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Influência da cultivar nas características físico-quimicas, sensoriais e biológicas de azeitonas verdes descaroçadas

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À minha mãe
À Ana

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ÍNDICE

CAPÍTULO 1. INTRODUÇÃO

CAPÍTULO 2. JUSTIFICAÇÃO E OBJECTIVOS

CAPÍTULO 3. EFFECT OF CULTIVAR ON SENSORY CHARACTERISTICS, CHEMICAL COMPOSITION AND NUTRITIONAL VALUE OF STONED GREEN TABLE OLIVES

CAPÍTULO 4. VOLATILE PROFILE OF STONED TABLE OLIVES FROM DIFFERENT VARIETIES BY HS-SPME AND GC/IT-MS.

CAPÍTULO 5. CULTIVAR EFFECT ON THE PHENOLIC COMPOSITION AND ANTIOXIDANT POTENTIAL OF STONED TABLE OLIVES

CAPÍTULO 6. DISCUSSÃO GERAL E CONCLUSÕES

Resumo

As azeitonas verdes descaroçadas, "*Alcaparras*", são um tipo de azeitona de mesa produzido de forma tradicional e muito apreciadas na região de Trás-os-Montes. De maneira geral, na sua produção o factor cultivar não tem sido tido em conta. Neste sentido, com o presente trabalho pretendeu-se avaliar a influência da cultivar nas características físico-químicas, sensoriais e biológicas deste tipo de azeitonas. Para tal, procedeu-se à preparação de diferentes lotes de "*alcaparras*" à escala laboratorial, com azeitonas das cultivares mais representativas da região, nomeadamente *Cv.* Cobrançosa, Madural, Negrinha de Freixo, Santulhana e Verdeal Transmontana, e avaliou-se a sua composição físico-química (humidade, gordura total, proteína total, cinzas e hidratos de carbono+fibras), valor energético, avaliação sensorial e quantificação de alguns componentes: ácidos gordos (GC/FID), tocoferóis (HPLC/FD), compostos voláteis (HS-SPME e GC/IT-MS) e compostos fenólicos (HPLC/DAD). Por último foi avaliada a actividade antioxidante das azeitonas de diferentes cultivares através dos métodos do efeito bloqueador dos radicais de DPPH e do Poder Redutor.

As "*alcaparras*" são maioritariamente constituídas por água (> 70%) e gordura (entre 12,5 e 20,1%). O valor energético variou entre as 154 e 212 kcal por 100g, com o menor valor registado em azeitonas produzidas com a *Cv.*Madural e maior na *Cv.* Verdeal Transmontana, sendo este valor influenciado principalmente pelo teor em gordura. O perfil em ácidos gordos é maioritariamente constituído por ácidos gordos monoinsaturados, sendo o ácido oleico o mais abundante (≥ 66,9%). A *Cv.* Negrinha de Freixo possui maior teor em tocoferóis (6,0 mg/kg), sendo o α-tocoferol o isómero mais abundante em todas as cultivares. O perfil em compostos voláteis das "*alcaparras*" é maioritariamente composto por aldeídos (> 74%) e, em menor quantidade, por álcoois, ésteres, cetonas, derivados de norisoprenóides, terpenos, sesquiterpenos e alcenos, num total de 42 compostos identificados. Foram identificados doze compostos fenólicos, sendo o hidroxitirosol o mais abundante e tendo a *Cv.* Cobrançosa reportado maior teor em compostos fenólicos totais (165,76 mg/kg). As azeitonas produzidas com as *Cvs.* Cobrançosa e Santulhana apresentaram maior actividade antioxidante (EC_{50} de 1,38 e 1,40 mg/ml para o poder redutor e 0,48 e 0,46 mg/ml para o DPPH). O teor em ácidos gordos, a composição em compostos voláteis e em compostos fenólicos, bem como a actividade antioxidante permitiram diferenciar as diferentes cultivares através de análises de componente principais e análises discriminantes lineares.

Sensorialmente, as azeitonas mais apreciadas pelos consumidores foram as produzidas com as *Cvs.* Verdeal Transmontana e Negrinha de Freixo com uma apreciação global de 6,7 e 5,9 respectivamente (escala de 1 a 9). A *Cv.* Verdeal Transmontana mostra assim uma grande apetência para a produção de azeitonas verdes descaroçadas uma vez que, para além da preferência por parte dos consumidores, paralelamente ao elevado teor em ácidos gordos monoinsaturados (especialmente oleico) que as caracteriza na generalidade, apresenta também um elevado teor em fenóis e é, entre as cultivares estudadas, uma das que possui maior poder antioxidante. Contudo, é de realçar que a genuinidade e tipicidade deste produto tradicional estará provavelmente relacionada com a mistura de azeitonas de diferentes cultivares, contribuindo cada uma para as características únicas deste produto.

Palavras-chave: "*Alcaparras*"; efeito da cultivar; avaliação nutricional; composição química; actividade antioxidante; compostos fenólicos; compostos voláteis; avaliação sensorial.

Abstract

Green stoned table olives, "*Alcaparras*", are a kind of table olives produced by a traditional method and are highly appreciated in Trás-os-Montes region. In a general way, in their production the effect of the olive cultivar is not considered. In this sense, with the present work was intended to evaluate the influence of cultivar in the physic-chemical, sensory and biological characteristics of this kind of table olives. For such approach, at laboratory scale, different lots of "*alcaparras*" were prepared using the most representative olive cultivars from the region, namely, *Cv.* Cobrançosa, Madural, Negrinha de Freixo, Santulhana and Verdeal Transmontana. Their physic-chemical composition (moisture, total fat, total protein, ash, carbohydrates+fiber), energetic value, sensory evaluation and the quantification of some compounds, like fatty acids composition (GC/FID), tocopherols (HPLC/FD), volatile compounds (HS-SPME and GC/IT-MS), and phenolic compounds (HPLC/DAD) were determined. The antioxidant activity of the olives from different cultivars was determined as well through the methods of scavenging effect of the free radicals of DPPH and reducing power.

"*Alcaparras*" table olives are mainly constituted by water (> 70%) and fat (between 12.5 and 20.1%). The energetic value vary from 154 and 212 kcal per 100 grams, reporting *Cv.* Madural the lowest value and *Cv.* Verdeal Transmontana the highest one, being this value influenced mainly by fat amount. The fatty acids profile is mainly composed by monounsaturated fatty acids, being oleic acid the most abundant (≥ 66,9%). *Cv.* Negrinha de Freixo has higher amounts of tocopherols (6.0 mg/kg), being α-tocopherol the most abundant isomer. The volatile compounds profile of "*alcaparras*" table olives is mainly composed by aldehydes (> 74%) and in minor amounts by alcohols, esters, ketones, norisoprenoids derivates, terpenic compounds, sesquiterpenes and alkenes, in a total of 42 compounds identified. Twelve phenolic compounds were identified, being hydroxytyrosol the most abundant, and *Cv.* Cobrançosa reported higher amounts of phenolic compounds (165.76 mg/kg). Table olives produced from *Cv.* Cobrançosa and Santulhana showed higher antioxidant activity (EC_{50} of 1.38 and 1.40 mg/ml for reducing power, and 0.48 and 0.46 mg/ml for DPPH method).

The fatty acids profile, the composition in volatile and phenolic compounds, as well as the antioxidant activity allowed the differentiation of the several olive cultivars through principal component analysis and linear discriminant analysis.

In the sensory evaluation, the olives most appreciated by the consumers were produced from *Cvs.* Verdeal Transmontana and Negrinha de Freixo, with a global appreciation of 6.7 and 5.9 respectively (scale from 1 to 9). The *Cv.* Verdeal Transmontana showed higher aptitude for the production of stoned green table olives, besides being preferred by the consumers, this cultivar reported high content of monounsaturated fatty acids (especially oleic acid) which generally characterize them, also presents high content of phenolic compounds and among the olive cultivars studied is one that present higher antioxidant power. However, is noteworthy that the genuineness and typicality of this traditional product is probably related with the blend of olives from different cultivars, contributing each one with unique characteristics to the product.

Keywords: “*Alcaparras*”; cultivar effect, nutritional evaluation; chemical composition; antioxidant activity; phenolic compounds; volatile compounds; sensorial evaluation.

Capítulo 1

Introdução

1.1. Introdução

Em Portugal a olivicultura desempenha um papel fundamental não só a nível económico mas também a nível social. A oliveira encontra-se distribuída por todo o território nacional ocupando actualmente uma vasta área que ronda os 380 000 hectares (INE, 2010) sendo, a seguir à vinha, a cultura mais dispersa. O olival está presente em cerca de 40% das explorações e ocupa quase metade da superfície destinada a culturas permanentes. A representatividade do olival no total da Superfície Agrícola Utilizável (SAU) é elevada (6,5%) e apenas os prados e pastagens ocupam uma superfície superior (INE, 2006).

A região de Trás-os-Montes é a segunda mais importante a nível nacional. Em 2005 comportava uma área aproximada de 75 800 hectares (24% da área de olival) (INE, 2006). Actualmente a região norte de Portugal, é responsável pela produção de cerca de 29,1% do azeite nacional (INE, 2010), sendo a esmagadora maioria proveniente da região Transmontana.

No que respeita à azeitona de mesa, apenas 3% da área total de olival é utilizada para a produção de azeitonas com esse fim. Destes, cerca de 43% estão localizados no Norte do país e em Trás-os-Montes, de onde sairam cerca de 54% da produção de azeitona de mesa na campanha de 2008/2009 (INE, 2010).

De entre os países da União Europeia, Portugal é o quarto maior produtor de azeitonas de mesa, atrás da Espanha, da Grécia e da Itália (COI, 2009). Nos últimos 10 anos a produção média anual de azeitona de mesa no país rondou as 12 000 toneladas, com uma variação anual entre 8 000 toneladas (campanha de 2005/2006) e 19 200 toneladas (campanha de 2006/2007) (Figura 1). Os dados disponíveis do Conselho Oleícola Internacional (COI) indicam que Portugal é deficitário neste género alimentício, uma vez que com excepção de duas campanhas (2006/2007 e 2009/2010), nas restantes o consumo foi superior à produção tendo ocorrido importação de países terceiros (COI, 2009).

Salienta-se, assim, um mercado sustentado por alguma importação (em média 230 toneladas anuais, considerando o mesmo período), onde a produção interna equivale a cerca de 1,7% (cerca de 12 000 toneladas anuais) da produção total de azeitonas de mesa em toda a União Europeia entre as campanhas de 2003/2004 e 2008/2009. Este valor, apesar de reduzido, é em grande parte sustentado pela produção na região de Trás-os-montes, demonstrando a importância desta cultura na região, bem como a necesidade de aumentar a sua produção, a procura interna e, principalmente, a sua exportação.

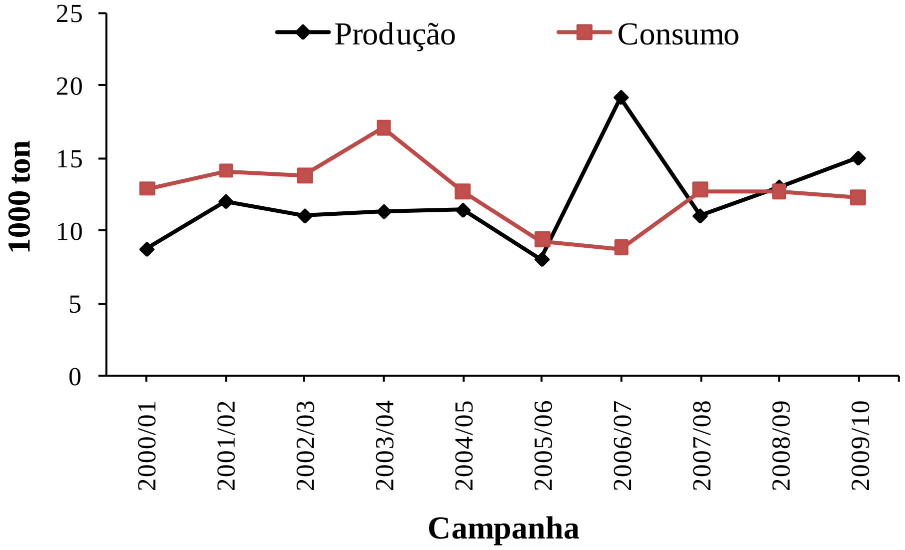

Figura 1. Evolução da produção e do consumo de azeitonas de mesa em Portugal nas últimas 10 campanhas. Adaptado a partir de dados do COI (2009).

Em Portugal, como no resto do mundo, a preparação de azeitonas de mesa segue três processos principais que serão descritos mais adiante. No entanto, a uma escala muito menor, ou regional, existem vários outros métodos de processamento, por vezes não incluídos nas estatísticas oficiais. No que respeita à região de Trás-os-Montes, a preparação de azeitonas verdes descaroçadas é um dos métodos mais utilizados, principalmente em azeitonas de início de estação.

1.2. Tipos de preparação de azeitonas de mesa

Contrariamente à maioria dos outros frutos, as azeitonas necessitam de sofrer uma série de alterações físico-quimicas para se tornarem edíveis pela remoção do amargor e do picante característico destes frutos. Qualquer processo tecnológico aplicado à azeitona para a produção de azeitona de mesa tem por principal objectivo levar à remoção desse amargor, cuja responsabilidade se deve maioritariamente à oleuropeína (Gómez *et al.*, 2006). Nos mercados internacionais existem maioritariamente três tipos de preparações comerciais: azeitonas de fermentação natural (estilo Grego), azeitonas verdes (estilo Espanhol ou Sevilhano) e as azeitonas pretas oxidadas (estilo Californiano ou Americano).

1.2.1. Fermentação natural

Para este tipo de preparação, os frutos normalmente são colhidos completamente maduros, mas não em demasia, uma vez que frutos colhidos no final da campanha, apesar de apresentarem uma excelente coloração a sua textura após processamento não é suficientemente firme (Goméz *et al.*, 2006). No entanto, de acordo com o grau de maturação dos frutos aquando da colheita e da região de produção, os frutos podem ter diversas tonalidades, desde avermelhada-escura, violeta, violeta-escura ou mesmo verde-escura e mesmo assim serem adequados para este tipo de processamento (Fernández *et al.*, 1997). Após transporte para as unidades industriais, as azeitonas são escolhidas e calibradas, sendo posteriormente lavadas para remover a sujidade superficial (Fernández *et al.*, 1997). Após lavagem, são colocadas em salmoura, com uma concentração de sal entre 8 e 10%, podendo utilizar-se concentrações inferiores (6%) em zonas mais frias (Gómez *et al.*, 2006). A partir desse momento dá-se início a uma fermentação natural, pela qual é responsável uma complexa microflora, composta essencialmente por leveduras e bactérias. A fermentação pode ser conduzida tanto em condições aeróbias como anaeróbias (Gómez *et al.*, 2006).Esta fermentação é demorada, essencialmente devido a dois factores: por um lado a lenta difusão de compostos fermentáveis através da pele da azeitona para o exterior, como por exemplo açucares, e por outro lado devido à presença de oleuropeína e outros compostos fenólicos que possuem actividade antimicrobiana (Sousa *et al.*, 2006). A fermentação pode ficar comprometida se não forem aplicados controlos físicos (arejamento, remoção do CO_2), químicos (controlo do pH e da concentração de NaCl) e microbiológicos (tipo e quantidade de microrganismos presentes no meio) (Fernández *et al.*, 1997; Gómez *et al.*, 2006). A remoção do amargor característico das azeitonas é conseguida apenas através da solubilização da oleuropeina na salmoura, sendo atingido um equilíbrio após 8-12 meses (Gómez *et al.*, 2006). Após fermentação, os frutos são oxidados por exposição ao ar de modo a melhorar a sua aparência e cor. Este passo não deve exceder as 48 horas de modo a não enrugar a superfície das azeitonas por desidratação (Gómez *et al.*, 2006). Depois de oxidadas, as azeitonas de mesa estão prontas para embalar e comercializar, sendo imersas na embalagem em nova salmoura que poderá provocar ou não uma nova fermentação (Fernández *et al.*, 1997). De modo a melhorar a conservação do produto final, pode ser aplicada uma pasteurização ou também poderão ser adicionados sorbato de potássio ou sorbato de sódio a 0,05%, expressos como ácido sórbico (Fernández *et al.*, 1997; Garcia *et al.*, 1986; Gómez *et al.*, 2006).

1.2.2. Estilo Espanhol ou Sevilhano

Neste tipo de preparação as azeitonas são colhidas verdes ou verde-amareladas. Após chegada à unidade fabril, são escolhidas e calibradas, sendo posteriormente mergulhadas numa solução com 2,0 a 5,0% de hidróxido de sódio (NaOH) com vista a remover quimicamente o amargor natural da azeitona. A concentração de NaOH adequada depende de vários factores: da temperatura, da cultivar e do grau de maturação dos frutos aquando do momento da colheita (Fernández *et al.*, 1997). Este tratamento prolonga-se até que a solução de NaOH penetre cerca de dois terços ou três quartos da distância entre a pele e o caroço. As azeitonas são posteriormente lavadas várias vezes com água, por períodos de tempo variáveis, para remover o excesso de NaOH presente (de Castro & Brenes, 2001). Após lavagem, as azeitonas são colocadas em salmouras com uma concentração de NaCl de aproximadamente 10%, onde se inicia uma fermentação láctica (Gómez *et al.*, 2006). A duração da fermentação depende essencialmente das características do tratamento alcalino prévio, da cultivar, da temperatura e da população microbiana existente no meio.

Nesta fermentação existem três fases distintas, nas quais a população microbiana varia. Numa primeira fase, há um crescimento de bactérias Gram-negativas não esporuladas (*Enterobacter cloacae, Citrobacter freundii, Klebsiella aerogenes, Flavobacterium diffusum, Aerochromobacter superficialis, Escherichia coli e Aeromonas* spp) que atingem um máximo após dois dias do início da fermentação, desaparecendo após 12-15 dias, sendo responsáveis pelas grandes quantidades de gás produzidas nos primeiros dias de fermentação. (Fernandes *et al.*, 1985). Na segunda fase, quando se atinge um pH de 6,0, há um crescimento rápido de leveduras e lactobacilus, havendo uma redução na população de bactérias Gram-negativas. A principal espécie de lactobacilos presente nesta fase é a *Lactobacillus plantarum*, no entanto também se identificam espécies dos géneros *Pediococcus* e *Leuconostoc*. A terceira e última fase dura até que todos os substractos fermentáveis se acabem, sendo o *Lactobacillus plantarum* a espécie dominante. Também se detecta a presença de leveduras nesta fase, que contribuem para o melhoramento das características organolepticas do produto final, sendo as seguintes espécies as mais representativas: *Hansenula anomala, Candida krusei* e *Saccharomyces chevalieri.*

Uma vez concluída a fermentação é efectuada uma calibração para posterior embalamento onde as azeitonas podem ser acondicionadas na salmoura onde fermentaram, numa nova salmoura, ou numa mistura de ambas. De modo a estabilizar e preservar o produto final, a embalagem é submetida a 15 unidades de pasteurização (15 minutos a 62,4ºC) (Sánchez *et al.*, 1989), de modo a eliminar a bactéria com maior resistência térmica capaz de crescer no meio do produto embalado, *Propionibacterium* (González *et al.*, 1982).

Este tipo de azeitonas tem diversas apresentações comerciais, desde inteiras, descaroçadas e recheadas com variados ingredientes.

1.2.3. Estilo Californiano ou Americano

Para este tipo de processamento o momento óptimo de colheita é muito vago, podendo-se incluir todos os frutos colhidos após a colheita das azeitonas destinadas ao processamento sevilhano e antes da colheita dos frutos destinados a processamento por fermentação natural (Fernández *et al.*, 1997), e desde que possuam uma polpa rija.

Para produzir este tipo de azeitonas pretas oxidadas, os frutos podem ser sujeitos directamente a processos de oxidação sem qualquer tipo de preservação. As azeitonas são sujeitas a tratamentos com soluções de NaOH (1 a 2%) que podem variar entre 2 e 5 tratamentos. A concentração das soluções de NaOH pode variar de acordo com a maturação dos frutos, a cultivar e a temperatura do tratamento e da penetração e velocidade desejada (Fernández *et al.*, 1997; Gómez *et al.*, 2006). A penetração da soda na azeitona é controlada de modo a que no primeiro tratamento o passe através simplesmente da pele do fruto. Nos tratamentos posteriores a penetração na polpa vai aumentando, até que se atinja o caroço no último tratamento (Fernández *et al.*, 1985). Entre cada tratamento, as azeitonas são suspensas em água intensamente arejada por ar injectado através de uma rede de tubos, de modo a oxidar uniformemente as azeitonas. Através de sucessivas suspensões em água com ar injectado a pele e polpa das azeitonas escurecem progressivamente devido à oxidação de *orto*-difenóis como o hidroxitirosol e o ácido cafeico (Brenes *et al.*, 1992; Garcia *et al.*, 1992). Após o último tratamento, as azeitonas sofrem sucessivas lavagens para remover o excesso de NaOH e baixar o pH da polpa para valores próximos de 8 (Fernández *et al.*, 1985).

A coloração negra obtida nas azeitonas é instável e pode perder-se ao longo da vida de prateleira do produto acabado. Para evitar a descoloração apenas é permitido o uso de gluconato ferroso e de lactato ferroso (García *et al.*, 1986). Os sais ferrosos são adicionados à última água de lavagem numa concentração de 100 ppm em ião ferro. A difusão do ferro na polpa estará completa após 10 horas de contacto, mas a etapa é prolongada e concluída após 24 horas de contacto (Garcia *et al.*, 2001). A partir deste ponto as azeitonas são calibradas e embaladas em diferentes contentores e banhadas em salmouras com cerca de 2 a 4% de NaCl e entre 10 a 40 ppm de ferro de forma a prevenir a deterioração da cor (Garrido *et al.*, 1995). Também podem ser adicionados sais de cálcio, de forma a melhorar a firmeza das azeitonas (García *et al.*, 1994; Romero *et al.*, 1995). Uma vez que o produto final apresenta uma acidez baixa, a preservação deste tipo de azeitonas de mesa pode passar pela adição de ácidos, como ácido láctico ou ácido glucónico, aplicando-se também pasteurizações (Gómez *et al.*, 2006).

1.2.4. Outros tipos de preparações

Além dos três principais tipos de preparações disponíveis no mercado, existem outros métodos utilizados na produção de azeitonas de mesa. A grande maioria destes processos alternativos podem ser considerados de importância regional ou local, como processos de fabrico artesanal, doméstico e tradicional.

Na Grécia existe um processo muito peculiar em que a produção não chega sequer para as necessidades locais, tal é a procura do produto. As azeitonas são produzidas a partir de uma variedade particular, *Cv.* Thrubolea, que cresce em algumas ilhas da Grécia. Estas azeitonas diferem das restantes, uma vez que em condições climáticas muito especificas da região, e sob acção de um fungo, *Phoma oleae*, as azeitonas perdem o amargor ainda na oliveira, sem ser necessário recorrer a fermentações. (Fernández *et al.*, 1997). Após colheita os frutos são desidratados ao sol e é-lhes adicionado sal para melhorar as características organolépticas e de conservação (Fernández *et al*., 1997).

Na região de Trás-os-Montes existe um tipo de azeitonas de mesa tradicional, conhecido como "*alcaparras*", que difere substancialmente na maneira como são fabricadas em relação aos três tipos já descritos.

Para este tipo de preparação, as azeitonas são colhidas ainda verdes, ou verde-amareladas, durante no início do Outono. Após colheita, as azeitonas são lavadas para remover a sujidade superficial e são quebradas de modo a retirar o caroço. A polpa é cortada em duas metades aproximadamente iguais, perpendicularmente ao maior eixo do fruto. A polpa é posteriormente colocada em água, sendo mudada várias vezes com o objectivo de remover o amargor (Sousa *et al*., 2006). De uma maneira geral as azeitonas ficam edíveis ao fim de uma semana. Após serem consideradas "doces", são escoadas para remover o excesso de água, sendo que, para fins comerciais, são mantidas em água salgada de modo a preservar o produto. Para consumo doméstico as "*alcaparras*" são temperadas a gosto com vários ingredientes, desde alho, sal, vinagre, azeite, ervas aromáticas, laranja, louro, entre outros.

Qualquer que seja o processamento tecnológico aplicado à azeitona, de modo a torná-la edível, existem modificações físico-quimicas que alteram a sua composição relativamente à matéria-prima. Os três principais tipos tecnológicos aplicados à azeitona influenciam a composição final da azeitona de mesa, principalmente a sua composição em compostos fenólicos.

1.3. Influência do processo tecnológico na composição e actividade antioxidante de azeitonas de mesa

1.3.1. Composição nutricional

De maneira geral, o processamento aplicado ao fruto para o tornar edível (azeitona de mesa), bem como a salmoura posterior, fazem aumentar os teores em humidade, cinzas e NaCl (Ünal & Nergiz, 2003). O aumento da quantidade de água está relacionado com as lavagens sucessivas dos frutos e imersões tanto em soluções alcalinas como salinas de modo a permitir uma correcta eliminação do amargor e fermentação dos frutos, respectivamente. Com a penetração do NaCl (presente nas águas de salmoura) na polpa dos frutos dá-se também um aumento significativo do teor em cinzas.

Na razão inversa, os açúcares redutores e totais desaparecem por completo até ao cessar das fermentações ou logo nos primeiros meses de armazenamento (Ünal & Nergiz, 2003). Isto deve-se ao facto de durante a fermentação haver uma difusão dos açúcares (compostos fermentáveis) através da película do fruto para o meio (Gómez *et al.*, 2006). Uma vez na salmoura, os açúcares serão utilizados pela flora existente como fonte de energia para o seu normal desenvolvimento e consequente fermentação (Kailis & Harris, 2007).

Também se verifica uma ligeira redução no teor de proteínas em alguns tratamentos, de fibras e do valor calórico (Ünal & Nergiz, 2003). Como os açúcares presentes nas azeitonas são quase completamente extraídos como fonte de energia para as leveduras e bactérias, aliado ao aumento percentual do teor em água, a densidade energética do produto processado diminui ligeiramente em relação à matéria-prima.

1.3.2. Composição em ácidos gordos

A fracção lipídica das azeitonas é naturalmente rica em triglicerídeos ricos em ácidos gordos monoinsaturados. Os ácidos gordos mais abundantes em azeitonas são o ácido oleico, claramente maioritário, sendo seguido dos ácido palmítico, linoleico e linolénico. O tipo de processamento aplicado para tornar as azeitonas edíveis não influencia significativamente o teor lipídico, pela sua natural insolubilidade na água de tratamento, bem como o perfil de ácidos gordos, pela adequada resistência à oxidação dos ácidos gordos monoinsaturados. Ünal e Nergiz (2003) observaram apenas ligeiras oscilações na quantidade dos ácidos gordos maioritários em azeitonas não processadas e processadas. No estilo Espanhol é de salientar a diminuição do teor de ácido palmítico e oleico e o aumento do ácido linoleico e do ácido esteárico. No entanto, no estilo Grego, o ácido esteárico diminui enquanto que o ácido linolénico aumenta ligeiramente o seu teor (Ünal & Nergiz 2003).

Contrariamente ao referido anteriormente, Sakouhi *et al*. (2008) reportaram diferenças significativas em relação a todos os ácidos gordos referidos anteriormente, diminuindo os seus teores após processamento. O rácio entre ácidos gordos poliinsaturados e ácidos gordos saturados aumenta após conclusão do processo produtivo, em consonância com Ünal e Nergiz (2003). A solidificação de alguns triglicéridos ricos em ácidos gordos mais saturados, de menor ponto de fusão, poderá estar na base destas perdas ligeiras, contribuindo para um aumento percentual da fracção polinsaturada por 100g de gordura.

1.3.3. Composição em tocoferóis

Os tocoferóis são componentes muito importantes das azeitonas de mesa, uma vez que possuem capacidades antioxidantes para a fracção lipídica, bem como propriedades nutricionais pela sua função vitamínica. Durante a preparação de azeitonas verdes de cultivares tunisinas (do tipo Espanhol), Sakouhi *et al.* (2008)mostraram que o teor em tocoferóis, nomeadamente α-tocoferol (isómero de tocoferol mais abundante em azeitonas) diminui com o processamento. Esta diminuição foi influenciada pela maturação do fruto e pelo factor cultivar, tendo sido registadas maiores diminuições em azeitonas pretas do que em azeitonas verdes da mesma cultivar (Sakouhi *et al.*, 2008). No entanto, Montaño *et al.* (2005), estudando várias etapas na produção de azeitonas do tipo Espanhol não verificaram efeitos significativos no teor de tocoferóis tanto no tratamento alcalino como na pasteurização. Já após 12 meses de armazenamento à temperatura ambiente as azeitonas apresentavam uma redução no teor de tocoferóis (Montaño *et al.*, 2005).

1.3.4. Composição em compostos fenólicos

De uma maneira geral, o processamento tecnológico leva à perda parcial dos compostos fenólicos, maioritariamente por hidrólise alcalina. As lavagens com água também provocam uma lixiviação dos compostos. Deste modo, a concentração e o tipo de compostos fenólicos presentes nas azeitonas tratadas e fermentadas difere substancialmente daqueles presentes em frutos crus.

Ben Othman *et al.* (2009) verificaram que, tanto por fermentação espontânea como por fermentação controlada de azeitonas Chétoui com diferentes estados de maturação, ocorreu perda de diversos compostos fenólicos. A oleuropeína foi o composto fenólico mais abundante nas azeitonas verdes, enquanto que o hidroxitirosol foi mais abundante em azeitonas mais maduras, com colorações diversas ou mesmo pretas, antes do processamento. Após processamento por fermentação natural, verificou-se que a fermentação controlada removeu maior quantidade de compostos fenólicos que a fermentação espontânea, tendo removido quantidades significativas de hidroxitirosol, oleuropeína e tirosol em todos os tipos de azeitonas (Ben Othman *et al.*, 2009). Na polpa dos frutos a redução de compostos fenólicos foi mais notória para os ácidos ferúlico e protocatecuico e para a oleuropeína. Simultaneamnte verificaram um aumento nos teores de hidroxitirosol e ácido cafeico, ambos os compostos formados, respectivamente, pela hidrólise da oleuropeína e pela degradação do verbascosídeo (Brenes *et al.*, 1992; Parinos *et al.*, 2007).

No mesmo tipo de processamento, Romero *et al.* (2004b) verificaram que, antes do início do processo, os fenóis mais representativos eram o hidroxitirosol-4-*β*-glucosido, a oleuropeína, o hidroxitirosol, o tirosol, o salidrosido e o verbascosídeo. No entanto passados 12 meses, o principal composto fenólico presente era o hidroxitirosol.

Romero *et al.* (2004a) demonstraram que as azeitonas processadas por fermentação natural (estilo Grego) apresentam maior conteúdo em compostos fenólicos do que as azeitonas pretas oxidadas (estilo Californiano). Ficou demonstrado que a cultivar e o tipo de apresentação das azeitonas condicionam o teor em compostos fenólicos (Romero *et al.*, 2004a).

No caso da produção de azeitonas verdes (estilo Espanhol) a oleuropeína é o composto fenólico maioritário antes do início do processamento (Brenes *et al.*, 1995), diminuindo o seu teor pela hidrólise das suas formas glucosiladas com hidróxido de sódio, dando origem à formação de hidroxitirosol durante o tratamento. A presença de tirosol também foi notada, provavelmente devido à hidrólise do ligstrosìdeo. O tratamento alcalino também provocou a diminuição nas quantidades de rutina e luteolina 7-glucosido e um aumento de ácido cafeico nas cultivares estudadas devido à hidrólise do verbascosideo (Brenes *et al.*, 1995). Uma vez mais o hidroxitirosol foi o composto fenólico em maior abundância no produto final.

No caso da produção de azeitonas pretas oxidadas, os principais compostos fenólicos presentes antes do início do processo eram a oleuropeína, o hidroxitirosol e aglíconas de oleuropeína. Após 4 meses em salmoura o teor de oleuropeína desceu drasticamente devido metabolismo bacteriano do meio fermentativo. Paralelamente foi observado um aumento nos derivados de oleuropeína e de hidroxitirosol (Marsilio *et al.*, 2001). O teor em tirosol também aumentou rapidamente durante o processo fermentativo e o de verbascosideo diminui. Neste processo a etapa de lavagem para remover o excesso de hidróxido de sódio pareceu ser a mais prejudicial, removendo grandes quantidades de todos os compostos fenólicos presentes (Marsilio *et al.*, 2001). Assim, verifica-se uma acentuada redução no teor de oleuropeína em todos os tipos de processamento. Isto deve-se maoritariamente à difusão dos compostos fenólicos para a salmoura, mas também à hidrólise das formas glucosiladas da oleuropeína pela presença de soda e/ou pela enzima β-glucosidase, produzida pelo *Lactobacillus plantarum* (Ciafardini *et al.*, 1994; Landete *et al.*, 2008), despolimerizando compostos fenólicos com elevado peso molecular em compostos fenólicos simples com baixo peso molecular (Ayed & Hamdi, 2003). Com isto há um aumento nos teores de hidroxitirosol através da hidrólise da oleuropeína mas também da hidrólise do hidroxitirosol-4-*β*-glucosido (Romero *et al.*, 2004b).

1.3.5. Composição em compostos voláteis

A formação de compostos voláteis advém de uma série de complexos mecanismos químicos que, no caso das azeitonas de mesa, envolvem microrganismos presentes no meio e responsáveis pela condução de processos fermentativos. Estes compostos voláteis podem afectar as propriedades organolépticas das azeitonas de mesa, especialmente o sabor e o aroma (Panagou & Tassou, 2006).

Na produção de azeitonas verdes pelo estilo Espanhol, os compostos voláteis mais abundantes são o etanol, metanol, 4-metil-1-pentanol, 1-pentanol, 2-pentanol, acetaldeído, acetato de etilo, acetato de isobutilo, acetato de hexilo, ácido isobutírico, ácido isovalérico e o ácido propiónico (Panagou & Tassou, 2006). Também se verificou que as suas concentrações variaram de acordo com a estirpe de levedura usada na fermentação, demonstrando que a formação e quantidade de compostos voláteis formados dependem em grande parte da constituição da microflora do meio (Panagou & Tassou, 2006). Os álcoois simples (etanol e metanol) foram também os principais compostos voláteis identificados por Montaño *et al.* (1990), além do acetaldeído, 2-butanol, n-propanol, acetona e acetato de etilo.

Através do estudo de azeitonas verdes já fermentadas pelo método Espanhol, Iraqi *et al*. (2005) demonstraram que a família dos aldeídos ((*Z*)-3-hexenal, metional e (*E*,*E*)-2,4-decadienal, (*E*,*Z*)-2,4-decadienal e (*E*)-2-decenal) foi a mais identificada e importante no perfil volátil deste tipo de azeitonas.

No caso da preparação de azeitonas de fermentação natural (estilo Grego), os principais compostos voláteis formados durante o processo fermentativo foram o etanol, metanol, acetaldeído e o acetato de etilo (Panagou *et al*., 2008; Fernández *et al*., 1985), em parte semelhante aos compostos voláteis identificados em azeitonas processadas pelo estilo Espanhol (Panagou & Tassou, 2006; Montaño *et al*., 1990).

1.3.6. Actividade antioxidante

As azeitonas de mesa apresentam na sua composição importantes micronutrientes, como é o caso dos tocoferóis e dos compostos fenólicos, que lhes conferem propriedades antioxidantes (Ben Othman *et al*., 2009; Sousa *et al*., 2006), O potencial antioxidante de azeitonas de mesa pode, no entanto, ser influenciado pelos diferentes processos tecnológicos aplicados às azeitonas. Um dos factores mais importantes é a perda de compostos fenólicos descrita anteriormente (tópico *1.3.4*).

Através do estudo da composição em compostos fenólicos e da actividade antioxidante em azeitonas de mesa provenientes de cultivares Portuguesas, e processadas através de diferentes estilos, Pereira *et al*. (2006) concluíram que o processamento influi no potencial antioxidante das azeitonas. As azeitonas de mesa com maior conteúdo em compostos fenólicos (processadas segundo fermentação natural), também apresentaram uma maior capacidade antioxidante em todos os métodos avaliados. As azeitonas pretas oxidadas (estilo Californiano) foram as que apresentaram menor teor em compostos fenólicos e respectivamente, menor capacidade antioxidante (Pereira *et al*., 2006). As azeitonas processadas segundo o estilo sevilhano apresentaram valores intermédios de compostos fenólicos e actividade antioxidante, comparativamente com os dois outros estilos.

Ben Othman *et al*. (2009) também verificaram que ao longo de fermentações espontâneas e fermentações controladas (fermentação natural) de azeitonas com várias tonalidades ocorre redução de compostos fenólicos e do potencial antioxidante.

Em súmula, parece claro que o tipo de processamento a aplicar às azeitonas tem influência directa nas características física, químicas e sensoriais do produto final. Para além disso, as características intrínsecas às azeitonas, nomedamente a cultivar e o estado de maturação, condicionam também o produto final.

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Capítulo 2

Justificação e objectivos

Justificação e objectivos

Em Trás-os-Montes, é produzido, de forma artesanal, um tipo de azeitonas verdes descaroçadas conhecidas localmente como "*alcaparras*". Contrariamente aos métodos comerciais disponíveis (azeitonas pretas oxidadas, azeitonas verdes e azeitonas de fermentação natural) estas azeitonas não sofrem qualquer processo fermentativo, sendo apenas sujeitas a tratamentos aquosos com vista à remoção do amargor natural da azeitona.

Inicialmente a produção de "*alcaparras*" era vista como uma forma de evitar desperdícios ao nível dos produtos do olival. Eram utilizados os frutos caídos da oliveira que já tinham um calibre e polpa que justificassem a sua produção. A maioria dos frutos caía devido à acção de pragas, nomeadamente a geração carpófaga da traça-da-oliveira que, ao sair do fruto, provocava a sua queda.

Produzidas a nível doméstico e para consumo próprio, a sua produção ocorria até meados do final do mês de Setembro. Com o passar do tempo as "*alcaparras*" adquiriram um estatuto económico importante para a subsistência de muitos produtores que aproveitaram para a tornar comercialmente rentável. Hoje em dia, devido à sua importância comercial e económica, para a produção deste produto artesanal já são recolhidos frutos sãos das árvores e o seu período de produção estende-se para além do final de Setembro.

No entanto, para a produção de "*alcaparras*", os produtores não têm em consideração a cultivar de azeitona, utilizando uma mistura das cultivares que dispõem no olival, sem saber quais as proporções e influência de cada cultivar no produto final. Da mesma forma, os estudos realizados até hoje em "*alcaparras*" foram feitos em amostras comerciais, nas quais não é tido em conta o possível efeito da cultivar de azeitona. Como tal, o objectivo principal deste trabalho, foi observar o efeito da cultivar na caracterização de "*alcaparras*", tendo sido estas produzidas a partir de 5 das principais cultivares de azeitona da região de Trás-os-Montes (*Cv.* Cobrançosa, Madural, Negrinha de Freixo, Santulhana e Verdeal Transmontana).

Os objectivos específicos deste trabalho foram:

i) Proceder à caracterização nutricional, determinando os teores em humidade, gordura bruta, proteína bruta, cinzas e hidratos de carbono+fibras de cada uma das cultivares, bem como proceder ao cálculo dos seus respectivos valores energéticos (Capítulo 3);

ii) Caracterização da fracção lipídica através da determinação do perfil em ácidos gordos por GC/FID e dos tocoferóis por HPLC/FD (Capítulo 3);

iii) Caracterização da componente sensorial das várias cultivares, através da avaliação de descritores recorrendo a um painel de consumidores não treinado (Capítulo 3);

iv) Determinação do perfil em compostos voláteis por HS-SPME e GC/IT-MS e sua relação com a avaliação sensorial (Capítulo 4);

v) Avaliação do potencial antioxidante de extractos aquosos das várias cultivares de “*alcaparras*” através dos métodos do poder redutor e do efeito bloqueador dos radicais livres de DPPH (Capítulo 5);

vi) Quantificação de compostos fenólicos individuais, por HPLC/DAD, em extractos aquosos das várias cultivares de “*alcaparras*” e sua relação com a actividade antioxidante registada (Capítulo 5).

Capítulo 3

Effect of cultivar on sensory characteristics, chemical composition and nutritional value of stoned green table olives

Effect of cultivar on sensory characteristics, chemical composition and nutritional value of stoned green table olives

Abstract

The effect of olive cultivar on sensory characteristics, chemical composition and nutritional value of traditional stoned green table olives "*alcaparras*" was studied. The most representative cultivars from Trás-os-Montes region, Portugal, (*Cv.* Cobrançosa, Madural, Negrinha de Freixo, Santulhana and Verdeal Transmontana) were studied. The results showed that, regardless the cultivar, water was the main constituent with values greater than 70%, followed by fat that varied between 12.5 and 20.1%. Carbohydrates amount was greater in *Cv.* Madural (9.2%) and those produced from *Cv.* Cobrançosa had higher level of nitrogenous compounds, with 1.4%. Ashes contents of table olives varied from 1.6 to 1.9%, without significant differences among cultivars. Moreover, one hundred grams of "*alcaparras*" provided an energetic value between 154 and 212 kcal, for *Cv.* Madural and Verdeal Transmontana respectively. Oleic acid was the main fatty acid detected (higher than 66.9%), followed by palmitic acid (10.8-13.3%) and linoleic acid (2.7-10.3%). A Linear Discriminant model was established based on the "*alcaparras*" table olives fatty acids profile. Three fatty acids ($C_{16:0}$; $C_{18:0}$ and $C_{18:3}$) and total SFA, MUFA and PUFA contents allowed distinguishing between the five olive cultivars studied, with overall sensitivity and specificity of 100%. The total content of vitamin E of the table olives varied from 3.5 and 6.0 mg/kg (for *Cv.* Santulhana and Negrinha de Freixo, respectively), being α-tocopherol the most abundant. The consumer's panel showed higher preference for the table olives of *Cv.* Verdeal Transmontana and Negrinha de Freixo, while *Cv.* Madural was negatively characterized in all the descriptors evaluated.

Keywords: *Olea europaea* L.; stoned table olives; olive cultivar; nutritional value, fatty acids, tocopherols.

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3.1. Introduction

Olive tree (*Olea europaea* L.) is one of the most important fruit trees in the Mediterranean Basin and is widespread through the entire region. Table olives world production is greatly agglomerated in this same region, being nearly half produced in the European Union countries, mainly in Spain, Greece, Italy and Portugal (IOOC, 2009). Well known sources of healthy compounds, table olives and olive oil are important components of the Mediterranean diet, being olive oil its main source of external fat (Schröder, 2007).

Table olives are the most popular agro-fermented food product and are consumed and enjoyed throughout the entire world. Consumers perception of quality is improving and nowadays an increased seek for healthier products can be observed worldwide. Mainly composed by monounsaturated fatty acids, table olives consumption can prevent and reduce the risk of cardiovascular diseases (Kastorini *et al.*, 2010). In addition, others minor constituents like tocopherols and phenolic compounds are responsible for antioxidant and antimicrobial properties (Sousa *et al.*, 2006), protecting the organism from diseases in which free radicals and pathogenic microorganisms are involved, preventing also the body from certain kinds of cancer (Owen *et al.*, 2004) and arthrosclerosis (Armstrong *et al.*, 1997).

To achieve an edible grade, table olives are mainly processed by three methods: Spanish-style green olives in brine, Greek-style naturally black olives in brine and Californian black ripe olives (Sabatini *et al.*, 2009). Other regional methods applied in the production of table olives are of smaller representativeness. In Trás-os-Montes, the Northeastern region of Portugal, it is produced a regional sort of green stoned table olives known as "*alcaparras*". These kind of green table olives differ from the main three kinds of preparations by the technological process. While the Spanish, Greek and Californian styles need to be subjected to lye treatments and/or fermentations in brine, "*alcaparras*" table olives are only subjected to aqueous treatments. The differences observed in the processes influence the chemical composition of the table olives by increasing the water content and salt levels due to NaCl penetration in the fruit (Gómez *et al.*, 2006), reduction of carbohydrates in the fruit due to consumption by the microorganisms in order to obtain energy (Kailis & Harris, 2007), and the loss of minor compounds like phenolic compounds (Brenes *et al.*, 1995; Marsilio *et al.*, 2001;

Romero *et al*., 2004). Table olives "*alcaparras*" are being studied by our research group in the last few years. Previous results obtained revealed that this kind of olives contains appreciable amounts of total phenolics, 5.58 - 29.88 mg GAE/g (Sousa *et al*., 2008), being the three flavonoidic compounds luteolin 7-O-glucoside, apigenin 7-O-glucoside, and luteolin identified in aqueous extracts (Sousa *et al*., 2006). "*Alcaparras*" aqueous extracts revealed inhibition of several microorganisms that may be causal agents of human intestinal and respiratory tract infections (Sousa *et al*., 2006) and appreciable antioxidant capacity against free radicals (Sousa *et al*., 2008). These works were carried out with commercial "*alcaparras*" which are a blend of several cultivars of the Trás-os-Montes region, since producers do not take in consideration the possible cultivar effect.

In this work "*alcaparras*" were produced in laboratory, following the same traditional method used by local producers, safeguarding the independence of five of the most representative olive cultivars of the region. To the best of the author's knowledge, this is the first time that the effect of cultivar in "*alcaparras*" table olives chemical composition, fatty acids and tocopherols profiles as well as in the sensorial characterization is studied.

3.2. Material and methods

3.2.1. Stoned table olives "Alcaparras" sampling and preparation

In this study, five of the most representative olive cultivars from Trás-os-Montes region were collected during September and October of 2006 from different olives groves subjected to similar agro-climatic conditions and agronomic practices. From each cultivar, five independent lots of olives, approximately of 5 kg each, were collected and immediately transported to the laboratory. At the laboratory, from each lot, approximately 2 kg of stoned table olives were prepared. For this, green or yellow-green healthy olive fruits were used, which were broken to separate the pulp from the stone. The pulp was placed into water during a week, daily changed, to remove olives bitterness. After the treatment, "*alcaparras*" table olives were frozen at -20° C until analysis, except for the sensorial analyses that took place in the first fifteen days after processing, being the table olives stored in the dark in 1.5 L volume glass containers and emerged in water. Each cultivar was processed in quintuplicate.

3.2.2. Sensorial evaluation

The sensorial evaluation was performed in individual cabins illuminated with a set of fluorescent lamps. Samples were codified with a three-digit combination and evaluated by a consumer's panel of 33 untrained volunteers. "*Alcaparras*" from each olive cultivar were evaluated using a preference test based on a nine-point hedonic scale (9 = like extremely and 1 = dislike extremely). Aroma, flavor, consistency and global appreciation were evaluated.

3.2.3. Chemical Analysis

3.2.3.1. Pulp Analysis

Moisture, total fat, ash and protein contents were analyzed in triplicate, at least. Moisture analysis was determined using approximately 5 g per test sample at 100 ± 2º C following AOAC 925.40 method (1995). Total fat content was determined in a Soxhlet apparatus according to AOAC 948.22 method, using petroleum ether as solvent with a minimum extraction time of 24 h (AOAC, 2000). The extracted fat was frozen at -20º C, for the fatty acids profile determination. Crude protein content was estimated by the Kjeldahl method (AOAC, 2000) and ash content was determined by incineration at 550 ± 15 ºC until constant weight was obtained (AOAC, 2000). Carbohydrate and fiber content was estimated by difference of the other components using the following formula: carbohydrate+fiber content = 100% - (% moisture + % protein + % fat + % ash). Energy was expressed as kilocalories, using the Atwater classical factors. Energy (kcal) = 4 x (g protein + g carbohydrate) + 9 x (g lipid).

3.2.4. Oil Analysis

3.2.4.1. Fatty acid composition

For fatty acid composition the oil extracted from total fat determination was used. Fatty acids were evaluated as their methyl esters after alkaline transesterification with methanolic potassium hydroxide solution (ISO, 2000) and extraction with n-heptane. The fatty acid profile was determined with a Chrompack CP 9001 Gas 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 capillary column coated with a 0.19 μ film of CP-Sil 88 (Chrompack). Helium was used as carrier gas at an internal pressure of 120 kPa. The temperatures of the detector and injector were 250 ºC and 230 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 (ISO, 1990) 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).

3.2.4.2. Tocopherol composition

Tocopherols were evaluated following the international standard ISO 9936 (2006), with some modifications as implemented by Amaral *et al*. (2005). Tocopherols and tocotrienols standards (α, β, γ and δ) were purchase from Calbiochem (La Jolla, San Diego, CA) and 2-Methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol (tocol) was from Matreya Inc. (Pleasant Gap, PA). A 50 mg amount of extracted fat was blended with an appropriate amount of internal standard (tocol) in a 1.5 mL of n-hexane and homogenized by stirring. Sample preparation was conducted in dark and tubes containing the samples were always wrapped in aluminum foil. The mixture was centrifuged for 5 minutes at 13000 g and the supernatant analyzed by HPLC. The liquid chromatograph consisted of a Jasco integrated system (Jasco Global, Japan) equipped with an AS-950 automated injector, a PU-980 pump, an MD-910 multiwavelength diode array detector and an FP-920 fluorescence detector (λ_{exc}= 290 nm and λ_{em}= 330 nm), connected in series. The chromatographic separation was achieved on a Supelcosil ™ LC-SI column (3 µm) 75 x 3.0 mm (Supelco, Bellefonte, PA), operating at constant room temperature (21 ºC). A mixture of n-hexane and 1,4-dioxane (98:2) was used as eluent, at a flow rate of 0.7 mL/min. Data were analyzed with the Borwin PDA Controller Software (JMBS, France). Tocopherols (α, β, γ, and δ) 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.

3.2.5. Statistical Analysis

3.2.5.1. Principal component analysis

Principal components analysis (PCA) was performed using the SPSS software, version 17.0 (SPSS, Inc.). It was applied as an unsupervised technique for reducing the number of variables (21 variables corresponding to 15 individual fatty acids and their different fractions – SFA, MUFA, PUFA and trans fatty acids) to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information, i.e., the five olive cultivars, Cobrançosa, Madural, Negrinha de Freixo, Santulhana and Verdeal Transmontana. Moreover, it allowed recognizing patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores).

The aim of the PCA is to produce components suitable to be used as predictors or response variables in subsequent analysis. The number of factors to keep in data treatment was evaluated by the Scree plot, taking into account the eigenvalues and the internal consistency by means of αCronbach’s value (Maroco, 2003; Rencher, 1995).

3.2.5.2. Linear discriminant analysis

A linear discriminant analysis (LDA) was performed using the SPSS software, version 17.0 (SPSS, Inc.). It was used as a supervised learning technique to classify the five olive cultivars according to their fatty acids profile. A stepwise technique, using the Wilk’s lambda method with the usual probabilities of F (3.84 to enter and 2.71 to remove), was applied for variable selection. (Maroco, 2003; Rencher, 1995; López *et al.*, 2008). To verify which canonical discriminant functions were significant, the Wilks' Lambda test was applied. To avoid overoptimistic data modulation, a leaving-one-out cross-validation procedure was carried out to assess the model performance. Moreover, the sensitivity and specificity of the discriminant model were computed from the number of individuals correctly predicted as belonging to an assigned group (Rencher, 1995; López *et al*., 2008).

3.2.5.3. Analysis of variance

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 17.0 (SPSS, Inc.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov-Smirnov with Lilliefors correction (if n>50) or the Shapiro-Wilk`s test (if n<50), and the Levene´s tests, respectively. All dependent variables were analyzed using a one-way ANOVA with or without Welch correction, depending if the requirement of the homogeneity of variances was fulfilled or not. The main factor studied was the effect of olive cultivar on the fatty acids profile, tocopherols content and sensorial evaluation. If a statistical significant effect was found, means were compared using Tukey´s honestly significant difference multiple comparison test or Dunnett T3 test also depending if equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

3.3. Results and discussions

3.3.1. Pulp analysis

In order to chemically characterize the pulp of the different cultivars of "*alcaparras*" table olives moisture, total fat, ash, crude protein, carbohydrates and the energy content were determined. The results obtained from such proximate chemical composition (grams per 100 g of fresh weight) are reported in Table 1.

Table 1. Proximate chemical composition (grams per 100g of fresh weight) of "*alcaparras*" samples from different cultivars.

Olive cultivar	Moisture	Crude Protein	Total fat	Ash	Carbohydrates	Energy (kcal)
Cobrançosa	74.2 ± 0.6 b	1.4 ± 0.0 d	16.5 ± 1.5 b	1.6 ± 0.0 a	6.3 ± 1.9	180 ± 7 b
Madural	75.2 ± 1.6 b	1.2 ± 0.0 c	12.5 ± 0.5 a	1.9 ± 0.0 b	9.2 ± 2.9	154 ± 8 a
Negrinha de Freixo	75.7 ± 3.7 b	0.9 ± 0.0 b	13.0 ± 1.0 a	1.7 ± 0.1 a	8.7 ± 2.7	155 ± 19 a,b
Santulhana	72.3 ± 1.7 a,b	0.8 ± 0.0 b	16.1± 1.1 b	1.7 ± 0.1 a	9.1 ± 1.9	184 ± 9 b
Verdeal Transmontana	70.1± 1.7 a	0.6 ± 0.0 a	20.1± 1.0 c	1.9 ± 0.1 b	7.3 ± 2.0	212 ± 9 c
***P* - value**	0.002(1)	< 0.001(1)	< 0.001(1)	< 0.001(1)	0.032 (2)	< 0.001(1)

a-eMeans within a line with different superscripts differ, $P < 0.05$.

(1)*P*-values are those for the effect of cultivar on the fatty acids profile of "*alcaparras*" table olives, from one-way ANOVA analysis. If there was a significant effect of cultivar on the fatty acids data, the means were compared by Tukey´s test, since equal variances could be assumed ($P > 0.05$ by means of Levene test).

(2)*P*-values are those for the effect of cultivar on the fatty acids profile of "*alcaparras*" table olives from one-way Welch ANOVA analysis. If there was a significant effect of cultivar on the fatty acids data, the means were compared by Dunnett T3´s test, since equal variances could not be assumed ($P < 0.05$ by means of Levene test).

Water was the major component in all "*alcaparras*" regardless the olive cultivar, with values higher than 70%. *Cv.* Negrinha de Freixo contained higher moisture while *Cv.* Verdeal Transmontana contained lower water content, with percentage values of 75.7 and 70.1%, respectively. Table olives fat content was the second most abundant component ranging from 12.5% to 20.1%, namely for *Cv.* Madural and Verdeal Transmontana, respectively. Despite the natural agro-biological factors influencing water content (Brescia *et al.*, 2007), the technological treatment applied increases osmotic processes, therefore raising the water content of olives and consequently reducing all the other components on a fresh weight basis, as can be observed for the fat content, which change during olives maturation (Brescia *et al.*, 2007). The most important factor that influences the amount of fat in olives is the olive cultivar, regulated by genetic factors (Di Bella *et al.*, 2007). Concerning "*alcaparras*" table olives, since they were harvested still green and due to the aqueous treatment applied, the differences among fat and water contents are higher.

Crude protein contents of "*alcaparras*" table olives varied between 0.6 and 1.4% (*Cv.* Verdeal Transmontana and Cobrançosa, respectively). Although presenting low protein content, some proteins from the oil bodies of the fruit pulp could be associated to some healthy characteristics (Hidalgo *et al.*, 2001).

Ash values were quite similar among all olive cultivars, varying from 1.6 to 1.9%. "*Alcaparras*" table olives are not implied in fermentative processes in brine that consequently increase salt levels in the olives due to NaCl retention. This fact could explain the lowest salt levels of "*alcaparras*" compared with those reported for other kinds of table olives, 4.4% and near 6% in green table olives (Lanza *et al.*, 2010; Ünal & Nergiz, 2003), and 4.5% in Kalamata table olives, and 5.9% in black table olives (Ünal & Nergiz, 2003). Moreover, ash content in table olives, besides increasing during fermentation also increases during ripening stage as demonstrated by Ajana *et al.* (1999), presenting lower levels in the earlier ripening stages. Such fact is in accordance with the ripening stages of the different cultivars of table olives that were hand-picked still green. A low content of ash also means low salt contents (sodium chloride) which is nutritionally more suitable. The consumption of high salt quantities is related with systolic and diastolic blood pressure increases, therefore increasing the risk of cardiovascular disease, particularly cerebral stroke and myocardial infarction risk (Hooper *et al.*, 2002).

In this study, carbohydrate contents include fiber content and being therefore higher than those reported for other table olives. Kailis and Harris (2007) reported carbohydrates contents between 8 and 12% for different raw olives, which are similar to those obtained in the present work for "*alcaparras*" table olives produced from different cultivars. Carbohydrates content in "*alcaparras*" table olives varied from 6.3 to 9.2%, respectively for *Cv.* Cobrançosa and Madural.

However, these levels are higher compared with those reported for other kinds of processed olives (5.4% in green table olives - Lanza *et al.*, 2010), being the total sugars and the reducing sugars absent in the final of three distinct processes studied by Ünal & Nergiz (2003). This difference could be explained by the technological factor. In fact, table olives that suffer fermentative processes are practically sugar free, since the microorganisms in the medium use the reducing sugars as an energy source (Kailis & Harris, 2007).

The energetic value per 100 g of ”*alcaparras*” table olives was accounted based on fat, protein and estimated carbohydrates amounts. *Cv.* Madural had the lowest energetic value (154 kcal) and *Cv.* Verdeal Transmontana showed the highest one (212 kcal). The differences in the energetic values of the “*alcaparras*” of the different cultivars are related with fat content which is genetically regulated (Di Bella *et al.*, 2007). This kind of table olives, compared to other potential fat sources provides lower caloric value, which turns them nutritionally advisable.

In a general way, the results obtained for the proximate chemical composition and energetic value of the different Portuguese cultivars of “*alcaparras*” table olives are in accordance with those reported in several works carried out with olives (Lanza *et al.*, 2010; Ünal & Nergiz, 2003).

3.3.2. Fatty acids composition

Fat composition of the different cultivars of “*alcaparras*” table olives was analyzed and the respective fatty acids profiles are given in Table 2. Just like with fat synthesis, the fatty acids composition of the different olive cultivars is mainly regulated by genetic factors but also depends, in lower amplitude, on pedological factors like the environment conditions (Di Bella *et al.*, 2007).

Fat can be classified as saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA), corresponding to the different nutritional fractions of fatty acids, including also *trans* isomers. As expectable, oleic acid ($C_{18:1c}$) was the most abundant fatty acid in all “*alcaparras*” table olives, independently of the olive cultivar, ranging from 66.9% (*Cv.* Madural and Santulhana) to 76.1% (*Cv.* Verdeal Transmontana). This same fatty acid was also the major one found in olive oils (around 60-80%) (Maggio *et al.*, 2009). Nutritionally MUFA are very important fatty acids since they can contribute to decrease the concentration of low density lipoprotein (LDL) cholesterol in the blood and at the same time possess the capacity to maintain or raise the concentration of high density lipoprotein (HDL) cholesterol (Lanza *et al.*, 2010).

Palmitic acid ($C_{16:0}$) was the main SFA determined, varying from 10.8 to 13.3%, corresponding respectively to *Cv.* Verdeal Transmontana and Negrinha de Freixo. Some studies indicate that diets rich in SFA fats could induce cardiovascular diseases, like cardiac arrhythmia (McLennan, 1993), due to the increase in the LDL-cholesterol concentration in the blood. “*Alcaparras*” table olives had a total SFA content lower than 17.9% (*Cv.* Cobrançosa).

PUFA contents varied from 3.5% (*Cv.* Negrinha de Freixo) to 11.6% (*Cv.* Madural). PUFA consumption helps to decrease LDL-cholesterol and HDL-cholesterol levels in the blood, contributing to reduce the incidence of cardiac arrhythmia (McLennan, 1993). Linoleic acid, the third most abundant fatty acid found, reported a higher variance among the olive cultivars varying from 2.7 to 10.3% (*Cv.* Negrinha de Freixo and Santulhana, respectively).

“*Alcaparra*s” table olives have a high oleic acid content, high oleic:palmitic acid (5.1-7.1 for *Cv.* Madural and Verdeal Transmontana) and MUFA:SFA (3.9-5.2 *Cv.* Cobrançosa and Verdeal Transmontana) ratios, altogether important factors indicating that moderate consumption of this kind of table olives associated to the Mediterranean diet can prevent the appearance of cardiovascular diseases.

Table 2. Fatty acid composition (percentage in the extracted fat) of "*alcaparras*" table olives from different cultivars (mean ± SD).

	Cobrançosa	**Madural**	**Negrinha de Freixo**	**Santulhana**	**Verdeal Transmontana**	***P* - value**
$C_{14:0}$	0.02 ± 0.01 a	0.03 ± 0.005 b	0.02 ± 0.01 a	0.02 ± 0.004 a	0.02 ± 0.005 a	$< 0.001^{(1)}$
$C_{16:0}$	12.9 ± 0.7 b,c	13.0 ± 0.26 b	13.3 ± 0.13 c	13.0 ± 0.21 b	10.8 ± 0.22 a	$< 0.001^{(2)}$
$C_{16:1}c$	0.90 ± 0.05 b	0.65 ± 0.02 a	1.30 ± 0.14 c	0.63 ± 0.04 a	0.64 ± 0.02 a	$< 0.001^{(2)}$
$C_{17:0}$	0.16 ± 0.01 b	0.06 ± 0.005 a	0.04 ± 0.004 c	0.06 ± 0.01 a	0.23 ± 0.02 d	$< 0.001^{(2)}$
$C_{17:1}$	0.24 ± 0.01 b	0.09 ± 0.006 a	0.11 ± 0.01 c	0.09 ± 0.005 a	0.35 ± 0.02 d	$< 0.001^{(2)}$
$C_{18:0}$	4.00 ± 0.53 e	2.44 ± 0.06 b	1.49 ± 0.10 a	2.77 ± 0.05 c	3.13 ± 0.11 d	$< 0.001^{(2)}$
$C_{18:1}c$	68.4 ± 1.63 b	66.9 ± 1.01 a	72.7 ± 0.99 c	66.9 ± 0.76 a	76.1 ± 0.70 d	$< 0.001^{(2)}$
$C_{18:2}cc$	6.75 ± 0.56 b	10.1 ± 0.25 c	2.66 ± 0.45 a	10.3 ± 0.63 c	2.86 ± 0.11 a	$< 0.001^{(2)}$
$C_{18:3}c$	1.06 ± 0.04 b	1.54 ± 0.05 c	0.83 ± 0.05 a	0.82 ± 0.06 a	0.82 ± 0.05 a	$< 0.001^{(1)}$
$C_{20:0}$	0.54 ± 0.05 d	0.41 ± 0.01 b	0.37 ± 0.02 a	0.49 ± 0.02 c	0.60 ± 0.03 e	$< 0.001^{(2)}$
$C_{20:1}c$	0.26 ± 0.03 a	0.33 ± 0.02 b	0.42 ± 0.04 c	0.33 ± 0.02 b	0.35 ± 0.03 b	$< 0.001^{(1)}$
$C_{22:0}$	0.14 ± 0.02 a,b	0.12 ± 0.02 a	0.14 ± 0.02 a	0.16 ± 0.02 b	0.19 ± 0.03 c	$< 0.001^{(1)}$
$C_{24:0}$	0.11 ± 0.02 b	0.10 ± 0.01 a,b	0.09 ± 0.01 a	0.10 ± 0.02 a,b	0.13 ± 0.01 c	$< 0.001^{(1)}$
SFA	17.9 ± 1.29 d	16.2 ± 0.26 c	15.5 ± 0.11 b	16.4 ± 0.55 c	15.0 ± 0.23 a	$< 0.001^{(2)}$
MUFA	69.8 ± 1.64 b	67.9 ± 1.02 a	74.4 ± 0.91 c	67.9 ± 0.76 a	77.5 ± 0.69 d	$< 0.001^{(2)}$
PUFA	7.82 ± 0.57 b	11.6 ± 0.30 d	3.50 ± 0.45 a	11.1 ± 0.67 c	3.7 ± 0.03 a	$< 0.001^{(2)}$
Trans isomers	0.04 ± 0.02	0.06 ± 0.02 a,b	0.07 ± 0.01 b	0.05 ± 0.01 a	0.05 ± 0.02 a	$< 0.001^{(2)}$

$^{a\text{-}e}$Means within a line with different superscripts differ, $P < 0.05$.

$^{(1)}$*P*-values are those for the effect of cultivar on the fatty acids profile of "*alcaparras*" table olives, from one-way ANOVA analysis. If there was a significant effect of cultivar on the fatty acids data, the means were compared by Tukey´s test, since equal variances could be assumed ($P > 0.05$ by means of Levene test).

$^{(2)}$*P*-values are those for the effect of cultivar on the fatty acids profile of "*alcaparras*" table olives from one-way Welch ANOVA analysis. If there was a significant effect of cultivar on the fatty acids data, the means were compared by Dunnett T3´s test, since equal variances could not be assumed ($P < 0.05$ by means of Levene test).

Moreover the results obtained are in accordance with those regulated for olive oil (EEC, 1991). Furthermore, the fatty acids profiles in the analyzed olive cultivars are similar to those obtained in olive oils produced in the region (Pereira *et al.*, 2002; Pereira *et al.*, 2004).

The unsupervised PCA method was applied to the fatty acids profiles recorded for the five cultivars of "*alcaparras*" table olives. Principal components analysis showed that 67.3% of the total variance of the data could be explained using only three principal components. Figure 1 shows the three-dimensional representation of the three principal components factor scores obtained from the five olive cultivars. As can be inferred by the results (Figure 1), the five olive cultivars could be separated in three different groups. The first principal component factor allowed the separation of *Cv.* Verdeal Transmontana (located in the negative region) from the remaining olive cultivars (placed in the positive region) mainly due to its higher contents of oleic acid ($C_{18:1c}$), MUFA, heptadecanoic acid ($C_{17:0}$) and 10-heptadecenoic acid ($C_{17:1c}$); the second factor separated *Cv.* Negrinha de Freixo (in the positive region) from the other olive cultivars (in the negative region) due to its higher contents on gadoleic acid ($C_{20:1c}$), palmitoleic acid ($C_{16:1c}$) and total *trans* fatty acids. The third principal component factor allowed the separation of *Cv.* Cobrançosa (in the positive region) from the other four olive cultivars (all represented in the negative region). Meanwhile, in Figure 1 can be inferred that a bigger group is represented in the positive region and negative region of the first and second factors, respectively, and all across the region of the third factor. This group is composed by *Cv.* Cobrançosa, Madural and Santulhana.

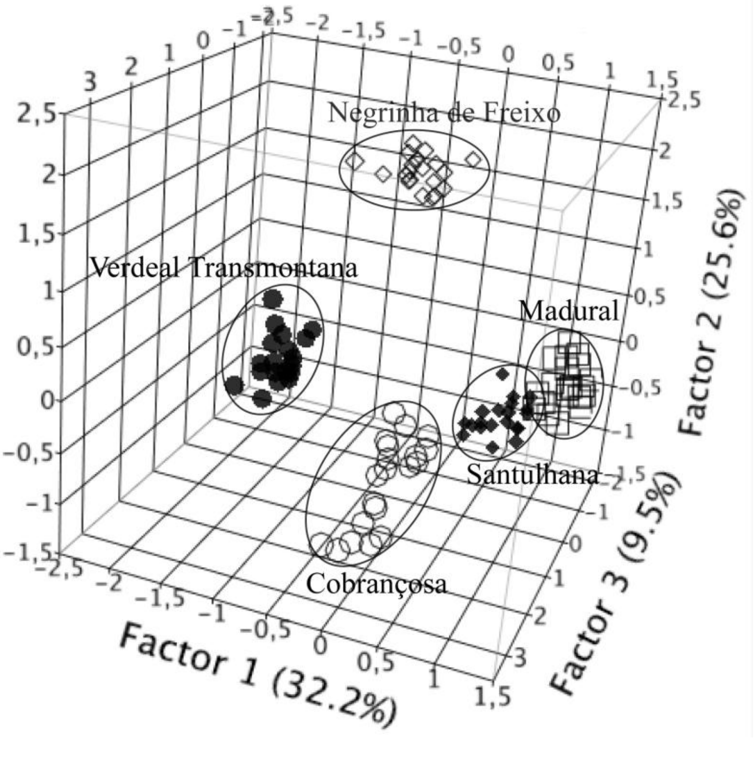

Figure 1: Principal components analysis using fatty acids data of the different cultivars of "*alcaparras*" table olives. The PCA factors explain 68.3% of the total variance.

Finally, the use of a stepwise LDA resulted in a discriminant model with four significant discriminant functions that explained 100% of the variance, although only the first two were used, since they explained 85.1% of the variance of the experimental data (the first explaining 50.2% and the second 34.9%).

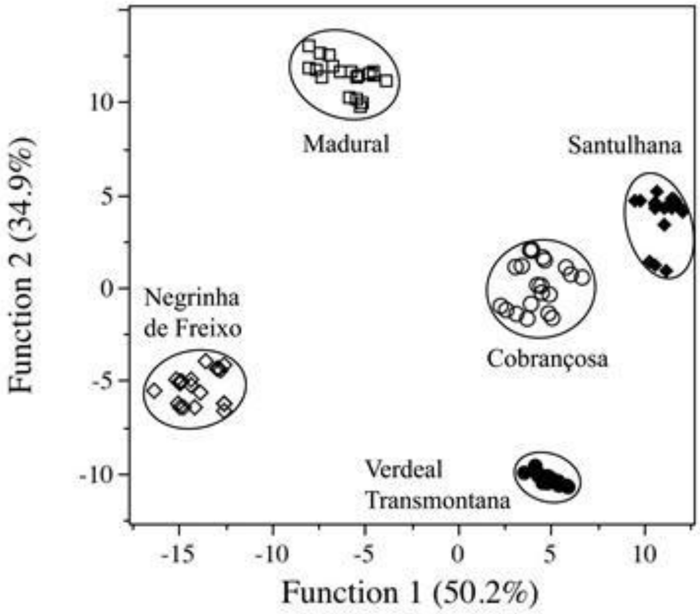

Figure 2: Linear discriminant analysis of the different cultivars of "*alcaparras*" table olives represented in a plane composed by the two main discriminant functions. The functions explain 85.1% of the total variance.

The model was based only in six variables: MUFA, PUFA, SFA, $C_{16:0}$, $C_{18:0}$ and $C_{18:3}$ and it showed a very satisfactory classification performance allowing to correctly classifying all the samples for the original groups as well as for the cross-validation procedure (sensitivities and specificities of 100%). The results obtained, showed that MUFA, PUFA, SFA, $C_{16:0}$, $C_{18:0}$ and $C_{18:3}$ allied to the application of LDA, could be used as a chemical marker of the olive cultivars, acting as an authenticity marker.

3.3.3. Tocopherols content

Three isomers of vitamin E, α-, β- and γ-tocopherol were identified in the different cultivars of "*alcaparras*" table olives, being the results shown in Table 3. α-Tocopherol was the most abundant vitamer of vitamin E found in all olive cultivars, varying from 2.26 and 5.66 mg/kg (fresh weight basis) in *Cv.* Santulhana and Negrinha de Freixo, respectively. Significant differences were found among the two olive cultivars referred (*P* = 0.034). As expectable, α-tocopherol is also the main vitamer found in olive oils (Cunha *et al*., 2006; Beltrán *et al*., 2010). α-Tocopherol possesses important antioxidant properties helping to defend the organism against the attacks of free radicals while protecting polyunsaturated fatty acids and acting as an efficient chain terminators in lipid autoxidation reactions (Kamal-Eldin & Andersson, 1997).

β-Tocopherol was present at very low concentrations, below 0.38 mg/kg (*Cv.* Cobrançosa), reporting *Cv.* Madural the lowest content (0.13 mg/kg). No significant differences (*P* = 0.250) were found among the five different cultivars within the results obtained. γ-Tocopherol of *Cv.* Santulhana had a significant (*P* < 0.001) high amount of this vitamer (0.96 mg/kg). Meanwhile, in the remaining olive cultivars values below 0.31 mg/kg were determined. Due to such fact, γ-tocopherol could be used as a chemical marker for *Cv.* Santulhana allowing its discrimination from the remaining cultivars.

Table 3. Tocopherol and tocotrienol contents (mg/kg of fresh weight) of "*alcaparras*" samples from different cultivars (mean ± SD).

Olive cultivar	**α-tocopherol**	**ß-tocopherol**	**γ-tocopherol**	**Total**
Cobrançosa	2.84 ± 0.64 a,b	0.38 ± 0.25	0.31 ± 0.16 b	3.53 ± 0.97
Madural	3.35 ± 1.65 a,b	0.13 ± 0.12	0.10 ± 0.09 b	3.59 ± 1.76
Negrinha de Freixo	5.66 ± 0.98 b	0.22 ± 0.08	0.13 ± 0.05 b	6.00 ± 1.03
Santulhana	2.26 ± 1.11 a	0.28 ± 0.04	0,96 ± 0.19 a	3.50 ± 1.34
Verdeal Transmontana	4.25 ± 1.13 a,b	0.20 ± 0.03	0.09 ± 0.01 b	4.54 ± 1.13
***P* - value**	0.034⁽¹⁾	0.250⁽²⁾	< 0.001⁽¹⁾	0.149⁽¹⁾

$^{a\text{-}b}$Means within a column with different superscripts differ, *P* < 0.05.

⁽¹⁾*P*-values are those for the effect of cultivar on the tocopherols profile of "*alcaparras*" table olives, from one-way ANOVA analysis. If there was a significant effect of cultivar on the tocopherols data, the means were compared by Tukey´s test, since equal variances could be assumed (*P* > 0.05 by means of Levene test).

⁽²⁾*P*-values are those for the effect of cultivar on the tocopherols profile of "*alcaparras*" table olives from one-way Welch ANOVA analysis. If there was a significant effect of cultivar on the tocopherols data, the means were compared by Dunnett T3´s test, since equal variances could not be assumed (*P* < 0.05 by means of Levene test).

Total contents of vitamin E varied from 3.5 to 6.0 mg/kg (*Cv.* Santulhana and Negrinha de Freixo respectively), which are very low amounts when compared to the reported in the literature for other green table olives (Montaño *et al.*, 2005; Sakouhi *et al.*, 2008)

It should be referred that α-tocopherol content decreases during storage of olive fruit, as reported by Pereira *et al.* (2002), as well as during processing to turn olives edible. In this study, the aqueous treatment applied to remove natural bitterness of table olives could also be responsible for removing significant amounts of several compounds, tocopherols included, because the olives were previously broken, while in other olive processing methods the olive fruits are processed intact.

3.3.4. Sensorial evaluation

Average values of the sensory parameters evaluated (aroma, consistency, flavour and global appreciation) are reported in Figure 3.

Considering the global appreciation *Cv.* Verdeal Transmontana and Negrinha de Freixo were the table olives preferred by the consumer's panel, with a respectively average score of 6.7 and 5.9 in a scale from 1 to 9.

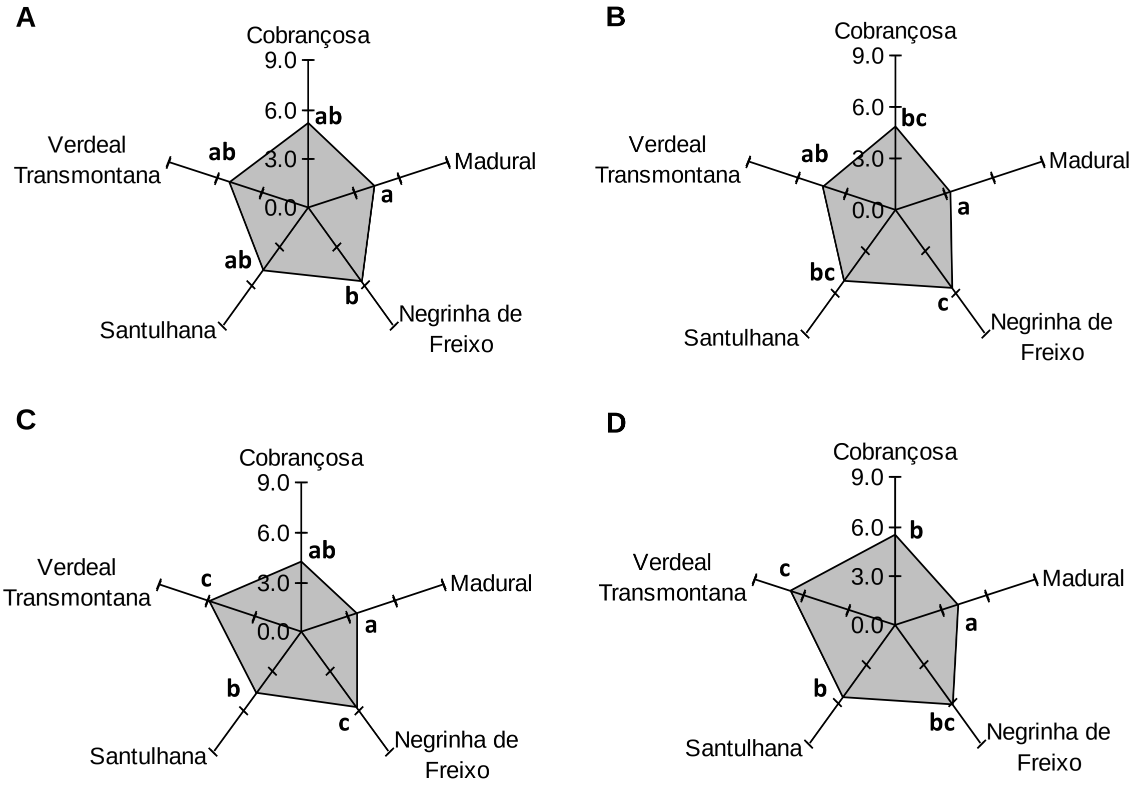

Figure 3: Representation of the sensorial characteristics (**A** – aroma; **B** – consistency; **C** – flavor; **D** – global appreciation) of five cultivars of "*alcaparras*" table olives.
P-values: Aroma – $P = 0.033^{(1)}$; Consistency, flavor and global appreciation – $P < 0.001^{(1)}$.
$^{a\text{-}d}$Means within the same descriptor figure, different superscripts differ, $P < 0.05$.
$^{(1)}$ *P*-values are those for the effect of olive cultivar on the sensorial evaluation from one-way ANOVA analysis. If there was a significant effect of olive cultivar on the sensorial evaluation data, then means were compared by Tukey´s test, since equal variances could be assumed ($P > 0.05$ by means of Levene test).

The olive cultivar Verdeal Transmontana presents table olives highly appreciated by the consumers, due to being fruity, fleshy and firm, what probably influenced the consumer's panel. Concerning to olives aroma consumer's panel showed preference by *Cv.* Negrinha de Freixo (5.5) and Cobrançosa (5.2). Significant differences were found mainly between the aroma of *Cv.* Negrinha de Freixo and Madural ($P = 0.033$). Olive's aroma, after visual contact, could be the most influencing factor in the consumer's acceptability towards a specific olive cultivar. It is related with both qualitative and quantitative compositions of volatiles (Sabatini *et al.*, 2008), and the fragrance transmitted derivates from an equilibrium of several chemical classes of volatile compounds. In a preliminary study, we evaluate the volatile profile of the five olive cultivars in study and we observe that "*alcaparras*" table olives are mainly composed by aldehydes, being hexanal the most abundant and followed by (*E*,*E*)-2,4-heptadienal and phenylacetaldehyde. These volatile compounds could be related to the consumer's preferences once that they are connoted with sensations highly appreciated by them. For example: hexanal is known as a compound that transmits green apple and cut grass sensations (Aparicio *et al*., 1996; Kiritsakis, 1998) and it is related to immature fruit characteristics; phenylacetaldehyde is associated to pungent and phenolic sensations (Angerosa *et al*., 2004), while (*E*,*E*)-2,4-heptadienal transmit fatty and nutty sensations (Ullrich & Grosch, 1998). Compounds like (*E*)-2-hexenal, norisoprenoids and terpenic compounds were also identified, being this compound related to bitter almonds and green fruity (Luna *et al*., 2006) floral and violet sensations.

The attributes related to the referred volatile compounds could lead the consumer's preferences towards the aroma of *Cv.* Negrinha de Freixo and Cobrançosa instead of other olive cultivars. However, such fragrance or aroma can be influenced by agronomic and technologic aspects that can affect the volatile fraction of table olives. The use of unhealthy fruits for table olives production, olive cultivar, fruit ripeness stage, climatic conditions, origin area, harvest method, olive fruit storage time, process applied to turn table olives edible, as well as genetic factors, can modify their volatile profile and consequently the consumer´s acceptance (Angerosa *et al*., 2004).

Concerning the consistency of the table olives, *Cv.* Negrinha de Freixo and Santulhana reported higher average values, 5.7 and 5.1, respectively.

In the remaining parameter evaluated (flavor), *Cv.* Verdeal Transmontana and Negrinha de Freixo were preferred by the consumer´s panel with a respectively score of 5.9 and 5.6.

Cv. Negrinha de Freixo was positively characterized in all the parameters evaluated as can be inferred by the Preference Map (Figure 4). This same olive cultivar is already used to process turning color in brine table olives in Portugal and due to its high quality it has been awarded with a "Protected Designation of Origin".

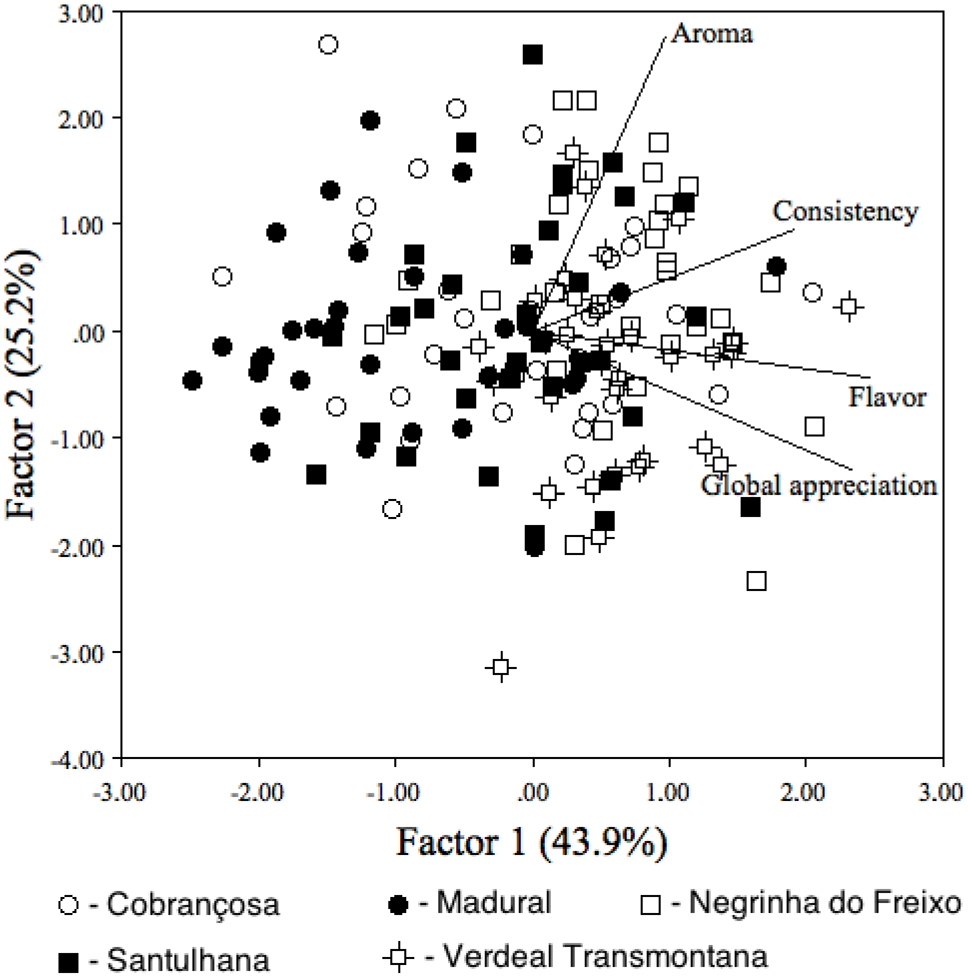

Figure 4: Internal preference map obtained by PCA of individual consumer preference ratings for the sensory parameters of the 5 olive cultivars. The PCA factors explain 69.1% of the total variance.

Based on the results obtained, *Cv.* Verdeal Transmontana is highly appreciated by the local consumers. This fact indicates that this cultivar could be used for table olive production.

On the other hand, *Cv.* Madural was negatively evaluated in all the sensorial parameters (Figure 4) and significant statistical differences were found between this olive cultivar and the remaining (Figure 3).

3.4. Conclusions

The results obtained clearly highlight the effect of olive cultivar in the chemical, nutritional and sensory characteristics of "*alcaparras*" table olives. Chemical composition, mainly the fat content and consequently the energetic value, are influenced by the olive cultivar. Fatty acids composition varies among the cultivars as well as the nutritional fractions, being MUFA the predominant fatty acids. The results showed that a linear discriminant model using the fatty acids profile (SFA, MUFA, PUFA, $C_{16:0}$, $C_{18:0}$ and $C_{18:3}$) could correctly identify the table olives cultivar, being an important tool for authenticity purposes. Despite being present in reduced amounts, tocopherols profile significantly differ, being α-tocopherol the most abundant one. *Cv.* Verdeal Transmontana and Negrinha de Freixo were the most appreciated by the consumer´s panel being positively characterized, while *Cv.* Madural was negatively characterized.

Compared to other fat sources, "*alcaparras*" table olives provide lower caloric values and are composed by healthy compounds like monounsaturated fatty acids and tocopherols. Included in the daily diet, "*alcaparras*" could contribute to a healthier nutrition, while preventing or reducing the risk of several modern diseases.

3.5. Literature cited

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Capítulo 4

Volatile profile of stoned table olives from different varieties by HS-SPME and GC/IT-MS

Volatile profile of stoned table olives from different varieties by HS-SPME and GC/IT-MS.

Abstract

The volatile composition of stoned table olives “*alcaparras*” produced from five of the most representative olive cultivars (*Cv*. Cobrançosa, Madural, Negrinha de Freixo, Santulhana and Verdeal Transmontana) of Trás-os-Montes region (Northeast of Portugal) was analytically characterized using HS-SPME/GC-IT-MS (headspace-solid phase microextraction/gas chromatography-ion trap- mass spectrometry).

Overall, forty two volatile compounds were identified, belonging to distinct chemical classes: 15 aldehydes, 7 esters, 5 alcohols, 5 sesquiterpenes, 4 norisoprenoids derivates, 3 monoterpenes, 1 ketone and 2 alkene. Aldehydes were the major chemical class identified in all olive cultivars studied (above 74% of all the volatile compounds identified). Hexanal, phenylacetaldehyde and (*E*,*E*)-2,4-heptadienal were the major volatile compounds identified.

With the results obtained from the volatile profile of the five olive cultivars was possible discriminating them trough a Principal Component Analysis (PCA). Both qualitative and quantitative fractions of “*alcaparras*” table olives were influenced by olive cultivar, which confers a single aroma. This fact certainly influences the consumer’s preference and acceptability towards a specific olive cultivar.

Keywords: *Olea europaea* L.;*“alcaparras”*; stoned table olives; HS-SPME/GC-IT-MS; volatile composition.

Malheiro, R.; Guedes de Pinho, P.; Casal, S.; Bento, A. & Pereira, J.A. (2011). Volatile profile of stoned table olives from different varieties by HS-SPME and GC/IT-MS. *Journal of the Science of Food and Agriculture*, **91**, 1693-1701..

4.1. Introduction

The olive fruit flavor, unique and pleasant, is probably the single most important characteristic that turn table olives so enjoyable by consumers (Sabatini & Marsilio, 2008) and directly influence the consumer's acceptability (Koprivnjak *et al.*, 2002). The global flavor is tightly related to both qualitative and quantitative compositions of volatiles (Sabatini *et al.*, 2008) which can contribute and influence the quality of table olives. Volatile compounds are responsible for the particular fragrance transmitted by table olives and such fragrance derivates from equilibrium of several volatile compounds, such as hydrocarbons, alcohols, aldehydes, ketones, esters and others[1]. Meanwhile, such fragrance or aroma can be influenced by agronomic and technologic aspects that can change the volatile fraction of table olives. The use of unhealthy fruits for table olives production, olive cultivar, fruit ripeness stage, climatic conditions, origin area, harvest method, olive fruit storage time, process applied to turn table olives edible, as well as genetic factors, can modify their volatile profile (Angerosa *et al.*, 2004; Ruíz *et al.*, 2005). The synthesis of volatile compounds during fruit development is reduced, but increases during ripening and also during the fermentation process (Kalua *et al.*, 2007).

Recently, the scientific interest on olive oil and table olives volatile characterization is strongly rising. Nevertheless, while a lot is known about the compounds responsible for olive oil aroma, the literature related to table olives volatiles is not so extensive. Some studies were carried out to evaluate the volatile composition of table olives in order to detect spoilage incidents (García-García *et al.*, 2004; Montaño *et al.*, 1990; Montaño *et al.*, 1992; Montaño *et al.*, 1993), to verify changes in the volatile profile during controlled fermentation process (Panagou & Tassou, 2006) and to differentiate olive cultivars (Gómez-Rico *et al.*, 2008).

In the last few years, our research group has been working with green stoned table olives, produced by a traditional method and known as "*alcaparras*" table olives in the Trás-os-Montes region (Northeast of Portugal). Several studies were conducted with this kind of table olives, such as antioxidant activity (Sousa *et al.*, 2008), phenolic compounds and antimicrobial potential (Sousa *et al.*, 2006), and more recently chemical composition, fatty acids composition and vitamin E determination.

This kind of table olives is produced during Autumn-Winter seasons using only green or yellow-green healthy fruits. The stone is removed and the pulp is placed into water until become edible. After this treatment, "*alcaparras*" are consumed plain, or flavored with garlic, olive oil, onion, herb spices, salt and other condiments. Meanwhile, the producers don't take into consideration the cultivar used to process this kind of olives, using a mixture of several cultivars from the region. The main objective of this work is the characterization of the volatile profile of "*alcaparras*" table olives produced from five of the most representative olive cultivars from "Trás-os-Montes" region, and to observe the cultivar effect.

4.2. Material and Methods

4.2.1. Stoned table olives "alcaparras" sampling and preparation

For this study, five of the most representative olive cultivars from Trás-os-Montes region were collected in September to October of the year of 2006 from different olive groves in Mirandela region subjected to similar agro-climatic conditions and agronomic practices. From each cultivar, five independent lots of olives, approximately of 5 kg each, were collected and immediately transported to the laboratory. At the laboratory, from each lot, approximately 2kg of stoned table olives were prepared. For this, green or yellow-green healthy olive fruits were used, which were broken to separate the pulp from the stone. The pulp was placed into water during a week, daily changed, to remove olives bitterness. After the treatment, "*alcaparras*" table olives were frozen at -20º C until analysis**.**

4.2.2. Standards

Reference compounds were purchased from several suppliers: 2-methylbutanal, pentanal, hexanal, (*E*)-2-hexenal, heptanal, octanal, (*E*)-2-octenal, (*E,E*)-2,4-nonadienal, geranylacetone, limonene, *ß*-cyclocitral, 6-methyl-5-hepten-2-one, hexanoic acid methyl ester, 2-methyl-1-butanol, 3-methyl-1-butanol, caryophyllene and *(E)*-3-hexen-1-ol were from Sigma (St. Louis, MO, USA); benzaldehyde, phenylacetaldehyde, (*E*)-2-

decenal and ß-ionone were obtained from SAFC (Steinheim, Germany); hexyl acetate and 1-hexanol were from Merck (Darmstdt, Germany); menthol was obtained from Fluka (Buchs, Switzerland); eucalyptol was obtained from Extrasynthese (Genay, France).

4.2.3. SPME Fibers

Several commercial fibers can be used to extract volatile compounds. According to bibliography, recommendations of supplier (Supelco, Bellefonte, PA), and our own experience (Guedes de Pinho *et al.*, 2009), the fiber used was coated with divinylbenzene/polydimethylsiloxane (DVB/PDMS), 65µm.

4.2.4. HS-SPME

For each cultivar, approximately 0.3 g of fresh olive, previously thawed were putted into a 15 mL vial with the addition of 3 mL of water. The vial was then sealed with a polypropylene cap with PTFE/silicon septum (Supelco). This mixture was stirred (280 rpm) at 40 ºC for 5 minutes. Then, the DVB/PDMS fiber was exposed to the headspace, and samples were stirred for 20 minutes (280 rpm at 40º C). Afterward, the fiber was pulled into the needle sheath, the SPME device was removed from the vial and inserted into the injection port of the GC system for thermal desorption. After 1 minute, the fiber was removed and conditioned in another GC injection port for 10 minutes, at 250 ºC. The same procedure was performed with a control sample containing only water.

4.2.5. Gas Chromatography-Ion Trap-Mass Spectrometry Analysis

HS-SPME analyses were performed using a Varian CP-3800 gas chromatograph equipped with a Varian Saturn 4000 mass selective detector and Saturn GC-MS workstation software version 6.8. A VF-5 ms (30 m × 0.25 mm × 0.25μm) column from Varian was used. A Stabilwax-DA fused-silica (60 m × 0.25 mm × 0.25 μm) column (Restek, USA) was used to check the identity of some compounds found in the first column. The injector port was heated to 220 ºC. The injections were performed in splitless mode. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of

1 mL/min. The oven temperature was set at 40 ºC for 1 min, then increased at 2 ºC/min to 220 ºC, and held for 30 min. All mass spectra were acquired in electron impact (EI) mode. Ionization was maintained off during the first minute. The ion trap detector was set as follows: the transfer line, manifold, and trap temperatures were 280, 50 and 180 ºC, respectively. The mass ranged from *m/z* 40 to 350, with a scan rate of 6 scan/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to autotune procedure. The maximum ionization time was 25000 μs, with an ionization storage level of *m/z* 35. Analyses were performed in full-scan mode.

Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic standards analyzed under the same conditions and by comparison of the retention indices (as Kovats indices) with literature data. MS fragmentation patterns were compared with those of pure compounds, and mass spectrum database search was performed using the National Institute of Standards and Technology (NIST) MS 05 spectral database. Confirmation was also conducted using a laboratory-built MS spectral database, collected from chromatographic runs of pure compounds performed with the same equipment and conditions. For quantification purposes, each sample was injected in triplicate, and the chromatographic peak areas (as kcounts amounts) were determined by a reconstructed full-scan chromatogram using for each compound some specific quantification ions: these corresponded to base ion (*m/z* 100% intensity), molecular ion (M^+), and another characteristic ion for each molecule. Hence, some peaks that could be co-eluted in full-scan mode (resolution value < 1) can be integrated with a value of resolution > 1.

4.2.6. Statistical Analysis

The HS-SPME analyses were performed in triplicate. Principal Component Analysis (PCA) was carried out using SPSS 17.0 software. PCA was applied for reducing the number of variables (8 variables corresponding to the chemical classes of the individual volatile compounds identified) to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information, i.e., the five olive cultivars, Cobrançosa, Madural, Negrinha de Freixo, Santulhana and Verdeal Transmontana. Moreover, it allowed recognizing patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores).

The aim of the PCA is to produce components suitable to be used as predictors or response variables in subsequent analysis. The number of factors to keep in data treatment was evaluated by the Scree plot, taking into account the eigenvalues, which should have: values greater than one for retaining the factor in the analysis, high values of total percentage of variance explained by the number of components selected internal consistency by means of α Cronbach's value, that should be positive (Maroco, 2003; Rencher 1995).

4.3. Results and discussions

The analysis of volatile compounds from "*alcaparras*" table olives produced with five Portuguese olive cultivars using HS-SPME/GC-IT-MS was performed. The assessment allowed identification of forty two compounds. The chromatographic profile of each variety is shown in Figure 1, while both qualitative and quantitative data (percentage of relative abundance) of volatile compounds of the five cultivars are described in Table 1. The forty two volatile compounds identified belong to different chemical classes: 5 alcohols (1-5), 15 aldehydes (6-20), 7 esters (21-27), 1 ketone (28), 4 norisoprenoid derivates (29-32), 3 monoterpenes (33-35), 5 sesquiterpenes (36-40), and 2 alkenes (41, 42) corresponding both to 3-ethyl-1,5-octadiene (Table 1).

All cultivars shown a similar volatile profile, nevertheless some differences were noticed considering qualitative and quantitative results (Table 1, Figures 1 and 2). *Cv.* Madural presented the highest number of the identified compounds (42), followed by *Cv.* Santulhana (41) and *Cv.* Cobrançosa and Negrinha de Freixo (37) (Table 1). *Cv.* Verdeal Transmontana reported the lowest diversity of compounds identified (33) (Table 1).

Table 1: Volatile compounds identified in "*alcaparras*" table olives processed with different cultivars, expressed in chromatographic area (mean ± standard deviation).

	Compound	**LRI**[a]	**QI (*m/z*)**[b]	**ID**[c]	**Area/1000 (S.D.)**[f]				
					Cobrançosa	**Madural**	**Negrinha de Freixo**	**Santulhana**	**Verdeal Transmontana**
Alcohols									
1	3-Methyl-1-butanol	827	55 / 70	S[d], MS[e]	0.65 ± 0.09	2.60 ± 0.46	0.46 ± 0.01	1.04 ± 0.11	0.41 ± 0.05
2	2-Methyl-1-butanol	831	55 / 70	S, MS	0.22 ± 0.03	0.81 ± 0.16	0.18 ± 0.03	0.46 ± 0.01	0.19 ± 0.05
3	1-Hexanol	917	56 / 69	S, MS	n.d.	0.86 ± 0.15	n.d.	0.93 ± 1.61	n.d.
4	(E)-3-Hexen-1-ol	950	67 / 82	S,MS	9.43 ± 0.98	8.26 ± 0.41	1.77 ± 0.19	17.04 ± 3.02	9.20 ± 0.14
5	(E)-2-Nonenol	1191	57 / 95	MS (86.1/86.8)	16.88 ± 2.58	9.49 ± 0.70	11.77 ± 0.96	13.04 ± 1.50	13.94 ± 1.14
			Σ of Alcohols		27.17 ± 3.53	21.72 ± 0.44	14.18 ± 1.03	32.52 ± 4.14	23.74 ± 0.97
Aldehydes									
6	3-Methylbutanal	742	57 / 58	MS (79.1/80.5)	4.27 ± 1.60	9.30 ± 1.88	1.88 ± 0.13	20.10 ± 0.78	8.47 ± 1.00
7	2-Methylbutanal	751	57 / 58	S, MS	20.26 ± 1.23	31.67 ± 3.31	5.89 ± 0.48	62.01 ± 1.30	22.98 ± 0.44
8	Pentanal	785	44 / 57 / 58	S, MS	5.42 ± 2.16	7.17 ± 0.41	3.63 ± 0.20	10.10 ± 0.81	n.d.
9	Hexanal	890	56 / 67 / 83	S, MS	261.48 ± 19.74	473.81 ± 75.20	108.88 ± 6.89	414.76 ± 47.07	60.80 ± 8.47
10	(E)-2-Hexenal	948	55 / 69 / 83	S, MS	20.26 ± 1.27	19.02 ± 0.28	54.97 ± 1.72	20.87 ± 4.59	23.30 ± 1.33
11	Heptanal	990	55 / 70 / 81	S, MS	45.62 ± 4.36	38.01 ± 3.72	19.63 ± 5.57	43.28 ± 2.39	20.27 ± 1.42
12	(Z)-2-Heptenal	1051	57 / 70 / 83	MS (85.9/91.9)	25.44 ± 2.38	17.53 ± 0.48	10.49 ± 0.26	29.32 ± 2.50	6.80 ± 2.64
13	Benzaldehyde	1057	77 / 105	S, MS	15.37 ± 1.27	20.27 ± 1.84	11.46 ± 0.31	25.39 ± 1.99	20.40 ± 1.43
14	(E,E)-2,4-Heptadienal	1085	53 / 81	MS (78.0/80.3)	58.55 ± 4.88	50.96 ± 6.87	24.66 ± 1.14	65.20 ± 9.84	30.52 ± 1.66
15	Octanal	1091	67 / 81 / 95	S, MS	0.45 ± 0.08	0.20 ± 0.02	0.35 ± 0.02	0.22 ± 0.11	0.30 ± 0.04
16	(E,E)-2,4-Nonadienal	1081	57 / 95	S, MS	0.46 ± 0.19	0.64 ± 0.20	0.19 ± 0.06	0.13 ± 0.00	0.00 ± 0.03
17	Phenylacetaldehyde	1137	91	S, MS	41.70 ± 15.03	61.99 ± 10.76	19.04 ± 3.64	92.95 ± 8.01	92.65 ± 14.93
18	(E)-2-Octenal	1151	70 / 93	S, MS	25.27 ± 2.33	18.69 ± 0.62	12.85 ± 0.10	26.80 ± 2.54	9.54 ± 0.32
19	Nonanal	1202	57 /81 / 82	MS (85.6/86.5)	24.47 ± 3.54	14.02 ± 1.04	17.40 ± 1.15	18.98 ± 2.01	19.92 ± 1.76
20	(E)-2-Decenal	1329	69	S, MS	3.40 ± 1.32	1.08 ± 0.16	2.67 ± 0.16	1.59 ± 0.30	1.41 ± 0.37
			Σ of Aldehydes		552.43 ± 43.68	764.37 ± 79.02	292.03 ± 3.59	831.81 ± 25.56	317.58 ± 15.86
Esters									

21	Ethyl 2-methylbutanoate	922	57 / 102	MS (80.3/91.7)	1.07 ± 0.16	1.21 ± 0.22	1.12 ± 0.06	0.98 ± 0.12	0.63 ± 0.10
22	3-Methyl-1-butanol acetate	959	70 / 87	MS (85.0/87.1)	n.d.	0.98 ± 0.15	n.d.	1.07 ± 0.13	n.d.
23	2-Methyl-1-butanol acetate	961	70 / 87	MS (82.2/86.7)	n.d.	0.45 ± 0.07	n.d.	0.46 ± 0.11	n.d.
24	Hexanoic acid methyl ester	1015	74 / 87	S, MS	2.04 ± 0.50	1.01 ± 0.51	n.d.	0.44 ± 0.45	n.d.
25	3-Hexenyl acetate	1092	67 / 82	MS (81.9/84.5)	n.d.	56.87 ± 1.63	21.06 ± 1.63	114.62 ± 10.84	n.d.
26	Hexylacetate	1100	56	S, MS	0.40 ± 0.23	6.56 ± 0.42	1.15 ± 0.38	2.03 ± 0.56	n.d.
27	Phenylethyl acetate	1325	91 / 102	MS (82.2/86.7)	n.d	0.26 ± 0.03	n.d.	0.11 ± 0.04	n.d.
			Σ of Esters		3.38 ± 0.64	67.32 ± 0.61	23.33 ± 1.18	119.71 ± 11.75	0.63 ± 0.10
Ketones									
28	6-Methyl-5-hepten-2-ona	1077	67 / 108	S, MS	24.69 ± 2.30	17.74 ± 0.45	39.45 ± 0.96	17.77 ± 1.68	21.25 ± 0.85
			Σ of Ketones		24.69 ± 2.30	17.74 ± 0.45	39.45 ± 0.96	17.77 ± 1.68	21.25 ± 0.85
Norisoprenoid derivates									
29	ß-Cyclocitral	1305	109 / 137 / 152	S, MS	0.71 ± 0.13	1.33 ± 0.07	0.62 ± 0.07	0.74 ± 0.06	0.75 ± 0.05
30	Cytral	1332	69 / 109	MS (87.2/91.8)	2.11 ± 0.37	2.19 ± 0.20	3.55 ± 0.25	2.38 ± 0.47	2.29 ± 0.06
31	Geranylacetone	1437	69 / 107 /136	S, MS	1.48 ± 0.09	1.60 ± 0.15	2.69 ± 0.18	1.00 ± 0.09	1.05 ± 0.31
32	ß-Ionone	1470	177	S, MS	0.33 ± 0.04	0.77 ± 0.05	0.22 ± 0.04	0.34 ± 0.03	n.d.
			Σ of Norisoprenoids derivates		4.63 ± 0.62	5.94 ± 0.44	7.08 ± 0.51	4.46 ± 0.58	4.09 ± 0.33
Monoterpenes									
33	Limonene	1119	67 / 93	S, MS	8.04 ± 1.03	9.81 ± 2.64	7.56 ± 0.17	6.32 ± 0.11	8.34 ± 0.43
34	Eucalyptol	1123	81 / 93 / 139	S, MS	0.89 ± 0.13	0.92 ± 0.14	1.21 ± 0.09	0.42 ± 0.06	0.98 ± 0.18
35	Menthol	1269	81 / 95 / 123	S, MS	0.06 ± 0.02	n.d.	0.23 ± 0.04	0.19± 0.02	0.33 ± 0.05
			Σ of Terpenes		9.05 ± 0.85	10.82 ± 2.71	9.12 ± 0.24	6.86 ± 0.17	9.65 ± 0.31
Sesquiterpenes									
36	α-Cubebene	1371	105 / 161	MS (88.0/88.8)	0.20 ± 0.03	2.79 ± 0.25	0.21 ± 0.03	0.15 ± 0.02	3.55 ± 0.29
37	(+)-Cyclosativene	1381	105 / 161	MS (89.1/90.1)	0.12 ± 0.02	0.23 ± 0.03	0.21 ± 0.02	2.97 ± 0.28	0.62 ± 0.10
38	Copaene	1384	105 / 161	MS (89.4/90.0)	1.92 ± 0.37	9.94 ± 0.08	3.07 ± 0.21	26.58 ± 0.93	12.84 ± 0.98
39	Caryophyllene	1403	91 / 133	S/MS	0.32 ± 0.11	1.04 ± 0.03	0.21 ± 0.06	0.29 ± 0.04	1.15 ± 0.05
40	α-Muurolene	1433	105 / 161	MS (92.8/94.4)	n.d.	0.24 ± 0.02	0.40 ± 0.02	4.80 ± 0.24	0.42 ± 0.10
			Σ of Sesquiterpenes		2.59 ± 055	14.24 ± 0.29	4.11 ± 0.15	34.80 ± 1.48	18.57 ± 1.30

Alkenes									
41	3-Etil-1,5-Octadiene	1044	69	MS (88.0/91.3)	0.53 ± 0.09	0.46 ± 0.05	0.26 ± 0.07	1.03 ± 0.09	1.04 ± 0.11
42	3-Etil-1,5-Octadiene	1053	69	MS (87.4/90.8)	2.10 ± 0.26	0.65 ± 0.01	0.59 ± 0.02	3.34 ± 0.25	2.00 ± 0.20
			Σ of Alkenes		2.63 ± 0.35	1.11 ± 0.06	0.85 ± 0.06	4.37 ± 0.33	3.04 ± 0.31

n. d. – not detected

[a] Linear Retention Index (Fit/Retrofit values-%) – determined in a VF-5ms column (30 m × 0.25 mm × 0.25 μm); [b] Quantification ions; [c] Identification method (fit/retrofit values, %); [d] Identified by comparison with reference compound; [e] Tentatively identified by NIST 05; [f] Area expressed as arbitrary units, S.D. = standard deviation of three assays.

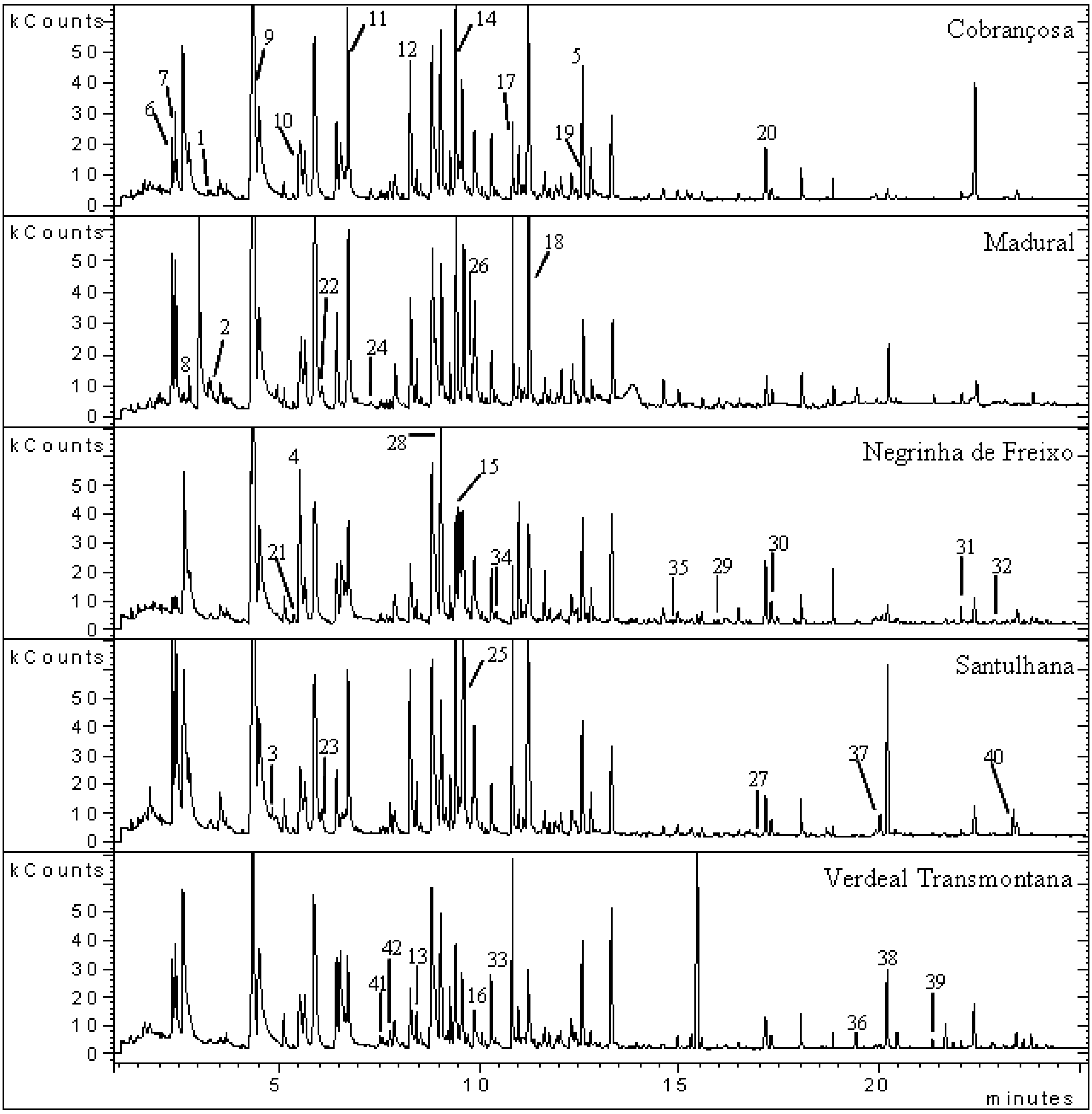

Figure 1: Chromatographic profile of "*alcaparras*" table olives processed with different cultivars by HS-SPME using divinylbenzene/PDMS fiber. Identification numbers correspond to those in Table 1.

Aldehydes were the major chemical class in all cultivars studied. In literature, aldehydes content can reach 50% of all identified volatile compounds in green olives and 75% in black olives[18]. In "*alcaparras*" table olives, aldehydes correspond to the greatest chromatographic peaks among all the volatile compounds identified, reporting

Cv. Cobrançosa higher content and by other hand *Cv.* Negrinha de Freixo the lowest one (Table 1, Figure 2).

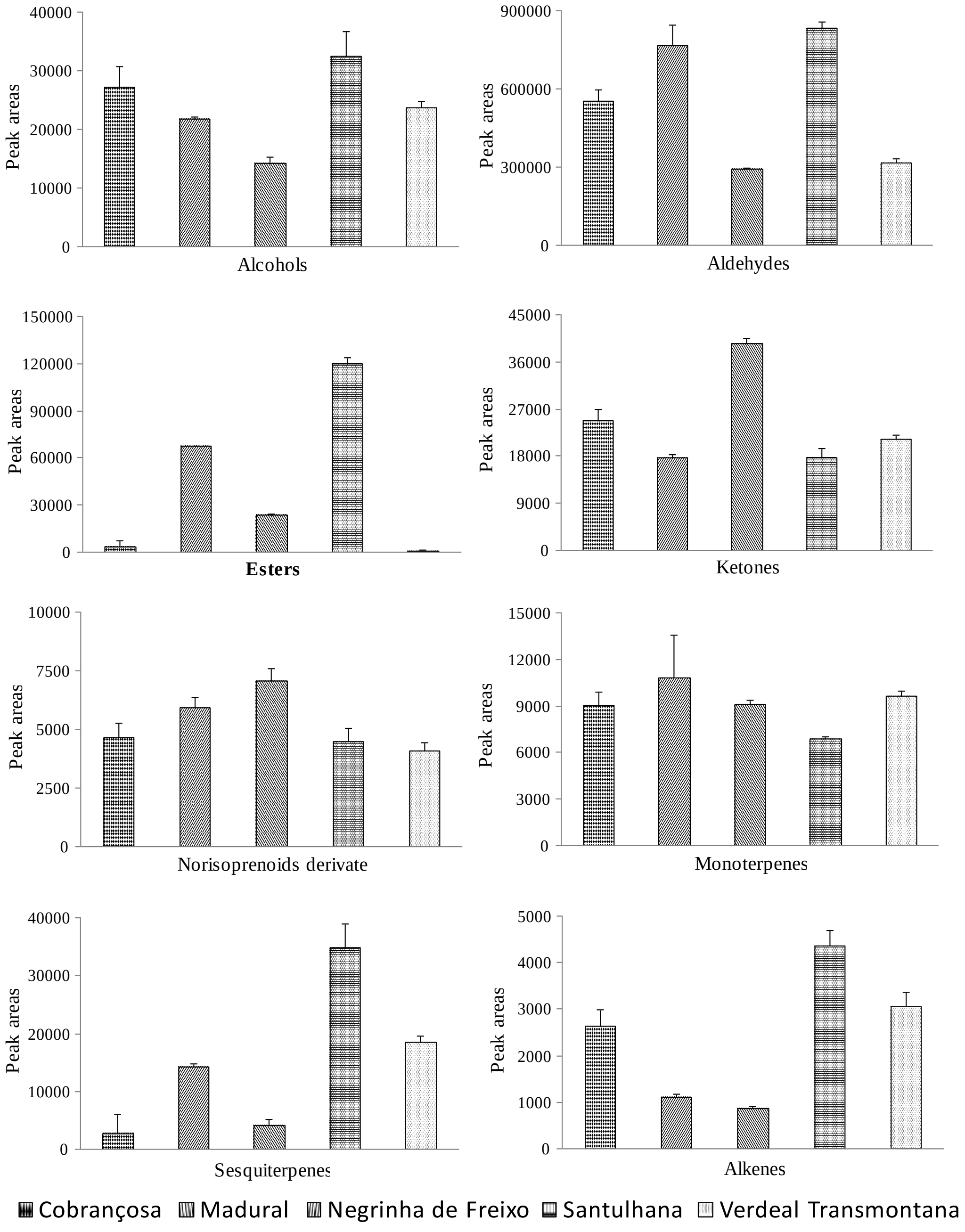

Figure 2: Sum of the area (arbitrary units) of the identified chemical classes (alcohols, esters, sesquiterpenes, norisoprenoids, aldehydes, monoterpenes, ketones and alkenes) of "*alcaparras*" table olives processed with different cultivars from Trás-os-Montes region.

Fifteen aldehydes were identified being hexanal the most abundant, not only among aldehydes but also among all the volatile compounds identified, presenting *Cv.*

Madural highest amounts. Hexanal is formed during fruit development trough the lipoxygenase pathway (LOX). The lipoxygenases are active on free unsaturated fatty acids like linoleic acid transforming it into respective 13-hydroperoxides becoming itself substrate for further enzymatic reactions. Then, 13-hydroperoxides are cleaved by hydroperoxyde lyases producing hexanal (Angerosa *et al.*, 1999; Cavalli *et al.*, 2004). Others aldehydes were present in significant amounts, (*E,E*)-2,4-heptadienal and phenylacetaldehyde. (*E,E*)-2,4-heptadienal reported the highest area values in all cultivars and phenylacetaldehyde showed a value of 23% in *Cv.* Verdeal Transmontana. Meanwhile, in the remaining olive cultivars studied, this aldehyde doesn't exceed 9% of the total compounds identified. Phenylacetaldehyde could be used as a authentication chemical marker for *Cv.* Verdeal Transmontana. Phenylacetaldehyde is formed from phenylalanine and is abundant in several fruits like tomato, strawberry and some grape varieties (Aubert *et al.*, 2005).

Aldehydes are very important in fruits and vegetables contributing to characteristic fragrances and flavors. Some aldehydes like (*E*)-2-hexenal and benzaldehyde showed antimicrobial and antifungal activity against a large number of microorganisms protecting the plant from pathogens (Kubo *et al.*, 1995; Vaughn *et al.*, 1993).

Alcohols are byproducts of some pathways where aldehydes are involved. Once formed the aldehydes suffer a series of enzymatic transformations mediated by isomerases and alcohol dehydrogenases forming C_6 alcohols (Cavalli *et al.*, 2004). C_6 volatile alcohols are also important components of the flavor of fruits, vegetables and leaves (Schwab *et al.*, 2008).

Alcohols were present in "*alcaparras*" table olives in small amounts. Five alcohols were identified being *(E)*-2-nonenol and *(E)*-3-hexen-1-ol the most abundant. *(E)*-3-Hexen-1-ol is produced in small amounts by the plants and it acts as an attractant to many predatory insects.

Among all the analyzed cultivars 7 esters were identified. *Cv.* Santulhana reported highest value of these compounds, being 3-hexenyl acetate the most abundant in this cultivar.

Norisoprenoids compounds are formed from the degradation of carotenoid molecules such as β-carotene, lutein, neoxanthin and violaxanthin (Kanasawud *et al.*, 1990) but also from the hydrolysis of glucoside molecules. Foods containing carotenoids could be subjected to norisoprenoids formation due to in vivo enzymatic degradation or postharvest thermal degradation (Mahattanatawee *et al.*, 2005). Norisoprenoids are C_9-C_{13} volatile compounds and are also characterized by very low olfactory perception thresholds which have a very important sensorial impact in aroma (Ferreira & Guedes de Pinho, 2004). Four norisoprenoids were identified in the studied olive cultivars, being the cytral the most abundant. This carotenoid derivate compound demonstrated antifungal activity and is effective against *Aspergillus flavus* spores avoiding their germination (Luo *et al.*, 2004). Some studies also reported that this volatile compound could effectively inhibit 14 bacteria and 12 fungi (Pattanaik *et al.*, 1997). β-Ionone and geranylacetone,two other norisoprenoid derivates, were found in "*alcaparras*" table olives. Both compounds, especially β-ionone were described as effective in inhibiting microbial growth in fresh-cut cantaloupe melon (Olusola & Richard, 2003). β-Ionone, geranylacetone and β-cyclocitral (present in small amounts,) play an important role in the plant defense against insects due to their repellent properties (Lwande *et al.*, 1999). On the other hand, the high antimicrobial properties of a great part of the identified compounds are in mind of previous works that revealed high antimicrobial activity of this kind of olives (Sousa *et al.*, 2006).

Only three monoterpenes were found, limonene, eucalyptol and menthol. Limonene is already known as a natural volatile compound which occurs naturally in citrus and other fruits. It has insecticidal and antimicrobial properties and is registered in 15 pesticide products used as insecticides and insect repellent (Hebeish *et al.*, 2008). In some studies, limonene is also believed to possess healthy properties once that is associated to the prevention of some kinds of cancer (Tsuda *et al.*, 2004).

Only one ketone was identified, 6-methyl-5-hepten-2-one having *Cv.* Negrinha de Freixo the highest levels of this ketone and *Cv.* Santulhana and Madural the lowest one. This compound is formed from carotenoides degradation (lycopene, γ-, δ-, and ζ-carotene) and is regarded as a marker compound for the degradation of lycopene (Creimer & Eichner, 2000). Ketones are also known as secondary products of oxidation from the degradation of fatty acids and hydroperoxydes formation leading to the development of off-flavors and odours (Richards *et al.*, 2005).

Two alkenes were identified, comparing the obtained retention indices (as Kovats indices) with those obtained by Oueslati *et al.* (2006), in all samples corresponding to both isomers of 3-ethyl-1,5-octadiene.

Sesquiterpenes are $C_{15}H_{24}$ compounds and in "*alcaparras*" table olives were present in low amounts. *Cv.* Verdeal Transmontana reported the highest sesquiterpenes amounts while *Cv.* Cobrançosa showed the lowest amount. Five of these compounds were tentatively identified by NIST 05 data base: α-cubebene, (+)-cyclosativene, copaene, caryophyllene and α-muurolene. The most abundant sesquiterpene was copaene with a maximum value of 3.23% on *Cv.* Verdeal Transmontana. Copaene is a mono-unsaturated sesquiterpene that has been already detected in Spanish olive oils, mainly from olives *Cv.* Hojiblanca (Guinda *et al.*, 1996) resulting as a chemical marker for such olive cultivar. Copaene occurs in a wide range of plant species including many host plants of *Ceratitis capitata*, the Mediterranean fruit fly (medfly), such as *Citrus* spp. (Dou, 2003; Nishida *et al.*, 2000). It is also a powerful attractant to male medflies (Flath *et al.*, 1994), being responsible for the enhanced mating success in such specie (Shelly, 2001).

To evaluate the variation of the volatile composition of "*alcaparras*" table olives produced from *Cv.* Cobrançosa, Madural, Negrinha de Freixo, Santulhana and Verdeal Transmontana, was performed a Principal Component Analysis (PCA) on the results obtained. With the PCA it was possible to distinguish and differentiate the five olive cultivars involved in this study. Figure 3A represents all the chemical variables, grouped by chemical classes in all the cultivars studied into a plane composed by the two principal components factors which contain 78.2% of the total variance. *Cv.* Negrinha de Freixo (NF) is represented in the positive region of the first principal component and in the negative region of the second principal component factor due to his high content in monoterpenes, norisoprenoids derivates and ketones. *Cv.* Verdeal Transmontana (VT) is located in both positive parts of the two principal components factors due to higher content in alcohols, alkenes and sesquiterpenes compounds. The remaining cultivars, *Cv.* Cobrançosa (C), Madural (M) and Santulhana (S) presented the highest similarity.

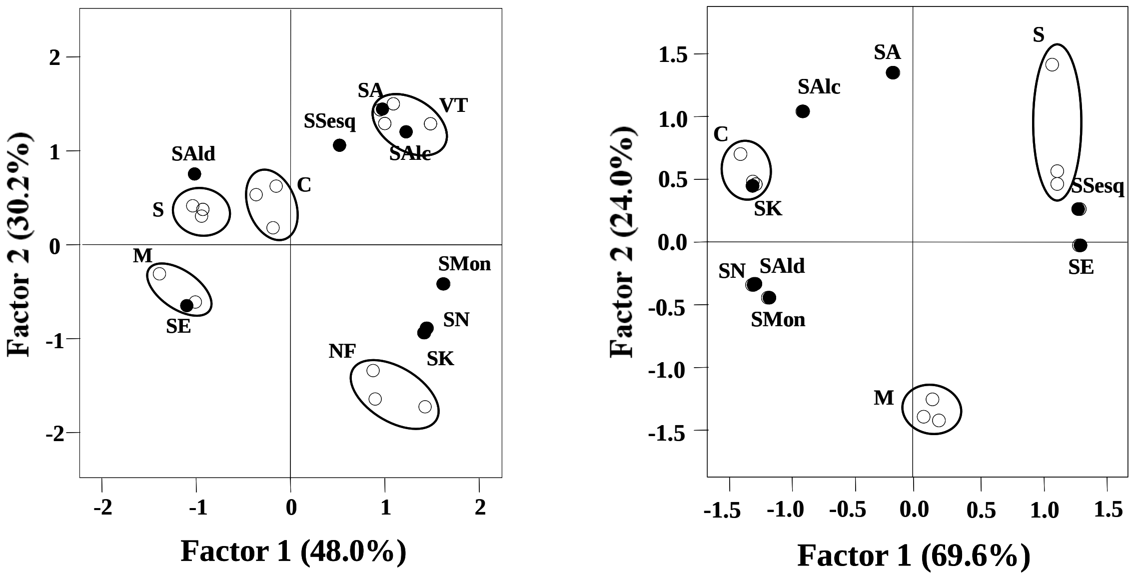

Figure 3: Principal component analysis of the volatile compounds analyzed by HS-SPME/GC-IT-MS grouped by chemical classes of "*alcaparras*" table olives processed with different cultivars (C - Cobrançosa; M - Madural; NF - Negrinha de Freixo; S - Santulhana; VT - Verdeal Transmontana). Variables: SAld – sum of aldehydes; SE – sum of ester compounds; SSesq – sum of sesquiterpenes; SA – sum of alkenes; SAlc – sum of alcohols; SMon – sum of monoterpenes; SN – sum of norisoprenoids derivates; SK – sum of ketones.

Hence, it was performed another PCA considering only the three olive cultivars. The results obtained from the second PCA are presented in another plane composed by two others principal components factors that contains 93.6% of all the total variance observed (Figure 3B). In this new PCA the three olive cultivars, Cobrançosa, Madural and Santulhana, are perfectly separated. *Cv.* Cobrançosa is discriminated due to higher content in ketones while *Cv.* Santulhana is characterized by higher content in sesquiterpenes and ester compounds.

Information regarding volatile composition of table olives is scarce compared to the existent about olive oil. Comparing the volatile profile of "*alcaparras*" table olives (mainly composed by aldehydes) with other works focused on table olives, we denote that such table olives are mainly composed by alcohols such as ethanol and 2-butanol, and also by acetic acid (Sabatini & Marsilio, 2008; Sabatini *et al.*, 2009).

The only works on volatile composition of table olives were carried out with olives prepared following the Spanish, Greek or Californian style, the three most common commercial preparations available in the international market (Panagou & Tassou, 2006). Such methods involve fermentative processes, mainly by lactic bacteria and yeasts, which enhance the final organoleptic properties of table olives. This is the reason why commercial table olives are mainly composed by alcohols and acetic acid, because the microorganisms involved produce mainly these compounds by several different biochemical pathways. Meanwhile, as described before, "*alcaparras*" table olives are produced following a traditional method being only subjected to aqueous treatment to remove olives bitterness. Such fact explains the reduced amount alcohols in the volatile profile, once that fermentative processes are not applied to turn this kind of table olives edible.

The volatile profile of "*alcaparras*" table olives are very different according to the variety used (Table 1). Such fact differentiates the sensory characteristics of each olive cultivar influencing their acceptability. The most abundant compounds are described in literature and are related to different sensory descriptors as follows: hexanal – green, apple, cut grass, green-sweet (Aparicio & Luna, 2002; Morales *et al.*, 1997); phenylacetaldehyde – pungent, phenolic (Spanier *et al.*, 2001); (*E*,*E*)-2,4-heptadienal – fatty, nutty (Ullrich & Grosch, 1988); 3-hexenyl acetate – green banana, fruity, green, green leaves, floral (Guth & Grosch, 1991; Morales *et al.*, 1997; Ramstad & Nestrick, 1980); 6-methyl-5-hepten-2-one – pungent, green (Morales *et al.*, 2005). The majority of the descriptors point to green and fruity sensations in accordance with the ripe stage of the olive fruits, once they were harvested still green, particularly in *Cv.* Verdeal Transmontana due to its later maturation. Volatile compounds have a great influence in the overall perception which transmits a unique and pleasant fragrance, being highly appreciated.

4.4. Conclusions

The volatile composition of "*alcaparras*" table olives is presented for the first time. Volatile profiling of "*alcaparras*" table olives was influenced by the olive cultivars used. In this work 42 volatiles were identified by GC-ITMS which were distributed through eight distinct chemical classes. "*Alcaparras*" table olives are mainly composed by aldehydes and also by others chemical classes present in minor content (less than 12%). Aldehydes are present in the highest levels and are related to green and fruity sensorial sensations. Depending on their presence and quantity, volatile compounds influenced the organoleptic characteristics of the olive cultivars as well as their sensorial perception, responsible for its unique flavor and aroma. By using Principal Component Analysis the olive cultivars were distinguished based on their volatile profiling.

The aqueous traditional method applied to process "*alcaparras*" table olives also contribute to obtain a different volatile profile from those obtained in commercial table olives, once that fermentative processes are not implicated in "*alcaparras*" table olives production.

Further studies should be developed to characterize volatile fraction of "*alcaparras*" table olives and the cultivar effect on the acceptation of table olives by consumers.

4.5. Literature cited

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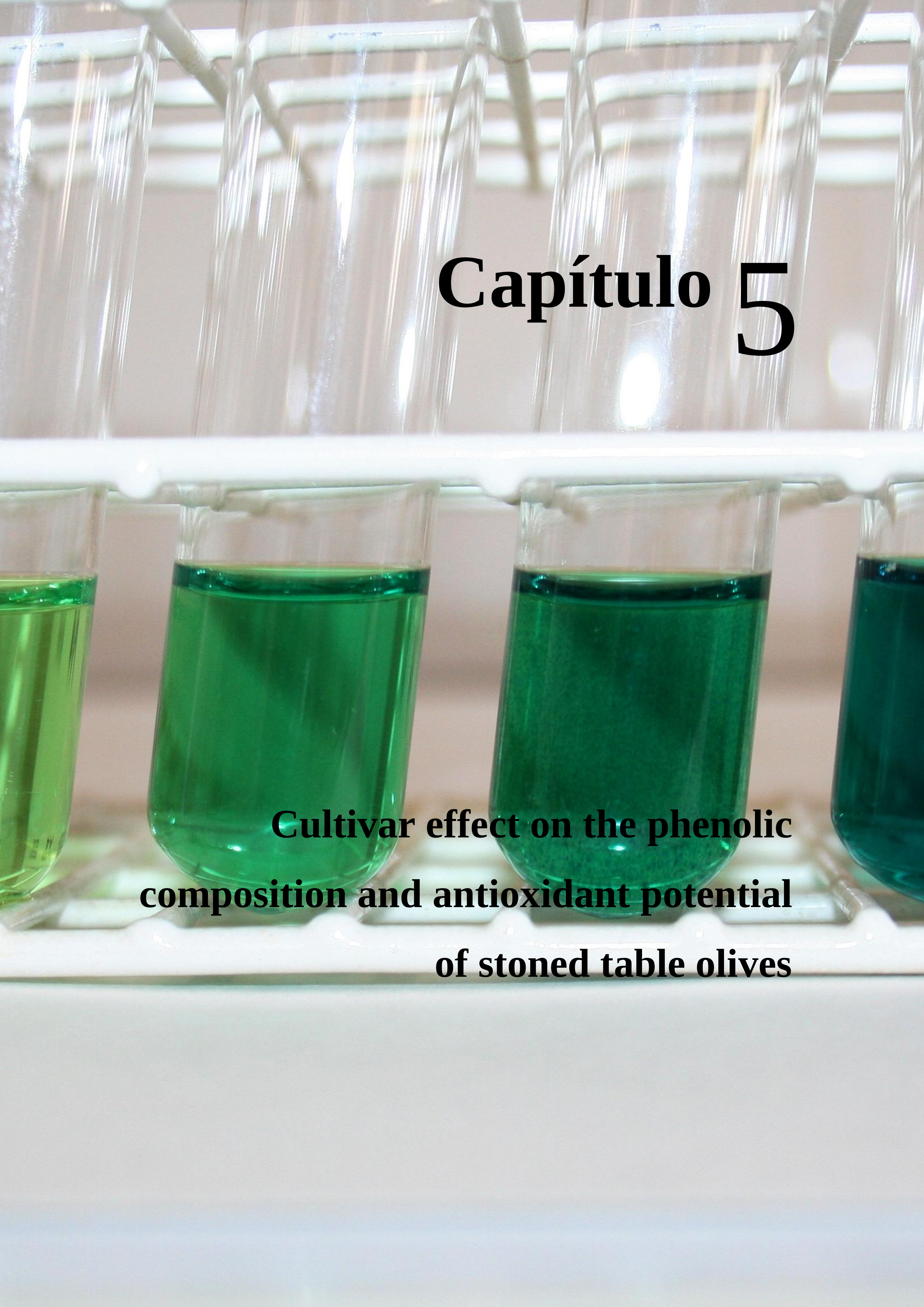

Capítulo 5

Cultivar effect on the phenolic composition and antioxidant potential of stoned table olives

Cultivar effect on the phenolic composition and antioxidant potential of stoned table olives.

Abstract

Stoned green table olives "*alcaparras*" prepared from five different varieties (*Cv.* Cobrançosa, Madural, Negrinha de Freixo, Santulhana and Verdeal Transmontana) were investigated concerning their phenolic composition and antioxidant potential. From each variety, five independent lots were prepared. The phenolic profile was determined by HPLC/DAD at 280 nm, and aantioxidant potential measured using the reducing power and scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals assays. Twelve phenolic compounds were identified, being hyrdoxytyrosol the most abundant one, followed by verbascoside and tyrosol. *Cv.* Cobrançosa and Santulhana reported higher content of phenolic compounds, with 165.76 and 163.66 mg/kg of fresh "*alcaparras*" table olives respectively. Regarding antioxidant activity, *Cv.* Santulhana and Cobrançosa showed higher EC_{50} values, lower than 1.40 and 0.48 mg/mL for reducing power and DPPH methods, respectively. Significant negative correlations were obtained between olive phenolics and EC_{50} values from the antioxidant activity. The direct contact of the pulp with water, characteristic of this processing method, eliminates important hydrossoluble compounds, being the cultivar used an important determinant for the final "*alcaparras*" composition in terms of ingested phenolic compounds and antioxidant activity.

Keywords: *Olea europaea* L.; stoned table olives; olive cultivar; phenolic composition, antioxidant potential.

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5.1. Introduction

Olea europaea L. products, mainly olive oil and table olives, are very important components of the Mediterranean diet (Boskou *et al.*, 2006). Their postulated health benefits seem to be intrinsically linked to the high monounsaturated fat content (Bianchi, 2003) and to minor constitutes like tocopherols and phenolic compounds (Montaño *et al.*, 2005).

Phenolic compounds are of great importance for the olive fruit, being responsible for important characteristics and properties, such as color, taste and texture (Marsilio *et al.*, 2001). Several reports also highlight their important antioxidant capacity (Ben Othman *et al.*, 2009), antimicrobial activity (Sousa *et al.*, 2006), and protection against micotoxins effects (Beekrum *et al.*, 2003).

Several phenolic compounds have been indentified in table olives, including oleuropein and hydroxytyrosol (Briante *et al.*, 2002), tyrosol (Briante *et al.*, 2002), rutin (Boitia *et al.*, 2001), quercetin (Obied *et al.*, 2007), as well as caffeic (Papadopoulos & Boskou, 1991), vanillic and σ- and ρ-coumaric acids (Brenes *et al.*, 1999), among others. Olives phenolic composition, however, is highly variable in both quality and quantity (Uccella, 2001, Vinha *et al.*, 2005), in the dependence of several factors: processing method (Romero *et al.*, 2004), irrigation regimes (Patumi *et al.*, 2002), cultivar (Romani *et al.*, 1999), and maturation degree (Ryan *et al.*, 1999). For instance, important changes are reported to occur in the phenolic fraction during olive fruit development, with depletion of oleuropein and increasing of tyrosol and hydroxytyrosol concentrations (Esti *et al.*, 1998; Ferreira *et al.*, 2002; Piga *et al.*, 2001).

Three kinds of table olives are more representative in the international market: Spanish-style green olives in brine, Greek-style naturally black olives in brine, and Californian black ripe olives (Blekas *et al.*, 2002; Sabatini *et al.*, 2009). All processing methods influence the phenolic composition of table olives reducing its content by different ways. In the Spanish-style green olive processing, Brenes et al. (1995) studied the changes in phenolic compounds and noticed that the NaOH treatment hydrolyzed oleuropein into hydroxytyrosol and elenolic acid glucoside, and that caffeic acid, oleuropein, and *p*-coumaric acid contents reduce during fermentation period, while tyrosol concentration remained constant (Brenes *et al.*, 1995). Marsilio *et al.* (2001) showed that Californian-style ripe olive processing also influences the phenolic composition. In particular, vanillic acid and oleuropein content decreased while tyrosol and hydroxytyrosol increased. Although the bacterial metabolism in the fermenting brine seems to play an important role, the washing step to remove the excess of NaOH (Marsilio *et al.*, 2001) was also the most implicated processing step. Romero *et al.* (2004) demonstrated that the main phenolic compounds before fermentation naturally black olives (Greek-style) were hydroxytyrosol-4-*β*-glucoside, oleuropein, hydroxytyrosol, tyrosol, salidroside, and verbascoside, while after 12 months the main phenolic was hydroxytyrosol, followed by hydroxytyrosol acetate, tyrosol, and tyrosol acetate.

"*Alcaparras*" are a kind of stoned green table olives processed by a traditional method in Trás-os-Montes region, highly appreciated and commercialized in local markets. For their production healthy green or yellow-green olive fruits are used, and, are broken to remove the stone. The pulp is immersed in water to remove natural bitterness being changed daily until achieve edible grade. Commercial "*alcaparras*" table olives, a blend of several olive cultivars, were already studied for their phenolic composition, with three flavonoidic compounds identified: luteolin 7-O-glucoside, apigenin 7-O-glucoside, and luteolin (Sousa *et al.*, 2006). They have also showed antioxidant properties and antimicrobial activity (Sousa *et al.*, 2008). Nevertheless, important variations were observed in their composition and sensorial attributes (data not published), highlighting the importance of a more dedicated work on the factors involved. Therefore, the present paper aimed to study the effects of olive cultivar on the phenolic composition and antioxidant activity of "*alcaparras*" produced by the traditional method in Trás-os-Montes region (Northeast of Portugal).

5.2. Material and Methods

5.2.1. Reagents and standards

Methanol, 2,2-diphenyl-1-picrylhydrazyl and iron (III) chloride were obtained from Sigma-Aldrich (St. Louis, USA). Methanol (HPLC grade), sodium dihydrogen phosphate dihydrate, potassium hexacyanoferrate (III), formic acid 98-100% were purchased from Merck (Darmstadt, Germany). Hydrochloric acid and di-sodium hydrogen phosphate 2-hydrate were obtained from Panreac (Barcelona, Spain). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). Hydroxytyrosol, tyrosol, chlorogenic acid, vanillic acid, syringic acid, verbascoside, luteolin 7-O-glucoside, oleuropein, rutin, apigenin 7-O-glucoside, quercetin and luteolin standards, used for phenolic profile identification were obtained from Extrasynthèse (Genay, France).

5.2.2. Stoned table olives "Alcaparras" sampling and preparation

For this study, five of the most representative olive cultivars (*Cv.* Cobrançosa, Madural, Negrinha de Freixo, Santulhana and Verdeal Transmontana) from Trás-os-Montes region were collected in September and October of 2006 from different olive groves subjected to similar agro-climatic and agronomic conditions. From each cultivar, five independent lots of olives, approximately of 5 kg each, were collected from several trees and immediately transported to the laboratory. At the laboratory, approximately 2 kg of stoned table olives were prepared from each lot. Only green or yellow-green healthy olive fruits were used, being manually broken to separate the pulp from the stone. The pulp was immersed in water during a week, daily changed, to remove olives bitterness. After the treatment, "*alcaparras*" table olives were frozen at -20º C and freeze dried (Ly-8-FM-ULE, Snijders) prior analysis.

5.2.3. Extraction preparation

For each sample, three freeze dried powdered sub-samples (~ 5 g; 20 mesh) were extracted with 250 mL of boiling water for 45 min and filtered through Whatman nº 4 paper. The aqueous extracts were weight, frozen, and lyophilized and again dissolved in water in concentrations ranging from 0.01 and 5 mg/mL for antioxidant activity assay and 50 mg/mL for phenolic profile evaluation.

5.2.4. Identification and quantification of phenolic compounds

Phenolic profile was performed by HPLC analysis on a Knauer Smartline separation module equipped with a Knauer smartline autosampler 3800, a cooling system set to 4ºC and a Knauer DAD detector. Data acquisition and remote control of the HPLC system was done by ClarityChrom® software (Knauer, Berlin, Germany). A reversed-phase Spherisorb ODS2 column was used (250 mm × 4 mm id, 5 µm particle diameter, end-capped Nucleosil C18 (Macherey-Nagel) maintained at 30 ºC (Gecko 2000)**.** The solvent system used was a gradient of water/formic acid (19:1) (A) and methanol (B), which were previously filtered and degasseddegassed and filtered. The flow rate was 0.9 mL/min with the following gradient: 5% B at 0 min, 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 40% B at 39 min, 45% B at 42 min, 45% B at 45 min, 47% B at 50 min, 48% B at 60 min, 50% B at 64 min and 100% B at 66 min. For the HPLC analysis the aqueous extracts were dissolved in methanol, in a reason of 50 mg/mL. All samples were filtered through a 0.2 µm Nylon membrane (Whatman) and 10 µL of each solution were injected. Chromatographic data was recorded at 280 nm. Spectral data from all peaks were accumulated in the 200–400 nm range. Phenolic compounds were identified by comparing the retention times and spectrums of the chromatographic peaks with those of authentic standards analyzed under the same conditions. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards.

5.2.5. Scavenging effect assay

The capacity to scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was monitored according to the method of Hatano *et al.* (1988). The extract solution (0.3 mL) was mixed with 2.7 mL of methanolic solution containing DPPH radicals (6×10^{-5} mol/L). The mixture was shaken vigorously and left to stand for 60 min at room temperature in dark (until stable absorbance values were obtained). The reduction of the DPPH-radical was measured by continuous monitoring of the absorption decrease at 517 nm.

DPPH scavenging effect was calculated as the percentage of DPPH discoloration using the following equation: % scavenging effect = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration in the solution.

5.2.6. Reducing power assay

The reducing power was determined according to a described procedure (Berker *et al*., 2007). The extract solution (1 mL) was mixed with 2.5mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 ºC for 20 min. After cooling, 2.5 mL of 10% trichloroacetic acid (w/v) were added and the mixture was centrifuged at 1000 rpm for 8 min (Centorion K24OR-2003 refrigerated centrifuge). The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm (higher absorbance readings indicate higher reducing power). Extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration in the solution.

5.2.7. Statistical analysis

A regression analysis, using Excel from Microsoft Corporation, was established between phenolic contents of the different olive cultivars and EC_{50} values obtained from the two antioxidant assays tested. A principal component analysis (PCA) and ANOVA were carried out using SPSS 17.0 software.

5.2.7.1. Analysis of variance

A regression analysis, using Excel from Microsoft Corporation, was established between phenolic contents of the different olive cultivars and EC_{50} values obtained from the two antioxidant assays tested. A principal component analysis (PCA) and ANOVA were carried out using SPSS 17.0 software.

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 17.0 (SPSS, Inc.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov-Smirnov with Lilliefors correction (if n>50), and the Levene´s tests, respectively. All dependent variables were analyzed using a one-way ANOVA with or without Welch correction, depending if the requirement of the homogeneity of variances was fulfilled or not. The main factor studied was the effect of olive cultivar on the phenolic compounds profile, EC_{50} values of the two antioxidant assays tested and extraction yield, and, if a statistical significant effect was found, means were compared using Tukey´s honestly significant difference multiple comparison test or Dunnett T3 test also depending if equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

5.3. Results and discussions

5.3.1. Identification and Quantification of Phenolic Compounds

The study of the phenolic composition of "*alcaparras*" table olives produced from different olive cultivars by HPLC/DAD revealed different qualitative and quantitative chemical profiles, in which twelve phenolic compounds were identified and quantified: hydroxytyrosol, tyrosol, chlorogenic acid, vanillic acid, syringic acid, verbascoside, luteolin 7-O-glucoside, oleuropein, rutin, apigenin 7-O-glucoside, quercetin and luteolin (Figure 1 and 2).

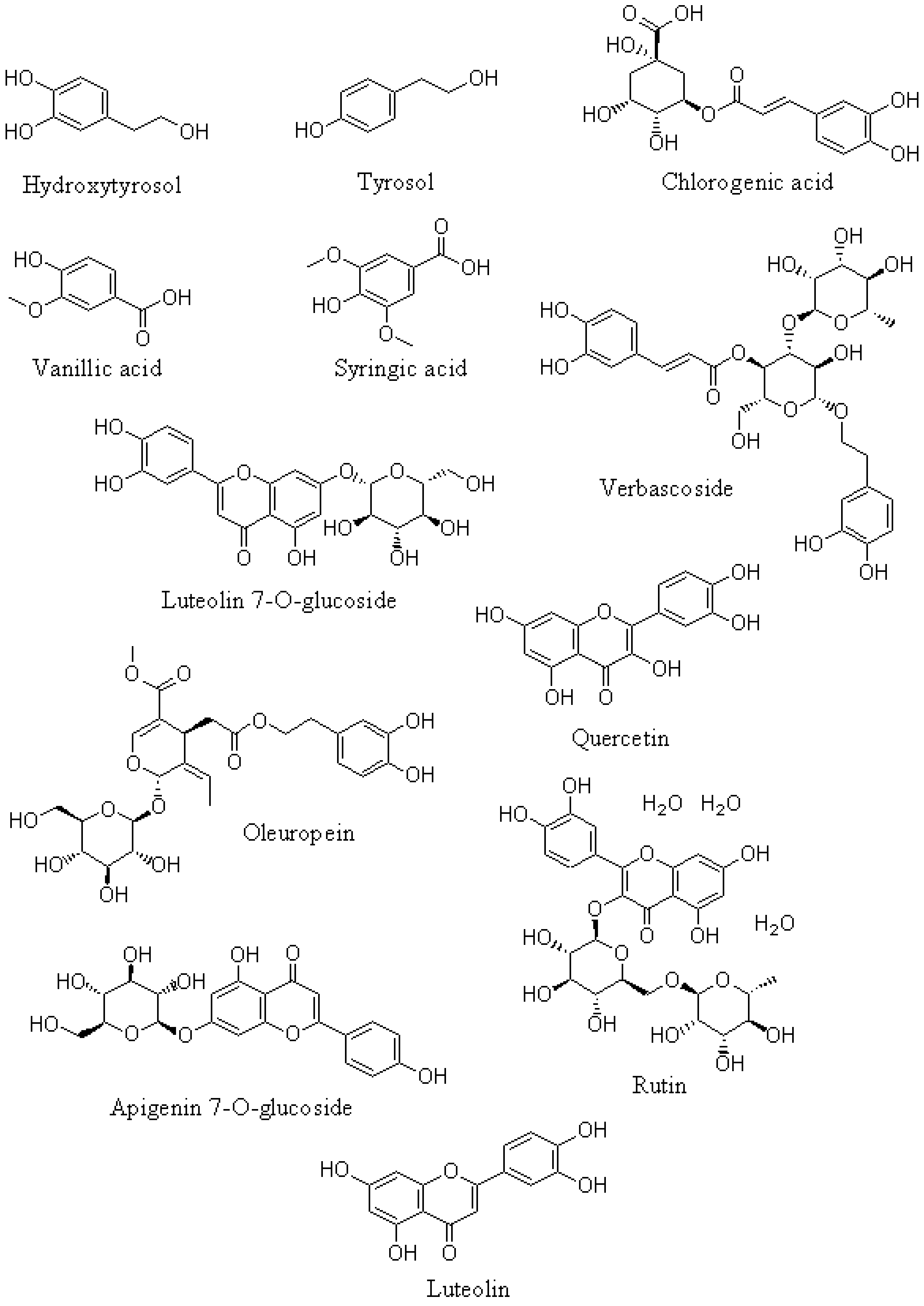

Figure 1. Chemical structures of the phenolic compounds analyzed.

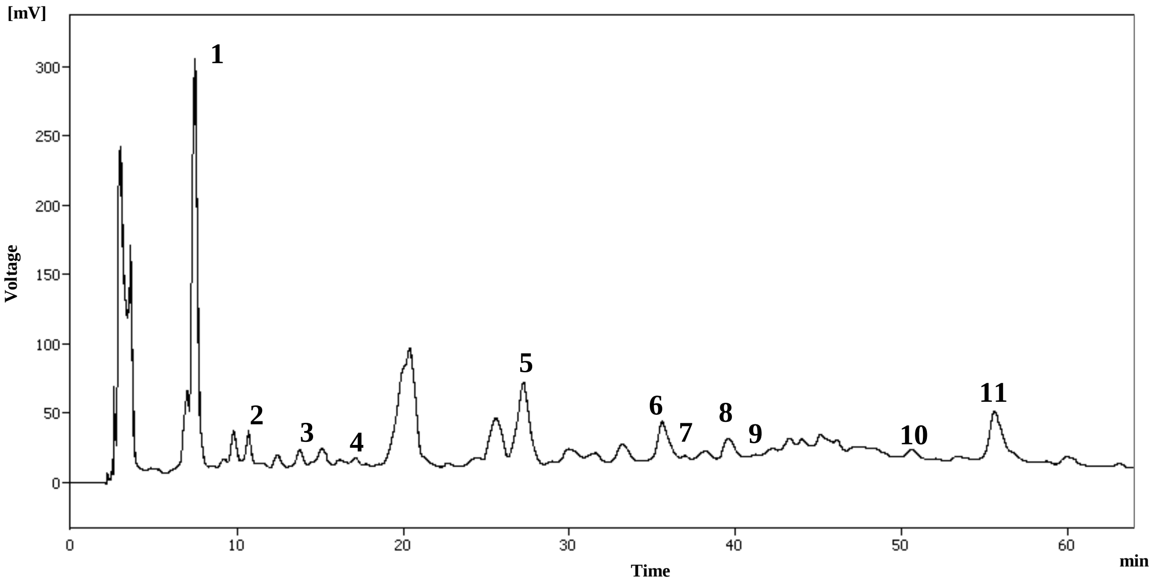

Figure 2. HPLC chromatogram of phenolic extracts of *Cv.* Cobrançosa. 1: hydroxytyrosol; 2: tyrosol; 3: clorogenic acid; 4: vanillic acid; 5: verbascoside; 6: luteolin 7-O-glucoside; 7: oleuropein; 8: rutin; 9: apigenin 7-O-glucoside; 10: quercetin; 11: luteolin.

Total and individual amounts of phenolic compounds are reported in Table 1, that are significantly affected ($P < 0.001$), with the exception of quercetin, by the olive variety used for table olive processing.

Table 1: Phenolic profile (mg/kg of fresh weight) of different cultivars of traditional stoned green table olives *"alcaparras"*.

Phenolic compound	Cobrançosa	Madural	Negrinha de Freixo	Santulhana	Verdeal Transmontana	*P* - Value
Hydroxytyrosol	75.27 ± 9.43 c	73.90 ± 21.55 b,c	24.73 ± 1.87 a	103.93 ± 8.46 b	84.41 ± 4.27 c	$< 0.001^{(1)}$
Tyrosol	11.20 ± 1.00 b	11.11 ± 4.20 a-c	5.48 ± 0.57 a	13.86 ± 1.52 c	13.49 ± 0.82 c	$< 0.001^{(1)}$
Chlorogenic Acid	1.36 ± 0.49 a,b	1.11 ± 0.34 a,b	0.84 ± 0.06 a	1.08 ± 0.11 b	1.29 ± 0.23 b	$0.001^{(1)}$
Vanillic Acid	tr.	-	tr.	-	-	-
Syringic Acid	tr.	-	tr.	-	-	-
Verbascoside	29.83 ± 8.34 b,c	6.22 ± 2.88 a	6.91 ± 3.53 a	28.39 ± 2.74 c	23.0 ± 0.68 b	$< 0.001^{(1)}$
Luteolin 7-O-glucoside	16.15 ± 2.13 b	tr.	2.49 ± 1.41 a	2.15 ± 2.63 a	3.49 ± 0.25 a	$< 0.001^{(1)}$
Oleuropein	tr.	tr.	-	-	19.89 ± 6.35	-
Rutin	13.97 ± 1.87 c	9.31 ± 1.17 b	14.45 ± 3.72 b,c	4.57 ± 2.37 a	tr.	$< 0.001^{(1)}$
Apigenin 7-O-glucoside	0.91 ± 0.82	tr.	2.10 ± 0.58	tr.	3.28 ± 0.65	$< 0.001^{(2)}$
Quercetin	6.39 ± 1.60	7.39 ± 2.35	tr.	5.99 ± 1.82	8.58 ± 0.60	$0.079^{(2)}$
Luteolin	7.49 ± 0.72 b	3.61 ± 1.19 a	7.54 ± 4.69 a,b	3.65 ± 1.12 a	1.92 ± 1.49 a	$< 0.001^{(1)}$
Total	165.76 ± 10.58 c	112.76 ± 22.81 b	66.45 ± 11.97 a	163.66 ± 16.62 c	160.24 ± 9.43 c	$< 0.001^{(1)}$

tr. – Traces. $^{a\text{-}c}$Means within a line with different superscripts differ, $P < 0.05$. (1)*P*-values are those for the effect of cultivar on the phenolic profile of "*alcaparras*" table olives from one-way Welch ANOVA analysis. If there was a significant effect of cultivar on the phenolic compounds data, then means were compared by Dunnett T3´s test, since equal variances could not be assumed ($P < 0.05$ by means of Levene test). $^{(2)}P$-values are those for the effect of cultivar on the phenolic profile of “*alcaparras*” table olives, from one-way ANOVA analysis. If there was a significant effect of cultivar on the phenolic compounds data, then means were compared by Tukey´s test, since equal variances could be assumed ($P > 0.05$ by means of Levene test).

Total phenolics ranged from 66.45 to 165.76 mg/kg (fresh weight), corresponding to *Cv.* Negrinha de Freixo and Cobrançosa, respectively (Table 1). Among the phenolic compounds identified, the most abundant were hydroxytyrosol, tyrosol and verbascoside. Depending on the olive cultivar, hydroxytyrosol comprised from 37 to 66% of all quantified phenolic compounds. Such results are in accordance with literature, once that hydroxytyrosol is the main phenolic compound in processed table olives (Romero *et al.*, 2004). This phenolic alcohol shown several biological properties, such as down-regulation of the immunological response (D´Angelo *et al.*, 2005), preventing human erythrocytes from oxidative damage induced by hydrogen peroxide (Zhang *et al.*, 2008), anti-inflammatory, antithrombotic, and hypochlolesterolemic effects in rats (Covas *et al.*, 2006; Deiana *et al.*, 2008; Visioli *et al.*, 1998). Rice-Evans *et al.* (1997) referred that acting as a free radical scavenger in olives, hydroxytyrosol could help preventing ageing and could reduce the damaging of iron- and nitric oxide-induced cytotoxicity.

Oleuropein, the main phenolic compound in fresh olive fruits (Vinha *et al.*, 2005), was also identified in *Cv.* Verdeal Transmontana “*alcaparras*”, comprising approximately 12% of all the phenolic compounds identified in this olive cultivar (19.89 mg/kg). The late maturation characterizing this cultivar could be responsible for higher amounts oleuropein at harvest time (not analyzed), being processed with green olives, the presence of oleuropein is expected in “*alcaparras*” table olives. In the remaining olive cultivars oleuropein was not found or present in vestigial amounts. Oleuropein is the main phenolic compound responsible for olives bitterness, and it is removed to turn olives edible, which explains low amounts of oleuropein in processed table olives. Meanwhile, oleuropein is hydrolyzed to hydroxytyrosol and tyrosol during fruit development (Ferreira *et al.*, 2002; Piga *et al.*, 2001), contributing to the presence of those compounds in table olives.

Some differences were noticed in the studied olive cultivars of “*alcaparras*” table olives. Syringic acid was only present in vestigial amounts in *Cv.* Negrinha de Freixo and Cobrançosa. Verbascoside vary in the studied samples from 6.22 to 29.83 mg/kg

(5.5 and 18% of all phenolic compounds identified) in *Cv.* Madural and Cobrançosa, respectively. Rutin and quercetin were identified in all olive cultivars but with vestigial amounts in *Cv.* Verdeal Transmontana and in *Cv.* Negrinha de Freixo, respectively. Such changes on both quantitative and qualitative fractions of phenolic compounds in the studied table olives are related to olive cultivar (Pereira *et al.*, 2006).

Some works studying the phenolic composition were conducted using Portuguese (Pereira *et al.*, 2006) and Greek (Boskou *et al.*, 2006) table olives. Comparing our results with those obtained in the mentioned studies, we have a poorer phenolic fraction. However, compared with commercial "*alcaparras*" table olives (blend of several cultivars) higher number of phenolic compounds were identified and the monocultivar "*alcaparras*" also presents higher phenolics content (Sousa *et al.*, 2006). Such fact could be explained due to the processing that "*alcaparras*" table olives are subjected to achieve edible grade. In opposition to the generalized table olives preparing methods, "*alcaparras*" are processed after being destoned, therefore more exposed to losses by lixiviation during the washing steps. While essential for bitterness removal, characteristic of green unripe olives, the loss of hydrossoluble compounds is inevitable. The cultivar phenolic amount is, therefore, of major importance for the residual amounts of phenolics in processed "*alcaparras*".

5.3.2. Antioxidant activity

The antioxidant activity of traditional stoned green table olives "*alcaparras*" was measured using two different chemical assays: reducing power and scavenging effect on DPPH free radicals. The results obtained are expressed as EC_{50} values (mg/mL) and are reported in Table 2.

Table 2: Extraction yield and EC_{50} values (mg/mL) of aqueous extracts of traditional stoned table olives, "*alcaparras*", from Cobrançosa, Madural, Negrinha de Freixo, Santulhana, and Verdeal Transmontana cultivars.

Cultivar	**Extraction yield (%)**	**Reducing power (EC_{50}^{a})**	**DPPH (EC_{50}^{b})**
Cobrançosa	10.11 ± 0.171 a	1.38 ± 0.165 a,b	0.48 ± 0.028 a
Madural	13.03 ± 0.435 b	1.47 ± 0.020 a,b	0.64 ± 0.044 b
Negrinha de Freixo	13.66 ± 0.253 b	3.08 ± 0.126 c	1.16 ± 0.107 d
Santulhana	9.88 ± 0.174 a	1.40 ± 0.070 a	0.46 ± 0.024 a
Verdeal Transmontana	9.74 ± 0.061 a	1.61 ± 0.035 b	0.76 ± 0.014 c
***P* - Value**	< 0.001$^{(1)}$	< 0.001$^{(1)}$	<0.001$^{(1)}$

^{a}EC_{50} (mg/mL): effective concentration at which the absorbance is 0.5;
^{b}EC_{50} (mg/mL): effective concentration at which 50% of DPPH radicals are scavenged.
$^{a\text{-}d}$Means within a column with different superscripts differ, $P < 0.05$.

(1)*P*-values are those for the effect of cultivar on the antioxidant potential and extraction yield of "*alcaparras*" table olives from one-way Welch ANOVA analysis. If there was a significant effect of cultivar on the antioxidant potential and extraction yield data, then means were compared by Dunnett T3´s test, since equal variances could not be assumed ($P < 0.05$ by means of Levene test).

In the extracts of the olive cultivars studied, a concentration-dependent activity for reducing power assay was observed (Figure 3).

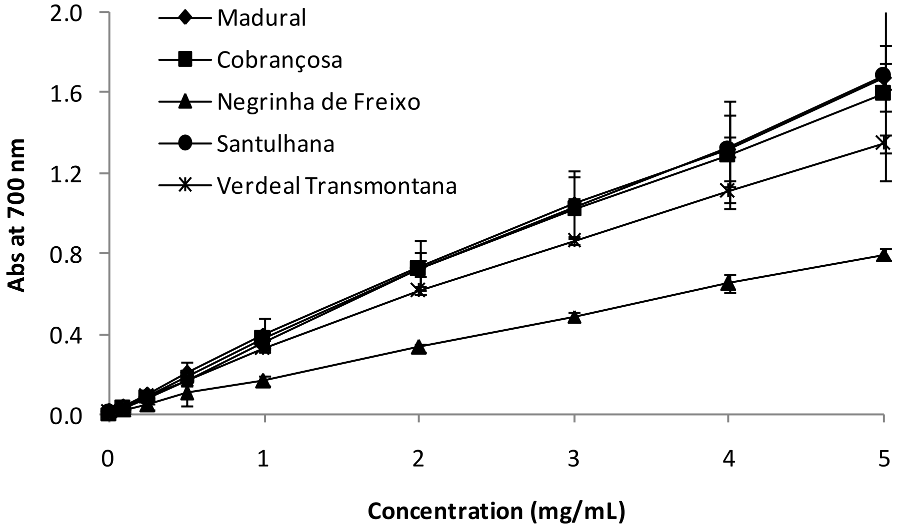

Figure 3. Reducing power values of different "*alcaparras*" table olives aqueous extracts (mean ± standard deviation, n=9).

Depending on the reducing power of the concentrations used the yellow color of the test solution changes to green and blue. This change is due to the presence of reducers, such as compounds with antioxidant properties, that leads to the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Pereira et al., 2006). For the reducing power method, "*alcaparras*" table olives showed high reducing powers at very low concentrations (<2mg/mL), except *Cv.* Negrinha de Freixo. *Cv.* Cobrançosa and Santulhana reported higher reducing power, which means higher antioxidant activity and lower EC_{50} values, 1.38 and 1.40 mg/mL, respectively. Meanwhile when the EC_{50} values were converted in the amount of olive pulp, less quantity was reported by *Cv.* Madural (0.044g). Such results are related to the extraction yields of each cultivar *Cv.* Cobrançosa and Santulhana reported 0.053 and 0.051g respectively. For *Cv.* Negrinha de Freixo were needed nearly 0.1g of olive pulp to obtain the EC_{50} value.

Regarding DPPH method, the scavenging effect of "*alcaparras*" aqueous extracts on DPPH free radicals also showed a concentration-dependent activity, especially for concentrations below 2 mg/mL (Figure 4). This method is an essential tool to access the antioxidant potential, more specifically, the antiradical activity of extracts. The scavenging activity of free radicals of DPPH was expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absorbance of extract at 517 nm (Figure 4).

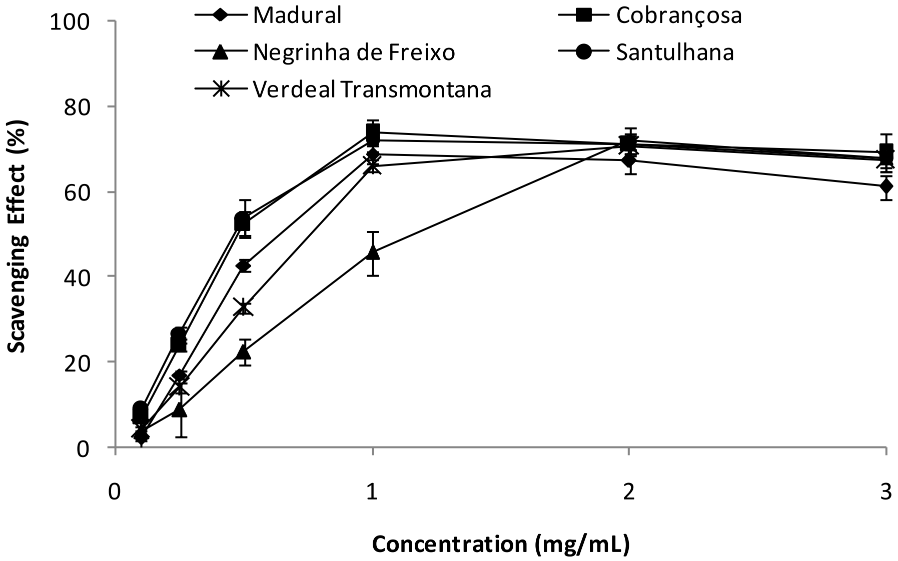

Figure 4. Scavenging effect on DPPH free radicals of different "*alcaparras*" table olives aqueous extracts (mean ± standard deviation, n=9).

Extracts from *Cv.* Cobrançosa and Santulhana displayed higher antioxidant activity, scavenging 50% of the free radicals of DPPH at very low concentrations [EC_{50} values: 0.48 (0.018g) and 0.46 mg/mL (0.017g) respectively (Table 2)]. Once more *Cv.* Negrinha de Freixo reported higher EC_{50} value, 1.16 mg/mL (0.035g), and consequently reported lower antioxidant activity.

In the aggregate of all the olive cultivars studied and with the results obtained in the two antioxidant assays, the antioxidant activity for the different olive cultivars followed the order *Cv.* Cobrançosa > Santulhana > Madural > Verdeal Transmontana > Negrinha de Freixo (Table 2). The results obtained in the antioxidant potential could be related, at least in part, to the phenolic compounds found in the different olive cultivars. Total phenolic content in the olive cultivars was reported as follows Cobrançosa > Santulhana > Verdeal Transmontana > Madural > Negrinha de Freixo (Table 1). Indeed, *Cv.* Negrinha de Freixo reported simultaneously lower total phenolics content and lower antioxidant activity, while *Cv.* Santulhana reported higher total phenolics content and higher antioxidant activity.

Comparing the antioxidant activity obtained in varietal stoned table olives with a previous work conducted with commercial ones by our research group (Sousa et al., 2008) similar results were observed on DPPH assay. Meanwhile, our results for reducing power assay demonstrated lower activity than commercial "*alcaparras*" (0.42 mg/mL). The same was observed when were compared with other kinds of Portuguese table olives, due to similar activity on the DPPH methods and worst results on reducing power method (Pereira et al., 2006).

The differences observed can be related to the aqueous treatment applied to turn the olives edible. Other fact that can explain the differences obtained is the possible existence of a potential synergy among the several cultivars that constitute the commercial "*alcaparras*" table olives. This relation may be responsible for higher antioxidant activity then isolated cultivars.

5.3.3. Correlation between phenolic composition and antioxidant activity

When a regression analysis was performed between the values of EC_{50} obtained in the antioxidant evaluation and the amounts of phenolic compounds found, hydroxytyrosol, tyrosol and verbascoside reported extremely significant correlations (*P* < 0.001) with the antioxidant activity presented by "*alcaparras*" extracts (Table 3).

Table 3: Correlation between phenolic compounds of "*alcaparras*" table olives and respective antioxidant activity.

	EC_{50} DPPH			**EC_{50} Reducing Power**		
Phenolic compound	**Equation**	**R^2**	**P^***	**Equation**	**R^2**	**P^***
Hydroxytyrosol	$y = -0.008x + 1.270$	0.650	***	$y = 0.373x + 0.050$	0.841	***
Tyrosol	$y = -0.052x + 1.274$	0.454	***	$y = -0.119x + 3.072$	0.404	***
Clorogenic Acid	$y = -0.322x + 1.071$	0.146	*	$y = -0.681x + 2.527$	0.108	n. s.
Vanillic Acid	$y = -0.202x + 0.717$	0.022	n. s.	$y = -0.518x + 1.784$	0.023	n. s.
Siringic Acid	$y = 0.120x + 0.664$	0.159	*	$y = 0.346x + 1.634$	0.220	**
Verbascoside	$y = -0.016x + 1.002$	0.408	***	$y = -0.028x + 2.229$	0.222	**
Luteolin 7-O-glucoside	$y = -0.017x + 0.792$	0.136	*	$y = -0.026x + 1.886$	0.052	n. s.
Oleuropein	$y = 0.001x + 0.670$	0.001	n. s.	$y = -0.016x + 1.836$	0.037	n. s.
Rutin	$y = 0.012x + 0.601$	0.065	n. s.	$y = 0.045x + 1.362$	0.153	*
Apigenin 7-O-glucoside	$y = 0.110x + 0.560$	0.281	**	$y = 0.198x + 1.463$	0.150	*
Quercetin	$y = -0.044x + 0.970$	0.224	**	$y = -0.152x + 2.654$	0.431	***
Luteolin	$y = 0.025x + 0.586$	0.081	n. s.	$y = 0.092x + 1.307$	0.186	*
Total phenolics	$y = -0.005x + 1.395$	0.617	***	$y = -0.011x + 3.279$	0.501	***

n. s. – not significant. $^{*}P \leq 0.05$ - significant correlation. $^{**}P \leq 0.01$ - very significant correlation. $^{***}P \leq 0.001$ - extremely significant correlation.

Although, this results doesn't mean that the minor phenolic compounds do not contribute to the overall antioxidant activity of "*alcaparras*" table olives, but in this case the major ones would definitively be the main intervenient. Several works demonstrated the antioxidant activity of hydroxytyrosol (D'Angelo et al., 2005; Obied et al., 2008; O'Dowd et al., 2004; Pereira-Caro et al., 2009; Visioli et al., 1998), tyrosol (Di Benedetto et al., 2007; Giovannini et al., 1999; González-Santiago et al., 2010; Owen et al., 2000) and verbascoside (Funes et al., 2009; Aldini et al., 2006), confirming that these compounds exhibits important antioxidant capacity.

Significant correlations were obtained for EC_{50} values of reducing power (R^2 = 0.501; *P* < 0.001) and DPPH (R^2 = 0.617; *P* < 0.001) assays. Correlations were also established between the individual phenolic compounds and the antioxidant assays tested.

Although other minor antioxidants could influenced and contribute to the results obtained, like α-tocopherol (Sakouhi et al., 2008), hydroxytyrosol is known to be one of the phenolic compounds with higher antioxidant capacity (González-Santiago et al., 2006).

5.3.4. Discrimination of olive cultivar based in phenolic composition and antioxidant activity

In order to access the variation of the phenolic composition and antioxidant activity of "*alcaparras*" table olives produced from *Cv.* Cobrançosa, Madural, Negrinha de Freixo, Santulhana and Verdeal Transmontana, a principal component analysis (PCA) was performed on the results obtained.

The PCA was applied in order to reduce the number of variables (13 variables corresponding to the phenolic compounds profile and antioxidant values for both methods) to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information. Moreover, it allowed recognizing patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores).

The aim of the PCA is to produce components suitable to be used as predictors or response variables in subsequent analysis. The number of factors to keep in data treatment was evaluated by the Scree plot, taking into account the eigenvalues, which should be greater than one for retaining the factor in the analysis, the total percentage of variance explained by the number of components selected and finally its internal consistency by means of αCronbach's value, that should be positive (Maroco, 2003; Rencher, 1995).

PCA showed that 63.2% of the total variance of the data could be explained using only two principal components. A two-dimensional plane of the two principal components factors scores obtained is shown in Figure 5.

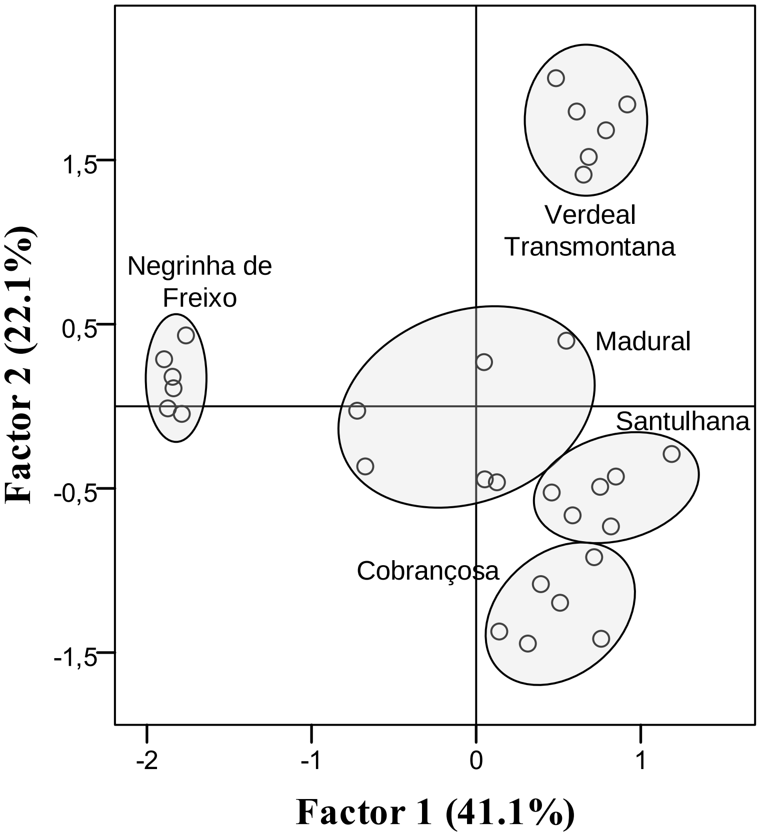

Figure 5. PCA of the phenolic compounds, total phenolic compounds, EC_{50} values of reducing power and DPPH methods in the olive cultivars studied. The plain contains 63.2% of the total variance.

The plane shows that the cultivars separation is possible. The first principal component factor separates the olive cultivars into two main groups, *Cv.* Negrinha de Freixo represented in the negative region, and the remaining olive cultivars represented mainly in the positive region. The second principal component factor allowed separating *Cv.* Verdeal Transmontana in the positive region from the remaining olive cultivars represented in the negative region, while *Cv.* Negrinha de Freixo and Madural are represented in both regions. *Cv.* Cobrançosa is mainly represented in the positive and negative regions of the first and second principal components respectively, due to higher content in and high contents of rutin and luteolin phenolic compounds. *Cv.* Santulhana represented above *Cv.* Cobrançosa is mainly characterized by higher concentration in hydroxytyrosol, tyrosol and high total phenolic compounds content. *Cv.* Verdeal Transmontana is shown in both positive regions of the two principal components due to being richer in oleuropein. *Cv.* Negrinha de Freixo is represented in the extreme negative region of the first principal component factor due to presenting higher EC_{50} values in both antioxidant assays, presenting lower antioxidant capacity. In the opposite region are represented *Cv.* Cobrançosa and Santulhana, once these cultivars reported lower EC_{50} values and higher antioxidant capacity.

5.4. Conclusions

The cultivar affects both quantitative and qualitative phenolic factions of these table olives, reporting unique and characteristic phenolic profile. These phenolic fractions also influenced and allowed to differentiate the total antioxidant activity observed in the cultivars. Both antioxidant potential and phenolic profile of the different cultivars of "*alcaparras*" table olives allowed differentiating them through PCA. With such results we can say that "*alcaparras*" table olives are a good source of important bioactive compounds, such as phenolic compounds which can contribute for the prevention of diseases in which free radicals are involved. A technological factor could be associated to the reduced amounts of the phenolic compounds found, like in the most other common methods available. According to our knowledge, this is the first time that the effect of the olive cultivar used to produce traditional green stoned "*alcaparras*" table olives in the antioxidant potential and in the phenolic profile is reported. Regarding antioxidant activity and phenolic composition, *Cv.* Negrinha de Freixo showed to be less suitable for this kind of technological process, while *Cv.* Cobrançosa and Santulhana reported better results. Meanwhile, in order to find the more adequate olive cultivar to produced "*alcaparras*" table olives, besides antioxidant potential and phenolic composition, the chemical composition, nutritional value and sensorial parameters should be considered as well.

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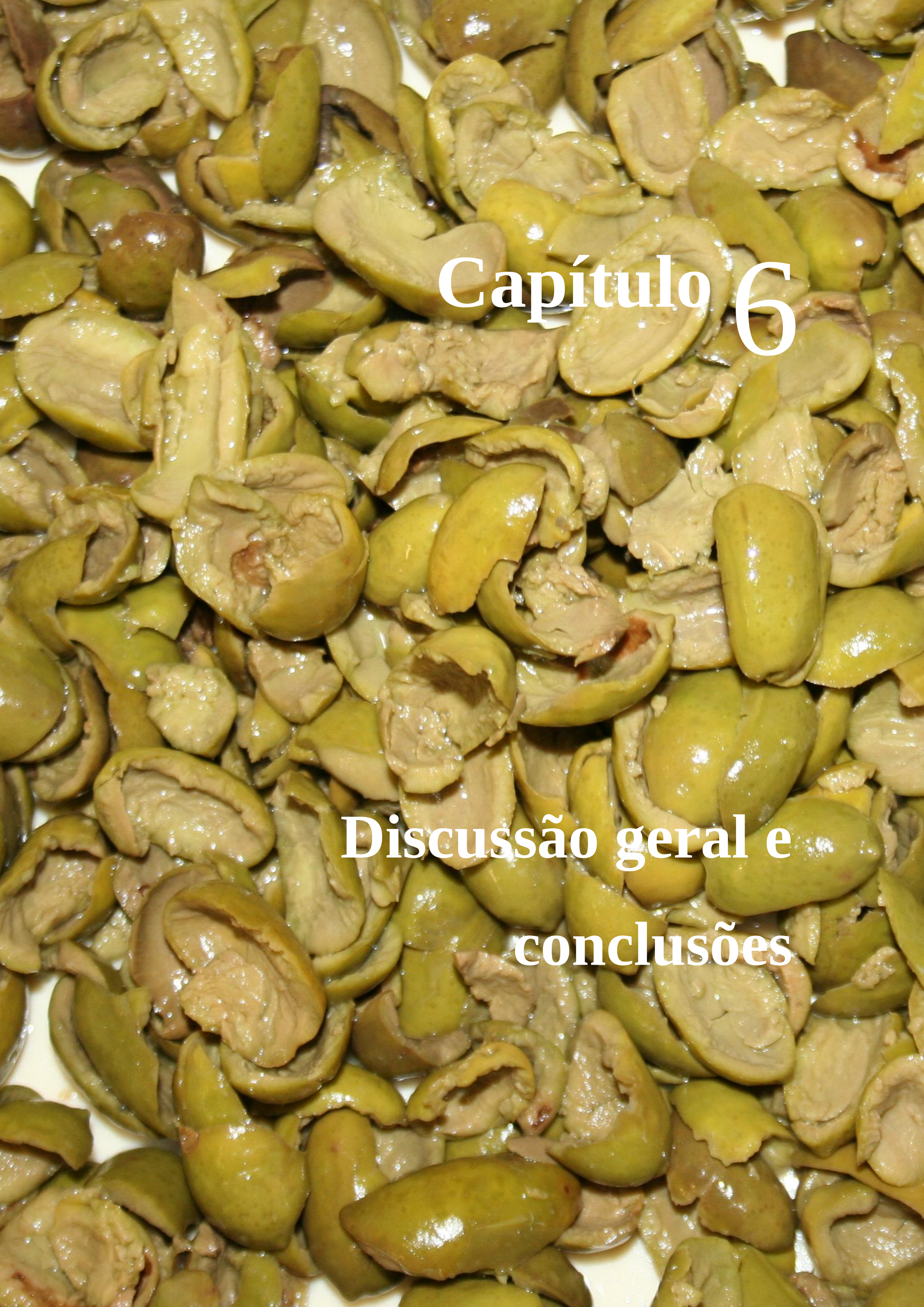
Capítulo 6

Discussão geral e conclusões



Discussão geral e conclusões

A produção de azeitonas verdes descaroçadas, "*alcaparras*", difere substancialmente dos três tipos comerciais mais representativos nos mercados internacionais (estilos Espanhol, Grego e Californiano) ao nível do processo produtivo. Este tipo de azeitonas não é sujeito a qualquer tratamento alcalino ou imerso em salmouras para a ocorrência de fermentações. O amargor característico dos frutos é retirado por imersões sucessivas em água, sendo a oleuropeína extraída da polpa por lixiviação. Desde logo este tipo de processamento confere características e composições distintas às azeitonas de mesa, nas quais a cultivar mostrou ser um factor influenciador.

A nível nutricional, as diferentes cultivares de "*alcaparras*" estudadas assemelham-se a outros tipos de azeitonas de mesa produzidas por diferentes métodos e estilos, excepto no teor em sal, substancialmente inferior neste tipo de azeitonas de mesa pela sua ausência no seu processamento. Este tipo de azeitonas de mesa é essencialmente composto por água e gordura, tendo a *Cv.* Verdeal Transmontana reportado o maior teor em gordura (20,1%). Embora este tipo de azeitonas de mesa seja uma fonte considerável de gordura, apresentam a vantagem de possuírem menor valor calórico comparativamente a outros tipos de azeitonas de mesa. Isto deve-se ao facto de os frutos de cada cultivar serem colhidos na altura de Setembro-Outubro, época em que os lípidos no interior do fruto não estão totalmente formados, possuindo os frutos ainda um elevado teor em humidade, o que consequentemente acarreta um menor valor calórico fornecido. Neste caso observou-se o efeito da cultivar na composição química e valor energético das "*alcaparras*", verificando-se valores calóricos entre 154 e 212 kcal/100 g de "*alcaparras*", respectivamente nas cultivares Madural e Verdeal Transmontana, e respectivamente as cultivares que apresentaram menor e maiores teores em gordura. A gordura das cultivares é um factor regulado geneticamente, intrínseco e característico de cada cultivar, levando a composições e valores nutricionais característicos.

Além de serem uma boa fonte de gordura, qualitativamente a gordura das "*alcaparras*" é excelente do ponto de vista nutricional. Independentemente da cultivar de azeitona que lhes deu origem, a gordura das "*alcaparras*" é maioritariamente composta por ácidos gordos monoinsaturados (MUFA > 67,9% em todas as cultivares) e apresentam um teor reduzido em ácidos gordos saturados (SFA). O ácido gordo maioritário foi o ácido oleico, tendo a cultivar Verdeal Transmontana apresentado maior teor (76,1%), tendo reportado também um maior rácio entre MUFA/SFA (5,17). Os perfis em ácidos gordos obtidos são em grande parte semelhantes aos dos azeites obtidos na região ("Azeite de Trás-os-Montes" D.O.P.) e característicos em relação a cada cultivar de azeitona estudada, podendo vir a ser uma ferramenta útil na detecção de adulterações e fraudes, actuando como marcadores de autenticidade.

A composição em tocoferóis apresentou valores inferiores aos normalmente reportados em diferentes azeitonas de mesa. O α-tocoferol foi o isómero mais abundante em todas as cultivares. A variação registada entre as cultivares advém da composição inicial em tocoferóis e possivelmente à reacção que cada uma tem em relação ao processo produtivo. A sua presença em quantidades reduzidas pode dever-se ao contacto com o ar aquando da etapa de descaroçamento dos frutos e em menor instância ao longo do processo de lixiviação dos compostos fenólicos. Parte dos tocoferóis poderão ter actuado como antioxidantes de modo a proteger os alvos lipídicos dos agentes pró-oxidantes tendo-se degradado e diminuído os seus teores. A cultivar Negrinha de Freixo apresentou maior teor em tocoferóis entre as cultivares estudadas (6,0 mg/kg de "*alcaparras*").

A nível sensorial, em praticamente todos os parâmetros avaliados (aroma, consistência, sabor e apreciação global), as cultivares Verdeal Transmontana e Negrinha de Freixo foram as preferidas pelo painel de consumidores. As aptidões da cultivar Negrinha de Freixo para a elaboração de azeitonas de mesa já eram conhecidas na região e a nível nacional, devido à existência da "Azeitona de Conserva Negrinha de Freixo D.O.P.", no entanto desconhecia-se tal facto quanto à cultivar Verdeal Transmontana. Esta preferência poderá estar relacionada com a composição destas cultivares, como o teor em gordura e em hidratos de carbono que tornam as azeitonas mais doces e suaves ao palato dos consumidores. Outro factor que está certamente relacionado com a preferência dos consumidores é a composição em compostos voláteis que influencia também a sua aceitabilidade. De entre os 42 compostos voláteis pertencentes a variadas famílias de compostos químicos, os compostos maioritários conotaram as cultivares com sensações verdes, a erva e frutos, facto que vai ao encontro do grau de maturação aquando da colheita dos frutos, principalmente na cultivar Verdeal Transmontana que é sobejamente conhecida por ter uma maturação tardia em relação às restantes cultivares. O perfil volátil obtido e sensorialmente perceptível pelos consumidores terá influenciado as suas preferências de encontro às cultivares Verdeal Transmontana e Negrinha de Freixo devido a um possível equilíbrio qualitativo e quantitativo entre as várias famílias de compostos identificados (álcoois, aldeídos, ésteres, cetonas, derivados de norisoprenóides, compostos terpénicos, sesquiterpenos e alcenos).

O perfil em compostos voláteis apresentado foi qualitativamente e quantitativamente característico de cada cultivar que tal como o perfil em ácidos gordos, permitiu realizar uma distinção entre as cultivares, tendo-se observado o efeito da cultivar uma vez mais.

Todas as cultivares de "*alcaparras*" demonstraram ter nas suas composições compostos com propriedades bioactivas, como é o caso dos compostos fenólicos. Verificou-se que o factor cultivar foi preponderante no perfil fenólico, uma vez que foram obtidos perfis característicos tanto em termos de tipo de compostos identificados como em termos das suas quantidades. As cultivares Cobrançosa e Negrinha de Freixo reportaram, respectivamente, maior e menor quantidade em compostos fenólicos por quilograma de "*alcaparras*" (165,76 e 66,45 mg/kg). Em relação a outro tipo de azeitonas de mesa, os teores dos diferentes compostos fenólicos são muito inferiores aos reportados. Como as cultivares foram colhidas ainda verdes, os compostos fenólicos caracteristicamente presentes em maiores quantidades poderiam ainda não se ter formado. Além disso, o processamento das "*alcaparras*" tem por vista a remoção de compostos fenólicos responsáveis pelo amargor das azeitonas que é devido principalmente à oleuropeína (composto fenólico maioritário em azeitonas verdes e precursor da formação de outros compostos fenólicos). Além da remoção da oleuropeína, a imersão em água poderá provocar uma lixiviação de outros compostos entre os quais compostos fenólicos.

As cultivares com maiores quantidades em compostos fenólicos demonstraram ter um potencial antioxidante mais elevado do que aquelas com menor teor. As cultivares Santulhana e Cobrançosa apresentaram maior actividade antioxidante, enquanto que a cultivar Negrinha de Freixo apresentou menor actividade antioxidante. Embora a cultivar Negrinha de Freixo tenha reportado maiores teores em tocoferóis que as restantes cultivares, este facto pode ser indicativo de que os compostos fenólicos possuem uma maior influência sobre a actividade antioxidante registada. Verificou-se então que a actividade antioxidante dos diferentes extractos foi influenciada pela cultivar que lhes deu origem e que está relacionada com a composição característica em compostos fenólicos. Sendo assim, a diferenciação entre as várias cultivares de azeitona foi obtida através da actividade antioxidante registada e os respectivos perfis em compostos fenólicos, podendo ser usados como outro potencial marcador de autenticidade.

Globalmente e através dos resultados obtidos pode-se afirmar que o factor cultivar deverá ser tido em conta aquando da produção de "*alcaparras*", não só como uma maneira de diversificar o produto, mas também uma maneira de o valorizar comercialmente. A cultivar Verdeal Transmontana foi a que melhor se adequou a este tipo de processamento. Apresentou um bom valor energético (212 kcal), com um teor de gordura considerável (20%), com um maior teor de ácidos gordos monoinsaturados e menor teor em ácidos gordos saturados entre as cultivares estudadas É a segunda cultivar que maior teor em vitamina E e sensorialmente foi a mais apreciada pelo painel de consumidores, apresentando um perfil em compostos voláteis equilibrado, com uma excelente actividade antioxidante e uma das cultivares com maior quantidade de compostos fenólicos.

Os dados obtidos neste trabalho contribuíram pela primeira vez para o estudo do efeito da cultivar na composição e actividade biológica de "*alcaparras*". Através dos resultados obtidos (ácidos gordos, compostos voláteis, perfil em compostos fenólicos e actividade antioxidante) e com o recurso ao uso de técnicas estatísticas (PCA e LDA) foi possível diferenciar e discriminar perfeitamente as cultivares em estudo. A informação obtida neste trabalho poderá abrir portas à uma possível criação de uma protecção especial como no caso já existente para a "Azeitona de Conserva Negrinha de Freixo" com Denominação de Origem Protegida.

No entanto outros trabalhos deverão ser conduzidos de modo a clarificar o efeito do prolongamento do tratamento aquoso nas características físicas e químicas de "*alcaparras*", bem como estudos para determinar qual o momento óptimo de colheita para a produção de "*alcaparras*" monocultivares e "*alcaparras*" comerciais.