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# Separation and Identification of Phenolic Compounds of Extra Virgin Olive Oil from *Olea europaea* L. by HPLC-DAD-SPE-NMR/MS. Identification of a New Diastereoisomer of the Aldehydic Form of Oleuropein Aglycone

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The phenolic fraction of a monovarietal extra virgin olive oil (EVOO) from *Olea europaea* L. var. Cornezuelo was studied by the hyphenated HPLC-DAD-SPE-NMR/MS techniques. This survey led to the identification of 25 main compounds. One was identified as a new diastereoisomer of the aldehydic form of oleuropein aglycone (AOA) and characterized by 1D and 2D NMR techniques. The relative configuration of this new AOA was determined as 5*R**,8*S**,9*S** on the basis of the results obtained from the combination of NOE experiments and Monte Carlo conformational search calculations. Assuming, as for the described diastereoisomers, that the new AOA comes from the natural oleuropein aglycone (OA), the absolute configuration was proposed as 5*S*,8*R*,9*R*.

**KEYWORDS: *Olea europaea* L.; olive oil; phenolic compounds; aldehydic form of oleuropein aglycone; HPLC-DAD-SPE-NMR/MS**

## INTRODUCTION

Olive oil represents the typical lipidic source of the Mediterranean diet, and its consumption is related to lower death rates, higher life expectancy, and a low incidence of several pathologies, including cardiovascular diseases and neurological disorders (*1*−*3*). In recent years its consumption has also spread remarkably outside the Mediterranean basin. The growing interest in olive oil may be partly related to its unique taste; however, particular interest is due to its nutritional properties. Phenolic compounds from olive oil are widely known due to their fundamental importance for its nutritional properties, sensory characteristics, and shelf life (*4*); because they have high antioxidant properties, they can confer a marked bitter taste or a sweet taste typical of some virgin olive oils (*5*). However, the biological properties of virgin olive oil's constituents have only been investigated in the past decade. Olive oil phenolics have beneficial biological activities such as altered lipid composition, platelet and cellular function, and microbiological activity, as well as a reduction in oxidative damage and inflammation in both human and animal (in vivo and in vitro) studies (*6*). Besides, some effects against cancer have been seen; that is, treatment of human colon adenocarcinoma cells with olive oil phenolics inhibits initiation, promotion, and metastasis of the colon carcinogenesis process (*7*). Also, some studies have been carried out in breast cancer, revealing antiproliferative effects by the action of crude extra virgin olive oil (EVOO) phenolic extracts and suggesting that the stereochemistry of EVOO-derived lignans and secoiridoids might provide an excellent and safe platform for the design of new anti-breast cancer drugs (*8*). With regard to secoiridoids and specifically oleuropein aglycones, Paiva-Martins et al. (*9*) have demonstrated recently the noteworthy protective role against oxidative injury in human cells of an oleuropein aglycone, specifically the 3,4-dihydroxyphenylethanol−elenolic acid dialdehyde. Considering all this, detailed knowledge of the phenolic compounds of olive oil becomes of high interest.

Over the years the determination of the phenolic fraction of EVOO has been widely studied, and the development of methodologies for its determination has been discussed extensively in the literature. Because of the need to carry out an individual identification of each phenolic compound present in the extract, spectrophotometric methods were replaced with separative techniques: GC (*10*), capillary electrophoresis (CE) (*11*, *12*), and, in particular, HPLC (*13*) coupled to different detectors such as ultraviolet (UV−vis) (*14*) electrochemical (*15*), fluorescence (*16*), or MS detection (*17*, *13*). In some cases, MS and MS/MS data cannot give detailed and conclusive information. Specifically, MS detection can hardly ever differentiate between isomers (*18*). However, NMR spectroscopy is a powerful technique that provides valuable structural information of molecules, leads to the complete characterization of compounds, and more importantly can distinguish between structural isomers and between diastereoisomers.

The hyphenation of HPLC and NMR spectroscopy allows the complete assignment and structure determination of analytes in complex mixtures, becoming an important technique in a lot of

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areas of knowledge (environmental, biomedical, pharmaceutical, food and natural products analysis, identification of drug metabolites) (*18*, *19*). When the concentration of an analyte is not sufficient for the performance of more sophisticated 2D NMR experiments (COSY, NOESY, HSQC, HMBC), which is in most cases necessary for the complete characterization of unknown compounds, the utilization of a post chromatography solid phase extraction (SPE) system allows the concentration of the analyte prior to NMR analysis, enhancing the sensitivity of the HPLC-NMR technique (*20*−*22*). Another advantage of its use is that it allows performance of the chromatography using just protonated solvents. A minimum amount of deuterated solvent is used afterward when the trapped compound is transferred to the NMR flow cell. An increasing number of studies of natural products using the online HPLC-SPE-NMR technique are available in the literature (*21*, *23*−*25*). Among them, a very interesting study of the phenolic fraction of EVOO using the hyphenated HPLC-NMR technique was carried out in 2005 by Christophoridou et al. (*26*).

This technique can be improved if MS detection is also coupled to the HPLC-DAD-SPE-NMR system. In this way the rapid and sensitive capabilities of MS will help (i) to find the peaks of interest in complex mixtures that afterward can be analyzed by NMR spectroscopy and (ii) to avoid the NMR analysis of those peaks corresponding to well-known analytes, which could represent an important saving of time. Furthermore, the MS results complement the NMR spectroscopic information, and in cases of highly symmetric molecules, knowing their molecular weight can be critical for their complete elucidation.

In the present work, we have studied in depth the phenolic fraction of EVOO using HPLC-DAD-SPE-NMR/MS. We have identified most of the compounds present in this fraction by MS and/or NMR spectroscopy. We have paid special attention to finding unknown isomeric forms of major phenolic constituents, in their identification and characterization, because different isomers may display different anticarcinogenic activities.

We have detected and completely elucidated by $^{1}H$ and $^{13}C$ NMR spectroscopy and MS a new diastereoisomer of the aldehydic form of oleuropein aglycone (AOA), one of the major phenolic compounds in EVOO, which could be related to the antiproliferative effects in breast cancer. In this work the two described diastereoisomers of AOA were also fully characterized by $^{1}H$ and $^{13}C$ NMR, which led to the correction of some previous NMR assignments.

## MATERIALS AND METHODS

**Chemicals.** Methanol and *n*-hexane were acquired from Panreac (Barcelona, Spain). Acetonitrile (supergradient HPLC grade), water (HPLC-MS grade), and acetic acid (96%, v/v) were purchased from Sharlau Chemie (Barcelona, Spain). For the NMR experiments, deuterated acetonitrile ($CD_3CN$, 99.8%) was obtained from Cortecnet (Voisins-le-Bretonneux, France).

**Sample Preparation.** The sample analyzed was a monovarietal EVOO of the Cornezuelo variety that had been shown to possess a high phenolic content. The phenolic fraction was isolated from a solution of 60 g of EVOO dissolved in 60 mL of hexane. A SPE method with Diol-cartridges was used (1 g, 6 mL; Supelco Bellefonte, PA), washing with hexane, to remove the nonpolar fraction of the oil, and recovering the polar fraction with methanol. The SPE protocol followed the conditions previously described (*12*).

**HPLC-DAD-SPE-NMR/MS Equipment.** HPLC analyses were performed using an Agilent 1200 series instrument (Agilent Technologies, Palo Alto, CA) equipped with a quaternary pump delivery system, degasser, autosampler, diode-array UV−vis detector (DAD) from Bruker-Biospin (Rheinstetten, Germany), and Esquire 6000 electrospray ionization (ESI) ion trap (IT) mass spectrometer (Bruker Daltonics, Bremen, Germany). Once the eluent left the HPLC column, it went through a BNMI (Bruker BioSpin, Rheinstetten NMR-MS Interface) split unit, such that 5% went through the mass spectrometer and 95% to the DAD. After the DAD, the eluent went through a Bruker/Spark Prospect 2 SPE unit (Bruker BioSpin and Spark, Emmen, The Netherlands). This SPE system was used to automatically trap the chromatographic peaks on Hysphere GP cartridges (2 mm i.d., 10 $\mu$m) after postcolumn addition of water using a Knauer K 120 HPLC pump (Berlin, Germany). The trapped peaks were dried with dry nitrogen gas and eluted with $CD_3CN$, using a flow probe of 60 $\mu$L of active volume, into an AVANCE 500 Bruker NMR spectrometer equipped with a high-sensitivity cryogenically cooled triple-resonance TCI probehead from Bruker BioSpin. The software used to control the whole process was HyStar 2.3 (Bruker Daltonics).

**HPLC-DAD-MS Analysis.** Chromatographic separation of phenolic compounds from EVOO was performed using a Gemini C18 column (5 $\mu$m particle size, 15 × 4.6 cm), operating at 25 °C, a flow rate of 0.5 mL/min, and an injection volume of 20 $\mu$L of the phenolic extract. The mobile phases used were phase A, water with 0.1% of acetic acid, and phase B, acetonitrile with 0.1% of acetic acid. The solvent gradient conditions used were as follows: 0−20 min, 95% A/5% B to 80% A/20% B; 20−30 min, 80% A/20% B to 70% A/30% B; 30−40 min, 70% A/30% B to 70% A/30% B; 40−50 min, 70% A/30% B to 65% A/35% B; 50−60 min, 65% A/35% B to 50% A/50% B; 60−70 min, 50% A/50% B to 5% A/95% B; 70−75 min, 5% A/95% B to 95% A/5% B. The chromatograms were registered with a DAD, set to operate at 240 and 280 nm wavelengths, and with a mass spectrometry detector (ESI-IT-MS).

The analyses carried out in the ESI-IT-MS were run in the negative ion mode, and the capillary voltage was set to 4000 V. The IT scanned at the $m/z$ 50−800 range at 13000 $m/z$/s during the separation and detection. The maximum accumulation time for the ion trap was set to 5000 $\mu$s, the target count to 10000, and the trap drive level to 42.8%. The ESI optimum conditions were a drying flow rate of 6 L/min at 300 °C using a nebulizing gas pressure of 40 psi.

**HPLC-DAD-SPE-NMR/MS Analysis.** Chromatographic separation and DAD and MS detection of phenolic compounds from EVOO were performed as described above. After the chromatographic separation and before the trapping into the SPE cartridges, a flow of water (1.5 mL/min) was added to the eluent by a second pump (makeup pump, K 120), to increase the polarity of the mobile phase and provide a higher retention of the peaks under study into the SPE cartridges. Before the trapping, the SPE cartridges were conditioned (500 $\mu$L of $CH_3CN$ at 6000 $\mu$L/min) and equilibrated (500 $\mu$L of $H_2O$ at 1000 $\mu$L/min). The trapping procedure was optimized with peak 21, as it was one of the major compounds in the phenolic extract of olive oil. Each peak was trapped from 5 to 10 times in the same cartridge, depending on its concentration in the initial extract. In this way the concentration of the compounds under study was increased before the NMR analyses were carried out. Once the chromatographic separation and multiple trapping were completed, the cartridges were dried with nitrogen gas for 30 min to remove the residual solvents, and afterward, the trapped fractions were transferred to the 60 $\mu$L flow cell of the NMR spectrometer with $CD_3CN$. The total amount of $CD_3CN$ used for the elution and transfer of each sample into the flow cell was of 390 $\mu$L.

$^{1}H$ (500.13 MHz) NMR spectra of eluted cartridges were recorded for most fractions using simple pulse acquisition sequence. When required, multiple solvent suppression with time-shared double presaturation of the residual water and organic solvent proton signal frequencies was performed. Shaped low-power radio frequency pulse and continuous-wave decoupling on the F2 channel for the decoupling of the $^{13}C$ satellites were utilized. All spectra were recorded at 25 °C and referenced to residual internal solvent peaks ($\delta_H$ = 1.93 ppm for $CD_2HCN$). The data were collected into 32K computer data points with a spectral width of 10 000 Hz. Ninety degree pulses were used with an acquisition time of 1.6 s, and the spectra were acquired by accumulation from 128 to 1024 scans. In some fractions a standard 2D COSY experiment was recorded.

The recovery of the measured trapped fraction into a vial was achieved by back flushing the flow cell with nitrogen gas.

**NMR Analyses of Chromatographic Fraction 21.** Chromatographic fraction 21 goes from the base peak chromatogram (BPC) time of 53.6 to 55.6 s (corresponding to chromatographic peak 21). The chromatography was repeated, and fraction 21 was trapped in the same SPE cartridge six times. $^{1}H$ (500.13 MHz) and $^{13}C$ (125.76 MHz) NMR spectra of

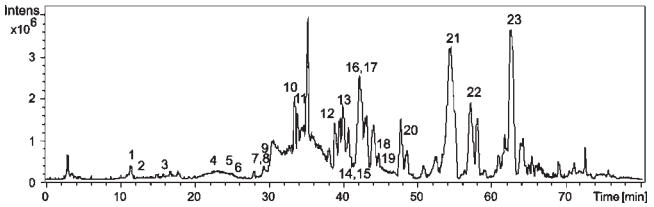


**Figure 1.** Base peak chromatogram of the phenolic fraction of EVOO obtained under the optimum chromatographic conditions. Peak numbers correspond to the compounds in **Table 1**.

the multiply trapped fraction were recorded on the AVANCE 500 Bruker spectrometer described above. A preliminary $^1$H NMR spectrum and a COSY experiment were performed in the hyphenated system, once the multiply trapped fraction 21 was transferred to the NMR cell via the flow probe, as described under HPLC-DAD-SPE-NMR/MS Analysis. Then the sample was recovered, transferred to a Bruker 2.5 mm microprobe NMR tube (Cortecnet, Voisins-le-Bretonneux, France), and analyzed by off-line NMR spectroscopy. The complete $^1$H and $^{13}$C NMR spectroscopy characterization of compounds **21a**, **21b**, **21c**, and **22** was achieved by means of the performance of 2D NMR experiments, such as 2D $^1$H−$^1$H COSY (correlated spectroscopy), $^1$H−$^{13}$C HSQC (heteronuclear single-quantum correlation), and $^1$H−$^{13}$C HMBC (heteronuclear multiple bond correlation) experiments. The $^{13}$C NMR chemical shifts were assigned by the HSQC and HMBC experiments. The presence of compound **22** in the same sample was due to a nonexact reproducibility of the chromatogram during the different chromatographic runs and trapping processes, which led to trap a part of the next eluting compound, **22**, in the same cartridge. For the study of the stereochemistry of the diastereoisomers of the AOA, selective 1D $^1$H−$^1$H NOESY experiments were recorded. NMR analyses were performed in $CD_3CN$ at 25 °C. $^1$H and $^{13}$C chemical shifts were referenced to residual internal solvent peaks ($\delta_H$ = 1.93 ppm and $\delta_C$ = 1.32 and 118.26 ppm for $CD_2HCN$). The patterns of peaks were reported as singlet (s), doublet (d), triplet (t), quintuplet (quin), double doublet (dd), double doublet of doublets (ddd), double triplet (dt), double quartet (dq), and multiplet (m).

All 2D experiments (COSY, TOCSY, HMQC, and HMBC experiments) were performed using standard Bruker pulse sequences under routine conditions.

**Computational Calculations.** Molecular mechanics calculations were done employing the Monte Carlo conformational search methodology (*27*,*28*). Monte Carlo calculations required 5000 searching steps, followed by geometric optimization up to 10000 iterations employing the Polak and Ribiere conjugated gradient algorithm (PRGC), energetic derivative convergence criterion, continuum $CH_3CN$ solvent model, and MM3* force field. Starting conformations were first minimized using the MacroModel minimization routine with the same parameters. The integrated software package Macromodel 9.0 and Maestro graphical interface were used (Schrödinger, Portland, OR; www.schrodinger.com).

## RESULTS AND DISCUSSION

**HPLC-DAD-SPE-NMR/MS Analysis.** The phenolic part of the EVOO from *Olea europaea* L. var. Cornezuelo was separated from the other components of the oil by means of a SPE procedure developed previously (*12*). The extract was used in the HPLC-DAD-SPE-NMR/MS study.

Initially, the chromatographic conditions and the trapping process were carefully optimized to be compatible with the MS conditions and to provide a good resolution of compounds, required to perform an adequate multiple trapping process. An initial HPLC-DAD-MS analysis was performed, where MS and UV data at two wavelengths (240 and 280 nm) were registered. **Figure 1** shows the MS base peak chromatogram (BPC) obtained at the optimum conditions, where 23 peaks were identified using the combination of UV and MS data together with the information found in other studies carried out previously on olive oil and its derivatives (*29*, *30*).

**Table 1.** Retention Times and MS Data of the Phenolic Compounds Identified in EVOO by HPLC-DAD-SPE-NMR/MS

| compd no. | compd name | $t_R$ (min) | *m*/*z* [M − H]$^-$ |
|---|---|---|---|
| **1** | hydroxytyrosol | 11.5 | 153.1 |
| **2** | dialdehydic form of elenolic acid lacking a carboxymethyl | 13.2 | 183.1 |
| **3** | tyrosol | 15.9 | 137.1 |
| **4** | hydroxy-D-oleuropein aglycone lacking a carboxymethyl | 23.2 | 335.1 |
| **5** | vanillin | 23.2 | 151.1 |
| **6** | *p*-coumaric acid | 25.3 | 163.1 |
| **7** | elenolic acid | 27.4 | 241.1 |
| **8** | hydroxytyrosol acetate | 29.4 | 195.1 |
| **9** | elenolic acid | 30.2 | 241.1 |
| **10** | hydroxy-D-oleuropein aglycone lacking a carboxymethyl | 33.4 | 335.1 |
| **11** | dialdehydic form of oleuropein aglycone lacking a carboxymethyl | 34.6 | 319.1[a] |
| **12** | luteolin | 38.8 | 285.3 |
| **13** | aldehydic form of ligstroside aglycone | 39.5 | 361.3 |
| **14** | dialdehydic form of ligstroside lacking a carboxymethyl | 41.5 | 303.1[a] |
| **15** | (+)-pinoresinol | 41.8 | 415.8[a] |
| **16** | aldehydic form of ligstroside aglycone | 42.1 | 361.3 |
| **17** | 10-hydroxy-oleuropein aglycone | 42.3 | 393.2 |
| **18** | 10-hydroxy- oleuropein aglycone | 44.6 | 393.2 |
| **19** | 3,4-dihydroxyphenylethyl-(2,6-dimethoxy-3-ethylidene)tetrahydropyran-4-yl]acetate | 45.6 | 365.3 |
| **20** | apigenin | 48.0 | 269.4 |
| **21a** | aldehydic form of oleuropein aglycone (5*S*,8*R*,9*S*), | 54.3 | 377.2[a,b] |
| **21b** | aldehydic form of oleuropein aglycone (5*S*,8*S*,9*S*), | 54. 3 | 377.2[a,b] |
| **21c** | aldehydic form of oleuropein aglycone (5*S*,8*R*,9*R*) | 54.3 | 377.2[a,b] |
| **22** | oleuropein aglycone | 57,3 | 377.2[a,b] |
| **23** | ligstroside aglycone | 62.4 | 361.3 |

[a] Identified also by 1H NMR spectroscopy (see Figure 1 of the Supporting Information). [b] Characterized by $^1$H and $^{13}$C NMR spectroscopy (see **Table 2**).

Then, a HPLC-DAD-SPE-NMR/MS analysis was performed, which allowed the study by NMR technique of the most intense fractions observed in the BPC. Ten consecutive chromatographic runs were performed following the method described above (HPLC-DAD-SPE-NMR/MS Analysis under Materials and Methods), and the most intense peaks (see peaks 11, 14, 15, 21, 22, and 23 in **Figure 1**) were multiply trapped onto the SPE cartridges. Because the concentration of most of the compounds was insufficient to perform a $^1$H NMR experiment, even with multiple trapping, only those fractions corresponding to the most intense peaks were trapped and analyzed afterward by NMR spectroscopy. Standard $^1$H and 2D COSY spectra were acquired for each multiply trapped fraction to confirm the structure of all the compounds; multiple-frequency solvent presaturation techniques were used when needed. **Table 1** and **Figure 2** summarize the structures of all characterized compounds, which include simple phenols, such as hydroxytyrosol (**1**), tyrosol (**3**), vanillin (**5**), *p*-coumaric acid (**6**), elenolic acid (**7**, **9**), and hydroxytyrosol acetate (**8**); flavonoids, such as luteolin (**12**) and apigenin (**20**); the lignan (+)-pinoresinol (**15**); and a large number of secoiridoids, among them oleuropein aglycone (**22**), ligstroside aglycone (**23**), and three different aldehydic forms of oleuropein aglycone (**21a**, **21b**, and **21c**) that were fully assigned and characterized (described below).

**Characterization of Chromatographic Fraction 21.** After the study of different fractions, it was observed that the $^1$H NMR spectrum corresponding to the multiply trapped fraction 21 was a mixture of several compounds. Its ESI-MS spectrum (**Figure 3c**)

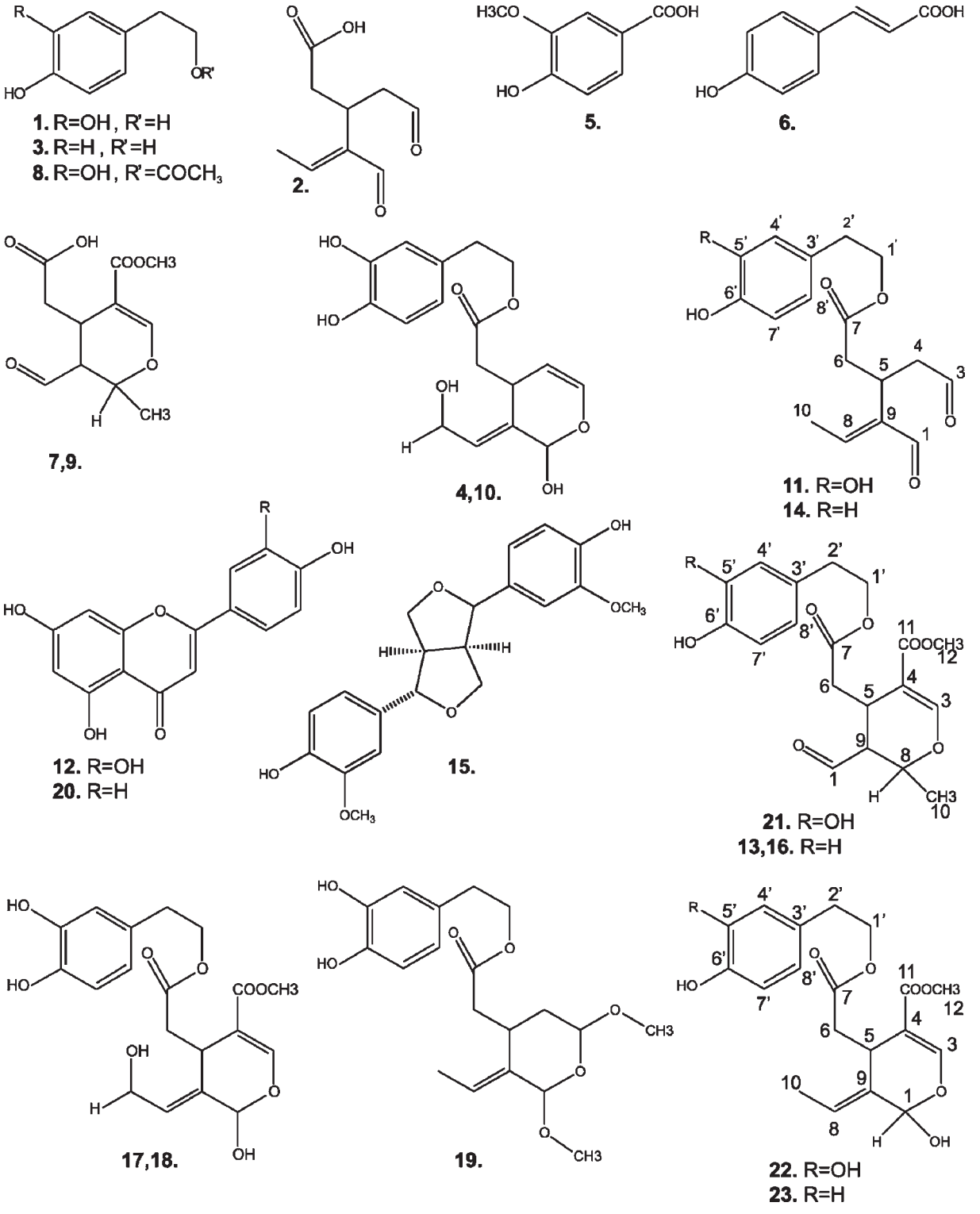


**Figure 2.** Chemical structures of the compounds identified.

shows two peaks at $m/z$ 377.2 $[M - H]^-$ and 755.2 $[2M - H]^-$ attributed to one or more isomers of oleuropein aglycone. A quick look at its $^1H$ NMR spectrum reveals the presence of four possible isomers of oleuropein aglycone (**Figure 3b**). At this point the study of this fraction by means of 2D NMR techniques was considered to identify and fully characterized them. The concerted use of 2D $^1H-^1H$ COSY and $^1H-^{13}C$ HSQC/HMBC experiments allowed the complete $^1H$ and $^{13}C$ chemical shift assignments of the four compounds within the mixture (**Table 2**; Figures 1−4 in the Supporting Information). Three diastereoisomers of the AOA (compounds **21a**, **21b** and **21c**) were identified, as well as oleuropein aglycone (OA) **22** (which corresponds to the next chromatographic peak and was partially trapped in the same SPE cartridge, see Materials and Methods). **Figure 3b** shows the $^1H$ NMR spectrum with the assignments for the four compounds and the amplified regions where H1, H3, and H10 (methyl) protons show up. Quantitative integration in the H3 proton region allowed the measurement of the relative ratio of **21a**/**21b**/**21c**, being 4.0:1.0:1.3. **Table 2** compiles $\delta$ ($^1H$ and $^{13}C$) and $J_{H,H}$ of the four isomers in parts per million and hertz, respectively. As in

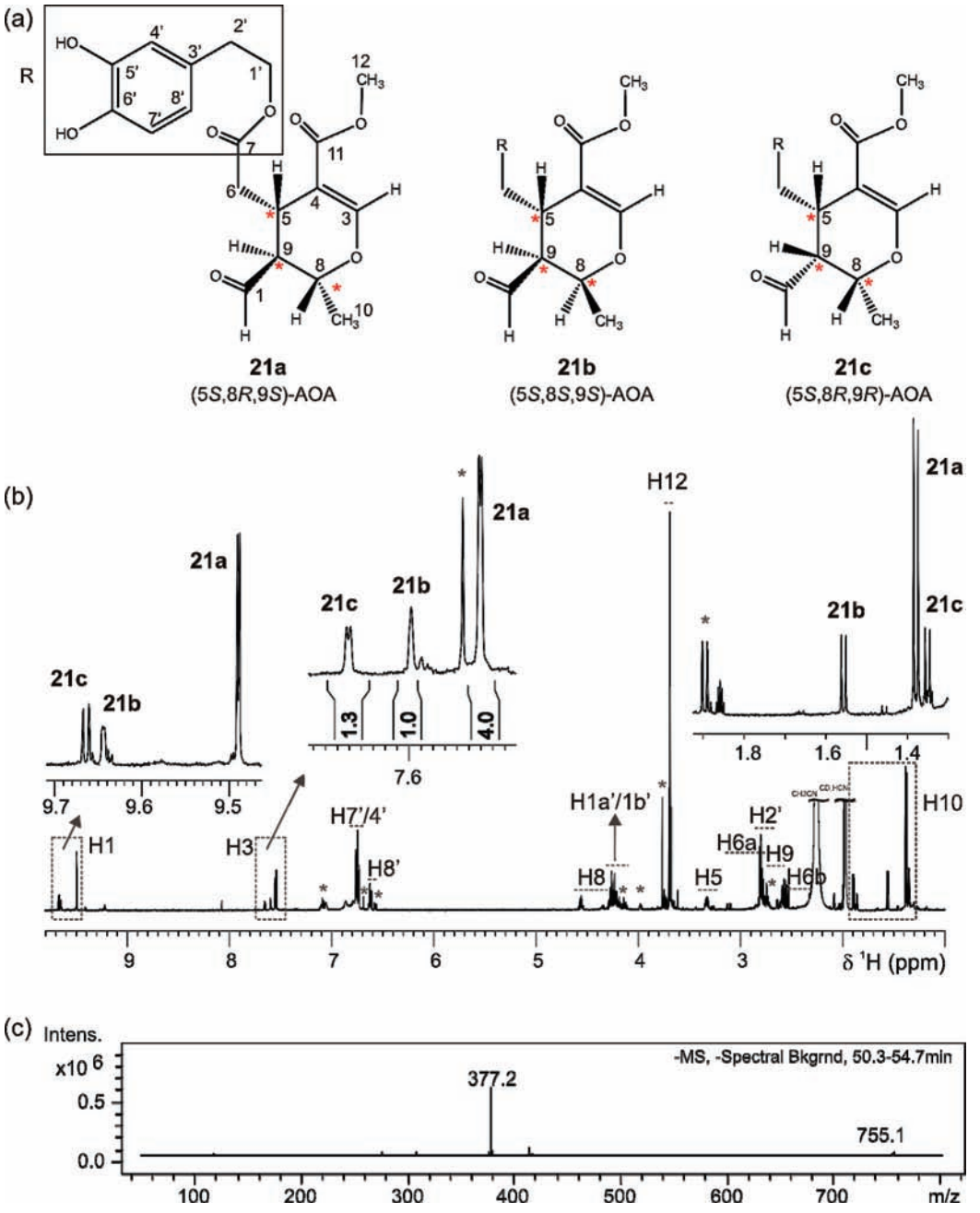


**Figure 3.** (**a**) Chemical structures and stereochemistry of compounds **21a**, **21b**, and **21c**. (**b**) $^{1}$H NMR spectrum of chromatogram fraction 21, dissolved in $CD_3CN$, acquired at 25.0 °C and a magnetic field of 500 MHz. $^{1}$H NMR assignments for compounds **21a**, **21b**, **21c** are shown, and signals corresponding to compound **22** are indicated with an asterisk (∗). The integration of H3 of each compound is also pointed out. (**c**) ESI(−)-MS spectrum of chromatogram fraction 21.

previous EVOOs studied (*26*, *31*), compound **21a** is the major AOA diastereoisomer in this EVOO.

As far as we know, only two diastereoisomers of AOA, (5*S*,8*R*,9*S*)-AOA and (5*S*,8*S*,9*S*)-AOA, have been reported previously, both of which were found in the leaves of *O. europaea* (*32*) and in the olive oil (*26*). $^{1}$H NMR data of compounds **21a** and **21b** (**Table 2**) are consistent with those described for diastereoisomers (5*S*,8*R*,9*S*)-AOA and (5*S*,8*S*,9*S*)-AOA, respectively, reported recently by Christophoridou et al. (*26*, *31*) (acquired at the same conditions of solvent and temperature). The only difference is for the value of the H8−H9 coupling constant, $J_{8,9}$ = 2.6 Hz (6.6 Hz for Christophoridou), which in our case fits that described previously by Gariboldi et al. (*32*) (2.5 Hz). Finally, $^{1}$H NMR data of compound **22** are consistent with the OA description (*26*, *31*, *32*). $^{13}$C NMR assignments of **21a**, **21b**, and **22** are also consistent with those described by Gariboldi et al. (*32*), except the difference observed between compounds **21a** and **21b** on δ (C4) and δ (C3′), which are exchanged with each other with respect to Gariboldi's results. This reassignment was made on the basis of $^{1}$H−$^{13}$C HMBC experiment, definitively proving the latter statement.

The stereochemistry of (5*S*,8*S*,9*S*)-AOA was first assigned by Gariboldi et al. in 1986, on the basis of the $^{1}$H NMR description of synthetic (5*S*,8*S*,9*S*)-methyl elenolate (ME) (*33*, *34*). In the same work Garilboldi et al. (*32*) proposed the stereochemistry of (5*S*,8*R*,9*S*)-AOA for the second diastereoisomer detected on the basis of the chemical shifts of C10 and H8 with respect to the former diastereoisomer and assuming a half-chair conformation of the dihydropyran ring. Then, the enantiopure (5*S*,8*R*,9*S*)-ME was

**Table 2.** Description of $^{1}H$ and $^{13}C$ NMR Chemical Shifts ($\delta$) and H,H-Coupling Constants ($^{3}J_{H,H}$ and $^{4}J_{H,H}$) of Compounds **21a**, **21b**, **21c**, and **22**[a]

| | **21a** | | | **21b** | | | **21c** | | | **22** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| atom | $\delta$ ($^{1}H$) (ppm) | $^{3/4}J_{H,H}$ (Hz) | $\delta$ ($^{13}C$) (ppm) | $\delta$ ($^{1}H$) | $^{3/4}J_{H,H}$ (Hz) | $\delta$ ($^{13}C$) (ppm) | $\delta$ ($^{1}H$) (ppm) | $^{3/4}J_{H,H}$ (Hz) | $\delta$ ($^{13}C$) (ppm) | $\delta$ ($^{1}H$) (ppm) | $^{3/4}J_{H,H}$ (Hz) | $\delta$ ($^{13}C$) (ppm) |
| **1** | 9.45 | d, $J_{1,9}$ = 1.5 | 201.34 | 9.61 | d, $J_{1,9}$ = 0.8 | 200.85 | 9.62 | d, $J_{1,9}$ = 4.1 | 202.82 | 4.20 | *b* | 106.80 |
| **3** | 7.51 | d, $J_{3,5}$ = 1.3 | 154.75 | 7.56 | s | 156.60 | 7.62 | d, $J_{3,5}$ = 2.0 | 156.50 | 7.52 | s | 155.30 |
| **4** | | | 107.11 | | | 108.07 | | | 108.11 | | | 112.10 |
| **5** | 3.29 | m, $J_{5,6b}$ = 9.7 $J_{5,9}$ = 4.9 $J_{5,6a}$ = 3.7 $J_{5,3}$ = 1.3) | 26.70 | 3.23 | m, $J_{5,6b}$ = 11.2 | 28.05 | 3.27 | m, $J_{5,3}$ = 2.0 $J_{5,9}$ = 6.1 $J_{5,6a}$ = 4.9 $J_{5,6b}$ = 9.9 | 31.07 | 3.94 | t, $J_{5,6b}$ = 4.7 $J_{5,6a}$ = 3.9) | 30.72 |
| **6a** | 2.76 | dd, $J_{6a,5}$ = 3.7 $J_{6a,6b}$ = 15,9) | 37.28 | 2.72 | dd, $J_{6a,5}$ = 2.9 $J_{6a,6b}$ = 16.4) | 38.59 | 3.08 | dd, $J_{6a,5}$ = 4.8 $J_{6a,6b}$ = 16.6) | 34.87 | 2.74 | dd, $J_{6a,5}$ = 3.9 $J_{6a,6b}$ = 16.6 | 38.55 |
| **6b** | 2.51 | dd, $J_{6b,5}$ = 9.7 $J_{6b,6a}$ = 15.9 | 37.28 | 2.26 | dd, $J_{6b,5}$ = 11.2 $J_{6b,6a}$ = 16.4 | 38.59 | 2.21 | dd, $J_{6b,5}$ = 9.9 $J_{6b,6a}$ = 16.6 | 34.87 | 2.59 | dd, $J_{6b,5}$ = 4.7 $J_{6b,6a}$ = 16.6 | 38.55 |
| **7** | | | 172.49 | | | 171.55 | | | 172.53 | | | 171.58 |
| **8** | 4.53 | quin, $J_{8,10}$ = 6.7 $J_{8,9}$ = 5,0 | 71.05 | 4.23 | dq, $J_{8,10}$ = 6.7 $J_{8,9}$ = 2.6 | 70.29 | 4.31 | dq, $J_{8,10}$ = 6.7 $J_{8,9}$ = 2.3 | 75.05 | 7.05 | qd, $J_{8,10}$ = 7.3 $J_{8,1}$ = 1.4 | 144.34 |
| **9** | 2.54 | dt, $J_{9,1}$ = 1.5 $J_{9,8}$ = 5.0 $J_{9,5}$ = 4.9 | 54.33 | 2.55 | dt, $J_{9,1}$ = 0.8 $J_{9,8}$ = 2.6 $J_{9,5}$ = 1.4 | 51.27 | 2.64 | ddd, $J_{9,1}$ = 4.1 $J_{9,8}$ = 2.3 $J_{9,5}$ = 6.1 | 52.13 | | | 127.14 |
| **10** | 1.34 | d, $J_{10,8}$ = 6.7 | 19.17 | 1.52 | d, $J_{10,8}$ = 6.7 | 17.67 | 1.31 | d, $J_{10,8}$ = 6.7 | 18.17 | 1.86 | dd, $J_{10,8}$ = 7.3 $J_{10,5/1}$ = 0.7 | 14.32 |
| **11** | | | 167.65 | | | 164.71 | | | 169.57 | | | 162.50 |
| **12** | 3.65 | s | 51.42 | 3.66 | s | 51.40 | 3.64 | s | 51.40 | 3.73 | s | 52.01 |
| **1a′** | 4.21 | dt, $J_{1a',1b'}$ = 14.6 $J_{1a',2}$ = 6.7 | 65.50 | 4.21 | dt, $J_{1a',1b'}$ = 14.6 $J_{1a',2}$ = 6.7 | 65.50 | 4.21 | dt, $J_{1a',1b'}$ = 14.6 $J_{1a',2}$ = 6.7 | 65.50 | 4.21 | dt[b] | 65.50 |
| **1b′** | 4.21 | dt, $J_{1b',1a'}$ = 14.6 $J_{1b',2}$ = 6.7 | 65.50 | 4.21 | dt, $J_{1b',1a'}$ = 14.6 $J_{1b',2}$ = 6.7 | 65.50 | 4.21 | dt, $J_{1b',1a'}$ = 14.6 $J_{1b',2}$ = 6.7 | 65.50 | 4.21 | dt[b] | 65.50 |
| **2′** | 2.76 | t, $J_{2',1a'}$ = 6.7 $J_{2',1b'}$ = 6.7 | 34.39 | 2.76 | t, $J_{2',1a'}$ = 6.7 $J_{2',1b'}$ = 6.7 | 34.39 | 2.76 | t, $J_{2',1a'}$ = 6.7 $J_{2',1b'}$ = 6.7 | 34.39 | 2.76 | dt[b] | 34.78 |
| **3′** | | | 130.55 | | | 130.55 | | | 130.55 | | | 130.00 |
| **4′** | 6.69 | d, $J_{4',8'}$ = 2.1 | 116.38 | 6.69 | d, $J_{4',8'}$ = 2.1 | 116.38 | 6.69 | d, $J_{4',8'}$ = 2.1 | 116.38 | 6.65 | d, $J_{4',8'}$ = 2.0 | 116.51 |
| **5′** | | | 145.10 | | | 145.10 | | | 145.10 | | | 145.10 |
| **6′** | | | 144.20 | | | 144.20 | | | 144.20 | | | 144.20 |
| **7′** | 6.72 | d, $J_{7',8'}$ = 8.1 | 115.85 | 6.72 | d, $J_{7',8'}$ = 8.1 | 115.85 | 6.72 | d, $J_{7',8'}$ = 8.1 | 115.85 | 6.70 | d, $J_{7',8'}$ = 8.1 | 115−116 |
| **8′** | 6.56 | d, $J_{8',7'}$ = 8.1 $J_{8',4'}$ = 2.1 | 121.19 | 6.56 | d, $J_{8',7'}$ = 8.1 $J_{8',4'}$ = 2.1 | 121.19 | 6.56 | d, $J_{8',7'}$ = 8.1 $J_{8',4'}$ = 2.1 | 121.19 | 6.53 | d, $J_{8',7'}$ = 8.1 $J_{8',4'}$ = 2.0 | 121.19 |

[a] Data correspond to the chromatogram fraction 21, dissolved in $CD_3CN$, and spectra were acquired at 25.0 °C and a magnetic field of 500 MHz. [b] Overlapped.

synthesized by Baggiolini et al. (*35*). The $^{1}H$ and $^{13}C$ NMR assignments for diastereoisomers **21a** and **21b** shown in **Table 2** are consistent with those described by Baggiolini (*35*) and MacKellar (*34*) for (5*S*,8*R*,9*S*)-ME and (5*S*,8*S*,9*S*)-ME, respectively. It is observed that chemical shifts are slightly high field shifted due to solvent effects.

To determine the relative stereochemistry of the novel compound **21c**, NOE experiments were performed together with theoretical calculations of the minimum energetic conformation of the diastereoisomeric forms of AOA. Monte Carlo molecular mechanics conformational search, with simulation of the solvent $CH_3CN$, was performed for the eight possible diastereoisomeric forms of AOA (see Materials and Methods for details). **Figure 4b** presents the baseline structures of the eight diastereoisomers (four couples of enantiomers). As shown, the dihydropyran ring adopts a half-chair conformation in each case. The relative position between

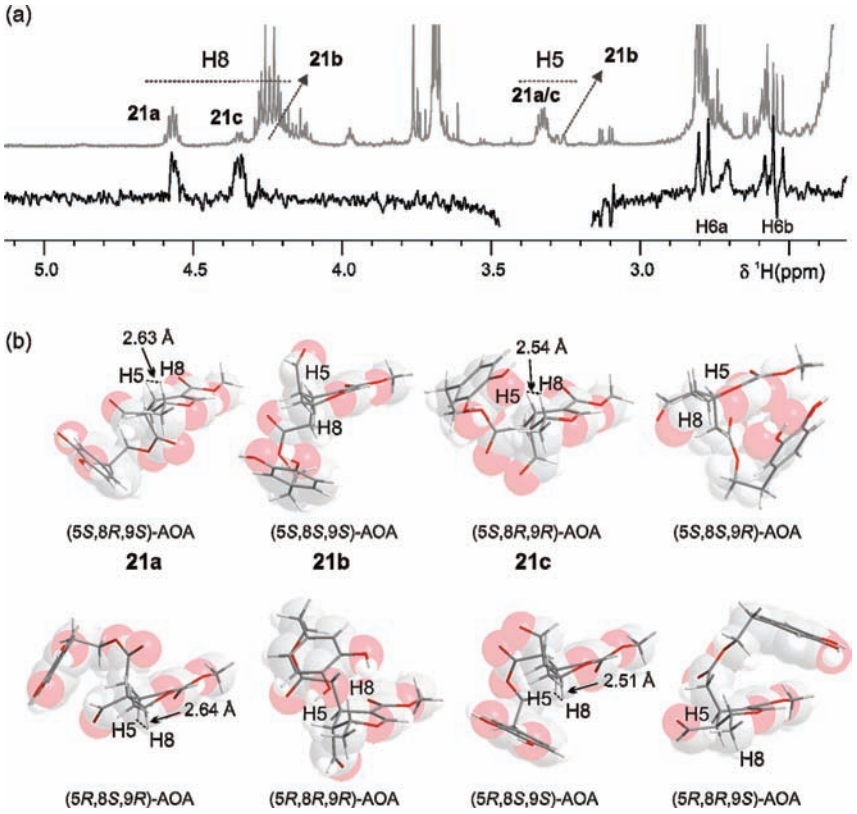


**Figure 4.** (**a**) $^1$H NMR spectrum and selective 1D NOESY spectrum with saturation of H5 signals of compounds **21a**, **21b**, and **21c**. NMR spectra correspond to chromatogram fraction 21, dissolved in $CD_3CN$, acquired at 25.0 °C and a magnetic field of 500 MHz. (**b**) Minimum energetic conformations obtained with Monte Carlo conformational search (Macromodel v. 9.0, MM3⋆ Force Field, Continuum Solvation, $CH_3CN$, simulated).

H5 and H8 in every structure is considered, and the theoretical distance between the two protons is shown in the figure. Different relative positions between these two protons is observed in the described diastereoisomers (and their enantiomers), *syn* for (5*S*,8*R*,9*S*)-AOA with a theoretical distance of 2.63 Å and *anti* for (5*S*,8*S*,9*S*)-AOA. Similar results are obtained for the other two couples of enantiomers, *syn* relative position for (5*S*,8*R*,9*R*)-AOA and its enantiomer (with theoretical distances of 2.54 and 2.51 Å, respectively) and *anti* for (5*S*,8*S*,9*R*)-AOA and its enantiomer. Considering this information, a selective 1D NOESY experiment was performed saturating the overlapped region where the H5 protons of the three compounds **21a**, **21b**, and **21c** resonate. The spectrum shows NOE between H5 and H8 of compounds **21a** and **21c**, but no NOE is observed for compound **21b** (**Figure 4a**). These results state that the theoretical model is consistent with the experimental data, because they are in agreement with the absolute configurations of **21a** and **21b** determined experimentally. On the basis of these results, the relative configuration of compound **21c** corresponds to that of the (5*R**,8*S**,9*S**)-AOA species (pair of enantiomers). Assuming, as for the described diastereoisomers (*32*), that **21c** comes from the natural OA (*32*, *36*), **21c** will correspond to (5*S*,8*R*,9*R*)-AOA.

In the present study the major constituents of the phenolic fraction of EVOO from *O. europaea* L. var. Cornezuelo were identified and characterized using the hyphenated HPLC-DAD-SPE-NMR/MS techniques. After the optimization of the chromatographic and trapping methods, the multiple UV, MS, and NMR detections led to the identification of 25 compounds, mainly simple phenols (hydroxytyrosol **1**, tyrosol **3**, vanillin **5**, *p*-coumaric acid **6**, elenolic acid **7** and **9**, and hydroxytyrosol acetate **8**), flavonoids (luteolin **12** and apigenin **19**), the lignan (+)-pinoresinol **15**, and a large number of secoiridoids (oleuropein aglycone **22**, ligstroside aglycones **23** and **24**, and aldehydic form of oleuropein aglycone **21** among others).

A complete NMR study of the complex chromatographic fraction 21 led to the differentiation of three diastereoisomers of the aldehydic form of oleuropein aglycone (**21a**, **21b**, and **21c**). The relative configuration of the new diastereoisomer was determined as (5*R**,8*S**,9*S**)-AOA on the basis of the results obtained from NOE experiments together with Monte Carlo conformational search calculations. Assuming, as for the described diastereoisomers (*32*), that **21c** comes from the natural OA (*32*,*36*), **21c** will correspond to the (5*S*,8*R*,9*R*)-AOA enantiomer. We think these results contribute toward a better knowledge of the polar fraction of extra virgin olive oil, and they could be of interest for future biological studies.

## ABBREVIATIONS USED

EVOO, extra virgin olive oil; AOA, aldehydic form of oleuropein aglycone; CE, capillary electrophoresis; UV, ultraviolet; SPE, solid phase extraction; DAD, diode-array UV−vis detector; ESI, electrospray ionization; IT, ion trap; $CD_3CN$, deuterated acetonitrile; BPC, base peak chromatogram; OA, oleuropein aglycone; COSY, correlated spectroscopy; HSQC, heteronuclear single-quantum correlation; HMBC, heteronuclear multiple bond correlation; ME, methyl elenolate; NOE, nuclear Overhauser effect.

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**Supporting Information Available:** An additional table and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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