Supplementary information

Metabolomic profiling of *Medicago sativa*-derived fungal endophytes and evaluation of their biological activities

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Abstract

This study aimed to discover the potential of Medicago sativa-derived fungal endophytes as a prospective source of bioactive metabolites. In the present study, three different strains of fungal endophyte Aspergillus terreus were isolated from leaves L, roots T and stems St of Medicago sativa to explore their biological and chemical diversity. These isolated fungi were exposed to different fermentation conditions by adding various chemical elicitors to their solid fermentation media. According to LC-HRESIMS-based metabolomics and multivariate analysis, each chemical treatment had a different effect on the chemical profiles of the fungi. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) proposed several compounds with anticancer action against MCF-7 (a human breast cancer cell line) and MDA-MB-231 (a human epithelial breast cancer cell line).

Keywords: Medicago sativa, Fungal endophytes, LC-MS metabolomics, *Aspergillus terreus*, anticancer, OPLS-DA.

1. Material and methods

1.1. Plant material

Image obtained from the book titled Medicinal Plants of the World [1]

1.2. Chemicals and reagents

The ethanol and sodium hypochlorite used in the subsequent sterilizing process were supplied by Egypt's El-Nasr Company for Pharmaceuticals and Chemicals. For the LC-MS study, we sourced the following solvents from Fisher Chemicals (Thermo Fisher Scientific Inc., Dublin, Ireland): water, methanol, and acetonitrile. The HPLC-grade compounds we used were of the highest quality. The Sigma-Aldrich Chemical Company (Arklow, Co. Wicklow, Ireland) supplied all the chemical elicitors. The anticancer substances MTT and dimethyl sulfoxide (DMSO) were purchased from Sigma Co. in St. Louis, Missouri, USA.

1.2. Isolation and identification of entophytic fungi

The protocol for isolating endophytic fungi from *M. sativa*, as outlined by Araujo et al [2], was employed with minor alterations. The roots, stems and leaves were initially rinsed with tap water for 10 minutes. El-Nasr Company for Pharmaceuticals and Chemicals in Egypt supplied the 70% ethanol and the 1% sodium hypochlorite used for the subsequent sterilization process that lasted 1 minute. Finally, they were washed three times with sterile distilled water. The sterile stems, leaves, and roots were sectioned into 0.5 cm^2 portions prior to their introduction into Petri dishes containing potato dextrose agar (PDA) at a pH of 6.0. The PDA contained potato extract (200 g/L), dextrose (20 g/L), and agar (15 g/L). Additionally, 250 mg/mL of streptomycin was added to the PDA for antibacterial purposes. To confirm that the surface disinfection was successful, samples of the final rinse water from the roots, stems and leaves fragments were also inoculated onto PDA. The Petri dishes were incubated at a temperature of 28 ± 2°C until fungal mycelia growth was observed on the plant samples. The plates were checked daily for up to 10 days after incubation and any visible fungi were isolated, purified, and stored at 4°C on PDA slopes for further identification based on their morphology [3, 4]. The protruding hyphae were transferred to a new PDA slant to purify the fungal endophytes. The endophytic fungi were identified based on their morphological features at the Centre of Mycology, Assiut University, Assiut, Egypt [4]. The fungi were identified by examining their macroscopic characteristics, such as the shape of their colonies and the production of pigments, as well as their microscopic features, such as the presence and arrangement of spores or other reproductive cells. The endophytes were stored at the Microbial Repository of the Botany and Microbiology Department at Minia University's Faculty of Science in Minia, Egypt. They were kept at a temperature of 4°C.

1.3. LC-HRMS analysis

Using an Accela high-performance liquid chromatograph (HPLC) equipped with a UV detector and an Exactive orbitrap high-resolution mass spectrometer from Thermo Fisher Scientific in Bremen, Germany, we investigated ethyl acetate extracts from these three fungal endophytes. The treatments were evaluated in triplicate at 280 and 360 nm after being dissolved in menthol at a dosage of 1 mg/ml. Media blanks were also made for comparison. A 75 mm x 3.0 mm x 5 mm C18 ACE column from Hichrom Limited in Reading, UK was used for the HPLC analysis. Both distilled water (A) and acetonitrile (B), both containing 0.1% formic acid, made up the mobile phase. Over the course of 30 minutes at a flow rate of 300 mL/min, the gradient program went from 10% to

100% acetonitrile (B). The concentration of acetonitrile (B) then stayed at 100% for 5 minutes before gradually dropping to 10% in 1 minute.
Before each injection, the column was regenerated with 10% acetonitrile (B) for 9 minutes. Each sample took 45 minutes to analyze completely. The tray was kept at 12° C, and the injection volume was 10µ L. Spray voltage was 4.5 kV, and capillary temperature was 320 °C; both positive and negative ESI ionization modes were used in high resolution mass spectrometry. The raw data was converted using Ms converter software to separate positive and negative ionization data. The data mining software MZmine 2.10 (Okinawa Institute of Science and Technology Graduate University, Japan) was used to deconvolve, peak-pick, align, deisotope, and formula-predict the obtained files. METLIN and the Dictionary of Natural Products (DNP) were searched for the compounds' identities. 2018: http://dnp.chemnetbase.com/faces/chemical/ChemicalSearch.xhtml.

1.4. Statistical and Multivariate Analysis

MetaboAnalyst software was used for all statistical analyses [4]. To find out how the samples varied in terms of their metabolite profiles, we used principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). All peak intensities were log10 converted to compare them. All variables were scaled to unit variance for PLS-DA obtained from the LC-MS data sets.

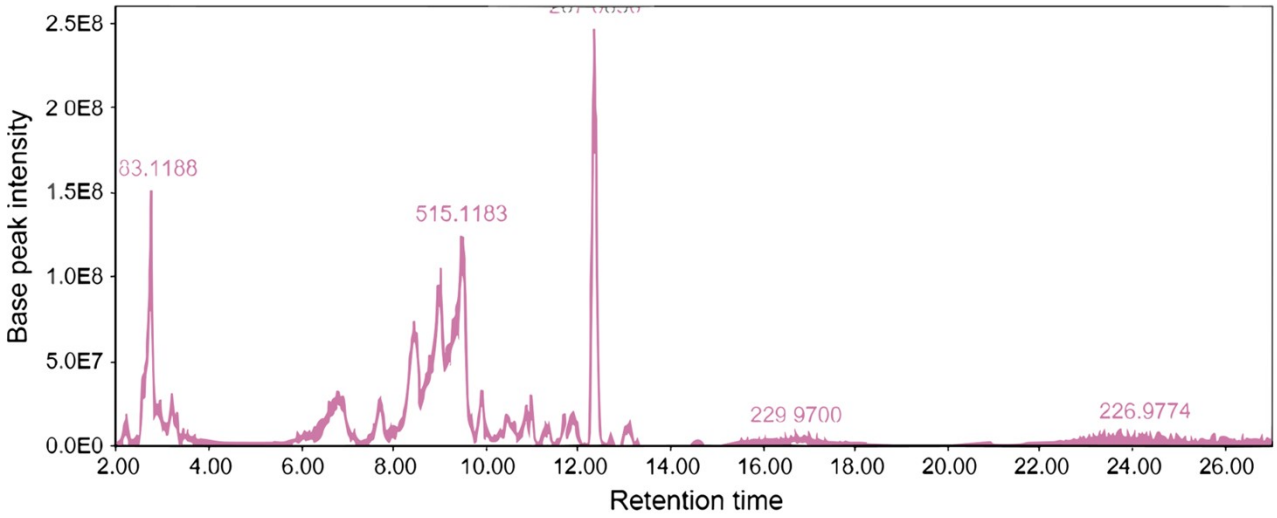
1.5. Anti-cancer activity of endophytic fungal extract

1.5.1 Cell line Both MCF-7 and MDA-MB-231 originate from human breast tumors. Unlike the latter, the former is known to be epithelial. We got these cell lines from ATCC by way of VACSERA in Cairo, Egypt. For the aim of evaluation, the conventional anticancer medication doxorubicin was used.
1.5.2. MTT assay [5]. To evaluate the potential growth-inhibiting effects of different drugs on the aforementioned cell lines for 21 samples, the MTT assay (a colorimetric test for assessing cell metabolic activity) was used. The MTT assay is a colorimetric method that involves the conversion of the yellow tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide into a purple formazan material. The succinate dehydrogenase enzyme located in healthy mitochondria catalyzes this process. The cell lines were cultured in RPMI-1640 media with 10% fetal bovine serum. Ten thousand cells were cultivated in each well of a 96-well plate to cultivate the cell lines. They spent 48 hours in a 37°C, 5% CO_2 incubator. The antibiotics penicillin (100 units /mL) and streptomycin (100 µg /mL) were added. The cells were then cultured for a further 24 hours after being treated with substances of varying doses. Following medication treatment for 24 hours, 20 µL of 5 mg mL^{-1} MTT solution was added and incubated for 4 hours. The purple formazan was dissolved by adding 100 µL of dimethyl sulfoxide (DMSO) to each well. Using a plate reader (EXL 800, USA), the absorbance at 570 nm was measured and recorded during the colorimetric experiment. (A_{570} of treated samples / A_{570} of untreated sample) x 100 gave the percentage of relative cell viability.

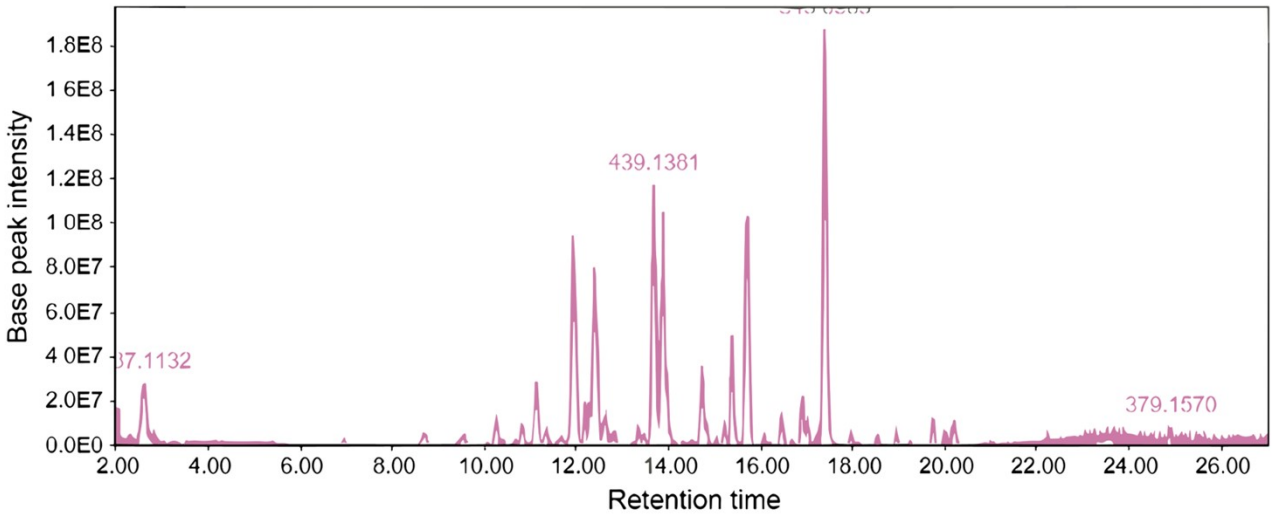
Results

LCHRMS chromatograms of the twenty-one extract obtained on fungal fermentation using different elicitors

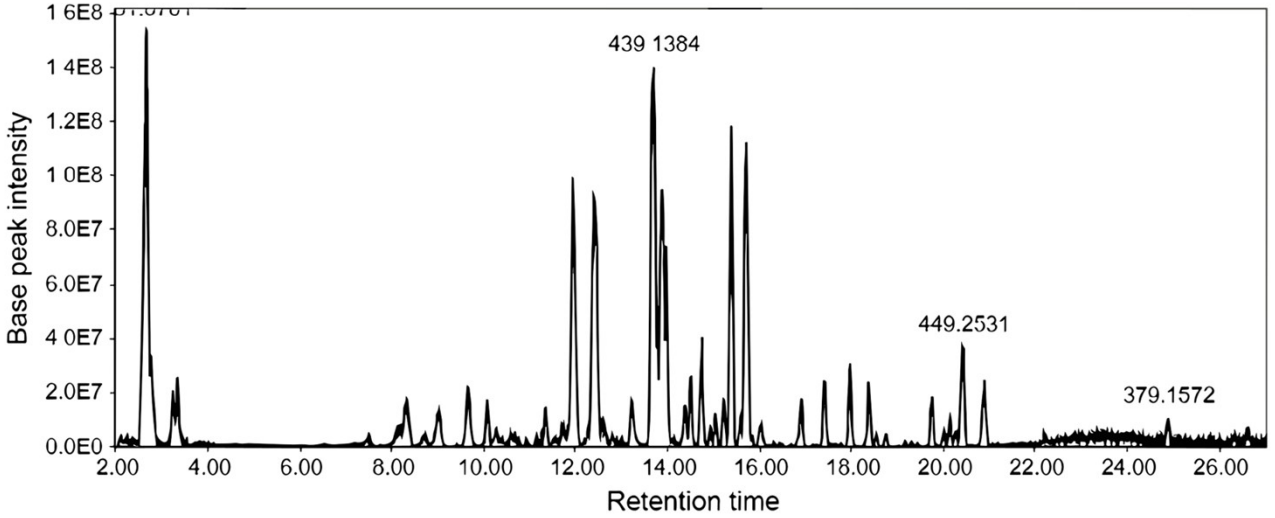
A1 refers to endophytic fungus obtained from *Medicago sativa* leaves
(glucoseamine E1, nicotinamide E2, sodium butyrate E3, erythromycin E4, cycloheximide E5 and tetracycline E6),



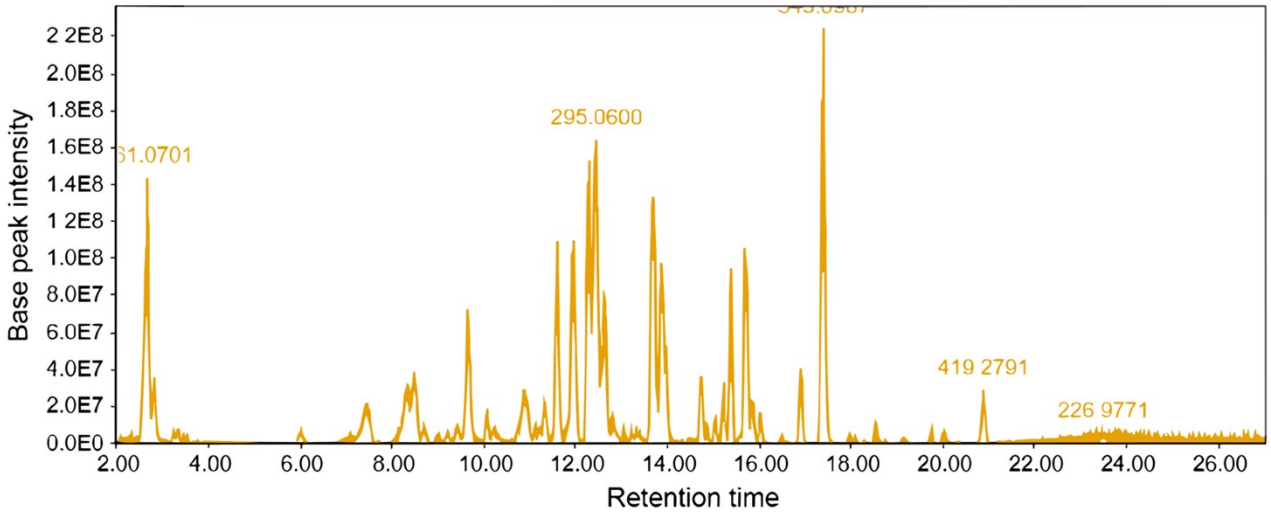
LC-HRMS positive mode total ion chromatogram of A1



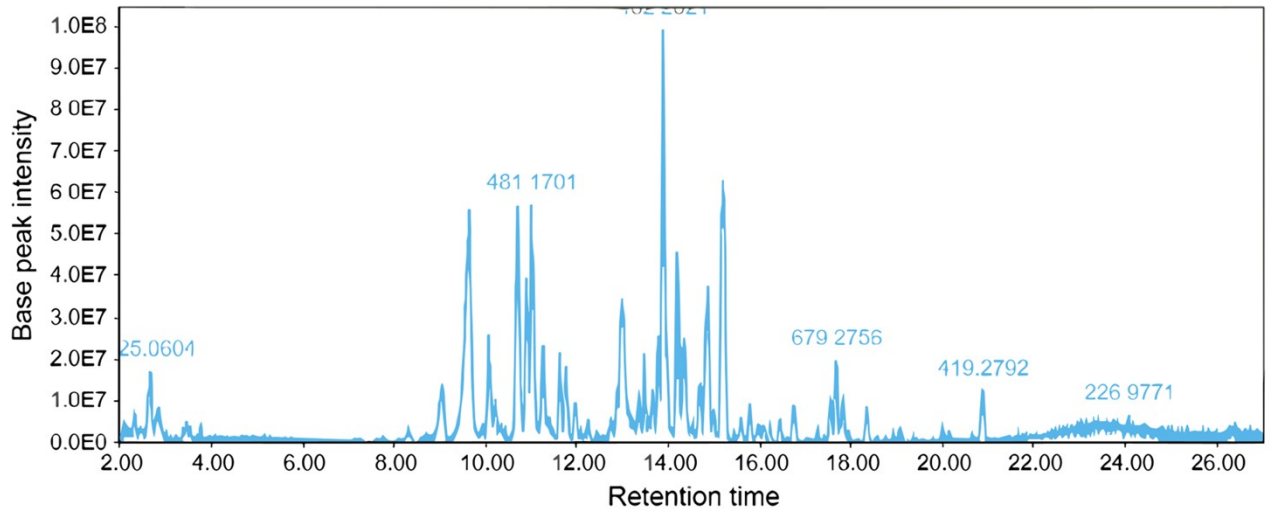
LC-HRMS positive mode total ion chromatogram of A1 E1



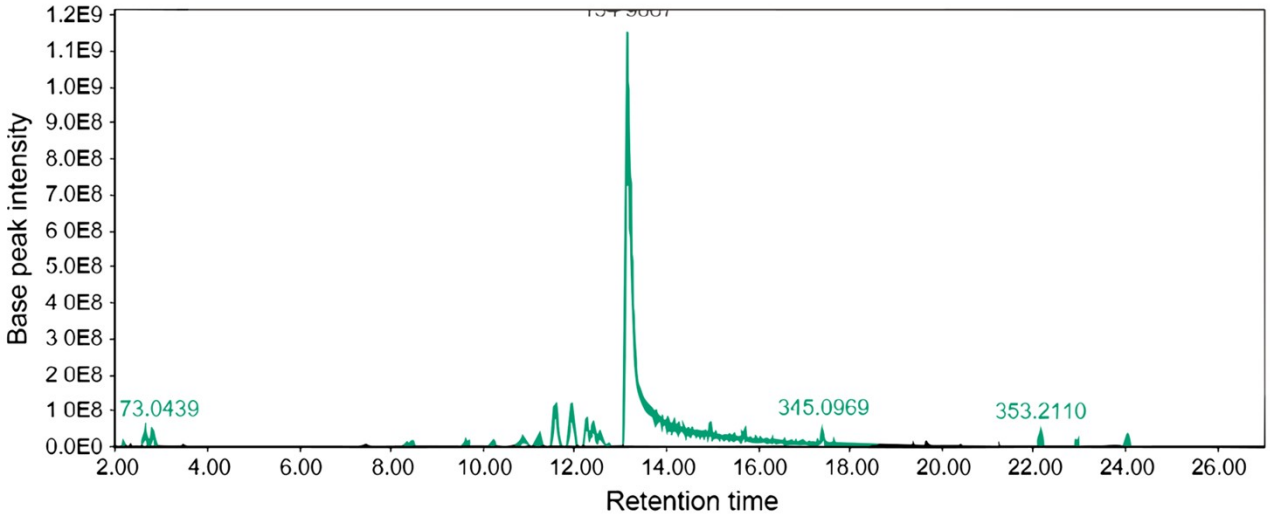
LC-HRMS positive mode total ion chromatogram of A1 E2



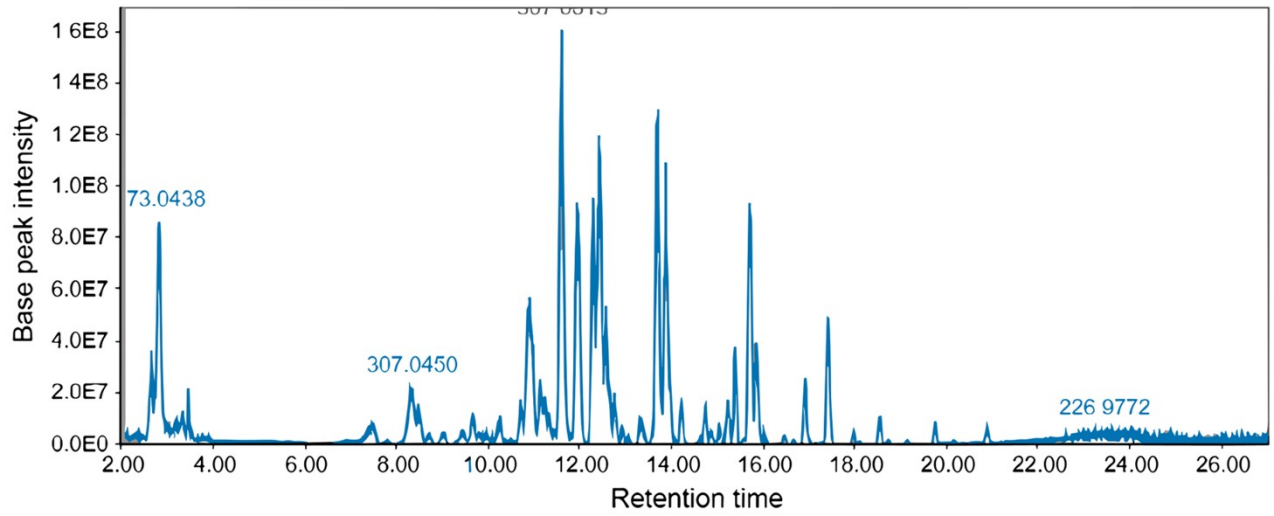
LC-HRMS positive mode total ion chromatogram of A1 E3



LC-HRMS positive mode total ion chromatogram of A1 E4



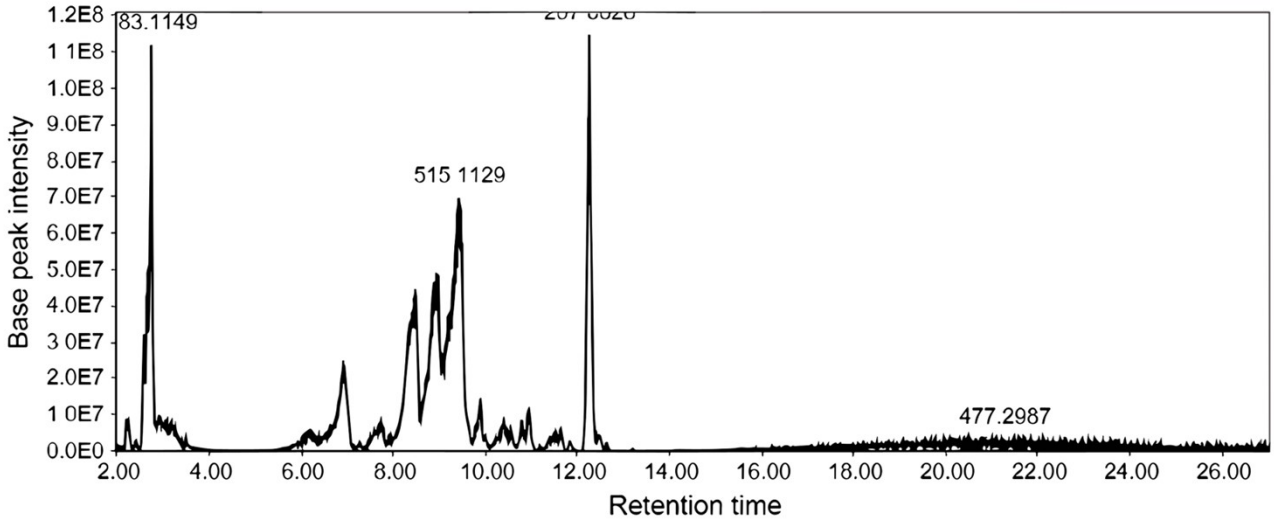
LC-HRMS positive mode total ion chromatogram of A1 E5



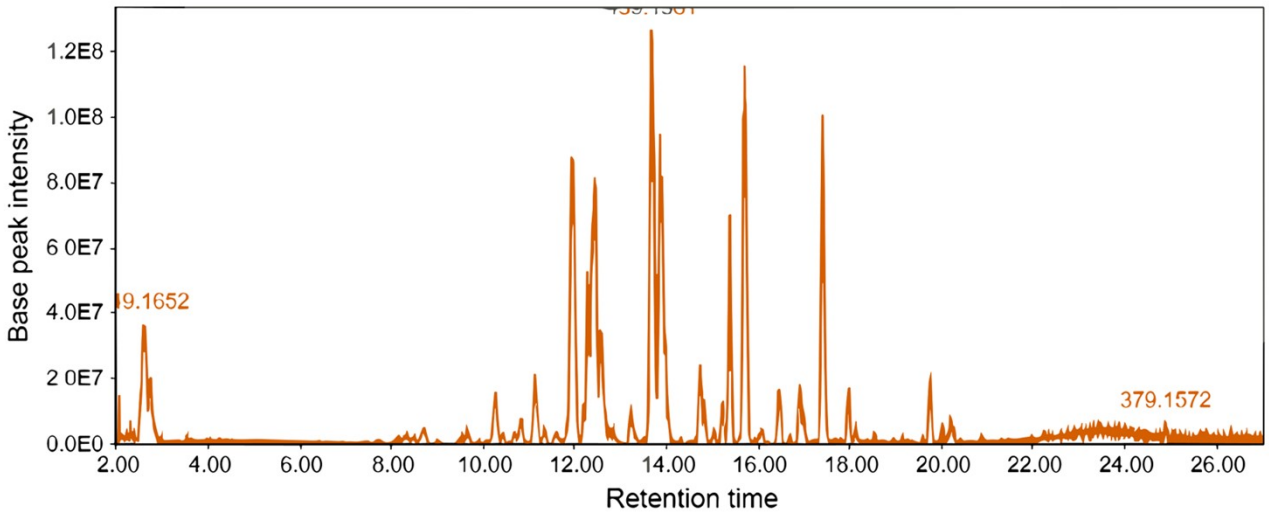
LC-HRMS positive mode total ion chromatogram of A1 E6

F2 refers to endophytic fungus obtained from *Medicago sativa* Stems

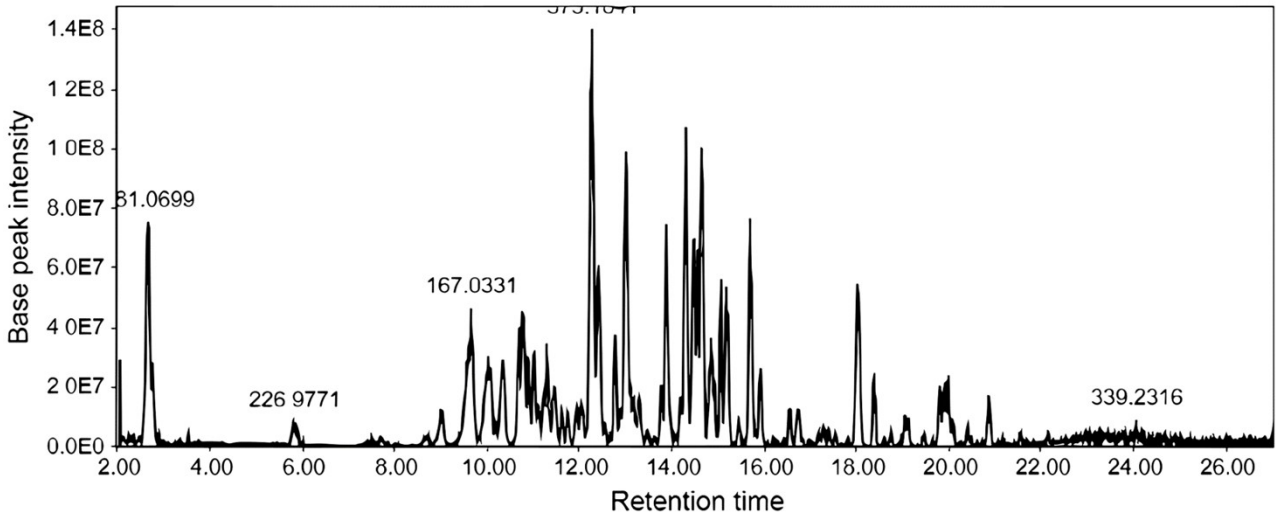
(glucoseamine E1, nicotinamide E2, sodium butyrate E3, erythromycin E4, cycloheximide E5 and tetracycline E6),



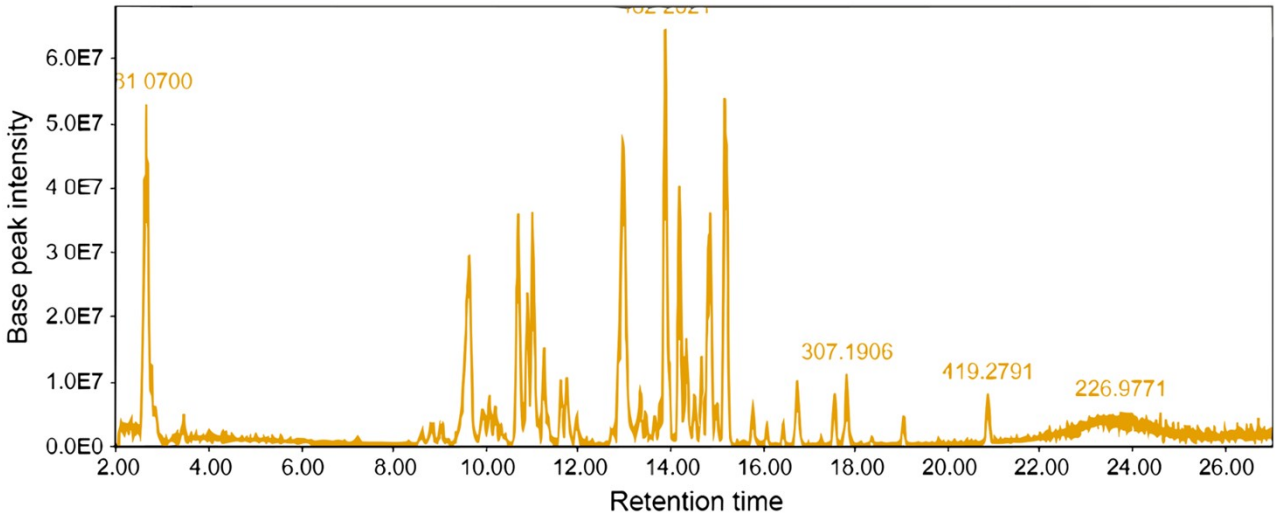
LC-HRMS positive mode total ion chromatogram of F2



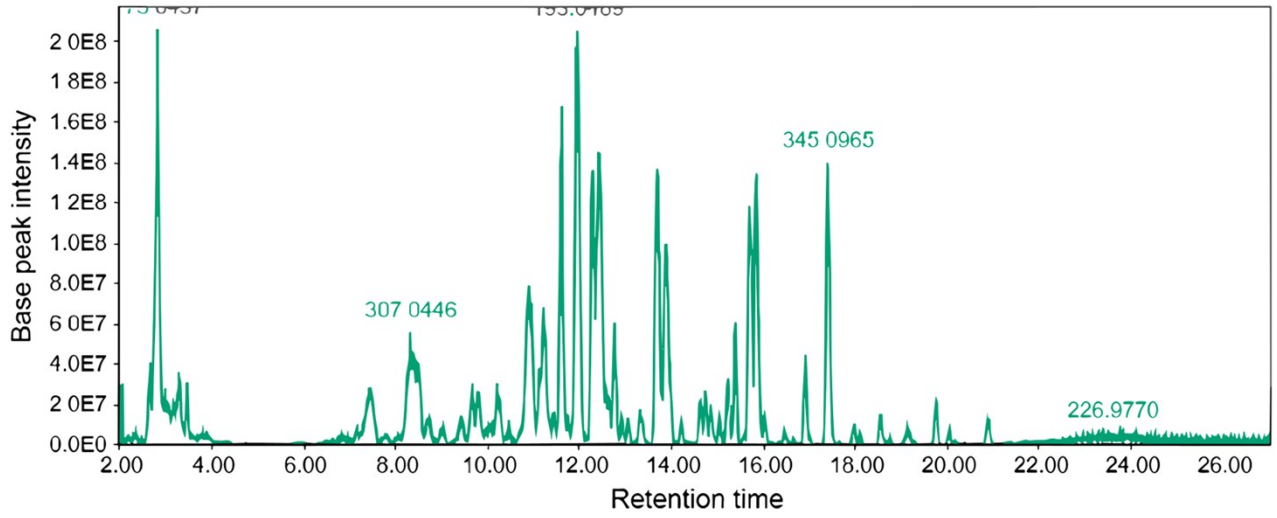
LC-HRMS positive mode total ion chromatogram of F2_ E1



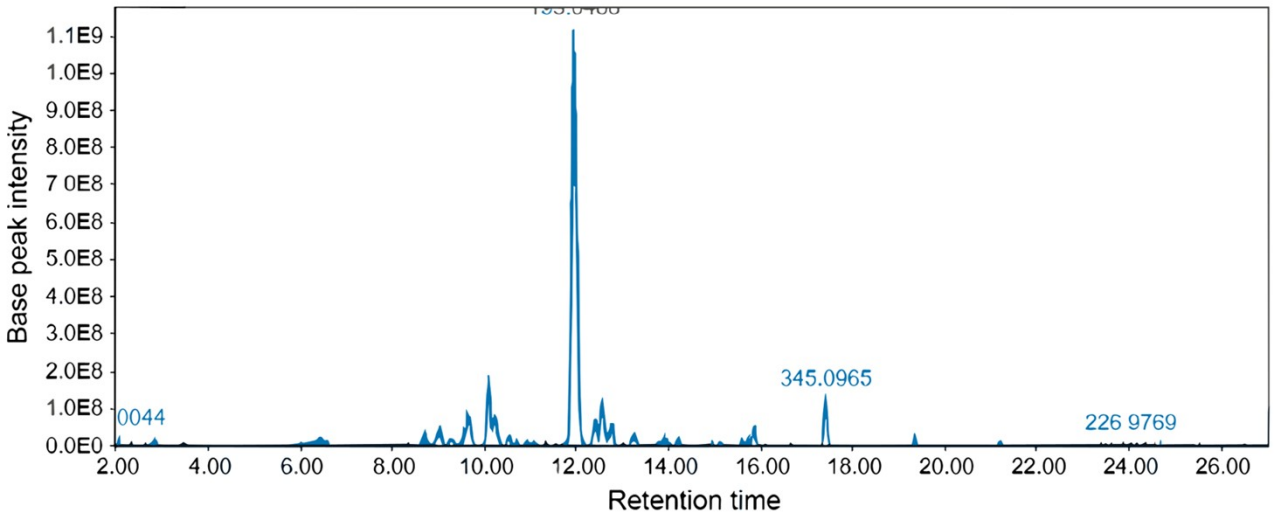
LC-HRMS positive mode total ion chromatogram of F2_ E2



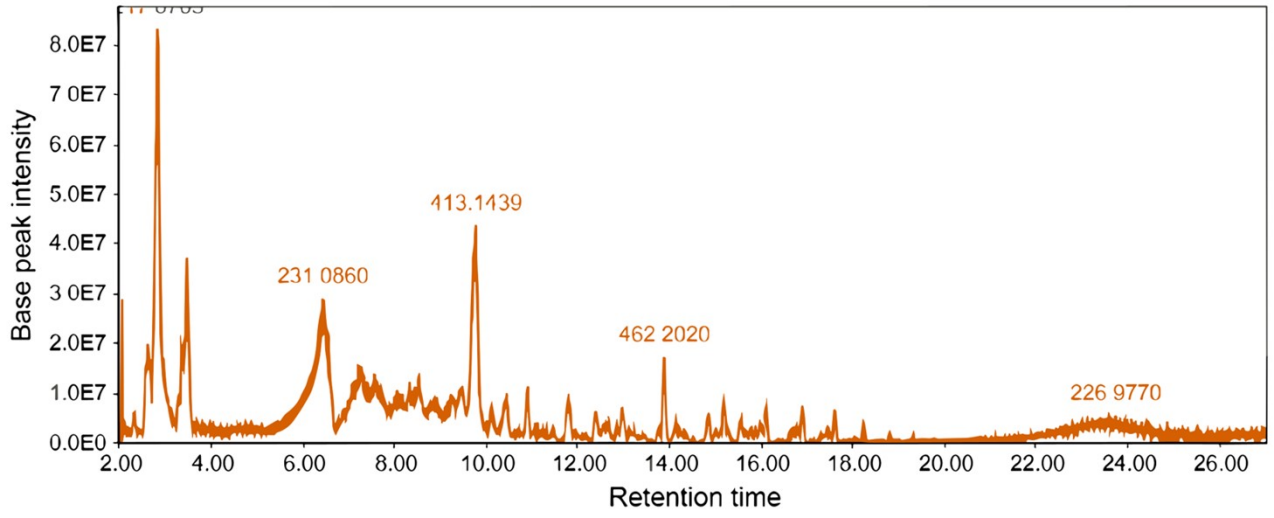
LC-HRMS positive mode total ion chromatogram of F2 _E3



LC-HRMS positive mode total ion chromatogram of F2_ E4



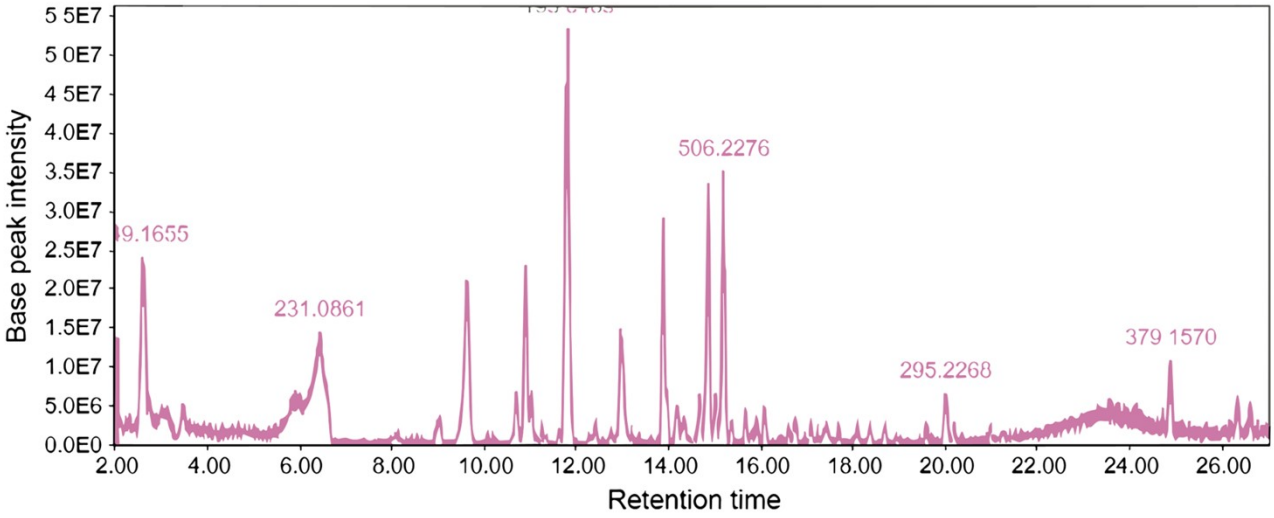
LC-HRMS positive mode total ion chromatogram of F2_ E5



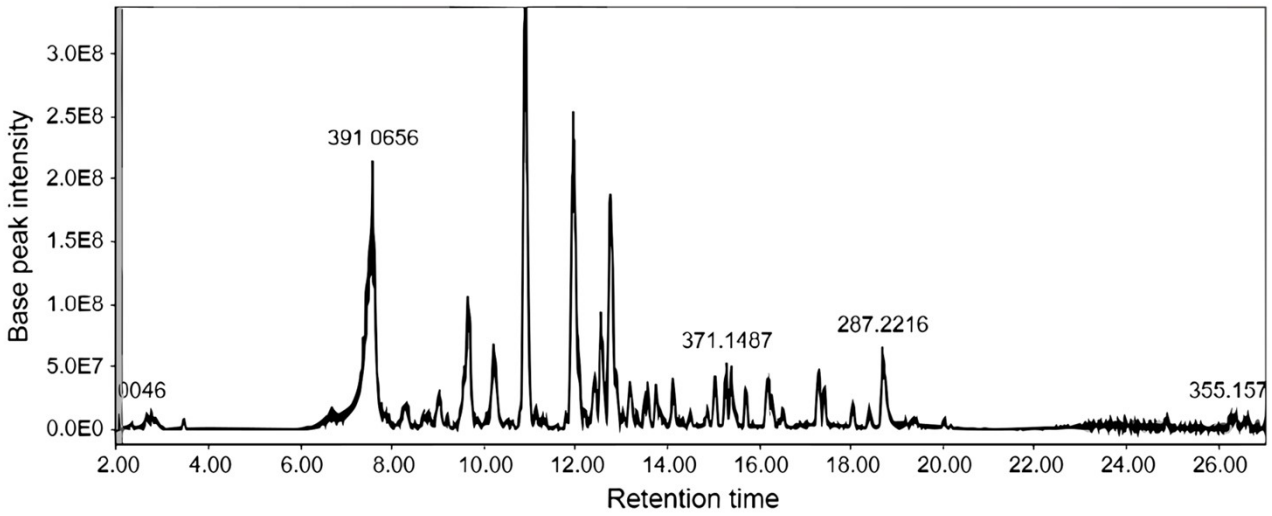
LC-HRMS positive mode total ion chromatogram of F2_ E6

A1B refers to endophytic fungus obtained from *Medicago sativa* roots

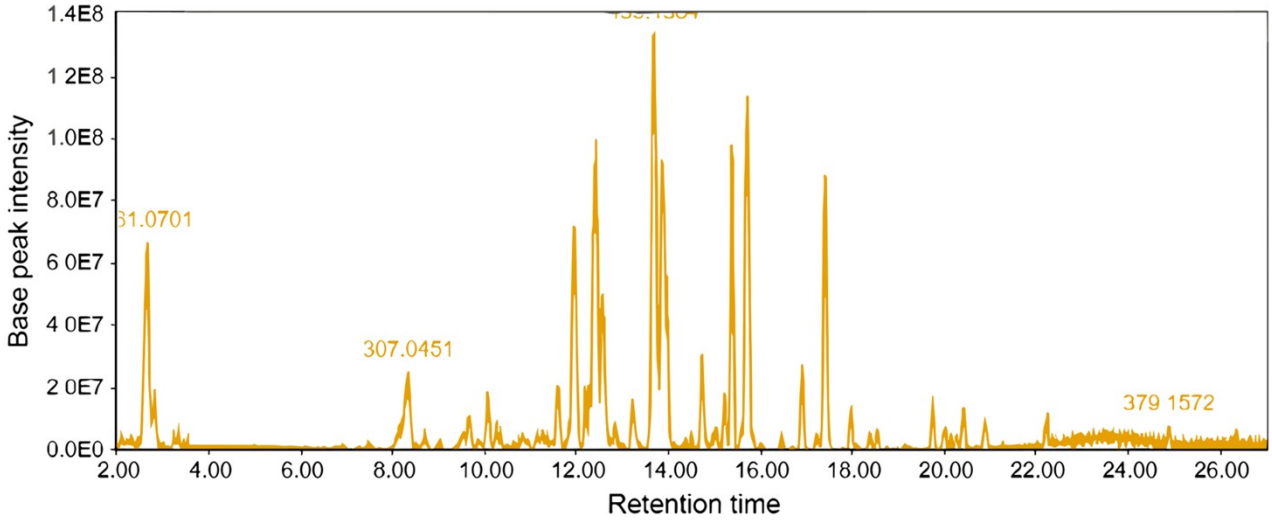
(glucoseamine E1, nicotinamide E2, sodium butyrate E3, erythromycin E4, cycloheximide E5 and tetracycline E6),



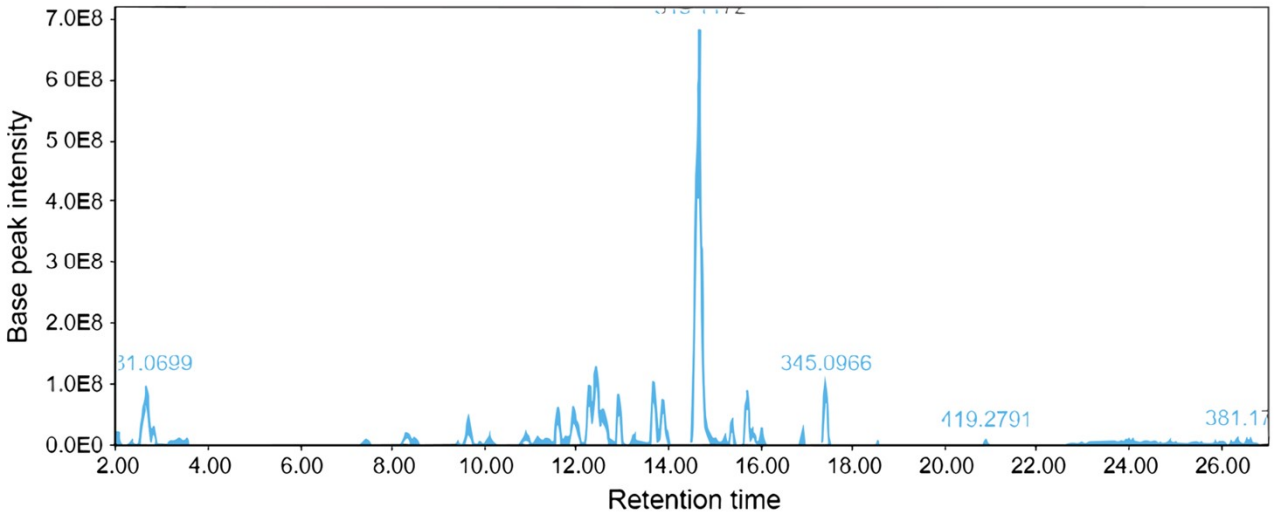
LC-HRMS positive mode total ion chromatogram of A1B



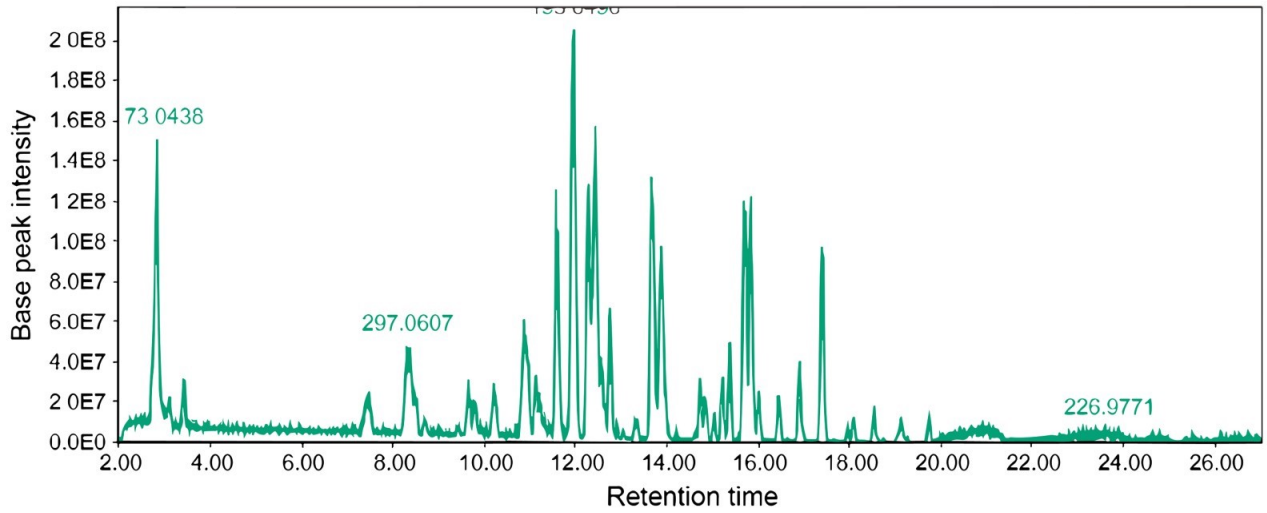
LC-HRMS positive mode total ion chromatogram of A1B E1



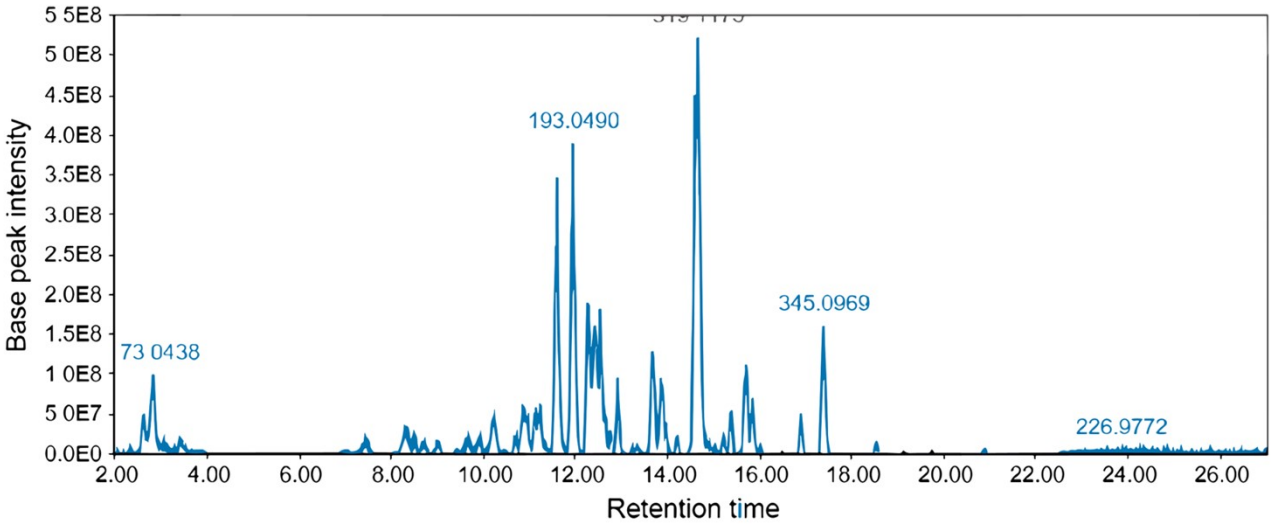
LC-HRMS positive mode total ion chromatogram of A1B E2



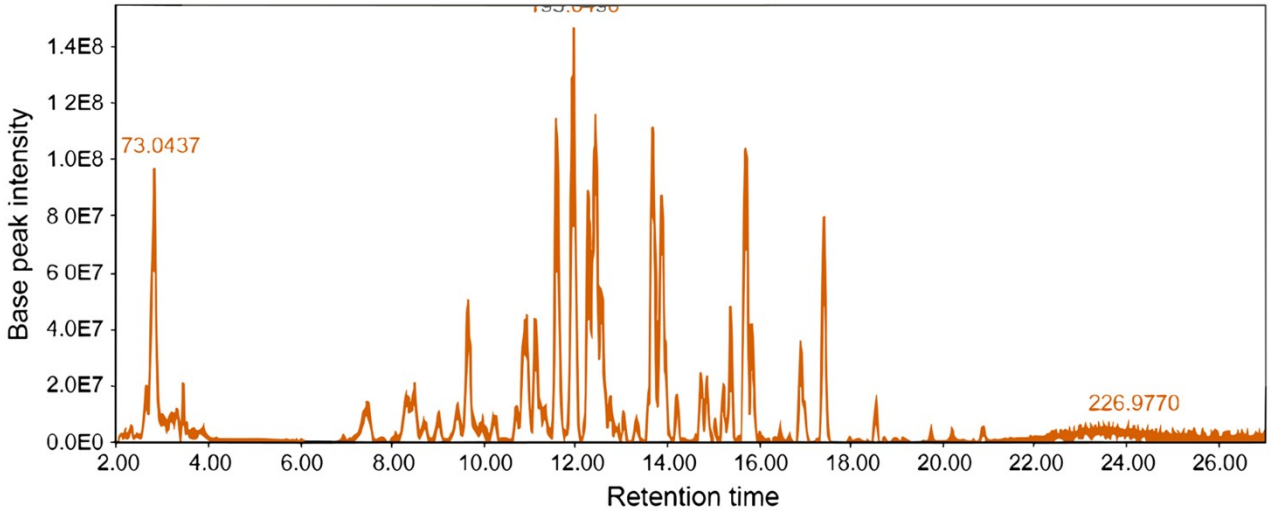
LC-HRMS postive mode total ion chromatogram of A1B E3



LC-HRMS positive mode total ion chromatogram of A1B E4



LC-HRMS positive mode total ion chromatogram of A1B E5



LC-HRMS positive mode total ion chromatogram of A1B E6

References

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