universidad de león

ESCUELA SUPERIOR Y TÉCNICA DE INGENIERÍA AGRARIA

INGENIERÍA DE BIOSISTEMAS

Characterization of wild and centenarian olive trees for their valorisation

Doctoral Thesis

Nuno Miguel de Sousa Rodrigues

Directors:

Professor Doutor José Alberto Cardoso Pereira

Professora Doutora Paula Cristina Santos Baptista

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Aos meus pais

À minha irmã

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Recomeça…

Se puderes,

Sem angústia e sem pressa.

E os passos que deres,

Nesse caminho duro

Do futuro,

Dá-os em liberdade.

Enquanto não alcances

Não descanses.

De nenhum fruto queiras só metade.

E, nunca saciado,

Vai colhendo ilusões sucessivas no pomar.

Sempre a sonhar e vendo

O logro da aventura.

És homem, não te esqueças!

Só é tua a loucura

Onde, com lucidez, te reconheças...

"Miguel Torga, Diário XIII"

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Abstract

The species *Olea europaea* L. is constituted by two varieties, the cultivated one - the olive tree, *O. europaea* subsp. *europaea* var. *europaea*; and the wild form - the wild olive tree or oleaster, *O. europaea* subsp. *europaea* var. *sylvestris*. These varieties were differentiated during the domestication process, and from the cultivated form a high number of cultivars evolved over the years. In the last decades, due to the crop intensification a few number of well adapted or productive cultivars have been used in the new olive plantations, leading to the abandonment of minor cultivars and to a loss of the olive genetic heritage. Nevertheless, in the last years, a niche of more informed and demanding consumers appeared, searching for olive oils that combine their richness in health promoters, like polyphenols, tocopherols and sterols, with a differentiated sensory profile. In this context, the general objective of this work was to characterize genetically and morphologically oleander populations and centenarian olive trees from the northeast of Portugal, as well as, to chemically and sensory evaluate the extracted oils, aiming the selection of plant specimens with the purpose of future valorization. Three oleander populations from Alijó, Moncorvo and Vila Nova de Foz Côa (VNFC) regions and 28 specimens of centenarian olive trees, grown in Mirandela region (Suções), some of them from known cultivars (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) were characterized.

The comparison of genetic diversity, structure and phylogenetic relationships within and among the oleaster and centenarian plants were studied using microsatellite markers. High genetic diversity was observed in both oleaster and centenarian plants, with no differences between them in the amount of genetic diversity. Population structure analysis suggests genetic differentiation between the studied varieties.

The oils of the three oleaster populations were evaluated considering their fatty acids, tocopherols, phenols and sterols composition. Globally, the chemical composition was very similar to that of olive oils and fulfilled the legal limits for virgin olive oil classification. Oleic (68.9-70.6%) was the main fatty acid, followed by palmitic (14.2-14.7%) and linoleic (7.87-9.88%). Tocopherol ranged between 263 and 503 mg/kg oil, being α-tocopherol the most representative (higher than 90%). High levels of total sterols were observed, from 1742 to 2198 mg/kg of oil, with a profile similar to olive oil. The contents of phenolic compounds were greater than 603 mg/kg and 14 compounds were identified. Ligstroside derivatives and oleuropein aglycon (and derivatives) were the most abundant ones. This work demonstrated that the chemical composition allowed

discriminating the different oil populations, showing that oleaster oils could be valorized, being a good source of bioactive compounds.

Also, in order to select specimens as potential producers of differentiated olive oils with high levels of phenolic compounds, and so, as possible candidates for multiplication or breeding programs, 28 centenarian trees were studied during four crop years (2014-2017). Thirteen phenolic compounds were identified and quantified, being hydroxytyrosol and tyrosol secoiridoids the predominant ones. Fifty per cent of the evaluated trees produced olive oils that could be labeled as "*Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress*". In particular, olive oils produced from trees nº24, 25 and 26 had, consistently, high phenolic contents, during the four studied crop years, being, therefore, good candidates to be used in breeding programs as producers of olive oils rich in phenolic compounds.

Six minor autochthonous olive cultivars produced from centenarian trees were also studied and characterized, aiming the selection cultivars that allowed producing differentiated olive oils. In this context, the quality parameters, sensory profile and oxidative stability were evaluated for two crop years (2016 and 2017); and, tocopherols contents and fatty acids composition, were determined during five consecutive crop years (2013-2017). All the oils produced were classified as Extra Virgin Olive Oils. Oils obtained from *cv*. Redondal presented the highest oxidative stability (OS), total phenols contents, and $C_{18:1}/C_{18:2}$ ratios. In contrast, *cv*. Madural oils presented the lowest levels of OS and $C_{18:1}/C_{18:2}$ ratios, strengthen the role of fatty acids levels in the OS. Finally, oils from *cv*. Verdeal had the lowest levels of total phenols. Regarding the sensory evaluation, the usual sensory notes of tomato, apple, dried fruit, fresh herbs, tomato leaves and cabbage were predominant in most cultivars, nevertheless some attributes were specific like banana and kiwi (*cv.* Madural) as well as cherry and apricot (*cvs.* Lentisca and Madural). The amounts of tocopherols, considering all years, followed the order *cvs*. Lentisca (456 mg/kg olive oil)≈ Redondal (404 mg/kg olive oil) > Madural (311 mg/kg olive oil) ≈ Rebolã (269 mg/kg olive oil) > Verdeal Transmontana (206 mg/kg olive oil) ≈ Verdeal (179mg/kg olive oil). Concerning the fatty acids profile, monounsaturated fatty acids allowed distinguishing different groups taking into account their relative abundance: *cvs.* Redondal (82.1%) ≈ Verdeal Transmontana (81.7%) > Lentisca (79.1%) > Verdeal (77.4%) > Rebolã (74.3%) > Madural (71.2%). The obtained results pointed out that, the oils extracted from the different cultivars, had a high consistent composition. Using different statistical tools, namely principal component analysis, discriminant analysis and hierarchical grouping analysis, it was possible to discriminate olive oil according to the cultivar and the crop

year. *Cvs.* Redondal and Lentisca showed the best results for the main evaluated parameters. Finally, the results obtained contributed to enhance the scarce knowledge of olive heritage, and may be further used to support the selection of olive cultivars for new plantations, based on their potential to produce oils with a more favorable chemical, sensory and bioactive profile, in which concerns the nutritional and oil quality points of view.

Keywords: Olive heritage, genetic, morphological and chemical characterization, sensory attributes, monovarietal olive oils, crop year influence.

Resumen

El olivo (*Olea europea* L.) es un árbol perenne que pertenece a la familia Oleaceae. Fue una de las primeras plantas domesticadas (Laaribi et al., 2017). Su cultivo se inició en Palestina hace cerca de 6000 años. Posteriormente, se dispersó por el norte de África y el Mediterráneo. En el último siglo, el olivo fue introducido en otros países sin tradición en la producción o el consumo de aceite donde el clima es muy semejante al clima Mediterráneo.

El olivo es el cultivo más ampliamente cultivado en el mundo. Ocupa actualmente aproximadamente 10,6 millones de hectáreas ((FAO, 2016), donde son producidas alrededor de 28,9 millones de toneladas de aceitunas (COI, 2018). Durante el proceso de domesticación no todas las plantas de adaptaron apropiadamente, lo que dio origen a dos variedades de *O. europea*, la forma cultivada – olivo (*O. europaea* subsp. *europaea* var. *europaea*), y la forma salvaje – acebuche u olivo salvaje (*O. europaea* subsp. *europaea* var *sylvestris*) (Besnard & Bervillé, 2000; Breton et al., 2008; Hannachi et al., 2013).

El acebuche presenta frecuentemente biotipo arbustivo o de pequeño árbol, pudiendo alcanzar los 15 metros de altura con grandes troncos. Esta planta no es cultivada porque sus características morfológicas no son favorables para la obtención de suficiente rentabilidad económica dado que presenta frutos pequeños, baja cantidad de grasa y un estado juvenil prolongado (Terral, & Arnold-Simard, 1996; Bouarroudj et al., 2016). A pesar de tratarse de una planta no cultivada ha sido utilizada en diversos estudios dado que puede desempeñar un papel muy importante en el desarrollo y selección de nuevos cultivares de olivo con calidad superior de aceite, además de a su alta adaptabilidad y supervivencia (Baccouri et al., 2010), así como a su rusticidad contra plagas y enfermedades. En Portugal, el acebuche se distribuye a lo largo de todo el país, principalmente en las regiones del centro y sur. Es una especie que vive en todos los tipos de suelos, especialmente en suelos básicos. Se desarrolla en lugares con buena exposición solar, rocosos y secos (Porto et al., 2018). En la región norte, a pesar de que el olivo es más abundante, también aparecen algunos ejemplares de acebuche en áreas próximas a ríos importantes como el Duero, el Sabor o el Côa. En el pasado, el aceite obtenido a partir de frutos de acebuche fue utilizado con fines medicinales, cosméticos y religiosos (Chiappetta et al., 2017) siendo actualmente objeto de estudio dado que la información sobre sus propiedades nutricionales es escasa (Belarbi et al., 2011). Se sabe apenas que el aceite de acebuche, similarmente a los aceites de oliva vírgenes, posee grandes cantidades de algunos compuestos menores como esteroles, compuestos fenólicos y tocoferoles (Dabbou et al., 2011).

El olivo en su forma cultivada, resultó de una selección de genotipos que poseen características agronómicas deseables como son frutos de tamaño grande y/o alta cantidad de grasa y fácil propagación vegetativa, ya sea directamente a través de estacas o a través de injertos en individuos silvestres (Chiappetta et al., 2015). Estos procesos de selección dieron lugar a los cultivares actuales de olivo. Su fruto, la aceituna, es una drupa compuesta por tres tejidos: el epicarpio (película), el mesocarpio (pulpa) y el endocarpio (hueso). El mesocarpio representa cerca del 70-90% del peso total del fruto, siendo que su tamaño depende fundamentalmente del cultivar, mientras que el hueso puede originar una variación del 10 al 30% del peso del fruto (Galanakis, 2011; Ghanbari et al., 2012; Peres et al., 2011). Otras características de la aceituna son su baja cantidad de azúcar (3,5-6,0%) y elevada cantidad de aceite acumulado durante la maduración (cantidad de aceite de 14-30%), una cantidad de proteínas en torno a 1,6 a 3,0%, siendo el resto constituido por fibra y minerales (Conde et al., 2008; Rallo et al., 2018). El aceite es extraído de las aceitunas frescas apenas por procesos mecánicos y físicos (molienda, batido y centrifugado) a temperaturas controladas y sin uso de solventes, lo que permite mantener algunos compuestos existentes en el fruto y que son de gran importancia como los compuestos fenólicos, tocoferoles, esteroles, pigmentos, etc. (Visioli, & Bernardini, 2013; Tsimidou, & Boskou, 2015). Es constituido mayoritariamente por ácidos grasos, siendo los principales el ácido oleico (55-83%), seguido por el ácido linoleico (3,5 a 21%), ácido palmítico (7,5 a 20%), ácido esteárico (0,5-5,0%) y el ácido linolénico (≤1%) (Reglamento de la Comisión Europea (CEE) 2568/91). Los ácidos grasos no se encuentran libres, sino esterificados con una molécula de glicerol, formando triglicéridos. El triglicérido más abundante es la trioleína, con valores entre 40-59%, seguido por linoleodioleína 12,5-20% y la palmitodioleína 12-20% (Boskou, 1996). En cuanto a los esteroles, el más abundante presente en el aceite de oliva es el β-sitosterol (≥ 93.0%), seguido del campesterol (≤ 4.0%) y el Δ-7-stigmastenol (≤ 0.5%) (Reglamento de la Comisión Europea (CEE) 2568/91 para la categoría de Aceite Virgen Extra). Los polifenoles son compuestos minoritarios pero que desempeñan un papel muy importante como preservadores del aceite de oliva y de la salud humana. Varios estudios muestran correlaciones positivas de la ingestión diaria de compuestos fenólicos en la salud. Su cantidad oscila generalmente entre 250 y 800 mg/kg de aceite de oliva donde los compuestos más abundantes son derivados de la oleuropeína y de los ligstrosídeos (Veneziani et al., 2018).En relación a los tocoferoles podemos encontrar vitámeros como α, β, γ y δ. Sus cantidades medias en el aceite de oliva son de 84 a 463 mg/kg de aceite. El α-tocoferol es el más abundante representando más del 90% de la cantidad total de tocoferoles (Beltrán et al., 2010).

El conocimiento acerca del patrimonio es escaso, sobre todo en lo que se refiere a cultivares minoritarios de expresión regional o local. Algunos de estos cultivares producen aceites de oliva distintos a nivel de la composición físico-química o a nivel sensorial. En ellos se pueden destacar notas de albaricoque, cereza verde, brócoli, piña, kiwi entre otras, que tornan el aceite de oliva más rico y diferenciado. El conocimiento de este patrimonio puede suponer un importante valor añadido, tanto para las poblaciones locales como para el consumidor dada su composición diferenciada y rica en compuestos benéficos para la salud y agradables para el olfato y al paladar.

En este sentido, este trabajo tuvo como objetivo general proceder a la caracterización de algunos ejemplares de olivos centenarios de la región de Trás-os-Montes y poblaciones de acebuche desde el punto de vista genético y físico-químico de los aceites de oliva extraídos de sus frutos durante diferentes años de producción. La información obtenida puede contribuir para la obtención de productos diferenciados y para la preservación y valorización de este rico patrimonio genético de la región.

Diseño experimental

La parte experimental del presente trabajo fue realizada de 2013 a 2018. El muestreo se realizó en diferentes localidades del Nordeste de Portugal. La zona de muestreo para el estudio de los olivos centenarios (*Olea europaea* ssp. *europaea* var. *europaea*) se sitúa cerca de Mirandela (Suções, N 41º 29 '26.628''; W 7º 15' 31.219'') en un olivar centenario (aproximadamente 250 años). Este olivar fue seleccionado debido a que sus árboles son de los más antiguos en la región y pertenecen a cultivares diferentes, siendo la mayoría desconocidos. De un total de 140 árboles que integran el olivar, se seleccionaron y marcaron individualmente 28 árboles. Los árboles se seleccionaron en función de su apariencia, estructura y espesura del tronco, con el objetivo de seleccionar los más antiguos y representativos de la diversidad del olivar. Las zonas de muestreo para el estudio de las poblaciones de acebuche (*O. europaea* subsp. *europaea* var. *sylvestris*) están localizadas en tres municipios diferentes del nordeste de Portugal: Moncorvo (N 41º 11 '57.498''; W 7º 5' 46.302''), Vila Nova de Foz Côa (N 41º 8'6.058''; W 7º 7'54.005'') y Alijó (N 41º 12'7.045''; W 7º 29'25.444''). En cada zona se seleccionaron aleatoriamente cuatro plantas diferentes de acebuche para el muestreo.

Muestreo

Caracterización morfológica y genética

De cada planta marcada (olivo y acebuche) se muestrearon dos tipos de muestras.

Para la caracterización genética fue seleccionada una rama joven sin señales de enfermedad o ataque de plagas por planta. En el laboratorio, se seleccionaron aleatoriamente diez hojas jóvenes de cada rama/planta, molidas en polvo fino en nitrógeno líquido y almacenadas a -80 °C hasta la extracción del ADN. La descripción del procedimiento usado se encuentra detallada en la sección de materiales y métodos del Capítulo 4.

Para la caracterización morfológica se seleccionó una muestra de aproximadamente 500 g de frutos de cada planta con un índice de maduración (IM) entre dos (IM 2) y tres (IM3), definidos conforme el color de la película. Los frutos con IM2 presentan machas rojas o violáceas en menos de la mitad de la aceituna, mientras que los frutos en que las manchas ocupan más de mitad de la aceituna se consideran IM3. De cada muestra se seleccionaron aleatoriamente 40 frutos. Estos fueron caracterizados morfológicamente de acuerdo con la norma de la Unión Internacional para la Protección de Nuevas Variedades Vegetales (UPOV) para cultivares de olivo (UPOV, 2011). Todas las muestras de frutos se caracterizaron usando parámetros biométricos aplicados al fruto y al hueso del mismo fruto. Una descripción más detallada del procedimiento usado se puede encontrar en el material y métodos del Capítulo 5.

Caracterización química

A lo largo de cinco años consecutivos de producción (2013 a 2017) se muestrearon aceitunas de cada uno de los 28 árboles referidos anteriormente. De cada árbol, se recogieron aproximadamente tres kilos de frutos sanos, entre IM 2 y IM3. Cada año el muestreo se realizó durante el mes de noviembre en los días 25 y 26 (2013), 10 y 11 (2014), 2 y 3 (2015), 07 y 08 (2016), y finalmente 13 y 14 (2017).

Fueron también muestreados cerca de dos kilos de frutos de acebuche el 10 y 11 de noviembre de 2016, de cuatro plantas por población.

Extracción de aceite

Para la extracción del aceite, los frutos fueron procesadas durante las primeras 24 horas tras la recolección. Fue realizada en una planta piloto de extracción Abencor (Comercial Abengoa SA, Sevilha, Espanha) con tres unidades principales: un molino, una termobatidora en la que el batido es realizado a temperatura controlada, y una centrífuga.

Los frutos fueron molidos formando una pasta homogénea. Se pesaron cerca de 700 g que fueron transferidos para un recipiente y batidos en la termobatidora (20 min) en un baño de agua a 25 ºC. Durante los 5 min finales de cada batido se adicionaron 100 mL de agua a 25 ºC para mejorar la separación del aceite. La mezcla fue centrifugada, decantada y el aceite recogido. A continuación, los aceites fueron preparados para análisis, filtrados con un papel de filtro (Whatman nº 4) en presencia de sulfato de sodio anhidro para eliminar las partículas sólidas y el agua residual. Los aceites se almacenaron en frascos oscuros de 125 mL, protegidos de la exposición lumínica y a temperatura ambiente.

Determinación de los aceites extraídos

Las muestras de aceite extraídas de los frutos de olivos centenarios y de acebuche se sometieron a diferentes análisis. En general, todos los ensayos se realizaron en triplicado en el plazo de dos meses después de la extracción. Los siguientes parámetros fueron evaluados:

- Compuestos fenólicos: aceites de olivos centenarios de los años 2014 y 2017 y aceite de acebuche.
- Composición de ácidos grasos: aceites de olivos centenarios de los años 2013 a 2017 y aceite de acebuche.
- Composición de tocoferoles: aceites de olivos centenarios de los años 2013 a 2017 y aceite de acebuche.
- Parámetros de calidad: aceites de olivos centenarios de los años 2016 y 2017.
- Análisis sensorial descriptivo: aceites de olivos centenarios de los años 2016 y 2017.
- Composición en esteroles: aceite de acebuche.
- Cantidad de fenoles totales: aceites de olivos centenarios de los años 2016 y 2017.
- Estabilidad oxidativa (Rancimat): aceites de olivos centenarios de los años 2016 y 2017.

La descripción detallada de los diferentes métodos se puede encontrar en las secciones de materiales y métodos del Capítulo 5, 6, 7, 8 y 9.

Capítulo 4. Diversidad genética e relación entre acebuches y olivos centenarios en el nordeste de Portugal

En Tras-os-Montes, nordeste de Portugal, el olivo es cultivado mayoritariamente olivares tradicionales, con baja densidad de plantas por hectárea, con bajos insumos, sin riego, y que mantiene las cultivares de olivos antiguas de hace muchos años (Duarte et al., 2008). Estos olivos tiene una grande importancia del punto de vista genético, agronómico, histórico y de mantenimiento del paisaje, y deben ser protegidos y valorizados como productos regionales de alto valor. Hasta el momento la caracterización genética de estos olivos centenarios nunca ha sido examinada contrariamente al que hay ocurrido en otras regiones de países Mediterráneos. Por otro lado, el proceso de domesticación del olivo no es claro a pesar de si aceptaren dos variedades distintas, los olivos cultivados (*Olea europaea* L. subsp. *europaea* var*. europaea*) como posible derivación del acebuche (*Olea europea* subsp. *Europea* var. *sylvestris*) (Besnard et al., 2014; 2017). La variación genética del acebuche y su relación con olivos centenarios nunca ha sido evaluada en la región de Tras-os-Montes (Portugal). En esta región, pueden ser encontradas poblaciones de acebuches que crecen sobre todo en las zonas más cálidas en terrenos incultos junto a los ríos Douro, Sabor e Côa. La caracterización de sus poblaciones puede tener importancia para futuros programas de mejora una vez que pueden ser fuente de alelos que confieren atributos importantes (Baldoni et al., 2006; Belaj et al., 2007; Erre et al., 2010). El estudio de sus relaciones con olivos centenarios puede abrir nuevo abordajes acerca del proceso histórico de creación de las cultivares de olivos regionales. En este estudio fueran usados marcadores microsatélites para examinar la diversidad genética, estructura y relaciones filogenéticas entre poblaciones y dentro de las poblaciones de acebuches y olivos centenarios cultivados en la región de Tras-os-Montes. Se elegirán tres poblaciones de acebuches distantes de los olivos centenarios. Se procuró contestar las preguntas: ¿es la variación genética de los acebuches superior à la encontrada en los olivos centenarios? ¿Son los acebuches genéticamente diferentes de las poblaciones de olivos centenarios? y ¿existe alguna evidencia de mescla entre olivos centenarios y acebuches a la escala regional? Como resultados del trabajo se observó una grande diversidad genética en ambos, los acebuches y los olivos centenarios, sin diferencias entre ellos en termos de diversidad genética. El análisis de estructura de poblaciones sugiere una diferenciación genética entre las variedades estudiadas.

Capítulo 5. Caracterización química del aceite de acebuche, *Olea europaea* var. *sylvestris* (Mill.) Lehr., de diferentes localizaciones del norte de Portugal

El acebuche u olivo salvaje, tiene gran interés como fuente de material genético para programas de mejora de olivos. Sin embargo, la información sobre la composición de su aceite es escasa. En el presente trabajo se realizó la caracterización del aceite de acebuche de tres poblaciones distintas del norte de Portugal, de los municipios de Moncorvo, Alijó y Vila Nova de Foz Côa. Se caracterizó su composición en ácidos grasos, tocoferoles, esteroles y compuestos fenólicos, junto a diferentes parámetros morfológicos de los frutos. La caracterización morfológica de los frutos y huesos se realizó de acuerdo a los parámetros de la norma de la Unión Internacional para la Protección de Nuevas Variedades Vegetales (UPOV) para la caracterización de cultivares de olivo (UPOV, 2011). Los resultados indicaron que el peso, longitud y anchura de los frutos fue superior en la población de Alijó y, generalmente menor en la población con VNFC. Se observaron variaciones en el formato de los frutos, desde frutos ovoides (Alijó) a alargados (Moncorvo y VNFC). Los frutos de acebuche tuvieron en general valores menores de peso, longitud y anchura en comparación con aceitunas procedentes de cultivares tradicionales del nordeste de Portugal como Cobrançosa, Cordovil, Madural y Negrinha de Freixo (Peres el al., 2011). Todas las poblaciones produjeron frutos asimétricos, centrados considerando la posición del diámetro transversal máximo en la posición B y con un ápice redondeado (Alijó) o truncado (Moncorvo y VNFC). En relación a los parámetros morfológicos del hueso, el peso varió entre 0.20 g (Alijó) y 0.28 g (VNFC), de forma similar a los resultados de Laaribi et al. (2017) para ejemplares antiguos de acebuche de Túnez Central Oriental (0.15 g a 1,23 g para huesos). Además, el peso fue de 3,5 a 6.0 veces menor que los pesos encontrados comúnmente para los huesos de cultivares de olivo de la misma región portuguesa (Peres et al., 2011). Los valores menores de longitud, anchura y forma de los huesos fueron obtenidos en VNFC, siendo consistentemente más elevados en la población de Alijó. Para los otros parámetros, la mayoría de los resultados fue semejante en las tres localizaciones, con pocas excepciones.

En cuanto al total de grasa de los frutos, expresada en peso fresco, los valores variaron entre 5,75 ± 0,51% en Moncorvo y 8,14 ± 0,49% en Alijó, inferiores a los valores comunes de otros cultivares de olivo.

En cuanto a la composición de ácidos grasos, el ácido oleico (C18:1) fue el principal ácido graso, variando entre 68,9% y 70,6%, con cantidades muy homogéneas entre las tres poblaciones estudiadas, en oposición a lo encontrado en otras regiones geográficas como

Túnez, donde variaron entre 47 y 72% (Hannachi et al., 2013) y entre 48,4% y 71,1% (Baccouri et al., 2008), o Argelia, con valores entre 64,7% y 76,1% (Bouarroudj et al., 2016). El ácido palmítico (C16:0), y el ácido linoleico (C18:2), que variaron entre 14,2 y 15,2% y entre 7,9 y 9,9%, fueron el segundo y tercero ácidos grasos más abundantes respectivamente. También presentaron valores semejantes en las diferentes localizaciones y las cantidades fueron más homogéneas que los observados por los autores mencionados anteriormente. La suma de ácidos grasos saturados (SFA), ácidos grasos monoinsaturados (MUFA) y ácidos grasos polinsaturados (PUFA) no presentaron diferencias significativas entre las tres poblaciones. La fracción más elevada correspondió a los MUFA, variando entre 71,9% (población Alijó) y 73,0% (población Moncorvo), seguida por los SFA (16,9-18,2%) y PUFA (8,8-10,9%). Al comparar estos resultados con la composición de ácidos grasos de los cultivares tradicionales portugueses (*cvs*. Cobrançosa, Madural y Verdeal Transmontana) (Gonçalves et al., 2012), presentaron perfiles semejantes. Además, todos los valores están de acuerdo con los valores máximos legales establecidos por el Reglamento de la Comisión Europea (CEE) 2568/91 para la categoría de Aceite Virgen Extra.

En cuanto a la composición en tocoferoles, el compuesto más abundante fue el α-tocoferol variando entre 263 y 458 mg/kg de aceite, con cantidades medias semejantes entre las tres poblaciones estudiadas (de 360,2 a 385,4 mg/kg de aceite). No obstante, los aceites de acebuche fueron significativamente diferentes ($P < 0,0010$) siendo que la mayor media de γ-tocoferol fue observada en los aceites VNFC (76 mg/kg de aceite) y la más baja en los aceites de la población de Moncorvo (27 mg/kg de aceite). El contenido de β-tocoferol fue muy consistente, variando entre 5,5 y 6,5 mg/kg de aceite. Las cantidades totales medias de tocoferoles encontradas en los aceites de acebuche de las poblaciones de Alijó y VNFC (439,4 y 467,6 mg/kg de aceite respetivamente) fueron significativamente mayores que las cantidades observadas en los aceites de la región de Moncorvo (392,5 mg/kg de aceite).

El perfil de tocoferoles fue semejante al aceite de oliva, siendo que el α-tocoferol representó más del 90% de la cantidad total de tocoferoles (Beltrán et al., 2010). Al comparar los resultados obtenidos en los aceites de acebuche con estudios similares realizados con aceites procedentes de variedades cultivadas de olivo, se observó que los aceites de las muestras estudiadas presentaron en general valores de α, β y γ-tocoferol superiores a los cultivares tradicionales (12,2-630 mg/k de aceite) (Tura et al., 2007; Beltrán et al., 2010).

En la composición encontrada para los aceites de acebuche, el β-sitosterol fue el principal esterol identificado seguido por el campesterol y estigmasterol. No fueron observadas diferencias entre las poblaciones para estos compuestos principales. Sin embargo, el porcentaje de Δ-7-estigmastenol (0,76%) en aceites la población de Alijó fue significativamente superior (P = 0,0384) que los valores observados para los aceites de las poblaciones de Moncorvo y VNFC. Los esteroles totales fueron notablemente superiores al límite mínimo legal (1000 mg/kg de aceite) para el aceite virgen extra y fueron significativamente superiores para aceites de Alijó (2199 mg/kg) e inferiores para Moncorvo (1742 mg/kg) (P = 0,0124). La cantidad de esteroles respeta los límites establecidos para el aceite virgen extra con excepción del Δ-7-estigmastenol (entre 0,61 y 0,76%), ligeramente superior al máximo legal (0,5%) definido en el Reglamento de la Comisión Europea (CEE 2568/91) para la categoría de Aceite Virgen Extra. Cabe destacar que los altos niveles de esteroles totales indican que los aceites presentaron una alta calidad. Además, valores bajos de alcoholes triterpénicos indican que los frutos también mostraron una buena calidad y que fueron aplicadas buenas prácticas de producción durante el proceso de extracción, es decir, una baja temperatura de extracción y reducido tiempo de batido. Asimismo, la fracción esterólica es un parámetro muy útil en la detección de adulteraciones, dado que puede ser considerado como marcador de origen botánico (Mohamed et al., 2018).

Las cantidades totales de esteroles cuantificados en los aceites de acebuche coincidieron con los encontrados en otros estudios con excepción de los niveles de β-sitosterol (Hannachi et al., 2013; Baccouri et al., 2018; Mohamed et al., 2018), lo que podrá estar relacionados con las diferentes metodologías analíticas utilizadas. Por otro lado, dado que el contenido de esteroles es influenciado por varios factores como el clima, cultivar y localización geográfica (Lerma-García et al., 2011), esos aspectos también pueden justificar las diferencias observadas en las cantidades de esteroles.

Se detectaron y cuantificaron 14 compuestos fenólicos en los aceites de acebuche obtenidos de las tres poblaciones. Los compuestos fenólicos identificados pertenecen a cinco grupos fenólicos: alcoholes fenólicos, flavonoides, secoiridóides agliconas, derivados de ácidos dihidroxibenzoicos y ácidos fenólicos. El grupo de los secoiridóides agliconas presentó mayor cantidad por los derivados de ligstrosídeo que varió entre 271 mg/kg (localización Moncorvo) y 359 mg/kg de aceite (localización VNFC) con diferencias significativas entre localidades. Sin embargo, el segundo compuesto más abundante, oleuropeína, presentó cantidades significativamente superiores (P = 0,0165) en los aceites de la región VNFC (58,9 mg/kg de aceite) que los cuantificados en los aceites de la localidad de Alijó (33,6 mg/kg de aceite).

Los aceites de acebuche poseen una cantidad considerable de compuestos fenólicos con una cantidad media que varió entre 600 y 750 mg/kg de aceite. Los valores obtenidos también fueron superiores a los observados por Bouarroudj et al. (2016) para aceites de acebuche argelinos y para variedades de aceitunas cultivadas en Portugal (Peres et al., 2016). Los resultados obtenidos son relevantes considerando que diferentes trabajos ya habían demostrado la importancia de los compuestos fenólicos en las características sensoriales, resistencia a la oxidación y efectos positivos para la salud de los aceites de oliva, siendo en este caso los aceites de acebuche también una buena fuente de estos compuestos.

Capítulo 6. Olivos centenarios como fuente de aceites ricos en compuestos fenólicos

Los compuestos fenólicos del aceite de oliva han recibido gran atención debido a su influencia en las características sensoriales y a las evidencias científicas de su efecto positivo en la salud. En este trabajo, fueron seleccionados 28 olivos centenarios, de los cuales durante cuatro años consecutivos (2014-2017) se recogieron aceitunas y se extrajo un total de 112 muestras de aceite de oliva, de las cuales fue caracterizada su fracción fenólica. Entre los cinco grupos de compuestos fenólicos identificados, los secoiridóides agliconas fueron el grupo principal, que incluyó principalmente la forma dialdeídica de la oleuropeína (oleaceina; 3,4-DHPEA-DEA), su forma monoaldeídica (3,4-DHPEA-EA) y los derivados equivalentes del ligstrosídeo (oleocantal - p-HPEA-DEA y p-HPEA-EA, respectivamente). Las cantidades medias para todos los derivados de oleuropeína variaron entre 32 (árbol 27) y 250 (árbol 24) mg de equivalentes de tirosol/kg de aceite, mientras que los derivados de ligstrosídeo variaron entre 85 (árbol 17) y 585 (árbol 26) mg de equivalentes de tirosol/kg de aceite. Los flavonoides fueron el segundo grupo más abundante. La cantidad fenólica en aceites de oliva depende de diferentes factores como el genotipo, el estado maduración de los frutos, las condiciones agro-climáticas, el año de producción y el origen geográfico (Tovar et al., 2002; Franco et al., 2014; Sánchez de Medina et al., 2015). En el presente trabajo, todos los árboles fueron cultivados con las mismas condiciones ambientales, sometidos a los mismos factores agronómicos y los frutos fueron recogidos con índices de maduración semejantes.

Las mayores cantidades fenólicas totales se observaron para los árboles 24, 26 y 28, con valores medios para los cuatro años de 611, 618 y 604 mg de equivalentes de tirosol/kg de aceite respectivamente. Asimismo, los valores más bajos fueron consistentemente obtenidos para los aceites de oliva extraídos de los árboles 2, 6, 9 y 27, con valores de 279, 285, 271 y 226 mg de equivalentes de tirosol/kg de aceite respetivamente. Estos resultados muestran que algunos de los árboles (por ejemplo, 24, 26 y 28) pueden ser candidatos interesantes para la multiplicación en programas de mejora con el objetivo de obtener aceites de oliva con altos niveles de compuestos fenólicos totales. Mediante Análisis de Componentes Principales (ACP), considerando la cantidad fenólica individual del aceite de oliva se discriminaron los aceites producidos a partir de los 28 olivos de acuerdo al año de producción, indicando una alta influencia del año de producción en la cantidad fenólica. Las tres primeras funciones principales (PC) explicaron más del 64% de la variabilidad de los datos (28,0%, 24,6% y 11,7% para PC1, PC2 y PC3, respectivamente). Los resultados también indicaron que el ácido ferúlico, los derivados del ligstrosídeo y la vanilina fueron los compuestos fenólicos que más contribuyeron para discriminar los aceites de oliva producidos en 2014. En relación al año 2015, el hidroxitirosol, el tirosol y el ácido vanílico fueron los que más influenciaron la composición fenólica del aceite de oliva, mientras que en 2016 el ácido cinâmico, la luteolina, el ácido *p*-cumárico y los derivados de oleuropeína fueron los principales compuestos en la discriminación. Finalmente, para 2017, todos los compuestos fenólicos fueron influenciados de forma semejante. Estos resultados están de acuerdo con los obtenidos por Köseoğlu et al. (2016) que refieren que las condiciones agroclimáticas influencian la composición fenólica de los aceites de oliva. Para una mejor identificación de los olivos con mayor potencial fenólico, los 112 aceites de oliva fueron divididos en 4 grupos diferentes, independientemente del año de recolección, de acuerdo a la suma de todos los compuestos fenólicos cuantificados (fenoles totales – FT) considerando las siguientes categorías: FT < 300 mg/kg de aceite, 300 ≤ FT <400 mg/kg de aceite, 400 ≤ FT <500 mg/kg de aceite y FT ≥ 500 mg/kg de aceite, todos en equivalentes de tirosol. El ACP de los compuestos fenólicos individuales mostró que los perfiles permitieron una diferenciación no supervisada satisfactoria de los aceites de oliva de acuerdo con los grupos FT. Los resultados indicaron que a pesar de la propuesta de 4 grupos, la diferenciación más evidente fue en aceites de oliva con un FT mayor de 500 mg/kg de aceite, siendo el ácido vanílico, el hidroxitirosol, el tirosol, el ácido p-cumárico y los derivados del ligstrosídeo los compuestos fenólicos que más influenciaron esta discriminación.

De acuerdo con el Reglamento UE 432/2012 (2012) y EFSA (2012), si un aceite de oliva tiene un mínimo de 5 mg de hidroxitirosol y sus derivados (por ejemplo, complejo de oleuropeína y tirosol) por 20 g de aceite de oliva, podrá ser usado en el rótulo la declaración nutricional "*los compuestos fenólicos del aceite de oliva contribuyen para la protección de los lípidos sanguíneos del estrés oxidativo*". No obstante, cabe destacar que esta clasificación es ambigua, sin una definición clara de qué compuestos fenólicos deben ser incluidos en dicha afirmación (Tsimidou, & Boskou, 2015). Utilizando un abordaje más conservador, es decir, apenas la suma del tirosol libre, del hidroxitirosol libre y de los derivados del hidroxitirosol (derivados de la oleuropeína y el acetato de hidroxitirosol), por 20g de aceite de oliva, conforme lo exigido por la declaración nutricional, los valores medios variaron entre 1,4mg/20g de aceite (árbol 27) y 9,1mg/20g de aceite (árbol 26), presentando una dispersión muy semejante entre la mayoría de los árboles.

Considerando todos los árboles evaluados, se observó que el 50% (14 árboles) produjo aceites de oliva con niveles superiores al mínimo requerido para la declaración nutricional. Los aceites obtenidos de esos árboles (nº 1, 4, 8, 11-14, 16, 19, 21, 24-26 y 28) pueden ser rotulados como "*los compuestos fenólicos del aceite de oliva contribuyen para la protección de los lípidos sanguíneos del estrés oxidativo*". En particular, los aceites de oliva obtenidos de tres de estos árboles (nº 24, 25 y 26), contenían consistentemente a los largo de los cuatro años por lo menos 50% más que el mínimo requerido, mostrando ser buenos candidatos para la selección. Por el contrario, los árboles nº 6, 17 y 27 presentaron menores cantidades de derivados de hidroxitirosol + tirosol + oleuropeína. Además de los beneficios para la salud, las cantidades fenólicas totales también pueden influenciar la evolución de la calidad del aceite de oliva durante el almacenamiento, siendo conocido que los aceites de oliva ricos en compuestos fenólicos poseen mayor plazo de validez debido a su mayor resistencia a la oxidación.

Capítulo 7. Búsqueda de aceites sensorialmente diferenciados preservando olivos centenarios de cultivares autóctonos

El patrimonio olivícola Mediterráneo se encuentra insuficientemente caracterizado, especialmente en lo que respecta a cultivares minoritarios, autóctonos y de poca expresión o de importancia localizada. En este capítulo fueron caracterizados química y sensorialmente aceites de cultivares minoritarios del Nordeste de Portugal (Lentisca, Madural, Rebolã, Redondal, Verdeal y Verdeal Transmontana) de árboles centenarios, con el objetivo de valorizar estos cultivares y de hallar aceites de oliva con características diferenciadas. Para su caracterización se determinaron parámetros de calidad durante dos años de recolección (2016 y 2017), siendo estos parámetros: acidez libre, índice de peróxidos, coeficientes de extinción a 232 y 270 nm (K_{232} y K_{270}) y evaluación sensorial. Fue realizado un análisis sensorial descriptivo de acuerdo con la norma establecida por el

Consejo Oleícola Internacional (COI/T.20/Doc. de 22 de noviembre de 2005); así mismo, fue determinada la composición de ácidos grasos, la cantidad en vitamina E, fenoles totales, estabilidad oxidativa y la relación entre el ácido oleico/ácido linoleico. Los resultados obtenidos muestran valores de acidez consistentemente bajos. En 2016, todas las muestras estaban dentro de un rango muy estrecho, de 0,15% a 0,23%, siendo que en 2017 la acidez aumento ligeramente, variando entre 0,26% e 0,32%. Todas las muestras presentaban valores inferiores al límite máximo del 0,8% establecido por el Reglamento de la Comisión Europea (CEE 2568/91) para la categoría de Aceite Virgen Extra. Para el índice de peróxido, también se observaron valores menores en 2016 variando de 1,4 (*cv*. Redondal) a 3,6 $mEqO_2$/kg de aceite (*cv*. Madural) mientras que en 2017, los valores variaron entre 3,0 (*cv*. Rebolã) y 5,2 $mEqO_2$/kg de aceite (*cv*. Lentisca). También en este caso todos los índices de peróxidos estaban por debajo del límite máximo de 20 $mEqO_2$/kg establecido por el Reglamento de la Comisión Europea (CEE 2568/91) para la categoría de Aceite Virgen Extra. Para el coeficiente de extinción K_{232} los valores fueron inferiores a 2,03 en ambos años, siendo el menor el observado en el *cv*. Redondal (2016, 1,31 ± 0,13), y el mayor en el *cv*. Verdeal (2016, 2,03 ± 0,02). Para el coeficiente de extinción K_{270} los valores variaron entre 0,11 (*cvs.* Rebolã y Verdeal Transmontana en 2016) y 0,23 (*cv*. Redondal, en 2017). Todos los valores del coeficiente de extinción estaban dentro de los límites legales establecidos por el Reglamento de la Comisión (CEE 2568/91) para la categoría de Aceite Virgen Extra, con excepción del *cv*. Redondal en 2017, siendo por tanto considerado apenas Aceite Virgen. Igualmente, en el análisis sensorial todos los aceites fueron clasificados como Aceites Virgen Extra sin defectos sensoriales y con intensidad media de notas frutadas superior a cero. A pesar de que hayan sido encontradas diferencias significativas para algunos parámetros de calidad ($P < 0,0001$) entre cultivares (en el mismo año de producción), y entre años (dentro del mismo cultivar), los niveles físico-químicos fueron bajos, indicando pequeñas hidrólisis y oxidación de los aceites de oliva. Esos resultados confirman que la correcta manipulación de los frutos posibilita la extracción de aceite de alta calidad, siendo que el substrato genético posee baja influencia sobre esos parámetros, y se encuentran en consonancia con los resultados obtenidos por otros autores (ej. Chiappetta et al., 2017; Xiang et al., 2017; Reboredo-Rodríguez et al., 2018). En la determinación de la estabilidad oxidativa se obtuvieron diferencias significativas entre los cultivares ($P <0,0001$), siendo que el año de recolección también tuvo influencia significativa en ese parámetro para los cultivares *cvs*. Madural y Rebolã ($P < 0,0001$). Consistentemente, los valores mayores (32,6 h en 2016 y 33,6 h en 2017) se observaron en el aceite del *cv.* Redondal y los menores (10,8 h en 2016 y 14,7 h en 2017) en el *cv*. Madural. Este parámetro representa gran importancia, dado que puede ayudar a prever el plazo de validez de los aceites de oliva y la capacidad de prevenir el proceso de oxidación. La gama global de valores obtenidos en la estabilidad oxidativa es semejante a los obtenidos por otros autores para diferentes aceites monovarietales, considerando condiciones analíticas análogas (ej. Tura et al., 2007). La cantidad de fenoles totales pareció ser significativamente influenciada por el cultivar ($P < 0{,}0001$), variando entre 75 y 135 mg de equivalentes de ácido cafeico/kg de aceite en 2016 y entre 164 y 363 mg de equivalentes de ácido cafeico/kg de aceite en 2017, con los valores más altos consistentemente observados en el *cv*. Redondal y los más bajos en el *cv*. Verdeal. La cantidad de fenoles totales también fue significativamente influenciada ($P < 0{,}0001$) por el año de recolección, con valores consistentemente más altos en 2017. Ese comportamiento era esperado, debido a que la cantidad final de compuestos fenólicos, que son metabolitos secundarios de las plantas, es influenciada por diversos factores como el cultivar, el origen geográfico, las condiciones agro-climáticas, el grado de maduración, el tiempo de espera antes de la extracción, el método de extracción y las condiciones de almacenaje (Dabbou et al., 2015; Gómez-Caravaca et al., 2016; Köseoğlu et al., 2016). Las cantidades de vitamina E dependieron de los dos factores estudiados: el cultivar de aceituna y el año de producción ($P < 0{,}0001$). Una vez más, el *cv*. Redondal fue más rico en vitamina E, con 309 mg/kg en 2016 y 354 mg/kg en 2017, mientras el *cv*. Verdeal fue el más pobre, con 140 mg/kg en 2016 y 172 mg/kg en 2017. Estos resultados están de acuerdo con los obtenidos para diferentes cultivares en diferentes partes del mundo, con cantidades medias que variaron entre 50 y 500 mg/kg de aceite, sugiriendo que la cantidad de vitamina E presenta una alta dependencia del cultivar de aceituna (Beltrán et al., 2010; Reboredo-Rodríguez et al., 2016; Tura et al., 2007). No obstante también es influenciada por otros factores como el proceso de maduración y las condiciones agro-climáticas. En el presente trabajo, la sequía severa observada durante el año 2017 pareció promover la síntesis de esos antioxidantes, probablemente como medio de protección de la planta contra el aumento del estrés ambiental. La composición en ácidos grasos es un parámetro de genuinidad de los aceites de oliva que puede ser usada para la discriminación de cultivares (Kritioti et al., 2018), mientras que el grado de insaturación determina la susceptibilidad oxidativa y consecuentemente la vida útil del aceite de oliva. La razón ácido oleico/ácido linoleico de las muestras de aceite de oliva fue influenciada significativamente ($P < 0{,}0001$) por el cultivar de aceituna y en algunos casos por la época de recolección. En ambos años el *cv*. Madural presentó menores ratios, con valores de 5,75 y 5,11 para 2016 y 2017 respetivamente. Los mayores ratios fueron obtenidos para el *cv*. Redondal, con 34,9 e 29,6 para 2016 e 2017 respetivamente. Según Cayuela-Sánchez et al. (2018), la genética, el clima y las condiciones agronómicas influencian la diversidad de ácidos grasos. En relación al perfil sensorial de los aceites de oliva se estableció una escala de intensidad graduada de 0 a 10. Todos los aceites de oliva presentaron una intensidad de afrutado superior a cero, sin ningún defecto sensorial, permitiendo su clasificación como Aceite Virgen Extra (Reglamento da Comisión Europea CEE 2568/91 de 11 de julio). En todas las muestras la sensación olfativa afrutada fue "verde" y no fueron encontradas diferencias significativas en relación a este parámetro, cuyas intensidades variaron entre 5,0 en los *cvs.* Rebolã y Verdeal y 5,9 en el *cv*. Madural. No obstante, fueron encontradas diferentes sensaciones de frutos. Todos los aceites de oliva presentaron una sensación de nota de tomate, con una amplia gama de intensidades percibidas que variaron entre 1,0 y 7,5. A pesar de ser estadísticamente semejantes para todos los cultivares, los mayores valores medios para este atributo fueron observados en el *cv*. Verdeal Transmontana (4,2) y los más bajos en el *cv*. Verdeal (3,0). Fueron observadas bajas intensidades para el aroma a manzana, habiendo sido constituidos dos grupos estadísticos diferentes ($P \leq 0,05$): intensidades más elevadas en el grupo constituido por los *cvs*. Madural, Rebolã, Redondal y Verdeal Transmontana, con medias en torno a 3,8 e intensidades inferiores en el grupo constituido por los *cvs*. Lentisca (3.1) y Verdeal (2.7). Para el aroma "banana", fueron obtenidos dos grupos estadísticos diferentes ($P \leq 0,05$), siendo este aroma característico de *cv*. Madural e Rebolã y ausente en los aceites Redondal y Verdeal. Todos los aceites de oliva presentaron "sensación a frutos secos" con valores similares entre 1,6 (*cv*. Verdeal) y 2,4 (*cv*. Redondal). Este último atributo es una nota típica que caracteriza el "*terroir*" de la región. En el presente trabajo, otras sensaciones de fruta fueron también percibidas por los probadores, incluyendo cereza verde, albaricoque y kiwi, clasificados como atributos positivos. La cereza verde fue perceptible en *cvs*. Lentisca y Madural con valores medios de 1,6 y 0,4, respectivamente, en cuanto a la sensación de albaricoque apareció en los *cvs.* Lentisca (1,7), Madural (0,5) y Verdeal Transmontana (0,1). Las notas de kiwi solo fueron perceptibles en el *cv*. Madural (0,4). Lukić et al. (2017) y Lukić et al. (2018) estudiaron aceites monovariatales de los *cvs*. Oblica Buža y Istarska bjelica y encontraron atributos sensoriales como manzana, tomate, almendra, hierbas aromáticas y achicoria cuyas percepciones difieren en función del cultivar. En relación a las sensaciones herbáceas, fueron encontrados atributos de hierba fresca, hojas de tomate y tomate (sensación de col cortada). Dentro de estos atributos la hierba fresca fue generalmente la nota dominante siendo significativamente diferente entre cultivares ($P \leq 0,05$). Los aceites del *cv*. Verdeal Transmontana fueron los que presentaron mayor valor medio (4,0) y el *cv*. Verdeal, menor

(2,5). Los resultados obtenidos para notas herbáceas coinciden con los obtenidos por otros autores para diversos cultivares (Lukić et al., 2018) y son los principales responsables por el aroma afrutado verde de los aceites de oliva. En cuanto a los atributos gustativos básicos, hubo diferencias estadísticamente significativas ($P < 0,05$) en las notas dulces y picantes. El aceite de los *cvs*. Redondal (1,6), Verdeal (1,6) y Verdeal Transmontana fueron significativamente menos dulces que el *cv*. Madural (3,3). Para el atributo picante fueron encontrados dos grupos significativamente distintos ($P \leq 0,05$), uno con valores mayores constituido por los *cvs.* Lentisca, Madural, Verdeal e Verdeal Transmontana, y otro con valores menores formado por el *cv*. Rebolã y Redondal. A pesar de la diversidad de valores observados para el amargo, no mostraron diferencias significativas. Valores similares fueron observaron para sabores básicos por Reboredo-Rodríguez et al., (2016) y Reboredo-Rodríguez et al., (2018) en aceites de oliva provenientes de cultivares autóctonos de Galicia (España). Los atributos gustativos incluían sensaciones afrutadas de tomate, manzana, banana y frutos secos. La sensación de tomate fue la más pronunciada, con valores medios de 1,0 a 7,6 en la que no fueron observadas diferencias significativas entre los cultivares. Las mismas tendencias se observaron para las sensaciones de manzana y nueces secas, semejantes en todos los cultivares. Esos atributos fueron bien perceptibles por los probadores en todos los aceites de oliva, pero no fueron observadas diferencias significativas entre cultivares, lo que podría ser atribuido al "*terroir*" de la región, una vez que también fueron encontrados en trabajos anteriores con otros cultivares (Rodrigues et al. 2018). Como en las sensaciones olfativas, también se encontraron otros atributos gustativos a fruta, como cereza verde, albaricoque y kiwi, pero fueron apenas percibidos en aceites de oliva de algunos cultivares. El atributo gustativo a cereza solo fue perceptible en los *cvs*. Lentisca (1,9) y Madural (0,3); la sensación de albaricoque apareció en los *cvs*. Lentisca (2,2), Madural (0,5) y Verdeal Transmontana (0,1); y notas de kiwi fueron perceptibles en los *cvs*. Madural (0,4), Lentisca (0,3), Rebolã (0,8) y Verdeal Transmontana (0,2). Las sensaciones herbáceas fueron dominadas por notas de hierbas frescas, hojas de tomate y col. Los valores de hierbas frescas y hojas de tomate fueron semejantes en todos los cultivares. Algunos autores (Caporale et al., 2004) indicaron que la sensación de hierbas frescas puede aumentar significativamente la percepción de amargo. Sin embargo, no fue observado en el presente trabajo, siendo probablemente dependiente del cultivar. La armonía es definida como una sensación general que combina todas las percepciones obtenidas por el probador. Su clasificación es indicativa del equilibrio entre las percepciones encontradas, siendo generalmente atribuidas puntuaciones más altas en aceites de oliva en los que son percibidas diferentes sensaciones, pero sin predominancia de ninguna. Fueron constituidos dos grupos significativamente diferentes ($P \leq 0,05$): el *cv*. Madural fue el que obtuvo el mayor valor, mientras que los *cvs*. Rebolã y Verdeal Transmontana obtuvieron los menores. En relación a las sensaciones olfato-gustativas los *cvs*. Lentisca y Madural fueron significativamente más complejos que los aceites del *cv*. Rebolã. El *cv*. Verdeal Transmontana originó aceites más persistentes mientras las *cvs*. Rebolã y Redondal presentaron los menores valores.

Capítulo 8. ¿Es la cantidad de tocoferoles del aceite de oliva dependiente del año de producción y del cultivar? Un estudio detallado con olivos centenarios.

Los tocoferoles son compuestos con elevada actividad biológica, con beneficios para la salud humana y que se pueden encontrar en aceites vegetales como el aceite de oliva, contribuyendo para su resistencia a la oxidación. En este trabajo, durante cinco años de producción consecutivos (2013-2017), se evaluó la cantidad de tocoferoles de aceites extraídos de aceitunas de seis cultivares (*cvs*. Lentisca, Madural, Rebolã, Redondal, Verdeal y Verdeal Transmontana) producidas por árboles centenarios. En todos los aceites de oliva analizados se detectaron tres isoformas de tocoferol (α, β e γ-tocoferoles), y su cantidad varió significativamente con el cultivar y el año de producción. Su proporción relativa fue casi constante entre años y cultivares, siendo la variación de α-tocoferol entre 94,5% y 98,2%, β-tocoferol entre 0,4% y 1,9% y γ-tocoferol entre 0,9% y 4,1%, con media de 96,8%, 1,1% e 2,2%, respetivamente. En este trabajo el α-tocoferol representó más del 94% de la cantidad total de tocoferol en todos los aceites de oliva evaluados lo que está de acuerdo con la literatura (Beltrán et al., 2010). Cuando fueron evaluadas cantidades absolutas (mg/kg de aceite) se encontró una mayor variabilidad. Para todos los cultivares evaluados, el α-tocoferol fue la principal isoforma, variando entre 118,4 mg/kg de aceite (*cv*. Verdeal) y 607,1 mg/kg de aceite (*cv*. Lentisca). En orden decreciente de contenido de α-tocoferol e independientemente del año de recolección el orden de los diferentes cultivares fue: *cvs*. Lentisca ≈ Redondal> Madural ≈ Rebolã> Verdeal Transmontana ≈ Verdeal. De hecho, los resultados indican que la cantidad de α-tocoferol fue significativamente influenciada por el cultivar de aceituna ($P \leq 0,001$). En relación a la concentración, el γ-tocoferol fue la segunda isoforma, variando entre 1,4 (*cv*. Verdeal Transmontana) y 17,3 mg/kg de aceite (*cv*. Lentisca). De la misma forma, las cantidades de γ-tocoferol fueron influenciadas significativamente por el cultivar de aceituna ($P \leq 0,001$)

en el siguiente orden decreciente: Lentisca > Redondal > Rebolã ≈ Madural ≈ Verdeal > Verdeal Transmontana. Finalmente, β-tocoferol fue la isoforma con las menores cantidades, variando entre 1,0 (*cv.* Verdeal) y 7,5 mg/kg de aceite (*cv*. Lentisca) ($P < 0,001$). Considerando la cantidad total de Vitamina E como la suma de las tres isoformas de tocoferoles previamente mencionadas, la cantidad varió entre 124,4 (*cv*. Verdeal) y 629,6 mg/kg de aceite (*cv*. Lentisca), siendo estas tres cantidades también significativamente dependientes del cultivar de aceituna ($P < 0,001$). El orden decreciente fue el mismo que para el α-tocoferol. A partir del análisis general del tocoferol en los seis cultivares, se puede inferir que los más prometedores para la obtención de aceites de oliva ricos en tocoferoles son los *cv*. Lentisca, seguido de *cv*. Redondal. Además, los perfiles de tocoferoles fueron utilizados para evaluar su capacidad de clasificar los aceites de oliva obtenidos de olivos centenarios de acuerdo con el cultivar de aceituna. De hecho, a partir del ACP, se puede observar que las cantidades de tocoferoles permitieron discriminar el aceite de acuerdo al cultivar de aceituna, utilizando tres componentes principales, lo que explicó 100% de la variabilidad de los datos (82,3%, 11,7% y 5,9% para PC1, PC2 e PC3, respetivamente). Para los *cvs*. Lentisca y Redondal, el β-tocoferol fue el que más contribuyó para agrupar los cultivares de olivo. Para el *cv*. Madural, el α-tocoferol fue el más influyente, mientras en el *cv*. Rebolã, γ-tocoferol tuvo la mayor influencia. En general para los aceites de oliva estudiados, la cantidad de tocoferol fue altamente dependiente del cultivar. El mapa de agrupamiento jerárquico reforzó lo anteriormente referido. Teniendo en cuenta la información sobre los dendrogramas, es evidente que el contenido de tocoferoles puede ser dividido en dos grupos, uno relativo al α-tocoferol y el otro compuesto por los β y γ-tocoferoles. Esta información permitió agrupar los aceites de oliva en cuatro grupos principales de acuerdo al cultivar de aceituna. El primer cluster consistió principalmente en aceites de los *cvs.* Lentisca, Madural y Redondal (cantidad más elevada de α-tocoferol y media a alta de las otras dos isoformas). El segundo grupo incluyó el *cvs*. Redondal, Madural e Rebolã (cantidad media a alta de las tres isoformas de tocoferol). Los tercero y cuarto clusters fueron constituidos principalmente por *cvs*. Verdeal e Verdeal Transmontana con algunos aceites de *cvs*. Redondal y Madural (cantidad media a baja de tocoferoles). Este análisis, a pesar de confirmar que el cultivar influenció en gran medida las cantidades de tocoferoles en aceites de oliva de árboles, también apuntó a que el año de recolección tiene un papel importante, dado que los cuatro cluster identificados contenían más de un cultivar. Considerando que las condiciones climáticas varían interanualmente y que este trabajo abarco cinco campañas consecutivas de producción, el efecto del año de recolección sobre la cantidad de las diferentes isoformas y el total de vitamina E fue también evaluado teniendo en cuenta todos los aceites independientemente del cultivar. En cuanto a la influencia del año de producción sobre la cantidad de las diferentes isoformas de tocoferol y de vitamina E: (i) los mayores niveles de α-tocoferol fueron registrados en 2013 y 2015, siendo significativamente superiores que en los otros años ($P \leq 0,0056$); (ii) los niveles de γ-tocoferol fueron significativamente superiores en 2013 (8,7 mg/kg de aceite) en comparación a los demás años ($P \leq 0,0001$), mientras que en 2014 fueron registradas las menores cantidades medias (4,1 mg/kg de aceite); (iii) en cuanto β-tocoferol, fueron observadas diferencias significativas entre años ($P \leq 0,0001$), con valores mayores en 2015 (4,55 mg/kg de aceite); (iv) para la vitamina E total, el orden decreciente de abundancia fue: 2013 ≈ 2015> 2017> 2016 ≈ 2014. Mediante una ACP se verificó que los perfiles de tocoferoles de aceites de olivos centenarios variaban de acuerdo al año de recolección independientemente del cultivar de aceituna. En general, las cantidades totales de tocoferoles encontrados (118,4 a 607,1 mg/kg) así como los de las diferentes isoformas confirman los valores de otros trabajos, a pesar de que en algunos casos fueran superiores (por ejemplo, Beltrán et al., 2010 Borges et al., 2017a; Noorali et al., 2017; Tura et al., 2007).

De forma general, se asume que la composición de los aceites de oliva varía interanualmente, en función de las condiciones climáticas. Sin embargo, la mayoría de las observaciones es apoyada en estudios de apenas dos o tres años. En el presente trabajo, con cinco años de producción evaluados, se observó una clara variación interanual. Beltrán et al. (2010) atribuyeron estas variaciones a los niveles pluviométricos, con mayores cantidades de tocoferol en los aceites de oliva en años más secos, a pesar de que esta tendencia no haya sido observada en el presente estudio. La cantidad de vitamina E presentó valores dentro de los límites de las declaraciones nutricionales permitidas para los aceites de oliva que, sobre ciertas condiciones (niveles establecidos en el Reglamento Europeo EU 1169/2011), por lo que pueden ser rotulados como "*Fuente de Vitamina E*". De acuerdo con este reglamento y considerando la recomendación de una ingesta diaria de 12mg, el consumo de 28,5 mL de aceite de olivos centenarios del *cv.* Lentisca evaluado en el presente trabajo, sería suficiente para atender las necesidades diarias de ingestión de vitamina E.

Capítulo 9. La composición en ácidos grasos del aceite de olivos centenarios es altamente dependiente del cultivar y año de producción

El aumento de consumo de aceite de oliva en las últimas décadas fue atribuido a sus propiedades benéficas para la salud, principalmente la composición de ácidos grasos con niveles elevados de ácidos grasos monoinsaturados. En el presente trabajo, durante cinco años de producción consecutivos (2013-2017), fue caracterizada la composición de ácidos grasos de aceites de oliva de seis cultivares autóctonos (Lentisca, Madural, Redondal, Rebolã, Verdeal y Verdeal Transmontana) producidos por árboles centenarios. Como esperado, el ácido oleico (C18:1) fue el principal ácido graso encontrado en todos los aceites de oliva de los diferentes cultivares y considerando los datos de los cinco años, su cantidad media varió entre 70,3% (*cv*. Madural) y 80,6% (*cv*. Redondal). Todos los años, las cantidades de ácido oleico fueron estadísticamente dependientes del cultivar de aceituna (P < 0,0001). Fueron identificados tres grupos de acuerdo a la cantidad de ácido oleico. El primer grupo con la mayor cantidad, constituido por aceites de los *cvs*. Verdeal Transmontana y Redondal y con valores medios superiores al 80%; un segundo grupo, con valores intermedios, que incluía *cvs*. Lentisca y Verdeal, con valores medios entre 78,0 y 75,8%; y un tercer grupo con las menores cantidades para los aceites del *cvs*. Madural (70,3%) y Rebolã (72,9%). El ácido palmítico (C16:0) fue el segundo ácido graso más abundante con valores más elevados en los aceites del *cv*. Verdeal, con un valor medio de los cinco años de 13,5%. Los valores menores fueron observados para el *cv*. Lentisca (valor medio de 10,4%). El ácido linoleico (C18:2), fue el tercer ácido graso más abundante. De acuerdo con los resultados obtenidos para los principales ácidos grasos de todas las muestras de los diferentes cultivares y para los cinco años de estudio se verificó que se cumplían los niveles establecidos por el Reglamento de la comisión Europea CEE 2568/91 de 11 de julio para la categoría de Aceite Virgen Extra. De acuerdo con el grado de saturación, en relación a la abundancia relativa de ácidos grasos monoinsaturados (MUFA), se identificaron cinco grupos significativamente diferentes (P ≤ 0,05) siendo que la cantidad varió con el cultivar de la siguiente forma: cv. Redondal (82,1%) ≈ *cv*. Transmontana Verdeal (81,7%) > *cv*. Lentisca (79,1%) > *cv*. Verdeal (77,4%) > *cv*. Rebolã (74,3%) > *cv*. Madural (71,2%). Una tendencia inversa fue observada para los PUFA, con una abundancia relativa de: *cv*. Madural (13,8%) > *cv*. Rebolã (10,1%) > *cv*. Lentisca (7,0%) > *cv*. Verdeal (5,9%) > *cv*. Verdeal Transmontana (3,5%) ≈ *cv*. Redondal (3,0%). Desde el punto de vista tecnológico, la presencia de valores altos de PUFA en los aceites vegetales disminuye su resistencia a la oxidación, afectando tanto a su vida útil como a su desempeño térmico. Fueron observados valores significativamente superiores para los SFA para *cv.* Verdeal (16,6%), seguido por el *cv*. Rebolã (15,5%), y los valores menores para el *cv*. Lentisca (13,8%). Los SFA de los *cvs* Madural, Redondal y Verdeal Transmontana presentaron cantidades relativas semejantes. Las cantidades globales de los diferentes grupos (ácidos grasos saturados e insaturados) son de gran relevancia debido a los impactos nutricionales y tecnológicos relacionados (Saad et al., 2012). Las cantidades de MUFA presentan correlaciones positivas con la estabilidad oxidativa de los aceites de oliva – aceites ricos en MUFA presentan en general mayor plazo de validez. Los MUFA también se correlacionaron con algunos atributos sensoriales, estableciéndose correlaciones positivas con el atributo amargo y negativas con el atributo dulce (García-Mesa et al., 2008, Youssef et al., 2011). Además, Youssef et al. (2011) se observó que una cantidad alta de SFA en aceites de oliva origina una mayor viscosidad y persistencia en la mucosa de la cavidad bucal, produciendo un efecto conocido como "sensación oleosa". Estos aspectos cualificaron la composición en ácidos grasos como índices de calidad de los aceites durante la producción, almacenamiento y comercialización (Prentki et al., 2012, Soto-Vaca et al., 2013). Globalmente, los resultados descritos en este trabajo están de acuerdo con los datos de la literatura, que indican que las cantidades de los diferentes grupos (MUFA, PUFA y SFA) son dependientes genéticamente del cultivar (ej. Kritioti et al. , 2018; Xiang et al., 2017). El año de recolección ejerció una influencia importante en los aceites de oliva obtenidos. En general, con excepción de *cv*. Lentisca, el año de recolección presentó un efecto significativo sobre la composición de ácidos grasos del aceite de oliva para los diferentes cultivares. La cantidad de ácido oleico (C18:1), el principal ácido graso, varió ligeramente con el año de recolección. En 2017 se verificó una mayor influencia del año, con valores significativamente inferiores a los valores máximos observados en los restantes años. Este hecho puede estar relacionado con la ocurrencia de precipitaciones y las temperaturas observadas en 2017. De hecho entre los cinco años estudiados, 2017 fue el año más seco, con un gran periodo sin lluvia, siendo considerado un año de sequía extrema. Considerando las lluvias de agosto, septiembre y octubre, se observaron menos de 30 mm de lluvia en 2017 mientras que en los demás años el total de lluvias de estos meses varió de 100 a 180 mm. Además, las temperaturas medias y máximas registradas durante octubre fueron mayores en 2017. El ácido palmítico también fue significativamente influenciado por el año de producción para todos los cultivares, con excepción del *cv*. Lentisca, a pesar de no haber sido observada ninguna tendencia. Otros autores observaron que la composición de los ácidos grasos dependía de las condiciones ambientales, especialmente de la temperatura desde el florecimiento hasta la recolección final (Borges et al., 2017b; Portarena et al., 2015), y la menor temperatura media durante el crecimiento de los frutos (Ceci et al. , 2017; Tena et al., 2017). Según Dabbou et al. (2010)y Mailer et al. (2010), los ácidos C18:1 y C16:0 son los que pueden ser más afectados por las condiciones ambientales. De los 14 ácidos grasos identificados y las cantidades totales de MUFA, PUFA y SFA, los ácidos que más contribuyeron para la clasificación no supervisada de los aceites de oliva por el cultivar fueron C16:0 y el total de SFA junto con C16:1, C17:1, C18:1, C20:1, C22:1, C16:1 y MUFA. Como puede ser fácilmente inferido, la composición en MUFA es la que más contribuyó para la diferenciación natural de aceite de oliva. La cantidad de C16:0 en conjunto con el MUFA, C17:1 y C18:1 fueron los más relevantes para diferenciar los aceites de los *cvs*. Redondal y Verdeal Transmontana de los *cv*. Lentisca, Madural, Rebolã y Verdeal. Por otro lado, los ácidos monoinsaturados C16:1, C20:1, C22:1 y SFA total fueron los que más contribuyeron para la diferenciación de los *cvs*. Lentisca, Madural, Rebolã e Verdeal.

Mediante el uso de herramientas estadísticas, se verificó que estos resultados demuestran claramente que dentro de cada clase de cultivar de aceituna, el año de recolección tiene un enorme impacto en la composición de ácidos grasos del aceite de oliva producido. Varios autores obtuvieron resultados semejantes con base en la influencia de las condiciones ambientales (Borges et al., 2017b, Farinelli & Tombesi, 2015; Reboredo-Rodríguez et al. al., 2015; Rodrigues et al., 2018; Yousfi et al., 2012). Cabe destacar que la formación de los subgrupos no supervisionados puede estar relacionada de forma provisoria con las diferentes condiciones climáticas observadas en 2013-2015 (alta cantidad de lluvias en octubre), 2014-2016 (lluvia distribuida a lo largo del periodo analizado y temperatura máxima alta) y 2017 (sequía y temperaturas altas durante toda el verano)

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Chapter 1

General Introduction

1. General Introduction

1.1. The olive tree and its geographical distribution

The olive tree (*Olea europaea* L.) is a perennial tree belonging to the family Oleaceae. This species is one of the earliest known agricultural settlements in the history (Laaribi et al., 2017). Its cultivation began in Palestine about 6000 years ago and, was probably one of the earliest domesticated trees (Terral et al., 2004; Breton et al., 2009). Later, different ancient civilizations were responsible for the spreading of the species throughout Africa and the west Mediterranean. After the discovery of America, olive cultivation spread to Peru, Argentina, Chile and Uruguay and to the north, to the coastal regions of Mexico and the United States where it found a favorable environment in the southern California (Cimato & Attilio, 2011). Recently, olive cultivation has also been introduced in other no traditional countries without custom in olives production and consumption, but where the environmental conditions are similar to the Mediterranean climate (Bouarroudj et al., 2016). Nowadays, olive cultivation can be found in South Africa, Australia, New Zealand, China and Brazil (Cimato, & Attilio, 2011), thus becoming one of the most extensively cultivated crop in the world (Figure 1.1). Nevertheless, the olive tree production is mainly based in the countries around the basin of the Mediterranean Sea, where approximately 97.9% of the olive tree plantation area is located (Rallo et al., 2018). Olive tree is one of the main crops with a long tradition and great environmental, economic and social importance.

Figure 1.1. Geographical distribution of olive growing areas (green areas). Adapted from Cimato, & Attilio (2011).

About 900 million olive trees are grown on approximately 10.6 million hectares worldwide (FAO, 2016). Approximately 28.9 million tons of olives are produced each year, of which 90% is processed into virgin olive oil, while the remaining 10% correspond to table olives (IOC, 2018). In Portugal, the olive tree has a long tradition. This country is the eighth largest producer of olive oil in the world and, according to the International Olive Council (IOC), is the fourth largest producer in the European Union (IOC, 2017). The Portuguese production has increased 94% over the last six years, with an average for the same period of 87.6 thousand tons, reaching the 135 thousand tons in the 2017 campaign. It is believed that the cultivation of olive trees reached the Iberian Peninsula during the second millennium before Christ (BC) (Terral et al., 2004). As the olive tree is a long life species, and has the ability to survive in adverse conditions, specimens of centenarians trees are found throughout the Mediterranean region (Baldoni et al., 2006; Moriondo et al., 2013) not only in the cultivated form but also as wild. During the domestication process, not all plants had a good adaptation to this process, originating two varieties of *O. europaea*, the cultivated forms, the olive tree (*O. europaea* subsp. *europaea* var. *europaea*) and the wild forms, the wild olive tree or oleaster (*O. europaea* subsp. *europaea* var. *sylvestris*) (Breton et al., 2006; Breton et al., 2008; Hannachi et al., 2013).

1.2. Wild form (*O. europaea* subsp. *europaea* var. *sylvestris*)

The oleaster or wild olive tree although often found as a shrub or small tree, it can reach up to 15 meters in height and large trunks. This species is not cultivated once its morphological characteristics are not favorable from an economic point of view, mainly due to the small fruits, low fat content and a very long juvenile stage (Bouarroudj et al., 2016). Nevertheless, in the past, there are abundant historical references of the use and production of olive oil from wild trees (Chiappetta et al., 2017). Historically, different parts of oleaster have been used, for different purposes like religious and/or recreational; as rootstock for olive cultivars; as combustible since the wood possesses a high calorific power; for oil extraction and, the leaves due to their various medicinal properties. Considering that oleaster plants possess high rusticity to different soils and lower susceptibility or higher resistance to pests and diseases, they can play an important role in the development and selection of new olive cultivars with high adaptability and survival skills, which may produce olive oils with high quality (Baccouri et al., 2010). Some authors reported ancient olive trees in wild forms in several Mediterranean countries, such as Algeria (Bouarroudj et al., 2016), Italy (Baldoni et al., 2006, Chiappetta et al., 2015, Chiappetta et al., 2017) and Tunisia (Baccouri et al., 2010; Hannachi et al., 2013). In the

Iberian Peninsula, oleaster is a well-adapted autochthonous species, being abundant throughout the Mediterranean zone especially in the southern part. In Portugal, oleaster is commonly distributed throughout the country (Figure 1.2), mainly in the central and southern regions. It is a species that grows on all types of soils, preferring basic soils. It occurs in places with good sun exposure, cork oak and holm oak and dry scrubland (Porto et al., 2018). In the northern region, although the olive tree in cultivated form is the most abundant, it is still possible to find some specimens of the wild olive in scrublands near important rivers such as Douro, Sabor and Côa.

In the past, oleaster oil was used for medicinal, cosmetic and religious purposes (Chiappetta et al., 2017). Nevertheless, the information available about its chemical composition and nutritional value is scarce. Dabbou et al. (2011) reported high levels of antioxidants and of oleic acid for some oleaster oils, highlighting its high potential as a source of phytochemicals (Bouarroudj et al., 2016). As far as we known, there is no information available concerning oils from Portuguese oleaster populations.

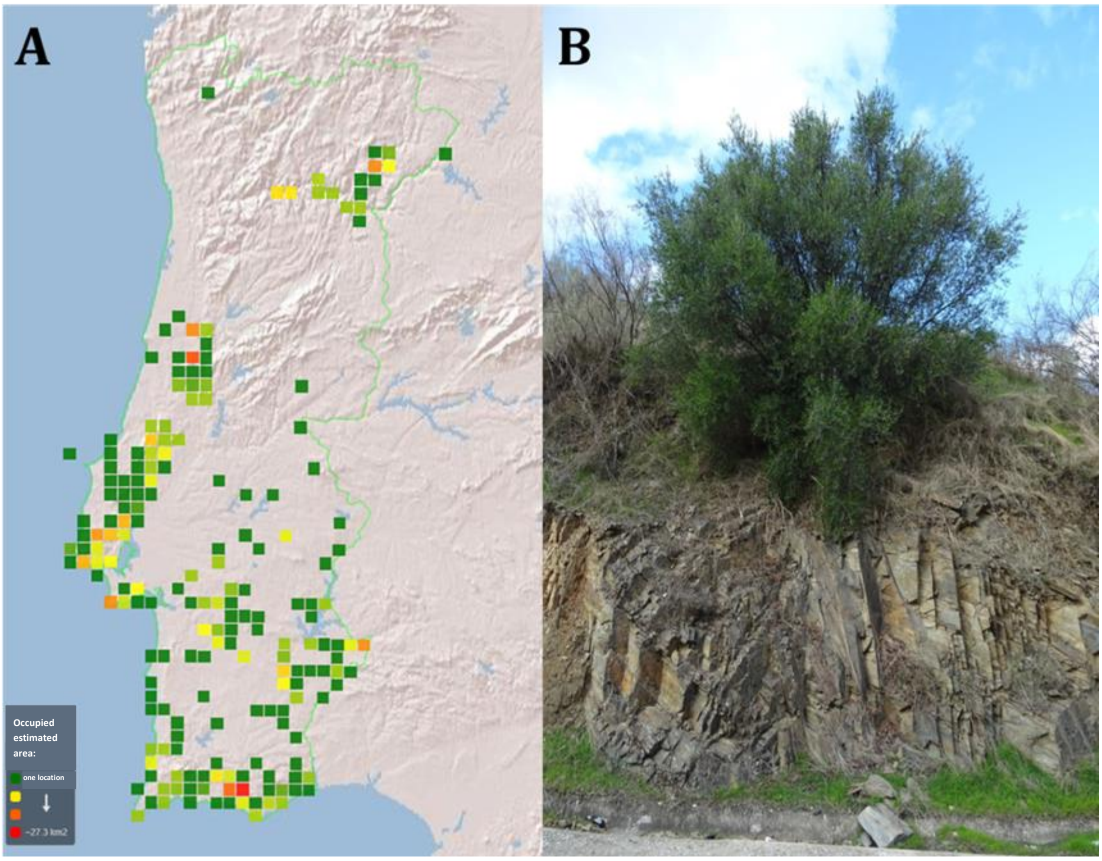

Figure 1.2. A- Geographical distribution of oleaster in Portugal, adapted from Porto et al. (2018); **B**- Oleaster plant in Vila Nova de Foz Côa region.

1.3. Cultivated form (*O. europaea subsp. europaea* var. *europaea*)

The process of domestication resulted in a selection of genotypes that have desirable technological/agronomic characteristics including: large fruit size; high ratio pulp/stone; high oil content; high oil quality; high resistance/tolerance to pests and diseases; high resistance/tolerance to particular conditions of stress (pH, salinity, drought, etc.); good adaptation to environmental conditions; and easy vegetative propagation, either by direct planting of cuttings or grafting in rootstocks (Chiappetta et al., 2015). However, the olive-growing heritage is still partly unknown, especially in the case of regional or local minority cultivars. Some of these cultivars produce diverse oils with different physicochemical compositions and sensory attributes, thus leading to a richer and differentiated olive oil. The knowledge of this heritage can be a great asset, both for the local populations and also for the consumer due to the differentiated composition, rich in compounds beneficial to the health and pleasant to the smell and the taste, which may contribute to obtain differentiated products and so, contributing to the preservation of the intrinsic rich genetic heritage of the region.

1.3.1. Olive fruit

The fruit of the olive tree is a drupe composed by exocarp (olive skin), mesocarp (pulp-edible portion) and endocarp (stone), which exhibits some morphological and physicochemical differences that make it distinct from the rest of drupes (Cabezas, 2011; Hammami et al., 2011). Usually, the mesocarp represents about 70-90% of the total fruit weight (Galanakis, 2011; Ghanbari et al., 2012; Peres et al., 2011), while the endocarp (stone) may differ from 10 to 30% of the fruit's weight. The fruit and endocarp sizes are dependent on the olive cultivar and growth conditions.

The different trees produce fruits with specific characteristics such as shape, size, color and weight, which allow to visually differentiating the olive cultivars (Figure 1.3). The growth and maturation process of the fruits can take about five months under normal climatic conditions, from the flowering to the harvest (Ghanbari et al., 2012), being the fruits size, as a whole, influenced by several factors, such as water availability, soil fertility, temperature or genetic information of each cultivar (Hammami et al., 2011).

Comparing olives with other well-known drupes (peaches, apricots, cherries and plums), olives have the particularity that cannot be directly consumed, due to the strong bitter taste caused by the presence of oleuropein and its derivatives (the predominant phenolic compounds) in the pulp (Vinha et al., 2005).

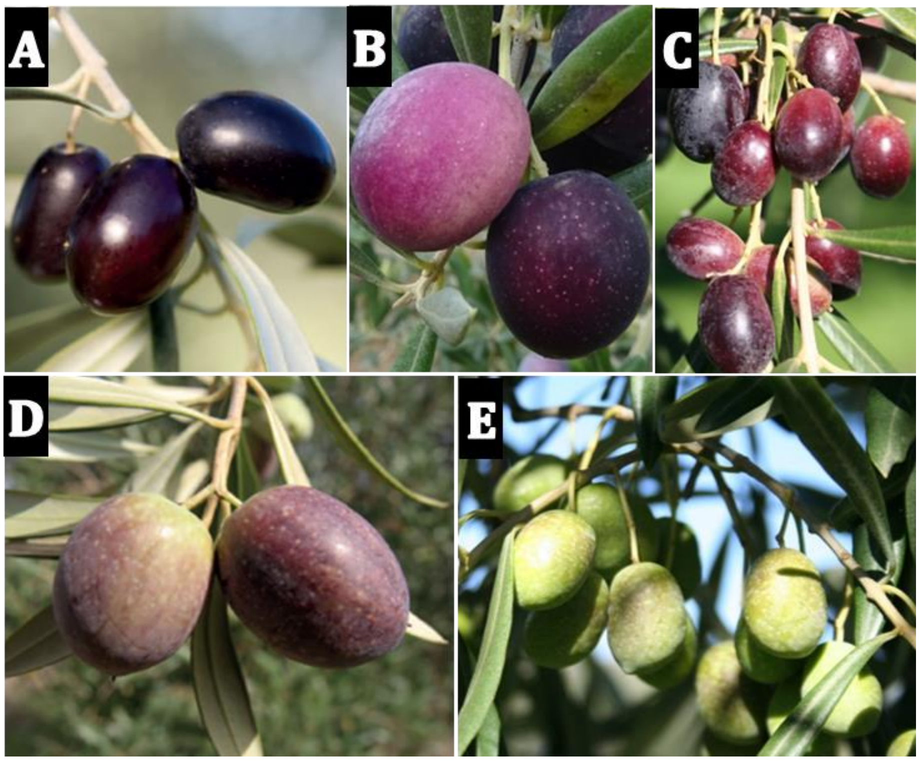

Figure 1.3. Appearance and shape of the different cultivars. **A**- *cv*. Madural; **B**- *cv*. Redondal; **C**- *cv*. Lentisca, **D**-*cv*. Santulhana, **E**-*cv*. Verdeal Transmontana.

Other characteristics that distinguish olives are the low sugar content (3.5-6.0%) and high oil amount (oil content of 14.0-30.0%) accumulated during maturation (Rallo et al., 2018). The main constituent of olives is water (more than 50.0%), possessing high levels of fibers (5.0-6.0%) and low protein contents (1.6-3.0%) (Conde et al., 2008). Usually, during the maturation process the color of olives changes from green to black or violet-brown but, these changes, are cultivar-dependent.

1.3.2. Olive oil

As mentioned before, olives are mainly produced for oil extraction. Virgin olive oil is extracted from healthy fresh olives only by mechanical and physical processes (milling, malaxation, and hydraulic presses, percolation or centrifugation of the olive paste), without the addition of any chemical solvents, at controlled temperatures that should be below 27ºC for the label indication of "*cold extraction*" (Commission Implementing Regulation (EU) Nº 29/2012 of 13 January 2012). This extraction process allows maintaining intact in the oil compounds, known for their high nutritional value and bioactivity, which exist in the fruits (Visioli & Bernardini, 2013; Tsimidou & Boskou, 2015). The biological benefits of olive oils are mainly linked to those compounds (phenolic, tocopherols, sterols, pigments, etc.) and a well-balanced fatty acid composition. Commercially, olive oil may be classified as Extra Virgin Olive Oil (EVOO), Virgin Olive Oil (VOO) and Lampante Olive Oil (LOO) (Commission Regulation (EEC 2568/91)). According to the same Regulation and subsequent updates olive oils are classified as EVOO if, simultaneously, fatty acid ethyl esters ≤ 30 mg/kg; free acidity ≤ 0.8% expressed in oleic acid; peroxide value ≤ 20 mEq O_2/kg of olive oil; specific coefficients of extinction at 232 nm (K_{232}) ≤ 2.50, specific coefficients of extinction at 270 nm (K_{270}) ≤ 0.22, and ΔK ≤ 0.01; waxes ≤ 150 mg/kg; and median intensity of sensory defects equal to 0 (zero) and median intensity of fruitiness greater than 0 (zero) (in an ordinal intensity scale ranging from 0 to 10). Olive oils would be classified as VOO if, at the same time, free acidity ≤ 2.0% expressed in oleic acid; peroxide value ≤ 20 mEq O_2/kg; specific coefficients of extinction at 232 nm (K_{232}) ≤ 2.60, specific coefficients of extinction at 270 nm (K_{270}) ≤ 0.25, and ΔK ≤ 0.01; waxes ≤ 150 mg/kg; and median intensity of sensory defect between 0 (zero) and 3.5 (in an ordinal intensity scale ranging from 0 to 10) and median intensity of fruitiness higher than 0 (zero). Olive oils would be classified LOO if at least one of the previous conditions is not fulfilled. But the last category (LOO) cannot be sold directly to consumers. .Fatty acids are the most abundant compounds in olive oil, from which monounsaturated fatty acids are the most outstanding. The main fatty acids are oleic acid (55-83%), followed by linoleic acid (3.5 to 21%), palmitic acid (7.5 to 20%), stearic acid (0.5-5.0%) and linolenic acid (≤1%) (levels established by the Commission Regulation (EEC 2568/91) for EVOO). Oleic acid is an unsaturated fatty acid that protects the human body against various types of diseases (Dewapriya et al., 2013; Medina & Tabernero, 2010). The linoleic and linolenic are essential fatty acids that cannot be synthetized by the human body and so, must be ingested being olive oil an important source of these compounds. Also, olive oil contains conjugated fatty acids, formed due to the linkage of glycerol to a set of three fatty acids forming the triacylglycerols. The most abundant are triolein (40-59%), linoleodiolein (12.5-20%) and palmitodiolein (12-20%) (Boskou, 1996). The sterol fraction in olive oil is largely dominated by β-sitosterol, which typically accounts for 75 to 90% of the sterols, and by a lower extent by Δ-5-avenasterol, which usually ranges between 5%-20%, although higher concentrations (up to 36%) have been reported for some cultivars (Boskou et al., 2006). The tocopherols, known generically as vitamin E, are other compounds of great importance that can be found in olive oils in four isoforms (α-, β-, γ- and δ-tocopherol) (Ahsan et al., 2015; Bramley et al., 2000). Usually, their amounts in olive oil is vary from 84 to 463 mg/kg of olive oil (Beltrán et al., 2010). Among them, α-tocopherol is the predominant vitamin E compound, in olive oil, representing 90-95% in most cultivars, whereas β and γ-tocopherols are found only in reduced amounts (Kalogeropoulos et al., 2017). Phenolic compounds constitute another relevant group of natural compounds found in olive oil, which content depends on the amount found on the olive fruits. They represent one of the most important and ubiquitous groups of plant metabolites, including from simple phenolic molecules to high molecular weight compounds, being their occurrence in plant foods extremely variable (Shahidi, & Ambigaipalan, 2015). The phenolic amount usually ranges from 250 to 800 mg/kg of olive oil, being oleuropein derivatives and ligstroside derivatives the most abundant (Veneziani et al., 2018).

Also, the colour of olive oil is a factor of acceptability among consumers, being the deep green colour usually highly appreciated by typical consumers of VOO. The olive oil contains green chlorophylls as well as feofitin, which is yellow. These pigments are responsible for the greenish coloration of certain olive oils. Carotenoids contribute also for the olive oil colour and possess antioxidant and pro-vitaminic A action. The main carotenoids present in olive oil are lutein, β-carotene and xanthophylls (Del Giovine, & Fabietti, 2005). On the other hand, the aroma of high quality EVOO is due to volatile compounds of diverse chemical nature: aldehydes, alcohols, esters, ketones, furans and hydrocarbons. Among them, aldehydes, alcohols and their corresponding esters are especially abundant and have been related to the "fresh green fruit odor" (Servili et al., 2009; Sánchez-Ortiz et al., 2018). A summarizing of the olive oil chemical composition can be found in Table 1.1.

However, olive oil chemical composition is affected by several factors like olive cultivar, fruit maturation, edaphoclimatic conditions, agronomic factors such as irrigation, fertilization, pests and diseases and the conditions used during the extraction period of the oil and storage (Cayuela et al., 2017; Kalogeropoulos et al., 2017).

Due to the beneficial nutritional properties of olive oil and its high economic value, the world consumption of olive oil has increased, especially in the last decades, leading to the search for new geographical areas for olive plantations, as well as for new production practices aiming to increase the productivity of olive oil per plant or area (Muzzalupo, 2012; Rufat et al., 2014). At present, and as previously mentioned, the olive tree is spreading from the Mediterranean area of origin to new production areas. However, the expansion of olive growing to new extensions around the world is based on the same commercial cultivars that are highly adaptable and productive. The increasing use of conventional cultivars has led to the replacement of old specimens, with low production, by highly productive ones.

Table 1.1. General chemical composition of olive oil.

Chemical Parameters	Amounts	References
Fatty acid composition		
Myristic acid (C14:0)	≤ 0.03%	Commission Regulation (EEC) 2568/91
Palmitic (C16:0)	7.50-20.00%	
Palmitoleic (C16:1)	0.30-3.50%	
Heptadecanoic (C17:0)	≤ 0.30%	
Heptadecenoic (C17:1)	≤ 0.30%	
Stearic (C18:0)	0.50-5.00%	
Oleic (C18:1)	55.00-83.00%	
Linoleic (C18:2)	2.50-21.00%	
Linolenic (C18:3)	≤ 1.00%	
Arachidic (C20:0)	≤ 0.60%	
Eicosenoic (C20:1)	≤ 0.40%	
Behenic (C22:0)	≤ 0.20%	
Lignoceric acid (C24:0)	≤ 0.20%	
Triglyceride Composition		
Triolein	40.0 – 59.0%	Boskou (1996)
Palmitodiolein	12.0 – 20.0%	
Dioleolinolein	12.5 – 20.0%	
Palmitooleolinolein	5.5 – 7.0%	
Dipalmitoolein	3.0 – 6.5%	
Stearodiolein	3.0 – 7.0%	
Sterols composition		
Cholesterol	≤ 0.5%	Commission Regulation (EEC) 2568/91
Brassicasterol	≤ 0.1%	
Campesterol	≤ 4.0%	
Stigmasterol	< campesterol	
β-Sitosterol apparent	≥ 93.0%	
Δ-7-Stigmastenol	≤ 0.5%	
Total Sterols	≥ 1000mg/kg	
Erythrodiol and uvaol	≤ 4.5%	
Tocopherols contents	84 to 463 mg/kg of olive oil	Beltrán et al., 2010
Phenolic compounds	250 to 800 mg/kg of olive oil	Veneziani et al., 2018
Chlorophylls	1 to 40 mg/kg of olive oil	Gandul-Rojas et al., 2000
Carotenoids	2 to 20 mg/kg of olive oil	Gandul-Rojas et al., 2000
Volatile compounds	8.06 to 34.24 mg/L of olive oil	Cherfaoui et al., 2018

This fact has contributed to the loss of the genetic identity of many minor cultivars. However, current trends of the high-quality EVOO market have led to an increasing seek for high-value differentiated products obtained from traditional cultivars (produced in non-intensive systems within specific geographic origins), products that are rich in bioactive compounds, which promote health, as well as for products with differentiated sensory characteristics (Bajoub et al., 2015, Krichene et al., 2010, Reboredo-Rodríguez et al., 2016). The combination of these factors is only found in traditional cultivars, and particularly by millennial and centenarian specimens grown in low-input systems, in specific terroirs (Reboredo-Rodríguez, et al., 2015). In this sense, several studies have been carried out by researchers with the objective of valuing the products obtained from these specimens and preserving the genetic identity kept for centuries.

1.4. Importance of centenarian olive trees

The olive tree in Portugal is a plant with a huge tradition. It is possible to find olive trees in all the territory but mainly in areas of the interior land since the soil and climate conditions allow the development of this cultivation in optimal conditions and to obtain high quality products. Different types of olive groves could be found (Figure 1.4).

Traditional olive groves, with a low numbers of plants per hectare (80-150 trees/ha), low inputs of production factors, non-irrigated, most of them ancient and from traditional olive cultivars; **intensive olive groves**, with a higher number of plants per hectare (300-500 trees/ha) and for which the cultural practices are more intensive, with considerable inputs of fertilizers, pesticides and irrigated when water is available; usually the age of this groves is less than 40 years old and a reduced number of cultivars are used, some of them foreign (eg., *cv*. Picual); and **high intensive or hedgerow olive groves**, with high number of plants per hectare (800-2000 trees/ha), with less than 20 years old, produced in an intensive way with high inputs of production factors (fertilizers, pesticides and water), highly mechanized, using foreign high productive cultivars, mainly *cvs.* Arbequina, Arbosana and Shikitita.

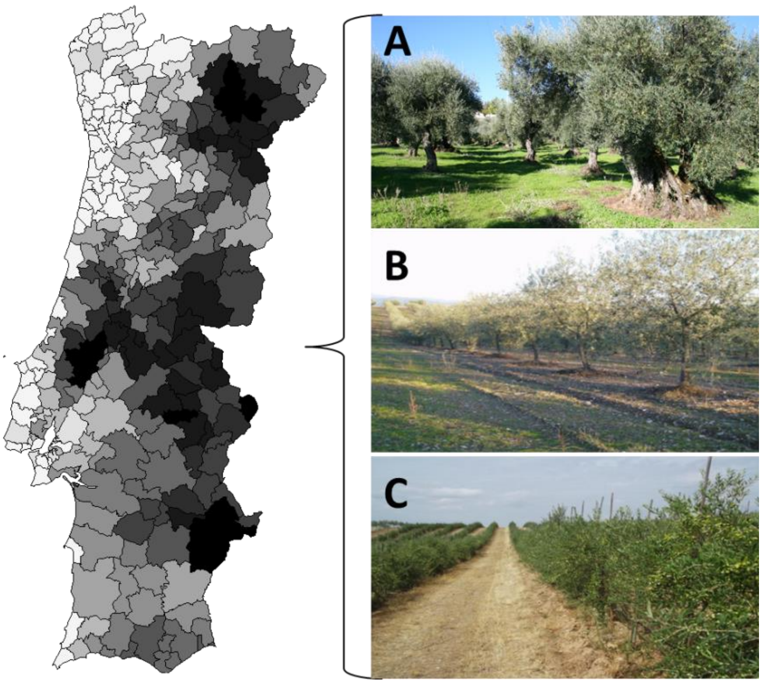

Figure 1.4. Map of the olive tree distribution in Portugal, and the tree main production systems. A- traditional olive grove; B- intensive olive grove; C- high intensive olive grove.

In the last decade, the number of high intensive olive groves increased in the Alentejo region due to the favorable conditions of soil, big areas and availability of water, being actually this region the first producing region of Portugal, representing more than 70% of the olive oil produced in the 2017 campaign.

Portugal has a great genetic heritage of olive germplasm represented by many "old" local cultivars, some of which have a restricted geographic distribution (Moreira, & Veloso, 2009). Olive trees are grown throughout the country, mainly in the interior, and represent 9.2% of the Utilized Agriculture Area (UAA). Such genetic variability contributes to the differentiation of the oil produced and determines the resilience of the crop. The capability to adapt to changes in the presence of biotic and abiotic factors such as pests, diseases and climate stresses is very important, particularly in areas where climate changes can become more serious. Trás-os-Montes, in the northeastern of the country, is the second Portuguese olive producing region, currently representing 12 to 15% of the national olive oil production. Although the region has lost its expression of production, olive cultivation continues to be an activity of great importance, not only from an economic point of view, but also a social, landscape and environmental point of view. In this region, traditional olive groves dominate being present in almost of 50% of the farms. There are a great number of locations with centenarian trees distributed for the entire region (Figure 1.5). In this region there is also a great diversity of olive cultivars, the best known being *cvs.* Cobrançosa, Verdeal Transmontana, Madural, Cordovil, Santulhana and Negrinha de Freixo. Nevertheless a high number of specimens are uncharacterized and their cultivars unknown.

The sustainability of the olive agroecosystems can be threatened by the emergence of modern systems of olive cultivation that have low genetic diversity. The identification of the existing genetic diversity is essential for the management and preservation of olive germplasm. This process should begin with morphological studies of the olive cultivars, such as the characteristics of the endocarp (Trujillo et al., 2014), which serve to discriminate olive trees. Methodologies involving molecular markers should complement the morphological characterization. In Trás-os-Montes region there is a great genetic heritage and landscape, but there is a great lack of knowledge especially regarding minor cultivars with regional or local expression.

Figure 1.5. Centenarian olive trees found on the region of Trás-os-Montes.

Throughout the region different cultivars of olives are recognized but, unfortunately, nobody possesses a true knowledge of their characteristics, lacking its formal and scientific characterization that would allow their further commercial exploitation. For the majority of these cultivars only ancient trees, many of them centenarians, exist. Some of these cultivars produce differentiated oils both with different physicochemical compositions and sensory attributes. Therefore, an urgent systematic study of these specimens is necessary to avoid larger genetic diversity losses. In this sense, the preservation and appreciation of this region's rich genetic heritage is of utmost relevance.

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Chapter 2

Objectives

2.1. General objective

Trás-os-Montes is the second Portuguese region of olive oil production. Traditionally, the quality of the olive oil produced in this region is highly recognized, which is probably related with the environmental characteristics of the region and with the extensive production system, with a great part of the olive production being made in traditional olive groves. But this quality is mostly related with the high olive genetic heritage of the region that could be at risk of being lost. There are a great number of groves with centenarian trees distributed through the entire region, with specimens belonging to unknown cultivars that could be a source of differentiated olive oils. On the other side, in the last years there has been a demand for differentiated products in the olive oil market, with oleaster oils representing the possibility of new products. In this context, the general objective of this work was to characterize genetically and morphologically oleaster populations and centenarian olive trees from the northeast of Portugal, as well as, to chemically and sensory evaluate the extracted oils, aiming the selection of plant specimens with the purpose of future valorization. The latter aspect is of the utmost importance, since centenarian specimens and the minor cultivars to which they belong give olive oils with unique sensory characteristics which must be exploited and valued.

2.2. Thesis structure

The thesis was structured in ten different chapters, respecting different matters, and, in six of them, the specific objective are mentioned.

Chapter 1. A brief overview about the *Olea europaea* L. origin and distribution, their importance in the world and in Portugal, a summary chemical characterization of oleaster, olives and olive oils, and the different olive production systems.

Chapter 2. The general objectives and structure of the thesis were presented.

Chapter 3. This chapter details the general experimental design of the work, sampling of plant materials, and oil extraction that were applied throughout the developed work.

Chapter 4. In this chapter the comparison of genetic diversity, structure and phylogenetic relationships within and among the oleaster populations from different locations and centenarian olive plants from the experimental grove were studied using 16 microsatellite markers.

Chapter 5. The chemical characterization of the oleaster oil obtained from three different populations of the north of Portugal (Alijó, Moncorvo and Vila Nova de Foz Côa) was made evaluating different constituents such as fatty acids, individual phenolic contents, tocopherols and sterols.

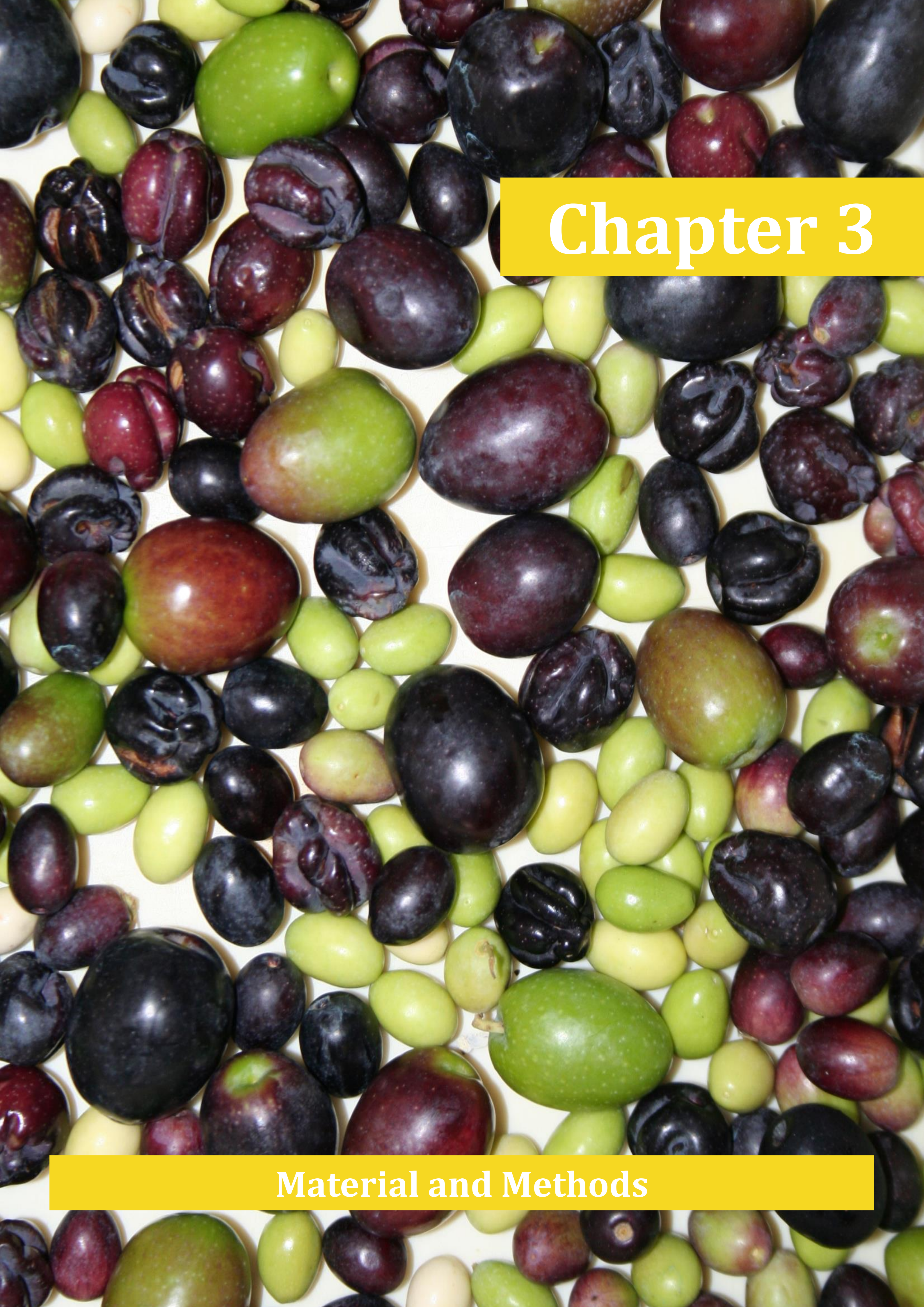
Chapter 6. The evaluation of the phenolic composition of olive oils extracted from centenarian olive trees, aiming to select potential producers of differentiated olive oils high rich in phenolics as possible candidates for inclusion in breeding programs, was presented in this chapter.

Chapter 7. Olive oils from six minor autochthonous olive cultivars (Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) produced from centenarian trees were chemical and sensorial characterized to contribute for their exploration and valorization as a means of preserving the olive heritage of the Trás-os-Montes region.

Chapter 8. In this chapter, the tocopherols contents of the olive oils of the six minor cultivars, along five crop years, were studied, in order to explore their diversity and future potential, taking into account the oil with the highest bioactive richness.

Chapter 9. The study of the fatty acid composition of the olive oil from the six minor cultivars from five consecutive crop years was made, aiming to select cultivars richer in oleic acid and monounsaturated fatty acids.

Chapter 10. The general conclusions of the work were presented.



Chapter 3

Material and Methods

Chapter 3

3. Experimental design

3.1. Field sites and trees selection

The experimental part of the present work was carried out from 2013 to 2018. Samples were collected in different locations of the Northeast of Portugal. The sampling site for the study of the centenarian olive trees (*Olea europaea* ssp. *europaea* var. *europaea*) is located near Mirandela (Suções, N 41º 29' 26.628''; W 7º 15' 31.219'') (Figure 3.1), in one centenarian grove (≈ 250 years). This grove was chosen because, as far as we known, it includes the oldest trees of the region, from diverse cultivars, being most of them unknown. From the total of140 trees that are in this grove, 28 trees were selected and individually marked. These trees were chosen because they seem to be the oldest and the most representative of grove diversity, based on their appearance, structure and trunk thickness.

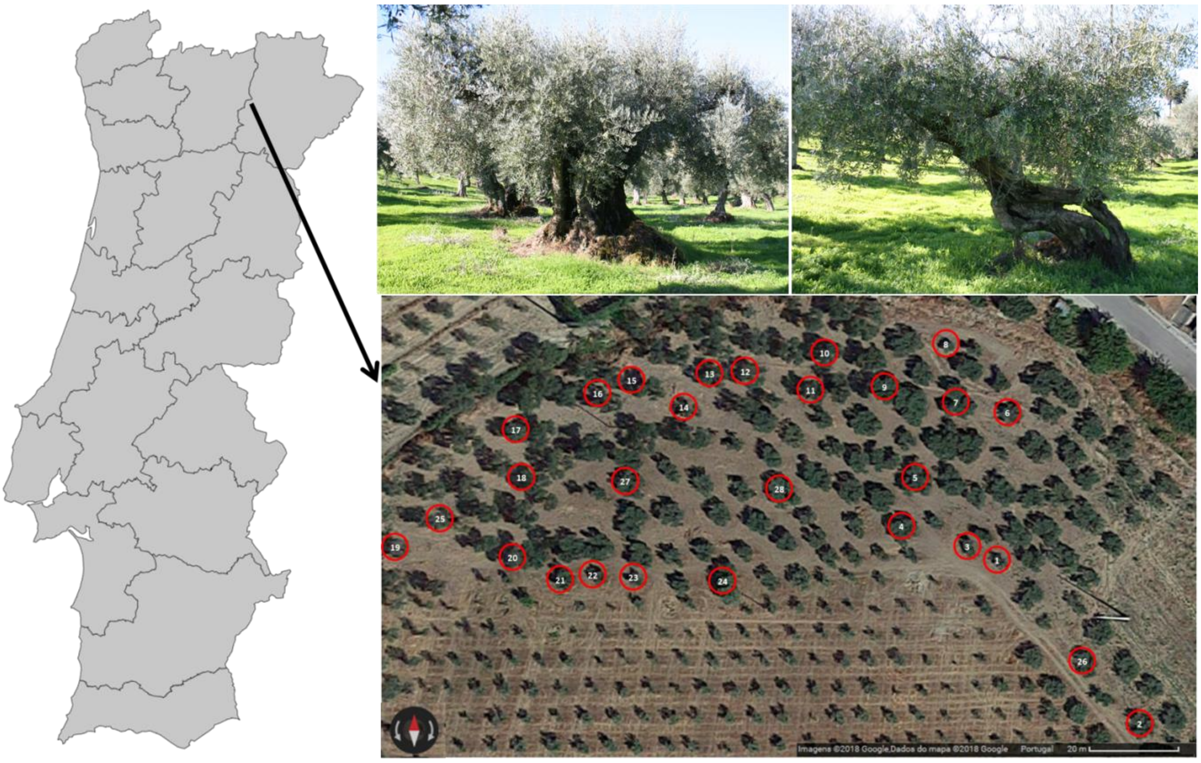


Figure 3.1. Geographical location of selected centennial olive grove, and distribution of the trees in the grove, in the northeast of Portugal, near Mirandela (Suções, N 41º 29' 26.628''; W 7º 15' 31.219'').

The sampling sites for the study of oleaster (*O. europaea* subsp. *europaea* var. *sylvestris*) populations are located in three different municipalities of the northeast of Portugal, namely *Alijó* (N 41º 12'7.045''; W 7º 29' 25.444''). *Moncorvo* (N 41º 11' 57.498''; W 7º 5'

46.302'') and *Vila Nova de Foz Côa* (N 41º 8'6.058''; W 7º 7'54.005'') (Figure 3.2). In each location, four different oleaster plants were randomly selected for samples collection.

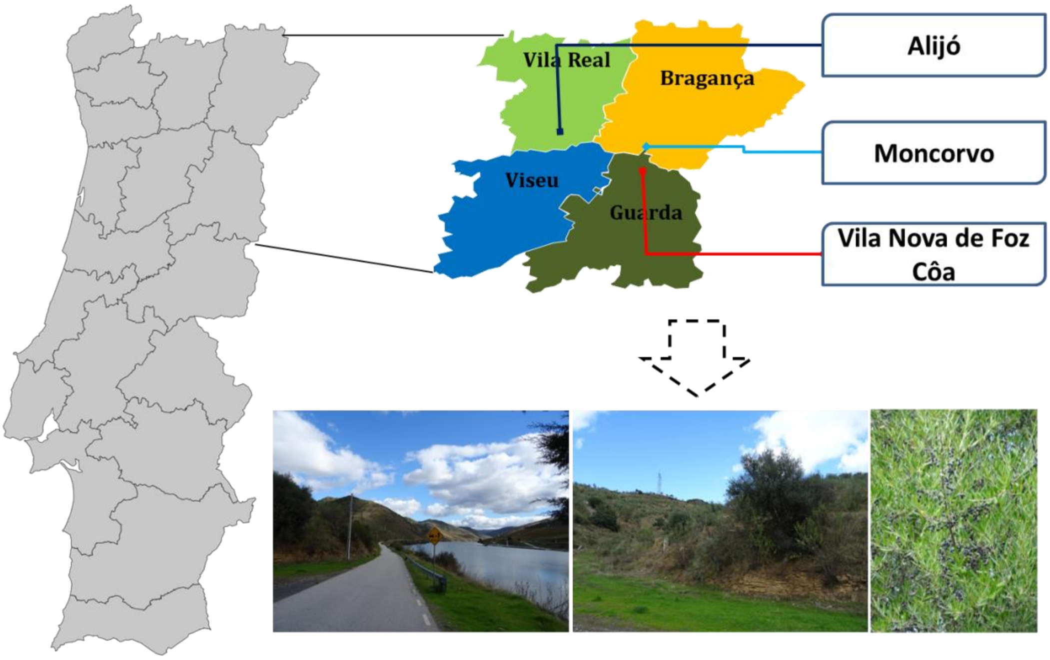


Figure 3.2. Geographical location of the regions where the oleaster populations were collected.

3.2. Sample collection

3.2.1. For morphological and genetic characterization

From each marked plant (olive tree and oleaster) two kind of samples were collected.
For the genetic characterization, from each plant, one young branch without signs of disease or pest infestation was collected. In the lab, from each branch/plant ten young leaves were randomly selected, ground to a fine powder in liquid nitrogen and further stored at −80°C until DNA extraction. The detailed description of the procedure used can be found in the material and methods section of the Chapter 4.
For morphological characterization, one sample of around 500g of olive fruits from each plant was collected. Fruits were harvested at maturity index (MI) of two (MI 2) and three (MI 3), which was defined based on fruit color of the skin. Fruits on an index of "IM 2" show their epidermis with red spots in less than an half of the fruit, whereas fruits showing the epidermis red or purple in more than an half of the olive were classified as "IM 3". From each sample 40 fruits were randomly selected for morphological characterization. This characterization was performed according to the International Union for the Protection of New Varieties of Plants (UPOV) guidelines for olive cultivars

(UPOV, 2011). All fruit samples were morphologically characterized using biometric parameters applied to both the fruit and the endocarp of the same fruit. A more detailed description of the procedure used can be found in the material and methods of Chapter 5.

3.2.2. For chemical characterization

Fruit samples from the 28 centenarian trees were taken along five consecutive crop years (from 2013 till 2017). From each tree, approximately three kilograms of healthy fruits were handpicked. The collected olive fruits had nearly the same maturity index (MI), ranging from two (MI 2) and three (MI 3). Thus, every year the harvest occurred during the month of November, namely on the 25th and 26th (in 2013); on the 10th and 11th (in 2014); on the 2nd and 3rd (in 2015); on the 07th and 08th (in 2016); and, on the 13th and 14th (in 2017).

Fruit samples from the oleaster populations were taken on 10th and 11th November 2016. In each location, four adult oleaster plants were randomly selected. From each plant, approximately two kilograms of healthy fruits were handpicked.

3.3. Oil extraction

Fruits were processed within 24 h of harvesting, in a pilot extraction plant with an Abencor analyzer (Comercial Abengoa S.A., Seville, Spain), with three main units: a mill, a thermobeater where malaxation takes place at controlled temperature, and a centrifuge. Fruits were milled, the paste was homogenized, and about 700 g were transferred to the thermobeater unit (20 min) for malaxation, using a thermostatic water bath at 25ºC. In the final 5 min of each malaxation, 100 mL of water at 25 ºC was added to enhance the oil separation. The mixture was centrifuged, decanted, and the oil collected. After that, the oils were prepared for analysis, being filtered (Whatman paper nº 4) over anhydrous sodium sulfate in order to remove the solid particles and residual water. The oils were stored in 125 mL dark bottles and protected from light exposition, at room temperature.

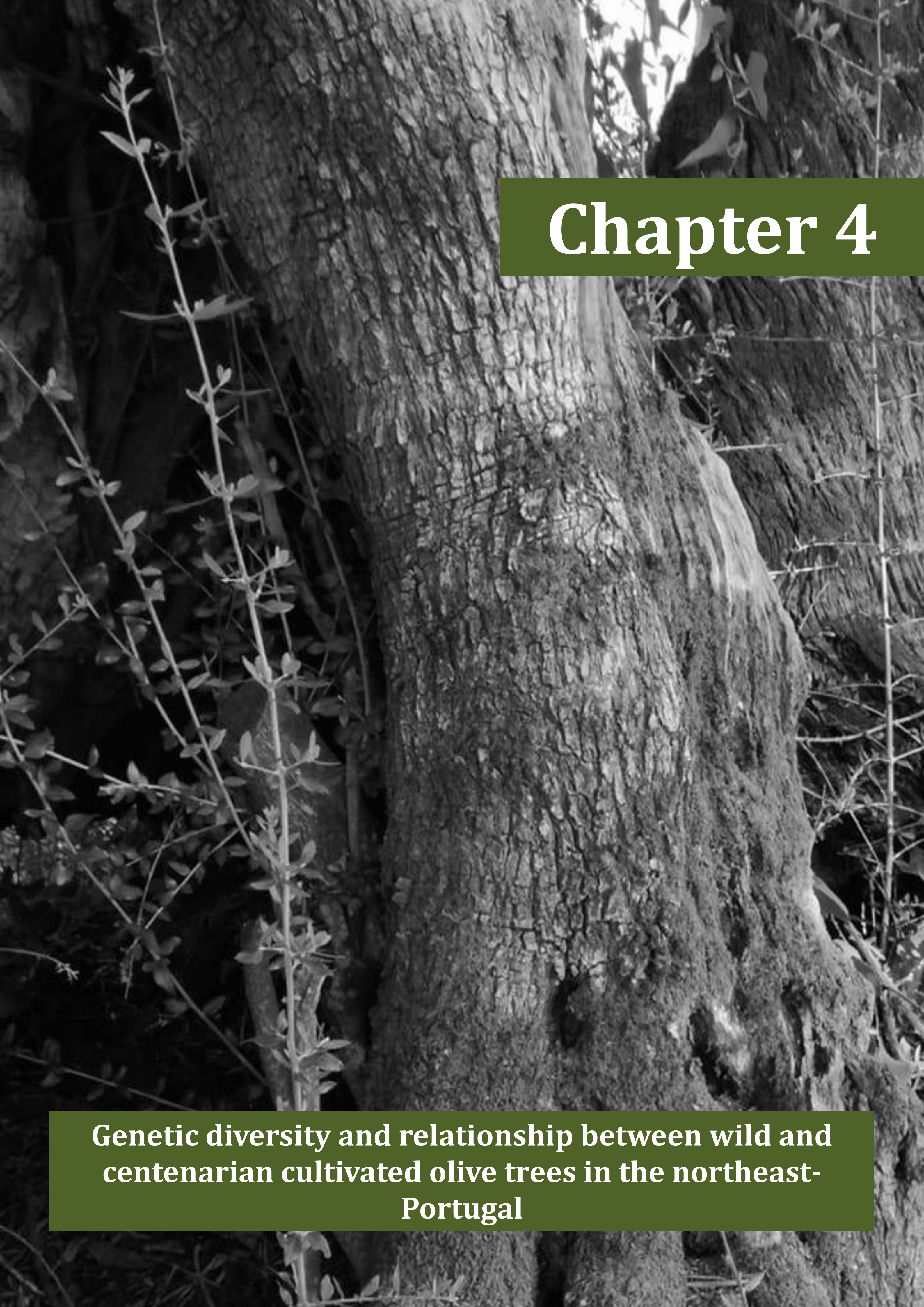
3.4. Evaluations in the oils

The different oils samples, from the centenarian specimens and oleaster plants, were subjected to different analysis. In general, all the assays were carried out in triplicate, within two months of extraction.

The following parameters were evaluated:

- Phenolic compounds: for the centenarian specimens of the crop years from 2014 to 2017 and oleaster oils;
- Fatty acids composition: for the centenarian specimens of the crop years from 2013 to 2017 and oleaster oils;
- Tocopherols composition: for the centenarian specimens of the crop years from 2013 to 2017 and oleaster oils;
- Quality parameters: for the centenarian specimens of the crop years 2016-2017;
- Descriptive sensory analysis: for the centenarian specimens of the crop years 2016 and 2017;
- Sterols composition: oleaster oils;
- Total phenols content: for the centenarian specimens of the crop years 2016 and2017;
- Oxidative stability (Rancimat): for the centenarian specimens of the crop years 2016 and 2017.

The detailed description of the different methods can be found in the material and methods sessions of Chapter 5, 6, 7, 8 and 9.



Chapter 4

Genetic diversity and relationship between wild and centenarian cultivated olive trees in the northeast-Portugal

Chapter 4

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Under preparation to be submitted

Chapter 4

Genetic diversity and relationship between wild and centenarian cultivated olive trees in the northeast-Portugal

Abstract

Two different of *Olea europaea* L. are known. The cultivated, the olive tree (*O. europaea* subsp. *europaea* var. *europaea*) and the wild, the oleaster (*O. europaea* subsp. *europaea* var. *sylvestris*). The comparison of genetic diversity, structure and phylogenetic relationships within and among the oleaster and centenarian plants were studied using microsatellite markers. In general, both centenarian olive tree and oleaster populations growing in Trás-os-Montes region present high genetic diversity, with no differences between them. Their genetic differentiation was significant. At least four olive genetic clusters, two of centenarian trees and two of oleaster specimens, were identified, providing evidence for the existence of a significant genetic structure in this region. Within centenarian olive trees several domestication processes might be happened in this region. Some of these trees seem to have originated from local oleasters, while other trees have an unknown origin. These findings open new questions that must be addressed. Future experiments should involve examining large scale patterns of genetic structure.

Chapter 4

Chapter 4

4.1. Introduction

The olive (*Olea europaea* L.) is one of the most economically important crops in Mediterranean countries, including Portugal. Nowadays, two different types of olive-growing areas are possible to distinguish in Portugal. In the southern (*i.e.* Alentejo region), the intensive olive monocultures have been replaced the traditional olive farming system (Tous et al., 2014), accounting already 75% of the total olive growing area of this region (INE 2016). In these new and/or reconverted plantations, new cultivars that are best adapted to this intensification system, have been introduced (Tous et al., 2014). "Arbequina" was the main cultivar introduced, representing 80% of the total growing area of this region (Tous et al., 2014). In contrast to southern, the biophysical characteristics of the northern related particularly with the climatic conditions, slope of the lands and orchard size, jeopardize the chances of olive intensification. Thus, most of the olive orchards in the northern of Portugal (*i.e.* Trás-os-Montes region) follow the traditional system (Duarte et al., 2008). This cropping system is associated with old or very old trees grown at low densities, and in non-irrigated conditions, that have been maintained by local communities for centuries (Duarte et al., 2008). These trees hold great genetics, agronomical, landscape and historical importance, and should be valorized as local production at high regional value. To date, the identity and genetic characterization of these centenarian olive trees have never been examined. Such studies have been already initiated in other Mediterranean countries, such as in Italy (Salimonti et al., 2013), Cyprus (Anestiadou et al., 2017), Spain (Diez et al., 2011), Israel and Palestine (Barazani et al., 2014), and have reveal that most ancient olive trees are unknown traditional cultivars, that might represent early stages in the domestication processes of the olive. Although the exact history of the olive tree domestication is unknown, it is widely accepted that the cultivated varieties (*Olea europaea* subsp. *europaea* var. *europaea*) were derived by the wild olive (*Olea europaea* subsp. *europaea* var. *sylvestris*), called oleaster (Besnard et al., 2014; Besnard et al., 2017). Previous studies suggest the existence of three geographic origins of cultivated olive trees, corresponding to the west, center and east Mediterranean Basin (Diez et al., 2012; Diez et al., 2015). Cultivated olive diversification seems to be derived not only from admixture between local oleasters and cultivated trees across these Mediterranean areas, but also from local independent domestication events (Besnard et al., 2013; Diez et al., 2015). Admixture between differentiated cultivated gene pools may also have contributed to olive diversification (Diez et al., 2015; Besnard et al., 2017).

Genetic variation of oleaster and their relationship with centenarian olives have never been evaluated in Trás-os-Montes region (Portugal). In this region, genuine oleaster populations can be found growing mostly in adverse conditions either in forest areas or in areas not far from olive groves (Porto et al., 2018). A better knowledge of the genetic diversity of these oleaster populations could have high importance for future olive breeding programs, because they could be a source of wild alleles for confer valuable traits (Baldoni et al., 2006; Belaj et al., 2007; Erre et al., 2010). The study of their relationship with centenarian olives may also bring new insights about the historical processes that produce locally adapted cultivars.

This study used microsatellite markers to examine the genetic diversity, structure and phylogenetic relationships within and among wild and centenarian olive trees growing in Trás-os-Montes region. Three oleaster populations, which were growing far from the centenarian olives (up to 340 km), were included to investigate the effects of scale on structure and differentiation of their populations. With this study we want to address the following questions: Is the genetic variation within oleaster higher compared to that found in centenarian olive populations? Is oleaster genetically different from centenarian cultivated olive trees? Is there any evidence for admixture between centenarian and wild populations at local and regional scale?

4.2. Material and methods

4.2.1. The studied populations

The sampling of centenarian olives (*Olea europaea* ssp. *europaea* var. *europaea*) were performed in one centenarian grove (≈ 250 years), located near Mirandela (Suçães, Table 4.1; Figure 4.1). This grove was chosen because, as far as we known, it includes the oldest trees of the region, from diverse cultivars, being most of them unknown. A total of 28 centenarian trees were selected from across the entire grove for sampling. These trees were selected because they seem to be the oldest and the most representative of the genetic diversity of this population, based on their appearance, structure and trunk thickness. For the sampling of oleaster (*Olea europaea* subsp. *europaea* var. *sylvestris*), four sites were selected (Table 4.1; Figure 4.1) in order to get different distances from the centenarian grove, ranging from short (< 35 km) to long distances (>340 km). The number of oleaster trees studied in each site was related to population size. Where possible, oleaster trees were sampled randomly along widely spaced transects through the collecting areas in order to represent their genetic diversity. The oleaster trees sampled were confirmed to be true wild olives based on phenotypic parameters of endocarp and fruit, and of their resembling shrubby phenotype of southeastern Mediterranean var. *sylvestris*. In each population, two branches with less than 2 years old from the mid-canopy were randomly harvested per individual. In the laboratory, the young leaves were detached from the branches and used for DNA extraction.

Table 4.1. Location and number of individual of the centenarian olive trees and oleaster populations analyzed.

Population	Localization	Sample size	Code	Coordinates
Centenarian olives	Mirandela (Suções)	28	AS	N 41º 29' 26.628''; W 7º 15' 31.219''
Oleaster	Alijó	6	WR	N 41º 12'7.045''; W 7º 29' 25.444''
	Moncorvo	4	WS	N 41º 11' 57.498''; W 7º 5' 46.302''
	Vila Nova de Foz Côa	4	WP	N 41º 8'6.058''; W 7º 7'54.005''
	Alentejo	4	WA	N 40° 45´ 36"; W 73° 59´ 2.4"

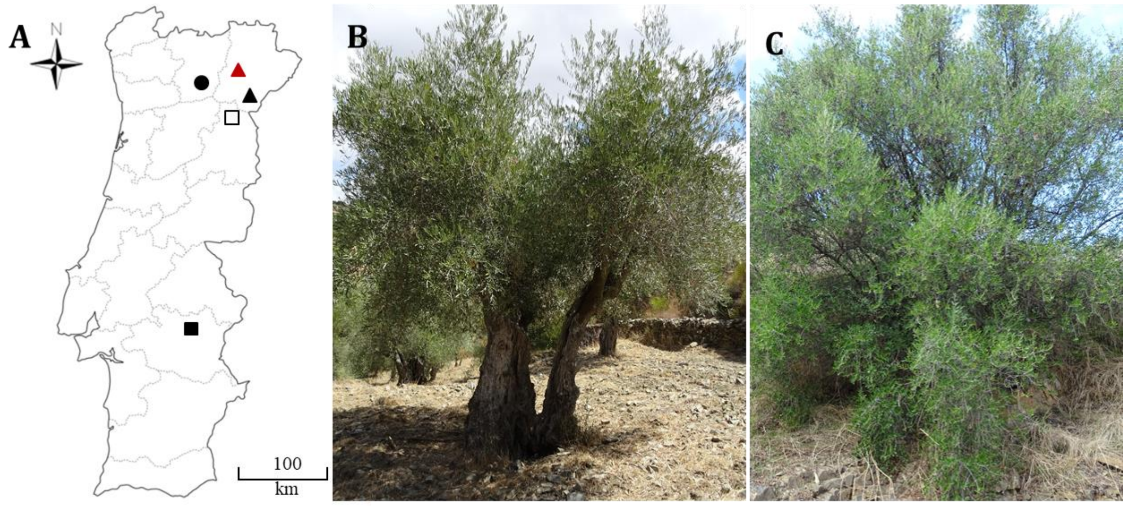

Figure 4.1. Map of Portugal indicating the olive (*Olea europaea* ssp. *europaea*) populations studied (a). Centenarian population is colored in red (▲) while oleaster populations are colored in dark (● - Alijó; ▲ - Moncorvo; □ - Vila Nova de Foz Côa; ■ - Alentejo). Example of (B) centenarian olive tree and (C) oleaster analyzed in this study.

4.2.2. SSR analysis

DNA was isolated from 100 mg of fresh young leaves ground in liquid nitrogen using the method described by Sá et al. (2011). DNA quality was checked on 0.8% agarose gel, and the DNA concentration was estimated using a microvolume spectrophotometer, mySPEC Twin (VWR, Germany). Previously published SSR markers that were proved to be suitable for the characterization of olive varieties (Sefc et al., 2000; Carriero et al. 2002; Cipriani et al., 2002; De la Rosa et al., 2002; Sabino Gil et al., 2006) were tested for the presence of genetic variation. Of these, 12 resulted in polymorphic, clear and scorable profiles and were used in this study for the genetic characterization of the samples (Table 4.2).

Table 4.2. SSR markers used, their expected size range and repeated motives.

SSR Marker	Expected Range	Repeat motif	References
GAPU-71B	117-140	$GA(AG)_6(AAG)_8$	Carriero et al., 2002
EM090	185-191	$(CA)_{10}$	De la Rosa et al., 2002
DCA09	162-205	$(GA)_{23}$	Sefc et al., 2000
UDO99-011	100-128	$(CT)_7(CA)_{10}(CT)_2(CA)_2CT(CA)_2CT(CA)_9$	Cipriani et al., 2002
DQ386912	186-203	$(CA)_9(GA)_2$	Sabino Gil et al., 2006
UDO99-043	172-221	$(GT)_{12}$	Cipriani et al., 2002
DCA16	122-200	$(GT)_{13}(GA)_{29}$	Sefc et al., 2000
DCA03	229-263	$(GA)_{19}$	Sefc et al., 2000
GAPU-101	184-218	$(GA)_8(G)_3(AG)_3$	Carriero et al., 2002
DCA14	172-189	$(CA)_{18}A_6(TAA)_7$	Sefc et al., 2000
DCA5	196-212	$(GA)_{15}$	Sefc et al., 2000
DCA18	159-185	$(CA)_4CT(CA)_3(GA)_{19}$	Sefc et al., 2000

PCR was conducted in a final volume of 20 µL containing 20 ng of DNA, 2 µl 5X colorless GoTaq® reaction buffer (Promega), 2.5 mM $MgCl_2$, 200 µM each dNTPs, 0.25 µM each primer (forward primer was labeled with either 6-FAM or HEX fluorescent dyes), and 0.03 units of GoTaq® DNA Polymerase (Promega). The PCRs were carried out in a MyCycler Thermal Cycler (Bio-Rad) with an initial denaturation at 94ºC for 4 min; followed by 35 cycles of 92ºC for 30 s, 50-55ºC for 30s, and 72ºC for 1 min; and then a final extension at 72ºC for 7 min. PCR products were separated by capillary electrophoresis that was outsourced to Stab Vida (http://www.stabvida.com/) and genotype profiles were evaluated in GeneMapper v. 4.1 (Applied Biosystems). The sizes of the amplified products were determined based on an internal standard included with each sample.

4.2.3. Data analysis

4.2.3.1. Genetic diversity. GenAlEx version 6.4 (Peakall, & Smouse, 2006) was used to characterized the genetic diversity of the populations analyzed by calculating several parameters for each microsatellite locus, such as number of different (Na) and effective (Ne) alleles, number of observed (Ho) and expected (He) heterozygosity, number of private alleles (Npa), Shannon's information index (I) and inbreeding coefficient (f).

4.2.3.2. Population structure and differentiation. The genetic structure of the oleaster and centenarian olive populations were analyzed using Principal Coordinate Analysis (PCoA) implemented in the program GenAlEx version 6.4 (Peakall, & Smouse, 2006) and also by the STRUCTURE, using the software Structure version 2.3.4 (Pritchard et al., 2000). This software applies a Bayesian clustering algorithm to identify subpopulations, and assign individuals to them. It sorts individuals into K clusters, according to their genetic similarity. For this analysis was applied the admixture model and no prior population information was used. To determine the optimal number of groups (K), 20 independent runs for K values ranging from 1 to 10 were performed with a burn-in length of 250,000 followed by 750,000 iterations (Markov chain Monte Carlo, MCMC). The optimal number of genetic clusters (K) was determined using the ad hoc statistic ΔK, based on the rate of change in the log probability of data between successive K values (Evanno et al., 2005).

The amount of genetic differentiation among centenarian olives and oleaster populations were assessed by estimating the fixation index F_{ST} (Wright, 1951), implemented in the GenAlEx version 6.4 (Peakall, & Smouse, 2006). The same software was also used to quantified the amount of gene flow (Nm) among populations

4.3. Results and discussion

4.3.1. There are differences in polymorphism level detected by each SSR marker between oleaster and centenarian olives

In this study was analyzed a total 46 genotypes (*i.e.* 28 centenarian olives and 18 oleaster) sampled in five populations, in Portugal. The 12 SSR markers used in this study were highly polymorphic. The average number of alleles per locus in the total of genotypes was 9.7, ranging from five (at locus GAPU-71B) to 13 (at loci UDO99-043 and DCA18) (Figure.

S4.1). Despite all the 12 SSRs were found to be polymorphic and able to distinguish the 46 genotypes, their ability to assess the genetic diversity on oleaster is different from the ones of centenarian olives. According to the diversity parameters estimated (Na, Ne, I, Ho and He), the most and the less polymorphic and informative loci among oleaster was DCA03 and EM090 while among centenarian olives was DCA18 and DCA5 (Table S4.1). A similar ability have been previously reported for these loci in several diversity analysis of Mediterranean olive tree populations (*e.g*. Baldoni et al., 2009; Díez et al., 2011; Cicatelli et al., 2013). Similarly, alleles private to the oleaster genotypes were represented mostly within loci DCA09, DCA16, DCA03 and DCA05, whereas among centenarian olives, DCA18, DCA16 and UDO99-043, were the loci that had the highest number of unique alleles.

4.3.2. Oleaster and centenarian olive genotypes showed similar amount of genetic diversity

The various genetic diversity parameters estimated for the whole oleaster and the whole centenarian olive genotypes exhibited high levels of genetic diversity, with minimal genetic differences between them (Table 4.3). Within the centenarian olives, the mean Na, Ne, I and Npa were 6.67, 3.45, 1.38 and 3.25, respectively; which were very similar to the ones estimated within oleaster (6.42, 3.95, 1.49 and 3.00, respectively). The high diversity values observed in this study are comparable to the ones reported previously either for wild (Erre et al., 2010; Belaj et al., 2010; Díez et al., 2015) or ancient cultivated olive trees (Erre et al., 2010; Díez et al., 2011; Díez et al., 2015) assessed with microsatellites, and thus confirming the high polymorphism of this species (*e.g*. Roubos et al., 2010). As far as we known, our study is the first ones providing novel insights into the genetic diversity of both oleaster and centenarian olive tree in Trás-os-Montes region. Based on our results, this region seems to represent a hot spot of both oleaster and centenarian olive genetic diversity, as similarly reported previously for Spain (Belaj et al., 2007; Díez et al., 2015). For both oleaster and centenarian olive tree populations the mean observed heterozygosity (Ho = 0.72 and 0.73) showed slightly higher values than the expected heterozygosity (He = 0.66 and 0.70) (Table 4.3), as reported in other studies (Erre et al., 2010; Díez et al., 2011). Accordingly to the heterozygosity excess, the f exhibited negative values (-0.07) within both oleaster and centenarian olive population, pointing to low levels of inbreeding.

Table 4.3. Genetic diversity within centenarian olives and oleaster (either in total or in each population, WR-WA): mean number (± SD) of different (Na) and effective (Ne) alleles, Shannon's information index (I), observed (Ho) and expected (He) heterozygosity, private alleles (Npa) and inbreeding coefficient (f).

		Na	Ne	I	Ho	He	Npa	f
Centenarian olives		6.67±2.02	3.45±1.32	1.38±0.38	0.72±0.27	0.66±0.16	3.25±0.69	-0.07±0.02
	Total	6.42±0.74	3.95±0.41	1.49±0.13	0.73±0.07	0.70±0.05	3.00±0.55	-0.07±0.02
	WR	4.58±1.56	3.40±1.20	1.28±0.42	0.67±0.28	0.66±0.18	1.00±0.35	-0.05±0.01
Oleaster	**WS**	2.50±1.62	2.12±1.28	0.78±0.54	0.69±0.43	0.45±0.29	0.25±0.13	-0.55±0.09
	WP	2.75±1.77	2.24±1.34	0.84±0.56	0.63±0.42	0.47±0.29	0.25±0.13	-0.36±0.13
	WA	1.83±0.58	1.79±0.51	0.55±0.34	0.75±0.45	0.38±0.23	0.33±0.19	-0.96±0.03

One possible explanation of such result might be related with the technique used to propagate the olive tree, which is mostly based on vegetative multiplication. As previously stated, asexual reproduction can be responsible for maintaining high heterozygosity levels or even for increasing heterozygosity by the accumulation of mutations over generations (Radosavljević et al., 2015). Once permanently fixed, the new mutations cannot be lost through genetic drift because of the presence of non-sexual (Radosavljević et al., 2015). In addition, both oleaster and centenarian olives are long-lived tree species and, therefore, the generation-number related decay of heterozygosity due to drift is to be expected to be relatively small (Kassa et al., 2017).

The genetic diversity of the four oleaster subpopulations (WR-WA) was found to differ (Table 4.3). The WR subpopulation showed the highest Na (4.58), Ne (3.40), I (1.28) and Npa (1.00), while the WA subpopulation had the lowest genetic diversity values (Na = 1.83, Ne = 1.79, I = 0.55). Curiously, these two subpopulations also differ strongly on the patterns of excess heterozygosity. In WA, the observed heterozygosity was noticeably higher than the expected (0.75 vs. 0.38, respectively), and the f value substantial negative (f = -0.96). A different pattern was noticed in the WA subpopulation, in which there was no noticeable difference between the Ho and He.

4.3.3. Most of the centenarian genotypes are well-differentiated from oleaster population genetic structure

The PCoA (Figure 4.2) and STRUCTURE (Figure 4.3) results demonstrated a clear genetic differentiation between oleaster and centenarian olive tree populations. However, several subgroups can be observed for these two populations indicating genetic variability among genotypes in each population. Within the centenarian olive tree population, it is possible to observed two genetic clusters either in the STRUCTURE at K≥2 or in the PCoA, in the second axis. Surprisingly, one of this subgroup (blue colored; Figure 4.3) showed a close relationship with oleaster genotypes from WR, WS and WP populations. This result suggests that these centenarian olive trees have probably originated from populations of local oleasters, still present in this region. These centenarian olive trees were probably selected directly from local oleasters, as previously suggested for other Mediterranean countries (Baldoni et al., 2006; Díez et al., 2015). In contrast, the second subgroup of centenarian olives (orange colored; Figure 4.3) is genetically well-differentiated from oleaster populations, leading to the conclusion that these centenarian genotypes did not develop from local oleaster. Thus, it is possible to see different domestication process within the centenarian olive trees growing in Trás-os-Montes region. Within oleaster population, two subgroups also appear at K≥3 (Figure 4.3) which was consistent with the analyses from PCoA (Figure 4.2). At K≥3 WA population separates from the rest of the oleaster populations. Geographically, WA population is located in the south of Portugal whereas the other oleaster populations are located in the North. The genetic separation of WA population from the others oleaster populations, as well as their highest heterozygosity levels (Table 4.3), may suggest that these trees represent genuine local oleasters. Due to their uniqueness, with genotypes likely to harbor novel alleles, these oleaster populations should be prioritized as source of germplasm for breeding.

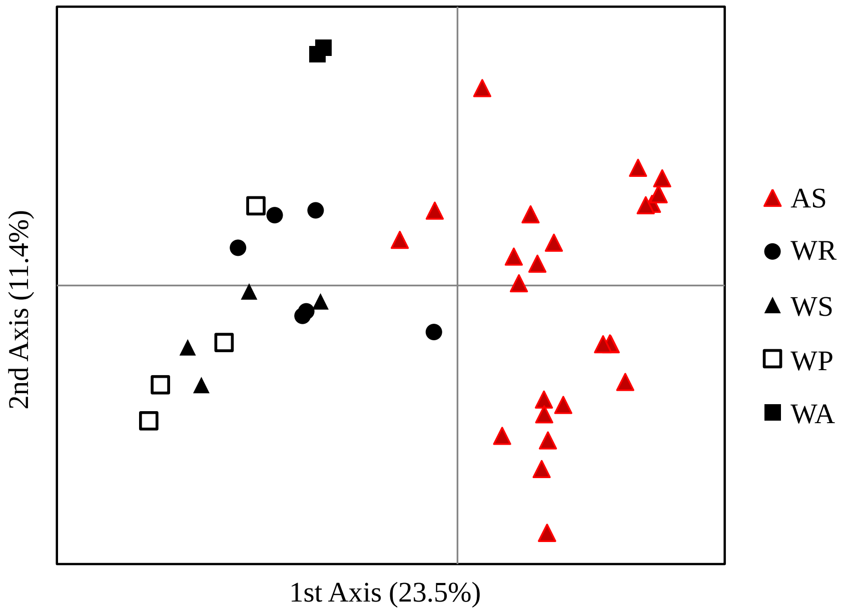

Figure 4.2. Principal Coordinates Analysis (PCoA) of SSR data showing the clustering of populations *Olea europaea* ssp. *europaea*: Centenarian population is colored in red (▲) while oleaster populations are colored in dark (● Alijó - WR; ▲ Moncorvo -WR; □ Vila Nova de Foz Côa - WP; ■ Alentejo - WA).

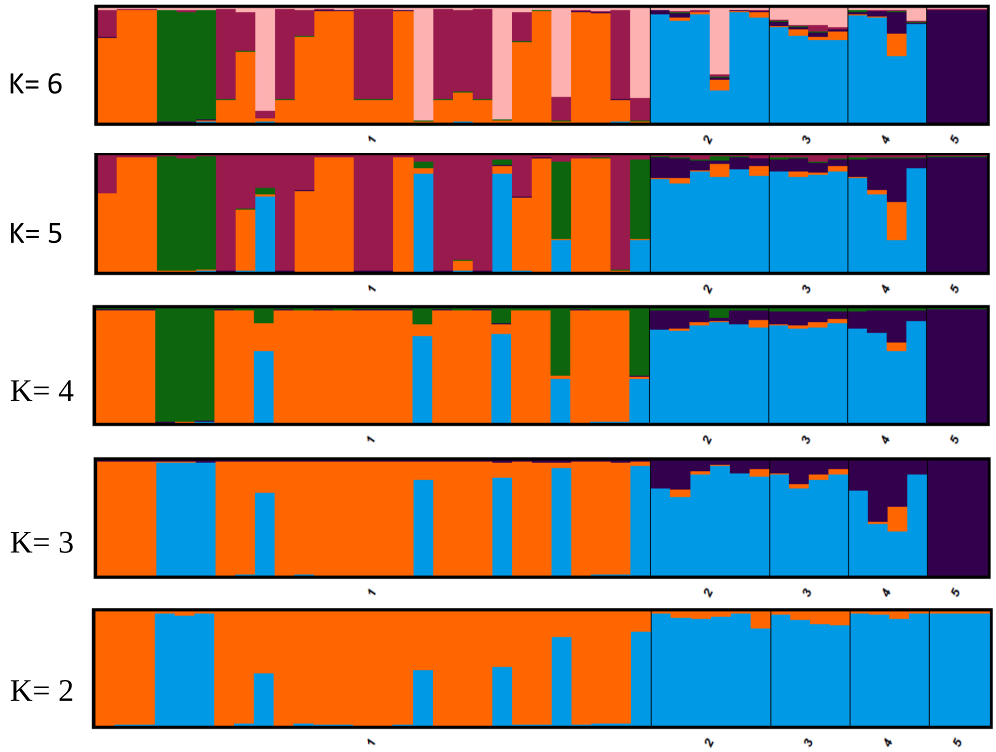

Figure 4.3. Bayesian model-based estimation of population structure for the 46 *Olea europaea* ssp. *europaea* genotypes in five populations (x-axis): 1-AS; 2- WR; 3- WS; 4- WP; 5- WA. Black line separates the five populations. The label of each population is indicated in table 1. The best K choice based on the ΔK method was K=4. Due to their biological relevance, other genetic structure K = 2, K = 3, K = 5 and K = 6 are also displayed.

4.3.4. Genetic Differentiation

The estimated genetic differentiation (Fst) and level of gene flow (Nm) among the two populations (*i.e* centenarian and oleaster olive tree) was 0.102 and 2.203, respectively. Comparisons made between subpopulations highlighted two different scenarios (Table 4): a) the comparison between centenarian *vs*. oleaster subpopulations indicated an increase of genetic differentiation with the increase of geographic distance. A strong genetic differentiation was found between the geographically separated populations WA and AS (Fst = 0.269); b) the comparison between oleaster subpopulations, indicated that geographic distance did not have greater effect on population genetic structure and differentiation. Oleaster subpopulations that are close to each other in distance (WP and WS: 5km apart) displayed a Fst value similar to the ones observed to populations that are further apart (WA and WP or WS: 310 km apart). It is more likely that differentiation of oleaster subpopulations might be caused by environmental selection (host specialization), as the external conditions were different where the two subpopulations occurred.

Table 4.4- Pairwise population Fst values. Centenarian population (AS), oleaster population located in Alijó (WR), Moncorvo (WS), Vila Nova de Foz Côa (WP) and Alentejo (WA).

	AS	WR	WS	WP	WA
AS	0.000				
WR	0.107	0.000			
WS	0.196	0.187	0.000		
WP	0.239	0.179	0.291	0.000	
WA	0.269	0.211	0.334	0.338	0.000

4.4. Conclusion

Overall, we have found high genetic diversity either within centenarian olive tree or within oleaster populations, growing in Trás-os-Montes region, with no differences between them. Their genetic differentiation was significant. At least four olive genetic clusters (2 of centenarian and 2 of oleaster) were identified, providing evidence for the existence of a significant genetic structure in the Trás-os-Montes region and supporting this region as an important genetic reservoir. Part of the centenarian olives growing in this region seem to have originated from local oleasters, while other part has an unknown origin. Therefore, our findings open new questions that must be addressed. Future experiments should involve examining large scale patterns of genetic structure

Aknowledgements

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Supplementary information

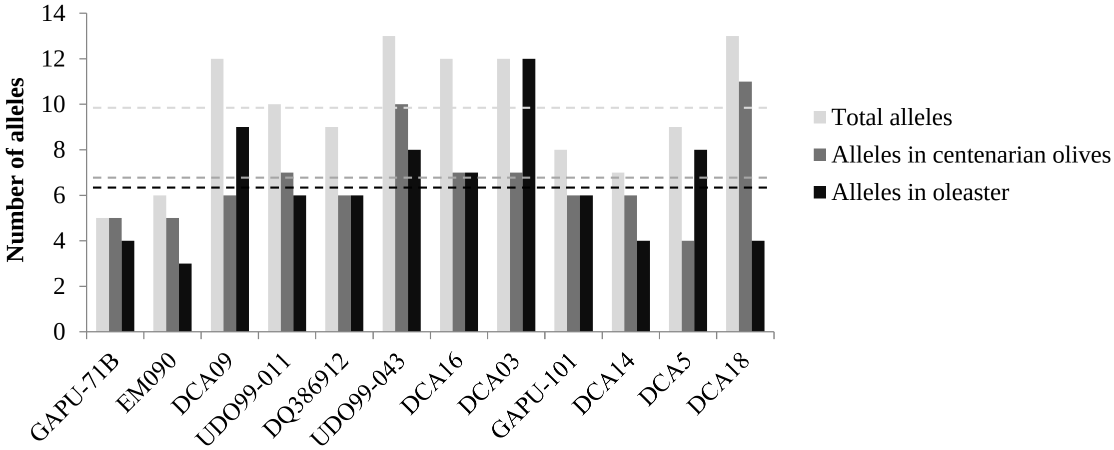
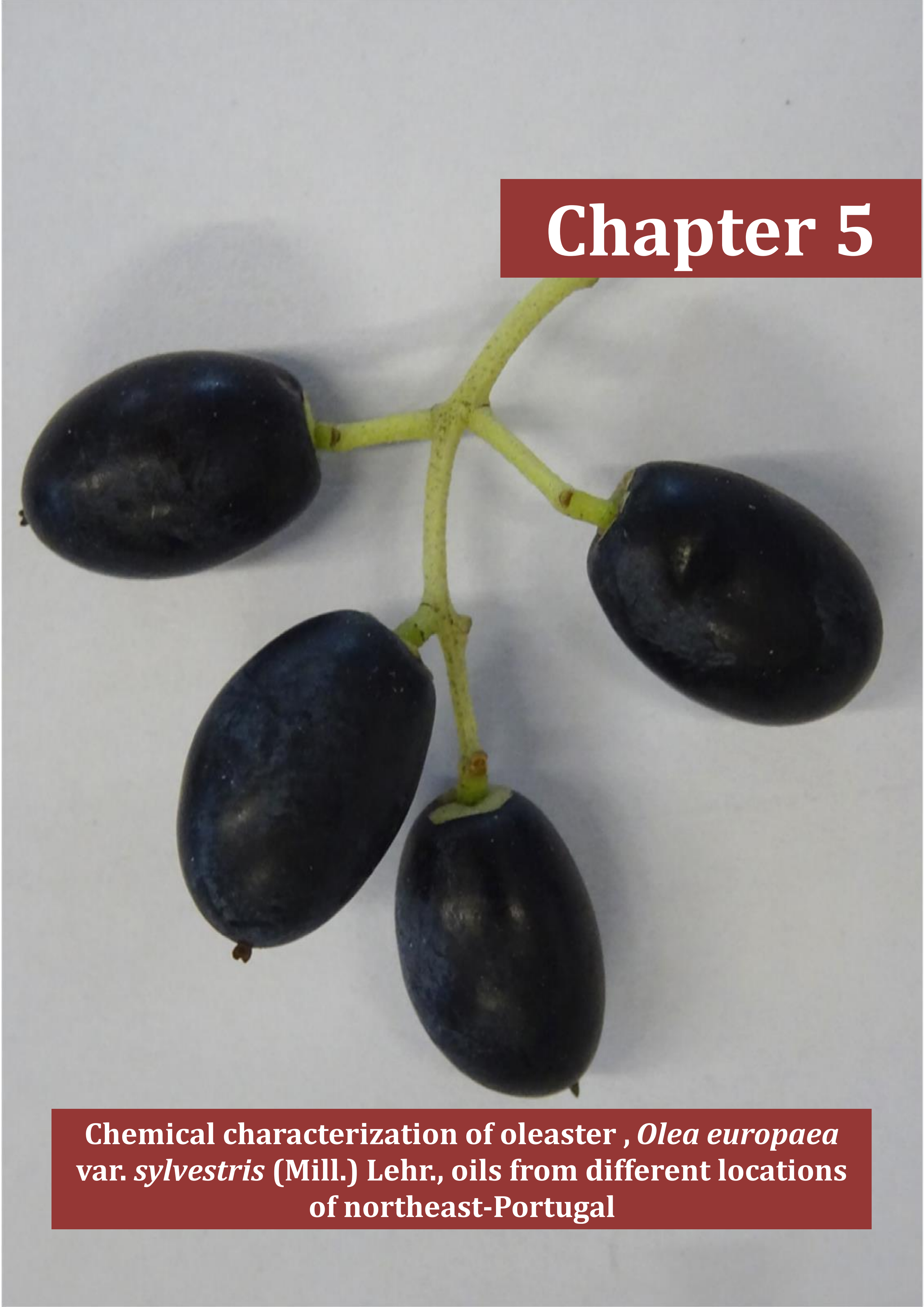


Figure S4.1. Number of alleles per locus found in the total of genotypes analyzed (total alleles), and in the centenarian (alleles in centenarian olives) and oleaster (alleles in oleaster) olives. The dashed lines indicate the average number of alleles in the total of genotypes (light grey), centenarian olives (dark grey) and oleaster (black).

Table S4.1. Summary statistics of genetic variation at 12 SSR loci in the whole centenarian olives and oleaster genotypes. N—Sample Size; Na—number of alleles; Ne—effective number of alleles; I - Shannon's Information Index; Ho—observed heterozygosity; He—expected heterozygosity; Npa—number of unique alleles. Differences between SSR loci are highlighted in bold.

		N	Na	Ne	I	Ho	He	Npa
Centenarian olives	GAPU-71B	28	5	4.03	1.49	**1.00**	0.75	1
	EM090	28	5	1.62	0.76	0.43	0.38	3
	DCA09	28	6	3.95	1.54	0.68	0.75	3
	UDO99-011	28	7	4.31	1.59	0.79	0.77	4
	DQ386912	28	6	2.95	1.30	0.68	0.66	3
	UDO99-043	28	10	3.54	1.65	0.89	0.72	5
	DCA16	27	7	3.34	1.41	0.96	0.70	5
	DCA03	28	7	4.59	1.66	0.79	0.78	-
	GAPU-101	23	6	3.55	1.44	**1.00**	0.72	2
	DCA14	28	6	1.96	1.04	0.39	0.49	3
	DCA5	28	<u>4</u>	<u>1.51</u>	<u>0.69</u>	<u>0.18</u>	<u>0.33</u>	1
	DCA18	28	**11**	**6.10**	**2.00**	0.89	**0.84**	**9**
Oleaster	GAPU-71B	15	4	2.20	1.02	0.67	0.54	-
	EM090	17	<u>3</u>	<u>1.27</u>	<u>0.43</u>	<u>0.24</u>	<u>0.22</u>	1
	DCA09	17	9	4.51	1.84	0.71	0.78	**6**
	UDO99-011	17	6	3.30	1.43	0.82	0.70	3
	DQ386912	17	6	4.78	1.69	0.71	0.79	3
	UDO99-043	17	8	4.62	1.71	0.71	0.78	3
	DCA16	11	7	3.72	1.60	0.36	0.73	5
	DCA03	16	**12**	**6.17**	**2.12**	0.75	**0.84**	5
	GAPU-101	12	6	4.88	1.67	**1.00**	0.80	2
	DCA14	5	4	2.94	1.19	**1.00**	0.66	1
	DCA5	11	8	5.63	1.87	0.82	0.82	5
	DCA18	5	4	3.33	1.28	**1.00**	0.70	2

Chapter 5



Chemical characterization of oleaster , *Olea europaea* var. *sylvestris* (Mill.) Lehr., oils from different locations of northeast-Portugal

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Submitted

Chemical characterization of oleaster, *Olea europaea* var. *sylvestris* (Mill.) Lehr., oils from different locations of Northeast-Portugal

Abstract

Oleaster (*Olea europaea* var. *sylvestris*), or wild olive tree, has great interest as source of genetic material for olive breeding programs. Nevertheless, information about its oil composition is scarce. In the present work, the characterization of oleaster oil from three different tree populations from the northeast-Portugal (Moncorvo, Alijó and Vila Nova de Foz Coa) was carried out, with respect to their composition in fatty acids, tocopherols, sterols and phenolic compounds, together with morphological parameters of the fruits. Fatty acid composition showed to be very similar to olive oil, constituted mainly by oleic acid (68.9-70.6%), followed by palmitic (14.2-14.7%) and linoleic acid (7.87-9.88%). α-tocopherol represented more than 90% of the tocopherols detected, with total tocopherol values between 263 and 503 mg/kg of oil. β-sitosterol was the major sterol, and total sterol ranged from 1742 to 2198 mg/kg of oil, again within olive oil regulation. Fourteen phenolic compounds, distributed by five families, were identified and quantified. Ligstroside derivatives and oleuropein aglycon (and derivatives) were the major ones, varying from 271 to 359 mg/kg of oil for the first, and 227 to 261 mg/kg of oil, relevant from an health point of view. The results indicate that the oil mechanically extracted from wild olive trees was highly consistent from a compositional point of view, meet all major requirements for olive oil classification, and was particularly rich in antioxidants that could be further explored in breeding programs to increase the amounts of bioactive compounds in cultivated olive oils.

Keywords: wild olive tree, fatty acids, tocopherols, sterols, phenolic compounds.

Chapter 5

5.1. Introduction

The olive tree, *Olea europaea* subsp. *europaea*, is a typical species of the Mediterranean basin, traditionally limited to Europe and Africa (Breton et al., 2008). This crop was one of the first to be domesticated, apparently in the Middle East, from where it spread to the west of the Mediterranean basin with human migrations (Breton et al., 2009). During this process, not all plants showed good adaptation to domestication, or characteristics considered of interest for selection, originating two varieties of *O. europaea*: the cultivated forms, the olive tree (*O. europaea* subsp. *europaea* var. *europaea*), and the wild forms, the oleasters (*O. europaea* subsp. *europaea* var *sylvestris*) (Besnard & Bervillé, 2000; Breton et al., 2006; Breton et al., 2008; Hannachi et al., 2013). Thus, cultivated olive tree and oleaster are considered by botanists two varieties of *O. europaea* subsp. *europaea* (Breton et al., 2008). The main phenotypic difference between both is that the flesh is thicker and the fruits are larger in most cultivated varieties (Breton et al., 2008). Nevertheless, other differences could be mentioned: oleasters have smaller leaves, prickly juvenile shoots in the lower branches, fruits with a lower ratio of pulp/endocarp and lower fat content, a longer juvenile stage and a greater ability to survive in harsh environments (Terral & Arnold-Simard, 1996; Bouarroudj et al., 2016).

Several studies have shown that olive oil obtained from cultured olive trees have beneficial effects on human health, with beneficial biological properties to the consumer (Tanjour, 2014). Motivated by this recognition, together with its exquisite sensorial attributes, the cultivated olive tree is now distributed throughout the world, with particular success in areas with a climate similar to that of the Mediterranean region (Bouarroudj et al., 2016). It is now one of the most consumed virgin edible oil in the world due to its good characteristics, such as its pleasant aroma and taste, and its high resistance to oxidation (Bouarroudj et al., 2016). As to *O. europaea* subsp. *europaea* var *sylvestris*, although not a cultivated species, it has been studied mainly due to its key role in the development and selection of new olive cultivars, with a recognized capacity for adaptation and survival and superior olive oil quality (Baccouri et al. al., 2010).

Olives obtained from the oleaster represent a distinctive element of the Mediterranean flora (Rubio de Casas et al., 2006). However, information regarding the oil extracted from these wild olive trees is scarce, in particular its composition (Hannachi et al., 2013; Baccouri et al., 2018). Some studies reported that oleaster oil has higher levels of antioxidants and oleic acid than the oil obtained from cultivated olive trees (Dabbou et al., 2011), highlighting the high potential of oleaster oil as a phytochemicals source, and as a possible alternative food and resource to improve the quality of olive oil (Bouarroudj et al., 2016).

In Portugal, oleaster is commonly distributed throughout the country, mainly in the central and southern regions. In the north region, although the olive tree in the cultivated form is dominant, specimens of oleaster can still be found in zones near important rivers like Douro, Sabor and Côa, not being used as fruits producers. Thus, in this context, the aim of the present work was to characterize the oil obtained by fruits of three oleaster populations from different municipalities located in the north of Portugal, Alijó, Moncorvo and Vila Nova de Foz Côa (VNFC), in terms of its composition in fatty acids, individual phenolic contents, tocopherols and sterols contents, to evaluate the possibility of using oleaster trees in breeding programs and also its oil as an innovative food rich in bioactive compounds.

5.2. Material and Methods

5.2.1. Sampling

Three different locations where oleaster (*O. europaea* L. ssp. *sylvestris* (Miller) Lehr synonym of *O. europaea* L. ssp. *oleaster* (Hoffmanns. & Link) Negodi) plants exist were chosen, being located in three municipalities of the northeast of Portugal, namely: Moncorvo (Foz do Sabor, N 41º 11' 57.498''; W 7º 5' 46.302''), Vila Nova de Foz Côa (VNFC) (Pocinho, N 41º 8'6.058''; W 7º 7'54.005'') and Alijó (Romaneira, N 41º 12'7.045''; W 7º 29' 25.444''). In each location, four adult oleaster plants with fruitification were randomly selected considering each plant as an independent tree. On November 2016, from each plant, approximately two kilograms of fruits were manually picked. All fruits were visually inspected and the fruits damaged or attacked by pests and diseases were discarded. From each olive sample, a sub-sample of 40 fruits was taken for morphological characterization and the remaining fruits were processed for oil extraction. All fruit samples were morphologically characterized using biometric parameters applied to both the fruit and the endocarp of the same fruit according to International Union for the Protection of New Varieties of Plants (UPOV) guidelines for *Olea europaea* (UPOV, 2011). The following parameters were evaluated: weight (g), length (mm), maximum diameter (mm), shape, symmetry in A and B positions, position of maximum transverse diameter at B position, apex in A position, base, nipple, number and dimension of the lenticules, color at full maturity, rugosity of surface, number of grooves on basal end and distribution of grooves on basal end. Fruit shape was calculated from the ratio between the maximum length/width, which may lead to a classification of the fruit shape as spherical (length/width ratio < 1.25), ovoid (1.25 ≤ length/width ratio ≤ 1.45) or elongated (length/width ratio > 1.45). For oil extraction, fruits were processed during the first 24 h after harvest, in a pilot extraction plant with an Abencor analyzer (Comercial Abengoa S.A., Seville, Spain), with three main units: a mill, a thermobeater where malaxation takes place at controlled temperature, and a centrifuge. Fruits were milled, the paste was homogenized, and about 700 g were transferred to the thermobeater unit (20 min) for malaxation, using a thermostatic water bath at 25ºC. In the final 5 min of each malaxation, 100 mL of water at 25 ºC was added to enhance the oil separation. The mixture was centrifuged, decanted, and the oil collected. After that, the oils were prepared for analysis, being filtered (Whatman paper nº 4) over anhydrous sodium sulfate in order to remove the solid particles and residual water. The oils were stored in 100 mL dark bottles and protected from light exposition, at room temperature. All the assays were carried out in triplicate within two months after extraction.

5.2.2. Fatty acids composition

Fatty acids were evaluated as their methyl esters after cold alkaline transesterification with methanolic potassium hydroxide solution (Commission Regulation (EEC 2568/91 of 11th July) and extraction with n-heptane. The fatty acid profile was determined with a Chrompack CP 9001 chromatograph equipped with a split-splitless injector, a FID detector, an autosampler Chrompack CP-9050 and a 50 m x 0.25 mm i.d. fused silica Select FAME capillary column (Varian). Helium was used as carrier gas at an internal pressure of 110 kPa. The temperatures of the detector and injector were 270 ºC and 250 ºC, respectively. The split ratio was 1:50 and the injected volume was of 1 µL. The results are expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area eluting between myristic and lignoceric methyl esters. A control sample (olive oil 47118, Supelco) and a fatty acids methyl esters standard mixture (Supelco 37 FAME Mix) was used for identification and calibration purposes (Sigma, Spain).

5.2.3. Tocopherols composition

Tocopherols composition was determined according to the ISO 9936 (2006), with some modifications as described by Rodrigues et al. (2012). Tocopherols standards (α-, β- and γ) were purchased from Sigma (Spain), while the internal standard 2-methyl-2-(4,8,12-

trimethyltridecyl)chroman-6-ol (tocol) was from Matreya Inc. (Pleasant Gap, PA, USA). Filtered olive oil (50 mg) was mixed with 10 μL of the internal standard solution (tocol, 100 μg/ml prepared with *n*-hexane). The mixture was centrifuged for 5 min at 13,000 rpm and the obtained supernatant analyzed by HPLC. The liquid chromatograph consisted of a Jasco integrated system (Japan) equipped with a Jasco LC-NetII/ADC data unit, a PU-1580 Intelligent Pump and a FP-920 fluorescence detector (λ_{exc}= 290 nm and λ_{em}= 330 nm). The chromatographic separation was achieved on a Supelcosil™ LC-SI column (3 μm) 75 x 3.0 mm (Supelco, Bellefonte, PA), operating at 23 ºC. A mixture of n-hexane and 1,4-dioxane (97.5:2.5) was used as eluent, at a flow rate of 0.7 mL/min. Data were analyzed with the ChromNAV Control Center - JASCO Chromatography Data Station (Japan). The compounds were identified by chromatographic comparisons with standards, considering the co-elution retention time and according to their UV spectra. Quantification was based on the internal standard method, using the fluorescence signal response and individual calibration curves for each tocopherol. Total vitamin E corresponded to the sum of the individual tocopherol contents.

5.2. 4. Sterols composition

The qualitative and quantitative sterol contents of the samples were determined in accordance with the official European analysis methods described in Annexes V and VI of Regulation EEC / 2568/91 of the Commission of the European Union. Briefly, oil samples were saponified with ethanolic potassium hydroxide solution in the presence of dehydrocholesterol as internal standard, followed by concentration of extracted unsaponifiables and separation on silica gel plates. The sterol fraction was extracted, silylated and analyzed on a GC–FID Thermo Finnigan (Milan, Italy), using a Zebron ZB-5HT Inferno (30m x 0.25 mm x 0.25 μL; Phenomenex, USA), with a temperature program from 250ºC to 280ºC. Helium (Gasin, Portugal) was used as carrier gas at an internal pressure of 0.6 ml/min. The results for individual sterols were reported in relative percentage and the total sterols amounts in mg/kg, as internal standard equivalents. Identification was achieved by comparing the retention times with commercial standards (Sigma-Aldrich, Germany) except for Δ^5-avenasterol and Δ^7-stigmastenol, tentatively identified by comparison with literature references. Apparent β-sistosterol, which is an important quality indicator, was calculated as the sum of Δ^5-avenasterol, clerosterol and β-sitosterol, as indicated in the EEC Regulation.

5.2.5. Phenolic Compounds

Phenolic compounds extraction was carried out according to the protocol of the International Olive Council (COI/T.20/Doc No 29/Rev.1 2017), with minor modifications. Briefly, the phenolic compounds were extracted with methanol/water solution (80:20, v/v), in the presence syringic acid as internal standard, followed by addition of *n*-hexane to the methanolic solution for removal of fat remains. The solution was concentrated under a gentle nitrogen stream (40ºC) and analyzed by high performance liquid chromatography (HPLC) with a diode array detector. Separation was accomplished on a C18 reversed-phase column (Kinetex C18 2.6 µm 100Å, 100 x 3.00 mm, Phenomenex), at 35ºC, using a gradient of water and acetonitrile, both with 0.1% of formic acid, at a flow rate of 0.8 mL. Peak identification was performed by comparing retention times and UV/Vis spectra (200 to 600 nm) with those of pure standards (tyrosol, hydroxytyrosol, vanillic acid, ferulic acid, *o*-coumaric acid, luteolin, cinnamic acid, apigenin, and oleuropein, from several suppliers). Ligstroside derivatives tentative identification was oriented by the COI method and available literature. For quantification, UV/Vis detection wavelengths were set to 280 nm (for simple phenols, vanillic acid, vanillin, and secoiridoids), 325 nm (for coumaric and ferulic acids), and 365 nm (for flavonoids). According to the COI guidelines, results were expressed as mg of tyrosol equivalents per kg of oil for each individual compound while the total phenols content corresponded to the sum of all individual compounds quantified.

5.2.6. Statistical analysis

One-way analysis of variance (one-way ANOVA) was applied to evaluate the existence of statistical significant effects of the sampling geographical location on the different physicochemical and sensory parameters of oils. Moreover, if a significant statistical effect was found ($P < 0.05$), the post-hoc multi-comparison Tukey's test was also applied aiming to identify the levels of each effect that were responsible for the detected significant effect. Also, depending on the parameters evaluated, boxplots were used to show the one-way ANOVA statistical results.

The possibility of using the physicochemical profiles of the oils to discriminate the three different geographical grown locations of the oleaster trees was further evaluated using Linear Discriminant Analysis (LDA), a supervised multivariate pattern recognition technique, coupled with the simulates annealing (SA) variable selection algorithm. The use of the SA algorithm allowed identifying which physicochemical parameters possessed the highest discrimination capability. For LDA, the values of the different parameters were centered and scaled minimizing data variability. The quality of the discrimination performance was assessed considering the correct classification rate for the original grouped data as well as for the internal cross-validation leave-one-out procedure (LOO-CV). Besides, the classification performance of the LDA-SA model was also graphically evaluated using 2-D plot of the two main discriminant functions, being plotted the class membership boundary lines established using the posterior probabilities, computed using the Bayes' theorem (which enables controlling overfitting issues) (Bishop, 2006).The statistical analysis was performed using the Subselect (Cadima et al., 2004; Cadima, Cerdeira, Silva, & Minhoto, 2012; Kuhn, & Johnson, 2013) and MASS (Venables, & Ripley, 2002) packages of the open source statistical program R (version 2.15.1), at a 5% significance level.

5.3. Results and discussions

5.3.1. Morphological characterization

The morphological characterization of the oleaster fruits and endocarps was performed according to UPOV parameters (Table 5.1). Fruit weight, length and width were all higher in the Alijó population, and usually lower in the VNFC population (Table 5.1). Variations of fruit shape were observed, from ovoid (fruits collected at Alijó location) to elongated ones (fruits collected at Moncorvo and VNFC locations). Compared with cultivated olives from traditional cultivars in the northeast of Portugal, as Cobrançosa, Cordovil, Madural and Negrinha de Freixo, oleaster fruits had in general lower fruit measures (Peres el al., 2011). As can be also seen in Table 5.1, all populations produced asymmetric fruits, centered considering the position of maximum transverse diameter at B position, and with a rounded apex. Some differences were obtained for the base parameter, which was either rounded (Alijó location) or truncated (Moncorvo and VNFC locations). For the remaining parameters (presence of nipple, number of lenticules, color when the fruit reaches the definitive color when mature) similar ranges were found among the three studied populations (Table 5.1). Concerning the morphological parameters of the endocarp, the stone weight varied from 0.20 g (Alijó location) to 0.28 g (VNFC location), similar to the lower results reported by Laaribi et al. (2017) for ancient native olive accessions in Central-Eastern Tunisia populations (i.e, 0.15 g to 1.23 g, for endocarps) and 3.5 to 6.0 times lower than the weights usually reported for the endocarps of cultivated olive cultivars of the same Portuguese region (Peres et al., 2011).

Table 5.1. Biometric and morphological characteristics of fruit and endocarp (mean ± standard deviation), in percentage (%) of occurrence, of oleaster fruits obtained from different geographic locations (Alijó, Moncorvo and Vila Nova de Foz Côa).

Olive Fruit	Weigth	Length (mm)	Width (mm)	Shape	Symm.A	T Diam.B	Apex.	Base	Nipple	Nº. of lent.	D. of lent.	Total Fat (% fresh matter)
Alijó	**1.08±0.38**	**15.20±2.41**	**10.54±1.47**	**1.45±0.16**	**Asymmetric**	**Central**	**Rounded**	**Rounded**	**Little evident**	**Very numerous**	**Small**	**8.14±0.49**
	(0.46-2.28)	(1.83-17.46)	(3.77-8.42)	Ovoid	66.2%	100.0%	76.9%	65.0%	100.0%	100.0%	100.0%	
Moncorvo	**0.72±0.26**	**13.34±1.09**	**8.93±1.51**	**1.52±0.25**	**Asymmetric**	**Central**	**Rounded**	**Truncated**	**Little evident**	**Very numerous**	**Small**	**5.75±0.51**
	(0.12-1.44)	(8.38-13.81)	(3.70-10.73)	Elongated	72.5%	100.0%	75%	81.2%	100.0%	100.0%	100.0%	
Vila Nova de Foz Côa	**0.59±0.27**	**12.18±2.04**	**8.33±1.21**	**1.55±1.28**	**Asymmetric**	**Central**	**Rounded**	**Truncate**	**Little evident**	**Very numerous**	**Small**	**6.81±0.85**
	(0.26-1.65)	(6.84-15.22)	(4.38-8.30)	Elongated	67.5%	100.0%	100.0%	85.0%	75.0%	75.0%	83.1%	

Stone	Weigth	Length (mm)	Width (mm)	Shape	Symm.A	Symm.B	T Diam.B	Apex. A	Base	Rugosity of surface	Nº. of grooves	Distribution of grooves
Alijó	**0.20±0.08**	**12.47±2.06**	**6.47±0.57**	**1.92±0.32**	**Asymmetric**	**Weakly asymmetric**	**Central**	**Rounded**	**Acute**	**Medium**	**Medium**	**Grouped around suture**
	(0.06-0.75)	(1.83-17.46)	(3.77-8.42)	Elliptic	81.2%	8.25%	81.2%	96.2%	58.75%	100.0%	63.1%	90.0%
Moncorvo	**0.24±0.06**	**11.20±0.92**	**6.00±0.76**	**1.89±0.23**	**Asymmetric**	**Weakly asymmetric**	**Central**	**Rounded**	**Rounded**	**Medium**	**Low**	**Grouped around suture**
	(0.07-0.49)	(8.38-13.81)	(3.70-10.73)	Elliptic	83.1%	87.5%	89.4%	73.8%	58.1%	100.0%	71.9%	81.9%
Vila Nova de Foz Côa	**0.28±1.03**	**9.96±1.66**	**5.63±0.75**	**1.77±0.20**	**Asymmetric**	**Weakly asymmetric**	**Central**	**Rounded**	**Rounded**	**Medium**	**Medium**	**Grouped around suture**
	(0.09-0.52)	(6.84-15.22)	(4.38-8.30)	Ovoid	74.4%	81.2%	100.0%	75.0%	54.4%	100.0%	54.4%	81.9%

Among the three populations, the lowest values for endocarps length, width and shape were obtained in VNFC population, being consistently higher in the Alijó population. For the other parameters the majority of the results were similar for the three locations, with few exceptions (Table 5.1). Concerning total fat contents, in fresh weight the values ranged between 5.75±0.51% in Moncorvo and 8.14 ±0.49% in Alijó (Table 5.1), lower than the values usual for olive cultivars.

5.3.2. Fatty acids composition

The fatty acids composition of the oils extracted from fruits of different oleaster populations are shown in Table 5.2. Oleic acid ($C_{18:1}$) was the major fatty acid, varying from 68.9% and 70.6%, with highly homogeneous contents between the three populations studied, in opposition to the ranges reported from other geographical regions like Tunisia, which varied from 47 to 72%, (Hannachi et al., 2013) and 48.4% to 71.1% (Baccouri et al., 2008), or Algeria, with values ranging from 64.7% to 76.1% (Bouarroudj et al., 2016). Also, the oleic acid contents were within the regulated values for virgin olive oil according to Commission Regulation (EEC 2568/91). Palmitic acid ($C_{16:0}$), ranging from 14.2 to 15.2%, and linoleic acid ($C_{18:2}$), varying from 7.9 to 9.9%, were the second and third major fatty acids, also with similar values in the different locations. Once again the content ranges are more homogeneous that those observed by the previously mentioned authors. Nevertheless, some minor fatty acids showed significant differences between the three population: stearic acid ($C_{18:0}$) was more abundant in the VNFC population than in Alijó and Moncorvo (P = 0.0102). In opposition, the amounts of linolenic acid ($C_{18:3}$) were significantly higher in the Alijó population (P = 0.0098), as was eicosenoic acid ($C_{20:1}$). The oleaster oils from Moncorvo were significantly richer in heptadecanoic ($C_{17:0}$) and heptadecenoic ($C_{17:1}$) acids, while VNFC showed the highest amounts of arachidic acid ($C_{20:0}$) (Table 5.2). The sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) had no significant differences between the different populations (Table 5.2). The highest fraction as expected were the MUFA, varying from 71.9% (Alijó population) and 73.0% (Moncorvo population), followed by SFA (16.9-18.2%) and PUFA (8.8-10.9%). When comparing these results with the fatty acid composition of Portuguese traditional cultivars (*cvs.* Cobrançosa, Madural, and Verdeal Transmontana) (Gonçalves et al., 2012), similar profiles could be found. Also, all the values are in agreement with the legal maximum values established by the Commission Regulation (EEC 2568/91) for olive oil.

Table 5.2. Fatty acids profile (%) of oils extracted from oleaster (mean ± standard deviation) collected from different geographical locations (Alijó, Moncorvo and Vila Nova de Foz Côa). Different lower case letters mean significant statistical differences at a 5% significance level (one-way ANOVA followed by the Tukey's multi-comparison test).

Fatty acid profile (%)	Olive oil[1]	Alijó	Moncorvo	Vila Nova de Foz Côa	One-way ANOVA (*P*-value)
Myristic acid (C14:0)	**≤ 0.03**	**0.02±0.01** (0.01-0.04)	**0.01±0.01** (0.01-0.03)	**0.02±0.01** (0.01-0.03)	0.6629
Palmitic (C16:0)	**7.5-20.0**	**14.24±2.22** (10.80-17.23)	**15.20±1.94** (13.38-18.39)	**14.68±2.45** (10.91-17.13)	0.4755
Palmitoleic (C16:1)	**0.3-3.5**	**2.54±1.38** (0.75-4.56)	**1.91±0.99** (1.26-3.61)	**2.03±0.87** (0.99-3.01)	0.2374
Heptadecanoic (C17:0)	**≤ 0.3**	**0.09±0.03^{b}** (0.06-0.15)	**0.13±0.06^{a}** (0.07-0.21)	**0.08±0.01^{b}** (0.07-0.08)	**0.0006**
Heptadecenoic (C17:1)	**≤ 0.3**	**0.14±0.06^{b}** (0.09-0.24)	**0.19±0.09^{a}** (0.09-0.30)	**0.10±0.01^{c}** (0.08-0.12)	**0.0010**
Stearic (C18:0)	**0.5-5.0**	**1.95±0.38^{b}** (1.48-2.51)	**2.02±0.18^{b}** (1.72-2.21)	**2.26±0.29^{a}** (1.97-2.73)	**0.0102**
Oleic (C18:1)	**55.0-83.0**	**68.90±1.87** (66.86-72.36)	**70.60±1.82** (67.52-72.39)	**69.98±2.27** (67.43-73.29)	0.0630
Linoleic (C18:2)	**2.5-21.0**	**9.88±4.13** (3.32-13.99)	**7.87±1.32** (5.79-9.39)	**8.62±1.39** (7.46-10.94)	0.1028
Linolenic (C18:3)	**≤ 1.0**	**1.06±0.04^{a}** (0.99-1.12)	**0.93±0.12^{b}** (0.76-1.10)	**0.99±0.15^{b}** (0.77-1.17)	**0.0098**
Arachidic (C20:0)	**≤ 0.6**	**0.37±0.02^{b}** (0.34-0.40)	**0.38±0.04$^{a.b}$** (0.33-0.44)	**0.39±0.02^{a}** (0.36-0.43)	**0.0212**
Eicosenoic (C20:1)	**≤ 0.4**	**0.29±0.02^{a}** (0.24-0.31)	**0.27±0.04^{b}** (0.21-0.31)	**0.26±0.04^{b}** (0.20-0.32)	**0.0467**
Behenic (C22:0)	**≤ 0.2**	**0.14±0.02** (0.11-0.17)	**0.15±0.02** (0.12-0.17)	**0.15±0.02** (0.12-0.18)	0.0755
Lignoceric acid (C24:0)	**≤ 0.2**	**0.07±0.02** (0.04-0.11)	**0.07±0.01** (0.05-0.09)	**0.07±0.03** (0.04-0.13)	0.9286
∑SFA	**N.D.[2]**	**16.9±1.91** (13.92-19.42)	**18±2.11** (15.74-21.46)	**17.69±2.26** (14.29-20.11)	0.3210
∑MUFA	**N.D.[2]**	**71.93±2.79** (66.96-77.28)	**73.01±0.98** (71.66-74.33)	**72.42±1.54** (70.52-75.10)	0.2926
∑PUFA	**N.D.[2]**	**10.94±4.17** (4.32-15.11)	**8.81±1.44** (6.56-10.49)	**9.61±1.49** (8.52-12.10)	0.0867

[1]reference values for olive oil according to the Commission Regulation (EEC) 2568/91 of 11th July

[2]not defined by Commission Regulation (EEC) 2568/91 of 11th July

As mentioned before, in the present work the variation in fatty acid profile showed high homogeneity between samples within and between locations, in disagreement with the literature for oleaster oils (Hannachi et al., 2013; Baccouri et al., 2008; Bouarroudj et al., 2016). This fact could probably be related with genetic factors, with more stable and homogeneous populations in the present study, and also imposed by more similar environmental conditions observed in the studied locations. It has been shown that the fatty acids composition varies slightly from region to region, related to the environmental condition, particularly with the lowest mean temperature observed during fruit growth (Ceci et al., 2017; Tena et al., 2017). Lower temperatures increase the amounts of oleic acid with an increase of 1 ºC causing up to 2% decrease on oleic amounts (Rondanini et al., 2011). The geographical region under study (northeast of Portugal) is colder than North Africa, probably contributing for the higher oleic acid and MUFA contents. Nevertheless, the climacteric conditions of the three locations of our work are very similar, being the observed differences between populations probably due to genetic factors.

5.3.3. Tocopherols composition

Three tocopherols isoforms, namely α-, β- and γ-tocopherol, were quantified in the oleaster oils (Table 5.3). The most abundant compound was α-tocopherol, ranging from 263 to 458 mg/kg, with similar average amounts between the three studied populations (from 360.2 to 385.4 mg/kg of oil). However, the oils were significantly different ($P <$ 0.0010) in terms of γ-tocopherol content: the highest average was observed in the VNFC oils (76 mg/kg of oil) and the lowest in the Moncorvo population oils (27 mg/kg of oil). β-tocopherol contents were highly consistent, ranging from 5.5 to 6.5 mg/kg of oil. As can be seen from Table 5.3, the mean total amounts of tocopherols found in the oleaster oils from Alijó and VNFC populations (varying between 439.4 and 467.6 mg/kg of oil respectively) were significantly higher than the amounts observed in oils from Moncorvo location (392.5 mg/kg of oil).

Table 5.3. Tocopherols contents (mg/kg of oil) of α-. β- and γ-tocopherols as well as of Vitamin E (total tocopherol content) found in oils extracted from oleaster (mean ± standard deviation) collected from different geographical locations (Alijó. Moncorvo and Vila Nova de Foz Côa). Different lower case letters mean significant statistical differences at a 5% significance level (one-way ANOVA followed by the Tukey's multi-comparison test).

Tocopherol contents (mg/kg of oil)	Alijó	Moncorvo	Vila Nova de Foz Côa	One-way ANOVA (*P*-value)
α -Tocopherol	**379.9±78.8** (296.1-503.3)	**360.2±60.1** (305.9-458.1)	**385.4±71.9** (263.3-440.2)	0.5738
β -Tocopherol	**6.5±3.8** (3.4-12.9)	**5.51±1.66** (3.71-7.43)	**6.26±1.12** (4.77-7.87)	0.5062
γ -Tocopherol	**53.0±20.8^{b}** (30.0-85.7)	**26.84±13.90^{a}** (9.23-46.71)	**75.95±33.30^{b}** (34.52-124.14)	**< 0.0001**
Σ Tocopherol	**439.4±84.1^{a}** (329.5-564.0)	**392.5±59.8^{b}** (343.1-485.4)	**467.6±99.6^{a}** (302.6-554.2)	**0.0438**

The tocopherol profile was similarly to that of olive oil, with α-tocopherol representing more than 90% of the total tocopherol content (Beltrán et al., 2010). The amounts quantified in the present work were much higher than those observed for oleaster oil from Algeria, which varied from 87 to 182 mg/kg of oil (Bouarroudj et al., 2016) and Turkey, with values lower than 40 mg/kg of oil (Mattans et al., 2014) but were of the same order of magnitude of those reported by Baccouri et al. (2008), for seven populations from Tunisia (309.5 to 781.8 mg/kg of oil); and by Dabbou et al. (2011) that analyzed two Tunisian samples and reported α-tocopherol contents of 313 to 390 mg/kg of oil. When comparing the results obtained in Table 5.3, for oleaster oils, with similar studies with olive oils obtained from cultivated varieties, it can be concluded that the oils from the studied oleaster samples presented, in general, higher values of α-, β- and γ-tocopherol contents, than the traditional ones (12.2-630 mg/k of olive oil) (Tura et al., 2007; Beltrán et al., 2010). Additionally, the amounts of γ-tocopherol observed in the present work were much higher, in some cases of five orders of magnitude, compared to those reported for varietal olive oils and also for oleaster oils from other regions. These differences could be attributed to genetic factors. In fact, Beltrán et al. (2010) and Baccouri et al., (2008) concluded that tocopherols are genetically regulated and are highly cultivar dependent. Tocopherols play important roles, acting as antioxidants, and therefore protecting lipids in human body and stored oils from oxidation and, in this sense, oleaster oils could be foreseen as important sources of these bioactive compounds, with high oxidative stability.

5.3.4. Sterols composition

The sterol composition found for the oleaster oils studied is given in Table 5.4. Among the sterols detected, β-Sitosterol was the main sterol identified, followed by campesterol and stigmasterol. No differences were observed between populations for these main compounds. Nevertheless, the percentage of Δ-7-Estigmastenol (0.76%) of oils from Alijó location was significantly higher (P = 0.0384) than the values observed for oils of Moncorvo and VNFC populations (Table 5.4). The percentage of triterpenic alcohols from Alijó location (0.47%) were significantly lower (P = 0.0265) than those observed for Moncorvo (0.75%) and VNFC (0.69%) populations. Also, total sterols were remarkably higher than the statutory minimum limit (1000 mg/kg of oil) for olive oil and were significantly (P = 0.0124) higher for Alijó samples (2199 mg/kg) and lower for Moncorvo location (1742 mg/kg). Indeed, the contents of all sterols respects the established limits for olive oil with the exception of Δ-7-Estigmastenol (varying 0.61 to 0.76%), slightly above the legal maximum (0.5%) defined in the Commission Regulation
(EEC 2568/91). It should be remarked that high levels of total sterols indicate that the oils are of high quality. Also, low values of triterpenic alcohols indicate that the fruits are of

good quality, and that good production practices were applied during the extraction process, namely a low extraction temperature and reduced time of malaxation. Also, the sterolic fraction is a very useful parameter in the detection of adulterations, since it can be considered as a botanic origin marker (Mohamed et al., 2018).

Table 5.4. Sterol compositions of oils extracted from oleaster (mean ± standard deviation) collected from different geographical locations (Alijó. Moncorvo and Vila Nova de Foz Côa). Different lower case letters mean significant statistical differences at a 5% significance level (one-way ANOVA followed by the Tukey's multi-comparison test).

Sterols composition	Olive oil[1]	Alijó	Moncorvo	Vila Nova de Foz Côa	One-way ANOVA (*P*-value)
Cholesterol	**≤ 0.5%**	**0.05±0.02[b]** (0.03-0.09)	**0.07±0.01[a]** (0.05-0.09)	**0.07±0.02[a]** (0.04-0.10)	**0.0040**
Brassicasterol	**≤ 0.1%**	**0.03±0.00[b]** (0.02-0.04)	**0.04±0.01[a]** (0.03-0.06)	**0.04±0.01[a]** (0.02+0.07)	**0.0012**
Campesterol	**≤ 4.0%**	**3.49±0.54** (3.12-4.42)	**3.69±0.30** (3.46-4.24)	**3.38±0.91** (2.17-4.59)	0.3807
Stigmasterol	**< camp**	**0.61±0.13** (0.46-0.77)	**0.79±0.60** (0.27-1.67)	**0.85±0.61** (0.37-1.89)	0.3599
β-Sitosterol apparent	**≥ 93.0%**	**94.14±0.94** (92.93-95.57)	**94.05±0.99** (92.51-95.13)	**94.19±1.46** (91.91-95.71)	0.9376
Δ-7-Stigmastenol	**≤ 0.5%**	**0.76±0.22[a]** (0.44-1.05)	**0.61±0.13[b]** (0.49-0.83)	**0.63±0.17[b]** (0.43-0.91)	**0.0384**
Erythrodiol and uvaol	**≤ 4.5%**	**0.47±0.21[b]** (0.17-0.77)	**0.75±0.39[a]** (0.19-1.14)	**0.69±0.27[a]** (0.39-1.08)	**0.0265**
Total Sterols	**≥ 1000mg/kg**	**2199±464[a]** (1651-2898)	**1742±280[b]** (1385-2144)	**1939±476[a,b]** (1492-2668)	**0.0124**

[1]reference values for olive oil according to the Commission Regulation (EEC) 2568/91 of 11th July β-sitosterol apparent = sum of Δ-5,23-estigmastadienol + clerosterol + β-sitosterol + sitostanol + Δ-5-avenasterol + Δ-5,24-estigmastadienol.

In this case, the composition was very similar to olive oil once it is a variety of the same species (*O. europaea* subsp. *europaea* var. *sylvestris*) and oleaster oil is a rich source of phytosterols. This fact is of major relevance, since several biological activities have been attributed to phytosterols, mainly those related with the reduction of cholesterol absorption levels in the blood, being sometimes used in the treatment of hypercholesterolemia (Hannachi et al., 2013). The amounts of total sterols quantified in the oleaster oils were in accordance with those reported in other studies, namely by Hannachi et al. (2013), Baccouri et al. (2018) and Mohamed et al. (2018), with the exception of the levels of β–sitosterol, which could be related with the analytical methodologies used. On the other hand, once sterol content is influenced by several factors, such as climate, cultivar, geographical location and production practices (Lerma-García et al., 2011) these aspects may also justify the differences observed in the sterols amounts.

5.3.5. Phenolic Compounds

Fourteen phenolic compounds were detected and quantified in the oleaster oils obtained from the three different populations. The identified phenolic compounds belong to five phenolic groups, namely, phenolic alcohols, flavonoids, secoiridoids aglycons, dihydroxybenzoic acids derivatives and phenolic acids (Table 5.5).

Table 5.5. Phenolic composition (mg/kg of oil) of oils extracted from oleaster (mean ± standard deviation) collected from different geographical locations (Alijó. Moncorvo and Vila Nova de Foz Côa). Different lower case letters mean significant statistical differences at a 5% significance level (one-way ANOVA followed by the Tukey's multi-comparison test).

Phenolic compound	Alijó	Moncorvo	Vila Nova de Foz Côa	One-way ANOVA (*P*-value)
Phenolic alcohols				
Hydroxytyrosol (3.4-DHPEA)	**3.3±0.5** (2.5-4.0)	**4.0±0.8** (2.455.2)	**4.3±3.1** (1.4-9.4)	0.2997
Tyrosol (*p*-HPEA)	**8.4±5.6** (3.4-18.6)	**8.0±6.1** (2.2-17.8)	**9.34±7.2** (2.8-21.6)	0.8271
Hydroxytyrosol acetate	**0.7±0.3^{a}** (0.3-1.4)	**0.3±0.2^{b}** (0.0-0.5)	**0.3±0.1^{b}** (0.2-0.4)	**< 0.0001**
Flavonoids				
Luteolin	**7.4±5.5** (2.4-16.0)	**8.3±0.6** (7.7-9.4)	**11.8±12.5** (1.3-32.4)	0.2687
Apigenin	**9.4±5.5** (4.7-19.2)	**12.0±2.5** (9.3-16.8)	**13.2±5.5** (8.6-22.2)	0.0787
Methyl-Luteolin	**0.6±0.4** (0.3-1.3)	**0.6±0.6** (0.2-1.6	**0.5±0.5** 0.2-1.4)	0.5789
Secoiridoids Aglycons				
Oleuropein aglycon (and derivatives)	**226.3±55.0** (164.2-310.1)	**233.3±108.3** (96.7-370.9)	**260.6±81.8** (151.7-362.1)	0.4845
Oleuropein	**33.6±17.0^{b}** (18.8-65.7)	**44.4±24.0$^{a.b}$** (12.9-75.0)	**58.9±29.2^{a}** (22.3-102.2)	**0.0165**
Ligstroside derivatives	**287.5±83.8^{a,b}** (173.3-389.9)	**270.6±95.9^{b}** (148.6-414.7)	**358.8±107.8^{a}** (179.5-441.4)	**0.0304**
Dihydroxybenzoic derivatives				
Vanillin	**1.1±0.6^{a}** (0.4-1.9)	**0.5±0.3^{b}** (0.0-0.9)	**0.6±0.5^{b}** (0.1-1.5)	**0.0077**
Phenolic acids				
p-Coumaric acid	**5.3±2.0^{b}** (1.9-7.1)	**10.7±8.8^{a}** (3.3-25.4)	**6.9±3.2$^{a.b}$** (2.5-11.1)	**0.0241**
o-Coumaric acid	**0.4±0.1** (0.3-0.5)	**0.6±1.0** (0.0-2.4)	**0.2±0.1** (0.1-0.3)	0.1114
Cinamic acid	**14.9±7.5^{b}** (6.0-26.4)	**14.7±4.8^{b}** (11.2-23.5)	**24.0±8.7^{a}** (12.3-37.3)	**0.0007**
Ferulic acid	**0.6±0.2** (0.4-0.9)	**0.6±0.4** (0.2-1.3)	**0.4±0.2** (0.2-0.7)	0.0932
∑ Phenols	**602.7±151.9** (392.9-752.2)	**612.0±216.8** (319.4-859.7)	**751.9±221.5** (401.9-942.9)	0.0705

The group of secoiridoids aglycons was the one present in greater quantities, imposed by the high ligstroside derivatives quantity that ranged from 271 mg/kg (Moncorvo location) and 359 mg/kg of oil (VNFC location), with statistical differences between both locations, and oleuropein aglycon (and derivatives) prevalence, with values between 227 mg/kg (Alijó location) and 261 mg/kg of oil (VNFC location), without statistical differences between locations. Nevertheless, the second most abundant compound, oleuropein, showed statistically higher contents (P = 0.0165) in the oils from VNFC location (58.9 mg/kg of oil) than those quantified in the oils from Alijó location (33.6 mg/kg of oil) (Table 5.5). In the group of phenolic alcohols, tyrosol (p-HPEA) ranged from 8.0 mg/kg of oil (Moncorvo location) and 9.4 mg/kg of oil (VNFC location). Together with hydroxytyrosol acetate, these were the only significantly different phenolic alcohols between populations, with highest values observed for Alijó location. Among the identified flavonoids, apigenin (9.4 to 13.2 mg/kg of oil) showed higher values compared to luteolin (7.4 to 11.8 mg/kg of oil) and methyl-luteolin, with statistical differences between oleaster populations. Similarly, luteolin and apigenin were the major flavonoids found in oleaster oils from Algeria (Bouarroudj et al., 2016). In the group of dihydroxybenzoic acids derivatives only vanillin was found, in significantly higher amounts for oils produced from Alijó population (1.06 mg/kg of oil). Four phenolic acids were identified, namely *p*-coumaric acid, *o*-coumaric acid, cinnamic acid and ferulic acid. Cinnamic acid was the most abundant phenolic acid, and its content was significantly higher in oils obtained at VNFC (24.0 mg/kg of oil), whereas, *p*-coumaric acid was significantly higher (P = 0.0241) in Moncorvo oleaster oils (10.7 mg/kg of oil). Oleaster oils possess a considerable amount of phenolic compounds, with the mean contents in the range of 600 to 750 mg/kg of oil. Again, the obtained values were higher to those observed by Bouarroudj et al. (2016) for Algerian oleaster oils, and for Portuguese cultivated olive varieties (Peres et al., 2016). These findings are quite relevant since different works previously demonstrated the importance of phenolic compounds on the sensory characteristics, resistance to oxidation and positive health effects of olive oils, being in this case oleaster oils also a good source of these compounds.

5.3.6. Oleaster oils discrimination according to the geographical location based on their physicochemical profiles

As previously discussed, the Portuguese oleaster oils studied showed physicochemical profiles (fatty acids, tocopherols, sterols and phenolic compounds) with similarities with those of oleaster oils from other countries (e.g., Algeria and Tunisia), as well as of cultivated olive trees (from Portugal and other countries). However, as also pointed out, for some chemical minor compounds (i.e., less abundant ones), significant statistical differences were detected among the three populations evaluated. Thus, LDA coupled with the SA algorithm (LDA-SA) was implemented to identify the chemical compounds that could be further used as chemical markers for each population studied (i.e., Alijó, Moncorvo, and VNFC locations) allowing discriminating the oleaster oils according to the geographical origin of the wild olive trees (Figure 5.1).

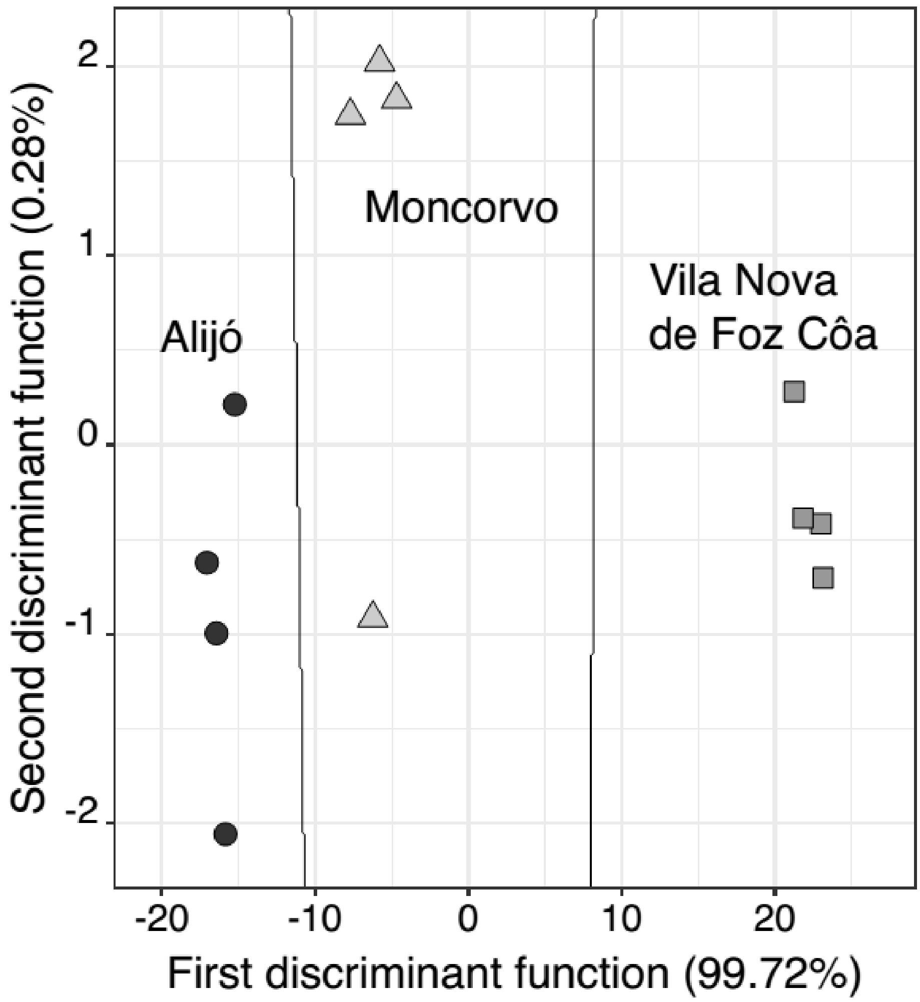

Figure 5.1. Discrimination of oleaster oils according to three geographical origins (Alijó, Moncorvo or Vila Nova de Foz Côa) using a LDA-SA model based on the contents of eicosenoic fatty acid; SFA; erythrodiol and uvaol; hydroxytyrosol acetate; and, cinnamic acid. The full lines represent the boundary lines based on the posterior probabilities calculated for each class membership.

The results showed that a LDA-SA model with two linear discriminant functions (explaining 99.72% and 0.28%, respectively) could be established based on the contents of five chemical compounds (i.e., eicosenoic fatty acid; SFA; triterpenic alcohols (erytrodiol + uval); hydroxytyrosol acetate; and cinnamic acid). The established model enabled the correct classification of 100% of the oils according to the location for the original grouped data. Also, for the LOO-CV procedure, a predicted correct classification rate of 100% was also obtained. These results clearly pointed out that, besides their relevance for health or sensory positive sensations, these chemical compounds and their contents may be further used as chemical location markers for oleaster oils.

5.4. Conclusions

Within the present work, we intended to characterize the oleaster olive oils obtained from three distinct locations in the northeast of Portugal. The results showed that the different populations presented some morphological differences but a very similar oil composition, with only slight variations in some fatty acids, tocopherols, sterols and phenolic compounds that allowed discriminating the oils according to the local of origin. On the other hand, oleaster oils presented a chemical profile very similar to olive oil, largely enriched in compounds such as tocopherols, sterols and phenolic compounds. Furthermore, it is clear that the region is an important factor in the chemical composition of studied oils. Although the profile is similar across all of them, the composition varied being some of them richer in antioxidant compounds than others, particularly the VNFC population, despite having the shorter fruits in the study. The rich composition of oleaster oil's indicates that the characterized populations could be included in breeding programs to produce olive oils rich in bioactive compounds. On the other hand, considering the market search for sensory and chemical differentiated products, the production of oleaster oils for commercial purposes could be envisaged.

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Chapter 6

Ancient olive trees as a source of olive oils rich in phenolic compounds

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Chapter 6

Ancient olive trees as a source of olive oils rich in phenolic compounds

Abstract

Olive oil phenolic compounds are receiving increased attention due to its influence on sensory characteristics and to scientific evidences of positive health effects. In this work, 28 ancient olive trees were selected and, during four consecutive crop years (2014-2017), oils were extracted and their phenolic fraction characterized. Hydroxytyrosol and tyrosol secoiridoids were the predominant groups, with contents between 32 to 496 mg of tyrosol equivalents/kg. Based on principal component analysis it could be concluded that the individual phenolic contents enabled the unsupervised grouping of olive oils by crop year. Furthermore, linear discriminant analysis allowed achieving sensitivities greater than 90%. It was shown that some specimens consistently allowed obtaining oils with high phenolic contents (≥500mg tyrosol equivalents/kg). The identification of centenarian specimens for breeding based on their potential to produce oils with high levels of healthy compounds is of utmost interest, contributing to preserve the genetic heritage.

Keywords: Phenolic compounds, health claims, crop year, olive heritage.

Chapter 6

6.1. Introduction

Olive oil is one of the most important components of the Mediterranean diet, due to its organoleptic characteristics, nutritional properties, and cultural influences (García-Vico et al., 2017; Fernández et al., 2018; Polari et al., 2018). Compared to other vegetable oils, olive oils greatest richness is mainly due to differences in the production process (Reboredo-Rodríguez et al., 2017). Olive oils are extracted from fresh olives only by mechanical and physical processes (milling, malaxation and centrifugation), which allows keeping intact the properties of the fruit, especially compounds related to its bioactive capacity (phenolics, tocopherols, sterols, pigments, etc.) (Visioli, & Bernardini, 2013; Tsimidou, & Boskou, 2015).

The biological benefits of health due to olive oil consumption are not only related to the high monounsaturated fat content. Indeed, several minor components also have important bioactive properties contributing to its nutritional value (Khymenets et al., 2011). Among these, polyphenols have recently received great attention (Khoddami et al., 2013; Shahidi, & Ambigaipalan, 2015), as they play a key role in human health, through its protective effects against neurodegenerative and cardiovascular diseases (Visioli, 2012; Olmo-García et al., 2017), while protecting the body from oxidative damage (Cicerale et al., 2010; García-Rodríguez et al., 2015). Different works have demonstrated the positive correlations between the daily intake of phenolic compounds in the Mediterranean diet and health (Fregapane, & Salvador, 2013; Aparicio-Ruiz et al., 2016; Vitaglione et al., 2015). Recently, EFSA (European Food Safety Authority) recognized an health claim associated with the contribution of "olive oil polyphenols" for the protection of blood lipids from oxidative stress, which is only allowed for "*olive oils containing at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil*" (Commission Regulation (EU) 432/2012, EFSA, 2012). This official recognition has increased the interest on the subject, particularly by the olive oil industry, aiming to produce olive oils that could hold that claim (Reboredo-Rodríguez et al., 2017; Veneziani et al., 2018). Different polyphenols can be found in virgin olive oils, mostly derivatives of tyrosol, hydroxytyrosol, 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid, together with lignans, secoiridoids and flavonoids (García-Villalba et al., 2010).

Although all are classified as phenolic compounds, not all are included in the above mentioned health claim. Oleuropein aglycon derivatives (dialdehyde and aldehyde forms) are usually the main phenolic compound in olives, derived from the enzymatic hydrolysis of oleuropein, but other hydroxytyrosol derivatives are also found, being these the main contributors for the health claim (Torres et al., 2018). However, the content of phenolic compounds in olive oil (both qualitative and quantitative) is strongly affected by diverse factors, giving rise to olive oils with diverse composition and quality over the years (García-Vico et al., 2017). Examples of that are cultivar, geographical origin, irrigation, edapho-climatic conditions, production processes, fruit maturation, harvesting methods, fruit freshness before extraction, extraction method and storage conditions (Tovar et al., 2002; Franco et al., 2014; Dabbou et al., 2015; Sánchez de Medina et al., 2015; Gómez-Caravaca et al., 2016; Köseoğlu et al., 2016). Thus, even under the most adequate productive and technological conditions, the same tree can give rise to olive oils with different compositions over the years, being difficult to identify the most adequate cultivars for ensuring a consistent bioactive richness if not studied over a long time-period. So far, most of the studies have only been focused on assessing olive oil phenolic composition over short time-periods (one or two years) and very few up to three years, which is not enough to really support the conclusions. On the other hand, in the last decades, there has also been a gradual loss of the genetic heritage potential in olive cultivation worldwide, with a substantial increase of only a minor number of cultivars, mostly based on their agricultural characteristics, particularly the high expected production yields. In a competitive rising marked, the search for innovation and product differentiation, such as the possibility of holding health claims, can only be achieved by re-orient the focus to the positive characteristics of the olive oils, rather than the productivity by itself. In this sense, the pursuit for olive oils richer in antioxidant compounds, particularly in phenolic compounds that may support the health claim, can be envisaged through the study and selection of specific olive tree genotypes. Focusing this search on ancient olive trees may also contribute to the future preservation of olive tree cultural heritage.

In this context, this work aimed to study the phenolic composition of olive oils produced from centenarian olive trees, aiming to characterize and possibly select those that enable a consistent production of olive oils richer in polyphenols over the years, contributing to olive oil valorization and probably ensuring health claims. For this, 28 centenarian olive trees, from one Portuguese olive grove, were selected and, during four harvest seasons (2014-2017), the olive oils polyphenol compositions were analyzed.

Unsupervised and supervised statistical techniques were used helping to classify/discriminate olive trees aiming to identify the most promising specimens in terms of health and nutritional claims.

6.2. Material and Methods

6.2.1. Sampling

6.2.1.1. Tree selection

The olive trees studied were grown in a centenarian olive grove (≈ 250 years) located in the northeast of Portugal, near Mirandela (Suçães, N 41º 29.425; W 7º 15.490). According to our best knowledge, this grove includes the oldest trees of the region, from diverse cultivars most of them unknown. The olive grove has 140 trees and, taking into account the tree appearance, structure and trunk thickness, which are classical indicators of the tree age, 28 distinct olive trees (20% of the trees), considered representative of the grove diversity, were selected and individually marked.

6.2.1.2. Harvest

Along four consecutive crop years (2014-2017), and from each tree, approximately three kilograms of fruits were manually picked. All fruits were visually inspected and the fruits damaged or attacked by pests and diseases were discarded. To avoid the influence of the maturity stage on the olive oil composition, harvest occurred always when the fruits were between the maturity stage (MI) two and three, which corresponds to the fruit epidermis with red spots in less than half of the olive (MI 2) and the fruit epidermis red or purple in more than half of the olive (MI 3) (Hermoso et al., 1991). Thus, every year the harvest occurred during the month of November, namely on the 10th and 11th days in 2014; on the 2nd and 3rd days in 2015; on the 07th and 08th days in 2016; and, on the 13th and 14th days in 2017.

6.2.1.3. Oil extraction

The fruits were processed in the first 24 h after harvest, in a pilot extraction plant with an Abencor analyzer (Comercial Abengoa S.A., Seville, Spain), with three main units: a mill, a thermobeater where malaxation takes place at controlled temperature, and a centrifuge. Olives were milled, the paste was homogenized, and about 700 g were transferred to the thermobeater unit (20 min) for malaxation, using a thermostatic water bath at 25ºC. In the final 5 min of each malaxation, 100 mL of water at 25 ºC was added to aid olive oil separation. The mixture was centrifuged, decanted, and the olive oil collected. After that, the oils were prepared for analysis, being filtered (Whatman paper nº 4) over anhydrous sodium sulfate in order to remove the solid particles and residual water. The olive oils were stored in 125 mL dark bottles and protected from light exposition, at room temperature. During the four years a total of 112 olive oils were analyzed. All the assays were carried out in triplicate within two months after extraction.

6.2.2. Phenolic Compounds

6.2.2.1. Extraction

Phenolic compounds extraction was carried out according to the protocol from the International Olive Council (COI/T.20/Doc No 29/Rev.1 2017), with minor modifications. Briefly, 0.4 g of olive oil were weighed in a 10 mL tube. An accurate amount of the internal standard solution (25 µL; syringic acid at 0.15 mg/mL in methanol/water 80/20 (v/v)) was added, and the sealed tube was vortexed for 30 seconds. The phenolic compounds were extracted with 2.5 mL of methanol/water solution (80:20, v/v), being the solution agitated during 30 sec in a vortex, followed by the addition of hexane (2.5 mL) for a more clear elimination of the fat, and followed again by vortex mixing for 5 min. Afterwards, the mixture was centrifuged at 5000 rpm for 5min. The lower phase (hydrophilic) was filtered through a 0.22 µm microfilter (PVDF). The solution was then taken to almost dryness under a gentle nitrogen stream (40ºC) and immediately reconstituted with 200 µL of methanol, being ready for injection in the HPLC system. All samples were extracted in duplicate.

6.2.2.2. HPLC analysis

The phenolic composition of the obtained olive oils was evaluated by high performance liquid chromatography (HPLC) with diode array detection (DAD) using an integrated HPLC system from Jasco (Japan) with a data transmitter (LC - NetII/ADC), two integrated pumps (PU - 4180), an auto-sampler (AS - 4050), oven (ECOM Eco2000, Czech Republic), and the DAD (MD – 4010). Separation was accomplished on a C18 reversed-phase column (Kinetex C18 2.6 µm 100Å, 100 x 3.00 mm, Phenomenex), at 35ºC, using a gradient of water and acetonitrile, both with 0.1% of formic acid, at a flow rate of 0.8 mL. Peak identification was performed by comparing retention times and UV/Vis spectra (200 to 600 nm) with those of pure standards (tyrosol, hydroxytyrosol, vanillic acid, ferulic acid, *o*-coumaric acid, luteolin, cinnamic acid, apigenin, and oleuropein, from diverse suppliers). Secoiridoids tentative identification was oriented by the COI method and available literature. For quantification, UV/Vis detection wavelengths were set to 280 nm (for simple phenols, vanillic acid, vanillin, lignans and secoiridoids), 325 nm (for coumaric and ferulic acids), and 365 nm (for flavonoids). Based on COI, results were expressed as mg of tyrosol equivalents per kg of olive oil for each individual compound while the total phenols content corresponded to the sum of all individual compounds quantified.

6.2.3. Statistical analysis

The possible effect of crop year (from 2014 to 2017) on the olive oil phenolic profile was evaluated using unsupervised and supervised multivariate pattern recognition techniques namely, principal component analysis (PCA) and linear discriminant analysis (LDA), based on the contents of the individual phenolic compounds. These two chemometric tools were also used to assess the possibility of using the same phenolic profiles to discriminate olive oils after regrouping samples into 4 groups based on their richness in total phenolic compounds (<300; 300 to 400; 400 to 500 and >500 mg tyrosol equivalents/kg), which can be related to possible olive oil health claims. For PCA, the individual phenolic contents were centered and scaled minimizing data variability. LDA was coupled with the meta-heuristic simulated annealing (SA) variable selection algorithm, aiming to identify the phenolic compounds that could enable olive oils discrimination according to the crop year or the phenolic richness (*i.e.*, which were most influenced by each factor), regardless the olive tree from which olives were collected. Indeed, SA algorithm is able to discard redundant variables (*i.e.*, in this case, phenolic compounds), selecting the most influent ones and so, maximizing the correct overall classification percentages (*i.e.*, maximizing the model predictive sensitivity) and minimizing possible noise effects (Bertsimas, & Tsitsiklis, 1992; Cadima et al., 2004; Kirkpatrick et al., 1983). The predictive performances of the LDA-SA models were evaluated considering the leave-one-out cross-validation (LOO-CV) and the repeated K-fold cross-validation (repeated K-fold-CV) techniques. In the repeated K-fold-CV, data was randomly split into K folds, being each of the folds left out in turn for internal-validation and the other K-1 folds used to establish the model. After all folds have been used for validation purposes, the K estimates are averaged to get the overall resampled estimate (Kirkpatrick et al., 1983). In this work the K-folds were set equal to 4, enabling the random formation of internal-validation subsets with 25% of the initial data. The procedure was repeated 10 times, which allowed putting the model under stress. The variables were scaled and centered before modeling to normalize the weight of each variable in the final linear classification model. The classification performance of each LDA-SA model was graphically evaluated by plotting the significant discriminant functions, which would allow visualizing the groups discrimination. Besides, sensitivity values were also calculated to quantitatively assess the discrimination performance.

The statistical analysis was performed using the Subselect (Cadima et al., 2004; Cadima et al., 2012; Kuhn, & Johnson, 2013) and MASS (Venables, & Ripley, 2002) packages of the open source statistical program R (version 2.15.1), at a 5% significance level.

6.3. Results and discussions

6.3.1. Phenolic compounds identification and quantification

In the present work, it was studied the phenolic profile of olive oils extracted during four crop years (2014-2017) from 28 individual centenarian olive trees, in a total of 112 olive oil samples, all classified as extra virgin olive oil (data not shown). Table 6.1 shows the mean contents ($\bar{x}$) and the respective standard deviations (*sd*) of each phenolic compound detected in the olive oils, which were organized in five phenolic groups, namely: phenolic alcohols (hydroxytyrosol and tyrosol), flavonoids (luteolin, apigenin and methyl luteolin), secoiridoids aglycons (sum of aldehydic form of oleuropein aglycone, dialdehydic and oxidized dialdehydic forms of decarboxymethyl oleuropein aglycone for oleuropein derivatives and sum of oxidised dialdehyde and dialdehyde forms of decarboxymethyl ligstroside aglycone, oxidised aldehyde, dialdehyde and hydroxylic forms of ligstroside aglycone for ligstroside derivatives), phenolic acids (*o*- and *p*-coumaric acid, ferulic acid and cinnamic acid) and dihydroxybenzoic derivatives (vanillic acid and vanillin). All the values are within the ranges reported for olive oils (Franco et al., 2014; Olmo-García et al., 2017; Alowaiesh et al., 2018). Nevertheless, the phenolic amount in olive oils are dependent of different factors such as genotype, fruit ripening stage, agro-climatic conditions, production year and geographical origin (Tovar et al., 2002; Franco et al., 2014; Sánchez de Medina et al., 2015). Aware of influence of diverse environmental factors in the olive oil phenolic composition, in the present work, all the trees were grown under the same environmental conditions, subjected to the same agronomic factors, and the fruits were collected at similar maturation indexes. Among the five phenolic compound groups identified, secoiridoids aglycons were the main group (Table 6.1), which included mainly the dialdehydic form of oleuropein (oleacein; 3,4-DHPEA-DEA), its monoaldehydic form (3,4-DHPEA-EA) and the equivalent ligstroside derivatives (oleocanthal - *p*-HPEA-DEA and *p*-HPEA-EA, respectively). The mean contents for all oleuropein derivatives varied between 32 (tree 27) and 250 mg of tyrosol equivalents/kg of olive oil (tree 24) while those of ligstrodide derivatives varied between 85 (tree 17) and 585 mg of tyrosol equivalents/kg of olive oil (tree 26). Flavonoids were the second group with higher contents (Table 6.1).

Table 6.1. A Phenolic composition (Phenolic alcohols, Flavonoids and Secoiridoid Aglycons)(mg of tyrosol equivalents/kg of olive oil) of EVOOs extracted from 28 centenarian trees (mean ± standard deviation) during four crop years (2014-2017).

A - Tree	Phenolic alcohols		Flavonoids			Secoiridoids Aglycons	
	Hydroxytyrosol	Tyrosol	Luteolin	Apigenin	Methyl luteolin	Oleuropein derivatives	Ligstroside derivatives
Tree 1	3.7±2.7	5.5±4.0	8.1±3.7	15.4±6.0	2.0±1.0	120.6±58.2	270.9±100.4
Tree 2	2.1±0.9	3.2±1.8	8.8±3.4	6.7±2.2	2.2±1.5	104.8±25.4	138.9±69.0
Tree 3	2.7±1.3	5.8±3.7	12.9±4.0	11.3±6.4	2.5±1.3	97.6±49.2	229.6±175.6
Tree 4	2.2±1.0	1.9±1.0	3.0±2.0	3.0±2.3	0.6±0.7	116.1±19.4	219.2±105.6
Tree 5	2.0±1.0	1.8±0.9	2.6±1.4	2.7±1.6	0.8±1.0	102.4±13.8	212.0±106.6
Tree 6	4.5±2.0	6.6±2.0	7.3±1.0	8.0±1.8	0.5±0.6	117.3±11.2	126.7±5.3
Tree 7	1.9±1.3	1.7±1.0	2.3±0.7	3.3±1.7	0.7±0.8	103.6±36.3	191.6±47.2
Tree 8	3.8±2.5	4.9±3.7	5.6±1.4	13.7±12.1	1.5±1.8	119.7±60.1	335.6±111.5
Tree 9	1.9±1.1	1.5±0.6	2.5±0.3	4.2±3.3	2.2±2.8	62.5±35.8	185.2±99.7
Tree 10	1.8±0.9	1.6±0.7	2.0±0.3	2.3±1.2	1.0±1.2	94.0±10.9	192.4±98.8
Tree 11	2.1±0.5	3.5±2.0	4.6±1.8	3.4±1.3	0.7±0.5	139.9±11.3	216.7±116.0
Tree 12	1.9±0.8	4.8±2.0	12.5±2.9	15.3±4.2	1.1±0.9	124.5±33.3	202.5±63.7
Tree 13	1.9±1.0	5.0±2.9	14.9±3.2	16.5±6.8	13.6±0.8	171.7±16.6	190.8±32.8
Tree 14	2.2±1.2	2.2±1.5	4.3±1.0	4.9±4.0	0.9±0.7	134.4±73.4	299.0±137.7
Tree 15	1.6±0.4	1.5±0.3	2.0±0.5	2.7±1.2	0.9±1.1	104.9±10.0	197.6±100.5
Tree 16	2.3±1.1	4.7±2.9	13.4±6.1	15.3±9.0	1.0±1.0	151.8±38.5	232.8±48.6
Tree 17	6.8±3.9	6.0±3.4	6.2±3.0	10.0±5.8	0.7±1.2	71.3±54.4	198.9±137.1
Tree 18	2.6±0.8	2.0±0.3	2.0±0.6	2.8±1.2	1.0±1.1	104.3±29.9	215.2±82.7
Tree 19	2.6±1.0	1.8±0.9	11.0±16.0	3.6±2.4	1.1±1.2	121.6±50.8	219.5±81.0
Tree 20	2.6±1.3	2.0±0.6	2.0±0.3	2.8±1.9	0.8±0.6	79.8±53.3	223.4±87.6
Tree 21	3.2±1.7	4.7±2.8	9.5±4.1	9.3±6.8	0.6±0.6	148.0±66.8	219.8±45.1
Tree 22	3.5±3.2	5.9±4.8	4.8±0.5	6.4±4.5	1.2±0.6	61.9±46.1	219.0±45.4
Tree 23	5.4±5.2	5.8±4.0	12.0±4.5	9.3±5.4	1.3±1.0	127.9±66.8	256.4±118.5
Tree 24	2.7±1.6	4.6±2.3	7.6±6.3	7.4±8.2	0.8±0.7	249.6±133.6	379.5±37.7
Tree 25	4.6±3.4	9.8±7.1	4.9±1.5	5.5±4.0	0.3±0.3	112.6±69.2	410.8±164.6
Tree 26	7.1±8.0	12.1±9.3	5.6±1.0	7.0±5.5	0.4±0.6	129.9±92.3	426.1±142.3
Tree 27	1.1±0.9	4.2±2.5	1.7±0.6	3.1±2.9	0.1±0.2	32.0±35.2	171.5±73.3
Tree 28	3.0±2.4	3.9±2.2	16.2±7.1	12.1±7.4	2.2±1.5	247.1±94.0	295.7±44.4
Median	2.3	2.5	4.6	4.8	0.7	109.8	201.1
Minimum	0.0	0.3	0.8	0.6	0.0	1.8	85.3
Maximum	20.0	26.4	37.3	31.6	6.8	414.1	591.4

Table 6.1. B Phenolic composition (Phenolic acids, Dihydroxybenzoic acid and Total Phenols) (mg of tyrosol equivalents/kg of olive oil) of EVOOs extracted from 28 centenarian trees (mean ± standard deviation) during four crop years (2014-2017).

B - Tree	Phenolic acids				Dihydroxybenzoic derivatives		Total Phenols
	p-Coumaric acid	Ferulic acid	*o*-Coumaric acid	Cinnamic acid	Vanillic acid	Vanillin	
Tree 1	3.3±2.3	0.7±0.6	0.4±0.4	4.0±4.1	3.4±1.8	2.7±2.4	441±90
Tree 2	3.5±3.2	0.3± 0.2	0.4±0.4	2.1±1.5	4.4±2.6	1.7±1.7	279±98
Tree 3	2.8±1.5	0.4±0.3	0.3±0.2	2.6±2.6	4.8±2.8	2.4±1.9	376±142
Tree 4	5.1±4.7	0.6±0.3	0.2±0.1	2.2±2.2	2.2±1.6	2.0±1.9	359±104
Tree 5	3.1±1.1	0.5±0.2	0.3±0.3	1.9±1.9	2.4±2.0	2.0±1.6	335±110
Tree 6	3.4±2.6	0.3±0.0	0.3±0.4	1.9±2.0	6.3±3.6	2.2±0.6	285±4
Tree 7	4.5±3.6	0.6±0.2	0.4±0.5	2.5±2.4	2.7±2.0	2.2±1.9	318±39
Tree 8	2.0±1.2	0.5±0.3	0.4±0.4	4.1±4.2	2.4±1.5	2.0±1.5	496±100
Tree 9	3.2±2.2	0.7±0.3	0.5±0.7	2.0±2.0	3.1±1.6	1.8±1.7	271±82
Tree 10	4.1±2.4	0.7±0.2	0.3±0.5	2.1±2.1	2.4±1.6	2.0±1.8	307±105
Tree 11	10.8±8.1	0.6±0.2	0.6±0.8	5.0±3.7	4.1±2.9	1.4±1.2	393±100
Tree 12	4.0±2.8	0.4±0.2	0.3±0.5	3.7±3.7	3.7±2.2	1.6±1.4	376±52
Tree 13	4.1±2.8	0.4±0.2	0.4±0.6	5.8±4.4	3.7±2.3	1.2±1.3	418±64
Tree 14	4.4±2.5	0.6±0.2	0.4±0.6	3.9±4.5	1.7±0.7	1.9±1.6	461±165
Tree 15	3.7±1.7	0.5±0.1	0.3±0.5	1.9±2.1	2.3±1.2	2.0±1.8	322±103
Tree 16	3.4±2.0	0.5±0.3	0.3±0.4	4.9±4.8	3.2±2.1	1.6±1.4	435±43
Tree 17	2.1±1.4	0.5±0.3	1.9±2.0	2.7±3.1	4.3±2.8	1.8±1.6	313±168
Tree 18	2.9±1.7	0.6±0.1	0.3±0.4	2.5±2.7	2.6±1.4	2.1±1.8	340±53
Tree 19	5.6±3.9	0.6±0.1	0.3±0.5	3.1±3.2	2.2±1.3	1.9±1.5	375±79
Tree 20	7.0±6.4	0.7±0.2	0.5±0.6	2.7±2.9	2.5±1.4	2.4±2.2	356±84
Tree 21	2.6±2.0	0.5±0.3	0.8±0.5	7.1±6.4	3.2±2.0	1.0±1.1	410±133
Tree 22	2.8±1.7	0.5±0.3	0.3±0.3	4.9±3.7	4.4±2.3	2.4±2.4	371±105
Tree 23	2.3±1.3	0.4±0.3	0.3±0.2	4.3±4.6	5.5±2.5	2.1±1.7	433±108
Tree 24	8.0±8.9	0.4±0.1	0.3±0.3	7.8±8.6	1.6±1.5	1.2±1.6	611±124
Tree 25	14.6±7.5	0.7±0.4	0.2±0.4	4.9±5.0	2.5±1.0	1.1±0.9	573±137
Tree 26	19.3±13.6	0.6±0.2	0.4±0.5	6.0±6.3	2.6±1.4	1.2±0.9	618±140
Tree 27	4.8±2.5	1.5±1.0	0.1±0.1	1.3±1.9	2.5±1.4	2.3±1.9	226±52
Tree 28	10.8±5.2	0.3±0.1	0.3±0.3	10.1±8.4	1.1±0.7	1.1±1.4	604±166
Median	3.2	0.5	0.1	2.8	2.9	1.4	365
Minimum	0.3	0.1	0.0	0.0	0.1	0.0	150
Maximum	40.4	2.3	5.1	19.8	9.5	5.8	810

The most representative compound in this class, apigenin, varied from 2.3 (tree 10) to 16.5 mg of tyrosol equivalents/kg of olive oil (tree 13). For the phenolic alcohols group, hydroxytyrosol ranged from 1.1 (tree 27) to 7.1 mg of tyrosol equivalents/kg of olive oil (tree 26), while tyrosol from 1.5 (tree 9) to 12.1 mg/kg of olive oil (tree 26). These low amounts are in accordance with the freshness of the extracted olive oils, as they increase with time by hydrolysis of secoiridoids. The group of phenolic acids included *o*- and *p*-coumaric, ferulic, and cinnamic acids; whereas in the group of dihydroxybenzoic acids two compounds were identified (vanillic acid and vanillin) (Table 6.1). Similar compounds and amounts were reported by Peres et al. (2016) in olive oils from the Portuguese varieties Galega Vulgar and Cobrançosa extracted at early ripening stages, by Veneziani et al. (2018) for olive oils from six Italian cultivars (Frantoio, Leccino, Gentile, Ogliarola garganica, Moraiolo and San Felice) and by Kotsiou, & Tasioula-Margari (2016) in Greek extra-virgin olive oils. The highest total phenolic contents were observed for the trees 24, 26 and 28 with average values for the four years of 611, 618 and 604 mg of tyrosol equivalents/kg of olive oil, respectively. On the contrary, the lowest contents were consistently obtained for the olive oils extracted from the trees 2, 6, 9 and 27, with values of 279, 285, 271 and 226 mg of tyrosol equivalents/kg of olive oil, respectively (Table 6.1). These results show that some of the trees (e.g., 24, 26 and 28) could be good candidates for multiplication and for breeding programs if the aim is to obtain olive oils with high levels of total phenolic compounds. As mentioned before, the phenolic contents in olive oil is affected by several factors such as genotype, maturation, geographic origin and olive genotype (Aparicio-Ruiz et al., 2016; Veneziani et al., 2018; Caruso et al., 2014). However, in the present work, since all trees were grown under the same conditions, the genotype is the main varying factor, suggesting that, as mentioned by Valls et al. (2015), the production of phenolic compounds is genetically regulated.

6.3.2. Effect of crop year on the phenolic composition

As previously stated, the phenolic profile of olive oil is also dependent of environmental conditions (Gómez-Caravaca et al. (2016), and this is among the less studied factors in the literature, due to the time required for reaching soundness conclusions. Thus, the influence of the crop year on the amounts of individual phenolic compounds found in the olive oils obtained from ancient olive trees during four consecutive crops was statistically evaluated. As can be seen from Figure 6.1, based on the olive oil's individual phenolic contents (Table 6.1), PCA allowed the unsupervised discrimination of the oils produced from the 28 olive trees according to the crop year, showing that the production year had a high influence on the phenolic fraction content. The first 3 principal component (PC) functions explained more than 64% of the data variability (28.0%, 24.6% and 11.7% for PC1, PC2 and PC3, respectively). The results also pointed out that ferulic acid, ligstroside derivatives and vanillin were the phenolic compounds that mostly contributed to discriminate olive oils produced in 2014. Regarding the year 2015, hydroxytyrosol, tyrosol and vanillic acid were those that most influenced the olive oil phenolic composition while in 2016, cinnamic acid, luteolin, *p*-coumaric acid and oleuropein derivatives were leading compounds in the discrimination. Finally, for 2017, all phenolic compounds were similarly influenced. These results are in agreement with the findings of Köseoğlu et al. (2016) which reported that the agro-climatic conditions influence the phenolic composition of olive oils.

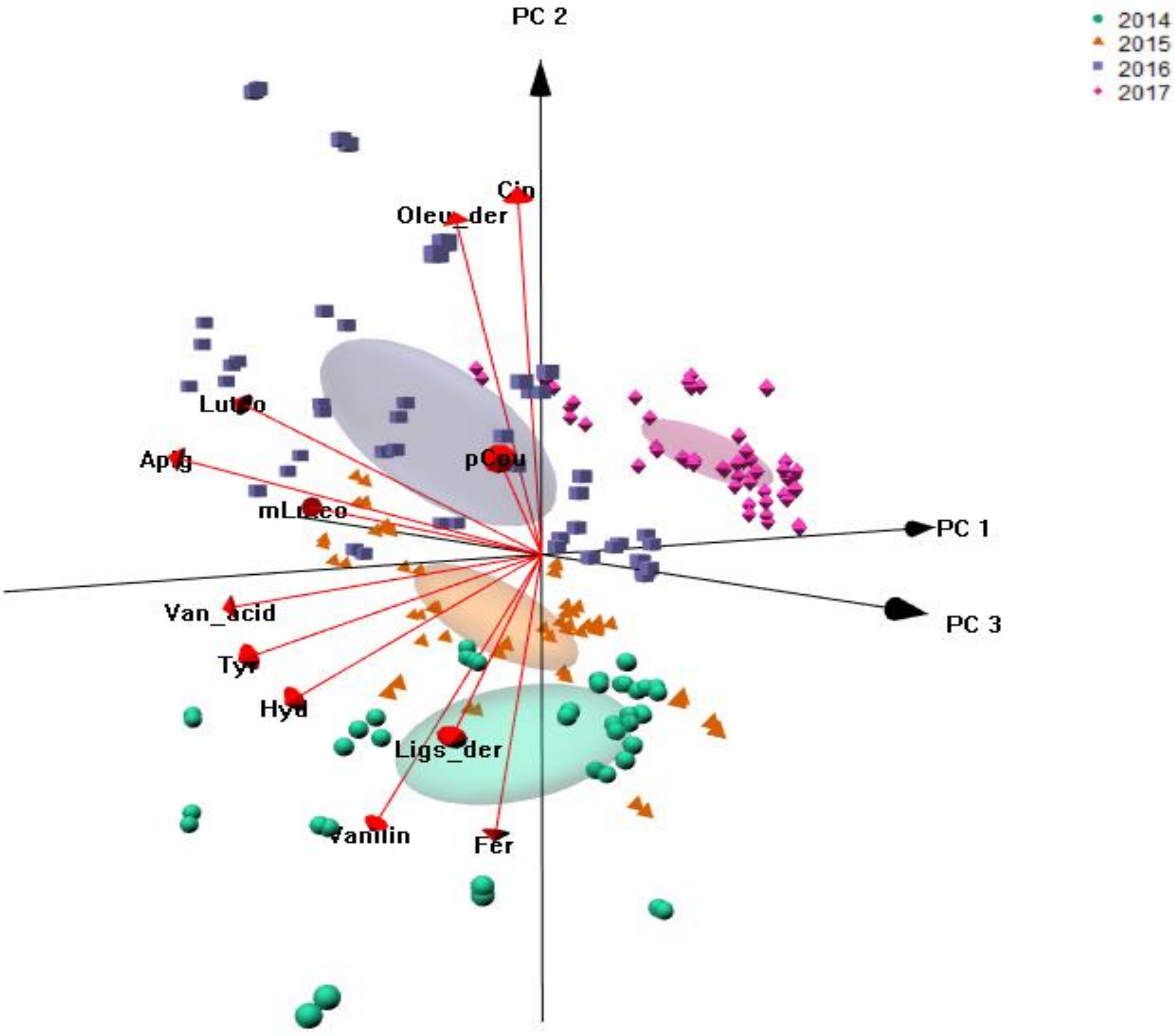

Figure 6.1. Principal component analysis (PC1: 28.0%, PC2: 24.6% and PC3: 11.7%): 3D plot showing the unsupervised pattern recognition according to crop year (2014-2017) based on the individual phenolic contents of olive oils obtained from olives collected from centenarian trees.

Our data showed that the different trends observed over the years have a strong influence of the climatic conditions (supplementary material, Figure 6.1), particularly the water amounts over the year since the temperatures ranges were quite similar. Also, the prominence of both free hydroxityrosol and tyrosol in 2015, seems to be attributed to an increased hydrolysis of secoiridoids. This year was characterized by an accelerated maturation, as can be depicted by the shorter harvest date to attain similar maturation degrees, and October was characterized by an intense rainfall. Both factors could have contributed to this phenomenon. The clear distinction of the 2017 phenolic pattern, with lower amounts of all compounds, can be attributed to the severe water shortage observed in the region (Figure 6.1). Although climatic stress is recognized as a favorable factor for an increased phenolic synthesis (Malheiro et al., 2015), the water shortage observed in that year at the budding process could probably had a negative influence in the amount of phenolic compounds in the fat, a situation that worth being explored in future studies.

Finally, LDA-SA was also applied to verify which of the phenolic compounds were more influenced by the crop year, by reducing the number of non-redundant variables.

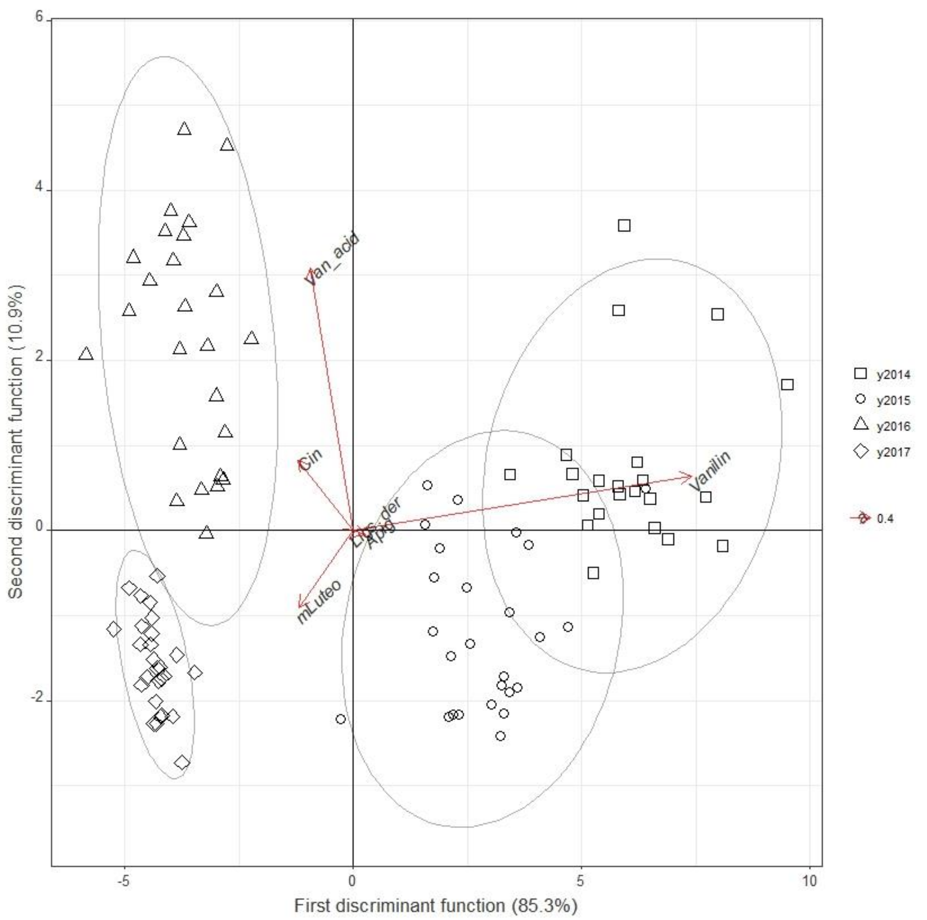

Figure 6.2. Linear Discriminant analysis (1st DF: 85.3%, and 2nd DF: 10.9%): 2D plot showing the discrimination of olive oil according to the production years based on the individual phenolic contents of oils obtained from olives collected from centenarian trees during four consecutive crop years (2014 to 2017).

A LDA-SA model, with three significant discriminant functions (explaining 85.3%, 10.9% and 3.8% of the data variability, respectively) was established based on the experimental contents of 6 phenolic compounds (importance of contribution: vanillin > vannillic acid > methyl luteolin > cinnamic acid > apigenin ≈ ligstroside derivatives).

The model allowed the correct classification of 96% of the original grouped data (Figure 6.2), 96% for the LOO-CV (4 of the 100 olive oils misclassified according to the crop year) and 94 ± 4% for the repeated K-fold-CV (4 folds × 10 repeats leading to 40 randomly runs, with sensitivities ranging from 79% to 100%).

From Figure 6.2 it can be stated that vanillin was the phenolic compound that mostly contributed for the differentiation of the olive oils obtained in 2014 and in 2015. On the contrary, cinnamic acid and vanillic acid were those that mostly contributed to the discrimination of the olive oils produced in 2016. Finally, for 2017, methyl luteolin was the phenolic compound that had the highest influence but the negative values on both components are in agreement with the previous observation of lower amounts of all phenolic compounds in that year.

These results showed the accuracy of the established multivariate linear classification approach, showing that the contents of the 6 abovementioned phenolic compounds could be used as chemical markers to discriminate olive oils according to crop year, confirming that the production year significantly influences the phenolic composition and nutritional quality of the olive oil. Additionally, it is important to stress that the most important phenolic compounds from the health claim point of view, that is, tyrosol, hydroxytyrosol and oleuropein derivatives presented a low inter-year variability within the same trees.

6.3.3. Olive oil discrimination according to phenolic classes and indirect tree classification

For a better identification of the olive trees with the highest phenolic potential, the 112 olive oils were split into 4 different groups, regardless the crop year, according to the sum of all quantified phenolic compounds (total phenolics - TP), namely TP < 300 mg/kg of olive oil, 300 ≤ TP < 400 mg/kg of olive oil, 400 ≤ TP < 500 mg/kg of olive oil and TP ≥ 500 mg/kg of olive oil, all in tyrosol equivalents. The PCA on the individual phenolic compounds showed that the profiles allowed a satisfactory unsupervised differentiation of the oils according to the TP groups (Figure 6.3).

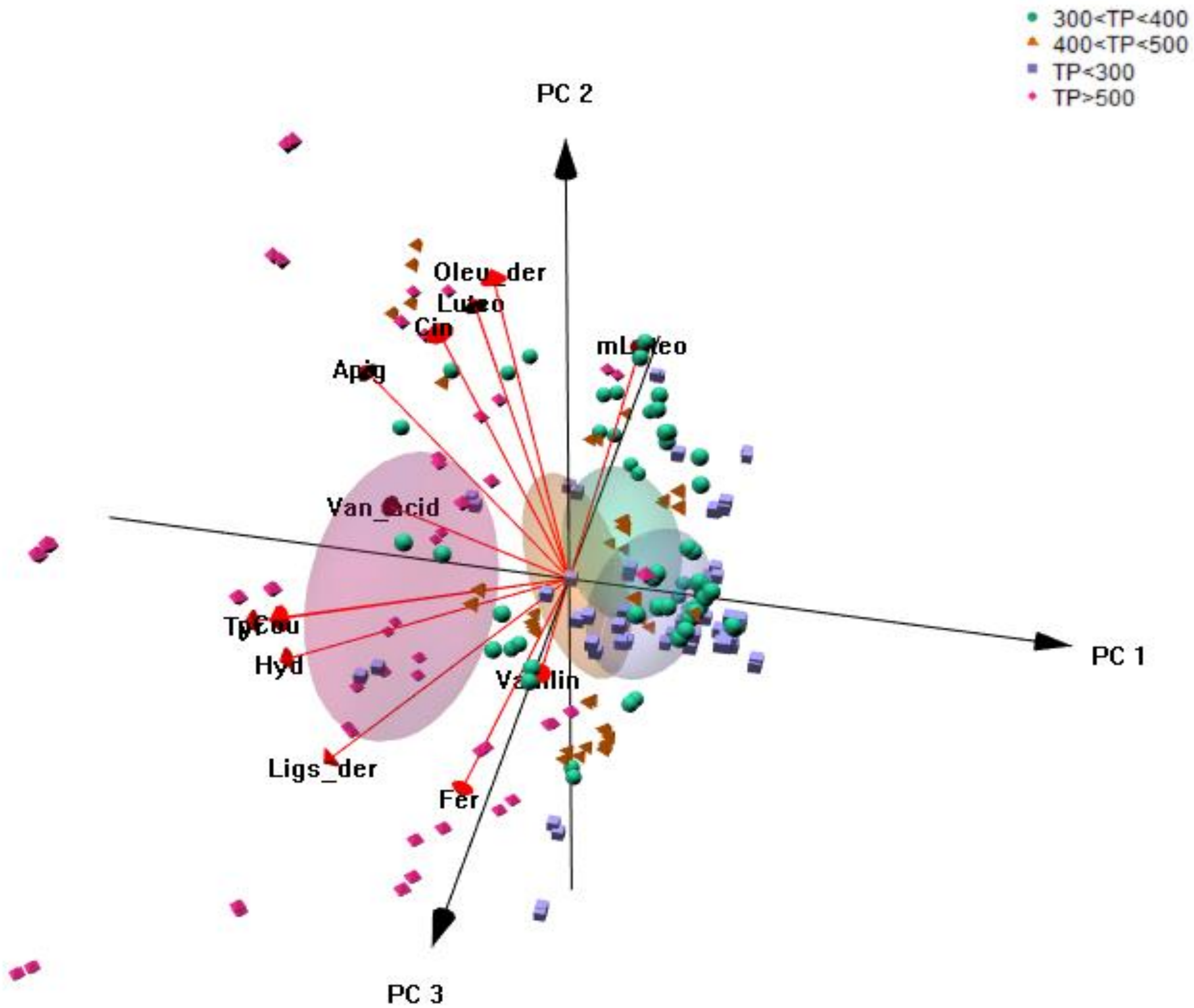

Figure 6.3. Principal component analysis (PC1: 28.0%, PC2: 24.6% and PC3: 11.7%): 3D plot showing the unsupervised pattern recognition according to the predefined groups (<300; 300-400; 400-500; > 500 mg tyrosol equivalents / kg) of olive oils obtained from olives collected during four consecutive crop seasons (2014-2017) based on their individual phenolic compounds.

The results pointed out that, although 4 groups were proposed, the most evident differentiation was from olive oils with a TP greater than 500 mg/kg of olive oil from the others, being the vanillic acid, hydroxytyrosol, tyrosol, *p*-coumaric acid and ligstroside derivatives the phenolic compounds that most influenced this unsupervised discrimination. The LDA-SA procedure allowed establishing a model with two discriminant functions (explaining 99.5% and 0.5% of the data variability, respectively) based on the contents of oleuropein derivatives and ligstroside derivatives. The classification model had sensitivities of 93% of the original grouped data (Figure 6.4), 92% for the LOO-CV (4 of the 100 olive oils misclassified according to the crop year) and 91 ± 6% for the repeated K-fold-CV (4 folds × 10 repeats leading to 40 randomly runs, with sensitivities ranging from 79% to 100%).

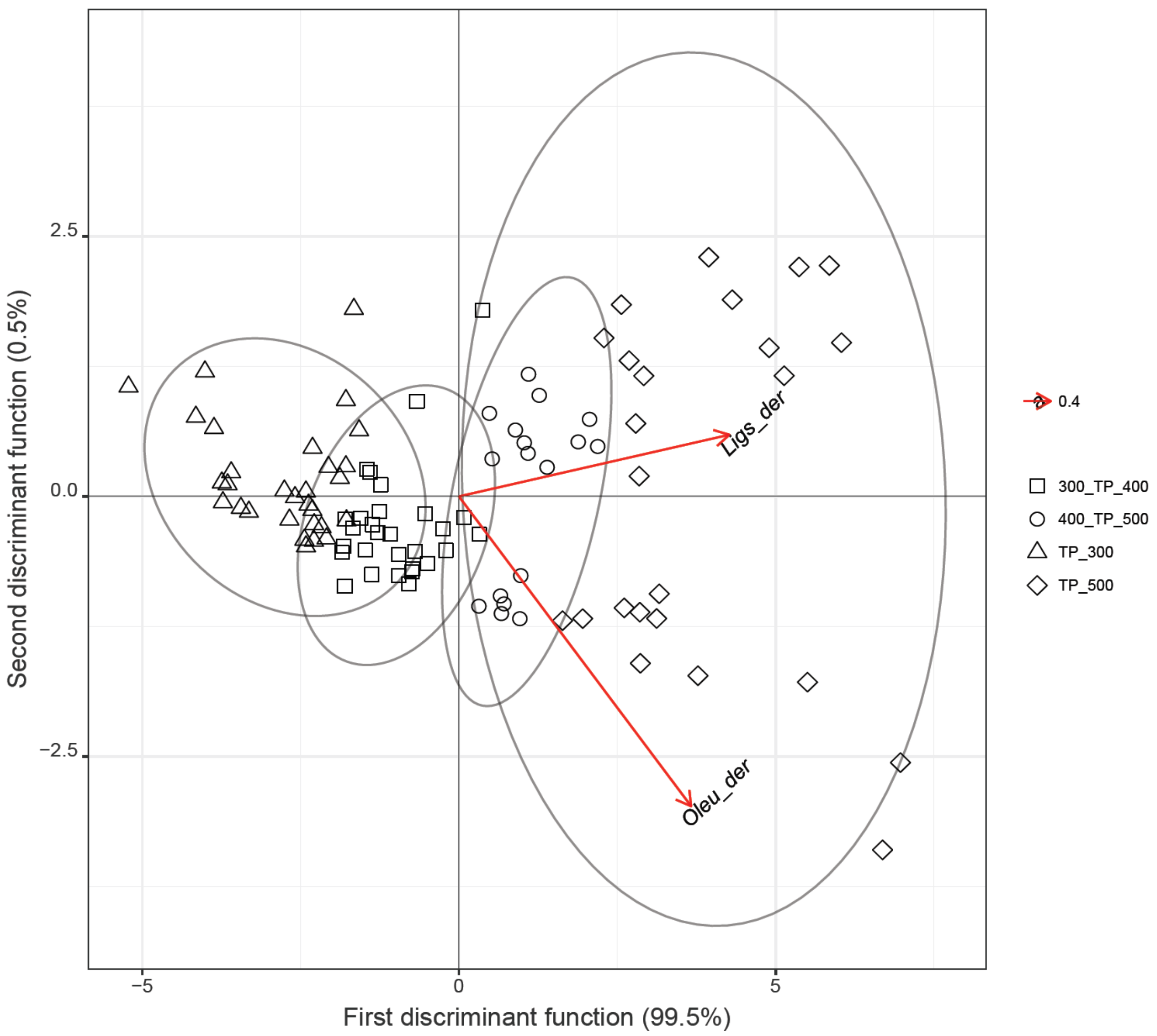

Figure 6.4. Linear Discriminant analysis (LDA1: 99.5%, and LDA2: 0.5%): 2D plot showing the discrimination of olive oil according to the predefined groups (<300; 300-400; 400-500; > 500 mg tyrosol equivalents / kg) of olive oils obtained from olives collected during four consecutive crop seasons (2014-2017) based on their individual phenolic compounds.

As can be inferred from Figure 6.4, the olive oil groups are located along the 1st discriminant function, and seem to be directly correlated (i.e., higher values of the 1st discriminant function corresponded to olive oils with higher overall phenolic contents). The overall results pointed out that, regardless the olive tree and the crop year, the TP content is mostly influenced by the individual secoiridoids aglycons, quantitative the most important phenolic group in the olive oils.

6.3.4. Health claims

According to EU Regulation 432/2012 (2012) and EFSA (2012), if an olive oil has a minimum of 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil, the claim of "*Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress*" could be used in the label. In this regard, it is worth mentioning that this classification is ambiguous, without a clear definition of what phenolic compounds should be included in the claim (Tsimidou, & Boskou, 2015). Using the most conservative approach, that is, solely the sum of free tyrosol, free hydroxytyrosol and hydroxytyrosol derivatives (oleuropein derivatives and hydroxytyrosol acetate), Figure 6.5 shows the mean amounts and standard deviation for the olive oils obtained from each tree in the four consecutive crop years studied per 20g of olive oil, as required by the health claim. The average values varied from 1.4mg/20 g of olive oil (tree 27) to 9.1mg/20g of olive oil (tree 26), with very similar dispersion between most of the trees.

Considering all trees evaluated, it was observed that 50% of them (14 trees) led to olive oils with levels higher than the minimum required for fulfilling the health claim. The olive oils obtained from those trees (tree nº 1, 4, 8, 11-14, 16, 19, 21, 24-26 and 28) could be labeled as "*Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress*".

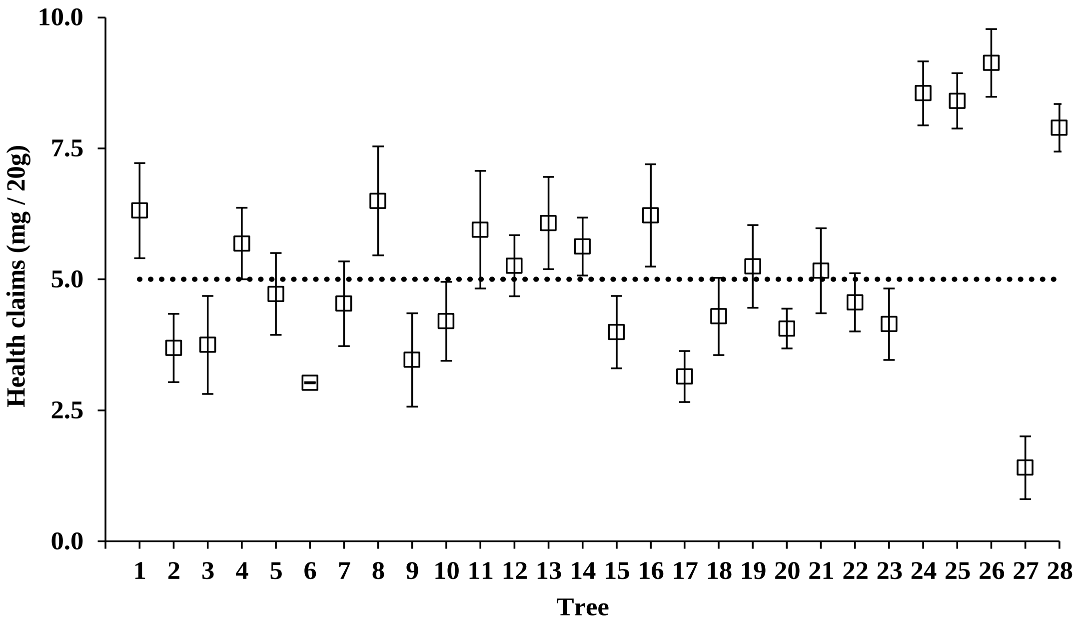

Figure 6.5. Fulfilment of the health claim (according to European Regulation EU 432/2012) based on the sum of free tyrosol, free hydroxityrosol and oleuropein derivatives (in mg of tyrosol equivalents/20g olive oil) for the 112 olive oils obtained from each of the selected 28 centenarian trees (mean ± standard error considering the four consecutive crop years evaluated).

In particular, olive oils obtained from three of these trees, namely trees nº 24, 25 and 26, contained consistently through the four years at least 50% more than the minimum required, representing good candidates for selection. On the contrary, trees nº 6, 17 and 27, showed the lowest amounts of "hydroxytyrosol + tyrosol + oleuropein derivatives" (Figure 6.5). Apart from the health benefits, the total phenolic amounts may also influence the olive oil quality evolution during storage, being known that olive oils rich in phenolic compounds possess greater shelf lives due to their higher resistance to oxidation.

6.4. Conclusions

With this work it was possible to clarify that the year of production is one of the main factor influencing the phenolic profile of olive oils, even if produced under the same agro-climatic conditions and extracted under the most adequate technological conditions. Therefore, for effective conclusions on the potential of certain specimens or cultivars for producing olive oil consistently rich in antioxidants it is necessary to study then over a huge period range. From the 28 specimens of centenarian trees selected for this study it was possible to identify specimens that gave olive oils with exceptional high content of phenolic compounds, consistent through different crop years, while other had no clear interest in this regard. These selected trees are potential candidates for breeding in order to obtain differentiated oils that could hold a health claim, promoting also to the future preservation of the genetic heritage.

Aknowledgements

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6.5. References

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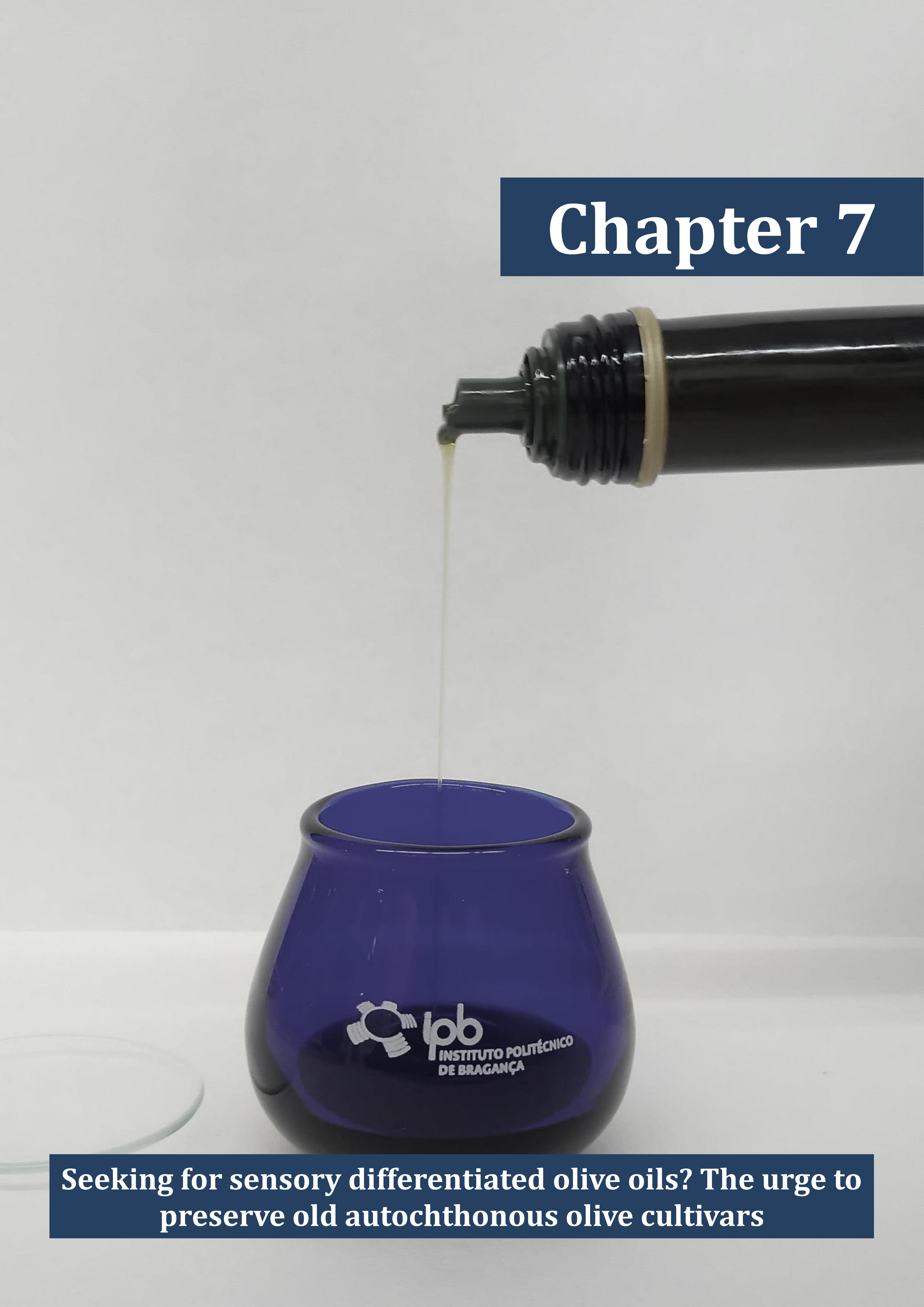
Chapter 7



Seeking for sensory differentiated olive oils? The urge to preserve old autochthonous olive cultivars

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Submitted

Chapter 7

Seeking for sensory differentiated olive oils? The urge to preserve old autochthonous olive cultivars

Abstract

Mediterranean olive heritage richness is poorly characterized. Olive oils from minor cultivars of Northeast-Portugal (Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) from centenarian trees were chemical and sensory characterized, aiming to identify autochthonous cultivars to produce differentiated olive oils. All oils, produced during two campaigns, were classified as extra virgin. *Cv.* Redondal showed the highest oxidative stability (OS), total phenols, vitamin E and $C_{18:1}/C_{18:2}$. Contrary, *cv.* Madural presented the lowest OS and $C_{18:1}/C_{18:2}$ ratios, supporting the importance of fatty acids on OS, while *cv*. Verdeal had the lowest total phenols and vitamin E contents. Sensory notes of tomato, apple, dry fruits, fresh herbs, tomato leaves and cabbage were predominant on most cultivars, whilst some attributes were more specific, such as banana and kiwi (Madural), cherry and apricot (Lentisca and Madural). The chemical and sensory diversity enabled the statistical discrimination of all cultivars and harvesting years.

Keywords: Olive heritage, centenarian trees, chemical characterization, sensory attributes, chemometric tools.

Chapter 7

7.1 Introduction

Olive oil is a product of great importance in Mediterranean countries. It is also one of the pillars of the Mediterranean diet. Due to its composition, nutritional properties and organoleptic characteristics, olive oil is an ingredient present in most dishes made and consumed daily. The origin of the olive tree is not well defined but it is believed that it began in the Asia Minor region, from where it spread to the Mediterranean basin, and from there to almost all continents. Therefore, in this region the existence of ancient olive trees, millenarian and centenarian, represents a very important genetic heritage that needs to be characterized, preserved and valorized. The productivity of these old specimens is usually low and not economically competitive. However, the current market trends of high quality extra virgin olive oil (EVOO) valorizes differentiated products from traditional cultivars (produced in non-intensive systems with specific geographical origins), products rich in health promoting bioactive compounds, as well as products with differentiated sensorial characteristics (Bajoub et al., 2015; Del Monaco et al., 2015; Garcia et al., 2012; Krichene et al., 2010; Reboredo-Rodríguez et al., 2016). The combination of these characteristics is only found in traditional cultivars, and particularly in millennial and centennial specimens grown in low input systems with specific "*terroirs*" (Reboredo-Rodríguez et al., 2015). In this context, olive oils extracted from olives produced by old trees (millenarian or centenarian) could have a great interest and acceptance, producing differentiated oils that could be competitive in the national and international markets (Salimonti et al., 2013). Nevertheless, the information available on old olive trees is very scarce. It is generally established that an olive cultivar, and consequently its genetic information, is a determining factor for the composition, nutritional value and sensory characteristics of olive oils (Bajoub et al., 2015; Fernandes et al., 2018; Köseoğlu et al., 2016). Some works demonstrated that olive cultivars influence the overall chemical composition of the obtained oils (Bajoub et al., 2015; Garcia et al., 2012; Köseoğlu et al., 2016; Lukić et al., 2018; Xian et al., 2017), whilst other demonstrated the influence of the cultivars in specific chemical families, as fatty acids (Krichene et al., 2010; Kritioti et al., 2018; Reboredo-Rodríguez et al., 2015), tocopherols (Beltran et al., 2010), phenolic compounds (Del Monaco et al., 2012), and sterols (Krichene et al., 2010). Among these, some minor compounds are of major importance for the sensory attributes, like polyphenols and volatiles, which are responsible for olive oil bitterness, pungency, and typical aroma (Aparicio-Ruiz et al., 2018; García-Vico et al., 2017; Servili et al., 2009). However, the final sensory profile has a far more complex nature, being also influenced by the soil characteristics, climate, tree health, fruit maturation at the time of harvest, olive harvesting process, olive storage conditions, extraction process and storage method of olive oil prior to packaging (Genovese et al., 2018; Lukić et al., 2018; Kanakis et al., 2013; Köseoğlu et al., 2016). Unfortunately, the cultivar effect on oils obtained from centenarian trees is not well documented, nor the "*terroir*" impacts. Trás-os-Montes is the second largest Portuguese olive-growing region, with a characteristic climate and soil that has contributed for the internationally recognized quality of the olive oils produced in this region. Also, a considerable number of ancient trees found in this region, some with more than 200 years, constitutes a great olive heritage that should be explored. These ancient trees could be considered a biodiversity reservoir of minor cultivars, unknown or poorly characterized. In this context, olive oils extracted from fruits of ancient trees were analyzed for chemical and sensory attributes over two consecutive harvest seasons (2016 and 2017) in order to contribute for its exploitation and valorization as a way to preserve the olive heritage of Trás-os-Montes region. The chosen trees belong to six cultivars, four of them (Lentisca, Rebolã, Redondal and Verdeal) characterized for the first time in the present work, and the other two still poorly known (Madural and Verdeal Transmontana).

7.2. Material and Methods

7.2.1. Sampling

One olive grove with centenarian trees (> 250 years) was selected near Mirandela (N 41º 29' 26.628''; W 7º 15' 31.219''), northeast of Portugal. Based on the tree appearance, structure and trunk thickness, 20 randomly trees were selected and marked. After morphological characterization of fruits and stones (see Supplementary Material Table S7.1), and with the help of experts from the Olive Producers Association of Trás-os-Montes and Alto Douro, the trees were identified as belonging to minor cultivars namely *cvs.* Lentisca (3 trees), Madural (3 trees), Rebolã (3 trees), Redondal (3 trees), Verdeal (2 trees) and Verdeal Transmontana (7 trees) (Figure 1). From each tree, during two crop years (2016 and 2017), approximately three kilograms of fruits were manually collected. To overcome the influence of the maturity stage on the olive oil composition, harvest occurred when the fruits were between the maturity stage two and three, when the fruit epidermis presents red spots in less than half the olive (MI 2) and the fruit epidermis is red or purple in more than half the olive (MI 3). Therefore, harvests took place on different dates in each year to grant similar maturity stages, taking place on the 07th and 08th November in 2016, and on the 13th and 14th November in 2017.

Figure 7.1. Olive fruits collected from centenarian trees from different cultivars (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana)

All fruits were inspected in order to avoid injured fruits attacked by pests or infested by diseases to maintain the maximum quality of the oils. The fruits were processed in the first 24 h after harvest, in a pilot extraction plant with an Abencor analyzer (Comercial Abengoa S.A., Seville, Spain), with three main units: a mill, a thermobeater where malaxation takes place at controlled temperature, and a centrifuge. Olives were milled, and about 700g of the homogenized paste were transferred to the thermobeater unit (25ºC, 20 min). In the final 5 min of each malaxation, 100 mL of water at 27 ºC was added to aid the olive oil separation. The mixture was centrifuged, decanted, and the olive oil collected. After that, the oils were filtered (Whatman paper nº 4) using anhydrous sulfate in order to remove solid particles and residual water. The olive oils were put in 125 mL dark bottles and stored in the dark at room temperature. All the assays were carried out in triplicate within two months after extraction.

7.2.2. Evaluation of quality parameters

All samples were analyzed following the European Union standard methods (Annexes II and IX in European Community Regulation EEC/2568/91 from 11th July and amendments), being assessed the following parameters: free acidity (FA, in % of oleic acid), peroxide value (PV, in $mEqO_2/Kg$), as well as specific coefficients of extinction at 232 nm and 270 nm (K_{232} and K_{270}) and the respective ΔK values. The sensory analysis of the different olive oil samples followed the same European Community Regulation and was performed by 8 trained panelists. For the determination of the descriptive profile a test sheet determined by International Olive Council (IOC) (COI/T.20/Doc. at the. 22 November 2005), with some modifications, was used. The intensities of the olfactory attributes were graded according to a continuous scale of intensity varying from 0 (without perceived sensory sensation) to 10 (maximum intensity of perceived sensory sensation), the intensity of fruity (mature or green), sensations of fruits, herbaceous sensations and harmony. The intensities of the gustatory-retronasal attributes were graded according to an intensity continuous ranging from 0 (in the sensory sensation perceived) to 10 (maximum intensity of the sensory sensation perceived), the intensity of fruity (mature or green), sweet, bitter, pungent, sensations of fruits, herbaceous sensations and harmony. The intensities of the overall sensory attributes were graded according to an intensity continuous scale ranging from 0 (in the sensory sensation perceived) to 10 (maximum intensity of the sensory sensation perceived), the complexity and the persistence were evaluated.

7.2.3. Fatty acids composition

Fatty acids were evaluated as their methyl esters after cold alkaline transesterification with methanolic potassium hydroxide solution (European Community Regulation EEC/2568/91 from 11th July). The fatty acid profile was determined with a Chrompack CP 9001 chromatograph equipped with a split-splitless injector, a FID detector, a Chrompack CP-9050 autosampler and a 50 m × 0.25 mm i.d. Select FAME fused silica capillary column coated (Agilent). Helium was used as carrier gas at an internal pressure of 110 kPa. The temperatures of the detector and injector were 250ºC and 230ºC, respectively. The split ratio was 1:50 and the injected volume was of 1 μL. The results were expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area eluting between myristic and lignoceric methyl esters. A control sample (olive oil 47118, Supelco) and a certified fatty acids methyl esters standard mixture (Supelco) were used for identification and calibration purposes (Sigma, Spain).

7.2.4. Vitamin E content

Vitamin E content was assessed as the sum of the individual tocopherol masses (α-, β- and γ-), which contents were determined according to the ISO 9936 (2006), with some modifications as described by Rodrigues et al. (2018a). Tocopherols standards (α-, β- and γ-) were purchase from Sigma (Spain), while the internal standard 2-methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol (tocol) was from Matreya Inc. (Pleasant Gap, PA, USA). Filtered olive oil (50 mg) was mixed with the internal standard solution (tocol) and dissolved in n-hexane. The mixture was centrifuged for 5 min at 13,000 rpm and the supernatant obtained analyzed by HPLC. The liquid chromatograph consisted of a Jasco integrated system (Japan) equipped with a Jasco LC-NetII/ADC data unit, a PU-1580 Intelligent Pump and a FP-920 fluorescence detector (λexc= 290 nm and λem= 330 nm). The chromatographic separation was achieved on a SupelcosilTM LC-SI column (3 μm) 75 x 3.0 mm (Supelco, Bellefonte, PA), operating at 23 ºC. A mixture of n-hexane and 1,4-dioxane (97.5:2.5) was used as eluent, at a flow rate of 0.7 mL/min. Data were analyzed with the ChromNAV Control Center - JASCO Chromatography Data Station (Japan). The compounds were identified by chromatographic comparisons with authentic standards, by co-elution and by their UV spectra. Quantification was based on the internal standard method, using the fluorescence signal response and individual calibration curves for each tocopherol. Total vitamin E corresponded to the sum of the individual tocopherol masses.

7.2.5. Total phenols content

Total phenols content was assessed by the methodology described by Capannesi et al. (2010) with some modifications, as follows: 2.5g of olive oil were diluted with 2.5mL of n-hexane (1:1 w/v) and extracted three times with 2.5mL of methanol/water (80:20; v/v), followed by 5 minutes of centrifugation at 5,000rpm. From the combined extract, 1 mL was taken for analysis, being added the same amount of Folin-Ciocalteau reagent and of Na_2CO_3 solution (7.5%), after which 7 mL of purified water were added. After homogenization, the samples mixtures were stored overnight (12-16 hours) in the dark, and the spectrophotometric analysis was performed at λ = 765 nm. A calibration curve of caffeic acid in methanol was made in concentration range of 0.04 to 0.18 mg/mL. The final results were expressed as mg of caffeic acid equivalents per kg of olive oil (mg CAE/kg).

7.2.6. Oxidative stability (Rancimat)

The oxidative stability (OS) was evaluated using a Rancimat 743 apparatus (Metrohm CH, Switzerland) following the methodology previous described by Rodrigues et al. (2016). Filtered, cleaned, dried air (20 L/h) was bubbled through the oil (3.00 g) heated at 120 ± 1.6ºC, with the volatile compounds being collected in water, and the increasing water conductivity continuously measured. The time taken to reach the conductivity inflection was recorded in hours, being assumed as the OS value.

7.2.7. Statistical analysis

One-way analysis of variance (one-way ANOVA) was applied to evaluate the existence of statistical significant effects of the tree cultivar or the harvest year in the different physic-chemical and sensory parameters of olive oils. Moreover, if a significant statistical effect was found ($P < 0.050$), the post-hoc multi-comparison Tukey's test was also applied aiming to identify the levels (i.e., olive tree cultivar or harvest season) of each effect that were responsible for the detected significant effect. Boxplots were used to show the one-way ANOVA statistical results. Finally, linear Pearson correlation coefficients (R-Pearson) were calculated to evaluate the existence of bivariate correlations between fatty acids contents and sensory attributes of olive oils. The possible influences of olive tree cultivar (*i.e.*, *cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal or Verdeal Transmontana) or harvest season (2016 and 2017) on the olive oil chemical and sensory characteristics of olive oil extracted from olives of centenarian trees was also evaluated using principal component analysis (PCA), an unsupervised multivariate pattern recognition technique. For PCA, the values of the different parameters were centered and scaled minimizing data variability. The statistical analysis was performed using the Subselect (Cadima et al., 2004; Cadima et al., 2012; Kuhn, & Johnson, 2013) and MASS (Venables, & Ripley, 2002) packages of the open source statistical program R (version 2.15.1), at a 5% significance level.

7.3. Results and discussions

7.3.1. Quality and Physicochemical Parameters

In order to evaluate the quality of the varietal olive oils obtained from minor cultivars centenarian trees, different quality parameters were determined during two crop years (2016 and 2017), namely free acidity (FA), peroxide value (PV), extinction coefficients at 232 and 270 nm (K_{232} and K_{270}) and quality grade classification according to the sensory evaluation (Table 7.1). The obtained results show a consistently low FA without a clear cultivar pattern. In 2016 all samples were within a very narrow range, from 0.15% to 0.23%, with *cv*. Redondal and *cv*. Verdeal Transmontana cultivars having the lowest FA and *cv*. Lentisca the highest. In 2017 the FA increased slightly, varying from 0.26% (*cvs.* Lentisca and Verdeal Transmontana) and 0.32% (*cv.* Redondal). According to the results obtained for this parameter, it was verified that there is a low global free acidity (from 0.2 to 0.3%), below the limit of 0.8% established by European Community Regulation EEC/2568/91 from 11th July for the EVOO category. It was noticed that in the harvest season of 2017 the results are globally higher than in 2016. This fact could be related with the extreme drought verified in 2017, during all season, that might have induced some increased hydrolysis in the fruit, but all values were generally low. For the peroxide value, indicative of oxidation, the lower values were also observed in 2016, ranging from 1.4 (*cv*. Redondal) to 3.6 mEqO_2/kg olive oil (*cv*. Madural) while in 2017, the values varied from 3.0 (*cv.* Rebolã) and 5.2 mEqO_2/kg olive oil (*cv.* Lentisca). Once again all PVs were below the 20 mEqO_2/kg maximum limit established by European Community Regulation EEC/2568/91 from 11th July for the classification of olive oil as EVOO. For the extinction coefficients, namely K_{232}, the values were lower than 2.03 in both years, with the lowest observed in *cv.* Redondal (2016, 1.31±0.13), as opposed to *cv.* Verdeal that presented the highest one (2016, 2.03±0.02). For the K_{270}, the values varied from 0.11 (*cvs.* Rebolã and Verdeal Transmontana in 2016) and 0.23 (*cv.* Redondal, in 2017). All extinction coefficient values were within the legal limits established by the European Community Regulation EEC/2568/91 from 11th July for EVOO, with the exception of *cv.* Redondal in 2017, being therefore considered VOO. All olive oils were submitted to a sensory analysis, being all classified as EVOO without sensorial defects, and with a mean fruity note intensity above zero. Although statistical differences were found for some quality parameters ($P < 0.0001$) between varieties (in the same harvest season), and between years (within the same variety), the physicochemical levels were generally low, indicative of small hydrolysis and oxidation of the oils.

Table 7.1. Free acidity (% oleic acid), peroxide value (mEq O_2/kg), specific extinction coefficients (K_{232} and K_{270}) of olive oils obtained from olives produced from centenarian olive trees of cultivars Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana, harvest years 2016-2017 (mean ± standard deviation).

Cultivar	Free Acidity			Peroxide Value			K_{232}			K_{270}			Sensory evaluation	
	2016	2017	*P*-value[2]	2016	2017	*P*-value[2]	2016	2017	*P*-value[2]	2016	2017	*P*-value[2]	2016	2017
Lentisca	0.23 ± 0.04^{a}	$0.26 \pm 0.03^{a,b}$	0.0598	$2.4 \pm 0.7^{a,b}$	5.2 ± 2.0	***0.0010***	1.77 ± 0.13^{b}	1.79 ± 0.28^{a}	0.4498	0.19 ± 0.04^{a}	$0.17 \pm 0.04^{b,c}$	0.2377	EVOO	EVOO
Madural	0.16 ± 0.03^{b}	$0.29 \pm 0.02^{a,b}$	***< 0.0001***	3.6 ± 1.0^{a}	4.8 ± 1.4	0.0555	1.55 ± 0.09^{c}	2.00 ± 0.19^{a}	***0.0002***	0.13 ± 0.02^{b}	$0.18 \pm 0.02^{b,c}$	***0.0002***	EVOO	EVOO
Rebolã	0.16 ± 0.03^{b}	$0.30 \pm 0.07^{a,b}$	***0.0004***	3.2 ± 1.0	3.0 ± 0.4	0.3181	$1.49 \pm 0.14^{c,d}$	$1.74 \pm 0.13^{a,b}$	***0.0045***	0.11 ± 0.04^{b}	0.15 ± 0.04^{c}	***0.0482***	EVOO	EVOO
Redondal	0.15 ± 0.03^{b}	0.32 ± 0.03^{a}	***< 0.0001***	1.4 ± 0.4^{b}	4.3 ± 2.9	***0.0178***	1.31 ± 0.13^{d}	1.90 ± 0.13^{a}	***< 0.0001***	0.12 ± 0.02^{b}	0.23 ± 0.03^{a}	***<0.0001***	EVOO	EVOO
Verdeal	$0.20 \pm 0.03^{a,b}$	$0.30 \pm 0.03^{a,b}$	***0.0015***	2.5 ± 0.0^{a}	4.0 ± 0.8	***0.0052***	2.03 ± 0.02^{a}	1.99 ± 0.20^{a}	0.3429	0.22 ± 0.01^{a}	$0.21 \pm 0.01^{a,b}$	0.2685	EVOO	EVOO
Verdeal Transmontana	0.15 ± 0.03^{b}	0.26 ± 0.04^{b}	***< 0.0001***	2.8 ± 0.8^{a}	3.6 ± 1.5	0.0693	1.34 ± 0.16^{d}	1.54 ± 0.11^{b}	***0.0004***	0.11 ± 0.02^{b}	0.16 ± 0.02^{c}	***< 0.0001***	EVOO	EVOO
***P*-value[1]**	***< 0.0001***	***0.0178***		***0.0004***	0.1600		***< 0.0001***	***< 0.0001***		***< 0.0001***	***<0.0001***		----	----

[1]For each column, a *P*-value < 0.05 (in bold and italic) means that, for each harvest year (2016 or 2017) the mean value of the evaluated parameter of at least one olive cultivar differs from the others, according to the one-way ANOVA results (in this case multiple-comparison tests were performed). In each column, different small letters mean significant statistical differences of the quality parameter under evaluation, at a 5% significance level (*P*-value < 0.05), according to multiple comparison Tukey's HSD test.
[2]For each line and for each olive cultivar, a *P*-value < 0.05 (in bold and italic) means that the mean value of the evaluated parameter varied significantly with the harvest year, according to t-Student test.

This is not surprising since the olive oils were fresh and obtained from healthy fruits, harvested manually at an ideal ripening stage, transported to the laboratory in a short time and extracted on the same day, minimizing the risk of hydrolysis and oxidation of fatty acids. These results confirm that a correct handling of the fruits enables to produce high quality olive oil, with the genetic ground having a low influence on these parameters, in line with the results obtained by other authors (e.g.: Chiappetta et al., 2017; Xiang et al., 2017; Reboredo-Rodríguez et al., 2018). Table 7.2 details the oxidative stability (OS), total phenols (TP), vitamin E, and the oleic acid/linoleic acid ratio of the olive oils. Significant differences between cultivars (P < 0.0001) were observed in the OS, with the harvest season having also a significant influence in this parameter for the *cvs.* Madural and Rebolã (P < 0.0001). Consistently, the highest values, 32.6h (2016) and 33.6h (2017) were observed in the olive oils of *cv.* Redondal, and the lowest ones, 10.8h (2016) and 14.7h (2017) in *cv*. Madural. This parameter is of high importance once it could help to predict the shelf life of the olive oils and the capacity to prevent the oxidation process. According to the obtained results the following order could be established for shelf life: Redondal > Verdeal Transmontana > Verdeal > Lentisca > Rebolã > Madural. The global range of OS values obtained is similar to the ones obtained by other authors for different monovarietal olive oils considering analogous analytical conditions (e.g. Tura et al., 2007). The TP content seemed to be significantly influenced by olive cultivar (P < 0.0001), ranging from 75 to 135 mg CAE/kg of olive oil in 2016 and from 164 to 363 mg CAE/kg of olive oil in 2017, with the highest values consistently observed in *cv.* Redondal and the lowest ones for *cv.* Verdeal. The TP contents were also significantly influenced (P < 0.0001) by harvest season with values consistently higher in 2017 (Table 7.2). This behavior was expected since the final amount of phenolic compounds, which are secondary plant metabolites, is influenced by diverse factors such as cultivar, geographical origin, agro-climatic conditions, degree of maturation, fruit freshness before extraction, extraction method and storage conditions (Dabbou et al., 2015; Gómez-Caravaca et al., 2016; Köseoğlu et al., 2016). Due to their sensory characteristics and antioxidant activity, phenolic compounds are important for the sensory profile of the olive oils, contributing to olive oil resistance towards oxidation, while constituting interesting antioxidant sources for olive oil consumers. Still, within the antioxidants, the role of vitamin E is also well known on human health.

Table 7.2. Oxidative stability (hours), total phenols (mg CAE/kg of olive oil), vitamin E (mg/kg of olive oil) and ratio $C_{18:1}$ / $C_{18:2}$ of olive oils obtained from olives produced from centenarian olive trees of cultivars Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana, harvest years 2016-2017 (mean ± standard deviation).

Cultivar	OS			Total Phenols			Vitamin E			$C_{18:1}/C_{18:2}$		
	2016	2017	*P*-value[2]	2016	2017	*P*-value[2]	2016	2017	*P*-value[2]	2016	2017	*P*-value[2]
Lentisca	21.4±7.1^{b,c}	16.7±2.7^{c}	0.0530	110±29^{a,b}	229±3^{a,b}	***< 0.0001***	242±75^{b}	335±139^{a}	0.0601	17.1±6.7^{c}	14.6±5.8^{b}	0.2229
Madural	10.8±1.2^{d}	14.7±0.4^{c}	***< 0.0001***	116±6.^{a,b}	231±20^{a,b}	***< 0.0001***	224±6^{b}	315±37^{a}	***< 0.0001***	5.8±0.2^{d}	5.1±0.2^{c}	***0.0001***
Rebolã	14.7±3.2^{c,d}	20.3±1.6^{c}	***0.0015***	97±12^{b}	252±30^{a}	***< 0.0001***	197±6^{b,c}	280±29^{a,b}	***< 0.0001***	9.9±0.8^{d}	8.6±2.6^{b,c}	0.1333
Redondal	32.6±1.4^{a}	33.6±1.4^{a}	0.2727	135±38^{a}	363±28^{a}	***< 0.0001***	309±11^{a}	354±12^{a}	***< 0.0001***	34.9±3.2^{a}	29.6±5.4^{a}	***0.0016***
Verdeal	19.3±0.3^{b,c,d}	19.9±0.6^{c}	0.0614	75.±18^{b}	168±15^{c}	***0.0001***	140±8^{c}	171±11^{b}	***0.0017***	16.9±0.5^{b,c}	12.2±1.0^{b,c}	***< 0.0001***
Verdeal Transmontana	23.2±5.3^{b}	26.6±6.1^{b}	0.0656	108±14^{a,b}	202±16^{b,c}	***< 0.0001***	161±7^{c}	198±29^{b}	***< 0.0001***	31.8±0.8^{b}	24.9±2.8^{a}	***< 0.0001***
***P*-value[1]**	***< 0.0001***	***< 0.0001***		***0.0038***	***< 0.0001***		***< 0.0001***	***< 0.0001***		***< 0.0001***	***< 0.0001***	

[1]For each column, a *P*-value < 0.05 (in bold and italic) means that, for each harvest year (2016 or 2017) the mean value of the evaluated parameter of at least one olive cultivar differs from the others, according to the one-way ANOVA results (in this case multiple-comparison tests were performed). In each column, different small letters mean significant statistical differences of the quality parameter under evaluation, at a 5% significance level (*P*-value < 0.05), according to multiple comparison Tukey's HSD test.
[2]For each line and for each olive cultivar, a *P*-value < 0.05 (in bold and italic) means that the mean value of the evaluated parameter varied significantly with the harvest year, according to t-Student test.

The results (Table 7.2) demonstrated that the amounts of vitamin E were dependent of both studied factors, the olive cultivar and harvest season (P < 0.0001). Again, *cv.* Redondal was richest in vitamin E, with 309 mg/kg in 2016, and 354 mg/kg in 2017, while *cv.* Verdeal was the poorest, with 140 mg/kg in 2016 and 172 mg/kg in 2017. These results agree in general with the ones obtained for different cultivars in different parts of the world, with average amounts varying from 50 to 500 mg/kg of olive oil, suggesting that the amount of vitamin E highly depends on the olive cultivar (Beltrán et al., 2010; Reboredo-Rodríguez et al., 2016; Tura et al., 2007) but also influenced by other factors like maturation process and agro-climatic conditions. In the present work, the severe drought observed during the year 2017 (data not shown) seems to promote the synthesis of these antioxidants, probably as a protection of the plant against the increased environmental stress.

The fatty acids composition is a genuineness parameter for olive oils according to the European Community Regulation EEC/2568/91 from 11th July and can also be used for cultivar discrimination (Kritioti et al., 2018), while the unsaturation degree settles the oxidative susceptibility, and consequently the shelf life of olive oils. The oleic/linoleic acid ratios of the olive oil samples were statistical influenced (P < 0.0001) by the olive cultivar and in some cases by the harvest season (Table 7.2). In both years, Madural was the cultivar that presented the lowest ratios, with values of 5.75 and 5.11 respectively for 2016 and 2017. On opposition, the highest ratios were obtained for *cv*. Redondal, with 34.9 and 29.6 for 2016 and 2017, respectively. In general, the $C_{18:1}/C_{18:2}$ ratio followed the order Redondal > Verdeal Transmontana > Verdeal > Lentisca > Rebolã > Madural, highly consistent with the results observed for the OS. It could also be observed that the ratios obtained in 2017 were lower than those of 2016, for all cultivars. This could be indicative that under high summer temperatures and drought (data not shown), as the ones observed in 2017, the fatty acids biosynthesis pathway is altered, probably in favour of linoleic acid. According to Cayuela-Sánchez et al. (2018), genetics, climate and agronomic conditions influence the diversity of fatty acids.

If all the information of the Table 7.2 is analysed together, it could be inferred that *cv.* Redondal is simultaneously the cultivar with the higher amount in antioxidants and also higher $C_{18:1}/C_{18:2}$ ratios, a combined situation that seems to be determinant for an increased OS. Conjugating both OS and the $C_{18:1}/C_{18:2}$ (mean values per cultivar for both years), a significant positive linear correlation was obtained (R-Pearson= +0.9704; P = 0.0013), pointing out that the OS of olive oils is promoted by higher $C_{18:1}/C_{18:2}$ ratios.

7.3.2. Descriptive Sensory Profile

The sensory profiles of the olive oils obtained from centenarian trees of minor cultivars Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana are shown in Figures 7.1, 7.2 and 3. In those figures, an average of both harvest seasons (2016-2017) of each attribute were presented once the global aim was to define the sensory profile of each cultivar. Figure 7.1 showed the olfactory profile of the different olive oils, which note intensity perception is graded from 0 to 10. All olive oils presented a fruity intensity higher than 0 without any sensory defect, allowing their classification as EVOO (European Community Regulation EEC/2568/91 from 11th July). In all samples the fruity olfactory sensation was "green" and no statistical differences were found regarding this parameter, which intensities varyied from 5.0 in the *cvs*. Rebolã and Verdeal to 5.9 in *cv.* Madural. Still, different fruits sensations were found (Figure 7.2). All oils presented a tomato note sensation, with a broad range of perceived intensities that varied from 1.0 to 7.5. Although statistically similar for all cultivars, the highest mean values for this attribute were observed in *cv.* Verdeal Transmontana (4.2) and the lowest in *cv.* Verdeal (3.0). Low intensities were observed for "apple" aroma and two statistical different groups could be found ($P \leq 0.05$): higher intensities were attributed to the group constituted by the *cvs.* Madural, Rebolã, Redondal and Verdeal Transmontana, with averages around 3.8, and lower to the group constituted by *cvs.* Lentisca (3.1) and Verdeal (2.7). For the "banana" aroma, two statistical different groups were also obtained ($P \leq 0.05$) (Figure 7.2), being this aroma characteristic of *cv.* Madural and Rebolã, and absent in Redondal and Verdeal olive oils. All oils presented "dry fruits sensation" with similar scores, between 1.6 (*cv.* Verdeal) to 2.4 (*cv.* Redondal). This latter attribute is a typical note that characterizes the "*terroir*" of the region. Rotondi et al. (2017) also considered dry fruits as a pattern when studied olive oils from different cultivars and their clones, which sensation was present in all samples. In the present work others fruit sensations were also perceived by the panelists, including green cherry, apricot and kiwi, classified as positive attributes. However, since their perceptions were residual and for a scarce number of samples, they were not statistically evaluated. Green cherry was noticeable in *cvs.* Lentisca and Madural with mean values of 1.6 and 0.4, respectively, while apricot sensation appeared in *cvs.* Lentisca (1.7), Madural (0.5) and Verdeal Transmontana (0.1). The kiwi notes were only perceptible in *cv.* Madural (0.4). Lukić et al. (2017) and Lukić et al. (2018) when studying monovarietal olive oils of *cvs.* Oblica Buža and Istarska bjelica, reported sensory attributes like apple, tomato, almond, aromatic herbs and chicory/rocket, which perceptions differ according to the cultivar.

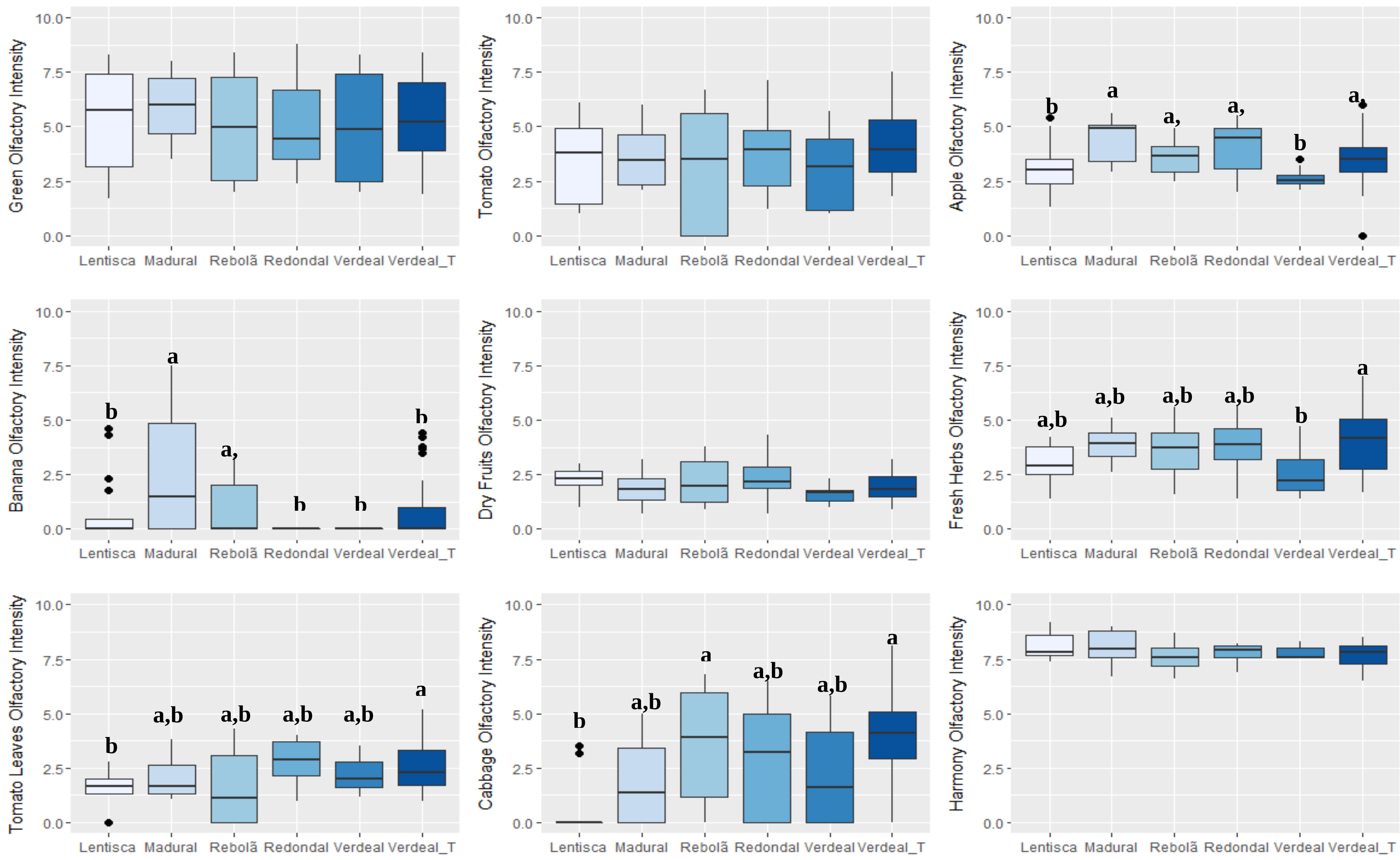

Figure 7.2. Boxplots of olfactory profile analysis (sensation of fruity, herbaceous sensations and harmony intensity) found in olive oils extracted from olives collected from centenarian trees from different cultivars (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) during two consecutiveharvest years (2016 to 2017). Different lowercase letters mean significant statistical differences at a 5% significance level (one-way ANOVA followed by the Tukey's multi-comparison test).

Concerning herbaceous sensations, attributes of fresh herbs, tomato leaves and cabbage (sensation of cut cabbage) were found. Within these attributes, fresh herbs were generally the dominant notes but statistically different between cultivars ($P \leq 0.05$). The olive oils from *cv.* Verdeal Transmontana presented the highest mean value (4.0) and the *cv.* Verdeal the lowest one (2.5). Similarly, cut cabbage sensation was significantly ($P \leq 0.05$) higher for *cv.* Verdeal Transmontana (3.8) and Rebolã (3.5) oils and lower for *cv.* Lentisca (0.4), with middle values for the remaining cultivars. For the "tomato leaves" attribute Verdeal Transmontana presented the highest values (2.6) while *cvs.* Lentisca and Rebolã (1.6) scored the lowest (Figure 7.2). The obtained results for herbaceous notes are in agreement with other authors for diverse cultivars (Lukić et al., 2018) and are the main responsible for the green fruity aroma of the olive oils. Figure 7.3 shows the values for fruity intensity and for the basic sweet, bitter and pungent attributes. All the analyzed olive oils showed similar fruity intensity, with mean values from 5.1 (*cv.* Rebolã) to 5.8 (*cv.* Verdeal Transmontana) (Figure 7.3). Concerning the basic gustatory attributes there were statistically differences ($P < 0.05$) in sweet and pungent notes. Olive oils from *cvs.* Redondal (1.6), Verdeal (1.6) and Verdeal Transmontana (1.5) were significantly less sweet than Madural (3.3). For pungent attribute two distinct significantly ($P \leq 0.05$) groups were found, one with higher values constituted by *cvs.* Lentisca, Madural, Verdeal and Verdeal Transmontana, and other with lower values formed by *cv.* Rebolã and Redondal. Despite the diversity of values observed for the bitter attribute, no significant differences were observed. Similar values of basic flavors were observed by Reboredo-Rodríguez et al., 2016 and Reboredo-Rodríguez et al., 2018 in olive oils from autochthonous olive cultivars from Galicia (Spain). García-Mesa et al. (2008) referred that basic sensations may vary according to the lipid composition, with matrices richer in monounsaturated fatty acids (MUFA) being more bitter and pungent than matrices rich in polyunsaturated acids (PUFA). However, our results did not follow this same tendency and no significant correlations were found between MUFA and bitter and pungent attributes. Nevertheless, a significantly negative correlation (R-Pearson= -0.8448; P= 0.0342) was determined between MUFA and the intensity of the sweet sensation. This attribute was also significantly positive correlated (R-Pearson= +0.9391; P= 0.0054) with PUFA and bitterness also showed a positive correlation with the amount of saturated fatty acids (R-Pearson= +0.8252; P= 0.0432). Gustatory attributes included fruity sensations of tomato, apple, banana and dry fruits (Figure 7.4). Tomato sensation was the most pronounced, with mean values from 1.0 to 7.6, but no significant differences were observed between cultivars.

A

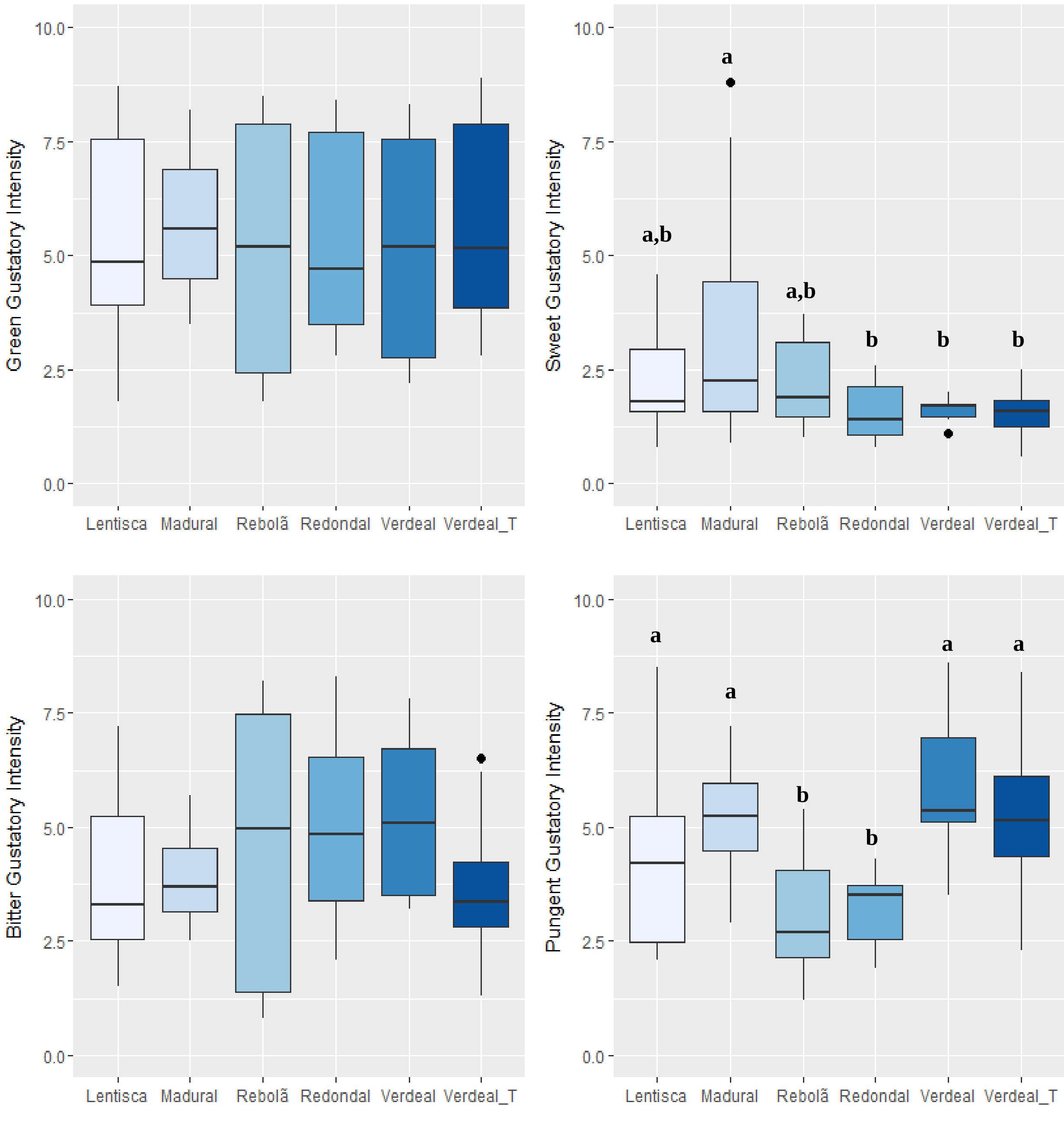

Figure 7.3. Boxplots of gustatory profile analysis (basic tastes intensity) found in olive oils extracted from olives collected from centenarian trees from different cultivars (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) during two consecutive harvest years (2016 to 2017). Different lowercase letters mean significant statistical differences at a 5% significance level (one-way ANOVA followed by the Tukey's multi-comparison test).

B

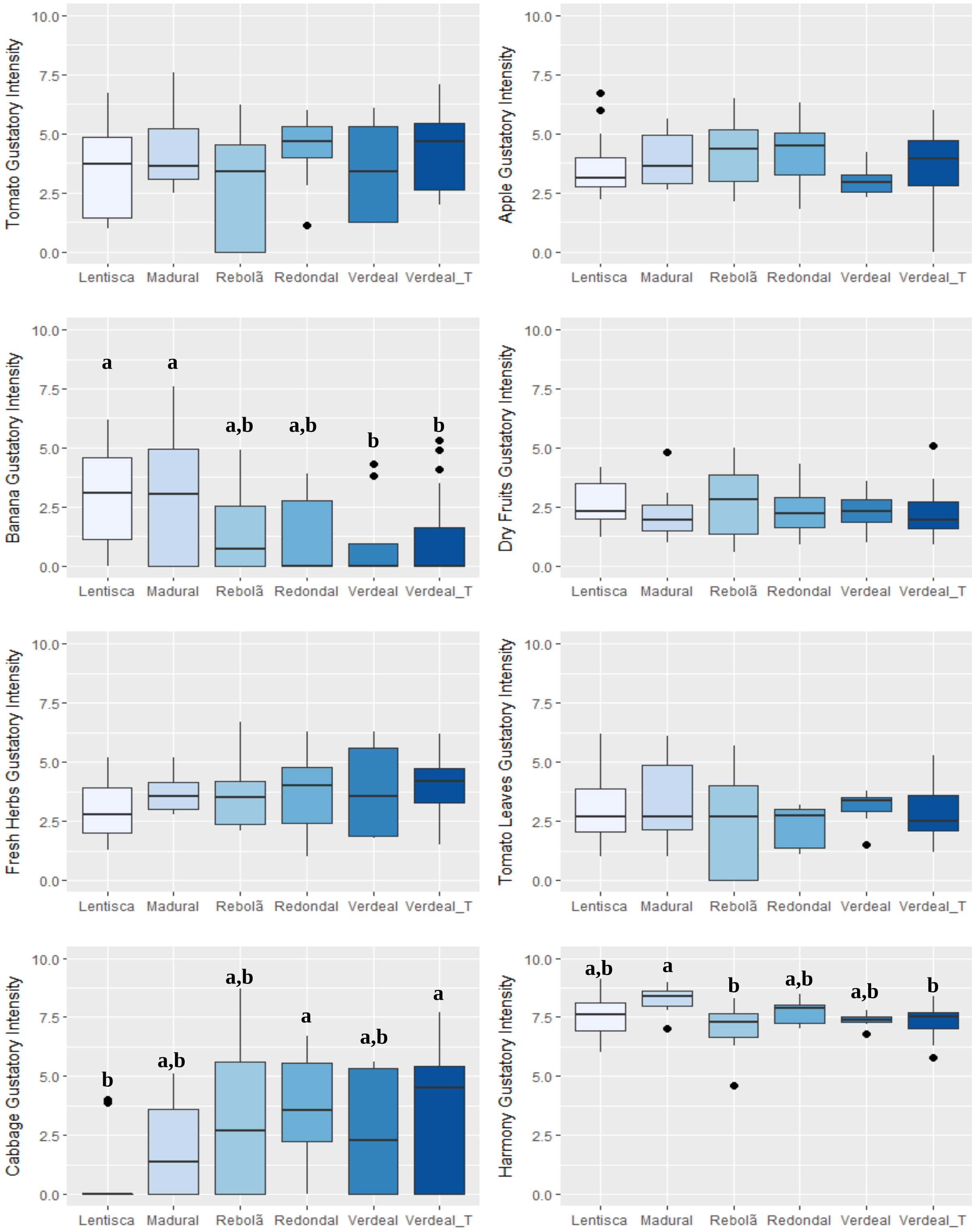

Figure 7.4. Boxplots of gustatory profile analysis (sensation of fruity, herbaceous sensations and harmony intensity) found in olive oils extracted from olives collected from centenarian trees from different cultivars (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) during two consecutive harvest years (2016 to 2017). Different lowercase letters mean significant statistical differences at a 5% significance level (one-way ANOVA followed by the Tukey's multi-comparison test).

The same trends were observed for apple and dry nut sensations, similar in all cultivars. These attributes were well perceptible by the panelists in all olive oils, but no statistical differences were observed between cultivars, which could be attributed to the "*terroir*" of the region since they were also reported in previous works with other cultivars (Rodrigues et al., 2018b).

A contrary situation was observed for banana gustatory attribute, with two significantly different groups observed ($P \leq 0.05$), being significant amounts detected in *cvs.* Madural (3.1) and Lentisca (3.0), whereas for Verdeal (1.0) and Verdeal Transmontana (0.9) the attribute is practically absent (Figure 7.4), and so, could be foreseen as potential markers of the former cultivars. As in olfactory sensations, other fruit gustatory attributes, like green cherry, apricot and kiwi were also found, but only perceived in olive oils of some cultivars. Cherry gustatory attribute was only noticeable in *cvs.* Lentisca (1.9) and Madural (0.3); apricot sensation appeared in *cvs.* Lentisca (2.2), Madural (0.5) and Verdeal Transmontana (0.1); and, kiwi notes were noticeable in *cvs.* Madural (0.4), Lentisca (0.3), Rebolã (0.8) and Verdeal Transmontana (0.2). Herbaceous sensations were dominated by notes of fresh herbs, tomato leaves and cabbage (Figure 7.3). The values of fresh herbs and tomato leaves were similar in all the cultivars. For cabbage sensation, significant differences ($P \leq 0.05$) were observed between cultivars, being higher values obtained for Verdeal Transmontana and Redondal, whilst this attribute was absent for Lentisca olive oils. Some authors (Caporale, et al., 2004) reported that the sensation of fresh herbs can significantly increase the perception of bitterness. Nevertheless, it was not observed in the present work, being probably cultivar dependent. Harmony is defined as an overall sensation that's combines all the obtained perceptions by the taster: its classification is indicative of the equilibrium between the perceptions found, being usually attributed higher scores when different sensations are perceived but without high dominance of any. Two statistical different ($P \leq 0.05$) groups were constituted: *cv.* Madural olive oils obtained the highest score whereas Rebolã and Verdeal Transmontana obtained the lowest (Figure 7.4).

At the level of gustatory-retronasal attributes (Figure 7.5) *cvs.* Lentisca and Madural were significantly more complex than the oils from *cv.* Rebolã, whihc was the less complex. Verdeal Transmontana originated more persistent olive oils whilst *cvs.* Rebolã and Redondal showed the lower persistence values. Also, Dias et al. (2016) conclude that olive oil persistence was influenced by cultivar.

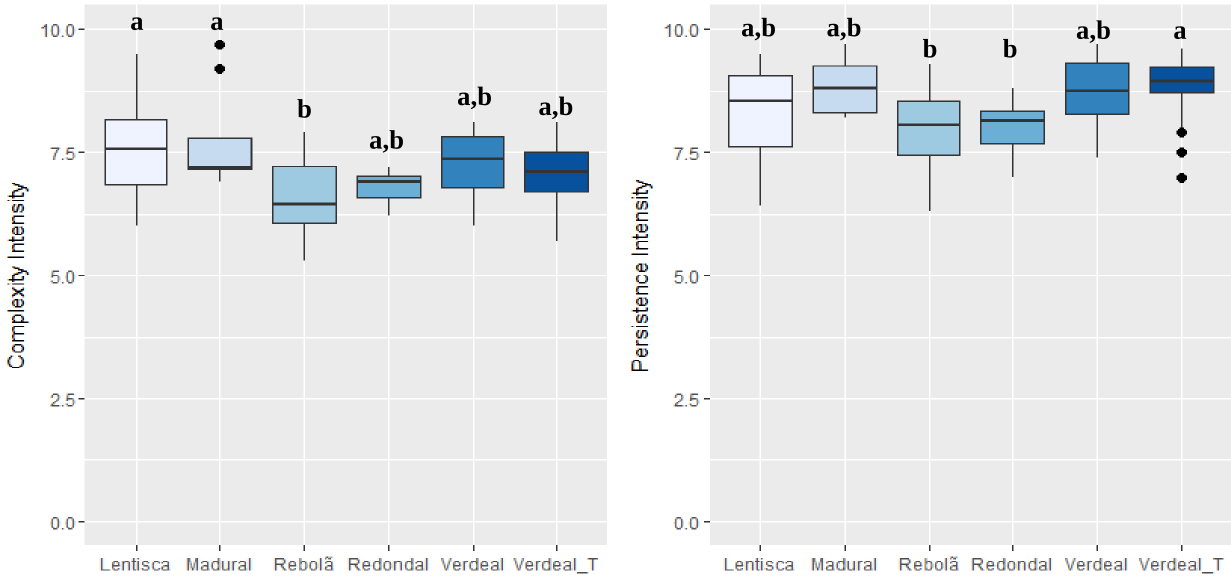

Figure 7.5. Boxplots of gustatory-retronasal attributes (Complexity and Persistence intensity) found in olive oils extracted from olives collected from centenarian trees from different cultivars (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) during two consecutive harvest years (2016 to 2017). Different lowercase letters mean significant statistical differences at a 5% significance level (one-way ANOVA followed by the Tukey's multi-comparison test).

7.3.3. Merging quality, physicochemical and sensory attributes towards the simultaneous discrimination of olive oil by harvest year and olive cultivar

The previous discussed effects of the harvest year and/or olive cultivar on the quality, physicochemical and perceived intensities of positive sensory attributes, pointed out the opportunity of simultaneously discriminating the olive oils produced from the centenarian trees according to the referred two factors by merging all the information collected, using a low-level fusion strategy. For that, a PCA was performed and, as can be inferred from Figure 7.6, the fused data allowed: (i) first, discriminating olive oils according to the harvest year, accordingly, olive oils produced on 2016 were located on the negative region of the 1st principal component, PC1, mainly due to the contribution of ΔK, $C_{18:1}/C_{18:2}$ and the sweet gustatory sensation, and those produced on 2017, located on the positive region of PC1, mainly due to the levels of free acidity, total phenols, olfactory and gustatory green and tomato sensations, and the bitter intensity attribute; and (ii) secondly, for each year, to differentiate olive oils based on the six olive cultivars evaluated, based on PC2 and PC3, due to the miscellaneous contribution of the assessed physicochemical and sensory parameters.

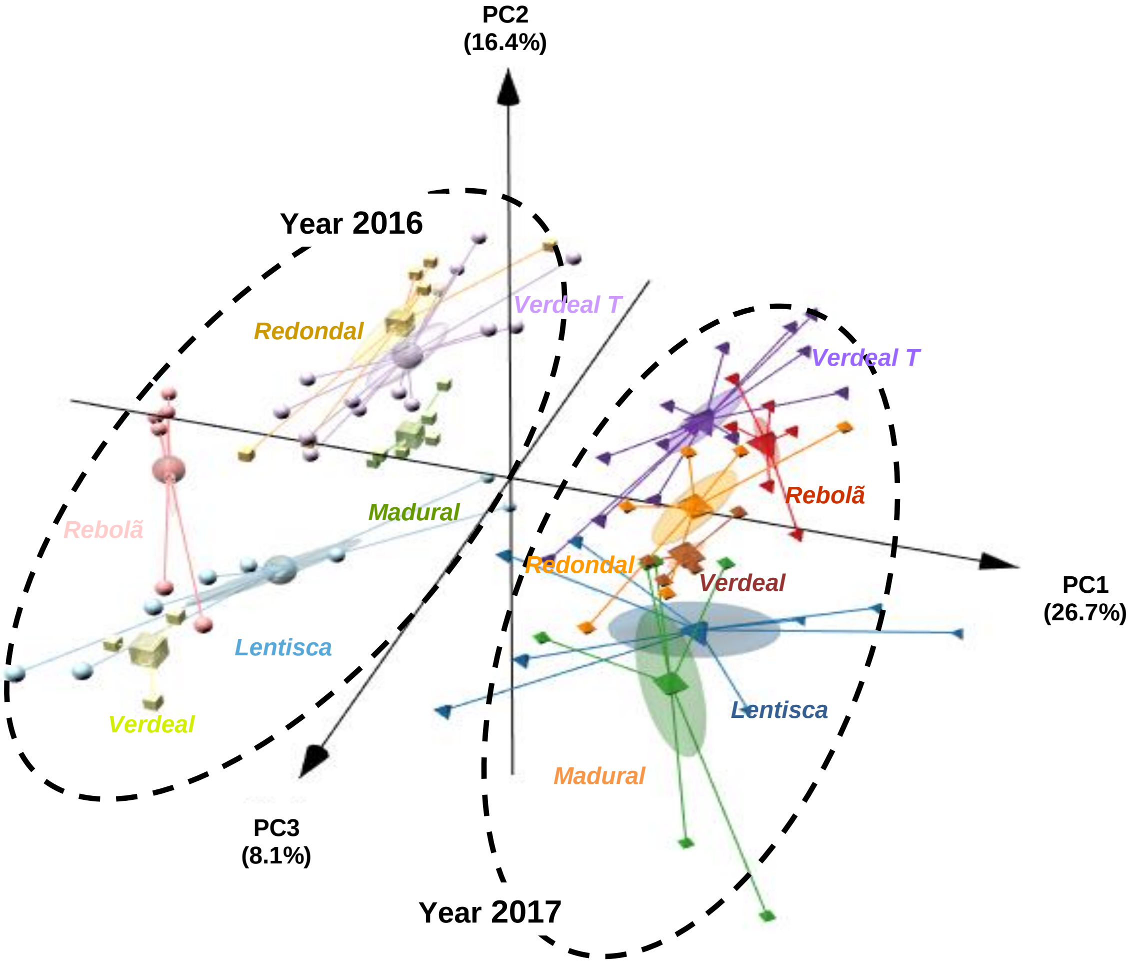

Figure 7.6. Principal component analysis (PC1: 26.7%, PC2: 16.4% and PC3: 8.1%): 3D plot showing the unsupervised pattern recognition according to olive cultivar (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) based on the quality and physicochemical parameters and sensory analysis profile contents found in olive oils obtained from olives collected from centenarian trees during two consecutive harvest years (2016 to 2017).

Since for each harvest year the space location of the olive oils belonging to the same olive cultivar substantially differ, with the exception of *cvs.* Verdeal Transmontana and Lentisca, possibly indicating that olive oils produced from the two latter cultivars were less prone to the known influence of climatic conditions. Globally, these findings are in agreement with the detailed study described in the previous sections concerning quality, physicochemical and sensory data, which pointed out the significant influences of both harvest year and olive cultivar on the overall composition and sensory profile of olive oils.

7.4. Conclusions

This work intended to characterize six minor olive cultivars produced from centenarian trees, aiming to contribute for their characterization, conservation and valorization as a way to preserve these cultivars as part of the genetic identity of the Trás-os-Montes region. Of the 20 specimens of centenary trees selected for this study, it was possible to identify specimens that gave olive oils of exceptional quality. From the point of view of the quality, all fulfilled the requirements to be classified as EVOO, showing the importance of the field and technological work. At the chemical level the cultivar effect was visible, with *cv.* Redondal having higher resistance to oxidation, higher total phenol content, total tocopherols and oleic/linoleic acid ratios. For the organoleptic point of view some of the obtained oils showed an exquisite profile, with rare notes like green cherry (Lentisca and Madural), apricot (Lentisca, Madural and Verdeal Transmontana) and kiwi (Lentisca, Madural, Rebolã and Verdeal Transmontana). These selected trees are potential candidates to obtain differentiated oils while promoting the future preservation of the region genetic heritage. Furthermore, linear correlations were found between sweet and bitter sensations and the mean levels of monounsaturated, polyunsaturated and/or saturated fatty acids, showing the influence of fatty acids on the olive oil organoleptic sensations. Finally, by merging the quality, physicochemical and sensory data allowed establishing a classification model that enabled assessing the olive oil according to the harvest year as well as to identify the olive cultivar of each oil evaluated.

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Table S1. Biometric and morphological characteristics of fruit and endocarp (mean ± standard deviation), of the different olive cultivars.

Olive Fruit	Weigth	Length (mm)	Width (mm)	Shape	Symm.A	T Diam.B	Apex.	Base	Nipple	Nº. of lent.	D. of lent.	Over color at full maturity
Lentisca	**2.30±0.71**	**19.61±1.88**	**13.91±1.86**	**1.42±0.13**	**Weakly asymmetric**	**Central**	**Rounded**	**Rounded**	**Absent**	**Little numerous**	**Small**	**Black**
	(0.87-4.91)	(13.90-25.04)	(9.76-21.43)	Ovoid	83.1%	71.2%	90%	67.5%	72.5%	80.5.0%	52.9%	100.0%
Madural	**2.47±0.63**	**17.93±5.30**	**13.80±1.75**	**1.28±0.30**	**Weakly asymmetric**	**Central**	**Rounded**	**Rounded**	**Absent**	**Very numerous**	**Small**	**Black**
	(1.35-4.85)	(8.76-26.30)	(10.22-20.13)	Ovoid	79.2%	69.2%	58.3%	51.7%	46.7%	50.0%	50.0%	100.0%
Rebolã	**4.38±1.52**	**23.83±2.92**	**17.24±2.63**	**1.39±0.10**	**Weakly asymmetric**	**Central**	**Rounded**	**Truncate**	**Little evident**	**Little numerous**	**Small**	**Black**
	(1.89-7.48)	(17.44-30.29)	(12.11-28.69)	Ovoid	60.8%	67.5%	82.5%	66.7%	53.3%	66.8%	98.8%	100.0%
Redondal	**5.85±1.30**	**25.08±2.89**	**19.52±1.51**	**1.29±0.12**	**Weakly asymmetric**	**Central**	**Rounded**	**Truncate**	**Little evident**	**Little numerous**	**Large**	**Black**
	(2.76-8.87)	(14.04-37.19)	(15.62-22.82)	Ovoid	80.0%	58.3%	65.8%	94.2%	45.8%	84.2%	62.5%	100.0%
Verdeal	**5.11±0.82**	**26.84±2.02**	**17.13±1.06**	**1.57±0.17**	**Symmetric**	**Central**	**Rounded**	**Truncate**	**Absent**	**Very numerous**	**Small**	Black
	(3.31-7.34)	(20.03-32.45)	(14.83-19.83)	Elongated	51.2%	91.2%	73.8%	53.8%	46.2%	53.8%	96.2%	100.0%
Verdeal Transmontana	**3.36±0.75**	**22.90±1.96**	**15.55±1.27**	**1.48±0.09**	**Weakly asymmetric**	**Central**	**Rounded**	**Rounded**	**Little evident**	**Very numerous**	**Large**	Black
	(1.82-6.12)	(14.05-28.98)	(12.70-23.27)	Elongated	75.4%	62.9%	93.9%	66.8%	52.5%	81.4%	79.6%	100.0%

Olive Stone	Weigth	Length (mm)	Width (mm)	Shape	Symm.A	Symm.B	T Diam.B	Apex. A	Base	Rugosity of surface	Nº. of grooves	Distribution of grooves
Lentisca	**1.09±0.90**	**15.32±1.65**	**7.91±0.87**	**1.94±0.15**	**Weakly asymmetric**	**Symmetric**	**Central**	**Acute**	**Rounded**	**Medium**	**High**	**Grouped around suture**
	(0.24-3.59)	(11.07-19.79)	(5.83-10.24)	Elliptic	65.0%	62.5%	86.9%	71.9%	48.1%	67.5%	63.8%	50.6%
Madural	**1.20±1.01**	**15.61±1.88**	**7.42±0.62**	**2.11±0.25**	**Weakly asymmetric**	**Symmetric**	**Central**	**Acute**	**Rounded**	**Medium**	**Medium**	**Uniform**
	(0.32-3.59)	(1.22-19.51)	(6.16-9.43)	Elliptic	52.5%	53.8%	70.0%	100%	51.0%	91.2%	53.8%	93.8%
Rebolã	**0.69±0.20**	**16.28±1.78**	**8.39±0.77**	**1.95±0.19**	**Weakly asymmetric**	**Symmetric**	**Central**	**Acute**	**Rounded**	**Medium**	**High**	**Uniform**
	(0.33-1.22)	(12.52-22.94)	(6.47-10.44)	Elliptic	48.3%	70.8%	73.3%	72.5%	48.3%	79.2%	71.7%	90.8%
Redondal	**0.93±0.22**	**16.83±1.92**	**9.27±1.15**	**1.83±0.20**	**Weakly asymmetric**	**Symmetric**	**Central**	**Acute**	**Rounded**	**Medium**	**High**	**Uniform**
	(0.30-1.42)	(10.27-21.21)	(6.66-17.80)	Elliptic	78.3%	94.2%	66.7%	70.8%	64.2%	65.8%	67.5%	84.2%
Verdeal	**0.82±0.11**	**23.73±4.42**	**8.21±0.40**	**2.90±0.55**	**Asymmetric**	**Weakly asymmetric**	**Central**	**Acute**	**Acute**	**Medium**	**High**	**Uniform**
	(0.61-1.18)	(15.51-32.45)	**(7.39-9.40)**	Elongated	58.8%	75.0%	85.0%	98.8%	56.2%	66.2%	85.0%	98.8%
Verdeal Transmontana	**0.74±0.48**	**17.15±1.53**	**8.08±0.60**	**2.13±0.16**	**Weakly asymmetric**	**Weakly asymmetric**	**Central**	**Acute**	**Acute**	**Medium**	**High**	**Uniform**
	(0.39-8.36)	(13.20-21.36)	(5.65-10.20)	Elliptic	61.8%	77.5%	65.7%	80.7%	59.6%	71.1%	64.6%	99.6%

Chapter 8

Are olive oil tocopherols content dependent on the crop season and cultivar? A detailed study with centenarian olive trees

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Submitted

Are olive oil tocopherols content dependent on the crop season and cultivar? A detailed study with centenarian olive trees

Abstract

Tocopherols are compounds with high biological activity, benefic for human health, that can be found in vegetable oils like olive oil, contributing for its resistance to oxidation. In this work, the tocopherols contents of olive oils extracted from centenarian olive trees of six cultivars (*cvs*. Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) were evaluated during five consecutive crop years (2013-2017). Three tocopherols isoforms (α-, β- and γ-tocopherols) were detected in all analyzed olive oils, and their content varied significantly with the cultivar and year of production. The highest amounts were found in *cv.* Lentisca (456±122 mg/kg olive oil), while the lowest were observed in *cv.* Verdeal (179±45 mg/kg olive oil). Crop year was the most influent factor, with the highest contents observed in 2013 and lowest in 2014. Principal component analysis and hierarchical clustering analysis allow differentiating the studied olive oils according to the olive cultivar or the production year, showing to be very robust pattern recognition tools for the study carried out. The overall results pointed out that tocopherol contents may be used as chemical markers to discriminate olive oils from centenarian trees either by olive cultivar or by crop year, being some cultivars identified as potential candidates for guaranteeing the production of olive oils rich in bioactive compounds.

Palavras-Chave: monovarietal olive oils, α-tocopherol, crop year, vitamin E, centenarian olive trees.

Chapter 8

8.1. Introduction

Virgin olive oil, obtained by mechanical and physical methods from fruits of the olive tree (*Olea europaea* L.), incorporates a huge chemical richness in terms of bioactive compounds (Boskou, 2015). Several studies showed that some of these micro-components play important roles in human health (Huang et al., 2008; López-Miranda et al., 2010; Nocella et al., 2018; Visioli et al., 2011). Indeed, olive oil consumption has been associated with lower incidence rates of coronary disease and cancer, improved digestive functions and brain aging delaying, among others (Abuznait et al., 2013; Huang et al., 2008; Psaltopoulou et al., 2011; Nocella et al., 2018; Visioli et al., 2011). Among these bioactive molecules, vitamin E, the generic name used for a group of lipid compounds, including at least four different tocopherols and tocotrienols (α-, β-, γ- and δ-)(Ahsan et al., 2000;Bramley et al., 2008) plays a key role in human health, acting as antioxidants in the lipid phase, capturing radicals in membranes and lipoprotein particles (Esterbauer et al., 1991; Pryor et al., 2000). In particular, α-tocopherol shows the highest biological activity, with numerous important clinical effects, regulating heme synthesis, inhibiting platelet aggregation and participating in the prevention of degenerative neuropathies (Bramley et al., 2000; Pryor et al., 2000).

Vitamin E is typically found in vegetables sources with high lipid contents, as seeds and nuts, where they act primarily as antioxidants, protecting the integrity of the lipids and increasing their oxidative stability (Bramley et al., 2000; Blekas et al., 1995). In olive oil, α-tocopherol is the predominant vitamin E compound, representing 90 to 95% in most cultivars (Kalogeropoulos, & Tsimidou, 2017) whereas β- and γ-tocopherols are only found in reduced amounts. However, the total amounts of vitamin E are variable, in a clear dependence of several factors, such as the cultivar, fruit maturation, edapho-climatic conditions, agronomic factors (e.g. irrigation, fertilization, pest and diseases incidence), conditions used during oil extraction, storage, etc. (Aguilera et al., 2005; Beltrán et al., 2010; Cayuela et al., 2017; Kalogeropoulos, & Tsimidou, 2017). From all these factors, the genetic cultivar seems to have a great influence in the contents and composition of tocopherols (García-González, & Aparicio, 2010; Tura et al., 2007). But, due to the great genetic richness this information remains unknown especially for the minor cultivars. Fruit maturation has also some importance, with α-tocopherol and total tocopherols decreasing with maturation (Beltrán et al., 2005). The edapho-climatic conditions are also recognized factors in olive oil compositional variability, in particular the year of production, which is known to significantly affect the content and composition of tocopherols due to stress conditions to which the plant is subjected throughout its vegetative cycle (Beltrán et al., 2010).

Recently, there has been an effort to find oils naturally rich in bioactive compounds. In this pursue, some traditional olive cultivars, even if with reduced geographical expression but with high biodiversity interest, as well as centenarian trees, can represent sources of these compounds that worth know and preserve. In the north of Portugal, and more specifically in the region of Trás-os-Montes, there is a great cultivar richness, largely unknown and uncharacterized, and a large quantity of centenarian olive trees, whose olive oils are potentially richer in bioactive compounds.

In this context, this work aimed to characterize the tocopherol profile and contents in olive oils extracted from olives produced by centenarian trees belonging to six minor olive cultivars along five production years (2013-21017), with the purpose to explore their diversity and potential for future breeding, taking into account the olive oil with higher bioactive richness. To the Authors' best knowledge, information about the influence of genetic diversity on the tocopherol contents is still scarce in the literature, or even inexistent concerning four of the minor cultivars studied herein (*cvs* Lentisca, Rebolã, Redondal and Verdeal).

8.2. Material and Methods

8.2.1. Sampling

In Trás-os-Montes region, near Mirandela (N 41º 29.425; W 7º 15.490), Northeast of Portugal, one olive grove with centenarian trees was selected. According to the property records the trees have more than 200 years old. Based on the tree appearance, structure and trunk thickness, twenty randomly trees were selected and marked. The trees belonging to different minor cultivars namely *cvs.* Lentisca (3 trees), Madural (3 trees), Rebolã (3 trees), Redondal (3 trees), Verdeal (2 trees) and Verdeal Transmontana (7 trees). During five consecutive crop years (from 2013 to 2017), approximately three kilograms of fruits were manually collected from each tree. To avoid the influence of the maturity stage on the olive oil composition the harvest occurred when the fruits were between the maturity stage two and three, that corresponds to the fruit epidermis with red spots in less than half the olive (MI 2) and the fruit epidermis red or purple in more than half the olive (MI 3). So, in every year the harvest occurred during the month of November, namely on the 25th and 26th days in 2013; on the 10th and 11th days in 2014; on the 2nd and 3rd days in 2015; on the 07th and 08th days in 2016; and, on the 13th and 14th

days in 2017. The fruits were extracted shortly after harvest, *i.e.*, less than 24 h after harvest, in a pilot extraction plant with an Abencor analyzer (Comercial Abengoa S.A., Seville, Spain), with three main units: a mill, a thermobeater where malaxation takes place at controlled temperature, and a centrifuge. Olives were milled, and the obtained paste was homogenized and about 700 g were transferred to the thermobeater unit (25 ºC, 20 min) for malaxation using a thermostatic water bath at 25 ºC. In the final 5 min of each malaxation, 100 mL of water at 27 ºC was added to aid the olive oil separation. The mixture was centrifuged, decanted, and the olive oil collected. After that, the oils were filtered (Whatman paper nº 4) using anhydrous sulfate in order to remove the solid particles and residual water. The olive oils were put in 125 mL dark bottles and stored in the dark at room temperature. All the assays were carried out between one and two months after extraction and were made in triplicate.

8.2.2. Vitamin E content and profile

Vitamin E contents were analyzed by HPLC with fluorescence detection, according to ISO 9936, with some modifications as detailed by Cruz and Casal, (2013), Tocopherols standards (α, β and γ) were purchase from Sigma (Spain), while the internal standard 2-methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol (tocol) was from Matreya Inc. (Pleasant Gap, PA). Individual standards purity was monitored by spectrophotometry (UV-1800, Shimadzu, Japan) based on their molar attenuation coefficients. N-hexane was HPLC grade from Sigma-Aldrich (Germany), 1,4-dioxane was from Sigma-Aldrich (p.a., USA).
Filtered olive oil (50 mg) was mixed with internal standard solution (tocol), diluted in n-hexane and homogenized. The mixture was centrifuged for 5 min at 13,000 rpm at room temperature and the supernatant obtained analysed by HPLC, using a normal phase silica column (SupelcosilTM LC-SI; 7.5 cm×3 mm; 3 mm) (Supelco, USA), conditioned at 25°C and eluted with a mobile phase of 1, 4-dioxane in n-hexane (2.5%, v/v), at a flow rate of 0.75 mL/min. Analyses were carried out using an integrated system with a data transmitter (Jasco LC – NetII/ADC, Japan), pumps (Jasco PU – 4180, Japan), an auto-sampler (Jasco AS – 4050, Japan), oven (ECOM Eco2000, Czech Republic), a DAD (Jasco MD – 4010, Japan), and fluorescence detector (FLD, Jasco FP – 4025, Japan) programmed for excitation at 290 nm and emission at 330 nm. Data were analyzed with the ChromNAV Control Center - JASCO Chromatography Data Station (Japan). The different compounds of vitamin E were identified by comparing the retention times with authentic standards, confirmed by their UV spectra and spectral purity and quantified by individual calibration curves, being expressed in mg/kg of olive oil.

8.2.3. Statistical analysis

One-way analysis of variance (one-way ANOVA) was applied to evaluate the existence of statistical significant effects of the tree cultivar or the crop year in the tocopherols contents of olive oils extracted using the same production techniques, from olives collected at similar maturation stages from centenarian olive trees grown in the same geographical area and under the same agricultural practices. Moreover, if a significant statistical effect was found ($P < 0.050$), the post-hoc multi-comparison Tukey's test was also applied aiming to identify the levels (i.e., olive tree cultivar or crop year) of each effect that were responsible for the detected significant effect. Finally, boxplots were used to show the one-way ANOVA statistical results.

The possible influences of olive tree cultivar (*i.e.*, *cvs*. Lentisca, Madural, Rebolã, Redondal, Verdeal or Verdeal Transmontana) or crop year (from 2013 to 2017) on the olive oil tocopherol profile of olive oil extracted from olives of centenarian trees was also evaluated using principal component analysis (PCA), an unsupervised multivariate pattern recognition technique. For PCA, the tocopherols' contents were centered and scaled minimizing data variability.

Finally, hierarchical clustering heatmap was used to verify if the tocopherols profiles could be used as chemical markers to differentiate the olive oil obtained from olives picked from different cultivars of centenarian olive trees. This false color image with dendograms is obtained by computing the distance (dissimilarity) between both rows (olive tree cultivar) and columns (tocopherols contents), being selected the Euclidean distance for matrix computation. This procedure uses a set of dissimilarities for the n objects being clustered. Initially, each object is assigned to its own cluster and then the algorithm proceeds iteratively, at each stage joining the two most similar clusters, continuing until there is just a single cluster. At each stage distances between clusters are recomputed by the Lance–Williams dissimilarity update formula according to the particular clustering method being used, being in this work chosen the complete/Ward's linkage method, used to find similar clusters, ensuring to obtain a monotone distance measure, avoiding inversions or reversals on the resulting dendrograms.

The statistical analysis was performed using the Subselect (Cadima et al., 2004; Cadima et al., 2012; Kuhn, & Johnson, 2013) and MASS (Venables, & Ripley, 2002) packages of the open source statistical program R (version 2.15.1), at a 5% significance level.

8.3. Results

A total of 100 olive oils, from six cultivars, were extracted and analyzed during five consecutive crop years. In all the olive oil samples evaluated, three isoforms of tocopherols, namely α-, β- and γ-tocopherol, were identified and quantified. Their relative proportion was almost constant between years and cultivars, with α-tocopherol ranging from 94.5% to 98.2%, β-tocopherol from 0.4% to 1.9%, and γ-tocopherol from 0.9% to 4.1%, with average amounts of 96.8%, 1.1% and 2.2%, respectively.

When quantified in absolute amounts (in mg/kg of olive oil), a higher variability was perceived. Figure 8.1 shows the average concentrations of each isoform and the total tocopherols contents for the six olive cultivars under study, considering simultaneously the five crop years. For all olive cultivars evaluated, α-tocopherol was the major isoform, varying between 118.4 mg/kg of olive oil (*cv.* Verdeal) and 607.1 mg/kg of olive oil (*cv.* Lentisca). Thus in descending order of α-tocopherol contents, and independently of the crop year, appears the *cvs.* Lentisca ≈ Redondal > Madural ≈ Rebolã > Verdeal Transmontana ≈ Verdeal (Figure 8.1). Indeed, the results pointed out that α-tocopherol contents were significantly influenced by olive cultivar ($P \leq 0.001$ for one-way ANOVA and/or Tukey's multi-comparison test; Figure 8.1).

The γ-tocopherol was the second isoform in terms of concentration, varying its contents between 1.4 (*cv.* Verdeal Transmontana) and 17.3 mg/kg of olive oil (*cv.* Lentisca). Similarly, the γ-tocopherol contents were significantly influenced by the olive cultivar ($P \leq 0.001$ for one-way ANOVA and/or Tukey's multi-comparison test; Figure 8.1) in the following descending order: Lentisca > Redondal > Rebolã ≈ Madural ≈ Verdeal > Verdeal Transmontana (Figure 8.1). Finally, β -tocopherol was the isoform with the lowest amounts, varying from 1.0 (*cv.* Verdeal) and 7.5 mg/kg olive oil (*cv.* Lentisca) ($P \leq 0.001$ for one-way ANOVA and/or Tukey's multi-comparison test; Figure 8.1). Considering the total content of Vitamin E as the sum of the three above-mentioned isoforms of tocopherols, the contents varied from 124.4 (*cv.* Verdeal) to 629.6 mg/kg of olive oil (*cv.* Lentisca), being these contents also significantly dependent of the olive cultivar ($P \leq 0.001$ for one-way ANOVA and/or Tukey's multi-comparison test; Figure 8.1). The decreasing content order was the same as for α-tocopherol.

From the overall tocopherol analysis in these six cultivars, it can be inferred that the centenarian *cv.* Lentisca trees, followed and *cv.* Redondal are the most promising for obtaining olive oils rich in tocopherols.

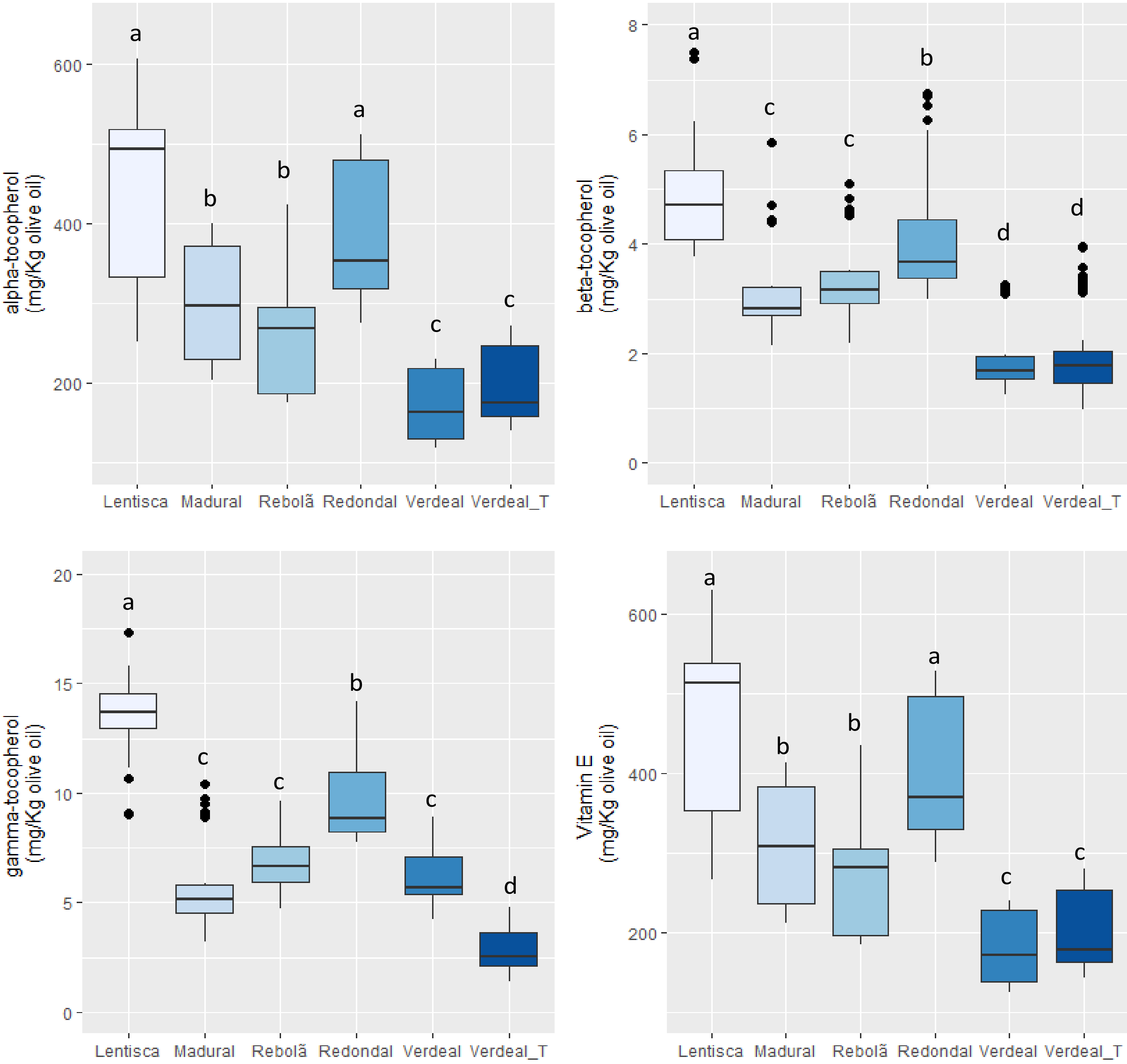

Figure 8.1. Boxplots of the contents (mg/kg of olive oil) of α-, β- and γ-tocopherols as well as of Vitamin E (total tocopherol content) found in olive oils extracted from olives collected from centenarian trees from different cultivars (*cvs*. Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) during five consecutive crop years (2013 to 2017). Different lowercase letters mean significant statistical differences at a 5% significance level (one-way ANOVA followed by the Tukey's multi-comparison test).

Also, the tocopherols profiles were further used to evaluate their capability for classifying the olive oils obtained from centenarian olive trees according to the olive cultivar. Indeed, from PCA (Figure 8.2) it could be observed that the tocopherols contents enabled to split the olive oils according to the olive cultivar, using three principal components (PC), which explained 100% of the data variability (82.3%, 11.7% and 5.9% for PC1, PC2 and PC3, respectively).

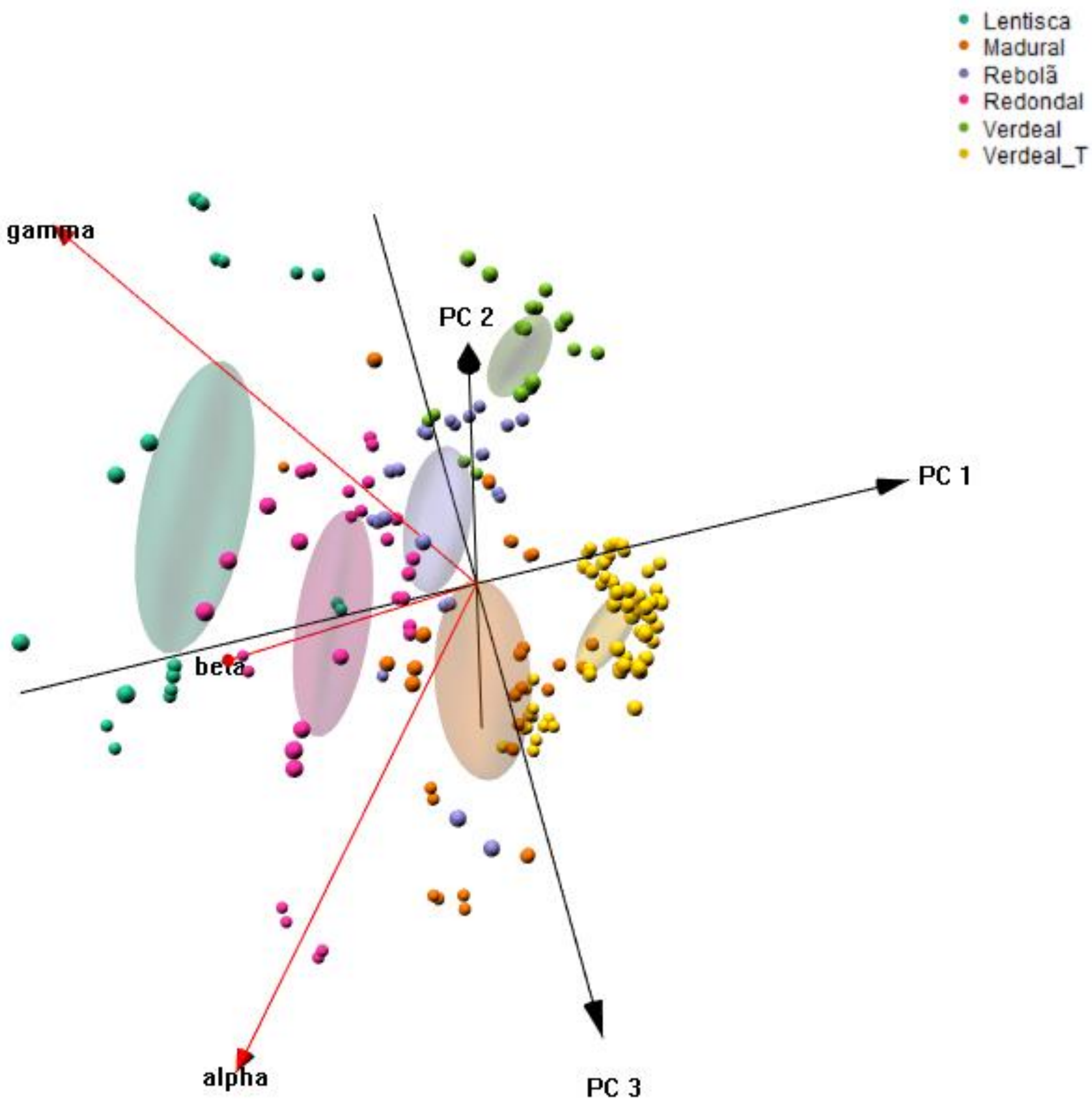

Figure 8.2. Principal component analysis (PC1: 82.3%, PC2: 11.7% and PC3: 5.9%): 3D plot showing the unsupervised pattern recognition according to olive cultivar (Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) based on the α-, β-and γ-tocopherols contents found in olive oils obtained from olives collected from centenarian trees during five consecutive crop years (2013 to 2017).

For *cvs.* Lentisca and Redondal, β-tocopherol was the tocopherol that most contributed to the olive cultivars clustering. For c*v.* Madural, α-tocopherol was the most influent vitamin, whereas in *cv.* Rebolã, γ-tocopherol had the greatest influence. Overall, for the olive oils studied, the tocopherol content was highly dependent on the cultivar. The hierarchical clustering heatmap shown in Figure 8.3 strengthen the previous findings. Taking into account the dendograms information it is clear that the tocopherols contents may be split into two groups, one concerning the α-tocopherol and the other comprising β- and γ –tocopherols. This information allowed grouping the olive oils into four main groups according to the olive cultivar. The 1st cluster mainly comprised olive oils from *cvs.* Lentisca, Madural and Redondal (highest contents of α-tocopherol and medium to high contents of the other two isoforms represented by yellow and light orange colors).

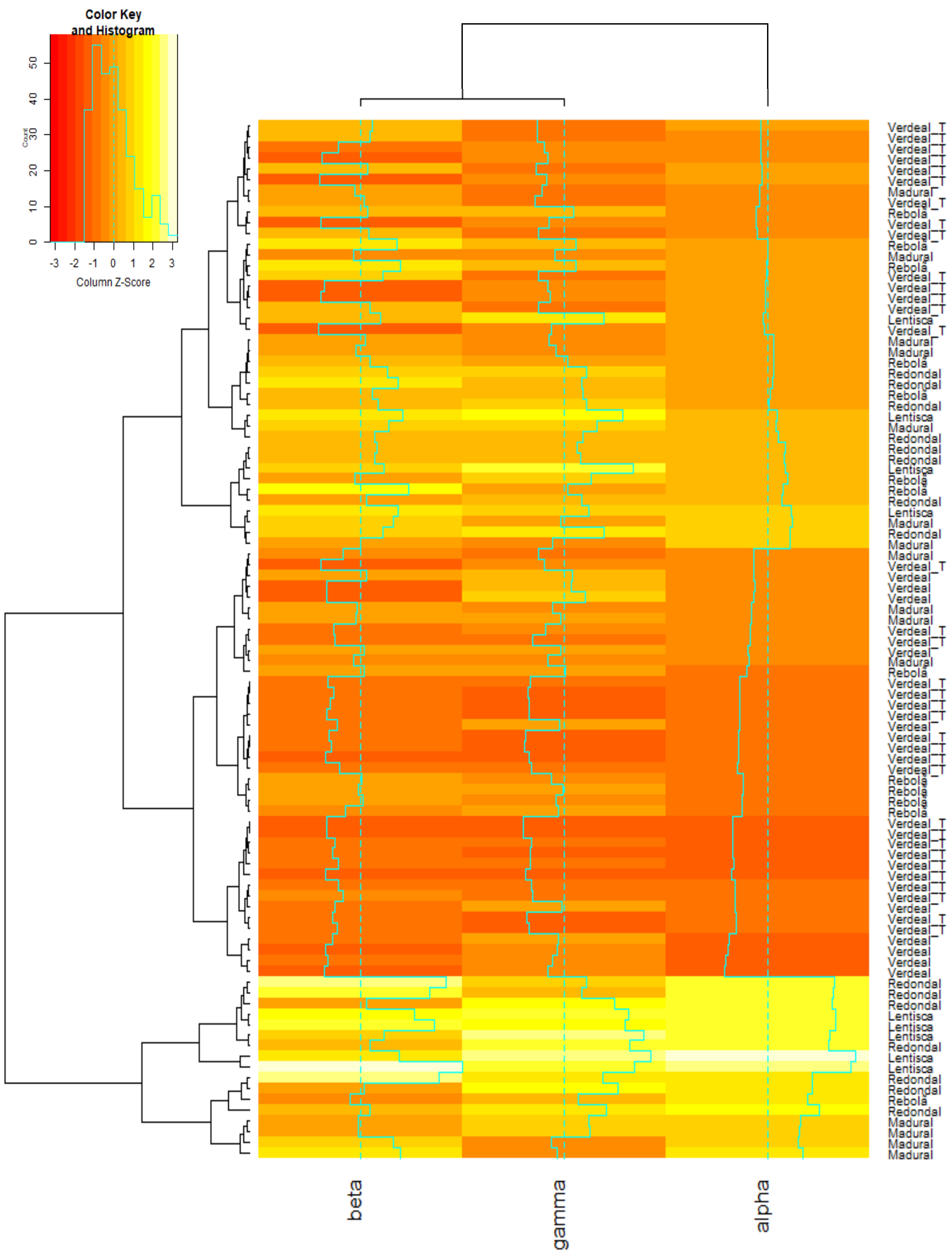

Figure 8.3. Hierarchical clustering heatmap (using Euclidean distances and Ward method) and respective dendograms for variables (α-, β-and γ-tocopherols contents) and olive oils obtained from centenarian olive trees of different cultivars (Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana), during five consecutive crop seasons (2013 to 2017).

The 2nd cluster included *cvs.* Redondal, Madural and Rebolã (medium to high contents of the three tocopherol isoforms, corresponding mainly to light orange and orange colors). The 3rd and 4th clusters were mainly constituted by *cvs.* Verdeal and Verdeal Transmontana with some olive oils of *cvs.* Redondal and Madural (medium to low tocopherols contents, represented by orange and red colors). This analysis, although confirming that the olive cultivar greatly influenced the tocopherols contents of olive oils from centenarian trees, also pointed out that the crop year has also an important role, since the 4 identified clusters contained more than one cultivar. Thus, considering that the climatic conditions vary from year to year, and that this work comprised five consecutive production campaigns, the effect of the crop year on the content of the different isoforms and the total of vitamin E was also evaluated, considering all the olive oils, regardless the olive cultivar. Figure 8.4 shows the effect of the crop year on the content of the different tocopherol isoforms and of vitamin E. For α-tocopherol, the highest levels were recorded in 2013 and 2015, being significantly higher ($P \leq 0.0056$, Tukey's multi-comparison test) than those determined for the other years. Thus, in decreasing order of abundance, 2013 ≈ 2015 > 2017 ≈ 2014 ≥ 2016 (Figure 8.4). Similar to α-tocopherol, the γ-tocopherol levels were significantly higher in 2013 (8.7mg/kg of olive oil) compared to the other crop years ($P \leq 0.0001$, Tukey's multi-comparison test), while in 2014 it was observed the lowest average amounts (4.1 mg/kg of olive oil). Also for β-tocopherol, statistically significant differences were observed between years ($P \leq 0.0001$, Tukey's multi-comparison test), with a higher significant content observed in 2015 (4.55 mg/kg of olive oil) (Figure 8.4). For the total vitamin E, in decreasing order of abundance, appeared 2013 ≈ 2015 > 2017 > 2016 ≈ 2014 (Figure 8.4).

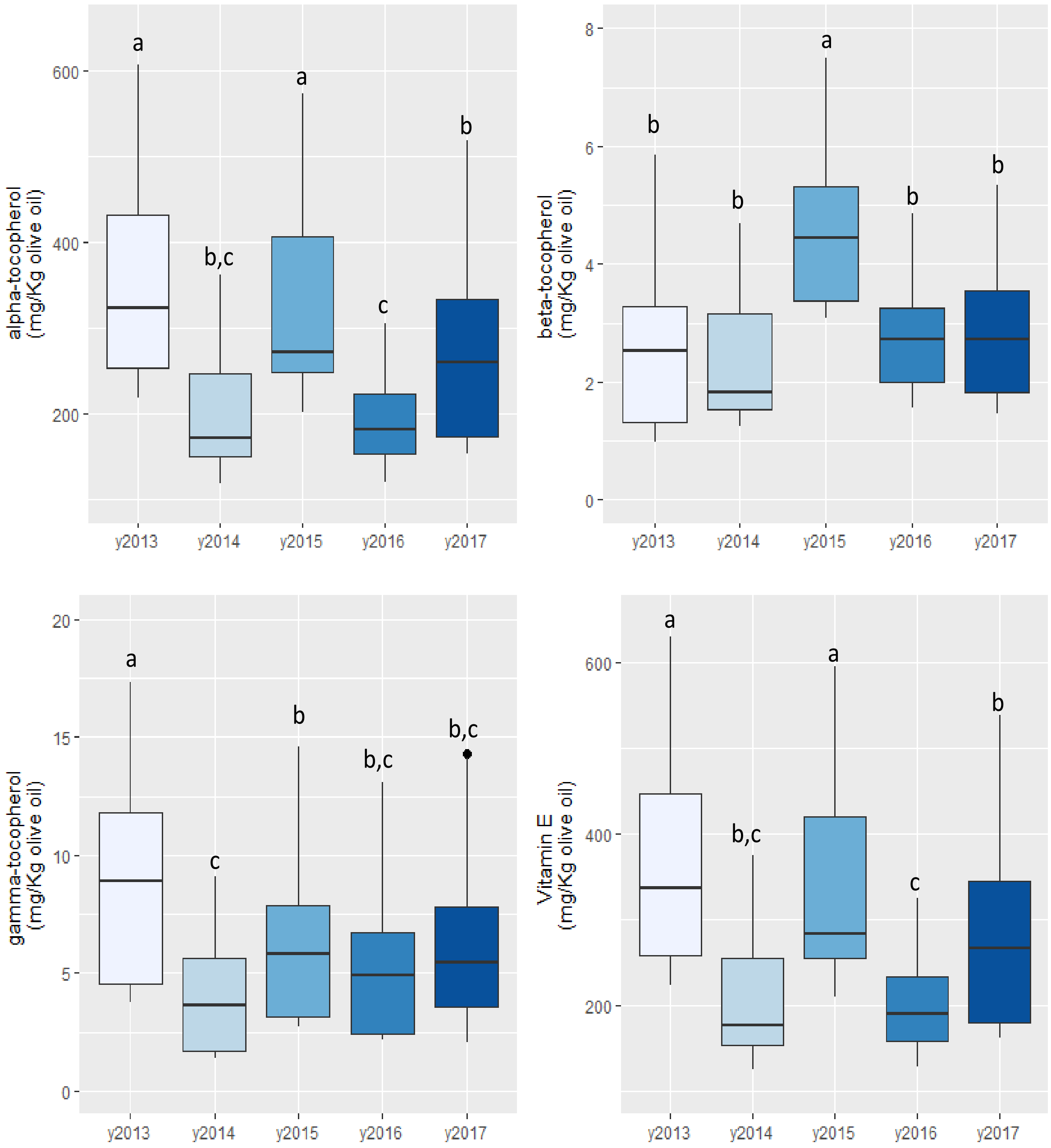

Figure 8.4. Boxplots of the contents (mg/kg of olive oil) of α-, β- and γ-tocopherols as well as of Vitamin E (total tocopherol content) found in olive oils extracted from olives collected from centenarian trees from different cultivars (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) during five consecutive crop years (2013 to 2017). Different lowercase letters mean significant statistical differences at a 5% significance level (one-way ANOVA followed by the Tukey's multi-comparison test).

The analysis allowed verifying that the tocopherols profiles of olive oils from centenarian trees changed according to the crop year, independently of the olive cultivar. This conclusion was further confirmed by the PCA (Figure 8.5).

As can be observed, olive oils may be partially grouped according to the crop year (although some overlapping of samples from different years may be observed), being the year 2013, 2015 and 2016 the most differentiated.

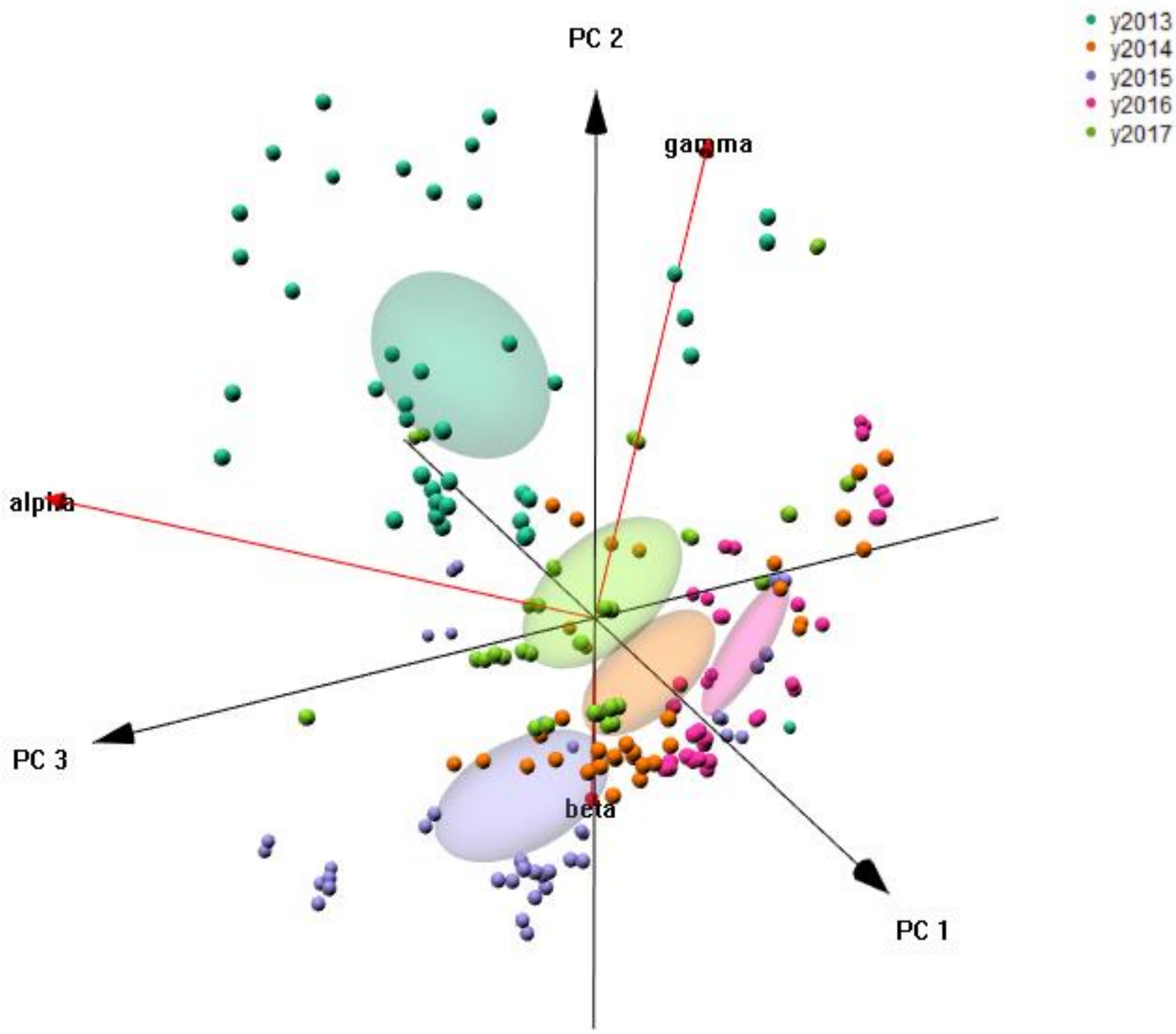

Figure 8.5. Principal component analysis (PC1: 82.3%, PC2: 11.7% and PC3: 5.9%): 3D plot showing the unsupervised pattern recognition according to crop year (2013 to 2017) based on the α-, β-and γ-tocopherols contents found in olive oils obtained from olives collected from centenarian trees of different cultivars (Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana).

8.3.1. Discussion

In the present work we intended to study the content of the tocopherol isoforms in olive oils extracted from fruits of different cultivars, collected from centenarian trees growing under the same orchard at the same agro-climatic conditions, during five consecutive crop years. It was our intention to explore the rich diversity of the olive tree germplasm for potential high bioactive contents tree selection.

In general, the total contents of tocopherols found (118.4 to 607.1 mg/kg), as well as those of the different isoforms, are in agreement with the literature values although, in some cases, were higher (eg Beltrán et al., 2010; Borges et al., 2017; Noorali et al., 2017; Tura et al., 2007). In this work, α-tocopherol represented more than 94% of the total tocopherol content in all the evaluated olive oils, which was also in accordance with the literature (Beltrán et al., 2010).

As mentioned in the introduction section, different factors can affect tocopherols content, such as cultivar, agronomic factors, environmental conditions, and maturation process. The olive oils studied were obtained from centenarian trees of six olive cultivars, namely *cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana, significantly differing the tocopherols amounts according to the olive cultivar (Figure 8.1). The dissimilarity of the tocopherols amounts also allowed identifying patterns within the olives oils that enabled the oils differentiation and clustering by cultivar (Figures 8.2 and 8.3). These results further support the evidence that genetic factors (*e.g.*, cultivar) have a great influence on the tocopherols concentrations found in olive oils. A study conducted with varietal olive oils from 18 Italian cultivars (Tura et al., 2007), showed that the total tocopherols contents, varying from 39.4 to 425.9 mg/kg, were mainly dependent of the olive cultivar, having the environmental condition lower or no significance. In the same way, the results from a study comprising 30 Spanish olive cultivars, with total tocopherols contents ranging from 84 to 463 mg/kg (Beltrán et al., 2010), also showed that the amounts of tocopherols in virgin olive oil was significantly genetic dependent (cultivar). Other works carried out in different countries such as Brazil (Borges et al., 2017), China (Xiang et al., 2017), Croatia (Špika et al., 2006), Greece (Boskou et al., 2006), Italy (Aguilera et al., 2005; Condelli et al., 2015), Spain (Beltrán et al., 2010; Franco et al ., 2014), Tunisia (Laroussi-Mezghani et al., 2016), and Turkey (Arslan et al., 2012; Uluata et al., 2016), reported similar tocopherols ranges.

Concerning Portuguese olive oils, a huge amount of information is available for one of the most spread olive cultivar, *cv.* Cobrançosa, with reported amounts of 99-313 mg/kg of olive oils, being these values dependent from the storage conditions of the olive fruits before extraction (Pereira et al., 2002), olive fly attack (Pereira et al., 2004), decreasing along the fruit maturation (Matos et al., 2007), and increasing with the addition of olive leaves during extraction (Malheiro et al., 2013). Also, Peres et al. (2016) reported that olive oils extracted from olives of *cv.* Cobrançosa and other typical cultivar (*cv.* Galega Vulgar), picked in early stages of maturation, had different tocopherols contents, but in lower amounts compared to those determined in this work for *cvs.* Lentisca, Madural and Redondal. Garcia et al. (2012) studied four typical cultivars (*cvs.* Cordovil, Carrasquinha, Verdeal and Negrinha de Freixo), with amounts within the expected range, from 201 mg/kg (*cv.* Negrinha do Freixo) to 391 mg/kg (*cv.* Cordovil). Additionally, these authors showed that there was a reduction trend with fruit maturation. Aware of these variations, we have eliminated this variability factor by using olives under the same maturation stage, in order to guarantee that the variations observed were strictly due to cultivar differences.

Although the effect of tree age on the oils composition was not an objective on this study, information available on this subject is scarce. There are many difficulties to develop and design a rigorous detailed study for this purpose, once it is not easy to assure the same genetic source of the plant material between different geographical origins. For a correct comparison, all trees should grow under the same edapho-climatic conditions and subjected to the same agronomic practices, being difficult to find groves under these conditions. Still, Chtourou et al. (2017) attempted to study the effect of tree age on the chemical composition of olive oils produced from a minor Tunisian cultivar, *cv.* Oueslati, under different maturation stages. The authors denoted some differences between adult and young trees, with a slight increase in total tocopherols in the older ones, but the effect of fruit maturation was more pronounced than tree age. To avoid this confounder, this study only used olive oils extracted from olives at the same maturation stage, being all trees fully adult ones.

There is a general consensus that the composition of olive oils varies from year to year, as a function of the climate conditions. However, most observations are only supported by studies conducted during two or three years. In the present study, with five consecutive crops evaluated, a clear inter-annual variation was observed. Beltrán et al. (2010) attributed these variations to rainfall levels, with higher tocopherol amounts in oils from drier crop years, although this trend was not observed in the present study.

The vitamin E content is part of the health claims allowed for olive oils which, under certain conditions (levels established in the European Regulation EU 1169/2011, may be labeled as "*Source of Vitamin E*". According to this regulation, and considering the recommendation of a daily intake of 12 mg, the ingestion of 28.5 mL of olive oil from centenarian trees of *cv.* Lentisca evaluated in the present work, would be sufficient to meet vitamin E daily intake requirements. For the other cultivars higher amounts would be required, namely 32.3 mL for *cv.* Redondal, 41.9 mL for *cv.* Madural, 48.4 mL for *cv.* Rebolã, 63.4 mL for *cv.* Verdeal Transmontana and 72.8 mL for *cv.* Verdeal. These values are of the same order of those reported in the literature, namely by Bayram et al. (2012), where a daily intake of 50 mL of olive oil was needed to meet the daily requirements of Vitamin E. Although other vegetable oils can be regarded as richer sources of vitamin E, particularly sunflower oil, it should be retained that the tocopherols are only part of the rich antioxidant pool of virgin olive oils. The simultaneous ingestion of other olive oil hydrophilic phenolic compounds extracted from the olive fruit, such as the hydroxityrosol derivatives, is also related with a recent health claim, as is the ingestion of monounsaturated fats (Reboredo-Rodriguez et al., 2017). Still regarding tocopherols, it should also be strengthen the olive oil richness in α-tocopherol, the most bioactive vitamin E isoform. Other isoforms show less clear positive associations for some health effects, namely γ-tocopherol (Cook-Mills et al., 2010), the main isoform in several vegetables oils, as soybean oil. In this sense, the search for the highest contents in these bioactive compounds has shown that some cultivars are stronger candidates for obtaining olive oils with higher health promoting activities and higher oxidative stability.

8.4. Conclusions

The work carried out showed that olive oils obtained from olives collected from centenarian trees of different cultivars (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) may be a good source of tocopherols, and a daily intake of 28.5 to 72.5 mL (depending on the cultivar) would guarantee the daily needs for vitamin E. Indeed, from a health point of view, olive oils from *cv.* Lentisca were the most promising ones. Overall, three tocopherol isoforms (α-, β- and γ -tocopherol) were found in all the olive oils, being α -tocopherol the main isoform. Both olive cultivar and crop year had a significant influence on the tocopherols profiles. Hopefully, the results reported in this work will attract the attention and increase the interest of olive producers to these minor and almost forgotten cultivars that are still grown in the region of Trás-os-Montes (Portugal). In fact, this study contributed to a better understand of the genetic heritage that exists in this Portuguese region, showing the possibility of selecting and propagating some of these minor olive cultivars with the aim of obtaining differentiated olive oils rich in antioxidant compounds.

Aknowledgements

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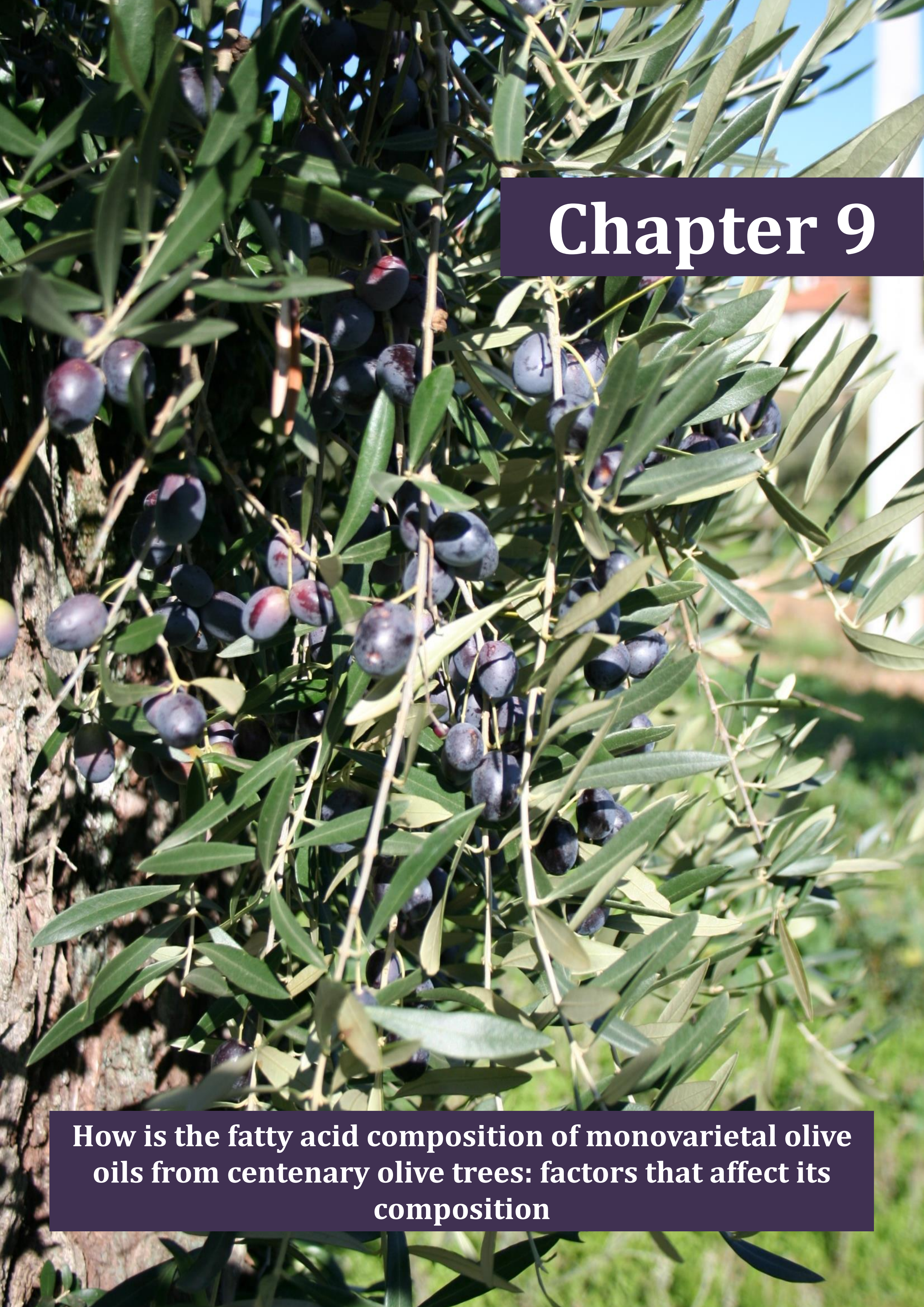
Chapter 9



How is the fatty acid composition of monovarietal olive oils from centenary olive trees: factors that affect its composition

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Submitted

Chapter 9

Fatty acids composition from olive oils of centenarian trees are highly dependent of olive cultivar and crop year

Abstract

The increase of olive oil consumption in the last decades has been driven by its nutritional, health and sensorial properties, being the former mainly due to its high levels of monounsaturated fatty acids. In this work, during five crop years (2013-2017), the fatty acids profiles of olive oils from six autochthonous cultivars (Lentisca, Madural, Redondal, Rebolã, Verdeal and Verdeal Transmontana) produced from centenarian trees grown in the same region were determined. Olive cultivar highly influenced the fatty acids relative contents, namely of oleic acid (70.3% for *cv.* Madural to 80.7% for *cv*. Redondal) and palmitic acid (10.4% for *cv*. Lentisca to 13.5% for *cv*. Verdeal). Similarly, crop year significantly influenced the individual fatty acid contents, despite being extracted from highly adapted tree specimens. Principal component analysis of fatty acids data enabled the unsupervised classification of olive oils by cultivar and, within each cultivar, by crop year. Furthermore, the levels of 9 fatty acids ($C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{17:1}$, $C_{18:0}$, $C_{18:1}$, $C_{20:0}$, $C_{20:1}$ and $C_{22:1}$) together with the polyunsaturated fatty acid contents, selected using the simulated annealing algorithm, allowed their correct classification, based on linear discriminant analysis, according to the olive cultivar, with an overall sensitivity of 92%, for leave-one-out cross-validation procedure. Globally, the cultivar effect superimposed that of crop year, showing that some cultivars, as Redondal and Verdeal Transmontana, have consistently high and homogeneous amounts of monounsaturated fatty acids, worth to be explored for future selection of cultivars, able to produce olive oils with increased nutritional value and less prone to oxidation.

Keywords: Olive heritage, chemical characterization, chemometrics, crop year influence.

Chapter 9

9.1. Introduction

Extra Virgin Olive Oil (EVOO) is a vegetable oil extracted from fresh and healthy olives (*Olea europaea* L.), by mechanical processes, respecting all good manufacturing practices of hygiene, safety and temperature. EVOO is a natural product that can be consumed directly in its raw state and enjoys worldwide recognition, thanks to its nutritional value and beneficial effects on health (Paolini et al., 2017). The high demand of this product results from the high content of monounsaturated fatty acids and the presence of minor components such as phytosterols, squalene, vitamins and antioxidants like polar phenols and tocopherols (Boskou, Blekas and Tsimidou, 2006). Within the monounsaturated fatty acids, oleic acid is the most abundant (55-83%), while palmitoleic and eicosenoic acid are present in reduced amounts. The remaining fatty acids include linoleic acid (3.5 to 21%), palmitic acid (7.5 to 20%) and linolenic acid (≤1%) (IOOC, 2013). Replacing saturated fats in the diet with unsaturated fats helps to maintain normal blood cholesterol levels (Regulation (EC) No 1924/2006). Oleic acid, in particular, seems to protect the human body against several types of diseases, having neurotrophic properties and slowing the progression of atheromatous lesion on the walls of the arteries by reducing LDL oxidation (Dewapriya et al., 2013; Medina & Tabernero, 2010).

However, the relative proportion of fatty acids in olive oil is not constant, but rather dependent on several factors associated with cultivar-genotype, and edaphoclimatic variables. Therefore, the nutritional and health benefits attributed to olive oil, as well as its sensorial properties, may vary greatly, depending on its fatty acids composition (García-Inza et al., 2018), and the amounts of other minor compounds, particularly antioxidants. The selection of cultivars that have consistently high oleic acids amounts, as well as a more clear understanding of the pedoclimatic factors that might influence oleic acid production in the olives, might help to provide interesting products from a nutritional and technological point of view. The effect of temperature on olive oil quality has been the subject of several studies, being known that the concentration of oleic acid is correlated with the average temperature (Orlandi et al., 2012; Rondanini et al., 2014), regulating fatty acid desaturases (Hernández et al., 2011). This fact was confirmed for *cv.* Arbequina, where the concentration of oleic acid in the olive oil had a linear negative correlation with the increase of the seasonal temperature in the range of 23-27 °C (Rondanini et al., 2011), as well as for *cv.* Arauco where the percentage of oleic acid in the whole fruit (endocarp and mesocarp) decreased by 0.7% $°C^{-1}$ with the increase of the average temperature in the 16-32 °C range during fruit growth (García-Inza et al., 2014). Other environmental parameters, as rainfall amounts and patterns, together with soil characteristics, agricultural practices and fruit ripening have already been studied and are known to influence olive oil composition (Bakhouche et al., 2013; Borges et al., 2017; Dabbou et al., 2011; Orlandi et al., 2012; Portarena et al., 2015). However, most studies are only performed during a reduced number of years, reducing the accuracy of the conclusions regarding both cultivar and environmental effects on olive oil composition.

There are some traditional olive cultivars, with reduced geographical expression but with a high biodiversity interest, that may represent high sources of oleic acid that are worth to know and to preserve. In the north of Portugal, and more specifically in the region of Trás-os-Montes, there is a great wealth of cultivars, largely unknown and uncharacterized, and a large number of centenary olive trees, whose oils are potentially richer in bioactive compounds, possessing a well-balanced composition from nutritional and quality point of views. In this context, during five consecutive crop years (2013-2017), the fatty acid composition of olive oils extracted from fruits produced by centenary trees belonging to six minor olive cultivars, some of them studied for the first time, were characterized. The effects of olive cultivar (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) and crop years (2013-2017) were studied using different chemometric tools aiming to identify the most promising ones in terms of oleic amounts and seasonal stability.

9.2. Material and Methods

9.2.1. Sampling

9.2.1.1. Samples

For this work, an olive grove with centenarian trees, located in the northeast of Portugal, near Mirandela (Suçães, N 41º 29' 26.628''; W 7º 15' 31.219''), was selected. Around 20% of the trees were chosen, respecting the proportion of each olive cultivar in the grove. A total of 20 trees of different minor cultivars were identified and marked, that included *cvs.* Lentisca (3 trees), Madural (3 trees), Rebolã (3 trees), Redondal (3 trees), Verdeal (2 trees) and Verdeal Transmontana (7 trees). From 2013 to 2017, in each crop year, and from each individual tree, approximately three kilograms of healthy olives were collected. In all crop years, harvest occurred at a similar maturity index (MI), between the MI 2 (fruit epidermis with red spots in less than half of the olive) and the MI 3 (fruit epidermis red or purple in more than half of the olive) (Hermoso, Uceda, García, Morales, Frias, & Fernandez, 1991). More precisely, harvest occurred on the 25th and 26th November in

2013; on the 10th and 11th November in 2014; on the 2nd and 3rd November in 2015; on the 07th and 08th November in 2016; and, on the 13th and 14th November in 2017.

In the first 24 h after harvest, fruits were extracted in a pilot extraction plant with an Abencor analyzer (Comercial Abengoa S.A., Seville, Spain).Olives were milled, the obtained paste was homogenized and about 700g were transferred to the thermobeater unit (25 ºC, 20 min) for malaxation at 25 ºC. In the final 5 min of each malaxation, water was added (100 mL of at 27 ºC) to aid in the olive oil separation process. The mixture was centrifuged, decanted, and the olive oil collected, filtered (Whatman paper nº 4 and anhydrous sulfate) and stored in the dark at room temperature in 125 mL dark bottles. All the assays below were carried out in triplicate within two months after extraction.

9.2.2. Fatty acids composition

Fatty acids were evaluated by gas chromatography (Chrompack CP 9001 with FID detection), after hydrolysis and conversion to methyl esters using cold alkaline transesterification with methanolic potassium hydroxide solution (European Community Regulation EEC/2568/91 from 11th July). The fatty acid profile was determined with a Separation was accomplished using a 50 m x 0.25 mm i.d. fused silica capillary column (CP-Sil 88,Varian) wuith helium as carrier gas at 110 kPa. The temperatures of the detector and injector were 250 ºC and 230 ºC, respectively.. The fatty acids composition is expressed in relative percentage of each fatty acid on the total fatty acids eluting between myristic and lignoceric methyl esters. A certified fatty acids methyl esters standard mixture (Supelco 37 Component FAME Mix) was used for identification and FID calibration purposes (Sigma, Spain).

9.2.3. Statistical analysis

One-way analysis of variance (one-way ANOVA) was applied to evaluate the existence of statistical significant effects of the tree cultivar or the crop year on the fatty acids composition of olive oils extracted using the same production techniques, from olives collected at similar maturation stages from centenarian olive trees grown in the same geographical area and under the same agricultural practices. Moreover, if a significant statistical effect was found ($P < 0.050$), the post-hoc multi-comparison Tukey's test was also further applied to identify the levels (i.e., olive tree cultivar or crop year) of each effect that were responsible for the detected significant effect. Boxplots were used to show the one-way ANOVA statistical results for each cultivar and all years evaluated, for saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids.

The influences of olive tree cultivar (*i.e.*, *cvs* Lentisca, Madural, Rebolã, Redondal, Verdeal or Verdeal Transmontana) or crop year (from 2013 to 2017) on the olive oil fatty acids composition of the olive oils extracted from olives of centenarian trees was also evaluated using principal component analysis (PCA), an unsupervised multivariate pattern recognition technique. For PCA, the fatty acids composition was centered and scaled minimizing data variability. The possibility of using the fatty acid composition of the oils to discriminate the different cultivars under study was further evaluated using Linear Discriminant Analysis (LDA), a multivariate supervised pattern recognition technique, along with the simulated annealing (SA) variable selection algorithm. The use of the SA algorithm allowed identifying which fatty acid parameters had the greatest discrimination power. For the LDA, the values of the different parameters were also centered and scaled, minimizing the variability of the data. The quality of discrimination performance was assessed by considering the correct classification rate for the pooled original data as well as for the internal single validation procedure (LOO-CV). The statistical analysis was performed using the Subselect (Cadima, Cerdeira, & Minhoto, 2004; Cadima, Cerdeira, Silva, & Minhoto, 2012; Kuhn, & Johnson, 2013) and MASS (Venables, & Ripley, 2002) packages of the open source statistical program R (version 2.15.1), at a 5% significance level.

9.3. Results and discussions

9.3.1. Effect of olive cultivar on fatty acid composition

In the present work the fatty acid composition of olive oils from minor cultivars, four of them not yet characterized (*cvs.* Lentisca, Rebolã, Redondal and Verdeal) and two poorly known (*cvs.* Madural and Verdeal Transmontana), produced from centenarian trees, were characterized during five consecutive crop years (2013-2017) (Table 9.1). For the main fatty acids all the samples of the different cultivars and throughout the five years of study fulfilled the levels established by the European Community Regulation EEC / 2568/91 from 11th July for EVOO. As expected, oleic acid ($C_{18:1}$) was the main fatty acid found in all cultivars, and, combining the data from the five years, its average amount ranged from 70.3% (*cv.* Madural) to 80.6% (*cv.* Redondal) (Table 9.1). In all years, the amounts of oleic acid were statistically dependent on the olive cultivar ($P < 0.0001$).

Table 9.1. Fatty acids composition (%) (mean ± standard deviation) of olive oils extracted from olives collected in centenarian trees of different cultivars (*cvs*. Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) during five consecutive crop years (2013 to 2017).

Fatty acids (%)	Year	*cv.* Lentisca	*cv.* Madural	*cv.* Rebolã	*cv.* Redondal	*cv.* Verdeal	*cv.* Verdeal Transmontana	*P*-value*
Palmitic acid ($C_{16:0}$)	**2013**	9.93±0.43^{C}	12.36±0.60$^{a;A}$	12.26±0.73$^{a,b;A}$	10.63±0.38$^{a,b;B,C}$	13.02±0.08$^{a;A}$	10.97±0.30$^{a,b;B}$	< 0.0001
	2014	9.91±0.43^{C}	11.66±0.28$^{b;B,C}$	12.76±0.47$^{a,b;B}$	10.20±0.28$^{b;C}$	14.82±2.14$^{a;A}$	11.10±0.70$^{a,b;B,C}$	< 0.0001
	2015	10.56±0.41^{C}	12.61±0.14$^{a;A}$	12.90±0.37$^{a;A}$	10.90±0.32$^{a;B,C}$	12.79±0.16$^{a;A}$	11.32±0.43$^{a;B}$	< 0.0001
	2016	10.15±1.04^{D}	11.28±0.32$^{b;B,C}$	11.98±0.40$^{b;A,B;}$	10.70±0.41$^{a,b;C,D}$	13.01±0.09$^{a;A}$	10.33±0.52$^{c;D}$	< 0.0001
	2017	11.01±1.44^{B}	11.12±0.11$^{b;B}$	13.04±0.17$^{a;A}$	11.19±0.24$^{a;B}$	13.99±0.15$^{a;A}$	10.70±0.55$^{b,c;B}$	< 0.0001
	P*-value	0.1650	< 0.0001	0.0018	0.0022	0.0453	< 0.0001	
	Mean (Min.-Max.)	**10.38 (9.06-12.51)**	**11.81 (10.83-13.06)**	**12.60 (11.43-13.31)**	**10.76 (9.93-11.49)**	**13.52 (12.70-16.73)**	**10.85 (9.76-11.87)**	
Palmitoleic acid ($C_{16:1}$)	**2013**	0.68±0.13^{B}	0.76±0.28$^{a;B}$	0.71±0.17$^{c;B}$	0.80±0.07$^{b;B}$	1.23±0.03$^{b;A}$	0.71±0.05$^{a;B}$	< 0.0001
	2014	0.61±0.16^{C}	0.48±0.01$^{b;C}$	1.30±0.03$^{a;A}$	1.03±0.04$^{a;B}$	1.39±0.05$^{a;A}$	0.58±0.07$^{b,c;C}$	< 0.0001
	2015	0.79±0.17^{C}	0.57±0.01$^{a,b;D}$	0.99±0.11$^{b;B}$	0.70±0.05$^{c;C,D}$	1.25±0.03$^{b;A}$	0.68±0.06$^{a,b;C,D}$	< 0.0001
	2016	0.69±0.24^{B,C}	0.48±0.01$^{b;D}$	0.83±0.05$^{b,c;B}$	0.76±0.07$^{b,c;B,C}$	1.18±0.02$^{b;A}$	0.59±0.10$^{c;C,D}$	< 0.0001
	2017	0.79±0.28^{B,C}	0.53±0.01$^{b;D}$	0.83±0.08$^{b,c;B}$	0.79±0.03$^{b,c;B,C}$	1.41±0.02$^{a;A}$	0.61±0.09$^{b,c;C,D}$	< 0.0001
	P*-value	0.5670	0.0033	< 0.0001	< 0.0001	< 0.0001	0.0002	
	Mean (Min.-Max.)	**0.72 (0.43-1.15)**	**0.56 (0.46-1.13)**	**0.92 (0.55-1.34)**	**0.80 (0.66-1.08)**	**1.29 (1.16-1.44)**	**0.64 (0.49-0.84)**	
Stearic acid ($C_{18:0}$)	**2013**	2.87±0.40^{B}	2.18±0.23$^{c;C}$	2.41±0.25$^{a,b;B,C}$	3.37±0.33$^{a;A}$	2.59±0.02$^{a;B,C}$	2.65±0.11$^{b;B}$	< 0.0001
	2014	2.69±0.23^{A}	2.44±0.04$^{b;A,B,C}$	1.84±0.04$^{c;D}$	2.27±0.15$^{c;C}$	2.35±0.09$^{b;B,C}$	2.60±0.14$^{b;A,B}$	< 0.0001
	2015	2.54±0.29^{A,C}	2.25±0.04$^{b,c;C,D}$	2.07±0.09$^{b,c;D}$	2.84±0.27$^{b;A}$	2.45±0.03$^{b;B,C}$	2.68±0.14$^{b;A,B}$	< 0.0001
	2016	2.70±0.18^{A,B}	2.31±0.10$^{b,c;C}$	1.97±0.10$^{b,c;C}$	2.88±0.42$^{a,b;A}$	2.38±0.03$^{b;B,C}$	2.70±0.25$^{b;A,B}$	< 0.0001
	2017	2.92±0.35^{A,B,C}	2.81±0.23$^{a;B,C}$	2.74±0.45$^{a;B,C}$	3.16±0.09$^{a,b;A,B}$	2.45±0.02^{b,C}	3.35±0.45$^{a;A}$	0.0004
	P*-value	0.1360	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	
	Mean (Min.-Max.)	**2.75 (2.11-3.42)**	**2.40 (1.89-3.10)**	**2.22 (1.80-3.37)**	**2.92 (2.13-3.77)**	**2.45 (2.26-2.63)**	**2.83 (2.33-3.89)**	
Oleic acid ($C_{18:1}$)	**2013**	77.04±2.49^{B}	70.52±1.82$^{a,b;C}$	72.60±2.54$^{a,b;C}$	80.41±0.26$^{b;A}$	76.85±0.18$^{a;B}$	80.39±0.24$^{b;A}$	< 0.0001
	2014	79.18±1.10^{B}	70.87±0.42$^{a;D}$	70.04±0.46$^{b;D}$	81.65±0.11$^{a;A}$	74.21±2.06$^{b;C}$	80.84±0.91$^{a,b;A,B}$	< 0.0001
	2015	77.59±2.45^{B}	69.04±0.52$^{b;D}$	73.26±1.81$^{a,b;C}$	80.82±0.32$^{a,b;A}$	76.77±0.34$^{a;B}$	80.25±0.48$^{b;A}$	< 0.0001
	2016	79.20±1.16^{B}	71.22±0.32$^{a;E}$	75.28±0.60$^{a;D}$	80.94±0.69$^{a,b;A}$	76.86±0.14$^{a;C}$	81.27±0.45$^{a;A}$	< 0.0001
	2017	77.12±1.18^{B}	69.68±0.57$^{a,b;D}$	72.36±2.05$^{b;C}$	79.32±0.96$^{c;A}$	74.09±0.36$^{b;C}$	79.35±0.59$^{c;A}$	< 0.0001
	P*-value	0.0856	0.0022	0.0019	< 0.0001	0.0005	< 0.0001	
	Mean (Min.-Max.)	**77.95 (74.22-80.55)**	**70.27 (68.29-72.90)**	**72.92 (69.60-75.80)**	**80.56 (78.11-81.76)**	**75.76 (72.37-77.06)**	**80.35 (78.30-82.14)**	
Linoleic acid ($C_{18:2}$)	**2013**	7.27±2.77^{B,C}	11.77±2.18$^{b;A}$	9.68±2.81$^{a,b;A,B}$	2.10±0.11$^{b;C}$	4.32±0.05$^{c;C}$	2.76±0.15$^{b;C}$	< 0.0001
	2014	5.56±1.63^{B}	12.08±0.31$^{a,b;A}$	11.74±0.92$^{a;A}$	2.12±0.01$^{b;C}$	5.25±0.04$^{b;B}$	2.31±0.12$^{c;C}$	< 0.0001
	2015	6.40±2.70^{C}	13.17±0.42$^{a,b;A}$	8.65±1.32$^{a,b;B}$	1.90±0.12$^{b;D}$	4.76±0.24$^{b,c;C}$	2.55±0.19$^{b,c;D}$	< 0.0001
	2016	5.33±2.03^{C}	12.39±0.49$^{a,b;A}$	7.82±0.45$^{b;B}$	2.06±0.16$^{b;D}$	4.56±0.12^{c,C}	2.55±0.06$^{b,c;D}$	< 0.0001
	2017	6.13±2.46^{C}	13.63±0.28$^{a;A}$	8.98±2.16$^{a,b;B}$	2.75±0.48$^{a;D}$	6.08±0.48$^{a;C}$	3.22±0.34$^{a;D}$	< 0.0001
	P*-value	0.6370	0.0253	0.0227	< 0.0001	< 0.0001	< 0.0001	
	Mean (Min.-Max.)	**6.15 (3.33-9.73)**	**12.61 (8.90-14.0)**	**9.17 (6.06-12.55)**	**2.19 (1.77-3.34)**	**4.99 (4.27-6.50)**	**2.74 (2.16-3.76)**	
Linolenic acid ($C_{18:3}$)	**2013**	0.93±0.20^{B,C}	1.27±0.19^{A}	1.17±0.23$^{a;A,B}$	0.90±0.07$^{a;C}$	0.96±0.06$^{a;B,C}$	0.81±0.06$^{a;C}$	< 0.0001
	2014	0.82±0.13^{C}	1.20±0.16^{A}	1.07±0.02$^{a,b;A,B}$	0.90±0.01$^{a;B,C}$	0.89±0.05$^{a,b;B,C}$	0.74±0.04$^{a,b;C}$	< 0.0001
	2015	0.89±0.23^{B}	1.23±0.06^{A}	0.92±0.04$^{b,c;B}$	0.88±0.05$^{a,b;B,C}$	0.87±0.01$^{b;B,C}$	0.76±0.04^{a,b,C}	< 0.0001
	2016	0.74±0.09^{C}	1.15±0.02^{A}	0.92±0.12$^{b,c;B}$	0.80±0.02$^{b,c;B,C}$	0.88±0.02$^{b;B}$	0.74±0.05$^{b;C}$	< 0.0001
	2017	0.83±0.14^{B,C}	1.11±0.03^{A}	0.86±0.04$^{c;B,C}$	0.77±0.04$^{c;C,D}$	0.94±0.03$^{a,b;B}$	0.74±0.07$^{b;D}$	< 0.0001
	P*-value	0.2930	0.1610	0.0014	< 0.0001	0.0134	0.0051	
	Mean (Min.-Max.)	**0.84 (0.65-1.19)**	**1.19 (1.00-1.41)**	**0.97 (0.80-1.37)**	**0.85 (0.73-0.97)**	**0.91 (0.84-1.01)**	**0.76 (0.65-0.89)**	

Arachidic acid ($C_{20:0}$)	**2013**	0.41 ± 0.04^{B}	$0.35\pm0.03^{b;C}$	$0.37\pm0.03^{b;B,C}$	$0.51\pm0.03^{a,A}$	$0.38\pm0.02^{a;B,C}$	$0.48\pm0.02^{b,A}$	< 0.0001
	2014	0.37 ± 0.06^{A}	$0.48\pm0.15^{a;A}$	$0.35\pm0.01^{b;A}$	$0.41\pm0.03^{b;A}$	$0.33\pm0.03^{b;A}$	$0.49\pm0.03^{a,b;A}$	0.0279
	2015	0.35 ± 0.05^{B}	$0.34\pm0.01^{b;B}$	$0.38\pm0.02^{b;B}$	$0.48\pm0.04^{a;A}$	$0.37\pm0.02^{a,b;B}$	$0.49\pm0.02^{b;A}$	< 0.0001
	2016	0.39 ± 0.06^{B}	$0.36\pm0.01^{b;B}$	$0.36\pm0.01^{b;B}$	$0.48\pm0.05^{a;A}$	$0.39\pm0.01^{a;B}$	$0.50\pm0.03^{a,b;A}$	< 0.0001
	2017	0.40 ± 0.07^{C}	$0.37\pm0.02^{a,b,C}$	$0.41\pm0.03^{a;B,C}$	$0.49\pm0.02^{a;A,B}$	$0.36\pm0.02^{a,b;C}$	$0.53\pm0.05^{a;A}$	< 0.0001
	P*-value	0.3670	0.0088	0.0005	0.0044	0.0092	0.0030	
	Mean (Min.-Max.)	**0.39 (0.29-0.48)**	**0.38 (0.30-0.68)**	**0.38 (0.34-0.44)**	**0.48 (0.39-0.54)**	**0.37 (0.30-0.41)**	**0.50 (0.44-0.61)**	
Eicosenoic acid ($C_{20:1}$)	**2013**	0.36 ± 0.10^{A}	$0.34\pm0.03^{a;A}$	$0.30\pm0.03^{a,b;A,B}$	$0.33\pm0.03^{b,c;A}$	$0.24\pm0.01^{b,c;B}$	$0.32\pm0.02^{b,c;A}$	0.0063
	2014	0.34 ± 0.09^{A}	$0.34\pm0.01^{a;A}$	$0.32\pm0.01^{a;A,B}$	$0.39\pm0.01^{a;A}$	$0.25\pm0.02^{a;B}$	$0.35\pm0.02^{a;A}$	0.0014
	2015	$0.33\pm0.10^{A;B}$	$0.32\pm0.01^{a,b;A,B}$	$0.30\pm0.01^{a;A,B}$	$0.36\pm0.02^{a,b;A}$	$0.26\pm0.01^{a,b;B}$	$0.34\pm0.01^{a,b;A}$	0.0230
	2016	$0.30\pm0.08^{A,B}$	$0.32\pm0.00^{a,b;A}$	$0.31\pm0.04^{a;A,B}$	$0.33\pm0.01^{b,c;A}$	$0.24\pm0.01^{a,b;B}$	$0.32\pm0.01^{b,c;A}$	0.0215
	2017	0.30 ± 0.08^{A}	$0.30\pm0.01^{b;A}$	$0.26\pm0.01^{b;A,B}$	$0.32\pm0.00^{c;A}$	$0.22\pm0.01^{c;B}$	$0.31\pm0.02^{c;A}$	0.0004
	P*-value	0.7560	0.0038	0.0040	< 0.0001	0.0006	< 0.0001	
	Mean (Min.-Max.)	**0.32 (0.22-0.45)**	**0.32 (0.28-0.39)**	**0.30 (0.24-0.37)**	**0.34 (0.29-0.40)**	**0.24 (0.21-0.28)**	**0.33 (0.28-0.36)**	

In each column and for each olive cultivar, different lower case letters mean significant statistical differences of the fatty acid relative percentage with the crop year, at a 5% significance level (*P*-value < 0.05), according to the multiple comparison Tukey's HSD test.

In each line and for each crop year, different capps letters mean significant statistical differences of the fatty acid relative percentage with the olive cultivar, at a 5% significance level (P-value < 0.05), according to the multiple comparison Tukey's HSD test.

* *P*-value < 0.05 meaning that the mean value of the evaluated fatty acid realtive percentage of at least one olive cultivar (for the lines) or of at least one crop year (for the columns) differs from the others, according to the one-way ANOVA results (in this case multiple-comparison tests were performed).

Three possible groups could be observed, one constituted by *cvs*. Verdeal Transmontana and Redondal olive oils, with the highest amounts, and mean values higher than 80%; one second group, with intermediate values, including *cvs.* Lentisca and Verdeal, means of 78.0 and 75.8%; and a third group with the lower amounts for the oils of *cvs.* Madural (70.3%) and Rebolã (72.9%) (Table 9.1). Palmitic acid ($C_{16:0}$) was the second major fatty acid (Table 9.1). Consistently, the highest values of this fatty acid were obtained for *cv*. Verdeal oil, with a mean value of 13.5% combining the five years studied. Also, in general, the lowest levels were observed for *cv*. Lentisca (mean value of 10.4%). For this fatty acid the amounts for *cvs*. Madural and Rebolã were similar, except for the 2017 crop year (Table 9.1). The amounts of the essential linoleic acid ($C_{18:2}$), the third in order of abundance, with the exception for *cv*. Redondal, significantly differ between the studied cultivars. The highest values, and in general with statistical differences between cultivars, were observed for *cv*. Madural olive oils (mean value of 12.6%), followed by *cv*. Rebolã (mean value of 9.2%), totally opposite to the average amounts found for Redondal (2.19%) and Verdeal Transmontana (2.74%), with *cv*. Lentisca and *cv*. Madural in between (Table 9.1). Surprisingly, in *cv*. Rebolã olive oils, the amount of stearic acid ($C_{18:0}$) (2.9%) was higher than the observed for linoleic acid (2.2%), contrarily to the observed for the other cultivars. Stearic acid ranged from 2-3% for all cultivars (Table 9.1).

Consistently, the amounts for the essential linolenic acid ($C_{18:3}$) was significantly higher for *cv*. Madural olive oils, with a mean value of 1.19%, whilst in the opposite place appeared *cv*. Lentisca (0.84%) and Verdeal Transmontana (0.76%) olive oils. The olive oils from oils from *cv*. Lentisca surpassed the maximum value of ≤ 1.0 established by the European Community Regulation EEC / 2568/91 from 11th July for EVOO while *cvs.* Lentisca, Rebolã (0.97%), Redondal (0.85%), Verdeal (0.91%) and Verdeal Transmontana were within this limit. Regarding palmitoleic acid ($C_{16:1}$), the highest value was observed for *cv*. Verdeal olive oil (1.27%), which was the only cultivar with levels greater than 1% (Table 9.1). In general, the lowest values were obtained for *cvs.* Madural (mean 0.56%) and Verdeal Transmontana (0.64%). The highest values of arachidic acid ($C_{20:0}$), were observed for Verdeal Transmontana (0.5%) and Redondal (0.48%) but were lower than the maximum (≤0.6%) established by the European Community Regulation EEC / 2568/91 from 11th July for EVOO. Also for eicosenoic acid ($C_{20:1}$) none the cultivars reach the maximum (≤0.4%) established by the same Regulation. The present results confirmed that the fatty acid composition is olive cultivar dependent. Some variations were observed between them, being the established fatty acid profile for each cultivar in agreement with the literature data for diverse olive cultivars (Borges et al., 2017; Kritioti et al., 2018; Portarena et al., 2015; Xiang et al., 2017; Wanga et al., 2018), although with different relative compositions. For example, for the main fatty acid (oleic acid), Xiang et al. (2017) found values ranging from 60.9 (*cv.* Barnea) to 74.0% (*cv.* Koreniki), and Wanga et al. (2018) from 62.2% (*cv.* Empeltre) to 74.5 % (*cv.* Cornicabra), when studying fatty acid composition of different cultivars. However, the comparison is not straightforward since besides different cultivars are involved, also different geographic regions are evaluated. Indeed, Borges et al. (2017) verified that for *cv.* Arbequina olive oils the geographic origin influences the fatty acid profile for the same cultivar. This variability factor was eliminated under the present study because all trees were grown under the same region. Therefore, the fatty acid variations observed are strictly dependent on the cultivar and the effect of climate on each cultivar. In Figure 9.1, the fatty acids contents according to their degree of saturation and considering the monounsaturated fatty acids (MUFA), allowing distinguishing five significantly different ($P \leq 0.05$) groups in terms of relative abundance: *cv.* Redondal (82.1%) ≈ *cv.* Verdeal Transmontana (81.7%) > *cv.* Lentisca (79.1%) > *cv.* Verdeal (77.4%) > *cv.* Rebolã (74.3%) > *cv.* Madural (71.2%). An inverse trend was observed for PUFA, with a relative abundance as follows: *cv.* Madural (13.8%) > *cv.* Rebolã (10.1%) > *cv.* Lentisca (7.0%) > *cv.* Verdeal (5.9%) > *cv.* Verdeal Transmontana (3.5%) ≈ *cv.* Redondal (3.0%) (Figure 9.1).

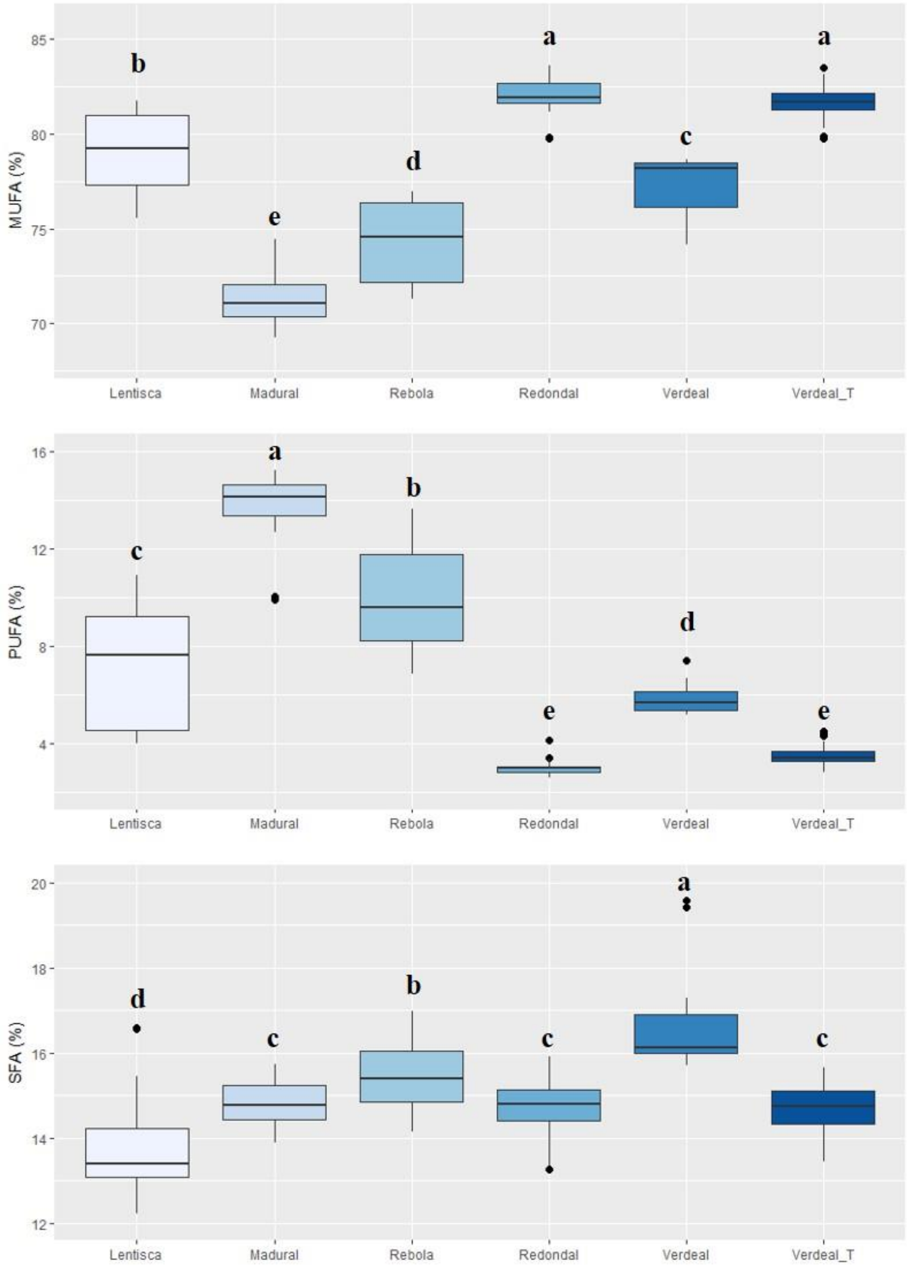

Figure 9.1. Boxplots of MUFA, PUFA and SFA (%) found in olive oils extracted from olives collected in centenarian trees of different cultivars (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) during five consecutive crop years (2013 to 2017). Different lowercase letters mean significant statistical differences at a 5% significance level (one-way ANOVA followed by the Tukey's multi-comparison test).

From a technological point of view, the presence of high PUFA amounts in vegetable oils decreases their resistance to oxidation, affecting both its shelf life and thermal performance. Significantly higher values for SFA were observed for *cv.* Verdeal (16.6%), followed by *cv*. Rebolã (15.5%), and the lowest values were observed for *cv.* Lentisca (13.8%).

For SFA *cvs* Madura, Redondal and Verdeal Transmontana presented similar relative amounts (Figure 9.1). The overall amounts of the different groups (saturated and unsaturated fatty acids) are of major relevance due to the related nutritional and technological impacts (Saad et al., 2012). Indeed, besides the previously mentioned health effect of MUFA, the MUFA and PUFA amounts are also correlated with the oxidative stability of oils, with higher shelf-life for olive oils richer in MUFA and usually lower for higher PUFA amounts. The amounts of MUFA are also known to be correlated with some sensory attributes, being stablished positive correlations with bitter attribute and negative ones with sweet attribute (García-Mesa et al., 2008, Youssef et al., 2011). Also, Youssef et al. (2011) observed that a high content of SFA in olive oils lead to a higher viscosity and persistence on the mucous of the oral cavity, producing an effect known as "fatty sensation", not sensory agreeable. All these reasons qualified fatty acids contents as quality indices of the oils during production, storage and trade (Prentki et al., 2012, Soto-Vaca et al., 2013). Globally, the results described in this work are in agreement with the literature data that considered the amounts of the different groups (MUFA, PUFA and SFA) genetically dependent and so, dependent of the cultivars (e.g. Kritioti et al., 2018; Xiang et al., 2017).

9.3.2. Effect of crop year on the fatty acids composition

For each cultivar and fatty acid, the relative amounts determined for each crop year are also given in Table 9.1. In general, with the exception of *cv*. Lentisca, the year of production showed a significant effect on the olive oil fatty acid composition for the different olive cultivars, but the cultivar effect was still prevalent. The amount of oleic acid ($C_{18:1}$), the major fatty acid, changed slightly with the crop year, and consistently, for the four cultivars, the values obtained in 2017 were significantly lower than the maximum values observed in other years. This fact could be related with the rain and temperatures observed in 2017 (Table 9.2). In fact, among the five years covered in this study, 2017 was the most dried year, with a large period without rain, being considered a year of extreme drought. Considering the rainfall of August, September and October, less than 30 mm of rain were observed in 2017 whereas, in the other years, the total rainfall of these months ranged from 100 to 180 mm (Table 9.2). Also the mean and maximum temperatures registered during October were higher in 2017, negatively influencing oleic acids contents, in agreement with literature findings (Orlandi et al. 2012; Rondanini et al., 2014; García-Inza et al., 2014).

Table 9.2. The mean temperature (minimum, mean and maximum) and total rainfall (mm) in the months of August, September and October of Mirandela region (northern Portugal) over five years (2013-2017).

Temperature records	Minimum	Mean	Maximum	Rainfall (mm)
2013				
August	16.76±2.18	25.58±9.33	34.40±3.39	0.00
September	14.21±2.19	22.38±8.80	30.54±3.85	30.00
October	11.00±3.76	16.44±6.41	21.88±2.89	111.40
2014				
August	14.26±2.64	22.71±8.91	31.16±2.64	0.40
September	14.45±4.52	21.01±7.58	27.57±4.52	84.80
October	10.93±3.03	17.07±6.84	23.21±3.03	77.20
2015				
August	14.54±2.56	23.21±9.43	31.88±4.36	20.20
September	11.92±3.01	19.51±8.31	27.11±3.47	50.40
October	9.80±3.32	15.21±6.23	20.62±2.71	109.40
2016				
August	15.75±2.45	25.13±9.87	34.51±3.21	32.20
September	12.87±2.78	21.44±9.65	30.01±5.47	24.20
October	9.9±2.70	16.26±7.19	22.63±3.76	41.60
2017				
August	14.99±2.82	24.41±9.99	33.83±3.42	1.20
September	10.83±3.06	19.87±9.62	28.92±3.12	0.20
October	8.91±3.68	17.7±9.96	26.49±5.35	28.60

Temperature seems to be particularly determinant between flowering and harvest (Borges et al., 2017; Portarena et al., 2015; Ceci et al., 2017; Tena et al., 2017). Oleic and palmitic acids are the fatty acids that seem to be more affected by the environmental conditions (Dabbou et al., 2010; Mailer et al., 2010). In the present study, palmitic acid was also significantly influenced by the crop year for all cultivars with the exception of *cv.* Lentisca (Table 9.1), although no marked trend was been observed.

An opposite trend compared to that observed for oleic acid, was found for linoleic acid, being the greatest values, for all cultivars, recorded in 2017. It was verified that there is a significant effect of the year on the cultivars, with the exception of the *cv.* Lentisca where no influence of the year on the composition in $C_{18:2}$ was detected. Also, for the other fatty acids, the year influenced their relative proportions (Table 9.1). In fact, colder years are described to increase oleic acid levels, as previously discussed, and this is usually accompanied by a reduction in the content of linoleic acid (Inglese et al., 2011,) being both fatty acids contents highly correlated. In addition to the climatic conditions considered in the present study, other factors such as agronomic aspects (maturation index, storage conditions, processing) or other environmental variables (light intensity, humidity, evapotranspiration, soil) may also influence the chemical activity and composition of olive oil (Romero et al., 2016, Rondanini et al., 2014). However, trying to mitigate some of those known effects, in this work, all the plants were cultivated in the same grove, subjected to similar agronomic and environmental conditions and harvested at a similar ripening index, being the variations observed mainly dependent of the cultivar and the environmental conditions of the harvest year.

9.3.3. Cultivar and crop year discrimination according to fatty acids composition

As can be inferred from Figure 9.2, the overall olive oil fatty acids profiles allowed the unsupervised differentiation of the oils according to the olive cultivar.

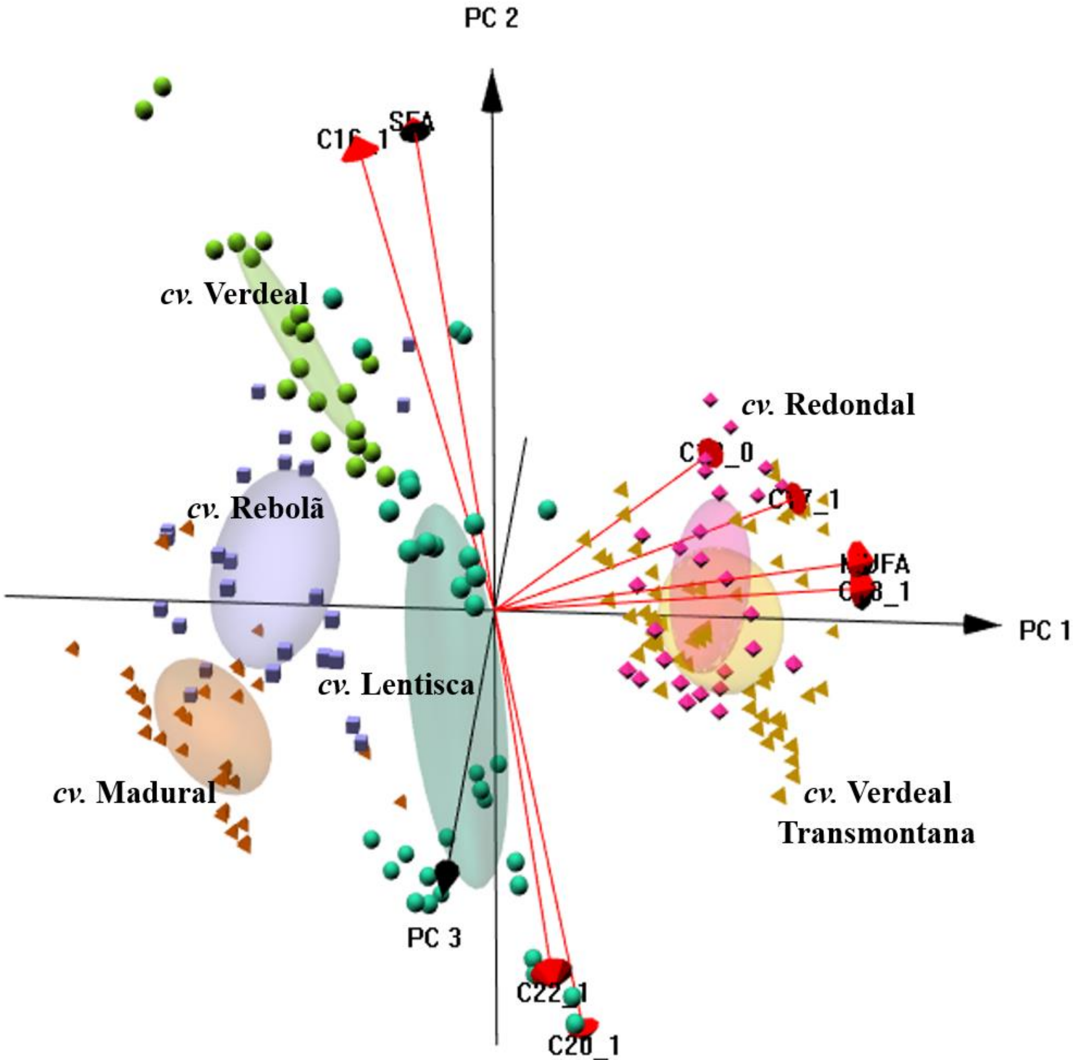

Figure 9.2. Principal component analysis (PC1: 44.2%, PC2: 19.7% and PC3: 9.7%) based on the fatty acids profile ($C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{17:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:0}$, $C_{20:1}$, $C_{22:0}$, $C_{22:1}$, $C_{24:0}$, SFA, PUFA and MUFA levels): 3D plot showing the unsupervised pattern recognition according to olive cultivar (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) based on fatty acids composition (%) found in olive oils obtained from olives collected in centenarian trees during five consecutive crop years (2013 to 2017).

The visualization of the PCA-3D plot, which 3 first principal components (PCs) explained 73.6% of the data variability), clearly shows that olive oils from *cvs.* Lentisca, Madural, Rebolã and Verdeal could be easily identified and distinguished form the other cultivars, being only a missing of olive oils from *cvs.* Redondal and Verdeal Transmontana.

Among the 14 fatty acids identified and the total SFA, MUFA and PUFA contents, the fatty acids that mostly contributed to the olive oils unsupervised classification by cultivar were $C_{16:0}$ and total SFA together with $C_{16:1}$, $C_{17:1}$, $C_{18:1}$, $C_{20:1}$, $C_{22:1}$, $C_{16:1}$ and total MUFA. As can be easily inferred, the composition in MUFA is the one that most contributed to the natural differentiation of the olive oils, pointing out the relevance of this fraction to the characterization of the studied olive oils. In more detail, the contents of $C_{16:0}$ together with total MUFA and the individual $C_{17:1}$ and $C_{18:1}$ were the most relevant for the differentiation of oils of *cvs.* Redondal and Verdeal Transmontana, from those of the other 4 cultivars (*cvs.* Lentisca, Madural, Rebolã and Verdeal). On the other hand, the monounsaturated acids $C_{16:1}$, $C_{20:1}$, $C_{22:1}$ and total SFA were those that mostly contributed to the differentiation of *cvs.* Lentisca, Madural, Rebolã and Verdeal. To further verify the usefulness of the fatty acids profile to discriminate olive oils by cultivar and to identify the fatty acids that could be tentatively used as chemical biomarkers of each cultivar, a LDA coupled to with the SA algorithm (LDA-SA) was implemented. The results (Figure 9.3) showed that, a LDA-SA model with three significant linear discriminant functions (LDs) allowed explaining 98.4% of the data variability (81.2%, 11.5% and 5.7%, respectively), based on the content of 4 saturated acids ($C_{16:0}$, $C_{17:0}$, $C_{18:0}$ and $C_{20:0}$), 5 monounsaturated compounds ($C_{16:1}$, $C_{17:1}$, $C_{18:1}$, $C_{20:1}$ and $C_{22:1}$) and total PUFA. The established model allowed the correct classification of 94% of the oils according to the cultivar for the original data grouped with a predicted correct classification rate of 92% for the LOO-CV procedure. These results confirmed that olive oil discrimination by cultivar can be successfully achieved using the oils fatty acids composition. Furthermore, for each cultivar evaluated, it was also evaluated the possibility of using the fatty acids compositions to identify the crop year.

For that PCA were carried out and, as can be seen from the PCA-3D plots shown in Figure 9.4, for each cultivar, oils could be discriminated according to the crop year. As can be inferred from the overall plotted data, the 5 crop years studied could be naturally grouped into 3 subgroups classes. One subgroup included the oils produced in 2017, which fatty acids composition was different from that of the oils produced in the other 4 studied years (2013-2016).

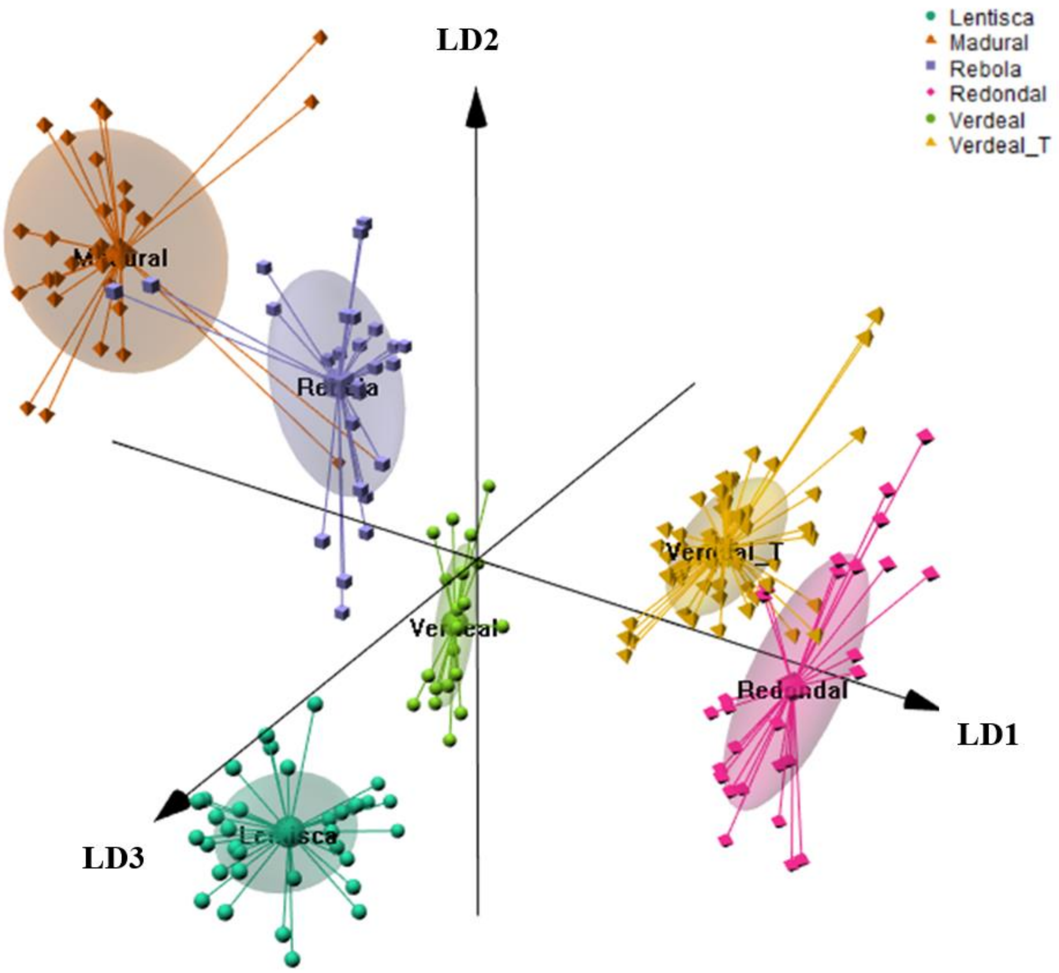

Figure 9.3. Linear Discriminant Analysis (1st DF: 81.2%, 2nd DF: 11.5%, and 3rd DF: 5.7%): 3D plot showing the discrimination of olive oil according to olive cultivar (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) based on the contents (%) of 9 fatty acids ($C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{17:1}$, $C_{18:0}$, $C_{18:1}$, $C_{20:0}$, $C_{20:1}$ and $C_{22:1}$) and PUFA (selected using the simulated annealing algorithm) found in olive oils obtained from olives collected in centenarian trees during five consecutive crop years (2013 to 2017).

The second subgroup comprised the oils produced in 2014 and 2016, being the last subgroup for the oils produced in 2014 and 2015. These results clearly point out that, within each olive cultivar class, crop year has a huge impact on the fatty acid composition of produced olive oil. It should be remarked that, the above-mentioned unsupervised subgroups formation could be tentatively related to the different climacteric conditions observed in 2013-2015 (high rain quantity in october), 2014-2016 (rain distributed along the analyzed period and high maximum temperature) and 2017 (dry and hot weather during all summer with the hottest temperature during this study), which can be inferred from the data shown in Table 9.2.

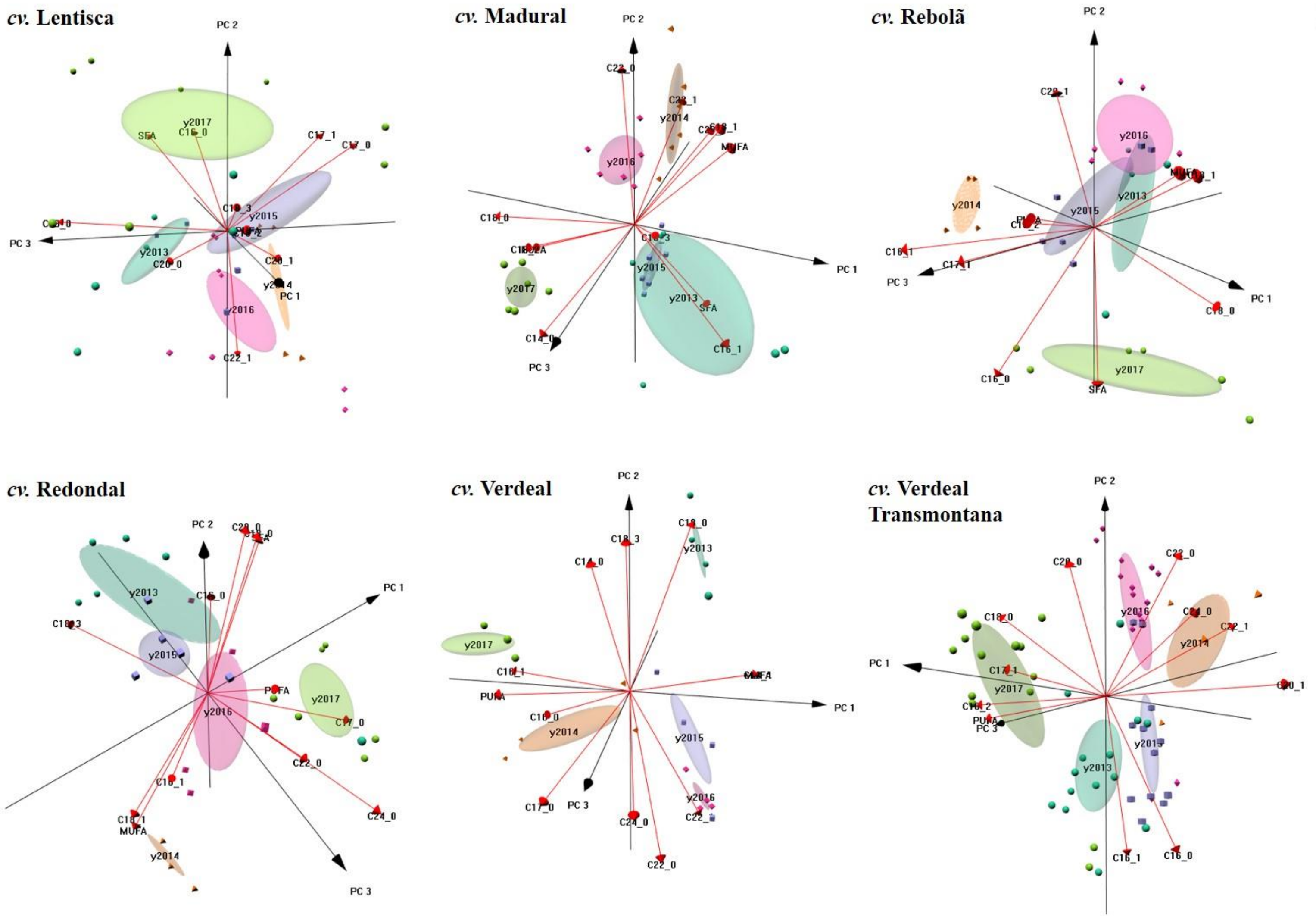

Figure 9.4. Principal component analysis for olive oils from each individual olive cultivar: 3D plot showing the unsupervised pattern recognition according to crop year (2013 to 2017) based on fatty acids profile (%) ($C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{17:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:0}$, $C_{20:1}$, $C_{22:0}$, $C_{22:1}$, $C_{24:0}$), SFA, PUFA and SFA levels found in olive oils obtained from olives collected in centenarian trees of different cultivars (*cvs.* Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana).

9.4. Conclusions

In the present work, oils obtained from centenarian specimens of six minority cultivars from Trás-os-Montes region, the second most important Portuguese olive region, were characterized regarding their fatty acids contents during five consecutive crop years, allowing to strengthen the findings achieved. Four of the six cultivars were studied for the first time (*cvs.* Lentisca, Rebolã, Redondal and Verdeal). Regarding the fatty acid composition, it was observed that oils from *cvs.* Redondal and Verdeal Transmontana were the richest in oleic acid, which could be indicative of a possible high shelf life and increased nutritional value. On the contrary, oils from *cv.* Madural had the lowest content in oleic acid, indicating a possible higher susceptibility to oxidation. The overall results also pointed out that both olive cultivar and crop year had marked effects on the oils fatty acid composition, which could be used as chemical biomarkers. Moreover, it was verified that the environmental conditions mainly influenced oleic acid and linoleic acid contents and that some authentic cultivars are not within regulated limited for linolenic acid, highlighting for the pertinence of this short limit. The obtained results contribute for enhancing the scarce knowledge of olive heritage, and may support the selection of olive cultivars for new plantations, based on their potential to allow a more favorable fatty acid composition in which concerns the nutritional and oil quality points of view.

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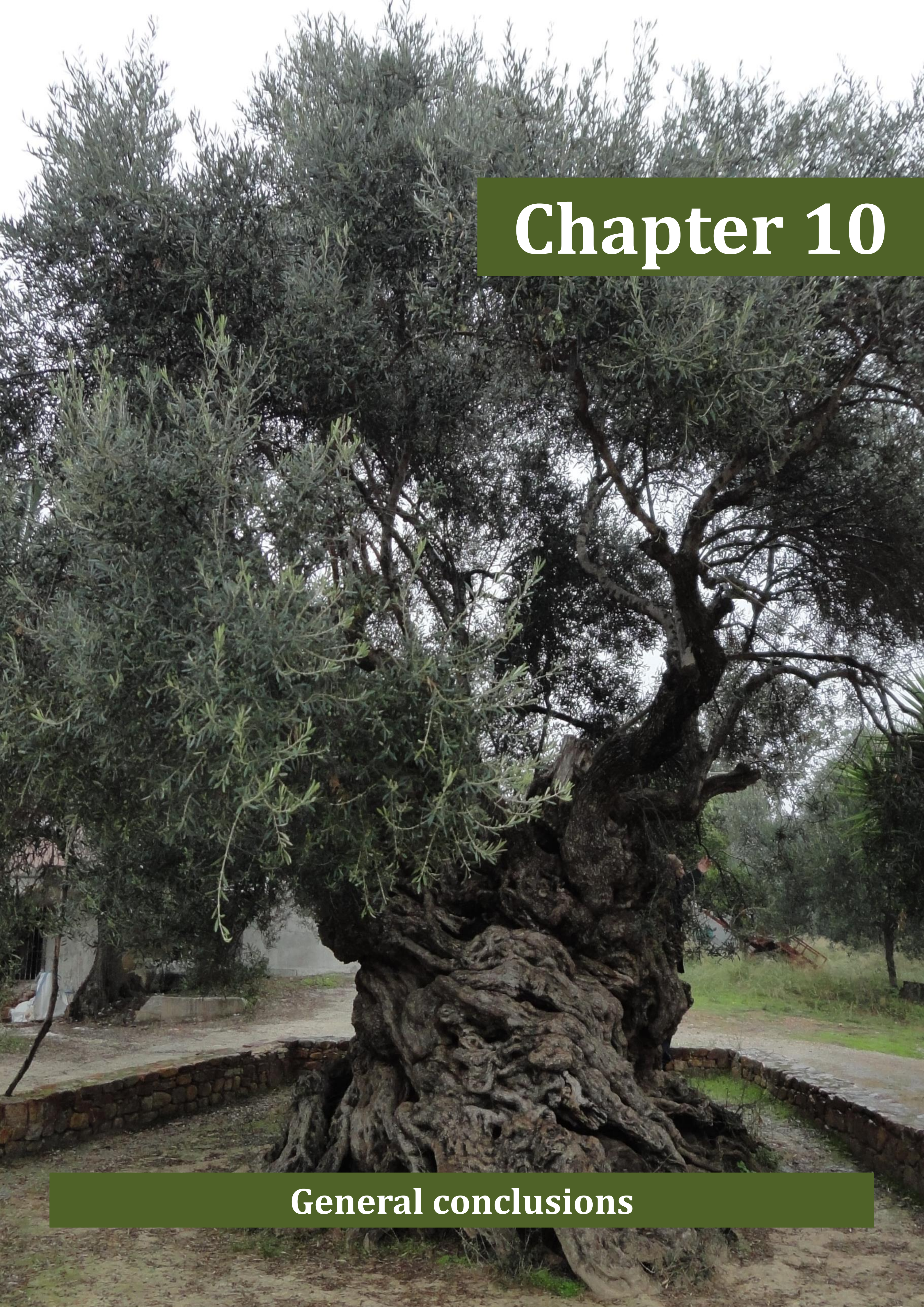
Chapter 10



General conclusions

Chapter 10

10.1. General Conclusions

This doctoral thesis contributed for the genetic and morphologic characterization of oleander populations and centenarian olive trees from the northeast of Portugal, as well as, to the establishment of chemical and sensory profiles of their oils with the purpose of future valorization. Therefore, it is concluded that:

- The evaluated oleasters and centenarian olive trees possess high genetic diversity, without differences between them in terms of genetic diversity.
- The study of the population structure analysis suggests genetic differentiation between oleander populations and centenarian olive trees.

- Oleaster oils from the different populations showed a very stable composition for the evaluated parameters (fatty acids, tocopherols, phenols and sterols). Nevertheless some few differences were observed between populations that allowed its discrimination by region of origin.
- The chemical profile of oleaster oils was very similar to that of olive oil, widely enriched with considerable amounts of tocopherols, sterols and phenolic compounds.
- The rich composition of oleaster oil indicates that the populations characterized could be included in breeding programs to produce oils rich in bioactive compounds.
- Given the market demand for differentiated sensory and chemical products, the production of oleaster oils for commercial purposes could be considered.

- From the centenarian trees, similar phenolic profiles were observed along the four years and for all the analyzed oil. Nevertheless their amounts were significantly influenced by the tree and crop year.
- Fifty per cent of the evaluated trees produced olive oils that could be labeled as "*Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress*".
- Some specimens, e.g. centenarian trees nº24, 25 and 26, consistently produced olive oils with high phenolics content, being good candidates to be used in breeding programs as producers of differentiated oils rich in phenolic compounds, contributing for the preservation and valorization of olive genetic heritage.
- The studied six minor cultivars (Lentisca, Madural, Rebolã, Redondal, Verdeal and Verdeal Transmontana) produced oils that fulfilled the requirements to be classified as extra virgin olive oils.

- Some of the obtained oils were sensory exquisite, with rare notes such as green cherry (*cvs*. Lentisca and Madural), apricot (*cvs*. Lentisca, Madural and Verdeal Transmontana) and kiwi (*cvs*. Lentisca, Madural, Rebolã and Verdeal Transmontana).
- The olive oils from *cv*. Redondal showed the highest oxidative stability, amounts of total phenol, and $C_{18:1}/C_{18:2}$ ratios.
- The olive oils from centenarian trees of the *cvs*. Lentisca and Redondal possesses high amounts in tocopherols, higher than 400 mg/kg olive oil, twice the observed for *cvs.* Verdeal and Verdeal Transmontana.
- *Cvs*. Redondal and Verdeal Transmontana olive oils were the richest in oleic acid (higher than 80%) and *cv*. Madural the poorest (around 70%).
- Crop year had significant influence in the amounts of tocopherols and some fatty acids, mainly oleic and linoleic acid.
- The application of different statistical tools, namely principal component analysis, discriminant analysis and hierarchical grouping analysis, to the obtained data allowed discriminating olive oil according to the cultivar and the crop year.

The integration of the results showed that the olive oils from centenarian trees of the c*vs.* Redondal and Lentisca had the best results for the main evaluated parameters. This conclusion could support the production of monovarietal olive oils from these cultivars that possesses simultaneously differentiate organoleptic characteristics and a very favorable chemical profile that allowed the use of health claims for fatty acids, phenolics and tocopherols.

Finally, the results obtained contributed to enhance the scarce knowledge of olive heritage, and may be further used to support the selection of olive cultivars for new plantations, based on their potential to produce oils with a more favorable chemical, sensory and bioactive profile, in which concerns the nutritional and oil quality points of view.

10.2. Conclusiones Generales

Esta tesis doctoral ha contribuido para la caracterización genética y morfológica de poblaciones de acebuche y olivos centenarios del nordeste de Portugal, bien como para el estabelecimiento del perfil químico y sensorial de sus aceites con el objetivo de valorización futura. Así, se ha concluido:

- Os acebuches e olivos centenarios evaluados en este estudio tiene alta diversidad genética, sin diferencias entre ellos en lo que respecta a la diversidad genética.
- El estudio de la estructura de la población sugiere diferenciación genética entre las poblaciones de acebuches y árboles de olivos centenarios.

- Los aceites de diferentes poblaciones de acebuches mostraran tener una composición mui estable en los parámetros evaluados (ácidos grasos, tocoferoles, fenoles y esteroles). Todavía fueran observadas algunas diferencias entre poblaciones que ha permitido su discriminación por región de origen.
- El perfil químico de los aceites de acebuche ha sido mui similar al del aceite de oliva, mui enriquecidos con considerable cantidad de tocoferoles, esteroles y compuestos fenólicos.
- La buena composición de los aceites de acebuche es indicativa que sus poblaciones pueden ser incluidas en programas de mejora para producir aceites ricos en compuestos bioactivos.
- Dada la demanda del mercado por productos diferenciados del punto de vista sensorial y químico, la producción de aceites de acebuches para fines comerciales podría considerarse.
- A partir de los árboles centenarios, para todo el aceite analizado se observaron perfiles fenólicos similares a lo largo de los cuatro años. Sin embargo, sus cantidades fueron significativamente influenciadas por el árbol y el año de cosecha.
- El cincuenta por ciento de los árboles evaluados produjo aceites de oliva que podrían etiquetarse como la alegación de la salud *"los compuestos fenólicos del aceite de oliva contribuyen para la protección de los lípidos sanguíneos del estrés oxidativo"*.
- Algunas de las árboles centenarios, por ejemplo los nº24, 25 y 26, han producido continuamente aceites de oliva con alto contenido fenólico, siendo buenos candidatos para ser utilizados en programas de mejoramiento como productores de aceites diferenciados ricos en compuestos fenólicos, contribuyendo para la preservación y valorización del patrimonio genético del olivo.

- Los seis cultivares menores estudiados (Lentisca, Madural, Rebolã, Redondal, Verdeal y Verdeal Transmontana) produjeron aceites que cumplían con los requisitos para ser clasificados como aceites de oliva extra virgen.
- Algunos de los aceites de oliva obtenidos fueron sensoriales exquisitos, con notas raras como cereza verde (*cv*. Lentisca y Madural), albaricoque (*cvs.* Lentisca, Madural y Verdeal Transmontana) y kiwi (*cvs.* Lentisca, Madural, Rebolã y Verdeal Transmontana).
- Los aceites de oliva de la *cv*. Redondal mostraron tener la mayor estabilidad oxidativa, las mayores cantidades de fenoles totales y las proporciones $C_{18:1}/C_{18:2}$ más altas.
- Los aceites de oliva de los árboles centenarios de los cvs. Lentisca y Redondal poseen altas cantidades de tocoferoles, más 400 mg/kg de aceite de oliva, el doble de lo observado para los *cvs*. Verdeal y Verdeal Transmontana.
- Los aceites de oliva de los *cvs*. Redondal y Verdeal Transmontana fueron los más ricos en ácido oleico (superior al 80%) y los del *cv*. Madural los más pobres (alrededor del 70%).
- El año de cosecha tuvo una influencia significativa en las cantidades de tocoferoles y algunos ácidos grasos, principalmente el ácido oleico y el linoleico.
- La aplicación de diferentes herramientas estadísticas, a saber, el análisis de componentes principales, el análisis discriminante y el análisis de agrupación jerárquica, a los datos obtenidos permitió discriminar el aceite de oliva según el cultivar y el año de cosecha.

La integración de los resultados mostró que los aceites de oliva de los árboles centenarios de los *cvs*. Redondal y Lentisca tuvieron los mejores resultados para los principales parámetros evaluados. Esta conclusión podría apoyar la producción de aceites de oliva monovarietales a partir de estos cultivares que poseen características organolépticas diferenciadas a la vez y un perfil químico muy favorable que permite el uso de declaraciones de propiedades saludables de ácidos grasos, fenólicos y tocoferoles.

Finalmente, los resultados obtenidos contribuyeron a mejorar el escaso conocimiento del olivar y pueden utilizarse para apoyar la selección de cultivares de olivo para nuevas plantaciones, en función de su potencial para producir aceites con un perfil químico, sensorial y bioactivo más favorable, en que se refiere a los puntos de vista de la calidad nutricional y del aceite.