Riscos e Alimentos

Portuguese Food Safety Authority Scientific Magazine

Olive oil and other vegetable oils

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Editorial

Pedro Portugal Gaspar
General Inspector of ASAE

The scientific area of ASAE has been to the citizens the less acknowledged of ASAE's services. This trend has been contradicted and has been receiving a strong commitment to this new management. The reason for this lies in the fact that, strategically, it allows to obtain useful information either from laboratory or from risk assessment procedures, which technically supports operational activity with statistical and scientific data.

In fact, ASAE, is the authority that gathers in terms of skills, all components of risk analysis, including assessment, management and communication, have to take an integrated approach for this purpose using all the tools available to make its performance adequate, consistent and proportionate to the risk. From this perspective, ASAE has a plan of activities on the scientific area that will allow this year, to conclude studies in the collaboration with the members of the Scientific Council and the Thematic Panels that will serve the interests of consumers and therefore contribute to a more complete evaluation of food safety. As it´s well known, science is not consistent with individualism, and only with discussion it´s possible to get consistent results.

So it is with great pride that I support the seventh edition of the scientific journal "Riscos e Alimentos", dedicated to olive oil, and once again had the valuable collaboration of the Scientific Council and thematic panels, as well as other authors, which magnify this publication. The theme, as mentioned in the first paragraph, relates to the laboratory information that was being collected in recent times and that led us to identify an issue to be highlighted in terms of risk communication.

I want to publicly thank all who contributed to the make possible the edition of this magazine.

Risk Assessment and Laboratory Activity in Food Safety Seminar

Graça Mariano
Director - DRAL/ASAE[1]
Department of Risk analysis and Laboratories/ASAE[1]

ASAE organized on 25 of June 25, 2014, on the campus of the Lumiar the Seminar entitled "Risk Assessment and Laboratory Activity in Food Safety". This seminar was attended by His Excellency the Deputy State Secretary and the Economy, Dr. Mathias Leonardo, as well General Inspector of ASAE, and also representatives of various National and European entities related to risk assessment and laboratory activities in food.

During the seminar was held the ceremony of inauguration, respectively of the Scientific Council of the ASAE[2] and members of the five Thematic Panels: Additives and Contaminants in the Food Chain; Food, Health and Animal Welfare; Nutrition and Food Allergies; Biological Hazards; Plant protection and GMOs. It is therefore considered that the organizational conditions are met for the Scientific Council assisted by thematic panels to have an active participation in the process of risk assessment, in particular in the provision of scientific advice in the area of food security.

The seminar consisted in four different topics with speakers from diverse public and private agencies:

1. Risk Assessment and Communication in Portugal (CC ASAE);
2. Scientific Cooperation in the European Panorama (EFSA[3], AECO SAN-Spain[4], ASAE);
3. Perspectives Laboratory Activities in Portugal (INIAV[5], GLOBALAB, SGS PT, ASAE, INFARMED[6], RELACRE[7] and Biopremier);
4. Accreditation in Laboratory Activity (IPAC[8], RELACRE, ASAE).

The collection and analysis of data to enable the characterization and assessment of risk in food safety, ensure public and transparent risk communication and the promotion and dissemination of food security information from consumers, are an integral part of the preventive arm of the ASAE . In this context, ASAE is the national liaison body, with its similar organizations at European and international level, acting as a focal point of EFSA in Portugal for technical and scientific issues related to assessment and risk communication in food safety. Therefore, EFSA was also represented in panel II, through the presence of Dr. Sergio Rodeia which presented the theme "Tools for scientific cooperation between EFSA and Portugal."

The aim of this seminar was to focus also on accreditation as a key tool to support the official control, both in terms of prevention and inspection. In this perspective, the last panel of the seminar reported the experiences of the accredited laboratories of ASAE and the different partners in the context of laboratory activity. As a corollary of this panel, it was identified the importance of accreditation in ensuring the credibility of the control of foodstuffs placed on the market. The laboratories of the ASAE has now 15 year story of accreditation, and a technical annex with 121 accredited methods.

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[1] DRAL/ASAE - Department of Risk analysis and Laboratories / Authority for Food and Economic Safety
[2] CC ASAE - Scientific Council of the Authority for Food and Economic Safety
[3] EFSA- European Food Safety Authority
[4] AECOSAN-Spain - Spanish Agency for Consumer Affairs, Food Safety and Nutrition
[5] INIAV - National Institute of Agricultural and Veterinary Research
[6] INFARMED - National Authority of Medicines and Health Products
[7] RELACRE - Association of Accredited Laboratories of Portugal
[8] IPAC - Portuguese Institute for Accreditation

Olive oil and health

Marina Rodrigues[1], Ana Ferreira[1], Marta Rocha[1], Patrícia Padrão[1], Pedro Graça[1]

[1] *Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto*

In the Mediterranean basin, olive oil, whole grains coupled to fruits, vegetables, nuts and moderate amounts of lean meats (preferably white), fish, red wine and dairy products, produce a dietary pattern promoter of health with clear benefits in the longevity of populations (1).

The Mediterranean Diet (MD), considered Intangible Heritage of Humanity by UNESCO in December 2013, was first described in the fifty to sixty years by Professor Ancel Keys, who observed a lower incidence of morbidity and mortality from coronary heart disease in populations from the Mediterranean region, compared to the U.S. and the countries of northern Europe (2, 3).

Indeed, at present the scientific evidence is growing regarding the association between the adoption of MD as a healthy food pattern and the decrease risk of developing neurodegenerative, cardiovascular and cerebrovascular diseases, diabetes mellitus type 2, obesity and several types of cancer (4-8).

As olive oil being a key component of the MD, the mentioned benefits should be attributed in part to its consumption by the populations of the Mediterranean as the main source of dietary fat, since it consists of several bioactive compounds (oleic acid, phenolic compounds, squalene, among others) that give this food very particular anti-inflammatory, antioxidant and anticancer properties (9-13), expressing its maximum protective characteristics in the "extra virgin" variety.

Indeed, initial work on the beneficial effects of the consumption of olive oil were attributed almost entirely to its high composition, about 78.6%, in monounsaturated fatty acids. However, and gradually their minor components were being valued, such as phospholipids, waxes, hydrocarbons, pigments, sterols (commonly called phytosterols), squalene (the most important hydrocarbon), tocopherols and phenolic compounds. Among these it is worth highlighting the phenolic alcohols (such as hydroxytyrosol and tyrosol), oleuropein and oleocantal for its strong bioactivity (14). Substances such as hydroxytyrosol and oleuropein are potent antioxidants and may explain the protective ability on the cell (15). In turn, the anti-inflammatory activity attributed to oleocantal appears to trigger a decrease of some inflammatory mediators (16, 17).

Thus, the consumption of olive oil should be encouraged favoring their consumption, not only in terms of culinary (cooked food and seasonings), but also at entrances or snacks. A good example is the Spanish rustic bread with tomato, garlic, oregano and olive oil instead of butter, pates and sauces with little nutritional benefit (18).

In summary, the consumption of olive oil, especially as part of the MD and a healthy lifestyle, has been associated with a decreased likelihood of the occurrence of some cancers, cardiovascular diseases, neurodegenerative diseases and diabetes mellitus type 2 (13, 20 , 21). Interestingly, some studies show that although olive oil is a fat and therefore with 9 kcal per gram, its use does not appear to increase the risk of developing overweight or obesety (22), and may even reduce the risk of childhood obesity in children (23). However these last extrapolations are still poorly supported. It is necessary to extend the research in this level.

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Pesticides in virgin olive oil: what is the risk?

Sara C. Cunha, José O. Fernandes
REQUIMTE, Laboratory of Food Science and Hydrology, Faculty of Pharmacy, University of Porto

Abstract

The control of diseases, pests and weeds in olive groves is often done with pesticides, therefore is not surprising the presence of pesticide residues in olive oils. Its occurrence mainly depends of the following factors: nature of the active molecule, application methods (dosage, frequency, mode of action and penetration), time between the last treatment and olive harvest and processing.

The aim of this short paper is to explain the importance of these factors to assess the safety of the olive oil. An attempt will also made to answer questions about whether virgin oils are more or less susceptible to the presence of pesticide residues compared to refined oil.

Introduction

Olive groves are often affected by pests (fruit fly, olive-kernel borer or olive moth, black scale, etc.) and diseases (anthracnose, peacock spot, tuberculosis), which are responsible by loses in olive production both at quantitative and quality level of raw material so consequently with repercussions in olive oil. Pest control of olive groves is therefore essential to ensure a continued profitability and quality. Usually, the fight against pests, diseases and weeds in olive groves is made making use of pesticides.

Pesticides include a huge number of substances with different chemical structure, usually highly toxic for humans, which have different functions and different biological action, and can be classified according to different criteria. The most common classification system divides pesticides into three large families according to the target organism: insecticides, fungicides and herbicides. Other groups with less significance are nematicides, molluscicides, acaricides and rodenticides. In the group of insecticides (some of them also exhibit nematicide and acaricide properties) are included compounds with different chemical structures, such as organophosphates, organochlorines, pyrethroids, and carbamates. Fungicides include, among others, compounds such as benzimidazoles, diazoles and tdithiocarbamates. Nitro compounds, carbamates, ureas and triazines are some classes of compounds belonging to the family of herbicides (Cunha, 2007).

Recently, European Union (EU) has approved four legislative texts, included in the Thematic Strategy on the Sustainable Use of Pesticides, with the following objectives: i) minimize the impact of pesticides on human health and the environment, ii) improve controls on the use and distribution of pesticides, iii) reduce the levels of harmful active substances to humans, iv) encourage the use of good agricultural practices, v) establish a transparent system for reporting and monitoring progress in meeting the previous measurements. The legislative package includes:

- UE regulation 1107/2009 concerning the placing of plant protection products on the market (which replaces Directive 91/414/EEC);
- UE directive 128/2009 on the sustainable use of pesticides;
- UE regulation 1185/2009 concerning statistics on pesticides;
- UE directive 2009/127/EC, an amendment to the machinery directive (2006/42/EC).

Despite the current legislation, the presence of pesticide residues in olive oil and other foods continues to be a risk to human health, since they can interfere with the reproductive systems and foetal development and most of them have the capacity to cause cancer (Gilden et al. 2010), representing a major public health issue. Data of 2011 in EU countries about pesticide residues, show that in a universe of 12 000 samples analysed, only in 53.4% of the samples no quantifiable residues were found, whereas 44.7% contained measurable residues within the legally allowed levels and only 1.9% show residues exceeding the respective MRL (EFSA 2014). The MRL corresponds to the upper legal level of a pesticide residue in or on food. In Portugal, the MRLs

are established by law according EU legislation (Regulation 396/2005); as a rule the MRLs are established for unprocessed foods. In the case of olive oil, the levels are those applied to olives for olive oil production. In olive groves, insecticides and fungicides are the most commonly pesticides used to control pests and fungal diseases, respectively. The insecticides used in olive groves includes fenthion, phosmet, dimethoate, methidathion, carbaryl, malathion, deltamethrin, which belong to the organophosphate, carbamate, organochlorine, and pyrethroide groups. Fungicides (eg. fosetyl-al, benomyl) often include phthalimides, triazoles, imidazoles, sulphonamides and other chemical classes (Tomlin, 2003). Other pesticide with large application in olive groves are herbicides such as sulfonylurea (eg. diphenyl ethers), shown in Table 1.

Table 1 - Pesticide residues in olive oil:

Pesticide	Chemical group	Action
Azinphos-ethyl	Organophosphate	Insecticide/Acaricide
Azadirachtin	Tetranortriterpenoid	Insecticide
Carbaryl	Carbamate	Insecticide
Chlorpyrifos	Organophosphate	Insecticide
a-Cyhalothrin	Pyrethroid	Insecticide
a-Cypermethrin	Pyrethroid	Insecticide
Deltamethrin	Pyrethroid	Insecticide
Diazinon	Organophosphate	Insecticide/Acaricide
Diflufenican	Pyrethroid	Insecticide
Dimethoate	Organophosphate	Insecticide/Acaricide
Diuron	Urea	Herbicide
Endosulfan sulfate	Organochlorine	Insecticide/acaricide
Ethion	Organophosphate	Acaricide/insecticide
Fenitrothion	Organophosphate	Insecticide
Fenthion	Organophosphate	Insecticide
Formothion	Organophosphate	Insecticide
Lindane	Organochlorine	Insecticide
Malathion	Organophosphate	Insecticide
Methidathion	Organophosphate	Insecticide/acaricide
Omethoate	Organophosphate	Insecticide
Oxyfluorfen	Diphenyl Ether	Herbicide
Parathion	Organophosphate	Insecticide
Phosmet	Organophosphate	Insecticide/Acaricide
Pirimiphos-methyl	Organophosphate	Insecticide/Acaricide
Terbuthylazine	Triazine	Herbicide
Terbutryn	Triazine	Herbicide
Trichlorfon	Organophosphate	Insecticide

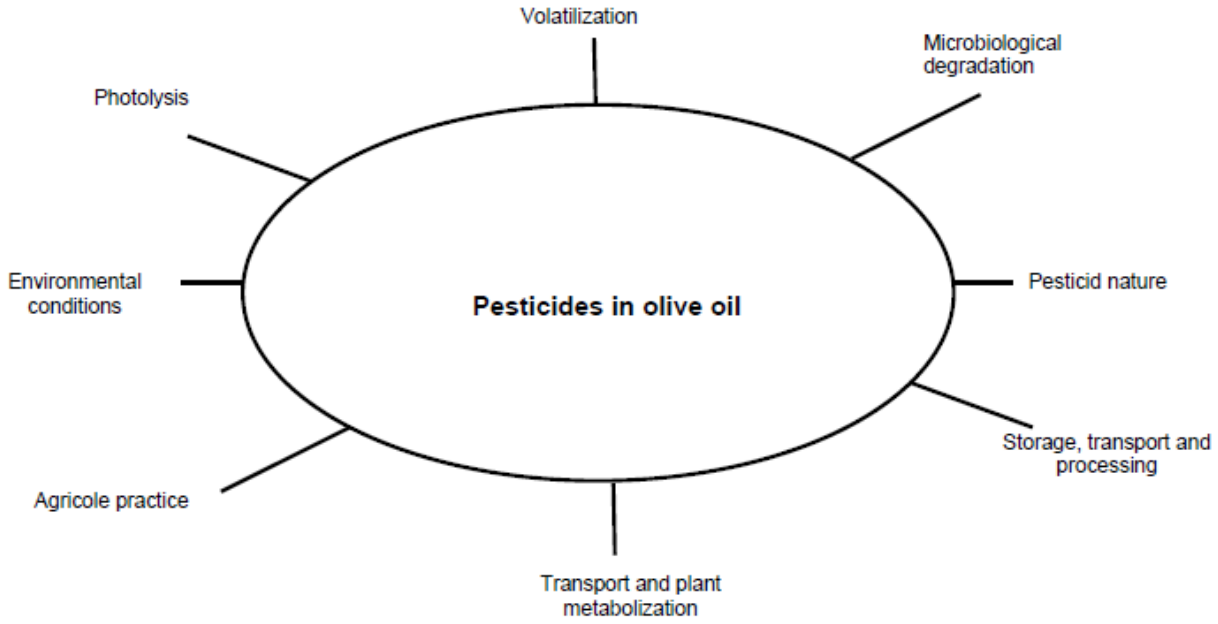
Following the application of pesticides to the crops, their path are subject to various factors, as illustrated in Figure 1, which determine it prevalence or dissemination in olives, such as the nature of the active molecule, the application conditions (dosage, frequency, mode of action and penetration), the time gap between the last treatment and the harvest the fruit, environmental conditions, and the type of storage, transportation and processing.

Generally, organophosphate pesticides have a higher toxic effect on pests as well as on mammals when compared to organochlorines and carbamates. Nevertheless, organophosphate pesticides are generally considered safe in the treatment of crops due to its relatively fast degradation, while organochlorines show high persistence in the environment and a greater capacity to bioaccumulate in the food chain.

Once applied the pesticide may be absorbed by the plant surface (surface waxy cuticle and roots), distributed to all tissues (systemic pesticide) or simple stay on the plant surface (contact pesticide). While still on the surface of the plant (leaves or fruits) the pesticide can undergo volatilization, chemical photolysis and/or microbiological degradation. All these processes can reduce the original concentration of the pesticide, although may also lead to the formation of metabolites, which can be more or less toxic than the original compound. Volatilization of the pesticide usually occurs immediately after field application. The process depends on the vapour pressure of the pesticide; pesticides with high vapour pressure tend to rapidly volatilize into the air, while those with low vapour pressure tend to remain longer in the plant surface. The rate of volatilization is also dependent on environmental factors such as temperature and wind speed. The higher the wind speed and the temperature, the faster is the evaporation of the pesticide. The photolysis occurs when pesticide molecules absorb the energy from sunlight, resulting in their degradation. The same reaction may occur indirectly as a result of the action of the pesticide products resulting from photolysis with other compounds. Some pesticides can be degraded by microbial metabolism. Microorganisms can use the pesticide as nutrients, breaking them into carbon dioxide and other metabolic products (Holland and Sinclair 2004, Keikotlhaile and Spanoghe 2011).

Even in identical application conditions (amount of active substance and olive groves), the level of pesticide residues in olives can vary widely between fruit, as found by Farris et al. (1992). The variability may be related to the size of the fruit, cultivar and ripening time.

Figure 1- Factors affecting level of pesticide residues in olive oil



During storage, transport and processing of agricultural products dissipation or concentration of pesticide residues may occur. Some pesticide residues are destroyed by food processing (heating and sterilization). In foods with a high fat content, however, its enrichment may occur.

Most agricultural products are consumed only after some processing. The cleaning and washing, are the initial steps in the majority of processing procedures, often reducing the levels of residues, particularly from non-systemic pesticides. Guardia Rubio et al. (2006) observed that washing performed routinely in oil mills was effective in removing the superficial contamination by herbicides present in olives harvested on the ground. However, even after washing, the olive oil obtained from ground olives showed herbicide residue concentrations higher than those obtained from tree olives.

In the virgin olive oil processing, after cleaning and washing the drupes to eliminate leaves and lands, processing methods comprise crushing and kneading following by pressing, whereby a mixture of oil and water, called must, is separated from the pulp. Virgin olive oils are obtained exclusively by mechanical or other physical main processes and grouped into three categories according to the free acidity degree and other parameters (extra virgin olive oil, virgin olive oil and lampante). On average 5 kg of olive groves are needed to obtain 1 liter of olive oil, therefore any pesticide residue in olives can theoretically concentrate 5 times. Farris et al. (1992) reported that the pesticides diazinon and methidathion behave in this way, whereas parathion and mecarbam, which also have high affinity for oil, have a 3-fold increase. However, in the same experiment, chlorpyriphos has about the same amount of residues in olives and olive oil, while dimethoate has a very low residue level in olive oil which may be due to its high water solubility. Similarly, Cabras et al. (1997) observed that the residues of azinphos-methyl, diazinon, methidathion, methyl-parathion, quinalphos and rotenone were higher in the oil than those found on the olives. Enrichment factors (residues in oil/ residues on olive) between 1.5 and 7 were established in function of the pesticide and the initial amount of residues on the olives. This concentration did not allow that the final levels exceeded MRLs established by law.

A comparative study conducted over three years in olive oil samples obtained from conventional Greek olive groves and organic olive groves concluded that in conventional ones olive residue levels remained constant over the three years, while in the groves from biological agriculture a decrease of 84% of fenthion and 90% of dimethoate was observed (Tsatsaki et al. 2003). In any case the MRLs established by legislation were not exceeded.

Other three-year study performed in Greece by Botitsi et al. (2004), concerning the determination of fenthion and its metabolites in 48 virgin olive oil samples supplied by different producers, found that all the levels were below the legally established MRLs. Similarly, Ballesteros et al. (2006) reported that in 15 virgin olive oil samples supplied by Andalusia producers the levels of dimethoate, chlorpyrifos-methyl and chlorpyrifos were below the MRLs legally established. Sánchez et al. (2006) detected phosmet residues in only one out of 25 virgin olive oil samples analysed, at a level lower than the LMR.

A study by Amvrazis and Albanis (2009) concerning the determination of 35 pesticides in 100 samples of Greek olive oil (29 commercial and 71 from producers of conventional agriculture), observed that 10% of samples of conventional olive groves did not contains detectable residues, while in the remaining samples 20 insecticides were detected. The highes levels coming from fenthion, dimethoate and endosulfan.

A more recent study in 2014 in Greece (Likudis et al. 2014) observed that 4 of the 70 samples analyzed with protected designation of origin or a protected geographical origin contained pesticide residue levels exceeding the MRLs (three samples contained fenthion and one parathion-methyl). The highest detection rates were observed for penconazole (n = 20) endolsulfan (n = 18) and flufenoxuron (n = 16).

In generally, virgin olive oils have higher levels of pesticide residues than refined oils (Ballesteros et al. 2006, Amvrazis and Albanis 2009, Cunha et al. 2010). These differences may be explained in part by the type of processing. Refined olive oils were obtained from virgin olive oils by refining methods involving the neutralization, discoloration and deodorizing steps. All these operations can contribute to lower pesticide residue levels found in the final product.

Although the levels of pesticide residues in olive oils were in most cases well below the MRLs established, recent studies show a prevalence of some compounds, which could have deleterious effects on human health. For example endosulfan, which use in olive groves is no longer allowed, was detected in 22 % of a total of 338 samples of Greek olive oil (Lentza-Rizo et al. 2001) and in 100% of a total of 31 samples of Spanish olive oil (Ferrer et al. 2005, Guardia-Rubio et al. 2006).

Another important aspect is that the occurrence of metabolites can be more worrying than the presence of the parent pesticides. Cunha et al. (2007a) performed the identification and quantification of fenthion and its metabolites in olives and olive oils from an olive grove where pesticide treatment was carried out at the recommended dosage. All samples (collected during the pre-harvest interval) had a common qualitative pattern, showing 4 identifiable substances (fenthion, fenthion sulfoxide, fenoxon and fenoxon sulfoxide). Fenthion and the 3 metabolites were still found in olives at harvest time. The same authors have studied the presence of metabolites of phosmet in olives obtained in similar conditions as described for fenthion. They found phosmet, phosmet-oxon, phthalimide, N-hydroxymethylphthalimide and phthalic acid in olives and the corresponding olive oil (Cunha et al., 2007b).

Conclusions

Studies performed in the determination of pesticide residues in olive oils show that there is a clear increase in the concentration of lipophilic pesticide residues when compared to the drupes. Several factors contribute to the dissipation or concentration of pesticides after it application in olive groves, such as nature of the active molecule, conditions of application, time interval between the last treatments and fruit harvest, environmental conditions, type of storage, transportation and processing. Based on the reported levels, human exposure to pesticides through the consumption of olives and olive oil is very low.

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Possible contaminants of frying oils

Tânia Gonçalves Albuquerque[a,b], Helena S. Costa[a,b], Ana Sanches-Silva[a,c], M. Beatriz P.P. Oliveira[b]

[a] *Unidade de Investigação e Desenvolvimento, Departamento de Alimentação e Nutrição, Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P.*

[b] *REQUIMTE/Faculdade de Farmácia da Universidade do Porto,* R. Jorge Viterbo Ferreira 228, Porto

[c] *Centro de Estudos de Ciência Animal, Universidade do Porto*

Introduction

The increased consumption of fried and pre-fried foods has been linked with an increase in the intake of fats and oils subjected to high temperatures. This has been influenced by social, economic and technical factors, since people spend less time in preparing food.

Oils and fats are usually processed to improve the quality, stability and safety. Despite the removal of a large amount of impurities from the oil, processing can often originate new contaminants that can cause additional health hazards to those who consume these foods.

The frying process provides a faster alternative cooking method, while it improves the sensory quality of food. The growth of industries that produce these foods has led to the development of new equipment for this purpose, both industrial and domestic, in which sometimes a large amount of oil is subjected to heating for long periods of time [1]. In the conventional frying cooking method, food is immersed into the frying oil/fat at a temperature of 180 ºC, which acts as the heat transfer medium. This form of heating is more efficient than cooking with hot air or cooking in water, since the temperature reached by the frying process is higher than those achieved by boiling water or by steam. Since part of the oil used for the heat transfer is absorbed by the food, thus becoming part of the food, the frying medium must have quality that needs to be maintained all over the process.

When the oil used in the frying process is heated for long periods, several complex reactions which produce a large number of compounds may occur, resulting in the degradation of the oil/fat. During these reactions, the functional, sensory and nutritional quality of the oil and the food can be modified, and it is not possible to continue to produce food with nutritional quality and safety.

An adequate intake of dietary fat is crucial for a healthy development. In addition, to the energy provided by the intake of fat, this nutrient also plays an important role with respect to our daily energy requirements and enables the absorption of fat-soluble vitamins. The recommended daily intake varies with age, health status and lifestyle of individuals. An excessive intake of fat has been linked as responsible for the increased risk of obesity, coronary heart disease and certain types of cancer. The mechanisms by which these diseases can be developed are complex and in many cases those mechanisms are still not fully understood.

Changes of frying oils during frying

Some of the chemical reactions that are involved in the frying oils degradation are: hydrolysis, oxidation, isomerisation and polymerisation. These chemical reactions can result in the production of free fatty acids, aldehydes, ketones, diglycerides and monoglycerides, *trans* isomers, and other compounds [2]. To avoid oxidation of oils and fats it is necessary to reduce the incidence of all factors that can induce this process, such as: keeping the minimum levels of energy (temperature and light), that are responsible for triggering the formation of free radicals process, avoiding the presence of trace metals into the oil, avoiding the contact with oxygen and preventing the formation of free radicals by means of antioxidants addition [3].

During the frying cooking method, the oil/fat undergoes a complex process of degradation yielding drastic changes in its structure. Therefore, it becomes necessary to use more stable oils and fats, among which palm oil and hydrogenated vegetable oils can be highlighted. These oils have low levels of polyunsaturated fatty acids (PUFA), which as a consequence, lead to the presence of considerable amounts of saturated fatty acids (SFA) and/or *trans* fatty acids (TFA) in

fried products. Currently, the production of high stability oils with low levels of PUFA, obtained by genetic modification of oilseeds, or oils with a high content of oleic acid, whose fatty acid composition and triglyceride is very different from conventional oils, has been increased [4]. During the frying process some toxic compounds that can be produced in the used oils/fats are, for example, 3- monochloropropane-1,2-diol (3-MCPD) and 4-hydroxy-2-*trans*-nonenal (HNE).

Trans fatty acids

TFA have been defined by different organizations, for instance *Codex Alimentarius* (1985) and EFSA (2010) [5, 6]. Although they are consistently the same, small differences may be found between them. According to *Codex Alimentarius*, TFA are "all the geometrical isomers of monounsaturated and polyunsaturated fatty acids having non-conjugated, interrupted by at least one methylene group, carbon-carbon double bonds in the *trans* configuration". EFSA specifies that *trans* polyunsaturated fatty acids "have at least one *trans* double bond and may therefore also have double bonds in the *cis* configuration".

Analytical methods are critical elements for the correct and accurate determination of TFA. Several techniques have been used to determine TFA content in foods and the most common are gas chromatography (GC) and reversed phase liquid chromatography (HPLC). In GC, either flame ionization detector (FID) or mass detector (MS) can be used, although MS detector allows a better confirmation of peak identities, not only comparison of the retention time with those of standards, but also by comparison of mass spectral information. FA have relatively high polarity, so they are generally derivatized in the corresponding fatty acid methyl esters which are non-polar derivatives [7]. With respect to chromatographic columns for TFA analyses, the most common are fused silica capillary columns of 100 m, since longer columns can reduce the risk of TFA isomers overlap [8].

There are three main sources for the origin of *trans* fats. The first source is the partial hydrogenation process which converts liquid vegetable oils into solid or semi-solid fats with appropriate melting properties suitable for products such as shortenings and margarines. The second source is the natural presence in fats from ruminant animals formed in their stomach by microbial hydrogenation of *cis*-unsaturated fatty acids. The third source of TFA is heat treatment [9].

The role of dietary fats and oils in human nutrition is one of the most complex and controversial areas of investigations in nutrition science. Dietary fat is perceived to be the "worst" of all the nutrients in promoting various diseases, like cardiovascular disease (CVD), diabetes, obesity, and certain types of cancers. According to the literature, TFA are associated with an undesirable effect on serum lipid profiles, and thus may increase the risk of CVD, being considered in this respect, worse than saturated fat [10]. A daily intake of 5 g of TFA was associated with an increase of 25% in the risk of coronary heart disease [11].

Over the years, several studies have been conducted in many countries to determine the levels of TFA in foods produced by industries, bakeries and fast food chains, with the objective of identifying the various food sources and to estimate the daily intake of TFA. According to Larqué et al. (2001), foods that contain partially hydrogenated fats contribute about 80% to 90% of the daily intake of TFA [12]. With respect to animal fats this contribution is much smaller, estimated at 2% to 8%. Refined oils have reasonably low TFA levels (1.0-1.5%), but the neutralization, especially in the preparation of fried foods, can make a significant contribution to the daily intake of TFA [13]. However, TFA are also produced during the preparation of margarines, when the PUFA from liquid oils are artificially hydrogenated to produce solid fats. Significant amounts of TFA are found in margarines, butters and some other types of industrial products that contain hydrogenated fat [14].

In Portugal, few studies in the last years, have determined TFA levels present in oils/fats. However, over the 90's, some studies were carried out by Portuguese researchers regarding the levels of TFA in various food groups, and some of the published results for margarines, oils and butters, are presented in Table 1.

Table 1. TFA methyl esters content (g/100 g of fat) in margarines, oils, butters and shortenings.

Foods	TFA[a] (g/100 g of fat)
Table margarines	0.23 – 14.8
Culinary margarines	0.95 – 13.1
Industrial margarines	3.15 – 12.9
"Shortenings"	0.07 – 16.9
Liquid fat for culinary	0.13
Butter	4.62 – 5.26
Vegetable oils	0.13 – 1.55
Cooking oils	0.14 – 0.25

Adapted from [15]

[a] The values are presented in g of fatty acids methyl esters per 100 g of fat.

In addition to the determination of fatty acids content in raw oils/fats, studies were also performed to assess the fatty acids composition of oils submitted to continuous frying operations [16]. In this study, frying tests were conducted in an oven up to 96h (using only the oil) to evaluate the formation of TFA in oils/fats that are commonly used in the frying process. Furthermore, the fatty acid profile of the oils used to fry potato chips, octopus fillets and meat rissoles was determined, as well as the fatty acid composition of the fried foods. It was concluded that the presence of TFA is dependent on the nature of the oil, i.e. soybean oil showed the highest levels compared to the frying oil. Nonetheless, frying oil showed more stability to frying. Moreover, it was also concluded that the fatty acid composition and the *trans* isomers of the frying bath, is a crucial factor to the final fat composition of the fried product [16].

3-monochloropropane-1,2-diol

3-MCPD is a food processing contaminant that belongs to a group of chemicals known as chloropropanols. These are chemical derivatives of glycerol, structurally characterized by the presence of one or two chlorine atoms. According to scientific literature, 3-MCPD is found in greater abundance in food, followed by 2-monochloro-1,2-diol (2-MCPD).

In recent years, there has been an increase in the number of studies related to the development of analytical methods for determining 3-MCPD levels in foodstuffs, as demonstrated by the recent scientific review of Crews et al. (2013) [17]. The analysis of these contaminants is extremely complex and there are two types of methods, direct and indirect. Direct methods allow the individual identification of esters of 3-MCPD. Usually these methods are preceded by solid phase extraction and determination of 3-MCPD is subsequently performed using GC methods mainly coupled to MS detector [18]. However, methods of high efficiency liquid chromatography coupled to mass spectrometry (LC-MS), using detectors as time of flight (TOF), orbitrap and triple quadrupole (MS/MS) have also been developed [19]. In indirect methods, the total concentration of 3-MCPD esters is measured as free 3-MCPD and, most of the times, it includes the addition of an internal standard, hydrolysis, neutralization, removal of fatty acids methyl esters, and finally the derivatized 3-MCPD is analyzed by GC/MS.

The International Agency for Research on Cancer has classified 3-MCPD as a possible human carcinogen (group 2B) [20]. There are few studies in the scientific literature, and many of them are controversial with regard to the effects of these contaminants on human health. However, in 2001, the "Scientific Committee on Food" concluded that 3-MCPD is a non-genotoxic carcinogen and established a tolerable daily intake for 3-MCPD of 2 µg/kg body weight [21]. The European Commission has established maximum levels of 20 µg/kg (for the liquid product containing 40% dry matter, corresponding to a maximum level of 50 µg/kg in the dry matter) for the occurrence of 3-MCPD in hydrolysed vegetable protein and soy sauce [22].

In the early 80's, the presence of 3-MCPD was detected in hydrolysed vegetable protein in soy sauce and similar products, which was formed as a product of the reaction of hydrochloric acid with triacylglycerols, phospholipids and glycerol present in vegetable oils [23]. Afterwards, it was found that 3-MCPD can also be present in other products which are thermally processed, such as pastry, malt-derived, smoked and/or cured fish or meat. In 2006, Zelinková et al. published the first study on the presence of 3-MCPD esters in fats and oils [24]. In Portugal, up to now, information regarding the content of this contaminant in food is still very limited or inexistent. In Table 2, some results from other countries concerning the levels of 3-MCPD in oils and fats are shown.

Table 2. Levels of 3-MCPD (µg/kg) in oils and fats.

Food	Range (µg/kg)	Reference
Refined olive oil	<300 – 2462	[24]
Milk fat, lard and poultry fat	<100 – 300	[25]
Margarine	400 – 4500	[25]
Frying oils (fresh and used)	500 – 5200	[25]
Refined vegetable oils	<300 – 1234	[24]
Refined vegetable oils	150 – 1880	[25]
Refined sunflower oil	100 – 2100	[26]
Refined salmon oil	700 – 13000	[26]
Refined palm oil	1100 – 10000	[26]

The quantification of the levels of these compounds in oils and fats is particularly important with respect to human health, since in addition to the oils/fats that contain very substantial amounts of 3-MCPD, these same oils/fats are often ingredients in processed foods. For example, palm oil

is commonly used in preparing foods for infants, pastry products and mayonnaise, among others. In recent years, significant improvements have been obtained regarding the development of analytical methods for determination of 3-MCPD, studies related with the formation mechanisms of these contaminants in foodstuffs, identification of new food sources, assessment of potential toxicity and development of processing techniques that minimize the formation of 3-MCPD and other chloropropanols.

4-hydroxy-2-*trans*-nonenal

HNE is a secondary lipid peroxidation product of linoleic acid and other omega-6 fatty acids [27, 28]. It is a toxic compound and has been related to atherosclerosis, low density lipoproteins oxidation, stroke, Parkinson's, Alzheimer's and Huntington's diseases, among others [29-31].

These aldehydes are formed as a result of degradation of the fatty acids in the presence of oxygen. When vegetable oils, particularly those that are rich in polyunsaturated fatty acids, are subjected to high temperatures, which occur with frying, the risk of formation of secondary products such as HNE is increased. Due to the toxicity of HNE, which can be absorbed by food, information regarding the mechanisms of formation of this compound, particularly in fats and oils subjected to high temperatures, is very important from the point of view of public health.

The analytical determination of HNE is mainly carried out using LC-MS or GC-MS techniques [32]. For GC-MS analyses, the sample requires derivatization, e.g. using pentafluorobenzil oxime, followed by silylation, and HNE may be detected with negative chemical ionization and quantified by comparison to the internal standard (deuterated HNE) [33, 34]. Methods that involve analysis by LC-MS have the advantage of not requiring derivatization, and thus the sample may be analyzed with less extraction intermediate steps [35, 36].

Boskou et al. (2006) reported results for HNE content in oils (sunflower, palm oil and vegetable oil) used to fry potatoes, as well as, for the fried foods, and found out that the presence of HNE is mainly related to the type of oil used for frying, and not so much with thermal deterioration of the oil [37]. In 2008, Han and Csallany compared butter and vegetable oils subjected to high temperatures for short periods of time, and subjected to lower temperatures but for longer periods of time [38]. The results were similar, and once more the fatty acid composition of vegetable oils is more important than the exposure time to the heat treatment itself, concluding that for oils/fats containing high levels of linoleic acid, the heat treatment must be performed using low temperatures to prevent the formation of HNE [38].

Scientific studies showed that HNE may be present in other foods than oils/fats of vegetable origin, like ham, bacon, and smoked sausages with varying contents between 3.77 and 95.2 mol/kg [39]. Csallany and Han (2012) have determined the levels of HNE in natural and imitation of Mozzarella cheeses, which were exposed to different temperatures and different heating times [40]. According to the obtained results, the formation of HNE was significantly lower in natural Mozzarella cheeses, which contains milk fat, in comparison with the imitation Mozzarella cheese that uses vegetable oils (high in linoleic acid) [40].

Therefore, since HNE is a toxic compound associated with negative effects on human health, more scientific research focusing on the determination of the content of this compound in other foods is needed, and the study of changes during processing that may lead to a decrease of its formation.

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Authentication of olive oils by DNA markers

Joana Costa, Isabel Mafra, M. Beatriz P. P. Oliveira

REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal (E-mail: beatoliv@ff.up.pt)

Introduction

Olive (*Olea europaea* L.) is an evergreen tree traditionally cultivated for olive oil and table olive production. Olive oil is the oily juice of the fruit that is mechanically separated from the other components of the pulp. Its fatty acid composition is characterised by a good balance between saturated, mono- and poly-unsaturated acids. This vegetable fat is considered unique among common vegetable oils since it can be consumed in its crude form, thus preserving vitamins and phenolic compounds of nutritional importance [1].

Over the last decades and especially in the past years, olive oil has occupied a very relevant position in human diet. Much appreciated by a significant percentage of world's population, this premium product is intrinsically related to the Mediterranean diet. Regarded as the most equilibrated diet, once it has been extensively reported as the model of healthy eating, being also associated with a lower risk of overall mortality and specific disorders such as cardiovascular disease, cancer, Parkinson's and Alzheimer's diseases, as well as other metabolic conditions. Widely related to the Mediterranean diet, olive oil is recognised as the primary source of fat, contributing to numerous health benefits like the improvement of the lipidic profile, the decreasing in insulin resistance and peripheral inflammation, and an improvement in oxidative stress processes and endothelial function (see review [2]). Besides all the potential health benefits and the nutritional value attributed to the consumption of olive oil, this fat is also largely appreciated by its particular taste and aroma.

Presently, because of the recommendations of the Food and Drug Administration (FDA), the American Heart Association, the European Society of Cardiology and other scientific organisations, regarding the inclusion of olive oil as main fat source in other diets rather than the Mediterranean, the demand for this premium product has been rising. Accordingly, the world's production of olive oil has increased more than 30% over the past 15 years. Confirming the high importance of olive oil in the Mediterranean area, almost 72% of olive oil production comes from Southern European countries. In 2012, Spain was the major producer of olive oil, followed by Italy and Greece, together accounting for more than 69% of the world's production. At a global scale and within the European Union (EU), Portugal occupies the eighth and fourth positions respectively, contributing with about 2.4% of the total olive oil production in 2012. In terms of global trade of vegetable fats, the latest available data (from 2011) established olive oil as the third most valuable crop, behind soybean and rapeseed [3].

Olive oil is considered a premium product with great economic importance, thus being highly susceptible to fraudulent practices. Because olive oil can be produced from a high number of different cultivars, which are responsible for its distinct chemical composition and sensorial characteristics, legislation has been created to protect both consumers and olive oil producers. Therefore, the EU has developed several instruments (appellation) such as Protected Designation of Origin (PDO) (Regulation (EC) No. 510/2006), which are used in olive oil labelling. Normally, the adulteration of olive oil with vegetable oils from different sources is considered mainly a problem of fraudulent practice. However, this practice can represent a real problem of public health for a rather significant portion of world's population, namely the sensitised/allergic individuals.

To verify labelling compliance, to ensure the quality/authenticity of this premium product and to ensure the protection of consumer's health, the development of highly sensitive and specific methods is of utmost importance. So far, both chemical and DNA-based methods have been extensively exploited to evaluate the authentication of olive oils. Considering the issues herein presented, this review will be mainly focused on the relevant topics related to the analysis of olive oils by means of DNA-based techniques.

DNA extraction from olive oils

Chemical analyses to determine fatty acids, sterols, phenols and/or secondary metabolites do not allow the exact identification of cultivars due to the significant effect of environmental conditions on the phenotype and, particularly on the chemical composition of olives. Hence, important efforts have been made to obtain unequivocal genetic profiles for olive oil cultivar using DNA analysis. Since a major constraint limitation, when applying molecular methods to olive oil, is obtaining satisfactory DNA extracts, most research work have dedicate much attention towards DNA extraction protocols (see review [4]).

The successful application of polymerase chain reaction (PCR) techniques is highly dependent on a critical step concerning DNA extraction and purification. In complex and highly processed food matrices, such as the case of olive and other vegetable oils, the stage of DNA extraction and purification is even more important. Adequate strategies are required to ensure efficient recovery of nucleic acids and the removal of potential PCR inhibitors. Substances such as polysaccharides, phenolic and other components are most frequently not entirely removed during classical extraction protocols, remaining as contaminants in the final DNA preparations. As consequence, the DNA amplification could be drastically affected or even completely inhibited by the presence of such components.

Considering that olive oil is a lipidic matrix, the extraction of DNA with proper quality parameters for PCR amplification is a hard task to accomplish, especially due to its low amount and integrity caused by the DNA nucleases present in this type of product. Until now, several methods have been described for extraction of amplifiable DNA from olive oils. The classical CTAB-based (cetyltrimethylammonium bromide) protocol, with or without modifications, is one of the most reported methods for DNA extraction from olive oil. Other protocols, namely several commercial kits have been successfully applied for the extraction of DNA from olive oils. Among those, the kit Wizard Magnetic purification system for food (Promega, Madison, WI, USA) used in several vegetable oils (including olive oil), the Nucleospin kits (Macherey -Nagel, Düren, Germany) employed in monovarietal olive oils and the QIAamp DNA Stool kit (Qiagen, Hilden, Germany) applied to filtered olive oils, are reported as the most adequate for extracting amplifiable DNA from lipidic matrices (olive and other vegetable oils) (see review [4]).

When the extraction regards olive oil, a wide range of starting amounts, from low (100 µL) to high amounts (500 g), with or without pre-concentration step, has been reported. Since olive oil is obtained by mechanical processes and without the need for refining treatments, this product is consumed in its crude form unlike other vegetable oils [1]. In this context, small starting amounts conjugated with the adequate DNA extraction procedure could allow obtaining extracts with proper quantity, integrity and purity for subsequent analysis by PCR-based methods. In addition to this, when amplifying DNA from olive oil, small DNA sequences should be targeted since some DNA degradation is to be expected.

Another issue of major importance is the storage period after milling olives. According to the literature, for a good traceability of olive oil, the sample should be as fresh as possible to ensure good repeatability and reliability of results, since it is well established that DNA can be damaged (oxidation processes) during long storage periods [5].

Identifying other species of origin in olive oils (adulteration)

Olive oil is one of the vegetable oils most likely to be a target of fraudulent practices since it is a premium product of higher commercial value than other vegetable oils. Several adulterants have been identified in virgin olive oils, varying from refined olive oil, deodorised virgin olive oils, olive pomace oil and synthetic olive oil-glycerol mixtures to almost all seed oils (e.g. maize, cottonseed, hazelnut, rapeseed and sunflower). In fact, the addition of expensive olive oils with less expensive and lower grade vegetable oils has been traditionally more than a potential problem in countries that produce seed oils and import olive oil (see review [6]).

The peculiar organoleptic features of olive oil associated with its well established beneficial health effects have increased its popularity and demand in the last years. The authentication of vegetable oils such as olive oil can be performed by a variety of methods, ranging from the classical physic-chemical techniques to the more current chromate-

graphic (gas, liquid, gas–liquid, quiral, silver-ion, mass, and supercritical fluid chromatographies), isotopic (stable carbon isotope ratio analysis, excitation-emission fluorescence and total synchronous fluorescence, pyrolysis-mass spectrometry), spectroscopic (infrared absorption, Raman scattering, nuclear magnetic resonance) (see review [6]) and molecular-based (PCR, amplified fragment length polymorphism - AFLP, random amplified polymorphic DNA - RAPD, simple sequence repeats - SSR, inter-simple sequence repeats - ISSR, single nucleotide polymorphism - SNP) methodologies (see reviews [4,7]).

Chromatographic and spectroscopic analysis of different families of compounds (fatty acids, triacylglycerols, phytosterols, tocopherols and tocotrienols, hydrocarbons, phenolic compounds, pigments and volatile compounds) are among the most used approaches for monitoring the quality and authenticity of olive oils (see review [6]). However, the majority of the available chemical methodologies are usually time consuming and labour intensive. In this sense, alternative DNA-based techniques have been successfully developed for the identification of different oil species added as adulterants to olive oil (see reviews [4,7]). These molecular approaches have particular interest from the point of view of the detection of allergenic oil matrices (e.g. hazelnut, peanut, soybean, among others) that can be incorporated in olive oils, posing a severe health risk for the allergic patients. Because the intentional addition of other vegetable oils rather than olive oil is a fraud, no information regarding the potential presence of allergenic ingredients is mentioned on the label. If the adulterated olive oil is consumed by sensitised/allergic individuals, they are at risk of suffering an allergic episode that can vary from mild to potentially fatal, depending on the severity of patient's allergy [8,9]. So far, literature describes some methodologies based on real-time PCR as very reliable, highly sensitive and specific tools, being of simple and time-effective performance for the rapid identification of adulterant vegetable oils in olive oil. More recently, the use of a real-time PCR coupled with the novel software of high resolution melting (HRM) analysis has also been reported as a fast and reliable methods for the discrimination of different species (e.g. hazelnut, peanut, soybean, among others) [9].

Identification of olive oil cultivars by DNA markers

One major aspect regarding the authenticity of olive oil is the determination of the cultivar(s) of origin. Since there is a great number of different olive cultivars, their chemical composition and sensorial characteristics can present very distinct patterns. Genetically identical cultivars are often designated by different names according to their place of origin (different countries or different regions within a country), which makes their identification/classification a hard task. In addition to this, the chemical composition and sensorial descriptors of cultivars are highly affected by environmental and agronomic aspects. Because some olive cultivars are considered of higher quality, certain olive oils have been awarded with certification brands PDO, which according to the EU legislation allows protecting both consumer's rights and a fair commercial trade. To verify labelling compliance of the origin of the cultivars used for olive oil production, several chemical methods are available in the literature (see review [6]), although presently the DNA-based methods have been considered the most adequate to overcome the problems related to environmental conditions of growth (see review [4]).

After overcoming the critical task of successfully extracting amplifiable DNA from olive oil, a number of markers such as SSR, AFLP, RAPD, ISSR and SNP have been proposed to verify the authentication of olive oils. Another relevant issue concerns the fact of olive oils are produced from monovarietal fruits or multiple cultivars, which increases the complexity of the matrix. Accordingly, the DNA-based techniques have been proving their usefulness for cultivars distinction. From the available DNA markers for the identification of olive cultivars, all methods have advantages and drawbacks, which are highlighted in Table 1. However, the choice of the better DNA marker is still a question of debate. The SNP seems to be one of the most promising markers since it allows distinguishing small differences among very similar individuals. Together with the SNP, the SSR markers are probably the most widely applied because of their high discriminatory power, although their reproducibility is sometimes compromised because some fragments, mainly of higher size, fail to amplify.

Table 1 Summary of the major advantages and drawbacks of the main molecular markers for olive oil authentication

Advantages	Drawbacks
SNP SNP	
✓ Easy performance and interpretation with the possibility of combining high-throughput technologies (*arrays)* ✓ A pplication to genotype olive cultivars ✓ Multiple SNP detection in single DNA analysis, ✓ Ability to distinguish small differences among very similar olive oil cultivars or others oil crop species, ✓ Apparently allows high correlation between olive leaves and olive oil, ✓ Application to assess the authenticity of monovarietal olive oils.	X Requires expensive technology (capillary electrophoresis, LDR-universal array, multiplex PCR coupled with microarray technology), χ More recently applied, less tested in olive oil matrices, χ Lack of information concerning the comparison with other single-locus markers.
SSR	
✓ Highly polymorphic and reliable markers ✓ Easy performance and interpretation ✓ Most employed DNA markers, great discriminatory power ✓ Identification of different olive oil cultivars ✓ Application to assess the authenticity of monovarietal olive oils.	χ Amplification of relatively high length DNA fragments χ Limited reproducibility when applied to olive oils due to the low DNA integrity
AFLP	
✓ Highly polymorphic and reliable markers without previous knowledge of the genome sequence ✓ One of the most used multi-locus DNA markers for the identification of olive oil cultivars ✓ Possibility of converting AFLP into SCAR markers ✓ Application to assess the authenticity of monovarietal olive oils.	X High complexity of the technique χ Difficult to analyse the numerous bands obtained, so not suitable for varietal oil mixtures χ Limited reproducibility when applied to olive oils due to the low DNA integrity
PCR	
✓ Species identification in crude and refined oils ✓ Detection of one or more DNA fragments, including GMO ✓ Detection of vegetable oil adulterations, ✓ Application of real-time PCR technique as a potential quantitative tool	χ DNA extraction is a critical step for its application to refined vegetable oils χ Unable to discriminate olive cultivars

Final Remarks

The growing interest towards the use of DNA markers for food authentication has been largely contributing for the development and application of molecular methods in food analysis, including olive oil matrices. The attention devoted to the development of effective DNA extraction protocols, applied to olive oil, has led to an upgrading in DNA recovery and quality. Conjugating good quality and purity DNA extracts with the amplification of small DNA targets seem to contribute to higher sensitivity and specificity. Besides these two factors, the time of storage is another issue of high relevance when applying any of the molecular approaches (see review [6]). Because most analytical parameters are dependent on environmental fluctuations, methods based on the genetic information have been proving their usefulness to discriminate olive cultivars, as well as promoting the identification of other plant species used as olive oil adulterants.

So far, several DNA markers have been successfully used for the identification of olive cultivars, with special highlight for SNP and SSR that have been classified as the most efficient markers.

In spite of all promising data regarding different DNA markers, much work is still need to define reference methods/ DNA markers for olive oil authentication.

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Olive Oil Adulteration

Ivonne Delgadillo
Departamento de Química/Universidade de Aveiro

Olive oil is perhaps the most emblematic food of Mediterranean Cuisine, and today much appreciated, not only in Europe but also, increasingly, in the rest of the world for its nutritional and organoleptic properties, as well as, for its beneficial action on health. Thus, olive growing and olive oil production are economic activities of great importance for the producing regions. The high prices of olive oils in the market and the increased demand have caused their falsification by addition of cheaper oils or even lower quality olive oils.

The International Olive Council (IOC) defines olive oils as those extracted from the fruit of the olive tree solely by mechanical means or by other physical means under conditions, particularly thermal conditions, that do not lead to product changes, and which have not undergone any treatment other than washing, decantation, centrifugation and filtration (IOC). Virgin olive oils fit for consumption, as they are, include:

- **Extra virgin olive oil**: virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 0.8 grams per 100 grams, and the other characteristics of which correspond to those fixed for this category in this standard

- **Virgin olive oil**: virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 2 grams per 100 grams and the other characteristics of which correspond to those fixed for this category in this standard.

- **Ordinary virgin olive oil**: virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 3.3 grams per 100 grams and the other characteristics of which correspond to those fixed for this category in this standard. This designation may only be sold direct to the consumer if permitted in the country of retail sale. If not permitted, the designation of this product shall comply with the legal provisions of the country concerned.

Refined Olive Oil is produced from virgin olive oil not fit for consumption as it is, and designated Lampante virgin olive oil (with free acidity, expressed as oleic acid, greater than 3.3 g/100g).

In the specific case of the European Union, the IOC categories coincide with the denomination of olive oil categories corresponding to the physical, chemical and organoleptic characteristics specified in the Annex to Regulation No 136/66/EEC and Commission Regulation (EEC) No 2568/91 of 11 July 1991, on the characteristics of olive oil and olive pomace, and methods of analysis. The new wording given by subsequent Regulations introduce some modifications in relation to the categories of olive oil and physical-chemical parameters. Commission Regulation (EC) No 1019/2002 states that "Olive Oil" contains refined olive oil and virgin olive oil, disappearing the category "ordinary virgin olive oil" of the IOC. The Commission Implementing Regulation (EU) No 1348/2013 updates some analytical methods and limit values for the characteristics of certain olive oils. It sets values of free acidity for "olive oil", which can not exceed 1.0g per 100g, while the olive oil with acidity greater than 2.0 g/100g is already considered lampante. Also, the acidity of refined olive oil may not exceed 0.3 g/100g.

The IOC and the European Directives have a detailed set and range of standards, in order to define and assist in the classification of olive oils and thus prevent adulteration or processing different from the mechanical extraction. These parameters include:

- Free fatty acids < 0.8%- expressed as oleic acid in 100g,
- Peroxide value < 20 mEq O2/ kg,
- Sterol composition,
- Stigmastadiene content,
- Wax content,
- UV Absorption (232 and 270 nm)- conjugated dienes and trienes,
- Equivalent carbon number (ECN) - equivalent carbon number equal to 42,
- Trans fatty acids,
- Triacylglycerol composition

In addition to the chemical characteristics, the organoleptic test is also an important parameter in the categorization of olive oil as extra virgin olive oil. The analysis requires a trained 8 to 12 tasters panel. To fulfil the requirements of extra virgin olive oil, the panel must determine that the oil

does not present taste defects, but also has certain organoleptic attributes.

The Commission Implementing Regulation (EU) No 1348/2013 adjusts the threshold values for stigmastadienes, waxes, myristic acid and alkyl esters of fatty acids and presents decision schemes for:

i) verification of compliance of an olive oil sample with the stated category, based on quality criteria such as acidity, peroxide value, spectrophotometric UV values, the organoleptic assessment and ethyl esters. Olive oils that have been classified as compliant are subject to the following decision scheme:

ii) compliance with purity criteria on the basis of 3,5-estagmadienes, *trans* isomers of fatty acids, fatty acid content, D ECN 42, sterol composition and total sterols, and waxes erythrodiol + uvaol.

It also introduces a scheme decision with more restrictive criteria for campesterol and delta-7-stigmastenol.

Despite the extensive list of parameters used to analytically confirm the authenticity of an olive oil, chemical similarity between the different vegetable oils hinders a quick verification of mixtures in small amounts. In the '90s were disjointed, in Italy and Spain, counterfeit networks of olive oil with almond and hazelnuts oils from Turkey. More recently were apprehended in Italy 25,000 L of genetically modified soya and sunflower oil, with added beta-carotene and chlorophyll, which was bottled as extra virgin olive oil.

The coarse forgeries are easily detected, either by the application of the methodologies indicated in international and European Directives or using sophisticated analytical techniques. Frankel published in 2010 an excellent review article on the *Chemistry of extra virgin olive oil, adulteration, oxidative stability and antioxidants*, with multiple references to the use of various instrumental techniques. The problem, however, arises if adulteration is performed with refined olive oil or olive oils of lower quality, such as deodorized olive oils by mild thermal treatment.

The free fatty acids, mono-, di-and triglycerides produced when the olives are stored before grinding are readily converted into alkyl esters by microbial esterification with ethanol and methanol. Deodorization removes unpleasant odors and these deodorized oils mixed with a fruity olive oil could, in theory, pass the test of a sensory panel. Being unrefined oils, most physicochemical parameters should be within the limits established. However, as alkyl esters are not removed by deodorizing, their presence can be considered as a good marker for low quality oil which was subjected to a gentle deodorization process (Pérez-Camino et al. 2008). Since April 1, 2014 the content of alkyl esters was introduced in Regulation 2568/91 (EEC) as a new parameter to assess the category of olive oil. There is now a limit of 75 mg / kg for total methyl and ethyl esters of fatty acids (Commission Implementing Regulation (EU) No 1348/2013).

In 2011 the University of California Davis published a paper in which reported that 73% of top brand extra virgin olive oils, had defects and were not approved in sensory tests by 2 panels of tasters recognized by the IOC, despite having been approved in most physical-chemical parameters. The group of researchers used two additional parameters, which are used in Germany and Australia respectively: diglycerides and pyropheophitins. These compounds, indicators of degradation were the most correlated with the negative opinion of the sensory panelists, as well as the UV measurements (Frankel et al, 2011).

The European Union recognizes that the adulteration of extra virgin olive oil, although not an issue of sanitary nature, is an economic fraud that penalizes the consumer, and has been since the 90s trying to define and improve legislation and methods for their detection. In December 2013 the European Commission launched a dedicated call for research and innovation projects on Authentication of Olive Oil (SFS-2014-14a). The call is intended to develop methodologies and / or protocols to detect unwanted processes, including deodorization and adulteration and to verify the quality of olive oils, especially extra virgin olive oil, based on advanced technologies. It is expected to find methodologies that will help to increase the competitiveness of Olive Oil.

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Food analysis and detection of fraud

***Maria Jesus Tavares*[1]*, Graça Campos*[2]**

[1] *Laboratório de Bebidas e Produtos Vitivinícolas/ASAE*

[2] *Laboratório de Físico Química /ASAE*

It is understood as fraud on goods, according to the legal definition of LD 28/84 the action of those who, with intent to mislead in business relationships, manufacture, process, import, export, has on deposit or on display for sale, selling or put into circulation by any other means, goods wich are:

a) counterfeit, fake or depreciated, making them go through authentic, unchanged or intact.

b) different kind or quality, or lower quantity wich affirm or appear to possess In the food industry this concept materializes in the alteration, and adulteration of the food or the addition or subtraction of components of foods that may have economic and / or consumer safety impacts, depending on whether the unfair competition or food security applies.

The higher or lower occurrence of fraud is conditioned by economic and social factors. Economic crises provide fraud as well as the free movement of goods and the increasing ease of communication that enhance networking worldwide.

Fraud has always existed and will always exist, however the corporate social responsibility has become an emerging topic, and there are international standards that describe it. The growing demand transparency in the actions of traders is something that follows the evolution of societies. Consumers are increasingly aware of the behavior of firms and controlling organisms, such sensitivity may take institutional form through consumers organizations, to promote and secure their rights which included the safety of their food. Also traders, especially in situations of economic crisis, demand controlling bodies the continuously defense of their rights with regard to ensuring the conditions of free competition.

It is therefore in this context of increasing social and economic importance of the fraud and its detection in demanding that the physico-chemical and sensory analysis of foods, applied to the detection of fraud is integrated. The ASAE, with its laboratories, as an organization that oversees and controls food, knows that fraudulent practices are always in development and tend to evolve according to the analytical capabilities of the controlling bodies. This requires that the analytical capabilities available in their laboratories are used in an integrated manner and the results assessed together. Thus, the physico-chemical and sensory analysis of foods must be understood as complementary and indispensable to characterize any type of food.

A classic example of economic fraud is the adding of water to milk or wine to increase its volume, thus allowing to obtain unfair profit. This type of fraud is economically relevant. Also a mixture of cow's milk cheeses declared as sheep or goat or mixture Sheep / Goat illustrates the case of economic fraud without being directly undermined food security. The commercial value of raw materials incorporated in cheese is determinant for profit. Of lower commercial value, cow's milk, is added to milk goats and / or sheep is a fraud that harms consumers in anticipation of the genuineness of the product that acquires and ensures greater profit to the producer. The legislation establishes as fraud detection of more than 1% of cow's milk mixed with milk of species declared.

Example of another type of fraud is the addition of a prohibited preservative or greater than the legal limit allowed (sulfites in meat juices or sorbic acid) to unduly prolong its life content. This type of fraud has economic relevance and also has relevance in terms of consumer safety potentially at risk when consuming these foods. Detection of fraud is usually a complex task that requires a joint interpretation of data that goes beyond compliance with legal limits, through the knowledge of manufacturing and technically permissible values and characterizing each type of food technologies.

Example: Olive Oil Fraud

Olive oil is an excellent product widely used in the Mediterranean diet has a beneficial effect on health due to its antioxidant properties, being rich in monounsaturated and polyunsaturated fatty acids, polyphenols and vitamins. For this reason and because it can be marketed in various categories is often subject to fraud, both in its classification as in its genuineness (Aduteration).

For the oil is marketed is necessary to submit in accordance with the legislated specifications, based on physico-chemical and sensory analyzes (Regulation (CE) 2568/91 and its amendments).

The physico chemical analyzes made in olive identifying two situations:

- Verification of the declared category - Quality criteria that depend on the manufacturing process, the quality of the olives, and changes with time and storage conditions, for example reaction temperature and with light and / or oxygen leading to an increase in acidity , the peroxides present and changing absortivities characteristics.

- Verification of identity - Purity criteria on which checks if the oil is unadulterated: mixed with olive pomace oil, or other refined oils, oleaginous seeds or other fats. The most relevant parameters for the purity criteria are the fatty acid profile, the stigmastadienes (adding refined), sterols and ECN 42 (mixed with fat from other sources); Waxes and Erythrodiol / uvaol (adding olive pomace oil)

The sensory analysis performed on the virgin olive oil samples intended to determine the median values of fruitiness and median of the defects.

Maximum values for these two parameters are stipulated taking into account the category are declared. As an example, indicate the values predicted for extra virgin olive oils, which may not have any defect and the median of the fruity must be greater than zero.

In the case of virgin olive oil and extra virgin olive oils there that complement the results obtained from the various parameters of physical chemistry with the results of sensory analysis since both are required to ensure specific quality category declared for the product analysis.

Biological diversity of the olive tree and olive oils authenticity

Miguel A. Faria*, Isabel M.P.L.V.O. Ferreira
REQUIMTE, Chemistry department, Faculty of Pharmacy, University of Porto
Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal
**corresponding author: mfaria@ff.up.pt*

Abstract

The olive tree (*Olea europaea* subsp. *europaea* var. *europaea*) is within the most ancient fruit tree cultivated in the Mediterranean countries. There are about 1200 cultivars, from 54 countries, which define the olive oil organoleptic characteristics together with the edaphoclimatic conditions of the region of origin. These typical features, more and more valued internationally, had been incorporated in the production of high quality olive oils elaborated to present specific characteristics highly dependent on the varietal composition either in blended or monovarietal oils. This products are thus of higher economic value and generally protected by international regulations. Consequently, the knowledge of the genetic diversity of the olive cultivars at the molecular level is of paramount importance in the development of genetic markers able to discriminate these same cultivars in protected olive oils as the PDO and PGI designations.

The present work aims at approaching the olive tree biological diversity at the international level and the way this is exploited in the development of DNA-based methodologies for the authentication of PDO olive oils, contributing thus to the economical valorisation of the olive oils and its affirmation as a protected product, legally controlled, of high economic value and providing wide health benefits to man.

The olive cultivars

The olive tree (*Olea europaea* L.) is an emblematic specie belonging to the *Oleaceae* family which includes also the genera *Fraxinus*, *Ligustrum*, *Jasminum* and *Syringa*. Accordingly to Grenn (Green, 2002) the specie comprises a botanical complex in which six sub-species can be identified: *cuspidata, laperrinei, maroccana, cerasiformis, guanchica e europaea*. This last, *Olea europaea* subsp. *europaea,* includes one wild form denominated *Olea europaea* subsp. *Europaea* var. *sylvestris*, and a cultivated forma *Olea europaea* subsp. *europaea* var. europaea*,* the only producer of edible fruits with agronomic interest (Chiappetta & Muzzalupo, 2012). This last form is thus the correct designation of the cultivated olive tree.

The origin of the olive cultivation had long been lost in time but there are some evidences that it started about 6 millennia in the Middle East region. It is believed that olive tree was primarily cultivated by Hamitic and Semitic people, inhabitants of southern Caucasus and in the west of Iran tablelands. There are also some linguistic evidences supporting this hypothesis as the term “zait”, derived from the Semitic language and used to denominate olive trees and related products, it is still today used in the Arabic language to designate olive as “zeitun”. It is also clear the similitude with the modern Portuguese words for olives “azeitona” and olive oil “azeite”. After the tree domestication and implementation in that region, population migration and maritime commerce have favoured the dissemination of the culture throughout all the Mediterranean countries where the olive culture is still of paramount importance as Greece, Italy, France, Spain and Portugal. During the maritime expansion the cultivation of the olive tree was also implemented in the new world by the hand of Spanish (Bartolini & Petruccelli, 2002) and Portuguese navigators.

The Mediterranean basin, being the traditional area for the olive cultivation, includes 95% of the olive orchards in the world. Notwithstanding, the olive tree continues its expansion to new areas as Australia, Argentina, Chile, USA, South Africa and even more exotic places as Hawaii (Chiappetta & Muzzalupo, 2012) mainly due to the recognition of olive oil exceptional nutritional characteristics as a source of beneficial fatty acids, phenolic compounds, vitamin E and carotenes.

Throughout the centuries several cultivars had aroused from spontaneous multiplication via sexual reproduction (i.e. seed germination). The olive tree is an alogamic specie which means their descendants are always originated by crossover between different plants. Plants obtained from seed germination will always express hybrid characteristics

reflecting the genetic variability of the progenitors (Peixe et al., 2013). Pollen grains produced by some tree can cover long distances until the fecundation of another plant which favours the genetic variability and the emergence of a high number of cultivars. Tree more adapted to the edaphoclimatic conditions in each local – the more productive, more resistant to diseases and producing fruits with more appreciated characteristics – were empirically selected by farmers and multiplied by vegetative means (Khadari & Bervillé, 2000). The more interesting plants are multiplied by cuttings given rise to clones (plants genotypically and phenotypically similar to the mother plant) which are then used in the orchards plantation.

From the complex genetic relations amongst the countless ancestral cultivars a high diversity have emerged and perpetuated until nowadays (Trujillo et al., 2013). This diversity configures a huge cultural and biological heritage that should be preserved and studied because it constitutes a putative source of solutions to future problems of olivicultture, due to foreseeable climatic changes and even as a source of new bioactive substances.

Accordingly to the international reference database of olive germplasm (http://www.oleadb.it/) there are about 1200 cultivars of olive trees, from 54 countries. At this database, created by Giorgio Bartolini from the "Istituto per la Valorizzazione del Legno e delle Specie Arboree", Italy, one can obtain information about the cultivars as the common name, synonyms, cultivation areas and the morphological descriptors used for visual identification. In the last 20 years however the technological developments, focusing mainly the plants productivity, have increased the risk of genetic erosion in olive trees due to the substitution of a high number of traditional cultivars by a few ones highly productive and more adapted to mechanical harvest (e.g. Arbequina). In this scenario, the identification and preservation of traditional cultivars is thus essential to assure the potential use of these trees in the future (Rallo et al., 2013). Cultivars preservation can only be achieved throughout the creation of germplasm banks were plants are planted, propagated, maintained and studied in *ex-sito* collections. This work has been done either in the Mediterranean countries or in the new producing regions as USA, Chile, South-Africa, Australia and China. There are presently about one hundred collections of olive cultivars all over the world (http://www.oleadb.it/collections). In Portugal, a few germplasm banks can be found regionally, dedicated to the collection and preservation of local cultivars, by qualified technicians. There is also a considerable collection of cultivars at the National Agronomical Station, Oeiras, and the National Reference Collection of Olive Cultivars is being constructed at the "Instituto Nacional de Recursos Biológicos", INRB, Elvas. The main objective of this last is the preservation and valorisation of the Portuguese autochthonous cultivars form the several producing regions in the country.

At the international level, one of the biggest and most important local for olive germplasm preservation is the World Olive Germplasm Bank of Cordoba (WOGBC), in Spain (Caballero & Río, 2008) were there are planted 499 accessions from 21 countries representing the worldwide genetic variability. The plantation in the same orchard of such genetic diversity will permit the development of a great variety of comparative assays amongst cultivars without the interference of edaphoclimatic variations. In addition, the cases of synonym (different names attributed to the same cultivar), homonym (the same name for different cultivars) and the incorrect labelling, very frequent between different producing countries, can be more easily solved. From 2003 a second world olive germplasm bank was also established in Tessaout, Morocco (Haouane et al., 2011).

Table 1 presents some examples of cultivars from 24 countries accordingly to the selection of the most representative ones presented in the World Catalogue of Olive Varieties (FAO). In the case of Portugal the most cultivated autochthonous cultivars are presented following the catalogue "Descrição das 22 variedades de Oliveira cultivadas em Portugal" (Leitão et al., 1986).

Tabela 1. *O. europaea* cultivars, representing 24 countries from both the Mediterranean basin and more recent producers

COUNTRY	CULTIVAR	COUNTRY	CULTIVAR
Albania	Kalinjot	**Italy**	Ascolana Tenera
Algeria	Azeradj		Biancolilla
	Blanquette de Guelma		Carolea
	Chemlal de Kabylie		Coratina
	Limli		Dritta
	Sigoise		Frantoio
Argentina	Arauco		Giarraffa
Chile	Azapa		Itrana
Cyprus	Ladoelia		Leccino
Croatia	Lastovka		Moraiolo
	Levantinka		Nocellara del Belice
	Oblica		Ottobratica
Egypt	Aggezi Shami		Pendolino
	Hamed		Rosciola
	Toffahi		Taggiasca
Slovenia	Bianchera	**Jordan**	Rasi'i
Spain	Arbequina	**Yugoslavia**	Zutica
	Bical	**Lebanon**	Soury
	Blanqueta	**Morocco**	Haouzia
	Carrasqueño de la Sierra		Menara
	Castellana		Meslala
	Changlot Real		Picholine marocaine
	Cornicabra	**Palestine**	Nabali Baladi
	Farga	**Portugal**	Carrasquenha
	Gordal de Granada		Cobrançosa
	Gordal Sevillana		Cordovil de Castelo Branco
	Hojiblanca		Cordovil de Serpa
	Lucio		Galega Vulgar
	Manzanilla Cacereña		Maçanilha Algarvia
	Morisca		Redondal
	Morrut		Azeiteira
	Picual		Conserva de Elvas
	Royal de Cazorla		Negrinha
	Sevillenca		Madural
	Verdial de Badajoz		Verdeal Transmontana
	Villalonga		Redondil
USA	Mission		Galega Grada de Serpa
France	Aglandau		Cordovil de Serpa
	Bouteillan		Verdeal Alentejana
	Grossane		Bical de Castelo Branco
	Lucques		Maçanilha Carrasquenha
	Picholine Languedoc	**Syria**	Abou-Satl
	Salonenque		Doebli
	Tanche		Kaissy
Greece	Adramitini		Sorani
	Amigdalolia		Zaity
	Chalkidiki	**Tunisia**	Chemlali de Sfax
	Kalamon		Chétoui
	Konservolia		Gerboui
	Koroneiki		Meski
	Mastoidis		Oueslati
	Megaritiki	**Turkey**	Ayvalik
	Valanolia		Çekiste
Iran	Fishomi		Domat
	Rowghani		Erkence
	Mari		Gemlik
Israel	Barnea		Izmir Sofralik
	Kadesh		Memecik
	Merhavia		Uslu

Olive oil diversity and varietal authentication

Olive oil is presently viewed by consumers as a high quality food product. Its economic value is dependent firstly on the attributed category (extra virgin, virgin, lampante). Notwithstanding, within the superior category of extra virgin olive oils the is a big variation in market value for different brands and types due to also different characteristics, as organoleptic uniqueness or the producing area of origin, which designation and labelling are legally protected within the PDO e PGI systems (CE regulation 2082/92). These products are obtained from defined varieties, in legacy defined percentages. Moreover, the emergence of monovarietal olive oils in the markets is increasing similarly to the succeeded in the wine market in the last years. The gourmet culture of varietal diversity in olive oils has been promoted in some countries, as Italy, resulting in a bigger diversification of olive oils which constitutes an attraction to more demanding consumers (Rotondi, Magli, Morrone, Alfei, & Pannelli, 2013). These last, are more attentive in the product characteristics, in its diversity and health benefits, which increases the commercial value of the product making it very attractive to adulteration practices.

The organoleptic uniqueness of an extra virgin olive oil is determined by genetic (cultivars), agronomic, environmental factors and by the technological processes used in oil extraction. Notwithstanding, the genetic matrix, defined by the constituent cultivars plays a very relevant role in sensorial characteristics (Rotondi, Alfei, Magli, & Pannelli, 2010). Nowadays olive oils are organoleptically evaluated by 13 attributes: 9 olfactory (fruity, green olive leaf, grass, almond, tomato, apple, artichoke, berries and aromatic herbs) and 4 gustative (fruity, bitter, pungent and fluidity). These attributes are quantitatively evaluated and an organoleptic profile is obtained for each of the oil samples (Rotondi et al., 2013). However, taking into account the biologic plant variability and the influence of environmental conditions, the organoleptic evaluation is not enough objective to assure olive oil authenticity. It is thus necessary the development of laboratorial tools able to identify the constituent varieties in the final product olive oil. This kind of control requires, therefore, methodologies permitting a discriminating power below the subspecies level, only possible throughout the DNA analysis, namely by focusing hypervariable regions between cultivars.

Methods for the molecular authentication in olive oils

The authenticity assessment of an olive oil, particularly the from the extra virgin category, is traditionally done by analytical laboratorial means by using chemical markers as sterols, phenols, fatty acids, triacylglycerols, volatiles, tocopherols, etc. However, the chemical composition of an olive oil is highly affected by environmental conditions which demand the use of alternative markers not dependent on environmental factors but instead only related to the varietal composition of the sample. DNA markers are known to be independent of environment or technological conditions thus these are the markers of choice for varietal composition evaluation in olive oils (Enferadi & Rabiei, 2013).

The main obstacle to the use of molecular markers in olive oil authentication is the DNA extraction step. Significant quantities of this molecule can be found in olive oils obtained by cold expression however, subsequent technological processes as filtrations and the natural enzymatic activity of nucleases, which degrade the DNA molecule, will induce the decrease of quantity and quality of nucleic acids present in the oil matrix (Consolandi et al., 2008).

When analysing packed olive oils from market shelves the DNA extracts obtained are low in quantity and the molecule is much degraded. Additionally, these extracts can also be rich in phenolic compounds that are strong inhibitors of the PCR (Polymerase Chain Reaction) reaction which is most of the times a crucial step in laboratorial protocols for the DNA analysis. The small quantity of the DNA obtained from olive oils is not the main drawback for the application of the molecular techniques. The main obstacle is in the high degradation presented by the extracted DNA which drastically decrease the information that can be obtained from its analysis (Enferadi & Rabiei, 2013). Several techniques had been proposed to surpass the difficulties of the DNA extraction with satisfactory results and good reviews about the subject had been published (Breton, Claux, Metton, Skorski, & Bervillé, 2004).

Once the first obstacle is overcome, i.e. the extraction of DNA with enough purity and integrity, there is the need to choose the most appropriate molecular markers. DNA markers are relatively short regions of the genome containing sequence variations permitting the discrimination of the cultivars. Most of these markers are previously amplified by

the PCR reactions and subsequently analysed following a wide range of laboratorial techniques. Many markers had already been tested in the varietal discrimination in olive oils as: AFLP (*Amplified Fragment Length Polymorphisms*), RAPD (*Random Amplified Polymorphic DNA*), ISSR (*Inter Single Sequence Repeats*), SSR (*Single Sequence Repeats or microsatellites*); SCAR (*Sequence Characterized Amplified Region*) e SNPs (*Single Nucleotide Polymorphisms*). Several review papers related to the type of molecular markers used in olive oil authentication were published in the last years from which I would point out two (Ben-Ayed, Kamoun-Grati, & Rebai, 2013; Bracci, Busconi, Fogher, & Sebastiani, 2011). Figure 1 summarizes the analytical workflow for the laboratorial assessment of olive oils authenticity by the use of molecular markers.

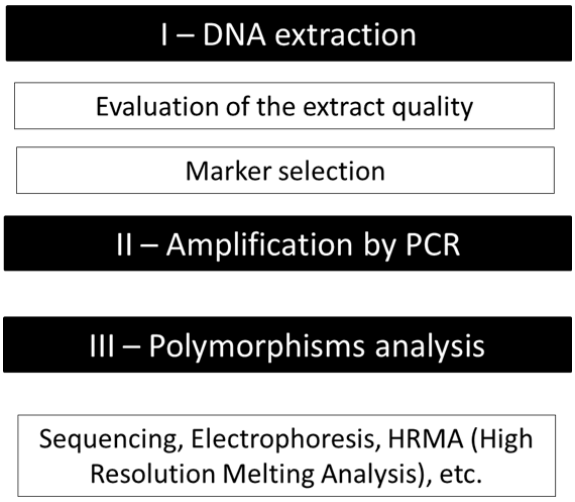

Figure 1. Laboratorial workflow of the analytical techniques used in the assessment of olive oil varietal authenticity.

Despite the high number of molecular markers that had been used in oils authentication, the most recent research works are focused on the application of SNP markers (Single Nucleotide Polymorphisms). SNPs are single base sequence variations in certain genome regions permitting the existence of different forms (alleles) in a normal population. They represent the most abundant forma of sequence variation in the genomes which makes a preferential target when the discrimination of highly similar individuals is required (Brookes, 1999). The identification of highly polymorphic SNPs is the main objective of very recent studies which are making use of cutting edge DNA sequencing techniques as the parallel or NGS (Next Generation Sequencing) applied to the massive sequencing of the most important international olive cultivars (Kaya et al., 2013; Pérez-Jiménez, Besnard, Dorado, & Hernandez, 2013). Once identified and tested these SNP markers can be analysed by using several techniques including the amplification of the genomic region containing the marker itself by PCR and the subsequent analysis by the most recent techniques as the HRMA (High Resolution Melting Analysis) (Faria, Magalhães, Nunes, & Oliveira, 2013).

The complete sequencing of the olive genome, the main objective of the International Olive Genome Consortium (http://olivegenome.karatekin.edu.tr/) is being performed as well as the resequencing of variable regions of considerable fractions of the genomes of the most important cultivars, which will make possible the development of new and more efficient markers for inter-varietal discrimination.

The emergence of olive oils with distinctive characteristics, based on mono-varietal production of made from a restrict group of cultivars, strongly increases the consumers interest on this high quality product and, as a consequence, its economic value. This had motivated the interest of several international research groups which have showed a considerable activity in the development of solution for olive oil effective authentication based on the most recent analytical technology. The development of a *sui generis* product, authentic and healthy, assured by the synergistic actions of the several stakeholders as producers, regulatory entities and researchers, will certainly warrant a promising future for the most important food product of the Mediterranean culture, the olive oil.

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The quality of olive oil: effects of storage and thermal processing

Susana Casal*, Carla Santos, Sara Cunha, José Alberto Pereira
REQUIMTE, Laboratório de Bromatologia, Departamento de Química e Hidrologia, Faculdade de Farmácia, Universidade do Porto
Autor correspondente: sucasal@ff.up.pt

Virgin olive oil is unique among commercial vegetable oils. A well-balanced chemical composition determines a high oxidative stability together with noble sensorial and health properties. Early studies attributed supported these health effects by its monounsaturated fatty acid content but, over the years, increasing proofs of the determinant roles of its minor constituents have raised. Today, we know that minor compounds that are extracted from the olive fruit, together with the triglycerides, are equally responsible for its health and technological properties (Pérez-Jiménez et al, 2007).

Extra-virgin olive oil is classified in accordance with its extractive method, purely physic and under low temperature, with its organoleptic characteristics and chemical composition (European regulation n.º299/2013). Such is among the few vegetable oils that can be consumed in its raw state. In opposition to all other commercial vegetable oils, virgin olive oil retains most of the compounds extracted from the fruit while others loose them during refining. Together with the removal of several chemical and sensorial "defects", refined vegetables oils are depleted of "freshness", as well as of sensorial attributes and identity. This is what makes virgin olive oils so delicate, requiring efficient handling and preservation, and at the same time so unique, by reflecting directly the qualities and defects of the raw materials and technological process applied.

Among the minor compounds that make virgin olive oil a unique fat in our diet, the phenolic compounds take a expressive part. These compounds have antioxidant properties and contribute to its delicate and pleasant flavor. The simultaneous presence of alfa-tocopherol (vitamin E), carotenoids, squalene, and sterols, among others (Figure 1), creates an efficient network, preserving olive oil from degradation, while giving consumers a pool of bioactive compounds with health potential (Rastrelli et al 2002). The antioxidant properties of its phenolic compounds, particularly oleuropein and hydroxityrosol (Figure 1), have been the most extensively studied. Olive oil phenolic compounds showed in experimental studies to act efficiently against lipids, DNA, and LDL oxidation (Covas, 2006), in order to delay the progression of atherosclerosis in animal models. Furthermore, there are also evidences that beta-sitosterol and the polyphenols in olive oil inhibit the formation of oxygen reactive species, reduce the susceptibility of LDL oxidation and erythrocyte membranes lipid peroxidation (Pérez-Jiménez et al, 2007).

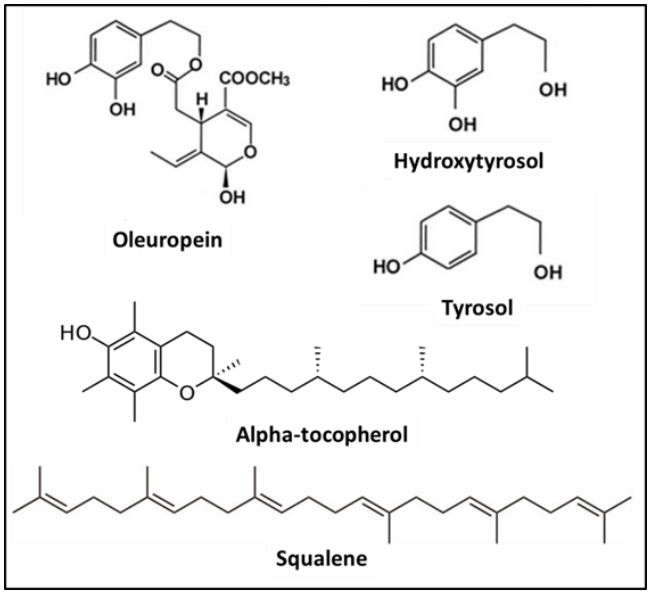

Figure 1: Minor components of high relevance in virgin olive oil

Unfortunately, in opposition to a good wine, olive oil quality does not increase with storage. From the moment lipids are removed from the olive fruit, quality begins to decrease mainly as a consequence of oxidative degradation and also hydrolytic degradation at low extend. Therefore, in order to assure consumers best interests, adequate attention has to be taken to four main points: olives quality, olive oil extraction, its storage conditions and, finally its adequate use. The first two are mainly determined by the olives producers and extractors, who grant the labeled information at the bottling moment. After that, bottled olive oil passes to sellers and buyers, being exposed to different environments, unfortunately not always the most adequate ones to preserve its

quality. These conditions arise mostly from reduced information, with the common believe that, independently from the storage conditions, the product inside the bottle retains the same properties from bottling moment until the labeled "best before" date. Nothing could be less truth. This review will focus mostly on olive oil storage and domestic use, highlighting the major alterations that inevitability occur with ageing and the most adequate measures to reduce them to the minimum.

Olive's quality and processing: Although not in the consumer hands, it is important to understand that even the best technological facilities cannot extract a good olive oil from defective olives. The presence of minor amounts of damaged olives can bring defects to an entire batch of olive oil, disabling immediately its classification as extra-virgin olive oil. Indeed, aged oils made from healthy olives may be of better quality than fresh oils made from damaged olive (Aparicio-Ruiz et al 2014). On the other hand, if inadequately processed, high quality olives can give rise to a low quality olive oil. Olive oil extraction should occur as soon as possible after harvest and be made within the short time possible to reduce hydrolysis and oxidation, and consequently loss of naturally present antioxidants. After pressing, the oil will be at its utmost freshness and ideal flavor. Subsequently, the oil will slowly begin to lose its natural color and flavor strength. The olives cultivar used and its maturity stage also contributes to the olive oil composition, in particular to the presence of phenolic compounds, known to be reduced during ripening, and also for sensorial attributes, reducing the fruitiness and increase the sweetness.

Olive oil storage: As with many other seasonal products, which are consumed through the year, olive oil must be stored. Therefore, after being extracted, olive oil is firstly preserved in bulk, being usually bottled only when sold for distribution. Without a legal restriction on its shelf-life, and knowing that it will ultimately depend on the olive oil composition and storage conditions, producers define usually the "best before" date based on the manufacture date, from 9 to 24 months, adjusted by the analytical characteristics presented at bottling time.

Even under proper storage conditions, olive oil oxidation occurs slowly but it's the main cause of virgin olive oil quality deterioration. As previously mentioned, olive oil shelf life is highly variable, depending on the olive variety, ripeness when pressed, care in processing, and storage, being hard to "guarantee" an accurate lifespan. Also, even the best extra-virgin olive oil can lose its properties if inadequately stored, either in the commercial shelves or at the consumer's home. Unfortunately, olive oil bottles are still seen directly exposed to bright light in commercial stores or even at consumers house, almost as decoration pieces, causing them to be already degraded when bought or consumed.

The polyunsaturated fatty acids are the main target of oxidation. In order to protect oxidative damages in the unsaturated fatty acids, olive oil natural antioxidants cooperate actively to counteract oxygen effects. Vitamin E, the most important and functional lipophilic antioxidant in olive oil is the first one to be oxidized. After vitamin E depletion, its oxidized products become partially pro-oxidants being able to further oxidize other antioxidant molecules such as squalene, generating a complex radical chain reaction, ultimately being unable to prevent polyunsaturated fatty acid oxidation. The loss of antioxidants during this process reduced the potential health effects, and the newly formed products can also give unpleasant smell and taste, therefore declassifying the virgin olive oil, and might also present some toxicity to consumers (Rastrelli et al, 2002)

Three main factors are responsible for bottled olive oil degradation: oxygen, light and temperature. In order to preserve it, olive oil should be stored in the dark, in the absence of oxygen and under cold temperatures. Oxygen will induce peroxidation of the unsaturated lipids, accelerated by warm temperatures, while light will induce photo-oxidation.

The container characteristics have a marked influence on both. Extra-virgin olive oil is commonly packed in glass, being increasingly sold also in plastic bottles, associated with lower prices. Colorless glass and plastic polymers have the disadvantage of enabling photo-oxidation. Only opaque or dark containers can grant efficient protection from light (Caponio et al 2005). The use of dark colored glass and plastic is increasing but true opaque containers are best. These, however, disable buyer to see the product, which is still regarded as suspicious by some. As to oxygen, only glass and stainless containers create effective barriers. In standard polythylene terephthalate (PET) bottles, after 3 months of storage the quality is already visibly lower than when duly

protected from both light and oxygen (Méndez and Falqué, 2007). PET permeability to oxygen can be reduced by a passive barrier technology, adding additional thin barrier film(s) over the PET, or by active technology, using PET impregnated with UV light blocking or oxygen scavenging substances in the molecular polymer matrix of the plastic. (Gambacorta et al, 2004, Cecchi et al, 2010). Nowadays, simple technologies are also available to reduce oxygen availability. These can pass from simply reducing the headspace to the most and/or filling it with as inert gas as nitrogen or argon.

However, once opened, the "barriers" created by the manufacture are compromised, particularly regarding oxygen availability. Indeed, each time a bottle is opened for use, fresh air will be in contact with the olive oil, creating a constant access to oxygen, and therefore to peroxidation. This phenomenon will further increase with use because the proportion of air in the bottle increases. Vitamin E loss, after 12 month of storage for instance, can increase from 25% in a full bottle to 90% in a half-filled one, either in clear or colorless glass (Rastrelli et al, 2002).

Olive oil usage: In order to better preserve extra virgin-olive oil attributes, one should consume it as is, as final seasoning in fresh salads, soups, etc. Still, one of the main uses of vegetable oils is for cooking, as roasting or frying. The elevated temperatures applied, inevitable increase lipids degradation, being no exception for olive oil. As previously mentioned, being rich in monounsaturated fatty acids and presenting a natural pool of antioxidants, olive oil is less prone to oxidation. However, while protecting olive oil during thermal processing, these compounds are degraded, and the potential health effects for the consumer may decrease. For instance, phenolic compounds as hydroxytyrosol and its derivatives can be reduced to half only by 10 min frying of fresh potatoes at 180ºC, while vitamin E is almost depleted after 3 to 6h of frying (Santos et al 2013). Therefore, if thermal processing is mandatory, temperature should be reduced to the minimum technology possible and for the shortest time. For prolonged heating, replenishment can be considered, as it will refresh the oil with fresh antioxidants, therefore preserving it more adequately. Still, phenolic compounds have some typical bitterness, leaving a characteristic flavor and aftertaste if the fried food. Therefore, for frying, "light" olive oils can be an interesting option, using either olive oils naturally with lower content of phenolic acids as occurs at full maturity, or using mixtures with refined olive oil, the commercial "olive oil" category. Indeed, it is economically advantageous to use lower grade olive oil and frequent replenishment under prolonged thermal processing (Santos et al, 2013).

Final remarks

Olive oil degradation can be reduced if adequate knowledge of its major weakness is known and effective measures are taken.

For sellers, special attention should be taken to the place were olive oil bottles are placed, with reduced direct light and cold temperatures, preferably with the external cardbox if the bottles are not light-protected.

For consumers, it is best to buy small quantities and store them in the dark, away from warm temperatures, and consume them during one crop season. Once opened, the bottle should be consumed fast in order to reduce the contact with oxygen. Extra-virgin olive oil is the best option for seasoning, bringing the most of olive oil potential health benefits to consumers. For thermal, processing, however, as these attributes will be partially lost, lower commercial olive oil grades may be favored.

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Olive oil and monounsaturated vegetables oils in food preparation: identical choices?

Carla Santos[a], Lucía García[a,b], Joana Martins[a], Rebeca Cruz[a], Sara Cunha[a], Susana Casal[a]

[a]REQUIMTE, Department of Chemical Sciences, Laboratory of Bromatology and Hidrology, Faculty of Pharmacy, University of Porto, Portugal; [b]Department of Physical and Analytical Chemistry, Faculty of Experimental Sciences, University of Jaén, Spain

Introduction

Modern society aims for fast and practical food preparation methods, with both tasteful and nutritive quality. Although consumers have acquired more information and consciousness on the associations between food, nutrition and health, in particular regarding lipids, the consumption of fried food continues to increase, essentially supported by its extraordinary sensorial attributes.

From the nutritional point of view, and according to Direção Geral da Saúde (2005), monounsaturated fatty acids (MUFA) are the most adequate ones to our body needs and its consumption is associated with a decrease in plasma cholesterol, particularly the low-density-lipid fraction designated as "bad" cholesterol, and the maintenance of cellular integrity. On a technological perspective, these are also more resistant to oxidation than the polyunsaturated ones, constituting interesting products to be used in thermal processing, since the more stable alternative, saturated fats, are harmful to our cardiovascular health.

Among the commercially available vegetable oils, the main suppliers MUFA are olive oil, followed by canola (rapeseed) and peanut oil (Decreto-Lei n.º106/2005). Therefore, these vegetable oils are highly recommended for frying. In Mediterranean countries, olive oil is the most used fat in domestic and industrial food preparation (Kalogianni et al, 2010; Carrageta, 2014), while peanut oil is recommended for use in Portuguese school canteens (Circular n.º3/DSEEAS/DGE/2013) and canola oil is very common in some Central and Eastern Europe countries (Booth and Gunstone, 2004).

Several cooking techniques may be selected when using vegetable oils, such as frying, roasting, or even microwave cooking. Deep-frying is the most utilized thermal processing type and it is characterized by immersion of food in oils or fats kept at high temperatures (up to 180°C) (Portaria n.º 1135/95), frequently with intermittent heating during prolonged time. In fact, frying involves a heat dehydration process with mass transfer, during which several physicochemical reactions occur between oil and air, and oil and food constituents. These reactions promote the desired organoleptic properties, with the formation of aroma compounds, color, and texture, while they may also reduce the quality of frying oil by hydrolysis, oxidation, polymerization, isomerization and cyclization (Choe and Min, 2007; Zhang et al, 2012; Santos et al, 2013).

In the present work, we propose to compare the thermal performance of monounsaturated vegetables oils, including olive oil, under real fresh potatoes frying conditions. Several oil oxidation parameters and some relevant nutritional components in potatoes, including vitamin C, fat incorporation and vitamin E, were determined, as well as contaminants resulting from food processing, such as acrylamide, and the general acceptance by a panel of tasters.

Materials and methods

Sampling

Fresh potatoes samples from *Fontane* variety, were cut in toothpicks and deep-fried during 6min. at 175°C in electric fryers (Tristar, FR-6929), simulating restaurant intermittent frying for 8h a day, in a total of 28h. Extra virgin olive oil, peanut oil and canola oil were used. Each 4h, oils and potatoes samples were collected for analysis, excepting in the first day, which occurred after 8h. Oil samples were stored at 4°C, in sealed vials under a nitrogen atmosphere. Potato samples were grinded (Flama, Cesar, Portugal). Some analysis were performed immediately and the remaining samples portions were stored at -20°C until further analysis.

Chemical analysis

In both, oils and potatoes, fatty acids and vitamin E were determined according to Casal et al (2010). For evaluation of oils oxidation state, the polar compounds were determined by high performance size exclusion chromatography (HPSEC) according to Márquez -Ruiz et al (1996) and Dobarganes et al (2000), and *p*-anisidine index in accordance with ISO

6885:2006. Incorporated fat in potato samples was extracted by Soxhlet method AOAC 945.16 and total vitamin C was quantified as ascorbic acid by high performance liquid chromatography with UV detection (HPLC-UV), after reduction of dehydroascorbic acid according to Van de Velde et al (2012) and Chebrolu et al (2012). Acrylamide was determined after derivatization with xanthydrol, followed by extraction of the xanthyl-acrylamide formed with ethylacetate and analyzed by gas chromatography-mass spectrometry (GC-MS). The extraction of volatiles was carried out using the Headspace Solid-Phase Microextraction technique, followed also by analysis by GC-MS.

Sensory analysis

Sensory analysis was performed by a panel of tasters and it was based on quantitative descriptive analysis to evaluate the sensory characteristics of the deep-fried potatoes in the three monounsaturated oils during the course of the study.

Results and discussion

Thermal resistance and harmful evidences

From the thermal performance point of view, it is important to verify the stability of fatty acids during frying time, in particular the stability of the MUFA fraction and the increase in trans fatty acids, inevitably formed under thermal tress . Indeed, the formation of *trans* fatty acids is used as an indicator of oil degradation (Talpur et al, 2012), being also associated with an increased incidence of cardiovascular diseases (Direção Geral da Saúde, 2005). Figure 1 compares the experimental results obtained with the three oils under study, all presenting different initial MUFA contents: higher in olive oil (74%), followed by canola oil (63%) and peanut oil (56%), being all in accordance with legislation (Regulamento (UE) n.º 299/2013; Decreto-Lei n.º106/2005). Furthermore, it is possible to verify in Figure 1A that the levels of MUFA remain stable during the whole study. In comparison with other vegetable oils with higher content of polyunsaturated fatty acids, as sunflower, maize or soybean, the higher prevalence of monounsaturated fatty acids is indeed of great importance for their thermal oxidative stability. Canola and peanut oils had higher initial contents of trans fatty acids than olive oil, due to the previous refining process, but the amounts were residual (0.18% and 0.26% against 0.01%). As expected, an increase of *trans* fatty acids occurred in all the oils during frying, being less significant in olive oil, allowing to infer a higher resistance of olive oil comparing with the others monounsaturated vegetables oils. Still, for increased frying times the final values were similar, around 0.4%.

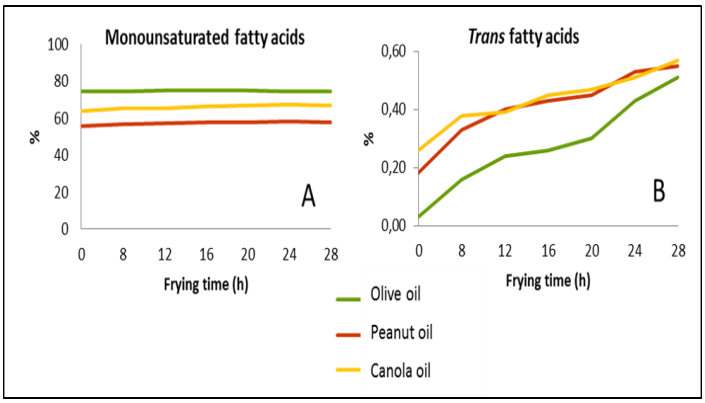

Figure 1. Evolution of the percentage of monounsaturated fatty acids (A) and *trans* (B) during frying time.

Thermal performance is also evaluated by the oils polar compounds content, also formed during frying. Under the common designation of polar compounds, several degradation products are included, namely those resulting from hydrolysis, oxidation and polymerization. According to the Portuguese law (Portaria n.º1135/95), the legal point of rejection for frying oils occurs when they reach 25% of total polar compounds. Figure 2 details the results obtained for total polar compounds (Figure 2A) and for the oxidized triacylglycerol fraction (figure 2B). The HPSEC analysis highlights the presence of oxidized triacylglycerols (Figure 2B), generated during thermal oxidation. The results show that olive oil presents greater stability to oxidation when compared to the others monounsaturated vegetable oils, without achieving the legal limit by the end of the study (Figure 2A). However, for the oxidized triacylglycerol fraction, representing diverse compounds with higher deleterious effects for consumer's health, an increase with frying time is observed for all the oils tested, ending up with equivalent levels of this parameter.

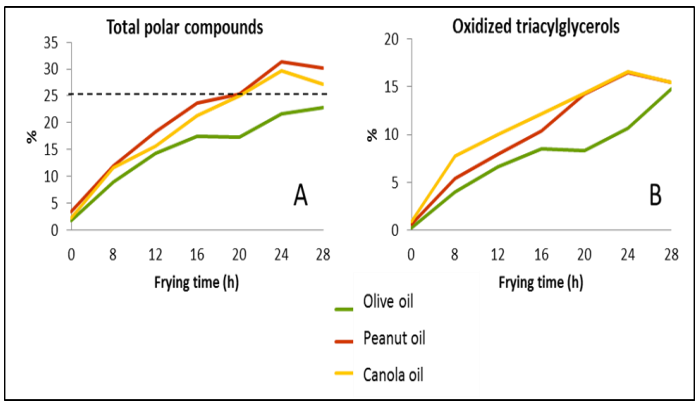

Figure 2. Evolution of total polar compounds percentage during frying time (A) and, within these, oxidized triacylglycerols (B).

The study of the nature and concentration of volatile compounds formed during frying, as a consequence of its thermal degradation, is relevant to understand the chemical reactions occurring during frying process (Takeoka et al, 1996). Among the great number of volatile compounds formed, a high prevalence of aldehydes (especially 2-alkenal and 2,4-alkadienals) formed due to thermal decomposition of hydroperoxides was observed (Fereidoon and Ying, 2005), whose total amounts are usually evaluated by the p-anisidine index, a fast method used to evaluate the extent of secondary oxidation *p*-anisidine mentioned above. Herein (Figure 3), a higher extent of secondary oxidation products was observed in canola oil, followed by peanut and olive oil. The analysis of volatiles emitted from potatoes also showed a higher formation of alkadienals, of recognized toxicity, being comparatively higher in canola oil and lower in olive oil (Katragadda et al, 2010).

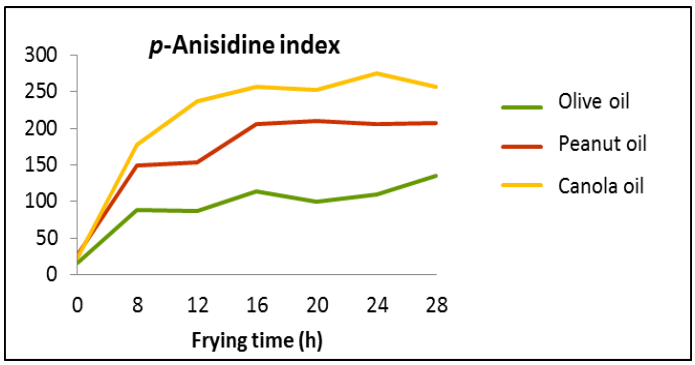

Figure 3. Evolution of *p*-anisidine index during frying time.

The formation of acrylamide during frying of potatoes has received much attention from the scientific community and consumers in general. In this study, the formation of acrylamide was similar between the three types of monounsaturated oils, corresponding to values in the 800-1200 µg/kg range, with identical results during frying time.

Nutritional benefits

From the nutritional point of view, the incorporation of fat in potatoes increases its caloric value, but simultaneously enriches them with oils components relevant to health, namely vitamin E and essential fatty acids.

Figure 4 refers to the % of fat incorporated with the three oils during frying time. Based on its analysis, regarding each individual frying time, it appears that potatoes deep-fried in olive oil and peanut oil absorb similar fat amounts, but smaller than potatoes deep-fried in canola oil. However, the fat content does not increase across the period of study.

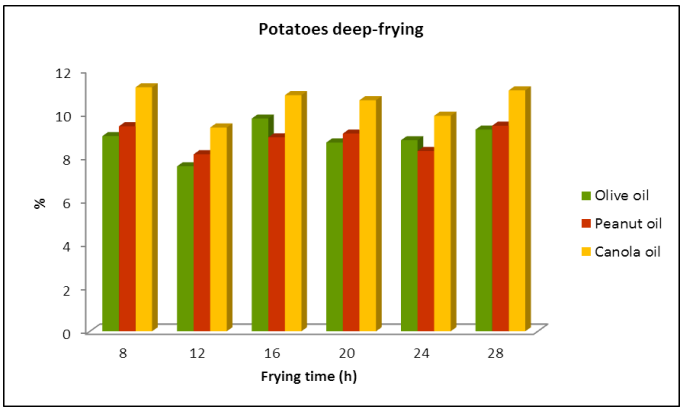

Figure 4. Evolution of potatoes fat percentage during frying time.

With frying, due to the reduction in the potatoes water amounts, and despite vitamin C thermo sensitivity, a slight increase of about 50% in comparison with raw potato was observed (Figure 5). However, there is a noticeable decrease in vitamin C by further increase of frying time, probably due to the increased oxidative *stress* in the heated oils, without distinction between them. Vitamin E is initially incorporated in a direct proportion of its content in the oils, higher in canola oil, followed by peanut oil and olive oil. However, there

is also a reduction with time in all frying oils, as expected (Figure 5).

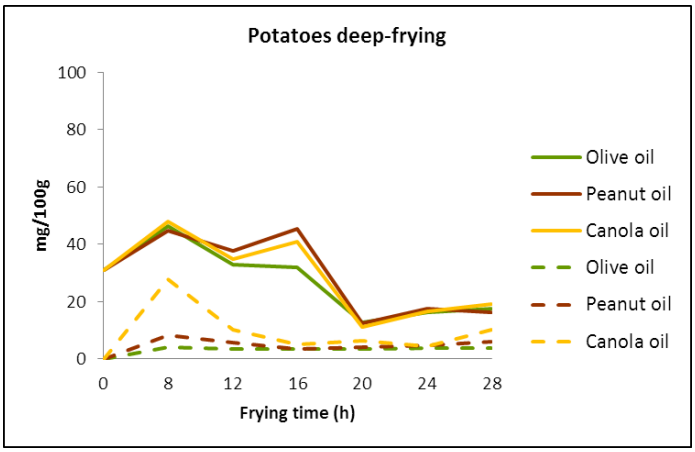

Figure 5. Evolution of vitamin C (solid line) and vitamin E (dashed line), in mg/100g during frying time.

With respect to sensorial analysis made by the tasters panel, no differences between potatoes deep fried in olive oil or the others monounsaturated vegetable oils. Thus, although having a high content of phenolic compounds compared to other vegetable oils, characterized by a more astringent taste (Servili et al, 2014), this factor did not constitute a rejection by the panel for potatoes fried in virgin olive oil.

Conclusion

Based on the results obtained in this study, it can be inferred that, although having a similar fatty acids composition, their thermal performance is quite distinct. Extra virgin olive oil showed greater stability to oxidation compared to peanut oil and canola oil, as explained by different thermal oxidation indicators.

From the consumer nutritional point of view, it is advisable to use the frying oils for up to 8h in order to keep vitamins C and E, without elevated amounts of oxidized products, being consequently healthier for consumers.

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Laurel Oil

Maria do Céu Costa
p1658@ulusofona.pt

Coordinator of Food Sciences and Phytochemistry (FSP) Group of the Research Center for Biosciences & Health Technologies a structure of COFAC (Cooperative Education and Cultural Animation, Crl.) founding body of the Universidade Lusófona de Humanidades e Tecnologias (ULHT)

Introduction

The use of the fruits of laurel in Madeira for the manufacture of a product known as oil of laurel is a result of harvesting and expression of ripe berries, which are harvested between September and November.

In popular tradition, the oil is traditionally considered (Obon & Rivera, 1995) a "miracle drug", curing almost all evils, internal and external, mainly as a healing agent for blood circulation problems, cases of gangrene and tetanus, rheumatism, stomach ailments, throat and liver.

A teaspoon of laurel oil, twice a month is popularly regarded (Personal Comm, 2001) the optimal dose for the prevention of cardiovascular diseases. The oil is applied and / or ingested as anti-infective preparation for treatment of stroke (long term use). Laurel oil is also used to rub the joints in order to reduce rheumatic pains. This type of oil is a fixed oil, extracted from one of the most abundant tree species in the endemic laurel forest on Madeira archipelagos (Figure 1), of the genus *Laurus* spp. (Rivas-Martínez et al., 2002), known as "Laurel", also endemic to the Azores and the Canary Islands. From the same species another oil type can also be extracted, a volatile oil, obtained by hydrodistillation of aerial parts (leaves and flowers) which is technically designated as essential laurel oil.

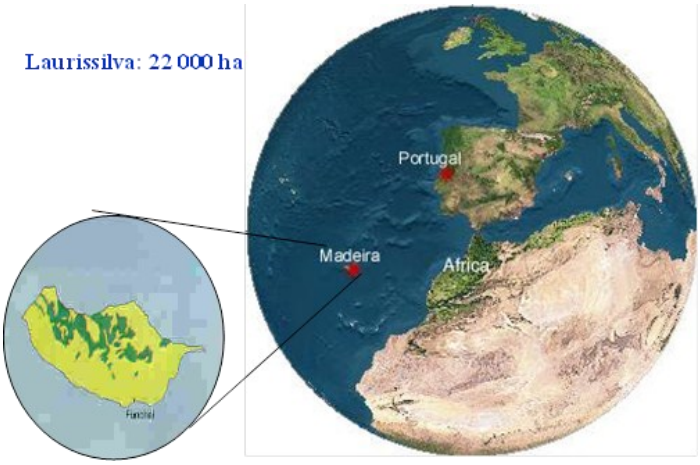

Figure 1. Laurel forest on Madeira, Portugal.

Taxonomy

For decades it was thought that existed in the Azores, Madeira and Canary the same kind of laurel*, Laurus azorica* (Seub.) Franco. But in 2002 it was determined that the species *Laurus azorica* (Seub.) Franco which is present in the Azores is distinct from that of the Madeira and Canary Islands (Figure 2) then classified as *Laurus novocanariensis* Rivas Mart., Lousã, Fern. Prieto, E. Dias, JC Costa & C. Aguiar (Rivas-Martínez et al., 2002). The name appears in the Latin binomial taxonomic nomenclature in italics, followed by the name (s) (s) of classifier (s), which in this case is a group of different names because it resulted from a tour of taxonomists. Different species, *Laurus* spp., grow spontaneously in the Macaronesia region and in particular on the island of Madeira, being the most abundant tree species, protected, with tendency to expand.

Figure 2. Madeira Laurel, *Laurus novocanariensis*, which grows in damp and dark places.

In the literature some scientific studies deal with the taxon *Laurus novocanariensis* Rivas Mart species, meaning that research groups have deposited in a herbarium a copy of each different crop that gave an analytical and pharmacological study (Ferrari et al, 2005; Castilho et al, 2005; ibid, 2005). There is also a patent granted for the use of a pharmaceutical composition based on Madeira laurel oil as an anti-inflammatory agent (Castilho, Rodrigues, Costa and Crow, 2002). Worthy of note is that when compared to *Laurus nobilis* L., this species is uncommon both in the Azores and Madeira and if it appears is grown in backyards and the like.

The medicinal flora of Madeira and Porto Santo consists of 259 species (Rivera & Obon, 1995) and the *Laurus novocanariensis* one of the 30 considered most important, have the largest list of registered uses in traditional practice. Morphological parts are used for therapeutic use, from fruit to fruit tea, essential oils, infusion of the leaves and the expression of fruits (berries) oil. This fixed oil is incorrectly called "laurel olive-oil" because it is extracted by mills identical to those applied in traditional olive oil production process, from the fruits, dark berries looking as olive (Figure 3).

Figure 3 - Production of fixed oil of Madeira laurel, *Laurus novocanariensis*.

Until 2002 (Castilho, Rodrigues Costa and Corvo, 2002), this oil had not been the subject of any study to characterize the type and level of constiuents, or setting out the relationship between chemical composition and therapeutic activities assigned to it. However, throughout Madeira island, this oil is extracted from the fruits, sold in the market, shops and pharmacies and applied by families. Wherever there is a community of Madeira immigrants seems maintained the tradition of its use in prophylactic and / or therapeutic intentions (Personal communication, 2000).

The study of the composition of the oil obtained by cold expression of the berries showed that consists mainly (ca. 85%) of triacylglycerols (TAG) with traces of free fatty acids (FFA) and diacylglycerols (4.5). Of the remaining 15%, volatile compounds are responsible for ca.10%, where *trans*-ocimene and germacrene D. The predominant oleic acid (30%) and linoleic acid (20%) are the major unsaturated fatty acids while lauric acid (18%) and palmitic acid (up 22.5%) are the major saturated fatty acids in the neutral lipid fraction. The oil has a sterol content of the same order of olive oil, with a predominance of β - sitosterol (84%). Two sesquiterpene lactones, costunolide and dehydrocostuslacton, represent 5% of the total composition (Castilho, Costa, Rodrigues and Supporter, 2005). These components, although a minority, are potentially essential for the anti-inflammatory activity, with effects potentially in synergy with the fatty acids present. In the last decade many studies have shown that, despite their toxicity, and beyond any traditional use as herbal medicine, isolated costunolide and dehydrocostunolide are interesting as models for the design of compounds with anticancer activity (Whipple *et al.* 2013).

Biological properties

Some studies have been conducted to clarify the therapeutic properties attributed to this oil. It was found that when administered orally to rats, the oil of Madeira laurel shows a significant inhibitory effect on the adjuvant arthritis model of rheumatoid rat (Castilho, Costa Rodrigues and Crow, 2002), where the reduction of swelling was highest at the higher dosage used (0.5 to 1 mL). Thus, a dose dependent effect was evidenced.

However, the product that is commonly used as a condiment in Madeira is the leaf of the laurel (*Laurus novocanariensis*) identical to the spontaneous continent bay leaf (*Laurus nobilis*), by contact with food to temper, usually in crude and especially meat. It is not reported as far as we know the traditional use of any oil of laurel as food flavoring.

In fact the chemical constituents of the leaves of *Laurus nobilis*, used as a condiment (Natural sources of flavorings (Rep No 1), 2000) are known as flavor molecules, although there are also reported some allergenic properties of some compounds of the oil present in minute quantities when the leaves are used to flavor. These compounds are methyleugenol (2.0-2.6 ppm); alkenyl benzenes (1,4-2.0% eugenol, methyleugenol 1.7-11.8%), eucalyptol (34-53%).

Consumer information for the use of products for oral administration based on *Laurus novocanariensis* depends on their classification as common food (as bay leaf) or another type of product adequately studied and characterized for introduction into the market.

When it comes to developing food products for beneficial use related to human physiology improvement, experts may apply for nutritional and health claims regulated at EU level by the EC Regulation n. º 1924/2006 of the European Parliament and of the Council of 20 December.

Should any evidence be provided of pharmacological characteristics, Decree-Law 176/2006, of 30 August (and subsequent updates, including review by the Decree-Law n. º 128/2013 of 5 September) applies since it is in force after the transposition of Community legislation, in particular Directive 2004/24/EC of, the European Parliament and of the Council of 31 March 2004, foreseeing the possibility of submission of applications for registration of traditional herbal medicinal products (articles 141 to 147). Traditional herbal medicines can only be the subject of this application for registration of which cumulatively:

a) have indications exclusively appropriate to herbal medicines and, given its composition and purpose, are intended and designed for use without the supervision of a physician for diagnosis, prescription or monitoring of treatment;

b) are intended to be administered solely in accordance with a specified strength and posology;

c) can be administered by one or more of the following routes: oral, external or inhalation;

d) are already reported with the object of long therapeutic use, according to the information or opinions mentioned in paragraph m) of paragraph 2 of the following article.;

e) are not demonstrably harmful when used as specified in accordance with the information and reputable enough;

f) can demonstrate, in accordance with existing enough and reputable information, pharmacological effects or efficacy plausible, taking into account the use and longstanding experience.

There is no monograph for Laurel (Madeira Laurel) as herbal medicine, which would be found in the main European Medicines Agency (cf. http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/landing/herbal_search.jsp&mid=WC0b01ac058001fa1d).
Hence, despite the numerous published *in vitro* pharmacological studies, more studies are necessary to understand the mode of action, and preclinical and toxicity tests as well as clinical trials are mandatory to clarify the whole therapeutic potential of the oil of *Laurus* spp. as a medicine, with a dossier Holder (MAH) to consecrate Quality and minimum requirements for ensuring safety of its use in accordance with international standards of good manufacturing and distribution. In the absence of this file, Madeira laurel oil should not be used for medicinal purposes. Commercialization of laurel oil also falls outside the regulation applicable to dietary supplements, as they are not intended to meet the need for a balanced diet.

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