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Total content of hydroxytyrosol and tyrosol in virgin olive oils: method optimization, validation and application

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Conteúdo total de hidroxitirosol e tirosol em azeites virgens: optimização, validação e aplicação do método analítico

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Dissertação apresentada à Escola Superior Agrária de Bragança
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À minha família

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ABSTRACT

Olive oil consumption increased worldwide and recognized beneficial health effects are largely related to the presence of minor phenolic compounds that promote the oxidative stability in olive oil, influence its organoleptic properties and contribute to the claim on the protection of blood lipids from oxidative stress, particularly hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol). However, there is no international regulation for their specific analysis, with a myriad of methods and conditions published over the years, most of them requiring increasingly sophisticated equipment. Many procedures have emerged over the years to simplify their determination, including the pre-hydrolysis of the bound forms of hydroxytyrosol and tyrosol derivatives followed by simpler quantification by high performance liquid chromatography. Thus, the present work aimed to internally optimize and validate a simple, high-throughput and reliable analytical method for the estimation of these compounds, with a particular focus on the hydrolysis conditions. For this purpose, an all-in-one acid hydrolysis/extraction method and subsequent analysis by chromatography was selected from the literature. The effectiveness of hydrolysis over time was tested with standard and samples, indicating that there is an effective need for a hydrolysis time of 6 h for completeness, neither with signs of significant degradation of the released phenolic alcohols nor of the internal standard. The chromatographic conditions were also optimized under isocratic elution, allowing for 6 min runs. For method validation, linearity was verified (0.1 - 19.0 μg mL^{-1} and r^2 = 0.9999 for both hydroxytyrosol and tyrosol) and low limits of detection and quantification were obtained for hydroxytyrosol (6.5/21.8 ng or 8.2/27.3 mg/kg of olive oil) and tyrosol (4.6/15.2 ng or 5.7/19.0 mg/kg of olive oil). Precision was assessed and all results showed a relative standard deviation below 1%. The method also showed excellent accuracy with recoveries above 99%. The method was applied to 103 olive oil samples from centenarian olive trees from the Côa Valley region, with variable total contents of hydroxytyrosol and tyrosol derivatives between 7.6 and 36.3 mg/20 g of olive oil, with results above the limit established for the use of the health claim, which demonstrates that the studied oils are of great interest.

Keywords: Phenolic compounds; hydroxytyrosol; tyrosol; olive oil; health claim; hydrolysis.

RESUMO

O consumo de azeite aumentou a nível mundial e os reconhecidos efeitos benéficos para a saúde estão largamente relacionados com a presença de compostos fenólicos minoritários que promovem a estabilidade oxidativa do azeite, influenciam as suas propriedades organoléticas e contribuem para a alegação de proteção dos lípidos do sangue contra o stress oxidativo, nomeadamente o hidroxitirosol e seus derivados (por exemplo, complexo oleuropeína e tirosol). No entanto, não há regulamentação internacional para suas análises específicas, com uma infinidade de métodos e condições publicados ao longo dos anos, a maioria deles exigindo equipamentos cada vez mais sofisticados. Muitos procedimentos surgiram ao longo dos anos para simplificar sua determinação, incluindo a pré-hidrólise das formas ligadas de hidroxitirosol e derivados de tirosol seguida de quantificação mais simples por cromatografia líquida de alta eficiência. Assim, o presente trabalho teve como objetivo otimizar e validar internamente um método analítico simples, de alto rendimento e confiável para a estimativa desses compostos, com foco particular nas condições de hidrólise. Para tanto, foi selecionado da literatura um método integrado de hidrólise/extração ácida e posterior análise por cromatografia. A eficácia da hidrólise ao longo do tempo foi testada com padrões e amostras, indicando que há necessidade efetiva de um tempo de hidrólise de 6 h para completude, sem sinais de degradação significativa dos álcoois fenólicos liberados ou do padrão interno. As condições cromatográficas também foram otimizadas sob eluição isocrática, permitindo corridas de 6 min. Para validação do método, foi verificada linearidade (0,1 - 19,0 μg mL^{-1} e r^2 = 0,9999 para hidroxitirosol e tirosol) e baixos limites de deteção e quantificação foram obtidos para hidroxitirosol (6,5/21,8 ng ou 8,2/27,3 mg/kg de azeite) e tirosol (4,6/ 15,2 ng ou 5,7/19,0 mg/kg de azeite). A precisão foi avaliada e todos os resultados mostraram um desvio padrão relativo abaixo de 1%. O método também apresentou excelente exatidão com recuperações acima de 99%. O método foi aplicado a 103 amostras de azeite de oliveiras centenárias da região do Vale do Côa, com teores totais variáveis de hidroxitirosol e derivados de tirosol entre 7,6 e 36,3 mg/20 g de azeite, com resultados acima do limite estabelecido para a utilização da alegação de saúde, o que demonstra que os azeites estudados são de grande interesse.

Palavras-chave: Compostos fenólicos; hidroxitirosol; tirosol; azeites; alegação de saúde; hidrólise.

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ABBREVIATIONS

ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
DAD	Diode Array Detector
EFSA	European Food Safety Authority
FAEE	Fatty Acid Ethyl Ester
HPLC	High-Performance Liquid Chromatography
Htyr	Hydroxytyrosol
IS	Internal Standard
ICH	International Conference on Harmonization
IOC	Internacional Olive Council
LDL	Low-Density Lipoproteins
LOD	Limits of Detection
LOQ	Limits of Quantification
MUFA	Monounsaturated Fatty Acids
RSD	Relative Standard Deviation
Tyr	Tyrosol
UV	Ultraviolet

CHAPTER I.

Introduction

I.1. OLIVE OIL

The olive tree (*Olea europaea* L.) is a plant belonging to the Oleaceae family, with main incidence in the Mediterranean region, which is responsible for about 98% of world cultivation, especially in Spain, Italy, Greece and Portugal. Food products derived from the olive tree, namely olive oil and table olives, have been widely studied due to the growing evidence of their beneficial effects on health such as reduced incidence of chronic diseases and prolonged longevity (Martínez-Zamora et al., 2021).

I.1.1. Production, classification and quality control

According to the International Olive Council (IOC, 2019), due to growing world consumption, olive oil production has tripled in the last 60 years, reaching more than 3 million tons in the 2017/18 harvest. Due to its lower extractive yield and quality requirements, the price of this vegetable oil tend to follow the oscillation between supply and demand, making olive oil an even more valuable commodity and a target of illicit manipulation and fraud in comparison to the remaining vegetable oils. As a result, its production and marketing in Europe is subjected to several regulations, which define specific parameters to guarantee its authenticity and, therefore, the safety of consumers (Conte et al., 2020).

As a form of differentiation, virgin olive oils are classified according to their quality degree (physicochemical composition and sensory characteristics), and can be considered as extra virgin, virgin and lampante. The extraction of virgin olive oils is performed only by mechanical and physical processes, including milling, malaxation and centrifugation. In this way, the bioactive properties of the fruit are maintained, including the preservation of phenolics compounds, tocopherols, sterols and pigments (Rodrigues et al., 2019; Tsimidou & Boskou, 2015).

Commission Regulation (EEC) No 2568/91 of 11 July 1991, frequently updated over the years, establishes the criteria used to verify the quality and purity of olive oil, the parametric limits, commercial category and the advised analytical methods. As for the quality characteristics, some analytical parameters are mandatory which include acidity, peroxide index, ultraviolet spectrophotometry analysis (extinction coefficients), sensory analysis and fatty acid ethyl esters (FAEE) content. About the purity characteristics that determine the product authenticity and assess if any irregular raw materials or illegal

procedures were used, one must consider the fatty acids and sterols profiling and the amount of waxes.

I.1.2. Variation in the olive oil composition

Olive oils have a qualitative and quantitative composition that varies in space and time, influenced by several factors, including agronomic, technological, storage and distribution variables (Gómez-Caravaca et al., 2016). Agronomic factors include the cultivar, environmental and edaphoclimatic conditions, geographic origin, agricultural and harvesting methods, stage of ripening of the olive at harvest and processing (Franco et al., 2014; Gómez-Caravaca et al., 2016).

The technological variables of olive oil extraction have also been the subject of several studies in order to optimize the retention of biologically active compounds (such as phenolics, tocopherols, sterols, pigments, among others) that increase the nutritional value of olive oil and influence the sensory experience (García-Vico et al., 2017; Rodrigues et al., 2018; Visioli & Bernardini, 2011). It is also known that other parameters can cause changes in the composition of oils, including storage and distribution conditions, such as time, temperature, light and packaging (Gómez-Caravaca et al., 2016).

I.1.3. Nutritional value

Olive oil is a widely appreciated food due to its cultural influences, organoleptic characteristics and nutritional properties, being a key element of the Mediterranean diet. (Martínez-Zamora et al., 2021). Adherence to this diet is associated with a reduced risk of morbidity and mortality (Bach-Faig et al., 2011; Knoops et al., 2004; Serra-Majem et al., 2020; Servili et al., 2014) by reducing the incidence of chronic-degenerative diseases, including cardiovascular problems, type 2 diabetes and even some types of cancer (Estruch et al., 2013; Karković Marković et al., 2019).

Based on their composition, olive oil can be divided into two fractions, the first of which is the major fraction (98-99%), mainly composed of triacylglycerols and also of free fatty acids, monoglycerols and diglycerols (Jerman Klen, 2014). The fatty acids profile has a high content of monounsaturated fatty acids (MUFA), more abundant in oleic acid, which is related to the reduction of the oxidation of low-density lipoproteins

(LDL) (Medina & Tabernero, 2010; Rodrigues et al., 2021). In addition, the consumption of MUFA in place of saturated fats helps to maintain normal blood cholesterol levels (Regulation (EC) No 1924/2006).

The second fraction is minority (1-2%) and includes over 230 chemical compounds, like phytosterols, aliphatic and triterpenic alcohols, squalene, volatile compounds and antioxidants (tocopherols and polar phenols) (Servili et al., 2014). Many of these minor components, with emphasis on phenolic compounds, have important bioactive properties contributing to its nutritional value (Franco et al., 2014).

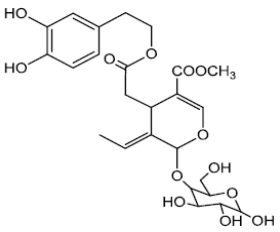
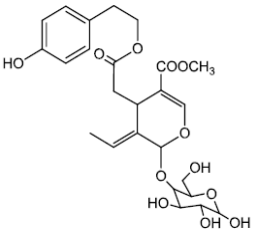
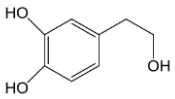
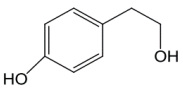
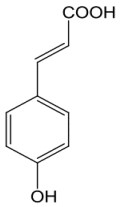
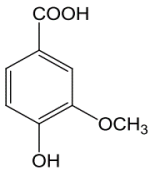
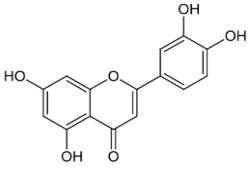
I.2. PHENOLICS COMPOUNDS

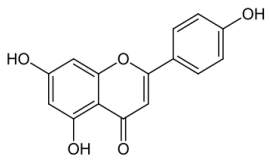
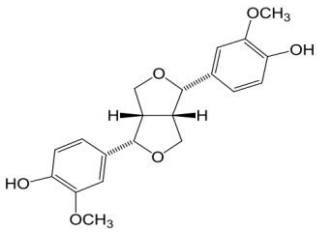
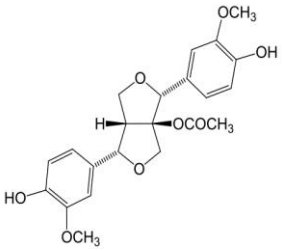
The hydrophilic phenols are secondary metabolites of plants, chemically defined as substances with an aromatic ring with one or more hydroxyl substituents. A second definition is related to metabolic origin, as substances derived from the shikimate pathway (responsible for the formation of phenylalanine and tyrosine aromatic amino acids) and from the phenylpropanoids metabolism (related of proteins production), being formed still in the plants (Ryan & Robards, 1998).

I.2.2. Classification and chemical structure

Polyphenols can be divided into several classes, depending on the number of phenolic rings and molecular binding elements. The most abundant classes of phenolic compounds in olives are represented in Table 1, and include secoiridoids (oleuropein and ligstroside derivatives), phenolic alcohols [Hydroxytyrosol (Htyr) and Tyrosol (Tyr)], phenolic acids, flavonoids and lignans (Montedoro et al., 1993; Purcaro et al., 2014).

Table 1. Chemical structure of representative phenolic compounds found in olives. (Source: adapted from Jerman Klen, 2014 and Romani et al., 2019).

Class	Phenolic compound	Molecular formula / CAS Number	Chemical structure
Secoiridoids	Oleuropein	$C_{25}H_{32}O_{13}$ / 32619-42-4	
	Ligstroside	$C_{25}H_{32}O_{12}$ / 35897-92-8	
Phenolic alcohols	Hydroxytyrosol	$C_8H_{10}O_3$ / 10597-60-1	
	Tyrosol	$C_8H_{10}O_2$ / 501-94-0	
Phenolic acids	*p*-Coumaric acid	$C_9H_8O_3$ / 501-98-4	
	Vanillic acid	$C_8H_8O_4$ / 121-34-6	
Flavonoids	Luteolin	$C_{15}H_{10}O_6$ / 491-70-3	

	Apigenin	$C_{15}H_{10}O_5$ / 520-36-5	
Lignans	Pinoresinol	$C_{20}H_{22}O_6$ / 487-36-5	
	Acetoxypinoresinol	$C_{22}H_{24}O_8$ / 81426-14-4	

Alagna et al. (2012) studied the metabolic and transcriptional profile of phenolic compounds during the ripening of olives, proposing the biosynthesis pathways of these molecules (Figure 1). These metabolic pathways are very complex, and the formation of phenolic compounds can change greatly depending on environmental stimuli (Ryan et al., 2002). In general, these molecules have interconnected metabolic pathways and, therefore, can be converted during fruit maturation, processing or storage (Jerman Klen, 2014). These pathways generally lead to the hydrolysis of compounds, converting them to Htyr or Tyr (Purcaro et al., 2014).

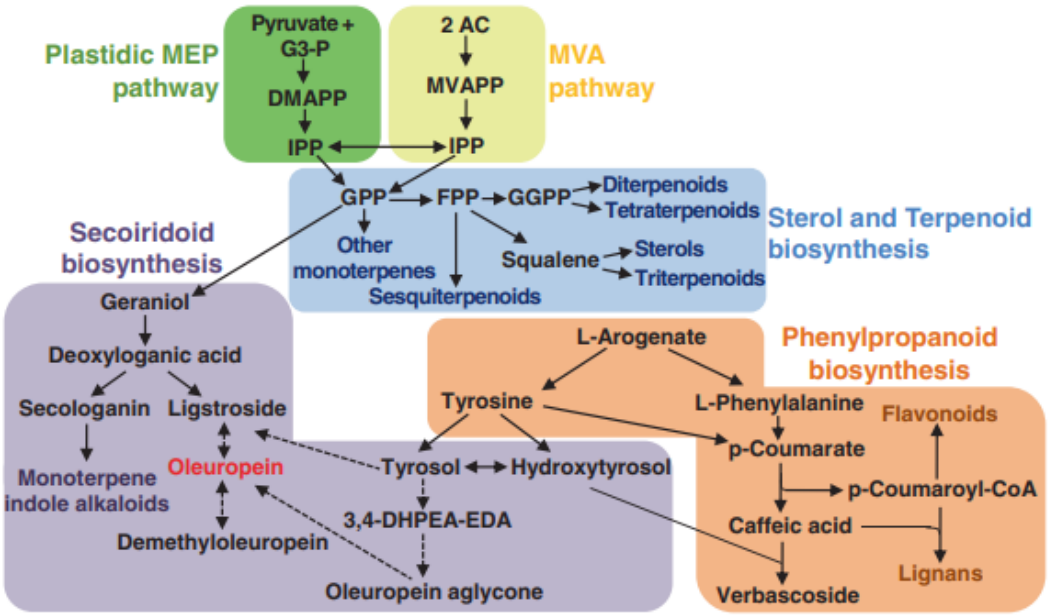


Figure 1. Schematic representation showing the production pathways of phenolic compounds in olive fruits. MEP: 2-C-methyl-d-erythritol 4-phosphate; G3P: Glyceraldehyde 3-phosphate; DMAPP: Dimethylallyl diphosphate; IPP: Isopentenyl diphosphate; MVA: Cytosolic mevalonate; AC: Acetyl-CoA; MVAPP: Mevalonate diphosphate; GPP: Geranyl diphosphate; FPP: Farnesyl diphosphate; GGPP: Geranyl geranyl pyrophosphate; 3,4-DHPEA-EDA: Oleacein. Dotted arrows indicate uncertain biosynthetic steps. (Source: Alagna et al., 2012).

In the process of olive oil production, some phenolic compounds are transferred in their original form, while others are transformed by various chemical and enzymatic reactions. This mainly depends on the nature of the compounds, the initial state of the fruit and the processing conditions and, as a consequence, the profile and amounts of phenolic compounds in olive oil can be highly variable (Herrera, 2007; Jerman Klen, 2014).

Oleuropein is the major phenolic compound in olive fruit, from the hydroxytirosol family. It is a compound linked to a sugar (glucose) and, during the fruit growth phase, its concentration reaches high levels. Ligstroside is the equivalent compounds with tyrosol moiety (Table 1). In virgin olive oil, oleuropein- and ligstroside-aglycones (without the sugar molecule) and their derivatives (mainly oxidation forms) appear in greater amounts, including their hydrolysis end products, Htyr and Tyr, respectively (Ryan et al., 2002; Vissers et al., 2002).

This can be explained by the fact that, during maturation, the activity of hydrolytic enzymes causes a decrease in oleuropein/ligstroside concentrations, while increasing the products of its hydrolysis (Figure 2) (Ryan et al., 2002; Tuck & Hayball, 2002). This change in the profile of phenolic compounds can also occur during olive oil storage, increasing the Htyr and Tyr concentration due to hydrolysis of the linked portions (Purcaro et al., 2014).

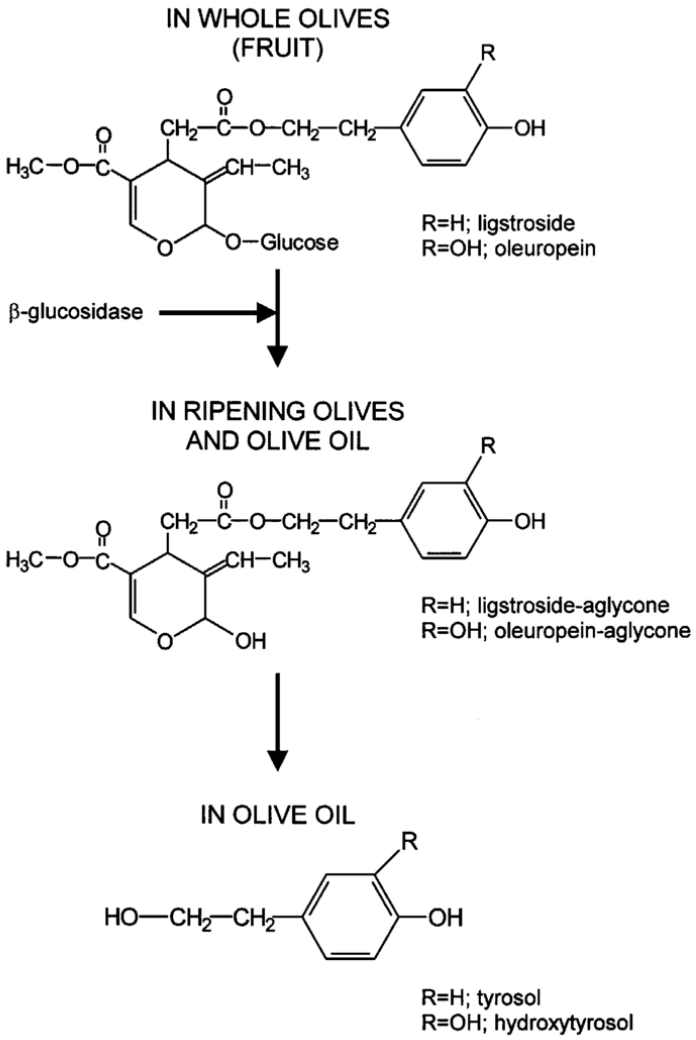


Figure 2. Structures of important phenols present in olives and olive oil, their degradation into aglycones during ripening, and hydrolysis of aglycones into hydroxytyrosol and tyrosol. (Source: Vissers et al., 2002).

I.2.3. Functions

Phenolics compounds may have several functions in plants, acting in plant growth, protection against photooxidation by ultraviolet (UV) light, fruit preservation (high oxidative stability and antimicrobial activity), as signaling molecules and on plant defense mechanism (Jerman Klen, 2014; Ryan & Robards, 1998).

In virgin olive oil, phenols contribute to its oxidative stability but also influence nutritional and organoleptic properties (Franco et al., 2014). Furthermore, these hydrophilic phenols are not found in other commercial oils and fats, unlike lipophilic phenols (Servili et al, 2014), due mostly to the fruit characteristics and losses during refining. In fact, the greatest interest in these compounds is related to their health properties, in particular the protective effects against neurodegenerative and cardiovascular diseases (Franco et al., 2014; Visioli & Bernardini, 2011). These properties stem from their ability to modulate certain cellular enzyme activities as hydrogen donators (AH), stopping oxidation chain reactions and oxidative damage caused by alkylperoxyl radicals ($ROO^{\bullet}$) formed at the beginning of lipid oxidation, resulting in a stable radical ($A^{\bullet}$) (Carrasco-Pancorbo et al., 2005; Jerman Klen, 2014; Lattanzio et al., 2006; Ryan & Robards, 1998), as shown in the reaction:

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

This interest in polyphenols from olive oil increased after the recognition by the European Food Safety Authority (EFSA) of the health claim for virgin olive oils containing at least 5 mg of Htyr and its derivatives (e.g. oleuropein complex and Tyr) per 20 g of olive oil, stating that “olive oil polyphenols contribute to the protection of blood lipids from oxidative stress” (Commission Regulation (EU) No 432/2012; EFSA, 2012; Rodrigues et al., 2019).

Htyr and Tyr (to a lesser extent) are thought to be responsible for most of the biochemical and pharmacological effects of olive polyphenols, both of which are absorbed in the intestine and can be detected in urine, plasma and LDL particles (Bonanome et al., 2000; Vissers et al., 2002). Although the metabolism and absorption mechanisms of phenols in the human body is still a little explored area, it is known that the precursor molecules, such as oleuropein, oleuropein-aglycone and oleacein for Htyr and listroside and oleocanthal for Tyr, are mostly hydrolyzed during gastrointestinal

digestion, generating Htyr and Tyr as a metabolite (Karković Marković et al., 2019; Serreli & Deiana, 2018).

I.3. ANALYTICAL METHODS FOR THE DETERMINATION OF PHENOLIC COMPOUNDS RELATED TO THE HEALTH CLAIM

Although there is a great interest in the quantification of phenolic compounds in olive oil to fulfill the health claim (Htyr and Tyr derivatives), there is no international regulation for the analysis of these compounds (Romero & Brenes, 2012). This is due mostly to the high complexity of this family of derivatives and absence of suitable commercial standards. Unfortunately, different results can be obtained from the type of methodology used (Tsimidou et al., 2019a).

Many procedures have been published and revised over the years, based on analysis by liquid chromatography, capillary electrophoresis and gas chromatography, mainly from the polar fraction of olive oil (Bendini et al., 2007; Cerretani et al., 2009; Gómez-Caravaca et al., 2015). Among them, the IOC proposed in 2009 the analysis of olive oil biophenols by isolating the polar fraction with methanol:water 80:20 (v/v) and performing the separation on high performance liquid chromatography (HPLC) (IOC, 2009). Quantification is achieved at 280 nm by summing the areas of chromatographic peaks and converting them into Tyr equivalents, using syringic acid as internal standard. However, these compounds show different responses in UV or diode array detectors (DAD), inducing different chromatographic proportions to the IS, not necessarily related to true mass amounts. Furthermore, in many cases several co-elutions may occur (e.g., between oxidized and non-oxidized products), in addition to poor resolution of secoiridoid aglycones (Romero & Brenes, 2012; Tsimidou et al., 2019a). Furthermore, this method ends up generating unreliable results that are far from the real concentration of Tyr and Htyr (Purcaro et al., 2014). These limitations have a negative effect on the commercial exploitation of the health claim (Tsimidou et al., 2019a).

In 2014, the IOC recognized that the existing analytical procedure was not adequate to the needs that have arisen with the new health claim (IOC, 2014). After that, publications with different approaches appeared, most of which sought to simplify the method through the hydrolysis of the bound forms of Htyr and Tyr, quantifying their total free forms (Romero & Brenes, 2012; Mastralexi et al., 2014; Purcaro et al., 2014; Ricciutelli et al., 2017; Bartella et al., 2018; Tsimidou et al., 2019b).

Still concerning the health claim related issues, one of the discussions raised was regarded to which compounds should be included in the calculation of phenolic compounds in olive oil. The question was scientifically answered by Tsimidou et al. (2018), which elucidates the need for consistent methods for the inclusion of all forms of Htyr and Tyr, as both have related biosynthesis pathways, and one can become the other in the human body.

An important factor is that the phenolic compounds profile can change during storage, as previously mentioned, increasing the Htyr and Tyr concentration due to hydrolysis of the linked portion (Purcaro et al., 2014). Thus, the analysis by hydrolysis of the bound forms and quantification of the free forms is the best way to avoid misinterpretations of the results, due to the change in the profile of phenolic compounds during storage (Purcaro et al., 2014). However, one cannot forget that after hydrolysis only a small portion of the original molecules is being quantified, and that under this direct approach the quantification will inevitably result in lower amounts than the ones that results from the mass quantification of the original molecules.

I.3.1. Total content of Htyr and Tyr after acid hydrolysis

Among the existing protocols in the literature, two are considered promising, from which several adaptations and variations emerged (Tsimidou et al, 2019b).

The first one was performed well before the emergence of the health claim by Mulinacci et al. (2006), who reported that only acid hydrolysis is able to release Htyr and Tyr without destroying them, unlike alkaline hydrolysis. In his protocol, the author first performs the extraction of the polar phase in the oils with ethanol:water 70:30 (v/v), water was acidified by formic acid (pH 2.5), followed by washing with hexane. Then, the hydrolysis of the bound forms is performed using H_2SO_4 1M, keeping the samples at 80ºC for 2h. Samples are diluted and filtered, then analyzed on HPLC/DAD at 280 nm. The eluents were formic acid (pH 3.2, A) and acetonitrile (B) with gradient application, in a total analysis time of 50 min.

Mastralexi et al. (2014) used the same hydrolysis and chromatographic separation procedure, but adapted the extraction solvents to methanol:water (60:40, v/v), approaching that suggested by the IOC (2009), namely methanol:water (80:20, v/v). Later, Tsimidou et al. (2019a) compared these two proportions of extraction solvents,

concluding that the one suggested by the IOC (2009) is superior in the application of the method.

In the second method, developed by Romero and Brenes (2012), the hydrolysis is performed directly on the oil, eliminating the pre-extraction step. For this purpose, the author kept the samples with 2M HCl for 6h under agitation and at room temperature. The extraction occurs after hydrolysis, with the filtering of the samples through a 0.22 μm pore size, and injected into the chromatograph. No washing of the extract with an apolar phase is performed. Separation was achieved in HPLC/DAD using an elution gradient with an initial composition of water:methanol solution (90:10, v/v) (water pH adjusted to 3.0 with phosphoric acid) until 100% methanol with 22 min total run.

Mastralexi et al. (2014) tested and compared procedures of Mulinacci et al. (2006) and Romero and Brenes (2012), concluding that both are adequate and effective. However, the authors considered the method of application of hydrolysis in the polar fraction to be of greater interest, rather than directly in the oil, since the first one includes the possibility of evaluating simultaneously the total phenolic profile before hydrolysis, according to the official method of the IOC (2009).

Tsimidou et al. (2019a) also compared both methods, concluding that the one proposed by Mulinacci et al. (2006) has a total sample preparation time 2.2-fold lower than that of Romero and Brenes (2012), as the latter includes 6 hours of hydrolysis. However, the method by Romero and Brenes (2012) ends up demanding fewer steps and less manpower, being considered even more simplified than the previous method.

I.3.2. Correction factors

When analyzing the hydrolyzed forms of phenolic compounds, the difference in molecular weights between the free and combined forms (i.e., between secoiridoids and their phenyl alcohols) should ideally be taken into account. However due to the inexistence of commercial standards, these calculations are not easy. Moreover, after hydrolysis, there is no possibility to know the specific molecules that originated the released phenolic acids, nor their proportion, making it virtually impossible to convert the phenolic alcohols mass into a true projection of the original biophenols amounts, the ones contributing to the health claim. Therefore, correction factors for Htyr (2.2) and Tyr (2.5) have been proposed to be applied (Mastralexi et al., 2014). These factors were obtained by dividing the average molecular mass of the best-known bound forms of Htyr and Tyr

(343 amu) and the molecular mass of Htyr (154 amu) and Tyr (138 amu). Correction factors are applied at the end of quantification, followed by the sum of Htyr and Tyr levels, resulting in a estimation of the polyphenol content to be considered for the health claim (Tsimidou et al., 2019b).

I.4. AIM OF THE WORK

Given the importance and obligation of analyzing the total content of Htyr and Tyr forms in olive oils as a parameter for evaluating their quality and the use of health claims, the aim of the present work is to internally validate a simple, high-throughput and reliable analytical method, including the free or combined forms of these compounds. In particular, we were concerned with the stability of the compounds over a extent hydrolysis period, including the internal standard, and the possibility to reduce this hydrolysis time to increase the method efficiency.

To achieve the general objective of the work, it was divided into specific objectives, namely:

1. Tentative optimization of the acid hydrolysis: evaluation of hydrolysis time, standard stability and phenolics conversion during hydrolysis;
2. Validation of the analytical method: evaluation of linearity, limits of detection (LOD) and quantification (LOQ), precision and accuracy;
3. Application of the validated method in analyses of olive oils in the region of Trás-os-Montes, in particular in the Côa Valley region, where this master's dissertation is included.

The development of this master's thesis was carried out at the Faculty of Pharmacy of the University of Porto. Consequently, the method implemented may, in the future, serve to complement several studies of virgin olive oil characteristics.

CHAPTER II.

Material and Methods

II.1. METHOD OPTIMIZATION, VALIDATION AND APPLICATION

II.1.1. Acid hydrolysis and extraction method optimization tests

First, the evaluation of hydrolysis time was performed to verify if the extraction method proposed by Romero and Brenes (2012) (using 2M HCl, at 25ºC for 6 h) is suitable for the purpose, particularly its completeness over time. For this, chromatographic analyses of an olive oil sample were achieved between 0 h and 7 h of hydrolysis, by analyzing, in triplicate, the released Htyr and Tyr hourly.

The second extraction test performed was the hydrolysis of an analytical standard of a secoiridoid (oleuropein), observing its conversion to the corresponding phenolic derivative (Htyr) over time and eventual losses in the conversion process. An oleuropein concentration equivalent to that of Htyr in olive oil samples was used, taking into account the differences in molecular weight.

Phenolic alcohols and internal standard stability were also evaluated under hydrolysis conditions. First, the stability of the internal standard was verified through tests containing only syringic acid. Hydrolysis and extraction were performed according to standard procedure (Romero and Brenes, 2012), and the chromatographic analysis was verified every hour (0 to 6 h), in triplicate.

Additionally, the stability of extracts after hydrolysis was also evaluated. For this, hydrolysis of the Htyr and Tyr standards was carried out at the concentrations used in the calibration curve, in triplicate. After the 6 h hydrolysis, the samples were injected immediately, and their concentration compared with equivalent standards without hydrolysis. Additionally, the solutions were reinjected after 24 h, kept in a refrigerated autosampler.

II.1.2. Validation of the analytical method

Validation of the analytical method was based on linearity, LOD, LOQ, precision and accuracy. For the calculation of LOD and LOQ, the method based on the standard deviation of a linear response and a slope (n = 8) was used. Method precision was calculated by intra- and inter-day assays, using one selected olive oil sample. The intra-day precision was determined by analyzing three replicates of the sample on the same day of analysis, and the test was repeated for a total of three non-consecutive days to calculate

the inter-day precision. The accuracy of the method was determined by spiking an olive oil sample at three levels (25, 50 and 100% of the expected values, in triplicate) with Htyr and Tyr standards.

II.1.3. Application of the validated method

After method validation, analyses of the 103 samples of oil from centenarian olive trees were performed in duplicate. To verify if the olive oils studied are suitable for the health claim, the results were also given in mg/20g of olive oil, before and after the application of the correction factor (2.2 for Htyr and 2.5 for Tyr).

II.2. REAGENTS AND STANDARDS

The eluents acetonitrile and methanol used in this study were HPLC grade (> 99% pure), and were obtained from Honeywell (North Carolina, USA). N-hexane and hydrochloric acid were analytical purity grade and were purchased from Carlo Erba Reagents (Val-de-Reuil, France) and VWR chemicals (Rosny-sous-Bois, France), respectively. Standards used were of the highest commercial purity available. Syringic acid, Htyr, Tyr and oleuropein were acquired from Sigma-Aldrich (St. Louis, USA).

II.3. SAMPLES

For the evaluation of hydrolysis time, a commercial extra virgin olive oil sample was used.

To carry out the analyses for method validation and application, 103 centenarian olive trees from the Côa Valley region (Northeast Portugal) were initially selected (Figure 3), with representative olive production in the 2020 harvest (Rodrigues et al., 2022). The olives were harvested according to the IOC guidelines (IOC, 2011), between two (MI 2) and three (MI 3) in the maturation index, and the fruits were processed in the first 24 h after harvest. The extraction of oils from the selected samples was carried out according to Rodrigues et al. (2022), in a pilot extraction plant with an Abencor analyzer (Spain) with three main units: a mill (MM100 from MC2., Spain), a thermobeater (Thermo-Mixer

TB-100 from MC2., Spain) and a centrifuge (Centrifugal Machine CF-100 from MC2., Spain) for solid-liquid separation.

Olives were milled and the paste was homogenized, and about 700 g was subjected to malaxation in the thermobeater unit (20 min), using a thermostatic water bath at 25 ºC. Then, the mixture was centrifuged and decanted, and the olive oil collected. After extractions, the obtained olive oils from the same tree were mixed in the same bottle. Once the extraction process was finished, the oils were prepared for analysis, and filtered (Whatman paper no. 4) over anhydrous sodium sulfate to remove the solid particles and residual water. The olive oil samples were stored in amber bottles in the dark, at room temperature, until the moment of analysis.

For the evaluation of accuracy, sample #70 was randomly selected among the 103 available.

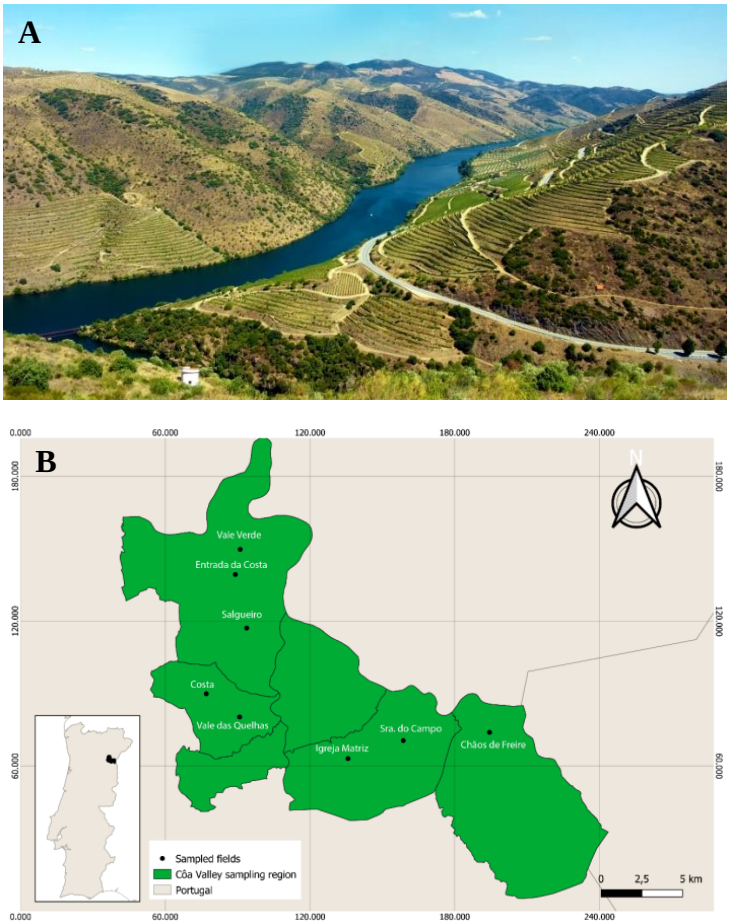


Figure 3. Côa Valley region in Northeast Portugal (A) and the selected sampling region (B). (Sources: **A-** https://www.agriterra.pt/Artigos/316435-Investigadores-vao-avaliar-o-impacto-das-alteracoes-climaticas-no-Coa.html, accessed on 09/26/2022; **B-** Rodrigues et al., 2022).

II.4. OLIVE OIL ACID HYDROLYSIS AND EXTRACTION

The method explored in this work was based on the one proposed by Romero and Brenes (2012), performed with some modifications. From each sample, 0.1 g were weighed into a plastic tube, 40 μL of syringic acid solution was added to act as internal standard (IS, 0.6 μg from a 0.15 mg/mL solution in methanol:water (80:20, v/v)). The hydrolysis initiated with the addition of 2.5 mL of a hydrochloric acid solution in methanol:water (80:20, v/v; 2 M), being the solution maintained at 25 ºC for 6 h of hydrolysis, under stirring (Multi RS-60, Latvia). Then, 2.5 mL of an acetonitrile:water solution (50:50, v/v) were added and further agitated during 30 sec in a vortex, followed by centrifugation for 3 min at 5,000 RPM (Labofuge Ae, Portugal). Then, 2 mL of this solution was washed with 2 mL of n-hexane for 30 s (vortex) for better removal of the remaining lipids, with phase separation aided by centrifugation at 5,000 RPM for 5 min. A portion of the lower phase (hydrophilic) was transferred to centrifuge tubes and centrifuged again at 13,000 RPM for 3 min. Finally, the supernatant was transferred to an injection vial and analyzed immediately.

II.5. HPLC ANALYSIS

Htyr and Tyr were separated by-reversed-phase HPLC with DAD detection (MD – 4010), using a Jasco integrated system (Japan) with a data transmitter (LC – NetII/ADC), a refrigerated auto-sampler (AS – 4050), two integrated pumps (PU –4180) and a column oven (ECOM Eco2000, Czech Republic). The chromatographic separation was achieved on a Gemini-NX C18 LC column (150 x 4.6 mm, 5 μm, 110 Å, Phenomenex) operating at 35 ºC under an isocratic system of water/acetonitrile (86:46, v/v), with 0.1% of formic acid, at a flow rate of 2 mL/min for 6 min. The injection volume used was 40 μL. The system was periodically cleaned for removal of non-polar retentions.

The compounds were identified by chromatographic comparisons with analytical standards and quantification was performed at 280 nm by the internal standard method, with individual calibration curves for Htyr and Tyr. The chromatographic results were expressed in mg of Htyr or Tyr per kg of olive oil, further converted to estimated phenols by application of the correction factors (2.2 for Htyr and 2.5 Tyr derivatives) according to Mastralexi et al. (2014).

II.6. STATISTICAL ANALYSIS

All treatments, tables and graphs were elaborated by Microsoft Office Excel 2016 software. The statistical analyses were performed using MASS package of the open-source statistical program R (version 4.1.0), at a 5% significance level. Normal distribution of the residuals and the homogeneity of variances were evaluated through the Shapiro-Wilk's and the Levene's test, respectively. Afterwards, the variables were studied using one-way analysis of variance (ANOVA). Moreover, if a statistically significant effect was verified, Tukey's test was also applied for means comparison.

CHAPTER III.

Results and Discussion

III.1. TENTATIVE OPTIMIZATION OF THE ACID HYDROLYSIS AND EXTRACTION METHOD

III.1.1. Evaluation of hydrolysis time

The analyses showed that the concentration of Htyr stabilizes after 6 hours of hydrolysis, while for Tyr, the maximum concentration is reached after 5 hours (Figure 4), considering the absence of significant statistical differences thereafter ($p > 0.05$). Therefore, the 6 h indicated by Romero and Brenes (2012) are indeed required to grant complete hydrolysis, and it was not possible to decrease the total hydrolysis time.

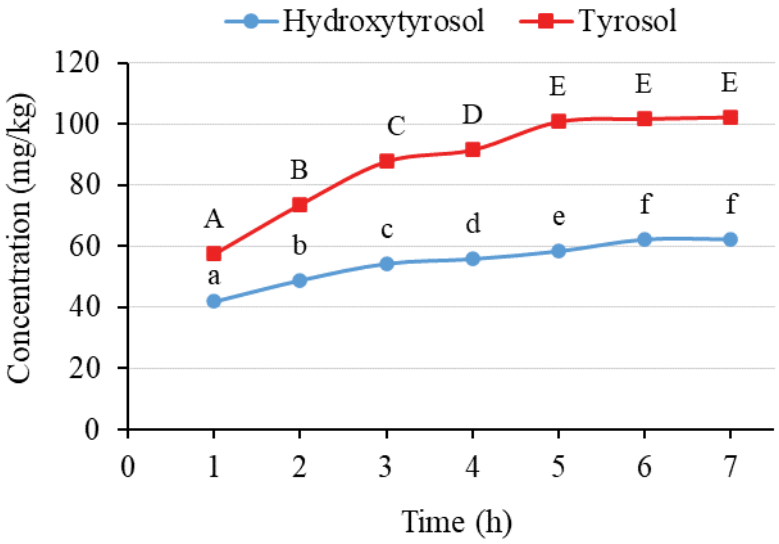


Figure 4. Hydroxytyrosol and tyrosol concentration over hydrolysis time in an olive oil sample. Different letters in each series show statistically significant differences ($p < 0.05$) from the given mean. Means were compared by Tukey HSD considering that the homogeneity of variances was confirmed by Levene's test.

III.1.2. Secoiridoids conversion during hydrolysis

In the second test performed, namely hydrolysis of an analytical standard of a secoiridoid (oleuropein), 100% of the oleuropein concentration at 0 h and 100% of the Htyr concentration at 6 h of hydrolysis were considered for the construction of the graph (Figure 5). The analysis was performed up to 6 h of hydrolysis, considering that this was the time previously validated (section III.1.1). The results demonstrate that the method

can truly perform the hydrolysis of phenolic compounds, with oleuropein disappearing after 6 hours of hydrolysis and with a recover of the expected Htyr amounts.

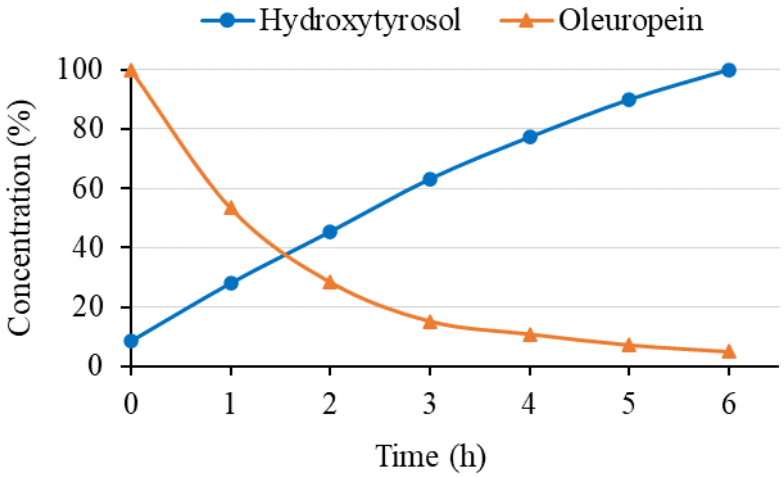


Figure 5. Hydrolysis of oleuropein and conversion to hydroxytyrosol over time using the proposed method.

III.1.3. Phenolic alcohols and internal standard stability

The internal standard content was compared in terms of chromatographic area with the initial time (0 h). The results showed that syringic acid undergoes a slight degradation over time, preserving 95.7% ± 3 of integrity after 6 hours of hydrolysis (Figure 6). Applying one-way ANOVA, a *p* value of 0.274 is obtained, which indicates that there are no significant differences between the concentration of the internal standard at the beginning and at the end of the hydrolysis.

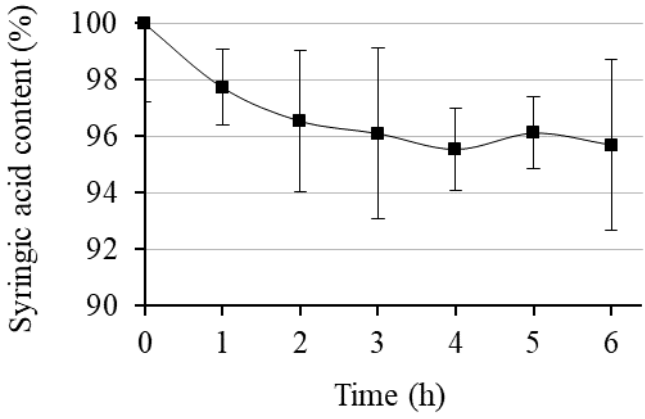


Figure 6. Internal standard stability over the hydrolysis time.

Sequentially, the evaluation of the stability of Htyr and Tyr in the extracts after hydrolysis was performed (Figure 7). Globally, no significant alteration was observed with the 6 h of hydrolysis for both compounds. However, a slight change in the slope of the Htyr calibration curve is observed after the 24 h period. This indicates that after hydrolysis, the extracts should be analyzed as soon as possible, so as not to influence the results. The implemented chromatographic method allows the extracts to be analyzed with only 6 min of run time, which allows the analysis of a large number of samples in a few hours.

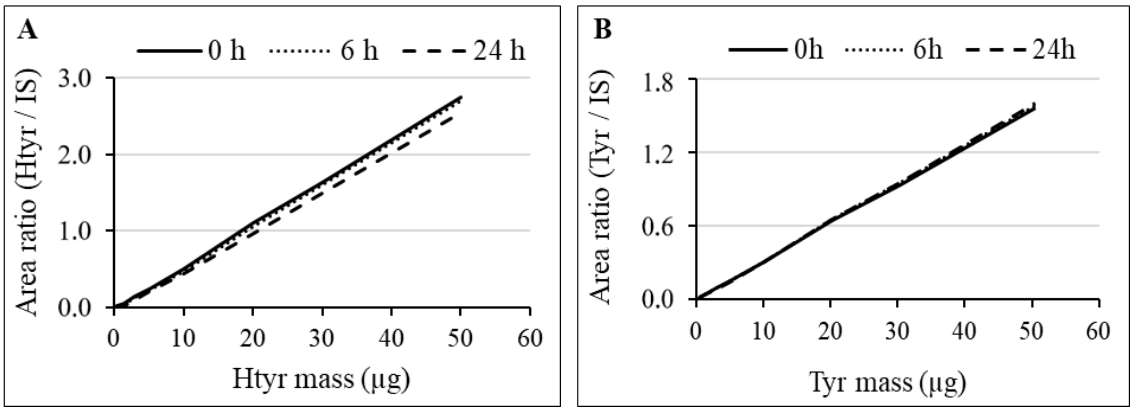


Figure 7. Extracts stability after hydrolysis using the standards hydroxytyrosol (A) and tyrosol (B). Htyr, Hydroxytyrosol; Tyr, Tyrosol; IS, Internal standard.

III.2. VALIDATION OF THE ANALYTICAL METHOD

Validation of the analytical method was based on linearity, LOD, LOQ, precision and accuracy. For Htyr and Tyr, eleven standard concentrations across the working range (Figure 8) were subjected to the complete hydrolysis/extraction and analysis procedure. A total of eight calibration curves were obtained through time, all with correlation coefficients greater than 0.999, confirming the method reliability (Table 2).

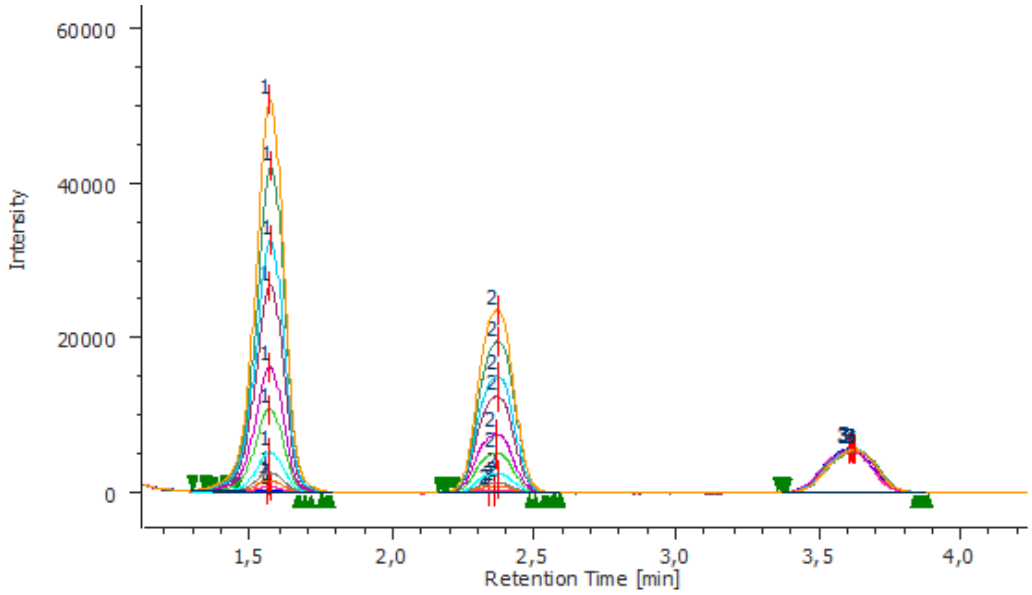


Figure 8. Chromatographic representation of a calibration curve (0.1 – 19.0 µg/mL) using the analytical method under validation. 1, Hydroxytyrosol; 2, Tyrosol; 3, Syringic acid (internal standard).

LOD is defined as "the lowest amount of analyte in the sample, which can be detected but not necessarily quantitated under stated experimental conditions", while LOQ can be quantitatively determined with suitable precision and accuracy (International Conference on Harmonization [ICH], 2005). In this work, a LOD/LOQ of 6.5/21.8 ng (8.2/27.3 mg/kg of olive oil) and 4.6/15.2 ng (5.7/19.0 mg/kg of olive oil) were achieved for Htyr and Tyr, respectively, for a 100 mg sample intake (Table 2).

The results obtained were very similar than those of Romero and Brenes (2012), which reached LOD/LOQ equivalents of 6.0/16.0 mg/kg for Htyr and 8.0/28.0 mg/kg for Tyr. The method applied in this work had some modifications in relation to that of Romero and Brenes (2012), particularly in the chromatographic method, replacing the elution gradient by an isocratic system, in addition to exchanging methanol for acetonitrile. Methanol causes the formation of gas in the system when the mobile phase is mixed. The switch to acetonitrile, together with the use of an isocratic system, resolved this issue. The modifications made the chromatographic method simple and fast, with a run time of only 6 min. The lows LOQ/LOD values achieved are able to detect and quantify minimal amounts of the compounds studied in olive oils, and also determine whether they are suitable for using the health claim.

Bellumori et al. (2019) found values between 14.5-445 mg/kg of Htyr and 55.5-362 mg/kg of Tyr among 64 samples of extra virgin olive oils. Tsimidou et al. (2019a) analyzed 30 different samples of virgin olive oils under different hydrolysis protocols, obtaining values between 8-365.5 mg/kg of olive oil for Htyr and 2.5-298 mg/kg of olive oils for Tyr (all results without correction factor). The results show that the content of phenolic compounds can be very variable depending on the olive oil sample, but the method chosen must be able to categorize it as fit for health claim. Thus, the LOD/LOQ values determined in this work are perfectly acceptable, and even samples with lower content of the compounds under analysis would still be quantifiable by the method to be validated.

Table 2. Method analytical parameters for hydroxytyrosol and tyrosol content in injected extract.

	Hydroxytyrosol	Tyrosol	Syringic acid
Retention time (min)	1.57	2.36	3.62
Retention time RSD (%, n = 11)	0.2	0.4	0.2
Correlation coefficient (r^2)	0.9999	0.9999	-
Slope	0.0581 ± 0.0002	0.0326 ± 0.0001	-
Working range (μg mL^{-1})	0.1 - 19.0	0.1 – 19.2	-
LOD (ng)	6.5	4.6	-
LOD (mg/kg)	8.2	5.7	-
LOQ (ng)	21.8	15.2	-
LOQ (mg/kg)	27.3	19.0	-

LOD, Limit of Detection; LOQ, Limit of Quantification; RSD, Relative Standard Deviation.

The results of precision obtained for both Htyr and Tyr (Table 3), and expressed as relative standard deviation (RSD), was below 1%, which demonstrates that our repeatability was comparatively lower (i.e. more sensitive) than Romero e Brenes (2012) (<3%).

Furthermore, the accuracy of the method showed a good recovery (~100%), as observed in Table 3, which were suitable considering the range 80–110% proposed by the Association of Official Agricultural Chemists (AOAC, 2016). Tsimidou et al. (2019b)

achieved the recovery of 117% for Htyr and 96.7% for Tyr, respectively, while Celano et al. (2018) reported recoveries between 98% for Htyr and 106% for Tyr.

Table 3. Method validation features of hydroxytyrosol and tyrosol total content.

Compounds	Precision		Accuracy (% ± SD, n = 3)		
	Intra-day RSD (%, n = 3)	Inter-day RSD (%, n = 3 × 3)	25%	50%	100%
Htyr	0.4	0.7	100.1 ± 0.1	99.9 ± 0.4	99.1 ± 0.3
Tyr	0.2	0.5	100.1 ± 0.2	100.3 ± 0.4	100.3 ± 0.2

Htyr, Hydroxytyrosol; Tyr, Tyrosol; RSD, Relative Standard Deviation; SD, Standard Deviation.

III.3. APPLICATION OF THE VALIDATED METHOD

The validated method was applied in the analysis of 103 samples of oil from olive trees in the region of Trás-os-Montes, in particular in the Côa Valley. Low standard deviations were obtained for each sample, supporting method reliability and applicability. The concentration of Htyr in the samples varied between 131 mg/kg and 470 mg/kg (average of 265 ± 60 mg/kg), and for Tyr between 34 mg/kg and 394 mg/kg (average of 139 ± 26 mg/kg). For the sum of the compounds, the total content was between 167 mg/kg and 773 mg/kg, without performing any conversions (Table 4). These results also reinforce the success obtained in the method validation, where LOD/LOQ values were obtained far below the total Htyr and Tyr content of the samples.

The values obtained were very close to the study performed by Rodrigues et al. (2019), who analyzed the phenolic composition of 28 ancient olive trees from Portugal during four consecutive seasons, finding values between 150 mg/kg and 810 mg/kg, through the sum of individual phenols. Bellumori et al. (2019) obtained results between 88 mg/kg and 778 mg/kg of italian olive oils. Garcia et al. (2003), found average values of 400 mg/kg when studying commercial virgin olive oils of the Picual, Hojiblanca, Arbequina and Cornicabra varieties, in the region of Spain. The amounts found in this work demonstrate that the olive oils studied are of great interest, due to the high content of phenolic compounds. It highlights the potential of the Côa Valley region, which is home to these olive trees that produce differentiated olives. This also underscores the importance of careful harvesting of olives and extraction of olive oils, which helps to preserve the phenolic characteristics.

Table 4. Total content of Htyr and Tyr (mg/kg of olive oil) in centenarian olive oil samples (average ± SD), applying the validation method.

Sample	Htyr	Tyr	Sample	Htyr	Tyr	Sample	Htyr	Tyr	Sample	Htyr	Tyr
1	221.9 ± 1.9	137.9 ± 0.9	**27**	466.4 ± 4.5	227.0 ± 2.1	**53**	287.9 ± 1.8	171.3 ± 0.7	**79**	217.7 ± 2.0	98.8 ± 0.8
2	291.6 ± 1.9	132.1 ± 1.1	**28**	310.3 ± 2.4	317.0 ± 2.7	**54**	288.8 ± 2.2	124.1 ± 0.0	**80**	265.7 ± 2.3	150.0 ± 0.3
3	170.1 ± 1.6	102.8 ± 0.7	**29**	321.7 ± 0.0	270.6 ± 0.0	**55**	240.5 ± 0.7	187.8 ± 1.8	**81**	203.0 ± 1.8	106.4 ± 0.5
4	213.8 ± 0.5	169.5 ± 1.5	**30**	153.7 ± 0.4	100.3 ± 0.0	**56**	360.1 ± 3.3	138.6 ± 0.2	**82**	282.4 ± 1.6	141.9 ± 0.5
5	235.9 ± 1.1	150.4 ± 1.0	**31**	240.2 ± 0.7	73.9 ± 0.6	**57**	176.3 ± 0.1	67.9 ± 0.5	**83**	247.9 ± 0.5	125.1 ± 0.6
6	249.5 ± 0.2	136.9 ± 0.9	**32**	181.3 ± 1.0	77.2 ± 0.2	**58**	384.4 ± 0.0	123.4 ± 1.9	**84**	251.6 ± 2.1	78.5 ± 0.8
7	164.7 ± 1.3	93.9 ± 0.4	**33**	304.3 ± 0.0	168.6 ± 1.0	**59**	227.2 ± 0.8	223.5 ± 1.1	**85**	230.1 ± 0.2	89.2 ± 0.1
8	233.4 ± 0.6	129.6 ± 0.9	**34**	280.7 ± 2.2	97.2 ± 0.2	**60**	249.7 ± 1.9	200.1 ± 1.3	**86**	233.7 ± 0.2	103.8 ± 0.1
9	243.4 ± 2.0	150.4 ± 0.2	**35**	325.0 ± 2.1	188.8 ± 0.2	**61**	259.9 ± 2.4	91.3 ± 0.7	**87**	192.1 ± 1.8	57.4 ± 0.1
10	311.3 ± 0.4	177.3 ± 1.4	**36**	301.8 ± 0.4	128.6 ± 0.2	**62**	210.5 ± 1.0	88.4 ± 0.2	**88**	256.0 ± 0.4	102.9 ± 0.4
11	229.4 ± 1.9	101.5 ± 0.8	**37**	263.2 ± 0.3	140.2 ± 1.2	**63**	190.1 ± 1.4	50.1 ± 0.0	**89**	247.9 ± 0.9	105.9 ± 0.2
12	194.0 ± 1.6	161.8 ± 0.2	**38**	359.8 ± 0.4	166.0 ± 1.3	**64**	355.9 ± 2.1	158.4 ± 0.1	**90**	299.7 ± 1.2	128.8 ± 0.3
13	196.9 ± 1.9	84.1 ± 0.5	**39**	236.7 ± 1.5	148.8 ± 0.5	**65**	295.9 ± 1.8	129.3 ± 0.9	**91**	131.2 ± 0.9	36.7 ± 0.3
14	275.7 ± 2.2	124.5 ± 0.9	**40**	396.5 ± 3.1	315.3 ± 0.8	**66**	264.4 ± 1.7	118.5 ± 1.0	**92**	252.3 ± 0.2	109.9 ± 0.5
15	207.9 ± 1.7	140.6 ± 1.1	**41**	226.1 ± 0.9	142.7 ± 0.2	**67**	237.4 ± 1.1	141.7 ± 0.5	**93**	286.8 ± 0.8	172.6 ± 0.1
16	161.8 ± 1.4	77.6 ± 0.4	**42**	242.1 ± 1.2	236.8 ± 1.3	**68**	265.9 ± 2.2	165.2 ± 1.0	**94**	310.8 ± 0.8	180.2 ± 0.8
17	373.9 ± 3.6	175.6 ± 1.7	**43**	271.3 ± 0.3	141.7 ± 0.7	**69**	284.1 ± 0.4	139.6 ± 0.1	**95**	219.1 ± 0.2	94.0 ± 0.4
18	340.8 ± 3.3	173.1 ± 0.3	**44**	234.1 ± 2.3	118.5 ± 0.9	**70**	156.9 ± 0.7	33.9 ± 0.1	**96**	255.2 ± 0.9	153.7 ± 0.0
19	325.0 ± 1.4	150.8 ± 0.8	**45**	297.2 ± 2.3	156.7 ± 0.1	**71**	243.2 ± 1.7	83.4 ± 0.4	**97**	253.8 ± 1.2	91.4 ± 0.5
20	214.0 ± 0.4	88.9 ± 0.6	**46**	387.3 ± 0.3	384.4 ± 1.1	**72**	222.5 ± 1.8	70.4 ± 0.5	**98**	215.8 ± 0.8	97.9 ± 0.0
21	268.7 ± 1.2	125.7 ± 0.1	**47**	283.2 ± 1.3	184.2 ± 0.9	**73**	198.7 ± 1.8	98.5 ± 0.8	**99**	309.3 ± 2.0	73.0 ± 0.1
22	348.3 ± 2.8	160.5 ± 1.0	**48**	319.5 ± 1.7	186.2 ± 1.3	**74**	289.8 ± 2.2	104.4 ± 0.3	**100**	233.0 ± 1.4	156.4 ± 0.7
23	264.0 ± 2.2	104.7 ± 0.1	**49**	308.9 ± 1.6	81.4 ± 0.5	**75**	324.6 ± 0.7	88.2 ± 0.1	**101**	265.6 ± 2.0	111.0 ± 0.3
24	322.5 ± 2.1	289.8 ± 1.9	**50**	297.5 ± 0.0	155.7 ± 0.2	**76**	279.1 ± 0.4	81.6 ± 0.3	**102**	258.5 ± 1.6	103.0 ± 0.5
25	317.6 ± 2.9	391.9 ± 2.2	**51**	304.6 ± 2.2	121.7 ± 0.6	**77**	308.1 ± 1.1	83.4 ± 0.6	**103**	309.2 ± 2.2	101.0 ± 0.6
26	358.9 ± 1.5	229.4 ± 1.1	**52**	211.2 ± 1.5	223.4 ± 2.0	**78**	240.3 ± 1.7	105.3 ± 0.8			

Htyr, Hydroxytyrosol; Tyr, Tyrosol; SD, Standard Deviation.

To verify if the olive oils studied are suitable for the health claim, the results were given in mg/20g of olive oil, before and after the application of the correction factor (2.2 for Htyr and 2.5 for Tyr) in Table 5. Even before the correction, only 4 of the 103 oils evaluated would be outside the limit of 5 mg/20 g of olive oil established for use of the health claim. After application of the correction, the total sum of phenolic compounds varies between 7.6 and 36.3 mg/20 g, which reinforces that the oils from the Côa Valley region are of great interest.

Table 5. Estimated total content of hydroxytyrosol and tyrosol (mg/20 g of olive oil) in centenarian olive oil samples (average ± SD), before and after the application of correction factors.

	Hydroxytyrosol	Tyrosol	Total	Total after correction
Range	2.6 - 9.4	0.7 - 7.9	3.3 - 15.5	7.6 - 36.3
Average ± SD	5.3 ± 1.2	2.8 ± 1.3	8.1 ± 2.2	18.6 ± 5.1

SD, Standard Deviation.

Despite the previous conclusions, analysis over the years is still necessary, considering the factors that influence olive production. The results obtained with this master's thesis will be integrated with the other results of the Olive Côa project, with the objective of valuing the centenarian olive trees in this region.

CHAPTER IV.

Conclusion

IV. CONCLUSION

Recognition by the EFSA of the health claim related to olive oil polyphenols gave rise to different methodologies for analyzing these compounds, with no international regulation for their quantification. This work was able to internally validate one of the analysis methodologies proposed in the literature for these compounds, applying it to Faculty of Pharmacy of the University of Porto.

The method was indeed an excellent choice for the analysis of the total content of Htyr and Tyr in virgin olive oils as it demonstrated good linearity over a wide range of work, very low limits, good precision and accuracy. The method adaptations brought even more added value, as it simplified the analysis and reduced the running time, increasing the high throughput.

In method application, the centenarian olive trees studied here proved to be of great interest, and the results obtained from this master's dissertation will be integrated with the other data obtained during the OLIVECOA project, with different analyses to be carried out during three consecutive years.

Finally, with the successful results obtained in the implementation, validation and application of the analytical method, it may, in the future, serves to complement several studies of virgin olive oil characteristics.

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