PosterREPRINT

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HIGH THROUGHPUT CHARACTERISATION OF *C*-GLYCOSIDIC FLAVONOIDS FROM BRAZILIAN *Passiflora* SPECIES USING LC-MS EXACT MASS MEASUREMENT AND IN-SOURCE CID

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INTRODUCTION

The pharmaceutical industry has utilised the abundant supply of natural products as a resource for drug discovery. Historically the process for natural product drug discovery has proved to be a time consuming process. As a means of illustrating how the natural product drug discovery cycle time can be reduced data, will be presented where serial and parallel LC-MS analysis has be performed using an oa-TOF. The area of interest is the *Passiflora* (Passifloraceae) species, which are utilised as phytomedicines (sedative / tranquillising). *Passiflora incarnata* L. (Passifloraceae) is widely known in Europe due to its sedative and tranquillising properties, but it does not grow well in the Brazilian climate. As an alternative to *Passiflora incarnata* Brazilian Pharmacopoeia indicates the species *Passiflora alata*; this was also found to be frequently substituted by *Passiflora edulis* (utilised in juices). Additionally, *P. caerulea* should also be studied due to its utilisation in Argentina and possibly also in South Brazil.

Medicinal *Passiflora* species contain flavonoids, mainly *C*-glycosylflavones (apigenin and luteolin derivatives, frequently occurring as isomers). LC-MS techniques such as CID (collision-induced dissociation) combined with exact mass measurement are important tools for unequivocal identification of flavonoid isomers in complex mixtures such as phytomedicines. In this study oa-TOF LC-MS full spectra acquisition has been performed, therefore it has been possible to distinguish and correctly assign the flavonoids of interest from degradation products due to the presence of other flavonoids, which also have the same luteolin-type or apigenin-type of skeletal structure. Also in-source CID has been carried out, to produce informative fragmentation, allowing for pseudo MS-MS data to be acquired. The experiments were performed using the dual electrospray LockSpray™ ion source and 5-Way MUX™ ion source. Oa-TOF is used as a tool for easy acquisition of exact mass (<5ppm) data; the system used for parallel exact mass LC-MS is illustrated in **Figure 1**. A schematic of an Oa-TOF is illustrated in **Figure 2** and a 5-Way MUX™ source is represented in **Figure 3** The aim of this work is unequivocal identification of flavonoid isomers in complex mixtures of *Passiflora* extracts.

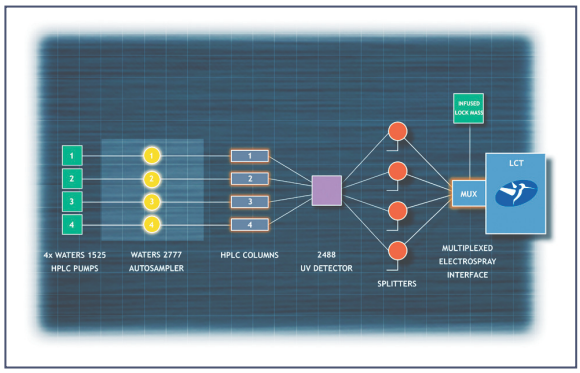

Figure 1. Oa-TOF parallel LC-MS analysis system schematic

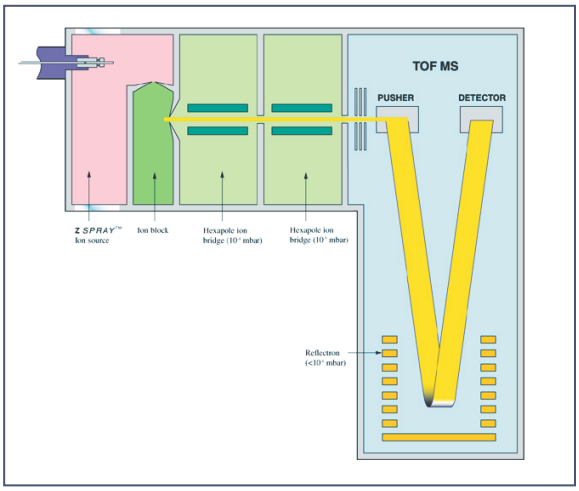

Figure 2. Oa-TOF schematic.

MICROMASS MS TECHNOLOGIES

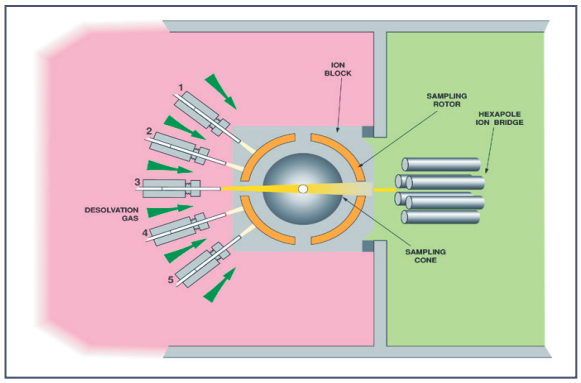

Figure 3. 5-WAY MUX™ schematic

EXPERIMENTAL

STANDARDS	Vitexin, orientin, isoorientin and rutin (obtained from Roth) methanolic solutions (0.25 ng/mL)
***Passiflora* SAMPLES**	**Vegetal material**: dried leaves of *Passiflora alata* and *P. edulis* (grown in Ribeirão Preto, SP, Brazil); *P. incarnata*, (supplied by CPQBA - Campinas - SP, Brazil) and *P. caerulea* (supplied by IAC - Campinas - SP, Brazil) **Sample preparation**: Extraction with ethanol-water (2:1 v/v, 1 g plant/10 mL solvent according to Brazilian Pharmacopoeia procedure) SPE clean-up (Sep-Pak RP-18, elution with 60% methanol - H_2O) and LC-MS analysis of hydromethanolic fraction (4 mg/mL)

	LC-MS CONDITIONS	
LC	**Column**	Symmetry RP-18 (250 mm x 4.6 mm x 5 mm) with guard column (2 cm x 3.9 mm x 5 mm) - Waters
	Column temperature	35° C
	Mobile phase	ACN (B) : H_2O (0.2% HCOOH) (A) Gradient: 0-10 min: 15% B; 10-40 min: 15-30% B; 40-50 min: 30-15% B
	Flow	1 mL/min - split 1:4 serial LC-MS 1 mL/min - split 1:10 parallel LC-MS
MS	**Instrument**	LC-TOF-MS, (Micromass) LCT equipped with LockSpray dual-electrospray ion source and 5-Way MUX™
	Mode	ESI: positive and negative
	ESI Voltage	+ve =3 kV -ve = 2.8kV
	Sample cone voltage	20 to 95 V
	Exact mass reference	Leucine enkephalin, $[M+H]^+$ =556.2771 $[M-H]^-$ = 554.2615
	M/Z range	100 to 800 Da

RESULTS

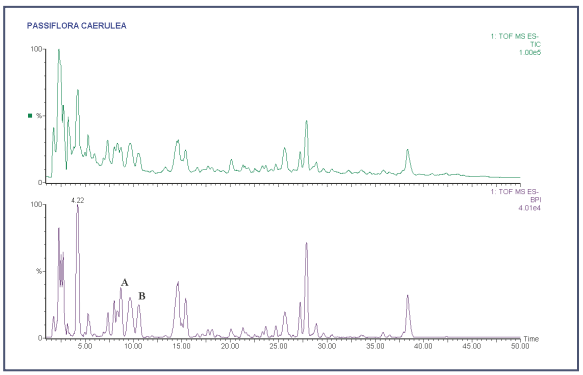

Figure 4. Negative ion mode Passiflora caerulea extract BPI and TIC, Peak A= isoorientin and Peak B= orientin.

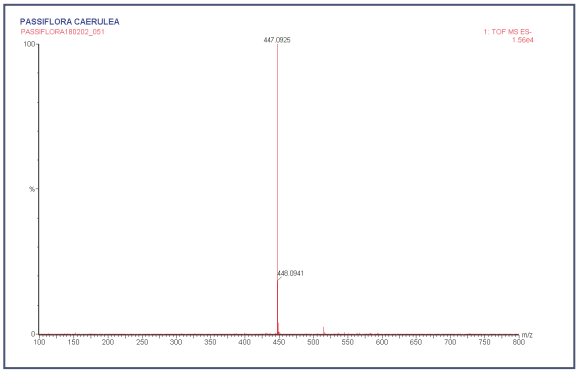

Figure 5. Negative ion mode Passiflora caerulea extract exact mass spectrum for isoorientin.

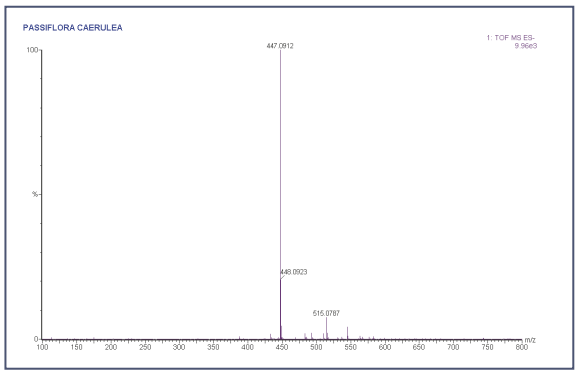

Figure 6. Negative ion mode Passiflora caerulea extract exact mass spectrum for orientin.

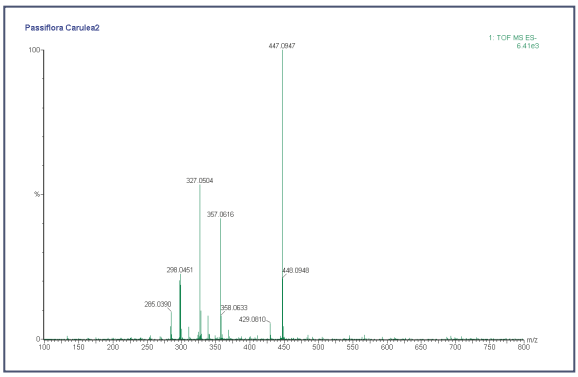

Figure 7. Negative ion mode exact mass in-source CID fragment ion mass spectrum for isoorientin extracted from Passiflora caerulea.

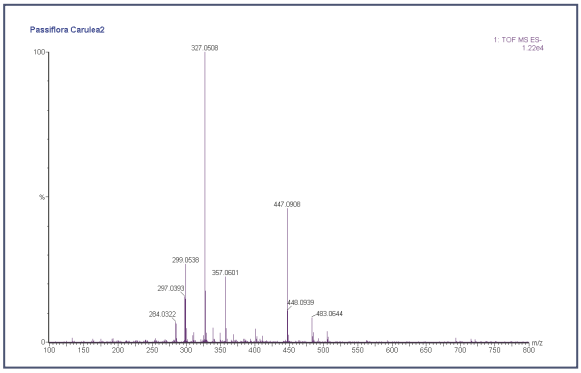

Figure 8. Negative ion mode exact mass in-source CID fragment ion mass spectrum for orientin extracted from Passiflora caerulea.

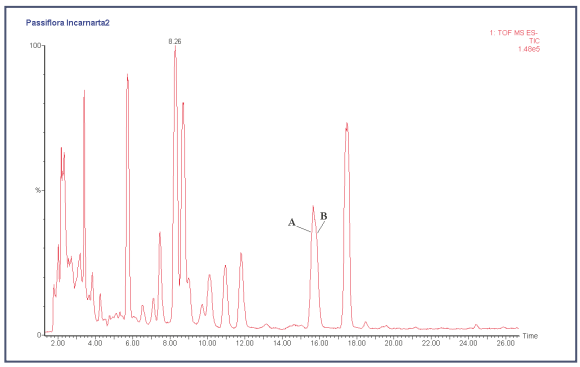

Figure 9. Negative ion mode expanded TIC (95V) for Passilfora incarnata; A) vitexin and B) isovitexin.

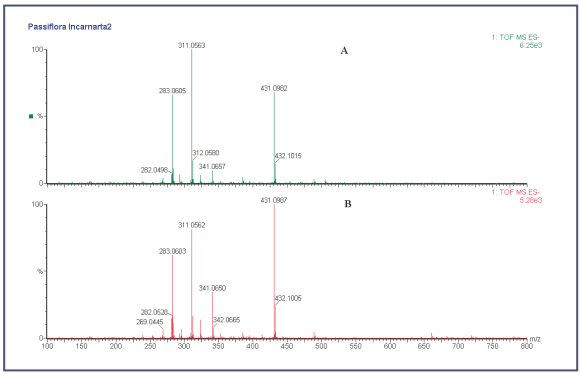

Figure 10. Negative ion mode exact mass in-source CID mass spectrum for A) vitexin and B) isovitexin extracted from Passiflora incarnata.

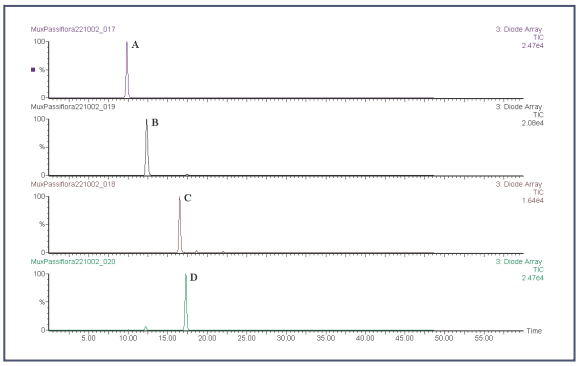

Figure 11. Parallel flavonoid LC-UV chromatograms λ= 337 nm A) isoorientin, B) orientin, C) vitexin and D) rutin.

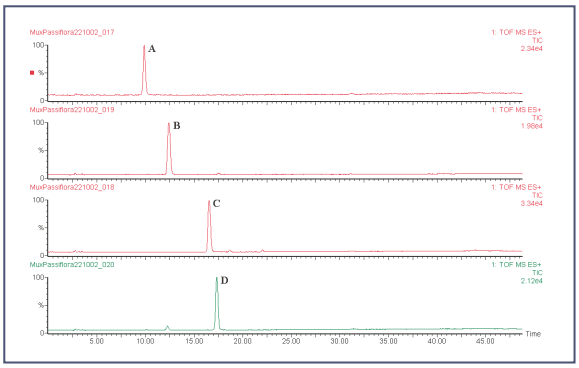

Figure 12. Parallel LC-MS positive ion mode TIC for flavonoid standards A) isoorientin, B) orientin, C) vitexin and D) rutin.

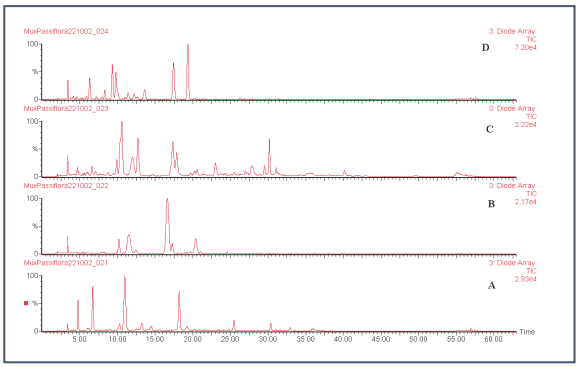

Figure 13. Parallel Passiflora extract LC-UV chromatograms λ =337 nm A) P. edulis, B) P. alata, C) P. caerulea and D) P. incarnata.

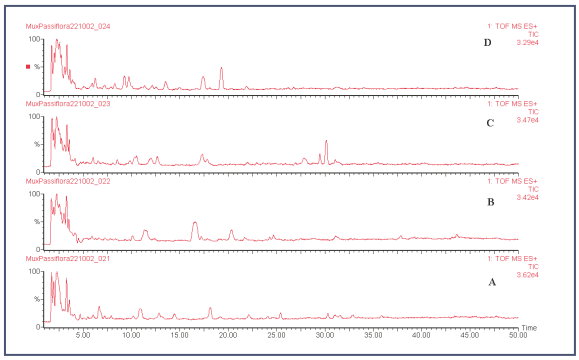

Figure 14. Parallel positive ion mode Passiflora extract MUX TIC's A) P. edulis, B) P. alata, C) P. caerulea and D) P. incarnata.

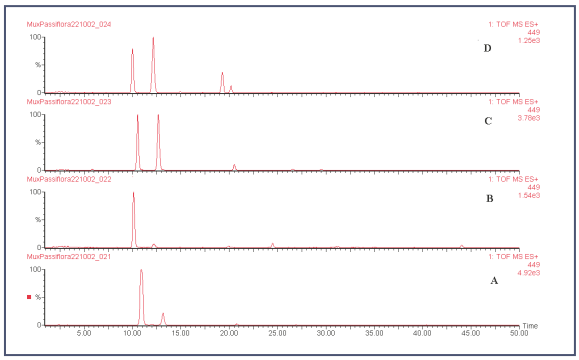

Figure 15. Parallel positive ion mode m/z 449 extracted mass chromatograms for A) P. edulis, B) P. alata, C) P. caerulea and D) P. incarnata.

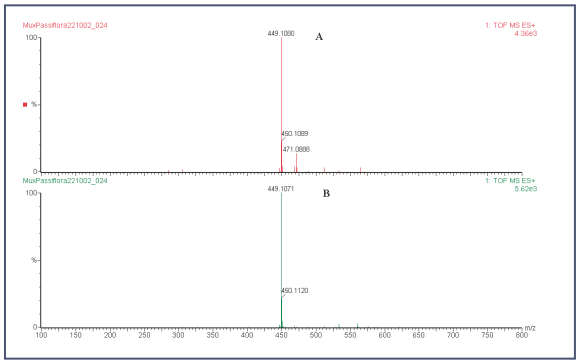

Figure 16. Exact mass spectra obtained using positive mode parallel analysis for A) orientin and B) isoorientin from the Passiflora incarnata extract.

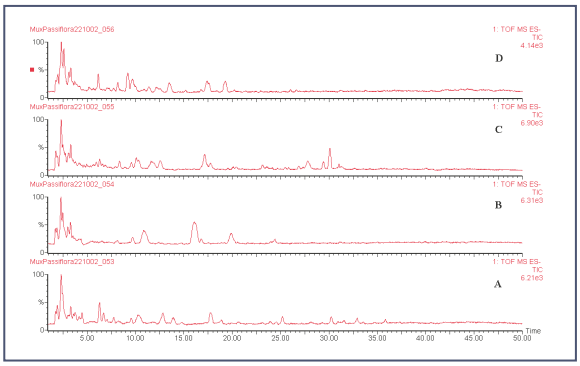

Figure 17. Parallel negative ion mode Passiflora extract MUX TIC's A) P. edulis, B) P. alata, C) P. caerulea and D) P. incarnata

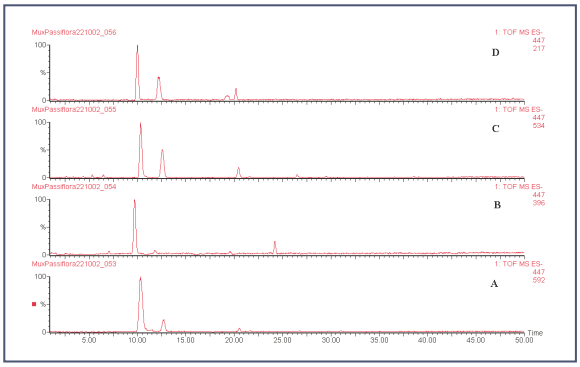

Figure 18. Parallel negative ion mode m/z 447 extracted mass chromatograms for A) P. edulis, B) P. alata, C) P. caerulea and D) P. incarnata

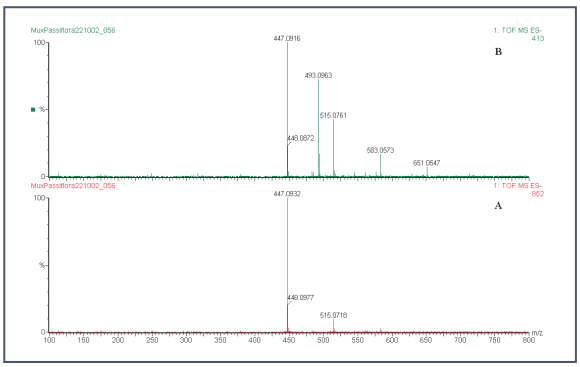

Figure 19. Exact mass spectra obtained using negative mode parallel analysis for A) orientin and B) isoorientin present within the Passiflora incarnata extract.

	Orientin	Isoorientin	Vitexin	Isovitexin
P.alata	+	+	absent	+
P.edulis	+	+	+	+
P.caerulea	+	+	+	+
P.incarnata	+	+	+	+

+ = Present

Table 1. Flavonoids detected by positive mode LC-CID-MS-MS on Passiflora extracts.

Table 2.Exact mass errors for flavonoids using positive and negative mode parallel real time LC-MS exact mass measurement.

Figure 4 illustrates the negative mode total ion chromatogram and BPI chromatogram acquired for the analysis of *Passiflora caerulea* extract, where the presence of isoorientin and orientin is illustrated. The exact mass spectrum for peak A (isoorientin) and peak B (orientin) are illustrated respectively in **Figure 5 and 6. Figure 7 and 8** respectively contain the negative mode in-source CID fragmentation mass spectra for isoorientin and orientin obtained from the extract of *Passiflora caerulea*. In **Figure 9** the *Passilfora incarnata* negative mode total ion chromatogram (95V) is presented, with the illustration of co-eluting vitexin (A) and isovitexin (B). The negative mode pseudo ms-ms mass spectra of peaks vitexin and isovitexin identified to be present in *Passiflora caerulea* (confirmation from positive ion mode in-source CID) can be viewed in **Figure 10**. Parallel LC-MS accurate mass measurement has been utilised to determine the applicability for rapid characterisation of natural products. Within **Figure 11** the LC-UV chromatograms obtained at λ=337nm can be seen. The positive mode TIC's obtained for the four standards, orientin, isoorientin, isovitexin and rutin injected in parallel are illustrated in **Figure 12**. Presented in **Figure 13** are the four λ=337nm LC-UV chromatograms acquired in parallel for the extracts of *Passiflora edulis, alata, caerulea* and *incarnata*. Shown in **Figure 14** are the four corresponding positive mode electrospray MUX TIC's. The four m/z 449 extracted mass chromatograms obtained for *Passiflora edulis, alata, caerulea* and *incarnata* can be viewed in **Figure 15**.

The positive ion mode exact mass spectra obtained for orientin and isoorientin present within the *Passiflora incarnata* extract are shown in **Figure 16**. Shown in **Figure 17** are the four corresponding negative mode electrospray MUX TIC's. The four m/z 447 extracted mass chromatograms obtained for *Passiflora edulis*, *alata*, *caerulea* and *incarnata* can be viewed in **Figure 18**. The accurate mass spectra obtained for orientin and isoorientin present within the *Passiflora incarnata* extract are shown in **Figure 19**.

The mass accuracy obtained for orientin, isoorientin, isovitexin and vitexin using parallel LC-MS real time exact mass measurement analysis in positive and negative ion mode is summarised in **Table 2**. Due to the low level of orientin present within *Passiflora alata*, ion statistics allowed for mass measurement within 10ppm, all other target flavonoids were real time mass measured within 5ppm using parallel LC-MS analysis. The general identification of these flavonoids on *Passiflora* extracts is summarised on **Table 1**. Using both positive and negative mode serial LC-MS analysis, accurate mass measurement of orientin, isoorientin, isovitexin and vitexin been achieved within 5ppm.

Using exact mass measurement with negative ion mode electrospray, orientin and isoorientin have been shown to be present in all the *Passiflora* extracts studied. Although partial chromatographic separation is achieved for isovitexin and vitexin, isovitexin eluting later than vitexin, from the exact mass measurements taken it can only be concluded that isovitexin has been shown to be present in the *Passiflora* extracts studied. Unlike positive ion mode the in-source CID using negative ion in-source CID fragmentation did not differentiate vitexin and isovitexin. However using negative ion mode it is possible to utilise negative ion mode electrospray with in source CID, under the conditions described as a "route" to the identification of the flavonoids orientin and isoorientin. Rutin was not determined to be present within the *Passiflora* extracts studied. The fragmentation routes have been presented Waters poster WP207.

DISCUSSION

Using oa-TOF LC-MS accurate mass measurement allowed for highly specific data to be acquired, where full spectra acquisition and accurate mass measurement for more than eighty major and minor components found in the some extracts of *Passiflora*. The increased selectivity of accurate mass measurement and accurate mass CID allowed for full confidence in flavonoid isomer assignment. Using the elemental composition calculator the most probable elemental formula was derived from the accurate mass spectrum, this further confirmed the identity of the flavonoid isomers of interest. The combination of accurate mass and in source CID allowed for unequivocal structural elucidation. Acquiring full mass spectra data fragment ion spectra also allowed isobaric degradation products of other flavonoids to be distinguished from the isoorientin, orientin, vitexin and isovitexin flavonoids. LC-MS and pseudo LC-MS-MS sample analysis in electrospray negative ion mode was performed for comparison with the results obtained in positive ion mode (presented in Waters posters WP207 and WP 245), in order to ascertain if additional confirmatory evidence could be obtained to identify the flavonoids of interest in this study. The application of increasing cone voltages to flavonoid standards (vitexin, orientin and isoorientin) provided characteristic fragment ion information, where the relative abundance of fragment ions was different for different flavonoid isomers.

Sample throughput was also increased by performing real time exact mass measurement of the extracts of interest using parallel LC-MS analysis in both positive and negative ion modes. Sample analysis was completed with a 75% reduction in analysis time with mass accuracy being maintained within 5ppm, except for one target flavonoid isomer where mass measurement was achieved within 10ppm.

CONCLUSIONS

- Parallel exact mass LC-MS analysis provides a route for high throughput screening of natural products.
- Analysis time was reduced by 75%.
- Real time accurate mass measurement within 5ppm has been achieved in both serial and parallel LC-MS analysis.
- Acquisition of full accurate mass spectral data allowed for isobaric apigenin and luteolin derivative degradation /fragment ions to be distinguished from ions of isovitexin, vitexin, isoorientin and orientin and hence correct assignment could be achieved rapidly.
- Characteristic assignment for 6-*C* and 8-*C* flavonoid glycosides isomers (vitexin and isovitexin) (orientin and isoorientin) has been possible using exact mass measurement and elemental composition calculation.
- Oa-TOF LC-MS can be used routinely to obtain mass spectral data within 5ppm for the characterisation of complex mixtures produced from phytomedicines.

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