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CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF NATURALLY OCCURRING CHLOROGENIC ACIDS BY ^1H NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND MASS SPECTROMETRY

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SUMMARY

The high-performance liquid chromatographic separation and identification of the phenolic compounds in extracts from coffee bean are described. The system consists of Fine SIL C_{18-5} analytical and preparative columns and gradient elution (solvent A, 10 m*M* H_3PO_4; solvent B, methanol). The seven chlorogenic acids isolated were identified by mass spectrometry and nuclear magnetic resonance spectroscopy. The isolated and identified compounds are 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-O-dicaffeoylquinic acid, 3,5-O-dicaffeoylquinic acid, 4,5-O-dicaffeoylquinic acid and 3-O-feruloylquinic acid.

INTRODUCTION

Green coffee beans contain considerable amounts of chlorogenic acids. These substances are known as neochlorogenic acid (4-O-caffeoylquinic acid)[1], band 510 (5-O-caffeoylquinic acid)[2], chlorogenic acid (3-O-caffeoylquinic acid)[3], isochlorogenic acid (a mixture of 3,4-O-dicaffeoylquinic acid, 3,5-O-dicaffeoylquinic acid and 4,5-O-dicaffeoylquinic acid)[4] and 3-O-feruloylquinic acid[5] (Table I). A number of other phenolic compounds have also been reported as present in coffee beans[6]. Traditionally the phenolic constituents of green coffee beans have been regarded as an important indicator of coffee beverage quality[7]. The relationship between these substances and the colour of roasted coffee infusion has long been recognized[8].

Various methods have been published for determining phenolics in coffee beans and other natural products. Most procedures involve paper chromatography[9], thin-layer chromatography[10], column chromatography[11] or counter-current distribution[12]. However, all the methods are tedious and time consuming. In addition, no simultaneous analysis of the phenolics is available. Recently, reversed-phase high-performance liquid chromatography (HPLC) has been shown to be applicable for the analysis of free hydroxycinnamic acids[11]. There are only a few studies on the

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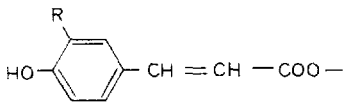
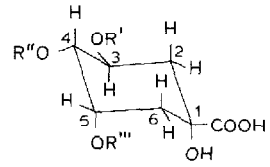
TABLE I

STRUCTURES OF HYDROXYCINNAMIC ACIDS AND HYDROXYCINNAMIC ACID DERIVATIVES



Caffeoyl - (C) R = OH
Feruloyl - (F) R = OCH_3



Compound	*R'*	*R''*	*R'''*
3-O-Caffeoylquinic acid (chlorogenic acid)	C	H	H
4-O-Caffeoylquinic acid	H	C	H
5-O-Caffeoylquinic acid	H	H	C
3,4-O-Dicaffeoylquinic acid	C	C	H
3,5-O-Dicaffeoylquinic acid	C	H	C
4,5-O-Dicaffeoylquinic acid	H	C	C
3-O-Feruloylquinic acid	F	H	H

separation of chlorogenic acids[13–15]. Stegen and Duijn have claimed the separation of nine chlorogenic acids and two free hydroxycinnamic acids of green coffee beans[15]. This paper describes the separation by reversed-phase HPLC of eleven phenolic compounds and the identification of the seven isolated compounds by mass spectrometry (MS) and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.

EXPERIMENTAL

Extraction

A 10-g amount of ground coffee beans (*Coffee canephora* var. Robusta) was extracted with 200 ml of 70% 2-propanol for 30 min at room temperature. The extraction was repeated four times. The combined extracts were concentrated to *ca.* 100 ml under the reduced pressure. The resulting aqueous solution was centrifuged for 30 min at 10,000 r.p.m. (8000 *g*), and the supernatant was filtered through a Millipore filter (pore size 0.45 μm). The filtrate was frozen and stored at −20°C in the dark until use.

Reversed-phase HPLC

All analytical and preparative separations were performed with Jasco Trirotar III high-performance liquid chromatograph equipped with a spectrophotometric detector Uvidex III. The UV detector was set at 325 nm. The analytical column (250 × 4.6 mm I.D.) and preparative column (250 × 7.2 mm I.D.) were filled with Fine SIL $C_{18\text{-}5}$ (Jasco, Japan) as stationary phase. Two solvents were used: A, 10 m*M* H_3PO_4; B, methanol.

For analytical separation a linear gradient was used: 0–15 min, 5–50% B in A; 15–30 min, 50–70% B in A. For preparative separation a combination of isocratic and linear gradient elution was used: 0–30 min, 5–50% B in A (linear gradient);

30–50 min, 50% B in A (isocratic); 50–55 min, 50–80% B in A (linear gradient); 55–70 min, 80% B in A (isocratic).

^1H NMR

The 360 MHz ^1H NMR spectra of the compounds were recorded on a Bruker WM 360 wb NMR spectrometer in dimethyl sulphoxide (DMSO) (*ca.* 0.1%, w/v), with tetramethylsilane (TMS) as an internal reference. Measurements were made at 25°C and 80°C. A 45° pulse width at 1.0 sec pulse interval was employed. The H–^{2}H exchange of samples was done by adding a drop of ^2H_2O to the DMSO. Chemical shifts were read relative to the internal standard. Decoupling experiments were performed using a homo-gated decoupling unit equipped with the spectrometer.

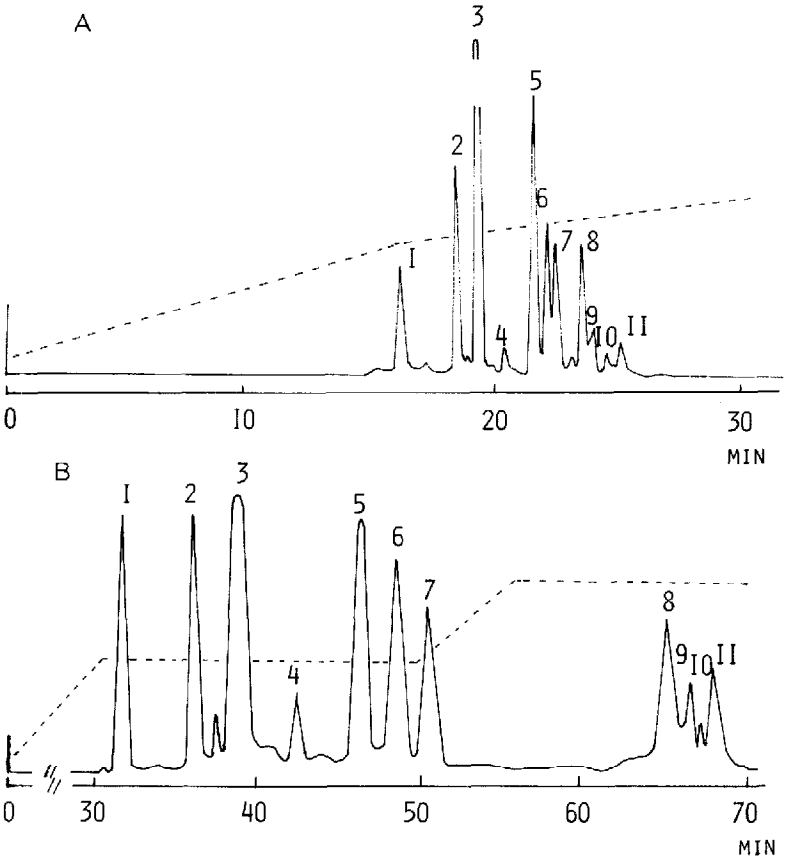


Fig. 1. (A) Analytical chromatogram of phenolic compounds in an extract of green robusta coffee beans. Conditions: column, Finepak SIL $C_{18\text{-}5}$ (5 μm), 25 × 0.46 cm I.D.; flow-rate, 1.0 ml/min; detector, 325 nm, 0.16 a.u.f.s.; injection volume, 5 μl; gradient elution (solvent A, 10 m*M* H_3PO_4; solvent B, methanol), from 5% B to 50% B in 15 min and then 70% B in 15 min. For peak identification, see Table III. (B) Preparative chromatogram of phenolic compounds in an extract of green robusta coffee beans. Conditions: column, Finepak SIL $C_{18\text{-}5}$ (5 μm), 25 × 0.72 cm I.D.; flow-rate, 1.0 ml/min; detector, 325 nm, 2.54 a.u.f.s.; injection volume, 4.0 ml; elution (solvent A, 10 m*M* H_3PO_4; solvent B, methanol), from 5% B to 50% B (linear gradient) in 30 min, then 50% B (isocratic) for 20 min, then from 50% B to 80% B (linear gradient) in 5 min, then 80% B (isocratic) for 15 min.

MS measurements

The FD-MS spectra were recorded on a GCMS-9020 DF-FD mass spectrometer equipped with a SCAP-1123 data aquisition system (Shimadzu, Kyoto, Japan).

RESULTS

Analytical and preparative HPLC

A chromatogram of phenolic compounds of green coffee beans on the analytical column is shown in Fig. 1A. Eleven peaks resulted from the linear gradient elution. Chlorogenic acid (3-O-caffeoylquinic acid) is the major phenolic constituent of green coffee beans. On the basis of its retention time and UV spectrum, it was readily assigned as peak 3. The UV absorption spectra of the other ten peaks were very similar to that of chlorogenic acid. We then attempted to isolate these compounds. By using a preparative column, we obtained a good separation with narrow and symmetrical peaks (Fig. 1B). The chromatogram showed an uncanny resemblance to that on the analytical column. Each peak was collected and examined for homogeneity by analytical HPLC. After rechromatography with an eluent containing distilled water in place of 10 m*M* H_3PO_4, the eluate was lyophilized. The white powder obtained was subjected to ^1H NMR spectroscopy and MS.

^1H NMR spectra

The ^1H NMR spectra of compounds I, II, III, VI, VII and VIII (corresponding

TABLE II

^1H NMR SPECTROSCOPY OF PHENOLIC COMPOUNDS IN EXTRACTS OF GREEN ROBUSTA COFFEE BEANS

Abbreviations: QA, quinic acid; CA, caffeic acid; FA, ferulic acid.

Parameter	*Position*	*Compound*									
		I	*II*	*III*	*V*	*VI*	*VII*	*VIII*	*QA*	*CA*	*FA*
Chemical shift (ppm)	COOH			12.40		12.40			12.32	12.11	12.11
	C-3-H	3.86	4.11	5.06	5.09	4.09	5.19	5.37	3.75		
	C-4-H	3.55	4.71	3.56	3.55	4.92	3.83	4.96	3.24		
	C-5-H	5.19	3.86	3.92	3.93	5.41	5.11	4.16	3.88		
	C_α-H	6.20	6.26	6.15	6.37	6.23	6.24	6.24		6.17	6.36
						6.19	6.26	6.15			
	C_β-H	7.46	7.48	7.41	7.49	7.45	7.47	7.48		7.41	7.49
							7.44	7.43			
	C-2'-H	7.02	7.04	7.03	7.29	7.02		7.02		7.02	7.28
	C-5'-H and C-6'-H	6.97	7.00	6.98	7.11	6.96		6.98		6.96	7.08
		6.76	6.77	6.77	6.80	6.75		6.74		6.76	6.79
	C-4'-OH	9.53	9.53	9.58	9.59	9.58	9.58	9.59		9.52	9.54
	C-3'-OH	9.14	9.12	9.15		9.13	9.16	9.13		9.12	
	OCH_3				3.82						3.81
Coupling constant (Hz)	$J_{\alpha,\beta}$	15.9	15.8	15.8	16.0	16.4	15.3	16.7		15.9	15.9
	$J_{5',6'}$	8.1	8.0	7.9	8.0					8.1	7.8

to peaks 1, 2, 3, 6, 7 and 8) were the sum of the spectra of caffeic acid and quinic acid, respectively, as shown in Table II. In the spectra of compounds I, II and III, the caffeic acid moiety was comparable with the quinic acid moiety, which indicated that positional isomers of monocaffeoylquinic acid were present. On the other hand, in the spectra of compounds VI, VII and VIII two moieties of caffeic acid were esterified with one moiety of quinic acid. They were positional isomers of dicaffeoylquinic acid. Similarly, the spectrum of compound V was the sum of the spectra of

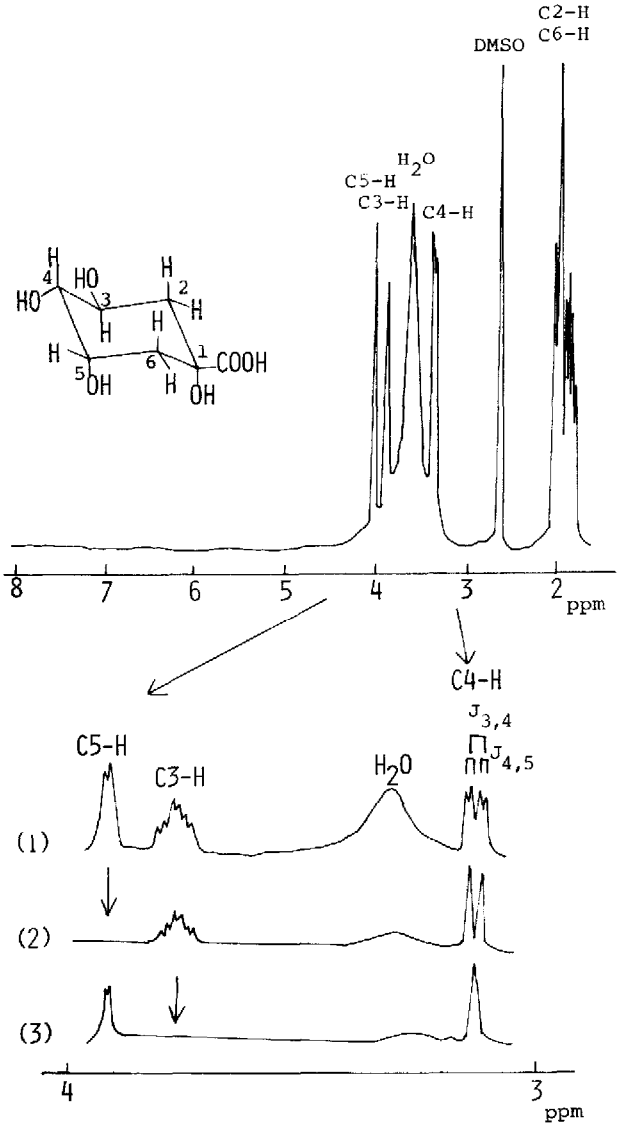


Fig. 2. Results of decoupling experiment on quinic acid. The ^1H NMR spectrum of quinic acid in DMSO (^2H_2O) is shown in the upper panel and the region of the C-3, C-4 and C-5 protons is shown expanded in the lower panel. The spectra are for (1) no irradiation, (2) irradiation at 3.88 ppm, and (3) irradiation at 3.75 ppm, respectively. Irradiated positions are indicated by arrows (↓).

TABLE III

IDENTIFICATION OF PHENOLIC COMPOUNDS IN EXTRACTS OF GREEN ROBUSTA COFFEE BEANS

Peak No.	*Compound*		*m/e*
1	(I)	5-O-Caffeoylquinic acid	354
2	(II)	4-O-Caffeoylquinic acid	354
3	(III)	3-O-Caffeoylquinic acid	354
5	(V)	3-O-Feruloylquinic acid	368
6	(VI)	4,5-O-Dicaffeoylquinic acid	516
7	(VII)	3,5-O-Dicaffeoylquinic acid	516
8	(VIII)	3,4-O-Dicaffeoylquinic acid	516

ferulic acid and quinic acid (Table II), indicating that compound V was the other related isomer, feruloylquinic acid. Decoupling experiments on quinic acid were performed to assign the C-3, C-4 and C-5 protons (Fig. 2). The assignments are based on the angular dependence of the coupling constant, *i.e.* $J_{3,4}$, the spin–spin coupling constant between a C-3 proton (axial) and a C-4 proton (axial) is larger than $J_{4,5}$ (axial–equatorial)[16]. As shown in Fig. 2, $j_{3,4}$ has a larger value (7.3 Hz) than $J_{3,5}$ (2.5 Hz). Similar decoupling results were obtained for all the isolated compounds, and the C-3, C-4 and C-5 protons were assigned accordingly. The peaks of the C-3, C-4 and C-5 protons in the seven isolated compounds are shifted downfield from those of quinic acid. It has been reported by Corse *et al.* that the paramagnetic chemical shifts have a direct relation to substitution on the hydroxyl groups of quinic acid[5]. The paramagnetic chemical shifts allowed us to determine the position of the ester bond in the quinic acid moiety of the chlorogenic acids. The identification of the compounds is summarized in Table III. Corse *et al.* reported the ^{1}H NMR spectrum (60 MHz) of a 3-O-feruloylquinic acid derivative (diacetone compound)[5]. In the present study we obtained the ^{1}H NMR spectrum of 3-O-feruloylquinic acid itself in DMSO. Its spectrum has characteristic peaks at 3.82 ppm (OCH_3) and 7.29 ppm (C-2′). The peak for the C-2′ proton (at 7.29 ppm) is shifted downfield from that of the caffeic acid moiety and so is useful for predicting the existence of the ferulic acid moiety. For all the compounds, spin–spin coupling constants (15Hz to 17Hz) of C_α and C_β protons were obtained; they clearly showed the presence of two or four *trans* vinyl protons from caffeic acid and ferulic acid moieties.

FD-MS spectra

Molecular weight information on the chlorogenic acids was obtained by FD-MS (Table III). It indicated that compounds I, II and III were positional isomers of monocaffeoylquinic acid (m/e = 354) and that compounds VI, VII and VIII were positional isomers of dicaffeoylquinic acid (m/e = 516). Compound V was shown to be methylated monocaffeoylquinic acid (m/e = 368).

DISCUSSION

Phenolic constituents of green coffee beans were resolved into eleven peaks on the reversed-phase HPLC column by gradient elution. The peak pattern was almost

same as that obtained by Stegen and Duijn[15]. Their identification was based only on the retention times, but this is inadequate for compounds in plant extracts[17]. Vande Casteele *et al.*[18]. insisted on the necessity of combined HPLC–MS for the proper identification of any of the substances studied. In the present study, we isolated seven compounds by preparative HPLC and identified them by ^{1}H NMR spectroscopy and MS.

The positional isomers of monocaffeoylquinic acid were eluted from reversed-phase columns in the sequence 5-, 4-, 3- by using gradient elution with 10 m*M* H_3PO_4 and methanol. The positional isomers of dicaffeoylquinic acid were eluted in the sequence 5,4-, 3,5-, 3,4-. The retention times of these compounds seem to be influenced by the interaction between the stationary phase and the caffeoyl residue. The elution order of the monocaffeoylquinic acid isomers indicates that the affinity increases in the following order: 3- > 4- > 5- substitution. On the other hand, the magnitude of the interaction of the dicaffeoylquinic acid isomers seems to be constitutive and additive, as shown by the elution order.

According to Corse *et al.*, acylation of the hydroxyl groups in quinic acid produces paramagnetic chemical shifts of 1.4–1.6 ppm[5]. The present results are consistent with their findings. Similar paramagnetic chemical shifts were also observed in ferulic acid. The formation of the ester bond also induces paramagnetic shifts for the protons on neighbouring carbon atoms (Table IV). The magnitude of the induced chemical shifts decreases with distance from substituted position. Isomers of dicaffeoylquinic acid have larger induced chemical shifts than those of monosubstituted acid. This may be due to the sum of the direct and long-range induced chemical shifts in the disubstituted acid. Assuming that these two effects are independent, the induced chemical shifts of dicaffeoylquinic acid can be established as follows:

$$\Delta^3_{3,4} = \Delta^3_3 + \Delta^3_4$$

where $\Delta^3_{3,4}$ is induced chemical shift of the C-3 proton of 3,4-O-dicaffeoylquinic acid, and Δ^3_3 and Δ^3_4 are those of 3-O-caffeoylquinic acid and 4-O-caffeoylquinic acid,

TABLE IV

INDUCED CHEMICAL SHIFTS BY FORMATION ESTER OF BONDS IN CHLOROGENIC ACIDS

Compound	*Induced chemical shifts (ppm)**		
	C-3	*C-4*	*C-5*
5-O-Caffeoylquinic acid	0.11	0.31	1.31
4-O-Caffeoylquinic acid	0.36	1.47	−0.02
3-O-Caffeoylquinic acid	1.31	0.32	0.04
4,5-O-Dicaffeoylquinic acid	0.34(0.47)**	1.68(1.78)**	1.53(1.29)**
3,5-O-Dicaffeoylquinic acid	1.44(1.42)**	0.59(0.63)**	1.23(1.35)**
3,4-O-Dicaffeoylquinic acid	1.62(1.67)**	1.72(1.79)**	0.28(0.02)**
3-O-Feruloylquinic acid	1.34	0.31	0.05

* Relative to quinic acid chemical shifts.

** The sums described in text are presented in parentheses.

respectively. The calculated values are presented in parentheses in Table IV. This treatment goes some way towards explaining the large induced chemical shifts of dicaffeoylquinic acids, and can be extended for the identification of quinic acid esters with other cinnamic acid derivatives.

CONCLUSION

The reversed-phase HPLC system presented here is well suited for the separation of chlorogenic acids. Green coffee beans contain a mixture of positional isomers of mono- and dicaffeoylquinic acid, whose separation with classical methods is elaborate and time-consuming. This technique allowed a clear separation of these compounds in non-purified extracts from cofee beans in a relatively short time. The ^{1}H NMR and MS measurements allowed the unequivocal identification of seven isolated compounds, and we obtained a series of ^{1}H NMR data about chlorogenic acids which will give important information to help in the identification of other quinyl esters of cinnamic acid derivatives.

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