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Supplementary file 1: References of pure standards used

Table S.1. References of pure standards

Substance	**Purity**	**Supplier**	**Batch number**	**Country of origin**
Andrographis paniculata extract	USP reference standard	USP Rockville, MD	FOI342	United States
Andrographolide	95%	Merck Sigma	102470954	United States
Quercetin hydrate	95%	Fluka	198952 1179	Switzerland
Neoandrographolide	95%	Sigma Aldrich	102482280	United States
Andrographiside	95%	Merck Sigma	102352205	United States

Supplementary file 2: Method of identification of *Andrographis paniculata*

UHPLC-PDA-HRMS/MS screening of standardized extract of Andrographis paniculata and commercial products

Untargeted analyses were performed on a Q-Exactive Focus mass spectrometer (Thermo Scientific, Bremen, Germany) with a heated electrospray interface (HESI-II) coupled to an ACQUITY UPLC (Waters, Milford, MA, USA). HESI conditions included a 2.5 kV source voltage, sheath gas flow of 55, auxiliary gas flow of 15, spare gas flow of 3.0, capillary temperature of 275 °C, and an S-Lens RF level of 45. Analysis occurred in positive and negative ion modes (*m/z* 120-1500 Da). Internal calibration used a standard mix in an acetonitrile/methanol/water solution with 1% formic acid. Separation employed the same column and chromatographic conditions as UHPLC-UV and UHPLC-MS measurements, detailed in the quantification part. The injection volume was 2 µL. Data-dependent MS/MS was performed on the three most intense ions, using a 1 Da isolation window and stepped collision energy of 15, 30, and 45 units. Full scans were acquired at 35,000 FWHM at m/z 200, and MS/MS scans at 17,500 FWHM with automated maximum injection time. The three most intense peaks were identified using exact mass and pure standards of andrographolide, neoandrographolide, and andrographiside. The UV spectrum of the andrographolide peak was recorded before quantification.

Supplementary file 3: Sample preparation

Samples were prepared by crushing capsules or tablets with a pestle and mortar. Around 1–5 mg of the homogenized sample was weighed and transferred to a 2 mL Eppendorf tube, then extracted with 1 mL of 100% MeOH. Glass beads (2µm) were added, and the mixture was ground in a Retsch MM200 ball mill (30 Hz, 4 min), then centrifuged (13,000 rpm, 3 min). The supernatant was collected, and the pellet was re-extracted with 500 µL MeOH. Both supernatants were combined (1.5 mL total) and evaporated to dryness using a vacuum concentrator prior to UHPLC analysis. The liquid products were simply diluted tenfold in water: methanol 50:50 solution (v/v) before injection.

Supplementary file 4: Method of quantification of andrographolide

UHPLC-UV and UHPLC-MS quantification

Analyses were carried out on an Acquity UPLC system (Waters, Milford, MA, USA) equipped with a Waters UPLC BEH C18 column (50 mm × 2.1 mm ID, 1.7 µm). Samples were kept at 10°C in an autosampler. The solvent system consisted of water: 0.1% formic acid (FA) for solvent A and acetonitrile: 0.1% FA for solvent B. The elution was performed in gradient mode with a flow rate 500 µL/min and the column was maintained at 40◦C in the dedicated compartment. The gradient steps were rigorously defined after geometrical gradient transfer from the standard Pharmacopeia HPLC method for quantification of andrographolide (22, 23). In details, B was held at 10% for 0.52 min; 10-38% of B was achieved in 1.33 min and maintained for 2.33 min; and finally, 38-50% of B was achieved in 0.84 min followed by re-equilibration step at 10% of B. This resulted in an andrographolide retention time of 2.1 min under the specified chromatographic conditions.

UV quantification was performed using a PDA detector (Waters, Milford, MA, USA) scanning the 190–300 nm range with a spectral resolution of 1.2 nm. Data acquisition was set at 20 points per second, ensuring a minimum of 10 data points per andrographolide peak for precise quantification.

MS quantification was performed on a Xevo TQ-S micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA) with an ESI interface operating in negative ionization mode. The source temperature was 150 ◦C, the desolvation temperature was 500 ◦C, the capillary voltage was 2.6 kV and the desolvation Gas Flow was 1000 L/Hr. Compound-dependent parameters were all optimized using the fluidics module with the flow state set in combined mode (direct infusion mixed with the LC flow). The cone voltage optimum was found to be at -25 V. The Dwell time was set at 25 msec. These parameters allowed the collection of at least 10 data points for the andrographolide chromatographic peak. For each *A. paniculata* containing product, quantification was performed in triplicate. Injection volume was 4 µL. Andrographolide was targeted with in single ion monitoring (SIM) mode. The SIM method was set at *m/z*: 395.00 ($[M+HCOO]^-$ adduct form of andrographolide) for the measurement of calibration points in both the pure standard and commercial samples.

Validation of methods of quantification of andrographolide

Table S.2. Linearity of calibration curves of Andrographolide with UPHLC-UV and UPHLC-MS quantification and determination of the LOD and LOQ

Quantification Method	Linear function	r^2	LOD	LOQ	Calibration ranges
UV	$y = 293.98x + 719.9$	0.998	2.59 (μg/mL)	8.33 (μg/mL)	10 – 250 μg/mL
MS	$y = 561.04x - 101.83$	0.999	0.21 (ng/mL)	0.70 (ng/mL)	1 – 40 ng/mL

r^2, Correlation coefficient; LOD, Limit of Detection; LOQ, limit of quantification

LOD and LOQ were automatically determined using the signal-to-noise factor measured by TargetLynx software. A concentration of andrographolide corresponding to a S/N ratio of 10 defined the LOQ.

Table S.3. Precision and accuracy of targeted UHPLC-MS (SIM method)

QC samples (*n*=6)	Concentration (ng/mL)	Measured	Precision (%, RSD)		Accuracy (%)
			Intra-day	Inter-day	
Low	0.75	0.70 ± 0.06	7.77	6.04	93.6
Mid	8	7.83 ± 0.64	8.22	10.62	97.9
High	20	19.40 ± 1.26	6.51	8.27	97.0

As no analyte-free matrix was available, the precision and accuracy of the method were assessed using QC samples corresponding to 3 concentrations (low, medium and high) of the measurement range. These samples were prepared from a stock solution independent of the solution required for their measurement. Intra-day measures were done on the same day whereas inter-day were assessed on two consecutive days. RSD were below 15% for every concentration range.

Matrix effects (ME) were assessed using a standard addition method in which 6 extracted products were spiked with a known concentration of andrographolide and measured against non-spiked products. The calculated ratio was 89.9%, indicating low ion suppression and that ME are acceptable.

Supplementary file 5: Identification of andrographolide and any co-eluate

The chromatogram of the reference standard of *Andrographis paniculata (Burm.f.) Nees*, is provided below, as well as the structure of the main component and pharmaceutically active substance, andrographolide. This plant belongs to the Acanthaceae family.

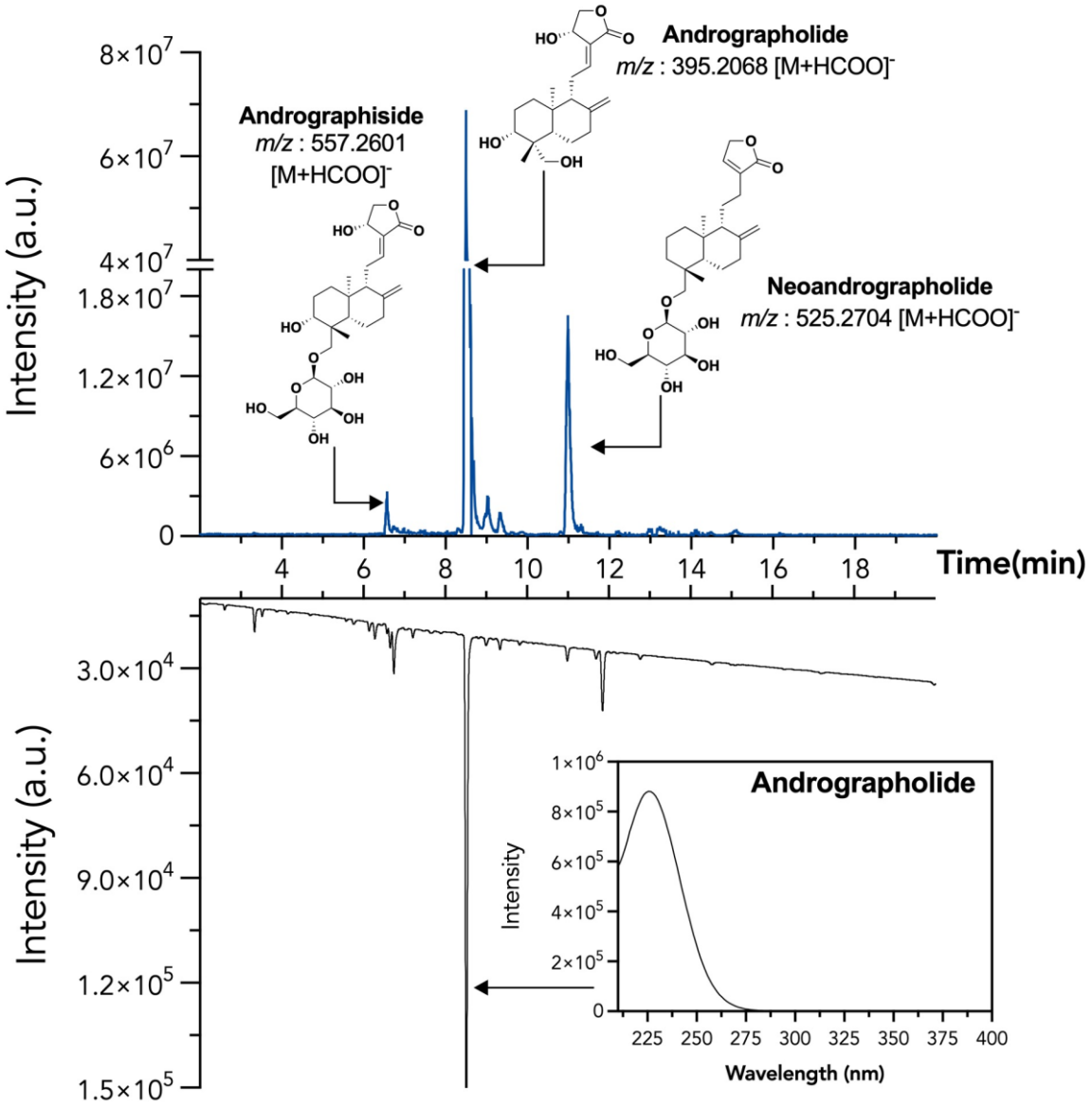
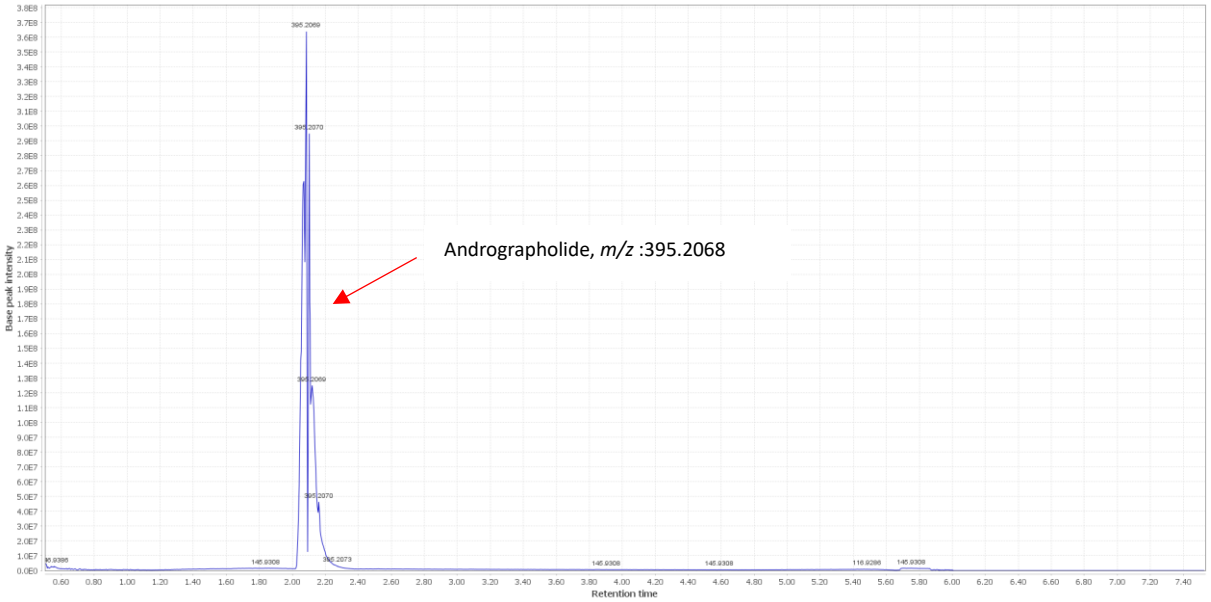


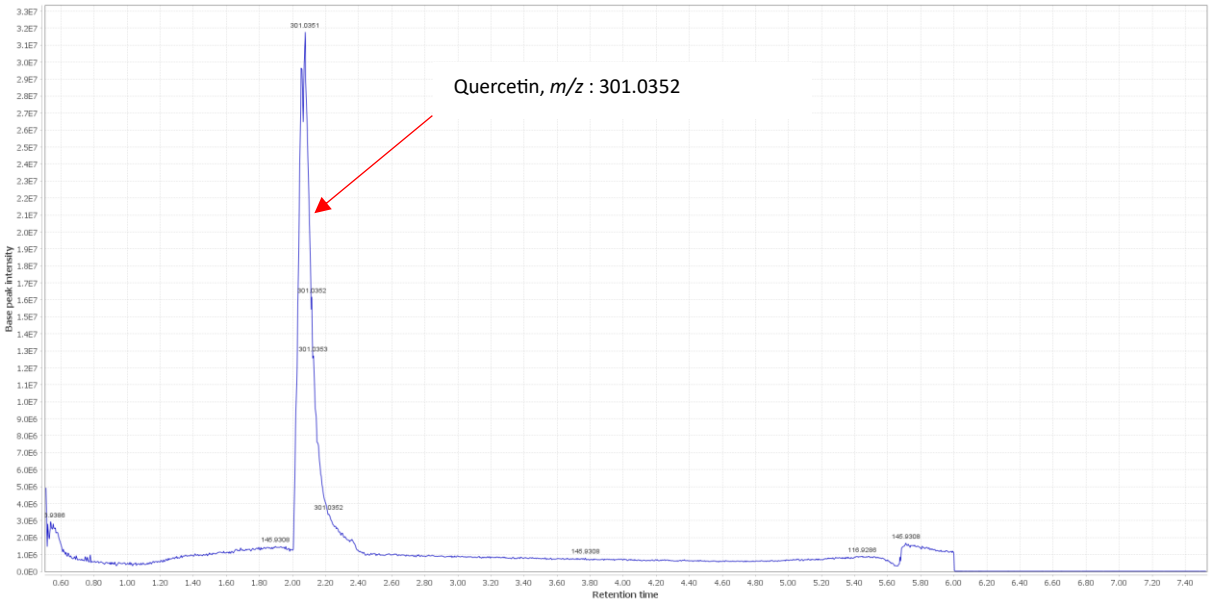
Figure S.1. UHPLC-PDA-HRMS analysis of the standardized Andrographis paniculata extract. The HRMS trace acquired in negative ionization mode (blue line) and the PDA trace (black line) are shown. The highest signal in each was that of andrographolide. In MS, andrographolide was observed as a formate adduct. The UV spectrum of andrographolide has been extracted from the UV chromatogram and is shown in the bottom right inset.

Further analyses were performed to determine if substances other than those contained in *Andrographis paniculata* could have coeluted. Below, you will find the chromatograms obtained with UHPLC-HRMS for the pure standard of andrographolide and quercetin, and the samples. Peaks were identified in samples using the results obtained with the pure standards and the reference standard.

Andrographolide pure standard (*m/z* : 395.2069)



Quercetine pure standard (*m/z* : 301.0351)



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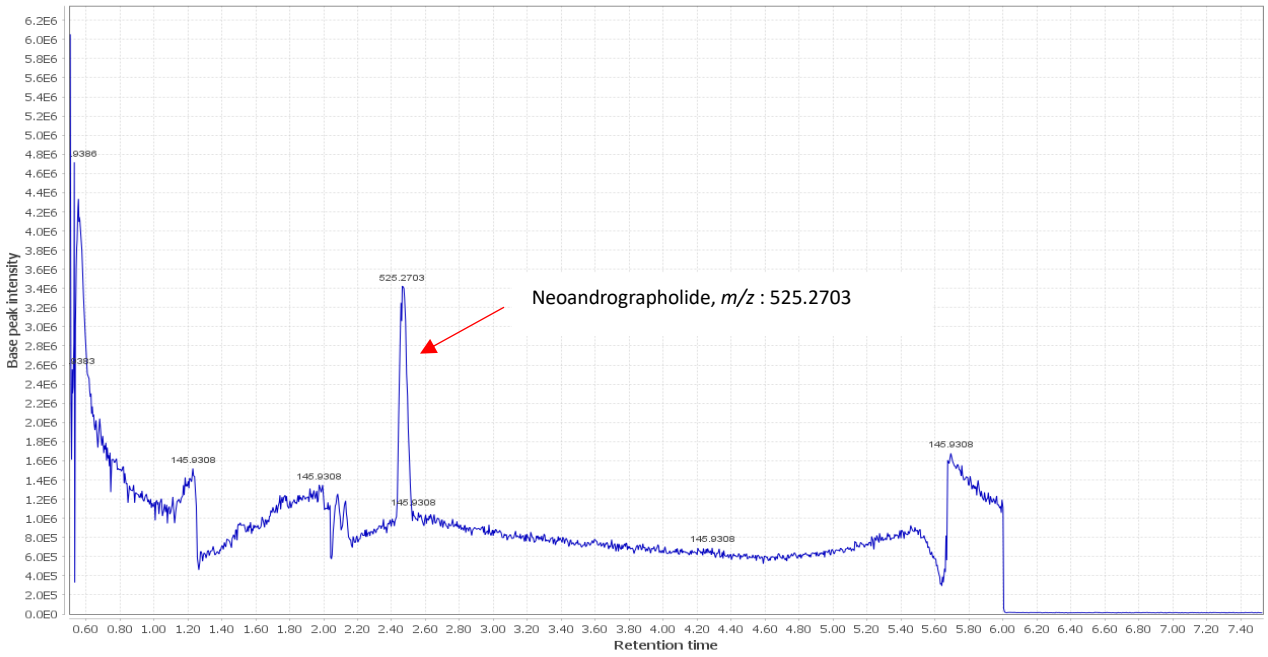
Base peak intensity
Retention time
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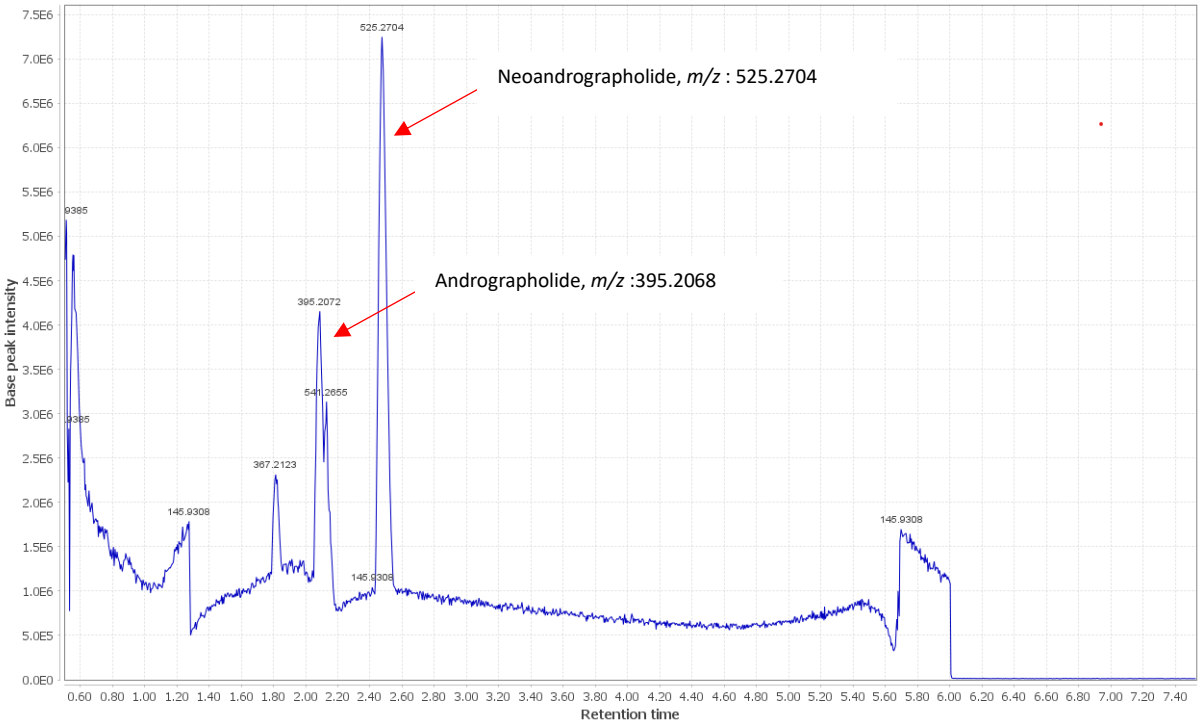
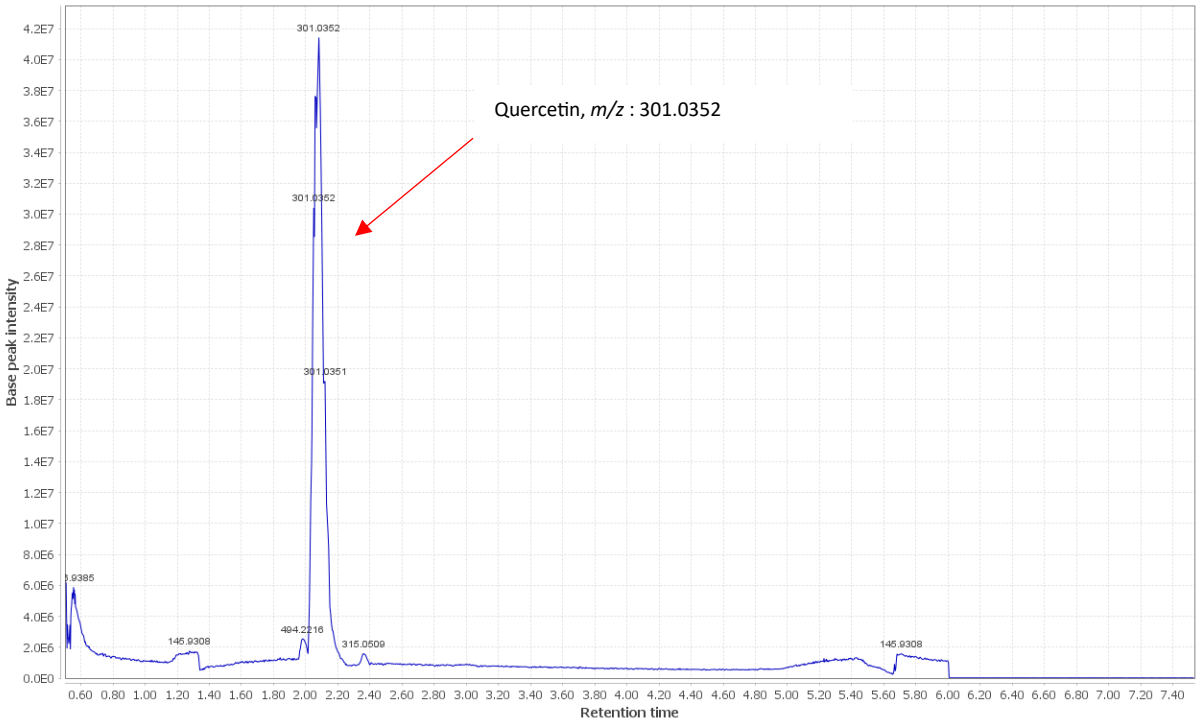
Neoandrographolide, m/z : 525.2703
145.9308

Ph2:


Base peak intensity
Retention time
525.2704
Neoandrographolide, m/z : 525.2704
Andrographolide, m/z :395.2068
395.2072
541.2655
367.2123
145.9308

Ph3:


Quercetin, *m/z* : 301.0352
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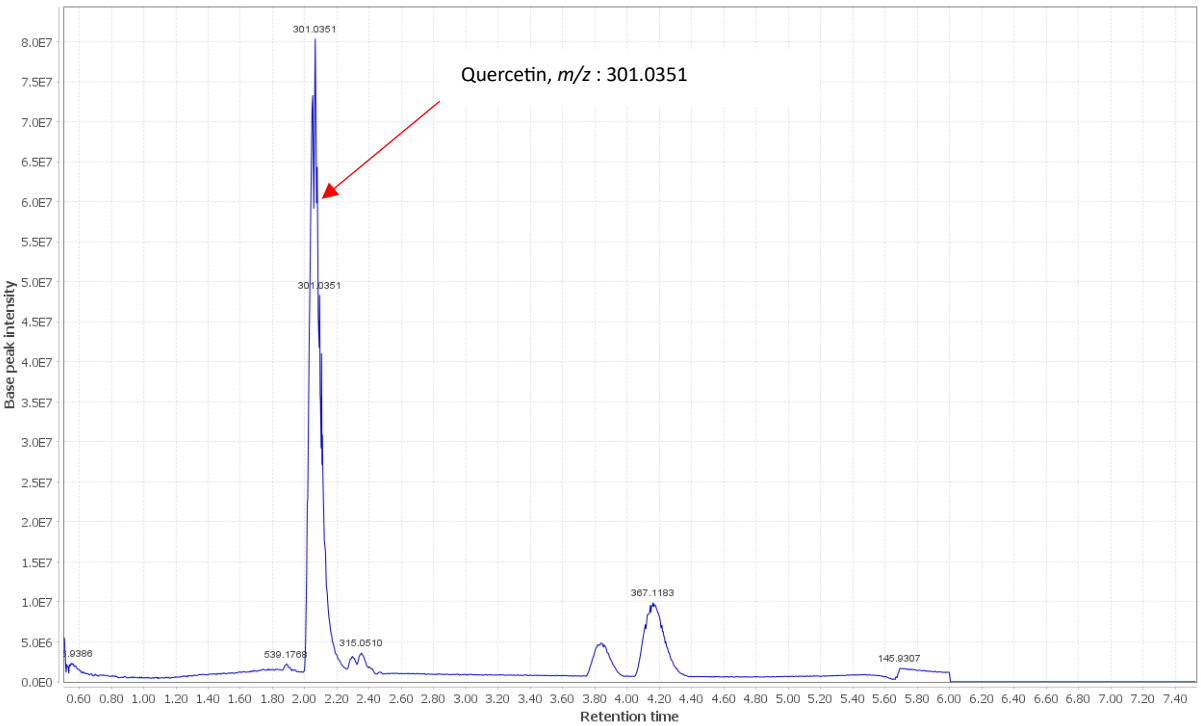
301.0352
301.0351
494.2216
315.0509
145.9308
145.9308
Base peak intensity
Retention time

Ph4:


Quercetin, *m/z* : 301.0351
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315.0510
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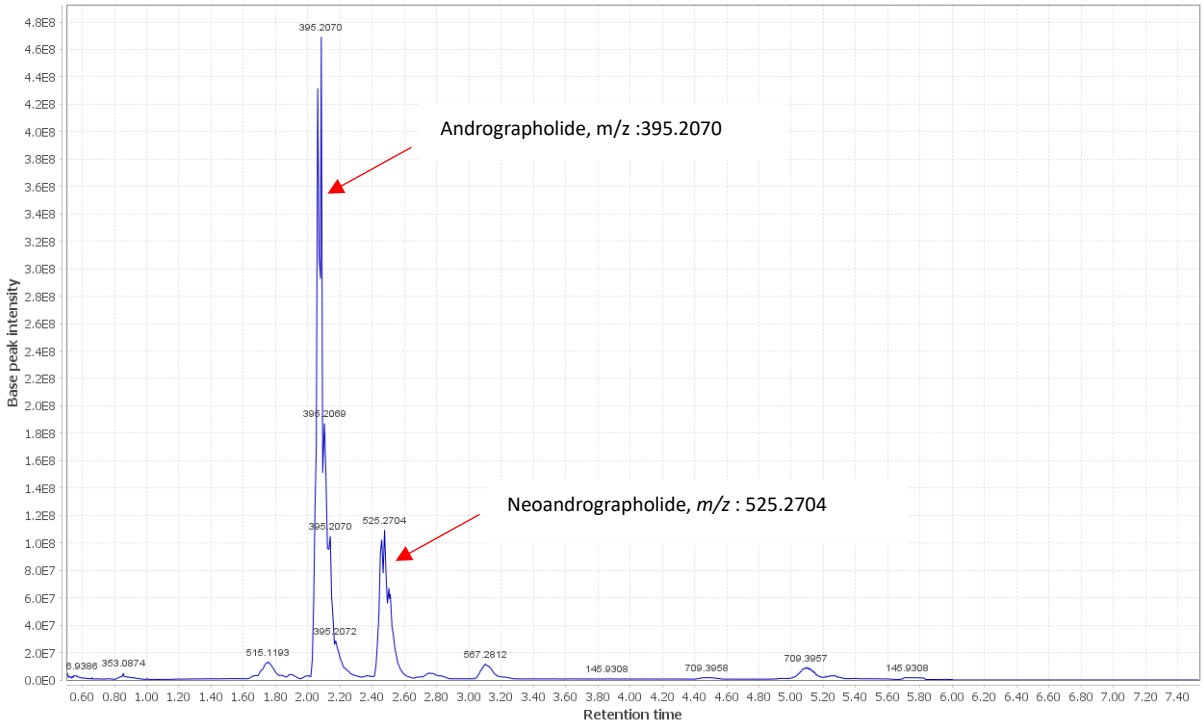
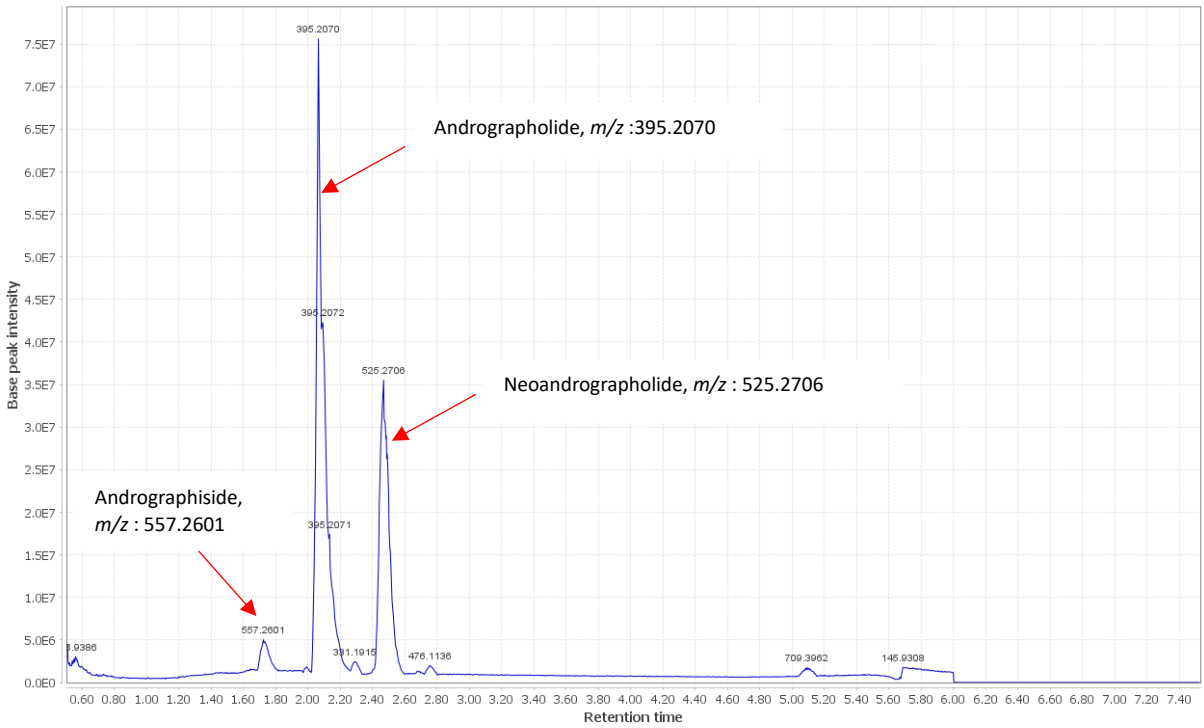
145.9307
Base peak intensity
Retention time

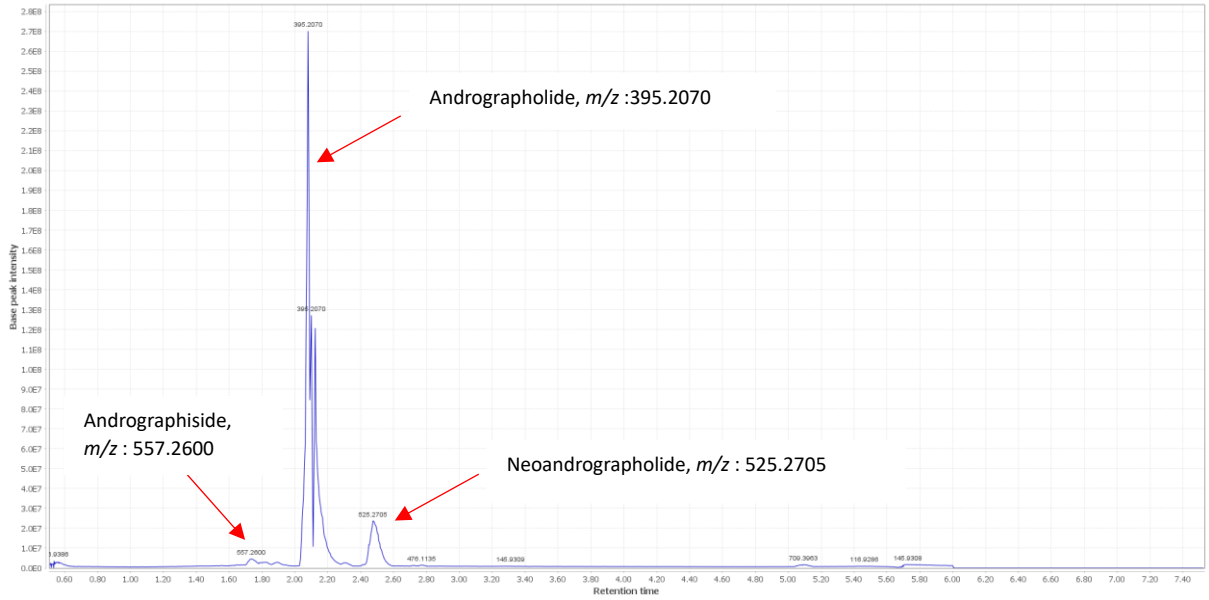
Ph5


Andrographolide, m/z :395.2070
Neoandrographolide, m/z : 525.2704
Base peak intensity
Retention time
395.2070
395.2069
395.2070
525.2704
395.2072
515.1193
567.2812
353.0874
145.9308
709.3958
709.3957
145.9308

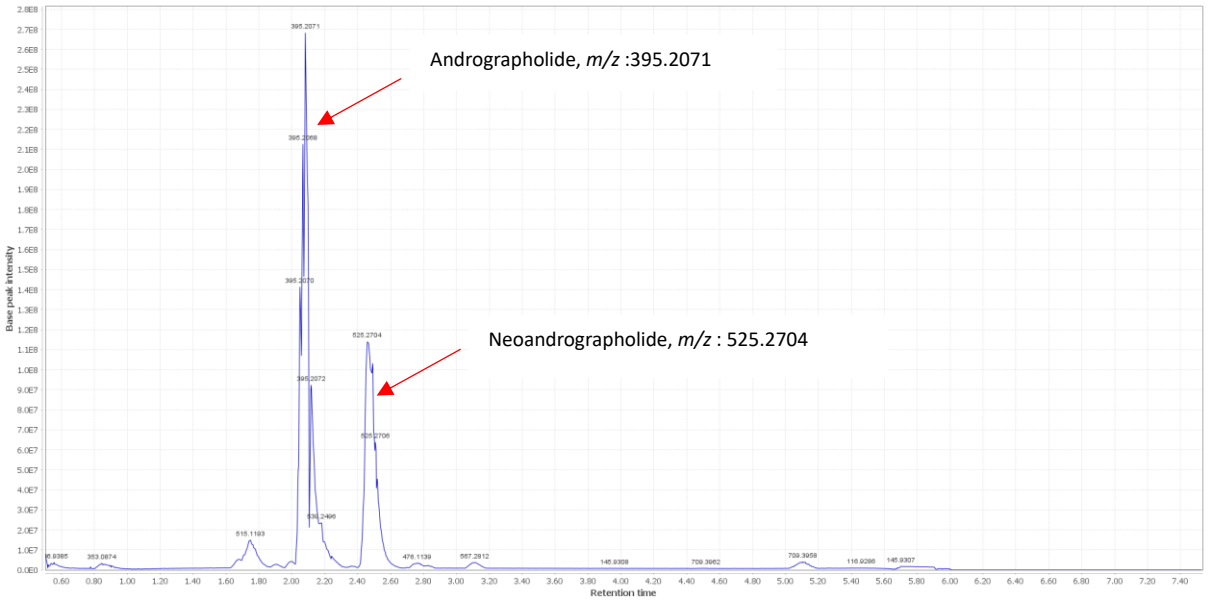
Ph6


Andrographolide, m/z :395.2070
Neoandrographolide, m/z : 525.2706
Andrographiside, m/z : 557.2601
Base peak intensity
Retention time
395.2070
395.2072
525.2706
395.2071
557.2601
331.1915
476.1136
709.3962
145.9308

Ph7


Andrographolide, *m/z* :395.2070
Andrographiside, *m/z* : 557.2600
Neoandrographolide, *m/z* : 525.2705
Base peak intensity
Retention time

Ph8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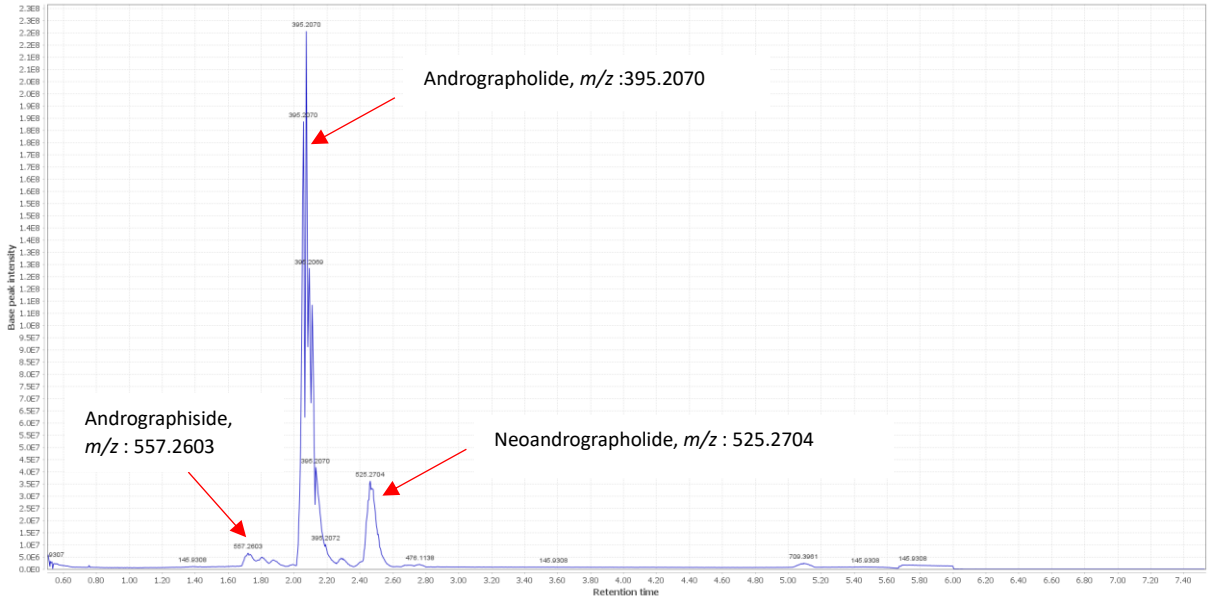
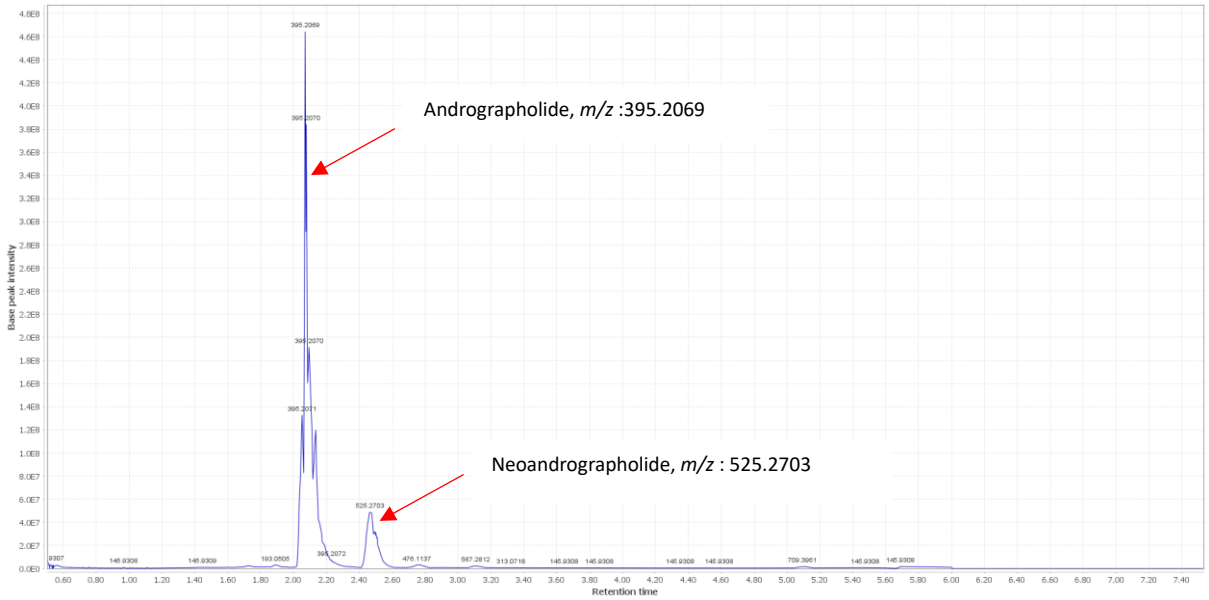
Neoandrographolide, *m/z* : 525.2704
Base peak intensity
Retention time

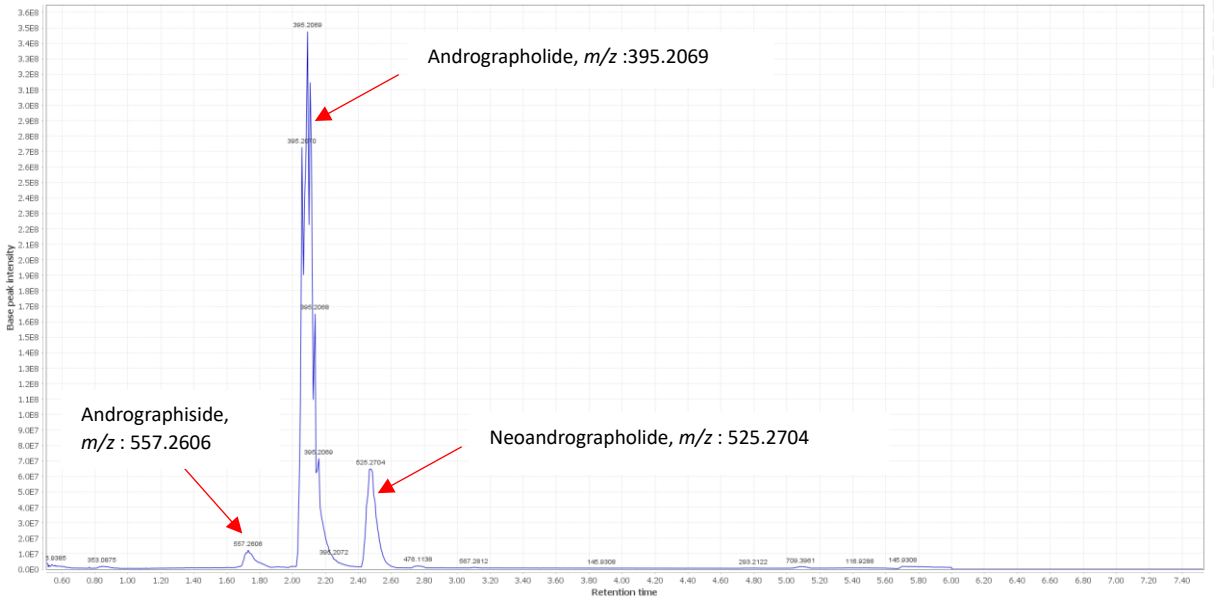
Ph9


Andrographolide, *m/z* :395.2070
Andrographiside, *m/z* : 557.2603
Neoandrographolide, *m/z* : 525.2704
Base peak intensity
Retention time

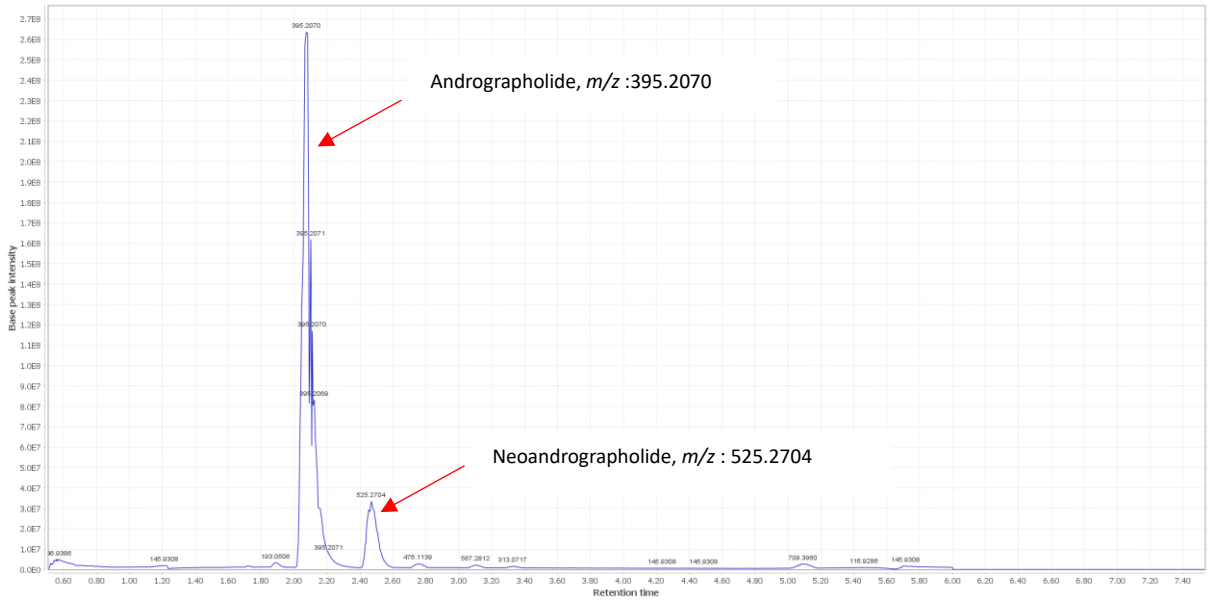
Ph10


Andrographolide, *m/z* :395.2069
Neoandrographolide, *m/z* : 525.2703
Base peak intensity
Retention time

Ph11


Andrographolide, *m/z* :395.2069
Andrographiside, *m/z* : 557.2606
Neoandrographolide, *m/z* : 525.2704
Base peak intensity
Retention time

Ph12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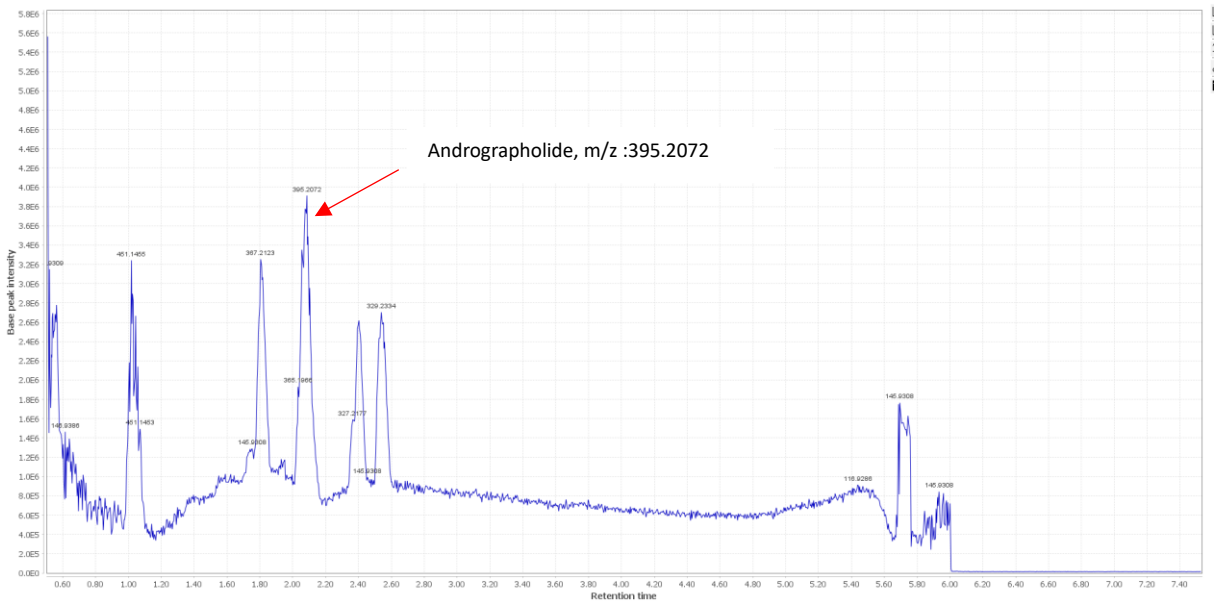
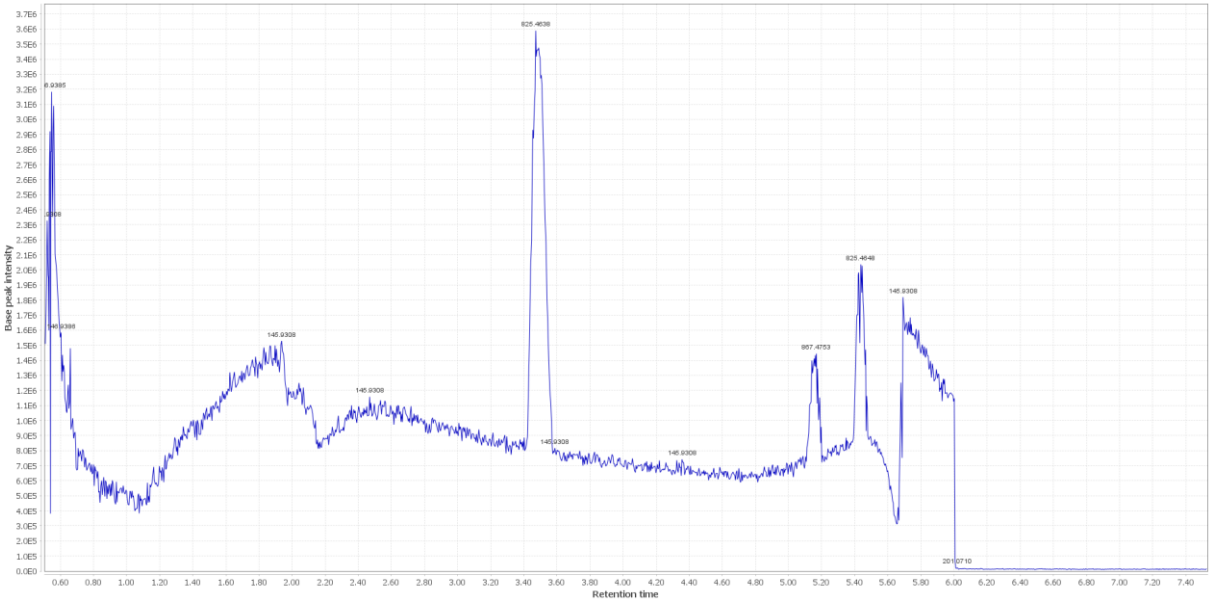
Neoandrographolide, *m/z* : 525.2704
Base peak intensity
Retention time

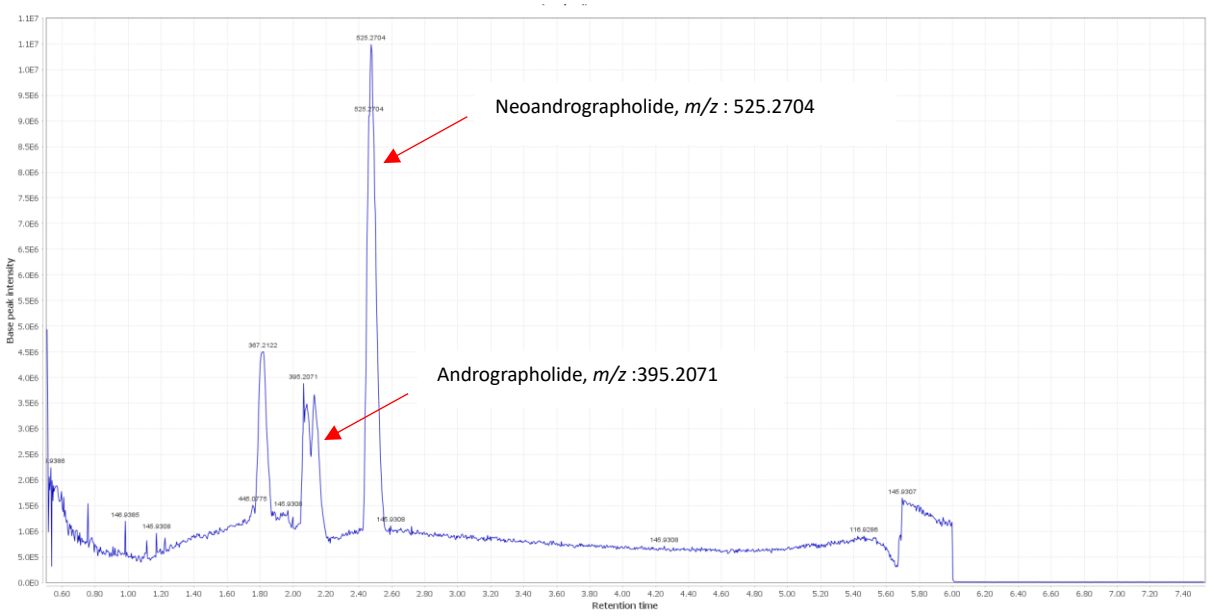
Ph13


Andrographolide, m/z :395.2072
Base peak intensity
Retention time

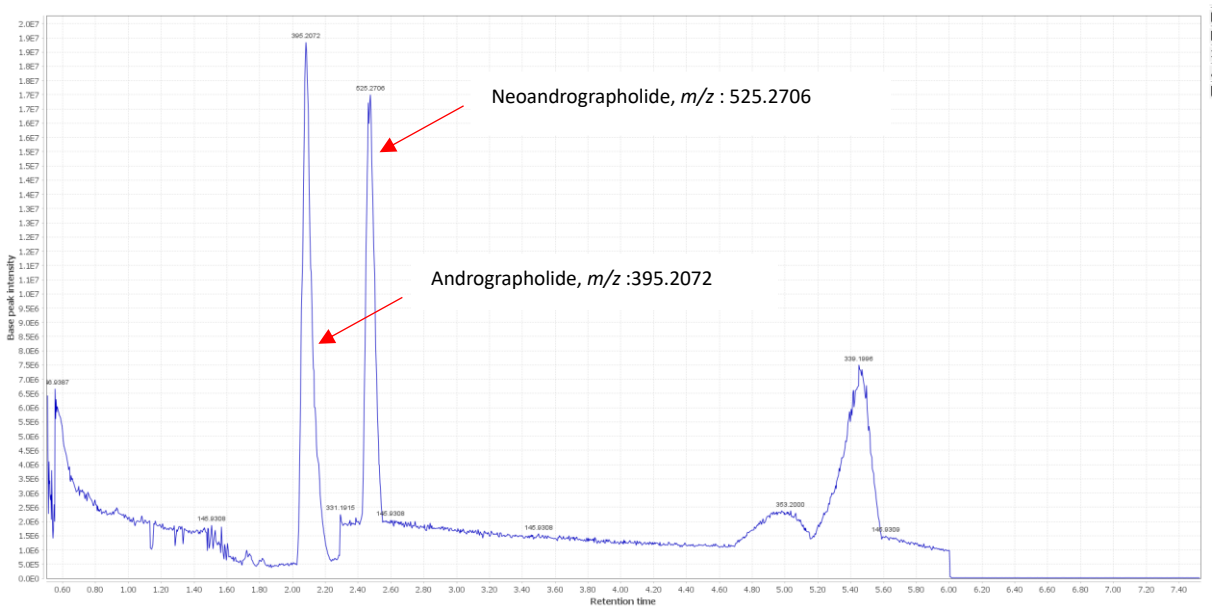
On1


Base peak intensity
Retention time

On2


Neoandrographolide, *m/z* : 525.2704
Andrographolide, *m/z* :395.2071
Base peak intensity
Retention time

On3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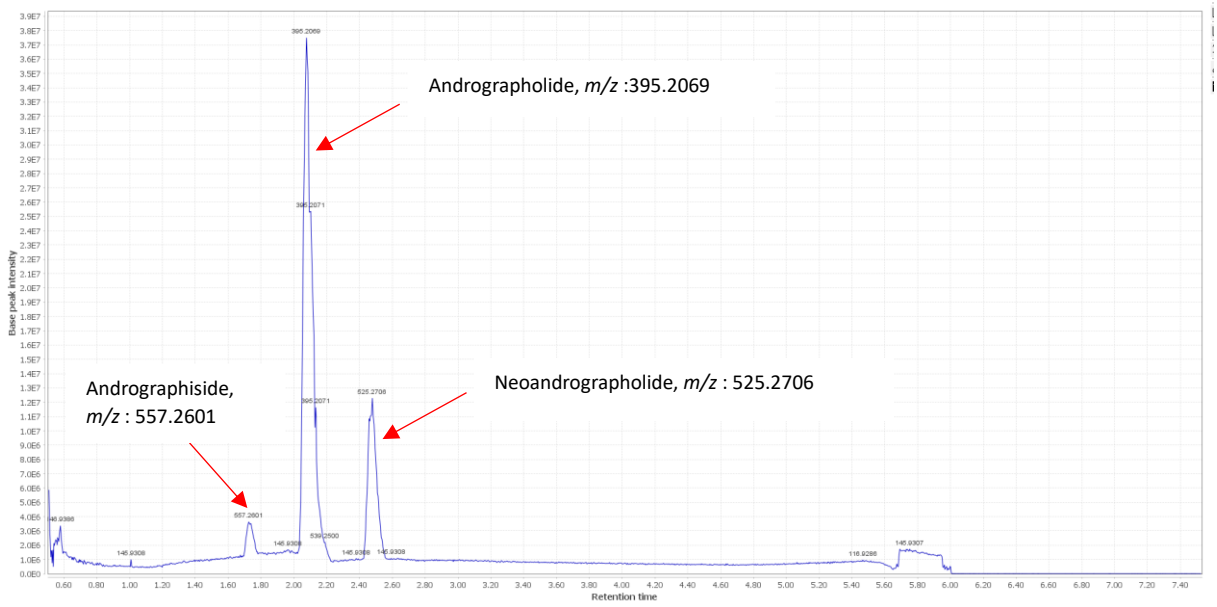
Base peak intensity
Retention time

On4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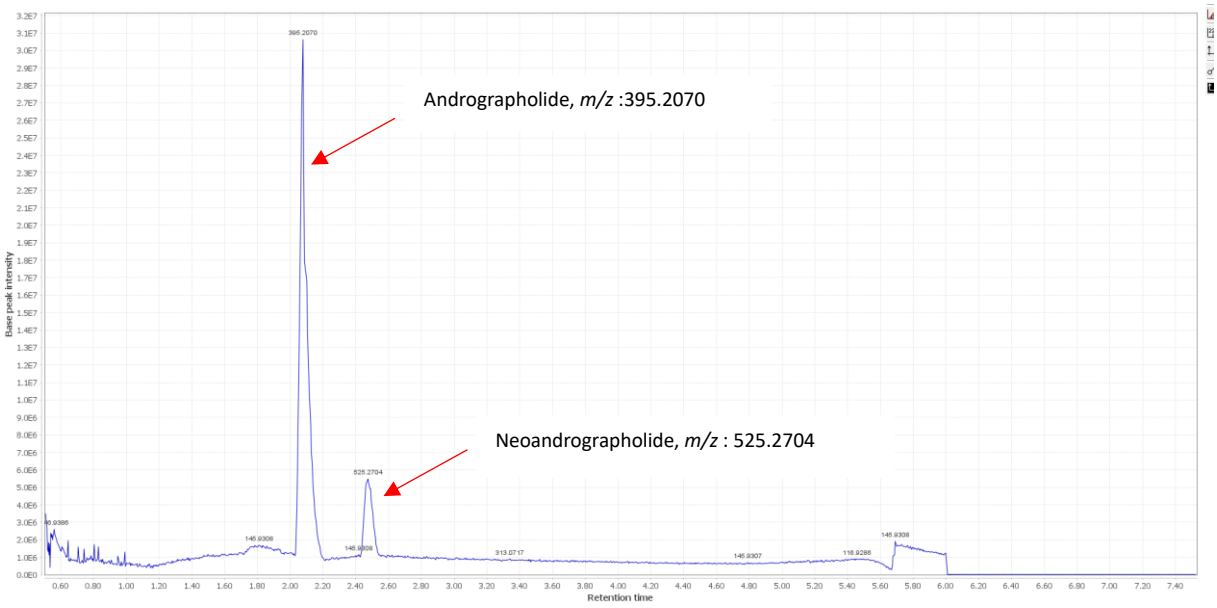
Base peak intensity
Retention time

On5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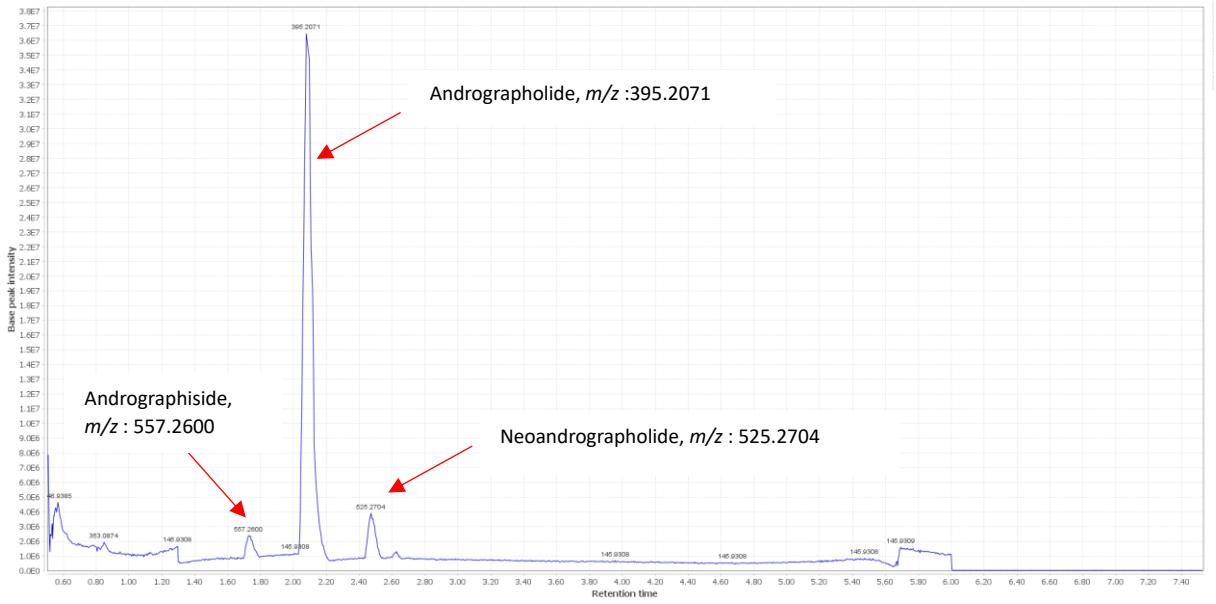
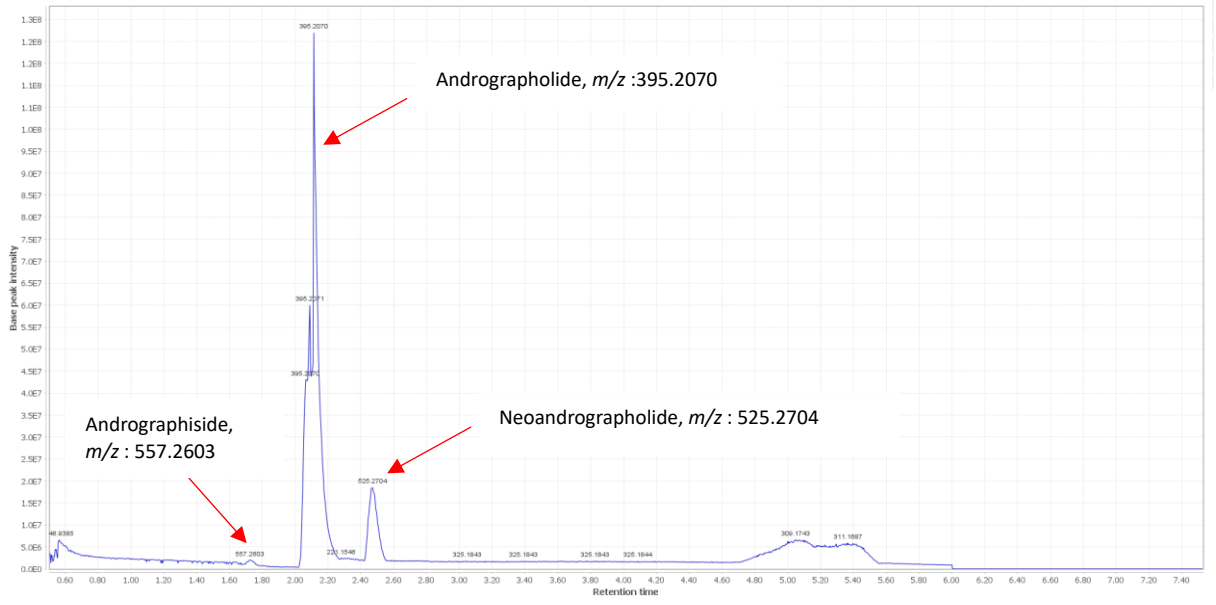
Neoandrographolide, *m/z* : 525.2704
Base peak intensity
Retention time

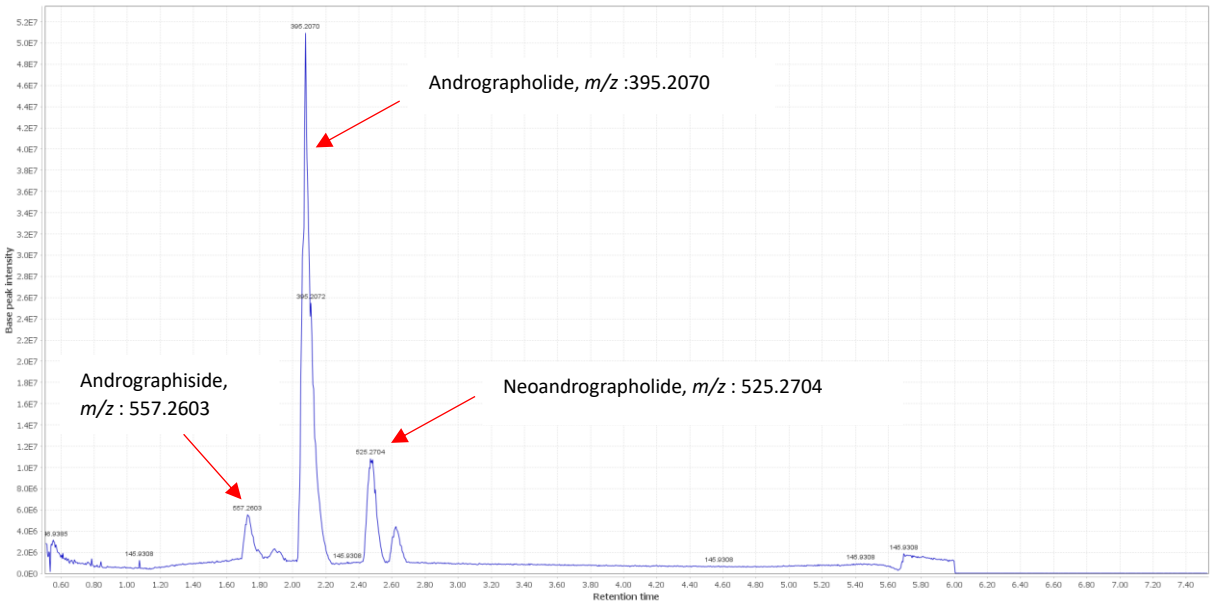
On6


Andrographolide, *m/z* :395.2071
Andrographiside, *m/z* : 557.2600
Neoandrographolide, *m/z* : 525.2704
Base peak intensity
Retention time

On7


Andrographolide, *m/z* :395.2070
Andrographiside, *m/z* : 557.2603
Neoandrographolide, *m/z* : 525.2704
Base peak intensity
Retention time

On8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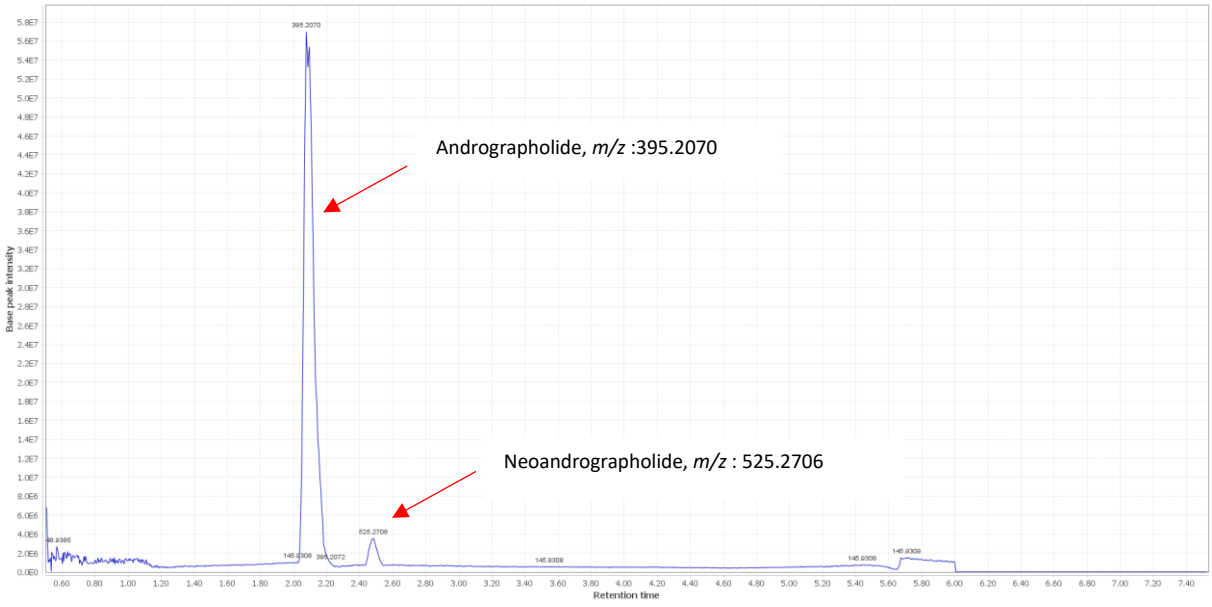
Base peak intensity
Retention time

On9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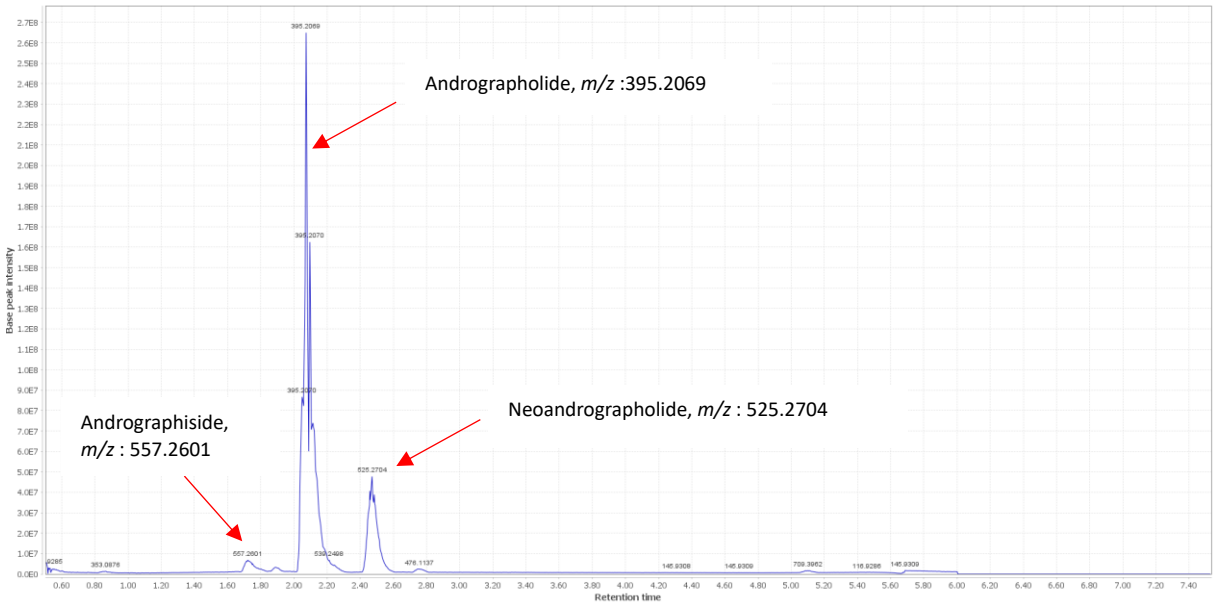
Base peak intensity
Retention time

On10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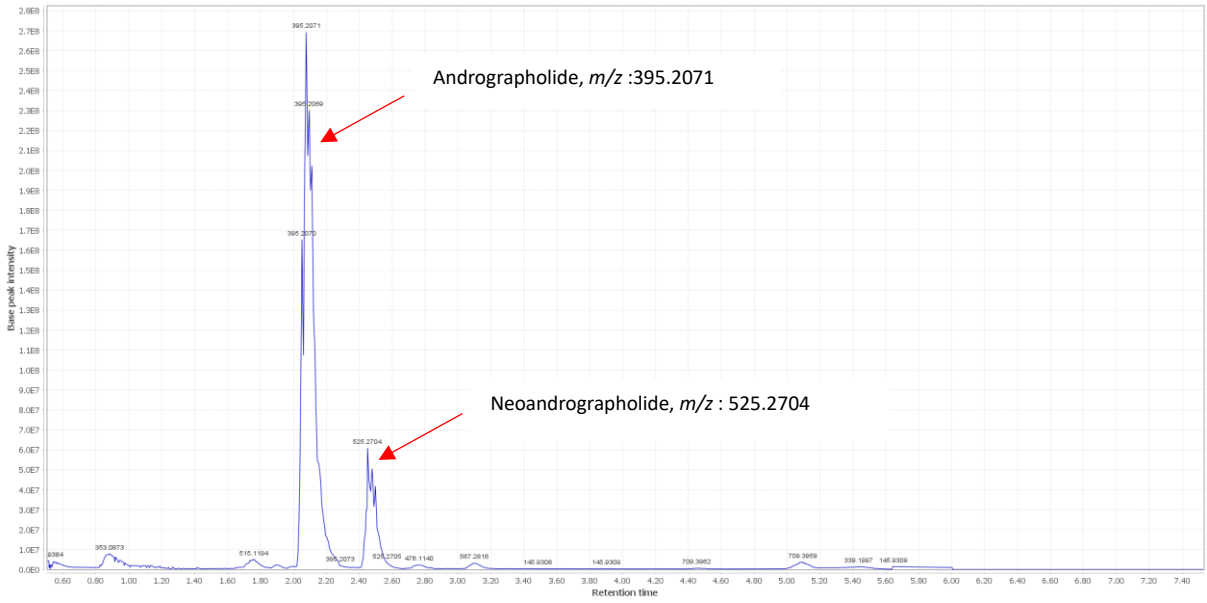
Neoandrographolide, *m/z* : 525.2704
Base peak intensity
Retention time

On11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Base peak intensity
Retention time

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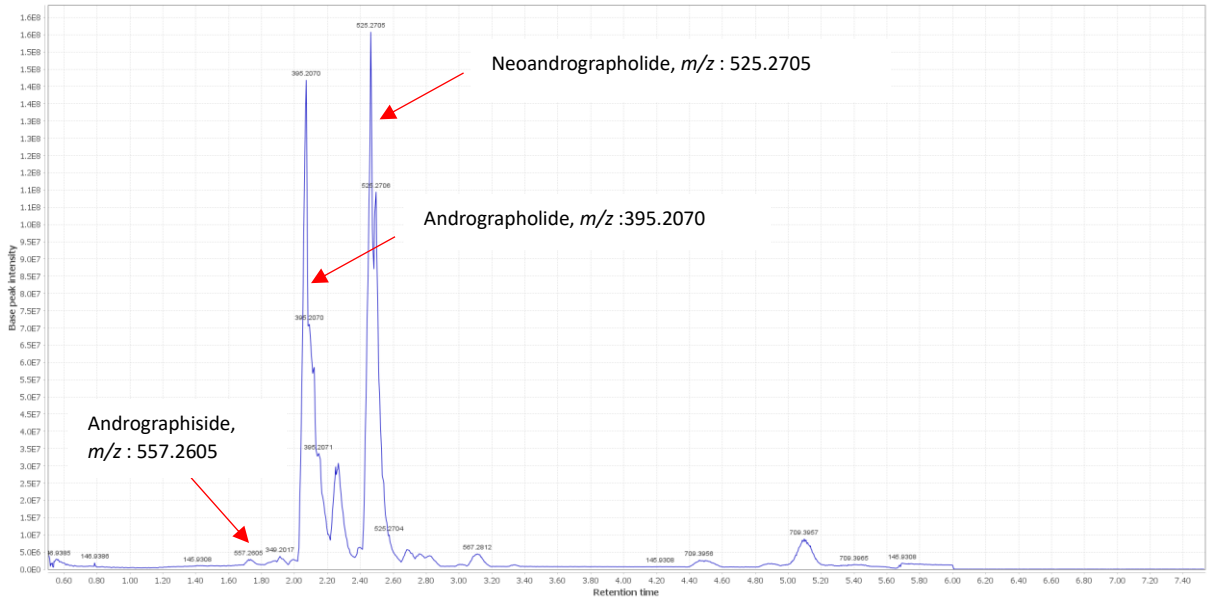
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Andrographolide, *m/z* :395.2070
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Base peak intensity
Retention time

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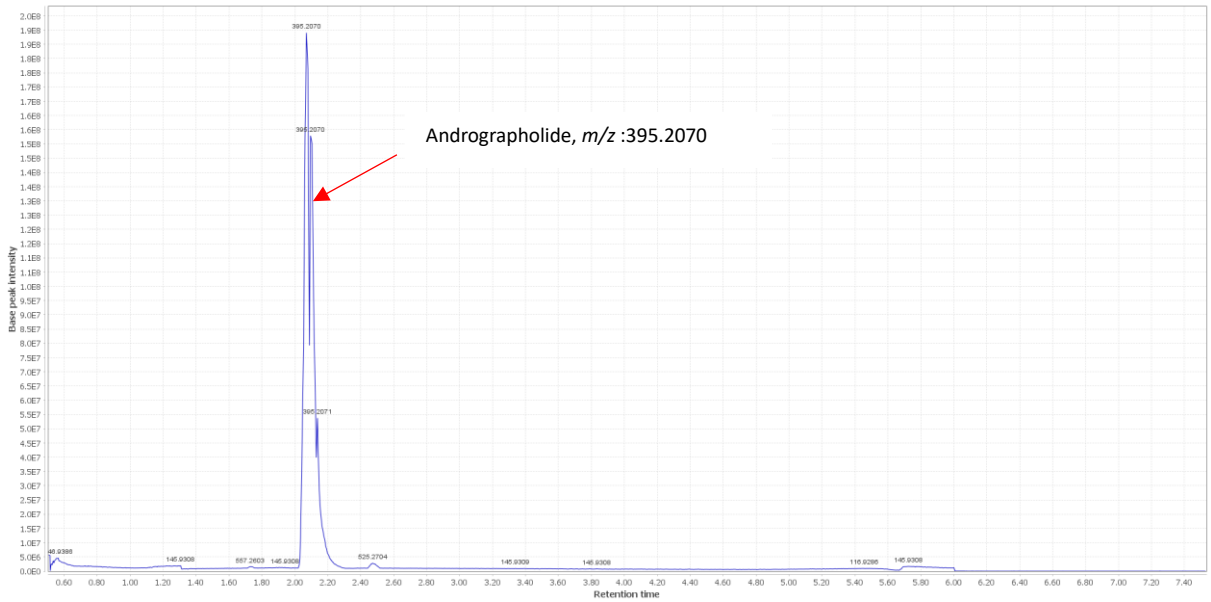
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Base peak intensity
Retention time

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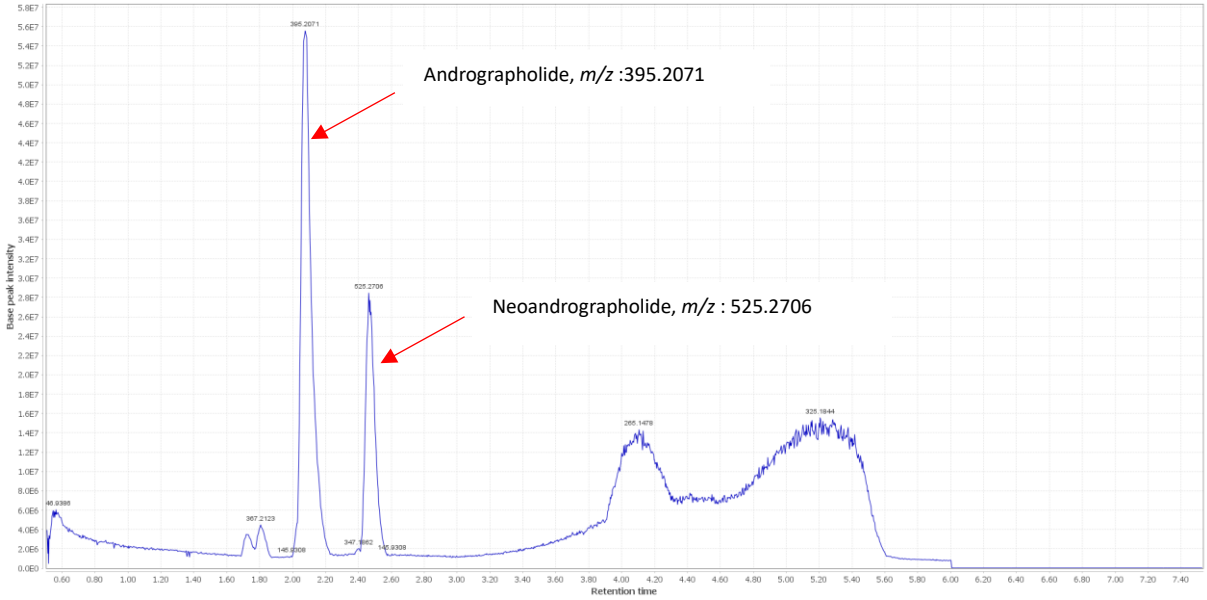
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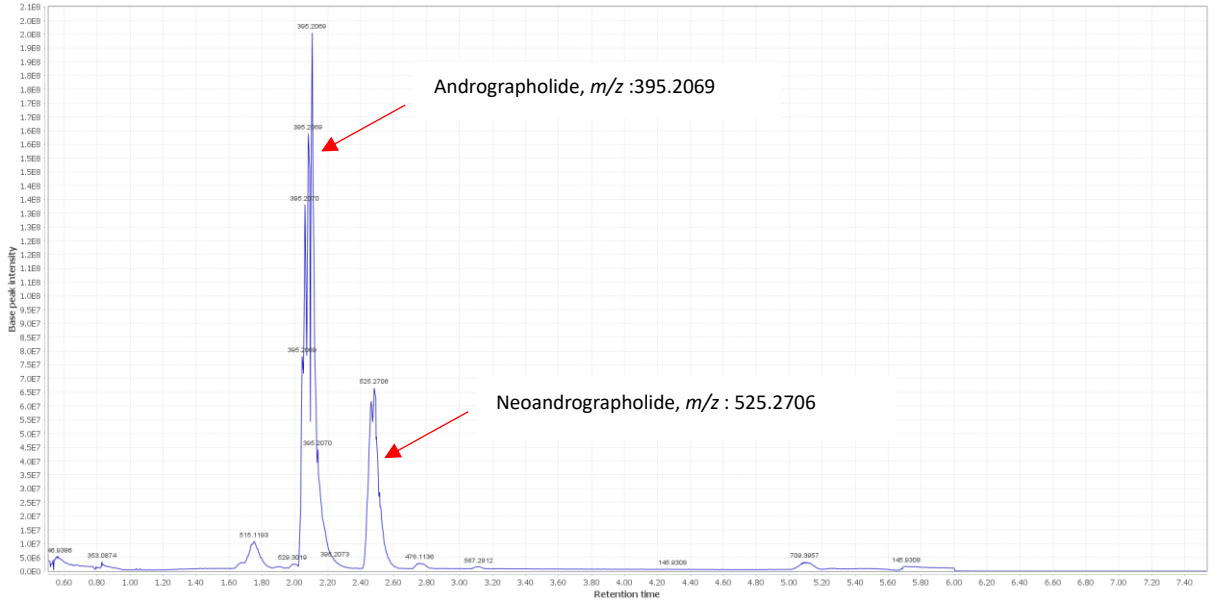
Neoandrographolide, *m/z* : 525.2706
Base peak intensity
Retention time

On15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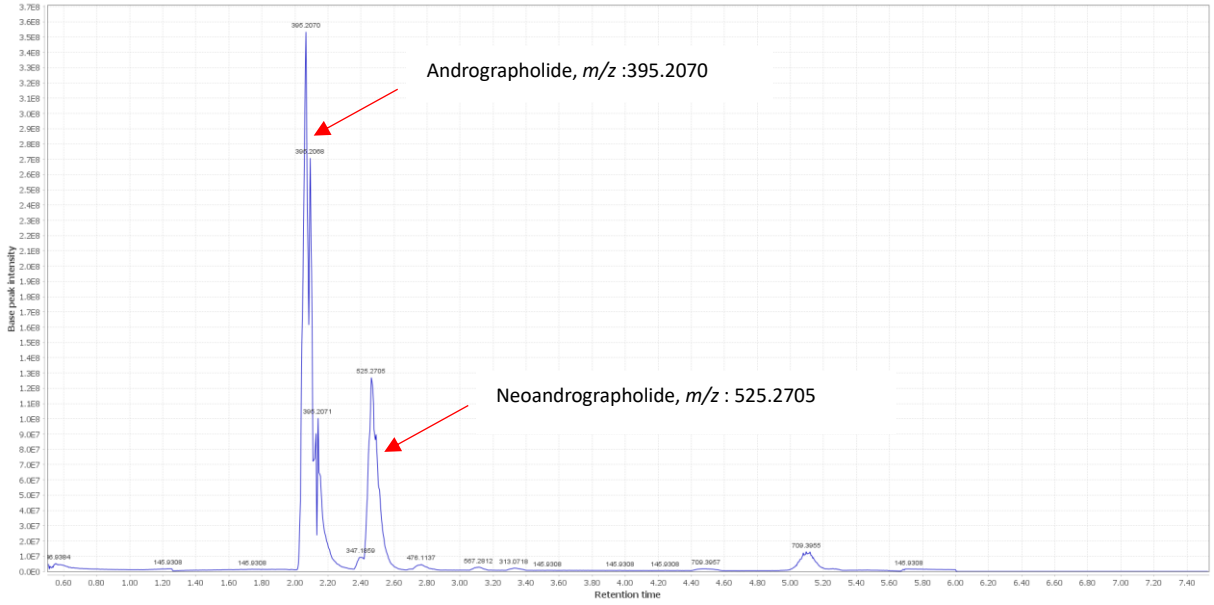
Neoandrographolide, *m/z* : 525.2706
Base peak intensity
Retention time

On16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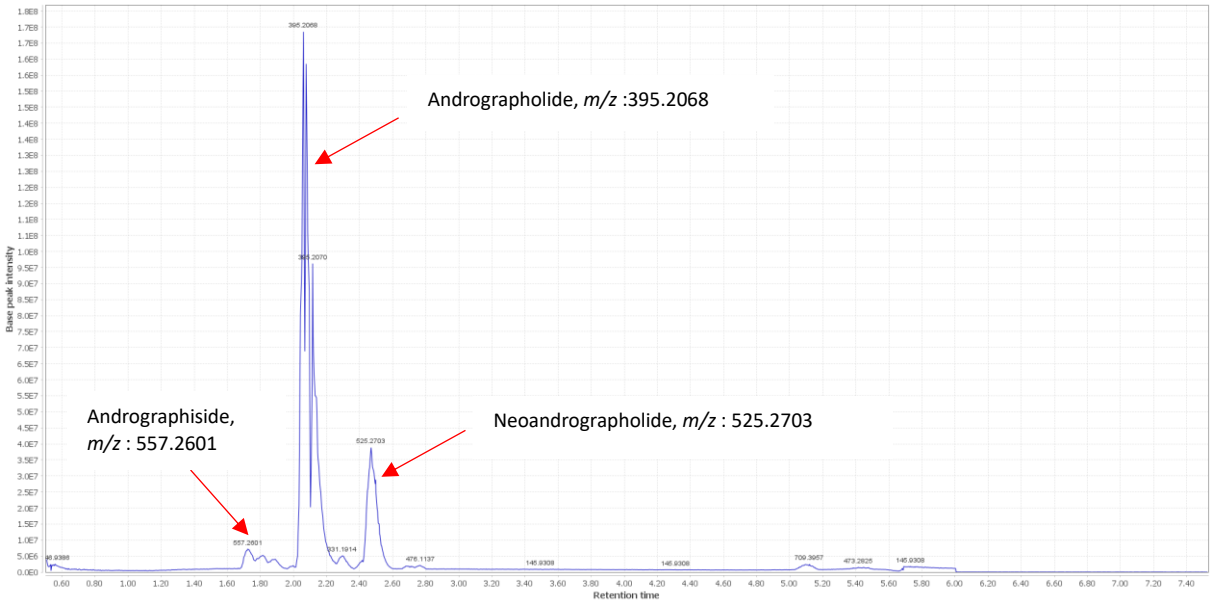
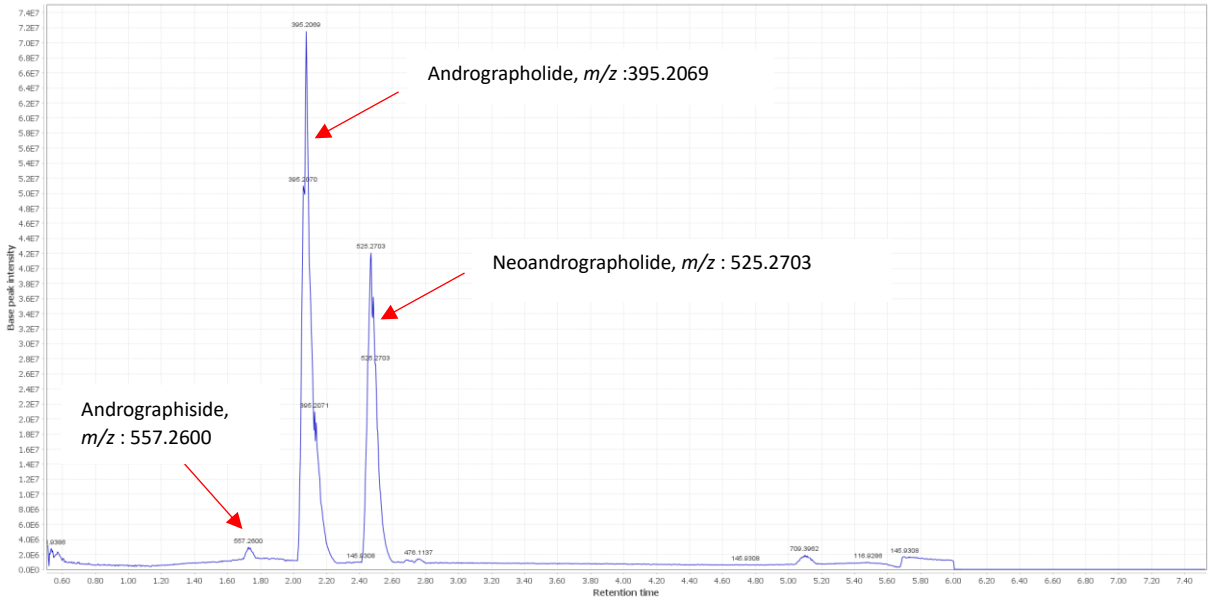
Neoandrographolide, *m/z* : 525.2705
Base peak intensity
Retention time

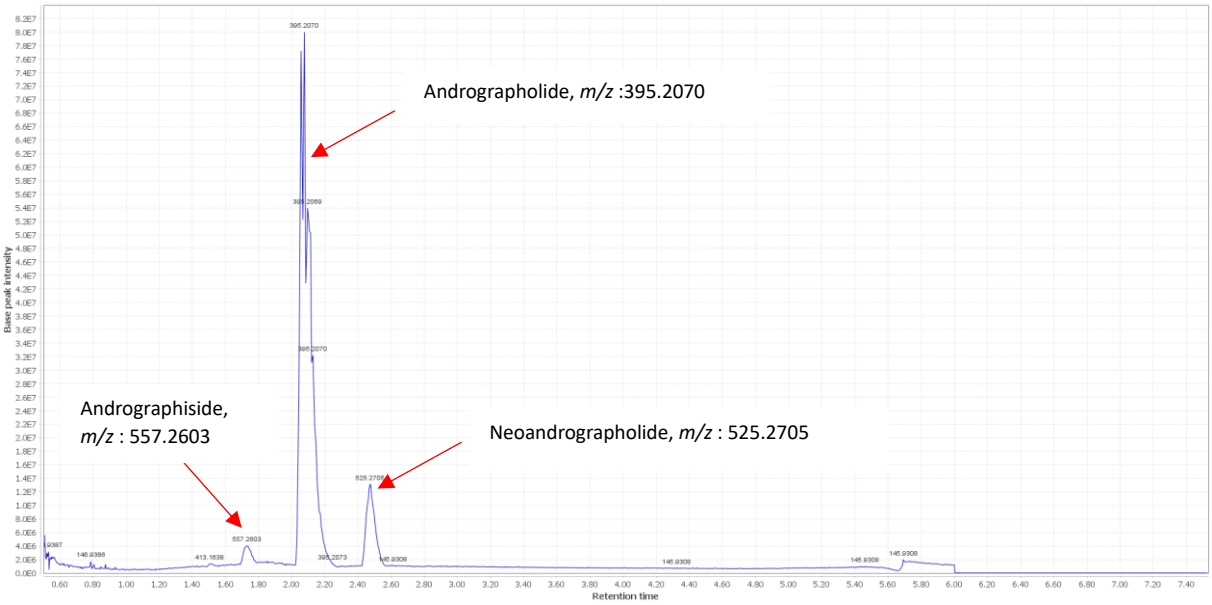
On17


Andrographolide, *m/z* :395.2068
Andrographiside, *m/z* : 557.2601
Neoandrographolide, *m/z* : 525.2703
Base peak intensity
Retention time

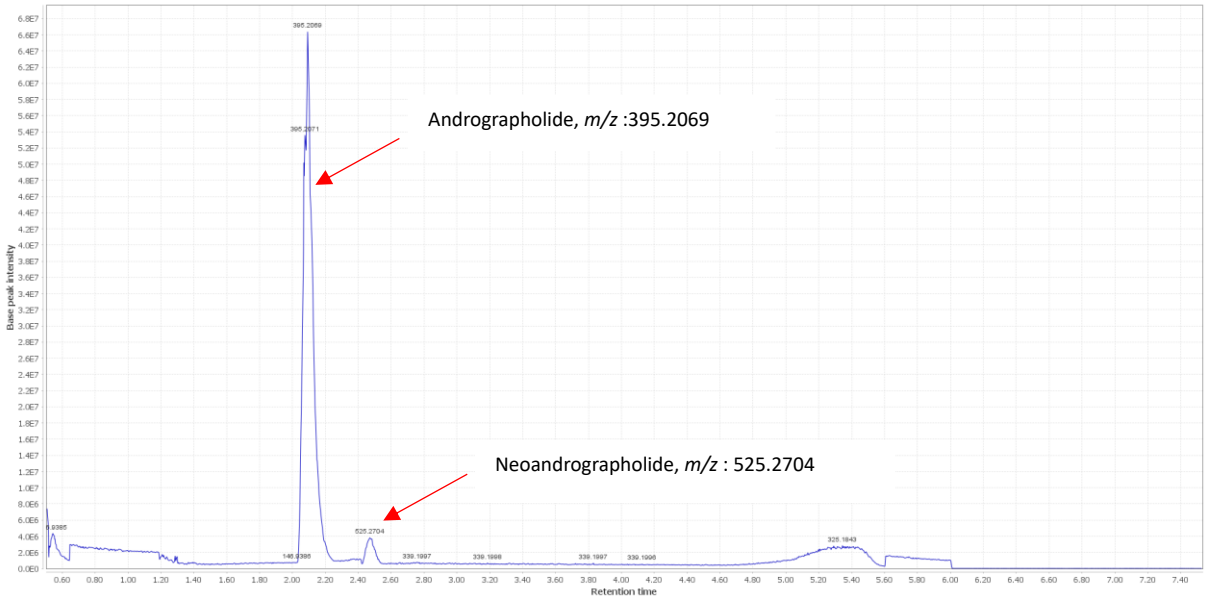
On18


Andrographolide, *m/z* :395.2069
Neoandrographolide, *m/z* : 525.2703
Andrographiside, *m/z* : 557.2600
Base peak intensity
Retention time

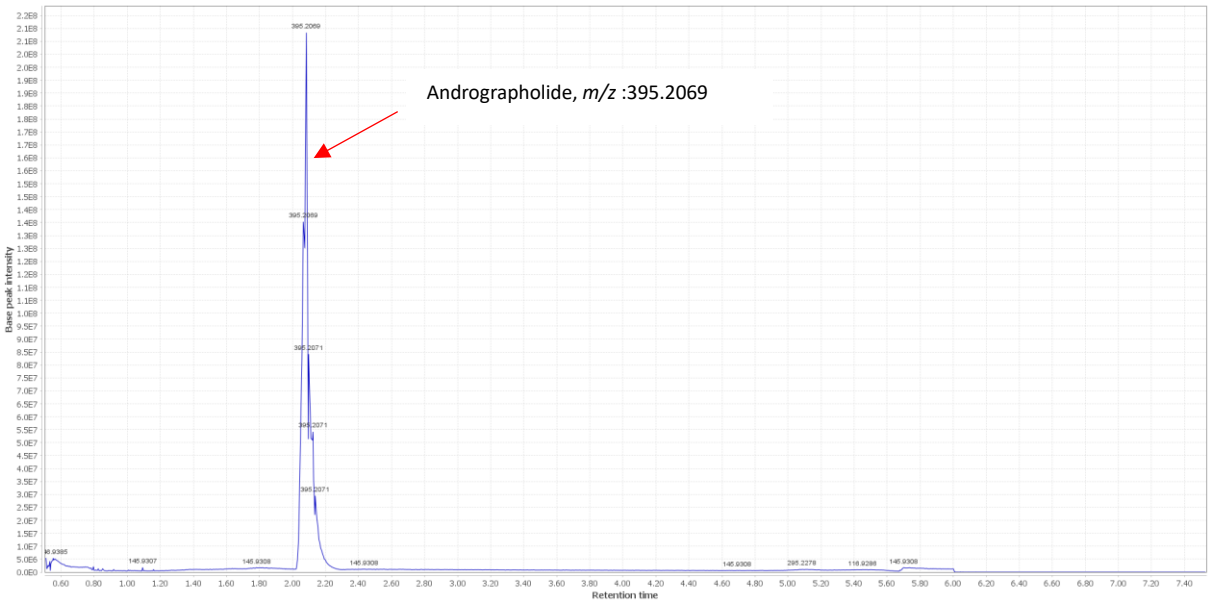
On19


Andrographolide, *m/z* :395.2070
Neoandrographolide, *m/z* : 525.2705
Andrographiside, *m/z* : 557.2603
Base peak intensity
Retention time

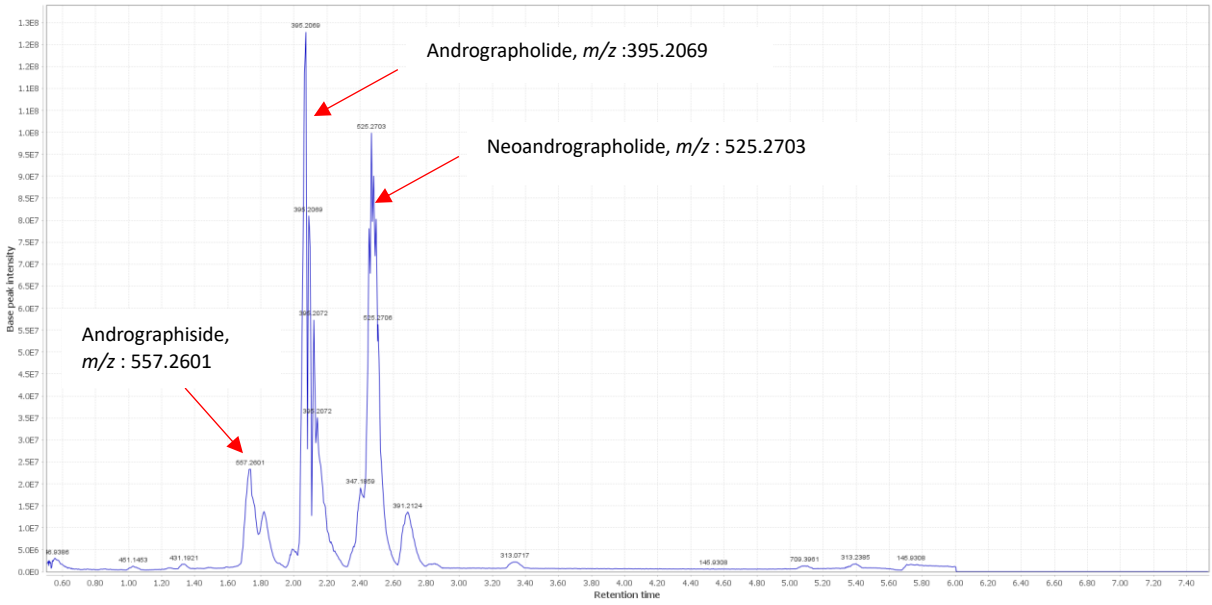
On20


Andrographolide, *m/z* :395.2069
Neoandrographolide, *m/z* : 525.2704
Base peak intensity
Retention time

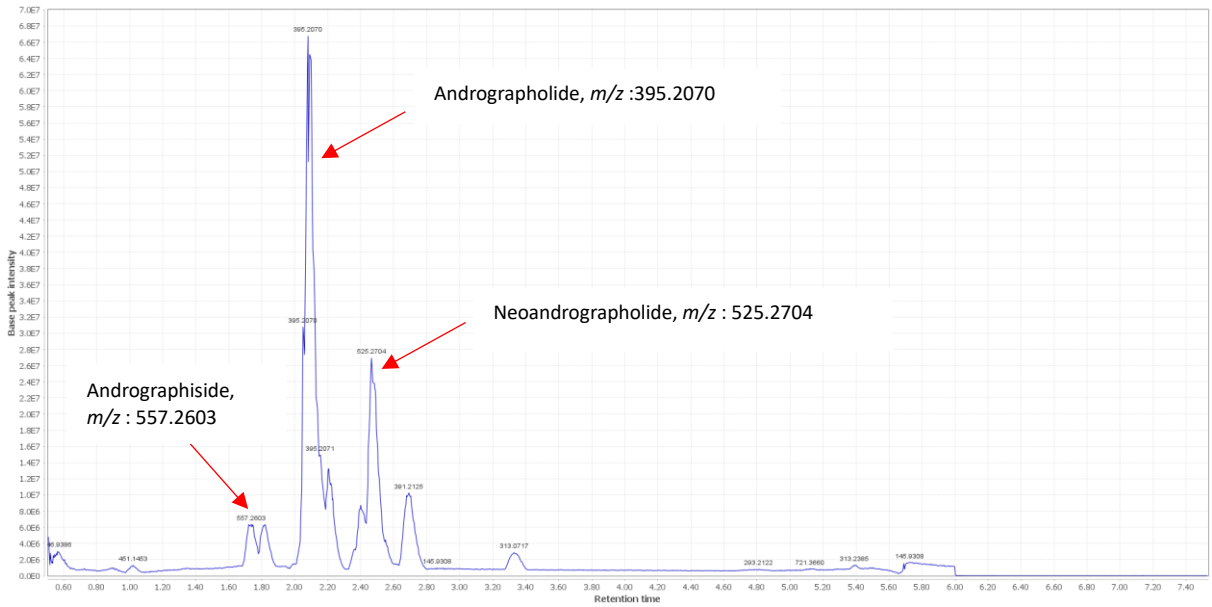
On21


Andrographolide, *m/z* :395.2069
Base peak intensity
Retention time

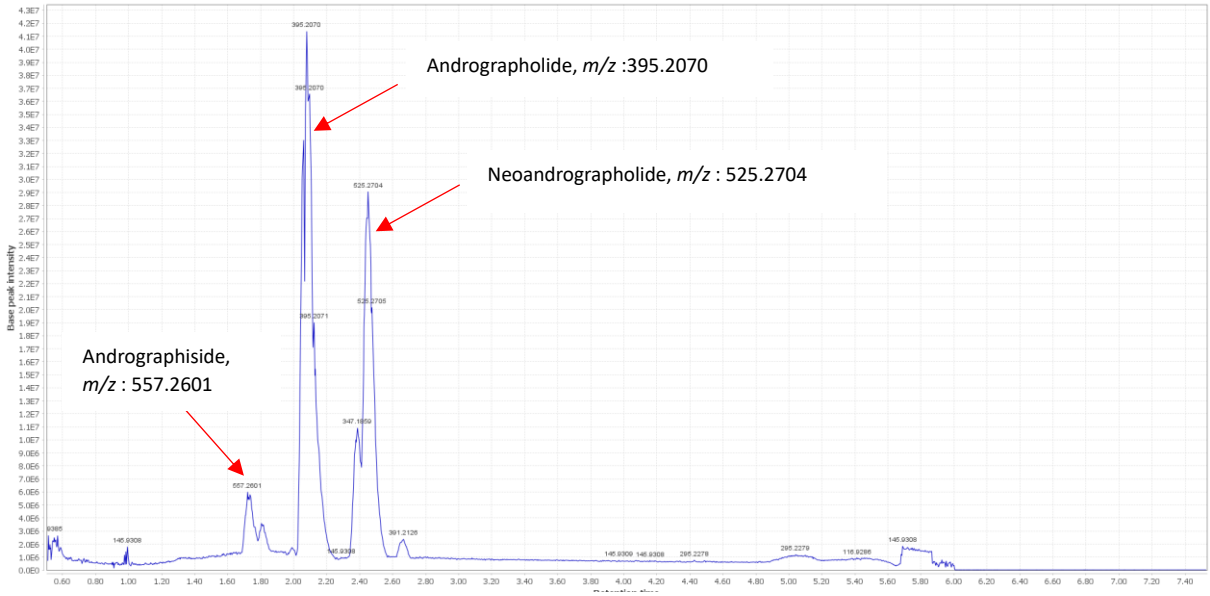
On22


Andrographolide, *m/z* :395.2069
Neoandrographolide, *m/z* : 525.2703
Andrographiside, *m/z* : 557.2601
Base peak intensity
Retention time

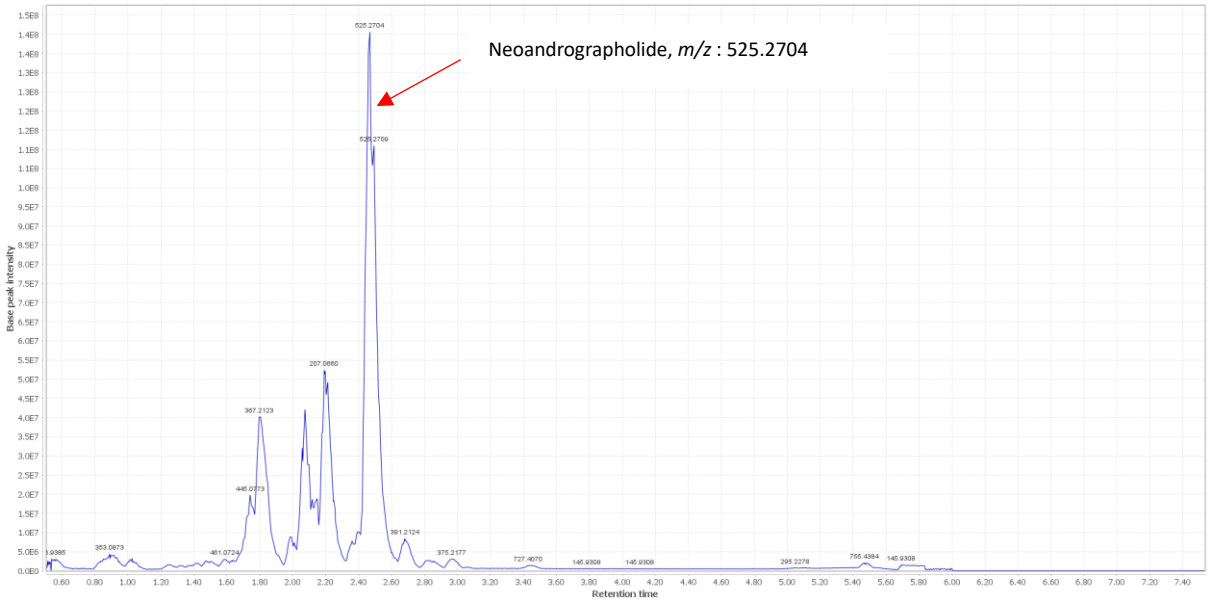
On23


Andrographolide, *m/z* :395.2070
Neoandrographolide, *m/z* : 525.2704
Andrographiside, *m/z* : 557.2603
Base peak intensity
Retention time

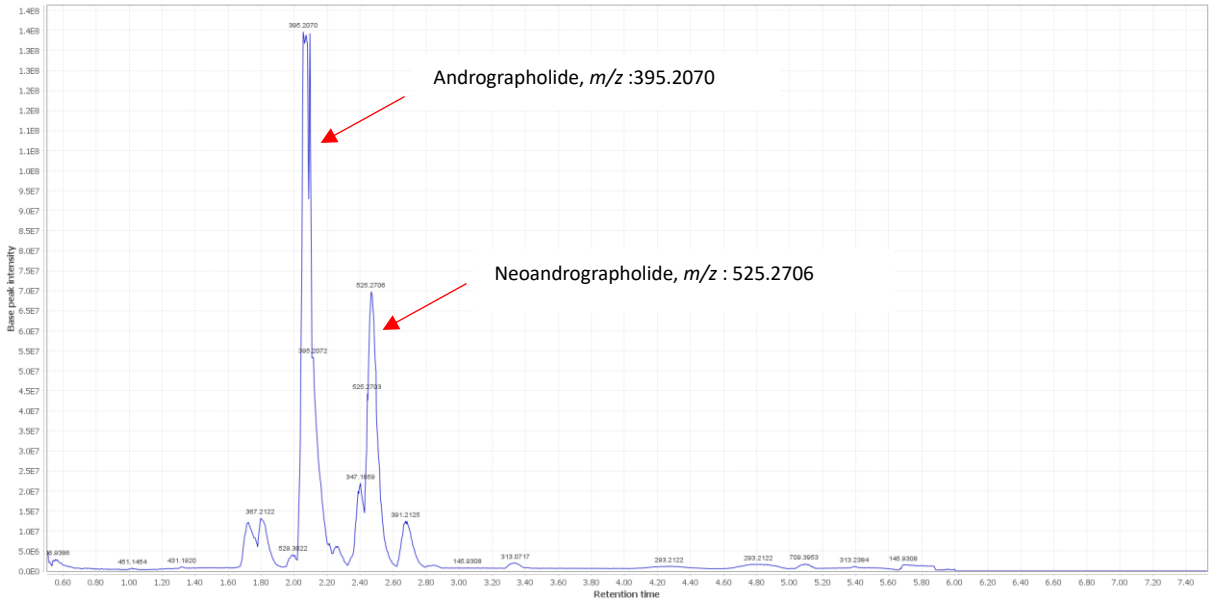
On24


Andrographolide, *m/z* :395.2070
Neoandrographolide, *m/z* : 525.2704
Andrographiside, *m/z* : 557.2601
Base peak intensity

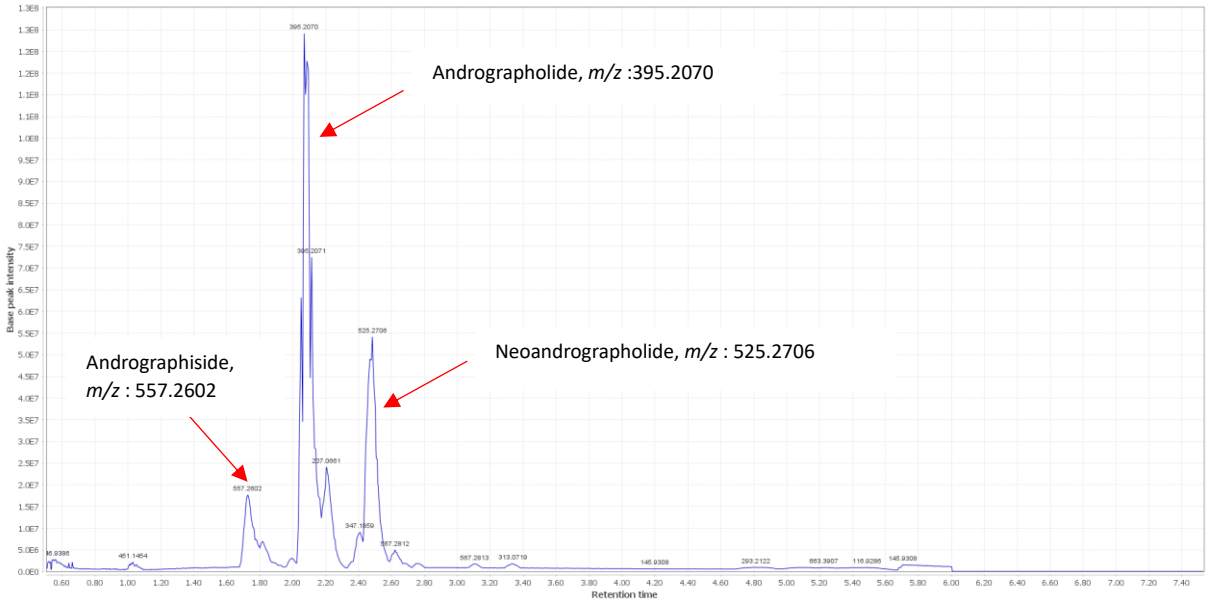
On25


Neoandrographolide, *m/z* : 525.2704
Base peak intensity
Retention time

On26


Andrographolide, *m/z* :395.2070
Neoandrographolide, *m/z* : 525.2706
Base peak intensity
Retention time

On27


Andrographolide, *m/z* :395.2070
Neoandrographolide, *m/z* : 525.2706
Andrographiside, *m/z* : 557.2602
Base peak intensity
Retention time

Supplementary file 6: Daily dose of andrographolide measured with UHPLC-MS and UHPLC-UV

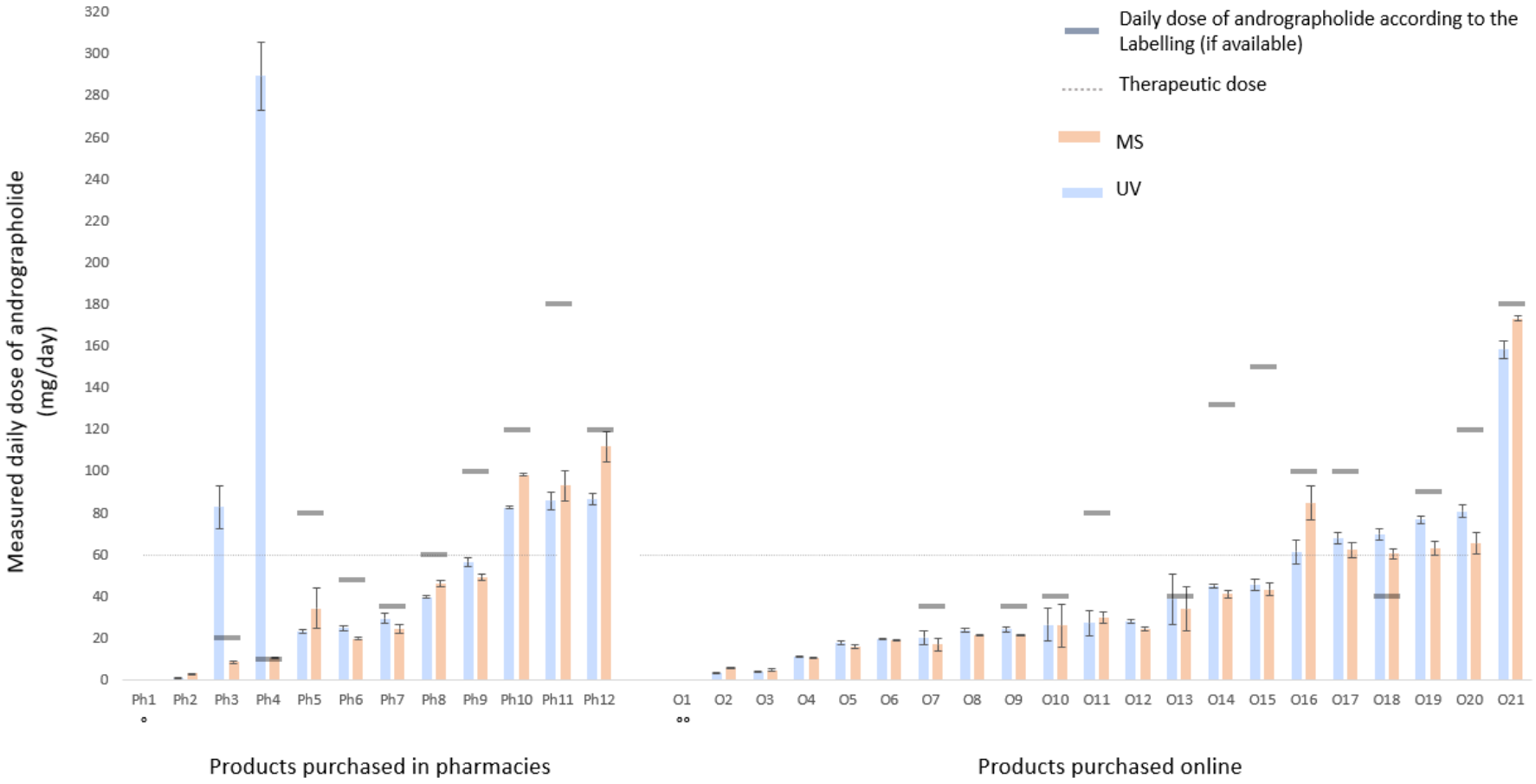


Figure S.2. ***Daily dose of andrographolide measured with UHPLC-UV versus those measured with UHPLC-MS for each product purchased in pharmacy or online. Measured levels are compared with those on the label (grey dash) and with the therapeutic reference dose (dotted line).*** *The data are from three products replicates ±SD. *: no information about daily serving, ** : no andrographolide detected.*

References

1. Challal S, Queiroz EF, Debrus B, et al. Rational and efficient preparative isolation of natural products by MPLC-UV-ELSD based on HPLC to MPLC gradient transfer. *Planta Medica* 2015; **81**(17): 1636-43.
2. Karioti A, Timoteo P, Bergonzi MC, Bilia AR. A Validated Method for the Quality Control of Andrographis paniculata Preparations. *Planta Med* 2017; **83**(14-15): 1207-13.