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Characterisation of Chlorogenic Acids by Simultaneous Isomerisation and Transesterification with Tetramethylammonium Hydroxide

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ABSTRACT

The use of tetramethylammonium hydroxide at room temperature is reported for the simple characterisation of trace amounts of chlorogenic acids belonging to the caffeoyl, p-*coumaroyl, feruloyl and dicaffeoylquinic acid subgroups. The assignments proposed have been confirmed by conventional proton NMR.*

INTRODUCTION

The chlorogenic acids (CGA) are a large family of esters formed between one or more residues of a phenolic acid (usually caffeic, ferulic or *p*-coumaric) and quinic acid (1L-1(OH),3,4,5-tetrahydroxycyclohexane carboxylic acid).

These compounds are widespread in plant tissues and many derived foods and beverages. At least 20 such compounds have been reported (Herrman, 1967; Clifford, 1985*a*, *b*) but only one member of the family seems to be

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available commercially. Using IUPAC nomenclature (IUPAC, 1976) and the system of abbreviations previously proposed (Clifford, 1985*a*, *b*) this is 5-caffeoylquinic acid (5-CQA) but it is normally supplied as chlorogenic acid, or, using the older numbering system, as 3-CQA.

The lack of reference compounds is a serious constraint to the study of these CGA. Reliable characterisation of a putative CGA requires complex multiple step chemical synthesis (e.g. see Clifford, 1985*b*; Kellard *et al.*, 1987) and/or isolation from a natural source and characterisation by NMR (Clifford, 1986). This paper reports a time-saving and cost efficient alternative based upon pre-column isomerisation and transesterification with tetramethylammonium hydroxide.

MATERIALS AND METHODS

Materials

Tetramethylammonium hydroxide (TMAH), caffeic acid, *p*-coumaric acid and ferulic acid were obtained from Aldrich Chemical Company, and 5-CQA from Sigma Chemical Company Ltd, Poole, UK. Cynarin (1,3-diCQA) was obtained from Roth GmbH KG Ltd, Karlsruhe, West Germany, but is no longer available. Other reagents and solvents were commercial items of good quality. 3-CQA, 4-CQA, 5-*p*CoQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA were isolated as described below.

Methods

Extraction of crude chlorogenic acids from green robusta coffee beans
Green coffee beans were frozen, ground and extracted in boiling 70% MeOH as previously described (Clifford, 1986; Clifford & Jarvis, 1988).

The extract was cleared with Carrez Reagent (1 ml Reagent A followed by 1 ml Reagent B) filtered (Whatman No. 1) and concentrated to saturation by evaporation at room temperature ($\sim 20°C$) and reduced pressure. Methanol was added dropwise to redissolve precipitated material.

Analytical HPLC

Analytical HPLC was performed conventionally using gradient elution from a 3 μ reversed phase packing as previously described (Clifford & Jarvis, 1988).

To ensure resolution of 1,5-diCQA and 3,5-diCQA a gradient of 20% to 22·5% acetonitrile in 0·5% formic acid over 30 min was also used as required.

Preparative HPLC

A Spectraphysics SP8700 XR gradient solvent delivery system was used in conjunction with a 25 cm × 8 mm semipreparative column packed with Spherisorb ODS1 5 μ (Hichrom Ltd) and protected with a 5 cm × 4·6 mm guard column containing the same packing material. Solvents were 0·5% formic acid in water (A) and 50% aqueous acetonitrile containing 0·5% formic acid (B) with a gradient designed to give good resolution of the peak required, as summarised below: 10% B to 15% B in 10 min; isocratic 15 min; 15% B to 25% B in 10 min; isocratic 15 min; 25% B to 40% B in 5 min; isocratic 5 min and reset. Flow rate, 2 ml min^{-1}.

Sample injection was via a 500 μl loop, with detection at 313 nm (Waters 440). Fractions, consisting of the centre of each peak of interest, were collected manually to achieve as high a purity as possible. After bulking, the fractions were evaporated to near dryness, transferred to small glass containers, and final traces of solvent removed with nitrogen gas.

The isolated material was weighed, dissolved in 70% MeOH, aliquoted as necessary and used for NMR and isomerisation studies as appropriate.

Isomerisation and transesterification

TMAH (X μl, 20% ethanolic) was added with immediate mixing to 10 $X\mu$l of a CGA solution (aqueous or methanolic) containing not less than 2·5 μg CGA. If the yellow colour did not appear immediately a second aliquot of $X\mu$l TMAH was added to overcome residual sample acidity, and the reaction allowed to proceed at room temperature (~20°C) for 5 min (monoacyl CGA) or 3 min (diacyl CGA). The reaction was terminated by the addition, with immediate mixing, of 2 $X\mu$l 3·5M acetic acid.

Where possible the value of X was set at 100 μl, but when necessary successful results were obtained with $X = 10$ μl. During the development of this procedure, preliminary investigations were performed, in which:

(1) the reaction temperature was varied (0°C, 20°C, 38°C)
(2) the reaction time was varied (0·25 min to 3 h)
(3) the concentration of 5-CQA was varied (25 μg/ml to 1 mg/ml)
(4) the ratio 5-CQA:TMAH v/v was varied (20:1 to 1:2)

Synthesis of methyl cinnamates

Methyl cinnamates were prepared in a Soxhlet apparatus from the corresponding cinnamic acids (caffeic, ferulic and *p*-coumaric) by refluxing for 30 min with azeotropic toluene, methanol, conc. hydrochloric acid (10:50:1 v/v/v). Magnesium sulphate, in a Soxhlet thimble, was used as a desiccant.

NMR

^{1}H-NMR spectra were obtained on a Brucker AC 300 operating at 300·14 MHz. Samples, dissolved in D_2O containing tetramethylsilane standard, were examined at room temperature.

RESULTS AND DISCUSSION

Conventional characterisation

Each CGA was checked for purity by analytical HPLC. In all cases a purity in excess of 91% and, in most cases, in excess of 95% was achieved as judged by peak area at 313 nm.

TABLE 1
Chemical Shifts for Protons at C3, C4 and C5 in the Quinic Acid Residue of various CGA, relative to free Quinic Acid

Compound	*Chemical shifts (ppm)*					
	C3, quartet		*C4, double doublet*		*C5, triple doublet*	
	δ	$\Delta\delta$	δ	$\Delta\delta$	δ	$\Delta\delta$
Quinic acid	4·22	—	3·70	—	4·02	—
3-CQA	**5·30**	**+1·08**	3·67	−0·03	4·11	+0·09
4-CQA	4·60	+0·38	**4·83**	**+1·13**	4·27	+0·25
5-CQA	4·37	+0·17	3·87	+0·07	**5·35**	**+1·33**
5-*p*CoQA	4·25	+0·03	3·71	+0·04	**5·25**	**+1·23**
5-FQA	4·14	−0·08	3·76	+0·06	**5·19**	**+1·17**
1,3-diCQA	**5·74**	**+1·52**	4·01	+0·31	4·15	+0·13
3,4-diCQA	**5·75**	**+1·53**	**5·25**	**+1·55**	4·25	+0·23
3,5-diCQA	**5·50**[a]	**+1·28**	4·06	+0·36	**5·45**[a]	**+1·43**
4,5-diCQA	4·40	+0·18	**5·15**	**+1·45**	**5·55**	**+1·53**

[a] Overlapping—shifts approximate.

Each CGA was characterised by proton NMR. The results were consistent with previous publications (Clifford, 1985*b*, 1986; Morishita, 1987). The chemical shifts for the protons at C3, C4 and C5 of the quinic acid residue are summarised in Table 1.

The methyl cinnamates were obtained quantitatively and were pure as judged by analytical HPLC. As their production employed a standard method, further characterisation was considered unnecessary.

Characterisation by precolumn treatment with TMAH

Preliminary studies showed that several interdependent reactions occurred when 5-CQA was treated with TMAH. So far no attempt has been made to define the kinetics of acyl migration (isomerisation), transesterification and degradation. The main products, identified by cochromatography with authentic material, are 3-CQA, 4-CQA, 5-CQA and methyl caffeate. A specimen chromatogram is shown in Fig. 1.

When mild conditions are employed (15s at 20°C, 5 min at 0°C) 5-CQA remains the dominant product. With slightly harsher conditions (5 min, 20°C) 3-CQA, 4-CQA, 5-CQA and methyl caffeate are present in similar quantities, while forcing conditions (1 h 20°C, 5 min 38°C) produce methyl caffeate as the major product. Once the four products have been formed in similar quantities these ratios are maintained, but total yield declines due to instability in base.

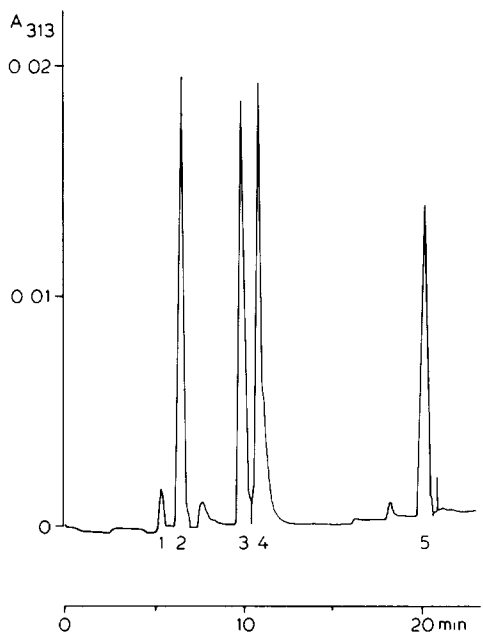

Fig. 1. Chromatograms of 5-CQA treated with TMAH for 5 min at 20°C. Peak 1 = 1-CQA (unconfirmed); Peak 2 = 3-CQA; Peak 3 = 4-CQA; Peak 4 = 5-CQA; Peak 5 = methyl caffeate. Reaction conditions—100 μl 5-CQA (75 μg/ml) plus 10 μl TMAH (20% ethanolic) at 20°C for 5 min. Reaction stopped by the addition of 20 μl of 3·5M acetic acid. Chromatographic conditions—6% acetonitrile in 0·5% formic acid to 40% acetonitrile in 0·5% formic acid linearly over 35 min on Spherisorb ODS1 3 μ; 20 μl injection; 0·01 AUFS; 313 nm.

TABLE 2
Yield of Products from TMAH Treatment (5 min, 20°C) of Monoacyl CGA

Starting material	*Yield of products (%)*				
	1-acyl	*3-acyl*	*4-acyl*	*5-acyl*	*Methyl cinnamate*
3-CQA		43	30	16	11
4-CQA		32	28	20	21
5-CQA		22	26	39	13
5-*p*CoQA	2[a]	25	22	29	22
5-FQA	8[a]	27	24	38	3

[a]Identity not confirmed.

Increasing the 5-CQA concentration in the range 25 μg/ml to 1 mg/ml, or the 5-CQA:TMAH ratio in the range 20:1 to 1:2 increased the rates of all reactions. While the initial 5-CQA concentration had little effect on the relative yield of products, increasing the TMAH:5-CQA ratio favoured the production of 3-CQA at the expense of 5-CQA.

Because of shortage of material this series of experiments was not applied to the other CGA, but the products ultimately obtained from 3-CQA, 4-CQA and 5-CQA were identical. However, slight differences were noted in the rates of reaction and relative yields as shown in Table 2. It would appear that 4-CQA is more readily converted to 3-CQA than to 5-CQA, and that of these three isomers 3-CQA is more resistant to acyl migration. Occasionally traces were seen of a component that might be 1-CQA, but this was not confirmed. 5-*p*CoQA and 5-FQA behaved very similarly to 5-CQA and gave the analogous products. Possibly the FQA were more resistant to transesterification as judged by the yield of methyl ferulate (see Table 2).

Four of the six possible diCQA isomers were similarly examined. The relative yields of products are shown in Table 3. In each case 3-CQA, 4-CQA, 5-CQA and methyl caffeate were present along with the starting material and at least one other diCQA isomer.

The diCQA isomers differed significantly in their susceptibility to acyl migration. Cynarin (1,3-diCQA) was the most resistant. Even after 10 minutes' treatment at 20°C only the second most resistant isomer, 3,4-diCQA, was observed. The three coffee bean diCQA isomers produced qualitatively identical mixtures but 3,5-diCQA and 4,5-diCQA were noticeably more susceptible to isomerisation than 3,4-diCQA. Even when using a shallow gradient chromatographic system known to resolve 3,5-diCQA and 1,5-diCQA in artichoke extracts (Clifford, unpublished; Adzet & Puigmacia, 1985) it proved impossible to detect either 1,4- or 1,5-diCQA in

TABLE 3
Yield of Products from TMAH (5 min, 20°C) Treatment of diCQA Isomers

Starting material	*Yield of products (%)*								
	1-CQA	*3-CQA*	*4-CQA*	*5-CQA*	*1,3-diCQA*	*3,4-diCQA*	*3,5-diCQA*	*4,5-diCQA*	*Methyl cinnamate*
1,3-diCQA	5[a]	2	2	2	67	16			5
3,4-diCQA		3	2	1		47	12	11	25
3,5-diCQA		7	4	2		20	11	14	42
4,5-diCQA		12	10	16		16	12	16	16

[a] Identity not confirmed.

any of the reaction mixtures. However, cynarin yielded a product which is thought to be 1-CQA at a concentration equalling that of the other three CQA isomers collectively.

By integrating these results with those obtained for the CQA isomers, it would appear that CGA bearing axial substituents (1 and/or 3) are rather more resistant to acyl migration and/or transesterification than those bearing equatorial substituents (4 and/or 5), with the axial-equatorial 3,4-diCQA and 3,5-diCQA being intermediate.

The relative retention times for the CGA, cinnamic acids and methyl cinnamates used in this study are presented in Tables 4 and 5 using three bases of comparison. These tabulations, while consistent with previous summaries (Clifford, 1985*b*) considerably extend the range of values available. The consistent effect on relative retention time of changing the identity of the acylating residue in the series caffeoyl (1·00), *p*-coumaroyl (1·22 ± 0·03) and feruloyl (1·35 ± 0·04) is clearly shown in Table 4. For a given cinnamoyl residue, a similarly consistent effect is observed (Table 5) as the position of esterification is changed—5-acyl (1·00), 4-acyl (0·92 ± 0·02), 3-acyl (0·69 ± 0·02) and tentatively 1-acyl (0·60 ± 0·03). Although so far only one series of diacyl CGA has been examined, it is reasonable to expect that other series will behave analogously.

It follows from these observations that transesterification with TMAH of an unknown CGA (at least one belonging to the caffeoyl, feruloyl, *p*-coumaroyl or dicaffeoyl subgroups) is all that is required to characterise the starting material with considerable certainty.

The subgroup is easily established from the identity of the methyl cinnamate, and since authentic methyl cinnamates are very easily produced this step is straightforward. Comparison of the retention time of the starting material with that of the isomers obtained, preferably, though not

TABLE 4
The Effect of the Identity of the Cinnamic Acid on the Relative Retention Time of CGA and Some Related Compounds

Position of esterification	*Identity of the Cinnamic acid*		
	Caffeic	p-*Coumaric*	*Ferulic*
1 acyl[a]	1·00	1·21	1·40
3 acyl	1·00	1·21	1·35
4 acyl	1·00	1·17	1·33
5 acyl	1·00	1·22	1·37
Cinnamic acid	1·00	1·26	1·37
Methyl cinnamate	1·00	1·25	1·29
Mean ± sd	1·00	1·22 ± 0·03	1·35 ± 0·04

[a] Identity not confirmed.
Chromatographic conditions—15 cm × 4·6 mm analytical column packed with 3 μ Spherisorb ODS1 plus a 5 cm × 4·6 mm guard column packed with Spherisorb 5 μ ODS1. Gradient 6% aqueous acetonitrile containing 0·5% formic acid to 40% aqueous acetonitrile containing 0·5% formic acid in 35 min.

TABLE 5
The Effect of Position of Esterification on the Relative Retention Time of CGA and Some Related Compounds

Position of esterification	*Subfamily*					*Mean ± sd* (A_1, *B and C*)
	CQA and diCQA	p*CoQA*		*FQA*		
	A_1	A_2	*B*	A_3	*C*	
1 acyl	0·64[a]	0·76[a]	0·63[a]	0·89[a]	0·68[a]	0·65 ± 0·03
3 acyl	0·68	0·85	0·68	0·94	0·72	0·69 ± 0·02
4 acyl	0·94	1·10	0·91	1·19	0·92	0·92 ± 0·02
5 acyl	1·00	1·22	1·00	1·30	1·00	1·00
Cinnamic acid	1·08	1·36	1·12	1·51	1·13	1·11 ± 0·01
Methyl cinnamate	1·70	2·13	1·75	2·20	1·70	1·72 ± 0·03
1,3-diacyl	1·22					
1,4-diacyl	—					
1,5-diacyl	1·67					
3,4-diacyl	1·59					
3,5-diacyl	1·67					
4,5-diacyl	1·78					

[a] Identity not confirmed.
A_1, A_2 and A_3 are relative to 5-CQA, B is relative to 5-*p*CoQA and C is relative to 5-FQA.
Chromatographic conditions as specified for Table 4.

necessarily, supported by reference to 5-CQA and its isomerisation products, permits the substitution pattern of the starting material to be deduced. Since natural extracts usually contain several of the isomers belonging to one subfamily (albeit in some cases possibly as artifacts of extraction) it would normally be possible to identify more than one unknown in the original extract by TMAH treatment of only one isolated component, and to achieve this with a smaller quantity of material than is normally required for NMR.

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