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New isobaric lignans from Refined Olive Oils as quality markers for Virgin Olive Oils

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ABSTRACT

Herein we describe the influence of olive oil refining processes on the lignan profile. The detection of new isobaric lignans is suggested to reveal frauds in commercial extra-Virgin Olive Oils. We analyzed five commercial olive oils by HPLC-DAD-TOF/MS to evaluate their lignan content and detected, for the first time, some isobaric forms of natural (+)-pinoresinol and (+)-1-acetoxypinoresinol. Then we analyzed partially and fully-refined oils from Italy, Tunisia and Spain. The isobaric forms occur only during the bleaching step of the refining process and remain unaltered after the final deodorizing step. Molecular dynamic simulation helped to identify the most probable chemical structures corresponding to these new isobars with data in agreement with the chromatographic findings. The total lignan amounts in commercial olive oils was close to 2 mg/L. Detection of these new lignans can be used as marker of undeclared refining procedures in commercial extra-virgin and/or Virgin Olive Oils.

1. Introduction

All over the world, the Mediterranean diet is recognized for its health benefits. Olive oil is one of the most important components of this diet (Buckland & Gonzalez, 2015) with special combination of fatty acids and bioactive minor constituents, which are particularly abundant in the highest quality Extra-Virgin Olive Oils (Beauchamp et al., 2005; Cecchi, Migliorini, Cherubini, Innocenti, & Mulinacci, 2015; Cecchi et al., 2013; Frankel, 2011; Grossi et al., 2013; Migliorini, Cherubini, Cecchi, & Zanoni, 2013; Salvini et al., 2006).

According to the International Olive oil Council (IOC), Virgin Olive Oils are obtained from olive fruit solely by mechanical or physical means under conditions that do not lead to alterations in the oil. Depending on their chemical and organoleptic properties, Virgin Olive Oils are classified as Extra Virgin Olive Oil (EVOO), Virgin Olive Oil (VOO), Ordinary Virgin Olive Oil (OVOO) or Lampante Virgin Olive Oil (LVOO). The oils belonging to this latter category need to be refined to make it edible.

VOOs and, above all EVOOs, are widely appreciated for their health benefits and sensorial properties. The health benefits are mainly related to a high presence of monounsaturated fatty acids, mainly oleic acid (Cohen, Epstein, Pittman, & Rivenson, 2000), and phenolic compounds (Coccia et al., 2014; Covas et al., 2006). The presence of these latter compounds has allowed the EFSA to approve the health claim, "the olive oil polyphenols contribute to the protection of blood lipids from oxidative stress" (EFSA Panel on Dietetic Products, 2011). The sensory properties are also influenced by this fraction together with the volatile compounds mainly derived from the lipoxygenase pathway (Andrewes, Busch, De Joode, Groenewegen, & Alexandre, 2003; Bendini et al., 2007; Gutièrrez-Rosales, Rios, & Gomèz-Rey, 2003). All these properties justify the higher price of the EVOOs and VOOs when compared with other edible oils (Tena, Wang, Aparicio-Ruiz, Garcìa-Gonzalez, & Aparico, 2015).

Oils of lower commercial value include Olive Oil (OO), which, according to the IOC, consists of a blend of Refined Olive Oil (ROO) and Virgin Olive Oils (IOC). The country of retail sale may

Abbreviations: EVOO, Extra Virgin Olive Oil; VOO, Virgin Olive Oil, as sub category of virgin olive oil; OVOO, Ordinary Virgin Olive Oil; LVOO, Lampante Virgin Olive Oil; OO, Olive Oil; ROO, Refined Olive Oil; cv, cultivar; EI, Extract ion; IOC, International Olive oil Council; EFSA, European Food Safety Authority.

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require a more specific designation; regarding Italy, Virgin Olive Oil used to prepare OOs has to be different from LVOO, according to European Regulation 1308/13 (2013).

Oil refining is a physical-chemical multi-step process applied to LVOOs and other common seed oils to make them edible. This process requires, at least, a deacidification treatment and a deodorization as last step. It has been reported that phenolic compounds disappear in olive oil after the refining process, with the exception of lignans, (+)-pinoresinol and (+)-1-acetoxypinoresinol (Garcìa, Ruiz-Mendez, Romero, & Brenes, 2006). Nevertheless, the amount of these lignans in VOOs or crude LVOOs is consistently higher than in the refined oils (Garcìa et al., 2006; Owen et al., 2004).

Due to their high price, EVOOs, VOOs and OOs are very attractive targets for fraudsters. The most common frauds are: i) adulteration consisting of mixture of different categories of olive oils; ii) adulteration consisting of mixture with other vegetable oils. Consequently, there is a continuous search for new markers to detect adulterations and to guarantee the quality and safety of EVOO (Tena et al., 2015).

The lignans are a group of phytochemicals widespread in plants; they belong to the class of phytoestrogens and are beneficial for human health (Fini et al., 2008). Some of them are typical of *Olea europaea* L. and, although their quantity in olive oils is comparable to other classes of phenolic compounds, they were discovered 10 years after the first studies appeared on the oleuropein derivatives (Brenes et al., 2000). Lignans are the most abundant phenolic compounds after secoiridoids in the Virgin Olive Oils (Bonoli, Bendini, Cerretani, & Lercker, 2004). Their concentration mainly depends on the cultivar while the milling process does not affect their amount in a significant manner (Servili et al., 2014). To date, it is not yet clear how they are transferred from olives into oils (Oliveras-Lopéz et al., 2008) and how their amount changes after the chemical/physical treatment of oil.

The principal lignan in almost all Virgin Olive Oils from different cultivars is (+)-1-acetoxypinoresinol with minor amounts of (+)-pinoresinol (Owen et al., 2004), hydroxypinoresinol and syringaresinol (Ballus et al., 2015). On the other hand, (+)-1-acetoxypinoresinol is the minor lignan compared to (+)-pinoresinol in the Picual cultivar oils, which represent approximately 25% of the world's production of olive oil.

(+)-Pinoresinol has also been proposed as a marker to authenticate from Picual (Brenes, Garcia, Rios, Garcia, & Garrido, 2002).

The main goals of this study were to investigate the influence of the olive oil refining process on the lignan profile and to propose the detection of new isobaric lignans as chemical markers of undeclared refining procedures in commercial EVOOs. We analyzed five Italian commercial OOs and three series of partially and fully-refined oils from Italian, Tunisian and Spanish industrial production. A mechanism for the formation of new isobaric lignans during the bleaching step is proposed by a comparison between the chromatographic findings and data from a dynamic molecular modeling study.

2. Materials and methods

2.1. Chemicals

All chemicals for the analyses were of analytical reagent grade: deionized water was produced by the Milli-Q-system (Millipore SA, Molsheim, France). Ethanol and *n*-hexane of analytical reagent grade and formic acid and acetonitrile of LC-MS grade were from J. T. Baker (Phillipsburg, New Jersey, USA). (+)-Pinoresinol from Sigma-Aldrich (Steinheim, Germany) was used as a standard compound. All stock solutions containing the standard (+)-pinoresinol were prepared in ethanol.

2.2. Samples

We sampled five Italian commercial OOs and three series of samples including oils collected at different stages of the refining process, as summarized in Table 1. The oils codified as "Italy", "Spain" and "Tunisia" are samples purchased from Italy, Spain and Tunisia respectively and kindly obtained by an Italian factory of commercial olive oils. Each sample was representative of an industrial batch and was kept in the dark and stored at room temperature until the time of analysis. All the analyzed samples were purchased in 2014–2015 and identified as follow: L for Crude Lampante Olive Oil; N for Neutral Oil; D for Bleached Oil and R for Refined Oil (Table 1).

2.3. Refining process

The Italian Lampante Olive Oil (L_{ITA}) was subjected to a first chemical deacidification at room temperature, to obtain an oil with an acidity of approximately 7.8% and to a second step of deacidification at 90 °C, giving an oil with an acidity of approximately 1.0% (N_{ITA}). In the following step the oil was bleached by using 1.5% of active earth at 90–100 °C and 40–50 mmHg (D_{ITA}). Finally, the oil was deodorized for 2.5 h at 230 °C and 1.5 mmHg (R_{ITA}).

The Spanish Lampante Olive Oil (L_{SPA}) was subjected to a physical treatment consisting of a degumming process (N_{SPA}). The obtained oil was bleached with 0.8% of active earth (D_{SPA}) and then deodorized for 2.5 h at 230 °C and 1.5 mmHg (R_{SPA}).

The Tunisian Lampante Olive Oil (L_{TUN}) was subjected to a chemical deacidification at 90 °C, to obtain an oil with an acidity

Table 1
List of the analyzed samples: in the different columns are indicated the provenience, the sample derived by a specific refining step, the codifying and a short description of the treatment applied to each refining step.

Provenience	Sample type	Code	Treatment
Italy	Crude Lampante Olive Oil	L_{ITA}	Crude Lampante Olive Oil; acidity ≈ 8.8%
	Neutral Oil	N_{ITA}	Chemical deacidification at room temperature and then at 90 °C; acidity ≈ 1.0%
	Bleached Oil	D_{ITA}	Bleaching with 1.5% of active earth at 90–100 °C and 40–50 mmHg
	Refined Oil	R_{ITA}	Deodorization for 2.5 h at 230 °C and 1.5 mmHg
Spain	Crude Lampante Olive Oil	L_{SPA}	Crude Lampante Olive Oil; acidity ≈ 9.0%
	Neutral Oil	N_{SPA}	Physical treatment: degumming
	Bleached Oil	D_{SPA}	Bleaching with 0.8% of active earth
	Refined Oil	R_{SPA}	Deodorization for 2.5 h at 230 °C and 1.5 mmHg
Tunisia	Crude Lampante Olive Oil	L_{TUN}	Crude Lampante Olive Oil; acidity ≈ 9.0%
	Neutral Oil	N_{TUN}	Chemical deacidification at 90 °C; acidity ≈ 1.0%
	Bleached Oil	D_{TUN}	Bleaching with 0.8% of active earth
	Refined Oil	R_{TUN}	Deodorization for 2.5 h at 230 °C and 1.5 mmHg

of approximately 1.0% (N_{TUN}). The obtained oil was bleached with 0.8% of active earth (D_{TUN}) and then deodorized for 2.5 h at 230 °C and 1.5 mmHg (R_{TUN}). The initial acidity values of all these oils are reported in Table 1.

The active earths used for the bleaching step are bleaching earths "CLINOLIP CS 1060" mixed with 10% powder active carbon "FILTRACARB SK1-P75.

2.4. Extraction of phenolic compounds from the oils

The extraction conditions to recover the phenolic fraction were the same of our previous works (Oliveras-Lopéz et al., 2007). Briefly, approximately 20 g of oil were extracted in 60 mL of the $EtOH/H_2O^+$ 70:30 solution by stirring for 30 min. The hydroalcoholic solution was brought to a pH = 3.2 with formic acid. The mixture was then defatted three times with 20 mL of hexane. During the defatting of samples L_{SPA}, N_{SPA}, L_{TUN}, N_{TUN}, the separation between the hydroalcoholic and hexane phase was incomplete so that it was necessary to use 60 mL of hexane for the first defatting step. The hydroalcoholic solution was evaporated under reduced pressure at approximately 35 °C and the residue was redissolved with 1.5 mL $EtOH/H_2O^+$ 70:30 solution. The sample was then centrifuged at 14,000 rpm and 10 °C, and the supernatant immediately used for chromatographic analysis.

To evaluate the efficiency of the extractive procedure on the recovery of lignans, a spiking test was also used. Two amounts of (+)-pinoresinol, 0.36 and 0.73 mg_{PIN}/kg_{OIL}, were added to D_{TUN} oil and the percentage of recovery was evaluated by TOF Mass Spectrometer.

2.5. HPLC/DAD/TOF-MS analysis of lignans

The analyses were performed using an HP 1100L Liquid Chromatograph. The detector was a DAD coupled to a TOF Mass Spectrometer equipped with an electrospray (ESI) interface (all from Agilent Technologies, Palo Alto, CA, USA). The analysis parameters were set using a negative ion mode with spectra acquired over a mass range of 100–800 *m*/*z*. The conditions of the ESI source were as follows: drying gas (N_2) temperature, 350 °C; drying gas flow-rate, 6 L/min; nebulizer, 20 psi; capillary voltage, 4000 V; fragmentation, 150 V; skimmer, 60 V. A 150 mm × 3 mm i.d., 2.7 μm Poroshell 120, EC-C18 column (Agilent, USA) equipped with a precolumn of the same phase was used; oven temperature 26 °C. The acquisition and data analysis were controlled using Agilent LC-MS TOF Software (Agilent, USA).

The solvents for the mobile phase were (A) 0.1% formic acid/water and (B) CH_3CN; the multi-step linear solvent gradient used was: 0–5 min 10–15% B; 5–15 min 15–30% B; 15–20 min 30–35% B; 20–23 min 35–40% B; 23–26 min 40–45% B; 26–32 min 45–100% B; 32–37 min 100% B; 37–42 min 100–10% B; equilibration time 10 min; flow rate 0.4 mL min^{-1}; injection volume 2 μL. The following wavelengths were simultaneously selected: 240 nm, 280 nm, 330 nm, 350 nm and 540 nm.

The detection of lignans was carried out at 280 nm by comparing their UV–vis and mass spectra and with the pure standard, (+)-pinoresinol. The TOF mass spectrometer was calibrated immediately before the analyses. No internal reference was used during the analyses. The accurate mass of the molecules was measured; the mass accuracy was checked by analyzing the (+)-pinoresinol standard in the same conditions used for the samples. To calculate the elemental compositions, a maximum difference of 10 ppm between calculated and measured was considered. A mass difference always less than 6 ppm was observed for (+)-pinoresinol and its possible isomers and for (+)-1-acetoxypinoresinol and its isobaric species calculated on the deprotonated molecular ion.

The lignans were quantified only in the fully refined oils (ROOs) because this category of oil is used to prepare the commercial OOs. The total amount was the sum of (+)-pinoresinol, (+)-1-acetoxypinoresinol and their isobaric forms; the data were expressed as mg_{PIN}/kg_{oil}. This molecule was used as external standard to build a five-point calibration curve at 280 nm, linearity range 0–1.21 μg and R^2 0.9999.

2.6. Dynamic molecular modeling

The chemical structures of the new isobaric forms of lignans were created starting from the structures of the natural (+)-pinoresinol and (+)-1-acetoxypinoresinol (Brenes et al., 2000; Owen et al., 2000) by using the Discovery Studio 3.5 Visualizer free program (Accelrys, San Diego, USA). The partial atomic charges were derived using the AM1-BCC method implemented in the ANTECHAMBER suite (Wang, Wang, Kollman, & Case, 2006). The energy minimizations and MD (Molecular Dynamic) were carried out using the SANDER module of AMBER 9 (Case et al., 2006) with the GAFF (Wang, Wolf, Caldwell, Kollman, & Case, 2004) force field. Molecular dynamic simulations were performed in implicit solvent using the Generalized Born Surface Area (GBSA) at constant pH. The constant pH molecular dynamics method has been implemented in SANDER (Mongan, Case, & McCammon, 2004). Before the dynamic simulation, 100 steps of steepest-descent and 900 steps of conjugate-gradient minimization on the entire complex were performed with a modified GB model with igb = 2 (Onufriev, Bashford, & Case, 2004), the surface area was computed and included in the solvation term, and a cutoff of 30 A° for non-bonded interactions was used. The system was then heated from −273.15 °C to 90 °C in 10 ps by holding the complex fixed with a harmonic constraint of a strength of 0.05 kcal/(mol $Å^2$). After the minimization and heating, to equilibrate the system, 0.1 ns of dynamic simulations, with the molecular constraint (strength of 0.01 kcal/(mol $Å^2$), were performed at constant temperatures of 27 and 90 °C. Finally, 3 ns dynamic simulations, with the complex constraint (strength of 0.01 kcal/(mol $Å^2$), were performed at a constant temperature of 27 and 90 °C with SHAKE turned on for bonds involving hydrogens, allowing a time-step of 2.0 fs. 300 conformations were collected during the simulation (one conformation every 10 ps). The stability of the different molecules was reported as the mean of 300 measurements and expressed in kcal/mol.

Data were subjected to analysis of variance using Microsoft Excel statistical software and F-Test ($P < 0.05$) was performed for statistical significance. The means were then compared by Fisher's LSD test by using the software DSAASTAT v. 1.1 (Onofri A., 2007).

3. Results and discussion

3.1. Phenolic profiling of commercial olive oils

Five commercial OOs were purchased and analyzed with the aim of evaluating their phenolic content, and especially their lignan content. As required by law, these oils are a mix of ROOs and Virgin Olive Oils and, due to the presence of these latter oils, it was expected to find several phenolic compounds. This hypothesis was confirmed only for three out of five samples highlighting at 280 nm the presence of several minor phenols. Surprisingly, the chromatographic profile at 280 nm of one sample was empty, while another oil showed four peaks in the typical chromatographic range of the phenolic compounds (Fig. 1A, rt 20–26 min). The UV and mass-TOF spectra of these analytes allowed us to identify (+)-pinoresinol and (+)-1-acetoxypinoresinol, together

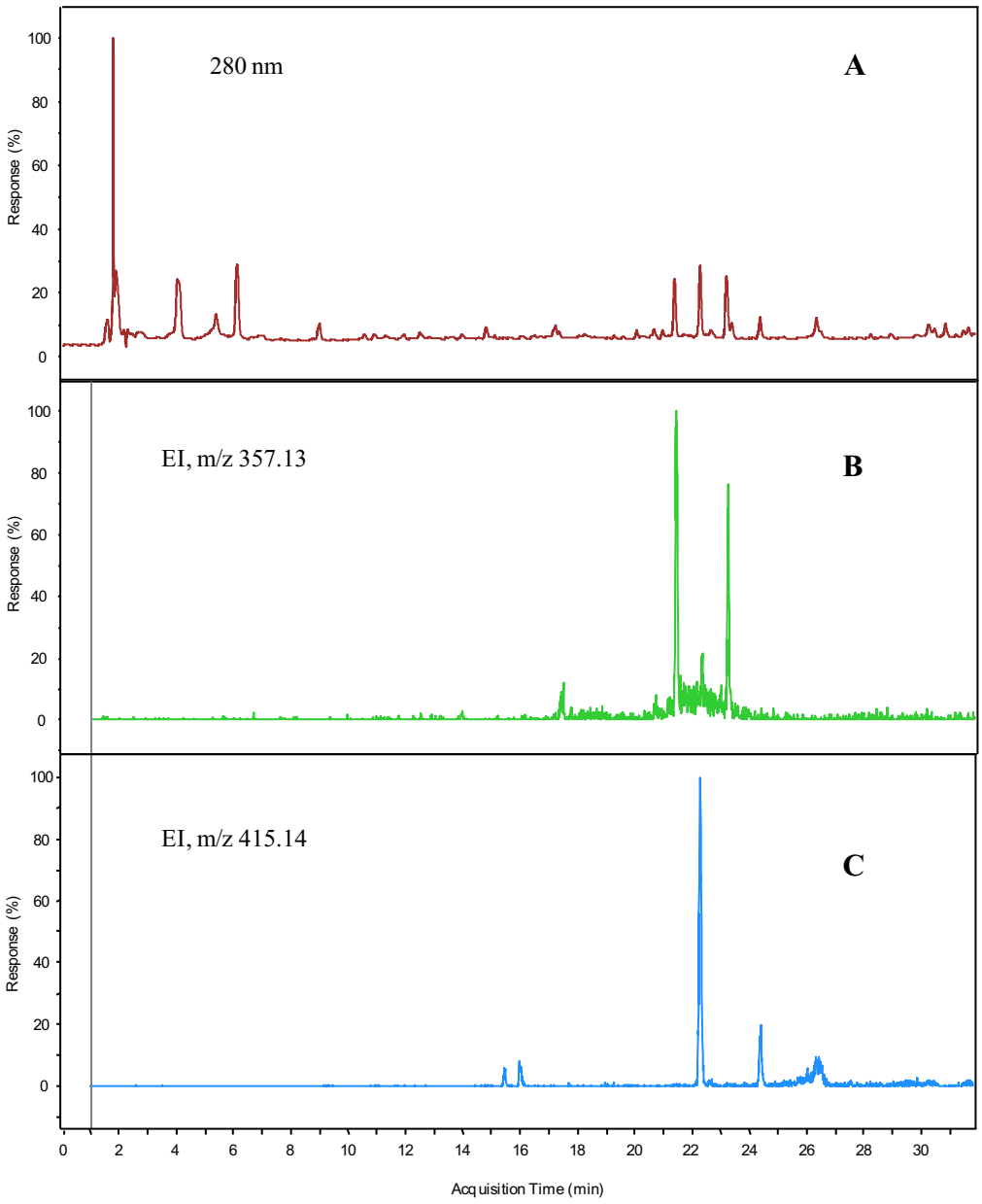

Fig. 1. Chromatographic profiles at 280 nm of an Italian commercial Olive Oil: (A), Extract Ions at *m/z* 357.13 for (+)-pinoresinol (B) and at 415.14 for (+)-1-acetoxypinoresinol (C).

with their isobaric forms as clearly shown by the Extract Ion profiles (EI) at *m/z* 357.13 and *m/z* 415.14 (Fig. 1B and C).

To the best of our knowledge, the presence of isobaric forms of lignans has never been described in VOOs and EVOOs derived only by mechanical means. Consequently it was hypothesized that their presence was induced by the refining process applied to LVOO. In light of this new evidence we decided to further investigate the origin of these new lignans by analyzing oil samples obtained after the different steps of the industrial refining process.

3.2. Phenolic profiles of partially and fully Refined Olive Oils

Aiming to work on a pool of representative samples obtained after industrial refining, three series of oils derived from different LVOO (Table 1) were extracted and analyzed by HPLC/DAD/MS-TOF. To date, only one work (Garcìa, Ruiz-Mendez, Romero, & Brenes, 2006) was focused on phenolic determination in partially and fully refined LVOO, with a minor attention to lignan fraction.

The use of TOF allowed us to detect the accurate mass of lignans, and confirm the presence of the new isobars, identifying the step responsible for this formation. Fig. 2A, B, C and D compare the profiles at 280 nm and the corresponding Extract Ions (*m/z* 357.13 and *m/z* 415.14) for L_{TUN}, N_{TUN}, D_{TUN} and R_{TUN}. It immediately appears that the number and intensity of all the peaks detected at 280 nm strongly decreases after every step of the refining process with a total reduction of approximately 90% during the whole process from L_{TUN} to R_{TUN}.

The very intense peak in chromatogram of D_{TUN} oil, (rt close to 27 min, Fig. 2C), absent in lampante oil, was probably formed during the bleaching step but it disappeared after deodorization presumably because of its volatility and/or low thermal stability. Analogous behavior was observed in the corresponding Italian oils. Regarding EI profiles at *m/z* 357.13 of L_{TUN} and N_{TUN}, the UV and

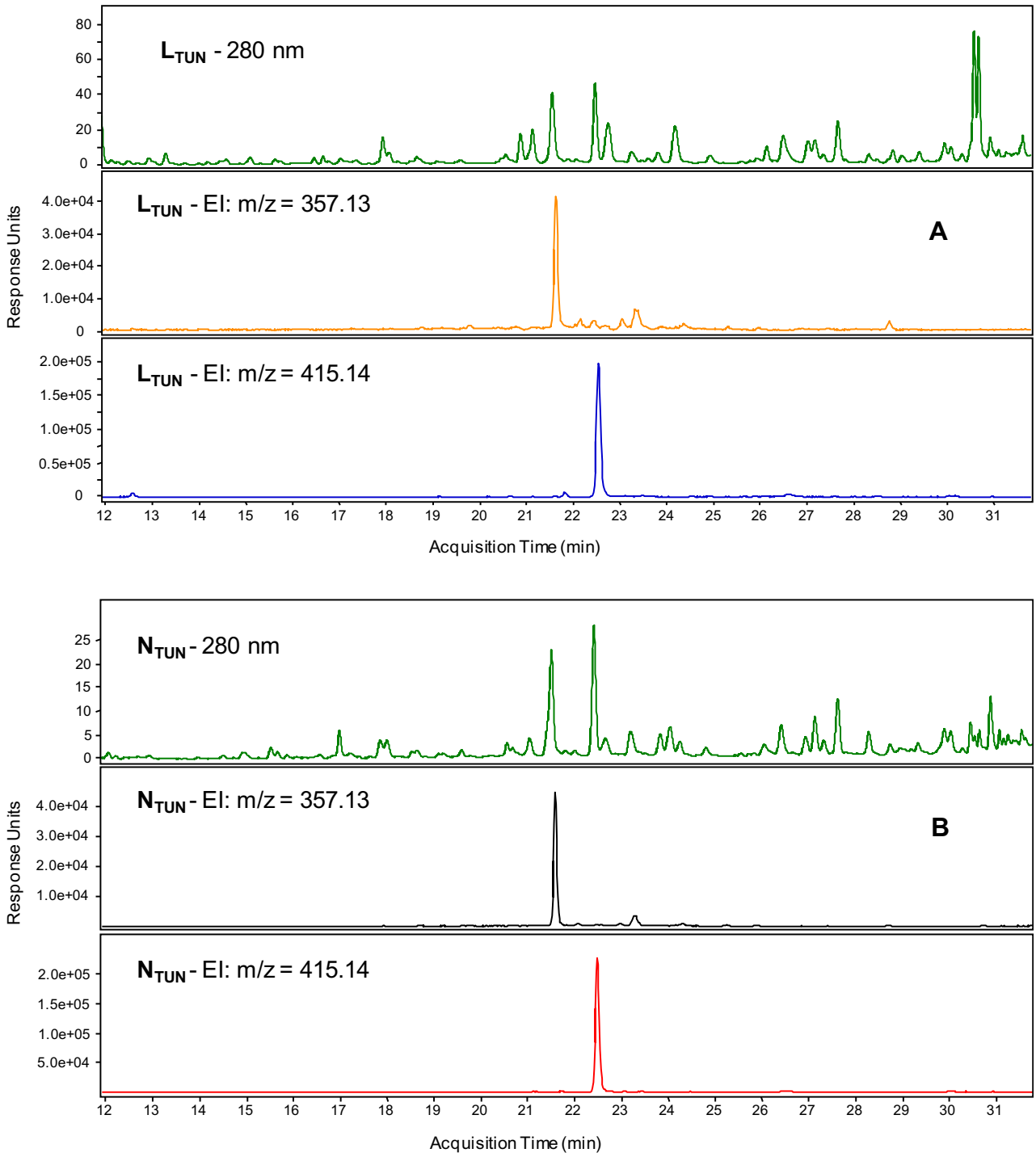

Fig. 2. Comparison between chromatograms at 280 nm, EI at *m/z* 357.13 and at *m/z* 415.14 for the Tunisian samples: L_{TUN} (A), N_{TUN} (B), D_{TUN} (C) and R_{TUN} (D).

mass spectra of the smaller peaks at rt 22–23.5 min did not fit with those of known phenols. Their further characterization was not carried out since was not part of the scope of this study.

The chromatograms at 280 nm for L_{TUN} (Fig. 2A) and N_{TUN} (Fig. 2B) show no qualitative differences but only a general decrease in the intensity of all the peaks. The corresponding EI chromatograms of Tunisian crude (2A) and neutral (2B) oils almost overlap, and only after the bleaching step (2C) do the isobaric analogues of (+)-pinoresinol and (+)-1-acetoxypinoresinol appear. According to previous data (Garcìa, Ruiz-Mendez, Romero, & Brenes, 2006), and confirmed by the lower intensity of N_{TUN} (Fig. 2C) *versus* D_{TUN} (Fig. 2B) in the EI profiles, refining consistently reduces the amount of all the phenolic compounds. Nevertheless, the bleaching step also induces a chemical modification of the remaining lignans. To the best of our knowledge, this effect has never been reported before. Finally, it should pointed out that the last step of the refining process, namely deodorization, does not induce further significant changes in the EI profiles.

Regarding the new isobaric lignans detected in D_{TUN}, the EI at *m/z* 315.13 (Fig. 2C) shows two peaks with comparable intensity, UV and mass spectra (Supplementary material, A-B) in agreement with those of (+)-pinoresinol. Two diagnostic ions, $[M-H]^-$ and its

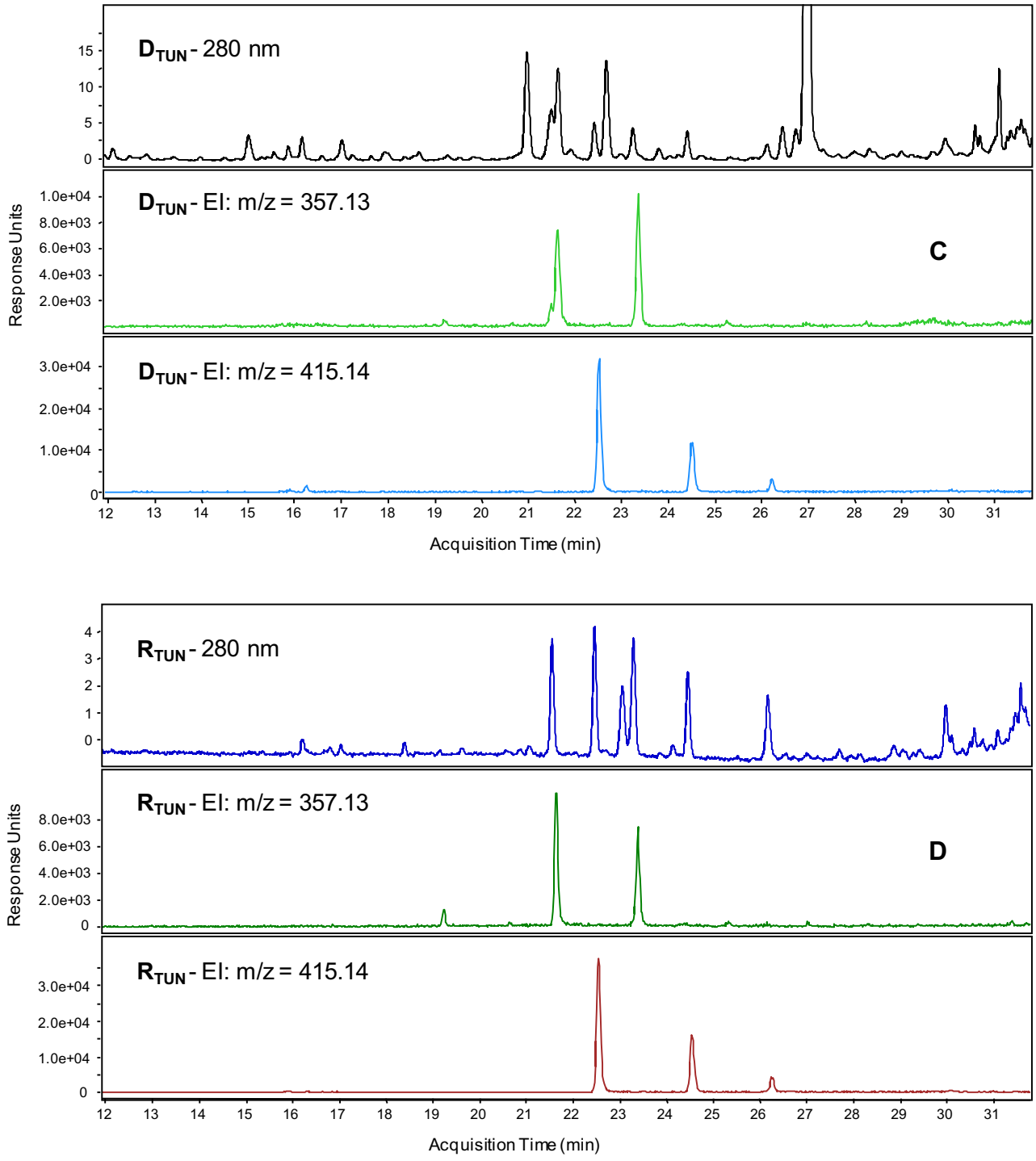

Fig. 2 (*continued*)

adduct with formic acid $[M+HCOOH]^-$, were detected for both the (+)-pinoresinol and its isobar at rt 23.3 min. Similarly, the EI profiles at *m/z* 415.14 (Supplementary material, C-D-E) pointed out three peaks with the same UV and mass spectra of (+)-1-acetoxypinoresinol. The diagnostic ions were again the $[M^-H]^-$ and the corresponding adducts with formic acid. Furthermore, two new isobars at rt 24.5 min and rt 26.2 min were detected.

The mass spectra in negative ionization mode of the natural lignans and of the three new isobaric forms are reported in Supplementary material. None of these spectra show the presence of fragment ions and the relative intensity of the molecular ion and its adduct with formic acid are very similar. Our findings in Tunisian oils highlight that the new isobaric forms of (+)-pinoresinol and (+)-1-acetoxypinoresinol are formed during the bleaching step of the refining process.

Regarding the Italian oils, the chromatogram at 280 nm of L_{ITA} presents several peaks attributable to phenolic compounds, with the most intense one corresponding to (+)-1-acetoxypinoresinol, and again the EI profiles confirmed the co-presence of (+)-pinoresinol and the absence of isobaric forms. The corresponding

profiles at 280 nm for neutral and bleached oils show the same variations in this series as previously described for the Tunisian oils. The UV and mass spectra of peaks in D_{ITA} and R_{ITA} confirm the presence of the same isobars already identified in the Tunisian oils and this formation is again induced by the bleaching step.

The third series of samples were from the refining process applied of a Spanish oil. The profile at 280 nm of the lampante oil (L_{SPA}) presents several peaks: the most intense one was (+)-pinoresinol while the peak of (+)-1-acetoxypinoresinol appeared consistently lower than the lampante Tunisian and Italian oils. This result suggests the presence of Picual, a typical Spanish cultivar in which the relative concentration of pinoresinol/acetoxypinoresinol is inverted compared to the majority of Virgin Olive Oils (Brenes et al., 2000) obtained from other cultivars in which the dominant lignan is always (+)-1-acetoxypinoresinol (Ballus et al., 2015).

The chromatograms for neutral and bleached oils show the same variations already described for the Tunisian and Italian series: the intensity of the peaks relating to (+)-1-acetoxypinoresinol and its isobaric forms is very low compared with (+)-pinoresinol, and the formation of isobaric forms of lignans occurred again during the bleaching step.

The next step focused on elucidating the structure of these new isobars by using a molecular dynamic simulation, whose rationale entails the following considerations. The mass spectra obtained by TOF are accurate enough to confirm the presence of the previously cited isobars, because ions at 315.13 *m/z* and 415.14 *m/z* in olive oil can be associated only with (+)-pinoresinol and (+)-1-acetoxypinoresinol, respectively. The extraction and purification of these isobars requires the treatment of several liters of bleached oil because their final content is very low (less than 2 mg/kg_{oil} as sum – see also next paragraph), their molecular weights are relatively high and the extractive yields are far away from 100%. Consequently, the dynamic molecular modeling at 27 and 90 °C was selected as a possible approach to acquire more information on the chemical structure of these new lignans. The chemical rearrangement behind this isomerization is discussed in the next paragraph.

3.3. Chemical rearrangement of lignans during the bleaching step

The UV spectra of new lignans, (+)-pinoresinol and (+)-1-acetoxypinoresinol, are identical indicating the presence of the same chromophore. MS-TOF analyses of isobaric forms showed exactly the molecular weight of the two natural precursors. In light of these findings no losses of groups of atoms and no definitive ring opening take place during this chemical rearrangement. To explain these isomerization it was hypothesized a ring opening/closing mechanism involving one of the C-O bond of the tetrahydrofuranic rings.

If this rearrangement involves the achiral carbons (C4 and C8, Fig. 3) the result is the re-formation of the natural lignan. On the other hand, if the rearrangement involves one chiral carbon (C2 or C6, Fig. 3), the re-formation of diastereomeric products is also possible. Taking into account the proposed chemical rearrangement and due to the presence of a C_2 axis perpendicular to the plane, we expect only one new isobar from (+)-pinoresinol. The chromatographic analyses (EI at $m/z = 357.13$ in Fig. 2C), agree with our hypothesis pointing out only one isobaric form. In the

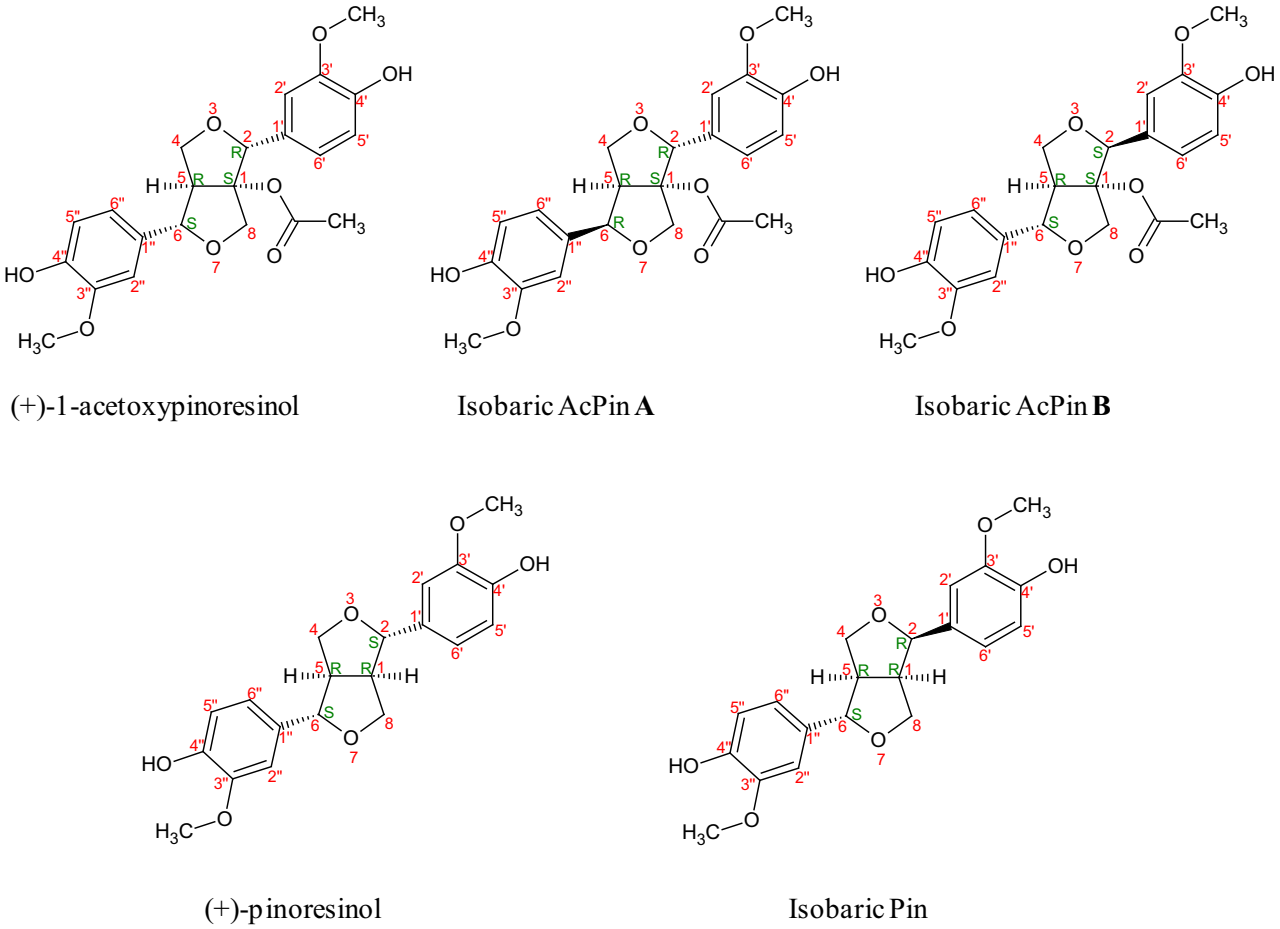

Fig. 3. Chemical structures of natural (+)-pinoresinol, (+)-1-acetoxypinoresinol and their new isobaric forms.

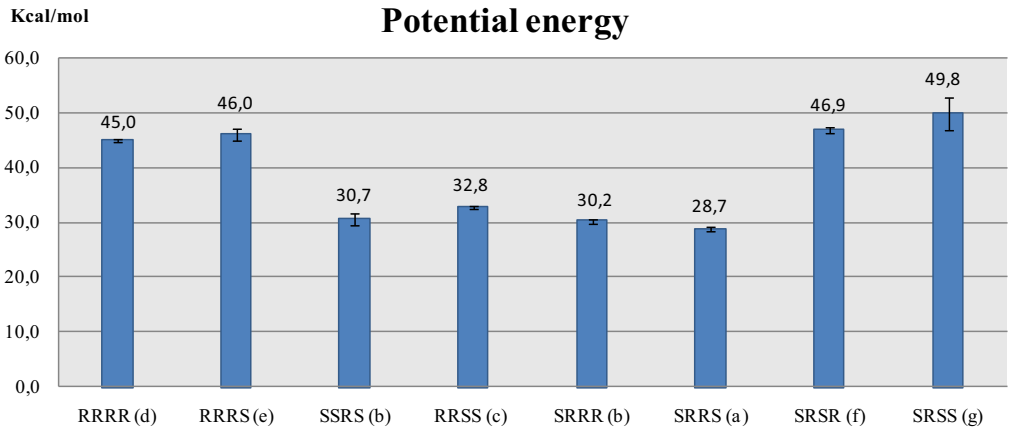

Fig. 4. Potential energy of all possible different diastereoisomers of (+)-1-acetoxypinoresinol from dynamic molecular modeling at 90 °C. Each isomers is identified by the configuration of C1, C2, C5 and C6 according to Fig. 3. Different letters (in brackets) point out significant differences by Fisher's LSD test.

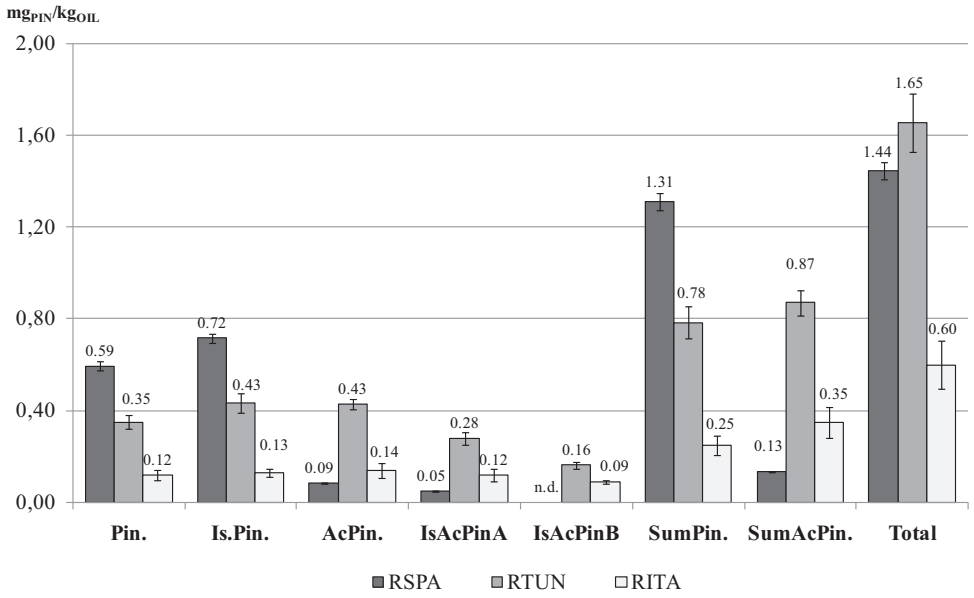

Fig. 5. Lignan content of refined oils; *Pin*, (+)-pinoresinol; *AcPin*, (+)-1-acetoxypinoresinol; *Is.Pin*, Isobar of pinoresinol; *Is.AcPinA*, Isobar A of acetoxypinoresinol; *Is.AcPinB*, Isobar B of acetoxypinoresinol; *SumPin*, sum of pinoresinol and its isobar; *SumAcPin*, sum of acetoxypinoresinol and its isobars; *Total*, sum of all lignans.

case of (+)-1-acetoxypinoresinol, no axis of symmetry is present in the molecule, so we can have really two new diastereoisomers from this lignan. Again, the chromatographic behavior agrees with the previous hypothesis (Fig. 2C, EI at m/z = 415.14) showing two isobaric forms.

The potential energies of all the possible diastereoisomers derived by the natural (+)-pinoresinol and (+)-1-acetoxypinoresinol, were calculated by applying dynamic molecular simulation. For (+)-1-acetoxypinoresinol with 4 chiral carbons and no symmetry axis, 16 (2^4) different diastereoisomers are possible, corresponding to 8 couples of enantiomers. The enantiomers are not distinguishable by chromatographic systems without chiral components and their potential energies are the same, consequently the structures of only eight diastereoisomers were considered for the calculations. The means of potential energies after dynamic simulations of the eight diastereoisomers are shown in Fig. 4. The more probable diastereoisomers were created starting from the natural (+)-1-acetoxypinoresinol (1*S*, 2*R*, 5*R*, 8*S*) by the inversion of *C*2 or C6 configuration, so obtaining two structures, called isobaricAcPinA and isobaricAcPinB (Fig. 3). The histogram highlights these two isobars as the most stable isomers with energy values comparable with that of the natural (+)-1-acetoxypinoresinol. These findings agree with the chromatographic results in EI profiles at m/z = 415.14 (Fig. 2C and D), where the natural form is the most abundant, followed by different amounts of only other two isobars. Similarly, for (+) pinoresinol the potential energy values (data no shown) are in agreement with the chromatographic results.

3.4. Determinations of lignans amount in refined oils (ROOs)

After the preliminary investigation on partially and fully refined LVOOs, we selected a pool of ROOs. Indeed, only this latter type of refined oils are used, in blends with Virgin Olive Oils, to obtain the commercial OOs destined for human consumption. We confirmed the possibility of carrying out this determination using an HPLC-DAD method at 280 nm without the need of more expensive and often unavailable mass detectors.

To evaluate the efficiency of the extractive procedure on lignan recovery, spiking tests were carried out using the D_{TUN} oil and

known amounts of (+)-pinoresinol. The results of these experiments, evaluated by the integration of the peak areas in the lignan EI profiles, highlighted a recovery of 95% when the spiking was 0.36 mg_{PIN}/kg_{OIL} and 98% when the spiking was 0.73 mg_{PIN}/kg_{OIL}. These results indicated that the extractive method guarantees almost the full recovery of lignans.

We selected the HPLC-DAD at 280 nm using (+)-pinoresinol as external standard with the aim of providing a simple analytical tool to estimate the lignan content in ROOs but also to detect frauds in the commercial OOs. Overall, the total amount of lignans in these oils was very low (Fig. 5) and always below 2 mg/kg_{OIL}; in R_{TUN} it was slightly higher than R_{SPA} and much higher than R_{ITA}; the (+)-pinoresinol content was higher than (+)-1-acetoxypinoresinol only in R_{SPA} oil in agreement with the hypothesis of a large presence of Picual cv.

4. Conclusions

To the best of our knowledge, isobaric forms of lignans have never been described in edible olive oils before this report. We have confirmed their presence in three series of olive oils of different origin and derived by an industrial refining process. We have also demonstrated how the bleaching step induces this isomerization. The proposed mechanism of rearrangement was confirmed by a dynamic molecular simulation which provided results in agreement with our analytical findings.

The isobaric forms of (+)-pinoresinol and (+)-1-acetoxypinoresinol, never detected before, can also be evidenced by the use of HPLC-DAD systems, although mass spectrometric analysis is recommended to definitively confirm their presence.

Further investigations on a wider number of commercial olive oils are required for evaluate the minimum amount of refined oil, illegally added to VOOs, that this method is able to detect. The validation of the analytical method is the next step to improve the study.

These new isobaric lignans are proposed, to the producers and the analysts responsible for the oil quality control, as chemical markers in detecting frauds regarding the application of undeclared refining procedures in extra-virgin or Virgin Olive Oils. The detection of these markers is possible by HPLC-DAD and without the need of more expensive mass spectrometric detectors. This aspect strongly facilitates the application of the method as a routine control for the oil quality in the next future

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.09.132.

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