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LC–MS^n analysis of the *cis* isomers of chlorogenic acids

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Abstract

The behaviour of *cis* isomers of selected mono- and di-acyl chlorogenic acids produced by UV-irradiation has been investigated by LC–MS^n. *cis* Isomers fragment identically to the more common *trans* isomers. *cis*-5-Acyl chlorogenic acids are more hydrophobic and elute later than their mono- or di-*trans* counterparts whereas the reverse is true for *cis*-3-acyl and *cis*-4-acyl chlorogenic acids. The *cis* isomers of 1,3-dicaffeoylquinic acid, the only 1-acyl chlorogenic acid investigated, are also more hydrophobic than the di-*trans* isomer. Coffee leaves had a proportionately greater content of *cis* isomers relative to *trans* isomers compared with coffee beans suggesting that UV-irradiation in vivo may also cause geometric isomerisation.
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Keywords: Caffeoylquinic acids; Chlorogenic acids; Coffee; *p*-Coumaroylquinic acids; Cynarin; Dicaffeoylquinic acids; Feruloylquinic acids; LC–MS^n; Leaves; UV-irradiation

1. Introduction

Classically, chlorogenic acids are a large family of esters formed between quinic acid and one to four residues of certain *trans*-cinnamic acids, most commonly caffeic, *p*-coumaric and ferulic: sinapic and dimethoxycinnamic acid also occur and in some plant species various aliphatic acids may replace one or more of the *trans*-cinnamic acid residues (Clifford, 2000, 2003; Clifford, Knight, Surucu, & Kuhnert, 2006). In the IUPAC system (–)-quinic acid is defined as 1L-1(OH),3,4/5-tetrahydroxycyclohexane carboxylic acid and that nomenclature is used throughout this paper (IUPAC, 1976). Although it is widely accepted that the known biosynthetic pathway produces *trans* isomers (Clifford, Jaganath, & Clifford, 2006), and these dominate most extracts, it has long been known that conversion to the *cis* geometry occurs readily, especially after exposure to UV light. (Kahnt, 1967). *cis* and *trans* isomers are easily resolved by chromatography on paper and thin layers of cellulose, but their behaviour during HPLC on reversed phase column packings has received far less attention (Möller & Herrmann, 1982). Recently, we reported the presence of a significant amount of *cis*-5-*p*-coumaroylquinic acid in Aster (Clifford, Zheng, & Kuhnert, 2006b) and in this paper we extend this study to the characterisation by LC–MS^n of several *cis* isomers of *p*-coumaroylquinic acids, caffeoylquinic acids, feruloylquinic acids and dicaffeoylquinic acids.

2. Materials and methods

2.1. Materials

Authentic standards were used where possible. 5-Caffeoylquinic acid was obtained from Sigma Chemical

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Company (Poole, Dorset, UK). Cynarin (1,3-dicaffeoylquinic acid) and 1,5-dicaffeoylquinic acid were obtained from LGC Promochem (Hatfield, UK). 4,5-Dicaffeoylquinic acid had previously been isolated and characterized in house (Clifford, Kellard, & Birch, 1989a, 1989b). When pure standards were not available commercial green coffee beans or cider were used as a convenient source of caffeoylquinic acids, feruloylquinic acids and dicaffeoylquinic acids, or *p*-coumaroylquinic acids, respectively. Coffee leaves collected in the botanic garden at Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, SP, Brazil and Centro de Café Alcides Carvalho, Instituto Agronômico de Campinas, Campinas, SP, Brazil were included in the study to compare the composition of tissues from the coffee plant that were naturally exposed to strong UV light (leaves) with tissues that were not (beans). The leaves were plucked, immediately transferred to liquid nitrogen, transported, and stored at −80 °C until freeze-dried at −50 °C for 40 h. (FreeZone® 1 Liter Benchtop Freeze Dryer System, Labconco Corporation, MO, USA) and ground to a fine powder.

2.2. Methods

2.2.1. Extraction

Methanolic extracts of commercial green coffee beans and coffee leaves were prepared as previously described (Clifford et al., 2006; Clifford, Wu, & Kuhnert, 2006a; Clifford, Wu, & Kuhnert, 2007; Clifford et al., 2006b). Samples (1 g) were extracted for 60 min with 20 min rinsing using 100 ml 70% v/v aqueous methanol in a Soxtec Advanti 2055 solid–liquid continuous extraction system (FOSS, Warrington, UK). The bulked extracts were treated with Carrez reagents (1 ml of reagent A plus 1 ml of reagent B) (Egan, Kirk, & Sawyer, 1981) to precipitate colloidal material, diluted to 100 ml with 70% v/v aqueous methanol and filtered through a Whatman No. 1 filter paper. The methanol was removed by evaporation with nitrogen and the aqueous extract stored at −12 °C until required, thawed at room temperature, centrifuged (1360*g*, 10 min) and used directly for LC–MS.

2.2.2. UV-irradiation

The methanolic extracts of green coffee bean and leaves were diluted with PureLab (Elga, Marlow, UK) water (1 + 2) and the standards were prepared at ca 100 μg/ml in 25% v/v aqueous methanol. Aliquots of cider, the methanolic extract of green coffee and the methanolic solutions of the pure standards (1 ml) were each transferred to a quartz cuvette, placed in a lightbox under a shortwave UV lamp (UPV, Upland, USA) and irradiated at 245 nm for 60 min.

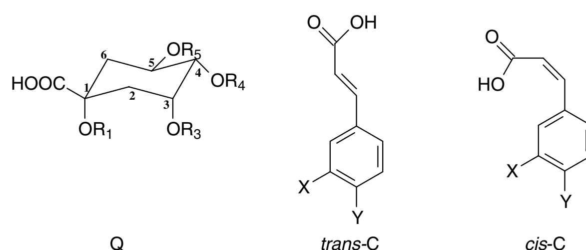
2.2.3. LC–MS^n

The LC equipment (ThermoFinnigan) comprised a Surveyor MS Pump, autosampler with 50 μl loop, and a PDA detector with a light-pipe flow cell (recording at 320, 280 and 254 nm, and scanning from 200 to 600 nm). This was interfaced with an LCQ Deca XP Plus mass spectrometer fitted with an ESI source (ThermoFinnigan). In order to discriminate unequivocally between the positional isomers of the various mono- and di-acyl chlorogenic acids MS data were obtained using targeted MS^2 mode for *p*-coumaroylquinic acids (*m*/*z* 337), caffeoylquinic acids (*m*/*z* 353) and feruloylquinic acids (*m*/*z* 367) and in targeted MS^3 mode (*m*/*z* 515 + 353) for dicaffeoylquinic acids (Clifford, Johnston, Knight, & Kuhnert, 2003; Clifford, Knight, & Kuhnert, 2005; Clifford et al., 2006b). MS operating conditions (negative ion) had been optimized using 5-caffeoylquinic acid with a collision energy of 35%, ionization voltage of 3.5 kV, capillary temperature 350 °C, sheath gas flow rate 65 arbitrary units, and auxiliary gas flow rate 10 arbitrary units.

Chlorogenic acid separations were achieved on a 150 × 3 mm column containing Kromasil phenylhexyl packing (Phenonemex, Macclesfield, UK). Solvent A was water:acetonitrile:glacial acetic acid (980:20:5 v/v, pH 2.68); solvent B was acetonitrile:glacial acetic acid (1000:5 v/v). Solvents were delivered at a total flow rate of 300 μl/min. The gradient profile for chlorogenic acid characterisation was 4% B to 33% B linearly in 45 min, a linear increase to 100% B at 50 min, followed by 5 min isocratic, and a return to 4% B at 60 min, and 5 min isocratic to re-equilibrate.

3. Results

All data for chlorogenic acids presented in this manuscript use the recommended IUPAC numbering system (IUPAC, 1976) and specimen structures are presented in



Name and abbreviation	R_1	R_3	R_4	R_5
1-*O*-cinnamoylquinic acid	C	H	H	H
3-*O*-cinnamoylquinic acid	H	C	H	H
5-*O*-cinnamoylquinic acid	H	H	H	C
4-*O*-cinnamoylquinic acid	H	H	C	H
1,3-di-*O*-cinnamoylquinic acid	C	C	H	H
1,4-di-*O*-cinnamoylquinic acid	C	H	C	H
1,5-di-*O*-cinnamoylquinic acid	C	H	H	C
3,4-di-*O*-cinnamoylquinic acid	H	C	C	H
3,5-di-*O*-cinnamoylquinic acid	H	C	H	C
4,5-di-*O*-cinnamoylquinic acid	H	H	C	C

Q = quinic acid; C = cinnamic acid;

X = H, Y = OH = *p*-coumaric acid; X = Y = OH = caffeic acid; X = OCH_3; Y = OH = ferulic acid

Fig. 1. The structure of caffeoylquinic and dicaffeoylquinic acids (IUPAC numbering) (IUPAC, 1976).

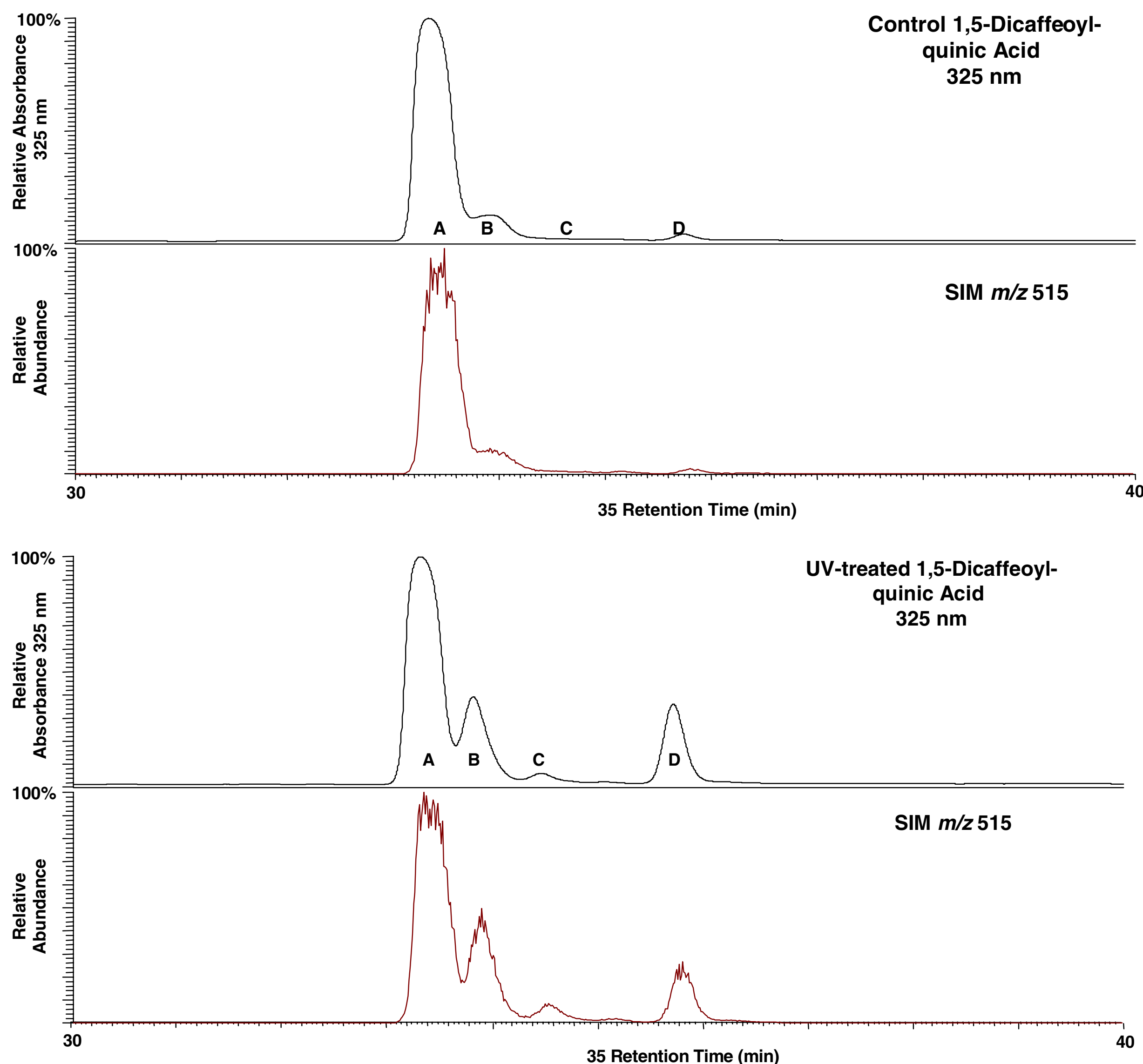


Fig. 2. A_{325} and SIM at m/z 515 for 1,5-dicaffeoylquinic acid before and after UV-irradiation for 1 h. A = di-*trans*-1,5-dicaffeoylquinic acid; B = first eluting mono-*cis*-1,5-dicaffeoylquinic acid; C = di-*cis*-1,5-dicaffeoylquinic acid; D = second eluting mono-*cis*-1,5-dicaffeoylquinic acid.

Fig. 1. A chromatogram of irradiated 1,5-dicaffeoylquinic acid is presented in Fig. 2. The void volume was assessed as 2.0 min by inspection of the UV trace. The calculated capacity factors for the *trans* isomers (k_{trans}) and putative *cis* isomers (k_{cis}) and the relative capacity factors (k_{cis}/k_{trans}) are presented in Table 1.

4. Discussion

LC–MS data for UV-irradiated samples were compared with their corresponding untreated controls. Attention was focussed on peaks seen at much increased concentration in the UV-irradiated samples. Those having a typical chlorogenic acid UV spectrum and an appropriate molecular ion were assigned as *cis* isomers of *p*-coumaroylquinic acids (m/z 337, λ_{max} 315 nm), caffeoylquinic acids (m/z 353, λ_{max} 325 nm), feruloylquinic acids (m/z 367, λ_{max} 325 nm) or dicaffeoylquinic acids (m/z 515, λ_{max} 325 nm). Interpretation of their MS^2 and MS^3 spectra using the hierarchical keys previously developed allowed each of these peaks to be assigned as a particular positional isomer (Clifford et al., 2003; Clifford et al., 2005).

It was immediately clear that geometrical isomerism did not influence the fragmentation behaviour of the positional isomers significantly thus confirming our previous observations of *cis*- and *trans*-5-*p*-coumaroylquinic acid (Clifford et al., 2006b). It was not possible to locate *cis*-3-feruloylquinic acid in the UV-irradiated green coffee extract, but each of the other eight mono-acyl chlorogenic acids investigated produced an easily detectable *cis* isomer. As previously observed (Clifford et al., 2006a; Möller & Herrmann, 1982) the *cis*-5-acyl isomers were appreciably more hydrophobic than their *trans* counterparts whereas for the three 4-acyl and two 3-acyl chlorogenic acids examined the

Table 1
Capacity factors and relative capacity factors for *cis* and *trans* isomers of chlorogenic acids

k_{cis} (min)	k_{trans} (min)	(k_{cis}/k_{trans})	Identity
14.3–2.0	14.8–2.0	0.96	3-*p*-Coumaroylquinic acid
21.2–2.0	24.3–2.0	0.86	4-*p*-Coumaroylquinic acid
28.9–2.0	23.2–2.0	1.27	5-*p*-Coumaroylquinic acid
9.7–2.0	9.9–2.0	0.98	3-Caffeoylquinic acid
14.5–2.0	17.6–2.0	0.80	4-Caffeoylquinic acid
21.5–2.0	15.6–2.0	1.44	5-Caffeoylquinic acid
n.d.	15.8–2.0		3-Feruloylquinic acid
21.3–2.0	23.0–2.0	0.92	4-Feruloylquinic acid
25.0–2.0	22.3–2.0	1.13	5-Feruloylquinic acid
	$k_{\text{di-}trans}$ (min)	($k_{cis}/k_{\text{di-}trans}$)	
	22.2–2.0		Di-*trans*-1,3-dicaffeoylquinic acid
24.3–2.0		1.10	A *cis*-1,3-dicaffeoylquinic acid
25.2–2.0		1.15	A *cis*-1,3-dicaffeoylquinic acid
	33.3–2.0		Di-*trans*-1,5-dicaffeoylquinic acid
33.6–2.0		1.01	A *cis*-1,5-dicaffeoylquinic acid
34.5–2.0		1.04	Di-*cis*-1,5-dicaffeoylquinic acid
35.8–2.0		1.08	A *cis*-1,5-dicaffeoylquinic acid
	33.7–2.0		Di-*trans*-3,4-dicaffeoylquinic acid
33.2–2.0		0.99	A *cis*-3,4-dicaffeoylquinic acid
34.2–2.0		0.96	A *cis*-3,4-dicaffeoylquinic acid
	34.4–2.0		Di-*trans*-3,5-dicaffeoylquinic acid
35.0–2.0		1.02	A *cis*-3,5-dicaffeoylquinic acid
35.6–2.0		1.04	A *cis*-3,5-dicaffeoylquinic acid
	36.1–2.0		Di-*trans*-dicaffeoylquinic acid
37.3–2.0		1.04	A *cis*-4,5-dicaffeoylquinic acid
39.6–2.0		1.10	Di-*cis*-4,5-dicaffeoylquinic acid
40.4–2.0		1.13	A *cis*-4,5-dicaffeoylquinic acid

n.d. = not detected.

reverse was true (Table 1). We suspected that the *cis*-5-acyl isomers were able to produce at least one hydrogen bond that does not occur in the *trans*-5-acyl isomers and that cannot form in *cis*-3-acyl and *cis*-4-acyl chlorogenic acids.

In order to rationalise the observed retention behaviour of the *cis* isomers, we performed some molecular modelling studies at the MM-2 level (Chem 3-D v6.0). Firstly we minimized the geometry of *cis*-methyl caffeic ester. The relative steric energy of the *cis-anti-syn* conformer was found to be lower than that of the corresponding alternative *cis–anti–anti* and *cis–syn–syn* conformers, where the first *anti*-descriptor describes the relative orientation of the ester carbonyl C=O relative to the aromatic ring and the second descriptor describes the relative orientation of the 3-OH group on the aromatic ring with respect to the ester substituent. The geometries are shown in Fig. 3.

With the optimized geometry of the *cis*-caffeic ester substituent established we continued minimizing the geometry of the *cis*-5-caffeoylquinic acid. A clear minimum structure was found that contains two distinct hydrogen bonds. Firstly, a hydrogen bond with a length of 199 pm could be observed between the 4-OH group of the quinic acid moiety and the ester carbonyl C=O of the caffeic acid substituent. This hydrogen bond appears to lock the ester substituent into a conformation in which a second hydrogen bond becomes possible between the phenolic 3′-OH and the carboxylic acid carbonyl of the quinic acid moiety with a length of 205 pm (Fig. 4). These two hydrogen bonds operating in concert appear to increase the hydrophobicity of the *cis*-5-caffeoylquinic acid derivative dramatically compared with its *trans*-isomer, 44% as judged by their capacity factors. In contrast the effect is weaker with the corresponding *cis*-*p*-coumaric ester (27%) and *cis*-ferulic ester (13%).

For the *cis*-*p*-coumaric and ferulic ester derivatives the first hydrogen bond between the quinic acid 4-OH and the ester carbonyl is still operational. The preferred geometry of the *cis*-ferulic methyl ester, however, changes to the *cis-anti-anti* conformation due to repulsion between the ester and ether OMe substituents in the methyl-5-feruloylquinate used for modelling. As a consequence in the minimized structure the distance between the phenolic 4-OH and the carboxylic acid carbonyl C=O increases to 470 pm. The increased hydrophobicity of the *cis*-ferulic and *p*-coumaric derivatives if compared with their *trans* counterpart could therefore be rationalized in three ways. Firstly, an extremely weak second hydrogen bond could be postulated, secondly a bridging water atom between the phenolic 4-OH and the carboxylic acid carbonyl could be postulated stabilizing this conformer or, finally, due to the first hydrogen bond the *cis* isomer exists in a more compact and therefore more hydrophobic structure.

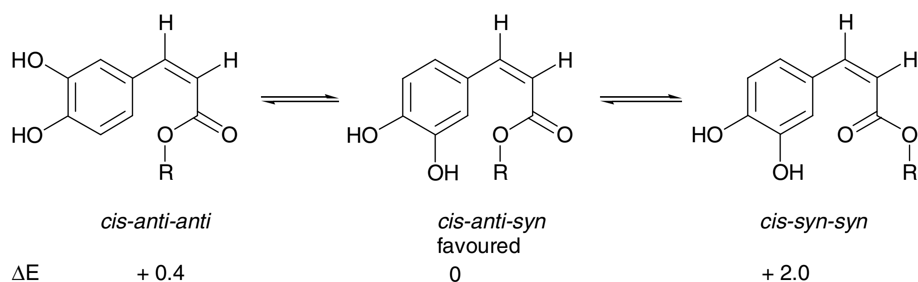


Fig. 3. Relative steric energies from MM-s minimization of *cis*-methyl caffeic ester.

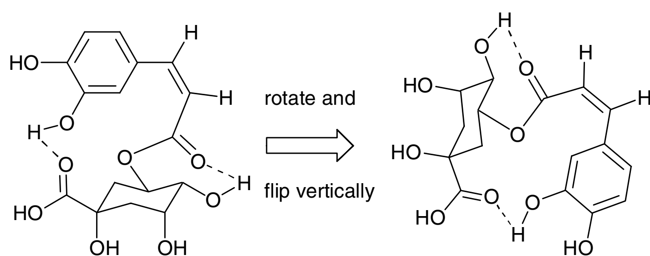


Fig. 4. MM-2 energy minimised structures showing hydrogen bonds in *cis*-5-caffeoylquinic acid between phenolic 3′-OH and O=C–OH (distance H–O=C of 205 pm) and between the quinic acid C4–OH and C=O of the caffeoyl substituent (distance H–O=C 199 pm).

UV-irradiation of pure 1,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid each produced three easily resolved products, two in a similar yield and one at a lower yield (Fig. 2). It was assumed on kinetic grounds that the minor component was the di-*cis* isomer and the others were the mono-*cis* isomers. In the case of pure 1,3-dicaffeoylquinic acid irradiation produced only two detectable *cis* isomers and it is assumed that the third coeluted with them or the starting material. Similarly, irradiation of the coffee bean extract produced only two resolvable *cis* isomers of 3,4-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid. The two *cis* isomers of 3,4-dicaffeoylquinic acid were overlain by di-*trans*-3,5-dicaffeoylquinic acid but were detected readily by the m/z 299 fragment ion that is produced only by 4-acyl (di)caffeoylquinic acids (Clifford et al., 2003, 2005).

In the case of 1,5-dicaffeoylquinic, 3,5-dicaffeoylquinic and 4,5-dicaffeoylquinic acids the eight *cis* isomers detected all eluted later than the di-*trans* isomers from which they were produced, but the effect on hydrophobicity was much weaker than seen for *cis*-5-caffeoylquinic acid. The two *cis* isomers of 3,4-dicaffeoylquinic acid are slightly more hydrophilic than the di-*trans* isomer consistent with the behaviour of 3-caffeoylquinic and 4-caffeoylquinic acid

Fig. 5. LC–MS traces (10–20 min) for an extract of *Coffea arabica* var. *Catuai* beans and leaves A = *trans*-3-caffeoylquinic acid; B = *trans*-5-caffeoylquinic acid; C = *trans*-4-caffeoylquinic acid; D = *cis*-5-caffeoylquinic acid.

and strongly implicating the 5-acyl moiety as contributing to the increased hydrophobicity of both mono- and di-acyl chlorogenic acids. However, the two resolved *cis* isomers of 1,3-dicaffeoylquinic acid were also more hydrophobic than the di-*trans* starting material suggesting that the caffeoyl residue at C1 may also hydrogen bond. For the di-*cis* isomer of 1,3-dicaffeoylquinic acid an interaction between the two caffeoyl residues seems plausible. Similarly, for the C1 *cis*-caffeoyl an interaction with the quinic acid carboxyl can be envisaged and the absence of a hydrogen bond in the other mono-*cis* isomer (C3 *cis*-caffeoyl) and consequent greater hydrophilicity may explain its failure to resolve from the di-*trans* starting material. Data to support this hypothesis were obtained by molecular modelling at the MM-2 level. In a minimised structure two hydrogen bonds could be localised for the for the *cis*-1 *trans*-3 and the di-*cis*-1,3-dicaffoylquinic acid, the first from the COOH functionality to the ester C=O of the *cis*-1-caffeoyl substituent, and the second from the phenolic 3′-OH of the *cis*-1-caffeoyl substituent to the ester C=O of the *cis*-3- or *trans*-3-caffeoyl substituent respectively at a distance of 205 pm. This finding supports the notion that a 1-*cis*-caffeoyl substituent increases the hydrophobicity if compared with its 1-*trans* counterpart. However, the results obtained from this molecular modelling exercise need to be treated with caution because at the MM-2 level π–π interactions between the two caffeoyl substituents are not considered and such interactions might be expected to influence the hydrophobicity.

Fig. 5 illustrates clearly the greater amount (ca × 3) of *cis*-5-caffeoylquinic acid relative to the *trans* isomer in an extract of coffee leaves compared with an extract of green coffee beans of the same variety. Moreover, in the leaf extract *cis*-5-caffeoylquinic acid is present at approximately the same concentration as *trans*-4-caffeoylquinic acid and at an appreciable greater concentration than *trans*-3-caffeoylquinic acid. Histochemical studies have indicated that caffeoylquinic and dicaffeoylquinic acids in coffee leaves are located adjacent to the chloroplasts where it is thought they provide protection against UV damage (Mondolot et al., 2006). Because the *cis* isomers are easily produced by exposure to UV light, and because exposure to UV light during sample preparation was the same for beans and leaves, we conclude that the greater content of *cis*-5-caffeoylquinic acid in leaves can be attributed to the more intense UV-irradiation to which that tissue is naturally exposed. However, since there is not such a pronounced relative increase in *cis*-3-caffeoylquinic acid and *cis*-4-caffeoylquinic acid relative to their *trans* isomers we suspect that stabilisation of the *cis*-5-caffeoylquinic acid by hydrogen bonding is also important.

Relatively large amounts of *cis*-5-*p*-coumaroylquinic acid and *cis*-5-caffeoylquinic acid have previously been observed in the flower buds of Aster (Clifford et al., 2006b) and tobacco leaves (Li et al., 2003) respectively, tissues that also are naturally more exposed to UV-irradiation than coffee beans. Whether the accumulation of the *cis*-5-acyl chlorogenic acids is of biological significance cannot be concluded from these experiments but it is possible that it favours the synthesis of coumarins formed by 2′-hydroxylation and ring closure of the *cis* isomer.

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