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Research Article

Can the High Performance Liquid Chromatographic (HPLC) Tracing of Biophenols Guide the Choice of Extra Virgin Olive Oils (EVOO) To Be Con-sumed "Raw"?

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Abstract

Extra Virgin Olive Oil (EVO) represents the main lipid intake of the Mediterranean Diet. It is considered a functional food as it is rich in polyphenols and antioxidant molecules, having multiple nutraceutical properties and being responsible for its bitter and spicy taste. Our body needs these EVO molecules, especially when taken "raw", while cooking tends to denature them. These water-soluble polyphenols together with the fat-soluble Vitamin E represent EVOO's antioxidant apparatus that protects the fat mass. EVOOs oxidize naturally and progressively, so their shelf life is of 18 months. In order to maintain their functional and nutritional properties, EVOOs must be stored away from light, heat and air, since its oxidation would accelerate. Prolonged exposure on the shelf would therefore cause a rapid impoverishment of its antioxidant defense system, producing rancidity in the taste and the formation of free radicals, which are harmful molecules to our body.

Since we are intaking this food "raw" in our daily diet, it is important to check its oxidative and functional state, in order to benefit from its properties. For this reason, samples of shelf-stable EVOOs, were sourced from both industrial and small Italian oil farms and were tested about nine months from their labeled expiration date, (50% of their shelf life, i.e.), and evaluated with High Performance Liquid Chromatography (HPLC). Important information on the "health state" of the selected "raw" EVO. The purpose of these exams is to verify whether there are any correlations, in order to suggest a better strategy for the consumer when purchasing "raw" EVOO.

Keywords: Extra Virgin Olive Oil; EVO; Polyphennols; High Performance Liquid Chromatogrphy; HPLC; Mediterrean Diet; Functional Foods

Introduction

EVO represents the primary fatty nutrient of the Mediterranean Diet with a 98% lipid content, rich in Oleic Acid, a medium-chain monounsaturated part of the Omega 9, with a good percentage of Polyunsaturated fatty acids, Omega 6 and Omega 3 in the ratio of 1:4-1:6. Saturated fatty acids such as stearic and palmitic acids are rarely present [1]. For these nutritional characteristics the European Food Safety Agency (EFSA) has approved four health claims to label EVOO, thereby emphasizing the human health benefits associated with the consumption of olive oil. Three of these health claims are not specific to EVOO and could be licit also for other edible oils including non-virgin or refined olive oils, as they concern:

the high content of oleic acid; the oil as a source of vitamin E; and the relatively high amounts of mono- and/or poly-unsaturated fatty acids [2,3]. Within EVOO's structure, is a non-saponifiable fraction, that is defined the "magic powder" which accounts for the remaining 2% content.This is made up of squalene, alcohols, fat-soluble vitamins, especially A and E, and water-soluble polyphenols. Vitamin E and polyphenols represent EVOO's antioxidant defense system. The latter also possess functional nutraceutical properties, which our body benefits through the "raw" intake. The polyphenols currently present in nature are about 8000. Those present in EVOO, that are water-soluble, are mainly represented by lignans, secoiroids and flavonoids [4]. They are also among the molecules responsible for the "chemistry of taste" by contributing fruity, astringent notes, or the bitter and spicy perceptions [5].

In particular, the last two express the typical taste sensations of EVO initially perceived in the mouth, while the latter in the aftertaste. Oleoeuropein gives the typical bitter taste, while Oleocanthal determines the spicy aftertaste note [6,7]. The more an EVO undergoes oxidative phenomena, the more these taste senses fade until vanishing completely, being replaced initially by a sweet taste and later by the rancid defect due to the oxidation of the lipid unprotected component.

These anti aging antioxidants, possess nutraceutical functional properties such as anti-inflammatory, antibacterial/viral, anti-allergic, estrogenic, anti-tumor and anti neurodegenerative [1,8]. Our body needs these essential molecules, which must be introduced through the daily diet by consuming "raw," EVO, so cooking at high temperatures largely denatures them. To identify the state of oxidation of an EVO, besides the everlasting tasting criteria, carried out by panels of professionals, objective tests are also done. For example, the total content of PEROXIDES, SPECTROPHOTOMETRY with U.V. with the detection of primary oxidation products (Peroxide Radical at K232 nm) and secondary oxidation products (Hydroperoxide Radical at K270 nm), Infrared Spectroscopy effective in quickly determining EVO's numerous quality parameters (loose acidity, number of peroxides, UV spectrophotometric indices, oxidative stability) [1,9-13].

Through a chemometric method, we can detect EVO's four main defects: moldy, heated, wined-up and rancid.

However, all these methods are unable to provide information on the change in polyphenolic content according to the state of oxidation, unlike HPLC.

Method and Materials

Thirty samples of Italian EVOOs were examined and compared, including 23 monocultivar and 4 multivarietal.

These samples were taken from small oil farms in various Italian regions. We also tested three samples of industrial oils from large retailers that were all produced with the "cold pressing" method, at temperatures below 27 ˚C. All the samples chosen had the same shelf life and were evaluated after 9 months from their packaging date, exactly halfway through their 18-month shelf life. Nine labels were BIO, of which five, were monocultivars and four were blends. The Selected monocultivars were:

Biancolilla (Sicily), Casaliva (Trentino Alto Adige), Cellina di Nardò (Puglia), Coratina (Puglia 5 samples), Ghiacciola (Emilia Romagna), Itrana (Lazio), Leccino (Tuscany), Moraiolo (Tuscany and Umbria 2 samples), Moresca (Sicily), Nocellara (Sicily), Nostrana di Brisighella (Emilia Romagna), Ogliarola (Puglia 2 samples),

Pendolino (Tuscany), Peranzana (Puglia), Raggiolo (Tuscany), Rosciola (Lazio), Taggiasca (Liguria).

The four EVOO Blended, were from the regions of Lazio, Umbria, Tuscany and Sicily. All samples were analyzed by phase HPLC in order to obtain the relevant biophenolic graphs.

Phenolic compounds were quantified by reversed, adapting the International Olive Council (IOC) method for the determination of olive oil biophenols [14,15]. Extraction and quantification of the phenolic compounds was carried out following an official methodology from the International Olive Council (see the original document for details and a representative spectrum).

The amounts of phenols reported represent the mean values for a specific type of cultivar. Through evaluation by HPLC both amounts of polyphenols and that of individual biophenols (mg/Kg) were analyzed. [9-13,16,17].

Those always present in EVOOs and represented in the graphs are:

Phenolic Alcohols : Hydroxytyrosol and Tyrosol Secoiridoids : Oleuropein Aglycone (3,4DHPEA-EA), Decarboxymethyl-Oleuropein Aglycone (3,4DHPEA-EDA), Ligstroside Aglycone (p-HPEA-EA) and Decarboxymethyl-Ligstroside Aglycone (p-HPEA-EDA);

Oleoeuropein is responsible for the bitter taste note, while Oleocanthal for the spicy flavour, Lignans (Pinoresinol , 1-Acetoxypinoresinol) Luteolin and Apigenin (Flavones). Their quantities were correlated to the type of *cultivar* and other agronomical parameters, such as the age of the trees and the areas they were located, as well as more technical variables, such as the interval time between harvesting and milling. The greater or lesser peak heights in the chromatographic traces represent the greater or lesser amount in each individual functional molecule.

The data received was then processed and correlated in relation to each other using both mathematical and statistical criteria. It thereby detected a normal range and its standard deviations capable of identifying samples with higher or lower amounts of polyphenols than the average. A score of +1 was then given if the amount of each individual polyphenol was above the standard deviation of the mathematical average, of 0 if within the medium range, and -1 if located below the lower standard deviation of the average, for each individual polyphenol peak. Subsequently, we summed all the scores we obtained from each individual sample. EVOOs that scored greater than +2 overall were considered to have a high antioxidant activity, although still rich in polyphenols (positioned above the green line).

Those scoring between -2 and +2 were considered average (included between the green and orange line), while those scoring between -3 and -5 were low in antioxidant activity (included within the red line). Finally, those that scored below -5, corresponding to the II standard deviation had very low activity, so they were highly oxidized and low-functional (located below the purple line).

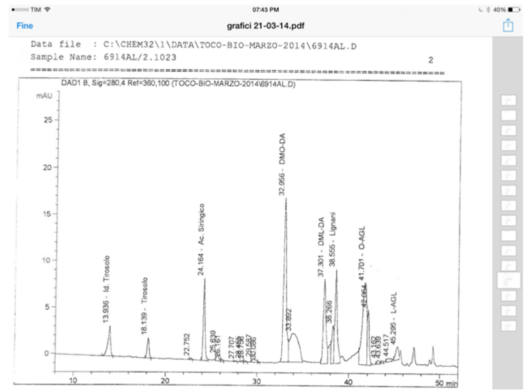
Results

Of the 30 EVO samples, 11of them (a 37%) showed an average antioxidant activity (the score was between +2 and -2), 10 were monocultivar and just 1 was multivarietal. The monocultivars did not belong to a territorial geographical subdivision (North, Central, South,or from the Islands), but were reffered to the various Italian regions examined. Four (4) samples (a 13%) were over the I standard upper deviation (> +2), consisting of 1 blend and 3 monocultivars, all coming from southern Italy, Puglia and Sicily. Of the 12 (40%) EVOOs that possessed a low antioxidant activity (between -3 and -5) none had a specific territorial connotation, 9 monocultivar, 1 multivarietal and 2 industrial. Lastly,we have 3 (10%) samples with very low activity (<-5) 1 mono, 1 multi and 1 industrial. From the graphs recieved, it can be stated that, as the total number of polyphenols decreases, the individual peaks decrease in height, thus resulting in the loss of EVO's antioxidant and functional properties. In the samples with total scores above -2, the reduction affects only a few peaks, while in those with scores below -2, their decrease appears evenly distributed. All nine BIO labels were within the range of +6 to -2, a fact that expresses an assurance when purchasing products suitable for "raw" consumption diets. Another interesting fact is that 15 EVOOs had a score below -3, 14 of them had a total polyphenol count below 280 mg/kg.

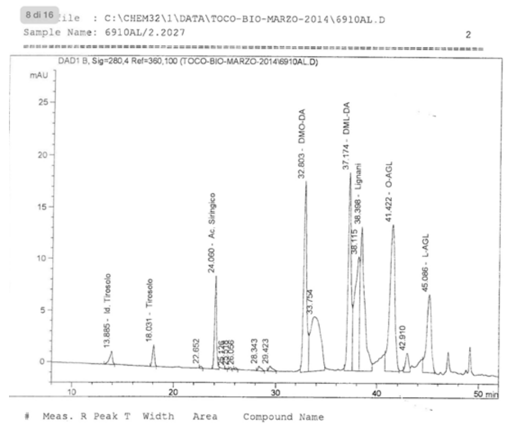
Furthermore, it was noticed, that the EVOOs having a total amount of polyphenols below 200 mg/kg, also presented a deterioration in taste, coming up with a sweet and a rancid hint taste.

The graphic schemes examined, show the following evident polyphenols:

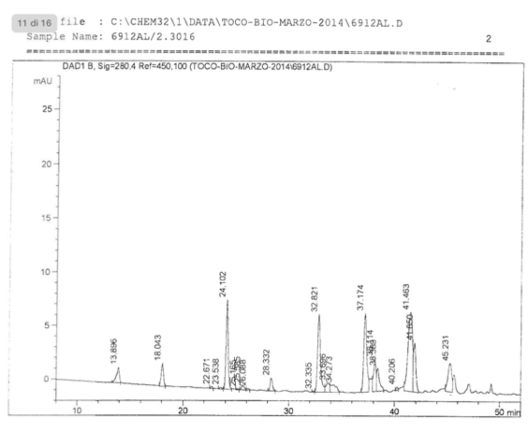
Hydroxytyrosol (3,4DHPEA), Tyrosol (p,HPEA), Decarboxymethyl-Oleuropein Aglycone (3,4DHPEH-EDA), Decarboxymethyl-Ligstroside Aglycone (p,HPEA-EDA, OC), Oluropein Aglycone (3,4DHPEA-ea), Ligstroside Aglycone (p,HPEA-EA),Lignans, Luteolin and Apigenin [18].



Graph A: Documents a biophenolic tracing of an EVO's normal "vitality" with medium oxidation state.



Graph B: Underlines a trace with elevated quantity of high peaks, therefore still rich in polyphenols, with a high level of antioxidant activity and consequently more functional for our bodies.



Graph C: refers to an EVO with a very low activity.

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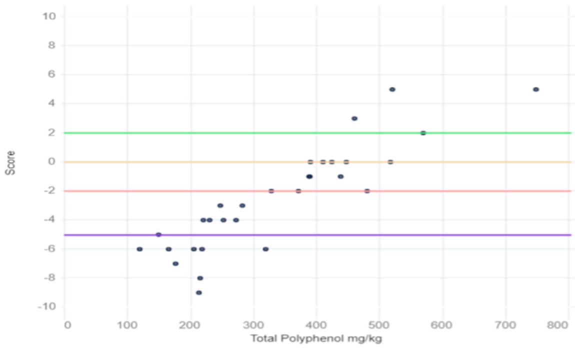
Therefore in the process of complete oxidation; note the widespread reduction in height of the various polyphenolic peaks compared to the tracings in graphs A and B. Interesting is the feedback in the difference in heights of each individual peak in EVOOs, having similar scores and belonging to the same range. This explains the different contribution quality in polyphenolic that varies from cultivar to cultivar.



Scores: Graph A,B,C.

In the eight samples, the graphs indicate a higher peak of Oleuropein, a biophenol with a proven anti-inflammatory action, which was more present in cultivars from central Italy (3 monovarietals and one blend) and from southern Italy (4 monovarietals). When studying lignans, the molecules with a phytoestrogenic action, the highest concentration was found in 7 samples of different monocultivars from southern Italy, but only one (1) from Northern Italy. These EVOOs have all shown to have low amounts of polyphenols both totally and individually, consequently resulting to be more oxidized, so they are not recommended for "raw" seasoning compared to others.

During the course of this research, other polyphenols were detected in some cultivars but not in traceable amounts, consequently, they were not able to develop a distinct peak. These may although, be able to determine a new chemical biodiversity among EVOOs and provide additional nutraceutical effects to our organism. Seventeen have been defined "variable" in addition to the 9 "fixed" ones, that are always present in the tracings. In addition, the use of polyphenolic tracings can be also useful to uncover sophisticated olive oil frauds, which are difficult to identify through regular routine chemical analysis, since it is impossible to reconstruct the "fixed" and "variable" polyphenolic EVOO's kit, in the laboratory.

Discussion

EVO is the "fat" ingredient on which the Mediterranean Diet is based on. In Italy alone, there are more than 600 monoultivars of Olive trees that produce biodiverse EVO, so it is correct to speak of EVOO. EVOOs are rich in antioxidant molecules such as Vitamin E and water-soluble biophenols that protect its nutritional integrity for months. Specifically, the water-soluble polyphenols are functional molecules with various properties. They can be antioxidant, therefore anti-aging by counteracting the free radicals produced by our body, anti-inflammatory, antibacterial and viral, estrogenic, anticarcinogenic, anti neurodegenerative, antiatherogenic and lastly, antiallergic.

This is the reason why they are of broad scientific and pharmacological interest. Commercially, nonsteroidal anti-inflammatory drugs already exsist, based on the biophenols that are extracted from the wastewater they are processed from. Thanks to these valuable molecules, EVO is not only considered a nutrient, but also a natural drug. In agreement with the EFSA indications, the Commission Regulation (EU) No. 432/2012 has authorized the EVOO specific health claims "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress", which can be used to label EVOO containing at least 5 mg of hydroxytyrosol and its derivatives (*e.g.*, oleuropein complexes, ligstroside and tyrosol) per 20 g of oil (polyphenols $\geq$ 250 mg kg^{-1}) [2,19,20].

Phenolic total compounds, which vary within a considerably wide range of concentrations (50-1000 mg kg^{-1}) in EVOO depending on olive genotype, elaiotechnical practices, process of oil extraction and storage conditions [4]. The phenolic compounds of EVOO, also referred to as "biophenols", are responsible of many among the health-promoting effects attributed to EVOO, mainly related to their antioxidant and radical scavenger properties [21,22].

Therefore, the EFSA health claim concerning phenolic compounds that has been introduced to classify EVOO on the market is still largely underutilized. Clearly, the failure to use the health claim penalizes the high-quality productions and limits the nutritional exploitation of EVOO benefits by consumers. Polyphenols are also among the molecules responsible for the "taste's chemistry" by providing fruity, astringent, bitter and spicy flavours. The last two are the typical EVOO tastes, the first, is savoured in the mouth, the latter, in the aftertaste [4,5,18, 21-24].

Oleoeuropein is responsible for the bitter taste note, while Oleocanthal for the spicy flavor. In a freshly produced EVO, the amount of biophenols are at their highest peak, but as time goes by, they decrease the protection of the fat mass, therefore increasing the oxidation. As the lipid content oxidizes, it goes rancid leading to anti-nutritional and harmful effects for our bodies, including the development of free radicals. The exposure of an EVO to light, sources of heat or oxygen, will accelerates its oxidation process.

EVO's worst enemy is light, because it triggers the chlorophyll process, consequently causing a photo-oxidation reaction toward which polyphenols cannot counteract. This unfortunately produces rancidity, causing the loss of its nutraceutical functional properties that are contributed by polyphenols. Oxygen induce the oxidative processes that cause the double bonds to fracture and the cutting of the unsaturated fatty acid molecules. These form saturated fatty acids, such as Caprylic and Caronic acids which are the ones that give the rancid odor. The pigments contained, such as the Carotenes and the Tocopherols, act directly on the oxygen molecole that when exposed to solar radiation, is very active and predisposed to producing free radicals. Tocopherols can also behave like the free radicals during the primary autoxidation phase, by blocking their chain production action from further radicals.

In the presence of light, chlorophylls and pheophtins have a harmful effect on the fatty acids, since they bring oxygen to its maximum reactivity state, by triggering the oxidative phenomena [23,25]. This explains why EVO must be consumed as soon as possible and not stored, unlike wine. In order to study the single quantity of the water-soluble polyphenol's majority contained in EVOOs, in relation to their temporal decay, a HPLC was used.

This methodical procedure is able to evaluate the different types of polyphenols by providing diagrams consisting of plots with a succession of peaks, the height of represents the amount present in the product, expressed in mg/Kg.

Through this method we are able to analyze the state of oxidation starting directly from the polyphenols, unlike the others normally used, which evaluate the same way the free radicals do, the molecules produced by oxidation. Through the HPLC the oxidative decay of EVOOs can be evaluated. This is expressed by the reduction in height of the peaks, that correspond to the polyphenols mostly present, which are nine, that are defined as "fixed". As a first analysis, it may appear complex to read the graphs because the biophenolic matrix is subject to many variables.

They are both environmental (microclimate, territory, plant varieties) and technical (crushing, production, storage, sophistication), which can also result in having significant variations among the EVOO diagrams from the same areas. This is precisely why the evaluation must take into account the total amount of these molecules and the individual peaks. In fact, a low calculation of the total polyphenols is not always an indication of a high oxidative state, since some cultivars possess lower numbers than others, resulting, however, more prone to deterioration over time and therefore to oxidation. The Taggiasca cultivar, for example, is the typical olive of the Liguria region, whose low polyphenolic content gives that "delicate taste" that consumers appreciate, since its not invasive to the palate because of the low level of bitterness and spiciness.

Graphically, this cultivar has a low peak of polyphenols which are responsible for bitterness, the Oleocanthal, and the spicy Oleoeurpein, while the others are statistically average. The use of mathematical/statistical evaluation criteria of individual peaks makes it possible to verify whether an EVO is truly oxidized or represents the characteristic of the cultivar in exam. This difference is represented in the first case by a homogeneous reduction of all peaks, while in the second, those maintained high, are the ones of greater magnitude.

Through the formulation of scores, four categories of EVOO were identified:

high (> +2), medium (<+2 and >-2) , low (< -2 and > -5) or very low antioxidant activity (<-5).

This distinction is important to indicate which EVOOs, can be consumed "raw" in our diet, in order to provide our bodies with both antioxidant and nutraceutical benefits, typical of the biophenols. When cooking food at high temperatures, we should use EVOOs with "low vitality" considering it would deteriorate. Using EVOOs with high or medium activity for this purpose, would make no sense and it would be a waste. The trace obtained with PLTC characterized by the succession of biophenolic peaks is similar to the human electrocardiographic examination, since both test the vitality.

While a succession of regular, high and "rhythmic" peaks represent a "healthy and vital" EVO, an irregular tracing, on the contrary, with low, "arrhythmic" peaks would represent the demise of the very oxidized EVOs when becoming almost flat, similar to that of a cardiopath patient. The HLPC study was able to test not only the nine mostly prevalent, "fixed" polyphenols, but an additional seventeen, "variable," present in some samples, in trace amounts. This confirms that there is a polyphenolic biodiversity between cultivar and cultivar, so all EVOs are not equal, even having different nutraceutical functional properties. For this reason some EVOOs could be recommended for the prevention of certain diseases or chosen for those pathological patients that require a special diet. On the other hand, the nutritionist's recommendation to use the "raw" EVOOs, in our daily diet, in the correct quantities, because it helps our body to fight the oxidative stress to which we are exposed daily. This oxidation is responsible for aging, the outbreak of cardio-circulatory, cancer and degenerative neurological diseases.

Based on what has been revealed in this study, which suggestions can be given to our consumers when choosing EVOO as a "raw" seasoning?. First of all, one must check whether the producer has specified the total polyphenol content on the label, which is now an option. If its not present, try to request it from the producer directly, if it was purchased on the farm.This information is usually indicated in the data sheet of the chemical analysis. If the total number of polyphenols is less than 200mg/Kg, EVO must be consumed raw very soon or otherwise, used for cooking.

In fact, as time goes by, Polyphenols are reduced by the natural oxidation phenomena and lose their functionality, as is shown in this study on samples examined 9 months after their commercial expiration. This happens especially if the quantity purchased is more than three liters, so one must consider it will take a longer consumption time, risking therefore, that this product will no longer give its benefits. We therefore suggest to purchase EVOOs that have polyphenol values above 300mg/Kg when bottled. Producers themselves should report the total amount of polyphenols on the label because it would be an important information for both the nutritionist and the consumer.

This would also be a good commercial lever by certifying its functionality. If you should not find this information anywhere, choose those EVOOs from the “raw” seasoning shelves, that have a packaging date as far behind the 18-month expiration date as possible. It is preferable, when possible, to buy EVOO directly from the producer, which will certainly guarantee a better preservation, considering that it is sheltered from the oxidizing factors such as light and heat, whereas in the supermarket it is well known that it will have a prolonged exposure to artificial lights and overheating. Although EVOO is contained in sealed glass bottles,of a green ultraviolet-absorbing color, this packaging is not sufficient to block out the oxygen and to prevent continuous exposure to artificial lights.

These factors would trigger the chlorophyll that produces a photo-oxidation reaction, that the polyphenols alone cannot stop. For this reason, when choosing to buy from stores or supermarkets, we suggest you purchase EVO that is in bottles lined with aluminum or choose dark colored bottles, being more resistant to both light and heat.

Monocultivar EVOOs often possess a higher polyphenol content so they last longer and are therefore preferable.

In the samples tested, the three monocultivars with the highest vitality were Coratina, Moresca and Cellina, all three coming from southern Italy. In spite of this indication, most consumers prefer the blends, because they have a lower amount of polyphenols, and are therefore often considered more enjoyable, balanced, and with a less aggressive taste for the bitter and spicy hints.

It is very important that consumers always taste the “raw” EVO they have bought in a teaspoon or in a paper cup, so that they can taste the bitter first and then the spicy aftertaste. If the chosen EVO has very little or zero intensity or a sweetish taste, it must be used only for cooking; consequently another label for the “raw” seasoning must be choosen. All of the three EVOOs samples from the large-scale retail distribution were found to have low and very low antioxidant activity, thus resulting suitable for cooking. In order to be sure of the functional, as well as nutritional validity of the product, we recommend the purchase of the monovarietal EVOO or blends Organic or DOP, to be guaranteed by strict specifications. These bottles can cost up to three times more than those produced in the large-scale distribution, and this often frightens and discourages the consumer, but who likewise easily spends the same amount of money on a labeled wine.

These people must be reminded that while a bottle of wine brings a few minutes of pleasure, a bottle of EVO, gives three weeks of health!

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