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Non-protein amino acids of *Bocoa* (Leguminosae; Papilionoideae)

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Abstract

The non-protein amino acids of the legume genus *Bocoa* (Papilionoideae; Swartzieae) were surveyed by LC–MS and GC–MS using extracts of herbarium leaf fragments. *Bocoa alterna* (Benth.) R.S. Cowan, *B. decipiens* R.S. Cowan, *B. limae* R.S. Cowan, *B. mollis* (Benth.) R.S. Cowan and *B. racemulosa* (Huber) R.S. Cowan were found to contain 2,4-methanoproline, 2,4-methanoglutamic acid, *cis*-1-amino-3-hydroxymethyl-cyclobutane-1-carboxylic acid and δ-*N*-acetylornithine. The former three compounds have otherwise only been reported from *Ateleia* and *Cyathostegia* and, therefore, the results support the relationship with these genera found in recent phylogenetic analysis of DNA sequence data. In contrast, *Bocoa viridiflora* (Ducke) R.S. Cowan was found to contain *trans*-5-hydroxypipecolic acid and *trans*-4-*cis*-5-dihydroxypipecolic acid, while *trans*-4-hydroxypipecolic acid and an unidentified compound were the major non-protein amino acids in *B. prouacensis* Aublet. The non-protein amino acid chemistry of these two species was therefore more similar to a representative of *Swartzia* examined, *S. macrosema* Harms, which also contained mono- and dihydroxypipecolic acids. The monotypic *Candolleodendron brachystachyum* (DC.) R.S. Cowan, considered related to *Bocoa*, accumulated *trans*-5-hydroxypipecolic acid. LC–MS data on flavonoids obtained from four of the extracts revealed the presence of flavone C-glycosides in *B. viridiflora* and *B. prouacensis* but only flavonoid *O*-glycosides in *B. alterna* and *B. mollis*. The chemical division of *Bocoa* concurs with studies of other character types and recent molecular phylogenies.

Keywords: *Bocoa*; *Candolleodendron*; *Swartzia*; Leguminosae; Swartzieae; Chemotaxonomy; Non-protein amino acids; Hydroxypipecolic acids

1. Introduction

Cryptic characters are becoming increasingly important in systematic botany to test relationships derived from phylogenetic analysis of DNA sequence data. One such cryptic character is the occurrence of non-protein amino acids, with the shared presence of uncommon compounds in taxa being thought to provide strong support of evolutionary affinities where there is other evidence to suggest a relationship (Bell, 1981).

In a recent phylogenetic analysis of the basal papilionoid legume tribe Swartzieae, using DNA sequences generated from the chloroplast *trn*L intron-spacer region (Ireland et al., 2000), four species of *Bocoa* were placed as sister to *Ateleia* and *Cyathostegia*. A fifth *Bocoa* species grouped with representatives of *Swartzia* in a sister clade. Although *Ateleia* and *Cyathostegia* were known to have close affinities, a potential relationship with *Bocoa* had not been suggested previously, as reflected by the classification of *Boccoa* in the *Swartzia* group and *Ateleia* and *Cyathostegia* in the *Ateleia* group of Swartzieae (Polhill, 1994); previously *Ateleia* and *Cyathostegia* were placed in a different tribe, Sophoreae (Polhill, 1981). From a chemical perspective, this relationship is interesting since unusual non-protein amino acids, containing the strained cyclobutane ring system, have been isolated from *Ateleia herbert-smithii* Pittier (Bell et al., 1980; Austin et al., 1987). The compounds concerned are 2,4-methanoproline (**1**), 2,4-methanoglutamic acid (**2**) and the alcohol of the latter, *cis*-1-amino-3-hydroxymethyl-cyclobutane-1-carboxylic acid (**3**) (Fig. 1). These non-protein amino acids have also been detected in *Cyathostegia* (Nash, 1986) but have not been found in any other plant. δ-*N*-Acetylornithine (**4**) has also been isolated from *Ateleia glazioviana* Baillon (Marona et al., 1993).

To test the relationship between *Bocoa* and *Ateleia*, we determined whether the rare '*Ateleia*-type' non-protein amino acids could be detected in any of the seven species of *Bocoa* recognised by Cowan (1974). The study used liquid chromatography–mass spectrometry (LC–MS)

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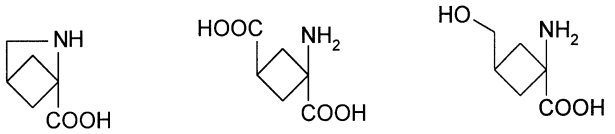

1 2 3

Fig. 1. Structures of '*Ateleia*-type' non-protein amino acids.

and gas chromatography–mass spectrometry (GC–MS) to analyse crudely purified extracts made from small leaf fragments of herbarium specimens. An example of *Swartzia* was also examined together with a specimen of the monotypic *Candolleodendron* since this genus is reminiscent of *Swartzia* and *Bocoa* (Cowan, 1966).

2. Results and discussion

2.1. LC–MS of non-protein amino acids in Ateleia

The non-protein amino acids reported from *Ateleia* were readily revealed by positive ion LC–MS analysis of a leaf extract of *A. herbert-smithii* using heptafluorobutyric acid as a volatile ion pair reagent to achieve chromatographic retention on a C18 polar end-capped column. Six major chromatographic peaks were observed in the base ion chromatogram (Fig. 2a), each due to a clear base ion being generated with four of these ions having the m/z values expected for the $[M+H]^+$ ions of the known compounds from *Ateleia*: 2,4-methanoproline, $[M+H]^+ = m/z$ 128 (**1**); 2,4-methanoglutamic acid, $[M+H]^+ = m/z$ 160 (**2**); *cis*-1-amino-3-hydroxymethyl-cyclobutane-1-carboxylic acid, $[M+H]^+ = m/z$ 146 (**3**); and δ-*N*-acetylornithine, $[M+H]^+ = m/z$ 175 (**4**). The other two major compounds generated $[M+H]^+$ ions at m/z 127 (**5**) and m/z 189 (**6**) and represent non-protein amino acids that have not previously been reported from the genus.

2.2. Non-protein amino acids of Bocoa

The LC–MS analyses of five of the *Bocoa* species, *B. alterna, B. decipiens, B. limae, B. mollis* and *B. racemulosa,* each showed chromatographic peaks having the same retention times and base ions as the four known non-protein amino acids, **1**–**4**, observed in the analysis of *A. herbert-smithii* (Fig. 2b). The CID spectra of these $[M+H]^+$ ions, obtained by MS/MS, were also the same as recorded from the *Ateleia* analysis, supporting their identification as these compounds. The relative peak areas produced by the $[M+H]^+$ ions in single ion chromatograms are given in Table 1. The unknown compound **5** was detected in all the above five species of *Bocoa* but **6** was absent. A compound (**7**) giving an $[M+H]^+$ ion at m/z 191 was, however, noted among the minor components.

None of the characteristic *Ateleia* non-protein amino acids could be detected in the analyses of *B. prouacensis* (Fig. 2c) or *B. viridiflora*. The LC–MS analysis of each of these species showed an early eluting chromatographic peak giving an $[M+H]^+$ ion at m/z 146, but the retention time of the peak was different for the two species and much less than obtained for **3** (which gives an $[M+H]^+$ ion at the same m/z value). The positive ion CID spectrum of the protonated compound in *B. viridiflora* (**9**) showed a good correlation with that reported for *trans*-5-hydroxypipeclic acid while the compound (**8**) in *B. prouacensis* showed a CID spectrum similar to *trans*-4 or *cis*-5-hydroxypipecolic acid (Kite, 1999). A smaller chromatographic peak giving a $[M+H]^+$ ion at m/z 162, the value expected for dihydroxypipecolic acids, was also present in both analyses. The retention times of these peaks (**13** and **14**) were similar in both analyses and, in positive ion MS–MS analysis, both showed the product ions expected for 4,5-dihydroxypipecolic acids. The relative intensities of product ions in the CID spectrum of **13** from *B. viridiflora* showed a good match with data reported for *trans*-4-*cis*-5-dihydroxypipecolic acid, the only 4,5-dihydroxypipecolic acid that can be assigned from its positive ion CID spectrum (Kite, 1999). A minor component (**10**) in *B. prouacensis* was assigned as *cis*-4-hydroxypipecolic acid from its CID spectrum.

To obtain confirmation of the identities of these hydroxy pipecolic acids, the extracts were reanalysed by negative ion LC–MS, using a normal phase column and no acidic ion pair reagent, and then by GC–MS following trimethylsilylation. The different negative ion CID spectra produced by 4- and 5-hydroxypipecolic acids (Kite, 1999) identified **8** as *trans*-4-hydroxypipecolic acid and confirmed the assignments of **9** and **10** given above. The negative ion CID spectrum also supported the identification **13** as *trans*-4-*cis*-5-dihydroxypipecolic acid since it showed a base ion at m/z 80. The correct GC retention indices and EI mass spectra of the trimethylsilyl ethers of these compounds were also obtained from GC–MS analyses (Kite and Hughes, 1997). However, in negative ion LC–MS/MS analysis, compound **14** in *B. prouacensis* gave a spectrum that was distinct from the published CID spectra of the four 4,5-dihydroxypipecolic acid epimers due to the presence of product ions at m/z 124 and 74: APCI–MS/MS (quadrupole ion-trap) precursor ion at m/z 160 $[M\text{-}H]^-$, m/z (rel. int.): 142 (100), 124 (10), 98 (8), 86 (25), 80, (4), 74 (21). The compound's trimethylsilyl derivative also gave an EI mass spectrum that differed from derivatised 4,5-dihydroxypipecolic acids, most noticeably by the presence of a fragment ion at m/z 144: EIMS (quadrupole ion-trap) 70 eV, m/z (rel. int.): 378 $[M+H]^+$ (7),

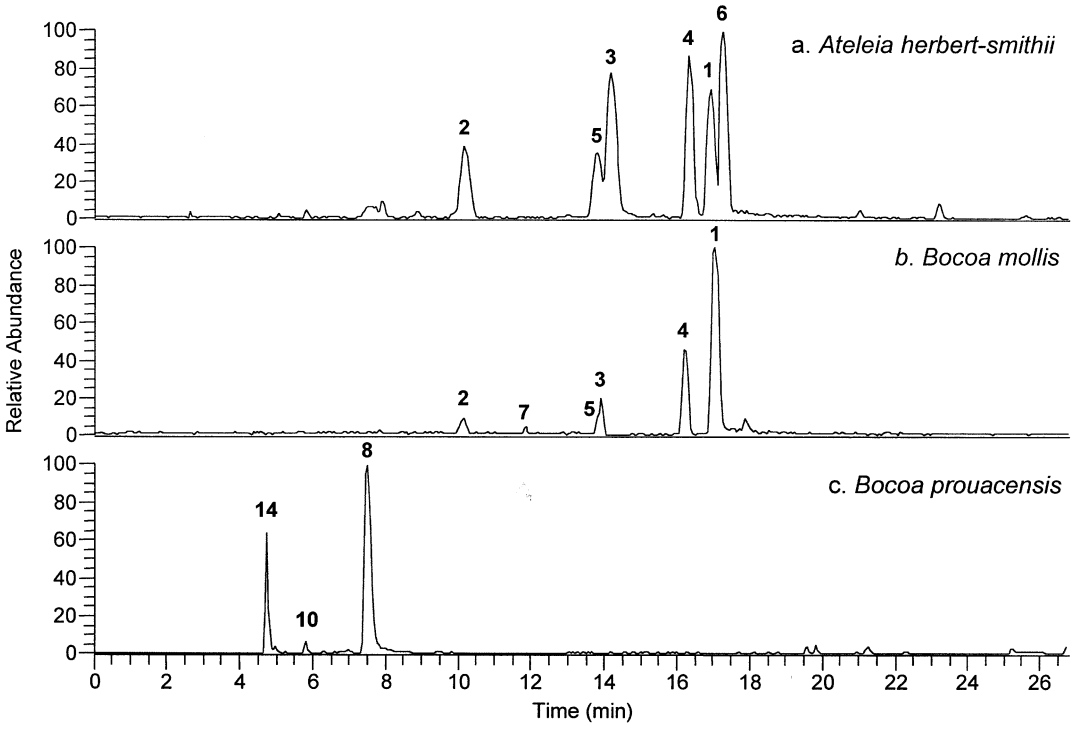

Fig. 2. Base ion chromatograms from the positive ion LC–MS analyses of (a) *Ateleia herbert-smithii* (b) *Bocoa mollis* and (c) *Bocoa prouacensis*. See Table 1 for peak identifications.

Table 1
Distribution of non-protein amino acids in species of *Bocoa* and related taxa. Figures refer to the peak area of the $[M+H]^+$ ion expressed as a percentage of the total peak area of all the $[M+H]^+$ ions monitored

Species	Compound No. $[M+H]^+$ (m/z)													
	1 128	**2** 160	**3** 146	**4** 175	**5** 127	**6** 189	**7** 191	**8** 146	**9** 146	**10** 146	**11** 146	**12** 162	**13** 162	**14** 162
Ateleia herbert-smithii	16	10	23	16	10	25	–	–	–	–	–	–	–	–
Bocoa alterna	52	6	16	22	2	–	3	–	–	–	–	–	–	–
Bocoa decipiens	44	3	24	23	2	–	4	–	–	–	–	–	–	–
Bocoa limae	58	8	30	2	2	–	–	–	–	–	–	–	–	–
Bocoa mollis (A)	42	12	17	20	3	–	6	–	–	–	–	–	–	–
Bocoa mollis (B)	57	6	11	23	2	–	1	–	–	–	–	–	–	–
Bocoa racemulosa	54	16	3	24	3	–	–	–	–	–	–	–	–	–
Bocoa prouacensis	–	–	–	–	–	–	–	65	–	6	–	–	–	29
Bocoa viridiflora	–	–	–	–	–	–	–	–	91	–	–	–	9	–
Candolleodendron brachystachyum	–	–	–	–	–	–	–	–	100	–	–	–	–	–
Swartzia macrosema	–	–	–	–	–	–	–	4	24	4	47	6	14	–

1 = 2,4-Methanoproline. **2** = 2,4-Methanoglutamic acid. **3** = *cis*-1-Amino-3-hydroxymethyl-cyclobutane-1-carboxylic acid. **4** = δ-*N*-Acetylornithine. **5** = Unknown. **6** = Unknown. **7** = Unknown. **8** = *trans*-4-Hydroxypipecolic acid. **9** = *trans*-5-Hydroxypipecolic acid. **10** = *cis*-4-Hydroxypipecolic acid. **11** = *cis*-5-Hydroxypipecolic acid. **12** = *trans*-4-*trans*-5-Dihydroxypipecolic acid. **13** = *trans*-4-*cis*-5-Dihydroxypipecolic acid. **14** = Unknown.

362 (2), 287 (6), 260 (53), 198 (5), 170 (15), 144 (21), 128 (15), 73 (100). The identity of **14**, therefore, remains unknown and sufficient material was not available to isolate it.

2.3. Non-protein amino acids of Candolleodendron

LC–MS and GC–MS analyses of the non-protein amino acid extract from *Candolleodendron brachystachyum* revealed that the species accumulated just one major compound which was identified as *trans*-5-hydroxypipecolic acid from its various mass spectral and chromatographic data, as described above. Similarly, *Swartzia macrosema* was found to contain a mixture of mono- and dihydroxypipecolic acids as listed in Table 1.

2.4. Flavonoids of Bocoa

Preliminary LC–MS data on flavonoids were only obtained for four of the specimens of *Bocoa* analysed.

Positive ion LC–MS analysis of the extracts of *B. alterna* and *B. mollis* revealed that each contained a single major flavonoid giving the same retention time and $[M+H]^+$ ion at *m*/*z* 433 in both species. It was assigned to a kaempferol-*O*-dirhamnoside by interpretation of the various CID spectra, as described by Grayer et al. (2000). Minor flavonoids detected in these species were also *O*-glycosides. Analysis of the extract of *B. viridiflora* revealed three major flavonoids, one giving an $[M+H]^+$ ion at *m*/*z* 595 and the other two both giving $[M+H]^+$ ions at *m*/*z* 579. The CID spectrum of the ion at *m*/*z* 595 showed the typical characteristics of a flavone C-glycoside (Grayer et al., 2000) and was assigned as vicenin-2 from its up-front CID spectrum. The other two flavonoids gave a loss of 146 amu from the protonated molecule in MS/MS to yield product ions at *m*/*z* 433, which showed typical C-glycoside losses in MS/MS/MS analyses. The up-front high energy CID of these compounds matched vitexin and isovitexin and thus they were assigned to vitextin and isovitexin *O*-rhamnosides. Analysis of the extract of *B. prouacensis* revealed the same three major flavonoids as in *B. viridiflora* and two less abundant compounds that were assigned as the flavone C-glycosides orientin and isoorientin from their CID spectra. A final prominent flavonoid peak in this extract gave an $[M+H]^+$ ion at *m*/*z* 741 and was assigned as a kaempferol *O*-triglycoside involving two rhamnosyl and one hexosyl units.

2.5. Taxonomic significance

The relationship between *Bocoa alterna*, *B. decipiens*, *B. mollis* and *B. racemulosa* with *Ateleia* and *Cyathostegia* found in the cladistic analysis of *trn*L sequence data (Ireland et al., 2000; Ireland, 2001) is supported by the presence of the rare *Ateleia*-type non-protein amino acids, **1**–**3**, in these species. In addition, *B. limae* (not included in the DNA sequence analysis) appears to be a member of this group of *Bocoa* species on the basis of its non-protein amino acid chemistry.

Ireland et al. (2000) also found that *B. viridiflora* emerged with *Swartzia* species in their analysis. This is also supported by the non-protein amino acid chemistry since *Ateleia*-type compounds could not be detected in this species which instead was found to accumulate hydroxypipecolic acids, like the representative of *Swartzia* examined. Ireland (2001) was unable to obtain suitable DNA from *B. prouacensis* for inclusion in the molecular analysis, but the accumulation of hydroxypipecolic acids and lack of *Ateleia*-type non-protein amino acids found in the present study suggests a closer affinity to *B. viridiflora* than to the other species of *Bocoa*, although the particular hydroxypipecolic acids synthesised by *B. viridiflora* and *B. prouacensis* were different. *Candolleodendron* also shows a chemical affinity with *B. viridiflora* and *B. prouacensis,* from its accumulation of a hydroxypipecolic acid.

The chemical division of *Bocoa* was also indicated from the preliminary flavonoid data obtained on four species, with both *B. prouacensis* and *B. viridiflora* containing mainly flavone C-glycosides whilst flavone C-glycosides could not be detected in *B. alterna* or *B. mollis*. Previous studies of other character types have also noted a division within the genus. Gasson (1996) found that the wood anatomy of *B. prouacensis* and *B. viridiflora* was more similar to that of *Swartzia* than *B. alterna*. Herendeen (1995) also recognised the same division in *Bocoa* and separated it into two groups for his cladistic analysis of morphology. One group included *B. prouacensis* and *B. viridiflora*, with opposite leaflets, and the other group contained *B. alterna*, *B. decipiens*, *B. limae* and *B. mollis*, with alternate leaflets. Ferguson and Schrire (1994), however, found a slightly different divide in *Bocoa* from pollen characters, with the pollen of *B. prouacensis* being more similar to that of *B. alterna* and *B. mollis* than *B. viridiflora*.

Nevertheless, given the rarity of the *Ateleia*-type non-protein amino acids, it is the chemical data that provide the most compelling 'non-molecular' character evidence for the division of *Bocoa,* with *B. alterna, B. decipiens, B. limae, B. mollis* and *B. racemulosa* forming a clear chemical group that shows a closer relationship to *Ateleia* and *Cyathostegia* than to *B. viridiflora* or *B. prouacensis*.

3. Experimental

3.1. Plant material

The herbarium specimens examined, with voucher details and weight of leaf material analysed, were as follows: *B. alterna* (Benth.) R.S. Cowan, *Jansen-Lacobs 4533* (K), 16 mg; *B. decipiens* R.S. Cowan, *Lobo et al. 334* (K), 18 mg; *B. limae* R.S. Cowan, *B. dos Santos 125* (K), 23 mg; *B. mollis* (Benth.) R.S. Cowan, *Andrade, Laurenio, Rodal & Figueiredo 20* (K), 25 mg (A) and *Brito & Pennington 318* (K), 29 mg (B); *B. prouacensis* Aublet, *Ijzerhart 69* (K), 50 mg; *B. racemulosa* (Huber) R.S. Cowan, *Ferreira et al 6990* (K), 26 mg; *B. viridiflora* (Ducke) R.S. Cowan, *Cid Ferreira 9602* (K), 31 mg; *Candolleodendron brachystachyum* (DC.) R.S. Cowan, *de Granville 6506* (K), 12 mg and *Swartzia macrosema* Harms, *T.D. Pennington & G. Tenorio 10754* (K), 15 mg. Leaf material of *Ateleia herbert-smithii* Pittier was obtained from a plant growing at the Royal Botanic Gardens, Kew (accession number 1997-6547), and freeze dried.

3.2. Preparation of extracts

Each leaf sample was ground with sand in 1.5 ml of 70% aqueous methanol using a pestle and mortar and left in the solvent overnight at room temperature. Following removal of the residue by centrifugation, 50 mg

of 100–200 mesh Dowex 50(H^+) ion exchange resin (Merck) was added and gently mixed. The liquid was removed and allowed to evaporate to dryness in a vial for later analysis for flavonoids. The resin, holding the amino acids, was washed repeatedly with water and then the amino acids were eluted by mixing with 0.5 ml of 2 M aqueous ammonia. The eluate was freeze dried and taken up in water such that the compounds extracted from 1 mg of dry leaf were present in 100 μl. The flavonoid samples were taken up in 70% methanol at the same concentration. The samples were filtered before analysis.

3.3. LC-MS of non-protein amino acids

The LC–MS system consisted of a high performance liquid chromatograph (Waters 600E) interfaced to a quadrupole ion-trap mass spectrometer (ThermoFinnigan LCQ Classic) via an atmospheric pressure ionisation (APCI) source (ThermoFinnigan). Separation of the non-protein amino acids on a reverse phase polar end-capped C18 column was achieved without their derivatisation using heptafluorobutyric acid (HFBA) as a volatile ion-pair reagent (Roberts and Hughes, 1998). The column used was a 250 mm x 4.6 mm (i.d.), 5 μm Aqua-C18 phase (Phenomenex) and the mobile phase consisted of 0.1% aqueous HFBA pH 2.2 and methanol mixed to produce a 1 ml/min flow containing a linear gradient of methanol as follows: 0 min 0%, 5 min 0%, 25 min 50%, 30 min 50%, 35 min 0%. The column was equilibrated for 10 min in the start conditions (i.e. 0.1% HFBA) before a sample was injected. The injection volume was 10 μl. The APCI source was operated in positive mode using the following conditions: vaporiser tube temperature, 450 °C; sheath and auxiliary nitrogen pressures, 80 and 20 psi; needle current, 5 μA; heated capillary temperature, 150 °C. The MS monitored ions in the range m/z 70–500 with alternating data-dependent MS/MS scans of the three or four most intense ions using an ion isolation width of 3 amu and collision energy of 35%. To obtain negative ion CID spectra of hydroxypipecolic acids detected, samples were reanalysed using a 150 mm x 4.6 mm (i.d.), 7 μm Adsorbosphere XL Carbohydrate XL normal phase column (Alltech Associates Inc.) and eluting with a linear gradient of 1.5 ml/min 90–70% aqueous acetonitrile over 30 min as described by Kite (1999). Except for the negative polarity, the APCI source conditions were as above. For MS/MS the ion isolation width was 3 amu and the collision energy was 16%.

3.4. GC–MS of trimethylsilyl derivatives

The sample remaining after LC–MS was freeze-dried and the compounds were trimethylsilylated by adding 30 μl of Sigma-Sil A reagent (1:3:9, trimethylchlorosilane/hexamethyldisilazane/pyridine; Sigma Chem. Co.) and heating at 70 °C for 20 min. One microlitre of this was analysed directly using a gas chromatograph (Perkin-Elmer Model 8500) interfaced to a quadrupole ion-trap mass spectrometer (ThermoFinnigan ITD 800). Chromatographic separation was achieved on a 25 m×0.22 mm (i.d.)×0.25 μm BPX5 capillary column (SGE Ltd.) using an oven temperature programme of 120–300 °C at 4 °C/min. Helium was used as the carrier gas at a head pressure of 20 psi. The MS recorded 70 eV EIMS in the range m/z 50–650 at a rate of 1 scan/s. Retention indices of compounds eluting from the column were calculated against a series of even carbon number *n*-alkanes (Supelco UK).

3.5. LC–MS of flavonoids

The flavonoid samples were analysed using the same LC–MS system and positive mode APCI source conditions as described above except the vaporiser temperature was raised to 550 °C. Separation was achieved on a 150 mm×4.6 mm (i.d.), 5 μm Luna C18 column (Phenomenex) using a 1 ml/min mobile phase gradient of 25–100% aqueous methanol (containing 1% acetic acid) over 25 min. The mass spectrometer was programmed to record the CID spectra of protonated flavonoid glycosides (by MS/MS), the CID spectra of product ions corresponding to the aglycone moieties of *O*-glycosides (by MS/MS/MS) and high energy CID spectra (by 'upfront' CID in the octapole lenses) as described by Grayer et al. (2000).

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