# NMR Spectroscopy: A Powerful Tool for the Analysis of Polyphenols in Extra Virgin Olive Oil

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## Abstract

Extra virgin olive oil (EVOO), a key component of the Mediterranean Diet, has aroused interest in recent years due to its health properties. Nuclear magnetic resonance (NMR) spectroscopy is an appropriate tool for the accurate quantification of minor compounds in complex food matrices, such as polyphenols in olive oil. Flavonoids, lignans, secoiridoids and phenolic acids and alcohols in EVOO have been identified and quantified by NMR. This review provides an overview of the major developments in the structural elucidation of polyphenol compounds in EVOO.

**Keywords:** flavonoids, lignans, secoiridoids, tyrosol, hydroxytyrosol, phenolic acids.

## INTRODUCTION

The interest in extra virgin olive oil (EVOO) is growing exponentially. Besides being a crucial component of the Mediterranean diet, appreciated for its organoleptic and nutritional attributes, there is growing evidence that EVOO has beneficial health effects. These are attributed mainly to its bioactive minor compounds, phenolic molecules [1,2,3].

Several classes of phenolic molecules (e.g., simple phenols, secoiridoids, lignans, and flavonoids) are responsible for the sensory and nutritional quality of olive oils while also having multiple pharmacological properties [4,5]. The total polyphenol content and profile of EVOO depend on many factors, including the olive cultivar, geographical origin, production and processing techniques, storage, as well as the age of the oil. While the total polyphenol content provides a quality measurement, identification of individual phenols can serve as a fingerprint to distinguish different EVOOs [6].

Nuclear magnetic resonance (NMR) spectroscopy is a potent tool for the qualitative and quantitative analysis of complex mixtures of small molecules in solution and has been used with great success in the field of food science in the last two decades [4,7]. NMR spectroscopy is particularly suitable for the investigation of complex matrices such as EVOO, and allows the quantification of minor components [8]. The qualitative and quantitative determination of the phenolic fraction by NMR have both been used to characterize EVOO varieties and geographical origin [9–12] as well as for sensory analysis [13]. The chromatographic analysis of a mixture of components requires calibration using standard compounds, many of which are unstable and/or not commercially available. In contrast, quantitative $^{1}$H Nuclear Magnetic Resonance (qHNMR) enables the quantification of

molecules in multicomponent solutions without the need for identical pure reference standards [14], and represents an alternative for accurate quantification of compounds in a complex food matrix [15].

## 1. LIGNANS AND FLAVONOIDS

NMR has proven to be a powerful tool to both identify and quantify lignans and flavonoids in EVOO samples. Despite their low concentration. These and other lignans and flavones identified in EVOO by NMR are shown in ***Figure 1***.

The lignans pinoresinol and 1-acetoxypinoresinol were identified in EVOO with a combination of GC-MS and semipreparative HPLC and NMR experiments using a Bruker AM-500 with $CDCl_3$ as the solvent. 1-Acetoxypinoresinol was identified on the basis of singlets at 1.703, 3.891, and 3.923 ppm, indicating the presence of an acetate ester and two aromatic methoxy groups. A product of deacetylation arising from alkaline hydrolysis showed a significant upfield shift for the carbon linked to the acetoxy group. Pinoresinol was identified due to its center of symmetry at C-2, and its stability to alkaline hydrolysis. The hydrogen from the heterocycle showed a chemical shift of 3.099 ppm, being a multiplet [16]. Similar results were reported in another study the same year, which also identified 1-acetoxypinoresinol and pinoresinol using MS, $^{1}H$ and $^{13}C$ spectra, without any hydrolysis [17]. These findings were confirmed by Pérez *et al.* (2010), who identified pinoresinol by simple $^{1}H$ experiments [18].

Syringaresinol was identified in EVOO by means of HPLC-SPE-NMR (Bruker Avance-600, $CH_3CN$). Syringaresinol showed a singlet at 3.81 ppm corresponding to twelve hydrogens from the four methoxy groups. In the same work, two flavones, luteolin and apigenin, were also determined. Apigenin showed one more aromatic proton than luteolin, with a duplet at 6.96 ppm corresponding to two identical protons [19].

NMR has been used to differentiate EVOOs according to the harvest year, cultivar and geographical origin. The markers with the greatest discriminating power include luteolin, apigenin, pinoresinol, 1-acetoxypinoresinol and syringaresinol [9]. This technique also proved useful to distinguish between EVOO, olive pomace and olive paste extracts, which have different concentrations of lignans and other phenolic compounds [20].

NMR experiments have effectively quantified lignans and flavonoids. The exact amount of pinoresinol and 1-acetoxypinoresinol extracted by preparative HPLC from EVOO was measured by comparing their signals with those of tyrosol [21]. A quantitative method based on $^{31}P$ NMR was developed and validated, involving the extraction and derivatization of polyphenols from EVOO before analysis. The derivatization is based on the quantitative reaction of the labile hydrogens of the phenolic hydroxyl and carboxyl groups with the phosphitylating reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane. The chemical shifts for each analyzed lignan and flavonoid (Bruker AMX500, $CDCl_3$) are summarized in ***Table 1*** [22]. The same group found this analytical method to be more precise than the usual chromatographic method [23]. They also developed an $^1H$ NMR analytical method to analyze the phenolic fraction using the oil extract without previous derivatization. The characteristic chemical shifts of lignans and flavonoids and their multiplicity (Bruker AMX500, DMSO-$d_6$) are summarized in ***Table 1*** [24].

MaxQ NMR can provide information on the qualitative and semi-quantitative polyphenol profile of EVOO. Characteristic chemical shifts and their maximum quantum yields (Bruker Avance III 500, $CD_3OD$) are shown in ***Table 1*** [25] . NMR is also a powerful tool for analyzing olive oil phenols without extraction or derivatization. Simple $^1H$ NMR with suppression of the strong lipid signals was used to quantify the phenols pinoresinol and 1-acetoxypinoresinol, using a Bruker Avance 400 spectrometer and $CDCl_3$ as the solvent. Their characteristic signals are also shown in ***Table 1*** [26].

## 2. SECOIRIDOIDS

Secoiridoids (SEC) are the most challenging phenolic compounds to quantify, mainly due to the formation of artificial peaks (SEC isomeric forms, acetals or hemiacetals) and/or broadened peaks [27]. Thus, derivatization reactions are frequently necessary [11,28,29]. The SEC group includes tyrosol (TY), hydroxytyrosol (OH-TY) and their derivatives (***Figure 2***).

### *2.1. Tyrosol and derivatives*

EVOO contains free TY and several well-known esterified derivatives of this compound, such as oleocanthal (OLC), the monoaldehydic form of ligstroside aglycone (*p*-HPEA-EA); oleokoronal, the stable enolic form of ligstroside aglycone; and the isomers 5*S*,4*R*- and 5*S*,4*S*- ligstrodials associated with ligstral, which constitutes the closed ring monaldehydic form of the ligstroside aglycone [26,30–32]. Ligstroside glycoside is almost completely absent in EVOO as it undergoes hydrolysis during EVOO extraction and storage, resulting in the ligstroside aglycone [19].

In 2012, Karkoula and colleagues [28] developed a method for the direct measurement of OLC levels in commercial EVOO by quantitative $^1$H NMR in $CDCl_3$ at 600 MHz. This involved the integration of doublets at 9.23 ppm, corresponding to the aldehydic protons, and using syringaldehyde as the internal standard. The same authors performed an NMR study using deuterated solvents and found that both oleacein (OLE) and OLC react with water and methanol to give hemiacetals and acetals. A spectrum corresponding to a single molecule was only produced when the solvents used were pure $CDCl_3$, *d*-ACN and DMSO, $CDCl_3$ being the best choice, as it does not overlap with the aldehydic protons [28]. Two years later this methodology was successfully used to quantify the ligstroside aglycone in monovarietal Greek and Californian EVOO by integrating doublets at 9.49 ppm [11]. The technique was recently applied to compare the OLC content of EVOO produced from Hojiblanca olives cultivated under organic and conventional systems [33].

NMR quantification of key trace analytes in a complex matrix can be difficult or even impossible when using the classic broad band excitation, due to the masking effect of very strong signals. These restrictions can be overcome by suppressing selected lipid signals. This multisuppression approach, allowing the detection of minor components along the entire spectrum (NOESYGPPS), [26] was employed to study minor components in monovarietal commercial Spanish EVOO (Arbequina, Arroniz, Cornicabra, Hojiblanca and Picual) with $CDCl_3$ at 400 MHz. The results showed the presence of TY (doublets at 6.780 and 7.065 ppm corresponding to the presence of aromatic protons at C-7 and C-4, respectively), OLC (a doublet at 9.223 ppm and a singlet at 9.640 ppm corresponding to C-1 and C-3, respectively) and ligstroside aglycone at 9.499 ppm (***Table 3***) [26]. Reported for oleokoronal were a doublet of doublets at 7.386 ppm (due to the proton in C-3 adjacent to a hydroxyl group), a doublet at 11.764 ppm (due to the proton of this hydroxyl group) and a doublet at 9.225 ppm (the aldehydic proton) (***Table 3***) [26]. In addition, the aldehydic proton in C-3 of 5*S*,4*R*- and 5*S*,4*S*-ligstrodial gave a doublet at 9.680 and 9.452 ppm, respectively (***Table 3***) [26]. This technique has also been used to predict the geographical origin of selected Italian and Greek olive oils [34] and to characterize Italian EVOO [10,35].

A new quantitative method involves selective excitation with a double pulsed field gradient "perfect echo" (SELDPFGPE) sequence, [36] consisting of selective experiments with pulses that only excite the region containing the minor analytes of interest, while excluding the spectral regions containing strong lipid signals [26]. An advantage of this method is the ultra-rapid screening (three minutes) of target compounds without any extraction, separation or derivatization [36]. In a recent study, the quantification of OLC and ligstroside aglycone of EVOO samples from all the major international brands available in supermarkets in California was achieved by the integration of the doublets at 9.23 ppm and at 9.49 ppm, respectively, in $CDCl_3$ at 600 MHz [36]. In another study, the enhanced detection of aldehydes in EVOO by means of band selective NMR spectroscopy allowed

the assignment of the two characteristic aldehydes of OLC by extending the DPFGSE technique to the 2D-HSQC experiments, in $CDCl_3$ at 500 MHz. The first aldehyde at 9.62 ppm was coupled with two aliphatic hydrogen atoms (at 2.97 and 2.73 ppm) and the second was in the region of 9.30-9.15 ppm, with aldehydic resonances showing very strong correlations with a single CH alkene signal (6.60 ppm) [7]. These signals were used for the quantification of OLC and ligstroside aglycone in Italian EVOO [37]. The selective excitation pulse NMR analysis was used recently to describe oleokoronal, the enol form of the ligstroside aglycone present in EVOO and therefore not an artifact [30]. In the same study, the aldehydic protons in C-1 of 5*S*,4*R*- and 5*S*,4*S*-ligstrodial showed a doublet at 9.212 and 9.218 ppm, respectively.

*2.2. Hydroxytyrosol and derivatives*

The free form of OH-TY as well as the acetate and esterified forms have been identified in EVOO [19]. Among the esterified derivatives (also known as SEC), oleuropein aglycone (OA), aldehydic oleuropein aglycone (AOA) and OLE are the most representative. Oleuropein glycoside (OG) is almost completely absent in EVOO because of its high solubility in water and its enzymatic hydrolysis during olive oil extraction [19].

Montedoro *et al.* (1993) were the first to apply NMR to identify the phenolic fraction of EVOO, resulting in the discovery of OLE [38]. Subsequently, OH-TY and OLE have been detected using $^{1}H$ NMR at 500.1 MHz and $^{13}C$ NMR at 125.7 MHz using the solvents DMSO-$d_6$ [24], $CD_3OD$ and $CDCl_3$ [16,20] (***Table 2, 3***). OG was detected using $CD_3OD$ as the solvent [16]. The isomer 5*S*,8*R*,9*R*-AOA was identified applying the same MHz values and $CD_3CN$ and performing NOE experiments [18]. In a study by Stella *et al.* (2005), operating at 600.13 MHz and using $CH_3CN$ as the solvent, two isomers of AOA were assigned by TOCSY (5*S*,8*R*,9*S* and 5*S*,8*S*,9*S*), as were the hemiacetalic forms of oleacein and elenolic acid derivatives (dialdehydic form of elenolic acid lacking a carboxymethyl, 5*S*,8*R*,9*S* and 5*S*,8*S*,9*S*

elenolic acid and methyl ester of the dialdehydic form of elenolic acid lacking a carboxymethyl group) [19]. In addition, Apostolis *et al.* (2017) identified the 5*S*,8*R*,9*S* and 5*S*,8*S*,9*S* isomers of elenolic acid, elenolic acid methylester, elenolic acid ethylester and AOA at 600 MHz in $CDCl_3$ [39] and a year later, the oleaceinic acid and an acidic derivative of the dialdehydic form of decarboxymethyl elenolic acid were identified using $^1H$ and $^{13}C$ NMR (1D and 2D) [40]. An $^1H$ NMR multisupression experiment was conducted without any prior extraction of the phenolic fraction, leading to the detection of OH-TY, OLE, AOA and the isomers 5*S*,4*R* and 5*S*,4*S*-oleuropeindial (400 MHz, $CDCl_3$). Furthermore, the authors identified specific signals of a recently described EVOO component, oleomissional (the stable enolic form of OA) (H-3 dd at 7.36 ppm and OH d 11.78 ppm) (***Table 3***) [26]. OH-TY, OH-TY acetate, OA and AOA have also been detected using MaxQ NMR at 500 MHz in a mixture of $(CD_3)_2SO$:$D_2O$:$CDCl_3$ (3:2:1) [25,41].

Other specific SEC have been identified: two oleuropeindials and an oleuropein-enolic form ($^1H$ and $^{13}C$ NMR at 300.13 and 75.42 MHz, respectively, using $CDCl_3$ as the solvent) [42] and 4-(acetoxyethyl)-1,2-dihydroxybenzene ($^1H$ and $^{13}C$ NMR at 300 and 75.4 MHz, respectively, using DMSO-$d_6$ as the solvent) (***Table 3***) [43].

The integration of the $^1H$ signals allows the quantification of the phenolic compounds. The signals used are: from 6.40 to 6.54 ppm for the total amount of OH-TY [24], and 9.19 ppm for OLE, 9.50 ppm for OA ($^1H$ NMR at 600 MHz in $CDCl_3$) [11,12,28,44].

## 3. PHENOLIC ACIDS AND ALCOHOLS

Only a few phenolic acids and alcohols have been identified and/or quantified by NMR (***Table 4***). Homovanillyl alcohol, vanillic acid, *p*-coumaric acid and vanillin (***Figure 3***) were quantified by $^{31}P$ NMR (202.2 MHz, $CDCl_3$) [19,24]. The signals used were 137.80 ppm for *p*-coumaric acid and 135.15 ppm for vanillic acid [9,22,23].

$^{1}$H NMR was also used to quantify phenolic alcohols and acids. The signals used are: 9.80 ppm for vanillin, 6.31 ppm for p-coumaric acid, and 3.76 ppm for homovanillyl alcohol [24]. Moreover, a $^{31}$P NMR method was first validated by Stella *et al.* (2001) using polyphenol model compounds of hydroxycinnamic and hydroxybenzoic acid derivatives [45].

## 4. POLYPHENOL FINGERPRINTING IN EVOO

Direct NMR approaches, although technically easy to setup and perform, can run into obstacles in the study of complex mixtures, mostly stemming from spectral overlaps. By increasing the spectral resolution, multidimensional correlation NMR experiments significantly help the spectral analysis of complex mixtures [46].

Stefano Caldarelli reported in 2011 an NMR protocol based on multiple-quantum spectroscopy for an analytical screening of phenolic molecules [41] and in 2018, they applied this technique to a three Italian EVOO with variable total polyphenol content [25]. The approach used could be easily extended for rapid qualitative and semi-quantitative screening of the polyphenol composition in many food products.

Advanced NMR methods, often based on sophisticated multidimensional (ND) schemes [47], have been developed to overcome the issues of signal overlap and high spectral density, which limit the applicability of 1D NMR for mixture analysis. A particularly useful method in the case of aromatic compounds is MaxQ NMR, based on multiple-quantum (MQ) NMR [48,49]. MaxQ NMR relies on the encoding of multiple-quantum coherences. Specifically, when the highest-quantum coherence (the MaxQ) present in a given spin system is encoded, the corresponding peak in the indirect dimension is reduced to a single line that resonates at a position given by the sum of the 1Q chemical shifts of the spins participating in the MaxQ. This radically simplifies the multiple-quantum dimension, allowing a differentiated identification of the compounds in the mixture [50]. Focusing on the signal

of the six-membered aromatic rings, a series of spectra ranging from 5Q to 3Q allowed similarities and differences in the polyphenol content to be determined [25]. This approach is easy to implement, reasonably fast, and sensitive. Most importantly, it does not require a physical separation of the polyphenols. It can be anticipated that the information obtained by this technique could be applied to the study of olive oil aging or adulteration, by monitoring temporal charges of specific polyphenols as a function of chemical and biological processes. MaxQ NMR databases could be easily built to further expand the applicability of this method for polyphenol analysis in various other mixtures of natural origin.

The combination of HPLC with NMR spectroscopy allows the complete assignment and structure determination of analytes in complex mixtures. It therefore constitutes an important technique for new compound identification both in food science and natural product analysis [51,52]. A novel hyphenated HPLC-DAD-SPE-NMR/MS technique led to the identification of 25 compounds of a monovarietal EVOO from *Olea europaea L.* var. Cornezuelo [18]. Standard $^{1}$H and 2D COSY spectra were acquired to confirm the structure of all the compounds. Among the 25 compounds, mainly simple phenols (OH-TY, TY, vanillin, *p*-coumaric acid, elenolic acid and, and OH-TY acetate), flavonoids (luteolin and apigenin), the lignan pinoresinol, and a large number of secoiridoids (OA, ligstroside aglycones, and AOA among others) were elucidated. The novelty of this work was the differentiation between three diastereoisomers of the AOA, assigned as AOA (5*S*,8*R*,9*S*), AOA (5*S*,8*S*,9*S*), and AOA (5*S*,8*R*,9*R*). The ESI-MS spectrum showed two peaks at *m/z* 377.2 $[M-H]^-$ and 755.2 $[2M-H]^-$ attributed to one or more isomers of OA. The use of 2D $^{1}$H-$^{1}$H COSY and $^{1}$H-$^{13}$C HSQC/HMBC experiments allowed the complete $^{1}$H and $^{13}$C chemical shift assignments of AOA diastereoisomers. NOE experiments were also performed, together with theoretical calculations of the minimum energetic conformation of the diastereoisomeric forms of AOA. A Monte Carlo molecular mechanics conformational search was performed for the eight possible diastereoisomeric

forms of AOA and allowed the structure of the three diastereoisomers of AOA to be corroborated. Although (5*S*,8*R*,9*S*)-AOA and (5*S*,8*S*,9*S*)-AOA had been previously reported in the leaves of *O. europaea* [24] and in olive oil [19], it was the first description of (5*S*,8*R*,9*R*)-AOA, made possible by the hyphenated HPLC-DAD-SPE-NMR/MS technique.

## CONCLUSIONS

Structural elucidation by NMR spectroscopy is a constantly evolving discipline, and in this review, we have attempted to provide an overview of the recent developments in its application to polyphenol compounds in EVOO. The examples provided demonstrate the tremendous potential of NMR techniques as an analytical method for multicomponent mixtures. To date, NMR techniques have contributed considerably to the identification of numerous structures in EVOO samples, whereas the next-generation NMR technology is already developing in several directions, including the improvement of stereochemical analysis.

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## FIGURE LEGENDS

***Figure 1.*** Chemical structures of lignans and flavonoids from EVOO

***Figure 2.*** Chemical structures of the most representative secoiridoids of EVOO

***Figure 3.*** Chemical structures of representative phenolic alcohols and acids in EVOO

***Table 1.*** $^{31}P$, $^{1}H$ and MaxQ NMR characteristic chemical shifts for flavonoids and lignans

***Table 2.*** $^{13}C$ NMR characteristic chemical shifts for secoiridoids

***Table 3.*** $^{1}H$ NMR characteristic chemical shifts for secoiridoids

***Table 4.*** $^{1}H$ NMR characteristic chemical shifts for phenolic alcohols and acids