QUANTIFICATION AND CHARACTERISATION OF C-GLYCOSIDIC FLAVONOIDS FROM NATURAL PRODUCTS USING Oa-Tof-MS

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INTRODUCTION

Previously LC-Tof instruments have been restricted by limited dynamic range; which leads to errors in quantification and mass measurement error at high concentrations. An oa-Tof (orthogonal acceleration time of flight) system with extended dynamic range capability that widens the utility of this technology for natural product profiling and quantification studies has been developed (Figure1). The bench top oa-Tof mass spectrometer used incorporates new hardware and software control technology to meet the increased analytical demands of the natural products arena. The highly specific data generated provides an extra degree of information that aids interpretation of the data. Real-time exact mass centroid data acquisition using positive ion electrospray has been performed for the study undertaken. The dynamic range enhancement (DRE) with this instrument operates routinely with the enhanced integral dual reference sprayer (LockSpray™ Figure 2). Both DRE and LockSpray operation are automated and transparent, the transformation in flexibility, allows the easy 24–7 operation of quadrupoles to be extended to oa-Tof. Dynamic range enhancement is simply "sensitivity switching" this novel approach allows the dynamic range to be increased (Figure 3). Three *Passiflora* species were profiled. They are utilised as phytomedicines in Brazil due to the sedative properties that are related to the presence of flavonoids in leaves. As a result of the importance of flavonoids and their glycosides to these species, the identification and/or structural determination of such compounds occurring in leaves play an important role. Hydroethanolic extracts of *P.incarnata*, *P.edulis* and *P.caerulea* were all analysed using oa-Tof LC-MS DRE. Using the Waters® Micromass® LCT Premier™ equipped with a integral dual ESI source, the presence of 6-C and 8-C flavonoid glycoside isomers (vitexin/isovitexin and orientin/isoorientin) have been determined using exact measurement and elemental composition calculation. This further allows for the specific identification of the species from which the flavonoids have been extracted. Utilising the functionality of oa-Tof low level analyte detection can be achieved when acquiring data over a wide mass range, exact mass measurement has been used as a tool for unequivocal identification of flavonoid isomers. Using isoorientin as the target flavonoid of interest, it has been possible to illustrate four orders of dynamic range and quantify the level of isoorientin in the plant extracts profiled.

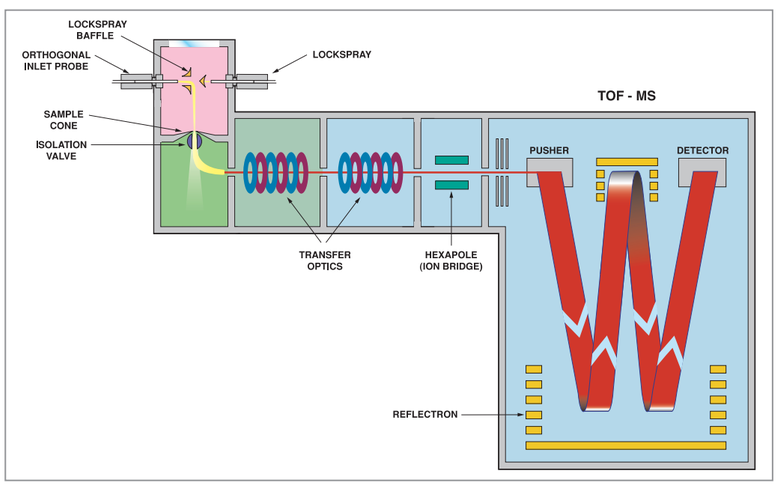

Figure 1. Schematic of dual resolution geometry oa-Tof (W mode > 10000 FWHM).

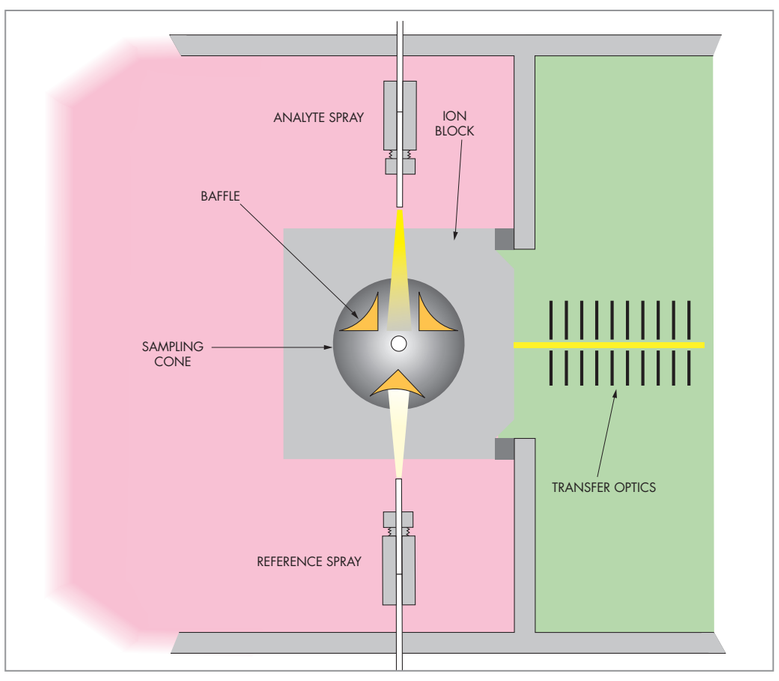

Figure 2. Schematic of the LCT Premier LockSpray source.

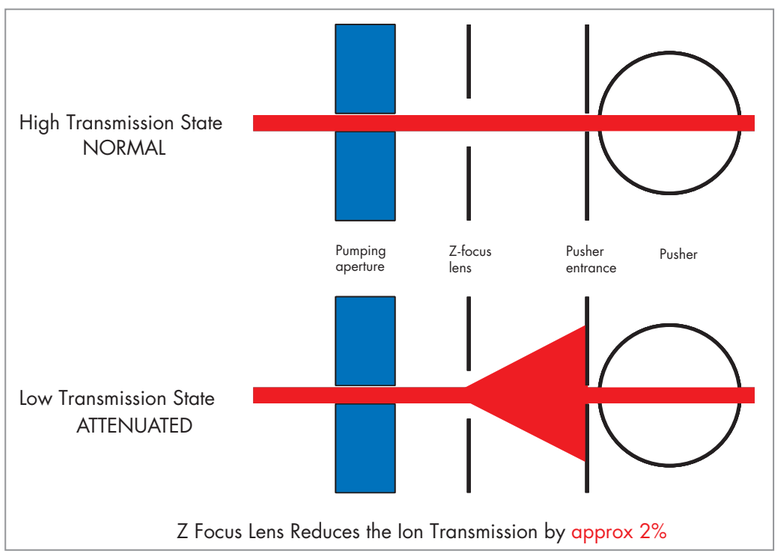

Figure 3. Schematic representation of DRE (Dynamic Range Enhancement).

EXPERIMENTAL

Mass Spectrometer	***HPLC***
LCT Premier™ oa-Tof	Waters® Alliance® HT System

HPLC

Column:	Waters Symmetry® C_{18} (250 mm x 4.6 mm, 5 µm particle size) with guard column (20 mm x 3.9 mm, 5 µm particle size).
Column temperature:	35 °C
Flow:	1 mL/min split 1:4
Mobile phase:	MeCN (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

MS

Ionization mode:	ESI Voltage -ve = 2.6 kV
Sample cone voltage:	100 V
Reference mass:	Electrospray; Leucine enkephalin [M-H]- = 554.2615
Acquisition parameters:	100–1000 m/z 1 second acquisition 5500 FWHM (V) 0.1 second inter acquisition delay

RESULTS

The true profiles of *Passiflora caerulea* and *incarnata* are presented in Figure 4. In Figure 5 the negative mode DRE m/z 431 extracted mass chromatogram for *Passiflora caerulea* is shown where flavonoid isomers A, B, C, D and E have an elemental composition C21H20O10. From Figure 6 the respective exact mass measurements for a single spectrum accumulation intensity, obtained for each flavonoid isomer can be seen. In each case the mass measurement error is shown and ranges between -1.5 and 1.0 ppm. A second example of DRE performance is shown in Figure 7, where the negative mode DRE m/z 577 extracted mass chromatogram for *Passiflora caerulea* flavonoid isomers A, B, C, D and E is illustrated. The elemental composition of these isomers being C27H29O14. The corresponding single spectrum accumulations and intensities are presented in Figure 8. From Table 1 it is shown that a series of flavonoid isomers were targeted in each respective Passiflora extract. Where identified to be present the combined peak intensity and exact mass measurement obtained are illustrated. The calibration curve obtained for target flavonoid isoorientin, for 1 pg/µL to 10000 pg/µL is shown in Figure 9.

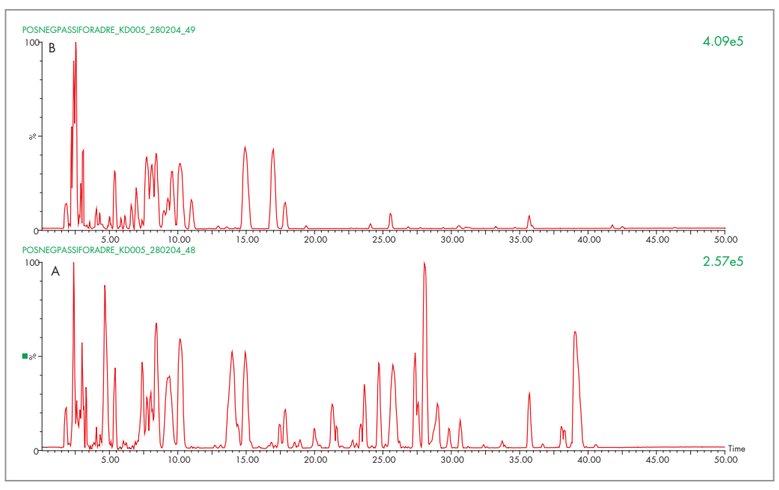

Figure 4. Negative mode DRE BPI chromatograms for Passiflora caerulea *(A) and* Passiflora incarnata *(B) 2 mg/mL extracts.*

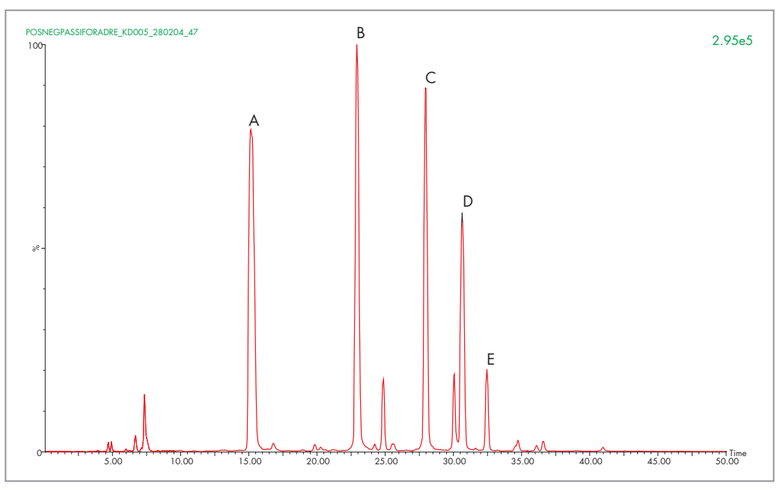

Figure 5. Negative mode DRE m/z 431 extracted mass chromatogram for Passiflora caerulea *where flavonoid isomers A, B, C, D and E have an elemental composition C21H20O10.*

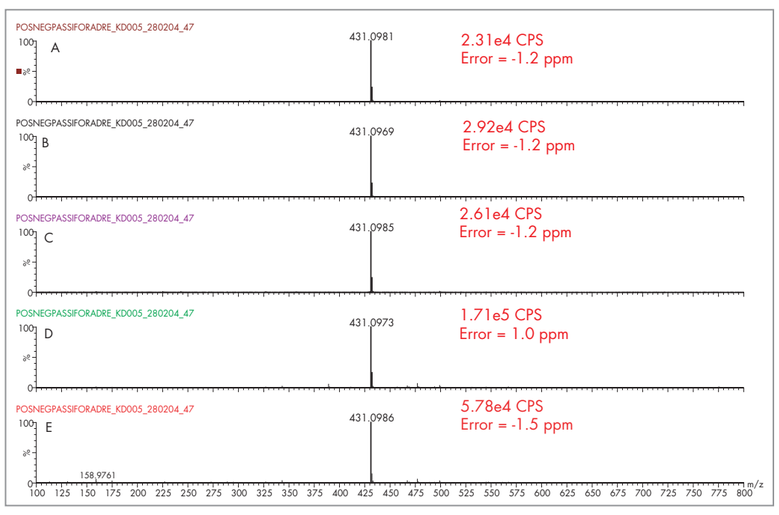

Figure 6. Negative mode DRE exact mass measurement errors and ion intensity responses for the flavonoid isomers A, B, C, D and E shown in Figure 5.

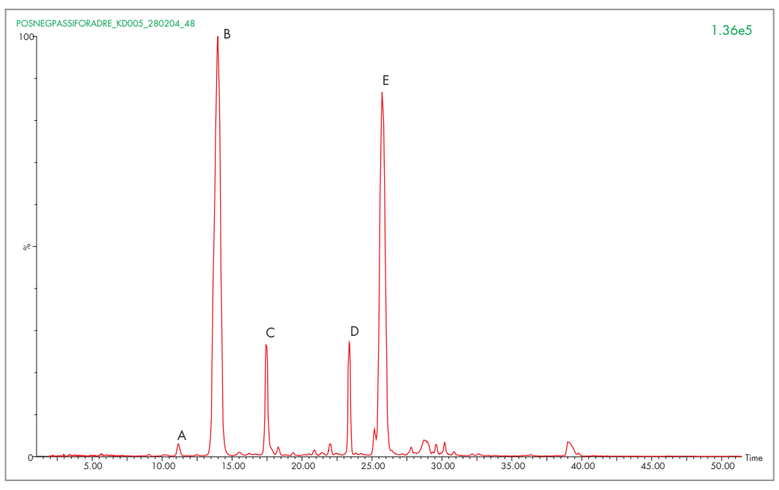

Figure 7. Negative mode DRE m/z 577 extracted mass chromatogram for Passiflora caerulea *where flavonoid isomers A, B, C, D and E have an elemental composition C27H29O14.*

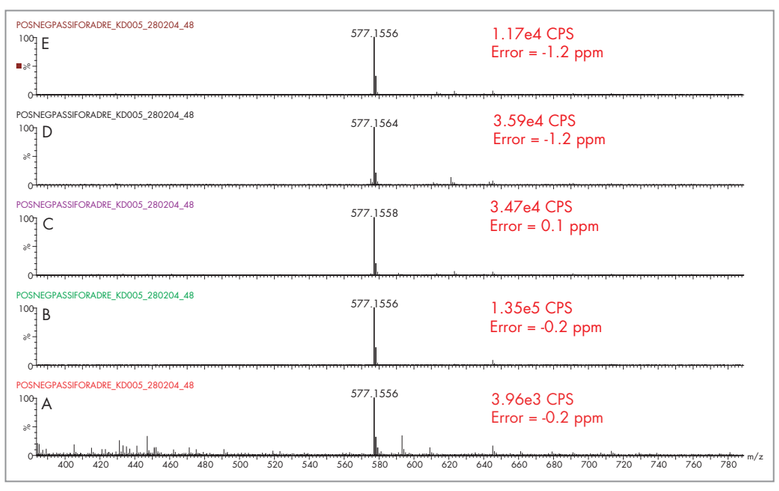

Figure 8. Negative mode DRE exact mass measurement errors and ion intensity responses for the flavonoid isomers A, B, C, D and E shown in Figure 7.

Elemental Composition	RT	PE CPS	Error PPM	PC CPS	Error PPM	PI CPS	Error PPM
C21H19O11	8.7	447.0924 850000	-0.8	447.0919 650000	-2.0	447.0926 600000	-0.4
	10.5	447.0936 510000	-2.0	447.0928 640000	0.1	447.0934 640000	1.4
	18.0	447.0931 130000	-0.3	447.0919 190000	-1.8	447.0928 130000	-0.2
C21H19O10	15.1	431.0977 980000	-0.2	431.0976 38000	-0.6	431.097 700000	-1.9
	22.9	431.0969 970000	-2.2	431.0973 400000	-1.2		
	27.9	431.0984 740000	1.2	431.0968 280000	-2.4		
	30.6	431.0973 590000	-1.3				
	32.5	431.0984 150000	1.3	431.0968 187000	-2.4		
C26H27O14	8.0	563.14 220000	-0.1	563.1411 130000	1.7	563.1391 210000	-1.7
	8.3	563.1405 160000	0.7	563.1407 310000	1.1	563.1397 410000	-0.7
C27H29O14	11.2	577.1567 500000	-0.1	577.1563 580000	1.0	577.155 14000	-1.2
	14.0			577.1564 580000	1.0		
	17.5			577.1563 120000	1.2		
	23.4			577.1563 110000	1.0		
	25.7			577.1564 460000	1.2		
C27H29O15	5.6	593.1519 310000	2.0	593.151 60000	0.6	593.1517 350000	1.7
	9.5	593.1513 120000	1.1	593.1511 480000	0.7	593.1504 200000	-0.4
	16.2	593.1506 220000	1.3				
C27H29O16	4.1	609.1462 180000	1.1	609.1469 55000	2.1	609.1472 110000	2.6
	6.6	609.1467 37000	1.8	609.1464 17000	0.9	609.1468 110000	2.0
	14.5	609.1456 42000	1.0	609.1459 100000	0.6		
RMS Error PPM			1.27		1.39		1.49

Table 1. Negative ion mode OpenLynx™ processed DRE results.
PE= *Passiflora edulis* PC=*Passiflora caerulea* PI= *Passiflora incarnata*
CPS= *Ion counts per second* RT= *Retention time*

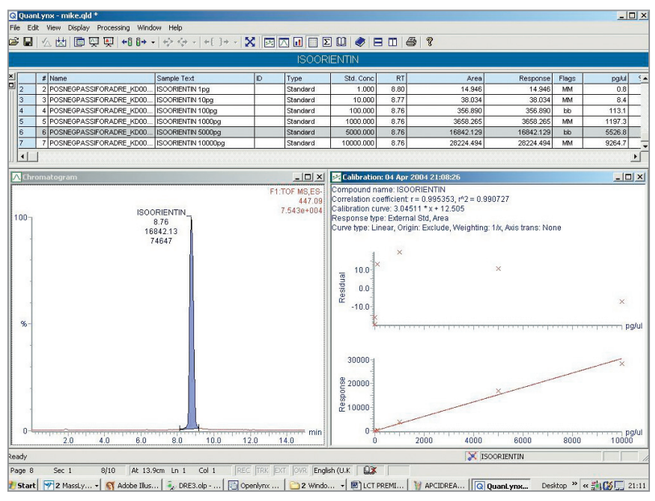

Figure 9. Calibration curve obtained for target flavonoid isoorientin, for 1 pg/µL to 10000 pg/µL.

DISCUSSION

Examples of data from the analysis of three different *Passiflora* species plant extracts has been presented to illustrate the functionality and performance of the LCT Premier™ in DRE mode. The analysis of the complex extracts illustrates how DRE can be used for routine profiling of different plant species, in effect profiling of any complex mixture. The exact mass of any component can be determined by simply selecting the top of the peak or combining the whole of the peak. The diversity of the component concentrations in the extracts analysed also shows a true response profile can be observed. The combination of DRE and enhanced sensitivity of the LCT Premier has enabled improved exact mass measurements to be obtained for all major and minor components. The extracts analyzed contain numerous flavonoid isomers, which have the same elemental composition. Illustration of how DRE has widened the analytical applicability of oa-Tof has been shown for m/z 577 extracted mass chromatogram obtained for the analysis of *Passiflora caerulea*. The mass measurement error obtained ranges from only 0.1 ppm to -1.2 ppm. It has been shown clearly that true exact mass measurement can be obtained without having to be selective, where the exact mass measurement errors and intensities for the average response obtained across the whole chromatographic peak have been presented. The exact mass measurements, ion intensities, peak areas and ppm errors were generated automatically using OpenLynx processing. The maximum average intensity observed was 850000 cps and the minimum observed was 1930 cps. Using OpenLynx processing, the RMS errors for *Passiflora edulis*, *caerulea* and *incarnata* were respectively 1.27 ppm, 1.39 ppm and 1.49 ppm. Four orders of linearity was obtained with a correlation coefficient of variation r2= 0.9953 and it is possible to see that at 10000 pg/µL there is some suppression in response due to saturation of the electrospray process. The concentrations of isoorientin determined to be in the 2 mg/mL extracts were determined to be; *Passiflora edulis* (11908 pg/µL), *Passiflora caerulea* (15384 pg/µL) and *Passiflora incarnata* (13822 pg/µL).

CONCLUSION

- Enhanced dynamic range has been achieved without compromising resolution, DRE acquisition has been performed routinely at > 5000 FWHM and > 10000 FWHM resolution.
- Four orders of linearity have been produced routinely using negative ion mode ES with a correlation coefficient of variation r^2= 0.9953.
- OpenLynx automatic data processing of three plant extracts have produced RMS errors of 1.27 ppm, 1.39 ppm and 1.49 ppm for the target analytes determined to present in each species.
- Mass measurement errors of < 3ppm have been routinely obtained for the combined response across the whole chromatographic peak in the order of 850000 cps using W mode.
- The LCT Premier with DRE functionality enables routine, easy and flexible operation exact mass measurement 24–7.
- Oa-Tof-MS with DRE can be used routinely for natural product profiling.