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Anabela da Conceição de Sousa

DETERMINAÇÃO DO PONTO ÓTIMO DE COLHEITA DAS CULTIVARES DE OLIVEIRA PERTENCENTES À DENOMINAÇÃO DE ORIGEM PROTEGIDA "AZEITE DE TRÁS-OS-MONTES"

Tese do 3º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas, especialidade de Nutrição e Química dos Alimentos

Trabalho realizado sob orientação de

Professora Doutora Susana Isabel Pereira Casal Vicente

e co-orientação de
Professor Doutor José Alberto Cardoso Pereira e
Professor Doutor Albino António Bento

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À MINHA MÃE

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Resumo

A olivicultura é uma atividade de grande relevância económica e social em Portugal. Trás-os-Montes é a segunda região produtora nacional, e é conhecida por possuir um grande património genético olivícola, que associado às condições edafoclimáticas da região permite a produção de azeites de excelente qualidade.

O presente trabalho teve por objetivo geral contribuir para a valorização dos azeites produzidos na região, com particular destaque para a área de influência da Denominação de Origem Protegida (DOP) "Azeite de Trás-os-Montes", nomeadamente ao nível da caraterização de azeites elementares de diferentes cultivares; da determinação do momento de colheita para as três cultivares de maior importância na região (Cobrançosa, Madural e Verdeal Transmontana) e da adição de especiarias e temperos no comportamento de azeites da Cv. Cobrançosa com vista à sua valorização.

Numa primeira fase foram caracterizadas dez cultivares de oliveira típicas da região, onde se incluíram as principais cultivares da DOP "Azeite de Trás-os-Montes", nomeadamente a Cobrançosa, a Madural e a Verdeal Transmontana, para além de outras com menor representatividade. Os resultados obtidos permitiram distingui-las do ponto de vista físico e químico e indicar as que poderão apresentar maior potencial para a produção de azeite e de azeitona de mesa. Esta diversidade e riqueza são importantes na genuinidade e diferenciação dos azeites produzidos na região e na manutenção do seu património genético.

No que respeita às três principais cultivares da DOP "Azeite de Trás-os-Montes", e com vista à otimização do ponto ótimo de colheita para maximização da sua qualidade, estudou-se em detalhe a composição fenólica e atividade antioxidante da azeitona ao longo da maturação. As três cultivares mostraram ser significativamente afetadas pela maturação, com os principais compostos fenólicos (oleuropeína e hidroxitirosol) a diminuírem drasticamente com a maturação, de forma proporcional à perda de atividade antioxidante da polpa. Os resultados demonstraram que o perfil e a evolução com a maturação são distintos entre as cultivares e que, para além dos compostos fenólicos avaliados, outros componentes da polpa deverão contribuir para a atividade antioxidante. Posteriormente, foram analisados em detalhe os parâmetros biométricos do fruto e químicos das azeitonas e do azeite obtidos em três anos distintos. Os resultados obtidos do ponto de vista agronómico e químico sugerem claramente datas distintas para a colheita das três cultivares. A cv. Madural, sendo mais sensível à oxidação e tendo um teor de lípidos relativamente constante ao longo da maturação, poderá beneficiar de uma apanha antecipada, logo no início da campanha em final de Outubro / início de

Novembro, com adaptações em função da data de floração anual. O azeite da cv. Cobrançosa é mais estável e apresenta uma elevada capacidade antioxidante, podendo ser colhido mais tarde, mas antes do final de Novembro. Finalmente, a cv. Verdeal Transmontana, devido à sua maturação mais lenta, elevado teor em compostos fenólicos e teor crescente de lípidos na polpa com a maturação, poderá ser apanhada no final da época, mas sempre antes das geadas características de Dezembro, onde a qualidade é drasticamente afetada. Este delineamento contribuirá para melhores práticas na apanha da azeitona na região, com impacto direto na qualidade dos azeites da DOP, com uma composição química mais equilibrada, com mais aromas verdes e frutados, mais estáveis e consequentemente com maior poder de conservação.

Por fim, e com o objetivo de dar resposta a uma tendência de diversificação dos produtos oferecidos na região, e conhecer o comportamento destes produtos, avaliou-se a qualidade, estabilidade e atividade antioxidante de azeites da cv Cobrançosa aromatizados com ervas aromáticas e especiarias. Verificou-se que a adição destes componentes não afeta significativamente a qualidade, mas em alguns casos pode afetar a estabilidade, com consequente redução do prazo de validade dos produtos.

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Abstract

Olive growing is an activity with great economic and social importance in Portugal. Trás-os-Montes region, the second most important producing area, is known for its olive genetic heritage which, associated with characteristic soil and climatic conditions, allows the production of excellent quality olive oils.

This study had the overall objective to contribute for the valorization of the olive oils produced in the region, with particular emphasis on the area of influence of the Protected Designation of Origin (PDO) "Azeite de Trás-os-Montes", in terms of characterization of elemental olive oils from different cultivars; selection of adequate harvest times for the three most important cultivars in the region (Cobrançosa, Madural and Verdeal Transmontana), and studying the effects of the addition of spices and seasonings to Cv Cobrançosa olive oil.

Initially, 10 typical olive cultivars of the region were characterized, including the main cultivars of the PDO "Trás-os-Montes olive oil", namely Cobrançosa, Madural and Verdeal Transmontana, in addition to other less representative ones. The results allowed distinguishing them from the physical and chemical points of view, while indicating which ones may present the greatest potential for the production of olive oil and table olives. This diversity and richness are important for authentication and differentiation of olive oil produced in the region, and maintenance of their genetic heritage.

With regard to the three main varieties of the PDO "Tras-os-Montes olive oil", and for optimization of the optimal harvest time for quality maximization, the phenolic composition and antioxidant activity of the olives was studied in detail over maturation. The three cultivars were shown to be significantly affected by maturation, with the main phenolic compounds (oleuropein and hydroxytyrosol) decreasing dramatically with maturation, proportionally to the loss of antioxidant activity of the pulp. The results showed that the profile and evolution during ripening are different between cultivars and that, in addition to the phenolic compounds evaluated, other components of the pulp might contribute to the observed antioxidant activity. The three cultivars were later analysed in detail for the biometric parameters of the fruit and chemical composition of the olives and olive oil obtained in three different years. The results of the agronomic and chemical characterization clearly suggest different dates for harvest of the three cultivars. Cv. Madural, being more sensitive to oxidation and having relatively constant lipid contents throughout maturation, may benefit from harvest early in the campaign, in late October / early November, with adaptations from the annual flowering dates. Cv Cobrançosa oil is more stable and has a higher antioxidant capacity and may therefore be harvested later,

but before the end of November. Finally, cv. Verdeal Transmontana, due to their slower maturation, high content in phenolic compounds and increased lipid content in the pulp with maturation, can be picked up at the end of the campaign, but always before the characteristics December frosts, where quality is dramatically affected. This information will contribute to best practices in olive picking in the region, with direct impact on the quality of PDO olive oils, with a more balanced chemical composition, with increased greener and fruity aromas and stability.

Finally, and in order to respond to a recent trend of diversification of products offered in the region, the quality of cv Cobrançosa olive oil flavoured with herbs and spices was studied. It was found that the addition of these components does not affect olive oil quality significantly, increases some nutritional features, but in some cases it may affect stability, with consequent reduction of the products shelf life.

Publicações e comunicações resultants do projeto de doutoramento

Publicações em revistas indexadas ao Journal Citation Reports da ISI Web of Knowledge:

Sousa, A.; Pereira, J.A.; Malheiro, R.; Bento, A.; Casal, S.. Contribution to the characterization of different olive cultivars from Trás-os-Montes region: morphological traits, quality and composition. Submetido **(Capítulo 3)**

Sousa, A.; Malheiro, R.; Casal, S.; Bento, A.; Pereira, J.A., 2014. Changes in antioxidant activity and phenolic composition of Cv. Cobrançosa olives through the maturation process. *Jornal of Funtional Foods*. 11, 20-29**. (Capítulo 4).**

Sousa, A.; Malheiro, R.; Casal, S.; Bento, A.; Pereira, J.A., 2015. Optimal harvesting period for cvs. Verdeal Transmontana and Madural, based on antioxidant potential and phenolic composition of olives. *LWT - Food Science and Technology*, 62, 1120-1126. (**Capítulo 5**).

Sousa, A.; Pereira, J.A.; Cruz, R.; Malheiro, R.; Bento, A.; Casal, S.. Optimal harvest moment for the three main olive cultivars in the Protected Designation of Origin "Azeite de Trás-os-Montes". Submetido **(Capítulo 6).**

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Abreviaturas e acrónimos

3,4-DHPEA (hydroxytyrosol)

3,4-DHPEA-EA (Oleuropein aglycone)

3,4-DHPEA-EDA (Dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol)

ANOVA (Analysis of variance)

cv. (cultivar)

$\mathbf{D_{max}}$ (maximum diameter)

$\mathbf{D_{min}}$ (minimum diameter)

DPPH (2,2-diphenyl-1-picrylhydrazil)

EVOO (Extra-virgin olive oil)

FA (Free acidity)

FAME (Fatty acids methyl esters)

FAOSTAT (Statistics Division of Food and Agriculture Organization)

FID (Flame ionization detector)

ha (hectare)

IOC (International Olive Council)

MI (Maturation index)

MUFA (Monounsaturated fatty acids)

p-HPEA (tyrosol)

p-HPEA-EDA (Dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol)

PC (Principal component)

PCA (Principal component analysis)

PUFA (Polyunsaturated fatty acids)

PV (Peroxide value)

SFA (Saturated fatty acids)

SPSS (Statistical Package for the Social Sciences)

Verdeal T. (Verdeal Transmontana)

VOO (Virgin olive oil)

PARTE I

Introdução Geral e Objetivos

Capítulo 1. Introdução e objetivos

Capítulo 2. Influência da maturação do fruto na composição e qualidade do azeite

CAPÍTULO 1.

1. Introdução Geral

1.1 Importância da oliveira no mundo

A oliveira, *Olea europea* L., é uma das árvores de fruto com grande importância socioeconómica a nível mundial, principalmente nos países da bacia mediterrânica, onde Portugal se insere. O setor olivícola representa um dos setores mais importante a nível económico e social nos países mediterrânicos, principalmente em países como Espanha, Itália, Grécia, Síria e Tunísia, os cincos maiores produtores mundiais (Figura 1.1). No entanto, nas últimas décadas, tem-se assistido a um aumento da área destinada à cultura e à produção em todo o mundo, com especial referência para o crescimento em países não tradicionalmente produtores, ou pequenos produtores como sejam por exemplo a Argentina, a Austrália e os EUA, atingindo uma superfície de cultura a nível global de mais de 8,6 milhões de ha. (FAOSTAT, 2015).

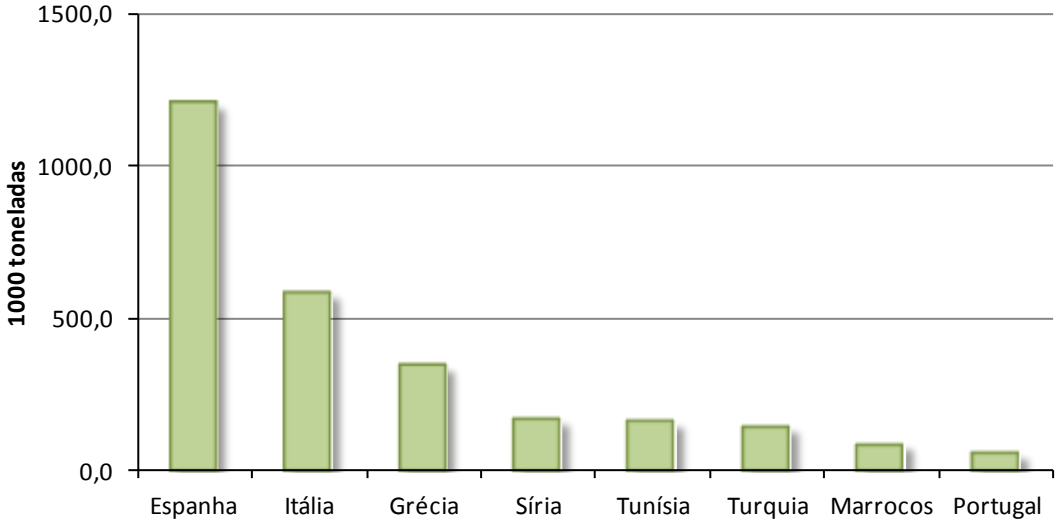

Figura 1. Principais países produtores (2000-2013) (FAOSTAT, 2015).

Pode considerar-se que os produtos do olival são diversos, contudo os mais comuns são claramente o azeite e depois para a azeitona de mesa. A produção de azeite tem vindo a aumentar ao longo dos anos (COI, 2014), embora com pequenas flutuações que são devidas maioritariamente a fatores ambientais (Figura 1.2.). A produção mundial de azeite na campanha 2013/14 foi de 3 270 500 toneladas. Foi também a segunda melhor campanha obtida até ao momento (a melhor foi a de 2011/12, que foram atingidas 3 321 000 toneladas de azeite). Os países membros do

Conselho Oleícola Internacional (COI) produziram 3 199 500 toneladas, a que correspondem 98% da produção mundial, cabendo aos países produtores europeus cerca de 77%, com 2 476 500 toneladas de azeite obtido (COI, 2014).

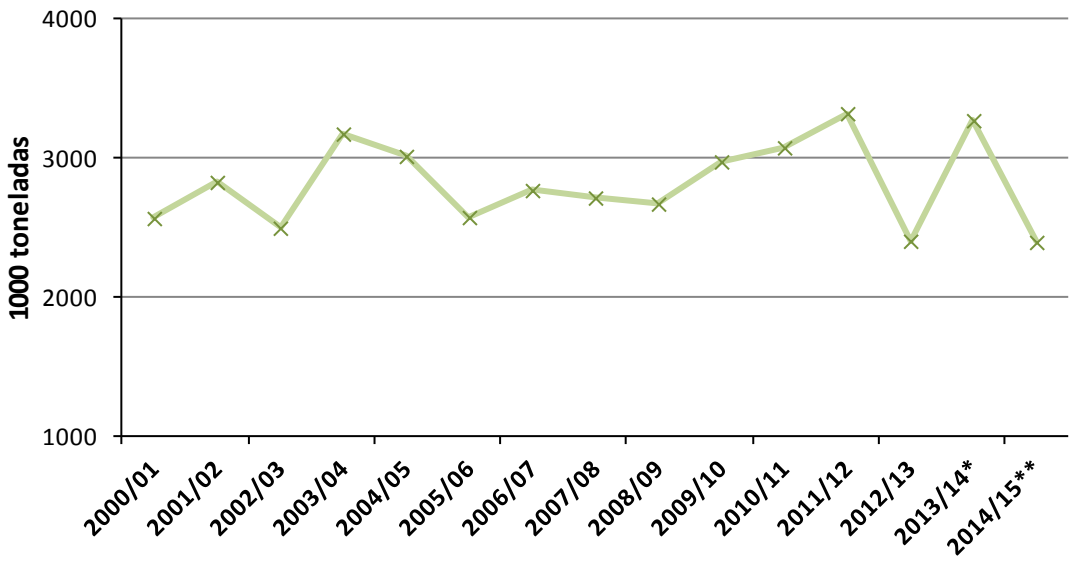

Figura 2. Evolução da produção mundial de azeite desde o ano 2000. (*dados provisórios; **dados previstos; COI, 2014)

No que respeita ao consumo global, verifica-se um aumento constante do consumo de azeite a nível mundial. A este fato não estará alheio, por um lado a existência de diferentes estudos que confirmam o impacto positivo para a saúde da ingestão de azeite virgem e por outro a existência de consumidores mais informados que têm vindo a descobrir as excelentes propriedades gastronómicas e nutricionais do azeite. De facto, existem estudos científicos que revelaram as potencialidades do azeite na proteção contra doenças cardiovasculares, na diminuição do risco de certos cancros e no retardar da evolução de certas doenças degenerativas (Visioli and Galli, 2002; Pérez-Jiménez et al, 2007). Estas propriedades estão intimamente relacionados com a composição do azeite e com a concentração de moléculas bioativas resultantes dos processos catabólicos e anabólicos que ocorrem durante o desenvolvimento do fruto.

Os ácidos gordos, componentes dos triglicéridos, são os constituintes mais abundantes no azeite e foram durante muitos anos considerados como sendo os principais responsáveis pelos seus efeitos benéficos para a saúde, sobretudo devido ao valor elevado da razão entre ácidos gordos monoinsaturados e ácidos gordos polinsaturados (Tripoli et al., 2005; Simopoulos AP 2002; Huang e Sumpio, 2008). Os ácidos oleico, linoleico e palmítico são os ácidos gordos mais abundantes no azeite,

(Owen et al., 2000) existindo um grande número de outros mas em percentagens reduzidas. Os outros constituintes que desempenham um papel relevante nas características desta gordura tão peculiar são: hidrocarbonetos (principalmente esqualeno), esteróis, álcoois alifáticos, tocoferóis e pigmentos (β-caroteno), bem como vários compostos fenólicos e voláteis (Boskou et al, 2006). Estes compostos têm sido considerados muito úteis na verificação da autenticidade do azeite e na caracterização dos azeites virgens monovarietais (Aparício e Luna, 2002; Pinelli et al., 2003; Matos et al., 2007) e são também responsáveis pelas propriedades sensoriais e pela elevada estabilidade oxidativa durante o armazenamento (Sánchez and Harwood, 2002a; Rotondi et al., 2004) sendo cada vez mais reconhecidos pela sua envolvência nos efeitos biológicos positivos (Martín-Peláez et al, 2013).

A variabilidade desta composição química depende da cultivar, das práticas agrícolas (rega, fertilização), das condições climatéricas, do momento de colheita, das condições de extração e de armazenamento do azeite (Lazzez et al., 2011; Aparicio e Luna, 2002; Gutiérrez et al, 1999).

1.2 Importância em Portugal

A nível nacional, tem-se assistido a uma grande transformação do sector, passando de uma produção inferior a 30000 toneladas de azeite na campanha de 2000/2001, período em que o País era altamente deficitário deste produto, para cerca de 90000 na última campanha, sendo que neste momento o País é autossuficiente em azeite (Figura 1.3.). Este aspeto denota por um lado um grande dinamismo do setor e por outro a uma aposta no olival como cultura, o que veio contrariar a tendência anterior em que ocorria redução da produção.

É de constatar que a produção nacional na campanha de 2013/2014 superou os valores registados nos anos anteriores, com uma produção de 91 600 toneladas de azeite (Figura 1.3), com um incremento superior a 300% (316%) face aos valores observados na campanha de 2002/03. Em Portugal, a oliveira é uma cultura que se encontra distribuída de Norte a Sul do País, especialmente nas regiões do interior.

De acordo com a edição de 2014 das estatísticas oficiais publicadas no “Inquérito à Estrutura das Explorações Agrícolas 2013”, o olival era, em termos de área, a principal cultura permanente, ocupando 48% da superfície destinada a culturas permanentes. Por outro lado, esta cultura tem sofrido um forte incremento, aumentando em termos de área 4,4 mil hectares de 2009 a 2013, o que mostra o forte dinamismo do sector. Em termos de área, o Alentejo é a principal região olivícola, com 49% da área destinada a esta cultura, seguida de Trás-os-Montes, com 22% da área nacional, Beira Interior, a que correspondem 18%, e Ribatejo e Oeste com 11%.

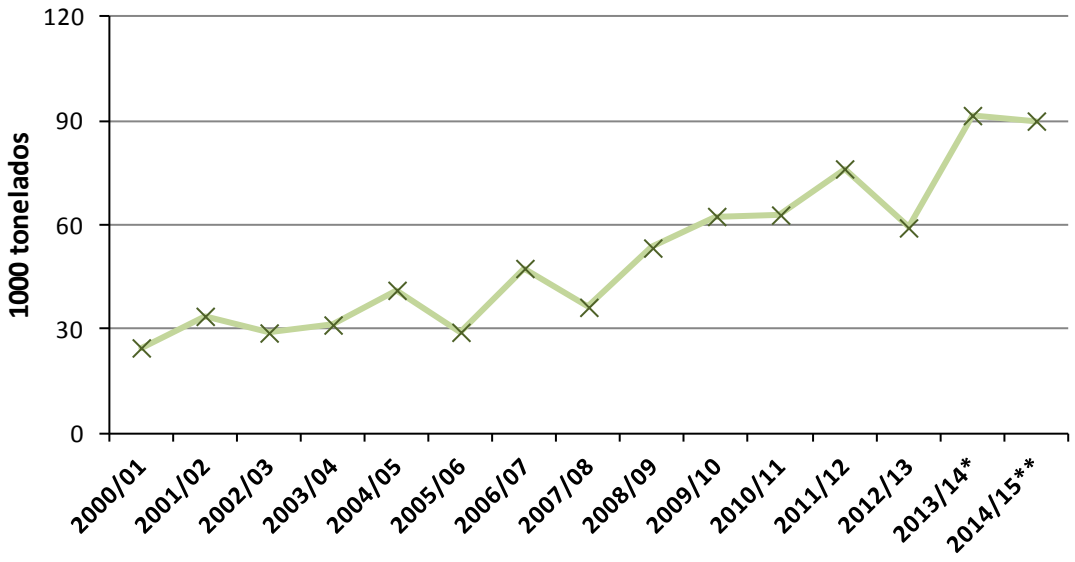

Figura 3. Evolução da produção de azeite em Portugal desde o ano 2000. (*dados provisórios; **dados previstos; COI, 2014).

A produção nacional de azeitonas é destinada sobretudo para a extração de azeite, com cerca de 96% das azeitonas destinadas a este fim, enquanto os restantes 4% são canalizados para a preparação de azeitonas de mesa (GPP, 2007).

Atualmente, o Alentejo concentra cerca de dois terços da produção nacional de azeite (Figura 1.4). Na última década e meia, assistiu-se, nesta região, a um forte investimento no olival, sobretudo em olivais novos conduzidos de forma intensiva, com elevado número de plantas por hectare, e com irrigação, beneficiando em grande parte do perímetro de rega de Alqueva.

Trás-os-Montes surge em segunda posição, com 15% da produção nacional (Figura 1.4). Aparentemente esta região perdeu uma grande importância em termos olivícolas nacionais, uma vez que em 2003 representava 34% da produção nacional, contudo, tal deve-se não à perda de produção, mas ao forte incremento do Alentejo, mantendo-se Trás-os-Montes a ser uma região olivícola de produção de azeites de excelência. Também com alguma importância são de destacar o Ribatejo e Oeste (6,3 %) a Beira Interior (5,6 %), a Beira Litoral (5 %). A figura 1.4 detalha a produção em termos de toneladas no ano de 2013.

Por outro lado, nos últimos anos, e uma vez que não há possibilidade de competir em termos de qualidade, tem havido um crescente interesse na certificação da origem geográfica dos produtos alimentares. A autenticidade destes produtos e a sua qualidade constituem fatores importantes na competitividade económica das regiões geográficas que os produzem.

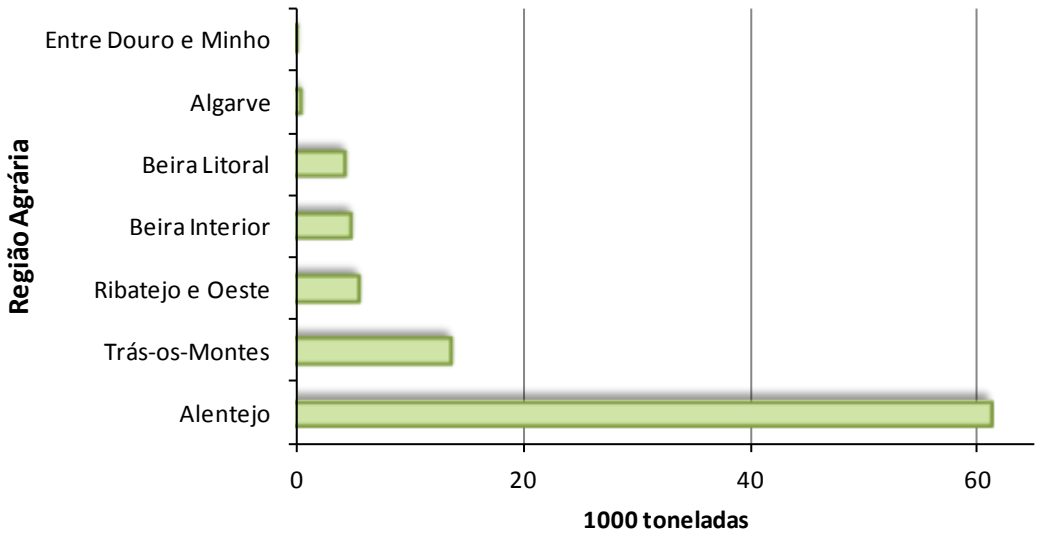

Figura 4. Produção de azeite por região agrária em 2013. (INE, 2013).

Os produtos do olival, como o azeite e a azeitona, são produtos importantes do ponto de vista económico, sendo recursos endógenos ligados ao território em muitas regiões. Esta atividade cria e mantém postos de trabalho, contribui para a manutenção de população no meio rural e gera também mais-valias do ponto de vista paisagístico. Todo este valor socioeconómico, aliado à reconhecida qualidade do azeite enquanto produto alimentar não só a nível nacional, mas também a nível mundial, levou à criação de diferentes Denominações de Origem Protegida (DOP) a nível nacional. No caso do azeite, esta denominação faz com que os azeites sigam especificações obrigatórias, tais como cultivar de azeitona, condições de apanha e transporte para o lagar, condições de laboração e as características do produto final. Para um azeite poder ser considerado DOP tem que apresentar as características que constavam do artigo 2º do regulamento (CEE) nº 2081/92, entretanto atualizado no Regulamento (CE) n.º 510/2006 do Conselho, de 20 de Março de 2006, relativo à proteção das indicações geográficas e denominações de origem dos produtos agrícolas e dos géneros alimentícios, e satisfazer as condições de um caderno de especificações, tal como era estipulado no artigo 6º do regulamento (CEE) nº 2082/92, entretanto revogado e substituído pelo Regulamento (CE) n.º 509/2006 do Conselho, de 20 de Março de 2006.

Os azeites DOP são originários de uma região geográfica delimitada, com solos e clima característicos, sendo produzidos apenas com azeitonas de certas cultivares. As características qualitativas e tipicidade que os distinguem de outros

azeites são também conferidas pelo “saber fazer” tradicional da região, no modo de condução das árvores, apanha da azeitona e extração do azeite. Atualmente existem seis regiões DOP de azeite em Portugal: Moura, Trás-os-Montes, Beira Interior, Norte Alentejano, Alentejo interior e Ribatejo. Estas regiões sempre foram reconhecidas pela produção de azeites de elevada qualidade.

O “Azeite de Moura”, produzido na margem esquerda do rio Guadiana, em que as cultivares dominantes são a Cordovil de Serpa, Galega Vulgar e Verdeal Alentejana, origina azeites muito frutados, amargos e picantes. Em Trás-os-Montes, as azeitonas são maioritariamente das cultivares Verdeal Transmontana, Madural, Cobrançosa e Cordovil, dando origem a azeites equilibrados com cheiro e sabor a fruto fresco, por vezes amendoado, e uma notável sensação de verde, amargo e picante. Os azeites da Beira Interior provêm de duas sub-regiões: Beira Baixa e Beira Alta. nos azeites da Beira Baixa predomina a cultivar Galega Vulgar, juntamente com a Bical e a Cordovil de Castelo Branco, originando azeites com cheiro e sabor complexos; em relação aos azeites da “Beira Alta”, a cultivar Galega Vulgar é substituída pelas cultivares Carrasquenha, Cobrançosa, Carrasquinha e Cornicabra. A DOP “Azeite do Ribatejo” é conhecida por possuir azeites doces devido à influência das cultivares Galega Vulgar e Lentisca. Nos azeites do Norte Alentejo a cultivar maioritária é Galega Vulgar juntamente com as Carrasquenha e Redondil em menor quantidade. O azeite obtido associa o frutado de azeitona a sensações fortes de maça e outros frutos maduros. Relativamente à DOP “Azeites do Alentejo Interior”, as cultivares típicas são a Galega Vulgar, a Cordovil de Serpa e a Cobrançosa, obtendo-se azeites mais suaves de amargo e picante.

1.3 A região de Trás-os-Montes e a DOP “Azeite de Trás-os-Montes”,

A olivicultura na região de Trás-os-Montes detém considerável importância a nível económico, social e ambiental. Esta região olivícola representa 22% da área nacional de olival, conforme referido, sendo a segunda Região Agrária a nível nacional, logo a seguir ao Alentejo (49%), mas contribui apenas com cerca de 15% da produção de azeite nacional (INE, 2013), fruto maioritariamente das condições geográficas da região, impeditivas do recurso à produção intensiva, e da elevada prevalência de pequenos e médios produtores.

Simultaneamente, devido às condições pedológicas e climatéricas da região, associadas às cultivares de oliveira tradicionais e às práticas culturais, o azeite e as azeitonas de mesa obtidos em Trás-os-Montes têm características únicas e são de excelente qualidade (Peres et al., 2011), sendo frequentemente alvo do

reconhecimento nacional e internacional em diversos concursos. Esta qualidade e genuinidade foram também reconhecidas pela criação referida da DOP “Azeite de Trás-os-Montes” para o caso do azeite, e pela DOP “Azeitona de mesa Negrinha de Freixo” para as azeitonas de mesa produzidas na região e que tenham por base a cultivar Negrinha de Freixo.

A área geográfica de produção (localização dos olivais, extração do azeite e seu acondicionamento) está circunscrita aos concelhos de Mirandela, Vila Flor, Alfândega da Fé, Macedo de Cavaleiros, Vila Nova de Foz Côa, Carrazeda de Ansiães e algumas freguesias dos concelhos de Valpaços, Murça, Torre de Moncorvo, Mogadouro, Vimioso e Bragança. Os azeites são extraídos de uma mistura de azeitonas das cultivares predominantes nesta região que são a Verdeal Transmontana, a Madural, a Cobrançosa, e em menor extensão a Cordovil, podendo ter outras cultivares minoritárias mas sempre em proporção inferior a 10%. Os azeites obtidos têm um perfil químico e sensorial caraterístico de onde se destaca um grande equilíbrio de sensações olfato-gustativas, caraterizado por notas frescas a azeitona, folhas de oliveira, erva e frutos secos verdes. Ao nível gustativo destacam-se as notas intensas de amargo e picante que se mantém na boca com grande persistência. Atualmente, tem-se verificado um aumento de plantações de olival com a cultivar “Cobrançosa”, justificado pela facilidade de propagação vegetativa, regularidade de produção, bom rendimento em azeite, baixa resistência do fruto ao desprendimento (facilidade na colheita mecânica) e produção de azeite de ótima qualidade.

Segundo o inquérito dirigido aos agrupamentos de produtores gestores de produtos qualificados como DOP/IGP/ETG, em 2012 existiam 6.000 explorações, totalizando uma área de olival de 12000ha que produziu 900000L de azeite certificado.

1.4 A importância da maturação na qualidade dos produtos

1.4.1 Composição da azeitona

A azeitona é uma drupa ovalada de cor verde que passa a violácea ou preto quando madura. É composta por três zonas bem definidas: o epicarpo ou pele, o mesocarpo ou polpa e o endocarpo ou caroço que envolve a amêndoa. Pesa entre 1,5 e 12 gramas e a polpa representa entre 70 a 88% do fruto. A azeitona é maioritariamente constituída por água, que representa mais de 50% do seu peso, e óleo – o azeite – que, dependendo da cultivar e do estado de maturação do fruto, ronda os 20% em peso fresco (Bianchi, 2003). O período de desenvolvimento e crescimento da azeitona é normalmente longo, completando o seu crescimento e

desenvolvimento em cerca de 6 a 7 meses (Hermoso et al., 2001). Nos primeiros 100 dias desenvolve-se rapidamente o endocarpo e faz-se a seleção natural dos frutos. No período que se segue, de 100-110 dias, dá-se um desenvolvimento rápido do mesocarpo e a chamada maturação verde, que ocorre com forte redução do conteúdo em clorofilas. Nesta fase, com o fruto já completamente desenvolvido, a polpa representa cerca de 70 a 90%, o endocarpo de 9 a 27% e a amêndoa de 2 a 3% (Hermoso et al., 2001). Quando as azeitonas ainda não estão maduras, a quantidade de água é maior do que a de óleo, invertendo-se esta situação gradualmente ao longo da maturação do fruto (Bianchi et al., 1994).

Do crescimento à maturação, a azeitona apresenta variações nos seus constituintes, alterações de tamanho, composição, cor, textura, sabor. O desenvolvimento do fruto e a maturação são uma combinação bioquímica e acontecimentos fisiológicos que ocorrem sob rigoroso controlo genético e a influência de várias condições ambientais

No ponto ótimo de colheita o mesocarpo contém cerca de 60% de água e teor em lípidos variável, dependendo da cultivar, correspondendo o restante a pequenas quantidades de hidratos de carbono, proteína, fibra e sais minerais. O endocarpo contém 10% de água, 30% de celulose, 40% de outros hidratos de carbono e cerca de 1% de lípidos. A semente tem 30% de água, lípidos e hidratos de carbono em proporções equivalentes (cerca de 30%) e 10% proteína (Conde et al., 2008; Connor e Fereres, 2005). No ponto ótimo de colheita pretende-se uma polpa de azeitona com um perfeito equilíbrio em ácidos gordos, tanto do ponto de vista nutricional como para a estabilidade oxidativa do azeite, bem como a maior atividade antioxidante possível, pelas mesmas razões, neste caso devido ao teor em compostos fenólicos (Conde et al., 2008).

Existem mais de 100 compostos fenólicos diferentes descritos em amostras de azeitona, em que os principais são o hidroxitirosol, tirosol e os seus derivados, verbascosídeo, lignanos e flavonoides (Obied et al., 2007; 2012). São potentes antioxidantes e desempenham um papel importante nas propriedades químicas, organoléticas e nutricionais do azeite virgem e da azeitona de mesa.

1.4.2 Evolução da maturação e como se alteram os diferentes constituintes

A qualidade do azeite é influenciada por vários fatores, entre os quais a cultivar e o estado de maturação dos frutos são dois dos mais importantes (Rotondi et al., 2004). Durante o amadurecimento ocorrem vários processos metabólicos nas

azeitonas, com consequente variação nos perfis de alguns componentes. Estas alterações são refletidas na qualidade do azeite, nomeadamente nas características sensoriais, estabilidade oxidativa e o seu valor nutricional.

A maturação conduz naturalmente a uma série de reações metabólicas que reduzem a quantidade de antioxidantes (fenóis, esteróis, pigmentos e tocoferóis) nas azeitonas e, consequentemente, em azeites (Jemai et al, 2009; Morello et al, 2004). Globalmente, à medida que o fruto amadurece, o óleo torna-se menos estável devido a um aumento em ácidos gordos polinsaturados e uma diminuição no teor em fenóis totais (Ayton et al., 2007; Dag et al., 2011), contudo as variações no teor em componentes minoritários contribuem no seu todo para as alterações verificadas.

A data apropriada de colheita no olival deve ser decidida de acordo com o estado de maturação das azeitonas e deve atender ao rendimento em gordura e à qualidade do azeite obtido. Muitas das vezes a sua determinação está dependente de um conjunto de aspetos que nada têm a ver com a quantidade e qualidade do azeite extraído. Destes aspetos, destaca-se a tradição, uma vez que em muitas regiões a colheita é tradicionalmente tardia, havendo um conjunto de provérbios populares como “Quem colhe antes do Natal deixa o azeite no olival” ou “Quem colhe antes de Janeiro deixa o azeite no madeiro”. Por outro lado a disponibilidade de abertura dos lagares de extração, havendo regiões onde nenhum lagar começa a laborar antes de 1 de Dezembro, noutras nenhum começa a atividade antes do dia de “Nossa Senhora da Conceição”, que é a 8 de Dezembro; ou ainda da disponibilidade de mão-de-obra uma vez que a colheita da azeitona faz parte de uma sequência de atividades agrícolas que normalmente começam com a vindima, passa para a apanha da castanha e só depois vai à colheita da azeitona. Assim, os métodos existentes para determinar o ponto ótimo de colheita utilizam critérios tradicionais mais do que científicos, uma vez que os estudos de determinação do momento de colheita são morosos e porque diferentes cultivares apresentarem um comportamento distinto (Matos et al., 2007). Geralmente, azeite obtido de azeitonas colhidas no momento ótimo contém 98% de ácidos lipídicos e 2% de compostos insaponificáveis, incluindo polifenóis, terpenos, pigmentos, tocoferóis e compostos voláteis diversos (Conde et al., 2008). O ácido oleico é o ácido gordo maioritário representando até 80% do total da composição lipídica. Outro ácido gordo presente é o polinsaturado ácido linoleico (2.5 – 20%) e o ácido palmítico com uma composição de 10 -20% (Conde et al., 2008).

1.4.3 Formação do azeite

O processo bioquímico de acumulação de lípidos no fruto da oliveira e os precursores para a sua síntese durante o período de maturação dos frutos têm recebido considerável atenção nos últimos anos (Nergiz et al., 2000). No geral, a qualidade do óleo sintetizado depende, entre outros fatores, da composição dos triacilgliceróis e é influenciada pela atividade das enzimas envolvidas na biossíntese dos mesmos durante a maturação (Sánchez e Harwood, 2002b). A biossíntese dos ácidos gordos ocorre dentro dos plastídeos, e inicia-se com a carboxilação da acetil-CoA a malonil-CoA (Sánchez e Harwood, 2002a). O ciclo prossegue com adição sequencial de dois átomos de carbono até ao palmitato que é posteriormente convertido noutros ácidos gordos, no âmbito da atividade de enzimas elongases e desaturases (Sakouhi et al., 2011). Esses ácidos gordos são utilizados por aciltransferases, no retículo endoplasmático, para a formação de triacilgliceróis de armazenamento (Sánchez e Harwood, 2002b).

Os compostos fenólicos são uma gama diversificada de metabolitos secundários derivados da via do chiquimato a partir de *L*-fenilanina ou *L*-tirosina (Cheynier, et al., 2013; Morelló et al., 2005). Os compostos fenólicos têm a sua origem no metabolismo fenilpropanóide, que passa pela conversão da *L*-fenilalanina em vários ácidos hidroxicinâmicos em quatro passos sequenciais. As enzimas que catalisam os passos individuais nesta sequência são, respetivamente, fenilalanina amónia liase, cinamato-4-hidroxilase e 4-cumarato-CoA ligase (Morelló et al., 2005). Os tocoferóis resultam da condensação de uma porção de um composto fenólico polar, o ácido *p*-hidroxifenilpiruvico, a partir da via chiquimato, e uma cadeia lateral poliprenil derivada do isopentenildifosfato produzido pela via 1-deoxi-D-xilulose-5- fosfato. A síntese de todos os tocoferois é iniciada pela conversão de ácido *p*-hidroxifenilpiruvico em ácido homogentísico, catalisada pela *p*-hidroxifenilpiruvico dioxigenase (Mène-Saffrané e Della Penna, 2010). A acumulação de compostos fenólicos varia fortemente com o estado fisiológico do fruto e é um resultado de um equilíbrio entre biossíntese e catabolismo.

1.4.4 Como evoluem alguns parâmetros com a maturação

1.4.4.1 Parâmetros de qualidade

A acidez é o resultado da presença de ácidos gordos livres obtidos por hidrólise e lipólise enzimática, sendo expresso em percentagem de ácido oleico, o ácido gordo maioritário no azeite. Este parâmetro é considerado um indicador da frescura do azeite

e da azeitona utilizada na produção do mesmo, sendo indiciadora de más práticas de fabrico ou de utilização de azeitona degradada. A deterioração do azeite é avaliada também pela sua oxidação, pelo índice de peróxido e pela absorvência no ultravioleta a 232 nm e a 270 nm. O índice de peróxido avalia a formação de hidroperóxidos, produtos de oxidação primária altamente instáveis. A absorvência no ultravioleta é uma medida da presença de dienos e trienos conjugados devido à formação de produtos primários e secundários da oxidação, respetivamente, sendo um indicador mais estável do que o anterior (Vichi et al., 2003). Todos estes parâmetros estão incluídos na legislação nacional e internacional, existindo limites máximos a cumprir para a classificação/desclassificação do azeite e contribuindo para a categorização do mesmo.

Apesar da estabilidade oxidativa não ser considerada um parâmetro padrão de qualidade, e por isso não estar regulamentado como os anteriores, pode ser usada como indicador do prazo de validade do azeite. Normalmente é avaliada pelo tempo de indução, ou seja, o período de tempo que decorre até ser atingido o ponto crítico da oxidação sob condições de oxidação forçadas. A estabilidade oxidativa é determinada, habitualmente, pelo método Rancimat e revela a resistência do produto à oxidação. A resistência à oxidação é atribuída, sobretudo, a dois fatores: a composição em ácidos gordos, que no caso do azeite se caracteriza por um valor elevado da razão entre ácidos gordos monoinsaturados e ácidos gordos polinsaturados e a presença de compostos minoritários com atividade antioxidante elevada, principalmente tocoferóis e polifenóis, mas também clorofilas e carotenóides (Matos et al, 2007).

Os parâmetros de qualidade não mostram usualmente diferenças significativas entre os azeites obtidos de azeitonas verdes e dos azeites obtidos a partir de azeitonas maduras (Salvador et al., 2001; Rotondi, et al., 2004; D’Imperio et al., 2010). Embora os dados mostrem um ligeiro aumento da acidez livre e uma ligeira diminuição no valor do índice de peróxido durante a maturação, essas diferenças não são usualmente significativas. O valor K_{232} diminuiu ligeiramente, em sintonia com o índice de peróxidos, enquanto o valor de K_{270} aumenta apenas ligeiramente nos azeites obtidos com azeitonas numa fase avançada na maturação. Contudo, Dag et al., (2011) e Yousfi et al. (2006) obtiveram resultados diferentes, com aumentos significativos ao longo da maturação em amostras das cultivares ‘Barnea’, ‘Arbequina’ e ‘Picual’, recomendando evitar a colheita tardia destas cultivares. Salienta-se, assim, a necessidade de estudar cada cultivar e situação de cultivo em particular e não extrapolar diretamente resultados obtidos em condições distintas.

1.4.4.2 Composição química

Ácidos gordos

Os ácidos gordos, componentes dos triacilgliceróis, são os constituintes mais importantes do azeite e os principais responsáveis pelos seus efeitos benéficos para a saúde, sobretudo devido ao valor elevado da razão ácidos gordos monoinsaturados/ácidos gordos polinsaturados. A composição em ácidos gordos depende da zona de produção, latitude, clima, cultivar de azeitona e o seu estado de maturação (Cunha et al., 2006; Boskou et al., 2006). Os ácidos oleico, linoleico e palmítico são os mais abundantes no azeite, entre muitos outros. Na tabela 1.1. pode ver-se a composição em ácidos gordos (limites de variação) que o azeite deve apresentar de acordo com, o Conselho Oleícola Internacional (COI) e o regulamento da Comissão Europeia nº2568/91.

O conhecimento da composição em ácidos gordos do azeite, tanto qualitativa como quantitativa, é de extrema importância, devido não só à sua caracterização mas também na deteção de possíveis adulterações desta gordura alimentar (Morales et al., 2000). Por exemplo, o estabelecimento de um nível máximo de ácido linolénico (polinsaturado) no azeite é considerado uma prioridade, uma vez que o seu conteúdo em relação aos ácidos gordos totais pode ser utilizado como um indicador da adulteração do azeite (Boskou et al., 2006). Simultaneamente, a quantidade de ácidos gordos trans, também legislados, permite distinguir entre as diversas categorias de azeite e validar uma possível adulteração do azeite pela presença de óleos refinados.

Vários estudos referidos na literatura descrevem que com a evolução da maturação a quantidade dos ácidos gordos saturados (palmítico e esteárico) diminui, e que os ácidos polinsaturados (PUFA) aumentam, enquanto a quantidade de ácido oleico, representando maioritários dos ácidos gordos monoinsaturados (MUFA), permanece constante ou mostra um ligeiro aumento. Sendo assim, a relação entre monoinsaturados e polinsaturados (MUFA/PUFA) diminui também ao longo da maturação, levando a um comprometimento da sua estabilidade oxidativa (Issaoui et al., 2011; Salvador et al., 2001; D'Imperio et al., 2010; Beltrán et al., 2004; Dag et al., 2011).

Tabela 1 Composição em ácidos gordos do azeite e os limites de variabilidade (COI, 2003; Reg. (CEE) nº2568/91).

Nome comum	Nomenclatura abreviada	%
Mirístico	C14:0	<0,05
Palmítico	C16:0	7,5 - 20,0
Palmitoleico	C16:1	0,3 - 3,5
Heptadecanóico	C17:0	≤ 0,3
Heptadecenóico	C17:1	≤ 0,3
Esteárico	C18:0	0,5 - 5,0
Oleico	C18:1	55,0 - 83,0
Linoleico	C18:2	3,5 - 21,0
Linolénico	C18:3	≤ 1,0
Araquidico	C20:0	≤ 0,6
Eicosenóico	C20:1	≤ 0,4
Beénico	C22:0	≤ 0,2
Erúcico	C22:1	não especificado
Lignocérico	C24:0	≤ 0,2

Compostos fenólicos

Os compostos fenólicos exibem funções e propriedades muito diversificadas no azeite. Começando pelos seus aspetos sensoriais, os fenóis são responsáveis pelos atributos positivos dos azeites o sabor amargo e picante (Servili et al., 2004). Em relação ao seu potencial farmacológico, os fenóis possuem atividade antioxidante, anti-inflamatória, efeitos nos sistemas cardiovasculares, imune, gastrointestinal, endócrino e respiratório. Além disso, intervêm no sistema nervoso central, e apresentam atividade antimicrobiana, anticancerígena e propriedades quimiopreventivas (Obied et al., 2012). Destes, os tocoferóis e tocotrienóis são importantes devido ao seu valor nutricional (vitamina E) e propriedades antioxidantes, pois protegem os componentes lipídicos presentes no azeite da oxidação. Constituem o grupo antioxidante lipofílico e destacam-se pela inibição eficaz da oxidação lipídica em todos os óleos vegetais atuando por dois mecanismos: doação de eletrões ou por captura do oxigénio singleto (Krichene et al, 2007).

Os principais fenóis detetados em produtos do olival incluem hidroxitirosol, tirosol e seus derivados secoiridóides (oleuropeína, aglícona de oleuropeína), verbascosídeo, lignanas e flavonóides (rutina e glicosídeos de luteolina e apigenina).

(Vinha et al., 2005; Malheiro et al., 2011). O decurso de maturação e o seu efeito na composição e conteúdo de compostos fenólicos em azeitonas e no azeite têm sido estudados em vários países e cultivares de azeitona, com observações semelhantes: os compostos fenólicos atingem um teor máximo nas azeitonas durante a fase "cherry", diminuindo drasticamente depois disso, durante a fase de maturação em que o fruto começa a mudar a cor para preto (Rotondi et al., 2004). A oleuropeína é o principal composto fenólico presente em azeitonas verdes e é responsável pelo seu amargor característico (Andrews et al., 2003). Este fenol apresenta elevada atividade antioxidante, tanto *in vivo* como *in vitro* (Speroni et al., 1998), mas com o amadurecimento da azeitona, o seu teor diminui drasticamente (Bouaziz et al., 2005;. Damak et al., 2008;. Jemai et al., 2009; Rotondi et al., 2004). Um dos seus principais derivados é o hidroxitirosol, que é também um dos mais ativos antioxidantes encontrados nos produtos do olival. Este composto também diminui com a maturação e esta tendência é apresentada em diversas cultivares e países. Morelló et al. (2004) declara que a diminuição do hidroxitirosol nas azeitonas pode ser provavelmente uma consequência de processos de hidrólise e de oxidação que ocorrem durante a maturação das azeitonas. Outros fenóis identificados em azeitonas incluem o tirosol, ácido vanílico, ácido cafeico, o ácido ρ-cumárico e verbascosídeo (Charoenprasert e Mitchell, 2012; Ryan e Robards, 1998; Savarese et al., 2007; Vinha et al., 2005), em conjunto com os compostos flavonóides, tais como a rutina, luteolina 7-O-glucósido e apigenina 7-O-glucósido, e vários pigmentos de antocianina (Savarese et al., 2007;. Vinha et al., 2005).

De entre os compostos fenólicos, os tocoferóis e tocotrienóis distinguem-se pela sua lipofilia e função vitamínica. Os tocoferóis e os tocotrienóis existem em quatro formas diferentes (α, β, γ e δ), que em conjunto têm a designação de vitamina E. No azeite virgem, cerca de 95% do teor total de tocoferóis corresponde a α-tocoferol (Matos et al., 2007). Em cultivares portuguesas Matos et al., (2007) determinaram tocoferóis em azeites com diferentes índices de maturação e verificaram que em qualquer das cultivares, o conteúdo de α- tocoferol diminui ao longo da maturação enquanto o isómero γ-tocoferol aumentou ligeiramente. Esta tendência foi também encontrada em estudos com cultivares internacionais (Aguilera et al., 2005; Beltrán et al., 2005).

1.4.4.3 Atividade antioxidante

As propriedades biológicas do azeite estão relacionadas com a presença de componentes minoritários, tais como esqualeno e fitoesteróis, e compostos antioxidantes, tais como tocoferóis e compostos fenólicos em geral (Baccouri et al, 2008). O teor de compostos fenólicos e de tocoferóis na azeitona, e consequentemente no azeite, depende de vários fatores: a cultivar de azeitona, solo, clima, irrigação, grau de maturação, sistema de extração e condições de processamento, embalamento, distribuição e armazenamento. (Boskou et al, 2006; Allalout et al, 2008). Entre os compostos antioxidantes naturais, os tocoferóis, o β-caroteno e os compostos fenólicos hidrofílicos têm um papel chave na prevenção da oxidação, estando relacionados com a estabilidade do azeite virgem durante o armazenamento. Em estudos de Gutiérrez et al (1999) e de Caponio et al (2001) foi descrita uma redução dos teores de compostos do azeite extra virgem (fenóis, tocoferóis, pigmentos) e também na estabilidade oxidativa em azeites produzidos com azeitonas com um maior grau de maturação. Os fenóis totais e os tempos de indução eram particularmente elevados em azeites produzidos com azeitonas verdes em relação aos azeites produzidos com azeitonas com maior grau de maturação (Boskou et al, 2006).

As alterações na composição dos componentes minoritários dos azeites ao longo da maturação vai provocar consequências inerentes ao nível da bioatividade, isto é, o potencial antioxidante dos produtos oleícolas vai diminuir. Para além de provocar alterações na estabilidade e, por conseguinte, no período de vida útil dos azeites, uma vez que a maturação conduz naturalmente a uma série de reações metabólicas que reduzem a quantidade de antioxidantes (fenóis, esteróis, pigmentos e tocoferóis) nas azeitonas e, consequentemente, nos azeites (Jemai et al, 2009; Morelló et al., 2004), do ponto de vista do consumidor origina igualmente redução nos potenciais efeitos benéficos decorrentes da ingestão destes compostos.

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CAPÍTULO 2.

2. Objetivos e estrutura do trabalho

A olivicultura é uma atividade de grande importância económica e social em Portugal, tendo nas regiões do interior do País uma importante relevância económica. Em territórios de baixa densidade populacional, como a região de Trás-os-Montes, em que as caraterísticas do relevo, estrutura fundiária, condições de solo, e disponibilidade de água, não permitem intensificação do cultivo, a produtividade do olival é na generalidade das vezes muito baixa o que compromete a sustentabilidade da cultura e das explorações agrícolas. Nestas condições, as explorações com olival terão que se afirmar, não pela quantidade de produto que produzem, mais apropriado para explorações intensivas, mas sim pela qualidade dos produtos que original. Neste sentido, nas últimas décadas tem havido um esforço dos produtores da região numa aposta em produzir produtos de elevada qualidade, tendo sido criada a Denominação de Origem Protegida “Azeite de Trás-os-Montes” com o objetivo de valorizar o azeite de elevada qualidade que se produz nesta região. A qualidade dos azeites extraídos na região estará relacionada quer com as caraterísticas edafo-climáticas quer com o importante património genético, com uma grande diversidade de cultivares, e a qualidade dos frutos aquando da colheita. De entre os fatores que mais influem na composição dos azeites está o momento de colheita da azeitona, pelo que a determinação do momento mais adequado de colheita para as cultivares maioritárias da DOP “Azeite de Trás-os-Montes”, isto é Cvs Cobrançosa, Madural e Verdeal Transmontana, é um dos aspetos da maior importância para a olivicultura da região. Por outro lado, a apetência do consumidor por produtos diferenciados requer que se proponham diferentes utilizações e novas aplicações ao azeite.

Neste sentido, os objetivos do presente trabalho foram:

- proceder a uma caraterização, ainda que preliminar, de um conjunto de cultivares de oliveira, através da caraterização biométrica dos seus frutos e endocarpos, bem como dos azeites extraídos;
- estudar o efeito da maturação dos frutos das três cultivares com maior importância na denominação de origem, isto é Cobrançosa, Madural e Verdeal Transmontana, ao nível da composição fenólica dos seus frutos e da atividade antioxidante, adaptando metodologias analíticas para a sua avaliação;

- acompanhar, em três campanhas de produção distintas, a evolução da maturação dos frutos, o rendimento em gordura, a resistência à oxidação, a qualidade e composição química da gordura, de forma a determinar um momento mais adequado de colheita, de forma a que seja maximizada a qualidade sem comprometer a quantidade de azeite extraído, para cada uma das três cultivares em estudo;

- avaliar de que forma a adição de diferentes especiarias e temperos, vulgarmente utilizados na preparação de azeites aromatizados, interfere ao nível da qualidade, resistência à oxidação, atividade antioxidante e composição química desse tipo de produtos.

Assim, a tese está estruturada em três partes, na primeira faz-se uma introdução geral acerca da importância da oliveira no mundo e em Portugal, quais os fatores que afetam a composição e qualidade, e de que forma o momento de colheita dos frutos interfere ao nível da composição química e qualidade dos azeites obtidos.

A segunda parte diz respeito à parte experimental propriamente dita, em que no primeiro dos cinco capítulos que compõem esta parte, é feita uma caraterização morfológica dos frutos e endocarpos, bem como dos azeites extraídos de 10 cultivares da região de Trás-os-Montes. Depois, nos capítulos 4 e 5, procedeu-se à implementação de uma metodologia por HPLC/DAD para a avaliação do teor em compostos fenólicos dos frutos, bem como à sua aplicação a frutos das cultivares Cobrançosa, Madural e Verdeal Transmontana, recolhidos em diferentes estados de maturação dos frutos, sendo também avaliada a evolução da capacidade antioxidante dos mesmos. No sexto capítulo, foi estudando, durante três campanhas de produção seguidas, o efeito da maturação em parâmetros biométricos, rendimento em gordura e composição do azeite, nas três cultivares, ao longo da maturação, de forma a fundamentar a decisão da época de colheita de frutos para obtenção de azeites de melhor qualidade. Por sua vez no sétimo capítulo estudou-se o efeito da adição de especiarias e temperos usados para aromatizar azeites, no comportamento de azeites da Cv. Cobrançosa.

Na terceira parte da tese, é feita uma discussão geral integrada dos resultados obtidos sendo também apresentadas as principais conclusões do trabalho desenvolvido.

PARTE II

Parte experimental

Capítulo 3. Contribution to the characterization of different olive cultivars from Trás-os-Montes region: morphological traits, quality and composition.

Capítulo 4. Changes in antioxidant activity and phenolic composition of Cv. Cobrançosa olives through the maturation process.

Capítulo 5. Optimal harvesting period for cvs. Verdeal Transmontana and Madural, based on antioxidant potential and phenolic composition of olives.

Capítulo 6. Optimal harvest moment for the three main olive cultivars in the Protected Designation of Origin “Azeite de Trás-os-Montes”

Capítulo 7. Aromatized olive oils: influence of flavouring in quality, composition, stability, antioxidants, and antiradical potential.

CAPÍTULO 3.

Contribution to the characterization of different olive cultivars from Trás-os-Montes region: morphological traits, quality and composition.

Anabela Sousa[†,§], José Alberto Pereira[†*]Ricardo Malheiro[†,§], Albino Bento[†], Susana Casal[§*],

[†]Mountain Research Centre (CIMO), School of Agriculture, Polytechnic Institute of Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal
[§]LAQV@REQUIMTE/Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, Porto University, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

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Abstract

Ten olive cultivars from the Trás-os-Montes region (Northeast of Portugal) were characterized: cvs. Bical, Borrenta, Cobrançosa, Cordovesa, Lentisca, Madural, Madural Negra, Negrinha de Freixo, Santulhana, and Verdeal Transmontana. All cultivars were studied regarding their morphological fruit traits (both olives and endocarps), olive oil quality (free acidity, K232 and K270), and composition (fatty acids, tocopherols and tocotrienols, and triglycerides).

Morphological characterization revealed differences among olive cultivars, particularly in cv. Lentisca, with smaller fruits. Some of these cultivars are used for table olives production due to their high pulp/stone ratio and pulp characteristics, while others are more suitable for olive oil extraction due to their high fat content. The extracted olive oils could be all classified as extra-virgin. The fatty acids profile was characteristic in each cultivar, allowing differentiation of all cultivars through chemometrics. Total vitamin E content varied significantly (46 and 148 mg/kg) of olive oil, as well as triolein content (38 to 64%), the most representative triglyceride in the olive oils. The characterization of these olive cultivars is important in order to guaranty the genuineness and authenticity of high quality olive oils produced in this region.

Keywords: cultivar characterization; olives; biodiversity; morphological traits; fatty acids; triglycerides.

1. Introduction

Olive growing, *Olea europaea* L., is spreading all around the world, due to the continuously increasing demand for olive products, mainly olive oil and also table olives. Olive oil consumption is increasing steadily during the last years, with an expected consumption of more than 2.8 million tons in the 2014-2015 season (IOC, 2015). According to Ryan et al. (1998), around 250 olive cultivars are considered by the International Olive Council to have commercial value for table olives and olive oil production, and around 2,500 cultivars are known worldwide. However, six main olive cultivars dominate the international markets: the Spanish cultivars Arbequina and Picual; the Italian cultivars Coratina, Frantoio, and Leccino; and the Greek cultivar Koroneiki (Vossen, 2007). Considering Spain as example, more than 90% of the recent planting olive orchards are from three main cultivars, Arbequina, Hojiblanca, and Picual. Several countries worldwide are using foreign olive cultivars, well adapted for intensive production schemes and with higher production yields, reducing therefore the proportion of traditional cultivars (IOC, 2000). With such practices, the space and proportion of autochthonous olive cultivars is reducing drastically, putting in danger olives biodiversity and some endemic cultivars need to be preserved and valorized.

Several low representativeness olive cultivars with high potentialities for olive oil production are yet to be explored, of high importance for olive cultivars diversity, and regional economies worldwide. Recently, different studies regarding the characterization of minor cultivars are being reported worldwide as a way to show their potentialities for olive oil production at regional level and to valorize them. For instance, minor cultivars from Tunisia (Manai-Djebali et al., 2012), wild olive trees in Pakistan (Anwar et al., 2013), cv. Nabali from Palestine (Abu-Reidah et al., 2013), some minor cultivars from Calabria in Italy (Runcio et al., 2008), and cvs. Ayvalik and Memecik from Turkey (Hyasoglu et al., 2010) were characterized regarding their oils quality, minor components and bioactivity. Simultaneously, several studies are being conducted, by molecular tools, to assess the genetic diversity of olives germplasm (Muzzalupo et al., 2014; Trujillo et al., 2014) and to avoid genetic erosion of traditional olive cultivars. In this sense, germplasm banks and collections were created to maintain all the information regarding genetic accessions of olive cultivars (Bartolini et al., 1998), being of extreme importance to avoid loss of important autochthonous olive cultivars around the world.

In Trás-os-Montes, a Portuguese region with a recognized history of high quality olive oil production, several olive cultivars are found, but only three are more frequently used for olive oil and table olives production: cvs. Cobrançosa, Madural, and Verdeal

Transmontana. These three cultivars are well characterized regarding their oils quality and composition (Matos et al., 2007a; Matos et al., 2007b). Nevertheless, several other cultivars are produced in the region, providing differentiated olive oils with excellent properties and quality. In this sense, in the present work it was intended to contribute for the characterization of minor olive cultivars from Trás-os-Montes region, regarding their fruits as well as their olive oil. For this study ten different cultivars from the region (cvs. Bical, Borrenta, Cobrançosa, Cordovesa, Lentisca, Madural, Madural Negra, Negrinha de Freixo, Santulhana, and Verdeal Transmontana) were selected. Morphological traits of olives as well as olive oils quality (free acidity, and specific coefficients of extinction) and composition (fatty acids, tocopherols, and triacylglycerols) were determined in the olive cultivars.

2. Material and methods

2.1. Sampling

The present study was conducted on ten olive cultivars from Trás-os-Montes region: cvs. Bical, Borrenta, Cobrançosa, Cordovesa, Lentisca, Madural, Madural Negra, Negrinha de Freixo, Santulhana, and Verdeal Transmontana. For each olive cultivar samples were collected from three independent olive trees (n = 3), in several olive orchards in Trás-os-Montes region in the 2009/2010 crop season.

2.2. Morphological characterization

For the morphological characterization, from each tree and olive cultivar 40 healthy olives were randomly collected around the tree and the following measures were taken: olives – weight (g), length (mm), maximum diameter (Dmax in mm) and minimum diameter (Dmin in mm); endocarps – weight (g), length (mm), Dmax (mm) and Dmin (mm). With the pulp and endocarp weight the ratio pulp/stone was calculated.

2.3. Moisture and fat content

Moisture and fat content were determined according to standard methods. Briefly, for moisture was determined by oven drying of 5 g of olive pulp per tree and cultivar, at 100±2 ºC, until constant weight. Total fat content was determined in a Soxhlet apparatus using petroleum ether as solvent with a minimum extraction of 24 h.

2.4. Olive oils extraction and sample preparation

The olive oils from the ten olive cultivars were extracted in triplicate using three samples of 1 kg each. The extraction of the olive oils was conducted within the first 24

h after collection. An Abencor analyser (Comercial Abengoa S.A., Seville, Spain) was used to process the olives in a pilot extraction plant. The unit consists of three essential elements: mill, thermobeater, and pulp centrifuge. The oil was separated by decanting, transferred into dark glass bottles and stored in the dark at 4 ºC. Before the analytical procedures, samples were dehydrated with anhydrous sodium sulphate and subsequently filtered through Whatmann no. 4 paper.

2.5. Quality parameters

The quality parameters assessed were free acidity (FA) and specific coefficients of extinction at 232 and 270 nm (K232 and K270), determined according to European Union standard methods (Annexes II and IX in the EEC/2568/91 from 11th July).

2.6. Fatty acids profile

Fatty acids were evaluated as their methyl esters after cold alkaline transesterification with methanolic potassium hydroxide solution (Annexes X, EEC/2568/91 from 11th July) and extraction with n-heptane. The fatty acid profile was determined with a Chrompack CP 9001 chromatograph equipped with a split-splitless injector, a FID detector, an autosampler Chrompack CP-9050 and a 50 m x 0.25 mm i.d. fused silica capillary column coated with a 0.19 µ film of CP-Sil 88 (Varian). Helium was used as carrier gas at an internal pressure of 110 kPa. The temperatures of the detector and injector were 250 ºC and 230 ºC, respectively. The oven temperature was programmed at 120 ºC during the first 3 min with an increase of 4 ºC/min until 220 ºC. The split ratio was 1:50 and the injected volume was of 1 µL. The results are expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area eluting between myristic and lignoceric methyl esters. A control sample (olive oil 47118, Supelco) and a fatty acids methyl esters standard mixture (Supelco 37 FAME Mix) was used for identification and calibration purposes (Sigma, Spain).

2.7. Tocopherols and tocotrienols composition

Tocopherols and tocotrienols composition was determined according to the ISO 9936 (2006), with the addition of an internal standard. Tocopherols and tocotrienols standards (α, β, γ and δ) were purchase from Calbiochem (La Jolla, San Diego, CA) and Sigma (Spain), while the internal standard 2-methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol (tocol) was from Matreya Inc. (Pleasant Gap, PA). Filtered olive oil (50 mg) was mixed with internal standard solution (tocol) and hexane and homogenized. The mixture was centrifuged for 5 minutes at 13000 rpm and the

solution analyzed by HPLC. The liquid chromatograph consisted of a Jasco integrated system (Japan) equipped with a Jasco LC – NetII/ADC data unit, a PU-1580 Intelligent Pump, a LG-1580-04 Quaternary Gradient Unit, a DG-1580-54 Four Line Degasser and an FP-920 fluorescence detector (λexc= 290 nm and λem= 330 nm). The chromatographic separation was achieved on a Supelcosil TM LC-SI column (3 µm; 75 x 3.0 mm; Supelco, Bellefonte, PA), operating at constant room temperature (23 ºC). A mixture of n-hexane and 1,4-dioxane (97.5:2.5) was used as eluent, at a flow rate of 0.7 ml/min. Data were analyzed with the ChromNAV Control Center - JASCO Chromatography Data Station (Japan). The compounds were identified by chromatographic comparisons with authentic standards, by co-elution and by their UV spectra. Quantification was based on the internal standard method, using the fluorescence signal response.

2.8. Triacylglycerols (TAGs) composition

The triacylglycerols composition was assessed according to the methodology of Cunha and Oliveira (2006a). A 0.2 g of olive oil sample from each olive cultivar was dissolved in 4 mL of acetone and homogenized by stirring. The mixture was filtered through a 0.22 µm disposable filter disk and analysed by HPLC. The chromatographic separation of the compounds was achieved with a Kromasil 100 C18 (5 µm; 250 × 4.6 mm) column from Teknokroma (Spain) operating at room temperature. The eluent used was a gradient of acetone (A) and acetonitrile (B). Elution was performed at a solvent flow rate of 1 mL/min with a linear gradient from 30% B to 25% B in 20 min., and to 20% at 35 min. (maintained for 20 min.) and returning to the initial conditions within 3 min. The effluent was monitored with an ELSD detector, with the following settings: evaporator temperature 40 ºC, air pressure 3.5 bar and photomultiplier sensitivity 6.

Standards of trilinolein (LLL), trimyristin (MMM), triolein (OOO), tripalmitin (PPP), tristearin (SSS), trilinolenin (LnLnLn), and tripalmitolein (PoPoPo) of purity greater than 98% and purchased from Sigma (St Louis, USA).the remaining peaks were identified according to the logarithms of α in relation to these homogeneous TAGs (Mottram et al., 1997). Quantification was obtained by relative percentage.

2.9. Statistical analysis

2.9.1. 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 21.0 (IBM Corporation, New York, U.S.A.). 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 analysed 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 factors studied were: the differences found in the parameters studied in the ten olive cultivars. 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.

2.9.2. Principal component analysis

Principal components analysis (PCA) was applied for reducing the number of variables in the ten olive cultivars to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information, i.e., the fatty acids profile of different olive cultivars from Trás-os-Montes region. Variables corresponding to 10 fatty acids and their different fractions (saturated, monounsaturated, polyunsaturated and trans fatty acids) were combined. PCA was performed by using SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.).

2.9.3. Linear discriminant analysis

A linear discriminant analysis (LDA) was used as a supervised learning technique to classify the ten 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. This procedure uses a combination of forward selection and backward elimination procedures, where before selecting a new variable to be included, it is verified whether all variables previously selected remain significant (Rencher, 1995; López et al., 2008). With this approach, it is possible to identify the significant variables among the fatty acids profile obtained for each sample. 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 sensibility 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). Sensibility was calculated by dividing the number of samples of a specific group correctly classified by the total number of samples belonging to that specific group. Specificity was calculated by dividing the number of samples of a specific group classified as belonging to that group

by the total number of samples of any group classified as belonging to that specific group. LDA was performed by using SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.).

3. Results and discussion

3.1. Morphological characterization

The ten olive cultivars were characterized regarding the morphological traits of their fruits and endocarps. These results are presented in Table 1 together with pulp/stone ratio, moisture and fat content. Olives weight varied significantly among olive cultivars ($P < 0.001$), between 1.08 g (cv. Lentisca) and 4.76 g (cv. Cordovesa). The cultivars Bical, Borrenta and Cordovesa were the ones who reported higher fruit weight. In the opposite trend, cv. Lentisca was by far the lighter olive cultivar, significantly different from all other nine cultivars in study (Table 1). Regarding length, it varied between 15.3 and 24.7 mm, in cvs. Lentisca and Bical, respectively. Dmax varied between 9.92 mm (cv. Lentisca) and 18.49 mm (cv. Borrenta), while Dmin varied between 7.66 mm (cv. Lentisca) and 13.96 mm (cv. Cordovesa). In all morphological parameters measured it was obvious that cv. Lentisca presents the lowest measures. As it can be inferred from Figure 1, this olive cultivar is recognized by its small fruits comparatively to the remaining olive cultivars in study. These observations were also checked in the morphological measures of the endocarps. Endocarps from cv. Lentisca reported always significantly lower measures comparatively to the remaining olive cultivars ($P < 0.001$ for all parameters; Table 1). Morphological data have a great importance once the correct characterization of olive cultivars and the data collected can be gathered and use for the creation of predicted models for the recognition of olive cultivars and guarantee the authenticity of the obtained products (Peres et al., 2011).

Other important information about olive cultivars is the pulp/stone ratio. It can reveal good cultivars for table olives processing, since higher pulp/stone ratio are desirable for table olives. In the cultivars studied, cv. Lentisca reported a lowest value (1.81), being therefore unsuitable for table olives processing due to the low amount of pulp. Higher pulp/stone ratios were found in cvs. Bical, Madural Negra, and Negrinha de Freixo, all of them with 5 times more pulp than stone. In fact, cv. Negrinha de Freixo is usually cultivated for table olives production, under the designation “Azeitona de Conserva Negrinha de Freixo”, a Protected Designation of Origin (PDO) in Trás-os-Montes region. Still regarding pulp/stone ratio, moisture content varied between 49.2% in cv. Verdeal Transmontana and 62.7% in cv. Negrinha de Freixo. This parameter is of

particular importance for industrial information about the oil yield, and also for cultivars comparison. Total fat content, always reported as percentage in dry weight, varied between 47.2% in cv. Lentisca, and 70.3% in cv. Bical. The fat amount and quality is a valuable information concerning the selection of the most productive cultivars for olive oil extraction. In this case, the cultivars that reported higher yield were Bical, Madural Negra (66.7%), Cordovesa (65.9%), and Verdeal Transmontana (62.2%). By the data obtained, it’s clear that cv. Lentisca is a cultivar with weak commercial potential, from the quantitative point of view, since it has small fruits with a low pulp/stone ratio, which turn it unsuitable for table olives processing. By other hand its low oil content also turn it unproductive for olive oil extraction.

Figure 1. Olives from the cultivars Cobrançosa (A), Lentisca (B), Madural (C), Negrinha do Freixo (D), Santulhana (E), and Verdeal Transmontana (F).

3.2. Olive oil quality

All ten olive cultivars oils were classified as extra-virgin olive oils regarding free acidity, K232 and K270. This means that for FA all oils were below 0.8%, the maximum legal values for extra-virgin olive oils (EVOO’s), and for K232 and K270 all values were below 2.50 and 0.22 for EVOO’s, respectively (EEC No 2568/91). These results attest the high quality of the oils obtained from the ten olive cultivars. .

Table 1. Morphological traits, moisture and fat content of fruits of olive cultivars from Trás-os-Montes region.

Fruit	**Weight (g)**	**Length (mm)**	**D_{max} (mm)**	**D_{min} (mm)**	**Pulp/stone ratio**	**Moisture (%)**	**Fat content (% dry weight)**
Bical	4.55±0.55 c	25.7±1.3 e	17.5±0.9 e,f	13.2±0.7 c,d	5.02±0.63 e-g	59.5±0.15	70.3±2.00
Borrenta	4.41±0.81 c	23.2±2.1 d	18.5±1.5 g	13.8±1.1 d,e	4.79±1.15 d-f	60.3±1.56	57.3±2.46
Cobrançosa	3.78±0.42 b	23.2±1.2 b,c	15.8±0.9 b	11.0±0.7 c	4.32±0.49 c	55.3±2.52	54.8±0.82
Cordovesa	4.76±0.53 c	24.8±1.3 e	18.1±1.1 f,g	14.0±0.9 e	4.69±0.67 d,e	56.1±1.01	65.9±0.97
Lentisca	1.08±0.14 a	15.3±1.8 a	9.9±0.5 a	7.7±0.4 a	1.81±0.40 a	53.3±0.48	47.2±4.33
Madural	3.34±0.41 b	22.7±1.2 d	15.0±0.8 b	10.9±0.8 b	4.31±0.62 d	58.3±0.02	48.4±0.08
Madural Negra	3.37±0.47 b	22.2±1.5 c,d	16.2±1.0 c,d	13.8±1.0 d,e	5.29±0.62 g	53.5±0.69	66.7±1.20
Negrinha de Freixo	3.50±0.52 b	20.7±1.4 b	16.9±1.0 d,e	12.9±1.0 c	5.23±0.69 f,g	62.7±1.24	52.1±0.51
Santulhana	3.71±0.61 b	21.4±1.5 b,c	16.9±1.1 d,e	13.2±1.1 c,d	4.74±0.69 d,e	55.9±0.67	55.1±1.90
Verdeal Transmontana	3.96±0.37 b	24.8±0.9 d	16.7±0.9 c	11.8±0.7 b	3.72±0.38 b	49.2±0.68	62.2±1.31
***P* value**	< 0.001[(1)]	< 0.001[(1)]	< 0.001[(1)]	< 0.001[(1)]	< 0.001[(1)]		
Endocarp	**Weight (g)**	**Length (mm)**	**D_{max} (mm)**	**D_{min} (mm)**			
Bical	0.76±0.10 e,f	18.8±1.1 d	8.2±0.5 b,c	6.8±0.5 c			
Borrenta	0.79±0.20 e-g	15.2±1.5 b	9.3±0.9 e	7.3±0.7 d			
Cobrançosa	0.71±0.08 d,e	17.6±0.9 c	8.3±0.6 b,c	6.7±0.4 c			
Cordovesa	0.85±0.12 g	18.0±1.0c,d	9.0±0.5 d,e	7.4±0.5 d			
Lentisca	0.39±0.06 a	13.4±1.8 a	6.8±0.5 a	5.4±0.9 a			
Madural	0.64±0.12 c,d	17.8±1.3 c	7.9±0.6 b	5.9±0.4 b			
Madural Negra	0.54±0.08 b	15.1±1.1 b	7.9±0.4 b	6.6±0.4 c			
Negrinha de Freixo	0.56±0.08 b,c	14.8±1.2 b	8.2±0.4 b	6.8±0.6 c			
Santulhana	0.65±0.11 d	15.5±1.3 b	8.6±0.6 c,d	6.8±0.4 c			
Verdeal Transmontana	0.84±0.08 f,g	18.7±2.1 c,d	8.9±0.5 d	6.3±0.4 c			
***P* value**	< 0.001[(1)]	< 0.001[(2)]	< 0.001[(1)]	< 0.001[(1)]			

In the same column mean values with different letters differ significantly ($P < 0.05$); [(1)] $P < 0.05$, by means of Levene test. *P* values are those from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed; [(2)] $P > 0.05$, be means of Levene test. *P* values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed.

3.3. Olive oils composition

3.3.1. Fatty acids profile

The fatty acids profile of the olive oils extracted from each of the ten cultivars under study was studied. Characteristic profiles were found, with significant differences between them ($P < 0.001$; Table 2). As expected, oleic acid (C18:1) was the main fatty acid, ranging from 68.6% in Madural Negra to 82.0% in cv. Verdeal Transmontana (Table 3), within regulated limits (EEC No 2568/91). Palmitic acid varied between 8.9% (cv. Santulhana) and 14.2% (in cv. Madural Negra), while linoleic acid varied significantly ($P < 0.001$), from 2.70 in cv. Lentisca, to 12.6% in cv. Borrenta. This is an important essential fatty acid (Spector & Kim, 2015) from the nutritional point of view, together with linolenic acid, but greater amounts of polyunsaturated fatty acids (PUFA) can compromise the oxidative stability of the oils (Kamal-Eldin, 2006).

Since olive oils are mainly composed by oleic acid, the main fraction is the monounsaturated fatty acids (MUFA). Besides oleic acid, others MUFA like palmitoleic acid (C16:1), heptadecenoic (C17:1) and eicosenoic (C20:1) were present in the olive oils, all within regulated limits. MUFA content varied between 70.0% in cv. Madural Negra and 83.2% in cv. Verdeal Transmontana. Saturated fatty acids (SFA) varied between 12.1% in Negrinha de Freixo and 16.9% in Madural Negra, mainly due to the high contents in palmitic acid, followed by reduced amounts of myristic acid (C14:0), heptadecanoic acid (C17:0), stearic acid (C18:0), behenic acid (C22:0), and lignoceric acid (C24:0). PUFA were restricted to two fatty acids, linoleic and linolenic acids, varying between 3.3% in cv. Verdeal Transmontana and 13.3% in cv. Borrenta (Table 2). *Trans* isomers were at very low extent in olive oils varying between 0.04 and 0.14%.

According to the results obtained it is possible to verify that the fatty acids profile was significantly different in the olive oils from the ten olive cultivars form Tras-os-Montes region. In this sense we applied chemometrics in order to verify if the fatty acids profile could be used to differentiate each cultivar. First we applied the fatty acids profile in a principal component analysis (PCA) (Figure 2A). It can be verified that each olive cultivar is represented individually, completely separated from other varieties. The two principal components (PC1 and PC2) represent 73.6% of the total variance of the data. The PC1 separates mainly cvs. Cobrançosa, Cordovesa, Madural and Madural Negra (in the positive region of PC1) from the remaining cultivars (represented in the negative region of PC1). The PC2 separates mainly cvs. Borrenta, Cobrançosa, Lentisa and Madural Negra (in the positive region of PC2) from cvs. Negrinha de Freixo, Santulhana, and Verdeal Transmontana.

Table 2. Fatty acids profile of monovarietal olive oils from Trás-os-Montes region (relative %).

Cultivar	**$C_{16:0}$**	**$C_{16:1}$**	**$C_{17:0}$**	**$C_{17:1}$**	**$C_{18:0}$**	**$C_{18:1}$**	**$C_{18:2}$**
Bical	11.6±0.09 e	0.74±0.01 e	0.06±0.00 b	0.08±0.00 a	2.64±0.01 e	74.0±0.08 e	9.49±0.04 d
Borrenta	13.6±0.07 g	0.78±0.00 e	0.06±0.00 b	0.09±0.00 a	2.29±0.00 b	69.3±0.08 b	12.6±0.01 h
Cobrançosa	11.0±0.03 d	0.65±0.00 d	0.21±0.00 e	0.29±0.00 c	4.34±0.01 i	74.5±0.02 f	7.62±0.03 c
Cordovesa	11.9±0.10 f	0.80±0.00 e,f	0.07±0.00 b,c	0.08±0.00 a	2.94±0.03 g	71.3±0.11 c	11.6±0.04 e,f
Lentisca	9.47±0.31 b	0.45±0.13 b,c	0.55±0.00 f	0.59±0.04 d	5.01±0.02 j	79.9±0.30 g	2.70±0.05 a
Madural	11.0±0.01 d	0.41±0.00 a,b	0.07±0.00 c	0.08±0.02 a	2.42±0.01 d	72.9±0.02 d	11.6±0.05 e
Madural Negra	14.2±0.06 h	0.88±0.01 g	0.16±0.00 d	0.24±0.00 b	2.33±0.01 c	68.6±0.08 a	12.2±0.01 g
Negrinha de Freixo	10.2±0.04 c	0.82±0.00 e,f	0.05±0.01 a	0.10±0.00 a	1.69±0.00 a	81.8±0.04 h	4.21±0.01 b
Santulhana	8.93±0.02 a	0.34±0.00 a	0.07±0.00 b,c	0.08±0.01 a	3.75±0.01 h	73.8±0.00 e	11.7±0.03 f
Verdeal Transmontana	10.2±0.05 c	0.52±0.01 c	0.21±0.00 e	0.32±0.01 c	2.77±0.00 f	82.0±0.04 h	2.74±0.00 a
***P* value**	< 0.001[(1)]	< 0.001[(2)]	< 0.001[(2)]	< 0.001[(2)]	< 0.001[(2)]	< 0.001[(2)]	< 0.001[(1)]
Cultivar	**$C_{18:3}$**	**$C_{20:1}$**	**$C_{22:0}$**	**SFA**	**MUFA**	**PUFA**	***Trans* isomers**
Bical	0.66±0.01 d	0.27±0.00 c,d	0.12±0.00 d,e	14.6±0.09 d	75.2±0.07 f	10.2±0.05 e	0.07±0.02 b
Borrenta	0.78±0.00 e	0.24±0.00 a,b	0.10±0.00 b,c	16.1±0.07 f	70.5±0.07 b	13.3±0.01 i	0.06±0.01 a,b
Cobrançosa	0.76±0.00 e	0.22±0.00 a	0.11±0.00 c,d	15.8±0.02 f	75.8±0.02g	8.38±0.03 d	0.07±0.00 b
Cordovesa	0.65±0.01 d	0.25±0.00 b	0.13±0.01 f	15.2±0.12 e	72.5±0.12 c	12.3±0.04 f	0.06±0.01 a,b
Lentisca	0.78±0.00 e	0.25±0.01 b,c	0.15±0.02 g	15.0±0.66 d,e	81.4±0.58 h	3.48±0.06 b	0.14±0.03 c
Madural	0.87±0.00 f	0.32±0.00 e	0.10±0.01 a-c	13.7±0.04 c	73.8±0.02 d	12.4±0.05 g	0.08±0.01 b
Madural Negra	0.76±0.01 e	0.24±0.00 a,b	0.08±0.00 a	16.9±0.07 g	70.0±0.07 a	13.0±0.02 h	0.07±0.00 b
Negrinha de Freixo	0.54±0.00 a	0.27±0.00 d	0.09±0.00 a,b	12.1±0.06 a	83.1±0.04 i	4.74±0.01 c	0.04±0.00 a
Santulhana	0.61±0.00 c	0.31±0.00 e	0.13±0.00 e,f	13.0±0.02 b	74.7±0.01 e	12.3±0.03 f	0.06±0.00 a,b
Verdeal Transmontana	0.58±0.00 b	0.32±0.02 e	0.13±0.00 f	13.4±0.05 b,c	83.2±0.05 i	3.32±0.01 a	0.07±0.01 b
***P* value**	< 0.001[(2)]	< 0.001[(2)]	< 0.001[(1)]	< 0.001[(2)]	< 0.001[(2)]	< 0.001[(1)]	< 0.001[(2)]

In the same column mean values with different letters differ significantly ($P < 0.05$); [(1)] $P > 0.05$, be means of Levene test. *P* values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed; [(2)] $P < 0.05$, by means of Levene test. *P* values are those from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed; SFA = $\Sigma\ C_{14:0} + C_{16:0} + C_{17:0} + C_{18:0} + C_{20:0} + C_{24:0}$; MUFA = $\Sigma\ C_{16:1} + C_{17:1} + C_{18:1} + C_{20:1}$; PUFA = $\Sigma\ C_{18:2} + C_{18:3}$; *Trans* isomers = $\Sigma\ C_{16:1}t + C_{18:1}t + C_{18:2}ct$

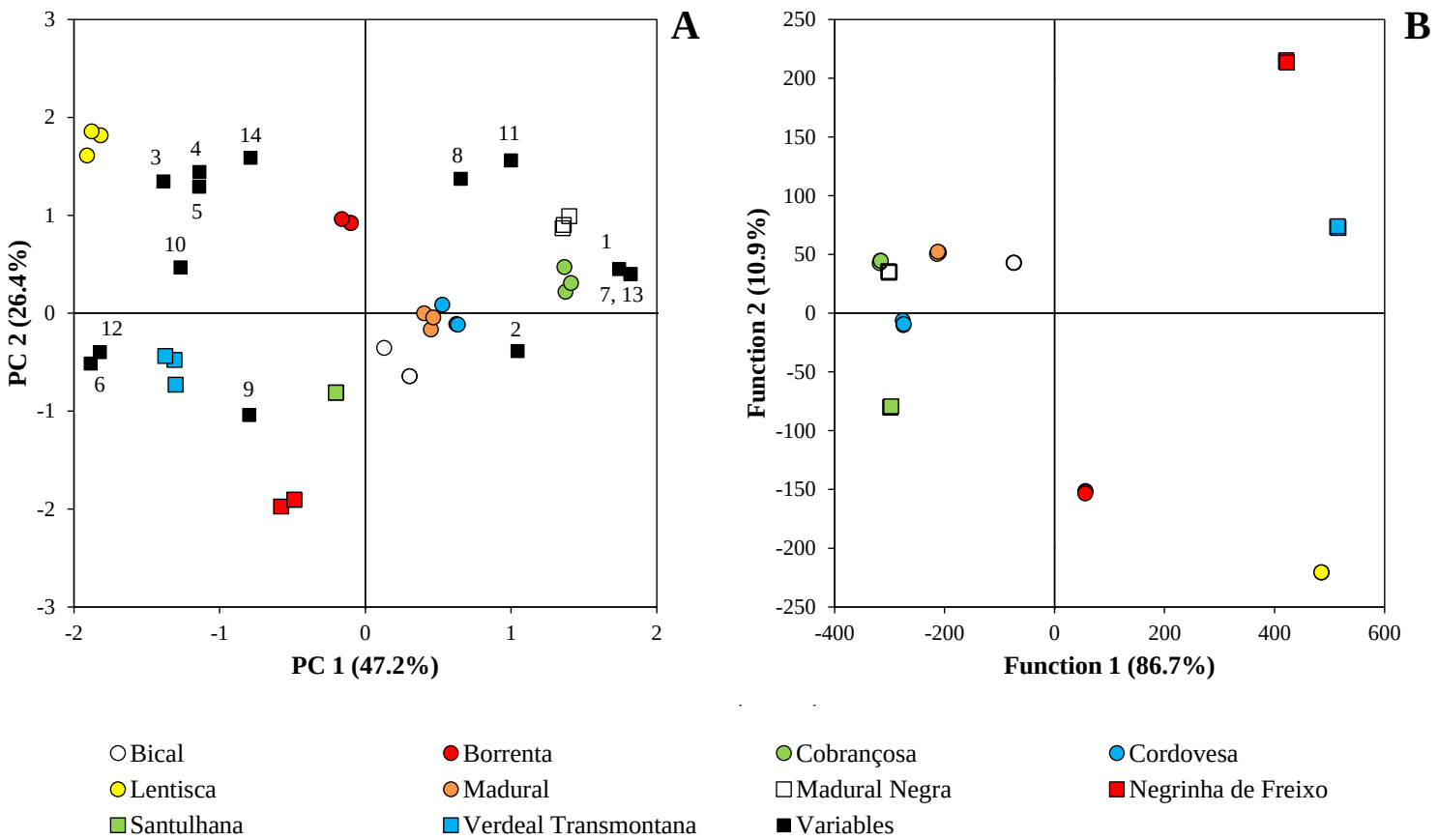

Figure 2. Principal component analysis (A) and linear discriminant analysis (B) obtained from the fatty acids profile of monovarietal olive oils from Trás-os-Montes region. The principal components (PCA) and discriminant functions (B) explain respectively 73.6 and 97.6% of the total variance. Variables used in PCA: 1 – C16:0; 2 – C16:1; 3 – C17:0; 4 – C17:1; 5 – C18:0; 6 – C18:1; 7 – C18:2; 8 – C18:3; 9 – C20:1; 10 – C22:0; 11 – SFA; 12 – MUFA; 13 – PUFA; 14 – *Trans* fatty acids.

Secondly, we applied the fatty acids profile obtained in the oils from the ten olive cultivars in a linear discriminant analysis (LDA). The stepwise LDA resulted in a discriminant model with six significant discriminant functions that explained 100% of the variance, although only the first two were used, since they explained 97.6% of the variance of the experimental data (the first explaining 86.7% and the second 10.9%) (Figure 2B). From the initial fourteen variables (in Table 1) the model was based in ten of the most discriminant variables. Those variables were palmitic, palmitoleic, heptadecanoic, heptadecenoic, stearic, oleic, linoleic, linolenic, and behenic acids, as well as SFA and PUFA. These variables 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, reporting sensitivities and specificities ratios of 100%. The obtained results in PCA and LDA are clearly indicative that fatty acids profile can be used for cultivars discrimination. Similar results were verified by Malheiro et al. (2012) working on the fatty acids profile of table olives from this region.

3.3.2. Tocopherols and tocotrienols composition

Tocopherols are important minor components of olive oil due to their dualistic function: vitamin and antioxidant. Four tocopherols (α-, β-, γ-, and δ-tocopherol) and two tocotrienols (α-, and γ-tocotrienol) were found in the olive oils from the ten olive cultivars (Table 3). α-Tocopherol was the main tocopherol found in olive oils, with amounts superior to 100 mg/kg in three cultivars: cv. Cordovesa (117.2 mg/kg), cv. Lentisca (119.8 mg/kg), and cv. Cobrançosa (130.4 mg/kg). The lowest amount was verified in cv. Madural Negra, with 34.4 mg/kg. γ-Tocopherol varied between 0.7 and 7.4 mg/kg in cvs. Verdeal Transmontana and Santulhana, respectively. β-Tocopherol and δ-tocopherol were present in low amounts in the olives, the first between 0.39 and 1.64 mg/kg (cvs. Madural Negra and Lentisca, respectively), and the second below 1 mg/kg in all cultivars (from 0.27 to 0.97 mg/kg, respectively in cvs. Verdeal Transmontana and Lentisca). Among tocotrienols, the most abundant was γ-tocotrienol, while α-tocotrienol was present in low amounts. γ-Tocotrienol varied significantly among olive oils ($P < 0.001$): cv. Negrinha de Freixo reported 16.0 mg/kg, while the oils from cv. Madural reported the lowest amount (3.7 mg/kg). Negrinha de Freixo olive oils reported a significant higher content in α-tocotrienol, with 2.33 mg/kg ($P < 0.001$), while the remaining olive cultivars reported values below 0.8 mg/kg (Table 3). Olive oils from cv. Negrinha de Freixo were those who reported higher content in tocotrienols.

Regarding total vitamin E content (as the sum of all tocopherols and tocotrienols), cv. Cobrançosa reported higher content, with 147.8 mg/kg, while cv. Madural Negra reported the lowest amount with 46.2 mg/kg (Table 3). The results obtained showed that some minor olive cultivars report considerable amounts of vitamin E, for instance cvs. Lentisca and Cordovesa reported 143.4 and 134.5 mg/kg of oil. Olive oils from Trás-os-Montes region, namely those from cvs. Cobrançosa, Madural and Verdeal Transmontana, reveal high content of vitamin E at several maturation indexes (Matos et al., 2007a). Regarding the remaining olive cultivars no studies were conducted so far, therefore this is the first report of tocopherols and tocotrienols content in those cultivars, as well as vitamin E content. Nevertheless our results are in accordance to those observed by Cunha et al. (2006b), that studied the tocopherols and tocotrienols composition of several commercial Portuguese olive oils, some of which from this producing region.

3.3.3. Triglycerides composition

The triglycerides composition of the olive oils from ten olive cultivars prevenient from Trás-os-Montes region is reported in Table 4. The main triglyceride present in the olive oil is triolein (OOO) with percentages varying between 38.1% in cv. Madural Negra, and 64.0% in cv. Verdeal Transmontana. The second most abundant triglyceride was palmitodiolein (POO) varying between 38.1% in cv. Santulhana, and 26.6% in Madural Negra. The third most abundant triglyceride was linodiolein (OLO), varying between 2.78% in cv. Lentisca and 19.2% in cv. Madural (Table 4), in a direct proportion to the linoleic acid content. Similar results on triglycerides profile were observed in commercial Portuguese olive oils (Cunha et al., 2006a). Regarding the variations observed among cultivars, the same variations were observed in Spanish olive oils from cultivars Cornicabra, Picual, Hojiblanca, and Arbequina (Aranda et al., 2004), therefore, olive cultivar is a preponderant factor that influence triglycerides composition.

Table 3. Tocopherols and tocotrienols (mg/kg) composition of monovarietal olive oils from Trás-os-Montes region.

Cultivar	α-Tocopherol	α-Tocotrienol	β-Tocopherol	γ-Tocopherol	β-Tocotrienol	γ-Tocotrienol	δ-Tocopherol	Total
Bical	91.8 ± 0.6 f	0.94±0.00 b-d	5.6±0.04 e	0.34±0.08 a	0.50±0.04 a-c	3.9±0.05 a,b	107±1 e	91.8 ± 0.6 f
Borrenta	43.0±0.1 b	1.02±0.12 c-e	2.3±0.14 c,d	0.38±0.00 a	0.40±0.11 a,b	4.5±0.09 a,b	54±1 b	43.0±0.1 b
Cobrançosa	130.4±1.1 i	1.21±0.14 e	5.8±0.21 e	0.45±0.20 a	0.77±0.09 c	7.0±0.35 d	148±1 h	130.4±1.1 i
Cordovesa	117.2±1.4 h	1.27±0.02 e	7.2±0.07 f	0.83±0.06 b	0.34±0.03 a	4.5±0.21 a,b	134±2 g	117.2±1.4 h
Lentisca	119.8±0.7 h	1.64±0.00 f	5.7±0.00 e	0.97±0.07 b	0.70±0.03 b,c	9.0±0.18 e	143±1 h	119.8±0.7 h
Madural	99.8±0.1 g	1.17±0.01 d,e	2.2±0.03 b,c	0.49±0.01 a	0.33±0.01 a	3.7±0.50 a	112±1f	99.8±0.1 g
Madural Negra	34.4±1.3 a	0.39±0.03 a	1.9±0.09 b	0.32±0.00 a	0.51±0.17 a-c	6.1±0.31 c,d	46±2 a	34.4±1.3 a
Negrinha de Freixo	84.4±0.2 e	0.91±0.02 b,c	2.6±0.00 d	0.94±0.02 b	2.33±0.04 d	16.0±0.42 f	113±1 f	84.4±0.2 e
Santulhana	49.8±0.3 c	0.77±0.08 b	7.4±0.08 f	0.49±0.08 a	0.34±0.08 a	5.0±0.42 b,c	66±1 c	49.8±0.3 c
Verdeal Transmontana	74.0±1.4 d	0.81±0.00 b,c	0.7±0.01 a	0.27±0.03 a	0.21±0.02 a	4.2±0.03 a,b	84±2 d	74.0±1.4 d
***P* value**	< 0.001[(1)]	< 0.001[(1)]	< 0.001[(1)]	< 0.001[(1)]	< 0.001[(1)]	< 0.001[(1)]	< 0.001[(1)]	< 0.001[(1)]

In the same column mean values with different letters differ significantly ($P < 0.05$); [(1)] $P > 0.05$, be means of Levene test. *P* values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed;

Table 4. Triglycerides composition (%) of monovarietal olive oils from Trás-os-Montes region.

	Bical	Borrenta	Cobrançosa	Cordovesa	Lentisca	Madural	Madural Negra	N. de Freixo	Santulhana	Verdeal Transm.
OLL	1.30±0.04	2.58±0.09	0.57±0.03	2.15±0.15	<0.01	2.44±0.03	2.23±0.08	0.15±0.02	2.21±0.00	<0.01
OOLn	0.57±0.03	0.60±0.03	0.56±0.01	0.47±0.02	0.66±0.01	0.77±0.07	<0.01	0.45±0.01	0.37±0.20	0.44±0.01
PLL	0.17±0.01	0.66±0.01	0.07±0.00	0.36±0.02	<0.01	0.23±0.01	0.53±0.01	<0.01	0.09±0.00	0.07±0.01
POLn	0.14±0.00	0.19±0.01	0.14±0.01	0.14±0.01	0.12±0.00	0.18±0.01	0.06±0.11	0.07±0.00	0.02±0.00	<0.01
OOL	15.3±0.08	16.8±0.10	11.5±0.03	18.0±0.29	2.78±0.01	19.2±0.32	18.0±0.30	7.29±0.27	19.0±0.44	3.68±0.00
PLO	5.18±0.07	8.03±0.07	3.58±0.01	6.85±0.18	0.76±0.02	5.76±0.10	8.79±0.10	1.51±0.06	4.48±0.04	0.85±0.00
PLP	0.19±0.01	0.40±0.01	0.28±0.00	0.28±0.01	0.48±0.01	0.17±0.01	0.50±0.02	0.04±0.04	0.09±0.00	0.22±0.00
OOO	47.6±0.11	39.2±0.14	51.2±0.15	42.4±0.21	59.3±0.21	45.5±0.29	38.1±0.32	63.2±0.50	49.2±0.08	64.0±0.13
POO	23.8±0.12	25.3±0.08	24.0±0.05	23.3±0.21	21.7±0.02	21.3±0.17	26.6±0.20	23.3±0.16	18.5±0.02	24.7±0.06
POP	1.59±0.03	2.25±0.02	1.37±0.02	1.61±0.03	1.01±0.02	1.15±0.02	2.37±0.01	1.12±0.05	0.68±0.02	1.19±0.01
GOO	0.07±0.00	0.04±0.01	0.08±0.01	0.05±0.01	0.53±0.01	0.05±0.00	0.03±0.01	0.07±0.02	0.05±0.00	0.19±0.00
SOO	2.56±0.05	1.74±0.03	5.12±0.02	2.67±0.07	9.49±0.28	1.90±0.08	1.45±0.01	1.48±0.03	3.76±0.06	3.48±0.05
POS	0.27±0.02	0.32±0.04	0.60±0.03	0.49±0.03	1.10±0.02	0.36±0.01	0.20±0.02	0.10±0.01	0.28±0.06	0.26±0.01
PPS	0.17±0.00	0.09±0.00	0.17±0.02	0.17±0.01	0.51±0.00	0.14±0.01	0.05±0.01	0.10±0.01	0.17±0.01	0.24±0.00
Others	1.26±0.06	1.88±0.10	0.80±0.05	1.17±0.05	1.85±0.04	0.94±0.03	1.10±0.06	1.25±0.02	1.15±0.08	0.85±0.06

4. Conclusions

The present work is a contribution for the characterization of minor cultivars from Trás-os-Montes region. According to the obtained results we can conclude that the different olive cultivars give origin to olive oils with high quality, and with differentiated composition but not all are adequate for the purpose, due to low fat content, while others might are more adequate for table olive production due to the high pulp to stone ratio. The fatty acids profile of the ten olive cultivars was capable to discriminate them by applying chemometrics. This type of studies is of high importance in order to avoid disappearance of cultural olive heritage, and to valorise traditional olive cultivars with low expression.

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CAPÍTULO 4.

Antioxidant activity and phenolic composition of Cv. Cobrançosa olives affected through the maturation process

Anabela Sousa[†,§], Ricardo Malheiro[†,§], Susana Casal[§*], Albino Bento[†], José Alberto Pereira[†*]

[†]Mountain Research Centre (CIMO), School of Agriculture, Polytechnic Institute of Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal
[§]LAQV@REQUIMTE/Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, Porto University, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

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Abstract

Maturation stage is a critical feature to obtain high quality olive products, with maximized bioactivity. In this study, phenolic composition and antioxidant activity of Cv. Cobrançosa through the maturation process were evaluated. The phenolic profile was assessed by HPLC/DAD, and antioxidant activity was studied through its reducing power and free-radical scavenging activity.

Total phenols varied from 34 to 1 g/kg, respectively, in the first and last sampling dates. Oleuropein, the main phenolic in the first stages of maturation, decreased drastically during ripening. At intermediate and high maturation stages hydroxytyrosol was the predominant phenol. Globally, the reducing capacity of Cv. Cobrançosa olive fruits decreased during the maturation process but its radical scavenging activity was only slightly altered. A principal components analysis corroborated the characteristic phenolic profile and changes experienced by the olive fruit during the maturation process. These results are important to maximise Cv. Cobrançosa olive products quality and biological properties.

Keywords: maturation process; Cv. Cobrançosa; phenolic profile; antioxidant activity.

1. Introduction

The increasing demand by consumers for healthier and safer foods is guiding food industry into a new path. From the implementation of improved food quality control and hazard prevention to new processing technologies, the natural bioactive properties of certain foods are becoming the focus of innovation and research. A refreshing attention is being devoted to natural products and to the technological needs for their bioactivity and potential health preventing effects maximization.

Olive products, namely virgin olive oil and table olives, and their by-products are among those products that have raised particular attention in recent years. Their unique chemical composition, mainly the richness in antioxidant compounds, is implicated in the positive health effects observed (Bendini et al. 2007; Bianco & Uccella 2000). This composition, however, is known to be influenced by several factors, particularly by the olives maturation stage (Charoenprasert & Mitchell 2012; Damak et al. 2008; Morelló et al. 2004). During ripening several metabolic processes occur, influencing the profile and amounts of olives bioactive compounds, including phenols, tocopherols, chlorophylls and carotenoids, as well as fatty acids and sterols (Matos et al. 2007). Among these, phenolic compounds are recognized as key components in olive products once they contribute with unique organoleptic characteristics and are also at least partially responsible for the documented bioactive properties (Caponio et al. 2001; Malheiro et al. 2011; Pereira et al. 2006). Besides conferring antioxidant properties to the olive products, phenolic compounds are also believed to decrease the risk of coronary diseases (Manna et al. 2002, Zbakh & Abbassi 2012), to prevent some kinds of cancer (Owen et al. 2000; Sepporta et al., 2014; Tripoli et al. 2005), while exhibiting antimicrobial and antiviral activities (Bisingnano et al. 1999).

The maturation process and its effect in the composition and content of phenolic compounds in olive fruits have been studied in several olive varieties and countries, with similar observations: the phenolic compounds reach a maximum content in the olive fruits during the “cherry” stage, decreasing drastically thereafter during the black maturation stage (Rotondi et al. 2004). Oleuropein is the main phenolic compound in green olive fruits and is responsible for their characteristic bitterness (Andrews et al. 2003). This phenol presents high antioxidant activity, both in vivo and in vitro (Speroni et al. 1998), but as the olive fruit becomes riper, oleuropein content drastically decreases (Bouaziz et al. 2005; Damak et al. 2008; Jemai et al. 2009; Rotondi et al. 2004). One of its main bioconvertion products, hydroxytyrosol, is fortunately also among the most active antioxidants found in olive products. Other phenols are found in olive fruits such as tyrosol, vanillic acid, caffeic acid, ρ-coumaric acid and verbascoside

(Charoenprasert & Mitchell 2012; Ryan & Robards 1998; Savarese et al. 2007; Vinha et al. 2005), together with flavonol compounds such as rutin, luteolin 7-O-glucoside and apigenin 7-O-glucoside, and several anthocyanin pigments (Savarese et al. 2007; Vinha et al. 2005).

Based on this knowledge, one can infer that the antioxidant capacity of olive products can be maximized if the olives are collected at the adequate stage. This stage however, will depend mostly on the cultivar, with singular particularities, and also on the local edaphoclimatic conditions. Therefore, data collected for other cultivars cannot be directly implemented in other geographical areas and, even for the same variety, the soil and weather condition, among others, will have a determinant influence.

Cobrançosa is the main cultivar used for the production of the Protected Designation of Origin (PDO) “Azeite de Trás-os-Montes” olive oil and table olives, in Northeast of Portugal. Other cultivars are also used, particularly Verdeal Transmontana and Madural, but in smaller amounts. Therefore, and in order to maximize this PDO olive oil antioxidant potential, a detailed study of its global antioxidant capacity and phenolic composition throughout maturation is a determinant step. So, the main purpose of the current work conducted with the Cv. Cobrançosa, is to study the effect of the maturation process in the phenolic profile and biological properties of the olive fruit, namely antioxidant potential, in order to maximise olive products quality and biological properties. From the author’s knowledge, this is the first maturation study being conducted in this region, which assumes a particular importance in the Portuguese panorama.

2. Material and methods

2.1. Reagents and standards

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

2.2. Sampling

In the present study, five representative olive trees from *Cv.* Cobrançosa were selected in an olive grove at Paradela, Mirandela region in the Northeast of Portugal, in the year of 2009. The orchard has 3 ha with a planting density of 7 x 7 m; trees have more than 40 years; the prune is made each three years; it is not irrigated and the soil is tilled 2–3 times each year. Five sampling dates (29th September, 13th and 27th October, and 10th and 18th November) were performed in order to monitor the maturation process, with the first date corresponding to unripe fruits (green colour), with intense bitterness and reduced oil content, and the latter to completely mature fruits (black colour). From each tree, approximately 1 kg of olive fruits were hand-picked all around the perimeter of the tree at the operator height. The samples were immediately transported to the laboratory and were frozen at -20 ºC and freeze dried (Ly-8-FM-ULE, Snijders) prior to extraction.

2.3. Identification and quantification of phenolic compounds

2.3.1. Extraction procedure

For each olive tree and sampling date, three powdered fruit sub samples were extracted three times as follow and using the residue of each extraction:~1.5g of sample stirring with 50 mL of methanol at 150 rpm for 1 h (room temperature) and filtered through a Whatman Nº.4 paper. The combined methanolic extracts were vacuum-evaporated (Stuart RE3000, UK) at 35 ºC and redissolved in methanol.

2.3.2. Chromatographic conditions

Phenolic profile was performed by HPLC analysis on a Knauer Smartline separation module equipped with a Knauer smartline auto sampler 3800 (with a cooling system set to 4 ºC) and a Knauer DAD detector 2800. 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 I.D., 5 µm particle diameter, end-capped Nucleosil C18 (Macherey-Nagel)) and its temperature was maintained at 30 ºC**.** The solvent system used was a gradient of water/formic acid (19:1, v/v) (A) and methanol (B) (Vinha et al. 2005), which were previously filtered and degassed. 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. All samples extracts were filtered

through a 0.2 µm Nylon membrane (Whatman) and 20 µL of each solution were injected. Chromatographic data were recorded at 280 nm. Spectral data from all peaks were accumulated in the 200–600 nm range. Phenolic compounds quantification was achieved by external standard calibration curves using authentic standards.

2.4. Antioxidant activity

2.4.1. Extraction procedure

For each sample, three freeze-dried powdered sub-samples (~5 g; 20 mesh) were extracted with 250 mL of water, under boiling for 45 min, and filtered through Whatman Nº. 4 paper. The aqueous extracts were frozen, freeze-dried, and weight. From the dry extract, water solutions ranging from 0.01 and 3 mg/mL were prepared for antioxidant activity assays.

2.4.2. Scavenging effect assay

The capacity to scavenge the free radical 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 decrease of absorption at 517 nm.

DPPH scavenging effect was calculated as a 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.

2.4.3. Reducing power assay

The reducing power was determined according to the method of Berker et al. (2007). The extract solution (1 mL) was mixed with 2.5 mL 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) was added; the mixture was centrifuged at 1000 rpm for 8 min (Centorion K24OR-2003). 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 at 700 nm. Extract concentrations providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration in the solution.

2.5. Statistical analysis

A regression analysis, using Excel from Microsoft Corporation, was established between each individual phenolic compound and the antioxidant activity recorded in both chemical assays. Another regression was also performed to observe the possible correlation between the maturation process and the phenolic profile and the antioxidant activity recorded.

Principal components analysis (PCA) was applied for reducing the number of variables (7 phenolic compounds - hydroxytyrosol, chlorogenic acid, verbascoside, oleuropein, rutin, apigenin 7-*O*-glucoside and luteolin; total phenols content; and EC_{50} values obtained from the two antioxidant assays, with a total of 10 variables) to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information, i.e., the influence of maturation process on the phenolic composition and antioxidant activity of *Cv.* Cobrançosa olive fruits. Moreover, it allowed recognizing patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores). PCA was performed by using SPSS software, version 21.0 (IBM Corporation, NY, USA).

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 maturation 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.

3. Results and discussion

3.1. Method validation

To validate the HPLC chromatographic method for phenolic quantification, a series of assays were performed, including the determination of linearity, LOD, LOQ, intra-day and inter-day precision, and recovery. The results are listed in Table 1. After studying the linearity range for each compound, 8 level calibration curves were constructed on a regular basis, always with high correlation coefficients (>0.999) (Table 1). The retention times (R_t) obtained for the phenolic compounds were: 8.4 min for hydroxytyrosol; 15.5 min for chlorogenic acid; 26.2 min for verbascoside; 38.3 min for oleuropein; 40.2 min for rutin; 41.6 min for apigenin 7-*O*-glucoside and 53.6 min for luteolin, with adequate stability (Table 1). The percentage variation coefficients (CV %) obtained for the R_t are shown in Table 1.

The limits of detection (LOD) and quantification (LOQ) were defined as the lowest concentrations in a sample that can be detected and quantified, being calculated as 3.3 and 10 times the standard deviation of the background noise divided by the slope of the calibration curves, respectively. The detection limits were lower than 0.004 mg/mL. The quantification limits ranged from 0.002 to 0.010 mg/mL, for verbascoside and oleuropein, respectively.

The intra-day precision was evaluated by assaying one sample (corresponding to the last sampling date) six times during the same day and the inter-day precision was determined by analysing the same sample in six different days. The method proved to be precise (intra-day precision ranging from 0.3 to 1.2%. and inter-day precision ranging from 0.3 to 9.7%) essential for conducting reproducible assays thought several months.

Accuracy of the method was assessed by the recovery percentage of phenols standards in the spiked samples. Two different concentration levels of individual phenolic standards were added to the sample before the extraction method, in triplicate. Recovery results are depicted in Table 1.

3.2. Identification and quantification of phenolic compounds

The phenolic composition of the methanolic extracts of *Cv.* Cobrançosa olive fruits in different maturity stages was assessed by HPLC/DAD. Seven phenolic compounds were identified and quantified, namely, hydroxytyrosol, chlorogenic acid, verbascoside, oleuropein, rutin, apigenin 7-*O*-glucoside and luteolin (Fig. 1).

Table 1. Chromatographic characteristics of the reported method.

Phenolic compounds	RT		Correlation coefficient (r^2)	Limits		Precision		Recovery
	min	CV(%) (n=10)		LOD (mg/mL)	LOQ (mg/ml)	Intra-day CV(%) (n=6)	Inter-day CV(%) (n=6)	Mean(%) (n=3)
Hydroxytyrosol	8.4	0.7	0.9997	0.002	0.003	0.5	1.1	96.8±1.4
Chlorogenic Acid	15.5	0.3	0.9990	0.003	0.004	1.2	2.6	96.9±2.3
Verbascoside	26.2	0.3	0.9985	0.001	0.002	0.4	9.7	87.4±1.3
Oleuropein	38.3	0.4	0.9996	0.004	0.010	-	-	99.0±1.2
Rutin	40.2	0.5	0.9995	0.002	0.004	0.7	2.6	87.2±2.0
Apigenin 7-O-glucoside	41.6	0.2	0.9992	0.003	0.003	0.3	0.3	91.9±7.0
Luteolin	53.6	0.2	0.9990	0.003	0.003	0.3	1.4	92.7±2.5

RT - retention time; LOD - limit of detection; LOQ - limit of quantification.

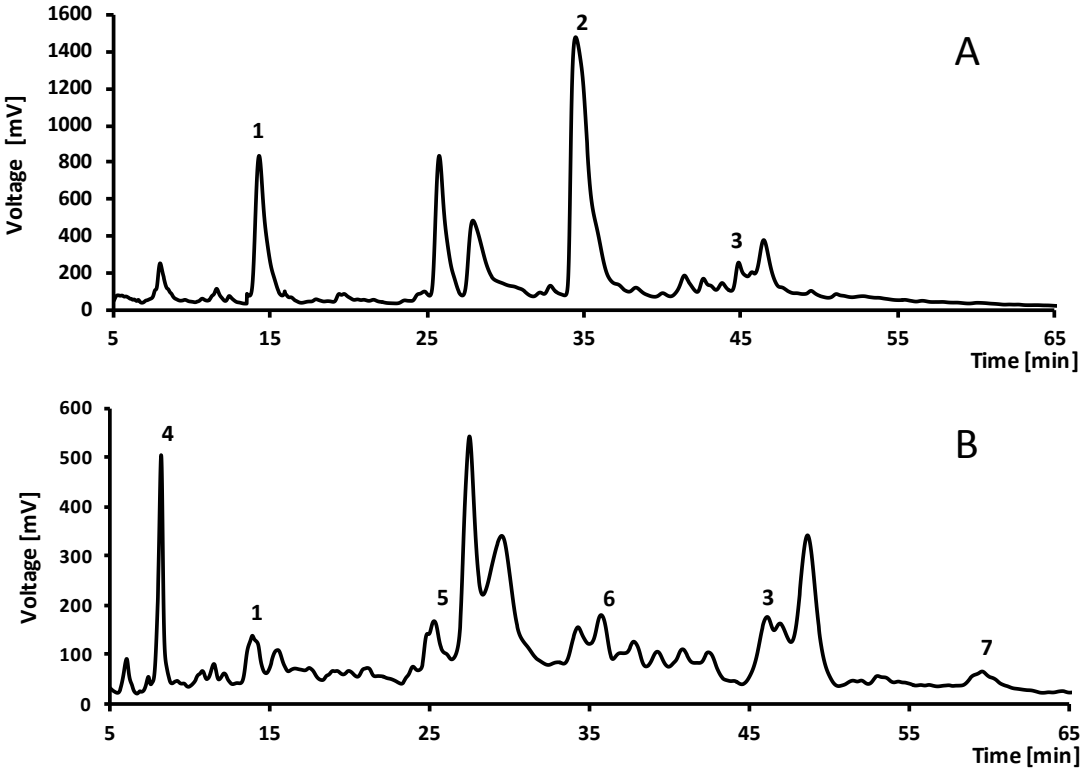

Figure 1. Chromatographic profile of methanolic phenolic extracts of *Cv.* Cobrançosa obtained by HPLC-DAD. (**1**) Chlorogenic acid; (**2**) Oleuropein; (**3**) Apigenin 7-*O*-glucoside; (**4**) Hydroxytyrosol; (**5**) Verbascoside; (**6**) Rutin; (**7**) Luteolin. A- the first sampling date (29th Sept.); B- the last sampling date (18th Nov.).

Total and individual amounts of phenolic compounds are reported in Table 2. Total phenolic content was severely influenced by the maturation process. Significant decline in total phenolics (P< 0.001) was observed from the first sampling date (29th Sept.) with near 34 g/kg to the last (18th Nov.) with less than 1 g/kg, on a fresh fruit pulp basis, corresponding only to 2% of the initial amounts. Such fact is related with the individual phenolic compounds content, particularly with the pattern observed for oleuropein. This phenolic, together with hydroxytyrosol and chlorogenic acid were the most abundant phenolic compounds in the olive fruits throughout the maturation process, results in accordance with the information available in literature for diverse cultivars (Gómez-Rico et al. 2008; Savarese et al. 2007; Vinha et al. 2005). In the two first sampling dates, with immature and astringent olives, oleuropein was the most abundant phenolic, attaining respectively 97.3% (32937 mg/kg) and 81.1% (3706 mg/kg) of the total phenols content, despite the abrupt reduction between these two dates. From this date its content decreased again deeply to below 1% in the 3rd and 4th collecting dates, being below the quantification limit in the last assay. .

Table 2. Phenolic profile (mg/kg of fresh weight) of olive fruits from *Cv.* Cobrançosa during the maturation process (mean ± standard deviation; n = 5).

Phenolic compound	29th Sept.	13th Oct.	27th Oct.	10th Nov.	18th Nov.	*P* - Value
Hydroxytyrosol	nq	672 ± 83 b,c	663 ± 4 b	614 ± 6 c	439 ± 13 a	< 0.001[(1)]
Chlorogenic acid	788 ± 12 c	nq	31 ± 2. b	29 ± 2 b	22 ± 4 a	< 0.001[(1)]
Verbascoside	nd	nd	nq	nq	66 ± 3	-
Oleuropein	32938 ± 204 d	3706 ± 167 c	254±24 b	126±59 a	nq	< 0.001[(1)]
Rutin	nd	9 ± 8 a	160 ± 16 c	127 ± 20 b	250 ± 4 d	< 0.001[(1)]
Apigenin 7-O-glucoside	131 ± 2 b	96 ± 8 a	88 ± 10 a	97 ± 5 a	131 ± 20 b	< 0.001[(2)]
Luteolin	nd	nd	nd	48 ±2 a	53 ± 4 b	< 0.001[(1)]
Total	33856±201 d	4564±217 c	1197 ± 40 b	1040 ± 70 a	960 ± 38 a	< 0.001[(1)]

[a-d]Means within a same line, with different superscripts, differ significantly, $P < 0.05$. [(1)]$P < 0.05$ by means of Levene test. *P* values from one-way Welch ANOVA analysis. Means were compared by Dunnett T3´s test, since equal variances could not be assumed. [(2)]$P > 0.05$ by means of Levene test. *P* values from one-way ANOVA analysis. Means were compared by Tukey´s test, since equal variances could be assumed.
nq – bellow LOQ; nd – bellow LOD

The pattern observed in the oleuropein is known to be due to several causes, including enzymatic bioconversion to diverse derivatives, including hydroxytyrosol (Amiot et al. 1986; 1989; Ryan et al. 2002). This phenolic is detected in olives with an intermediate or advanced maturation. Indeed, hydroxytyrosol content was below our LOQ in the first sampling date, increasing abruptly to 0.6 g/kg in the second one (13th Oct.). It remained constant throughout all collecting dates and decline slightly to 0.4 g/kg in the last sampling date (18th Nov.). Although derived from oleuropein by hydrolysis, their amounts are not correlated, as hydroxytyrosol increase was not proportional to oleuropein decrease. Hydroxytyrosol has been extensively studied regarding its antioxidant properties and potential health beneficial effects, with increased bioactivity when compared to oleuropein (Obied et al. 2005; Ryan and Robards 1998). Chlorogenic acid content also decreases over the maturation, from 787 mg/kg in the first sampling date to 21.75 mg/kg in the last date (Table 2). In the second sampling date (13th Oct.) chlorogenic acid content was below our LOQ, and consequently we were unable to quantify it.

Regarding other minor phenolics, verbascoside was not detectable in immature fruits (Table 1), as also observed by Ryan and Robards (1998) and Vinha et al. (2005), being only quantified in the last sampling date with 65.73 mg/kg. Some authors suggest that the formation of verbascoside may be also related with the partial degradation of oleuropein, which could explain the later appearance of verbascoside in olive fruits (Ryan and Robards 1998). The presence of rutin has been reported in other olive cultivars (Bouaziz et al. 2005; Cardoso et al. 2005; Gómez-Rico et al. 2008; Ryan et al. 2002). A clear increase in the concentrations of this compound during fruit maturation was observed, from 89.94 mg/kg to 249.51 mg/kg. Similar results were reported by Gómez-Rico et al. (2008), who found equivalent values for rutin in some Spanish cultivars. Many biological effects have been attributed to this flavonoid, which shows antioxidant, anti-inflammatory, anti-thrombotic, cytoprotective, vasoprotective and antimicrobial activities (Savarese et al. 2007).

Globally, the major differences were observed between the 2nd and 3rd sampling dates, corresponding to the beginning of the reddish spots, marked by the reduction in oleuropein and appearance of hydroxytyrosol and rutin. The 3rd and 4th sampling dates presented similar amounts of total phenolic compounds, with oleuropein decreasing and other minor phenolics increasing slightly. The last sampling date, however, was clearly distinct regarding its phenolic profile, with the absence of oleuropein and appearance of luteolin and verbascoside, this last already quantified in the earlier week. Our results are in line with those previously reported by Damak et al. (2008), Jemai et al. (2009), Morelló et al. (2004), Morelló et al. (2005), who showed that the

major phenolic compounds in olive drupe (hydroxytyrosol and oleuropein) followed the same trends during maturation.

A regression analysis was done in order to try to establish correlations between the data obtained in the phenolic profile and antioxidant activity with the maturation process of *Cv*. Cobrançosa olive fruits (Table 3). The contents of verbascoside, rutin and luteolin were extremely positively correlated ($P < 0.001$) with the maturation process and for hydroxytyrosol a very positive significant correlation was established ($0.001 < P < 0.01$). For chlorogenic acid, oleuropein, and total phenols content extremely significant negative correlations were confirmed (Table 3), once their content decrease as the olive fruit become riper. No correlation was established for apigenin.

Table 3. Correlation between the phenolic composition, and antioxidant activity of olive fruits from *Cv.* Cobrançosa with the maturation process.

	Fruit maturation process		
Phenolic compounds	**Equation**	**R^2**	***P***
Hydroxytyrosol	$y = 81.9x + 231.7$	0.206	**
Chlorogenic Acid	$y = -150.4x + 625.1$	0.479	***
Verbascoside	$y = 13.1x + 26.3$	0.499	***
Oleuropein	$y = -6945.6x + 28241.5$	0.585	***
Rutin	$y = 53.6x - 35.4$	0.834	***
Apigenin 7-*O*-glucoside	$y = -0.5x + 111.3$	0.001	n.s.
Luteolin	$y = 15.5x - 26.2$	0.768	***
Total phenols	$y = -6930.7x + 29113.4$	0.583	***
Antioxidant activity			
EC_{50} DPPH	$y = 0.005x + 0.144$	0.151	**
EC_{50} Reducing power	$y = 0.040x + 0.349$	0.444	***

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

3.3. Antioxidant activity

Besides the phenolic maturation trends observed and discussed above, other compounds with antioxidant activity are known to be present in the olive fruits. In order to better understand the global antioxidant capacity throughout maturation and the

phenolics significance within it, the antioxidant potential of *Cv.* Cobrançosa olive aqueous extracts was measured by two different assays: scavenging activity on DPPH radicals and reducing power.

In the reducing power assay, a ferric ion-based total antioxidant capacity assay, the presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form by donating an electron. A concentration-dependent reducing activity was observed (Fig. 2), with a linear increase in the absorbance's up to the 3 mg/mL tested, for all sampling dates (Fig. 2).

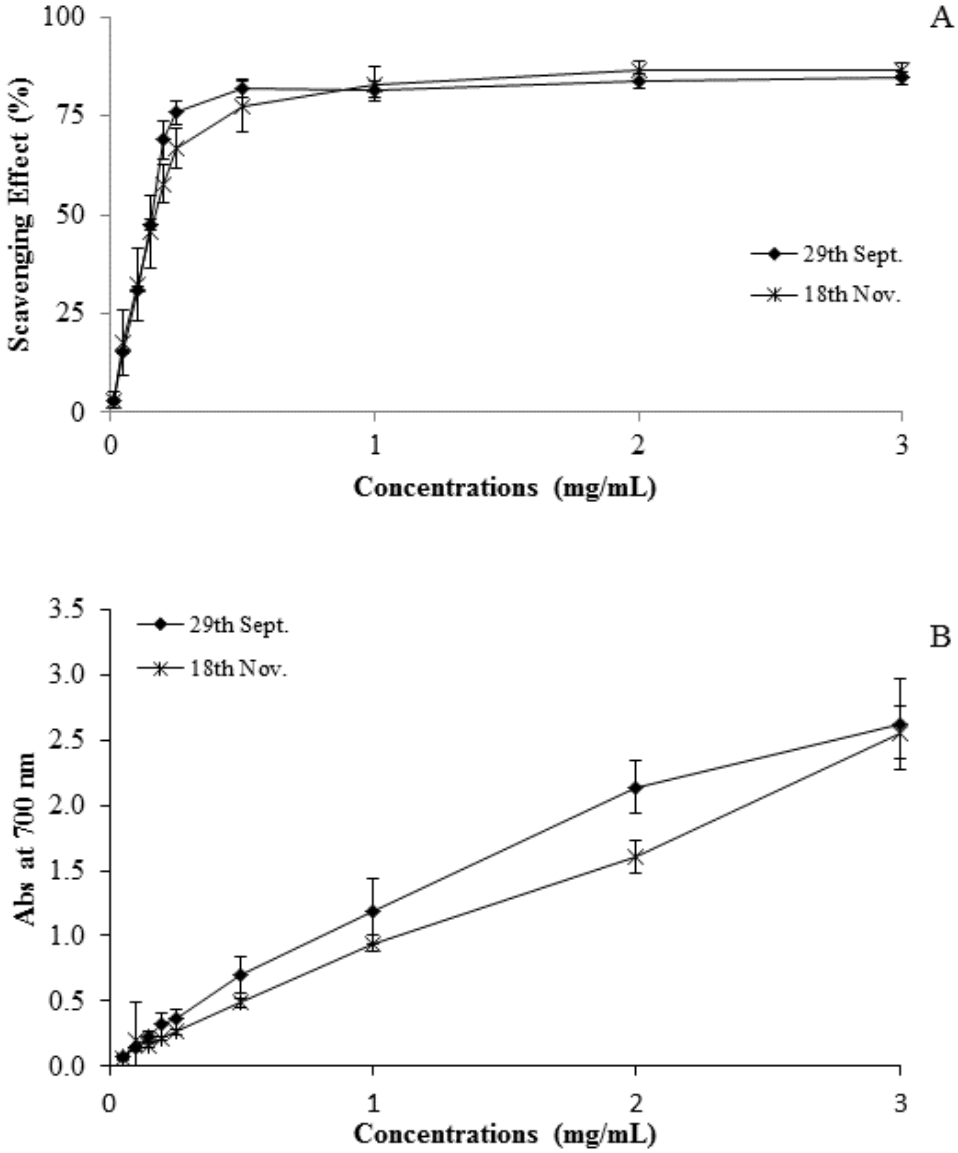

Figure 2. Scavenging effect on DPPH radicals (A) and reducing power (B) of Cv. Cobrançosa aqueous extracts in the first (29th Sept.) and last (18th Nov.) sampling dates (mean ± standard deviation; n = 5).

However, earlier sampling dates presented higher slopes than late ones, indicating the presence of higher content of compounds with effective reducing capacity in the aqueous extracts obtained from the olives. This observation is more clear when the EC_{50} values are compared (Fig. 3), with 0.36 mg/mL in the first sample (29th Sept.), increasing to the last sampling date (18th Nov.), with a significant higher value of 0.53 mg/mL ($P < 0.001$), the highest value obtained. This increase in the EC_{50} is indicative of a lower content of compounds with reducing capacity in the same mass of aqueous extracts. Knowing that the extract yield also decreased with maturation, from 45% in the first sampling date to 33% in the last ones, the magnitude of the differences observed further increases during maturation.

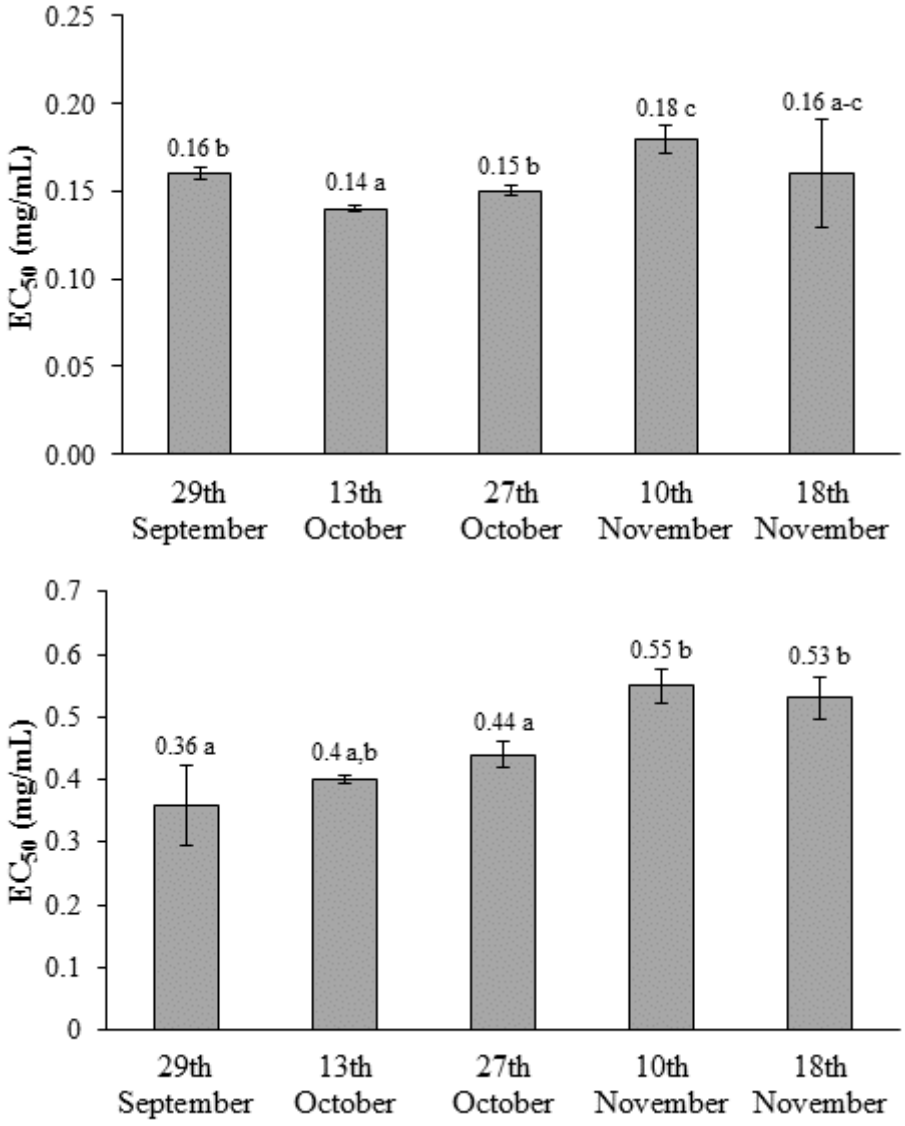

Figure 3. EC_{50} values of DPPH (A - effective concentration at which 50% of DPPH radicals are scavenged) and reducing power (B - effective concentration at which the absorbance is 0.5) chemical assays of Cv. Cobrançosa aqueous extracts during the maturation process (mean ± standard deviation; n = 5).

The decrease in the reducing capacity followed a similar trend to the total phenolic compounds, as previously detailed. Indeed, phenolic compounds are recognized as the major antioxidant compounds in olive extracts, and their redox properties are attributed to their phenolic hydroxyl groups and conjugated double bonds, with the ability to break the free radical chain by donating electrons. However, while total phenolics decreased almost 98% through sampling dates, the reducing capacity decreased only about 66%. This might be derived from the distinct redox effectiveness of each phenolic compound, not only due to the number and position of free and esterified hydroxyl groups but also from the structural relationships between the different parts of their chemical structure, the presence or absence of glycosidic moieties, the glycosylation site, etc.

Indeed, the high amounts of oleuropein were only partially accompanied by increasing amounts of hydroxytyrosol, but the latter has a recognized higher antioxidant activity than the formed on a mass basis. Also, despite being chlorogenic acid an important antioxidant in the first sampling date, its disappearance was accompanied by the formation of rutin and luteolin, highly effective flavonoids, as well as verbascoside, among the phenolics with higher antioxidant activity due to its two catechol structures. Simultaneously, one cannot disregard that phenolic compounds are not acting alone, and synergies might occur within the phenolic pool (Benavente-García et al., 2000), as well as with other non-phenolic compounds with the ability to react with Fe^{3+}, as sugars (Menz & Vriesekoop, 2010), organic acids (Lopez et al., 2005), peptides (Zamora et al., 2001), etc.

A regression analysis was tested in order to observe if the individual phenolic compounds could be related with the antioxidant activity recorded. Indeed, for the reducing power EC_{50} values, only apigenin 7-*O*-glucoside was not correlated ($y = -8.2E^{-4}x + 0.560$; $R^2 = 0.040$; $P > 0.05$). Verbascoside was positively correlated ($y = 0.001x + 0.456$; $R^2 = 0.115$; $0.01 < P < 0.05$), and the remaining phenols were all extremely correlated ($P < 0.001$). Total phenols content ($y = -4.3E^{-6}x + 0.506$; $R^2 = 0.420$; $P < 0.001$), oleuropein ($y = -4.3E^{-6}x + 0.502$; $R^2 = 0.419$; $P < 0.001$) and chlorogenic acid ($y = -1.8E^{-4}x + 0.501$; $R^2 = 0.398$; $P < 0.001$) were those with highest correlations for the antioxidant activity of the olives extracts. This means that higher contents are related with lower EC_{50} values, and therefore higher antioxidant activity displayed, as previoulsly discussed. As expected, extremely significant correlations ($P < 0.001$) were also observed with the reducing power (Table 3).

The ability to scavenge radicals by donation of hydroxyl groups was evaluated by the DPPH assay. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation, therefore of particular importance in lipids.

Considering the olives extracts collected at the first sampling date, an increase in the scavenging effect from 2.98% to 84.60% was observed when the concentration increased from 0.01 mg/mL to 1 mg/mL, remaining constant for increased amounts, indicative of that low amounts are sufficient for maximum activity, and therefore a high scavenging effect is expected. In opposition to the reducing power, the maturation process brought reduced change in the EC_{50} values of DPPH method, varying between 0.14 mg/mL in the 13th Oct. sample and 0.18 mg/mL in the 10th Nov. sample, but without a clear pattern (Table 2). In a general way, the first samples reported lower EC_{50} values while the last ones reported higher EC_{50} values, indicative of a lower antioxidant potential with increased maturity, with statistical significance (P< 0.001). The different evolution in comparison with the reducing power results might be an indication of the higher radical scavenging activity of the compounds extracted present from the last sampling dates, in opposition to the former ones. In particular, the presence of the mentioned o-dihydroxy (catechol) structures together with the presence of both 3- and 5-hydroxyl groups, as in rutin and luteolin, maximizes radical-scavenging capacity and strongest radical absorption (Benavente-García et al., 2000), derived as being 2.5 more actives than vitamins C or E, while for oleuropein the strongest structural entity are the cathecol structures alone, therefore less effective. Vitamin E was not evaluated under the present work but it is a recognized as a powerful antioxidant in olives, with a determinant part in the preservation of the lipid moiety. Indeed, it has also a phenolic basis, and, despite being insoluble in water, the presence of other olive pulp compounds might have co-extracted it partially, being also a potential candidate for the observed overall effects in the two assays.

When regression was tested, from the seven phenolic compounds identified, only luteolin was extremely correlated with the EC_{50} values obtained in the DPPH method ($y = 4.1E\text{-}4x + 0.152$; $R^2 = 0.273$; $P < 0.001$), stressing the importance of this flavonoid in the effects observed. For the remaining phenols, their content was not correlated with the results obtained for the DPPH antioxidant method ($P > 0.05$). In opposition to the reducing power, only very significant correlations ($0.01 < P < 0.05$) were established between the EC_{50} values of DPPH and maturation stage.

The results obtained demonstrates that green olive fruits (from the first sampling date) possess higher antioxidant potential than black olives (from the last sampling date), being the maturation process a key intervenient in the bioactive properties of olive fruits, particularly regarding its reductive potential. Such results are in accordance with the antioxidant activity verified during the maturation of several other olive cultivars (Bouaziz et al. 2004; Damak et al. 2008; Jemai et al. 2009). The antioxidant potential

observed could be relatedwith the phenolic composition of the extracts but other components could also have an important contribution.

3.4. Discrimination of maturation stage based in the phenolic composition and antioxidant activity

In order to verify if the phenolic composition and antioxidant activity of *Cv.* Cobrançosa olive fruits could classify the different sampling dates during the maturation process, a PCA was performed. The PCA showed that 78.0% of the total variance of the data used could be explained by using only two principal components (Fig. 4). Through the results obtained from the PCA it is inferred that it's possible to differentiate the five sampling dates into four specific groups. The same observation was obtained by applying a stepwise linear discriminant analysis, where the discriminant model was capable to classify all the five samples in study according to their maturation stage (data not showed). A curious observation is also noticed in Fig. 4, being the sampling dates represented in a clockwise direction.

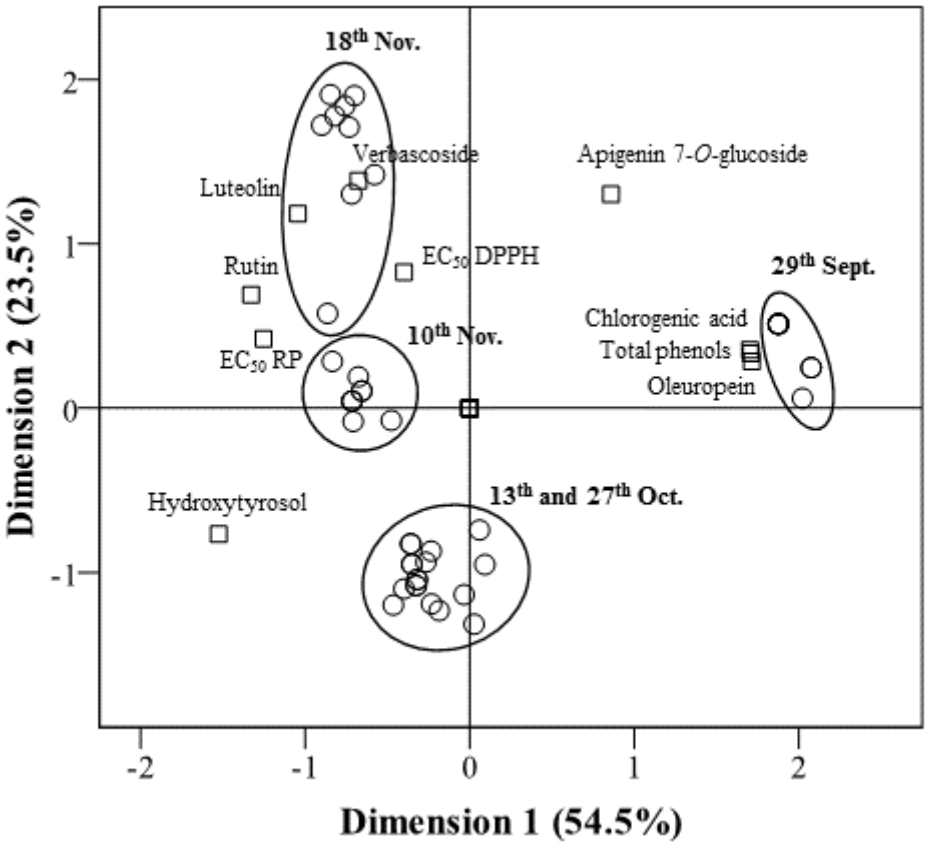

Figure 4. Principal components analysis obtained from the phenolic composition and EC_{50} values of DPPH and reducing power methods of olive fruits from Cv. Cobrançosa during the maturation process. The PCA factors explain 78.0% of the total variance.

In the positive regions of both principal components are represented the fruits sampled at 29th September (first sampling date) separated from the remaining sampling dates by the first principal component. Fruits from this sample were characterized by higher content in oleuropein, chlorogenic acid and in total phenolic content (Fig. 4 and Table 2). Both samples from October (13th and 27th) are represented mainly in both negative regions of the principal components due to their high content in hydroxytyrosol, Samples collected in November (10th and 18th November) are mainly represented in the negative region of the first principal component and in the positive region of the second principal component. These samples were characterized by higher verbascoside, rutin, and luteolin contents. The samples from November are represented in the extreme opposite from the sample of September, another fact that differentiate the first sample from the last ones concerning mainly antioxidant potential and total phenols content. Samples from November were those who reported higher EC_{50} values for both antioxidant chemical assays, which means lower antioxidant activity and lower total phenols content. By other hand samples from 29th September were those who reported higher antioxidant activity in part related with the high total phenols content present in the fruits from the beginning of maturation.

This data emphasises that during the maturation, the phenolic composition of olive fruits changes continuously conferring a characteristic phenolic profile that could influence in a decisive way the bioactive properties of the olive fruits, as observed in the antioxidant potential. It also indicated that the phenolics are among the main but are not the only hydrophilic antioxidant compounds in olive fruits.

4. CONCLUSIONS

With the present study, for the first time it was possible to report the phenolic composition and antioxidant activity of *Cv.* Cobrançosa olives during the maturation process. Important changes occurred in olives concerning their phenolic composition. Oleuropein, the main phenolic compound in green olives, decreased drastically during the maturation, while hydroxytyrosol increased and was the main phenolic in ripe olives. Total phenols content dropped to near 2% when the first stage was compared with the last. Antioxidant activity was influenced by the individual phenolics, being established correlations between both parameters and with the maturation process. During maturation the reductive capacity decreased, mainly due to the decrease in the content of oleuropein but the formation of new phenolics with increased reductive capacity and particularly radical scavenging activity reduced the reduction magnitude

from the *in vitro* teste point of view. The changes observed in both qualitative and quantitative fractions of phenolic compounds as well as in the antioxidant activity during the maturation, allowed their discrimination, which corroborated the unique phenolic profile in each stage of the maturation process and its contribution to the overall activity. The results collected from this work are a useful contribution for the characterization of one of the most important olive cultivars from the PDO “Azeite de Trás-os-Montes”. Information regarding the influence of maturation in the composition and bioactive properties of olives are of major importance once that can help us to improve table olives and olive oil composition, and the most important of all, allows us to better estimate the optimum harvest time. However, further studies are requested in order to completely understand the full impact of maturation in olive fruits composition. Nutritional studies, sensory evaluations, and further bioactive properties are among those included in ongoing studies.

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CAPÍTULO 5.

Optimal harvesting period for cvs. Madural and Verdeal Transmontana , based on antioxidant potential and phenolic composition of olives

Anabela Sousa[†,§], Ricardo Malheiro[†,§], Susana Casal[§*], Albino Bento[†], José Alberto Pereira[†*]

[†]Mountain Research Centre (CIMO), School of Agriculture, Polytechnic Institute of Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal
[§]LAQV@REQUIMTE/Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, Porto University, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

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Abstract

In the present study we propose to determine an approximate optimum harvesting period for table olives and olive oil of two Portuguese olive cultivars (Madural and Verdeal Transmontana) based on phenolic modifications (HPLC/DAD) and antioxidant activity (scavenging capacity on 2,2-diphenyl-1-picrylhydrazyl and reducing power). Samples were collected from almost edibility to slightly over-mature. The sum of polyphenols, as well as its most abundant components oleuropein and hydroxytyrosol, decreased during this maturation period, more intensively in Madural than Verdeal Transmontana. In their green stages an antioxidant potential loss was gradually observed in both olive cultivars, while in the latter purple-black phases a slight increase in the antioxidant activity was observed. Both phenolic profile and antioxidant activity were highly correlated with the maturation process. A principal component analysis showed the particular effect of maturation in both olive cultivars.

Based on the acquired knowledge we can advance that, for these cultivars and geographical region, olives harvest for table olives, traditionally collected sooner, can be performed in the middle of September. For olive oil harvesting can occur in the first days of November, giving priority to cv. Madural rather than Verdeal Transmontana, in order to enhance the bioactivity, phenolic composition and stability of olive oils.

Keywords: maturation process; olive cultivar; phenolic profile; antioxidant activity.

1. Introduction

Olive products are increasingly popular worldwide, not only for their unique sensorial characteristics but also for the beneficial health effects associated with their consumption, particularly within the Mediterranean diet. An array of olive components have been linked to its beneficial properties: a balanced fatty acid profile, sterols, tocopherols, pigments like chlorophylls and carotenoids, and a very important group of components - the phenolic compounds. Indeed, several biological functions and properties are ascribed to phenolic compounds, particularly within olive products. Apart from their natural roles in plant chemical defense mechanism, as common to other species, they are particularly important for the olive products sensorial attributes, particularly oleuropein for its bitterness (Andrews, Busch, Joode, Groenewegen, & Alexandre, 2003), being also associated with other positive sensorial attributes, such as the spicy, pungency and bitter ones (Dierkes, Krieger, Duck, Bongartz, Schmitz, & Hayen, 2012). Obied et al. (2012) reviewed the pharmacology of olives biophenols and discussed their antioxidant, anti-inflammatory, cardiovascular, immunomodulatory, gastrointestinal, endocrine, respiratory, autonomic, central nervous system, antimicrobial, chemotherapeutic, anticancer and chemopreventive effects/properties. Based on these potential benefits, olive products phenolic compounds should be maximized, with careful attention to keep a balanced sensorial profile for consumer's acceptability.

Several aspects are known to influence olives phenolic composition, with direct repercussions on its derived products, in particular: i) olive cultivar (Malheiro, Sousa, Casal, Bento, & Pereira, 2011); ii) geographical origin (Vinha et al., 2005); iii) agricultural practices (Tovar, Motilva, & Romero et al., 2001); and iv) maturation process (Bouaziz, Chamkha, & Sayadi, 2004; Morelló, Romero, & Motilva, 2004; Ryan, Robards, & Lavee, 1999). The maturation process assumes a special importance when high quality olives are intended for future processing. During olives maturation a series of metabolic and enzymatic reactions prompts a decrease in many phenolic compounds. Indeed, advanced maturation results in a clear reduction of positive sensorial attributes and oxidative stability due to the decline on photosynthetic pigments (chlorophylls and carotenoids) and phenolic compounds (Morelló et al., 2004), directly influencing olive products quality. Several studies devoted to the study of phenolic composition of olives during maturation indicate that phenols content increases progressively during the so-called green-phase, corresponding to the fruit growth period. When olives are purple and black the phenols content decrease sharply (Morelló et al., 2004).

Madural, Verdeal Transmontana, and Cobrançosa, are the main cultivars used for the production of the Protected Designation of Origin (PDO) “Azeite de Trás-os-Montes” olive oil, in Northeast of Portugal. These cultivars account for more than 90% of olives cultivation area in this region and are also cultivated in others olive producing regions of Portugal. There is a lack of information on the chemical characteristics of Madural and Verdeal Transmontana olives as regards to antioxidant capacity and phenolic composition throughout maturation. The aim of this investigation is to study the effect of the maturation process in the phenolic profile and biological properties of the olive fruit, particularly its antioxidant potential, in order to maximize olive products quality and biological properties, being, for the author’s knowledge, the first report of this kind in these two olive cultivars.

2. Material and methods

2.1. Reagents and standards

Methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH) 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 dihydrate were obtained from Panreac (Barcelona, Spain). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). Hydroxytyrosol, chlorogenic acid, verbascoside, oleuropein, rutin, apigenin 7-O-glucoside and luteolin standards, used for phenolic profile identification were obtained from Extrasynthèse (Genay, France).

2.2. Sampling

Five representative olive trees from cvs. Madural and Verdeal Transmontana were selected in an olive grove at Paradela, Mirandela (Northeast of Portugal), in 2009. Olive grove characteristics: 3 ha; planting density of 7 × 7 m; trees more than 40 years old; pruned every three years; rain-fed; soil tilled 2–3 times/year. Five sampling dates (29th September, 13th and 27th October, and 10th and 18th November) were chosen to monitor the maturation process, corresponding to potentially edible olives from slightly green to over-mature ones. From each tree and sampling date olives were handpicked (1 kg). Samples were divided in two parts, one part used for maturation index estimation and moisture content (oven drying at 105ºC), and the remaining olives

depulped, frozen at -20 ºC and freeze-dried (Ly-8-FM-ULE, Snijders) for subsequent chemical analysis. Maturation index (MI) was determined on each olive cultivar and sampling date as described by Hermoso, Uceda, Frias, and Beltrán (2001).

2.3. Identification and quantification of phenolic compounds

2.3.1. Extraction procedure

For each olives sample, three powdered pulp fruit sub samples (~1.5 g; sieve size 0.841 mm) were extracted by stirring with 50 mL of methanol, for 1 h at 150 rpm, and filtered through Whatman Nº. 4 paper. The residue was re-extracted similarly with three additional 50 mL portions of methanol. The combined methanolic extracts were vacuum-evaporated (Stuart RE3000, United Kingdom) at 35 ºC, redissolved in methanol, filtered through a 0.2 µm Nylon membrane (Whatman) and analyzed by HPLC. Previous tests with hydro-methanolic and water extracts were also assayed according to the above extraction conditions. Once methanolic extract profile comprises more phenolic compounds of several different polarities than the others, it was chosen for the quantification purposes.

2.3.2. Chromatographic conditions

Phenolic profile was performed by HPLC analysis on a Knauer Smartline separation module equipped with a Knauer smartline autosampler 3800 (with a cooling system set to 4 ºC) and a Knauer DAD detector 2800. A reversed-phase Spherisorb ODS2 column was used (250 mm × 4 mm I.D., 5 µm particle diameter, end-capped Nucleosil C18 (Macherey-Nagel)) and its temperature was maintained at 30 ºC. The solvent system used was a 66 minutes gradient program of formic acid/water (50 mL/L) (A) and methanol (B) at 0.9 mL/min (Vinha et al. 2005). Spectral data from all peaks were accumulated in the 200–600 nm range. Phenolic compounds quantification was performed at 280 nm and achieved by external standard calibration curves using authentic standards.

2.4. Antioxidant activity

2.4.1. Extraction procedure

For each sample, three freeze dried powdered sub-samples (~5 g; sieve size 0.841 mm) were extracted with 250 mL of water, under boiling for 45 min, and filtered through Whatman Nº. 4 paper (Malheiro et al 2011). The aqueous extracts were frozen, lyophilized, and weighed. From the dry extract, aqueous solutions ranging from 0.01 and 3 g/L were prepared for antioxidant activity assays.

2.4.2. Scavenging effect assay

The capacity to scavenge DPPH free radicals was monitored according to the method of Hatano, Kagawa, Yasuhara, and Okuda (1988) with modifications. The extract solution (0.3 mL) was mixed with 2.7 mL of methanolic DPPH radicals (6×10-5 mol/L) solution. The mixture was shaken vigorously, monitoring continuously the absorbance decrease at 517 nm, read against a blank, until stable absorbance values were obtained. DPPH scavenging effect was calculated as a percentage of DPPH discoloration using the following equation: % scavenging effect = [(ADPPH-AS)/ADPPH] × 100, where AS is the absorbance of the solution when the sample extract has been added at a particular level, and ADPPH is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (EC50) was calculated and converted to pulp mass based on the extract weight at 2.4.1.

2.4.3. Reducing power assay

The reducing power was determined according to the method of Berker, Güçlü, Tor, and Apak (2007). The extract solution (1 mL) was mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (10 g/L). The mixture was incubated at 50 ºC for 20 min. After cooling, 2.5 mL of trichloroacetic acid (100 g/L) was added, the mixture was centrifuged at 145 g for 8 min (Centorion K24OR- 2003). The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of a solution of ferric chloride (1 g/L), and the absorbance was measured at 700 nm. Extract concentrations providing 0.5 of absorbance (EC50) were calculated from the graph of absorbance at 700 nm against extract concentration in the solution and converted to fresh pulp mass.

2.5. Statistical analysis

Regression analysis, an analysis of variance (ANOVA), and a principal component analysis (PCA) were performed using SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.).

3. Results and discussion

3.1. Identification and quantification of phenolic compounds

Phenolic composition of methanolic extracts of olives from cvs. Madural and Verdeal Transmontana during the maturation process were assessed by HPLC/DAD. In both cultivars, seven phenolic compounds were identified during maturation: one phenolic alcohol (hydroxytyrosol), two flavones (apigenin 7-O-glucoside and luteolin), a caffeoyl phenylethanoid glycoside (verbascoside), one secoiridoid (oleuropein), one phenolic acid (chlorogenic acid), and a flavonol (rutin) (Figure 1).

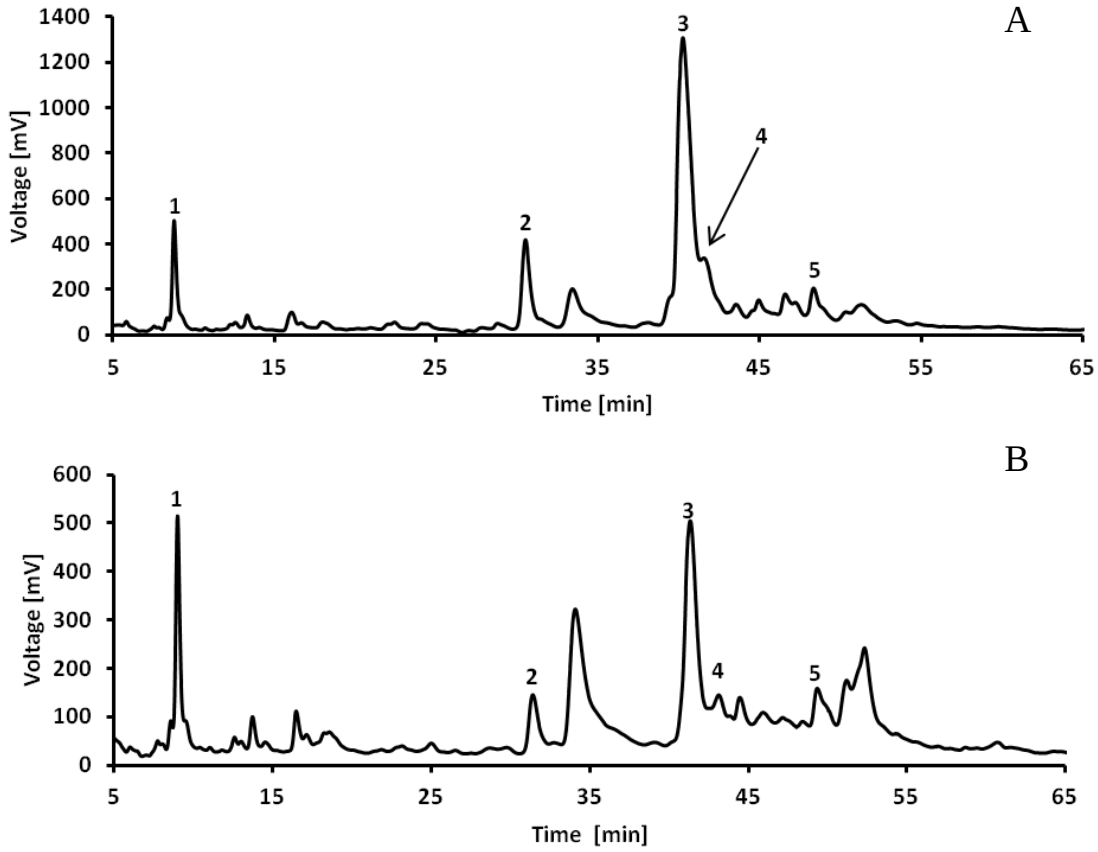

Figure 1. Chromatographic phenolic profile of olives methanolic extracts from cvs. Madural (Fig. 1A) and Verdeal Transmontana (Fig. 1B), in the first sampling date (29th Sept.), obtained by HPLC-DAD at 280 nm. (1) Hydroxytyrosol; (2) verbascoside; (3) oleuropein; (4) rutin; (5) apigenin 7-O-glucoside.

Olive cultivar and harvest date had a marked influence on the phenolic content, both individually (expressed as mg of phenolic compound/kg of fresh olive fruit) and as the sum of polyphenols (Table 1). Phenolic content decreased continually with olives

maturation, with a specific trend according to the olive cultivar assessed. Madural olives presented higher sum of polyphenols at first sampling date (29th Sept.), with nearly 39 g/kg, while cv. Verdeal Transmontana had approximately 15 g/kg (Table 1), having both an external green epidermis and a maturation index of 1. Significant losses ($P < 0.001$) were observed in both cultivars during maturation, achieving 98.7% in cv. Madural (494 mg/kg in the last sampling date) and 95.5% in cv. Verdeal Transmontana (667 mg/kg in the last sampling date), when olives were purple or black. Interestingly, while Madural was characterized by higher sum of polyphenols in the green stages, from the third picking date forward cv. Verdeal Transmontana presented higher sum of polyphenols amounts than the former.

The loss of phenols is mainly determined by oleuropein content, the main phenolic component of olives, in accordance with studies on diverse cultivars (Vinha et al., 2005; Damak, Bouaziz, Ayadi, Sayadi, & Damak, 2008; Gómez-Rico, Fragapane, & Salvador, 2008). In both olive cultivars, a high concentration of this secoiridoid was observed in the first sampling date (29th Sept.) with Madural olives reporting 36 g/kg and Verdeal Transmontana 13 g/kg (Table 1). Such high oleuropein contents during olives green phaseare expected, as olives growth phase is characterized by an accumulation of oleuropein (Charoenprasert & Mitchell, 2012). Thereafter, oleuropein content in olives diminishes with variable rates, in parallel with an external color change from green to purple and black olives, with low amounts of oleuropein usually present in ripe olives. This transformation seems to occur at expenses of enzymatic activity, including enzymes present in the fruit, like polyphenol oxidase (Ortega-García, Blanco, Peinado, & Peragón, 2008) and β-glucosidase (Gutierrez-Rosales, Romero, Casanovas, Motilva, & Mínguez-Mosquera, 2012).

Madural and Verdeal Transmontana olives presented a similar reduction trend, but with different patterns between them. Indeed, while Madural reported a continuous drop on oleuropein content until the last sampling date, with only 0.7% of the content on the first sampling date (263 mg/kg), olives from cv. Verdeal Transmontana presented lower loss of oleuropein content through this sampling period. This observation should be a direct consequence of its slower maturation process, particularly visible from the third sampling date forward, where both color and MI are lower in cv. Verdeal Transmontana. Also, at this same sampling date, cv. Verdeal Transmontana had nearly 1 g/kg, almost three times more than cv. Madural. At the last sampling date, corresponding to over mature olives, Verdeal Transmontana olives had 3.3% of oleuropein present in the first sampling date (431 mg/kg).

Table 1. Phenolic profile (mg/kg of fresh weight) of olives from cvs. Madural and Verdeal Transmontana during the maturation process (mean ± standard deviation; n = 5).

Samples	Fruit color	MI*	Phenolic compounds							
			Hydroxytyrosol	Chlorogenic acid	Verbascoside	Oleuropein	Rutin	Apigenin 7-*O*-glucoside	Luteolin	Sum of polyphenols (g/kg)
Madural.										
29th Sept.	Green	1	830 ± 110 b	-	968 ± 82 c	36375 ± 3436 c	484 ± 111 c	171 ± 30 c	-	39 ± 4 d
13th Oct.	Green	1.04	100 ± 24 a	-	-	950 ± 151 b	271 ± 82 b	34 ± 7 b	11 ± 3 b	1.4 ± 0.2c
27th Oct.	Green-purple	2.27	86 ± 11 a	-	-	332 ± 59 a	213 ± 78 b	30 ± 8 b	12 ± 3 b	0.7± 0.1 b
10th Nov.	Black	3.91	70 ± 20 a	-	166 ± 49 b	298 ± 72 a	113 ± 36 a	27 ± 7 b	8 ± 2 a,b	0.7± 0.1a,b
18th Nov.	Black	5.02	83 ± 5 a	-	36 ± 2 a	263 ± 121 a	87 ± 20 a	17 ± 2 a	7 ± 1 a	0.5± 0.1a
Verdeal T.										
29th Sept.	Green	1	752 ± 18 d	-	311 ± 27 b	13097 ± 219 e	515 ± 35 d	126 ± 6 c	-	14.8± 0.2d
13th Oct.	Green	1	256 ± 2 b	60.7 ± 0.7 d	98 ± 3 a	595 ± 64 b	251 ± 15 c	57 ± 2 b	-	1.3± 0.1b
27th Oct.	Green	1.06	299 ± 1 c	18 ± 0 c	-	934 ± 3 d	169 ± 17 b	102 ± 1 d	45 ± 1 b	2± 0c
10th Nov.	Purple	2.95	174 ± 17 a	16 ± 1 b	-	878 ± 8 c	171 ± 7 b	93 ± 6 c	19 ± 3 a	1.4± 0.0b
18th Nov.	Purple-black	3.28	119 ± 45 a	12 ±3 a	-	431 ± 15 a	98 ± 41 a	6 ± 1 a	-	0.7± 0.1a

*Maturation index; [a-e] Means within the same column and cultivar , with different letters, differ significantly at $P < 0.05$

Similar results were reported by Jemai, Bouaziz, and Sayadi (2009), who found equivalent values and trends for oleuropein in two Tunisian olive cultivars, decreasing from 3.3 g/kg fresh olive to 0.16 g/kg in cv. Dhokar and from 5.7 to 3.8 g/kg in cv. Chemlali. Gomez-Rico et al. (2008) reported the same in cv. Arbequina, decreasing oleuropein from 2.23 to 0.06 g/kg during fruit ripening.

Concerning hydroxytyrosol, the second most abundant phenolic compound identified (Table 1), it also decreased during maturation in both olive cultivars. Following a similar pattern to oleuropein, cv. Verdeal Transmontana olives presented higher hydroxytyrosol amounts than Madural olives (Table 1). This trend is similar to that presented by cvs. Arbequina, Farga and Morrut from Spain (Morelló et al., 2004) and cv. Chétoui from Tunisia (Damak et al., 2008). In fact, Morelló et al. (2004) propose that the decrease of hydroxytyrosol in olives may be probably a consequence of hydrolysis and oxidation processes which occur during olives maturation. When only the last two months are compared, from almost edibility (green-table olives) to over-mature olives, a decrease in hydroxytyrosol is usually found (Bouaziz et al., 2004; Ryan et al., 1999).

Verbascoside, a caffeoyl phenylethanoid glycoside, was present in both olive cultivars, mainly in the green phase and later in the black phase. In cv. Madural it has a significant presence in the first sampling date (968 mg/kg), appearing only latter in the black phase. In cv. Verdeal Transmontana verbascoside was only identified in the two first sampling dates (311 and 98 mg/kg, respectively), in the green phase. From the results observed in both olive cultivars, it appears that verbascoside is present at higher concentration in the beginning of maturation, during the green phase, decreasing its content in the turnover and purple phases and then increases in the final stages of maturation, when olives became black, in accordance with Morelló et al. (2004) for Arbequina, Farga and Morrut olive cultivars in Spain, orMalik and Bedford (2006) in green Arbequina olives under north-America soil.

Rutin and apigenin 7-O-glucoside were present in both olive cultivars and in all sampling dates. Their contents decreased continuously during olives maturation with similar values between cultivars (Table 1).

Luteolin was present in higher amounts in mature green olives, just before the turnover and purple phase, when it’s content start to decrease. In the case of cv. Verdeal Transmontana luteolin was not identified in higher stages of maturation. In this same olive cultivar, chlorogenic acid was identified from the second until the last sampling date, varying between 61 and 12 mg/kg, being undetectable in cv. Madural.

3.2. Antioxidant activity

By using a water extract and a high solvent/sample ratio we have achieved higher efficiencies in the antioxidant assays tested than with the methanolic extracts used for phenolic compounds quantification by HPLC. Therefore, other molecules might also contribute to the global antioxidant activity, of major interest for the definition of the maturity stage with higher potential bioactivity.

The results obtained in the antioxidant activity were dependent on the concentration tested, maturation stage and olive cultivar assessed (Fig. 2).

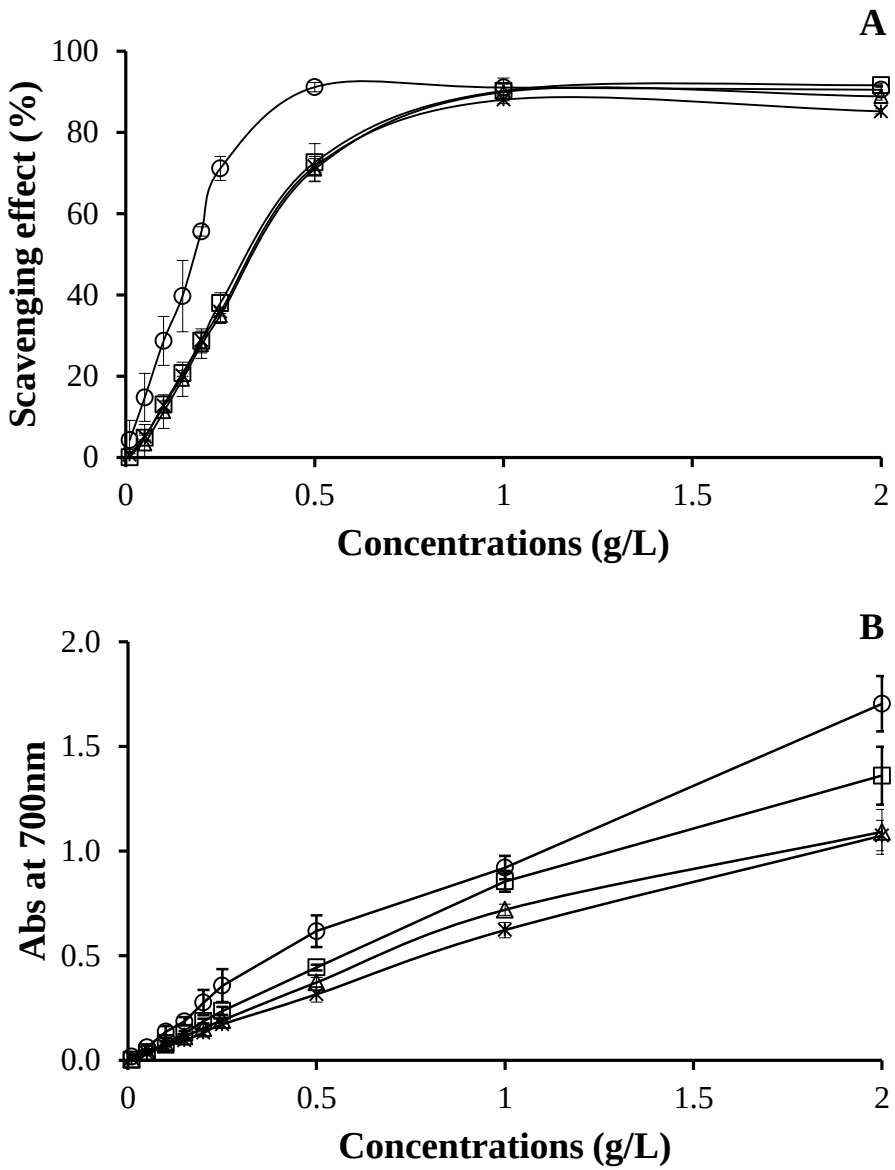

Figure 2. Antioxidant properties of aqueous extracts of olives from cvs. Madural and Verdeal Transmontana at first (29th Sept.) and last (18th Nov.) sampling dates, assessed by the scavenging effect on DPPH free radicals (Fig. 2A) and reducing power (Fig. 2B) (mean ± standard deviation) (○ 29th Sept. Madural; □ 29th Sept. Verdeal Transmontana; × 18th Nov. Madural; △ 18th Nov. Verdeal Transmontana;).

Concerning EC_{50} values of both methods and in the two olive cultivars, a similar trend was observed, with an increase in the beginning of maturation, and a slight decrease in the last harvest periods, corresponding to an increased antioxidant activity. It appears that olives lose antioxidant capacity in the beginning of maturation, which is plausible due to drastic losses in phenolic compounds with antioxidant potential, as oleuropein (Table 1), but when olives start to turn purple-black a slight increase in antioxidant potential was observed (Table 2).

Table 2. EC_{50} values (g/L) of DPPH and reducing power chemical assays of aqueous extracts of olives from cvs. Madural and Verdeal Transmontana, during the maturation process, expressed in fresh olive pulp mass (mean ± standard deviation).

Samples	DPPH	Reducing power
Madural.		
29th Sept.	0.18 ± 0.01 a	0.39 ± 0.08 a
13th Oct.	0.39 ± 0.02 b	0.90 ± 0.07 b
27th Oct.	0.44 ± 0.03 d	1.15 ± 0.05 c
10th Nov.	0.34 ± 0.01 b	0.72 ± 0.04 c
18th Nov.	0.35 ± 0.01 c	0.80 ± 0.06 d
Verdeal Transmontana		
29th Sept.	0.34 ± 0.02 a	0.57 ± 0.02 a
13th Oct.	0.19 ± 0.03 b	0.58 ± 0.05 b,c
27th Oct.	0.38 ± 0.02 d	0.84 ± 0.06 d
10th Nov.	0.50 ± 0.09 d	0.75 ± 0.03 c
18th Nov.	0.35 ± 0.02 c	0.68 ± 0.02 b

In each column, within the same olive cultivar during the maturation process, values with different letters differ significantly ($P < 0.05$).

When both cultivars are compared, cv. Madural presented always lower antioxidant capacity for the same sampling date. Such results may be related to the advanced maturation of olives from cv. Madural relatively to those from cv. Verdeal Transmontana which presents a slower maturation process. Our results are in accordance to those obtained by Bouaziz et al., 2004, Damak et al. (2008), and; Jemai et al., 2009), who observed different antioxidant capacities correlations with the sum of polyphenols during maturation for Chétoui, Chemlali and Dhokar Tunisian olive cultivars. These observations support that olive cultivar, rather than the edaphoclimatic conditions, might have a determinant effect on the phenolic pattern and antioxidant activity.

The antioxidant activity displayed was, at least partially, related with the phenolic composition of olives during maturation, but probably also with other hydrophilic compounds, some of which responsible for olives pigmentation, such as anthocyanins, belonging to the same flavonoid family as rutin. According to Romero, Brenes, García, García, and Garrido (2004) the loss of green coloration and the appearance of purple-black pigmentation during olives maturation, are a direct consequence of an increase of monomeric anthocyanins, mainly cyanidin 3-glucoside and cyanidin 3-rutinoside, being also varietal dependent (Ryan, Antolovich, Prenzler, Robards, & Lavee et al., 2002).

3.3. Correlation between phenolic composition, antioxidant activity and olives maturation process

Regression analysis was done as an attempt to establish correlations between the data obtained in the phenolic profile and antioxidant activity with the maturation process of cvs. Madural and Verdeal Transmontana (Table 3). The results obtained showed that phenolic composition and sum of polyphenols were extremely negatively correlated with the maturation process ($P \leq 0.001$ for all individual phenolic compounds and sum of polyphenols in both olive cultivars). This means that with the advance of the maturation process the contents of individual and sum of polyphenols decreased (equations and R^2 at Table 3). Such evidences, also take effect on antioxidant activity. EC_{50} values obtained in DPPH and reducing power assays were positively correlated with the maturation process in both cultivars

Such data suggests that besides being dependent on the phenolic composition of the extracts, the antioxidant activity was also dependent on other compounds associated with the maturation stage of olives.

Table 3. Correlation of phenolic composition, and antioxidant activity with the maturation process of olives from cvs. Madural and Verdeal Transmontana.

	Madural			**Verdeal Transmontana**		
Phenolic compounds	**Equation**	**R^2**	***P***	**Equation**	**R^2**	***P***
Hydroxytyrosol	$y = -12.34x + 564.7$	0.552	***	$y = -10.68x + 606.3$	0.739	***
Chlorogenic Acid	-	-	-	$y = -1.25x + 68.81$	0.762	***
Verbascoside	$y = -12.79x + 966.2$	0.984	***	-	-	-
Oleuropein	$y = -590.5x + 23469.5$	0.553	***	$y = -202.2x + 8605.5$	0.549	***
Rutin	$y = -7.53x + 435.7$	0.753	***	$y = -7.23x + 434.6$	0.793	***
Apigenin 7-*O*-glucoside	$y = -2.53x + 123.7$	0.597	***	$y = -1.48x + 116.5$	0.406	***
Luteolin	$y = -0.14x + 14.17$	0.371	***	-	-	-
Sum of polyphenols	$y = -626.5x + 25198.9$	0.555	***	$y = -227.3x + 10032.8$	0.577	***
Antioxidant activity						
EC_{50} DPPH	$y = 0.002x + 0.273$	0.253	***	$y = 0.003x + 0.275$	0.237	***
EC_{50} Reducing Power	$y = 0.005x + 0.648$	0.150	**	$y = 0.003x + 0.593$	0.318	***

n. s. – not significant; ** $P \leq 0.01$ (very significant correlation); *** $P \leq 0.001$ (extremely significant correlation).

3.4. Discrimination of maturation stage based in the phenolic composition and antioxidant activity

With the data acquired in the present work, a PCA was performed. IOlives from both cultivars collected in the first sampling date are separated from the remaining samples (Figure 3). Such evidence is related with the higher contents of hydroxytyrosol, verbascoside, oleuropein, rutin, apigenin 7-O-glucoside, and sum of polyphenols in the olives (Figure 3; Table 1). Olives from cv. Verdeal Transmontana were characterized by chlorogenic acid, mainly olives collected at 13th Oct., the second sampling date. Olives from both cultivars in the first sampling date and olives of cv. Verdeal Transmontana from the second sampling date, are represented in the positive region of first dimension (principal component – PC1), in association with high phenolic content, and apart from the remaining samples, which are all represented in the negative region of PC1. Madural olive olives from second (13th Oct.) and third (27th Oct.) sampling dates were characterized by higher EC_{50} values in both antioxidant assays. Such fact means that Madural olives possess lower antioxidant properties at 13th and 27th October, comparatively to Verdeal Transmontana olives. These samples are represented in the extreme opposite region comparatively to both samples from the first sampling date and Verdeal Transmontana olives from the second sampling date. This happens due to the lower EC_{50} values reported in the beginning of maturation, also related with a higher content of phenolic compounds with antioxidant properties.

Luteolin characterized mainly Verdeal Transmontana olives from the third and fourth (10th Nov.) sampling dates, due to higher content on this flavone (Figure 3; Table 1). Madural olive olives from fourth and fifth (18th Nov.) sampling dates as well as Verdeal Transmontana olives from third to fifth sampling dates are represented more closely, due to lower variability of the data on this olives.

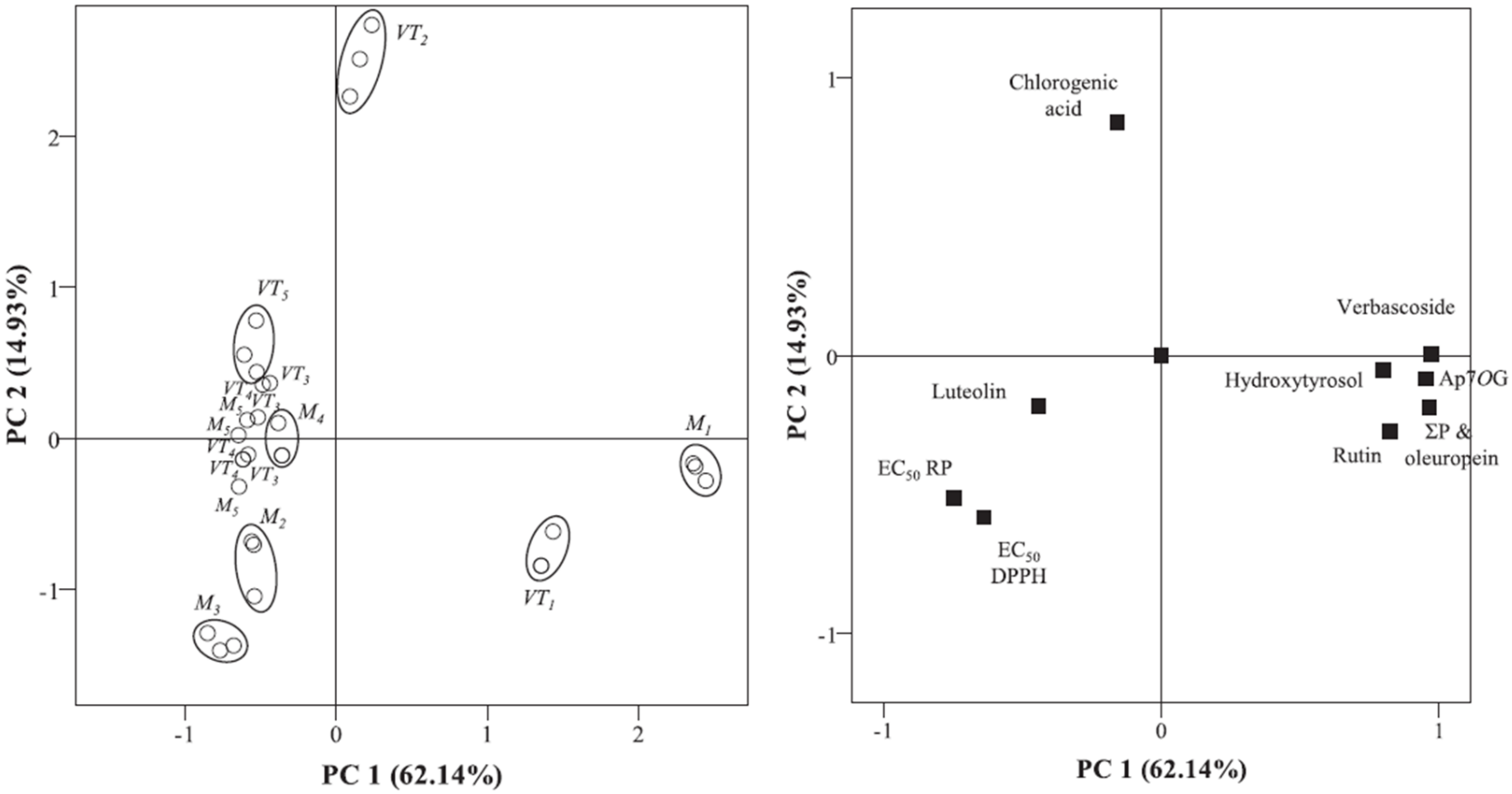

Figure 3. Principal components analysis obtained from the phenolic composition and EC_{50} values of DPPH and reducing power (RP) methods of olives from cvs. Madural and Verdeal Transmontana during the maturation process. The PCA components explain 79% of the total variance. ΣP – sum of polyphenols; Ap7OG – apigenin 7-O-glucoside; 1 – 29th Sept.; 2 – 13th Oct.; 3 – 27th Oct.; 4 – 10th Nov.; 5 – 18th Nov.

3.5. Proximate optimum harvesting period of olives

Besides the data obtained in the phenolic profile and antioxidant activity, the optimum harvesting period must be based on the type of olive product desired.

Particularly, a detailed and careful attention must be given to the adequate picking date of olives for olive oil extraction. Olives harvesting period depend on the type of olive oil, either monovarietal or a blend of cultivars. Olive oil from cv. Madural is recognized as a “sweet” and smooth olive oil, with little notes of spicy and bitter. This is related with the low phenolic amounts of olives, as described previously (Table 1), which will be in lower amounts in olive oils. On the opposite, cv. Verdeal Transmontana olive oils are characterized as very spicy, strong and connoted with cut grass and green sensations. Furthermore, olive oils from cv. Verdeal Transmontana are chemically more stable than Madural olive oils due to their phenolic composition, antioxidant properties and fatty acids profile, mainly MUFA/PUFA ratio (monounsaturated and polyunsaturated fatty acids) (Pereira, Casal, Bento, & Oliveira, 2002). The combination of cv. Madural and Verdeal Transmontana olives with a third olive cultivar, cv. Cobrançosa, is used within the P.D.O. (Protected Designation of Origin) olive oil “Azeite de Trás-os-Montes”. In this case, with a blend of several olive cultivars, each one with distinctive function in the final product, the determination of harvesting period is critical, since all three cultivars possess distinct maturation stages but should be picked simultaneously. According to Gonçalves, Malheiro, Casal, Torres, and Pereira (2012) from the beginning of November forward, olives oil content is stabilized, which means that no further oil is formed in olives. Connecting this physiological fact with the data obtained in the phenolic composition and antioxidant activity, we suggest that the proximate optimum harvest period for cvs. Verdeal Transmontana and Madural for “Azeite de Trás-os-Montes” P.D.O. olive oil, should occur in the beginning of November, despite the tradition to prolong it into December. At the proposed date, phenolic composition is balanced in cv. Verdeal Transmontana, with 1.3 g/kg and near 0.9 g/kg of oleuropein. On that same period antioxidant activity increases slightly, which will enhance the antioxidant activity of the obtained olive oil. Knowing that part of the phenolic compounds is lost during the physical and mechanical steps of olive oil extraction process, reducing therefore the antioxidant potential of the final olive oil, these higher initial contents will support these losses and grant final olive oils with increased antioxidant activity and stability. In the beginning of November, a good combination between phenolic content and antioxidant activity is observed that surely influences sensory characteristics with the increase of positive attributes mainly fruity, bitter and pungent.

4. Conclusions

Phenolic composition of olives from cvs. Madural and Verdeal Transmontana are considerably affected by maturation process. The secoiridoid oleuropein and the phenolic alcohol hydroxytyrosol were the main phenolic compounds found in the olive cultivars, decreasing significantly during olives maturation. Phenolics content affected the antioxidant activity of olive pulp together with the maturation process but other molecules might also participate in the olives global antioxidant activity. The present work allowed estimating an optimum harvesting period for two important cultivars from Northeast of Portugal. Such knowledge will contribute for better practices in olive growing and in order to pass to olive products as much as possible bioactive compounds contributing the quality and properties of olive products.

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CAPÍTULO 6.

Optimal harvest moment for the three main olive cultivars in the Protected Designation of Origin "Azeite de Trás-os-Montes"

Anabela Sousa[1,2], José Alberto Pereira[1]*, Rebeca Cruz[2], Ricardo Malheiro[1,2], Albino Bento[1], Susana Casal[2]*

[†]Mountain Research Centre (CIMO), School of Agriculture, Polytechnic Institute of Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal
[§]LAQV/REQUIMTE/Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, Porto University, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

Submitted

Abstract

Olives maturation is one of the most important factors influencing the olive oils quality, being therefore important to establish optimum proximate harvest moments. With this purpose, the three main olive cultivars in the Trás-os-Montes DOP (Cobrançosa, Madural and Verdeal Transmontana) were studied during three consecutive crop seasons for phenological stages and olive oil quality, based on its composition, antioxidant properties and oxidative stability. Olive cultivar and sampling date, as well as crop season influenced olive oil quality, corroborating the importance to establish this kind of studies in more than one crop season. It has been established that cv. Madural, with faster maturation and lower oxidative stability, should be harvested in late October, followed by cv. Cobrançosa in November, while Verdeal Transmontana, with a slower maturation rate and increased phenolic content, can be harvested latter, but before the typical December frosts, as these will inevitable compromise olive oil quality.

Keywords: Olive oil; olive cultivar; harvest date; quality; yield.

1. Introduction

Olive oil is one of the most promising vegetable oils produced worldwide, supported by its recognized health effects (Covas et al., 2006) and increased consumption (IOC, 2015), a huge attention has been recently driven to this traditional product. The Mediterranean basin is the most important olive oil producing region worldwide compressing around 96% of the obtained olive oil for the 2014/2015 crop season (IOC, 2015).

Olive oil quality is the result of several determinant factors, beginning already in the field. Indeed, the climate conditions, the geographical area, the olive cultivar, and the ripening stage influence its chemical composition and therefore quality as well. While the first two parameters are usually outside the control of an established producer, the harvest date is, year by year, the most challenging decision to be taken. In Portugal, this is still one of the most important aspects influencing olive oil quality. In the majority of the cases, the harvest periods takes several months, beginning in late October and being extended in some cases up to February in some olive producing regions. While some social aspects might contribute to these decisions, as manual labor shortage, overbooking of extraction facilities has been one of the most conditioning factors. However, a huge investment is being made to increase the number and quality of these extraction facilities, supported by national and international funds, raising the possibility to decide the harvest date based on maximized potential quality of the olive oil.

Portuguese producers are now more focused on yields and quality, to gain international competitively both by price and by high-quality. Indeed, the quality of Portuguese extra-virgin olive oils (EVOO) is increasingly internationally recognized, attaining important awards in international competitions. Trás-os-Montes (Northeast of Portugal) have been one of the most important Portuguese producing regions. Despite being unable to grant the same productive yields as the southern regions of the country, due to its climate, soil morphology, and traditional productive systems, its quality is recognized worldwide, with a delicate yet complex flavor, and a balanced taste, with green, bitter, spicy and sweet notes. As recognition of its quality and particular characteristics, a Protected Designation of Origin (PDO) was created for this olive oil, with the designation of “Azeite de Trás-os-Montes”. This PDO olive oil is the result of a blend of olives from different cultivars that are traditionally growth in this region, with at least 90% of the olives being from cvs. Cobrançosa, Madural and Verdeal Transmontana, processed and prepared in this specific region, using traditional production methods as regulated (Council Reg. 510/06, Regulation (EU) No 1151/2012).

Olive oils produced it the beginning of the crop season are usually of superior quality than those extracted at advanced maturation, the later characterized by lower shelf life and sensorial attributes (Baccouri et al., 2008; Herrera et al., 2012), but oil yield follows usually an opposite trend. Indeed, maturation is accompanied by several physical and chemical changes in the drupe that will influence oil yield and composition, namely its fatty acids ratios, amount of antioxidants, vitamins, pigments, phenolics, among others (Matos et al., 2007a; Sousa et al., 2014; 2015). The extension and path of these alterations, however, is highly characteristic of each cultivar, and, within a single cultivar, it will also depend closely on the edaphoclimatic conditions. Therefore, in order to decide the best harvest date for maximized chemical and sensorial quality without compromising yield, a detailed study on all contributing parameters through an extended period of time within possible harvest dates, preferably during different years, is necessary to verify evolution patterns.

Based on the exposed, the main objective of this work is to study the phenological and chemical changes verified during maturation in the three main cultivars of “Azeite de Trás-os-Montes” PDO olive oil, cvs. Cobrançosa, Madural and Verdeal Transmontana. These three cultivars, have particular physiological characteristics, and originate olive oils with different attributes. The most common practice is to collect and process all the cultivars together but, in order to maximize the quality and shelf life of the PDO, it is important to determine the best harvest date for each cultivar. Also, no definitive conclusion can be drawn from a single crop season as frequently published. Therefore, the maturation process of the three cultivars in the “Azeite de Trás-os-Montes” PDO was assessed during three consecutive crop seasons, focusing on oil yield and olive oil quality, in order to provide data to support decisions regarding harvest dates.

2. Materials and methods

2.1. Data collection and samples

In the present study, five representative olive trees from each olive cultivar (Cobrançosa, Madural, and Verdeal Transmontana) were selected in an olive grove at Paradela, Mirandela (Northeast of Portugal - 41º32’35.72’’N; 7º07’27.17’’W), and sampled in the years of 2009, 2010 and 2011. The orchard has 3 ha with a planting density of 7 × 7 m; trees are more than 40 years old; pruning is conducted every 3 years; it is not irrigated and the soil is tilled two to three times each year.

For each cultivar, from the beginning of April to the harvest period, the phenological growth stages were evaluated according the methodology proposed by Colbrant and Fabre (1972) with some modifications according Sanz-Cortés et al. (2002). The correspondences are C: Inflorescence buds open and flower cluster development starts; D1: flower cluster totally expanded and floral buds start to open; D2: Corolla larger than the calyx; F: flowering; G: petals falling; H: fruit set; I: fruit growth; and J: maturation. Five sampling dates were performed in 2009, extended to 9 sampling dates in 2010 and 2011. The first date considered for oil extraction and analysis corresponded to unripe fruits (green color), and the latter to completely mature fruits (black color). Therefore, in 2010 and 2011, two sampling dates were performed previously to the first one considered for oil extraction, and the last two were picked after the collection period of the producer.

From each selected tree, approximately 1 kg of olives were hand-picked all around the perimeter of the tree at the operator height. The samples were immediately transported to the laboratory and processed for oil extraction. An extra portion of 150 g was reserved for the physical measurements and for the water and fat content analysis.

2.2. Physical measurements

Ten fruits from each of the five trees, for each cultivar/year, were evaluated for total weight and for the pulp and stone weights and ratio. The pulp was further processed for moisture and fat content as described below.

2.3. Pulp Analysis

Moisture was determined at 100 ± 2 °C (~5 g test sample) following AOAC 925.40 (1995) method. Total fat was extracted according to AOAC 948.22 method, using with petroleum ether, in a Soxhlet apparatus, for 24 h (AOAC 2000). Total fat was estimated after drying at 100 ± 2100 ± 2 °C, until constant weight. Results are expressed on a fresh basis (FW) and on a dry weight (DW). The evolution of the fat amount per fruit was estimated based on the average values for pulp and fat amounts per tree.

2.4. Oil extraction

The extraction of the olive oils was conducted within the first 24 h after harvest. An Abencor analyzer (Comercial Abengoa S.A., Seville, Spain) was used to process the olives in a pilot extraction plant. The unit consists of three essential elements: mill, thermobeater, and a pulp centrifuge. The oil was separated by decanting, transferred

into dark glass bottles and stored in the dark at 4 °C. Before the analytical procedures, the samples were dehydrated with anhydrous sodium sulfate and subsequently filtered through Whatman no. 4 paper. In the 2009 crop season the oils were manually extracted in the laboratory, with reduced extraction efficiency. Therefore, the Abencor system, as described above, was used in the 2010 and 2011 crop seasons.

2.5. Quality parameters

The olive oil samples extracted were evaluated for the most common quality parameters, namely acidity (FA), peroxide value (PV) and specific extinction coefficients at 232 and 270 nm (K_{232} and K_{270}), all according to the official methods described in the EEC Regulation 2568/91.

2.6. Oxidative stability (Rancimat)

The oxidative stability was estimated by measuring the oxidation induction time, on a Rancimat 743 apparatus (MetrohmCH, Switzerland). Filtered, cleaned, dried air (20 L/h) was bubbled through the oil (3.0 g), heated at 120 ± 1.6 °C, with the volatile compounds being collected in deionized water, and the increasing water conductivity continuously measured (ISO 6886:2006).

2.7. Fatty acid composition

Fatty acids were evaluated as methyl esters, in accordance with EEC Regulation 2568/91, after alkaline transesterification with methanolic potassium hydroxide solution and extraction with *n*-heptane. The fatty acid profile was determined by GC-FID (Chrompack CP 9001, Middelburg The Netherlands) equipped with a split-splitless injector, and a 50 m × 0.25 mm i.d. CP-Sil 88 column (manufactured by Chrompack and available from Varian Inc.). Helium was used as carrier gas at an internal pressure of 120 kPa. The results are expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area. A fatty acids methyl esters standard mixture (Supelco 37 FAME Mix) was used for identification and calibration purposes (Sigma, Spain).

2.8. Tocopherol composition

Tocopherols were evaluated following the ISO 9936:2006 international standard, with some modifications. Briefly, an accurate oil amount (ca. 50 mg) was blended with an appropriate amount of internal standard (tocol, Matreya, Inc.) in *n*-hexane (1.5 mL), homogenized by stirring, centrifuged at 13,000 g and analyzed by HPLC. The liquid chromatograph consisted of a Jasco integrated system (Japan)

equipped with an AS-950 automated injector, a PU-980 pump, and an FP-920 fluorescence detector (λex = 290 nm and λem = 330 nm). The chromatographic separation was achieved on a Supelcosil TM LC-SI (3 µm) 75 × 3.0 mm (Supelco, Bellefonte, PA,USA), operating at constant room temperature (21 °C). A 98:2 mixture of *n*-hexane and 1,4-dioxane was used as eluent, at 0.7 mL/min. Data were processed by 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 for the establishment of calibration curves for each compound**.**

2.9. Radical scavenging activity (RSA)

Olive oil samples were analyzed for their antiradical activity by two chemical assays: DPPH (2,2-diphenyl-1-picrylhydrazyl) radical and ABTS (2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)) radical.

In DPPH assay the method applied was performed accordingly to that described by Kalantzakis et al. (2006). Briefly, olive oil was diluted in ethyl acetate (100 µL/mL of ethyl acetate), mixed with a DPPH solution with a concentration of 1×10^{-4} mol/L in ethyl acetate. The mixture was then homogenized and kept in the dark for 30 min for reaction. After that the absorbance was registered at 515 nm against a blank solution.

The ABTS method was applied according to that describe by Sanchez et al. (2007), based on the capacity of a sample to inhibit the ABTS radical, generated by chemical reaction with potassium persulfate ($K_2S_2O_8$). To 25 mL of ABTS solution (7 mmol/L) 440 mL of $K_2S_2O_8$ were added (140 mmol/L), being the solution kept in darkness during 12 to 16 h at room temperature in order to form the radical. An accurate volume of the previous solution was diluted in ethanol until an absorbance of 0.70 ± 0.02 at 734 nm. Once the radical was formed, 2 mL of the ABTS radical solution were mixed with 100 mL of oil and the absorbance measured at 734 nm.

The capacity of the oils to inhibit DPPH and ABTS radicals was measured applying the following formula: % scavenging effect = $[(A_{FR} – A_S)/A_{FR}] \times 100$, where AS is the absorbance of the solution when the sample is present, and A_{FR} is the absorbance of the free radical solution, DPPH or ABTS solutions in this case.

2.10. Statistical Analysis

All analyses were performed using SPSS software, version 22.0 (IBM Corporation, New York, USA), as detailed below.

The outcomes of this work are presented as mean values and standard deviation from duplicate analysis of each sample. Aiming to perform an analysis of variance, normal distribution of the residuals and the homogeneity of variances were evaluated through the Shapiro Wilk's test (sample size < 50) and the Levene's test, respectively. Furthermore, in order to assess the effect of crop's season (year and maturation time), a Pearson's correlation was established between these independent variables and each parameter analyzed.

Finally, a principal components analysis (PCA) was conducted aiming to reduce the number of variables that adequately summarize the effect of the different varieties and maturation stages on the olives nutritional composition and biometric features.

3. Results and discussion

3.1. Phenological evolution

The phenological stages were monitored in the three olive cultivars during three consecutive crop seasons, in order to verify possible differences between them from flowering thruogh their maturation process. The obtained results are reported in Figure 1. Two major observations could be retained: cv. Madural has a faster maturation process, while cv. Verdeal Transmontana reported a slower maturation process. In the 2009 crop season petals start to fall down (phonological stage G) around in the first week of June in cvs. Cobrançosa and Madural, while in cv. Verdeal Transmontana it was verified one week later (Figure 1). In the same season, fruits from cv. Madural start to ripe (phonological stage I) at the second week of October, while in cvs. Cobrançosa and Verdeal Transmontana the fruits were still green. In the 2010 crop season, all phonological stages were similar in three olive cultivars until the first week of October. At that period olives from cv. Madural start to change color, and at the second week some of the fruits were completely ripe. Olives from cvs. Cobrançosa and Verdeal Transmontana start to change color at the end of October, and until the end of the sampling dates the fruits weren’t ripe. In the 2011 crop season similar observations were recorded to those from the 2010 crop season. Fruits from cv. Madural developed earlier, change color and become ripe earlier than cvs. Cobrançosa and Verdeal Transmontana as well. However, in the 2011 crop season the fruit set was earlier but fruits development was longer than usual, since fruits also start to change color and ripe only at the third week of November in October and at the beginning of November for cvs. Cobrançosa and Verdeal Transmontana.

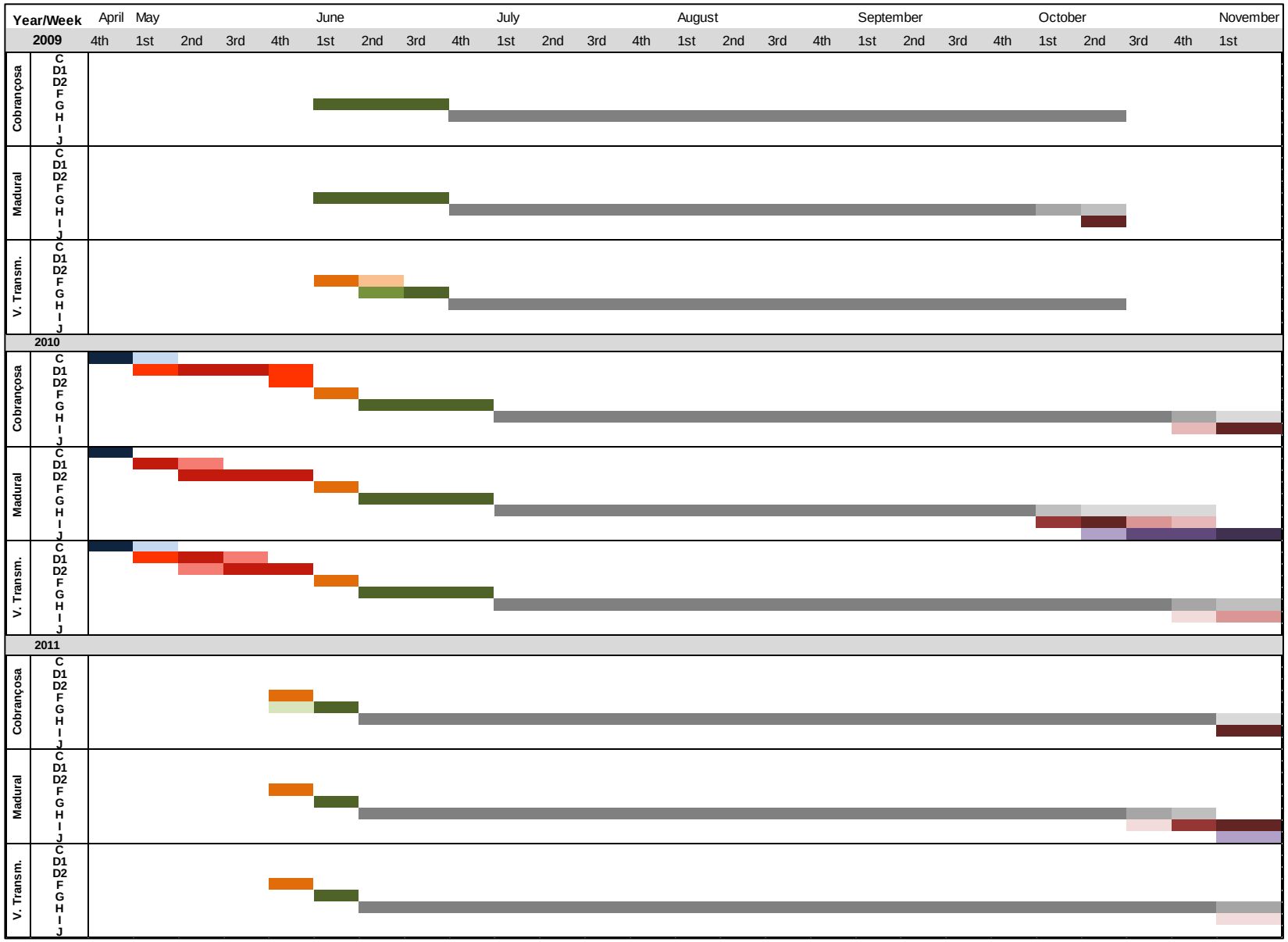

Figure 1. Phenological stages of cvs. Cobrançosa, Madutal and Verdeal Transmontana, from 2009 to 2011 crop seasons.

3.2. Biometric parameters

Fruit size is an important biometric measure, being a potential estimator for productivity. However, due to the different characteristics of each cultivar, particularly stone size within the fruit, the pulp/stone ratio gives a more reliable value of the effective pulp mass. Figure 2 details the evolution of the average pulp/stone ratio on the three cultivars, according to the crop season assessed.

It is easily perceived that the same cultivar has a distinct evolution pattern each year, and that the three cultivars follow similar patterns within a year but not between different years. Within the three crop seasons, cv. Cobrançosa fruit mass varied between 1.31 g to 4.03 g, with a pulp/stone ratio varying from 1.84 to 4.53, this later achieved in the 2010 crop season, 185 after flowering (1st December), with almost stabilized ratios from the 149th day forward (26th October). For cv. Madural, the fruits had similar sizes, from 1.37 to 3.54 g, with a pulp/stone ratio of 1.59 to 5.04 (Figure 2), achieved in the 2011 crop season, 175 days after flowering (18th November), slightly sooner than cv. Cobrançosa, but highly stable from the 153th day after flowering. Finally, cv. Verdeal Transmontana fruits varied from 1.84 to 4.38 g, with a pulp/stone ratio of 1.62 to 4.53 in the 2010 crop season, but with stabilized ratios from the 158th up to the 185th day after flowering (the 1st December in the 2010 crop season). The maximum fruit mass and pulp/stone ratios in the three crop seasons were achieved on similar days after flowering, with a maximum deviation of 10 days. From these dates forward, the fruits mass and the pulp/stone ratio decreased. This was a direct consequence of a gradual moisture loss (Table 1), almost perceived from the beginning of sampling dates, and a cumulative formation of oil in the fruit. Consequently, the oil content on a mass basis is almost constant through time, with oil formation being shaded by moisture decrease.

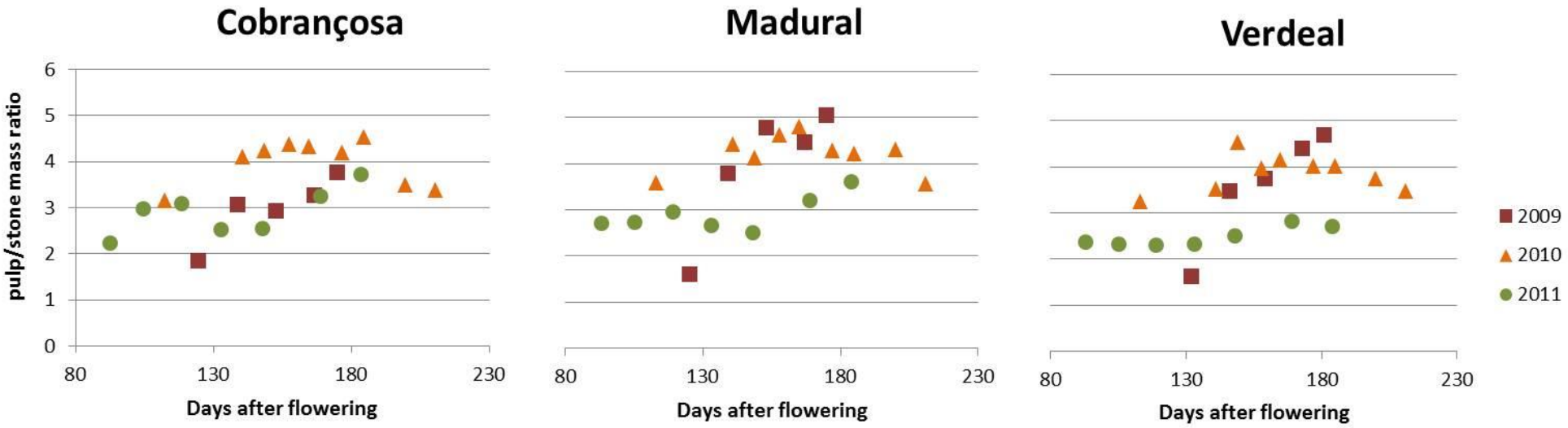

Figure 2. Pulp/stone mass ratio in cvs. Cobrançosa, Madural and Verdeal Transmontana, in 2009, 2010 and 2011 crop seasons.

Table 1. Quality parameters, composition, antioxidant activity and oxidative stability of olive oils extracted from cvs. Cobrançosa, Verdeal and Madural in 2009, 2010 and 2011 crop seasons.

Cobrançosa	2009					2010					2011					
sampling date (days after flowering)	29/9 (125)	13/10 (139)	27/10 (153)	10/11 (176)	18/11 (175)	26/10 (149)	4/11 (158)	11/11 (165)	23/11 (177)	1/12 (185)	9/11 (133)	23/11 (148)	30/11 (169)	07/12 (184)	14/12 (191)	21/12 (198)
moisture (%)	46 ± 4	67 ± 1	66 ± 2	63 ± 3	65 ± 3	71 ± 2	66 ± 1	67 ± 1	64 ± 1.5	60 ± 1	60 ± 2	60 ± 2	59 ± 3	58 ± 2	52 ± 1	49 ± 0
pulp oil (% DW)	24 ± 7	39 ± 13	37 ± 2	41 ± 8	47 ± 1	55 ± 3	57 ± 1	57 ± 3	57 ± 1	57 ± 3	57 ± 2	56 ± 4	58 ± 1	61 ± 3	63 ± 3	63 ± 2
C16:0 (%)	12.5 ± 0.2	13.1 ± 0.6	13.2 ± 0.5	12.0 ± 0.3	11.4 ± 0.4	11.8 ± 0.3	11.2 ± 0.1	10.9 ± 0.0	10.3 ± 0.0		11.5 ± 0.1	11.2 ± 0.0	10.8 ± 0.1	10.9 ± 0.0	10.6 ± 0.0	10.4 ± 0.1
C18:0 (%)	3.9 ± 0.2	3.7 ± 0.3	3.4 ± 0.1	4.5 ± 0.2	4.8 ± 0.7	3.7 ± 0.0	4.3 ± 0.0	4.5 ± 0.0	4.8 ± 0.0		4.3 ± 0.1	4.4 ± 0.0	4.4 ± 0.0	4.3 ± 0.0	4.3 ± 0.0	4.5 ± 0.0
C18:1 (%)	73.7 ± 1.2	72.8 ± 1.4	71.6 ± 0.8	72.6 ± 0.5	72.2 ± 0.3	74.3 ± 0.6	73.5 ± 0.1	73.6 ± 0.2	73.9 ± 0.1		71.1 ± 0.4	71.9 ± 0.2	72.8 ± 0.3	72.4 ± 0.1	73.4 ± 0.0	73.1 ± 0.2
C18:2 (%)	5.8 ± 0.7	6.3 ± 0.9	7.6 ± 0.5	7.0 ± 0.6	7.7 ± 0.5	6.3 ± 0.0	6.8 ± 0.1	7.1 ± 0.1	6.9 ± 0.0		9.1 ± 0.1	8.5 ± 0.1	8.3 ± 0.0	8.8 ± 0.0	8.1 ± 8.5	8.5 ± 0.1
C18:3 (%)	1.1 ± 0.0	1.1 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0		0.9 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.0
MUFA/PUFA	11 ± 1	10 ± 2	8 ± 1	9 ± 1	9 ± 0	10 ± 0	10 ± 0	9 ± 0	10 ± 0		7 ± 0	8 ± 0	8 ± 0	8 ± 0	8 ± 0	8 ± 0
α-tocopherol (mg/kg)						309 ± 5	234 ± 3	235 ± 4	247 ± 9		188 ± 16	174 ± 10	178 ± 15	192 ± 4	190 ± 11	202 ± 8
γ-tocopherol (mg/kg)						6 ± 0	6 ± 0	7 ± 0	8 ± 0		14 ± 1	12 ± 1	10 ± 1	13 ± 0	12 ± 1	15 ± 0
Rancimat (h)						15 ± 2	14 ± 2	13 ± 1	13 ± 0	14 ± 1	20 ± 1	21 ± 0	22 ± 0	21 ± 1	24 ± 0	
DPPH (% inhibition)						86 ± 4	90 ± 3	91 ± 2	84 ± 2	88 ± 1	95 ± 1	95 ± 0	94 ± 1	93 ± 1	94 ± 1	
ABTS (% inhibition)						96 ± 1	94 ± 1	96 ± 1	96 ± 1	95 ± 1	99 ± 1	99 ± 0	98 ± 1	89 ± 1	86 ± 1	
Madural	2009					2010					2011					
moisture (%)	42 ± 2	66 ± 1	67 ± 2	64 ± 1	63 ± 0	62 ± 2	58 ± 2	54 ± 2	58 ± 3	55 ± 3	65 ± 2	63 ± 1	63 ± 1	58 ± 0	52 ± 1	49 ± 0
pulp oil (% DW)	21 ± 5	35 ± 7	47 ± 2	54 ± 6	49 ± 4	53 ± 1	52 ± 1	52 ± 1	63 ± 2	60 ± 5	61 ± 5	57 ± 3	51 ± 6	53 ± 4	45 ± 7	48 ± 4
C16:0 (%)	14.0 ± 0.0	12.2 ± 0.4	11.9 ± 0.5	10.9 ± 0.4	10.8 ± 0.1	12.2 ± 0.2	11.7 ± 0.0	11.4 ± 0.0	10.9 ± 0.0		11.3 ± 0.0	10.8 ± 0.2	10.5 ± 0.1	10.5 ± 0.0	10.1 ± 0.1	10.0 ± 0.1
C18:0 (%)	3.0 ± 0.2	3.3 ± 0.2	2.8 ± 0.1	2.6 ± 0.2	2.4 ± 0.1	2.3 ± 0.0	2.3 ± 0.0	2.3 ± 0.0	2.3 ± 0.0		3.3 ± 0.0	2.9 ± 0.1	2.9 ± 0.0	2.7 ± 0.0	2.7 ± 0.0	2.8 ± 0.1
C18:1 (%)	73.6 ± 0.1	69.0 ± 0.2	67.9 ± 0.7	69.9 ± 0.7	69.1 ± 0.4	69.4 ± 0.1	69.6 ± 0.2	70.5 ± 0.0	70.3 ± 0.1		67.9 ± 0.1	67.9 ± 0.4	68.0 ± 0.0	68.6 ± 0.0	69.7 ± 0.2	69.1 ± 0.2
C18:2 (%)	6.1 ± 0.0	12.5 ± 0.5	14.4 ± 1.0	13.7 ± 1.2	14.7 ± 0.5	12.2 ± 0.1	12.0 ± 0.0	11.9 ± 0.0	12.2 ± 0.0		14.4 ± 0.0	14.6 ± 0.1	14.9 ± 0.0	14.5 ± 0.1	13.8 ± 0.0	14.5 ± 0.2
C18:3 (%)	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.0	1.2 ± 0.0	1.1 ± 0.0	1.2 ± 0.0		1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
MUFA/PUFA	10 ± 0	5 ± 0	4 ± 0	5 ± 0	4 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0		4 ± 0	4 ± 0	4 ± 0	4 ± 0	5 ± 0	4 ± 0
α-tocopherol (mg/kg)						289 ± 3	256 ± 2	227 ± 2	235 ± 6		172 ± 6	166 ± 7	188 ± 3	173 ± 5	189 ± 3	212 ± 5
γ-tocopherol (mg/kg)						3 ± 0	3 ± 0	3 ± 0	3 ± 0		10 ± 1	7 ± 0	8 ± 0	6 ± 0	9 ± 0	10 ± 0
Rancimat (h)						5 ± 0	9 ± 1	7 ± 0	7 ± 0	7 ± 0	11 ± 0	10 ± 0	10 ± 0	11 ± 1	10 ± 0	
DPPH (% inhibition)						69 ± 1	92 ± 1	80 ± 3	83 ± 3	83 ± 2	93 ± 2	80 ± 3	76 ± 0	78 ± 3	90 ± 2	
ABTS (% inhibition)						89 ± 1	91 ± 0	90 ± 0	89 ± 0	90 ± 1	99 ± 0	98 ± 1	97 ± 0	87 ± 1	86 ± 1	
Verdeal Transmontana	2009					2010					2011					
moisture (%)	40 ± 3	64 ± 0	64 ± 5	62 ± 1	62 ± 1	66 ± 3	62 ± 2	59 ± 1	58 ± 3	55 ± 3	61 ± 1	59 ± 2	59 ± 1	58 ± 2	51 ± 2	49 ± 3
pulp oil (% DW)	20 ± 9	39 ± 4	51 ± 2	56 ± 2	54 ± 3	55 ± 5	67 ± 1	67 ± 1	63 ± 2	60 ± 5	51 ± 5	60 ± 3	57 ± 5	56 ± 4	58 ± 4	59 ± 1
C16:0 (%)	12.1 ± 0.9	12.8 ± 0.9	12.7 ± 1.0	13.0 ± 0.2	12.8 ± 0.4	11.5 ± 0.2	10.8 ± 0.0	10.5 ± 0.0	10.1 ± 0.0		11.3 ± 0.4	10.4 ± 0.2	10.2 ± 0.0	10.0 ± 0.0	10.1 ± 0.1	10.2 ± 0.1
C18:0 (%)	3.1 ± 0.1	3.2 ± 0.1	2.9 ± 0.0	2.8 ± 0.1	2.9 ± 0.1	3.4 ± 0.0	2.5 ± 0.0	2.6 ± 0.0	2.7 ± 0.0		4.0 ± 0.2	3.4 ± 0.1	3.3 ± 0.0	3.3 ± 0.0	3.4 ± 0.1	3.4 ± 0.0
C18:1 (%)	77.8 ± 0.6	76.4 ± 1.1	77.3 ± 1.8	76.2 ± 0.8	76.3 ± 0.4	75.9 ± 0.1	79.8 ± 0.1	80.5 ± 0.0	80.5 ± 0.0		74.7 ± 0.2	78.1 ± 0.5	78.5 ± 0.1	79.3 ± 0.2	78.5 ± 0.3	79.3 ± 0.2
C18:2 (%)	2.8 ± 0.0	4.1 ± 0.1	3.8 ± 1.0	4.8 ± 0.4	5.1 ± 0.1	5.1 ± 0.1	2.4 ± 0.1	2.4 ± 0.0	2.5 ± 0.0		5.5 ± 0.1	3.8 ± 0.1	3.8 ± 0.3	3.2 ± 0.0	3.8 ± 0.1	3.4 ± 0.3
C18:3 (%)	1.0 ± 0.2	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.9 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0		0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
MUFA/PUFA	21 ± 1	16 ± 0	16 ± 1	15 ± 1	14 ± 0	13 ± 0	26 ± 0	26 ± 0	26 ± 0		12 ± 0	17 ± 0	17 ± 1	20 ± 0	18 ± 1	20 ± 1
α-tocopherol (mg/kg)						236 ± 0	154 ± 0	141 ± 0	140 ± 0		141 ± 14	126 ± 6	131 ± 6	121 ± 5	119 ± 1	116 ± 6
γ-tocopherol (mg/kg)						6 ± 6	5 ± 5	5 ± 5	5 ± 5		6 ± 0	3 ± 0	3 ± 0	3 ± 1	3 ± 0	2 ± 1
Rancimat (h)						15 ± 2	14 ± 2	13 ± 1	13 ± 0	14 ± 1	26 ± 3	30 ± 4	26 ± 2	40 ± 1	35 ± 1	
DPPH (% inhibition)						72 ± 4	62 ± 2	73 ± 3	70 ± 0	60 ± 3	91 ± 1	80 ± 6	67 ± 6	90 ± 1	93 ± 0	
ABTS (% inhibition)						91 ± 1	91 ± 2	88 ± 2	91 ± 2	91 ± 1	97 ± 1	95 ± 2	95 ± 0	89 ± 1	86 ± 1	

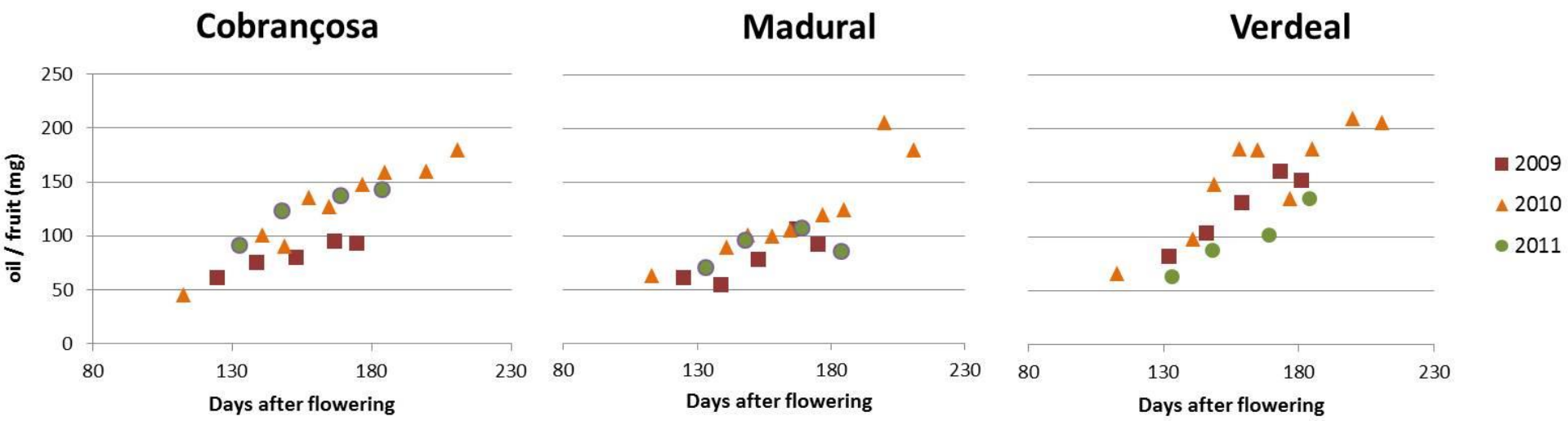

Figure 3. Oil mass per fruit in cvs. Cobrançosa, Madural and Verdeal Transmontana, in 2009, 2010 and 2011 crop seasons.

The oil content, expressed on a dry basis, is not constant through years (Table 1). On the 2009 crop season it was higher in cv. Verdeal Transmontana and lower in cv. Madural, while in 2011 crop season cv. Cobrançosa exhibited higher oil amounts on a mass basis. Knowing that the fruit mass is different between cultivars, in order to verify true yield, the effective oil content evolution per fruit, by combining the oil amount per fruit and the fruit mass, is detailed in Figure 3. Here, an increase on the oil per fruit is noticeable through time, particularly in cvs. Cobrançosa and Madural, with similar values through different years. Verdeal Transmontana exhibited higher variability but achieved the highest oil amounts per fruit. Also, for cvs. Cobrançosa and Verdeal, and except in the 2010 crop season, the oil increase rate per fruit did not increase approximately from the 150 days after flowering onward, corresponding to the last week of October or first of November, within commonly practiced harvest dates. Only cv. Madural exhibited an increase in the oil content in late harvest (2010 crop season), but these dates are usually outside the common harvest dates, with overmature olives, and the oil is usually associated with increased acidity and lower sensorial quality and shelf life (Baccouri et al., 2008; Herrera et al., 2012). The results obtained for the three cultivars regarding moisture and oil content are in agreement with those reported by Gonçalves et al. (2012) that studied this parameters in the same olive cultivars in the 2007 crop season.

3.3. Olive oil quality, composition and properties

The extracted oil was analysed for the most common quality parameters (FA, PV, K_{232}, and K_{270}). FA was low on all samplings, globally ranging from 0.2 to 0.5%, without perceived different between cultivars or years, a probable direct consequence of the healthy olives and fast extraction applied, as generally recommended. In addition, the peroxide value (PV) was within regulated limits, always below 20 meq.O_2/kg. However, interesting variations were observed between cultivars and years. The 2010 crop season was characterized by low PV on all sampling, from 3 to 7, while the values almost doubled in the 2011 crop season, varying from 6 to 16. Also, cv. Cobrançosa had always the lowest values (3 to 12), followed by cv. Verdeal Transmontana (5 to 12), while cv. Madural had the highest ones (6 to 16). The PV also varied with time, but without a constant pattern. Cobrançosa and Madural olive oils had their PV decreased with sampling dates, increasing in the later sampling dates, while cv. Cobrançosa exhibited an opposite pattern, increasing in intermediate samplings and reducing in the last ones (200-210 days). The absorptivities were also within the limits regulated for extra virgin olive oil (EVOO) category and no pattern was observed (data not shown). The results obtained in our study regarding quality parameters are in

accordance to those obtained by Matos et al. (2007b), however our samples could be classified as EVOO's, while some samples from Matos et al. (2007b) exceed some legal maximum values to be classified as EVOO's.

The chemical composition (fatty acids profile and tocopherols content), as well as antioxidant activity (DPPH and ABTS) and oxidative stability are detailed in Table 1. The fatty acids were generally within the EEC Regulation 2568/91 limits and were highly constant between years. Clear differences were observed between cultivars, as already expected based on previous works (Pereira et al. 2002; Matos et al., 2007a; Gonçalves et al., 2012). In particular, the highest oleic acid amounts were observed in cv. Verdeal Transmontana (76-80%), followed by cvs. Cobrançosa (71-74%), and Madural (68-71%). Linoleic acid was always higher in cv. Madural (12-15%), followed by cv. Cobrançosa (6-9%), with cv. Verdeal Transmontana presenting the lowest amounts (3-6%). Linolenic acid varied from 0.7 to 1.0% in cv. Verdeal Transmontana, from 0.9% to 1.1% in cv. Cobrançosa and from 1.0 to 1.2% in cv. Madural, slightly outside the limits of the EEC Regulation 2568/91, but apparently a typical characteristic of this cultivar. Globally, despite the constant saturated/unsaturated ratio through all samplings, cultivars and years (0.2; not shown), the MUFA/PUFA ratio varied slightly with cultivars but only minor alterations were perceived with time (Table 1). These highest ratios were observed in cv. Verdeal Transmontana, as a consequence of the highest oleic acid amounts and lower linoleic and linolenic ones, highly different from those presented by both cvs. Cobrançosa and Madural, this latter with the lowest values.

Regarding vitamin E content in EVOO, no reference limits are described, but its presence is associated with quality and shelf-life due to its inherent antioxidant activity. Vitamin E was characterized mostly by the presence of α-tocopherol, followed by γ-tocopherol (Table 1). Both β-tocopherol and α-tocotrienol were only present in minor amounts (not shown). Only the 2010 and 2009 crop seasons were analysed for this parameters due to the reduced amounts of oils extracted in the 2009 crop season, as explained. Globally, the 2011 crop season had lower amounts of vitamin E than the one from 2010, for all the cultivars. This is in accordance with the PV values, higher in 2011. On a comparative basis, cv. Cobrançosa had the highest amounts, closely followed by cv. Madural, while cv. Verdeal Transmontana had lower amounts (Table 1), as an inverse association to the MUFA/PUFA ratio previously discussed. Being cv. Verdeal Transmontana the cultivar with the lowest unsaturation ratio, it is somewhat expected that it could have naturally less vitamin E content, while the other cultivars, more prone to oxidation, need more antioxidant protection. In cv. Verdeal Transmontana, the amounts from two consecutive crops were even similar, indicating

that the vitamin E presence should be produced in proportion to the fat composition, with highest polyunsaturation degree (Madural > Cobrançosa > Verdeal) correlated with the vitamin E content on the fat. With maturation, and except for the first 2010 sampling, only small variations were observed for cvs. Cobrançosa and Madural, decreasing initially to recover on later samplings. Verdeal Transmontana, however, had its contents decreased with time. No variations were perceived for γ-tocopherol with time but lower amounts were also detected in cv. Verdeal Transmontana.
To understand the coordinated effect of the oil content and pulp mass on the vitamin E amounts, we have further evaluated the amount of vitamin E per fruit (data not sown). In opposition to the trends observed per oil, these were highly constant with time, indicative that vitamin E synthesis is probably adjusted to the fruit needs on a mass basis, and therefore its antioxidant activity might be important not only for the oil but for the pulp as well.

In order to have an indicator of oxidative stability, we have determined the oxidation time by the Rancimat test. Although not being a true indicator for shelf life, nor high temperature processing resistance, it is generally used for stability and, on a comparative basis, could give interesting information. Indeed, the oxidative stability predicted by the test was significantly different between cultivars, and years, but less with maturation. The lowest stability was observed in cv. Madural, with 5 to 9 hours in the 2010 crop season, stabilized at 7 hours around the 150th day, and varying from 10 to 11 hours in the entire 2011 crop season. Cobrançosa varied from 13 to 15 hours in 2010, and from 20 to 24 hours in 2011, with an apparent slight tendency to increase with time. Verdeal Transmontana had the highest oxidative stability, ranging from 13 to 15 hours in 2010, without important variations, and from 26 to 40 hours in 2011, with a tendency to increase on the latest samplings dates, already in December.

Therefore, the observed stability seems to be a direct consequence of the fat acid profile characteristic of each cultivar, particularly the already discussed MUFA/PUFA ratio. The higher stability observed in the 2011 crop season for all cultivars is not associated with vitamin E amounts, smaller in the 2011 crop, nor with the PV observed, higher in 2011. Indeed, the olive oils extracted in 2011 had all apparently higher oxidation degrees (higher PV, lower vitamin E), but the oxidative stability under the Rancimat test was generally higher. Other factors, therefore, could be implicated in the oxidative stability observed.

We have further evaluated the antioxidant capacity of the extracted oils by two different tests (Table 1). The ability to scavenge free radicals by donation of hydroxyl groups, one of the known mechanisms by which antioxidants inhibit lipid oxidation, was

evaluated by the DPPH assay. The results are present directly as percentage of inhibition efficiency. Globally, higher oxidative efficiency was observed in the 2011 crop season against the DPPH radical, but the evolution with time was variable. Cobrançosa had a lower variability with time, with higher efficiencies than the ones observed for cvs. Madural and Verdeal, by this order, and with higher inhibition efficiency observed between the 158 and 165th days in the 2010 crop season, corresponding to the beginning of November. The values in the 2011 crop season were highly constant with time. For cv. Madural, the DPPH inhibition efficiency increased in the beginning of November (2010), reducing and stabilizing thereafter. In the 2011 crop season similar efficiencies were obtained, with higher values in the beginning of November, decreasing thereafter with a high increase observed in the last sampling date, in mid-December. Finally, cv. Verdeal Transmontana showed always the lowest efficiency, with higher values in mid-November in the 2010 crop season (165-177th days after flowering). In the 2011 crop season, higher efficiencies were verified later (184-191th day after flowering) probably because flowering also began latter this year, indicating that the weather could also have a determinant part in the antioxidant composition of the fruits. The lowest inhibition capacity in cv. Verdeal Transmontana could be associated with the lower vitamin E content in this cultivar.

The antioxidant efficiencies observed by the ABTS assay were higher than those observed in the DPPH, consistently with the observation of Floeger et al (2011) for a variety of fruits and vegetables. Also, steady values were observed during the entire 2010 samplings, up to the beginning of December, with 185 days after flowering, similarly to the 2011 crop season. From this point forward (2011 crop season) the efficiency is reduced, consistently on all cultivars. Also, higher values were obtained for all the cultivars in the 2011 crop season, as previously observed for the DPPH results and for the oxidative stability.

The antioxidant capacity tested under these assays is usually associated with the phenolic compounds, including lipophilic ones, as the tocopherols, but mostly hydrophilic phenolics, the main antioxidants in olive pulp (Owen et al., 2000). We have previously studied the evolution of these compounds during maturation in the fruit pulp (Sousa et al, 2014; 2015). Despite the variations in the individual phenolic compounds quantified, a huge decrease was observed in total hydrophilic phenolics in the green to purple fruit transition, corresponding to the beginning of October, with small reductions thereafter. Therefore, those results are not directly correlated with the ones observed here, in the extracted oil from the same olive fruits. Also, higher amounts were found in cv. Verdeal Transmontana fruits, followed by cv. Cobrançosa, stabilizing around 1 g/kg

fresh pulp from the mid October forward, and latter cv. Madural, with the lower amounts, but also stable within these dates. This shows that the antioxidant capacity of the extracted oil is not proportional to the phenolic content in the fruits, and that one cannot predict the antioxidant capacity of the oils on this basis. However, the higher antioxidant content in the pulp could protect the oil longer before extraction, particularly in cv. Verdeal Transmontana cultivar.

3.4. Global variability

From the results discussed previously, a high variability was observed between years and cultivars, while variations with maturation were less perceived for the majority of the components. However, this was our major objective: to define the best date or time span for harvest for each cultivar individually. In order to cross yield with quality and find a possible variability pattern, we have performed a PCA analysis with the global physical and chemical data, independent of the year (Figure 4). The two first components are able to explain almost 75% of the total variability, and samples were clearly grouped by cultivar, supporting that the differences between cultivars are indeed higher than those observed within each cultivar in different years and maturity stages.
Madural is positioned in the left side of Component 1, mostly due to the higher content of linolenic and linoleic acids together with α-tocopherol (Figure 4). Indeed, this antioxidant is the main responsible for the polyunsaturated fatty acid protection and therefore this association is perfectly understandable, as previously discussed. To the right, cv. Verdeal Transmontana presented higher amounts of oleic acid, fat amount and oxidative stability. The clear separation of cv. Cobrançosa from cv. Verdeal Transmontana is mostly due to the higher antioxidant activity (ABTS and DPPH) in cv. Cobrançosa. These observations are consistent with the previous discussion and with published data, where cv. Madural is characterized by higher polyunsaturated acids, and therefore, lower oxidative resistance (Matos et al., 2007a; 2007b)

Therefore, due to the reduced variability observed and discussed previously, each cultivar was studied individually for correlations with each parameter analysed through time, with all years taken together. Table 2 resumes the Pearson correlations verified with time after flowering for the three cultivars, independently of the year.

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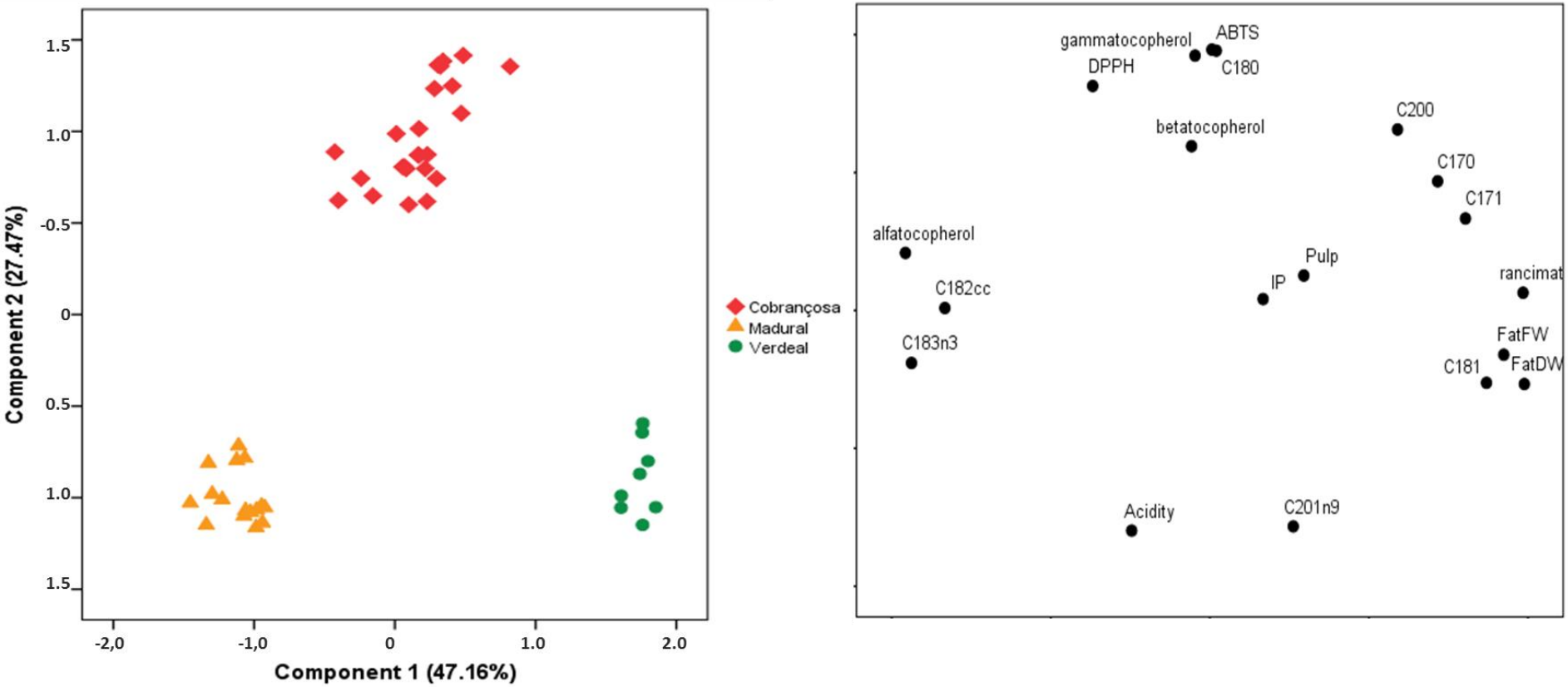

Figure 4. Principal component analysis from the data obtained in the three olive cultivars during the three years.

Table 2. Pearson correlations between several analytical parameters of the extracted olive oils and days after flowering.

	Cobrançosa	Madural	Verdeal T.
Moisture	-.321**	-.122	-.152
Pulp/stone ratio	.386**	.243	.154
Fat (DW)	.644**	.472**	.433**
$C_{16:0}$	-.759**	-.924**	-.570**
$C_{17:0}$	-.551**	.387**	.224
$C_{18:0}$	.505**	-.084	.219
$C_{20:0}$	-.168	-.480**	.354**
$C_{16:1}$	-.222	-.436**	-.285*
$C_{17:1}$	-.654**	.204	.271*
$C_{18:1}$	-.124	-.406**	.309*
$C_{18:2}$	.737**	.666**	.005
$C_{18:3}$	-.651**	-.398**	-.295*
α-tocopherol	-.695**	-.467**	-.801**
β-tocopherol	.806**	.890**	.716**
γ-tocopherol	.705**	.703**	.077
Free acidity	.587**	-.651**	-.567**
Peroxide Index	.531**	.502**	.482**
K_{232}	.588**	.627**	-.179
K_{270}	-.407	.495*	-.492*
ΔK	-.364	-.076	.154
Oxidative stability	.669**	.541**	.684**
Antioxidant activity (DPPH)	.422**	.136	.441**
Antioxidant activity (ABTS)	-.533**	-.141	-.258

**. Correlation is significant at the 0.01 level (2-tailed).

Most of the parameters presented similar evolutions through time, as a clear reduction in palmitic and linolenic acid, together with α-tocopherol. On the opposite trend, all cultivars present an increase in oxidative stability, β-tocopherol, and peroxide value. The remaining parameters, however, present different correlations with time. Particular attention could be given to K_{232}, whose value decreases in cv. Verdeal Transmontana, indicating that its harvest could be indeed prolonged in comparison with the remaining cultivars. Also, the antioxidant activity evaluated by the DPPH test increases in cvs. Cobrançosa and Verdeal Transmontana but only slightly in cv.

Madural, indicating potential losses for this activity, as well as an increase in the K_{270}, also indicative of oxidation, and a potential concern for EVOO classification.

Globally, cv. Madural presented the lower oxidative stability and it decreased with time. This is consistent with an increase in oxidative parameters, as the K_{232} and particularly the K_{270}. The benefits on the antioxidant activity from delaying harvest time were the lowest among the three cultivars. This cultivar is also the one with the lowest yield on oil per fruit, and the increase with time is reduced. Therefore, this cultivar could benefit from early harvest, reducing the degradation of the oil.

The highest correlation between time and fat content was observed in cv. Cobrançosa, indicating that this cultivar yield is strongly dependent on the harvest date. Therefore, knowing that all the parameters presented a high stability up to around the 170th days after flowering, corresponding to the late November, this cultivar could benefit from being collected only after cv. Madural.

Finally, cv. Verdeal Transmontana showed the strongest oxidative stability, increasing with time, while both K_{232} and K_{270} presented a negative trend, indicative of its strong stability despite the low vitamin E content and low performance under the antioxidant activity assays. The higher phenolic content of the drupes (Sousa et al 2014) could contribute to this increased resistance, but its fatty acid composition is certainly the main determinant. Being cv. Verdeal Transmontana one of the cultivars with the highest potential oil yield, and the fat amount per fruit stabilizing around the 180th day after flowering (beginning of December), this cultivar can be left for harvest latter. This is also in accordance with its slower maturation rate (see Figure 1).

4. Conclusions

The agronomic data obtained in this study suggests different dates for each of the three main cultivars in the “Azeite de Trás-os-Montes” PDO. Madural, being more prone to oxidation and having a lower yield than cv. Cobrançosa or cv. Verdeal, could benefit from earlier harvest, around the beginning of November, with adjustments based on flowering dates. Cobrançosa oil is more stable, and presented the highest antioxidant activity. As the quality benefits from the 150th day forward are reduced, this cultivar should be collected before the end of November. Finally, cv. Verdeal has a slower maturation process and its oil is the more stable to oxidation. Its composition stabilizes soon but the oil content per fruit increases steadily, with a latter crop potentially increasing yield without quality loss. Therefore, this cultivar could be

harvested after Cobrançosa, in late November or even in the beginning of December, avoiding the typical December frosts, as these will inevitably deteriorate the olive oil.

This information is of major importance for the farmers and highlights the importance or treating each cultivar separately for maximized quality and yield. However, climate changes and potential pest attacks cannot be disregarded, and adjustments should be made when the conditions observed under this three-year study change.

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CAPÌTULO 7.

Aromatized olive oils: influence of flavouring in quality, composition, stability, antioxidants, and antiradical potential

Anabela Sousa[†,§], Susana Casal[§*],Ricardo Malheiro[†,§], Hugo Lamas[†], Albino Bento[†], José Alberto Pereira[†*]

[†]Mountain Research Centre (CIMO), School of Agriculture, Polytechnic Institute of Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal
[§]LAQV@REQUIMTE/Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, Porto University, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

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Abstract

In the present work different flavourings (garlic, hot chili peppers, laurel, oregano and pepper) commonly used in Mediterranean cuisine were added to olive oils from Cv. Cobrançosa. Flavouring influence in olive oils quality, fatty acids profile, tocopherols and tocotrienols composition, antiradical activity, total phenols content and oxidative stability were evaluated.

Garlic addition induced an increase in free acidity values (from 0.6 to 0.8%), but the remaining quality indices weren´t negatively affected. Fatty acids profile changed but values remained under the limits of extra-virgin olive oils. Olive oils were nutritionally enriched due to the increase in vitamin E, mainly in oils flavoured with hot chili pepper (198.6 mg/kg). Antioxidant properties were influenced as well. Total phenols content decreased in all flavoured olive oils (control with 345.7 mg CAE/kg; oregano 293.8 mg CAE/kg) but the capability to counteract oxidation was generally improved (control with 9.4 h and oregano with 10.4 h). The addition of flavouring influenced quality, composition and olive oils characteristics being possible to separate them according to the flavouring used by applying chemometrics.

Keywords: *Olea europaea* L.; fatty acids; tocopherols; total phenols oxidative stability.

1. Introduction

According to recent statistics published by the International Olive Council (IOC) the olive oil consumption is increasing in recent years, being predicted to achieve a worldwide consumption level above 3 million tons in 2014 (IOC, 2013). Undoubtedly olive oil sensorial characteristics and health claims are associated with this increase. Besides being a key ingredient of the Mediterranean diet and cuisine, olive oil is related with many health benefits, including the prevention of many modern life-style diseases, like some kinds of cancer (Assmann et al., 1997; Owen, Haubner, Würtele, Hull, Spiegelhalder, & Bartsch, 2004) and cardiovascular diseases (Covas, 2007; Fitó et al., 2005).

Consumers are now more informed than ever regarding food products, increasingly demanding for top quality, healthy, and innovative products. In the olive sector, quality products with healthy characteristics have been a constant over the years. Concerning innovation, the recent introduction of flavoured or gourmet olive oils in the market have been the route followed by some industrials. Several kinds of flavourings are used to aromatize olive oils: essential oils (mint and thyme); fruits (apple, banana, bitter-orange and orange, lemon, mandarin); herbs (basil, estragon, fennel, juniper, laurel, lavender, mint, oregano, rosemary, sage, thyme); mushrooms (porcini mushrooms and other truffes); nuts (almonds, hazelnuts, pine nuts); spices (clove, ginger, nutmeg); and vegetables (dried tomatoes, garlic, hot chili peppers, onions, pepper). These flavourings could be added to the olive oil after its extraction, with a defined period of maceration to aromatize the oil, or can be mixed directly with the olive fruits and extracted simultaneously.

The addition of aromatizers to the olive oil influences several characteristics and properties. Their inclusion improves olive oils sensorial characteristics, but the concentration must be kept at low or moderate levels in terms of sensorial acceptability by consumers in order to avoid over-aromatization (Kandylis et al., 2011; Matsakidou, Blekas & Paraskevopoulou, 2010), particularly for some intense spices (Akçar & Gümüşkesen, 2011; Antoun & Tsimidou, 1997; Moldão-Martins, Beirão-da-Costa, Neves, Cavaleiro, Salgueiro, & Beirão-da-Costa, 2004). Their quality and shelf-life could be affected as well, since the incorporation of antioxidant and/or pro-oxidant compounds influence olive oils stability. By studying quality indices during storage of flavoured olive oils, Baiano, Terracone, Gambacorta and La Notte (2009) observed that those with garlic retained their indices below the maximum allowed for extra-virgin olive oils. Gambacorta, Faccia, Pati, Lamacchia, Baiano, and La Notte (2007) reported that the addition of different concentrations of garlic, hot pepper, oregano, and rosemary at long term improved the stability of the olive oils. Some works studied the changes in the oxidative status of

flavoured olive oils to verify the efficiency of flavourings bioactive properties and their contribution to olive oils stability. Aromatic plants like rosemary and thyme were capable to protect the oil from thermal oxidation (Ayadi, Grati-Kamoun, & Attia, 2009). Meanwhile lemon and thyme at high concentrations (80 g/kg of oil) weren´t efficient to protect the olive oils from thermo-oxidative processes at the smoking point as observed by Issaoui, Flamini, Hajaij, Cioni, and Hammami (2011). The addition of different flavourings is also known to induce the presence and survival of some microorganisms (moulds, yeast and bacteria) according to the concentration and aromatizer used (Ciafardini, Zullo, & Peca, 2004).

With the present work we intend to contribute for the existent knowledge on flavoured olive oils by studying common flavourings in the Mediterranean cuisine (garlic, hot chili pepper, laurel, oregano and pepper). In this sense we studied the effect of those herbs and spices in the quality parameters (free acidity, peroxide value, K232, K270 and ΔK), fatty acids profile, and tocopherols and tocotrienols content. Total phenols content, antiradical scavenging activity, and oxidative stability were also evaluated to observe the possible role of the flavourings in the bioactive potential and capability to counteract the oxidative reactions in the olive oils.

2. Materials and methods

2.1. Samples

Monovarietal Cobrançosa extra virgin olive oil from the crop season of 2010/11 was used (composition and properties before spices addition reported in Table 1). The herbs and spices selected were based in the flavourings most commonly used in the Mediterranean cuisine: *Allium sativum* (garlic), *Capsicum frutescens* L. (hot chili pepper), *Laurus nobilis* L (laurel), *Origanum vulgare* L. (oregano), and *Piper nigrum* L. (pepper). All the flavourings were obtained from local markets and were incorporated dried as is in the olive oils (with exception of garlic which was added fresh). After herbs and spices incorporation (10 g/L of olive oil) the olive oils were stored during three months at room temperature (protected from light exposure in static positions) in order to allow a better maceration and extraction of the flavourings into the olive oil. One group was used as control, with no added flavourings. After this storage period the olive oils were dehydrated with anhydrous sodium sulphate, filtered through Whatman no. 4 paper and used for the analytical determinations.

Table 1. Quality parameters, sensorial analysis, composition, bioactivity and stability of cv. Cobrançosa olive oil before the addition of different spices.

FA (%)	0.6±0.0	**$C_{16:0}$**	10.49 ± 0.23
PV (meq. O_2/kg)	2.8 ± 0.3	**$C_{16:1}$**	0.66 ± 0.03
K_{232}	2.10 ± 0.08	**$C_{17:0}$**	0.14 ± 0.02
K_{270}	0.13 ± 0.00	**$C_{17:1}$**	0.21 ± 0.02
ΔK	-0.004 ± 0.001	**$C_{18:0}$**	2.75 ± 0.06
α-Tocopherol (mg/kg)	184 ± 0.4	**$C_{18:1}$**	74.45 ± 0.25
α-Tocotrienol (mg/kg)	n. d.	**$C_{18:2}$**	9.58 ± 0.11
β-Tocopherol (mg/kg)	0.9 ± 0.1	**$C_{20:0}$**	0.41 ± 0.03
γ-Tocopherol (mg/kg)	4.0 ± 0.1	**$C_{20:1}$+$C_{18:3}$**	1.04 ± 0.05
Total vitamin E (mg/kg)	189 ± 0.5	**$C_{22:0}$**	0.13 ± 0.01
DPPH (μmol/L TE)	144 ± 8	**SFA**	13.87 ± 0.30
ABTS (μmol/L TE)	300 ± 4	**MUFA**	75.37 ± 0.20
Total phenols (mg CAE equiv./kg)	352 ± 18	**PUFA**	10.61 ± 0.06
Oxidative stability (h)	10.6 ± 0.1	**Sensory analysis**	EVOO

n. d. – not detected; EVOO – extra virgin olive oil according to European Community Regulation EEC/2568/91 and all subsequent amendments.

2.2. Quality parameters determination

The quality parameters assessed were free acidity (FA), peroxide value (PV) and specific coefficients of extinction at 232 and 270 nm (K_{232}, K_{270}, and ΔK). All the mentioned quality parameters were determined according to European Union standard methods (Annexes II and IX in European Community Regulation EEC/2568/91 from 11th July).

2.3. Fatty acids composition

Fatty acids were evaluated as their methyl esters after cold alkaline transesterification with methanolic potassium hydroxide solution (Annexes II and IX in European Community Regulation EEC/2568/91 from 11th July) and extraction with n-heptane. The fatty acid profile was determined accordingly to the method described by Malheiro, Casal, Lamas, Bento and Pereira (2012).

2.4. Tocopherols and tocotrienols composition

Tocopherols and tocotrienols composition was determined according to the ISO 9936 (2006), with some modifications as described by Malheiro, Casal, Teixeira, Bento, and Pereira (2013). Tocopherols and tocotrienols standards (α, β, γ and δ) were purchase from Calbiochem (La Jolla, San Diego, CA) and Sigma (Spain), while the internal standard 2-methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol (tocol) was from Matreya Inc. (Pleasant Gap, PA). Filtered olive oil (50 mg) was mixed with internal standard solution (tocol) and

homogenized. The mixture was centrifuged for 5 minutes at 13000 rpm and the supernatant obtained analyzed by HPLC.

The chromatographic conditions are those reported by Malheiro et al. (2012) and Malheiro et al. (2013). The compounds were identified by chromatographic comparisons with authentic standards, by co-elution and by their UV spectra. Quantification was based on the internal standard method, using the fluorescence signal response.

2.5. Radical scavenging activity (RSA)

Olive oil samples with different flavouring were analysed for their antiradical activity by two chemical assays: DPPH (2,2-diphenyl-1-picrylhydrazyl) radical and ABTS (2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)) radical.

In DPPH assay the method applied was performed accordingly to that described by Kalantzakis, Blekas, Pegklidou, and Boskou (2006) and Malheiro, Casal, Lamas, Bento and Pereira (2012). Briefly, olive oil was diluted in ethyl acetate (100 µL/mL of ethyl acetate) was mixed with a DPPH solution with a concentration of 1×10^{-4} mol/L in ethyl acetate. The mixture was then homogenised and kept in the dark for 30 minutes for reaction. After that the absorbance was registered at λ = 515 nm against a blank solution.

The ABTS method was applied according to that describe by Sánchez, González, García-Parrilla, Granados, Serrana, and Martínez (2007), based on the capacity of a sample to inhibit the $ABTS^{\cdot+}$ radical. The $ABTS^{\cdot+}$ radical was generated by chemical reaction with potassium persulfate ($K_2S_2O_8$). To 25 mL of ABTS (7 mmol/L) were added 440 µL of $K_2S_2O_8$ (140 mmol/L), being the solution kept in darkness during 12-16 h at room temperature in order to form the radical. An accurate volume of the previous solution was diluted in ethanol until an absorbance of 0.70 ± 0.02 at λ = 734 nm. Once the radical was formed 2 mL of the $ABTS^{\cdot+}$ radical solution were mixed with 100 µL of oil and the absorbance measured at λ = 734 nm.

For both methods a trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) calibration curve was prepared for a concentration range of 0 - 350 µmol/L, and the inhibition percentage obtained for the samples was interpolated to calculate the concentration in trolox equivalents (µmol/L TE).

2.6. Total phenols content

Total phenols content was assessed by the methodology described by Capannesi, Palchetti, Mascini, and Parenti (2010) with some modifications. For total phenols content 2.5 g of olive oil were diluted in a reason 1:1 with n-hexane, and extracted with 2.5 mL methanol/water (80:20; v/v) three times, being the mixture centrifuged during 5 minutes at 2600 g. From the combined extract 1 mL was added with the same amount of Folin-

Ciocalteau reagent and Na_2CO_3 (7.5%), to which 7 mL of purified water were added. After homogenization, the mixture was stored overnight and spectrophotometric analysis was performed at λ = 765 nm

For quantification purposes a calibration curve of caffeic acid in methanol was performed in concentration range 0.04-0.18 mg/mL. The calibration curve was treated on the same way for oil analysis. The final results were expressed as mg of caffeic acid equivalents per kg of olive oil (mg CAE/kg).

2.7. Oxidative stability

The oxidative stability was measured in a Rancimat 743 apparatus (Metrohm CH, Switzerland): To 3 g of olive oil heated at 120 ± 1.6 ºC was incorporated air (filtered, cleaned, and dried) at a reason of 20 L/h. The resulting volatile compounds were collected in water, and the increasing water conductivity was continuously measured. The time taken to reach the conductivity inflection was recorded.

2.8. Statistical analysis

The results reported in this study are the averages of at least six replicates per olive oil category (n = 6).

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) and a principal component analysis were performed using the SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.). ANOVA statistical tests were performed at a 5% significance level.

A regression analysis, using Excel from Microsoft Corporation, was established between the total vitamin E and TPC of the flavoured olive oils with the data obtained in the RSA and oxidative stability of the same samples.

3. Results and discussion

3.1. Quality parameters

In order to assess the effects of different spices in the quality of olive oil, free acidity (FA), peroxide value (PV), specific coefficients of extinction at 232 and 270 nm (K_{232} and K_{270}), and ΔK were determined. Concerning FA, values varied between 0.6% (olive oils flavoured with red chili pepper, laurel and oregano), and 0.8% in the olive oils flavoured with garlic (Table 2). The addition of garlic increased significantly the FA values ($P < 0.001$) comparatively to the others spices added and to control olive oils. Gambacorta et al. (2007) also verified that FA values increased when garlic was added to Italian extra virgin olive oils, while observing the same tendency for hot pepper and oregano.

Table 2. Effect of the addition of different spices to olive oil on the quality parameters (mean ± standard deviation; n = 6).

	FA (%)	PV (meq.O_2/kg)	K_{232}	K_{270}	ΔK
Control	0.6 ± 0.0 a	4.9 ± 0.6 b	2.65 ± 0.15 c	0.14 ± 0.01 a	-0.004 ± 0.001 a,b
Garlic	0.8 ± 0.1 b	2.7 ± 0.4 a	2.11 ± 0.12 a	0.14 ± 0.01 a,b	-0.003 ± 0.001 a
Hot chili	0.6 ± 0.1 a	5.0 ± 0.0 b	2.29 ± 0.23 a,b	0.16 ± 0.01 c,d	-0.007 ± 0.003 b
Laurel	0.6 ± 0.1 a	4.8 ± 0.3 b	2.56 ± 0.27 b,c	0.15 ± 0.01 b-d	-0.004 ± 0.001 a,b
Oregano	0.6 ± 0.2 a	2.9 ± 0.4 a	2.09 ± 0.12 a	0.15 ± 0.01 a-c	-0.004 ± 0.001 a
Pepper	0.6 ± 0.2 a	5.0 ± 0.0 b	2.30 ± 0.20 a,b	0.16 ± 0.01 d	-0.006 ± 0.003 b
***P* value**	< 0.001*	< 0.001*	< 0.001*	< 0.001**	< 0.001*
Pooled SD[a]	0.018	0.236	0.189	0.011	0.002

Values within the same column with different letters differ significantly ($P < 0.05$); *$P<0.05$, by means of Levene test. *P* values from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed; **$P>0.05$, by means of Levene test. *P* values from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed.
[a]Pooled standard deviation

However no differences were observed from control samples after 90 days of storage but these authors used garlic extracts instead of fresh garlic, as in our case. The registered rise in the FA values in our study could be related to increased enzymatic activity that promotes lipolytic reactions in the olive oil, or simply by the increased presence of water in fresh garlic. In opposition, other study reported that the combined extraction of olives with dehydrated garlic leads to a decrease in the olive oils FA values (Baiano et al., 2009). Concerning the remaining spices tested (hot chili peppers, laurel, oregano, and pepper), no significant increases in the FA values (P = 0.536) relatively to control olive oils were observed.

The formation of primary compounds of oxidation was assessed by the PV. Olive oils with garlic and oregano were those who reported lower PV, with 2.7 and 2.9 meq.O_2/kg of oil, respectively (Table 2). These two olive oils reported significant lower PV comparatively to the remaining oils (P < 0.001), and even against control olive oils (4.9 meq.O_2/kg), meaning that their inclusion improves the oils stability towards the formation of primary products of oxidation. Oils with hot chili peppers, laurel and pepper, besides reporting higher PV than garlic and oregano, were not significantly different (P = 0.437) from control olive oils. Their inclusion was apparently not beneficial to the olive oils oxidative stability neither was it harmful. The results obtained in the PV are in consonance with those obtained in the K_{232}, another parameter that allows evaluating the formation of primary oxidation compounds. Once more olive oils with garlic and oregano reported lower K_{232} values, 2.09 and 2.11 respectively (Table 2). Unflavoured samples reported significantly higher K_{232} values than oils with garlic and oregano, 2.65 (P < 0.001). Baiano et al., (2009) while extracting olive oil with garlic, lemon, oregano, hot pepper, and rosemary, verified that garlic was the only flavouring that reported lower PV and K_{232} values than unflavoured olive oil, while the remaining spices and herbs increased significantly its value.

Regarding the formation of secondary products of oxidation, we proceed to the determination of the coefficient of extinction at 270 nm (K_{270}), since these compounds absorb in the 270 nm region and their presence is indicative of extensive oxidation. In this case unflavoured olive oils reported the lowest K_{270} values together with those olive oils with garlic, 0.14, while oils with hot chili pepper and pepper reported significantly higher values, 0.16 (P < 0.001). The same tendency was observed in the ΔK values. The addition of red chili pepper extracts obtained by supercritical fluid extraction also increased K_{232} and K_{270} values of Portuguese olive oils (Gouveia, Duarte, Beirão da Costa, Bernardo-Gil, & Moldão-Martins, 2006). When Baiano et al. (2009) tested the combined extraction of different spices with Peranzana olive fruits, higher primary products of oxidation (PV) were observed in the olive oils with hot pepper, in contradiction

to the results obtained in our work. Still, the amount of product used per olive oil volume should be implicated, as increased concentrations of hot pepper revealed to be pro-oxidant, increasing PV and K_{232} and K_{270} values (Baiano et al., 2009). Meanwhile, for long term storage, hot pepper extracts counteract quite well the formation of oxidation products when compared to unflavoured controls (Gambacorta et al., 2007).

Regarding the results obtained in the present study, some flavoured olive oils could not be considered as extra virgin olive oils, according to the European legislation (European Community Regulation EEC/2568/91 and all subsequent amendments). In particular, olive oils with garlic exceed the 0.8% of free acidity and some samples exceed the maximum legal value for K_{232} values (2.50). Particular attention must be given to these two quality parameters in order to avoid the declassification of the olive oil from the extra virgin or virgin categories.

3.2. Fatty acids profile

The fatty acids profile was assessed in the olive oils flavoured with herbs and different spices. Their detailed composition is reported in Table 3. In all samples, oleic acid ($C_{18:1}$) was the most abundant fatty acid, followed by palmitic acid ($C_{16:0}$) and linoleic acid ($C_{18:2}$). Oleic acid content increased with the addition of herbs and spices ($P < 0.001$). Its values ranged from 74.47% in the control olive oils to 75.09% in the pepper samples. Respecting to $C_{16:0}$ and $C_{18:2}$, the incorporation of the herbs and spices in the olive oil decreased significantly their content ($P < 0.001$ and $P = 0.009$ respectively). Control olive oils reported 10.80% of $C_{16:0}$, while the flavoured ones presented values equal or below to 10.40% (Table 3). Olive oils with laurel were those who reported lower $C_{16:0}$ content, 10.19%. In the case of $C_{18:2}$ control olive oils contained 9.70% and olive oils with pepper were those with lower content (9.15%). Among the individual fatty acids, the addition of herbs and spices didn't influence significantly the amounts of heptadecanoic acid ($C_{17:0}$), 10-heptadecenoic acid ($C_{17:1}$) and eicosanoic acid ($C_{20:0}$) ($P = 0.470$; $P = 0.549$; and $P = 0.121$ respectively). However some fatty acids fractions, like SFA (saturated fatty acids), and MUFA (monounsaturated fatty acids), were significantly affected by the addition of herbs and spices to the olive oil ($P < 0.001$ for SFA and $P = 0.001$ for MUFA). The results obtained revealed that the addition of flavourings decrease significantly SFA content, a decrease that varied between 0.30% in oils with garlic and 0.45% in those oils flavoured with laurel. By other hand the amounts of MUFA were significantly increased with the addition of laurel and oregano.

Table 3. Fatty acids profile (%) of olive oils flavored with different spices (mean ± standard deviation; n = 6).

	Control	Garlic	Hot chili	Laurel	Oregano	Pepper	*P* value	Pooled SD[a]
$C_{16:0}$	10.80 ± 0.23 c	10.40 ± 0.18 b	10.31 ± 0.11 a,b	10.19 ± 0.11 a	10.20 ± 0.13 a	10.26 ± 0.13 a,b	< 0.001**	0.155
$C_{16:1}$	0.67 ± 0.04 b	0.63 ± 0.04 a	0.62 ± 0.03 a	0.62 ± 0.03 a	0.61 ± 0.01 a	0.64 ± 0.03 a,b	< 0.001**	0.052
$C_{17:0}$	0.14 ± 0.03	0.14 ± 0.02	0.15 ± 0.04	0.14 ± 0.03	0.13 ± 0.02	0.14 ± 0.02	0.470**	0.050
$C_{17:1}$	0.20 ± 0.02	0.21 ± 0.02	0.20 ± 0.02	0.20 ± 0.01	0.21 ± 0.02	0.20 ± 0.02	0.549**	0.047
$C_{18:0}$	2.79 ± 0.05 b	2.73 ± 0.05 a	2.79 ± 0.12 a,b	2.80 ± 0.10 a,b	2.81 ± 0.15 a,b	2.84 ± 0.13 a,b	0.013*	0.104
$C_{18:1}$	74.47 ± 0.24 a	74.76 ± 0.47 a,b	74.78 ± 0.28 a,b	74.96 ± 0.22 b	74.97 ± 0.29 b	75.09 ± 0.60 a,b	< 0.001*	0.362
$C_{18:2}$	9.70 ± 0.16 b	9.59 ± 0.12 a,b	9.51 ± 0.16 a,b	9.46 ± 0.39 a,b	9.45 ± 0.26 a,b	9.15 ± 0.66 a	0.009*	0.349
$C_{20:0}$	0.39 ± 0.03	0.39 ± 0.03	0.41 ± 0.04	0.38 ± 0.02	0.39 ± 0.01	0.41 ± 0.02	0.121*	0.048
$C_{20:1}$+$C_{18:3}$	0.95 ± 0.09 a	1.02 ± 0.07 b	1.09 ± 0.05 b,c	1.12 ± 0.04 c	1.09 ± 0.06 c	1.14 ± 0.03 c	< 0.001**	0.071
$C_{22:0}$	0.06 ± 0.05 a	0.15 ± 0.03 b	0.13 ± 0.05 b	0.14 ± 0.02 b	0.16 ± 0.03 b	0.14 ± 0.02 b	< 0.001*	0.053
SFA	14.10 ± 0.16 b	13.80 ± 0.20 a	13.78 ± 0.12 a	13.65 ± 0.19 a	13.72 ± 0.14 a	13.77 ± 0.17 a	< 0.001**	0.162
MUFA	75.31 ± 0.27 a	75.54 ± 0.57 a,b	75.56 ± 0.25 a,b	75.77 ± 0.22 b	75.80 ± 0.32 b	76.00 ± 0.65 a,b	0.001*	0.394
PUFA	10.61 ± 0.27	10.51 ± 0.39	10.64 ± 0.15	10.58 ± 0.38	10.41 ± 0.50	10.21 ± 0.71	0.603*	0.443

Values within the same line with different letters differ significantly ($P < 0.05$); *$P<0.05$, by means of Levene test. *P* values from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed; **$P>0.05$, by means of Levene test. P values from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed. [a]Pooled standard deviation

Control olive oils reported 75.31% while olive oils with pepper contained higher MUFA amounts, about 76%. Concerning PUFA the addition of herbs and spices didn´t influenced significantly their content (*P* = 0.603). PUFA content was higher in the olive oils with hot chili pepper (10.64%), reporting the olive oils with pepper lower content (10.21%). Regardless of the variations observed, the results obtained are still in accordance with the maximum permitted levels in order to be considered as extra-virgin olive oils (European Community Regulation EEC/2568/91 and all subsequent amendments).

3.3. Tocopherols and tocotrienols composition

Tocopherols are important components of olive oil since they play a dualistic role. By one hand they exhibit important nutritional properties due to their vitaminic function (vitamin E) and by other they contribute to the stability of the oils since they are ascribed with valuable antioxidant properties (Blekas, Tsimidou, & Boskou, 1995; Warner 2005). Therefore their characterization in flavoured olive oils is essential and this kind of information is scarce in the literature available. In the olive oils analysed, three tocopherols (α-, β-, and γ-tocopherol) and one tocotrienol (α-tocotrienol) were found (Table 4). As expected for olive oils, α-tocopherol was the main vitamin E isoform found. Its content varied between 174.6 mg/kg in the oils with laurel and 192.5 mg/kg in the olive oils flavoured with hot chili peppers. In fact the amounts of α-tocopherol in the oils with hot chili peppers was significantly higher comparatively to the olive oils with garlic, laurel and pepper (*P* = 0.003). Concerning γ-tocopherol, unflavoured olive oils were the only samples that reported values below 4 mg/kg, while the remaining samples reported higher values comprised between 4.09 and 4.38 mg/kg, again with higher amounts by using hot chili peppers (Table 4). β-tocopherol values were all below 1 mg/kg, with significant higher values with garlic and hot chili pepper (*P* = 0.004) comparatively to control olive oils. α-Tocotrienol was only present in the oils flavoured with oregano and hot chili pepper, being absent in the control samples. The addition of hot chili pepper influenced all the isoforms of vitamin E, increasing their content. Consequently total vitamin E of the olive oils was significantly higher (*P* = 0.003) in those flavoured with hot chili peppers, with 198.6 mg/kg.

Table 4. Tocopherols and tocotrienols (mg/kg of oil) composition of olive oils flavoured with different spices (mean ± standard deviation; n = 6).

	α-Tocopherol	α-Tocotrienol	β-Tocopherol	γ-Tocopherol	Total Vitamin E
Control	181.7 ± 3.5 a,b	n.d.	0.84 ± 0.1 a	3.8 ± 0.2 a	186.4 ± 3.7 a,b
Garlic	179.2 ± 2.6 a	n.d.	0.94 ± 0.1 b	4.1 ± 0.1 b	184.2 ± 2.6 a
Hot chili	192.5 ± 11.5 b	0.76 ± 0.1 a	0.95 ± 0.1 b	4.4 ± 0.4 b	198.6 ± 11.8 b
Laurel	174.6 ± 7.8 a	n.d.	0.86 ± 0.1 a,b	4.2 ± 0.3 a,b	179.7 ± 8.0 a
Oregano	181.6 ± 10.9 a,b	0.83 ± 0.1 b	0.90 ± 0.1 a,b	4.1 ± 0.3 a,b	187.4 ± 11.3 a,b
Pepper	177.8 ± 9.8 a	n.d.	0.93 ± 0.1 a,b	4.3 ± 0.4 b	183.0 ± 10.0 a
***P* value**	0.003*	0.046**	0.004**	< 0.001*	0.003*
Pooled SD[a]	8.426	0.223	0.081	0.313	8.668

Values within the same column with different letters differ significantly ($P < 0.05$); *$P<0.05$, by means of Levene test. *P* values from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed; **$P>0.05$, by means of Levene test. P values from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed. [a]Pooled standard deviation

3.4. Radical scavenging activity (RSA) and total phenols content (TPC)

The radical scavenging activity of the olive oils flavoured with herbs and spices was measured by two chemical assays: the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) scavenging assay , and the 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) free radical scavenging assay (Table 5). These two methods are essential in measuring the antioxidant potential of the samples since in the presence of antioxidants they become more stable and a discoloration is observed in both methods, leading to an absorbance decrease which is indicative of higher antioxidant potential. For the DPPH assay olive oils with pepper and hot chili peppers reported higher antioxidant activity, with 144.5 and 143.1 µmol/L TE respectively, without significant differences from control samples (P = 0.198). Still, only olive oils with garlic had significant lower values (P < 0.001) comparatively with the control olive oils (Table 5). Concerning the capacity of the olive oils to scavenge the free radicals of ABTS, control olive oils were those that reported higher antioxidant activity, 296.3 µmol/L TE, while olive oils with oregano, hot chili pepper, and pepper revealed lower capacity to scavenge $ABTS^{\cdot+}$ (Table 5). Baiano et al. (2009) also observed that the addition of herbs and spices to olive oils decrease its antioxidant potential, with beneficial effects being observed only at long-term storage. The results obtained in the two methods used to evaluate the RSA of the flavoured olive oils are in accordance with usual values obtained for such vegetable oil (Sánchez et al., 2007).

Table 5. Radical scavenging activity (DPPH and $ABTS^{\cdot+}$, µmol/L TE), total phenols content (mg caffeic acid equiv./kg of olive oil) and oxidative stability (hours) of olive oils flavored with different spices (mean ± standard deviation; n = 6).

	DPPH	$ABTS^{\cdot+}$	Total phenols	Oxidative stability
Control	140.8 ± 3.0 b-d	296.3 ± 2.8 c	345.7 ± 15.2 b	9.4 ± 0.1 a,b
Garlic	126.8 ± 11.4 a	295.8 ± 3.9 b,c	325.6 ± 38.9 a-c	9.8 ± 0.2 a-c
Hot chili	143.1 ± 1.5 d	290.9 ± 2.6 a	336.3 ± 19.8 b,c	10.1 ± 0.7 b,c
Laurel	133.6 ± 4.0 a,b	294.0 ± 2.4 b,c	317.8 ± 37.2 a-c	9.2 ± 0.4 a
Oregano	137.4 ± 4.3 a-c	293.3 ± 2.3 a,b	293.8 ± 23.6 a	10.4 ± 0.5 c
Pepper	144.5 ± 9.6 c,d	293.4 ± 3.7 a,b	326.0 ± 14.3 c	9.8 ± 0.5 a-c
***P* value**	< 0.001*	< 0.001*	< 0.001*	< 0.001**
Pooled SD[a]	7.439	4.141	26.73	0.453

[a]Pooled standard deviation

Concerning olive oils total phenols (TPC), they varied in the following order: unflavoured olive oils (345.7 mg/kg) > hot chili peppers (336.3 mg/kg) > pepper and garlic (326 mg/kg) > laurel (317.8 mg/kg) > and oregano (293.8 mg/kg) (Table 5). The

incorporation of herbs and spices was not beneficial to the olive oils phenolic composition and the consequent expected bioactivity. This same observation was verified by Baiano et al. (2009) by adding different flavourings to Italian olive oils. This author reported losses around 150 mg/kg when extracting the olive oil with garlic and lost 130 mg/kg and 100 mg/kg with oregano and hot peppers extraction, respectively. However, when studying the aromatization of Tunisian olive oils, Ayadi et al. (2009) observed that the addition of basil increased significantly the TPC.

When the data obtained in the methods of RSA were correlated with those obtained in the TPC, a significant positive correlation was established for the DPPH method (R^2 = 0.136; $P < 0.05$). Meanwhile, no correlation was established for the data obtained in the ABTS (R^2 = 0.001; $P > 0.05$). The low R^2 values demonstrate that phenolic compounds don't contribute decisively for the antioxidant potential displayed by the samples. This data strengthens the hypothesis that other compounds different from phenolics and/or synergic reactions could play an important function in the olive oil antioxidant properties, and tocopherols could be one of those groups of compounds. Furthermore antioxidant compounds present in the herbs and spices may differ significantly (Baiano, Gambacorta, & La Notte, 2010) as well as their antioxidant potential which may have influenced the results obtained.

3.5. Oxidative stability

The oxidative stability is an important parameter in the analysis of vegetable oils. With this determination it is possible to verify the preservation status of the oils as well as their predictive resistance to oxidative processes. In this study we intend to verify if the addition of herbs and different spices influences the resistance to oxidation under low-heating and oxidative stress. Comparatively to control samples all the flavoured olive oils tested reported slightly higher oxidative stability, except laurel (Table 5). Olive oils flavoured with oregano reported significantly higher resistance to oxidation ($P < 0.001$) than the control ones, with 10.4 and 9.4 h, respectively. The introduction of herbs and spices improved the oxidative stability of the olive oils, a fact already witnessed in the quality indices, mainly PV and K_{232}. Despite being apparently correlated with the total vitamin E content (R^2 = 0.213; $P < 0.01$), which means that higher vitamin E amounts leads to a higher oxidative stability in the olive oils, this oxidative stability is usually more associated with increased phenolic contents, which was not observed in the present study (R^2 = 0.062; $P > 0.05$) (Aparicio et al., 1999; Baldioli et al., 1996). Therefore, other compounds extracted from the spices could motivate these findings, including for instance, sesquiterpenes, triterpenes, alkaloids or even ascorbic acid, deserving further attention in future studies. Several authors also report improvements in olive oils stability

by the addition of herbs and spices, mainly after a storage period (Antoun & Tsimidou, 1997; Ayadi et al., 2009; Gambacorta et al., 2007).

The addition of herbs and spices to olive oil after its extraction brought changes in their quality, composition, bioactive properties and stability of the olive oil, accordingly to the type of flavouring used, as represented in Figure 1. A PCA (principal component analysis) was applied to the quality indices (free acidity, peroxide value, K_{232}, K_{270}, and ΔK), tocopherols and tocotrienols content, the data obtained in the RSA, total phenols content and oxidative stability. PCA allowed explaining 63.7% of the total variance of the data by using three principal components (Fig. 1).

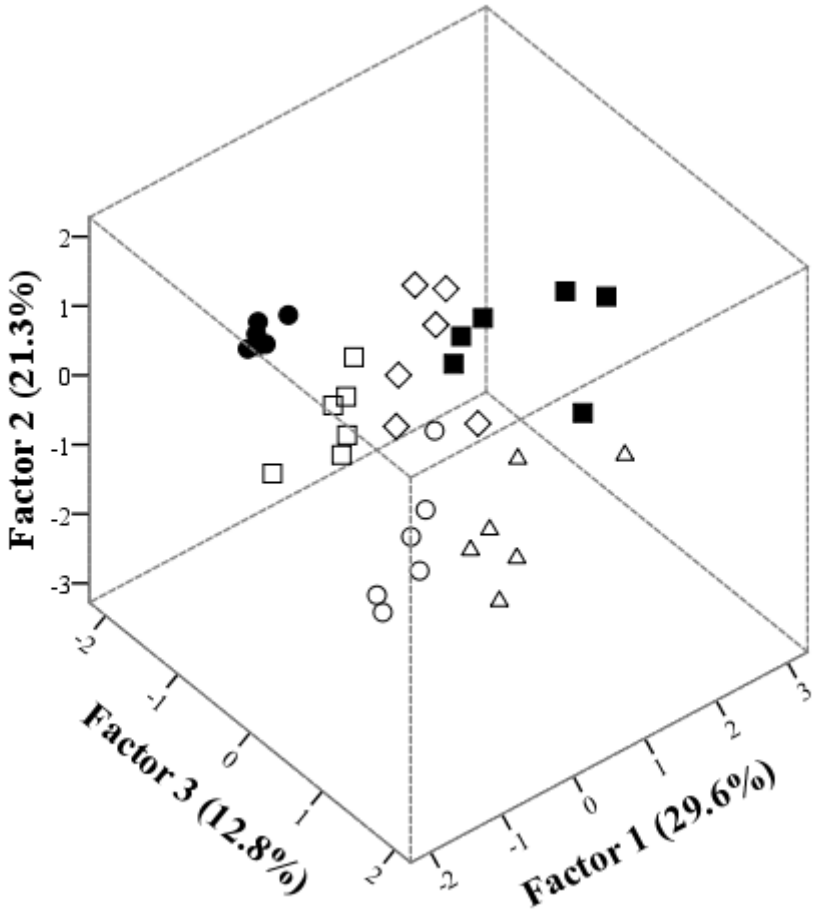

Figure 1. Principal component analysis (PCA) of flavored olive oils obtained by using quality parameters data (free acidity, peroxide value, K_{232}, K_{270} and ΔK), tocopherols and tocotrienols content, oxidative stability, antiradical activity (DPPH and ABTS) and total phenols content. The PCA factors explain 63.7% of the total variance of the data (○ control; ● garlic; △ oregano; □ laurel; ◇ pepper; ■ hot chili).

In Figure 1 it is possible to verify the formation of groups according to the flavouring used to aromatize the olive oils. Unflavoured olive oils represented a distinctive group in the negative regions of the first and third principal components factor scores and in the positive region of the second factor score. These olive oils were characterized by higher phenolic compounds and higher values in K_{232}. Olive oils flavoured with hot chili peppers, represented in the positive regions of the three principal components factors, contributed with increased vitamin E values. Concerning garlic, flavoured olive oils were characterized by higher free acidity values and lower PV, and are represented in the opposite direction of those oils with hot chili pepper and pepper. Olive oils with oregano were capable to contribute with the highest oxidative stability and reported the highest amounts of α-tocotrienol.

4. Conclusions

The present study is a contribution for the characterization of flavoured olive oils concerning their quality, composition, antioxidant properties and stability. From the results obtained we concluded that the addition of herbs and spices didn´t affect olive oils quality, with the exception of fresh garlic which increase free acidity values. Hot chili peppers increased the content of all the isoforms of vitamin E, increasing also the nutritional value. The antioxidant activity measured by two radical scavenging methods revealed that some flavourings decrease olive oil bioactive properties. Total phenols content also decreased with the addition of flavourings, and their amount was correlated with the results observed in the DPPH radical scavenging method. Vitamin E was correlated with the results obtained in the oxidative stability of the olive oils, generally increasing by the addition of the herbs and spices, exception made for of olive oils flavoured with laurel. We also concluded, by applying a PCA that the addition of different flavourings affected on its own distinctive way the quality, composition, and properties of the olive oils.

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PART III

Discussão geral e Conclusões

Capítulo 8. Discussão geral

Capítulo 9. Conclusões

CAPÍTULO 8.

Discussão geral

A olivicultura é uma atividade importante na região de Trás-os-Montes, onde o património genético da oliveira é rico e diversificado. Neste sentido, no capítulo 3, procedeu-se à caraterização de dez cultivares de oliveiras da região, no que respeita às características morfológicas dos seus frutos e endocarpos, relação polpa/caroço, teor de gordura e caraterização do azeite extraído. As cvs. Bical, Borrenta e Cordovesa foram as que apresentaram os frutos com maior massa, enquanto na cv. Lentisca se registaram os frutos mais leves, significativamente diferentes das outras nove cultivares em estudo. Também no comprimento foram registadas grandes diferenças, variando entre 15,3 e 24,7 mm para as cvs. Lentisca e Bical, respetivamente. Em todos os parâmetros morfológicos avaliados, a cv. Lentisca foi a que apresentou valores mais baixos. A relação polpa/caroço é um parâmetro importante, uma vez que pode dar a noção de uma maior aptidão de determinada cultivar para a preparação de azeitonas de mesa, caso tenha uma relação elevada, ou para extração de azeite, caso essa relação não seja tão favorável. Contudo, outros parâmetros como sejam a rigidez da polpa e a sua apetência para o processo tecnológico e o teor em gordura da polpa, são aspetos da enorme importância. No presente trabalho, a relação de polpa/caroço mais elevada foi encontrada nas cvs. Bical, Madural Negra e Negrinha de Freixo. Na verdade, a última cultivar (cv. Negrinha de Freixo) é conhecida pelas suas excelentes caraterísticas e aptidão tecnológica para a produção de azeitona de mesa, sendo cultivada em grande medida para esse fim. As suas excelentes caraterísticas e a genuinidade e tipicidade das azeitonas de mesa que produz têm sido reconhecidas, sendo que a única Denominação de Origem Protegida de azeitona de mesa que existe na região de Trás-os-Montes tem por base esta cultivar, sob a designação DOP "Azeitona de Conserva Negrinha de Freixo".

A quantidade de gordura é uma informação valiosa sobre a escolha das cultivares mais produtivas para extração do azeite, contudo esta informação deve ser conjugada com a informação acerca da qualidade dessa gordura. Neste caso, as cultivares que obtiveram maior rendimento foram Bical, Madural Negra, Cordovesa e Verdeal Transmontana (62,2%). Pelos resultados obtidos, pode inferir-se que a cv. Lentisca é uma cultivar que, para além de produzir frutos pequenos, com baixa relação polpa/caroço, a sua polpa também é pobre em azeite em comparação com as restantes cultivares, mostrando-se sem apetência para a produção de azeitona de mesa e com

fraco poder de produção para o caso do azeite. Estas informações têm sido reconhecidas no campo, uma vez que os agricultores estão naturalmente a abandonar esta cultivar de azeitona, sendo já muito raras as árvores em algumas zonas da região.

De uma maneira geral, em termos de qualidade, os azeites obtidos das 10 cultivares são de boa qualidade, tendo sido todos classificados na categoria de "Azeite Virgem Extra" relativamente aos parâmetros avaliados e de acordo com as gamas de valores que constam no Regulamento Europeu (REG nº 2568/91 e alterações subsequentes). No que respeita à composição em ácidos gordos, o ácido oleico (C18:1) foi o ácido gordo maioritário, como espectável, variando de 68,6%, na cv. Madural Negra, a 82,0% na cv. Verdeal Transmontana. Os valores obtidos encontram-se dentro dos limites regulamentados (Reg CEE nº 2568/91 e suas alterações subsequentes). O ácido palmítico variou entre 8,9% (cv. Santulhana) e 14,2% (em cv. Madural Negra), enquanto o ácido linoleico apresentou maior variabilidade, sendo claramente inferior na cv. Lentisca, com 2,7%, em comparação com as restantes, tendo sido superior na cv. Borrenta, com 12,6%. Este ácido gordo, juntamente com o ácido linoleico, são de grande importância nutricional uma vez que são ácidos gordos essenciais. Contudo é de realçar que o grau de insaturação dos ácidos gordos tem uma influência negativa ao nível da estabilidade oxidativa do azeite, diminuindo esta com o teor em ácidos gordos insaturados, e como consequência reduzindo também o tempo de armazenamento e tempo de prateleira dos azeites obtidos. Os resultados obtidos na análise componentes principais e análise linear discriminante indicam claramente que o perfil de ácidos gordos pode ser usado para a discriminação dos azeites das 10 cultivares.

Os tocoferóis são importantes componentes menores de azeite devido à sua função dualista: vitamina e antioxidante. Nas 10 cultivares estudadas, foram encontrados quatro tocoferóis (α-, β-, γ-, e δ-tocoferol) e dois tocotrienóis (α- e γ-tocotrienol). O α-tocoferol foi o principal tocoferol encontrado nos azeites, representando mais de 90% do total. Quanto ao conteúdo total de vitamina E (soma de todos os tocoferóis e tocotrienóis), a cv. Cobrançosa mostrou ter o teor mais elevado, enquanto a cv. Madural Negra apresentou o menor teor. Os resultados obtidos mostraram que algumas cultivares de oliveira, consideradas minoritárias, apresentam teores consideráveis e vitamina E, como sejam as cvs. Lentisca e Cordovesa. Por outro lado constata-se também que entre os valores mais elevados registados neste parâmetros, se encontram as três cultivares maioritárias da DOP "Azeite de Trás-os-Montes", nomeadamente as cvs. Cobrançosa, Madural e Verdeal Transmontana. Também como seria de esperar a trioleína (OOO), foi o triacilglicerol registados em maior quantidade, com teores a variar entre os 38,1%, na cv. Madural Negra, e os 64,0%, na cv. Verdeal Transmontana.

Nos Capítulos 4 e 5 procedeu-se ao estudo da composição fenólica das três cultivares dominantes da DOP "Azeite de Trás-os-Montes", nomeadamente a Cobrançosa, a Madural e a Verdeal Transmontana. Foram identificados e quantificados sete compostos fenólicos. Um fenol, o hidroxitirosol; duas flavonas, a apigenina-7-O-glucósido e a luteolina; um glicósido feniletanóide, o verbascosídeo; um secoiridóide, a oleuropeína; um ácido fenólico, o ácido clorogénico; e um flavonol, a rutina. O teor de compostos fenólicos totais foi severamente influenciado pelo processo de maturação, diminuindo drasticamente logo da primeira para a segunda data de colheita. Este comportamento foi idêntico nas três cultivares em estudo. A perda de compostos fenólicos ao longo da maturação é essencialmente determinada pelo teor de oleuropeína, que é o componente fenólico principal nas azeitonas. Nas duas primeiras datas de amostragem, em que as azeitonas se encontram verdes e com um sabor muito adstringente, a oleuropeína foi o composto fenólico mais abundante, com cerca de 33 g/kg na cv. Cobrançosa, 36 g/kg na cultivar Madural e 13 g/kg na cv. Verdeal Transmontana, na primeira data, e cerca de 3 g/kg, 1 g/kg e 0,6 g/kg, na segunda data de colheita, respetivamente. Verificou-se também que o teor em oleuropeína continuou a descer, em paralelo com uma mudança de cor dos frutos de verde a preto, para desaparecer completamente na última data de amostragem nas azeitonas da cv. Cobrançosa. Esta diminuição estará relacionada com a atividade enzimática no fruto, como sejam a polifenol oxidase e a β-glicosidase. Globalmente, as maiores diferenças foram observadas entre a segunda e a terceira datas de amostragem, correspondente ao início da viragem da cor, marcada pela redução da oleuropeína e ao surgimento do hidroxitirosol e da rutina. A última data de amostragem, no entanto, foi claramente distinta, com ausência de oleuropeína e aparecimento na cv. Cobrançosa da luteolina e do verbascosídeo.

Na avaliação da atividade antioxidante da polpa, observou-se uma relação de dependência entre a concentração de extrato testada, o estado de maturação e a cultivar. No que diz respeito a valores foi observada uma tendência semelhante à verificada para os compostos fenólicos, mostrando os extratos atividade superior no início da maturação que foi diminuindo ao longo das datas de colheita. As alterações ocorridas estarão relacionadas com a diminuição do teor em compostos fenólicos desde o início da maturação até à última data de amostragem. O aparecimento de alguns derivados da oleuropeína a partir da mudança de cor com atividade antioxidante superior parece contribuir para o efeito verificado, uma vez que se nota uma ligeira melhoria neste parâmetro, mas possivelmente outros compostos químicos poderão estar igualmente envolvidos na atividade verificada.

No capítulo 6, o objetivo principal foi estudar as mudanças fenológicas e químicas verificadas durante a maturação nas três principais cultivares do azeite DOP "Azeite de Trás-os-Montes ", cvs. Cobrançosa, Verdeal Transmontana e Madural, de modo a definir a altura ideal para a colheita de cada cultivar individualmente. Na verdade, a prática comum na região consiste na apanha simultânea de todas as oliveiras por produtor, perdendo-se assim qualidade. O processo de maturação das três cultivares foi avaliado durante três épocas de colheita consecutivas, com foco na produção e qualidade do azeite, a fim de fornecer dados aos produtores para apoiar decisões sobre datas de colheita adequadas.

O estudo detalhado dos estádios fenológicos permitiu verificar claramente que a cv. Madural tem um processo de maturação mais rápido, enquanto cv. Verdeal Transmontana tem um processo de maturação mais lento. Ao analisar os parâmetros biométricos dos frutos é notório que uma mesma cultivar tem um padrão de evolução distinto em cada ano, e que as três cultivares seguem padrões semelhantes dentro de um ano, mas não entre os diferentes anos. O teor em gordura em base seca, por exemplo, não é constante ao longo de anos. Já a qualidade do azeite extraído esteve sempre dentro dos limites regulamentares, mas a sua composição apresentou variações entre cultivares, principalmente na composição em ácidos gordos, compostos fenólicos e atividade antioxidante, e entre anos, nomeadamente no teor em gordura e parâmetros de oxidação. Em particular, a cv. Verdeal Transmontana apresenta maior quantidade de ácido oleico (76-80%), o que lhe dá estabilidade oxidativa, e a cv. Madural em ácido linoléico (12-15%), naturalmente um foco de oxidação mais precoce. A colheita de 2011 teve menor quantidade de vitamina E do que a de 2010, para todas as cultivares, em sintomia com os valores mais elevados de índice de peróxidos em 2011, o que demonstra a existência de variabilidade entre anos de colheita, mas no geral a produção de vitamina E parece seguir a composição em ácidos gordos, sendo mais elevada quanto maior o teor de insaturação do azeite. Parece assim constituir um parâmetro de proteção, ajustado pela própria planta, mas variável em função do ano. A estabilidade oxidativa foi claramente diferente entre cultivares e anos, mas variou menos com a maturação, indicador que as características químicas de cada cultivar deverão ter aqui um papel determinante. A menor estabilidade foi observada na cv. Madural, seguida da cv. Cobrançosa e por fim da cv. Verdeal Transmontana, com valores excecionais (até 40 horas) e com tendência a aumentar nas últimas datas de amostragens, já em dezembro.

Nos últimos anos, têm-se verificado um aumento da oferta de azeites aromatizados no mercado. Esta tendência surgiu por um lado para aumentar a diferenciação e oferta de

produtos da fileira olivícola, e por outro para conseguir valorizar azeites que, apesar das suas características sensoriais e químicas permitirem a sua classificação nas categorias comerciais de azeite virgem extra e de azeite virgem, não têm grande fator diferenciador. Por outro lado, e uma vez que por vezes ao nível da produção não há capacidade de colheita da azeitona e extração do azeite atempadamente, os azeites resultantes têm pouco frutado, amargo e picante, e necessitam de ser valorizados de outra forma. Assim, no capítulo 7 estudou-se de que forma a adição de diferentes especiarias e temperos, vulgarmente utilizados na preparação de azeites aromatizados, interfere ao nível da qualidade, resistência à oxidação, atividade antioxidante e composição química desse tipo de produtos.

O azeite utilizado na preparação foi sempre o mesmo e tratava-se de um azeite monovarietal de cv. Cobrançosa classificado na categoria comercial de azeite virgem extra, pelo que as alterações ocorridas foram devidas ao agente aromatizante. Verificou-se que a adição de alho induziu um aumento dos valores de acidez livre (0,6-0,8%), o que poderá estar relacionado com o fator de o alho fresco ter algum teor em água o que pode desencadear mecanismos de hidrólise de ácidos gordos. Este aspeto é de particular importância, visto que a utilização de temperos ou especiarias que não estejam estabilizados, por exemplo microbiologicamente, e que tenham atividade de água elevada, pode levar a que ocorram estes fenómenos. Por outro lado verificou-se também que após aromatização alguns dos azeites não poderiam ser considerado como azeite virgem extra por excederem o valor máximo legal para valores de K_{232} de acordo com a legislação europeia (Reg. CEE 2568/91 e todas as alterações posteriores), o que estará relacionado com a oxidação acelerada originada por algumas das substâncias adicionadas

O perfil de ácidos gordos foi alterado, mas os valores permaneceram dentro dos limites considerados normais para azeites virgens extra. Os ácidos gordos saturados e ácidos gordos monoinsaturados, foram significativamente afetados pela adição de ervas e especiarias ao azeite. Os resultados obtidos revelaram que a adição de aromatizantes levou à diminuição do teor em ácidos gordos saturados, entre 0,30% nos azeites com alho e 0,45% nos azeites aromatizados com louro. Por outro lado, as quantidades de monoinsaturados aumentaram significativamente com a adição de louro e orégão. Os azeites foram nutricionalmente enriquecidos devido ao aumento do teor em vitamina E, principalmente, em óleos aromatizados com malagueta. Detetaram-se também alterações ao nível da atividade antioxidante, havendo uma diminuição do conteúdo de fenóis totais em todos os azeites aromatizados em relação ao controlo. Contudo, a capacidade de neutralizar a oxidação foi em geral aumentada.

Este tipo de azeites podem ser alternativas interessantes do ponto de vista comercial, pela inovação e mais-valia que trazem ao setor. Ao permitirem o escoamento de azeites que de outra forma seriam vendidos a preços mais baixos, permitem rentabilizar os produtos do olival. Por outro lado, do ponto de vista nutricional são produtos enriquecidos.

CAPÍTULO 9.

Conclusões

Os resultados obtidos no presente trabalho contribuíram para a valorização dos azeites produzidos na região de Trás-os Montes, com particular destaque para a área de influência da Denominação de Origem Protegida (DOP) "Azeite de Trás-os-Montes". Destaca-se o contributo na caraterização de azeites elementares de diferentes cultivares, na determinação do momento ótimo de colheita para as três cultivares de maior importância na região (Cobrançosa, Madural e Verdeal Transmontana) e na avaliação efeito da adição de temperos e especiarias ao azeite de forma a valorizá-lo.

Pode concluir-se que todas as cultivares de oliveiras estudadas originam azeites de qualidade, com rendimentos diferenciados, e possivelmente com diferentes aptidões, ou maioritariamente para azeite ou para azeitona de mesa. A composição química é diferenciada mas está dentro dos parâmetros admissíveis para as categorias comerciais de azeite, com maior ou menor estabilidade oxidativa como resultado da sua composição química.

Durante a maturação das três principais cultivares, a oleuropeína, o principal composto fenólico em azeitonas verdes, diminui drasticamente, enquanto o hidroxtirosol aumenta, sendo o principal composto fenólico em azeitonas maduras. Em azeitonas maduras, os fenóis totais podem diminuir até cerca de 2% quando se comparou a primeira data de colheita. A atividade antioxidante é influenciada pela variação teor em compostos fenólicos individuais, sendo possível estabelecer corelações entre alguns parâmetros, contudo considera-se que outros componentes presentes no azeite deverão igualmente contribuir para o efeito verificado.

Em relação à definição do momento ótimo de colheita, pode concluir-se que a cv. Madural, sendo mais sensível à oxidação e tendo um teor de lípidos relativamente constante ao longo da maturação, deverá ser colhida logo no início da campanha, no final de Outubro / início de Novembro, com adaptações em função da data de floração anual. O azeite da cv. Cobrançosa é claramente mais estável à oxidação, podendo ser colhida a azeitona a seguir à cv. Madural, de preferências ainda em Novembro, permitindo a colheita da cv Verdeal Transmontana no máximo no início de dezembro. A cv. Verdeal Transmontana, devido à sua maturação mais lenta, elevado teor em compostos fenólicos e teor crescente de lípidos na polpa ao longo da maturação, a sua colheita pode ser mais tardia mais para o final da época, devendo contudo ser salvaguarda a sua proteção das

geadas típicas de dezembro puma vez que afetam a qualidade do azeite de forma irreversível.

Esta informação resultou de um trabalho detalhado e sistemático realizado durante três épocas de colheita, reunindo assim as variações características típicas de cada cultivar e da região. Contudo, as variações climáticas e potenciais ataques de pragas não podem ser desconsideradas, e devem ser feitos ajustes quando as condições observadas se alteram.

Informações sustentadas sobre a influência da maturação nas propriedades bioativas das azeitonas e do seu azeite são de grande importância, uma vez que permitem moldar a sua composição, tornando-a mas equilibrada em termos de componentes lipídicos e atividade antioxidante, originando azeites com maior estabilidade e atributos sensoriais distintos. O impacto sensorial destas alterações poderá não ser rapidamente aceite por todos os consumidores, habituados a azeites mais neutros e descaracterizados, mas o reconhecimento das vantagens do ponto de vista nutricional será certamente um fator favorável na decisão dos mais informados e preocupados com a sua saúde.

A qualidade, estabilidade e atividade antioxidante de azeites da cv Cobrançosa aromatizados com temperos e especiarias, uma prática crescente num mercado que procura valorizar-se pela diversidade de produtos do olival, permitiu concluir que a adição destes componentes não afeta significativamente a qualidade do ponto de vista regulamentar, mas em alguns casos pode afetar a sua estabilidade, com consequente redução do prazo de validade. A introdução destes produtos no mercado deverá por isso ser cuidadosa, principalmente no ponto de vista da determinação do seu prazo de validade.

Os resultados obtidos nesta tese são de grande importância para os agricultores da região de Trás-os-Montes, e destaca a importância de tratar cada cultivar separadamente para maximizar a qualidade e rendimento, uma prática ainda pouco comum na região. Implicará alterações no saber fazer e justes inclusive nas datas de laboração dos lagares e na disponibilidade para a apanha, mas o resultado será certamente compensador, do ponto de vista da qualidade dos azeites de Trás-os-Montes e da sua projeção nacional e internacional.