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Study of the Chemical Composition of Essential Oils by Gas Chromatography

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1. Introduction

Essential oils are complex mixtures, constituted by terpenoid hydrocarbons, oxygenated terpenes and sesquiterpenes. They originate from the plant secondary metabolism and are responsible for their characteristic aroma.

The various applications of essential oils account for the great interest in their study. Such applications may be found in the cosmetic industry, as ingredients of fragrances, decorative cosmetic, fine fragrances and flavouring, in the food industry, as aromas and flavours, in the pharmaceutical industry, as active components of medicines and as antibacterials/antimicrobials, and in aromatherapy. At present, there are many studies in which they are used as intermediaries in fine chemistry reactions, among other applications.

The most common methods used for the industrial extraction of these oils are steam-distillation, extraction with solvents and expression. Their selection will depend on the characteristics of the material from which the oil will be extracted, since they can be present in different parts of the plant, like the roots, the stem, the leaves, the fruits and/or the seeds.

Once the oils are obtained, the fundamental contribution of the organic chemistry to the industry resides in their characterisation, as their chemical composition may vary even whithin one botanical species. These variations might be due to the presence of different chemotypes, according to the plant adaptation to the surrounding environment, as well as its state of development. We have to take into account the fact that it is the composition of the essential oils what provides their intrinsic properties and economic value.

The development of chromatographic techniques has allowed us to make considerable progress in the study of the chemical composition of essential oil. Gas Chromatography (GC) is, by all means, the best method, due to its simplicity, rapidity and efficiency, for both the identification and quantification of essential oil components and composition variations.

The aim of this chapter is to describe the different applications of GC, starting from the quality control up to the identification and quantification of the chemical components of essential oils from different aromatic species that grow in the northeast of Argentina, which have emerged as a result of years of experience in this topic.

In regards to the applications of GC, different studies will be presented, among which we will discuss the optimization of the operational conditions used to separate the different components, the analysis of the variation in the composition of regional essential oils, the measurement, using internal standards, of oils as well as oil modification by fractional vacuum distillation, and the study of semi-synthesis reactions to obtain high added-value compounds, starting from oils or their components.

2. Essential oils

Essential oils are natural products that plants produce for their own needs other than nutrition (i.e. protection or attraction). In general, they are complex mixtures of organic compounds that give characteristic odour and flavour to the plants. They are mainly made up by monoterpenes and sesquiterpenes whose main metabolic pathway is through mevalonate leading to sesquiterpenes and from methyl-erythritol leading to monoterpenes. They are located in different parts of the plant. They can be found in the root such as that of the vetiver grass (*Vetiveria zizanioides*), in stems like that of peteribi wood (*Cordia trichotoma*) and incense, in leaves like in eucalyptus trees (*Eucalyptus citriodora*), citronella (*Cymbopogon nardus*), chinchilla (*Tagetes minuta*) and lemon grass (*Cymbopogon citratus*), in flowers like lavenders (*Lavandula officinalis*), in fruit like lemon, orange (*Citrus spp.*) and even in seeds as in the case of anise (*Pimpinella Anisum*), coriander (*Coriandrum sativum*) and pepper (*Piper nigrum*), among others (Baser, 2010). They can work as internal messengers, like defense substances or plant volatiles aimed at natural enemies but also to attract pollinating insects to their host (Harrewijn et al., 2001).

Essentials oils are accumulated in cells, secretory cavities or glandular hairs of plants. They are globules with impermeable cells (stomata) whose interior have essentials oils. In the case of citrus, stomata can be observed at first sight because they are macroscopic. Apart from superior plants, some land and sea animals, insects, mushrooms and microorganisms are also known for the biosynthesis of similar volatile compounds (Berger, 2007).

In general, essential oils have a nice smell, that is why they are used in different industries, especially in perfumes (fragancias and lotions), in foodstuff (like flavoring and preservatives) and in pharmaceutical products (therapeutic action) (Zygadlo & Juliani, 2000).

There are different methods for essential oil extraction. One of the most common is steam-distillation since it allows for the separation of slightly volatile, water-inmiscible substances by means of low temperature distillation, being of particular use when the components boil at high temperature (higher than 100ºC) and are susceptible to decomposition below this temperature. Although this methodology presents several advantages, it is necessary to bear in mind that it is not just a simple steam dragging business. The release of the components present in the stomas is caused by cell-wall rupture as a result of the higher pressure and the oil content expansion of the cell generated by heat. The steam flow gets in through the stomas, breaks them and eventually drags the essential oil (Baser, 2010).

In a nutshell, steam-distillation consists of steaming as a result of a straight current of steam water, which heats the mix as well as it decreases the boiling temperature because of the higher steam tension inherent in water to those of volatile components in essential oils. The steam coming from the distillator gets cold in a condenser and, finally, the inmiscible mix gets separated in a clarifier or Florentine flask. This methodology is more convenient than

organic solvent extraction or straight distillation as water steam has a lower cost compared to organic solvents. Also, it avoids oil heating or the use of sophisticated equipment. Nonetheless, the extraction method depends, among other factors, on the kind of material to be processed and the location of the components within the vegetable structure according to the species and botanical family (Bandoni, 2000).

The extraction technique with organic solvents is based on the distribution balance or selective dissolution of the oil within two inmiscible phases. The starting point is the fresh or dessicated vegetable material. However, the dessicated material, even when the extraction is done at room temperature and in darkness, incurs in a partial loss of the product, because of the steaming of most of the volatile components of the oil. The extraction can be carried out with volatile solvents, for instance petroleum ether, n-hexane, among others. In simple extractions, solid solvents and high quality fat can be used for the extraction of essential oils of flower leaves like violets, jasmine or roses (enfleurage).

In order to get oil from citrus fruits, such as oranges, lemons or tangerines, cold expression is preferred, due to the thermal instability of the main constituents of the essential oil. The oil cells are located below the epicarp surface. The fruit must be washed and sliced into two halves, the pulp withdrawn and then the peel must be softly pressed to break the oil glands, which can be removed with water.

Once the oil is obtained, it must be dried with anhidrous $Na_2 SO_4$, then filtered and stored for its ulterior study or separation of the components (inert atmosphere and protected by light). The oil analysis entails the determination of its physical properties (refractive index, density, and optical rotation) and chemical properties (acid index, esters, carbonyl compounds, fenols, primary and tertiary alcohols), as well as the separation and identification of its major components.

Although the essential oils have a great number of components, the ones of commercial interest are generally those composed of one or two major components, which provides them with accurate features. Nonetheless, in some cases, the minor components are also important because they might provide the oils with a of exquisite perfume, that is why this kind of material must be handled with care. The extraction, preservation and conditioning of such a material are very important in order not to alter its composition. Otherwise the market price would drop sharply. For the same reason, it is vital to study the composition of these oils and, in some cases, even those components present at milligram or nanogram amounts. The organic chemistry, mainly through GC and mass spectroscopy, has a leading role in this area.

From the chemical point of view, the essential oil composition frequently changes in different parts of the plant. Quite often, between the different organs of the plant, phytochemical polymorphism can be produced. As an example, in *Origanum vulgare* ssp. hirtum, polymorphism could be detected, even within one individual plant, between different oil glands of a single leaf (Johnson et al., 2004). However, this kind of polymorphism is not very usual, being the difference in the oil composition between glands usually related to gland age (Grassi et al., 2004; Johnson et al., 2004; Novak et al., 2006; Schmiderer et al., 2008). In general, the different growth stages of the plant create variations in the oil composition within the same organ of the plant (Chamorro et al., 2008).

Polymorphism is also often found when the essential oil composition of individual plants of one species is compared (intraspecific variation, "chemotype") and is based on the genetic background of the species. Sometimes the difference in the complex composition of two essentials oils of one kind are difficult to assign to specific chemotypes or to differences that arise as a result of the plant response to environmental conditions, for instance, different growth locations.

Generally speaking, genetic differences are much higher than those caused by varying environmental conditions. However, many intraspecific polymorphisms are unlikely to have been detected yet or have been recently described, even for essential oils widely used, such as those present in sage (Novak et al., 2006).

Due to its soil and climate characteristics, Argentina is a prime area for growing aromatic plants, both native and foreign. In the northeastern region comprising the provinces of Chaco, Formosa, Corrientes and Misiones, species that grow well contain large percentages (60-90%) of oxygenated monoterpenes such as citronellal, dehydrotagetone tagetone, α-pinene and β-pinene, among others. An important source of those compounds are species like *Eucalyptus citriodora*, *Cymbopogon spp. Tagetes spp.* and *Schinus spp.*, which have been studied in various aspects by GC and mass spectroscopy.

As a starting point for the study of essential oils, it is necessary to define the best working conditions in order to get the right separation, identification and quantification of its components. To exemplify the methodology used in regards to this matter, we will describe work done on *Cymbopogon winterianum*.

Within the several uses of this methodology, we can mention the study of the variation of the oil composition in terms of the different stages of the plant, as well as the different origins. In this regard, the methodology used in the study of two effective biocides will be described, the essential oil of *Tagetes minuta* L (Zygadlo et al., 1993), which presents variations in its composition in the different parts of the plant in different stages of its vital cycle; as well as the results obtained in the study of essential oils of *Schinus molle* in the City of Resistencia, Chaco, presenting diverse variations in connection with plants of other origins (Maffei & Chialva, 1990; Menendez et al., 1996).

Also be quantified by gas chromatography, reaction products of semi-synthesis of terpenes from low economic value, such as obtaining isopulegol from the cyclization of citronellal, a major component of essential oils of *Eucalyptus citriodora* and *Cymbopogon winterianus*.

3. Determination of chromatographic conditions

The aim of the selection and definition of chromatographic conditions is to achieve a proper separation of the components of the oil, both for the qualitative analysis, as also for the proper quantification. To do so, well resolved peaks and not distorted ones, good relation signal-noise and horizontal base line with absence of drift, must be obtained for each one of the components.

To accomplish this objective, a correct selection of the column is key: the ones which are the most used for essential oils are the polar, and in particular, have chiral stationary phases those essential oils which have components of interest that present optical isomerism.

In general, for the development and selection of stationary phases, it must be considered, among other things, the thermal and chemical stability of the column, the selectivity in the separation of the components, the lining or coating surface, the diameter of the column, as well as the incorporation of more specific components to the stationary phase, or the use of different technologies to optimize the phase available to the specific regions of analyses that require better resolution (Marriott et al., 2001). From all the factors that should be considered in the selection of stationary phases, the thermal stability of the column is of less importance, since the essential oils are eluted prior to the rank of highest temperature defined by the high-temperature GC. However, a greater column stability also implies that the column will be stable over a longer period, and this will translate into a better reproducibility in long-term analysis, which leads to a more precise analytical characterization.

The variable that the analyst most frequently handles at the time of separation of the components of essential oils is perhaps the working temperature.

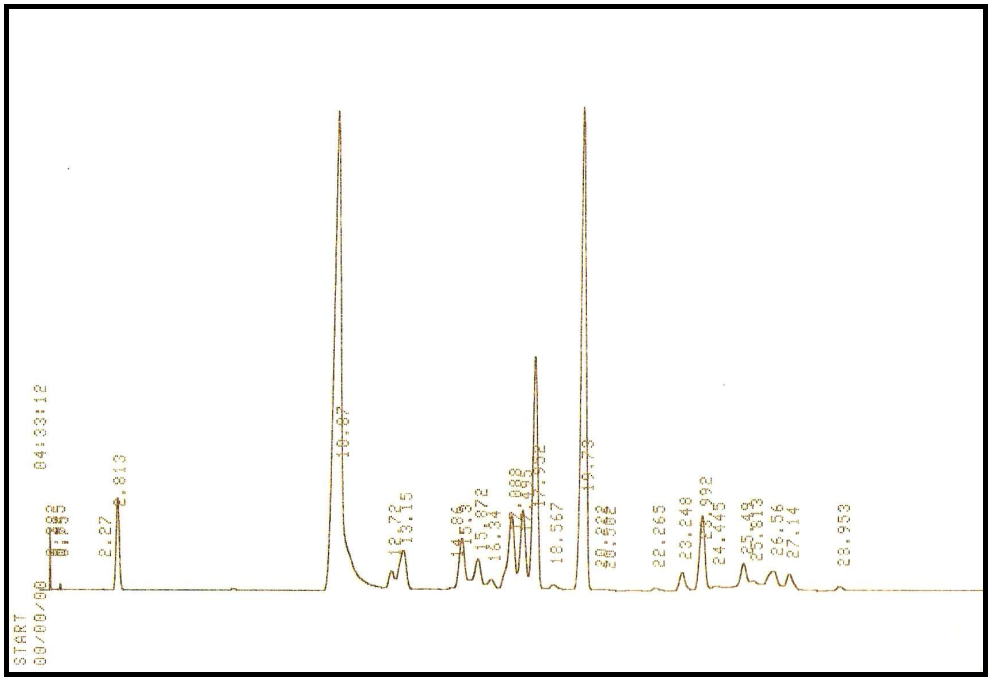

Fig. 1. Chromatogram of citronella essential oil with column temperature of 60 °C for 5 minutes, then a ramp with an increase of 5 °C/minute up to 200 °C, finally leaving this temperature constant for 10 minutes.

Given that essential oils are mixtures of compounds of different molecular weights, from the most volatile hydrocarbons of ten carbon atoms, called monoterpenes, to oxygenated compounds of 15 atoms of carbon, or sesquiterpenes, it is necessary to start with low temperatures that allow the separation of the most volatile ones, then raise it 5 °C or 10 °C per minute to reach the temperature of 200 °C to achieve the elution of the heaviest terpenoids (Francisco et al., 2008).

If the chromatography was performed isothermally at low temperature (60 °C), the components were not separated from each other, migrating as a single, broad peak and employing long elution times. If, on the other hand, the isothermal run was done at high temperatures (200 °C), the peaks were slender and the elution time shorter but proper peak migration and separation was not achieved.

Figure 1 shows a chromatogram of the essential oils of citronella (*Cymbopogon winterianus*) in which the column temperature was kept at 60 °C for 5 minutes, then ramped at 5 °C/minute up to 200 °C and finally kept for 10 minutes at that same temperature. The run was done using a SHIMADZU GC 14B chromatograph, equiped with a Mega Bore DB-WAX P/N 125-7032 column (30 m in length x 0.53 mm i.d. x 1 µm), a Flame Injector Detector (FID) with an operating temperature of 220 °C and an injector with a temperature of 180 °C, manual injection and nitrogen as gas carrier.

4. Identification of essential oils components

Tagetes minuta L. is an native aromatic plant from South America, which is well known in the Province of Chaco (Argentine), and it is vulgarly called "chinchilla" (Parodi, 1959). It grows naturally from spring until it practically disappears with the beginning of the winter, developing its complete life cycle within this period of time. This oil has wide applications as flavoring and perfume (Vasudevan et al., 1997). In addition, it is well known for its biocide properties (Zygadlo, 1994).

With that purpose, vegetable material, from different locations of the Province of Chaco, was collected during the fall. It is during this season that the plant is likely to be used for essential oil extraction. The oil obtained by steam-distillation, independently from non-bloomed plant leaves, from bloomed plant leaves and from flowers of *T. minuta*.

The identification of the essential oil components were carried out by gas chromatography-mass spectrometry (GC-MS) using a gas chromatograph Agilent 6890 with selective mass detector Agilent 5973, a capillary column of HP-5MS (30 m x 0.25 mm i.d. x 0.25 µm film thickness) and a split/splitless injector, with an automatic injection system ALS Agilent 7683, and the Library NIST Mass Spectral Search Program, version 1.6d. Working conditions were: split injection; ratio 70:1, injecting 0.2 µl. The test was carried out at 250ºC, the oven at initial 50ºC for 2 minutes increasing 10 ºC per minute till 200ºC was reached. The flow was of 0.7 µl/min at a constant speed of 30 cm/s with a 250ºC interface.

Figure 2 shows a chromatogram of the essential oil of *Tagetes minuta*, in which efficient separation of different components, including geometric isomers such as cis and trans-tagetenone (peaks 6 and 7) was obtained. Figure 3 and 4 show the mass spectrum of both these isomers. As it can be seen, both have the same formula and molecular weight (150) and yet show very different mass spectrum. In the same manner, we could identify all the other components of the oil.

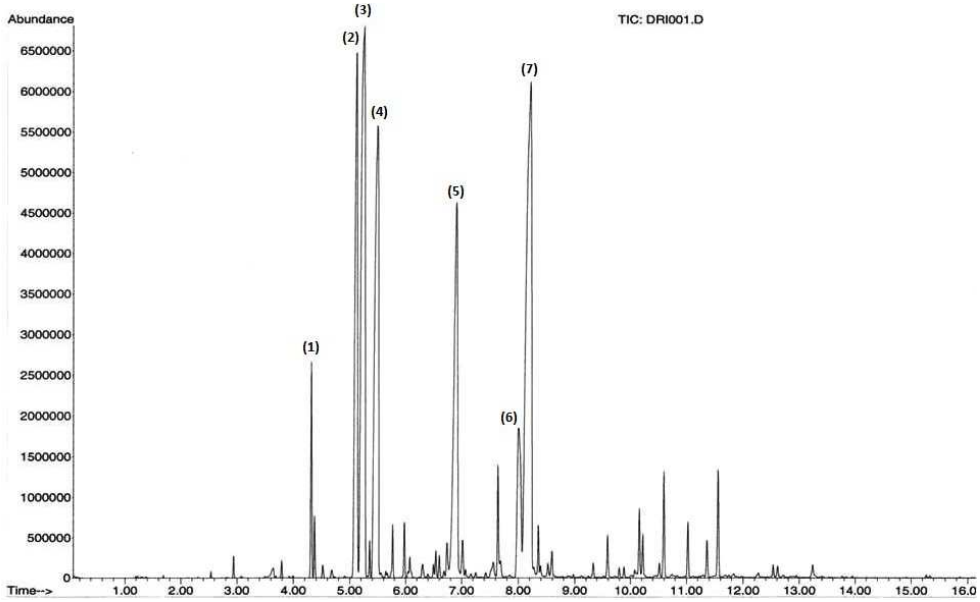

Fig. 2. *Tagetes minuta* essential oil chromatogram carried out using a gas chromatograph-mass spectrometry Agilent 6890 with a capillary column of HP-5MS. Components: β-phelandrene (1), limonene (2), β-ocimene (3), dihydrotagetone (4), tagetone (5), cis-tagetenone (6) and trans-tagetenone (7).

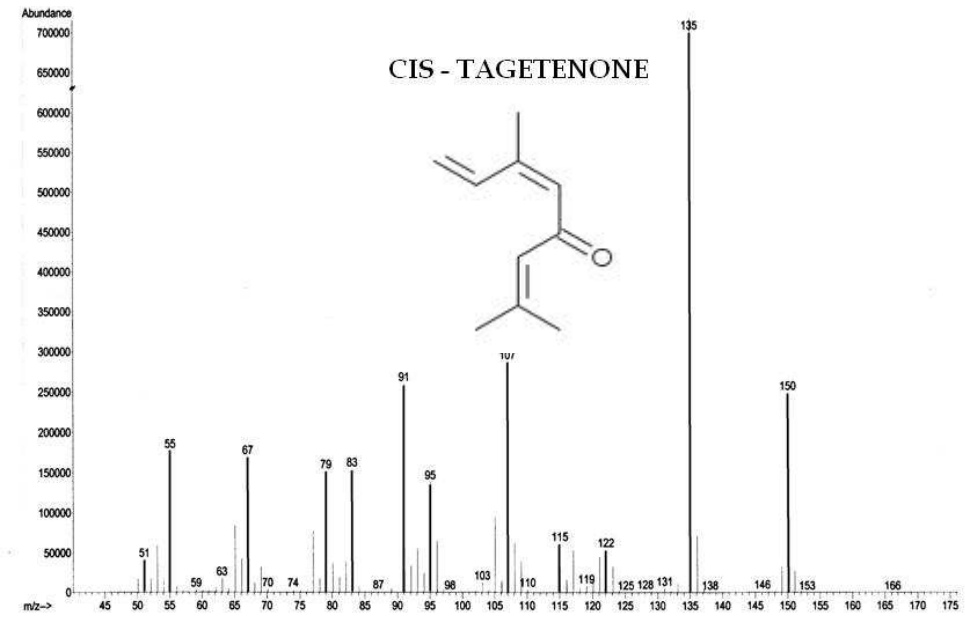

Fig. 3. Mass spectrum of cis-tagetenone.

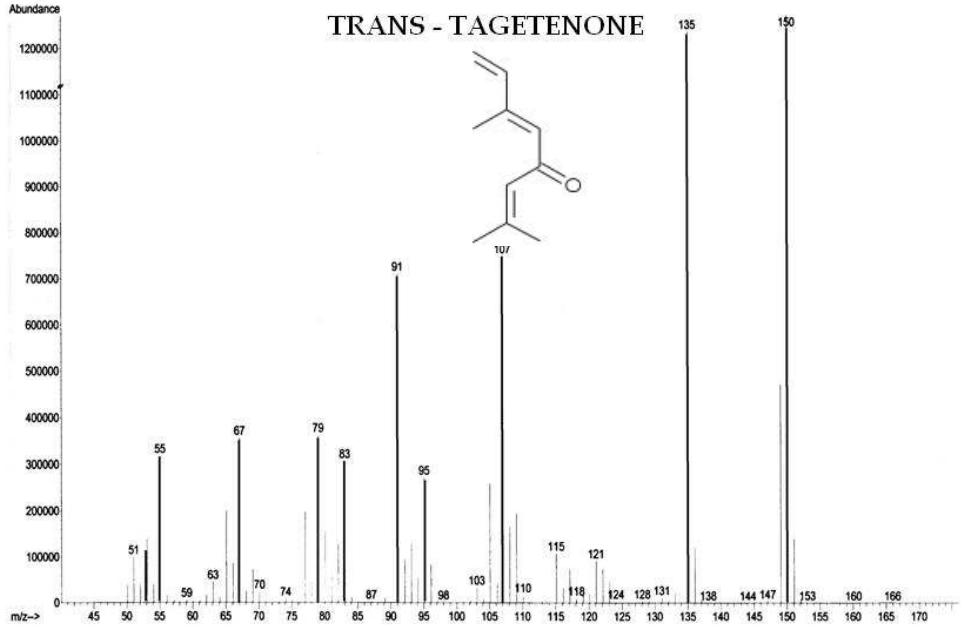

Fig. 4. Mass spectrum of trans-tagetenone.

Various studies on *T. minuta* reported that there are variations in the essential oil composition according to the harvesting location (Zygadlo et al., 1993), the growth stage (Moghaddam et al., 2007) the different parts of the plant (Weaver et al., 1994) and the different chemotypes (Gil et al., 2000). All these facts suggest that a deep study of the native *T. minuta* essential oils is necessary at the regional level.

Given that the applications of essential oils, such as the biocide action, are based on their composition, these variations may explain presence or absence of a certain effect, depending on the material used. With this purpose, we studied the composition of the essential oil of *T. minuta* at different stages of the plant life cycle.

The oil were obtained by steam-distillation, independently, from non-bloomed plant leaves, from bloomed plant leaves and from flowers of *T. minuta*. The essential oil obtained was analyzed qualitatively and quantitatively by means of GC. The quantitative data were determinated from the peak percentage areas. A Shimadzu GC 14B gas chromatograph equiped with a Mega Bore DB-WAX P/N 125-7032 (30 m x 0.53 mm i.d. x 1 μm film thickness) column and FID detector. The column was programmed as follows: 60ºC during 5 minutes, increased to 200°C at 5°C/min. The injector temperature was 180°C and detector temperature was 220°C. Peak area percentages were calculated with a Shimadzu C-R6A Chromatopac Integrator without including response factors or internal standards.

Figure 5 shows a comparison of the chromatograms obtained for the essential oil of *T. minuta* obtained from a) leaves from non-bloomed plants, b) leaves from bloomed plants and c) flowers, whose components had been previously identified by GC-MS. The variations in the relative proportions of the six components identified are clearly shown.

An analysis of the relative composition of flowers oil, shows that the major components are β-ocimene and tagetenone, at the expense of a considerable decrease of dihydrotagetone (major component of leaves from non-bloomed plant). This might explain the increased biocidal activity of flower oil, since its effectiveness is usually associated with the presence of tagetenona in it.

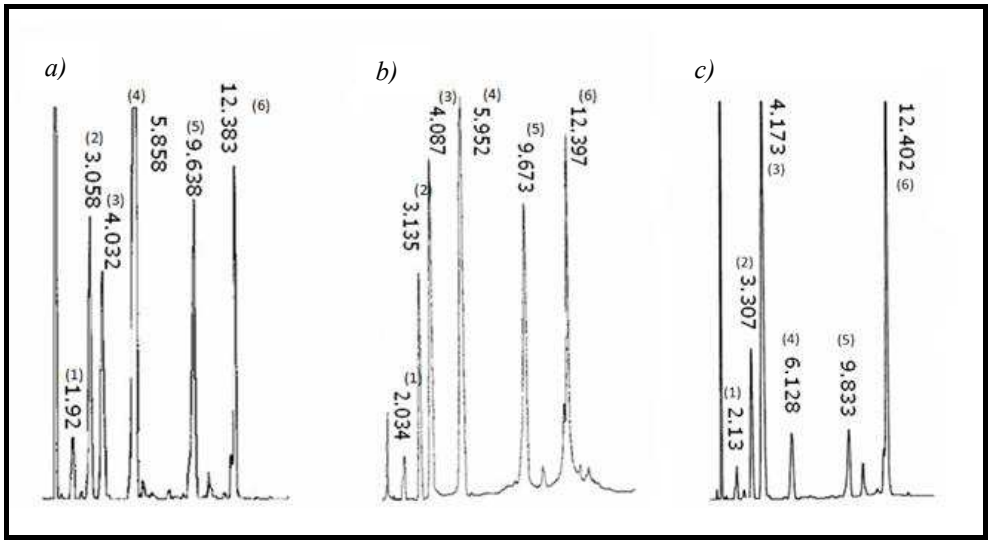

Fig. 5. Cromatograms of *Tagetes minuta* essential oil from *a)* leaves non-bloomed plant *b)* leaves bloomed plant and *c*) flowers. Components: β-phelandrene (1), limonene (2), β-ocimene (3), dihydrotagetone (4), tagetone (5) and tagetenone (6).

Peak N°	**Component**	**leaves non-bloomed plant oil %**	**leaves bloomed plant oil %**	**leaves bloomed / fructified plant oil %**	**flower oil %**	**flower oil %**	**flower and seed oil %**
		March	**April-May**	**June**	**April**	**May**	**June**
1	β-phelandrene	2.6	2.1	2.2	0.8	1.2	1.4
2	limonene	12.9	13.1	11.8	4.4	6.4	8.6
3	β-ocimene	11.1	17.6	15.4	**47.4**	**38.2**	**31.2**
4	dihydrotagetone	**47.0**	**28.1**	**38.3**	1.5	6.6	11.7
5	tagetone	15.9	18.2	12.3	3.7	8.9	11.8
6	tagetenone	8.8	18.1	11.5	**36.0**	**34.0**	**28.7**

Table 1. Variation of the chemical composition of the essential oil from *T. minuta* L. leaves and flowers depending on the growth stage.

Schinus molle L. (Common name: bolivian Pepper, molle, aguaribay, huaribay, false pepper, peruvian mastic, anacahuita), plant species of the family Anacardiaceae, native to the Peruvian Andes (Huerta et al., 2010), is widely cultivated in tropical and subtropical countries (Wimalaratne, et al., 1996). The *Schinus molle* L. is widely spread outside their original geographical ranges, grows in North and Central America, Africa, Middle East and is cultivated around the Mediterranean in southern Europe. In Argentina, it is widely used as an urban tree because of its resistance to pollution, easy and economical spread and little need for irrigation.

The leaves of this tree are an important raw material for the extraction of essential oil used in folk medicine as they have antimicrobial, antispasmodic, antipyretic, antifungal and cicatrizing properties (Marongiu et al., 2004, Ferrero et al. 2006; Hayouni et al. 2008; Maffei & Chialvo, 1990). Likewise, it is noteworthy as a repellent and its utlization as a bioinsecticide (Ferrero et al., 2007). All these properties are associated with the presence of certain components in the essential oil, whose composition can change according to several factors: growth stage, geographical factors (location), ecological (habitat), genetic variability (chemotype), the extraction process, etc. (Bandoni, 2000).

GC coupled with mass spectrometry proved to be a useful application to identify variations in the composition of essential oil and make comparative analysis, in view of their possible use originated on the properties described before. Essential oils obtained by steam-distillation from *Schinus molle* leaves and young tree branches from the city of Resistencia, Province of Chaco were compared with oils from trees of different backgrounds (Maffei & Chialvo, 1990, Menendez et al., 1996, Barroso et al., 2011; Guala et al., 2009).

With this purpose, these oils were analyzed using Shimadzu GC 14B gas chromatograph equipped with a Mega Bore DB-WAX P / N 125-7032 column (30 mx 0.53 mm id x 1 micron film thickness) and a FID detector.

Identification of the components was performed with an Agilent 6890 gaseous chromatograph with Agilent 5973 mass detector, a HP-5MS capillary column (30 mx 0.25 mm id x 0.25 um film thickness) a split / splitless injector, the ALS Agilent 7683 automatic injector, and Library NIST Mass Spectral program.

The major components identified in the essential oil of *S. molle* of Resistencia city were α-pinene (11.5%), β-pinene (14.71%), limonene (9.17%), α-ocimene (3.1%), germacrene D (3.6%), γ-cadinene (6.9%), δ-cadinene (4.9%) and epi-bicyclosesquiphelandrene (18.6%), as shown in the Figure 6 and Table 2. However, the composition of these oils differ in their main components compared to data reported from other sources, such as Liguria (Italy), whose main components are α-phellandrene (30%) and elemol (13.25%) (Maffei & Chialvo, 1990), Uruguay with 30% of Biciclogermacreno (Menendez et al., 1996), state of Rio Grande do Sul in southern Brazil with 40% of limonene (Barroso et al., 2011) and Santa Fe (Argentina) whose major component is limonene (40%) (Guala et al., 2009).

The differences in the oil composition of *S. molle* from distinct sources, probably attributed to different geography and plant chemotypes, strongly support for the need for a thorough evaluation of the biocidal properties of these oils obtained from different locations.

compounds	Chaco (%) Arg.	Santa Fe (%) Arg.	Brazil (%)	Uruguay (%)	Italy (%)
α-pinene	**11.51**	**11.7**	2.70	-	1.46
canfene	0.45	0.3		-	tr
β-pinene	**14.71**	**12.7**		**13.95**	0.10
sabinene		45.0	5.85	**12.92**	0.67
β-myrcene	0.49	2.4	0.83	**5.46**	tr
α-felandrene	-			-	**30.24**
limonene	**9.18**	3.8	**41.87**	0.88	9.27
β-felandrene	-			0.30	**9.63**
γ-terpinene		2.9		1.13	-
β-ocimene	0.16			-	-
α-ocimene	3.17			-	-
linalool				0.71	-
caryophillene	1.05	1.2	**15.60**	**7.68**	0.08
terpinen-4-ol		4.4		**10.57**	0.03
azulene Derivative	3.71			-	-
α-humelene				0.57	0.39
α-terpineol				1.25	-
germacrene D	**3.57**		**8.86**	**12.08**	**5.21**
bicyclogermacrene			**11.59**	**29.20**	-
isoledene	3.93			-	-
γ-cadinene	**6.87**			-	-
δ-cadinene	**4.90**			1.26	1.71
copaene	0.26			-	0.02
elemene					0.03
caryophilene oxide				0.53	
spathulenol	0.99			-	
eudol	1.11			-	
germacrone				0.75	
unidentified sesquiterpene alcohol	3.40			-	
epi-bicyclosesquiphelandrene	**18.60**				
A-cadinol	1.62				tr
elemol					**13.25**
neril hexanoate					2.94
γ-eudesmol					3.24
γ-cadinol					4.70
unidentified sesquiterpenes	6.50				1.96

Table 2. Chemical composition of essential oils of *Schinus molle* Argentina, Uruguay, Brazil and Italy (tr: traces).

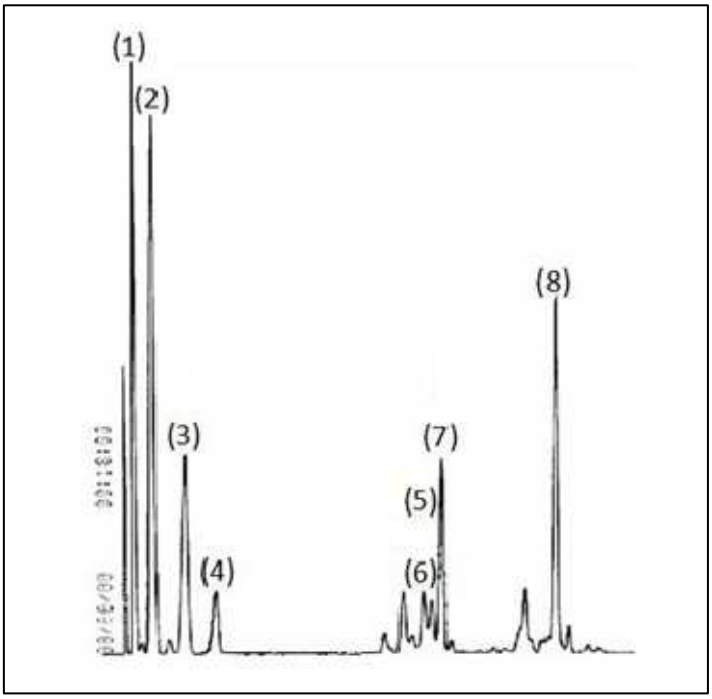

Fig. 6. Chromatogram of essential oil from *Schinus molle*. Components: α-pinene (1), β-pinene (2), limonene (3), α-ocimene (4), germacrene D (5), γ-cadinene (6), δ-cadinene (7) and epi-byciclosesquiphelandrene (18.6%).

5. Quantification of essential oil components

In order to quantify a component by GC, it is possible to use the methods of either the internal or external standard. For the particular case of essential oils, one can frequently use the method of relative concentrations related to the total area of the peaks, due to the similarity of reaction factors that the main components of the terpene family have. What follows is a brief description of each one of them, its advantages and limitations.

The relative area method results in a percentage relation of the area corresponding to each component with regards to the total area of the chromatogram, understanding this as the addition of the individual areas of each one of them, as it is shown in the following equation (1) (Orio et al., 1986)

$$\%\text{relative of the component} = \frac{\text{component area}}{\text{Total area}} \text{x } 100 \quad (1)$$

The external standard method is easy to apply, and is the habitual basis for numerous analytical determinations. It allows us to calculate the concentration or percentage of mass of one or many constituents that appear separated in the chromatogram, even in the presence of unsolved peaks.

Number of peak	Time (min)	Area	Relative concentrations (%)
1	0.382	6679	0.10
2	2.730	176430	2.74
3	9.065	2343024	36.45
4	10.070	53522	0.83
5	10.385	177509	2.76
6	11.472	184517	2.87
7	11.888	119067	1.85
8	12.618	541246	8.42
9	12.837	689214	10.72
10	13.760	1464544	22.79
11	15.648	70466	1.10
12	15.990	282347	4.39
13	16.763	197766	3.08
14	17.333	121177	1.89
Total		6.427.508	100

Table 3. Individual areas, their addition and the relative percentage of each component, corresponding to the chromatogram in Figure 3.

Such process is based on the comparison of two chromatograms, one of which corresponds to the established standard and the other one to the component being studied. In such comparisons, we have to dilute the standard (purity ≥ 95.0 % from Aldrich) of the component to be determined. For example, (±)- Citronella GC (Zambón et al, 2011). A known volume of what has been diluted is injected and the reference area (Aref) and the corresponding peak in the chromatogram is measured. Then, without changing any of the analysis conditions, an identical volume of the sample is injected and the area corresponding to the sample (Asample) is measured. Since the volume in both cases is equal, there is proportionality between the areas, which depends on the injected masses and the concentrations. Thus, we can determine the sample concentration with the equation (2) (Rouessac et al., 2003)

$$\text{Sample concentration} = \frac{\text{Asample}}{\text{Aref}} \text{x reference concentration} \quad (2)$$

To exemplify this methodology we show the results of the quantitative study of citronella (the major component of the essential oil of *Cymbopogon winterianus*), during the reaction of the cyclization to isopulegol with acid heterogeneous catalysis. The samples corresponding to tests 1 and 2 show the remnants of citronella after the cyclization of this component to isopulegol, while samples 3 and 4 show initial citronella.

It is important to take into account that, if only one point corresponding to the standard is used, the calibration curve is considered to go through the origin, so the precision will be acceptable if the concentrations of the standard and the samples are similar. This can be considerably improved by using many identical injections for both, the component and the standard. However, instead of taking a great number of measurements, it is preferable to work with calibration with various points, called multilevel calibration.

Sample	Area	Concentration (%P/P)
standard of (±)-Citronellal	42624105	95.0
Sample 1	43072780	96.0
Sample 2	41726755	93.0
Sample 3	13035302	29.05
Sample 4	12373866	27.58

Table 4. Areas and citronellal concentrations (initial and remnant) of the reaction of cyclization of isopulegol, by means to the external standard method.

Sample	Mass isopulegol (g)	Mass solvent (g)	Mass of internal standard (g)	Area of internal standard	Area isopule-gol	Relation A isopulegol/ A internal standard	Concentra-tion of isopulegol (Mol)
M0	0.0189	0.1139	0.0361	809262	442226	0.55	0.56
M1	0.0439	0.1115	0.0353	902541	1729214	1.92	1.14
M2	0.0720	0.1158	0.0365	667942	2032979	3.04	1.67
M3	0.0893	0.1060	0.0366	610626	2220885	3.64	1.93
M4	0.1141	0.1038	0.0344	633292	3020415	4.77	2.24

Table 5. Calibration curve of isopulegol by means of the internal standard method.

The internal standard method is based on the use of the relative response factor of each component to be measured with respect to a marker introduced as reference. This avoids the imprecision related to the injected volumes, which is a disadvantage of the previous method. However, it requires the addition of a component to a sample dilution. In general, a calibration curve is built by applying different solutions of increased concentrations of the standard analyte with a constant quantity of internal standard. When injecting such samples, we obtain the relation between the areas of the analyte and the internal standard; then, it is marked in a graph according to the concentration of analyte in each solution. By means of interpolation in the graphic, we get the relation of the areas of an unknown sample, which has to contain the same quantity of internal standard.

The selection of an appropriate internal standard is very important. It has to be pure and must not be present in the sample at the beginning; its elution peak must be well solved in relation to those that conform the chromatogram of the sample; the retention times must be next to those of the solute to be measured; its concentration has to be near or superior to the rest of the solutes, so that it allows for a linear answer of the detector, and must be inert in relation to the constituents of the sample.

Table 5 shows an example of a calibration curve for (-) – isopulegol (purity > 99% GC, addition of enantiomers) where tert-butanol was used as solvent, and the internal standard was methyl ethyl ketone. Figure 7 shows the obtained calibration curve.

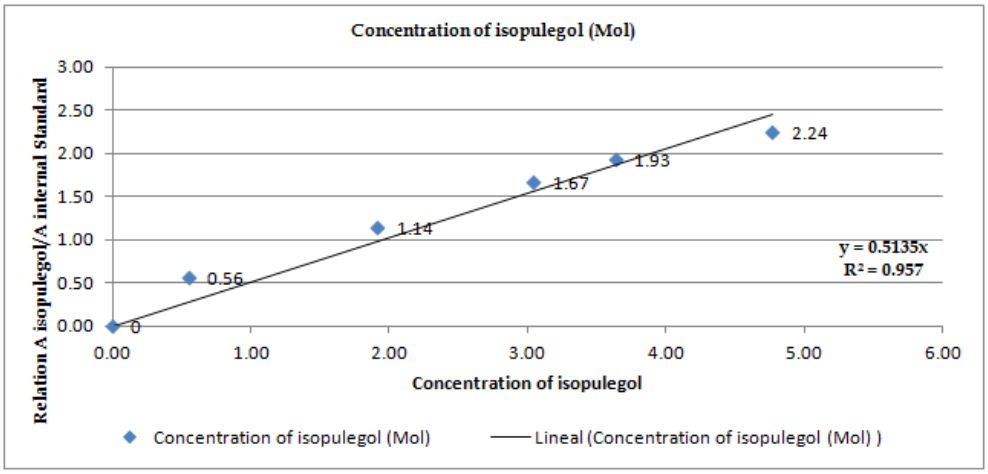

Fig. 7. Calibration curve of isopulegol by means of the external standard method.

By using the obtained calibration curve, it is posible to calculate the isopulegol moles in unknown samples.

Samples	Area internal standard	Area of isopulegol	Relation A isopulegol/A internal standard	Concentrations of isopulegol of unkown samples
Sample 1	733318	140553	0.19	0.44
Sample 2	789175	363884	0.46	0.55

Table 6. Concentration of isopulegol (expressed in moles) in unknown simples.

It is important to remark that the use of automatic injectors increases the reproducibility in the injection, which has allowed for the spread in the use of external standards because of its simplicity. However, in the case of manual injection, the internal standard method turns out to be simple and adaptable to everyone.

6. Conclusion

In this chapter, we described the importance of the technique of gas chromatography in the study and analysis of essential oils. It is important to remark that they have extremely varied industrial applications, such as food, pharmaceutical, aromas, flavors, organic synthesis, among other.

This technique has become an indispensable tool for the control of essential oils, as prices of these vary depending on their composition and distribution of components, and the oils do so according to their origin, growth stages, chemotypes, among others.

Currently, there are new stationary phases which significantly improve the separation of the components, especially the most important are chirals phases, which allow for the separation of compounds with optical isomerism, which creates an especially important step for the chemical and pharmaceutical industries.

When searching for the correct separation of the peaks, it is important to consider the selection of detectors. Notably, the most commonly used for identification of the components is the mass spectrometer (MS), while for the quantification of its simplicity and specificity toward organic compounds is the flame ionization detector (FID).

It is important to note that the components of essential oils are what provide them with their intrinsic properties and for which they are used productively.

As important as identifying the components, quantifying them, such as the variation in the distribution of mixture components, modifies the particular properties of essential oils.

As developed in this chapter, there are several measurement techniques, such as the relative percentages, internal or external standards. Each has advantages and disadvantages and are applicable to quantifying components that have previously been identified.

Through the study of the essential oils by GC-MS, we have been able to appreciate the value of this technique in the identification and quantification of their components. With this technique, it was possible to show that the composition of the essential oils of *Tagetes minuta* varies in different parts of plants, as well as in different stages of growth. It was also found that *Schinus molle* essential oil, an important biocide, shows large differences in composition, depending on the origin of plants, been this data a key for assessing their biocidal properties. In a similar way, this technique becomes important in the quantification of oil components and even more when added value products are developed from them, such as the cyclization of citronellal to isopulegol.

Argentina is an important exporter of essential oils because it has favorable agro-climatic characteristics for the development of aromatic plants, both native and foreign. The main destination of these products are European countries that demand high levels of quality and is this sense GC is the technique that ensures compliance with the specifications agreed between the parties.

Then, in a highly demanding market such as essential oils is gas chromatography the technique that provides the industry and science with simplicity, speed and efficiency for the characterization and quantification of components.

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Gas Chromatography in Plant Science, Wine Technology, Toxicology and Some Specific Applications

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The aim of this book is to describe the fundamental aspects and details of certain gas chromatography applications in Plant Science, Wine technology, Toxicology and the other specific disciplines that are currently being researched. The very best gas chromatography experts have been chosen as authors in each area. The individual chapter has been written to be self-contained so that readers may peruse particular topics but can pursue the other chapters in the each section to gain more insight about different gas chromatography applications in the same research field. This book will surely be useful to gas chromatography users who are desirous of perfecting themselves in one of the important branch of analytical chemistry.

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