Part II

A. **Extraction of essential oil from *Ocimum basilicum* L.and *Pimpinella anisum* L.calli and assessment of the yield.**

B. **Gas chromatography- Mass spectral analysis (GC/MS) of the extracted oils.**

Materials and methods

A. Materials

1. Plant material

   Dried calli of *O. basilicum* L. and *P. anisum* L. obtaind from tissue cultures for each.

2. Equipment

   a. Clevenger – type apparatus.

   b. GC/MS; Gas chromatography: GC- Hewlett-Packard 7890A apparatus. Mass spectrometer: MS 5975C VL MSD with Triple-Axis detector- Automatic sampler- 7683B.

B. Methods

Extraction of essential oils

Media were separated out and the air dried calli of *O. basilicum* and *P. anisum* were submitted to hydrodistillation for 3 hours, using a Clevenger-type apparatus, according to the European Pharmacopoeia (Council of Europe, 2007). A simple Clevenger-type apparatus contained a 1,000 ml flask, a condenser, and a measuring tube with stopcock, a return tube for the aqueous part of the distillate connects the bottom of the measuring tube and the vertical tube. A round bottom flask of 1,000 ml capacity is packed with the dried calli. Approximately 250 ml distilled water is added and heated by means of an electric heating mantel. The distillate was condensed as a mixture of essential oil and water in the graduated oil trap, where the oil had been separated from the water and the oils were estimated as percent dry weight (v/w) of calli. The resulted oils were dried over anhydrous sodium sulfate and stored at -20ºC in glass vials until analysis.

The yield of essential oil from 4-weeks old till 20- weeks old calli

To determine the relation between age of harvested calli from suspension cultures and production of essential oils, calli in suspension culture at different ages of from 4-week old calli till 20- week old for both *O. basilicum* and *P. anisum* were collected , dried, weighed, steam distilled, and the collected volumes of oils were determined using a suitable oil trap.

Table V. The yield of essential oil of *O. basilicum* L.

Dry weight of callus and aerial parts of *O. basilicum* (gm).	Age of callus and aerial parts of *O. basilicum* (weeks).	Volatile oil yield (ml)	ml of volatile oil /100gm dry weight (%)
340	4	2.38	0.7
415	8	3.74	0.90
450	12	3.95	0.87
330	16	2.4	0.72
320	20	2.5	0.78

Table VI. The yield of essential oil of *P. anisum* L.

Dry weight of callus, aerial parts and roots of *P. anisum* (gm).	Age of callus, aerial parts and roots of *P. anisum* (weeks).	Volatile oil yield (ml)	ml of volatile oil /100gm dry weight (%)
385	4	8.47	2.2
380	8	8.76	2.3
390	12	8.85	2.22
380	16	8.5	2.23
350	20	8.7	2.5

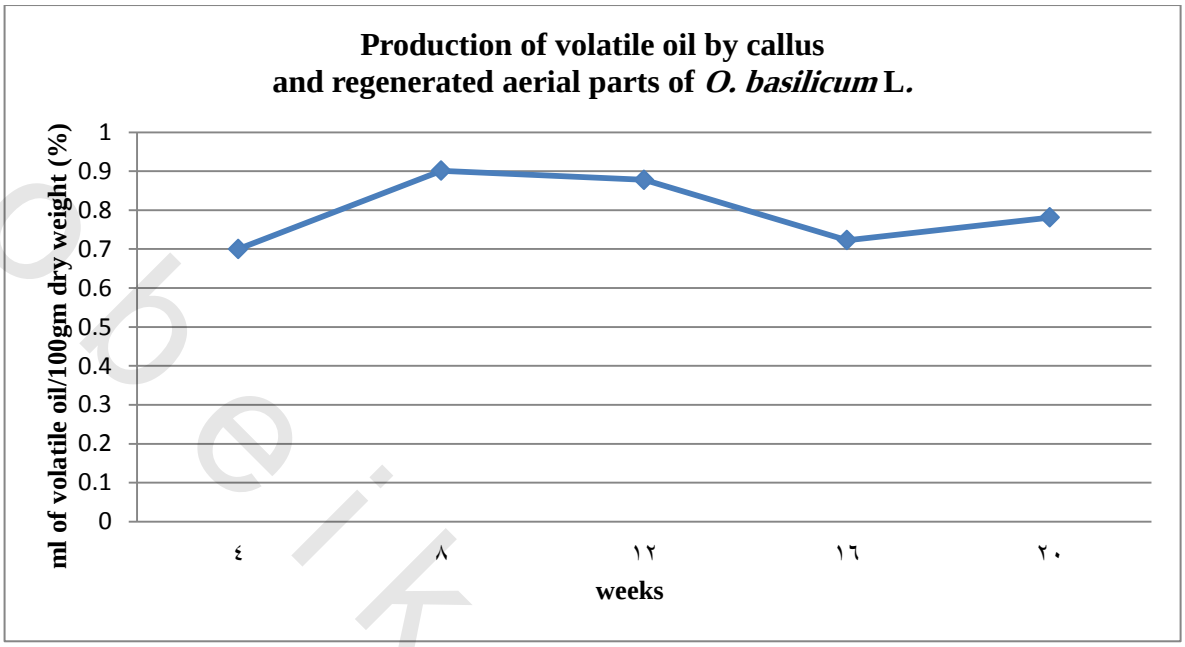


Figure11. The yield of essential oil of *O. basilicum* L.

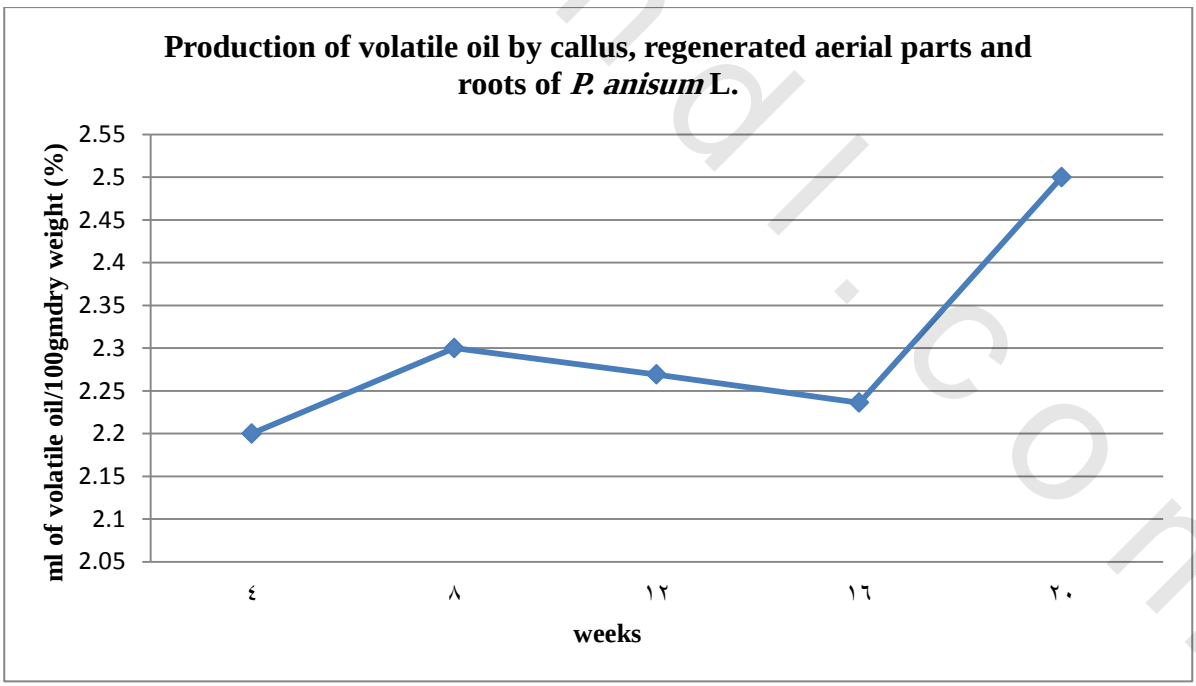


Figure12. The yield of essential oil of *P. anisum* L.

Gas chromatography / Mass spectrometry (GC/MS) analysis for the extracted oils

Sample preparation for GC/MS analysis

Essential oils of *O. basilicum* L. and *P. anisum* L. were diluted 10 times in hexane and mixed well. After phase separation upper layers were taken to autosampler vials with no treatment and 0.2µl was directly injected to GC/MS for both oils.

Conditions of analysis

The identification of volatile constituents was conducted by gas-chromatography (GC-MS) technique – GC 7890A apparatus with Mass spectrometer–MS 5975C VL MSD with Triple-Axis Detector (Agilent MSD ChemStation software, version G1701EA E.02.00493 for GC and GC/MS analysis)- Automatic sampler – 7683B Series injector and split/split less injection system operating in electronic ionization mode achieved by electron impact at 70 eV. The HP-5MS capillary column (30 m x 250 µm, and 0.25µm film thickness). In: Front SS Inlet He, Out: Vacuum Flow 3ml/min., Pressure107.29 kPa, Average velocity 15cm/sec. Helium was used as carrier gas (purity 99.999%) with flow rate of 1ml/min. The mass spectrometer had a vacuum compensation ON, solvent delay time 4 min to avoid the solvent peak, split ratio 1:100 and electronic pressure control on. The mass spectrum of each peak was recorded in the total ion current mode of the mass spectrometer within a mass scan range of 50-650 m/z. The temperature program was as follows: Injection port temperature, 300°C; Temperature of MS detector 280°C, column oven temperature 80°C and gradient programmed at 15°C/min to 320°C, kept constant for 13 min, 1min hold time and 30 min was the final time, transfer line temperature was 280 °C.

Identification of compounds

The components of the oils were identified by matching of their mass spectral fragmentation patterns with those reported in computerized MS-data bank spectral libraries (NIST 98 and WILEY 138). The compounds identified are reported to have match quality > 90% with respect to the experimental spectrum. The compounds are arranged in order to GC elution on HP-5MS capillary column. For quantification purpose, relative area percentages were obtained by electronic integration on GC peak areas without the use of correction factors. However, the identification using mass spectral library alone is not always possible since some structural molecular fragment cannot be distinguished solely on the basis of MS data, for instance differences in the number or position of carbon skeleton branching, isomeric systems, etc. Therefore, to increase the reliability of the analytical results, it is necessary to utilize both MS data and retention indices identities as identification criteria [138]. The molecular weights of the compounds were confirmed by comparison of their GC retention indices (RI) on apolar column with those reported in literature data. The RIs were calculated

according to the RI Van den Dool and Kratz equation relative to the retention times of a series of n-alkanes [139].

Kovats retention index

Kovats retention index (shorter Kovats index, retention index; plural retention indices) is a concept used in gas chromatography to convert retention times into system-independent constants. Tables of retention indices can help identify components by comparing experimentally found retention indices with known values [140]. For temperature programmed chromatography, the Kovats index is given by the following equation:

$$I = 100 \times \left[n + (N - n) \frac{t_{r(unknown)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}} \right]$$

Where:

$I =$ Kovats retention index,
$n =$ the number of carbon atoms in the smaller n-alkane,
$N =$ the number of carbon atoms in the larger n-alkane,
$t_r =$ the retention time.

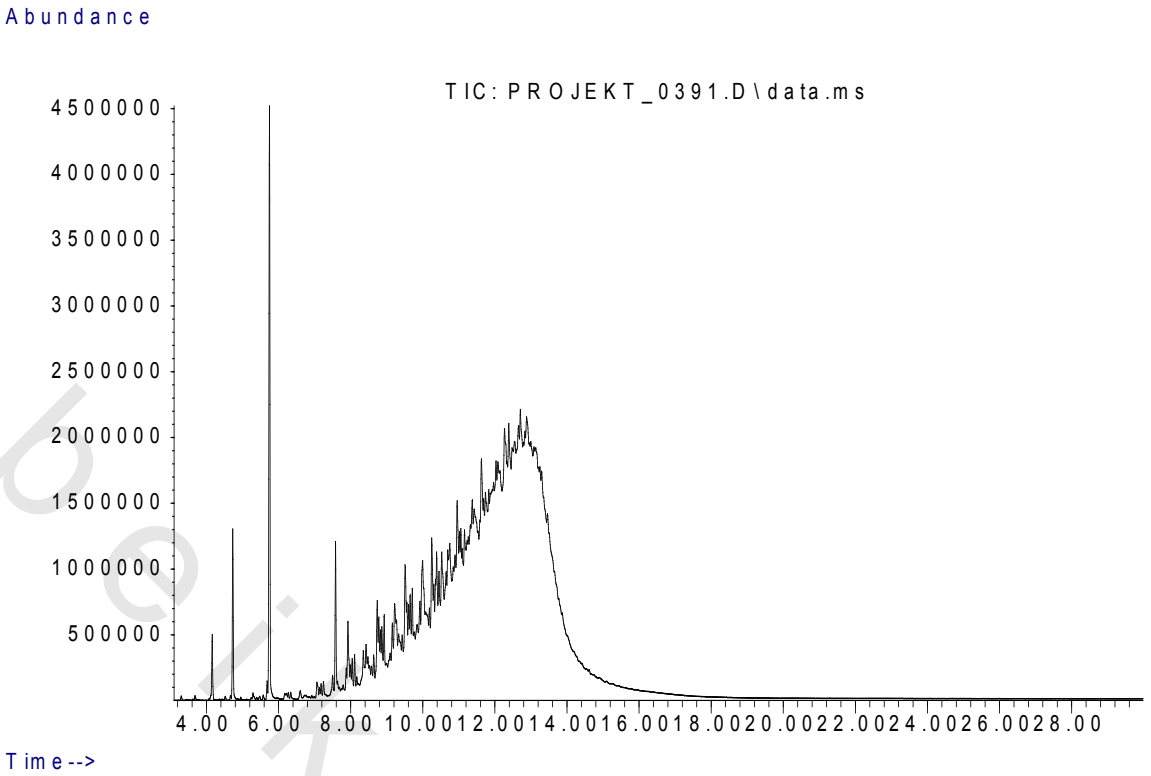


Figure13. GC/MS chromatogram of the essential oil of *O. basilicum* L.

Table VII. Chemical structure of compounds identified in essential oil of *O. basilicum* L. calli [141-144, 147].

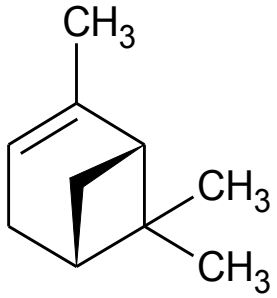
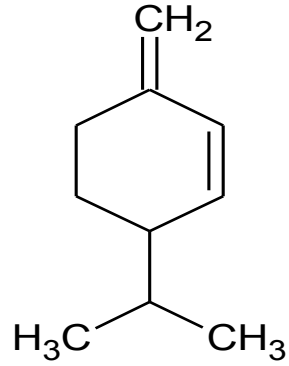
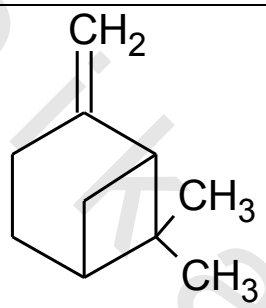
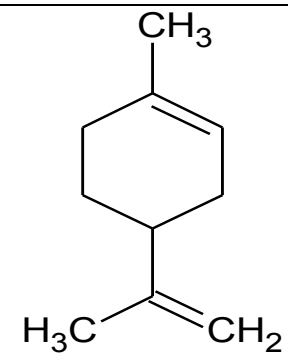
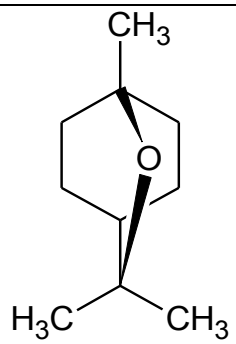
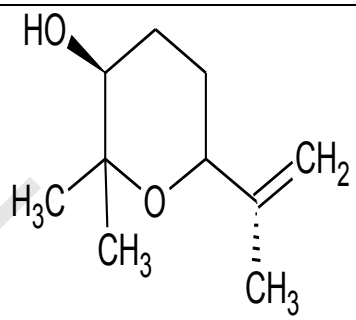
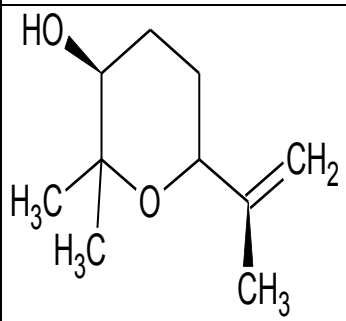
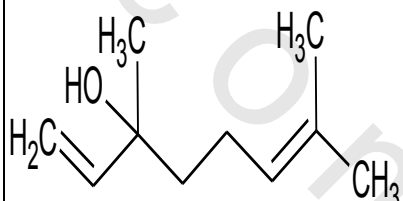
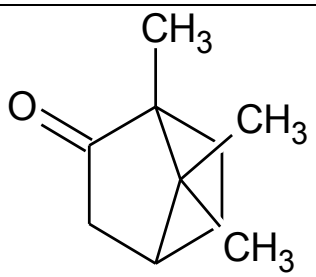
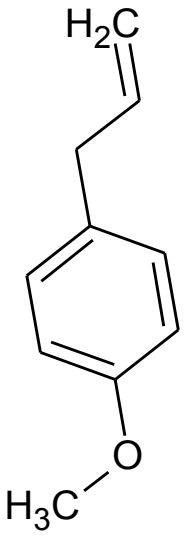
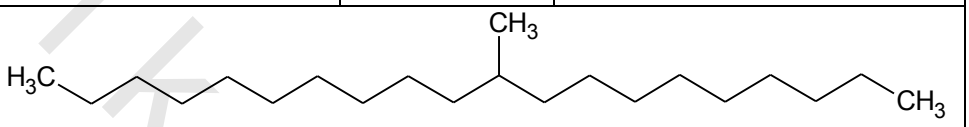
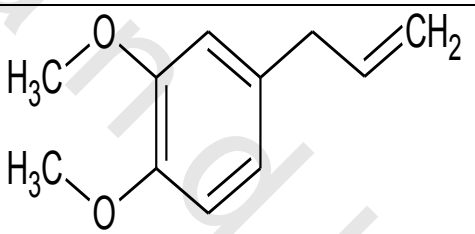
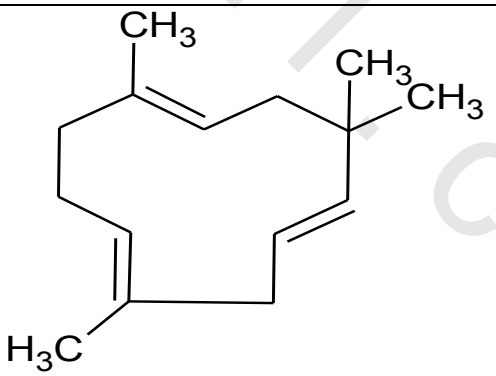
Compound name	Chemical structure	Compound name	Chemical structure
α-Pinene.		β-Phellandrene	
β- Pinene.		D- Limonene	
1, 8- Cineol		*Cis*- linalool oxide.	
Trans- linalool oxide		Linalool	

Table VII. Continued

Camphor		Estragole	
10-Methylicosane			
Methyl eugenol			
α-Caryophyllene			

Mass spectral analysis and fragmentation pattern of compounds identified in essential oil from *O. basilicum* L. tissue culture.

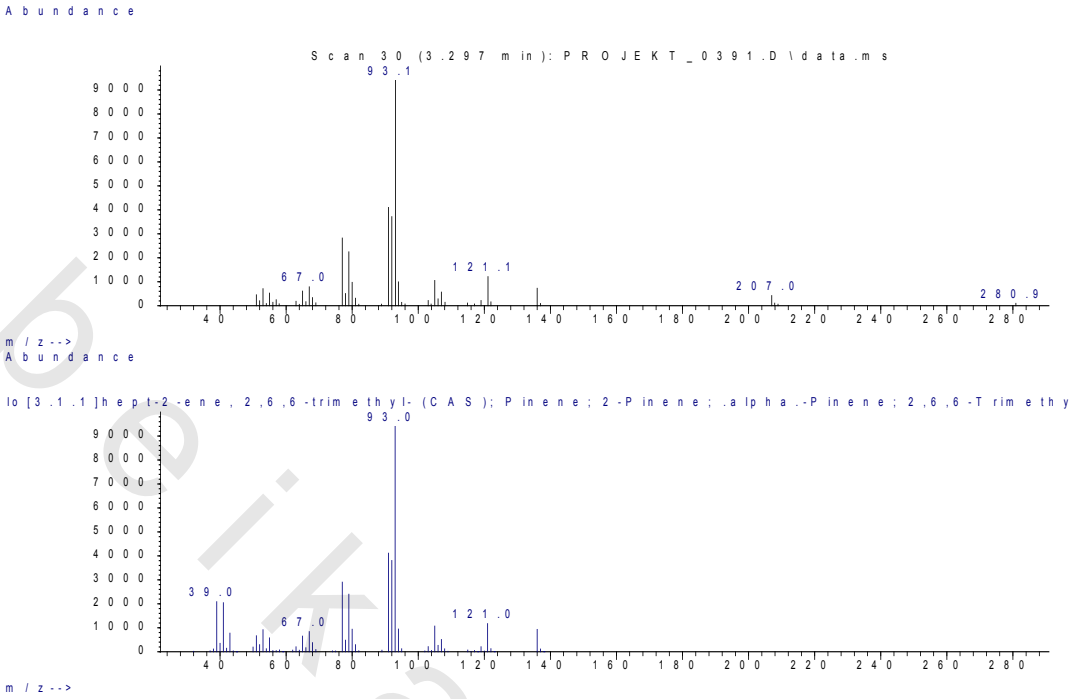


Figure 14. Mass spectral analysis and fragmentation pattern of α-pinene

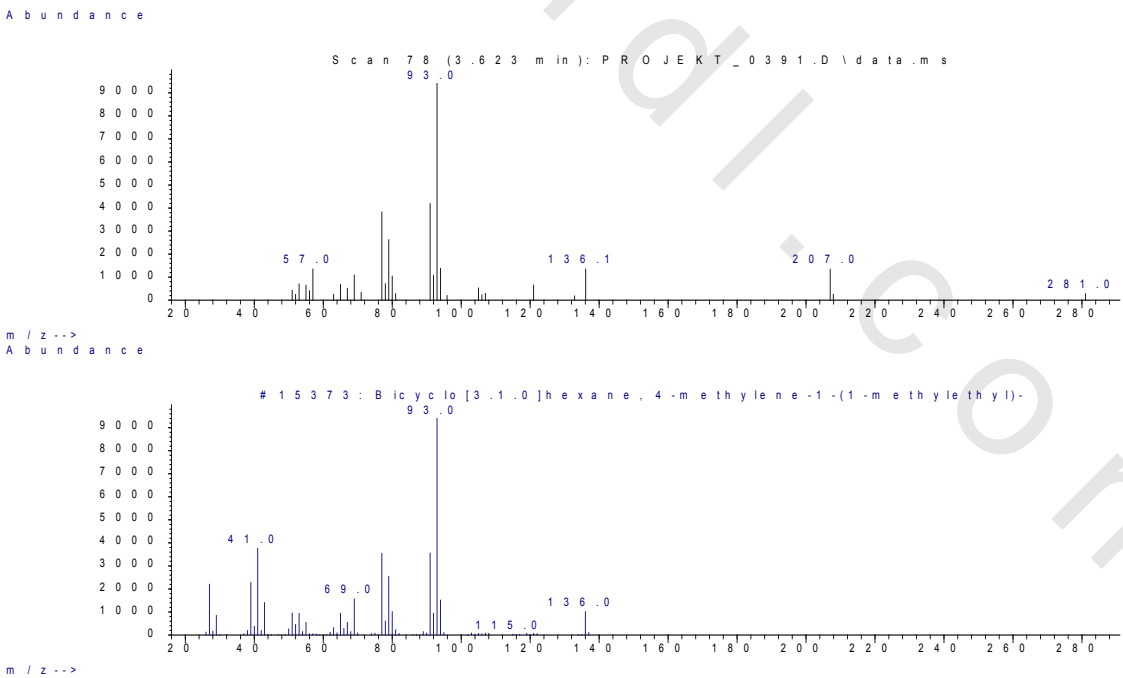


Figure 15. Mass spectral analysis and fragmentation pattern of β-phellandrene.

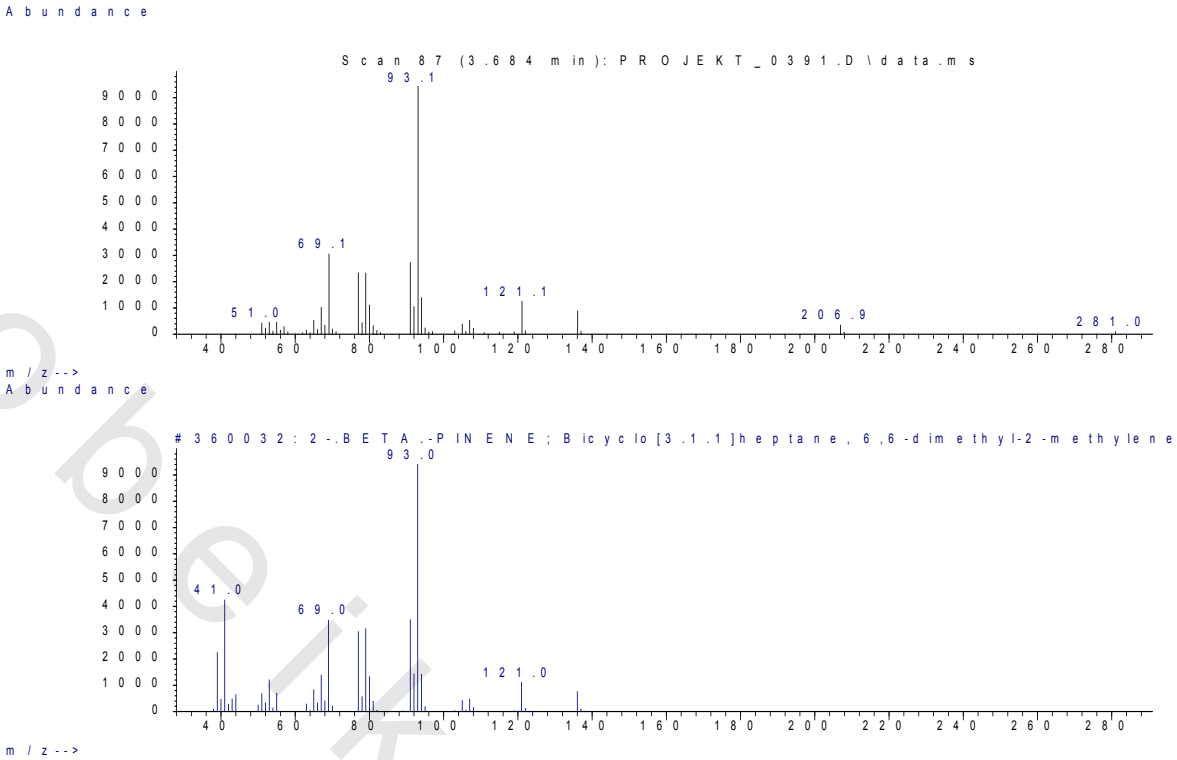


Figure 16. Mass spectral analysis and fragmentation pattern of β-pinene.

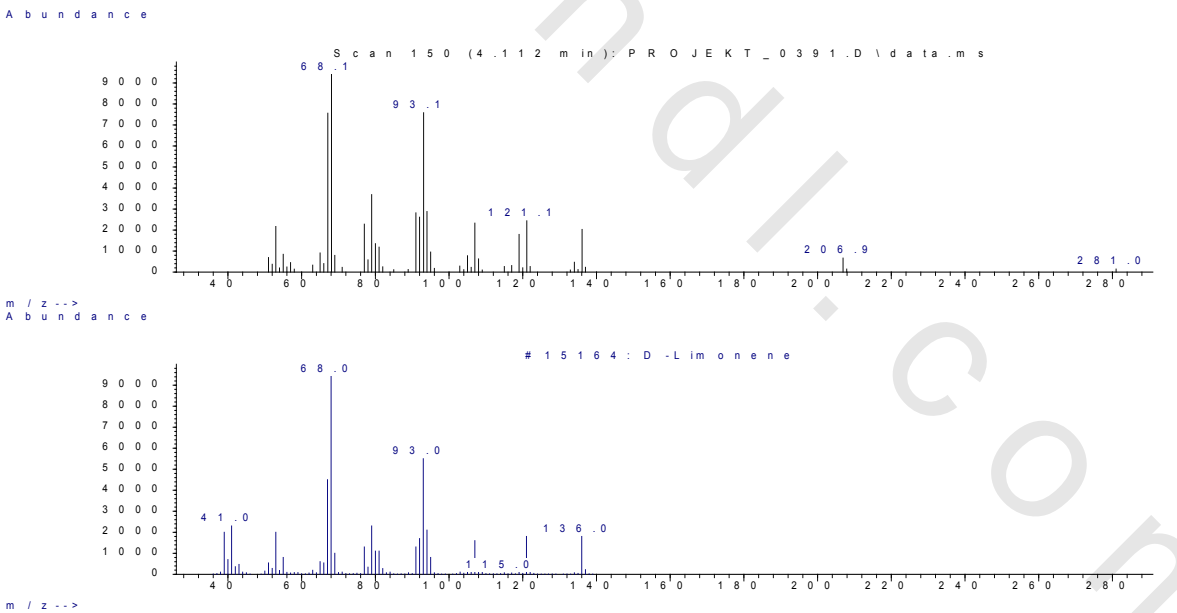


Figure 17. Mass spectral analysis and fragmentation pattern of d-limonene.

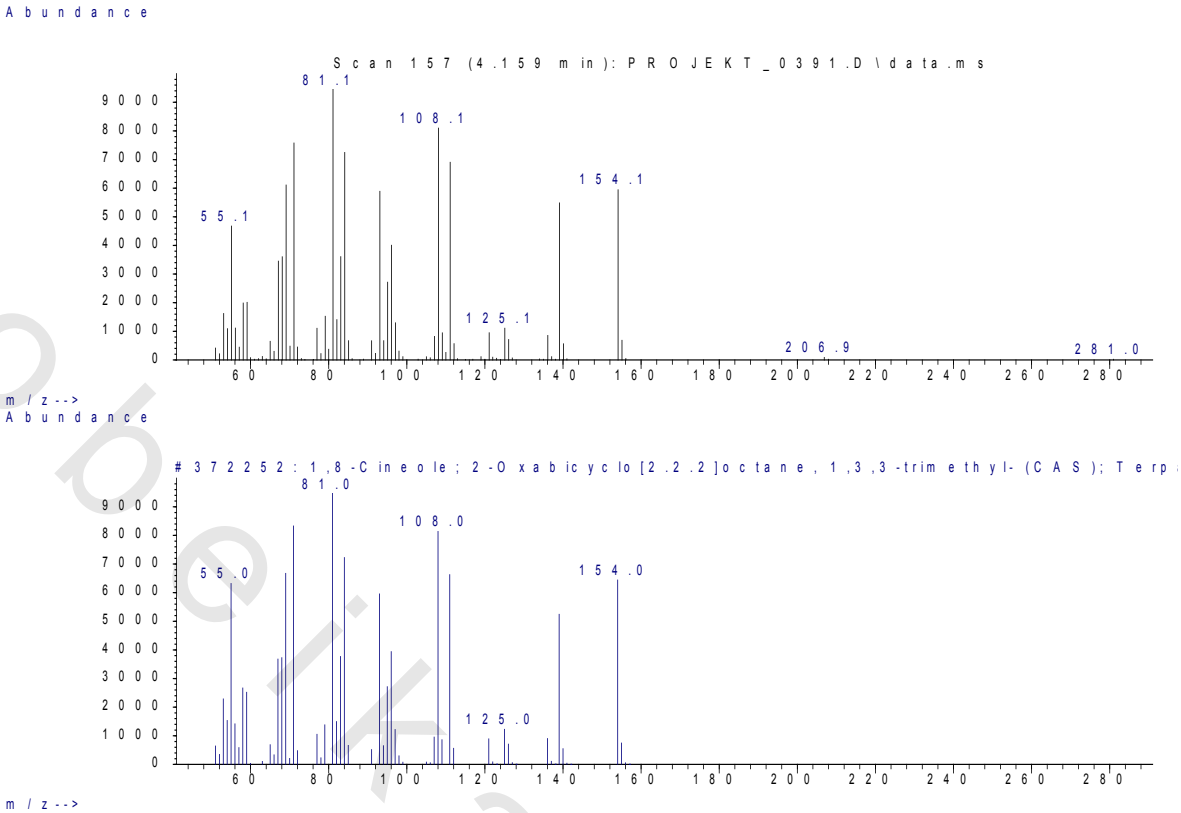


Figure 18. Mass spectral analysis and fragmentation pattern of 1, 8- cineol.

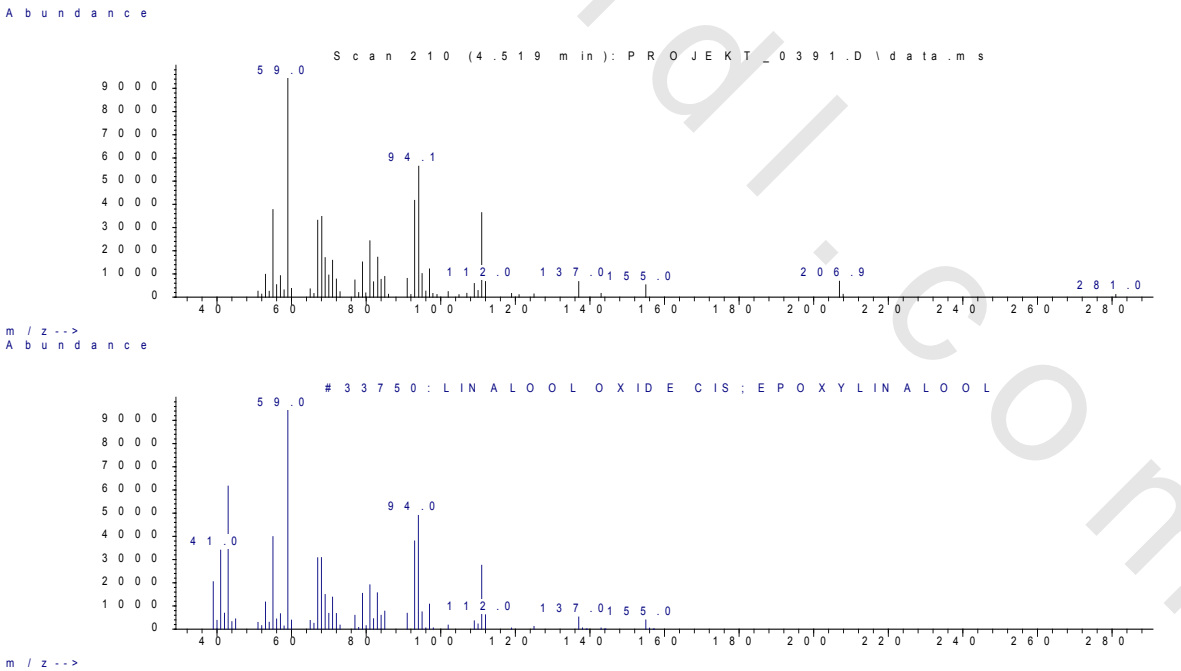


Figure 19. Mass spectral analysis and fragmentation pattern of *cis*-linalool oxide.

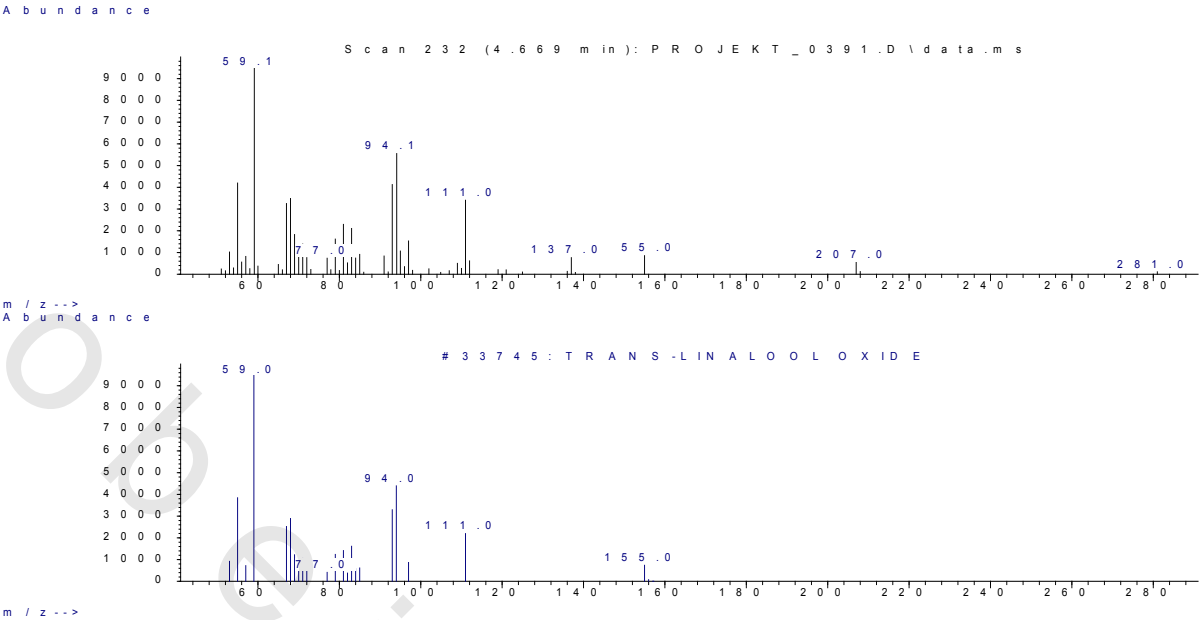


Figure 20. Mass spectral analysis and fragmentation pattern of *trans*-linalool oxide.

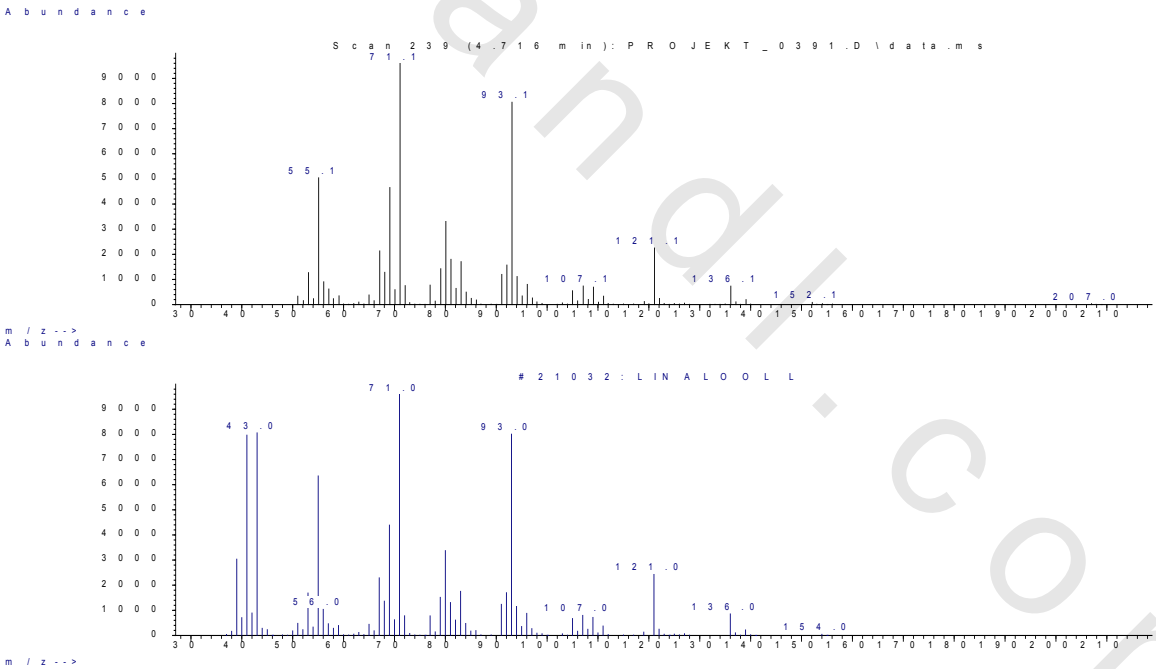


Figure 21. Mass spectral analysis and fragmentation pattern of linalool

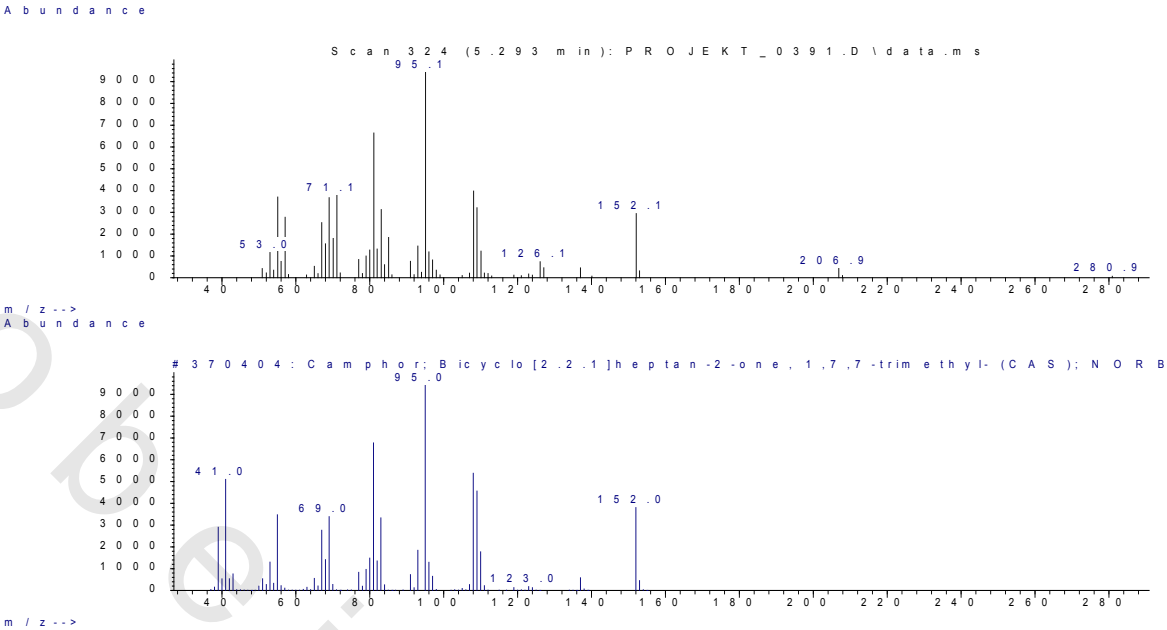


Figure 22. Mass spectral analysis and fragmentation pattern of camphor.

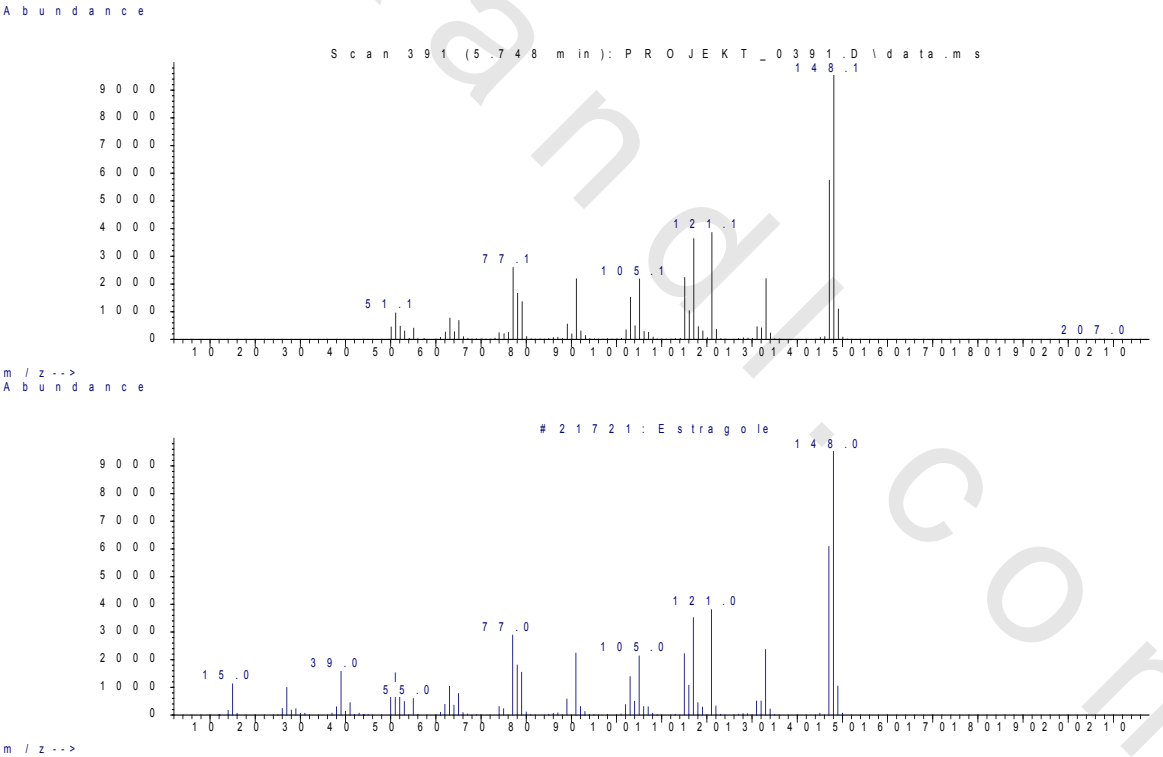


Figure 23. Mass spectral analysis and fragmentation pattern of estragole.

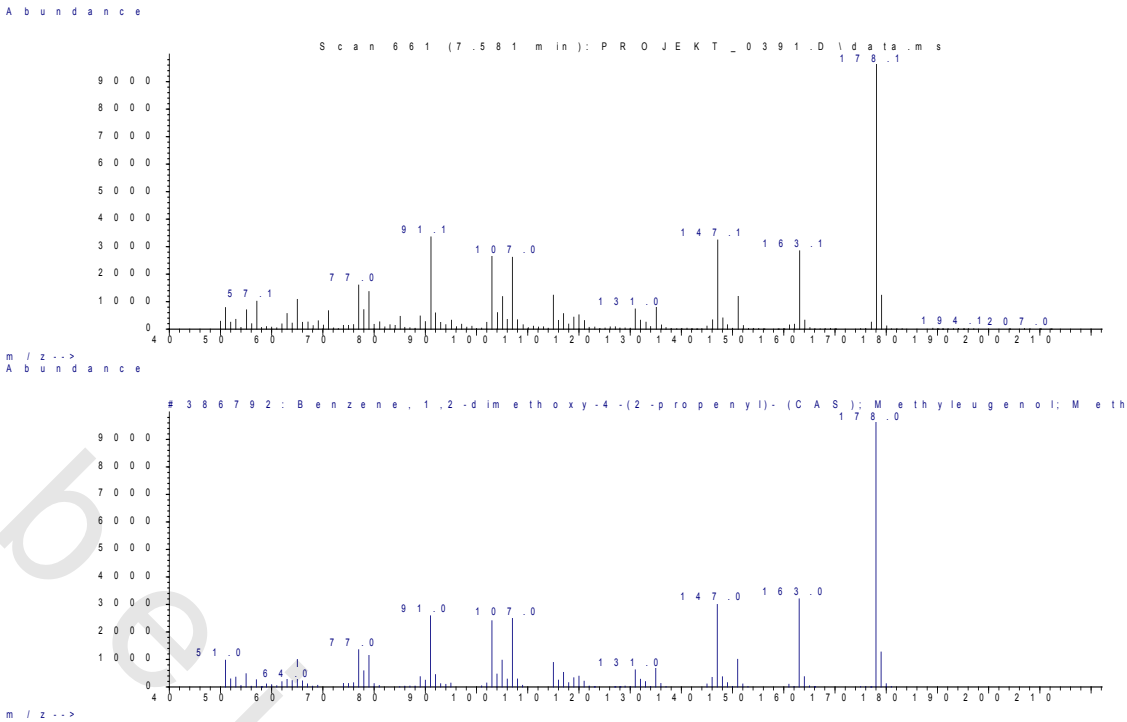


Figure 24. Mass spectral analysis and fragmentation pattern of methyleugenol

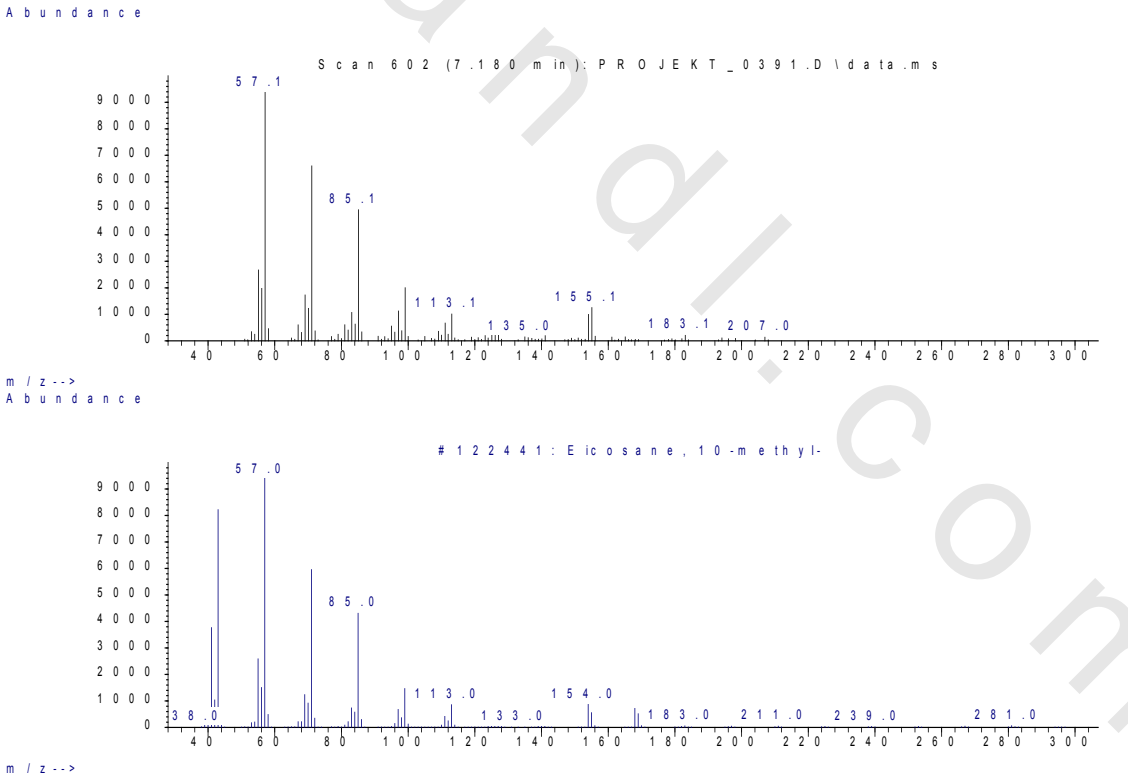


Figure 25. Mass spectral analysis and fragmentation pattern of 10-methylicosane.

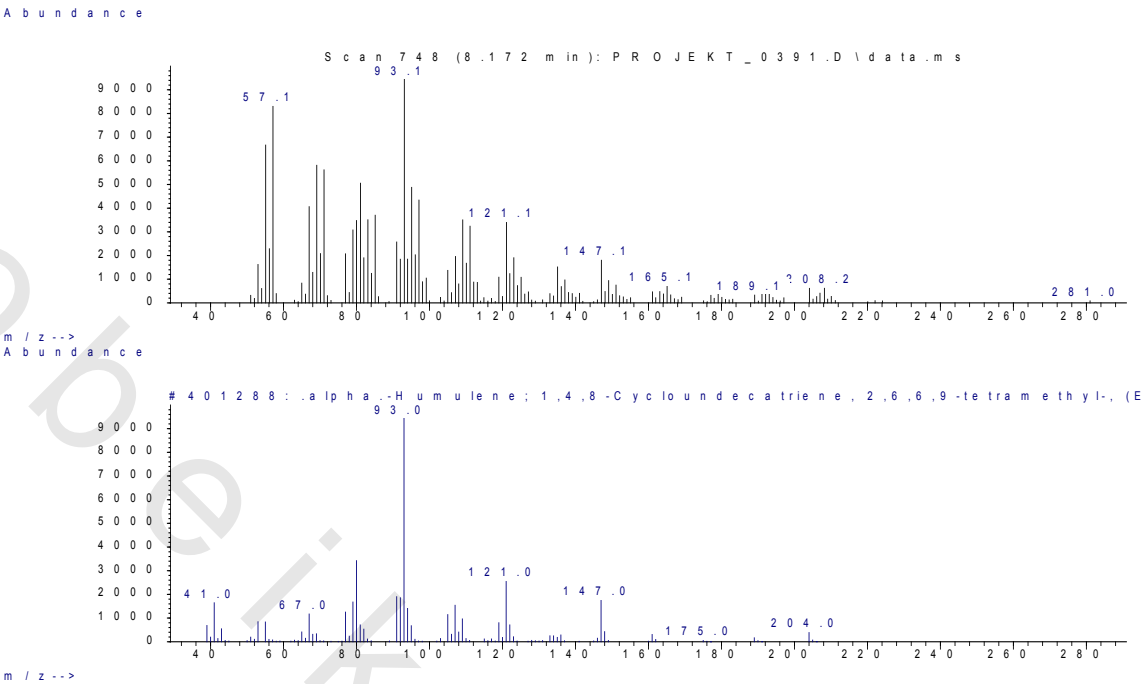


Figure 26. Mass spectral analysis and fragmentation pattern of α-caryophyllene.

Table VIII. Chemical composition of essential oil of *O. basilicum* L. calli using GC/MS.

Compound	t_R(min)	Relative percentage (%)	RI(Exp)	RI(Lit)
α-Pinene	3.297	0.052	934	932[141]
β-Phellandrene	3.626	0.014	1019	1023[141]
β-Pinene	3.684	0.066	976	973[141]
d-Limonene	4.112	0.014	1034	1032[142]
1,8-Cineol	4.159	5.25	1030	1034[143]
Cis-linalool oxide	4.519	0.49	1069	1063[144]
Trans-linalool oxide	4.669	0.52	1081	1077[144]
Linalool	4.716	47.6	1090	1086[141]
Camphor	5.293	1.295	1130	1136[145]
Estragole	5.748	11.9	1180	1183[146]
10-Methylicosane	7.180	1.4	2038	2041[147]
Methyl eugenol	7.581	11.165	1398	1401[148]
α-Caryophyllene	8.172	2.03	1426	1425[149]
Compounds not identified		18.204		

$t_{R.}$: Retention time
RI (Exp): Relative retention indices calculated against homologous series of n-alkanes (C_9-C_{20}) on HP-5MS non-polar column.
RI (Lit): Relative retention indices reported in literature.

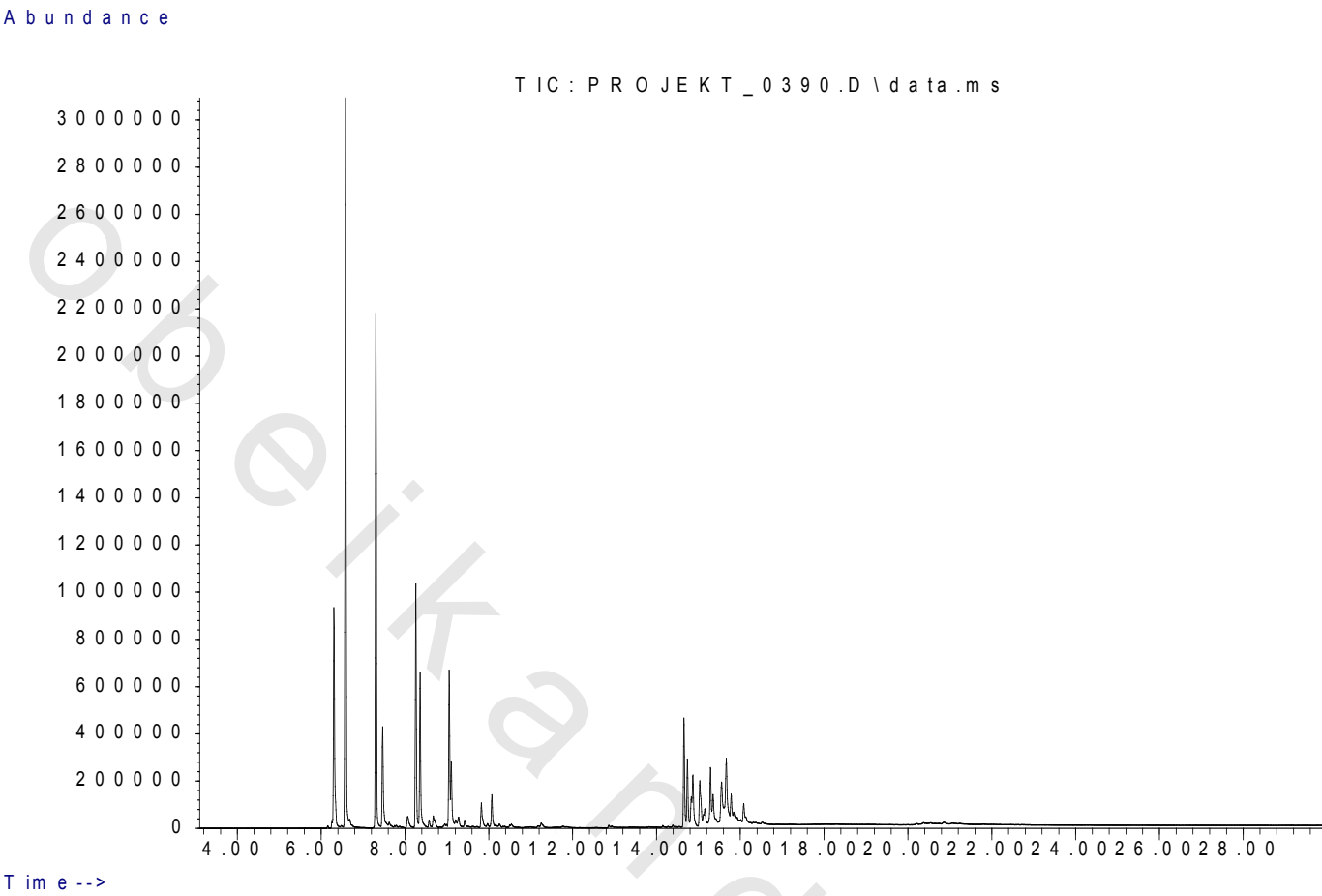
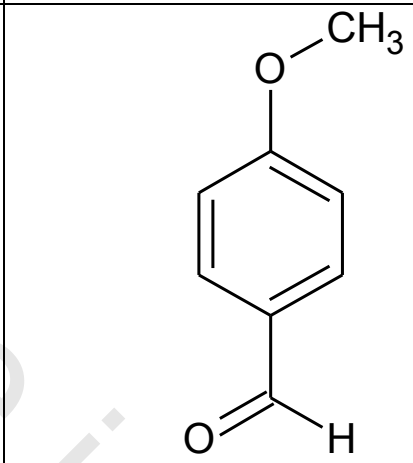
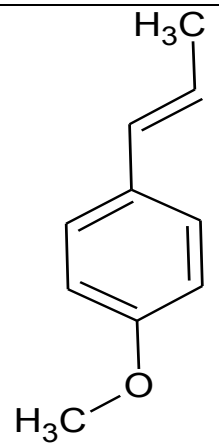
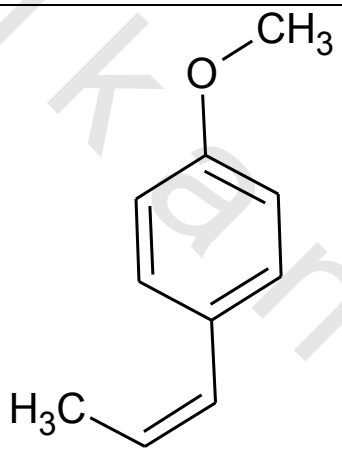
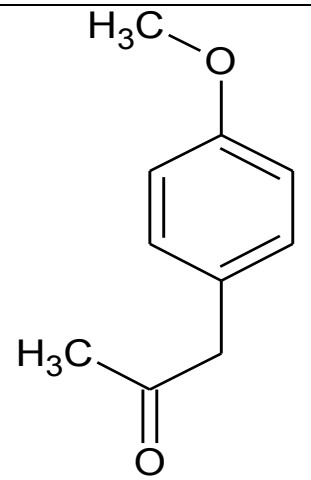
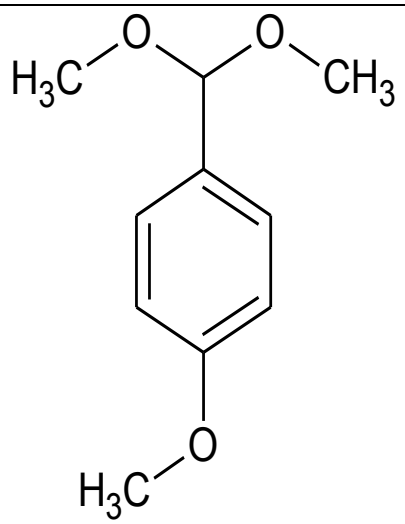
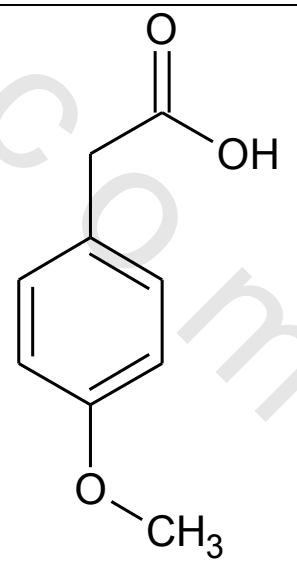


Figure 27. GC/MS chromatogram of essential oil of *P. anisum* L.

Table IX. Chemical structure of compounds identified in essential oil of *P. anisum* L. calli [150-155].

Compound name	Chemical structure	Compound name	Chemical structure
p-Anisaldhyde		*Trans*-anethole	
Cis- anethole		*p*-Anisyl acetone	
p-Anisaldhyde dimethylacetal		*p*-Anisic acid	

Mass spectral analysis and fragmentation pattern of compounds identified in essential oil from *P. anisum* L. tissue culture.

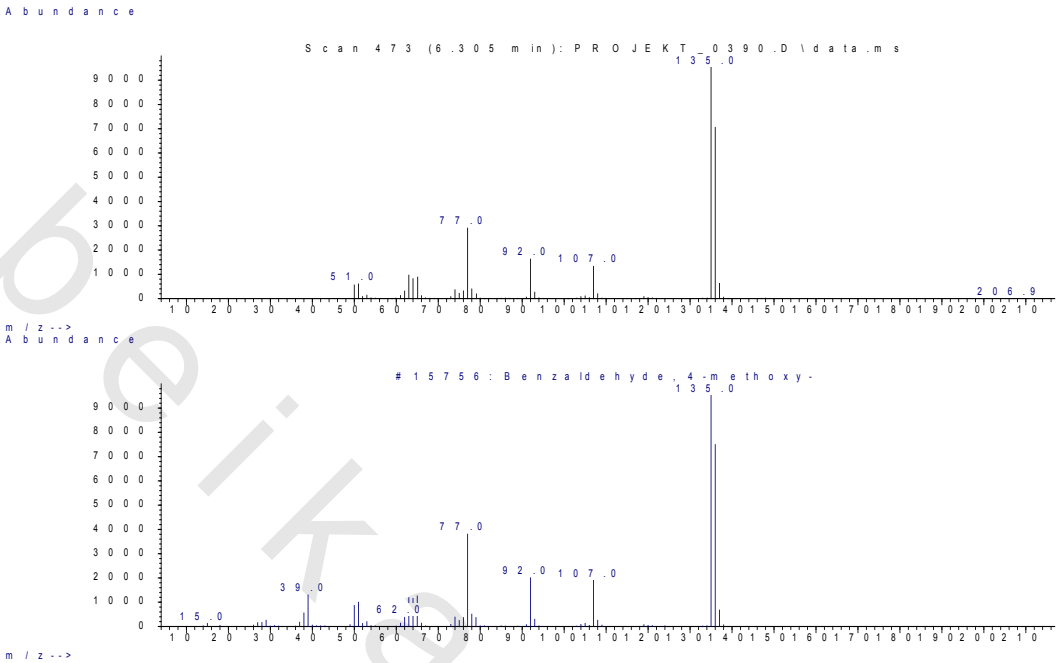


Figure 28. Mass spectral analysis and fragmentation pattern of *p*-anisaldhyde.

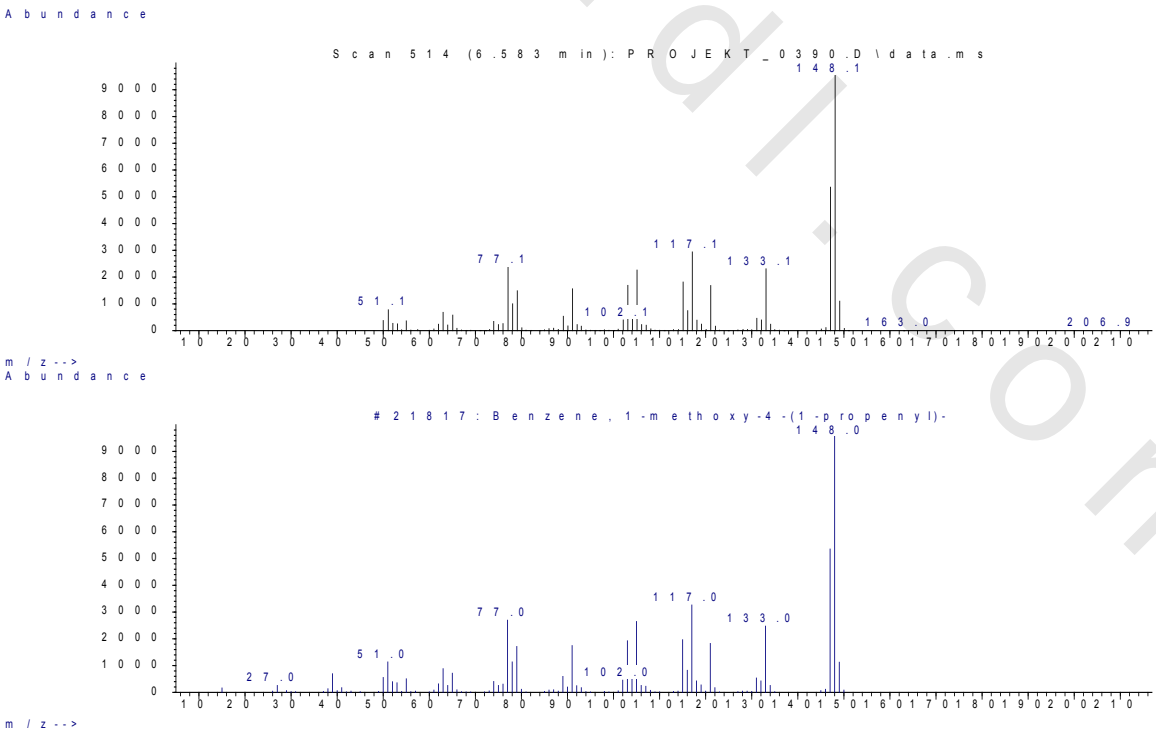


Figure 29. Mass spectral analysis and fragmentation pattern of *trans*-anethole.

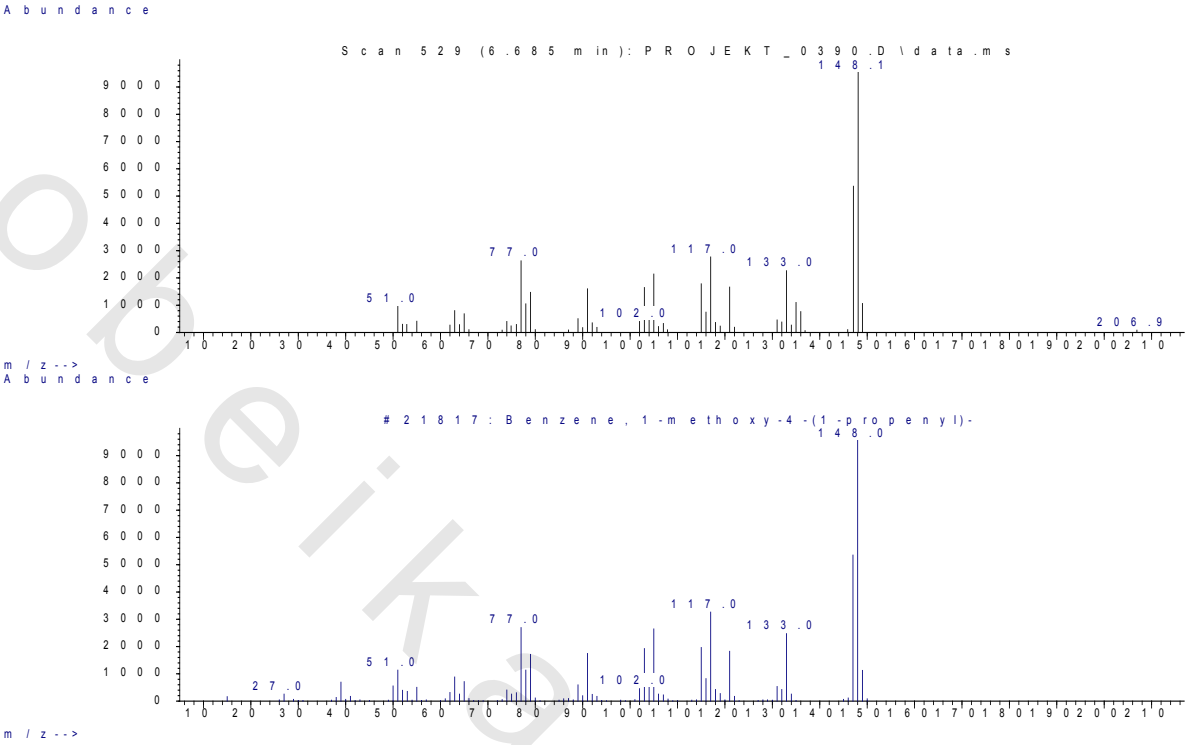


Figure 30. Mass spectral analysis and fragmentation pattern of *cis* -anethole.

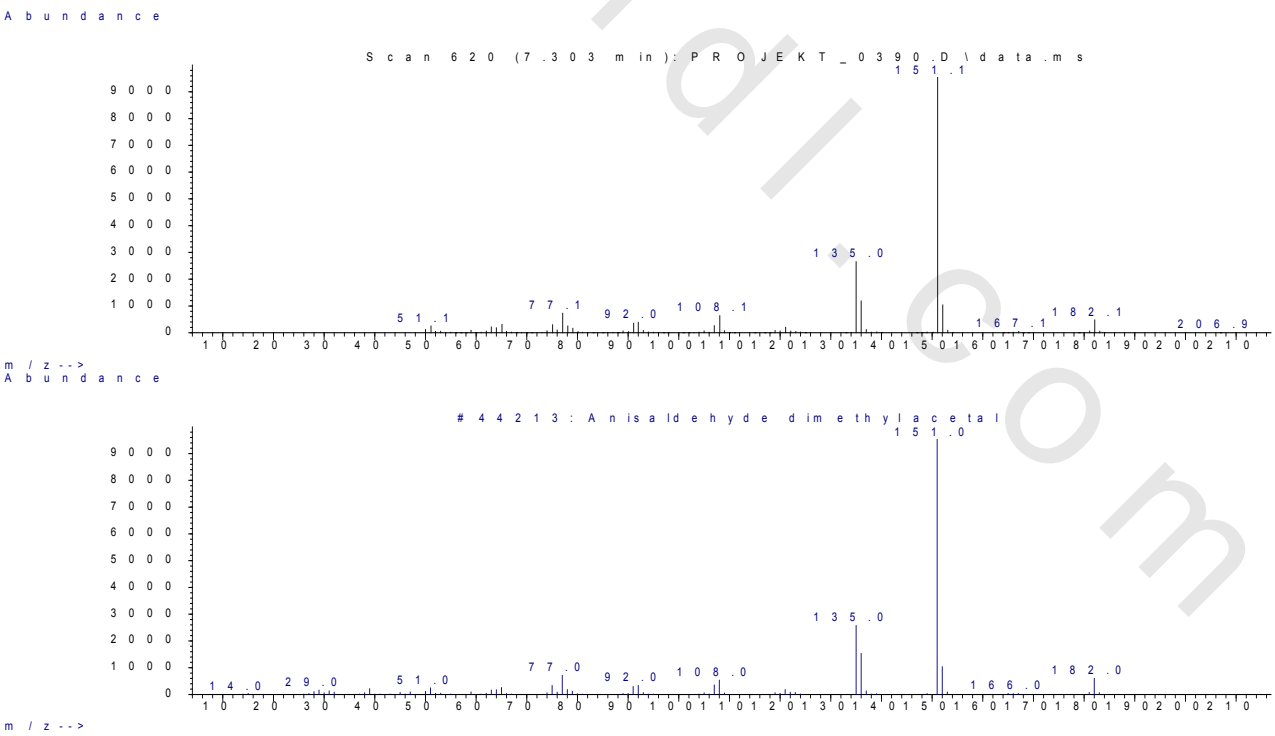


Figure 31. Mass spectral analysis and fragmentation pattern of *p*-anisaldhyde dimethyl acetal.

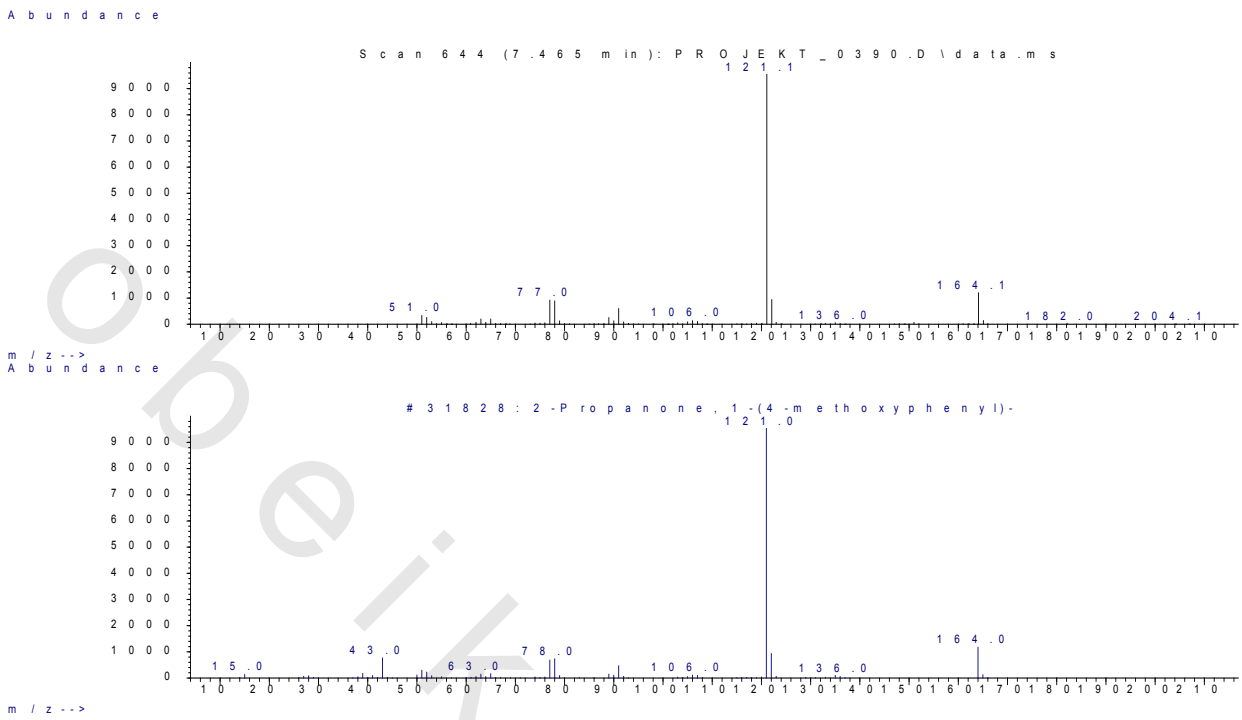


Figure 32. Mass spectral analysis and fragmentation pattern of *p*-anisyl acetone.

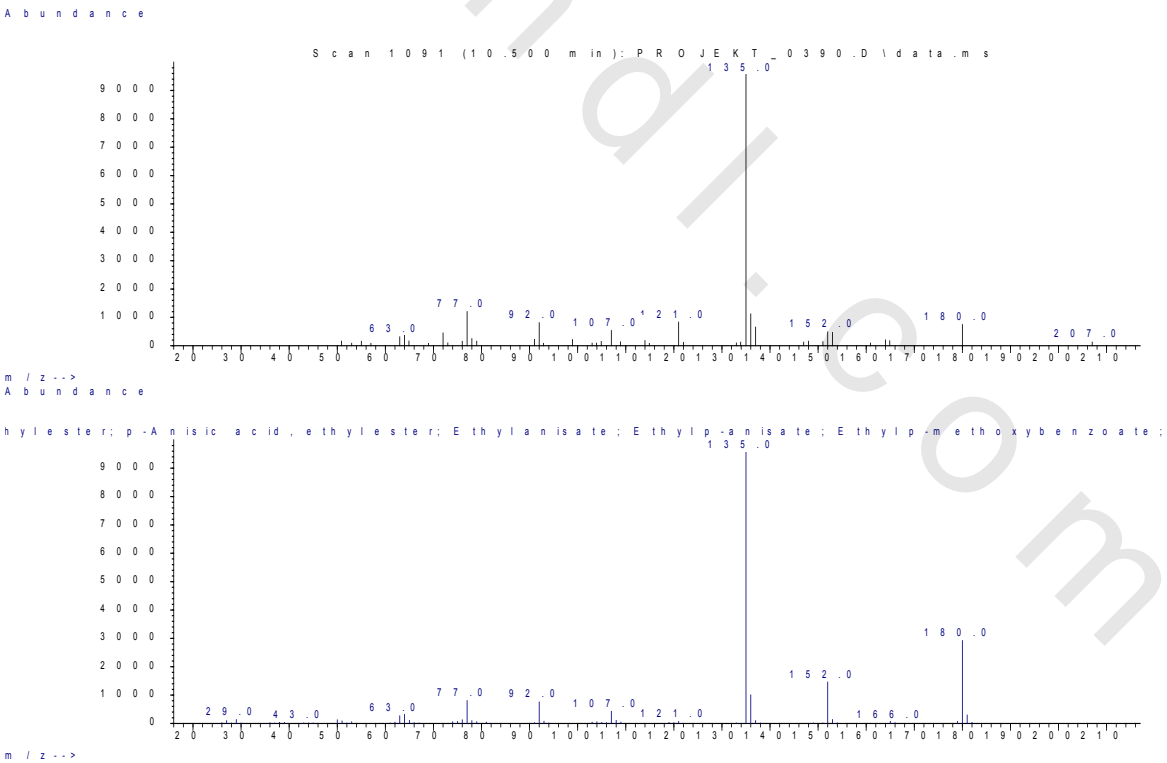


Figure 33. Mass spectral analysis and fragmentation pattern of *p*-anisic acid.

Table X. Chemical composition of essential oil of *P. anisum* L. calli using GC/MS.

Compound	t_R (min)	Relative percentage (%)	RI(Exp)	RI(Lit)
p-Anisaldhyde	6.305	17.2	1240	1236[150]
Trans-anethole	6.583	49.6	1280	1279[151]
Cis-anethole	6.685	1.108	1253	1258[152]
p-Anisaldhyde dimethyl acetal	7.303	15.5	1293	1296[153]
p-Anisyl acetone	7.465	7.8	1472	1473[154]
p-Anisic acid	10.5	2.3	1352	1350[155]
Compounds not identified		6.46		

$t_{R:}$: Retention time
RI (Exp): Relative retention indices calculated against homologous series of n-alkanes (C_9-C_{20}) on HP-5MS non-polar column.
RI (Lit): Relative retention indices reported in literature.

Results and discussion

Productivity curve shown in [Figure11] expresses the relation between production of essential oil in suspension culture and the age of calli for *O. basilicum.* We can notice that the yield increased from 4-weeks old calli to obtain the maximum yield at 8-weeks old (0.9%), then the yield gradually decreased to become 0.87% at 12- weeks old and the lowest yield was 0.72% at 16- weeks old. Finally at 20 weeks old calli the yield began to increase again to be 0.78%. So, the yield of essential oil obtained by extraction of calli of *O. basilicum* on the basis of callus dry weight (v/w), was approximately between (0.72- 0.9%) and that exceeds the pharmacopoeial requirements for the wild plant (not less than 0.5%).

Productivity curve shown in [Figure12] expresses the relation between production of essential oil in suspension culture and the age of calli for *P. anisum.* We can notice that the yield increased gradually from 2.2 % at 4-weeks old calli and that was the lowest yield to become 2.3% at 8- weeks old ones and there was only a slight decrease in the yield at 12- weeks old calli. Then the yield increased again from16-weeks old calli to obtain the highest yield at the age of 20 –weeks old (2.5 %). So, the yield of essential oil obtained by extraction of calli of *P. anisum* on the basis of callus dry weight (v/w), was approximately between (2.2- 2.5%) and that exceeds the yield mentioned in European Pharmacopoeia 2007(Ph Eur. 2007) for the wild plant (not less than 2%).

From the previously mentioned data, we can deduce that plant tissue culture provided us with overproducer cell lines for both *O. basilicum* and *P. anisum* even for the lowest yield. So, plant tissue culture may be a promising field to obtain larger quantities of rarely produced secondary metabolites.

The developed analytical system made it possible to identify the major and minor phytochemical components of the extracted essential oils as well as their related analogues clearly. Results of GC/MS for essential oils extracted from calli of *O. basilicum* and *P. anisum* are shown in [Figures 13-33] and [TablesVIII and X]. The components of the oils were identified by matching of their mass spectral fragmentation patterns with those reported in computerized MS-data bank spectral libraries and earlier publications. However, the identification using mass spectral library alone is not always possible since some structural molecular fragment cannot be distinguished solely on the basis of MS data. Therefore, to increase the reliability of the analytical results, it is necessary to utilize both MS data and retention indices identities as identification criteria [138].

From quantification data shown in [Tables VIII and X] we can deduce that thirteen compounds were identified in essential oil of *O. basilicum*, and the major components were linalool (47.6%), estragole (11.9%), methyleugenol (11.165%) and1,8-cineol (5.25%). For essential oil extracted from *P. anisum* six compounds are clearly identified with *trans*- anethole as the major component (49.6%), *p*-anisaldhyde (17.2%), anisaldhyde dimethyl acetal (15.5%), *p*- anisyl acetone (7.8%), *p*- anisic acid (2.3%), and *cis*- anethole (1.108%).

Furthermore, 10- methylicosane and anisaldhyde dimethyl acetal were new compounds appeared for the first time in essential oil of *O. basilicum* and *P. anisum*

respectively, and that explains the effect of suspension culture on production of secondary metabolites.