CRISTIANO AUGUSTO BALLUS

CHEMICAL CHARACTERIZATION AND ANTIOXIDANT CAPACITY OF EXTRA-VIRGIN OLIVE OILS FROM BRAZIL AND OTHER COUNTRIES USING ELECTROPHORETIC, CHROMATOGRAPHIC AND SPECTROMETRIC TECHNIQUES

CARACTERIZAÇÃO QUÍMICA E CAPACIDADE ANTIOXIDANTE DE AZEITES DE OLIVA EXTRAVIRGEM PROVENIENTES DO BRASIL E DE OUTROS PAÍSES UTILIZANDO TÉCNICAS ELETROFORÉTICAS, CROMATOGRÁFICAS E ESPECTROMÉTRICAS

CAMPINAS

2014

UNIVERSIDADE ESTADUAL DE CAMPINAS

Faculdade de Engenharia de Alimentos

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Thesis presented to the Faculty of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Ph.D. in Food Science.

Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Ciência de Alimentos.

Supervisor/*Orientadora*: Profª. Dra. Helena Teixeira Godoy

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELO ALUNO CRISTIANO AUGUSTO BALLUS E ORIENTADA PELA PROFª. DRA. HELENA TEIXEIRA GODOY

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Assinatura do Orientador

CAMPINAS

2014

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Engenharia de Alimentos
Márcia Regina Garbelini Sevillano - CRB 8/3647

B214c

Ballus, Cristiano Augusto, 1985-
Chemical characterization and antioxidant capacity of extra-virgin olive oils from Brazil and other countries using electrophoretic, chromatographic and spectrometric techniques / Cristiano Augusto Ballus. – Campinas, SP : [s.n.], 2014.

Orientador: Helena Teixeira Godoy.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos.

1. Azeite de oliva extravirgem. 2. Compostos fenólicos. 3. Cromatografia líquida. 4. Eletroforese capilar. 5. Espectrometria de massas. I. Godoy, Helena Teixeira. II. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Caracterização química e capacidade antioxidante de azeites de oliva extravirgem provenientes do Brasil e de outros países utilizando técnicas eletroforéticas, cromatográficas e espectrométricas
Palavras-chave em inglês:
Extra-virgin olive oil
Phenolic compounds
Liquid chromatography
Capillary electrophoresis
Mass spectrometry
Área de concentração: Ciência de Alimentos
Titulação: Doutor em Ciência de Alimentos
Banca examinadora:
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Data de defesa: 25-02-2014
Programa de Pós-Graduação: Ciência de Alimentos

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ABSTRACT

Consumption of extra-virgin olive oil (EVOO) is highly recommended for its benefits to human health. In Brazil, consumption of EVOO, which is imported from other countries, is increasing annually. In the last years, Brazil started to produce EVOO, although in an experimental way. In chapter 1, a literature review highlighting the most relevant studies on the chemical composition and the health benefits of the extra-virgin olive oil is presented. In chapter 2, the aim was to determine the total phenolic content (TPC) and the antioxidant capacity (AC), as well as the correlation between TPC and each one of the four AC methods, of 15 EVOO brands, each one in three batches, resulting in 45 samples. TPC was evaluated by Folin-Ciocalteu reagent method, while the AC was assessed using FRAP, ABTS, DPPH$^{\bullet}$ and ORAC assays. The TPC varied from 70 to 297 mg GAE kg^{-1}, FRAP from 114 to 1557 µmol TE kg^{-1}, ABTS from 0.5 to 1.9 mmol TE kg^{-1}, DPPH$^{\bullet}$ from 72 to 1129 µmol TE kg^{-1}, and ORAC from 1.1 to 12.9 µmol TE g^{-1}. High and significant correlation was found between the TPC and each one of the AC methods evaluated in this study (FRAP, r^2 = 0.8904; p < 0.001; ABTS, r^2 = 0.7837; p < 0.001; DPPH$^{\bullet}$, r^2 = 0.7908; p < 0.001; ORAC, r^2 = 0.7431; p < 0.001). Therefore, most of the EVOO brands presented a considerable TPC and high AC values. In chapter 3, the aim was to optimize the separation of 17 phenolic compounds already detected in EVOO. A Doehlert matrix experimental design was used, evaluating the effects of pH and electrolyte concentration. Resolution, runtime and migration time relative standard deviation values were used as responses. Derringer's desirability function was used to simultaneously optimize all 37 responses. The 17 peaks were separated in 19 minutes using a fused-silica capillary (50 µm internal diameter, 72 cm of effective length) with an extended light path and 101.3 $mmol.L^{-1}$ of boric acid electrolyte (pH 9.15, 30 kV). The method was validated and applied to 15 EVOO samples found in Brazilian supermarkets. In chapter 4, the aim was to determine the phenolic compounds, tocopherols and fatty acids contents of 17 monovarietal EVOOs produced in Minas Gerais state,

during two crop years. Compounds identified comprised palmitic acid (6-12.6%), palmitoleic acid (0.2-2.5%), stearic acid (1.6-2.2%), oleic acid (70.8-84.3%), linoleic acid (3.2-11.7%), α-linolenic acid (0.6-1.4), arachidic acid (0.4-0.8%), 9-eicosenoic acid (0.4-0.9%), tyrosol (NQ-155.21 mg kg^{-1}), (+)-pinoresinol (2.89-22.64 mg kg^{-1}), hydroxytyrosol (ND-37.74 mg kg^{-1}), luteolin (ND-2.23 mg kg^{-1}), α-tocopherol (28.92-232.93 mg kg^{-1}), β-tocopherol (ND-9.56 mg kg^{-1}), γ-tocopherol (ND-18.75 mg kg^{-1}). Some of these monovarietal EVOOs presented results similar to those described in the literature. The aim of chapter 5 was to determine the phenolic compound contents of Brazilian EVOO, using rapid-resolution liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (RRLC-ESI-TOF-MS). A total of 25 EVOO samples from Rio Grande do Sul, Santa Catarina and Minas Gerais states and two crops, were analyzed. It was possible to identify and quantify 20 phenolic compounds, belonging to the phenolic alcohol, secoiridoid, lignan and flavonoid classes. EVOOs from Coratina (364 mg kg^{-1}), Arbosana (255 mg kg^{-1}) and Grappolo (228 mg kg^{-1}) varieties presented the highest total phenolic contents. The results showed that Brazilian EVOOs are promising concerning the total phenolic contents, since the values were comparable to those from high-quality EVOOs produced in other countries.

Keywords: extra-virgin olive oil; chemical composition; phenolic compounds; capillary electrophoresis; RRLC-ESI-TOF-MS; optimization.

RESUMO

O consumo de azeite de oliva extravirgem (EVOO) é altamente recomendado por seus benefícios à saúde humana. No Brasil, a ingestão de EVOO, o qual é importado de outros países, vem aumentando anualmente. Nos últimos anos, o Brasil começou a produzir EVOO, porém de maneira experimental. No capítulo 1 foi apresentada uma revisão bibliográfica destacando os estudos mais relevantes acerca do composição química e dos benefícios à saúde do azeite de oliva extravirgem. No capítulo 2, o objetivo foi determinar o teor de fenólicos totais (TFT) e a capacidade antioxidante (CA), bem como a correlação entre o TFT e cada um dos quatro métodos de CA, de 15 marcas de EVOO, cada qual em três lotes, resultando em 45 amostras. O TFT foi avaliado pelo método do reagente de Folin-Ciocalteu, enquanto a CA foi determinada pelos ensaios de FRAP, ABTS, DPPH$^{\bullet}$ e ORAC. O TFT variou de 70 a 297 mg EAG kg^{-1}, FRAP de 114 a 1557 µmol ET kg^{-1}, ABTS de 0,5 a 1,9 mmol ET kg^{-1}, DPPH$^{\bullet}$ de 72 a 1129 µmol ET kg^{-1}, e ORAC de 1,1 a 12,9 µmol ET g^{-1}. Houve elevada e significativa correlação entre o TFT e cada um dos métodos de CA (FRAP, r^2 = 0,8904; p < 0,001; ABTS, r^2 = 0,7837; p < 0,001; DPPH$^{\bullet}$, r^2 = 0,7908; p < 0,001; ORAC, r^2 = 0,7431; p < 0,001). Portanto, a maioria das marcas de EVOO apresentaram considerável TFT e elevados valores de CA. No capítulo 3, o objetivo foi otimizar a separação de 17 compostos fenólicos previamente detectados em EVOO. Foi utilizado um planejamento experimental Doehlert, avaliando-se o pH e a concentração do eletrólito. Resolução, tempo de corrida e coeficientes de variação dos tempos de migração foram as respostas. A função de desejabilidade de Derringer foi utilizada para otimizar simultaneamente as 37 respostas. Os 17 picos dos compostos foram separados em 19 minutos em capilar de sílica fundida (50 µm diâmetro interno, 72 cm comprimento efetivo) com bulbo estendido e eletrólito ácido bórico 101,3 mmol L^{-1} (pH 9,15, 30 kV). O método foi validado e aplicado em 15 amostras comerciais de EVOO. No capítulo 4, o objetivo foi determinar o teor de compostos fenólicos, tocoferóis e ácidos graxos de 17 EVOO monovarietais produzidos em Minas

Gerais, durante duas colheitas. Foram quantificados os ácidos palmítico (6-12,6%), palmitoleico (0,2-2,5%), esteárico (1,6-2,2%), oleico (70,8-84,3%), linoleico (3,2-11,7%), α-linolênico (0,6-1,4), araquídico (0,4-0,8%), 9-eicosenoico (0,4-0,9%) e os compostos tirosol (NQ-155,21 mg kg^{-1}), (+)-pinoresinol (2,89-22,64 mg kg^{-1}), hidroxitirosol (ND-37,74 mg kg^{-1}), luteolina (ND-2,23 mg kg^{-1}), α-tocoferol (28,92-232,93 mg kg^{-1}), β-tocoferol (ND-9,56 mg kg^{-1}) e γ-tocoferol (ND-18,75 mg kg^{-1}). Em geral, os resultados foram similares aos descritos na literatura. O objetivo do capítulo 5 foi determinar o teor de compostos fenólicos em EVOO brasileiros por cromatografia líquida de rápida resolução acoplada à espectrometria de massas por tempo de voo com ionização por *electrospray* (RRLC-ESI-TOF-MS). Foram analisadas 25 amostras de EVOO do Rio Grande do Sul, Santa Catarina e Minas Gerais e duas colheitas. Foram identificados e quantificados 20 compostos fenólicos das classes dos alcoóis fenólicos, secoiridoides, lignanas e flavonoides. Os teores de compostos fenólicos totais nos EVOOs destacaram-se nas variedades Coratina (364 mg kg^{-1}), Arbosana (255 mg kg^{-1}) e Grappolo (228 mg kg^{-1}). Desta forma, os EVOOs brasileiros são promissores no que se refere ao teor de compostos fenólicos totais, visto que os valores são comparáveis àqueles dos EVOOs de elevada qualidade produzidos em outros países.

Palavras-chave: azeite de oliva extravirgem; composição química; compostos fenólicos; eletroforese capilar; RRLC-ESI-TOF-MS; otimização.

SUMÁRIO

"Nobody ever figures out what life is all about, and it doesn't matter. Explore the world. Nearly everything is really interesting if you go into it deeply enough."

Richard P. Feynman

"Somewhere, something incredible is waiting to be known."

Carl Sagan

"You have no responsibility to live up to what other people think you ought to accomplish. I have no responsibility to be like they expect me to be. It's their mistake, not my failing."

Richard P. Feynman, *Surely You're Joking, Mr. Feynman!*

Dedico este trabalho

aos meus pais, Virgínio e Maria Eli,

às minhas irmãs, Patrícia e Bianca,

ao Sérgio, meu grande amigo,

e a todos que já foram meus professores

nesta longa jornada.

AGRADECIMENTOS

À minha orientadora, professora Dra. Helena Teixeira Godoy, por todo seu apoio, carinho, ensinamentos e, principalmente, pela amizade, da qual sinto muito orgulho. Você é uma pessoa muito especial em todos os sentidos, um exemplo que irei carregar por toda a minha vida. Muito obrigado por tudo.

Aos meus pais, Virgínio e Maria Eli, e às minhas irmãs, Patrícia e Bianca, por todo o amor, carinho, por estarem sempre ao meu lado, compreendendo a ausência e me incentivando a seguir sempre em frente. Devo tudo a vocês.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela concessão da bolsa de Doutorado (processo nº 140965/2010-6).

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de estudos de Doutorado Sanduíche no Exterior (PSDE, processo nº 1580-12-4).

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), pela aprovação do projeto (processo nº 12/04523-5), cujo financiamento tornou possível a aquisição dos materiais e reagentes para a realização de parte dos experimentos desta tese.

Ao professor Dr. Roy Edward Bruns, do Instituto de Química da UNICAMP, pelo seu profissionalismo, disponibilidade e apoio na supervisão dos planejamentos experimentais e na análise estatística dos dados, bem como na redação dos artigos. Sua experiência e dedicação foram imprescindíveis para a execução do nosso trabalho.

Aos membros da banca examinadora, Dr. Renato Grimaldi, Dra. Marisa Aparecida Bismara Regitano D'Arce, Dra. Carla Beatriz Grespan Bottoli, Dr. Jesuí Vergilio Visentainer, Dr. Severino Matias de Alencar, Dra. Marina Franco Maggi Tavares e Dr. Fábio Cesar Gozzo, pelas valiosas correções e sugestões que colaboraram muito para o aprimoramento deste trabalho.

Ao Sérgio, meu grande amigo e companheiro, que esteve ao meu lado durante todo o período do doutorado, sempre disponível para me ajudar no que

fosse necessário. Ter te conhecido foi um presente inestimável, pelo qual serei eternamente grato.

À professora Dra. Juliana Azevedo Lima-Pallone, minha grande amiga, pela amizade, pelo carinho, pelo apoio em inúmeras situações e por todos os bons momentos que passamos nestes quatro anos.

Às minhas colegas e amigas tão especiais, Marla, Ju Hashimoto, Sabrina e Ana Paula, por todos os momentos maravilhosos que compartilhamos, bem como pelo apoio e suporte nos momentos de necessidade. Sem vocês, chegar até aqui teria sido muito mais difícil.

Aos colegas do Laboratório de Análise de Alimentos, Adriana e Francisco (Chico), pela inestimável ajuda na execução dos experimentos, bem como pelos momentos de alegria e descontração que disfrutamos juntos. À Milene, por toda a sua ajuda durante o processo da bolsa para o Doutorado Sanduíche e solicitação do visto, e principalmente durante nossa etapa final, quando nos preparávamos para a defesa. Agradeço também aos demais colegas, Alane, Carlos, Cíntia, Dani Neves, Dani Pane, Dani Rodrigues (Dani Bio), Danilo, Elenice, Janclei, Jessica, Leonardo, Maria Rosa, Mateus, Patrícia Souza, Paula, Pollyane, Stefany, Suélen, Tayse, Thais, Vivian e Wellington, às técnicas Renata, Marcela e Miriam e ao professor Dr. Marcelo Alexandre Prado, por tornarem o laboratório um local tão agradável de se trabalhar.

Ao professor Dr. Antonio Segura-Carretero, da *Universidad de Granada* (Espanha), por ter me aceitado em seu laboratório e por todo o apoio durante o período em que estive no CIDAF (*Centro de Investigación y Desarrollo del Alimento Funcional*). Ao professor Dr. Alberto Fernández-Gutiérrez, chefe do grupo de pesquisa *FQM-297: Environmental, Biochemical and Foodstuffs Analytical Control Research Group*, do Departamento de Química Analítica da *Universidad de Granada*. Ao Hakim, meu grande amigo, por todos os momentos que compartilhamos em Granada. *¡Tu eres un amigo muy especial para mi, y estoy seguro de que tendrás mucho éxito en tu vida!* À Rosa, por ser esta pessoa tão especial e por tudo que aprendi com ela. *¡Te admiro mucho!* Às meninas

Carol, Andreia, Nassima, Celia, Cecilia, Maria Del Mar, Ana Gómez, Patricia e Arantxa, vocês foram muito importantes para mim e me ajudaram muito em diversos momentos. *Muchas gracias por todo, ¡guapas!* Agradeço também aos demais colegas do CIDAF, Isa, Jesús, Ana López, Salvador, María de la Luz, Ibrahim, Cristina, David, Ihsan e Ameni pelo carinho e disponibilidade. Agradeço a todos pelo ano maravilhoso em que trabalhamos e nos divertimos juntos. *¡Granada y vosotros estáis en mi corazón!*

Ao Dirceu, pelo exemplo de profissional dedicado e sempre disposto a ajudar. Seu trabalho foi extremamente importante e facilitou a condução dos experimentos descritos neste trabalho.

Ao pessoal da Secretaria de Pós-Graduação, em especial ao Cosme e ao Marcos, por todo o suporte e paciência, principalmente durante o processo de defesa.

Ao pessoal da Secretaria do DCA, Marcos, Jardete, Guiomar e todos os demais, que foram muito importantes durante todo o período do doutorado.

A todos que já foram meus professores durante esta longa jornada acadêmica.

E agradeço também a todos que, mesmo não sendo citados, colaboraram para a realização deste trabalho e para que eu pudesse chegar até aqui.

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INTRODUÇÃO GERAL

Estudos realizados ao longo das últimas décadas demonstraram que o azeite de oliva extravirgem (EVOO) proporciona diversos benefícios à saúde, de maneira que o seu consumo vem aumentando significativamente em vários países, inclusive no Brasil. Entre os principais responsáveis por estes efeitos destacam-se o elevado teor de ácido oleico (18:1n-9), a presença de ácido α-linolênico (18:3n-3) e os compostos fenólicos, que agem como antioxidantes e sequestradores de radicais livres. O azeite de oliva extravirgem é obtido do fruto da oliveira (*Olea europaea* L.) somente por meios mecânicos, sem nenhum outro tratamento além de lavagem, filtração, decantação ou centrifugação. Desta forma, os componentes minoritários provenientes do fruto da oliveira, entre eles os compostos fenólicos, permanecem no produto final, ao contrário de outros óleos vegetais que foram submetidos aos processos de refino.

O Brasil é um grande importador de azeite de oliva da Argentina e de países da Europa, sendo que, entre janeiro e setembro de 2013, o Brasil importou 50 mil toneladas de azeite de oliva, o que resultou em gastos de US$259 milhões. De 2005/6 a 2011/2, o consumo de azeite de oliva no Brasil cresceu a uma taxa de 17,7% ao ano. Em relação aos azeites de oliva extravirgem importados para comercialização no Brasil, existem diversos trabalhos que avaliaram a qualidade, a ocorrência de adulterações, bem como a determinação de diferentes classes de compostos que constituem sua composição química, como triacilgliceróis, ácidos

graxos e fitosteróis. Entretanto, não foram encontrados estudos que avaliassem a capacidade antioxidante e o teor de compostos fenólicos destes azeites de oliva.

Considerando-se os gastos bastante elevados para a importação do azeite de oliva extravirgem, bem como o fato de que isto resulta em um produto mais caro ao consumidor, surgiu o interesse de cultivar oliveiras no Brasil, de forma a produzir azeite de oliva genuinamente nacional, o qual inclusive poderá apresentar um preço inferior aos produtos importados encontrados no mercado interno. Existem hoje no Brasil algumas tentativas no que se refere ao cultivo experimental de oliveiras para a posterior produção de azeite de oliva nacional. A EMBRAPA - CPACT (Empresa Brasileira de Pesquisa Agropecuária - Centro de Pesquisa Agropecuária de Clima Temperado) em Pelotas, no Rio Grande do Sul, está desenvolvendo um projeto chamado "Introdução e Desempenho Agronômico de Cultivares de Oliveiras no Rio Grande do Sul e em Santa Catarina". A área cultivada com oliveiras no Rio Grande do Sul é de aproximadamente 400 ha, onde as cultivares mais promissoras para extração de azeite são Arbequina, Koroneïki, Arbosana e Picual. Em Santa Catarina, a EPAGRI (Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina) também está conduzindo estudos com oliveiras, nas regiões oeste e extremo oeste do estado. Em Minas Gerais, com o auxílio das pesquisas da EPAMIG (Empresa de Pesquisa Agropecuária de Minas Gerais), há a produção de azeitonas e azeite de oliva no estado. O cultivo está sendo conduzido em 400 ha, com 200.000 plantas cultivadas em 50 municípios

A produção experimental de azeite de oliva extravirgem no Brasil vem apresentando resultados promissores, de maneira que uma futura produção em escala industrial torna-se bastante provável. Com base nisso, é de fundamental importância caracterizar a composição química destes azeites nacionais, pois a qualidade dos mesmos está diretamente relacionada às diversas classes de componentes químicos que constituem o produto. Até o momento, não foram encontrados na literatura nenhum estudo apresentando dados a respeito dos teores de compostos fenólicos e tocoferóis dos azeites de oliva extravirgem produzidos a partir de diferentes cultivares e em diferentes regiões dos estados brasileiros do Rio Grande do Sul, Santa Catarina e Minas Gerais. Existem apenas alguns poucos trabalhos em revistas brasileiras da área de ciências agrárias com os resultados de análises de acidez, índice de peróxidos e composição em ácidos graxos para algumas amostras de azeite de oliva extravirgem do estado de Minas Gerais.

Com base em todas estas informações, o objetivo deste trabalho inicialmente foi analisar a capacidade antioxidante e o teor de compostos fenólicos em amostras de EVOO disponíveis ao consumidor brasileiro, as quais são importadas principalmente de países da América do Sul e Europa. Para a determinação do teor de compostos fenólicos, foi otimizado um método de separação de 17 compostos fenólicos por eletroforese capilar de zona com detector de arranjo de diodos. A avaliação da capacidade antioxidante em EVOOs que são consumidos atualmente pela população brasileira será de grande valia, visto que será possível determinar se o EVOO disponível ao consumidor

apresenta valores para este parâmetro que sejam compatíveis com o que se espera deste tipo de produto. Os resultados da quantificação de compostos fenólicos em EVOOs comerciais serão importantes, considerando-se que estes compostos estão relacionados aos efeitos benéficos proporcionados pelo consumo regular de EVOO, bem como estão intimamente relacionados com a capacidade antioxidante total destes produtos.

Em seguida, de posse dos primeiros EVOOs produzidos no Brasil, o objetivo deste estudo foi ampliado para englobar a caracterização de compostos majoritários e minoritários dos azeites de oliva extravirgem brasileiros. Assim, foram analisados os teores de compostos fenólicos, ácido graxos e tocoferóis em EVOOs produzidos no estado de Minas Gerais, nos anos de 2010 e 2011, utilizando-se diferentes técnicas analíticas, como cromatografia em fase gasosa, cromatografia líquida de alta eficiência e eletroforese capilar. Finalmente, amostras de EVOOs de três estados brasileiros, Rio Grande do Sul, Santa Catarina e Minas Gerais, do anos de 2011 e 2012, foram analisadas para a determinação do perfil qualitativo e quantitativo de compostos fenólicos por cromatografia líquida acoplada à espectrometria de massas. A análise de compostos majoritários e minoritários nos EVOOs produzidos no Brasil será de fundamental importância para determinar de maneira mais abrangente a qualidade dos azeites brasileiros, permitindo avaliar se os resultados encontram-se de acordo com as diretrizes nacionais e internacionais, bem como para diferenciá-los dos azeites de oliva provenientes de outros países. Além disso, será o primeiro passo para a construção de uma identidade para os EVOOs produzidos nos

diferentes estados do Brasil, permitindo avaliar de que forma a localização geográfica, bem como as características climáticas e outros fatores locais, alteram a composição química dos EVOOs brasileiros.

CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

Composição química do azeite de oliva extravirgem e benefícios à saúde provenientes do seu consumo regular

CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

Composição química do azeite de oliva extravirgem e benefícios à saúde provenientes do seu consumo regular

Cristiano Augusto Ballus, Helena Teixeira Godoy

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1.1. Azeite de oliva extravirgem (EVOO)

A oliveira (*Olea europaea* L.), conforme é conhecida atualmente, teve sua origem há cerca de 5.000 anos na região que hoje corresponde à antiga Pérsia e Mesopotâmia. Posteriormente, a oliveira se espalhou a partir destes países para os territórios mais próximos que hoje correspondem à Síria e à Palestina (HARWOOD & APARICIO, 2000). O azeite de oliva extravirgem, obtido a partir da prensagem a frio de azeitonas frescas, é um importante óleo comestível presente na dieta Mediterrânea, o qual é reconhecido atualmente pelos seus potenciais benefícios à saúde (FRANKEL, 2010).

O azeite de oliva extravirgem é único entre os diferentes tipos de óleos vegetais, pois é obtido do fruto das oliveiras (*Olea europaea* L.) somente por meios mecânicos. Este tipo de processamento retém os compostos minoritários originalmente presentes no fruto da oliveira, diferentemente de outros óleos vegetais, onde estes compostos geralmente são removidos durante as diferentes etapas de refino (CARRASCO-PANCORBO et al., 2004; CARRASCO-PANCORBO et al., 2005). O fruto da oliveira (azeitona) é classificado como uma drupa, sendo que o mesocarpo responde por 70-90% do peso total, o endocarpo por 9-27% e a semente por 2-3%. O mesocarpo contém cerca de 30% de óleo, e a semente é constituída por 27% de óleo. O óleo proveniente do mesocarpo corresponde a mais de 95% do óleo total extraído do fruto (CONDE, DELROT & GERÓS, 2008).

A legislação brasileira define azeite de oliva virgem como o produto obtido do fruto da oliveira (*Olea europaea* L.) somente por processos mecânicos ou

outros meios físicos, em condições térmicas que não produzam alteração do azeite e que não tenha sido submetido a outros tratamentos além de lavagem, decantação, centrifugação e filtração (BRASIL, 2005). Essa definição está de acordo com as diretrizes do *Codex Alimentarius* para o azeite de oliva (CODEX STAN 33, 2009).

Atualmente, as cultivares de oliveiras mais comumente cultivadas para a produção do azeite de oliva extravirgem são Arbequina, Manzanilla e Picual (Espanha), Barnea (Israel), Koroneiki (Grécia) e Grappolo, Frantoio e Leccino (Itália). A cultivar Arbequina é, de longe, a mais universalmente cultivada (GARCÍA-GONZÁLEZ & APARICIO, 2010).

1.2. Obtenção do azeite de oliva extravirgem

Depois de serem colhidas nos olivais, as azeitonas são depositadas em uma moega, a qual está conectado a uma esteira. É necessário realizar a remoção das folhas e uma etapa de lavagem, para remover todo material estranho às azeitonas que podem causar danos aos equipamentos ou contaminar o produto final. Em seguida, a prensagem é o primeiro passo principal no processamento da azeitona. O objetivo da prensagem é romper as células do mesocarpo para facilitar a liberação do óleo a partir dos vacúolos. A prensagem é realizada em um grande recipiente no qual duas ou três rodas muito pesadas giram em altas velocidades, esmagando as azeitonas. Depois que as azeitonas foram prensadas, a pasta resultante é homogeneizada. Homogeneização ou malaxação implica em agitar a pasta de azeitona devagar e constantemente por cerca de 30 min. O

objetivo desta operação é aumentar a porcentagem de óleo disponível. Ela também ajuda na coalescência das pequenas gotas de óleo para formar gotas maiores, facilitando assim a separação de fases entre o óleo e a água, bem como ajuda a desfazer as gotas de emulsão óleo/água. Os malaxadores diferem em tamanho e formato. São feitos de aço inoxidável, para evitar oxidação e reações indesejáveis. O aparato básico consiste em cubas cilíndricas com pás rotatórias e parede dupla. Uma hélice rotatória com múltiplas asas mistura a pasta, geralmente em baixa velocidade (19-20 rpm). Para azeitonas que atingiram o estádio de maturação, 20-30 min de malaxação são suficientes e, para maior eficiência, os malaxadores possuem parede dupla, para circulação de água quente. Um aumento na temperatura resulta em menor viscosidade do óleo e maior produção de azeite de oliva. Entretanto, a temperatura da água não deve ser superior a 30ºC para prevenir a destruição de compostos voláteis, mudança na coloração do óleo para avermelhado e aumento da acidez. Os efeitos negativos são evitados através do acoplamento de termostatos aos malaxadores. A centrifugação é um processo relativamente novo para a separação do óleo da pasta de azeitona. É baseada nas diferenças de densidade entre os constituintes da pasta de azeitonas (azeite de oliva, água e sólidos insolúveis). A separação é obtida através de uma centrífuga horizontal. Após a prensagem e a malaxação, o azeite de oliva está ou completamente livre ou na forma de pequenas gotículas dentro de microgéis, ou emulsificado na fase aquosa. O azeite de oliva livre é separado pela centrífuga, enquanto o óleo preso nos microgéis é liberado pela adição de mais água. Após a separação do azeite de oliva, é necessário realizar mais uma centrifugação, agora

em uma unidade de centrifugação vertical que gira a baixas velocidades. A fase líquida é distribuída sobre a superfície total em finas camadas e a centrifugação resulta em uma separação final entre o óleo e a água e outras substâncias (KAPELLAKIS, TSAGARAKIS & CROWTHER, 2008). Na **Figura 1.1** está representada esquematicamente uma planta moderna para extração de azeite de oliva extravirgem.

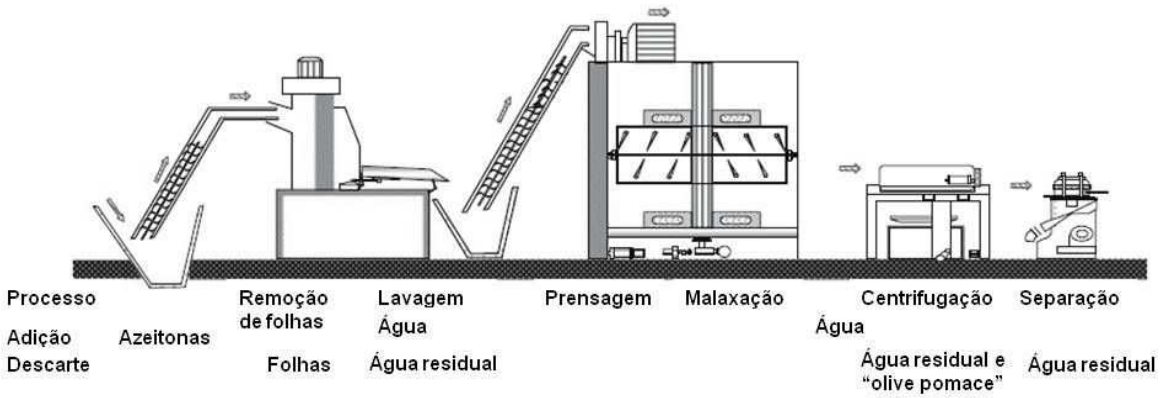


Figura 1.1. Planta de extração de azeite de oliva extravirgem moderna. Fonte: Adaptado de Kapellakis, Tsagarakis & Crowther (2008).

1.3. Azeite de oliva extravirgem e saúde

Nos últimos 30 anos o interesse no uso culinário do azeite de oliva vem aumentando, principalmente pelas virtudes da dieta mediterrânea e seus efeitos benéficos à saúde (SERVILI & MONTEDORO, 2002; CARRASCO-PANCORBO et al., 2005).

É importante mencionar, entretanto, que se deve tomar cuidado com o termo “dieta mediterrânea”, já que não há uma única, mas sim várias “dietas mediterrâneas”, visto que os países ao longo do Mediterrâneo possuem diferentes

religiões, tradições econômicas e culturais, e a dieta é afetada por todos estes fatores (SIMOPOULOS, 2001). Existe uma significativa correlação entre as dietas mediterrâneas e uma redução nas taxas de mortalidade por diversas causas, além dos efeitos favoráveis desta dieta sobre os níveis das lipoproteínas, vasodilatação, resistência à insulina, capacidade antioxidante e síndrome metabólica. Os estudos indicam que há nove componentes de grande importância nas dietas mediterrâneas: elevada razão de ácidos graxos monoinsaturados/saturados (derivada do alto consumo de azeite de oliva); consumo moderado de etanol (principalmente vinho); elevado consumo de legumes; alto consumo de cereais (principalmente cereais não refinados e pães); alto consumo de frutas; elevado consumo de vegetais; baixo consumo de carne e produtos cárneos; consumo moderado de leite e produtos lácteos; elevado consumo de peixe e produtos derivados (SAURA-CALIXTO & GOÑI, 2009).

O consumo de azeite de oliva extravirgem pode contribuir para a menor incidência de doenças coronárias e câncer de cólon e de próstata (OWEN et al., 2000a; BENKHALTI et al., 2002; MURKOVIC et al., 2004; PERONA, CABELLO-MORUNO & RUIZ-GUTIERREZ, 2006; SÁNCHEZ et al., 2007; LEE et al., 2008). Uma das explicações para esse fato seria a sua capacidade de diminuir os efeitos deletérios dos radicais livres. Radicais livres e outros compostos reativos são gerados por substâncias químicas exógenas ou processos metabólicos endógenos em alimentos ou no corpo humano. Estes radicais podem causar danos oxidativos, os quais possuem um papel patológico importante em algumas

doenças humanas como aterosclerose, câncer e artrite (ZULLO & CIAFARDINI, 2008).

1.4. Produção e consumo do azeite de oliva extravirgem

A produção mundial de azeite de oliva foi de aproximadamente 2,934 milhões de toneladas por ano no período entre 2006/7 e 2011/12. A União Europeia foi responsável pela maior parte da produção mundial, com 73,6% de todo o azeite de oliva produzido no mundo neste período. Entre os países da União Europeia, no período entre 2006/7 e 2011/12 a Espanha foi a primeira colocada, com 1,297 milhões de toneladas por ano (60% da produção da UE), seguida por Itália, com 476,7 mil toneladas por ano (22,1% da produção da UE) e Grécia, com 319,7 mil toneladas por ano (14,8% da produção da UE) (IOC, 2013). Juntos, os países do Mediterrâneo produzem cerca de 98% de todo o azeite de oliva comercializado no mundo. Para alguns países como a Tunísia, apesar de a produção total ser menor que a de outros países, a importância econômica da exportação de azeite de oliva para a sua pauta de importações chega a 38% (HARWOOD & APARICIO, 2000).

Em 2012, o Brasil importou 51 mil toneladas de azeite de oliva, a um custo de US$210 milhões. No período compreendido entre janeiro e setembro de 2013, o Brasil já importou 50 mil toneladas de azeite de oliva, o que resultou em gastos da ordem de US$259 milhões. Assim, houve um aumento nos gastos com importação de azeite de oliva de 23,7% em relação ao ano de 2012, sendo considerados apenas os nove primeiros meses de 2013 (CONAB, 2013). De

2005/6 a 2011/12, o consumo de azeite de oliva no Brasil aumentou em média 17,7% ao ano, passando de 26 mil toneladas em 2005/6 para 68 mil toneladas em 2011/12. A previsão para 2012/2013 é de um consumo de 70 mil toneladas de azeite de oliva. Entretanto, mesmo com estes números, o Brasil foi responsável por apenas 1,7% do consumo mundial e 7,3% da importação mundial de azeite de oliva por ano entre 2006/6 e 2011/12, enquanto que EUA, por exemplo, foram responsáveis por 9% do consumo mundial e 39% da importação mundial de azeite de oliva por ano neste mesmo período (IOC, 2013).

Atualmente, todo o azeite de oliva comercializado no Brasil é proveniente de importações. Pontualmente, existem algumas experiências com a produção de oliveiras no Brasil, em microclimas favoráveis à cultura, como é o caso de algumas regiões da Serra da Mantiqueira, nos estados de Minas Gerais e São Paulo, e regiões do sul do Brasil, como nos estados do Rio Grande do Sul e Santa Catarina. Na maioria dos casos, a introdução do material genético ocorreu via imigrantes portugueses, sucedidos pelos imigrantes espanhóis e italianos (BERTONCINI, TERAMOTO & PRELA-PANTANO, 2010).

No Rio Grande do Sul, a EMBRAPA - CPACT (Empresa Brasileira de Pesquisa Agropecuária - Centro de Pesquisa Agropecuária de Clima Temperado) aprovou, em 2005, o projeto “Introdução e desempenho agronômico de cultivares de oliveira no Rio Grande do Sul e em Santa Catarina”, que implantou 25 unidades experimentais de observação no estado. A área cultivada com oliveiras no Rio Grande do Sul é próxima a 400 ha, onde as cultivares mais promissoras para extração de azeite são Arbequina, Koroneiki, Arbosana e Picual. Em Santa

Catarina, os estudos com oliveiras estão sendo conduzidos pela EPAGRI (Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina). Em 2005, houve aprovação de projeto para coleta do material genético já existente no estado e para compra de novo material genético. Atualmente, oito áreas tiveram sucesso para implantação da cultura, e estão situadas em municípios do oeste e extremo oeste de Santa Catarina, entre eles Chapecó, Campo Erê, São Lourenço e Caçador. Em Minas Gerais, a introdução das oliveiras iniciou-se em 1930, na cidade de Maria da Fé, por imigrantes portugueses. O cultivo de azeitonas passou por diversos insucessos mas, atualmente, com o auxílio das pesquisas da EPAMIG (Empresa de Pesquisa Agropecuária de Minas Gerais), há produção de azeitonas e azeite de oliva no estado. O cultivo está sendo conduzido em 400 ha, com 200.000 plantas cultivadas em 50 municípios, sendo 50% Arbequina; 20% Grappolo; 10% Maria da Fé; e outros 10% distribuídos entre Arbosana, Koroneiki e Ascolano (BERTONCINI, TERAMOTO & PRELA-PANTANO, 2010).

1.5. Composição química do azeite de oliva extravirgem

1.5.1. Componentes majoritários

1.5.1.1. Triacilgliceróis

Os triacilgliceróis respondem por 98% da composição do azeite de oliva extravirgem (CARRASCO-PANCORBO et al., 2005) e são formados a partir de uma única molécula de glicerol combinada com três ácidos graxos. A estrutura

molecular destes compostos, incluindo a distribuição dos ácidos graxos entre as diferentes posições estereoespecíficas do esqueleto de glicerol, controla a funcionalidade de óleos e gorduras como ingredientes dos alimentos, influenciando propriedades físicas como a estrutura cristalina e o ponto de fusão. Além disso, possuem importantes efeitos fisiológicos como componentes da dieta humana, sendo uma importante fonte de ácidos graxos essenciais. A falta de balanceamento dos triacilgliceróis pode conduzir a diversas desordens, como doenças coronárias, obesidade ou dislipidemia (LERMA-GARCÍA et al., 2011).

Para se referir aos triacilgliceróis, os pesquisadores utilizam siglas constituídas por três letras, cada uma simbolizando qual o ácido graxo que está ligado em qual posição da molécula de glicerol. Por exemplo, **POO** é a sigla para o triacilglicerol composto por ácido **P**almítico na posição 1, ácido **O**leico na posição 2 e outro ácido **O**leico na posição 3. Assim, O se refere ao ácido oléico; P ao ácido palmítico; L ao ácido linoléico; S ao ácido esteárico; e Ln ao ácido linolênico.

Os triacilgliceróis encontrados em maior quantidade no azeite de oliva são OOO (40-59%), POO (12-20%), OOL (12,5-20%), POL (5,5-7%) e SOO (3-7%). Pequenas quantidades de POP, POS, OLnL, LOL, OLnO, PLL, PLnO e LLL já foram detectadas. Triacilgliceróis constituídos apenas de ácidos graxos insaturados não foram relatados na literatura, bem como os tri-insaturados contendo três ácidos α-linolênicos (BOSKOU, 2006).

1.5.1.2. Ácidos graxos

A principal característica do azeite de oliva é a abundância de ácido oleico (OA, 18:1n-9), que varia entre 55 e 83% do total de ácidos graxos (CARRASCO-PANCORBO et al., 2005; SERVILI et al., 2009). Este elevado teor de OA é importante porque ele é menos susceptível à oxidação do que os ácidos graxos poli-insaturados que predominam em outros óleos, o que colabora para sua estabilidade (OWEN et al., 2000b). A elevada proporção de OA ajuda a reduzir os níveis de colesterol LDL ("*low-density lipoprotein"*) e aumentar os níveis de colesterol HDL ("*high-density lipoprotein"*) no sangue. Isto é extremamente importante, visto que as lipoproteínas HDL transportam o colesterol das artérias para o fígado, enquanto que as lipoproteínas LDL transportam o colesterol a partir do fígado, depositando-o nas artérias, onde pode causar aterosclerose e, assim, aumentar o risco de doenças cardiovasculares. Alguns compostos fenólicos e o ácido α-linolênico (LNA, 18:3n-3) colaboram para reduzir o risco de doenças do coração, diminuir a pressão sanguínea e proteger contra a formação de placas nas artérias (COVAS, 2007; CONDE, DELROT & GERÓS, 2008; CICERALE et al., 2009).

A composição em ácidos graxos do azeite de oliva é fortemente influenciada por diversos fatores, tais como a cultivar, o estágio de maturação do fruto e o local de origem, podendo ser utilizado para diferenciar azeites de oliva de diferentes variedades de azeitonas e conforme a origem geográfica (MONTEALEGRE, ALEGRE & GARCÍA-RUIZ, 2010).

1.5.2. Componentes minoritários

1.5.2.1. Compostos fenólicos

O clima distinto da bacia mediterrânea, caracterizado por tempo quente e irradiação prolongada de luz solar, permitiu o desenvolvimento de plantas, tais como as oliveiras, cujos frutos precisam de uma elevada proporção de moléculas antioxidantes. A síntese de compostos fenólicos como antocianinas, flavonoides e ácidos fenólicos, de fato, é ativada pela irradiação de luz branca, e resulta em frutos de coloração escura que, assim, protegem a si mesmos dos efeitos nocivos da prolongada exposição à luz solar (VISIOLI, BELLOMO & GALLI, 1998; VISIOLI & GALI, 2002). Os compostos fenólicos também agem nas plantas como antimicrobianos, fotorreceptores, atrativos visuais e repelentes de predadores, como herbívoros (PIETTA, 2000; NICHENAMETLA et al., 2006).

Os compostos fenólicos possuem um importante papel na qualidade do azeite de oliva, visto que contribuem significativamente para a estabilidade oxidativa do mesmo. Além disso, os fenólicos são os principais responsáveis pelo sabor amargo, adstringência e pungência do azeite de oliva (RODRÍGUEZ-MÉNDEZ, APETREI & DE SAJA, 2008; INAREJOS-GARCIA et al., 2009). Em geral, assume-se que os responsáveis pelo estímulo “pungente” e “amargo” do azeite de oliva virgem sejam os compostos tirosol, hidroxitirosol e seus derivados (ESTI et al., 2009; SERVILI, et al., 2009). O azeite de oliva virgem apresenta uma notável resistência à oxidação, a qual tem sido relacionada à sua composição em

ácidos graxos e aos elevados níveis de antioxidantes naturais, tais como os compostos fenólicos hidrofílicos e lipofílicos (PAPADIMITRIOU et al., 2006).

Existem pelo menos 36 compostos fenólicos hidrofílicos estruturalmente distintos já identificados no azeite de oliva virgem. Nem todos os fenólicos estão presentes em cada azeite de oliva virgem, e há variação na concentração de fenólicos entre os azeites de oliva virgem (CICERALE et al., 2009). Basicamente, costuma-se agrupar os compostos fenólicos nas seguintes categorias: 1) fenóis, ácidos fenólicos e ácidos fenilacéticos; 2) ácidos cinâmicos, cumarinas, isocumarinas e cromonas; 3) lignanas; 4) flavonoides; 5) ligninas; 6) taninos; 7) benzofenonas, xantonas e estilbenos; 8) quinonas; 9) betacianinas. A maioria dos compostos fenólicos é encontrada na natureza em uma forma conjugada, principalmente com moléculas de açúcares (CARRASCO-PANCORBO et al., 2005).

Os compostos fenólicos já identificados e quantificados em azeite de oliva pertencem às classes de fenil-etil-álcoois (como hidroxitirosol e tirosol), ácidos fenólicos (como o ácido *p*-cumárico, ácido vanílico, etc.), lignanas [(+)-pinoresinol e (+)-1-acetoxipinoresinol], secoiridoides (vários derivados agliconas da oleuropeina e ligstrosídeo) e flavonoides (luteolina e apigenina) (CARRASCO-PANCORBO et al., 2006). Entre os compostos fenólicos mencionados, aqueles pertencentes à classe dos secoiridoides são característicos dos frutos da oliveira e do azeite de oliva virgem produzido a partir destes (OBIED et al., 2008). A aglicona oleuropeina e seus derivados (*o*-difenóis) estão presentes em grande quantidade no azeite de oliva virgem e possuem uma comprovada atividade contra

radicais livres (GALLINA-TOSCHI et al., 2005; BENDINI et al., 2007; CONDE, DELROT & GERÓS, 2008). Hidroxitirosol e oleuropeina exibiram atividade antioxidante mais efetiva do que os tocoferóis e alguns antioxidantes sintéticos aprovados para uso em alimentos, como o butil hidroxitolueno (BHT) (MEDINA et al., 1999; BENDINI et al., 2007).

Tirosol, hidroxitirosol e seus derivados secoiridoides respondem por cerca de 90% do teor total de fenólicos do azeite de oliva virgem. Hidroxitirosol é o fenólico antioxidante mais potente do azeite de oliva, cuja atividade biológica tem estimulado pesquisas sobre o seu provável papel na proteção cardiovascular (OWEN et al., 2000b; O'DOWD et al., 2004; DE LA TORRE, 2008; BROUK & FISHMAN, 2009; CHOE & MIN, 2009). As **Figuras 1.2** a **1.5** apresentam as estruturas dos principais compostos fenólicos do EVOO.

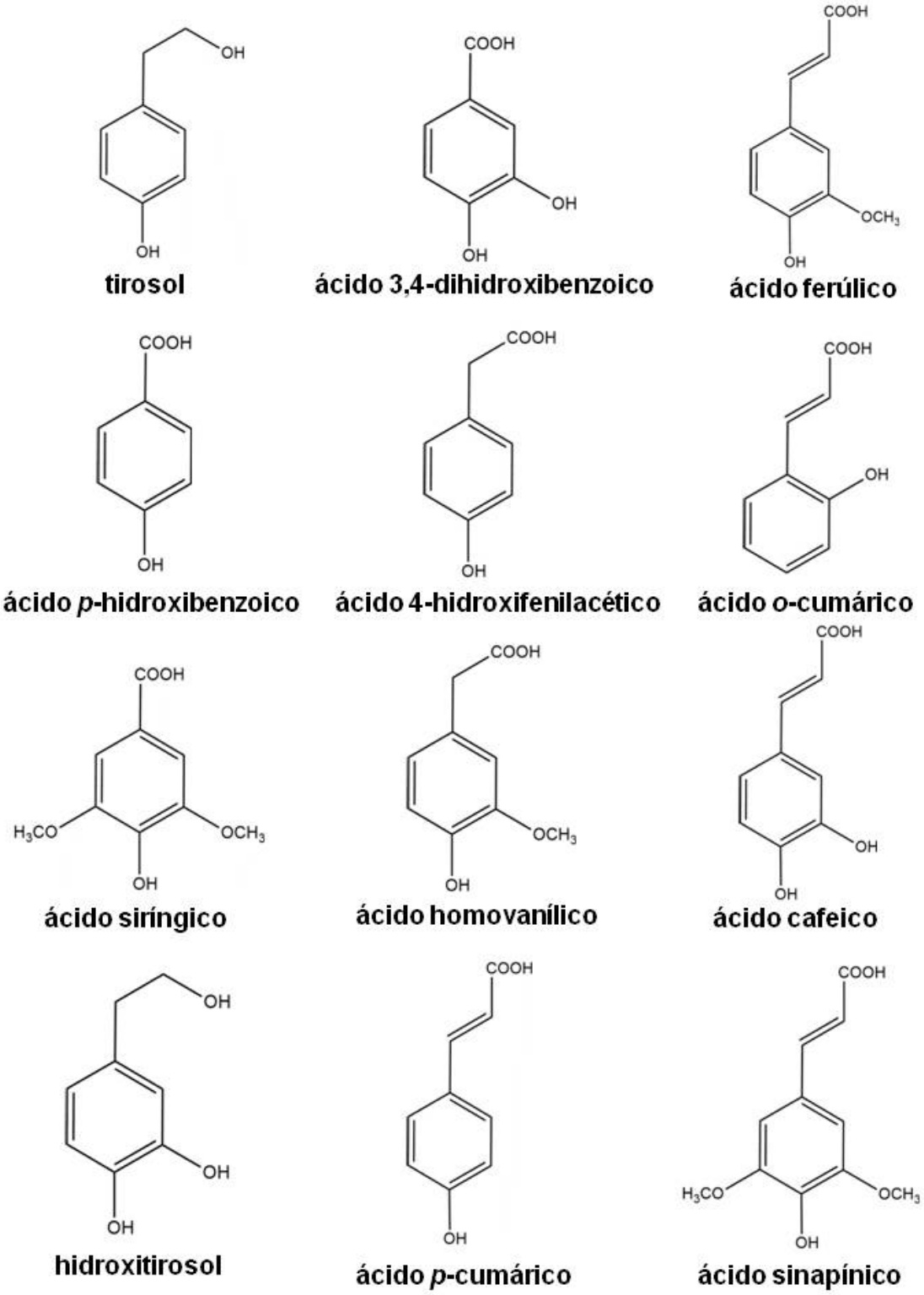
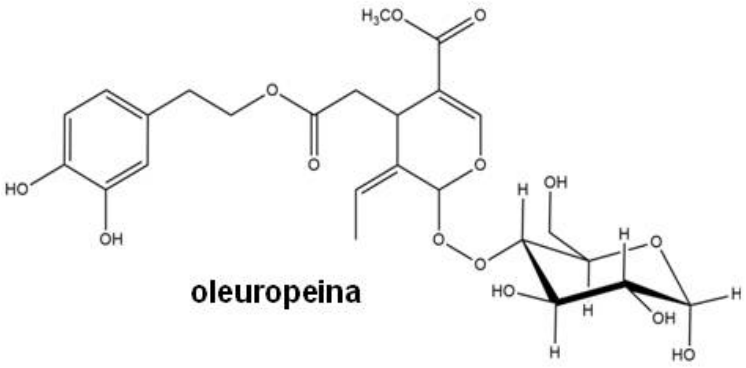
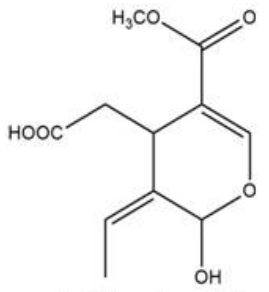
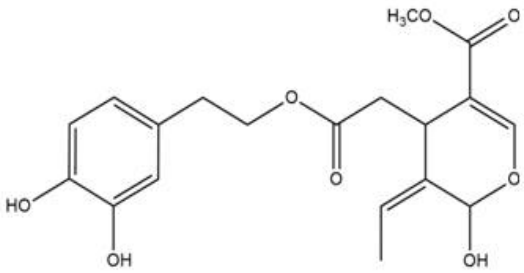
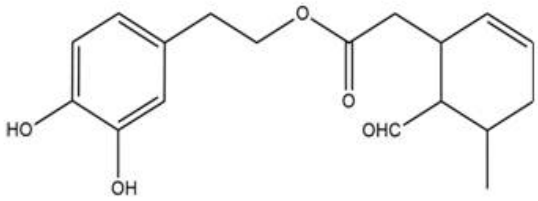


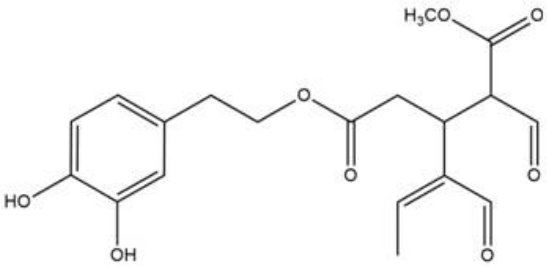
Figura 1.2. Estrutura de compostos fenólicos previamente identificados em azeite de oliva extravirgem.

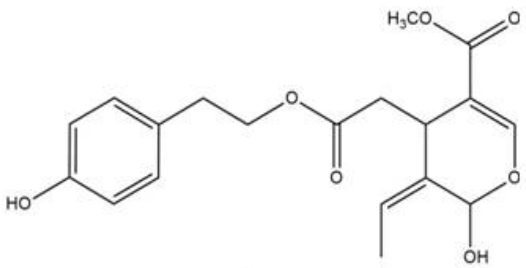












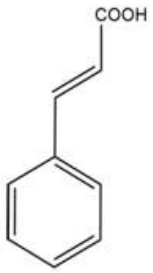


Figura 1.3. Estrutura de compostos fenólicos previamente identificados em azeite de oliva extravirgem (continuação).

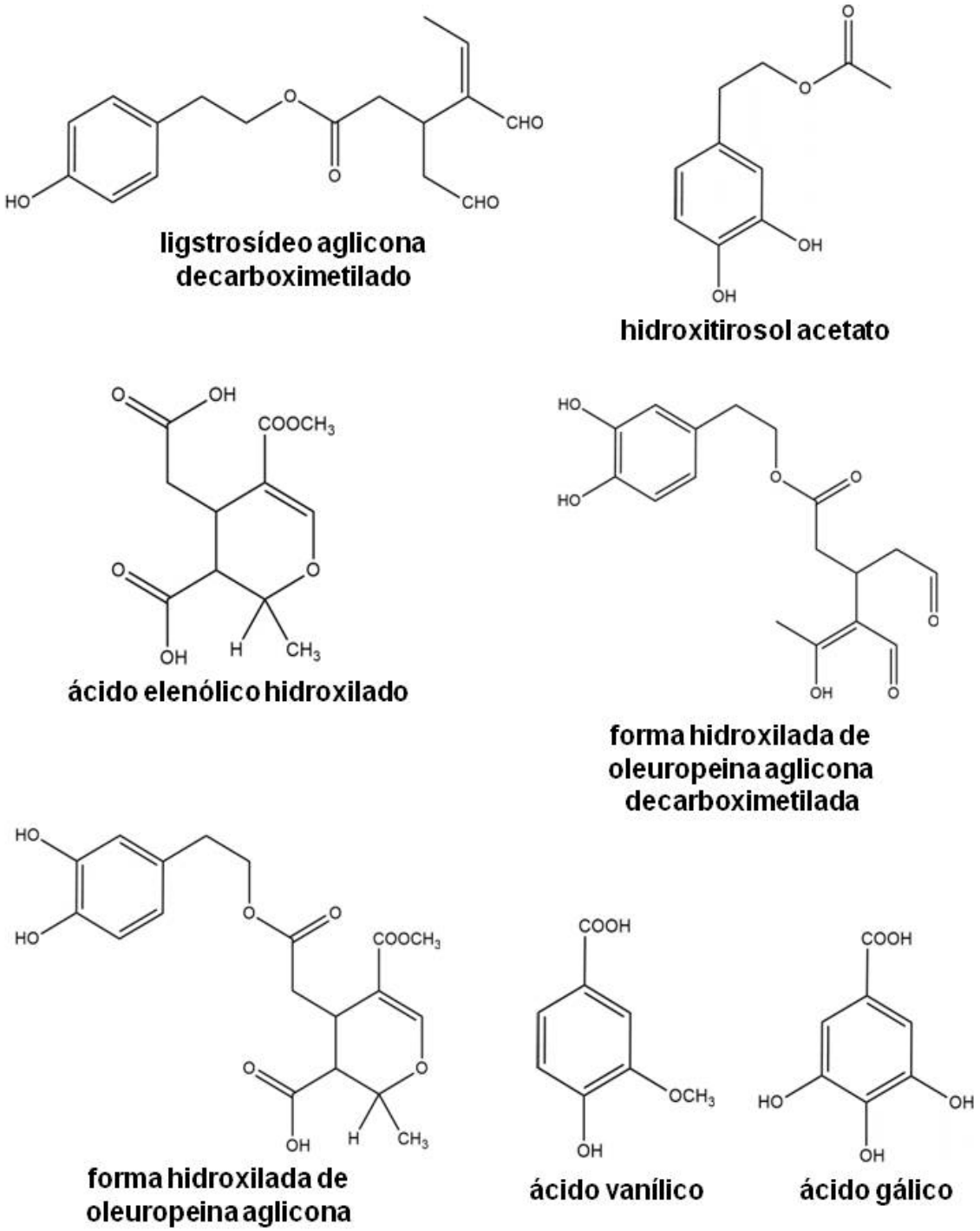


Figura 1.4. Estrutura de compostos fenólicos previamente identificados em azeite de oliva extravirgem (continuação).

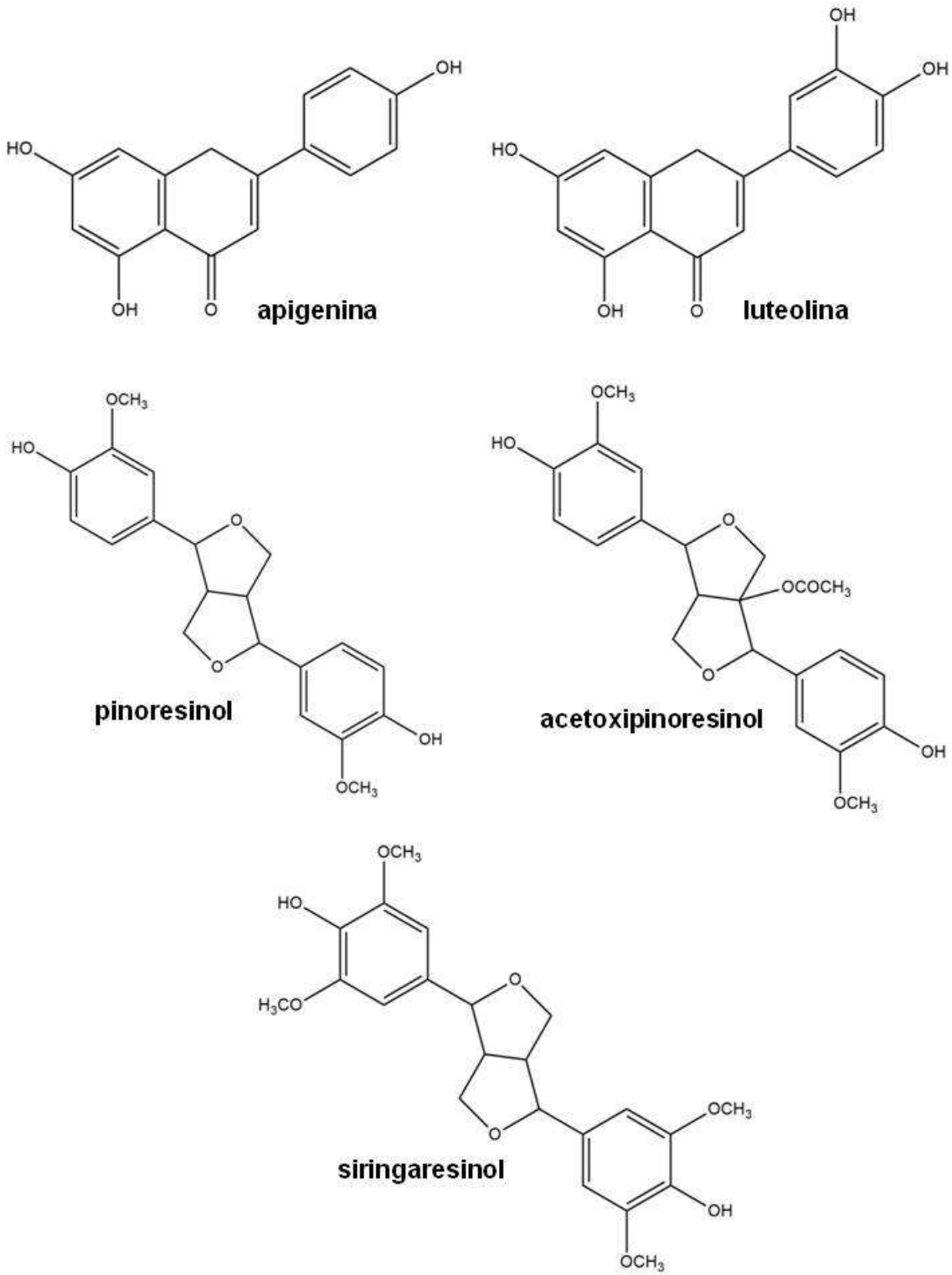


Figura 1.5. Estrutura de compostos fenólicos previamente identificados em azeite de oliva extravirgem (continuação).

1.5.2.2. Tocoferóis

Tocoferóis são componentes funcionais particularmente importantes em alimentos. Possuem propriedades de vitamina E e apresentam capacidade antioxidante, a qual protege os tecidos do corpo contra os efeitos negativos causados por radicais livres que resultam dos vários processos metabólicos. Entre todos os homólogos do tocoferol, o α-tocoferol apresenta o potencial biológico mais elevado (LÓPEZ ORTÍZ, PRATS MOYA & BERENGUER NAVARRO, 2006; HOUNSOME et al., 2008).

O α-tocoferol é um nutriente essencial para os seres humanos, visto que é necessário para a prevenção dos sintomas de deficiência de vitamina E, incluindo neuropatia periférica e anemia hemolítica. Trata-se de um antioxidante lipofílico capaz de impedir a reação em cadeia de formação dos radicais livres em membranas e lipoproteínas, assim como nos alimentos. Devido a este potencial antioxidante e a diversas outras funções em nível molecular, acredita-se que ele diminua o risco de doenças cardiovasculares e de certos tipos de cânceres (SCHWARTZ, OLLILAINEN, PIIRONEN & LAMPI, 2008; TRABER & STEVENS, 2011).

Pesquisas relacionadas à ocorrência e aos níveis de tocoferóis em azeite de oliva extravirgem demonstraram que, dos oito isômeros conhecidos, o α-tocoferol responde por cerca de 90% do teor total de tocoferóis, sendo encontrado na forma livre. Existe uma elevada amplitude na faixa de concentração do α-tocoferol, sendo que os níveis dependem da cultivar e de fatores tecnológicos

(BOSKOU, 2006). Na **Figura 1.6** pode ser visualizada a estrutura dos quatro principais isômeros de tocoferóis encontrados no azeite de oliva extravirgem.

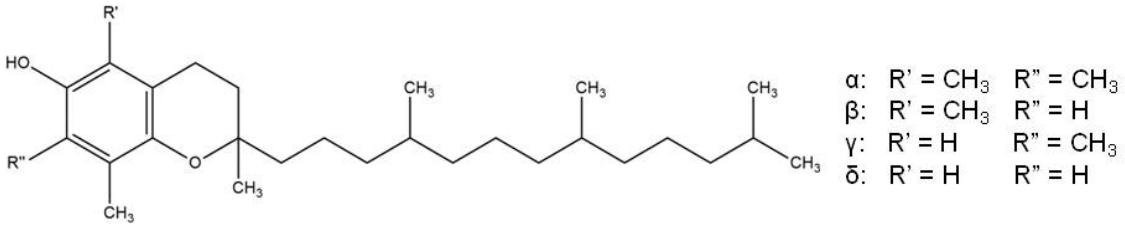


Figura 1.6. Estrutura dos quatro principais isômeros de tocoferóis presentes em azeite de oliva extravirgem.

1.5.2.3. Fitosteróis

Esteróis de plantas, também denominados de fitosteróis, compreendem a maior proporção da fração insaponificável dos óleos vegetais. Eles são derivados biossintéticos do esqualeno, formando o grupo dos triterpenos. São importantes constituintes das células vegetais, controlando a fluidez e a permeabilidade da membrana plasmática (AZADMARD-DAMIRCHI, 2010). Estruturalmente, os esteróis de plantas são similares ao colesterol, com pequenas diferenças na posição relativa dos grupos etil e metil. Os esteróis mais comuns na dieta são o β-sitosterol, o campesterol e o estigmasterol (GUPTA et al., 2011).

O teor total de fitosteróis em azeites de oliva extravirgem varia entre 1000 mg kg^{-1} e 2000 mg kg^{-1}. Podem ser encontrados na forma livre ou esterificados. Os principais componentes da fração esterólica do azeite são β-sitosterol, Δ^5-avenasterol e campesterol. Outros esteróis foram encontrados em menores quantidades ou em traços, entre eles o estigmasterol, brassicasterol, clerosterol,

ergosterol, sitostanol, campestanol, Δ^7-avenasterol, Δ^7-colestenol, Δ^7-campestenol, Δ^7-estigmastenol, $\Delta^{5,23}$-estigmastadienol, $\Delta^{5,24}$-estigmastadienol, $\Delta^{7,22}$-ergostadienol, 24-metileno-colesterol e 22,23-dihidrobrassicasterol. Estudos sobre a composição de esteróis demonstraram que o β-sitosterol responde por 75-90% da fração esterólica total do azeite, enquanto o Δ^5-avenasterol varia entre 5 e 20%. Campesterol e estigmasterol foram quantificados em torno de 4% e 2% da fração de esteróis, respectivamente. Cerca de 10 a 40% do total de esteróis encontram-se esterificados. A composição de esteróis e o teor total dos mesmos são afetados pela cultivar, época da colheita, grau de amadurecimento do fruto, tempo de estocagem dos frutos antes da extração do óleo, processamento e fatores geográficos (BOSKOU, 2006).

1.5.2.4. Carotenoides

Carotenoides são compostos isoprenoides que possuem uma estrutura hidrocarbônica com ligações duplas conjugadas, as quais determinam muitas das propriedades e atividades relacionadas a estes compostos. A maioria dos carotenoides descritos possui 40 átomos de carbono. Podem ser divididos em carotenos (carotenoides contendo somente carbono e hidrogênio) e xantofilas (carotenoides que também possuem funções oxigenadas, como grupos epóxido, hidroxila, acetato, carbonila e carboxila, entre outros). Na natureza, os carotenoides podem ser encontrados livres ou associados a outros compostos, tais como ácidos graxos, açúcares e proteínas (MELÉNDEZ-MARTÍNEZ, VICARIO & HEREDIA, 2007).

Os carotenoides não podem ser sintetizados pelos tecidos dos animais, embora as células animais possam modificá-los quimicamente para assimilação. Assim, estas moléculas devem ser obtidas por meio da dieta. Os potenciais benefícios de uma dieta rica em carotenoides estão sendo reportados por estudos recentes que demonstraram seus papéis como antioxidantes e como agentes que podem prevenir doenças cardiovasculares e patologias degenerativas do olho, bem como o valor de provitamina A dos carotenoides com um anel β-ionona. Sua presença em azeite de oliva depende de fatores genéticos dos frutos da oliveira (variedade), do estágio de maturação dos frutos, condições ambientais, ano da colheita, do processo de extração e das condições de estocagem. Os pigmentos da classe dos carotenoides respondem pela cor amarela do azeite, sendo que os compostos majoritários são a luteína e o β-caroteno. Os carotenoides, juntamente com os compostos fenólicos e com os tocoferóis, proporcionam uma elevada estabilidade oxidativa aos azeites de oliva, bem como possuem ações sinergísticas como antioxidantes e anticarcinogênicas, em concentrações fisiológicas (GIUFFRIDA et al., 2011). A fração de carotenoides pode também incluir diversas xantofilas (violaxantina, neoxantina, luteoxantina, anteraxantina, mutatoxantina e β-criptoxantina). A proporção entre os dois carotenoides majoritários (luteína e β-caroteno) está relacionada à cultivar da oliveira (BOSKOU, 2006).

1.5.2.5. Clorofilas

As clorofilas são complexos de magnésio derivados de porfina, que é uma estrutura macrocíclica completamente insaturada que contém quatro anéis

pirrólicos ligados por uma única ponte de carbono. As porfinas substituídas são chamadas de porfirinas, que se referem a qualquer pigmento tetrapirrólico macrocíclico no qual os anéis pirrólicos estão unidos por pontes metina e o sistema de ligações duplas forma uma configuração cíclica e conjugada (DAMODARAN, PARKIN & FENNEMA, 2010).

As clorofilas (clorofilas *a* e *b*) são os pigmentos responsáveis pela cor verde característica da azeitona. Os pigmentos clorofilas, juntamente com os carotenoides, estão associados às membranas tilacoides dos cloroplastos, que por sua vez estão presentes nos tecidos fotossintéticos ativos da drupa, do epicarpo e da polpa, em quantidade proporcional à atividade de fotossíntese (ROCA & MÍNGUEZ-MOSQUERA, 2001).

Quando o EVOO é extraído a partir das azeitonas, ocorre uma grande perda das clorofilas, sendo que menos de 20% do teor presente nos frutos realmente permanece no óleo após a extração (GALLARDO-GUERRERO et al. (2002). Do ponto de vista qualitativo, o perfil de clorofilas do EVOO é determinado pelos pigmentos que estavam presentes inicialmente nos frutos e pelos derivados formados durante a extração do óleo. As clorofilas *a* e *b*, presentes no fruto, são convertidas irreversivelmente em pigmentos mais estáveis, as feofitinas, onde o íon central de Mg^{2+} do anel porfirina foi substituído por dois átomos de H^{+}. Estas alterações estruturais no cromóforo das clorofilas afeta a cor do óleo, a qual muda de verde brilhante para marrom, e finalmente para amarelo (GIULIANI, CERRETANI & CICHELLI, 2011).

As clorofilas são importantes não apenas por influenciarem a cor do EVOO, que é uma das principais características do azeite, mas também possuem alguns efeitos negativos. Clorofilas e feofitinas possuem ação pró-oxidante em presença de luz. Estes pigmentos agem como catalisadores na formação do oxigênio singlete, o qual pode reagir diretamente com as ligações duplas dos ácidos graxos oleico, linoleico e α-linolênico, produzindo espécies reativas de oxigênio (LANFER-MARQUEZ, BARROS & SINNECKER, 2005). Em contrapartida, alguns estudos demonstram que as clorofilas podem exercer diversas atividades benéficas, como anti-inflamatória, antimutagênica, sequestradora de radicais livres e de inibição da cristalização do oxalato (HUA KAO, JU CHEN & HUEI CHEN, 2011).

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CHAPTER II

ARTICLE

Antioxidant capacity and total phenolic content of extra-virgin olive oils consumed by the Brazilian population

CHAPTER II

Antioxidant capacity and total phenolic content of extra-virgin olive oils consumed by the Brazilian population

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***This manuscript was submitted to the journal "Food Research International".**

ABSTRACT

Hydrophilic extracts of extra-virgin olive oil (EVOO) contain a large number of phenolic compounds, including simple phenols, lignans, flavonoids and secoiridoids, which exhibit antioxidant properties. In Brazil, the consumption of EVOO has increased in the last years, since its health benefits are now well known by the population. The aim of this study was to determine the total phenolic content and the total antioxidant capacity, as well as their correlations, of 15 EVOO brands available to Brazilian population, each one in three batches, resulting in a total of 45 samples. Total phenolic content was evaluated by Folin-Ciocalteu reagent method, while the antioxidant capacities were assessed using FRAP, ABTS, DPPH$^{\bullet}$ and ORAC assays. The total phenolic contents varied from 70.05 to 297.37 mg GAE kg^{-1}, FRAP results varied from 114.83 to 1557.35 µmol TE kg^{-1}. In the ABTS assay, results ranged from 0.53 to 1.90 mmol TE kg^{-1}. For DPPH$^{\bullet}$, results were in the range of 72.15 to 1129.46 µmol TE kg^{-1}. The overall ORAC values were between 1.14 and 12.90 µmol TE g^{-1}. It was found a high and significant correlation between the total phenolic content and each one of the four antioxidant capacity methods evaluated in this study (FRAP, r^2 = 0.8904; p < 0.001; ABTS, r^2 = 0.7837; p < 0.001; DPPH$^{\bullet}$, r^2 = 0.7908; p < 0.001; ORAC, r^2 = 0.7431; p < 0.001). Therefore, most of the EVOO brands available to Brazilian population presented a high total phenolic content and strong antioxidant capacity.

Keywords: extra-virgin olive oil; Folin-Ciocalteu; antioxidant capacity; correlation.

2.1. Introduction

The Mediterranean diet includes the consumption of noticeable amounts of extra-virgin olive oil (EVOO). To be an EVOO, it must be obtained from the fruit of the olive trees solely by mechanical or other physical means, under conditions that do not lead to alteration in the oil and without any treatment other than washing, decantation, centrifugation, or filtration (Baiano et al., 2009). As a result, EVOO contains a large number of phenolic compounds, including simple phenols, lignans, flavonoids and secoiridoids, which exhibit antioxidant properties (Carrasco-Pancorbo et al., 2005).

Antioxidants are compounds or systems that delay autoxidation by inhibiting formation of free radicals or by interrupting propagation of the free radical by one (or more) of several mechanisms: (1) scavenging species that initiate peroxidation, (2) chelating metal ions so that they are unable to generate reactive species or decompose lipid peroxides, (3) quenching $O_2^{\bullet -}$ preventing formation of peroxides, (4) breaking the autoxidative chain reaction and/or (5) reducing localized O_2 concentrations. The most effective antioxidants are those that interrupt the free radical chain reaction (Brewer, 2011).

Antioxidant capacities of samples might be influenced by several factors, such as test system, and could not be fully described by one single method. In addition, most natural antioxidants are multifunctional. Therefore, a reliable antioxidant evaluation protocol requires to perform different antioxidant capacity assessments to take into account various mechanisms of antioxidant action (Fu et al., 2011). Methods commonly used to determine the total antioxidant capacity fall

into two major groups: assays based on a single electron transfer (SET), monitored through a change in color as the oxidant is reduced, and assays based on a hydrogen atom transfer reaction (HAT), where the antioxidant and the substrate (probe) compete for free radical. Among the SET methods there are Trolox equivalent antioxidant capacity (TEAC) assay, also known as ABTS assay, the ferric reducing antioxidant power (FRAP), and the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging capacity assay. The most employed HAT method is the oxygen radical absorbance capacity (ORAC) assay (Huang, Ou, & Prior, 2005; Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2009).

In Brazil, the consumption of EVOO has increased in the last years, since its health benefits are now well known by the population. However, there are no studies about the antioxidant capacities of these EVOOs. The aim of this study was to determine the total phenolic content and the total antioxidant capacity of EVOO samples commercialized in Brazil. Four antioxidant capacity methods were chosen (FRAP, ABTS, $DPPH^{\bullet}$ and ORAC), and their correlation with the total phenolic content of the EVOO samples were also determined.

2.2. Material and methods

2.2.1. Chemicals and apparatus

Hexane and methanol p. a. (Synth, Brazil) were used for samples extraction. TPTZ (2,4,6-tripyridyl-*s*-triazine) (Fluka, USA), $FeCl_3.6H_2O$ (Vetec, Brazil), HCl (Synth, Brazil), acetic acid (Qhemis, Brazil), sodium acetate (Synth, Brazil), Folin-

Ciocalteu reagent (Dinâmica, Brazil), sodium carbonate (Synth, Brazil), ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] (Sigma-Aldrich, USA), potassium persulfate (Synth, Brazil), ethanol HPLC grade (J. T. Baker, USA), DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich, USA), K_2HPO_4 (J. T. Baker, USA), phosphoric acid 85% p. a. (Ecibra, Brazil), AAPH [2,2'-azobis(2-amidino-propane) dihydrochloride] (Sigma-Aldrich, USA) and fluorescein disodium (Synth, Brazil) were the chemicals used for the antioxidant capacity assays. Gallic acid and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) standards were acquired from Sigma-Aldrich (USA).

Spectrophotometric measurements for total phenolic content, FRAP, ABTS and DPPH$^{\bullet}$ methods, all in the visible region of the electromagnetic spectrum, were performed using a UV-1600 spectrophotometer (Pro-Análise, Brazil), while the fluorescence monitoring for the ORAC assay was achieved using a NOVOstar microplate reader (BMG Labtech, Germany).

2.2.2. Samples

The samples of extra-virgin olive oil (EVOO) were acquired in supermarkets of Campinas (São Paulo, Brazil), in a total of 15 brands, each one in three different batches, resulting in 45 samples. Each batch was composed by mixing the content of two bottles of that batch. All the samples were from European or South American countries, Spain, Portugal, Greece, Italy and Argentina, and they were analyzed before their expiration dates. The maximum acidity, as described in the label, varied from 0.5 to 0.8%. Brands were coded using numbers from 1 to 15,

and the batch was coded adding numbers 01, 02 or 03 to the brand number. Thus, the sample with the code 101 refers to brand 1, batch 01, and so on.

2.2.3. Preparation of EVOO polar extracts

EVOO polar extracts were prepared according to Nakbi et al. (2010). Briefly, 2.5 g of each EVOO were weighed in a centrifuge tube, and then 5 mL of hexane p. a. and 5 mL of methanol:water (60:40, v/v) were added. This mixture was vigorously vortexed for 2 min and, after that, the tubes were centrifuged for 5 min at 5,000 *g*. The polar phase (in the bottom) was removed, filtered through 0.45 μm membranes (Millipore, France), and then submitted to the total phenolic content analysis and to all the four antioxidant capacity methods.

2.2.4. Total phenolic content by Folin-Ciocalteu reagent

The analysis of the total phenolic content by the method of Folin-Ciocalteu reagent was realized according to the procedure described by Singleton, Orthofer, & Lamuela-Raventos (1999). In this procedure, 0.5 mL of EVOO polar extract was mixed with 2.5 mL of Folin-Ciocalteu reagent (diluted 1:10 in ultrapure water), being kept in the darkness during 5 min. Afterwards, 2 mL of sodium carbonate 7.5% were added and the tubes were incubated in the dark for 2 hours. Then, the absorbance at 760 nm was measured. The quantitative results were calculated using an analytical curve of gallic acid and expressed as mg of gallic acid equivalents per kg of EVOO sample (mg GAE kg^{-1}).

2.2.5. Antioxidant capacity assays

2.2.5.1. Ferric-reducing antioxidant power (FRAP) assay

The FRAP assay was realized according to Benzie, & Strain (1996). In a test tube, 320 µL of the EVOO polar extract and 2.4 mL of the FRAP reagent were mixed. The FRAP reagent consisted in a mixture of 300 mmol L^{-1} sodium acetate buffer solution (pH 3.6), 10 mmol L^{-1} of TPTZ solution in HCl 40 mmol L^{-1}, and 20 mmol L^{-1} of $FeCl_3.6H_2O$ solution, in a volume ratio of 10:1:1, respectively. After adding the FRAP reagent, the tubes were placed into a water bath at 37ºC for 15 min. The absorbance of the mixture was measured at 593 nm 4 min after the incubation period. For the quantification step, a Trolox analytical curve was constructed, and the results were expressed as µmol of Trolox equivalents per kg of EVOO sample (µmol TE kg^{-1}).

2.2.5.2. ABTS radical cation decolorization assay

The ABTS method was performed according to the procedure described by Re et al. (1999). At first, ABTS was dissolved in water to a 7 mmol L^{-1} final concentration. This solution was mixed with a 2.45 mmol L^{-1} potassium persulfate solution, in a volume ratio of 1:1, and then incubated in the dark for 12-16 h at room temperature, in order to produce a stock solution of the radical cation ($ABTS^{\bullet+}$). $ABTS^{\bullet+}$ working solution was prepared by diluting the stock solution with ethanol HPLC grade until reaching an absorbance of 0.700 ± 0.020 at 734 nm and equilibrated to 30ºC. For the sample analysis, 30 µL of the EVOO polar extract was

added to a test tube and mixed with 3 mL of the $ABTS^{\bullet+}$ radical cation working solution ($A_{734\ nm}$ = 0.700 ± 0.020). Absorbancec readings were taken at 734 nm after 6 min. Quantification was conducted using a Trolox analytical curve and the results were expressed as mmol of Trolox equivalents per kg of EVOO sample (mg TE kg^{-1}).

2.2.5.3. Radical scavenging activity by DPPH• assay

$DPPH^{\bullet}$ assay was performed following the methodology described by Brand-Williams, Cuvelier, & Berset (1995) and Nakbi et al. (2010). A 3.9 mL aliquot of a 6 x 10^{-5} mol L^{-1} $DPPH^{\bullet}$ methanolic solution was mixed with 100 µL of EVOO polar extract. $DPPH^{\bullet}$ absorbance was monitored at 515 nm during one hour, at 25ºC. The quantification was made through a Trolox analytical curve, and the results were expressed as µmol of Trolox equivalents per kg of EVOO sample (µmol TE kg^{-1}).

2.2.5.4. Oxygen Radical Absorbance Capacity (ORAC) assay

The antioxidant capacity measured by the ORAC assay followed the procedure presented by Ou, Hampsch-Woodill, & Prior (2001) and modified by Huang, Ou, Hampsch-Woodill, Flanagan, & Prior (2002) and Dávalos, Gómez-Cordovés, & Bartolomé (2004). AAPH reagent (1.08 g) was completely dissolved in 10 mL of 75 mmol L^{-1} phosphate buffer (pH 7.4), resulting in a final concentration of 400 mmol L^{-1}. Fluorescein stock solution (10 mmol L^{-1}) was prepared in 75 mmol L^{-1} phosphate buffer (pH 7.4) and was kept in the dark at 4ºC. Fluorescein working

solutions (0.1 mmol L^{-1}) were daily prepared by diluting the stock solution with 75 mmol L^{-1} phosphate buffer (pH 7.4). The reaction consisted in rapidly mixing 20 µL of the EVOO polar extract, 120 µL of fluorescein working solution and 60 µL of AAPH in a 96-well polypropylene plate, using a multichannel pipet. The microplate was immediately placed in the reader and the fluorescence recorded every minute for 80 min. Fluorescence readings were obtained at λ_{Ex} = 485 nm and λ_{Em} = 520 nm. Quantification was performed using a Trolox analytical curve. To construct the curves, it was necessary to plot the Trolox concentrations (X) against the net area under the fluorescence decay curve, net AUC (Y). The AUC was calculated as

$$AUC = 0.5 + \sum_{i=1}^{i=79} f_i / f_0 \ + 0.5(\frac{f_{80}}{f_0})$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to a standard or a sample was calculated by subtracting the AUC corresponding to the blank (AUC_{sample} - AUC_{blank}). The results were expressed as µmol of Trolox equivalents per g of EVOO sample (µmol TE g^{-1}).

2.2.6. Statistical analysis

The means obtained as the results of each analysis were compared using ANOVA and Tukey test, at 95% confidence level. A correlation analysis between

the total phenolic content and each one of the antioxidant capacity assays was also performed. All the statistical analysis was carried out using the software Statistica 7.0 (Statsoft, USA).

2.3. Results and discussion

2.3.1. Total phenolic content of EVOO polar extracts

The total phenolic content of EVOO polar extracts was estimated by the method of Folin-Ciocalteu reagent, using gallic acid as the standard for quantification. Data about the linear regression for the analytical curve used for total phenolic content quantification are summarized in **Table 2.1**.

Table 2.1. Analytical curves used for determining the extra-virgin olive oil total phenolic content and antioxidant capacity.

Methods	Standard	Linearity	Equation	r^2
Total Phenolic Content	Gallic Acid	10 - 70 mg L^{-1}	$y = 0.0134\ x - 0.0229$	0.9993
FRAP	Trolox	3 - 118 µmol L^{-1}	$y = 0.0042\ x + 0.0244$	0.9962
ABTS	Trolox	378 - 1367 µmol L^{-1}	$y = 0.0460\ x - 2.8235$	0.9946
DPPH	Trolox	68 - 942 µmol L^{-1}	$y = 0.1002\ x + 3.4176$	0.9990
ORAC	Trolox	131 - 665 µmol L^{-1}	$y = 0.0349\ x + 0.7893$	0.9957

All the results for the total phenolic content of the EVOO polar extracts can be seen in **Table 2.2**. The contents varied from 70.05 to 297.37 mg GAE kg^{-1}, within a difference of 4-fold, while the mean value was 186.30 mg GAE kg^{-1}. The

lowest content was found for sample 1401, from Argentina, and the highest content was for sample 203, from Spain. In **Figure 2.1E** is presented a graph with the comparison of the mean total phenolic content of each brand analyzed (obtained as the mean of the three batches).

Table 2.2. Total phenolic contents and antioxidant capacities of 45 extra-virgin olive oil samples (mean ± standard deviation, n = 3).[a]

Samples	Country	Total Phenolics Content (mg GAE kg^{-1})	FRAP (µmol TE kg^{-1})	ABTS (mmol TE kg^{-1})	DPPH$^{\cdot}$ (µmol TE kg^{-1})	ORAC (µmol TE g^{-1})
101	Greece	134 ± 1 b	388 ± 13 b	1.19 ± 0.02 b	312 ± 9 b	2 ± 1 b
102	Greece	119 ± 2 c	414 ± 41 b	1.18 ± 0.01 b	258 ± 10 c	3.1 ± 0.3 a,b
103	Greece	158 ± 6 a	713 ± 64 a	1.4 ± 0.1 a	355 ± 6 a	4 ± 1 a
201	Spain	226 ± 4 c	912 ± 14 c	1.5 ± 0.1 b	629 ± 30 c	7 ± 1 b
202	Spain	261 ± 15 b	1151 ± 43 b	1.8 ± 0.1 a	974 ± 63 b	9 ± 2 b
203	Spain	297 ± 11 a	1557 ± 20 a	1.89 ± 0.04 a	1129 ± 13 a	12.9 ± 0.4 a
301	Portugal	198 ± 3 a	864 ± 45 a	1.2 ± 0.1 b	573 ± 7 b	7 ± 1 a
302	Portugal	185 ± 3 a	828 ± 16 a,b	1.27 ± 0.02 b	625 ± 26 a	6.6 ± 0.5 a
303	Portugal	191 ± 11 a	785 ± 16 b	1.6 ± 0.1 a	542 ± 17 b	7 ± 1 a
401	Spain	237 ± 9 a	982 ± 51 a	1.69 ± 0.01 a	646 ± 34 a	10.4 ± 0.1 a
402	Spain	176 ± 9 b	699 ± 24 b	1.61 ± 0.03 b	545 ± 30 b	6 ± 1 b
403	Spain	160 ± 3 b	623 ± 24 b	1.37 ± 0.05 c	526 ± 11 b	5.7 ± 0.3 b
501	Portugal	141 ± 2 b	457 ± 17 c	1.03 ± 0.02 b	405 ± 14 b	4 ± 1 b
502	Portugal	182 ± 4 a	665 ± 20 a	1.17 ± 0.02 a	532 ± 35 a	7 ± 1 a
503	Portugal	142 ± 8 b	538 ± 18 b	1.20 ± 0.03 a	412 ± 20 b	5.3 ± 0.4 a,b
601	Portugal	222 ± 8 a	841 ± 48 a	1.47 ± 0.03 a	649 ± 26 a	7.6 ± 0.5 a
602	Portugal	186 ± 4 b	644 ± 19 b	1.55 ± 0.05 a	554 ± 35 b	5 ± 1 b
603	Portugal	166 ± c	510 ± 35 c	1.3 ± 0.1 b	454 ± 18 c	5.3 ± 0.5 b
701	Portugal	257 ± 13 b	1077 ± 36 c	1.73 ± 0.04 a	857 ± 48 b	9 ± 1 a
702	Portugal	286 ± 2 a	1157 ± 27 b	1.7 ± 0.1 a	1004 ± 17 a	8.6 ± 0.4 a
703	Portugal	271 ± 9 a,b	1234 ± 14 a	1.8 ± 0.1 a	1035 ± 4 a	9 ± 2 a
801	Spain	118 ± 1 a	339 ± 24 b	0.53 ± 0.02 b	326 ± 15 a	2 ± 1 a
802	Spain	110 ± 2 b	426 ± 16 a	1.03 ± 0.01 a	183 ± 12 b	2 ± 1 a
803	Spain	110 ± 1 b	261 ± 3 c	1.05 ± 0.05 a	78 ± 4 c	1.8 ± 0.2 a

[a] Means followed by the same letter in the column showed no significant difference ($p < 0.05$) by the Tukey test, for the comparison among the three batches of a same brand.

Table 2.2. Continued.[a]

Samples	Country	Total Phenolics Content (mg GAE kg^{-1})	FRAP (μmol TE kg^{-1})	ABTS (mmol TE kg^{-1})	DPPH$^{\cdot}$ (μmol TE kg^{-1})	ORAC (μmol TE g^{-1})
901	Greece	141 ± 4 b	353 ± 31 c	1.14 ± 0.03 b	297 ± 14 b	3.2 ± 0.4 a
902	Greece	146 ± 5 b	451 ± 8 b	1.2 ± 0.1 b	316 ± 19 b	3.3 ± 0.1 a
903	Greece	171 ± 3 a	678 ± 46 a	1.35 ± 0.04 a	453 ± 12 a	4.0 ± 0.9 a
1001	Portugal	255 ± 3 a	696 ± 9 c	1.49 ± 0.03 b	255 ± 10 b	4.6 ± 0.4 b
1002	Portugal	239 ± 6 b	766 ± 26 b	1.6 ± 0.1 a	555 ± 19 a	6 ± 1 a,b
1003	Portugal	218 ± 1 c	849 ± 9 a	1.7 ± 0.1 a	528 ± 23 a	6.2 ± 0.3 a
1101	Spain	218 ± 5 a	829 ± 27 a	1.73 ± 0.04 a	513 ± 9 a	5 ± 1 a
1102	Spain	215 ± 3 a	837 ± 10 a	1.6 ± 0.1 a	498 ± 13 a	5.3 ± 0.3 a
1103	Spain	205 ± 3 b	749 ± 13 b	1.6 ± 0.1 a	504 ± 34 a	5.2 ± 0.2 a
1201	Italy	271 ± 6 a	1270 ± 39 a	1.67 ± 0.03 b	874 ± 31 a	7.2 ± 0.5 a
1202	Italy	235 ± 2 c	1152 ± 31 a,b	1.5 ± 0.1 b	769 ± 34 b	5.8 ± 0.4 b
1203	Italy	253 ± 3 b	1019 ± 96 b	1.9 ± 0.1 a	849 ± 51 a,b	6.1 ± 0.5 a,b
1301	Portugal	197 ± 1 a	725 ± 15 b	1.35 ± 0.02 b	513 ± 8 a	5.0 ± 0.3 a,b
1302	Portugal	197 ± 2 a	785 ± 8 a	1.6 ± 0.1 a	526 ± 31 a	6 ± 1 a
1303	Portugal	184 ± 4 b	751 ± 22 a,b	1.6 ± 0.1 a	561 ± 16 a	4.1 ± 0.4 b
1401	Argentina	70 ± 1 c	115 ± 10 c	0.90 ± 0.03 b	72 ± 4 c	1.1 ± 0.3 b
1402	Argentina	140 ± 1 a	474 ± 6 a	1.1 ± 0.1 a	328 ± 14 a	3.2 ± 0.1 a
1403	Argentina	88 ± 3 b	266 ± 13 b	0.82 ± 0.01 b	133 ± 8 b	1.2 ± 0.2 b
1501	Spain	156 ± 7 a	516 ± 13 a	1.1 ± 0.1 a	388 ± 32 a	2.4 ± 0.5 a
1502	Spain	129 ± 3 b	428 ± 23 b	1.05 ± 0.04 a,b	327 ± 20 b	2 ± 1 a
1503	Spain	125 ± 2 b	330 ± 27 c	0.98 ± 0.03 b	300 ± 6 b	1.8 ± 0.2 a

[a] Means followed by the same letter in the column showed no significant difference ($p < 0.05$) by the Tukey test, for the comparison among the three batches of a same brand.

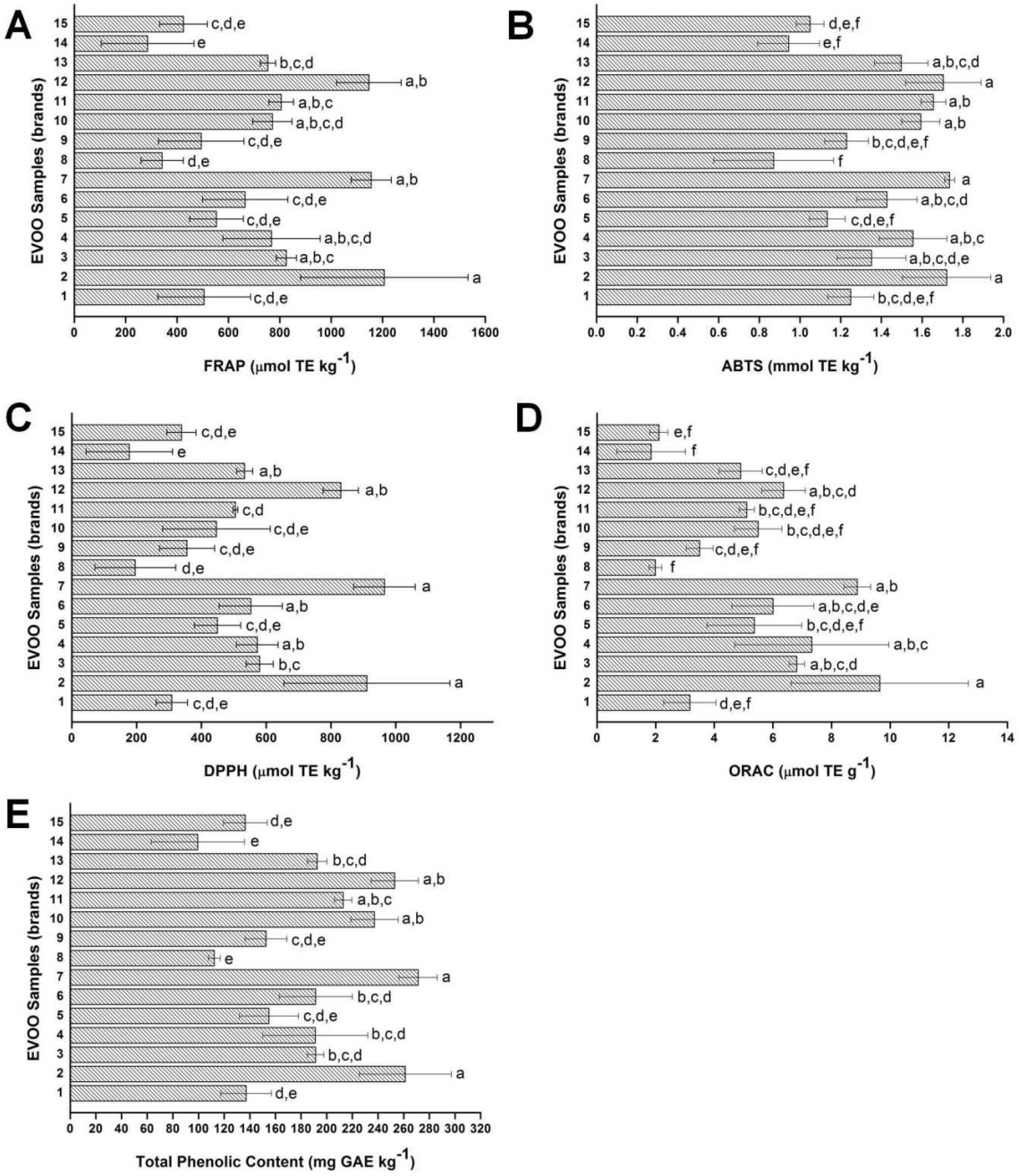

Figure 2.1. Mean results of three batches for the 15 extra-virgin olive oil brands analyzed in this study. A) FRAP assay (μmol of Trolox equivalents per kg); B) ABTS assay (mmol of Trolox equivalent per kg); C) DPPH$^{\bullet}$ assay (μmol of Trolox equivalent per kg); D) ORAC assay (μmol of Trolox equivalents per g); E) Total Phenolic Content (mg of gallic acid equivalents per kg). Bars followed by the same letters showed no significant difference ($p < 0.05$) by the Tukey test.

Ninfali, Aluigi, Bacchiocca, & Magnani (2001) reported total phenolic contents for Italian EVOOs ranging from 50 to 236 mg GAE kg^{-1}. Andjelkovic et al. (2008) reported total phenolic contents for several Spanish and French EVOOs ranging from 63 to 239 mg GAE kg^{-1}. EVOOs from Chétoui and Chemlali varieties cultivated in Tunisia, presented total phenolic content between 158 and 395 mg kg^{-1} (Nakbi et al., 2010). Loizzo, Di Lecce, Boselli, Menichini, & Frega (2012) studied EVOOs from Frantoio variety in Italy and found total phenolic contents between 109 and 250 mg GAE kg^{-1}. In the work from Fuentes, Báez, Bravo, Cid, & Labra (2012) a number of EVOOs samples from Chile, Spain, Italy and Argentine was assayed, and the results for total phenolic content were between 69 and 186 mg kg^{-1}, but expressed as caffeic acid rather than gallic acid equivalents. The results obtained in all these studies agree with those obtained for the EVOOs commercialized in Brazil. However, it is possible to find ranges significantly different in the literature, as in the work from Samaniego Sánchez et al. (2007), where total phenolic contents between 1085.0 and 1406.0 mg GAE kg^{-1} were found for 39 samples of Picual EVOO (Spain). There were also observed significant differences among the three batches of a same brand for almost all the brands analyzed, with exception of sample 3 (**Table 2.2**). The broad range found in literature for the total phenolic content of EVOOs can be understood since it is well known there are substantial variations in the composition and concentration of phenolic compounds that may be caused by factors such as variety, region grown, agricultural techniques, maturity of the fruit at harvest and processing (Cicerale, Lucas, & Keast, 2012).

2.3.2. Antioxidant capacity of EVOO samples

In **Table 2.1**, data about linearity range, equation and r^2 of the analytical curves used to determine the antioxidant capacity by all the four methods are exhibited. In **Table 2.2** it is possible to find the results for the four antioxidant capacity assays of the 45 EVOO samples analyzed in this study.

In summary, FRAP results varied from 114.83 to 1557.35 µmol TE kg^{-1}. In the ABTS assay, results ranged from 0.53 to 1.90 mmol TE kg^{-1}. For DPPH$^{\bullet}$, results were in the range of 72.15 to 1129.46 µmol TE kg^{-1}. The overall ORAC values were between 1.14 and 12.90 µmol TE g^{-1}. As previously seen for the total phenolic contents, the antioxidant capacities also varied among the batches of a same brand (**Table 2.2**). Mean results of the four antioxidant capacity methods, obtained from the three batches for each one of the 15 brands analyzed, are presented in **Figure 2.1A-D**. Observing the figures, it is possible to see that all the antioxidant capacity methods presented the same results profile. The EVOO extracts with the highest total phenolic contents also presented the highest antioxidant capacities, despite of the method employed.

FRAP, ABTS and DPPH$^{\bullet}$ methods are classified as single electron transfer (SET) reaction based assays, as well as the previously discussed total phenolic content by the Folin-Ciocalteu reagent (FCR) method. The difference among Folin-Ciocalteu, ABTS and FRAP methods rely in the pH. Total phenolics is carried out under basic conditions, in order to allow the phenolic proton to dissociate, leading to a phenolate anion, which is capable of reducing FCR. ABTS method is carried out at neutral pH, while FRAP assay under acidic (pH 3.6) conditions. DPPH$^{\bullet}$ was

believed to involve hydrogen atom transfer reaction, since it was observed that the rate-determining step of its reaction consists of a fast electron transfer process from the phenoxide anions to $DPPH^{\bullet}$. In this case, the hydrogen atom abstraction from a phenolic compound by DPH becomes a marginal reaction path, because it occurs very slowly in strong hydrogen-bond-accepting solvents, such as methanol and ethanol (Huang, Ou, & Prior, 2005). Thus, it is easy to understand why the results of all the SET methods presented the same behavior, despite of exhibiting different absolute values. This different absolute values originate from this differences in the reaction medium, and also because the structure of the compounds to be reduced by SET reaction are very different among the four SET methods.

In this way, the correlation between total phenolic content and each one of the four antioxidant capacity methods was calculated, to obtain experimental evidences of what was presented in the previously paragraph. The results are presented in **Figure 2.2A-D**.

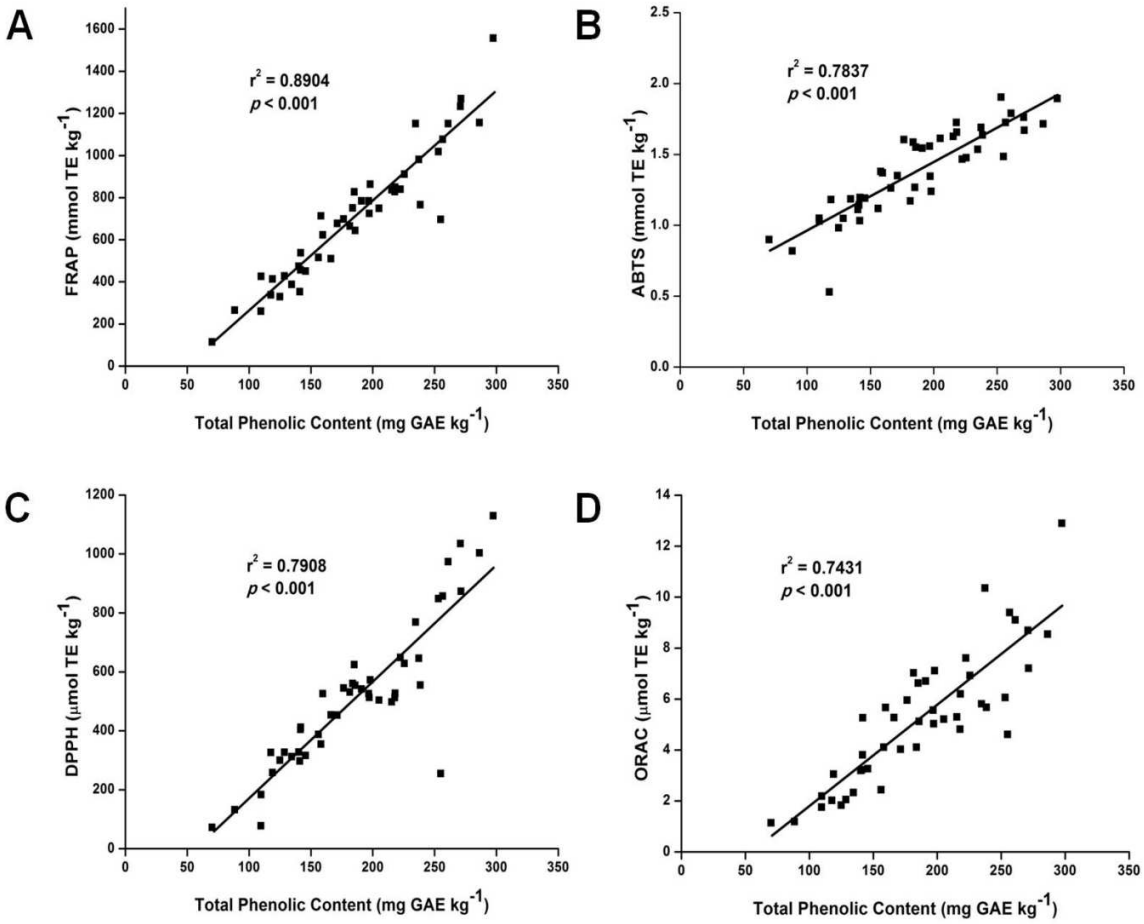

Figure 2.2. Correlation between the total phenolic content and each one of the four antioxidant capacities of the extra-virgin olive oil samples. **A)** FRAP assay; **B)** ABTS assay; **C)** DPPH$^{\bullet}$ assay; **D)** ORAC assay.

As expected, there was a high and significant correlation between total phenolic contents and FRAP (r^2 = 0.8904; p < 0.001; **Figure 2.2A**); total phenolic contents and ABTS (r^2 = 0.7837; p < 0.001; **Figure 2.2B**); and total phenolic contents and DPPH$^{\bullet}$ (r^2 = 0.7908; p < 0.001; **Figure 2.2C**). It is possible to say that the compounds present in EVOO polar extracts are efficient electron donors, despite of the reaction medium conditions and the compounds to be reduced. Similar correlations were observed between total phenolic contents and ABTS or DPPH$^{\bullet}$ for EVOO polar extracts in the study of Samaniego Sánchez et al. (2007).

However, it is believed that the hydrogen atom transfer (HAT) is a key step in the radical chain reaction. Therefore, an extract with compounds that react by HAT will be more effective as radical chain-breaking antioxidant capacity. The ORAC method is classified as a hydrogen atom transfer (HAT) reaction based assay (Huang, Ou, & Prior, 2005). The EVOO polar extracts also presented high ORAC values, indicating that the polar compounds extracted from EVOO are able to react by the HAT mechanism. In addition, the total phenolic contents and the ORAC results presented a high an significant correlation (r^2 = 0.7431; p < 0.001), as can be seen in **Figure 2.2D**. High and significant correlations between total phenolic contents and ORAC values, with similar r^2 and p values, were already reported in the literature for EVOO extracts (Ninfali, Aluigi, Bacchiocca, & Magnani, 2001; Ninfali, Bacchiocca, Biagiotti, Servili, & Montedoro, 2002; Samaniego Sánchez et al., 2007). As a result, it can be said that polar compounds from EVOOs, mostly phenolic compounds, are able to react by SET or HAT mechanisms, being this latter the most important for a real effect of scavenging

free radicals in a radical chain reaction. It was proved before that the phenolic compounds from EVOO mainly act as chain breakers by donating a radical hydrogen to alkylperoxyl radicals (ROO) formed during the initiation step of lipid oxidation, being hydroxytyrosol the phenolic compound from EVOO that exhibited the strongest antioxidant capacity in vitro (Carrasco-Pancorbo et al., 2005), which agrees with the ORAC results and with the correlation between total phenolics and ORAC observed in this study.

2.4. Conclusions

Total phenolic contents and total antioxidant capacity of 45 EVOO samples commercialized in Brazil were determined and it was the first study to analyze antioxidant properties of the EVOO consumed by the Brazilian population.

The results indicated that the total phenolic content and the antioxidant capacities determined by different methods are comparable to those values previously described in the literature. It was possible to verify that there is a significant variation in the results among batches of a same brand for the total phenolic contents and antioxidant capacities.

Moreover, a high and significant correlation was found between the total phenolic content and each one of the four antioxidant capacity methods evaluated in this study. Thus, the phenolic compounds that compose the EVOO polar fraction possess a significant antioxidant effect *in vitro*.

Therefore, most of the EVOO brands available to Brazilian population presented high total phenolic content and, consequently, their antioxidant capacity

was also very strong, and then, these EVOO can help providing several health benefits to those who consume them regularly.

2.5. Acknowledgments

We would like to thank FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo (process no. 08/57548-0), CNPq – Coordenação Nacional de Desenvolvimento Científico e Tecnológico, and CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, for their financial support.

2.6. References

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CHAPTER III

ARTICLE

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Doehlert design-desirability function multi-criteria optimal separation of 17 phenolic compounds from extra-virgin olive oil by capillary zone electrophoresis

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***Manuscript published in the journal "Food Chemistry"**.

BALLUS, C. A.; MEINHART, A. D.; CAMPOS Jr., F. A. S.; BRUNS, R. E.; GODOY, H. T. Doehlert design-desirability function multi-criteria optimal separation of 17 phenolic compounds from extra-virgin olive oil by capillary zone electrophoresis. **Food Chemistry**, v. 146, p. 558-568, 2014.

ABSTRACT

In Brazil, the consumption of extra-virgin olive oil (EVOO) is increasing annually, but there are no experimental studies concerning the phenolic compound contents of commercial EVOO. The aim of this work was to optimize the separation of 17 phenolic compounds already detected in EVOO. A Doehlert matrix experimental design was used, evaluating the effects of pH and electrolyte concentration. Resolution, runtime and migration time relative standard deviation values were evaluated. Derringer's desirability function was used to simultaneously optimize all 37 responses. The 17 peaks were separated in 19 minutes using a fused-silica capillary (50 μm internal diameter, 72 cm of effective length) with an extended light path and 101.3 mmol.L^{-1} of boric acid electrolyte (pH 9.15, 30 kV). The method was validated and applied to 15 EVOO samples found in Brazilian supermarkets.

Keywords: capillary zone electrophoresis; phenolic compounds; extra-virgin olive oil; Doehlert matrix; Derringer's desirability function; method optimization.

3.1. Introduction

It has been postulated that the components of extra-virgin olive oil (EVOO) in the Mediterranean diet, a diet which is largely vegetarian in nature, can contribute to a lower incidence of coronary heart disease and prostate and colon cancers (Murkovic, Lechner, Pietzka, Bratacos, & Katzogiannos, 2004). EVOO contains a significant if minor phenolic portion, which has been shown to possess anti-microbial, antioxidant and anti-inflammatory properties, *in vivo* and *in vitro* (Landete, 2012). Therefore, the phenolic components of EVOO are of particular interest for human health. At least 36 phenolic compounds have been identified in EVOO to date, and there are substantial variations in the composition and concentration of these phenolic compounds, which may be caused by any numbers of factors such as variety, region grown, agricultural techniques, maturity of the fruit at harvest, and processing (Cicerale, Lucas, & Keast, 2012).

In Brazil, the demand for EVOO is increasing every year, mostly due to the fact that the population has became aware of its health benefits with regular consumption. Nevertheless, there are no experimental studies concerning the phenolic compound content of commercial EVOO in Brazil.

Food analysis always demand robust, efficient, sensitive and cost-effective analytical methods to ensure the safety, quality and, increasingly, the traceability of foods. There is also a growing need for research on nutritional and functional properties of foods, as a result of an increasing public concern about this subject (Castro-Puyana, García-Cañas, Simó, & Cifuentes, 2011).

Capillary electrophoresis (CE) is increasingly recognized as a versatile analytical tool for the routine determination of a wide variety of phenolic compounds in different samples due to its separation efficiency, resolution, analysis time and low consumption of samples and reagents (Ignat, Volf, & Popa, 2011). Capillary zone electrophoresis (CZE) is the simplest of all the CE techniques. Analytes are separated inside a narrow bore capillary containing only a buffer solution across which a voltage is applied creating an electric field and different migration velocities depending on charge and size. Buffer pH is crucial for the dissociation of analytes (Rabanes, Guidote Jr., & Quirino, 2012). Most of the CZE methods developed for phenolic compound separations use basic solutions as an electrolyte, controlled by a borate buffer. The borate buffer can form a complex with the phenolic compound hydroxyl groups altering the charge and size, and improving separation (Ballus, Meinhart, Oliveira, & Godoy, 2012; Bizzotto et al., 2012).

In the development of a CE method, several factors affect the separation of the analytical signals. For food samples, which present a large number of analytical peaks, many of which must be separated, the optimization process must take into account all the critical separations simultaneously. As such, multi-criteria methods are very convenient to use, if accurate response surfaces have been determined from statistically designed experiments (Meinhart et al., 2010; Ballus, Meinhart, Bruns, & Godoy, 2011; Meinhart, Ballus, Bruns, Lima Pallone, & Godoy, 2011). In the literature, several studies have successfully separated food compounds by capillary electrophoresis using different combinations of factorial design, variables

and number of responses (Orlandini, Giannini, Pinzauti, & Furlanetto, 2008; Fukuji, Tonin, & Tavares, 2010; Hevia et al., 2010; Orlandini, Gotti, Giannini, Pasquini, & Furlanetto, 2011; Palabiyik, Caglayan, & Onur, 2011; Vignaduzzo, Vera-Candioti, Castellano, & Goicoechea, 2011; Hefnawy, Sultan, Al-Johar, Kassem, & Aboul-Enein, 2012).

In this work, a capillary electrophoresis method coupled to a diode array detector (CE-DAD) for the separation of 17 phenolic compounds already detected in the EVOO polar fraction was optimized. As the basis for this new study our previous laboratory work, Ballus et al. (2011), separating 13 phenolic compounds (tyrosol, oleuropein glycoside, hydroxytyrosol, cinnamic acid, luteolin, apigenin, ferulic acid, caffeic acid, *p*-coumaric acid, vanillic acid, 3,4-dihydroxybenzoic acid, gallic acid and *p*-hydroxybenzoic acid) was used. Our choice of adding four new compounds, (+)-pinoresinol, syringic acid, sinapinic acid and *o*-coumaric acid, to those previously studied was based on new literature data about phenolic composition of EVOO samples (Frankel, 2010; Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011), and also with the aim of including all the main phenolic classes found in this matrix. A multi-criteria optimization, using the Doehlert matrix design and Derringer's desirability function, was used to optimize this work. A Doehlert matrix was chosen because it allows the study of the various factors at a number of levels, and is attractive for resolving problems where specific information about the system indicates some factors deserve more attention than others (Ferreira et al., 2007). This was exactly what was learned from our previous work (Ballus et al., 2011), where pH exhibited the most complex

behavior and had the greatest influence on peak-pair separation. Furthermore, it was decided to evaluate not just the peak-pair resolutions and runtime, but also the stability of each condition by measuring the relative standard deviation (RSD) of migration time for all compounds. As a result, this is the first optimization study simultaneously evaluating such a large number of experimental responses (37) including resolution (18), runtime, and stability (18). After optimization, the method was carefully validated and applied to 15 commercial EVOO samples from Brazil supermarkets.

3.2. Material and methods

3.2.1. Chemicals

Hexane p.a. (Synth, Brazil), methanol p.a. (Synth, Brazil) and methanol HPLC grade (J. T. Baker, USA) were purchased as well as boric acid (Ecibra, Brazil) and sodium hydroxide p.a. (Nuclear, Brazil). Water was purified in a Milli-Q system (Millipore, USA). Standards of tyrosol (*TYR*), gallic acid (*GAL*), *p*-coumaric acid (*p-CUM*), *p*-hydroxybenzoic acid (*p-HYD*), caffeic acid (*CAF*), 3,4-dihydroxybenzoic acid (*3,4-D*), cinnamic acid (*CIN*), vanillic acid (*VAN*), ferulic acid (*FER*), luteolin (*LUT*) and apigenin (*API*) were purchased from Sigma-Aldrich (USA). Hydroxytyrosol (*HYD*) was obtained from Cayman Chemical (USA), oleuropein glycoside (*OLE*) from Extrasynthèse (France), syringic acid (*SYR*), sinapinic acid (*SIN*) and *o*-coumaric acid (*o-CUM*) standards from Chem Service (USA), and (+)-pinoresinol (*PIN*) was purchased from Arbo Nova (Finland).

The standard stock solutions were prepared by dissolving the appropriate amount of each compound in methanol HPLC grade to a final concentration of 2 $g.L^{-1}$ for (+)-pinoresinol, 1 $g.L^{-1}$ for hydroxytyrosol and also for luteolin, 0.4 $g.L^{-1}$ for apigenin, and 5 $g.L^{-1}$ for the other 13 compounds. Then the solutions were filtered through a 0.45 µm Millipore PVDF membrane (Millipore, USA), stored at –18ºC and protected from light.

To execute the optimization experiments, a working methanol:water solution (30:70, v/v) containing 39.6 $mg.L^{-1}$ of each analyte was prepared. The vials with the working solutions were placed under ultrasound for 5 min before injection to remove air bubbles.

3.2.2. Equipment

An Agilent G1600AX (Agilent Technologies, Germany) capillary electrophoresis system equipped with a diode array detector (DAD), automatic injector and temperature control system adjusted to 25ºC was used in this study. A fused-silica capillary of 50 µm internal diameter and 72 cm of effective length with extended light path (Agilent Technologies, Germany) was also used. Detection was at 210 nm and data treatment was performed with HP ChemStation software.

New capillaries were activated and conditioned by washing under 1 bar pressure using 1 $mol.L^{-1}$ NaOH for 30 min, followed by 10 min of ultra-pure water. At the end of the day, the capillary was washed for 5 min with 1 $mol.L^{-1}$ NaOH and

5 min with ultra-pure water. The capillary was stored in ultra-pure water during the night.

3.2.3. Experimental design and data treatment

To obtain an initial evaluation of compound separation, solutions were injected using the method previously described in Ballus et al. (2011). Co-elution among additional four compounds [(+)-pinoresinol, sinapinic acid, syringic acid and *o*-coumaric acid] occurred when the 17 compounds of interest were injected into this system; the other 13 separated as previous described (Ballus et al., 2011). So, a new multivariate optimization procedure to achieve separation of all 17 compounds in the same run, with the lowest runtime and best stability was executed.

Previously (Ballus et al., 2011), voltage had little impact on runtime and none on separation, whilst pH was the most significant variable affecting phenolic compound separation. The electrolyte concentration also had a significant effect on the peak-pair resolutions for some compounds. As a consequence, it was decided to use only the pH and the boric acid concentration (BOR) as variables for the new optimization procedure with the voltage fixed at the highest value (30 kV) allowed by the instrumentation. Moreover, a different kind of experimental design was chosen for this new optimization. Since the separation of the phenolic compounds in this system had a clear dependence on pH, this variable was examined more closely than, for example, the boric acid concentration. In this case, the Doehlert design is very useful, allowing one variable to be studied at more levels than the

other. So, since there were two variables to be optimized, the Doehlert design was constructed with five levels for pH and three for BOR. The levels varied from 40 to 120 mmol.L^{-1} (BOR), and from 9 to 10.5 (pH), respectively. All subsequent analysis were at 0.5 Pa for 5 s (injection), 25ºC and 210 nm. The design center point was executed in triplicate resulting in a total of nine experiments, which were injected in random order. Before running an experimental design condition, the capillary was conditioned for five min with 1 mol.L^{-1} NaOH, five min with ultra-pure water and 10 min with the running electrolyte corresponding to that condition. The conditioning among runs of a same experimental design condition consisted of 1 min of 1 mol.L^{-1} NaOH + a 1 min wait + 1 min of ultra-pure water + 1 min of electrolyte + a 1 min wait + 1 min of electrolyte, totaling six minutes conditioning between runs of a same experimental design condition.

The phenolic standards mixture was injected twice followed by seven injections of a same EVOO polar extract containing all 17 compounds. The polar extract was obtained as described in section *3.2.6*. Thus, nine injections were performed for each experimental design condition (n = 9). The first injection was used to calculate resolution and runtime, while all nine injections were used to determine migration time RSD.

Elementary resolution (R_s) was chosen as one of the responses (Breitkreitz, Jardim & Bruns, 2009) and calculated between peak pairs, which co-eluted under at least one of the design conditions. Resolution values were calculated using:

$$R_s = \frac{2(t_2 - t_1)}{w_2 + w_1}$$

for which t_1 and t_2 are migration times and w_1 and w_2 are the corresponding widths of the bases of the pair of adjacent peaks.

Run time was defined as migration time for the last compound plus one minute. Another set of responses consisted of the relative standard deviation (RSD, %) of the migration times for the 17 peaks and of the electrosmotic flow (solvent), with the objective of evaluating method stability after successive injections and also studying the matrix effect on the separation and stability.

The models were validated by mean of the Analysis of Variance (ANOVA) at the 95% confidence level. Then, the optimum conditions to separate all 17 peaks were determined by examining response contour graphs and using the multi-criteria response technique of Derringer and Suich. Desirability values were established for each individual response and they were combined into their recommended global desirability function (Derringer, & Suich, 1980). The individual desirabilities were defined to maximize the resolutions and to minimize the runtime and the migration time RSD values. Data treatment was carried out using the

Design Expert 6.0.10 (Minneapolis, USA) software. The predicted conditions were submitted to an experimental verification, carried out in triplicate.

3.2.4. Capillary electrophoresis method validation

The method was validated as prescribed by the Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis IUPAC/ISO/AOAC International (Thompson, Ellison, & Wood, 2002). The limit of detection (LOD) was determined through successive dilutions of the standard mixture, until peaks with a signal-to-noise ratio close to three were achieved. The limit of quantification (LOQ) was also determined through successive dilutions, being defined as the concentration that gives peaks with a signal-to-noise ratio close to six. The intra-assay precision was also verified at the limit of quantification (n = 7). System linearity was studied individually for each compound with calibration curves made up of seven points, prepared in triplicate and injected randomly. A lack of fit test for each calibration curve was performed. The intra-assay precision was determined by injecting a solution containing the 17 phenolic compounds at three different concentrations. This procedure was carried out seven consecutive times in one day for each concentration. The inter-assay precision was determined by repeating this procedure on three consecutive days. The first concentration consisted of 10 $mg.L^{-1}$ of tyrosol and oleuropein glycoside, and 5 $mg.L^{-1}$ of the other 15 compounds. The second concentration consisted of 28 $mg.L^{-1}$ of tyrosol and oleuropein glycoside, and 14 $mg.L^{-1}$ of the other compounds. The third

concentration consisted of 42 $mg.L^{-1}$ of tyrosol and oleuropein glycoside, and 21 $mg.L^{-1}$ for the remaining compounds.

3.2.5. EVOO samples

The EVOO samples were acquired in supermarkets of Campinas (São Paulo, Brazil) and comprised 15 different brands. They were coded by numbering them from 1 to 15, and these codes were used throughout this study when referring to the samples. The maximum acidity, as described on the label, varied from 0.2 to 0.8% in oleic acid. All the samples available were imported from other countries, Spain, Portugal, Italy, Greece and Chile, and they were analyzed before their expiration dates. Most of the labels did not display any information about the varieties of olives from which the olive oil had been extracted. Only sample nº 2 (Hojiblanca) and samples nº 10 and nº 11 (Arbequina) presented the olive variety for the consumer. These three samples came from Spain.

3.2.6. Phenolic compound extractions from the EVOO samples

The procedure for phenolic compound extractions was based on the works of Pirisi, Cabras, Cao, Migliorini, & Muggelli (2000) and Bonoli, Montanucci, Gallina Toschi, & Lercker (2003). Hexane (1 mL) and methanol:water (60:40, v/v, 2 mL) were added to EVOO (2 g) in a centrifuge tube. This mixture was stirred for 2 min in a vortex apparatus, and the tube was centrifuged at 5,000 rpm for 5 min. The methanol:water layer was separated and the extraction repeated twice. The extracts were combined and evaporated to dryness at 39ºC under reduced

pressure. Samples were re-dissolved in methanol:water (30:70, v/v, 1 mL) and filtered through a 0.45 μm PVDF membrane (Millipore, USA) before capillary electrophoresis analysis. All the samples were extracted in triplicate (n = 3). Peak identification in the samples was achieved by comparing the migration time and the UV spectra to those obtained for the phenolic compound standards, as well as by spiking.

A recovery assay was also performed at two concentration levels, to estimate the trueness of the extraction technique, since there are no certified reference materials (CRM) for these kinds of compounds in this matrix. The first level consisted of the addition of 14 $mg.kg^{-1}$ of tyrosol and oleuropein glycoside, and 7 $mg.kg^{-1}$ for the others compounds. For the second level, 18 $mg.kg^{-1}$ of tyrosol and oleuropein glycoside, and 10 $mg.kg^{-1}$ of the other compounds were added. The appropriate volume of standard mixture was added to EVOO samples and the tubes were stirred for five min. The whole extraction procedure was completed as described above. The results were calculated as the percentage of recovery (%) for each compound. The recovery assays were executed in triplicate, for each concentration level (n = 3).

3.3. Results and discussion

3.3.1. Models calculation and validation

Table 3.1 summarizes all the results obtained in the Doehlert design experiments for the large number of responses evaluated in this study. For resolution, 18 responses were investigated, considering all the peaks that co-eluted at least once in the design experiments. It must be pointed out that this is the first work to deal with such a large number of resolutions to be optimized simultaneously. Among the 17 compounds, *o*-coumaric acid, apigenin and luteolin were the most susceptible to variations in the experimental conditions, each being involved in four co-elutions, followed by sinapinic acid, ferulic acid and *p*-coumaric acid, with three co-elutions each. Only cinnamic acid did not co-elute under any of the experimental conditions examined. **Table 3.1** also contains the results for runtime and migration time RSD for the compounds, giving 37 responses to be considered in the optimization procedure.

From the results presented in **Table 3.1**, models for all 37 responses were calculated as well as verified for regression significance and possible significant model lack of fit to the experimental data. Complete linear and quadratic models were calculated for each of the 37 response values and applied in the optimization process. However, only the significant coefficients at the 95% confidence level are given in **Table 3.2**. The model for the electro-osmotic flow (solvent) migration time RSD was not significant indicating it did not depend on pH or BOR levels and could be removed from the optimization procedure. All the other models presented

significant regressions, but some of them also presented significant lack of fit (ANOVA, 95%). Five out of the 18 models for resolution presented lack of fit (SYR/FER, syringic acid/ferulic acid; TYR/OLE, tyrosol/oleuropein glycoside; FER/LUT, ferulic acid/luteolin; LUT/o-CUM, luteolin/*o*-coumaric acid; and o-CUM/VAN, *o*-coumaric acid/vanillic acid). The model for runtime (RTIM) also suffered from lack of fit, as did seven models for the migration time RSD values (HYD, hydroxytyrosol; CIN, cinnamic acid; API, apigenin; LUT, luteolin; CAF, caffeic acid; GAL, gallic acid; and 3,4-D, 3,4-dihydroxybenzoic acid).

Table 3.1. Doehlert design experiments and the results obtained for each one of the thirty seven responses.

Experiment	Variables [a]		Resolution (R_S) [b]																	RTIM (min) [b]
	pH	BOR (mmol L^{-1})																		
	(x_1)	(x_2)	EOF/TYR	SYR/FER	p-CUM/API	TYR/OLE	o-CUM/p-CUM	3,4-D/GAL	SIN/LUT	p-HYD/3,4-D	SIN/FER	FER/LUT	LUT/o-CUM	o-CUM/API	LUT/API	SIN/API	o-CUM/VAN	SYR/CAF	p-CUM/CAF	PIN/HYD	
1	0	0	26.77	2.30	29.52	20.20	5.39	7.01	2.61	3.57	25.04	26.15	40.52	27.98	14.59	12.78	13.11	17.87	0.00	28.05	21.06
2	0	0	21.20	2.04	31.90	21.60	7.22	6.83	-1.25	-1.24	26.40	27.70	41.11	24.01	16.92	16.52	13.73	25.56	4.68	33.41	20.09
3	0	0	23.92	2.29	37.31	21.68	7.03	7.78	1.99	3.77	26.31	27.01	41.73	28.33	15.28	14.01	13.99	21.68	1.46	30.94	20.94
4	1	0	43.41	6.11	57.16	-10.07	-3.36	-14.17	19.47	30.71	25.51	41.46	69.16	60.09	19.00	-1.21	2.08	-0.83	-30.21	-14.98	28.31
5	0.5	0.866	36.57	4.54	56.06	1.19	1.67	-1.93	19.96	33.91	31.22	48.72	79.40	58.48	24.85	5.58	7.43	6.74	-21.71	0.71	41.78
6	-1	0	3.56	-1.59	-1.32	28.25	9.46	-10.25	-48.24	-49.15	20.15	-28.61	-23.99	-10.12	-14.19	35.05	15.59	74.33	61.93	80.15	15.99
7	-0.5	-0.866	10.05	0.00	8.80	29.33	8.80	7.07	-22.56	-21.08	16.75	-2.13	1.91	0.00	1.91	21.62	14.98	35.75	25.58	45.86	13.22
8	0.5	-0.866	15.87	4.51	34.70	0.83	0.73	-0.68	9.64	9.57	19.53	26.59	47.79	38.16	12.97	3.81	7.49	7.12	-6.53	4.32	14.94
9	-0.5	0.866	8.94	-1.51	15.05	29.93	12.49	15.40	-34.73	-32.78	27.62	-0.81	7.67	1.63	6.30	33.12	20.13	58.07	35.56	73.34	23.63

Experiment	Variables [a]		Migration time RSD values (%) [b]																	
	pH	BOR (mmol L^{-1})																		
	(x_1)	(x_2)	EOF	TYR	PIN	OLE	HYD	SIN	CIN	SYR	FER	o-CUM	p-CUM	API	VAN	LUT	p-HYD	CAF	GAL	3,4-D
1	0	0	0.49	1.20	2.66	0.51	0.41	1.40	0.85	1.66	1.35	2.56	1.81	0.35	1.75	0.82	1.25	1.58	0.65	1.75
2	0	0	0.11	0.66	2.15	0.31	0.67	1.00	0.76	1.38	1.03	1.96	1.47	0.23	1.40	0.87	1.99	1.23	0.67	1.29
3	0	0	0.53	1.22	3.24	0.53	0.64	1.88	0.63	2.12	1.78	3.21	2.53	0.51	2.39	0.77	3.07	1.35	0.61	1.47
4	1	0	0.41	2.00	4.32	1.78	3.61	5.48	4.58	8.17	8.78	10.92	11.98	9.87	10.81	8.84	9.26	11.91	12.80	16.48
5	0.5	0.866	0.29	2.64	7.28	0.59	2.48	4.11	2.64	5.76	4.74	9.73	7.45	0.94	7.09	2.41	11.59	2.87	3.21	2.71
6	-1	0	0.22	0.31	0.07	0.41	0.61	0.40	0.77	0.45	0.42	0.51	0.55	0.52	0.49	0.88	0.54	1.27	1.32	1.40
7	-0.5	-0.866	0.29	0.35	1.08	0.52	0.85	0.30	1.07	0.25	0.48	0.27	0.31	0.87	0.32	1.46	0.32	1.87	1.84	2.24
8	0.5	-0.866	1.92	2.57	2.88	2.85	3.69	3.92	4.29	4.34	4.46	4.36	4.86	5.16	5.12	5.29	5.66	6.92	6.97	8.16
9	-0.5	0.866	0.56	0.93	1.27	0.46	0.54	1.27	0.68	1.53	1.42	1.77	1.89	0.74	1.80	0.85	2.03	0.96	0.79	0.97

[a] Codified values of experimental factors: $x_1 = (pH - 9.75)/0.75$; $x_2 = ([BOR] - 80)/46.2$; BOR, boric acid concentration.

[b] Responses: EOF/TYR, electroosmotic flow/tyrosol; SYR/FER, syringic acid/ferulic acid; p-CUM/API, *p*-coumaric acid/apigenin; TYR/OLE, tyrosol/oleuropein glycoside; o-CUM/p-CUM, *o*-coumaric acid/*p*-coumaric acid; 3,4-D/GAL, 3,4-dihydroxybenzoic acid/gallic acid; SIN/LUT, sinapinic acid/luteolin; p-HYD/3,4-D, *p*-hydroxybenzoic acid/3,4-dihydroxybenzoic acid; SIN/FER, sinapinic acid/ferulic acid; FER/LUT, ferulic acid/luteolin; LUT/o-CUM, luteolin/*o*-coumaric acid; o-CUM/API, *o*-coumaric acid/apigenin; LUT/API, luteolin/apigenin; SIN/API, sinapinic acid/apigenin; o-CUM/VAN, *o*-coumaric acid/vanillic acid; SYR/CAF, syringic acid/caffeic acid; p-CUM/CAF, *p*-coumaric acid/caffeic acid; PIN/HYD, (+)-pinoresinol/hydroxytyrosol; RTIM, runtime; EOF, electroosmotic flow; TYR, tyrosol; PIN, (+)-pinoresinol; OLE, oleuropein glycoside; HYD, hydroxytyrosol; SIN, sinapinic acid; CIN, cinnamic acid; SYR, syringic acid; FER, ferulic acid; o-CUM, *o*-coumaric acid; p-CUM, *p*-coumaric acid; API, apigenin; VAN, vanillic acid; LUT, luteolin; p-HYD, *p*-hydroxybenzoic acid; CAF, caffeic acid; GAL, gallic acid; 3,4-D, 3,4-dihydroxybenzoic acid. RSD, relative standard deviation.

Table 3.2. Significant model coefficients, their standard errors, and ANOVA summary, considering the statistical significance of the regression and the lack of fit. [a]

Responses [b]	Indicated Model	Significant coefficients ± standard error						Regression Significance	Model Fit
		Intercept	A (pH)	B (BOR)	A^2	B^2	AB	($p < 0.05$)	($p > 0.05$)
Resolutions (R_S)									
EOF/TYR	Linear	21.14 ± 2.02	18.86 ± 3.49	-	-	-	-	0.0040	0.1363
SYR/FER	Linear	2.08 ± 0.20	4.32 ± 0.35	-	-	-	-	< 0.0001	**0.0378**
p-CUM/API	Linear	29.91 ± 1.62	30.64 ± 2.81	7.97 ± 2.81	-	-	-	<0.0001	0.3986
TYR/OLE	Linear	15.88 ± 2.10	-22.31 ± 3.64	-	-	-	-	0.0026	**0.0117**
o-CUM/p-CUM	Linear	5.49 ± 0.69	-7.42 ± 1.20	-	-	-	-	0.0023	0.1496
3,4-D/GAL	Quadratic	7.21 ± 0.26	-12.32 ± 0.26	2.05 ± 0.26	-9.17 ± 0.40	-	-5.53 ± 0.51	0.0001	0.6460
SIN/LUT	Quadratic	-	37.05 ± 2.79	-	-15.50 ± 4.41	-	-	0.0063	0.0635
p-HYD/3,4-D	2FI	-2.52 ± 2.21	42.84 ± 3.82	-	-	-	20.80 ± 7.64	0.0005	0.1079
SIN/FER	Quadratic	25.92 ± 0.38	2.85 ± 0.38	6.51 ± 0.38	-3.08 ± 0.61	-	-	0.0024	0.6393
FER/LUT	Quadratic	26.95 ± 1.17	36.40 ± 1.17	6.77 ± 1.17	-20.53 ± 1.85	-	12.01 ± 2.34	0.0004	**0.0499**
LUT/o-CUM	Quadratic	41.12 ± 3.34	50.65 ± 3.34	10.79 ± 3.34	-18.53 ± 5.28	-	-	0.0041	**0.0037**
o-CUM/API	Linear	25.40 ± 1.97	39.24 ± 3.42	-	-	-	-	< 0.0001	0.1069
LUT/API	Quadratic	15.60 ± 0.75	16.00 ± 0.75	4.70 ± 0.75	-13.19 ± 1.18	-	-	0.0011	0.3482
SIN/API	Linear	15.70 ± 0.99	-19.65 ± 1.71	-	-	-	-	< 0.0001	0.2561
o-CUM/VAN	Linear	12.06 ± 0.90	-7.87 ± 1.55	-	-	-	-	0.0063	**0.0187**
SYR/CAF	Quadratic	21.70 ± 1.93	-38.38 ± 1.93	6.33 ± 1.93	15.05 ± 3.05	-	-13.10 ± 3.85	0.0019	0.6603
p-CUM/CAF	Quadratic	2.05 ± 1.19	-45.61 ± 1.19	-	13.81 ± 1.88	-	-14.53 ± 2.38	0.0003	0.6846
PIN/HYD	2FI	31.31 ± 1.33	-50.74 ± 2.30	6.89 ± 2.30	-	-	-17.95 ± 4.59	< 0.0001	0.2604
Runtime (min)									
RTIM	2FI	22.22 ± 0.71	7.42 ± 1.23	10.75 ± 1.23	-	-	9.48 ± 2.45	0.0005	**0.0368**
Migration time RSD values (%)									
EOF	2FI	-	-	-	-	-	-	**0.0946**	0.2140
TYR	Linear	1.32 ± 0.18	1.22 ± 0.31	-	-	-	-	0.0221	0.2129
PIN	Linear	2.77 ± 0.39	2.72 ± 0.68	-	-	-	-	0.0126	0.1377
OLE	Quadratic	0.45 ± 0.16	0.86 ± 0.16	-0.67 ± 0.16	-	-	-1.27 ± 0.32	0.0249	0.0685
HYD	Quadratic	0.57 ± 0.25	1.80 ± 0.25	-	1.54 ± 0.40	-	-	0.0243	**0.0364**
SIN	Linear	2.19 ± 0.27	2.77 ± 0.47	-	-	-	-	0.0029	0.1879
CIN	Quadratic	0.75 ± 0.19	2.13 ± 0.19	-	1.93 ± 0.30	1.25 ± 0.30	-	0.0068	**0.0367**
SYR	Quadratic	1.72 ± 0.20	3.96 ± 0.20	0.78 ± 0.20	2.59 ± 0.31	-	-	0.0016	0.5795
FER	Quadratic	1.39 ± 0.23	4.00 ± 0.23	-	3.21 ± 0.37	-	-	0.0023	0.3721
o-CUM	Quadratic	2.58 ± 0.37	5.48 ± 0.37	1.98 ± 0.37	3.14 ± 0.58	-	-	0.0036	0.3980
p-CUM	Quadratic	1.94 ± 0.31	5.50 ± 0.31	1.21 ± 0.31	4.32 ± 0.49	-	-	0.0021	0.4223
API	Quadratic	0.36 ± 0.66	3.86 ± 0.66	-	4.84 ± 1.05	-	-	0.0324	**0.0049**
VAN	Quadratic	1.85 ± 0.24	5.12 ± 0.24	1.00 ± 0.24	3.80 ± 0.38	-	-	0.0013	0.8747
LUT	Quadratic	0.82 ± 0.35	3.55 ± 0.35	-	4.04 ± 0.55	-	-	0.0078	**0.0026**
p-HYD	Linear	3.97 ± 0.72	5.39 ± 1.25	-	-	-	-	0.0102	0.1147
CAF	Quadratic	1.39 ± 0.51	4.71 ± 0.51	-	5.20 ± 0.80	-	-	0.0102	**0.0137**
GAL	Quadratic	0.64 ± 0.53	5.08 ± 0.53	-	6.42 ± 0.84	-	-	0.0085	**0.0004**
3,4-D	Quadratic	1.51 ± 1.01	6.30 ± 1.01	-	7.43 ± 1.60	-	-	0.0297	**0.0058**

[a] Bold values in Regression Significance means this model does not present significant regression. Bold values in Model Fit means this model presents lack of fit.

[b] Responses: EOF/TYR, electroosmotic flow/tyrosol; SYR/FER, syringic acid/ferulic acid; p-CUM/API, *p*-coumaric acid/apigenin; TYR/OLE, tyrosol/oleuropein glycoside; o-CUM/p-CUM, *o*-coumaric acid/*p*-coumaric acid; 3,4-D/GAL, 3,4-dihydroxybenzoic acid/gallic acid; SIN/LUT, sinapinic acid/luteolin; p-HYD/3,4-D, *p*-hydroxybenzoic acid/3,4-dihydroxybenzoic acid; SIN/FER, sinapinic acid/ferulic acid; FER/LUT, ferulic acid/luteolin; LUT/o-CUM, luteolin/*o*-coumaric acid; o-CUM/API, *o*-coumaric acid/apigenin; LUT/API, luteolin/apigenin; SIN/API, sinapinic acid/apigenin; o-CUM/VAN, *o*-coumaric acid/vanillic acid; SYR/CAF, syringic acid/caffeic acid; p-CUM/CAF, *p*-coumaric acid/caffeic acid; PIN/HYD, (+)-pinoresinol/hydroxytyrosol; RTIM, runtime; EOF, electroosmotic flow; TYR, tyrosol; PIN, (+)-pinoresinol; OLE, oleuropein glycoside; HYD, hydroxytyrosol; SIN, sinapinic acid; CIN, cinnamic acid; SYR, syringic acid; FER, ferulic acid; o-CUM, *o*-coumaric acid; p-CUM, *p*-coumaric acid; API, apigenin; VAN, vanillic acid; LUT, luteolin; p-HYD, *p*-hydroxybenzoic acid; CAF, caffeic acid; GAL, gallic acid; 3,4-D, 3,4-dihydroxybenzoic acid.

As with Ballus et al. (2011), pH was the strongest factor affecting peak-pair resolutions in the phenolic compound separations, presenting the most complex behavior. All the pH coefficients for the resolution models were significant and most of them had high values. The different algebraic signs of the pH coefficients mean that, while some peak-pair resolutions increased with increasing pH, other peak-pairs lost resolution. This explains the difficulty in separating a large number of compounds in the same run using a single pH value. Also, the curvature effects given by the squared coefficients (**Table 3.2**) for pH were significant for the (3,4-D/GAL, 3,4-dihydroxybenzoic acid/gallic acid), (SIN/LUT, sinapinic acid/luteolin), (SIN/FER, sinapinic acid/ferulic acid), (FER/LUT, ferulic acid/luteolin), (LUT/o-CUM, luteolin/*o*-coumaric acid), (LUT/API, luteolin/apigenin), (SYR/CAF, syringic acid/caffeic acid) and (p-CUM/CAF, *p*-coumaric acid/caffeic acid) resolution models.

Boric acid concentration displayed simpler behavior with only positive coefficients. In all cases an increase in the boric acid concentration improved separation between the compound pairs within the design domain.

The effects of the BOR and pH interaction were significant for some of the resolution models, also presenting different algebraic signs. This can be explained by the complex mechanism of the phenolic compound separations in borate buffers. In this system, pH and the formation of borate-hydroxyl groups had a role in the resolution of compounds being separated.

The RSD of migration times increased at higher pH values, and this factor was significant for all the models of this response. Also, the curvature effect for pH

was significant for most of the migration time RSD models. So, in general, it was better to work at lower pH values to ensure greater stability for the phenolic compound migration times. The boric acid concentration had little effect on the migration time RSD values.

3.3.2. Multi-criteria optimization using Derringer's desirability function

None of the Doehlert experimental conditions resulted in the separation of all 17 phenolic compounds. The next step was to use Derringer's desirability function to optimize the separation, employing the models for the 37 responses simultaneously. All the models were combined to search for the maximum resolution between peak-pairs in the shortest runtime with the best migration time stabilities (lower migration time RSD values).

Before searching for the optimum set of experimental conditions using the desirability function, it was necessary to evaluate the models that presented lack of fit. Two approaches were used. The first trial was carried out using all the models within the whole experimental region of the design (between -1.00 and 1.00 for the two variables). To define the desirability function, lower and upper limits for each response were specified, based on experimental data, respectively. With these parameters, only two possible conditions with maximum resolutions and minimum runtime and migration time RSD values were predicted. These were largely the same and one was chosen to perform an experimental verification in triplicate (pH = -0.25; BOR = 0.40). The global desirability for this condition was 0.70. The codified values obtained correspond to real values of pH = 9.56 and BOR = 98.5

mmol.L^{-1}. On execution, the 17 phenolic compounds did not separate. Furthermore, there were huge discrepancies between predicted and observed values. This can easily be explained by the use of all models including those with a lack of fit leading to the erroneous prediction of optimal conditions. Even though it corresponds to a high desirability value, these conditions did not work adequately when running real experiments in the laboratory.

After this, another desirability search was performed but more detailed observations of the models with lack of fit were performed first. Of the 13 models suffering from lack of fit, eight presented a mean square lack of fit/mean square pure error ratio between one and four times higher than the critical F value at the 95% confidence level. Just five models (LUT/o-CUM, luteolin/*o*-coumaric acid; API, apigenin; LUT, luteolin; GAL, gallic acid; and 3,4-D, 3,4-dihydroxybenzoic acid) showed a mean square lack of fit/mean square pure error ratio higher than the critical F value (between 9 and 132 times higher). All the electropherograms and resolution data were inspected to find the experimental region where responses presented attractive values for optimization. After this, desirability function optimization using all models was carried out restricting the experimental region to that sub-region (between -1.00 and 0.00 for pH and between 0.00 and 1.00 for BOR). Again, for all responses, lower and upper limits were defined as those obtained experimentally. However, optimization criteria for responses from the five models with lack of fit were chosen so the predicted values were "in range" since the "out of range" predictions were not expected to be accurate. As a result, 12 sets of conditions were predicted. To select the best conditions among the 12,

those presenting poor predicted resolutions were eliminated. After inspection of all the predicted responses, just two conditions seemed promising. Both were basically the same, and one was chosen to perform experimental verification in triplicate (pH = -0.80 and BOR = 0.46, with a desirability value of 0.45). These codified values correspond to the laboratory values of pH = 9.15 and BOR = 101.3 $mmol.L^{-1}$. These experiments confirmed the separation of all 17 phenolic compounds with good resolution, short runtime and the best stability. **Table 3.3** summarizes the optimization data using Derringer's desirability function, and the predicted and observed response values for the optimal condition. **Figure 3.1** presents the resulting electropherogram with the 17 phenolic compounds separated in 19 min.

The experimental values were very close to the predicted ones, with few exceptions (p-CUM/API, *p*-coumaric acid/apigenin; p-HYD/3,4-D, *p*-hydroxybenzoic acid/3,4-dihydroxybenzoic acid; o-CUM/API, *o*-coumaric acid/apigenin; SIN/API, sinapinic acid/apigenin; and RTIM, runtime), as can be seen in **Table 3.3**. These differences may have arisen from restrictions in the experimental region that could compromise the models, even for those that did not present lack of fit. Nevertheless, the observed values resulted in adequate compound separation with only some co-elution of syringic and ferulic acids. The resolution of this peak pair did not improve within this experimental region. However method applicability is not seriously affected, since both compounds are found in only a few EVOO samples. In summary, the separation of the 17 phenolic compounds from EVOO was performed in only 19 min, using a fused-silica capillary of 50 μm internal diameter

and 72 cm of effective length with extended light path, 101.3 mmol.L^{-1} of boric acid electrolyte at 9.15 pH, 30 kV, 25ºC, injection of 0.5 Pa for five seconds, and detection at 210 nm.

Table 3.3. Desirability criteria, predicted optimal variables and responses, and experimentally observed responses for the predicted variables.

Variables and Responses [a]	Desirability criteria for variables and responses			Predicted Variables (Codified)	Predicted Responses	Observed Responses (Mean ± SD, n = 3) [b]
	Goal	Lower Limit	Upper Limit			
Variables						
pH	is in range	-1.00	0.00	-0.80		
BOR	is in range	0.00	1.00	0.46		
Resolutions (R_S)						
EOF/TYR	maximize	3.56	43.40		8.77	6.22 ± 0.12
SYR/FER	maximize	-1.59	6.10		-1.56	-1.94 ± 0.14
p-CUM/API	maximize	-1.31	57.20		9.24	3.67 ± 0.71
TYR/OLE	maximize	-10.06	29.92		33.75	34.66 ± 0.71
o-CUM/p-CUM	maximize	-3.36	12.49		12.01	11.51 ± 0.48
3,4-D/GAL	maximize	-14.17	15.40		14.21	13.95 ± 1.44
SIN/LUT	maximize	-48.24	19.95		-44.36	-44.11 ± 1.17
p-HYD/3,4-D	maximize	-49.15	33.91		-42.56	-48.89 ± 6.88
SIN/FER	maximize	16.75	31.22		24.15	23.76 ± 1.29
FER/LUT	maximize	-28.61	48.72		-17.32	-19.83 ± 2.58
LUT/o-CUM	is in range	-23.99	79.40		-12.03	-14.85 ± 1.34
o-CUM/API	maximize	-10.12	60.10		-2.87	-8.47 ± 0.40
LUT/API	maximize	-14.20	24.85		-5.11	-6.91 ± 1.58
SIN/API	maximize	-1.21	35.05		33.09	38.49 ± 1.41
o-CUM/VAN	maximize	2.07	20.13		19.00	18.74 ± 0.75
SYR/CAF	maximize	-0.83	74.30		69.90	74.49 ± 5.69
p-CUM/CAF	maximize	-30.20	61.90		52.48	54.53 ± 5.87
PIN/HYD	maximize	-14.98	80.14		81.45	81.49 ± 1.12
Runtime (min)						
RTIM	minimize	13.22	41.78		17.81	19.51 ± 0.11
Migration time RSD values (%)						
EOF	minimize	0.11	1.92		0.52	0.28 ± 0.19
TYR	minimize	0.30	2.64		0.44	0.32 ± 0.20
PIN	minimize	0.07	7.28		1.23	0.60 ± 0.33
OLE	minimize	0.30	2.84		0.47	0.32 ± 0.18
HYD	minimize	0.40	3.69		0.37	0.56 ± 0.20
SIN	minimize	0.30	5.47		0.15	0.76 ± 0.35
CIN	minimize	0.63	4.58		0.53	0.66 ± 0.18
SYR	minimize	0.25	8.16		0.71	0.82 ± 0.42
FER	minimize	0.42	8.77		0.71	0.85 ± 0.40
o-CUM	minimize	0.27	10.92		0.49	0.78 ± 0.40
p-CUM	minimize	0.30	11.97		0.82	0.95 ± 0.44
API	is in range	0.22	9.86		0.74	0.62 ± 0.32
VAN	minimize	0.31	10.80		0.76	1.00 ± 0.48
LUT	is in range	0.76	8.83		0.76	0.68 ± 0.21
p-HYD	minimize	0.31	11.59		0.71	1.11 ± 0.50
CAF	minimize	0.96	11.90		1.07	1.05 ± 0.24
GAL	is in range	0.60	12.79		0.86	1.05 ± 0.25
3,4-D	is in range	0.96	16.47		1.23	1.15 ± 0.26

[a] BOR, boric acid concentration; EOF/TYR, electroosmotic flow/tyrosol; SYR/FER, syringic acid/ferulic acid; p-CUM/API, *p*-coumaric acid/apigenin; TYR/OLE, tyrosol/oleuropein glycoside; o-CUM/p-CUM, *o*-coumaric acid/*p*-coumaric acid; 3,4-D/GAL, 3,4-dihydroxybenzoic acid/gallic acid; SIN/LUT, sinapinic acid/luteolin; p-HYD/3,4-D, *p*-hydroxybenzoic acid/3,4-dihydroxybenzoic acid; SIN/FER, sinapinic acid/ferulic acid; FER/LUT, ferulic acid/luteolin; LUT/o-CUM, luteolin/*o*-coumaric acid; o-CUM/API, *o*-coumaric acid/apigenin; LUT/API, luteolin/apigenin; SIN/API, sinapinic acid/apigenin; o-CUM/VAN, *o*-coumaric acid/vanillic acid; SYR/CAF, syringic acid/caffeic acid; p-CUM/CAF, *p*-coumaric acid/caffeic acid; PIN/HYD, (+)-pinoresinol/hydroxytyrosol; RTIM, runtime; EOF, electroosmotic flow; TYR, tyrosol; PIN, (+)-pinoresinol; OLE, oleuropein glycoside; HYD, hydroxytyrosol; SIN, sinapinic acid; CIN, cinnamic acid; SYR, syringic acid; FER, ferulic acid; o-CUM, *o*-coumaric acid; p-CUM, *p*-coumaric acid; API, apigenin; VAN, vanillic acid; LUT, luteolin; p-HYD, *p*-hydroxybenzoic acid; CAF, caffeic acid; GAL, gallic acid; 3,4-D, 3,4-dihydroxybenzoic acid.
[b] SD, standard deviation

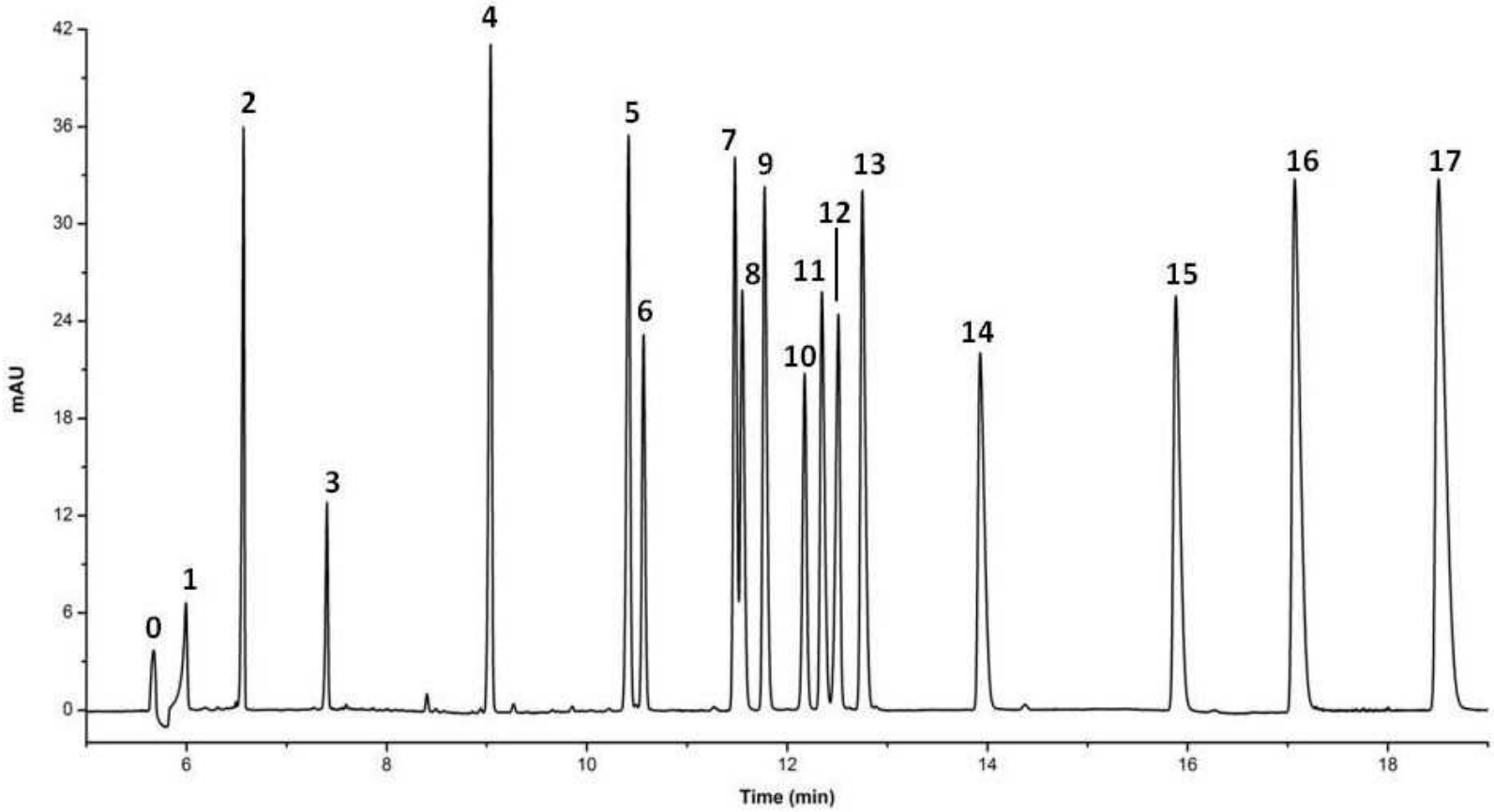

Figure 3.1. Electropherogram for the optimal separation of seventeen phenolic compounds. Fused-silica capillary of 50 μm internal diameter and 72 cm of effective length with extended light path, 101.3 mmol.L^{-1} of boric acid electrolyte at 9.15 pH, 30 kV, 25ºC, injection of 0.5 Pa for 5 seconds, and detection at 210 nm. Peak identification: 0, solvent; 1, tyrosol; 2, (+)-pinoresinol; 3, oleuropein glycoside; 4, hydroxytyrosol; 5, cinnamic acid; 6, sinapinic acid; 7, syringic acid; 8, ferulic acid; 9, *o*-coumaric acid; 10, apigenin; 11, *p*-coumaric acid; 12, luteolin; 13, vanillic acid; 14, *p*-hydroxybenzoic acid; 15, caffeic acid; 16, gallic acid; 17, 3,4-dihydroxybenzoic acid.

3.3.3. Method validation

Before validation, it was noted that even though RSD migration time values for the nine injections were very low at the optimal condition, co-elution between cinnamic and sinapinic acids as well as between luteolin and vanillic acid occurred after just three injections. This is because separation of all 17 phenolic compounds was only achieved in a very narrow pH range, and even a small modification in electrolyte pH lead to variations in migration times. After a few injections, the high voltage applied promoted changes in electrolyte pH and, consequently, in migrations of the compounds. Since the peaks were very close, even a small change in migration time led to co-elution of some compounds. As injections were performed using the same electrolyte, the sinapinic acid and luteolin peaks started to move towards each other resulting in co-elution after the third injection. So, all system vials (electrolyte, NaOH, water) were changed after three successive injections, avoiding the co-elution problem. Other conditioning protocols were also evaluated, but none eliminated undesired peak displacement.

The results obtained after performing validation are presented in **Tables 3.4** and **3.5**. All parameters were evaluated according to the Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis IUPAC/ISO/AOAC International (Thompson, Ellison, & Wood, 2002).

Table 3.4. Figures of merit for method validation for the separation of 17 phenolic compounds from extra-virgin olive oil.

Compounds	LOD ($mg.L^{-1}$) [a]	LOQ ($mg.L^{-1}$) [a]	Intra-assay Precision LOQ (n = 7) [a]	Linearity ($mg.L^{-1}$)	Equation	r^2	Lack of Fit Test ($p > 0.05$)
Tyrosol	2.50	4.38	8.31	9.2 - 45.8	$y = 0.772x - 0.75$	0.9902	0.216
(+)-Pinoresinol	0.60	1.20	10.57	4.4 - 22.0	$y = 2.124x - 2.40$	0.9941	0.404
Oleuropein glycoside	2.37	4.14	9.41	8.7 - 43.4	$y = 0.844x - 1.77$	0.9973	0.356
Hydroxytyrosol	1.20	2.10	6.86	4.4 - 22.0	$y = 2.240x - 2.00$	0.9963	0.383
Cinnamic acid	1.19	2.08	9.63	4.4 - 21.8	$y = 3.030x - 0.82$	0.9917	0.650
Sinapinic acid	1.19	2.09	7.69	4.4 - 21.9	$y = 1.433x - 1.58$	0.9932	0.260
Syringic acid	1.18	2.07	9.77	4.3 - 21.7	$y = 2.049x - 3.39$	0.9969	0.224
Ferulic acid	1.18	2.07	8.39	4.3 - 21.7	$y = 2.316x - 1.45$	0.9962	0.337
o-Coumaric acid	1.19	2.08	8.07	4.4 - 21.8	$y = 2.683x - 1.93$	0.9946	0.380
Apigenin	1.48	2.58	8.83	5.4 - 27.1	$y = 2.448x - 5.24$	0.9835	0.176
p-Coumaric acid	1.21	2.11	7.81	4.4 - 22.1	$y = 2.549x - 1.71$	0.9949	0.797
Luteolin	1.20	2.10	8.89	4.4 - 22.0	$y = 2.984x - 2.44$	0.9810	0.147
Vanillic acid	1.20	2.10	5.63	4.4 - 22.0	$y = 3.198x - 3.80$	0.9952	0.496
p-Hydroxybenzoic acid	1.23	2.16	5.89	4.5 - 22.6	$y = 3.160x + 0.15$	0.9919	0.690
Caffeic acid	1.24	2.16	7.05	4.5 - 22.7	$y = 3.617x - 3.59$	0.9952	0.158
Gallic acid	1.25	2.18	9.95	4.6 - 22.9	$y = 5.380x - 5.72$	0.9953	0.051
3,4-Dihydroxybenzoic acid	0.66	1.32	10.83	4.8 - 24.2	$y = 7.362x - 6.99$	0.9949	0.088

[a] LOD, limit of detection; LOQ, limit of quantification.

Table 3.5. Precision results for the separation method of 17 phenolic compounds from extra-virgin olive oil and recovery results for the liquid-liquid extraction method.

Compounds	Intra-assay Precision (%, n = 7) [a]			Inter-assay Precision (%, n = 3) [a]			Recovery (%, mean ± SD, n = 3) [b]	
	First Level	Second Level	Third Level	First Level	Second Level	Third Level	First Level	Second Level
Tyrosol	2.75	2.38	2.79	2.11	0.93	2.79	82.20 ± 7.61	76.68 ± 0.09
(+)-Pinoresinol	2.66	2.15	1.90	3.89	5.03	5.17	78.99 ± 1.69	78.51 ± 3.56
Oleuropein glycoside	3.19	2.42	1.85	9.74	10.02	1.45	95.37 ± 0.64	91.20 ± 3.12
Hydroxytyrosol	4.30	2.04	2.31	5.55	8.62	0.99	80.93 ± 1.16	83.98 ± 4.81
Cinnamic acid	3.30	2.08	1.80	6.78	8.90	0.39	67.06 ± 0.36	59.87 ± 2.66
Sinapinic acid	2.91	3.12	2.08	3.03	10.88	8.95	88.52 ± 1.32	90.31 ± 6.32
Syringic acid	4.80	3.23	2.11	13.50	8.59	0.98	95.97 ± 0.66	92.92 ± 6.34
Ferulic acid	2.94	2.85	2.11	6.66	6.03	0.85	98.53 ± 4.10	87.08 ± 3.03
o-Coumaric acid	3.75	2.19	2.70	8.18	11.13	0.59	91.43 ± 1.01	87.06 ± 5.09
Apigenin	3.84	3.30	4.68	5.82	8.41	16.45	102.06 ± 1.33	99.44 ± 5.99
p-Coumaric acid	2.42	2.63	2.24	5.47	6.54	4.73	91.42 ± 2.92	88.69 ± 3.78
Luteolin	2.38	3.12	2.30	12.78	4.25	3.25	77.60 ± 4.07	79.37 ± 3.67
Vanillic acid	3.93	2.30	1.83	7.96	8.51	0.69	96.43 ± 0.34	90.71 ± 4.56
p-Hydroxybenzoic acid	4.13	2.46	2.43	5.48	5.09	1.33	90.35 ± 0.74	85.79 ± 4.17
Caffeic acid	3.23	2.32	1.99	6.77	9.56	1.01	92.54 ± 1.22	88.05 ± 3.07
Gallic acid	3.60	1.94	1.89	11.64	13.44	1.25	90.78 ± 0.05	84.67 ± 3.96
3,4-Dihydroxybenzoic acid	3.00	2.30	1.87	5.87	10.54	1.17	92.03 ± 0.13	88.29 ± 2.96

[a] Concentration of the standards. First Level: tyrosol and oleuropein glycoside: 10.0 $mg.L^{-1}$; others compounds: 5.0 $mg.L^{-1}$; Second Level: tyrosol and oleuropein glycoside: 28.0 $mg.L^{-1}$; others compounds: 14.0 $mg.L^{-1}$; Third Level: tyrosol and oleuropein glycoside: 42.0 $mg.L^{-1}$; others compounds: 21.0 $mg.L^{-1}$.

[b] Concentration of the standards added to the sample: First Level: 14.0 $mg.kg^{-1}$ of tyrosol and oleuropein glycoside; 7.0 $mg.kg^{-1}$ for the others compounds; Second Level: 18.0 $mg.kg^{-1}$ of tyrosol and oleuropein glycoside; 10.0 $mg.kg^{-1}$ for the others compounds. SD, standard deviation.

The limits of detection were between 0.66 $mg.L^{-1}$ [(+)-pinoresinol] to 1.25 $mg.L^{-1}$ (gallic acid). The exceptions were tyrosol (2.5 $mg.L^{-1}$) and oleuropein glycoside (2.37 $mg.L^{-1}$), which had higher limits of detection arising from lower molar absorptivity at 210 nm. The values for intra-assay precision at the limit of quantification were below 10% facilitating precision even at low concentrations. It was not possible to employ the on-line pre-concentration used in our previous work (Ballus et al., 2011). Increasing injection volume (large-volume sample stacking, LVSS), caused the tyrosol peak to split. So, reverse electrode polarity stacking mode (REPSM) was attempted. However, all combinations of reverse voltage values and times led to a reduction in tyrosol. It is possible that any reverse voltage application would be enough to push some of the tyrosol molecules out of the capillary, back to the injection vial. In this case, ionic strength-mediated stacking was used, since it naturally occurs in this system, given that the standards and samples were dissolved in methanol:water (30:70).

After determining the linear range for each compound, residual plots and lack of fit tests were used to demonstrate the data were not heteroscedastic and presented and adequate fit.

The inter-assay precision, evaluated at three different concentrations, was below 5% for all compounds and levels examined. For the inter-assay precision, also assessed at three levels, most compounds had RSD values below 5%, while some fell in the 5-10% range, and a few between 10 and 15%. Most of the values with more than 10% variation were from lower concentrations where greater variations would be expected to occur.

Recovery assays, at two concentration levels, presented adequate values for most compounds, in the 90 to 102% range. Tyrosol, (+)-pinoresinol, hydroxytyrosol, luteolin and cinnamic acid, with recoveries between 60 and 80%, were exceptions. Bendini, Bonoli, Cerretani, Biguzzi, Lercker, & Gallina Toschi (2003), using exactly the same LLE extraction procedure, obtained similar recovery values. Cinnamic acid, with the poorest recovery (67.1%) of the 17 compounds, also had a low recovery value in the previous study (73.6%). Carrasco Pancorbo, Cruces-Blanco, Segura-Carretero, & Fernández-Gutiérrez (2004) and Hrncirik, & Fritsche (2004), using a LLE method slightly different from the one used here but with the same solvent (methanol:water 60:40, v/v), obtained similar recoveries to those observed here.

3.3.4. Phenolic compound contents of EVOO samples

The optimized and validated separation method was applied to the analysis of 15 EVOO brands commercialized in Brazil. **Table 3.6** presents the results. Using the optimized extraction method, and despite the LOQ for some compounds, it was possible to quantify tyrosol, (+)-pinoresinol, hydroxytyrosol, apigenin and luteolin in these samples. Apigenin was above the limit of quantification in just two samples (11 and 15), being detected but not quantified in all other samples. Luteolin was not quantified in only three samples (1, 3 and 7). The other three compounds were quantified in all samples. **Figure 3.2** presents representative electropherograms for the polar extracts of the EVOO samples. The electropherogram for sample nº 1 can be seen in **Figure 3.2A**, while the one for the same extract spiked with the 17

phenolic compound standards is shown in **Figure 3.2B**, which also shows the selectivity of the method in a polar extract. In **Figure 3.2C**, the electropherogram for sample nº 15 is presented and shows the greater quantities of luteolin and apigenin found. The (+)-pinoresinol capillary elution region has been enlarged in all the figures to show the good resolution with closely eluting compounds.

In this study, tyrosol was determined in the 5.69 and 23.81 $mg.kg^{-1}$ range, while (+)-pinoresinol occurred in concentrations from 1.77 to 6 $mg.kg^{-1}$. For hydroxytyrosol, concentrations ranged from 3.87 to 36.3 $mg.kg^{-1}$, apigenin between an unquantified lower level and 4.1 $mg.kg^{-1}$ and luteolin from unquantified to 5.73 $mg.kg^{-1}$. Bendini et al. (2003) found a tyrosol contents of 49.2 $mg.kg^{-1}$ and 62.8 $mg.kg^{-1}$ for hydroxytyrosol in virgin olive oil, using the same LLE extraction, and a CE-DAD separation method. Gómez Caravaca, Carrasco Pancorbo, Cañabate Díaz, Segura Carretero, & Fernández Gutiérrez (2005) found 2.62 $mg.kg^{-1}$ of tyrosol and 10 $mg.kg^{-1}$ of (+)-pinoresinol in Spanish Arbequina EVOO, and also 3.78 $mg.kg^{-1}$ of tyrosol and 6.97 $mg.kg^{-1}$ of (+)-pinoresinol in Spanish Hojiblanca EVOO, using CE-DAD and a different LLE procedure. Carrasco-Pancorbo, Gómez-Caravaca, Cerretani, Bendini, Segura-Carretero, & Fernández-Gutiérez (2006), employing solid-phase extraction (SPE) and CE-DAD, determined 7.3 $mg.kg^{-1}$ for tyrosol, 3 $mg.kg^{-1}$ for (+)-pinoresinol, 0.66 $mg.kg^{-1}$ for luteolin and 2.2 $mg.kg^{-1}$ for apigenin, in Spanish Hojiblanca EVOO, as well as 7.7 $mg.kg^{-1}$ for tyrosol, 4.95 $mg.kg^{-1}$ for (+)-pinoresinol, 2.45 $mg.kg^{-1}$ for luteolin and 1.26 $mg.kg^{-1}$ for apigenin in Spanish Arbequina EVOO. Carrasco-Pancorbo, Gómez-Caravaca, Segura-Carretero, Cerretani, Bendini, & Fernández-Gutiérrez (2009) analyzed Italian and

Spanish extra-virgin olive oil using SPE and CE-DAD. Italian samples contained 0.47-49.61 $mg.kg^{-1}$ tyrosol; 0.01-11.78 $mg.kg^{-1}$ (+)-pinoresinol; and 4.52-84.34 $mg.kg^{-1}$ hydroxytyrosol. Spanish EVOO contained 3.42-13.45 $mg.kg^{-1}$ tyrosol; unquantified-14.09 $mg.kg^{-1}$ (+)-pinoresinol, and 1.28-16.57 $mg.kg^{-1}$ hydroxytyrosol. All these literature results are consistent with those described in this study, despite differences in the extraction methods employed by each.

Table 3.6. Phenolic compound contents in extra-virgin olive oil samples (mean ± standard deviation, n = 3).

Samples	Country	Phenolic Compounds Content (mg.kg^{-1})				
		Tyrosol	(+)-Pinoresinol	Hydroxytyrosol	Apigenin	Luteolin
1	Portugal	9.01 ± 0.34	2.99 ± 0.16	7.62 ± 0.19	nq [a]	nq
2	Spain	16.29 ± 0.82	2.28 ± 0.16	22.26 ± 3.38	nq	3.76 ± 0.46
3	Spain	14.21 ± 1.03	3.51 ± 0.27	8.64 ± 1.18	nq	nq
4	Chile	5.69 ± 0.09	1.99 ± 0.08	4.47 ± 0.11	nq	3.08 ± 0.10
5	Italy	19.71 ± 0.83	2.52 ± 0.24	17.94 ± 2.25	nq	2.25 ± 0.25
6	Greece	6.53 ± 0.65	2.95 ± 0.20	3.87 ± 0.32	nq	2.50 ± 0.26
7	Italy	23.81 ± 1.60	2.87 ± 0.19	17.35 ± 0.79	nq	nq
8	Greece	17.76 ± 0.18	4.31 ± 0.05	11.38 ± 0.23	nq	2.74 ± 0.12
9	Portugal	16.12 ± 1.07	2.84 ± 0.23	10.28 ± 0.87	nq	2.07 ± 0.19
10	Spain	8.87 ± 0.31	3.14 ± 0.03	6.91 ± 0.08	nq	3.82 ± 0.14
11	Spain	11.86 ± 1.32	1.77 ± 0.20	15.54 ± 2.08	2.36 ± 0.28	4.15 ± 0.76
12	Italy	19.27 ± 3.39	2.86 ± 0.30	17.65 ± 0.48	nq	2.13 ± 0.28
13	Spain	16.71 ± 1.53	3.25 ± 0.32	10.61 ± 0.86	nq	2.45 ± 0.23
14	Portugal	23.52 ± 0.79	2.98 ± 0.06	25.38 ± 0.33	nq	1.84 ± 0.04
15	Chile	15.19 ± 0.88	6.00 ± 0.45	36.30 ± 0.30	4.10 ± 0.28	5.73 ± 0.27

[a] nq, below the limit of quantification.

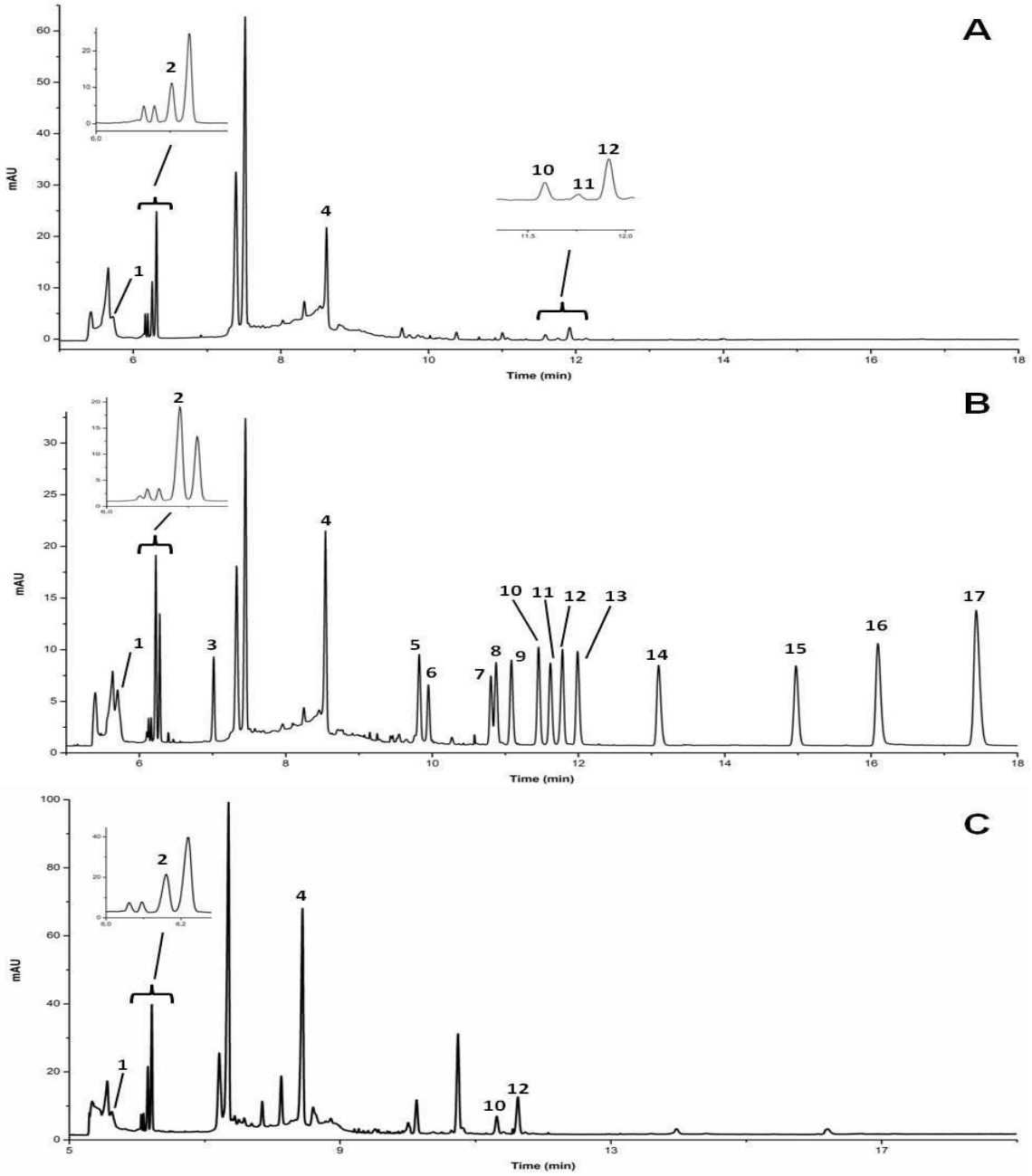

Figure 3.2. Representative electropherograms obtained for the polar extracts of the extra-virgin olive oil samples. **A**) Electropherogram for the polar extract of sample nº 1. **B**) Electropherogram for the polar extract of sample nº 1 spiked with the seventeen phenolic compound standards. **C**) Electropherogram for the polar extract of sample nº 15. Some regions of the electropherograms were enlarged to better visualize some of the compounds detected in the samples. Electrophoretic conditions and peak identification can be seen in **Figure 3.1**.

3.4. Conclusions

A nine experiment Doehlert design used with Derringer's desirability function resulted in the separation of the 17 phenolic compounds previously detected in EVOO, in only 19 minutes. The Doehlert design with more pH levels than boric acid electrolyte solution concentrations permitted accurate response surface analyses. This proved to be very important since the peak separations were very sensitive to pH. Although the optimal pH value found here (pH 9.15) was close to the value used to separate 13 peaks previously (pH 10.2), the difference (pH 1.05) is two third of the range investigated (i.e. proportionally large) and demonstrates this sensitivity to pH. On the other hand, the optimal boric acid electrolyte concentration (101.3 mmol L^{-1}) is twice the concentration used previously (50 mmol L^{-1}).

This approach appears to be useful for dealing with optimization of peak separation, saving time and reagents as well as allowing mathematical analysis of the influence of each factor. Moreover, this is the first time this group of phenolic compounds has been separated using capillary electrophoresis and multivariate optimization. Furthermore, simultaneous evaluation of 37 responses, optimal peak resolution, run time and migration time stability has not previously been subjected to multivariate analysis and optimization such as described here.

The method was successfully validated and applied to 15 EVOOs commercialized in Brazil. This is the first study to quantify these phenolic compounds in EVOO, which is becoming an important part of the Brazilian diet.

3.5. Acknowledgments

We thank FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo (process no. 08/57548-0), CNPq - Coordenação Nacional de Desenvolvimento Científico e Tecnológico and CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, for their financial support.

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CHAPTER IV

ARTICLE

A quantitative study on the phenolic compound, tocopherol and fatty acid contents of monovarietal extra-virgin olive oils produced in the southeast region of Brazil

CHAPTER IV

A quantitative study on the phenolic compound, tocopherol and fatty acid contents of monovarietal extra-virgin olive oils produced in the southeast region of Brazil

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***This manuscript was submitted to the journal "Food Research International".**

ABSTRACT

Consumption of extra-virgin olive oil (EVOO) is highly recommended due to its human health benefits. Brazil is now beginning to experimentally produce EVOO, and there are no data on its chemical profile. The aim of this work was to determine the phenolic compound, tocopherol and fatty acid contents of 17 monovarietal EVOOs produced from olive varieties cultivated in the southeast region of Brazil during two crop years. The chemical composition of Brazilian EVOO resembles that found in the literature for well-established EVOOs. The analyzed compounds comprised palmitic acid (6-12.6%), palmitoleic acid (0.2-2.5%), stearic acid (1.6-2.2%), oleic acid (70.8-84.3%), linoleic acid (3.2-11.7%), α-linolenic acid (0.6-1.4), arachidic acid (0.4-0.8%), 9-eicosenoic acid (0.4-0.9%), tyrosol (NQ-155.21 mg kg^{-1}), (+)-pinoresinol (2.89-22.64 mg kg^{-1}), hydroxytyrosol (ND-37.74 mg kg^{-1}), luteolin (ND-2.23 mg kg^{-1}), α-tocopherol (28.92-232.93 mg kg^{-1}), β-tocopherol (ND-9.56 mg kg^{-1}), and γ-tocopherol (ND-18.75 mg kg^{-1}). There was a significant difference in the contents of almost all of the analyzed compounds between the two crop years. Principal component analysis demonstrated that some varieties can be differentiated from one another by chemical composition. The results indicated that some Brazilian monovarietal EVOOs are promising and that further studies will help to improve the quality of Brazilian EVOO.

Keywords: extra-virgin olive oil; phenolic compounds; tocopherols; fatty acids; principal component analysis.

4.1. Introduction

Extra-virgin olive oil (EVOO) is a product of the extraction of olive fruit and is considered to be one of the best sources of fatty acids and natural antioxidants such as phenolic compounds and tocopherols. Its nutritional properties are highly valued for their positive effects on human health. The chemical composition of EVOO consists primarily of monounsaturated (MUFA), polyunsaturated (PUFA) and saturated fatty acids (SFA), mainly in the form of esters with glycerol (triacylglycerols), which represents more than 98% of its total content. Important minor components in olive oil are sterols, hydrocarbons, phenolic compounds, tocopherols, volatile compounds, terpenols, terpenic acids, free glycerol, free fatty acids, and mono and diacylglycerols. As a result, olive oil constitutes a complex multi-component matrix and its analysis is not an easy task (Dais, & Hatzakis, 2013; Del Coco et al., 2013).

Because the olive tree has been cultivated for thousands of years in the Mediterranean, EVOO is one of the main components of the Mediterranean diet. It is highly appreciated all over the world for its taste and aroma, as well as for its nutritional properties (López-Cortés, Salazar-García, Veláquez-Martí, & Salazar, 2013). Different cultivars, pedoclimatic conditions of the orchards, and varying agricultural practices, together with olive ripeness and olive oil extraction techniques, result in a great diversity of olive oil chemical profiles (García-González, & Aparicio, 2010).

All of the EVOO consumed in Brazil is imported from European (Portugal, Spain, Italy and Greece) and South American (Argentina and Chile) countries. As a

result, the price of EVOO in Brazil is relatively high, and a considerable part of the population does not have access to this important and healthy vegetable oil. Brazil is beginning to cultivate olives and to produce olive oils to offer a product with lower prices to the consumer in the near future and to create new opportunities for Brazilian agribusiness. However, it is of great importance to determine if the chemical composition of the Brazilian EVOO is similar to high quality EVOO produced in European, South American and other countries that possess more experience in this field.

Thus, the aim of this work is to provide the first data on the phenolic compound, tocopherol and fatty acid contents of Brazilian EVOOs produced in the southeast region of Brazil. In fact, these were the first EVOOs extracted in the country, and the results of this work will be helpful to agronomists in their search for the best-adapted and optimal EVOO-producing olive varieties.

4.2. Material and methods

4.2.1. Chemicals and standards

Hexane p.a. and methanol p.a. were purchased from Synth (Brazil). Methanol, acetic acid and isopropanol were all of HPLC grade and were purchased from J. T. Baker (USA), while HPLC grade hexane was from Mallinckrodt (USA). Boric acid (Ecibra, Brazil), sodium chloride p.a. (Nuclear, Brazil), sodium hydroxide p.a. (Nuclear, Brazil), butylated hydroxytoluene, BHT, (Sigma-Aldrich, USA) and boron trifluoride-methanol complex (20% solution in methanol) (Merck, Germany)

were also used in these studies. Water was purified in a Milli-Q system (Millipore, USA). Standards of tyrosol, gallic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, caffeic acid, 3,4-dihydroxybenzoic acid, cinnamic acid, vanillic acid, ferulic acid, luteolin and apigenin were acquired from Sigma-Aldrich (USA). The hydroxytyrosol standard was obtained from Cayman Chemical (USA). The oleuropein standard was purchased from Extrasynthèse (France). The syringic acid, sinapinic acid and *o*-coumaric acid standards were acquired from Chem Service (USA). The (+)-pinoresinol standard was purchased from Arbo Nova (Finland). Standards of α-, β-, γ-, and δ-tocopherols, and the standards of C_4 to C_{24} methyl esters (FAME Mix), were acquired from Supelco (USA).

Standard stock solutions of phenolic compounds were prepared by dissolving the appropriate amount of each compound in HPLC grade methanol to a final concentration of 2 g L^{-1} for (+)-pinoresinol, 1 mg L^{-1} for hydroxytyrosol and luteolin, 0.4 g L^{-1} for apigenin, and 5 g L^{-1} for the other 13 compounds. Tocopherol standard stock solutions were prepared by dissolving them in HPLC grade hexane containing 0.01% BHT, at the concentrations of 25 g L^{-1} for the α, γ-, and δ-tocopherol isomers, and 50 g L^{-1} for the β-tocopherol isomer. The fatty acid methyl ester standard stock solution was prepared in HPLC grade hexane. Standard stock solutions were filtered through a 0.45 µm Millipore PVDF membrane (Millipore, USA), stored at –18ºC and protected from light. Vials containing working solutions were placed under ultrasound for five min before injection.

4.2.2. EVOO Samples

A total of 17 EVOO samples were obtained from Maria da Fé Experimental Farm of the Agricultural and Livestock Research Corporation of the State of Minas Gerais (EPAMIG). Maria da Fé is a city situated in the micro-region of Serra da Mantiqueira in the south of Minas Gerais state (latitude: 22º 18' 28" S; longitude: 45º 22' 30" W; altitude: 1276 m above sea level). Using the Köppen-Geiger climate classification system, Maria da Fé has a temperate highland tropical climate with dry winters (Cwb). The mean annual temperature is 17ºC and fluctuates between 10.1ºC (minimum) and 23.3ºC (maximum), while the mean annual rainfall is approximately 1738.6 mm.

The samples consisted of EVOOs produced from different olive varieties during two different years. From the 2010 crop, samples of MGS Grap 561 (Grappolo 561), Cornicabra, Tafahi 390, Grappolo 575, Arbequina, Alto D'Ouro, Negroa, MGS Neblina and JB1 varieties were available. From the 2011 crop, samples of MGS Mariense (Maria da Fé), Mission, Grappolo 575, Arbequina, Alto D'Ouro, Negroa, MGS Neblina and JB1 varieties were evaluated.

An Abencor® system (Suárez, Aranda, Mendoza, & Rey, 1975) adapted for obtaining sufficient olive oil to perform the chemical analysis was employed for olive oil extraction. Olives from each variety (10 kg) were washed with water to remove impurities and leaves prior being milled in a metallic mill. The olive paste was heated to 28ºC (Sánchez, Pacheco, Rubia, Sánchez, & Pereira, 2005) during the homogenizing process, which was performed in a domestic mixer using two types of movements, translation and rotation, during an interval of 50-60 minutes.

Using an analytical balance, 450 g of the olive paste were transferred to a high rotation centrifuge and subjected to centrifugation at 4200 rpm, allowing EVOO separation. The EVOO phase was separated and placed into plastic packages, where remained for 60 minutes to allow residual sedimentation. Finally, EVOOs were transferred to amber glass bottles.

After arriving at the laboratory, all samples were maintained under refrigeration (4ºC) and protected from light until analysis.

4.2.3. Sample preparation and extraction procedures

4.2.3.1. Fatty acids

Approximately 100 mg of each extra-virgin olive oil sample was weighed into test tubes, with the subsequent addition of 4 mL of 0.5 mol L^{-1} NaOH solution in methanol. The tubes were heated in a 100ºC water bath for approximately 8 min until a transparent solution was obtained. After cooling, 3 mL of a 12% BF_3 solution in methanol was added and the tubes were heated again in a 100ºC water bath for 3 min. After cooling, 4 mL of saturated NaCl solution was added with agitation. Next, 4 mL of hexane was added with vigorous agitation. Then, the tubes were left to rest to allow phase separation, and 1 μL from the upper layer was injected into the gas chromatograph. Each sample was prepared in triplicate (n = 3). This procedure was adapted from the work of Joseph, & Ackman (1992).

4.2.3.2. Phenolic compounds

The phenolic compound extraction procedure was based on the work of Pirisi, Cabras, Cao, Migliorini, & Muggelli (2000) and Bonoli, Montanucci, Gallina Toschi, & Lercker (2003). Approximately 2 g of EVOO was weighed into a centrifuge tube and 1 mL of hexane and 2 mL of methanol:water (60:40, v/v) were added. This mixture was stirred for 2 min in a vortex apparatus, and the tube was then centrifuged at 5000 rpm for five minutes. The methanol:water layer was separated and the extraction was repeated twice. The extracts were combined and evaporated to dryness at 39ºC under reduced pressure. Samples were resuspended in 1 mL of methanol:water (30:70, v/v) and filtered through a 0.45 μm PVDF membrane (Millipore, USA) before analysis by capillary electrophoresis. All samples were extracted in triplicate (n = 3).

4.2.3.3. Tocopherols

For the tocopherol analysis, extra-virgin olive oil samples were diluted in hexane (0.1 g in 10 mL of hexane containing 0.01% BHT), filtered through a 0.22 μm PVDF membrane (Millipore, USA), and then directly injected into the column in the HPLC system. This sample preparation was based on the work of Dionisi, Prodolliet, & Tagliaferri (1995), Guinazi, Milagres, Pinheiro-Santa'Ana, & Chaves (2009) and Pinheiro-Sant'Ana et al. (2011). Samples were prepared in triplicate (n = 3).

4.2.4. Separation methods

4.2.4.1. Fatty acids

A Varian 3800 Gas Chromatograph (Varian Inc., USA) equipped with a flame ionization detector (FID), a split/splitless injector (split ratio 1/80) and a fused-silica capillary column with 90% cyanopropyl-modified polysiloxane (100 m length, 0.25 mm i.d., 0.25 μm film thickness) (NST BIS-6025025, Nano Separation Technologies, Brazil) was used for the determination of fatty acid methyl esters (FAMEs). The optimized parameters were: injector temperature (240°C); detector temperature (240°C); carrier gas, hydrogen, flow rate 1.4 mL min^{-1}; detector gases flow rate (H_2 / N_2 / Synthetic Air – 30 / 30 / 300 mL min^{-1}); oven temperature program (197°C for 23 min, increasing to 225ºC at a rate of 20ºC per min, and kept at the final temperature for 15 min). Peak identification was accomplished by comparing the retention time of the standards with those of the peaks observed in the samples separated under the same conditions. The peak area results are expressed as the percentage of the total FAME peak area. Chromatographic conditions were adapted from Tanamati et al. (2010). Samples were injected in triplicate.

4.2.4.2. Phenolic compounds

An Agilent G1600AX (Agilent Technologies, Germany) capillary electrophoresis system equipped with a diode array detector (DAD) and an

automatic injector and temperature control system adjusted to 25ºC was used in this study. The method used for the analysis of phenolic compounds by capillary zone electrophoresis with diode array detection was optimized in the work from Ballus, Meinhart, Campos Jr., Bruns, & Godoy (2014). A fused-silica capillary with a 50 µm internal diameter and 72 cm of effective length with an extended light path (Agilent Technologies, Germany) was used, as well as an electrolyte consisting in 101.3 mmol L^{-1} boric acid solution at pH 9.15, a voltage of 30 kV and an injection of 50 mbar for 5 s. Detection was performed at 210 nm and data analysis was performed with the HP ChemStation software. New capillaries were activated and conditioned by washing under 1 bar of pressure with 1 mol L^{-1} NaOH for 30 min followed by 10 min of water. At the end of each day, the capillary was washed for 5 min with 1 mol L^{-1} NaOH and then for 5 min with water. The capillary was stored in water overnight. Peak identification was achieved by comparing the migration time and the UV spectra of each peak to those obtained for the phenolic compound standards, as well as by co-chromatography. Samples were injected in triplicate.

4.2.4.3. Tocopherols

Separation of tocopherols was achieved using an Agilent 1100 HPLC (Agilent Technologies, Germany) coupled to a fluorescence detector, quaternary pump system, automatic injector and oven to control the column temperature. Method conditions were based on the work of Pinheiro-Sant'Ana et al. (2011). A 150 mm x 4.6 mm x 3.0 µm normal phase column (Hypersil Silica, Thermo,

Germany) was used. The mobile phase consisted of an isocratic system composed of hexane:isopropanol:acetic acid (98.9:0.6:0.5) with a flow rate of 1.0 mL min^{-1}. The temperature was maintained at 30ºC, and the injection volume was 100 µL. Fluorescence detection was performed at λ_{Ex} 290 nm and λ_{Em} 330 nm. Compounds were identified by comparing the retention times of the compounds found in the samples to those in the tocopherol standards separated under the same conditions, as well as by co-chromatography. Samples were injected in triplicate.

4.2.5. Method validation

4.2.5.1. Fatty acids

The method precision for the separation of fatty acid methyl esters was evaluated through intra- (n = 7) and inter-day (n = 3) instrumental precisions by injecting a methylated EVOO sample.

4.2.5.2. Phenolic compounds and tocopherols

Both methods were validated as prescribed by the Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis IUPAC/ISO/AOAC International (Thompson, Ellison, & Wood, 2002). The limit of detection (LOD) was determined through successive dilutions of the standard mixture until peaks with a signal-to-noise ratio near three were reached. The limit of quantification (LOQ) was also determined through successive dilutions, being defined as the concentration that resulted in peaks with a signal-to-noise ratio near six. The intra-day

instrumental precision was verified at the limit of quantification (n = 7 for phenolic compounds and n = 10 for tocopherols). System linearity was studied individually for each compound with calibration curves consisting of seven points, in which standards were prepared in triplicate and injected randomly. A lack of fit test for each calibration curve was performed. The intra-day instrumental precision was determined by injecting a solution containing the 17 phenolic compounds or the 4 tocopherols, at three different concentration levels. This procedure was performed 7 consecutive times in one day (phenolic compounds) and 10 consecutive times in one day (tocopherols) for each concentration level. The inter-day instrumental precision was determined by repeating this procedure on three consecutive days. For the phenolic compounds, the first concentration level consisted of 10 mg L^{-1} of tyrosol and oleuropein, and 5 mg L^{-1} of the other 15 compounds. The second level consisted of 28 mg L^{-1} of tyrosol and oleuropein, and 14 mg L^{-1} of the other compounds. The third level consisted of 42 mg L^{-1} of tyrosol and oleuropein, and 21 mg L^{-1} of the remaining compounds. For tocopherols, the first level consisted of 75 ng of α-tocopherol, 11.3 ng of β-tocopherol, 9.9 ng of γ-tocopherol, and 2.6 ng of δ-tocopherol. The second level consisted of 250 ng of α-tocopherol, 37.5 ng of β-tocopherol, 33.1 ng of γ-tocopherol, and 8.8 ng of δ-tocopherol. The third level consisted of 425 ng of α-tocopherol, 63.8 ng of β-tocopherol, 56.3 ng of γ-tocopherol, and 14.9 ng of δ-tocopherol.

A recovery assay was also performed at two concentration levels to estimate the validity of the extraction technique because there are no certified reference materials (CRM) for both compound classes in EVOO. The first level

consisted of the addition of 13 mg kg^{-1} of tyrosol and oleuropein, and 6 mg kg^{-1} for the other phenolic compounds. For the second level, 20 mg kg^{-1} of tyrosol and oleuropein, and 10 mg kg^{-1} of the other phenolic compounds were added. For analysis of tocopherol recovery, the first level consisted of 70 mg kg^{-1} of α-tocopherol, 10 mg kg^{-1} of β- and γ-tocopherols, and 2.5 mg kg^{-1} of δ-tocopherol. The second level consisted of 200 mg kg^{-1} of α-tocopherol, 30 mg kg^{-1} of β-tocopherol, 25 mg kg^{-1} of γ-tocopherol, and 7 mg kg^{-1} of δ-tocopherol. When determining the phenolic compound or tocopherol recovery, the required volume of each of the compound standard solution at each concentration level was added to the EVOO samples and the tubes were stirred for 5 min. Then, the whole sample preparation procedure was executed as described in section *4.2.3.2* or *4.2.3.3*. The percentage of recovery (%) was calculated for each compound, taken into account and subtracting the original content of the compounds that were found in the EVOO samples. The recovery assays were executed in triplicate for each concentration level (n = 3).

4.2.6. Statistical analysis and chemometrics

The means obtained for each compound determined in the extra-virgin olive oil samples were compared using ANOVA and Tukey tests at a 95% confidence level. In the cases where samples from two crop years were analyzed, the results were compared between years and between different varieties from the same crop year. The statistical analysis was performed using the Statistica 7.0 (Statsoft, USA) software. A Principal Component Analysis (PCA) was also performed to better

visualize the differences in the analyzed compounds between samples as well as any correlations between variables. PCA was carried out using Pirouette 3.11 (Infometrix, USA).

4.3. Results and discussion

4.3.1. Method validation results

Figure 4.1 displays representative electropherogram and chromatographic profiles obtained for Brazilian EVOO samples. For the fatty acid separation method, the values for intra-day instrumental precision were approximately 5% for the major compounds (palmitic acid, 16:0 – 5.7%; stearic acid, 18:0 – 2%; oleic acid, 18:1n-9 – 0.8%; and linoleic acid, 18:2n-6 – 1%). For the minor fatty acids, the values were higher than 5%, but this was expected because the concentrations were very low and the instrumental precision is generally worse in this range (palmitoleic acid, 16:1n-7 – 7.8%; α-linolenic acid, 18:3n-3 – 6.2%; arachidic acid, 20:0 – 7.9%; and 9-eicosenoic acid, 20:1n-11 – 14.4%). The inter-day values for all analyzed fatty acids were satisfactory (palmitic acid, 16:0 – 3.4%; palmitoleic acid, 16:1n-7 – 9.5%; stearic acid, 18:0 – 1.3%; oleic acid, 18:1n-9 – 0.6%; linoleic acid, 18:2n-6 – 0.4%; α-linolenic acid, 18:3n-3 – 2.3%; arachidic acid, 20:0 – 3.8%; and 9-eicosenoic acid, 20:1n-11 – 6.7%).

Tables 4.1 and **4.2** present the results for the parameters evaluated during the method validation for the separation and quantification of phenolic compounds and tocopherols.

The intra- and inter-day instrumental precisions were below 5% for both separation methods, with the exception of some phenolic compounds in the first concentration level assayed, which were between 5% and 10%, as is expected for this concentration range. All intra-day instrumental precision results conducted at the limit of quantification were below 10%. The calibration equations presented adequate fits ($p > 0.05$) in the concentration ranges used in this work. In addition, both extraction procedures presented good recovery results. For phenolic compounds, the recovery ranged from 70 to 130%, while for tocopherols it was between 92 and 118%. All of these results are comparable to those obtained in the validation carried out by the authors that originally developed both methods (Pinheiro-Sant'Ana et al., 2011; Ballus, Meinhart, Campos Jr., Bruns, & Godoy, 2014).

In summary, the results for the method and extraction validations indicate that all three separation and detection methods can be efficiently and confidently used to separate and quantify the compounds under assay in this study.

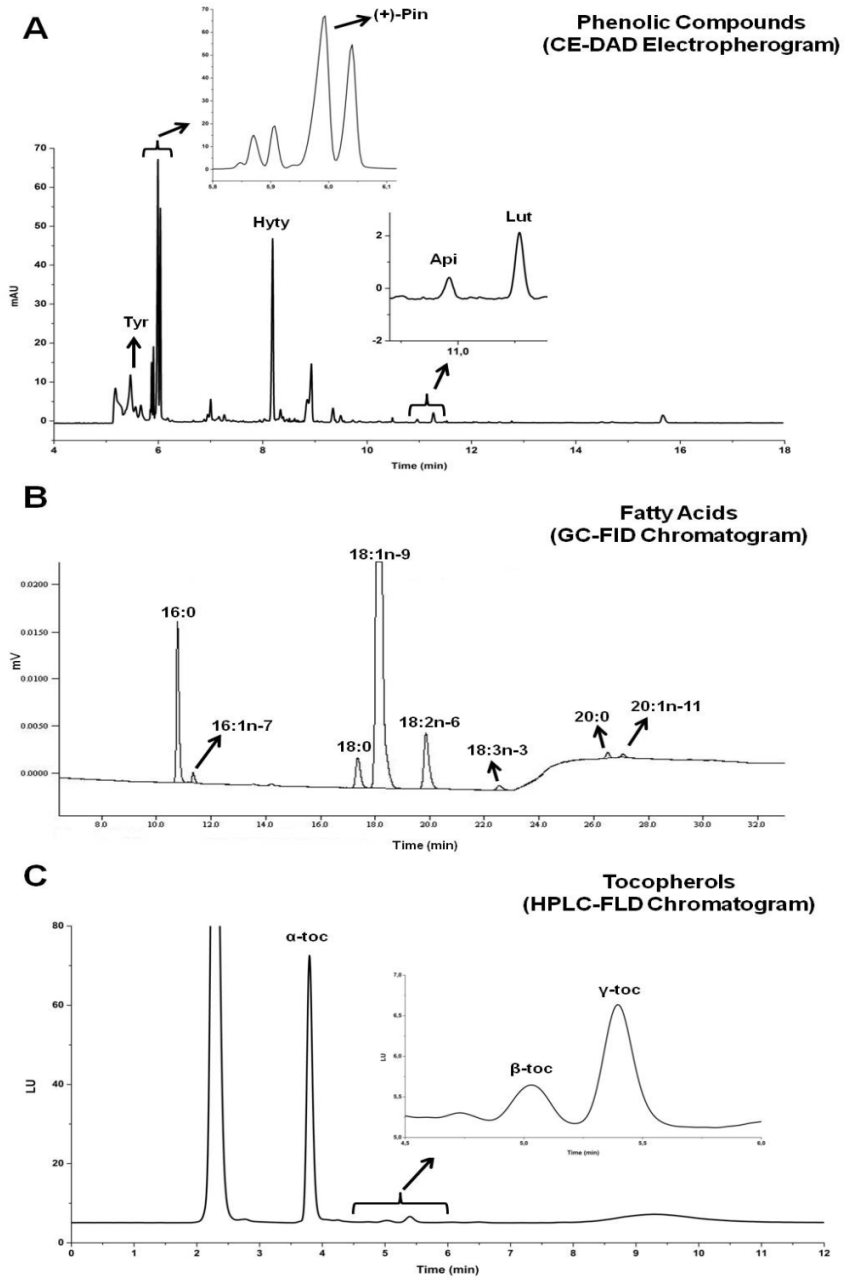

Figure 4.1. Representative electropherogram for the analysis of phenolic compounds (**A**), and representative chromatographic profiles for the analysis of fatty acids (**B**) and tocopherols (**C**) in Brazilian extra-virgin olive oil (Negroa variety, 2011 crop). Peak identification: Tyr, tyrosol; (+)-pin, (+)-pinoresinol; Hyty, hydroxytyrosol; Api, apigenin; Lut, luteolin; 16:0, palmitic acid; 16:1n-7, palmitoleic acid; 18:0, stearic acid; 18:1n-9, oleic acid; 18:2n-6, linolenic acid; 18:3n-3, α-linolenic acid; 20:0, arachidic acid; 20:1n-11, 9-eicosenoic acid; α-toc, α-tocopherol; β-toc, β-tocopherol; γ-toc, γ-tocopherol.

Table 4.1. Figures of merit for the validation of the phenolic compound and tocopherol separation methods.

Phenolic Compounds	LOD ($mg.L^{-1}$) [a]	LOQ ($mg.L^{-1}$) [a]	Intra-day Precision LOQ (n = 7) [a]	Linearity ($mg.L^{-1}$)	Equation	r^2	Lack of Fit Test ($p > 0.05$)
Tyrosol	2.50	4.38	5.53	8.3 - 41.7	$y = 0.820x - 0.81$	0.9907	0.136
(+)-Pinoresinol	0.60	1.20	9.78	4.0 - 24.0	$y = 2.284x - 1.40$	0.9910	0.743
Oleuropein glycoside	2.37	4.14	4.87	7.9 - 47.4	$y = 0.862x - 1.17$	0.9932	0.234
Hydroxytyrosol	1.20	2.10	5.67	4.0 - 24.0	$y = 2.212x - 0.71$	0.9902	0.474
Cinnamic acid	1.19	2.08	5.20	4.0 - 19.8	$y = 3.283x + 1.08$	0.9911	0.316
Sinapinic acid	1.19	2.09	8.60	4.0 - 19.9	$y = 2.154x - 1.11$	0.9911	0.121
Syringic acid	1.18	2.07	9.63	3.9 - 19.7	$y = 2.553x - 2.82$	0.9955	0.104
Ferulic acid	1.18	2.07	1.36	3.9 - 23.7	$y = 2.609x - 0.79$	0.9905	0.531
o-Coumaric acid	1.19	2.08	3.57	4.0 - 19.8	$y = 2.968x - 0.55$	0.9937	0.546
Apigenin	1.48	2.58	6.06	4.9 - 24.6	$y = 3.312x - 0.72$	0.9919	0.383
p-Coumaric acid	1.21	2.11	9.18	4.0 - 20.1	$y = 2.912x - 0.16$	0.9956	0.092
Luteolin	1.20	2.10	4.77	4.0 - 20.0	$y = 3.745x - 1.40$	0.9952	0.286
Vanillic acid	1.20	2.10	6.35	4.0 - 20.0	$y = 3.665x - 1.91$	0.9967	0.084
p-Hydroxybenzoic acid	1.23	2.16	4.23	4.1 - 20.5	$y = 3.383x + 1.00$	0.9952	0.414
Caffeic acid	1.24	2.16	9.79	4.1 - 20.6	$y = 4.050x - 1.84$	0.9961	0.465
Gallic acid	1.25	2.18	8.89	4.2 - 20.8	$y = 6.035x - 4.23$	0.9946	0.362
3,4-Dihydroxybenzoic acid	0.66	1.32	4.75	4.4 - 22.0	$y = 8.552x - 3.63$	0.9941	0.560
Tocopherols	**LOD ($\mu g.L^{-1}$) [a]**	**LOQ ($\mu g.L^{-1}$) [a]**	**Intra-day Precision LOQ (n = 10) [a]**	**Linearity (ng)**	**Equation**	**r^2**	**Lack of Fit Test ($p > 0.05$)**
α-Tocopherol	40.20	80.40	6.11	75.00 - 375.00	$y = 2.168x - 63.65$	0.9990	0.247
β-Tocopherol	18.75	37.50	5.15	11.25 - 48.75	$y = 1.438x - 3.88$	0.9992	0.051
γ-Tocopherol	16.55	33.10	8.40	16.54 - 49.63	$y = 1.903x - 8.35$	0.9993	0.295
δ-Tocopherol	4.40	8.80	8.28	4.39 - 13.18	$y = 8.599x - 8.53$	0.9995	0.469

[a] LOD, limit of detection; LOQ, limit of quantification.

Table 4.2. Instrumental precision results for the separation of phenolic compounds and tocopherols from extra-virgin olive oil and recovery results for both extraction methods.

Phenolic Compounds	Intra-day Precision (%, n = 7) [a]			Inter-day Precision (%, n = 3) [a]			Recovery (%, mean ± SD, n = 3) [b]	
	First Level	Second Level	Third Level	First Level	Second Level	Third Level	First Level	Second Level
Tyrosol	5.28	4.62	3.32	2.27	2.88	2.85	70 ± 6	83.8 ± 0.4
(+)-Pinoresinol	6.03	2.93	1.58	3.76	2.12	0.73	86 ± 6	73 ± 5
Oleuropein glycoside	4.78	2.52	2.11	4.12	1.30	0.12	91 ± 5	79 ± 5
Hydroxytyrosol	5.36	2.47	2.29	5.67	2.03	1.18	81 ± 1	86 ± 8
Cinnamic acid	6.55	3.53	2.40	2.61	1.77	0.93	72 ± 11	75 ± 4
Sinapinic acid	9.12	4.48	2.39	2.84	1.96	1.76	86 ± 7	84 ± 2
Syringic acid	8.92	3.59	3.27	6.65	2.05	1.15	96 ± 7	90 ± 2
Ferulic acid	7.24	3.84	2.85	5.12	1.57	0.86	86 ± 4	101 ± 3
o-Coumaric acid	8.11	3.27	2.99	4.97	2.39	0.38	92 ± 8	90 ± 3
Apigenin	7.50	3.40	7.15	7.83	0.84	4.80	121 ± 1	130 ± 11
p-Coumaric acid	8.77	2.84	2.39	3.28	2.04	1.73	99 ± 8	93 ± 2
Luteolin	7.46	3.07	5.02	7.18	5.35	6.23	82 ± 8	76 ± 6
Vanillic acid	8.94	3.18	2.93	5.02	2.35	1.33	99 ± 8	95 ± 3
p-Hydroxybenzoic acid	6.49	3.47	2.76	2.26	2.50	1.30	94 ± 9	90 ± 2
Caffeic acid	8.26	2.97	2.71	3.36	1.86	0.58	96 ± 9	89 ± 4
Gallic acid	7.16	2.79	2.61	6.42	1.94	1.06	88 ± 7	78 ± 6
3,4-Dihydroxybenzoic acid	7.30	3.25	2.34	4.02	1.60	0.79	96 ± 9	89 ± 4

Tocopherols	Intra-day Precision (%, n = 10) [a]			Inter-day Precision (%, n = 3) [a]			Recovery (%, mean ± SD, n = 3) [b]	
	First Level	Second Level	Third Level	First Level	Second Level	Third Level	First Level	Second Level
α-Tocopherol	3.06	0.89	0.64	6.68	0.49	1.93	114 ± 14	111 ± 4
β-Tocopherol	1.76	1.32	0.69	1.98	0.50	2.33	92 ± 5	95 ± 2
γ-Tocopherol	3.43	1.25	0.51	8.29	2.72	4.09	102 ± 8	102 ± 2
δ-Tocopherol	3.54	0.66	0.43	9.63	4.09	3.84	118.2 ± 0.5	114 ± 0.4

[a] Concentration of the standards. First level: tyrosol and oleuropein: 10.0 mg L^{-1}; other phenolic compounds: 5.0 mg L^{-1}; α-tocopherol: 75 ng; β-tocopherol: 11 ng; γ-tocopherol: 10 ng; δ-tocopherol: 3 ng; Second level: tyrosol and oleuropein: 28.0 mg L^{-1}; other phenolic compounds: 14.0 mg L^{-1}; α-tocopherol: 250 ng; β-tocopherol: 38 ng; γ-tocopherol: 33 ng; δ-tocopherol: 9 ng; Third level: tyrosol and oleuropein: 42.0 mg L^{-1}; other phenolic compounds: 21 0 mg.L^{-1}; α-tocopherol: 425 ng; β-tocopherol: 64 ng; γ-tocopherol: 56 ng; δ-tocopherol: 15 ng.

[b] Concentration of the standards added to samples: First level: 14.0 mg kg^{-1} of tyrosol and oleuropein; 7.0 mg kg^{-1} for the other phenolic compounds; α-tocopherol: 70 mg kg^{-1}; β-tocopherol and γ-tocopherol: 10 mg kg^{-1}; δ-tocopherol: 2.5 mg kg^{-1}; Second level: 18.0 mg kg^{-1} of tyrosol and oleuropein; 10.0 mg kg^{-1} for the other phenolic compounds; α-tocopherol: 200 mg kg^{-1}; β-tocopherol: 30 mg kg^{-1}; γ-tocopherol: 25 mg kg^{-1}; δ-tocopherol: 7.0 mg kg^{-1}. SD, standard deviation.

4.3.2. Fatty acid composition

Eight fatty acids were identified in the samples of Brazilian EVOO: palmitic acid (16:0), palmitoleic acid (16:1n-7), stearic acid (18:0), oleic acid (18:1n-9), linoleic acid (18:2n-6), α-linolenic acid (18:3n-3), arachidic acid (20:0) and 9-eicosenoic acid (20:1n-11). The quantitative results are presented in **Table 4.3**. Some of the varieties were only analyzed for one crop year (MGS Grap 561, Cornicabra, Tafahi 390, MGS Mariense and Mission) because EVOO production is still experimental and the yield of extracted EVOO was sometimes very low and thus not available for this study.

The overall ranges observed in this study, 6-12.6% for palmitic acid (16:0), 0.2-2.5% for palmitoleic acid (16:1n-7), 1.6-2.2% for stearic acid (18:0), 70.8-84.3% for oleic acid (18:1n-9), 3.2-11.7% for linoleic acid (18:2n-6), 0.6-1.4 for α-linolenic acid (18:3n-3), 0.4-0.6% for arachidic acid (20:0), and 0.4-0.8% for 9-eicosenoic acid (20:1n-11), are compatible with those specified for an EVOO by the Codex Alimentarius (Codex Stan 33, 2003). In general, it was noted that, as the content of palmitic acid (16:0) increased, the content of oleic acid (18:1n-9) decreased. One hypothesis to explain this correlation is that, after the production of palmitoyl-ACP (16:0 linked to an acyl carrier protein) during fatty acid synthesis in olives, two pathways could be followed: hydrolysis to palmitate (16:0) and ACP, or a further condensation reaction producing stearoyl-ACP (18:0-ACP). Stearoyl-ACP can then be converted to oleoyl-ACP (18:1n-9-ACP) by action of a desaturase. The transcription of desaturase increases during ripening. Thus, in general, more oleic acid is produced as the olive matures, resulting in less palmitic acid because it is

the substrate for oleic acid production (Conde, Delrot, & Gerós, 2008). Another inverse correlation occurred between the oleic (18:1n-9) and linoleic (18:2n-6) acid contents. This was also observed in the work of Rondanini, Castro, Searles, & Rousseaux (2014) for Arbequina and Arauco varieties cultivated in Argentina. During ripening, these varieties presented a linear increase in the linoleic acid (18:2n-6) concentration and a linear decrease in the oleic acid (18:1n-9) content of the olives. Something similar may have happened to the Arbequina and MGS Neblina olives varieties because the EVOO extracted from them presented higher linoleic acid contents than was observed for the other varieties.

Figure 4.2 presents a comparison of the fatty acid results between the two crop years for the varieties for which samples were available (Grappolo 575, Arbequina, AltoD'Ouro, Negroa, MGS Neblina and JB1).

In general, the fatty acid composition did not change significantly between crops. The fatty acids most affected by the crop year were linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), while arachidic acid (20:0) and 9-eicosenoic acid (20:1n-11) were not affected. There was also a significant difference between the different varieties from the same crop year for all eight fatty acids analyzed.

Table 4.3. Fatty acid contents of Brazilian extra-virgin olive oil samples (mean ± standard deviation, n = 3).

Varieties	Fatty Acid Contents (% of relative area) per crop year [a]							
	16:0		16:1n-7		18:0		18:1n-9	
	2010	2011	2010	2011	2010	2011	2010	2011
Grappolo 575	11.1 ± 0.2 A a [b]	9.8 ± 0.2 B a	0.6 ± 0.1 A c	0.69 ± 0.04 A d	2.06 ± 0.01 A a,b	2.02 ± 0.02 B a,b	77.8 ± 0.2 B c,d	81.0 ± 0.1 A a
Arbequina	11 ± 1 A a	9.8 ± 0.2 A a	1.4 ± 0.2 A b	1.2 ± 0.1 A c,d	1.63 ± 0.04 A e	1.69 ± 0.05 A c	75 ± 1 A e	75.7 ± 0.1 A c
Alto D`Ouro	12 ± 1 A a	11 ± 1 A a	2.3 ± 0.2 A a	2.0 ± 0.2 A a,b	1.61 ± 0.03 A e	1.59 ± 0.05 A c	79 ± 1 A c	80 ± 1 A a,b
Negroa	13 ± 1 A a	11 ± 1 A a	2.5 ± 0.2 A a	2.2 ± 0.2 A a,b	1.66 ± 0.04 A e	1.58 ± 0.01 B c	78 ± 1 A c,d	79 ± 1 A a,b
MGS Neblina	11.15 ± 1 A a	12 ± 1 A a	1.1 ± 0.1 A b	1.1 ± 0.1 A c,d	2.0 ± 0.1 A b	1.87 ± 0.05 A b	74 ± 1 A e	70.8 ± 0.5 B d
JB1	12.0 ± 0.4 A a	1 ± 1 A a	2.4 ± 0.1 A a	2.0 ± 0.2 B a,b	1.67 ± 0.03 A d,e	1.64 ± 0.04 A c	79.3 ± 0.4 A c	80 ± 1 A a,b
MGS Grap 561	7.0 ± 0.5 b	NA	0.16 ± 0.03 d	NA	1.88 ± 0.03 c	NA	82.0 ± 0.3 b	NA
Cornicabra	12 ± 1 a	NA	2.2 ± 0.1 a	NA	1.80 ± 0.05 c,d	NA	77 ± 1 d	NA
Tafahi 390	5.9 ± 0.4 b	NA	0.33 ± 0.01 c,d	NA	2.20 ± 0.02 a	NA	84.3 ± 0.3 a	NA
MGS Mariense	NA [c]	12 ± 2 a	NA	1.6 ± 0.4 b,c	NA	2.2 ± 0.1 a	NA	78 ± 2 b,c
Mission	NA	12 ± 1 a	NA	2.5 ± 0.3 a	NA	1.55 ± 0.04 c	NA	79 ± 1 a,b

Varieties	Fatty Acid Contents (% of relative area) per crop year							
	18:2n-6		18:3n-3		20:0		20:1n-11	
	2010	2011	2010	2011	2010	2011	2010	2011
Grappolo 575	6.78 ± 0.02 A d	4.65 ± 0.01 B c	0.60 ± 0.01 B d,e	0.65 ± 0.01 A d,e	0.54 ± 0.01 A a,b	0.56 ± 0.04 A a,b	0.59 ± 0.04 A a,b,c	0.7 ± 0.1 A a
Arbequina	9.6 ± 0.1 A b	9.71 ± 0.05 A b	0.68 ± 0.01 A c	0.7 ± 0.1 A b,c	0.53 ± 0.03 A a,b	0.58 ± 0.03 A a,b	0.6 ± 0.1 A a,b	0.6 ± 0.1 A a,b
Alto D`Ouro	3.57 ± 0.03 B g	4.0 ± 0.1 A d	0.56 ± 0.01 B e,f	0.74 ± 0.01 A b,c	0.43 ± 0.03 A b	0.48 ± 0.05 A b,c	0.5 ± 0.1 A b,c	0.45 ± 0.05 A b,c
Negroa	3.50 ± 0.02 B g	3.98 ± 0.05 A d	0.55 ± 0.05 B e,f	0.76 ± 0.03 A b	0.42 ± 0.04 A b	0.44 ± 0.02 A b,c	0.37 ± 0.04 A c	0.5 ± 0.1 A b,c
MGS Neblina	9.8 ± 0.3 B a	11.7 ± 0.1 A a	0.87 ± 0.02 B a	1.36 ± 0.01 A a	0.6 ± 0.1 A a	0.6 ± 0.1 A a	0.6 ± 0.1 A a,b	0.51 ± 0.05 A a,b,c
JB1	3.23 ± 0.02 B h	3.76 ± 0.04 A e	0.51 ± 0.01 B f	0.68 ± 0.01 A c,d	0.43 ± 0.04 A b	0.47 ± 0.02 A b,c	0.46 ± 0.01 A b,c	0.44 ± 0.03 A b,c
MGS Grap 561	7.37 ± 0.04 c	NA	0.79 ± 0.01 b	NA	0.45 ± 0.04 a,b	NA	0.8 ± 0.1 a	NA
Cornicabra	5.59 ± 0.02 e	NA	0.72 ± 0.01 c	NA	0.49 ± 0.03 a,b	NA	0.47 ± 0.05 b,c	NA
Tafahi 390	5.26 ± 0.01 f	NA	0.62 ± 0.02 d	NA	0.57 ± 0.02 a	NA	0.8 ± 0.1 a	NA
MGS Mariense	NA	4.7 ± 0.1 c	NA	0.67 ± 0.02 c,d,e	NA	0.5 ± 0.1 a,b,c	NA	0.4 ± 0.1 b,c
Mission	NA	3.56 ± 0.04 f	NA	0.61 ± 0.02 e	NA	0.39 ± 0.02 c	NA	0.38 ± 0.02 c

[a] Fatty acid nomenclature: 16:0, palmitic acid; 16:1n-7, palmitoleic acid; 18:0, stearic acid; 18:1n-9, oleic acid; 18:2n-6, linoleic acid; 18:3n-3, α-linolenic acid; 20:0, arachidic acid; 20:1n-11, 9-eicosenoic acid.
[b] Significant differences in the same column are indicated with different lowercase letters (comparison among varieties, $p < 0.05$). Significant differences in the same row are indicated with different uppercase letters (A-B) (comparison between crop years, $p < 0.05$).
[c] NA, not available.

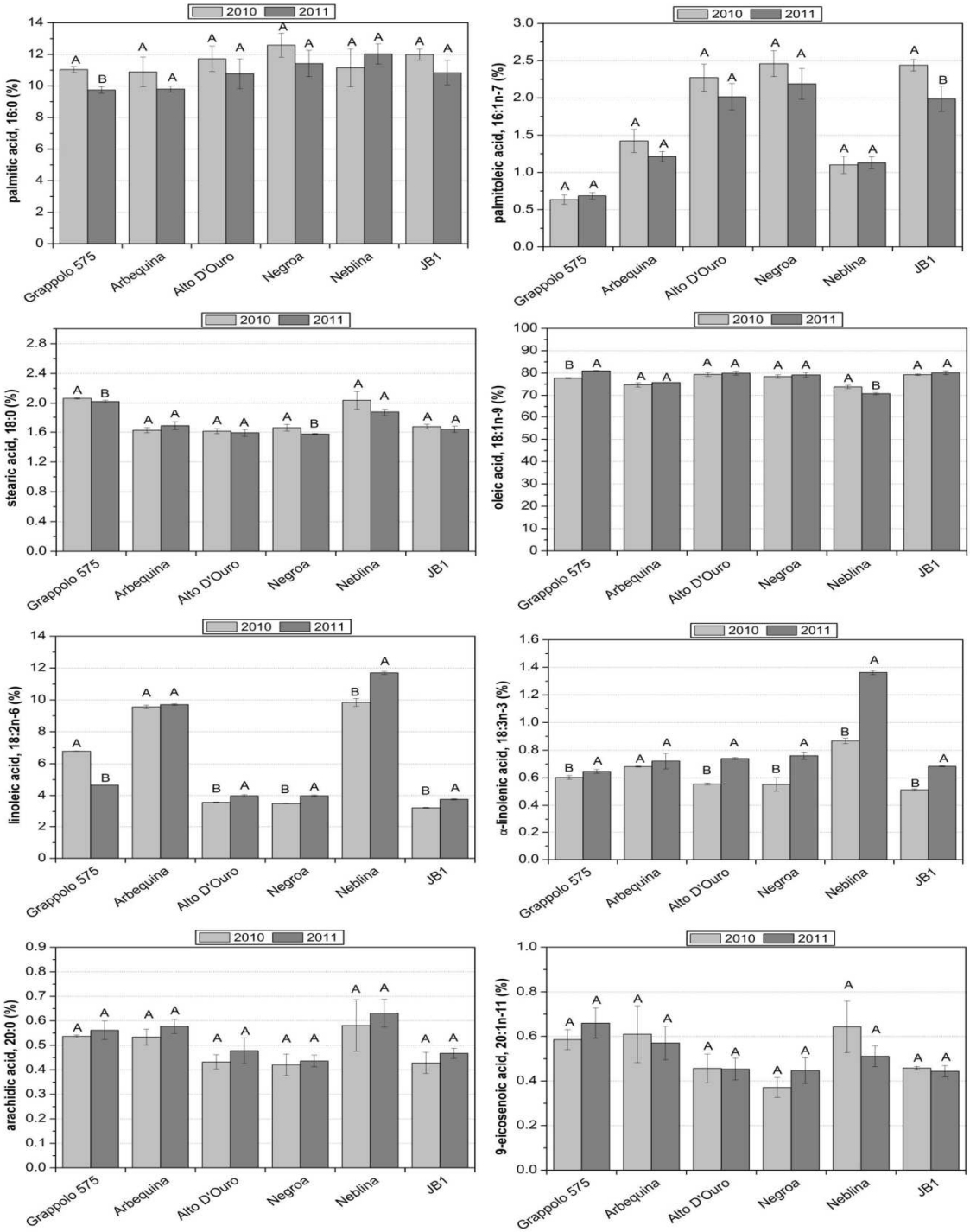

Figure 4.2. Comparison between the fatty acid contents (% of relative area) of Brazilian extra-virgin olive oil from two different varieties over two crop years. Bars followed by the same uppercase letters showed no significant difference ($p < 0.05$) by the Tukey test, for the crop year comparisons.

4.3.3. Phenolic compound composition

The phenolic compounds presented high variation among the different varieties and between crop years. This class of compounds is strongly affected by a large number of agronomical and technological factors, which explains the pronounced variability in the phenolic compound contents.

Table 4.4 contains the data for phenolic compounds from all EVOO samples. Four phenolic compounds were quantified in the samples of Brazilian EVOO, tyrosol, (+)-pinoresinol, hydroxytyrosol and luteolin. (+)-Pinoresinol was quantified in all 17 samples. Tyrosol was quantified in all except for three samples. Hydroxytyrosol was quantified in six samples, while luteolin was quantified in just two samples. The ranges were from NQ to 155.21 mg kg^{-1} for tyrosol, from 2.89 to 22.64 mg kg^{-1} for (+)-pinoresinol, from ND to 37.74 mg kg^{-1} for hydroxytyrosol, and from ND to 2.23 mg kg^{-1} for luteolin. Several papers have reported the content of phenolic compounds in EVOO from around the world, and the contents found for Brazilian EVOOs were similar to those from the literature. Some of the authors consulted were Bendini et al. (2003), Gómez-Caravaca, Carrasco-Pancorbo, Cañabate Díaz, Segura-Carretero, & Fernández Gutiérrez (2005), Carrasco-Pancorbo et al. (2006), Carrasco-Pancorbo et al. (2009), Flores, Romero-González, Frenich, & Vidal (2012), Godoy-Caballero, Acedo-Valenzuela, & Galeano-Díaz (2012), Godoy-Caballero, Galeano-Díaz, & Acedo-Valenzuela (2012), and Monasterio, Fernández, & Silva (2013). The only value that was not comparable to the literature, and thus is most likely an outlier, was the concentration of 155.21 mg kg^{-1} for tyrosol in the MGS Neblina (2010 crop)

sample. Such a content of tyrosol could be explained, for example, if extensive hydrolysis of the phenolic compound ligstroside aglycone had occurred because it is an ester of elenolic acid with tyrosol (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011). There is no commercially available standard for ligstroside aglycone, so it was not possible to evaluate its content in the EVOO samples to test this hypothesis. However, the same sample from the following year (MGS Neblina, 2011 crop) presented a lower tyrosol concentration of 7.51 mg kg^{-1}, which further suggests that the value from the 2010 crop must be an outlier.

There was a significant difference between the two crops, as seen in **Figure 4.3**. Almost all phenolic compound contents were significantly affected by the crop year. This pronounced sensitivity is easy to understand because, while the content of phenolic compounds in olives has a strong genetic base, it is also strongly affected by pedoclimatic production conditions, agronomic techniques and fruit ripening. Moreover, technological factors, mainly milling and malaxation, have a critical influence on the final content of phenolic compounds in EVOO (Conde, Delrot, & Gerós, 2008; El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011).

Table 4.4. Phenolic compound and tocopherol contents of Brazilian extra-virgin olive oil samples (mean ± standard deviation, n = 3).

Varieties	Phenolic Compound Contents (mg kg^{-1}) per crop year							
	Tyrosol		(+)-Pinoresinol		Hydroxytyrosol		Luteolin	
	2010	2011	2010	2011	2010	2011	2010	2011
Grappolo 575	15 ± 2 A b [a]	11.3 ± 0.3 B c,d	3.1 ± 0.2 B d	5.3 ± 0.4 A c	ND [d]	ND	NQ	NQ
Arbequina	6 ± 1 B b	9.4 ± 0.7 A c,d,e	3.3 ± 0.5 B d	5.9 ± 0.3 A c	ND	14 ± 1 c	NQ	NQ
Alto D`Ouro	5.4 ± 0.3 B b	27 ± 3 A b	13 ± 2 B a	22 ± 2 A a	5.7 ± 0.1 B	21 ± 2 A b	NQ	NQ
Negroa	4.45 ± 0.05 B b	14.6 ± 0.2 A c	7.9 ± 0.1 B b	22 ± 2 A a	NQ	38 ± 5 a	NQ	NQ
MGS Neblina	155 ± 16 A a	7 ± 4 B d,e	7 ± 1 A b,c	7 ± 1 A c	ND	ND	2.2 ± 0.2 A	1.8 ± 0.1 B
JB1	4.9 ± 0.2 A b	5.3 ± 0.2 A e	15 ± 2 B a	23 ± 3 A a	ND	21 ± 2 b,c	NQ	NQ
MGS Grap 561	8.3 ± 0.6 b	NA	2.9 ± 0.1 d	NA	ND	NA	NQ	NA
Cornicabra	NQ [b]	NA	9 ± 1 b	NA	ND	NA	NQ	NA
Tafahi 390	NQ	NA	3.6 ± 0.3 c,d	NA	NQ	NA	NQ	NA
MGS Mariense	NA [c]	37 ± 1 a	NA	8.1 ± 0.3 c	NA	ND	NA	ND
Mission	NA	10 ± 1 c,d,e	NA	17 ± 1 b	NA	18 ± 1 b,c	NA	NQ

Varieties	Tocopherol Contents (mg kg^{-1}) per crop year					
	α-Tocopherol		β-Tocopherol		γ-Tocopherol	
	2010	2011	2010	2011	2010	2011
Grappolo 575	69 ± 1 A d	31 ± 1 B e	9.5 ± 0.4 A a	8.3 ± 0.4 B b	9.6 ± 0.3 A e	7.9 ± 0.4 B b
Arbequina	62.0 ± 0.3 B e	201 ± 7 A b	5.1 ± 0.1 B e	7.6 ± 0.1 A b	3.9 ± 0.3 B f	5.4 ± 0.2 A d
Alto D`Ouro	108 ± 2 B b	205 ± 3 A b	5.2 ± 0.1 B d,e	6.1 ± 0.2 A c,d	11.4 ± 0.3 A b,c,d	11.63 ± 0.04 A a
Negroa	96 ± 4 B c	233 ± 5 A a	5.84 ± 0.02 A c,d	6.2 ± 0.3 A c	11.0 ± 0.1 A c,d	11.3 ± 1 A a
MGS Neblina	29 ± 2 B g	140 ± 4 A c	ND	7.0 ± 0.4 a	ND	ND
JB1	93 ± 5 B c	201 ± 2 A b	5.9 ± 0.3 A b,c	5.4 ± 0.3 A d	12 ± 1 A b,c	11.0 ± 0.3 A a
MGS Grap 561	137 ± 2 a	NA	9.6 ± 0.2 a	NA	12.5 ± 0.3 b	NA
Cornicabra	59 ± 1 e	NA	6.5 ± 0.2 b	NA	10.1 ± 0.2 d,e	NA
Tafahi 390	51 ± 3 f	NA	5.8 ± 0.2 c,d	NA	19 ± 1 a	NA
MGS Mariense	NA	31 ± 2 e	NA	6.5 ± 0.2 c	NA	6.6 ± 0.2 c
Mission	NA	127 ± 3 d	NA	5.9 ± 0.1 c,d	NA	11.1 ± 0.5 a

[a] Significant differences in the same column are indicated with different lowercase letters (comparison among varieties, $p < 0.05$).
Significant differences in the same row are indicated with different uppercase letters (A-B) (comparison between crop years, $p < 0.05$).
[b] NQ, below the limit of quantification.
[c] NA, not available.
[d] ND, below the limit of detection.

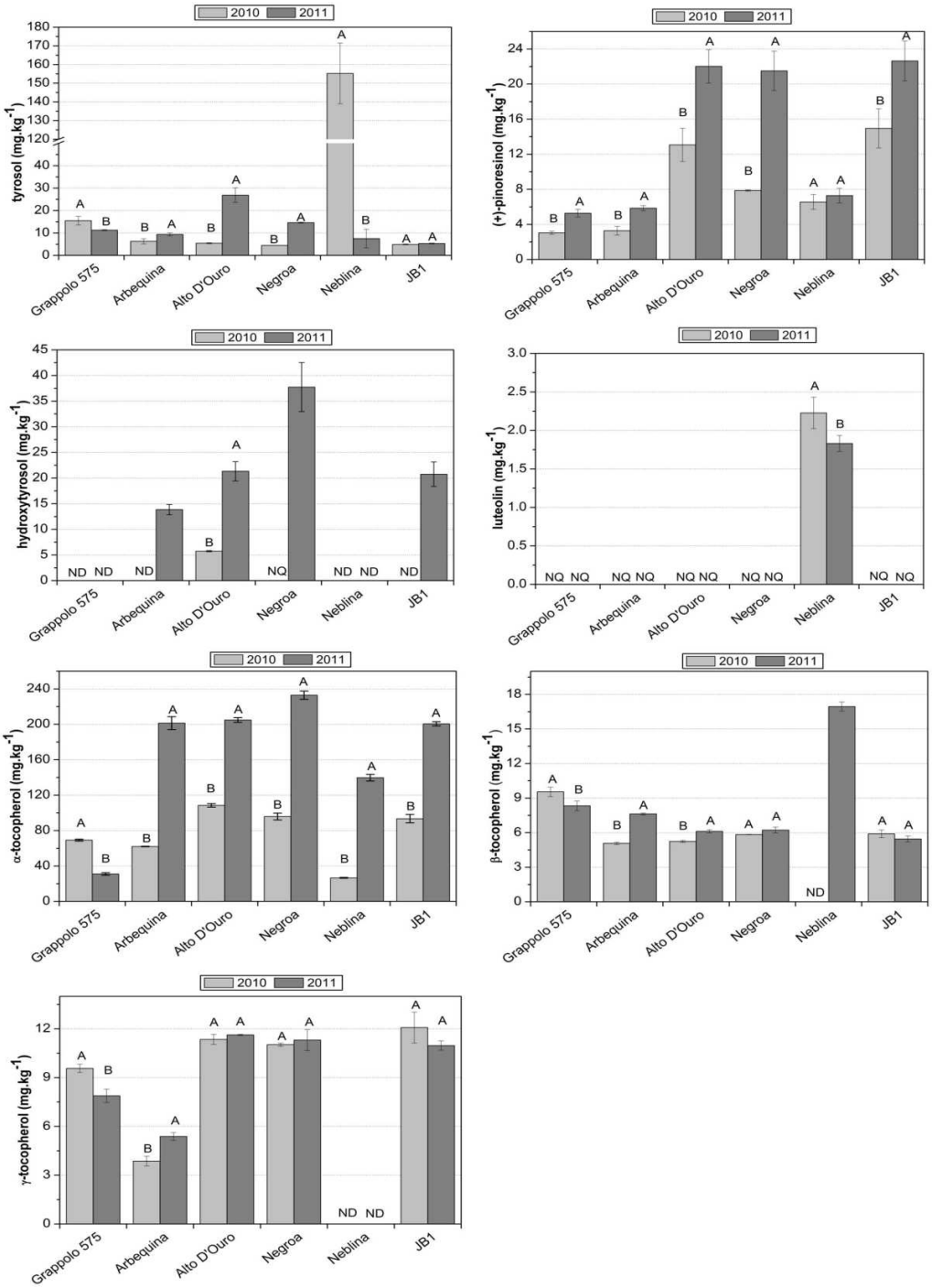

Figure 4.3. Comparison between the phenolic compound and tocopherol contents of Brazilian extra-virgin olive oil obtained from different varieties over two crop years. Bars followed by the same uppercase letters showed no significant difference ($p < 0.05$) by the Tukey test, for the crop year comparisons.

Some of the other phenolic compounds assayed were detected, but they were present below the limit of quantification. Apigenin was detected in the samples of Grappolo 575 (2010 and 2011 crops), Arbequina (2010 and 2011 crops), Negroa (2010 and 2011 crops), MGS Neblina (2010 and 2011 crops), JB1 (2010 and 2011 crops), Cornicabra (2010 crop), Tafahi 390 (2010 crop), Alto D'Ouro (2011 crop), MGS Mariense (2011 crop) and Mission (2011 crop). *p*-Coumaric acid was detected in the samples of MGS Neblina (2010 and 2011 crops), Arbequina (2011 crop) and Grappolo 575 (2011 crop). Vanillic acid was detected in the samples of Alto D'Ouro (2010 crop), MGS Neblina (2010 and 2011 crops), JB1 (2010 and 2011 crops), Mission (2011 crop), Arbequina (2011 crop) and Grappolo 575 (2011 crop). *p*-Hydroxybenzoic acid was detected only in the Arbequina (2010 crop) sample, while 3,4-dihydroxybenzoic acid was detected in the MGS Neblina (2010 crop) and JB1 (2011 crop) samples.

4.3.4. Tocopherol composition

Three tocopherol isomers were detected and quantified in the samples of Brazilian EVOO, α-tocopherol, β-tocopherol and γ-tocopherol. **Table 4.4** presents the results for all EVOO samples, while **Figure 4.3** displays a comparison between the crop years for the six varieties for which the two years were available.

The α-tocopherol isomer was quantified in all 17 samples ranging from 28.92 to 232.93 mg kg^{-1}, while β-tocopherol ranged from 5.07 to 9.56 mg kg^{-1}, but was not detected in the MGS Neblina (2010 crop) sample. γ-Tocopherol ranged from 3.87 to 18.75 mg kg^{-1} and was only undetectable in the MGS Neblina (2010

and 2011 crops) sample. These contents are compatible with those from the literature because α-tocopherol varies from trace up to 300 mg kg^{-1} and the concentrations of β- and γ-tocopherols vary from trace to 25 mg kg^{-1} (Ghanbari, Anwar, Alkharfy, Gilani, & Saari, 2012). Because α-tocopherol is the main tocopherol isomer found in EVOOs, the most promising Brazilian varieties with respect to its content were Arbequina, Alto D'Ouro, Negroa and JB1, which had contents of greater 200 mg kg^{-1} (2011 crop). From **Figure 4.3** it is clear that the tocopherol contents of each of the three detected isomers were significantly different between crop years. When studying the Arbequina variety, Benito et al. (2013) reported that the content of α-tocopherol significantly decreased in EVOO as olive ripening progressed.

4.3.5. Chemometrics

A data matrix for PCA was constructed using all of the replicates (n = 51) and all 14 variables. The variables comprised the eight fatty acids (palmitic, palmitoleic, stearic, oleic, linoleic, α-linolenic, arachidic and 9-eicosenoic acids), the three tocopherols (α-, β-, and γ-tocopherol), and the phenolic compounds tyrosol, (+)-pinoresinol and hydroxytyrosol. Luteolin was not considered because it was only quantified in one sample. When the samples presented values below the limit of quantification but above the limit of detection for tyrosol, hydroxytyrosol, β-tocopherol and γ-tocopherol, the value of the limit of quantification (calculated in mg kg^{-1} of sample) was used in the matrix, while in cases where the sample

presented values below the limit of detection, the limit of detection itself (also calculated in mg kg^{-1} of sample) was used in the matrix for PCA.

The main PCA results are illustrated in **Figure 4.4**. Seven principal components were sufficient to explain 96.7% of the data variation. Together the first and second principal components accounted for 63.0% of the explained variance. When plotting the scores graph for these two principal components, it was possible to visualize the behavior depicted in **Figure 4.4-B**. Some varieties grouped separately from the others, as seen for Grappolo, Arbequina, Tafahi and MGS Neblina. Arbequina also exhibited the smallest difference between crops because all values are very close, while there is a large separation between crop years for MGS Neblina. Cornicabra and MGS Mariense grouped together, which indicates that they possess a very similar chemical composition with respect to the compounds used in the PCA. The last group visible in the scores graph contains the Negroa, Alto D'Ouro, JB1 and Mission varieties, and it was not possible to differentiate them based on the contents of the compounds analyzed in this work. The profile of variables shown in the loadings graph (**Figure 4.4-A**) can help to explain this behavior. MGS Neblina presented high levels of 18:3n-3, 18:2n-6 and tyrosol, and this lead to its separation from the other samples. Arbequina also exhibited high contents of 18:2n-6. Tafahi contains the highest quantities of 18:1n-9 and γ-tocopherol. The Negroa, Alto D'Ouro, JB1 and Mission samples are correlated with the compound 16:0, 16:1n-7, (+)-pinoresinol, hydroxytyrosol and α-tocopherol. The loading graph also shows an inverse correlation between the

variables 16:0 and 18:1n-9 and between 18:1n-9 and 18:2n-6, as previously observed in section *4.3.2* for the fatty acid results.

Figure 4.4-C is a Mahalanobis distance graph, which is normally used to verify the presence of outliers. In fact, the MGS Neblina variety must be considered differently from the others varieties studied, as it is clearly outlier because it is outside the normality region for this set of evaluated samples. This suggests that the chemical composition of MGS Neblina is completely different from the other Brazilian EVOOs, mostly because this variety was the only sample that did not contain β-tocopherol in the 2010 crop year and was also the only sample that did not contain γ-tocopherol for both crop years. Moreover, it was the only sample to possess a luteolin content above the limit of quantification. As such, in the future it would be interesting to conduct experiments to understand what changes in its metabolism lead to these significant differences. Additionally, it seems that, when considering the tocopherol content of Brazilian EVOOs, MGS Neblina would not be the best choice of variety to be used in large scale EVOO production.

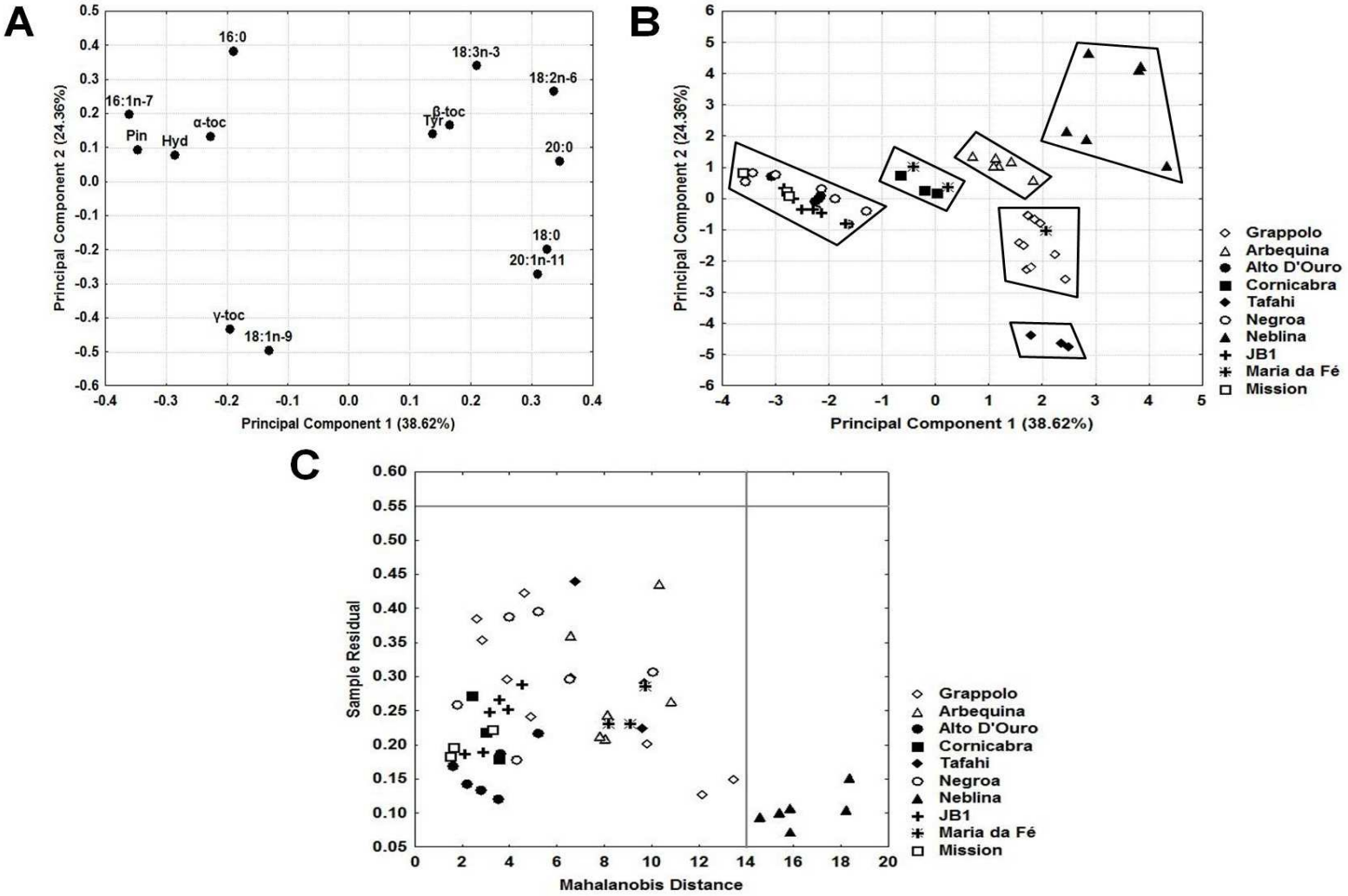

Figure 4.4. Results of the principal component analysis, showing the first and the second principal components for the response values. (**A**) Loadings graph; (**B**) Scores graph; (**C**) Mahalanobis distance graph.

4.4. Conclusions

In this work, the phenolic compound, tocopherol and fatty acid contents of monovarietal Brazilian EVOOs were evaluated using different analytical techniques that were validated to obtain reliable data. This study is the first report on the chemical composition of Brazilian EVOOs, and it will be of paramount importance for everyone involved in EVOO research in Brazil and other countries.

The results indicated that the fatty acid composition of all EVOOs is in accordance with the internationally accepted ranges for this type of product and that the phenolic compound and tocopherol isomer contents are also compatible with literature data. Some varieties, such as Arbequina, Alto D'Ouro, Negroa and JB1 are promising candidates for good quality EVOO with respect to the overall chemical composition as analyzed in this work. For the Grappolo, Cornicabra, Tafahi, MGS Mariense and Mission varieties, more samples should be analyzed because only one crop year was available for this study, and it is difficult to make a reliable evaluation with this amount of data. The MGS Neblina variety should be given more attention to understand why it was so different from the other Brazilian EVOOs in this study. The significant differences found between the two crops are also a reminder that it is important to control all of the factors that affect EVOO chemical composition.

As a matter of fact, this work was only the beginning. More research is needed to improve the chemical quality of Brazilian EVOO, including sensorial evaluation, leading to a product that will genuinely attract consumers' attention.

4.5. Acknowledgments

We would like to thank FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo (process no. 12/04523-5), CNPq - Coordenação Nacional de Desenvolvimento Científico e Tecnológico, and CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, for their financial support for this study.

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CHAPTER V

ARTICLE

Phenolic compounds profile of Brazilian extra-virgin olive oils by rapid resolution liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (RRLC-ESI-TOF-MS)

CHAPTER V

Phenolic compounds profile of Brazilian extra-virgin olive oils by rapid resolution liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (RRLC-ESI-TOF-MS)

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***This manuscript was submitted to the *Food Chemistry* journal.**

ABSTRACT

In recent years, agronomical researchers started to cultivate several olive (*Olea europaea* L.) varieties in different regions of Brazil, aiming to produce extra-virgin olive oil (EVOO). As there is no data about phenolic profile of the first Brazilian EVOO samples, the aim of this work was to determine the phenolic compound contents of these samples, using rapid-resolution liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (RRLC-ESI-TOF-MS). A total of 25 EVOO samples from Arbequina, Koroneiki, Arbosana, Grappolo, Manzanilla, Coratina, Frantoio and MGS Mariense varieties, from three different Brazilian states (Rio Grande do Sul, Santa Catarina and Minas Gerais) and two crops (2011 and 2012), were analyzed. It was possible to identify and quantify 20 phenolic compounds, belonging to the phenolic alcohol, secoiridoid, lignan and flavonoid classes. The results showed that Brazilian EVOOs are promising concerning the total phenolic contents, since the values were comparable to those from high-quality EVOOs produced in other countries. Coratina (364 mg kg^{-1}), Arbosana (255 mg kg^{-1}) and Grappolo (228 mg kg^{-1}) presented the highest total phenolic contents. New studies will be necessary, since the EVOO production in Brazil is still experimental, and this data will be useful to the Brazilian EVOO development and improvement.

Keywords: EVOO; phenolic compounds; RRLC-ESI-TOF-MS; principal component analysis.

5.1. Introduction

Extra-virgin olive oil (EVOO) is obtained from the olive (*Olea europaea* L.) fruits, only by mechanical extraction. It is edible without previous physical-chemical treatments and, for its nutritional and sensory qualities, it is a prime component of the Mediterranean diet (Baiano, Terracone, Viggiani, & Del Nobile, 2013).

Chemical composition of EVOO consists of approximately 98% of triacylglycerols and 2% of minor components, that include more than 230 compounds such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants (Servili et al., 2009). Antioxidants in EVOO are represented by tocopherols, pigments and phenolic compounds. Hydrophilic phenolic compounds such as phenolic acids, phenolic alcohols, hydroxy-isochromans, flavonoids, secoiridoids and lignans are especially important in EVOO quality. Among these secondary metabolites, secoiridoids and lignans are present in the highest contents (El Riachy et al., 2011).

EVOO has a well-balanced composition of fatty acids, with small amounts of palmitate, and it is highly enriched in oleic acid. This makes it both fairly stable against auto-oxidation and suitable for human health (Conde, Delrot, & Gerós, 2008). Caramia, Gori, Valli, & Cerretani (2012) reviewed the main effects of a diet rich in EVOO on the human health and which are the compounds responsible for those effects. It has been shown that EVOO acts in the prevention and/or reduction of hypercholesterolaemia, serum lipoprotein levels and atherosclerosis (by its phenolic compounds content); hypertension, cardiovascular diseases and thrombotic risk (by its tyrosol, hydroxytyrosol, oleic acid and hydroxyl-oleic acid

contents); oxidation and oxidative stress (by its high level of oleic acid and lack of excess of linoleic acid as well as by its α-linolenic acid, phenolic compounds and tocopherols contents); obesity and type 2 diabetes (by its oleic acid, α-linolenic acid, phenolic compounds, carotenoids and tocopherols contents); inflammatory processes (by its oleocanthal content, also known as decarboxymethyl ligstroside aglycone), and cancer (by its oleuropein and oleic acid contents).

EVOO production is concentrated in the Mediterranean countries, being Spain, Italy and Greece those that present the highest production volume. Nevertheless, today the production and consumption of olive oil are moving slowly but inexorably beyond the Mediterranean countries, and olive trees are being planted in countries as far from the Mediterranean basin as New Zealand and Argentina (García-González, & Aparicio, 2010). Following this trend, Brazil started to experimentally cultivate olive trees from different varieties, in order to study the effects of its edapho-climatological conditions on the olive productivity, and then find the varieties that best adapt to them. All the EVOO found in Brazil is imported from European and South American countries, because there is no commercial EVOO production in Brazil nowadays, even though its consumption is increasing annually. Since there is a large market to be explored with the EVOO business, Brazil now has its first extra-virgin olive oils, obtained from those olive trees recently cultivated in different regions of the country. However, there is a lack of knowledge about its chemical composition.

With all these in mind, the main goal of this work was to determine the phenolic compounds profile of the Brazilian EVOOs already available in the

country, which were experimentally obtained from several olive varieties cultivated in different regions of Brazil. To reach this objective, it was used rapid-resolution liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (RRLC-ESI-TOF-MS) to separate, identify and quantify the phenolic compounds profile. To the best of our knowledge, this is the first study carried out about the phenolic composition of Brazilian EVOOs from different regions of the country using such a powerful analytical tool.

5.2. Material and methods

5.2.1. Chemicals and standards

Methanol p.a., hexane p.a., sodium hydroxide p.a. and sodium acetate p. a. were purchased from Panreac (Barcelona, Spain). Acetic acid p.a., methanol and isopropanol LC-MS grade were from Fischer Scientific (Leicestershire, UK). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). The standards of tyrosol and apigenin were purchased from Sigma-Aldrich (St. Louis, MO, USA), while oleuropein, luteolin, hydroxytyrosol and dihydrocaffeic acid were from Extrasynthèse (Lyon, France), (+)-pinoresinol was from Arbo Nova (Turku, Finland), and quinic acid was from Acros Organics (New Jersey, USA).

The phenolic and other polar compounds standard stock solutions were prepared by dissolving the appropriate amount of each compound in methanol LC-MS grade to a final concentration of 1 g L^{-1}. The solutions were filtered through a

0.22 µm Fisherbrand cellulose membrane (Fisher Scientific, Pittsburgh, PA, USA), stored at –80ºC and protected from light.

5.2.2. EVOO Samples

Samples consisted of 25 EVOOs produced from different olive varieties cultivated in three Brazilian states, Rio Grande do Sul (RS), Santa Catarina (SC) and Minas Gerais (MG). Rio Grande do Sul and Santa Catarina are located in the South region of Brazil, while Minas Gerais is located in the Southeast region. From Rio Grande do Sul and Santa Catarina states, EVOOs from olives cultivated in two different crop years (2011 and 2012) were available, while from Minas Gerais there were samples only from 2012 crop. After arriving at the laboratory, all the samples were maintained under cold refrigeration (4ºC) and protected from light, until the moment of the analysis.

5.2.2.1. EVOO samples from Rio Grande do Sul (RS)

The EVOO samples from Rio Grande do Sul state were provided by the Brazilian Agricultural Research Corporation - Agricultural Research Center for Temperate Climate (EMBRAPA - CPACT). Olive trees from Manzanilla, Grappolo, Arbequina and Koroneiki varieties (2011 crop) were cultivated in Dom Pedrito, a city located near to the border with Uruguay (latitude: 31º 19' 53" S; longitude: 54º 06' 25" W; altitude: 212 m above sea level). The annual mean temperature in Dom Pedrito is 19.5ºC, while the annual mean rainfall was 95.4 mm. The soil class was orthic hypochromic luvisol. All the olive trees were about seven years old. Olive

trees from Arbequina and Koroneiki varieties (2011 and 2012 crops) and from Coratina and Frantoio varieties (2012 crop) belonged to the Active Germplasm Bank of EMBRAPA - CPACT and were cultivated in the city of Pelotas (latitude: 31º 40' 48.48" S; longitude: 52º 26' 42.71" W; altitude: 57 m above sea level). For 2011 crop, the annual mean rainfall was 104 mm, the annual mean temperature was 18ºC, while for 2012 crop, the annual mean rainfall was 124 mm and the annual mean temperature was 19ºC. The soil class was red-yellow argisol. The olive trees were five years old in 2011 crop and, consequently, six years old in 2012. For the oil extraction, all the olives were processed within approximately 14 h after having been collected. Moments before starting the process, the olives that presented visible damages originated from plagues or diseases were removed and discarded and, then, the olive fruits were washed with water. The oil extraction was performed using a TEM SPREMOLIVA 10 mill (Toscana Enologica Mori, Tavarnelle Val di Pesa, FI, Italy), that employs a cold extraction in two phases. About 30 days after extraction, the EVOOs were filtered to eliminate impurities.

5.2.2.2. EVOO samples from Santa Catarina (SC)

EVOO samples from Santa Catarina state were obtained from the Agricultural Research and Rural Extension Corporation of the State of Santa Catarina (EPAGRI). From 2011 crop, EVOOs from Grappolo, Arbequina, Arbosana and Koroneiki varieties were available, as well as an EVOO consisting of a mixture of Arbequina/Arbosana varieties (50:50, v/v), here named "Mixture of varieties". From 2012 crop, EVOOs from Arbequina, Arbosana, Koroneiki and the Mixture of

varieties were studied. In 2011, the olive trees were 4.3 years old. The olive trees were cultivated in the city of Chapecó (latitude: 27º 05' 45" S; longitude: 52º 37' 04" W; altitude: 670 m above sea level). The annual mean temperature in Chapecó was 19.6ºC (ranging from 19.4 to 24.1ºC) in 2011 and 20.6ºC (ranging from 20.6 to 25.6ºC) in 2012. The annual mean rainfall was 2243 mm in 2011 and 1364 mm in 2012. Before the oil extraction, all the solid dirt was removed manually and the olives were washed with water. The oil extraction was performed using a TEM SPREMOLIVA 10 mill (Toscana Enologica Mori, Tavarnelle Val di Pesa, FI, Italy), that employs a cold extraction in two phases.

5.2.2.3. EVOO samples from Minas Gerais (MG)

A total of six EVOO samples were obtained from olive trees cultivated by the Maria da Fé Experimental Farm of the Agricultural and Livestock Research Corporation of the State of Minas Gerais (EPAMIG). Maria da Fé is a city situated into the micro-region of Serra da Mantiqueira, in the south of Minas Gerais (MG) state (latitude: 22º 18’ 28” S; longitude: 45º 22’ 30” W; altitude: 1276 m above sea level). Considering the Köppen-Geiger climate classification system, Maria da Fé has a temperate highland tropical climate, with dry winters (Cwb). The annual mean temperature is 17ºC, ranging from 10.1ºC (minimum) to 23.3ºC (maximum), while the annual mean rainfall is about 1738.6 mm. From EPAMIG were available the monovarietal EVOOs Grappolo 575, Grappolo 541, Koroneiki, Arbequina, Arbosana and MGS Mariense, all from 2012 crop.

For the olive oil extraction, it was employed an Abencor® system (Suárez, Aranda, Mendoza, & Rey, 1975), adapted for obtaining sufficient olive oil to perform the chemical analysis. Olives from each variety (10 kg) were washed with water in order to remove impurities and leaves prior to being milled into a metallic mill. The olive oil was heated up to 28ºC (Sánchez, Pacheco, Rubia, Sánchez, & Pereira, 2005) during the homogenizing process, that was realized into a domestic mixer, using two types of movements, translation and rotation, during an interval of 50-60 minutes. By using an analytical balance, 450 g of EVOO sample was transferred to a high rotation centrifuge and submitted to a centrifugation process under 4200 rpm, allowing the EVOO separation. The EVOO samples were packed into plastic packages, where it has remained during 60 minutes for an eventual residual sedimentation. In the end, the EVOOs were transferred to amber glass bottles.

5.2.3. Phenolic compounds extraction procedure

Phenolic compounds were extracted from the EVOO samples based on a liquid-liquid extraction procedure described in the work from Taamalli, Román, Zarrouk, Segura-Carretero, & Fernández-Gutiérrez (2012). Firstly, 2.5 g of EVOO sample were weighed in a centrifuge tube, and then 50 µL of internal standard (dihydrocaffeic acid, 25 mg L^{-1}) was added, with subsequent agitation during 2 min, addition of 5 mL of hexane and then agitation for 1 min more. After, 5 mL of methanol:water (60:40, v/v) was added, and this mixture was vortexed during 2 min and centrifuged at 3500 rpm during 10 min. The resulting polar extract (bottom

phase) was evaporated to dryness in a rotary evaporator under reduced pressure and temperature of 39ºC. The residue was re-dissolved in 0.25 mL of methanol:water (50:50, v/v) and filtered through a 0.22 µm Fisherbrand cellulose membrane (Fisher Scientific, Pittsburgh, PA, USA) before injection into the chromatographic system. All the samples were extracted in triplicate (n = 3).

5.2.4. Experimental conditions for separation, identification and quantification of EVOO phenolic and other polar compounds

An Agilent 1200 Series Rapid Resolution LC system (RRLC) (Agilent Technologies, Waldbronn, Germany), equipped with a vacuum degasser, a binary pump, an autosampler and a diode-array detector (DAD) was used to perform phenolic and other polar compounds separation. Analysis was carried out in a Zorbax Eclipse Plus C_{18} column (Agilent Technologies, Palo Alto, CA, USA) with 150 mm x 4.6 mm i.d. x 1.8 µm particle diameter employing a gradient of water with 0.25% acetic acid as eluent A and methanol as eluent B in the following steps: 0 min, 5% B; 7 min, 35% B; 12 min, 45% B; 17 min, 50% B; 22 min, 60% B; 25 min, 95% B; 27 min, 5% B, and then a conditioning cycle of 5 min with the same conditions for the following analysis. The flow rate was 0.80 mL min^{-1}, and the column temperature was maintained at 25ºC. The injection volume into the chromatographic system was 10 µL.

Separated compounds were monitored using a micrOTOF^{TM} orthogonal-accelerated time-of-flight mass spectrometer (TOF-MS) (Bruker Daltonik, Bremen, Germany) equipped with an electrospray ionization (ESI) interface (model G1607A,

Agilent Technologies, Palo Alto, CA, USA), working in negative ion mode. The adjusted parameters for the ESI interface comprised capillary voltage, +4 kV; drying gas temperature, 190ºC; drying gas flow, 9 L min^{-1}; nebulizing gas pressure, 2 bar; and end plate offset, -0.5 kV. For ion transfer, the adjusted values were capillary exit voltage, -120 V; skimmer 1, -40 V; hexapole 1, -23 V; RF hexapole, 50 Vpp; and skimmer 2, -22.5 V. All spectra were acquired into 50-1000 *m/z* mass range.

The flow coming from the RRLC into the MS detector was split in a 1:3 ratio with a flow splitter, in order to achieve stable electrospray ionization and reproducible results. External calibration of TOF-MS was performed using a syringe pump (74900-00-05 Cole Palmer, Vernon Hills, Illinois, USA) equipped with a Hamilton syringe (Reno, NV, USA) and directly connected to the interface. The sodium acetate clusters calibration solution, prepared using 5 mmol L^{-1} sodium hydroxide and water:isopropanol 1:1 (v/v) with 0.2% of acetic acid, was injected into the TOF-MS at the beginning of the run, and all the spectra were calibrated prior to phenolic compounds identification, in the high precision calibration (HPC) regression mode. By using this method, an exact calibration curve based on numerous cluster masses, each differing by 82 Da ($NaC_2H_3O_2$), was obtained. Due to the compensation of temperature drift in the micrOTOF^{TM}, this external calibration provided accurate mass values for a complete run, without the need for a dual sprayer set-up for internal mass calibration.

The accurate mass data for the molecular ions were processed using the software Data Analysis 4.0 (Bruker Daltonik, Germany), which provided a list of

possible elemental formulae by using the GenerateMolecularFormula Editor. This latter uses a CHNO algorithm providing standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalent, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma Value) to increase confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions was established at 5 ppm (Bringmann et al., 2005). The tolerance in the mSigma value is usually established at 50, although it is influenced by co-eluting analytes or matrix compounds, so it may be higher in some cases (Peters, Bolck, Rutgers, Stolker, & Nielen, 2009).

All these experimental conditions for the separation and identification of EVOO phenolic compounds were obtained from the study carried out by Lozano-Sánchez et al. (2010).

5.2.5. Method validation

In order to obtain better reproducibility in the phenolic compounds quantification, it was decided to use an internal standard (IS) early in the extraction phase. The compound that was chosen as IS was dihydrocaffeic acid, since it was not present in EVOO samples, and did not co-elute with the other phenolic compounds. The volume necessary to obtain a dihydrocaffeic acid final concentration of 5 mg L^{-1} was added to the standards and to the EVOO samples. Seven phenolic and other polar compounds standards were used for the

quantification step, namely quinic acid, hydroxytyrosol, tyrosol, (+)-pinoresinol, oleuropein, luteolin and apigenin.

Limits of detection (LOD) and quantification (LOQ) of the RRLC-ESI-TOF-MS method were estimated as being 3 and 10 times the signal to noise ratio, respectively. Intra-day instrumental precision was determined injecting a solution containing the seven phenolic standards 10 consecutive times in one day. Inter-day instrumental precision was determined repeating the inter-assay procedure in three consecutive days. The linearity was verified individually for each compound, with analytical curves made up of five equally spaced points. A lack of fit test for each analytical curve was performed as recommended by Danzar, & Currie (1998).

5.2.6. Statistical analysis

Means obtained for the sum of each phenolic compound class (phenolic alcohols, secoiridoids, lignans and flavonoids), as well as for the total phenolic content, were compared using ANOVA and Tukey test, at 95% confidence level. The statistical analysis was done using the software Statistica 7.0 (Statsoft, USA). In addition, it was performed a Principal Component Analysis (PCA) in order to better visualize the samples behavior towards the analyzed compounds as well as their correlations. PCA was carried out using Pirouette 3.11 (Infometrix, USA).

5.3. Results and discussion

5.3.1. Results for the method validation

In **Table 5.1** are presented the figures of merit evaluated during the chromatographic method validation. The results for intra-day and inter-day instrumental precisions were very satisfactory, since they remained in the range of 5-10%. The analytical curves presented an adequate fit when submitted to the lack of fit test ($p > 0.05$) and can reliably be used for the phenolic compounds quantification. Both limits of detection and quantification were very low, and comparable to those obtained by Lozano-Sánchez et al. (2010) when they first optimized this chromatographic separation.

5.3.2. Phenolic and other polar compounds identification in the Brazilian EVOO samples

Phenolic and other polar compounds identification was carried out by comparing the retention times and the MS spectral data from EVOO samples to those obtained for the standards. Concerning the compounds for which there were not commercial standards available, they were identified by the interpretation of their mass spectrum provided by the TOF-MS and also by using information previously reported in the literature for EVOO phenolic compounds (Lozano-Sánchez et al., 2010; Lozano-Sánchez et al. 2011; Bakhouche et al., 2013). In **Table 5.2** are summarized the 20 phenolic and other polar compounds tentatively

identified in the Brazilian EVOO samples, including retention times, molecular formula, experimental and calculated *m/z*, error and mSigma values. In **Figure 5.1** are presented the base-peak chromatograms (BPC) for six Brazilian EVOOs.

Table 5.1. Figures of merit for the RRLC-ESI-TOF-MS method validation.

Compounds	LOD [a] (μg mL^{-1})	LOQ [a] (μg mL^{-1})	Intra-day Precision (%, n = 10)	Inter-day Precision (%, n = 3)	Linearity Range (μg mL^{-1})	Equation	r^2	Lack of Fit Test ($p > 0.05$)
Quinic acid	0.018	0.060	3.48	5.18	0.36 - 16.43	$y = 0.371\,x + 0.08$	0.9938	0.090
Hydroxytyrosol	0.032	0.106	2.33	7.65	6.79 - 19.64	$y = 0.272\,x - 0.50$	0.9955	0.118
Tyrosol	0.020	0.066	4.70	4.05	6.79 - 19.64	$y = 0.014\,x + 0.04$	0.9865	0.274
(+)-Pinoresinol	0.008	0.027	2.46	6.93	0.36 - 19.64	$y = 0.278\,x - 0.07$	0.9920	0.618
Oleuropein	0.002	0.008	2.81	2.18	0.36 - 19.64	$y = 0.749\,x + 0.49$	0.9913	0.133
Luteolin	0.007	0.022	4.31	4.43	0.36 - 19.64	$y = 0.436\,x + 0.34$	0.9924	0.175
Apigenin	0.001	0.004	3.07	3.47	0.36 - 16.43	$y = 2.620\,x - 1.01$	0.9878	0.140

[a] LOD, limit of detection; LOQ, limit of quantification.

Table 5.2. Main phenolic compounds identified in a representative extract of Grappolo EVOO variety by RRLC-ESI-TOF-MS.

Peak	Compound	Retention Time (min)	Molecular Formula	*m/z* calculated	*m/z* experimental	Error (ppm)	mSigma
1	Quinic acid	2.0	$C_7H_{12}O_6$	191.0561	191.0562	0.4	4.0
2	Hydroxytyrosol	7.7	$C_8H_{10}O_3$	153.0557	153.0560	-1.5	6.2
3	Tyrosol	9.6	$C_8H_{10}O_2$	137.0608	137.0602	4.5	7.5
4	Hydroxytyrosol acetate	14.2	$C_{10}H_{12}O_4$	195.0663	195.0662	0.2	13.1
5	Elenolic acid	14.8	$C_{11}H_{14}O_6$	241.0718	241.0722	-1.6	21.1
6	Hydroxy elenolic acid	15.5	$C_{11}H_{14}O_7$	257.0667	257.0669	-0.9	3.5
7	Decarboxymethyl oleuropein aglycone	16.1	$C_{17}H_{20}O_6$	319.1187	319.1185	0.6	4.1
8	Hydroxy decarboxymethyl oleuropein aglycone	16.4	$C_{17}H_{20}O_7$	335.1136	335.1138	-0.5	7.2
9	Syringaresinol	17.9	$C_{22}H_{26}O_8$	417.1555	417.1549	1.5	8.4
10	(+)-Pinoresinol	18.6	$C_{20}H_{22}O_6$	357.1344	357.1340	1.0	10.2
11	Acetoxypinoresinol	19.1	$C_{22}H_{24}O_8$	415.1398	415.1413	-3.6	4.1
12	Decarboxymethyl ligstroside aglycone	19.0	$C_{17}H_{20}O_5$	303.1238	303.1236	0.7	11.4
13	Hydroxy decarboxymethyl ligstroside aglycone	19.6	$C_{17}H_{20}O_6$	319.1187	319.1186	0.4	11.6
14	10-Hydroxy oleuropein aglycone	23.0	$C_{19}H_{22}O_9$	393.1191	393.1207	-4.0	3.3
15	Oleuropein aglycone	22.9	$C_{19}H_{22}O_8$	377.1242	377.1252	-2.7	2.7
16	Luteolin	23.4	$C_{15}H_{10}O_6$	285.0405	285.0409	-1.7	3.2
17	Hydroxypinoresinol	24.7	$C_{20}H_{22}O_7$	373.1293	373.1282	2.8	9.8
18	Ligstroside aglycone	25.4	$C_{19}H_{22}O_7$	361.1293	361.1297	-1.2	3.0
19	Apigenin	25.6	$C_{15}H_{10}O_5$	269.0455	269.0458	-1.0	2.6
20	Methyl oleuropein aglycone	26.2	$C_{20}H_{24}O_8$	391.1398	391.1385	3.3	44.3

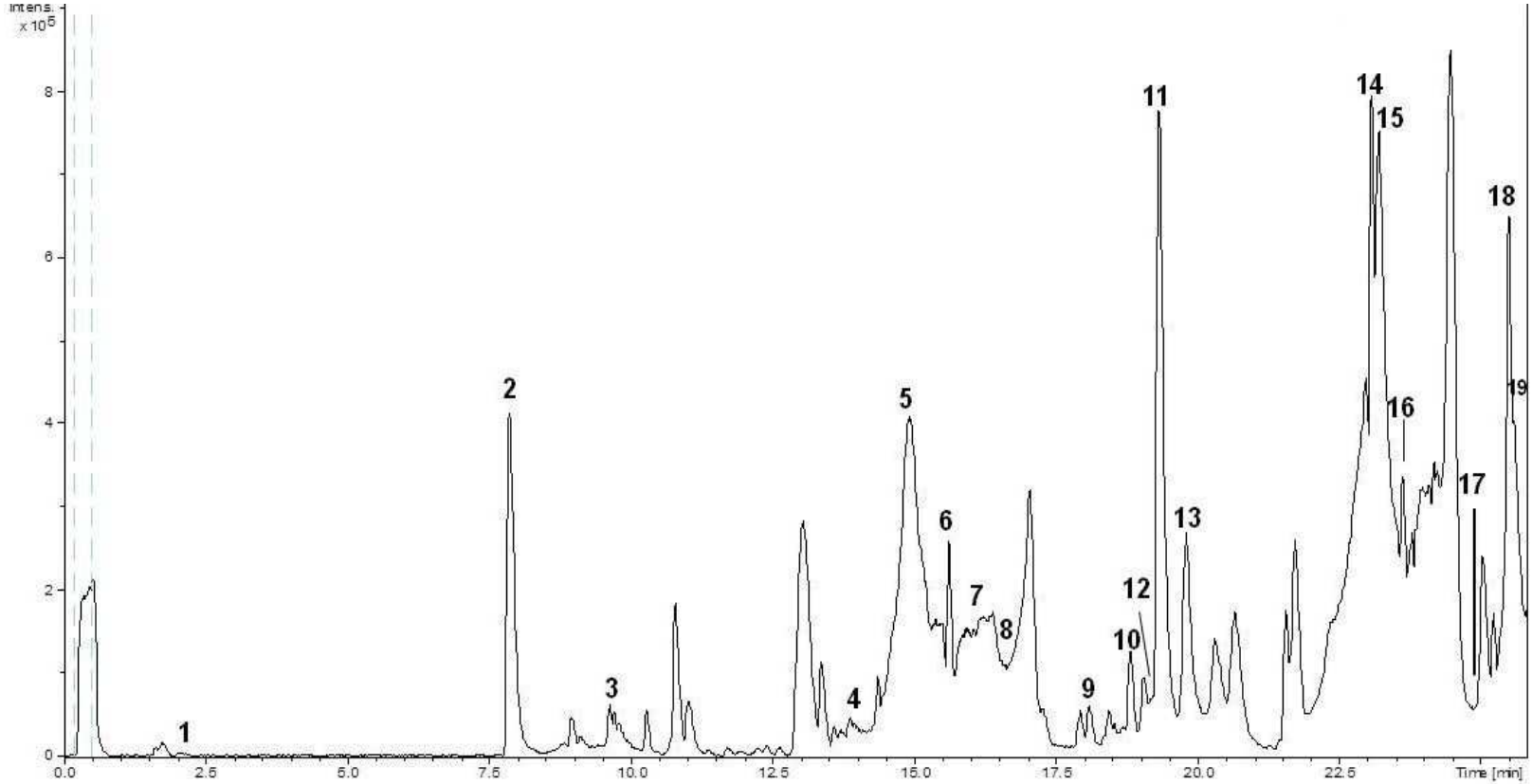

Figure 5.1. Base-peak chromatogram (BPC) of a representative Grappolo (Dom Pedrito, RS, 2011 crop) EVOO polar extract, obtained by RRLC-ESI-TOF-MS. Peak identification is the same as provided in **Table 5.2**.

Among the phenolic alcohols, it was possible to identify hydroxytyrosol and tyrosol, as well as a hydroxytyrosol derivative known as hydroxytyrosol acetate. From the secoiridoid group, oleuropein and ligstroside aglycones were identified, as well as their decarboxymethylated and hydroxylated derivatives (decarboxymethyl oleuropein aglycone, decarboxymethyl ligstroside aglycone, hydroxy decarboxymethyl oleuropein aglycone, hydroxy decarboxymethyl ligstroside aglycone and 10-hydroxy oleuropein aglycone). Elenolic acid and its hydroxylated derivative, hydroxy elenolic acid, were also detected in the EVOO samples. Regarding the lignans, (+)-pinoresinol and its derivatives, hydroxypinoresinol and acetoxypinoresinol, were identified. Moreover, the compound syringaresinol, another lignan, was identified in all the EVOO samples. The flavones luteolin and apigenin, belonging to the flavonoids group, were present in all the EVOO samples analyzed in this study. It was also possible to identify another polar compound, quinic acid, in a large number of Brazilian EVOOs.

5.3.3. Phenolic and other polar compounds quantification in Brazilian EVOO samples

Quantification of the phenolic and other polar compounds was carried out by RRLC-ESI-TOF-MS, using internal calibration with dihydrocaffeic acid (DCA) as the internal standard (IS). Quinic acid, hydroxytyrosol, tyrosol, (+)-pinoresinol, luteolin and apigenin were quantified by their own analytical curves, obtained from their commercial standards. Other phenolic compounds tentatively identified in the samples and which do not present commercial standards available had their

content estimated by using the analytical curves of the compounds with similar chemical structure. Hydroxytyrosol acetate content was estimated by using the analytical curve for hydroxytyrosol, while the analytical curve for (+)-pinoresinol was employed to estimate the concentrations of the other lignans (acetoxypinoresinol, syringaresinol and hydroxypinoresinol). Oleuropein analytical curve was used to estimate the contents of all secoiridoids (elenolic acid, hydroxy elenolic acid, decarboxymethyl oleuropein aglycone, hydroxy decarboxymethyl oleuropein aglycone, decarboxymethyl ligstroside aglycone, hydroxy decarboxymethyl ligstroside aglycone, 10-hydroxy oleuropein aglycone, oleuropein aglycone, ligstroside aglycone and methyl oleuropein aglycone).

Quantitative results for the individual phenolic and other polar compounds of the Brazilian EVOOs are summarized in **Table 5.3**.

Table 5.3. Phenolic compound contents in Brazilian extra-virgin olive oil samples (mean ± standard deviation, n = 3).

	Arbequina						Manzanilla
	Dom Pedrito, RS 2011	Pelotas, RS 2011	Pelotas, RS 2012	Chapecó, SC 2011	Chapecó, SC 2012	Maria da Fé, MG 2012	Dom Pedrito, RS 2011
Quinic acid	0.2 ± 0.1	0.02 ± 0.01	NQ [b]	NQ	0.03 ± 0.01	0.10 ± 0.02	0.05 ± 0.01
Hydroxytyrosol	1.4 ± 0.1	1.6 ± 0.1	0.48 ± 0.03	0.20 ± 0.01	0.33 ± 0.01	1.5 ± 0.1	3.0 ± 0.1
Tyrosol	2.2 ± 0.2	1.5 ± 0.2	2.4 ± 0.2	ND	NQ	1.2 ± 0.1	5.4 ± 0.4
Hydroxytyrosol acetate	1.8 ± 0.1	1.6 ± 0.1	0.75 ± 0.04	0.26 ± 0.01	0.45 ± 0.01	1.29 ± 0.05	0.74 ± 0.02
Elenolic acid	8 ± 1	6 ± 2	1.8 ± 0.1	0.27 ± 0.03	0.13 ± 0.04	1.40 ± 0.1	2.4 ± 0.2
Hydroxy elenolic acid	0.76 ± 0.03	2.7 ± 0.2	0.08 ± 0.01	0.10 ± 0.01	NQ	0.03 ± 0.01	0.27 ± 0.02
Decarboxymethyl oleuropein aglycone	14 ± 1	20 ± 3	0.06 ± 0.01	ND	NQ	29 ± 1	0.61 ± 0.04
Hydroxy decarboxymethyl oleuropein aglycone	2.7 ± 0.1	15 ± 2	NQ	ND	NQ	0.96 ± 0.04	0.11 ± 0.01
Syringaresinol	1.17 ± 0.05	1.2 ± 0.1	1.5 ± 0.1	2.0 ± 0.1	2.5 ± 0.4	1.8 ± 0.1	1.08 ± 0.05
(+)-Pinoresinol	1.7 ± 0.1	1.6 ± 0.2	3.0 ± 0.4	4.0 ± 0.3	3.3 ± 0.4	3.8 ± 0.1	1.35 ± 0.05
Acetoxypinoresinol	11.4 ± 0.5	10 ± 1	16 ± 2	27 ± 2	21 ± 4	11 ± 1	8.4 ± 0.4
Decarboxymethyl ligstroside aglycone	1.4 ± 0.1	1.6 ± 0.1	0.24 ± 0.03	ND	NQ	0.57 ± 0.04	0.91 ± 0.02
Hydroxy decarboxymethyl ligstroside aglycone	5 ± 1	10 ± 2	0.09 ± 0.01	ND	ND	ND	1.2 ± 0.1
10-Hydroxy oleuropein aglycone	0.09 ± 0.01	0.5 ± 0.1	ND	ND	NQ	ND	2.2 ± 0.1
Oleuropein aglycone	1.7 ± 0.1	2.9 ± 0.3	1.0 ± 0.1	ND	0.5 ± 0.1	2.5 ± 0.1	22 ± 1
Luteolin	4.6 ± 0.2	6.0 ± 0.4	10 ± 2	2.4 ± 0.3	6.8 ± 0.5	11 ± 1	4.29 ± 0.02
Hydroxypinoresinol	0.51 ± 0.02	0.43 ± 0.04	0.57 ± 0.05	0.32 ± 0.03	1.2 ± 0.1	0.36 ± 0.02	0.46 ± 0.02
Ligstroside aglycone	0.27 ± 0.02	0.4 ± 0.1	0.23 ± 0.03	ND	0.10 ± 0.03	0.12 ± 0.01	1.9 ± 0.1
Apigenin	0.30 ± 0.01	0.44 ± 0.03	0.8 ± 0.1	0.22 ± 0.02	0.88 ± 0.05	0.8 ± 0.1	0.35 ± 0.01
Methyl oleuropein aglycone	ND [a]	ND	NQ	ND	ND	ND	ND

	Koroneiki						Coratina
	Dom Pedrito, RS 2011	Pelotas, RS 2011	Pelotas, RS 2012	Chapecó, SC 2011	Chapecó, SC 2012	Maria da Fé, MG 2012	Pelotas, RS 2012
Quinic acid	0.19 ± 0.02	0.41 ± 0.03	NQ	0.13 ± 0.01	0.03 ± 0.01	NQ	0.08 ± 0.01
Hydroxytyrosol	0.43 ± 0.02	0.62 ± 0.04	1.2 ± 0.1	4.9 ± 0.1	3.3 ± 0.2	4.6 ± 0.2	12.3 ± 0.5
Tyrosol	10 ± 1	4.7 ± 0.5	4.3 ± 0.2	4.2 ± 0.3	2.1 ± 0.3	3.1 ± 0.2	10.5 ± 0.3
Hydroxytyrosol acetate	0.26 ± 0.01	ND	0.47 ± 0.01	0.39 ± 0.02	1.0 ± 0.1	ND	ND
Elenolic acid	7 ± 1	10 ± 2	26 ± 2	2.9 ± 0.1	6 ± 1	45 ± 2	37 ± 3
Hydroxy elenolic acid	0.58 ± 0.04	1.6 ± 0.2	0.39 ± 0.03	0.7 ± 0.1	0.63 ± 0.03	0.80 ± 0.04	1.0 ± 0.1
Decarboxymethyl oleuropein aglycone	0.05 ± 0.01	0.11 ± 0.02	2.7 ± 0.2	NQ	33 ± 6	28 ± 1	91 ± 3
Hydroxy decarboxymethyl oleuropein aglycone	NQ	NQ	0.09 ± 0.01	0.09 ± 0.01	2.9 ± 0.3	1.03 ± 0.05	4.6 ± 0.4
Syringaresinol	0.77 ± 0.04	0.7 ± 0.1	1.5 ± 0.1	1.29 ± 0.02	0.9 ± 0.1	0.47 ± 0.01	0.6 ± 0.1
(+)-Pinoresinol	1.1 ± 0.1	0.7 ± 0.1	1.7 ± 0.1	2.47 ± 0.05	1.2 ± 0.1	2.1 ± 0.1	0.9 ± 0.1
Acetoxypinoresinol	6.0 ± 0.5	3.8 ± 0.3	12 ± 1	17 ± 1	5.4 ± 0.4	6.4 ± 0.3	4.7 ± 0.3
Decarboxymethyl ligstroside aglycone	1.4 ± 0.2	1.5 ± 0.2	2.6 ± 0.3	NQ	1.9 ± 0.2	0.98 ± 0.03	39 ± 3
Hydroxy decarboxymethyl ligstroside aglycone	1.8 ± 0.1	8 ± 2	0.8 ± 0.1	0.09 ± 0.01	1.5 ± 0.1	0.34 ± 0.02	6 ± 1
10-Hydroxy oleuropein aglycone	0.07 ± 0.01	0.5 ± 0.1	0.69 ± 0.04	7 ± 1	5.5 ± 0.8	2.04 ± 0.05	2.7 ± 0.3
Oleuropein aglycone	0.8 ± 0.1	1.7 ± 0.1	40 ± 3	12 ± 1	50 ± 4	54 ± 2	115 ± 9
Luteolin	5 ± 1	5 ± 1	10 ± 2	5 ± 1	4 ± 1	8.8 ± 0.5	5 ± 1
Hydroxypinoresinol	0.07 ± 0.01	ND	ND	ND	ND	ND	ND
Ligstroside aglycone	1.6 ± 0.2	1.9 ± 0.2	2.6 ± 0.3	1.2 ± 0.1	2.4 ± 0.2	1.3 ± 0.1	35 ± 3
Apigenin	0.6 ± 0.1	0.7 ± 0.1	1.7 ± 0.2	0.7 ± 0.2	0.5 ± 0.1	0.49 ± 0.04	0.31 ± 0.04
Methyl oleuropein aglycone	ND	ND	ND	ND	ND	ND	NQ

[a] ND, below the limit of detection.
[b] NQ, between the limit of detection and the limit of quantification.

Table 5.3. (Continued)

	Arbosana			Mixture Arbequina/Arbosana		Frantoio
	Chapecó, SC 2011	Chapecó, SC 2012	Maria da Fé, MG 2012	Chapecó, SC 2011	Chapecó, SC 2012	Pelotas, RS 2012
Quinic acid	0.9 ± 0.1	0.03 ± 0.01	0.3 ± 0.1	0.10 ± 0.01	NQ	0.03 ± 0.01
Hydroxytyrosol	0.8 ± 0.1	2.19 ± 0.05	ND	0.32 ± 0.01	3.34 ± 0.03	0.29 ± 0.01
Tyrosol	7 ± 1	4.7 ± 0.4	ND	0.89 ± 0.02	3.4 ± 0.1	2.4 ± 0.1
Hydroxytyrosol acetate	1.5 ± 0.1	2.33 ± 0.03	0.27 ± 0.01	0.61 ± 0.02	0.69 ± 0.02	0.25 ± 0.01
Elenolic acid	ND [a]	7.0 ± 0.1	0.5 ± 0.1	0.04 ± 0.01	3.1 ± 0.2	0.12 ± 0.02
Hydroxy elenolic acid	0.16 ± 0.02	0.58 ± 0.04	NQ [b]	NQ	1.1 ± 0.1	NQ
Decarboxymethyl oleuropein aglycone	ND	0.60 ± 0.05	NQ	ND	26 ± 1	ND
Hydroxy decarboxymethyl oleuropein aglycone	ND	0.03 ± 0.01	ND	ND	2.6 ± 0.1	ND
Syringaresinol	13 ± 1	2.9 ± 0.1	3.0 ± 0.2	2.3 ± 0.1	0.67 ± 0.01	1.35 ± 0.05
(+)-Pinoresinol	16.7 ± 0.5	4.0 ± 0.1	5.3 ± 0.3	7.0 ± 0.1	0.56 ± 0.01	2.5 ± 0.1
Acetoxypinoresinol	196 ± 4	29.1 ± 0.5	21 ± 1	26 ± 1	1.86 ± 0.04	31.5 ± 0.3
Decarboxymethyl ligstroside aglycone	ND	0.05 ± 0.01	NQ	ND	8.9 ± 0.5	ND
Hydroxy decarboxymethyl ligstroside aglycone	ND	0.04 ± 0.01	ND	ND	8 ± 1	NQ
10-Hydroxy oleuropein aglycone	ND	1.0 ± 0.1	ND	ND	14 ± 1	ND
Oleuropein aglycone	ND	15 ± 1	NQ	NQ	55 ± 1	NQ
Luteolin	16 ± 1	5.4 ± 0.3	4 ± 1	5.9 ± 0.1	2.7 ± 0.3	4.2 ± 0.3
Hydroxypinoresinol	ND	1.07 ± 0.01	ND	ND	ND	ND
Ligstroside aglycone	ND	2.10 ± 0.05	0.05 ± 0.01	ND	13.3 ± 0.2	0.63 ± 0.02
Apigenin	4.0 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	0.98 ± 0.05	0.21 ± 0.02	0.48 ± 0.02
Methyl oleuropein aglycone	ND	ND	NQ	ND	ND	ND

	Grappolo				MGS Mariense
	Dom Pedrito, RS 2011	Chapecó, SC 2011	Maria da Fé, MG 2012 [c]	Maria da Fé, MG 2012 [d]	Maria da Fé, MG 2012
Quinic acid	0.03 ± 0.01	1.40 ± 0.05	1.2 ± 0.1	NQ	0.09 ± 0.01
Hydroxytyrosol	4.1 ± 0.2	0.67 ± 0.03	0.53 ± 0.01	2.4 ± 0.2	2.4 ± 0.1
Tyrosol	6 ± 1	ND	6 ± 1	2.0 ± 0.3	2.9 ± 0.4
Hydroxytyrosol acetate	0.49 ± 0.02	ND	ND	ND	ND
Elenolic acid	14 ± 3	9 ± 1	7.3 ± 0.1	71 ± 5	10 ± 1
Hydroxy elenolic acid	0.80 ± 0.04	0.52 ± 0.02	0.26 ± 0.01	2.1 ± 0.1	0.06 ± 0.01
Decarboxymethyl oleuropein aglycone	1.0 ± 0.1	0.69 ± 0.03	0.22 ± 0.01	36 ± 3	8 ± 1
Hydroxy decarboxymethyl oleuropein aglycone	0.15 ± 0.01	0.04 ± 0.01	NQ	2.0 ± 0.1	0.09 ± 0.01
Syringaresinol	0.59 ± 0.03	0.78 ± 0.02	0.54 ± 0.02	0.33 ± 0.03	0.85 ± 0.04
(+)-Pinoresinol	1.0 ± 0.1	0.87 ± 0.04	2.4 ± 0.1	1.5 ± 0.2	3.4 ± 0.1
Acetoxypinoresinol	7.6 ± 0.5	3.15 ± 0.05	14 ± 1	1.4 ± 0.2	10.6 ± 0.3
Decarboxymethyl ligstroside aglycone	0.66 ± 0.04	1.5 ± 0.1	0.08 ± 0.01	2.0 ± 0.2	0.47 ± 0.03
Hydroxy decarboxymethyl ligstroside aglycone	0.67 ± 0.03	2.8 ± 0.1	0.07 ± 0.01	1.1 ± 0.1	ND
10-Hydroxy oleuropein aglycone	3.0 ± 0.1	0.33 ± 0.01	0.37 ± 0.01	12 ± 2	0.67 ± 0.04
Oleuropein aglycone	35 ± 4	1.30 ± 0.02	8.8 ± 0.4	87 ± 8	27 ± 2
Luteolin	2.2 ± 0.2	1.01 ± 0.03	8 ± 1	4 ± 1	5 ± 1
Hydroxypinoresinol	0.25 ± 0.01	0.29 ± 0.02	0.07 ± 0.01	ND	ND
Ligstroside aglycone	3.0 ± 0.1	2.9 ± 0.1	0.59 ± 0.02	3.1 ± 0.3	1.08 ± 0.04
Apigenin	0.15 ± 0.01	0.12 ± 0.01	0.52 ± 0.04	0.18 ± 0.03	0.25 ± 0.02
Methyl oleuropein aglycone	0.12 ± 0.01	ND	ND	0.04 ± 0.01	0.09 ± 0.01

[a] ND, below the limit of detection.
[b] NQ, between the limit of detection and the limit of quantification.
[c] Grappolo 575, Maria da Fé, MG, 2012 crop.
[d] Grappolo 541, Maria da Fé, MG, 2012 crop.

Siringaresinol, (+)-pinoresinol, acetoxypinoresinol, luteolin and apigenin were the only compounds quantified in all the Brazilian EVOO samples assayed in this study. The overall content, in mg kg^{-1}, for the individual phenolic or other polar compounds was in the range of NQ-1.40 for quinic acid; ND-12.15 for hydroxytyrosol; ND-10.46 for tyrosol; ND-2.33 for hydroxytyrosol acetate; ND-71.13 for elenolic acid; NQ-2.65 for hydroxy elenolic acid; ND-90.58 for decarboxymethyl oleuropein aglycone; ND-15.08 for hydroxy decarboxymethyl oleuropein aglycone; 0.33-13.02 for siringaresinol; 0.56-16.68 for (+)-pinoresinol; 1.38-195.98 for acetoxypinoresinol; ND-38.69 for decarboxymethyl ligstroside aglycone; ND-9.58 for hydroxy decarboxymethyl ligstroside aglycone; ND-14.10 for 10-hydroxy oleuropein aglycone; ND-114.94 for oleuropein aglycone; 1.01-16.10 for luteolin; ND-1.22 for hydroxypinoresinol; ND-34.80 for ligstroside aglycone; 0.12-3.95 for apigenin; ND-0.12 for methyl oleuropein aglycone. These results agree with data of a number of recent papers on phenolic compounds of EVOOs varieties cultivated in different countries as Spain, Italy, Australia and USA (Lozano-Sánchez et al., 2010; Bayram et al., 2012; Loizzo, Di Lecce, Boselli, Menichini, & Frega, 2012; Bakhouche et al., 2013). For Arbequina variety, there is more available data about phenolic compounds than for others varieties, and Brazilian Arbequina EVOOs, in general, presented lower contents of secoiridoids like elenolic acid, decarboxymethyl oleuropein aglycone and decarboxymethyl ligstroside aglycone than those from Spain, for example. Servili et al. (2004) reported that, due to the agronomic and technological aspects of olive production, that strongly affect their occurrence, the definition of the average concentration of hydrophilic phenols in

EVOO is rather difficult, and their concentration may range between 40 and 900 mg kg^{-1}.

In **Figure 5.2** the results obtained for the four main phenolic compound classes found in the EVOOs, phenolic alcohols, lignans, secoiridoids and flavonoids are presented. To build those graphs, the sum of the individual compounds that belongs to each of the evaluated classes was employed. Also, the total phenolic content was calculated by summing the contents of all the individual phenolic ompounds, with exception of quinic acid, for each sample.

EVOO from Coratina variety (Pelotas, RS, 2012 crop) showed the highest phenolic alcohols content, 22.72 mg kg^{-1}, followed by Grappolo (Dom Pedrito, RS, 2011 crop) with 10.77 mg kg^{-1} and Koroneiki (Dom Pedrito, RS, 2011 crop) with 10.50 mg kg^{-1}, as seen in **Figure 5.2-A**. The lowest phenolic alcohols contents were found in Arbequina (Chapecó, SC, 2011 and 2012 crops) and Arbosana (Maria da Fé, MG, 2012 crop).

Regarding the lignans (**Figure 5.2-B**), EVOO from Arbosana variety (Chapecó, SC, 2012 crop) contained 225.68 mg kg^{-1}, which was far beyond the ones that had been found for the other samples. The second highest value was 37.06 mg kg^{-1}, also for Arbosana variety (Chapecó, SC, 2011). The main responsible for this high lignans content of Arbosana (Chapecó, SC, 2012 crop) was the compound acetoxypinoresinol, detected in a concentration of 195.98 mg kg^{-1}. Alagna et al. (2012) observed acetoxypinoresinol contents approximately ten times higher than those of pinoresinol in a minor Italian olive variety, Dolce d'Andria. The lowest lignans contents were detected in the samples Mixture of

Arbequina/Arbosana (Chapecó, SC, 2012 crop), Grappolo 571 (Maria da Fé, MG, 2012 crop) and Grappolo (Chapecó, SC, 2011 crop).

It is possible to see in **Figure 5.2-C** that Coratina variety (Pelotas, RS, 2012 crop) showed the highest secoiridoids content, 329.84 mg kg^{-1}. Grappolo 571 (Maria da Fé, MG, 2012 crop) and Koroneiki (Maria da Fé, MG, 2012 crop) also presented high concentration of secoiridoids, 215.99 and 135.57 mg kg^{-1}, respectively. On the other hand, the secoiridoids of the samples Mixture of Arbequina/Arbosana (Chapecó, SC, 2011 crop), Arbosana (Chapecó, SC, 2011 crop), Arbequina (Chapecó, SC, 2011 and 2012 crops), Arbosana (Maria da Fé, MG, 2012 crop) and Frantoio (Pelotas, RS, 2012 crop) were present in concentrations lower than 1 mg kg^{-1}.

Flavonoid total contents are exhibited in **Figure 5.2-D**. Arbosana (Chapecó, SC, 2011 crop) clearly had the highest flavonoids concentration, 20.04 mg k^{-1}. Other significant flavonoid contents were detected in Arbequina (Maria da Fé, MG, 2012 crop), 11.73 mg kg^{-1}; Koroneiki (Pelotas, RS, 2012 crop), 11.70 mg kg^{-1}; and Arbequina (Pelotas, RS, 2012 crop), 10.67 mg kg^{-1}. In opposition, samples of Grappolo (Chapecó, SC, 2011 crop) and Grappolo (Dom Pedrito, RS, 2011 crop) were the samples with the lowest flavonoid contents 1.13 and 2.31 mg kg^{-1}, respectively.

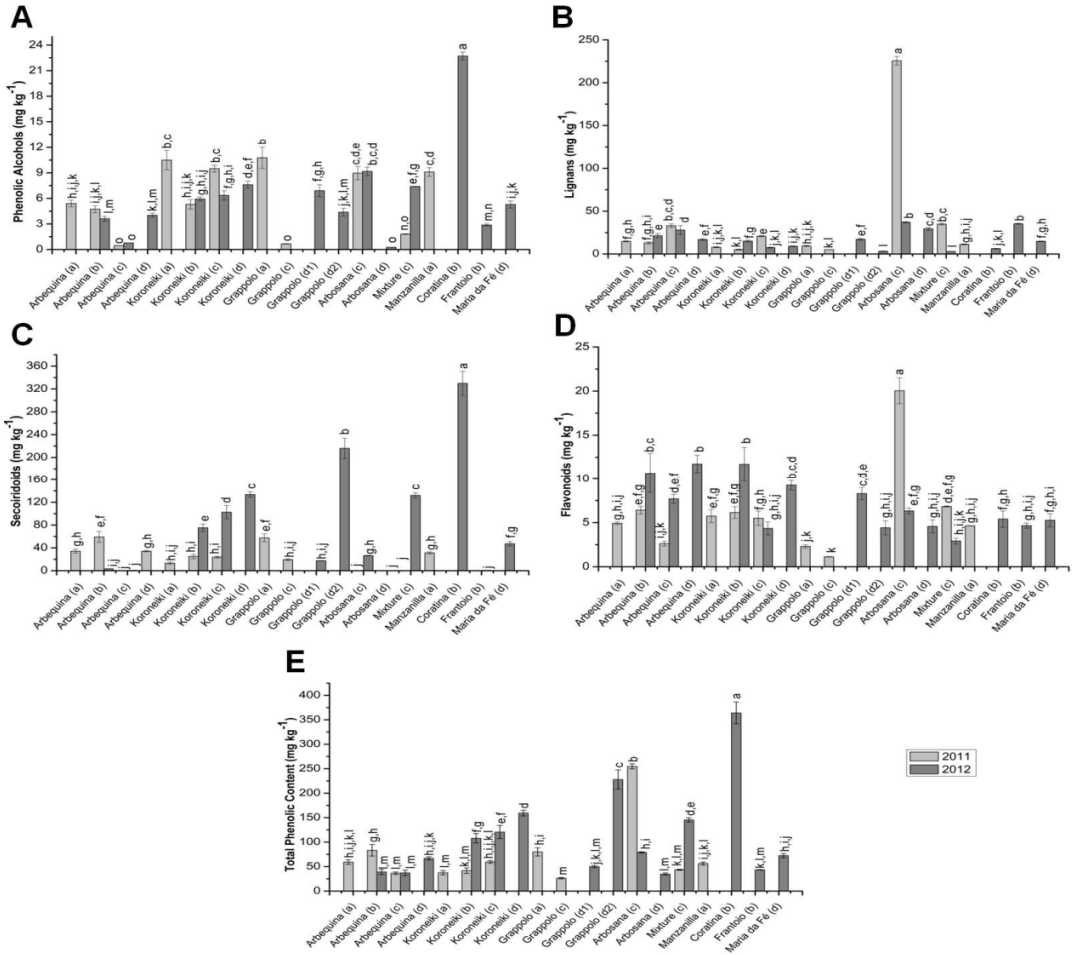

Figure 5.2. Phenolic alcohols (**A**), Lignans (**B**), Secoiridoids (**C**), Flavonoids (**D**) and Total Phenolic Contents (**E**), in mg kg^{-1}, of Brazilian extra-virgin olive oils. Columns followed by the same letters showed no significant difference ($p < 0.05$) by the Tukey test. The letter between parenthesis after the variety name in the "x" axis refer to the place of origin: (a) Dom Pedrito, RS; (b) Pelotas, RS; (c) Chapecó, SC; (d), Maria da Fé, MG; (d1) Maria da Fé, MG, Grappolo 575; (d2) Maria da Fé, MG, Grappolo 541.

It seems to exist an inverse correlation between secoiridoid and lignan contents. The samples of Arbosana (Chapecó, SC, 2011 crop) and Frantoio (Pelotas, RS, 2012 crop), which presented some of the lowest secoiridoid contents, contained some of the highest lignan concentrations. For Grappolo 571 (Maria da Fé, MG, 2012 crop), its high secoiridoids value was opposite to its low concentration of lignans. For Coratina variety (Pelotas, RS, 2012 crop), that was responsible for the highest secoiridoids value, only a few mg kg^{-1} of lignans concentration was detected.

Finally, the highest total phenolic content was found for the EVOO from Coratina variety (Pelotas, RS, 2012 crop), 364 mg kg^{-1} (**Figure 5.2-E**). In fact, Coratina EVOOs are known for their high phenolic content, that also gives a marked "bitter and pungent" taste (Gambacorta et al., 2010). Other promising Brazilian monovarietal EVOOs concerning to the total phenolic content were Arbosana (Chapecó, SC, 2011 crop), with 255 mg kg^{-1}; Grappolo 571 (Maria da Fé, MG, 2012 crop), with 228 mg kg^{-1}; Koroneiki (Maria da Fé, MG, 2012 crop), with 159 mg kg^{-1}; Koroneiki (Chapecó, SC, 2012), with 121 mg kg^{-1}; and Koroneiki (Pelotas, RS, 2012), with 108 mg kg^{-1}. In this case, each Brazilian state has at least two varieties capable to produce an EVOO with appreciable contents of phenolic compounds. However, as the EVOO production in Brazil is still done in an experimental scale, phenolic compound contents observed in this study showed great variation between crops, and new studies will be necessary to reach a better control of agronomical conditions that affects phenolic compound contents, and also when the EVOO extraction migrates to the industrial scale in the future.

5.3.4. Chemometrics

Before applying the Principal Component Analysis (PCA) to the EVOO phenolic compound data, it was decided to execute one PCA for each Brazilian state separately, since the production and extraction conditions of the samples were not the same for the three states. In effect, when the PCA was performed with all the samples at the same time, the only samples that classified separately were the three with the highest total phenolic contents, Coratina (Pelotas, RS, 2012 crop), Arbosana (Chapecó, SC, 2011 crop) and Grappolo 571 (Maria da Fé, MG, 2012 crop). In this case, useful information obtained from the PCA was limited and difficult to be interpreted.

Thus, three data matrix [(*I,J*), where *I* is the number of lines and *J* is the number of columns] for PCA were constructed, using all the replicates as the samples and 17 variables (phenolic compounds contents, in mg kg^{-1}): RS matrix (*30,17*); SC matrix (*27,17*); and MG matrix (*18,17*). Even though 20 phenolic or other polar compounds were identified, the compounds quinic acid, hydroxypinoresinol and methyl oleuropein aglycone were not included in the PCA analysis, since quinic acid is not considered a phenolic compound and hydroxypinoresinol and methyl oleuropein aglycone were not detected in most of the samples. When the sample presented values below the limit of quantification but above the limit of detection, the value of the limit of quantification (calculated in mg kg^{-1} of sample) was used in the matrix, while in those cases where the sample presented values below the limit of detection, the limit of detection itself (also

calculated in mg kg^{-1} of sample) was used in the matrix for the PCA. The results of the PCA for each Brazilian state are presented in **Figure 5.3**.

For RS state PCA, seven principal components were used into the model, which explained 97.7% of the variance. The score and loading plots for the two first principal components (PCs) are showed in the **Figures 5.3a** and **5.3b**, respectively. With these two PCs, 64.2% of variance was explained, and it was possible to visualize a clear separation between almost all the samples, with the exception of Arbequina (Pelotas, 2012 crop) and Frantoio (Pelotas, 2012 crop) as well as Manzanilla (Dom Pedrito, 2011 crop) and Koroneiki (Dom Pedrito, 2011 crop). The phenolic composition of these two sets of samples resulted similar, and then the samples were grouped together. Coratina (Pelotas, 2012 crop) was separated by its correlation with high contents of some secoiridoids and phenolic alcohols, while Arbequina (Pelotas, 2011 crop) was grouped separately by its high hydroxylated derivatives of decarboxymethyl oleuropein aglycone, elenolic acid and decarboxymethyl ligstroside aglycone. The other samples were more correlated to high contents of lignans and flavonoids, being pushed to the left and below in the scores graph. PCA results also reassure the significant difference in the phenolic contents between the two crops, considering the same variety in the same city, since these samples did not group together.

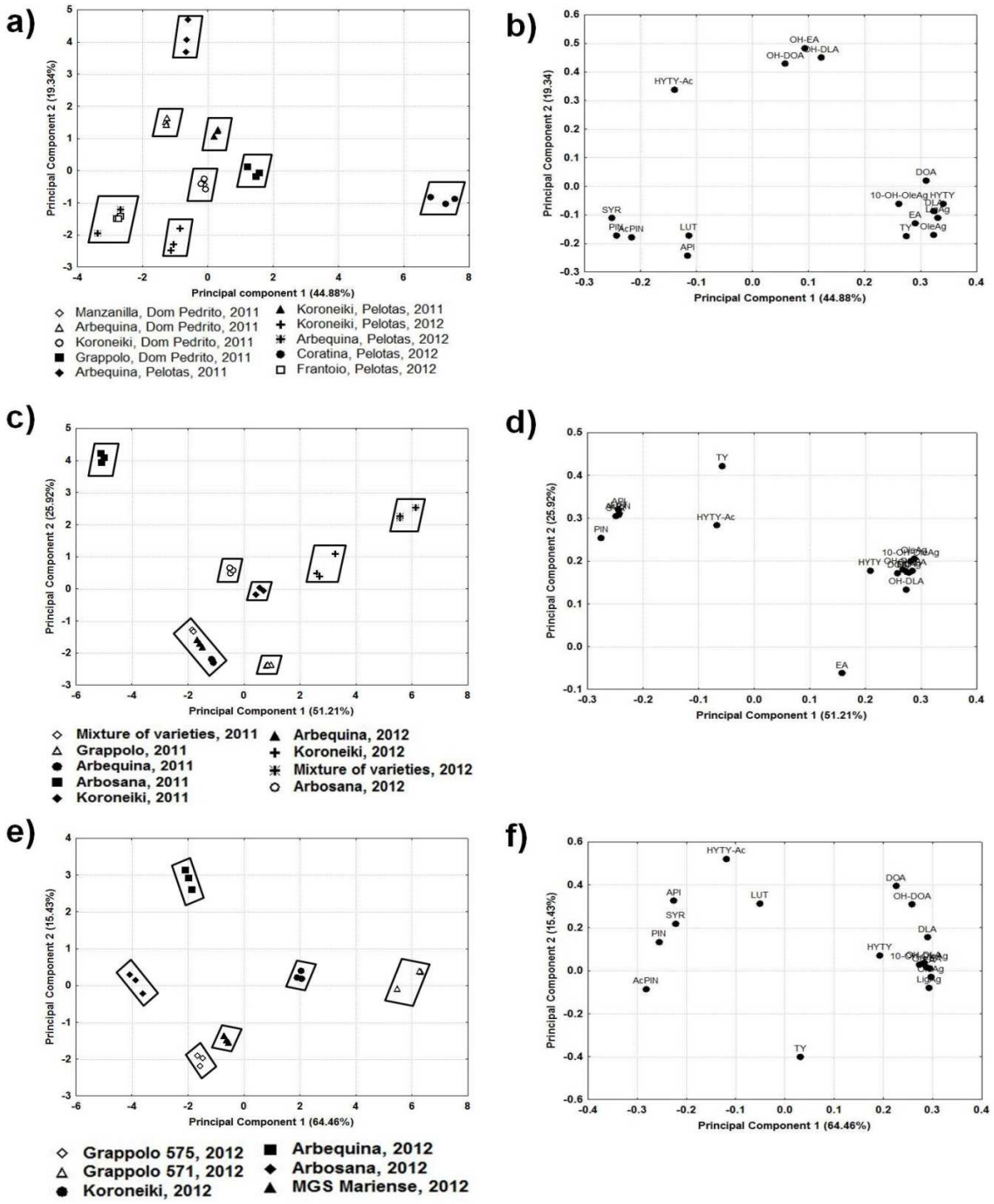

Figure 5.3. Results of the Principal Component Analysis, showing the first and the second principal components for the responses values. **a)** scores graph, Rio Grande do Sul state; **b)** loadings graph, Rio Grande do Sul state; **c)** scores graph, Santa Catarina state; **d)** Loadings graph, Santa Catarina state; **e)** scores graph, Minas Gerais state; **f)** loadings graph, Minas Gerais state.

Scores and loadings graphs for the PCA of SC state can be seen in **Figures 5.3c** and **5.3d**, respectively. The model used was constructed with seven PCs, explaining 99.7% of the variance. For the two first PCs, used to plot the score and loading graphs, the explained variance was of 77.1%. It was possible to visualize that almost all the samples presented an unique phenolic compounds profile, since they grouped separately from each other, with exception of Arbequina (Chapecó, 2011 and 2012 crops) and the Mixture of varieties (Chapecó, 2011 crop), that were located very close to each other in the scores graph. This behavior can be explained since these samples did not present or present low contents of tyrosol, and they are inversely correlated to the tyrosol concentration, as can be seen in the loadings graph, where tyrosol is located in the region of higher loading values. Also, the absence of tyrosol and the high content of elenolic acid were the responsible for Grappolo (Chapecó, 2011 crop) position in the scores graph. Arbosana (Chapecó, 2011 crop) was classified separately by its high lignans and low secoiridoids contents, while the Mixture of varieties can be differentiated by its high secoiridoids and low lignans contents. Here it was also observed a significant difference between the two crops, as it has happened to the RS samples.

Considering the PCA results for MG state (**Figures 5.3e** and **5.3f**), it was possible to see that all the samples classified separately. The model was composed by five PCs, explaining 99.5% of data variance. PCs one and two were responsible for 79.9% of variance explanation. Main results observed from the score and loading graphs are that Grappolo 575 (Maria da Fé, 2012 crop) and MGS Mariense (Maria da Fé, 2012 crop) were positioned very close, meaning that

they possess a similar phenolic compounds profile. Grappolo 571 (Maria da Fé, 2012 crop) was very different from the other MG EVOOs thanks to its high secoiridoids content, while Arbosana (Maria da Fé, 2012 crop) can be recognized by its high lignans concentration. Arbequina (Maria da Fé, 2012) was characterized by a high flavonoids concentration in opposition to low levels of almost all the other phenolics.

5.4. Conclusions

Rapid-resolution liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (RRLC-ESI-TOF-MS) was successfully employed in the first characterization of the Brazilian EVOO phenolic compounds. It was possible to detect and quantify or estimate the concentrations of 20 phenolics belonging to the main class of those compounds already described for EVOO samples. Coratina variety, cultivated in Rio Grande do Sul state, showed the highest phenolic compounds content, comparable to high-quality EVOOs from countries with more experience in its production.

In general, Brazilian EVOOs presented phenolic profiles significantly different, as well as between the two crops assayed in this study. Even though some variation is expected, since phenolic compounds are affected by a large number of edapho-climatic and extraction conditions, it is possible that this fluctuation can also be related to the fact that both olives and EVOO production in Brazil are still experimental and need to be improved.

In this way, data generated by this study will be very useful, helping the agronomists in their work towards the obtainment of high-quality Brazilian genuine EVOO. Moreover, the results presented here for the Brazilian EVOO phenolic composition will be available to all those researchers involved in the EVOO chemical characterization in different parts of the world, considering that its production is spreading to several new places and the work into EVOO phenolics characterization is still far from conclusion.

5.5. Acknowledgments

We thank FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo (process no. 12/04523-5) and CNPq - Coordenação Nacional de Desenvolvimento Científico e Tecnológico, for their financial support for this study. The author C. A. Ballus also would like to thank CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES Foundation, Brazil), for having granted him a PSDE scholarship (process no. 1580-12-4).

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CONCLUSÃO GERAL

Após a avaliação do teor de compostos fenólicos totais e da capacidade antioxidante total de 45 amostras de EVOO comerciais, distribuídas por 15 marcas, com três lotes analisados de cada uma delas, foi possível concluir que muitas delas são comparáveis aos EVOOs de outras partes do mundo em relação a estes parâmetros analíticos. Houve correlação entre o teor de compostos fenólicos totais e os quatro métodos de capacidade antioxidante utilizados neste trabalho, indicando que quanto maior o teor de compostos fenólicos, maior será a capacidade antioxidante do EVOO, independente do método utilizado para realizar as medições.

O emprego de técnicas estatísticas multivariadas permitiu atingir a separação de 17 compostos fenólicos previamente descritos em amostras de EVOO por eletroforese capilar com detector de arranjo de diodos. Foi a primeira vez que um número elevado de respostas, 37 ao total, foram avaliadas simultaneamente, com o objetivo de alcançar a maior resolução entre os pares de picos que sofreram coeluição nas diferentes condições experimentais, bem como o menor tempo de corrida e o menor coeficiente de variação nos tempos de migração dos compostos fenólicos. O método foi validado e aplicado com sucesso a 15 marcas comerciais de EVOOs, fornecendo os primeiros dados a respeito do teor de compostos fenólicos de alguns dos azeites de oliva consumidos pelos brasileiros.

De posse dos resultados para as análises de compostos fenólicos, tocoferóis e ácidos graxos em amostras de EVOO produzidas no Brasil, mais precisamente no estado de Minas Gerais, pode-se afirmar que muitas das variedades de oliveiras cultivadas neste Estado são bastante promissoras e resultaram em EVOOs de excelente qualidade, quando comparados aos azeites de oliva produzidos em países com uma longa e comprovada experiência neste assunto. Foi detectada uma grande variação entre a composição química dos EVOOs provenientes de duas colheitas diferentes, sendo que novos estudos serão necessários para que os agrônomos envolvidos no cultivo das oliveiras e na extração do azeite de oliva possam definir e estabelecer as melhores condições agronômicas e tecnológicas envolvidas na produção do EVOO de Minas Gerais.

A cromatografia líquida de rápida resolução acoplada à espectrometria de massas foi aplicada com sucesso na identificação e quantificação de aproximadamente 20 compostos fenólicos em EVOOs provenientes de três estados brasileiros, Rio Grande do Sul, Santa Catarina e Minas Gerais. Este trabalho foi o primeiro a determinar o teor de todas as principais classes de compostos fenólicos do EVOO, alcoóis fenólicos, secoiridoides, lignanas e flavonoides, em amostras oriundas de diferentes regiões do Brasil, sendo mais um passo importante para a caracterização química deste produto que pode vir a ser muito importante para o país em um futuro não muito distante.