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Genetic Improvement of Olives, Enzymatic Extraction and Interesterification of Olive Oil

Fabiano Jares Contesini[1,*], Camilo Barroso Teixeira[1], Paula Speranza[1], Danielle Branta Lopes[1], Patrícia de Oliveira Carvalho[2], Hélia Harumi Sato[1] and Gabriela Alves Macedo[1]

[1]*Laboratory of Food Biochemistry, Department of Food Science, College of Food Engineering, State University of Campinas (UNICAMP), Campinas, SP,*

[2]*Multidisciplinar Laboratory, University São Francisco, Bragança Paulista, SP, Brazil*

1. Introduction

Extra virgin olive oil (EVOO) contains a wide range of bioactive compounds which give it its particular aroma and taste. It is a well-known key component in the traditional Mediterranean diet and due to its high levels of phenolics and unsaturated fatty acids, it is believed to be associated with good health and a relatively long life (De Faveri et al., 2008). The phenolic compounds have the ability to reduce the oxidative modification of low-density lipoproteins (Fitó et al., 2005), which play a key role in the development of atherosclerosis and coronary heart disease. Moreover, olive oil is very resistant to peroxidation (Najafian et al., 2009), a fact conferring great oxidative stability to the product (Bendini et al., 2006). The sustainable development of the agriculture and food industry is dependent upon powerful biotechnological tools which meet the demands of the new urbanized population. The improvement in the properties of EVOO is a good example of how useful the application of biotechnology to improve food quality is.

The olive oil extraction process is extremely important for its quality. During this step, the content of some components is significantly altered, depending on the extraction technique employed. A new process for the extraction of olive oil that has been studied is the addition of enzyme preparations during malaxation. This reduces the complexing of hydrophilic phenols with polysaccharides, increasing the concentration of free phenolic compounds in the olive paste and their consequent dissolution in the oil and waste waters during processing (De Faveri et al., 2008). The enzymes most used in the extraction of EVOO are microbial pectinases and cellulases, which hydrolyse the cell wall of the olive fruits, liberating the oil and phenolic compounds. This method has some advantages compared to traditional methods, giving higher oil and phenolic compound extraction yield. It also involves lower energy costs and possibly provides an oil with improved health properties

* Corresponding Author

due to the liberation of the phenolic compounds. Microbial lipases can also be used to synthesize structured lipids from olive oil triacylglycerols.

This review discusses mainly the genetic improvement of *Olea europaea* to achieve higher quality EVOO, with lower production costs and greater productivity. Additionally, it reports on the use of enzymes to improve the extraction of virgin olive oil from olives and the enzymatic synthesis of lipids based on olive oil triacylglycerols.

2. Genetic modification of olive cultivars: Crossbreeding

Improvement of the cultivars is one of the major targets of olive biotechnology. The recent diffusion of the olive outside its traditional cultivation area, the Mediterranean basin, together with a continuous trend for industrial modernization, has recently increased the demand for improved cultivars. As a result, clonal selection and crossbreeding programmes have been applied in olive growing countries, aiming at selecting genotypes with improved characteristics. The desirable characteristics are early bearing, resistance to pests and to abiotic stress (such as frost and drought), limited alternate bearing and suitability for intensive cultivation and mechanical harvesting. In relation to the product, the search is for high-quality production with respect to the organoleptic characteristics of both the fruits and oils, and finally a high content of bioactive substances that may favourably affect human health (Fabbri et al., 2009). Olive crossbreeding programmes have provided new genotypes with a wide range of variation for all the characteristics, including the oil composition (Belaj et al., 2010). This technique has been used to generate new cultivars from traditional ones, which are used as the genitors. For instance, in Tunisia, an olive breeding programme started in 1989 with a cross between Tunisian and foreign cultivars. This created new cultivars with a quality of oil superior to that of *Chemlali* (the main olive cultivar in the country, characterized by low levels of oleic acid) and characteristics close to the standards of the international market (Baccouri et al., 2007; León et al., 2011; Manai et al., 2007; Manai et al., 2008; Rjiba et al., 2010). The large variability in all the components of olive oil in these advanced selections suggests that diversity in olive oil composition could be obtained in any crossbreed progeny. Therefore, any breeding programme provides diversity of the oils.

In Spain a breeding programme began in 1992 to obtain new olive cultivars with some of the following advantages: early bearing, high productivity and oil content, resistance to peacock eye (*Spilocaea oleagina*, Cast), suitability for mechanical harvesting and high olive oil quality (León et al., 2004a).

León et al. (2011) selected fifteen genotypes from crosses between the cultivars *Arbequina*, *Frantoio* and *Picual* on the basis of their agronomic characteristics. In this work, the main components of the olive oil were characterized and compared with their genitors. A wide range of variation was observed for all the fatty acids and minor components, and for the related characteristics evaluated, with significant differences between the genotypes, except for the β-tocopherol content. The values obtained in the selections have extended the range of variation of the three genitors for all the characteristics evaluated. The selections showed the highest average values for tocopherols, polyphenols and the C18:1 content, respectively. The breeding procedures used to obtain these selections including crossing, the forced growth protocol and an initial seedling evaluation, are all described (León et al., 2004a; León et al., 2004b; Santos-Antunes et al., 2005).

A wide range of variation was observed for all the fatty acids, minor components and related characteristics evaluated by León et al. (2011). The fatty acid C18:1 was the predominant fatty acid in all the selections, with values ranging from 62 to 81%. Together with C16:0 and C18:2, it accounted for more than 94% of the total fatty acid composition, on average. The genotypes producing olive oils with high oleic acid percentages could be of particular interest for planting in low latitude locations, where the oleic acid content tends to be too low (Ripa et al., 2008). Of the minor components, α-tocopherol represented more than 90% of the total tocopherols, whereas the total polyphenol content varied widely from 67 to 1033 mg/kg. A wide range of variation was also obtained for stability, with values ranging from 16 to 195 h.

The statistical analysis showed that genotypic variance was the main contributor to the total variance for all the fatty acids and ratios evaluated, with significant differences between the genotypes in all cases. In fact, the effect was significant for all the fatty acids, except C18:3, all the minor components and related characteristics evaluated, α-tocopherol and stability, but was lower for the other characteristics. Several studies have demonstrated that the quality of olive oil is greatly determined by genetic (cultivars) factors. For instance, in the Germplasm Banks of Catalonia and Cordoba, Tous et al. (2005) and Uceda et al. (2005), respectively, showed that more than 70% of the variation in the fatty acids (except for C18:3) and several minor components, such as the polyphenol content, bitter index (K225) and oil stability, was due to genetic effects. It should be noted that many other factors including pedoclimatic aspects, olive ripeness, olive harvesting methods and the olive extraction system have also been reported as quality indicators of virgin olive oil (Aguilera et al., 2005; Guerfel et al., 2009; León et al., 2011).

Ayton et al. (2007) found a stronger relationship between the polyphenols content and oil stability when individual cultivars were analyzed separately, which suggests that the relationship between induction time and total polyphenol content is different for each cultivar. In another study (León et al., 2011), the ranking of the cultivars was different for the polyphenols content and oil stability, which could suggest that not only the total polyphenol content, but also different polyphenol profiles in the different cultivars could have distinct antioxidant effects. Similar results have been reported for the analysis of the composition and oxidative stability of virgin olive oil from selected wild olives (Baccouri et al., 2008). The correlation between the different fatty acids also agrees with what was previously reported for olive cultivar collections and breeding progenies (León et al., 2004a).

Significant differences between the genotypes obtained for crosses between *Arbequina*, *Frantoio* and *Picual* were observed for the fatty acid composition, minor components and related characteristics. The multivariate analysis allowed for the classification of the genotypes into four groups according to their olive oil compositions. Further work will be required to determine the best selections to adapt to different environmental conditions, as well as the optimal harvesting periods in terms of optimal oil quality (León et al., 2011).

Ripa et al. (2008) evaluated oil quality, in terms of fatty acid composition and content in phenolic compounds, for many new genotypes previously selected in a breeding programme and cultivated in three different locations of central and southern Italy. The availability of data from many genotypes cultivated in all three locations allowed quantitative analyses of the genetic and environmental effects on the oil quality traits studied. The acidic composition varied greatly both with genotype and with environment, and so did the concentration in phenols, though the effect of genotype on phenols was not significant. The fatty acid

composition appeared predominantly under genetic control while the environmental effect explained 0.31 of the total variance. The oil content in phenolic compounds, instead, had lower heritability (0.29) and was more affected by the environment, which explained 0.50 of the total variance. Few genotypes were selected as the best for each location, but none performed best in all locations. This suggests that, in olives, the highest oil quality is difficult to achieve with a single genotype in different environments, due to a strong or even predominant effect of the environment on some quality traits. More likely, combinations of genotypes and territories can produce oils with high and typical quality.

3. The use of enzymes in the extraction of olive oil

The most commonly used method for the extraction of olive oil is the mechanical process, however some of the non-extracted oil remains in the solid residue or cake. The majority of the oil is located in the vacuoles as free oil, but oil dispersed in the cytoplasm is not extractable and is therefore lost in the waste (Najafian et al., 2009). Therefore the cell walls must be destroyed to effectively recover the oil enclosed in the cell. The use of enzymes has been studied for the hydrolysis of the different types of polysaccharides in the cell wall structure (Chiacchierini et al., 2007). The major polysaccharides found in the cell wall of the olive fruit were pectic polysaccharides and the hemicellulosic polysaccharides xyloglucan and xylan (Vierhuis et al., 2003).

Several innovating biotechnological techniques have been studied to obtain high-quality oils and/or improve product outputs. They include the use of microorganisms (Kachouri & Hamdi, 2004, 2006) or enzymes (Vierhuis et al., 2001) during different steps of the oil processing procedure. Several enzyme processing aids have been successfully tested for olives in recent years (De Faveri et al., 2006; García et al., 2001). Different enzymes are naturally present inside the olive fruit, but are strongly deactivated during the pressing phase, most likely due to the formation of oxidized phenols bonding to the enzyme prosthetic group (Vierhuis et al., 2001). In this case, the addition of suitable enzymes to the olive paste during the mixing step was proposed as a tool to replace the deactivated natural ones (Ranalli et al., 2001). Furthermore, the enzyme complexes are water-soluble and after the application, they are found in the olive mill waste waters, indicating that the oil composition is not modified (Chiacchierini et al., 2007).

Ranalli et al. (2003a) estimated the composition of three types of olive oil (Caroleo, Coratina and Leccino) extracted by the application of the Bioliva enzymatic complex. During extraction, the action of the enzymes on the fruit tissues resulted in the release of greater amounts of oil and other constituents, which dissolved in the oily phase (Ranalli et al., 2003b). The enzymatic application resulted in an increase in several key compounds, such as phenols, tocopherols, and flavour compounds, without changing the natural parameters related to product authenticity (waxes, sterols, triterpene alcohols, fatty acids and triacylglycerol composition).

The loss of phenols during processing can be attributed to interactions between the polysaccharides and phenolic compounds present in the olive pastes (Servili et al., 2004). Studies show that the addition of commercial enzyme preparations during the malaxation can reduce the complexation of hydrophilic phenols with polysaccharides. It increases the concentration of free phenols in the olive paste and their consequent release into the oil and waste waters during processing (De Faveri et al., 2008).

A mixture of three enzyme formulations was tested by Aliakbarian et al. (2008) to improve the yield and the quality of the olive oil obtained from the Italian cultivar Coratina. Since no single enzyme is adequate for the efficient maceration and extraction of oil from olives, pectinase, cellulase and hemicellulase were essential for this purpose (Chiacchierini et al., 2007; De Faveri et al., 2008). A homogeneous mixture of the three different enzyme formulations was used at the beginning of the malaxation step in the proportions 33.3:33.3:33.3% (v/v/v). This choice was suggested by the higher efficacy of these enzymes in releasing phenolics into the oil when working as a ternary system (A:B:C), rather than in binary combinations (A:B, A:C, B:C) (De Faveri et al., 2008). In summary, A is a complex formulation containing pectinase plus cellulase and hemicellulase; B shows equilibrated pectinase–hemicellulase activity; C is a pectolytic enzyme. The enzymes selected are naturally present inside the olive fruit, but are strongly deactivated during the critical pressing step, presumably because of the oxidation (Chiacchierini et al., 2007). The highest levels of total polyphenols (874 $\mu g_{CAE}/g_{oil}$), antiradical power (25.1 $\mu g_{DPPH}/\mu L_{extract}$) and o-diphenols ($\mu g_{CAE}/g_{oil}$) were all reached at the highest enzyme concentration (25 mL/kg_{paste}). Moreover, the highest oil extraction yield (17.5 $g_{oil}/100\ g_{paste}$) was reached with the longest malaxation time (t = 150 min), always with the highest enzyme concentration.

4. Enzymatic synthesis of structured lipids

The enzymatic synthesis of structured lipids is relatively new in lipid modification. Although enzymes have been used for several years to modify the structure and composition of foods, they have only recently become available for large-scale use, mainly because of the high cost. Within this context lipases are reported for the enzymatic synthesis of structured lipids. They have the ability to carry out hydrolytic reactions, but the manipulation of the reaction at low water levels permits their use also for the synthesis of triacylglycerols. These enzymes can be successfully used in the production of lipids structured for medical purposes (De Araújo, 2011).

Enzymatic modification of olive oil triacylglycerols has been discussed by Boskou (2006, 2009). The development of techniques for the preparation of oils and fats from enzyme-modified olive oil is an attractive prospect for the food industry, given the high oxidative stability of the product at frying temperatures and the health enhancing properties of this material (Criado et al., 2007).

Nunes et al. (2011) produced structured triacylglycerols containing medium chain fatty acids, by the acidolysis of virgin olive oil (VOO) with caprylic or capric acids in a solvent-free media or in n-hexane, catalyzed by immobilized lipases from *Thermomyces lanuginosa, Rhizomucor miehei* and *Candida antarctica*. The results indicated that the incorporation was always greater for capric than for caprylic acid, but for both acids, higher incorporation was always attained in solvent-free media. All the biocatalysts presented 1,3-regioselectivity. The lipases from *Rhizomucor miehei* and *Candida antarctica* were the biocatalysts presenting the highest operational stability, together with high incorporation levels and low acyl migration in the batch production of structured lipids by the acidolysis of VOO with caprylic or capric acids. Therefore, these biocatalysts seem to be the most adequate for the implementation of a process aimed at the production of triacylglicerols containing medium and long fatty acids (MLM) rich in caprylic and capric acids. The structured triacylglycerol obtained from VOO has oleic acid at the sn-2 position, indicating a better absorption, whilst medium chain fatty acids will mainly be esterified at the external positions of the TAG molecules.

Criado et al. (2007) have also studied the enzymatic interesterification of extra virgin olive oil with a fully hydrogenated palm oil to produce lipids with desirable chemical, physical and functional properties. The sn-1,3 non-specific immobilized lipases from Candida antarctica and two sn-1,3 specific immobilized lipases from *Thermomyces lanuginosus* and *Rhizomucor miehei* were employed as the biocatalysts. The authors concluded that the oxidative stability increased when the percentage of TAG containing multiple fully saturated residues increased. In all the cases studied, the stability of the physical blend was higher than that of the reaction products. The final products were considered as plastic over wider temperature ranges. The large amount of unsaturated residues present in these samples, primarily oleic acid residues, was the factor leading to the extended plasticity range of these interesterified mixtures.

5. Conclusion

The improvement of the properties of extra virgin olive is crucial, considering the extensive number of functional compounds present in this oil. Genetic improvement techniques are the option showing the most promising results. The genetic modification of olive cultivar crossbreeding is focused on solving agronomic and commercial problems, such as the control of fruit ripening and increase in the oil content and quality. Another improvement suggested by different research groups is the use of enzymes added to the paste to improve extraction of the oil and the bioactive compounds it contains. Enzymes, such as microbial lipases, can also be used to synthesize different high-value products from tryacylglicerol of olive oil. It is possible to obtain structured lipids that have functional and technological properties suitable for applications in the food, pharmaceutical and oleochemical industries. This can be considered as a "green" industrial process, taking into account that these compounds are currently synthesized by chemical processes that use catalysts and generate byproducts.

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