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The cinnamoyl–amino acid conjugates of green robusta coffee beans

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Abstract

Methanolic extracts of green robusta coffee beans have been analysed for cinnamoyl amides by electrospray LC–MS^n. Evidence is presented for the presence of *p*-coumaroyl-*N*-tyrosine, feruloyl-*N*-tyrosine, feruloyl-*N*-tryptophan and caffeoyl-*N*-phenylalanine, in addition to the previously reported *p*-coumaroyl-*N*-tryptophan, caffeoyl-*N*-tryptophan and caffeoyl-*N*-tyrosine. These compounds are found at higher levels in Angolan coffees compared with coffees of other origins. Caffeoyl-*N*-phenylalanine has previously been reported in Lady Fern (*Athyrium filix-femina*) but *p*-coumaroyl-*N*-tyrosine, feruloyl-*N*-tyrosine and feruloyl-*N*-tryptophan seem not to have been reported elsewhere. A combination of negative ion and positive ion LC–MS^2 is a convenient procedure for characterising cinnamoyl amides as the former gives an MS^2 base peak defining the amino acid and the latter an MS^2 base peak defining the cinnamic acid in the conjugate.

Keywords: Caffeoyl-*N*-phenylalanine; Caffeoyl-*N*-tryptophan; Caffeoyl-*N*-tyrosine; Cinnamoyl-amides; Coffee; *p*-Coumaroyl-*N*-tryptophan; *p*-Coumaroyl-*N*-tyrosine; Feruloyl-*N*-tryptophan; Feruloyl-*N*-tyrosine; LC–MS^n

1. Introduction

Green coffee beans are characterised by their significant content of cinnamate conjugates. The best known conjugates are those with quinic acid, collectively known as chlorogenic acids (CGA) (Clifford, 2000; Clifford, 1999). The CGA are accompanied in robusta coffee beans by several conjugates with amino acids, for example *N*-β-caffeoyl-L-tryptophan (Morishita et al., 1987), and *N*-β-*p*-coumaroyl-L-tryptophan (Murata, Okada, & Homma, 1996). Robusta coffees from Angola also contain *N*-β-caffeoyl-L-tyrosine (Clifford, Kellard, & Ah-Sing, 1989) and a second similar but incompletely characterised compound, designated Angola II (Correia, Leitao, & Clifford, 1995). With the advent of LC–MS^n and its proven efficacy in characterising CGA (Clifford, Johnston, Knight, & Kuhnert, 2003) it was considered timely to re-examine a selection of commercial robustas, including some from Angola, to investigate whether as-yet-unknown amino acid conjugates contribute to the complement of CGA-like substances. These CGA-like substances have been exploited chemotaxonomically in *Coffea* and *Psilanthus* (Clifford, 1986), and for commercial robusta coffee beans they might also serve as an indicator of geographic origin (Clifford & Jarvis, 1988). Such cinnamoyl amides have been reported also in a wide range of plant material botanically unrelated to coffee (Adam, 1995; Lin, Kuo, & Chou, 2000; Negrel & Javelle, 1997; Sanbongi, Osakabe, Natsume, Takizawa, Gomi, & Osawa, 1998; Schmidt, Grimm, Schmidt, Scheel, Strack, & Rosahl, 1999; Takii et al., 1999; Watanabe, 1999; Wu, Chang, Ko, & Teng, 1995).

2. Materials and methods

2.1. Samples

The Angolan green coffee beans examined were identical to those used in the original study in this laboratory (Clifford et al., 1989) along with a selection from other sources, as used in a study of a possible relationship between the content of CGA-like substances and geographical origin (Clifford & Jarvis, 1988), which had

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all been kept in a dry and dark storage room at ca. 20 °C.

Green robusta coffee beans were frozen overnight (−12 °C), ground in a hammer mill to pass 0.7 mm, and 500 mg extracted (4 × 25 ml, 25 min each) with 70% v/v aqueous methanol using an HT1043 solid–liquid continuous extraction system (Tecator, Bristol, UK) (Balyaya & Clifford, 1995). The bulked extracts were treated with Carrez reagents (1 ml reagent A plus 1 ml 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 at room temperature from an aliquot (ca. 3 ml) by evaporation with nitrogen (N-Evap-111, Organomation Associates Inc., Berlin, MA, USA) and the aqueous extracts were stored at −12 °C until required, thawed at room temperature, centrifuged at 1360*g*, filtered through a 0.42 μm filter, and used directly for LC–MS.

2.2. LC–MS^n

The LC equipment (ThermoFinnigan, San Jose, CA, USA) comprised a Surveyor MS Pump, autosampler with 20 μl loop, and a PDA detector with a light-pipe flow cell (recording at 320, 280 and 254 nm, and scanning from 240 to 600 nm). This was interfaced with an LCQ Deca XP Plus mass spectrometer, fitted with an ESI source (ThermoFinnigan, San Jose, CA, USA) and operating in zoom scan mode, for the accurate determination of parent ion m/z, and in data-dependent, turboscan, MS^n mode to obtain fragment ion m/z. MS operating conditions (negative ion) had been optimised using 5-caffeoylquinic acid (Sigma Chemical Company, Poole, Dorset, UK) with a collision energy of 35%, ionisation voltage of 3.5 kV, capillary temperature 350 °C, sheath gas flow rate 65 arbitrary units, and auxiliary gas flow rate 10 arbitrary units. The positive ion tune file was prepared using *N*-benzoyl-glycine (Sigma Chemical Company, Poole, Dorset, UK) and a collision energy of 92%. Positive ion operating conditions were ionisation voltage of 5.5 kV, capillary temperature 350 °C, sheath gas flow rate 65 arbitrary units, and auxiliary gas flow rate 10 arbitrary units. The scan range was from m/z 125 to m/z 1000 in both polarities. All analyses were repeated a minimum of three times.

CGA separation was achieved on a 150 × 3 mm column containing Luna 5 μm 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^{-1}. The gradient profile was 4% B to 33% B linearly in 90 min, a linear increase to 100% B at 95 min, followed by 5 min isocratic, and a return to 4% B at 105 min, and 5 min isocratic to re-equilibrate.

3. Results

Mass fragmentation data in negative and positive ion mode are summarised in Table 1. The deduced fragment structures are illustrated in Figs. 1 and 2. Selected mass spectra are presented in Figs. 4–7.

4. Discussion

4.1. General

The previously reported tryptophan (caffeoyl and *p*-coumaroyl) and tyrosine (caffeoyl) conjugates were located on the chromatogram obtained from an extract of an Angolan robusta coffee bean by a combination of their molecular ions and UV spectra. In zoom-scan mode the molecular masses predicted from the parent

Table 1
Positive and negative ion MS data for cinnamoyl–amino acid conjugates in extracts of green Robusta coffee beans

		Positive ion		Negative ion			
		Parent ion	MS^2 base peak	Parent ion	MS^2 base peak	MS^3 base peak	MS^4 base peak
Compound	*N*	m/z	m/z	m/z	m/z	m/z	m/z
Tryptophan conjugates							
p-Coumaroyl	3	351.3	147.6	349.8	229.9	186.0	142.9
Caffeoyl	3	367.3	163.7	365.7	229.4	186.2	142.5
Feruloyl	6	381.6	177.5	379.8	229.4	186.1	142.6
Tyrosine conjugates							
p-Coumaroyl	6	328.3	147.4	326.5	206.0	163.8	119.4
Caffeoyl	3	344.6	163.8	342.7	206.8	164.0	119.5
Feruloyl	6	358.8	177.9	356.9	206.8	n.d.	n.d.
Phenylalanine conjugates							
Caffeoyl	6	328.0	163.8	327.0	190.4	147.0	103.1

n.d. not detected.

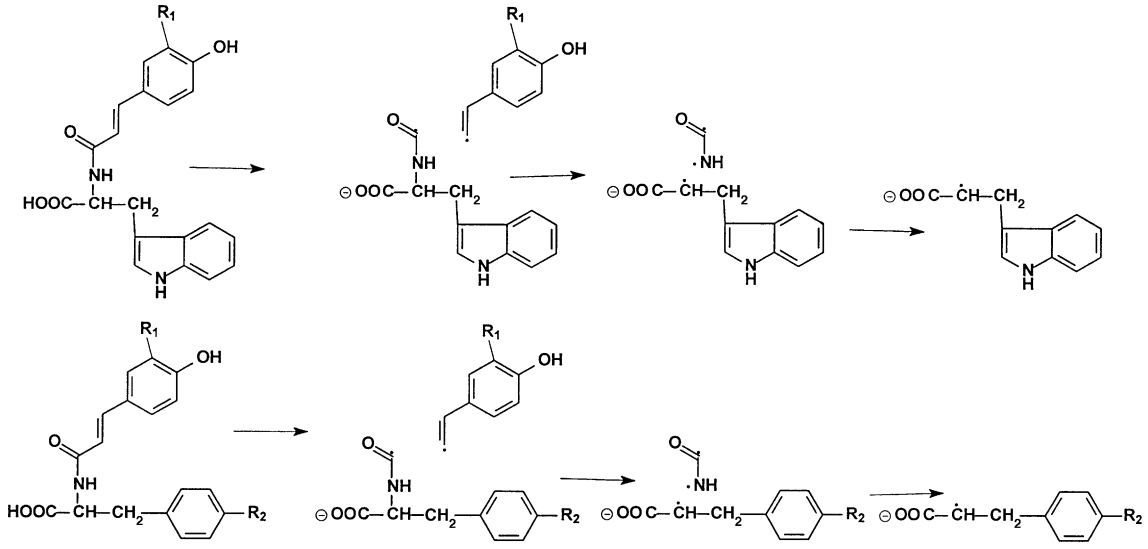

Fig. 1. Negative ion fragmentation of cinnamoyl amides. $R_1 = H = p$-Coumaroyl derivative: $R_1 = OH =$ Caffeoyl derivative: $R_1 = OCH_3 =$ Feruloyl derivative: $R_2 = OH =$ Tyrosine derivative: $R_2 = H =$ Phenylalanine derivative.

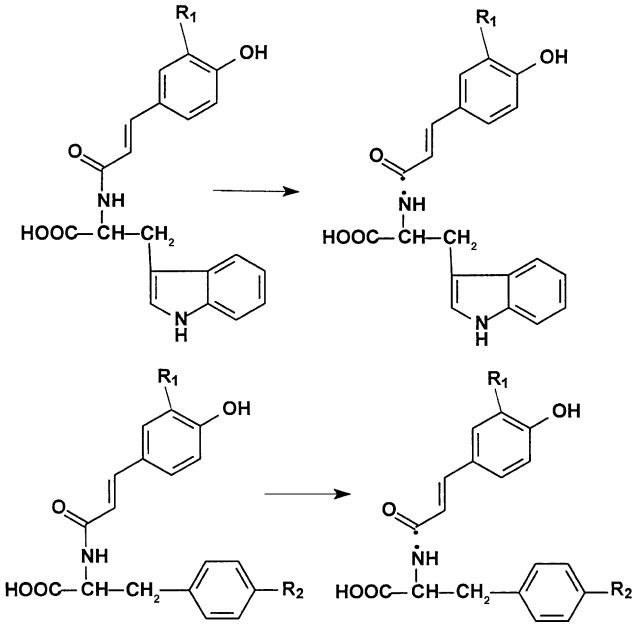

Fig. 2. Positive ion fragmentation of cinnamoyl amides. $R_1 = H =$ p-Coumaroyl derivative: $R_1 = OH =$ Caffeoyl derivative: $R_1 = OCH_3 =$ Feruloyl derivative: $R_2 = OH =$ Tyrosine derivative: $R_2 = H =$ Phenylalanine derivative.

ions were within 0.05 to 0.07 mass units of the theoretical accurate masses.

In negative ion mode, the MS^2 base peaks of these three compounds were characteristic of the amino acid at $\sim m/z$ 229.5 for tryptophan and $\sim m/z$ 206.5 for tyrosine, corresponding to the loss of a vinylphenol fragment characteristic of the cinnamic acid residue (i.e., ~136 m.u. for caffeic acid, and ~120 m.u. for p-coumaric, respectively). Subsequent fragmentation involves de-amidation, yielding MS^3 base peaks at $\sim m/z$ 186.0 and $\sim m/z$ 164.0, respectively, followed by a second decarboxylation yielding an MS^4 base peak at $\sim m/z$ 143.8 and $\sim m/z$ 119.5, respectively. In positive ion mode, the MS^2 base peaks are characteristic of the cinnamoyl residue at $\sim m/z$ 147.5 for p-coumaroyl and m/z 163.5 for caffeoyl.

Accordingly, the collected negative ion and positive ion spectra were searched using SIM for additional chromatographic peaks showing either the relevant fragment ions or the analogous fragments to be expected from conjugates involving other amino compounds that are known to occur as cinnamoyl conjugates in plants other than coffee, i.e., 5-hydroxytryptophan (m/z 245.5), dihydroxyphenylalanine (m/z 212.5), serotonin (m/z 201.5), phenylalanine (m/z 190.5), tyramine (m/z 162.5), aspartic acid (m/z 159.5), tryptamine (m/z 146.5), and aminobutyric acid (m/z 139.5). This strategy led to the detection of Compounds **I–IV**.

4.2. Putative tyrosine conjugates

Compounds **I** and **II** both showed (Figs. 3 and 4) negative ion MS^2 base peaks at $\sim m/z$ 206.8, and positive ion MS^2 base peaks at $\sim m/z$ 147.6 and $\sim m/z$ 177.6, suggesting that these are p-coumaroyl and feruloyl derivatives of tyrosine, respectively. Zoom scans indicated accurate masses of 327.10 and 357.10, that respectively are 0.01 or 0.02 mass units lower than the calculated accurate masses for p-coumaroyl-N-tyrosine and feruloyl-N-tyrosine. Due to the weak signal, it was not possible to obtain negative ion MS^3 and MS^4 data for feruloyl-N-tyrosine and, therefore, confirmatory positive ion data for this compound are presented in Fig. 5. The very broad λ_{max} in the range 280–310 nm for Compound **I** and a primary λ_{max} at

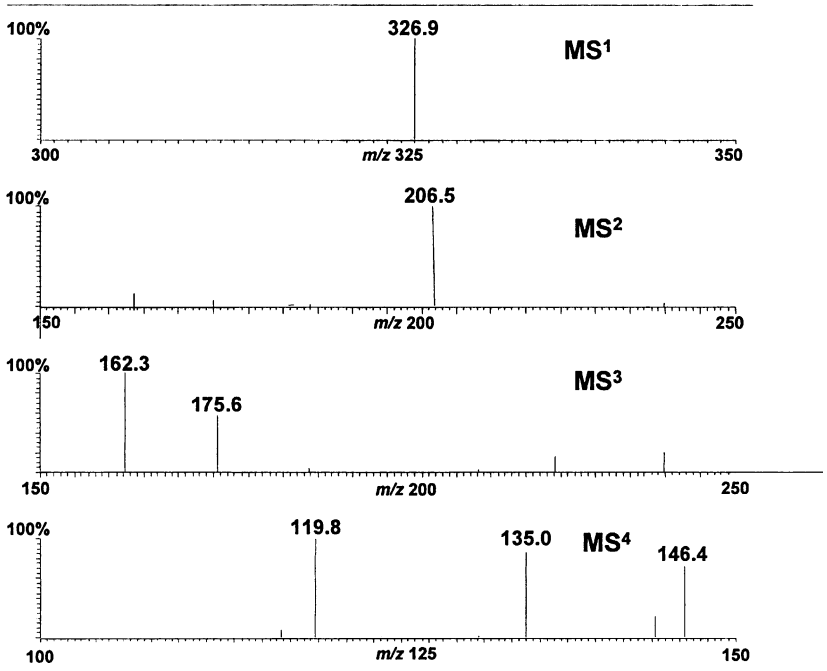

Fig. 3. Negative ion MS^4 spectra for *p*-coumaroyl-tyrosine.

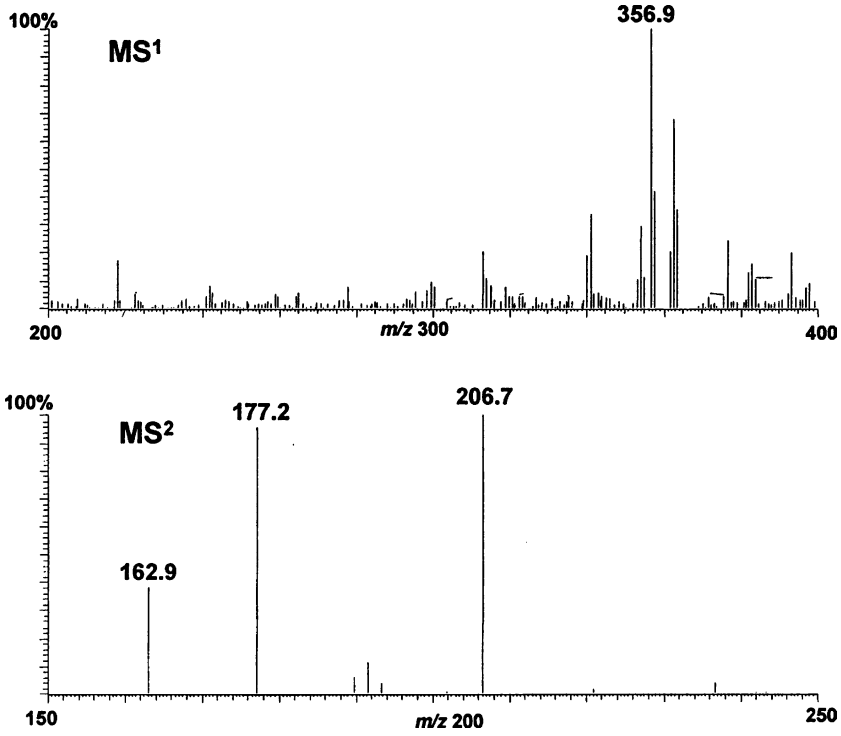

Fig. 4. Negative ion MS^2 spectra for feruloyl-tyrosine.

320 nm and a secondary λ_{max} at 290 nm (80%) for Compound **II** are consistent with these assignments. The relative retention times of 1.20 and 1.35 compared with caffeoyl-*N*-tyrosine are consistent with the known relative hydrophobicity of the corresponding quinic acid conjugates (Clifford, 2003; Clifford, Kellard, & Birch, 1989). *p*-Coumaroyl-*N*-tyrosine was present at a greater concentration in Angolan beans and is almost certainly the compound previously referred to as Angola II (Clifford & Jarvis, 1988; Correia et al., 1995).

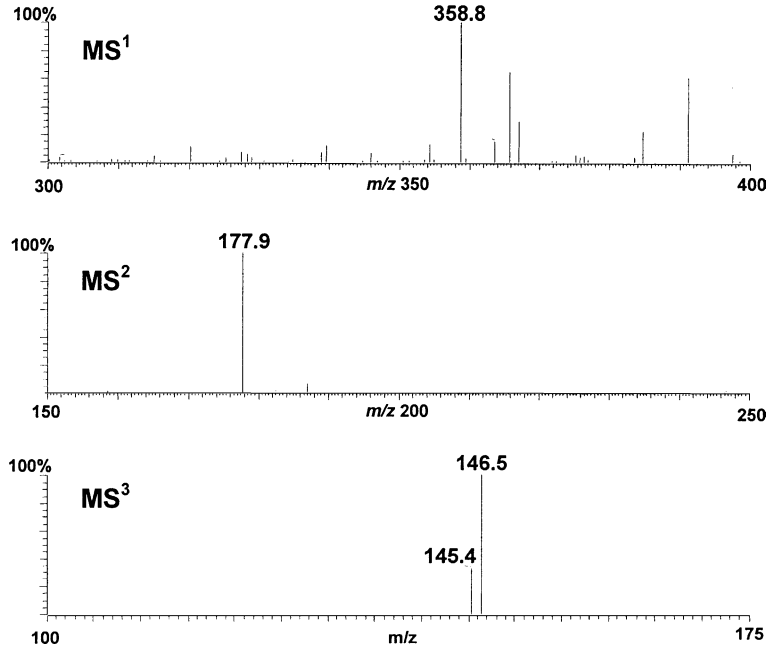

Fig. 5. Positive ion MS^3 spectra for feruloyl-tyrosine.

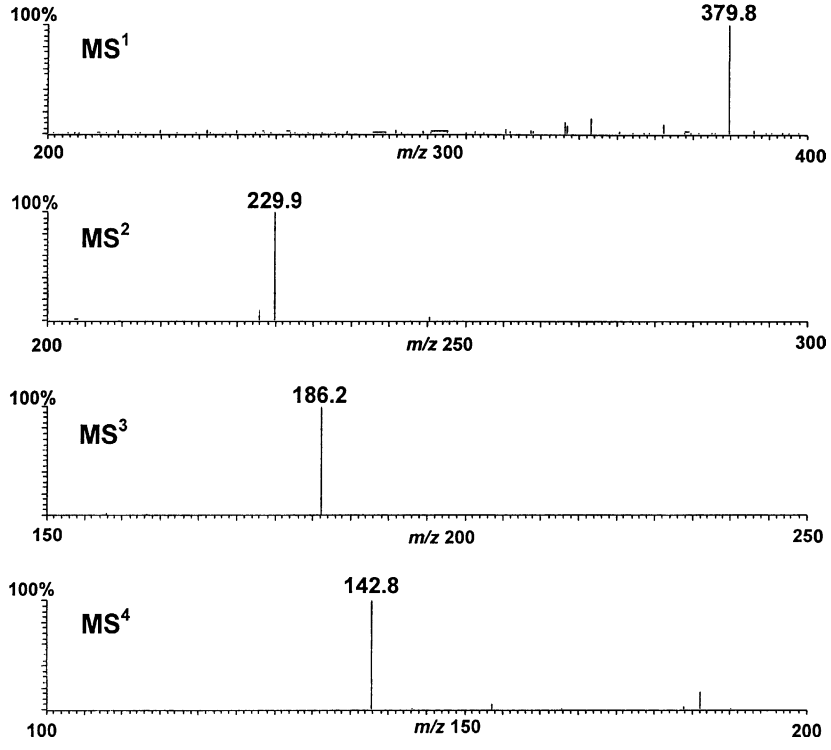

Fig. 6. Negative ion MS^4 spectra for feruloyl-tryptophan.

4.3. Putative tryptophan conjugates

Compound **III** showed (Fig. 6) a negative ion MS^2 base peak at $\sim m/z$ 229.4, and a positive ion MS^2 base peak at $\sim m/z$ 177.9, suggesting that this is a feruloyl derivative of tryptophan. A zoom scan suggested a molecular mass of 380.19 compared with a calculated accurate mass of 380.14. The UV spectrum with a primary λ_{max} at 320 nm and a secondary λ_{max} at 290 nm (95%) and RRT values of 1.61 compared with caffeoyl-*N*-tryptophan and 1.04 compared with *p*-coumaroyl-*N*-tryptophan are consistent with this assignment. So far as

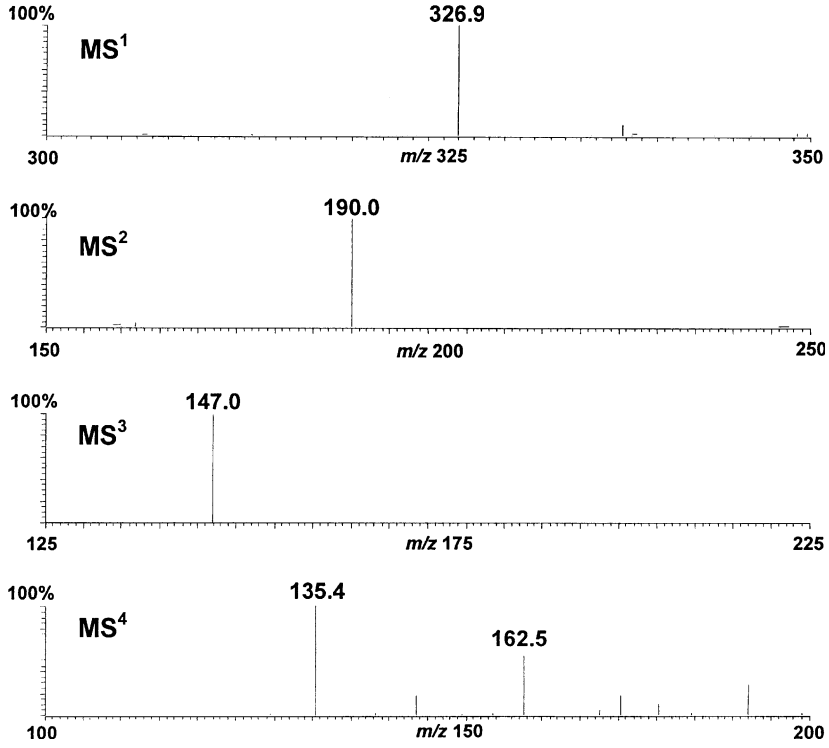

Fig. 7. Negative ion MS^4 spectra for caffeoyl-phenylalanine.

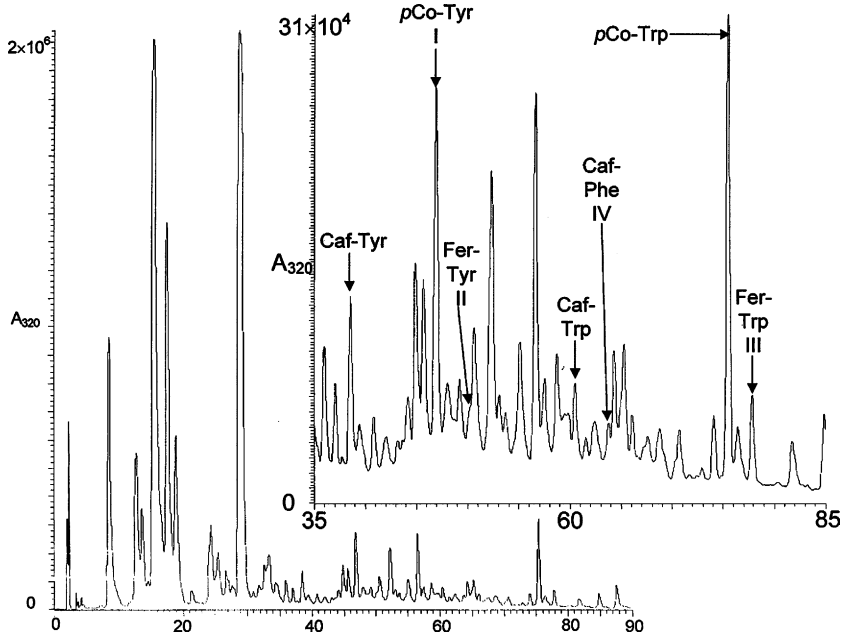

Fig. 8. Chromatogram at 320 nm of a methanolic extract of a green Tanzanian coffee bean. The insert shows the 35–85 min portion enlarged approximately ×6. Caf-Tyr = caffeoyl-tyrosine; *p*Co-Tyr = *p*-coumaroyl-tyrosine; Fer-Tyr = feruloyl-tyrosine; Caf-Trp = caffeoyl-tryptophan; *p*Co-Trp = *p*-coumaroyl-tryptophan; Fer-Trp = feruloyl-tryptophan; Caf-Phe = caffeoyl-phenylalanine.

we are aware, Compounds **I–III** have not previously been reported in nature.

4.4. Conjugates with other amino acids

Compound **IV** showed (Fig. 7) a negative ion MS^2 base peak at $\sim m/z$ 190.4, and a positive ion MS^2 base peak at $\sim m/z$ 163.8, suggesting that this is a caffeoyl derivative of an amino acid 16 m.u. smaller than tyrosine. Zoom scan suggested a molecular mass of 327.13 that is 0.02 m.u. larger than the calculated accurate mass for caffeoyl-*N*-phenylalanine. The retention time of 1.35 relative to caffeoyl-*N*-tyrosine is consistent with the more hydrophobic nature of the amino acid residue.

This compound has been reported in Lady Fern (*Athyrium filix-femina*) (Adam, 1995) but not in coffee so far as we are aware. *p*-Coumaroyl and feruloyl analogues were sought by SIM but were not found.

4.5. Content of cinnamoyl amides

The precise quantification of these cinnamoyl amides was not a primary objective of this study. Previous investigations have shown that commercial robusta coffees are very variable in their content of these substances, with those from Angola being significantly richer than those from other sources (Clifford & Jarvis, 1988). Re-examination of these previously published data, in the light of the structural characterisations reported here, indicates that, on a dry matter basis, unroasted Angolan robustas could contain 1.3–4.8 g/kg caffeoyl-*N*-tyrosine and 1.9–3.7 g/kg *p*-coumaroyl-*N*-tyrosine compared with 30.0–44.4 g/kg of the major chlorogenic acid (5-CQA) (Clifford & Jarvis, 1988; Correia et al., 1995). Robusta coffees of other geographic origins have been reported to contain 4.5–6.7 g/kg caffeoyl-*N*-tryptophan, up to 1.8 g/kg caffeoyl-*N*-tyrosine, and up to 1.2 g/kg *p*-coumaroyl-*N*-tryptophan compared with 35–49 g/kg 5-CQA (Balyaya & Clifford, 1995; Correia et al., 1995). Based on these previous studies, the contents of feruloyl-*N*-tyrosine, feruloyl-*N*-tryptophan and caffeoyl-*N*-phenylalanine do not exceed some 0.4 g/kg, even in Angolan robustas (Clifford & Jarvis, 1988). Assuming a similar molar absorption coefficient for 5-CQA and each of the cinnamoyl amides, then, based on Fig. 8, the current data are in general agreement with these previous publications, except that the content of caffeoyl-*N*-tryptophan is somewhat lower.

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