Waters

INCREASING THE RATE OF THROUGHPUT OF NATURAL PRODUCT CHARACTERISATION USING EXACT MASS MEASUREMENT WITH POLARITY SWITCHING AND PARALLEL ANALYSIS

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INTRODUCTION

Due to the component make up of natural product extracts containing non-polar and polar compounds long HPLC gradients analysis times are required to achieve good chromatographic resolution for the analyte separation. For the study undertaken initially the analysis time was one hour for a plant extract. Analysis of four flavonoid standards and four *Passiflora* extracts required a total analysis time of eight hours for LC-MS data to be acquired. In order to increase productivity, a 5-Way MUX™ source (Figure 3) was interfaced to an oa-Tof (Figure 1) allowing the independently pumped eluents from 4 LC columns to be analysed in parallel on the same mass spectrometer (Figure 2). Another route to increasing sample throughput utilises polarity switching, which enables analytes that ionise in positive or negative ion modes to be detected in one single analysis run. Historically performing polarity switching was more challenging, due to the technology used. The previous power supply stabilisation time required one second, when the polarity was switched. New technological advances enable the power supplies to rapidly reach a stable equilibrium; hence the LCT Premier™ polarity-switching rate has been improved by 80%, and can now switch at 0.2 seconds. The fast switching time makes polarity switching more amenable to chromatographic time frames, with very good mass accuracy being obtained in both positive and negatives modes. The combination of polarity switching with full spectra acquisition and high sensitivity provides an efficient route to detecting unknowns. A selection of flavonoids previously determined to be present in the extracts of *Passiflora edulis* were targeted to illustrate the exact mass measurement performance using polarity switching. The analytes of interest were detected in both positive and negative electrospray mode, hence further increasing beyond doubt, the confidence in the elemental compositions generated for the flavonoids identified. Real time exact mass centroid data acquisition has been performed using both polarity switching and parallel analysis combined with oa-Tof to illustrate a route to faster natural product characterisation.

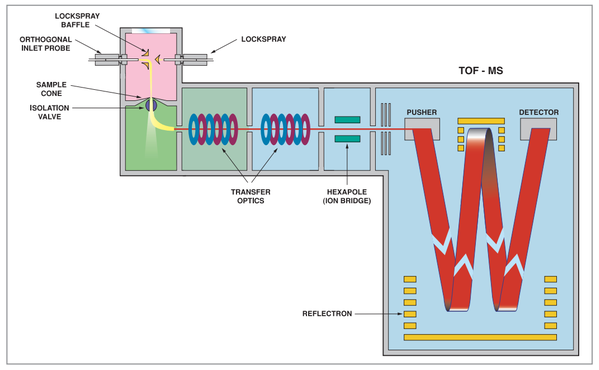

Figure 1. Schematic of oa-Tof (W mode > 10000 FWHM).

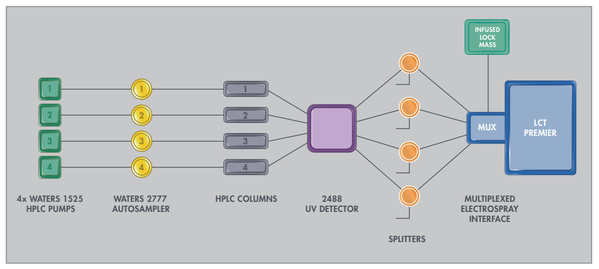

Figure 2. Oa-Tof parallel LC-MS analysis system schematic.

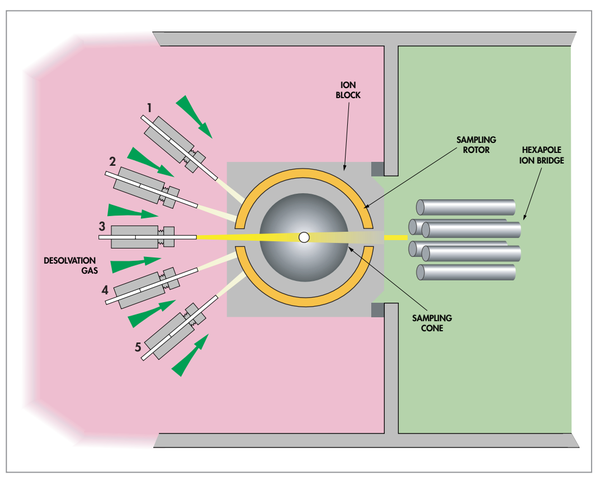

Figure 3. 5-WAY MUX™ schematic.

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) (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

Ionisation Mode: ESI Voltage +ve = 3 kV, -ve = 2.7 kV

Sample cone voltage: 100 V

Reference mass: Leucine enkephalin
$[M+H]^+$ = 556.2771 $[M-H]^-$ = 554.2615

Acquisition Parameters: 100–1000 m/z; 1 second acquisition (LockSpray); 0.15 sec acquisition (MUX); 0.2 second polarity switching inter scan delay; 0.05 MUX inter accumulation delay

RESULTS

Presented in Figure 4 are the parallel LC-oa-Tof-MS positive ion electrospray MUX BPI (base peak ion) chromatograms obtained for the four *Passiflora* extracts profiled. The corresponding parallel LC-UV λ=330 nm chromatograms are shown in Figure 5. From Table 1 the exact mass measurement obtained for the target flavonoids are shown for both positive and negative ion mode parallel analysis. The LCT Premier polarity switching performance is illustrated in Figure 6 with PDA data acquired for the profiling of *Passiflora edulis*. Examples of the exact mass measurement performance obtained using polarity are shown for flavonoids kaempferol-3-rutinoside and isoorientin in Figures 7 and 8 respectively. For kaempferol-3-rutinoside (B), in positive mode the exact mass measurement error obtained was within (0.6 mDa) 1 ppm and in the case of negative an error of (-0.6 mDa) -1 ppm was recorded. In the case of isoorientin (A), mass measurement errors of -1 ppm (-04 mDa) were acquired in negative mode and -1.1 ppm (-0.5 mDa) with positive mode using polarity switching. Table 2 illustrates examples of the exact mass measurement results and elemental compositions obtained for flavonoids determined to be present in *Passiflora edulis* using polarity switching. A comparison of the peak area response obtained in polarity switching mode and single polarity mode is shown for kaempferol-3-rutinoside in Figure 9.

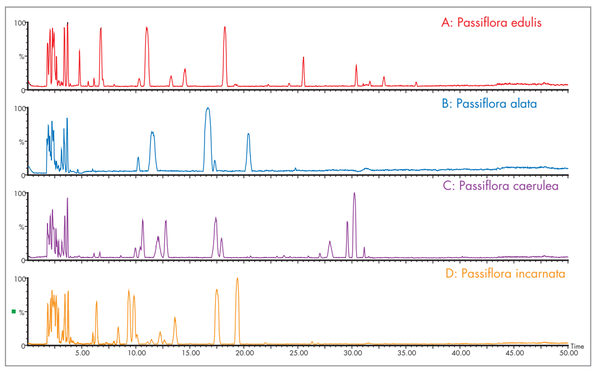

Figure 4. Parallel LC-MS positive ion mode Passiflora extract MUX BPI's (A) P.edulis, (B) P.alata, (C) P.caerulea and (D) P.incarnata.

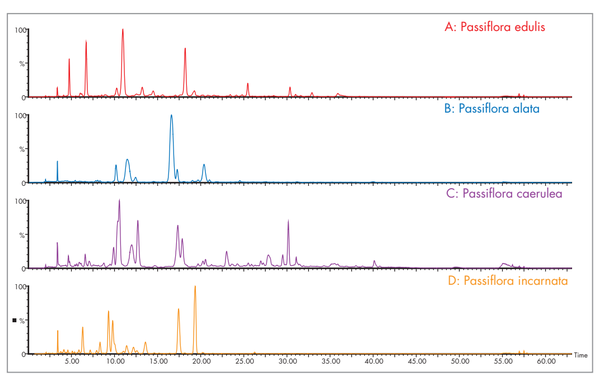

Figure 5. Parallel LC-UV λ=330nm chromatograms for Passiflora extracts (A) P.edulis, (B) P.alata, (C) P.caerulea and (D) P.incarnata.

Parallel Analysis +ve mode	Mass Measurement Errors			
	Isoorientin	Orientin	Vitexin	Isovitexin
Passiflora edulis	2.9 ppm	2.3 ppm	-	1.3 ppm
Passiflora alata	1.6 ppm	3.4 ppm	-	1.7 ppm
Passiflora caerulea	3.1 ppm	4.3 ppm	-	1.0 ppm
Passiflora incarnata	0.0 ppm	1.3 ppm	-	3.8 ppm
Parallel Analysis -ve mode	**Mass Measurement Errors**			
	Isoorientin	Orientin	Vitexin	Isovitexin
Passiflora edulis	0.3 ppm	2.9 ppm	-	2.2 ppm
Passiflora alata	1.2 ppm	7.9 ppm	-	0.6 ppm
Passiflora caerulea	0.7 ppm	4.3 ppm	-	1.2 ppm
Passiflora incarnata	1.1 ppm	2.6 ppm	-	4.8 ppm

Table 1. Exact mass errors for flavonoids using positive and negative mode parallel real time LC-MS exact mass measurement.

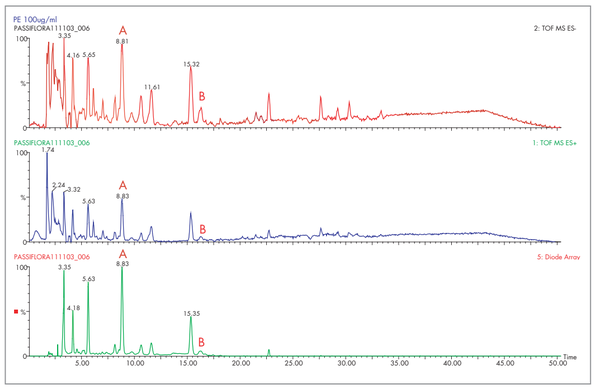

Figure 6. Positive and negative mode total ion chromatograms, with PDA chromatogram acquired for analysis of a 100 µg/mL plant extract using the LCT Premier in polarity switching mode.

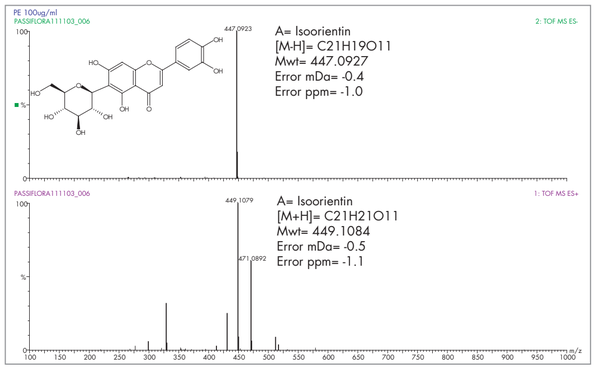

Figure 7. Positive and negative mode exact mass spectra for kaempferol-3-rutinoside (B) of Figure 6.

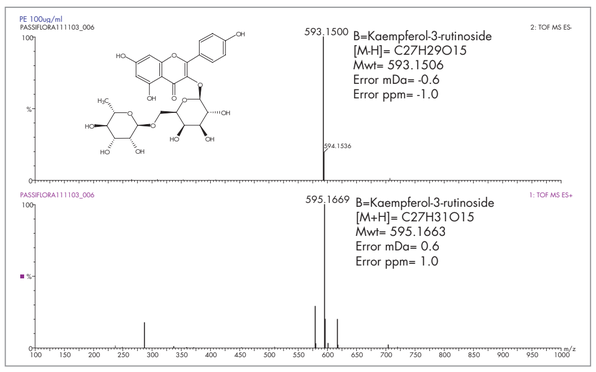

Figure 8. Positive and negative mode exact mass spectra for isoorientin (A) of Figure 6.

Compound	Elemental Composition	$[M+H]^+$ Calculated M/Z	$[M+H]^+$ Mass Measured	Error	$[M+H]^-$ Calculated M/Z	$[M+H]^-$ Mass Measured	Error
Isoorientin	$C_{21}H_{20}O_{11}$	449.1084	449.1079	-0.5 mDa -1.1 ppm	447.0927	447.0923	-0.4 mDa -1.0 ppm
Orientin	$C_{21}H_{20}O_{11}$	449.1084	449.1081	-0.3 mDa -0.6 ppm	493.0982*	493.0986*	0.4 mDa 0.8 ppm
Isovitexin	$C_{21}H_{20}O_{10}$	433.1135	433.1143	0.8 mDa 1.9 ppm	431.0978	431.0971	-0.7 mDa -1.7 ppm
Kaempferol -3-rutinoside	$C_{27}H_{30}O_{15}$	595.1663	595.1669	0.6 mDa 1.0 ppm	593.1506	593.1500	-0.6 mDa -1.0 ppm
RMS Error				**0.8 ppm**			**1.17 ppm**

Table 2: Exact mass measurement obtained for flavonoids determined to be present in the plant extract analysed, using the LCT Premier in polarity switching mode.

**For orientin in negative ion mode the formate adduct was observed.*

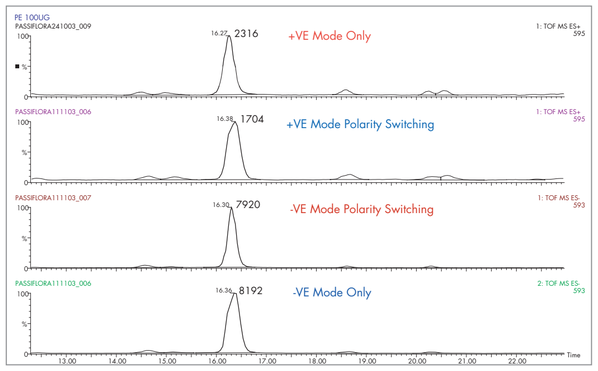

Figure 9. Area responses for positive mode m/z 595 and negative mode m/z 593 extracted mass chromatograms, with corresponding polarity switching chromatograms for kaemferol-3-rutinoside.

DISCUSSION

Using oa-Tof parallel LC-MS accurate mass measurement allowed for highly specific data to be acquired, where full spectra acquisition and exact mass measurement for more than eighty major and minor components found in the some extracts of *Passiflora*. The total analysis time for *Passiflora* species is sixty minutes. The increased selectivity of exact mass measurement allowed for full confidence in flavonoid isomer assignment. Using the elemental composition calculator the most probable elemental formula was derived from the exact mass spectrum, this further confirmed the identity of the flavonoid isomers of interest. Acquiring full mass spectra data allowed isobaric degradation products of other flavonoids to be distinguished from the isoorientin, orientin, vitexin and isovitexin flavonoids. Sample throughput was increased by performing real time exact mass measurement of the extracts of interest using parallel LC-MS analysis, with a 75% reduction in analysis time and exact mass being maintained within 5 ppm. Using positive/negative switching it is possible to observe in one single analysis, if there is a difference in the profile of the positive and negative TIC's. The case may be that some analytes may only be seen in one polarity mode; hence polarity switching provides a rapid screening method. The enhanced polarity switching time has enabled the chromatographic integrity to be maintained in both positive and negative LC/MS modes. From the experiment performed, it is possible to see that negative ion mode has provided more sensitivity. Also it is clear from comparing the PDA chromatogram, that LC/MS provides more information. Using polarity switching a wealth of information can be obtained in one analysis from the combination of oa-Tof full spectra acquisition with exact mass measurement.The RMS ppm exact mass measurements obtained over a sixty-minute acquisition time for isoorientin, orientin, isovitexin and kaempferol-3-rutinoside in positive mode were 0.8 ppm and in negative mode the RMS error obtained was less than 1.2 ppm. The effect of polarity switching on sensitivity can be seen from the peak areas for kaempferol-3-rutinoside, where on average between positive and negative modes greater than eighty percent of the sensitivity has been maintained.

CONCLUSION

- Parallel exact mass LC-MS analysis provides a route for high throughput screening of natural products providing a 75% increase in sample throughput.
- 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 3 ppm for the characterisation of complex mixtures produced from phytomedicines.
- Polarity switching combined with exact mass measurement and full spectra acquisition provides a time efficient route to the detection of unknowns with a reduction of 50% analysis time.
- Using a plant extracts of *Passiflora* species, the flavonoids isoorientin, orientin, kaempferol-3-rutinoside and isovitexin have been successfully detected using positive/negative mode switching.
- Analysis has been performed using centroid acquisition mode and exact mass measurement performed in real time with polarity switching.
- 0.8 RMS ppm error has been obtained for isoorientin, orientin, kaempferol-3-rutinoside and isovitexin in positive mode.
- 1.17 RMS ppm error has been obtained for isoorientin, orientin, kaempferol-3-rutinoside and isovitexin in negative mode.
- LockSpray independent reference sampling enables the routine acquisition of highly specific data using polarity switching.