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# Characterisation of Caffeoylferuloylquinic Acids by Simultaneous Isomerisation and Transesterification with Tetramethylammonium Hydroxide

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*ABSTRACT*

*It is suggested that green robusta coffee beans contain at least three, and possibly up to six, caffeoylferuloylquinic acid isomers (CFQA). This suggestion is based upon the action of tetramethylammonium hydroxide (TMAH) upon the putative CFQA. The deduced structural assignments are not in complete agreement with previously published data, particularly recent data obtained by proton NMR. These apparently conflicting interpretations are critically discussed, and an explanation therefore, is proffered.*

## INTRODUCTION

The use of tetramethylammonium hydroxide (TMAH) has recently (Clifford *et al.*, 1989*a*) been reported as a precolumn derivatising reagent to facilitate the characterisation of the commoner chlorogenic acids (CGA). Four CGA subgroups—the caffeoylquinic acids (CQA), the *p*-coumaroylquinic acids (*p*CoQA), the feruloylquinic acids (FQA) and the dicaffeoylquinic acids

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(diCQA)—have been studied. It was established that the TMAH treatment of any member of these four subgroups resulted in the formation of a characteristic series of positional isomers and the corresponding methyl cinnamate. The reversed phase chromatogram of these products, when compared with the corresponding chromatograms of the appropriate methyl cinnamates, and of similarly treated, commercially available 5-CQA, permitted the easy identification of the particular CGA originally treated.

This paper reports the extension of that study to microgram quantities of the comparatively unknown caffeoylferuloylquinic acids (CFQA).

## MATERIALS AND METHODS

TMAH was obtained from the Aldrich Chemical Company, and 5-CQA from the Sigma Chemical Company Limited, Poole, UK. Cynarin (1,3-diCQA) was obtained from Roth GmbH Ltd, Karlsruhe, West Germany, but is no longer supplied. The methyl cinnamates, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA were prepared as previously described (Clifford *et al.*, 1989*a*). The CFQA were isolated as described below, and all other reagents were normal commercial items of good quality.

The grinding of the green coffee beans, extraction of crude CGA, the analytical HPLC and proton NMR were performed as previously described (Clifford *et al.*, 1989*a*). The preparative HPLC was performed using the same equipment, but required a modified gradient profile—20% B for 30 min isocratic, 20% B to 25% B in 5 min, 20 min isocratic, 25% B to 20% B in 5 min, and reset.

Aliquots (100 $\mu$l) of the isolated components containing, respectively, some 60 $\mu$g of component 10, 7 $\mu$g of component 13, or 17 $\mu$g of component 15, were treated with 10 $\mu$l of 20% ethanolic TMAH for 2 min at 20°C.

UV spectra of the isolates were recorded in 70% MeOH against a 70% MeOH blank using a Kontron Uvikon 860 recording spectrophotometer.

## RESULTS AND DISCUSSION

It should be noted that this publication uses the IUPAC (1976) numbering system for chlorogenic acids, with the system of abbreviations proposed by Clifford (1985*a*, *b*). *Quotations from other publications have been amended to this system where necessary.*

The fractions used in this study were collected in microgram quantities while coincidentally preparing diCQA for physico-chemical studies, and there was insufficient material to obtain proton NMR spectra at 300 MHz.

These fractions have previously been referred to as components 10, 13 and 15 (Clifford & Jarvis, 1988; Clifford *et al.*, 1989*b*). Each had a UV spectrum similar to 5-CQA with lambda max. in the region 320–321 nm. They eluted in a very crowded region of the preparative chromatogram, and even after optimisation of the gradient profile to separate the diCQA, it was not possible to collect these components in pure form. Analytical HPLC indicated that component 10 contained some 5% of both component 11 and 4,5-diCQA; component 13 contained some 15% each of components 11 and 12 plus a trace of component 10; and component 15 contained some 10% each of components 14 and 16.

Figure 1 is a composite prepared from analytical chromatograms of components 10, 13 and 15 before, and of component 10 after, TMAH treatment. The quantitative results are shown in Table 1, along with the identity of the products and their relative retention times in two chromatographic systems. These results show clearly that components 10,

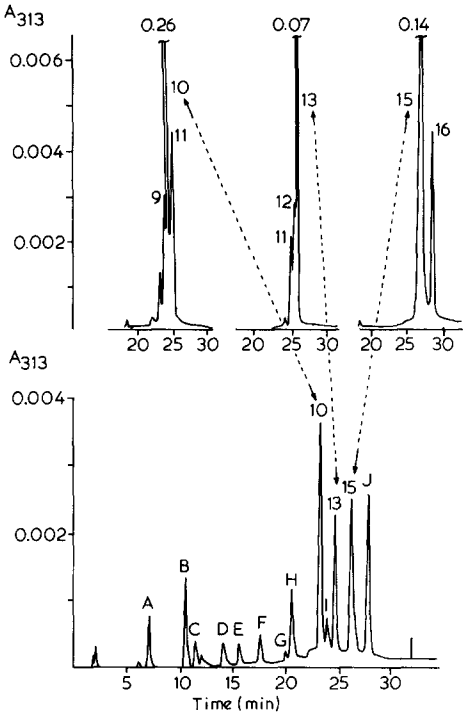


**Fig. 1.** A composite chromatogram of components 10, 13 and 15 before and component 10 after TMAH treatment. A = 3-CQA; B = 4-CQA; C = 5-CQA; D = 4-FQA; E = 5-FQA; F = unknown product; G = 3,4-diCQA; H = MeC; I = unknown product; J = MeF; 10 = putative 3,4-CFQA; 11 and 12 = unknown components; 13 = putative 3,5-CFQA; 14 = unknown component; 15 = putative 4,5-CFQA; 16 = unknown component. Chromatographic conditions as given in previous section.

13 and 15 are easily interconverted by treatment with TMAH, thus suggesting, by analogy with the results previously obtained (Clifford *et al.*, 1989*a*), that they are members of the same CGA subgroup.

Similarly, the consistent production of methyl caffeate with three CQA isomers at a yield essentially equalling that of methyl ferulate and two FQA isomers (3-FQA coelutes with 4-CQA) provides a strong indication that the original components were CFQA isomers.

In theory up to twelve CFQA isomers may occur. However, since the

**TABLE 1**

The Relative Retention Times, Yield and Identity of the Products obtained by TMAH Treatment of Components 10, 13 and 15

| *Product* | *Relative retention time* | | | *Percentage yield component* | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Normal gradient* | *Shallow gradient* | | *10* | | *13* | | *15* | |
| | | | | *b* | *a* | *b* | *a* | *b* | *a* |
| 3-CQA | 0·63 | | | | 4 | | 4 | | 3 |
| 4-CQA | 0·92 | | | | 8 | | 3 | | 1 |
| 5-CQA | 1·00 | | | | 2 | | 1 | | 7 |
| 4-FQA | 1·23 | | | | 2 | | 1 | | 2 |
| 5-FQA | 1·31 | | | | 2 | | 1 | | 3 |
| 3,4-diCQA | 1·72 | 1·00 | 1·00 | | + | | | | |
| 3,5-diCQA | 1·81 | 1·05 | 1·26 | | + | | | | |
| 1,5-diCQA | 1·81 | 1·05 | 1·31 | | | | | | |
| 4,5-diCQA | 1·95 | 1·13 | 1·66 | 5 | + | | | | |
| 3,4-CFQA | 2·03 | 1·00 | 1·00 | 90 | 21 | + | 22 | | 18 |
| 3,5-CFQA | 2·15 | 1·06 | 1·26 | | 12 | 70 | 15 | | 7 |
| 4,5-CFQA | 2·29 | 1·13 | 1·65 | | 20 | | 17 | 80 | 19 |
| MeC | 1·78 | | | | 6 | | 3 | | 8 |
| MeF | 2·43 | | | | 17 | | 8 | | 17 |
| 11 | 2·09 | | | 5 | 3 | 15 | 8 | | 8 |
| 12 | 2·13 | | | | | 15 | | | |
| 14 | 2·22 | | | | | | 15 | 10 | |
| 16 | 2·33 | | | | | | | 10 | |
| Total C* | | | | | 21 | | 9 | | 15 |
| Total F** | | | | | 21 | | 10 | | 22 |

* The sum of MeC, all CQA isomers and the coeluted 3-FQA.

** The sum of MeF, 4-FQA and 5-FQA.

*a* = after treatment with TMAH.

*b* = before treatment with TMAH.

coffee plant does not seem able to synthesise 1-acyl-CGA, it is reasonable to expect only up to six CFQA isomers in coffee beans. Using abbreviations that are consistent not only with IUPAC (1976) numbering, but also with the abbreviations previously proposed and generally used for the commoner CGA (Clifford, 1985*a*, *b*) these six CFQA isomers may be referred to as set out below:

| *Isomer triplets* | *Isomer pairs* | | |
|---|---|---|---|
| | *3,4-CFQA* | *3,5-CFQA* | *4,5-CFQA* |
| Low C triplet | 3-C, 4-FQA | 3-C, 5-FQA | 4-C, 5-FQA |
| Low F triplet | 3-F, 4-CQA | 3-F, 5-CQA | 4-F, 5-CQA |

It has been shown recently that, at low pH values, the known CGA elute from reversed phase chromatographic materials in a consistent and predictable manner (Clifford *et al.*, 1989*a*). The factors primarily responsible for determining the sequence and the relative retention times are the identity, number and position on the quinic acid residue, of the acylating cinnamic acids. Such consistent behaviour permitted the probable behaviour of the CFQA to be predicted as follows:

(1) the low C and low F isomer pairs will have virtually identical hydrophobicity and will be very difficult to separate;

(2) the two CFQA triplets will elute in the same sequence as the coffee bean diCQA triplet (3,4-diCQA, 3-5-diCQA and 4,5-diCQA), with each triplet having very similar retention times relative to the fastest moving member of each;

(3) since the insertion of a 3′-methyl ether into a CQA isomer (producing the corresponding FQA isomer) causes an increase in retention time of 35–36%, it would be expected that the similar insertion of a 3′-methyl ether into only one of the caffeoyl residues of a diCQA isomer (producing one of the two corresponding CFQA isomers) will cause a retardation of some 17 to 18%.

The results presented in Fig. 1 and Table 1 show clearly that components 10, 13 and 15 could not be resolved into two components each, but that the triplet did indeed have relative retention times that were essentially identical to those for the diCQA triplet. By manipulation of the data in Table 1 it can be seen that components 10, 13 and 15 are retarded by 18%, 19% and 17% relative to 3,4-diCQA, 3,5-diCQA and 4,5-diCQA, respectively. These results are completely consistent with the predictions, and it is therefore

proposed that components 10, 13 and 15 consist, respectively, of the two 3,4-CFQA isomers, the two 3,5-CFQA isomers and the two 4,5-CFQA isomers.

The possible occurrence in green robusta coffee beans of at least one CFQA isomer was suggested originally by Corse *et al.*, (1965) on the basis of an NMR signal for a 3'-methyl ether observed while examining an impure diCQA preparation. Van der Stegen & Van Duijn (1980) suspected the existence of two similar mixed esters after the analysis of the partial acid hydrolysates of two fractions isolated from green robusta coffee beans. These earlier observations are consistent with those currently reported. In contrast, Iwahashi *et al.* (1985) claim to have resolved 4-F, 5-CQA and 4-C, 5-FQA very easily using a chromatographic system essentially identical to that employed in the current study (Iwahashi *et al.*, 1985; Morishita, 1987). Critical evaluation of their data is made difficult by inconsistencies in their two papers. In the first, peak IX was assigned as 4-F, 5-CQA and peak XI as 4-C, 5-FQA with peak X unidentified. In the second, peak X was referred to as 4-F, 5-CQA and peak IX was not identified.

Since their proton NMR spectra for these preparations (Morishita, 1987) clearly showed four *trans* vinyl protons, there is no doubt that these components were diacyl-CGA. The presence in each spectrum of signals for three protons associated with a methyl ether implies that their isolates were indeed CFQA isomers. However, for the reasons set out below, it is suggested that their assignments to specific isomers may be in error.

With pure preparations of CGA at moderate concentrations there is rarely any difficulty in distinguishing between the protons at C3 (quartet) and C5 (double doublet). However, at low concentrations, especially of impure compounds, peak distortion may lead to confusion since the C3-acyl and C5-acyl protons in diacyl-CGA have similar resonances (5·45 to 5·75 ppm—Clifford *et al.*, 1989*a*; 5·11 to 5·41 ppm—Morishita, 1987), and in some cases even overlap. The resonances for these two protons are similar also when the associated hydroxyls are free (4·25 to 4·40 ppm—Clifford *et al.*, 1989*a*; 4·09 to 4·17 ppm—Morishita, 1987).

For 3,4-diCQA, Morishita (1987) reported signals for the C3 and C5 protons at 5·41 and 4·09 ppm, respectively. However, for the earliest eluting CFQA (IX or X?) signals at 4·17 and 5·41 ppm were assigned, respectively, to the protons at C5 and C3 (Iwahashi *et al.*, 1985). Since the peaks for these protons were distorted, and those for the C3 proton swamped by the signal for water, it is quite possible that these assignments were in error, *and should perhaps be reversed.*

Iwahashi *et al.* (1985) further supported their assignments by comparing the chemical shifts of the $H_{alpha}$ protons in free caffeic and ferulic acids with the chemical shifts for the corresponding protons in various CQA and FQA. These workers suggested that these relative chemical shifts indicated not

only whether the $H_{alpha}$ proton was associated with a caffeoyl or a feruloyl residue, but also the position at which the particular cinnamoyl residue was attached to the quinic acid—i.e. that by this parameter it was possible to distinguish between the low C and low F isomer pairs.

That one can distinguish between the $H_{alpha}$ protons in caffeoyl and feruloyl residues is not disputed. The ability to identify which cinnamoyl residue is attached where, however, is questioned. On the basis of their data, to make such a distinction, it would be necessary to distinguish reliably between relative chemical shifts of −0·01 to −0·02 ppm (5-C), 0·02 ppm (5-F), 0·02 to 0·08 ppm (3-C) and 0·07 to 0·09 ppm (4-C). Moreover, it would be necessary to do this without access to the corresponding values for 3-F and 4-F. Experience in this laboratory indicates that these relative chemical shifts cannot be measured at 300 MHz with the precision required when working with dilute and impure solutions. *Conclusions based on such measurements are therefore weakly based and may be in error.* It is suggested therefore that peak XI of Iwahashi *et al.* is more likely to be a mixture of the two 4,5-CFQA isomers rather than an essentially pure preparation of 4-C, 5-FQA, and that the earliest eluting CFQA isomer (IX or X?) is more likely to be a mixture of the two 3,4-CFQA isomers.

## CONCLUSIONS

Green robusta coffee beans contain at least three, and probably six, CFQA isomers. These compounds show chromatographic behaviour which is entirely consistent with the behaviour of the other CGA subgroups. It is suggested that the NMR interpretations of CFQA structure reported by previous workers (Iwahashi *et al.*, 1985; Morishita, 1987) may be in error. However, the unequivocal resolution of these discrepancies must await the isolation of the CFQA in greater quantity and purity.

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