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A NEW ULTRA RAPID SCREENING METHOD FOR OLIVE OIL HEALTH CLAIM EVALUATION USING SELECTIVE PULSE NMR SPECTROSCOPY

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1 INTRODUCTION

The traditional Mediterranean diet, which is continuously attracting the interest of the scientific community for its health protecting activities, is based on the daily consumption of olive oil as the major source of lipids.[1-3] One of the most important class of constituents of olive oil are the secoiridoid polyphenolic derivatives which present an increasing potential for health protection.[4,5] The European Union legislation based on the scientific opinion of EFSA[6] has permitted specific health claims related to the levels of specific phenolic compounds found in olive oil (5 mg per 20 g dose or 250 mg/Kg). The key compounds that are responsible for the recognized health claim "protection of blood lipids from oxidative stress" are hydroxytyrosol, tyrosol and their derivatives. For this reason the accurate measurement of the levels of those compounds in olive oil is very important. As of today there is no officially adopted method for their measurement because of well known technical difficulties. Hydroxytyrosol and tyrosol are found in olive oil mainly as the following esterified derivatives: oleacein (3,4-DHPEA–EDA), monoaldehydic form of oleuropein aglycon (3,4-DHPEA-EA), oleocanthal (p-HPEA-EDA) and the monoaldehydic form of ligstroside aglycon (p-HPEA-EA) which possess significant biological activities, as previously summarized.[7,8] There are several works concerning the chromatographic analysis of those compounds (HPLC-UV or LCMS)[9-12] but their accuracy is questionable because as we have recently described[7] oleocanthal and oleacein react with methanol and water which are commonly and officially[13] used for the extraction of polyphenols and as constituents of the mobile phase during their analysis, leading to the formation of several artifacts and making the analysis very difficult. To overcome these problems that make the chromatographic analysis complicated and questionable we recently developed a simple and rapid method using quantitative NMR (qNMR) including a simple extraction step to increase the concentration of the analytes and reduce the bulk lipid matrix.[8]

Nuclear Magnetic Resonance (NMR) spectroscopy is well suited for quantitative analyses of complex chemical mixtures. 1D ^{1}H NMR typically provides the highest sensitivity with excellent linear response to component concentrations. Quantitation of key trace analytes in the presence of very strong signals from the bulk matrix can however be problematic or even impossible, depending on concentration, using typical broad band excitation. This is due to dynamic range limitations of the analog to digital converter

(ADC), especially on older instruments. These limitations can be overcome by the use of band selective shaped pulses to excite only the region containing the minor analytes while excluding the regions containing strong matrix signals which would exceed the ADC range. Region selective 1D ^{1}H-NMR methods for quantitation of aldehydes in honey and terpenes in olive oil have been described.[14] These utilize double pulsed field gradient spin echo (DPFGSE) sequences with band-selective refocusing pulses. In cases where the selectively excited region contains two or more J-coupled spins, significant anti-phase magnetization can occur, reducing the integrated signal intensities of these relative to uncoupled spins. This coupling evolution can be removed by utilizing the recently reported "perfect echo" (PE) sequence.[15]

In this framework, we envisaged the utilization of selective excitation with a double pulsed field gradient perfect echo method (SELDPFGPE) to analyze the aldehydes in olive oil without the need for concentration of these analytes. The method was developed targeting all the four major secoiridoid derivatives of hydroxytyrosol and tyrosol.

An analysis of the aldehyde region in Sicilian extra virgin olive oils utilizing DPFGSE has been reported,[16] although the authors did not identify oleocanthal, oleacein, or the aglycons of oleuropein and ligstroside as the observed components nor were the components quantitated.

The developed method in combination with the previously reported qNMR method[8] was applied to the study of 100 commercial olive oil samples from all the major brands available in supermarkets in California offering a good estimation of the average levels of the secoiridoid aldehydes that are available to the consumers. The varieties presenting the highest concentrations of the studied compounds were recognized and presented herein.

2 MATERIALS AND METHODS

2.1 Extra Virgin Olive Oil samples

The commercial extra virgin olive oil samples used in the study were obtained from olives (*Olea europaea* L.). The samples were purchased from major super markets in the San Francisco and Sacramento area in November 2013. The samples were bottled in 2013 and were coming from the 2012-2013 harvest season. 40 samples were produced in California, 26 in Italy, 11 in Greece, 7 in Spain, 1 in Morocco, 1 in Argentina, 1 in Chile, 1 in France and 12 were labelled as Mediterranean mixtures.

2.2 Reference Compounds

Oleocanthal, oleacein, oleuropein aglycon and ligstroside aglycon were isolated from an olive oil extract as previously described[7,8] and their purity was >98%. Syringaldehyde (98% purity) used as internal standard (IS) was purchased from Sigma–Aldrich (Steinheim, Germany). IS solution for extracted oil was prepared in acetonitrile at a concentration of 0.5 mg/mL and kept in refrigerator. Prior to use the IS solution was left to come to room temperature. All NMR solvents used throughout the experiments were obtained by Sigma–Aldrich.

2.3 NMR Analysis of Olive Oil without Extraction

225 mg olive oil (ca 250 μL) were mixed with 500 μL $CDCl_3$ containing syringaldehyde as internal standard (50 μg/mL) and transferred to a 5mm NMR tube. The DPFGPE sequence

was executed utilizing a 2.6 ms 180 degree reburp selective refocusing pulse, affording a 2400 Hz excitation window from 11 to 7 ppm. Data from 16 scans were collected for a total experiment time of 3 min. The spectra were phase corrected automatically using TopSpin software (Bruker). Accurate integration was performed manually for the peaks of interest. The experiments were performed using a Bruker Avance 600 MHz with cryoprobe.

2.4 Calibration Curves and Quantitation

Calibration curves were prepared by addition of known quantities of oleocanthal, oleacein, oleuropein aglycon or ligstroside aglycon to a selected commercial olive oil that was found to be free of all secoiridoid derivatives and following the above described measurement method. The standard compounds were dissolved in $CDCl_3$ containing I.S. and then mixed with the oil. The quantitation was based on the integration ratio between the aldehydic proton signal of syringaldehyde at 9.81 ppm and the aldehydic protons of oleocanthal at 9.23 ppm, oleacein at 9.21 ppm, oleuropein aglycon at 9.50 ppm and ligstroside aglycon at 9.49 ppm.

2.4.1 Standard and spiked solutions. Stock standard solutions of oleocanthal, oleacein, oleuropein aglycon and ligstroside aglycon were prepared in $CDCl_3$ at the 3 mg/mL level and were kept in refrigeration. Prior to use the stock solution were let to come to room temperature. Spiked olive oil samples were prepared to give concentrations of each analyte at 5, 20, 75, 150 and 300 mg/Kg by mixing appropriate volumes of the stock standard solutions with 225 mg of olive oil and $CDCl_3$ containing I.S. The mixture was homogenized using a vortex mixer for 30 sec and then transferred to a 5 mm tube for NMR analysis.

2.5 Method Validation

The method was checked for the linearity, accuracy [evaluated as the relative percentage error % (Er%), defined as (assayed concentration−nominal concentration)/(nominal concentration)×100], sensitivity [evaluated as the limits of Detection (LOD) and Quantitation (LOQ)].

*2.5.1 Linearity***.** Spiked olive oil samples were prepared to give concentrations of oleuropein aglycon and ligstroside aglycon at the 10, 20, 40, 80, 160 and 320 mg/Kg and were analyzed for the determination of the linearity. The relationship of the integration ratio of the analytes versus the internal standard and the corresponding concentration of the spiked olive standards was determined by linear regression analysis.

2.5.2 Accuracy. Spiked olive oil samples at three concentration levels of both aglycons, 20, 75 and 150 mg/Kg, were analyzed in order to determine the accuracy of the method.

2.5.3 Limits of detection and quantitation. The LOD and LOQ were determined running six blank samples of olive oil free of secoiridoids and measuring the background response at the chemical shift of each analyte. A signal-to-noise (*S*/*N*) ratio of 3:1 and 10:1 were used for the calculation of the LOD and LOQ, respectively.

2.6 Olive Oil Extraction and Sample Preparation and NMR Spectral Analysis of Extracted Oil.

The analysis of extracted oil was performed as previously described.[8] Briefly: Olive oil (5.0 g) was mixed with cyclohexane (20 mL) and acetonitrile (25 mL) and the mixture was homogenized using a vortex mixer for 30 sec and centrifuged at 4,000 rpm for 5 min. A part of the acetonitrile phase (25 mL) was collected, mixed with 1.0 mL of a syringaldehyde solution (0.5 mg/mL) in acetonitrile and evaporated under reduced pressure using a rotary evaporator. The residue was dissolved in $CDCl_3$ (750 μL) and an accurately measured volume of the solution (550 μL) was transferred to a 5 mm NMR tube.^{1}H-NMR spectra were recorded at 600 MHz (Bruker Avance 600) with cryoprobe. Typically, 50 scans were collected into 32K data points over a spectral width of 0-16 ppm with a relaxation delay of 1 s and an acquisition time of 1.7 s.Prior to Fourier transformation an exponential weighing factor corresponding to a line broadening of 0.3 Hz was applied.The spectra were phased corrected and integrated automatically using TopSpin software (Bruker). Accurate integration was performed manually for the peaks of interest.

3 RESULTS AND DISCUSSION

3.1 Method Development.

3.1.1 Selection of NMR solvent. The selection of $CDCl_3$ as solvent for NMR analysis of olive oil was based on the observation that it does not react with the analytes and that it presents a well resolved set of peaks corresponding to the aldehydic protons of the studied compounds between 9.1 and 9.8 ppm. It should be emphasized that methanol and water which are commonly[11,12] and officialy[13] used for the extraction of phenolics from olive oil react immediately with the dialdehydic form of oleocanthal or oleacein leading to the corresponding acetals or hemiacetals.[7] It should be emphasized that a large number of compounds identified in HPLC-UV or LCMS chromatograms of olive oil extracts are obviously artifacts produced by that type of reactions. All previous studies using those type of solvents for quantitative analysis should be reconsidered. Other studied deuterated solvents like acetonitrile, acetone or CD_2Cl_2 gave overlapping signals of the analytes and were considered as not appropriate.

3.1.2 Selection of internal standard. The choice of syringaldehyde as internal standard and of $CDCl_3$ as solvent for the NMR measurement was based on reasons explained previously.[7]

3.1.3 NMR spectral analysis of target compounds in extra virgin olive oil. The spectrum region between 9.1 and 9.8 ppm in the spiked oil that was used for the method development was clearly resolved making feasible the integration of the corresponding peaks and their comparison with the peak of the internal standard. Oleuropein aglycon (3,4-DHPEA-EA) and ligstroside aglycon (p-HPEA-EA) were quantified by integrating doublets at 9.50 ppm and at 9.49 ppm respectively. Oleocanthal and oleacein were measured at 9.23 ppm and 9.21 ppm respectively.

3.1.4 Development of calibration curves and validation. The calibration curves were constructed by the addition of known quantities in a specifically selected olive oil sample which did not contain any of the analytes. The method was validated for accuracy and sensitivity.

Linearity: Good linearity was achieved for all analytes for concentration ranging from 20 to 300 mg/Kg, with satisfactory correlation coefficients, r^2 >0.995.

Accuracy: The estimated accuracy values with the proposed method are within acceptable levels for the four analytes (Er%<10) and the method could be considered as accurate.Sensitivity: The sensitivity of the method as presented by its limit of detection (LOD) and the limit of quantification (LOQ) were found to be 5 mg/Kg and 20 mg/Kg, respectively, for all compounds.

3.1.5 Overlapping limitations. We observed two types of signal overlap which limit the application of the selective excitation method. In the first case, the peaks of oleuropein and ligstroside aglycons are overlapped by the signal of conjugated aliphatic aldehydes like hexenal (9.5 ppm doublet). The overlapping problem is more serious in the case of aged oils with increased levels of hexenal which is a derivative of oxidative decomposition of lipids. This type of problem is not significant in the case of fresh oils.

The second type of overlapping occurs in the 9.20-9.21 ppm region. This is the region where the peaks of oleocanthal and oleacein are observed as two well separated doublets. When the oil contains only those two dialdehydic compounds there is no problem of overlapping (Figure 1). However, if the oil contains compounds like the oleuropein and ligstroside aglycon dialdehyde forms there is overlap that can be revealed only after comparison with the spectrum of the extracted oil (Figure 2).

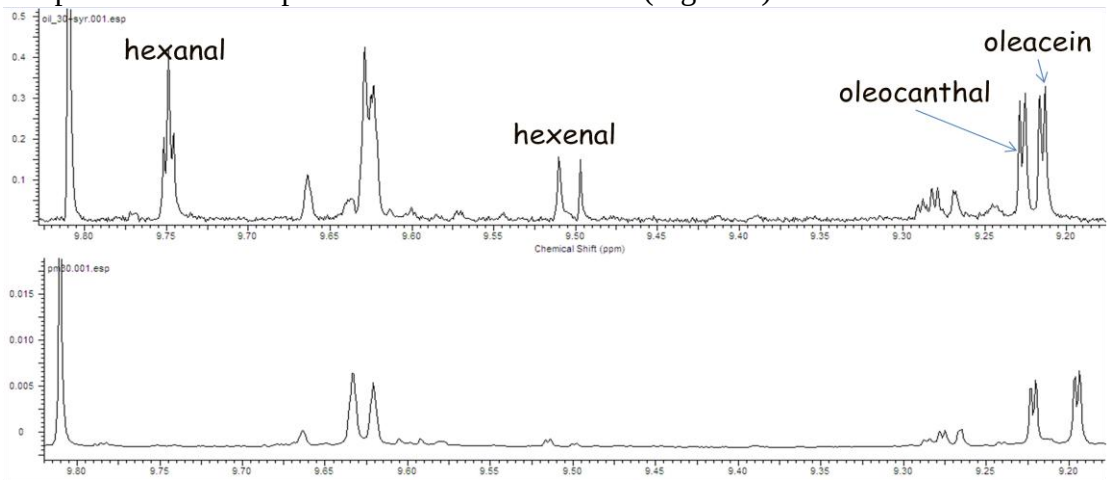

Figure 1 *Comparison of spectra using selective pulse without extraction (up) and after extraction (down). This case does not present overlapping limitations*

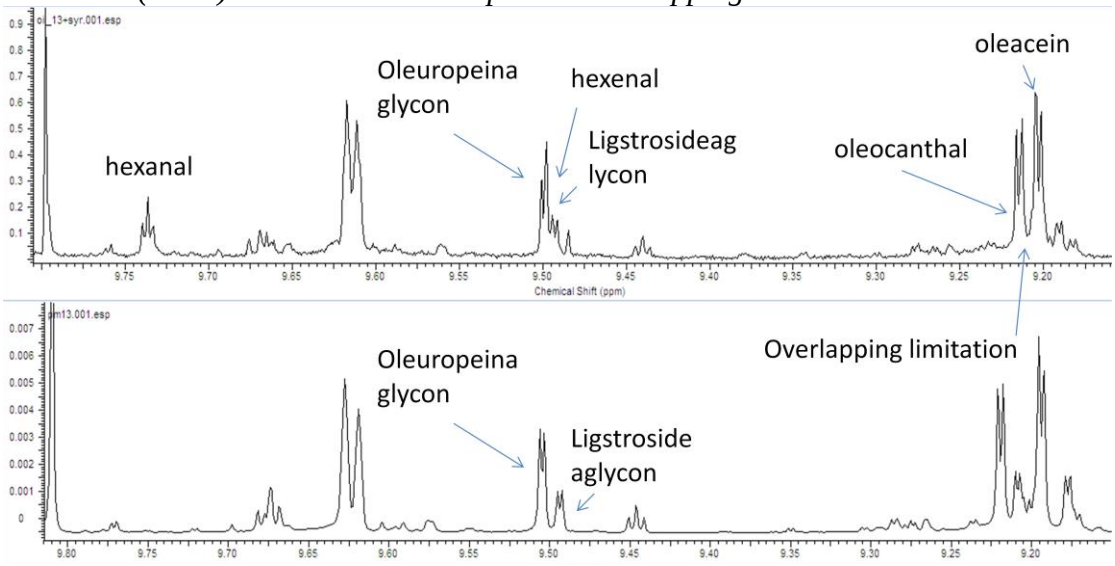

Figure 2 *Comparison of spectra using selective pulse without extraction (up) and after extraction (down). This case presents two types of overlapping limitations*

In most studied oils those compounds are found in small quantities and do not create a significant problem, however there are oils with high concentrations which may lead to a false overestimation of oleacein and oleocanthal levels. The overlapping problem can be overridden by 2D qNMR which is currently under investigation.

3.2 Method Application

The selective pulse method presents important advantages in comparison to alternative chromatographic or spectroscopic methods. The oil sample can be quantitatively analysed without any treatment (e.g extraction, derivatization, separation etc), without the use of standards and without the risk of decomposition or isomerization as happens during chromatographic analysis.[8] Moreover it is the fastest available method, with a total needed time of less than 5 min for sample preparation, spectrum acquisition, integration and quantitation. However, as explained above, the method presents some overlapping limitations that in some cases may lead to overestimation of some compounds. For this reason, this method is currently useful mainly as a screening tool. More specifically it is the most rapid and easy way to discriminate the oils that do not fulfill the European Union criteria for polyphenol content and health claims. Even if it does not give accurate results in all oils, it can definitely be used to exclude oils from further evaluation.

In this framework, during the current study the olive oil samples were first screened with the selective pulse and those presenting concentration close or higher than 250 mg/Kg were re-examined following the previously validated qNMR method[8] after extraction of olive oil to achieve accurate measurement. The samples showing lower content were excluded from any further accurate measurement saving significant amount of time and effort. The combined method with first the selective pulse measurement and second the extraction step, was applied to the study of 100 commercial olive oil samples from all the major brands available in supermarkets in California (San Francisco and Sacramento area).

The quantification results for each compound together with data about variety and geographic origin of the highest content oils are provided in Table 1.

A wide variation concerning the concentrations of all four secoiridoids was recorded. The concentration of each one ranged from non-detectable to 402 mg/Kg and the sum of the four major secoiridoids (D3) from non-detectable to 1232 mg/Kg. More than 50% of the studied samples failed to offer more than 250 mg/Kg of hydroxytyrosol or tyrosol derivatives and only 22 samples (5 Italy, 1 Spain, 1 Greece, 15 California) showed hydroxytyrosol derivatives (oleacein+oleuropein aglycon)> 250 mg/Kg as required by the EU health claim regulation. This result emphasizes the need for appropriate labeling of olive oils. The present study offers a good estimation of the average levels of the secoiridoid aldehydes that are available to the consumers by commercially available oils.

One interesting observation concerning the role of the variety on the chemical profile of the olive oil polyphenols was that some varieties showed consistently increased concentration of specific compounds. More specifically, all oils produced exclusively by California Mission variety or even containing a part coming from Mission variety showed high levels of oleuropein aglycon, which is a compound with promising activity against Alzheimer disease.[17] The highest concentration was recorded at 397 mg/Kg coming from a Mission sample from Berkeley Olive grove. The cv. Mission from California seems to be highly interesting since in all studied samples the major secoiridoid was oleuropein aglycon. Moreover, in all Mission samples the concentration of oleocanthal and oleacein was lower than that of oleuropein and ligstroside aglycons confirming our previous

observation that there are least two distinct biosynthetic pathways leading to the domination of each group of compounds.

Table 1. List of the 22 out of 100 commercial samples showing the highest content in secoiridoid polyphenols and satisfying the EU regulation for health claim. Oils are listed according their total measured content in secoiridoid polyphenols.

Origin	**Variety**	**Oleuropein aglycon mg/Kg**	**Ligstroside aglycon mg/Kg**	**Oleocanthal mg/Kg**	**Oleacein mg/Kg**
California	70% Mission, Leccino, Frantoio	352.5	155.1	314.5	400.6
Greece/Antiparos	Koroneiki	95.2	65.1	347.2	281.4
California	Manzanillo, Mission	163.3	65.6	231.9	318.1
California/Oroville	Mission	397.2	162.5	69.0	69.0
California/Oroville	Mission	355.1	52.0	107.9	107.0
California	Not mentioned	285.8	103.2	125.2	176.3
California/Yolo	Leccino, Pendolino, Moraiolo, Frantoio	179.4	74.1	352.4	257.7
California	Barouni	197.3	133.4	233.5	233.0
Italy	Not mentioned	149.5	139.2	402.3	250.4
California	Ogliarola, Barese, Biancolilla, Cerasuola	197.3	89.3	181.1	193.9
Northern California	Mission	275.7	82.8	34.7	49.6
California/Yolo	Picual, Ascolano, Koroneiki, Pendolino, Leccino, Frantoio	87.2	47.8	271.1	236.4
California/Yolo	Leccino	83.2	41.7	301.3	235.3
Italy	Not mentioned	124.9	174.5	266.7	167.6
California	Arbequina	13.2	7.0	218.9	277.8
California	Arbequina, arbosana, koroneiki	0	1.8	147.6	284.2
Italy	Not mentioned	132.3	161.2	342.9	145.0
California/Marin county	Frantoio, leccino, pendolino, maurino, coratina, leccio del corno	119.9	69.9	135.5	152.8
Italy	Coratina	128.6	100.0	287.8	141.9
Spain	Not mentioned	195.3	132.4	98.5	67.2

California	Arbosana	17.6	8.9	167.6	241.4
Italy	Not mentioned	130.7	105.5	226.0	125.7

In most Italian and Greek oils, the dominating compound was oleocanthal and in almost all cases the ratio between oleocanthal and oleacein (index D2=oleacein/oleocanthal) was lower than 1. Interestingly in most of the samples coming from the Spanish varieties Arbequina and Arbosana the D2 index was higher than 1 and oleacein was the dominating derivative. It confirms our previous observation that D2 seems to be dependent on the olive tree variety probably due to genetic reasons and independent of the olive mill procedure.

A final observation is that the selective pulse NMR method offers a new alternative way for direct observation of aldehydes related to lipid oxidation and identification of rancid oils without sensory evaluation. The peak at 9.5 ppm corresponding to conjugated aliphatic aldehydes, like hexenal, could be used as a marker of rancidity. Indeed, oils showing high levels of that peak were evaluated in all cases as rancid oils by a sensory panel (data not presented).

4 CONCLUSION

Although according to their label all the studied samples were considered as extra virgin olive oils, the observed significant variation of the concentration of the bioactive polyphenolic secoiridoids confirms our previous conclusion[8] that there is need of a new type of classification of EVOO especially related to possible health claims of those compounds. D3 index is more accurate and specific than the commonly used total polyphenols index (expressed as gallic acid or caffeic acid equivalents) and could become a new standard for the characterization of olive oil healthfulness.

qNMR is a powerful tool for the measurement of the specific polyphenols required by the EU legislation. The application of the selective pulse for that purpose is a step towards the use of NMR as a high throughput screening method that can be routinely used by the olive oil industry for the discrimination and evaluation of hundreds of samples in a single day.

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ABSTRACT
A new quantitative method for ultra rapid screening of olive oil was developed targeting to the levels of secoiridoid aldehydes (oleocanthal, oleacein, oleuropein aglycon and ligstroside aglycon) which constitute the major phenolic compounds related to the health claims of olive oil according to European Union regulations. Using selective pulse NMR spectroscopy, it was possible to measure the levels of the four compounds directly in 250 μL of olive oil without any extraction, separation or derivatization in only three minutes. In some cases the developed method presented some overlapping limitations that currently make it useful mainly for the rapid rejection of the oils that do not fulfil the EU criteria for polyphenol content and health claims. In the current study, the method was applied to100 commercial olive oil samples from all the major international brands available in supermarkets in California. The olive oil samples were first rapidly screened with the selective pulse and those presenting concentration <250 mg/Kg were rejected and the rest were re-examined following a previously validated qNMR method which includes an extraction step of oil and achieves accurate measurement. A wide variation concerning the concentrations of all four secoiridoids was recorded across the commercial samples. The concentration of each one ranged from non-detectable to 402 mg/Kg and the sum of the four major secoiridoids from non-detectable to 1232 mg/Kg. More than 50% of the studied samples failed to offer more than 250 mg/Kg of hydroxytyrosol or tyrosol derivatives emphasizing the need for appropriate labelling of olive oils.