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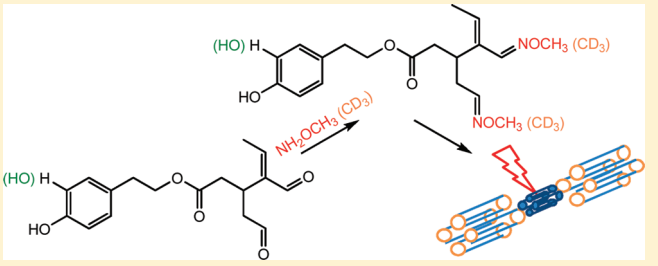
High-Throughput Assay of Oleopentanedialdheydes in Extra Virgin Olive Oil by the UHPLC−ESI-MS/MS and Isotope Dilution Methods

Leonardo Di Donna, Hicham Benabdelkamel, Fabio Mazzotti, Anna Napoli, Monica Nardi, and Giovanni Sindona*

Dipartimento di Chimica, Università della Calabria, via P. Bucci, cubo 12/C, I-87030 Arcavacata di Rende (CS), Italy

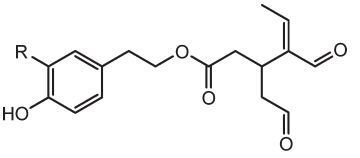
ABSTRACT: The quality of extra virgin olive oil is associated with the presence of microcomponents whose healing effects have been proved in some special cases. The enzymatic hydrolysis of oleuropein and ligstroside, and of their demethylated analogues, affords four different pentanedialdehydes, and for one of which, 2-(4-hydroxyphenyl)ethyl (3*S*,4*E*)-4-formyl-3-(2-oxoethyl)hex-4-enoate, also known as oleocanthal, an anti-inflammatory effect was quite recently carefully assessed. Extra virgin olive oil is now worldwide considered as a functional food whose daily intake, as for the Mediterranean diet, helps consumers in keeping a constant level of nonsteroidal anti-inflammatory drug (NSAID) in the blood. The presence of these active principles provides, therefore, olive oil with an important added value. In the framework of the actions of the recently funded Agrifood Regional Center, which should coordinate the scientific research and production worlds, an absolute analytical method was developed for the mass spectrometric detection of the two most abundant NSAIDs, Tyr-OLPD and HTyr-OLPD (oleopentanedialdehydes (OLPDs) conjugated to *p*-hydroxyphenylethanol and 3,4-dihydroxyphenylethanol, respectively), by UHPLC-ESI-MS/MS.



Olive oil is one of the most important constituents of the Mediterranean diet; its constant intake may provide potential health benefits to humans, lowering the incidence of cardiovascular diseases.[1−4] It has been suggested that this effect is largely due to the antioxidant properties of phenolic compounds present in the oil.[5,6] Recent reports have demonstrated that the antioxidant properties of olive oil are related to phenolic glycoside compounds such as the most abundant secoiridoid which possess the oleuropein and ligstroside aglycons[7] and their deacetoxylated homologues. The oleuropein phenolic glycoside, which is present in large amounts in the drupes,[8−12] can be easily hydrolyzed to its constituent aglycon either during fruit maturation or during the processing of the oil. Among these aglyconic products formed, it was established that oleopentanedialdehydes (OLPDs) conjugated to 3,4-dihydroxyphenylethanol (HTyr-OLPD, **2**; Chart 1) and *p*-hydroxyphenylethanol (Tyr-OLPD, **1**), also known as oleocanthal, originated from demethylation of oleuropein and ligstroside.[13−20] These aglyconic products may account for up to 55% of the total phenolic fraction.[16−20]

The aglycons mentioned above afford geometric isomers due to the keto−enolic tautomeric equilibrium that involves ring-opening.[21−24] Compound **1** was identified for the first time in olive oil by Montedoro et al.[25] from NMR, IR, and UV data. Andrewes and co-workers[26] independently reported that **1** was responsible for the throat irritation associated with some extra virgin olive oils. Beauchamp and colleagues[27] have demonstrated that oleocanthal did indeed possess anti-inflammatory activity due to its dose-dependent ability to inhibit the cyclooxygenase (COX) enzymes in the prostaglandin biosynthesis (inflammatory) pathway similarly to ibuprofen. Interestingly, it has also been demonstrated that the dose-dependent anti-inflammatory properties oleocanthal exhibits in vitro are mimicked by its dose-dependent irritation in the oral cavity;[27] Oreset et al.[28] showed that oleocanthal and its derivatives decreased lipolysaccharide-induced NOS2 synthesis in chondrocytes, without significantly affecting cell viability at lower concentration; furthermore, oleocanthal possesses other potential benefits such as neuroprotective properties because of the nonsteroidal anti-inflammatory and antioxidant activities.[29,30]

Chart 1



1, R= H Tyr-OLPD

2, R = OH HTyr-OLPD

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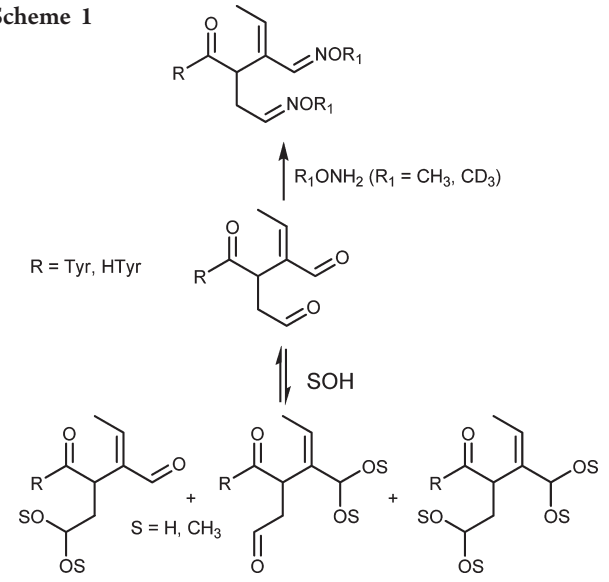
Hence, it is essential to determine the concentration of Tyr-OLPD and HTyr-OLPD both for their potential healing effects and also for their potentiality as markers of the quality of olive oils. The number of species at equilibrium when **1** and **2** interact with water or other polar solvents[12] (Scheme 1) affects the assay of OLPD active principles since they strictly depend on the selected method and on the environmental conditions. Among the few methods introduced for the assay of Tyr-OLPD, some of those based on liquid chromatography (LC) coupled to different detectors[25−27,31] are worth mentioning. These approaches, indeed, suffer by the limitation of the previously mentioned equilibria, detected by mass spectrometry.[12,32−34] In LC conditions, in fact, a reliable quantitation of each of the species at equilibrium, likely separated in the chromatographic stage, should be considered. Accordingly, a robust, affordable, and accurate analytical method requires the blocking of the OLPD's aldehyde functionality in situ directly in the oil and/or during online assessment.

The main goal of this novel method is based on the in situ chemical derivatization[35] of the whole set of molecules into stable alkyloxime derivatives such as *O*-methoxypentanedialdoxime-Tyr (**3**) and *O*-methoxypentanedialdoxime-HTyr (**4**; Scheme 2). The assay is performed using liquid chromatography coupled with mass spectrometry. The reaction with methoxyamine is fast and quantitative; it allows the concomitant use of a stable isotope standard which improves both the precision and accuracy of the measurements, thus reducing the drawbacks that may arise from the calibration procedure, sample preparation, and matrix effects.[36−41] The developed method has been tested on the determination of **1** and **2** in virgin olive oils using the stable isotope labeled d_6-*O*-methoxypentanedialdoxime-HTyr (**5**) as an internal standard; the measurements were carried out with low-energy collision dissociation tandem mass spectrometric analysis (CID-MS/MS) using the MRM mode by following the ion current generated by specific fragmentation pathways.

Scheme 1



MATERIALS AND METHODS

Chemicals. Solvents and reagents were commercially available (Sigma-Aldrich, St. Louis, MO).

HTyr-OLPD (**2**) standard was obtained by literature methods.[42] Pure HTyr-OLPD (50 mg) was kept frozen upon usage.

Oil Samples. Five extra virgin olive oils were stored in amber glass bottles at room temperature until analysis. O-1, O-2, O-3, and O-4 were obtained from GABRO srl, within the Olisafe project; the other olive oil sample (O-5) was purchased at a local store.

Synthesis of d_3-*O*-Methylhydroxylammonium Chloride ($CD_3NH_2 \cdot HCl$). *N-d_3-Methoxyphthalimide.* Anhydrous potassium carbonate (2.21 g, 16.0 mmol) was added slowly to a stirred solution of *N*-hydroxyphtalimide (4.10 g, 25.0 mmol) in dimethyl sulfoxide (37 mL). CD_3I (6.04 g, 2.65 mL, 42.5 mmol) was added dropwise to the red-brown solution at a rate such that the temperature did not exceed 30 °C. The reaction was stirred at room temperature for 24 h and then poured into cold water and left to stand at 0−5 °C for about 30 min. The obtained crystals were filtered, washed with water (3 × 20 mL), and dried under vacuum to a constant mass (3.34 g, 18.9 mmol).

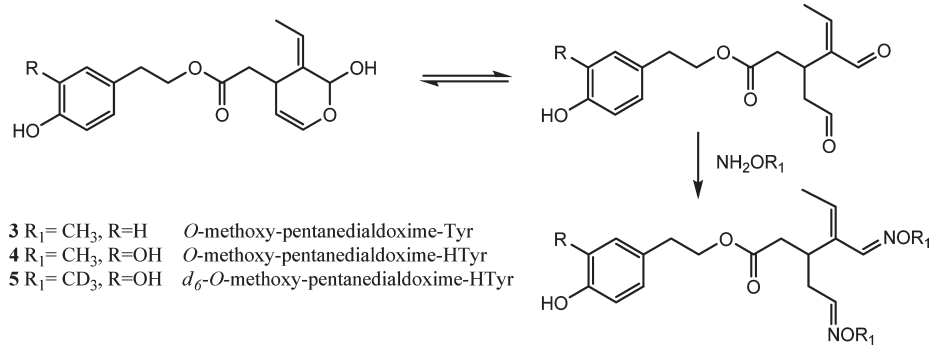
d_3-O-Methylhydroxylammonium chloride ($CD_3NH_2 \cdot HCl$) was synthesized according to the literature[43] The isotopic distribution, checked by high-resolution mass spectrometry, was d_2 = 1% and d_3 = 99%.

Standard and Sample Preparation. *Derivatization Procedure.* A 100 μL volume of a standard solution of **1** at 1000 mg/L was added to 1 mL of a 1.5 M solution of *O*-methylhydroxylammonium chloride in methanol and the resulting solution heated at 55 °C for 60 min. Finally, the mixture containing *O*-methoxypentanedialdoxime-HTyr (**4**) was cooled, centrifuged at 4000 rpm for 5 min, and diluted 10 times with CH_3OH/H_2O (70/30).

In analogy, a stock solution of d_6-*O*-methoxypentanedialdoxime-HTyr (**5**) was prepared using d_3-*O*-methylhydroxylammonium as the derivatizing agent (Scheme 2).

Sample Preparation. A 100 mg portion of olive oil was mixed with 900 μL of a 1.5 M solution of *O*-methylhydroxylammonium chloride. The mixture was stirred at 55 °C for 60 min; after cooling, the reaction mixture was centrifuged at 8000 rpm for 1 min, and then 100 μL of supernatant was mixed with 100 μL of a 10 mg/L concentration of the internal standard dissolved in CH_3OH/H_2O (70/30). The mixture was diluted to 1 mL with a solution of

Scheme 2



3 R_1= CH_3, R=H *O*-methoxy-pentanedialdoxime-Tyr
4 R_1= CH_3, R=OH *O*-methoxy-pentanedialdoxime-HTyr
5 R_1= CD_3, R=OH d_6-*O*-methoxy-pentanedialdoxime-HTyr

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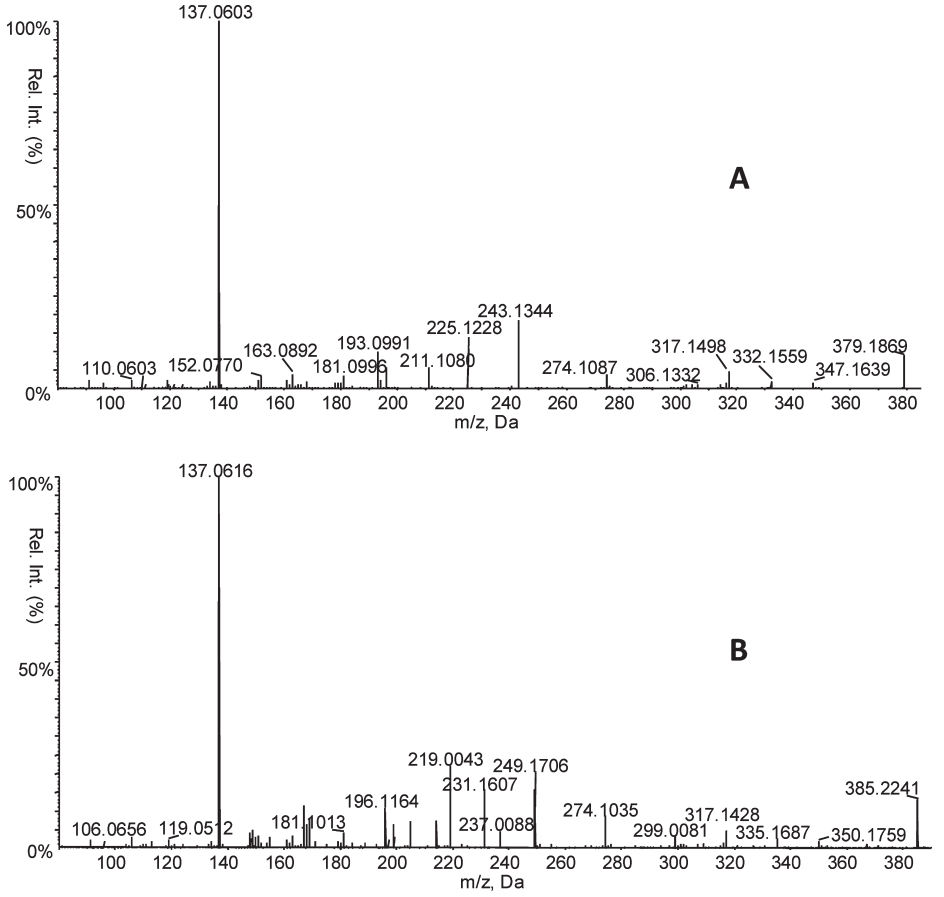


Figure 1. HRESI(+) MS/MS spectra of compounds **4** (A) and **5** (B).

CH_3OH/H_2O (70/30); the resulting solution was mixed thoroughly by vortex for 30 s twice to allow homogeneous distribution of the standards; at the end the mixture was diluted (1/10), filtered through a 0.22 μm filter, and then injected into the instrument.

Mass Spectrometry. LC−MS analysis was carried out using a Thermo Scientific UHPLC instrument coupled to a TSQ Quantum Vantage (Thermo Fisher Scientific, San José, CA) triple-stage quadrupole mass spectrometer. The chromatographic separation was achieved using a C_{18} reversed-phase analytical column, Hypersil GOLD (2.1 × 50 mm, 1.9 μm particle size, Thermo Fisher Scientific). The elution gradient consisted of mobile phases (A) H_2O (0.1% TFA) and (B) CH_3OH. The linear gradient used was the following: at t = 0.0 min, 70% A and 30% B; at t = 1.0 min, 70% A and 30% B; at t = 5.0 min, 2% A and 98% B; at t = 8 min, 2% A and 98% B; at t = 9.0 min, 70% A and 30% B; at t = 11.0 min, 70% A and 30% B. The flow rate was set at 0.4 mL/min, and the sample injection volume was 12 μL. A further switching valve located on the mass spectrometer was used to divert the LC flow to waste for the initial 3 min of each injection to protect the MS source from contamination. All valve positions and instrument parameters were controlled by Xcalibur software, version 2.0.0 (Thermo Fisher Scientific).

Mass spectrometric analysis was performed on a triple-quadrupole mass analyzer fitted with a heated electrospray ionization (HESI II) source operating in positive ion mode. The following working conditions were applied: spray voltage, 4.8 kV; vaporizer and capillary temperatures, 200 and 300 °C, respectively; sheath and auxiliary gas at 30 and 10 arbitrary units (au), respectively. The collision gas was argon used at a pressure in the collision cell (Q2) of 1.5 mTorr, and the mass resolution at the first (Q1) and third (Q3) quadrupoles was set at 0.7 Da at full width at half-maximum (fwhm). The S-lens rf amplitude was kept at 103 V, while the collision energy (CE) was optimized individually per compound. The multiple reaction monitoring (MRM) mode was used to quantify the analytes: the assay of oleopentanedialdehydes was performed following two transitions per compound, the first one for quantitation and the second for confirmation, in particular for **3** the transitions m/z 379 → m/z 137 (assay, CE = 27 eV) and m/z 379 → m/z 274 (confirmation, CE = 22 eV), while for compound **4** the reactions m/z 363 → m/z 121 (assay, CE = 27 eV) and m/z 363 → m/z 258 (confirmation, CE = 22 eV). Instrument control and data processing were carried out by means of Xcalibur software.

The total LC−MS/MS method run time was 11 min.

High-resolution electrospray ionization (HRESI) experiments were carried out in a hybrid Q-Star Pulsar-i (MSD Sciex Applied Biosystem, Toronto, Canada) mass spectrometer equipped with an ion spray ionization source. Samples were introduced by direct infusion (3 μL/min) of the sample containing the analyte (5 ppm), dissolved in a solution of 0.1% acetic acid and acetonitrile/water (50/50), at the optimum ion spray (IS) voltage of 4800 V. The source nitrogen (GS1) and the curtain gas (CUR) flows were set at pressures of 20 and 25 psi, respectively, whereas the first declustering potential (DP1), the focusing potential (FP), and the second declustering potential (DP2) were kept at 50, 220, and 10 V relative to ground, respectively.

Analytical Parameters. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated by applying

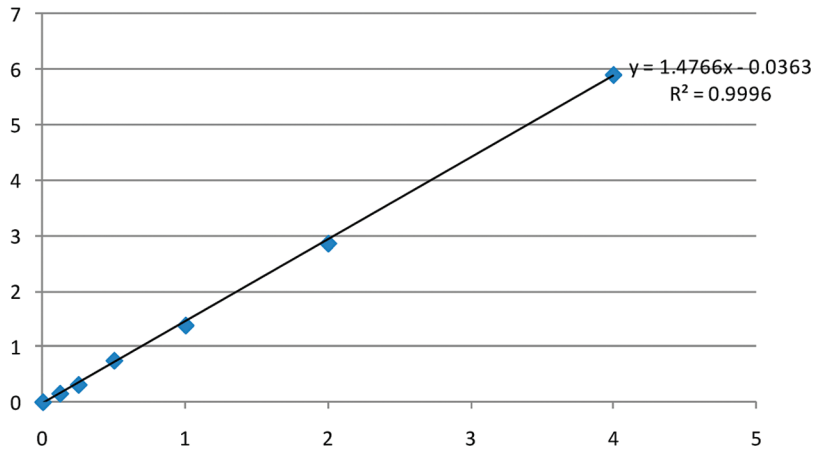


Figure 2. Calibration curve of six standard solutions of HTyr-OLPD and internal standard d_6-HTyr-OLPD.

eqs 1 and 2, following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry. S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the signal of the blank oil (seed oil), and σ_{RB} is the standard deviation.

$$S_{LOD} = S_{RB} + 3\sigma_{RB} \quad (1)$$

$$S_{LOQ} = S_{RB} + 10\sigma_{RB} \quad (2)$$

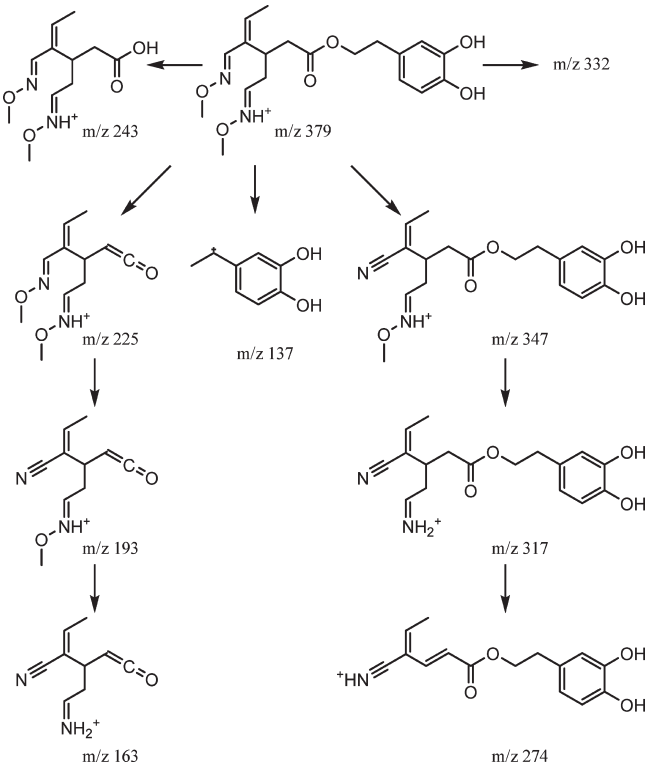
RESULTS AND DISCUSSION

The ESI(+) MS/MS spectrum of **4** is characterized by a small number of fragment ions, and diagnostic structures are shown in Figure 1A and Scheme 3. The fragmentation of the protonated molecule $[M + H]^+$ at m/z 379 (Scheme 3) leads to the formation of the ion at m/z 137, corresponding to the hydroxytyrosol cationic moiety; on the other hand, the phenolic portion may be lost as neutral to give rise to the ion at m/z 243 by a classical McLafferty rearrangement. The ion at m/z 225 may be formed by the direct cleavage of the precursor ion at the ester moiety of the molecule, which may subsequently lose methanol and formaldehyde to provide the ions at m/z 193 and m/z 163, respectively. The same series of reactions take place on the precursor ion, producing the species at m/z 347 and m/z 317, which arise from the loss of methanol and formaldehyde. The latter ion subsequently fragments to the ion at m/z 274, whose hypothesized structure is given in Scheme 3.

Another reaction channel with respect to the methoxime moieties affords the fragment ion at m/z 332, which may result from the loss of one methoxyamine molecule. The proposed reaction pathways described above were confirmed by the CID-MS/MS spectrum of the deuterated species **5**, which shows a shift of 3 or 6 amu (Figure 1B).

The MRM methodology was carried out by monitoring the ion current of two different fragmentation pathways. The reaction used for the assay of HTyr-OLPD corresponds to the breakdown of the protonated molecular ions of **4** and its d_6 isomer **5** to give rise to the fragment ions at m/z 137 (Scheme 3). The second transition, used only to confirm the elution of analytes, is that leading to the ion at m/z 274 from either unlabeled or labeled protonated molecular ions. In analogy, the assay of Tyr-OLPD was performed using the transition leading to the tyrosol ion (m/z 121) from the parent ion.

Scheme 3



The calibration curve, used for the assay of either Tyr-OLPD or HTyr-OLPD in olive oils, has been obtained by injecting the standard solutions of *O*-methoxypentanedialdoxime-Tyr and internal standard d_6-*O*-methoxypentanedialdoxime-HTyr. The former was used at concentrations ranging from 0.012 to 0.400 ppm, while the latter was kept constant at 0.100 ppm. Figure 2 shows the linearity in the selected range of concentrations.

Table 1 shows the amount of **1** and **2** in different oils obtained from the local market together with the parameters of repeatability. It must be stressed that the assay is performed directly on the oil without any further step of extraction. In fact, the oil is only diluted with solvent and mixed with the derivatizing agent. The yield of derivatization is quantitative, so no recovery problems may affect the methodology.

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Table 1. Amount of Tyr-OLPD and HTyr-OLPD in Extra Virgin Olive Oil Samples

extra virgin olive oil	[Tyr-OLPD] (ppm)	RSD (%)	[HTyr-OLPD] (ppm)	RSD (%)
O-1	71.63 ± 6.74	9.41	68.50 ± 4.79	7.00
O-2	106.35 ± 9.98	9.38	95.41 ± 10.59	11.10
O-3	166.17 ± 10.44	6.28	132.14 ± 5.15	3.90
O-4	109.32 ± 12.08	11.05	22.59 ± 3.81	16.86
O-5	91.36 ± 5.63	6.16	37.53 ± 6.17	16.44

Table 2. Reproducibility, Recovery, LOQ, and LOD Values for the Proposed Method

Tyr-OLPD		reproducibility RSD[a] (%)	
LOQ (ppm)	7.19	O-1	O-5
LOD (ppm)	5.19	6.65	15.7
recovery	>99%		
HTyr-OLPD		reproducibility RSD[a] (%)	
LOQ (ppm)	8.87	O-1	O-5
LOD (ppm)	5.90	4.77	4.12
recovery	>99%		

[a] The reproducibility of the measurements was determined by extracting each sample three times over a period of one week.

Table 3. Accuracy Values for Two Distinct Samples of Seed Oil Fortified with 2

concn (ppm)	calcd concn (ppm)	accuracy (%) (average)	RSD (%) (average)
0.020	0.022 ± 0.003	110.0	13.63
0.300	0.304 ± 0.016	101.3	2.34

Two olive oil samples (O-1, O-5) were analyzed in triplicate to check the reproducibility of the measurements. The values of the relative standard deviation (RSD; %) of the calculated concentration (Table 2) show a satisfying degree of reproducibility; Table 2 also displays the values of LOQ and LOD, which are in the low parts per billion range for both the examined compounds.

The accuracy of the method was also checked on two seed oil samples spiked with a known amount of HTyr-OLPD: in particular, compound **2** was spiked at 0.020 and 0.300 ppm into 100 mg of seed oil samples before the derivatization reaction; Table 3 shows that the value of accuracy for the two samples ranged from 101% to 110%.

CONCLUSIONS

An innovative and reliable platform has been presented allowing specialized laboratories to assay two nonsteroidal anti-inflammatory drug natural molecules in an important aliment of widespread use such as olive oil. Dialdehydes **1** and **2** are involved in a series of chemical equilibria, reported in Scheme 1, in which they are interconverted either in solution and/or in the ionic plasma during the formation of the charged particle.[12] Moreover, the concentration of the equilibrating species depends on the environmental conditions, e.g., solvents, temperature, and internal energy of each single species. Therefore, a reliable assay of Tyr-OLPD and HTyr-OLPD would be impossible in these experimental conditions. Hence, the uniqueness and novelty of the proposed method is based on the expected reactivity of the methoxyamines over aliphatic dialdehydes, which shifts the equilibria toward the irreversible formation of the methyloxime derivatives (Scheme 1). Finally, the use of labeled internal standard characterizes the accuracy of the method. The certification of the quantities of HTyr-OLPD and Tyr-OLPD in olive oil should be adverted to consumers, thus favoring the production and introduction in the market of good-quality foodstuff.

AUTHOR INFORMATION

Corresponding Author

*Phone: +39-0984-492046. Fax: +39-0984-493307. E-mail: sindona@unical.it.

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