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GABRIEL DESCHAMPS FERNANDES

DETECTION OF SOPHISTICATED FRAUD IN OLIVE OIL USING OFFICIAL METHODOLOGIES AND DNA MOLECULAR MARKERS

DETECÇÃO DE FRAUDES SOFISTICADAS EM AZEITE DE OLIVA UTILIZANDO METODOLOGIAS OFICIAIS E MARCADORES MOLECULARES DE DNA

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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 drgree of Doctor in Food Technology.

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 Tecnologia de Alimentos.

Orientador: Prof. Dr. DANIEL BARRERA ARELLANO

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RESUMO

O azeite de oliva é a fração lipídica dos frutos da oliveira, obtida exclusivamente por processos físicos, que apresenta alto valor agregado tanto pelas suas características organolépticas quanto pelo potencial nutracêutico. O consumo de azeite de oliva cresce continuamente, tanto em países tradicionalmente consumidores quanto naqueles considerados novos mercados. O crescimento e a abrangência do mercado levaram ao desenvolvimento de legislações capazes de garantir a autenticidade e a qualidade do produto comercializado. No Brasil, terceiro maior importador mundial de azeite de oliva, a recente legislação inclui parâmetros de qualidade e identidade, que classificam o azeite de oliva em distintas categorias e permitem a detecção de fraudes. Entretanto, ao mesmo tempo em que se avança no desenvolvimento de ferramentas capazes de detectar as fraudes, novas modalidades de adulteração vão aparecendo, como as chamadas fraudes sofisticadas, que são de difícil detecção. Dentre elas, destacam-se a adição de óleos de amêndoa doce, avelã, noz pecan e de azeite de abacate. Assim, este trabalho teve por objetivo desenvolver ferramentas para a detecção de fraudes sofisticadas em azeite de oliva. Para tanto, as fontes de adulteração foram caracterizadas quimicamente, por meio de métodos oficiais e reconhecidos pelo Comitê Oleícola Internacional. Esta caraterização teve como principais objetivos aprimorar o conhecimento quimico destas fontes de fraudes e buscar marcadores químicos para detecção das fraudes. Porém, quimicamente as fontes de fraudes são muito semelhantes ao azeite de oliva não sendo possível estabelecer marcadores químicos viáveis para a detecção destas fraudes. Em uma segunda etapa deste trabalho buscou-se desenvolver ferramentas baseadas em marcadores moleculares de DNA, capazes de detectar as fraudes. Utilizou-se PCR em tempo real como ferramenta de analítica o que possibilitou a detecção de cerca de 5% de adição de óleos de amêndoa doce, avelã, noz pecan e de azeite de abacate ao azeite de oliva. Esta abordagem de detecção de fraudes sofisticadas foi uma das principais contribuições do trabalho.

Palavras-chaves: Azeite de Oliva, Fraude, Marcadores moleculares.

ABSTRACT

Olive oil is the lipid fraction of the fruit of the olive tree, obtained exclusively by physical processes, with high added value both for its organoleptic characteristics as the nutraceutical potential. Olive oil consumption is raising continuously, both in traditional countries as those new markets. The growth and breadth of the market led to the development of legislation capable of guaranteeing the authenticity and quality of the product. In Brazil, world's third largest importer of olive oil, recent legislation includes quality and identity parameters that classify olive oil in different categories and allow detection of fraud. However, while it advances in the development of tools capable of detecting fraud, new forms of adulteration are appearing, such as the sophisticate frauds, which are very difficult to detect. Among them, include the addition of sweet almond oil, hazelnut, pecan nut and avocado oil. This work aimed to develop tools for the detection of sophisticated fraud in olive oil. Therefore, the sources of adulterations were chemically characterized, through official and recognized methods. This characterization has as main goal to improve the chemical knowledge of these frauds sources and seek chemical markers for the detection of fraud. However, chemically fraud sources are very similar to olive oil is not possible to establish viable chemical markers for detecting such fraud. In a second stage of this study we sought to develop tools based on molecular markers of DNA capable of detecting fraud. It was used real-time PCR as an analytical tool which allowed detection of about 5% addition of sweet almond, hazelnut, pecan and avocado oils to olive oil. This detection approach of sophisticated fraud was one of the main contributions.

Keywords: Olive Oil, Fraud, Molecular Markers.

SUMÁRIO

INTRODUÇÃO GERAL

INTRODUÇÃO GERAL

O consumo de azeite de oliva cresce constantemente, não apenas em países tradicionalmente consumidores, mas também em mercados emergentes, como o Brasil, onde o conhecimento sobre este alimento é escasso e a qualidade e pureza dos azeites pode ser questionadas (FRANKEL, et al., 2011; OLIVA, 2012; INMETRO, 2015).

Atualmente, o Brasil é o terceiro maior importador mundial de azeite de oliva, atrás apenas dos Estados Unidos e da União Européia (UE). Nas safras de 2013/14 foram importadas 72,5 mil toneladas, enquanto que em 2014/15 a cifra alcançou 66,5 mil toneladas de azeite. Embora o Brasil seja um dos principais mercados importadores de azeites fora da Europa, o consumo per capita, cerca de 0,2 kg/ano, ainda é muito baixo quando comparado a países como a Espanha e a Itália, com consumo de 23 kg/habitante/ano (OLIVA, 2012). Pequenas produções nos estados do Rio Grande do Sul e Minas Gerais têm contribuído para o início do cultivo nacional de oliveiras e da produção de azeite de oliva. Seja importado ou nacional, este produto apresenta um elevado preço no mercado, em parte justificado pelos custos de produção, somados a uma alta qualidade organoléptica e nutracêutica.

Para ampliar ganhos econômicos são introduzidas adulterações que consistem em misturar ao azeite de oliva, outros tipos de óleos, gorduras e/ou azeites de menor qualidade e custo. Atualmente, este problema deixa de ser simplesmente um risco potencial para ser uma realidade, sobretudo nos países importadores de azeite de oliva e produtores de outros tipos de óleos. Embora à primeira vista possa parecer simplesmente um problema de fraude econômica, há implicações à saúde, uma vez que nas misturas podem ser

utilizados óleos capazes de provocar reações alérgicas, como é o caso do óleo de avelã (BREMER; SMITS, HAASNOOT, 2009). Também se produzem fraudes pela adição de azeites de oliva de baixa qualidade submetidos a determinados processos de purificação (“refino suave”), resultando em um produto comercializado com menor qualidade que a esperada. Este procedimento é extremamente prejudicial para os mercados emergentes de azeite de oliva, uma vez que os novos consumidores adquirem o azeite por seus benefícios à saúde e suas qualidades organolépticas, recebendo, entretanto, um produto que não lhes proporciona os benefícios esperados. Em países produtores e que também são grandes consumidores (Espanha, Itália e Grécia), a adulteração geralmente é realizada com óleo de bagaço ou azeites de oliva refinados ou ainda outros suavemente desodorizados, além da adição de óleos de avelã e amêndoas. Em outros países, as adulterações incluem óleos de menor valor, como algodão, soja, colza/canola e girassol, de acordo com a disponibilidade e produtividade de cada país.

Para garantir a autenticidade do azeite de oliva é necessário que os organismos nacionais e internacionais, envolvidos na comercialização e fiscalização, regulamentem adequadamente a qualidade e a pureza do azeite de oliva. As regulamentações devem ser uniformes e oferecer definições claras das distintas categorias de azeite de oliva, além de metodologias e técnicas instrumentais precisas, exigindo altos níveis de controle para os exportadores. Em 2012, o Brasil deu um importante passo para o combate das fraudes, lançando a Instrução Normativa 001/2012 do Ministério da Agricultura, Pecuária e Abastecimento (MAPA), que visa classificar e fiscalizar o azeite de oliva importado e/ou produzido no Brasil. O Conselho Oleícola Internacional

(COI), um organismo das Nações Unidas que reúne os países produtores de azeite de oliva, bem como a Comissão da União Européia (UE), adotam em seus regulamentos oficiais métodos analíticos e valores para determinados parâmetros físico-químicos que permitem detectar a maioria das fraudes. Entretanto, a ampla disponibilidade de fontes lipídicas e a falta de conhecimento difundido quanto à composição química destas fontes, juntamente com melhorias nos processos industriais de obtenção e refino, têm possibilitado misturas fraudulentas mais complexas e de difícil detecção, chamadas de fraudes sofisticadas. Estas, em geral, são adulterações não detectáveis quando os níveis de inclusão estão abaixo de 30%. Dentre as principais fontes de fraudes sofisticadas em azeite de oliva destacam-se os óleos de avelã, amêndoa, nozes e azeite de abacate.

Mesmo com o intenso trabalho de combate a fraudes por parte dos organismos regulatórios, aliado a novas ferramentas de detecção desenvolvidas pela academia e instituições de pesquisa, a divulgação de escândalos envolvendo fraudes em azeite de oliva é constante (PROTESTE, 2013). O periódico *The Olive Oil Times* é um dos pioneiros na divulgação em massa destes eventos (The Olive Oil Times, http://www.oliveoiltimes.com/). A farmacopéia americana mantém livre acesso a um centro de registro de fraudes em alimentos, incluindo azeite de oliva (USP, 2016).

Nos últimos anos, a incorporação de novos métodos analíticos às regulamentações internacionais, como o método global de análise de triacilgliceróis, tem permitido combater grande parte das adulterações em azeite de oliva (IOC, 2013). Além disso, técnicas baseadas em marcadores moleculares de DNA têm sido desenvolvidas com o intuito de abranger,

principalmente, as fraudes que não são detectadas pelos métodos químicos. Alguns trabalhos têm também direcionando uso de marcadores moleculares de DNA para a detecção e certificação de azeites monovarietais (MONTEALEGRE, 2010).

O presente trabalho tem por objetivo desenvolver ferramentas baseadas em marcadores moleculares de DNA para a detecção de fraudes sofisticadas em azeite de oliva, adicionado de óleos de amêndoa doce, avelã e noz pecan e de azeite de abacate.

Para tanto, foi realizada uma revisão bibliográfica sobre o assunto e o trabalho prático-teórico foi dividido em duas etapas. A primeira corresponde à caracterização química dos óleos e azeites fontes de fraude, compreendendo dois artigos: Artigo 1 - *New Approach on Nut Oils Characterization: Almond, Hazelnut and Pecan Nut* e Artigo 2 - *Chemical Characterization of Monovarietal and Commercial Avocado Oils*. A segunda etapa também foi dividida em dois artigos e está relacionada ao estabelecimento de metodologias baseadas na análise de marcadores moleculares de DNA para a detecção das fraudes em questão, compreendendo o Artigo 3 - *Application of DNA markers on the detection of fraud in virgin olive oil: Sweet Almond, Hazelut, Pecan Nut presence* e o Artigo 4 - *Virgin olive oil adulteration with avocado oil: new challenge for DNA markers*. Por fim, apresenta-se uma discussão geral que relaciona os resultados obtidos nos quatro artigos com o objetivo central do trabalho que é a detecção de fraudes em azeite de oliva, encerrando efetivamente com as principais conclusões obtidas ao logo desta tese.

REVISÃO BIBLIOGRÁFICA

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1. AZEITE DE OLIVA

O azeite de oliva consiste na fração lipídica obtida diretamente das azeitonas, frutos da planta *Olea europaea* L., família *Oleaceae*, mediante a utilização exclusiva de processos físicos como pressão, centrifugação, lavagem, decantação e filtração, em condições térmicas controladas, que não ocasionem quaisquer alterações ao azeite (BRASIL, 2012). O azeite de oliva apresenta uma série de características e componentes que o diferenciam do restante dos óleos e gorduras vegetais comestíveis. Dentre as principais diferenças pode-se citar a alta porcentagem de ácidos graxos monoinsaturados, como o oléico, e a presença de compostos fenólicos característicos, como tirosol e hidroxitirosol. Esta composição química confere ao azeite de oliva propriedades nutricionais benéficas à saúde dos consumidores, principalmente relacionadas com a prevenção de doenças cardiovasculares (VARELA, RUIZ-ROSO, 2000).

Em 2012, o MAPA publicou a primeira Instrução Normativa (IN) que regulamenta a classificação do azeite de oliva no Brasil. A IN 01/2012 está baseada em regulamentações internacionais como as do Comitê Oleícola Internacional (COI), da União Européia (UE) e do *Codex Alimentarius* (CODEX) (CODEX, 1981; BRASIL, 2012; IOC, 2015). O estabelecimento desta instrução normativa foi um grande passo na regulamentação da qualidade do azeite de oliva no Brasil. Sendo o Brasil o terceiro maior importador mundial, o estabelecimento de normas de identidade e qualidade para a comercialização visa proteger o mercado consumidor, além de viabilizar de forma muito clara a

classificação dos azeites que recentemente têm sido produzidos no Brasil (FAO, 2015; IOC, 2015).

Estão descritas na IN 001/2012 do MAPA (BRASIL, 2012) as diferentes categorias de azeites de oliva disponíveis comercialmente no Brasil. A primeira instância de classificação está dividida em cinco grupos: (1) *azeite de oliva virgem*, que é obtido diretamente de azeitonas por meio de processos físicos; (2) *azeite de oliva refinado*, produzido por meio do refino de azeite de oliva virgem de baixa qualidade; (3) *óleo de bagaço de oliva refinado*, o produto obtido do bagaço de oliva (subproduto da extração de azeite de oliva virgem) por meio de solvente e, posteriormente, refinado; caso este seja adicionado de azeite de oliva virgem passa então a ser comercializado como (4) *óleo de bagaço de oliva*; e por fim o denominado apenas de (5) *azeite de oliva*, oriundo da mistura de azeite de oliva virgem com azeite de oliva refinado (FIGURA 1).

Os grupos azeite de oliva, azeite de oliva refinado, óleo de bagaço de oliva e óleo de bagaço de oliva refinado não possuem classificações subsequentes, são apenas de tipo único. Entretanto, o azeite de oliva virgem deve ser classificado, de acordo com a qualidade, em três tipos: (4) a*zeite de oliva extra virgem*, que consiste no azeite obtido de azeitonas sadias e recém colhidas, considerado o azeite de oliva de máxima qualidade; (5) a*zeite de oliva virgem*, que possui qualidade ligeiramente inferior; e (6) *azeite lampante*, de baixíssima qualidade. Os azeites de oliva extra virgem e virgem apresentam características organolépticas que permitem o consumo sem passar por nenhum processo de refino. Seu consumo torna-se cada vez mais popular, principalmente devido aos benefícios à saúde. Porém, devido à baixa qualidade, o azeite de oliva lampante necessita de um processo de refino para

ser consumido. Após o refino, o azeite de oliva lampante passa a ser classificado como azeite de oliva refinado (EFSA Panel on Dietetic Products 2011; BRASIL, 2012).

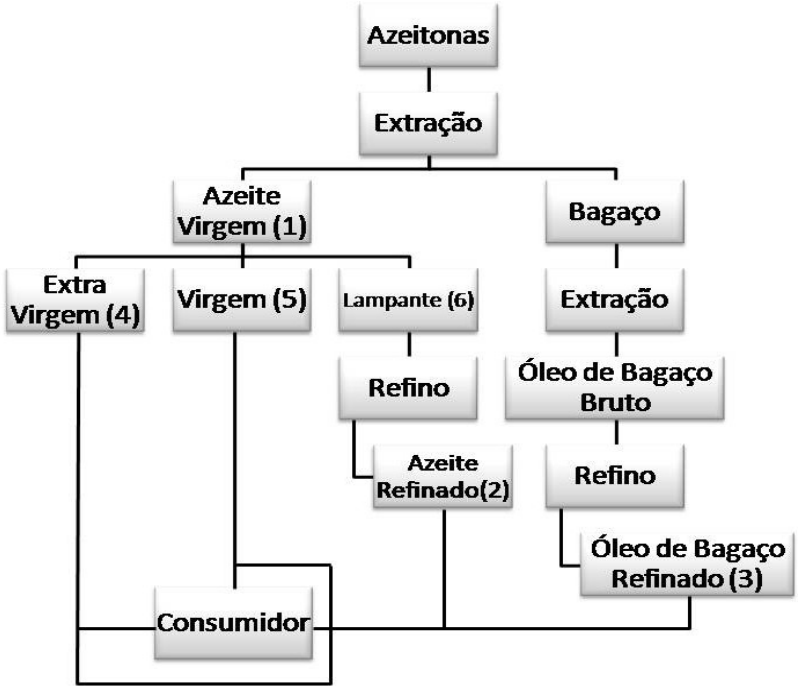

Figura 1: Fluxograma dos tipos comerciais de azeite de oliva de acordo com a IN 001/2012 do MAPA. Fonte: BRASIL (2012).

1.1. OBTENÇÃO DE AZEITE DE OLIVA

Os processos de obtenção do azeite de oliva, de maneira geral, são precedidos pela colheita e lavagem das azeitonas. Na sequencia, como o conteúdo lipídico encontra-se, basicamente, na polpa do fruto, é necessário um processo de moagem para rompimento das estruturas celulares e liberação do azeite. A extração propriamente dita pode seguir por três métodos diferentes: prensagem, percolação e centrifugação. Cabe ressaltar que, de acordo com as legislações nacional e internacionais atualmente vigentes, a obtenção do azeite

de oliva deve se dar unicamente por processos físicos, sem a utilização de solventes (IOC, 2006a; BRASIL, 2012).

1.1.1 Colheita e lavagem

A operação de colheita tem um papel muito importante no processamento, na qualidade e nas características químicas e organolépticas do azeite de oliva, sendo o período de maturação das azeitonas um dos principais fatores. Azeitonas colhidas verdes (no início do período de maturaçõ) são mais resistentes ao processo de extração devido à estrutura do fruto verde (maiores teores de pectina e lignina). Desta maneira, a extração do azeite é mais difícil e os rendimentos de extração são menores em frutos verdes, quando comparados aos rendimentos de extração de azeite a partir de frutos maduros. Sensorialmente, as azeitonas verdes são responsáveis por azeites com maiores conteúdos de clorofila e compostos fenólicos (antioxidantes). A concentração destes compostos resulta em azeites mais amargos e picantes que apresentam um frescor no consumo, com notas sensoriais de tomate fresco e grama cortada. Contudo, azeites produzidos a partir de azeitonas maduras possuem coloração dourada, são mais adocicados e com notas sensoriais amendoadas (PETRAKIS, 2006).

O sistema implantado na colheita é outro fator relevante na obtenção de azeite de oliva, pois deve manter ao máximo a integridade dos frutos, uma vez que eventuais injúrias desencadeiam processos fermentativos indesejáveis. Estes processos podem ocasionar defeitos organolépticos, que são irreversíveis e depreciam a qualidade do azeite de oliva, como por exemplo, o defeito avinagrado (APARICIO, HARWOOD, 2003).

O sistema mais tradicional de colheita é a colheita manual, mais eficiente em manter a integridade das azeitonas. Entretanto, os volumes atuais de produção exigiram o desenvolvimento de métodos mecanizados. Dentre os mais difundidos, estão o de varas vibratórias, onde utiliza-se uma haste vibratória para golpear os ramos da árvore derrubando as azeitonas que são recolhidas em uma tela disposta no solo. Outro sistema vibratório consiste de um equipamento que é conectado ao caule da oliveira e vibra toda a planta fazendo com que as azeitonas caiam e sejam recolhidas em uma tela. Atualmente, o sistema que permite maior automação consiste de uma colheitadeira mecânica empregada em sistemas de cultivo intensivo (APARICIO, HARWOOD, 2003; PETRAKIS, 2006).

Após coletadas, as azeitonas devem ser lavadas e processadas o mais rápido possível, pois o armazenamento por longos períodos também possibilita o estabelecimento de processos fermentativos e favorece reações de hidrólise e oxidação que podem modificar as características químicas e organolépticas do azeite de oliva. A lavagem tem como principal objetivo a retirada de sujidades aparentes e redução da carga microbiana. Assim, diminui-se a possibilidade de ocorrer fermentação da pasta e, consequentemente, o aparecimento de defeitos fermentativos (DI GIOVACCHINO, 2000; APARICIO, HARWOOD, 2003).

1.1.2 Métodos de extração de azeite de oliva

1.1.2.1 Prensagem

O método de prensagem, ilustrado esquematicamente no fluxograma na FIGURA 2, inicia pela moagem das azeitonas (com caroço), geralmente realizada em moinhos de pedra. Este tipo de moinho consiste de grandes pedras de granito redondas ou cônicas que rolam sobre as azeitonas esmagando-as e liberando o azeite do interior dos vacúolos. No próprio moinho ocorre a etapa de malaxagem, que consiste em unir as pequenas gotas de azeite, dispersas na pasta de azeitona, formando gotas maiores e melhorando os rendimentos de extração. A separação das frações sólida e líquida da pasta se dá por meio da prensagem da pasta de azeitonas em um sistema descontínuo de prensas hidráulicas, onde a massa é disposta em meio a discos vazados, por onde escorre o azeite quando a força de prensagem é exercida. A fração líquida é então coletada em um tanque de decantação para a separação do azeite e da água (naturalmente presente nas azeitonas) (DI GIOVACCHINO, 2000; APARICIO, HARWOOD, 2003; PETRAKIS, 2006).

O método de prensagem é o método mais tradicional de extração de azeite de oliva, entretanto, é um processo demorado e ocorre de forma descontínua, não sendo capaz de atender à demanda mercadológica de azeite de oliva. Além disso, durante o processo, ocorre a deposição de massa de azeitona nas entranhas dos discos de prensagem (incapaz de ser removida mesmo com intensas lavagens) resultando na ocorrência de processos fermentativos e reduzindo a qualidade do azeite produzido.

Figura 2: Fluxograma do método de extração de azeite de oliva por prensagem. Fonte: Adaptado de PETRAKIS (2006).

1.1.2.2 Percolação

O método de percolação inicia-se pela moagem contínua (geralmente em moinhos de martelo), malaxagem, percolação e uma centrifugação vertical final para eliminar o resto de água presente no azeite. O processo está baseado na diferença da tensão superficial entre a água e o azeite. Desta forma, quando uma lâmina de inox é inserida na pasta de azeitona, o azeite recobre a lâmina e, no momento em que a lâmina deixa a pasta, carrega consigo o azeite. O processo de percolação foi automatizado em um processo contínuo por meio do sistema Sinolea®. Este método não teve uma boa aderência mercadológica, pois os rendimentos de extração são menores que 80% (DI GIOVACCHINO, 2000; PETRAKIS, 2006; VEA, 2015).

1.1.2.3 Centrifugação

De modo a atender às demandas de mercado, bem como, melhorar o rendimento e a qualidade do azeite produzido, foi desenvolvido o processo de extração por centrifugação. A moagem das azeitonas é feita em um moinho de martelos, seguida pela malaxagem. Neste caso, a malaxagem é essencialmente importante, pois o moinho de martelos reduz o tamanho das gotas de azeite e ainda dispersa as gotas em uma pasta emulsionada. Deste

modo, na malaxação ocorre a quebra da emulsão promovendo a agregação das microgotas de azeite. Neste método, para o processo de extração, foram desenvolvidos dois sistemas, o sistema de três fases e o sistema de duas fases (DI GIOVACCHINO, 2000; APARICIO, HARWOOD, 2003; PETRAKIS, 2006).

No sistema de três fases, à pasta de azeitonas batida, adiciona-se cerca de 50 a 100% de água (dependendo da relação da umidade inicial/umidade de trabalho). Esta pasta obtida, é então submetida a uma centrifugação horizontal (centrífuga decantadora) para separação sólido/líquido (bagaço/azeite e água). Posteriormente, a fração líquida é novamente centrifugada, dessa vez em uma centrífuga vertical de pratos, separando a água e o azeite (FIGURA 3). É importante salientar que azeites obtidos por este método são menos picantes e amargos, pois parte dos compostos fenólicos do azeite migram para a fração de água. Além disso, a água resultante deste processo de extração carrega grande quantidade de matéria orgânica. Desta maneira, o volume de resíduos que necessitam de tratamento é muito grande, tornado-se uma desvantagem do sistema (PARASKEVA, DIAMADOPOULOS, 2006).

Mediante, principalmente, a questão ambiental envolvida no processo de três fases, foi desenvolvido o sistema de duas fases que não contempla a adição de água à massa batida. Neste caso, pode-se adicionar talco (material inorgânico inerte) na etapa de malaxagem, facilitando a quebra da emulsão e, consequentemente, a extração. Após a malaxagem a pasta é centrifugada, em uma centrífuga decantadora, separando o bagaço (contendo o talco) da fração líquida. Assim como no sistema de três fases, a fração líquida é novamente centrifugada, em uma centrífuga vertical, para a efetiva

separação do azeite e da água de vegetação (água intrínseca do fruto). Apesar da geração de água como resíduo nesta etapa, as quantidades são muito menores que no sistema de três fases, facilitando o tratamento de resíduos (FIGURA 3) (DI GIOVACCHINO, 2000; APARICIO, HARWOOD, 2003; PETRAKIS, 2006).

Após a obtenção do azeite por centrifugação, passa-se à decantação e filtração para a separação de possíveis sólidos suspensos e microgotas de água na fração lipídica. Estes processos têm como objetivo reduzir a possibilidade de hidrólise e processos fermentativos durante a estocagem, que contribuem para a redução da qualidade do produto final (APARICIO, HARWOOD, 2003).

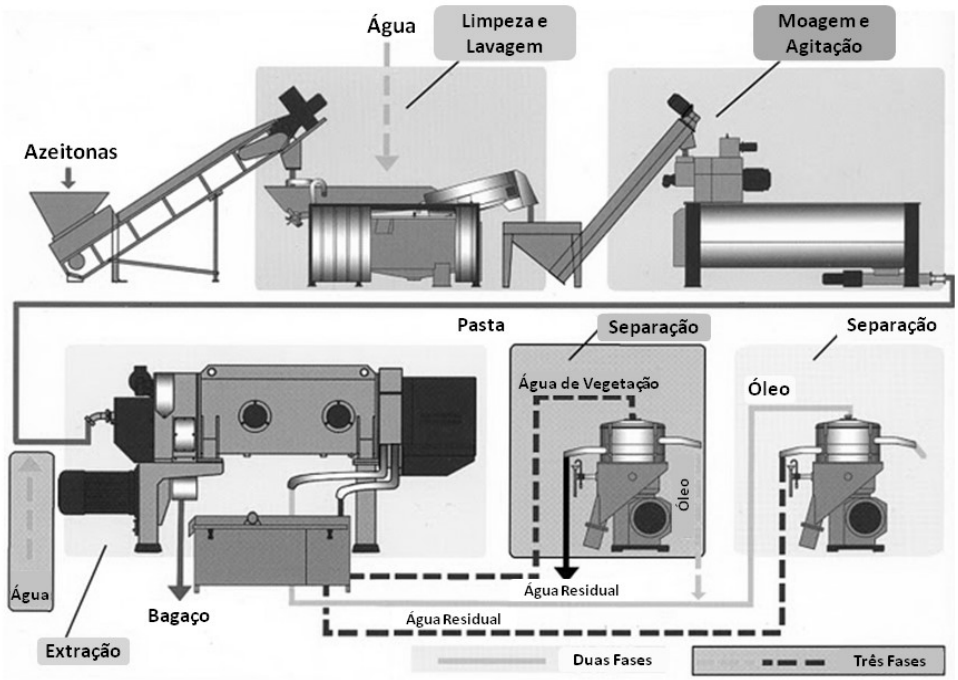

Figura 3: Fluxograma da obtenção do azeite de oliva, por meio do processo de centrifugação, nos sistemas de duas e três fases. Fonte: Adaptado de Vivante (2012).

1.2. OBTENÇÃO DO ÓLEO DE BAGAÇO

Como complemento da cadeia produtiva do azeite de oliva pode-se ainda, extrair a fração lipídica contida (retida) no bagaço. Para tanto, ele deve ser imediatamente seco para cessar as reações enzimáticas e a atividade microbiológica. Por ação do calor e da redução de atividade de água durante a secagem, as enzimas e microorganismos são inibidos e as atividades de deterioração, principalmente a hidrólise (que gera ácidos graxos livres, aumentando a acidez do azeite) e a fermentação, são inibidas (DI GIOVACCHINO, 2000).

Após a secagem, o bagaço passa por um processo de extração que utiliza hexano como solvente, obtendo-se o óleo de bagaço bruto, ao qual se aplica um processo de refino químico, originando o óleo de bagaço refinado. Para a comercialização e consumo, como óleo de bagaço de oliva, deve-se adicionar ao óleo de bagaço refinado 20% de azeite de oliva extra virgem ou virgem de acordo com as legislações (DI GIOVACCHINO, 2000; IOC, 2015; BRASIL, 2012).

1.3. CARACTERIZAÇÃO E CLASSIFICAÇÃO DE AZEITE DE OLIVA

Apesar do grande número de variedades de oliveiras cultivadas em todo o mundo, as sínteses biológicas dos componentes da fração lipídica (azeite) seguem uma determinada rota de formação. Portanto, os critérios utilizados para a caracterização de azeite de oliva estão baseados na determinação de seus componentes fundamentais, cujos conteúdos seguem uma lei básica de distribuição, estando relacionada com sua origem e atributos de qualidade. Assim, torna-se possível o estabelecimento de uma composição

média, com limites bem estabelecidos, abrangentes o suficiente para compreender a variabilidade de composição da espécie (APARÍCIO, HARWOOD, 2003).

Para tanto, foram estabelecidas diversas legislações, no âmbito nacional e internacional, contendo os parâmetros de identidade e qualidade do azeite de oliva. A recente legislação brasileira IN 001/2012 do MAPA fundamentou-se em organismos regulatórios como o COI, o CODEX e a UE para a determinação dos limites legais de composição. O COI, além de regulamentar os critérios legais para a caracterização e classificação do azeite de oliva mundialmente, gerencia a balança comercial relacionada ao azeite de oliva e subprodutos (CODEX, 1981; IOC, 2006, BRASIL, 2012). Na Tabela 1 encontram-se os parâmetros de qualidade e identidade para classificação de azeite de oliva de acordo com a IN 001/2012 (BRASIL, 2012).

As análises atualmente aplicadas ao controle de qualidade e identidade do azeite de oliva são baseadas em técnicas oficiais, ou seja, reconhecidas por renomados órgãos das áreas de química, óleos e gorduras e, especialmente, de azeite de oliva. Dentre os quais podemos citar o COI, a ISO (*International Organization for Standardization*), e a AOCS (*American Oil Chemists' Society*).

Em se tratando das análises que objetivam o controle de identidade de azeite de oliva, as metodologias envolvem procedimentos laboriosos e que despendem tempo excessivo na realização. Entretanto, estas são consideradas oficiais, uma vez que atendem aos requisitos de validação e são facilmente aplicáveis em laboratórios sem estrutura sofisticada.

Tabela 1: Critérios para classificação de Azeite de Oliva de acordo com a IN 001/2012 do MAPA

Grupo		Azeite de oliva virgem			Azeite de oliva	Azeite de oliva refinado	Óleo de bagaço de oliva	Óleo de bagaço de oliva refinado
Tipo		Extra Virgem	Virgem	Lampante				
Acidez Livre (%)		≤ 0,8	≤ 2,0	> 2,0	≤ 1,0	≤ 0,3	≤ 1,0	≤ 0,3
Índice de Peróxidos (mEq/Kg)		≤ 20,0		*	≤ 15,0	≤ 5,0	≤ 15,0	≤ 5,0
Extinção específica	K 270	≤ 0,22	≤ 0,25	*	≤ 0,9	≤ 1,1	≤ 1,7	≤ 2,0
	ΔK	≤ 0,01		*	≤ 0,15	≤ 0,16	≤ 0,18	≤ 0,20
	K 232	≤ 2,5	≤ 2,6	*	*			
Estigmastadienos (mg/Kg)		≤ 0,15		≤ 0,5	*			
Ceras (mg/Kg)		≤ 250		≤ 300	≤ 350		> 350	
Diferença ECN 42		≤ 0,2		≤ 0,3	≤ 0,3		≤ 0,5	
Composição em Ácidos Graxos (%)								
C14:0		≤ 0,05						
C16:0		7,5 - 20,0						
C16:1		0,3 - 3,5						
C17:0		≤ 0,3						
C17:1		≤ 0,3						
C18:0		0,5 - 5,0						
C18:1		55,0 - 83,0						
C18:2		3,5 - 21,0						
C18:3		≤ 1,0						
C20:0		≤ 0,6						
C20:1		≤ 0,4						
C22:0		≤ 0,2					≤ 0,3	
C24:0		≤ 0,2						
C18:1 t		≤ 0,05		≤ 0,1	≤ 0,2		≤ 0,4	
C18:2 t + C18:3 t		≤ 0,05		≤ 0,1	≤ 0,3		≤ 0,35	
Esteróis totais (mg/Kg)		> 1000					> 1,600	> 1,800
Composição de esteróis (%)								
Colesterol		≤ 0,5						
Campesterol		≤ 4,0						
Estigmasterol		< do que o observado para o Campesterol						
Brassicasterol		≤ 0,1					≤ 0,2	
β- Sitosterol + Δ-5,23-estigmastadienol + Clerosterol + β- Sitostanol + Δ-5-Avenasterol + Δ-5,24-estigmastadienol		≥ 93,0						
7- Estigmastenol		≤ 0,5						
Eritrodiol e Uvaol		≤ 4,5					> 4,5	
Características Sensoriais								
Mediana do Defeito		0	≤ 3,5	> 3,5	*			
Mediana do Frutado		> 0		0	*			

*Não se aplica. Fonte: Adaptado de BRASIL (2012).

1.3.1 Parâmetros de Identidade

A determinação de alguns compostos químicos, que compreendem os parâmetros de identidade, representam a impressão digital do azeite de oliva. Estas características são aplicadas tanto para a identidade quanto para a detecção das possíveis fraudes de azeite de oliva com outros azeites, óleos e gorduras.

Os componentes majoritários do azeite de oliva (~98%) contemplam os triacilgliceróis e os ácidos graxos, enquanto que os minoritários (~2%) consistem de esteróis, alcoóis, ceras e hidrocarbonetos. Os ácidos graxos são compostos de cadeia carbônica linear, saturada ou insaturada com um grupo de ácido carboxílico na extremidade. Os ácidos graxos são comumente designados de acordo com o número de carbonos, a quantidade de duplas ligações e a isomeria geométrica das moléculas (sendo necessário indicar apenas a isomeria *trans*). Também é comumente indicado o carbono correspondente à posição da insaturação na cadeia carbonica, contando a partir do carbono ômega (último carbono da cadeia). O ácido oleico, por exemplo, possui uma cadeia com 18 carbonos, 1 dupla ligação na posição 9 e isomeria cis, sendo representado por C18:1w9; enquanto que o ácido elaídico possui uma cadeia com 18 carbonos, 1 dupla ligação na posição 9 e isomeria *trans*, sendo portanto C18:1 *trans w9*. A esterificação de três ácidos graxos a uma molécula de glicerol resulta em uma molécula de triacilglicerol. Tanto a composição em ácidos graxos como a distribuição dos ácidos graxos nas moléculas de triacilgliceróis, esta última avaliada pelo incremento de ECN42 (triacilgliceróis com número de carbono equivalente igual a 42), inferem sobre as vias enzimáticas envolvidas na sintese destes compostos. As vias de síntese de ácidos graxos e triacilgliceróis possuem certa variação interespecífica, possibilitando a exploração destes parâmetros para determinar a identidade do azeite de oliva (SCRIMGEOUR, 2005; MOREDA, PÉREZ-CAMINO, CERT, 2003; IOC, 2015).

Os compostos minoritários são os mais utilizados como índices de autenticidade por serem características intrínsecas de determinadas espécies,

ou ainda, por terem sofrido alguma transformação durante o processamento que permite inequivocamente detectar algumas fraudes.

Dentre os compostos mioritários, os esteróis são componentes muito importantes como parâmetros de identidade do azeite de oliva, pois fazem parte da fração insaponificável e expressam significativas variações interespecíficas. Estas moléculas possuem uma estrutura comum com um núcleo esteroide composto de quatro anéis carbônicos, três aneis com seis e um com cinco carbonos. Diferem, entretanto, quanto à presença de determinadas ligações duplas e quanto à conformação e composição da cadeia lateral. Assim, tem-se o *β-sitosterol,* o *campesterol* e o *estigmasterol* como os esteróis (fitosteróis) mais representativos na fração insaponificável dos óleos e gorduras de origem vegetal (LEHNINGER et al., 2002; APARICIO, HARWOOD, 2003; MOREDA, PEREZ-CAMINO, CERT, 2004). Durante o processo de refino, por efeito das terras ácidas, na etapa de clarificação, ou da desodorização, pode ocorrer a desidratação dos esteróis, especificamente do β-sitosterol, produzindo 3,5-estigmastadieno, outro importante parâmetro de identidade para o azeite de oliva, uma vez que azeites de oliva virgens não devem conter estigmastadienos (DOBARGANES et al., 1999).

As determinações de eritrodiol, uvaol, alcoóis triterpênicos, ceras, ésteres de ácidos graxos de cadeia longa e alcoóis de cadeia longa, também são consideradas parâmetros de identidade. Estes compostos, que estão presentes na pele das azeitonas, migram para a fração de óleo, apenas na extração com solventes ou com altas temperaturas, como no caso da extração de óleo de bagaço de oliva (MOREDA, PEREZ-CAMINO, CERT, 2004).

Alguns parâmetros de identidade, embora estejam contidos na legislação nacional, são considerados ultrapassados, pois são determinações muito amplas, não suficientemente discriminatórias. Este é o caso do índice de iodo e do índice de refração, que relacionam o teor de insaturações do azeite; o delta K, que caracteriza a homogeneidade de absorção ultravioleta do azeite de oliva; o índice de saponificação e a matéria insaponificável, que determinam as relações dos componentes saponificáveis (acilgliceróis, ácidos graxos livres e ésteres) e dos componentes insaponificáveis (esteróis, hidrocarbonetos, alcoóis, compostos fenólicos, etc.) (APARICIO, HARWOOD, 2003).

1.3.2 Parâmetros de Qualidade

Além da identidade do azeite é sumamente importante determinar a qualidade do azeite a ser produzido e/ou comercializado. Assim, a legislação nacional estabelece classes de azeite de oliva virgem diferenciadas pelos parâmetros de qualidade. Dentre as classes determinadas pela legislação nacional, tem-se o *azeite de oliva extra virgem,* o *azeite de oliva virgem* e o *azeite de oliva lampante*, sendo o primeiro de maior qualidade e o último de menor qualidade (inclusive considerado impróprio para o consumo como azeite não refinado) (BRASIL, 2012).

Um dos parâmetros de qualidade mais evidenciado pelo mercado consumidor é o índice de acidez, que determina a quantidade de ácidos graxos livres, ou seja, o grau de hidrólise dos triacilgliceróis. O índice de peróxidos e a extinção específica no ultravioleta estão relacionados com os processos oxidativos que envolvem a degradação dos azeites. Por meio da análise de índice de peróxidos, determinam-se os compostos primários de oxidação

lipídica (hidroperóxidos), enquanto que na extinção específica, determinam-se principalmente os compostos secundários de oxidação lipídica (dímeros e polímeros). Estas determinações estão diretamente relacionadas com problemas nos processos de armazenamento e envase do azeite (TSIMIDOU, 2006).

Os ésteres alquílicos de ácidos graxos, compostos formados pela esterificação dos ácidos graxos livres, produzidos pela hidrólise dos triacilgliceróis, com alcoóis derivados de processos fermentativos (metanol e etanol), estão intimamente relacionados com a qualidade do fruto do qual se obtém o azeite. Deste modo, frutos que tenham sofrido ataque de pragas e processos prolongados de armazenamento são passiveis de sofrerem processos fermentativos, resultando em elevados teores de ésteres alquílicos de ácidos graxos, em particular ésteres etílicos. Azeites armazenados em contato com material orgânico em decomposição (borras), por efeito de uma decantação deficiente, apresentam teores de ésteres etílicos aumentados (PEREZ-CAMINO et al., 2008). A presença de borras de decantação está também relacionada ao teor de umidade e às impurezas insolúveis, que tambem são parâmetros de qualidade.

Um dos parâmetros de qualidade mais importantes é a avaliação sensorial, que engloba a detecção de atributos de qualidades e defeitos organolépticos no azeite. As análises de classificação e de fiscalização de azeite são realizadas por um painel de provadores treinados de acordo com rígidas metodologias estabelecidas pelo COI. De modo geral, o treinamento e formação de um painel sensorial de análise leva cerca de um ano para ser concluído. Desta maneira, o painel avalia os atributos de odor e sabor, tanto os

positivos quanto os negativos (defeitos), discriminando a intensidade de cada atributo. Por fim, a mediana destas intensidades caracteriza o azeite em questão (BRASIL, 2012; IOC, 2015b).

2. DETECÇÃO DE FRAUDES EM AZEITES DE OLIVA

Os mercados, consumidor e produtor, têm evoluido muito nos últimos anos, de modo que o trabalho de melhoramento genético com o intuito de ampliar as fronteiras de cultivo das oliveiras tem se intensificado principalmente no quesito de adaptação edafoclimática das variedades e até mesmo no desenvolvimento de novas variedades. Ao longo dos anos, este avanço permitiu uma maior flexibilização dos parâmetros de identidade do azeite de oliva, uma vez que em algumas variedades a composição química sofre muitas influências edafoclimáticas. Desta maneira, a ampliação do mercado, o alto valor agregado e as amplas faixas de composição aceitas pela legislação permitiram o estabelecimento de práticas de mercado fraudulentas, consistindo na adição de outros óleos, gorduras e azeites ao azeite de oliva autêntico. Estas fraudes não têm apenas implicações econômicas, mas também nutricionais, uma vez que podem ser adicionados óleos que apresentam características alergenicas ou até mesmo pelo simples fato de o consumidor buscar os benefícios nutracêuticos do azeite de oliva e não encontrá-los em azeites adulterados (APARICIO, 2000; ANGEROSA, CAMPESTRE, GIANSANTE, 2006).

A farmacopéia americana mantém um banco de dados de fraudes em alimentos onde é possível buscar por eventos registrados ou até mesmo reportar a ocorrência de uma fraude. O primeiro registro de fraude em azeite de

oliva deste banco de dados foi em 1962 e até hoje soma-se um total de 303 registros, sendo que só em 2015 foram realizados 10 registros de fraudes em azeite de oliva. São inúmeros os tipos de fraudes, que vão desde a adição de óleo mineral até a adição de azeite de oliva refinado ao azeite extra virgem. Nos últimos anos, tem se intensificado o uso de óleo de girassol alto oleico e óleo de avelã. De modo geral, as adoções dos métodos e parâmetros oficiais pelos organismos de controle inibiram significativamente a incidência destas fraudes (USP, 2016).

2.1. DETECÇÃO DE FRAUDES DE QUALIDADE

A detecção de fraudes relacionadas à qualidade, aquelas onde se comercializam produtos de baixa qualidade como se fossem produtos de alta qualidade, é bastante consistente, tanto no que diz respeito aos métodos físico-químicos, quanto aos métodos sensoriais. Os parâmetros de acidez, índice de peróxidos e absorção no ultravioleta (K) são bastante difundidos, inclusive no mercado consumidor, porém, não são unicamente suficientes. A análise sensorial para a detecção dos defeitos organolépticos, juntamente com a análise de ésteres alquílicos, são parâmetros complementares e também de extrema importância para garantir a qualidade do azeite comercializado (IOC, 2015).

Um dos casos mais críticos de fraudes em azeite de oliva é a adição de azeites de oliva lampantes que tenham sido submetidos a refinos suaves (processamentos em condições leves), a baixas temperaturas e a vácuo, que eliminam todos os compostos voláteis responsáveis pelos defeitos organolépticos (sem produção de estigmastadienos). Os organismos oficiais

têm uma grande preocupação com os impactos e a magnitude desta fraude, já que se submetem a este processo azeites de oliva com defeitos organolépticos e/ou com atributos sensoriais indesejáveis. Desta maneira, azeites que seriam lampantes, são submetidos à neutralização e desodorização em condições leves de processamento e, posteriormente, podem ser adicionados a um azeite de oliva extra virgem, sem que sejam detectados pelos métodos oficiais vigentes.

No entanto, a quantificação dos ésteres alquílicos de ácidos graxos permite a detecção dos azeites suavemente desodorizados, quando o defeito original deste azeite é de caráter fermentativo. Isto é possível, pois os ésteres alquílicos não são eliminados nas condições empregadas na desodorização suave. Entretanto, ainda existe a possibilidade de utilizar deodoratos (azeites suavemente desodorizados) provenientes de azeites com defeitos organolépticos não fermentativos, ou seja, que não produzem incremento de ésteres alquílicos, como ranço. Assim, os chamados “deodoratos desenhados”, não podem ser detectados pela presença de ésteres alquílicos (PÉREZ-CAMINO; MOREDA et al., 2002; PEREZ-CAMINO; CERT et al., 2008).

2.2. DETECÇÃO DE FRAUDES DE IDENTIDADE

As fraudes de identidade estão relacionadas à adição de óleos e azeites de outras fontes ao azeite de oliva e ao óleo de bagaço de oliva, ou ainda à adição de óleo de bagaço de oliva e/ou azeite de oliva refinado ao azeite de oliva virgem.

O método mais tradicional para a detecção da adição de óleos e azeites de outras fontes ao azeite de oliva e ao óleo de bagaço de oliva é a

determinação da composição em ácidos graxos. Entretanto, o aparecimento de sementes melhoradas e/ou modificadas geneticamente, cujos óleos apresentam composições em ácidos graxos similares à do azeite de oliva, diminuiu significativamente a eficiência do método de determinação de ácidos graxos para detectar a autenticidade do azeite de oliva. Neste ponto de vista, a determinação de esteróis permanece efetiva, uma vez que o melhoramento genético modifica a composição em ácidos graxos, mas não altera a composição em esteróis. Porém, a indústria de fraudes caminha a passos muito largos e vem desenvolvendo métodos para retirar os esteróis dos óleos, produzindo os chamados “óleos desesterolizados” cuja adição não pode ser facilmente detectada por meio do método de determinação de esteróis.

O fato dos óleos comerciais serem em sua grande maioria refinados, faz com que a determinação do 3,5-estigmastadieno seja uma prova inequívoca da adição de qualquer óleo e/ou azeite refinado em azeite de oliva virgem. Contudo, atualmente, os métodos de refino e desesterolização são mais suaves e produzem menores quantidades de estigmastadienos, reduzindo a efetividade dos métodos de detecção de fraudes. A determinação de ceras, eritrodiol e uvaol, por sua vez, permite a detecção de azeites extraídos por solventes, pois, estes compostos estão presentes em grandes quantidades na pele das azeitonas e migram para o azeite durante a extração com solventes (MARIANI, VENTURINI, GROB, 1995; MARIANI, VENTURINI, 1997; CERT, MOREDA, 1998; DOBARGANES et al., 1999; PEREZ-CAMINO et al., 2008).

Os dois métodos mais recentes adicionados às legislações internacionais para o controle de fraudes em azeite de oliva são o incremento de ECN42 e o Método Global. Ambos estão baseados na lógica de síntese dos

triacilgliceróis nas oliveiras, uma vez que as oliveiras não produzem ou produzem em baixíssimas quantidades triacilgliceróis sem a presença de um ácido oleico. Deste modo, o método de ∆ECN42 calcula a composição em triacilgliceróis com ECN42 teórica, a partir da composição em ácidos graxos, e compara com a composição de triacilgliceróis com ECN42 real, obtida experimentalmente por meio da análise de triacilgliceróis em HPLC (*High Performance Liquid Chromatography*). Segundo a IN 01/2012 do MAPA, esta diferença deve ser menor ou igual a 0,2 (BRASIL, 2012; IOC, 2010; 2015).

O método global, oficialmente chamado de “método global para detecção de óleos e azeites estranhos em azeite de oliva”, é muito mais abrangente, pois não avalia apenas ECN42 e sim a composição em triacilgliceróis como um todo. O método em si, consiste de uma serie de algoritmos matemáticos, chamados critérios, que comparam tanto a composição em triacilgliceróis teórica com a experimental, quanto a relação entre os diferentes triacilgliceróis em uma amostra. O azeite analisado deve passar por todos os critérios do método para ser considerado genuíno (IOC, 2013; MOREDA, PÉREZ-CAMINO, CERT, 2003).

É importante salientar que os parâmetros de identidade e qualidade são complementares para a caracterização e classificação do azeite de oliva, sendo, portanto, necessário a análise de todos os parâmetros legais para as devidas afirmações.

2.3. FRAUDES SOFISTICADAS EM AZEITE DE OLIVA

Apesar do constante desenvolvimento de metodologias analíticas para detecção das diversas fraudes, o aprimoramento dos que buscam meios

fraudulentos também se sofistica, a ponto dos métodos analíticos perderem seu poder de detecção total ou parcialmente. Neste sentido, as fraudes sofisticadas são aquelas não detectadas pelos métodos oficiais quando a adição do óleo de adulteração for inferior a 30%.

Dentre as principais fontes de fraudes sofisticadas em azeite de oliva destacam-se os óleos de avelã (*Corylus avellana*), noz (*Juglans regia*) e amêndoa (*Prunus dulcis*) e o azeite de abacate (*Persea americana*). Embora os estudos voltados à caracterização destas fontes lipídicas sejam limitados, sabe-se que suas composições químicas são muito semelhantes a do azeite de oliva. Estas características dificultam o estabelecimento de marcadores químicos específicos para detectar as fraudes.

A seguir, serão descritas as principais características das fontes de fraudes em questão e, na TABELA 2, podem ser observadas informações adicionais quanto à composição química destas fontes de fraudes.

2.3.1 Avelã (*Corylus avellana* L.)

A avelã é um fruto seco indeiscente proveniente da aveleira ou avelaneira, árvore proveniente da Europa e do Leste da Ásia, onde é amplamente cultivada. O principal produtor mundial é a Turquia, seguida pela Itália e os EUA (FAO, 2013). O fruto da aveleira consiste de uma semente envolta por uma casca dura e fibrosa. A semente, muito apreciada para consumo *in* natura, contém cerca de 50% de lipídios. O óleo de avelã é extraído da semente por prensagem, tanto em prensas hidráulicas como em *expeller*, podendo ou não ser refinado (KAMAL-ELDIN, MOREAU, 2009).

A utilização do óleo de avelã em produtos alimentícios abrange desde a mistura em produtos a base de chocolate, originando produtos de valor agregado, até o uso de óleo de avelã não refinado em saladas, que vem crescendo juntamente com a tendência de *gourmetização* do mercado (NATTRESS et al., 2004; KAMAL-ELDIN, MOREAU, 2009). Embora seja amplamente utilizado na indústria de alimentos, o óleo de avelã contém alguns compostos de caráter alergênicos relacionados com proteínas. Dentre os principais fatores alergênicos estão o Cor a1 (proteínas da família Pr10), Cor a8 (proteína de transferência de lipídios), Cor a9 (globulina 11S) e Cor a14 (albumina 2S), sendo que, este último é utilizado como marcador sorológico para a identificação de alergia (MARTÍN-HERNÁNDEZ, BÉNET, OBERT, 2008; ELLER, MORTZ, BINDSLEV-JENSEN, 2016). Devido ao elevado risco de reações alérgicas, os órgãos regulatórios tornaram obrigatório indicar, nas embalagens a presença de avelã em alimentos (BRASIL, 2015). De modo a garantir a eficiência da fiscalização dos alimentos, métodos analíticos têm sido constantemente desenvolvidos, sendo que as análises de DNA têm se destacado ultimamente (PLATTEAU et al., 2011; MADESIS, 2013; COSTA, MAFRA, OLIVEIRA, 2015). Neste sentido, pode-se citar o desenvolvimento de um *kit* para a detecção de alérgenos em alimentos por meio da análise de marcadores moleculares de DNA descrito na patente EP2226395A1. Arlorio e colaboradores (2010) relataram o risco de reações alérgicas desencadeadas por azeites de oliva adulterados com óleo de avelã, exemplificando, desta maneira, que a detecção de fraudes de azeite de oliva com óleo de avelã não é apenas de importância econômica, mas também de saúde.

A composição química do óleo de avelã é muito semelhante ao azeite de oliva, tendo o ácido oleico como principal ácido graxo, o β-sitosterol como principal esterol e um baixo conteúdo de tocoferóis (TABELA 2) (MATTHÄUS, ÖZCAN, 2012). As fraudes de azeite de oliva com óleo de avelã estão entre as mais relatadas e estudadas atualmente (USP, 2016). No caso do óleo de avelã, com os métodos oficiais (esteróis, ΔECN42), o nível de detecção é superior a 30%. Entretanto, a aplicação do método global permitiu alcançar níveis de detecção em torno de 12% (CERT, MOREDA, 2000; MOREDA, PÉREZ-CAMINO, CERT, 2003; IOC, 2010; 2013).

Além dos métodos oficiais, outras ferramentas têm sido propostas para a detecção de fraudes de azeite de oliva com óleo de avelã. Dentre os marcadores químicos, tem se estudado a presença de lupeol (AZADMARD-DAMIRCHI, SAVAGE, DUTTA, 2005; AZADMARD-DAMIRCHI, 2010), de filbertona (BLANCH, 1998; FLORES et al., 2006), a composição de esteróis esterificados (CERCACI et al., 2003; MARIANI et al., 2006), bem como, modelos matemáticos relacionando a composição química do azeite de oliva e do óleo de avelã (GARCÍA-GONZÁLEZ et al., 2007). Outras tecnologias também têm sido estudadas, como a ressonância de prótons (GARCÍA-GONZÁLEZ et al., 2004; MANNINA et al., 2009; AGIOMYRGIANAKIA, PETRAKIS, DAISA, 2010) e a espectrometria de massas (CALVANO et al., 2012).

Apesar de amplamente documentada, a detecção, a níveis seguros (econômicos e de saúde), de óleo de avelã em azeite de oliva ainda não está completamente estabelecida. As dificuldades estão relacionadas, principalmente, às amplas faixas de composição do azeite de oliva e do óleo de

avelã, o que exige que outras ferramentas sejam exploradas a fim de complementar as possibilidades existentes.

2.3.2 Noz Pecan (*Carya illinoinensis*)

A noz pecan, apesar de muito parecida com a nogueira comum (*Juglans regia*), pertence a outro gênero e é originária da América do Norte (EUA), mas teve boa adaptação em países da América do Sul, além de outros países do hemisfério sul como a Austrália e a África do Sul. O fruto da noz pecan é do tipo drupa e seco, geralmente agrupado em cachos de até sete frutos, onde a maturação é indicada pela separação do epicarpo do fruto (WAKELING et al., 2001; BOUALI et al., 2014).

A noz pecan é geralmente consumida *in natura* ou adicionada a tortas e bolos. Industrialmente, é utilizada como matéria-prima para pastas e ainda adicionada a chocolates e *spreads*. Os aromas da noz pecan tanto *in natura* como tostada, são sensorialmente pronunciados e muito apreciados pelo setor gastronômico (MAGNUSON et al., 2016). O fruto da noz pecan contém alta concentração de compostos antioxidantes, presentes tanto nas sementes como na casca. Devido à presença destes compostos a noz pecan foi incluída pelo Food and Drug Administration (FDA) (2003) na lista de alimentos que possuem efeitos benéficos à saúde, incentivando, assim, o consumo frequênte destas sementes (PRADO et al., 2013; DOMÍNGUEZ-AVILA et al., 2015).

Apesar dos inúmeros benefícios dados pela presença de compostos antioxidantes, a noz pecan assim como a noz comum, contém proteínas capazes de desencadear reações alérgicas nos consumidores. Em 2003, a noz

pecan ocupava o quarto lugar em maior número de ocorrências de alergia entre as nozes, no *ranking* americano (SICHERER, MUNOZ-FURLONG, SAMPSON, 2003). Dentre as proteínas causadoras de alergia presentes na noz pecan, os grupos mais comuns são Car i 1 (albumina 2S), Car i 4 (legumina 11S) e Car i 7 (7S globulina), sendo que os genes relacionados a estes grupos de proteínas são mais expressos principalmente no período ótimo de maturação dos frutos (MATTISON et al., 2013). O monitoramento da declaração e presença destes alergenos no mercado alimentar é de suma importância para assegurar a saúde do consumidor. Dentre os métodos disponíveis para estas análises, o diagnóstico por meio da amplificação de regiões específicas de DNA, relacionados à expressão destas proteínas, têm se mostrado viável (LÓPEZ-CALLEJA et al., 2015).

A semente de noz pecan pode conter mais de 60% de lipídios, sendo o óleo obtido tanto por prensa hidráulica como por prensa do tipo *expeller*, e apresentando baixa acidez e alta estabilidade oxidativa. Em relação à composição, o principal ácido graxo é o ácido oleico, o que o diferencia muito do óleo de noz comum, que é composto predominantemente por ácido linoleico. Quanto aos compostos minoritários, os mais representativos são os fitoesteróis, sendo o β-sitosterol o principal composto (TABELA 2) (RYAN et al., 2006; MOODLEY, KINDNESS, JONNALAGADDA, 2007; ORO et al., 2009; BOUALI et al., 2014).

Pouco tem se estudado quanto à utilização de óleo de noz pecan como fonte de fraudes em azeite de oliva. Entretanto, estudos recentes têm relatado a utilização de espectroscopia de Raman para a detecção de misturas de óleos de noz pecan com outros óleos de sementes vegetais (CARMONA et

al., 2015). Pelos estudos até o momento publicados, nota-se grande semelhança entre o óleo de noz pecan e o azeite de oliva. Tal semelhança possibilita o estabelecimento de fraudes que não podem ser facilmente detectadas. Isso reforça a necessidade de maiores estudos quanto à composição do óleo de noz pecan, bem como a busca de marcadores bioquímicos que possam detectar níveis mínimos de inclusão desse óleo no azeite de oliva.

2.3.3 Amêndoa (*Prunus dulcis*)

O fruto da amendoeira é do tipo drupa, ou seja, contém apenas uma semente, envolvida por endocarpo lenhoso, resultando em um conjunto comumente conhecido como amêndoa. Apesar de ser originária do norte da África, o maior produtor mundial de amêndoas é o EUA, sendo a produção concentrada no estado da Califórnia (ABDALLAH et al., 1998; SATHE et al., 2008).

As amêndoas são amplamente utilizadas na indústria de alimentos, tanto em pedaços quanto como na forma de cremes e pastas; principalmente em alimentos com alto teor de lipídios, como chocolates (ALTAN et al., 2011; BEHMADI et al., 2015). A inserção de amêndoas e óleo de amêndoas na alimentação tem sido fortemente embasada por estudos que demonstram efeitos benefícios à saúde. Dentre os principais efeitos estão a prevenção de doenças cardiovasculares, por meio do aumento de HDL aliado a redução de LDL e triacilgliceróis séricos; e os efeitos antidiabéticos, por meio da inibição da proteína tirosina fosfatase-1B (FDA, 2003; AHMAD, 2010; HYSON, SCHNEEMAN, DAVIS, 2002; QURESHI et al., 2016).

Entretanto, as amêndoas fazem parte de um grupo de alimentos classificados pela ANVISA e outros órgãos reguladores, como potenciais causadores de reações alérgicas (BRASIL, 2015). As reações alérgicas relacionadas ao consumo de amêndoas são causadas por grupos de proteínas como: Pru du 1 (proteínas PR-10), Pru du 2S albumina (proteínas de transporte de lipídios), Pru du 5 (proteínas ribossomais ácidas 60S) e Pru du 6 (amandinas) (COSTA et al., 2012; COSTA, MAFRA, OLIVEIRA, 2012b). Assim como para a avelã, ferramentas analíticas têm sido desenvolvidas para a detecção de amêndoas em alimentos, viabilizando mecanismos de fiscalização com o objetivo de assegurar a saúde do consumidor. Dentre as ferramentas desenvolvidas, as técnicas de biologia molecular, envolvendo a amplificação e detecção de regiões específicas de DNA, se destacam quanto a viabilidade de aplicação, seletividade e alta sensibilidade de detecção, sendo capazes de detectar traços de amêndoas em diferentes matrizes alimentares (PAFUNDO, GULLÌ, MARMIROLI, 2009; COSTA, MAFRA, OLIVEIRA, 2012b; European Patent EP2226395A1).

As sementes de amêndoas são compostas por mais de 50% de lipídios, sendo que a extração do óleo ocorre normalmente com o uso de *expeller* a cerca de 40ºC, alcançando rendimentos próximos a 80% (MARTÍNEZ et al., 2013). Os óleos de amêndoas possuem baixa acidez e alta estabilidade oxidativa, podendo ser consumidos sem a necessidade de refino e adição de antioxidantes. Quanto à composição química destacam-se a alta concentração de ácido oléico e o β-sitosterol como principal fitosterol (TABELA 2) (ASKIN et al., 2007; SATHE et al., 2008; FERNANDEZ-CUESTA et al., 2012; KARATAY et al., 2014).

A composição química do óleo de amêndoa se assemelha muito à composição química do azeite de oliva, o que torna muito difícil a detecção deste óleo no azeite de oliva. Devido à dificuldade de detecção, é uma fraude pouco relatada. Além disso, os parâmetros de identidade atualmente aplicados pelos organismos regulatórios, não apontam indícios sobre a fraude de azeite de oliva com óleo de amêndoas. O método global, por meio de algoritmos relacionados à composição de triacilgliceróis pode contribuir para esta detecção. Entretanto, até o momento, nenhum estudo foi ainda conduzido neste sentido. O desenvolvimento de ferramentas para a detecção de óleo de amêndoa em azeite de oliva tem implicações econômicas, nutricionais e de saúde.

2.3.4 Abacate (*Persea americana*)

O abacateiro, originário da América Central, está taxonomicamente dividido em três raças, Mexicana, Guatemalense e Caribe. Depois de muitos anos de cultivo, o cruzamento destas três raças originou inúmeros cultivares de abacate, cujas características físicas das frutas e composição química são muito diversas. A fruta do abacateiro é na verdade um pseudofruto, uma vez que a parte carnosa trata-se do desenvolvimento do pedúnculo floral e não do ovário da flor (POPENOE, 1941; ASHWORTH, CLEGG, 2003).

De acordo com os dados da FAO, o México é o maior produtor mundial de abacate, sendo responsável por cerca de 30% da produção mundial. O Brasil é o terceiro maior produtor mundial, tendo sua produção concentrada no estado de São Paulo (FAOSTAT, 2013; TANGO, CARVALHO, SOARES, 2004). De modo geral, o consumo de abacate vem sendo

incentivado e, continuamente, relacionado com possíveis benefícios à saúde. O fruto possui alto valor nutricional, é altamente energético, contendo lipídios, proteínas e vitaminas lipossolúveis. Além disso, apresenta alta capacidade antioxidante, que está intimamente relacionada à presença de compostos fenólicos, carotenóides, flavonóides, vitaminas e, mais recentemente, as acetogeninas presentes nas diferentes partes do fruto (VILLA-RODRÍGUEZ et al., 2011; RODRÍGUEZ-SÁNCHEZ et al., 2013; VINHA, MOREIRA, BARREIRA, 2013; DUARTE et al., 2016).

Dentre os mais variados cultivares de abacate, apenas alguns são utilizados industrialmente para a obtenção do azeite de abacate. Os mais cultivados para este fim são os cultivares Hass e Fuerte, que possuem conteúdo lipídico superior a 30%. Já os cultivares Bacon e Pinkerton também apresentam potencial para aplicação industrial, sendo entretanto, menos difundidos comercialmente (MOHAMEED et al., 1997; TANGO, CARVALHO, SOARES, 2004). No fruto do abacateiro a fração lipídica está concentrada no mesocarpo, no interior dos idioblastos, que são células cuja estrutura da parede é bastante complexa, composta de duas camadas de celulose separadas por uma camada de suberina (PLATT, THOMSON, 1992).

O estabelecimento de métodos de extração para azeite de abacate tem sido foco de muitos estudos. Em geral, os estudos enfocam tanto o pré-tratamento da polpa quanto a obtenção da fração lipídica. Dentre os sistemas de pré-tratamento estão a secagem da polpa, por calor ou liofilização; e a ação da pectinase, para facilitar a liberação do azeite. Em relação à obtenção da fração lipídica, os métodos baseiam-se desde a extração com solventes, até a prensagem e uso de CO_2 supercrítico (FREITAS, YAHYA, LAGO, 1998;

BOTHA, McCRINDLE, 2003; MORENO et al., 2003; ORTIZ et al., 2004; REDDY, MOODLEY, JONNALAGADDA, 2012; SANTOS et al., 2014; SANTANA et al., 2015; BARROS et al., 2016). Entretanto, o método de extração que atualmente apresenta-se mais viável à aplicação industrial é a centrifugação. De maneira muito semelhante ao método de extração de azeite de oliva, este método consiste da moagem, da malaxagem da pasta e uma centrifugação horizontal, para a separação sólido/líquido, seguida de uma centrifugação vertical para a separação do azeite da água de vegetação (EYRES, SHERPA, HENDRIKS, 2001; WONG, REQUEJO-JACKMAN, WOLF, 2010; COSTAGLI, BETTI, 2015).

O azeite de abacate é predominantemente monoinsaturado, sendo o ácido oleico seu principal componente. Quanto aos compostos minoritários, a quantidade de fitoesteróis é bastante expressiva (>2500 $mg.Kg^{-1}$), sendo o β-sitosterol o majoritário (TABELA 2) (SALGADO et al., 2008; MASSAFERA, COSTA, OLIVEIRA, 2010; FLORES, PEREZ-CAMINO, TROCA, 2014; REDDY, MOODLEY, JONNALAGADDA, 2014; RUEDA et al., 2014; SANTOS et al., 2014; SANTANA et al., 2015). Por vezes, o azeite de abacate é considerado um produto de mercado paralelo ao mercado de frutas e, em algumas ocasiões, até um subproduto. Deste modo, a qualidade das frutas que são destinadas à extração de azeite, é muitas vezes questionável, fazendo com que a qualidade do azeite produzido também o seja. Woolf e colaboradores (2009) sugeriram o estabelecimento de padrões de qualidade para o azeite de abacate. Entretanto, não há uma legislação específica que regulamente sua comercialização. Ainda neste sentido, alguns trabalhos sugerem o estabelecimento de ferramentas analíticas para a detecção de fraudes em

azeite de abacate, sendo que a espectroscopia de infravermelho tem sido a mais estudada dentre as ferramentas analíticas (QUIÑONES-ISLAS et al., 2013; LUMAKSO et al., 2015a,b; ROHMAN et al., 2016).

Embora estudos de adulteração de azeite de oliva com azeite de abacate não tenham sido descritos até o momento, sabe-se que os dois azeites são muito semelhantes e que por vezes o azeite de abacate é comparado ao azeite de oliva e até mesmo sugerido como substituto (SALGADO et al., 2008; BERASATEGI et al., 2012). Em 2012, a polícia espanhola desmascarou um esquema de fraudes de azeite de oliva que envolvia misturas com óleo de avelã e azeite de abacate. O esquema criminoso foi descoberto apenas por suspeitas de fraudes fiscais, uma vez que os métodos analíticos vigentes não são efetivos para a detecção destas fraudes sofisticadas (BUTLER, 2012).

Por todo o exposto até agora, é clara a necessidade de desenvolvimento de estratégias para abordar o estudo destas fraudes, utilizando-se métodos mais modernos, sensíveis e efetivos. Conforme já citado, estes métodos implicam no uso de equipamentos que permitam a quantificação de compostos específicos presentes nos óleos vegetais (HOLCAPEK, JANDERA, 2001; FASCIOTTI, PEREIRA, 2010; GARCIA-VILLALBA et al., 2011). Dentre os métodos utilizados para estes desenvolvimentos estão a espectrometria de massas, a espectroscopia de infravermelho e a análise de marcadores moleculares de DNA, que é o foco deste trabalho.

Tabela 2: Composição química das fontes de fraudes sofisticadas em Azeite de Oliva

Fonte		Abacate	Avelã	Noz Pecan	Amêndoa
Teor de Óleo (g/.100g^{-1})		5.3 - 31.1	8.1 - 64.1	58.0 -74.0	25.2 - 60.8
Composição em Ácidos Graxos (%área)	C14:0	0.02 - 1.5	-	0.05 - 0.1	0 - 0.07
	C16:0	12.5 - 35.2	4.1 - 6.8	4.3 - 7.6	4.4 - 15.8
	C16:1	2.8 - 18.0	0.10 - 0.70	0.1 - 0.2	0.4 - 2.5
	C18:0	0.2 - 1.5	1.6 - 2.8	1.8 - 2.8	0.2 - 2.5
	C18:1	36.9 - 74.0	72.8 - 83.5	40.6 - 62.1	50.4 - 81.2
	C18:2	6.1 - 21.2	6.5 - 16.6	27.2 - 50.3	6.2 - 37.1
	C18:3	0.3 - 1.0	0.1 - 0.3	0.6 - 1.9	0 - 11.1
	C20:0	0.1	0 - 0.4	0.3	0.04 - 0.07
Fitosteróis Totais mg.kg^{-1}		-	1242 - 6031	2200	2769 - 2777
Composição em Fitosteróis (%área)	Colesterol	0.3 - 2.3	0.8 - 2.3	-	-
	24-metilen-colesterol	-	0 - 0.1	-	-
	Campesterol	4.9 - 6.6	4.8 - 7.4	2.7	-
	Campestanol	0 -0.7	0 - 0.2	-	-
	Stigmasterol	0.8 - 1.5	1.3 - 2.1	17.9	-
	7-camersterol	-	0 - 0.4	-	-
	Clerosterol	0 - 13.4	0.9 - 1.3	-	-
	β - sitosterol	71.8 - 83.7	78.1 - 82.4	82.8	55.9 - 84.6
	Sitostanol	0 - 6.0	1.8 - 3.6	-	-
	5 - avenasterol	1.8 - 7.0	1.3 - 5.2	-	8.5 - 28.2
	5,24-stigmastadienol	-	0.3 - 1.1	-	-
	7-avenasterol	-	0.3 - 2.3	-	-
	7avenastenol	-	0.5 - 1.9	-	-
Tocóis Totais mg.100g^{-1}		13.0 - 20.0	25.8 - 69.8	70.0 - 152.0	-
Composição em Tocóis (%do total de tocóis)	α - tocoferol	85.0 - 98.0	53.8 - 90.5	48.0 - 55.0	-
	β - tocoferol	15.0	2.1 - 4.2	10.0 - 12.0	-
	γ - tocoferol	-	3.1 - 41.9	-	-
	δ - tocoferol	0.5	-	38.0 - 45.0	-
	α - tocotrienol	-	0 - 7.1	-	-
Referências		TANGO, et al., 1972; CANTO, SANTOS, TRAVAGLINI,1980; TANGO, et al., 2004; SALGADO, et al., 2008; REDDY, MOODLEY, JONNALAGADDA, 2012; FLORES, PEREZ-CAMINO, TROCA, 2014; VILLA-RODRİGUEZ, et al., 2014; RUEDA, et al., 2014; MATTHÄUS, ÖZCAN, 2012; BENITEZ-SÁNCHEZ, LEÓN-CAMACHO, APARICIO, 2003; AZADMARD-DAMIRCHI, SAVAGE, DUTTA, 2005, AZADMARD-DAMIRCHI, 2010; MOODLEY, KINDNESS, JONNALAGADDA, 2007; KAMAL-ELDIN, MOREAU, 2009; PRADO, et al., 2013; BOUALI, et al. 2014; RYAN, et al. 2015; ABDALLAH, AHUMADA, GRADZIEL,1998 ; ASKIN, et al., 2007 ; SATHE, et al., 2008 ; CELIK, BALTA, 2010; FERNANDEZ-CUESTA, et al., 2012; KARATAY, et al., 2014			

- Valores não determinados e/ou ensaios não realizados nos trabalhos de referência

3. DETECÇÃO DE FRAUDES POR MARCADORES MOLECULARES DE DNA

Devido às limitações relacionadas ao estabelecimento de marcadores de composição química, o combate às fraudes em azeite de oliva tem adotado ferramentas inovadoras na área de óleos e gorduras. A recente utilização de ferramentas de análise de DNA tem se destacado como uma potencial metodologia de rastreabilidade e de combate a fraudes

(MONTEALEGRE, ALEGRE, GARCIA-RUIZ, 2010; BEN-AYED, KAMOUN-GRATI, REBAI, 2013; DANEZIS et al., 2016).

As ferramentas de análise de DNA consideram o fato de que cada organismo vivo possui um genoma único, que está replicado em praticamente todas as suas células. Existem determinadas regiões do genoma que são extremamente conservadas em um gênero, espécie e/ou cultivar. Outro fator positivo para a aplicação destas ferramentas é que o código genético não sofre interferências diretas das condições edafoclimáticas (COSTA, MAFRA, OLIVEIRA, 2012a; PASQUALONE et al., 2016).

De modo geral, as técnicas consistem na **extração** do DNA contido nos azeites e óleos, seguida pela **amplificação** de regiões do DNA, identificando **marcadores** específicos para cada azeite/óleo, e por fim, a **análise** dos fragmentos de DNA gerados. A condução adequada de cada etapa é determinante para o sucesso do método e seu efetivo uso no monitoramento da qualidade do azeite de oliva (COSTA, MAFRA, OLIVEIRA, 2012; BEN-AYED, KAMOUN-GRATI, REBAI, 2013; DANEZIS et al., 2016; PASQUALONE et al., 2016).

3.1 EXTRAÇÃO DE DNA

Nos óleos e azeites, o DNA está contido em células dispersas, em pequenos fragmentos de tecido e/ou ainda, podem estar livres na matriz lipídica. De modo geral, a quantidade de DNA é muito baixa; além disso, o grau de degradação do DNA é alto, principalmente por ação de nucleases presentes no azeite. Assim, extrair um DNA com qualidade de óleos e azeites é uma tarefa desafiadora e muito importante para o sucesso das técnicas que visam

analisar o DNA presente nestas matrizes (MUZZALUPO, PERRI, 2002; PASQUALONE et al., 2016).

Neste sentido, diversos procedimentos foram avaliados, desde protocolos tradicionais de extração de DNA até *kits* comerciais com diferentes princípios de funcionamento, com o foco de obter um DNA de boa qualidade para amplificação. Previamente a lise celular e a extração de DNA, alguns trabalhos sugerem realizar uma etapa de concentração do material em suspensão, com centrifugação em velocidades superiores a 7000 x g por períodos de ±30 min e a temperatura de ±4ºC (MUZZALUPO, PERRI, 2002; TESTOLIN, LAIN, 2002; MONTEMURRO et al., 2015). Recentemente, Muzzalupo e colaboradores (2015) introduziram a utilização de uma membrana com afinidade para ácidos nucléicos (Hybond™-N+, 0.45µm) como ferramenta para concentração e captura de DNA.

Dentre os protocolos tradicionais de lise celular e extração de DNA mais estudados estão aqueles baseados no uso do CTAB (*cetyltrimethylammonium bromide*) desenvolvido originalmente por Doyle e Doyle (1990) para a extração de DNA de plantas. Algumas variantes incluíram lavagens com hexano, clorofórmio e clorofórmio:álcool isoamílico para diminuir os inibidores de PCR (reação em cadeia da polimerase) (MUZZALUPO, PERRI, 2002; TESTOLIN, LAIN, 2002; GIMÉNEZ et al., 2010; GANOPOULOS et al., 2013; HE et al., 2013; RAIETA, MUCCILLO, COLANTUONI, 2015; KALOGIANNI et al., 2015; BAZAKOS et al., 2016).

Em relação aos *kits* comerciais para extração de DNA, dois princípios têm sido bastante aplicados, o de partículas magnéticas que adsorvem as moléculas de DNA e o de colunas retentoras de DNA. O *Wizard*

Magnetic DNA Purification System for Food (Promega, Madison, USA) é o método de partículas magnéticas mais utilizado, apresentando bom desempenho tanto para azeites como para azeitonas (TESTOLIN, LAIN, 2002; WU et al., 2011; LI et al., 2012; ZHANG et al., 2012). Dentre os métodos de colunas, os mais relatados estão o *Gene Elute Plant Kit* (Sigma Aldrich) (ALBA et al., 2009; MONTEMURRO et al., 2015), *QIAamp DNA stool* (Qiagen) (TESTOLIN, LAIN, 2002; KUMAR et al., 2011; BEN-AYED et al., 2012), *NucleoSpin Plant kit* (Macherey–Nagel) (PAFUNDO et al., 2010; PASQUALONE et al., 2012) e *DNeasy Plant Mini Kit* (Qiagen) (KUMAR et al., 2011). Em geral, os métodos que utilizam colunas de afinidade para extração resultam em um DNA mais purificado, ou seja, com menos inibidores de PCR.

Independente do método, a quantidade de amostra inicial de azeite é outro ponto bastante variável, iniciando em 50 µL e indo até 200 mL. Embora não seja uma regra, a utilização de volumes maiores é normalmente justificada para a aplicação da técnica em azeites refinados, onde a qualidade do DNA obtido nem sempre está adequada. Assim, a escolha dos métodos de preparo, extração e a quantidade de amostra utilizada, impacta diretamente na quantidade, qualidade e, principalmente, na reprodutibilidade do método, que, atualmente, é um dos maiores desafios destas metodologias.

3.2 AMPLIFICAÇÃO DE DNA

A amplificação dos fragmentos de DNA está fundamentada na reação da polimerase em cadeia (PCR, *Polymerase Chain Reaction*). A reação foi desenvolvida em 1983 por Kary Mullis, com o objetivo de mimetizar a síntese de cópias do material genético *in vitro* e desde então serve como base

para os métodos de biologia molecular. Nesta reaçãode PCR ocorre a amplificação de regiões específicas do DNA flanqueadas por fragmentos de DNA iniciadores chamados *primers* (BEM-AYED, KAMOUN-GRATI, REBAI, 2013).

Em suma, ocorre a desnaturação da dupla fita de DNA em fitas simples de DNA, por meio do aumento de temperatura (±90°C), permitindo assim o anelamento dos *primers* nas regiões complementares da fita, dado por uma leve diminuição da temperatura (±60°C). Na sequência, torna-se a aumentar a temperatura (±75°C) possibilitando a ação da polimerase, enzima que sintetizará uma nova fita de DNA complementar à região flanqueada pelos *primers*. O estabelecimento de diversos ciclos desta reação gera quantidades suficientes de cópias para serem posteriormente analisadas. A região de anelamento e a especificidade dos *primers* dependem do tipo de marcador molecular a ser analisado (KUBISTA et al., 2006).

3.3 MARCADORES MOLECULARES DE DNA

Os marcadores moleculares são regiões de DNA que estabelecem quais os conjuntos de organismos pertencem a uma mesma linhagem evolutiva. Desta maneira, possibilitam diferenciar organismos, espécies, gêneros e outras classes taxonômicas. Dentre os marcadores moleculares de DNA mais difundidos, destacam-se a amplificação randômica de DNA polimórfico (RAPD, *Random Amplified Polymorphic DNA*); a amplificação de região com sequência caracterizada (SCAR, *Sequence Characterized Amplified Region*); polimorfismo de fragmentos de DNA amplificados (AFLP, *Amplified Fragment Length Polymorphism*); polimorfismo de fragmentos por ação de

enzimas de restrição (RFLP, *Restriction Fragment Length Polymorphism*); amplificação de regiões entre simples sequências repetidas (ISSR, *Inter Simple Sequence Repeat*); os microssatélites ou amplificação de simples sequências repetidas (SSR, *Simple Sequance Repeat*) e o polimorfismo de um único nucleotídeo (SNP, *Single nucleotide polymorphysm*) (COSTA, MAFRA, OLIVEIRA, 2012a; BEN-AYED, KAMOUN-GRATI, REBAI, 2013; PASQUALONE et al., 2016).

Entretanto, nem todos os marcadores têm demonstrado potencial para aplicação em azeite de oliva. Na sequência, são descritos os princípios, as vantagens e desvantagens dos marcadores mais utilizados e com maior potencial de aplicação para a análise de azeite de oliva.

3.3.1 RAPD - Amplificação randômica de DNA polimórfico (*Random Amplified Polymorphic DNA*)

Esta técnica consiste na amplificação de regiões aleatórias de DNA, utilizando uma sequência de *primers* genéricos. Os *primers* podem se anelar em diferentes regiões do DNA alvo, podendo, portanto, gerar uma quantidade diversa de sequências, tanto em número como em tamanho. Ao final, avalia-se o padrão de amplificação dos fragmentos gerados. Assim, quando se compara a presença ou ausência de determinados fragmentos em diferentes amostras, pode-se estabelecer a relação de diversidade entre as amostras (WILLIAMS et al., 1990). A técnica possui muitas vantagens, sendo a principal delas a de poder ser aplicada sem informações genéticas prévias do organismo de estudo, além de ser simples, rápida e barata (COSTA, MAFRA, OLIVEIRA,

2012; BEN-AYED, KAMOUN-GRATI, REBAI, 2013; PASQUALONE et al., 2016).

Em azeite de oliva, marcadores do tipo RAPD são utilizados para estudos de variabilidade e correlação genética entre indivíduos e populações. Em geral, os trabalhos relatam baixa reprodutibilidade destes métodos para aplicação em rastreabiliade e combate a fraudes (MUZZALUPO, PERRI, 2002; BEN-AYED, KAMOUN-GRATI, REBAI, 2013).

3.3.2 SSR – Amplificação de simples sequências repetidas (*Simple Sequence Repeat*)

Este marcador consiste em pequenas sequências repetidas de DNA (2-6pb) encontradas ao longo do genoma. De forma semelhante ao RAPD, os *primers* se anelam nas sequências repetidas por todo o genoma. Entretanto, no caso dos SSR as sequências nucleotídicas devem ser conhecidas e altamente específicas. O padrão de amplificação gerado permite estabelecer a relação de diversidade de forma comparativa entre as amostras. Dentre as vantagens do uso de SSR estão o alto grau de polimorfismo detectado e a alta especificidade (COSTA, MAFRA, OLIVEIRA, 2012; BEN-AYED, KAMOUN-GRATI, REBAI, 2013; PASQUALONE et al., 2016).

Este tem sido o marcador molecular mais utilizado em azeite de oliva. A maioria dos SSR é utilizado para avaliar o grau de variabilidade genética entre as cultivares. A partir destas informações é possível estabelecer certa rastreabilidade quanto aos azeites de oliva monovarietais (TESTOLIN, LAIN, 2002; ALBA et al., 2009; VIETINA et al., 2011; BEN-AYED et al., 2012;

PASQUALONE, et al., 2012; MONTEMURRO et al., 2015; RAIETA, MUCCILLO, COLANTUONI, 2015).

3.3.3 SNP – Polimorfismo de um único nucleotídeo (*Single nucleotide polymorphysm*)

Os SNPs consistem de variações de um único nucleotídeo em uma região do genoma, quando comparadas sequências de diversos organismos relacionados. Deste modo, a partir do alinhamento de diversas sequências homólogas é possível desenhar *primers* específicos, capazes de identificar apenas a sequência de interesse. A possibilidade de desenhar um par de *primers* altamente específico é a principal vantagem deste marcador. Entretanto, para isto, é necessário um conhecimento prévio das sequências dos genes com os quais se pretende trabalhar e esta é uma das principais desvantagens (COSTA, MAFRA, OLIVEIRA, 2012; BEN-AYED, KAMOUN-GRATI, REBAI, 2013). Vale ressaltar que atualmente as bases de dados, como NCBI (National Center for Biotechnology Information), disponibilizam a sequência de muitos genes, o que minimiza a desvantagem deste marcador.

A utilização de SNPs tem se mostrado muito efetiva para a determinação de autenticidade, tanto para azeites monovarietais como para outros óleos e azeites adicionados ao azeite de oliva (TESTOLIN, LAIN, 2002; BAZAKOS et al., 2012; KALOGIANNI et al., 2015; BAZAKOS et al., 2016).

3.4 ANÁLISE DOS FRAGMENTOS DE DNA

As sequências de DNA, após amplificadas e/ou fragmentadas, necessitam ser analisadas. As ferramentas disponíveis para a análise dos

fragmentos de DNA se baseiam geralmente em três princípios, a análise da presença e do tamanho dos fragmentos e a análise direta ou indireta da composição química dos fragmentos. A seguir, são destacadas as ferramentas de análise de DNA mais utilizadas em estudos com azeite de oliva.

3.4.1 Eletroforese

A técnica de eletroforese consiste na separação das moléculas de acordo com a difusão através de um gel, utilizando um campo elétrico como força motriz. Normalmente, como suporte de difusão, utiliza-se gel de agarose ou poliacrilamida e como as moléculas de DNA têm carga negativa, difundem-se para o polo positivo. Além disso, a separação também está relacionada com o tamanho dos fragmentos a serem analisados, de modo que os fragmentos menores difundem-se mais rapidamente que os fragmentos maiores. A visualização das bandas de DNA ocorre devido a moléculas que intercalam-se na fita de DNA e fluorescem sob luz ultravioleta (SLATER et al., 2000).

Com o objetivo de aumentar a capacidade de operação e a viabilidade de aplicação, bem como a resolução da separação, foi desenvolvida a eletroforese capilar. Neste caso, o suporte de difusão é fixado na parede de um capilar, e o campo elétrico é aplicado nas extremidades deste capilar. Para realizar a detecção dos fragmentos, na extremidade final do capilar encontra-se um detector de fluorescência. Este detector emite um sinal sempre que um fragmento de DNA cruza o campo ótico do detector. Tanto na eletroforese em gel plano, quanto na eletroforese capilar, é possível determinar a concentração de fragmentos de uma determinada banda, por meio da intensidade do sinal fluorescente (HELLER, 2001).

Para a análise de azeite de oliva, a eletroforese é aplicada sempre que se deseja avaliar os padrões, tanto de amplificação quanto de fragmentação, do DNA de uma amostra. Deste modo, geralmente a análise por eletroforese está associada a marcadores moleculares como RAPD e SSR (MUZZALUPO, PERRI, 2002; ZHANG et al., 2012; BEN-AYED et al., 2012; RAIETA, MUCCILLO, COLANTUONI, 2015; BAZAKOS et al., 2016.)

3.4.2 PCR em Tempo Real (Real Time-PCR)

Esta técnica consiste, basicamente, no acompanhamento em tempo real da reação de PCR. Neste sentido, utiliza-se uma molécula fluorescente que se intercala na molécula de DNA, sendo o SYBR Green o mais utilizado. Desta maneira, quanto maior for a concentração de DNA no meio, mais fluorescente ele ficará. Na prática, a reação inicia-se sem fluorescência e, à medida que são geradas cópias do DNA alvo, a fluorescência aumenta. Como a reação ocorre exponencialmente, a fluorescência também aumenta exponencialmente. Quanto maior a quantidade de DNA alvo no início da reação, mais cedo a fluorescência será detectada, de modo que o número de ciclos necessários para que a fluorescência seja detectada é diretamente proporcional à concentração inicial de DNA. Construindo uma curva de calibração, transforma-se a PCR em tempo real em PCR quantitativa (REBRIKOV, TROFIMOV, 2006).

A PCR em tempo real geralmente está associada a ensaios de diagnóstico, por meio da amplificação com *primers* específicos. Desta maneira, apresenta-se como a ferramenta mais apropriada para a detecção de fraudes, uma vez que a resposta é bastante específica e rápida (GIMÉNEZ et al., 2010;

DEBODE et al., 2012; HE et al., 2013). Outro segmento onde a PCR em tempo real vem sendo utilizada é na quantificação de expressão gênica, permitindo a comparação fenotípica entre as cultivares e/ou espécies (BEN-AYED, KAMOUN-GRATI, REBAI, 2013).

3.4.3 Análise da curva de dissociação (*Melting curve*)

Nesta análise, avalia-se a dissociação da dupla fita de DNA de acordo com o incremento de temperatura. Deste modo, obtém-se a temperatura de dissociação, ou *melting point*, que corresponde à temperatura na qual 50% das fitas de DNA amplificadas dissociam-se. Esta temperatura é influenciada tanto pelo tamanho das fitas de DNA como também pela composição de nucleotídeos da mesma, uma vez que T-A (Timina e Adenina) interagem por duas ligações de hidrogênio, enquanto C-G (Citosina e Guanina) por três. Assim, fragmentos que possuem maiores concentrações de C e G apresentarão maiores temperaturas de dissociação do que fragmentos de mesmo tamanho que apresentem maiores conteúdos de A e T. Esta técnica é muito utilizada em associação à PCR em tempo real e à amplificação de regiões específicas, possibilitando a caracterização do fragmento amplificado (WITTWER, 2009; GANOPOULOS et al., 2013; VIETINA et al., 2011; MONTEMURRO et al., 2015).

Por fim, é importante ressaltar que as técnicas de biologia molecular estão em constante desenvolvimento. Portanto, a associação de diferentes métodos, bem como a implementação de novas técnicas têm amplo potencial de aplicação acadêmico, fiscal e industrial.

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ARTIGO 1 - New Approach on Nut Oils Characterization: Almond, Hazelnut and Pecan Nut

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New Approach on Nut Oils Characterization: Almond, Hazelnut and Pecan Nut.

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Abstract

The aim of this work was to characterize the major and minor compounds of laboratory extracted and commercial oils from sweet almond, hazelnut and pecan nut. Oils from sweet almond, hazelnut and pecan nut were obtained by means of an expeller system, while the corresponding commercial oils were provided from Vital Âtman (BR). The content of triacylglycerols, fatty acids, aliphatic and terpenic alcohols, desmethyl- methyl- and dimethylsterols, squalene and tocopherols were determined. Oleic, palmitic and linoleic acids were the main fatty acids. Desmethylsterols were the principal minor compounds being β-sitosterol the most abundant component. Low amounts of aliphatic and terpenic alcohols were also found. The major tocopherol in hazelnut and sweet almond oils was α-tocopherol, whereas γ-tocopherol

prevailed in pecan nut oil. Principal component analysis made it possible for us to differentiate among samples, as well as to distinguish between commercial and laboratory extracted oils. Heatmap highlighted the main variables featuring each sample. All these results together have brought a new approach on nut oil characterization.

Keywords: Sweet almond; Hazelnut; Pecan nut; Chemical characterization.

Introduction

Nuts belong to various plant families, although they have special common features such as high oil content and large seed size, when compared to other oilseed species. Almonds (*Prunus dulcis*, family Rosaceae), hazelnuts (*Corylus avellana*, family Betulaceae) and pecan nuts (*Carya illinoinensis*, family Juglandaceae) are part of the main group of tree nuts and nut oil sources. In many parts of the world, such as the Mediterranean countries and North America, tree nuts are besides an important oil crop, an essential dietary component, acting as sources of energy and functional nutrients. Actually, nut oils have been widely enjoyed for food applications, mainly due their particular flavour and, more recently, because of their relationship with health-promoting effects. Besides, tree nut oils are also widely used in the cosmetic industry [1, 2].

The almond group is composed of two species, namely *Prunus dulcis* (sweet almonds) and *Prunus amara* (bitter almond). Almond oil is extracted mainly from sweet almonds, which contain around of 50% oil. This extraction is commercially conducted by cold press and/or solvent extraction [3]. According to FAO [4], USA is the main almond producer in the world (~37% of the total

production) followed by Spain (~11%) and Australia (~7%). Chemically speaking, sweet almond oil has been described as an unsaturated oil, being oleic acid (O, C18:1) the main fatty acid (~65%)[5], with β-sitoterol as the most representative sterol, and α-tocopherol as the major tocopherol [6, 7]. Table 1 shows the detailed composition of almond oil based on bibliographic research [1-3,5-7].

Hazelnut (*Corylus avellana*) is a nut included in the Mediteranean diet, whose main producer worldwide is Turkey, with just about 72% of the total production in 2012 [8]. Hazelnut kernels contain about 60% oil, which is obtained by cold press and/or solvent extraction. Hazelnut oil has been frequently compared to olive oil due their similar compositions: oleic acid as the main fatty acid and β-sitoterol as the main minor compound (Table 1) [9, 10]. Actually, hazelnut oil is commonly used in the cosmetic industry, although its current prominence as health-promoting oil has increased its use as food and even gourmet oil.

Pecan nut (*Carya illinoinensis*) originates from USA but is well adapted in several countries including Australia, South Africa and different parts of South America. Nowadays, more than 80% of the total world production is produced in the USA. The nut contains 70% oil, which is easily extracted by means of an expeller-press. Also in pecan nuts, oleic acid is the predominant fatty acid (~60%), and curiously, γ-tocopherol has been reported as the main tocopherol, as can be seen in Table 1 [11-13].

Nut oils are getting an outstanding position as gourmet and health-promoting oils, both for their sensory characteristics of flavor, and for their nutritional characteristics. Actually, as far as the nutritional characteristics are

concern, the high amount of oleic acid, as well as phytosterols, allow frequent comparisons of these oils with olive oil. However, nut oils have also been indicated as inducers of allergic reactions in consumers [14].

The aim of this work is to characterize the major and minor compounds of laboratory extracted and commercial nut oils from sweet almond, hazelnut and pecan nut in order to increase knowledge about the chemical composition of nut oils as well as to establish, based on a statistical approach, the main compounds which would allow distinguishing among oil samples and their origin.

Table 1: Bibliographic information on the chemical composition of almond, hazelnut and pecan nut oils. References [1-3, 5-7, 9, 11, 26-33]

Nut Oil		Almond	Hazelnut	Pecan Nut
Oil Amount (g/.100g^{-1})		25.1 - 60.7	8.10 - 67	58-74
Fatty Acid Composition (%area)	Myristic acid - 14:0	0 - 0.07	0 - 0.1	0.05 - 0.09
	Palmitic acid - 16:0	4.7 - 15.8	4.85 - 6.50	6.49 - 7.64
	Palmitoleic acid - 16:1	0.1 - 2.5	0.16 - 0.3	0.11 - 0.21
	Stearic acid - 18:0	0.3 - 2.5	2.7- 3.80	2.23 - 2.80
	Oleic acid - 18:1	50.4 - 81.2	76.30 - 82.7	49.6 - 62.1
	Linoleic acid - 18:2	6.21- 37.1	6.50 - 14.00	27.2 - 37.71
	Linolenic acid - 18:3	0 - 11.1	0.10 - 0.50	1.47 - 1.94
	Arachidic acid - 20:0	0.04 - 0.2	0 - 0.20	0.34
Triacylglycerol Composition (%area)	LLLn		0.1	-
	LLL	8.7	3.7	-
	OLLn	0.1	0.5	-
	OLLn	27.6	12.3	-
	OLnO	-	0.7	-
	LLP	4.8	1.6	-
	OLO	28.0	28.2	-
	LOP	11.3	5.2	-
	PLP	0.5	0.2	-
	OOO	13.3	36.5	-
	SLO	1.8	1.4	-
	OOP	2.7	6.1	-
	SOO	0.6	2.8	-
Total Sterols mg.kg^{-1}		2178 - 2777	1096 - 6031	1899
Sterol Composition (%area)	Cholesterol	-	0.8 - 2.3	-
	24-methylene-cholesterol	-	0 - 0.1	-
	Campesterol	2.5	4.8 - 7.4	2.7
	Campestanol	-	0 - 0.2	-
	Stigmasterol	2.5	1.3 - 2.1	17.9
	Δ7-campesterol	-	0 - 0.4	-
	Δ5,23-stigmastadienol+ Clerosterol	-	0.9 - 1.3	-
	β-Sitosterol	55.9 - 95.1	78.1 - 90.4	82.8
	Sitostanol	-	1.8- 3.6	-
	Δ5-avenasterol	8.5 - 28.2	1.3 - 5.2	-
	Δ5,24-stigmastadienol	-	0.3 - 1.1	-
	Δ7-Stigamastenol	-	0.3 - 2.3	-
	Δ7-avenasterol	-	0.5 - 1.9	-
Squalene mg.100g^{-1}		95.0	186.0 - 371.0	152.0
Total Tocopherol mg.100g^{-1}		451.0	25.8 - 69.8	180.0
Tocopherol and Tcotrienol composition (%of total content)	α - tocopherol	97.3	53.8 - 90.6	12.0
	β - tocopherol	-	2.1 - 4.2	-
	γ - tocopherol	2.8	3.1 - 41.9	168.0
	δ - tocopherol	-	-	-
	α - tocotrienol	-	0 - 7.1	-

P - palmitic acid; S - stearic acid; O - oleic acid; L - linoleic acid; Ln - linolenic acid
- Not determined and/or evaluated by the authors

Material and Methods

Chemicals

Acetone, diethyl ether, hexane, propionitrile, and tetrahydrofuran (THF) were supplied by VWR International (West Chester, PA, USA). Silica solid phase extraction (Si-SPE) cartridges were from Varian (EA Middelburg, The Netherlands). Potassium hydroxide (KOH) was from Panreac (Montcada I Reixac, Barcelona, Spain). Hexamethyldisilazane, pyridine, trimethylchloroxilane and standards of α, γ, β, and δ-tocopherol were from Merck (Merck Group, Darmstadt, Germany). Standards of 5-α-cholestan-3β-ol, squalane and n-eicosanol were from Sigma-Adrich Co. (St. Louis, MO, USA). All chemical reagents were at least analytical grade.

Samples

Nuts from sweet almonds, hazelnuts and pecan nuts were obtained in local grocery stores in Brazil. Commercial nut oils from sweet almonds, hazelnut and pecan nut were provided by Vital Âtman (Uchoa, SP, Brazil). Both nuts and nut oils were properly stored at 4°C until extraction and analysis.

Extraction of nut oils

For each extraction, 1000 g nuts were milled in a knife mill and the obtained products were taken to an expeller press system, Komet Oil Press (IBG Monforts Oekotec GmbH & Co.KG., Germany) and then filtered through filter paper to remove any solid material. The oil obtained was stored at 4°C until analysis.

Chemical Characterization

Fatty acid composition

The fatty acid composition was determined according to IUPAC Standard Methods [15, 16], as the composition of fatty acid methyl esters (FAME) by GC. Trans-esterification of the oils was carried out with KOH in methanol at a concentration of 2 mol/L. The chromatographic analysis was done using an Agilent 5890 GC system (Agilent Technologies, Santa Clara, California) equipped with an automated liquid sampler (1 µL injections), split injector (1:50 split ratio), polar capillary column (SPTM-2380 – poly(90% biscyanopropyl−10% cyanopropylphenyl)siloxane, 60m × 0.25mm internal diameter (i.d.) x 0.20µm film thickness) and flame ionization detector (FID). Hydrogen was used as carrier gas at a flow rate of 1.0 mL/min. The initial oven temperature was 180 ºC, and the temperature gradient was from 180 ºC to 220 ºC at 3 ºC/min. The detector and injector temperatures were 225 ºC and 250 ºC, respectively. Peak identification was carried out by means of comparation with a standard chromatogram provided by method. Data were described as fatty acid profiles by peak area normalization.

Triacylglycerol composition

This determination was done following the procedure established by Moreda, Pérez-Camino and Cert [17]. For oil purification, a Si-SPE cartridge was washed without vacuum with 6mL hexane. After that, a solution of the oil (0.12g) in 0.5mL hexane was added. The solution was pulled through the cartridge and then eluted with 10mL hexane-diethylether (87:13 v/v) solution.

The eluted solvents were evaporated to dryness under reduced pressure at room temperature. The residue was dissolved in 2mL acetone. For triacylglycerol (TAG) analysis 10µL of this solution was injected directly, using the auto-sampler (508 system), in a RP-HPLC system. The separations were done on a Merck Li-Chrospher 100 RP-18 column (250mm × 4mm i.d. x 4µm particle size) thermostated at 20°C. The liquid chromatograph (Beckman Coulter, Fullerton, CA, USA) was equipped with a pumping unit (118 solvent module) and propionitrile was used as mobile phase at a flow rate of 0.6mL/min. Detection was done with a PerkinElmer 200 RI detector. Identification of triacylglycerol peaks were done by comparation with chromatograms stablished by authors' method. Data were processed by peak area normalization and expressed as TAG percentage.

Sterol composition and aliphatic alcohols

Sterols and aliphatic alcohols are components of the unsaponifiable fraction. Therefore, removing the saponifiable compounds previously to their determination is essential. In this line, the samples were analyzed according to the methodology proposed by the International Olive Council [18, 19]. Summarizing: 5g of oil was saponified with 50 mL ethanolic KOH solution, at a concentration of 2 mol/L, during 1 h under reflux. The unsaponifiable compounds were then extracted with diethyl ether (3 x 80mL) and the organic phase washed with distillated water until complete neutralization. After drying, unsaponifiable matter was fractionated by thin layer chromatography (TLC) using silica plates impregnated with KOH. The plate was developed twice with a mixture of petroleum ether:diethyl ether (87:13, v/v). After separation, three

bands could be observed, corresponding to desmethylsterols, methylsterols together with aliphatic alcohols, and dimethylsterols. Each band was then scratched off and extracted with hot chloroform and diethyl ether. The solutions were evaporated until dryness, derivatized with 500 µL of a 1:3:9 (v/v/v) trimethylchloroxilane:hexamethyldisilazane:pyridine solution, and analyzed by GC. The gas chromatograph (Agilent 6890N) was equipped with an automated liquid sampler (1 µL injections), split injector (1:50 split ratio), a fused silica low-polarity capillary column (poly(5% diphenyl−95% dimethyl)siloxane, 30 m × 0.25 mm i.d. × 0.25 µm film thickness) and FID. The oven program for the determination of desmethylsterols was set isothermally at 260 °C. Hydrogen was used as carrier gas at a flow rate of 1 mL/min. For the analysis of the second and the third fractions a temperature gradient was applied: starting at 220 °C (2 min) until 295 °C at 2 °C/min. The temperatures of the injector and detector were 300 °C. The quantitative determinations were done using the internal standards α-cholestanol for desmethylsterols and n-eicosanol for aliphatic alcohols, methyl- and dimethylsterols. Data were always expressed as the total (mg/kg of oil) of each compound class, and the profile of each class was described as the percentage of area of each compound within the class, according to method recommendation. Peak assignments were carried out by retation time calculation and comparation with chromatogram as decribed in each method.

Squalene

This procedure derives from that published previously [20]. Oil samples (0.04 g) together with 40µL internal standard (squalane 5 mg/mL) were

dissolved in 1mL hexane and saponified at room temperature with 200 µL methanolic KOH at a concentration of 2 mol/L. After separation (by gravity), the upper phase was washed with 3 x 400 µL ethanol:water 1:1, v/v, and 1 µL of the supernatant analyzed by GC. GC analyses were carried out with an Agilent 6890N Gas Chromatograph (Agilent Technologies, Santa Clara, California) equipped with an automatic liquid sampler, split injection (20:1), and a FID. The conditions for the GC assays were: DB5-HT column (5% diphenyl-95% dimethylpolysiloxane); 30m x 0.25mm i.d. x 0.10µm film; (Agilent Technologies, Santa Clara, California), hydrogen carrier gas at 0.8mL/min. The oven worked isothermally at 250ºC for 10 minutes. The injector and detector temperatures were 300ºC and 345ºC, respectively. Peak identification was conduced by rtation time calculation, based on internal standard. The quantitative evaluation was carried out using squalane as internal standard, and the data expressed in mg/kg of oil.

Tocopherols

Tocopherols were determined following IUPAC Standard Method 2432 [21], according to which 10 mg oil was diluted in 1 mL hexane and directly injected in a liquid chromatograph with a Si-column (250mm × 4mm i.d. x 4µm particle size). The elution solvent was a mixture of hexane:2-propanol (99:1, v/v) at a flow rate of 1mL/min. Detection was done by means of a RF-10AXL Shimadzu fluorescence detector, setting excitation at λ = 290 nm and emission at λ = 330nm. Analytical curve for quantitative and qualitative determinations was performed by mean of injections of tocopherol standards at concentrations

between 4-6 µg/mL in hexane. Results were expressed in mg/kg of oil for each tocopherol compound.

Stigmastadiene

In order to verify the presence of refined oils, stigmastadienes were determined only in commercial samples. The method described in COI/T.20/Doc. no. 11 was followed for this determination [22]. Oil samples (20g) together with 1 mL internal standard solution (3,5-cholestadien, 20 µg/mL) were saponified with 75 mL alcoholic KOH (10%) during 30 min under reflux. The unsaponifiable compounds were then extracted with hexane (2 x 100 mL), and the organic phase neutralized washing it with an ethanol-water (1:1) solution. The solvent was then evaporated until dryness in a rotary evaporator at 30 °C. After this preparation, the unsaponifiable matter obtained was fractionated by silica gel column chromatography using hexane as mobile phase. The first 30 mL eluated were discarded and the following 40 mL were collected, dried and injected in the chromatograph. The GC system was an Agilent 6890N Gas Chromatograph (Agilent Technologies, Santa Clara, California) equipped with an automatic liquid sampler, split injection (15:1), and a FID. The parameters for the GC assays were: DB5-HT column (5% diphenyl-95% dimethylpolysiloxane; 30 m x 0.25 mm i.d. x 0.10 µm film; Agilent Technologies, Santa Clara, California), 1.0 µL injection volume and hydrogen as carrier gas at 1mL/min. The injector and detector temperatures were 300 ºC and 320 ºC, respectively. The oven temperature program was: 235 °C for 6 minutes, rising at 2°C/min up to 285°C. For peak identification were evaluated the retation time along with comparation with the standard chromatogram decribed in the method.For

quantitative evaluation, 3,5-cholestadiene was used as internal standard. Data were expressed in mg/kg.

Statistical analysis

In order to verify significant differences ($p<0.05$) among samples, for each feature, an ANOVA test was accomplished in Metaboanalyst 3.0 web-based tool [23]. After that, multivariate statistical analysis was performed with full information of the chemical characterization; data files were saved as .csv format and uploaded into the Metaboanalyst 3.0 web-based tool. A principal component analysis (PCA) was performed considering all features and their relationship. To make the features more comparable a range scaling (mean-centered and divided by the value range of each variable) was applied.

Additionally, cluster hierarchical analysis was also performed, using a word clustering algorithm and a Euclidean distance measure. The cluster was then plotted with a heat map composed by the 15 most important characteristics of the samples. These characteristics were selected by random forest analysis using random features selection from a bootstrap sample until the best grouping was reached.

Results and Discussion

Major component, fatty acid and triacylglycerol profiles.

Fatty acids composition is the most common feature for fat and oil characterization. It is related to oxidative stability as well as to some nutritional characteristics. Fatty acid profile has been widely described for almonds, hazelnuts and pecan nuts, as Table 1 shows. However, in this work it was possible to bring some new information about this feature (Table 2), as it is the case of the description of some isomers present in each sample. According to ANOVA analysis the amount of ω-9 palmitoleic acid (C16:1ω-9),ω-7 and ω-11 oleic acids (C18:1ω-7, C18:1ω-11), behenic acid (C22:0), and lignoceric acid (C24:0) did not differ statistically among the samples ($p>0.05$), whereas for all other fatty acids a statistical difference was found ($p\leq0.05$) for this set of samples.

For all the samples, ω-9 oleic acid (C18:1ω-9) was the main fatty acid, being its concentration around 80% in hazelnut, 70% in pecan nut and 60% in sweet almond. Other isomers of oleic acid like C18:1ω-7 and C18:1ω-11 were also found, although their sum never reached 2% of the total fatty acids. In the case of palmitoleic acid isomers, their sums did not exceed 1% in each sample, and C16:1ω-7 was the most abundant one. Linoleic acid (C18:2ω-6) was the second most abundant fatty acid, with the highest presence in sweet almond, almost 30%, followed by pecan nut with around 20%, and hazelnut with approximately 10%.

Table 2 provides the complete fatty acid composition for both sweet almond and hazelnut. These results are in general within the ranges showed

Table 1. Exceptions can be found regarding pecan nut, where lower amounts of palmitic (C16:0), linoleic (C18:2ω-6), and linolenic (C18:3ω-3) acids were found, as well as higher concentrations of stearic (C18:0) and oleic (C18:1) acids. Although statistically different, the fatty acid compositions of the three nut oils are quite similar, reminding the similarities between olive and avocado oils, both of them fruit oils [24, 25].

Although the fatty acid composition is quite similar the distributions of the fatty acids in the triacylglycerol molecules are very different (Table 2). While in hazelnut and pecan nut oil samples there is a clear predominance of PLP+OOO+PoPP, in sweet almond samples this predominance is equally shared between PLP+OOO+PoPP and OOL+LnPP. Another particularity of the sweet almond is the amount of OLL, near 18%, whereas it does not reach 5 % and 10% in hazelnut and pecan nut, respectively.

The presence of SOL (around 12%) is a particularity of hazelnut and it does not exceed 3% in sweet almond and pecan nut, in contrast to POO, which does not surpass 1% in hazelnut, and is around 7% and 8% in sweet almond and pecan nut, respectively. Fatty acid and TAG profiles are complementary feature since TAG profile shows how fatty acids are grouped.

Table 2: Fatty acid and triacylglycerol composition of Sweet almond, Hazelnut and Pecan nut oil laboratory and commercial samples.

Sample		Extracted Sweet Almond Oil		Commercial Sweet Almond Oil		Extracted Hazelnut Oil		Commercial Hazelnut Oil		Extracted Pecan Nut Oil		Commercial Pecan Nut Oil	
Fatty Acid Composition %area													
Lauric - 12:0		0.02	0.00	ND	-	ND	-	ND	-	ND	-	ND	-
Miristic - 14:0		0.04	0.00	0.04	0.00	0.03	0.00	0.03	0.00	0.04	0.00	0.03	0.00
Pentadecanoic - 15:0		0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00
Palmitic - 16:0		6.60	0.08	6.62	0.02	5.57	0.04	5.55	0.01	5.61	0.07	5.57	0.02
Palmitoleic ω9 - 16:1 ω9		0.03	0.00	0.03	0.00	0.03	0.00	0.03	0.00	0.03	0.00	0.02	0.00
Palmitoleic ω7 - 16:1 ω7		0.43	0.00	0.43	0.00	0.19	0.00	0.23	0.00	0.07	0.00	0.08	0.00
Palmitoleic ω11 - 16:1 ω11		0.02	0.00	0.02	0.00	0.01	0.00	0.01	0.00	ND	-	ND	-
Margaric - 17:0		0.05	0.00	0.05	0.00	0.05	0.00	0.03	0.00	0.07	0.00	0.07	0.00
Margaroleic - 17:1		0.12	0.00	0.12	0.00	0.08	0.00	0.07	0.00	0.06	0.00	0.06	0.00
Stearic - 18:0		1.34	0.02	1.33	0.00	2.48	0.01	1.85	0.02	2.87	0.02	2.58	0.01
Oleic ω9 - 18:1 ω9		60.22	0.59	59.70	0.20	80.64	0.12	78.34	0.10	67.55	0.22	70.96	0.01
Oleic ω7 - 18:1 ω7		1.17	0.86	1.69	0.06	1.44	0.06	1.80	0.10	1.31	0.08	1.22	0.02
Oleic ω11 - 18:1 ω11		0.16	0.03	0.20	0.02	0.21	0.02	0.13	0.04	0.27	0.10	0.16	0.04
Linoleic trans - 18:2 t		0.02	0.00	0.02	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00
Linoleic - 18:2 ω6		29.58	0.13	29.54	0.14	8.76	0.02	11.53	0.06	20.67	0.06	17.97	0.08
Linoleic isomer - 18:2		ND	-	ND	-	0.05	0.00	0.04	0.01	0.19	0.01	0.12	0.01
Linolenic - 18:3 ω3		0.04	0.00	0.04	0.00	0.11	0.01	0.09	0.00	0.80	0.01	0.70	0.01
Arachidic - 20:0		0.07	0.00	0.07	0.00	0.13	0.00	0.10	0.00	0.12	0.00	0.12	0.00
Gondoic - 20:1		0.07	0.00	0.06	0.00	0.17	0.00	0.15	0.00	0.29	0.00	0.28	0.00
Behenic - 22:0		0.02	0.00	0.02	0.00	0.03	0.00	0.02	0.00	0.02	0.00	0.02	0.00
Lignoceric - 24:0		ND	-	0.01	0.00	ND	-	ND	-	ND	-	0.01	0.00
TAG Composition %area													
LLL	ECN 42	2.88	0.07	2.93	0.04	0.90	0.02	0.81	0.00	2.63	0.03	2.19	0.08
OLLn + PoLL		0.25	0.03	0.29	0.03	0.08	0.03	0.08	0.00	0.68	0.01	0.58	0.04
PLLn		0.11	0.01	0.10	0.02	ND	-	ND	-	0.14	0.04	0.17	0.03
OLL	ECN 44	18.05	0.07	18.04	0.45	3.42	0.65	4.55	0.03	9.71	0.20	8.33	0.05
OOLn + PoOL		0.49	0.03	0.38	0.13	0.57	0.12	0.32	0.05	0.99	0.05	1.02	0.21
PLL + PoPoO		2.49	0.01	2.29	0.23	0.63	0.20	0.55	0.01	1.74	0.09	1.67	0.26
POLn + PPoPo + PPoL		0.38	0.08	0.27	0.03	ND	0.00	0.05	0.01	0.27	0.07	0.17	0.01
OOL + LnPP	ECN 46	26.34	0.17	26.76	0.16	14.04	0.79	18.89	0.33	23.24	0.28	21.11	0.26
PoOO+SLL + PLO		10.75	0.17	10.78	0.20	3.55	0.10	3.60	0.26	6.03	0.16	5.99	0.40
PoOP + SPoL + POLn + SPoPo		0.34	0.06	0.34	0.01	0.42	0.17	0.57	0.06	0.16	0.03	0.38	0.26
PLP + OOO + PoPP	ECN 48	27.39	0.29	27.21	0.26	55.68	2.22	53.63	0.04	38.75	0.11	42.89	0.69
SOL		1.96	0.05	1.89	0.26	13.41	0.32	12.05	0.12	2.60	0.14	2.19	0.19
POO		7.00	0.29	6.96	0.14	ND	-	0.33	0.00	8.12	0.16	8.51	0.20
POP	ECN 50	0.17	0.00	0.23	0.02	1.08	0.54	0.57	0.01	0.50	0.05	1.12	0.07
SOO		1.40	0.05	1.53	0.17	5.68	0.88	3.66	0.02	4.07	0.27	3.35	0.10
POS + SSL	ECN 52	ND	-	ND	0.00	0.53	0.08	0.32	0.04	0.36	0.04	0.32	0.03

Bold numbers - mean; Normal numbers - standard deviation; ND - not detected

P - palmitic acid; Po - palmitoleic acid; S - stearic acid; O - oleic acid; L - linoleic acid; Ln - linolenic acid

Minor compounds, Unsaponifiable matter

Minor components are commonly known as the fingerprint of some vegetable oils. In this way, they have been widely related to oil identity in many regulations [24]. Sterols are the most abundant class of compounds in the unsaponifiable matter [20]. In this work, desmethyl-, methyl- and dimethylsterols have been analyzed. Desmethylsterols are the most commonly analyzed group.

Total amount of desmethylsterols was higher for both commercial and extracted sweet almond oils. As expected, β-sitosterol was the main desmethylsterol, followed by Δ5-avenasterol, which was higher in pecan nut oil (around 15%) than in sweet almond and hazelnut oils, around 9 % and 5%, respectively (Table 3). Among all samples, cholesterol and stigmasterol concentrations did not show significant statistical differences ($p>0.05$).

In the case of methysterols (Table 3), citrostadienol is the main species in all samples. Sweet almond oil shows higher obtusifoliol presence (reaching 28%) than Hazelnut and Pecan nut oils, whose concentrations do not exceed 20% and 10%, respectively. Total amount of methysterols is more than three times bigger in pecan nut oil samples than in the other samples. Regarding dimethysterol, the total amount in pecan nut oil was more than six times higher than in the other samples, reaching 200 $mg.kg^{-1}$, while in sweet almond and hazelnut oils it was around 30 $mg.kg^{-1}$. For Sweet almond and hazelnut oils the main dymethysterol was 24-methylencycloartanol, followed by butyrospermol in hazelnut oil, while in sweet almond oil the profile changed between commercial and extracted samples. However, in pecan nut oilsamples it is possible to see a very particular behavior, since in pecan nut oil samples the main dimethylsterol was cycloartenol, reaching 70% of the total dimethylsterols.

Squalene is a terpenic hydrocarbon, precursor of sterols, which has been highlighted due its health benefits [20]. In general, the total amount of squalene in commercial oils was always higher than in laboratory extracted samples (Table 3).

Table 3: Minor compounds of Sweet almond, Hazelnut and Pecan nut oil samples: desmethyl-, methyl- and dimethylsterols, terpenic alcohols, squalen, aliphatic alcohols, and tocopherols.

Sample	Extracted Sweet Almond Oil		Commercial Sweet Almond Oil		Extracted Hazelnut Oil		Commercial Hazelnut Oil		Extracted Pecan Nut Oil		Commercial Pecan Nut Oil	
Desmethylsterols % area												
Cholesterol	**0.25**	0.03	**0.13**	0.02	**0.22**	0.01	**0.18**	0.01	**0.35**	0.06	**0.16**	0.01
Brasicasterol	**ND**	-	**0.06**	0.03	**ND**	-	**ND**	-	**ND**	-	**ND**	-
24-methylene-cholesterol	**0.04**	0.00	**0.03**	0.00	**ND**	-	**ND**	-	**0.29**	0.00	**0.30**	0.01
Campesterol	**2.46**	0.02	**2.53**	0.02	**5.21**	0.04	**4.85**	0.02	**4.45**	0.00	**4.16**	0.03
Campestanol	**0.13**	0.04	**0.09**	0.01	**0.39**	0.01	**0.26**	0.01	**0.09**	0.00	**0.12**	0.06
Stigmasterol	**0.91**	0.00	**0.86**	0.01	**0.91**	0.01	**0.88**	0.03	**0.90**	0.01	**0.86**	0.01
Δ7-campesterol	**0.78**	0.47	**0.83**	0.00	**ND**	-	**ND**	-	**ND**	-	**ND**	-
Δ5,23-stigmastadienol+Clerosterol	**1.40**	0.05	**1.37**	0.00	**0.96**	0.02	**0.81**	0.03	**0.89**	0.04	**1.06**	0.00
β-Sitosterol	**77.42**	0.37	**77.28**	0.13	**81.39**	0.03	**81.79**	0.03	**75.51**	0.09	**74.86**	0.01
Sitostanol	**1.91**	0.09	**1.52**	0.04	**3.54**	0.09	**2.55**	0.03	**0.57**	0.05	**0.36**	0.01
Δ5-avenasterol	**9.89**	0.03	**9.93**	0.04	**4.37**	0.04	**5.44**	0.03	**14.94**	0.03	**15.81**	0.09
Δ5,24-stigmastadienol	**1.48**	0.02	**1.64**	0.04	**0.83**	0.02	**1.02**	0.02	**1.07**	0.00	**1.27**	0.03
Δ7-Stigamastenol	**1.94**	0.04	**2.30**	0.00	**1.45**	0.04	**1.32**	0.04	**0.24**	0.01	**0.40**	0.00
Δ7-avenasterol	**1.39**	0.03	**1.44**	0.01	**0.73**	0.01	**0.89**	0.01	**0.69**	0.01	**0.65**	0.00
Total mg.kg^{-1}	**2870.48**	29.24	**2804.31**	21.85	**1787.82**	2.94	**1807.72**	5.28	**1791.44**	14.58	**1746.01**	3.21
Methylsterols %area												
Obtusifoliol	**26.57**	0.35	**28.81**	0.17	**18.53**	0.30	**14.39**	0.67	**6.82**	0.17	**6.48**	0.15
Gramisterol	**13.46**	0.16	**12.18**	0.58	**6.72**	1.28	**5.83**	0.24	**13.88**	0.25	**8.98**	0.02
Citrostadienol	**59.96**	0.00	**59.02**	1.22	**74.75**	1.11	**79.78**	1.85	**79.31**	0.15	**84.54**	0.56
Total mg.kg^{-1}	**29.73**	0.57	**33.86**	0.65	**43.09**	0.10	**39.85**	0.40	**110.78**	2.76	**111.09**	2.81
Dimethylsterols %area												
Dammaradienol	**7.44**	0.66	**6.37**	0.68	**2.05**	0.32	**3.04**	0.55	**0.17**	0.02	**0.56**	0.01
Taraxerol	**4.55**	0.30	**3.90**	0.62	**9.38**	0.48	**9.02**	0.46	**2.15**	0.24	**3.06**	0.11
α+β Amyrina	**24.97**	1.01	**6.89**	1.22	**5.13**	0.39	**5.61**	0.10	**3.76**	0.17	**3.62**	0.07
Butyrospermol	**ND**	-	**ND**	-	**29.37**	0.20	**28.21**	0.68	**6.15**	0.02	**1.55**	0.04
Cycloartenol	**11.91**	0.24	**18.67**	1.48	**7.49**	0.41	**8.19**	0.12	**78.69**	0.03	**75.58**	0.31
24-methylencycloartanol	**51.13**	0.42	**64.18**	1.41	**46.58**	0.45	**45.94**	0.81	**9.08**	0.05	**15.64**	0.08
Total mg.kg^{-1}	**28.35**	4.16	**32.57**	2.55	**29.20**	0.45	**26.11**	1.43	**214.69**	11.51	**192.51**	1.28
Squalene mg.Kg^{-1}	**96.43**	0.59	**113.11**	5.37	**340.05**	1.39	**431.72**	5.34	**298.83**	5.10	**320.81**	1.63
Terpenic Alcohols %area												
Phytol	**71.65**	0.09	**73.00**	1.26	**49.58**	7.04	**55.66**	1.04	**70.68**	1.01	**44.60**	1.15
Geranylgeraniol	**28.35**	0.09	**27.00**	1.26	**50.42**	7.04	**44.34**	1.04	**29.32**	1.01	**55.40**	1.15
Total mg.kg^{-1}	**9.74**	0.36	**9.61**	0.63	**26.54**	4.06	**20.37**	2.42	**66.57**	0.65	**36.64**	0.12
Aliphatic Alcohols %area												
C22-OH	**23.13**	0.62	**11.29**	2.05	**3.71**	0.57	**8.30**	0.67	**8.56**	0.31	**15.91**	0.60
C23-OH	**2.56**	0.24	**1.46**	0.34	**ND**	-	**ND**	-	**ND**	-	**ND**	-
C24-OH	**29.66**	0.90	**19.29**	6.26	**20.21**	0.48	**16.43**	1.58	**21.30**	0.24	**39.60**	2.87
C25-OH	**7.70**	0.24	**7.37**	3.11	**4.47**	0.05	**0.00**	0.00	**3.36**	0.17	**6.25**	0.76
C26-OH	**40.31**	1.83	**30.81**	18.03	**55.10**	1.91	**33.93**	2.64	**13.16**	0.04	**24.47**	1.75
C27-OH	**ND**	-	**ND**	-	**9.38**	0.22	**41.31**	1.00	**ND**	-	**ND**	-
C28-OH	**ND**	-	**ND**	-	**3.96**	0.05	**3.42**	0.50	**55.22**	2.29	**11.37**	0.29
Total mg.kg^{-1}	**5.55**	0.27	**6.05**	1.69	**31.70**	0.20	**12.40**	0.33	**24.88**	0.95	**12.85**	0.03
Tocopherols mg.kg^{-1}												
α-Tocopherol	**233.40**	0.40	**236.06**	4.71	**227.95**	9.81	**205.35**	13.22	**5.08**	0.00	**5.07**	0.13
β-Tocopherol	**8.45**	0.45	**8.52**	0.30	**9.97**	0.16	**7.12**	0.70	**ND**	-	**ND**	-
γ-Tocopherol	**10.10**	0.10	**10.12**	0.37	**102.00**	0.61	**8.26**	0.42	**306.34**	2.83	**197.09**	2.08
δ-Tocopherol	**0.87**	0.07	**0.91**	0.15	**5.31**	0.13	**0.78**	0.08	**2.38**	0.14	**1.57**	0.18
Total mg.kg^{-1}	**252.83**	1.03	**255.60**	5.53	**345.22**	9.23	**221.51**	14.42	**313.80**	2.70	**203.73**	1.77

Bold numbers - mean; Normal numbers - standard deviation; ND - not detected

Terpenic alcohols presence in Sweet almond oil was below 10 mg.kg^{-1}, whereas it went beyond 20 mg.kg^{-1} in hazelnut oil and 30 mg.kg^{-1} in pecan nut oil. Actually, it was higher than 60mg/kg in the extracted sample of

pecan nut. In the case of aliphatic alcohols in Sweet almond samples the total amount did not exceed 7 $mg.kg^{-1}$, while in Hazelnut and Pecan nut oils the total amount was above 12 $mg.kg^{-1}$, being the content in extracted samples higher than in commercial oils (Table 3). Differences can be noted in the profile of both terpenic and aliphatic alcohols, and seem to be related to sample processing and origin, although there are not concrete evidences to prove it. Tocotrienols were not present in any of the samples, however, the total amount of tocopherols exceeded 200 $mg.kg^{-1}$ in all cases, with no statistical difference ($p>0.05$). The amount of δ-tocopherol was not statistically different as well. When it comes to sweet almond and hazelnut oils, the main compound was α-tocopherol (Table 3), the same as in most vegetable oils. Regarding Pecan nut samples, the main tocopherol was γ-tocopherol, which is also present in walnut and corn oils [20].

Statistical grouping analysis

A principal component analysis (PCA) was performed in order to establish a statistical relationship among samples. FIGURE 1 shows results plotted by means of principal component PC1 vs. PC2 (1A) and PC2 vs. PC3 (1B). PC1 explain 47.3% of the sample variances while PC2 and PC3 explain 35.8 % and 6.4%, respectively. According to the results on Figure 1A, PC1 clearly isolates sweet almond samples (PC1 < 0 zone) from hazelnut and pecan nut oils (PC1 >0 zone), while PC2 separates hazelnut (PC2 > 0 zone) from pecan nut samples (PC2 < 0 zone). Figure 1B reflects the important influence of PC3, which disconnects the extracted (PC3 > 0 zone) from the commercial (PC3 < 0 zone) samples.

Cluster hierarchical analysis results (FIGURE 2) reaffirm the closer relationship between hazelnut and pecan nut oils, while sweet almond oil is clearly isolated. Observing the heatmap in Figure 2, there is a reflex of previous results and discussion, once it highlights the 15 main compounds responsible for the distinction of the samples, considering the relationship of all samples and features. In this way, for sweet almond samples obtusifoliol, damaradienol, C16:1ω-7 acid, OLL+LnPP, C18:2t acid, and Δ5,24-stigmastadienol were the main features selected through random forest analysis. For pecan nut oil, C20:1 acid, cycloartenol, Δ5-avenasterol, OLL+PoLL, PLLn, and C17:0 acid were the most important characteristics, whereas hazelnut oil, β-sitosterol, C18:1ω-9 acid, and campesterol were the main ones.

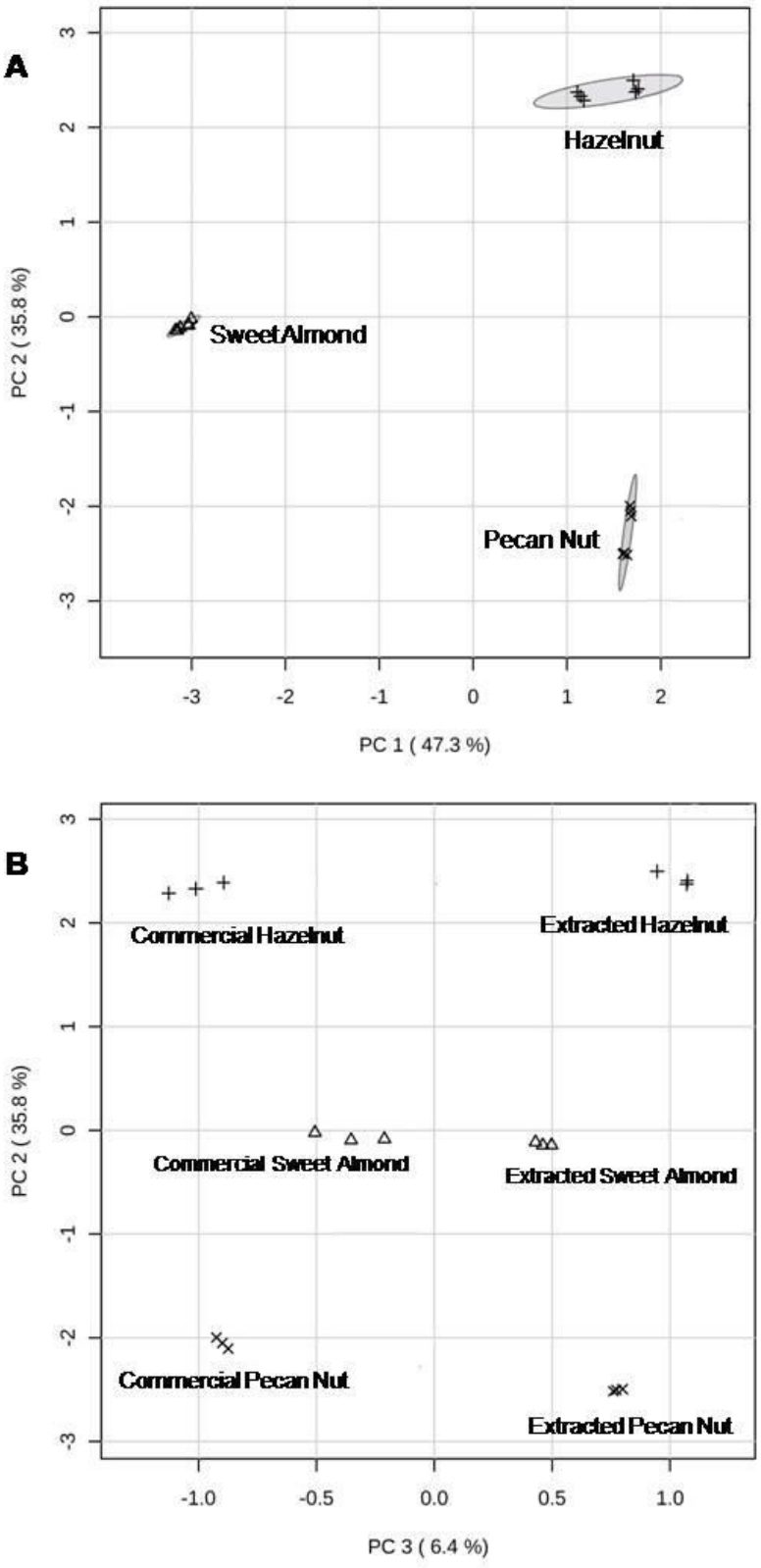

Figure 1: Principal component analysis score plot for all analyzed samples. PC1 x PC2 (A); PC2 x PC3 (B).

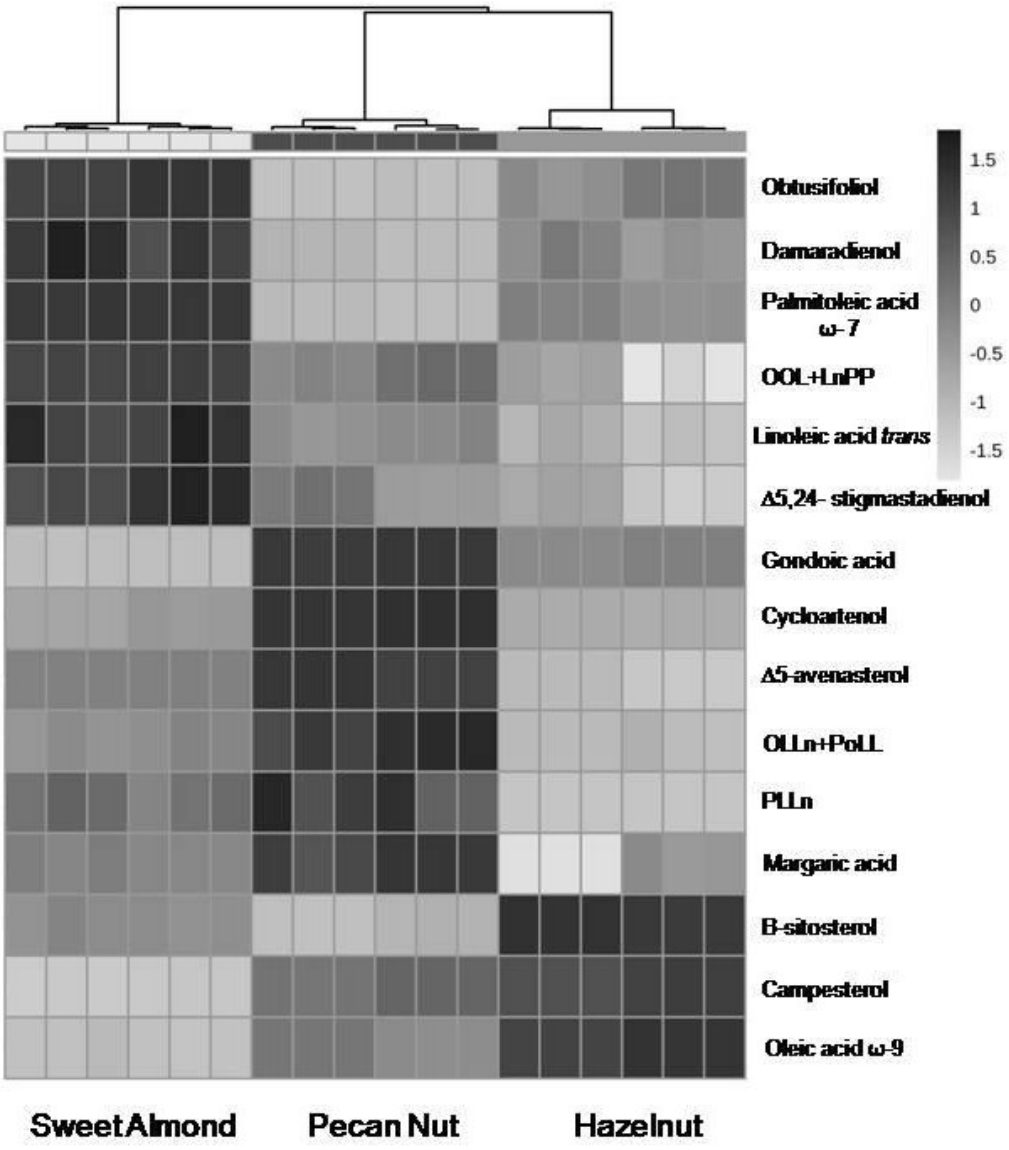

Figure 2: Cluster hierarchical analysis and heat map of the fifteen more important features forsample grouping, selected by Random forest statistical tool.

Conclusion

Oils from sweet almond, hazelnut and pecan nut are monounsaturated fats whose fatty acid composition is dominated by oleic acid. For the first time the presence of ω-7, ω-9, and ω-11 isomers of palmitoleic and oleic acids have been described in these kinds of oils. In general, when TAG are formed, those with oleic acids prevail, even though a distinctive profile could be described for Sweet almond oil, in a way that this may help to differentiate it from hazelnut and pecan nut oils.

β-sitosterol (desmethylsterols) was the main minor compound found, and the amount of Δ5-avenasterol one of the most distinguishing features of pecan nut oil. The high concentrations of total methyl- and dimethylsterols were important particularities of Pecan nut oils, as well as the higher quantity of γ-tocopherol, since in hazelnut and sweet almond oils the main tocopherols was α-tocopherol.

This work also describes for the first time the presence in nut oils of other minor compounds like terpenic and aliphatic alcohols.

Using multivariate statistical analysis it was possible to establish relationships among samples, and carry out sample grouping. In this sense, hazelnut oils resulted to be chemically closer to pecan nut oils than to sweet almond oils. From these data analysis it was also possible to differentiate commercial from extracted oils. Heatmap highlighted the components that are more important for the distinction of the samples considering the relationship among all samples and features.

This work brings a new approach on the characterization of nut oils. It performs a complete characterization of minor and major identity parameters, as well as establishing the relationship among samples and features.

All this information may be taken in account for establishing regulatory recommendations and laws.

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ARTIGO 2 - Chemical Characterization of Monovarietal and Commercial Avocado Oils

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Chemical Characterization of Monovarietal and Commercial Avocado Oils

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Abstract

This work aimed to characterize the major and minor compounds of monovarietal and commercial avocado oils. Monovarietal oils from the Bacon, Fuerte, Hass, and Pinkerton cultivars were obtained by means of an Abencor® system, while commercial oils from Brazil, Chile, Equator and New Zealand, were locally purchased. The content of triacylglycerols, fatty acids, aliphatic and terpenic alcohols, desmethyl- methyl- and dimethylsterols, squalene and tocopherols were determined. The main triacylglycerols were those with ECN48. Besides, oleic, palmitic and linoleic fatty acids prevailed. Desmethylsterols were the principal minor compounds. Low amounts of aliphatic and terpenic alcohols were also found. Squalene concentrations were higher in Bacon, Fuerte and Pinkerton oils than in the other oils. The most abundant tocopherol was α-tocopherol. Principal component analysis made it possible to differentiate between commercial and monvarietal oils. Heat map highlighted the main variables featuring to each sample. Finally, this work brings a new approach on avocado oil characterization.

Keywords: Avocado oils, chemical characterization, monovarietal oils, commercial oils.

1. Introduction

Avocado oil is extracted from the pulp of *Persea americana* fruits. In the fruit the oil is stored as a single oil droplet in the idioblast cells, which are dispersed in the mesocarp (pulp). The idioblast wall structure is very complex, and it is basically composed by two cellulose layers separated by a suberin layer (Platt & Thomson, 1992). This morphology hampers oil extraction. Recently, avocado oil studies have been focused on its healthy and nutritional characteristics, which are mainly due to its high content in monounsaturated fatty acids such as oleic acid (Lerman-Garber, Ichazo-Cerro & Zamora-Gonzáles, 1994).

According to FAO, Mexico is the world's largest producer. In 2012, this country produced 1.31 million tons of avocado fruits, around 30% of the world stock (FAOSTAT, 2013). However, the avocado oil industry grows slowly because this is just a side-industry of the fresh-fruit business.

Taxonomically, *P. americana* species is separated in three distinct races, commonly known as Mexican, Guatemalan and West Indian (Popenoe, 1941). Over many years of cultivation the crossing of the three races resulted in the development of a large variety of avocado cultivars, however, due to differences in the oil content, only a few of them are used as industrial oil sources (Tango, Carvalho & Soares, 2004). In this way, normally Hass (from Guatemala race) and Fuerte (from the crossing of Guatemala and Mexican races) varieties are commonly utilized for oil extraction, with an oil contents

around of 30%. Among others cultivars with potential application in oil industry we can cite Pinkerton and Bacon varieties, both coming from Guatemalan and Mexican race crossing (Mohameed, et al., 1997; Ashworth & Clegg, 2003).

Although several methods have been proposed for avocado oil obtaining, including enzyme, solvent and supercritical fluid extraction (Freitas, Yahya & Lago, 1998; Moreno, Dorantes, Galíndez & Guzmán, 2003; Botha, 2004; Ortiz, Dorantes, Gallndez & Cárdenas, 2004), the market trend toward more “natural” oils has put them aside. Actually, it is cold pressed virgin oils what consumers are looking for. In this way, the industrial extraction of avocado oil has become quite similar to that of olive oil, being the main differences related to skin removal, stoning and malaxation, which in avocado oil extraction is conducted at higher temperature (45 ºC) and during longer time (>60 min) than in olive oil obtaining. These changes are needed due to the obvious differences between the avocado and the olive fruits. Besides, the structure of idioblast cells also makes oil extraction more difficult than in the case of the olive mesocarp. The final pulp-water-oil separation is made through two centrifugation steps by means of a decanting and a polish centrifuge (Eyres, Sherpa & Hendriks, 2001; Wong, Requejo-Jackman & Woolf, 2010).

The fatty acid composition of avocado oil has been reported in several studies, describing oleic acid (C18:1ω9) as the main one. This fact has been used as support for studies that want to prove the beneficial health effects of avocado oil, such as reduction of diabetes effects, oxidative stress on mitochondrial membrane and cardiovascular disease markers (Ortiz-Avila, et al., 2013; Carvajal-Zarrabal, Nolasco-Hipolito, Aguilar-Uscanga, Melo-Santiesteban, Hayward-Jones & Barradas-Dermitz, 2014).

In order to achieve the standardization of avocado oil, Woolf and coworkers (2009) proposed a classification according to processing and quality parameters: extra virgin, high quality category with low sensory defects; virgin, with low quality features and some sensory defects; pure, a kind of refined avocado oil; and blend, when the oil is mixed to other vegetable oils. These authors suggested a typical fatty acid (FA) profile composed by palmitic (C16:0) 10-25%, palmitoleic (C16:1) 2-8%, stearic (C18:0) 0.1-0.4%, oleic (C18:1) 60-80%, linoleic (C18:2) 7-20%, and linolenic (C18:3) 0.2-1% acids. In addition, the tocopherol content was established to be between 70-190 $mg \cdot kg^{-1}$. However, in our bibliographic search we found other ranges for this identity parameters: palmitic 10.0-35.2%, palmitoleic 2.8-16.1%, stearic 0.2-1.5%, oleic 36.9-74%, linoleic 6.1-21.2%, and linolenic 0.3-2.1% acids, together with tocopherols 130-200 $mg \cdot kg^{-1}$ (Tango, Costa, Antunes & Figueiredo, 1972; Canto, Santos & Travaglini, 1980; Werman & Neeman, 1987; Martínez Nieto, Barranco-Barranco & Moreno-Romero, 1992; Moreno, Dorantes, Galíndez & Guzmán, 2003; Tango, Carvalho & Soares, 2004; Salgado, Danieli, Regitano-D'arce, Frias & Mansi, 2008). Other features have also been cited like the sterol composition and content, with β-sitosterol as the main sterol (71.8–93.05%) and low amounts of clerosterol, avenasterol, campesterol and sitostanol, and the triacylglycerol (TAG) profile, where OOP, OOO and OOL (O, oleic acid; P, palmitic acid; L, linoleic acid) were the most abundant ones (Hierro, Tomás, Fernández-Martín & Santa-María, 1992; Martínez Nieto, Barranco-Barranco & Moreno-Romero, 1992; Salgado, Danieli, Regitano-D'arce, Frias & Mansi, 2008). A depth study focused on the composition of major and minor compounds of avocado oil has not been carried out yet.

Therefore, the aim of this work is to characterize the major and minor compounds of monovarietal avocado oils from Bacon, Fuerte, Hass and Pinkerton cultivars and commercial avocado oils from Brazil, Chile, Equator and New Zealand, so as to increase knowledge about the chemical composition of avocado oil.

2. Material and Methods

2.1 Chemicals

Acetone, diethyl ether, hexane, propionitrile, and tetrahydrofuran (THF) were supplied by VWR International (West Chester, PA, USA). Potassium hydroxide (KOH) was from Panreac (Montcada I Reixac, Barcelona, Spain). Silica solid phase extraction (Si-SPE) cartridges were from Varian (EA Middelburg, The Netherlands). Standards of 5-α-cholestan-3β-ol, squalane and n-eicosanol were from Sigma-Adrich Co. (St. Louis, MO, USA). Hexamethyldisilazane, pyridine, trimethylchloroxilane and standards of tocopherols were from Merck (Merck Group, Darmstadt, Germany). All chemical reagents were at least analytical grade.

2.2 Samples

Fruit samples of Bacon, Fuerte, Hass and Pinkerton cultivars were provided by Instituto de Hortofruticultura Subtropical y Mediterranea La Mayora (IHSM-CSIC, Málaga-Spain). Commercial avocado oil samples from has variety were obtained in local grocery stored from Brazil, Chile (2, named “Chile A” and “Chile B”), Equator, and New Zealand. They were properly storage at 4°C until analysis.

2.3 Extraction of monovarietal avocado oils

For each extraction, 500 g mature avocados, without seeds, were milled in a knife mill and the paste was taken to extraction by means of an Abencor® system malaxer and centrifuge (MC2 Ingenierıa Sistemas, Seville, Spain) followed by an additional centrifugation step. Malaxation was carried out below 40°C during 40min, with talc addition (~10g.100g^{-1} paste). Distillated

water, (20mL.100g^{-1} paste) was added after 10 min of starting the malaxation process. The first centrifugation was carried out in the Abencor® system centrifuge at 3000rpm during 60s. The paste was then split out and the liquid phase was further centrifuged in a bench centrifuge (5000rpm, 10min). The oil obtained was filtered and stored at 4°C until analysis.

2.4 Chemical Characterization

2.4.1 Fatty acid composition

The fatty acid composition was determined as the composition of fatty acid methyl esters (FAME) by GC, according to IUPAC Standard Methods (IUPAC, 1987a,b). Transesterification of the oils was carried out with 2N KOH in methanol. The chromatographic analysis was done using an Agilent 5890 GC system (Palo Alto, CA, USA) equipped with split injector (1:50 split ratio), automated sampler (1μL injections), polar capillary column (SPTM-2380 – poly(90% biscyanopropyl−10% cyanopropylphenyl)siloxane, 60m × 0.25mm internal diameter (i.d.) x 0.20μm film thickness) and FID. Hydrogen was used as carrier gas at a flow rate of 1.0mL·min^{-1}. The detector and injector temperatures were 225 and 250°C, respectively. The initial oven temperature was 180°C, and the temperature gradient was from 180 to 220°C at 3°C·min^{-1}. Data were described as fatty acid profile by peak area normalization. Peak identification was made by comparation with the standard chromatogram provided by method.

2.4.2 Triacylglycerol composition

This determination was done according to Moreda, Pérez-Camino and Cert (2003). Oil samples were purified using a Si-SPE cartridge. The cartridge was washed without vacuum with 6mL hexane. After that a solution of the oil (0.12g) in 0.5mL hexane was added. The solution was pulled through the cartridge and then eluted with 10mL hexane-diethylether (87:13 v/v) solution. The eluted solvents were evaporated to dryness under reduced pressure at room temperature. The residue was dissolved in 2mL acetone. For (TAG) analysis this solution (10µL) was injected directly using the auto-sampler (508 system) in a RP-HPLC system. The separations were done on a Merck Li-Chrospher 100 RP-18 column (250mm × 4mm i.d. x 4µm particle size) thermostated at 20°C. The liquid chromatograph (BeckmanCoulter, Fullerton, CA, USA) was equipped with a pumping unit (118 solvent module) and propionitrile was used as the mobile phase at a flow rate of 0.6mL·min^{-1}. Detection was done with a PerkinElmer 200 RI detector. Triacilglycerol peak assignment was carried out by means of comparation with elution time and standards chromatograms described by the authors. Data were processed by peak area normalization and expressed as TAG percentage.

2.4.3 Sterol composition and aliphatic alcohols

Sterols and aliphatic alcohols are minor components of the oil unsaponifiable fraction. Therefore, it is advisable to remove the saponifiable compounds previously in order to get better analytical results. In this line we followed the methodology proposed by the International Olive Council (IOC, 2013; IOC 2015). In short: samples of 5g of oil were saponified under reflux with

50mL 2N ethanolic KOH solution during 1h. The unsaponifiable compounds were then extracted with diethyl ether (3 x 80mL) and the organic phase neutralized by means of washing it with distillated water. The residue (unsaponifiable matter) was fractionated by silica TLC using plates impregnated with potassium hydroxide. The plate was developed twice with a mixture of petroleum ether:diethyl ether (87:13, v/v). Three fractions were obtained: desmethylsterols, methylsterols together with aliphatic alcohols, and dimethylsterols. Each of them was scratched off and extracted with hot chloroform and diethyl ether. The solution was evaporated until dryness, derivatized with 500µL of the 1:3:9 (v/v/v) trimethylchloroxilane:hexamethyldisilazane:pyridine admixture, and analyzed by GC. The gas chromatograph (Agilent 6890N) was equipped with a fused silica low-polarity capillary column (poly(5% diphenyl−95% dimethyl)siloxane, 30m × 0.25mm i.d. × 0.25µm film thickness), and FID. The oven program for the determination of desmethylsterols (first fraction) was set isothermally at 260°C, with a 1:50 split ratio. Hydrogen was used as carrier gas at a flow rate of 1mL·min−1. For the second and the third fractions a temperature gradient was applied: starting at 220°C (2min) until 295°C at 2°C.min-1. The temperatures of the injector and detector were 300°C. The quantitative determination was done using the internal standards α-cholestanol for desmethylsterols and n-eicosanol for aliphatic alcohols, methyl- and dimethylsterols. Data were always expressed as the total (mg.kg-1) of each compound class, and the profile of each class was described as the percentage of each compound within the class, according to method recommendation. Peak identification was performed by retation time calculation and comparation with chromatogram as decribed in each method.

2.4.4 Squalene

The procedure derives from that published previously (Lanzón, Guinda, Albi & De la Osa, 1995; Gómez-Coca, Pérez-Camino & Moreda, 2015). 0.04 g Oil together with 40µL internal standard (squalane 5mg·mL^{-1}) were dissolved in 1mL hexane and saponified at room temperature with 200µL 2N methanolic KOH. Two phases appear, and after washing the upper part with 3 x 400µL ethanol:water 1:1, v/v, 1µL of the supernatant was analyzed by GC. Gas chromatography analyses of squalene were carried out with an Agilent 6890N Gas Chromatograph (Agilent Technologies, Santa Clara, California) equipped with an Agilent 7683B Automatic Liquid Sampler and a flame ionization detector (FID). Acquisition of data was done with the Agilent ChemStation for GC System program. The conditions for the GC assays were: DB5-HT column (5% diphenyl-95% dimethylpolysiloxane; 30m x 0.25mm i.d. x 0.10µm film; Agilent Technologies, Santa Clara, California), 1.0µL injection volume, hydrogen carrier gas at 0.8mL·min^{-1} and 20:1 split injection. The oven temperature program was: 250ºC for 10 minutes. The injector and detector temperatures were 300ºC and 345ºC, respectively. The quantitative evaluation of squalene was carried out using squalane as internal standard, and the data expressed in mg·kg^{-1}. For peak identification were evaluated the retation time along with comparation with the standard chromatogram decribed in the method

2.4.5 Tocopherols

Tocopherols were determined according to IUPAC Standard Method 2432 (1987c). Oil samples were diluted in hexane (10mg·mL^{-1}) and directly injected in a liquid chromatograph fitted with a Si-column (250mm × 4mm i.d. x

4µm particle size). The elution solvent was a hexane:2-propanol (99:1, v/v) mixture at a flow rate of 1mL·min-1. Detection was done by fluorescence (RF-10AXL Shimadzu fluorescence detector), setting excitation and emission at λ = 290 and λ = 330nm, respectively. For quantitative determinations a calibration curve was performed by mean of injections of tocopherol standards at concentrations between 4−6µg·mL^{-1} in hexane and the data, corresponding to each tocopherol compound, expressed in mg.kg^{-1}. The retation tme of standards was used for qualitative analysis.

2.4.6 Stigmastadiene

Stigmastadienes were determined only in the commercial samples in order to verify the presence of refined oils. The method described in COI/T.20/Doc. no. 11 was used on this determination (IOC, 2001). Thus, 20g oil together with 1mL internal standard solution (3,5-cholestadien, 20µg.m^{-1}) were saponified under reflux with 75mL alcoholic KOH (10g.100g^{-1}) during 30min. The unsaponifiable matter was then extracted with hexane (2 x 100mL), and the organic extract washed with a ethanol-water (1:1) solution until neutral pH. The solvent was then evaporated until dryness in a rotary evaporator at 30°C. After this preparation the residue (unsaponifiable matter) was fractionated in a silica column using hexane as mobile phase. The first eluate (30mL) was discarded and the following one (40mL) collected, dried and injected in the chromatograph. The GC system utilized was an Agilent 6890N Gas Chromatograph (Agilent Technologies, Santa Clara, California) equipped with an Agilent 7683B Automatic Liquid Sampler and FID. The parameters for the GC assays were: DB5-HT column (5% diphenyl-95% dimethylpolysiloxane; 30m

x 0.25mm i.d. x 0.10µm film; Agilent Technologies, Santa Clara, California), 1.0µL injection volume, hydrogen carrier gas at 1mL.min-1 and 15:1 split injection. The oven temperature program was: 235°C for 6 minutes, then rising at 2°C.min-1 up to 285°C. The injector and detector temperatures were 300ºC and 320ºC, respectively. The quantitative evaluation of 3,5-stigmastadien was carried out using 3,5-cholestadieneas internal standard. Data acquisition was done with the Agilent ChemStation for GC System program. Data were expressed in mg.kg-1. Peak identification was conduced by rtation time calculation, based on internal standard.

2.5 Statistical analysis

A multivariate statistical analysis was performed with full information of the chemical characterization; the tables of data were saved as .csv files and uploaded into the Metaboanalyst 3.0 web-based tool (Xia, Mandal, Sinelnikov, Broadhurst, & Wishart, 2012). A principal component analysis (PCA) was performed finding the directions that best explain the variance in a data set. The data are summarized in fewer variables called components, which are the weighted average of the original variables. A range scaling (mean-centered and divided by the value range of each variable) was used to make the features more comparable.

In order to select the most important features for sample grouping we used the random forest tool, which builds grouping trees using random features selection from a bootstrap sample until the best grouping is reached. In this work we plotted the heat map with the ten more important features to distinguish the sample.

3. Results and Discussion

3.1 Major compounds, fatty acids and triacylglycerols profile.

Fatty acid profile is the most common parameter for oil characterization. It is widely described in many books and legislation (IOC, 2015; CX/FO, 2013a,b). Table 1 shows the fatty acid profile of all avocado oil samples. As expected, the main fatty acid was oleic acid (C18:1ω9), which reached the highest concentration in Fuerte cultivar (64.62±0.20%). In contrast, the lowest oleic acid presence was found in the Brazilian commercial avocado oil (45.18±0.10%). The C18:1ω7 isomer was also observed (7.87±0.55% - 10.08±0.32%). Palmitc acid (C16:0) was the second most abundant fatty acid, within a range between 11.64±0.13% (Chile A) and 21.05±0.06% (Brazil). Linoleic acid (C18:2) fluctuated between 8.25±0.02% in Pinkerton and 16.50±0.04% in Chile B, while palmitoleic acid (C16:1ω7) varied from 3.99±0.05% in Chile B to 11.41±0.02% in Brazil. The ω9 and ω11 isomers of palmitoleic acid were also detected. However, the ω7 isomer was always above 91% of the sum of the three of them. Another important characteristic is the C18:1ω9/C18:1ω7 ratio, which was always above 5. The presence of C18:1 and C16:1 isomers (ω7, ω9 and ω11) in avocado oil was firstly described in this work.

All results obtained in fatty acid composition are within the ranges already described for avocado oil, cited in the Introduction. However, looking at the quality standards proposed by Woolf and coworkers (2009) some results do not match with those described in this work. Brazilian sample stands out due to three parameters (oleic, palmitoleic, and stearic acids), while Chile A and B are

different as far as linolenic and stearic acids are concerned. On the other hand, Equator, Fuerte and Pinkerton samples differed from the published data due to their stearic acid content. Therefore, since the samples are in accordance with other's work and even some of the parameters measured for some of the monovarietal samples are out of the established ranges, is possible to consider the need of more comprehensive studies and probably improving the standard table.

Distribution of fatty acids in TAG is another important identity parameter related with the saponifiable fraction of lipids. The experimental TAG profiles are described in Table 1. For all samples the ECN48 and ECN46 TAG groups were the most abundant ones. Brazil sample had the most different profile due the high amount of palmitic and palmitoleic fatty acids. TAG profile of Brazil sample was very particular, being the peak corresponding to POO+SOL the biggest one (21.14±0.98%), followed by OOO+PLP+PoPP (18.47±0.94%) and almost by equal amounts of PoOP+SPoL+POLn+SPoPo (10.48±0.31%) and SLL+PLO (10.44±0.33%). For the other samples the highest peaks matched OOO+PLP+PoPP, and ranged from 27.61±0.20% in Hass to 40.67±0.40% in Fuerte. Other important TAG were POO+SOL, which varied between 17.72±0.31% in Chile B and 24.98±0.35% in Chile A, and OLL+LnPP which ranged from 8.72±0.10% to 16.98±0.25%. Although Brazil sample was the most different one, its TAG profile was closer than those of the other samples to those given by Hierro, Tomás, Fernandez-Martin and Santa-María (1992). Finally, the TAG profiles of samples other than Brazil cultivar were very close to the TAG profile of olive oil described by Moreda and coworkers (2003).

Table 1: Fatty acids and triacylglycerols compositions of avocado oil samples.

Sample		Monovarietal Avocado Oil								Commercial Avocado Oil									
		Bacon		Fuerte		Hass		Pinkerton		Brazil		Chile A		Chile B		Equator		New Zealand	
Fatty Acid Profile %area																			
Miristic - 14:0		0.02	0.00	0.03	0.00	0.03	0.00	0.03	0.00	0.04	0.00	0.04	0.01	0.06	0.00	0.03	0.00	0.03	0.00
Pentadecanoic - 15:0		0.02	0.00	0.02	0.00	0.02	0.00	0.02	0.00	0.01	0.00	0.03	0.01	0.04	0.01	0.03	0.00	ND	-
Palmitic - 16:0		12.16	0.04	12.37	0.01	18.17	0.02	16.93	0.03	21.05	0.06	11.64	0.13	12.65	0.10	16.17	0.18	13.39	0.17
Palmitoleic w-9 - 16:1 w9		0.09	0.00	0.11	0.00	0.09	0.00	0.05	0.00	0.10	0.00	0.16	0.05	0.13	0.01	0.09	0.00	0.10	0.00
Palmitoleic w-7 - 16:1 w7		6.57	0.01	4.03	0.01	7.58	0.00	7.33	0.05	11.41	0.02	3.13	0.09	3.99	0.05	6.54	0.09	4.83	0.06
Palmitoleic w-11 - 16:1 w11		0.10	0.00	0.08	0.00	0.12	0.00	0.08	0.00	0.22	0.01	0.14	0.08	0.13	0.02	0.13	0.02	0.08	0.00
Margaric - 17:0		0.02	0.01	0.02	0.00	0.01	0.00	0.08	0.07	0.03	0.00	0.05	0.03	0.02	0.01	0.02	0.01	0.03	0.02
Margaroleic - 17:1		0.10	0.00	0.08	0.00	0.08	0.00	0.10	0.00	0.08	0.00	0.10	0.01	0.08	0.00	0.08	0.00	0.10	0.01
Margaroleic Isomer - 17:1		0.01	0.00	0.01	0.00	0.03	0.00	0.01	0.00	0.03	0.00	0.01	0.01	0.02	0.00	ND	-	ND	-
Stearic - 18:0		0.38	0.01	0.51	0.01	0.37	0.00	0.43	0.01	0.51	0.00	0.79	0.04	0.50	0.03	0.56	0.01	0.37	0.03
Oleic w-9 - 18:1 w9		61.72	0.30	64.62	0.20	51.76	0.04	57.39	0.18	45.18	0.10	61.86	0.22	55.98	0.09	57.14	0.29	61.54	0.45
Oleic w-7 - 18:1 w7		9.83	0.22	8.95	0.17	9.80	0.06	8.53	0.17	7.89	0.02	7.87	0.55	8.44	0.12	8.68	0.13	10.08	0.32
Oleic w-11 - 18:1 w11		ND	-	ND	-	ND	-	ND	-	0.17	0.02	ND	-	ND	-	ND	-	ND	-
Linoleic trans - 18:2 t		ND	-	ND	-	ND	-	ND	-	0.01	0.00	0.04	0.02	ND	-	ND	-	ND	-
Linoleic - 18:2		8.30	0.02	8.46	0.02	11.12	0.01	8.25	0.02	12.30	0.05	12.73	0.10	16.50	0.04	9.78	0.03	8.80	0.07
Linolenic - 18:3		0.44	0.00	0.47	0.00	0.59	0.00	0.56	0.00	0.62	0.00	1.03	0.01	1.15	0.00	0.54	0.00	0.49	0.01
Arachidic - 20:0		0.04	0.00	0.05	0.00	0.04	0.00	0.04	0.00	0.07	0.00	0.11	0.01	0.07	0.02	0.06	0.01	0.04	0.01
Gondoic - 20:1		0.19	0.00	0.18	0.00	0.18	0.00	0.16	0.00	0.20	0.00	0.22	0.00	0.20	0.03	0.17	0.02	0.12	0.05
Behenic - 22:0		0.01	0.00	ND	-	ND	-	ND	-	0.03	0.00	0.06	0.02	0.03	0.01	ND	-	ND	-
Lignoceric - 24:0		ND	-	ND	-	ND	-	ND	-	0.05	0.00	ND	-	ND	-	ND	-	ND	-
TAG Profile %area																			
Unknown I		0.04	0.01	0.04	0.00	0.05	0.00	0.03	0.01	0.16	0.01	ND	-	ND	-	0.11	0.01	0.10	0.02
LLL		0.32	0.08	0.28	0.00	0.40	0.00	0.24	0.01	0.66	0.04	0.84	0.03	1.09	0.04	0.41	0.03	0.37	0.07
OLLn	ECN 42	0.40	0.09	0.20	0.01	0.50	0.01	0.34	0.00	1.00	0.08	0.58	0.00	1.17	0.11	0.36	0.03	0.33	0.02
PoLL		0.08	0.02	0.06	0.00	0.12	0.02	0.12	0.00	0.28	0.00	0.15	0.01	0.25	0.07	0.10	0.02	0.07	0.03
PLLn		0.03	0.03	0.00	0.00	0.06	0.01	0.09	0.03	0.09	0.01	0.13	0.01	0.24	0.01	0.03	0.01	ND	-
OLL		1.36	0.10	1.58	0.08	1.47	0.01	0.83	0.00	1.67	0.04	3.24	0.09	5.60	0.03	1.74	0.05	1.68	0.08
OOLn + PoOL	ECN 44	3.30	0.00	2.45	0.17	3.40	0.10	2.04	0.56	4.41	0.18	2.45	0.04	3.88	0.10	3.15	0.01	2.77	0.14
PLL + PoPoO		1.48	0.18	0.70	0.04	1.71	0.05	1.47	0.10	3.45	0.16	1.48	0.03	2.45	0.02	1.70	0.11	1.40	0.29
POLn + PPoPo + PPoL		1.89	0.57	0.91	0.01	2.46	0.00	2.42	0.05	3.51	0.07	1.08	0.09	1.21	0.03	1.47	0.04	1.18	0.28
Unknown II		ND	-	ND	-	ND	-	0.73	0.10	0.99	0.02	ND	-	ND	-	ND	-	ND	-
OOL + LnPP		11.67	0.24	12.76	0.32	11.32	0.11	8.72	0.10	8.34	0.26	12.53	0.22	16.98	0.25	10.83	0.13	11.40	0.77
PoOO	ECN 46	8.88	1.17	5.80	0.01	7.63	0.08	7.55	0.05	9.31	0.22	4.40	0.19	5.19	0.32	8.07	0.00	7.69	0.20
SLL + PLO		7.86	2.18	6.02	0.02	9.81	0.23	6.70	0.08	10.44	0.33	7.66	0.19	8.57	0.51	7.84	0.09	7.16	0.50
PoOP + SPoL + POLn + SPoPo		5.57	0.88	3.16	0.04	6.39	0.06	6.47	0.12	10.48	0.31	2.35	0.12	2.14	0.13	5.37	0.19	4.23	0.92
PLP + OOO + PoPP	ECN 48	32.78	5.37	40.67	0.40	27.61	0.20	34.78	0.10	18.47	0.94	32.72	0.07	29.89	0.16	28.66	0.27	32.84	3.48
SOL+POO		21.09	1.34	21.82	0.32	22.57	0.14	21.90	0.18	21.14	0.98	24.98	0.35	17.72	0.31	24.72	0.22	24.14	1.09
POP	ECN 50	3.22	1.34	2.97	0.24	4.54	0.02	5.01	0.06	5.73	0.48	3.95	0.44	2.89	0.03	4.51	0.05	4.01	0.52
SOO		ND	-	0.61	0.01	ND	-	0.60	0.01	ND	-	1.13	0.12	0.75	0.02	0.94	0.16	0.68	0.14
POS + SSL	ECN 52	ND	-	ND	-	ND	-	ND	-	ND	-	0.32	0.07	ND	-	ND	-	ND	-

Bold numbers - mean; Normal numbers - standard deviation; ND - not detected
P - palmitic acid; Po - palmitoleic acid; S - stearic acid; O - oleic acid; L - linoleic acid; Ln - linolenic acid

3.2 Minor compounds, Unsaponifiable components

Minor compounds are widely related to fats and oils identity, they are normally found in the unsaponifiable matter (Gómez-Coca, Pérez-Camino & Moreda, 2015). Sterols are the most representative class of unsaponifiable components and among them desmethylsterols are the most commonly analyzed. However, in this work also methyl- and dimethylsterols were determined (Table 2). The total amount of desmethylsterols in avocado oil was very high ranging from 3828.78±11.67 $mg.kg^{-1}$ in Chile A to 7611.88±0.91 $mg.kg^{-1}$ in Hass. When the profile of desmethylsterols was evaluated, β-sitosterol was the most abundant one with concentrations between

80.56±0.08% (Fuerte) and 86.03±0.03% (New Zealand). The second most important sterol was Δ5-avenasterol (4.36±0.03 - 9.26±0.03%), followed by campesterol (3.71±0.01 - 6.09±0.03%). The amount of the major sterols is very close to that cited for avocado oil. Unfortunately, the detailed composition, as shown in Table 2, has not been reported before (Salgado, Danieli, Regitano-D'arce, Frias & Mansi, 2008; Gómez-Coca, Pérez-Camino & Moreda, 2015).

Regarding methylsterols, Pinkerton was the sample with the highest concentration (1091.66±2.84mg.kg^{-1}) whereas Chile A had the lowest one (287.98±7.32mg.kg^{-1}). Within this class, citrostradienol was the predominant compound ranging from 71.21±0.21% (Equator) to 83.04±0.23% (Bacon). Dymethylsterols were the most variable class of compounds within sterol group, were founded values from 39.68±1.56mg.kg^{-1} in Chile B to 545.33±25.84mg.kg^{-1} in Pikerton. Bacon was the only sample in which 24-methylencicloartenol content is relevant (74.47±0.58%) since in the other cases cicloartenol was always the main compound oscillating between 50.77±0.62% in Pinkerton and 70.43±3.24% in Chile B.

Squalene health effects have already been described, mainly due to olive oil and shark liver oil consumption (Newmark, 1997; Zhang, Yeung, Huang & Chen, 2002). In Table 2, is possible to compare the amount of squalene in avocado oil to corn, and olive oils (Gómez-Coca, Pérez-Camino & Moreda, 2015). In the samples under study, squalene concentrations were between 190.52±5.40mg·kg^{-1} (New Zealand) and 1366.64±6.52mg·kg^{-1} (Fuerte).

Phytol and geranylgeraniol are primary alkenols with terpenic skeletons. Total concentrations were below 100mg·kg^{-1} for all samples. Chile B oil was the sample with the highest amount, 92.36±16.46mg·kg^{-1}, and New

Zealand oil the sample with the lowest presence, 41.68±2.04mg.Kg^{-1}. Geranylgeraniol was normally the compound in the highest proportion among all of them (54.97±3.14% - 79.44±2.47%), except for the case of Chile A oil, in which the proportion of the two compounds were almost the same.

The aliphatic alcohol profile is dominated by molecules with even C-atom number, like C22-OH, C24-OH, C26-OH, and C28-OH. In Bacon, Brazil, Fuerte, Hass, New Zealand and Pinkerton cultivars the main alcohol was C22-OH (31.59±0.09% to 43.75±6.35%), whereas in Chile A and B samples, C26-OH was the main one (21.34±4.18% and 31.59±1.36%, respectively). Only in Equator variety, C28-OH stood out among the others (27.28±6.79%).

Table 2: Minor compounds of avocado oil samples: desmethyl-, methyl- and dimethylsterols, terpenic alkenols, squalen, aliphatic alcohols, and tocopherols.

Sample	Monovarietal Avocado Oil								Commercial Avocado Oil									
	Bacon		Fuerte		Hass		Pinkerton		Brazil		Chile A		Chile B		Equator		New Zealand	
Desmethylsterols %area																		
Cholesterol	**0.19**	0.00	**0.34**	0.10	**0.17**	0.01	**0.17**	0.00	**0.15**	0.00	**0.19**	0.00	**0.20**	0.01	**0.15**	0.01	**0.21**	0.01
Brasicasterol	**0.03**	0.01	**0.03**	0.00	**0.02**	0.00	**0.03**	0.01	**0.02**	0.00	**ND**	-	**ND**	-	**ND**	-	**ND**	-
24-methylene-cholesterol	**0.30**	0.00	**1.94**	0.00	**0.95**	0.01	**0.56**	0.00	**0.54**	0.00	**0.68**	0.00	**1.59**	0.03	**1.59**	0.01	**0.61**	0.00
Campesterol	**3.71**	0.01	**4.62**	0.02	**5.88**	0.01	**6.00**	0.01	**5.99**	0.03	**5.30**	0.01	**6.09**	0.03	**5.04**	0.02	**4.70**	0.00
Campestanol	**0.05**	0.00	**0.04**	0.00	**0.04**	0.00	**0.04**	0.00	**0.07**	0.01	**0.07**	0.00	**0.06**	0.00	**0.05**	0.00	**0.05**	0.01
Stigmasterol	**0.40**	0.01	**0.15**	0.00	**0.13**	0.00	**0.11**	0.00	**0.27**	0.01	**0.47**	0.01	**0.33**	0.02	**0.18**	0.01	**0.19**	0.00
7-campesterol	**0.16**	0.00	**0.19**	0.01	**0.19**	0.01	**0.14**	0.00	**0.42**	0.27	**0.10**	0.01	**0.18**	0.01	**0.87**	0.68	**0.25**	0.00
5,23-stigmastadienol +Clerosterol	**1.97**	0.02	**2.04**	0.05	**2.08**	0.01	**2.01**	0.01	**1.89**	0.02	**1.80**	0.00	**1.80**	0.02	**1.72**	0.13	**1.72**	0.01
b-Sitosterol	**82.60**	0.03	**80.56**	0.08	**82.95**	0.06	**84.08**	0.08	**84.77**	0.21	**84.93**	0.04	**83.80**	0.09	**82.36**	0.33	**86.03**	0.03
Sitostanol	**0.58**	0.04	**0.57**	0.04	**0.46**	0.01	**0.41**	0.03	**0.58**	0.02	**0.80**	0.02	**0.81**	0.08	**0.56**	0.03	**0.54**	0.01
5-avenasterol	**9.16**	0.03	**8.81**	0.03	**6.63**	0.07	**5.86**	0.01	**4.85**	0.01	**4.36**	0.03	**4.65**	0.00	**7.04**	0.12	**5.37**	0.01
5,24-stigmastadienol	**0.34**	0.00	**0.30**	0.04	**0.25**	0.00	**0.22**	0.01	**0.21**	0.00	**0.75**	0.01	**0.27**	0.00	**0.21**	0.05	**0.07**	0.00
Stigamastadienol	**0.17**	0.01	**0.15**	0.00	**0.08**	0.00	**0.13**	0.01	**0.09**	0.02	**0.36**	0.03	**0.06**	0.00	**0.07**	0.00	**0.11**	0.01
7-avenasterol	**0.34**	0.01	**0.26**	0.00	**0.16**	0.00	**0.24**	0.00	**0.13**	0.00	**0.19**	0.01	**0.15**	0.00	**0.16**	0.00	**0.14**	0.01
Total mg.kg^{-1}	**5376.33**	47.86	**6798.42**	3.88	**7611.88**	0.91	**6723.05**	62.06	**4889.06**	41.72	**3828.78**	11.67	**5490.61**	56.90	**5545.14**	10.60	**4656.95**	9.94
MethylSterols %area																		
Obtusifoliol	**5.48**	0.01	**7.29**	0.03	**7.56**	0.07	**7.60**	0.03	**8.02**	0.02	**10.98**	0.54	**9.14**	0.46	**9.59**	0.04	**10.04**	0.03
Gramisterol	**11.48**	0.02	**20.24**	0.14	**18.46**	0.05	**20.05**	0.02	**18.16**	0.07	**12.69**	0.31	**14.99**	0.00	**19.39**	0.05	**15.84**	0.01
Citrostadienol	**83.04**	0.23	**72.46**	0.15	**73.98**	0.05	**72.34**	0.08	**73.82**	0.19	**76.33**	0.50	**75.87**	0.02	**71.02**	0.21	**74.13**	0.64
Total mg.kg^{-1}	**769.39**	6.55	**854.20**	14.90	**657.30**	8.66	**1091.66**	2.84	**388.35**	9.13	**287.98**	7.32	**344.16**	12.52	**502.78**	18.66	**388.06**	14.67
Dimethylsterols %area																		
Butyrospermol	**0.95**	0.19	**6.63**	0.59	**0.95**	0.04	**0.90**	0.06	**0.97**	0.07	**2.30**	2.30	**ND**	-	**0.52**	0.04	**1.02**	0.07
Cicloartenol	**24.57**	0.39	**59.02**	0.43	**67.41**	0.24	**50.77**	0.62	**70.22**	0.17	**68.12**	5.71	**70.43**	3.24	**53.70**	3.72	**57.91**	1.69
24-methylencycloartenol	**74.47**	0.58	**34.35**	1.01	**31.64**	0.21	**48.34**	0.68	**28.82**	0.24	**29.58**	3.41	**29.57**	3.24	**45.78**	3.76	**41.07**	1.62
Total mg.kg^{-1}	**340.62**	6.96	**538.58**	8.30	**392.38**	20.71	**545.33**	25.84	**247.65**	4.78	**192.32**	#####	**39.68**	1.56	**200.98**	28.61	**272.72**	11.63
Squalen mg.kg^{-1}																		
Squalen mg.kg^{-1}	**1327.33**	0.00	**1366.64**	6.52	**778.76**	7.12	**1314.73**	12.05	**359.93**	0.54	**924.82**	39.80	**350.86**	0.76	**670.05**	9.34	**190.52**	5.40
Terpenic Alkenols %area																		
Phytol	**27.02**	3.44	**36.72**	1.53	**38.30**	3.35	**20.56**	2.47	**25.85**	4.02	**50.72**	2.35	**35.62**	5.71	**24.43**	1.47	**45.03**	3.14
Geranylgeraniol	**72.98**	3.44	**63.28**	1.53	**61.70**	3.35	**79.44**	2.47	**74.15**	4.02	**49.28**	2.35	**64.38**	5.71	**75.57**	1.47	**54.97**	3.14
Total mg.kg^{-1}	**67.56**	6.17	**87.34**	0.34	**74.21**	3.02	**84.22**	0.29	**69.96**	1.14	**45.61**	1.01	**92.36**	16.46	**51.27**	1.12	**41.68**	2.04
Aliphatic Alcohol %area																		
C22-OH	**43.75**	6.35	**41.80**	0.23	**41.79**	2.97	**38.57**	0.70	**31.59**	0.09	**18.61**	7.51	**24.51**	5.80	**23.32**	1.23	**36.43**	1.01
C23-OH	**ND**	-	**ND**	-	**ND**	-	**ND**	-	**ND**	-	**3.60**	0.07	**ND**	-	**2.43**	0.22	**ND**	-
C24-OH	**17.06**	2.76	**18.71**	1.17	**ND**	-	**21.16**	0.51	**20.24**	0.06	**28.31**	0.20	**19.72**	1.19	**18.94**	1.29	**18.22**	2.25
C25-OH	**9.62**	6.27	**4.33**	0.23	**4.82**	0.20	**8.18**	0.10	**2.88**	0.12	**4.78**	0.26	**7.24**	0.48	**3.58**	0.46	**5.53**	0.54
C26-OH	**16.69**	0.80	**18.84**	0.45	**31.24**	0.73	**20.72**	1.65	**21.34**	4.18	**31.59**	1.36	**28.79**	2.35	**24.46**	4.94	**14.40**	0.96
C27-OH	**ND**	-	**ND**	-	**ND**	-	**ND**	-	**ND**	-	**ND**	-	**ND**	-	**ND**	-	**ND**	-
C28-OH	**12.87**	3.65	**16.31**	1.62	**22.15**	2.04	**11.36**	0.53	**23.95**	4.02	**6.77**	1.53	**19.74**	4.16	**27.28**	6.79	**25.42**	4.75
Total mg.kg^{-1}	**5.65**	1.21	**6.55**	0.45	**4.91**	0.70	**5.99**	0.31	**3.13**	0.56	**14.41**	1.09	**5.47**	2.21	**3.45**	0.12	**2.77**	0.30
Tocopherols mg.kg^{-1}																		
α-Tocoferol	**51.90**	0.04	**103.11**	6.07	**86.75**	0.62	**45.62**	0.19	**0.31**	0.01	**7.40**	0.05	**39.14**	3.46			**0.15**	0.01
β-Tocoferol	**ND**	-	**1.13**	0.18	**3.37**	0.93	**3.95**	0.10	**0.90**	0.01	**8.07**	0.06	**1.65**	0.05			**0.79**	0.01
γ-Tocoferol	**71.61**	0.57	**20.35**	1.22	**9.02**	0.09	**13.71**	0.56	**6.08**	0.06	**12.30**	0.24	**4.15**	0.02	**ND**		**2.42**	0.04
δ-Tocoferol	**14.98**	0.13	**16.90**	1.32	**26.84**	0.01	**44.10**	0.13	**2.00**	0.02	**3.37**	0.07	**ND**	-			**1.63**	0.04
Total mg.kg^{-1}	**138.50**	0.73	**141.50**	4.15	**125.98**	0.41	**107.39**	0.71	**9.30**	0.10	**31.14**	0.07	**44.94**	3.52			**4.99**	0.10

Bold numbers - mean; Normal numbers - standard deviation; ND - not detected

The presence and the concentration of tocopherols are closely related to both identity and quality, since these molecules have antioxidant activity and may indicate the resistance of oil to oxidation as well as the fact of having been exposed to oxidation conditions. The total amount of tocopherols (Table 2) in commercial samples was very low when compared to monovarietal oils. In the case of Equator cultivar, tocopherols were not possible to quantify and in others commercial samples were lower than 50mg·kg^{-1}. However, in monovarietal samples tocopherol concentration ranged from 107.39±0.71mg·kg^{-1} to 141.50±4.15mg·kg^{-1}. This fact can be related to irregular or long time

storage in the grocery store. Generally, α-tocopherol was the main species found, except in Bacon in which γ-tocopherol was the most abundant one. Tocotrienols were not found in these samples.

To the best of our knowledge, these approaches in avocado oil characterization (detailed characterization of major and minor compounds) have never been published, which make both the election of relevant characterization parameters and the subsequent discussions, difficult. In this line, we performed a PCA in order to find relationships among samples, and to determine which parameters are more important to establish this relationship.

After the statistical analysis described in the Material and Methods section, the distribution graphic based on component 1 and component 2 was chosen, whose sum explained more than 50% of data variance. As Figure 1 shows, component 1 clearly separates monovarietal and commercial oils. Chile A and B samples are closer from each other than in the other cases; the same trend is observed with Equator and New Zealand cultivars, whereas Brazil variety seems to be the most different one. These changes can be related to pedoclimatic effect, since they were cultivated/produced in different countries. Among monovarietal samples, it is possible to see that they differ from each other, but that there are no discrepancies in the distribution.

Based on this distribution another PCA analysis was performed to confirm if the difference between commercial and monovarietal samples were statistically significant. Thus we plotted the PCA displaying the region of 95% of confidence for monovarietals and commercial groups (Figure 2). It is clear that the Hass sample belongs to both groups, it happens once that the commercial samples of avocado oil were produced from Hass variety (as labeling declared).

Keeping up statistical analysis the heat map in Figure 3 shows the ten most significant features for grouping the samples. Looking at the characteristic already described, they match with the features selected by random forest selection. More specifically, we can see that stearic and linoleic acids have strong influence on Chile A and B samples, while palmitic acid has great effect on Brazil sample. Finally, Bacon differentiation is influenced by γ-tocopherol, while Pinkerton is influenced by δ-tocopherol.

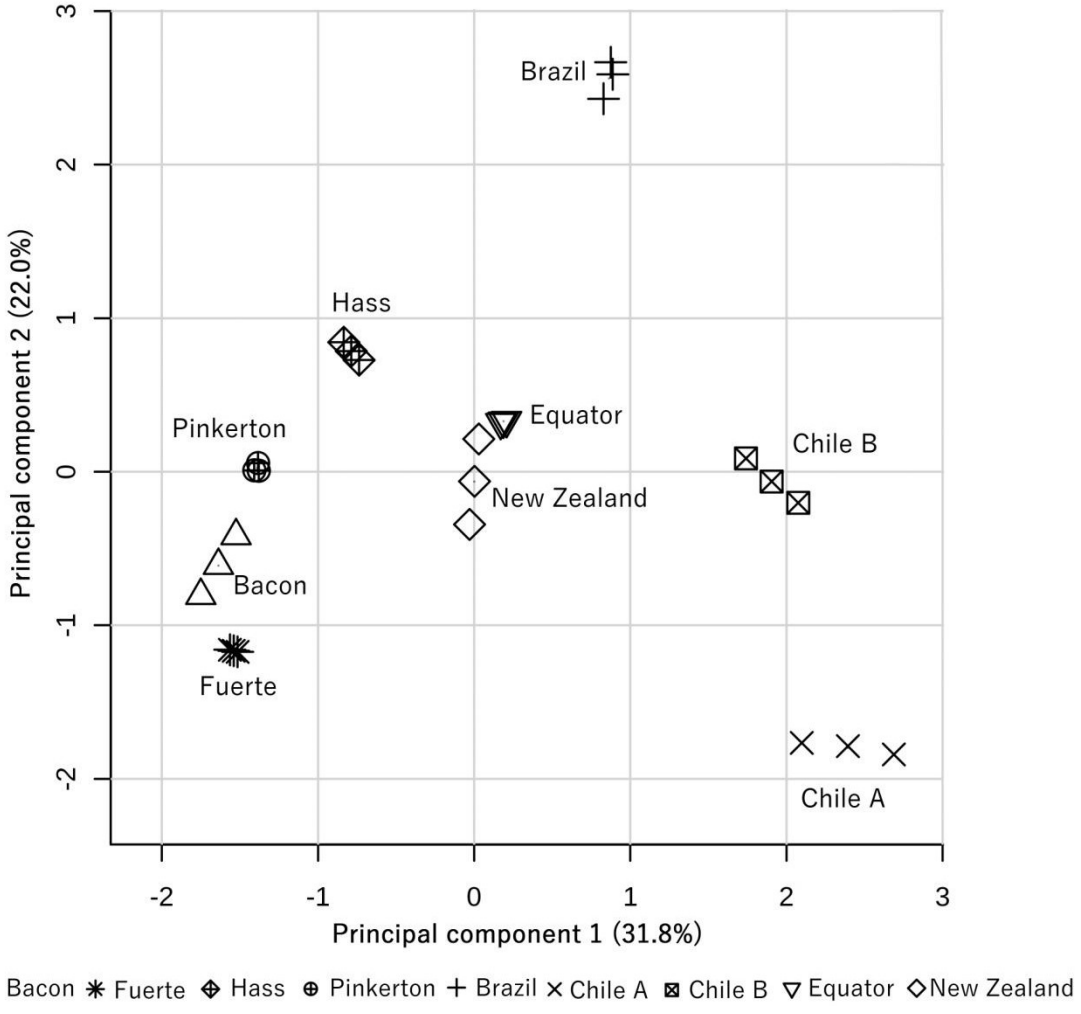

Figure 1: Principal component analysis scores plot for all analyzed samples, PC1 x PC2.

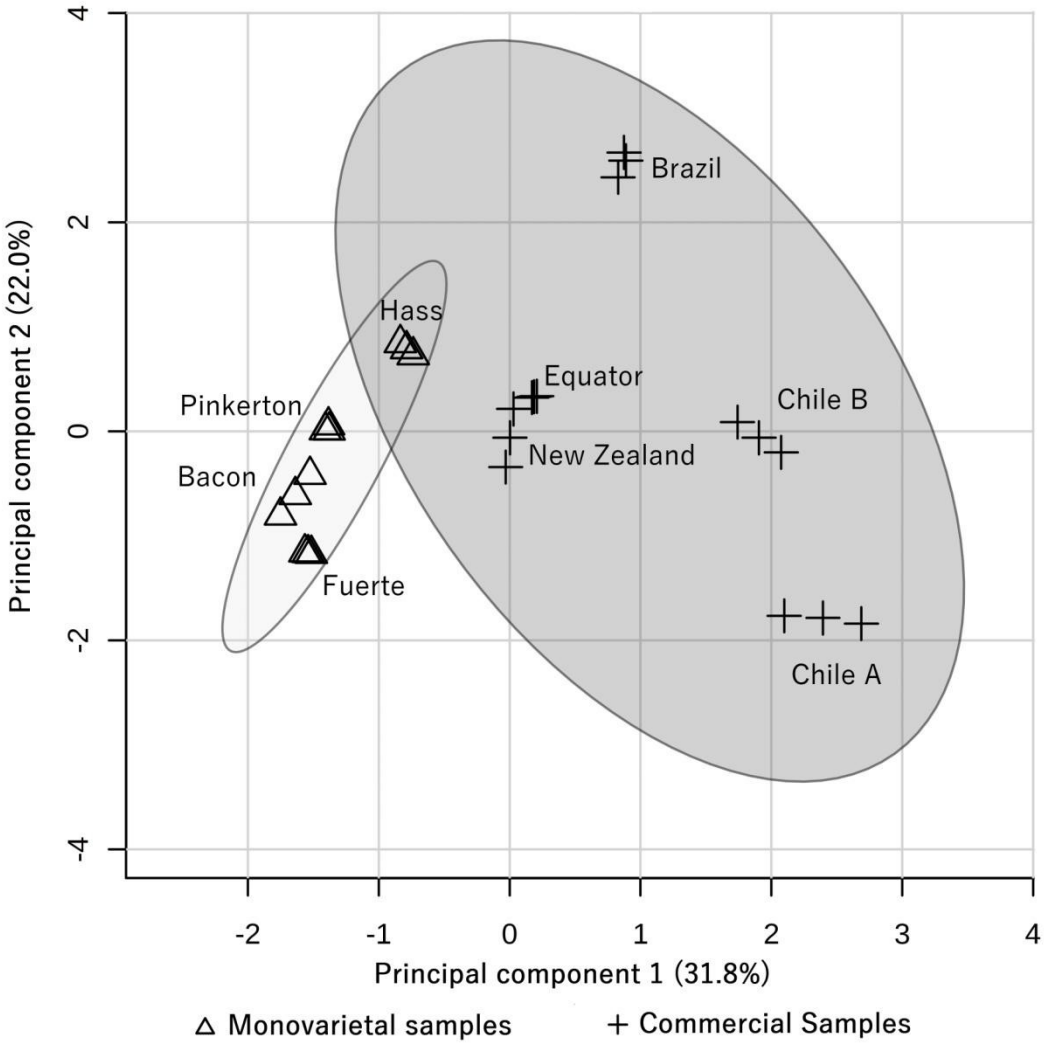

Figure 2: Principal component analysis scores plot grouping monovarietal and commercial samples with 95% confidence region, PC1 x PC2.

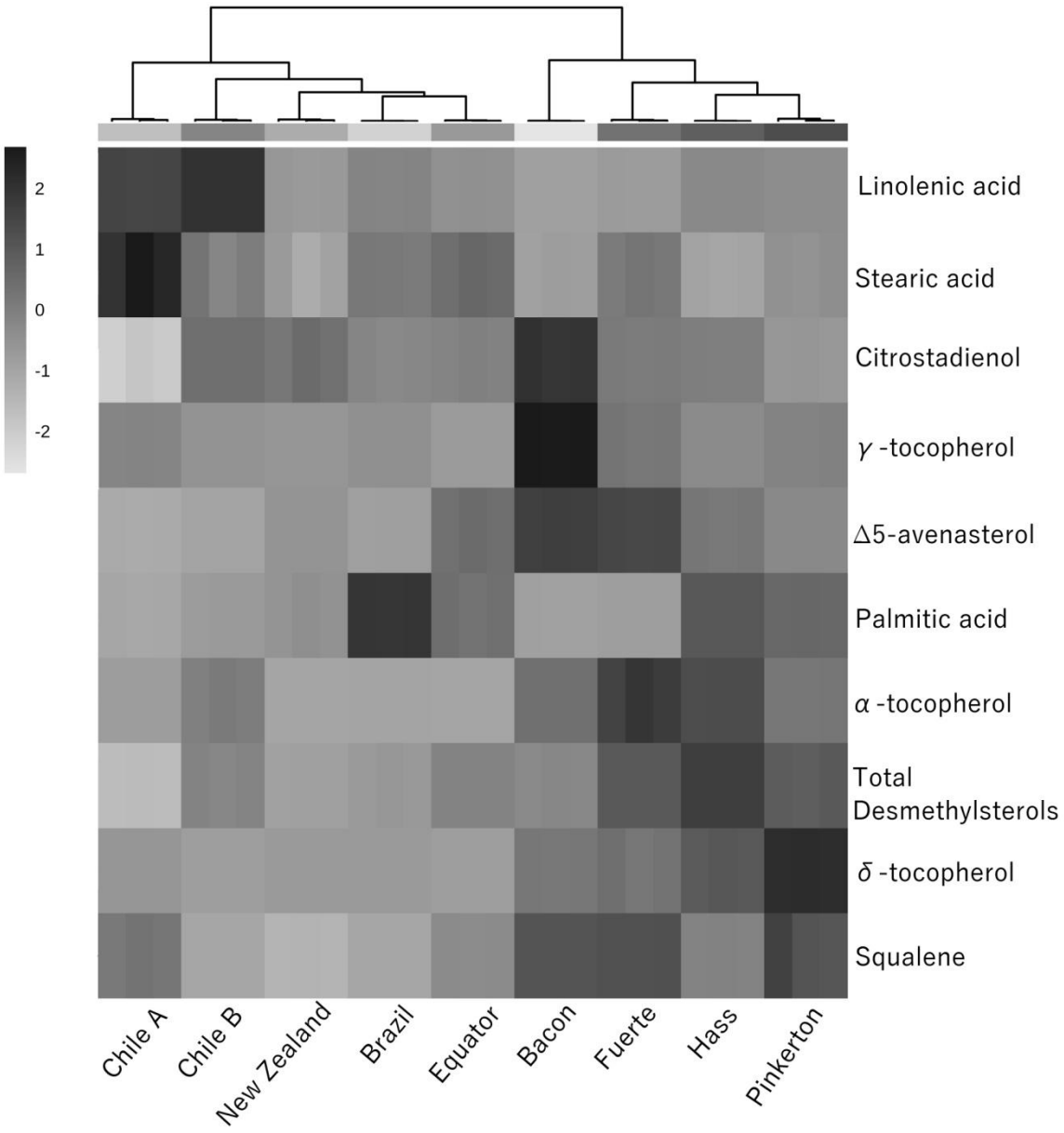

Figure 3: Heat map of the ten more significant features to distinguish the samples, selected by Random forest statistical tool.

4. Conclusions

Avocado oil can be considered to be mainly monounsaturated oil, being oleic acid the main fatty acid. TAG composition is also dominated by oleic acid. For the first time the presence of ω7, ω9 and ω11 isomers of oleic and palmitoleic acids were described for avocado oil.

Desmethylsterols were described, being β-sitosterol the main molecule we found. The squalene concentrations were higher in Bacon, Fuerte and Pinkerton oils than in Hass and commercial oils, such avocado oils may be suggested as sources of squalene. Tocopherols were also found, mainly α-tocopherol and γ-tocopherol, however, tocotrienols were not found.

Minor compounds like methyl- and dimethylsterols, terpenic alkenols, and the aliphatic alcohols were described to first time in this work.

From these data it was also possible to distinguish which features were more important for sample differentiation. The Hass variety is related to commercial samples since the commercial samples were labeled as produced from Hass cultivar. Heat map and principal component analysis revealed differences among the samples, mainly related to commercial oils. Such differences should be taken in account for establishing regulatory laws.

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ARTIGO 3 - Application of DNA markers in the detection of fraud in virgin olive oil with sweet almond, hazelut and pecan nut oils.

G.D. Fernandes, R.B. Gómez-Coca, M.C. Pérez-Camino, J.M. Martínez-Rivas, W. Moreda, D. Barrera-Arellano.

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Application of DNA markers on the detection of fraud in virgin olive oil with Sweet Almond, Hazelut and Pecan Nut oils.

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Abstract

Virgin olive oil chemical composition is very similar to that of sweet almond, hazelnut and pecan nut oils what makes the detection of frauds with nut oils in virgin olive oil hardly possible when using current chemical methodology. The objective of this work was to develop a detection tool based on DNA markers through which the addition of nut oils in virgin olive oil becomes evident. For this development were used primers initially described for the detection of allergens in foods. Primer specificity was checked for both fruit oil and nut oil samples. Finally the primer efficiency was checked in spiked virgin olive oil samples where sweet almond, hazelnut and pecan nut oils were present at concentrations of 10, 5, and 1%. A limit of detection of 5% for this kind of adulterants in virgin olive oil was established.

Keywords: Olive oil fraud, Sweet almond oil, Hazelnut oil, Pecan nut oil, DNA marker.

1. Introduction

Virgin olive oil consumption has increase noticeably mainly due to its high nutraceutical value related, to the high antioxidant concentration among many other things. Furthermore, its especial organoleptic characteristics have made it be widely appreciated in the so-called “gourmet” market. However, the knowledge on virgin olive oil beneficial properties is still quite limited in a number of non-Mediterranean countries (EFSA, 2011, Frankel, et al. 2011).

According to the definition set by international regulating organizations, virgin olive oil is that oil obtained from fruits of *Oleaeuropea* L. using only physical processes in which particular control is given to thermal conditions in a way that they do not cause any modification in the oil (IOC, 2015). In 2012 the International Olive Oil Council registered a world production of about 2.4 million tons virgin olive oil (IOC, 2014). Interestingly, for the same period the IOC recorded a consumption of 2.9 million tons virgin olive oil. Clearly that 500000 ton difference points out an obvious situation of fraud. Actually, the large availability of new sources of fats and oils, as well as the improvement on the refining process have enabled the presence of undetected fakes (Butler,2012).

The IOC has been intensely focused on ensuring both authenticity and quality of virgin olive oil. Official regulations aim to classify virgin olive oil in any of the categories officially recognized and to detect possible frauds, based on analytical methods and specific ranges for physical-chemical and sensory parameters (IOC, 2015).

Generally, virgin olive oil fraud is related to the addition of low quality olive oil or of fats and oils from sources other than olive oil to high quality extra virgin olive oil. This represents not only an economic fraud but also a public health problem since some nuts used to this adulteration may cause allergic reactions. Actually, almond, hazelnut and pecan nut oils, the most representative high oleic acid nut oils, having allergen characteristics (Ben-Ayed, Kamoun-Grati & Rebai, 2013, Bremer, Smits & Haasnoot, 2009, Fernandes, Gómez-Coca, Pérez-Camino, Moreda, Barrera-Arellano, 2015).

Almond (*Prunus dulcis*) contains 50% oil. Its chemical composition is very similar to that of virgin olive oil, being oleic acid (C18:1, O) the most abundant fatty acid (~65%), and β-sitoterol the most representative sterol (Sathe, Seeram, Kshirsagar, Heber, Lapsley, 2008, Fernández-Cuesta, Kodad, Socias, Velasco, 2012). Regarding the allergenic characteristics of sweet almond oil, the main genes involved are those codifying for Pru du 1 11s globulin, (NCBI accession, X78119), Pru du 2S albumin (NCBI accession, P82944), Pru du conglutin (NCBI accession, P82952) and Pru du 4 (NCBI accession, Q8GSL5) (Robotham, et al., 2005).

Hazelnut (*Corylus avellana*) oil is a well-known olive oil adulterant, mainly due to its importance as part of the Mediterranean diet (Kamal-Eldin, Moreau, 2009). Turkey, an important virgin olive oil producer, is also the largest hazelnut producer in the world, with a production of 72 % (FAOSTAT, 2013). Hazelnut oil has been frequently compared with virgin olive oil due their similar chemical composition (Amaral, Casal, Citová, Santos, Seabra, Oliveira, 2006). Actually, several methods have been suggested in order to detect hazelnut oil in virgin olive oil, however, only the global method have been applied as a

regulation step and some combinations of olive and hazelnut oils can still be made and pass undetected. Allergenic characteristics of hazelnut oils are related to factors Cor a 1 Bet-v1 related protein (NCBI accession, Z72439), Cor a 2 (porfilin) and Cor a 9 (NCBI accession, Q8W1C2) (Robotham, et al., 2005).

Pecan nut (*Carya illinoinensis*) oil is mainly produced in the USA (~80% of the total world production). It contains almost 70% of oil being oleic acid the main fatty acid (~60%). However, γ-tocopherol has been reported as the main tocopherol in pecan nut oil, although it is not a good identity parameter as far as fraud detection is concerned since tocopherols are easily degraded (Oro, Bollini, Arellano, Block, 2009). An important allergen component have also been described for this kind of oil: Car i 1 (NCBI accession, AAO32314) (Robotham, et al., 2005).

Molecular Biology techniques have been applied for the detection of these allergens in foods. In 2010 Marmiroli and coworkers published the patent of a kit for their detection in foods (patent EP2226395A1). At the same time several methods for DNA oil extraction and PCR (Polimerase Chain Reaction) amplification were developed mainly for olive oil analysis; these procedures use DNA markers such as sequence repeat (SSR) markers (Pasqualone, Di Rienzo, Nasti, Blanco, Gomes & Montemurro, 2013), commonly associated to electrophoresis separation, or real-time PCR with high resolution melting point (HRM), to detect the adulteration of virgin olive oil with seed oils and/or monovarietal olive oils (Kumar, Kahlon & Chaudhary, 2011, Wu, Zhang, Han, Wang, Wang, Ju & Chen,2011, Ganopoulos, Bazakos, Madesis, Kalaitzisc & Tsaftarisa, 2013, He, et al., 2013, Agrimonti, Vietina, Pafundo & Marmiroli, 2011).

The aim of this work is to apply molecular markers for the detection of allergens in foods through the recognition of adulteration of virgin olive oil with other oils such as virgin nut oils from sweet almond, hazelnut and pecan nut, offering in this way a solution to both economic and public health problems.

2. Material and Methods

2.1 Chemicals and Samples

Heptane was purchased from VWR International (West Chester, PA, USA), DNeasy Plant Mini Kit, from Qiagen (Turnberr, CA, USA), and Maxima SYBR Green qPCR Master Mix from Thermo Scientific (Waltham, MA, USA). Primers were synthesized by Thermo Fisher Scientific (São Paulo, SP, BR). Olive fruits, sweet almonds, hazelnuts and pecan nuts were purchased in Brazilian groceries while authentic virgin olive oil was provided directly by Spanish producers. Pure virgin oils from sweet almonds, hazelnuts and pecan nuts were provided by Vital Âtman (Uchoa, SP, Brazil) (Bauru, SP, BR).

2.2 DNA Extraction

The protocol described in DNeasy Plant Mini Kit, from Qiagen, was used for DNA extraction from olive fruits, sweet almonds, hazelnuts and pecan nuts. In short: cell disruption was carried out with 200 mg of fresh milled samples with lysis buffer and RNase A, at 65ºC during 10 min. After that, a salt-precipitation procedure of proteins and polysaccharides was performed and the precipitate removed by centrifugation. Supernatant was then filtered through the QIAshredder column. Through the addition of binding buffer DNA binds to the DNeasy spin column membrane while two ethanol wash steps efficiently remove contaminants. Finally, pure DNA was eluted with a small volume of water.

Regarding the DNA extraction from oil samples, the samples were homogenized during 30min. After that, 100mL oil and 50mL heptane were mixed in an Erlenmeyer flask together with 500µL lysis buffer. This mixture was

then placed under strong agitation (1500rpm, magnetic bar) during 30min, and centrifuged at 10000rpm (1 h). The lower phase was transferred to a microtube and any vestige of solvent was evaporated under nitrogen flow. 4µL RNAse A was added and incubated at 65ºC during 10min. From this point, the protocol of DNeasy Plant Mini Kit was followed again starting with the addition of the buffer for salt-precipitation of proteins and polisacharides. Supernatant was taken through the filtration unit (QIAshredder column) and then bound to DNeasy spin column by the addition of binding buffer. Bound DNA was washed with ethanol and eluted in a small volume of sterile distilled water (SDW). In order to confirm that the extracted DNA could be amplified we performed a real time PCR with a universal JR1-JR2 pair of primers.

2.3 Primers

For the development of this work, were used the pairs of primers described in the EP2226395A1 (Table1) patent for the detection of allergens in foods, (Marmiroli, Gulli', Pafundo, 2010). For sweet almond and hazelnut samples the pairs of primers described from *Prunus dulcis* and *Corylus avellana* were applied, respectively. However, the refered patent does not describe any primer for pecan nut. So, the ability of a pair of primers described for walnut (*Juglans regia*) to amplify for pecan nut was checked. It is very important to say that walnut oil is not one of the high oleic acid oils, although its linoleic acid and linolenic acid contents are very important. This is why it is not necessary to apply DNA analysis for the detection of this particular oil on the detection of olive oil adulteration.

Table 1: Description of the PCR Primers

Specificity	Pair	Forward 5'-3'	Length (pb)	CG %	Reverse 5'-3'	Length (pb)	CG %	Product Legth (pb)	Target Gene
Sweet Almond	EPA1-EPA2	GAATGTGAACGCCCACTCAG	20	55.0	ATCTCCGTTCTCGTTCACCAC	21	52.3	76	Pru du 1 11s globulin
Hazelnut	EPC1-EPC2	CAAAGGTGACCATGAGGTGGAT	22	50.0	ACCTTTTCTTTGCCACCCTTAAT	23	39.1	54	Cor a 1 Bet-v1 related protein
Pecan Nut	EPN1-EPN2	ATTCGCAGAAGCTGGTTCTAGA	22	45.0	CTTCCAGCGACAAATGCTAGAG	22	50.0	81	Jug r1 2s albumin
Universal	JR1-JR2	GATCCCGGACGTAATCCTG	19	57.0	ATCGTACCGAGGGTTCGAAT	20	50.0	-	tRNA-Gly

2.4 Real time PCR conditions

PCR reactions were carried out in duplicate in Real Time CFX384 (Bio-Rad, Hercules, CA, USA) working with Hard-Shell® Thin-Wall 384-Well Skirted PCR Plates (Bio-Rad, Hercules, CA, USA). The reaction volume was 13 µL per well, which contained 6.25 µL of Maxima SYBR Green qPCR Master Mix, 1.25µL of each forward and reverse primers, 3.25 µL of SDW and 1 µL of DNA. The thermal cycling conditions consisted of an initial denaturation step of 95ºC for 3min, followed by 40 cycles of 95ºC for 10s, and 60 or 65ºC for 20s. This anealing temperature depends on the primer Tm. Melting curve analysis was also performed following the final step of the PCR, from 60 through 95ºC at $0.5ºC.s^{-1}$. Data were processed in CFX Manager 2.1 (Bio-Rad, Hercules, CA, USA).

2.5 Experimental procedure

2.5.1 Primer specificity

In order to verify the specificity of primers experiments of real time PCR with primers described in Table 1 were performed. EPA1-EPA2, EPC1-EPC2 and EPN1-EPN2 were applied to DNA obtained from olives, sweet almonds, hazelnuts and pecan nuts. All experiments monitored both

amplification and melting point curve, and were conducted in duplicate using two different annealing temperatures, 60 and 65ºC.

In order to check their performance against a DNA from oil samples, after the confirmation of primers specificity, they were applied to DNA extracted from olive, sweet almond, hazelnut and pecan nut oils.

2.5.2 Detection of the adulteration of virgin olive oil with nut oils

The last experiments aimed to check the ability to detect sweet almond oil, hazelnut oil, or pecan nut oil in virgin olive oil. Therefore, virgin olive oil samples were spiked with the corresponding oils at concentrations of 10, 5, and 1% (w/w). Samples were then homogenized and the DNA extracted according to the procedure described in Section 2.2. Real time PCR was performed with specific primers for each sample. Specific amplifications and melting curves were also monitored. All this procedures were done in triplicate.

3. Results and Discussion

3.1 Primer Specificity

The first step of this work was to verify the specificity of the primers described in the patent. In order to do that each pair of primer was tested against each sample and also against virgin olive oil. Initially, the tests were carried out with DNA extracted from olive fruits, sweet almonds, hazelnuts and pecan nuts, in two annealing temperatures: 60 and 65ºC.

For the EPA1-EPA2 pair, originally designed from *Prunus dulcis*, no specificity was observed at 60ºC. However, it was very specific for sweet almond samples at 65ºC (Figura1). Actually, at this last temperature DNA from sweet almond was detected with a medium threshold cycle (Cq) of 18.46 cycles and a medium maximum fluorescence (Fmax) of 5749.99 RFU. The melt curve analysis of the amplicon showed a melting temperature (Tm) of 78.0ºC.

Regarding the EPC1-EPC2 pair, designed for *Corylus avellana*, when tested in DNA extracted from hazelnut samples there was not specificity at 60ºC but it appeared to be specific at 65ºC, In this case, the observed Cq was of 21.08 cycles and the Fmax was 3913.28 RFU . In the melt curve the amplicon Tm was 74.5ºC (Figura 2).

EPN1-EPN2 pair was initially designed from *Juglans regia* (walnut), and seemed to be specific for pecan nut samples with a Cq of 27.33 cycles and a Fmax of 2555.26 RFU. The amplicon Tm was of 77.0ºC (Figura 3). At 65ºC (annealing temperature) this pair of primers did not show any amplification.

Once the primer specificity was confirmed in fruit/nut samples the same experiments were performed in DNA extracted from oil samples. It is important to keep in mind that these experiments were conducted only in the

optimal annealing temperature for each pair of primers, and that the DNA extracted from each oil sample was checked against the JR1-JR2 universal primer pair, obtaining in all cases positive results.

In general, all primers were equally specific when applied to DNA from oil samples. EPA1-EPA2 pair amplifies only in DNA extracted from sweet almond samples, being the observed Cq of 23.65 cycles and the Fmax of 5107.13 RFU. The same amplicon (Tm 78.0ºC) was observed in sweet almond fruit and oil samples. Amplification for the EPC1-EPC2 pair was registered only in hazelnut oil samples with a Cq of 24.38 cycles, Fmax of 3911.13, and an amplicon of Tm 74.5ºC. For the EPN1-EPN1 pair, which amplifies only for pecan nut samples, the obtained results were: Cq = 31.34 cycles, Fmax= 1017.36 RFU, and amplicon Tm = 77.0ºC.

After checking the specificity of each primer for sweet almonds, hazelnuts and pecan nuts, all of them were used for the detection as adulterants of these oils in virgin olive oil.

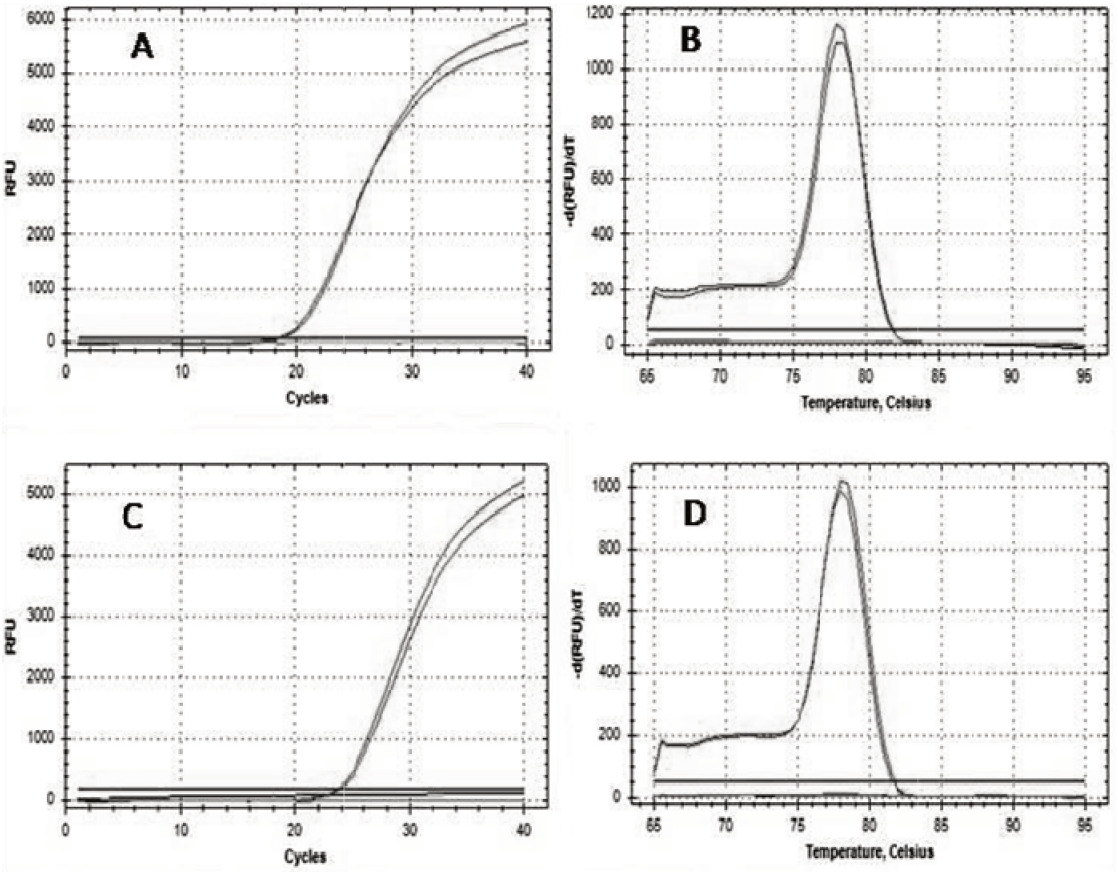

Figure 1: Results on the experiment for checking the specificityof the EPA1-EPA2 pair of primers: (A) Amplification curve when applied to fruit/nut samples; (B) Melt curve of the amplicon when applied to fruit/nut samples; (C) Amplification curve in oil samples; (D) Melt curve of the amplicon in oil samples.

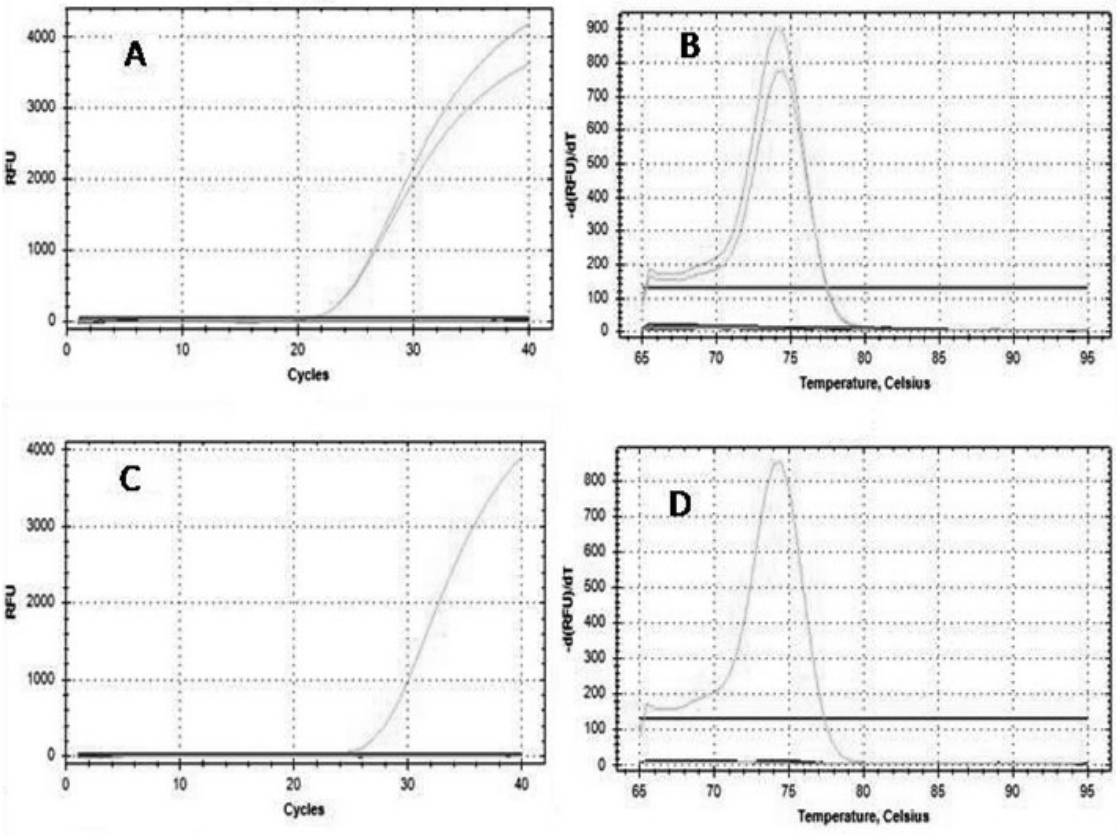

Figure 2: Results on the experiment for checking the specificity of the EPC1-EPC2 pair of primers (A) Amplification curve when applied to fruit/nut samples; (B) Melt curve of the amplicon when applied to fruit/nut samples; (C) Amplification curve in oil samples; (D) Melt curve of the amplicon in oil samples.

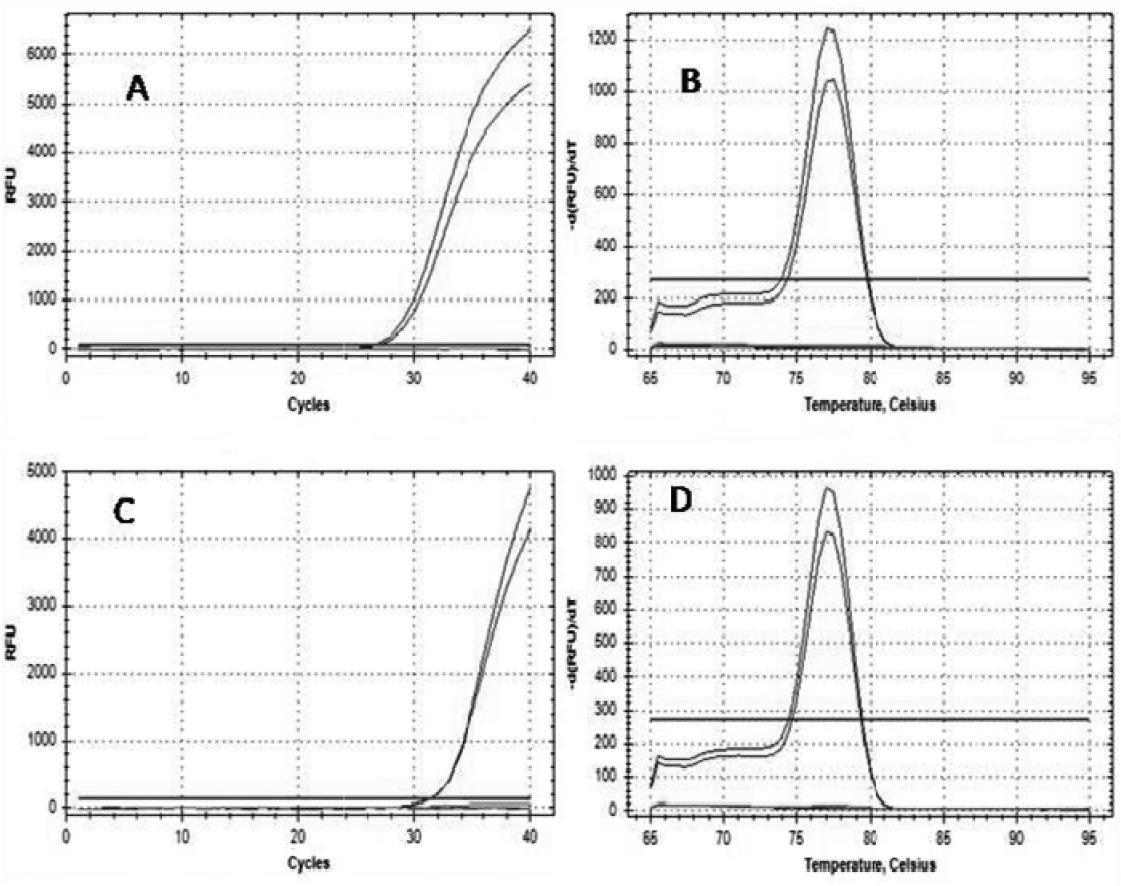

Figure 3: Results on the experiment for checking the specificity of the EPN1-EPN2 pair of primers:(A) Amplification curve when applied to fruit/nut samples; (B) Melt curve of the amplicon when applied to fruit/nut samples; (C) Amplification curve in oil samples; (D) Melt curve of the amplicon in oil samples.

3.2 Detection of adulteration of virgin olive oil with nut oils

These experiments aimed to detect the addition of virgin nut oils in virgin olive oil. In other words, our goal was to check if the extraction method along with each specific pair of primers would be able to detect the presence of each nut oil in olive oil (Figure 2).

Using the EPA1-EPA2 pair of primers in virgin olive oil, detection of sweet almond oil was possible when it was present at least at 1 %

concentration (Cq = 30.51 ± 0.97 cycles; Fmax= 1263.32 RFU), which is actually a very low amount of adulterant. For the samples spiked with 5% sweet almond oil, Cq was 28.02 ± 0.52 cycles and Fmax was 3619.19 ± 226.33 RFU, whereas if sweet almond oil was present at 10%, Cq was 25.55 ± 0.15 cycles and Fmax was 5236.26 ± 202.72 RFU. For all spiked samples the amplicon has a Tm of 78.0ºC.

The EPC1-EPC2 pair was then applied for the detection of virgin hazelnut oil in virgin olive oil. In this case the limit of detection was 5%. The samples with 10% of hazelnut oils showed a Cq of 25.09 ± 0.19 cycles and Fmax of 2549.65 ± 204.43 RFU, while the sample with 5% showed a Cq of 33.89 ± 0.92 cycles and a Fmax of 452.20 ± 54.47 RFU. For both, 10% and 5% samples the amplicon Tm was 74.5 ºC. Amplification was not observed in the samples in which hazelnut oil presence was 1%.

Finally, the EPN1-EPN2 pair was applied to detect virgin pecan nut oil in virgin olive oil, showing a limit of detection of 5% too. In this way, for samples with 10% pecan oil addition Cq was 30.89 ± 0.21 cycles and Fmax 2953.96 ± 58.08 RFU, whereas when pecan oil was present at 5 % Cq was 34.20 ± 0.47 and Fmax 763.04 ± 52.45 RFU.

These experiments demonstrate the high performance of the method that has been described to detect low levels of virgin nut oils in virgin olive oil. Differences among the limit of detection for the samples may be related to variations in the DNA concentration and quality. In this way, 5% adulterant may be considered as the general limit of detection of this DNA-based marker method.

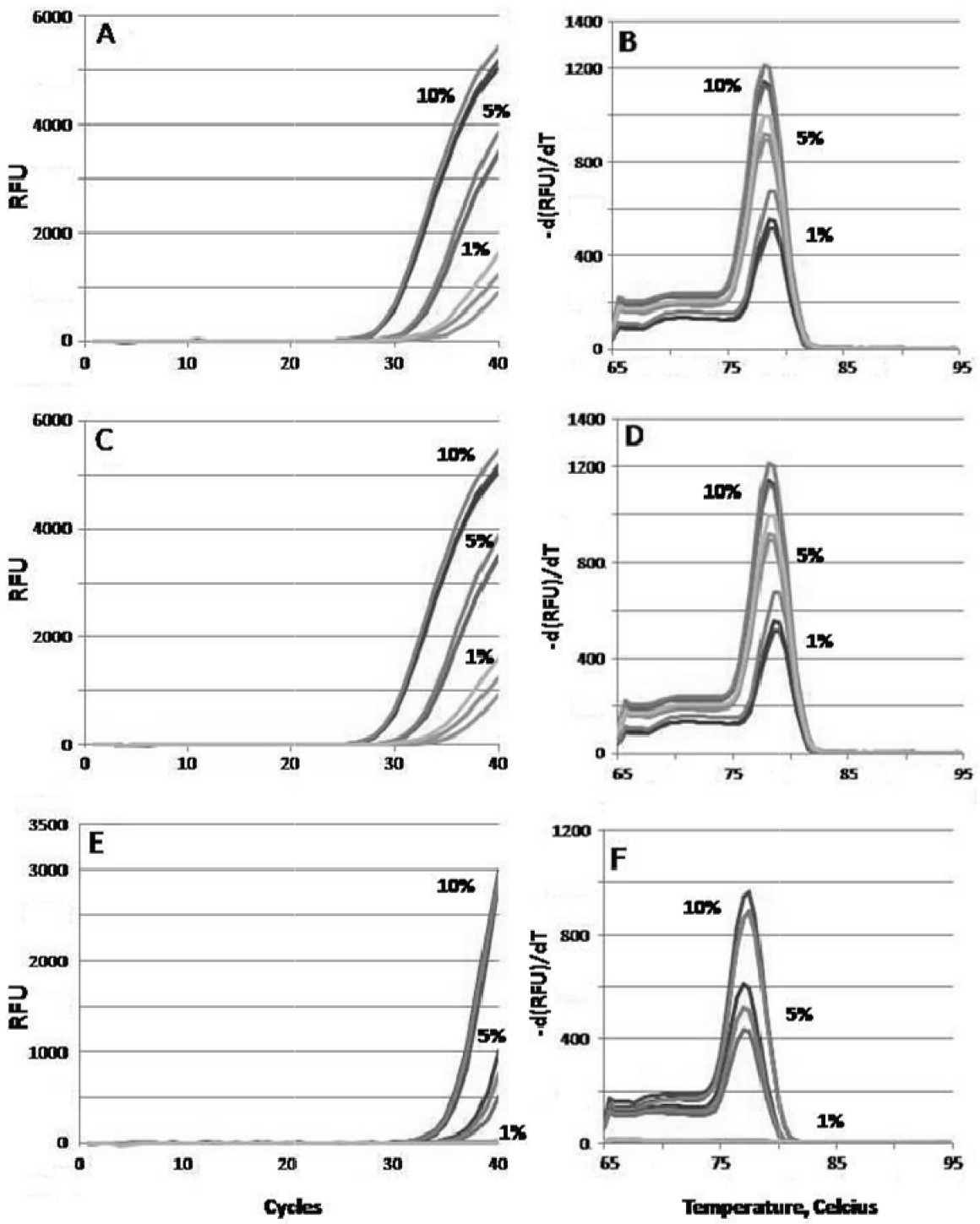

Figure 4: Results on the detection of virgin olive oil admixtures with nut oils at 10, 5, and 1 % concentrations: (A) Amplification curve for the EPA1-EPA2 pair of primers when in adulterations with sweet almond oil, and (B) melt curve of the product reaction. (C) Amplification curve for the EPC1-EPC2 pair of primers when in adulterations with hazelnut oil, and (D) melt curve of

the product reaction. (E) Amplification curve for the EPN1-EPN2 pair of primers when in adulterations with pecan nut oil, and (F) melt curve of the product reaction.

4. Conclusions

Specificity of the primers described in the EP2226395A1 patent was confirmed for DNA extracted from olive fruits, hazelnuts, sweet almonds, pecan nuts, as well as their respective oils, via amplification and melt curves in RT-PCR.

It was possible to detect the adulteration of olive oil with 5% of hazelnut and pecan nut oils, while the detection limit for the adulteration of virgin olive oil with sweet almond was down to a 1 % level. However, based on all results we decided to consider a general limit of detection of 5%, since there is a large variation in the amount and the quality of the DNA present in oil samples.

Finally, this work brings an important point of view on the detection of virgin olive oil health and economic fraud: the use of Molecular Biology technics to evidence the presence of food allergens and extraneous oils, respectivelly.

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ARTIGO 4 - Virgin olive oil adulteration with avocado oil: new challenge for DNA markers.

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Virgin olive oil adulteration with avocado oil: new challenge for DNA markers.

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Abstract

The detection of fraudulent mixtures of avocado oil and olive oil by the current chemical methodology, established by Regulatory Organizations, is very difficult due to the similarity between the chemical compositions of both oils. Thus, this work has as a goal the development of a detection tool based on DNA markers. A method for DNA extraction from oil samples was carried out together with the design of four pairs of avocado specific primers. Primer specificity was tested for both, fruit/nut and oil samples. At the end of this experiments only one pair of primers proved to be specific and with high performance for application to avocado oil samples. To check the suitability of the method for the detection of olive oil adulteration with avocado oil, olive oil samples with 10, 5 and 1 % of avocado oil were prepared and their DNA

extracted and amplified by the TP1-CP1 primer pair. Results showed the feasibility of detecting avocado oil when present at concentrations of 5 %.

Keywords: Olive oil fraud, Avocado oil, DNA extraction, DNA detection.

1. Introduction

Olive oil is obtained from fruits of *Olea europea* L., using only physical methods under controlled thermal conditions which do not cause any modification in the oil (IOC, 2015). Olive oil is a high added-value food mainly due the high nutraceutical and organoleptic importance besides the elevated production cost. Olive oil consumption has increased remarkably in several non-traditionally consuming countries where the 'knowledge' of this ingredient, in terms of food culture, is limited (EFSA, 2011, Frankel, et al. 2011).

According to International Olive Oil Council (IOC, 2014) in 2012 the olive oil world production was about 2.4 million tons, with the Mediterranean countries as the main producers. However, for that same period olive oil consumption was around 2.9 million tons. Therefore, about 500.000 tons were not produced but still were consumed, what points out to a clear situation of fraud.

Generally olive oil fraud consist of the mixture of high quality virgin olive oil with low quality olive oil or another kind of vegetable oil or animal fat. Although, it seems to be a simple problem of economical fakes, there are also health world problems, since that can be mixed oils able to cause allergic reactions, as in case of hazelnut oil (Ben-Ayed, Kamoun-Grati & Rebai, (2013), Bremer, Smits & Haasnoot, 2009).

In order to ensure the authenticity and also the quality of olive oil, the IOC, an organization belonging to theUnited Nations (UN) included a number of sensory and physical-chemical parameters, with their corresponding method of analysis, which allow the detection of certain fraudulent practices (IOC, 2015). However, advances in the knowledge of virgin olive oil chemical composition and improvements on the refining processes, together with the large availability of new sources of fats and oils have enabled undetected fakes.

In 2012, Spanish police released an international scheme of olive oil fraud involving avocado, palm and sunflower oils, which was only detected by means of missing information on tax documents, meaning that analytical methods were not effective on the detection of this fraud (Butler, 2012).

Avocado oil is extracted from the pulp of *Persea americana*fruits, normally from the Hass and Fuerte varieties, through a system similar to that used for olive oil (Wong, Requejo-Jackman & Woolf, 2010). Avocado oil chemical composition is quite similar to that of olive oil, which makes very difficult the detection of avocado oil-virgin olive oil add mixtures (Woolf, et al., 2009, Fernandes, Gómez-Coca, Pérez-Camino, Moreda & Barrera-Arellano, 2015a).

Recently, some Molecular Biology techniques have been applied in oil analysis, mainly in the field of olive oil (Agrimonti, Vietina, Pafundo & Marmiroli, 2011). Several papers describe DNA extraction from oil and its amplification through PCR (Polimerase Chain Reaction), using DNA markers such as sequence repeat (SSR) markers to identify the DNA of species present in the samples (Pasqualone, Di Rienzo, Nasti, Blanco,Gomes & Montemurro, 2013). Different types of DNA analysis have been also used to detect the

adulteration of olive oil with seed oils and/or monovarietal olive oils, as it is the case of electrophoresis separation, or real-time PCR with or without high resolution melting point (HRM) (Kumar, Kahlon&Chaudhary, 2011, Wu, Zhang, Han, Wang, Wang, Ju & Chen, 2011, Ganopoulos, Bazakos, Madesis, Kalaitzisc & Tsaftarisa, 2013, He et al., 2013).

This work aimed to develop a high throughput method to detect the addition of virgin avocado oil to virgin olive oil, using single nucleotide polymorphism (SNP) as species specific DNA marker and real time PCR for DNA amplification and detection.

2. Material and Methods

2.1 Chemicals and Samples

Heptane was supplied by VWR International (West Chester, PA, USA).DNeasy Plant Mini Kit, from Qiagen (Turnberr, CA, USA),.Maxima SYBR Green qPCR Master Mix provided by Thermo Scientific (Waltham, MA, USA). Primers were synthesized by Thermo Fisher Scientific (São Paulo, SP, BR). Olives and avocado fruit, as well as hazelnuts and pecan nuts were purchased in Brazilian grocery stores. Virgin olive oil was purchased directly from producers in Sevilla, Spain, and virgin avocado oil from Jaguacy (Bauru, SP, BR).

2.2 DNA Extraction

DNA extraction from avocado, olive fruits, hazelnuts and pecan nuts was carried out following the protocol suggested by DNeasy Plant Mini Kit, from Qiagen. Fresh samples (200mg) were disrupted with RNAse A-added lysis

buffer, at 65ºC during 10min. After that proteins and polysaccharides were salt-precipitated with a second buffer and separated by centrifugation. Supernatant was then taken to the QIAshredder column (filtration unit). Binding buffer was added to promote the binding of the DNA to the DNeasy spin column. In this way DNA was selectively bound to the membrane while contaminants could be efficiently removed. Finally, pure DNA was eluted with a small volume of sterile distilled water (SDW).

For DNA extraction from oil samples, were first homogenized during 30 min, after that, an aliquot (100mL) was placed in an Erlenmeyer flask and 50 mL heptane were added together with 500 µL of lysis buffer. The mixture was placed under strong agitation with magnetic bar (1500rpm, 30 min), and then centrifuged at 10000 rpm during one hour. The aqueous (lower) phase was transferred to a 2 mL microtube and any vestige of solvent was evaporate under gentle nitrogen flow. 4 µL RNAse A was added and incubated at 65ºC during 10min. From this point on the procedure of the DNeasy Plant Mini Kit was followed adding the buffer for salt-precipitation of proteins, and polisacharides removed by centrifugation. Supernatant was taken to the filtration unit (QIAshredder column) and then bonded to DNeasy spin column by the addition of the binding buffer. Bound DNA was washed with ethanol and eluted with a small volume of SDW. In order to confirm that the extracted DNA could be amplified we performed a RT-PCR with an universal pair of primers JR1-JR2.

2.3 Primer design

Primers for Persea Americana specific amplification were designed by selecting the gene of ribulose-1,5-bisphosphate carboxylase/oxygenase

large subunit (rbcL) from avocado (*Persea americana*, accession NCBI JF966620) and olive (*Olea europaea*, accession NCBI DQ673304).Hazelnut (*Corylus avellana*, accession NCBI AY263929) and pecan nut (*Carya illinoinensis*, accession NCBI U00436), due their chemical composition (Fernandes, Gómez-Coca, Pérez-Camino, Moreda & Barrera-Arellano, 2016b), are also species with which olive oil illegal mixtures may be made., Therefore they were selected in order to guarantee primer specificity. Genes were aligned using ClustalX and SNPs were chosen after the criteria of being allocated at the end of primer sequence (Figure 1). In this way the forward specific primers were obtained, whereas the reverse primers were designed usingNCBI/Primer-BLAST to generate a product of 100-200 bp and tohave a Tm (melting temperature) with a difference with respect to the forward primer (Tm = 60-65ºC) no more than 1ºC, and a length of 19-23 bp.Finally, four pairs of specific primers were designed for avocado oil: TP1-CP1; TP2-CP2; CP3-TP3 and CP4-TP4 (Table 1).

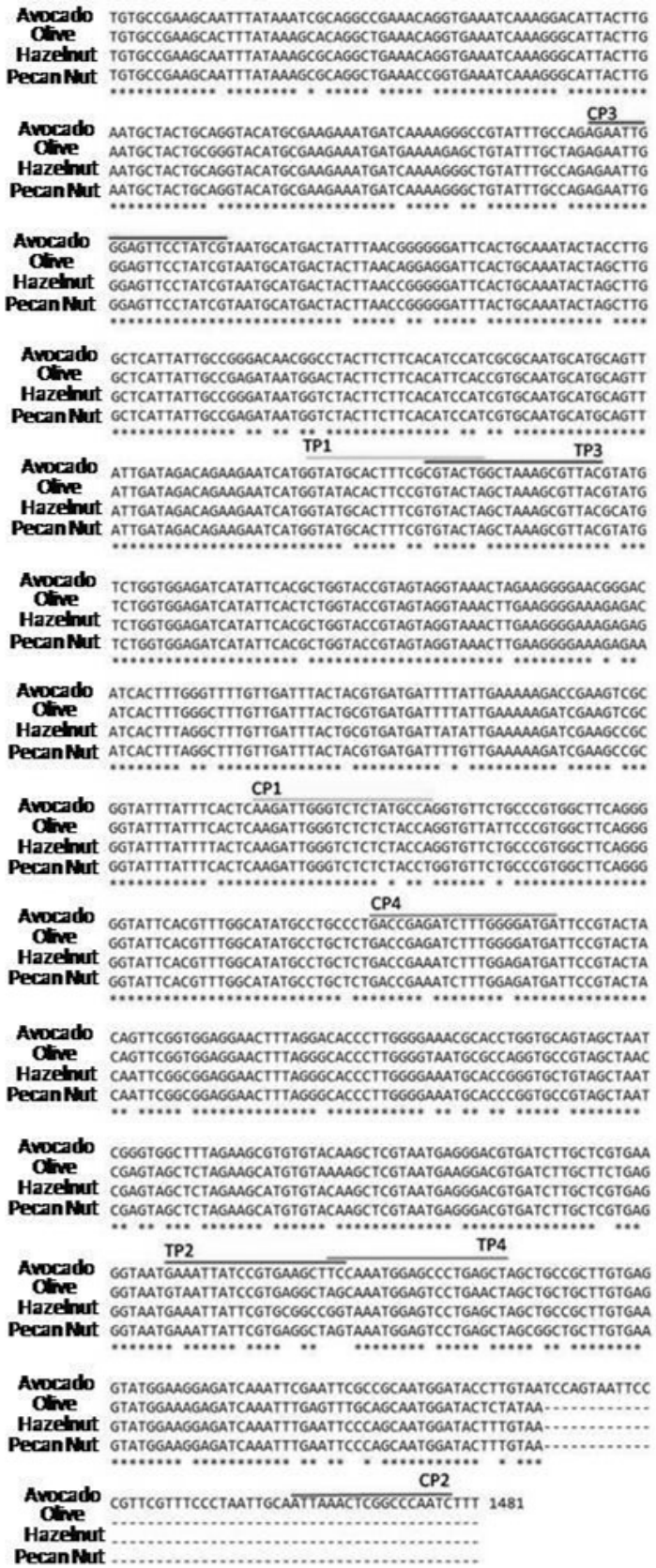

Figure 1: Primer location in SNPs of the gene of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) from avocado (*Persea*

americana, accession JF966620), aligned with the same gene of olive (Olea europaea, accession DQ673304); hazelnut (*Corylus avellana*, accession AY263929) and pecan nut (*Carya illinoinensis*, accession U00436).

Table 1: Description of PCR primers.

Pair	Forward 5'-3'	Length (pb)	CG %	Tm (°C)	Reverse 5'-3'	Length (pb)	CG %	Tm (°C)	Product Legth (pb)	Product Tm (°C)	Target Gene
TP1-CP1	GTATGCACTTTCGCGTACTG	20	50.0	65.0	TGGCATAGAGACCCAATCTT	20	45.0	65.0	194	78.5	
TP2-CP2	GAAATTATCCGTGAAGCTTC	20	40.0	65.0	GATTGGGCCGAGTTTAAT	18	61.0	65.0	182	80.0	RuBisCO large
CP3-TP3	AGAATTGCGAGTTCCTATCG	20	45.0	65.0	GTAACGCTTTAGCCAGTACG	20	50.0	65.0	152	-	subunit
CP4-TP4	GACCGAGATCTTTGGGGATGA	21	55.0	65.0	AGCTCAGGGCTCCATTTGGA	20	55.0	65.0	195	81.0	
JR1-JR2	GATCCCGGACGTAATCCTG	19	57.0	60.0	ATCGTACCGAGGGTTCGAAT	20	50.0	60.0	Depends on the sample		tRNA-Gly

2.4 Real time PCR conditions

PCR reactions were carried out in duplicate in Real Time CFX384 (Bio-Rad,Hercules, CA, USA) working with Hard-Shell® Thin-Wall 384-Well Skirted PCR Plates (Bio-Rad,Hercules, CA, USA).Reactionmix, 13µL per well, contained 6.25 µL of Maxima SYBR Green qPCR Master Mix, 1.25 µL of each (forward and reverse) primer, 3.25 µL of SDW, and 1 µL of DNA. The thermal cycling conditions consisted of an initial denaturation step at 95ºC for 3 min, followed by 40 cycles at 95ºC for 10 s and 60-65ºC for 20s; this annealing temperature depends on the primer Tm. Melting curve analysis was also performed following the final step of the PCR, from 60 through 95 ºC at 0.5ºC/s. Data were processed in CFX Manager 2.1 (Bio-Rad,Hercules, CA, USA).

2.5 Experimental procedure

2.5.1 Primer specificity

In order to verify the specificity of primers experiments of RT-PCR with designed primers TP1-CP1; TP2-CP2; CP3-TP3, CP4-TP4 were performed and applied to DNA obtained from avocado, olive fruits, hazelnuts and pecan nuts. All experiments were conducted in duplicate using two different annealing temperatures, 60 and 65ºC. Both amplification and melting point curve were monitored to determine primer specificity.

The primers that showed specificity in the previous experiments were applied to the DNA extracted from olive and avocado oils to check their performance against DNA from oil samples.

2.5.2 Detection of adulteration of olive oil with avocado

The ability to detect avocado oil in olive oil through DNA extraction from the oil mixture, and specific amplification of the adulterant was checked by spiking samples of olive oil with avocado oil in amounts of 10, 5 and 1% (w/w). Spiked oil samples were homogenized and the DNA extracted according to the procedure described above (Section 2.2). Real time PCR was performed with specific primers selected previously. To check the suitability of the procedure, amplification and melting curves were monitored. All these steps were conducted in triplicate.

3. Results and Discussion

3.1 Primer Specificity

The main question when performing these experiments is a relatively simple one: Does the pair of primers specifically amplify DNA from avocado oil? Thus, to access the first information about the specificity of primers designed for avocado rbcL gene (JF966620) primers were tested against DNA extracted from olive fruits, avocado, hazelnuts and pecan nuts. Although this work aimed to detect the presence of avocado oil in olive oil we also checked the specificity against other sources of virgin oils that could also be used to adulterate virgin olive oil. In this way, the possibility of false positive results signaling the presence of avocado oil in olive oil was minimized. The experiments were carried out in two annealing temperatures, 60 and 65ºC. The TP2-CP2 pair of primers did not amplify at any of the temperatures for neither DNA samples therefore it was discarded. The CP4-TP4 pair amplified for all the samples at both 60 and 65ºC annealing temperatures. Figure 1A shows the amplification graphic of CP4-TP4 for all samples in the annealing temperature of 65ºC; the best amplification occurs for avocado samples with a medium cycle threshold (Cq) of 17.55 cycles and a maximum fluorescence medium (Fmax) of 6191.23 RFU. For olive oil DNA the Cq was 29.76 cycles and the Fmax 4103.15 RFU. For nut samples DNA there was also amplification by CP4-TP4: for hazelnut DNA the Cq was 33.12 cycles and Fmax 2564.19 RFU; for pecan nut DNA, Cq was 33.62 and Fmax 2596.71 RFU. For all samples the same reaction product was observed, as shown in Figure 1. The melt temperature (Tm) of all products was 81ºC. The CP4-TP4 pair of primers was discarded due to its lack of specificity.

TP1-CP1 and CP3-TP3 pairs were not specific for avocado oil at the annealing temperature of 60ºC on the contrary they were specific for those samples at 65ºC, (Figure 2). Actually, TP1-CP1 showed a Cq of 24.96 cycles and Fmax of 3442.89 RFU, while CP3-TP3 showed a Cq of 28.65 cycles and Fmax of 1488.03. The amplification product for TP1-CP1 had a Tm of 78.5 ºC, whereas that for CP3-TP3 had a Tm of 80.0 ºC. As these pairs of primers were specific for avocado oil samples we kept them for the next experiment, where the ability of TP1-CP1 and CP3-TP3 to amplify DNA extracted from avocado oil was checked.

It is important to keep in mind that DNA extracted from each oil sample was checked against the JR1-JR2 universal primer pair, obtaining in all cases positive results. The CP3-TP3 pair did not amplify the DNA extracted from avocado oil samples. However, TP1-CP1 amplified this same DNA very well (Figure 2), showing the same amplification product than for fruits (78.5 ºC, 28.19 cycles and, 2830.60 RFU). This pair of primers keeps its specificity in the DNA extracted from oil samples, in other words, it does not amplify in olive and nut oil samples. In this way TP1-CP1 was the pair of primers selected for the next step of this work: detection of the addition of avocado oil in olive oil.

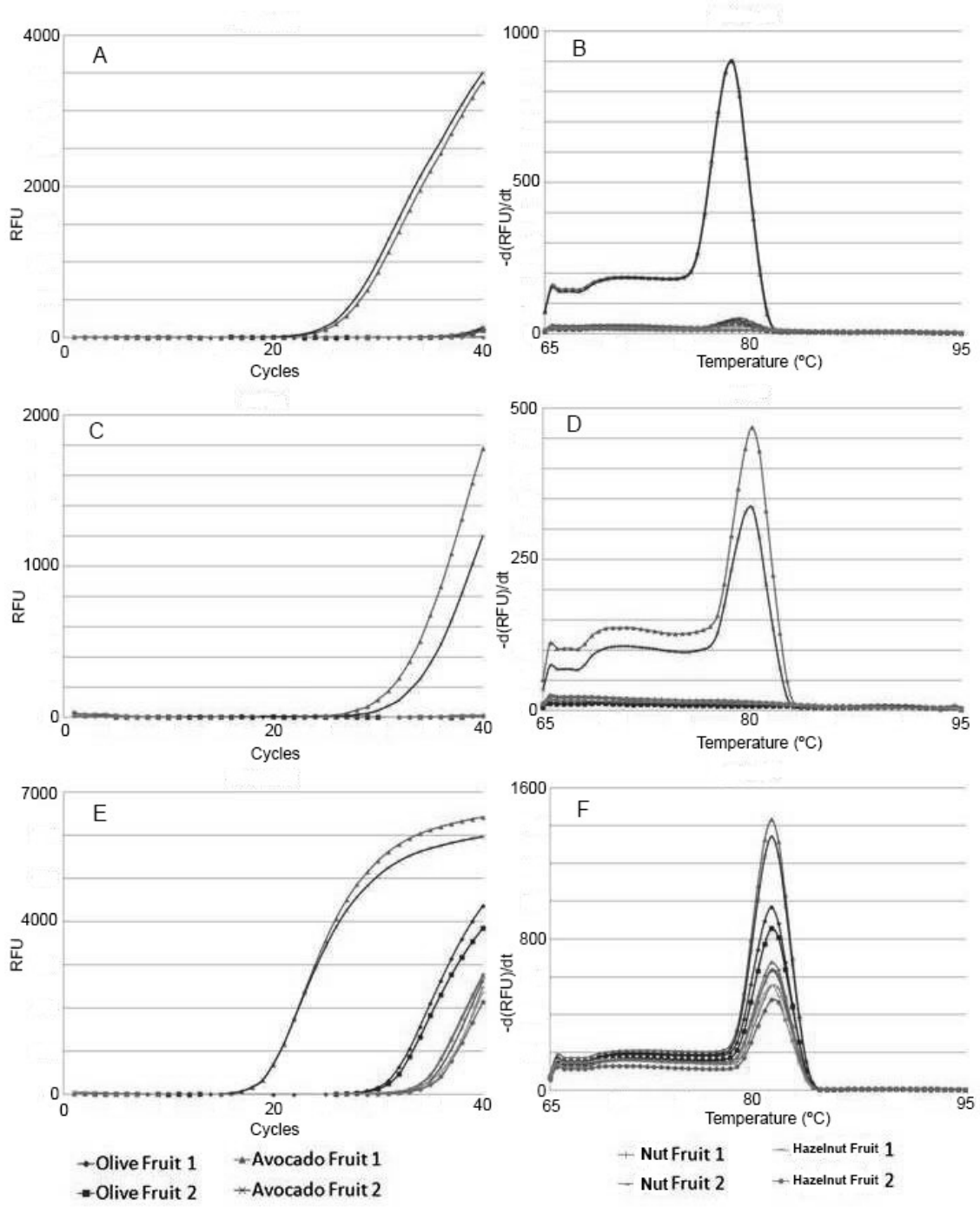

Figure 2: Results of the experiment for checking the primer specificity in fruit/nuts samples. (A) Amplification curve for the TP1-CP1 pair; (B) Melt curve for the TP1-CP1 product reaction; (C)Amplification curve for the CP3-TP3 pair; (D) Melt curve for the CP3-TP3 product reaction;(E)Amplification curve for the CP4-TP4 pair; (F) Melt curve for the CP4-TP4 product reaction.

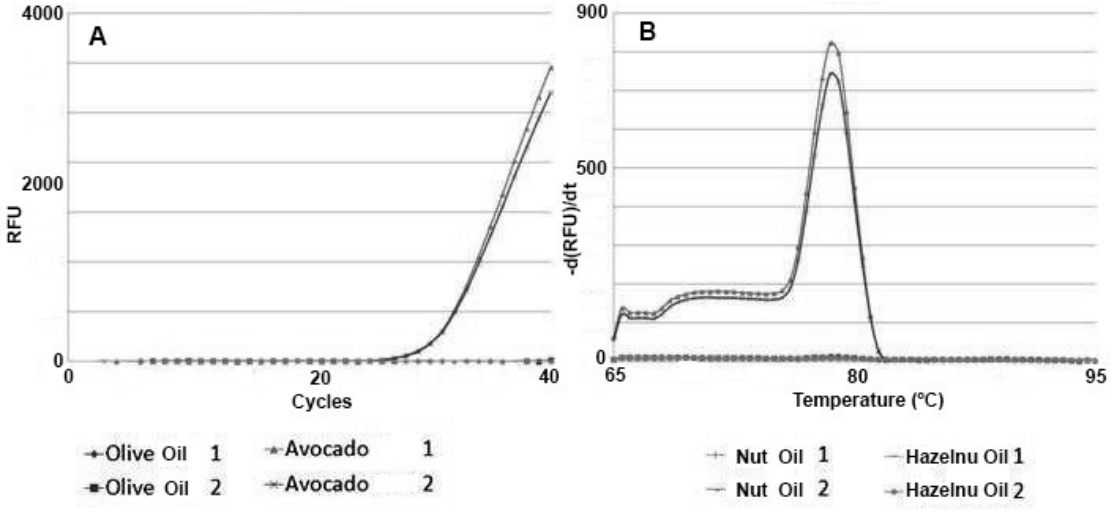

Figure 3: Results of the experiment for checking the primer specificity in oil samples. (A) Amplification curve for the TP1-CP1 pair; (B) Melt curve for the TP1-CP1 product reaction.

2.2 Detection of adulteration of olive oil with avocado oil

These experiments were conducted in order to detect the addition of virgin avocado oil in virgin olive oil. It sought to discover if the extraction method together with the pair of primers selected would be able to detect the presence of avocado oil in olive oil. Samples with 10, 5, and 1% olive oil were prepared and analyzed in triplicate.

The mixture in which avocado oil was present at 10% was perfectly identifiable in the three repetitions, since the amplification product was clearly the one with Tm 78.5 ºC, and the experiments showed Cq of 30.47 ± 0.27 cycles and Fmax of 1477.74 ± 83.23 RFU. Samples with 5 % avocado oil were also detectable, (the product of Tm 78.5 ºC was confirmed). However, the detection had a high Cq (35.60 ± 0.57 cycles) and a low Fmax(269.47 ± 47.16 RFU), which indicated that the detection limit was close. This indication was

confirmed when working with the virgin olive oil sample with the lowest avocado oil presence (1%). In this case the adulteration could not be detected.

Therefore, the detection limit for this method and this pair of primers was set at 5% avocado oil adulteration in virgin olive oil. Although 5% may seem high, this is the first method that proposes a solution to the problem of adulteration of virgin olive oil with avocado oil.

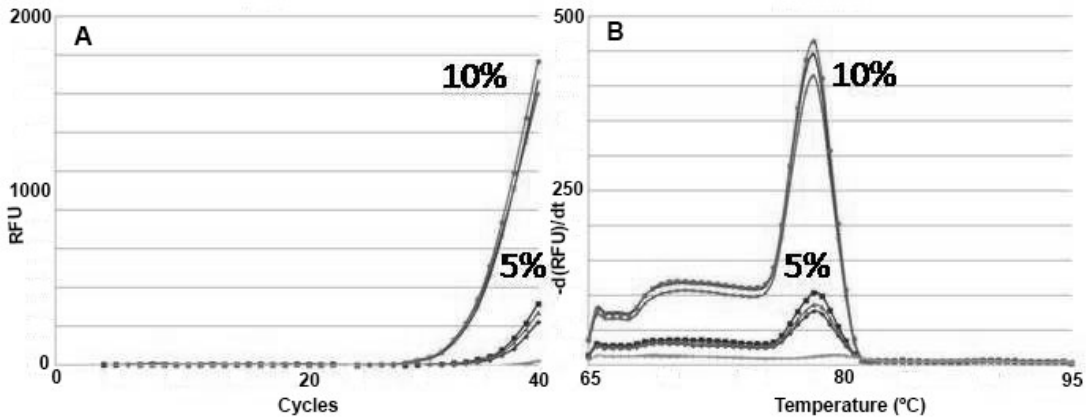

Figure 4: Results for the detection of olive oil fraud with avocado oil. (A) Amplification curve for the TP1-CP1 pair applied in spiked samples of virgin olive oil with 10, 5, and 1% virgin avocado oil; (B) Melt curve of the product reaction for the TP1-CP1 CP1 pair applied in spiked samples of virgin olive oil with 10, 5, and 1% virgin avocado.

4. Conclusions

Four specific pair of primers for avocado oil were designed in this work, however, according to primer specificity only two pairs of them, TP1-CP1 and CP3-TP3, are specific and could be used for application in oil samples. Besides, only the TP1-CP1 pair showed specificity and high application performance for DNA extracted from oil samples.

In order to guarantee the suitability of the method for the detection of avocado oil adulteration in olive oils, it was applied in spiked samples of virgin olive oil where avocado oil was present at concentrations of 10, 5, and 1%. In this way we could detect the addition of 10 and 5% virgin avocado oil in virgin olive oil.

Finally, this work brings a new approach on the detection of olive oil fraud. For the first time the application of SNP on the detection of virgin olive oil frauds has been demonstrated together with the detection of avocado oil in virgin olive oil, a problem which had not been faced up to date.

Acknowledgements

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DISCUSSÃO GERAL

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A detecção de fraudes baseada na adição de outros óleos ao azeite de oliva depende do conhecimento das características químicas dessas fontes lipídicas. Desta maneira, os órgãos regulatórios vêm trabalhando para identificar marcadores de composição química, bem como disponibilizar métodos analíticos capazes de diferenciar o azeite de oliva autentico do adulterado. É o caso da utilização do ácido linoleico e linolênico para detectar fraudes com óleos de soja e linhaça; colesterol para inferir sobre fraudes com gorduras animais; os $\Delta 7$ esteróis para a presença de óleos de sementes e ainda a presença de 3,5-estigamastadieno para a presença de óleos e azeites refinados.

Mesmo conhecendo a composição das fontes de fraude e estabelecendo os marcadores químicos, não é possível afirmar com exatidão qual seria a quantidade de óleo/azeite adicionado em uma fraude, bem como, não é possível estabelecer exatamente qual o limite de detecção de fraude dos métodos utilizados. Estas limitações estão diretamente relacionadas à variabilidade de composição dos diferentes óleos e azeites, aliadas à ampla faixa de composição do azeite de oliva, aceita pela legislação. Vale ressaltar também que estas informações não dependem apenas da composição da fonte usada para fraudar, mas também da composição química do azeite a ser adulterado.

No artigo 1 foram analisados óleos virgens de amêndoa doce, avelã e noz pecan, tanto amostras de origem comerciais como amostras extraídas por prensagem. Contrapondo a composição em ácidos graxos com a faixa de composição descrita na IN 01/2012 MAPA para azeite de oliva, apenas as

amostras de amêndoa doce encontram-se fora da legislação devido ao alto teor de ácido linoleico, cerca de 29%. Entretanto, este marcador não deve ser efetivamente utilizado como parâmetro para detecção de fraudes, uma vez que, uma simples mistura com um azeite de oliva baixo em ácido linoleico seria suficiente para tornar esta fraude não detectável.

Para estas fontes, a composição em esteróis, que é muitas vezes mencionada como a impressão digital das espécies oleaginosas, também não seria efetiva, uma vez que são sutis as diferenças em relação ao que está previsto na legislação. Por outro lado, também seriam facilmente corrigidas por meio de misturas com azeites de oliva puros, tanto em relação ao perfil de esteróis quanto à quantidade de esteróis totais.

Dos parâmetros legislados nacionalmente o que se demonstraria mais eficiente para a detecção de fraude seria o incremento em ECN42, uma vez que as amostras de amêndoa doce e noz pecan apresentam elevados teores de LLL. Entretanto, a mistura com azeites de oliva genuínos, com baixos teores de LLL, poderiam ainda impossibilitar esta detecção. O método global seria o método que, baseado nas características químicas, detectaria estes azeites adulterados com maior eficiência, entretanto, este método ainda não esta previsto pela legislação brasileira e ainda assim, ficaríamos dependentes da eficiência de apenas um método.

Os perfis de metil e dimetilesteróis, alcoóis alifáticos e alcoóis terpênicos, apesar de mostrarem informações importantes quanto às características químicas destes óleos, não seriam eficientes para a detecção de fraudes. As quantidades totais destes compostos são muito baixas e seriam facilmente encobertas pela mistura com azeite de oliva. O perfil de tocoferóis

seria outra característica bastante singular, principalmente a presença de γ-tocoferol na amostra de noz pecan. Entretanto, os tocoferóis não são confiavelmente aplicados na detecção de fraudes, pois são facilmente degradados pela ação da luz e do calor.

No artigo 2 encontra-se a caracterização química dos azeites de abacate. Neste caso foi possível caracterizar tanto amostras monovarietais de abacate quanto amostras comerciais. A extração do azeite de abacate se dá de maneira muito semelhante àquela do azeite de oliva, utilizando a centrifugação. Isso facilita o uso do azeite de abacate na adulteração de azeite de oliva, pois os dois podem ser extraídos nas mesmas instalações. Avaliando a sua composição em ácidos graxos, nota-se que a presença de ácido palmitoleico é bastante característica deste azeite. Entretanto, este não é um parâmetro suficiente para a comprovação da adulteração do azeite de oliva, uma vez que este pode conter até 3,5% de ácido palmitoleico. Apenas a titulo de exemplo, poderíamos adicionar cerca de 40% de azeite de abacate à um azeite de oliva, baixo em ácido palmitoleico (0,3%), sem que fosse possível detectar a fraude por meio da composição em ácidos graxos.

Em relação aos esteróis, praticamente todos os parâmetros atendem à legislação nacional para azeite de oliva e, consequentemente, não seriam aplicáveis para a detecção desta fraude.

Continuando a avaliar os parâmetros presentes nas legislações, para o caso do azeite de abacate, a utilização do incremento em ECN42 não seria eficiente, uma vez que a presença dos triacilgliceróis de ECN42 é muito baixa em azeites de abacate, de modo muito semelhante ao azeite de oliva. O perfil de triacilgliceróis do azeite de abacate como um todo é muito semelhante

ao perfil de triacilgliceróis do azeite de oliva. Este é um fator que está relacionado à semelhança na fisiologia de armazenamento e síntese lipídica nas duas espécies. Portanto, a fraude com azeite de abacate é muito tentadora, uma vez que a semelhança no perfil de triacilgliceróis diminui a eficiência do método global, que embora menos eficiente do que para as outras fraudes, ainda é a melhor opção analítica.

Para os outros parâmetros analisados, como o perfil dos metil e dimetilesteróis, alcoóis alifáticos e triterpênicos, tocoferóis e esqualeno embora contemplem informações importantíssimas para a caracterização do azeite de abacate, não agregaram informações para a detecção de fraude de azeite de oliva com azeite de abacate.

A avaliação das características químicas presentes nos artigos 1 e 2 reitera a questão de que, apesar do extensivo esforço por parte dos órgãos regulatórios e dos programas de combate a fraudes, ainda existem fraudes de difícil detecção, ou até mesmo indetectáveis, mediante aos métodos atualmente regulamentados. Este fato reitera o conceito das fraudes sofisticadas. Ainda que, aparentemente, as fraudes sofisticadas não tenham dominado o mercado, é fato que não há ferramentas legais cabíveis à detecção destas adulterações. Isso fica mais evidente ao aobservar a notícia citada no artigo 4, que retrata o fato das autoridades espanholas só descobrirem uma fraude com azeite de abacate devido às falhas fiscais cometidas pelo sistema de fraude utilizado na ocasião.

Mediante este cenário, a segunda parte desta tese (artigos 3 e 4) objetivou desenvolver ferramentas baseadas em marcadores moleculares de DNA para o combate de fraudes sofisticadas em azeite de oliva.

A detecção das fraudes com óleo de amêndoas, avelã e nozes em alimentos é bastante difundida no mercado alimentício, devido ao caráter alergênico destes frutos secos. Desta maneira, no artigo 3 buscou-se adaptar *primers* previamente desenvolvidos para a detecção destas espécies em alimentos a base de cereais e chocolate, descritos na patente EP2226395A1, na detecção de fraudes de azeite de oliva com óleos de amêndoa doce avelã e noz pecan.

Para viabilizar a análise de DNA nas amostras de óleos e azeites trabalhamos com um método rápido de extração de DNA de óleos e azeites desenvolvido por uma equipe de pesquisadores colaboradores no Instituto de La Grasa (método ainda não publicado). Tendo portanto, acesso ao DNA de cada óleo, a especificidade dos *primers* foi confirmada por meio de PCR em tempo real. Posteriormente certificamos a eficácia do método como um todo, extraindo o DNA e analisando amostras adulteradas de azeite de oliva com óleos de amêndoa doce, avelã e noz pecan. Ao final estabeleceu-se um limite de detecção de 5% para os óleos virgens em questão. Sabe-se que este limite é sempre dependente da quantidade de DNA inicial nas amostras utilizadas para a adulteração, mas também leva-se em consideração que existem ferramentas de biologia molecular capazes de possibilitar o acesso a quantidades menores de DNA e que poderiam ser aplicadas em projetos futuros.

No artigo 4 que trata da detecção de fraudes em azeite de oliva pela adição de azeite de abacate utilizando marcadores moleculares de DNA, tomou-se uma abordagem diferente. Não haviam *primers* específicos descritos para abacate que pudessem ser aplicados neste trabalho, portanto, foi

necessário desenharmos os *primers* baseados nos bancos de dados de informação genômica de abacate. Deste modo, foram desenhados quatro pares de *primers* capazes de anelar e amplificar parte do gene da subunidade L da enzima RuBisCo. Estes *primers* foram então submetidos aos testes de especificidade, sendo que apenas dois pares de *primers* foram específicos para abacate. Após o teste de eficácia mediante o azeite de abacate apenas um par de *primers* mostrou-se efetivo para ser aplicado como marcador molecular para a detecção de fraudes com azeite de abacate em azeite de oliva. Por fim, o limite de detecção experimental também foi de 5% de azeite de abacate virgem em azeite de oliva virgem, fazendo desta, a única ferramenta atualmente disponível para esta detecção.

De modo geral, nota-se que os métodos baseados em análise de marcadores moleculares de DNA podem contribuir muito como ferramentas adicionais para a detecção de fraudes de identidade em azeite de oliva, principalmente no caso das fraudes sofisticadas onde muitas vezes os parâmetros químicos perdem eficácia.

CONCLUSÕES GERAIS

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Com o intuito de aprofundar o conhecimento das fontes de fraudes sofisticadas em azeite de oliva, amostras de óleos e azeites provenientes de amêndoa doce, avelã, noz pecan e abacate foram caracterizados quanto a parâmetros referentes à composição química.

Em relação à composição de ácidos graxos todas as amostras são prediominantemente compostas por ácido oléico, de maneira muito semelhante ao azeite de oliva. Pela primeira vez os isômeros ω-7, ω-9 e ω-11 dos ácidos oléico e palmitoleico foram detalhadamente descritos para estas amostras. Como reflexo da composição em ácidos graxos, os principais triacilgliceróis foram os triacilgliceróis que contem ácido oleico.

β-sitosterol (desmetilesterol) foi o principal esterol para todas as amostras. As smostras apresentaram altos teores de desmetilesteróis e baixos teores de metil e dimetilesteróis, alcoóis alifáticos e triterpenicos e tocoferóis. Dentre os compostos minoritários, destacaram-se os elevados teores de esqualeno nos azeites comerciais e monovarietais de abacate.

Após a caracterização química das fontes de fraudes não foi possível estabelecer marcadores químicos capazes de serem aplicados de maneira eficiente na detecção de fraudes com estas fontes lipídicas no azeite de oliva.

A anállise de marcadores moleculares de DNA possibilitou estabelecer uma ferramenta para a detecção destas fraudes sofisticadas.

Para fraudes de azeite de oliva com óleos de amêndoa doce, avelã e noz pecan, os *primers* da patente EP2226395A1 (previamente descrita para

detecção de alérgenos em alimentos) tornaram possível a detecção de até 5% de adição.

Porem, para o azeite de abacate foram desenvolvidos *primers* específicos. A especificidade dos primers desenvolvidos foi testada e a ferramenta foi aplicada para a detecção de até 5% de azeite de abacate virgem em azeite de oliva virgem.

Por fim, este trabalho contribuiu para ressaltar a análise de marcadores moleculares de DNA para o combate de fraudes, propondo o desenvolvimento e disseminação desta ferramenta como complementar à análise regulatória de azeite de oliva.

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