5. STUDYING THE INFLUENCE OF ELICITORS AND PRECURSOR FEEDING ON THE ACCUMULATION OF POLYPHENOLS IN SUSPENSION CULTURES

Elicitation experiment

1. Preparation of elicitors and precursor stock solutions

- **Elicitors**

a. **Chitosan:** stock solution of chitosan (500 mg/L) was prepared by dissolving 25 mg in 50ml of 1% glacial acetic acid in MS media supplemented with 1.5 mg/L BA, 0.5 mg/L NAA and 30g/L sucrose.

b. **Sodium nitroprusside(SNP):** stock solution of SNP (2500 µM) was prepared by dissolving 65.4 mg in 100 ml MS media supplemented with 1.5 mg/L BA, 0.5 mg/L NAA and 30 g/L sucrose.

c. **Copper sulphate ($CuSO_4$):** stock solution of $CuSO_4$ (1 mM) was prepared by dissolving 12.5 mg in 50 ml MS media supplemented with 1.5 mg/L BA, 0.5 mg/L NAA and 30 g/L sucrose.

d. **Methyl jasmonate (MeJA):** MeJA was purchased as a 95% aqueous solution, 1 mg/ml stock solution was prepared by dissolving 51.0975 µl MeJA equivalent to 50 mg in 50 ml 50% ethanol (prepared by mixing 25 ml absolute ethanol with 25 ml MS media supplemented with 1.5 mg/L BA, 0.5 mg/L NAA and 30 g/L sucrose).

- **Precursors**

Phenylalanine: stock solution of phenylalanaine (50 mM) was prepared by dissolving 1.078g in 100 ml MS media supplemented with 1.5 mg/L BA, 0.5 mg/L NAA and 30g/L sucrose.

Upon frequent subculture, the suspension cultures become dense (applesauce-like consistency with different sizes of cell aggregates). In order to reduce the size of the plant cell aggregates and to prepare a fine cell suspension[87], 1000 ml of suspension culture was homogenized in a sterilized 1 litre blender for 5 seconds. The homogenized culture **(Figure 5)** was then used as a mother stock by transferring 10 ml packed cell volume (PCV) to a 250ml Erlenmeyer flask containing 90 ml fresh medium. Three culture flasks were used for each concentration, while running suitable control without elicitor addition. Elicitors such as chitosan and $CuSO_4$ were added to MS medium and the pH was adjusted before autoclaving. While the precursor and the other two elicitors SNP and MeJA were added to autoclaved medium after filter sterilization using 0.22 µm filters. The elicitors and precursors were selected based on earlier studies as described previously in literature review. Thus, MeJA solution was used at 0, 5, 10 and 20 mg/L[88] whereas chitosan used at concentrations 0, 5, 25 and 50 mg/L[89], SNP used concentrations were 0, 50, 100 and 250 µM [90]and $CuSO_4$ concentrations were 0, 2, 4 and 8 µM[91],

while the amino acid precursor phenylalanine was used at concentrations 0, 1, 2 and 3 mM [88]. Treatments were added aseptically on day 0 to the suspension cultures in each of the cultured flasks. Cultures were incubated at 25±2 °C under photo-period (16-h light/8-h dark; daylight fluorescent tubes; 50 µmol/m^2/s) with continuous agitatation at 120 rpm on orbital shaker. After 15 days incubation with different elicitors, precursor and controls **(Figure 6 - Figure 10)**, the calli were harvested by centrifugation for the determination of dry weight and extraction of total polyphenolics. The calli extracts were evaluated from chemical and biological points of view and compared to that of the locally cultivated plant extracts.

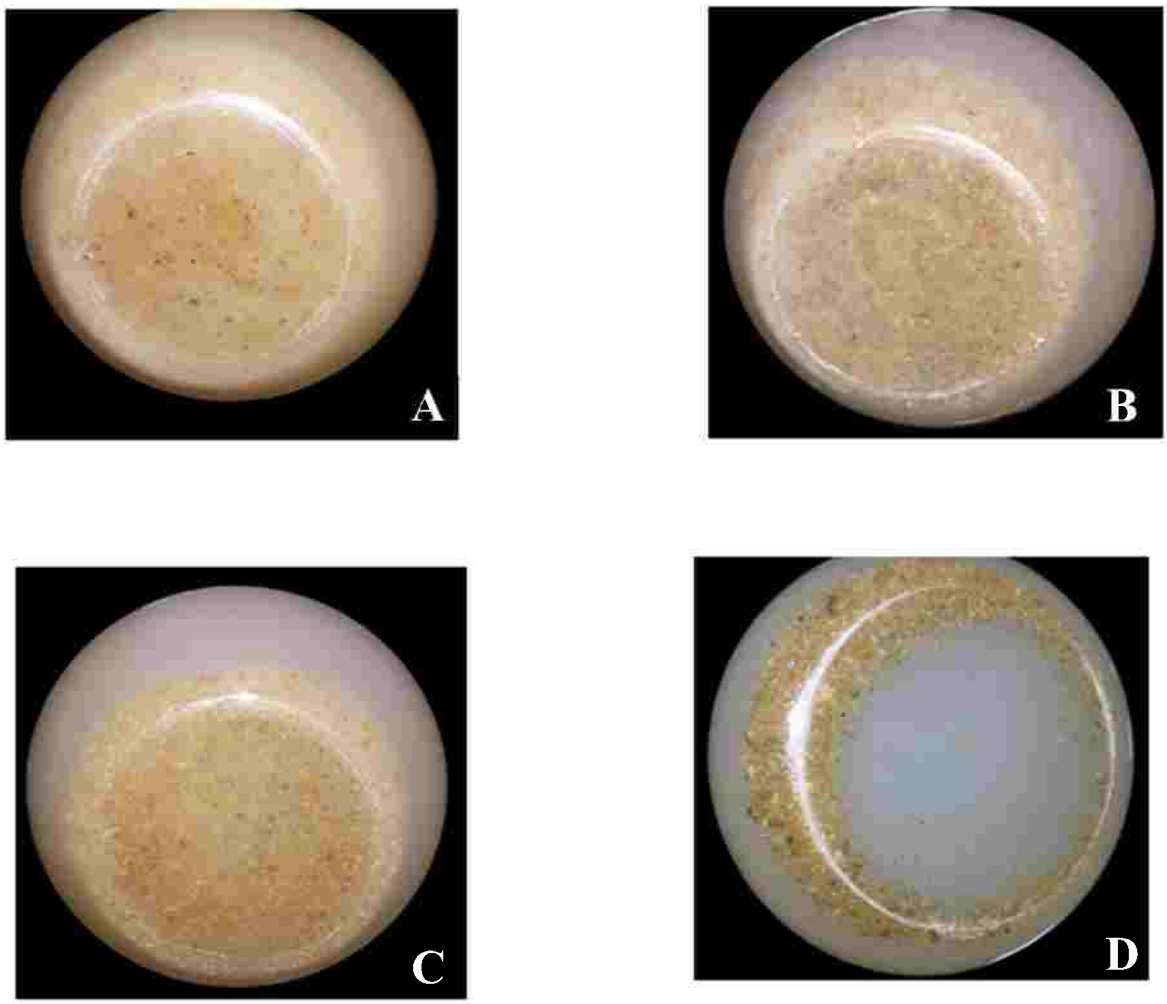

Figure 6: Suspension cultures growing on MS liquid medium supplemented with 1.5 mg/L BA and 0.5 mg/L NAA containing phenylalanine (Precursor)

Flasks A, B, C and D were incubated with 1, 2, 3 and zero mM phenylalanine respectively for 15 days.

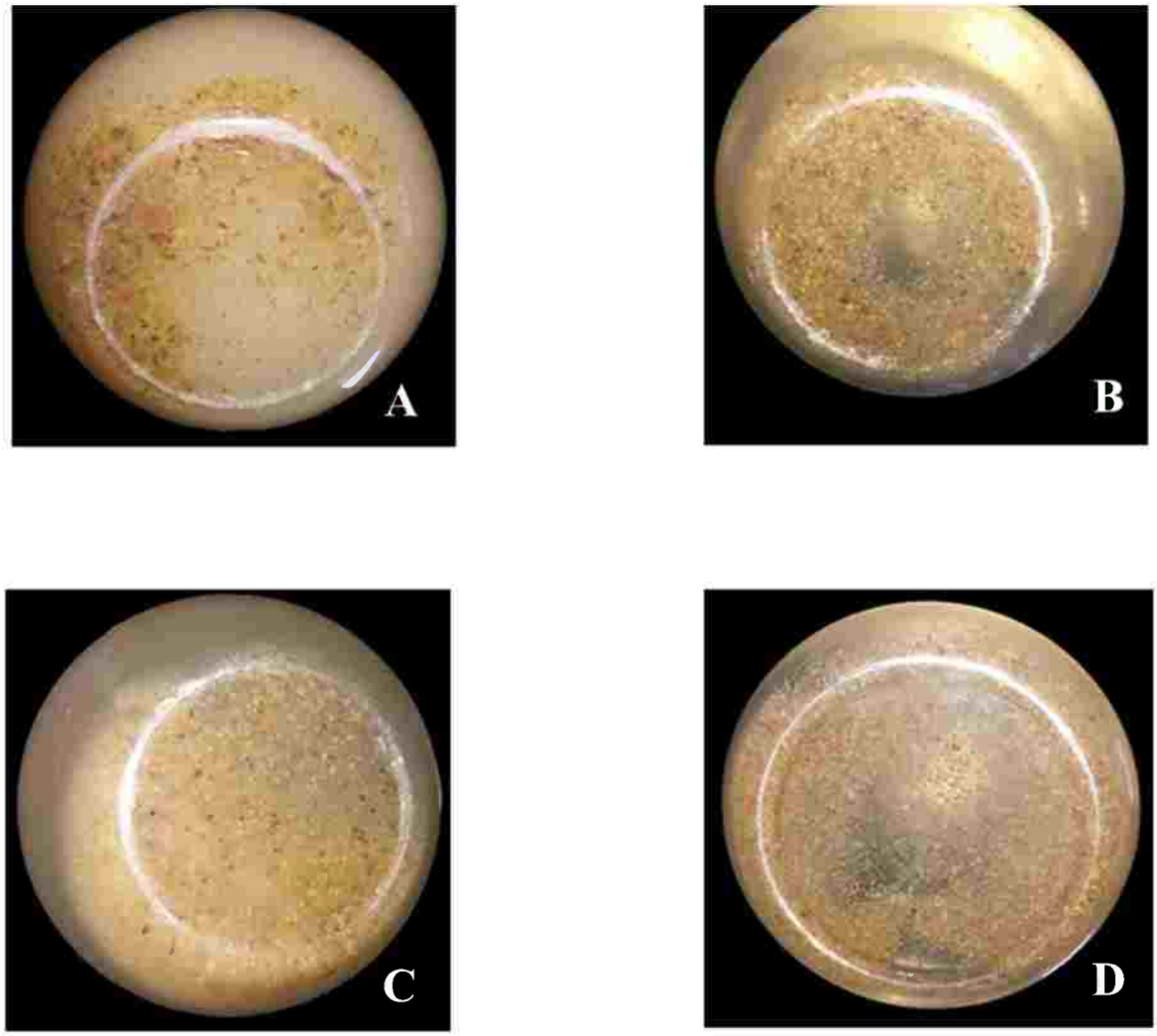

Figure 7: Suspension cultures growing on MS liquid medium supplemented with 1.5 mg/L BA and 0.5 mg/L NAA containing chitosan

Flasks A, B, C and D were incubated with 5, 25, 50 and 0 mg/L chitosan respectively for 15 days.

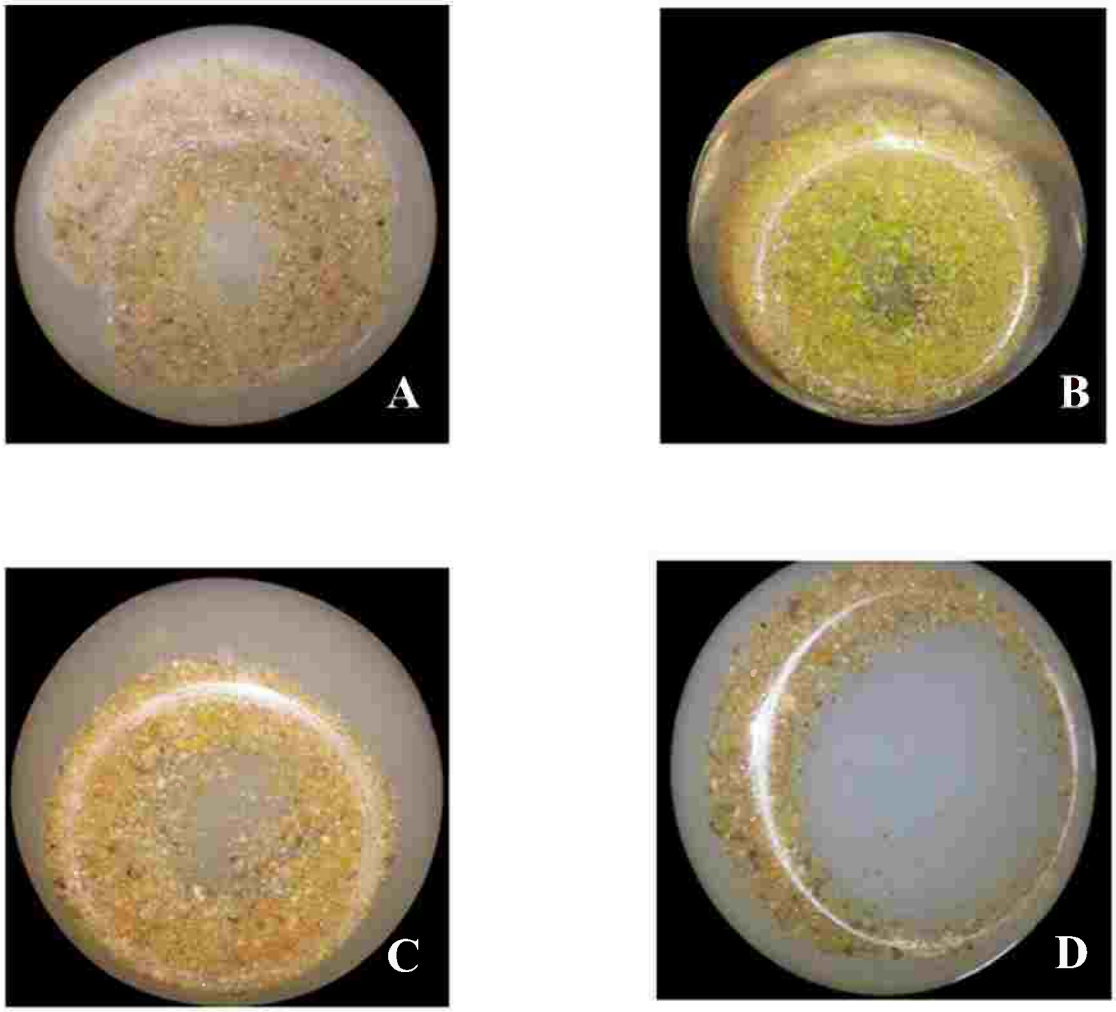

Figure 8: Suspension cultures growing on MS liquid medium supplemented with 1.5 mg/L BA and 0.5 mg/L NAA containing $CuSO_4$

Flasks A, B, C and D were incubated with 2, 4, 8 and 0 μM $CuSO_4$ respectively for 15 days.

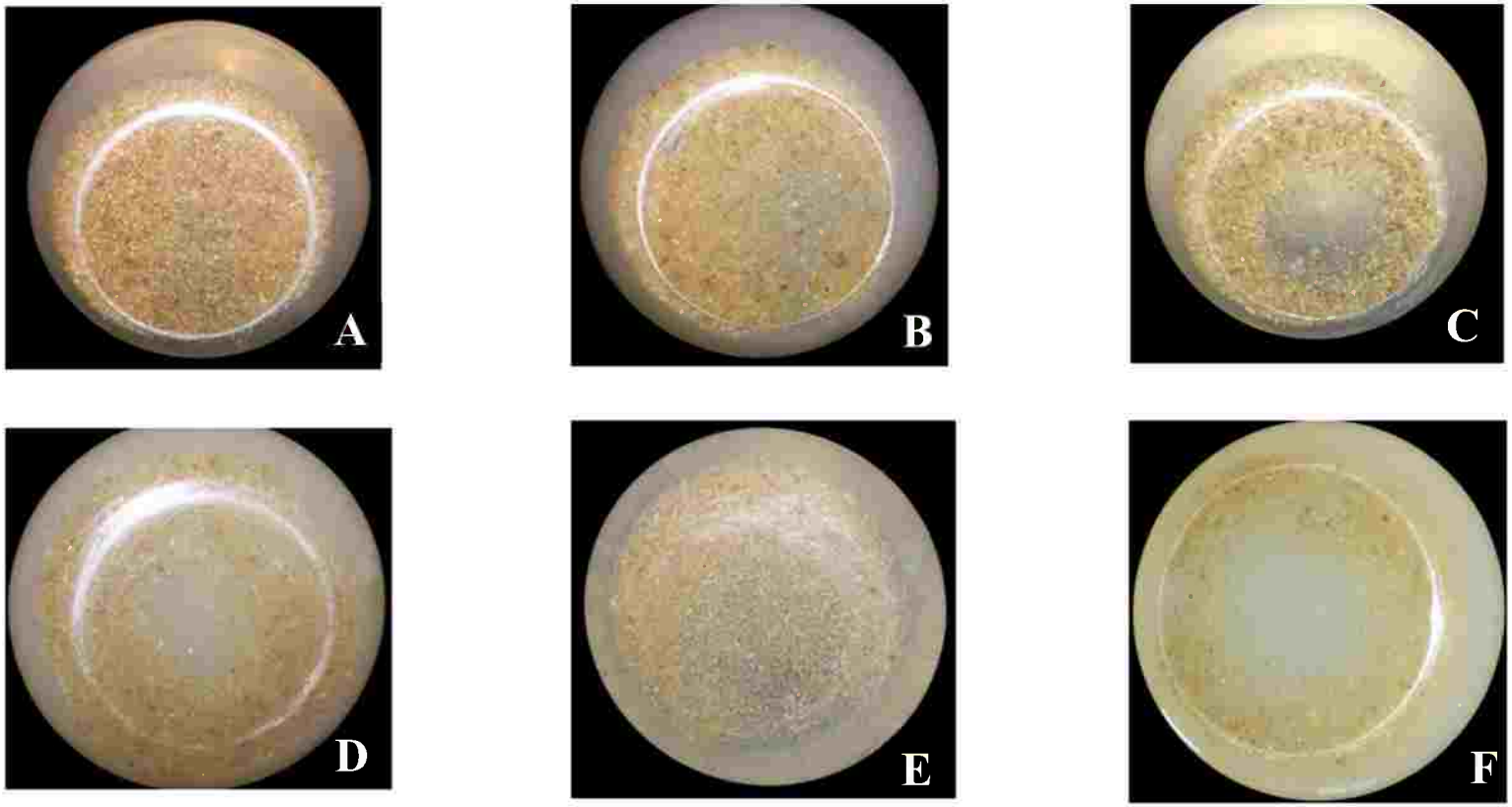

Figure 9: Suspension cultures growing on MS liquid medium supplemented with 1.5 mg/L BA and 0.5 mg/L NAA containing methyl jasmonate

Flasks A, B and C were incubated with 5, 10 and 20 mg/L MeJA respectively for 15 days.

Flasks D, E and F are the corresponding controls for the stated concentrations respectively

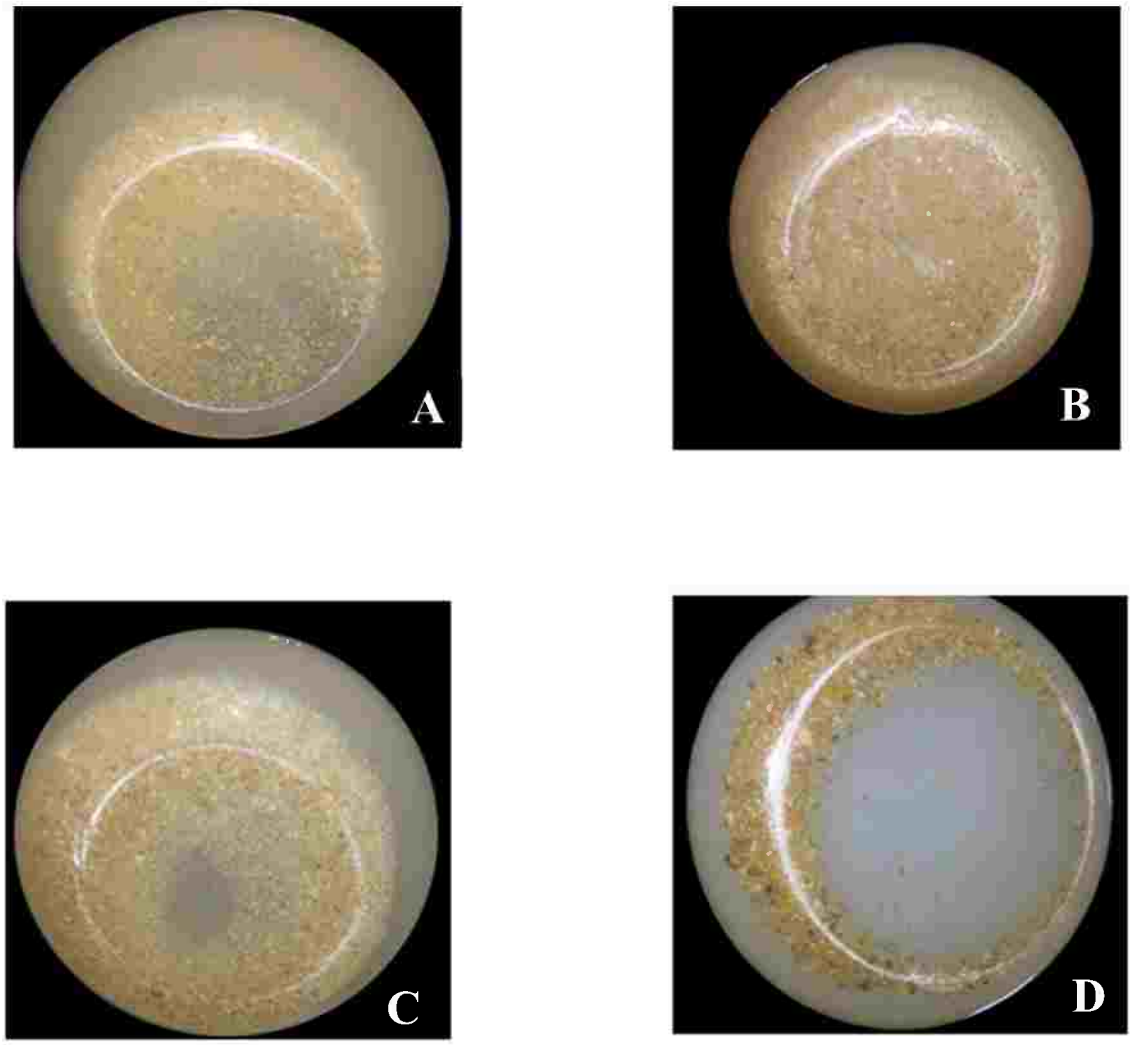

Figure 10: Suspension cultures growing on MS liquid medium supplemented with 1.5 mg/L BA and 0.5 mg/L NAA containing sodium nitroprusside

Flasks A, B, C and D were incubated with 50, 100, 250 and 0 µM SNP respectively for 15 days.

2. Extraction of polyphenols

Calli were stored frozen (-80 °C) and their dry weight was recorded after lyophilisation, dried tissues were ground in a homogenizer immediately prior extraction. Crude extraction of polyphenols was carried out in 50 ml falcon tubes, 200 mg powdered tissues were weighed into falcons and were extracted for 30 minutes with 1800 µl 70% ethanol at 23 °C in

an ultrasonic water bath[92,93]. All samples were centrifuged at 4,000 rpm for 15 minutes and the supernatants were collected and stored at-80 °C for lyophilization. A stock solution (2 mg/ml) of each extract was prepared in 7% ethanol and filtered using a 0.2 µm syringe filter to be used in further assays.

3. Estimation of total polyphenol contents in calli tissues using Folin-Ciocalteu reagent

The assay describes a microplate-adapted colorimetric total phenolics assay that utilizes Folin–Ciocalteu (F–C) reagent. The F–C assay relies on the transfer of electrons in alkaline medium from phenolic compounds to blue phosphomolybdic/phosphotungstic acid complexes, which are determined spectroscopically at 765 nm. The total phenolic content was expressed as gallic acid equivalents in mg/g of dry matter[94].

- **Method**

Twenty µl of each extract, gallic acid standard concentrations (200-6.25µg/L) or 7% ethanol (solvent blank) was placed in a 96 well plate to which 40 µl 10% F–C reagent were added, the plate was gently shaken, then 160 µl 700mM Na_2CO_3 was added into each well and the plate was incubated at room temperature for 2 hours. The color intensity was assayed using a microplate reader spectrophotometer adjusted at 765 nm. Total phenolics were calculated as gallic acid equivalents using the regression equation in **Figure 11** between gallic acid standards and A_{765}[94].Results were presented in **Figure 12.**

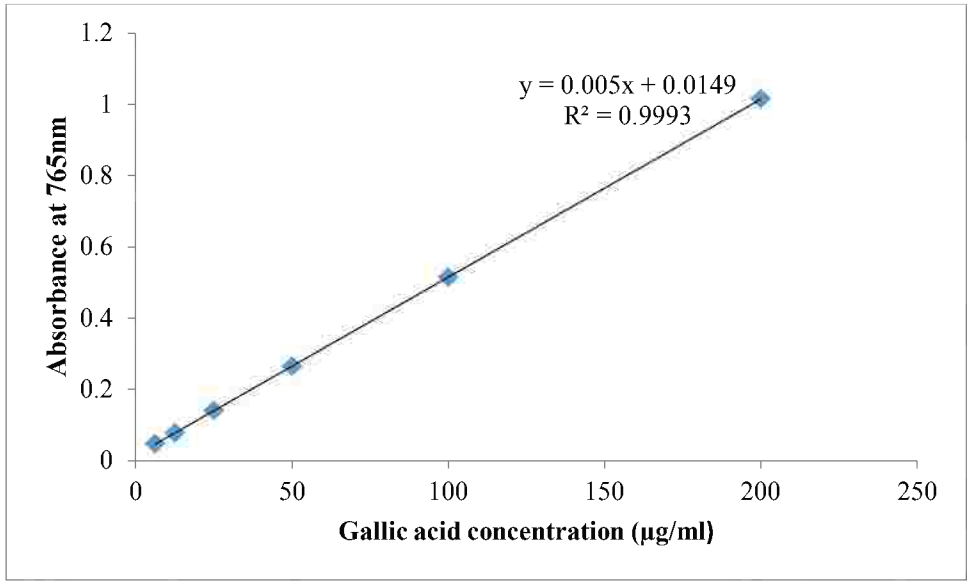

Figure 11: Standard curve (absorbance versus concentration) of gallic acid

4. LC-MS method for identification and quantification of caffeic acid derivatives in *Echinacea* extracts

LC-MS/MS analysis was performed in *the Center of Pharmaceutical and Fermentation Industry Development, City for Scientific Research and Technology Application, Borg El-Arab, Alexandria, Egypt.*

- Stock solutions of caffeic, caftaric, chlorogenic and cichoric acids were prepared in methanol: water (1:1 v/v) at a concentration of 200ng/ml, these stocks were used for the preparation of calibration standards.
- For the calibration standards the stock solution was further diluted to obtain working solutions in a range from 0.1 to 20 ng/ml for caffeic acid, from 0.2 to 20 ng/ml for caftaric acid, from 0.05 to 20 ng/ml for chlorogenic acid and from 0.1-25 ng/ml for cichoric acid.
- For the internal standard (IS), a stock solution of ferulic acid was prepared in methanol:water (1:1 v/v) at a concentration of 2 µg/ml, it was further diluted upon addition to reference standard solution or sample extract to reach a final concentration of 20 ng/ml.
- Concentration of caffeic, caftaric, chlorogenic and cichoric acids in each extract was calculated using the regression equation in **Figures (13-16)** respectively, between concentration in (ng/ml) and peak area of each reference standard/peak area of IS.
- High-performance liquid chromatography (HPLC) was performed using ekspert^{TM} ultraLC system (Dublin, CA, USA). The analytical column was a Synergi 4u Fusion-RP 80A column (4 µm, 150 × 3 mm) maintained at 30◦C. The mobile phase composed of 0.1 % formic acid in water (Glass distilled water was further purified using Milli-Q water purification system (Millipore, Bedford, MA, USA) and HPLC grade methanol (50:50). The elution was isocratic along the run (5.50 min). The flow rate was 600 µL/min, and the injection volume was 10 µL. The UPLC system was coupled on-line to AB SCIEX Triple Quad 5500 mass spectrometer equipped with Turbo V^{TM} ion source operated in a negative ion mode. The turbo gas temperature was set at 500 ◦C and Turbolon needle voltage was adjusted to 5500 V[95]. Data acquisition and processing was performed using Analyst software 1.6 (AB Sciex, Darmstadt, Germany). Results were presented in **Table 2**. LC/MS/MS analysis data of standard references were presented from **Figure 21** to **Figure 35**.

Results and discussion

1. Total polyphenol contents in calli tissues

To the best of our knowledge, no reports have been recorded earlier on callus induction from this particular plant organ (root) or species (*E.purpurea*) so this will be the first recorded data on *Echinacea purpurea* root callus induction and suspension cultures elicitation.

Table 1: Different *Echinacea* extracts involved in the study

Sample	Identity Code
Alcoholic extracts of untreated callus extract	Callus
Alcoholic extracts of cultivated plant	Cultivated
Alcoholic extracts of 1 mM Phenylalanine treated calli	Phe 1
Alcoholic extracts of 2 mM Phenylalanine treated calli	Phe 2
Alcoholic extracts of 3 mM Phenylalanine treated calli	Phe 3
Alcoholic extracts of 5 mg/L methyl jasmonate treated calli	MeJA 5
Alcoholic extracts of 10 mg/L methyl jasmonate treated calli	MeJA 10
Alcoholic extracts of 20 mg/L methyl jasmonate treated calli	MeJA 20
Alcoholic extracts of 50 µM sodium nitroprusside treated calli	SNP 50
Alcoholic extracts of 100 µM sodium nitroprusside treated calli	SNP 100
Alcoholic extracts of 250 µM sodium nitroprusside treated calli	SNP 250
Alcoholic extracts of 2 µM Copper sulphate treated calli	$CuSO_4$ 2
Alcoholic extracts of 4 µM Copper sulphate treated calli	$CuSO_4$ 4
Alcoholic extracts of 8 µM Copper sulphate treated calli	$CuSO_4$ 8
Alcoholic extracts of 5 mg/L chitosan treated calli	Chitosan 5
Alcoholic extracts of 25 mg/L chitosan treated calli	Chitosan 25
Alcoholic extracts of 50 mg/L chitosan treated calli	Chitosan 50
Alcoholic extracts of ethanol treated calli (used as a control for methyl jasmonate different treatments)	EtOH 0.5, 1, 2
Alcoholic extracts of acetic acid treated calli (used as a control for chitosan different treatments)	Control acetic acid

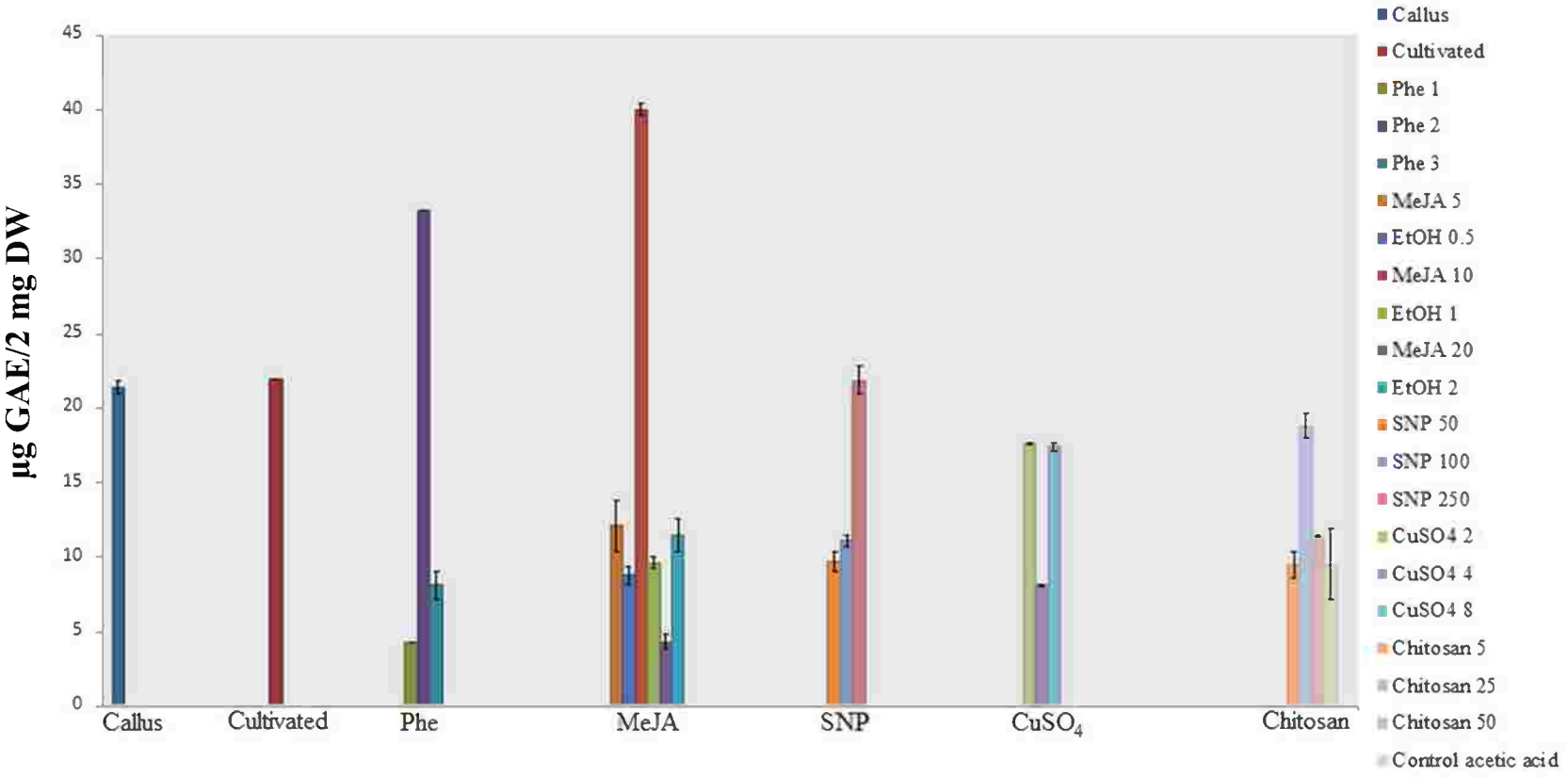

Figure 12: Total polyphenol content (expressed as µg GAE/2 mg DW) of treated *Echinacea purpurea* calli extracts compared with untreated callus and cultivated plant extract.

Results were presented as Mean ± SEM of three parallel measurements. Statistical evaluation was carried out by one-way analysis of variance (ANOVA). Statistical significance is expressed as $p < 0.05$

This study was conducted to enhance one of the main active components of *Echinacea purpurea* CADs and total polyphenolics through performing some treatments on callus tissues. A comparative analysis between the treated calli, untreated calli and the intact plant from chemical and biological point of view was performed to investigate differences and similarities between different treatments and to assess the impact of increase in total polyphenolic content on biological activity of the *Echinacea*. Data shown in **Figure 11** and **Table 4 (in appendix)** indicates that the highest polyphenolic content was achieved and detected in 70% hydroalcoholic extracts of calli treated with **10 mg/L MeJA,** with a statistically significant **1.81-fold** increase with a total yield of 40.02 µg GAE/2 mg dry extract as compared to the cultivated plant which contains 22.02 µg GAE/2 mg dry extract and a statistically significant **1.86-fold** increase as compared to the extracts of untreated *Echinacea* calli which contains 21.42 µg GAE/2 mg dry extract.

While extracts from calli treated with 2 mM Phe shows a statistically significant 1.5-fold increase in polyphenolic content with a total yield of 33.22 µg GAE/2 mg dry extract in comparison with the cultivated plant extracts and a statistically significant 1.55-fold increase in comparison with extracts of untreated calli .Precursor feeding has been an obvious and popular approach to increase secondary metabolite production in plant cell cultures. The concept is based upon the idea that any compound, which is an intermediate, in or at the beginning of a biosynthetic route stands a good chance of increasing the yield of the final product. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases[96]. In this concern Mobin *et al*[32] studied the effect of aromatic amino acid Phe on adventitious root cultures of *E. purpurea* grown in presence of 0.5 mM Phe which resulted in a 1.29 fold increase in the phenolic content in comparison with the control cultures.

Polyphenolic contents detected in calli treated with **250 µM SNP** showed insignificant increase in comparison with cultivated plant and insignificant 1.023 fold increase in comparison with the untreated calli**.** The other tested elicitors treatments showed low productivity of polyphenolics in comparison with the cultivated plant extracts.

2. Identification and quantification of caffeic acid derivatives in *Echinacea* extracts using LC-MS method

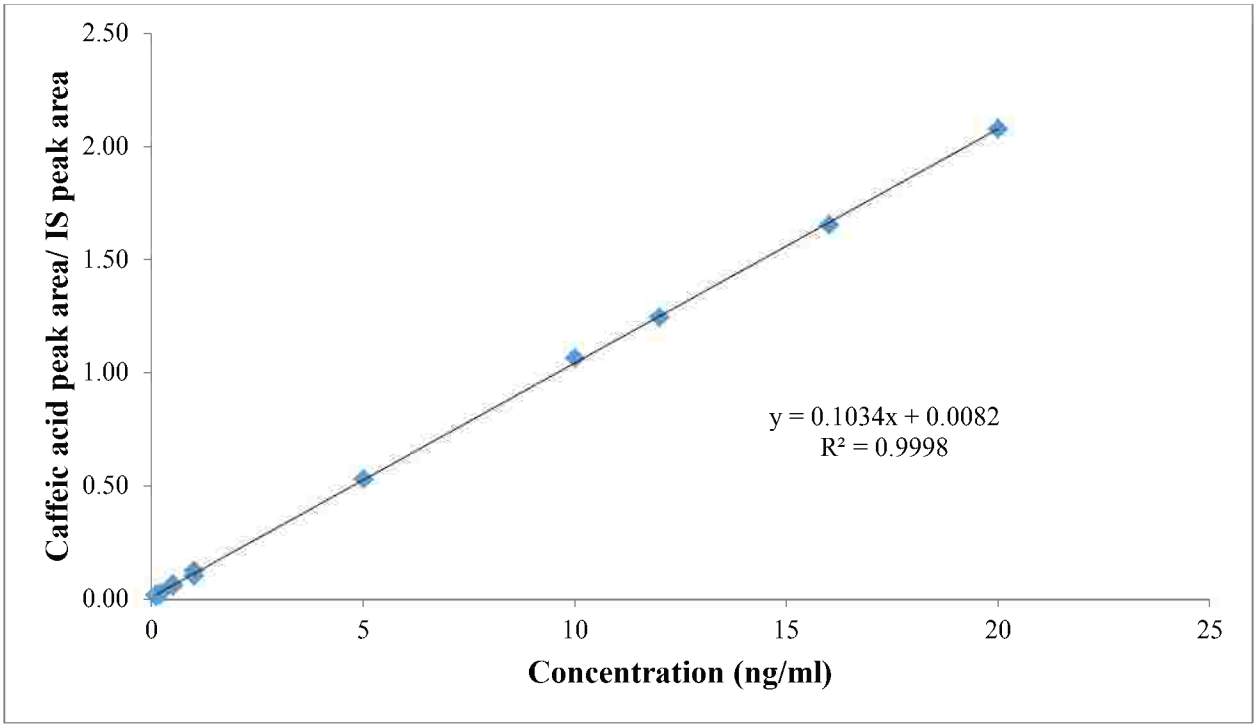

Figure 13: Calibration curve of caffeic acid

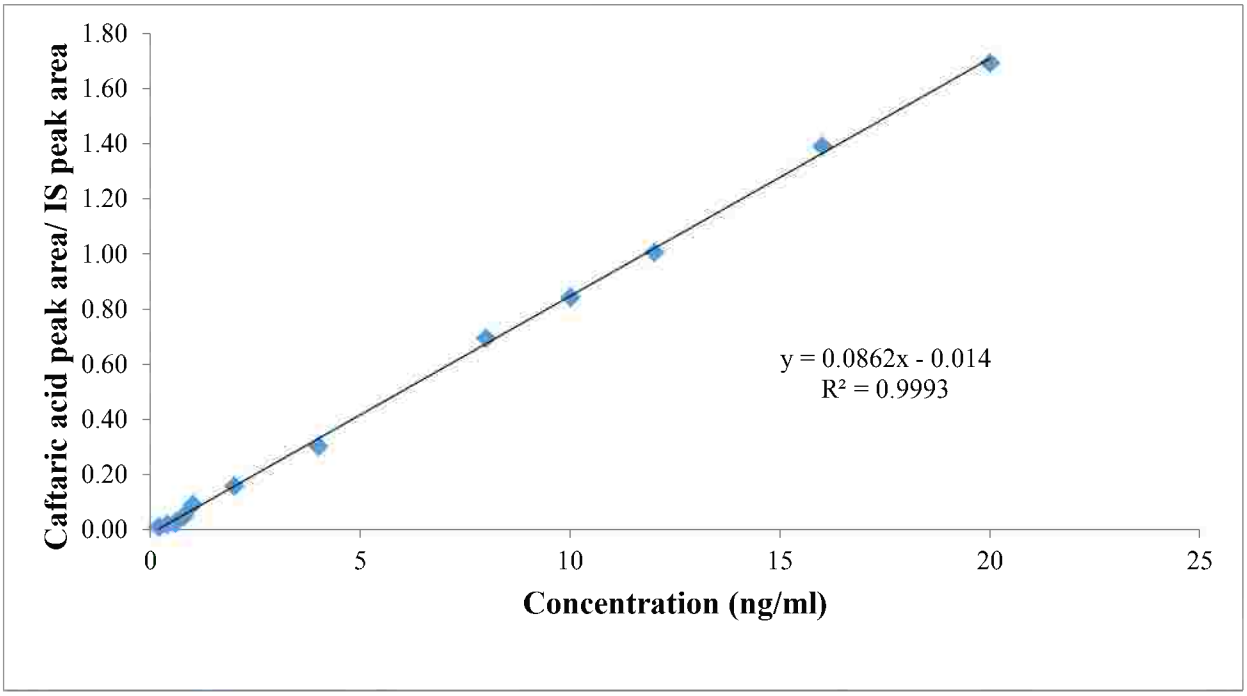

Figure 14: Calibration curve of caftaric acid

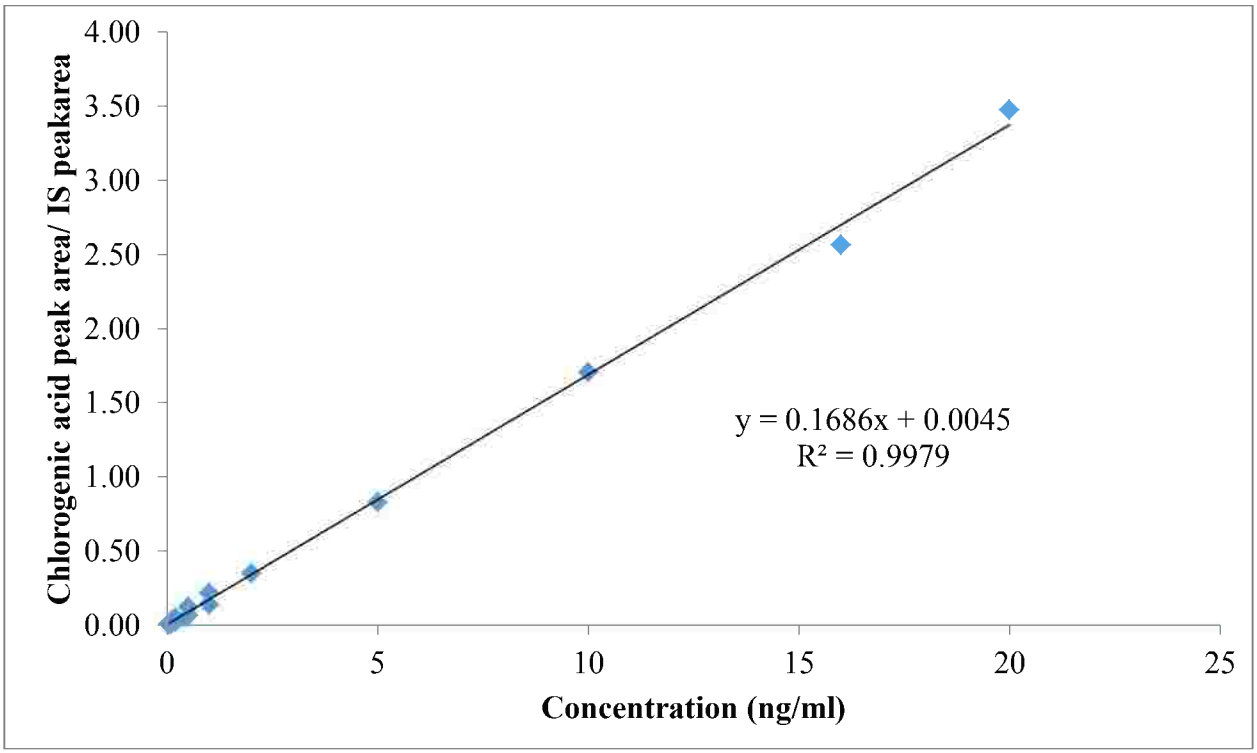

Figure 15: Calibration curve of chlorogenic acid

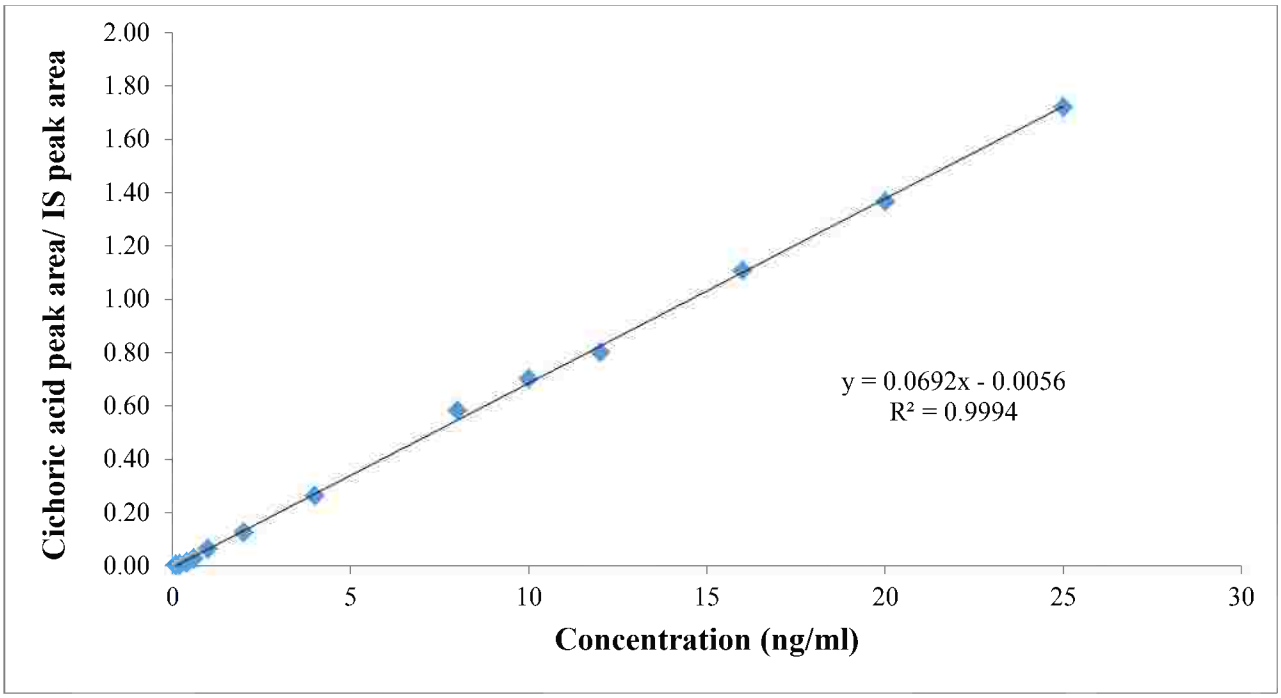

Figure 16: Calibration curve of cichoric acid

Table 2: Concentrations of phenolic acids detected in *Echinacea purpurea* extracts using LC/MS/MS

Sample Name	Final concentration (ng/mg extract)			
	Caffeic	Caftaric	Chlorogenic	Cichoric
Callus	**199.23**	138.22	**237.87**	179.96
Cultivated	7.26	1128.55	4.56	1028.87
Phe 2	1.36	5.82	0.71	6.22
MeJA 10	6.10	14.97	**38.99**	53.65
SNP 50	0.87	4.72	5.07	9.47
MeJA 5	1.81	6.38	14.89	31.00
EtOH 0.5	1.40	5.11	11.34	9.88
$CuSO_4$ 2	6.38	26.81	**85.87**	**276.53**
EtOH 1	0.60	1.85	0.69	0.71
Control acetic acid	0.53	0.93	0.20	0.26
$CuSO_4$ 4	**94.99**	16.65	**96.64**	8.98
Chitosan 5	0.49	0.46	0.08	0.13
Chitosan 50	0.10	0.67	0.52	0.53
SNP 250	1.05	3.92	0.91	1.83
Phe 1	0.20	2.01	0.43	0.77
MeJA 20	0.61	1.93	2.02	2.55
Phe 3	0.71	5.54	1.75	23.70
SNP 100	0.63	5.86	10.13	9.28
Chitosan 25	0.17	0.52	0.17	0.21
EtOH 2	0.34	4.25	3.10	6.51
$CuSO_4$ 8	**46.05**	20.49	**121.31**	25.07

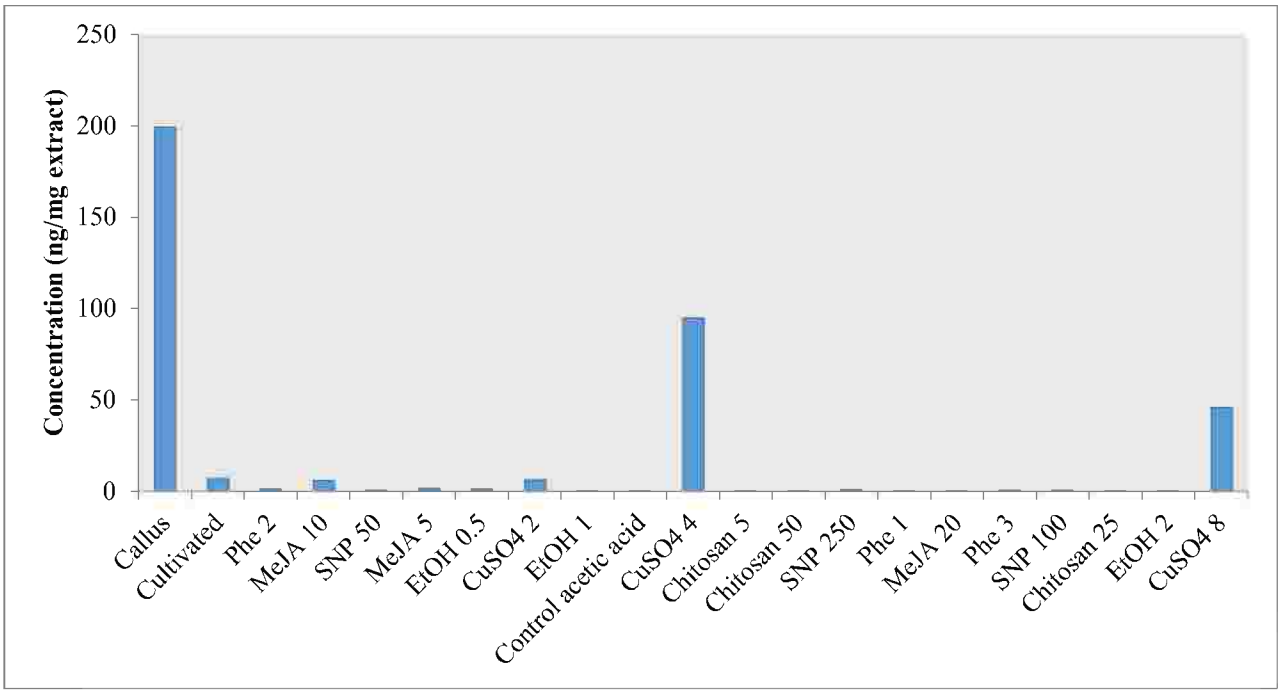

Figure 17: Caffeic acid concentration (expressed as ng/mg extract) of treated *Echinacea purpurea* extracts compared with untreated callus and cultivated plant extract

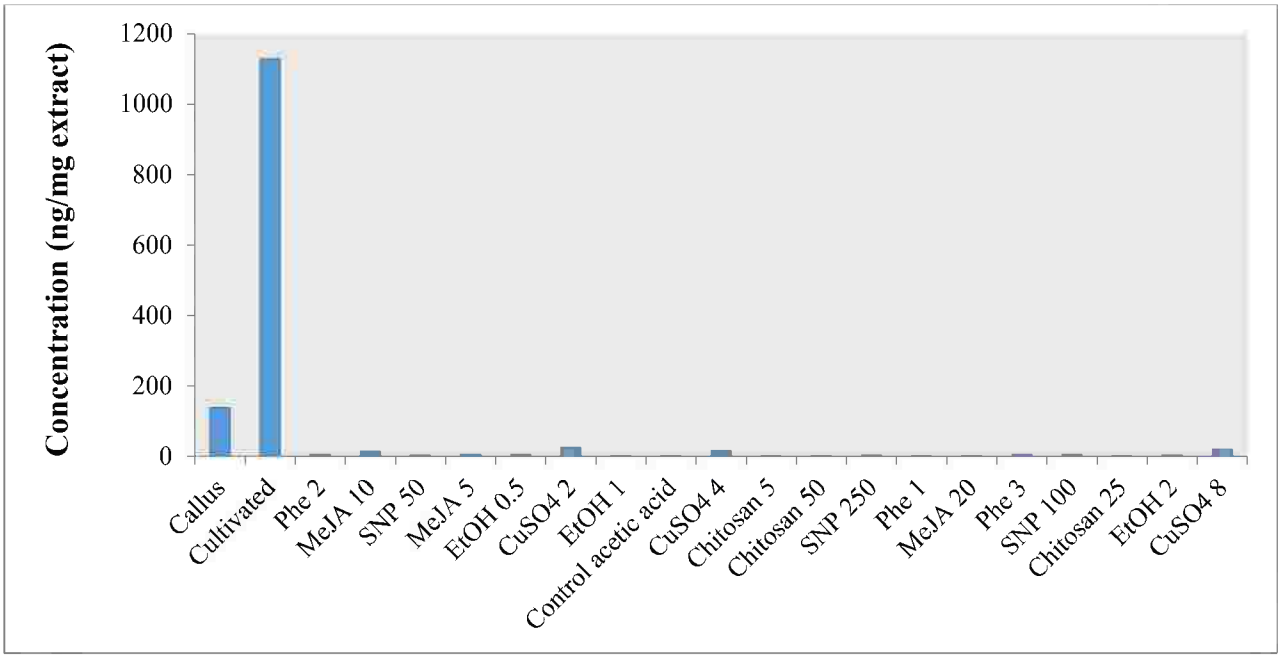

Figure 18: Caftaric acid concentration (expressed as ng/mg extract) of treated *Echinacea* extracts compared with untreated callus and cultivated plant extract.

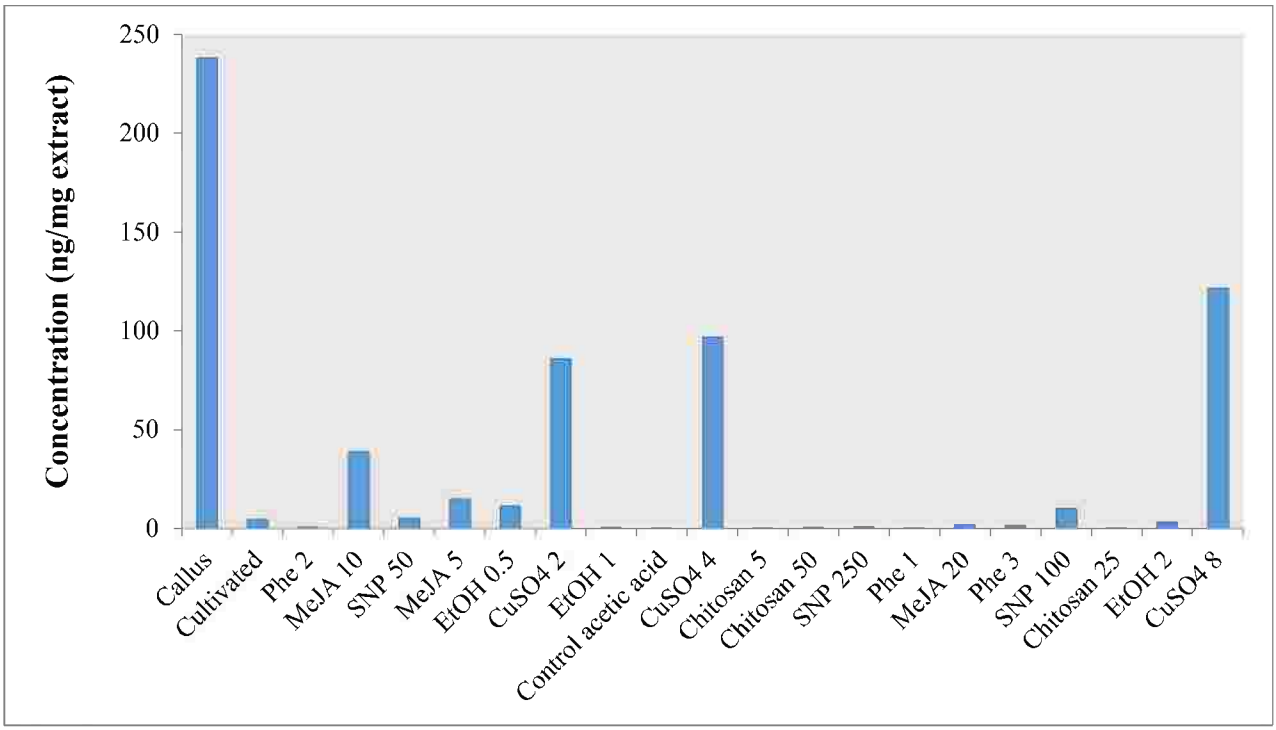

Figure 19: Chlorogenic acid concentration (expressed as ng/mg extract) of treated *Echinacea* extracts compared with untreated callus and cultivated plant extract.

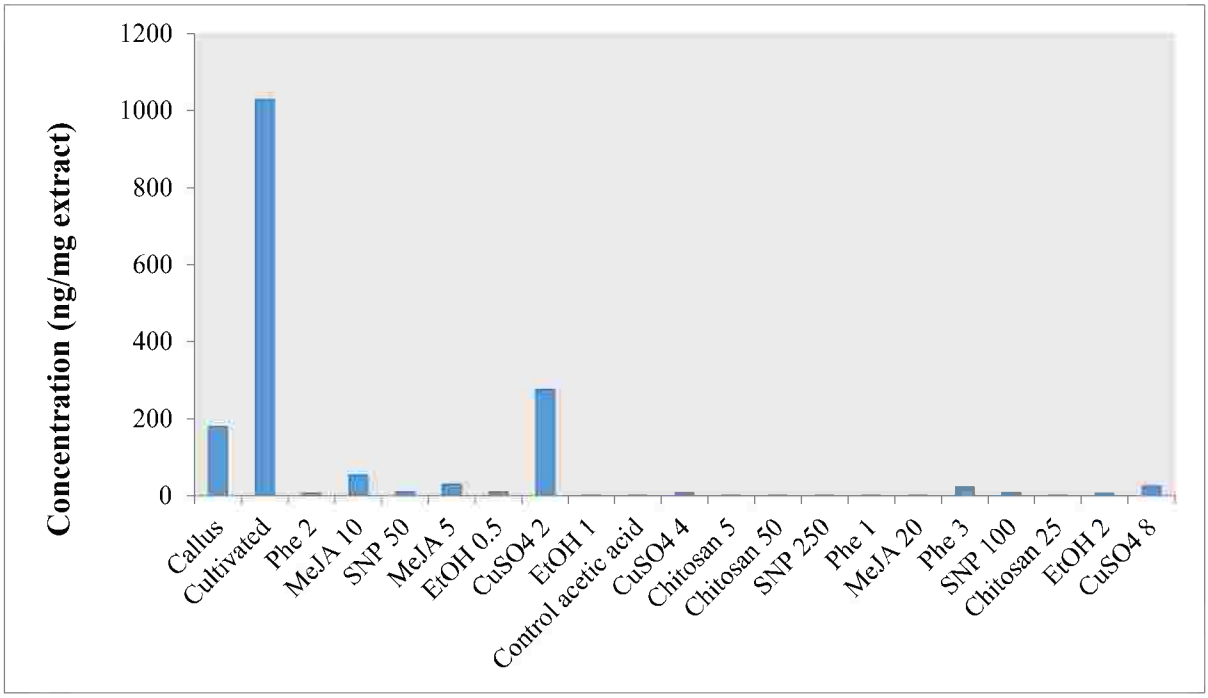

Figure 20: Chicoric acid concentration (expressed as ng/mg extract) of treated *Echinacea* extracts compared with untreated callus and cultivated plant extract

a. LC/MS/MS analysis data of standard reference caffeic acid

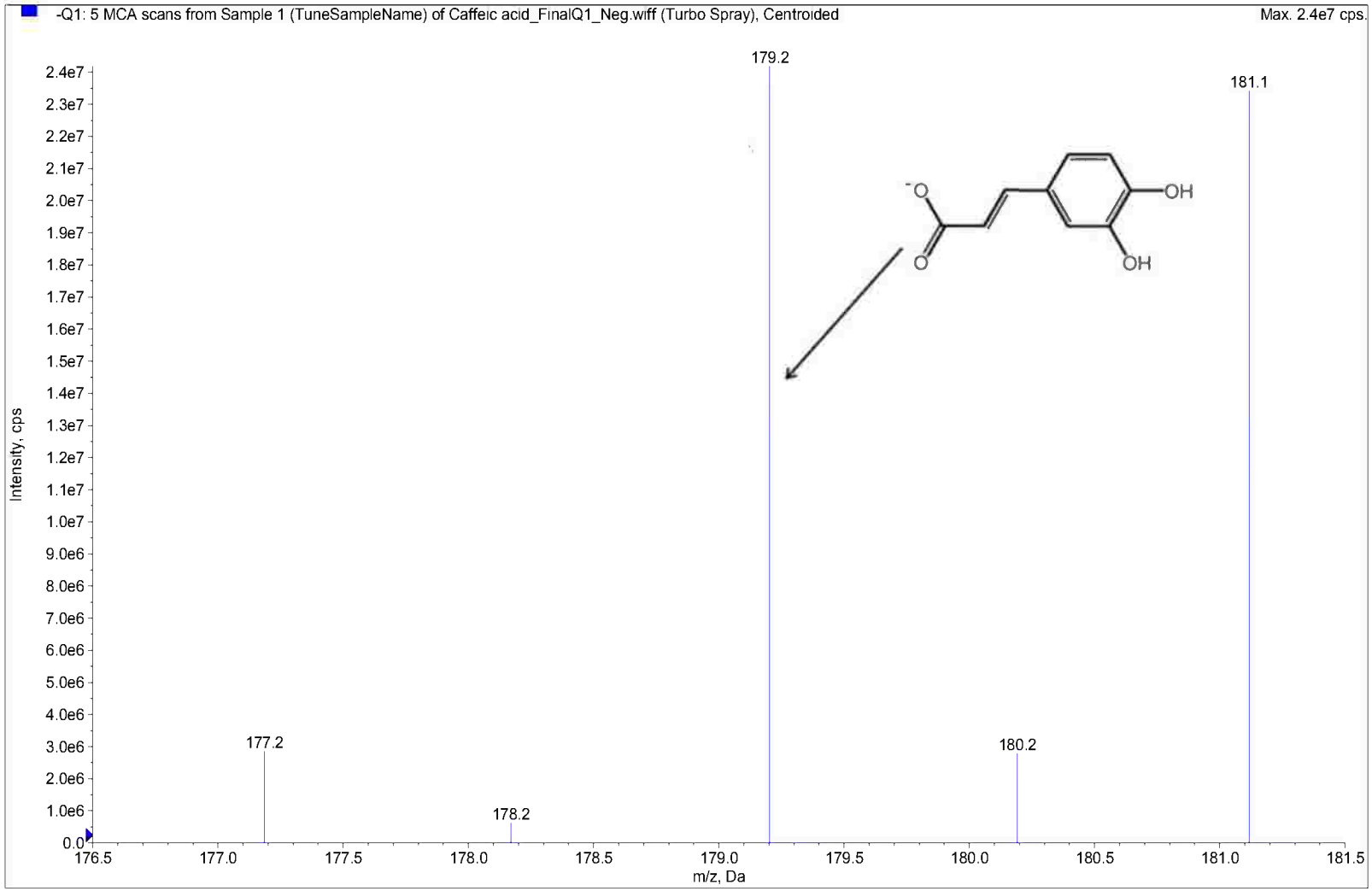

Figure 21: Negative ion mode of parent ion (caffeic acid)

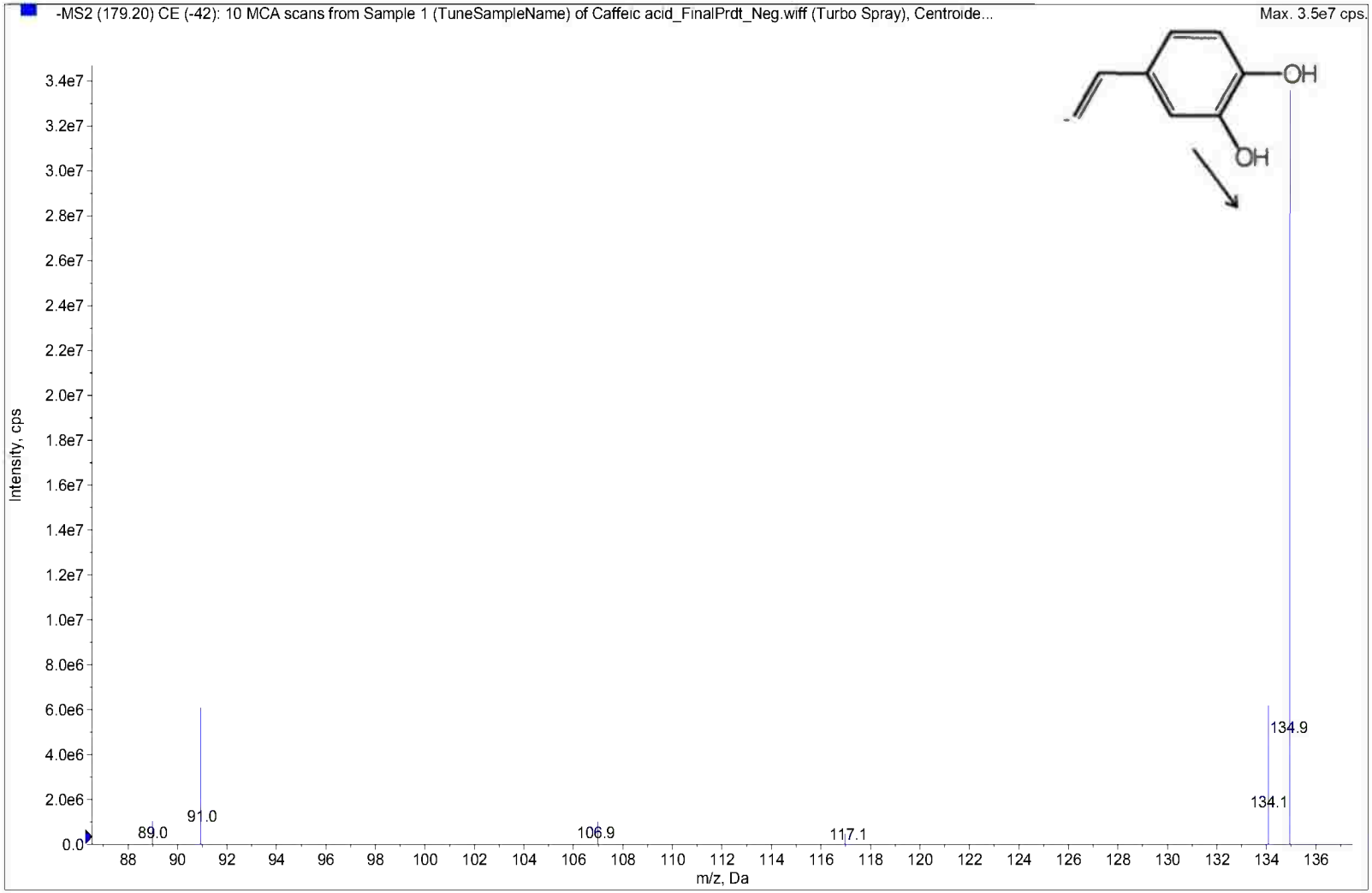

Figure 22: Negative ion mode of product ion (caffeic acid)

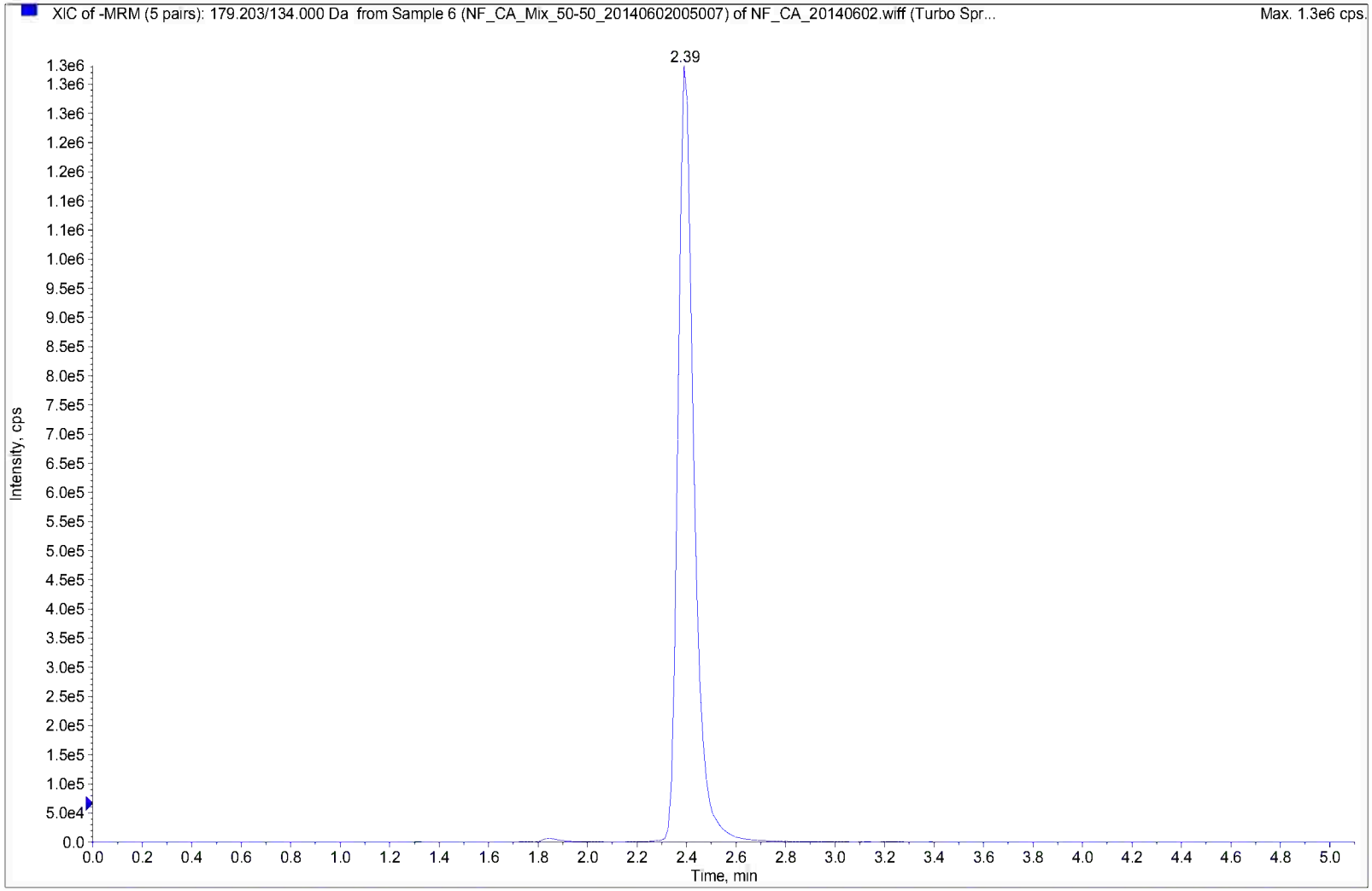

Figure 23: HPLC chromatogram of caffeic acid R_t at 2.39 minutes

b. LC/MS/MS analysis data of standard reference caftaric acid

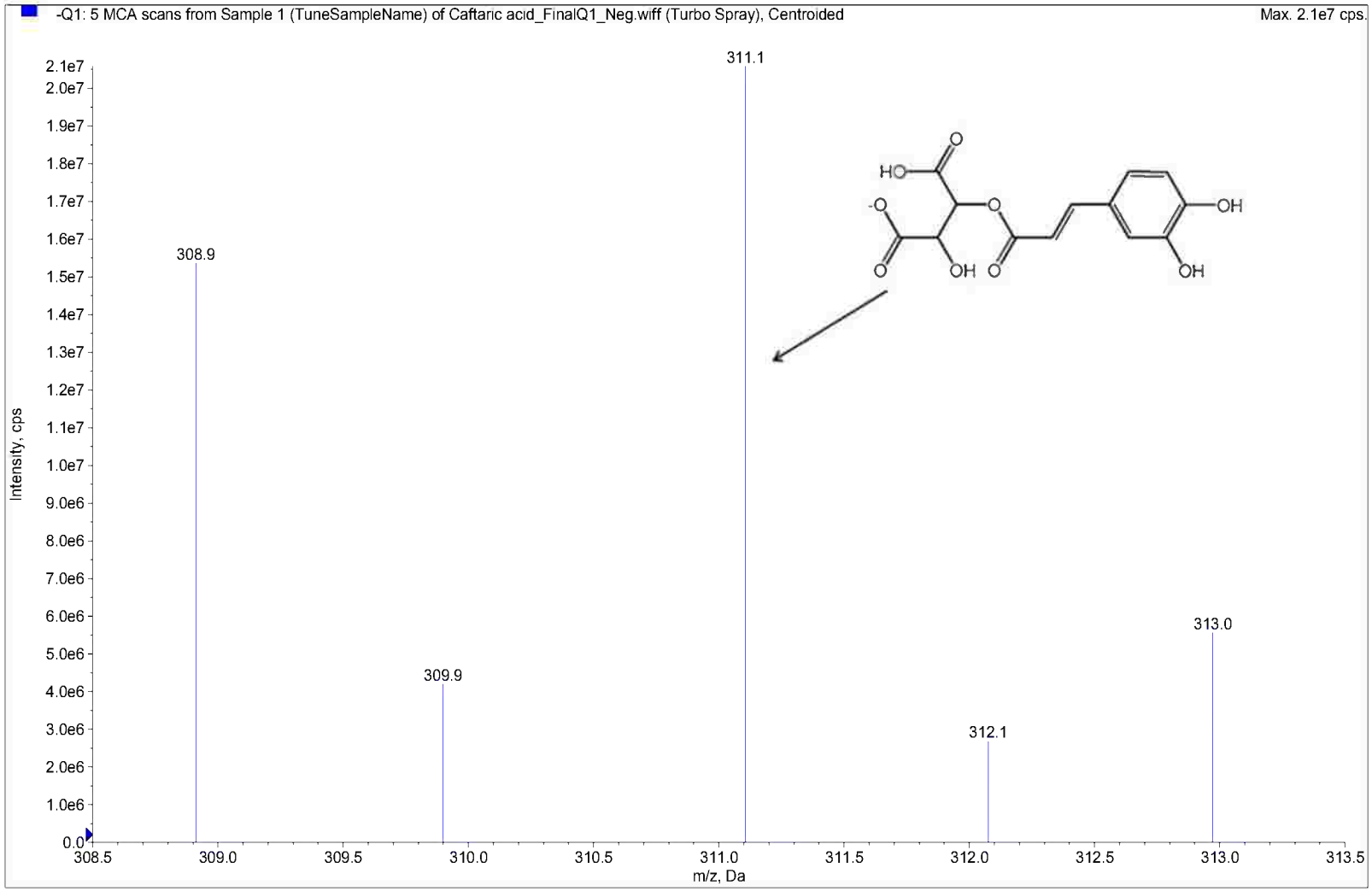

Figure 24: Negative ion mode of parent ion (caftaric acid)

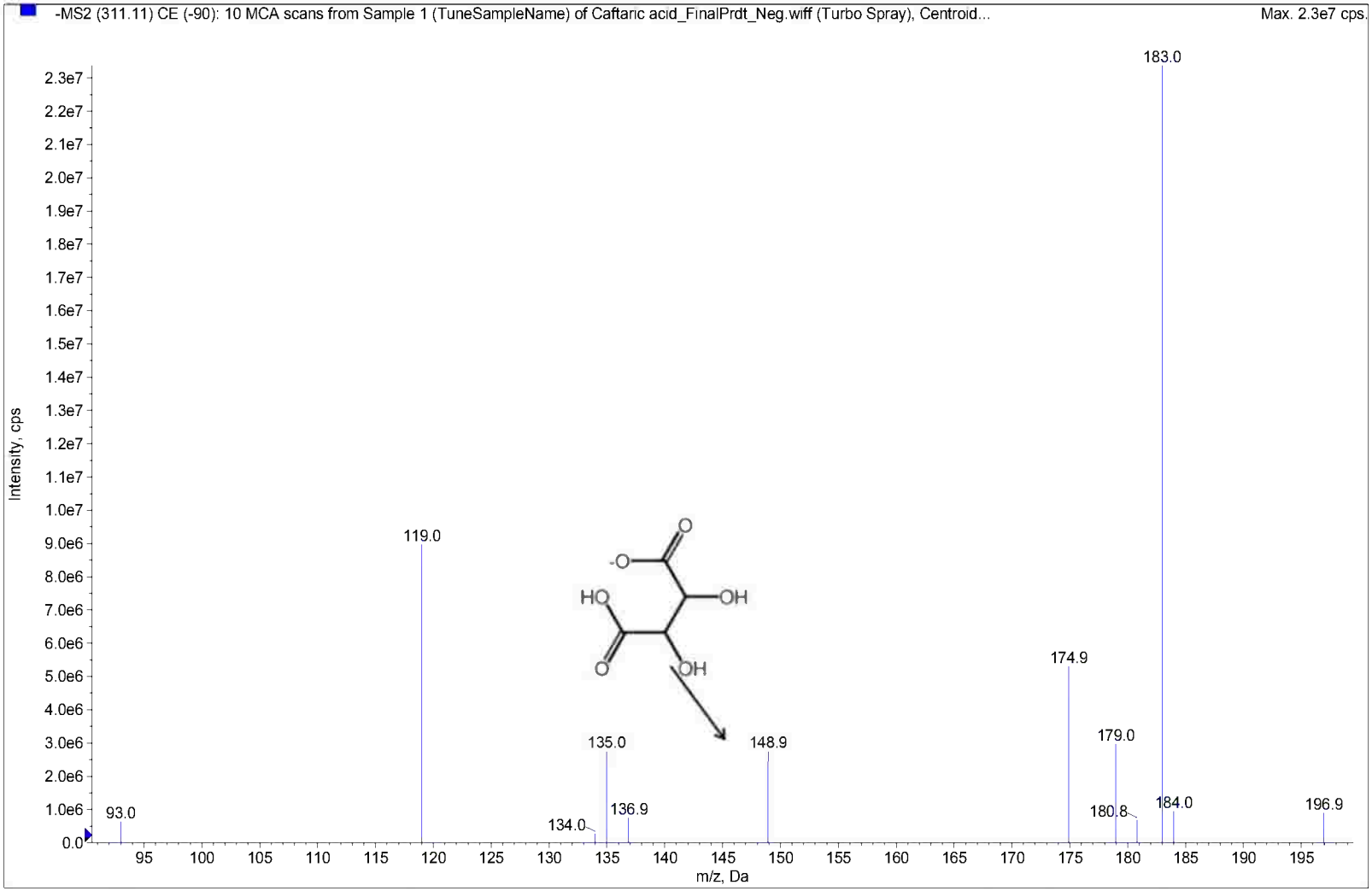

Figure 25: Negative ion mode of product ion (caftaric acid)

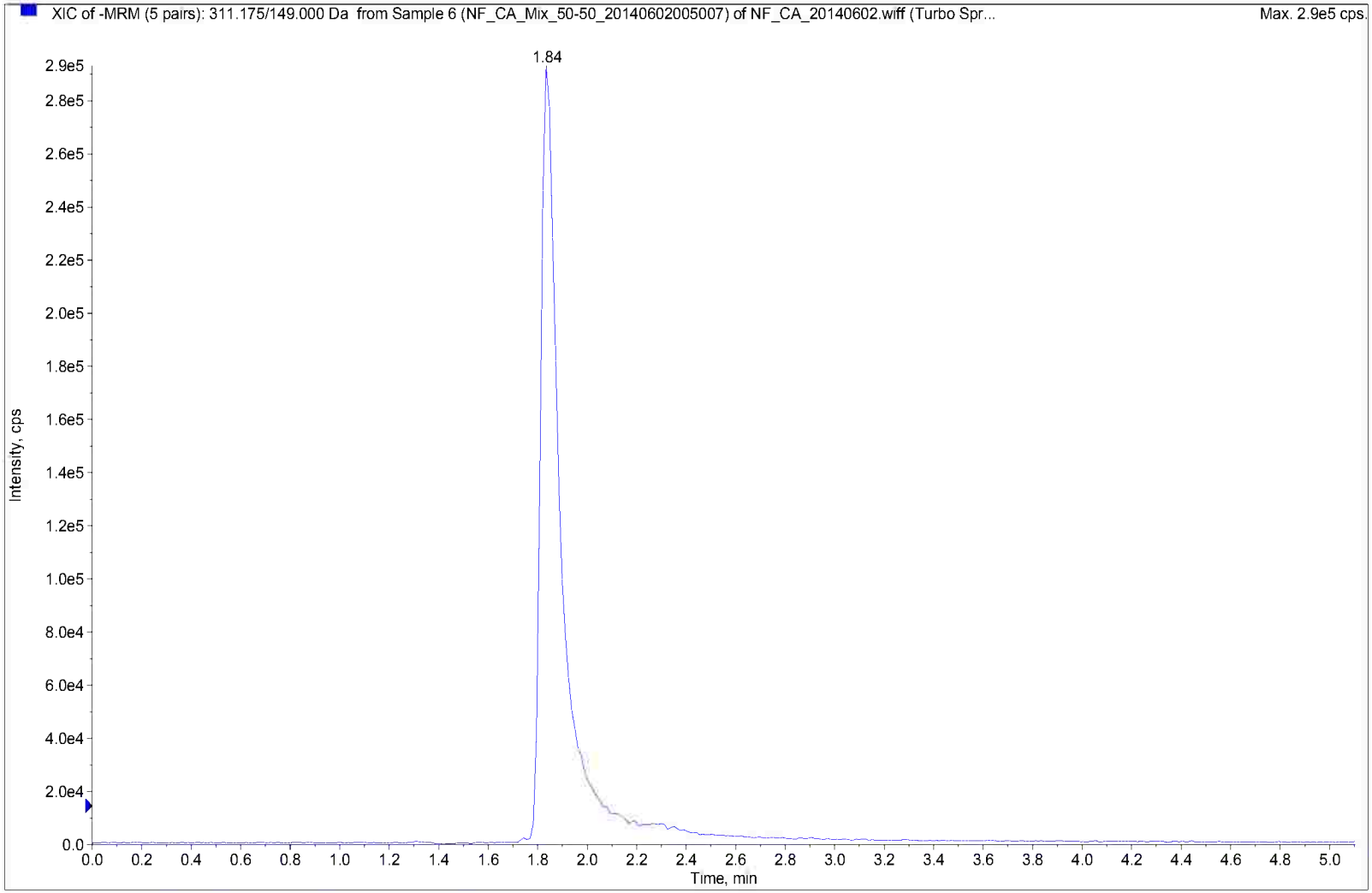

Figure 26: HPLC chromatogram of caftaric acid R_t at 1.84 minutes

c. LC/MS/MS analysis data of standard reference chlorogenic acid

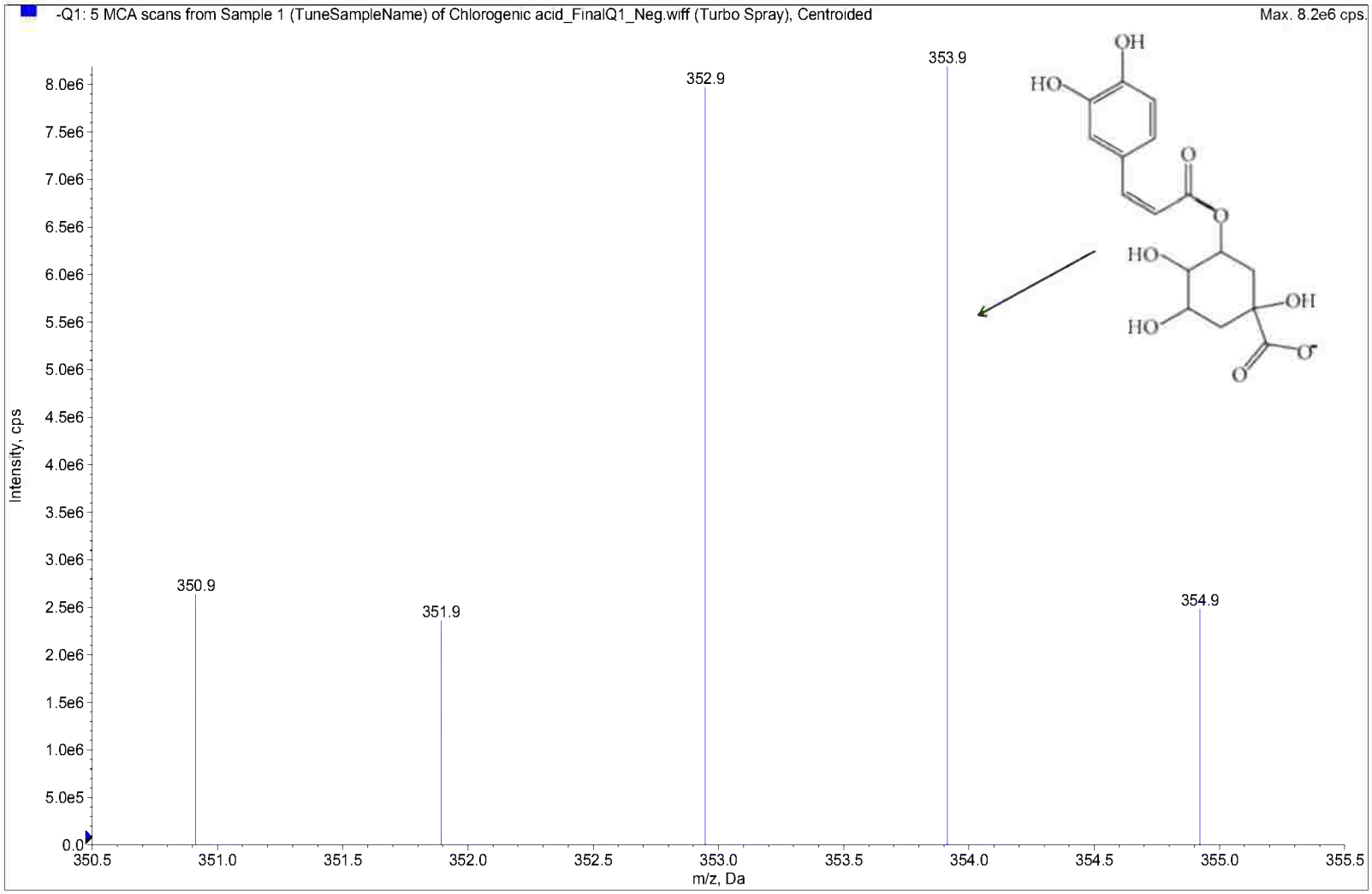

Figure 27: Negative ion mode of parent ion (chlorogenic acid)

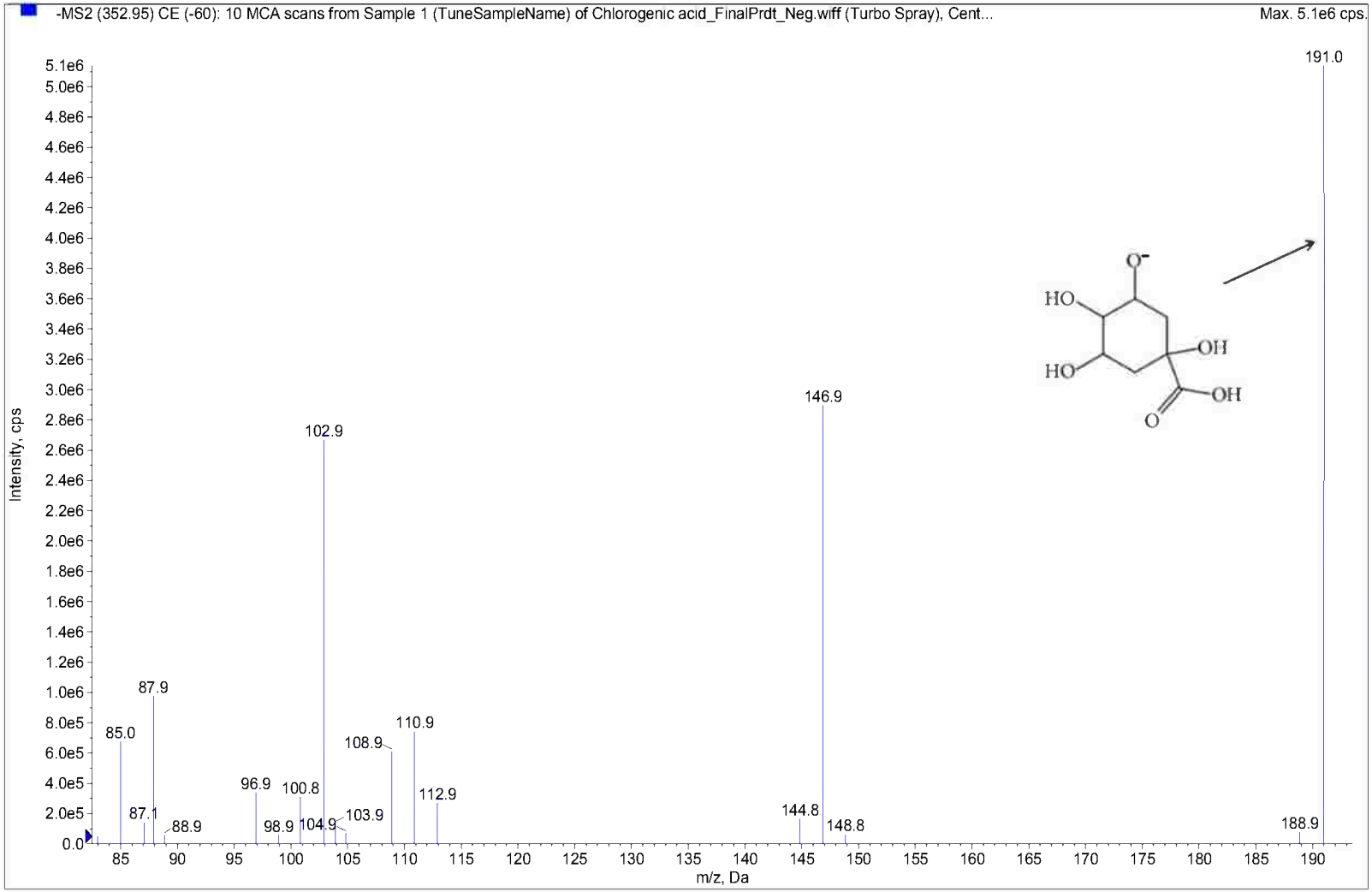

Figure 28: Negative ion mode of product ion (chlorogenic acid)

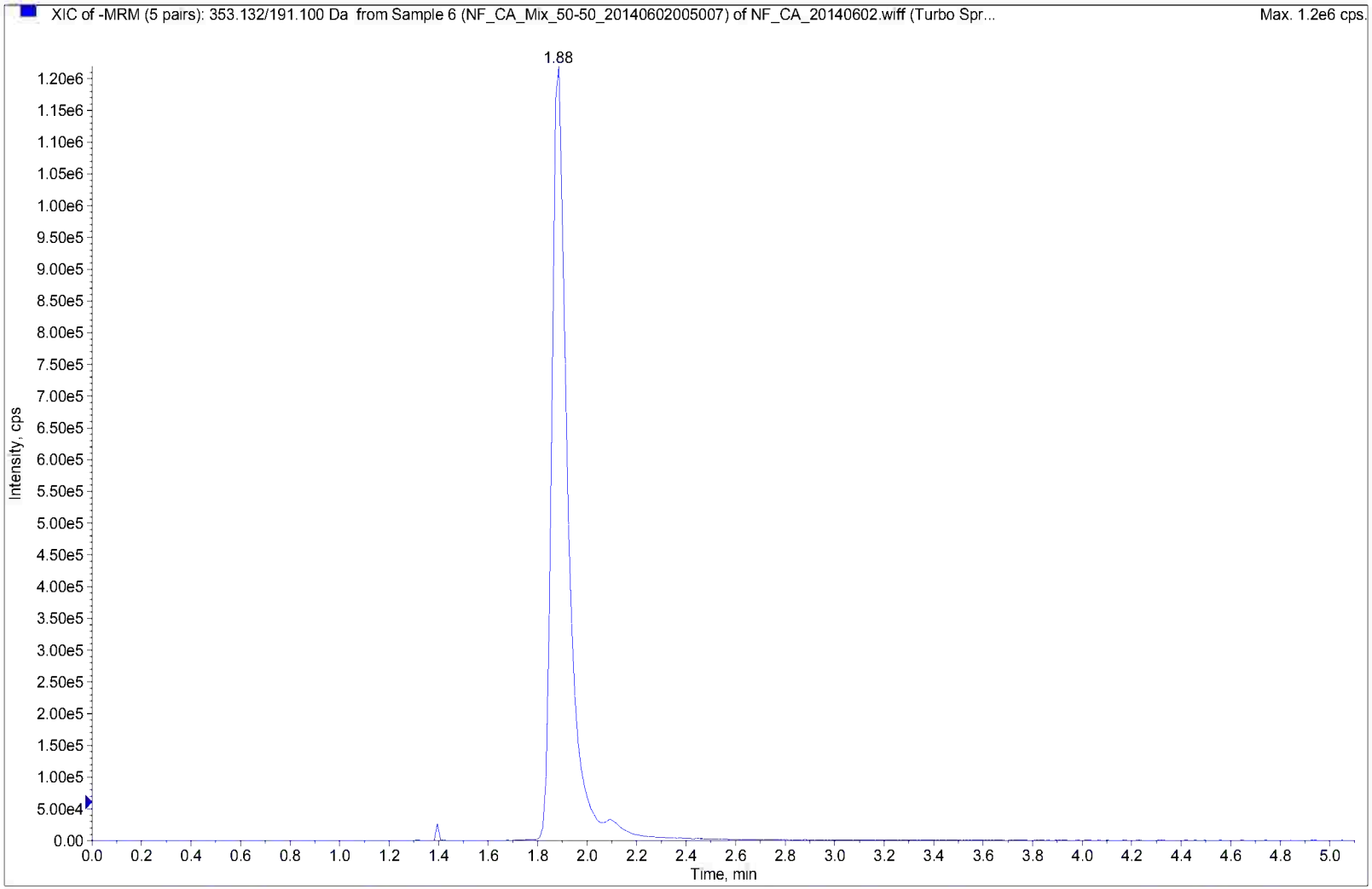

Figure 29: HPLC chromatogram of chlorogenic acid R_t at 1.88 minutes

d. LC/MS/MS analysis data of standard reference cichoric acid

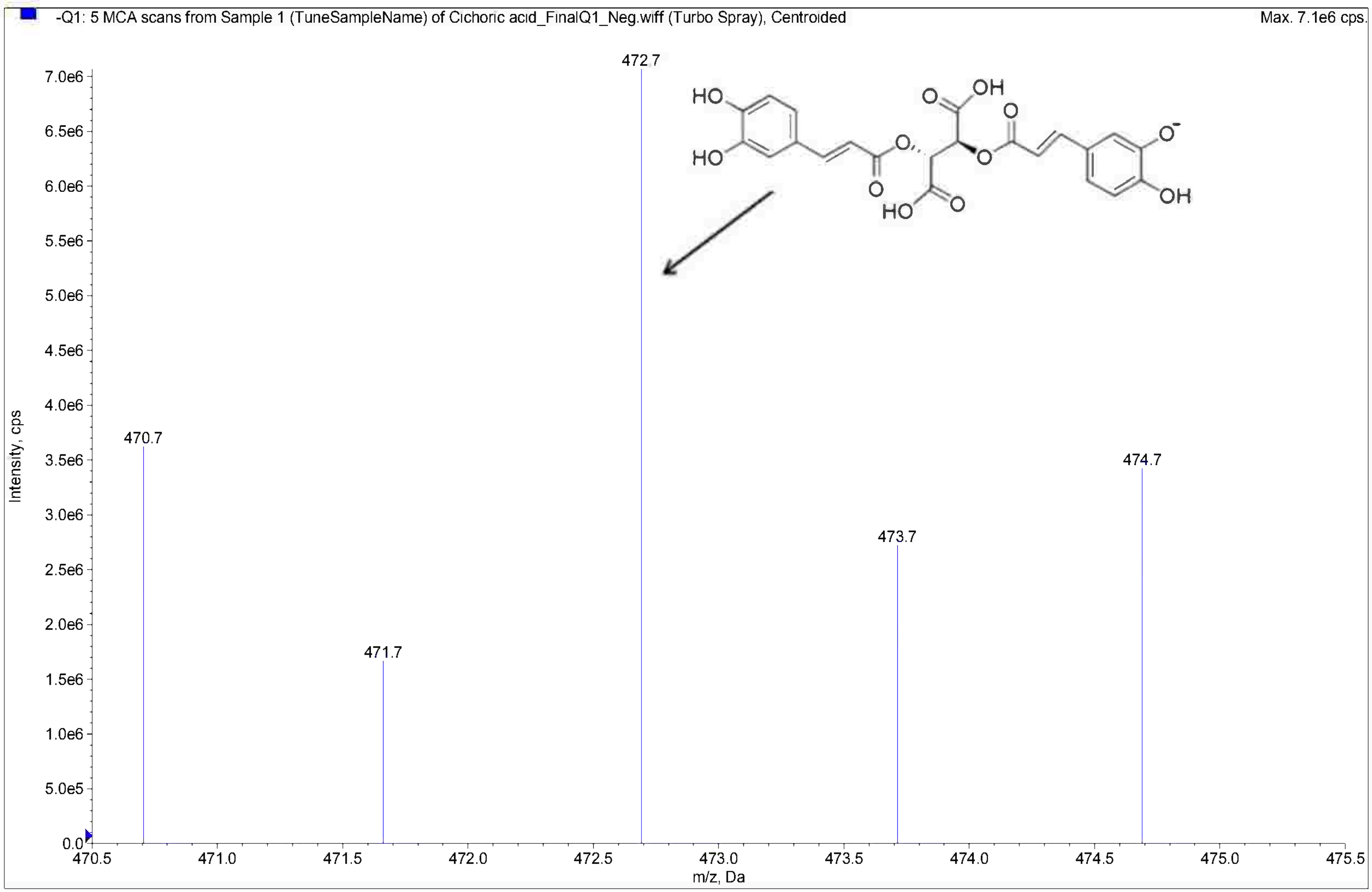

Figure 30: Negative ion mode of parent ion (cichoric acid)

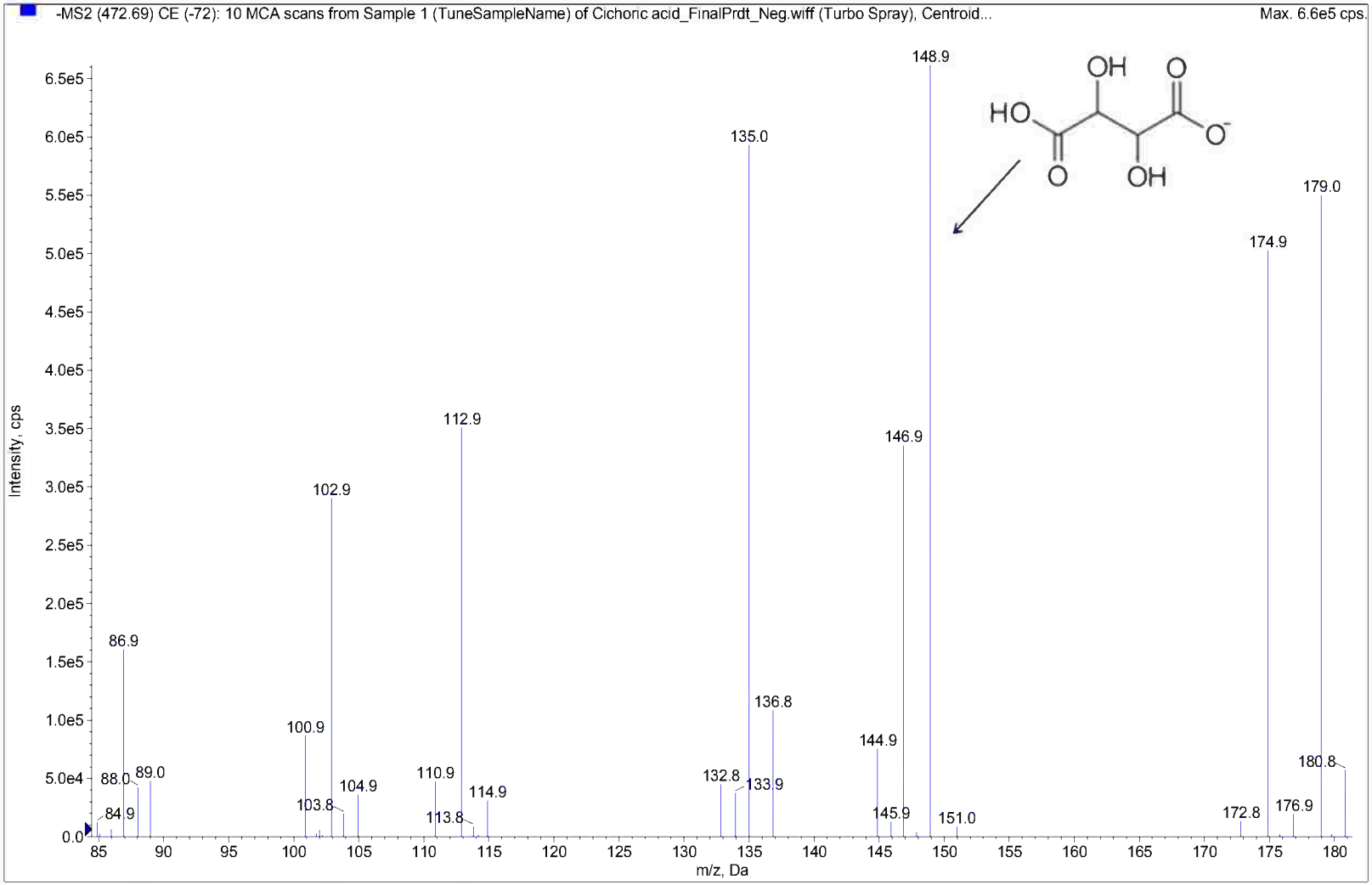

Figure 31: Negative ion mode of product ion (cichoric acid)

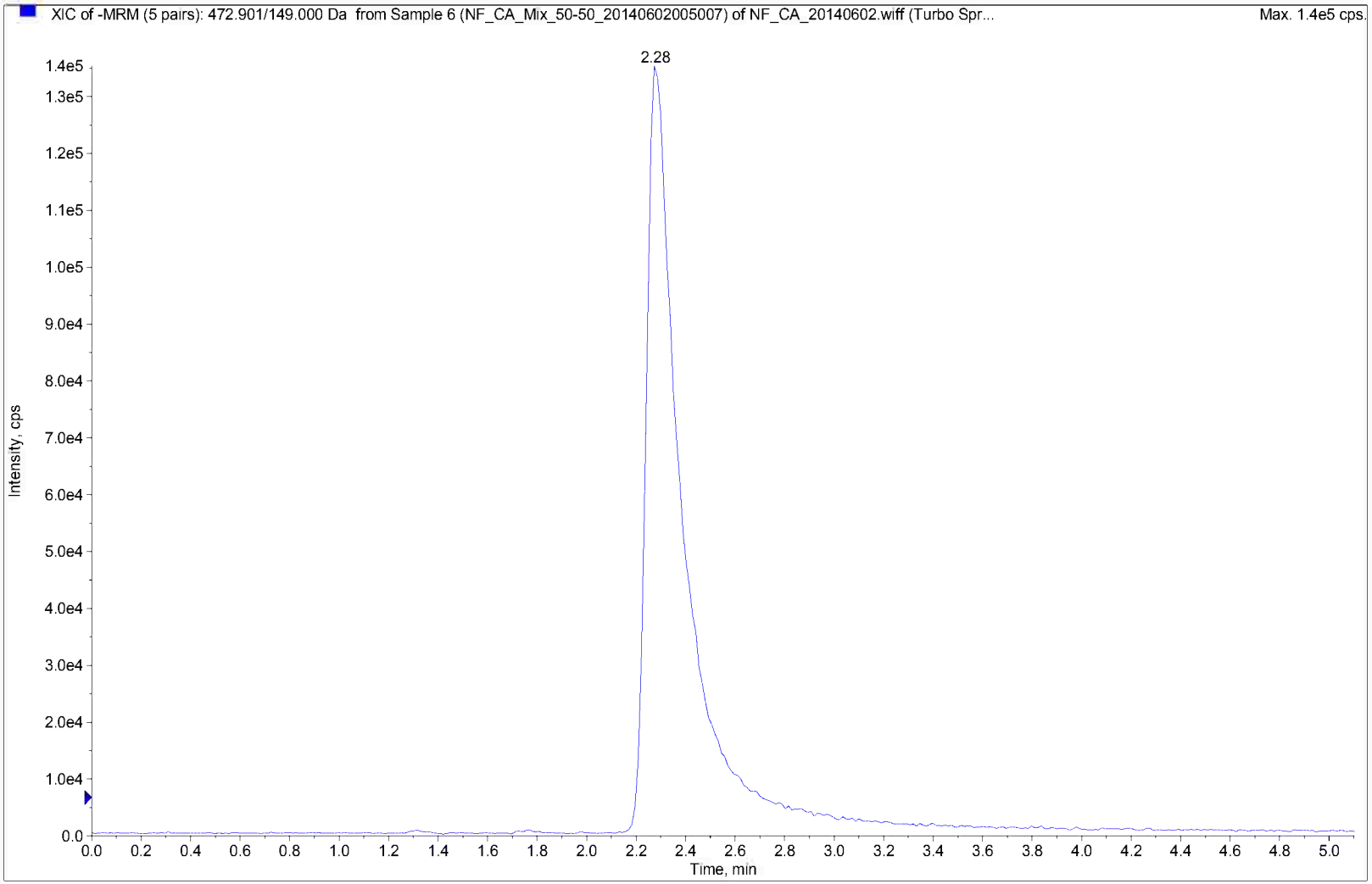

Figure 32: HPLC chromatogram of cichoric acid R_t at 2.28 minutes

e. LC/MS/MS analysis data of internal standard reference ferulic acid

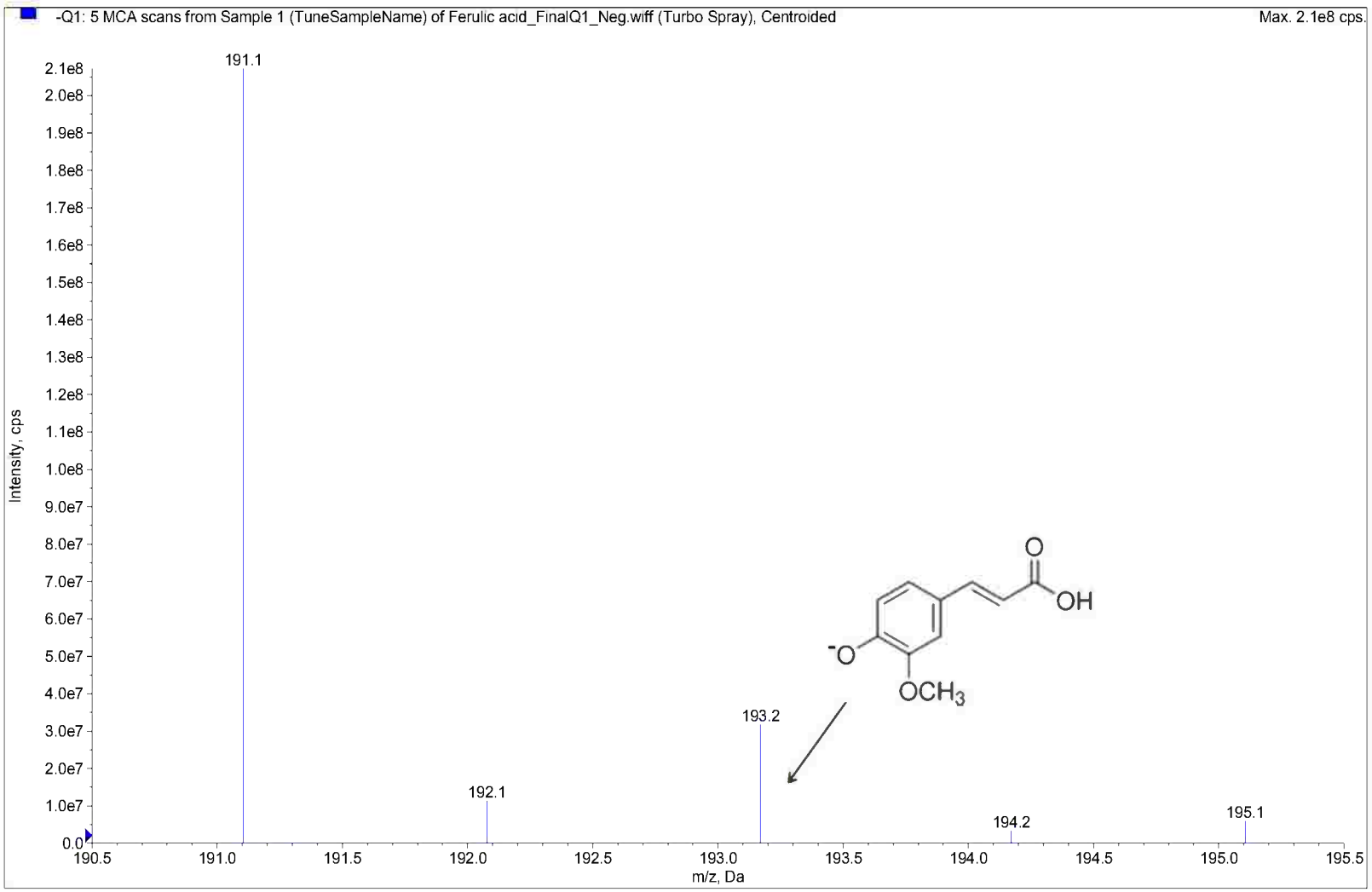

Figure 33: Negative ion mode of parent ion (ferulic acid)

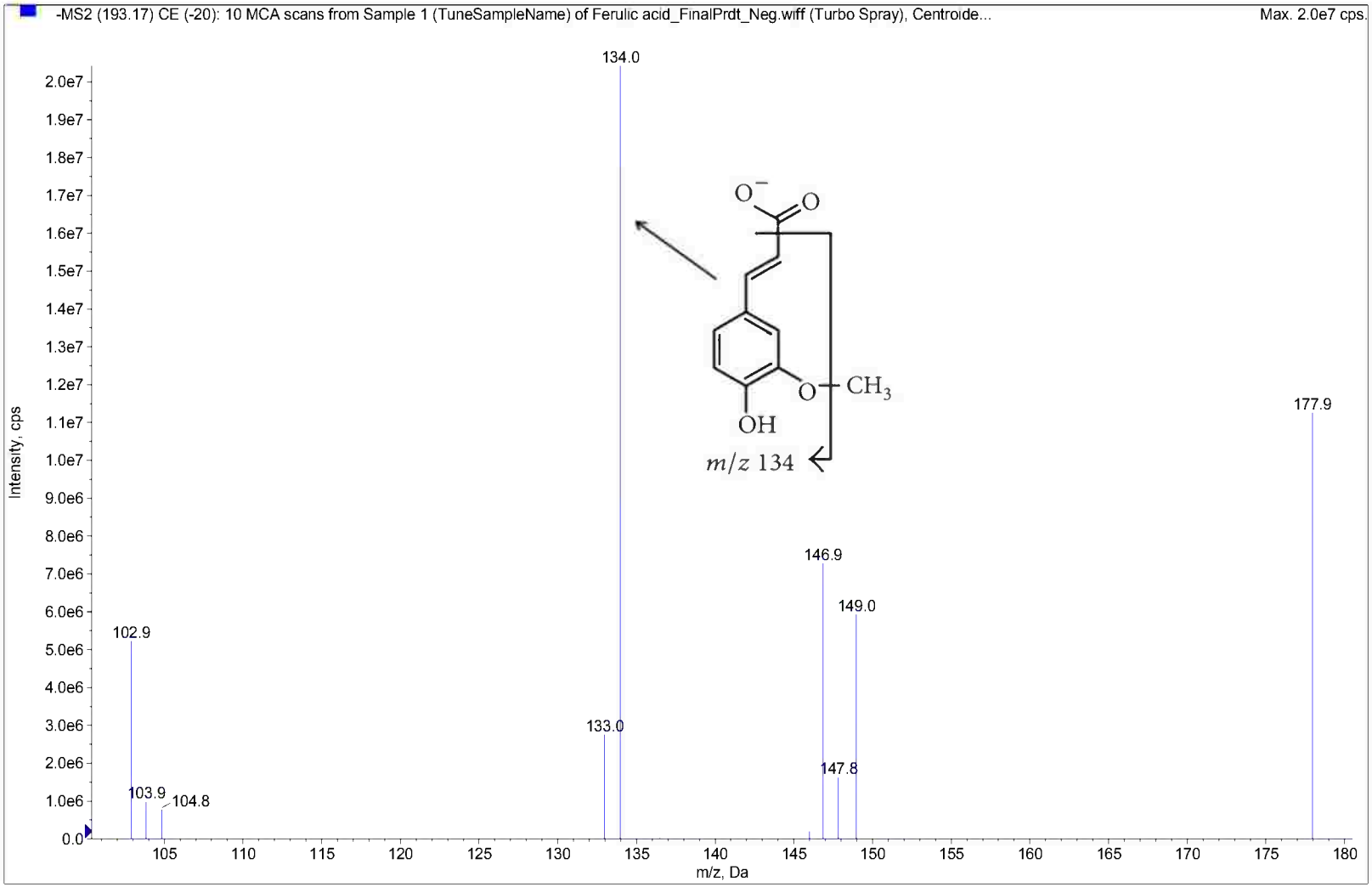

Figure 34: Negative ion mode of product ion (ferulic acid)

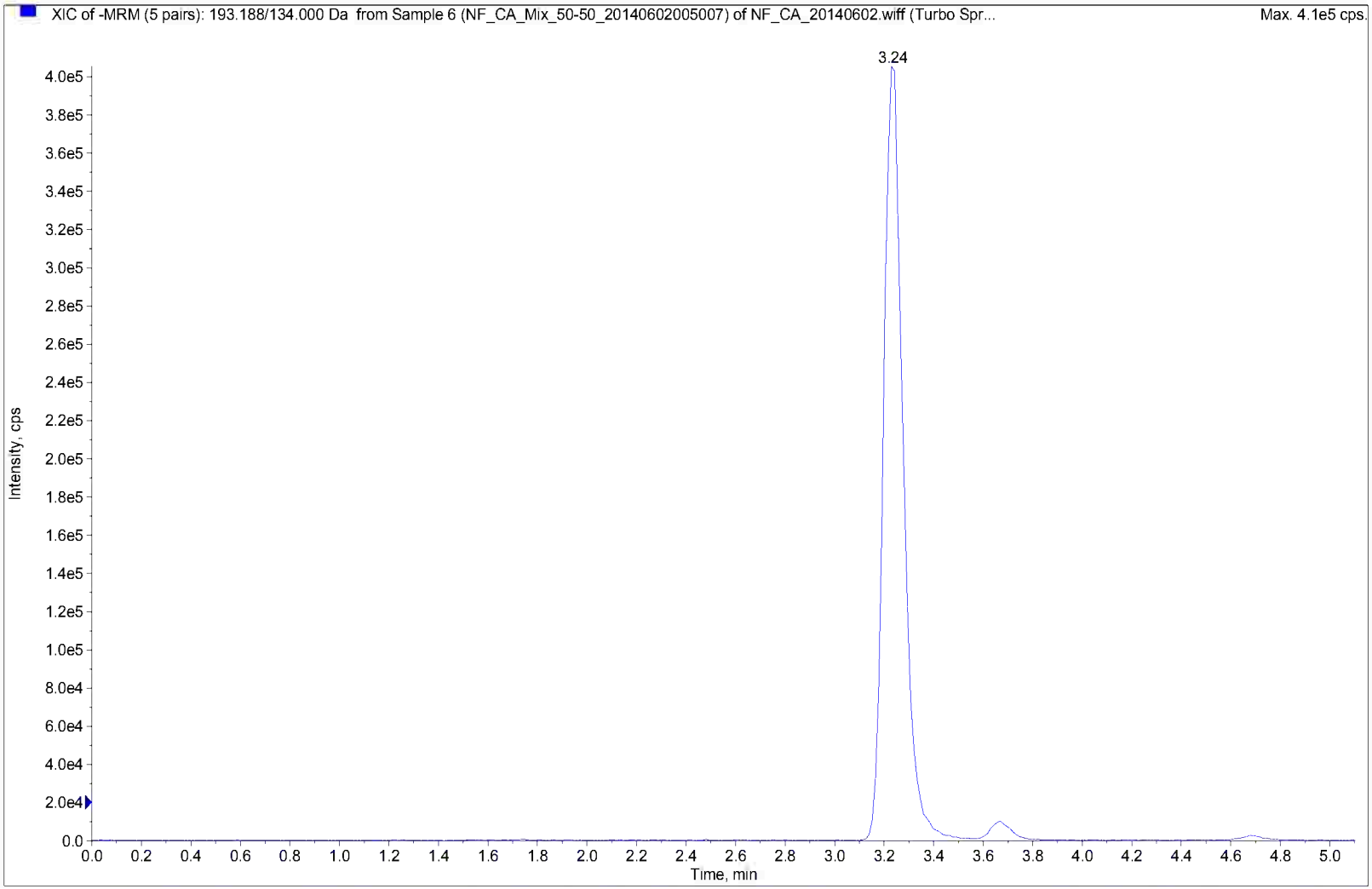

Figure 35: HPLC chromatogram of ferulic acid R_t at 3.24 minutes

Phenolic acids were analyzed using LC/MS/MS after *Echinacea* calli were treated with MeJA , Phe, $CuSO_4$, Chitosan and SNP for 15 days in comparison with the untreated calli and the cultivated plant using phenolic acids standards as reference and ferulic acid as an internal reference standard. Caffeic acid was eluted at 2.39 minutes **(Figure 23)** under previously mentioned experimental conditions, mass spectral analysis of caffeic acid revealed a molecular ion of *m/z* 179.2 **(Figure 21)** the MS-2 product-ion *m/z* 134 **(Figure 22).** Caftaric acid was eluted at 1.84 minutes **(Figure 26)** under previously mentioned experimental conditions, mass spectral analysis of caftaric acid revealed a molecular ion of *m/z* 311.1 **(Figure 24),** the MS-2 product-ion *m/z* 149 **(Figure 25).** Chlorogenic acid was eluted at 1.88 minutes **(Figure 29)**under previously mentioned experimental conditions, mass spectral analysis of chlorogenic acid revealed a molecular ion of *m/z* 353**(Figure 27)** the MS-2 product-ion *m/z* 191.1 **(Figure 28).** Chicoric acid was eluted at 2.28 minutes **(Figure 32)** under previously mentioned experimental conditions, mass spectral analysis of chicoric acid revealed a molecular ion of *m/z* 472.9 **(Figure 30);** the MS-2 product-ion *m/z* 149 **(Figure 31).** Ferulic acid was eluted at 3.24 minutes **(Figure 35)**under previously mentioned experimental conditions, mass spectral analysis of ferulic acid revealed a molecular ion of *m/z* 193.18 **(Figure 33);** the MS-2 product-ion *m/z* 134 **(Figure 34)**. The product ions previously mentioned for each phenolic acid was chosen for quantitation as it was the most abundant ion with the least background and acceptable sensitivity compared to any other product ions formed resulting from its fragmentation. Not all of the caffeic acids identified were observed in all the calli extracts, the detailed accumulation of caffeic acid, caftaric acid, chlorogenic acid and chicoric acid was recorded in **Table 2.**

Untreated calli recorded the highest concentration of caffeic acid with **27.6-fold** higher than the cultivated plant, while calli treated with **4 µM $CuSO_4$** recorded **13.19-fold** higher than the cultivated plant and calli treated with **8 µM $CuSO_4$** with **6.3-fold** higher than the cultivated plant **(Figure 17).** Caftaric acid content was reduced by all treatments compared with untreated callus which means the *in vitro* callus content of caftaric acid is diminished compared to the caftaric acid content naturally occurring in the intact cultivated plant **(Figure 18)**, same goes to cichoric acid the levels expressed *in vitro* in untreated calli is diminished as compared with the cultivated plant, but it is worth to mention that the calli treated with **2 µM $CuSO_4$** show higher levels (**1.5-fold**) greater than the untreated calli **(Figure 20).** Chlorogenic acid concentration was highly elevated in untreated callus tissue as compared to the cultivated plant, **52.16-fold** increase in concentration was recorded, followed by **26.6-fold** increase recorded by extracts from calli treated with **8 µM $CuSO_4$**, **21.19-fold** increase in extracts of **4 µM $CuSO_4$** treated calli, **18.83-fold** increase in extracts of **2 µM $CuSO_4$** treated calli and **8.5-fold** increase recorded by **10 mg/L MeJA** treated calli **(Figure 19).**