A1

Preparation of stock solution and reagents for DPPH assay

i. Plant sample (Stock solution)

A stock solution of 20 mg/ml of each extract was prepared and wrapped in aluminium foil. The crude methanol and its fractionated extracts (hexane and ethyl acetate) were dissolved in methanol whilst the water extracts were dissolved in distilled water.

ii. DPPH (1,1-diphenyl-2-picrylhydryl)

A stock of DPPH (Sigma) in methanol was prepared at concentration of 8 mg/ml (0.08 gm DPPH + 10 ml methanol). The stock solution was kept in flask wrapped in aluminium foil.

iii. Ascorbic acid/ Buthylated hydroxyanisole (BHA)

Ascorbic acid and BHA were used as the positive reference standards in the DPPH assay. A stock of ascorbic acid / BHA (Sigma) in methanol was prepared at a concentration of 400 μg/ml (0.04 gm ascorbic acid / BHA + 100 ml methanol). The stock solution was kept in flask wrapped in aluminium foil.

A2

Preparation of stock solution and reagents for Reducing power assay

i. Stock solutions

Stock solution of 5 mg/ml, 10 mg/ml, 15 mg/ml, and 20 mg/ml of each extract was prepared and wrapped using an aluminum foil. The crude methanol and its fractionated extracts (hexane and ethyl acetate) were dissolved in methanol whilst the water extracts were dissolved in distilled water.

ii. Potassium ferricyanide (1 %, w/v)

Potassium ferricyanide 1 % was prepared by dissolving 0.1 g of Potassium ferricyanide (Sigma) in 10 ml distilled water. The solution was kept in centrifuge tube and wrapped in an aluminium foil.

iii. Trichloroacetic acid (10 %, w/v)

TCA was prepared by dissolving 50 g of TCA (Sigma) in 500 ml distilled water. The solution was kept in flask wrapped in an aluminium foil.

iv. Ferric chloride (0.1 %, w/v)

Ferric chloride solution 0.1 % was prepared by dissolving 0.01 g of ferric chloride (Sigma) in 10 ml distilled water. The solution was kept in centrifuge tube and wrapped in aluminium foil.

v. 0.2 M Phosphate buffer pH6.6

a. 0.2 M Monobasic stock

Prepared by dissolving 13.90 g of sodium phosphate monobasic (NaH_2PO_4, Sigma) in 500 ml distilled water.

b. 0.2 M Dibasic stock

Prepared by dissolving 26.825 g of sodium phosphate dibasic heptahydrate (Sigma) in 500 ml distilled water.

0.2 M Phosphate buffer pH 6.6 was prepared by adding 62.50 ml 0.2 M monobasic stock and 37.50 ml 0.2 ml dibasic stock in 200 ml distilled water. The pH of the solution was calibrated to pH 6.6

A3

Preparation of stock solutions and reagents for β-carotene bleaching assay

i. Stock solutions

Stock solutions of 4 mg/ml, 8 mg/ml, 16 mg/ml and 20 mg/ml of each extract was prepared and wrapped in aluminium foil. The crude methanol, hexane and ethyl acetate extracts were dissolved in methanol. The water fraction was dissolved in distilled water.

ii. 0.2 mg/ml β- carotene solution

0.2 mg/ml of β- carotene solution was prepared by dissolving 0.002 g of β- carotene in 10 ml chloroform. The solution was kept in a centrifuge tube and wrapped in aluminium foil.

iii. Tween 80 and Linoleic acid

Tween 80 (polyooxyethylene sorbitan monolaurate); Linoleic acid is obtained from Sigma. Best kept in ice throughout experiment and kept in -20˚C when not in use.

Antioxidant activity (%) = $(R_{control} - R_{sample}) / R_{control} \times 100\%$

Calculation:

For example, antioxidant activity (%) for BHA at concentration of 20 mg/ml,

$$= \frac{(5.685 \times 10^{-4}) - (4.195 \times 10^{-5})}{(5.685 \times 10^{-4})} \times 100$$

$$= 92.62\ \%$$

A4

Chemicals and media for Neutral Red cytotoxicity assay

RPMI 1640 medium, M199 medium, McCOY'S 5A medium, EMEM medium, sodium bicarbonate , N-2-hydroxyethyl-piperazine-n-2-ethane-sulfonic acid , Foetal Bovine Serum (FBS), sodium pyruvate , trypsin/EDTA, phosphate buffered saline (PBS), Penicillin/streptomycin solution, amphotereicin B, neutral Red (NR), dimethyl sulfoxide (DMSO), analytical grade, ethanol (EtOH), analytical grade, glacial acetic acid, analytical grade, distilled H2O, 0.20 μm filter membrane.

Preparation of media and solution:

i. Preparation of basic medium (RPMI 1640 Medium, M199 Medium, McCOY'S 5A Medium and Basic Eagle Minimum Essential Medium (MEM))

The medium was prepared by dissolved medium (RPMI 1640 Medium (Sigma); M199 Medium (Sigma); McCOY'S 5A Medium (Sigma) and EMEM powder (Sigma)), 2.0 g of sodium bicarbonate ($NaHCO_3$, Merck, Germany) and 0.5206 g of N-2-hydroxyethyl-piperazine-n-2-ethane-sulfonic acid (HEPES, 2 mM/l, Merck, Germany) in 1000 ml with distilled water. The pH of the medium is calibrated to pH 7.4 (Hanna Instruments 8417). The medium was then filtered sterilized using 0.20 μm filter membrane (Schleicher & Schuell) and stored at 4℃ for up to 3 months.

ii. Complete growth medium (Cells Maintenance)

10% supplement Medium (RPMI 1640, McCOY'S 5A Medium, M199 Medium) were prepared by using 90 ml of basic medium, supplement with 10 ml inactivated Foetal Bovine Serum (FBS, PAA Lab, Austria), 100 µg/ml penicillin/ streptomycin (PAA Lab, Austria) and 50 µg/ml of Amphostat B (PAA Lab, Austria). While 10% supplement of MEM medium were prepared by using 87 ml of basic medium(MEM), supplement with 10 ml inactivated Foetal Bovine Serum (FBS), 100 µg/ml penicililin/ streptomycin (PAA Laboratories), 50 µg/ml of Amphostat B (PAA Lab,Austria) and 100 µl of sodium pyruvate (Sigma). The mediums were filtered sterilized using a 0.22 µm filer membrane (Shcleicher & Schuell). The colours of the medium were reddish orange and were kept at 4˚C for up to 2 weeks.

iii. Revival medium

Revival medium was prepared as complete growth medium described above, except revival medium was supplemented with 20 % instead of Foetal Bovine Serum.

iv. Cryopreservation medium

Cryopreservation medium was prepared by 50 % Foetal Bovine Serum (FBS, PAA Lab, Austria), 40 % basic culture medium and 10 % dimethylsulfoxide (DMSO) as cryoprotectant.

Preparation of solutions

i. Phosphate Buffered Saline (PBS)

The Phosphate Buffered Saline (PBS) was prepared using 1.52 g sodium hydrogen orthophosphate anhydrous (Na_2HPO_4, Merck, Germany,), 0.58 g potassium dihydrogen phosphate (KH_2PO_4, Merck, Germany) and 8.5 g sodium chloride (NaCl, BDH Analar) were dissolved in distilled water and the volume was made up to 1000 ml. The pH of the buffer was adjusted to 7.2 using a pH meter (Hanna Instruments 8417). The buffer was then filtered using a 0.2 µm filter membrane and autoclaved for 15 min at 15 psi 121˚C and finally stored at room temperature.

ii. Trypsin-EDTA

Trypsin-EDTA solution was prepared by dissolving 0.25 g trypsin (Amresco, USA) and 0.03 g EDTA (Sigma, USA) in 100 ml of distilled water. The solution was sterilized by filtration using a 0.22 µm filter membrane and stored at -20˚C.

iii. 0.4 % Tryphan Blue

0.4 % Tryphan Blue solution was prepared by dissolving 0.2 g tryphan blue in 50 ml distilled water.

iv. Stock of Crude Extract (20 mg/ml)

Stock of crude plant extract was prepared by dissolving 0.02 g plant extract in 1 ml DMSO in 1.5 ml micro-centrifuge tube. The extract was mixed homogenous using vortex mixer and then stored at -20˚C.

v. Maintenance of cells:

An estrogen positive (ER^+) human mammary adenocarcinoma cells (MCF-7); human epidermal carcinoma of cervix cell line (CaSki); human colon carcinoma cells (HT29) and human lung cancer cell line (A549) are grown as a mono-layer in RPMI 1640 medium with 10 % heat-inactivated Foetal Bovine Serum, antibiotics (100 000 U/l penicillin and 1 mg/l streptomycin). Human Colon Carcinoma cells (HCT-116) will be grown as a mono-layer in McCOY'S 5A medium with 10 % heat-inactivated Foetal Bovine Serum, antibiotics (100 000 U/l penicillin and 1 mg/l streptomycin) and normal human lung fibroblast (MRC-5) in MEM medium with 10 % heat-inactivated Foetal Bovine Serum, antibiotics (100 000 U/l penicillin and 1 mg/l streptomycin) in 25 ml tissue culture flask. The culture was incubated in 5 % CO_2 incubator (Shel Lab water-jacketed) kept at 37 ˚C in a humidified atmosphere. The culture was sub-cultured every 2 or 3 days and routinely checked under and inverted microscope (IMT-2 Olympus, Japan) for any contamination. Vialibility of the cell was checked before and after treatment by the tryphan blue exclusion dye method. Frozen cell stocks were stored in liquid nitrogen (- 196 ˚C) prior to use.

vi. Subcultivation of cells:

Adherent cells were attached and formed a single layer in the culture flask. The cells were examined under inverted microscope to check for any microbial contamination and also the attachment of the cell on the tissue culture flask. If the cells were attached, the culture medium was discarded. The confluent cells were washed twice using 6 to 7 ml of phosphate saline buffer (PBS) to remove all the traces of serum. The cells were detached from the flask by incubating in 1 ml 25 % tyrpsin-EDTA solution and 3 ml of PBS solution for 5-10 minutes at 37 ˚C ,and then slightly be to release the cells from attachment. The floating cells were transferred into a centrifuge tube that contained 1 ml

of complete medium and centrifuged for 5 minutes at 1000 rpm. The supernatant was removed and 3 to 4 ml of complete medium was added to the pellet by gently pipetting.

The cells were separated or divided and transferred into different flask which containing 4ml of culture media. The flask then further incubated at 37˚C in a 5 % CO_2 incubator.

vii. Revival of cells:

Revival of cells was done by taking the provial of cells from liquid nitrogen and plunged into a beaker of ice. The cells were put into a 37 ˚ C water bath for quick thawing. The cap of the vials was kept out of the water when thawing to reduce the possibility of contamination. The vial was immediately removed from the water bath once the contents are thawed, and 70 % ethanol is spraying on the vial to remove the contaminants. The cells were then transferred into 1 ml of 10% supplement RPM1 1649 medium or McCOY'S 5A Medium or MEM medium in a centrifuge tube (Falcon, USA). Then spin the mixture at 1000 rpm for 5 minutes. Supernatant was removed and the pellet was resuspended in 5- 7 ml of complete growth medium and then pipet gently into a 25 ml tissue culture flask (Falcon, USA) and incubated in a 25ml tissue culture flask at 37˚C in a 5 % CO_2 incubator.

viii. Cryopreservation of cells:

Cell line stocks were preserved and stored in liquid nitrogen. Cell suspension was spun down with 1000 rpm for 5 minutes using a bench centrifuge (Clements 2000) after the medium was discarded. 50 % FBS was added to 40 % basic culture medium to give approximately 1 x 10^7 cells/ml, followed by 10 % dimethylsulfoxide (DMSO) as cryoprotectant. The mixture was stored in cryopreservation vial and placed in vapour

phase of liquid nitrogen at least 4 hours. The vial was then transferred into cryocane of liquid nitrogen (-196˚C) and stored in cold room (4˚C).

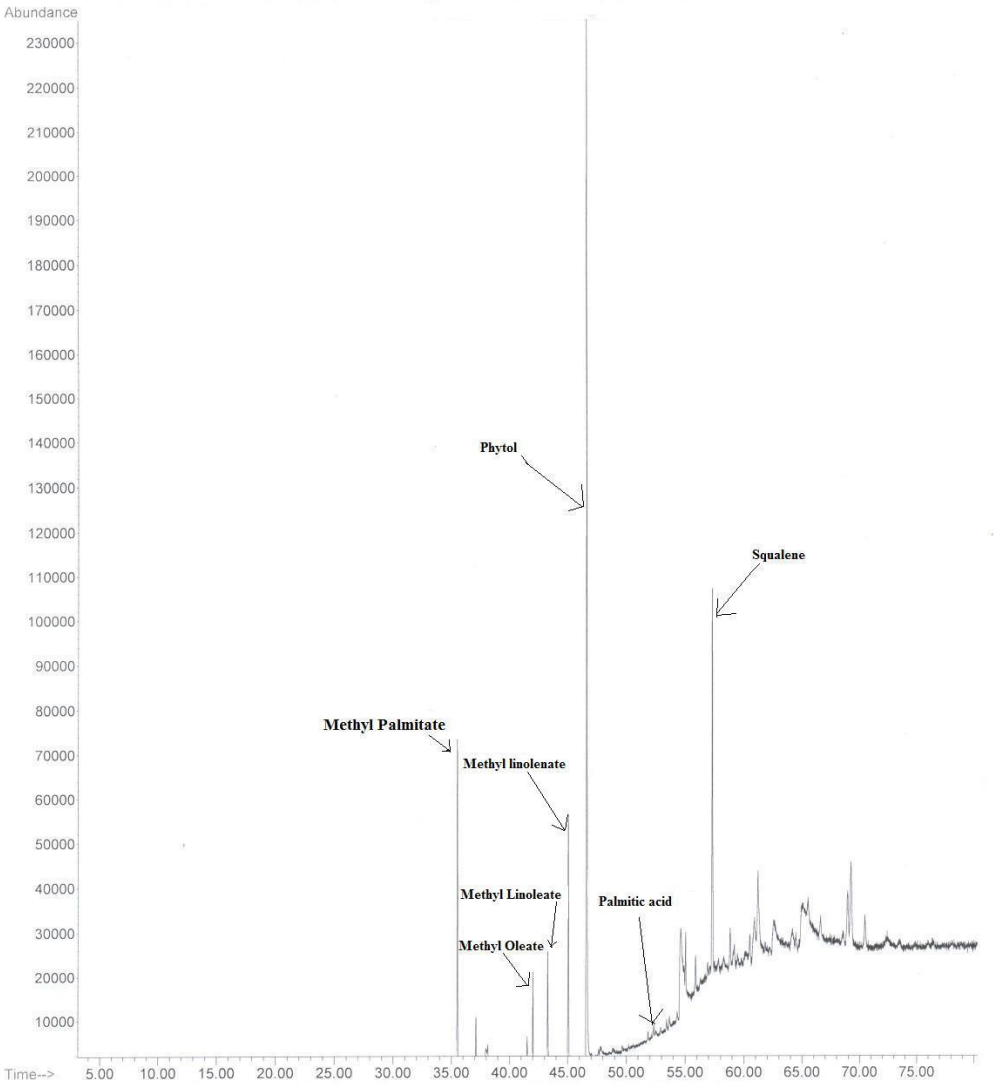


Figure A5: The total ion chromatogram of the hexane fraction of *P. oleracea*

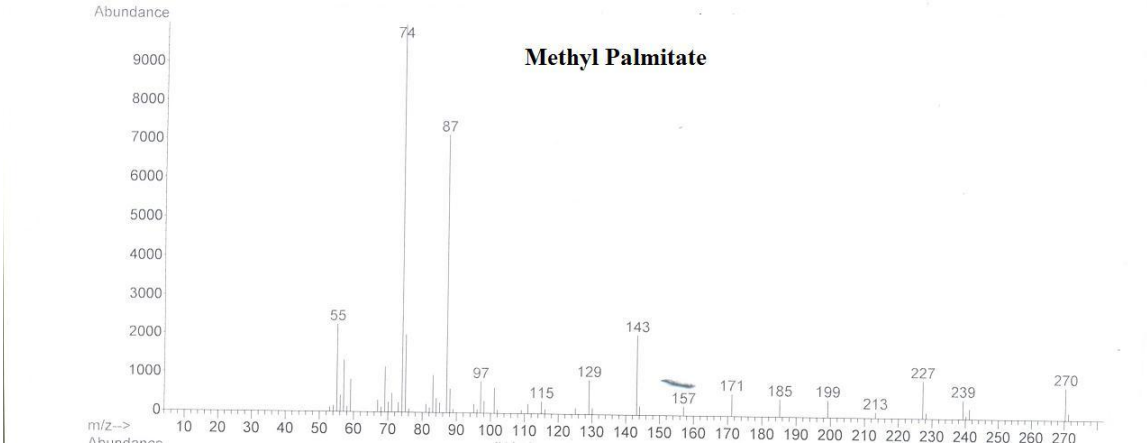


Figure A6: Mass spectrum of methyl palmitate

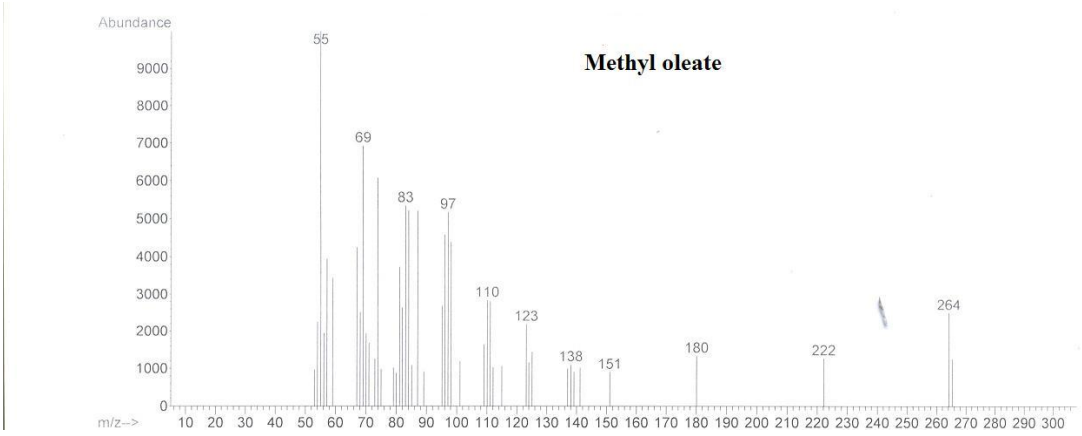


Figure A7: Mass spectrum of methyl oleate

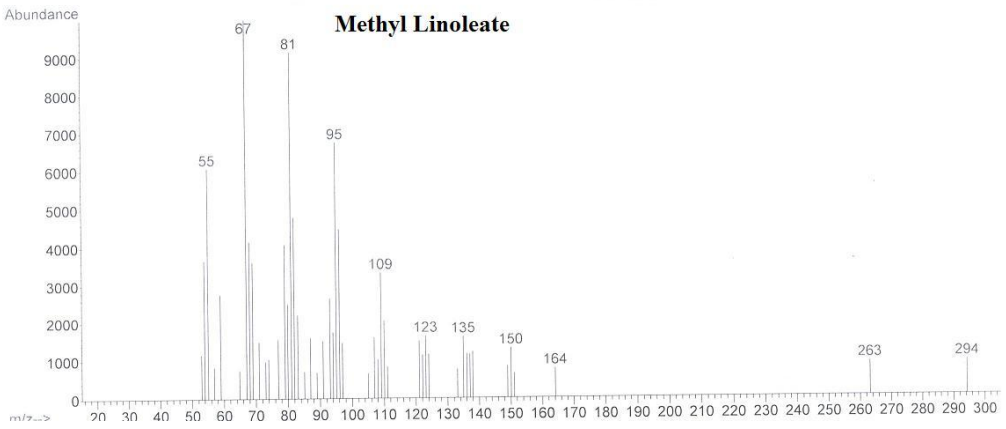


Figure A8: Mass spectrum of methyl linoleate

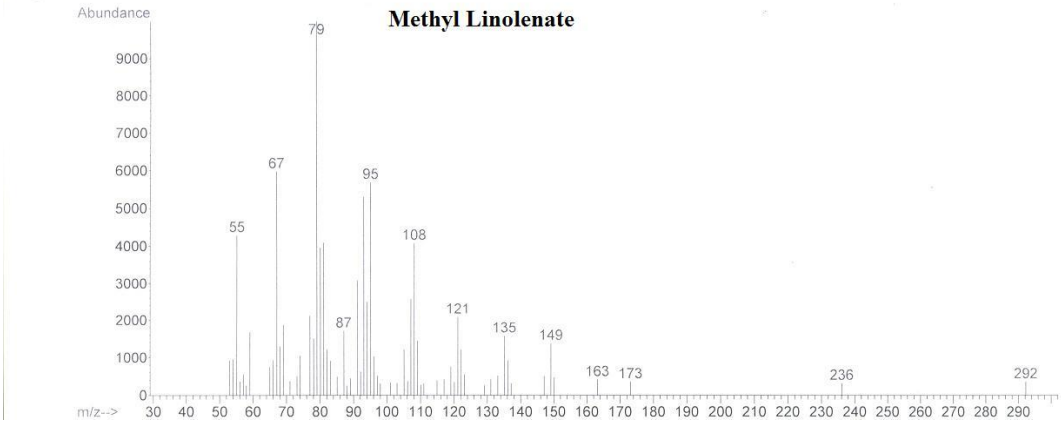


Figure A9: Mass spectrum of methyl linolenate

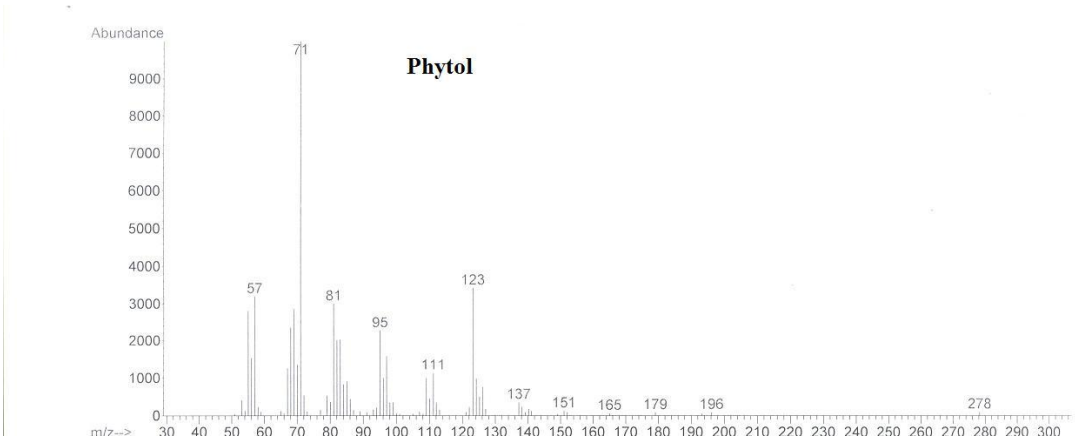


Figure A10: Mass spectrum of phytol

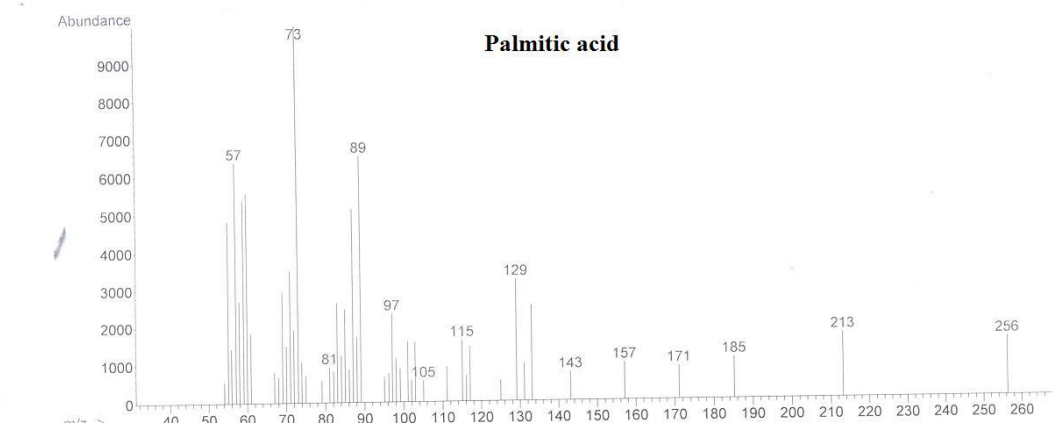


Figure A11: Mass spectrum of palmitic acid

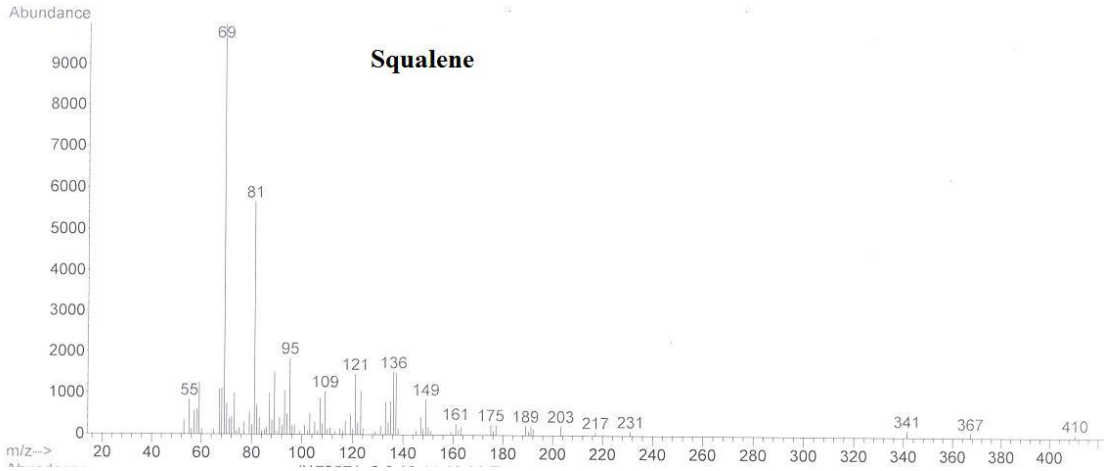


Figure A12: Mass spectrum of squalene

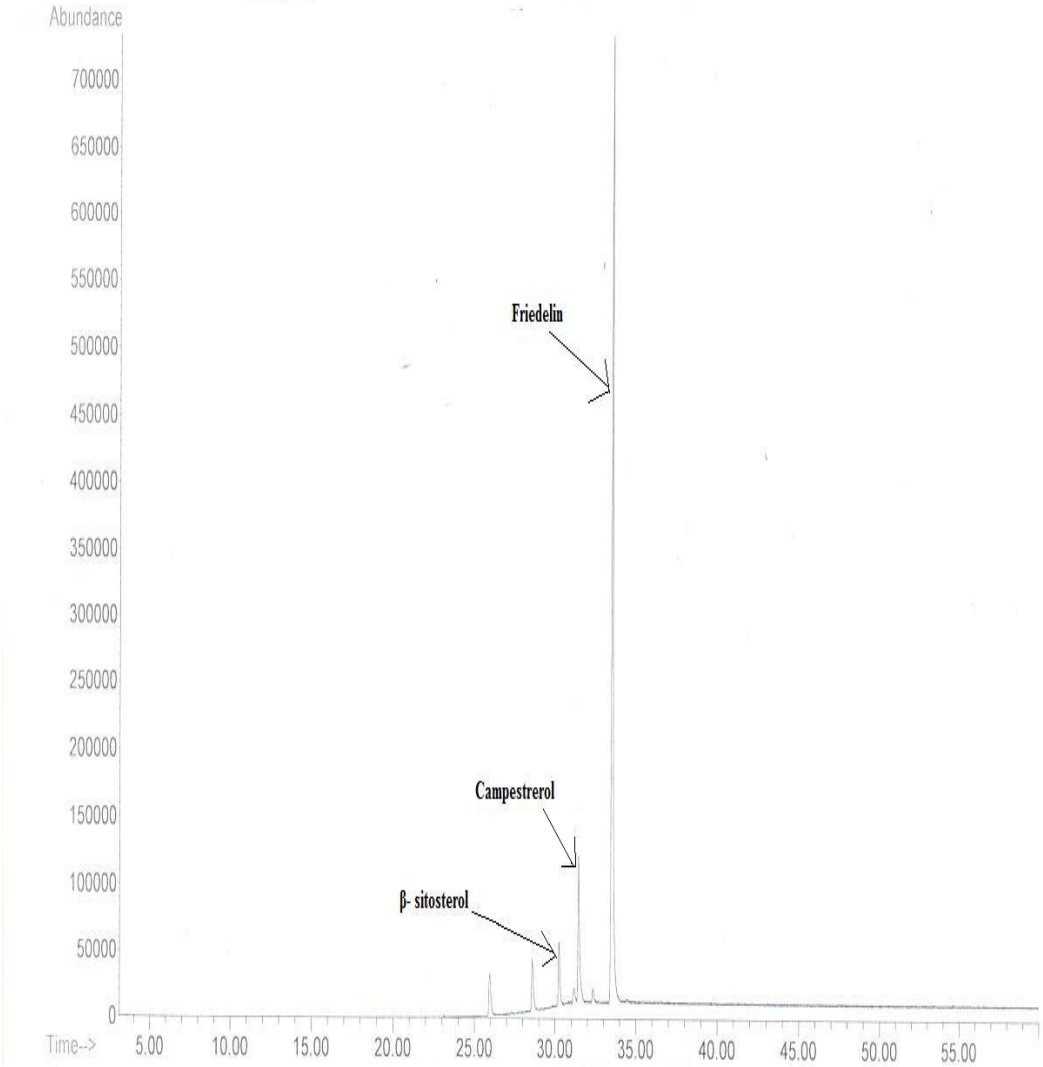


Figure A13: The total ion chromatogram of mixture (I)

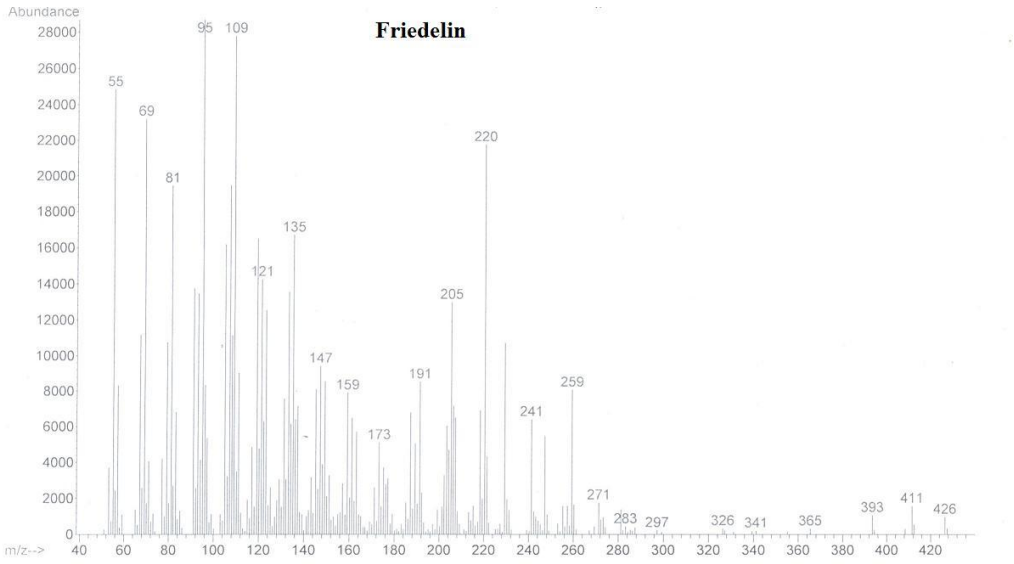


Figure A14: Mass spectrum of friedelin from mixture (I)

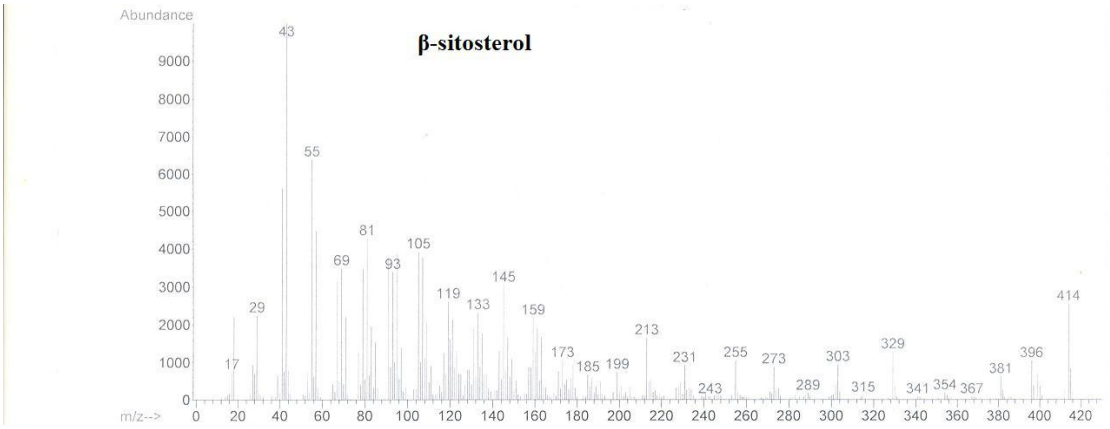


Figure A15: Mass spectrum of β-sitosterol from mixture (I)

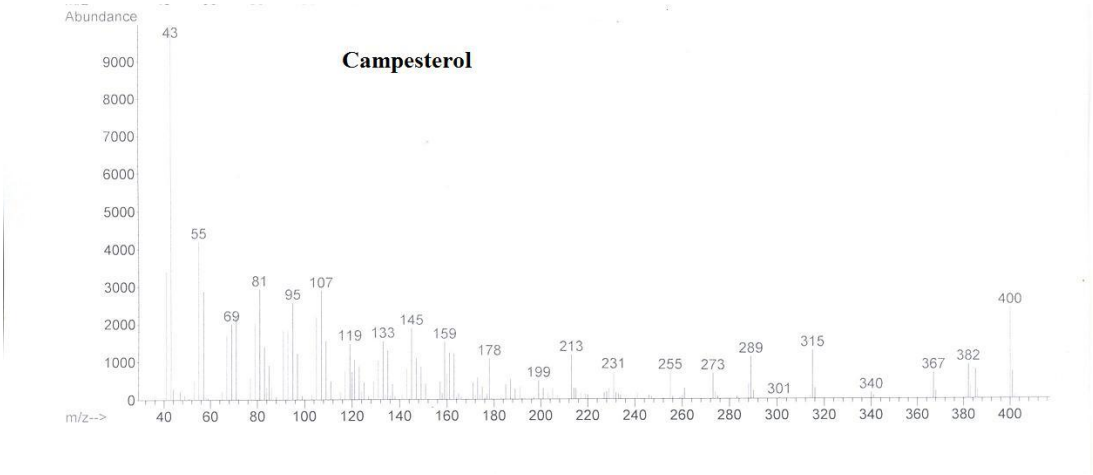


Figure A16: Mass spectrum of campesterol

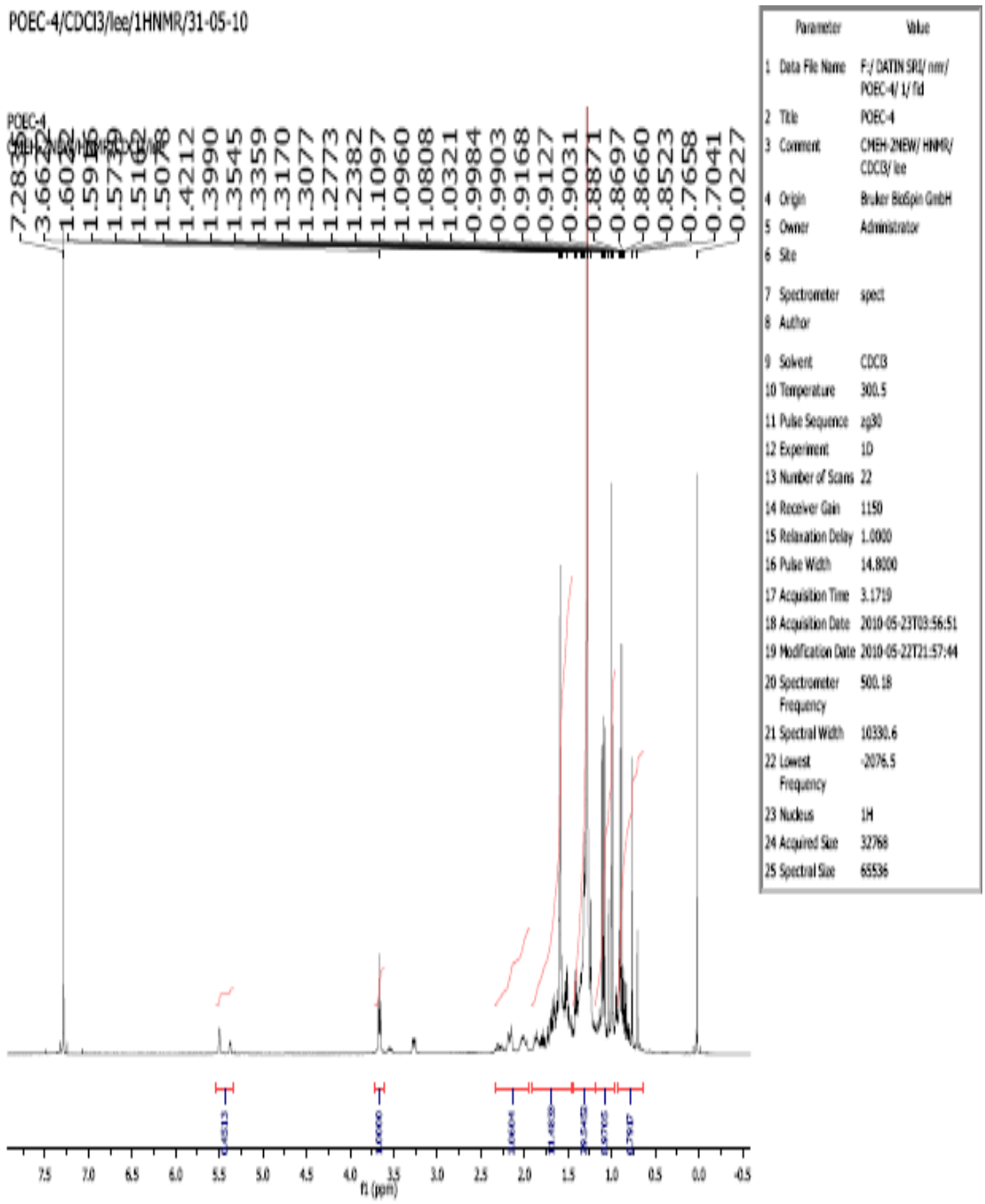


Figure A17: ^{1}H NMR spectrum of mixture (I)

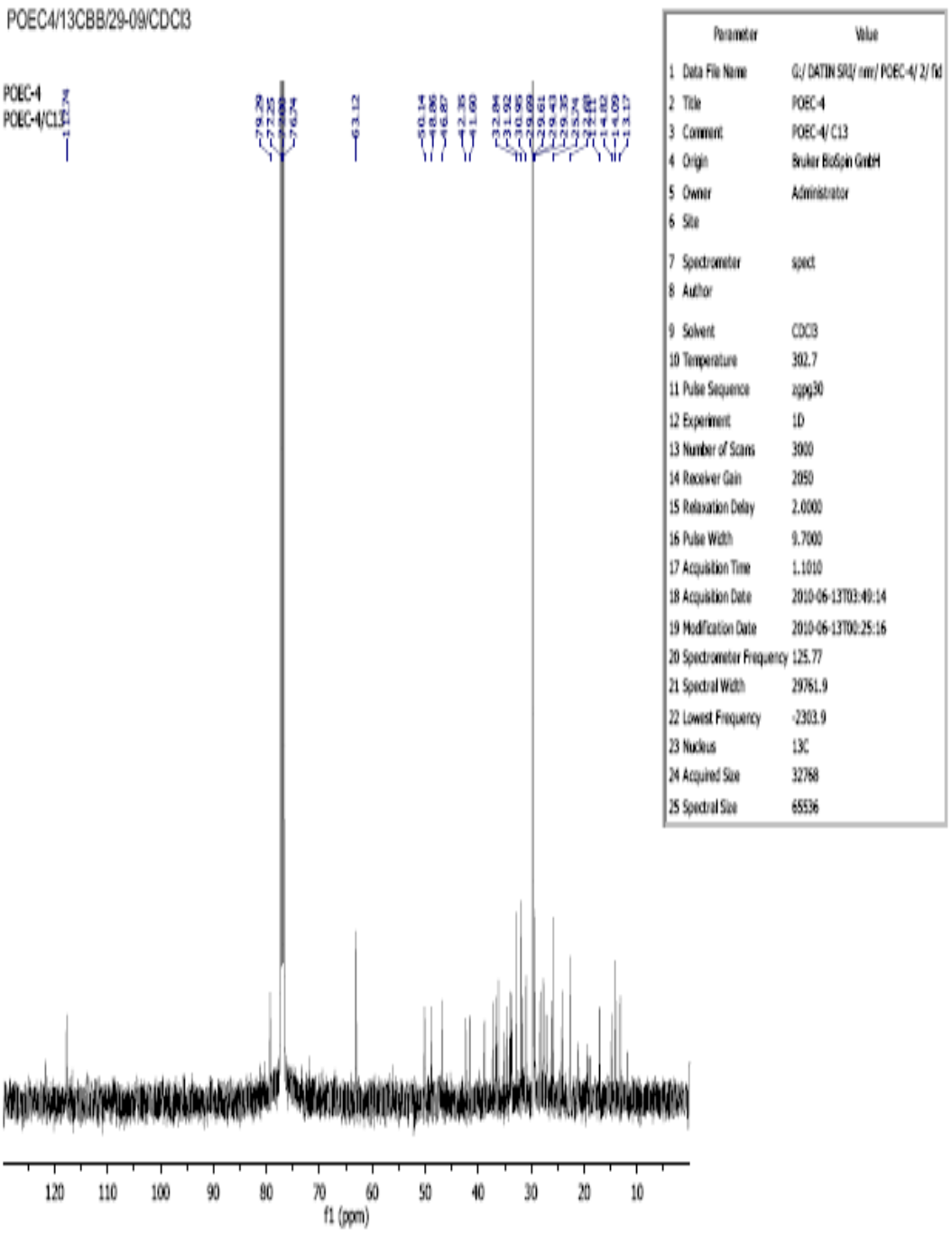


Figure A18: ^{13}C NMR spectrum of mixture (I)

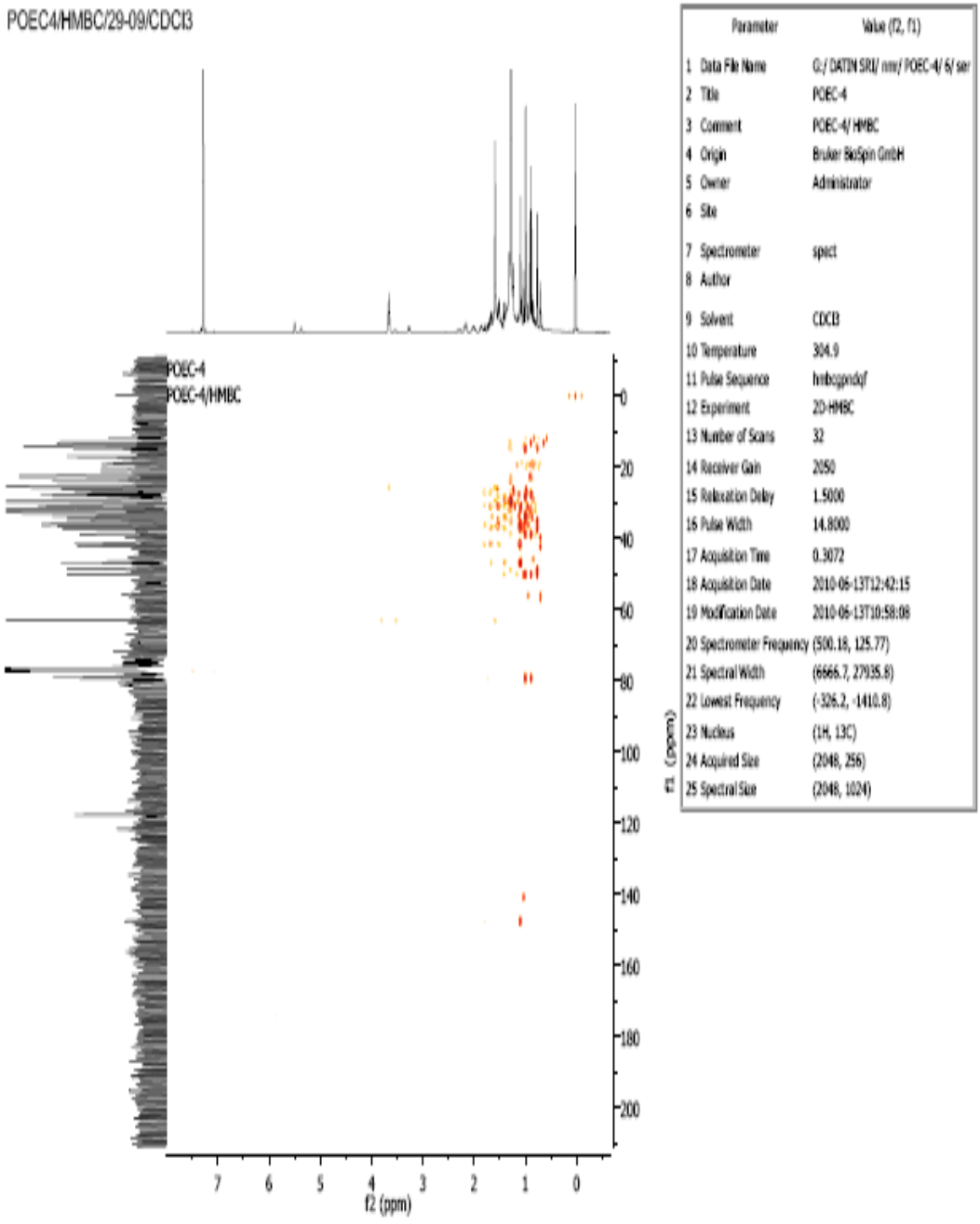


Figure A19: HMBC spectrum of mixture (I)

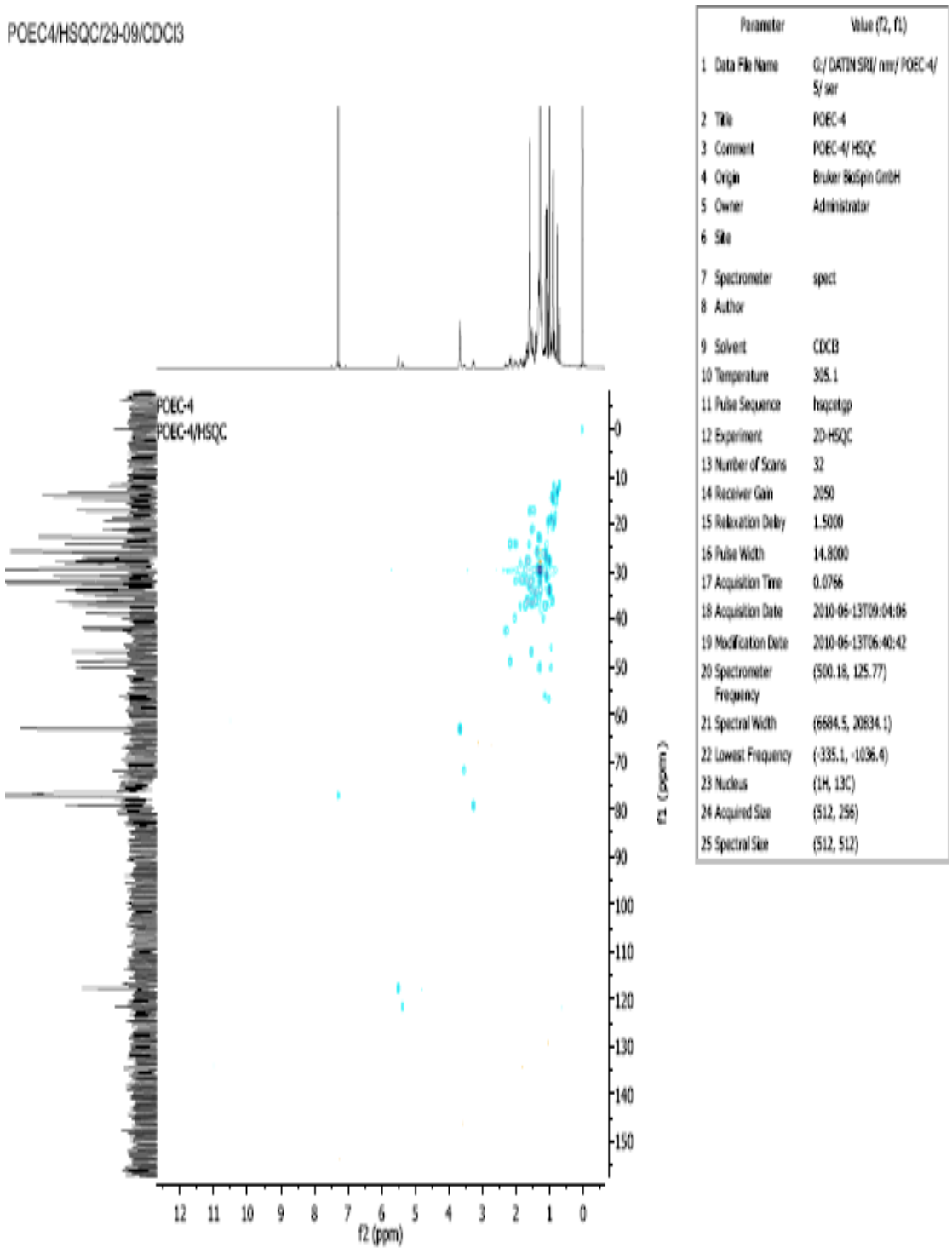


Figure A20: HSQC spectrum of mixture (I)

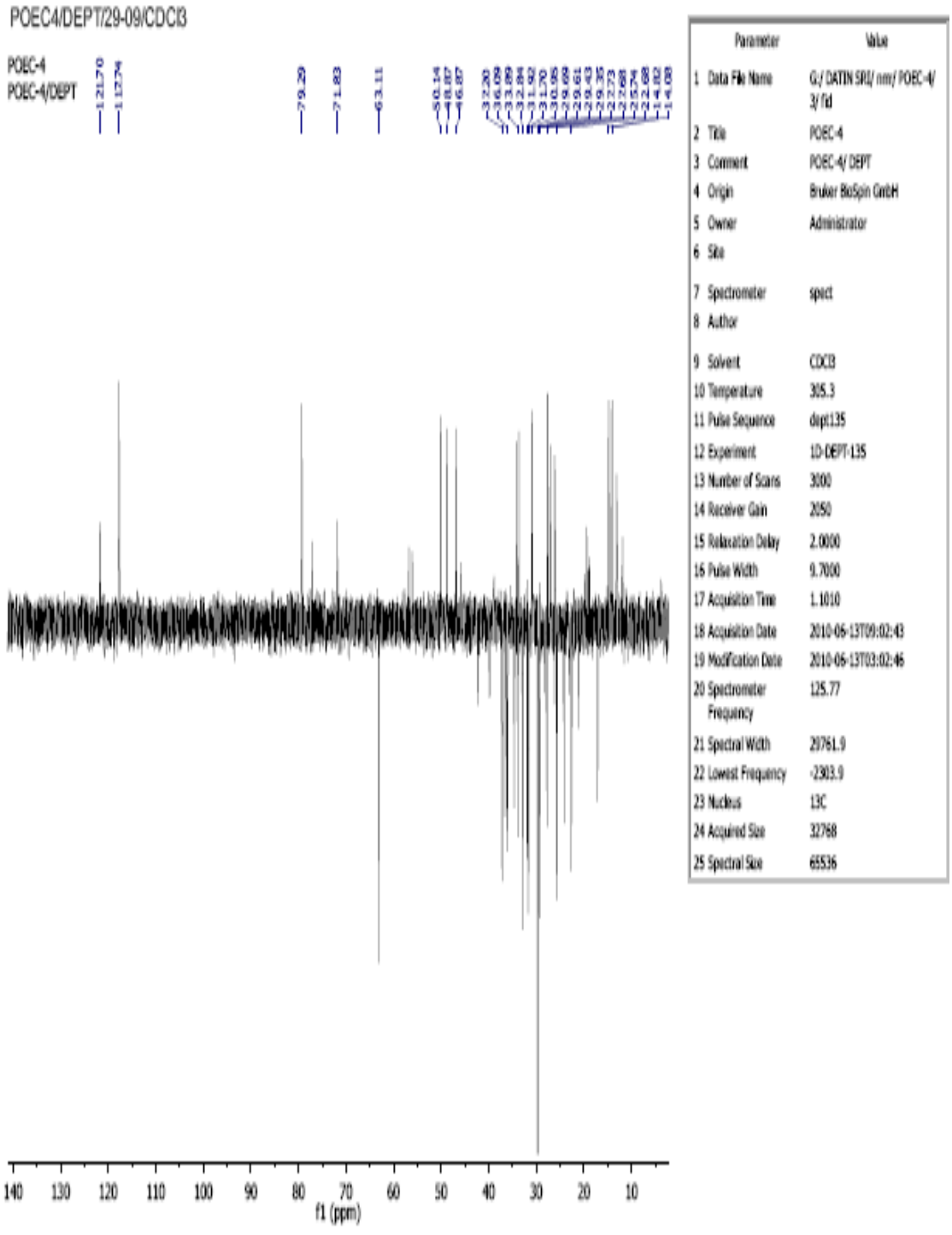
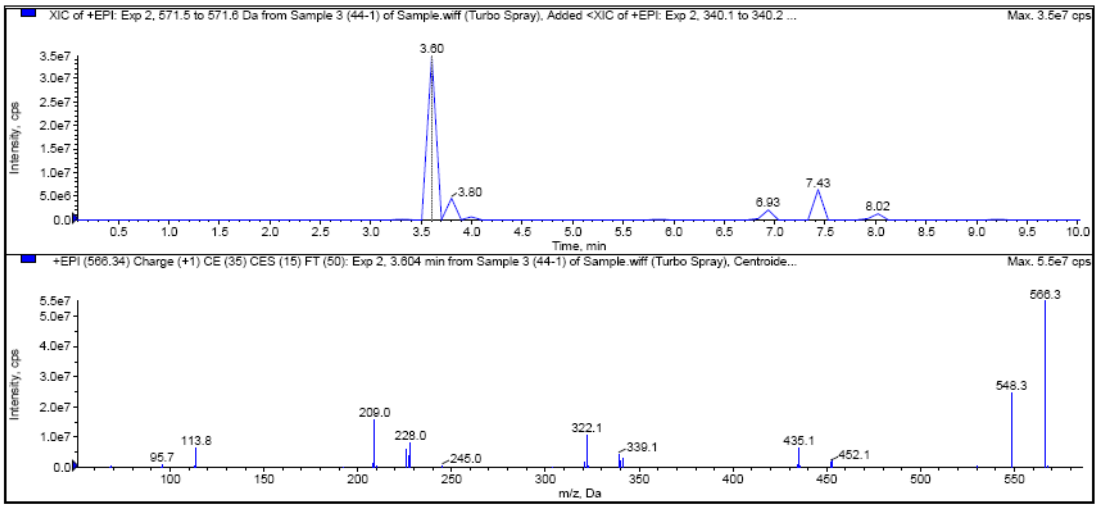
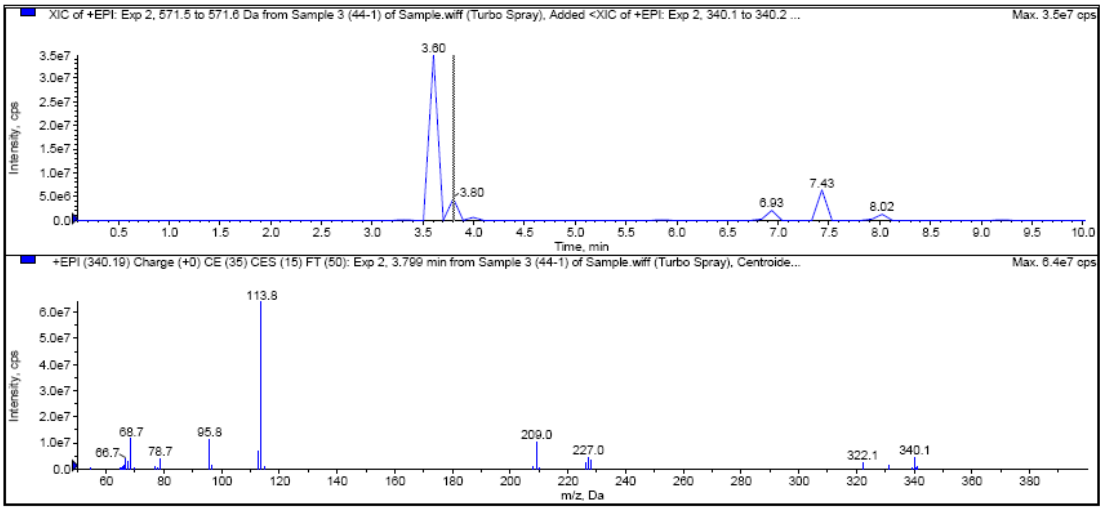


Figure A21: DEPT spectrum of mixture (I)





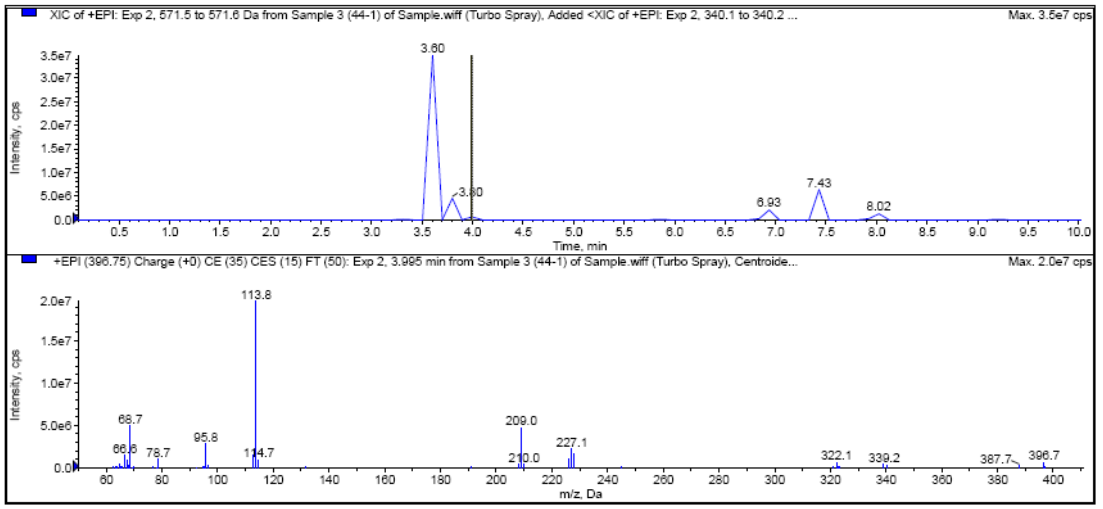


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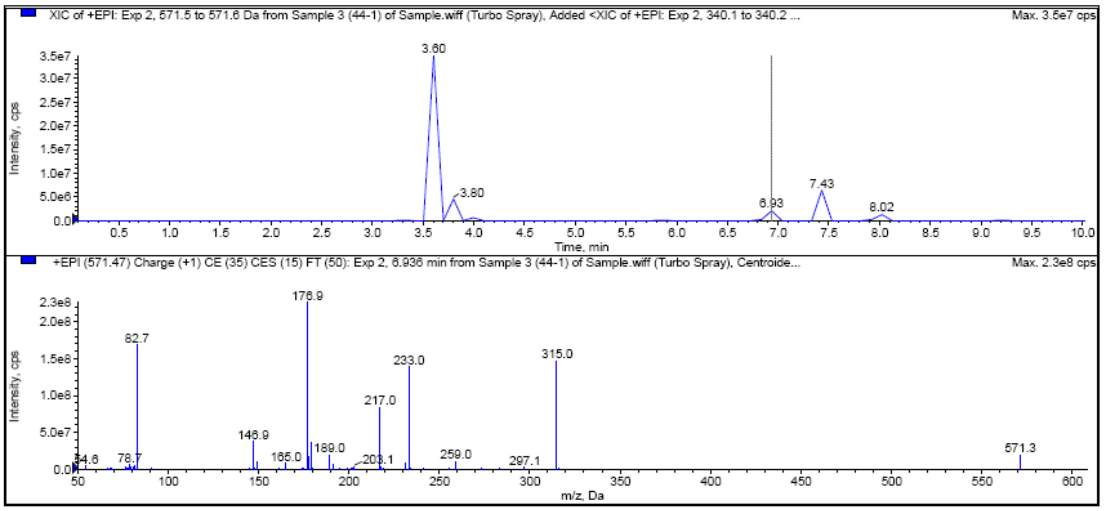
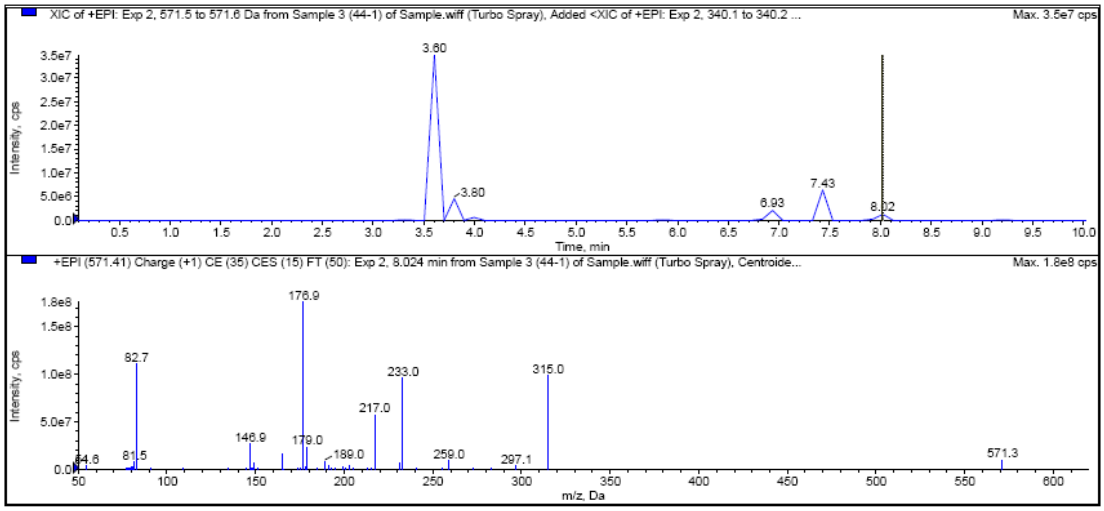
Figure A22: Chromatogram of mixture (II) and the mass spectra of the compounds in the chromatogram.

Figure A22, continued





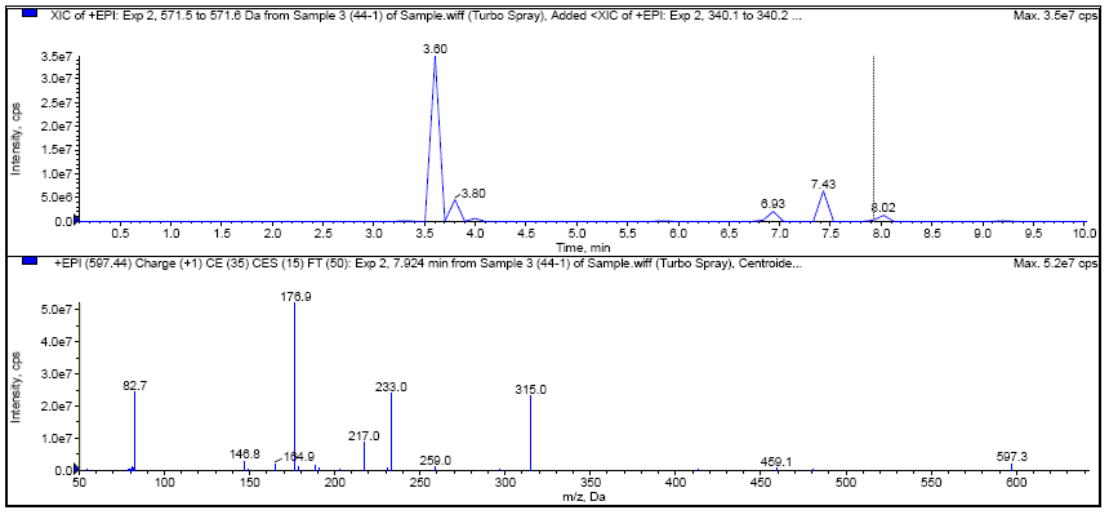


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Figure A22, continued

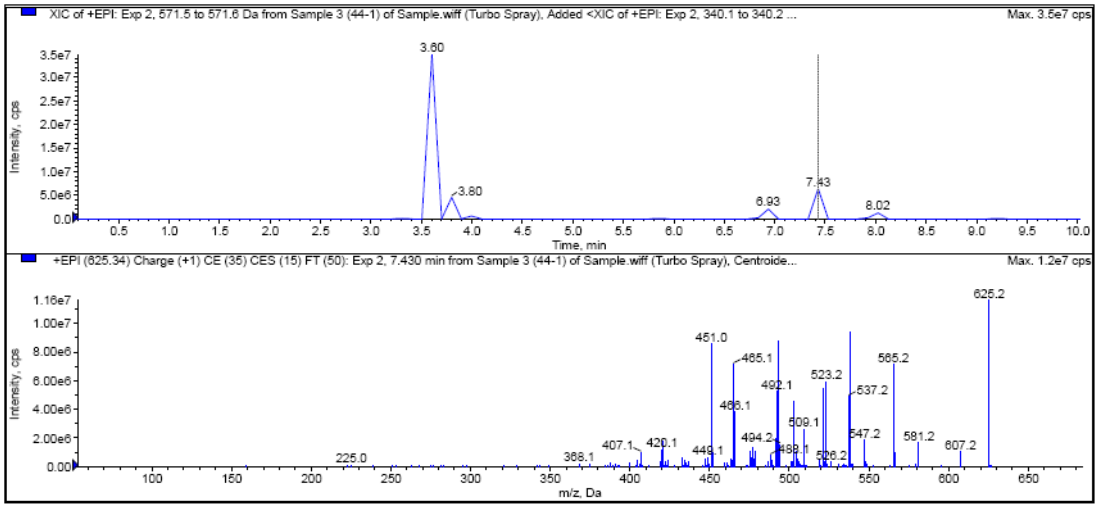


Figure A22: Chromatogram of mixture (II) and the mass spectra of the compounds in the chromatogram.

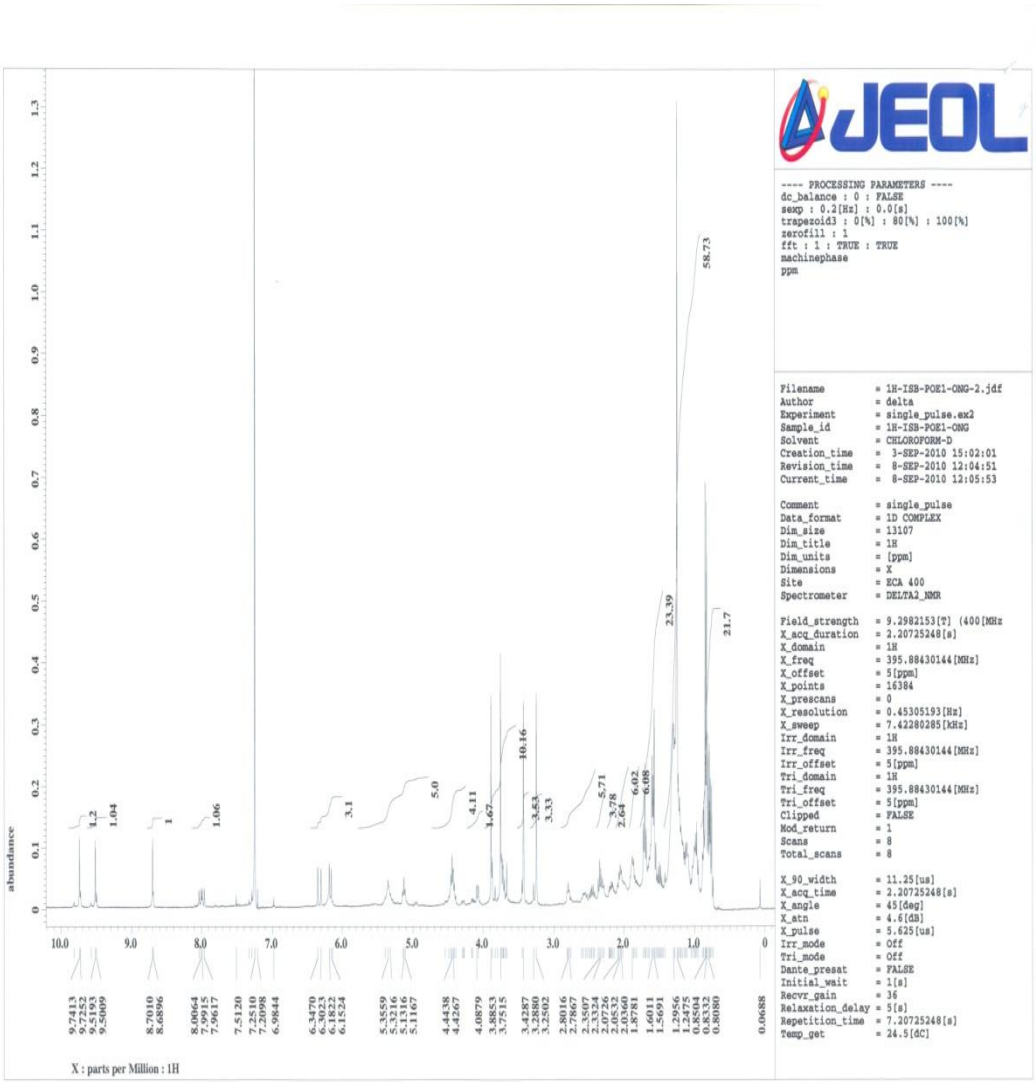
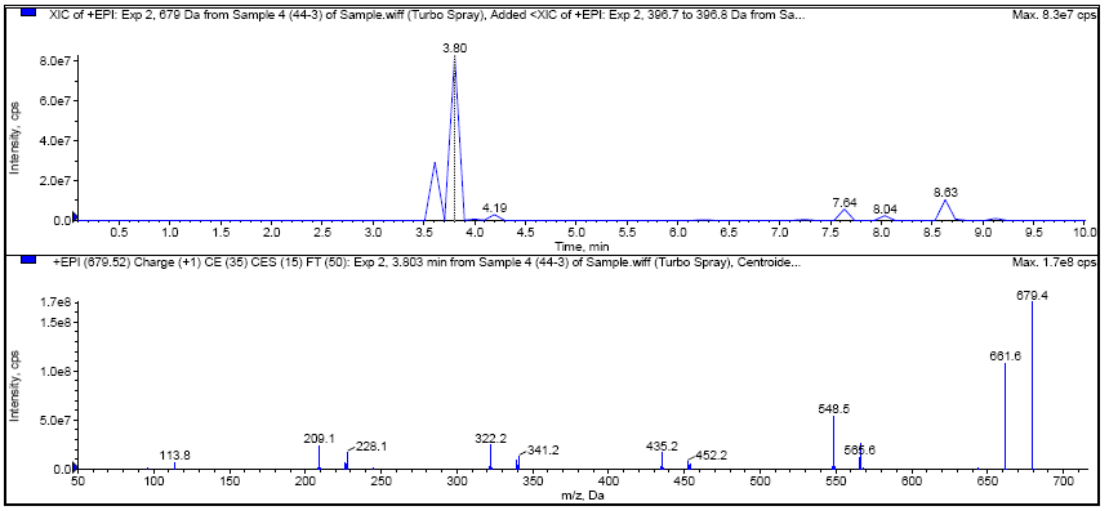
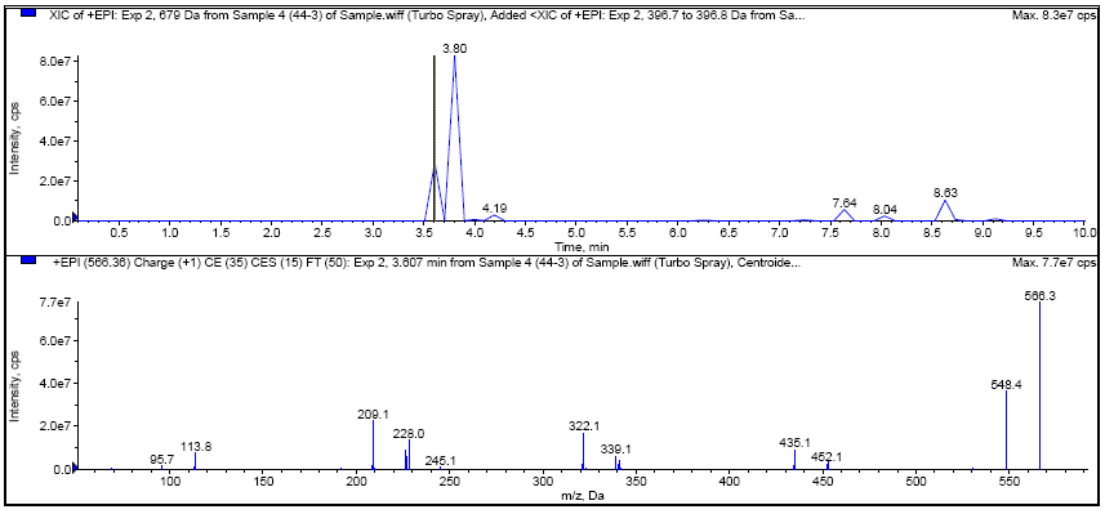


Figure A23: ^{1}H NMR spectrum of mixture (II)





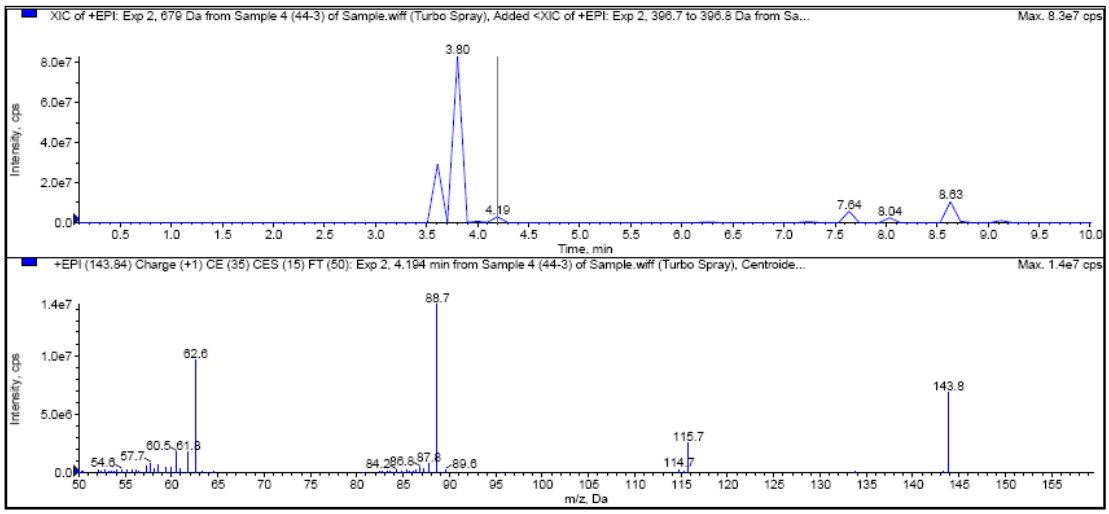


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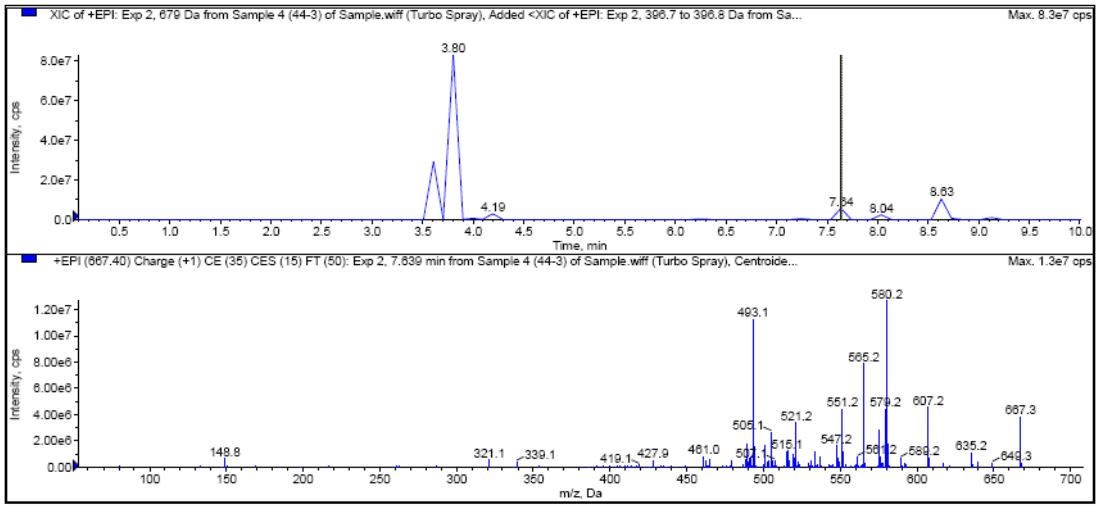
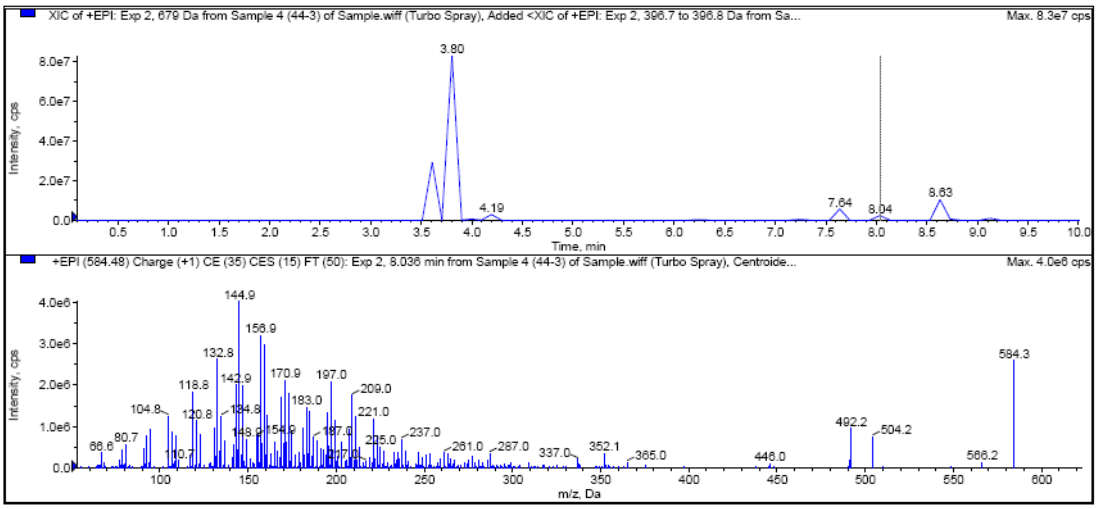
Figure A24 : Chromatogram of mixture (III) and the mass spectra of the compounds in the chromatogram .

Figure A24, continued





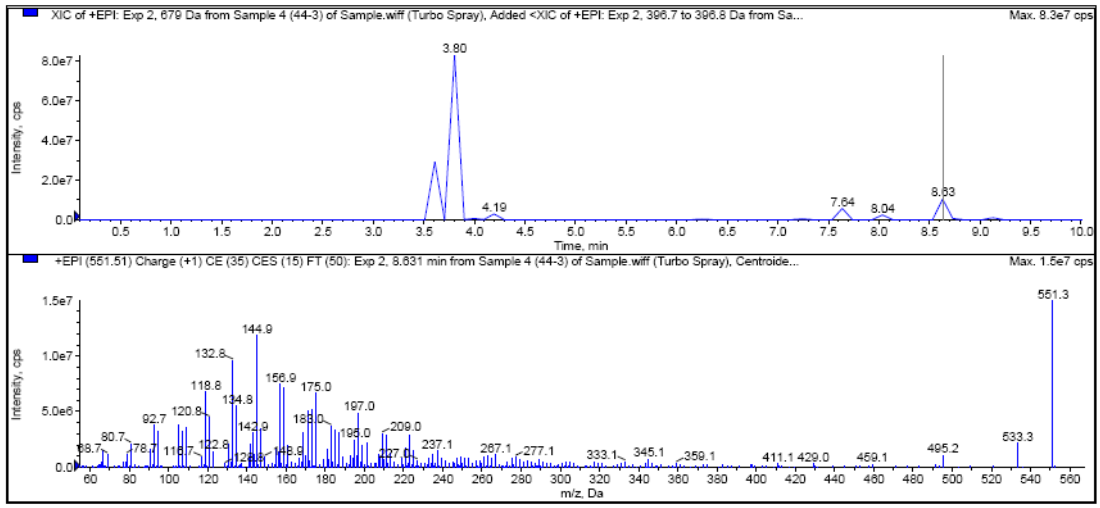


Figure A24 : Chromatogram of mixture (III) and the mass spectra of the compounds in the chromatogram .

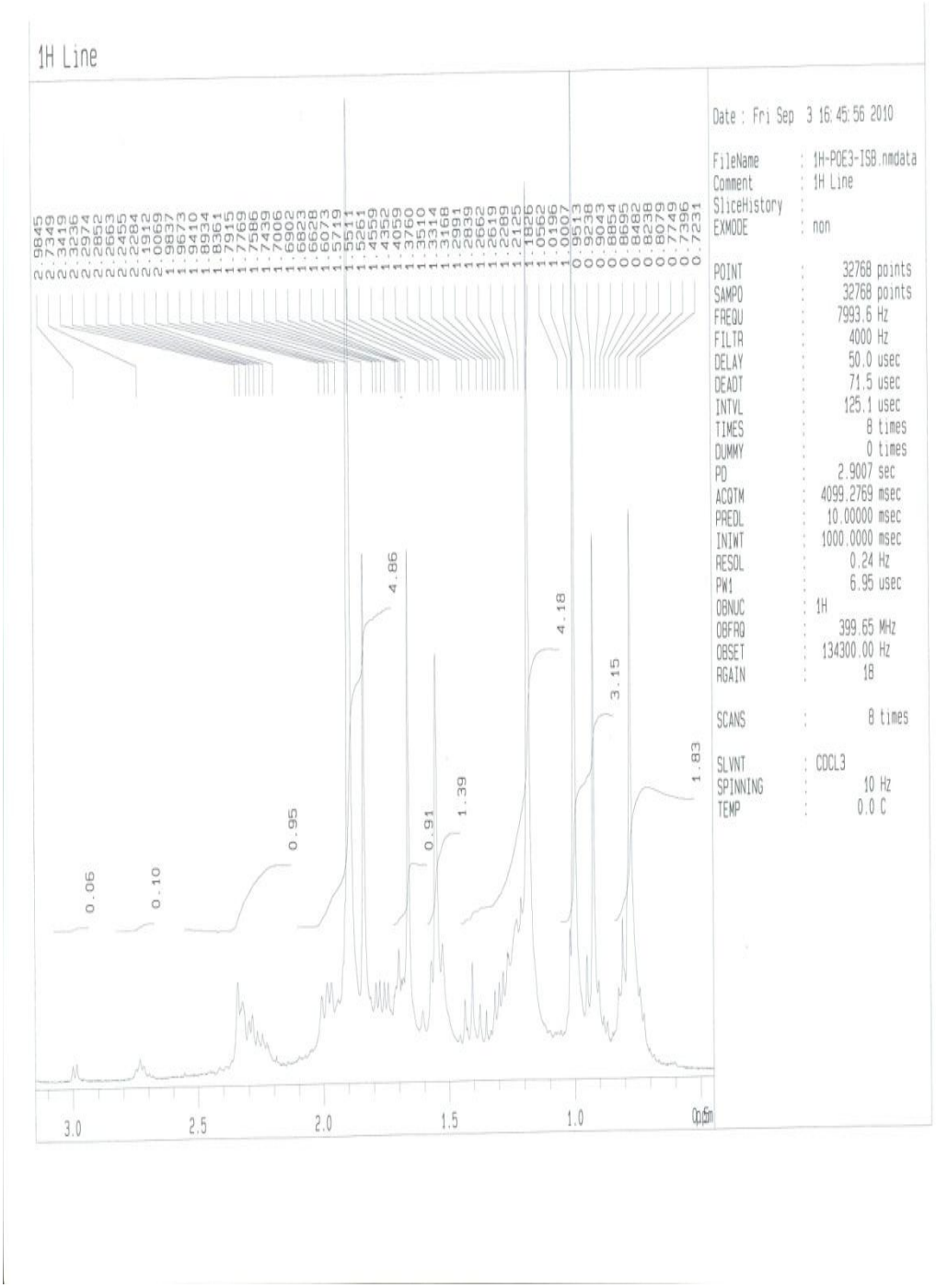


Figure A25: ^{1}H NMR spectrum of mixture (III)

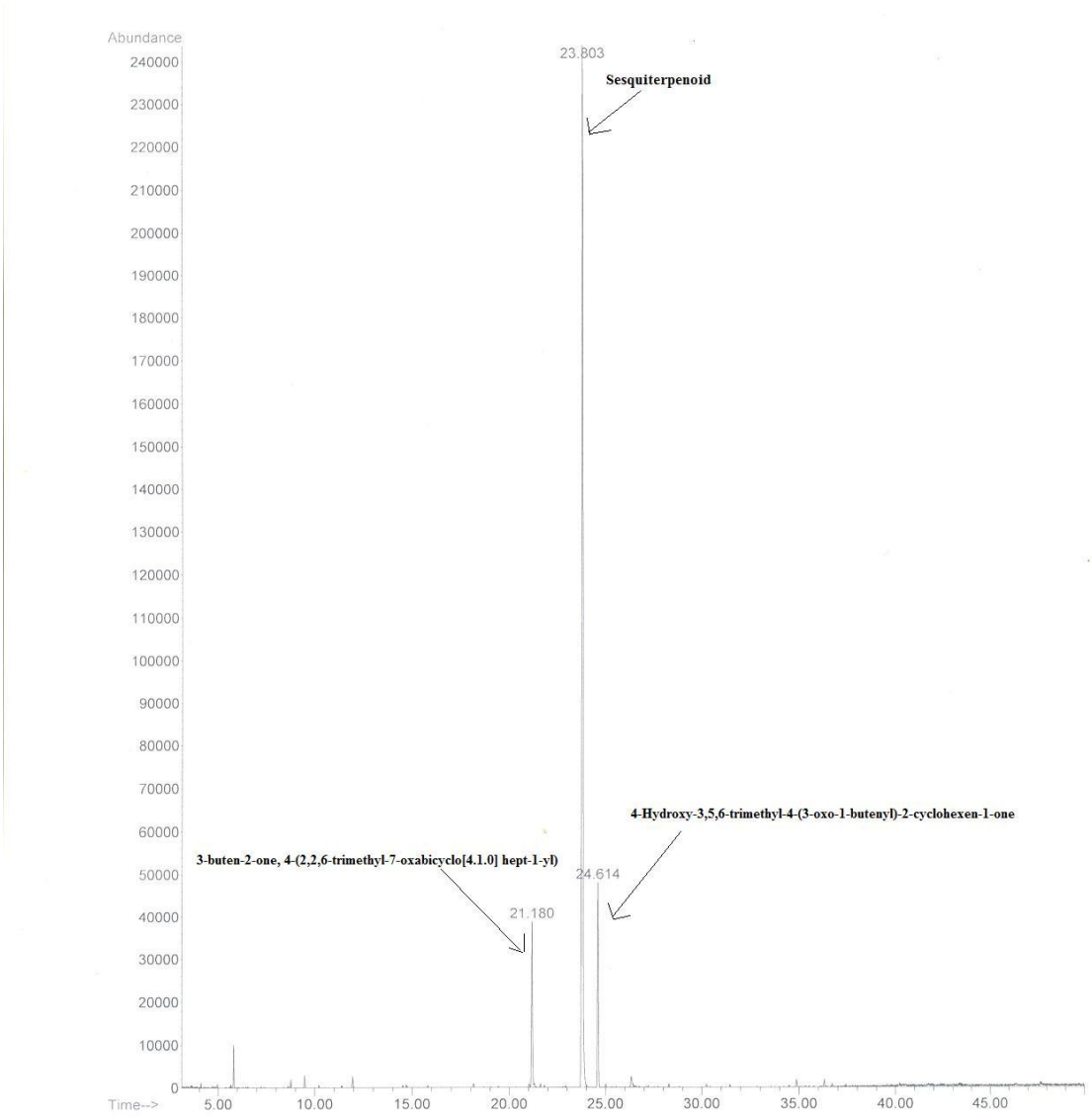


Figure A26: The total ion chromatogram of mixture (IV)

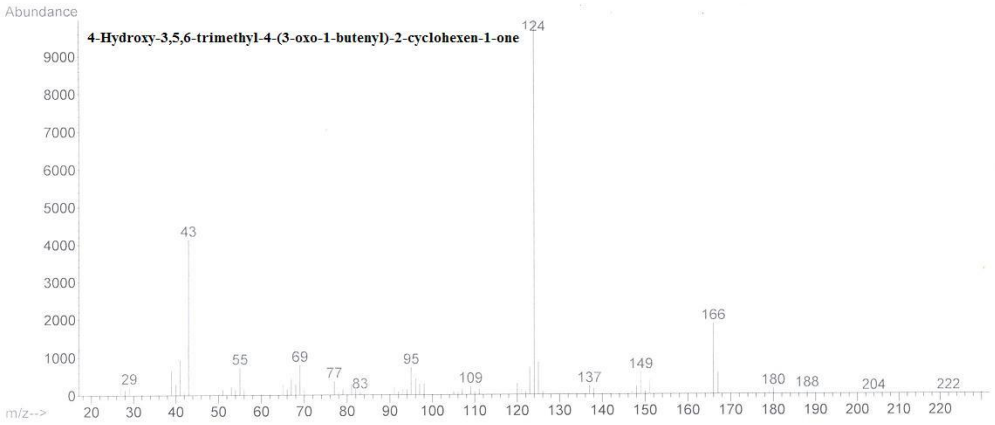


Figure A27: Mass spectrum of 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-2-cyclohexen-1-one from mixture (IV)

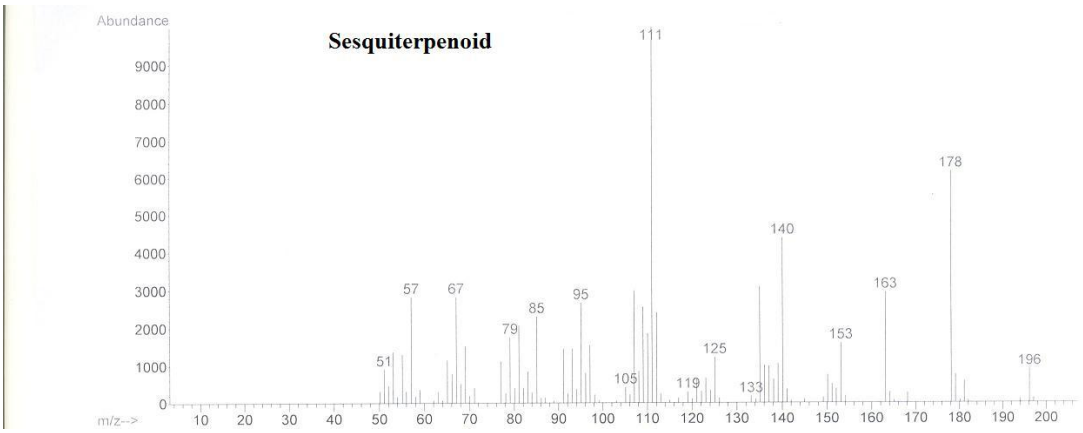


Figure A28 : Mass spectrum of sesquiterpene from mixture (IV)

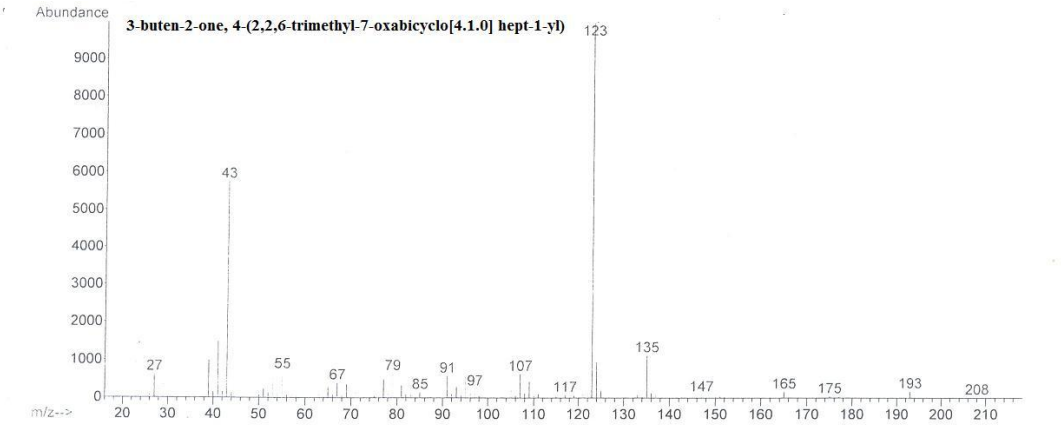


Figure A29: Mass spectrum of 3-buten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0] hept-1-yl) from mixture (IV)

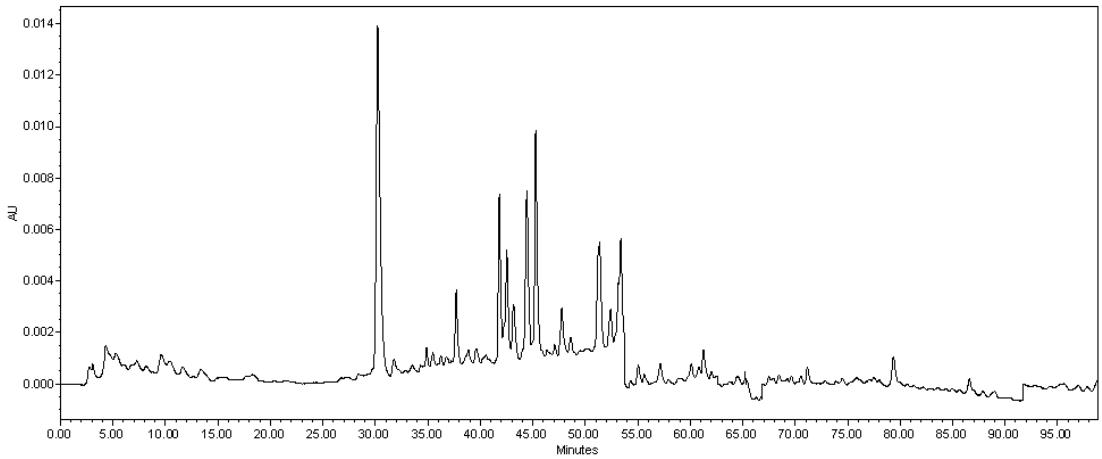


Figure A30 : HPLC chromatogram for ethyl acetate fraction of *P.oleracea*

Table A31 : HPLC separation method for ethyl acetate fraction of *P.oleracea*

Time (min)	Mobile Phase
0 – 70	80.0 (A) – 20.0% (B)
70 – 95	15.0 (A) – 85.0% (B)
95 – 96	80.0 (A) – 20.0% (B)

(A) : water
(B) : acetonitrile