INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior Agrária

Caracterização Quimica e Actividade Biológica de Azeitonas Verdes Descaroçadas "*alcaparras*" Produzidas em Trás-os-Montes

Anabela da Conceição de Sousa

Dissertação apresentada à Escola Superior Agrária de Bragança
para obtenção do Grau de Mestre em Qualidade e Segurança Alimentar

Orientado por

Prof. Doutor José Alberto Cardoso Pereira

Prof. Doutora Susana Isabel Pereira Casal Vicente

Esta dissertação inclui as críticas e sugestões feitas pelo Júri

Bragança
2008

O trabalho que se apresenta teve o apoio financeiro de:

Projecto INTERREG III A MOABEPE - *Identificación de los agentes patógenos y beneficiosos de los principales cultivos de las regiones fronterizas Tras Os Montes y Castilla y León para la realización de estrategias de control razonadas"*

À Constança

À Vitória

Agradecimentos

Ao entregar este trabalho, é com o maior prazer, que agradeço a todos os que de alguma forma contribuíram para a sua realização.

Em primeiro lugar ao meu orientador, Professor Doutor José Alberto Pereira, da Escola Superior Agrária, pela grande ajuda ao longo do trabalho laboratorial e escrito, permanente disponibilidade, incentivo e amizade demonstrada.

À Professora Doutora Susana Casal, do Serviço de Bromatologia da Faculdade de Farmácia do Porto, pela ajuda, disponibilidade permanente e sugestões criticas ao longo do trabalho.

À Professora Doutora Beatriz Oliveira, responsável do Serviço de Bromatologia da Faculdade de Farmácia do Porto, pela simpatia e facilidades concedidas para a realização de parte do trabalho laboratorial.

Ao Professor Doutor Albino Bento, da Escola Superior Agrária, pela simpatia e esforço para garantir condições materiais e financeiras para o bom desenvolvimento do trabalho.

À Professora Doutora Letícia Fernandes, da Escola Superior Agrária, pelas facilidades laboratoriais e ensinamentos na parte da actividade antimicrobiana.

À Professora Doutora Paula Andrade e sua equipa, do Serviço de Farmacognosia da Faculdade de Farmácia do Porto, pela determinação dos compostos fenólicos.

À Professora Doutora Isabel Ferreira, pela simpatia e conhecimentos transmitidos especialmente na parte da actividade antioxidante.

Aos meus colegas, elementos do Laboratório de Fitotécnia da Escola Superior Agrária, Susana Pereira, Ivo Oliveira e Ricardo Malheiro pelo apoio, incentivo e boa disposição sempre demonstrada.

À Lillian Barros, Ana Paula Pereira e Daniela Correia pela amizade e pelo acompanhamento, disponibilidade e ajuda constantes na parte experimental deste trabalho.

Finalmente ao Ricardo e à minha família, pelo apoio e incentivo constante ao longo da realização deste trabalho.

ÍNDICE

Resumo

As azeitonas de mesa descaroçadas "*alcaparras*" são um tipo de azeitonas produzidas de forma tradicional e muito apreciadas em Trás-os-Montes. Neste trabalho pretendeu-se contribuir para a sua caracterização em termos nutricionais, químicos e de actividade biológica (poder antioxidante e actividade antimicrobiana). O trabalho incidiu em 30 amostras recolhidas ao longo de três anos consecutivos.

Na caracterização nutricional procedeu-se à determinação da humidade, proteína bruta, gordura total, cinzas, hidratos de carbono e valor energético. Verificou-se que as "*alcaparras*" são maioritariamente constituídas por água (entre 60,4 e 81,0%), e gordura, (de 7,6 a 29,3%). Para o estudo da composição química foi avaliado na gordura extraída o perfil em ácidos gordos por GC/FID e o teor em tocoferóis por HPLC/FD, enquanto que na polpa da azeitona foram determinados os compostos fenólicos por HPLC/DAD. A composição em ácidos gordos é semelhante à do azeite da região com predominância dos ácidos gordos monoinsaturados, o ácido oleico foi maioritário (≈ 77,7%) seguido do palmítico (≈ 13,1%) e do linoleico (≈ 3,8%). O teor em tocoferóis variou entre 30,7 mg e 106,3 mg/kg de gordura, sendo o α-tocoferol mais abundante. Detectou-se e quantificou-se também a presença de três flavonóides (luteolina 7-*O*-glucosido, apigenina 7-*O*-glucosido e luteolina) na polpa da azeitona.

Para avaliação do poder antioxidante, pelos métodos do poder redutor e efeito bloqueador de radicais livres de DPPH, e teor em polifenóis totais, pelo método de Folin-Ciocalteau, foram testados diferentes solventes e temperaturas de extracção tendo a água à ebulição mostrado ser o método mais eficaz. O teor de fenóis totais variou entre 5,58 e 29,88 mg equiv. ácido gálico/g, enquanto os valores de EC_{50} estiveram compreendidos entre 0,36 e 1,64 mg/ml, para o poder redutor, e 0,34 e 1,72 mg/ml para o efeito bloqueador de radicais livres DPPH.

A actividade antimicrobiana foi testada com bactérias Gram-positivo e Gram-negativo e leveduras, tendo revelado os extractos de "*alcaparra*" inibição no crescimento quer das bactérias Gram-positivo e Gram-negativo, com excepção da *Pseudomonas aeruginosa* a concentrações de 0,05 mg / ml de extracto. Contudo não foi observada qualquer actividade em leveduras.

Palavras-chave: azeitonas de mesa descaroçadas; "*alcaparras*"; avaliação nutricional; composição química; potencial antioxidante; actividade antimicrobiana.

Abstract

"*Alcaparras*" are stoned halved olives traditionally produced and largely appreciated in "Trás-os-Montes", northeast of Portugal. This work aim was to contribute for their nutritional evaluation, chemical characterization, and screening of potential biological activities (antioxidat and antimicrobiological). The analyses were performed on 30 samples, collected in local markets over three consecutive years.

Samples were characterized for their water content, crude protein, fat, ash and carbohydrates, and their caloric value estimated. "*Alcaparras*" are mainly constituted by water (60.4% to 81.0%) and fat (7.6% to 29.3%). The extracted fat was further characterized for fatty acids by GC/FID and tocopherols by HPLC/FD, while pulp phenolic compounds were evaluated by HPLC/DAD. The fatty acid composition is similar to that presented by local olive oil, with monounsaturated fatty acids as main constituents (≈77.7% of oleic acid), followed by palmitic (≈13.1%) and linoleic (≈3.8%) acids. Total tocopherol amounts in the extracted fat varied between 30.7 mg/kg and 106.3 mg7kg, with α-tocoferol as dominant isomer. Three flavonoids were detected: luteolin 7-*O*-glucoside, apigenin 7-*O*-glucoside and luteolin.

For antioxidative potential evaluation, by both reducing power and radical scavenging effect on DPPH, and for total phenol content estimation, by the Folin-Ciocalteau method, different solvents and extractive temperatures were tested, with boiling water attaining highest efficiency. Total phenol content varied between 5.58 and 29.88 mg of gallic acid equivalents/g. The reducing power EC_{50} was between 0.36 and 1.64 mg/ml while for scavenging effect varied from 0.34 to 1.72 mg/ml.

The antimicrobial activity was tested against gram-positive and gram-negative bacteria and against fungi. The 0.05mg /ml "*alcaparra*" olive extracts inhibited all tested gram-positive and negative bacteria, with the exception of *Pseudomonas aeruginosa*. No inhibitory activity was detected for the fungal species tested.

Key-words: stoned table olives; "alcaparras"; nutritional value; chemical composition; antioxidant potential; antimicrobial activity.

Capítulo 1

Introdução

1.1. Introdução

A oliveira (*Olea europea* L.) é uma das árvores de fruto mais importantes nos países mediterrânicos. Desta espécie são utilizados diferentes produtos, sendo o azeite e as azeitonas de mesa os mais comuns. Contudo outras utilizações são também usuais. As folhas de oliveira, por exemplo, para além dos usos fitoterapêuticos tradicionais, são também utilizadas na alimentação animal, como fertilizante orgânico e como fonte de extracção de compostos para diversas aplicações. Também o bagaço de azeitona, resultante das unidades de extracção do azeite, tem múltiplas aplicações, que vão desde a sua utilização para extracção de óleo de bagaço de azeitona, ou de compostos específicos, à utilização como fertilizante ou fonte de energia para centrais de biomassa. As águas de vegetação, resíduos das indústrias de preparação de azeitonas de mesa e de extracção de azeite podem ser valorizadas através da extracção de compostos como o hidroxitirosol.

No que respeita às azeitonas de mesa, a sua preparação é uma tradição de longa data nos países da bacia do mediterrâneo, onde Portugal se inclui. Mais recentemente esta indústria tem tido grande desenvolvimento noutros países como a Turquia e Estados Unidos. As azeitonas são produtos fermentados e, como tal, o processo de fermentação e o seu controlo, físico, químico e microbiológico, são uma base fundamental para o conhecimento e melhoria da sua preparação, armazenamento e segurança do produto final (Fernández et al., 1997).

Os produtos vegetais em salmoura, incluindo as azeitonas de mesa, podem definir-se como os produtos em cuja preparação e preservação se combinam a salga, a fermentação e/ou acidificação. Segundo Fernández et al. (1997), este processo apresenta várias vantagens. Permite a preservação de matérias-primas perecíveis por um longo período de tempo. Também envolve um pequeno gasto energético, pois os factores conservantes são os efeitos combinados da força iónica, do pH baixo e da acidez orgânica, não sendo por norma necessária a utilização de tratamentos térmicos para a sua estabilização. Há ainda a referir a manutenção de propriedades nutricionais devidas a substâncias lábeis e de propriedades físicas, tais como a cor e a textura. Por último, a fermentação/salga permite obter produtos com características sensoriais muito especiais e bem definidas, tão do agrado do consumidor.

Para as azeitonas se tornarem comestíveis é necessário que ocorram alterações físico-químicas que alterem ou eliminem parcialmente o amargor que é característico dos frutos frescos. Este processo pode ser realizado tradicionalmente, por repetidas imersões das azeitonas em água corrente ou em soluções diluídas de sal. Neste processo tradicional podem ser adicionadas ervas aromáticas, tais como orégãos ou tomilho, que contribuem com os seus aromas para o sabor do produto final e que podem, adicionalmente, contribuir com os seus óleos essenciais para a preservação das azeitonas. No entanto, a produção de azeitonas de mesa a uma escala industrial usa outros sistemas para remover o amargor e para embalar o produto final nas diversas apresentações existentes no mercado.

Na actualidade, a produção de azeitonas de mesa atingiu já um elevado volume à escala mundial e representa uma parte substancial da economia de vários países. Consequentemente, o regulamento do comércio deste produto atraiu a atenção de organizações nacionais e internacionais. O Conselho Oleícola Internacional (COI, 2004) descreveu os diversos processos tecnológicos que podem ser usados na elaboração das azeitonas de mesa e regulamentou as suas formas de apresentação e categorias de qualidade. À semelhança, muitos países com produção de azeitona desenvolveram as suas próprias normas internas. Em Portugal vigora a Norma Portuguesa-3034 (1987) – *Derivados de frutos e de produtos hortícolas. Azeitonas de mesa. Definição, classificação, características, acondicionamento e marcação.* A norma define as diferentes apresentações comerciais e estabelece as categorias de qualidade apropriadas, de modo mais ou menos semelhante ao estabelecido pelo COI.

1.2. Caracterização da azeitona de mesa

1.2.1. Definição

Segundo a Norma Portuguesa NP – 3034 (1987), entende-se por "Azeitonas de mesa" o produto preparado a partir de frutos de variedades apropriadas da espécie *Olea europaea sativa* Hoffg Link, em estado de maturação conveniente, submetidos a tratamentos e operações que assegurem as suas características e boa conservação.

Ainda de acordo com a mesma Norma, na escolha das variedades a utilizar deve ter-se em conta o volume e forma do fruto, a boa proporção de polpa em relação ao caroço e as características da polpa, nomeadamente sabor, firmeza e facilidade de separação do caroço. Não devem ser usados os frutos tratados com pesticidas cujos resíduos não possam ser eliminados ou reduzidos a teores inócuos.

1.2.2. Classificação

Existem diferentes tipos ou classificações de azeitona de mesa que dependem sobretudo do grau de maturação dos frutos, do processo de preparação das azeitonas e/ou da sua forma de apresentação.

Contudo, o COI, entidade que a nível internacional regula o sector dos azeites e da azeitona de mesa, apenas considera três tipos de azeitona de mesa (Anónimo, 2004):

. **Azeitonas verdes** – obtidas a partir de frutos colhidos durante o período de amadurecimento, apresentado a cor verde, e que tenham atingido o tamanho normal. A cor do fruto pode variar de verde a amarelo-palha.

. **Azeitonas mistas** – frutos colhidos antes de atingirem a completa maturação, na altura da mudança de cor. A cor do fruto pode variar desde tons rosados a acastanhados.

. **Azeitonas pretas** – frutos colhidos no momento em que atingiram a maturação completa ou ligeiramente antes de a atingir. A cor do fruto pode ir de negro-avermelhado a castanho-escuro, passando por tons violáceos.

Contudo a classificação apresentada acima apenas diz respeito ao tipo de azeitonas que estão na origem da azeitona de mesa. Por sua vez, a Norma Portuguesa NP – 3034 (1987), classifica as azeitonas de mesa de acordo com o processo tecnológico que lhe deu origem, em:

. **Azeitonas verdes curadas em salmoura –** tratadas com solução alcalina, seguida de uma fermentação láctica natural total (à sevilhana) ou parcial, em salmoura. No caso das azeitonas serem submetidas à fermentação parcial, a sua conservação subsequente pode ser assegurada por

esterilização ou pasteurização, por conservante, por refrigeração ou por tratamento com azoto ou gás carbónico, sem salmoura.

. **Azeitonas verdes ao natural em salmoura –** em qualquer tratamento prévio por solução alcalina e conservadas por fermentação natural.

. **Azeitonas mistas curadas em salmoura –** conservadas por fermentação natural em salmoura, por tratamento térmico ou por ambos, após tratamento por solução alcalina.

. **Azeitonas mistas ao natural em salmoura –** conservadas por fermentação natural após tratamento directo com salmoura.

. **Azeitonas escurecidas por oxidação –** conservadas a partir de frutos que não atingiram a completa maturação, escurecidos por oxidação após tratamento alcalino. Estas azeitonas devem ser acondicionadas em salmoura e preservadas por esterilização.

. **Azeitonas pretas curadas em salmoura –** conservadas por fermentação natural, por um ou por vários dos seguintes processos, em salmoura, esterilização, pasteurização ou por agentes de conservação após tratamento alcalino.

. **Azeitonas pretas ao natural –** conservadas por fermentação natural através de um ou vários dos processos seguintes: em salmoura, por esterilização, por pasteurização ou por agentes de conservação após tratamento directo com salmoura. Conservam um gosto mais pronunciado do que as azeitonas curadas, com um ligeiro amargor.

. **Azeitonas pretas com sal –** conservadas por polvilhação com sal ou por camadas alternadas de azeitonas e sal.

Ainda de acordo com a NP – 3034 (1987) e as Normas do Codex Alimentarius (Anónimo, 1987), as azeitonas de mesa podem ser classificadas quanto à sua forma de apresentação em:

. **Azeitonas inteiras –** frutos com a conformação natural, não descaroçados, com ou sem pedúnculos.

. **Azeitonas retalhadas –** frutos inteiros, mas com a polpa golpeada.

. **Azeitonas descaroçadas –** frutos com a conformação natural aos quais foi retirado o caroço.

. **Azeitonas recheadas –** frutos descaroçados e com recheio.

. **Azeitonas recheadas em metades –** frutos recheados e cortados em duas partes aproximadamente iguais.

. **Azeitonas recheadas em rodelas –** frutos recheados e cortados em fatias sensivelmente com a mesma espessura.

. **Azeitonas em pedaços –** frutos descaroçados e cortados em bocados.

. **Outras formas –** frutos que correspondem às especificações da Norma, mas com apresentação diversa das anteriores.

1.2.3. Métodos de preparação

A azeitona é uma drupa ovalada de cor verde que passa a violácea ou preto quando madura. 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 variedade e do estado de maturação do fruto, ronda os 20% em peso fresco (Bianchi, 2003). Este fruto, tal como é recolhido da árvore, não se encontra apto para ser consumido, sendo necessário uma série de modificações para reduzir o seu amargor e o torne edível (Jimenéz et al., 1995; Ciafardini et al., 2005). O amargor é devido ao elevado teor em compostos fenólicos, especialmente a oleuropeína (Ryan & Roberts, 1998), que actuam também como inibidores de algumas leveduras que realizam a fermentação láctica.

São vários os métodos utilizados no processamento da azeitona de mesa, sendo os mais comuns a fermentação ao natural, o método Sevilhano ("estilo Espanhol") e o método Californiano ("estilo Americano"). Os diferentes tipos de tratamento têm por objectivo produzir no fruto uma série de transformações, consequência de processos físico-químicos e microbiológicos, que o fazem adquirir as características organolépticas de cor, sabor e textura tão apreciadas universalmente, permitindo

simultaneamente a sua conservação por um espaço de tempo prolongado, em condições óptimas para a sua posterior comercialização (Bianchi, 2003).

Para a preparação de **azeitonas de fermentação natural**, os frutos colhidos são transportados para a unidade industrial, onde são escolhidos e calibrados. Posteriormente as azeitonas são lavadas com água para remover a sujidade superficial e colocadas em salmoura com uma concentração de sal entre 6% a 10%, ou ligeiramente inferior (Nychas et al., 2002). A fermentação é conduzida predominantemente por leveduras (Nout & Rombouts, 2000) e é um processo lento devido simultaneamente à presença da oleuropeína e à lenta difusão dos açúcares e de outros compostos solúveis dos frutos para a salmoura. De acordo com o estado de maturação do fruto, podem originar azeitonas pretas ao natural, quando as azeitonas que lhe deram origem são colhidas em estado de maturação avançado e as azeitonas se encontravam todas negras, ou azeitonas mistas ao natural quando o seu estado de maturação não era homogéneo nem se encontrava em estado tão avançado. No caso das azeitonas pretas ao natural, no fim do processo de fermentação são expostas ao ar para haver uma melhoria da cor. Após estes processos as azeitonas são classificadas por tamanho e embaladas em nova salmoura podendo ou não sofrer fermentação (Fernández et al., 1997).

Nas **azeitonas verdes**, também denominadas azeitonas verdes curadas em salmoura, azeitonas verdes **estilo Sevilhano ou Espanhol**, as azeitonas são colhidas quando atingem uma cor verde-amarelada, e submetidas a um tratamento anaeróbio com hidróxido de sódio (NaOH) durante umas horas. A concentração de NaOH a usar varia consoante a temperatura, a cultivar e o grau de maturação dos frutos (Fernández et al., 1997). A solução alcalina é eliminada e as azeitonas são lavadas repetidamente com água, que remove parte da oleuropeína e os seus produtos de hidrólise, outros polifenóis e alguns dos açúcares fermentáveis (Fernández et al., 1997). De seguida, os frutos são colocados em salmoura, contendo 7% a 10% de NaCl, e sofrem uma fermentação láctica (Asehraou & Faid, 1993; Nout & Rombouts, 2000). Uma vez fermentadas, as azeitonas são seleccionadas e classificadas por tamanhos para serem embaladas como inteiras, descaroçadas ou recheadas com diversos ingredientes, podendo ou não ocorrer uma pasteurização.

As **azeitonas pretas oxidadas**, ou também conhecidas por **estilo Californiano ou Americano** são azeitonas já em estado de maturação adiantado (mistas ou pretas) submetidas a tratamento aeróbico com NaOH para eliminação dos compostos amargos e

uniformização da cor. Antes do processamento as azeitonas podem, se necessário, ser conservadas vários meses sob condições anaeróbias numa salmoura. No entanto, durante este período ocorre fermentação, sobretudo devido ao crescimento de leveduras (Bianchi, 2003). As azeitonas são tratadas com três a cinco soluções de hidróxido de sódio (1% a 2%), por períodos de tempo variável, para conseguir uma penetração progressiva do NaOH desde a polpa até ao caroço (Fernández et al., 1997). No fim de cada tratamento alcalino as azeitonas são lavadas com água, à qual é injectado ar sob pressão. Este tratamento alcalino aeróbio permite o escurecimento progressivo tanto da pele como da polpa do fruto. Depois de obtida a cor desejada continuam a ser lavadas e arejadas até se atingir aproximadamente um pH 8 (Marsilio et al., 2001). Posteriormente, é adicionado à última água de lavagem, 0,1% de gluconato ferroso para estabilizar a cor alcançada na oxidação. Finalmente as azeitonas são escolhidas e classificadas de acordo com o calibre dos frutos. As azeitonas são embaladas numa salmoura contendo 3% de NaCl, e como o produto final, independentemente da sua forma de apresentação, é um alimento de baixa acidez, deve ser conservado mediante esterilização (Fernández et al., 1997).

1.3. Composição química

A composição química e as propriedades físicas da azeitona são factores importantes que determinam a qualidade do produto final, e são fortemente influenciados pela cultivar, condições climáticas, estado de desenvolvimento e maturação do fruto na altura da colheita. O processamento tecnológico, nomeadamente o tratamento alcalino e a fermentação, provocam alterações físico-químicas na azeitona de mesa, afectando os seus constituintes lipídicos, fenóis, açúcares e sais.

A polpa da azeitona de mesa é maioritariamente constituída por a água, de 70 a 75%, e uma fracção lipídica que varia entre 14 a 15% nas azeitonas verdes e é cerca de 30% do peso da polpa nas azeitonas pretas (Fernández et al., 1997; Conde et al., 2008; Sakouhi et al., 2008). Apresenta um teor relativamente baixo em açúcares (2-5%) e proteína (3%) sendo o restante constituído por fibra e cinzas (Conde et al., 2008). O conteúdo em hidratos de carbono inclui ainda polissacarídeos e substâncias pécticas, determinantes na qualidade textural da polpa da azeitona. Durante o processamento e

armazenamento da azeitona, as substâncias pécticas são hidrolisadas por enzimas pectinolíticas, o que provoca uma diminuição da firmeza do fruto (Bianchi, 2003).

A fracção lipídica das azeitonas de mesa apresenta uma composição similar à do azeite, predominando os ácidos gordos monoinsaturados, com o ácido oleico como maioritário, e um teor em ácido gordos saturados que não ultrapassam 15% do total de lipidos (Conde et al., 2008; Sakouhi et al., 2008). Na sua maioria os ácidos gordos encontram-se na forma de triglicéridos e a quantidade em ácidos gordos livres é, de maneira geral, baixa (Bianchi, 2003). Dissolvidos nesta fracção detectaram-se também compostos com actividade antioxidante e vitamínica, como sejam os tocoferóis, sendo o α-tocoferol, o vitâmero maioritário (Montaño et al., 2005; Sakouhi et al., 2008).

O principal açúcar livre presente na polpa é a glucose (1 a 3% do peso da polpa) seguido da frutose (0,1 a 1,1%) e pequenas concentrações de xilose, sacarose e manitol (Nout & Rombouts, 2000). Os açúcares solúveis diminuem ao longo do processo de maturação do fruto, e constituem a principal fonte de energia e carbono para os microrganismos envolvidos na fermentação da azeitona de mesa (Fernández et al., 1997; Nout & Rombouts, 2000).

Em termos de ácidos orgânicos, os ácidos oxálico, succínico, málico e cítrico existem numa percentagem entre 1,2 a 2,1% do peso da polpa em matéria seca, e a sua concentração aumenta até à maturação, momento em que estabilizam (Bianchi, 2003).

As azeitonas contêm uma grande variedade de compostos fenólicos, com um papel importante nas propriedades químicas, organolépticas e nutricionais do azeite virgem e das azeitonas de mesa (Rodríguez et al., 2009). Os polifenóis ou fenóis livres e os seus glucósidos representam 1 a 3% do peso da azeitona. As classes mais importantes de compostos fenólicos em azeitonas de mesa, incluem ácidos fenólicos, os álcoois fenólicos, flavonóides e secoiridoides (Ryan & Roberts, 1998; Brenes et al., 1999; Soler-Rivas et al., 2000; Owen et al., 2000; Owen et al., 2003; Vinha et al., 2005). O perfil fenólico da azeitona de mesa é muito complexo e pode variar tanto na qualidade como na quantidade de compostos fenólicos (Uccella, 2001), dependendo dos métodos de processamento (Romero et al., 2004), da cultivar (Esti et al., 1998; Brenes et al., 1999; Vinha et al., 2005), do clima (Salvador et al., 2001), dos regimes de rega (Romero et al., 2002) e do grau de maturação do fruto (Gutiérrez et al., 1999; Morelló et al., 2003; Conde et al., 2008).

Os principais polifenóis encontrados nas azeitonas não processadas são o secoiridoide oleuropeína, o hidroxitirosol e tirosol, sendo o primeiro o mais abundante (Uccella, 2001; Bianchi, 2003; Blekas et al., 2002; Conde et al., 2008). A oleuropeína é a responsável pelo sabor amargo das azeitonas, e a sua expressão na azeitona vai diminuindo ao longo da maturação (Ryan & Robards, 1998). Paralelamente o teor de hidroxitirosol vai aumentando, torna-se o composto fenólico maioritário nas azeitonas maduras (Romero et al., 2002). Também foram identificados os fenóis verbascósido, rutina (flavonóide), luteolina 7-*O*-glucósido (flavonóide), e as antocianinas cianidina 3-glucósido e cianidina 3-rutinósido. Apesar destes fenóis estarem presentes em quase todas as cultivares de azeitona, cada cultivar tem um perfil fenólico característico (Esti et al., 1998). Durante o processamento da azeitona os fenóis sofrem transformações químicas, e de um modo geral a sua concentração na azeitona diminui. Estes compostos fenólicos desempenham um papel importante na fermentação devido às suas propriedades antimicrobianas, especialmente contra bactérias lácticas e no desenvolvimento da cor do fruto durante a maturação e no processo de escurecimento das azeitonas pretas oxidadas (Nout & Rombouts, 2000).

1.4. Actividade biológica

1.4.1. Actividade antioxidante

Durante o metabolismo celular são formadas espécies reactivas de oxigénio que quando presentes em concentrações elevadas têm acção tóxica no organismo humano (Satoh et al., 2005). As células possuem defesas intracelulares, nomeadamente enzimas (superóxido dismutase, catalase ou glutationa peroxidase) para as proteger contra níveis excessivos de radicais livres. Uma protecção adicional pode ser fornecida com a adição exógena de compostos tais como vitaminas (A, E, β-caroteno), minerais (selénio, zinco) ou proteínas (transferrina, albumina) (Ostrovidov et al., 2000), ácido lipóico, carotenóides (β-caroteno) e flavonóides (ex. apigenina e luteolina) entre outros (Valko et al., 2007).

Alguns produtos naturais possuem na sua composição compostos com actividade antioxidante que podem ser úteis no auxílio do sistema protector endógeno, podendo ser

utilizados como nutracêuticos (Kanter, 1998). Nessa perspectiva, os antioxidantes presentes na nossa dieta assumem uma grande importância como possíveis agentes protectores que ajudem a corpo humano na redução dos danos oxidativos. Os fitoquímicos são classificados como compostos bioactivos provenientes de diferentes partes das plantas, tais como, sementes, cereais, vegetais, frutos, folhas, raízes, especiarias e ervas (Ramarathnam et al., 1995, Skerget et al., 2005). Os polifenóis também pertencem à categoria dos fitoquímicos e são os antioxidantes mais abundantes da nossa dieta (Visioli et al., 2002). A sua capacidade antioxidante é importante para a nutrição humana porque contribuem para o aumento da resistência celular ao stress oxidativo (Saija & Ucella, 2001). Muitos estudos referem o papel protector dos fenóis na oxidação de lipoproteínas de baixa densidade (Andrikopoulos et al., 2002) e nas alterações oxidativas devidas aos radicais livres e outras espécies reactivas (Soler-Rivas et al., 2000). De facto, os polifenóis são benéficos para a saúde humana, estando a sua actividade antioxidante associada a menores incidências de doença coronária (Keys, 1995; Simopoulos, 2001; Tapiero et al., 2002; Trichopoulou & Legiou, 1997), de certos tipos de cancro (Kris-Etherton et al., 2002; Simopoulos, 2001; Trichopoulou & Legiou, 1997) e de processos inflamatórios (Tapiero et al., 2002; Trichopoulou & Legiou, 1997).

A crescente preocupação dos consumidores com a sua saúde e qualidade de vida, bem como algumas restrições legais em alguns países (Paradiso et al., 2009), tem levado a que as diversas pesquisas científicas se concentrem na possibilidade de reduzir o uso de antioxidantes químicos, substituindo-os por compostos de origem natural, principalmente na indústria alimentar (Laguerre et al., 2007). Tem-se assim verificado um interesse crescente pelos produtos e subprodutos da azeitona, devido principalmente às suas propriedades antioxidantes. Nestes incluem-se os carotenóides, tocoferóis e compostos fenólicos que, ao agirem por diferentes mecanismos, complementam-se numa defesa eficaz contra as espécies reactivas (Morelló et al., 2005). Apesar de existirem alguns estudos sobre a composição quantitativa e qualitativa dos fenóis da azeitona de mesa (Blekas et al., 2002; Romero et al., 2002; Pereira et al., 2006), há pouca informação disponível acerca do potencial antioxidante destes compostos da azeitona.

Owen et al. (2003) investigaram a capacidade antioxidante de dois tipos de azeitona de mesa Italianas em salmoura, uma preta e outra verde, pelo método da hipoxantina/xantina oxidase, e concluíram que as azeitonas pretas, com concentrações

mais elevadas de compostos fenólicos, apresentavam maior actividade antioxidante que as azeitonas verdes.

Boskou et al. (2006) determinaram a capacidade antioxidante total de cinco variedades de azeitona de mesa Gregas, usando o método do DPPH (2,2-difenil-1-picrilhidrazil) e verificaram que estas são uma boa fonte de polifenóis, cuja quantidade varia de acordo com o tipo de azeitona, e que o conteúdo quantitativo e qualitativo destes em cada tipo de azeitona de mesa diferencia-se da capacidade antioxidante total.

Em azeitonas de mesa portuguesas, Pereira et al. (2006) demonstraram que o processamento das azeitonas influi na sua capacidade antioxidante. Por outro lado, azeitonas com Denominação de Origem Protegida (DOP) de fermentação natural demonstraram possuir maior poder antioxidante que as azeitonas pretas oxidadas.

1.4.2. Actividade antimicrobiana

Nas últimas décadas têm-se multiplicado os casos de resistência de microrganismos aos antibióticos. Esta situação, a par do uso generalizado de drogas imunossupressoras e do aumento de infecções fúngicas, justificam a necessidade de descobrir e/ou desenvolver novos agentes antimicrobianos. A maior parte destes estudos estão a incidir nas propriedades antimicrobianas de princípios activos de derivados de plantas, tais como, especiarias e óleos essenciais, que têm vindo a ser utilizados desde sempre na medicina tradicional para tratar infecções (Cowan, 1999).

A oliveira tem sido também alvo de estudos para pesquisa de agentes com acção antimicrobiana. De uma maneira geral, essa acção encontra-se relacionada com o elevado teor de compostos fenólicos. Este grupo de compostos, para além das propriedades antioxidantes comprovadas, tem demonstrado possuir actividade antimicrobiana. Os compostos fenólicos existem nos diferentes órgãos da planta, especialmente nas folhas e frutos, e nos produtos daí resultantes, como sejam a azeitona de mesa e o azeite. (Hoult & Payá, 1996; Ryan & Robards, 1998; Rauha et al., 2000; Puupponen-Pimia et al., 2001; Proestos et al., 2005; Pereira et al., 2006, 2007).

Vários são os trabalhos desenvolvidos sobre a actividade antimicrobiana em produtos do olival, nomeadamente com folhas de oliveira (Markin et al., 2003; Pereira et al., 2007), com os frutos (Nychas et al., 1990), com as azeitonas de mesa (Pereira et

al., 2006; Medina et al., 2007) e produtos da sua fermentação (Medina et al., 2007) com as águas residuais (Capasso et al., 1995), azeite (Romero et al., 2007), águas russas (Ramos-Comenzana et al., 1996) e compostos isolados, tais como a oleuropeína (Bisignano et al., 1999; Furneri et al., 2002), o hidroxitirosol (Bisignano et al., 1999) e os aldeídos alifáticos (Battinelli et al., 2006).

Pereira et al. (2006) estudaram a actividade antimicrobiana de azeitonas de mesa portuguesas e verificaram que os extractos destas azeitonas inibiam o crescimento das bactérias *Bacillus cereus*, *Bacillus subtillis*, *Staphylococcus aureus*, *Escherichia coli* e *Klebsiella pneumoniae*.

Bisignano et al. (1999) demonstraram que o hidroxitirosol e a oleuropeína inibem ou atrasam o crescimento de uma vasta gama de bactérias e fungos (Soni et al., 2006), como *Haemophilus influenzae*, *Moraxella catarrhalis*, *Salmonella typhi*, *Vibrio parahaemolyticus* e *Staphylococcus aureus*. A oleuropeína, também inibe o crescimento de *Mycoplasma hominis*, *Mycplasma fermentans*, *Mycplasma pneumoniae* e *Mycplasma pirum* (Furneri et al., 2002).

Os aldeídos hexanal, nonanal, (E)-2-hexenal, (E)- 2-heptenal, (E)-2-octenal e (E)-2-nonenal, existentes na azeitona revelaram actividade antifúngica contra *Trycophytum mentagrophytes*, *Microsporum canis* e *Candida* spp (Battinelli et al., 2006).

Os compostos fenólicos da azeitona de mesa que exibem actividade antimicrobiana contra bactérias e fungos, podem ser usados como aditivos alimentares alternativos em detrimento de compostos químicos.

1.5. Referências bibliográficas

Andrikopoulos, N. K.; Kaliora, A.C.; Assimopoulou, A. N. & Papageorgiou, V. P. (2002). Biological activity of some naturally occurring resins, gums and pigments against *in vitro* LDL oxidation. *Phytotherapy Research*, *17*, 501-507.

Anónimo, (1987). Codex Standard for Table Olives (Codex Stan 66-1981 (Rev. 1-1987)). Codex Alimentarius.

Anónimo (2004). Trade Standard Applying to Table Olives. International Olive Oil Council COI/OT/NC nº. 1, Dezembro de 2004.

Asehraou, A. & Faid, M. (1993). Etude physico-chimique et microbiologique des olives vertes désamérisées en fermentation. *Actes Institute Agronomique et Veterinaire*, *13*, 21-26.

Battinelli, L.; Daniele, C.; Cristiani, M.; Bisignano, G.; Saija, A. & Mazzanti, G. (2006). In vitro antifungal and anti-elastase activity of some aliphatic aldehydes from *Oleae europaea* L. fruit. *Phytomedicine*, 13, 558-563.

Bianchi, G. (2003). Lipids and phenols in table olives. *European Journal of Lipid Science and Technology*, *105*, 229-242.

Bisignano, G.; Tomaino, A.; Lo Cascio, R.; Crisafi, G.; Uccelle, N. & Saija, A. (1999). On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol. The *Journal of Pharmacy and Pharmacology*, *51*, 971-974.

Blekas, G.; Vassilakis, C.; Harizanis, C.; Tsimidou, M. & Boskou, D. G. (2002). Biophenols in table olives. *Journal of Agricultural and Food Chemistry, 50*, 3688-3692.

Boskou, G.; Salta, F. N.; Chrysostomou, S.; Mylona, A.; Chiou, A. & Andrikopoulos, N. K. (2006). Antioxidant capacity and phenolic profile of table olives from the Greek market. *Food Chemistry*, *94*, 558-564.

Brenes, M.; García, A.; García, P.; Rios, J. J. & Garrido, A. (1999). Phenolic compounds in Spanish olive oils. *Journal of Agricultural and Food Chemistry*, *47*, 3535-3540.

Capasso, R.; Evidente, A.; Schivo, L.; Orru, G.; Marcialis, M. A. & Cristinzio, G. (199). Antibacterial polyphenols from olive oil mill waste waters. *Journal of Applied Bacteriology*, *79*, 393-398

Ciafardini, G.; Zullo, B. A. & Cioccia, G. (2005). Effectof lipase producing yeast on the oily fraction of microbiologically debittered table olives. *European Journal of Lipid Science and Technology, 107*, 851–856

Conde, C.; Delrot, S. & Gerós, H. (2008). Physiological, biochemical and molecular changes occurring during olive development and ripening. *Journal of Plant Physiology*, *165*, 1545-1562.

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Review*, *12*, 564-582.

Esti, M.; Cinquanta, L. & La Notte, E. (1998). Phenolic compounds in different olive cultivars. *Journal of Agricultural and Food Chemistry, 46*, 32-35.

Fernández, A. G.; Díez, M. J. F. & Adams, M. R. (1997). *Table olives. Production and processing*. Chapman & Hall. London, UK.

Furneri, P. M.; Marino, A.; Saija, A.; Uccella, N. & Bisignano, G. (2002). In vitro antimycoplasmal activity of oleuropein. *International Journal of Antimicrobial Agents*, *20*, 293-296.

Gutiérrez, F.; Jímenez, B.; Ruíz, A. & Albi, M. A.(1999). Effect of olive ripeness on the oxidative stability of virgin olive oil extracted from the varieties Picual and Hojiblanca and on the different components involved. *Journal of Agricultural and Food Chemistry*, *47*, 121–127.

Hoult, J. & Payá, M. (1996). Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential. *General Pharmacology, 27*, *4*; 713-722.

Jimenéz, A.; Guillén, R.; Sánchez, C.; Fernández-Bolaños, J. & Heredia, A. (1995). Changes in texture and cell wall polysaccharides of olive fruit during "spanish green olive" processing. *Journal of Agricultural and Food Chemistry*, *43*, 2240-2246,

Kanter, M. (1998). Free radicals, exercise and antioxidant supplementation. *Proceedings of Nutrition Society*, *57*, 9-13.

Keys, A. (1995). Mediterranean diet and public health: personal reflections. *American Journal for Clinical Nutrition*, *61*, 1321-1323.

Kris-Etherton, P. M.; Hecker, K. D.; Bonanome, A.; Coval, S. M.; Binkoski, A. E. & Hilpert, K. F. (2002). Bioactive compounds in foods: Their role in the prevention of cardiovascular disease and cancer. *The American Journal of Medicine*, *113* (9B), 71-88.

Laguerre, M., Lecomte, J., Villeneuve, P. (2007). Evaluation of the ability of antioxidants to counteract lipid oxidation: Existing methods, new trends and challenges. *Progress in Lipid Research*, *46*, 244-282.

Markin, D.; Duek, L. & Berdicevsky, I. (2003). *In vitro* antimicrobial activity of olive leaves. *Mycoses*, *46*, 132-136.

Marsilio, V.; Campestre, C. & Lanza, B. (2001). Phenolic compounds change during California-style ripe olive processing. *Food Chemistry*, *74*, 55-60.

Medina, E.; Brenes, M.; Romero, C.; Garcia, A. & Castro, A. (2007). In vitro activity of olive oil polyphenols against *Helicobacter pyroli*. *Journal of Agricultural and Food Chemistry, 55*, 680-686.

Montaño, A.; Casado, F. J.; Castro, A.; Sánchez, A. H. & Rejano, L. (2005). Influence of processing, storage time, and pasteurization upon the tocopherol and amino acid contents of treated green table olives. *European Food Research and Technology*, *220*, 255-260.

Morelló, J.R.; Motilva, M.J.; Ramo, T. & Romero, M.P. (2003). Effect of freeze injuries in olive fruit on virgin olive oil composition. *Food Chemistry, 81*, 547-553.

Morelló, J.R.; Vuorea, S.; Romero, M.P.; Motilva, M.J. & Heinonen, M. (2005). Antioxidant Activity of Olive Pulp and Olive Oil Phenolic Compounds of the Arbequina Cultivar. *Journal of Agricultural and Food Chemistry, 53***,** 2002-2008.

Norma Portuguesa (NP) 3034, 1987. *Derivados de frutos e de produtos hortícolas. Azeitonas de mesa. Definição, classificação, características, acondicionamento e marcação.*

Nout, M. J. R. & Rombouts, F. M. (2000). Fermented and Acidified Plant Foods, *in* Lund, B. M., Baird-Parker, T. C., Gould, G. W. *The Microbiological Safety and Quality of Food*. Volume I. Aspen Publishers, Inc. Gaithersburg, Maryland.

Nychas, G. J. E.; Panagou, M. L.; Waldron, K. W. & Tassou, C. C. (2002). Microbial colonization of naturally black olives during fermentation and associated biochemical activities in the cover brine. *Letters in Appplied Microbiology*, *34*, 173-177.

Nychas, G. J. E.; Tassos, S. C. & Board, R. G. (1990). Phenolic extract from olives: inhibition of *Staphylococcus aureus*. *Letters in Applied Microbiology*, *13*, 217-220.

Ostrovidov, G.; Franck, P.; Joseph, D.; Martarello, L.; Kirsch, G.; Belleville, F.; Nabet, P. & Dousset, B. (2000). Screening of new antioxidant molecules using flow cytometry. *Journal of Medicinal Chemistry*, *43,* 1762-1769.

Owen, R. W.; Haubner, R.; Mier, W.; Giacosa, A.; Hull, W. E.; Spiegelhalder, B. & Bartsch, H. (2003). Isolation, structure elucidation and antioxidant potential of the major phenolic and flavonoid compounds in brined olive drupes. *Food and Chemical Toxicology*, *41*, 703-717.

Owen, R. W.; Mier, W.; Giacosa, A.; Hull, W. E.; Spiegelhalder, B. & Bartsch, H. (2000). Phenolics compounds and squalene in olive oils: the concentration and antioxidant potencial of total phenols, simple phenols, secoiridoids , lignans and squalene. *Food and Chemical Toxicology.*, *38*, 647-659.

Paradiso, V., Summo, C., Pasqualone, A. & Caponio, F. (2009). Evaluation of different natural antioxidants as affecting volatile lipid oxidation products related to off-flavours in corn flakes. *Food Chemistry*, *113*, 543-549.

Pereira, A. P.; Ferreira, I. C. F. R.; Marcelino F.; Valentão P.; Andrade, P. B., Seabra R.; Estevinho, Letícia; Bento A. & Pereira, J. A. (2007). Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L. *Cv*. Cobrançosa) leaves. *Molecules, 12*, 1153-1162.

Pereira, J. A.; Pereira, A.P.; Ferreira, I.C.F.R.; Valentão, P.; Andrade, P.; Seabra, R.; Estevinho, L. & Bento, A. (2006). Phenolics compounds, antioxidant potential and microbial activity of table olives from Portugal. *Journal of Agriculture and Food Chemistry, 54*, 8425-8431.

Proestos, C.; Chorianopoulos, N.; Nychas, G.-J. E. & Komaitis, M. (2005). RP-HPLC analysis of the phenolic compounds of plant extracts. Investigation of their antioxidant capacity and antimicrobial activity. *Journal of Agriculture and Food Chemistry*, *53*, 1190-1195.

Puupponen-Pimiä, R.; Nohynek, L.; Meier, C.; Kähkönen, M.; Heinonen, M.; Hopia, A. & Oksman-Caldentey, K. M. (2001). Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology*, *90*, 494-507.

Ramarathnam, N.; Osawa, T.; Ochi, H. & Kawakishi, S. (1995). The contribution of plant food antioxidants to human health. *Trends in Food Science and Technology*, *6*, 75-82.

Ramos-Comenzana, A.; Juárez-Jiménez, B. & Garcia-Pareja, M.P. (1996). Antmicrobial activity of olive mill waste-waters (alpechin) and biotransformed olive oil mill wastewater. *International Biodeterioration & Biodegradation, 38*, 283-290

Rauha, J.-P.; Remes, S.; Heinonen, M.; Hopia, A.; Kähkönen, M.; Kujala, T.; Pihlaja, K.; Vuorela, H. & Vuorela, P. (2000). Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology, 56,* 3-12.

Rodríguez, G.; Lama, A.; Trujillo, M.; Espartero, J. L. & Fernández-Bolañosa, J. (2009). Isolation of a powerful antioxidant from *Olea europaea* fruit-mill waste: 3,4-Dihydroxyphenylglycol. *LWT - Food Science and Technology*, *42*, 483–490.

Romero, C.; Brenes, M.; Yousfi, K.; Garcia, P.; Garcia, A. & Garrido, A. (2004). Effect of cultivar and processing method on the contents of polyphenols in table olives. *Journal of Agricultural and Food Chemistry*, *52*, 479-484.

Romero, C.; Medina, E.; Vargas, J.; Brenes, M. & Castro, A. (2007). Main antimicrobial compounds in table olives. *Journal of Agricultural and Food Chemistry, 55*, 9817-9823.

Romero, M. P.; Tovar, M. J.; Girona, J. & Motilva, M. J. (2002). Changes in the HPLC phenolic profile of virgin olive oil from young trees (*Olea europaea* L. *Cv. Arbequina*) grown under different deficit irrigation strategies. *Journal of Agricultural and Food Chemistry*, *50*, 5349-5354.

Ryan. M. & Robards, K. (1998). Phenolic compounds in olives. *Analyst, 123*, 31R-44R

Saija, A. & Ucella, N. (2001). Olive biophenols: functional effects on human wellbeing. *Trend in Food and Science & Technology*, *11*, 357-363.

Sakouhi, F.; Harrabi, S.; Absalon, C.; Sbei, K.; Boukhchina, S. & Kallel, H. (2008). α-Tocopherol and fatty acids contents of some Tunisian table olives (*Olea europea* L.): Changes in their composition during ripening and processing. *Food Chemistry, 108*, 833-839

Salvador, M. D.; Aranda, F. & Fregapane, G. (2001). Influence of fruit ripening on "Cornicabra" virgin olive oil quality. A study of four successive crop seasons. *Food Chemistry*, *73*, 45–53.

Satoh, E.; Tohyama, N. & Nishimura, M. (2005). Comparison of the antioxidant activity of roasted tea with green, oolong, and black teas. *International Journal of Food Sciences and Nutrition, 56*, 551-559.

Simopoulos, A. (2001). The Mediterranean Diets: What is so important about the diet of Greece? The scientific evidence. *Journal of Nutrition*, *131,* 3065S-3073S.

Skerget, M.; Kotnik, P.; Hadolin, M.; Hras, A.R.; Simonic, M. & Knez, Z. (2005). Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chemistry*, *89*, 191-198.

Soler-Rivas, C.; Espín, J. C. & Wichers, H. J. (2000). Oleuropein and related compounds. *Journal of the Science of Food and Agriculture*, *80*, 1013-1023.

Soni, M.G.; Burdock, G.A.; Christian, M.S.; Bitler, C.M. & Crea, R. (2006). Safety assessment of aqueous olive pulp extract as an antioxidant or antimicrobial agent in foods. *Food and Chemical Toxicology, 44,* 903–915.

Tapiero, H.; Tew, K. D.; Nguyen Ba, G. & Mathé, G. (2002). Polyphenols: do they play a role in the prevention of human pathologies? *Biomedicine & Pharmacotherapy*, *56*, 200-207.

Trichopoulou, A. & Legiou, P. (1997). Healthy traditional Mediterranean diet: An expression of culture, history and lifestyle. *Nutrition Reviews*, *55,* 373-389.

Uccella, N. (2001). Olive biophenols: novel ethnic and technological approach. *Trend in Food and Science & Technology*, *11*, 328-339.

Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M.; Mazur M. & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry and Cell Biology*, *39*, 44-84.

Vinha, A. F.; .Ferreres, F.; Silva, B. M.; Valentão, P.; Gonçalves, A.; Pereira, J. A.; Oliveira, M. B. P. P.; Seabra, R. M. & Andrade, P. B. (2005). Phenolic profiles of Portuguese olive fruits (*Olea europaea* L.): influence of cultivar and geographical origin. *Food Chemistry*, *89***,** 561-568.

Visioli, F.; Poli, A. & Galli, C. (2002). Antioxidant and other biological activities of poliphenols from olives and olive oil. *Medicinal Research Reviews*, *22***,** 65-75.

Capítulo 2

Justificação e objectivos

Justificação e objectivos

As azeitonas verdes descaroçadas em pedaços, também chamadas de "*alcaparras*", são um tipo de azeitona de mesa produzido de forma tradicional, na região de Trás-os-Montes. A produção de "*alcaparras*", muito apreciadas pelos consumidores, ocorre no Outono e a sua comercialização é feita nos mercados locais de toda a região.

Inicialmente, a produção deste tipo de azeitonas era vista como um aproveitamento de frutos caídos naturalmente da oliveira iniciando-se a produção de "*alcaparras*" quando a azeitona atingia calibre e polpa suficiente, e que corresponde a de meados a finais de Setembro. Geralmente, a queda dos frutos resultava maioritariamente da saída dos adultos da geração carpófaga da traça-da-oliveira (*Prays oleae* Bern.). Este insecto, que se desenvolve dentro do caroço da azeitona, consumindo a amêndoa, pouco prejudica o desenvolvimento normal do fruto provocando no entanto a sua queda ao sair para o exterior. Neste período o fruto encontra-se praticamente com o tamanho final, dependendo das cultivares em estudo e das condições culturais do olival, contudo não tem uma quantidade de gordura que justifique a sua extracção para azeite.

Actualmente, dada a importância comercial e económica que esta especialidade tem vindo a atingir, para a produção de "*alcaparras*" são recolhidas azeitonas da árvore e a época de produção é mais alargada no tempo não se limitando aos finais de Setembro.

Para o seu processamento, as azeitonas são colhidas verdes, partidas com a ajuda de um martelo, usualmente de madeira, ocorrendo a separação do caroço e da polpa da azeitona que fica quebrada em duas ou mais partes desiguais. A polpa da azeitona é colocada em água que é trocada diariamente até que as azeitonas percam o amargor ou sejam consideradas "doces". Nesta altura é-lhe adicionado sal e são temperadas com laranja, louro, ervas aromáticas, cebola ou alho consoante o gosto de cada um.

Apesar de ser um produto típico da região de Trás-os-Montes, não eram conhecidos até ao momento quaisquer estudos nesta matriz. Neste sentido, foi objectivo principal deste trabalho contribuir para uma caracterização das azeitonas descaroçadas tipo "*alcaparras*" produzidas na região de Trás-os-Montes. Como uma primeira abordagem para uma eventual protecção de valorização do produto como seja por

exemplo a criação de uma Denominação de Origem Protegida de "Azeitonas Descaroçadas em Pedaços tipo *Alcaparra*".

Os objectivos específicos do trabalho foram:

- Proceder à caracterização nutricional, pela determinação do teor em humidade, teor em gordura bruta, teor em proteína bruta, teor em cinzas e em hidratos de carbono, bem como a avaliação do valor energético de diferentes amostras provenientes da região em três anos consecutivos (Capítulo 3);
- Caracterizar a fracção lípidica em termos de composição em ácidos gordos individuais por GC/FID e tocoferóis por HPLC/FD (Capítulo 3);
- Optimizar da metodologia de extracção de compostos bioactivos para a avaliação do teor em fenóis totais e actividade antioxidante pelos métodos do poder redutor e do efeito bloqueador de radicais livres de DPPH, em diferentes amostras de "*alcaparras*" provenientes da região (Capítulo 4);
- Quantificação de compostos fenólicos, por HPLC/DAD, em extractos aquosos de "*alcaparras*" e avaliar o efeito dos extractos em diferentes microorganismos considerados patogénicos para o homem (Capítulo 5).

Capítulo 3

Chemical characterization and nutritional value of traditional stoned table olives "*alcaparras*" produced in the Northeast of Portugal

Chemical characterization and nutritional value of traditional stoned table olives "*alcaparras*" produced in the Northeast of Portugal

Submitted

Abstract: In the present work the chemical composition and nutritional value of stoned table olives "*alcaparras*" produced and commercialized in Trás-os-Montes region (Northeast of Portugal) were investigated. These olives are largely consumed in winter season and no study concerning their characterization has been performed. For this purpose, during three consecutive years (2004-2006) a total of 30 "*alcaparras*" samples (10 per year) were investigated in what concerns their nutritional characterization (moisture, crude protein, total fat, ash, carbohydrates, and energy), fatty acid composition (GC/FID) and tocopherols content (normal-phase HPLC/FD). Water was their major constituent ranging from 60.4% to 81.0%, followed by fat that varied between 7.6% and 29.3%. The medium amount for carbohydrates was 7.9% and for protein and ash were respectively 1.1% and 3.3%. One hundred grams of "*alcaparras*" table olives presented an average energetic value of 178 kcal. Thirteen fatty acids were detected and quantified, being oleic acid the most abundant (medium value of 77.7±2.0%), followed by palmitic acid (13.0±1.0%), and linoleic acid (3.8±1.7%). The samples showed a total content of tocopherol of extracted oil from 30.7 mg/kg to 106.3 mg/kg, being α-tocopherol the most abundant.

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

3.1. Introduction

In the last decades an increasing interest in olive products has been noticed, not only for olive oil, the finest olive product, but also for table olives. This fact is due to their chemical composition which confers them important biological properties.

Table olives are important constituents of Mediterranean diet. Epidemiological studies corroborate this importance and demonstrate that this kind of diet has significant protective effects on human health and is associated with lower risk of several diseases, including some kinds of cancer (e. g. breast and colon cancers) (Bogani *et al*., 2007) and coronary heart disease (Keys, 1995; Trichopoulou & Lagiou, 1997).

According to the Trade Standard Applying to Table Olives (COI/OT/NC nº1, 2004) table olives are the product "prepared from the sound fruits of varieties of the cultivated olive tree (*Oleae europaea* L.) which are chosen for their production of olives whose volume, shape, flesh-to-stone ratio, fine flesh, taste, firmness and ease of detachment from the stone make them particularly suitable for processing". According to the ripeness stage of the fruit, trade preparation, styles and sizing different kinds of table olives can and should be classified. Stoned halved olives are one of the styles considered by the Trade Standard.

In Portugal, different kinds of table olives are produced by traditional ways. In the Trás-os-Montes region (Northeast of Portugal), the second national producing area, stoned halved olives known as "*alcaparras*" are largely produced by local growers, commercialized in the local market and consumed flavoured with herb spices, onion, garlic, vinegar and olive oil, mostly in winter season. This kind of table olives are processed from green or yellow-green healthy olive fruits, which are broken using a wood hammer, being the pulp and stone separated. The pulp is sliced into two approximately equal parts, perpendicularly to the major axis of the fruit, and placed in water, changed three or four times during a week. This treatment removes the bitterness and the olives can be salty, flavoured and consumed.

Different works studied table olive and the influence of some aspects in its composition, such as trade preparations (Hassapidou et al., 1994; Jiménez et al., 2000;

Marsilio et al., 2001a; Montaño et al., 2005) ripening time (Georget et al., 2001; Sakoui et al., 2008), variety (Jiménez et al., 2000; Marsilio et al., 2001b; Montaño et al., 2003; Sakoui et al., 2008), and agronomical aspects (Marsilio et al., 2006). Table olives are mainly constituted by water followed by fat, carbohydrates, proteins, and ash.

From a nutritional point of view, table olives are well-known sources of compounds with beneficial relevance. These benefits are associated to fatty acid composition, mainly monounsaturated fatty acids (Bianchi, 2003; Ribavora et al., 2003), and to minor constituents, such as tocopherol, phenolic compounds and phytosterols (Montaño et al., 2005; Simopoulos, 2001).

Particular attention was been given to polyphenolic composition (Blekas et al., 2002; Marsilio et al., 2001, Romero et al., 2002; Romero et al., 2004; Pereira et al., 2006). However, few studies are known in respect to table olive tocopherols. Tocopherols are important lipophilic constitutes once they have both antioxidant and vitamin action (Ranalli & Angerosa, 1996). In particular, many studies describe α-tocopherol as having a protective action on human health against different pathologies and contribute to minimize the adverse effects of inflammatory diseases defending the body against free radicals (Cheeseeman & Slater, 1993; Doelman, 1989; Kamal-Eldin & Andersson, 1997). In olive products, tocopherols also contribute to an increased stability against oxidation (Aparicio *et al*., 1999; Deiana *et al*., 2002 Psomiadou & Tsimidou., 2002).

In the last years our research group began the study of "*alcaparras*" table olives. This kind of olives revealed appreciable amounts of total phenolics, 5.58 - 29.88 mg GAE/g (Sousa et al., 2008), being the three flavonoidic compounds luteolin 7-O-glucoside, apigenin 7-O-glucoside, and luteolin identified in aqueous extracts (Sousa et al., 2006). "*Alcaparras*" aqueous extracts revealed inhibition of several microorganisms that may be causal agents of human intestinal and respiratory tract infections (Sousa et al., 2006) and appreciable antioxidant capacity against free radicals (Sousa et al., 2008).

As far as we know no study concerning their fatty acid and tocopherols characterization has been done. Thus, the aim of the present work was to contribute for the primary chemical characterization of "*alcaparras*" table olives produced and commercialized in Trás-os-Montes region (Northeast of Portugal) in respect to chemical and nutritional value. For this purpose, a total of 30 samples, produced in three different

years (2004, 2005 and 2006), were bought at regional markets, and studied for nutritional value, individual fatty acids and tocopherols contents.

3.2. Materials and Methods

3.2.1. Samples

Thirty stoned halved table olives *alcaparras*" samples were studied during the three years of the study (2004-2006). In each year, between October and December, ten samples were obtained in the traditional market. The samples were purchased in different municipalities of Trás-os-Montes region: Bragança, Mirandela, Carrazeda de Ansiães and Macedo de Cavaleiros. In each sample, with approximately 1 kg, the excess of brine was removed and "*alcaparras*" table olives were put in plastic bags, and frozen to -20º C, until the analysis.

3.2.2 Samples preparation

For proximate chemical composition of "*alcaparras*" (moisture, protein, fat, ash), the samples were chopped in a 643 MX mill (Moulinex, Spain).

For oil extraction the samples were further triturated in an Ultra-Turrax (T-25). The olive paste was homogenized and warmed in a water bath (35ºC), during 30 minutes, and the oil extracted by centrifugation (5000 r.p.m./30 minutes) two times. The oil was decanted, filtered in the presence of anhydrous sodium sulphate, and stored at 4ºC in aluminium foil wrapped falcon tubes, until the analyses.

3.2.3. Pulp analysis

Moisture, total fat, ash and protein contents were analyzed in duplicate. Moisture was determined at 100 ± 2º C (~5 g test sample) by AOAC 925.40 method (AOAC, 1995). Total fat content was determined in a Soxhlet apparatus according to AOAC 948.22 method using petroleum ether as solvent with a minimum extraction time of 24 h

(AOAC, 1995). Crude protein content was estimated by the macroKjeldahl method (AOAC 2000) and ash content was determined by incineration at 550±15 ºC until consistent weigh (AOAC 2000). Carbohydrate content was estimated by difference of the other components using the following formula: carbohydrate content = 100% - (% moisture + % protein + % fat + % ash). Energy was expressed as kilocalories, using the factors mentioned in the Portuguese Law (Dec.-Lei nº 167/2004): Energy (kcal) = 4 x (g protein + g carbohydrate) + 9 x (g lipid).

3.2.4. Oil analysis

3.2.4.1. Fatty acids composition

Fatty acids were evaluated as their methyl esters after alkaline transesterification with methanolic potassium hydroxide solution (ISO 5509) and extraction with *n*-heptane. The fatty acid profile was analyzed with a Chrompack CP 9001 Chromatograph equipped with a split-splitless injector, a FID, an autosampler Chompack CP-9050 and a 50m x 0.25 mm i.d. fused silica capillary column coated with a 0.19μ film of CP-Sil 88 (Chrompack). Helium was used as carrier gas at an internal pressure of 120kPa. The temperatures of the detector and injector were 250 and 230, respectively. The split ratio was 1:50 and the injected volume 1μL. The results are expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area (ISO 5508). The fatty acid methyl esters standard mixture (Supelco 37 FAME Mix) was used for identification (Sigma, Spain).

3.2.4.2. Tocopherols composition

Tocopherols were evaluated following the international standard ISO 9936 (2006), with some modifications as implemented by Amaral et al (2005). A 50mg amount of extracted fat was blended with an appropriate amount of internal standard (tocol) in a 1.5 ml volume of n-hexane and homogenized by stirring. Sample preparation was conducted in dark and tubes containing the samples were always wrapped in aluminum

foil. The mixture was centrifuged for 5 minutes at 13000g and the supernatant analyzed by HPLC. The liquid chromatograph consisted of a Jasco integrated system (Japan) equipped with an AS-950 automated injector, a PU-980 pump, an MD-910 multiwavelength diode array detector and an FP-920 fluorescence detector (λ_{exc}= 290 nm and λ_{em}= 330 nm), connected in series. The chromatographic separation was achieved on a Supelcosil ^{TM} LC-SI (3 μm) 75 x 3.0 mm (Supelco, Bellefonte, PA), operating at constant room temperature (21ºC). A mixture of n-hexane and 1,4-dioxane (98:2) was used as eluent, at 0.7 mL/min. Data were analyzed in the Borwin PDA Controller Software (JMBS, France). Tocopherols (α, β, γ, and δ) were identified by chromatographic comparisons with authentic standards, by co-elution and by their UV spectra. Quantification was based on the internal standard method, using the fluorescence signal response.

3.3. Results and discussion

3.3.1. Pulp analysis

Table 1 shows the proximate chemical composition (grams per 100 g of fresh weight) of "*alcaparras*" samples from the three different years of production. Water was the major component, varying from 60.3% to 81.0%, with a medium value of 72.5±5.4%. The total fat amount ranged from 7.6% to 29.3% with marked differences observed between the years of study. In 2004, the medium value was 14.6%, in 2005 was 18.7%, whereas in 2006 this value was lower (12.7%). The differences observed in water and fat content could be attributed to different factors. The major factor is certainly technological, since a higher time of processing in water will increase the water content with a consequent decline in the fat percent. On the other hand, the olive cultivar is the most important feature that influences the amount of fat in olives and is regulated by genetic factors (Di Bella, 2007).

Usually, in "*alcaparras*" processing, the producers don't select specific olive cultivars and this aspect could explain the obtained variability. Also, the amount of water and fat varies along ripening and fat increasing during the maturation process (Gutiérrez et al., 1999). In accordance, table olives processed in September showed

lower fat amount that olives prepared in November. Crude protein laid below 1.7% in all samples (medium value of 1.1±0.3%). Ash content was the parameter presenting higher variations, with values from 1.0 to 8.3%. This fact can be explained by the use of salted water for their preservation. Despite being an important factor for microbial safety, high values are nutritionally unsuitable.

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

Samples	Moisture	Crude protein	Total fat	Ash	Carbohydrates	Energy (kcal)
2004						
1	74.8±0.5	-	13.4±0.0	-	-	-
2	74.5±0.3	-	14.2±0.0	-	-	-
3	78.3±0.5	-	14.3±0.0	-	-	-
4	71.7±1.6	-	17.6±0.0	-	-	-
5	75.2±0.7	-	13.8±0.0	-	-	-
6	66.8±3.3	-	15.7±0.0	-	-	-
7	74.1±1.6	-	13.1±0.0	-	-	-
8	70.1±1.5	-	22.0±0.0	-	-	-
9	72.6±1.5	-	9.1±0.0	-	-	-
10	73.7±1.8	-	12.6±0.0	-	-	-
Average	**73.2±3.1**	-	**14.6±0.0**	**-**	**-**	-
2005						
1	72.1±0.4	0.9±0.0	13.3±1.6	5.6±0.0	8.0±1.8	155.6
2	71.0±2.8	1.4±0.0	21.4±0.2	1.8±0.1	4.4±2.7	215.8
3	73.3±0.9	0.9±0.0	10.4±3.6	3,6±0.0	11.7±2.7	144.3
4	72.2±1.3	0.8±0.0	10.8±0.8	3.1±0.3	13.1±0.3	152.7
5	71.8±0.6	0.8±0.0	13.7±4.6	4.7±1.1	9.1±4.2	162.4
6	70.2±0.9	0.9±0.0	18.5±1.0	3.6±0.5	6.9±0.5	197.0
7	62.5±0.2	1.7±0.0	28.4±4.3	1.8±0.8	5.5±3.4	284.4
8	60.4±0.5	1.5±0.0	29.3±2.1	5.1±0.5	3.2±2.1	282.5
9	60.3±1.6	1.7±0.0	22.8±0.1	2.9±0.1	12.4±1.5	261.0
10	65.7±0.7	1.5±0.0	18.7±0.8	1.7±0.0	12.5±0.1	224.1
Average	**67.5±5.9**	**1.2±0.4**	**18.7±6.8**	**3.4±1.4**	**8.7±3.6**	**212.5±61.8**
2006						
1	77.3±0.5	1.3±0.0	10.5±1.7	2.8±0.1	7.1±1.3	128.1
2	66.7±0.5	1.4±0.0	17.8±0.4	2.3±0.1	11.8±0.9	213.0
3	75.4±1.3	1.0±0.0	15.8±0.2	1.0±0.0	7.7±0.3	177.0
4	76.4±1.6	1.0±0.0	14.2±0.4	1.5±0.1	8.2±0.2	164.6
5	78.1±1.3	0.8±0.0	9.5±0.7	8.3±0.0	4.2±1.6	105.5
6	81.0±1.3	1.0±0.0	7.6±0.2	3.3±0.1	6.9±1.9	100.0
7	78.7±0.3	1.1±0.0	10.8±3.2	4.6±0.1	6.2±2.7	126.4
8	73.3±0.7	1.2±0.0	17.6±0.9	1.2±0.0	6.6±0.7	189.6
9	79.3±1.3	1.0±0.0	9.1±0.2	4.6±0.1	6.7±0.5	112.7
10	77.3±1.3	0.9±0.0	14.3±0.4	2.1±0.0	6.0±0.7	156.3
Average	**76.3±4.0**	**1.1±0.2**	**12.7±3.7**	**3.2±2.2**	**7.1±2.0**	**147.3±38.5**

Carbohydrates represent in medium terms 7.8%. Based on the fat, protein and estimated carbohydrates amounts, an energetic value of 178 kcal was accounted per 100 g of "*alcaparras*" table olives. Nevertheless, high variations are reported in relation to energetic value, ranging from 100 and 284 kcal, related essentially with the quantity of fat in each sample. In a general way the obtained results were in agreement with previous works in diversified table olives.

3.3.2. Fatty acids composition

Table 2 details the fatty acid composition (percentage) for the samples studied within the three years. Oleic acid ($C_{18:1}c$) is the predominant one, ranging from 77.1% to 81.9%. Palmitic acid ($C_{16:0}c$) was the second most abundant fatty acid, followed by linoleic acid, a pattern observed in the three years.

These results are totally in accordance with those regulated for olive oil (European Regulation 2568/91). The composition of fatty acids in the analyzed samples, as expected, showed a similar composition to the olive oils produced in the region (Pereira et al., 2002, 2004). Nevertheless, and despite the homogeneous results presented for most fatty acids within the three years, some samples presented a slightly higher content on linoleic acid, especially higher in sample 4 in 2004.

The high variations occurred between samples were probably dependent on the olive cultivar. The different fatty acid composition, like in fat production, is genetically regulated and depends in low amplitude to the environmental conditions (Di Bella et al., 2007).

The estimation of the different nutritional fractions (Saturated Fatty Acids – SFA; Monounsaturated Fatty Acids - MUFA, Polyunsaturated Fatty Acids - PUFA and *trans* isomers) of fatty acids of "*alcaparras*" samples from different years are presented in Table 3. The MUFA were the major group, mainly due to the contribution of oleic acid, being similar within the three years of study with 72.1% to 81.7% of the fatty acids content. SFA represented less than 18.2% and the medium value for the samples analysed in 2004, 2005 and 2006, was respectively 15.4, 16.4 and 16.5%. Concerning to the PUFA, with the exception of the sample 4 of 2004, their amount represented less

Table 2. Fatty acid composition (percentage) of oil extracted from "*alcaparras*" samples from three different years of production (mean ± SD)

Samples	$C_{14:0}$	$C_{16:0}$	$C_{16:1c}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1c}$	$C_{18:2cc}$	C_{20}	$C_{18:3c}$	$C_{20:1}$	$C_{22:0}$	$C_{24:0}$
2004													
1	0.02±0.00	12.95±0.03	0.99±0.00	0.06±0.01	0.08±0.00	2.24±0.00	79.13±0.02	2.99±0.01	0.19±0.00	0.67±0.00	0.39±0.00	0.16±0.00	0.09±0.00
2	0.02±0.01	12.98±0.03	0.98±0.04	0.07±0.00	0.09±0.00	2.21±0.00	79.35±0.02	2.86±0.00	0.18±0.00	0.63±0.00	0.38±0.00	0.15±0.00	0.08±0.00
3	0.01±0.00	13.58±0.01	0.71±0.00	0.06±0.00	0.08±0.00	2.15±0.00	77.75±0.03	4.11±0.03	0.26±0.01	0.72±0.00	0.34±0.00	0.11±0.00	0.08±0.00
4	0.02±0.00	12.76±0.01	0.83±0.00	0.06±0.00	0.08±0.00	2.66±0.00	70.93±0.01	10.81±0.00	0.34±0.01	0.69±0.00	0.28±0.00	0.13±0.00	0.06±0.00
5	0.02±0.00	12.63±0.01	0.83±0.01	0.07±0.01	0.08±0.00	2.19±0.01	79.28±0.02	3.39±0.03	0.22±0.01	0.69±0.01	0.35±0.00	0.14±0.00	0.08±0.00
6	0.02±0.01	11.93±0.02	0.80±0.00	0.21±0.01	0.32±0.00	2.67±0.00	79.55±0.03	2.97±0.01	0.19±0.01	0.69±0.00	0.33±0.00	0.13±0.01	0.08±0.00
7	0.01±0.00	12.98±0.04	0.99±0.01	0.08±0.00	0.09±0.00	1.97±0.00	79.12±0.06	3.30±0.05	0.21±0.00	0.65±0.00	0.36±0.00	0.12±0.00	0.08±0.00
8	0.02±0.00	12.46±0.01	0.96±0.01	0.08±0.02	0.10±0.01	1.84±0.02	80.46±0.05	2.70±0.03	0.17±0.00	0.59±0.00	0.37±0.00	0.12±0.00	0.07±0.00
9	0.01±0.00	10.01±0.03	0.49±0.00	0.24±0.00	0.34±0.00	2.91±0.01	81.85±0.04	2.80±0.01	0.18±0.00	0.54±0.00	0.32±0.00	0.16±0.00	0.08±0.00
10	0.01±0.00	12.62±0.15	0.78±0.01	0.12±0.00	0.15±0.00	2.82±0.01	76.61±0.07	5.19±0.03	0.33±0.01	0.75±0.00	0.30±0.01	0.19±0.11	0.09±0.04
Average	**0.02±0.01**	**12.49±0.94**	**0.84±0.15**	**0.11±0.07**	**0.14±0.10**	**2.34±0.36**	**78.43±2.80**	**4.11±2.39**	**0.23±0.06**	**0.66±0.06**	**0.34±0.03**	**0.14±0.02**	**0.08±0.01**
2005													
1	0.01±0.00	13.17±0.12	1.24±0.01	0.05±0.00	0.10±0.00	1.99±0.01	78.80±0.09	3.06±0.01	0.37±0.00	0.62±0.00	0.34±0.00	0.11±0.00	0.03±0.03
2	0.02±0.00	12.34±0.15	1.14±0.02	0.16±0.01	0.30±0.01	3.10±0.00	76.84±0.01	4.31±0.18	0.49±0.00	0.73±0.01	0.29±0.01	0.14±0.00	0.04±0.02
3	0.01±0.00	13.73±0.23	1.16±0.00	0.06±0.00	0.10±0.00	2.06±0.04	78.00±0.06	3.18±0.06	0.38±0.01	0.70±0.01	0.33±0.00	0.13±0.01	0.05±0.04
4	0.02±0.00	12.10±0.05	0.97±0.01	0.06±0.00	0.08±0.00	2.84±0.03	77.03±0.77	5.06±0.69	0.46±0.00	0.81±0.06	0.30±0.00	0.14±0.01	0.03±0.02
5	0.02±0.00	13.19±0.02	1.13±0.00	0.05±0.00	0.07±0.00	2.45±0.05	77.57±0.13	3.86±0.06	0.42±0.00	0.68±0.00	0.30±0.00	0.13±0.01	0.03±0.01
6	0.01±0.00	13.44±0.01	0.95±0.01	0.06±0.01	0.08±0.00	2.67±0.04	75.68±0.10	5.58±0.04	0.40±0.00	0.65±0.01	0.25±0.00	0.11±0.01	0.02±0.01

Table 2. Continuation

7	0.01±0.00	13.59±0.09	0.67±0.00	0.06±0.00	0.08±0.00	2.47±0.01	76.57±0.17	4.80±0.08	0.41±0.00	0.77±0.00	0.32±0.00	0.13±0.00	0.03±0.00
8	0.01±0.00	10.84±0.03	0.60±0.00	0.27±0.01	0.40±0.00	3.36±0.04	79.36±0.10	3.15±0.01	0.57±0.01	0.79±0.00	0.31±0.02	0.17±0.00	0.05±0.00
9	0.01±0.00	14.04±0.28	0.81±0.01	0.22±0.00	0.35±0.00	3.24±0.15	76.38±0.73	3.16±0.29	0.50±0.01	0.73±0.01	0.29±0.02	0.14±0.01	0.02±0.00
10	0.01±0.00	12.36±0.00	0.80±0.00	0.16±0.00	0.25±0.00	3.34±0.06	74.85±0.09	6.46±0.02	0.44±0.01	0.86±0.00	0.23±0.00	0.12±0.00	0.02±0.00
Average	**0.01±0.00**	**12.88±0.95**	**0.95±0.21**	**0.12±0.08**	**0.18±0.13**	**2.75±0.51**	**77.11±1.37**	**4.26±1.17**	**0.44±0.06**	**0.73±0.07**	**0.30±0.03**	**0.13±0.02**	**0.03±0.01**
2006													
1	0.07±0.02	14.81±0.13	0.9±0.06	0.08±0.02	0.11±0.00	2.20±0.07	75.38±0.05	4.33±0.04	0.43±0.01	1.02±0.01	0.36±0.00	0.14±0.01	0.16±0.07
2	0.04±0.02	12.25±0.07	1.03±0.01	0.06±0.01	0.12±0.01	1.82±0.02	79.75±0.29	2.85±0.02	0.43±0.01	0.84±0.01	0.42±0.01	0.15±0.00	0.07±0.04
3	0.06±0.03	13.58±0.23	1.15±0.03	0.06±0.01	0.10±0.02	2.35±0.12	78.36±0.87	2.92±0.04	0.49±0.01	0.74±0.01	0.39±0.00	0.17±0.01	0.13±0.04
4	0.11±0.02	13.90±0.08	1.18±0.01	0.06±0.01	0.18±0.10	2.46±0.02	76.76±0.05	3.13±0.16	0.54±0.04	0.79±0.01	0.39±0.02	0.22±0.09	0.19±0.01
5	0.10±0.02	14.51±0.36	1.05±0.02	0.10±0.06	0.12±0.03	2.30±0.02	77.02±0.32	2.72±0.02	0.49±0.01	0.81±0.02	0.38±0.01	0.17±0.03	0.19±0.03
6	0.05±0.02	13.32±0.12	0.97±0.02	0.23±0.01	0.41±0.01	2.77±0.04	76.38±0.11	3.75±0.01	0.50±0.01	0.93±0.02	0.36±0.01	0.14±0.01	0.11±0.03
7	0.04±0.01	13.72±0.17	1.02±0.02	0.06±0.01	0.11±0.01	1.75±0.03	78.88±0.21	2.35±0.05	0.43±0.02	0.97±0.01	0.42±0.01	0.16±0.02	0.13±0.04
8	0.03±0.01	13.74±0.06	1.03±0.03	0.07±0.01	0.12±0.00	1.90±0.04	78.03±0.34	3.02±0.02	0.43±0.02	0.93±0.01	0.4±0.01	0.15±0.01	0.18±0.08
9	0.02±0.00	13.30±0.10	1.11±0.01	0.06±0.00	0.12±0.01	2.18±0.03	77.80±0.23	3.29±0.04	0.49±0.02	0.81±0.01	0.41±0.01	0.17±0.01	0.17±0.07
10	0.03±0.00	14.30±0.07	1.01±0.01	0.05±0.00	0.11±0.00	1.70±0.00	78.32±0.01	2.21±0.01	0.45±0.02	1.02±0.00	0.41±0.00	0.15±0.01	0.20±0.01
Average	**0.05±0.03**	**13.66±0.76**	**1.05±0.08**	**0.08±0.06**	**0.15±0.09**	**2.15±0.34**	**77.79±1.30**	**3.06±0.55**	**0.47±0.04**	**0.88±0.09**	**0.39±0.02**	**0.16±0.03**	**0.14±0.06**

than 5.0%. The *trans* fatty acids had a very limited occurrence (less than 0.14%). With a PFA:SFA ratio near 0.3 and a (MUFA+PUFA):SFA of 5, the "*alcaparras*" lipid fraction in mainly characterized by a high oleic acid content, within that reported for olive oil, exhibiting, therefore, the same nutritional benefits of the former.

Table 3. SFA, MUFA, PUFA and *trans* isomers (%) of "*alcaparras*" samples from three different years of production (mean ± SD).

Samples	SFA	MUFA	PUFA	Trans isomers
2004				
1	15.71±0.01	80.59±0.02	3.65±0.01	0.05±0.00
2	15.68±0.05	80.79±0.05	3.49±0.00	0.04±0.00
3	16.26±0.01	78.87±0.03	4.83±0.02	0.04±0.00
4	16.37±0.01	72.12±0.01	11.46±0.00	0.05±0.00
5	15.34±0.02	80.55±0.02	4.08±0.03	0.04±0.00
6	15.23±0.03	81.06±0.03	3.66±0.02	0.06±0.00
7	15.45±0.05	80.56±0.06	3.95±0.05	0.05±0.00
8	14.77±0.01	81.89±0.04	3.29±0.03	0.06±0.01
9	13.60±0.03	83.00±0.04	3.34±0.01	0.07±0.00
10	16.18±0.09	77.84±0.08	5.94±0.02	0.05±0.00
Average	**15.43±0.77**	**79.75±2.86**	**4.77±2.41**	**0.05±0.01**
2005				
1	15.77±0.09	80.51±0.08	3.69±0.01	0.03±0.00
2	16.33±0.13	78.59±0.04	5.04±0.18	0.04±0.00
3	16.46±0.12	79.63±0.07	3.87±0.05	0.04±0.00
4	15.67±0.02	78.42±0.77	5.88±0.75	0.04±0.01
5	16.32±0.05	79.11±0.12	4.54±0.06	0.04±0.01
6	16.74±0.07	76.99±0.12	6.23±0.04	0.04±0.01
7	16.73±0.09	77.67±0.17	5.57±0.08	0.03±0.00
8	15.32±0.08	80.71±0.07	3.94±0.01	0.03±0.00
9	18.21±0.47	77.85±0.76	3.89±0.29	0.05±0.00
10	16.52±0.08	76.11±0.10	7.32±0.01	0.05±0.00
Average	**16.41±0.78**	**78.56±1.46**	**4.99±1.21**	**0.04±0.01**
2006				
1	17.85±0.12	77.41±0.04	4.69±0.04	0.00±0.00
2	14.27±1.03	81.73±0.29	3.26±0.02	0.04±0.03
3	16.32±0.90	80.35±0.88	3.30±0.04	0.05±0.03
4	17.38±0.40	78.90±0.16	3.59±0.07	0.03±0.02
5	17.94±0.37	79.12±0.27	3.11±0.03	0.03±0.01
6	17.07±0.13	78.69±0.13	4.10±0.02	0.04±0.02
7	16.11±0.08	80.99±0.19	2.77±0.06	0.06±0.01
8	16.49±0.26	80.11±0.31	3.42±0.03	0.14±0.05
9	16.31±0.11	79.83±0.21	3.70±0.05	0.11±0.02
10	16.91±0.02	80.47±0.02	2.62±0.01	0.00±0.00
Average	**16.46±1.25**	**79.89±1.27**	**3.44±0.55**	**0.05±0.05**

3.3.3. Tocopherols contents

Table 4 gives the results obtained for the tocopherols contents. In the first year of sample collection, 2004, the tocopherols analysis were not performed immediately, and the measures taken to conserve the samples were ineffective, resulting in lower amounts when compared with the following years. In 2005 and 2006 the analysis where performed within a short period. Therefore, the values reported for the 2004 samples will not be taken into account for discussion purposes. All samples presented the four tocopherol isomers α, β γ and δ, although this last in vestigial amounts. The more representative was α -tocopherol, again in accordance with the values reported for olive oil.

Table 4. Tocopherol contents (milligrams per kilogram) of oil extracted from "*alcaparras*" samples from two different years of production (mean ± SD).

Samples	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Total
2005					
1	67.4±1.0	1.7±0.2	1.2±0.1	0.3±0.0	70.7±1.2
2	68.7±0.2	0.7±0.0	9.7±0.3	0.4±0.0	79.7±0.2
3	77.1±0.3	1.2±0.0	1.0±0.1	0.1±0.0	79.3±0.4
4	78.5±1.5	1.1±0.2	3.4±0.2	0.1±0.0	83.1±1.8
5	65.8±0.7	1.0±0.1	2.2±0.2	0.1±0.0	69.1±0.9
6	52.5±1.7	1.0±0.1	2.3±0.4	0.1±0.0	55.8±2.1
7	47.8±0.3	1.0±0.1	7.3±0.2	0.3±0.0	56.4±0.5
8	62.5±2.9	0.8±0.1	0.7±0.2	0.1±0.0	64.2±3.2
9	81.5±3.9	1.4±0.2	2.7±0.4	0.1±0.0	85.7±4.3
10	57.4±5.1	1.0±0.1	2.5±0.2	0.2±0.0	60.4±6.1
Average	**65.9±11.0**	**1,0±0.3**	**3.3±2.9**	**0.2±0.1**	**70.4±11.0**
2006					
1	60.8±3.8	7.0±0.5	3.0±0.1	0.3±0.0	71.2±4.2
2	45.0±2.0	1.2±0.1	1.0±0.1	0.4±0.0	47.6±2.1
3	66.3±2.7	0.6±0.0	3.0±0.2	0.1±0.0	70.1±2.8
4	58.1±3.5	1.0±0.1	3.2±0.4	0.1±0.0	62.5±3.9
5	83.3±4.1	0.7±0.0	4.4±0.2	0.3±0.0	88.8±4.3
6	87.7±4.6	1.4±0.1	2.3±0.2	0.1±0.0	91.6±4.9
7	53.9±0.3	1.4±0.0	0.9±0.1	0.3±0.2	56.4±0.1
8	30.7±0.6	1.2±0.0	1.5±0.0	0.2±0.1	33.7±0.5
9	55.7±4.4	0.7±0.0	3.9±0.3	0.5±0.0	60.8±4.6
10	106.3±4.2	1.6±0.2	1.1±0.1	0.6±0.0	109.5±4.4
Average	**64.8±21.6**	**1.7±1.8**	**2.4±1.2**	**0.3±0.2**	**69.2±21.9**

The mean value of total tocopherols content was around 70 mg per kilogram of extracted oil, with values ranging from 33.4mg to 112.7 mg/kg. These values are

generally lower than those reported for olive oil (Pereira et al., 2002). Taking into account the preparation method of the "*alcaparras*" and exposure of the inner fat to oxidation upon removal of the inner stone, a reduction in the tocopherol content is justifiable.

The sample mentioned earlier as containing as unexpected high amount of linoleic acid (2004:4) is also characterized by a higher amount of γ-tocopherol than expected from olive oil alone. This isomer is characteristically higher in other vegetable oils, namely soybean oil (Amaral et al., 2006). The addition of this or other oil with similar composition in terms of tocopherol profile, might also justify the increase in the linoleic acid, characteristically higher in these vegetable oils.

3.4. Conclusion

In conclusion, according to the obtained results we can denote that "*alcaparras*" could have an important nutritional value on our diet, once that they may constitute a good source of healthy fat, with high amount of MUFA, and tocopherols, and a relatively low caloric intake. Some studies suggest that these compounds can be useful in the prevention of diseases in which free radicals are implicated, given that tocopherols have both antioxidant and vitamin action. In addition, and as far as we know, this is the first report considering the chemical characterization and nutritional value of *alcaparras*"; further studies are needed to clarify the role of different olive cultivars and their ripening stage used to make "*alcaparras*" in its phenolic composition and its chemical characterization.

Acknowledgements

The authors are grateful to INTERREG III A Program, Project MOABEPE for financial support of this work.

3.5. References

Amaral, J.; Casal, S.; Alves, M.R.; Seabra, R.M. & Oliveira, B.P.P. (2006). Tocopherol and tocotrienol content of hazelnut cultivars grown in Portugal. *Journal of Agricultural and Food Chemistry*, *54*, 1329-1336.

Amaral, J.S., Casal, S., Oliveira, M.B. & Seabra, R.M. (2005). Development and Evaluation of a Normal Phase Liquid Chromatographic Method for the Determination of Tocopherols and Tocotrienols in Walnuts. *Journal of Liquid Chromatography & Related Technologies*, *28*, 785–795.

AOAC. *Official Methods of Analysis of AOAC International*, 17th ed.; Horwitz, W., Ed.; AOAC: Arlington, VA, 2000; Vol. II, 40, pp 1-3.

AOAC. Official Methods of Analysis, 16th ed.; Association of Official Analytical Chemists: Arlington, VA, 1995.

Aparicio, R.; Roda, L.; Albi, M.A. & Gutiérrez, F. (1999). Effect of various compounds on virgin olive oil stability measured by Rancimat. *Journal of Agricultural and Food Chemistry, 47*, 4150-4155.

Bianchi, G. (2003). Lipids and phenols in table olives. *European Journal of Lipid Science and Technology*, *105*, 229-242.

Blekas, G., Vassilakis, C., Harizanis, C., Tsimidou, M., & Boskou, D.G. (2002). Biophenols in table olives. *Journal of Agriculture and Food Chemistry*, *50*, 3688-3692.

Bogani, P.; Galli, C.; Villa, M. & Visioli, F. (2007). Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. *Atherosclerosis, 190*, 181–186

Cheeseeman, K. H.; & Slater, T. F., 1993. An introduction to free radical biochemistry. *British Medicine Bulletin*, **49**, 481-493.

Commission Regulation nº 2568/91 from 11th July, *Off. J. Eur. Commun*., **1991**, L 248, 1-82.

Deiana, M.; Rosa, A.; Cao, C.F.; Pirisi, F.M.; Bandino, G. & Dessì, M.A. (2002). Novel approach to study oxidative stability of extra virgin olive oils: Importance of α-Tocopherol concentration. *Journal of Agricultural and Food Chemistry*, *50*, 4342-4346.

Di Bella, G.; Maisano, R.; La Pera, L.; Lo Turco, V.; Salvo, F. & Dugo, G. (2007). Statistical characterization of Sicilian olive oils from the Peloritana and

Maghrebian zones according to the fatty acid profile. *Journal of Agricultural and Food Chemistry*, *55*, 6568-6574.

Doelman, 1989. In Anclair, C.; & Emerit, C. J., (Eds.). *Antioxidant therapy and Preventive Medicine,* (p. 9). New York: Plenum Press.

Georget, D.M.R.; Smith, A.C. & Waldron, K.W. (2001). Effect of ripening on the mechanical properties of Portuguese and Spanish varietes of olive (*Olea europaea* L). *Journal of the Science of Food and Agriculture*, *81*, 448-454.

Gutiérrez, F., Jímenez, B., Ruíz, A., & Albi, M.A. (1999). Effect of olive ripeness on the oxidative stability of virgin olive oil extracted from the varieties Picual and Hojiblanca and on the different components involved. *Journal of Agriculture and Food Chemistry*, *47*, 121–127.

Hassapidou, M.N.; Balatsouras, D.D. & Manoukas, A.G. (1994). Effect of processing upon the tocopherol and tocotrienol composition of table olives. *Food Chemistry*, *50*, 111-114.

ISO 5508: 1990. Animal and vegetable fats and oils. Analysis by gas chromatography of methyl esters of fatty acids.

ISO 5509: 2000. Animal and vegetable fats and oils – Preparation of methyl esters of fatty acids.

ISO 9936: 2006. Animal and vegetable fats and oils – Determination of tocopherol and tocotrienol contents by hugh-performance liquid chromatography.

Jiménez, A.; Rodríguez, R.; Fernández-Caro, I.; Guillén, R.; Fernández-Bolaños, J. & Herdedia, A. (2000). Dietary fibre content of table olives processed under different European styles: study of Physico-chemical caracteristics. *Journal of the Science of Food and Agriculture*, *80*, 1903-1908.

Kamal-Eldin, A.; & Andersson, R. A. (1997). Multivariate study of the correlation between tocopherol content and fatty acid composition in vegetable oils. *Journal of American Oil Chemists' Society*, *74*, 375-380.

Keys, A. (1995). Mediterranean diet and public health: Personal reflections. *American Journal of Clinical Nutrition*, *61*, 1321S-1323S.

Marsilio, V., Campestre, C., & Lanza, B. (2001a). Phenolic compounds change during California-style ripe olive processing. *Food Chemistry*, *74*, 55-60.

Marsilio, V.; Campestre, C.; Lanza, B. & De Angelis, M. (2001b). Sugar and polyol compositions of some European olive fruit varieties (*Oleae europaea* L.) suitable for table purposes. *Food Chemistry*, *72*, 485-490.

Marsilio, V.; d`Andria, R.; Lanza, B.; Russi, F.; Iannucci, E.; Labini, A. & Morelli, G. (2006). Effect of irrigation and lactic acid bacteria inoculants on the phenolic fraction, fermentation and sensory characteristics of olive (*Olea europaea* L. cv. Ascolana tenera) fruits. *Journal of the Science of Food and Agriculture*, *86*, 1005-1013.

Montaño, A.; Casado, F. J.; Castro, A.; Sánchez, A. H. & Rejano, L. (2005). Influence of processing, storage time, and pasteurization upon the tocopherol and amino acid contents of treated green table olives. *European Food Research and Technology*, *220*, 255-260.

Montaño, A.; Sánchez, A.H.; Casado, F.J., De Castro, A. & Rejano, L. (2003). Chemical profile of industrially fermented green olives of different varieties. *Food Chemistry*, *82*, 297-302.

Pereira, J. A., Casal, S., Bento, A., & Oliveira, M.B.P.P. (2002). Influence of the olive storage period on oil quality in the portuguese "Cobrançosa", "Madural" and "Verdeal Transmontana" cultivars. *Journal of Agriculture and Food Chemistry*, *50*, 6335-6340.

Pereira, J. A.; Alves, R.; Casal, S. & Oliveira, M.B.P.P. (2004). Effect of olive fruit fly infestation on the quality of olive oil from cultivars Cobrançosa, Madural and Verdeal Transmontana. *Italian Journal of Food Science, 16*, 355-365.

Pereira, J. A.; Pereira, A.P.; Ferreira, I.C.F.R.; Valentão, P.; Andrade, P.; Seabra, R.; Estevinho, L. & Bento, A. (2006). Phenolics compounds, antioxidant potential and microbial activity of table olives from Portugal. *Journal of Agriculture and Food Chemistry, 54*, 8425-8431.

Psomiadou, E. & Tsimidou, M. (2002) Stability of virgin olive oil. 1. Autoxidation studies. *Journal of Agriculture and Food Chemistry*, *50*, 716-721

Ranalli, A. & Angerosa, F. (1996). Integral centrifuges for olive oil extraction. The qualitative characteristics of products. *Journal of the American Oil Chemical Society, 73*, 417-422.

Ribavora, F; Zanev, R.; Shishkov, S. & Rizov, N. (2003). α-Tocopherol, fatty acids and their correlations in Bulgarian food stuffs. *Journal of Food Composition and Analysis*, *16*, 659-667.

Romero, C., Brenes, M., Yousfi, K., García, P., García, A., & Garrido, A. (2004). Effect of cultivar and processing method on the contents of polyphenols in table olives. *Journal of Agriculture and Food Chemistry*, *52*, 479-484.

Romero, C., García, P., Brenes, M., García, A., & Garrido, A. (2002). Phenolic compounds in natural black Spanish olive varieties. *European Food Research Technology*, *215*, 489-496.

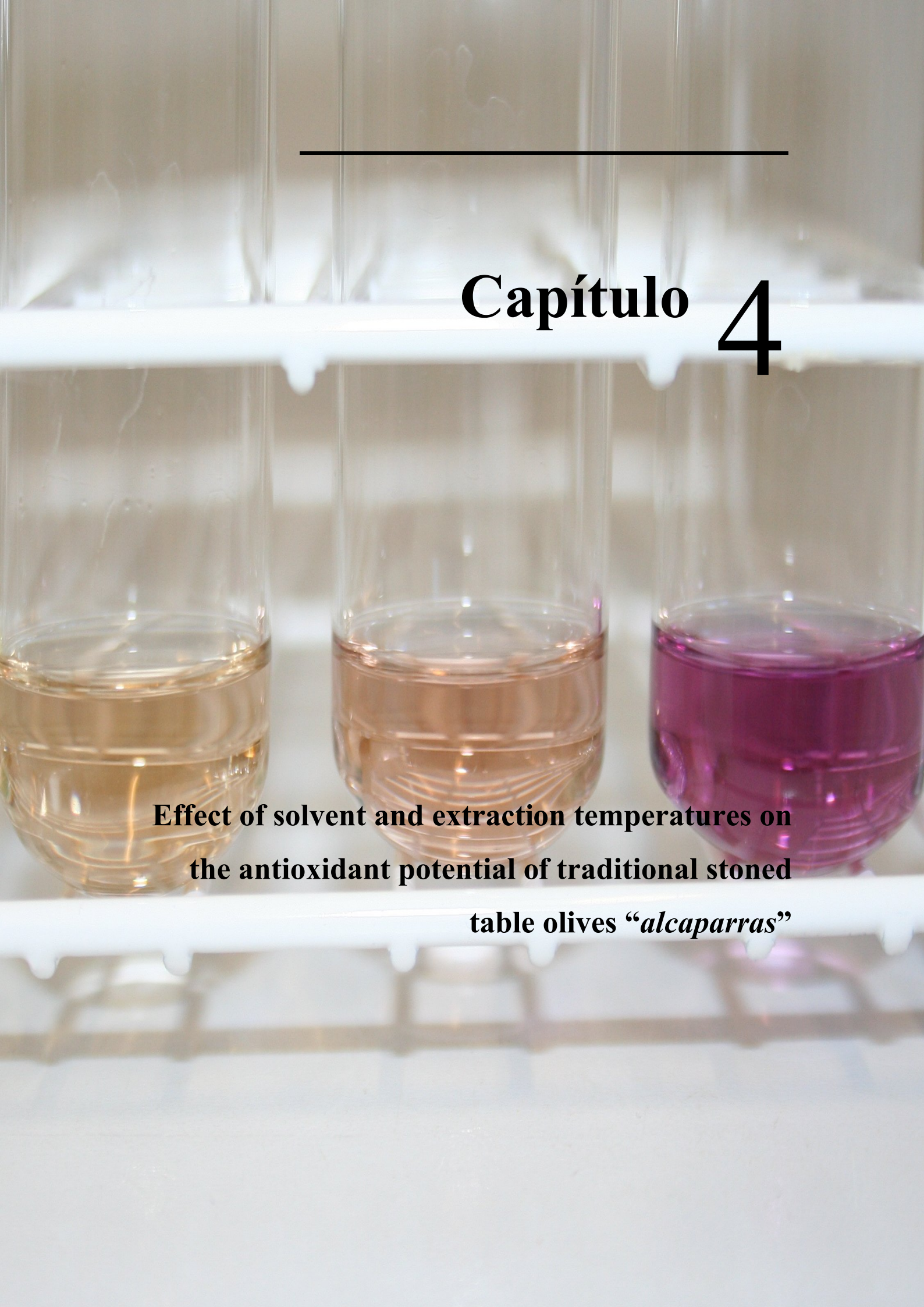
Sakoui, F.; Harrabi, S.; Absalon, C.; Sbei, K.; Boukhchina, S. & Kallel, H. (2008). α-Tocopherol and fatty acids contents of some Tunisian table olives (*Olea europea* L.): Changes in their composition during ripening and processing. *Food Chemistry*, *108*, 833-839.

Simopoulos A.P. (2001). The Mediterranean diets: what is so special about the diet of Greece? The scientific evidence. *Journal of Nutrition, 131*, 3065S–3073S.

Sousa, A., Ferreira, I.C.F.R., Calhelha, R.C., Andrade, P.B., Valentão, P., Seabra, R., Estevinho, L., Bento, A., & Pereira, J.A. (2006). Phenolics and antimicrobial activity of traditional stoned table olives "*alcaparra*". *Bioorganic & Medicinal Chemistry, 14*, 8533-8538.

Sousa, A..; Ferreira, I.C.F.R.; Barros, L.; Bento, A. & Pereira, J. A. (2008). Antioxidant potential of traditional stoned table olives "Alcaparras": influence of the solvent and temperature extraction conditions. *LWT - Food Science and Technology, 41*, 739-745

Trichopoulou, A., & Lagiou, P. (1997). Healthy traditional Mediterranean diet: An expression of culture, history and lifestyle. *Nutrition Reviews*, *55*, 383-389.



Capítulo 4

Effect of solvent and extraction temperatures on the antioxidant potential of traditional stoned table olives "*alcaparras*"

Effect of solvent and extraction temperatures on the antioxidant potential of traditional stoned table olives "*alcaparras*"

LWT – Food Science and Technology, **41** (2008), 739-745

Abstract: This paper reports the first approach to the antioxidant potential evaluation of traditional stoned table olives "*alcaparras*". This kind of olives is largely produced and consumed in Trás-os-Montes region (Northeast of Portugal). Different solvents and temperature extraction conditions were employed in order to achieve the best method to obtain phenolic compounds and a higher antioxidant activity. The optimum method (water at boiling temperature) was applied on ten samples from the traditional market. The total phenol content ranged between 5.58 mg Gallic Acid Equivalents (GAE)/g and 29.88 mg GAE/g and Effective Concentration (EC_{50}) values were in the range 0.36 - 1.64 mg/mL and 0.34 - 1.72 mg/mL for reducing power and radical scavenging effect, respectively. A significantly negative linear regression was observed between the total phenol content found in the samples and its antioxidant activity.

Keywords: stoned table olives; extraction conditions; phenols; antioxidant potential

4.1. Introduction

Mediterranean diet has been associated with a lower risk of coronary heart disease and certain kinds of cancer, e.g. breast and colon cancers (Keys, 1995; Trichopoulou & Lagiou, 1997). This diet is rich in fruits, vegetables, whole grains, fish, low-fat dairy products and monounsaturated fats (Panagiotakos et al., 2006). In this diet, olive oil is a main source of fat, in daily cooked dishes and salads, and table olives are also largely consumed.

According to the Trade Standard Applying to Table Olives (COI/OT/NC nº1, 2004) table olives are defined as the product prepared from the sound fruits of varieties of the cultivated olive tree (*Olea europaea* L.) that are chosen for their production of olives whose volume, shape, flesh-to-stone ratio, fine flesh, taste, firmness and ease of detachment from the stone make them particularly suitable for processing". Different kinds of table olives should be classified according to the ripeness stage of the fruit, trade preparation, styles and sizing. Stoned halved olives are one of the styles considered by the Trade Standard already referred.

In the Northeast of Portugal, stoned halved olives known as "*alcaparras*" are largely produced by the local growers, commercialized in the local market and consumed flavoured with herb spices, onion, garlic, vinegar and olive oil. "*Alcaparras*" are processed from green or yellow-green healthy olive fruits, which are broken using a wood hammer, being the pulp and stone separated. The pulp is sliced into two approximately equal parts, perpendicularly to the major axis of the fruit, and placed in water, which changes three or four times during a week. This treatment removes the bitterness and the olives can be salted, flavoured and consumed.

In the past few years, the suspected toxicity of some synthetic compounds used in food has raised the interest in natural products (Fukushima & Tsuda, 1985; Stone et al., 2003). Some industries, such as those related to food additive production, cosmetics, and pharmaceuticals, have increased their efforts in obtaining bioactive compounds from natural products by extraction and purification. Antioxidant compounds can increase shelf life by retarding the process of lipid peroxidation, which is one of the

major reasons for deterioration of food products during processing and storage (Halliwell, 1997; Halliwell & Gutteridge, 1999).

There is an increasing interest in olive products and by-products, due to their antimicrobial and antioxidant properties. Recently our research group demonstrated a strong antimicrobial activity of *alcaparras*" extracts against several microorganisms that may be causal agents of human intestinal and respiratory tract infections (Sousa et al., 2006). Concerning to the antioxidant activity, olive oil polyphenols revealed good properties (Gordon et al., 2001; Paiva-Martins et al., 2003) and olive leaves have been referred as a source of several antioxidants (Bouaziz & Sayadi, 2005; Briante et al., 2002; Meirinhos et al., 2005; Ranalli et al., 2006; Ferreira, Barros et al., 2007). Also table olives have been studied in its polyphenol composition (Blekas et al., 2002; Marsilio et al., 2001; Romero et al., 2002; Romero et al., 2004; Pereira et al., 2006) and antioxidant activity (Boskou et al., 2006; Owen et al., 2003; Pereira et al., 2006). Nevertheless, as far as we know, it is the first time that a study on the antioxidant potential of stoned table olives "*alcaparras*" is reported.

Herein, we intended to found the most appropriate solvent for extracting phenols, in relation to other constituents, from "*alcaparras*" table olives and to correlate their levels with antioxidant activity of the extracts obtained. The best extraction method was applied to ten samples from the tradition market of four different municipalities of Trás-os-Montes region, and their reducing power and scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals were investigated and correlated with their total phenolic content.

4.2. Materials and Methods

4.2.1. Samples

Ten different samples of stoned table olives "*alcaparras*" were obtained in the traditional market. The samples were from different municipalities of Trás-os-Montes region: Bragança (samples B1 – B5), Mirandela (M1 and M2), Carrazeda de Ansiães (CA1 and CA2) and Macedo de Cavaleiros (MC).

4.2.2 Standards and reagents

BHA (2-*tert*-butyl-4-methoxyphenol), α-tocopherol and gallic acid were purchase from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA). Methanol was obtained from Pronalab (Lisboa, Portugal). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

4.2.3. Sample preparation

The samples (~ 100 g) were dried in a stove (Memmert Schwabach 854, 1994) at 30ºC for three days and stored at 4ºC protected from light until further use.

For the extraction method optimization, a fine dried powder (20 mesh) of sample B1 (5g) was extracted using four different conditions:

i) Extraction with 250 mL of boiling water (bt) for 45 min and filtered through Whatman nº 4 paper. The aqueous extract was frozen and lyophilized.

ii) Stirring with 100 mL of water at room temperature (rt) at 150 rpm for 24h and filtered through Whatman nº 4 paper. The residue was then extracted with two additional 100 mL portions of water, as described earlier. The combined aqueous extracts were frozen and lyophilized.

iii) Extraction using a Soxhlet extractor for 8h with 250 mL of methanol (MeOH). The methanolic extract was evaporated at 40 ºC to dryness.

iv) Stirring with 100 mL of methanol at room temperature at 150 rpm for 24h and filtered through Whatman nº 4 paper. The residue was then extracted with two additional 100 mL portions of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 ºC to dryness.

All the extracts were redissolved in the corresponding solvent at a concentration of 50 mg/mL, and analysed for their content in phenols.

The first extraction method was employed in the preparation of ten different samples for analysis of their antioxidant activity.

4.2.4. Determination of total phenol content

Phenolic compounds concentration in the extracts was estimated by a colorimetric assay based on procedures described by Singleton and Rossi (1965) with some modifications. Briefly, 1 mL of sample was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of saturated Na_2CO_3 solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (Analytik Jena 200-2004 spectrophotometer). Gallic acid was used for constructing the standard curve (0.01-0.4 mM). The results are expressed as mg of gallic acid equivalents/g of extract (GAEs).

4.2.5. Reducing power assay

The reducing power was determined according to the method of Oyaizu (1986). The extract solution (2.5 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 2.5 mL of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 1000 rpm for 8 min (Centorion K24OR- 2003 refrigerated centrifuge). The upper layer (5 mL) was mixed with 5 mL of deionised water and 1 mL of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm (higher absorbance indicates higher reducing power). Extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration in the solution. BHA and α-tocopherol methanolic solutions were used as standards.

4.2.6. Scavenging effect assay

The capacity to scavenge the "stable" free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was monitored according to the method of Hatano et al. (1988). The extract solution (0.3 mL) was mixed with 2.7 mL of methanolic solution containing DPPH radicals ($6x10^{-5}$ mol/L). The mixture was shaken vigorously and left to stand for 60 min in the 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 discolouration using the 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. BHA and α-tocopherol methanolic solutions were used as standards.

4.2.7. Statistical analysis

For each extraction method conditions, nine assays were performed using the sample B1. The differences between treatments (solvent and temperature conditions) in each parameter were analyzed using one-way analysis of variance (ANOVA) followed by Tukey s HSD Test with $\alpha = 0.05$. This treatment was carried out using SAS v. 9.1.3 program. All the antioxidant activity assays were carried out in triplicate and the results are shown as mean values and standard deviation. The regression analysis between phenol contents and EC_{50} values for reducing power and scavenging activity was conducted using the same statistical package.

4.3. Results and discussion

4.3.1. Influence of the extraction conditions in the "alcaparras" antioxidant potential

In order to achieve the best extraction conditions, the total phenol content, reducing power and DPPH radical scavenging activity were evaluated for the stoned table olives *alcaparras*" (sample B1) obtained using two different extraction solvents (water and methanol) and two different temperatures (room and boiling temperatures) (Table 1).

Table 1. Reducing power and scavenging effect EC_{50} values (mg/mL), and total phenols content (mg/g) of "*alcaparras*" table olives extracts obtained using different solvents and temperatures in the extractions[a]. In each row different letters mean significant differences ($p< 0.05$) between different conditions. bt H_2O – boiling water; rt H_2O – room temperature water; bt MeOH – boiling methanol; rt MeOH – room temperature methanol.

	bt H_2O	rt H_2O	bt MeOH	rt MeOH
Extraction yield (%)	7.20±0.25 b	5.82±0.04 c	32.22±12.95 a	19.38±0.81 a
Total phenols content	15.48±1.05 a	3.48±0.68 c	4.86±1.79 b	5.90±0.21 b
Reducing power (EC_{50}[b])	0.42±0.03 c	4.21±0.55 a	1.97±0.73 b	1.51±0.13 b
DPPH (EC_{50}[c])	0.47±0.03 c	4.16±1.04 a	1.55±0.52 b	1.55±0.13 b

[a] Each value is expressed as mean ± standard deviation (n=9).
[b] EC_{50} (mg/mL): effective concentration at which the absorbance is 0.5.
[c] EC_{50} (mg/mL): effective concentration at which 50% of DPPH radicals are scavenged.

In this study, using methanol as solvent no significant differences ($p > 0.05$) were observed either in room temperature or in boiling temperature. The total phenol content and the EC_{50} values for reducing power and scavenging activity obtained after table olive sample extraction at both temperatures were very similar. However, a higher reproducibility of the results was observed in the case of extraction at room temperature (lower standard deviation values).

The extraction procedure using water at room temperature was significantly ($p < 0.05$) least efficient (very low total phenol content, 3.48 mg/g dry extract, and higher EC_{50} values, 4.21 mg/mL for reducing power and 4.16 mg/mL for scavenging effect), while boiling water proved to be the most adequate solvent extracting a significantly (*p*

< 0.05) higher amount of phenols in relation to total extracted olive constituents (15.48 mg/g dry extract). Accordingly, the EC_{50} values obtained using the last conditions were significantly lower (0.42 mg/mL for reducing power and 0.47 mg/mL for scavenging effect).

Figure 1 shows the reducing power of *alcaparras*" extracts obtained using both solvents at boiling and at room temperature, as a function of their concentration. The presence of reducers (i.e. antioxidants) caused the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) monitored at 700 nm.

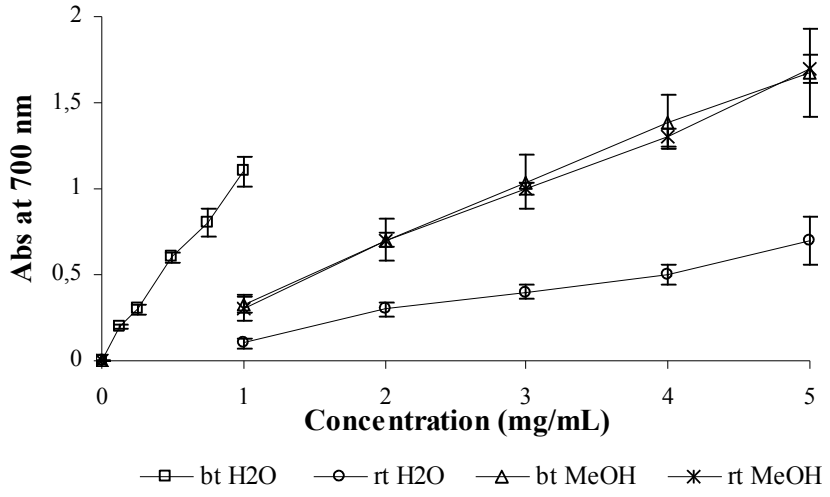


Figure 1. Reducing power values of "*alcaparras*" table olives extracts obtained using water and methanol at boiling and room temperature. Each value is expressed as mean ± standard deviation (n=9). bt H_2O – boiling water; rt H_2O – room temperature water; bt MeOH – boiling methanol; rt MeOH – room temperature methanol.

The reducing power was found to increase with the amount of extract dissolved in the respective solvent. Aqueous extract obtained at room temperature showed the lowest reducing power values (0.70 at 5 mg/mL). It must be emphasized that aqueous extracts obtained at boiling temperature presented the highest reducing power values (1.10 at 1mg/mL). The extracts obtained using methanol at boiling and room temperature presented similar reducing power (~1.5 at 5 mg/mL). Despite the different extraction conditions, the "*alcaparras*" extracts reducing power was always higher than reducing power of BHA (0.12 at 3.6 mg/mL) and α-tocopherol (0.13 at 8.6 mg/mL).

Antioxidant molecules can quench DPPH free radicals and convert them to a colourless/bleached product (i.e. 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation (Ferreres et al., 2006). The scavenging effect of *alcaparras*" extracts obtained using both solvents at boiling and room temperatures was also examined (Figure 2); results are expressed as the ratio percentage of the absorbance decrease of DPPH radical solution in the presence of extract at 517 nm to the absorbance of DPPH radical solution at the same wave length.

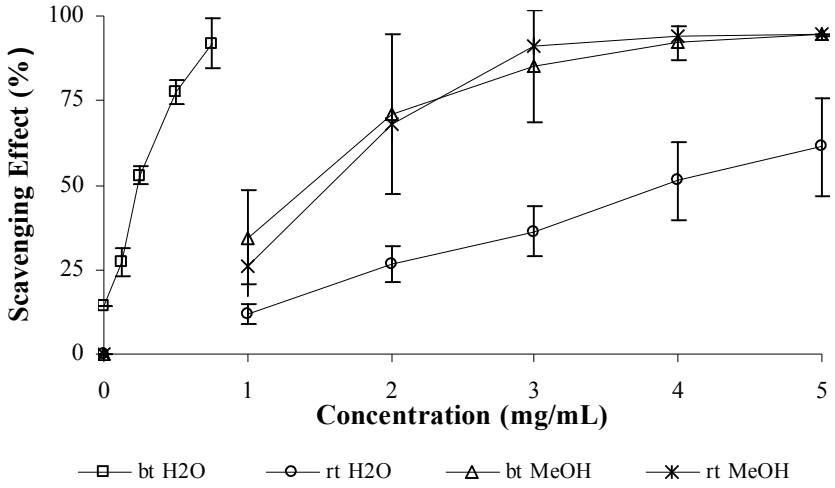


Figure 2. Scavenging effect of "*alcaparras*" table olives extracts obtained using water and methanol at boiling and room temperature. Each value is expressed as mean ± standard deviation (n=9). bt H_2O – boiling water; rt H_2O – room temperature water; bt MeOH – boiling methanol; rt MeOH – room temperature methanol.

From the analysis of Figure 2 we can observe that the extracts scavenging effects on DPPH radicals increase when the concentration increase. Once more, aqueous extract obtained at boiling temperature scavenged DPPH radicals more effectively (92.0% at 1 mg/mL) than aqueous extract obtained at room temperature (61.3% at 5 mg/mL). The extracts obtained using methanol at boiling and room temperature presented the same scavenging effect (94.6% at 5 mg/mL). These values were comparable to the scavenging effect obtained for the standards BHA (96% at 3.6 mg/mL) and α-tocopherol (95% at 8.6 mg/mL).

In our study, water at boiling temperature was the most effective solvent for the antioxidants extraction, providing higher total phenol content, and therefore a higher antioxidant potential. Previous studies on the influence of different extracting solvents in the yield of phenols extracted from other plant materials and in antioxidant activity of the extracts obtained have been reported (Cheung et al., 2003; Sun & Ho, 2005). Nevertheless, our results were not in agreement with that of the above mentioned authors who observed a better scavenging activity and higher phenolic content in methanolic or acetone extracts.

4.3.2. Antioxidant potential of several "alcaparras" samples

The most efficient conditions for the phenols extraction (boiling water) were applied to ten stoned table olives "*alcaparras*" from the traditional market. Table 2 shows the reducing power and scavenging effect EC_{50} values (mg/mL), and total phenol content (mg/g dry extract) of the extracts obtained from ten olive samples.

EC_{50} values obtained for both methods were lower than 1.8 mg/mL, and in the order of CA2<B1<MC<B4<M2<B2<B5~CA1<B3<M1. The extract of sample CA2 (from Carrazeda de Ansiães) showed the highest phenolic content (29.88 mg/g) and the highest antioxidant potential, presenting the lowest EC_{50} values (0.36 mg/mL for reducing power and 0.34 mg/mL for scavenging effect). Otherwise, the extract of sample M1 (from Mirandela) showed the lowest phenolic content (5.58 mg/g) and the highest EC_{50} values (1.64 mg/mL for reducing power and 1.72 mg/mL for scavenging effect). The "*alcaparras*" table olive consumption needed for the intake of phenols in order to achieve the same antioxidant capacity depends on the sample used. For example, if we choose two samples bought in the same municipality (CA1 and CA2) to reach the same antioxidant capacity it is necessary to consume three times more olives from the sample CA1 than from the sample CA2. This fact is probably related to the olive variety used to make "*alcaparras*". It is known that in the Northeast of Portugal the olive varieties have different fatty acids profiles, different levels of monounsaturated fatty acids (especially oleic acid) (Pereira et al., 2002a; Pereira et al., 2002b) and different phenol profile (Vinha et al., 2005) which is related to its antioxidant properties (Carrasco-Pancorbo et al., 2005; Owen et al., 2000). The olive ripening is also a factor

Table 2. Reducing power and scavenging effect EC_{50} values (mg/mL), and total phenol content (mg/g) of ten different "alcaparras" table olives samples.

Samples	Total phenol content	Reducing power (EC_{50})					DPPH (EC_{50})				
		Experimental	Polynomial	Error (%)	Linear	Error (%)	Experimental	Polynomial	Error (%)	Linear	Error (%)
Bragança (B1)	**16.63**	**0.49**	0.39	20	0.66	36	**0.40**	0.30	25	0.64	60
Bragança (B2)	**9.54**	**0.88**	0.96	9	1.00	13	**0.89**	0.97	9	1.02	14
Bragança (B3)	**6.58**	**1.40**	1.30	6	1.14	18	**1.71**	1.40	18	1.18	31
Bragança (B4)	**14.59**	**0.58**	0.52	11	0.76	31	**0.42**	0.44	6	0.75	79
Bragança (B5)	**7.51**	**0.97**	1.19	23	1.09	13	**0.93**	1.25	35	1.13	22
Mirandela (M1)	**5.58**	**1.64**	1.44	12	1.18	28	**1.72**	1.56	10	1.23	29
Mirandela (M2)	**11.90**	**0.59**	0.73	24	0.89	51	**0.65**	0.69	7	0.89	38
Mac Cavaleiros (MC)	**16.00**	**0.51**	0.43	15	0.69	13	**0.44**	0.34	22	0.67	53
Car. Ansiães (CA1)	**7.51**	**1.15**	1.19	4	1.09	5	**1.07**	1.25	17	1.13	6
Car. Ansiães (CA2)	**29.88**	**0.36**	0.37	2	0.04	88	**0.34**	0.37	9	0.08	76

could have influence in the antioxidant potential. During the olive maturation, some changes occur in the fruit composition, especially on the polyphenols: oleuropein decreases and tyrosol and hydroxytyrosol increase (Beltrán et al., 2005; Gutiérrez et al., 1999; Salvador et al., 2001; Skevin et al., 2003).

A significantly negative linear correlation was established between phenol content and EC_{50} reducing power values (determination coefficients 0.639; $p < 0{,}001$) (Figure 3). This negative linear correlation proves that the sample with highest total phenol content shows higher reducing power and lower EC_{50} values (sample CA2), while the sample with lowest total phenol content presents lower reducing power and higher EC_{50} values (sample M1). A similar situation was observed when we established a correlation between the total phenol content and scavenging effect values (determination coefficients 0.573; $p < 0{,}001$) (Figure 4). The obtained data were also adjusted to a

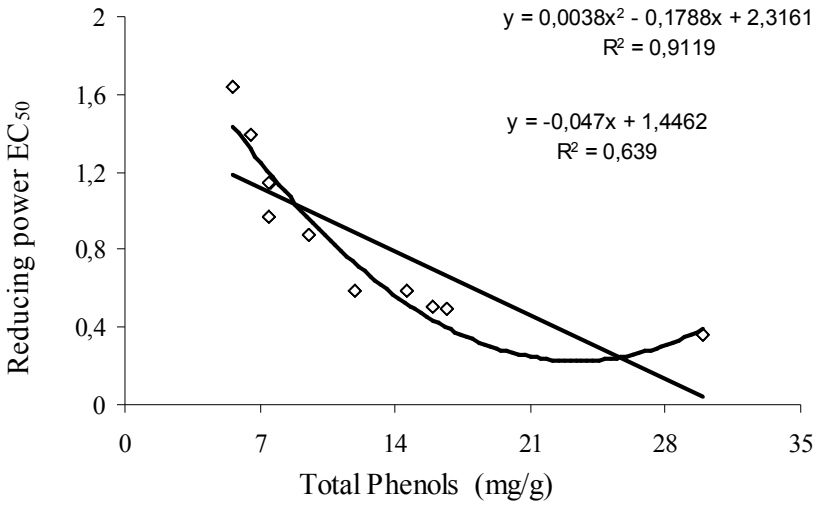


Figure 3. Correlation established between total phenol content and reducing power values.

polynomial curve (Figs. 3 and 4) which gave a better approximation, with r^2 of 0.912 and 0.878 for reducing power and DPPH radical scavenging effect EC_{50} values, respectively. On the basis of the previously established relationships between EC_{50} values and total phenol, the EC_{50} values were estimated according polynomial and linear curves (Table 2), and compared to experimental values through the error percentage. As can be observed, the values were similar and the error found was, in most cases, less than 10%, being lower in the case of polynomial approximation. This

information is particularly useful when the total phenol content is known because it allows the estimation of EC_{50} values instead of their experimental determination.

In conclusion, extraction with water at boiling temperature proved to be a better method to extract phenolic antioxidant compounds from *alcaparras*" table olives, than methanol at room or boiling temperature. A significantly negative linear regression was established between the total phenol content found in the samples and its antioxidant activity (reducing power and radical scavenging effect on DPPH radicals).

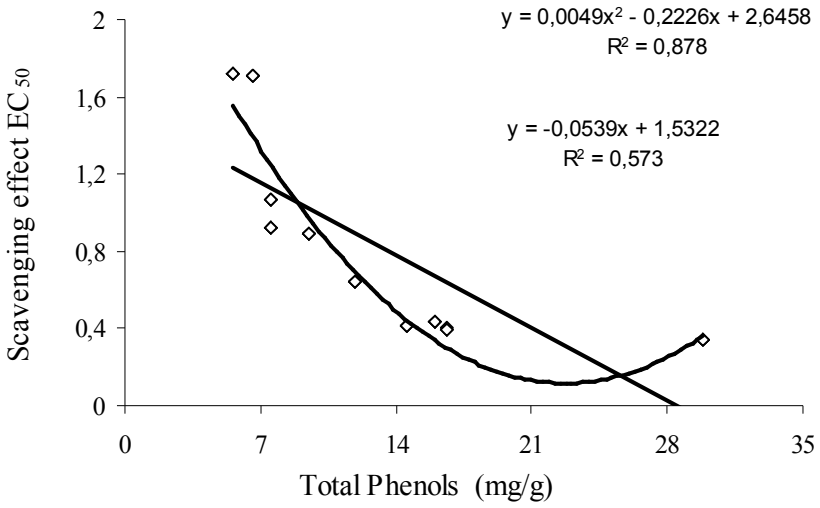


Figure 4. Correlation established between total phenol content and scavenging effect.

The results obtained in the present work denote that stoned table olives "*alcaparras*" may constitute a good source of healthy compounds or phenols intake in the diet, suggesting that it could be useful in the prevention of diseases in which free radicals are implicated. In addition, and as far as we know, this is the first report considering the antioxidant potential of "*alcaparras*" table olives; furthers studies are need to clarify the role of different olive cultivars and their ripening stage used to make "*alcaparras*" table olives in its phenolic composition and antioxidant potential.

Acknowledgements

The authors are grateful to INTERREG III A Program, Project MOABEPE for financial support of this work.

4.4. References

Beltrán, G., Aguilera, M.P., Del Rio, C., Sanchez, S., & Martinez, L. (2005). Influence of fruit ripening process on the natural antioxidant content of Hojiblanca virgin olive oils. *Food Chemistry*, *89*, 207-215.

Blekas, G., Vassilakis, C., Harizanis, C., Tsimidou, M., & Boskou, D.G. (2002). Biophenols in table olives. *Journal of Agriculture and Food Chemistry*, *50*, 3688-3692.

Boskou, G., Salta, F.N., Chrysostomou, S., Mylona, A., Chiou, A., & Andrikopoulos, N.K. (2006). Antioxidant capacity and phenolic profile of table olives from the Greek market. *Food Chemistry*, *94*, 558-564.

Bouaziz, M., & Sayadi, S. (2005). Isolation and evaluation of antioxidants from leaves of a Tunisian cultivar olive tree. *European Journal of Lipid Science and Technology*, *107, 497-504.*

Briante, R., Patumi, M., Terenziani, S., Bismuto, E., Febbraio, F., & Nucci, R. (2002). *Olea europaea* L. leaf extract and derivatives: antioxidant properties. *Journal of Agriculture and Food Chemistry*, *50*, 4934-4940.

Carrasco-Pancorbo, A., Cerretani, L., Bendini, A., Segura-Carretero, A., Carlo, M., Gallina-Toschi, T., Lercker, G., Compagnone, D., & Fernández-Gutiérrez, A. (2005). Evaluation of the Antioxidant Capacity of Individual Phenolic Compounds in Virgin Olive Oil. *Journal of Agriculture and Food Chemistry*, *53*, 8918-8925.

Cheung, L. M., Peter, C. K., & Vincent, E. C. (2003). Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry*, *81*, 249–255.

Ferreira, I.C.F.R., Barros, L; Soares, M.E.; Bastos, M.L., & Pereira, J.A. (2007). Antioxidant activity and phenolic contents of *Olea europaea* L. leaves sprayed with different copper formulations. *Food Chemistry*, *103*, 188-195.

Ferreres, F., Sousa, C., Vrchovska, V., Valentão, P., Pereira, J. A., Seabra, R.M., & Andrade P. B. (2006). Chemical composition and antioxidant activity of tronchuda cabbage internal leaves. *European Food Research and Technology, 222,* 88-98.

Fukushima, N.S., & Tsuda, H. (1985). Carcinogenity and modification of the carcinogenic response by BHA, BHT and other antioxidants. *Critical Reviews in Toxicoogy*, *15*, 109-150.

Gordon, M.H., Paiva-Martins, F., & Almeida, M. (2001). Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. *Journal of Agriculture and Food Chemistry*, *49*, 2480-2485.

Gutiérrez, F., Jímenez, B., Ruíz, A., & Albi, M.A. (1999). Effect of olive ripeness on the oxidative stability of virgin olive oil extracted from the varieties Picual and Hojiblanca and on the different components involved. *Journal of Agriculture and Food Chemistry*, *47*, 121–127.

Halliwell, B. (1997). Antioxidants in human health and disease. *Annual Review of Nutrition*, *16*, 33–50.

Halliwell, B., & Gutteridge, J. M. C. (1999). *Free radicals in biology and medicine*. Oxford University Press: United Kingdom.

Hatano, T., Kagawa, H., Yasuhara, T., & Okuda, T. (1988). Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *Chemical and Pharmaceutical Bulletin*, *36*, 2090-2097.

Keys, A. (1995). Mediterranean diet and public health: Personal reflections. *American Journal of Clinical Nutrition*, *61*, 1321S-1323S.

Marsilio, V., Campestre, C., & Lanza, B. (2001). Phenolic compounds change during California-style ripe olive processing. *Food Chemistry*, *74*, 55-60.

Meirinhos, J., Silva, B.M., Valentão, P., Seabra, R.M., Pereira, J.A., Dias, A., Andrade, P.B., & Ferreres, F. (2005). Analysis and quantification of flavonoidic compounds from Portuguese olive (*Olea europaea* L.) leaf cultivars. *Natural Product Research*, *19*, 189-195.

Owen, R. W., Haubner, R., Mier, W., Giacosa, A., Hull, W.E., Spiegelhalder, B. & Bartsch, H. (2003). Isolation, structure and antioxidant potential of the major

phenolic and flavonoid compounds in brined olive drupes. *Food and Chemical Toxicology*, *41*, 703-717.

Owen, R. W., Mier, W., Giacosa, A., Hull, W. E., Spielgelhalder, B., & Bartsch, H. (2000). Phenolic compounds and squalene in olive oils: The concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. *Food Chemistry Toxicology*, *38*, 647-659.

Oyaizu, M. (1986). Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucosamine. *Japonese Journal of Nutrition*, *44*, 307-315.

Paiva-Martins, F., Gordon, M.H., & Gameiro, P. (2003). Activity and location of olive oil phenolic antioxidants in liposomes. *Chemistry and Physics of Lipids*, *124*, 23-36.

Panagiotakos, D.B., Pitsavos, C., & Stefanadis, C. (2006). Dietary patterns: A Mediterranean diet score and its relation to clinical and biological markers of cardiovascular disease risk. *Nutrition, Metabolism and Cardiovascular Diseases*, *16*, 559-568.

Pereira, J. A., Casal, S., Bento, A., & Oliveira, M.B.P.P. (2002a). Influence of the olive storage period on oil quality in the portuguese "Cobrançosa", "Madural" and "Verdeal Transmontana" cultivars. *Journal of Agriculture and Food Chemistry*, *50*, 6335-6340.

Pereira, J. A., Oliveira, M. B. P. P., Casal, S., & Alves, M.R. (2002b). Discrimination of varietal olive oils of the portuguese cultivars Cobrançosa, Madural and Verdeal based on their fatty acids composition. *Acta Horticulturae*, *586*, 591-594.

Pereira, J. A.; Pereira, A.P.; Ferreira, I.C.F.R.; Valentão, P.; Andrade, P.; Seabra, R.; Estevinho, L. & Bento, A. (2006). Phenolics compounds, antioxidant potential and microbial activity of table olives from Portugal. *Journal of Agriculture and Food Chemistry, 54*, 8425-8431.

Ranalli, A., Contento, S., Lucera, L., Febo, M.D., Marchegiani, D., & Fonzo, V.D. (2006). Factors affecting the contents of Iridoid Oleuropein in olive leaves (*Olea europaea* L.). *Journal of Agriculture and Food Chemistry*, *54,* 434-440.

Romero, C., García, P., Brenes, M., García, A., & Garrido, A. (2002). Phenolic compounds in natural black Spanish olive varieties. *European Food Research Technology*, *215*, 489-496.

Romero, C., Brenes, M., Yousfi, K., García, P., García, A., & Garrido, A. (2004). Effect of cultivar and processing method on the contents of polyphenols in table olives. *Journal of Agriculture and Food Chemistry*, *52*, 479-484.

Salvador, M.D., Aranda, F., & Fregapane, G. (2001). Influence of fruit ripening on "Cornicabra" virgin olive oil quality. A study of four successive crop seasons. *Food Chemistry*, *73*, 45–53.

Singleton, V.L., & Rossi, J.A. jr.. (1965). Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16*, 144-158.

Skevin, D., Rade, D., Strucelj, D., Mokrovcak, Z., Nederal, S., & Bencic, D. (2003). The influence of variety and harvest time on the bitterness and phenolic compounds of olive oil. *European. Journal of Lipid Sciences and. Technology*, *105*, 536–541.

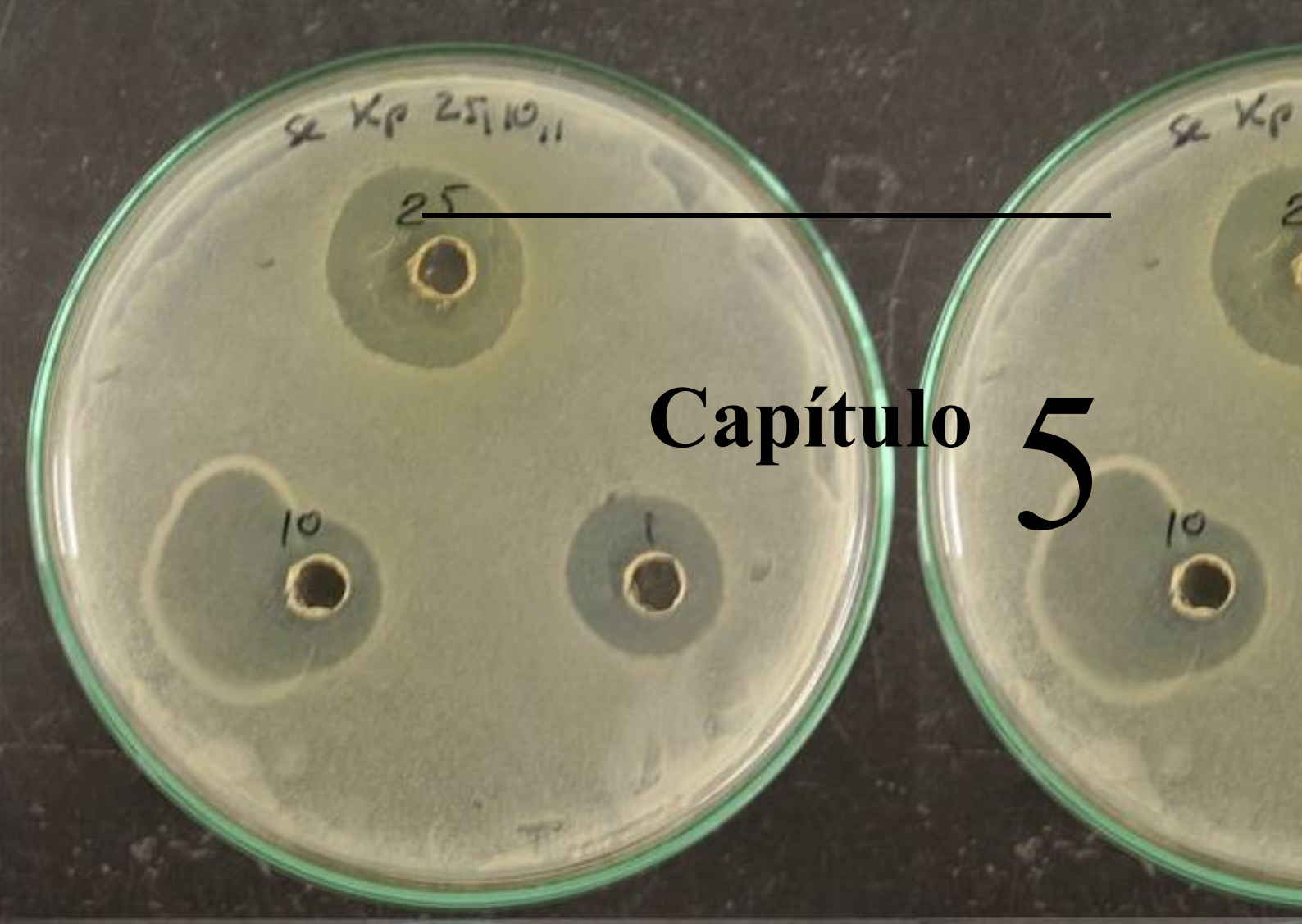
Sousa, A., Ferreira, I.C.F.R., Calhelha, R.C., Andrade, P.B., Valentão, P., Seabra, R., Estevinho, L., Bento, A., & Pereira, J.A. (2006). Phenolics and antimicrobial activity of traditional stoned table olives "*alcaparra*". *Bioorganic & Medicinal Chemistry, 14*, 8533-8538.

Stone, W.L., Leclair, I., Ponder, T., Bagss, G., & Barret-Reis, B. (2003). Infants discriminate between natural and synthetic vitamin E. *American Journal of Clinical Nutrition*, *77*, 899-906.

Sun, T., & Ho, C. T. (2005). Antioxidant activities of buckwheat extracts. *Food Chemistry*, *90*, 743–749.

Trichopoulou, A., & Lagiou, P. (1997). Healthy traditional Mediterranean diet: An expression of culture, history and lifestyle. *Nutrition Reviews*, *55*, 383-389.

Vinha, A. F., Ferreres, F., Silva, B.M., Valentão, P., Gonçalves, A., Pereira, J. A., Oliveira, M.B.P.P., Seabra, R.M., & Andrade, P.B. (2005). Phenolic profiles of Portuguese olive fruits (*Olea europaea* L.): influence of cultivar and geographical origin. *Food Chemistry*, *89*, 561-568.



Capítulo 5

Phenolics and Antimicrobial Activity of Traditional Stoned Table Olives "*alcaparra*"

Phenolics and Antimicrobial Activity of Traditional Stoned Table Olives "*alcaparra*"

Bioorganic and Medicinal Chemistry, **14** (2006), 8533-8538

Abstract: In the present work we report the determination of phenolic compounds in "alcaparra" table olives by reversed-phase HPLC/DAD, and the evaluation of their extract *in vitro* activity against several microorganisms that may be causal agents of human intestinal and respiratory tract infections, namely Gram positive (*Bacillus cereus*, *B. subtilis* and *Staphylococcus aureus*), Gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*) and fungi (*Candida albicans* and *Cryptococcus neoformans*). Three flavonoidic compounds were identified and quantified: luteolin 7-*O*-glucoside, apigenin 7-*O*-glucoside and luteolin. At low concentrations (0.05 mg/mL) "alcaparra" extract revealed significant inhibition of both Gram positive and Gram negative bacteria growth, with exception of *P. aeruginosa*. Nevertheless, no antifungal activity was observed at the tested concentrations.

Keywords: Luteolin, Luteolin 7-*O* glucoside, Apigenin 7-*O* glucoside, "Alcaparra", Table olives, Antimicrobial activity.

5.1. Introduction

In the Mediterranean basin, olive oil, together with red wine, fruits, vegetables and fish, are important constituents of the diet and are considered major factors in preserving a healthy and relatively disease-free population. In fact, epidemiological data show that the Mediterranean diet has significant protective effects against cancer and coronary heart disease (Keys, 1995; Trichopoulou & Lagiou, 1997). Olive products, typical components of the Mediterranean diet, contain high concentrations of complex phenols, which are endowed with strong antioxidant activity (Baldioli et al., 1996; Litridou et al., 1997). The most important classes of phenolic compounds in olive products, namely table olives, include phenolic acids, phenolic alcohols, flavonoids and secoiridoids (Ryan & Roberts, 1998; Brenes et al., 1999; Soler-Rivas et al., 2000; Owen et al., 2000; Owen et al., 2003; Vinha et al., 2005).

Many researches have demonstrated that the above mentioned bio compounds are able to inhibit or delay the rate of growth of a range of bacteria and microfungi (Cowan, 1999), so that they might be used as alternative food additives or in integrated pest management programs.

The increasing resistance to antibiotic represents the main factor justifying the need to find and/or develop new antimicrobial agents. Thus, some studies have been focused on the antimicrobial agents and on the antimicrobial properties of plant-derived active principles (such as spices and essential oils), which have been used for a long time in traditional medicine to overcome infections (Cowan, 1999). The use of crude extracts instead of isolated constituents may be more appropriate since a specific compound could be bioactive but in the presence of other compounds present in the extracts could change its properties.

Recent studies on antimicrobial activity of olive products were developed, namely with olive leaves (Markin et al., 2003), olive fruits (Nychas et al., 1990) and their pure compounds, such as oleuropein (Bisignano et al., 1999; Furneri et al., 2002) and hydroxytyrosol (Bisignano et al., 1999), and aliphatic aldehydes (Battinelli et al., 2006). Hydroxytyrosol and oleuropein have been shown to inhibit or delay the rate of growth of a range of bacteria and microfungi like *Haemophilus influenza*, *Salmonella typhi*,

Vibrio parahaemolyticus, and *Staphylococcus aureus* (Bisignano et al., 1999). Also, oleuropein inhibited the growth of *Mycoplasma hominis*, *M. fermentans*, *M. pneumoniae*, and *M. pirum* (Furneri et al., 2002). Aldehydes, such as hexanal, nonanal, (E)-2-hexenal, (E)-2-heptenal, (E)-2-octenal, and (E)-2-nonenal revealed antifungal activity against *Trycophyton mentagrophytes*, *Microsporum canis,* and *Candida* spp. (Battinelli et al., 2006).

In the Northeast of Portugal, stoned halved olives (table olives), known as alcaparra", are largely produced and commercialized in the local market, constituting an important agro-economic factor for the local producers. This kind of table olives are processed from green or yellow-green healthy olive fruits, which are broken using a wood hammer, being the pulp and stone separated. The pulp is sliced into two approximately equal parts, perpendicularly to the major axis of the fruit, and placed in water, daily changed during a week to remove its bitterness. As far as we know, no reports on the nature and/or concentrations of phenolic compounds in "alcaparra" olive and on its antimicrobial activity exist.

Therefore, in the present work, the phenolic compounds of this kind of Portuguese table olives were determined. A screening of "*alcaparra*" extracts antibacterial activities against Gram positive (*Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*) and Gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*) and of antifungal capacity (*Candida albicans*, *Cryptococcus neoformans*) was also performed.

5.2. Experimental

5.2.1. "*Alcaparra*" *sample*

"Alcaparra" traditional stoned olives were purchased at a local market in Northeast Portugal and frozen. The sample was then lyophilized, reduced to a fine powder (20 mesh) and stored in an exsiccator protected from light.

5.2.2 Identification and quantification of phenolic compounds

5.2.2.1. Standards: The standards used were from Sigma (St. Louis, MO, USA) or Extrasynthèse (Genay, France). Methanol and formic acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA) before use.

5.2.2.2. Extraction of phenolic compounds: The extraction was achieved as previously reported (Tshikalange et al., 2005) with somemodification: each sample (ca. 1.5 g) was thoroughly mixed with methanol until complete extraction of these compounds (negative reaction to NaOH 20%). The methanolic extract was filtered, evaporated to dryness under reduced pressure (40 ºC) and redissolved in methanol (2 mL) of which 20 μL were injected for HPLC analysis.

5.2.2.3. HPLC-DAD system for analysis of phenolic compounds*:* Chromatographic separation was carried out as reported previously (Vinha et al., 2002), with an analytical HPLC unit (Gilson), using a Spherisorb ODS2 column (250 x 4.6 mm, RP-18, 5 μm particle size, Merck, Darmstadt, Germany) with the solvent system water–formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 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, 100% B at 66 min and 100% B at 68 min. The flow rate was 0.9 mL/min. Detection was achieved with a diode array detector, and chromatograms were recorded at 280, 320 and 500 nm. Spectral data from all peaks were accumulated in the range 200–600 nm. The data were processed on a Unipoint® system software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards at 350 nm.

5.2.3. Antimicrobial Activity

5.2.3.1. Materials and Reagents: Ampicillin and cycloheximide (analytical standard compounds), were of the highest available quality, and purchased from Merck (Darmstadt, Germany). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

5.3.3.2. Extract preparation: Powdered sample (5g) was extracted with 250 mL of boiling water for 45 min and filtered through Whatman nº 4 paper. The aqueous extract was frozen, lyophilized and redissolved in water at a concentration of 50 mg/ml, and analysed for their antimicrobial activity.

5.3.3.3. Microorganisms and culture conditions: The bacterial strains used were *Bacillus cereus* CECT 148, *B. subtilis* CECT 498, *Staphylococus aureus* ESA 7, *Escherichia coli* CECT 101, *Pseudomonas aeruginosa* CECT 108, and *Klebsiella pneumoniae* ESA 8. The fungi strains used were *Candida albicans* CECT 1394 and *Cryptococcus neoformans* ESA 3. Microorganisms CECT were obtained from the Spanish type culture collection (CECT) of Valencia University, while microorganisms ESA were clinically isolated strains identified in Microbiology Laboratory of Escola Superior Agrária de Bragança.

Microorganisms were cultured aerobically at 37 ºC in nutrient agar medium for bacteria, and at 30 ºC (Scientific 222 oven model, 2003) in sabouraud dextrose agar medium for fungi. Before experimental use, cultures from solid medium were subcultivated in liquid media, incubated and used as the source of inoculums for each experiment.

5.3.3.4. Preliminary assays for antimicrobial activity: A screening of antibacterial activities with three Gram - (*E. coli*, *P. aeruginosa* and *K. pneumoniae*) and three Gram + bacteria (*B. subtilis*, *B. cereus* and *S. aureus*) was performed; antifungal activity (*C. albicans*, *C. neoformans*) was also assessed, and the minimal inhibitory concentrations (MICs in mg/mL) were determined by an adaptation of the agar streak dilution method based on radial diffusion (Hawkey & Lewis, 1994; Ferreira et al., 2004). Suspensions of the microorganism were prepared to contain approximately 10^8 cfu/mL, and the plates containing agar medium were inoculated (100 μL). A 50 μL volume of each sample (2-50 mg/mL) was pipetted into a hole (depth 3 mm, diameter 4 mm) made in the centre of the agar. Under the same conditions, different concentrated solutions of ampicillin (antibacterial) and cycloheximide (antifungal) were used as control standards. The assays with the standards were carried out using DMSO solutions because water could not dissolve the compounds. After toxicity assays this solvent showed to be not toxic (did not inhibited per si microorganisms growth).

The MIC was considered to be the lowest concentration of the tested sample to inhibit growth of bacteria or fungi on the plate, after 24h. The diameters of the inhibition zones corresponding to the MICs were measured in millimeters with an accuracy of 0.5 mm using a ruler. Each inhibition zone diameter was measured three times (three different plates) and the average was taken. A control using only inoculation was also carried out.

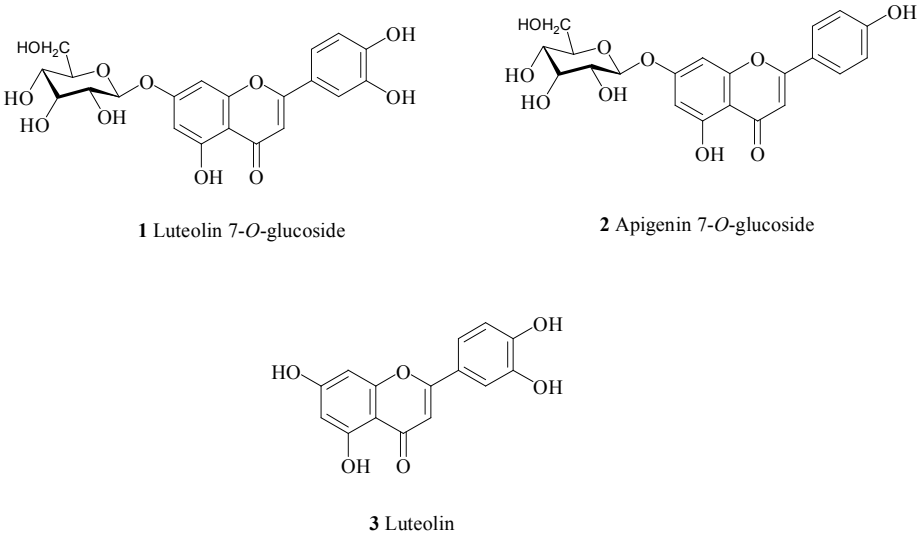
5.3.3.5. Test assays for antibacterial activity: A macro-broth-dilution technique was used to determine the growth inhibition of the susceptible bacteria (*B. subtilis*, *B. cereus*, *S. aureus*, *E. coli*, and *K. pneumoniae*) to "*alcaparra*" previously screened using the radial diffusion on agar. A nutrient medium (10g tryptone, 5g meat extract, water to 1 L) was used to prepare the inocula after being sterilized in an autoclave (P Selecta model, 2002) at 121 ºC for 20 min). Erlenmeyer flasks (50 ml) with 10 ml of the liquid culture medium were inoculated with the bacteria suspension (10^8 cfu/ml) and each extract concentration to be tested (0.05- 10 mg/mL) was added. Incubation was carried out for 24 hours at 37 ºC in a rotary shaker (Stuart Scientific SI50 model, 2001) at 150 rpm. The growth of bacteria cultures was monitored by measuring optical density at 540 nm in a UV–visible spectrophotometer (Varian Cary 50 Scan model, 1998). Controls were carried out in the same conditions but in the absence of sample extract. All assays were carried out under aseptic conditions. Values for bacteria growth rate were obtained by linear regression analysis in at the exponential growth range in the graphs of optical density at 540 nm versus incubation time. The equation curve slope was considered the rate of bacteria growth and was expressed relatively to the control (100%). IC_{50} values (extract concentration which inhibits 50% of bacterial growth) were also determinate.

5.3. Results and discussion

5.3.1. Phenolic compounds in "alcaparra" olive

"*Alcaparra*" table olive presented a chemical profile composed of three identified phenolic compounds: luteolin 7-*O*-glucoside, apigenin 7-*O*-glucoside and luteolin (Figure 1 and 2). Three unidentified flavonoidic compounds (a, b and c) and one hydroxycinnamic derivative were also detected in the methanolic extract (Figure 2). The

existence of several other compounds, namely hydroxytyrosol, tyrosol, 5-*O*-caffeoylquinic acid, verbascoside, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, oleuropein, rutin and quercetin 3-*O*-rhamnoside, described in olive fruits (Vinha et al., 2005), was also checked, but it were not detected in the analysed sample. Other extraction solvents were used, namely methanol, hydromethanol and water, presenting all the extracts the same qualitative composition and similarities in the quantitative analysis. As far as we know, it is the first time that information about phenolic compounds in "*alcaparra*" stoned table olives is reported.



1 Luteolin 7-*O*-glucoside

2 Apigenin 7-*O*-glucoside

3 Luteolin

Figure 1. Flavonoid compounds identified in the "*alcaparra*" sample.

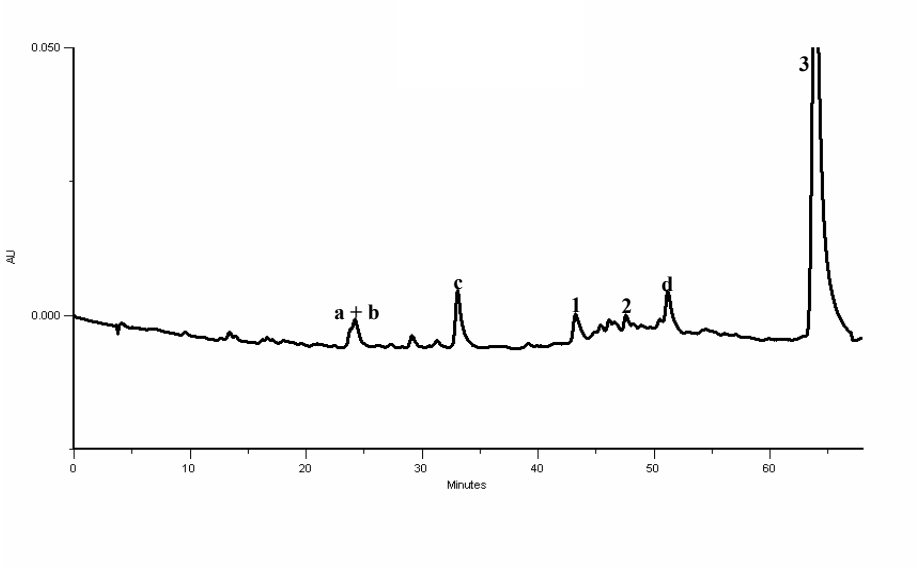


Figure 2. HPLC phenolic profile of "alcaparra" table olive sample. Detection at 320 nm. (1) luteolin 7-*O*-glucoside; (2) apigenin 7-*O*-glucoside; (3) luteolin; (a), (b), (c) unidentified flavonoidic derivatives; (d) unidentified hydroxycinnamic acid derivative.

The quantification of the identified phenolics (Table 1) revealed that luteolin was the compound present in the highest amount, corresponding to ca. 91% of total amount of identified compounds, while apigenin 7-*O*-glucoside was the minor one (ca. 1% of total phenolics).

Several studies on phenolic composition of other different kinds of table olives, namely Spanish-style green olives in brine, naturally or turning colour table olives in brine, Kalamata olives in brine and oxidized table olives in brine were performed (Marsilio et al., 2001; Blekas et al., 2002; Owen et al., 2003; Romero et al., 2004; Boskou et al., 2006). These studies showed that the major phenolic compounds present in table olives were tyrosol, hydroxytyrosol and oleanolic acid and that their concentration depended of the degree of maturation and the treatment method of olive drupe till they become edible.

Table 1. Phenolic compounds content in table olive "alcaparra" sample (mg/kg, dry basis)[a].

Compound		**Mean**	**SD**
1	luteolin 7-*O*-glucoside	9.3	0.1
2	apigenin 7-*O*-glucoside	1.4	0.0
3	luteolin	111.7	2.5
	Σ	122.4	

[a] Results are expressed as mean of three determinations. SD: standard deviation. ∑: sum of the determined phenolic compounds

"*Alcaparra*" table olives show a singular phenolic profile that is most probably related with the technological process to which they are subjected. As mentioned above, the bitterness of "*alcaparra*" is removed in the pulp washing, and this may justify the absence of secoiridoids like oleuropein. In addition, glycosidic phenolic compounds and other polar compounds presenting higher water solubility may also be extracted during this process, leading to its loss. So, due to its less polarity, the existence of a very high content of luteolin in "*alcaparra*" table olives is not surprising.

5.3.2. Antimicrobial assays

The "*alcaparra*" aqueous extract was screened for its antimicrobial activity against *B. cereus*, *B. subtilis, S. aureus* (Gram +), *E. coli*, *P. aeruginosa, K. pneumoniae* (Gram -) bacteria, and *C. albicans* and *C. neoformans* (fungi). Aside from concerns with food quality degradation, these microorganisms may be causal agents of intestinal infections in humans. The minimal inhibitory concentration (MIC) values for bacteria and fungi (Table 2) were determined as an evaluation of the antimicrobial activity of the tested "*alcaparra*" sample.

The extract inhibited all the tested bacteria, with the exception of *P. aeruginosa* (Gram -). *B. cereus* and *K. pneumoniae* were the most sensitive Gram + and Gram - bacteria, respectively (Table 2). However, the tested fungal species *C. albicans* and C. *neoformans* were resistant to "*alcaparra*" extract.

As expected, the MICs of antibacterial (ampiciline) and antifungal (cycloheximide) control standards were lower than those for "*alcaparra*" extract. Usually, pure active compounds reveal to have more activity than crude extracts.

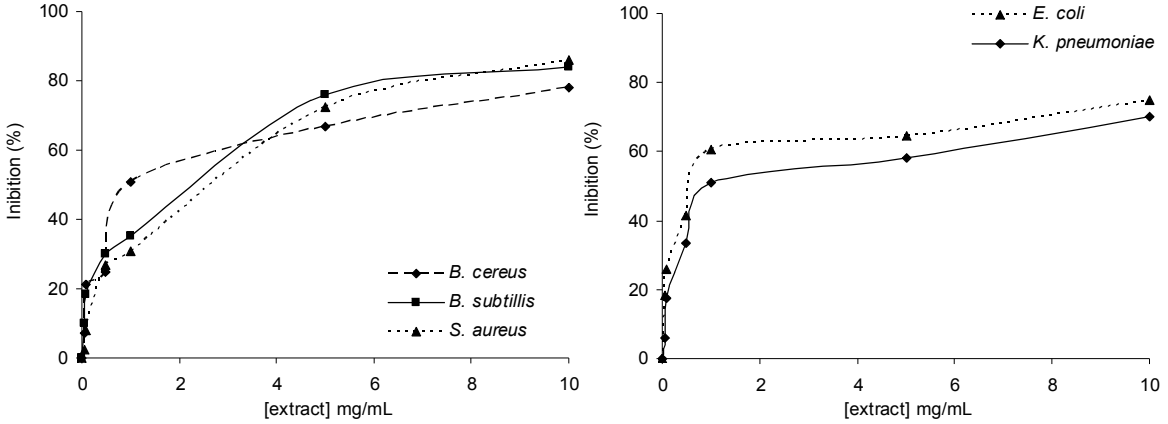


Figure 3. Inhibition (%) of bacterial growth by different concentrations of "Alcaparra" extract.

Only the microorganisms that exhibited susceptibility in the screening performed in solid medium were submitted to assays in liquid medium, in order to determine their growth rates.

Table 2. Antimicrobial activity of the "alcaparra" extract.

	MIC (mg/mL)							
Samples	*B. cereus*	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*K. peumoniae*	*C. albicans*	*C. neoformans*
Alcaparra	25	25	25	50	50	10	50	50
	(+ + + +)	(+ +)	(+ +)	(-)	(+ + +)	(+ +)	(-)	(-)
Ampicillin	0.00313	0.0125	0.00625	0.00625	0.00625	0.00625	NT	NT
	(+ + + +)	(+ + + +)	(+ + + +)	(+ + + +)	(+ + + +)	(+ + + +)		
Cycloheximide	NT	NT	NT	NT	NT	NT	0.0125	0.00625
							(+ +)	(+ + + +)

No antimicrobial activity (-), inhibition zone < 1 mm. Slight antimicrobial activity (+), inhibition zone 2-3 mm. Moderate antimicrobial activity (+ +), inhibition zone 4-5 mm. High antimicrobial activity (+ + +), inhibition zone 6-9 mm. Strong antimicrobial activity (+ + + +), inhibition zone > 9 mm. Standard deviation ± 0.5 mm. NT- Not tested.

Table 3. IC_{50} values of "*alcaparra*" extract.

Bacterial species	IC_{50} (mg/mL)
B. cereus	0.981
B. subtilis	2.445
S. aureus	2.841
E. coli	0.722
K. pneumoniae	0.813

The values obtained suggested a broad antimicrobial activity of "*alcaparra*" extract in a concentration-dependent manner, against the tested bacterial strains. According to the percentage of inhibition of bacteria growth in the presence of different extract concentrations (Figure 3) "alcaparra" extract presented antibacterial capacity in the order *E. coli* > *K. pneumoniae* > *B. cereus* > *B. subtilis* > *S. aureus* (Table 3).

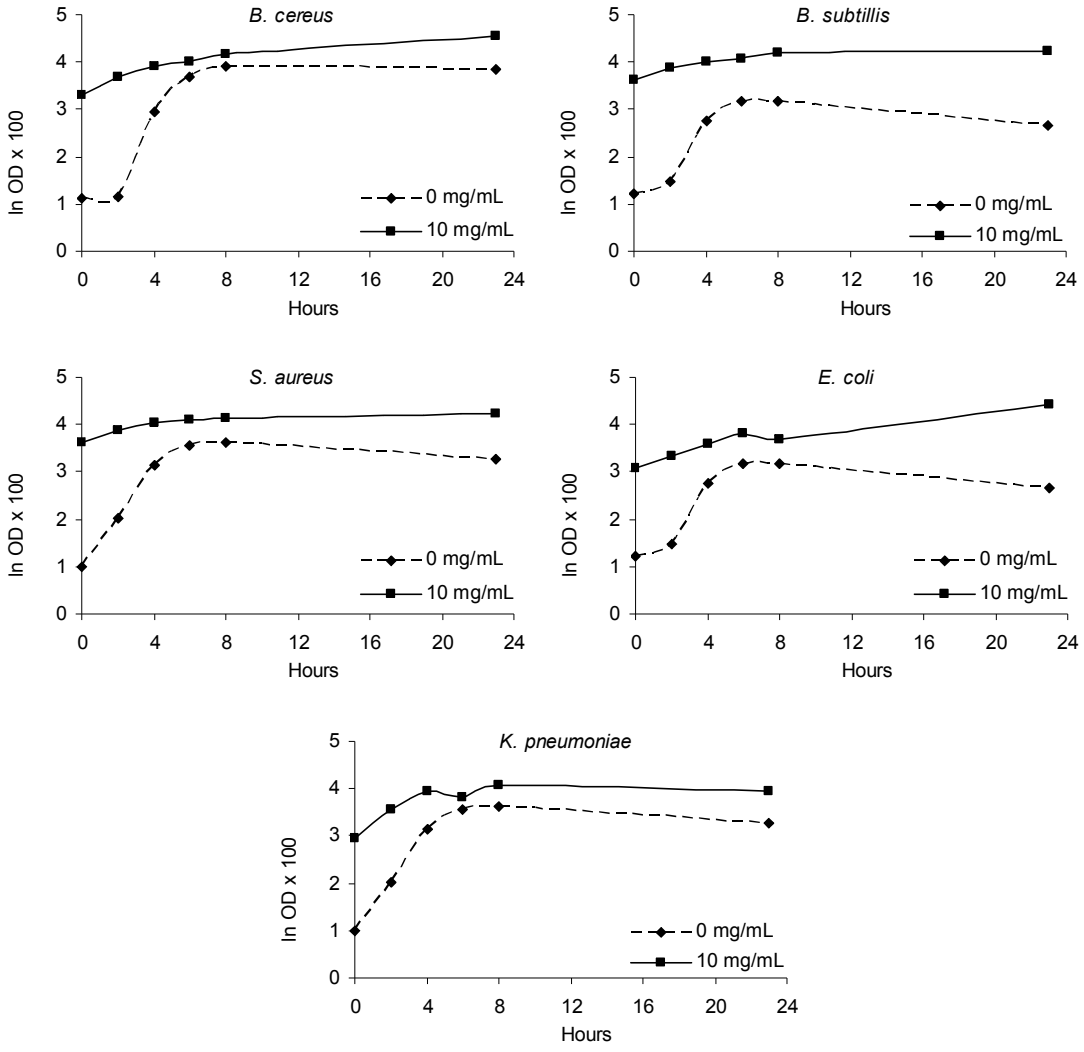


Figure 4. Bacterial growth in the presence of "*alcaparra*" extract (10 mg/mL) and in the absence of extract (0 mg/mL) along the incubation period.

Certainly the chemical composition of "*alcaparra*" extract conditioned the antibacterial effects observed. The high content of luteolin and the other phenolic compounds identified in the extract might contribute for its antimicrobial properties. In fact, the antimicrobial action of phenolics is well-known and it is related to their ability to denature proteins, being generally classified as surface-active agents Denyer & Stewart, 1998). They act by causing the leakage of cytoplasmic constituents such as protein, glutamate or potassium and phosphate from bacteria, which may be due to disruption of cell peptidoglycan or damage of the cell membrane. The mode of action of phenolic compounds has been shown to be concentration dependent (Cowan, 1999; Furneri et al., 2002). In particular, luteolin has been described to possess antimicrobial activity against several bacterial species (Basile et al., 1999; Sato et al., 2000; Cottiglia et al., 2001; Tshikalange et al., 2005).

In conclusion, data obtained in this study demonstrate that "alcaparra" table olive may be a good candidate for employment as antimicrobial agent against bacteria responsible for human gastrointestinal and respiratory tract infections. These results are particularly important against *S. aureus*, a microorganism extensively studied due to its ability to produce enterotoxins, and against *E. coli*, both exceptionally resistant to a number of phytochemicals. Thus, it seems that dietary intakes of "*alcaparra*" may lower the risk of bacterial infections particularly in the intestinal tract, mainly due to the protective action provided by its phenolic compounds.

5.4. References and notes

Baldioli, M.; Servili, M.; Perretti, G. & Montedoro, G. F. (1996). Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. *Journal of the American Oil Chemists' Society*, *73*, 1589-1593.

Basile, A.; Giordano, S., López-Sáez, J.A. & Cobianchi, R.C. (1999). Antibacterial activity of pure flavonoids isolated from mosses**.** *Phytochemistry*, *52*, 1479-1482.

Battinelli, L.; Daniele, C.; Cristiani, M.; Bisignano, G.; Saija, A. & Mazzanti, G. (2006). In vitro antifungal and anti-elastase activity of some aliphatic aldehydes from *Olea europaea* L. fruit. *Phytomedicine*, *13,* 558-563.

Bisignano, G.; Tomaino, A.; Lo Cascio, R.; Crisafi, G.; Uccella, N. & Saija, A. (1999). On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol. *The Journal of Pharmacy and Pharmacology*, *51*, 971-974.

Blekas, G., Vassilakis, C., Harizanis, C., Tsimidou, M., & Boskou, D.G. (2002). Biophenols in table olives. *Journal of Agriculture and Food Chemistry*, *50*, 3688-3692.

Boskou, G., Salta, F.N., Chrysostomou, S., Mylona, A., Chiou, A., & Andrikopoulos, N.K. (2006). Antioxidant capacity and phenolic profile of table olives from the Greek market. *Food Chemistry*, *94*, 558-564.

Brenes, M.; García, A.; García, P.; Rios, J.J. & Garrido, A. (1999). Phenolic compounds in Spanish olive oils. *Journal of Agricultural and Food Chemistry*, *47*, 3535-3540.

Cottiglia, F.; Loy, G.; Garau, D.; Floris, C.; Casu, M.; Pompei, R. & Bonsignore, L. (2001). Antimicrobial evaluation of coumarins and flavonoids from the stems of *Daphne gnidium. Phytomedicine*, *8*, 302-305

Cowan, M.C. (1999). Plants products as antimicrobial agents. *Clinical Microbiology Reviews, 12*, 564-582

Denyer, S.P. & Stewart, G.S.A.B. (1998). Mechanisms of action of disinfectants. *International Biodeterioration & Biodegradation*, *41*, 261-268.

Ferreira, I.C.F.R.; Calhelha, R.C.; Estevinho, L. M.; Queiroz, M-J.R.P. Screening of antimicrobial activity of diarylamines in the 2,3,5-trimethylbenzo[b]thiophene series: a structure–activity evaluation study. *Bioorganic & Medicinal Chemistry Letters*, *14*, 5831-5833.

Furneri, P.M.; Marino, A.; Saija, A.; Uccella, N. & Bisignano, G. (2002). *In vitro* antimycoplasmal activity of oleuropein. *International Journal of Antimicrobial Agents*, *20*, 293-296.

Hawkey P.M.; Lewis, D.A. *Medical Bacterology- A Practical Approach. Oxford University*, UK, 1994; pp 181-194.

Keys, A. (1995). Mediterranean diet and public health: Personal reflections. *American Journal of Clinical Nutrition*, *61*, 1321S-1323S.

Litridou, M.; Linssen, J.; Schols, H.; Bergmans, M.; Posthumus, M.; Tsimidou, M. & Boskou, M. (1997). Phenolic compounds in virgin olive oils: Fractionation by solid phase extraction and antioxidant activity assessment *Journal of the Scince and Food and Agriculture, 74*, 169-174.

Markin, D.; Duek, L. & Berdicevsky, I. (2003). In vitro antimicrobial activity of olive leaves. *Mycose*s, *46*, 132-136.

Marsilio, V., Campestre, C., & Lanza, B. (2001). Phenolic compounds change during California-style ripe olive processing. *Food Chemistry*, *74*, 55-60.

Nychas G.J.E.; Tassos, S.C. & Board, R.G. (1990). Phenolic extract from olives: inhibition of *Staphylococcus aureus***.** *Letters in Applied Microbiolology*, *10*, 217-220.

Owen, R. W., Haubner, R., Mier, W., Giacosa, A., Hull, W.E., Spiegelhalder, B. & Bartsch, H. (2003). Isolation, structure and antioxidant potential of the major phenolic and flavonoid compounds in brined olive drupes. *Food and Chemical Toxicology*, *41*, 703-717.

Owen, R. W., Mier, W., Giacosa, A., Hull, W. E., Spielgelhalder, B., & Bartsch, H. (2000). Phenolic compounds and squalene in olive oils: The concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. *Food Chemistry Toxicology*, *38*, 647-659.

Romero, C., Brenes, M., Yousfi, K., García, P., García, A., & Garrido, A. (2004). Effect of cultivar and processing method on the contents of polyphenols in table olives. *Journal of Agriculture and Food Chemistry*, *52*, 479-484.

Ryan. M. & Robards, K. (1998). Phenolic compounds in olives. *Analyst, 123*, 31R-44R

Sato, Y.; Suzaki, S., Nishikawa, T.; Kihara, M.; Shibata, H. & Higuti, T. (2000). Phytochemical flavones isolated from *Scutellaria barbata* and antibacterial activity against methicillin-resistant *Staphylococcus aureus. Journal of Ethnopharmacolology, 72*, 483-488.

Soler-Rivas, C.; Espín, J.C. & Wichers, H.J. (2000). Oleuropein and related compounds. *Journal of the Science of Food and Agriculture*, *80*, 1013-1023.

Trichopoulou, A., & Lagiou, P. (1997). Healthy traditional Mediterranean diet: An expression of culture, history and lifestyle. *Nutrition Reviews*, *55*, 383-389.

Tshikalange, T.E.; Meyer, J.J.M. & Hussein, A.A. (2005). Antimicrobial activity, toxicity and the isolation of a bioactive compound from plants used to treat sexually transmitted diseases. *Journal of Ethnopharmacology, 96*, 515-519.

Vinha, A. F., Ferreres, F., Silva, B.M., Valentão, P., Gonçalves, A., Pereira, J. A., Oliveira, M.B.P.P., Seabra, R.M., & Andrade, P.B. (2005). Phenolic profiles of Portuguese olive fruits (*Olea europaea* L.): influence of cultivar and geographical origin. *Food Chemistry*, *89*, 561-568.

Vinha, A. F.; Silva, B.M.; Andrade, P.B.; Seabra, R.M.; Pereira, J. A. & Oliveira, M.B.P.P. (2002). Development and evaluation of an HPLC/DAD method for the analysis of phenolic compounds from olive fruit. *Journal Liquid Chromatography & Related Technologies*, *25*, 151-160.

Capítulo 6

Discussão geral e conclusões

Discussão geral e conclusões

As azeitonas de mesa descaroçadas em pedaços, denominadas de "*alcaparras*", são um tipo de azeitona processada de forma tradicional na região de Trás-os-Montes. Contrariamente aos restantes tipos de azeitonas, durante o processamento das "*alcaparras*" não ocorre nenhum tipo de fermentação. O amargor característico de frutos não processados não é eliminado ou "biotransformado" pela presença de microrganismos mas sim por passagens sucessivas das azeitonas por água para que ocorra a lavagem dos compostos responsáveis pelo amargor que são, na sua generalidade, hidrossolúveis em água.

Nutricionalmente este tipo de azeitonas apresenta uma composição muito semelhante às restantes azeitonas de mesa, visto que a matéria-prima que lhes dá origem é a mesma. Contudo, e uma vez que os frutos utilizados no seu processamento são colhidos da oliveira sem que o azeite se encontre completamente formado, usualmente durante o mês de Setembro e Outubro, este tipo de azeitonas são menos ricas em gordura. Por outro lado também como apresentam um elevado teor em humidade, quer pela época em que são colhidas quer pela forma de processamento, são usualmente menos calóricas que as restantes (em média 178 kcal/100 g de azeitonas).

A sua composição em ácidos gordos, em tudo semelhante à do azeite produzido na região e que se insere na região da Denominação de Origem Protegida "Azeite de Trás-os-Montes DOP", conferem-lhe propriedades nutricionais únicas, com um elevado teor em ácidos gordos monoinsaturados, onde predomina o ácido oleico (representando em média 77,7% da gordura), e um teor reduzido em ácidos gordos saturados.

As "*alcaparras*" contêm na sua composição também uma quantidade apreciável de compostos com elevada actividade biológica como os tocoferóis. Este compostos, importantes precursores da vitamina E, apresentam elevada capacidade protectora de oxidação das gorduras, contribuindo para o aumento da estabilidade das azeitonas produzidas e, quando ingeridos, para um aumento do teor em antioxidantes no organismo, protegendo-o de eventos oxidativos.

Apesar dos compostos fenólicos presentes na azeitona serem hidrosolúveis e serem "lavados" ou degradados durante o processo de obtenção das "*alcaparras*", estas azeitonas contém ainda um teor elevados em fenóis totais. Deste grupo de compostos foram identificados três importantes flavonóides: luteolina 7-O-glucosido, apigenina 7-

O-glucosido e luteolina. São sobejamente conhecidas as propriedades e acção protectora que os compostos fenólicos exercem no organismo humano quando ingeridos contribuindo para a prevenção de diversas patologias de entre as quais se destacam vários tipos de cancro.

Os extractos aquosos obtidos de "*alcaparras*" mostraram ser eficientes na extracção de compostos bioactivos comparativamente à extracção com solventes. A avaliação da actividade antioxidante desta fracção, testada por dois métodos diferentes, mostrou que este tipo de azeitonas possui um elevado poder antioxidante, estando esse poder correlacionado com o teor em fenóis totais.

Quando avaliada a acção dos extractos de "*alcaparras*" sobre diferentes microorganismos capazes de causar doenças no homem, estes mostraram ser inibidores do crescimento de alguns tipos de bactérias quer Gram-positivo quer Gram-negativo.

A composição química das "*alcaparras*" e a elevada actividade biológica (antioxidante e antimicrobiana) que revelaram possuir, vem demonstrar que este tipo de azeitonas quando ingeridas, e incluídas numa dieta mediterrânica, apresentam elevados benefícios para a saúde dos seus consumidores, aumentando-lhe as defesas do organismo quer contra agentes de stress oxidativo quer contra organismos patogénicos.

O trabalho apresentado contribuiu para uma primeira caracterização das azeitonas verdes descaroçadas tipo "*alcaparra*" produzidas na região de Trás-os-Montes. A informação obtida pode servir de base para uma possível criação de uma protecção especial, como seja uma Denominação de Origem Protegida. Contudo factores como sejam a cultivar, o período de colheita das azeitonas, e o processo tecnológico são aspectos que justificam a variabilidade detectada nas amostras avaliadas e que necessitam de ser clarificados em trabalhos posteriores.