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Departamento de Química Analítica. Facultad de Ciencias

TESIS DOCTORAL:

ASSESMENT OF CHEMICAL AND MORPHOLOGICAL VARIATION OF SPANISH MARJORAM, SPANISH SAGE AND SPIKE LAVENDER

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ASSESMENT OF CHEMICAL AND MORPHOLOGICAL VARIATION OF SPANISH MARJORAM, SPANISH SAGE AND SPIKE LAVENDER

Presentada por Inés Méndez Tovar para optar al grado de doctora por la Universidad de Valladolid

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“Success is not built on success. It's built on failure. It is built on frustration. Sometimes it is built on catastrophe”

A mi familia

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ABSTRACT

Traditionally aromatic and medicinal plants have been collected from the wild. However, wild collection does not allow high quality plant material to be collected and produces deforestation thus threatening the species harvested. It should be added that strict rules are published to regulate the quality of the volatiles. Additionally, when the plant material is intended for medicinal use, the effectiveness of the different active substances such as polyphenols and the concentration thereof, might varies hugely from one individual to another. The need to find high quality specimens and to cultivate the selected individuals not only increases the yield and the quality of collected plant material but also enables sustainable development and conservation of the species. In addition to that, some of the parameters for cultivating these species are unknown. In order to overcome such problems, the chemical variability of the secondary metabolites and morphological variation of *Lavandula latifolia* Medik., *Salvia lavandulifolia* Vahl. L. and *Thymus mastichina* L. has been evaluated to find high quality individuals.

In view of the great chemical variability that occurs both in the composition of essential oils and polyphenols for the different studied species, as well as its morphology, and given that these features are primarily ruled by genetic characters being the environmental factors a weaker and less influential factor, the ability to select organisms with characteristics and qualities desired by the market is a real possibility for these species. The establishment of plot assays demonstrated that these species can be easily cultivated avoiding wild collection, allowing traceability and offering the possibility to control agronomic parameters such as irrigation doses or treatments that maximize the yield. Moreover the selection of organisms with outstanding features led to an increase of crop productivity and gets better the economical profit, which could be very beneficial to the industry.

RESUMEN

El presente estudio se llevó a cabo para apoyar al sector agronómico y para superar los problemas actuales que el mercado de las plantas aromáticas y medicinales (PAM) tiene hoy en día. El problema principal se debe a que las PAM se han recolectado tradicionalmente de forma silvestre. La recolección silvestre no permite la recogida de material vegetal de alta calidad y produce la deforestación, amenazando las especies recolectadas. Hay que añadir que existen estrictas regulaciones en materia de calidad para la comercialización de los aceites esenciales. Además, en el caso de que el material vegetal sea para uso medicinal, la eficacia de las diferentes sustancias activas, tales como polifenoles, y la concentración de los mismos, puede variar de un individuo a otro. La selección de ejemplares de alta calidad y el cultivo de especímenes seleccionados, no sólo aumenta el rendimiento y la calidad del material vegetal recolectado, sino que también permite el desarrollo sostenible y la conservación de la especie. También cabe añadir que algunos de los parámetros para cultivar estas especies son desconocidos. Para superar estos problemas, la variabilidad química de los metabolitos secundarios y la variación morfológica de *Lavandula latifolia* Medik., *Salvia lavandulifolia* Vahl. L. y *Thymus mastichina* L. se ha evaluado con el fin de encontrar individuos de alta calidad. En vista de la gran variabilidad química que se produce tanto en la composición de los aceites esenciales y los polifenoles para las diferentes especies estudiadas, así como en su morfología, y dado que estas características dependen principalmente de factores genéticos, siendo los factores ambientales un factor menos influyente, la capacidad de seleccionar organismos con características y cualidades deseadas por el mercado es una posibilidad real para estas especies. El establecimiento de ensayos demostró que estas especies pueden cultivarse fácilmente evitando la recolección silvestre y permitiendo la trazabilidad del producto. Del mismo modo, ofreciendo la posibilidad de controlar los parámetros agronómicas tales como dosis de riego o tratamientos que maximicen el rendimiento. Además, la selección de organismos con características deseadas por el mercado conduce a una mejora de la productividad de los cultivos y a un aumento del beneficio económico, que podría ser muy beneficioso para la industria.

General Introduction

1.1 Aromatic and medicinal plants overview

The medicinal and aromatic plants (MAPs) sales are a large worldwide market, as can be seen through the following figures. The volume of MAPs traded globally for the period between 1991 and 2003 was quantified by Lange, D. (2006) as 467.000 t and for a value of US$ 1.2 billion. Another conclusion that can be drawn from the available data is that the world traded volume of MAPs has shown a steady increase in the last year as reflected in the increase in exports and imports in the last decades. The 12 leading MAPs importing countries, imported a total of 320.650 t for a value of 930.520.000 US$ for the period between 1991 and 2003 while the amount of MAPs imported for the period between 2004 to 2008 was 338.173 for a value of 1.004.692.000 US$ (Lubbe and Verpoorte 2011). The same tendency is shown in export figures for recent years. The exports of the 12 top leading exporters accounted for 368.200 t for a value of 807.977.000 US$ for the period 1991-2003 while the exports for 2004-2008 increased to 416.807 t for a value of 923.728.000 US$ (Lubbe and Verpoorte 2011). With respect to the leading exporters of MAPs in the world between 1991 and 2003, China, Hong Kong, India, Mexico and Germany were the countries that exported larger volumes while China, India, Mexico and Egypt were the top exporters from 2004 to 2008. On the other hand China, Morocco, India and Egypt were the leading suppliers for European countries between 2008 and 2012 (CBI 2013). This steady increase in the consumption of plants is something that has also taken place in Spain. Increased consumption of aromatic plants in Spain has been reflected in increased imports and increased national cultivation in recent years. In last decades Spain has been in the top 10 world leading importing countries of MAPs positioning itself in tenth and ninth place. The volume of imported MAPs in Spain between 1991 and 2003 was 9.850 t for the value of 2.7648.000 US$ while the volume of imported plants between 2004 and 2008 was 15.670 t for the value of 44.337.000 US$ (Lubbe and Verpoorte 2011). This upward trend continues today as shown in the following figure.

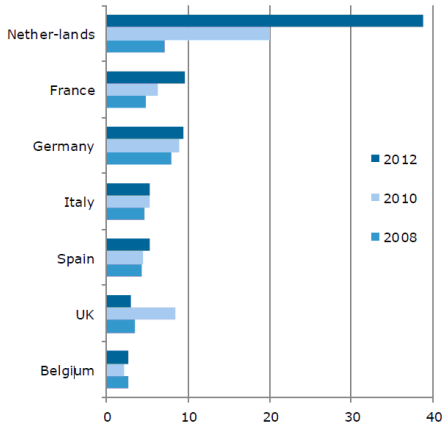

Figure 1: Leading European importers of MAPs 2008-2012 in 1.000 t

Source: CBI

Spain has not only increased imports of MAPs but has also increased the national land dedicated to growing this kind of crops and in recent years the production of aromatic plants has increased from 2.180 ha in 2004 to 9.810 ha in 2014 (MAGRAMA 2014).

The main markets where MAPs are sold in the world are medicinal, food, cosmetic and perfume industry. The most commonly used herbal medicines from plants in the world are: *Echinacea purpurea*, *Panax ginseng*, *Serenoa repens*, *Ginkgo biloba*, *Hypericum perforatum*, *Valeriana officinalis*, *Allium sativum*, *Tanacetum parthenium*, *Ephedra sinica*, *Cimicifuga racemosa* and *Piper methysticum*. Other examples of most commonly traded species used to isolate target compounds are *Papaver somniferum*, *Digitalis lanata*, *Atropa belladonna*, *Taxus* spp., *Cinchona* spp., *Catharanthus roseus* and *Artemisia annua* amongst others. Some examples of the most frequently used natural colorants for food and cosmetics are *Tagetes spatula*, *Juglans regia*, *Lawsonia inermis*, *Isatis tinctoria*, *Curcuma longa*, *Capsicum annuum*, *Vitis vinifera* or *Spinacia oleracea*. Finally some examples of the most used species for the extraction of essential oils in the world with a production above 100 tons (in decreasing quantity): *Citrus sinensis*, *Mentha arvensis*, *Citrus limon*, *Eucalyptus globulus*, *Mentha x piperita*, *Cymbopogon winterianus*, *Syzygium aromaticum*, *Cinnamomum micranthum*, *Citrus aurantifolia*, *Lavandula* x *intermedia*, *Pogostemon cablin*, *Mentha spicata*, *E. citriodora*, *Chamaecyparis funebris*, *Litsea cubeba*, *Juniperus virginiana*, *Illicium verum*, *Citrus reticulate* and *Vetiveria zizanoides.* Essential oil crops with a production from 100 to 50 tons are *Lavandula latifolia*, *Salvia sclarea*, *S. officinalis*, *S. lavandulifolia*, *Pimenta dioica*, *Thymus zygis*, *T. vulgaris*, *Origanum majorana* and *Ocimum basilicum* (Lubbe and Verpoorte 2011).

In Spain the demand for plants for extraction of essential oils is higher than the demand for medicinal plants; however, the number of companies interested in medicinal plants is also substantial (CBI 2013) . Some of the most used and consumed species of MAPs in the Spanish industry are *Mentha x piperita*, *Melissa officinalis*, *Papaver somniferum*, *Chamaemelum nobile*, *Equisetum arvense*, *Thymus* spp., *Rosmarinus officinalis*, *Salvia officinalis*, *S. lavandulifolia*, *Pimpinella anisum*, *Lavandula latifolia*, *Matricaria chamomila*, *Ginkgo biloba*, *Tilia spp*, *Juniperus communis*, *Passiflora incarnata*, *Eucalyptus globulus* and *Crocus sativus* which are mainly wild harvested or imported. Some of them are also cultivated in small amounts with the exception of *L. angustifolia* and lavandin which are widely farmed (Arraiza Bermúdez-Cañete 2006; Palos and Gorgues 2002). The MAPs production of the different Spanish regions can be seen in the following figure:

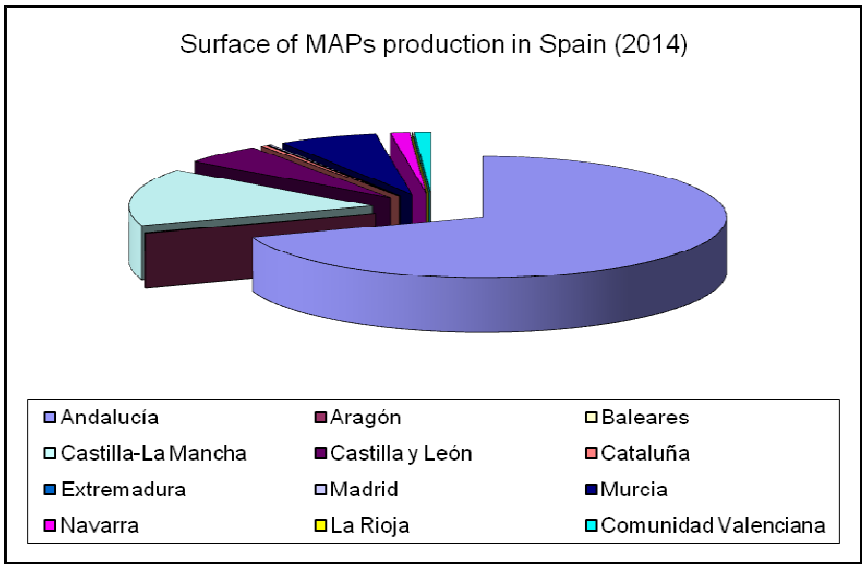

Figure 2: Surface of MAPs production in Spain 2014.
Source ANIPAM

Many of the MAPs sold in the world market are collected from their natural habitat. In the world between 60 and 90% of the raw material used by companies selling products obtained from aromatic and medicinal plants come from wild collection (Laird et al. 2002).

In Spain *Thymus mastichina* is one of the plants most collected from the wild of Spain and 95% of the annual production of this species comes from wild collection, while only 5% of the production is under culture. For *Lavandula latifolia* 30% of the production comes from wild collection and 70% is under culture and for *Salvia lavandulifolia* 20% comes from wild collection while 80% is harvested from crops.

Lavandula latifolia, Salvia lavandulifolia and *Thymus mastichina,* were selected for this study because they are all characteristic plants growing in Spain and they are important for the world and for the Spanish MAPs industry. Regarding annual production of these species, in Spain in 2014 the production was of 20.11 ha for *Thymus mastichina,* Andalusia being the main producer, 136.42 ha for *Salvia lavandulifolia* and *Salvia officinalis* and 595.51 ha for *Lavandula angustifolia* and *Lavandula latifolia* (ANIPAM, 2014).

L. latifolia and *S. lavandulifolia* are among the 20 most traded plants for essential oil extraction worldwide, with an annual production of 50 to 100 tons per year (Lubbe and Verpoorte 2011). Taking into consideration that an important part of these three species are collected from the wild in Spain, and considering that the surface cultivated nowadays is really small and the production per hectare is low, and taken into account that these species are of high interest for

the world market with an increasing demand for them, Spain is the perfect place to increase the surface of cultivation and the yield of production per hectare and thus decrease the wild collection and at the same time increase the quality of the products.

The wild collection of MAPs has a series of associated problems that are enumerated below:

- Misunderstanding characterisation of species and mixing of similar species.
- Lack of knowledge about the kind of active ingredients and their quality.
- Lack of homogeneity of the product.
- Lack of traceability.
- Sometimes the amount of plant material collected from the wild cannot fulfill the market's needs.
- Wild collection can threaten species. Some plants are scarce in nature and human pressure reduces their numbers.
- Different methods to increase productivity and maximize yield, such as irrigation techniques or changes in cultivation parameters, cannot be applied to wild plants.

In view of these problems caused by wild collection all of which lead to a loss of biodiversity and in view of the net balance between imports and exports, there is an increasing need to study the growing of MAPs of industrial interest to enhance their cultivation and to promote their sustainable development and biodiversity. The increase of the value of MAPs has led to the need to strengthen the sector. In recent years the consumption of MAPs has increased in Spain. Imports are greater than exports; however a large number of plant species are naturally growing in Spain (it being the country with the highest biodiversity in Europe), hence this country has the suitable weather and environmental conditions for the sustainable production of MAPs. Besides the lack of farmed medicinal and aromatic plant species and the lack of a proper plant material selection, it implies a loss of productivity and biodiversity.

The search for individuals with enhanced properties is the key to increase the production per ha and to improve the quality of the plant material through breeding programmes.
The benefits that can be achieved through cultivation and breeding include the followings:

- Avoiding the threat to the species and encouraging the protection of nature.
- Estimating the production volumes enabling planning of production.
- Possibility of implanting traceability systems and establishing controls on plant material quality, thus avoiding adulteration or fraud.

- The use of the appropriate irrigation dose or satisfactory agronomic techniques can be applied to improve production.

There is great variability (inter and intra-population) in wild species, different populations have different selection pressures and therefore can manifest morphological and chemical differences. Moreover, different individuals within each population show differences due to the different genetic combinations that each has accumulated. The diversity is crucial for carrying out breeding programmes. Therefore, it is important to study the biodiversity through its characterization, evaluation, and conservation and make this available for breeders, in order to better use these genetic resources. In this way new genotypes of wild species, with key genes will be found allowing development of new cultivars for agricultural production.

1.2. Secondary metabolites

Plants are able to synthesize many kinds of compounds such as carbohydrates, lipids, proteins and vitamins. Some of these compounds are synthesized for the survival, growth and reproduction of plants, that is, they are part of the primary metabolism of plants and are present in all of them. Besides these compounds, plants are also capable of synthesizing secondary metabolites which are often derived from primary metabolites.

Secondary metabolites are natural compounds produced by certain organisms. They are called "secondary" because they are not directly involved in the survival of the living beings. However, they play an indispensable role for living organisms and plants. Unlike primary metabolites, which are common to all plants, secondary metabolites are characteristic and change from species to species. These compounds are commonly stored in particular parts of the plant as vacuoles or secretory structures such as thorns, spikes, trichomes, glandular or stinging hairs, laticifers, ducts etc.

Although nowadays the exact functions of some secondary metabolites are still unknown, these compounds can be toxic or beneficial for the rest of living beings. They are mainly involved in defence mechanisms working as allelochemical products acting against herbivores, microorganisms, pests, pathogens or competitive plants. They can act as internal messengers and also confer on plants characteristics such as a colour or an odour; and thus frequently attract pollinators for seed and pollen dispersion.

A simple classification of these compounds based on their biosynthetic origin includes alkaloids, terpenoids and phenols.

1.2.1 Alkaloids

Alkaloids are nitrogenous organic molecules. Many of them are toxic or have pharmacological activity. Examples of these groups of compounds are morphine synthesized by the opium poppy (*Papaver somniferum*), cocaine isolated from the coca plant (*Erythroxylum coca* and *Erythroxylum novogranatense*) or caffeine synthesised by *Coffea arabica*. The species studied here do not contain alkaloids.

1.2.2 Terpenoids

These are also known as isoprenoids and are derivatives from isoprene which is a small molecule of five carbon atoms. They are hydrocarbons with additional functional groups and the name terpene comes from turpentine, one of the main plant raw materials used in former times consisting mainly of terpenes. Depending on the number of isoprenes forming a terpenoid they can be classified as follow:

- Hemiterpenoids: 1 isoprene unit (C_5)
- Monoterpenoids: 2 isoprene units (C_{10})
- Sesquiterpenoids: 3 isoprene units (C_{15})
- Diterpenoids: 4 isoprene units (C_{20})
- Sesterterpenoids: 5 isoprene units (C_{25})
- Triterpenoids: 6 isoprene units (C_{30})
- Tetraterpenoids: 8 isoprene units (C_{40})
- Polyterpenoid: with a larger number of isoprene units

The cyclic ether 1,8-cineol, also known as eucalyptol, is the most important terpenoid for the species of interest being the main compound for *L. latifolia, S. lavandulifolia* and *T. mastichina* essential oils and it is a characteristic compound for many species of the Lamiaceae family, also being the main compound for eucalyptus genera and thus also known as eucaliptol. Other important terpenoids very abundant in the species under study are camphor, linalool, borneol and α and β-pinene.

1.2.3 Phenols

Phenols are compounds formed by a hydroxyl group (-OH) bonded to an aromatic hydrocarbon group (C_6H_5OH). They can be synthesised through two main synthetic pathways: the shikimate pathway and the acetate pathway (Harborne 1993). When this structure element occurs several times in a molecule, the molecules may be called polyphenol. They can be classified taking into consideration their chemical nature:

- Phenolic acids

Phenolic acids are organic compounds derivated from benzoic or cinnamic acid. In general they include phenols that have a phenolic ring and carboxylic acid functionality. Depending on their origin they can be classified as follow:

- Hydroxycinnamic acid
- Hydroxybenzoic acid

- Flavonoids

Flavonoids are molecules formed by 15 carbon atoms ($C_{15}H_{10}O_2$) containing two phenyl rings and one heterocyclic ring. They are synthesized through the phenyl-propanoid metabolic pathway and in this process the amino acid phenylalanine produced 4-coumaroyl-CoA. It is a very diverse family of compounds. The changes suffered after the biosynthetic pathway it can lead to one type of compounds or another (Boros et al. 2010):

- Anthocyanins
- Flavonols
- Flavanones
- Chalcones
- Flavones

- Lignans

Lignans are a kind of polyphenols mainly found in seeds in small concentrations and built up of C_6C_3 units.

- Stilbenoids

Stilbenoids are hydroxylated derivatives of stilbene with a C_6-C_2-C_6 structure.
Rosmarinic acid is the main polyphenolic compound found in the 3 studied species and a characteristic compound for the Lamiaceae species (Lamaison et al. 1990). Although there is a huge variability in polyphenolic compounds in the species of interest some characteristic compounds like caffeic acid and chlorogenic acid are common to the three species. Luteolin, apigenin and their derivatives are also compounds frequently present in *Salvia*, *Lavandula* and *Thymus* genera (Boros et al. 2010; Spiridon et al. 2011) and salvinolic acid derivatives, carnosol and carnosic acid are very characteristic phenols from *Salvia* genus (Johnson 2011; Zimmermann et al. 2011).

1.3 *Lamiaceae* family

The *Labiatae* family, also known as *Lamiaceae* or *Labiataceae* comprises 186 genera and around 5600 species (Morales 2010b). It is a cosmopolite family spread worldwide but with few representations in the tropical rain forest. Species belonging to this family are commonly aromatic, because these plants are able to synthesize volatile compounds and they are also an important source of a large amount of polyphenols.

1.3.1 Botanical description

Plant habit and cycle: bushy or herbaceous. They can be perennial or annual.
Root: often tuberous.
Stem: generally with quadrangular shape.
Leaves: mainly simple, opposite or whorled and without stipules.
Inflorescence: formed by verticillasters.

Flowers: generally pentamer and hermaphrodite.

Calyx: five sepals soldiers, forming a bell-shaped calyx often bilabiate.

Corolla: formed by five petals welded grouped into two lips. The upper lip is formed of two petals and the lower lip by three petals.

Androecium: It consists of four stamens, however *Salvia* genus has two. When it has four stamens, it has two long ones and two short one fused to the corolla.

Gynoecium: bicarpelar, the ovary is divided into four lobes, owing to the presence of false walls. The style is gynobasic and thready and the stigma is bifid.

Fruit: schizocarp, indehiscent, monospermic and little endosperm.

Botanical formula: ↓ K(5) [C(5) A4+1º] G2

1.3.2 Uses and Properties

The importance of MAPs is based on their uses and properties. Plants from the *Lamiaceae* family are mainly used as ornamentals, for the extraction of essential oils, for the food industry, as nutraceuticals or for phytotherapy. Essential oils are mainly used by industries of aromas and flavours and their uses are mostly related to perfume and cosmetic production. Other uses are aromatherapy, cleaning products or insect and moth repellents. Some examples of the most used essential oils of the *Lamiaceae* with an annual production exceeding 100 tons are species of the genus *Mentha* such as cornmint (*M. arvensis*, menthol production), peppermint (*M. piperita*), scotch spearmint (*M. spicata*) and native spearmint (*M. gracilis*) as well as lavandin (*Lavandula* x *intermedia*) among others. With a production of between 50-100 tons per year the outstanding species would be: spike lavender (*Lavandula latifolia*), sage (*Salvia sclarea*, *S. officinalis* and *S. lavandulifolia*), thyme (*Thymus vulgaris* and *T. zygis*) and marjoram (*Origanum majorana*) (Lubbe and Verpoorte 2011) among others.

In the food industry, MAPs are mainly used as dried herbs in order to enhance colour, taste, smell and/or act as a preservative. The genus *Mentha* for example is often used in drinks; thyme and rosemary are used in snacks, olives, dressings as oils or vinegars etc. As preservatives, thyme and oregano are often used as a marinade for meats or cheeses to slow the appearance of microorganisms and to increase the life of the product.

Many species of the *Lamiaceae* family are also marketed as phyto-pharmaceutical products. The medicinal properties of this plant family are varied depending on the genus in particular. Several species of the genera belonging to this family such as *Mentha*, *Rosmarinus*, *Origanum*, *Thymus*, *Lavandula* for example, have multiple properties such as antibacterial (Okoh et al. 2010), antifungal (Adam et al. 1998), antiviral (Tada et al. 1994), anti-spasmodic (Babaei et al. 2008) and anti-inflammatory (Conforti et al. 2008) etc.

1.4 *Lavandula* genus: *Lavandula latifolia* Medik.

1.4.1 *Lavandula* genus

Lavandula belongs to the subfamily *Nepetoideae* and it is formed by a total of 39 species. It is a group of typical Mediterranean plants, also which also spreads in the Macaronesian region and is dispersed throughout northern Africa, southern Asia and Arabian Peninsula (Morales 2010a).

The plants of this genus are typical of limestone soils and are shrubby perennial or short-lived herbaceous plants. Leaves can be entirely or deeply dissected and even absent in some species. The species of this family are characterized by having an inflorescence composed of compact terminal spikes of flowers that are held on a peduncle. The corolla is usually lilac, violet or blue. The most important species of this genus are *Lavandula angustifolia*, *L. latifolia* and the hybrids created from both (Lavandins).

1.4.2 Uses and properties

Lavandula species are mainly grown for the perfumery industry. Their essential oils are widely used in cosmetics and perfumes and in recent years its use for aromatherapy has increased. Its medicinal use is less widespread but is also important, especially its sedative properties. Infusions of lavender are a common product in the market and there are several scientific studies on its efficacy to cause relaxation and sleepiness avoiding the side effects that some medications can cause. Kasper et al (2010) proved that essential oils extracted from *Lavandula* genus can improve the quality of sleep and reduce anxiety and stress without side effects. Among other medicinal properties are its tonic and antispasmodic action (Vanaclocha and Cañigueral 2003). In addition, anticancer properties are also attributed to this species due to the content of perillyl alcohol (Gerhard 2002). Spike lavender also has traditional pharmacological actions such as tonic, antiseptic, antispasmodic, organoleptic corrective properties (Vanaclocha et al. 1992) and hypoglycemic effects (Gamez et al. 1988).

In general, *Lavandula* species also have antimicrobial effects. This property is variable within the species and spike lavender, containing a high proportion of camphor, has the most powerful effect (Lis-Balchin 2002b). Some species also have antifungal properties. Hanamanthagouda et al. (2010) proved that *L. pinnatata* essential oils can inhibit the growth of several bacterial and fungal strains. The effectiveness of *Lavandula* as an anti-moth product is also unquestionable, since most of the manufactured products contain this species, López et al. (2007) analysed the antifungal activity of spike lavender against *Rhyzopus solanifer*, although this species was not the most effective species of the extracts tested, it showed inhibition. Rota et al. (2004) tested Spike lavender essential oil against *S. thypimurim*, *S. flexneri*, *L. monocytogenes*, *S. aureus* showing inhibition for all the strains.

On the other hand, *Lavandula* genus species also have considerable antioxidant properties. Gülçin et al. (2004) for example, found a very good antioxidant result for *L. stoechas* L. and Spiridon et al. (2011)analysed *L. angustifolia* and although it was less active than *Origanum vulgare* and *Melissa officinalis*, results were also significant.

1.4.3 *Lavandula latifolia* Medik.

L. latifolia grows naturally in Spain. It appears at very different altitudes, some plants are found high up in the mountains and others at low altitude (Lis-Balchin 2002a). This species is also distributed throughout France, Italy and the former Yugoslavia(Morales 2010a).

As its name suggests (*latifolia*), this species is characterized by broader leaves than other plants belonging to the genus *Lavandula*. It is a shrub with a woody base and a thick root that can have a length of up to 40 cm. The stem is woody and also about 10 to 15 cm tall. Flowers have different colorations within blue-violet range and are grouped into glomeruli. The corolla is bilabiate and tubular and between 8-10 cm long. Flower spikes are formed by cymes that are supported by bracteoles of about 3 or 4 mm. The fruit is a dark tetrachaenium with 4 seeds of about 2 mm. The leaves are greenish and greyish and vary from lanceolate to spatulate. The stem is often trident-shaped branched although in many cases there is no other branching. They can reach a height of over 20 cm and the inflorescence has a variable size ranging from 4 to 10 cm. The bracts look different to the vegetative leaves. The flowering date varies with geography and is mainly between the months of July and September.

1.4.4 Variability and chemical composition studies of *L. latifolia*

Although spike lavender is a plant of great commercial interest and the chemical composition of the species is very important for the market, the number of studies related to its chemical composition is not very abundant, unlike what happens with *T. mastichina* and *S. lavandulifolia*.

One of the oldest scientific studies which report the volatile composition of this species is from De Pascual-T et al. (1983) who studied part of the compounds known at present, finding 1,8-cineol, linalool and camphor as main compounds (as is usual for this species). Muñoz-Bertomeu et al. (2007) studied seven wild population of Spike lavender, finding 28 compounds and classifying their essential oils as high, intermediate and low content of linalool. These essential oil types were respectively correlated to the Supra-, Meso- and Thermo- Mediterranean bioclimatic belts and 1,8-cineol, linalool and camphor were found to be the main compounds. In a similar study, Herraiz-Penalver et al. (2013) found the same three compounds as principles,

identifying a total of 40 compounds and finding a certain relationship between the chemical composition of the essential oils and their geographical origin.

On the other hand, Eikiani et al. (2008) compared different methods for the extraction of spike lavender essential oil, using superheated water extraction, hydrodistillation and soxhlet extraction, obtaining as a result that the superheated water extraction method was quicker and more selective for oxygenated constituents. Last but not least, Barazandeh et al. (2002) studied *L. latifolia* cultivated in a garden in Iran, in this study the plant was brought from Europe and cultivated for 30 years in the garden. Unlike other studies, Barazandeh et al. (2002) and Eikiani *et al*. (2008) found borneol instead of camphor as one of the three main compounds.

Although there are many studies regarding the volatile compounds of essential oils of spike lavender, Spanish sage and Spanish marjoram, there is very little literature concerning the polyphenol content of these three species. Shimizu et al. (1990) studied the anti-inflammatory effect of *L. latifolia* finding three polyphenols with this effect: coumarin, 7-methoxycoumarin and trans-phytol. Lamaison et al. (1990) studied rosmarinic acid content of several Apiaceae, Boraginaceae and Lamiaceae species finding an amount of 700 mg/100 g DW of rosmarinic acid in spike lavender. Although there are few polyphenols studies on *L. latifolia* and these are not up to date, the studies on lavandin are more complete and updated, like the work of Torres-Claveria et al. (2007) detecting a total of 32 polyphenols in lavandin waste using liquid chromatography coupled to ionspray mass spectrometry (LC/MS/MS).

1.5 Salvia genus: *Salvia lavandulifolia* Vahl.

1.5.1 *Salvia* genus

Salvia is a cosmopolitan and diverse genus which comprises about 900-1000 species. It is a genus distributed worldwide and more than half of the species are found in Central America and South America(Sáez 2010; Walker and Sytsma 2007). Of all the species of the genus, the most cultivated and most important in the world market is *S. officinalis* and is popularly known for its use in food, as an ornamental and a medicinal plant. *S. sclarea, S. officinalis, S. lavandulifolia* are widely used for essential oil extraction (Lubbe and Verpoorte 2011) and other commercial sage species also include *S. fruticosa, S. verbenaca,* and *S. tomentosa* (Kintzios 2004).

It is a plant that can be multiplied by seeds, cuttings, layering and division rooted feet (Muñoz 1987) and is able to grow in diverse habitats. The colour of the corolla can be blue, red or white and less commonly yellow. Unlike the rest of *Labiatae*, the androecium consists of two stamens.

1.5.2 Uses and properties

The genus *Salvia* is mainly used as an ornamental plant, for its medicinal properties or for essential oil extraction. As regards its medical properties, it is noted by its memory enhancing properties (Eidi et al. 2006; Tildesley et al. 2003) and anti-inflammatory action (Dweck 2005; Kintzios 2004). Sage infusions are also traditionally used from dry or fresh plant parts as a remedy for ailments such as abdominal pains, coughs, sore throat, stomatitis, gingivitis, toothaches, diarrhoea, stomach ache, diabetes, hypertension, rheumatism, skin diseases, perspiration, fever; as a carminative, spasmolytic; antiseptic/bactericidal; astringent; as a gargle or mouthwash against the inflammation of the mouth, tongue and throat; a wound-healing agent; in skin and hair care; and against rheumatism and sexual debility in treating mental and nervous conditions (Kintzios 2004; Vanaclocha and Cañigueral 2003; Veličković et al. 2003).

Besides these medical uses, several species of the genus have also shown antioxidant activity (Asensio-S-Manzanera et al. 2011; Lu and Foo 2001). Ramos et al. (2010) demonstrated that due to the existence of polyphenols *Salvia* genus shoes a DNA protective effect.

As is common for the Lamiacea family, biocide properties inhibiting bacteria and fungi have also been proved (Longaray Delamare et al. 2007; Pitarokili et al. 2003). Giner et al. (2012) studied the effect of a mixture of plant extracts containing *S. lavandulifolia, R. officinalis* and *T. mastichina*. The mixture was tested against microorganisms that usually appear in tomato soup, showing inhibition of the bacteria: *E. coli*, *S. enterica*, *E. aerogenes*, *B. cereus*, *S. aureus*. The same mixture was also studied by Vegara et al. (2011) finding inhibition of *E. coli* and *S. aureus.*

With regard to the essential oils, Santana et al. (2012) also proved the effectiveness of the essential oils of Spanish sage against fungi demonstrating inhibition for *Fusarium moniliforme***,** *F. oxysporum* and *F. solani* and antifeedant activity for *L. decemlineata, S. littoralis, M. persicae* and *R. padi*. Usano-Alemany et al. (2012) tested the essential oil extracted from Spanish sage in the different seasons of the year against several bacteria (*E. coli, B. subtilis, B. polymyxa, E. aerogenes, P. fluorescens, S. marcescens, Salmonella* sp) and fungi (*C. albicans*) finding inhibition for all the microorganisms tested, however, the essential oils coming from autumn and winter produced higher inhibition on the growth of most of the bacteria used.

Pierozan et al. (2009) found good results against *B. subtilis*, *E. faecalis*, *M. luteus*, *S. aureus*, *S. mutans,* finding that essential oil of Spanish sage is more effective as a bacteriostatic agent against Gram-positive microorganisms.

On the other hand *Salvia* has also been used as a drug target for Alzheimer showing positive effects in patients with healthy memory or dementia (Akhondzadeh et al. 2003; Perry et al. 2003). *S. lavandulifolia* has shown anticholinesterase activity and a protective effect against Alzheimer (Perry et al. 2003; Savelev et al. 2003; Tildesley et al. 2003) demonstrating also anti-inflammatory, oestrogenic and sedative effects all of which were relevant to the treatment of Alzheimer's disease.

1.5.3 *Salvia lavandulifolia* Vahl.

This species can be found in the calcareous mountains from the western Mediterranean area or in limestone, marl and gypsiferous soils at a height between 200 and 2000 meters, however it is more common to find it between 600 and 1000 m of altitude (Burillo Alquézar 2003). It is grows in Spain, southern France and north-west Africa(Sáez 2010). It is a shrub with woody stems at the base that reaches a height of 20 to 65 cm. The leaves are simple petiolates and the corolla is frequently pink, blue or violet with a height of around 15 to 40 mm. Androecium is formed by two stamens. This plant can withstand shallow soils and inhospitable environmental conditions; however it does not resist the excess of water.

The essential oil production is around 13.90 L/ha (Mossi et al. 2011). This species presents a huge morphological and chemical variability and the blossom period extends from June to September depending on the geographical location and in Spain it extends from late June to early August.

Subspecies (Sáez 2010):

- Subsp. *lavandulifolia.* Usually a hairy calyx with teeth smaller than 3 mm. Widespread in the centre and East of Spain.
- Subsp. *vellerea*. Usually persistent bracts, size of teeth of the calyx between 3 and 5 mm. It grows in the East and South of the Iberian Peninsula.
- Subsp. *mariolensis*. Bracts usually not persistent, size of teeth of the the calyx between 0.7 and 2.2 mm. It grows only in the East of Spain.

- Subsp. *blancoana*. Open and branched inflorescence, early deciduous bracts, calyx with indumentum composed mainly by glanduliferum hairs; corolla size between 24 and 40 mm. Characteristic of Spain and Northern Africa.
- Subsp. *oxyodon*. Glabrescent calyx with teeth larger than 3 mm. Spread through the East of Spain.

1.5.4 Variability and chemical composition studies

Although there is a huge chemical variability for *S. lavandulifolia* essential oil, there are no chemotypes clearly accepted. However Jordan et al. (2009) classified several samples of *S. lavandulifolia* subsp. *vellerea* in different chemotype groups because there was a great chemical variability regarding the main compounds.

With current methodologies and chromatographic techniques there is a large amount of secondary plant metabolites that can be known, and *Salvia* presents a huge variability of these compounds.

With respect to the chemical composition of *S. lavandulifolia*, what characterizes this species is the absence of α and β-thujone which are chemical markers that distinguish *S. lavandulifolia* from *S. officinalis* (Guillén et al. 1996; Herraiz-Penalver et al. 2010; Mathe et al. 2006; Perry et al. 2003). However, some authors have found large amounts of these compounds in Spanish sage. For example, Mossi et al. (2011) and Pierozan et al. (2009) found a content of these compounds of 18.95%±7.7 for α-thujone and 19.96%±3.5 for β-thujone. On the other hand, Guillen et al. (1996) found a content of α-thujone of 11.07%. This is not typical of this species; however this could be due to hybridization with other species of *Salvia*, to particular environmental conditions that favor the formation of thujone, or misidentification of specimens.

Usano-Alemany et al. (2012) studied the seasonal influence on the yield and in the chemical composition obtaining an important variation both in the yield and in the chemical composition during the different seasons, finding as main compounds: 1,8-cineol (21.4-33.8%); limonene (5.6-10.4%); α-pinene (10.5-17.5%); β-pinene (6.0-17.3%); myrcene (t-10.0%); camphor (6.1-9.4%); and *trans*-caryophyllene (4.0-8.5%), which varied according to the seasons. Contrary to what happens with other species (e.g. *T. mastichina* or *L. latifolia*), where the main compounds are stable and remain as principal for that species, for Spanish sage major compounds vary widely depending on the collection site, the environmental conditions etc. Herraiz-Peñalver et al. (2010) found a similar composition analysing 20 wild populations of this species, obtaining a huge range of variability for the main compounds (1,8-cineol 6.4-34.5%; limonene 0.8-16.2%;

α-pinene 6.7-23.2%; *β*-pinene 3.8-19.2%; camphor 0-15.4%). The values mentioned values represent the composition of essential oil extracted from the whole plant. However, Schmiderer et al. (2008) in a more detailed study, described the composition of the different parts of this species finding that leaves and calyces are characterized by the main compounds, 1,8-cineol, camphor and *β*-pinene, and by the sesquiterpenes *α*-humulene and *β*-caryophyllene and the corolla shows the highest proportion of borneol, but a low concentration of camphor.

As already mentioned, there are many studies on the composition of the essential oils of Spanish Sage, but there is little and outdated literature on *S. lavandulifolia* polyphenols. Lu and Foo (2002) made a complete review concerning the polyphenols dectected in several *Salvia* species finding several publication from various authors on Spanish Sage polyphenols between 1974 and 1999 reporting several compounds such as rosmarinic acid, apigenin, genkwanin, 7,4´-dimethyl ether, luteolin, chrysoeriol, hispidulin, cirsimaritin, nepetin, eupatorin and 6,7,3´,4´-tetramethyl ether.

1.6 Thymus genus: *Thymus mastichina* L.

1.6.1 *Thymus* genus

Thymus belongs to the subfamily Nepetoideae. It comprises by a total of 220 species and it extends across Eurasia, the coast of Greenland, the Macaronesian region, North Africa and the mountains of Abyssinia and Yemen (Morales 2010b). It is a genus characteristic of the Iberian Peninsula and it includes several endemic species from there. The flowering phase begins between March and June depending on the species in particular. Multiplication can be done by seed, vegetative division or cuttings-feet. It grows in a warm temperate climate and mountain climate and is resistant to frost and drought but not to excess humidity and mainly grows on limestone or clay soils (Muñoz 1987). The calyx is bilabiate, reaches between 3 and 8 mm and the colours range between green, red or whitish. The corolla is also bilabiate and ranges from 2 to 18 mm, the colour can be white, yellow, pink or purple.

1.6.2 Uses and properties

Thymus is known since ancient times and used for medicinal and culinary purposes. Currently the plants belonging to this genus have a remarkable importance in the pharmaceutical and perfume industry and are also used for ornamental purposes.

The most used and produced species for essential oil extraction are *Thymus vulgaris* and *Thymus zygis*, mainly cultivated in Europe for its ornamental and medicinal properties and with a worldwide annual production of between 50-100 t (Lubbe and Verpoorte 2011).

There is great variability in chemical composition and frequently different chemotypes are found in some species of the genus. The characteristic compounds from genus *Thymus*, are as in the Lamiacea family, secondary metabolites mainly classified in groups of terpene derivatives and polyphenols which confer on this genus several properties:

Regarding its medicinal properties, *Thymus* L. is distinguished by its spasmolytic properties. As well as its ability to act as an antiseptic, anti-inflammatory and antioxidant (Vanaclocha et al. 1992). Albano et al. (2011) showed that *T. mastichina* has a high ability to inhibit 5-lipoxygenase and thus demonstrate anti-inflammatory activity. Besides, Gordo et al. (2012) found that extract of *T. mastichina* may have a protective effect against colon cancers.

The antiseptic properties coupled with their ability to act as an antioxidant have resulted in several novel applications in the food industry as for example their use as livestock feed for the transfer to cheese and milk for beneficial active substances (Boutoial et al. 2013) or its uses as preservative incorporated to food packaging acting as active protecting film avoiding food contamination by microorganisms (Mehdizadeh et al. 2012) and extending the life of the product (Kykkidou et al. 2009). Another new use found for the aqueous extract of *Thymus* is the ability to prevent alcohol toxicity. Tests were performed on mice proving that the use of aqueous extracts of *Thymus* disinfect wounds liver and brain damage caused by alcohol abuse (Shati and Elsaid 2009).

In recent decades there has also been a growing search for natural biocides to give them a practical use such as the application thereof as pesticides, medicines, antiseptics etc. Among the different biocide action is their use as antibacterial (Marino et al. 1999), antifungal (Reddy et al. 1998), insecticides (Mansour et al. 2000) or antiviral products (Nolkemper et al. 2006). The biocide properties of *T. mastichina* have been amply demonstrated by several authors showing its ability to act as bactericide, insecticide and fungicide.

With regard to the antibacterial activity of *T. mastichina*, Faleiro et al. (2003), demonstrated that its essential oil inhibited *C. albicans*, *E. coli*, *L. monocytogenes*, *P. mirabilis*, *Salmonella* sp, *S. aureus*. Moreover, Vegara et al. (2011) found that some components of *T. mastichina* can reduce *E. coli* and *S. aureus*. In the same way, Pouvova et al. (2008) found inhibition of *C.*

michiganensis using *T. mastichina* essential oils. Concerning its insecticidal properties, Pavela et al. (2005) found that essential oils of *T. mastichina* were highly toxic against larvae of *S. littoralis* with $LD_{50} \leq 0.05$ µl/larvae. Regarding the fungicide properties of this plant species, Fraternale et al. (2003) found antifungal activity against eight pathogenic fungi of the genus *Fusarium* (*F. culmorum*, *F. graminearum*, *F. poae* , *F. avenaceum*, *F. equiseti*, *F. semitectum*, *F. sporotrichoides*, *F. nivale*) and López-Escudero et al. (2007) proved that this plant is highly effective in reducing the viability of *V. dahliae* microsclerotia. Furthermore, Pina-Vaz et al. (2004) found antifungal activity of essential oil of *T. mastichina* against several *Candida* species and Leal et al. (2013) found that extracts of *T. mastichina* may be particularly useful against *A. fumigatus.*

1.6.3 *Thymus mastichina* L.

Thymus mastichina L. is an endemic species from the Iberian Peninsula with simple and opposite leaves. Flowers are zygomorphic and bilabiate and are grouped in clusters of white color. The androecium has four external stamens and the gynoecium has an ovary divided into four parts. It has entomogamous pollination and seeds are dispersed by autochory. It grows in sandy and siliceous soils and the flowering period extends from late May and early June to late July, early August.

Subspecies (Morales 2010b)

- Subsp. *mastichina* when the calyx is larger than 5 mm; inflorescences are bigger than 10 mm in diameter and bracts are oblong-ovate or elliptic.
- Subspecies *donyanae* when the calyx has a size equal to or less than 5 mm. inflorescences are smaller than 10 mm of diameter and bracts are widely ovate.

The subspecies *donyanae* is only present in the southwest of the Iberian Peninsula (Doñana and Algarve) while the subspecies *mastichina* extends throughout the Iberian Peninsula but it is less abundant on the northwest coast of Spain and the Mediterranean coastline.

1.6.4 Variability and chemical composition studies

Regarding its chemical composition, this species is classified into three chemotypes with two compounds as major constituent (Adzet et al. 1977).

- The first and most widespread chemotype contains 1,8-cineol as main compound.

- The second chemotype contains linalool as main compound
- The third chemotype is the intermediate chemotype with both compounds as principles (1,8-cineol-linalool).

There are many phytochemical studies conducted with *T. mastichina* regarding the composition of its essential oil because of its great commercial interest. Most studies are focused on the chemical characterization and variability of the species and some even characterized the composition of the subspecies (Salgueiro et al. 1997). Miguel et al. (2004b) for example, studied the effect of harvesting date and the chemical variability between wild and cultivated individuals.The composition between different organs of plants was studied in *T. mastichina* by Miguel et al. (2004a), who found different composition in flowers and leaves from different populations. The essential oil yield was lower in leaves than in flowers and leaves had a higher content of camphor.

The chemical composition of *T. mastichina* was in some studies correlated with the biological activity of the essential oil (Faleiro et al. 2003). On the other hand the relative percentages of volatiles in the essential oil of this species have shown correlation with environmental parameters such us humidity (1997). The variability in the chemical composition produced by using various extraction technique was studied by Jimenez et al. (1999) who studied the difference between continuous subcritical water extraction and hydrodistillation.

The antioxidant activity of *T. mastichina* extracts was reported by Bentes et al. (2009) and regarding the *T. mastichina* polyphenols, Gordo et al. (2012) identified 9 compounds (sakuranetin, sterubin, oleanolic acid, usolic acid, luteolin, beta-sitosterol, rosmarinic acid, 6-hydroxyluteolin-7-O-beta-glucopyranoside and hydroxiapigenin-7-O-beta-glucopyranoside) and proved their anti-cancer activity. On the other hand, Sánchez-Vioque et al. (2013) and Delgado et al. (2014) studied the waste product obtained with *T. mastichina* after the distillation process. The list of tentative major compounds identified by LC–MS in *T. mastichina* solid residues from Soxhlet and ultrasound-assisted extractions by Sánchez-Vioque et al. (2013) were: quercetin glucoside, luteolin glucoside, rosmarinic acid, apigenin-7-O-glucoside, quercetin, luteolin derivative, luteolin, naringenin, carnosol, apigenin and kaempferol. On the other hand, Delgado et al. (2014) found in the methanolic extracts of the waste product: rosmarinic acid as the most abundant polyphenol, followed by methoxysalicylic acid, apigenin, kaempferol and luteolin.

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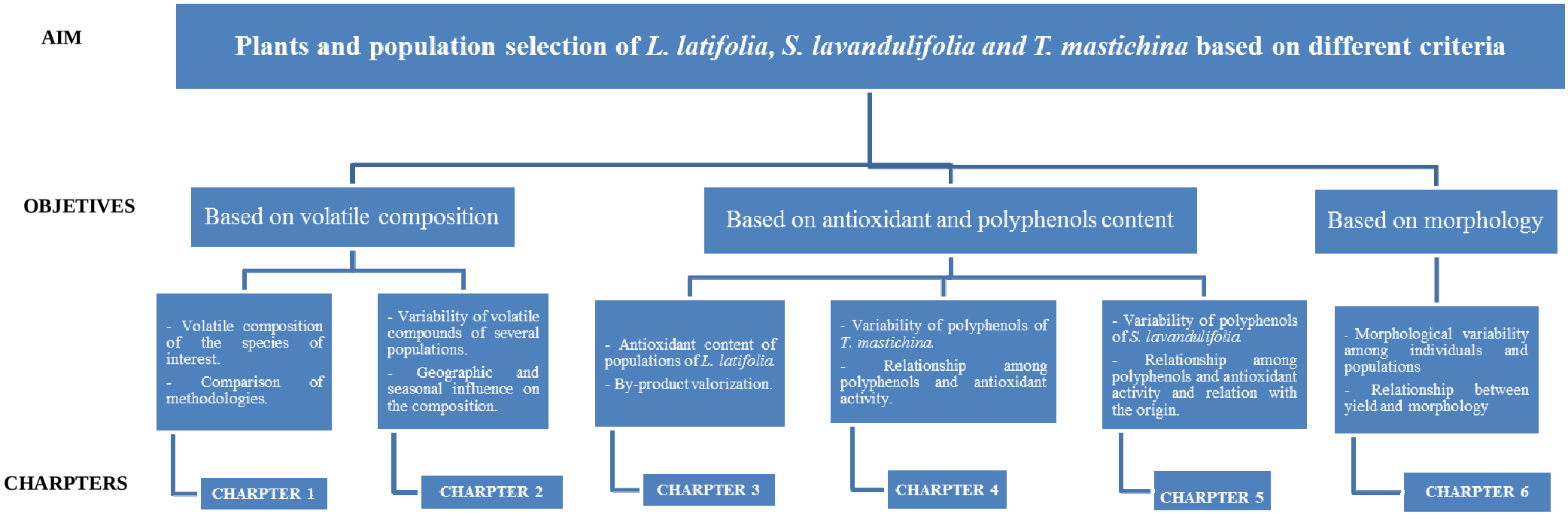
Objectives and thesis outline

Scope of the thesis


AIM
Plants and population selection of L. latifolia, S. lavandulifolia and T. mastichina based on different criteria
OBJETIVES
Based on volatile composition
Based on antioxidant and polyphenols content
Based on morphology
- Volatile composition of the species of interest.
- Comparison of methodologies.
- Variability of volatile compounds of several populations.
- Geographic and seasonal influence on the composition.
- Antioxidant content of populations of L. latifolia.
- By-product valorization.
- Variability of polyphenols of T. mastichina.
- Relationship among polyphenols and antioxidant activity.
- Variability of polyphenols of S. lavandulifolia.
- Relationship among polyphenols and antioxidant activity and relation with the origin.
- Morphological variability among individuals and populations
- Relationship between yield and morphology
CHARPTERS
CHARPTER 1
CHARPTER 2
CHARPTER 3
CHARPTER 4
CHARPTER 5
CHARPTER 6

2.1. MOTIVATION AND PURPOSE

Traditionally aromatic and medicinal plants have been collected from the wild. However, wild collection does not allow high quality plant material to be collected and produces deforestation thus threatening the species harvested. It should be added that strict rules are published to regulate the quality of the volatiles. Additionally, when the plant material is intended for medicinal use, the effectiveness of the different active substances such as polyphenols and the concentration thereof, might varies hugely from one individual to another. The need to find high quality specimens and to cultivate the selected individuals not only increases the yield and the quality of collected plant material but also enables sustainable development and conservation of the species. In addition to that, some of the parameters for cultivating these species are unknown. In order to overcome such problems, the chemical variability of the secondary metabolites and morphological variation of *Lavandula latifolia* Medik., *Salvia lavandulifolia* Vahl. L. and *Thymus mastichina* L. has been evaluated to find high quality individuals.

The plants under study seem to have a wide morphological and chemical variability in their natural habitat. This variation reflects adaptation to the local environmental factors showing the degree of relation between the environmental conditions and the population genotypes and also offering information about the evolutionary stability of a species.

Many species have been barely prospected because their analysis is time-consuming and implies long journeys. However, this process makes it possible to classify the germoplasm into groups and offers the market the possibility of selecting the specimens or populations with the most desired features.

The analysis of this variability is the necessary basis for domestication and cultivation, for selecting high performance individuals and thus for commercial exploitation.

2.2. OBJECTIVES

The main objective of the thesis was the analysis of variability in a broad sense of *L. latifolia*, *S. lavandulifolia* and *T. mastichina* in order to select high quality plant material. The study of this variability has been addressed from different points of view with the purpose of achieving the main objective:

a) Evaluation and optimization of the methodology necessary to carry out the analysis of variability.

b) Evaluation of the variability of volatile compounds composition of wild populations.

c) Study of the polyphenols profile and the antioxidant activity of different populations.

d) Examination of the influence of different factors on the essential oils composition and polyphenols production such as environmental factors.

e) Study of the morphological variability of different populations in order to perform an agronomic selection of each species.

2.3. THESIS OUTLINE

In the General Introduction, a generic overview regarding aromatic and medicinal plant production and current uses in Spain and Castilla y León is presented as well as a brief description of the species of interest, the secondary metabolites classification and the uses of the selected species.

In Scope of the thesis the objectives of the thesis and the thesis outline are presented.

Chapter 1 shows a comparison of the classical method for the extraction of volatile compounds from plants (hydrodistillation), with other methods such as micro-distillation and extraction with organic solvent and ultrasounds. Hydrodistillation is the method used industrially, but requires a larger amount of plant material and a longer process. In order to see if the methods are comparable and reduce the time of analysis and the amount of plant material required, other two methods were studied. The objective was to find a suitable methodology for an easier and quicker study of plant material.

In Chapter 2, the variability of volatile compounds of the essential oils of several wild populations of *L. latifolia*, *S. lavandulifolia* and *T. mastichina* collected in Castilla y León has been evaluated, studying the geographic and annual influence on the composition of essential oils.

A large volume of waste is generated each year by the distillery industry, which is a major environmental problem and reuse and revaluation of by-products can help to improve the

economic performance of the industries in an environmental friendly way. **Chapter 3** includes the antioxidant content of several wild populations of *L. latifolia* collected in Castilla y León, as well as the antioxidant content of plant by-products generated after distillation which could be revaluated for their antioxidant content. It also includes the variability of several polyphenols analysed in the waste.

Once the chemical variability of wild populations had been evaluated, the study began of three plot assays (one for each species) established in ITACyL to subjet all the populations and individuals under the same environmental conditions. Populations were chosen trying to maintain maximum variability found in previous surveys. Intra-population variability of populations was kept collecting 25 plants per population and reproducing them through vegetative propagation. The trials were planted in a randomized block design with three replications.

The target of these plot assays was to subject all populations and individuals to the same environmental conditions and to study the variability among and within populations from a chemical and morphological point of view.

Chapter 4 contains a study of the variability of the polyphenols of 14 populations of *T. mastichina* cultivated in the plot assay, as well as the study of the antioxidant potential and its relationship with the main polyphenols of this species.

Chapter 5 includes a study of the polyphenolic variability of seven *Salvia lavandulifolia* populations cultivated in the experimental field.

With regard to the need to improve the agronomic parameters, **Chapter 6** includes a study of the morphology of *T. mastichina* and the morphological variability among individuals and populations.

The **Conclusions** present the general closures.

Chapter 1

Volatile fraction differences for Lamiaceae species using different extraction methodologies

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Volatile fraction differences for Lamiaceae species using different extraction methodologies..

Abstract:

To achieve a detailed chemical characterization and to find the changes in the composition the volatiles of *Lavandula latifolia*, *Salvia lavandulifolia* and *Thymus mastichina* were analyzed through GC-FID/MS and a total of 47, 48 and 48 compounds were identified, respectively. 1,8-cineol+limonene was the main compound in the three species. Three extracting methodologies were used: hydrodistillation (HD), microdistillation (MD) and dichloromethane extraction (EX). The amount of volatile compounds was affected by the kind of method used and by the kind of species analyzed, thus, *S. lavandulifolia* produced a higher amount of volatiles with the EX and *T. mastichina* with MD. HD showed significantly lower amount of volatiles for the three species. With HD and MD a higher amount of *β*-pinene was obtained. MD produced a higher proportion of 1,8-cineol+limonene. Canonical discrimination function was done using the two most predictable compounds to distinguish among techniques revealing the method used for each species.

Keywords: hydrodistillation, microdistillation, solvent extraction, spike lavender, Spanish sage and Spanish marjoram.

3.1. Introduction

The *Lamiaceae* family, rich in essential oil producing species, comprises 186 genera and around 5000 species. *Lavandula*, *Salvia*, and *Thymus* contribute a total of 39, 900 and 220 species, respectively (Morales et al. 2010), to this family. Many different species belonging to these three genera are used in perfumery, cosmetics, medicine and the food industry. *Lavandula latifolia* Medik. (LL), *Salvia lavandulifolia* Vahl. (SL) and *Thymus mastichina* L. (TM), as part of the mentioned genera, were selected for the study due to their high commercial importance. TM is endemic to the Iberian Peninsula and all three species are characteristic wild plants from Spain.

The uncontrolled collection of aromatic and medicinal plants from the wilderness is a global issue. More than 60% of the plants that are marketed around the world are collected from their

natural environment (Laird 2005), which poses a threat for many species. Currently, Medicinal and Aromatic Plants (MAPs) selection programmes are essential tools to guarantee traceability and to monitor cultivation parameters. The running of selection programmes requires large samples. The analysis of the volatile compounds of these plant species is also necessary to assess the established quality parameters. Hydrodistillation is the most common technique used for the extraction of volatile compounds, and is accepted by the European Pharmacopeia to market essential oils. However, the amount provided by a single plant is sometimes too small to be hydrodistilled, and hydrodistillation of a large number of samples is a lengthy process.

Alongside hydrodistillation (HD), there are other techniques such as microdistillation (MD) and solvent extraction (EX) which can be carried out with smaller samples of plant material (Baser et al. 2001) and also take less processing time than HD (Da Porto et al. 2009). These techniques could be useful to run selection programmes. As reported by other studies (Da Porto et al. 2009; Özek et al. 2006; Özek et al. 2005), different extraction methods usually lead to changes in the volatile composition. When using other techniques such as MD and EX to analyze a high number of samples for the chemical characterization of volatiles in a short time span, knowledge of the kind of changes that occur in the composition of volatiles and of the differences among methods is essential.

In order to achieve a detailed chemical characterization of LL, SL and TM, and to detect possible changes in chemical composition, the extraction of the volatile compounds was carried out using three different techniques: HD, MD and EX.

3.2. Materials and Methods

Plant materials

Representative samples of *Lavandula latifolia*, *Salvia lavandulifolia* and *Thymus mastichina* were collected from the region of Castile and León, Spain, at the time of full blossom, between June and August 2011. *Lavandula latifolia* was collected from Cevico Navero (Palencia) on 9 August 2011, from an altitude of 876 m, and within the coordinates 415657N and 0042228W; *Salvia lavandulifolia* was collected from Cevico Navero (Palencia) on 6 June 2011, from an altitude of 916 m, within the coordinates z415221N and 0041136W; and *Thymus mastichina* was collected from Carrocera (León) on 16 June 2011, from an altitude of 1029 m, within the coordinates 424942N and 0115213W.

The samples were air-dried at room temperature and stored away from light. Flowers and leaves were separated from the stems and used for further analysis. The analyses were based on homogeneous samples of plant materials from each species.

Voucher specimens of the samples were deposited in the herbarium of the botanic area (PALAB) of the Yutera Campus, University of Valladolid (specimen codes: LL-11/11, SL-11/11 and TM-37/11).

Hydrodistillation

Each species was distilled in a modified Clevenger-type apparatus. HD was performed on an amount of 2g and repeated four times for each species. The volume of the flask was 500 mL, to which 200 mL of distilled water were added. The distillation was performed for 1 hour, following the methodology of Schmiderer *et al*. (2008), and the sample was kept at -20 °C until further analysis. Biphenyl was used as internal standard with a concentration of 0.1 mg/mL.

Microdistillation

Four replicate analyses were performed using an Eppendorf MicroDistiller® apparatus (Eppendorf, Hamburg, Germany). An amount of 400 mg of plant material was weighed in a sample vial and 10 mL of distilled water were added. In the collecting vial, 500 mg of sodium chloride and 300 µl of dichloromethane were added. Sample vials were heated at 20ºC/minute until reaching 108ºC. This temperature was maintained for 15 minutes and then increased at the same rate until reaching 112ºC, and maintained for 45 minutes ending with a post run of 3 minutes. Collection vials were kept at a temperature of -2ºC throughout the MD process. When the process was finished, 1 mL of water was added to the collection vials and the layer containing the volatile fraction was collected with a pipette and transferred into a GC vial. Biphenyl was used as internal standard at a concentration of 0.2 mg/mL. The sample was kept at -20°C until further analysis.

Solvent extraction

An amount of 500 mg of plant material was added to 3 mL of dichloromethane and the sample was introduced in an ultrasonic water bath at room temperature for 1 hour. Biphenyl with a

concentration of 0.1 mg/mL was used as internal standard. The extracts were filtered with cellulose wadding in a Pasteur pipette and analysed by GC.

GC-MS and GC-FID analysis

The analysis of the samples was carried out using a GC-FID (6890N Network GC system Agilent Technologies, Palo Alto, CA, USA) equipped with a FID and fitted with a DB-5 narrow bore column (10 m x 0.1 mm id. x 0.17 μm film thickness). Helium was used as carrier gas. The front inlet was kept at 260ºC with a split ratio of 100:1. Injection volume was 0.2 μl. Temperature program: 60ºC for 1 minute; 60-85ºC at a rate of 8ºC/minute; 85-280ºC at a rate of 15ºC/minute; 280-300ºC at 30ºC/minute, and maintained at 300ºC for 5 minutes.

The GC-MS analysis was carried out using a GC-MS HP 6890 coupled with a HP 5972 MSD (Hewlett-Packard, Palo Alto, CA, USA). The GC was equipped with a DB-5MS capillary column (30 m x 0.25 mm id. x 0.25 μm film thickness). Helium (2 mL/minute) was used as carrier gas. GC oven temperature programme was 60ºC for 4 minutes, which was raised to 100ºC at 5ºC/minute, and then from 100ºC to 280ºC at 9ºC/minute. Liner Retention Indices (LRI) were calculated using homologous n-alkane hydrocarbons (Mixture of alkanes for gas chromatography, Sigma-Aldrich, Vienna, Austria). The different compounds of the volatile fraction were identified by comparison of the mass spectra of every compound with a mass spectra library of essential oils, and confirmed by comparison of retention indices from literature (Adams 2007).

Statistical analysis

The statistical method used was analysis of variance (ANOVA) with SPSS 15, and the average values were compared using the Tukey B test at $P<0.05$. Discriminant function analysis using the stepwise method and selecting the two most predictive variables for each plant species was performed to uncover the extraction methodologies.

3.3. Results and Discussion

***Lavandula* l*atifolia* Medik**

In LL, a total of 47 compounds were identified, of which 38 were monoterpenes and 9 were sesquiterpenes (Table 1). The main compound detected in LL was 1,8-cineole+limonene (since

both compounds were not totally separated in all the samples, they are always mentioned together). The relative percentage of the total peak area (Table 2) of 1,8-cineole+limonene was 38±1.47%, 42.6±1.88% and 37.4±1.63% for HD, MD and EX, respectively. The other main compounds detected in LL were linalool (35.8±0.24%, 34.4±2.1% and 32.6±1.36% in HD, MD and EX, respectively), and camphor (8.6±0.66%, 9.1±0.54% and 9±0.18% for HD, MD and EX, respectively).

In agreement with other authors, these three compounds have also been found as principal compounds in analyses of LL (Muñoz-Bertomeu et al. 2007; Salido et al. 2004) with similar relative percentages. Muñoz-Betomeu *et al*. (Muñoz-Bertomeu et al. 2007) analyzed separately leaves and flowers of LL finding 1,8-cineol (46.8-54.6%) and camphor (34.9-43.5%) as main compounds for leaves and 1,8-cineol (20.8-47.8%), linalool (15.1-54.7%) and camphor (11.4-18.6%) as main compounds for the flowers. Salido *et al*. (Salido et al. 2004) found percentages of linalool of 27.2–43.1%, 1,8-cineole of 28.0–34.9% and camphor of 10.8–23.2%. However, Eikani *et al*. (2002), (29.6%, 22.8% and 14.2% for linalool, 1,8-cineol and borneol, respectively) and Barazandeh *et al*. (2008) (30.6-31.9%, 18.8-20.9% and 8.9-10.1% for linalool, 1,8-cineol and borneol, respectively) found borneol, instead of camphor as one of the three main compounds.

***Salvia lavandulifolia* Vahl**

For SL, 36 monoterpenes and 12 sesquiterpenes were identified (Table 2). The main compound found in SL was 1,8-cineole+limonene (20.9±0.77%, 26±1.1%, and 20.6±0.98% for HD, MD and EX, respectively). Other abundant compounds were viridiflorol (13±1.37%, 7.1±0.4%, and 12.4±0.66% for HD, MD and EX, respectively) α-pinene (8.6±2.32%, 9.2±0.69%, and 7.6±0.44% for HD, MD and EX, respectively) camphor (8.6±0.82%, 13.9±1.07%, and 12.9±1.55% for HD, MD and EX, respectively) myrcene (8.8±0.39%, 7.6±0.74%, and 6.4±0.17% for HD, MD and EX, respectively) and camphene (7.3±1.46%, 7.5±0.34%, and 6±0.34% for HD, MD and EX, respectively).

The different studies about Spanish Sage composition concluded that this species has great intraspecific chemical variability and the main compounds were also very variable depending on the population studied (Herraiz-Penalver et al. 2010; Jordan et al. 2009). The most abundant compounds detected through SL by Herraiz-Peñalver *et al*. (2010) were 1,8-cineole (6.4-34.5%), α-pinene (6.7-23.2%) and β-pinene (3.8-19.2%). They also found important amounts of camphor (0-15.4%) and viridiflorol (0.1-9.7%). Jordán *et al*. (2009) analyzed volatile

compounds for different populations of *Salvia lavandulifolia* subsp. *Vellerea* finding camphor (23.7-32.7%) and 1,8-cineol (16.7-40.7%) as main compounds and other abundant compounds were very variable within the populations studied.

***Thymus mastichina* L.**

In TM, 34 monoterpenes and 14 sesquiterpenes were identified (Table 2). The main compounds was 1.8-cineol+limonene (61.6±2.5%, 69.3±0.3% and 64±1.01% for HD, MD and EX, respectively). Linalool (6±0.67%, 4.6±0.45% and 4.1±0.24% for HD, MD and EX, respectively) and *β*-pinene (5.7±0.5%, 4.4±0.06% and 3.7±0.06% for HD, MD and EX, respectively) were also among the main compounds of the volatile fraction studied.

There are three known chemotypes for TM (Salgueiro et al. 1997): that with 1,8-cineole as main constituent, that with linalool as its main compound, and the intermediate chemotype 1,8-cineole/linalool. All the analyzed samples correspond to 1.8-cineol chemotype, which is the commercial one. The percentage of 1,8-cineol and linalool of the studied samples are similar to those obtained by Miguel *et al*. (2004) for the 1,8-cineol chemotype samples with ranges for 1,8-cineol of 44.2-69.2% for the leaves and 39.4-54.6% for the flowers and ranges for linalool of 0.9-6.3% for the leaves and 1.3-13.7% for the flowers.

Comparison of the different extraction methods

Although, as regards the different extraction methodologies used, no significant differences were observed in the case of many compounds, in other cases an increase in the amount of certain specific compounds could be observed depending on the methodology. Namely, MD always showed significantly higher amounts ($P<0.05$) of 1,8-cineole+limonene and terpinen-4-ol and many of the monoterpenes were significantly higher ($P<0.05$) in HD and MD in comparison with EX as for example *α*-pinene, *β*-pinene and *γ*-terpinene. Caryophyllene oxide was significantly lower ($P<0.05$) in MD and *β*-caryophyllene was always higher for EX.

In accordance with other studies (Özek et al. 2006; Özek et al. 2005), MD and HD showed more qualitative similarities. The total percentages of monoterpene were similar for HD and MD, while for the three plants EX showed a significantly lower percentage ($P<0.05$) of monoterpene. The composition of the volatile fraction obtained with EX was the most different. The use of EX enables the extraction of semi-volatile and non-volatile compounds with higher boiling points that cannot be isolated with HD (Richter and Schellenberg 2007) or with MD. As a

result, the sesquiterpene fraction obtained was higher in solvent extracts than in the other two techniques. This is illustrated by the results, where the percentage of total sesquiterpenes obtained (especially hydrocarbon sesquiterpenes) was significantly higher ($P<0.05$) in EX as compared to MD and HD. Furthermore, the percentage of identified compounds was around 10% lower in EX as compared to HD and MD because a higher proportion of compounds which eluted in the sesquiterpene region was obtained with EX, and several of them could not be identified because sesquiterpenes are more difficult to detect (Merfort 2002; Shellie et al. 2002).

It should also be noted that although HD and MD have a more similar composition, MD always showed the lowest sesquiterpene content of the three methods.

Reactions such as oxidation, isomerization, cyclization or dehydrogenation (Turek and Stintzing 2012) can lead to rearrangements in volatile compounds. In HD and MD, changes are mostly due to the high temperatures required and the long-term exposure to water of the samples required, which may lead to the oxidization, or even destruction, of certain compounds. An example of such change is sabinene hydrate, which suffers isomerization and hydrolysis as a result of prolonged contact with water and heat (Pop and Barth 2001). This makes the content of sabinene hydrate significantly lower ($P<0.05$) in HD and MD with respect to the content in EX for the three plant species. Another example of these reactions is the decrease of α-humulene and β-caryophyllene resulting in increased caryophyllene oxide contents due to oxidation. As shown in the tested samples, the ratio between α-humulene and β-caryophyllene and caryophyllene oxide yielded a higher proportion of caryophyllene oxide for MD and HD as compared to EX.

Regarding the total production of volatile compounds obtained Tables 3, 4 and 5 show the quantification of the main compounds expressed in mg per g of plant material. Total production was higher with EX and MD with differences among the different species, while HD showed the lowest production for all three species. This is probably a result of the fact that the solubility of certain essential oil components in water at boiling temperature can lead to the reduction, or even the disappearance, of some compounds. For LL no significant differences were found for EX and MD. However, when EX was applied to SL, the amount of volatiles obtained was significantly higher than that resulting from MD, since the proportion of sesquiterpenoids is higher in this species than in the other two. For TM the opposite happened.

Uncovering volatile extraction methodology

Canonical discrimination function (CDF) for the volatile fraction of the studied species (Figure 3) was conducted to obtain a predictive model for the extraction methodologies. The outcome proved that it is possible to reveal the way in which the volatile compounds of these species were extracted. The two most predictive variables were selected for each plant species; the most important predictors for LL were *γ*-terpinene, *cis*-sabinene hydrate. For SL the most powerful discriminating compounds were terpinolene and viridiflorol and for TM, germacrene D and terpinolene. The function also showed that, despite the similarities between HD and MD, these two techniques can also be differentiated through a CDF. The results prove the capability of multivariate statistical methods to reveal (unknown) information about the volatile fraction. Multivariate statistical methods are well documented by Oms-Oliu *et al*. (2013) as a useful tool to identify similarities and differences among crop varieties, to know the state of ripening or growth of plants and fruits or to determine the authenticity of food among many other examples, and they could be also useful to detect differences among extraction methodologies.

3.4. Conclusions

Depending on the species tested, the application of different methodologies led to very different results in the composition of the volatile fraction. TM showed few differences between extraction methodologies, while SL showed important differences using EX. Some compounds yielded no significant differences as far as the method applied was concerned, while others produced higher contents with a particular technique.

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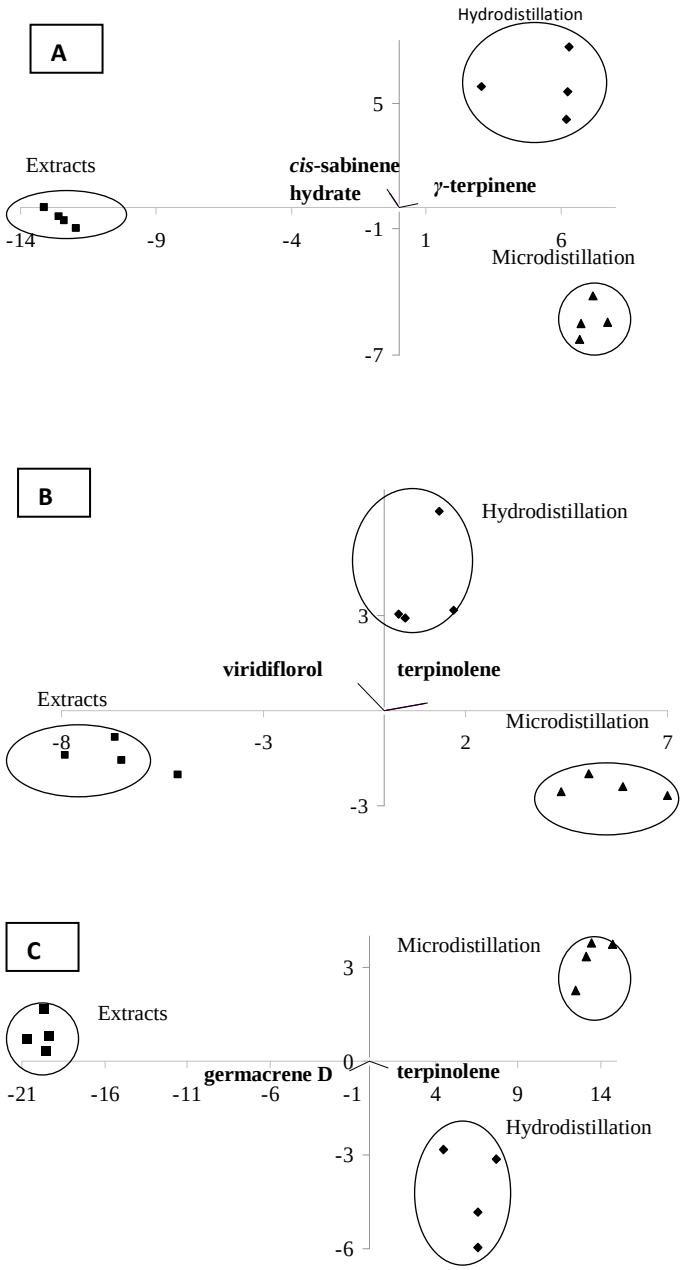

Figure 3: Canonical Discrimination Function for the two most predictive variables for *L. latifolia* (*γ*-terpinene, *cis*-sabinene hydrate) in chart A, *S. lavandulifolia* (terpinolene, viridiflorool) in chart B and *T. mastichina* (Germacrene D and terpinolene) in chart C.

Table 2. Relative percentage of total peak area of the volatiles obtained with Hydrodistillation (HD), Microdistillation (MD) and Solvent Extraction (EX).

RIs[a]	LRI[b]	Compound[c]	LL% HD		LL% MD		LL% EX		SL% HD		SL% MD		SL% EX		TM% HD		TM% MD		TM% EX	
		Monoterpene hydrocarbons																		
926	927	tricyclene	tr		tr		tr		0.3	±0.09	0.3	±0.01	0.3	±0.04	tr		tr		-	
930	931	*α*-thujene	tr		tr		tr		0.3	±0.07	0.3	±0.01	0.3	±0.06	0.2	±0.02	0.2	±0.01	0.1	±0.01
939	**938**	***α*-pinene**	1.5	±0.19	1.3	±0.08	1.1	±0.13	**8.6**	**±2.32**	**9.2**	**±0.69**	**7.6**	**±0.44**	3.6	±0.30	3.0	±0.03	2.5	±0.04
954	953	camphene	0.6	±0.09	0.5	±0.03	0.4	±0.03	**7.3**	**±1.46**	**7.5**	**±0.34**	**6.0**	**±0.34**	0.2	±0.00	0.2	±0.01	0.2	±0.01
960	958	thuja-2.4(10)-diene	tr		tr		tr		-		-		-		-		-		-	
975	976	sabinene	0.6	±0.08	0.4	±0.02	0.5	±0.04	0.5	±0.04	0.4	±0.05	0.5	±0.11	3.6	±0.32	2.6	±0.06	2.5	±0.11
979	980	*β*-pinene	2.0	±0.26	1.7	±0.06	1.5	±0.12	5.4	±0.54	5.1	±0.64	4.1	±0.66	**5.7**	**±0.50**	**4.4**	**±0.06**	**3.7**	**±0.06**
990	991	myrcene	0.5	±0.05	0.4	±0.01	0.4	±0.03	**8.8**	**±0.39**	**7.6**	**±0.74**	**6.4**	**±0.17**	2.0	±0.18	1.5	±0.03	1.2	±0.06
1002	1006	*α*-phellandrene	tr		tr		tr		tr		tr		tr		tr		tr		tr	
1017	1020	*α*-terpinene	0.2	±0.02	0.2	±0.01	tr		0.4	±0.05	0.3	±0.03	0.3	±0.02	0.3	±0.03	0.2	±0.01	0.1	±0.01
1026	1028	*p*-cymene	0.2	±0.03	0.2	±0.01	0.1	±0.01	2.9	±0.30	2.4	±0.25	1.8	±0.22	0.3	±0.03	0.1	±0.12	0.2	±0.03
1031	**1037**	**1,8-cineole+limonene**	**38.0**	**±1.47**	**42.6**	**±1.88**	**37.4**	**±1.63**	**20.9**	**±0.77**	**26.0**	**±1.10**	**20.6**	**±0.98**	**61.6**	**±2.50**	**69.3**	**±0.30**	**64.0**	**±1.01**
1037	1042	*ß-cis*-ocimene	0.3	±0.03	0.2	±0.02	0.2	±0.01	0.2	±0.01	0.2	±0.04	0.2	±0.04	tr		tr		tr	
1050	1053	*ß-trans*-Ocimene	tr		tr		tr		tr		tr		-		1.0	±0.08	0.7	±0.11	0.7	±0.05
1059	1064	*γ*-terpinene	0.3	±0.03	0.3	±0.01	tr	±0.01	1.2	±0.16	1.0	±0.12	0.8	±0.14	0.5	±0.05	0.4	±0.02	0.1	±0.01
		Oxygenated monoterpene																		
1070	1071	*cis*-sabinene hydrate	0.6	±0.07	0.3	±0.03	1.3	±0.06	0.2	±0.04	0.3	±0.04	0.6	±0.04	0.7	±0.09	0.5	±0.05	1.3	±0.03
1072	1075	*cis*-linalool oxide	tr		0.2	±0.03	0.4	±0.01	-		-		-		tr		tr		0.1	±0.00
1086	1083	*trans*-linalool oxide	tr		0.1	±0.02	0.4	±0.07	-		-		-		-		tr		0.1	±0.01
1088	1091	terpinolene	0.3	±0.02	0.2	±0.01	tr		0.3	±0.02	0.2	±0.01	0.2	±0.01	0.2	±0.02	0.1	±0.00	0.1	±0.00
1096	**1099**	**linalool**	**35.8**	**±0.24**	**34.4**	**±2.10**	**32.6**	**±1.36**	0.4	±0.04	0.6	±0.05	0.7	±0.03	**6.0**	**±0.67**	**4.6**	**±0.45**	**4.1**	**±0.24**
1102	1102	*α*-thujone	-		-		-		-		-		-		tr		tr		tr	
1114	1118	*ß*-thujone	-		-		-		0.2	±0.04	0.1	±0.03	0.2	±0.03	-		-		-	
1121	1124	*cis*-p-menth-2-en-1-ol	tr		tr		tr		-		-		-		tr		tr		tr	
1026	1127	*α*-campholenal	0.2	±0.01	0.1	±0.01	0.1	±0.01	tr		tr		tr		tr		tr		tr	
1040	1138	nopinone	0.1	±0.01	0.1	±0.02	0.2	±0.01	-		-		-		tr		tr		0.1	±0.01
1042	1144	*trans*-sabinol	0.4	±0.04	0.2	±0.18	0.3	±0.03	1.4	±0.36	1.6	±0.50	1.8	±0.90	0.2	±0.01	0.2	±0.01	0.1	±0.01
1046	**1148**	**camphor**	**8.6**	**±0.66**	**9.1**	**±0.54**	**9.0**	**±0.18**	**8.6**	**±0.82**	**13.9**	**±1.07**	**12.9**	**±1.55**	0.1	±0.01	0.1	±0.01	0.2	±0.01
1154	1159	sabina ketone	tr		tr		0.1	±0.00	-		-		-		tr		tr		tr	
1159	1162	isopulegol	-		-		-	-	0.2	±0.02	0.3	±0.07	0.3	±0.03	-		-		-	
1164	1165	pinocarvone	0.4	±0.03	0.3	±0.04	0.4	±0.02	tr		tr		-		0.1	±0.00	0.1	±0.01	0.1	±0.02
1169	1170	borneol	2.2	±0.20	2.2	±0.21	2.5	±0.28	3.7	±0.37	5.1	±0.57	4.8	±0.38	1.2	±0.07	1.5	±0.03	1.9	±0.06
1177	1181	terpinen-4-ol	0.5	±0.08	0.7	±0.03	0.3	±0.01	0.3	±0.07	0.7	±0.04	0.3	±0.06	0.5	±0.09	0.8	±0.03	0.3	±0.02
1182	1186	*p*-cymen-8-ol	-	-	-	-	-	-	tr		tr		0.1	±0.02	-		-		-	
1188	1190	*α*-terpineol	1.0	±0.13	0.9	±0.03	1.0	±0.03	0.2	±0.02	0.4	±0.12	0.3	±0.05	3.2	±0.12	4.0	±0.11	4.8	±0.20
1192	1192	hexyl butanoate	tr		tr		tr		-		-		-		-		-		-	
1195	1197	myrtenal	0.5	±0.04	0.5	±0.04	0.6	±0.09	tr		tr		tr		0.2	±0.02	0.1	±0.01	0.1	±0.03
1205	1209	verbenone	0.1	±0.01	0.2	±0.02	0.5	±0.05	-		-		-		-		-		-	
1216	1221	*trans*-carveol	0.1	±0.01	0.1	±0.01	0.2	±0.02	-		-		-		-		-		-	
1239	1233	isobornyl formate	0.2	±0.03	0.1	±0.01	0.1	±0.00	tr		tr		-		-		-		-	
1236	1237	hexyl-2-metyl-butanoate	0.1	±0.01	0.1	±0.01	0.3	±0.02	-		-		-		-		-		-	
1244	1242	hexyl-*iso*-valerate	0.1	±0.00	0.1	±0.01	0.1	±0.00	-		-		-		-		-		-	
1285	1286	isobornyl acetate	-		-		-		tr		tr		tr		-		-		-	
1288	1289	bornyl acetate	-		-		-		2.7	±0.15	1.8	±0.34	1.8	±0.16	-		-		-	
1290	1292	*trans*-sabinyl acetate	-		-		-		2.5	±0.65	1.2	±0.69	1.6	±0.41	-		-		-	
1294	1295	2-undecanone	-		-		-		tr		tr		tr		-		-		-	
1299	1301	carvacrol	-		-		-		0.2	±0.05	0.2	±0.03	0.2	±0.08	-		-		-	
1317	1320	*δ*-terpenyl acetate	-		-		-		-		-		-		0.4	±0.07	0.2	±0.01	0.2	±0.02
1349	1353	*α*-terpenyl acetate	-		-		-		-		-		-		1.2	±0.25	0.7	±0.06	0.8	±0.08
1381	1381	geranyl acetate	-		-		-		-		-		-		tr		tr		0.2	±0.03
1476	1480	geranyl propanoate	-		-		-		tr		tr		0.1	±0.07	-		-		-	
		Sesquiterpene hydrocarbons																		
1376	1387	*α*-copaene	-		-		-		0.2	±0.02	0.1	±0.01	0.1	±0.08	-		-		-	
1388	1389	*β*-burbonene	-		-		-		-		-		-		0.1	±0.01	0.1	±0.00	0.2	±0.01
1390	1393	*β*-elemene	-		-		-		-		-		-		tr		tr		tr	
1409	1424	*α*-gurjunene	-		-		-		0.2	±0.03	0.1	±0.03	0.4	±0.04	tr		tr		tr	
1419	1435	*β*-caryophyllene	0.8	±0.27	0.3	±0.10	2.2	±0.25	2.0	±0.18	1.2	±0.23	4.2	±0.44	1.5	±0.10	1.0	±0.15	2.2	±0.13
1456	1459	*trans-ß*-farnesene	0.2	±0.06	0.1	±0.02	0.8	±0.07	0.2	±0.03	0.1	±0.03	0.5	±0.10	tr		tr		0.1	±0.02
1454	1470	*α*-humulene	-		-		-		1.5	±0.15	0.9	±0.15	2.4	±0.17	0.1	±0.01	0.1	±0.01	0.3	±0.04
1485	1495	germacrene D	0.3	±0.13	0.1	±0.03	1.3	±0.25	-		-		-		0.4	±0.04	0.3	±0.02	1.0	±0.03
1513	1527	*γ*-cadinene	0.1	±0.04	tr		0.3	±0.04	0.2	±0.04	0.1	±0.04	0.5	±0.03	tr		tr		0.1	±0.01
1523	1534	*δ*-cadinene	tr		tr		tr		0.5	±0.09	0.3	±0.08	1.0	±0.06	0.1	±0.00	tr		0.1	±0.03
		Oxigenated sesquiterpene																		
1548	1561	elemol	-		-		-		-		-		-		1.3	±0.08	0.9	±0.10	1.6	±0.21
1568	1586	palustrol	-		-		-		0.2	±0.06	tr	±0.03	0.1	±0.09	-		-		-	
1578	1594	spathulenol	-		-		-		0.6	±0.06	0.4	±0.14	0.6	±0.06	0.4	±0.03	0.2	±0.03	1.0	±0.25
1583	1600	caryophyllene oxide	1.2	±0.31	0.6	±0.13	1.2	±0.12	3.0	±0.35	1.8	±0.33	2.8	±0.22	1.0	±0.09	0.7	±0.08	1.4	±0.10
1592	**1612**	**viridiflorol**	-		-		-		**13.0**	**±1.37**	**7.1**	**±0.40**	**12.4**	**±0.66**	1.2	±0.42	0.6	±0.06	1.3	±0.33
1602	1623	ledol	0.1	±0.04	tr		0.1	±0.03	0.2	±0.03	0.1	±0.02	0.2	±0.04	-		-		-	
1632	1635	*γ*-eudesmol	-		-		-		-		-		-		0.5	±0.07	0.3	±0.05	0.8	±0.16
1646	1656	torreyol	0.6	±0.19	0.3	±0.06	0.6	±0.06	-		-		-		-		-		-	
1685	1693	*α*-bisabolool	0.8	±0.22	0.3	±0.05	1.0	±0.03	-		-		-		-		-		-	
		Monoterpene hydrocarbons	44.4	±0.77	48.0	±2.05	41.8	±1.96	56.9	±4.40	60.6	±2.59	48.9	±2.09	79.1	±1.10	82.5	±0.34	75.5	±0.96
		Oxigenated monoterpenes	51.3	±0.60	50.1	±1.72	50.6	±1.36	21.3	±2.23	27.1	±1.62	25.9	±1.40	14.0	±0.72	13.2	±0.51	14.3	±0.31
		Sesquiterpene hydrocarbons	1.4	±0.52	0.6	±0.16	4.6	±0.57	4.9	±0.50	2.9	±0.54	9.2	±0.66	2.4	±0.15	1.6	±0.19	4.2	±0.14
		Oxigenated sesquiterpene	2.8	±0.76	1.3	±0.24	2.9	±0.20	16.9	±1.82	9.4	±0.85	16.1	±0.77	4.4	±0.60	2.7	±0.22	6.0	±0.78
		Total identified	98.6	±0.33	99.0	±0.12	91.3	±0.28	92.9	±0.84	94.0	±0.94	76.3	±0.71	95.7	±0.36	96.3	±0.24	82.0	±0.73

[a]Retention Index from literature. [b]Linear Retention Index relative to n-alkanes on non-polar column. [c]Order of elution in non-polar column (DB-5). tr: traces, less than 0,1%, -, not detected, ±: standard deviations. In bold the main compounds. LL: *Lavandula latifolia*. TM: *Thymus mastichina*. SL: *Salvia lavandulifolia*.

Table 3. Main volatile compounds quantification expressed in milligrams of terpenoids per gram of plant material (content > 0.5 mg/g in any of the three methods) and statistical significance of *Lavandula latifolia*.

LRI	Compound	HD mean		STD	MD mean		STD	EX mean		STD
1037	1,8-cineole+limonene	6.5	a	±0.94	12	b	±1.82	8.8	a	±0.59
Total Monoterpene hydrocarbons		7.6	a	±0.96	13.5	b	±2.05	9.9	a	±0.68
1099	linalool	6.1	a	±0.63	9.7	a	±1.60	7.7	a	±0.63
1148	camphor	1.5	a	±0.26	2.6	b	±0.31	2.1	ab	±0.16
1170	borneol	0.4	a	±0.05	0.6	b	±0.06	0.6	b	±0.06
Total Oxygenated monoterpenes		8.8	a	±1.02	14.1	b	±2.05	11.9	b	±0.84
1435	*β*-caryophyllene	0.1	a	±0.03	0.1	a	±0.03	0.5	b	±0.07
Total Sesquiterpene hydrocarbons		0.2	a	±0.06	0.2	a	±0.05	1.1	b	±0.17
Total Oxygenated sesquiterpenes		0.5	a	±0.08	0.4	a	±0.08	0.7	b	±0.02
Total identified		17.1	a	±1.87	28.1	b	±4.00	23.6	b	±1.38
Total compounds		17.3	a	±1.85	28.4	b	±4.02	25.9	b	±1.47

HD, hydrodistillation; MD, microdistillation; EX, solvent extraction; STD, standard deviation. a, b and c: significant differences among methods.

Table 4. Main volatile compounds quantification expressed in milligrams of terpenoids per gram of plant material (content > 0.5 mg/g in any of the three methods) and statistical significance of *Salvia lavandulifolia*.

LRI	Compound	HD mean		STD	MD mean		STD	EX mean		STD
938	*α*-pinene	0.8	a	±0.22	1.2	b	±0.16	2.2	c	±0.16
953	camphene	0.7	a	±0.14	1	b	±0.11	1.8	c	±0.12
980	*β*-pinene	0.5	a	±0.06	0.7	a	±0.13	1.2	b	±0.23
991	myrcene	0.8	a	±0.04	1	a	±0.08	1.9	b	±0.08
1028	*p*-cymene	0.3	a	±0.03	0.3	a	±0.05	0.5	b	±0.05
1037	1,8-cineole+limonene	1.9	a	±0.05	3.4	b	±0.40	6.1	c	±0.44
Total monoterpene hydrocarbons		5.2	a	±0.48	8.0	b	±0.88	14.6	c	±0.95
1144	*trans*-sabinol	0.1	a	±0.03	0.2	a	±0.07	0.5	b	±0.25
1148	camphor	0.8	a	±0.06	1.8	b	±0.20	3.8	c	±0.49
1170	borneol	0.3	a	±0.03	0.7	b	±0.07	1.4	c	±0.14
1289	bornyl acetate	0.2	a	±0.02	0.2	a	±0.03	0.5	b	±0.04
1292	trans-sabinyl acetate	0.2	a	±0.1	0.2	a	±0.08	0.5	a	±0.13
Total oxygenated monoterpenes		1,9	a	0.17	3.5	b	±0.22	7.4	c	±0.47
1435	*β*-caryophyllene	0.2	a	±0.01	0.2	a	±0.02	1.2	b	±0.12
1470	*α*-humulene	0.1	a	±0.01	0.1	a	±0.01	0.7	b	±0.07
Total sesquiterpene hydrocarbons		0.4	a	±0.04	0.4	a	±0.05	2.7	b	±0.22
1600	caryophyllene oxide	0.3	a	±0.03	0.2	a	±0.03	0.8	b	±0.07
1612	viridiflorol	1.2	a	±0.11	0.9	a	±0.08	3.6	b	±0.11
Total oxygenated sesquiterpene		1.5	b	±0.14	1.2	a	±0.10	4.7	c	±0.17
Total identified		9.0	a	±0.15	13.1	b	±0.98	29.4	c	±1.07
Total compounds		10.0	a	±0.31	14.0	b	±0.91	38.6	c	±1.32

HD, hydrodistillation; MD, microdistillation; EX, solvent extraction. STD, standard deviation. a, b and c: significant differences among methods.

Table 5. Main volatile compounds quantification expressed in milligrams of terpenoids per gram of plant material (content > 0.5 mg/g in any of the three methods) and statistical significance of *Thymus mastichina*.

LRI	Compound	HD mean		STD	MD mean		STD	EX mean		STD
938	*α*-pinene	0.8	b	±0.06	1	c	±0.05	0.5	a	±0.04
976	sabinene	0.8	b	±0.06	0.8	b	±0.06	0.5	a	±0.05
980	*β*-pinene	1.3	b	±0.08	1.4	b	±0.08	0.8	a	±0.06
991	myrcene	0.4	b	±0.03	0.5	b	±0.03	0.3	a	±0.03
1037	1,8-cineole+limonene	13.7	a	±1.86	22.3	b	±1.17	14.1	a	±0.67
Total monoterpene hydrocarbons		17.6	a	±1.99	26.6	b	±1.43	16.7	a	±0.88
1099	linalol	1.3	b	±0.04	1.5	b	±0.17	0.9	a	±0.08
1170	borneol	0.3	a	±0.04	0.5	c	±0.03	0.4	b	±0.03
1190	*α*-terpineol	0.7	a	±0.09	1.3	c	±0.08	1.1	b	±0.11
Total oxygenated monoterpenes		3.1	a	±0.18	4.2	b	±0.25	3.2	a	±0.25
1435	*β*-caryophyllene	0.3	a	±0.03	0.3	a	±0.04	0.5	b	±0.05
Total sesquiterpene hydrocarbons		0.5	a	±0.05	0.5	a	±0.06	0.9	b	±0.08
Total oxygenated sesquiterpene		1.0	a	±0.16	0.9	a	±0.07	1.3	b	±0.21
Total identified		22.2	a	±2.27	32.2	b	±1.63	22.1	a	±1.37
Total compounds		23.4	a	±2.37	33.5	b	±1.67	27.0	a	±1.84

HD, hydrodistillation; MD, microdistillation; EX, solvent extraction. STD, standard deviation. a, b and c: significant differences among methods.

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Chapter 2

Variability in essential oil composition of wild populations of *Lamiaceae* species collected in Spain

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Variability in essential oil composition of wild populations of Labiatae species collected in Spain

Abstract

Essential oils of 11 populations of *Thymus mastichina* (L.) L., 10 populations of *Salvia lavandulifolia* Vahl and 12 populations of *Lavandula latifolia* Medik., collected in full bloom during 3 years (2009, 2010 and 2011) were analysed by GC-FID/MS to study the variability among populations and the influence of the year of harvest in the essential oil composition. One factor ANOVA for population origin and year of harvest, and Principal Component Analyses (PCA) using the main compounds as set of observations were carried out for each species. For *T. mastichina* all the samples were 1,8-cineol chemotype (58.52-68.82%), however the linalool content showed a great range of variation (1.16-10.24%). 1,8-cineol (6.21-33.69%), camphor (2.85-22.44%) and *β*-pinene (5.11-19.85%) were the main compounds for *S. lavandulifolia* and 1,8-cineol (30.57-54.09%) and linalool (15.82-45.94%) for *L. latifolia* essential oils. Populations from *T. mastichina* and *S. lavandulifolia* from different years appeared mainly grouped in the PCA figures while *L. latifolia* populations showed no clustering. *T. mastichina* was the least environmentally influenced species, showing mainly differences among populations. *S. lavandulifolia* also had small differences among campaigns and higher differences within populations. Conversely, *L. latifolia* showed a higher percentage of differences in the volatile composition depending on the year of harvest but genotypic variability was also observed. In conclusion, the variability of the essential oil composition is largely dependent on the population studied having genetic factors a greater influence than the environmental factors. However, environmental factors are also influencing the essential oils composition and must be taken into account.

Keywords: Essential oils, *Lavandula latifolia, Salvia lavandulifolia, Thymus mastichina*.

4.1. Introduction

Lamiaceae is a family of great diversity which comprises a large number of species with aromatic and medicinal properties. *Thymus mastichina* (L.) L. (Spanish marjoram) distributed over most of the Iberian Peninsula (Spain and Portugal), *Salvia lavandulifolia* Vahl (Spanish sage) characteristic-growing plant of Spain, SE France and NW Africa and *Lavandula latifolia* Medik. (spike lavender), growing wild in the Iberian Peninsula, France, Italy and the West-Balkan States, are part of this family (Morales *et al.*, 2010).

The essential oil of these three species is extracted and used for the perfumery, cosmetic and aromatherapy industry. *S. lavandulifolia* and *L. latifolia* essential oils are among the 20 most traded in the world with a global annual production of 50-100 t (Lubbe and Verpoorte, 2011). Furthermore, Spain and Portugal are the biggest producers of *T. mastichina* essential oil. The dried leaves of these species are used for phytotherapy because of their medicinal properties. The food industry also employs them as flavourings, colourings or antioxidants.

These species have been harvested from nature since ancient times, but wild collection presents a set of problems such as: misidentification of the material, lack of rural labor, heterogeneous and insufficient production, impossibility to certify the material as organic products and absence of awareness of the chemical composition. In addition, consumers sometimes prefer wild plants because they are considered more natural and crop parameters to cultivate this plant material are often unknown. However, cultivation of these species results in a homogeneous production, protecting them and preventing the threat of the habitat that occurs with wild collection. Cultivation also enables the development of rural areas where climatic conditions allow the growth of these species. Nowadays, they are already domesticated, but still an important part of the production comes from wild plants. Especially *T. mastichina*, since 90% of the production in the Iberian Peninsula is harvested from its natural habitat. As industry requires material with a specific quality, some standards have been established. Achieving quality parameters established in the pharmacopeias and ISO quality standards is not possible if the material is not cultivated and previously selected.

Essential oils are complex mixtures of volatile compounds produced by plants as secondary metabolites. The production of these secondary metabolites can be influenced by several factors. According to Figueiredo *et al*. (2008) these factors include: physiological variations, environmental conditions, geographic variations, genetic factors, evolution, storage, etc.

Many studies have been conducted to analyse how these various factors influence the production and composition of essential oils, with the aim of optimizing the culture conditions, knowing the adequate time of harvest and obtaining the quality essential oils required by the industry. With respect to physiological variations, different organs of the plant may produce differences in the composition of the essential oils. Miguel *et al*. (2004a) found different composition in flowers and leaves of *T. mastichina* and Schmiderer *et al*. (2008) obtained differences within the volatile fraction of calyx, corolla and anthers of *S. lavandulifolia*.

Environmental factors are also a parameter to be taken into account, Curado *et al*. (2006) obtained several correlations between the essential oil composition of *Lychnophora ericoides* Less. and different environmental factors and Salgueiro *et al*. (1997) found a correlation between the linalool content of *T. mastichina* and Atlantic humidity. Other authors have proved that seasonal variation influence the essential oil composition as showed Miguel *et al*. (2004b) for Spanish marjoram. There are three known chemotypes for this species: 1,8-cineol, linalool and an intermediate chemotype (Adzet et al., 1977), and Salgueiro *et al*. (1997) found that the geographical variation of *T. mastichina* was related to the different chemotypes.

The above mentioned studies refer to examples that may cause changes in the chemical composition. However, no long term assays have been conducted to study which chemical compounds remain stable during different harvesting campaigns and which compounds can be influenced by weather conditions in each year of harvest.

In order to study the influence of the year of harvest and the geographical origin of population in the variability of the essential oils of *T. mastichina*, *S. lavandulifolia* and *L. latifolia* species, several wild populations were collected in the field during three consecutive years (2009, 2010 and 2011) for further analysis.

4.2. Materials and Methods

Plant material

Representative samples of 11 populations of *T. mastichina*, 10 population of *S. lavandulifolia* and 12 populations of *L. latifolia*, were collected during full blossom phase between June and August for three consecutive years (2009, 2010 and 2011) in Castilla y León, Spain.

All the samples were air-dried at room temperature and kept from light. Flowers and leaves were separated from the stems and used for further analysis. Collection data of the populations are given in Table 6. Voucher specimens from all the samples were deposited in the herbarium of the botanic area (PALAB) in the Campus of Palencia, University of Valladolid.

Hydrodistillation

The essential oils were isolated from 180 g of dried material of each population by hydrodistillation in 2 L of water for 150 min, using a Clevenger-type apparatus.

GC-MS and GC-FID analysis

Essential oils were analysed with a GC-MS and a GC-FID. GC-FID system (6890N Network GC system Agilent Technologies, Palo Alto, CA, USA) was equipped with a DB-5 narrow column (10 m x 0.1 mm x 0.1 μm). Helium was used as carrier gas. Front inlet was kept at 260ºC with a split ratio of 100:1. The volume of sample injection was 0.2 μL. The temperature program was, 60ºC for 1 min; 60-85ºC at a rate of 8ºC/min; 85-280ºC at a rate of 15ºC/min; 280-300ºC at 30ºC/min, and held at 300ºC for 5 min.

The GC-MS analysis was carried out with a GC-MS HP 6890 coupled with a HP 5972 MSD (Hewlett-Packard, Palo Alto, CA, USA). The GC was equipped with a DB-5ms capillary column (30m x 0.25 mm x 0.25 μm film thickness). The carrier gas was Helium (2 mL/min). GC oven temperature program was: 60ºC for 4 min, rising to 100ºC at 5ºC/min, and then from 100ºC to 280ºC at 9ºC/min. The volatile compounds were identified by comparison of the mass spectra of every compound with a mass spectra library of volatile compounds and confirmed by comparison of the retention indices from literature (Adams, 2007). Some of the compounds were additionally checked by reference compounds. All the compounds are expressed in peak area percentage.

Statistical analysis

The statistical analyses of the data were done with SPSS program version 15.0 (SPSS, 2006). One factor ANOVA was done for population and year of harvest as sources of variation and a Duncan test was performed to analyse the differences among harvesting years for each species. In order to assess the patterns of variation, PCA was done by simultaneously considering the main compounds (choosing the compounds with an average higher than 1%, deleting those which were not significant, neither for population nor for year in the previous ANOVA). For

this analysis the characters were initially scaled to make their variances equal. In the multivariate space that they defined, a new set of axes was then chosen so that the variance on each axis was as large as possible but at right angles to the preceding ones. The coefficient of each data point on each new axis was a weighted sum of its coefficients on the original axes.

4.3. Results

Thymus mastichina (L.) L. variability

In *T. mastichina*, a total of 63 compounds were identified (Table 7). The compounds were mainly oxygenated monoterpenes (82.40±1.09%). Ten compounds showed averages higher than 1%: *α*-pinene, sabinene, *β*-pinene, myrcene, 1,8-cineol, limonene, *β-trans*-ocimene, linalool, *α*-terpineol and *α*-terpinyl acetate, being limonene, *β-trans*-ocimene, linalool and *α*-terpinyl acetate, whose showed the higher variability. The main compound was 1,8-cineol in all samples (58.52-68.82%), so all the studied populations belong to 1,8-cineol chemotype.

In relation to the population variation, a total of 39 compounds out of the 63 identified were statistically significant (Table 7). In contrast, in relation to the year of harvest, the variability of each compound was statistically significant for only five compounds: *α*-thujene, sabinene, myrcene, *α*-campholenal and *trans*-sabinyl acetate. The year 2009 was characterized by a significantly higher amount of myrcene than the other two years and by a higher amount of sabinene than 2011. The year 2010 was characterized by a significantly lower amount of *α*-thujene than the other two campaigns and the year 2011 was distinguished by higher *trans*-sabinyl acetate than the other harvesting years and higher *α*-campholenal than 2009.

All compounds whose average was greater than 1% were used for PCA. The first three principal components explain 81.21% of the total variability. The Figure 1 (A and B) showed that samples of some populations are grouped in a close area demonstrating a small variability among the different harvesting years: TM-1, TM-17, TM-33, TM-37 and TM-42.

Axis 1 explains 41.26% of the variability. The populations located in the left part of the graph A are characterized by a higher content of linalool (TM-17, TM-1 and TM-23). On the other hand, the positive part of axis 1 is characterized by populations with higher content of *β*-pinene (TM-42, TM-12, TM-33).

Axis 2 explains 21.38% of the variability; the populations located in the upper part of the graph A are characterized by high contents of *α*-terpineol. TM-33 and TM-17 showed high contents of *α*-terpineol (4.77% and 4.48%, respectively). However, the negative part of the axis 2 is characterized by a higher content of *α*-terpinyl acetate. TM-42 and TM-12 were characterized by the highest content of *α*-terpinyl acetate (2.89% and 2.52%, respectively).

Axis 3 explains 18.57% of the variability; populations located in the positive part of axis 1 and the negative part of axis 3 were characterized by high content of 1,8-cineol. TM-42 and TM-33, were the populations with the largest amount of 1,8-cineol with 67.31% and 65.74%, respectively. TM-29, TM-17 and TM-1, were the populations with the lowest amount of 1,8-cineol with 59.54%, 60.18% and 60.94%, respectively.

***Salvia lavandulifolia* Vahl variability**

In Spanish sage, a total of 60 volatile compounds were identified (Table 8). The compounds were mainly monoterpenes (34.24±3.83% monoterpene hydrocarbons and 50.79±3.84% oxygenated monoterpenes) but also an important amount of sesquiterpenes is characteristic of *S. lavandulifolia* (4.88±1.25% sesquiterpene hydrocarbons and 6.79±1.99% oxygenated sesquiterpenes). The main compounds were 1,8-cineol (18.74±6.15%), camphor (11.13±4.85%) *α*-pinene (9.70±2.22%) and *β*-pinene (9.47±3.74%), although other 11 compounds obtained averages higher than 1% (camphene, myrcene, *o*-cymene, limonene, *γ*-terpinene, borneol, isobornyl acetate, *β*-caryophyllene, *α*-humulene, caryophyllene oxide and viridiflorol). Of the three species studied, *S. lavandulifolia* was the species which presented the greatest variability ranges, especially for: camphene 0.98-10.01%, camphor 2.85-22.42%, isobornyl acetate 0.09-2.32%, caryophyllene oxide 0.79-5.69% and viridiflorol 0.34-7.16%.

The variability of the essential oil compounds in relation to the population showed statistical significance for 24 compounds (40% of the total). However, there was almost no statistical significance with respect to the variability among the years of harvest (only for sabinene, nerol and *α*-muurulol). The year 2010 was characterized by a significantly higher content of sabinene while the year 2011 was characterized by a significantly lower content of nerol and *α*-muurulol. Thus, the variability found in *S. lavandulifolia* depends mainly on the origin of populations studied as in *T. mastichina*. However, for this species the variability in composition is very high, and although, part of the variability can be explained by the population studied, the percentage of compounds explaining this variability is lower than for *T. mastichina*, so it exists a significant part of the variability of the composition of *S. lavandulifolia* essential oil that cannot be explained by the factors studied.

Fifteen compounds of Spanish sage essential oil obtained averages higher than 1%, however, only nine of these compounds showed statistically significant variability for origin of population (camphene, *β*-pinene, 1,8-cineol, camphor, borneol, *iso*-bornyl acetate, *β*-caryophyllene, caryophyllene oxide and viridiflorol). The ACP (Figure 2) taking into account those 9 compounds explains 71.27% of the variability with the first two principal components. Axis 1 explains more than a half of the variability (57.48%) and represents mainly the variability in camphene, 1,8-cineol, camphor, borneol and isobornyl acetate. The populations represented on the negative part of axis 1 are characterized by a higher content of 1,8-cineol: SL-4 (29.37%), SL-3 (26.20%) and SL-9 (20.78%) are grouped in this part of the graph. At he same time, SL-4 and SL-3 are characterized by lower contents of camphene (1.69% and 3.95%, respectively), camphor (3.83% and 6.25%) and borneol (1.45% and 2.55%, respectively).

***Lavandula latifolia* Medik. variability**

A total of 55 compounds were identified in *L. latifolia* (Table 9), mainly oxygenated monoterpenes (89.96%±1.74%) The main compounds of this plant species were 1,8-cineol (41.96±5.48%), linalool (30.34±7.69%) and camphor (9.27±2.46%), but other compounds showed averages higher than 1%: *α*-pinene, *β*-pinene, 1,8-cineol, linalool, camphor, borneol and *α*-terpineol. Variability in these compounds was lower than in *T. mastichina* and *S. lavandulifolia*.

The variability of the identified compounds of spike lavander is influenced by the two analysed factors: the year of harvest and the population origin. For the year of harvest as dependent variable, a total of 16 compounds showed statistical significance and for the population, a total of 15 compounds were statistically significant. From the three studied species, *L. latifolia* was the most influenced by the year of harvest and least influenced by the population as source of variation.

With respect to the main compounds, the year 2011 was characterized by a lower content of *α* and *β*-pinene, sabinene, *α*-terpineol and myrcene. In contrast, *cis* and *trans*-linalool oxide and carvone were higher for the campaign of 2011. The compound *α*-muurulol was higher for the season of 2009, showing no significant differences between the 2010 and 2011 campaigns.

As limonene was not significant neither for year or population, therefore seven compounds were used in PCA (Figure 3). The PCA explains 75.40% of the variability through principal components 1 and 2. Axis 1 explains 50.16% of the total variability. The right part of axis 1 is

characterized by a high content of 1,8-cineol, α-pinene, β-pinene and α-terpineol while the left area of the plot is characterized by high contents of linalool. LL-4 and LL-9 are characterized by low contents of 1,8-cineol (35.35% and 35.89%, respectively) and high contents of linalool (38.57%, 42.18%, respectively) while LL-2 is characterized by high contents of 1,8-cineol (47.25%) and low contents of linalool (22.53%).

Axis 2 explains 25.24% of the variability; the positive area is characterized by high contents of camphor and borneol, therefore LL-5 is characterized by high contents of camphor (13.47%). The lower part of the plot is characterized by higher contents of α-terpineol, linalool and β-pinene. Almost all the samples collected in the campaigns of 2009 and 2010 appear in the negative part of the axis or very close to it (except LL-5 population that seems to be less influenced by seasonality) and the content of α-terpineol and β-pinene are significantly higher for these campaigns in comparison with 2011.

4.4 Discussion

Chemical variability for the different species

The physiological aspects affecting essential oil production show that many species have great differences in essential oil composition, while others have a more or less stable composition (Figueiredo et al., 2008). Of the three species studied, *S. lavandulifolia* was the species which presented the greatest variability ranges. *T. mastichina* and *L. latifolia* are mainly constituted by oxygenated monoterpenes while *S. lavandulifolia* has a more variable composition. For the species Spanish marjoram and spike lavender the main components do not change, although they are highly variable in content, however, to Spanish sage there are more compounds considered as main components and these are present or not depending on the population origin. This huge variability has been studied and confirmed in *S. lavandulifoila* by other authors (Herraiz-Peñalver et al., 2010; Jordán et al., 2009a). This variability could be the reason why several chemotypes have been identified in this plant species (Jordán et al., 2009a) as well as in other species of the genus *Salvia* (Lamien-Meda et al., 2009; Mockutë et al., 2003; Novak et al., 2006).

Minor variability was found among the *T. mastichina* samples. Although interpopulation variability was high for the two compounds that determine the chemotype, all the analysed samples belongs to chemotype 1,8-cineol (Adzet et al., 1977; Salgueiro et al., 1997). *L. latifolia* populations showed less variability than *T. mastichina* and *S. lavandulifoila*, in agreement with

the fact that no chemotypes are described for spike lavender, even though the main compounds (1,8-cineol, linalool and camphor) exhibited the greatest variability, as well as previous studies (Herraiz-Peñalver et al., 2013).

Variability of the volatile compounds among populations

The chemical composition of the essential oils extracted from plants is mainly controlled by the genotype. Although in *T. mastichina* all the studied samples were 1,8-cineol chemotype, around 60% of the compounds identified in this species showed significant differences with the population as dependent variable, meaning that the chemical variability of this species is mainly agenotypic characteristic of each population.

In the case of *S. lavandulifolia*, around 39% of the identified compounds showed significant differences with respect to the population origin. Hence, the variability of Spanish sage is mainly related to the genotype; however, the great variability shown by this species is not as dependent on the population as in Spanish marjoram and other factors seem to affect the chemical variability. This could be caused by other factors, not taken in to account, that could influence the chemical composition, like non-homogeneity within the populations, differences in the phenological stage (Porres-Martínez *et al*., 2014), the morphological development or changes in the flowers/leaves ratio. The composition of flowers and leaves is sometimes very different, Usano-Alemany (2012) found a marked difference for leaves and flowers essential oil composition of *S. lavandulifolia* and contents of linalool for flowers was 35% while leaves presented around 1%. To prevent such variability, it is very important to select morphologically homogeneous plant material.

For *L. latifolia*, only about 30% of the identified compounds showed significant differences depending on the population. The main compounds showing significant differences among populations were camphene, *β-cis*-ocimene, pinocarvone and borneol. Borneol content seems to be related to the population studied; in connection with this, Barazandeh *et al*. (2002) found a population of *L. latifolia* containing borneol (14.2%) in higher amount than camphor (6.9%), however there are no chemotypes described for this species. The content of borneol in leaves of *L. latifolia* is greater than the content in the flowers (Muñoz-Bertomeu et al., 2007). It could be that populations with a higher content of borneol present a higher amount of leaves or larger leaves.

Variability of the volatile compounds within harvesting campaigns

For *T. mastichina* and *S. lavandulifolia*, the chemical variability with respect to the year of harvest was very small; only five and three compounds, respectively, were statistically significant. In contrast, *L. latifolia* was the most affected species in relation to the harvesting year and then probably more influenced by the weather conditions that occurred in each year of the harvest season.

Sabinene showed significant differences with respect to the year of harvest in the three species studied, in the same way, the compounds α-muurulol and myrcene were also statistically significant in two of the studied species, so it is likely that these compounds are less related to genetic factors and more influenced by the environmental conditions of each year or by the phenological stage in which the plant is collected.

Essential oil production and others secondary metabolites are in general extremely dependent on weather (Figueiredo et al., 2008). Weather conditions influence the arid terrain, humidity and irrigation water among other factors. As an example, Boira and Blanquer (1998) found in *Thymus piperella* L. that the chemotype *p*-cymene/γ-terpinene/carvacrol shows a positive correlation with the aridity index and Jordán *et al*. (2009b) found changes in the volatile compounds of *T. zygis* subsp. *gracilis* (Boiss.) R. Morales shrubs cultivated under different watering levels. These environmental factors affect each species differently and even have different effects within the same species. Ložienė & Venskutonis (2005), changing the environmental conditions, found in *T. pulegioides* L. two types of plants: (a) plants preserving their volatile composition and (b) plants which significantly changed their volatile compounds.

Furthermore, the phenological stage in which the plant is collected can greatly affect the composition. Although all the samples were collected in full blossom phase, sometimes there are great differences in the maturation of the flowers even in the same plant. For example, the compound myrcene can decrease from 1.2% to 0.9% from floral budding to the flowering stage and increase again to 1.3% when the seed is formed (Nejad Ebrahimi et al., 2008) and Milos et al. (2001) found in *Satureja montana* L. an increase of this compound from 5% to 6.7% from July to September. Likewise, sabinene can change from 0.7% in floral budding and completely disappear in the flowering stage and seed formation (Nejad Ebrahimi et al., 2008). Because of this, selecting the right period for harvest with a comprehensive monitoring of the maturation of the flower is important for obtaining the desired composition.

Another important factor to consider is the different composition of flowers and leaves. The climatic conditions of each year, might also lead to different development of flowers or leaves changing flower/leaf ratio of the different campaigns. The compounds *α*-pinene, *β*-pinene, sabinene and *α*-terpineol were significantly lower for the campaign of 2011 in *L. latifolia* and all these compounds are higher in flowers of *L. latifolia* than in leaves (Muñoz-Bertomeu et al., 2007). Sabinene and myrcene were also significantly lower for the season of 2011 in *T. mastichina* and it is reported for this species that both compounds are higher in flowers than in leaves (Miguel et al., 2004a). Thus, the climatic conditions of 2011 could promote lower flower development by increasing the percentage of leaves in the collected plant material.

Uses of the essential oils, selection and quality

Once the variability in the chemical composition of the different species populations is known, a process of selection of plants to cultivate could begin thus ending the massive wild collection that threatens the conservation of the species. As the primary use of the essential oils of these plant species is intended for perfume and cosmetic industries, plant selection should be as close as possible to meeting the quality standards (ISO, 2003, 2009, 2012) set by the industry for these three species which were established to facilitate assessment of their quality (Table 10).

For *T. mastichina* most of the samples met the quality parameters for all compounds given in the standard except for linalool acetate where the majority of the samples showed a range below that required and only 13% of the samples reached the quality range for this compound. For *S. lavandulifolia* and in agreement with Herraiz-Peñalver *et al*. (2010) the quality standard is more difficult to reach due to the great variability of this species. The most restrictive ranges for the samples analysed were camphor (only 13% of the samples reached the standard), *trans*-sabinyl acetate (only 20% of the samples reached the standard), and limonene (the majority of the samples presented higher percentages than the recommended in the standard). For the *L. latifolia* samples analysed, in general they reached the quality recommended ranges, however the content of linalool is, for half of the samples, higher than suitable by the standards of quality and *trans*-*α*-bisabolene was not detected.

4.5. Conclusions

As may be observed from the results obtained for the three species, the variability is largely dependent on the populations studied meaning that genetic factors have greater influence in comparison with the environmental factors and it is possible to select populations with desirable

composition. However, environmental factors which can produce differences in the phenological stage or morphological development can also have an effect on the chemical variability. Furthermore, the control of the environmental factors, as well as choosing the right period of harvest are really important to obtain a desired composition, especially for *L. latifolia.* Standardising the plant material, thus providing maximum homogeneity, is essential for quality production.

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Table 6. Geographical coordinates of the studied populations

Population	Species	Locality (province)	Altitude	Longitude	Latitude
TM-1	*Thymus mastichina*	Toro (Zamora)	704	413122N	052424W
TM-12	*Thymus mastichina*	Tordesillas (Valladolid)	675	412933N	050054W
TM-14	*Thymus mastichina*	Truchas (León)	957	421325N	061512W
TM-17	*Thymus mastichina*	Almazán (Soria)	933	412826N	023538W
TM-23	*Thymus mastichina*	Riaza (Segovia)	814	411527N	032805W
TM-29	*Thymus mastichina*	Serranillos (Ávila)	1190	402040N	045415W
TM-33	*Thymus mastichina*	Perandones (León)	507	423407N	064604W
TM-37	*Thymus mastichina*	Carrocera (León)	1029	424942N	115213W
TM-42	*Thymus mastichina*	Saldaña (Palencia)	816	423158N	044326W
TM-43	*Thymus mastichina*	Boñar (León)	1017	425143N	051816W
TM-44	*Thymus mastichina*	Lerma (Burgos)	945	415838N	032908W
SL-2	*Salvia lavandulifolia*	Velamazán (Soria)	932	412905N	024722W
SL-3	*Salvia lavandulifolia*	Burgo de Osma (Soria)	917	413435N	030148W
SL-4	*Salvia lavandulifolia*	Langa de Duero (Soria)	846	413524N	031929W
SL-5	*Salvia lavandulifolia*	Moral de Hornuez (Segovia)	1130	412714N	033756W
SL-6	*Salvia lavandulifolia*	Fuentidueña (Segovia)	844	412641N	035738W
SL-7	*Salvia lavandulifolia*	Olivares del Duero (Valladolid)	866	413749N	042847W
SL-8	*Salvia lavandulifolia*	Santibáñez del Val (Burgos)	945	415838N	032908W
SL-9	*Salvia lavandulifolia*	Valle de Sedano (Burgos)	725	424344N	034551W
SL-11	*Salvia lavandulifolia*	Cevico Navero (Palencia)	916	415221N	041136W
SL-12	*Salvia lavandulifolia*	Reinoso de Cerrato (Palencia)	876	415657N	042228W
LL-1	*Lavandula latifolia*	Dévanos (Soria)	940	415406N	015501W
LL-2	*Lavandula latifolia*	Velamazán (Soria)	932	412905N	024722W
LL-3	*Lavandula latifolia*	Moral de Hornuez (Segovia)	1130	412715N	033756W
LL-4	*Lavandula latifolia*	Fuentidueña (Segovia)	844	412641N	035738W
LL-5	*Lavandula latifolia*	Sedano (Burgos)	830	424118N	034413W
LL-6	*Lavandula latifolia*	Santibáñez del Val (Burgos)	945	415838N	032908W
LL-7	*Lavandula latifolia*	Gumiel de Izán (Burgos)	888	414623N	034040W
LL-8	*Lavandula latifolia*	Quintanilla de Onésimo (Valladolid)	796	413714N	042009W
LL-9	*Lavandula latifolia*	Aguilar de Campoo (Palencia)	913	424533N	041350W
LL-10	*Lavandula latifolia*	Tejado (Soria)	920	413340N	021326W
LL-11	*Lavandula latifolia*	Cevico Navero (Palencia)	916	415221N	041136W
LL-12	*Lavandula latifolia*	Reinoso de Cerrato (Palencia)	876	415657N	042228W

Table 7. Minimum, maximum and average of the compounds found in the essential oil of 11 populations of *Thymus mastichina* L. collected in 2009, 2010 and 2011 expressed in peak area percentage (%). Statistical significance for year and population variables.

LRI[a]	Tentative compound[b]	min	max	mean	st	Population	2009		2010		2011		Year
927	tricyclene	0.00	0.11	0.02	0.03	ns	0.02	a	0.02	a	0.01	a	ns
930	*α*-thujene	0.04	0.26	0.15	0.04	ns	0.16	a	0.12	b	0.17	a	*
938	***α*–pinene**	**2.58**	**3.78**	**3.06**	**0.24**	*	**3.10**	**a**	**3.02**	**a**	**3.07**	**a**	**ns**
955	camphene	0.05	1.82	0.43	0.49	***	0.45	a	0.43	a	0.43	a	ns
977	**sabinene**	**2.36**	**3.63**	**2.84**	**0.29**	**ns**	**3.04**	**a**	**2.81**	**ab**	**2.71**	**b**	*
981	***β*-pinene**	**3.89**	**5.08**	**4.44**	**0.32**	**	**4.50**	**a**	**4.42**	**a**	**4.41**	**a**	**ns**
991	**myrcene**	**1.36**	**2.05**	**1.61**	**0.15**	**ns**	**1.72**	**a**	**1.58**	**b**	**1.55**	**b**	*
1005	*α*-phellandrene	0.00	0.13	0.07	0.03	*	0.07	a	0.07	a	0.06	a	ns
1020	*α*-terpinene	0.14	0.28	0.20	0.04	*	0.19	a	0.19	a	0.21	a	ns
1029	*p*-cymene	0.16	0.51	0.27	0.09	***	0.27	a	0.25	a	0.29	a	ns
1040	**limonene**	**1.23**	**6.11**	**2.95**	**1.18**	***	**3.34**	**a**	**2.76**	**a**	**2.84**	**a**	**ns**
1041	**1,8-cineol**	**58.52**	**68.82**	**63.60**	**2.65**	***	**63.22**	**a**	**63.12**	**a**	**64.38**	**a**	**ns**
1045	*β*-*cis*-ocimene	0.00	0.25	0.10	0.06	***	0.10	a	0.11	a	0.09	a	ns
1151	***β*-*trans*-ocimene**	**0.18**	**2.75**	**1.20**	**0.72**	***	**1.17**	**a**	**1.25**	**a**	**1.18**	**a**	**ns**
1062	*γ*-terpinene	0.25	0.61	0.37	0.08	***	0.36	a	0.35	a	0.39	a	ns
1074	*cis*-sabinene hydrate	0.39	0.75	0.63	0.08	*	0.64	a	0.64	a	0.61	a	ns
1076	*cis*-linalool oxide	0.00	0.19	0.06	0.04	***	0.06	a	0.05	a	0.06	a	ns
1087	terpinolene	0.09	0.26	0.14	0.03	ns	0.15	a	0.14	a	0.15	a	ns
1088	*trans*-linalool oxide	0.00	0.30	0.05	0.06	ns	0.05	a	0.04	a	0.07	a	ns
1100	**linalool**	**1.16**	**10.24**	**3.96**	**2.14**	***	**3.50**	**a**	**4.37**	**a**	**3.92**	**a**	**ns**
1012	*β*-thujone	0.00	0.14	0.04	0.04	ns	0.03	a	0.06	a	0.04	a	ns
1130	*cis*-*p*-Menth-en-1-ol	0.00	0.15	0.07	0.04	ns	0.07	a	0.08	a	0.07	a	ns
1133	*α*-campholenal	0.00	0.05	0.01	0.02	ns	0.01	b	0.01	ab	0.02	a	*
1146	nopinone	0.00	0.08	0.04	0.03	ns	0.05	a	0.03	a	0.04	a	ns
1148	*trans*-sabinol	0.05	0.23	0.14	0.03	ns	0.12	a	0.14	a	0.14	a	ns
1154	camphor	0.00	0.85	0.16	0.20	***	0.15	a	0.17	a	0.15	a	ns
1164	sabina ketone	0.00	0.09	0.03	0.03	ns	0.03	a	0.02	a	0.04	a	ns
1169	pinocarvone	0.00	0.13	0.10	0.02	ns	0.09	a	0.11	a	0.09	a	ns
1171	*δ*-terpineol	0.49	1.99	0.95	0.40	***	0.94	a	0.95	a	0.96	a	ns
1174	borneol	0.44	1.80	0.86	0.37	***	0.85	a	0.86	a	0.87	a	ns
1185	terpinen-4-ol	0.52	1.13	0.77	0.12	**	0.74	a	0.78	a	0.78	a	ns
1191	*p*-cymen-8-ol	0.00	0.08	0.04	0.02	ns	0.05	a	0.03	a	0.04	a	ns
1198	***α*-terpineol**	**2.36**	**5.21**	**3.70**	**0.78**	***	**3.78**	**a**	**3.67**	**a**	**3.67**	**a**	**ns**
1205	myrtenal	0.04	0.20	0.09	0.03	ns	0.08	a	0.10	a	0.09	a	ns
1210	verbenone	0.00	0.03	0.01	0.01	ns	0.01	a	0.00	a	0.01	a	ns
1223	*trans*-carveol	0.00	0.10	0.03	0.03	ns	0.03	a	0.03	a	0.02	a	ns
1228	nerol	0.00	0.13	0.02	0.03	**	0.03	a	0.03	a	0.02	a	ns
1234	isobornyl formate	0.00	0.14	0.02	0.04	**	0.02	a	0.02	a	0.02	a	ns
1242	neral	0.00	0.09	0.04	0.02	ns	0.05	a	0.04	a	0.04	a	ns
1253	linalool acetate	0.00	0.58	0.14	0.14	**	0.16	a	0.14	a	0.12	a	ns
1271	geraniol	0.00	0.11	0.05	0.03	ns	0.06	a	0.06	a	0.04	a	ns
1283	isobornyl acetate	0.00	0.92	0.13	0.22	***	0.13	a	0.12	a	0.14	a	ns
1292	*trans*-sabinyl acetate	0.00	0.03	0.01	0.01	ns	0.00	b	0.00	b	0.01	a	**
1315	*δ*-terpinyl acetate	0.05	1.03	0.45	0.29	***	0.47	a	0.47	a	0.41	a	ns
1350	***α*-terpinyl acetate**	**0.18**	**3.21**	**1.40**	**0.91**	***	**1.48**	**a**	**1.44**	**a**	**1.30**	**a**	**ns**
1379	geranyl acetate	0.00	0.39	0.05	0.08	ns	0.08	a	0.03	a	0.04	a	ns
1389	*β*-bourbonene	0.00	0.27	0.07	0.05	**	0.06	a	0.08	a	0.06	a	ns
1413	*α*-gurjunene	0.00	0.11	0.03	0.03	***	0.03	a	0.04	a	0.03	a	ns
1427	*β*-caryophyllene	0.29	1.29	0.83	0.28	***	0.79	a	0.89	a	0.78	a	ns
1441	*β*-gurjunene	0.00	0.05	0.01	0.01	**	0.01	a	0.01	a	0.01	a	ns
1463	*trans*- *β*-farnesene	0.00	0.04	0.01	0.01	**	0.01	a	0.01	a	0.00	a	ns
1469	*allo*-aromandendrene	0.02	0.19	0.09	0.04	**	0.08	a	0.10	a	0.08	a	ns
1489	germacrene D	0.06	0.93	0.23	0.18	***	0.23	a	0.26	a	0.20	a	ns
1502	bicyclogermacrene	0.11	0.43	0.27	0.09	***	0.26	a	0.28	a	0.27	a	ns
1520	*γ*-cadinene	0.00	0.14	0.05	0.03	ns	0.05	a	0.05	a	0.04	a	ns
1524	*δ*-cadinene	0.00	0.14	0.06	0.04	**	0.06	a	0.06	a	0.06	a	ns
1555	elemol	0.16	1.17	0.62	0.26	***	0.60	a	0.65	a	0.59	a	ns
1586	spathulenol	0.00	0.23	0.08	0.06	ns	0.05	a	0.10	a	0.08	a	ns
1593	caryophyllene oxide	0.08	0.63	0.32	0.13	**	0.28	a	0.37	a	0.31	a	ns
1596	viridiflorol	0.14	1.11	0.44	0.20	**	0.42	a	0.48	a	0.41	a	ns
1605	ledol	0.00	0.22	0.09	0.05	*	0.08	a	0.10	a	0.09	a	ns
1635	*γ*-eudesmol	0.00	0.05	0.00	0.01	ns	0.01	a	0.00	a	0.00	a	ns
1652	*α*-murulol	0.00	0.31	0.10	0.07	*	0.10	a	0.11	a	0.08	a	ns
	Monoterpene hydrocarbons	11.25	16.06	13.09	1.07	**	13.52	a	12.89	a	12.93	a	ns
	Oxigenated monoterpenes	79.60	84.05	82.40	1.09	*	82.07	a	82.21	a	82.86	a	ns
	Sesquiterpene	0.77	2.59	1.63	0.44	***	1.58	a	1.77	a	1.54	a	ns
	Oxigenated sesquiterpenes	0.93	2.69	1.65	0.45	*	1.54	a	1.81	a	1.57	a	ns
	Total identified	97.74	99.50	98.77	0.36		98.72		98.68		98.90		

[a]Linear retention index relative to n-alkanes in non-polar column. [b]Order of elution in non-polar column DB-5. In bold the 10 compounds used for the PCA. st: standard deviation. Population and Year: ns= non significant, * P<0.05, ** P<0.01, *** P<0.001. Different letter for the campaigns (2009, 2010 and 2011) means significant differences.

Table 8. Average, maximum and minimum amount of the compounds found in the essential oil of 12 populations of *Salvia lavandulifolia* from 2009, 2010 and 2011 expressed in peak area percentage (%).Statistical significance for year and population variables.

LRI[a]	Tentative compounds[b]	min	max	mean	st	Population	2009		2010		2011		Year
927	tricyclene	0.02	0.49	0.29	0.12	***	0.30	a	0.28	a	0.30	a	ns
930	α-thujene	0.25	1.22	0.40	0.17	ns	0.36	a	0.38	a	0.46	a	ns
939	α-pinene	6.60	15.07	9.70	2.22	ns	10.35	a	9.36	a	9.39	a	ns
956	**camphene**	**0.98**	**10.01**	**6.17**	**2.57**	***	6.34	**a**	5.96	**a**	6.20	**a**	**ns**
959	thuja-2,4 (10)-diene	0.00	0.03	0.01	0.01	ns	0.01	a	0.01	a	0.01	a	ns
977	sabinene	0.29	1.36	0.85	0.23	ns	0.81	b	1.01	a	0.74	b	*
982	**β-pinene**	**5.11**	**19.85**	**9.47**	**3.74**	*	8.90	**a**	11.06	**a**	8.44	**a**	**ns**
991	myrcene	3.16	8.28	5.83	1.60	ns	6.29	a	5.12	a	6.09	a	ns
1008	α-phellandrene	0.00	0.12	0.05	0.03	ns	0.05	a	0.05	a	0.06	a	ns
1020	α-terpinene	0.14	0.50	0.22	0.07	ns	0.23	a	0.19	a	0.24	a	ns
1029	*o*-cymene	0.48	3.00	1.23	0.49	ns	1.20	ab	1.02	b	1.48	a	ns
1038	limonene	4.76	17.91	9.14	2.91	ns	9.10	a	8.59	a	9.73	a	ns
1039	**1,8-cineol**	**6.21**	**33.69**	**18.74**	**6.15**	***	18.79	**a**	17.94	**a**	19.50	**a**	**ns**
1040	β-*cis*-ocimene	0.04	0.17	0.07	0.03	*	0.07	a	0.08	a	0.06	a	ns
1051	γ-terpinene	0.54	2.08	1.35	0.34	ns	1.38	a	1.45	a	1.21	a	ns
1074	*cis*-sabinene hidrate	0.33	0.70	0.49	0.08	ns	0.50	a	0.47	a	0.48	a	ns
1087	terpinolene	0.11	0.36	0.21	0.06	**	0.22	a	0.22	a	0.21	a	ns
1100	linalool	0.46	0.94	0.69	0.12	ns	0.72	a	0.67	a	0.68	a	ns
1123	β-thujone	0.00	0.14	0.02	0.04	ns	0.01	a	0.02	a	0.04	a	ns
1133	α-campholenal	0.04	0.15	0.09	0.03	*	0.10	a	0.09	a	0.09	a	ns
1145	*trans*-pinocarveol	0.11	3.64	0.50	0.75	ns	0.23	a	0.88	a	0.40	a	ns
1153	**camphor**	**2.85**	**22.44**	**11.13**	**4.85**	***	11.06	**a**	11.54	**a**	10.77	**a**	**ns**
1169	pinocarvone	0.05	0.15	0.09	0.03	**	0.09	a	0.09	a	0.09	a	ns
1178	**borneol**	**0.74**	**7.72**	**3.90**	**1.47**	**	3.93	**a**	3.85	**a**	3.93	**a**	**ns**
1185	terpinen-4-ol	0.52	1.20	0.74	0.18	*	0.78	a	0.74	a	0.72	a	ns
1197	α-terpineol	0.35	2.63	0.97	0.48	ns	0.89	a	0.98	a	1.06	a	ns
1201	myrtenal	0.02	0.11	0.06	0.02	**	0.06	a	0.07	a	0.06	a	ns
1209	verbenone	0.00	0.06	0.01	0.01	ns	0.01	a	0.01	a	0.01	a	ns
1223	*trans*-carveol	0.00	0.10	0.05	0.02	**	0.06	a	0.06	ab	0.04	b	ns
1234	isobornyl formate	0.00	0.13	0.06	0.05	ns	0.05	a	0.02	a	0.05	a	ns
1228	nerol	0.01	0.10	0.06	0.02	ns	0.07	a	0.07	a	0.04	b	**
1249	carvone	0.00	0.07	0.04	0.02	ns	0.04	a	0.04	a	0.04	a	ns
1253	linalool acetate	0.00	0.24	0.13	0.06	ns	0.12	a	0.13	a	0.14	a	ns
1284	**isobornyl acetate**	**0.09**	**2.32**	**1.12**	**0.52**	*	1.00	**a**	1.05	**a**	1.30	**a**	**ns**
1290	*trans*-sabinyl acetate	0.00	3.48	0.36	0.78	ns	0.03	a	0.68	a	0.36	a	ns
1315	δ-terpinyl acetate	0.00	0.01	0.00	0.00	ns	0.00	a	0.00	a	0.00	a	ns
1349	α-terpinyl acetate	0.00	0.34	0.07	0.08	ns	0.09	a	0.06	a	0.07	a	ns
1352	α-cubebene	0.00	0.16	0.09	0.05	ns	0.09	a	0.08	a	0.11	a	ns
1389	β-bourbonene	0.00	0.12	0.03	0.03	*	0.02	a	0.04	a	0.02	a	ns
1392	β-elemene	0.00	0.02	0.00	0.01	ns	0.00	a	0.00	a	0.01	a	ns
1413	α-gurjunene	0.00	0.22	0.06	0.05	ns	0.05	a	0.06	a	0.08	a	ns
1427	**β-caryophyllene**	**1.06**	**4.29**	**2.32**	**0.77**	*	2.35	**a**	2.47	**a**	2.15	**a**	**ns**
1437	β-gurjunene	0.00	0.06	0.02	0.02	ns	0.02	a	0.01	a	0.02	a	ns
1447	aromadendrene	0.00	0.11	0.03	0.03	ns	0.04	a	0.02	a	0.04	a	ns
1464	α-humulene	0.74	2.19	1.38	0.38	ns	1.35	a	1.39	a	1.39	a	ns
1470	*allo*-aromandrene	0.02	0.09	0.06	0.02	*	0.06	a	0.06	a	0.07	a	ns
1478	α-muurulene	0.01	0.35	0.14	0.08	*	0.10	a	0.15	a	0.16	a	ns
1489	germacrene D	0.00	0.16	0.05	0.04	ns	0.04	a	0.07	a	0.04	a	ns
1491	β-selinene	0.00	0.20	0.04	0.04	ns	0.05	a	0.04	a	0.04	a	ns
1520	γ-cadinene	0.00	0.41	0.10	0.13	*	0.07	a	0.12	a	0.11	a	ns
1524	δ-cadinene	0.02	0.78	0.14	0.15	ns	0.13	a	0.12	a	0.17	a	ns
1559	elemol	0.00	0.20	0.07	0.05	**	0.07	a	0.05	a	0.08	a	ns
1581	palustrol	0.00	0.08	0.02	0.02	***	0.02	a	0.02	a	0.02	a	ns
1587	spathulenol	0.00	2.91	0.31	0.54	ns	0.06	b	0.28	ab	0.59	a	ns
1593	**caryophyllene oxide**	**0.79**	**5.69**	**2.55**	**1.06**	*	2.49	**a**	2.76	**a**	2.39	**a**	**ns**
1606	**viridiflorol**	**0.34**	**7.16**	**3.63**	**1.97**	***	3.77	**a**	3.56	**a**	3.56	**a**	**ns**
1617	ledol	0.00	0.18	0.09	0.02	*	0.02	a	0.03	a	0.02	a	ns
1635	γ-eudesmol	0.00	0.13	0.03	0.03	ns	0.03	a	0.03	a	0.03	a	ns
1652	α-muurulol	0.00	0.44	0.24	0.14	ns	0.29	a	0.28	a	0.15	b	*
1656	β-eudesmol	0.03	0.28	0.15	0.05	ns	0.16	a	0.16	a	0.12	a	ns
	Monoterpene hidrocarbons	27.37	44.72	34.24	3.83	ns	34.83	a	34.43	a	33.41	a	ns
	Oxigenated monoterpenes	39.41	59.18	50.79	3.84	ns	49.40	a	49.40	a	51.10	a	ns
	Sesquiterpene hydrocarbons	2.37	7.79	4.88	1.25	*	4.37	a	4.62	a	4.39	a	ns
	Oxigenated sesquiterpenes	3.68	11.09	6.79	1.99	**	6.92	a	7.16	a	6.96	a	ns
	Total identified	92.65	97.37	95.79	0.77		95.52		96.00		95.86		

[a]Linear retention index relative to n-alkanes in non-polar column. [b]Order of elution in non-polar column DB-5. In bold the 9 compounds used for the PCA. st: Statistical significance of the variables Population and Year. ns: no significant; *:P<0.05; **: P<0.01; ***: P<0.001. Different letter for the campaigns (2009, 2010 and 2011) means significant differences.

Table 9. Average, maximum and minimum amount of the compounds found in the essential oil of 12 populations of *Lavandula latifolia* collected in 2009, 2010 and 2011 expressed in peak area percentage (%).Statistical significance for year and population variables.

LRI[a]	Tentative compound[b]	min	max	mean	st	Population	2009		2010		2011		Year
927	tricyclene	0.00	0.34	0.05	0.06	ns	0.05	a	0.06	a	0.05	a	ns
932	*α*-thujene	0.00	0.21	0.03	0.05	ns	0.03	a	0.04	a	0.02	a	ns
939	***α*-pinene**	**0.66**	**2.93**	**2.01**	**0.55**	**ns**	2.40	**a**	2.01	**b**	1.61	**c**	***
954	camphene	0.21	0.76	0.49	0.12	***	0.49	a	0.49	a	0.48	a	ns
958	thuja-2.4(10)-diene	0.02	0.12	0.06	0.02	ns	0.05	a	0.06	a	0.07	a	ns
976	sabinene	0.31	1.14	0.75	0.19	*	0.88	a	0.75	a	0.62	b	**
980	***β*-pinene**	**1.35**	**3.58**	**2.49**	**0.55**	**ns**	2.95	**a**	2.52	**b**	2.01	**c**	***
991	myrcene[c]	0.39	0.87	0.63	0.12	ns	0.71	a	0.67	a	0.50	b	***
1005	*α*-phellandrene	0.00	0.20	0.04	0.04	ns	0.05	a	0.06	a	0.03	a	ns
1020	*α*-terpinene	0.09	0.23	0.16	0.03	*	0.16	a	0.15	a	0.17	a	ns
1028	*p*-cymene	0.03	0.33	0.19	0.05	ns	0.19	a	0.19	a	0.18	a	ns
1038	limonene	0.69	2.80	1.32	0.42		1.30	a	1.14	a	1.52	a	ns
1036	**1,8-cineole**	**30.57**	**54.09**	**41.96**	**5.48**	*****	42.20	**a**	40.09	**a**	43.60	**a**	**ns**
1040	*β-cis*-ocimene	0.13	0.72	0.28	0.14	***	0.31	a	0.25	a	0.28	a	ns
1053	*β-trans*-Ocimene	0.00	0.18	0.06	0.04	*	0.07	a	0.05	a	0.05	a	ns
1064	*γ*-terpinene	0.18	0.49	0.27	0.06	ns	0.27	a	0.26	a	0.27	a	ns
1071	*cis*-sabinene hydrate	0.28	0.81	0.50	0.15	*	0.57	a	0.48	ab	0.44	b	ns
1078	*cis*-linalool oxide	0.00	0.48	0.08	0.09	ns	0.04	b	0.04	b	0.16	a	***
1092	*trans*-linalool oxide	0.00	0.43	0.06	0.08	ns	0.02	b	0.02	b	0.13	a	***
1091	terpinolene	0.09	0.44	0.20	0.06	ns	0.20	a	0.21	a	0.19	a	ns
1098	**linalool**	**15.82**	**45.94**	**30.34**	**7.69**	*****	26.76	**b**	33.42	**a**	30.84	**ab**	**ns**
1124	*cis-p*-menth-2-en-1-ol	0.00	0.09	0.04	0.03	ns	0.05	a	0.04	a	0.04	a	ns
1128	*α*-campholenal	0.03	0.19	0.12	0.04	*	0.13	a	0.11	a	0.13	a	ns
1146	nopinone	0.08	0.18	0.12	0.02	ns	0.12	ab	0.11	b	0.14	a	ns
1149	*trans*-sabinol	0.19	0.44	0.31	0.06	ns	0.31	ab	0.28	b	0.33	a	ns
1148	**camphor**	**3.45**	**16.14**	**9.27**	**2.46**	**	9.35	**a**	8.50	**a**	9.94	**a**	**ns**
1164	sabina ketone	0.00	0.11	0.05	0.03	ns	0.04	a	0.04	a	0.06	a	ns
1166	pinocarvone	0.19	0.50	0.31	0.07	ns	0.31	a	0.29	a	0.34	a	ns
1171	**borneol**	**1.03**	**3.07**	**1.61**	**0.40**	***	1.57	**a**	1.62	**a**	1.63	**a**	**ns**
1181	terpinen-4-ol	0.45	0.83	0.63	0.09	*	0.62	a	0.62	a	0.66	a	ns
1190	*p*-cymen-8-ol	0.05	0.33	0.17	0.05	ns	0.18	ab	0.15	b	0.20	a	*
1193	hexyl butanoate	0.00	0.14	0.05	0.04	*	0.03	a	0.06	a	0.04	a	ns
1192	***α*-terpineol**	**0.85**	**1.83**	**1.34**	**0.26**	**ns**	1.52	**a**	1.42	**a**	1.08	**b**	***
1197	myrtenal	0.17	0.67	0.33	0.11	ns	0.34	a	0.33	a	0.33	a	ns
1209	verbenone	0.07	0.19	0.13	0.03	ns	0.13	b	0.11	b	0.16	a	**
1221	*trans*-carveol	0.00	0.09	0.03	0.03	ns	0.05	a	0.02	a	0.03	a	ns
1232	isobornyl formate	0.00	0.12	0.06	0.03	ns	0.05	a	0.05	a	0.07	a	ns
1239	hexyl-2-metyl-butanoate	0.06	0.18	0.12	0.03	ns	0.12	ab	0.14	a	0.11	b	*
1246	hexyl-*iso*-valerate	0.00	0.20	0.09	0.05	ns	0.07	a	0.07	a	0.11	a	ns
1249	Carvone	0.02	0.29	0.11	0.05	ns	0.09	b	0.09	b	0.15	a	***
1260	linalool acetate	0.00	0.06	0.01	0.02	ns	0.01	a	0.01	a	0.01	a	ns
1424	*α*-gurjunene	0.00	0.55	0.07	0.09	ns	0.07	a	0.05	a	0.08	a	ns
1435	*β*-caryophyllene	0.19	0.85	0.44	0.17	**	0.48	a	0.44	a	0.38	a	ns
1451	*trans- β*-farnesene	0.04	0.22	0.11	0.05	ns	0.12	a	0.12	a	0.08	b	*
1485	germacrene D	0.07	0.25	0.15	0.05	ns	0.17	a	0.15	ab	0.13	b	ns
1512	*cis-α*-bisabolene	0.00	0.14	0.05	0.03	ns	0.06	a	0.06	a	0.04	a	ns
1517	γ-cadinene	0.05	0.22	0.11	0.05	ns	0.14	a	0.10	b	0.09	b	*
1524	*δ*-cadinene	0.00	0.20	0.07	0.04	ns	0.09	a	0.05	b	0.06	b	*
1559	Elemol	0.00	0.04	0.01	0.01	ns	0.00	a	0.01	a	0.01	a	ns
1585	germacrene-D-4-ol	0.00	0.12	0.02	0.02	ns	0.03	a	0.02	a	0.02	a	ns
1595	caryophyllene oxide	0.26	0.89	0.46	0.16	ns	0.43	ab	0.39	b	0.55	a	*
1604	viridiflorol	0.00	0.31	0.03	0.08	ns	0.01	a	0.02	a	0.08	a	ns
1616	ledol	0.00	0.11	0.05	0.03	ns	0.05	a	0.04	a	0.05	a	ns
1652	*α*-muurulol	0.16	0.73	0.38	0.12	ns	0.48	a	0.35	b	0.32	b	***
1691	*α*-bisabolool	0.00	0.84	0.23	0.16	**	0.29	a	0.20	a	0.19	a	ns
	Monoterpene hidrocarbons	3.34	9.81	6.89	1.45	ns	7.96	a	6.99	b	5.74	c	***
	Oxigenated monoterpenes	86.63	94.10	89.96	1.74	ns	88.46	c	90.03	b	91.40	a	***
	Sesquiterpene hydrocarbons	0.53	1.68	0.98	0.31	*	1.14	a	0.97	a	0.85	a	ns
	Oxigenated sesquiterpenes	0.65	2.01	1.17	0.35	ns	1.29	a	1.02	a	1.21	a	ns
	Total identified	98.00	99.50	99.01	0.31		98.84		99.00		99.20		

[a]Linear retention index relative to n-alkanes on non-polar column. [b]Order of elution in non-polar column DB-5. In bold the 7 compounds used for the PCA. st: Statistical significance of the variables Population and Year. ns= no significant * P<0.05, ** P<0.01, *** P<0.001. Different letter for the campaigns (2009, 2010 and 2011) means significant differences.

Table 10. Quality ranges from the International Standard Organization for *Thymus mastichina, Salvia lavandulifolia* and *Lavandula latifolia*.

ISO 4728:2003		**ISO 3526:2005**		**ISO 4719:2012**	
T. mastichina	(%)	*S. lavandulifolia*	(%)	*L. latifolia*	(%)
α-pinene	1-4.5	*α*-pinene	4.0-11.0	limonene	0.5-3.0
β-pinene	2.0-5.0	sabinene	0.1-3.0	1,8-cineol	16.0-39.0
limonene	1.0-6.0	limonene	2.0-5.0	linalool	34.0-50.0
1,8-cineol	30.0-68.0	1,8-cineol	11.0-30.0	linalyl acetate	t-1.6
linalool	3.0-48.0	linalool	0.3-4.0	*α*-terpineol	0.2-2.0
camphor	0.1-2.0	camphor	15.0-36.0	*trans*-*α*-bisabolene	0.4-2.5
δ-terpineol	0.2-2.0	borneol	1.0-5.0		
borneol	0.1-1.8	terpinen-4-ol	0-2		
terpinen-4-ol	0.2-1.2	linalyl acetate	0.1-5.0		
linalyl acetate	0.2-4.0	sabinyl acetate	0.5-9.0		
β-caryphyllene	0.5-1.5				

%: Peak area percentage

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Chapter 3

By-product of *Lavandula latifolia* Medik. essential oil distillation as source of natural antioxidants

Méndez-Tovar I., Herrero B., Pérez-Magariño S., Pereira J.A., Asensio-S-Manzanera M.C. (**2014**). By-product of *Lavandula latifolia* essential oil distillation as source of antioxidants. *Journal of Food and Drug Analysis*, 23, 225-233.

By-product of *Lavandula latifolia* essential oil distillation as source of antioxidants.

Abstract

The objective of the work was to evaluate the antioxidant properties of *Lavadula latifolia* wastes. Samples of 12 populations collected in 2009 and 2010, were hydrodistilled and by-products were analysed through Folin-Ciocalteu, free radical scavenging activity (DPPH) and the ferric reducing antioxidant power (FRAP) methods. Rosmarinic acid, apigenin and luteolin contents were analysed by HPLC-DAD. Mean of total phenols ranged from 1.89±0.09 to 3.54±0.22 mg GA eq/g DW. Average value of the EC50 for scavenging activity ranged from 5.09±0.17 to 14.30±1.90 mg/mL and the variability of the EC50 in FRAP ranged from 3.72±0.12 to 18.55±0.77 mg/mL. Annual variation was found and environmental conditions of 2009 were more favourable. The population Sedano showed the highest antioxidant power. Rosmarinic acid and apigenin content, contributed to the antioxidant properties of the waste. In conclusion, the by-product of the distillation industry could be valorising as source of natural antioxidants.

Keywords: Spike Lavender, by-product, antioxidant activity, total phenol content, interpopulation variability.

5.1. Introduction

The genus *Lavandula* is a member of the Lamiaceae (Labiatae) family which comprises 39 species. This genus has a wide distribution from the Macaronesic region to all the Mediterranean regions and is scattered throughout the North of Africa, the Arabian Peninsula and South Asia reaching India (Morales et al. 2010). In particular, spike lavender (*L. latifolia* Medik.), is a shrub plant that measures between 50 and 70 cm and blossoms in mid-July (Upson 2002). *L. latifolia* is mainly found in limestone rocks or dry pastures on sunny hillsides, in basic substratum and alluvial sands (between 20 and 2050 m) being typical in the Iberian Peninsula, France, Italy and former Yugoslavia (Morales et al. 2010).

Since ancient times, the main uses of *Lavandula* genus have been medicinal, ornamental, for flavourings, to obtain essences, as a condiment and a disinfectant due to its antiseptic properties. In relation to that, the capacity of *Lavandula* oil to act as a bactericidal and bacteriostatic agent and also its antifungal properties have been studied and proved in recent years (Hanamanthagouda et al. 2010).

Lavandula x intermedia Emeric ex Loisel., *L. angustifolia* Mill. and *L. latifolia* Medik. are the most widely used (Morales et al. 2010). Nowadays the main uses of the genus are for medicinal purposes and distillation to obtain essential oils used in the perfume industry. Among the medicinal properties are: anti-inflammatory (Hajhashemi et al. 2003), antispasmodic, anticonvulsant (Gilani et al. 2000) and sedative properties, improving the quality of sleep and reducing anxiety and stress (Field et al. 2008; Kasper 2010).

Spike lavender is used in the perfume industry to extract the essential oil from the plant, generating a residue of several tons because there is a worldwide production of between 50-100 tons every year (Lubbe and Verpoorte 2011). The by-product of the distillation is a problem for the distilling industry due to its huge volumes. In some industries the waste biomass is used for energy or for compost (Schmidt 2010). The disadvantages of this recycling system is that, for industries, recycling the by-product to energy requires a huge investment, and recycling to composting is not always satisfactory due to the antigerminative properties of some aromatic plants (Martino et al. 2010), which may also be transferred by the plant residue. Aerial parts of the plants of the *Lavandula* genus have also the ability to act as a natural antioxidant (Gülçin et al. 2004) acting as a free radical scavenger with a diverse content of polyphenols (Spiridon et al. 2011). Torres Claveira *et a*l. (Torras-Claveria et al. 2007) found an important amount of polyphenols remaining in the by-product of lavandin (Lavandula x intermedia Emeric ex

Loiseleur) finding rosmarinic acid as main compound and an important presence of flavones (apigenin, luteolin, and chrysoeriol). This indicates that other residues may also contain polyphenols and could be valued for it.

To increase the value of *L. latifolia* by-product, it could be used as a source of natural antioxidants. These natural antioxidants could be extracted for animal feed or as a natural food preserver in the food industry. The toxicity of food preservatives like butylated hydroxytoluene known as BHT has been widely proved in animals (Reed et al. 2001), and the toxicity of butylated hydroxyanisole (BHA) has also been studied (Jos et al. 2005). Although recent studies by the European Food Safety Authority (EFSA 2012a; b) reported that the acceptable daily intake of BHA and BHT are not generally exceeded, the safety of these authorised and widely used additives is still controversial. Antioxidant intake and searching for new sources of natural antioxidants are a priority nowadays. This is the reason why numerous studies on the antioxidant content of plants have been carried out in recent years (Chiu et al. 2013; Spiridon et al. 2011; Zheng and Wang 2001).

In order to exploit spike lavender as a source of natural antioxidants, it is essential to know the variability in the antioxidant content of different populations of *L. latifolia*, so as to select those with a high content of antioxidants. The objective of this work was to study the variability of the antioxidant capacity and polyphenols content among populations and seasons in the aerial parts of the plant and in the hydrodistilled residue. In this way, it would be possible to revalue the waste of the distilling industry, thus reducing production costs and preserving the environment. Antioxidants and phenols are influenced by climatic conditions (Lamien-Meda et al. 2010), therefore the populations were studied during two years.

5.2. Materials and methods

Plant material

The aerial parts of 12 wild populations of *L. latifolia* were collected during the blossom phase in the summer of 2009 and 2010 in twelve locations from Castilla y León (Spain).
Voucher specimens of these populations were deposited in Botany Laboratory, herbarium of campus of Palencia (PALAB).

In Table 11 the province, locality and geographical coordinates of the sampling sites are indicated. The plant material was dried for four weeks at room temperature, in the dark, after

being collected. When the drying process was finished, leaves and flowers were separated from the stems and only the mix of leaves and flowers was used for further analysis. Part of the raw plant was ground by a grinder type ZM 1 (Retsch, Germany) and preserved in a glass flask for further analyses.

Chemical and Reagents

Methanol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu reagent was supplied by Panreac (Barcelona, Spain). Sodium carbonate and trichloroacetic acid (TCA) were obtained from Fluka (Steinheim, Switzerland). Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and ferric chloride [$FeCl_3.6H_2O$] were supplied by Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffer (pH 6.6) was prepared from sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) supplied by Merck (Darmstadt, Germany) and disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), supplied by Panreac (Barcelona, Spain).

Distillation process

The essential oils were isolated from 180 g of dried material of each population by hydrodistillation in 2 L of water for 150 min, using a Clevenger-type apparatus. The by.product obtained (waste plant material obtained after removing the essential oils through distillation process) was dried in an oven at 32 ºC for 48 hours and used for further analysis. The dry by-product was ground by a grinder type ZM 1 (Retsch, Germany) and preserved in a glass flask for further analyses.

Preparation of extracts

For each population, three homogeneous samples of 0.5 g of ground plant material or ground by-product were mixed with 15 mL of methanol. After addition of methanol the mixture was vigorously shaken for 5 min and allowed to decant for 1 hour. Subsequently, the methanol extracts were separated and stored at -18 ºC until analysis of total phenol content and antioxidant activity by DPPH and FRAP methods.

Determination of total phenol content

The total phenol content of the extracts was measured using the Folin-Ciocalteu method based on a colorimetric assay described by Singleton and Rossi (1965). One mL of each extract (with a concentration of 5 mg/mL for the plant and 10 mg/mL for the by-product) was mixed with

1 mL of Folin-Ciocalteu reagent. After 2 min, 1 mL of saturated solution of sodium carbonate was added and finally 7 mL of milliQ water were added. After 90 min of reaction in the dark at room temperature, the absorbance was measured at 725 nm in a Jasco V530 UV/VIS Spectrophotometer. The content of total phenols in each extract was determined through a calibration curve of gallic acid (0.01-0.08 mM; correlation coefficients > 0.99). The total phenol content was expressed as mg of gallic acid equivalent/g of dry weight plant or dry weight by-product (mg GAE/g).

Free radical scavenging activity (DPPH method)

The free radical scavenging activity was determined using the method reported by Pereira *et al*. (2008).

The analysis was carried out with 300 μL of six different concentrations of methanolic-aqueous dissolutions (0.66-16.66 mg/mL) mixed with 2.7 mL of DPPH radical (6 x 10^{-5} mol/L in methanol). A blank solution was also prepared with 300 μL of milli-Q water and 2.7 mL of DPPH solution. The mixture was vigorously shaken and allowed to rest for 60 min in the dark at room temperature. A colorimetric evaluation was then carried out in the spectrophotometer at 517 nm. The free radical scavenging was measured as a percentage of DPPH decolouration following the equation:

$$\%\text{Scavenging effect} = [(A_{DPPH} - A_S)/A_{DPPH}]x100$$

where A_{DPPH} is the absorbance of the blank solutions and A_S is the absorbance of each sample concentration tested. The extract concentration providing 50% inhibition (EC_{50}) was calculated. Lower EC_{50} value means a higher antioxidant activity.

Ferric Reducing Antioxidant Power (FRAP)

Six different concentrations of the methanolic-aqueous dissolutions (0.66-16.66 mg/mL) were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) $K_3[Fe(CN)_6]$ solution. The mixtures were incubated for 20 min in a water bath at 50°C. The incubated mixtures were allowed to cool at room temperature. Once cooled 2.5 mL of 10% (w/v) trichloroacetic solution (TCA) was added. The solutions were mixed thoroughly, aliquots of 2.5 mL were withdrawn and 0.5 mL of 0.1% (w/v) $FeCl_3.6H_2O$ solution was added. The absorbance was measured at 700 nm. The same procedure was followed for a blank but with milli-Q water

instead of the sample. The extract concentrations needed to provide an absorbance of 0.5 (EC_{50}) were calculated. Lower EC_{50} value means a higher antioxidant activity.

HPLC analysis

For HPLC analysis, 0.5 g of every sample of by-product were solved in petroleum ether during 24 hours, then filtered and extracted in a soxhlet with methanol as solvent for 150 min. The methanol extracts were concentrated under vacuum at 50 ºC using a rotary evaporator and then were re-suspended in 5 mL of acetonitrile/water (1/1).

HPLC analysis was performed in an Agilent Technologies 1200 series High Performance Liquid Chromatograph (HPLC) with a Diode Array Detector (DAD) and equipped with a Zorbax Eclipse XDB-C18 column (150 mm x 4.6 mm d.i., 5 µm; Agilent Technologies). The column was thermostated at 25 ºC and the injection volume was 20 µL. The solvents were (A) water:acetic acid (98:2) and (B) acetonitrile. The flow rate was 1.2 mL min^{-1}. The linear gradient used was: from 10 to 22% B in 10 min, from 22 to 38% B in 2 min, isocratic for 5 min, finally from 38 to 100% B in 2 min and then isocratic for 5 min. Compound identification was done by comparing their retention times and UV-visible spectra with their respective pure standards at a wavelength of 254, 280 or 350 nm depending on the maximum absorption of each compound. The phenolic compounds were quantified using the external standard method and the respective calibration curve of each quantified phenolic compound.

Statistical analysis

The statistical analysis of the data was done with SPSS programme version 15.0 (IBM 2006). A general ANOVA was done to check differences between distilled and non-distilled plant material. The ANOVAs by plant material were performed to evaluate populations and seasons. The Pearson correlation coefficients among total phenol content, free radical scavenging activity and ferric reducing antioxidant power were also determined. To study the relationship between antioxidant capacity and phenolic compounds in the by-product, a stepwise regression was performed.

5.3. Results

Total phenol content

The total phenol content in the non-distilled plant material was higher than the phenol content in the by-product for all studied populations (Table 12). The phenol content in the non-distilled material ranged from 2.67±0.14 (LL-2) to 8.27±0.50 (LL-8) mg GAE/g of dry plant for 2009 and from 2.40±0.12 (LL-1) to 6.83±1.74 (LL-6) mg GAE/g of dry plant for 2010. For the by-product, the phenol content of the populations ranged from 1.89±0.09 (LL-3) to 3.54±0.22 (LL-5) mg GAE/g of dry by-product for the samples collected in 2009 and from 1.97±0.16 (LL-3) to 2.60±0.23 (LL-9) mg GAE/g of dry by-product for 2010. The loss of phenols by hydrodistillation ranged between 24 and 89% depending on the population and the season.
Table 13 shows the sum of squares from the analysis of variance expressed as percentages and indicating the contribution to the variability for the studied factors (population, season and interaction between season and population) and residual express the percentage of the variability not explained by these factors. The effect of the season on total phenol content and the effect of genotypic variability were statistically significant, but the effect of the season was much lower than that of population effect. The interaction between year and population also had a strong effect on phenol content (Table 13), this means that the annual season variation have been not the same for all the populations. The percentages of influence of each variable for both plant and by-product showed that the population was the most important variable followed by interaction between season and population and being the season the less influential variable.

Comparing the results obtained with other authors is neither simple nor accurate because the results vary depending on the extraction method used and the solvent used for the different concentrations of plant material (Krishnaiah et al. 2011). However, comparison with other studies is necessary to have more representative results. For other *Lavandula* species such as *L. angustifolia,* Miliauskas *et al*. (2004) measured the total phenol content in methanolic extracts obtaining 5.4±0.2 mg GAE/g of plant extract, a very similar result to that obtained in this study for *L. latifolia*.

Free radical scavenging activity (DPPH)

The scavenging activity of the aqueous-methanolic dissolutions in the non-distilled plant material was higher than in the by-product for all studied populations (Table 12). The value of the extract concentrations providing a 50% inhibition of DPPH free radicals varied from

1.85±0.05 mg/mL (LL-8) to 4.56±0.15 mg/mL (LL-1) for the non-distilled plant material collected in 2009 and from 2.94±0.09 (LL-8) mg/mL to 5.69±0.35 mg/mL (LL-1) for the samples collected in 2010. For the by-product, the average value of EC_{50} ranged from 6.33±0.10 (LL-5) to 11.90±0.05 (LL-8) mg/mL for the samples collected in 2009 and from 8.48±0.51 mg/mL (LL-12) to 14.30±1.90 mg/mL (LL-3) mg/mL for the samples collected in 2010. For the different populations studied, the increase in the EC_{50} for the by-product in comparison with the plant material ranged from 29 to 84% depending on the population and season.

The effect of the season on scavenging activity was much lower than that of genotypic variability, however both factors were statistically significant, as well as the interaction between year and population. Average values of scavenging activity for samples collected in 2009 showed higher antioxidant capacity than populations collected in 2010 however, checking every population in some cases the results were more favourable in 2009 and in other cases in 2010 because the interaction between year and population was very important and the samples were collected in different places under different weather and ecological conditions.

The lowest EC_{50} were shown by LL-8 (1.85±0.05 mg/mL for 2009 and 2.94±0.09 mg/mL for 2010) and LL-5 (2.83±0.04 mg/mL for 2009 and 3.07±0.08 mg/mL for 2010) for both seasons in the non-distilled plant material. For the by-product, LL-7 and LL-5 showed the highest antioxidant capacity in 2009 with 5.09±0.17 and 6.33±0.14 mg/mL, respectively, and LL-12 and LL-5 in 2010 with 8.48±0.51 mg/mL for LL-12 and 9.21±0.61 mg/mL for LL-5. Miliauskas *et al* (2004) found an inhibition of 35.4±1.7% using a methanol extract concentration at 2.5 mg/mL for *L. angustifolia* which is very similar to those results obtained with the non-distilled plant of *L. latifolia*, since concentrations between 1.85±0.05 to 5.69±0.35 mg/mL provided an inhibition of 50%.

Ferric Reducing Antioxidant Power (FRAP)

The samples analysed with FRAP method showed more antioxidant activity for the non-distilled plant extracts than for the by-product for all the analysed populations the same as with the previous methods (Table 12), except for LL-9 in 2009 where the antioxidant power was higher in the by-product. The variability of the EC_{50} value of the reducing power in the studied populations ranged from 1.79±0.01 mg/mL (LL-8) to 6.21±0.03 mg/mL (LL-1) in the non-distilled plant material collected in 2009 and from 3.33±0.16 mg/mL (LL-9) to 5.83±0.44 mg/mL (LL-1) for the material collected in 2010. For the by-product the data ranged from 3.72±0.12 mg/mL (LL-7) to 9.37±0.51 mg/mL (LL-1) for the samples collected in 2009 and from 7.88±0.61 mg/mL (LL-5) to 18.55±0.77 mg/mL (LL-1) for 2010. The increase in the EC_{50}

for the by-product in comparison with the plant material ranged from 13 to 79% depending on the population and year of harvest.

Like for total phenol content and scavenging activity, the effect of the population on reducing power was much higher than that of season, although both were statistically significant, as was the interaction between year and population (Table 13). Samples collected in 2009 showed higher antioxidant power than samples collected in 2010.

The populations with higher FRAP were LL-8 and LL-5 for 2009 and LL-9 and LL-8 for 2010 with 1.79±0.01, 2.17±0.06, 3.33±0.16 and 3.36±0.10 mg/mL, respectively, for the non-distilled plant. LL-7 and LL-5 in 2009, and LL-5 and LL-8 in 2010 with 3.72±0.12, 4.35±0.12, 7.88±0.61 and 7.97±0.60 mg/mL were the populations with higher FRAP respectively, for the by-product.

Gülçin *et al*. (2004) measured the FRAP in aqueous and ethanolic extracts of *L. stoechas* obtaining values of EC_{50} around 0.04 mg/mL for the ethanolic extracts and around 0.06 mg/mL for the aqueous extracts, showing reducing power values higher than those obtained for *L. latifolia* in our work.

As has already been obtained by other authors (Miliauskas et al. 2004; Torras-Claveria et al. 2007), a negative correlation was found between the phenol content and the EC_{50} values for DPPH antioxidant capacity (Table 14), both in the non-distilled plant extracts and in the by-product (-65.92%, P=0.0220, and -44.92%, P=0.0277, respectively. The lower EC_{50} values, the higher antioxidant capacity). There was also a negative correlation between the phenol content and the EC_{50} values for reducing power method as other authors found (Parejo et al. 2002) but this correlation was only found for the non-distilled plant (-46.51 %, P=0.0220) and not for the by-product. The correlation between EC_{50} values for FRAP and DPPH method was statistically significant for both materials (plant and by-product) with a correlation of 64.20% ($P<0.0007$) for the plant and a correlation of 72.70% ($P<0.0001$) for the by-product.

HPLC analysis

The three phenolic compounds identified in spike lavender hydrodistilled residue were: apigenin, luteolin and rosmarinic acid (Table 15). Rosmarinic acid was the predominant phenolic compound in all populations regardless of the year assessed, although, its variation is important from year to year. The average content of all samples was higher in 2009 (189.18 mg/100g of dry residue) than in 2010 (159.11 mg/100g), however, in some populations the

opposite occurs: LL-3, LL-8, LL-10 and LL-12. High contents of rosmarinic acid is a characteristic of the species of *Lamiaceae* (Janicsák et al. 1999; Komes et al. 2011).

Variations in the content of major phenolic compounds identified were highly significant ($P<0.01$) for all three compounds, among the collected samples. The same as for antioxidant capacity analysis, these data indicate that the presence of polyphenols in the hydrodistilled residue of *L. latifolia* depends on the population and the environmental characteristics.
The results of the stepwise regression between antioxidant capacity and phenolic compounds are shown in Table 16. This regression model was done to predict the influence of the polyphenols on the antioxidant capacity of the by-product. For total phenol content, no variable met the 0.05 significance (Pr>F) for entry into the model, which means that other phenolic compounds not measured and present in methanolic extracts of waste of spike lavender are influencing the amount of total phenols. In the case of scavenging effect and ferric reduction antioxidant power, rosmarinic acid was ranked before the apigenin, for both of the models, which explain 51.88 and 40.16% (Partial R-Square) of the variability, respectively. Despite this, the models explained only 57.88 and 63.54% (Model R-Square) of the variability, respectively, indicating that, although these two compounds are responsible for part of the antioxidant activity of spike lavender waste, there are other compounds that affect this biological activity.

5.4. Discussion

Methanolic extracts of the by-product showed a considerably lower antioxidant activity than the plant material before distillation. The results found are in agreement with those found by Chizzola *et al*. (2008) who found a smaller amount of antioxidants in the by-products of leaves of *Thymus vulgaris* than in the non-distilled plant material. Water soluble compounds and essential oil are extracted from the original plant material by the distillation process, and some of these compounds also contribute to the total antioxidant activity (Katalinic et al. 2006). Moreover, the essential oil of spike lavender contains a proportion of antioxidants (Chia-Wen et al. 2009), and due to that the by-product obtained after distillation showed less antioxidant power. On the other hand, phenolic compounds are in general sensitive to prolonged exposure to heat and could be degraded with thermal treatments (Chipurura et al. 2010) such as hydrodistillation and the industrial process of essence extractions.

Although the waste of the distilling industry showed less antioxidant activity than the original plant, it would be possible to recover appreciable amounts of antioxidants from hydrodistilled residue. Rosmarinic acid presence in this residue is a characteristic of *Labiatae* family (Komes

et al. 2011), and our results confirm that this phenolic acid, as well as apigenin, contribute to the antioxidant properties of the by-product. However, these compounds do not explain all the antioxidant activity of the samples; other phenolic compounds detected in these samples but not identified could also have antioxidant properties.

There was a considerable variability in the total phenol content, in the antioxidant capacity data and in the FRAP among the populations studied. For all cases, except for the total phenol content of the by-product of 2010 which presented medium values, the population LL-5 from Sedano (Burgos) is among the three populations with greater antioxidant capacity and phenol content for the two seasons both in the non-distilled material and in the by-product. By contrast, the population LL-1 from Dévanos (Soria) is among the three populations showing less antioxidant activity for both years and for the three colorimetric methods except for the phenol content of the by-product of 2009. This preliminary study showed that the population LL-5 could be selected by its highest antioxidant properties.

Phenols are secondary metabolites which are formed by the plant in stress conditions such as drought, competition with other plants and infection (Kirakosyan et al. 2003; Manach et al. 2004) among other aspects. The interannual variation was statistically significant in all cases although it was less important than population variation. Conditions in 2009 proved to be more favourable than those of 2010 due to a set of variables such as rainfall and temperatures. Irrigation causes a reduction in the antioxidant content of plants and fruits (Patumi et al. 2002), and given that the 2009 season was a dry summer in Castilla y León with low rainfall and high drought conditions with respect to the overall mean, this could be responsible for the higher antioxidant activity of the samples collected in 2009.

Data of the latitude, longitude and altitude were collected for every population however there were no correlation between these parameters and the experimental data.

Other species of the Lamiaceae family noted for antioxidant activity could be: *Mentha longifolia* L. ssp. *longifolia* with an EC_{50} for antioxidant activity of 57.4±0.5 mg/L, and with a phenol content of the extract of 45 mg GAE/g of dry extract (Güllüce et al. 2007); *Origanum vulgare* with an EC_{50} for the antioxidant activity in methanol extracts of 9.5±0.5 mg/L and with a phenol content of 220 mg GAE/g dry extract (Şahin et al. 2004). The antioxidant activity of *L. latifolia* in comparison with the aforementioned species does not stand out, but it could be considered that the by-product of *L. latifolia* is a source of natural antioxidants.

5.5. Conclusions

Distillation process of *L. latifoli*a produces a non profitable waste and this by-product generated could be valorised and used as a source of natural antioxidants. In order to offer to the industry a homogeneous product, it would be necessary to standardise the production condition and to look at the factors that influence the antioxidant content. This study concludes that populations, seasons and population by season interaction influence the antioxidant properties of the *L. latifolia* by-product.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Table 11. Geographical coordinates of collected populations of *Lavandula latifolia*.

Populations	Province	Locality	Latitude (N)	Longitude (W)	Altitude (m)
LL-01	Soria	Dévanos	41º54'06"	1º55'01"	968
LL-02	Soria	Velamazán	41º29'05"	2º47'22"	936
LL-03	Segovia	Moral de Hornuez	41º27'15"	3º37'56"	1133
LL-04	Segovia	Fuentidueña	41º26'41"	3º57'38"	844
LL-05	Burgos	Sedano	42º41'18"	3º44'13"	750
LL-06	Burgos	Santibáñez del Val	41º58'38"	3º29'08"	953
LL-07	Burgos	Gumiel de Izán	41º46'23"	3º40'40"	899
LL-08	Valladolid	Quintanilla de Onésimo	41º37'14"	4º20'09"	879
LL-09	Palencia	Aguilar de Campoo	42º45'33"	4º13'50"	913
LL-10	Soria	Tejado	41º33'40"	2º13'26"	1066
LL-11	Palencia	Cevico Navero	41º52'21"	4º11'36"	916
LL-12	Palencia	Reinoso de Cerrato	41º56'57"	4º22'28"	876

Table 12. Total phenol content, EC_{50} of scavenging activity, and EC_{50} of ferric reduction antioxidant power, of the plant and the by-product of the 12 population of *Lavandula latifolia*.

Population	Plant material 2009			By-product 2009		
	TP	**DPPH**	**FRAP**	**TP**	**DPPH**	**FRAP**
	mg GAE/g	**EC_{50} mg/mL**	**EC_{50} mg /mL**	**mg GAE/g**	**EC_{50}mg/mL**	**EC_{50}mg/mL**
LL-1	3.70±0.21	4.56±0.15	6.19±0.24	2.55±0.39	8.25±1.14	9.37±0.51
LL-2	2.67±0.14	4.28±0.38	3.92±0.13	1.91±0.34	10.55±1.05	7.45±0.31
LL-3	3.77±0.18	2.94±0.26	2.93±0.05	1.89±0.09	10.41±0.48	7.61±0.24
LL-4	3.13±0.28	4.39±0.16	3.36±0.23	2.09±0.13	8.97±0.30	6.17±0.07
LL-5	5.15±0.46	2.83±0.04	2.17±0.06	3.54±0.22	6.33±0.14	4.35±0.12
LL-6	3.70±0.44	3.82±0.07	3.39±0.03	2.05±0.12	8.03±0.45	5.21±0.15
LL-7	4.36±0.11	3.62±0.08	3.24±0.02	2.62±0.09	5.09±0.17	3.72±0.12
LL-8	8.27±0.50	1.85±0.05	1.79±0.01	2.00±0.11	11.90±0.05	8.43±0.17
LL-9	4.93±0.20	3.18±0.08	6.21±0.03	2.45±0.16	8.23±0.59	5.61±0.08
LL-10	5.07±0.33	3.61±0.96	2.79±0.28	2.13±0.12	9.67±0.41	7.25±0.24
LL-11	4.09±0.22	3.86±0.10	3.28±0.07	2.36±0.07	8.42±0.53	5.76±0.27
LL-12	4.00±0.26	3.86±0.11	3.42±0.16	2.15±0.13	10.14±0.37	7.44±0.14
Population	**Plant material 2010**			**By-product 2010**		
	TP	**DPPH**	**FRAP**	**TP**	**DPPH**	**FRAP**
	mg GAE/g	**EC_{50}mg/mL**	**EC_{50}mg/mL**	**mg GAE/g**	**EC_{50}mg/mL**	**EC_{50}mg/mL**
LL-1	2.40±0.12	5.69±0.35	5.83±0.44	2.14±0.16	12.63±0.86	18.55±0.77
LL-2	3.06±0.22	3.19±0.05	3.67±0.15	2.11±0.13	11.26±1.21	10.85±0.99
LL-3	3.79±0.33	3.24±0.02	3.64±0.13	1.97±0.16	14.30±1.90	11.03±1.72
LL-4	4.50±0.49	3.59±0.16	3.91±0.32	2.00±0.12	13.87±0.67	11.03±0.63
LL-5	6.30±0.36	3.07±0.08	3.55±0.39	2.36±0.17	9.21±0.61	7.88±0.61
LL-6	6.83±1.74	4.06±0.44	3.82±0.43	2.29±0.14	11.20±0.39	9.65±0.30
LL-7	4.57±0.42	3.22±0.21	3.98±0.19	2.16±0.05	10.29±0.52	9.38±0.74
LL-8	4.79±0.36	2.94±0.09	3.36±0.10	2.49±0.23	9.22±0.65	7.97±0.60
LL-9	4.05±0.35	3.11±0.08	3.33±0.16	2.60±0.23	9.99±0.66	8.70±0.78
LL-10	3.29±0.30	4.01±0.26	4.47±0.26	2.52±0.30	9.64±1.09	9.49±1.08
LL-11	2.88±0.18	4.83±0.28	5.39±0.22	2.57±0.28	9.81±1.22	8.35±1.38
LL-12	3.16±0.17	5.33±0.16	4.42±0.23	2.50±0.20	8.48±0.51	8.71±0.43

TP: total phenol content. **DPPH EC_{50}:** extract concentration providing a 50% inhibition of scavenging activity. **FRAP EC_{50}:** extract concentration needed to provide an absorbance of 0.5 of ferric reduction antioxidant power. **mg GAE/g:** mg of gallic acid equivalents per g of dry matter.

Table 13. Percentages of the sum of squares obtained in the analysis of variance by type of plant material using population, year and year by population interaction as sources of variation.

	df	Total phenols		df	DPPH		df	FRAP	
Plant material									
Population	11	55.57	***	11	70.19	***	11	58.77	***
Year	1	0.85	***	1	2.76	***	1	6.62	***
Year x Population	11	33.71	***	11	18.88	***	11	32.11	***
Residual	192	9.87		48	8.18		120	2.50	
By-product									
Population	11	50.04	***	11	37.04	***	11	48.03	***
Year	1	0.80	**	1	20.85	***	1	35.28	***
Year x Population	11	29.98	***	11	31.28	***	11	12.66	***
Residual	192	19.21		48	10.83		120	4.02	

****** values significant at P< 0.01; ******* values significant at P< 0.001 **df:** degrees of freedom**. DPPH:** Radical scavenging activity. **FRAP:** Ferric reducing antioxidant power.

Table 14. Coefficients of Pearson correlation among total phenols, free radical scavenging activity and ferric reducing antioxidant power.

		Plant		
		TP	**DPPH**	**FRAP**
By-product	**TP**		-0.6592	-0.4652
			P<0.0005	P<0.0220
	DPPH	-0.4492		0.642
		P<0.0277		P<0.0007
	FRAP	-	0.727	
			P<0.0001	

TP: total phenols. **DPPH:** free radical scavenging activity. **FRAP:** ferric reducing antioxidant power. **P:** statistical significance.

Table 15. Phenolic compounds identified in by-product of 12 *Lavandula latifolia* populations (results expressed in mg/100 g of dry extract).

Population code		LL-1	LL-2	LL-3	LL-4	LL-5	LL-6	LL-7	LL-8	LL-9	LL-10	LL-11	LL-12	Average	St dev	Min	Max
Apigenin	2009	3.25	3.18	3.43	3.14	3.16	3.10	3.41	3.30	3.29	3.40	5.59	3.57	3.49	0.68	3.10	5.59
	2010	1.78	1.29	0.77	0.54	1.64	1.31	0.77	0.54	0.77	0.49	0.58	0.33	0.90	0.48	0.33	1.78
Luteolin	2009	4.94	4.91	4.59	5.55	4.39	4.56	4.70	5.29	6.04	6.70	10.26	6.26	5.68	1.62	4.39	10.26
	2010	4.00	5.91	3.25	3.92	3.88	2.87	2.98	3.61	2.73	3.64	3.42	2.08	3.52	0.94	2.08	5.91
Rosmarinic acid	2009	180	200	119	193	255	215	304	158	223	170	134	119	189	56	119	304
	2010	90	138	142	133	154	129	218	191	180	224	137	173	159	39	90	224

Table 16. Stepwise regression analysis taking scavenging activity (DPPH) and Ferric Reduction Antioxidant Power (FRAP) as dependent variables and the polyphenol content of apigenin, luteolin and rosmarinic acid as independent variables.

	Step	Variable Entered	Partial R-Square	Model R-Square	F-value	Pr>F
DPPH						
	1	Rosmarinic acid	0.5188	0.5188	23.72	<0.001
	2	Apigenin	0.1166	0.6354	6.72	0.017
FRAP						
	1	Rosmarinic acid	0.4016	0.4016	14.77	0.0009
	2	Apigenin	0.1772	0.5788	8.83	0.0073

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Chapter 4

Contribution of the main polyphenols of *Thymus mastichina* to its antioxidant properties L

Méndez-Tovar I., Sponza S., Asensio-S-Manzanera MC., Schmiderer C. Novak J. (**2015**). Contribution of the main polyphenols of *Thymus mastichina* subsp. *mastichina* to its antioxidant properties. *Industrial Crops and Products*, 66, 291-298.

Contribution of the main polyphenols of *Thymus mastichina* subsp. *mastichina* to its antioxidant properties.

Abstract

The antioxidant activity and phenol content of 14 populations of *Thymus mastichina* grown in an experimental plot, was analysed by DPPH, FRAP and Folin-Ciocalteu method to define their antioxidant activity. Polyphenols were analysed by HPLC-DAD and the relationship between polyphenols and antioxidant capacity was established. Populations means for DPPH activity ranges were 44-98 mg TE/g DW while FRAP antioxidant capacity was 52-115 mg TE/g DW. Total phenol content ranged between 11 and 38 mg of CAE/g DW for the different populations. The polyphenols identified were: chlorogenic acid, caffeic acid, rosmarinic acid, luteolin glucoside and luteolin. The main polyphenols were rosmarinic acid, ranging from 1.7 to 43 mg/g DW, one unidentified polyphenol designated as Peak 3 (0.53-15 LE eq mg/g) and luteolin glucoside ranging from 0.96-19 LE eq mg/g. Rosmarinic acid contributed mainly to the FRAP antioxidant capacity and to the total phenols, while peak 3 contributed mainly to the DPPH assay. Luteolin, chlorogenic acid and caffeic acid had a range of 0-2.7, 0.07-2.2, 0-0.46 mg/g DW, respectively. The study showed high intra-populations variability and above all high inter-populations variability. The Carrocera population had the highest antioxidant activity and amount of phenols and it could be selected for its content.

Keywords

Spanish marjoram, DPPH, FRAP, total phenol content, cultivated plants.

6.1. Introduction

Thymus mastichina L. is an endemic species from the Iberian Peninsula belonging to the Lamiaceae family. It is divided into two subspecies: *T. mastichina* subsp. *mastichina* and *T. mastichina* subsp. *donyanae* (Morales 2002). The first subspecies grows in most of Spain and Portugal, while *T. mastichina* subsp. *donyanae* is found only in the southwest of the Iberian Peninsula. Spanish marjoram is a species rich in essential oil and its main essential oil compounds are 1,8-cineole and linalool (Salgueiro et al. 1997).

Since ancient times, this plant species has been used as a food spice and currently has numerous applications (Barros et al. 2010). In the field of food, it is mainly used as an infusion or as a dressing for olives; in the field of medicine, for its antitussive and antiseptic properties, and in the perfume and cosmetic industry, its essential oil is used as an ingredient in perfumes, creams, soaps etc. The biological activities of this species have also been investigated recently showing anti-inflammatory (Albano and Miguel 2011), anti-carcinogenic activity (Gordo et al. 2012) and antifungal properties (Leal et al. 2013).

T. mastichina is also known for its antioxidant properties. The existence of polyphenols such as rosmarinic acid and luteolin was reported in this plant species (Gordo et al. 2012) as well as in other species from the genus *Thymus* (Costa et al. 2012). The analysis of the antioxidant activity of essential oils in *T. mastichina* with colorimetric methodologies has been widely studied by several authors (Bentes et al. 2009; Galego et al. 2008; Miguel et al. 2007; Miguel et al. 2005; Salgueiro et al. 1997) however, the antioxidant activity in plant extracts of *T. mastichina*, has only been reported by Barros *et al*. (2010) when evaluating samples from Bragança (Portugal) and by Albano *et al*. (2011) on analysing samples from the Algarve (Portugal).

The antioxidant variability among populations coming from different origins has not yet been evaluated in *T. mastichina.* This might be because it is an endemic species adapted to the Iberian Peninsula however its study is very important for the growers. The high variability that may exist among different origins of the same species can be a problem for the commercialization of this plant species. Furthermore environmental and external factors play a secondary role for the qualitative and quantitative accumulation of secondary metabolites (Horwath et al. 2008). Antioxidant variability may occur by seasonal variation and is also influenced by environmental factors such as weather conditions or temperature (André et al. 2009; Skrzypczak-Pietraszek and Pietraszek 2012). The evaluation of the variability in

antioxidants under the same environmental factors, finding populations or individual plants with high antioxidant activity is an important issue to standardize plant raw material for production and to further optimize it by plant breeding.

This work aims to evaluate the variability in antioxidant activity and main polyphenols composition of *T. mastichina* from Spain. Fourteen populations were studied in order to find specimens with high antioxidant levels and to know the contribution of the phenolic profile to the antioxidant properties of the species. The samples were analysed using colorimetric methodologies and HPLC-DAD analysis.

6.2. Materials and Methods

Plant Material

The samples of this study come from a field trial comprising fourteen populations of *T. mastichina* located in the Instituto Tecnológico Agrario de Castilla y León (ITACyL) in Valladolid (Spain). The populations came from several localities in Spain (Table 17); they were multiplied by vegetative reproduction establishing the assay in 2010 (Asensio S.Manzanera et al. 2010). To avoid the influence of environmental and external factors, all the samples were placed in the same experimental field under the same environmental and weather conditions.

All the samples were collected at the beginning of the flowering phase during June and early July 2012. The flowers and leaves of 7-10 individual plants of each population were collected separately and air dried at room temperature in a dark room and conserved under these conditions until extraction.

Chemicals and solvents

Luteolin, caffeic acid, chlorogenic acid, rosmarinic acid, 2,2 diphenyl 1 picrylhydrazyl radical (DPPH) and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma-Aldrich (Steinheim, Germany). Folin-Ciocalteu reagent, methanol (p.a.), sodium acetate trihydrate, sodium nitrite, and ferric chloride hexahydrate were obtained from Merck (Darmstadt, Germany). Trolox was purchased from Fluka (Denmark, Germany). Sodium carbonate anhydrous, sodium hydroxide, hydrochloric acid, acetic acid, acetonitrile and HPLC grade methanol were obtained from Carl Roth (Karlsruhe, Germany).

Extraction

The extracts were prepared from 100 mg of dry flowers and leaves of *T. mastichina* that were previously ground. After an extraction in 16 mL methanol 50% for one hour at room temperature in an ultrasonic bath, the samples were filtered using Pasteur pipettes covered with cotton on their tip and stored in a cooling room (+4°C) until analysis.

Free radical scavenging activity method (DPPH)

The free radical scavenging activity was measured by DPPH radical, following the methodology described by Lamien-Meda *et al*. (2010). The extracts were diluted using 200 µL of the original extract and filled up to 500 µL with methanol 50%. 10 µL of this dilution was adjusted to 100 µL of methanol and mixed with 100 µL of DPPH solution (0.015%). The microplate was covered with parafilm, incubated in darkness for 30 min. and was then read at 490 nm with a microplate reader (BIO-RAD Tokyo, Japan). The calibration curve was prepared with six different concentrations of trolox (0-2.48µg/mL) using the highest concentration as a blank. Every result is a mean of a quadruplicate analysis and is expressed as mg of trolox equivalent per gram of dry weight (mg TE/g DW).

Ferric reduction antioxidant power assay (FRAP)

The FRAP assay was carried out to evaluate the capacity of the extracts to reduce ferric ion (Fe3+) to ferrous ion (Fe2+) and produce ferrous-tripyridyltriazine. The colour of the samples changes depending on the reducing power of each extract. Ferric chloride hexahydrate ($FeCl_3 6H_2O$) dissolved in methanol (6.26 mg in 10 mL of MeOH) and tripyridyltriazine (TPTZ) (31.2 mg of TPTZ in 10 mL of HCL 40 mM) were prepared before analysis. 25 mL of acetic acid buffer (pH=3.6) were mixed with 2.5 mL of TPTZ solution and 2.5 mL of chloride hexahydrate to form the working reagent. 1 µL of the original extract was mixed with 180 µL of working reagent and 23 µL of H_2O. Trolox was used as a standard to prepare the calibration curve with a total of eight different concentrations (0-400 µg/mL). 6 µL of trolox was mixed with 180 µL of working reagent and 18 µL of H_2O. The blank was performed with 180 µL of working reagent and 24 µL of H_2O. The microplate was covered with parafilm and incubated in darkness for 5 min then read in the microplate reader (BIO-RAD Tokyo, Japan) at 595 nm. The results are expressed in mg TE/g DW and are presented as means of quadruplicate analyses.

Total phenol content

The Folin-Ciocalteu method to determine the total content of phenols was carried out according to the methodology of Chizzola *et al*. (2008). The original extract solution was diluted (100 µL of the original extract mixed with 300 µL methanol 50%) and 10 µL of this dilution were used for analysis mixing it with 225 µL of H_2O, 5 µL of Folin-Ciocalteu reagent and 10 µL of Na_2CO_3 solution (35% in H_2O). A calibration curve with eight different concentrations (0-23 µg/mL) of caffeic acid was performed using 10 µL of each concentration. A blank was performed using 10 µL of methanol 50%. The microplate was covered with parafilm and incubated in darkness for 30 min. Then the plate was measured at 750 nm with a microplate reader model 450 (BIO-RAD Tokyo, Japan). Every analysis was carried out in quadruplicate and the results are expressed as mg of caffeic acid equivalents per gram of dry weight (mg CAE/ g DW).

High performance liquid chromatography analysis (HPLC-DAD)

The analyses of polyphenols of the *T. mastichina* extracts were carried out with a HPLC system from Water Corporation (Mildford, MA). The system was equipped with a quaternary pump, an auto-sampler (Waters 717) and a photo diode array detector (DAD Detector Waters 996). The column used was reverse phase symmetry C18 (4.6 mm x 150 mm, 5µm pore size) Luna 5u (Phenomenex). The two mobile phases used were: 1% acetic acid/acetonitrile (85:15 v/v) as solvent A and methanol as solvent B. The gradient elution profile started with A-B (90:10). Solvent B was gradually increased to 100% with a flow rate of 1.5 mL/min at 30 min and kept constant for 10 min. The identification of polyphenols was achieved by comparison of their retention times and UV spectra with those of reference standards. Detection was performed at 330 nm for rosmarinic acid, chlorogenic acid, luteolin and caffeic acid. Calibration curves were made for each standard with the concentrations range 1-100 µg/mL except for unknowns, which were quantified in luteolin equivalent (mg LE/g DW).

Statistics

Statistical analyses by analysis of variance (ANOVA) were carried out with SPSS 15 (SPSS Inc., Chicago, IL) and the average values were compared using Tukey B test at $P<0.05$. Correlation between methods and compounds detected was calculated through Pearson correlation coefficients. A stepwise regression was done to build a predictive model using colorimetric methods as dependent variables and chlorogenic acid, caffeic acid, peak 3, luteolin glucoside, rosmarinic acid and luteolin as independent variables.

6.3. Results and Discussion

Antioxidant activity. DPPH and FRAP

DPPH and FRAP antioxidant capacities of the different populations studied are represented in box-plot graphs in Figures 4A and 4B. Populations with high DPPH free radical assay also showed high FRAP antioxidant activity and vice versa with the exception of the Hontanar population, which showed relatively high FRAP antioxidant capacity (86 mg TE/g DW) and medium-low DPPH activity (55 mg TE/g DW). Both methods showed a high significant correlation of 0.755 ($P<0.01$) (Table 18).

The species showed very high variability among individual plants analysed with a range for DPPH assay from 18 to 149 mg TE/g DW and a range for FRAP antioxidant capacity from 30 to 154 mg TE/g DW.

High inter-population variability was also found. The average content of the populations with the highest amount of antioxidants was twice as high as the average of populations with a lower content of antioxidants. Saldaña, Lerma and Carrocera were the populations with the highest DPPH assay with mean values of 98, 88, 78 mg TE/g DW, respectively. These three populations plus Hontanar and Ponferrada were the populations with the highest FRAP antioxidant capacity: Lerma (115 mg TE/g DW), Carrocera (96 mg TE/g DW), Hontanar (86 mg TE/g DW), Saldaña (83 mg TE/g DW) and Ponferrada (79 mg TE/g DW). On the other hand Serranillos was the population with the lowest values for DPPH assay (44 mg TE/g DW) and FRAP antioxidant capacity (52 mg TE/g DW).

As regards intra-population variability, Lezuza and Moral de Calatrava showed the lowest variability with similar values among the different individual plants of the same population. The range for Lezuza was from 21 to 69 mg TE/g DW for DPPH free radical assay and from 50 to 89 mg TE/g DW for FRAP antioxidant capacity. For Moral de Calatrava, FRAP antioxidant capacity varied from 58 to 104 mg TE/g DW and DPPH assay from 37 to 72 mg TE/g DW. By contrast, the Saldaña population showed high variability; DPPH antioxidant content ranged from 50 to 149 mg TE/g DW and FRAP antioxidant capacity ranged from 33 to 134 mg TE/g DW.

Some individual plants showed extremely high levels of FRAP antioxidant capacity, as for example a plant from Carrocera with 145 mg TE/g DW, a plant from Hontanar

(147 mg TE/g DW) and two plants from Lerma (154 and 143 mg TE/g DW). The two plants from Lerma were also amongst the plants with the highest DPPH free radical assay (128 and 117 mg TE/g DW). Furthermore, in 4 plants from Saldana very high DPPH values were identified with 106, 110, 132 and 149 mg TE/g DW. In general, all the specimens studied presented medium to high antioxidant content.

On comparing these results with the results obtained by Chizzola *et al*. (2008) using the same methodology for *T. vulgaris* leaves, DPPH and FRAP content of *T. mastichina* was comparable to that of the leaves of *T. vulgaris*.

Total phenols

Total phenol results (mg of CAE/g DW) are shown in Figure 4C. The total phenol content shown by this plant species had high variability ranging from 6.8 to 56.4 mg CAE/g DW for all the individual plants analysed.

On the population level, there was again high variability. The populations with a higher average content of total phenols were Saldaña (37.7 mg CAE/g DW), Carrocera (32.7 mg CAE/g DW) and Lerma (31.8 mg CAE/g DW) while the populations with the lowest content of phenols were Ponferrada (18.8 mg CAE/g DW), Villacastín (19.5 mg CAE/g DW) and Lezuza (21.2 mg CAE/g DW). In general, populations with a high content of DPPH and FRAP also showed high content of total phenols as for example Saldaña, Carrocera and Lerma. However, this behaviour did not occur in all populations and there were some exceptions such as Serranillos and Ponferrada. While Serranillos showed the lowest DPPH and FRAP antioxidant capacity and a medium content of total phenols, Ponferrada showed the lowest total phenols content and medium values of DPPH and FRAP antioxidant capacity.

With regard to the intra-population variability, low variability in total phenols, was shown by Lezuza and Moral de Calatrava, the same populations that showed the lowest variability in DPPH and FRAP antioxidant capacity. The content of phenols for Lezuza ranged from 15.6 to 27.0 mg CAE/g DW and for Moral de Calatrava from 15.4 to 27.5 mg CAE/g DW. On the other hand, the population Saldaña with a range from 17.7 to 56.4 mg CAE/g DW showed the highest intra-population variability as well as for DPPH and FRAP.

The highest total phenol contents were obtained in two plants from Saldaña (56.4 and 53.6 mg CAE/g DW, respectively), one plant from Lerma (46.5 mg CAE/g DW), one from Tordesillas (44.7 mg CAE/g DW) and one from Carrocera (44.6 mg CAE/g DW). Three of

these plants also presented one of the best DPPH and FRAP antioxidant capacities. The lowest content of phenols was found in a plant from Villacastín (6.8 mg CAE/g DW), in one from Ponferrada (9.13 mg CAE/g DW) and also in a plant from Riaza (10.4 mg CAE/g DW).

It is not easy to compare these results directly with those obtained by other authors for the following reasons: the antioxidant content is highly influenced by the type of solvent used (Chizzola et al. 2008; Sánchez-Vioque et al. 2013), temperature and number of extractions (Barros et al. 2010), sonication (Pingret et al. 2013) etc and also because sometimes the results are expressed as gallic acid equivalent, trolox equivalents or caffeic acid equivalents making results not directly comparable. However, the comparison may offer a rough idea of the potential of a particular species. Barros *et al*. (2010) obtained values of total phenols in *T. mastichina* inflorescences from 47.7 to 165.3 mg GAE/g DW depending on the extraction method used. Phenol content was much higher than that of the populations studied probably due to prolonged contact time between plant material and solvent and the double extraction performed. Regarding other species from the genus *Thymus,* Alizadeh *et al*. (2013) found a total phenol content ranging from 18.8 to 19 (mg GAE/g DW) in the aerial parts of *T. daenensis* subsp. *daenensis* Celak and Jabri-Karoui *et al*. (2012) found values of 15.06±0.73 mg GAE/g DW in Tunisian *T. capitatus*. The total phenol content from Ponferrada (18.83 mg CAE/g DW) and Villacastín (19.45 mg CAE/g DW) was similar to these results.

The average content of total phenols of all the plants studied (26.17±9.06 mg CAE/g DW) is slightly lower than that obtained by Chizzola *et al*. (2008) in *T. vulgaris* leaves where the average content varies from 23.1±6.4 to 51.7±15.0 mg CAE/g DW depending on the extraction method used. Jordan *et al*. (2009) obtained a range of values that varied with watering levels from 108.5±19.2 to 135.2±22.9 mg GAE/g DW in the distillation residue of *T. zygis* subsp *gracilis*. This is a very high antioxidant content taking into account that it was measured in the by-product after the distillation process.

Total phenol content and DPPH antioxidant capacity presented a highly significant correlation of 0.855 ($P<0.01$) (Table 18), thus indicating that the polyphenols presented in *T. mastichina* have a high concentration of phenol that act as antioxidants. Moreover, the correlation between total phenol content and FRAP was considerably high showing a great capacity of this plant species to act as a reducing agent (correlation FRAP-Total phenol content: 0.682 ($P<0.01$)).

Phenols composition of the populations

The polyphenols detected and identified in the *T. mastichina* populations were rosmarinic acid, luteolin, chlorogenic acid, caffeic acid and luteolin glucoside with a range of 1.7-43, 0-2.7, 0.07-2.2, 0-0.46 mg/g DW and 0.96-19 mg LE/g DW, respectively. Another important compound present in *T. mastichina* HPLC-profiles could not be identified and is named as peak 3. The range of variability of this compound was from 0.53-15 mg LE/g DW.
In general, rosmarinic acid was the main compound in the samples studied as is usual in the genus *Thymus* (Adzet et al. 1988; Boros et al. 2010). However, in some particular samples, luteolin glucoside was higher than rosmarinic acid as can be observed in the chromatographic profiles (Figure 5A and 5B). Peak 3 was also found in large amounts (Figure 4H) but never higher than rosmarinic acid. In relation to the samples in which rosmarinic acid is not the main compound, Sánchez-Vioque *et al*. (2013) measured residues of *T. mastichina* obtaining a chromatographic profile in which two compounds were detected with a higher area percentage than rosmarinic acid (9%); these compounds were tentatively identified as luteolin glucoside (17%) and quercetin glucoside (21%).

Of the 128 individual plants analyzed, the general trend of the species was a profile such as that in Figure 5A (rosmarinic acid as main compound), 76.6% of the samples analysed presented this profile. In Figure 5B (luteolin glucoside as main compound), it occurred only in 11 individual plants (8.6% of samples analysed) in the populations of Riaza, Saldaña and Villacastín. On the other hand, some samples showed an intermediate situation (14.8% of samples analysed) and were characterized by individual plants with similar contents of rosmarinic acid and luteolin glucoside as was the case of some samples of the Villacastín, Serranillos and Toro populations.
Thymus is a genus which hybridizes easily and produces hybrids between different sections (De Feo et al. 2003). Polyphenol profiles were proven to be useful in detecting hybridizations in *Thymus* (Horwath et al. 2008) and the differences observed among profiles in this study could be due to hybridizations.

The variability of rosmarinic acid was very high both among populations and among plants in the same population. Populations with the highest amount of rosmarinic acid were Lerma, Carrocera and Saldaña with average values of 29, 23 and 22 mg/g DW, respectively. These populations also showed the highest DPPH and FRAP antioxidant capacity and very high total phenol content. Individual plants with a very high content of rosmarinic acid were two from Saldaña (35 and 33 mg/g), one from Serranillos (35 mg/g), one from Carrocera (36 mg/g) and two from Lerma (39 and 38 mg/g). With regard to the lower content, one plant from Villacastín

was the individual plant with the lowest amount of rosmarinic acid (1.7 mg/g). This plant also presented a low content of luteolin glucoside (2.3 LE eq mg/g) and was the sample with the lowest DPPH and FRAP antioxidant capacity and the lowest content of phenols of all the plants studied.

Luteolin glucoside also presented high inter-population variability. The populations with the highest average value of this compound were the same as for rosmarinic acid (Saldaña, Lerma and Carrocera) with an average content of 12 mg LE/g, 10 mg LE/g and 9 mg LE/g, respectively. The variability range for all the analysed plants was from 1.0 LE eq mg/g to19 LE eq mg/g. The plants with the highest content were 3 plants from Saldaña with a content of 19 LE eq mg/g, 18 LE eq mg/g and 16 LE eq mg/g, respectively and one plant from Riaza with a content of 18 LE eq mg/g.

Peak 3 was not the main compound in any of the individual plants. However, several individual plants showed very high contents of this compound, mainly in some individual plants of Saldaña and Lerma where the contents ranged from 4.0 LE eq mg/g to 16 LE eq mg/g. The populations with the highest amount of peak 3 were: Saldaña, Lerma, Carrocera and Lezuza with a mean content of 7.6 LE eq mg/g, 6.7 LE eq mg/g, 4.8 LE eq mg/g and 4.7 LE eq mg/g , respectively.
Luteolin was detected in all the samples analysed except in one. Sanchez-Vioque *et al*. (2013) also detected luteolin in *T. mastichina* waste. The populations with the highest average content of luteolin were Moral de Calatrava and Riaza with a content of 1.9 mg/g and 1.7 mg/g, respectively, and the population with the lowest average content was Hontanar with 0.6 mg/g.

Chlorogenic acid was present in all individual plants analysed, showing low content and low variability. The average content of populations ranged from 0.07 mg/g to 2.2 mg/g. The chlorogenic acid content of *T. mastichina* is for most of the samples higher than the content reported in *T. capitatus* (0.29 mg/g) by Jabri-Karoui *et al*. (2012).

In most individual plants of the populations from Riaza, Saldaña and Serranillos, caffeic acid was either absent or detected in very small amounts. For the other populations, caffeic acid amounts were not significantly different with a mean value in each population that varies from 0 to 0.54 mg/g. The content of caffeic acid (0.10 mg/g) found in *T. capitatus* dried flowers by Jabri-Karoui *et al*. (2012) was similar to some of the individual plants of *T. mastichina* tested.

Contribution of the polyphenols to the antioxidant properties of *Thymus mastichina L.*

Pearson linear coefficient among polyphenols and colorimetric methods.

Pearson linear correlation coefficients among main polyphenols and colorimetric methods are given in Table 18.

All of these plants presented medium to high antioxidant capacity and medium to high phenol content, but not all of the individual plants with the highest rosmarinic acid content are amongst the samples that presented the highest values for the colorimetric methods. Peak 3, which in some samples is present in large amounts, seems to increase the antioxidant capacity and phenols content of *T. mastichina* samples.
The correlation between rosmarinic acid and the colorimetric methods was considerably high with an r=0.78, (P<0.01) for FRAP antioxidant capacity, r=0.77, (P<0.01) for DPPH antioxidant capacity and r=0.76, (P<0.01) for total phenol content (Table 18).

The luteolin glucoside presented an important correlation with the colorimetric methods (Table 18) especially with DPPH antioxidant capacity (0.78, P<0.01). Peak 3 had higher correlation (Table 18) with DPPH antioxidant capacity (0.84, P<0.01) than rosmarinic acid and also important correlations with total phenols (0.72, P<0.01) and FRAP antioxidant capacity (0.63, P<0.01). As for luteolin, there was no correlation between the content of this compound and colorimetric methods (Table 18).

Stepwise regression among polyphenols and colorimetric methods.

Stepwise regression analysis among polyphenols and colorimetric methodologies is shown in Table 19.

The stepwise regression analysis showed that for DPPH antioxidant capacity, the best predictor was peak 3 which explains 70% (P<0.0001) of the total variability (Table 19), while rosmarinic acid only explains 10.8% (P<0.0001). These two compounds with luteolin and the luteolin glucoside explained in total 84% of the model.

Rosmarinic acid content is the best predictor of FRAP antioxidant capacity with an R-square in the stepwise regression of 60% (P<0.0001). Peak 3, caffeic acid and luteolin also contributed to the prediction of FRAP antioxidant capacity explaining in total 68%.

Rosmarinic acid is also the main compound contributing to the total phenols and explaining 58% ($P<0.0001$) in the model. Peak 3 also made an important contribution (11%, $P<0.0001$) and together with luteolin glucoside these three compounds explain 69% of the model.

The contribution of polyphenols to the antioxidant properties of *Thymus mastichina* varies depending on the colorimetric method used. The general trend is that samples with high DPPH activity also have high FRAP antioxidant capacity and high total phenols; however, this does not always happen because there is great variability among samples and populations.

Peak 3 appears to contribute more to the capacity to capture free radicals than to the capacity to act as a reducer of ferric to ferrous ion. For example, the population Hontanar showed high FRAP antioxidant capacity and medium-low DPPH assay having a relatively low content of peak 3 (3.4 LE eq mg/g) and a medium content of rosmarinic acid (17 mg/g).

6.4. Conclusions

In conclusion, samples with higher antioxidant capacity also had higher polyphenol content but the different polyphenols contributed in a different way. Some populations showed high intra-population variability while others showed a similar composition among individual plants. The populations from Saldaña, Lerma and Carrocera were the populations with a higher amount of polyphenols and antioxidant components in the samples studied and they could be selected for their higher content. Both compounds; rosmarinic acid and peak 3 are mainly responsible for the antioxidant properties of *Thymus mastichina* L.

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Figure 4: Box-plot graphs of DPPH (A), FRAP (B), total phenols (C), rosmarinic acid (D), chlorogenic acid (E), caffeic acid (F), luteolin (G), peak 3 (H) and luteolin glucoside (I) of the *Thymus mastichina* populations.

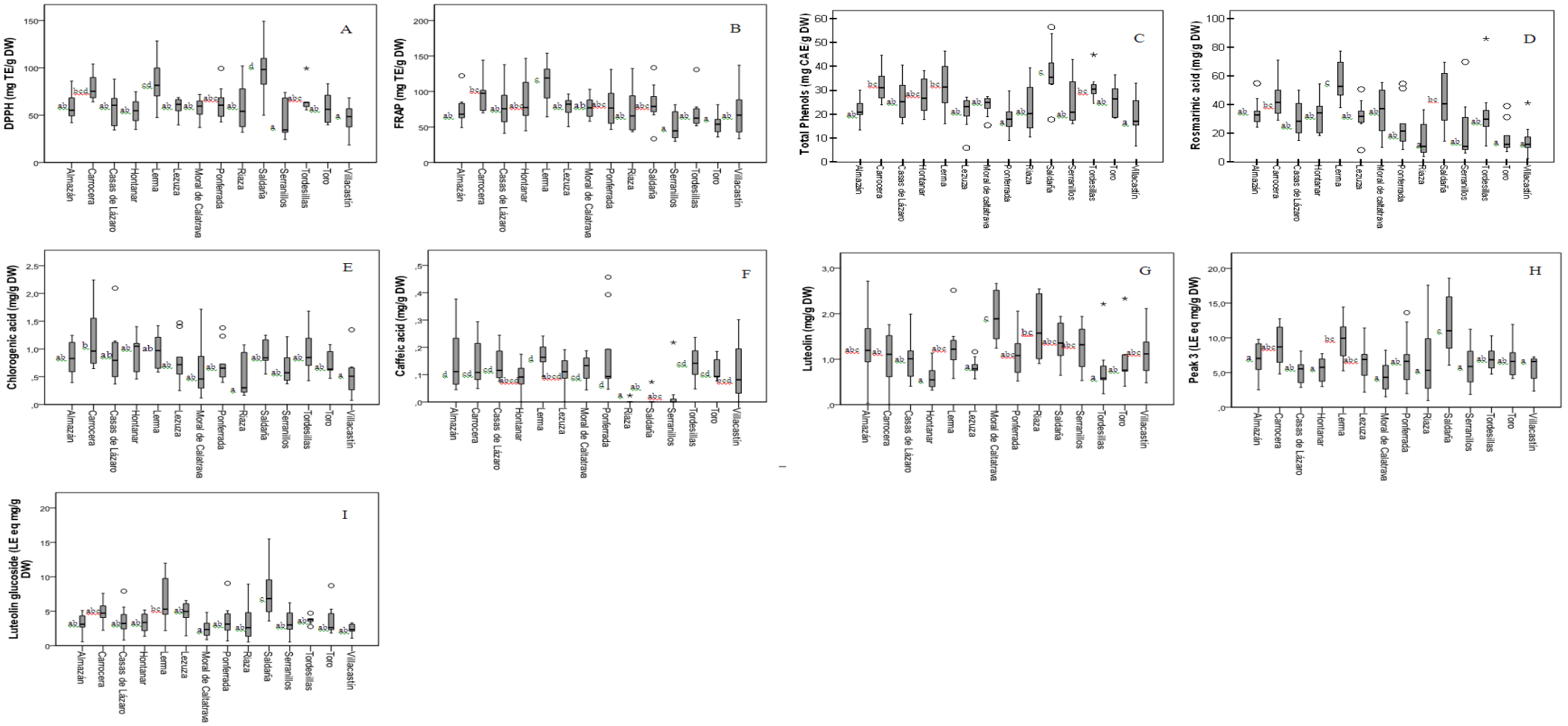

Figure 5: Chromatogram of Saldaña 1-c (A) and Riaza 1-c (B). 1: Chlorogenic acid, 2: Caffeic acid, 3: Unknown 1 “peak 3”, 4: Luteolin glucoside, 5: Rosmarinic acid, 6: Luteolin at 330 nm.

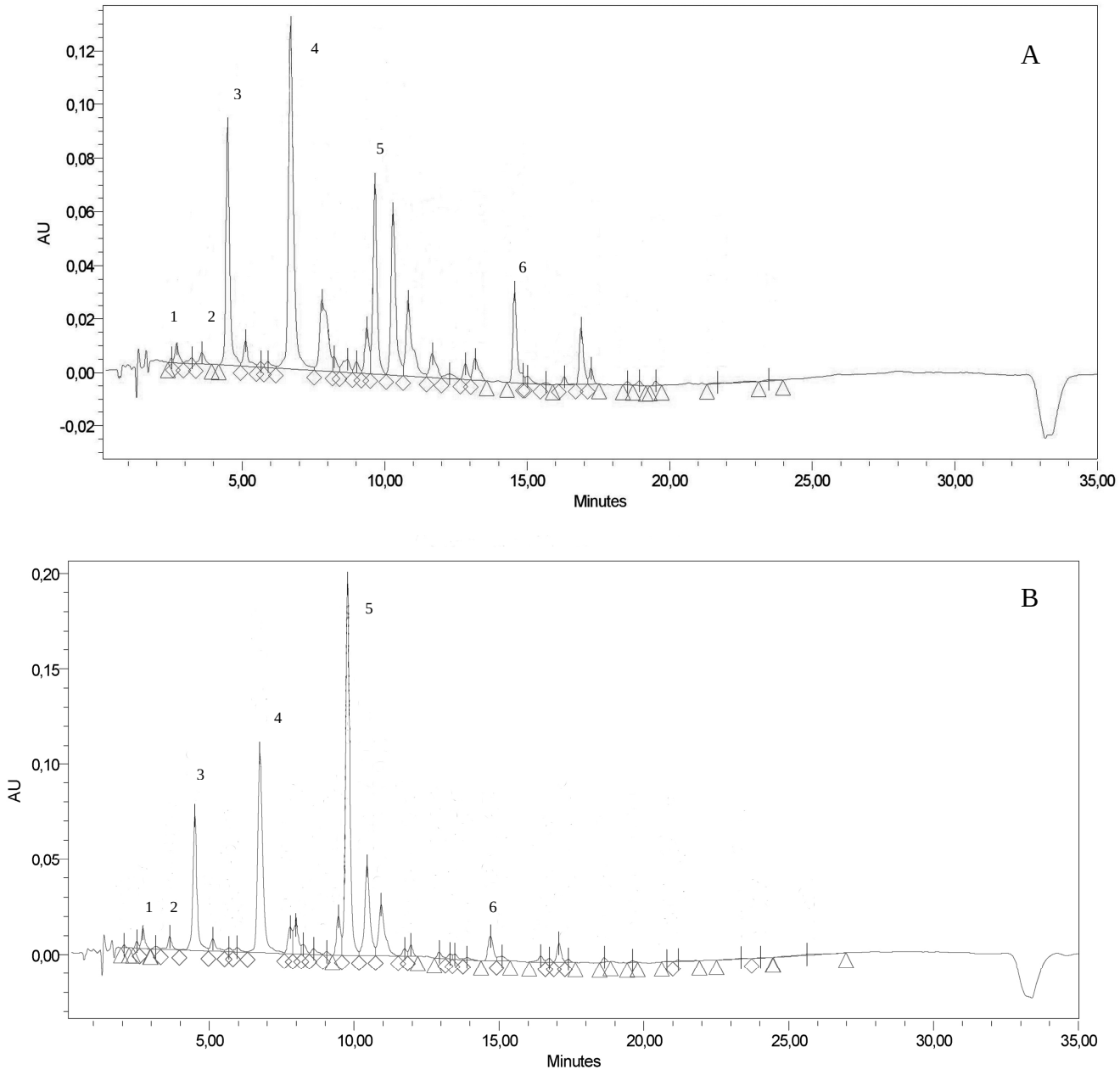

Table 17. Data of the origins of the analyzed *Thymus mastichina* populations.

Origin	Number of individual plants analyzed	Coordinates		Altitude
Almazán (Soria)	10	412826N	0023538W	933
Carrocera (León)	10	424942N	0115213W	1029
Casas de Lázaro (Albacete)	10	384423N	0021321W	827
Hontanar (Toledo)	10	393052N	0043419W	848
Lerma (Burgos)	8	415838N	0032908W	945
Lezuza (Albacete)	9	385456N	0022308W	1054
Moral de Calatrava (Ciudad Real)	10	385036N	0033356W	730
Ponferrada (León)	10	423407N	0064604W	507
Riaza (Segovia)	8	411527N	0032805W	814
Saldaña (Palencia)	10	423158N	0044326W	816
Serranillos (Ávila)	9	402040N	0045415W	1190
Tordesillas (Valladolid)	7	412933N	0050054W	675
Toro (Zamora)	9	413122N	0052424W	704
Villacastín (Segovia)	8	404622N	0042253W	1056

Table 18: Pearson correlation coefficients between the antioxidative test and the identified polyphenols.

	DPPH	FRAP	TP
FRAP	0.755**	-	-
TP	0.855**	0.682**	-
Chlorogenic acid	0.532**	0.579**	0.570**
Caffeic acid	0.258**	0.412**	0.179*
Peak 3	0.835**	0.630**	0.722**
Luteolin glucoside	0.781**	0.549**	0.682**
Rosmarinic acid	0.770**	0.776**	0.759**
Luteolin	0.129	0.150	0.057

DPPH: Free Radical Scavenging Activity. FRAP: Ferric reducing antioxidant power. TP: Total phenols. *: Significant correlation at P<0.05. **: Significant correlation at P<0.01.

Table 19: Stepwise regression analysis taking scavenging activity (DPPH), ferric reduction antioxidant power (FRAP) and total phenols (TP) as dependent variables and the polyphenol contents as independent variables.

Dependent variable		Partial	Model	F-Valor
DPPH	**Compound**	**R-Square**	**R-Square**	**Pr>F**
	Peak 3	0.6976	0.6976	<.0001
	Rosmarinic acid	0.1075	0.8051	<.0001
	Luteolin	0.0253	0.8304	<.0001
	Luteolin glucoside	0.0082	0.8386	0.0136
FRAP				
	Rosmarinic acid	0.6018	0.6018	<.0001
	Peak 3	0.0385	0.6403	0.0004
	Caffeic acid	0.0236	0.6639	0.0038
	Luteolin	0.0203	0.6842	0.0058
Total phenols				
	Rosmarinic acid	0.5764	0.5764	<.0001
	Peak 3	0.1063	0.6828	<.0001
	Luteolin glucoside	0.0107	0.6935	0.0394

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Chapter 5

Polyphenols variability of *Salvia lavandulifolia* populations from Spain

I. Méndez-Tovar, J. Novak, S. Sponza & M.C. Asensio-S.-Manzanera.

Polyphenol variability of *Salvia lavandulifolia* Vahl. Spanish populations

Abstract

This study analyses the polyphenol profile of 86 samples belonging to 7 Spanish populations of *Salvia lavandulifolia* harvested from a plot assay. Samples were studied through reverse phase HPLC-DAD with the aim to evaluate inter and intra-population variability of the polyphenols profile and the antioxidant potential. Rosmarinic acid (6-43 mg/g DW) and luteolin-7-O-glucoside (0-36 mg/g DW) were found as main compounds. Chlorogenic acid (0.08-1.7 mg/g), caffeic acid (0.11-1.2 mg/g), apigenin-7-O-glucoside (0.08-2.3 mg/g DW), luteolin (0-0.92 mg/g DW), apigenin derivative (0-5.5 mg/g DW), betulinic acid (0-3.5 mg RAE/g DW) and carnosic acid (0-0.7 mg RAE/g DW) were also identified. A canonical discrimination function was done to classify the populations attending to their polyphenolic profile similarities and differences, showing that all populations presented characteristic phenolic profiles. Inter-population variability was always higher than the variability within the populations and some compounds are characteristic of each population and then related with the geographical origin. Phenolic content and antioxidant activity were analysed through Folin-Ciocalteu, radical scavenging content (DPPH) and ferric reducing antioxidant power (FRAP) methodologies. Moratalla showed the lowest antioxidant properties and the lowest rosmatinic acid content while La Sentiu de Sió population was highlighted by highest antioxidant properties.

Keywords

Spanish sage, *Salvia lavandulifolia*, antioxidant variability, polyphenols profile.

5.1. Introduction

Salvia lavandulifolia Vahl., a perennial shrub belonging to the *Lamiaceae* family, is growing in the west Mediterranean area and distributed mainly in Spain and south-west of France. It is also known as Spanish sage. It has woody stems at the base and reaches a height of 20 to 65 cm. It has simple and petiolate leaves and the corolla is frequently pink, blue or violet (Sáez 2010). It is a species mainly used as tee, food flavouring and for essential oil production. Spanish sage plant extracts are interesting because of its polyphenolic and antioxidant content (Lu and Foo 2002) and, unlike to the numerous studies on essential oils about this species (Herraiz-Penalver et al. 2010; Porres-Martínez et al. 2013; Usano-Alemany et al. 2014a; Usano-Alemany et al. 2014b), there is only little and old bibliography about the polyphenolic variability of Spanish sage (Cañigueral et al. 1986, 1989).

S. officinalis intake is widely extended throughout the world since ancient times because its numerous medicinal properties based on their active ingredients, thus because of its secondary metabolites content. It is the most used species of the genera; however, the existence of high amount of thujones in *S. officinalis* makes the consumption of this species less healthy than Spanish sage intake due to the thujone toxicity (Pelkonen et al. 2013). *S. lavandulifolia* is characterized by the absence or by very low contents of α and β-thujone (Guillén et al. 1996; Herraiz-Penalver et al. 2010; Mathe et al. 2006; Perry et al. 2003).

Rosmarinic acid is the main and most important compound found in of *S. officinalis* and the interest on this compound is based on its antioxidant, anti-inflammatory, astringent, antibacterial and antiviral properties (Petersen et al. 2013). Other of the most important compound in relation with the antioxidant properties of *S. officinalis* is carnosic acid (Okamura et al. 1994; Frankel et al. 1996) and although is found in lower amounts than other polyphenols, a strong antioxidant power is reported for this compound (Baskan et al. 2007; Okamura et al. 1994).

The existence of rosmarinic acid in *S. lavandulifolia* has been also reported by Cañigueral et al. (1989), however as far as we know the presence of carnosic acid in Spanish sage has never been reported.

The chemotaxonomical studies allow using differencies and similarities in the biochemical compositions of plant species to classify and identify them. The study of the polyphenolic profile is a tool that also allow to distinguish species (Horwath et al. 2008) being an extraordinary tool for establishing traceability in plant material (Siracusa and Ruberto 2014) and offering information that cannot be appreciate with the data of plant morphology. Therefore, polyphenolic profile could be

used as a measure of variability among populations and individuals. Since the overall objective of the study is to select high quality Spanish sage specimens in relation to its antioxidant activity, the study of variability of this species could be useful for population and individual selection.

With the aim of characterizing the populations, studying the polyphenolic variability and knowing their potential as antioxidant, the polyphenolic profile, the total phenol content, radical scavenging activity and ferric reducing antioxidation power were measured in seven populations of *S. lavandulifolia*. The rosmarinic acid quantification and the search for canosic acid acid in Spanish sage, might be useful to check if Spanish sage consumption could be and alternative to *S. officinalis* intake.

5.2. Materials and Methods

Plant Material

A total of 86 samples of *S. lavandulifolia* individual plants coming from 7 populations, were previously collected from the wild in different localities of Spain (Table 20). The samples were multiplied by vegetative reproduction and placed under culture in a plot assay located on the property of Zamadueñas in the Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid (Spain). For every population, between 6 and 19 samples from individual plants were collected during the summer of 2011 in full flower. All the samples were air-dried at room temperature and kept from light. Flowers and leaves were separated from the stems and used for further analysis. Voucher specimens of each population are deposited in the herbarium of the botanic area (PALAB) in the Yutera Campus , University of Valladolid.

Extractions

Each sample was extracted with 100 mg of ground flowers and leaves of *S. lavandulifolia* with 16 mL of MeOH:H_2O (1:1) for an hour in an ultrasound bath at room temperature and subsequently filtered for analysis.

High Performance Liquid Chromatography Analysis

Polyphenols from *S. lavandulifolia* were analysed through HPLC system from Water Corporation (Mildford, MA). The system was equipped with a Waters 717 plus autosampler, a Waters 616 quaternary pump and a Waters 996 Photodiode Array Detector. The column used was reverse phase

symmetry Luna C18 (4.6 mm x 150 mm, 5µm pore size). For the mobile phases the eluent A was composed by 1% acetic acid/acetonitrile (85:15 v/v) and solvent B by pure methanol HPLC grade. The gradient of elution started with A-B (90:10). Solvent B was gradually increased to 100% with a flow rate of 1.5 mL/min at 30 min and kept constant for 10 min more. Identification of the polyphenol compounds was achieved by comparing their retention time values and UV_{max} spectra with those of the reference standards. Detection was performed at 330 nm for chlorogenic acid, caffeic acid, luteolin-7-O-glucoside, apigenin-7-O-glucoside, rosmarinic acid, apigenin drivative, luteolin and apigenin; and 280 nm were used for betulinic acid and carnosic acid. Standard curves were made from each standard at the concentrations of 1-100 µg/mL. Apigenin derivative, betulinic acid, carnosic acid and non-identified compounds were quantified as rosmarinic acid equivalent (mg RAE/g DW).

Total Phenolics

Colorimetric methods were done following the methodology described by Lamien-Meda et al. (2010). To determine the total content of phenols, 100 µL of each extracted sample were diluted with 300 µL of MeOH:H_2O (1:1). 10 µL of this dilution was used for analysis and mixed with 225 µL of H_2O, 5 µL of Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) and 10 µL of Na_2CO_3 solution (26% w/w) in a 96-well microplates (Thermo Scientific, Vienna, Austria). A calibration curve with several caffeic acid (Sigma-Aldrich, Steinheim, Germany) concentrations was done and 10 µL of each concentration was mixed with H_2O, Folin-Ciocalteu reagent and sodium carbonate solution (Carl Roth, Karlsruhe, Germany) under the same procedure followed for the samples. A blank was done using 10 µL of MeOH:H_2O (1:1). The microplate was covered with parafilm and incubated in the dark for 30 minutes and subsequently measured at 750 nm with a microplate reader model 450 (BIO-RAD Tokyo, Japan). Every analysis was carried out in quadruplicate and the results are expressed as mg of caffeic acid equivalents per gram of dry weight (mg CAE/g DW).

Radical Scavenging Activity (DPPH)

100 µL of the original extract were diluted with 300 µL of MeOH:H_2O (1:1), then 10 µL of every dilution were mixed with 100 µL methanol and 100 µL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.015%). The microplate was covered with parafilm and incubated in the dark for 30 min. The microplate was measured at 490 nm with a BIO-RAD microplate reader. The calibration curve was prepared with six different concentrations of Trolox, using a higher concentration of it as a blank. Every result is a mean of a quadruplicate analysis and is expressed as mg of Trolox equivalent per gram of dry weight (mg TE/g DW).

Ferric reducing antioxidant power (FRAP)

25 ml of acetic acid buffer (pH 3.6), 2.5 ml of 2,4,6-tripyridyl-s-triazine (TPTZ) solution (0.0312 g of TPTZ in 10 ml HCL 40 mM) and 2.5 ml of $FeCl_3 6H_2O$ (0.054 g in 10 ml H_2O) were mixed to prepare the working reagent solution.

1 µL of the original extract was used for the analysis and mixed with 180 µL of working reagent solution and with 23 µL of H_2O. Trolox (6.26 mg in 10 ml MeOH) was used as a standard to prepare the calibration curve with eight different concentrations. 6 µL of every concentration were mixed with 180 µL of working reagent and 18 µL of H_2O. Also a blank was performed with 180 µL of working reagent and 24 µL of H_2O. The microplate was covered with parafilm and incubated in the dark during 5 min. The samples were measured in the microplate reader with a wavelength of 595 nm. The results are expressed in mg of Trolox Equivalent per gram of Dry Weight (mg TE/g DW) and are presented as means of quadruplicate analyses.

Statistics

An analysis of variance (ANOVA) was carried out with SPSS 19 (IBM Inc., Chicago, IL) and the average values were compared using Tukey B test at $P<0.05$. Canonical discrimination function was done for all the detected compounds with the aim to find the differences and similarities among populations, to classify them and to study the inter-population variability. Coefficients of variation were calculated for every compound detected in each population to study the intra-variability of the compounds within the populations.

5.3. Results and Discussion

Polyphenolic compounds

The polyphenols ranges for the identified compounds of the studied populations are shown in Table 21. The main polyphenols (compounds c and e in Figure 6) were rosmarinic acid, present in all the samples and ranging from 6 to 43 mg/g DW, and luteolin-7-O-glucoside, ranging from 0 to 36 mg/g DW and present in almost all the samples in high amounts (66% of the samples presented more than 10 mg/g DW), however, it was absent in some specimens from La Sentiu de Sió and Tendilla populations.

On comparing the results with the existing bibliography about *S. lavandulifolia* polyphenols, Cañigueral et al. (1986; 1989) identified in methanolic extracts of Spanish sage rosmarinic acid and luteolin-7-O-glucoside as main compounds, finding also apigenin and luteolin. However nepetin, 3-O-*β*-glucosil-quercitina, 4´-O-diglucuronil-luteolin and 7-O-(6-O-L-ramnosil-*β*-D-glucosil)-luteolin identified by Cañigueral et al. were not measured in the present study.

Rosmarinic acid and luteolin-7-O-glucoside were also the main compounds for *S. officinalis* species with a similar variability ranges for both compounds. Lamien-Meda et al. (2010) found a variation of rosmarinic acid from 6 to 47 mg/g DW in the accesions analysed in 2007 and a range from 5 to 25 mg/g DW for the accesions analysed in 2008. Zimmermann et al. (2011) showed a variability range for rosmarinic acid from 1.2 to 30 mg/g DW and a range for luteolin-7-O-glucoside from 3.8 to 17 mg/g DW in water extractions. Kontogiani et al. (2013) obtained a content of rosmarinic acid of 10 mg/g DW and Cvetkovikj et al. (2013) found a variability of rosmarinic from 0.9 to 22 mg/g DW. Grzegorczyk et al. (2007), found contents of rosmarinic acid in *S. officinalis* ranging from 7.23 to 30.9 mg/g DW.

Kostic et al. (2015) found contents of rosmarinic acid for *S. verbenaca* ranging from 1.32 to 94 mg/g DW depending on the kind of extract done and Martins et al. (2015) found content a little bit higher than the content found by other authors for *S. officinalis*, ranging from 73.9 to 93.4 mg/g DW and conversely it is worth to highlight the lower amount of rosmarinic acid found by Başkan et al. (2007) in comparison with the other authors ranging from 2.50 to 4.01 mg/g.

Caffeic acid and chlorogenic acid were present in all the 86 analysed samples and the variability ranges were from 0.08 mg/g DW to 1.70 mg/g DW and from 0.11 mg/g DW to 1.22 mg/g DW, respectively.

The content o caffeic acid found by Dincer et al. (2012) in *S. fruticosa* were similar than those found in the present study for *S. lavandulifolia* ranging form 1.58 to 2.04 mg/g DW. However, the content of chlorogenic acid quantified by Dincer et al. (2012) was a little bit lower than that found for *S. lavandulifolia*, ranging for *S. futicosa* from 0.042 to 0.149 mg/g DW.

The Tendilla population had no apigenin, on the other hand, the compounds luteolin and apigenin were detected in almost all other samples, but always in little amounts, with a variability range from 0-0.92 mg/g DW and 0-0.83 mg/g DW for luteolin and apigenin, respectively. Further compounds identified were apigenin-7-O-glucoside (0.11-3.09 mg/g DW) and apigenin derivative (0-10.33 mg/g DW) as well as the diterpenoids carnosic acid (0-3.55 mg RAE/g DW) and betulinic acid (0-0.71 mg/g DW). The apigenin content found by Dincer et al. (2012) in *S. fruticosa* ranged from

0.54 to 0.76 mg/g DW being similar than those found for *S. lavandulifolia* while the contents of luteolin were a little bit higher for *S. fruticosa* ranging from 0.90 to 1.15 mg/g DW.

The diterpene carnosic acid which is considered a common compound for *S. officinalis* (Cvetkovikj et al. 2013; Kontogianni et al. 2013; Lamien-Meda et al. 2010; Zimmermann et al. 2011), was present in *S. lavandulifolia* in the whole population of la Sentiu de Sió (0.3-0.7 mg RAE/g DW), in some samples of Tuixent, Guixers and in Tendilla population in little amounts (0-0.2 mg RAE/g DW) and it was absent in all other samples. The carnosic acid content found in *S. officinalis* by other authors is higher than those found for *S. lavadulifolia*. For example, the content of carnosic acid found by Başkan et al. (2007) ranged from 2.99 to 7.16 mg/g and Grzegorczyk et al. (2007), found a range from 0 to 8.68 mg/g DW. Lamien-Meda et al. (2010) found a range content of 0.5 to 4.3 mg/g DW.

Of the non-identified compounds, the most abundant, with average values for all the populations higher than 0.5 mg RAE/g DW, were peak 11, ranging from 0 to 7.26 mg RAE/g DW and present in La Sentiu de Sió population. Peak 20 ranging from 0 to 1.65 mg RAE/g DW present in almost all the samples in similar amounts and peak 23 ranging from 0 to 2.88 mg RAE/g DW with contents higher than 1 mg RAE/g DW in almost all the samples of La Sentiu de Sió, Tuixent and Guixers populations.

Chemical variability of the populations

Figure 7 shows function 1 and 2 of a canonical discrimination analysis using all the compounds detected in the populations. The three first functions explain 83.4% of the variance. The first function explains 54.9% of the variance and the most discriminate standardized coefficient were found for the variables peak 8, peak 27, peak 32 and peak 34. The second function explains 15.7% of the variability being the most discriminant compounds peak 14, peak 25, peak 32 and peak 34. Function 3 explains 12.9% of the variance and the most differentiating variable was peak 7.

It can be appreciated that the different samples of every population appeared in a close area, indicating that differences among populations are bigger than differences among samples of the same population. La Sentiu de Sió and Letur seem to be the most different populations while Guixers and Tuixent are the most similar and located in a close area. As shows Table 20, Guixers and Tuixent were originally populations growing in closer localities with a similar altitude in the origin (Table 20).

Some other differences among populations can be observed. For example Letur is characterized by the peak 8 which is a compound only present in this population in high contents (2.2-6.6 mg RAE/g DW). On the other hand, Moratalla is characterized by the lower amount of rosmarinic acid with a mean content of 11±3.92 mg/g DW and a high content of apigenin-7-O-glucoside with an average value of 1.3±1.0 mg/g DW. Peak 32 and 34 were only present in Moratalla population, ranging from 0.1 to 2.8 mg RAE/g DW and to 0.1 to 1 mg RAE/g DW, respectively. La Sentiu de Sió was characterized by a high content of rosmarinic acid (21.1±4.88 mg/g DW), the highest content of peak 11 (2.5±2.08 mg RAE/g DW) and the highest content of peak 23 with 2.0±0.5 mg RAE/g DW. Tuixent stand out by the highest rosmarinic acid content (30.0±8.07 mg/g DW) and the lowest amount of apigenin-7-O-glucoside (0.3±0.1 mg/g DW); and Guixers is characterized by the highest amount of luteolin-7-O-glucoside (with a mean value of 8.9±0.95 mg/g DW) and clorogenic acid (1.1±0.3 mg/g DW). Compound 7 was characteristic of Guixers and was present in all the samples of this population (0.6-1.5 mg RAE/g DW). Guixers was also characterized by the highest amount of peak 20 (1.0±0.2 mg RAE/g DW). Tendilla was characterized by the compound 14 which was present only in this population in high amounts (ranging from 1.6 to 4.1 mg RAE/g DW) and in one sample of La Sentiu de Sió (0.1 mg RAE/g DW). Tendilla was also characterized by the highest amount of the peak 5 (0.81±0.14 mg RAE/g DW). This compound only appeared in Tendilla and in Tuixent population (0.3±0.17 mg RAE/g DW).

The highest variability was found for minoritary compounds in all the populations except for Tuixent and Tendilla which presented the highest intra variability for compounds with relatively high concentrations. The coefficients of variation were calculated for each compound in every population to analyse the intra-population variability (Table 21). The compounds with higher variation for Aliaguilla population were peak 1, peak 26 and peak 30. For Letur population peak 4 and peak 20 were the most variable. For Moratalla peak 4 and peak 35 presented the highest variability. Peak 1 was the most variable for Guixers. For Tuixent population peak 10, peak 15, peak 18 and carnosic acid presented the highest variability. In La Sentiu de Sió population peak 15 and luteolin were the most variable compounds and for Tendilla peak 2 and luteolin presented the highest rate of variation.

The lower intra-variability in comparison with the inter-population variability and the existence of compounds which are characteristic of every population demonstrated that some polyphenols are related to the genotype of the population; therefore, the phenolic profile of *S. lavandulifolia* also provides information on their geographical origin.

Antioxidant potential of the populations

With regard to total phenols, the results varied from 16 mg CAE/g DW to 50 mg CAE/g DW (Table 23). Moratalla showed values significantly lower ($p<0.05$) than the other populations with a mean value of 23±6.8 mg CAE/g DW. For the other populations there were no significant differences ($P<0.05$), values ranged from 32 to 39 mg CAE/g DW and Letur was the population with the highest content of total phenols 39±4.1 mg CAE/g DW followed by La Sentiu de Sió 37±7.9 mg CAE/g DW.

Scavenging activity range for the analysed samples was from 31 mg TE/g DW to 165 mg TE/g DW. DPPH value for Moratalla population was significantly lower ($p<0.05$) than for the other populations with a mean value of 62±22 mg TE/g DW. The other populations ranged from 88 to 117 mg TE/g DW and La Sentiu de Sió was the population with the highest value (117±28 mg TE/g DW).

For FRAP analysis, the samples ranged from 25 mg TE/g DW to 106 mg TE/g DW and also Moratalla was significantly lower ($p<0.05$) than the other populations (38±12 mg TE/g DW). La Sentiu de Sió presented the highest value (76±20 mg TE/g DW) while the other five populations ranged from 46 to 69 mg TE/g DW.

Moratalla was the population with the lower antioxidant capacity. Despite being a population with important intra-variability, the maximum values do not reach the levels achieved by other samples from other populations. The remaining populations are not significantly different; this may be due to the considerable high intra-variability found among the samples analyzed from each population, making it possible to select high quality individuals in each population.

Comparing these results with other authors, Lamien-Meda et al. (2010), following the same methodology, found for *S. officinalis* similar variability range for DPPH analysis (62-132 mg TE/g DW), however FRAP analysis and total phenols showed a higher potential for *S. officinalis* with a range for FRAP analysis from 76 mg TE/g DW to 246 mg TE/g DW. Chizzola et al. (2008) also used the same methodology for *Thymus vulgaris* finding lower values for DPPH analysis (18-88 mg TE/g DW) and similar values for FRAP (18-91 mg TE/g DW) and total phenols (15-81 CAE/g DW).

Relation among polyphenolic compounds and colorimetric methodologies

Table 24 summarizes the correlations between colorimetric methodologies and peaks. Only the peaks which presented a positive or negative correlation are included. The total sum of polyphenols is highly correlated with the colorimetric methodologies, indicating that for *S. lavandulifolia* a higher sum of polyphenols means a higher antioxidant activity.

The most powerful positive correlation was found for rosmarinic acid, carnosic acid and apigenin derivative. Although polyphenolic compounds are known by their antioxidant potential (Romanova et al. 2000). They can produce synergism and antagonism among compounds. Wang et al. (2013) for example, found antagonism and synergism among antioxidant capacities from fruits, legumes and vegetables when they were mixed and Freeman et al. (2010) demonstrated the synergism and antagonism effect for several polyphenolic compounds (chlorogenic acid, hesperidin, luteolin, myricetin, naringenin, p-coumaric acid, and quercetin) by measuring the antioxidant activity of different mixtures.

Moratalla was the populations with the lowest antioxidant potential. This is likely because is the population with the lowest amount of rosmarinic acid (11±3.92 mg/g DW) and the only population which presented the compounds 32 (1±0.77 mg RAE/g DW) and 34 (0.3±0.25 mg RAE/g DW). Both compounds seem to act as oxidative compounds with a correlation of -0.476**, -0.400**, -0.463** for compound 32 and a correlation of -0.504**, -0.431** and -0.489** for compound 34 for DPPH, FRAP and TP, respectively (Table 24).

On the other hand, the population La Sentiu de Sió which stands out by their antioxidant potential was the population with the highest amount of carnosic acid. As the correlations showed (0.472**, 0.478**, 0.322** for DPPH, FRAP and TP, respectively) and as it is supported by other authors (Baskan et al. 2007; Okamura et al. 1994), this is a compound with a strong antioxidant power. The population of La Sentiu de Sió is also characterized by the highest amounts of peak 11, 12, 21 and 23 and all these four compounds presented a positive correlation with the colorimetric methodologies.

5.4. Conclusions

The phytochemical study of seven populations of Spanish sage has demonstrated that their polyphenolic profiles are related with the geographical origin of plant populations. The population of Moratalla is an option to discard, due to its lower antioxidant and romarinic acid contents,

significantly lower than the other populations. In the other populations there is considerable variability range so that the selection of high quality individuals is possible in each population, highlighting La Sentiu de Sió which was the population with the highest antioxidant properties presenting high contents of rosmarinic acid and considerable presence of carnosic acid. The population of Letur is also highlited by a high amount of phenols.

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Table 20: Data of the origins of the *Salvia lavandulifolia* populations.

Locality	Province	Plants per population	Original	cordinates	Original altitude
Aliaguilla	Cuenca	6	394446N	0012110W	1090
Guixers	Lérida	18	420903N	0014155E	1388
La Sentiu de Sió	Lérida	19	414824N	0005247E	248
Letur	Albacete	8	381427N	0021012W	1185
Moratalla	Murcia	12	381135N	0021122W	1165
Tendilla	Guadalajara	6	403231N	0025703W	858
Tuixent	Lérida	17	421437N	0013151E	1031

Table 21: Amounts of detected compounds in the 7 populations of *Salvia lavandulifolia*.

				Aliaguilla					Letur					Moratalla					Guixers					Tuixent					La Sentiu de Sió					Tendilla				
	UV_{max}	RT	λ	min	max	av	std	Cv	min	max	av	Std	Cv	min	max	av	std	Cv	min	max	av	std	Cv	min	max	av	std	Cv	min	max	av	std	Cv	min	max	av	std	Cv
a	330.3	2.4	330	0.2	1.5	0.7 **bc**	0.5	0.7	0.5	1.3	0.8 **cd**	0.3	0.3	0.1	0.6	0.4 **a**	0.2	0.4	0.6	1.7	1.1 **d**	0.3	0.3	0.2	1.4	0.6 **ab**	0.4	0.6	0.1	0.8	0.3 **a**	0.2	0.5	0.6	1.5	1.0 **cd**	0.3	0.3
1	330.3	3.0	330	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	0.6 **b**	0.6	1	0.2	2.3	0.8 **b**	0.5	0.7	-	0.2	t **a**	0.1	-	0.1	0.2	0.2 **a**	0.1	0.5
b	330.3	3.6	330	0.2	0.8	0.5 **bc**	0.3	0.6	0.2	0.5	0.3 **ab**	0.1	0.4	0.1	0.4	0.2 **a**	0.1	0.5	0.3	1.2	0.6 **c**	0.3	0.4	0.2	1.1	0.6 **c**	0.2	0.4	0.1	0.4	0.2 **a**	0.1	0.4	0.1	0.3	0.2 **a**	0.1	0.4
2	256.8, 349.4	4.0	330	-	-	-	-	-	-	-	-	-	-	-	1	0.1 **a**	0.3	2.8	-	-	-	-	-	-	-	t	-	-	-	-	-	-	-	-	-	1	0.2 **a**	0.4	2
3	282.8, 344.7	4.8	330	-	0.2	0.1 **b**	0.1	0.8	0.1	0.4	0.2 **bc**	0.1	0.5	-	0.3	0.2 **bc**	0.1	0.6	0.2	0.5	0.3 **c**	0.1	0.2	-	0.5	0.3 **c**	0.2	0.5	-	0.3	0.1 **ab**	0.1	0.9	-	-	-	-	-
4	273.3, 344.7	6.5	330	-	0.3	0.1 **ab**	0.1	1	-	0.3	0.1 **ab**	0.1	1.3	-	0.4	0.1 **ab**	0.1	1.4	-	0.2	t **ab**	0.1	-	-	0.2	0.1 **ab**	0.1	0.9	-	0.7	0.2 **b**	0.2	1	-	-	-	-	-
5	273.3, 332.7	6.9	330	-	0.1	t **a**	0.1	-	-	-	t a	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.7	0.3 **b**	0.2	0.6	-	0.1	t **a**	-	-	0.6	1	0.8 **c**	0.1	0.1
6	268.6, 347.1	7.1	330	-	0.3	0.1 **a**	0.1	1	0.4	0.8	0.6 **b**	0.2	0.3	0.3	1.4	0.8 **b**	0.3	0.4	-	-	-	-	-	-	-	-	-	-	0.2	1.7	0.8 **b**	0.4	0.5	-	0.1	t **a**	0.1	-
7	256, 347	7.5	330	-	0.6	0.2 **b**	0.3	1.3	-	-	-	-	-	-	-	-	-	-	0.6	1.5	1.0 **c**	0.3	0.3	-	-	-	-	-	-	-	-	-	-	-	0.1	t **a**	-	-
c	256.8, 349.4	7.6	330	6.5	13	9.5 **a**	2.6	0.3	6.9	19	9.7 **a**	4.2	0.4	2.7	15	9.9 **a**	3.8	0.4	24	36	27.5 **b**	2.9	0.1	1.1	22	14.8 **a**	6.6	0.4	0	23	10.1 **a**	7.9	0.8	0	19	10.1 **a**	6.2	0.6
8	282.8, 339.9	9.1	330	-	-	-	-	-	2.6	6.6	5.1 **a**	1.4	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	268.6, 339.9	9.2	330	-	0.5	0.3 **a**	0.2	0.7	-	-	-	-	-	-	-	-	-	-	0.2	0.7	0.4 **ab**	0.1	0.3	0.1	0.4	0.2 **a**	0.1	0.4	-	0.4	0.2 **a**	0.1	0.7	-	4.8	0.9 **b**	1.9	2.1
d	273.3, 330.3	9.6	330	0.2	1.6	0.9 **abc**	0.5	0.6	0.3	1.1	0.7 **ab**	0.3	0.4	0.4	3.1	1.3 **c**	1	0.7	0.3	0.6	0.4 **a**	0.1	0.2	0.1	0.6	0.3 **a**	0.1	0.4	0.3	0.9	0.6 **ab**	0.2	0.3	0.8	1.2	1.0 **bc**	0.2	0.2
e	245.0, 330.3	10.0	330	9	17	13.4 **ab**	2.9	0.2	10	20	15.1 **abc**	3	0.2	6	18	11 **a**	3.9	0.4	17	26	21.3 **c**	2.4	0.1	16	43	30 **d**	8.1	0.3	13	29	21.1 **c**	4.9	0.2	13	21	17.4 **bc**	3.5	0.2
10	271, 328	10.5	330	-	-	-	-	-	-	-	-	-	-	-	1.2	0.6 **a**	0.5	0.9	-	0.5	t **a**	0.1	-	-	2.5	0.6 **a**	0.9	1.5	-	1.2	0.4 **a**	0.4	1	-	0.6	0.3 **a**	0.2	0.7
f	268.6, 337.5	11.0	330	0.1	1.2	0.6 **a**	0.4	0.6	0.2	0.8	0.5 **a**	0.2	0.4	0.3	2.3	0.9 **a**	0.7	0.8	0.2	0.5	0.3 **a**	0.1	0.2	0.1	0.4	0.2 **a**	0.1	0.5	0.2	0.7	0.5 **a**	0.1	0.2	0.6	0.9	0.8 **a**	0.1	0.2
11	268.6, 335.1	11.3	330	-	-	-	-	-	-	-	-	-	-	-	2.2	0.9 **a**	0.8	0.8	0.1	0.9	0.5 **a**	0.2	0.5	-	2.3	0.2 **a**	0.6	2.8	-	7.3	2.5 **b**	2.1	0.8	-	-	-	-	-
12	280.4	12.6	330	-	0.2	0.1 **a**	0.1	1	-	0.2	0.1 **a**	0.1	1	-	0.1	t **a**	-	-	-	0.3	t **a**	0.1	-	-	0.1	t **a**	-	-	-	0.2	0.1 **a**	0.1	0.9	-	0.6	0.1 **a**	0.2	2
13	268.6, 342.3	13.0	330	-	0.1	t **a**	-	-	-	0.1	t **a**	-	-	-	-	-	-	-	-	0.1	t **a**	0.1	-	-	0.5	0.2 **ab**	0.2	0.8	-	1.2	0.4 **b**	0.4	0.9	-	-	-	0	-
14	245, 268.6, 342.3	13.1	330	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	t **a**	-	-	-	4.1	1.6 **b**	1.8	1.1
15	271, 328	13.3	330	0.1	1.2	0.7 **b**	0.4	0.5	0.2	1.2	0.7 **b**	0.3	0.5	0.2	1.2	0.7 **b**	0.3	0.5	0.3	0.5	0.4 **ab**	0.1	0.2	-	0.4	0.1 **a**	0.1	1.3	-	0.6	0.2 **a**	0.3	1.3	-	2.2	0.8 **b**	1	1.3
16	280.4	14.2	330	-	0.1	t **ab**	0.1	-	-	0.1	t **a**	0	-	-	-	-	-	-	-	0.1	t **a**	0	-	-	0.1	t **a**	-	-	-	0.3	0.1 **ab**	0.1	1.1	-	1	0.2 **b**	0.4	2
17	271	14.7	330	-	0.1	t **a**	-	-	-	0.2	t **a**	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
g	256.8, 349.4	14.9	330	-	0.2	0.1 **a**	0.1	0.9	0.1	0.3	0.1 **a**	0.1	0.7	0.1	0.9	0.3 **b**	0.2	0.8	0.1	0.3	0.2 **ab**	0.1	0.3	0	0.2	0.1 **a**	0.1	0.8	0	0.6	0.1 **a**	0.1	1.4	0.1	0.2	0.1 **a**	0.1	0.6
18	292.3	16.0	330	-	0.3	0.1 **a**	0.1	1.3	-	0.2	0.1 **a**	0.1	0.9	-	0.1	t **a**	0.1	-	-	0.2	0.1 **a**	0.1	0.7	-	0.4	0.1 **a**	0.1	1.4	-	-	-	-	-	-	0.3	0.1 **a**	0.1	1
19	278.1	16.8	330	0.1	0.7	0.3 a	0.3	0.8	-	0.3	0.1 **a**	0.1	0.8	-	0.3	0.1 **a**	0.1	1	0.2	0.8	0.4 **bc**	0.2	0.5	0.1	2	0.7 **c**	0.5	0.7	-	0.1	t a	-	-	-	0.2	0.1 **a**	0.1	1
h	268.6, 335.1	17.4	330	t	0.2	0.1 **b**	0.1	0.7	-	0.2	0.1 **ab**	0.1	0.8	-	0.8	0.3 **c**	0.2	0.7	0.1	0.2	0.2 **b**	0	0.2	-	0.2	0.1 **ab**	0.1	0.8	-	0.2	0.1 **ab**	0.1	0.7	-	-	-	-	-
20	282.8, 332.7	19.3	330	0.3	1.2	0.8 **bc**	0.4	0.5	-	1	0.3 **a**	0.4	1.2	0.2	1.7	0.8 **bc**	0.4	0.5	0.6	1.5	1.0 **c**	0.2	0.2	0.3	0.8	0.4 **ab**	0.2	0.4	0.3	1.5	0.7 **abc**	0.3	0.4	0.3	0.6	0.4 **a**	0.2	0.5
21	282.8, 332.7	19.7	330	-	-	-	-	-	-	0.2	t **a**	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.1	0.6 **b**	0.3	0.4	-	-	-	-	-
22	278, 330	21.3	330	-	0.1	t **a**	-	-	-	0.1	t a	-	-	-	0.2	0.1 **a**	0.1	0.9	-	-	-	0	-	-	0.1	t **a**	-	-	-	0.2	t a	0.1	-	-	-	-	-	-
23	278.1, 332.7	22.6	330	-	2.3	0.7 **ab**	0.8	1.2	-	0.5	0.4 **a**	0.2	0.5	-	1.2	0.7 **ab**	0.3	0.5	0.9	1.9	1.3 **c**	0.3	0.2	0.5	1.9	1.1 **bc**	0.4	0.4	1.4	2.9	2.0 **d**	0.5	0.3	0.4	0.8	0.7 **ab**	0.2	0.3
24	278.1, 332.7	22.8	330	-	0.4	0.1 **a**	0.2	1.9	-	0.2	0.1 **a**	0.1	0.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	247.3, 292.3	23.1	330	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.3	0.2 **a**	0.1	0.4	-	0.4	0.2 **a**	0.1	0.5
26	247.3, 292.3	23.6	330	-	0.9	0.3 **a**	0.3	1.1	0.1	0.3	0.2 **a**	0.1	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	254.4, 294.7	23.8	330	-	0.6	0.1 **a**	0.3	2.5	0.1	0.6	0.3 **b**	0.1	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	249.7, 292.3	24.9	330	-	-	-	-	-	-	0.2	t **a**	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.1 **b**	0.1	1
29	209.7, 451.4	26.3	280	0.1	0.2	0.1 **c**	0	0.3	-	0.3	0.1 **c**	0.1	1	-	0.1	t **ab**	-	-	0.1	0.2	0.1 **bc**	0	0.4	-	0.4	0.1 **abc**	0.1	0.7	0.1	0.3	0.2 **d**	0	0.2	-	-	-	0	-
30	207.4, 273.7	26.9	280	-	1	0.2 **b**	0.4	2.1	0.4	1.1	0.8 **c**	0.3	0.4	0.1	0.3	0.1 **ab**	0.1	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
j	202, 226, 278	27.1	280	0.3	0.8	0.4 **ab**	0.2	0.5	0.1	0.3	0.3 **ab**	0.1	0.3	-	-	-	-	-	0.4	0.9	0.4 **ab**	0.1	0.3	0.3	3.5	0.6 **b**	0.8	1.3	0.2	0.8	0.3 **ab**	0.1	0.4	0.1	0.1	0.1 **a**	0	0
31	212, 249.7	27.7	280	-	0.2	t **ab**	0.1	-	-	0.1	0.1 **bc**	0	0.3	-	-	-	-	-	-	-	-	-	-	-	0.3	0.1 **c**	0.1	0.8	-	-	-	-	-	-	-	-	-	-
32	266.2	27.9	280	-	-	-	-	-	-	-	-	-	-	0.1	2.8	1.0 **a**	0.8	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	294.7, 351.8	28.2	280	-	0.2	t **a**	0.1	-	-	0.5	0.3 **b**	0.2	0.5	-	-	-	-	-	-	-	-	-	-	-	0.2	t **a**	0.1	-	-	-	-	-	-	-	-	-	-	-
k	207.4, 282.8	28.5	280	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	t **ab**	-	-	-	0.2	0.1 **b**	0.1	0.9	0.3	0.7	0.5 **c**	0.1	0.3	-	-	-	-	-
34	266.2	28.9	280	-	-	-	-	-	-	-	-	-	-	0.1	1	0.3 **a**	0.3	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	212, 249.7	29.2	280	-	0.6	0.3 **b**	0.3	0.8	-	-	-	-	-	-	0.4	0.1 **a**	0.1	1.3	-	-	-	-	-	-	0.4	0.1 **a**	0.1	1	-	-	-	-	-	-	-	-	-	-
36	202.7, 275.7	29.6	280	-	0.1	t **a**	-	-	-	-	-	-	-	-	0.5	t **a**	0.2	-	-	0.5	0.2 **b**	0.1	0.6	-	0.1	t **a**	0	-	-	-	-	-	-	-	-	-	-	-

UVmax: Ultra violet maximum absorption. **RT**: Retention time. **λ**: Wavelength of measurement. **min**: minimum. **max**: maximum. **av**: average. **std**: standard deviation. Cv: coefficient of variation. **a**: clorogenic acid, **b**: cafeic acid, **c**: luteolin-7-O-glucoside, **d**: apigenin-7-O-glucoside, **e**: rosmarinic acid, **f**: apigenin derivative, **g**: luteolin, **h**: apigenin **i**: betulinic acid, **j**: carnosic acid. Number from 1 to 36: Detected polyphenols not identified. Compounds a, b, c, d, e, f, g and h are expressed in mg/g DW. Compounds i, j and h are expressed in mg/g DW. Compounds i, j and compound number 1 to 36 are compound number 1 to 36 are expresed as mg/g of rosmarinic acid equivalent DW. t: traces <0.01 mg/g of rosmarinic acid equivalent DW.

Table 22: Maximum, minimum, average and deviation for the antioxidant activity colorimetric methodologies.

	All the population				Aliaguilla				Letur				Moratalla				Guixers				Tuixent				La Sentiu de Sió				Tendilla			
	MIN	**MAX**	**AV**	**STD**	**MIN**	**MAX**	**AV**	**STD**	**MIN**	**MAX**	**AV**	**STD**	**MIN**	**MAX**	**AV**	**STD**	**MIN**	**MAX**	**AV**	**STD**	**MIN**	**MAX**	**AV**	**STD**	**MIN**	**MAX**	**AV**	**STD**	**MIN**	**MAX**	**AV**	**STD**
TP	16	50	33	±7.5	30	35	33 **b**	±1.5	32	44	40 **b**	±4.1	16	36	23 **a**	±6.8	27	42	32 **b**	±4.4	23	45	35 **b**	±6.6	24	50	37 **b**	±7.9	29	40	35 **b**	±4.8
DPPH	31	165	96	±24.8	95	109	103 **b**	±4.8	71	126	101 **b**	±17.3	31	91	62 **a**	±22.3	80	113	98 **b**	±9.4	63	118	88 **b**	±15.6	68	165	117 **b**	±28	84	119	100 **b**	±11.7
FRAP	25	106	60	±18.1	60	72	67 **b**	±4.1	49	65	58 **ab**	±6	25	59	38 **a**	±12	58	87	69 **b**	±8.7	33	61	46 **a**	±8.2	37	106	76 **b**	±20.3	46	67	57 **ab**	±7.5

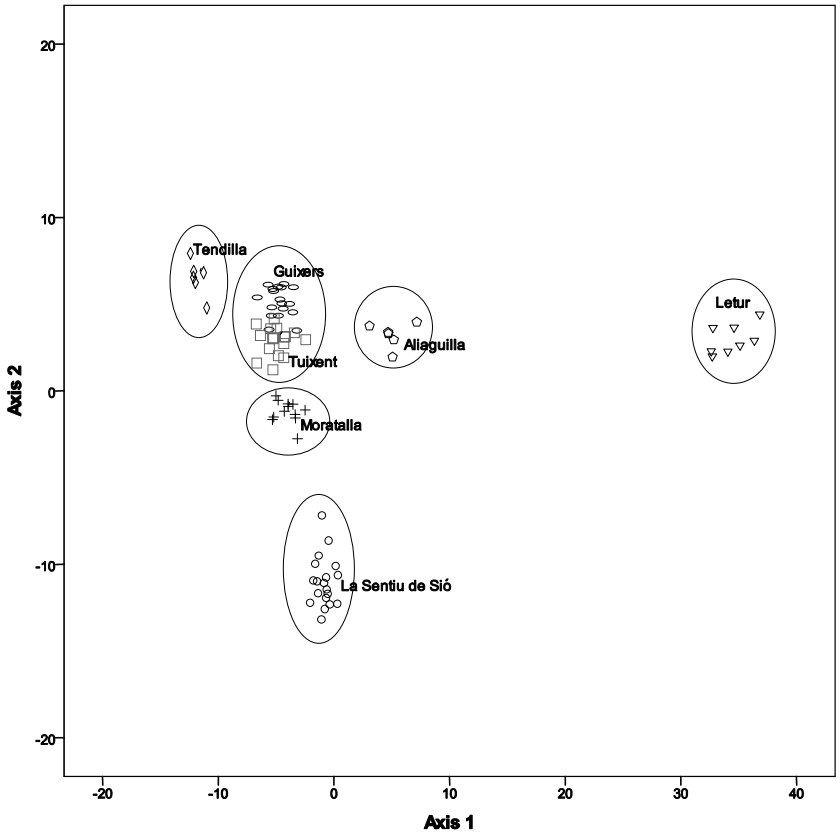
MIN: Minimun, **MAX:** Maximun, **AV:** Average, **STD:** Standard deviation. **TP:** Total phenols, **DPPH:** Scavenging activity and **FRAP:** Ferric reducing antioxidant power

Table 23. Significative correlations between methods and polyohenols.

	DPPH	FRAP	TP
FRAP	0.866**	-	-
TP	0.841**	0.665**	-
b	-	-	0.227*
4	0.352**	0.279*	0.230*
5	-	-0.217*	-
6	0.228*	-	-
7	-	0.269*	-
8	-	-	0.284**
e	0.418**	0.268*	0.536**
10	-	-0.223*	-
f	0.401**	0.401**	0.339**
11	0.322**	0.291**	-
12	0.297**	0.284**	0.281**
13	0.302**	0.224*	0.314**
15	-0.218*	-	-
16	0.233*	0.249*	0.273*
g	-0.270*	-	-0.306**
h	-0.325**	-	-0.405**
20	-	0.324**	-
21	0.342**	0.348**	-
23	0.402**	0.434**	-
25	0.368**	0.275*	-
29	0.384**	0.387**	0.272*
j	-	-	0.244*
31	-	-0.242*	-
32	-0.476**	-0.400**	-0.463**
33	-	-	0.250*
k	0.472**	0.478**	0.322**
34	-0.504**	-0.431**	-0.489**
SUM	0.526**	0.402**	0.606**

TP: total phenols, **DPPH**: scavenging activity and **FRAP**: ferric reducing antioxidant power, **SUM**: total sum of all detected polyphenols, **P**: peak, **b**: cafeic acid, **e**: rosmarinic acid, **f**: apigenine derivative, **g**: luteolin, **h**: apigenin **i**: betulinic acid, **j**: carnosic acid. Number from **1** to **34**: detected polyphenols not identified.

Figure 6: Canonical discrimination function for the polyphenols profile of the different *S. lavandulifolia* populations.



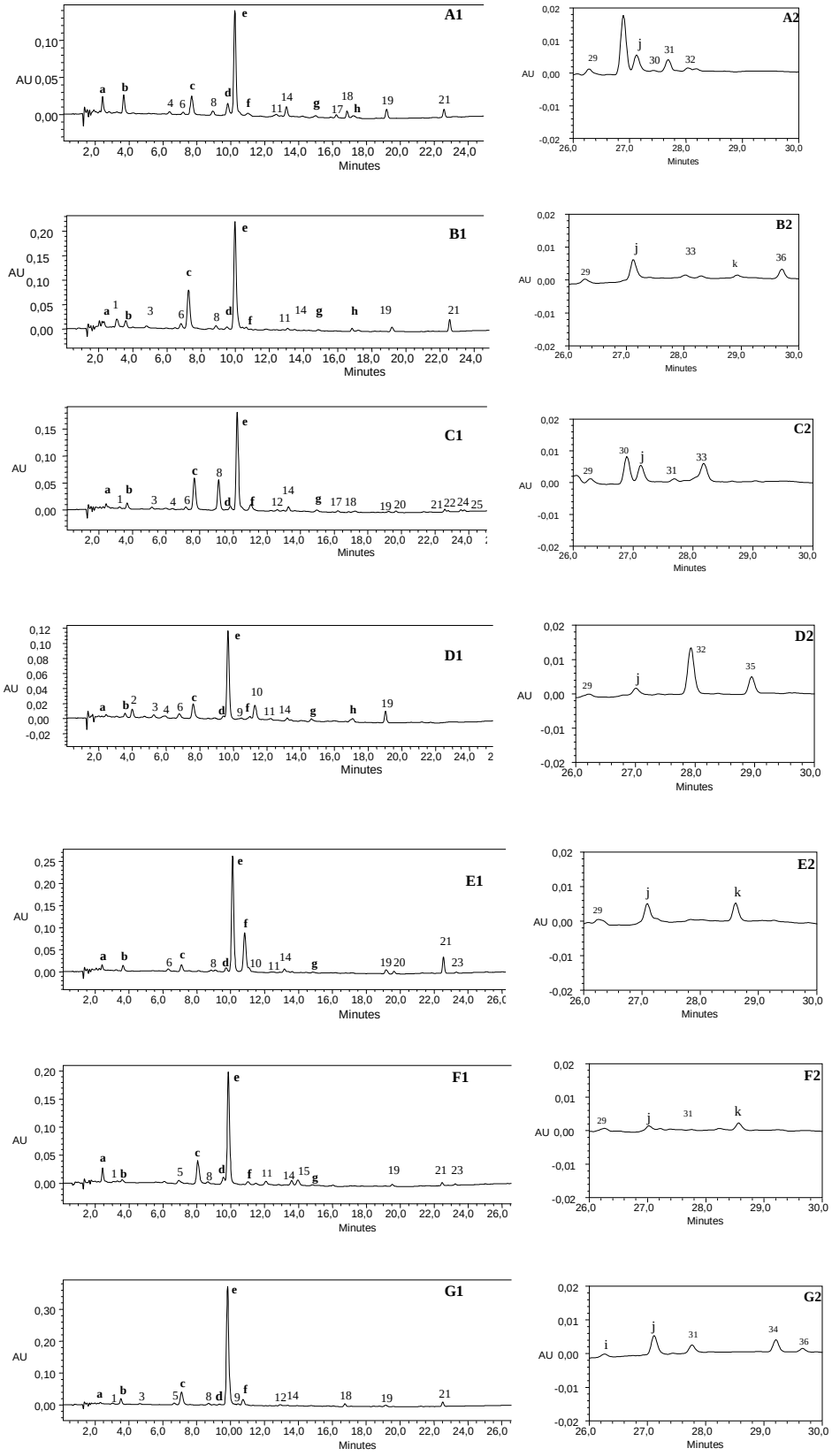


Figure 7: Examples of chromatograms of each population of *S. lavandulifolia*. Left chromatograms measurements at λ=330, right chromatograms measurements at λ=280. **A1**/**A2**: Aliaguilla. **B1**/**B2**: Guixers. **C1**/**C2**: Letur. **D1**/**D2**: Moratalla. **E1**/**E2**: La Sentiu de Sió. **F1**/**F2**: Tendilla. **G1**/**G2**: Tuixient. **a**: clorogenic acid, **b**: cafeic acid, **c**: Luteolin-7-o-glucoside, **d**: apigenin-7-O-glucoside, **e**: Rosmarinic acid, **f**: apigenin derivative, **g**: luteolin, **h**: apigenin, **i**: betulinic acid, **j**: carnosic acid. Numbers from **1** to **36**: Detected polyphenols not identified.

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Chapter 6

Variation in morphological traits among *Thymus mastichina* (L.) L. populations

I. Méndez-Tovar, H. Martín, Y. Santiago, A. Ibeas, B. Herrero & M.C. Asensio-S.-Manzanera. (**2015**). Variation in morphological traits among *Thymus mastichina* (L.) L. populations. *Genetic Resources and Crop Evolution*, 1-11.

Variation in morphological traits among *Thymus mastichina* (L.) L. populations

Abstract

Thymus mastichina (L.) L. is an endemic species of the Iberian Peninsula and one of the most collected of wild plants in Spain. Despite the huge amount of possibilities for the use of *T. m.* subsp. *mastichina* as an industrial crop, cultivation is almost absent and no selection has been made. To identify the most useful and discriminating traits and to study the morphological variability of Spanish marjoram, a total of 16 populations were studied during 2011 and 2012 and 20 morphological traits (15 quantitative and 5 qualitative) were used for characterization. Analysis of variance, Principal Component Analysis, correlations among traits and cluster analysis have been carried out for data processing. Intra and inter-population variability were highly statistically significant for all the quantitative traits, revealing the polymorphism of the species, although variability among populations was greater than variability within populations showing that populations are morphologically distinguished from each other. Number of flowers per flower head, number of flower heads per branch, length/width of the bract, and bract width were the most variable traits. The most valuable traits for characterization were related to the bract form and size, leaves and capitula. The ranges of morphological variation for the cultivated specimens compared with references to wild specimens in the bibliography, showed slightly larger sizes of leaves, bracts and inflorescences and smaller sizes of calyces and corollas. Cluster analysis classified the populations into 4 groups. Populations from group 3 (CLM4, TM23 and TM43) were highlighted by the biggest flower heads and they may be more productive for essential oil extraction.

Keywords

Morphological variability, morphological traits, Spanish marjoram, phenotypic diversity, *Thymus mastichina*

8.1. Introduction

Thymus mastichina (L.) L., an endemic species from the Iberian Peninsula, is characterized by simple and opposite leaves and by zygomorphic and bilabiate flowers as part of the family Labiatae. Its calyx tube terminates in 5 teeth with long cilia which give the plant a feathery appearance and the white flowers are grouped in flower heads or capitula. It grows in sandy and siliceous soils and, in the Iberian Peninsula, flowers from late May and early June to late July and early August.

The species can be classified into two subspecies: subsp. *mastichina* characterized by calyx longer than 5 mm, inflorescences larger than 10 mm in diameter and oblong-ovate or elliptic bracts; and subspecies *donyanae* R. Morales characterized by calyx with a size equal to or less than 5 mm, inflorescence smaller than 10 mm in diameter and bracts widely ovate (Morales 2010). The subspecies *donyanae* is only present in Huelva and Seville (South-West Spain) and in Algarve (South-East Portugal) while the subspecies *mastichina* extends throughout all the Iberian Peninsula with lower presence in the northwest coast of Spain and the Mediterranean coastline (Morales 2010).

With regard to its chemical composition, 1,8-cineol and linalool are defined as main constituent and three chemotypes are known depending on its relative content (Miguel et al. 2004; Salgueiro et al. 1997). The most widespread chemotype contains 1,8-cineole as main compound. The second chemotype contains linalool as principal component and the third chemotype is intermediate with both volatiles as main compounds (1,8-cineole-linalool).

T. mastichina, known as Spanish marjoram, is remarkable for its medicinal properties including antiseptic, digestive, antirheumatic and antitussive effect (Vanaclocha and Cañigueral 2003). It is also used as an aromatic plant in the perfume and cosmetic industry, as a food seasoning and as a honey plant. In the last decades, its extracts and its essential oils have also been widely studied for their bactericide, insecticide and fungicide properties (Faleiro et al. 2003; Fraternale et al. 2003; Pavela 2005; Vegara et al. 2011). Despite the huge amount of possibilities for using *T. mastichina* as an industrial crop, significant efforts have not been made for its domestication and selection although it could be a very good alternative in the development of rural areas in the Iberian Peninsula. *T. mastichina* is cultivated in Spain as an essential oil plant, although mainly wild populations are used for distillation (Henelt and IPK 2001).The study of the morphological variability of this species is essential if it is to be domesticated. Also, description

of the morphological characteristics is the usual methodology accepted from a legal point of view for the patenting and registration of varieties.

Multivariate statistical methods have been successfully used to classify quantitative and qualitative variations in many crop species (Berdahl et al. 1999; Bhargava et al. 2007; Khadivi-Khub et al. 2012) and it is also useful to study the variability of wild populations (Delgado et al. 2010). This study is the first to report morphological traits analysis within an elevated number of populations of an endemic plant from the Iberian Peninsula such as *T. mastichina*, observed during two years under cultivation.

The present study was conducted with the aim of identifying the most useful traits for discrimination among the populations, finding the relationship among them, and evaluating features to identify the individuals with good potential for exploitation. It was also conducted to determine the extent of genetic diversity for effective germplasm management and proper utilization in selecting programs.

8.2. Materials and Methods

Plant Material

The experimental material comprised 16 wild populations of *T. m.* subsp. *mastichina* collected in Spain (Table 24). These populations were studied in a randomized block design trial with three replications during two seasons (2011 and 2012) in the experimental field of the Instituto Tecnológico Agrario de Castilla y León (ITACyL), Valladolid (Spain). The experimental plot was 5.25 m^2 with row-to-row and plant-to-plant distance of 0.75 m and 0.20 m, respectively. A total of 91 individual plants from 14 populations and 168 specimens from 16 populations were analysed in 2011 and 2012, respectively. The data of several plants from each population were recorded for 20 morphological traits as described in Table 25. Voucher specimens of each population were deposited in the herbarium of the botanic area (PALAB) in the Yutera Campus of the University of Valladolid.

Statistical analysis

Data of individual plants of 14 populations for quantitative traits recorded in 2011 were used in analysis of variance (ANOVA) in order to determine the population and population per plant effect, representing inter and intra-population variability, respectively. Coefficients of variation

(CVs %) were determined as indicators of variability. Correlations between the traits were determined using the Pearson correlation coefficients. In order to identify the most useful traits for discrimination among the specimens, Principal Component Analysis (PCA) was done by simultaneously considering all the variables. Afterwards, influence of the year and relationships among the populations were investigated by ANOVA and PCA, respectively, using data recorded in 2011 and 2012 for the traits selected above. To bring out the patterns of similarity and dissimilarity, the mean data of two seasons were subjected to cluster analysis using Ward's method to group the 16 populations. All analyses were performed using SPSS 15 statistics software (IBM Inc., Chicago, IL).

8.3. Results and discussion

Identification of the most useful traits for discrimination among the populations

The highest CVs for the traits analysed were recorded for: number of flowers per flower head (48.4%), number of flower heads per branch (46.8%), length/width of the bract (36.5%), and bract width (27.4%) showing a high degree of diversity (Table 26).

Analysis of variance exhibited highly significant differences for all the 15 quantitative traits among the 14 populations indicating the presence of high degree of morphological variations among the populations studied. For all the traits analysed, the intra-population variability is also significant as expected for wild populations. In all traits, the inter-population variability has a greater importance than the inter-population variability, except for the trait L/W of the flower head, indicating that populations are morphologically distinguished from each other, despite the great variability shown by plants within the same population.

There was an important correlation between lengths of leaves and bracts (50.6, $P<0.001$) and the length of the bracts also presented correlation with the length of the calyx (44.5, $P<0.001$) and corolla (31.3, $P<0.001$) (Table 27). Width of the bract showed negative correlations with the number of flower heads per branch (-48.8, $P<0.001$) and greater length of the branches indicated a higher number of flowers per flower head (32.0, $P<0.001$). The number of flowers per flower head was correlated with the length and the width of the leaves (-23.7, $P<0.05$ and 31.3, $P<0.01$, respectively), however there was no correlation between the size of the flower head and the size of bracts and leaves of *T. mastichina* and these three traits can be useful for selecting plant material because essential oils are mainly found in these parts of this plant species, although flowers present a higher amount of essential oil than leaves (Faleiro et al. 2003).

In order to assess the patterns of variation, PCA was done by simultaneously considering all the variables. For this analysis the characters were initially scaled to make their variances equal. In the multivariate space that they defined, a new set of axes was then chosen so that the variance on each axis was as large as possible but at right angles to the preceding ones. The coefficient of each data point on each new axis was a weighted sum of its coefficients on the original axes. The variance on each axis is called latent root or autovalue; the percentage of total variance that each represents and the coefficients used in the weighted sum (loadings or eigenvectors) are presented in Table 26. The first seven principal components (PCs) explained 74% of the variability present among the individual plants studied for the 20 traits under study. PC1, accounting for 20.2% of the variation, had the trait form of the bract and width of the bract as the variables with the largest positive coefficients, while the relation between length and width of the bract and leaf showed the largest negative coefficients. The second axis (PC2) explained 15.5% of the variability with the traits length and width of the flower head with the largest positive coefficients and the relation length/width of the leaf and leaf length with the largest negative coefficient. PC3 explained 11.8% of the variability with the bract length as the most powerful coefficient, while PC4 explained 8.6% of the variability showing the relation length/width of the flower head the weightiest coefficient. PC5 explained 6.7% of the variability with bract length as main trait. In view of the results, the most important traits are those related to the leaves and bracts as well as the size and form of the capitulum.

As there are good correlations between bract form and bract length, and width and leaf form with leaf length and width, these qualitative traits were not selected to study the diversity of the species. Traits related to the seeds were also rejected because their influence on the variability was low and they were not related to the parameters of plant yield. There was a high correlation between the number of flowers per flower head and the length and width of the capitula, so the number of flowers was not taken into account since registering this feature is time-consuming. Finally, traits related to the branch, despite being important as parameters of yield, were rejected because of the high variability found even in the same sample plant.

Morphological variability

Once the 11 biometrical traits were selected, another ANOVA and PCA were done to analyse the morphologic variability of the species for the samples collected in 2011 and 2012.
Population was the most important source of variation for all studied traits, except for leaf length (Table 27). Size of the flower head and length of the leaves seem to be more influenced

by environment. For those characters which the percentage of the sum of squares of residuals was large, the intrapopulation variation was larger.

The first three principal components (PCs) explained 83.3% of the variability present among the populations studied. PC1 contributed with 38.5% of the variation, PC2 with 33.6% and PC3 with 11.2%. Figure 8A shows axes 1 and 2 and figure 8B represents axes 1 and 3.

In figure 8A, the populations located in the upper right part of the plot, are characterized by bigger flower heads (CLM4, TM43 y TM23). Populations located in the upper left part of the plot are populations with large and narrow leaves and bracts (CLM1, CLM2 y TM29), and populations located down and right in the plot are characterized by shorter and wider leaves and bracts (TM37, TM39, TM42 y TM14). In figure 8B, populations located in the upper part of the plot have larger corollas (TM1, TM38, CLM1) and populations in the right corner are also characterized by larger calyxes (TM43, TM39).

Cluster analysis was carried out and it allowed the assessment of similarity or dissimilarity and clarified some of the relationships among the 16 populations studied (Figure 9). The dendogram showed that the germplasm collected could be divided into four groups. The mean values of populations included in each group are presented in Table 28. Each group had some specific characteristics, in Figure 9 the first group encompasses the populations CLM1, CLM2 and TM29 characterized by large and narrow leaves and bracts. Group 3 encompasses the populations CLM4, TM23 and TM43 and is noted for the biggest flower heads and the largest calyces. Populations in group 4 (TM14, TM17, TM37, TM39 and TM42) showed short and wide leaves and bracts. The remaining populations are clustered in group 2 and characterized by small leaves and flower heads.

The traits investigated in this study are very important from a commercial point of view, as they have direct and indirect effects on foliage and flower production and presumably on its essential oil production. It is well known that essential oil is produced in the aerial parts of the aromatic plants, mainly in flowers and leaves in the case of *Thymus* species (Faleiro et al. 2003; Guillen and Manzanos 1998; Hazzit and Baaliouamer 2009). Also, the same as in other Labiatae species, Spanish marjoram contains more essential oil in flowers than in leaves (Miguel et al. 2004). Therefore, the populations grouped within group 3 (CLM4, TM23 and TM43) are the most desirable populations because of their large leaves, bracts and inflorescences (and consequently, a greater number of flowers) and they could result in a higher production of essential oil per unit area.

Cultivation and selection of T. mastichina

Comparing the ranges of variability of the studied samples with the ranges of variation published by Morales et al. (2010) for wild *T.mastichina*, none of the studied leaves (0.87 to 1.92 cm for length and 0.22-0.55 cm for width) had smaller size than wild plants (0.5 to 1.3 cm for length and 0.1-0.4 cm for width), however 40% of the leaves were larger and 50% were wider than wild samples. With regard to the bracts (0.45-1.15 cm for length and 0.12-0.72 cm for width) 14% of the samples were shorter and 5% were narrower than wild bracts (0.6-0.8 cm for the length and 0.3-0.6 cm for the width); on the other hand, 18% of the samples were larger and 13% were wider than wild specimens. With respect to calyces (0.40-0.77 cm for the studied specimens) only 5% of them were bigger than wild calyces (0.55-0.7 cm) and 54% of the studied samples were shorter than wild specimens. With regard to corollas (with a range for the studied samples of 0.30-0.74 cm), 7% of the samples were above the range for wild specimens and 49% of the samples were below the range (ranging for wild specimens from 0.5 to 0.65 cm). The inflorescences of *mastichina* subspecies vary in their natural habitat from 1 to 1.8 cm. The cultivated samples presented a 7% of samples under the range and 19% of the samples above the range for wild inflorescences.

The results show that cultivation of the species increases slightly the sizes of leaves, bracts and inflorescences. Cultivation also decreases the sizes of calyces and corollas, probably due to bigger inflorescences and competence among flowers. The increase of the size of these organs of the plant is another advantage offered by the cultivation of *T. mastichina*.

T.mastichina is one of the 10 most collected plants from the wild in Spain. 95% of the Spanish marjoram production comes from wild collection and this could be a threat for the species and a loss of profit, because of the lack of selected material and the lack of control of the culture parameters to increase the production. In view of the huge profits generated by this species (Douglas et al. 2005), there is a definite need for its selection and genetic improvement to develop high yielding clones.

In the process of domestication of any species, genetic diversity among germplasm plays the major role, since it opens the way to select the best specimens. Phenotypic variation within populations can have important consequences on the biomass and essential oil production. The study reported that the most variable traits are related to the size of leaves, bracts and capitulum. These traits influence biomass development and therefore essential oil production. Selecting programmes should search for specimens with larger branches, bigger leaves and bracts and bigger flowers and flower heads. On the other hand, the leaf size is not related to the size of the

capitulum, and it would be possible to obtain plants with bigger leaves and flower heads. The establishment of *T. mastichina* crops in Spain could help with the sustainable development of the species promoting its conservation.

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Table 24. Data of the origins of the studied populations of *Thymus mastichina.*

Population	Locality	Province	Longitude	Latitude	Altitude	Plants evaluated 2011	Plants evaluated 2012
CLM1	Lezuza	Albacete	385456N	022308W	1054	3	10
CLM2	Moral de Calatrava	Ciudad Real	385036N	033356W	730	8	12
CLM3	Hontanar	Toledo	393052N	043419W	848	-	12
CLM4	Casas de Lázaro	Albacete	384423N	021321W	827	2	11
TM1	Toro	Zamora	413122N	052424W	704	-	9
TM12	Tordesillas	Valladolid	412933N	050054W	675	6	7
TM14	Truchas	León	421325N	061512W	957	9	12
TM17	Almazán	Soria	412826N	023538W	933	3	11
TM23	Riaza	Segovia	411527N	032805W	814	6	8
TM29	Serranillos	Ávila	402040N	045415W	1190	9	12
TM33	Ponferrada	León	423407N	064604W	507	9	12
TM37	Carrocera	León	424942N	115213W	1029	9	12
TM38	Villacastín	Segovia	404622N	042253W	1056	6	8
TM39	Lerma	Burgos	415838N	032908W	945	3	8
TM42	Saldaña	Palencia	423158N	044326W	816	9	12
TM43	Boñar	León	425143N	051816W	1017	9	12

Table 25. Qualitative and quantitative traits studied in *Thymus mastichina*.

Trait		Abbreviation	Nº of data per plant	Unit	Scale		
Qualitative Traits					1	3	5
Leaf	Form	SL	1	-	Elliptic	Intermediate	Lanceolate
Bract	Form	SB	1	-	Long	Intermediate	heart-shaped
	Pilosity	HB	1	-	Low	Medium	High
Seed	Colour	SC					
	Form	SF					
Quantitative Traits							
Leaf	Length (L)	LL	20	cm			
	Width (W)	LW	20	cm			
	L/W	LL/LW	20	-			
Branch	Length	BLH	3	cm			
	Nº of flower head per branch	FHB	3	-			
Flower head	Length (L)	FHL	10	mm			
	Width (W)	FHW	10	mm			
	L/W	FHL/ FHW		-			
	Nº of flowers per flower head	NFH	3	-			
Bract	Length (L)	BL	6	cm			
	Width (W)	BW	6	cm			
	L/W	BL/BW	6	-			
Flower	Corolla length	CL	6	cm			
	Calix size	CS	6	cm			
Seeds	100-seed weight	WS	1	g			

Table 26. Mean, range, coefficients of variation (CVs) and main components for 20 morphological traits in 14 populations of *Thymus mastichina* collected in 2011.

Morphological traits		Unit	Mean	Range	CVs (%)	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Qualitative traits												
Leaf	**Form**		-		-	0.2736	0.0957	-0.0281	0.2530	-0.1561	-0.1649	-0.5013
Bract	**Form**		-		-	0.3607	-0.1745	-0.1815	-0.0664	0.2913	0.0974	0.2526
	Pilosity		-		-	-0.0336	0.2363	0.0731	-0.0624	0.3733	-0.1238	0.3978
Seed	**Colour**		-		-	-0.1091	-0.0908	-0.2171	0.0901	0.5260	-0.0891	-0.3976
	Form		-		-	-0.0136	-0.3743	-0.0265	-0.1663	-0.0050	0.1271	-0.0842
Quantitative traits												
Leaf	**Length (L)**	**cm**	1.29	0.87-1.92	17.5	-0.2548	-0.0588	0.3257	0.1611	0.1957	0.3892	0.1898
	Width (W)	**cm**	0.40	0.22-0.55	16.5	0.2621	-0.0104	0.1896	0.3414	-0.1353	0.4290	-0.1424
	L/W		3.37	2.24-5.84	24.9	-0.3843	-0.3822	0.7895	-0.1808	0.2414	-0.0328	0.3068
Branch	**Length**	**cm**	35.58	16.33-49.17	20.4	0.0764	0.2273	-0.1595	0.1838	-0.0003	0.5093	0.2012
	Nº flower head		36.27	8.67-92	46.8	-0.2879	0.1842	-0.1245	0.0415	-0.1256	-0.0202	0.0937
Flower head	**Length (L)**	**cm**	1.56	0.74-3.10	25.4	0.1455	0.3876	0.3338	-0.2562	0.0576	-0.0649	-0.0220
	Width (W)	**cm**	1.44	0.72-2.09	20.9	0.1920	0.3607	0.1989	0.2482	0.2292	-0.2392	0.0667
	L/W		1.10	0.73-2	20.1	-0.0433	0.0639	0.1582	-0.6289	-0.1989	0.1999	-0.1598
	Nº of flowers		69	20-196	48.4	0.2557	0.3290	0.1852	-0.2521	0.0715	0.1422	-0.1735
Bract	**Length (L)**	**cm**	0.71	0.45-1.15	18.5	-0.1013	-0.2077	0.4692	0.0880	0.6482	0.1047	0.0396
	Width (W)	**cm**	0.43	0.12-0.72	27.4	0.3463	-0.3066	0.1102	-0.1413	0.1521	0.0287	0.1681
	L/W		1.85	0.94-4.75	36.5	-0.3856	0.1775	0.1126	0.2082	-0.1509	0.0363	-0.1161
Flower	**Corrolla lenght**	**cm**	0.49	0.30-0.74	20.8	0.0767	-0.2204	0.2061	0.0704	-0.3610	-0.2445	0.4475
	Calyx length	**cm**	0.54	0.40-0.77	14.0	-0.0258	-0.2088	0.3442	0.1084	0.2490	0.1023	-0.2686
Seed	**100-seed weight**	**g**	0.03	0.02-0.06	26.7	0.0311	-0.1103	0.3280	0.0844	-0.0546	-0.3645	-0.1852
Autovalue						4.0	3.1	2.4	1.7	1.3	1.2	1.0
% variability explained						20.2	15.5	11.8	8.6	6.7	6.1	5.2
Variability accumulated						20.2	35.7	47.5	56.1	62.7	68.8	74.0

Table 27. Pearson correlation coefficients among the 20 studied traits of *Thymus mastichina*.

		Quantitative traits															*Qualitative traits*			
		Leaf			**Flower**		**Branch**				**Flower head**				**Bract**		**Seed**	**Leaf**	**Bract**		**Seed**
Quantitative traits		**LL**	**LW**	**LL/LW**	**CL**	**CS**	**BLH**	**FHB**	**FHL**	**FHW**	**FHL/FHW**	**NFH**	**BL**	**BW**	**BL/BW**	**WS**	**SL**	**SB**	**HB**	**SC**
Leaf	**LW**	14.4																		
	LL/LW	**60.4**	**-65.8**																	
Flower	**CL**	-1.5	10.5	-8.4																
	CS	**27.0**	9.2	10.4	15.1															
Branch	**BLH**	-9.5	**25.2**	**-23.9**	-14.1	-15.9														
	FHB	10.4	**-24.0**	**25.8**	-3.8	-10.7	10.6													
Flower head	**FHL**	-9.6	16.4	-18.0	-11.0	-0.9	19.5	-5.9												
	FHW	-11.6	20.7	**-24.5**	-4.0	6.7	**22.9**	4.0	**68.4**											
	FHL/FHW	-2.1	-2.2	2.1	-11.1	-7.7	-1.2	-0.4	**47.4**	**-28.5**										
	NFH	**-23.7**	**31.3**	**-38.5**	**-22.7**	-10.4	**32.0**	-18.0	**78.8**	**52.4**	**37.6**									
Bract	**BL**	**50.6**	10.6	**22.9**	**31.3**	**44.5**	**-25.2**	-7.9	-8.9	-5.5	-5.9	-12.0								
	BW	-15.4	**30.8**	**-33.3**	**30.3**	18.1	-8.2	**-48.8**	-0.4	-4.3	3.2	16.8	**23.9**							
	BL/BW	**41.0**	**-25.8**	**46.4**	-14.4	8.2	-2.9	**39.3**	-6.8	-1.3	-6.9	**-28.9**	**22.1**	**-80.0**						
Seed	**WS**	15.4	10.6	-0.3	**21.5**	**25.2**	**-25.9**	-18.1	6.9	4.8	2.3	-3.1	**28.9**	11.0	0.5					
Cualitative traits																				
Leaf	**SL**	**-31.8**	**26.2**	**-44.5**	6.3	-10.7	8.2	-14.8	14.5	**37.0**	**-25.0**	19.8	-1.0	**25.5**	-20.3	-1.0				
Bract	**SB**	**-36.4**	**28.1**	**-44.2**	3.9	-15.6	18.1	**-38.9**	-3.2	1.5	-5.7	14.6	**-24.2**	**71.1**	**-78.1**	-13.3	21.0			
	HB	2.4	-7.3	4.9	-14.7	-11.0	9.4	9.8	**30.2**	**26.8**	4.9	**23.1**	-7.7	13.0	5.1	-8.4	-4.9	2.1		
Seed	**SC**	2.3	-20.4	14.2	-20.1	9.2	-5.6	12.9	**-28.6**	-8.2	-21.2	**-23.5**	-8.6	-9.9	0.9	-6.0	-17.2	5.0	-5.2	
	SF	1.1	-7.0	9.6	11.4	**23.3**	-20.0	**-22.5**	**-37.8**	**-51.1**	8.7	-21.2	-21.3	**28.6**	-13.9	0.1	-3.0	12.8	-16.3	3.8

In bold significant correlations at P<0.05

Table 28. Percentages of the sum of squares obtained in the analysis of variance using population, year and year x population interaction as sources of variation.

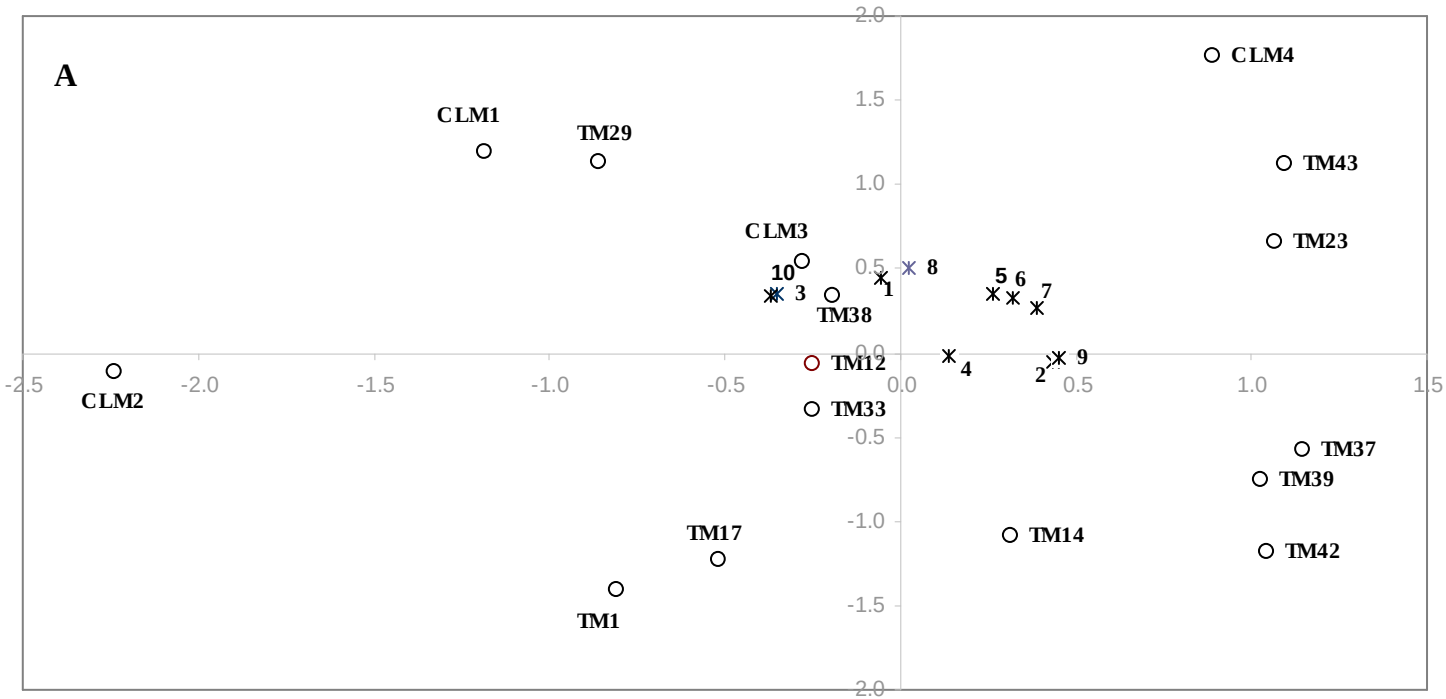
		Population		Year		Population x year		Residual
Leaf	Length (L)	17.23	***	31.68	***	6.44	***	39.40
	Width (W)	24.84	***	0.03	ns	2.99	ns	69.17
	L/W	33.83	***	25.33	***	3.43	ns	36.70
Flower head	Length (L)	14.33	***	2.48	**	9.68	**	76.53
	Width (W)	21.41	***	12.46	***	11.28	***	60.35
	L/W	4.44	ns	2.81	ns	4.38	ns	88.26
Bract	Length (L)	32.24	***	0.20	ns	8.76	**	59.27
	Width (W)	24.93	***	0.83	ns	6.51	ns	70.04
	L/W	34.67	***	3.79	***	6.49	*	59.15
Flower	Corolla length	12.14	**	0.86	ns	5.38	ns	82.56
	Calyx length	13.38	**	0.25	ns	7.92	ns	77.30

*, ** and *** Statistical significance at P<0.05, 0.01 and 0.001 level, respectively. Ns: non-significant.

Table 29. Mean values of *Thymus mastichina* populations included in each group.

Trait*		Overall mean			Group 1				Group 2				Group 3				Group 4			
Leaf	Length (L)	1,06	±	0,11	1,20	±	0,06	a	0,99	±	0,12	b	1,16	±	0,03	a	1,00	±	0,03	b
	Width (W)	0,40	±	0,04	0,35	±	0,04	b	0,37	±	0,03	b	0,42	±	0,01	a	0,43	±	0,03	a
	L/W	2,78	±	0,44	3,50	±	0,17	a	2,73	±	0,27	b	2,82	±	0,17	b	2,37	±	0,18	c
Flower head	Length (L)	16,07	±	1,24	15,31	±	1,57	b	16,12	±	0,58	b	17,80	±	0,29	a	15,43	±	0,99	b
	Width (W)	15,46	±	1,08	14,35	±	1,01	b	15,54	±	0,67	b	16,90	±	0,24	a	15,19	±	0,91	b
	L/W	1,05	±	0,03	1,08	±	0,03	a	1,05	±	0,02	ab	1,06	±	0,04	ab	1,02	±	0,02	b
Bract	Length (L)	0,72	±	0,07	0,76	±	0,09	a	0,72	±	0,06	ab	0,80	±	0,05	a	0,66	±	0,02	b
	Width (W)	0,44	±	0,05	0,39	±	0,02	b	0,41	±	0,03	b	0,48	±	0,01	a	0,49	±	0,03	a
	L/W	1,74	±	0,28	2,12	±	0,04	a	1,84	±	0,15	b	1,71	±	0,14	b	1,42	±	0,08	c
Flower	Corolla length	0,52	±	0,03	0,49	±	0,04	ns	0,53	±	0,04	ns	0,52	±	0,05	ns	0,52	±	0,02	ns
	Calyx length	0,55	±	0,03	0,55	±	0,02	b	0,54	±	0,02	b	0,59	±	0,02	a	0,55	±	0,02	b

*Different letter in each row means differences among mean values. ns: non-significant


A
CLM1
TM29
CLM3
10
3
TM38
8
1
5
6
7
CLM4
TM43
TM23
TM12
4
2
9
CLM2
TM33
TM37
TM39
TM17
TM14
TM42
TM1
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
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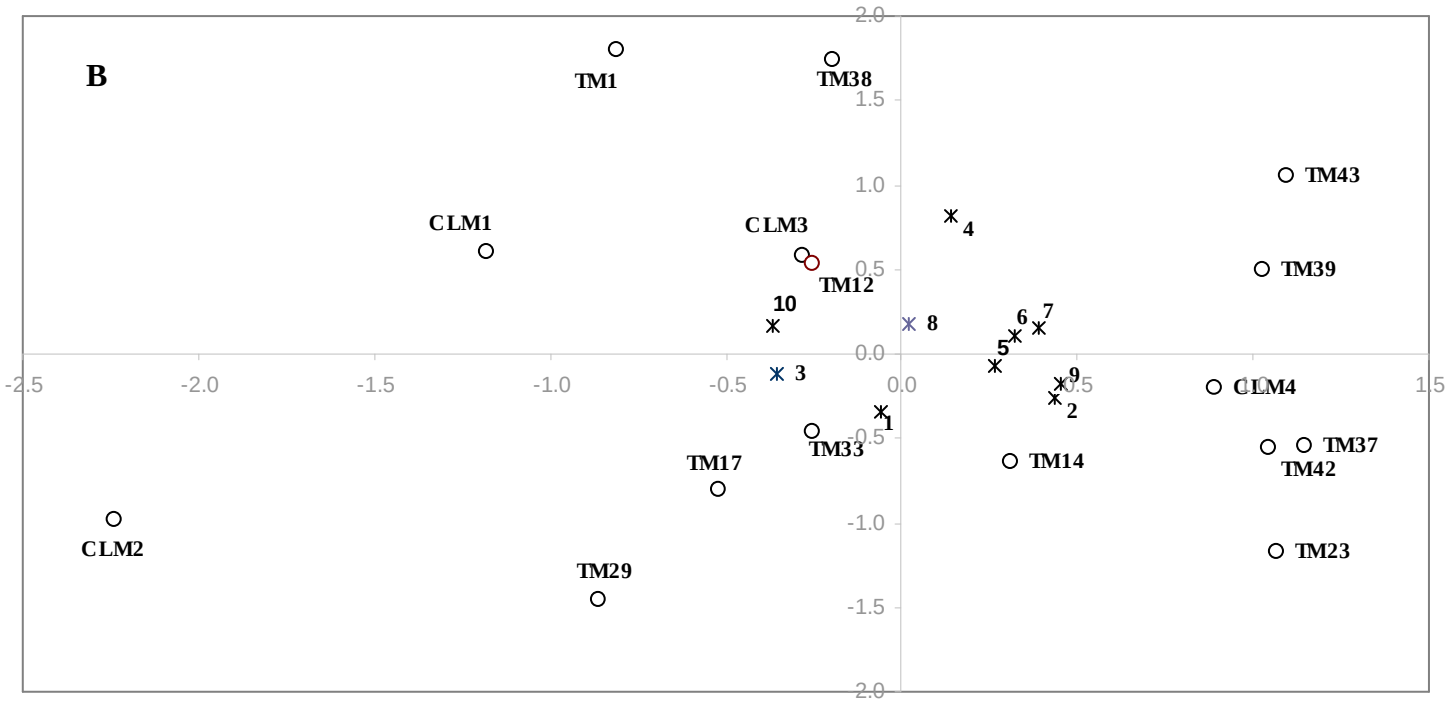

Figure 8: A, representation of axes 1 and 2 of ACP. **B**, representation of axes 1 and 3 of ACP. **1**: Leaf length, **2**: Leaf width, **3**: Leaf Length/Width, **4**: Corolla length, **5**: Calyx length, **6**: Flower head length, **7**: Flower head width, **8**: Bract length, **9**: Bract width, and **10**: Bract Length/Width.

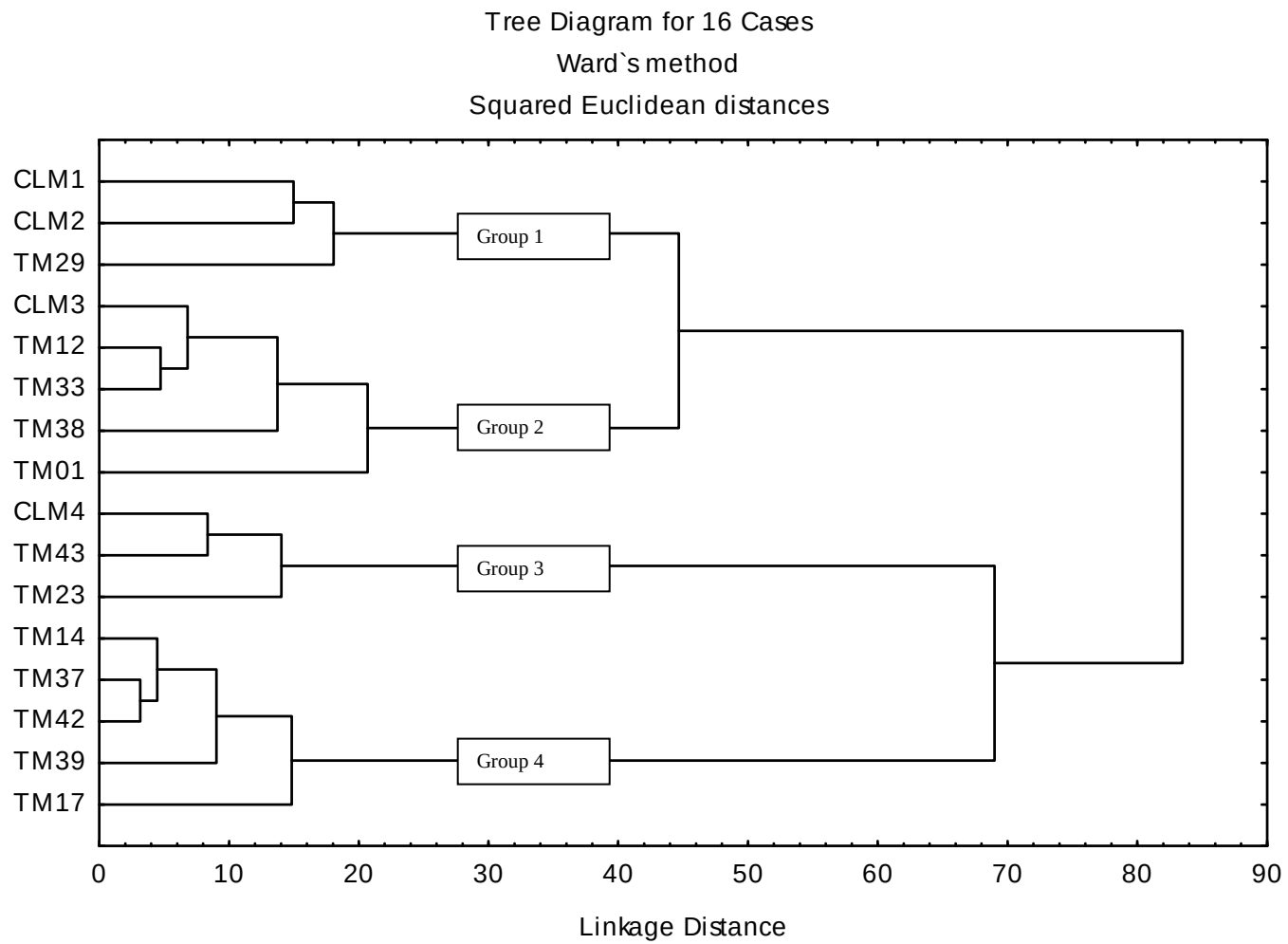

Figure 9: Cluster analysis using Ward's method to group the 16 populations separated in four groups.

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General Conclusions

8. General conclusions

In view of the great chemical variability that occurs both in the composition of essential oils and polyphenols for the different species studied as well as their morphology and given that these features are primarily ruled by genetic characters, with the environmental factors being a weaker and less influential factor, the ability to select organisms with characteristics and qualities desired by the market is a real possibility for these species. The establishment of plot assays demonstrated that these species can be easily cultivated avoiding wild collection and allowing traceability and offering the possibility of controlling agronomic parameters such as irrigation doses or treatments that maximize the yield. Moreover the selection of organisms with outstanding features leads to an increase of crop productivity and improves economic profit, which could be very beneficial to the industry.

To carry out the analysis of the chemical variability, **Chapter 1** shows a comparison of methodologies for analysing the similarities and differences between the methods studied and their suitability for each species. Depending on the species tested, the application of different methodologies led to different results in the composition of the volatile fraction. TM showed few differences between extraction methodologies, while SL showed important differences using EX. Some compounds yielded no significant differences as far as the method applied was concerned, while others produced higher contents with a particular technique.

In view of the huge amount of by-products generated by the essential oil industry, a valorization of such products can lead to the increasing of the profits of the industry in an environmental friendly way. **Chapter 2** concludes that the non-profitable waste produced by *L. latilofia* essential distillation could be valorized and used as a source of natural antioxidants. Populations, seasons, and population by season interaction influence the antioxidant properties of the *L. latifolia* by-product.

As may be observed from the results obtained for the three species (**Chapter 3)**, the variability of essential oils volatiles is largely dependent on the populations studied, meaning that genetic factors have greater influence in comparison with the environmental factors and it is possible to select populations with a desirable composition and the plant material can be standardised, thus providing maximum homogeneity, which is essential for quality production. However, environmental factors which can produce differences in the phenological stage or morphological development can also have an effect on the chemical variability. Furthermore, the control of the

environmental factors, as well as choosing the right period of harvest are also important to obtain a desired composition, especially for *L. latifolia.*

As for essential oils volatiles, polyphenols also showed great variability (**Chapters 4 and 5)**. For *Thymus mastichina,* samples with higher antioxidant capacity also had higher polyphenol content but the different polyphenols contributed in a different way to the antioxidant properties of this species. Some populations showed high intra-population variability while others showed a similar composition among individual plants. The populations from Saldaña, Lerma and Carrocera were the populations with a higher amount of polyphenols and antioxidant components in the samples studied and they could be selected for their higher content. Both compounds; rosmarinic acid and peak 3 are mainly responsible for the antioxidant properties of *Thymus mastichina* L **(Chapter 4)**. For *Salvia lavandulifolia*, the phytochemical study of seven populations of Spanish sage has demonstrated that the polyphenolic profiles were related to the geographical origin of plant populations and can be a useful tool to elucidate the origin of plant populations **(Chapter 5)**. The population of Moratalla showed le lowest antioxidant properties, while La Sentiu de Sió was highlighted by higher antioxidant content.

Finally, morphology also presented an important variability as shown in *Thymus mastichina* **(Chapter 6)**. In the process of domestication of any species, genetic diversity among germplasm plays the major role, since it opens the way to select the best specimens. Phenotypic variation within populations can have important consequences on the biomass and essential oil production. The study reported that the most variable traits are related to the size of leaves, bracts and capitulum. These traits influence biomass development and therefore essential oil production. Selection programmes should search for specimens with larger branches, bigger leaves and bracts and bigger flowers and flower heads. On the other hand, the leaf size is not related to the size of the capitulum, and it would be possible to obtain plants with bigger leaves and flower heads. In view of the high incidence of wild collection of this particular plant species, the establishment of *T. mastichina* crops in Spain could help with the sustainable development of the species thus promoting its conservation.

Resumen en español

10 INTRODUCCIÓN GENERAL

10,1 Visión general de las plantas aromáticas y medicinales

El mercado de las plantas aromáticas y medicinales (PAM) es un gran mercado, siendo el volumen comercializado a nivel mundial para el período entre 1991 y 2003 de 467.000 t, por un valor de 1,2 mil millones US$ (Lange, 2006). Otra de las conclusiones que se pueden obtener con los datos disponibles es que el volumen de PAM con el que se comercia en el mundo ha mostrado un aumento constante en los últimos años. Este aumento del volumen queda reflejado en el aumento de las exportaciones e importaciones en las últimas décadas. Los 12 principales países importadores de PAM, importaron un total de 320.650 t por un valor de 930.520.000 US$ para el período comprendido entre 1991 y 2003, frente a la cantidad de PAM importada para el período de 2004 a 2008 que fue de 338.173, por un valor de 1.004.692.000 US$. La misma tendencia se muestra en las cifras de exportación de los últimos años. Los volúmenes de ventas de los 12 principales países exportadores representan un total de 368.200 t por un valor de 807.977.000 US$ para el período de 1991 a 2003, mientras que las exportaciones de 2004 a 2008 aumentaron a 416.807 t por un valor de 923.728.000 US$ (Lubbe y Verpoorte, 2011).

Este aumento constante del consumo de plantas aromático-medicinales es algo que también se ha producido en España. Dicho aumento se ha visto reflejado en el incremento de las importaciones y el aumento del cultivo nacional en los últimos años. España ha estado en las últimas décadas en el top 10 de los principales países importadores de PAM en el mundo posicionándose entre el décimo y noveno puesto entre 1991 y 2008. El volumen de PAM importadas en España entre 1991 y 2003 fue de 9.850 t por un valor de 27.648.000 US$, mientras que dicho volumen fue de 15.670 t entre 2004 y 2008, por un valor de 44.337.000 US$ (Lubbe y Verpoorte, 2011). España no sólo ha aumentado las importaciones de PAM, sino que también ha aumentado las hectáreas de terreno nacional dedicada a cultivar este tipo de especies y en los últimos años la producción de plantas aromáticas se ha incrementado de 2.180 ha en 2004 a 9.810 ha en 2014 (MAGRAMA, 2014).

Los principales sectores en los que se comercia con PAM en el mundo son las industrias farmacéuticas, alimentarias, cosméticas y perfumeras. En España, la demanda de plantas para extracción de aceites esenciales es mayor a la demanda de plantas medicinales aunque el número de empresas interesadas en la comercialización de plantas medicinales también es sustancial (CBI, 2013). Algunas de las especies más utilizadas y consumidas de PAM en la industria española son: *Mentha piperita*, *Melisa officinalis*, *Papaver somniferum*,

Chamaemelum nobile, *Equisetum arvense*, *Thymus* spp., *Rosmarinus officinalis*, *Salvia officinalis*, *S. lavandulifolia*, *Pimpinella anisum*, *Lavandula latifolia*, *Matricaria chamomile*, *Ginkgo biloba*, *Tilia* spp., *Juniperus comunis*, *Passiflora incarnata*, *Eucaliptus globulus* y *Crocus sativus* que son principalmente recolectadas de forma silvestre e importadas aunque también se cultivan en pequeñas cantidades, y *L. angustifolia* y lavandín que son ampliamente cultivadas (Arraiza, 2006; Palos y Gorgues, 2002).

La mayor parte del mercado mundial de PAM es recolectada de forma silvestre. En el mundo, entre el 60 y el 90% de la materia prima utilizada por las empresas que venden productos obtenidos a partir de plantas aromáticas y medicinales provienen de recolección silvestre (Laird et al., 2002).

Thymus mastichina es una de las plantas más recolectadas de forma silvestre en España para su explotación industrial ya que el 95% de la producción anual de esta especie vegetal proviene de la recolección en su hábitat natural, mientras que sólo el 5% de la producción se obtiene de cultivos. Para *Lavandula latifolia* el 30% de la producción viene de la recolección silvestre y el 70% es cultivado y para *Salvia lavandulifolia* un 20% proviene de la recolección silvestre mientras que el 80% se obtiene de su cultivo (datos de la empresa Esencias Martín Lozano).
La recolección silvestre de PAM tiene una serie de problemas asociados que se enumeran a continuación:

- Identificación equivocada a la hora de caracterizar las especies y mezcla de especies similares.
- Falta de conocimiento sobre los ingredientes activos y su calidad.
- Falta de homogeneidad del producto.
- Falta de trazabilidad.
- Algunas veces el volumen de material vegetal silvestre recolectado no alcanza las necesidades del mercado.
- La recolección silvestre puede ser una amenaza para la protección de las especies. Algunas plantas son escasas en la naturaleza y la presión humana reduce su número.

En vista de estos problemas causados por la recolección silvestre, los cuales conducen a una pérdida de la biodiversidad y en vista de un mayor volumen de las importaciones en relación con las exportaciones, hay una creciente necesidad de aumentar la superficie de cultivo en España y de estudiar los parámetros de cultivo de plantas aromáticas y medicinales de interés industrial, con el fin de mejorar su cultivo y promover el aprovechamiento sostenible, maximizando el rendimiento de cultivo. El aumento del valor de las PAM ha llevado a la necesidad de fortalecer el sector. Las importaciones son mayores que las exportaciones y el

clima y las condiciones ambientales de España (y de la Península Ibérica en general) son las idóneas para la producción sostenible de PAM.

Una de las dificultades que encuentra el cultivo de PAM es la falta de material vegetal seleccionado adecuado, lo cual dificulta una explotación económica rentable.

Los beneficios que se pueden lograr a través del cultivo incluyen los siguientes:

- Evitar la amenaza de la especie en cuestión y fomentar la protección de la naturaleza.
- Estimar los volúmenes de producción que permitan una planificación.
- Posibilidad de implantar sistemas de trazabilidad y establecer controles sobre la calidad del material vegetal, evitando la adulteración o el fraude.
- Utilizar dosis de riego adecuadas y técnicas agronómicas que permiran mejorar la producción.

Existe una gran variabilidad (inter e intra-poblacional) en las especies silvestres. Diferentes poblaciones han sido sometidas a diversas presiones de selección, por lo que se pueden manifestar diferencias. Por otra parte, distintos individuos dentro de cada población, muestran diferencias debidas a las combinaciones genéticas que cada uno ha acumulado. Esta diversidad es esencial para llevar a cabo programas de selección de material vegetal. También es importante el estudio de la biodiversidad a través de su caracterización, evaluación y conservación para ponerla a disposición de los agricultores, con el fin de utilizar mejor estos recursos genéticos. De esta manera se pueden encontrar nuevos genotipos de especies silvestres que permitan el desarrollo de nuevas variedades para la producción agrícola.

Lavandula latifolia, *Salvia lavandulifolia* y *Thymus mastichina* han sido seleccionadas para este estudio, ya que todas ellas son especies de plantas características de España y como se mencionó anteriormente, son de gran importancia para la industria tanto a nivel mundial como nacional. La producción anual de estas especies en España en el año 2014 fue de 20,11 ha de *T. mastichina* siendo Andalucía el principal productor, 136,42 ha de *S. lavandulifolia* y *S. officinalis* y de 595,51 ha para *L. latifolia* y *L. angustifolia* (ANIPAM, 2014). Por otro lado, anualmente se extrae en España entre 3 y 4 t de aceite esencial de *T. mastichina*, alrededor de 8 t de aceite esencial de *S. lavandulifolia* y 8 t de *L. latifolia* (datos de Industria Martín Lozano). *L. latifolia* y *S. lavandulifolia* además se encuentran entre las 20 plantas más utilizadas para la extracción de aceite esencial en el mundo, con una producción anual de 50 a 100 t por año (Lubbe y Verpoorte, 2011).

10.2. Metabolitos secundarios

Las plantas son capaces de sintetizar muchos tipos de compuestos tales como carbohidratos, lípidos, proteínas y vitaminas. Algunos de estos compuestos son sintetizados para la supervivencia, el crecimiento y la reproducción de las plantas, es decir, que son parte del metabolismo primario de las plantas y están presentes en todas ellas. Además de estos compuestos, las plantas también son capaces de sintetizar metabolitos secundarios que a menudo derivan de metabolitos primarios.

Los metabolitos secundarios son compuestos naturales producidos por un determinado organismo. Se les llama "secundarios" porque no están directamente involucrados en la supervivencia de los seres vivos, sin embargo juegan un papel indispensable para los seres vivos y las plantas son productoras de metabolitos secundarios por excelencia. A diferencia de los metabolitos primarios, que son comunes a todas las plantas, los secundarios son característicos y cambian de una especie a otra. Estos compuestos se almacenan habitualmente en determinadas partes de la planta, principalmente en las vacuolas o estructuras secretoras como espinas, pinchos, tricomas, pelos glandulares, laticíferos, conductos, etc.

Aunque hoy en día las funciones exactas de algunos metabolitos secundarios son aún desconocidas, estos compuestos pueden ser tóxicos o beneficiosos para el resto de los seres vivos. Están involucrados principalmente en los mecanismos de defensa, actuando como productos alelo-químicos en defensa de herbívoros, microorganismos, parásitos, patógenos o plantas competitivas. También pueden actuar como mensajeros internos y conferir a la planta características tales como color u olor; es por ello que con frecuencia actúan como atrayentes de polinizadores o atraen animales para la dispersión de semillas.

Una clasificación simple de estos compuestos basada en su origen biosintético, incluye alcaloides, terpenoides y fenoles.

-Alcaloides: Los alcaloides son moléculas orgánicas nitrogenadas que suelen ser tóxicas o tener actividad farmacológica. Algunos ejemplos de este grupo de compuestos son la morfina sintetizado por la adormidera (*Papaver sonniferum*), la cocaína aislado de la planta de coca (*Erythroxylum coca* y *Erythroxylum novogranatense*) o la cafeína sintetizada por *Coffea arabica*. Las especies objeto de este estudio no contienen alcaloides.

-Terpenoides: también conocidos como isoprenoindes, son derivados de la molécula de isopreno formada por cinco carbonos. Se trata de hidrocarburos a los que se adhieren grupos funcionales y su nombre proviene de la trementina. Dependiendo del número de isoprenos que conforman un terpenoide se establece la siguiente clasificación:

- Hemiterpenoides: 1 unidad de isopreno (C5)
- Monoterpenoides: 2 unidades de isopreno (C10)
- Sesquiterpenoides: 3 unidades de isopreno (C15)
- Diterpenoides: 4 unidades de isopreno (C20)
- Sesterterpenoides: 5 unidades de isopreno (C25)
- Triterpenoides: 6 unidades de isopreno (C30)
- Tetraterpenoids: 8 unidades de isopreno (C40)
- Politerpenoides: con un mayor número de unidades de isopreno

El 1,8-cieneol es un éter cíclico también conocido como eucaliptol. Es el terpenoide más importante de las especies estudiadas, siendo el compuesto principal de *L. latifolia*, S. *lavandulifolia* y *T. mastichina*, así como también es un compuesto característico de muchas especies de la familia Lamiacea y del género Eucaliptus y de ahí su nombre. Otros terpenoindes importantes y muy abundantes en estas especies son el alcanfor, el linalol, el borneol y elα y β-pineno.

-Fenoles: los fenoles son compuestos formados por un grupo hidroxilo (-OH) unido a un hidrocarburo aromático (C_6H_5OH). Estos están principalmente sintetizados a través de dos rutas biosintéticas: la ruta del ácido shikímico y la vía del ácido malónico (Harborne, 1989), y cuando están formadas por más de un fenol son llamados polifenoles. Si los clasificamos atendiendo a su naturaleza química podemos distinguir:

- *Ácidos fenólicos:* los ácidos fenólicos son compuestos orgánicos derivados de ácido benzoico o cinámico. En general, incluye sustancias que tienen un anillo fenólico y una función orgánica de ácido carboxílico. Según su origen se pueden clasificar como sigue: acido hidroxicinámico y acidos hidroxibenzoicos.

- *Flavonoides:* los flavonoides son moléculas formadas por 15 átomos de carbono ($C_{15}H_{10}O_2$) que contienen dos anillos de fenilos y un anillo heterocíclico. Se sintetizan a través de la vía biosintética de los flavonoides en cuyo proceso, el aminoácido fenilalanina produce 4-coumaroyl-CoA. Se trata de una familia de compuestos muy diversos y dependiendo de los cambios a los que se someten estás moléculas tras la ruta biosintética, se puede originar un tipo

de compuestos u otro (Boros et al., 2010a.): antocianos, flavonoles, flavanones, chalconas, flavonas.

- *Lignanos:* los lignanos son un tipo de polifenoles que se encuentra principalmente en las semillas en pequeñas concentraciones y están constituidos por unidades de C_6C_3.

- *Estilbenoides:* los estilbenoides, son compuestos hidroxilados derivados del estilbeno y tienen una estructura química C6-C2-C6.

El ácido rosmarínico es el principal compuesto polifenólico que se encuentra en las 3 especies estudiadas y además se trata de un compuesto característico de la familia de las Lamiaceae (Lamaison et al., 1990). Aunque hay una gran variabilidad en compuestos polifenólicos en las especies de interés, algunos compuestos característicos que cabe destacar son el ácido cafeico y el ácido clorogénico comunes a las tres especies. La luteolina, la apigenina y sus derivados son también compuestos que con frecuencia están presentes en los géneros de *Salvia, Lavandula* y *Thymus* (Boros et al, 2010b; Spiridon et al, 2011a.). Por otro lado, los derivados del ácido salvinólico, el carnosol y el ácido carnósico son muy característicos del género *Salvia* (Johnson, 2011; Zimmermann et al, 2011).

10.3 Familia Lamiaceae

La familia Labiatae, también conocida como Lamiaceae o Labiataceae, comprende 186 géneros y alrededor de 5.600 especies (Morales et al., 2010). Es una familia muy cosmopolita y está extendida en todo el mundo, pero con poca representación en el bosque tropical lluvioso. Las especies que pertenecen a esta familia, son comúnmente aromáticas ya que estas plantas son capaces de sintetizar compuestos volátiles y son una importante fuente de una gran variedad de polifenoles.

10.3.1 *Lavandula latifolia* Medik

L. latifolia pertenece al género *Lavandula* y a la subfamilia Nepetoidae. Este género está formado por un total de 39 especies y es un grupo de plantas típicas del Mediterráneo que también se extiende por la región Macaronésica y está presente de forma dispersa por el norte de África, el sur de Asia y la Península Arábiga (Morales, 2010).

Las plantas de este género son típicas de suelos de piedra caliza y son generalmente plantas herbáceas de ciclo corto plantas arbustivas perennes. Las hojas pueden ser enteras, dentadas o varias veces divididas e incluso ausentes en algunas especies. Las especies de esta familia se caracterizan por tener una inflorescencia compuesta por flores pedunculadas agrupadas en espigas terminales. La corola suele ser lila, violeta o azul.

L. latifolia es una especie que crece de forma natural en España y puede encontrarse tanto en montañas altas como a baja altura (Lis-Balchin, 2002a), además también se distribuye a lo largo de Francia, Italia y la antigua Yugoslavia (Morales, 2010).

Como su nombre indica (*latifolia*), esta especie se caracteriza por unas hojas más anchas que otras plantas pertenecientes al género *Lavandula.* Se trata de un arbusto con base leñosa y raíz gruesa que alcanza hasta 40 cm. El tallo es leñoso y con una altura de entre 10 y 15 cm. Las flores tienen diferentes coloraciones dentro del rango azul-violeta y se agrupan en glomérulos o espigas. La corola es bilabiada y tubular y con una longitud de entre 8 y 10 cm, las flores agrupadas en espigas terminales son soportadas por bractéolas de unos 3 o 4 mm. El fruto es un tetraquenio oscuro formado por 4 aquenios o núculas de unos 2 mm. Las hojas son de color verdoso a grisáceo y varían de lanceoladas a espatuladas. El tallo está a menudo ramificado en forma de tridente aunque en otros muchos casos no hay ramificación. Estos pueden alcanzar una altura de más de 20 cm y la inflorescencia tiene un tamaño variable que oscila entre 4 y 10 cm. Las brácteas aparecen diferentes a las hojas vegetativas y la fecha de floración es variable, teniendo lugar entre los meses de julio a septiembre.

Variabilidad y estudios sobre la composición química de *L. latifolia*

Aunque *L. latifolia* es una planta de gran interés comercial y la composición química de las especies es muy importante para el mercado, el número de estudios referentes a la composición química de esta especie no son muy abundantes, a diferencia de lo que sucede con *T. mastichina* y *S. lavandulifolia*.

Uno de los estudios científicos más antiguos que recogen información sobre la composición química de esta especie es de De Pascual-T et al. (1983), que estudió parte de los constituyentes de *L. latifolia* conocidos en la actualidad, encontrando 1,8-cineol, linalol y alcanfor como compuestos principales (como es habitual para esta especie). Muñoz-Bertomeu et al. (2007) estudiaron siete poblaciones silvestres de *L. latifolia,* identificando un total de 28 compuestos y clasificando sus aceites esenciales como aceites con alto, intermedio y bajo contenido de linalol. Esta clasificación de aceites esenciales se correlaciona con los pisos bioclimáticos supra, meso y

termo mediterráneos. Estos autores obtuvieron 1,8-cineol, linalol y alcanfor como compuestos principales del aceite esencial. En un estudio similar, Herraiz-Peñalver et al. (2013) encontraron también estos tres compuestos como principales, además identificaron un total de 40 compuestos y encontraron cierta relación entre la composición química de los aceites esenciales y su origen geográfico.

Por otro lado, Eikiani et al. (2008) compararon diferentes métodos para la extracción de aceite esencial de *L. latifolia* utilizando extracción por sobrecalentamiento de agua, hidrodestilación y extracción con soxhlet, obteniendo como resultado que el método más rápido y más selectivo para componentes oxigenados fue el método de extracción por sobre calentamiento de agua. Por último, Barazandeh et al. (2002) y Eikiani et al. (2008) encontraron borneol en lugar de alcanfor como uno de los tres compuestos principales.

Aunque hay varios estudios sobre los compuestos volátiles de los aceites esenciales de *L. latifolia*, *S.lavandulifolia* y *T. mastichina*, hay muy poca literatura publicada sobre el contenido de polifenoles de estas tres especies. Shimizu et al. (1990) estudiaron el efecto antiinflamatorio de *L. latifolia* encontrando dicho efecto en tres polifenoles de *L. latifolia*: cumarin, 7-metoxicumarin y trans-fitol. Lamaison et al. (1990) estudiaron el contenido de ácido rosmarínico de varias especies de Apiaceae, Boraginaceae y Lamiaceae encontrando una cantidad de 700 mg/100 g MS de ácido rosmarínico en *L. latifolia*. Aunque existen pocos estudios sobre los polifenoles de*L. latifolia* y no son recientes, sí que existe bibliografía más completa y actualizada para el lavandín, como por ejemplo el estudio de Torres-Claveira et al. (2007) que detectaron un total de 32 polifenoles en residuos de lavandín, utilizando cromatografía líquida acoplada a espectrometría de masas Ion-Spray como técnica analítica (LC/MS/MS).

10.3.2*Salvia lavandulifolia* Vahl

Salvia es un género cosmopolita y diverso que comprende aproximadamente entorno a 900-1000 especie. Se trata de un género distribuido por todo el mundo, encontrándose más de la mitad de las especies en América Central y América del Sur (Morales, 2010; Walker y Sytsma, 2007). De todas las especies de este género, el más cultivado y el más importante en el mercado mundial es *S. officinalis* y es popularmente conocido por su uso alimentario, ornamental y como planta medicinal. *S. sclarea, S. officinalis* y *S. lavandulifolia* son ampliamente utilizados para la extracción de aceite esencial (Lubbe y Verpoorte, 2011) y otras especies comerciales de *Salvia* que cabe incluir son *S. fruticosa*, *S. verbenaca* y *S. tomentosa* (Kintzios, 2004). Esta planta

puede multiplicarse por semillas, esquejes, acodo y división de pie (Muñoz, 1987) y es capaz de crecer en hábitats muy diversos. El color de la corola puede variar entre la gama del azul, rojo o blanco y, menos habitual, de color amarillo. A diferencia del resto de las labiadas, el androceo consta de dos estambres.

Salvia lavandulifolia Vahl perteneciente a dicho género, puede encontrarse en las montañas calizas del Mediterráneo occidental o en suelos de sustrato calizo, margoso o yesífero a una altura entre 200 y 2.000 metros, sin embargo, es más común encontrar que entre 600 y 1.000 m de altitud (Burillo, 2003). Esta especie también conocida como Salvia española es característica de España, del sur de Francia y del noroeste de África (Sáez, 2010). Se trata de un arbusto con tallos leñosos en la base que alcanza una altura de entre 20 y 65 cm. Tiene hojas simples y pecioladas y la corola suele ser de color rosa, azul o violeta con una longitud de 15 a 40 mm. El androceo está formado por dos estambres. Esta planta puede soportar suelos poco profundos así como también condiciones ambientales inhóspitas; sin embargo, no soporta el exceso de agua.
El rendimiento de producción de aceite esencial es de alrededor 13,90 L/ha (Mossi et al., 2011). Esta especie presenta una gran variabilidad tanto morfológica como química y el período de floración se extiende desde junio hasta septiembre dependiendo de la localización geográfica, floreciendo en España desde finales de junio hasta principios de agosto.
Subespecies (Sáez, 2010):

- subsp. *lavandulifolia*. Por lo general cáliz piloso con dientes inferiores a 3 mm. Se extiende principalmente por el centro y el este de España.
- subsp. *vellerea*. Por lo general las brácteas son persistentes, el tamaño de los dientes del cáliz oscila entre 3 y 5 mm. Crece en el este y sur de la Península Ibérica.
- subsp. *mariolensis*. Las brácteas suele ser persistente con un tamaño de dientes del cáliz entre 0,7 y 2,2 mm. Sólo crece en el este de España.
- subsp. *blancoana*. Inflorescencia abierta y ramificada, brácteas pronto caedizas, cáliz con indumento formado principalmente por pelos glanduliferos; el tamaño de la corola oscila de entre 24 y 40 mm. Característica de España y del norte de África.

subsp. *oxyodon*. Cáliz glabrescente con dientes mayores de 3 mm. Se extiende por el este de España.

Variabilidad química y estudios previos sobre su composición

Aunque existe una gran variabilidad química para *S. lavandulifolia*, no hay quimotipos claramente reconocidos. Sin embargo, Jordán et al. (2009) clasificaron varias muestras de *S.*

lavandulifolia subsp. *vellerea* en diferentes grupos atendiendo a los compuestos volátiles predominantes.
Con las actuales metodologías y técnicas cromatográficas hay una gran cantidad de metabolitos secundarios de las plantas que pueden identificarse, y *Salvia* presenta una enorme variabilidad de estos compuestos.

Con respecto a la composición química de *S. lavandulifolia*, lo que caracteriza a esta especie es la ausencia de α y β tuyona considerándose estos compuestos como marcadores químicos que permiten distinguir *S. lavandulifolia* de *S. officinalis* (Mathe, et al. 2006; Guillen, et al. 1996). Sin embargo, algunos autores han encontrado grandes cantidades de estos compuestos en la salvia española. Por ejemplo, Mossi et al. (2011) y Pierozan et al. (2009) encontraron un contenido de estos compuestos de 18,95% ± 7,7 para la α-tuyona y 19,96% ± 3,5 para β-tuyona. Por otra parte, Guillén et al. (1996) encontró un contenido de α-tuyona del 11,07%. Esto no es habitual para esta especie; sin embargo, esto podría ser debido a la hibridación con otras especies del género *Salvia*, a condiciones ambientales particulares que favorecen la formación de tuyonas o a una identificación errónea de las muestras.

Usano-Alemany et al. (2012) estudiaron la influencia estacional en el rendimiento y en la composición química de salvia española, obteniendo una variación importante tanto en el rendimiento como en la composición química durante las diferentes estaciones del año y encontrando como compuestos principales: 1,8-cineol (21,4-33,8%); limoneno (5.6 a 10.4%); α-pineno (10,5-17,5%); β-pineno (6,0 a 17,3%); mirceno (t-10,0%); alcanfor (6.1 hasta 9.4%); y *trans*-cariofileno (4,0-8,5%), variando de acuerdo a las estaciones. Al contrario de lo que ocurre con otras especies (por ejemplo con *T. mastichina* o con *L. latifolia*), donde los principales compuestos son estables y permanecen como compuestos principales para esa especie, los compuestos principales para salvia española varían ampliamente, dependiendo del lugar de recolección, las condiciones ambientales, etc. Herraiz-Peñalver et al. (2010) analizaron un total de 20 poblaciones silvestres de esta especie, obteniendo un enorme rango de variabilidad de los compuestos principales (1,8-cineol 6,4-34,5%; limoneno 0,8-16,2%; α-pineno 6,7-23,2%; β-pineno 3,8 a 19,2%; alcanfor 0-15,4%). Los valores mencionados representan la composición del aceite esencial extraído de la planta entera. Sin embargo, Schmiderer et al. (2008) en un estudio más detallado, describe la composición de las diferentes partes de la planta, mostrando que las hojas y los cálices tienen como compuestos principales característicos 1,8-cineol, alcanfor y β-pineno, y los sesquiterpenos α-humuleno y β-cariofileno, mientras que la corola muestra una mayor proporción de borneol, pero una baja concentración de alcanfor.

Como ya se ha mencionado, hay muchos estudios sobre la composición de los aceites esenciales de salvia española, sin embargo hay muy poca literatura y bastante anticuada con respecto a los polifenoles *S. lavandulifolia*. Lu y Foo (2002) hicieron una amplia revisión sobre los polifenoles de varias especies del género *Salvia*. Los compuestos polifenólicos recogidos en dicha revisión para Salvia española fueron extraídos de diversos artículos publicados entre 1974 y 1999, siendo los compuestos principales identificados: ácido rosmarínico, apigenina, genkwanin, 7,4' dimetil eter, luteolina, chrysoeriol, hispidulin, cirsimaritin, nepetin, eupatorin y 6,7,3', éter 4 'tetrametil.

10.3.3 Thymus mastichina L.

El género *Thymus* pertenece a la subfamilia Nepetoidae. Este género está formado por un total de 220 especies y se extiende a través de Eurasia, la costa de Groenlandia, la región Macaronésica, África del Norte y las montañas de Abisinia y Yemen (Morales, 2002). Alguna de las especies del género son características de la Península Ibérica incluyendo varias especies endémicas. La fase de floración comienza entre marzo y junio, dependiendo de la especie en particular. La multiplicación puede hacerse por semilla, por división vegetativa, por esquejes o por pies. Crece en clima de montaña y clima templado-cálido y es resistente a las heladas y la sequía, pero no soporta el exceso de humedad y crece principalmente en suelos de piedra caliza o arcillosa (Muñoz, 1987). El cáliz es bilabiado, alcanza entre 3 y 8 mm y los colores varían desde la gama de verde, rojos hasta la gama de blancos. La corola también es bilabiada y tiene un tamaño que varía desde 2 hasta 18 mm, el color varía entre blanco, amarillo, rosa o púrpura.
Thymus mastichina L. perteneciente a dicha familia es una especie endémica de la Península Ibérica con hojas simples y opuestas. Las flores son zigomorfas y bilabiadas y se agrupan en glomérulos de color de blanco. El androceo tiene cuatro estambres externos y el gineceo tiene un ovario dividido en cuatro partes. La polinización es entomógama y las semillas se dispersan por autocoria. Crece en suelos arenosos y silíceos y el período de floración se extiende desde finales de mayo, principios de junio hasta finales de julio, principios de agosto.

Subespecies: (Morales et al. 2010)

- subsp. *mastichina* cuando el cáliz es mayor de 5 mm; las inflorescencias tienen más de 10 mm de diámetro y las brácteas son oblongo-ovadas o elípticas.
- Subsp. *donyanae* cuando el cáliz tiene un tamaño igual o inferior a 5 mm. Las inflorescencia son menores de 10 mm de diámetro y las brácteas son ampliamente ovadas.

- La subespecie *donyanae* sólo está presente en el suroeste de la Península Ibérica (Doñana y Algarve), mientras que la subespecie *mastichina* se extiende por toda la Península Ibérica, pero es menos abundante en la costa noroeste de España y la costa mediterránea.

Variabilidad y estudios de composición química

En cuanto a su composición química, esta especie se clasifica en tres quimiotipos, con dos compuestos como constituyente principales (Adzet et al., 1977).

- El quimiotipo más extendido es el que contiene 1,8-cineol como compuesto principal.
- El segundo quimiotipo contiene linalol como compuesto principal
- El tercer quimiotipo es el intermedio con ambos compuestos como principales (1,8-cineol y linalol).

Hay muchos estudios fitoquímicos realizados sobre *T. mastichina* en lo referente a la composición química de su aceite esencial, debido a su gran interés comercial. La mayoría de los estudios se centran en la caracterización química y la variabilidad de las especies y algunos incluso estudian la composición de las subespecies (Salgueiro et al., 1997). Miguel et al. (2004b) por ejemplo, estudió la variación química en diferentes etapas de crecimiento y la variabilidad química entre individuos con sustrato fertilizado y no fertilizado. La composición química del aceite esencial no varió en las diferentes etapas vegetativas, sin embargo el rendimiento de aceite esencial fue mayor en plena floración. Respecto a los sustratos, el no fertilizado produjo un mayor rendimiento de aceite esencial y un menor porcentaje de linalool.

Las diferencias de composición entre los diferentes órganos de *T. mastichina,* ha sido estudiada por Miguel et al. (2004a), obteniendo una composición diferente para flores y hojas de distintas poblaciones. También encontraron que el rendimiento de aceite esencial fue menor en las hojas que en las flores y las hojas tenían un mayor contenido de alcanfor.

La composición química de *T. mastichina* también ha sido correlacionada con la actividad biológica del aceite esencial (Faleiro et al., 2002). Por otro lado, los porcentajes relativos de los compuestos volátiles del aceite esencial mostraron correlación con la humedad ambiental (Salgueiro et al., 1997).

La variabilidad en la composición química producida por el uso de distintas técnicas extractivas, fue estudiada por Jiménez et al. (1999) observando las diferencias que se producían entre la extracción de agua subcrítica continua y la hidrodestilación.

Bentes et al. (2009), estudiaron la capacidad antioxidante de esta especie y con respecto a los polifenoles de *T. mastichina*, Gordo et al. (2012) identificaron 9 compuestos (sakuranetin, sterubin, ácido oleanólico, ácido usolico, luteolina, *β*-sitosterol, ácido rosmarínico, 6-hidroxiluteolina-7-O-beta-glucopiranósido y hydroxiapigenin-7-O-beta-glucopiranósido) y probaron su actividad anti-cancerígena. Por otra parte, Sánchez-Vioque et al. (2013) y Delgado et al. (2014) estudiaron el residuo obtenido a partir de *T. mastichina* tras el proceso de destilación. La lista de los compuestos principales tentativos identificados por Sánchez-Vioque et al. (2013) con LC-MS en residuos sólidos de *T. mastichina* extraídos por Soxhlet y usando extracción asistida con ultrasonidos fueron: quercetina glucósida, luteolina glucósida, ácido rosmarínico, apigenina-7-O-glucósido, quercetina, derivado de luteolina, naringenina, carnosol, apigenina y kaempferol. Por otro lado, Delgado et al. (2014) encontraron en los extractos metanólicos de los residuos estudiados, ácido rosmarínico como compuesto más abundante seguido de ácido metoxisalicílico, apigenina, luteolina y kaempferol.

11 ÁMBITO DE TRABAJO

11.1 Motivación y finalidad

Las plantas aromáticas y medicinales se han recolectado tradicionalmente en su hábitat natural. Sin embargo, la recolección silvestre no permite la recogida de material vegetal de alta calidad y produce deforestación, amenazando las especies recolectadas. Además, cabe añadir que existen estrictas regulaciones con respecto a los estándares de calidad de los compuestos volátiles. Por otra parte, en el caso de que el material vegetal sea para uso medicinal, la eficacia de las diferentes sustancias activas, tales como polifenoles, y la concentración de los mismos, puede variar de un individuo a otro. La necesidad de encontrar ejemplares de alta calidad y ponerlos en cultivo, no sólo aumenta el rendimiento y la calidad del material vegetal recolectado sino que también permite el desarrollo sostenible y la conservación de la especies. También cabe añadir que como consecuencia de la recolección silvestre, los parámetros de puesta en cultivo de estas especies son en gran parte desconocidos. Para buscar una solución a este conjunto de problemas, la variabilidad química de los metabolitos secundarios y la variación morfológica de *Lavandula latifolia* Medik., *Salvia lavandulifolia* Vahl. L. y *Thymus mastichina* L. se ha evaluado con el fin de encontrar individuos de alta calidad.

Las plantas objeto de estudio parecen tener una amplia variabilidad morfológica y química en su hábitat natural. Esta variación refleja la adaptación de dichas especies a los factores ambientales

locales mostrando el grado de relación entre las condiciones ambientales y los genotipos de las diversas poblaciones, así como también ofrecen información acerca de la estabilidad evolutiva de las especies.

Muchas especies han sido apenas prospectadas porque su análisis requiero mucho tiempo e implica un largo trayecto. Sin embargo, este proceso permite clasificar el germoplasma en grupos y ofrece la posibilidad de seleccionar los especímenes o poblaciones con las características más deseadas por la industria y los consumidores.

El análisis de esta variabilidad es la base necesaria para la domesticación y el cultivo, para la selección de individuos de alto rendimiento y por lo tanto para la explotación comercial.

11.2. Objetivos

El objetivo principal de la tesis se centra en el análisis de la variabilidad en amplio sentido de *Lavandula latifolia* Medikus, *Salvia lavandulifolia* Vahl. y *Thymus mastichina*L. con el fin de seleccionar material vegetal de alta calidad.

Diferentes trabajos de investigación se han llevarán a cabo para alcanzar el objetivo principal:
a) Evaluar y optimizar la metodología necesaria para llevar a cabo el análisis de la variabilidad.
b) Evaluar la variabilidad de los compuestos volátiles de las poblaciones silvestres de *Lavandula latifolia* Medikus, *Salvia lavandulifolia* Vahl. L. y *Thymus mastichina* L.
b) Estudiar los polifenoles y el potencial antioxidante de las poblaciones silvestres de *Lavandula latifolia* Medikus, *Salvia lavandulifolia* Vahl. L. y *Thymus mastichina* L.
c) Examinar la influencia de diferentes factores en la producción de aceites esenciales y polifenoles tales como los factores ambientales, la estacionalidad o la morfología.

11.3. Resumen tesis

La introducción general recoge una visión genérica sobre la producción de plantas aromáticas y medicinales y usos actuales en Castilla y León, en España y en el mundo. También incluye la clasificación de metabolitos secundarios, una breve descripción de las especies de interés y los usos y estudios previos recogidos en la bibliografía de las especies seleccionadas.

En el apartado de motivación y ámbito de trabajo de la tesis, se presentan los objetivos y el esquema del estudio a realizar.

El **Capítulo 1** muestra una comparación del método clásico para la extracción de compuestos volátiles a partir de plantas (hidrodestilación), con otros métodos tales como la micro-destilación y extracción con disolvente orgánico y ultrasonidos. La hidrodestilación es el método utilizado industrialmente, pero requiere una mayor cantidad de material vegetal y un proceso más largo. Con el fin de ver si los métodos son comparables y reducir el tiempo de análisis y la cantidad de material vegetal necesario, se comparó la hidrodestilación con otros dos métodos. El objetivo fue encontrar una metodología adecuada para un estudio más fácil y rápido de material vegetal.

En el **capítulo 2**, la variabilidad de los compuestos volátiles de los aceites esenciales de varias poblaciones silvestres de *L. latifolia*, *S. lavandulifolia* y *T. mastichina* recogido en Castilla y León fue evaluada, estudiando también la influencia geográfica y anual sobre la composición de los aceites esenciales.

Cada año se genera por la industria de la destilería un gran volumen de residuos, lo cual constituye un importante problema medioambiental. La reutilización y revalorización de subproductos puede ayudar a mejorar el rendimiento económico de las industrias de forma respetuosa con el medio ambiente. El **Capítulo 3** incluye un estudio sobre el contenido de antioxidante de varias poblaciones silvestres de *L. latifolia* recolectadas en Castilla y León, así como también incluye el estudio de los polifenoles y capacidad antioxidante de los residuos que se generan después del proceso de destilación, y que podrían ser valorizados por su contenido en antioxidantes.

Una vez que la variabilidad química de las poblaciones silvestres fue evaluada, tres campos experimentales (una para cada especie) se establecieron con el objetivo de estudiar los especímenes y poblaciones bajo las mismas condiciones ambientales. Las poblaciones fueron elegidas tratando de mantener la máxima variabilidad encontrada en las prospecciones silvestres realizadas previamente. La variabilidad intra-población de las poblaciones se mantuvo recolectando 25 plantas por población y multiplicándolas por propagación vegetativa. Los ensayos se establecieron en un diseño de bloques al azar con tres repeticiones.

El objetivo de estos ensayos fue el de someter a todas las poblaciones a las mismas condiciones ambientales y así poder estudiar la variabilidad inter e intra poblacional desde el punto de vista químico y morfológico.

El **Capítulo 4** contiene un estudio de la variabilidad de los polifenoles de 14 poblaciones de *T. mastichina* cultivadas en el ensayo experimental, así como el estudio del potencial antioxidante y la relación con los principales polifenoles de esta especie.

El **Capítulo 5** incluye un estudio de la variabilidad polifenólica de siete poblaciones de *Salvia lavandulifolia* cultivadas en el ensayo d campo.

Con respecto a la necesidad de mejorar los parámetros agronómicos, el **Capítulo 6** incluye un estudio de la morfología de *T. mastichina* analizando la variabilidad morfológica entre individuos y poblaciones.

Las conclusiones presentan los resultados generales.

12. CAPÍTULOS

12.1 Capítulo 1: Volatile fraction differences for Lamiaceae species using different extraction methodologies.

Para lograr una caracterización química detallada, los compuestos volátiles de *Lavandula latifolia*, *Salvia lavandulifolia* y *Thymus mastichina* fueron analizados mediante GC-FID/MS. Un total de 46, 47 y 48 compuestos fueron identificados en *L, latifolia*, *S, lavandulifolia* y *T. mastichina*, respectivamente. 1,8-cineol+limoneno resultó ser el compuesto mayoritario en las tres especies.

Las tres metodologías de extracción utilizadas fueron: hidrodestilación (HD), microdestilación (MD) y extracción con diclorometano (EX). Hidrodestilación es la técnica más común utilizada para la extracción de compuestos volátiles, y es la técnica aceptada por la Farmacopea Europea para comercializar aceites esenciales. Sin embargo, la cantidad de material de una única planta es a veces demasiado pequeña para su hidrodestilación, y la hidrodestilación de un gran número de muestras requiere mucho tiempo. MD y EX son técnicas que necesitan menos tiempo de procesado y menos cantidad de muestra, por lo que pueden ser útiles para ejecutar programas de selección. Los programas de selección son herramientas esenciales para encontrar ejemplares de alto rendimiento que puedasn maximizar el beneficio económico. Para llevar a cabo dichos programas de selección, un gran número de muestras debe ser analizado.

Diferentes métodos de extracción producen generalmente cambios en la composición volátil. Para poder utilizar las técnicas de MD y EX y poder acortar así el tiempo de análisis y el volúmen de muestra, los cambios que se producen en la composición química de las distintas extracciones deben ser estudiados.

La cantidad de compuestos volátiles obtenida se vio afectada por el tipo de método utilizado y por las especies analizadas, es por ello que, *S. lavandulifolia* produjo una mayor cantidad de compuestos con EX, mientras que *T. mastichina* dio lugar a una mayor cantidad de volátiles con MD. Por otra parte, HD produjo una cantidad de compuestos volátiles significativamente menor para las tres especies. Con el método HD se obtuvo una mayor cantidad de *β*-pineno. MD produjo una mayor proporción de 1,8-cineol+limoneno. Se realizó una función de discriminación canónica utilizando los dos compuestos más predecibles en cada método y revelando de este modo las técnicas utilizadas para cada especie.

12.2 Capítulo 2: Variabilidad en la composición del aceite esencial de poblaciones silvestres de Labiadas recolectadas en España

Los aceites esenciales de 11 poblaciones de *Thymus mastichina* (L.) L., 10 poblaciones de *Salvia lavandulifolia* Vahl y 12 poblaciones de *Lavandula latifolia* Medik., recogidos en plena floración en su hábitat natural durante 3 años (2009, 2010 y 2011), fueron analizados por GC-FID/MS con el fin de de estudiar la variabilidad entre las distintas poblaciones, así como también la influencia del año de cosecha en la composición del aceite esencial. Se utilizó ANOVA de un factor para el estudio de la influencia del tipo de población de origen y del año de la cosecha. Además también se realizó un Análisis de Componentes Principales (ACP) en el cual se utilizaron los compuestos principales de las especies de estudio como conjunto de observaciones para cada especie.

Para la especie *T. mastichina* ,todas las muestras fueron quimiotipo 1,8-cineol (58,52%-68,82%), sin embargo, el contenido de linalol mostró un rango de variación importante (1,16 a 10,24%). 1,8-cineol (6,21-33,69%), alcanfor (2,85-22,44%) y *β*-pineno (5,11 a 19,85%) fueron los principales compuestos para *S. lavandulifolia*, mientras que 1,8-cineol (30,57-54,09%) y linalol (15,82-45,94%) lo fueron para los aceites esenciales de *L. latifolia*. Las poblaciones de *T. mastichina* y de *S. lavandulifolia*s e agruparon principalmente en el ACP en función del origen de la población mientras que para *L. latifolia*, las poblaciones no mostraron apenas agrupación poblacional.

T. mastichina fue la especie menos influenciada por los factores ambientales, mostrando principalmente diferencias entre poblaciones. *S. lavandulifolia* mostró pequeñas diferencias entre campañas de recolección mientras que las mayores diferencias se dieron en función del origen de la población. Por el contrario, en *L. latifolia* se observaron mayores diferencias en la composición volátil en función del año de cosecha, aunque también se observó variabilidad genotípica.

En conclusión, la variabilidad de la composición del aceite esencial de las especies estudiadas está principalmente influida por el tipo de población por lo que los factores genéticos tienen un mayor efecto que los factores ambientales. Sin embargo, los factores ambientales también influyen en la composición de los aceites esenciales y por tanto, deben ser tenidos en cuenta.

12.3 Capítulo 3: Subproductos de la destilación de aceites esenciales de *Lavandula latifolia* como fuente de antioxidantes

La industria dedicada a la destilación de aceites esenciales genera cada año un gran volumen de residuos, lo cual es un importante problema ambiental. La reutilización y revalorización de estos subproductos pueden ayudar a mejorar el rendimiento económico de las industrias de forma respetuosa con el medio ambiente.

El objetivo de este trabajo fue evaluar las propiedades antioxidantes de los residuos de *Lavandula latifolia* obtenidos después de destilación del aceite esencial. Las muestras de 12 poblaciones silvestres de *Lavandula latifolia*, fueron recogidas durante 2009 y 2010, fueron hidrodestiladas y extracciones metanólilcas de sus subproductos fueron analizados utilizando el método de Folin-Ciocalteu (contenido en fenoles totales), el método de captura de radicales libres (DPPH) y el método del poder antioxidante reductor de hierro (FRAP). Ácido rosmarínico, apigenina y luteolina fueron identificados y cuantificados por cromatografía de líquidos (HPLC) con detector Diodo Array (DAD) con el objetivo de correlacionar el contenido en dichos polifenoles con la capacidad antioxidante del residuo.

La media del contenido de fenoles totales osciló de 1,89±0,09 mg equivalentes de ácido gálico/g de peso seco hasta 3,54±0,22 mg equivalentes de ácido gálico/g de peso seco. El valor medio de la concentración activa de sustancia que causó el 50% de la respuesta máxima (EC_{50}) para el método DPPH varió desde 5,09±0,17 mg/mL hasta 14,30±1,90 mg/mL y la variabilidad de la EC_{50} en FRAP osciló de 3,72±0,12 mg/mL hasta 18,55±0,77 mg/mL. Como resultado se obtuvo que la variabilidad de las muestras fue en parte debida a la variación anual, encontrando

que las condiciones ambientales de 2009 fueron más favorables respecto a la producción de antioxidantes. Las plantas recogidas en Sedano mostraron el mayor poder antioxidante. Los resultados mostraron que el ácido rosmarínico y la apigenina contribuyeron a las propiedades antioxidantes de los residuos de *L. latifolia*. En conclusión, el subproducto de la industria destiladora podría valorizarse como fuente de antioxidantes naturales.

12.4 Capítulo 4: Contribución de los principales polifenoles del *Thymus mastichina* subsp. *mastichina*a sus propiedades antioxidantes.

La actividad antioxidante y el contenido de polifenoles de muestras procedentes de plantas individuales pertenecientes a 14 poblaciones de *Thymus mastichina* cultivadas en una parcela experimental, fueron analizadas por los métodos de DPPH, FRAP y Folin-Ciocalteu para definir su actividad antioxidante. Además, los polifenoles fueron analizados por HPLC/DAD y se estableció una relación entre polifenoles y capacidad antioxidante. A lo mejor podrías incluir mínimamente el análisis stad´sitico que se hizo

La capacidad de captación de radicales libres (DPPH) varió para las distintas poblaciones desde 44 hasta 98 mg equivalentes de Trolox (TE)/g de materia seca (MS), mientras que el poder eductor (FRAP) osciló de 52 a115 mg TE/g MS. El contenido de fenoles totales varió entre 11 y 38 mg de equivalentes de ácido cafeico (CAE)/g MS para las diferentes poblaciones. Los polifenoles identificados fueron: ácido clorogénico, ácido cafeico, ácido rosmarínico, glucósido de luteolina y luteolina. Los principales polifenoles fueron el ácido rosmarínico, que osciló de 1,7 hasta 43 mg/g MS, un polifenol no identificado designado como Pico 3 (0,53-15 mg equivalentes de luteolina (LE)/ g MS) y el glucósido de luteolina que varió desde 0,96 hasta 19 mg LE/g. El ácido rosmarínico contribuyó principalmente alpoder reductor FRAP y a los fenoles totales, mientras que el pico 3 contribuyó principalmente a la capacidad de reducción de radicales libres DPPH. La luteolina, el ácido clorogénico y el ácido cafeico presentaron un rango de variación de 0–2,7; 0,07–2,2; 0–0,46 mg/g MS, respectivamente. El estudio mostró una alta variabilidad intra-poblacional y sobre todo una gran variabilidad inter-poblacional. La población de Carrocera tuvo la mayor actividad antioxidante y el mayor contenido de fenoles por lo que podría ser seleccionada entre las demás como fuente de antioxidantes naturales.

12.5 Capítulo 5: Variabilidad en el perfil polifenólico de *Salvia lavandulifolia* Vahl.

Salvia lavandulifolia Vahl. es un arbusto perenne perteneciente a la familia Lamiaceae caracteristico del oeste mediterránea y dividida en cinco subespecies: lavandulifolia, oxyodon, blancoana, vellerea y mariolensis (Saéz et al., 2010). En medicina popular y fitoterapia esta especie se utiliza por sus efectos espasmolíticos, actividad antibacteriana (Baricevic y Bartol, 2000), propiedades hipoglucemiantes (Jiménez et al., 1986.; Zarzuelo et al, 1990) (Cabo J et al., 1986) además de tener eficacia en el tratamiento de la enfermedad de Alzheimer (Perry et al., 2001) por ser un potenciador de la memoria (Tildesley et al., 2003) debido a la inhibición de la enzima acetilcolinesterasa.

A diferencia de los numerosos estudios sobre los aceites esenciales de *S. lavandulifolia* (Herraiz-Peñalver et al, 2010;.. Porres-Martínez et al, 2014; Usano-Alemany et al, 2014A;.. Usano-Alemany et al, 2014b), existe muy poca y antigua bibliografía sobre la variabilidad polifenólica de esta especie (Adzet et al, 1988;.. Cañigueral et al., 1989; Escudero et al, 1983).Sin embargo *S. lavandulifolia* presenta la ventaja de no tener tuyonas o tener un bajo ccontenido (<2%) de α y β-thujones (Mathe, et al. 2006; Guillen, et al. 1996; Herraiz-Peñalver et al, 2010; Perry et al, 2000). De modo que su ingesta como infusión o para su uso medicinal podría ser más benificioso debido a la toxicidad que presentan las tuyonas.

El perfil de polifenoles de 86 muestras pertenecientes a 7 poblaciones españolas de *S. lavandulifolia*cosechadas en un ensayo experimental, establecido a partir de poblaciones silvestres, fue estudiado por cromatografía líquida de alta eficacia de fase reversa con detector Diodo Array (HPLC-DAD).

El ácido rosmarínico (6-43 mg/g) y la luteolina-7-O-glucósido (0-36 mg/g de materia seca (MS)) resultaron ser los compuestos principales de dichas muestras. Ácido clorogénico (0,08 a 1,7 mg/g MS), ácido cafeico (0,11 a 1,2 mg/g MS), apigenina-7-O-glucósido (0,08 a 2,3 mg /g MS), luteolina (0 a 0,92 mg/g MS), genkwanin (0-5,5 mg/g MS), ácido betulínico (0-3,5 mg equivalentes de ácido rosmarínico (RAE)/g MS) y ácido carnósico (0-0,7 mg RAE/g MS) también fueron identificados.

Los resultados fueron analizados mediante una discriminación canónica y la figura resultante mostró que todas las poblaciones presentaron perfiles fenólicos característicos. El análisis de varianza (ANOVA) demostró que la variabilidad entre poblaciones fue siempre mayor que la

variabilidad dentro de las poblaciones y algunos compuestos resultaron ser característicos de cada población y por tanto relacionados con su origen geográfico.

La capacidad antioxidante fue analizada mediante tres metodologías: el contenido en fenoles totales fue analizado a través del método de Folin-Ciocalteu, el método de captación de radicales libres (DPPH) y el método del poder antioxidante reductor de hierro (FRAP), con el objetivo de correlacionarlos con los perfiles polifenólicos de las poblaciones. Los distintos coeficientes de correlación de Pearson entre polifenoles detectados y metodologías colorimétricas mostraron que los compuestos contribuyeron de manera diferente a las propiedades antioxidantes de la especie.

12.6 Capitulo 6: Variabilidad de características morfológicas de poblaciones de *Thymus mastichina*

Thymus mastichina (L.) L. es una especie endémica de la Península Ibérica y una de las especies más recolectadas en España de forma silvestre para su uso industrial. A pesar de la gran cantidad de posibilidades para el uso de *T. mastichina* subsp. *mastichina* como cultivo industrial, el cultivo está casi ausente y no se ha realizado ninguna selección de material vegetal. Para identificar los caracteres más útiles y discriminatorios y para estudiar la variabilidad morfológica de mejorana española, se han estudiado un total de 16 poblaciones recolectadas en 2011 y 2012 cualtivadas en una parcela experimental. Se han estudiado un total de 20 características morfológicas (15 cuantitativas y 5 cualitativas) las cuales fueron utilizadas para su caracterización. Un análisis de varianza, un análisis de componentes principales y correlaciones entre los caracteres morfológicos, así como un análisis cluster se han llevado a cabo para el análisis de los datos.

La variabilidad intra e inter-poblacional fueron estadísticamente muy significativas para todos los caracteres cuantitativos, revelando que la especie cuenta con un gran polimorfismo. Por otra parte, la variabilidad entre las poblaciones fue mayor que la variabilidad dentro de las poblaciones mostrando que las poblaciones cuentan con una distinción morfológica entre ellas. El número de flores del capítulo, el número de capítulos por rama, la relación longitud/anchura de la bráctea, y la anchura de las brácteas fueron los caracteres que más variación presentaron. Los rasgos más valiosos para la caracterización fueron los relacionados con la forma y tamaño de las brácteas, hojas y capítulos. Los rangos de variación morfológica de los ejemplares cultivados objeto de este estudio, fueron comparados con especímenes silvestres cuyos datos están recogidos en bibliografía, mostrandoque las hojas de especímenes cultivados, las brácteas

y las inflorescencias son ligeramente más grandes que los de especímenes silvestres y además los ejemplares cultivados mostraron menores tamaños de cálices y corolas. El el análisis de cluster ordenó las poblaciones en cuatro grupos. Las poblaciones del grupo tres (CLM4, TM23 y TM43) destacaron por tener unos capítulos más grandes por lo que podrían ser más productivos para la extracción de aceite esencial dado que las flores contienen mayor cantidad de aceite esencial que las hojas.

13. CONCLUSIONES

Como conclusión general, este estudio demuestra que existe una alta variabilidad en las especies estudiadas. Esta variabilidad es diferente dependiendo de la especie y de las variables analizadas. De este modo, se ha observado que la variabilidad encontrada en SL es mayor que la variabilidad encontrada en TM y LL. Debido a la existencia de esta gran variabilidad, queda demostrado por tanto, la posibilidad de seleccionar organismos con características y cualidades de alta calidad que permitan aumentar la producción y que puedan ser comercializadas.

En primer lugar, para llevar a cabo este estudio, el **capítulo 1** muestra una comparación de diferentes métodos de extracción y su idoneidad para cada especie. Dependiendo de la especie estudiada, el uso de distintas metodologías dio lugar a resultados diferentes en la composición de la fracción volátil. TM y LL mostraron pocas diferencias entre las metodologías de extracción, mientras que en SL se observaron diferencias importantes al utilizar el método EX. Algunos compuestos volátiles no mostraron diferencias significativas entre los distintos métodos utilizados mientras que otros mostraron un mayor rendimiento con una metodología concreta.

En el **capítulo 2** se evaluaron poblaciones silvestres, encontrando que los factores genéticos fueron más importantes en TM y SL que en LL. Este estudio concluye que la mayor parte de la variabilidad encontrada en la composición de volátiles del aceite esencial está relacionada con el componente genético propio de cada especie, lo cual va a permitir seleccionar material vegetal con propiedades idóneas para la industria.

En cuanto a la variabilidad encontrada en el perfil polifenólico y en la capacidad antioxidante, el genotipo es también un factor determinante. En la evaluación de estas características sobre poblaciones silvestres de espliego (**capítulo 3**) queda patente que unas poblaciones presentan mejores características antioxidantes que otras. De igual forma, en el estudio de poblaciones cultivadas de TM (**capítulo 4**) y SL (**capítulo 5**) se observa que el perfil polifenólico es

característico de cada población, así como las propiedades antioxidantes que este perfil les confiere. Al mismo tiempo, queda demostrado en estos dos capítulos que la variabilidad intrapoblacional es también importante, y un factor a tener en cuenta en los futuros procesos de selección necesarios para domesticar estas especies.

Por otro lado, los resultados obtenidos en el **capítulo 3**, destacan la posibilidad de que el residuo hidrodestilado pueda ser revalorizado y utilizado como fuente de antioxidantes naturales.

Se estudió también la influencia de los factores ambientales sobre la variabilidad de estas especies. En los capítulos 2 y 3 se estudió el efecto del año de recolección sobre la composición química del aceite esencial (**capítulo 2**) y sobre las propiedades antioxidantes y el contenido en ciertos polifenoles (**capítulo 3**), encontrando que, aunque en menor proporción que la componente genética, los factores ambientales también tienen efecto sobre la composición química de estas especies, y que las diferentes poblaciones no responden de la misma forma a las variaciones anuales. Estos resultados ponen de manifiesto la necesidad de tener en cuenta la influencia ambiental en el proceso de selección de estas plantas.

Por último, la morfología también presentó una variabilidad importante como se muestra en el **capítulo 6**. En el proceso de domesticación de las especies, la diversidad genética entre germoplasma juega el papel más importante, ya que abre el camino para seleccionar los mejores ejemplares. La variabilidad fenotípica de las distintas poblaciones puede tener consecuencias importantes sobre la biomasa y la producción de aceite esencial. Este estudio concluyó que los caracteres morfológicos que presentaron mayor variabilidad en *Thymus mastichina* están relacionadas con el tamaño de las hojas, las brácteas y el capitulo. Estos caracteres influyen en el desarrollo de la biomasa y la producción de aceite esencial. Los programas de selección genética deben buscar especímenes con ramas, hojas, brácteas, flores y capítulos más grandes. Por otro lado, el tamaño de la hoja no está relacionado con el tamaño del capítulo, de modo que sería posible obtener plantas con hojas y capítulos más grandes. En vista de la alta recolección silvestre de esta especie vegetal en particular, el establecimiento de cultivos de *T. mastichina* en España podría ayudar con el desarrollo sostenible de esta especie promoviendo su conservación.

Annexed I: Lamiaceae photography's.

a.1) b.1) c.1) d.1) e.1) f.1)

a.2) b.2) c.2) d.2) e.2) f.2) g.2)

a.3) b.3) c.3) d.3) e.3) f.3)

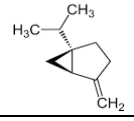
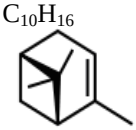
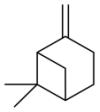
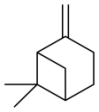
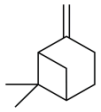
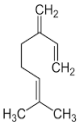
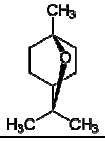
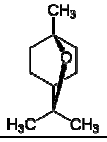
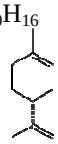
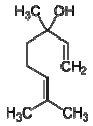
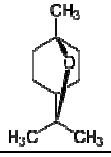
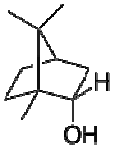
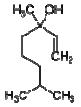
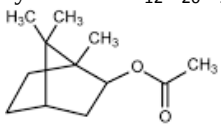
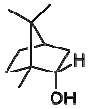
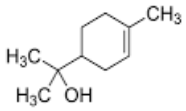
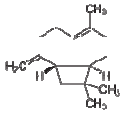
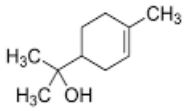
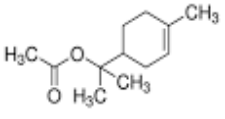
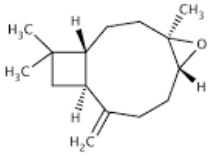
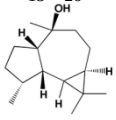
a.1): *Thymus mastichina* inflorescence, b1): *T. mastichina* leaves, c1) Samples of *T. mastichina* with yellow stems, d1) Samples of *T. mastichina* with red stems, e1) and f1) seeds of *T. mastichina*.

a2): *Salvia lavandulifolia* inflorescence, b.2): *S. lavandulifolia* tetrachaenium detail, c.2): *Salvia lavandulifolia* verticilaster, d.2): *S. lavandulifolia* leaves, e.2): *S. lavandulifolia* bracts, f.2): *S. lavandulifolia* inflorescence, g.2): *S. lavandulifolia* seeds.

a.3): *Lavandula latifolia* inflorescence, b.3) and c3): *L. latifolia* leaves, d.3) *L. latifolia* verticilaster, e.3): *L. latifolia* shrub, f.3): *L. latifolia* seeds.

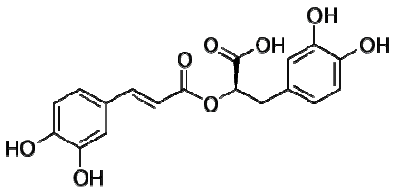
Anex. 2 Structural formula of the secondary metabolites studied

a. Volatiles compounds

Thymus mastichina	*Salvia lavandulifolia*	*Lavandula latifolia*
sabinene $C_{10}H_{16}$ 	Camphene $C_{10}H_{16}$ 	*α*-pinene $C_{10}H_{16}$ 
β-pinene $C_{10}H_{16}$ 	*β*-pinene $C_{10}H_{16}$ 	*β*-pinene $C_{10}H_{16}$ 
Myrcene $C_{10}H_{16}$ 	1,8-cineol $C_{10}H_{18}O$ 	1,8-cineol $C_{10}H_{18}O$ 
limonene $C_{10}H_{16}$ 	camphor $C_{10}H_{16}O$ 	linalool $C_{10}H_{18}O$ 
1,8-cineol $C_{10}H_{18}O$ 	borneol $C_{10}H_{18}O$ 	Camphor $C_{10}H_{16}O$ 
linalool $C_{10}H_{18}O$ 	isobornyl acetate $C_{12}H_{20}O_2$ 	borneol $C_{10}H_{18}O$ 
α-terpineol $C_{10}H_{18}O$ 	β-caryophyllene $C_{15}H_{24}$ 	α-terpineol $C_{10}H_{18}O$ 
α-terpinyl acetate $C_{12}H_{20}O_2$ 	caryophyllene oxide $C_{15}H_{24}O$ 	
	viridiflorol $C_{15}H_{26}O$ 	

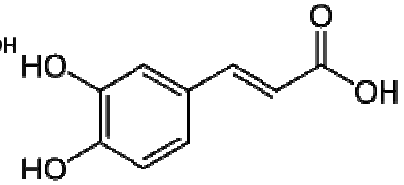
b. Main polyphenols

b1) Phenols



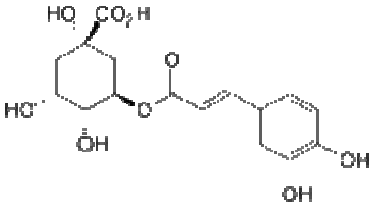
Rosmarinic acid

$C_{18}H_{16}O8$



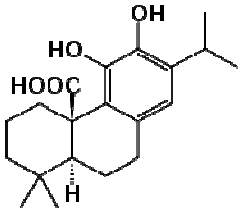
Ácido cafeico

$C_9H_8O_4$



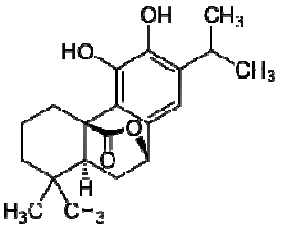
Ácido clorogénico

$C_{16}H_{18}O_9$



Carnosic acid

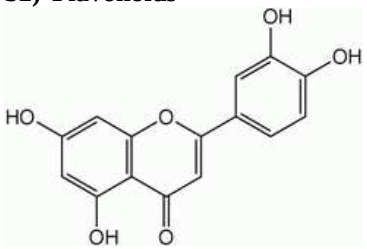
$C_{20}H_{28}O_4$



Carnosol

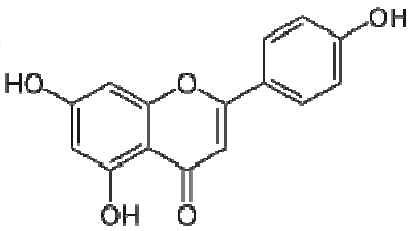
$C_{20}H_{26}O_4$

b2) Flavonoids



Luteolina

$C_{15}H_{10}O_6$



Apigenina

$C_{15}H_{10}$

ACKNOWLEDGEMENT

Aunque una tesis la entrega una sola persona, es en realidad la suma del trabajo de muchas personas. Es por ello que quiero agradecer a todos los que han colaborado aportando su ayuda, su tiempo y su conocimiento.

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Gracias a Mona Scharft por su amistad y su compañía durante mis 3 estancias en Viena y a Arianna de Mori por los buenos recuerdos que me trae la última estancia "*Combattiamo all'infinito. Combattiamo senza curarci di quanto ci costa, delle sconfitte che incassiamo, dell'improbabilità del successo. Combattiamo fino all'ultimo respiro. Non è una questione di coraggio. L'incapacità di arrendersi è un dato caratteriale. Forse è semplicemente stupida fame di vita*" Yann Martel (La vita di Pi).

Muchas gracias a Barbara Knowles por haber leido pacientemente todos los documentos y haberme aconsejado con la gramática.