

Application Note

Quantitative Analysis of C-Glycosylflavonoids Using oa-ToF

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Abstract

In this study, data is presented that will illustrate the enhanced performance of the Waters Micromass LCT Premier orthogonal acceleration time of flight (oa-Tof) mass spectrometer, where Dynamic Range Enhancement (DRE) functionality is used to extend the analytical applicability of oa-Tof. The data presented shows that four orders of linearity in electrospray mode can be achieved routinely. Plant extracts of 2 mg/mL concentration have been used to illustrate the response for the major and minor components present. The corresponding PDA data is also presented to illustrate the maintenance of chromatographic integrity. It is shown that the improved data quality achieved with the LCT Premier allows routine, automated data processing of exact mass data using OpenLynx Software.

Introduction

As increased regulation comes in to place within the global herbal/natural medicines market, it will be necessary to produce standardized products. Evidence of reproducible component profile will be required. In addition, each individual component will have to be quantified. Analysis using oa-Tof will provide a full spectra profile with low detection limits. Quantitative analysis can also be performed routinely with Dynamic Range Enhancement functionality on the LCT Premier, which is enabled through a combination of hardware and software improvements. The enhanced performance obtained using DRE can be applied to numerous application areas, such as metabonomics, metabolite identification, impurity profiling, plant medicine research, environmental monitoring, clinical screening, and toxicology application arenas, as well as numerous other application areas.

Plant extracts at concentrations of 2 mg/mL have been utilized to illustrate the response obtained on the LCT Premier for a plethora of major and minor components. The high-duty cycle of ToF can now be exploited for qualitative and quantitative studies, generating full spectra at high mass accuracy (<3 ppm RMS). In the case of the minor components present, enhanced sensitivity has resulted in better ion statistics, and hence improved exact mass measurement. For the major components, DRE has enabled exact mass measurements of <3 ppm RMS to be obtained where previously detector saturation would have produced a shift to a lower mass, and hence incorrect mass measurement.

The LCT Premier is shown in Figure 1; a schematic of it with the analyzer in W geometry is illustrated in Figure 2. DRE operates routinely with the enhanced integral LockSpray source shown in Figure 3. It is now possible to use reference compound concentrations over a wide intensity range, providing flexibility of instrument operation. A compound that ionises in both positive and negative modes can be used, even

though the response level obtained in either mode is different. Previously it was necessary to maintain a reference response below 200 counts per second. DRE and LockSpray are transparent to the user. These new flexible operation enhancements allow the easy 24-7 operation of quadrupole MS to be matched.



Figure 1. Alliance 2795/LCT Premier System featuring integral LockSpray.

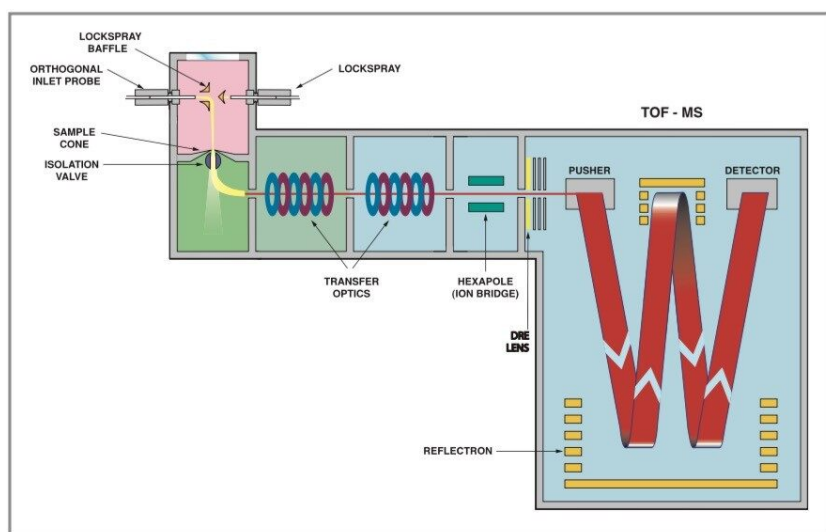


Figure 2. Schematic of dual resolution geometry oa-ToF (W mode > 10000 FWHM).

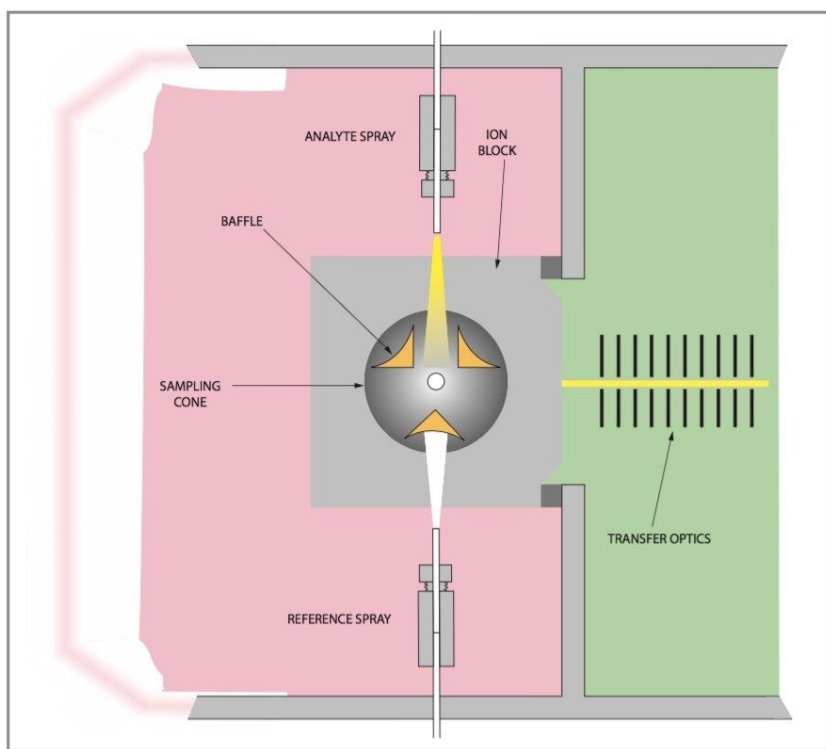


Figure 3. Schematic of the LCT Premier LockSpray source.

The mechanism by which DRE performs is represented schematically in Figure 4. Dynamic range enhancement is simply "sensitivity switching" which allows the dynamic range to be increased without compromising resolution (>10000 FWHM). During an acquisition the voltage applied to the DRE lens is switched rapidly between two values, resulting in high and low intensity data being produced for alternating ion accumulations. The transmission switch allows any saturated high intensity data to be transparently replaced by low intensity data, which has been corrected for the transmission switch. The result is a normal spectrum that includes high intensity peaks, which would have been saturated without DRE. DRE has been shown to produce 4 orders of dynamic range routinely.

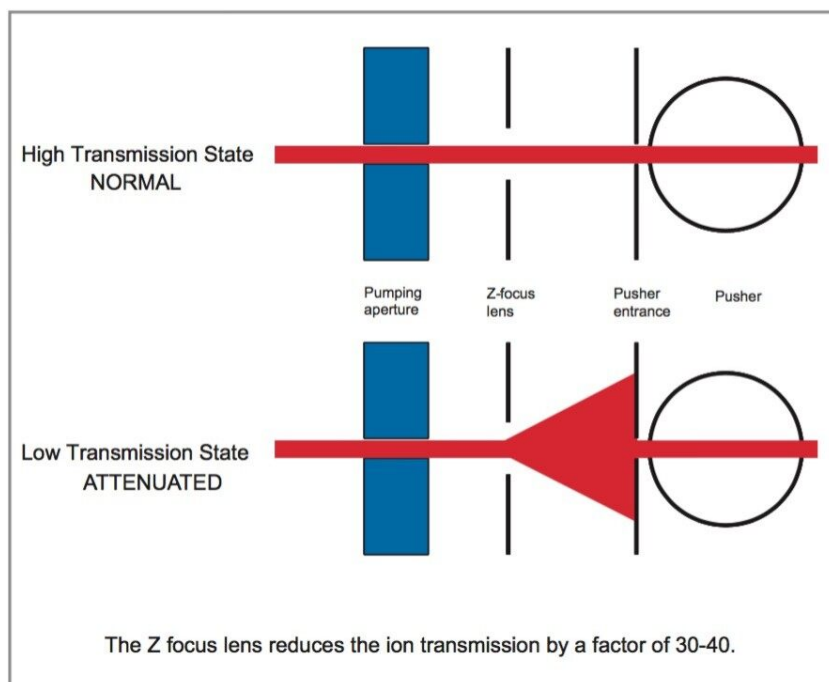


Figure 4. Schematic representation of DRE (Dynamic Range Enhancement).

Experimental

HPLC Conditions

HPLC system:	Waters Alliance HT 2795 Separations Module
Column:	Waters Symmetry C ₁₈ (250 mm x 4.6 mm, 5 µm particle size) with guard column (2 mm x 3.9 mm, 5 µm particle size)
Column temperature:	35 °C
Flow rate:	1 mL/min - split 1:4

Mobile phase:	A: H ₂ O (0.2% HCOOH), B: MeCN
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Gradient:	0-10 min: 15% B 10-40 min: 15-30% B 40-50 min: 30-15% B
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MS Conditions

Mass spectrometer:	Waters Micromass LCT Premier oa-ToF
Ionization mode:	ESI Voltage +ve =3.0 kV, -ve= 2.6 kV
Sample cone voltage:	100 V
Reference mass:	Electrospray; Leucine enkephalin, [M+H] ⁺ =556.2771, [M-H] ⁻ =554.2615
Resolution:	10500 FWHM

Results and Discussion

An example of data from the analysis of three different plant extracts is presented to illustrate the functionality and performance of the LCT Premier using DRE. 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. In previous applications it would have been necessary to select a section of the chromatographic peak that was below 25000 cps. Data acquisition was performed in W mode geometry with resolution >10500 FWHM. From Figure 5, it can be seen that the UV data and the DRE data correspond and chromatographic integrity is maintained. Also in the negative mode BPI chromatogram the maximum response obtained was 471000 counts. The diversity of the component concentrations in the extracts analysed is further illustrated in Figure

6, where a true response profile can be observed. The flavonoid isomer ratios obtained are not distorted due to detector saturation, and simple visualization of the chromatograms for each extract enables each extract to be identified. 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.

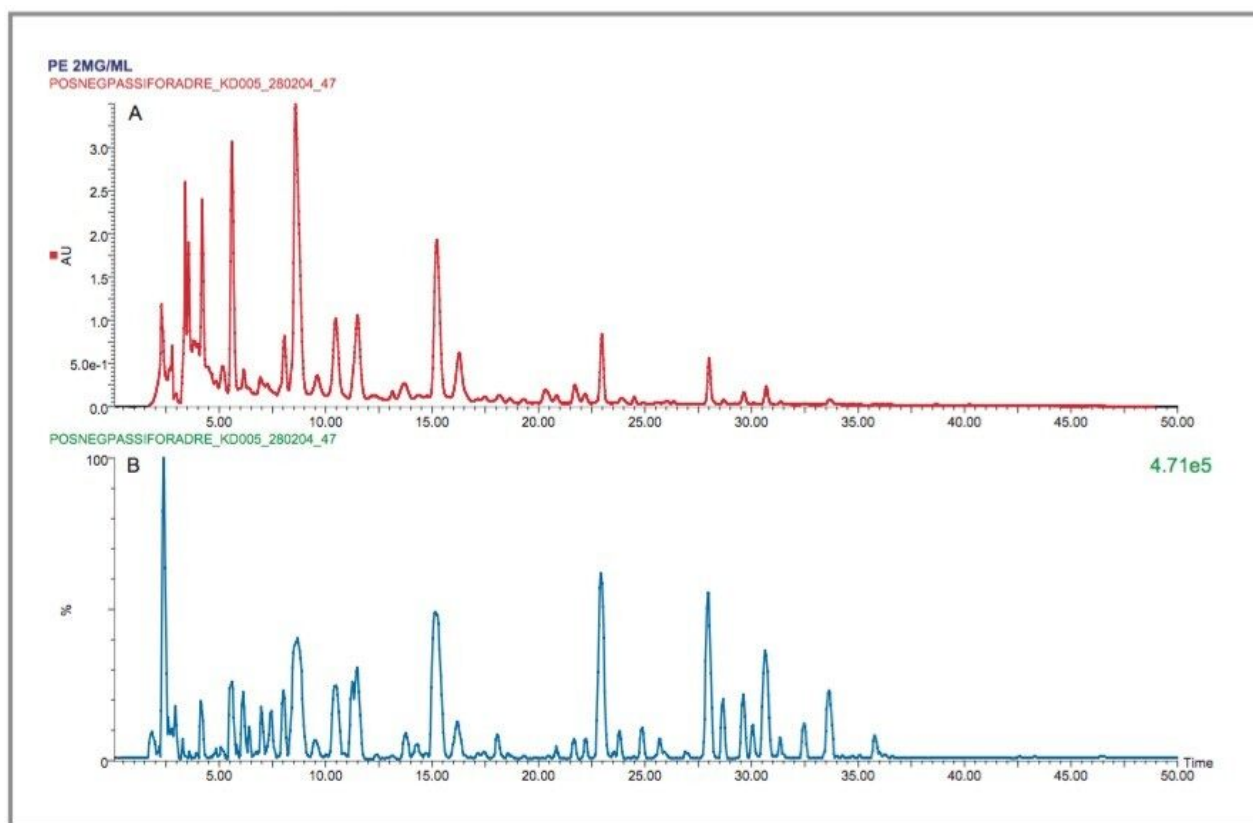


Figure 5. Negative mode DRE BPI chromatogram (B) and corresponding UV chromatogram (A) for a 2 mg/mL extract of *Passiflora edulis*.

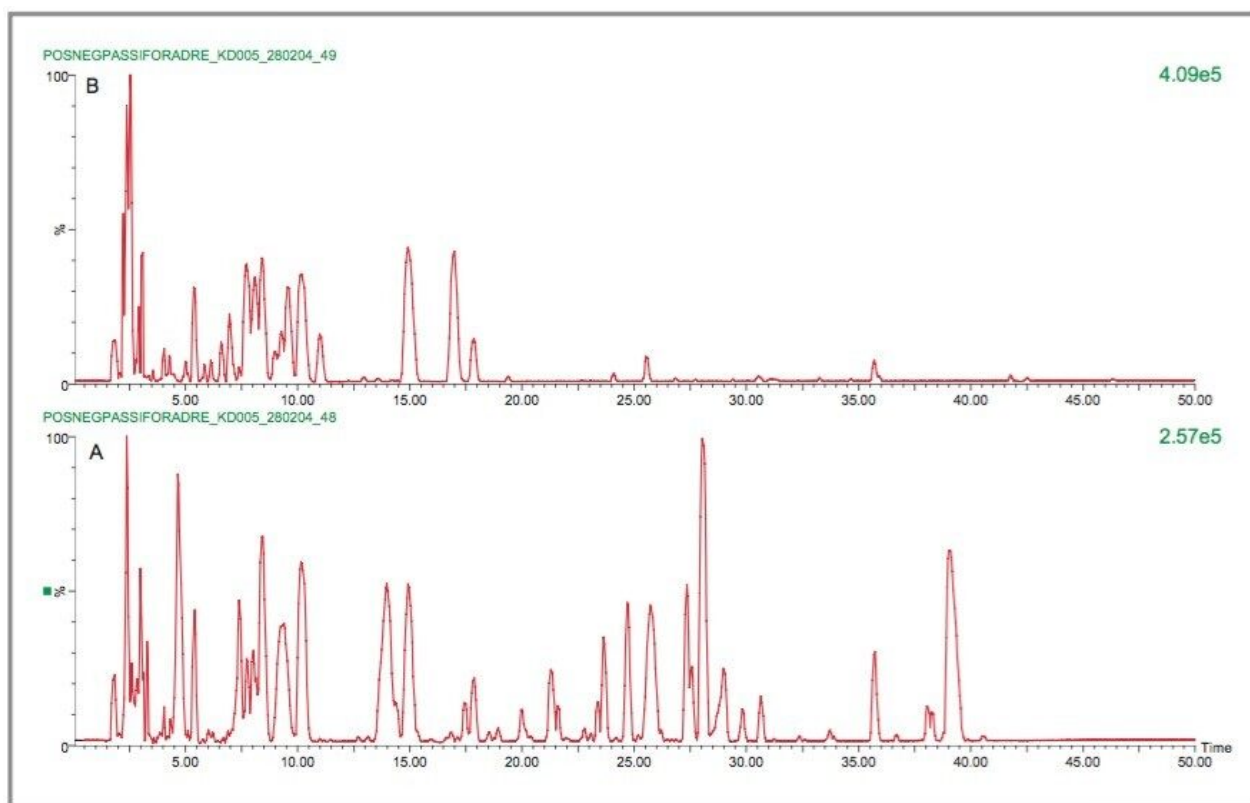


Figure 6. Negative mode DRE BPI chromatograms for *Passiflora caerulea* (A) and *Passiflora incarnata* (B) 2 mg/mL extracts.

The extracts analyzed contain numerous flavonoid isomers, which have the same elemental composition. Differentiation of these isomers has previously been illustrated.¹ The m/z 447 extracted mass chromatogram obtained for the analysis of *Passiflora caerulea* is shown in Figure 7. The peaks labelled A, B and C vary in response. The exact masses obtained at the peak top and ion count response are presented in Figure 8. The actual exact mass is 447.0927 for $[M-H]^-$ and elemental composition is $C_{21}H_{20}O_{11}$ for the three labelled components.

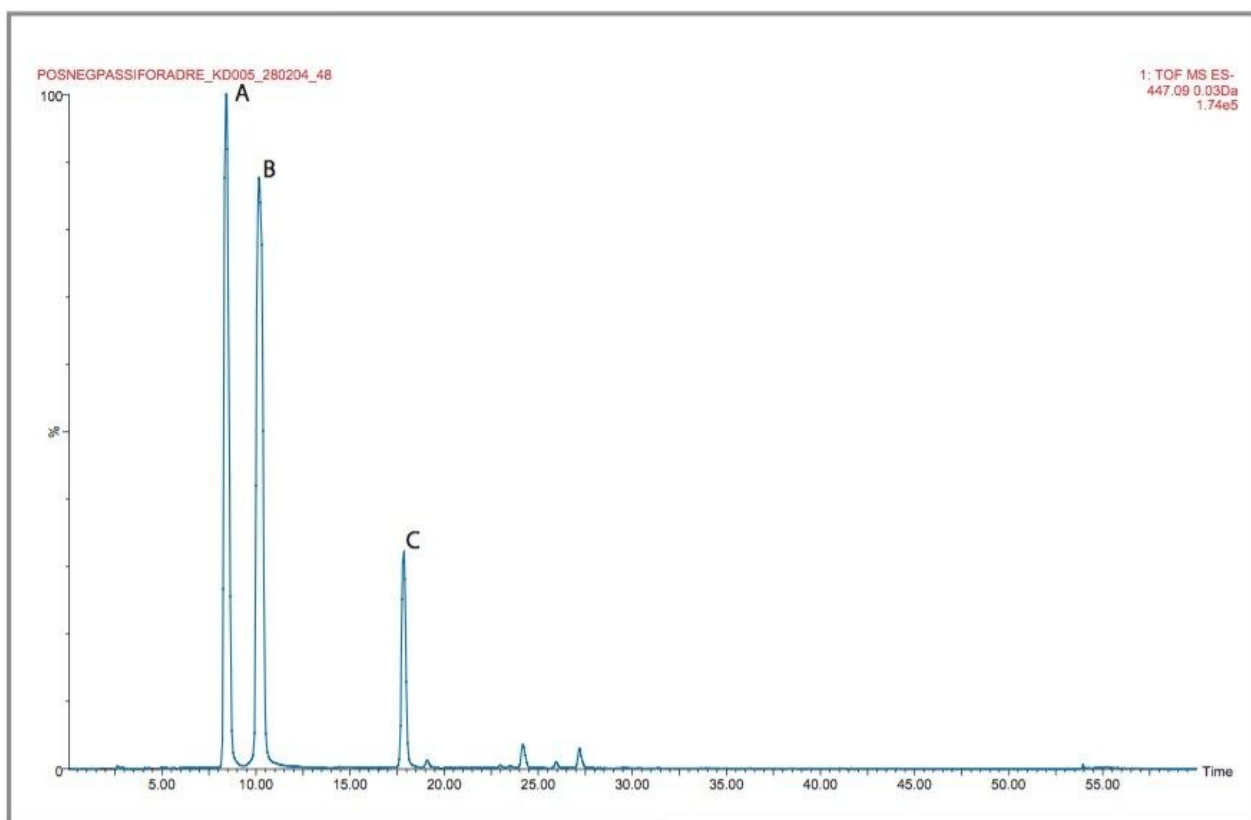


Figure 7. Negative mode DRE m/z 447 extracted mass chromatogram for *Passiflora caerulea* where flavonoid isomers A, B and C have an elemental composition $C_{21}H_{20}O_{11}$.

As can be seen from Figure 8, the lowest response is 49000 cps, and the maximum is 174000 for a single ion accumulation. A maximum error of -1.5 ppm was obtained. Without DRE it would not have been possible to take the exact mass measurement from the peak top. In every example shown, saturation would have occurred and the exact mass at the peak top would not have been correct. A second selection of flavonoid isomers is shown in Figure 9 for the m/z 431 extracted mass chromatogram from *Passiflora caerulea*. Five of the major isomers were selected and labelled A–E respectively.

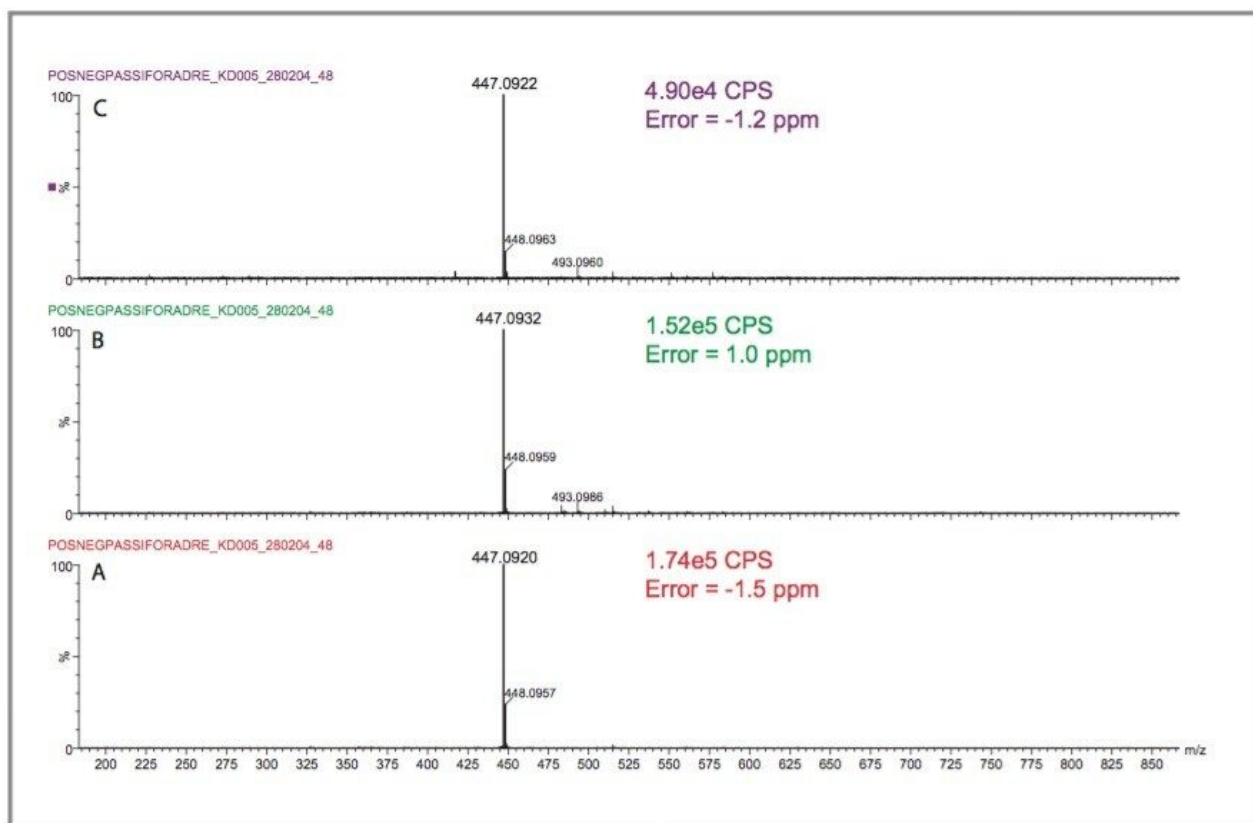


Figure 8. DRE mode negative ion exact mass measurement errors and ion intensity response obtained for the components illustrated in Figure 7.

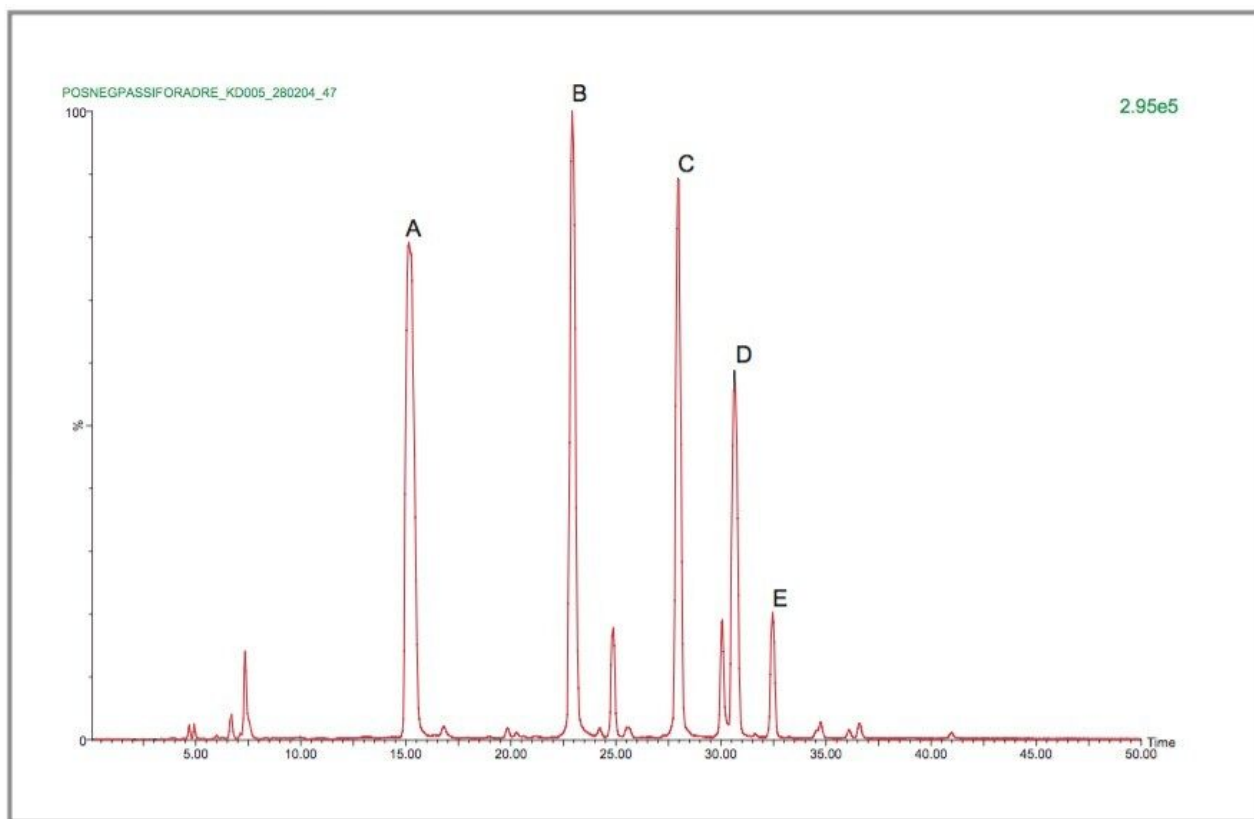


Figure 9. Negative mode DRE m/z 431 extracted mass chromatogram for *Passiflora caerulea* where flavonoid isomers A, B, C, D, and E have an elemental composition $C_{21}H_{20}O_{10}$.

The intensity of a single ion accumulation is presented for each compound from Figure 9. In each case the mass measurement error is shown, and here it ranges between -1.5 and 1.0 ppm. As can be seen for isomers A to E of Figure 10, responses are now obtained in the range of 23100 to 171000 cps for the maximum response. This response illustrates improved sensitivity, better ion statistics and hence better mass accuracy for the minor components of these complex extracts. In addition exact mass measurement has been obtained for the most abundant components, so saturation of the detector has not occurred. The elemental composition for these isomers is $C_{21}H_{20}O_{10}$; the actual mass for the de-protonated m/z 431 $[M-H]^-$ ion is 431.0978. Excellent exact mass measurement performance and reproducibility is illustrated using DRE.

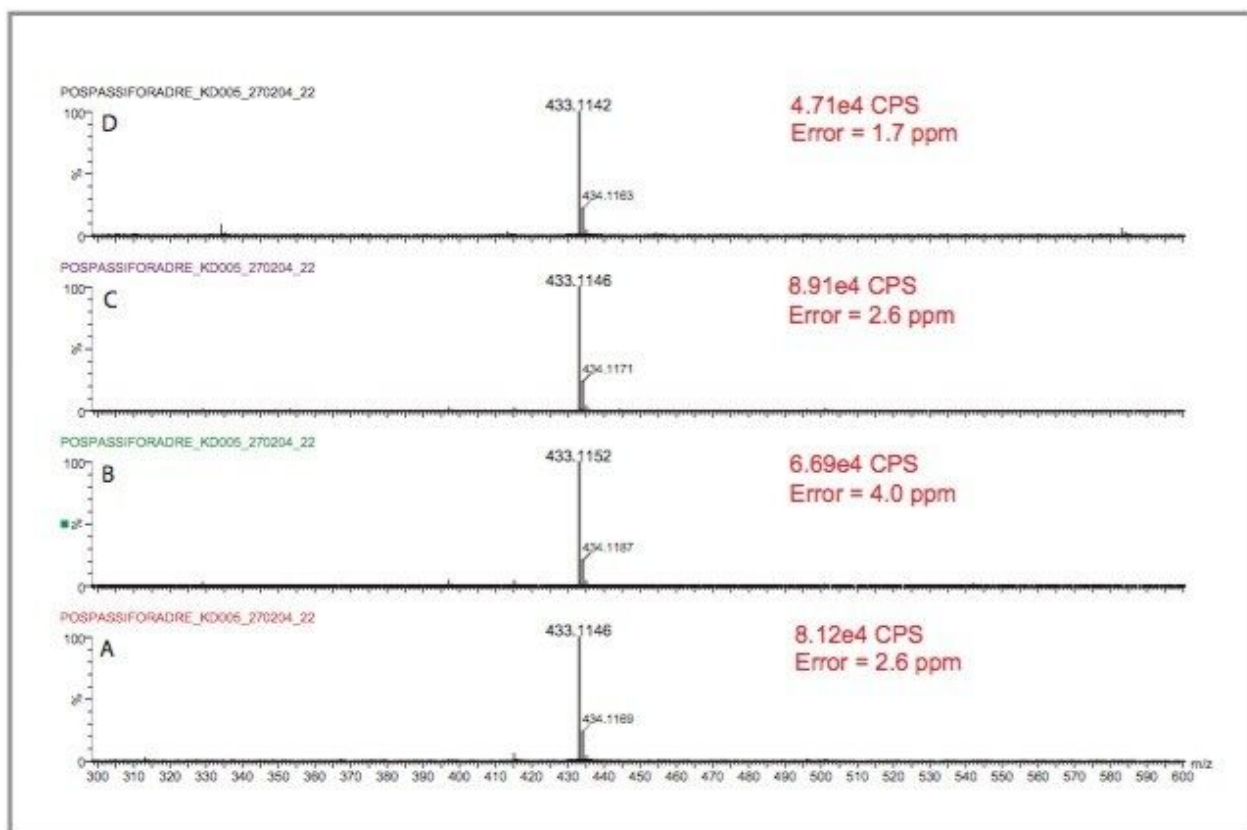


Figure 10. Negative mode DRE exact mass measurement errors and ion intensity responses for the flavonoid isomers A, B, C, D, and E shown in Figure 9.

Further illustration of how DRE has widened the analytical applicability of oa-ToF is shown in Figure 11, the m/z 577 extracted mass chromatogram obtained for the analysis of *Passiflora caerulea*. Five major and minor components have been labelled A–E in Figure 11, and the respective exact mass spectra obtained for a single ion accumulation at the peak top are presented in Figure 12. The mass measurement error obtained range from only 0.1 ppm to -1.2 ppm. The calculated mass for the de-protonated species is 577.1557. Using DRE exact mass measurement on the LCT Premier, performance has surpassed the LCT performance, allowing a standard of analysis previously not possible. The introduction of DRE also enables easier data processing using OpenLynx, (an automated data processing tool for peak detection and extraction of spectra) since the data can now be processed in a manner to incorporate the whole chromatographic peak, rather than having to determine a set of parameters to process the data in the correct intensity range. In the past, it was necessary to predetermine an intensity range where detector saturation did not occur, and several spectra on the tail of the chromatographic peak would be averaged to obtain an exact mass measurement. Automatic processing of LCT Premier DRE data can be achieved by simply integrating the whole chromatographic

peak, enabling an average of all the exact mass spectra to be acquired.

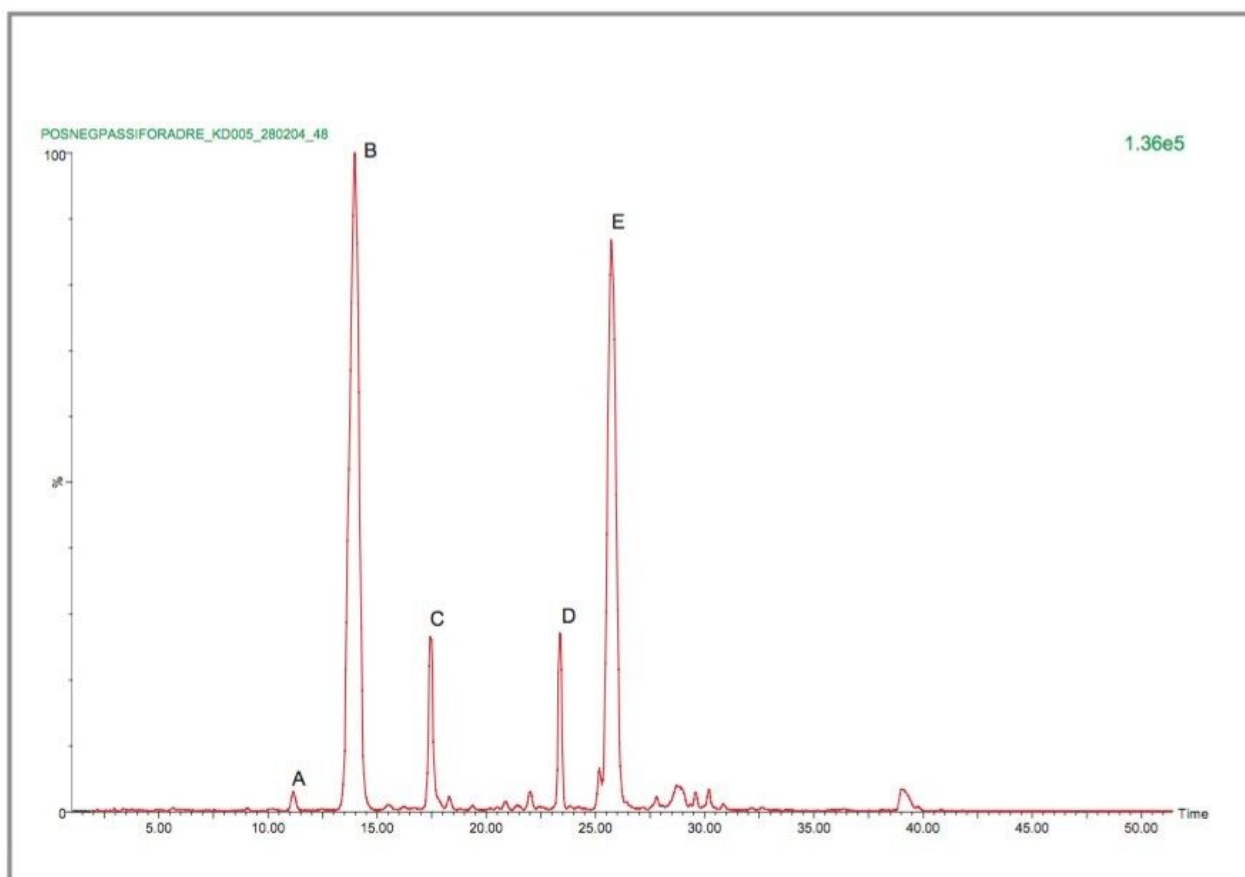


Figure 11. Negative mode DRE m/z 577 extracted mass chromatogram for *Passiflora caerulea* where flavonoid isomers A, B, C, D, and E have an elemental composition $C_{27}H_{29}O_{14}$.

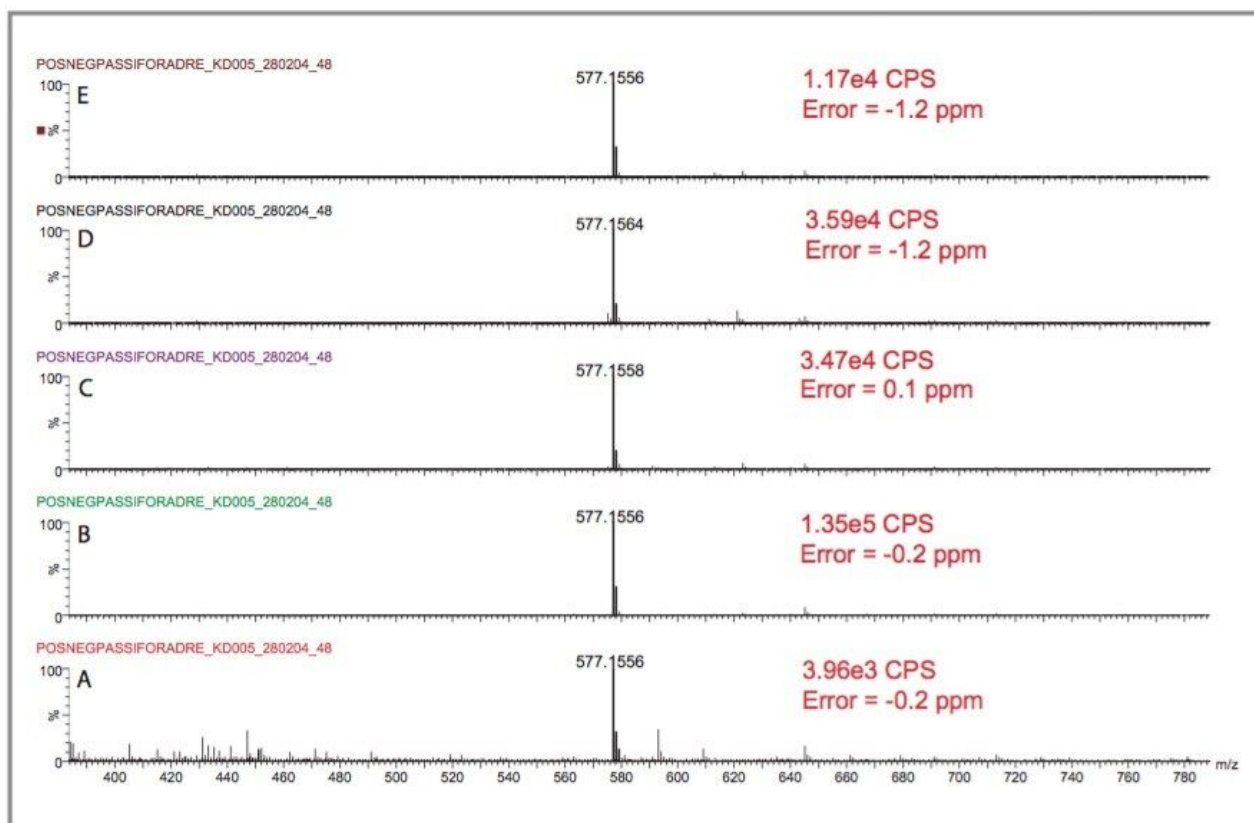


Figure 12. Negative mode DRE exact mass measurement errors and ion intensity responses for the flavonoid isomers A, B, C, D, and E shown in Figure 11.

In Table 1, the exact mass measurement and intensities obtained at the top of each peak has been recorded for manually processed data, simply by selecting the chromatographic peak top. Three extracts have been analysed and in each case the presence of six target flavonoids has been determined. Where detected, the intensity response and mass measured are shown. The maximum intensity acquired was 292000 cps and the minimum observed was 1930 cps. Using manual processing for the target flavonoid, RMS errors for *Passiflora edulis*, caerulea and incarnata were respectively 1.95 ppm, 1.46 ppm, and 1.34 ppm, clearly illustrating that the true exact mass measurement can be obtained without having to be selective. Table 2 shows the exact mass measurement errors and intensities for the average response obtained for the whole peak 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.

Response Obtained at Peak Top DRE W Mode

Elemental Composition	RT	PE CPS	Error PPM	PC CPS	Error PPM	PI CPS	Error PPM
$C_{21}H_{19}O_{11}$	8.7	447.0920 183000	-1.6	447.0915 174000	-2.8	447.0924 165000	-0.8
	10.5	447.0916 116000	-2.5	447.0931 153000	0.8	447.0936 145000	1.9
	18.0	447.0929 40700	0.4	447.0919 56100	-1.9	447.0931 55800	0.8
$C_{21}H_{19}O_{10}$	15.1	431.0981 231000	0.6	431.0985 134000	1.6	431.0973 177000	-1.2
	22.9	431.0969 292000	-2.1	431.0975 119000	-0.7		
	27.9	431.0985 261000	1.6	431.0969 65600	-2.1		
	30.6	431.0973 271000	-1.2				
	32.5	431.0988 57800	1.8	431.0965 7870	-3.1		
$C_{26}H_{27}O_{14}$	8.0	563.1411 109000	1.8	563.1400 70100	-0.1	563.1399 159000	-0.3
	8.3	563.1382 47200	-3.3	563.1412 56100	2.0	563.1396 142000	-0.9
$C_{27}H_{29}O_{14}$	11.2	577.1570 145000	2.2	577.1556 3960	-0.2	577.1552 1930	-0.9
	14.0			577.1556 135000	-0.2		
	17.5			577.1558 34700	0.1		
	23.4			577.1564 35900	1.2		
	25.7			577.1556 117000	-0.2		
$C_{27}H_{29}O_{15}$	5.6	593.1517 119000	1.8	593.1510 99100	0.6	593.1512 128000	0.9
	9.5	593.1509 31800	0.4	593.1512 24400	0.9	593.1503 66200	-0.6
	16.2	593.1516 60500	1.6				
$C_{27}H_{29}O_{16}$	4.1	609.1466 92600	1.7	609.1464 32000	1.4	609.1469 46400	2.2
	6.6	609.1472 21000	2.7	609.1465 9360	1.5	609.1470 55200	2.4
	14.5	609.1473 11600	2.9	609.1460 36100	0.7		
RMS Error PPM			1.95		1.46		1.34

Table 1. Negative ion mode manually processed DRE results.

PE= *Passiflora edulis*, PC= *Passiflora caerulea*, PI= *Passiflora incarnata*, CPS= Ion counts/second, RT= Retention time

OpenLynx DRE W Mode Results

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 380000	-0.6	431.0970 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	563.1400 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	577.1550 140000	-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	593.1510 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 2. Negative ion mode automatically processed DRE results using OpenLynx software.

Figure 13 shows the calibration curve obtained for 50 µL injections of the standard isoorientin at

PE= *Passiflora edulis*, PC= *Passiflora caerulea*, PI= *Passiflora incarnata*, CPS= Ion counts/second, RT= concentrations of 1 pg/µL, 10 pg/µL, 100 pg/µL, 1000 pg/µL, and 10000 pg/µL. Four orders of linearity were Retention time

obtained with a correlation coefficient of variation $r^2 = 0.9953$. From Figure 13, it is possible to see that at 10000 pg/ μ L there is some suppression in response due to saturation of the electrospray process. The concentration of isoorientin in the 2 mg/mL extracts were determined to be *Passiflora edulis* (11908 pg/ μ L), *Passiflora caerulea* (15384 pg/ μ L), *Passiflora incarnata* (13822 pg/ μ L).

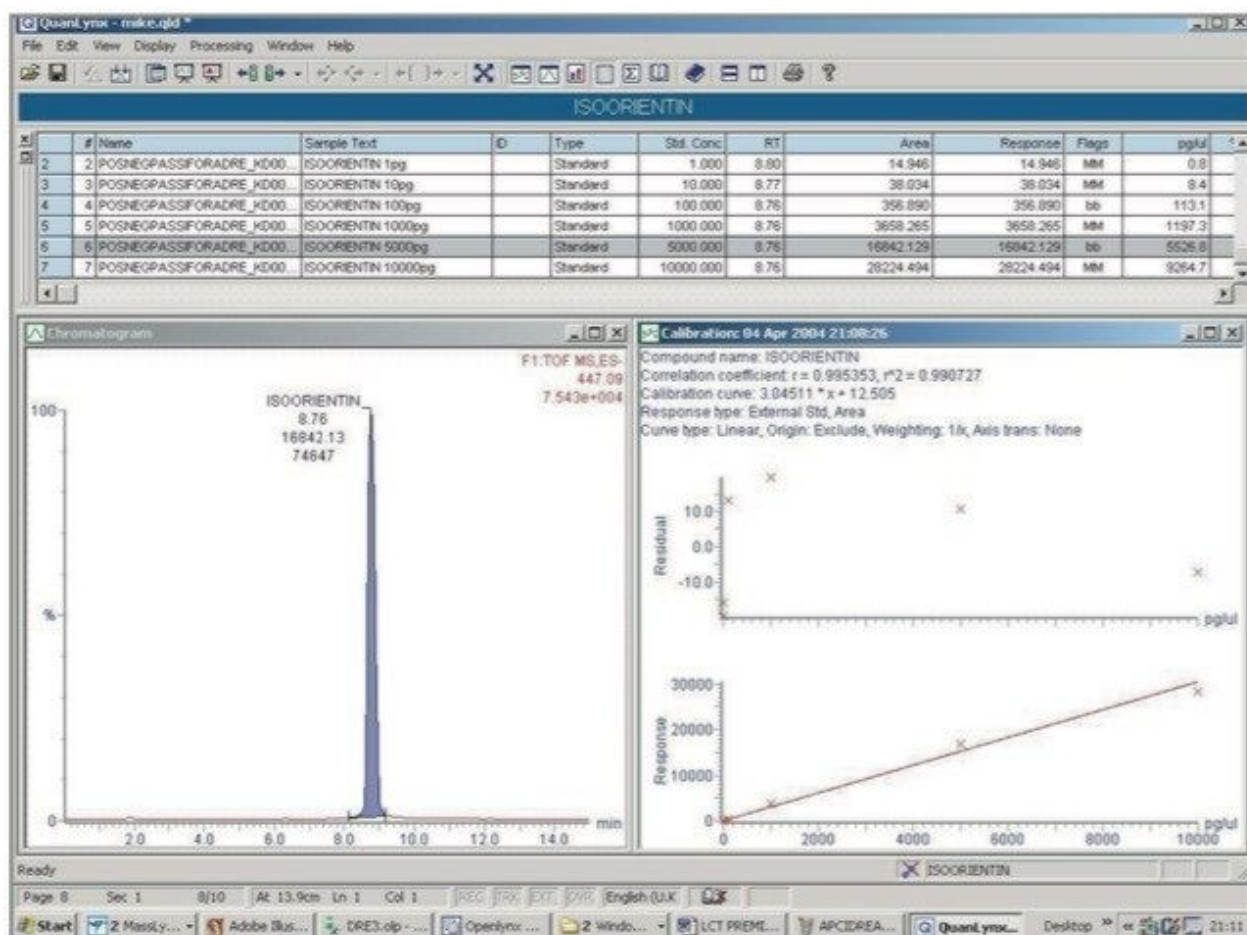


Figure 13. Calibration curve obtained for target flavonoid isoorientin, for 1pg/ μ L to 10000 pg/ μ L.

The analysis was also performed in positive ion DRE mode. Examples of the target flavonoids have been selected and the extracted chromatograms for *Passiflora caerulea* are presented in Figure 14 along with the corresponding mass spectra. As can be seen from Figure 14, all the intensities are above 25000 cps, where saturation of the detector would have previously taken place. Simply selecting the highest response has enabled the correct elemental compositions to be determined within an error range of 3.7 ppm to -0.8 ppm. From Figure 15 and 17 it can be seen that from just selecting the top of the chromatographic peaks routine exact mass measurement can be obtained using DRE.

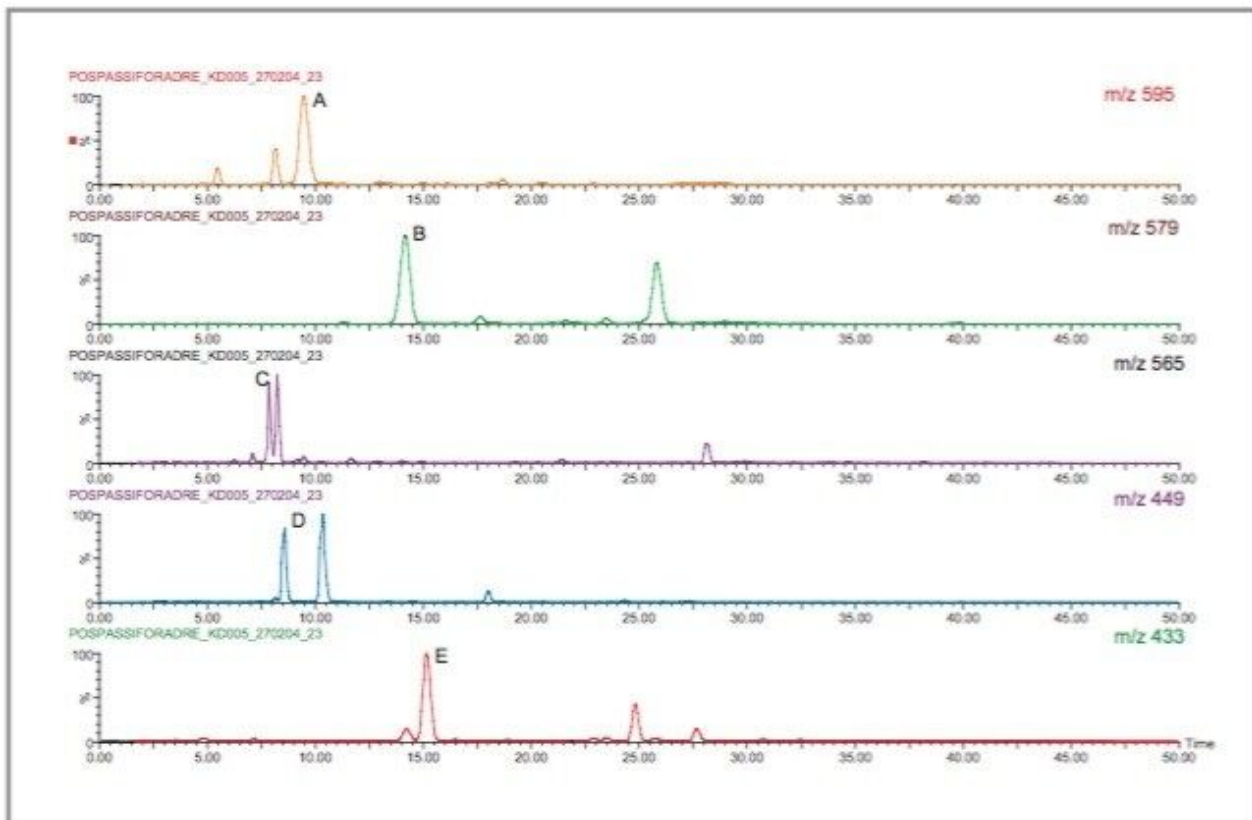


Figure 14. Extracted mass chromatograms m/z 595, m/z 579, m/z 565, m/z 449, and m/z 433 for the *Passiflora caerulea* extract.

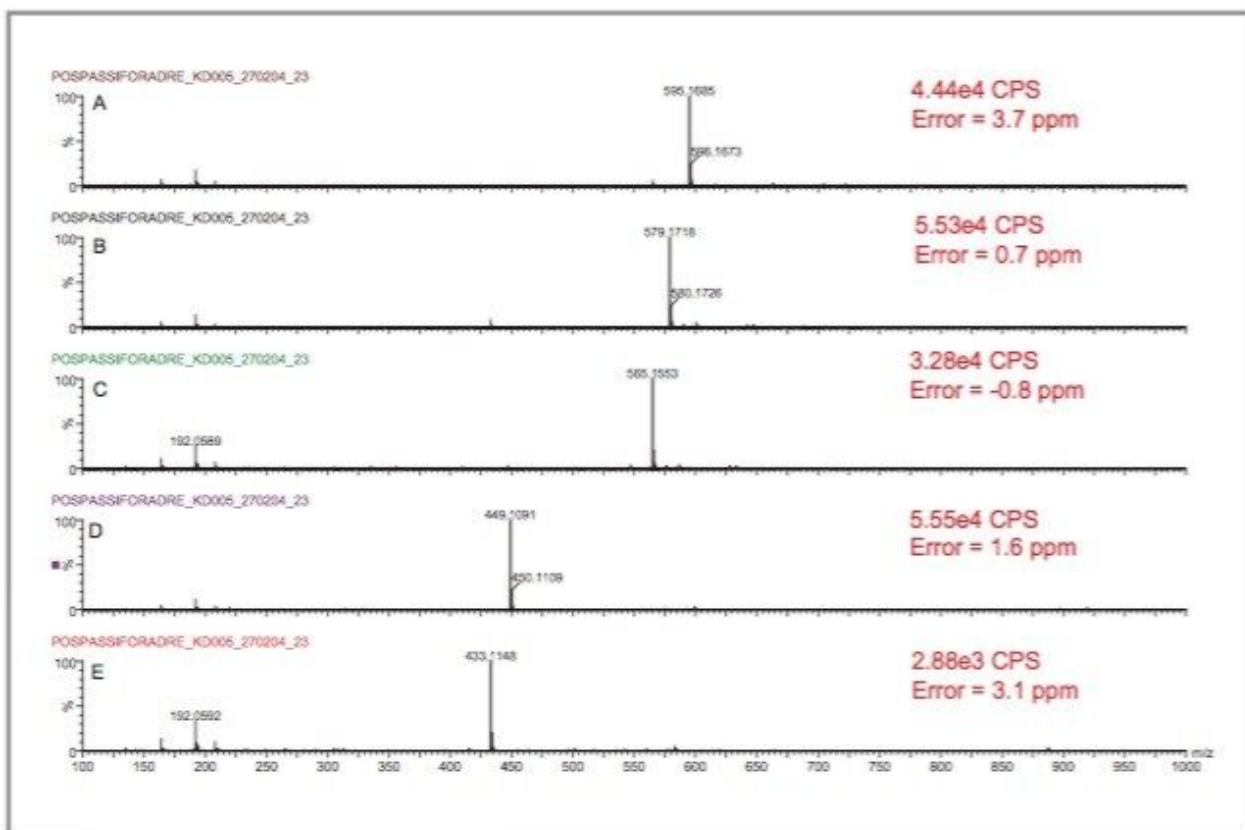


Figure 15. Exact mass spectra and ion intensity responses for obtained for target flavonoids present in *Passiflora caerulea* extract using positive ion mode electrospray DRE.

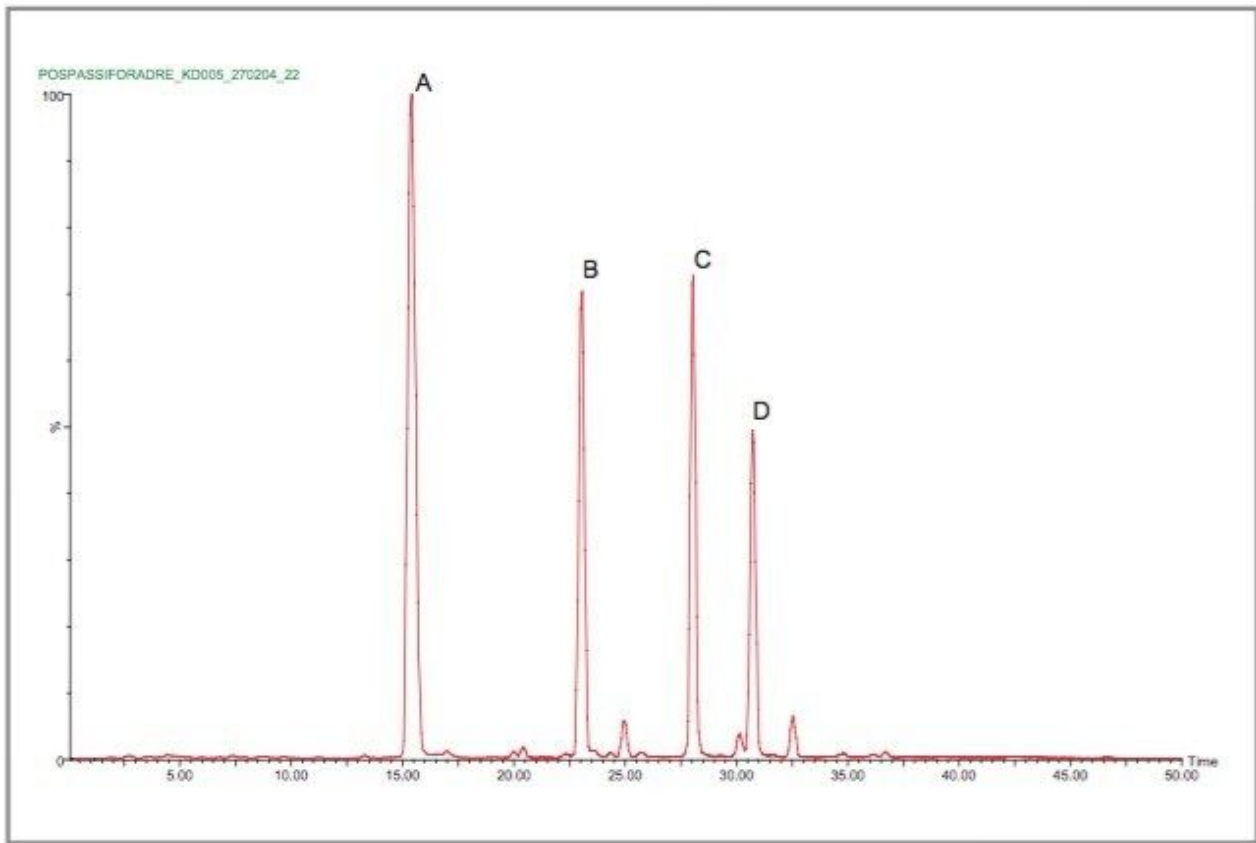


Figure 16. Positive mode m/z 433 extracted mass chromatogram for the *Passiflora edulis* extract.

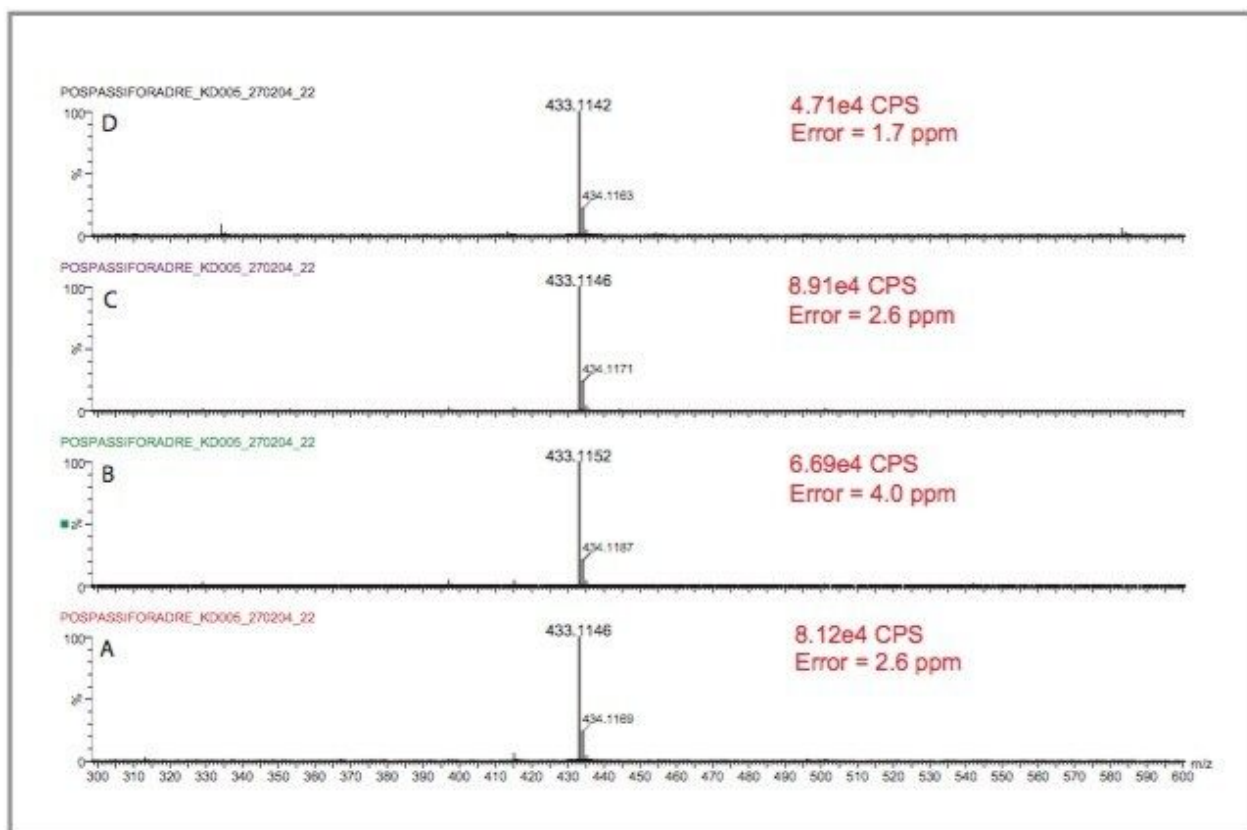


Figure 17. Positive mode exact mass spectra and ion intensity responses for the flavonoid isomers labelled A-D in m/z 433 extracted mass chromatogram for the *Passiflora edulis* shown in Figure 16.

Conclusion

- Enhanced dynamic range has been achieved without compromising resolution; DRE acquisition has been performed routinely at >10000 FWHM resolution.
- Four orders of linearity have been produced routinely and a correlation coefficient of variation $r^2 = 0.9953$ has been illustrated.
- Manual data processing of three plant extracts has produced RMS errors of 1.95 ppm, 1.45 ppm, and 1.34 ppm for the target analytes determined to be present in each species.
- OpenLynx automatic data processing of three plant extracts has produced RMS errors of 1.27 ppm, 1.39 ppm, and 1.49 ppm for the target analytes determined to be present in each species.

- Mass measurement errors of <3ppm have been routinely achieved for single ion accumulations in the order of 250000 cps using W mode.
- Mass measurement errors of <3ppm have been routinely achieved 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.

References

1. Identification of Flavonoids from Passiflora Species Using LC-MS Exact Mass Measurement and In-Source CID, Waters Application Note 720001137EN

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