically active nitrogenous components other than caffeine have suitable therapeutic effects for cancer[4] and dementias.[5,6] Thus, the biologically active substances contained in the coffee have been of particular interest. Their amount and composition in the coffee brew are dependent on the type of coffee bean and on the degree of roasting. An accurate analysis of the active substances contained in coffee brew is of considerable importance for clarifying the relation between the pretreatment conditions of coffee beans and the compositions of the active substances. These active ingredients have been analyzed by gas chromatography,[7] liquid-chromatography,[8,10,13,14] thin-layer chromatography,[9] bioassay techniques[11] and high gel filtration chromatography.[12] However, a more convenient and rapid analytical technique with the development of an instrument is desirable for the determination of active nitrogenous components. The subjects for analyzing the biologically active substances are to simplify the extraction process of the active material separately from the sample, to reduce the analysis time with good resolution of resemble materials in properties each other, to optimize the analysis conditions, and to deal quickly with a large number of samples. Among the various methods, a high-performance liquid chromatography (HPLC) is the most promising. Especially, an HPLC/diode-array system provides advantages in setting the optimum pretreatment and measurement conditions. More recently, Casal *et al.*[15,16] have determined trigonelline, nicotinic acid and caffeine in coffee brew by using an HPLC/diode-array system and an ODS-2 column at 268, 264 and 276 nm, respectively.

In the present work, in order to establish the technique for separating biologically active compounds under a simple condition, we studied the simultaneous quantitative analysis of nicotinic acid, trigonelline, caffeine, quinolinic acid and pyrogallic acid in several roasted coffees by an HPLC/diode-array system with home-made sol-gel and ODS-2 columns at the same wavelength of 210 nm. In addition, the elution behavior of these compounds in the brew extracted from commercially available coffee beans was investigated.

Experimental

Materials

Green and roasted coffee beans were purchased from Key Coffee Co. Quinolinic acid, nicotinic acid, tannic acid, pyrogallic acid and caffeine from Wako Pure Chemicals Co. (Osaka, Japan) and trigonelline from Sigma (St. Louis, MO, USA) were used without further purification.

Sample preparation

The four coffee beans (arabica species of columbia, brazil, guatemala and indonesia robusta) were roasted in five degrees of light, medium, city, full city and French, respectively. The roasting of green coffee beans was carried out at 230˚C for 10 – 30 min. The colors of light and medium (American style) roasts are light brown and chestnut, respectively. However, the city type is the standard roast, being called the New York style, and the full city is deeper roast than city. French is the deepest grade, roasting at the highest temperature, called the French style. A 25-g portion of roasted coffee bean was ground for 12 s on a Philip Mill and Drip coffee maker. The coffee brew was obtained by extraction from the grinding coffee powder with

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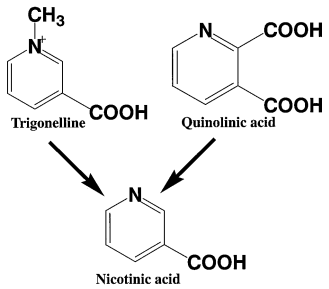

Scheme 1

distilled hot water (300 ml) on the coffee maker. After the coffee brew was freeze-dried, the extract was dissolved with a 300 ml of water–methanol (1:3).

HPLC analysis

The prepared sample solutions were analyzed by using HPLC systems (Shimazu Co.) composed of an intelligent pump (LC-10AT VP, flow rate of 1 ml min^{-1}), a UV-VIS detector (SPD-10A VP (210 nm, 0.05 AU/FS)), a diode array detector (SPD-M 10A VP, and a neo-pack ODS-2 column (pore size: 12 nm, 4.6 mm i.d. × 150 mm, Nishio Co.)) or a sol-gel column (pore size: 10 nm, 4.6 mm i.d. × 150 mm, Nishio Co.) set in a column oven (CTO-10AS VP (40˚C)). A mobile phase was methanol 50 v/v% (pH 7.5) containing a 10 mM phosphate buffer solution adjusted to pH 6.3 with 10 mM KOH. Quantification was carried out using an absolute calibration curve method with standard solutions of nicotinic acid, trigonelline, pyrogallic acid, caffeine and qunolinic acid.

Results and Discussion

HPLC analysis of biologically active substances

The separation method of nitrogenous components, such as nicotinic acid, trigonelline and quinolinic acid in roasted coffees by HPLC, has not been established. Both compounds are difficult to detect at the same wavelength because of the similar structures. Since both trigonelline and quinolinic acid,[12] which are easily thermally decomposed to nicotinic acid, are slightly contained in the roasted coffee bean, the separation of these compounds has been complicated (Scheme 1).

Though three components of trigonelline, nicotinic acid and caffeine have been detected at different wavelengths using the HPLC/diode-array system with the ODS-2 column by Casal,[15,16] we investigated the optimum conditions for separating biologically active substances, such as nicotinic acid, trigonelline, pyrogallic acid, caffeine and quinolinic acid, using the sol-gel column at the same wavelength. To determine the optimum detection wavelength, a three-dimensional chromatogram of a mixture of pure quinolinic acid, nicotinic acid, trigonelline, pyrogallic acid and caffeine was measured using a diode/array detector and a sol-gel column, as shown in Fig. 1. Figure 1 indicates that biologically active compounds can be detected simultaneously at 210 nm. Figure 2 illustrates the chromatograms obtained using various columns at 210 nm.

In all of the columns, only caffeine was clearly separated at retention times of 4 – 6 min. It's retention was considerably shortened compared with the retention time of *ca.* 20 min reported by Casal.[15,16] Three component peaks of quinolinic acid, nicotinic acid and trigonelline were overlapped when the ODS-2 columns were used, particuraly, the pyrogallol acid

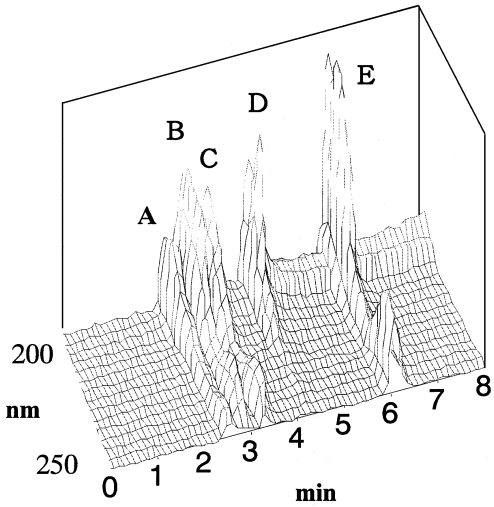

Fig. 1 Three-dimensional chromatograms of (A) quinolinic acid, (B) nicotinic acid, (C) trigonelline, (D) pyrogallic acid, and (E) caffeine (two sol-gel columns 4.6 mm i.d. × 150 mm × 2).

being undetectable. In the case of sol-gel column, the five components of quinolinic acid, nicotinic acid, trigonelline, pyrogallic acid and caffeine were clearly separated, where these resolutions were dramatically improved, especially by connecting the two sol-gel columns, although the retention time became slightly longer. The resolution (R_s) of these components by the sol-gel column was calculated[17] using

$$R_s = 2(t_2 - t_1)/1.70(W_1 + W_2), \quad (1)$$

where t_1 and t_2 are the retention times of compounds 1 and 2, respectively and W_1 and W_2 denote the peak widths at the middle point of compounds 1 and 2, respectively. Generally, the separation of each compound is judged to be good if $R_s > 1$. The resolution values of the five components calculated for the ODS-2 and sol-gel columns are summarized in Table 1. As shown in Table 1, the sol-gel column provides a better resolution for each peak in the chromatogram of the mixture than the ODS-2 column. When two sol-gel columns were connected, the R_s value was above 1.05, and each chemical compound was clearly separated. Taguchi,[18] Casal[15,16] and Maier[19] have reported on the separation of mixtures containing trigonelline, its derivative and caffeine using ODS and ODS-2 columns. In their experiments, the detector was set at different wavelengths of 260 – 270 nm for each compound, and a long time of 20 – 30 min has been required for their separations. In the present work, the simultaneous determination of quinolinic acid, nicotinic acid, trigonelline, pyrogallic acid and caffeine was achieved at a fixed wavelength of 210 nm and at a high speed of 7 min by using the sol-gel column. Thus, the chromatogram can be obtained under the simple operating conditions. The sol-gel column is very effective for the analysis of alkaloids in an aqueous elution system.

Characterization of fillers

The particles of the ODS-2 and sol-gel columns were prepared from a totally porous silica-gel. The ODS-2 particles were introduced as an octadecyl group ($C_{18}H_{37}$) onto the active surface through a silanol group. On the other hand, sol-gel particle was fabricated by chelation from tetra-alkylsilika of a starting material, and then the residual hydroxyl groups were end-capped with trimethyl chlorosilane. Figure 3 exhibits

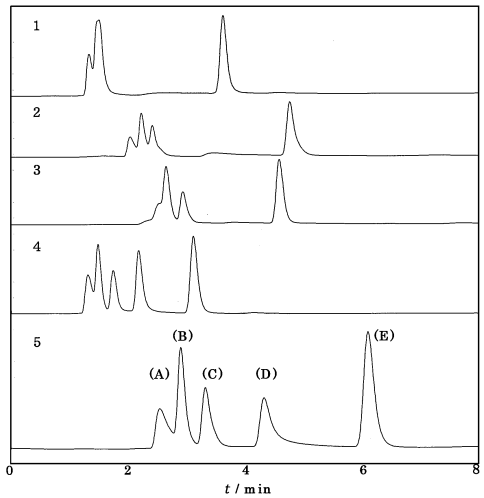

Fig. 2 Chromatograms of the nitrogen compounds using sol-gel and ODS-2 columns. (A) Quinolinic acid, (B) nicotinic acid, (C) trigonelline, (D) pyrogallic acid, (E) caffeine. 1, ODS-2 (4.6 mm i.d. × 150 mm); 2, ODS-2 (5.6 mm i.d. × 150 mm); 3, ODS-2 × 2 (4.6 mm i.d. × 150 mm); 4, sol-gel (4.6 mm i.d. × 150 mm); 5, sol-gel × 2 (4.6 mm i.d. × 150 mm).

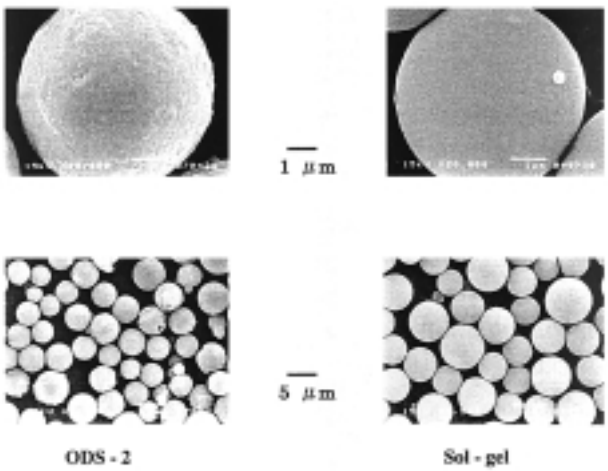

Fig. 3 SEM images of the filler particles.

scanning electron microscopic (SEM) images of the fillers ODS-2 and sol-gel columns; the characteristics of each particle are summarized in Table 2. The particle size of the sol-gel filler is smaller than that of the ODS-2 filler and its surface is very smooth. The surface of sol-gel particles are completely alkylated by the special end-capping compared with ODS. The separating efficiency of the sol-gel column prepared as noted above is relatively high, and hence the chemical compound with a similar structure might be clearly separated. In addition, a sol-gel filler can be employed in a wider range of the mobile phase than ODS-2, and is considerably effective for separation in the aqueous solution because of the durability for the comparative aqueous medium.

Separation of the biologically active substances in coffee brew

The simultaneous determination of quinolinic acid, nicotinic acid, trigonelline, pyrogallic acid and caffeine in the roasted coffee on the market was carried out by an HPLC/diode-array system under the optimum conditions described above. As

Table 1 Resolution of the mixture for various columns

	R_s value		
	Sol-gel (4.6 mm i.d. × 2)	Sol-gel (4.6 mm i.d.)	ODS-2 (5.6 mm i.d.)
Qunolinic acid	1.05	0.5	0.45
Nicotinic acid	1.05	0.5	0.45
Trigonelline	1.24	0.76	0.55
Pyrogallic acid	1.95	1.29	—
Caffeine	3.22	2.76	—

Table 2 Particle size, pore size and number of theoretical plates of sol-gel and ODS-2 columns

Column	Particle size/ μm	Pore size/ nm	Number of theoretical plates
ODS-2	5	12	*ca*. 10000
Sol-gel	5	10	*ca*. 8000

shown in Fig. 4, each peak was confirmed under the same conditions as the measurement of a standard sample, and a tannin acid was also newly detected. When the ODS-2 column was employed, the separations of quinolinic acid, nicotinic acid and trigonelline were interfered by an overlapping of tannic acid elution. However, by using the sol-gel column their active components could be separated with independence to each other at 210 nm. Other interference was found to be absent from the absorption spectra measured by the HPLC/diode-array system. However, the retention times of nicotinic acid, tannin acid, quinolinic acid were close to each other in the mobile phase of water:methanol = 50:50 v/v. When the water content of the mobile phase was increased as water:methanol = 60:40 v/v (pH 7.5),[20] the separation of these compounds was considerably improved with regard to the resolution and the elution speed, as shown in Fig. 4. Because quinolinic acid, which should thermally decomposes to nicotinic acid during roasting, was hardly detected in the coffee brew prepared, the standard of quinolinic acid was added to the coffee brew sample to confirm the separation. No detection of pyrogallic acid suggests that pyrogallic acid was not formed from the tannin under the present roasting conditions. The peak of quinolinic acid was absent, except for the brazil light roasted coffee. This work demonstrates that the biologically active substances in coffee can be simultaneously determined at a single wavelength with better resolution and more rapid elution than when using the conventional method with an ODS column.

Effect of roasted degrees on the compositions of biologically active substances in coffee brew

Coffee brew was analyzed by an HPLC/diode-array system under the conditions described above. Table 3 indicates the content of caffeine, trigonelline and nicotinic acid in coffee brew extracted from beans with the five degrees of roasting. Torigonelline in coffee brew decreases with increasing degree of the roast. The increase of nicotinic acid suggests its formation during roasting, particularly in the full city and French type, because of a very small quantity in the native green coffee bean. The caffeine contents of several roasted coffees also showed a similar tendency with that of nicotinic acid. These results indicate that this decomposition can be characterized by the content ratio of the trigonelline to the

Table 3 Trigonelline, nicotinic acid and caffeine contents of roasted coffee (mg/200 ml)

Sample	Trigonelline						Nicotinic acid						Caffeine					
	roast degree						roast degree						roast degree					
	Green	Light	Medium	City	Fullcity	French	Green	Light	Medium	City	Fullcity	French	Green	Light	Medium	City	Fullcity	French
Colombia	52.7	39.4	49.5	30.5	31.3	35.2	0.1	3.9	5.4	2.8	8.9	7.7	170.7	281.7	276.3	277.0	314.0	323.7
Brazil	58.7	86.2	71.6	39.7	32.4	29.7	0.1	1.0	2.2	1.3	6.1	7.4	175.3	326.7	322.4	315.6	322.2	319.1
Indonesia	76.4	61.6	63.2	25.6	27.8	24.0	0.6	0.6	5.0	4.8	6.4	5.6	200.5	318.4	315.7	319.1	321.1	324.3

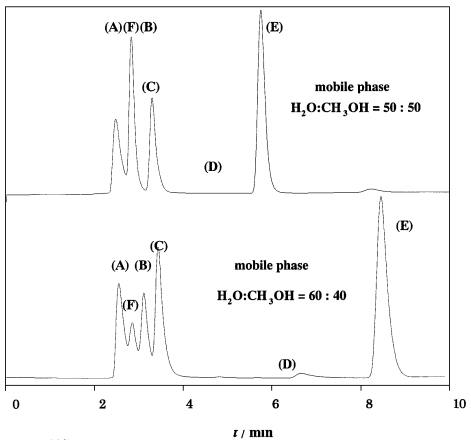

Fig. 4 Chromatograms of the nitrogen compound in the coffee brew of brazil light roasted coffee beans using sol-gel columns. (A) Quinolinic acid, (B) nicotinic acid, (C) trigonelline, (D) pyrogallic acid, (E) caffeine, (F) tannic acid.

nicotinic acid for any roasted sample because the nicotinic acid found in the roast coffee was essentially derived from the trigonelline in the green coffee during roasting. Actually, the main components of the green bean is a fatty acid,[21] which is called a coffee oil existing as a droplet. The above-mentioned component seems to be wrapped in this oil droplet.[22] The component inside the roasted bean is fluidized and the volatile component is discharged as a bubble. Then, the green bean cell is destroyed and exudation of the oil begins. Therefore, these compounds may be readily extracted even with hot water. The nicotinic acid in the green bean extracted with hot-water yielded a very low level of 0.6 – 3.6 mg/100 g. When the green beans were roasted, the nicotinic acid increased to a level of 3.6 – 53.4 mg/100 g because of a progressive demethylation of trigonelline and decarboxylation of qunolinic acid.

The coffees prepared from deeply roasted beans gave a high level of nicotinic acid as vitamin B; the maximum value in the coffee brew was 8.9 mg/200 ml. Since an indispensable quantity is 17 mg day^{-1} in the case of an adult, we can obtain sufficient vitamin B by drinking two cups of coffee.

Conclusions

In this study, a simultaneous quantitative analysis of the biologically active substances, such as quinolinic acid, nicotinic acid, trigonelline, pyrogallic acid, and caffeine, in the coffee brew became feasible by the HPLC/diode-array system with the home-made sol-gel column at a single wavelength of 210 nm. The most efficient condition of the R_s value was above 1.05 when two sol-gel columns were connected. In addition, the elution behavior of nicotinic acid in brew extracted from commercially available coffee beans suggests a thermal decomposition process during roasting, and indicated the maximum value for full city roasted coffee. The roasted degree of green coffee beans was a significant factor governing the contents of the biologically active substances in the coffee brew.

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