Acknowledgments

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Abstract

The wild species Antirrhinum linkianum shows striking differences with the laboratory line of A. majus 165E in four volatile organic compounds (VOCs), i.e. methyl benzoate, methyl cinnamate, ocimene and acetophenone. Although the scent profiles of both species are complex, the segregation of the four aforementioned VOCs in the F2 population of a recombinant inbred line under construction of Antirrhinum majus x A. linkianum could be explained by mendelian genetics. The current work aims to confirm the identified genetic segregation by use of the F3 population.

A F3 population of 93 plants was analysed for VOC emission. The volatiles were trapped by headspace collection using Gerstel twisters and analysed by GC-MS via thermal desorption. The corresponding profiles were analysed.

Analysis of the GC-MS profiles confirmed the mendelian segregation found in the F2 population. Furthermore at least one plant with a high level of emission in the F2 had daughter plants with very low or no emission in the F3. This confirmed the segregation of a wild type active allele. These results ruled out an environmental effect in the F2 determination and confirmed the identified genetic segregation being correct. Some discrepancies found were presumably caused by allele instability in the gene involved in acetophenone synthesis

Abstract Dutch

De wilde soort Antirrhinum linkianum toont opvallende verschillen met de laboratoriumlijn van A. majus 165E in vier vluchtige organische componenten (VOCs), methylbenzoaat, methylcinnamaat, ocimeen en acetofenon. Hoewel de geurprofielen van beide soorten complex zijn kan de segregatie van de vier eerder genoemde VOCs in de F2 populatie van een recombinante inteeltlijn in aanbouw van Antirrhinum majus x A. Linkianum, verklaard worden door de Mendeliaanse genetica. Het doel van dit werk was om door het gebruik van de F3 populatie te controleren of de geïdentificeerde genetische segregatie correct was.

Een F3 populatie van 93 planten werd geanalyseerd op VOCs-emissies. De vluchtige stoffen werden gevangen door headspace collectie met behulp van Gerstel twisters en geanalyseerd met GC-MS via thermische desorptie.

Analyse van GC-MS profielen bevestigde de gevonden mendeliaanse segregatie in de F2 populatie . Bovendien had minstens één plant met een hoog emissieniveau in de F2 dochterplanten met zeer lage of geen emissie in de F3 populatie. Dit bevestigt de segregatie van een wild-type actief allel. Deze resultaten sluiten een milieueffect in de F2 uit en tonen aan dat de geïdentificeerde genetische segregatie juist was. Enkele waargenomen onjuistheden werden hoogstwaarschijnlijk veroorzaakt door allel instabiliteit in het gen dat tussenkomt in de acetofenon synthese.

1 Introduction

Floral scent is a key factor in the interaction between flowers and insects. Insect are attracted by the emitted scent of flowers and provide pollination (Reinhard, Srinivasan, Guez, & Zhang, 2004; Wright, Lutmerding, Dudareva, & Smith, 2005). The emitted scent of the plants can also function as a defence (Wink, 2003).There is a large diversity of floral scent in quantity and identity (Knudsen, 2006) causing a difference in attraction of insects (Wright et al., 2005). Difference in quantity can also be found in a single genus of plants and reflects in a variety of phenotypes.

A science team of the Escuela Técnica Superior de Ingeniería Agronómica (ETSIA) of the Universidad Politecnica de Cartagena (UPCT) analysed the phenotypic space of the Antirrhinum genus. The Antirrhinum genus comprises about 25 species having its centre of genetic diversification in the Iberian Peninsula. The research team revealed that all species had a different scent profile. A phylogenetic analysis of the scent profiles uncovered that the scent profiles correspond to the molecular phylogenetic structure of the genus (Wilson & Hudson, 2011). In a sub research a recombinant line of Antirrhinum majus and A. linkianum was created as they show contrasting emission of several volatile compounds (VOCs). The major differences are found in four volatile compounds, methyl benzoate, methyl cinnamate, ocimene and acetophenone. The F2 population of the line confirmed that the emission phenotypes found correspond to simple mendelian segregations of loci involved in synthesis of single compounds (Ruiz Hernández, Egea Cortines, & Weiss, n.d.). Prof. Dr. Marcos Egea Gutiérrez-Cortines is the coordinator of the research and also the external promoter for the master's thesis.

1.1 Problem statement

The determination of the phylogenetic structure of the genus by scent analysis is only possible if the molecular functions of the genes are known and a well-developed map is available (Stubbe, 1966). Accordingly, confirming the accuracy of the molecular functions of the genes is necessary for the collaborating research and all the further research which is based on described pathways in this master's thesis.

From an economic point of view, understanding these pathways can provide flowers with a better insect attraction ability, for agronomical use, or a better scent bouquet, for ornamental crops (Pichersky & Dudareva, 2007). The aroma and flavour of all foods consumed directly depend on the volatiles present. It is for these reasons that the study of the aromas is a field with important surveys.

The effects of the mutations on the pathways can contribute to the knowledge of the pathways. The production of acetophenone is not yet described and the study on the mutation in the gene of this volatile will contribute to elucidate a new step. The study of methyl benzoate, ocimene and cinnamate will confirm the already known conversion steps as no loss of function alleles have been obtained yet (N Dudareva et al., 2003)(Natalia Dudareva et al., 2000; Jeremy Kapteyn et al., 2007).

The above mentioned study of the volatiles should be preceded by a study of the mendelian segregation. It is important to know how many genes there are involved in the synthesis of the different volatiles.

1.2 Objective

The aim of the present project is to analyse the levels of emission of the volatiles in plants from the F3 population comprising progeny from the F2 population. If the decrease in volatiles concentration is caused by mutations the mutations should segregate by mendelian laws. Thus the decrease in concentration of the volatiles will be seen in a number of plants, according to specified ratios. The ratios in which the decrease of volatile is expressed resemble the number of alleles that are interacting. The ratios can differ from the mendelian ratios due to epistasis (Cordell, 2002) and fixed homozygosis. The master's thesis will also concentrate on the putative homozygotes occurring in the F2 population and confirm their homozygosis in the F3 population.

2 Literature review

This literature review provides information for a better understanding of the master's thesis. Firstly the Antirrhinum species is described. Secondly the pathway of the aforementioned VOCs is discussed. Finally the basics of genetics and mendelian segregation are explained.

2.1 Antirrhinum species

The Antirrhinum genus (snapdragon) consists of 25 species with their natural habitat located in the Mediterranean region, South East of Europe and the North of Africa (Vargas, Carrió, Guzmán, Amat, & Güemes, 2009) (see figure 1). Antirrhinum is cultivated as an ornamental crop for centuries and is wide spread. In the colder regions of Europe, Antirrhinum is used as an annual plant as it does not survive the cold conditions.

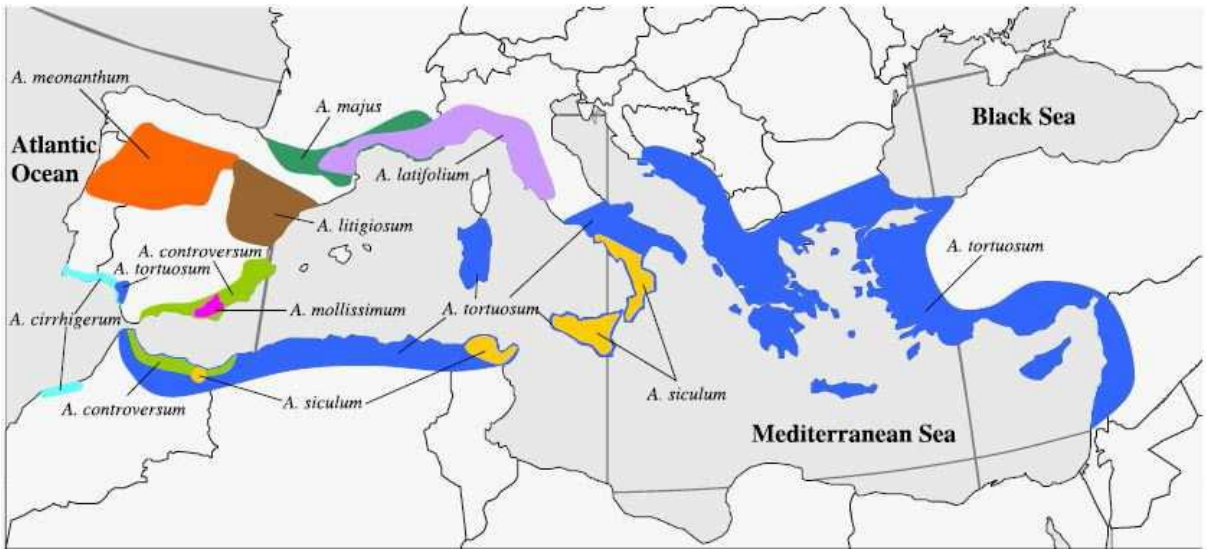

Figure 1 Distribution of the Antirrhinum species across Europe and the North of Africa. (Vargas et al., 2009)

The colour of the flowers include both pastel shades and bright colours, as some bicolours, in shades of white, yellow, red, pink, orange, peach and purple. The glossy dark green leaves are lance shaped. The plants are divided into three groups based upon height: dwarf plants (20-30 cm), intermediate plants (30-60 cm) and tall plants (60 – 90 cm). Figure 2 shows the difference in height between a dwarf plant and a tall Antirrhinum plant.

Figure 2 The difference in height between a dwarf Antirrhinum plant and a tall Antirrhinum plant

The Antirrhinum majus subspecies, also called " the common snapdragon" is frequently used for biological and developmental studies (Yama & Aum, 2004). It emerged as a model organism for the first studies of plants (Darwin, 1868). This is due to its diploid inheritance, simple operation in culture and variability in morphology and colour of flowers. Over time, these features have led to the formation of a considerable research infrastructure environment.

For this study a recombinant line of Antirrhinum majus E165 x A. linkianum as parental lines was used, which shows drastic differences in the production of four major VOCs, methyl benzoate, methyl cinnamate, ocimene and acetophenone. The E165 stands for a mutation of the original A. majus. The mutant is not affecting the scent production whereas A. majus E165 can be seen as a wild type. Previous research shows a relative high emission of ocimene, acetophenone and methyl benzoate of A. majus compared to A. linkianum. Contrariwise the A. linkianum produces a relative high amount of methyl cinnamate compared to A. majus. The comparison is based on the average of data obtained by multiple measurements (see table 1). (Weiss et al., unpublished results).

Table 1 Comparison of emitted volatiles for A. majus and A. linkianum

	E165 A. majus ($\mu g\ g_{fw\ (fresh\ weight)}^{-1}\ 24h^{-1}$)	A. linkianum ($\mu g\ g_{fw}^{-1}\ 24h^{-1}$)
ocimene	13,97	10,89
acetophenone	33,52	0,00
methyl benzoate	19,55	0,04
methyl cinnamate	0,31	2,28

2.2 Scent production

Snapdragon relies on insects for his pollination. Insects are attracted by the emitted scent of the flowers (Reinhard et al., 2004)(Wright et al., 2005). All the pathways of scent production and their regulation in snapdragon are not yet fully discovered and understood. Understanding these pathways can provide flowers with a better insect attraction ability, for agronomical use, or a better scent bouquet, for ornamental crops (Pichersky & Dudareva, 2007). The master's thesis is one of the first steps in the process of the confirmation and discovery of steps in the scent production pathways.

The below discussed pathways were established by multiple studies in which the enzymes were extracted and their function confirmed in vitro. The behaviour of the enzymes in vitro can differ from the in vivo processes. As previously mentioned, this master's thesis is the first step in the confirmation of the described pathways. The confirmation is done by studying the in vivo changes, caused by mutations. This study will use the described pathways as starting points.

Methyl benzoate and acetophenone are produced in the benzenoid pathway which is previously described for Petunia (Boatright et al., 2011). However SAMT, the enzyme which converts benzoic acid to methyl benzoate, described for the petunia pathway, is absent in snapdragon. The lack of this enzyme is an important factor to consider during the research for the production of methyl benzoate in snapdragon (Negre, Kolosova, Knoll, & Kish, 2002). The production of the volatiles methyl benzoate and ocimene, a monoterpene, are previously described (Natalia Dudareva et al., 2000 and 2003). The phenylpropanoids pathway and the specific conversion step to methyl cinnamate is recently described (Vogt, 2010)(Jeremy Kapteyn et al., 2007).

The scent production responds to a diurnal emission with the maximum emission during the day (Kolosova, Gorenstein, Kish, & Dudareva, 2001). This is related to the type of pollinators, day or night, characteristic for each plant (Klahre et al., 2011). The emission starts after anthesis and raises the first 4 days. The emission is at the utmost between day 4 and 6, thereafter it declines (Weiss et al., un published results). The regulation of the scent emission defined the sampling method. The scent had to be captured at the same period of time during the day, using flowers of the same age.

The secretion process of the VOCs is not yet fully understood. The lipophilic propriety probably contributes to the secretion mechanism. The four volatiles are lipophilic and so insoluble in water (Caissard, Joly, & Bergougnoux, 2004).

2.3 Genetics

This master's thesis needs to confirm the mendelian segregation of the emission phenotypes. The confirmation is necessary to exclude that environmental factors rather than mutations are responsible for the change in phenotypes. (Lobo, 2008). This master's theses wills determine at the same time how many different genes are mutated and which plants are homozygotes.

2.3.1 Mendelian segregation

Mendelian segregating alleles behave as recessive or dominant. The dominant alleles will show a phenotype. The recessive alleles will only manifest their phenotype as homozygotes (Chial, 2008). However the appearance of a mutation is not always expressed as simple as dominance and recessive. The heterozygote plants can differ from the dominant homozygote plant. The possible forms are partial-, co- and over-dominance. (Miko, 2008).

2.3.2 Epistasis

The term 'epistatic' was first used in 1909 by Bateson to describe a masking effect whereby a variant or allele at one locus prevents the variant at another locus from manifesting its effect. It can be easily explained as dominant and recessive interactions between two genes that have different loci. The presence of epistasis, the interaction between genes, is a concern because the effect of a locus is altered or masked by the effect of another locus. Thus the detection capability of the first locus will be reduced by the joint effects, and the two loci will be hidden by their interaction. If there are more than two loci involved , the situation is further complicated by the possibility of multiple interactions between different loci (Cordell, 2002).

Epistatic genes behave as mendelian genes for segregation or recombination during sexual reproduction. Their presence is detected by changes in the standard segregation ratios. In the case of crossing two independent genes, according to Mendel's third law, the expected phenotype ratio would be 9:3:3:1. In the case of epistatic genes, the ratios found are variable, such as: 9:4:3 ; 9:7 ; 12:4; 12:3:1 ; 9:6:1 or 15:1 (Phillips, 1998).

Unfortunately, there is no direct correspondence between biological and statistical epistasis models. The deduction of biological results from the statistical results is complicated and is limited, so prior knowledge of the etiology is necessary. That is why molecular research is a fundamental key (Cordell , 2002).

2.3.3 Quantitative genetics

Although many traits can be explained by mendelian genetics, in natural populations many traits show a quantitative segregation with several loci explaining together the complete phenotypic variability. In most cases, due to the different effects of different alleles, the description of major and minor genes depends on the observed quantitative contribution. (Lynch & Walsch, 1998).

Current thinking is that natural variation found in wild species and populations tend to be quantitative in nature. Explanations of phenotypic variability by simple mendelian genetics would require a genetic structure based on single loci, and strong loss of function alleles.

3 Material and methods

3.1 Cultivation and collection of the plants

The homozygous lines A. majus 165E, growing in the United Kingdom (Schwarz-Sommer et al., 2010) and A. majus sbsp linkianum (Linkianum), a Western Iberian endemism growing mainly in dune systems (Rothm, Ártabra, & Amenazado, 2013) are crossed with each other, resulting in F1 plants. Then the flowers are selfed during 2 generations and cultivated using the single seed method giving the F3 population. The single seed method means that only one randomly chosen offspring is cultivated, and it is a standard method to obtain recombinant inbred lines (Eshed & Zamir, 1995; Wisman, Cardon, Fransz, & Saedler, 1998). An exception is made for some parental plants, part of the F2 generation, which are showing interesting scent profiles. The interesting lines are 126, 9, 86 and 13 and multiple descendants are cultivated. For distinguishing the offspring, an extra letter after the number is indicated.(Weiss et al., unpublished results)

The F3 population used in this thesis was developed on the farm Tomás Ferro (La Palma) UPCT. The use of a closed greenhouse excludes a possible pollination by insects. The F3 population consisted of 134 plants in total. A total of 93 samples were taken from plants during the time span of the project between February and the end of May 2014. The flowers were tagged at a stage in which they were still closed, roughly 36 hours before anthesis (see figure 3). The tag was marked with the date and the flowers were collected three days after tagging. The flowers were collected by hand in a way that a small part of the peduncle was included.

Figure 3 Tagged flowers in the closed stage. The tag records the date of tagging that corresponds to 48 hours before anthesis

3.2 Collection of the VOCs

The flowers were weighted before analysis. The headspace scent collection is executed in a small glass desiccator in which a beaker with the flowers is placed. A Gerstel twister, (GerstelGmbH&Co.) a magnetic stir bar of 10 mm length coated with 0.5 mm polydimethylsiloxane that had previously been conditioned, was attached to the beaker using a paperclip (see figure 4). The scent collection was performed in most cases with two flowers from the same plant in one glass beaker. The desiccator remained in a versatile environmental test chamber from Sanyo (MCR-350 H) during 24 hours (see figure 4). The growth chamber had a 12 hour day/night cycle with a temperature of 23°C during the day and 18°C during the night.

Figure 4 Left: flowers in a beaker with a Gerstel twister attached. Right: The beakers placed in desiccators in a growth chamber for scent collection.

3.3 GC-MS analysis

The volatile constituents in the flowers of the plants were separated and qualitatively identified by capillary gas chromatography/mass spectrometry (GC-MS). Scent profiles were resolved on a 6890 gas chromatograph coupled to a 5975 inert XL mass selective detector (Agilent Technologies) equipped with a thermal desorption unit, a cooled injector system (CIS 4) and a multi- purpose sampler (MPS2) (Gerstel GmbH & Co. KG). The GC separation was performed on an HP-5MS UI capillary column (Agilent Technologies), 30 m, length 0.25 mm, internal diameter x 0.25 lm (film) in constant pressure mode. The oven temperature was sequentially increased from 50 to 70°C at 5°C per min, held for 1 min, and thereafter increased to 240°C at 10°C per min, with a holding time of 15 min. The inlet operated in solvent vent mode with a split ratio of 1:50. Chromatographic-grade helium was used as the carrier gas. The stir bar was thermally desorbed in the thermal desorption unit using the following desorption temperature program: initial temperature of 40°C, ramping at 100°C per min until 150°C, and a holding time of 5 min. The transfer temperature was 300°C, working in splitless desorption mode. The volatiles thermally desorbed were cryo-focused in the cooled injector system inlet at -100°C using liquid nitrogen, with a carrier gas flow of 50 ml min^{-1}. After cryo- focusing was completed, the volatiles were transferred into the capillary column by heating the CIS4 inlet at a rate of 10°C sec^{-1} to 150°C (holding time 3 min). Mass spectra were collected in the scan range m/z 30–450. The measurements were performed using an electron bombardment ion source with electron energy of 70 eV. The transfer line, source and quadrupole temperatures were set at 280, 230 and 150°C, respectively. The chromatograms and mass spectra were evaluated using ChemStation software (G1791CA, version D.03.00; Agilent Technologies). Chromatographic peak identification was performed by library matching using the Standard Reference Database Wiley 10th NIST 2011b (National Institute of Standards and Technology). (Manchado-Rojo, Delgado-Benarroch, Roca, Weiss, & Egea-Cortines, 2012)

3.4 Analysis of standards

The concentration of the VOCs was calculated using a calibration curve. Standards from ocimene (Sigma-Aldrich product, code: W353901), acetophenone (Sigma-Aldrich, product code: 42163), methyl benzoate (Sigma-Aldrich, product code: 18344) and methyl cinnamate (Sigma-Aldrich, product code: 96410) were made in methanol as solvent (Panreac, product code: 361091). The concentration of ocimene ranged from 25 to 1250 ppm. The concentration of the other standards ranged from 50 to 2500 ppm. An injection volume of 0.5 µl was used. The standards were directly injected using a split/splitless injector (Agilent Technologies). A calibration curve was made using the statistical processing software of the GC-MS.

3.5 Analysis of samples

First, the results of the samples were automatically integrated using the retention times of the standards. The concentration of the VOCs was calculated using the calibration line.

Secondly, a threshold value for the categorization of the samples into low or high emission producing plants was determined. This threshold value was statistically calculated. A 95% confidence interval was calculated for a random survey of the scent concentration of a 165E A. majus population. The confidence interval corresponds with the phenotypic space of scent emission that takes into account the emission variability. The plants which produced a VOC with a concentration under the lower limit (threshold) of ocimene, acetophenone and methyl benzoate were categorised as homozygote mutants as those three VOCs are high in A. majus. The plants which produced a higher amount than the threshold were categorised as the dominant producing phenotype. Plants producing higher amounts than the upper limit would correspond to transgressive segregation typical for this type of experiments. In this case the upper limit of the methyl benzoate confidence interval was used as the threshold. Due to an insufficient amount of plants a confidence interval for A. linkianum could not be established.

Thirdly, the results of the mendelian segregation were statistically analysed using the RStudio software, version 0.97.336. The segregations were calculated using the chi-square test. The null hypothesis (defined as a specific segregation) was rejected in the event that the P value was less than or equal to 0.05.

Finally the emission of the multiple seed offspring was compared with their parentals of the F2 generation.

4 Results

4.1 Qualification and quantification of the VOCs using GC-MS spectrum and standards

The retention times were revealed with the scent profile of a plant (126B from the F3) with high emission in all VOCs (see figure 5). The qualification was done by a library search (see 3.1.3) in which the MS-spectrums were compared with the library spectrum (see annex). Analysis showed that the peaks of ocimene, methyl benzoate and methyl cinnamate were situated between 7 and 9 minutes. Under the used separation conditions, acetophenone was found with retention times ranging between 12 and 14 minutes. The enlargements (figure 6 and 7) allow a better distinction of the peaks and an exact identification of the retention times.

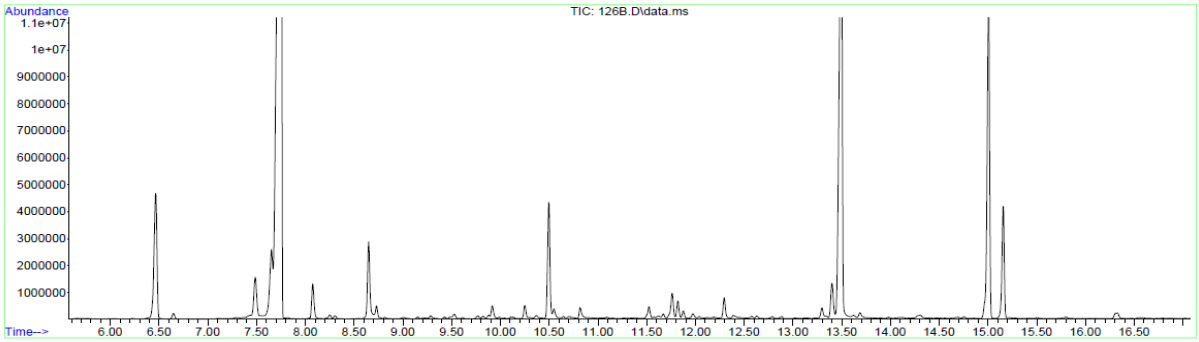

Figure 5 The complete GC spectrum of the emission of line 126B, showing the peaks of the volatiles with the corresponding retention times in minutes.

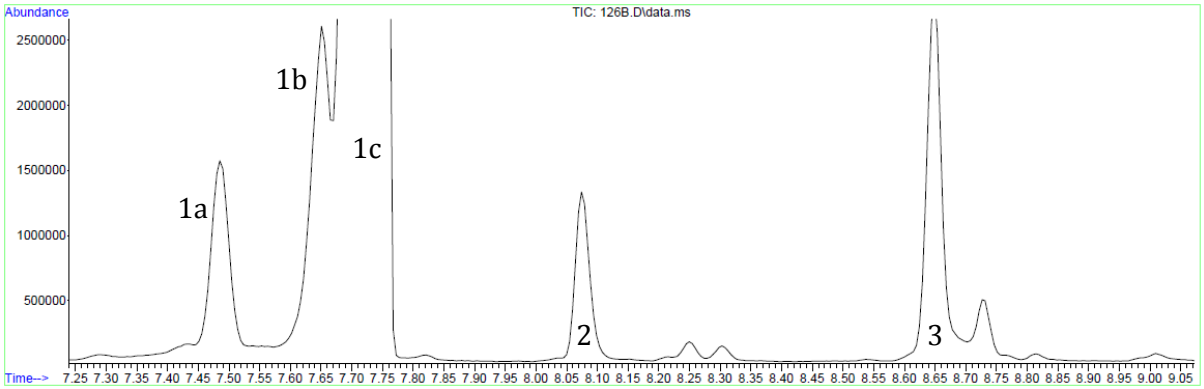

Figure 6 Enlarged interval of line 126B's GC spectrum ranging from 7 to 9 minutes. The peaks numbered by 1, 2 and 3 correspond to ocimene, acetophenone and methyl benzoate respectively.

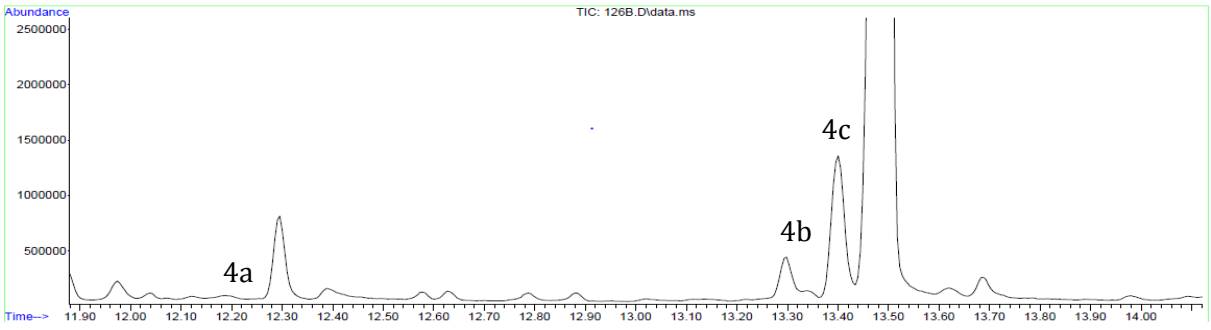

Figure 7 Enlarged interval of line 126B's GC-spectrum ranging from 12 to 14 minutes. The three peaks correspond to methyl cinnamate. 4b and 4c are the same compounds which were separated during analysis due to high concentration.

The peaks numbered by 1, 2 and 3 in figure 6 correspond to ocimene, acetophenone and methyl benzoate respectively. For ocimene multiple peaks were found, which can be expected as ocimene consists of multiple isomers (Zviely & Li, 2013) each having a different retention time. Multiple peaks, numbered by 4, were also found for methyl cinnamate (figure 7). Peak 4b was due to separation of peak 4c caused by too high concentration. The first methyl cinnamate peak (4a) was identified as an isomer. Table 2 shows the retention time of the different peaks.

Table 2 Retention times of the VOC's

Number	Name	Retention time (min)
1	Ocimene	7,485 7,655 7,748
2	Acetophenone	8,079
3	Methyl benzoate	8,649
4	Methyl cinnamate	12,295 13,400 13.,497

4.2 Analysis of the standards and comparison with the sample

The standards were used to confirm the peaks and their corresponding retention times (figure 8) as well as to make the calibration curve. As a constant pressure setup was used in the GC-analysis, the retention times of the standards correspond with those of the samples. For ocimene a mixture of E and Z isomers was used as standard which does not allow a classification of the peaks in the sample regarding the isomeric form. However only the sum of the isomers is interesting and therefore a specific identification of the isomers was not required. The peak at 7.748 min is automatically integrated together with the two other peaks as the integration interval was chosen to comprise the three peaks. The peaks of acetophenone and methyl benzoate correspond nicely with their standard and were integrated automatically. The methyl cinnamate standard was a pure solution of the E isomeric form. The standard displayed a peak with retention time at 13.465 min and corresponds to the sample peak. The peak at 12.295 min could be classified as the Z form and is not included in the automatic integration.

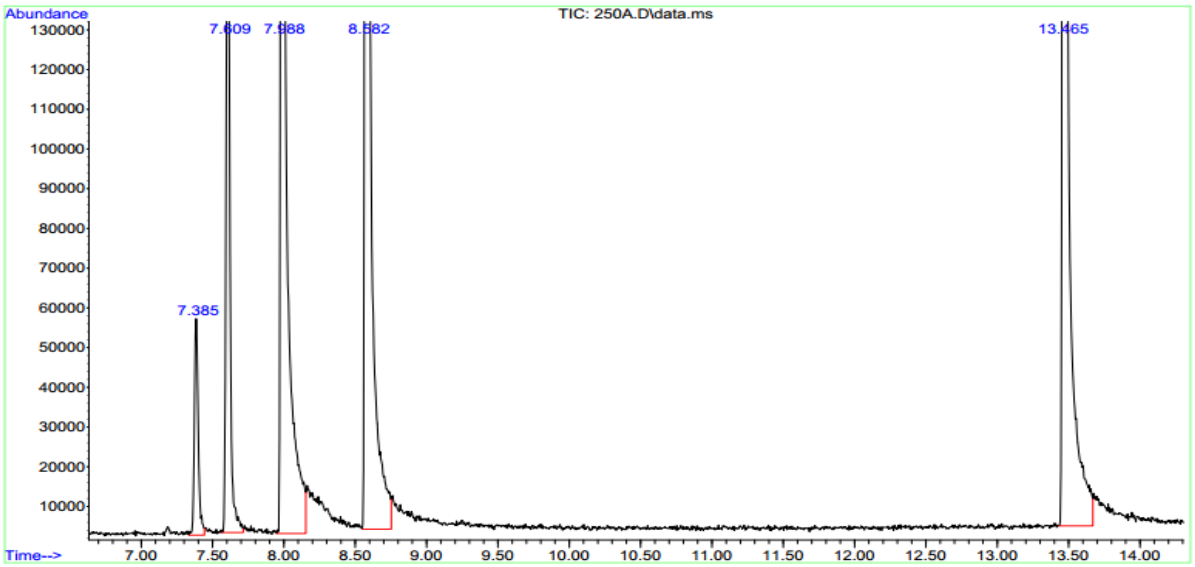

Figure 8 GC spectrum of the VOCs standards; retention time in minutes

Table 3 shows the obtained areas of the standards together with their concentration. A regression analysis was performed with the GC-MS integrated software. The library search report is shown in 7.3.

Table 3 Calibration data obtained by analysis of the standards

Injection concentration	Quantity (µg)	Area first ocimene peak	Area second ocimene peak	
25 ppm	0,0125	12825	36139	
50 ppm	0,025	31735	92224	
125 ppm	0,0625	102704	333595	
250 ppm	0,125	271063	830324	
500 ppm	0,25	708214	1947168	
1250 ppm	0,625	2080089	5072658	
Injection concentration	Quantity (µg)	Area acetophenone	Area methyl benzoate	Area methyl cinnamate
50 ppm	0,0125	50426	159311	122718
100 ppm	0,025	228359	405971	355290
250 ppm	0,0625	914563	1281567	1480800
500 ppm	0,125	2331850	2858321	3712154
1000 ppm	0,25	5182808	6129143	8473693
2500 ppm	0,625	12828367	14546767	21479506

4.3 Statistical determination of the threshold

The confidence interval was calculated with the results of the random survey of line 165E A. majus (see 3.1.5). Table 4 shows the calculated concentrations.

Table 4 Emission of VOCs obtained from a 165E A. majus population which are used to calculate the phenotypic space

Name	Ocimene (µg g_{fw}^{-1} $24h^{-1}$)	Acetophenone (µg g_{fw}^{-1} $24h^{-1}$)	Methyl benzoate (µg g_{fw}^{-1} $24h^{-1}$)	Methyl cinnamate (µg g_{fw}^{-1} $24h^{-1}$)
1	1,29	0,37	0,26	0,04
2	13,49	1,74	3,36	0,08
3	9,18	1,16	1,44	0,06
4	5,95	0,91	1,45	0,05
5	9,43	1,01	0,98	0,05
6	4,84	0,80	1,24	0,04
7	2,11	0,78	0,71	0,06
8	6,28	1,06	0,53	0,06
9	3,45	0,53	0,17	0,05
10	1,18	0,27	0,16	0,03
12	4,22	0,97	1,44	0,03
13	2,08	0,37	0,16	0,04
14	3,23	0,44	0,27	0,04
15	4,03	0,61	0,59	0,03
16	1,53	0,63	1,88	0,04
17	3,43	1,37	2,06	0,05

Table 5 shows the 95% confidence interval which corresponds with the calculated phenotypic space of the 165E A. majus species. The lower limit of the confidence interval of methyl benzoate was negative caused by a big spread of the data. After dismissing the outliers the lower limit remained negative. Therefore an 80% confidence interval for methyl benzoate was calculated. Weiss et al., unpublished results show that 9G is a double mutant genotype which was categorised as a wild type phenotype. This result favoured the suggested smaller confidence interval.

Table 5 95 % confidence range of the phenotypic space of 165E A. majus with the exception of methyl benzoate which is an 80% confidence interval

	Ocimene ($\mu g\ g_{fw}^{-1}\ 24h^{-1}$)	Acetophenone ($\mu g\ g_{fw}^{-1}\ 24h^{-1}$)	Methyl benzoate ($\mu g\ g_{fw}^{-1}\ 24h^{-1}$)	Methyl cinnamate ($\mu g\ g_{fw}^{-1}\ 24h^{-1}$)
upper limit	7,53	1,38	1,72	0,08
lower limit	0,73	0,17	0,10	0,02

The samples were categorized (see annex) using the confidence interval (see 3.1.4).

4.4 Results of scent analysis in the F3 population

The F3 population was grown using the single seed procedure (see 3.1.1). In the F3, segregation with a higher number of homozygotes than the F2 was expected. Table 6 shows the categorised plants and the corresponding mendelian segregation

Table 6 Categorized plants with the corresponding mendelian segregations calculated using a Chi square test. If the P value exceeds 0.05 the suggested mendelian segregation is accepted

	Nº plants producing	Nº plants that do not produce	Chi square test for mendelian segregation	P value
Ocimene	59	34	3:1	0.01004
Acetophenone	88	5	15:1	0.7278
Methyl benzoate	64	29	3:1	0.1685
Methyl cinnamate	85	8	13:3	0.01217

The identified segregations of the four VOCs correspond to those of the F2. Both methyl benzoate and acetophenone adjust to a single and double mutant genetic structure. In contrast, ocimene and methyl cinnamate have more complex segregations that can be explained by the possible protein complex found in methyl cinnamate synthase (J Kapteyn et al., 2007) and the number of ocimene synthase genes present in the Antirrhinum genome (Natalia Dudareva et al., 2003).

4.5 Comparison of descendants in the F3 population and their parental

The F2 population contained plants which were found to produce either large amounts of scent, or to have a major scent compound absent from their profile. Multiple descendants of those plants were cultivated and analysed in the F3 population (see 3.1.1). The differences between the parentals and descendants give more information about the segregation and potential homozygosis.

Table 7 Comparison of descendants in the F3 population and their parentals with mendelian meaning

Name	Previous phenotype	Descendants ratio low/high	mendelian meaning
126	Ocimene high	1/3	Heterozygote segregating
	Acetophenone high	4	Homozygote fixed
	Methyl benzoate high	2/2	Heterozygote segregating
	Methyl cinnamate high	4	Homozygote fixed
9	Acetophenone low	0/7	Possible revertant
	Methyl benzoate low	7/0	Homozygote fixed
86	Methyl cinnamate low	2/2	Heterozygote segregating
13	Acetophenone high	0/4	Homozygote fixed
	Methyl cinnamate high	0/4	Homozygote fixed

5 Conclusion

The statistical approach as categorisation method appears to be functional. Calculation of the phenotypic space of A. linkianum, using this statistical approach contributes to a better categorisation and even reveals heterozygosis if the phenotypic spaces are not overlapping. If the phenotypic spaces are overlapping it can provide a way to diminish the false positive or false negative results. This statistical approach is suggested to be used in further research.

The results confirm the mendelian segregation (3:1) of acetophone and methyl benzoate. The results show also that ocimene and methyl cinnamate have more complex segregations which can be explained by the possible protein complex found in methyl cinnamate synthase (J Kapteyn et al., 2007), and the number of ocimene synthase genes present in the Antirrhinum genome (Natalia Dudareva et al., 2003).

The fact that line 9 shows a parental lacking acetophenone production whereas all descendants produce high levels of this VOC indicates two possible scenarios. One being a cross pollination with an acetophenone producing plant. A second possibility is the existence of an unstable allele involved in acetophenone synthesis. As the F3 plants from line 9 did not produce methyl benzoate a cross pollination can be ruled out and an unstable allele of acetophenone synthase in the genetic background seems to be the cause.

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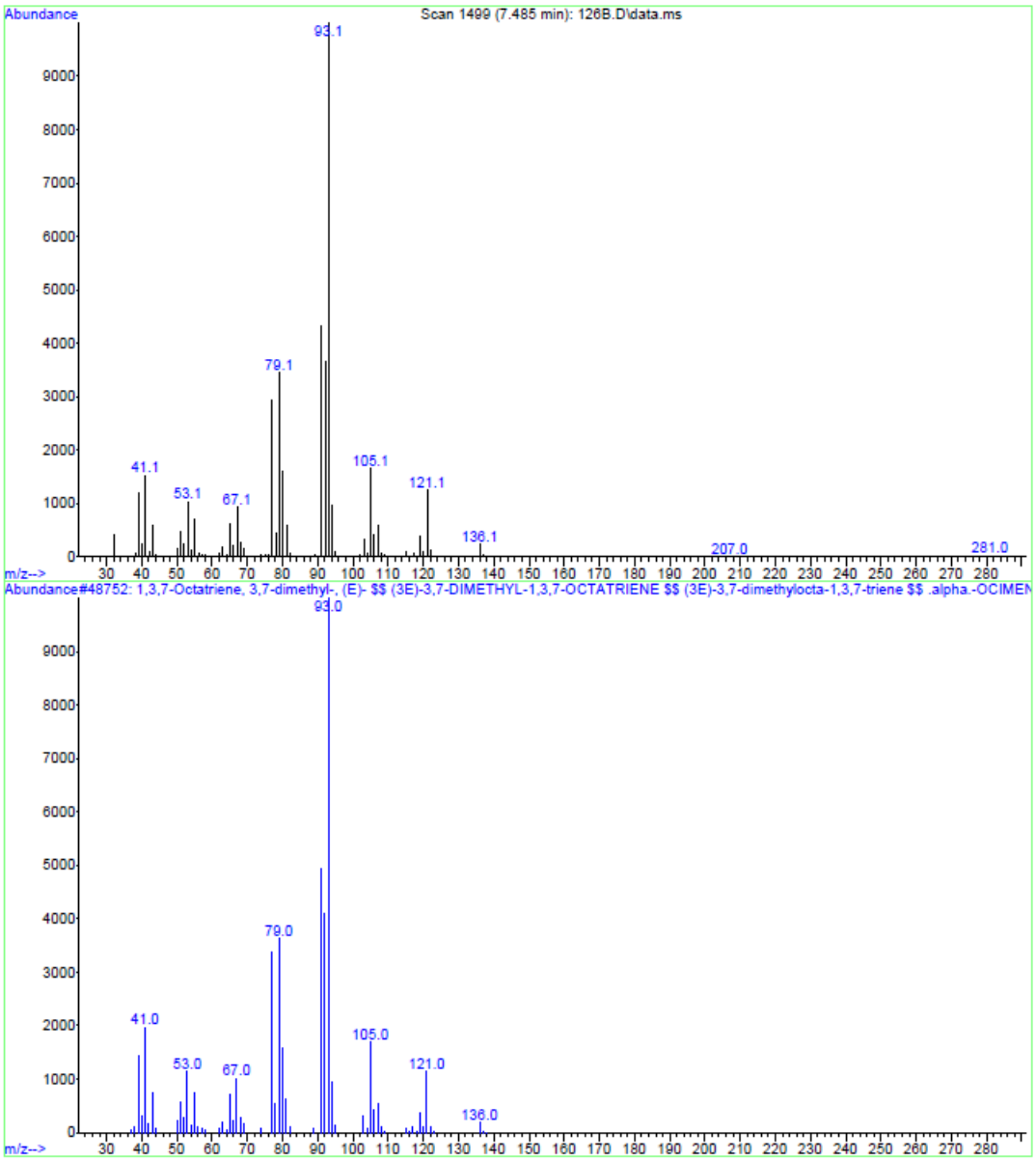
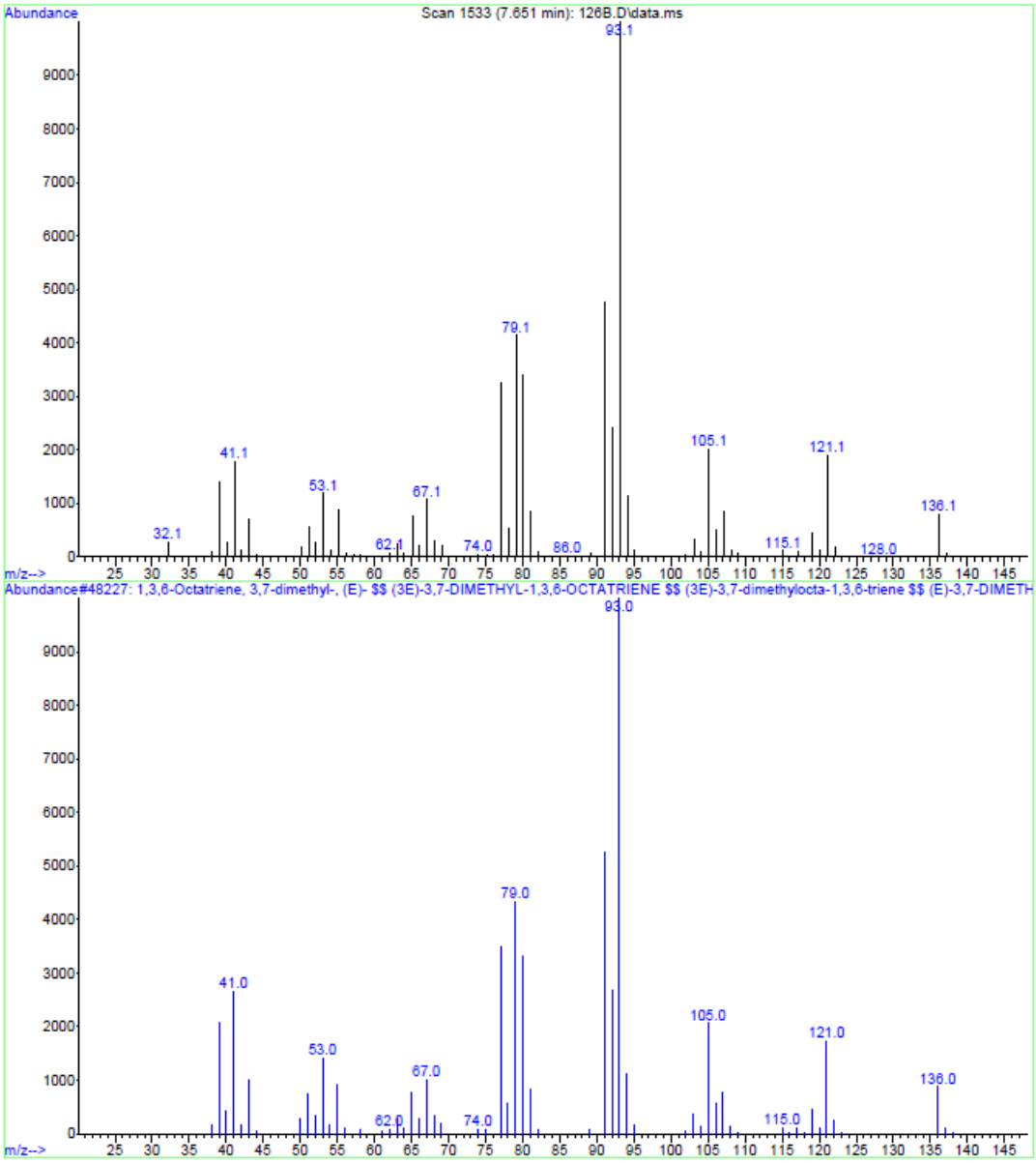
7 Annex

7.1 Comparison between MS-spectrum of line 126B and library

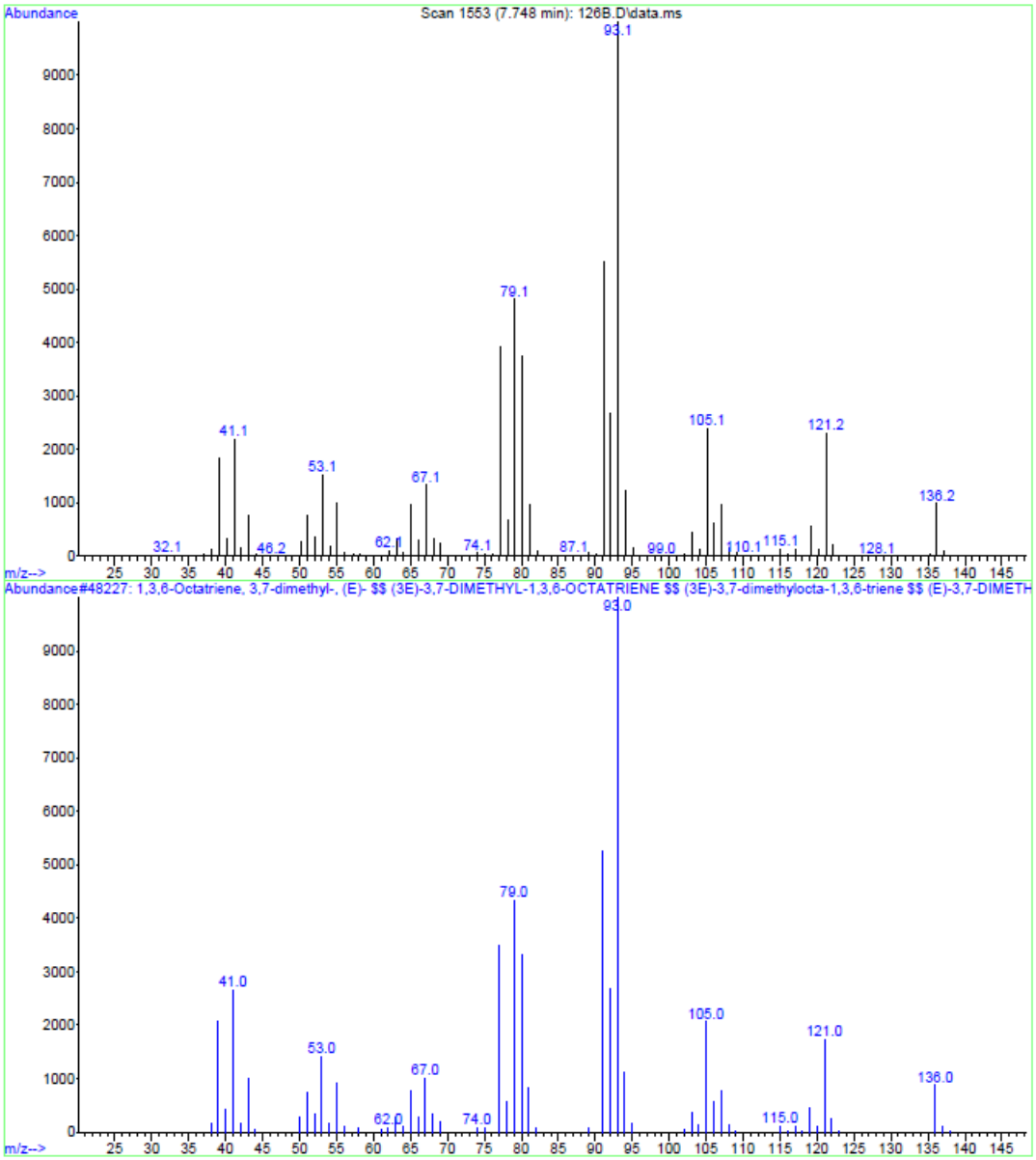
7.1.1 First ocimene peak



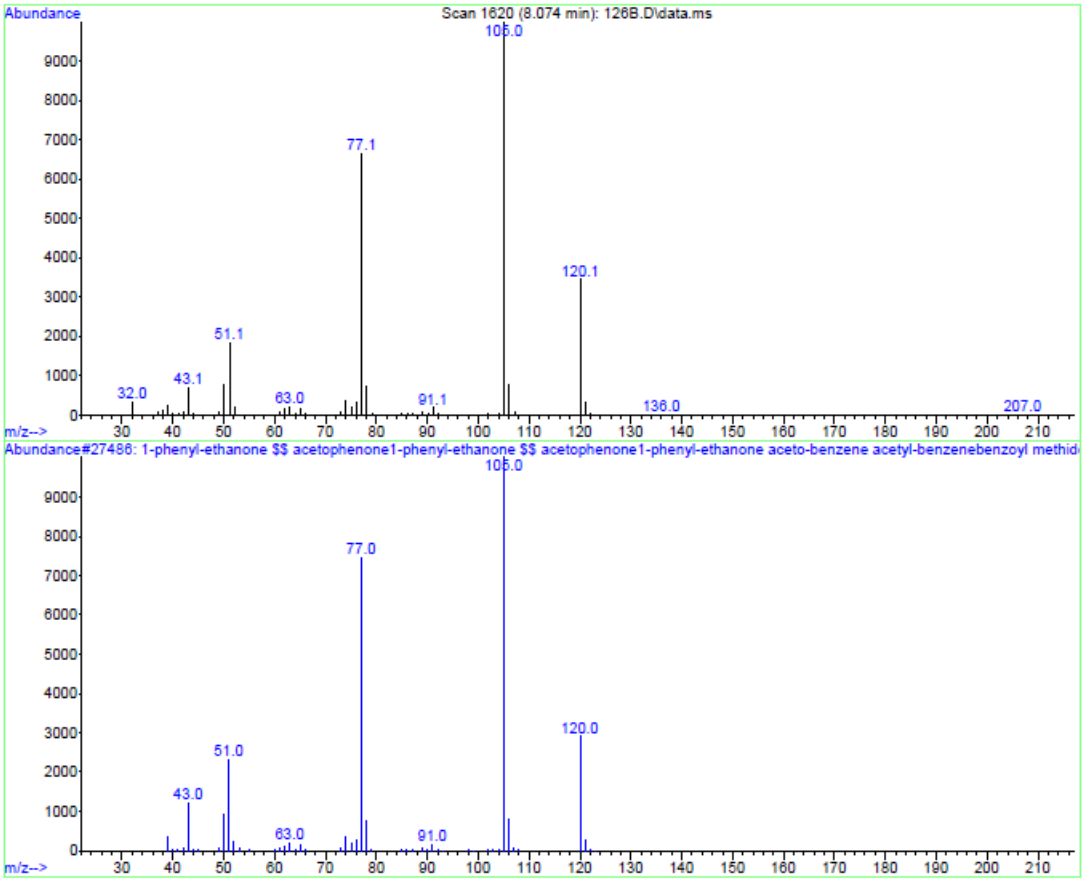
7.1.2 Second ocimene peak



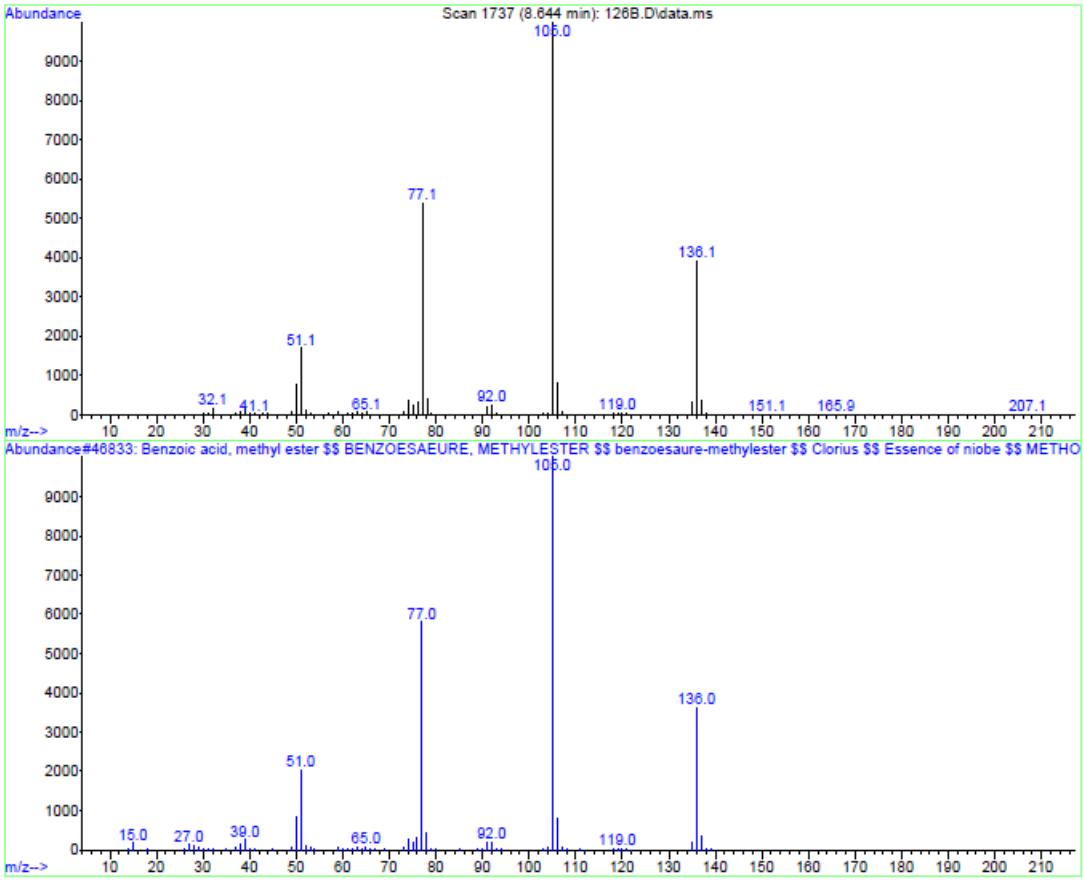
7.1.3 Third ocimene peak



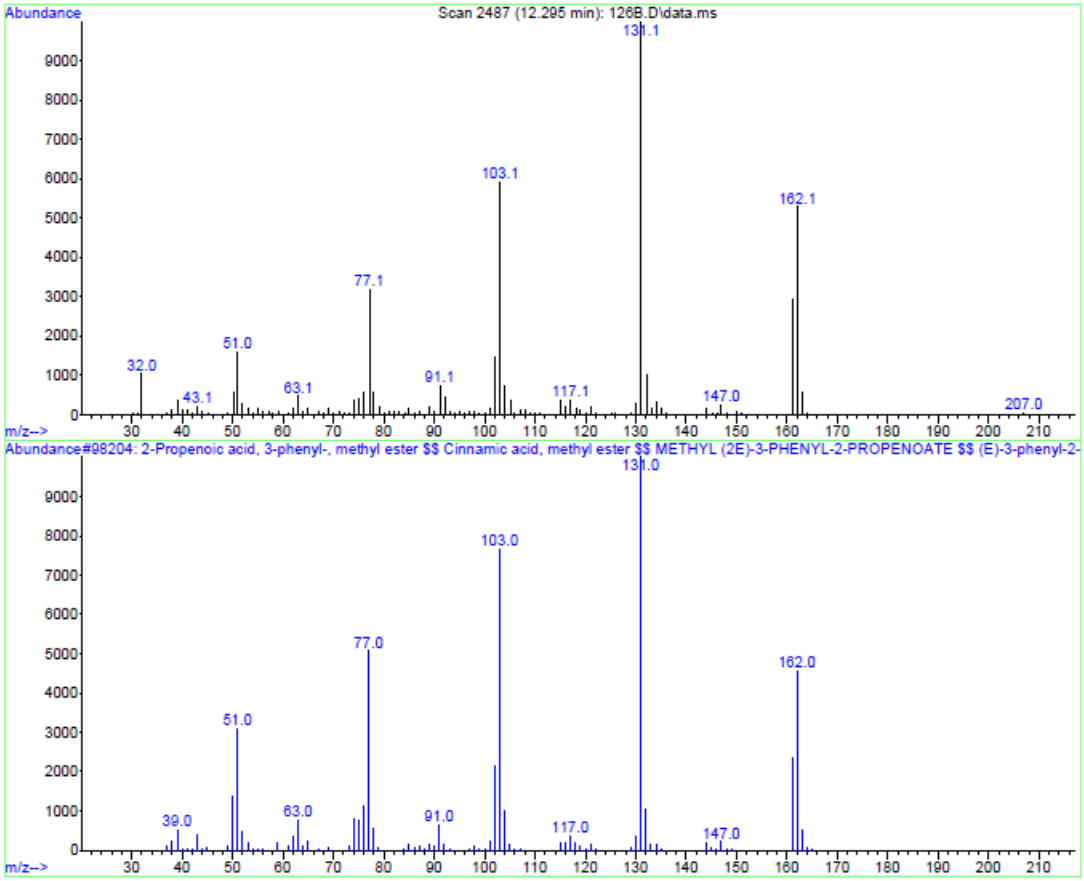
7.1.4 Acetophenone peak



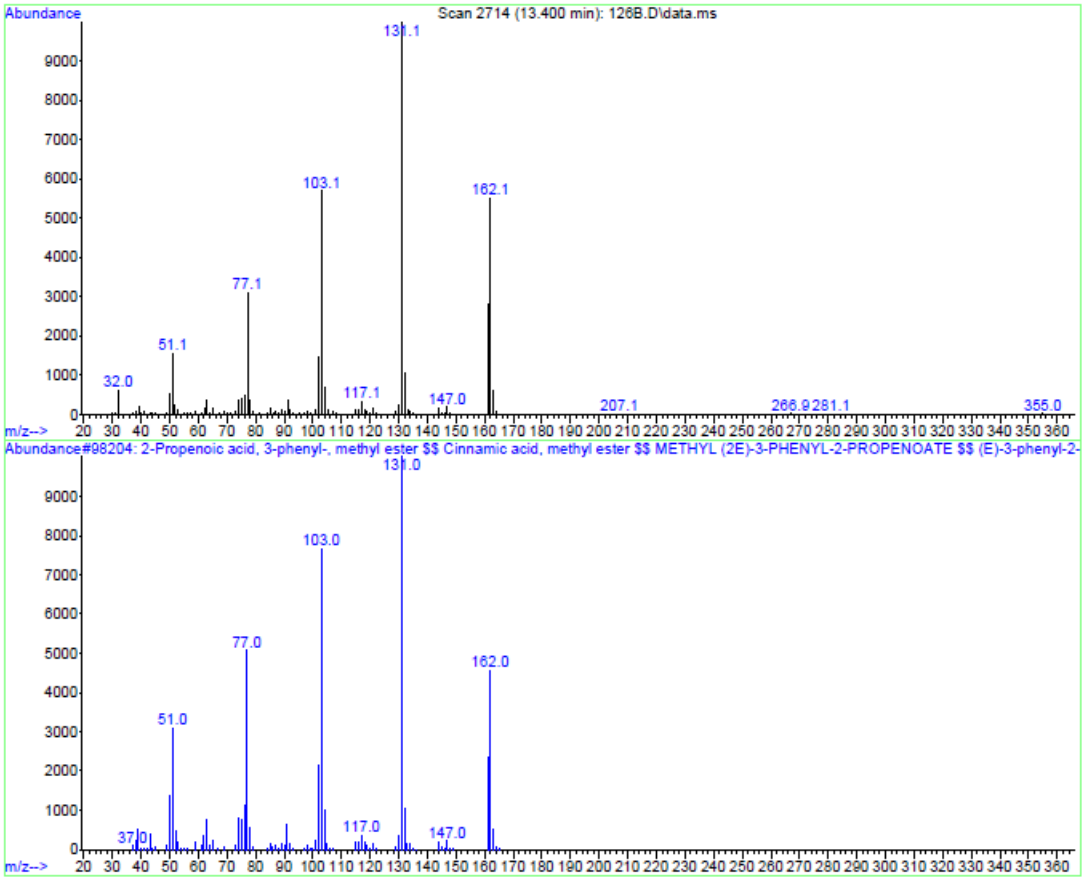
7.1.5 Methyl benzoate peak



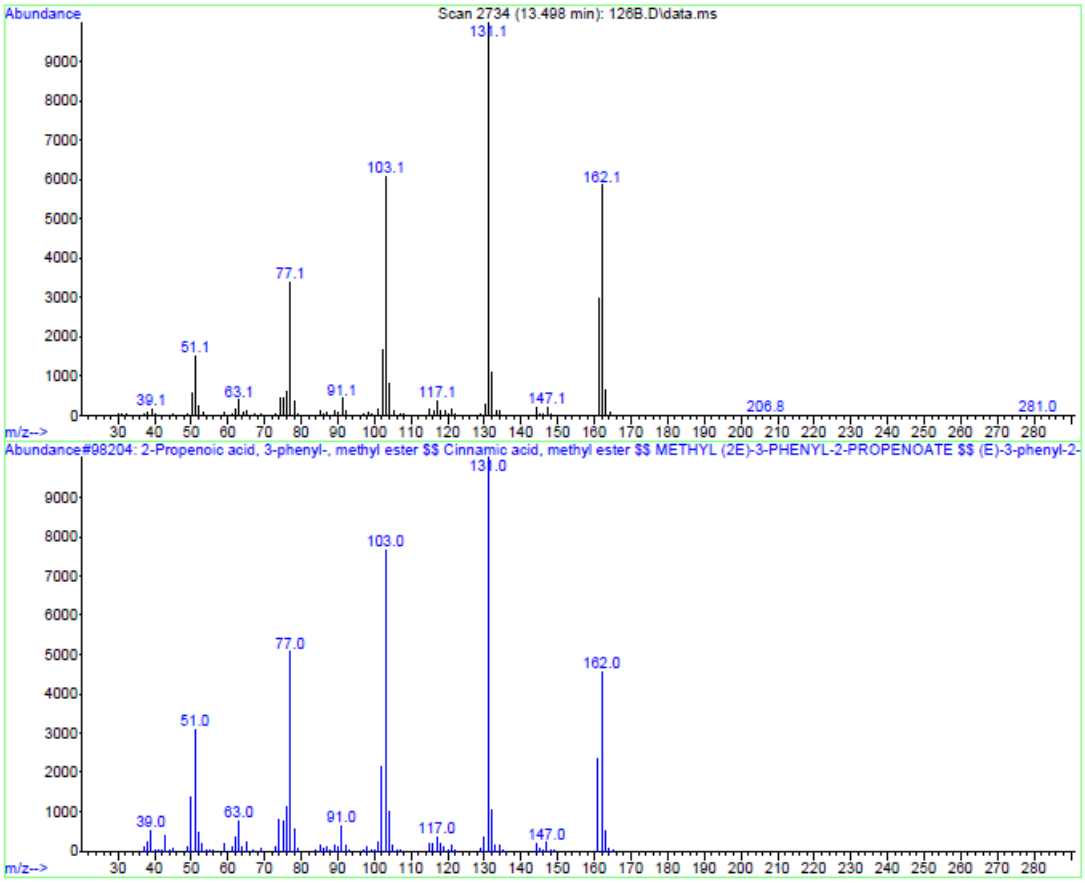
7.1.6 First methyl cinnamate peak



7.1.7 Second methyl cinnamate peak



7.1.8 Third methyl cinnamate peak



7.2 Library search report of the standard 250a

RT (min)	Hit Name	Quality	CAS Number
7,385	1,3,7-Octatriene, 3,7-dimethyl-, (E)- $$ (3E)-3,7-DIMETHYL-1,3,7-OCTATRIENE $$ (3E)-3,7-dimethylocta-1,3,7-triene $$ .alpha.-OCIMENE $$ 1,3,7-OCTATRIENE, 3,7-DIMETHYL- $$ 2,6-DIMETHYL-1,5,7-OCTATRIENE $$ 3,7-DIMETHYL-1,3,7-OCTATRIENE $$ CIS-OCIMENE	97	006874-10-8
	1,3,6-Octatriene, 3,7-dimethyl-, (E)- $$ (3E)-3,7-DIMETHYL-1,3,6-OCTATRIENE $$ (3E)-3,7-dimethylocta-1,3,6-triene $$ (E)-3,7-DIMETHYLOCTA-1,3,6-TRIENE $$ (E)-Ocimene $$ - $$ .beta. OCIMENE Y $$ .beta.-(E)-OCIMENE $$ .beta.-CIS-OCIMENE $$ OCIMENE-X	97	003779-61-1
	trans-.beta.-Ocimene $$ 1,3,6-Octatriene, 3,7-dimethyl-, (E)- $$ .beta.-trans-Ocimene $$ trans-3,7-Dimethyl-1,3,6-Octatriene $$ Ocimene, trans-.beta.- $$ (E)-Ocimene $$ trans-Ocimene $$ (3E)-3,7-Dimethyl-1,3,6-octatriene $$ .beta.-(E)-Ocimene	96	003779-61-1
	trans-.beta.-Ocimene $$ 1,3,6-Octatriene, 3,7-dimethyl-, (E)- $$ .beta.-trans-Ocimene $$ trans-3,7-Dimethyl-1,3,6-Octatriene $$ Ocimene, trans-.beta.- $$ (E)-Ocimene $$ trans-Ocimene $$ (3E)-3,7-Dimethyl-1,3,6-octatriene $$ .beta.-(E)-Ocimene	95	003779-61-1

7,609	1,3,6-Octatriene, 3,7-dimethyl-, (E)- $$ (3E)-3,7-DIMETHYL-1,3,6-OCTATRIENE $$ (3E)-3,7-dimethylocta-1,3,6-triene $$ (E)-3,7-DIMETHYLOCTA-1,3,6-TRIENE $$ (E)-Ocimene $$ - $$ .beta. OCIMENE Y $$ .beta.-(E)-OCIMENE $$ .beta.-CIS-OCIMENE $$ OCIMENE-X	98	003779-61-1
	1,3,6-Octatriene, 3,7-dimethyl-, (Z)- $$ .beta.-cis-Ocimene $$ cis-.beta.-Ocimene $$ cis-3,7-Dimethyl-1,3,6-octatriene $$ Ocimene, cis-.beta.- $$ (Z)-Ocimene $$ cis-Ocimene $$ (3Z)-3,7-Dimethyl-1,3,6-octatriene $$ .beta.-Ocimene, (Z)-	97	003338-55-4
	.beta.-Ocimene $$ 1,3,6-Octatriene, 3,7-dimethyl- $$ Ocimene $$ 3,7-Dimethyl-1,3,6-octatriene $$ beta-Ocimene	97	013877-91-3
	1,3,7-Octatriene, 3,7-dimethyl- $$ 2,6-Dimethyl-1,5,7-octatriene $$ 3,7-Dimethyl-1,3,7-octatriene # $$ .alpha.-Ocimene $$ 3,7-Dimethylocta-1,3,7-triene $$ alpha-Ocimene	96	000502-99-8
7,988	1-phenyl-ethanone $$ acetophenone1-phenyl-ethanone $$ acetophenone1-phenyl-ethanone aceto-benzene acetyl-benzenebenzoyl methide hypnonemethyl phenyl ketone $$ Ethanone, 1-phenyl- $$ .alpha.-Acetophenone $$ 1-Phenyl-1-ethanone $$ aceto-benzene	95	000098-86-2
	1-phenyl-ethanone $$ acetophenone1-phenyl-ethanone $$ acetophenone1-phenyl-ethanone aceto-benzene acetyl-benzenebenzoyl methide hypnonemethyl phenyl ketone $$ Ethanone, 1-phenyl- $$ .alpha.-Acetophenone $$ 1-Phenyl-1-ethanone $$ aceto-benzene	94	000098-86-2
	1-phenyl-ethanone $$ acetophenone1-phenyl-ethanone $$ acetophenone1-phenyl-ethanone aceto-benzene acetyl-benzenebenzoyl methide hypnonemethyl phenyl ketone $$ Ethanone, 1-phenyl- $$ .alpha.-Acetophenone $$ 1-Phenyl-1-ethanone $$ aceto-benzene	93	000098-86-2
	1-phenyl-ethanone $$ acetophenone1-phenyl-ethanone $$ acetophenone1-phenyl-ethanone aceto-benzene acetyl-benzenebenzoyl methide hypnonemethyl phenyl ketone $$ Ethanone, 1-phenyl- $$ .alpha.-Acetophenone $$ 1-Phenyl-1-ethanone $$ aceto-benzene	91	000098-86-2
8,582	Benzoic acid, methyl ester $$ BENZOESAEURE, METHYLESTER $$ benzoesaure-methylester $$ Clorius $$ Essence of niobe $$ METHOXYCARBONYL-BENZENE $$ Methyl benzenecarboxylate $$ Methyl benzoate $$ Methyl ester of benzoic acid $$ methyl phenylcarboxylate	95	000093-58-3
	Benzoic acid, methyl ester $$ Methyl benzoate $$ Clorius $$ Methyl benzenecarboxylate $$ Niobe oil $$ Oil of Niobe $$ Essence of niobe $$ Methylester kyseliny benzoove $$ Oxidate le $$ UN 2938 $$ Methyl ester of benzoic acid $$ Oniobe oil	95	000093-58-3

	Benzoic acid, methyl ester $$ BENZOESAEURE, METHYLESTER $$ benzoesaure-methylester $$ Clorius $$ Essence of niobe $$ METHOXYCARBONYL-BENZENE $$ Methyl benzenecarboxylate $$ Methyl benzoate $$ Methyl ester of benzoic acid $$ methyl phenylcarboxylate	95	000093-58-3
	Benzoic acid, methyl ester $$ Methyl benzoate $$ Clorius $$ Methyl benzenecarboxylate $$ Niobe oil $$ Oil of Niobe $$ Essence of niobe $$ Methylester kyseliny benzoove $$ Oxidate le $$ UN 2938 $$ Methyl ester of benzoic acid $$ Oniobe oil	94	000093-58-3
13,465	2-Propenoic acid, 3-phenyl-, methyl ester $$ Cinnamic acid, methyl ester $$ METHYL (2E)-3-PHENYL-2-PROPENOATE $$ (E)-3-phenyl-2-propenoic acid, methyl ester $$ (E)-3-phenylacrylic acid methyl ester $$ (E)-Methyl cinnamate $$ MC $$ methyl cinnamate	97	000103-26-4
	(E)-3-phenyl-2-propenoic acid, methyl ester $$ 2-Propenoic acid, 3-phenyl-, methyl ester, (E)- $$ Cinnamic acid, methyl ester, (E)- $$ METHYL (2E)-3-PHENYL-2-PROPENOATE $$ (E)-3-phenylacrylic acid methyl ester $$ (E)-Methyl cinnamate $$ MC	95	001754-62-7
	2-Propenoic acid, 3-phenyl-, methyl ester $$ Cinnamic acid, methyl ester $$ METHYL (2E)-3-PHENYL-2-PROPENOATE $$ (E)-3-phenyl-2-propenoic acid, methyl ester $$ (E)-3-phenylacrylic acid methyl ester $$ (E)-Methyl cinnamate $$ MC $$ methyl cinnamate	95	000103-26-4
	2-Propenoic acid, 3-phenyl-, methyl ester $$ Cinnamic acid, methyl ester $$ Methyl cinnamate $$ Methyl cinnamylate $$ Methyl 3-phenylpropenoate $$ Methyl 3-phenyl-2-propenoate $$ Methyl 3-phenylacrylate $$ Methyl ester of cinnamic acid $$ NSC 9411	95	000103-26-4

7.3 Categorised samples

Ocimene ($\mu g\ g_{fw}^{-1}\ 24h^{-1}$)		Acetophenone ($\mu g\ g_{fw}^{-1}\ 24h^{-1}$)		methyl benzoate ($\mu g\ g_{fw}^{-1}\ 24h^{-1}$)		methyl cinnamate ($\mu g\ g_{fw}^{-1}\ 24h^{-1}$)	
34C	0,10	34C	0,05	35A	0,00	23	0,03
13D	0,13	96	0,10	126A	0,00	86C	0,04
80	0,21	30	0,10	F	0,00	43B	0,05
9A	0,22	105	0,13	9A	0,01	112A	0,05
49	0,23	1	0,14	13D	0,01	80	0,06
F	0,23	Lower limit	0,17	34C	0,01	115	0,06
43B	0,23	9A	0,16	9F	0,02	86A	0,07
77	0,27	112	0,18	9D	0,02	111	0,07
34	0,28	80	0,18	122	0,02	upper limit	0,08
34A	0,31	32B	0,18	9B	0,03	131	0,08
1	0,32	9C	0,19	5	0,03	109	0,09
113	0,33	3	0,19	9C	0,03	43D	0,10
135	0,34	86	0,21	38A	0,03	1	0,10
135	0,41	80	0,25	1	0,03	9A	0,10
89	0,42	39	0,26	34	0,04	62	0,11
35A	0,42	77	0,27	135B	0,04	126C	0,14
141	0,44	43B	0,31	75A	0,05	86	0,15
13D	0,44	62	0,36	129A	0,05	9C	0,16
126C	0,47	13D	0,43	9E	0,05	86 plus R	0,17
13A	0,49	60	0,45	96	0,05	34A	0,19
60	0,51	34	0,47	129B	0,05	135B	0,20
129	0,57	63	0,49	108B	0,06	32A	0,20
112A	0,57	111	0,57	108A	0,06	112B	0,21
75A	0,58	13D	0,61	32A	0,07	38A	0,23
32A	0,58	128	0,62	126D	0,07	112	0,23
13C	0,59	64	0,63	112	0,07	13D	0,24
134	0,60	23	0,81	F	0,08	129A	0,24
109	0,61	89	0,87	34A	0,09	32C	0,25
96	0,61	86	0,99	32B	0,09	U	0,27
30	0,64	34A	1,10	Lower limit	0,10	108B	0,30
105	0,64	86A	1,15	9G	0,11	132	0,30
117	0,68	13C	1,18	126C	0,11	129 A	0,33
35	0,71	U	1,27	87	0,13	F	0,38
62A	0,71	Upper limit	1,38	129	0,14	62A	0,39
Lower limit	0,73	49	1,36	35	0,14	32B	0,43
13A	0,78	112A	1,51	13A	0,15	108A	0,50
78C	0,78	134	1,54	35	0,15	9G	0,52
39	0,80	135	1,66	129 A	0,17	135	0,53
86	0,81	112B	1,74	13C	0,21	77	0,54
13B	0,82	86 plus R	1,81	135	0,24	F	0,57
111	0,82	113	1,90	120	0,26	135	0,61
80	0,83	F	1,95	134	0,27	88	0,62
35	0,89	86C	1,98	49	0,27	35	0,65
112	0,93	126C	2,02	43B	0,28	87	0,66
135D	1,02	120	2,07	13A	0,29	129	0,75

135B	1,04	40	2,10	132	0,30	13C	0,77
126B	1,12	13A	2,32	78C	0,30	128	0,78
120	1,16	43D	2,46	13D	0,31	9B	0,89
129 A	1,29	113	2,46	U	0,31	9E	0,92
126A	1,31	78C	2,51	135D	0,32	9D	0,97
9B	1,32	87	2,60	77	0,34	86	0,98
129B	1,34	9F	2,63	141	0,34	39	0,98
38A	1,37	126D	2,64	13B	0,34	122	1,14
112B	1,40	13A	2,79	126B	0,35	34C	1,15
75	1,42	13B	2,89	112B	0,35	129b	1,16
129b	1,45	F	2,90	125	0,37	113	1,20
131	1,56	135B	2,92	62A	0,38	113	1,21
128	1,58	122	3,00	117	0,38	120	1,23
9C	1,63	135	3,20	88	0,39	75A	1,25
32B	1,71	141	3,26	113	0,41	3	1,36
126B	1,74	129A	3,30	135	0,44	60	1,38
125	1,78	120	3,30	32B	0,46	75	1,39
86C	1,97	35A	3,35	113	0,50	126B	1,59
120	2,21	126B	3,53	120	0,50	63	1,60
87	2,22	126A	3,61	40	0,50	30	1,81
U	2,34	62A	3,74	89	0,57	A	1,84
43D	3,14	75	3,75	32C	0,59	35	1,98
23	3,30	117	3,78	128	0,63	129B	2,02
9F	3,36	35	3,80	86	0,71	9F	2,05
122	3,86	135D	3,88	39	0,80	120	2,06
40	3,92	131	4,00	3	0,81	13D	2,10
113	4,07	9G	4,14	23	0,86	96	2,15
63	4,97	129	4,22	62	0,89	5	2,17
9G	5,34	129 A	4,33	109	0,92	78C	2,19
F	5,62	125	4,36	75	0,94	13A	2,26
3	5,66	115	4,49	126B	0,96	135D	2,26
86A	5,97	108B	4,51	63	0,98	49	2,27
5	6,19	129B	4,61	105	1,02	141	2,32
86	6,34	35	4,74	115	1,02	13A	2,32
86 plus R	7,24	5	4,85	131	1,08	13B	2,43
upper Limit	7,53	108A	5,19	A	1,25	35A	2,81
9E	7,59	32A	5,23	86 plus R	1,36	34	3,09
64	8,08	75A	5,37	64	1,39	32B	3,10
129A	8,31	129b	5,39	86A	1,40	40	3,10
32C	8,74	9E	5,97	80	1,42	126A	3,27
88	8,74	109	6,13	112A	1,49	117	3,53
62	9,77	32C	6,71	86C	1,67	126D	3,75
126D	9,99	88	6,89	Higher limit	1,70	125	3,76
9D	10,15	38A	7,52	86	1,79	64	4,00
A	10,89	9B	7,90	80	1,79	105	4,14
108B	11,29	9D	8,00	111	1,87	89	4,14
132	12,26	A	8,55	43D	2,09	126B	4,81
115	13,38	132	9,68	129b	2,42	134	5,24

108A	14,97	126B	15,96	30	2,47	80	5,40
32B	28,01	32B	22,31	60	2,77		

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Ascertaining the mendelian segregation of four volatile organic compounds produced in flowers of a recombinant inbred line of Antirrhinum majus x Antirrhinum linkianum

Richting: **master in de industriële wetenschappen: biochemie**
Jaar: **2014**

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Datum: **11/06/2014**