Simultaneous analysis of natural pigments and E-141i in olive oils by Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

This work describes the development of an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) method for the determination of carotenoids (ß–carotene, lutein, ß–criptoxanthin, neoxanthin, violaxanthin) and chlorophylls, as well as their related compounds (chlorophyll A and B, pheophytin A and B and the banned dyes Cu–pyropheophytin A, Cu–pheophytin A and B) in olive oils. For this purpose, the feasibility of electrospray (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) for the ionization of these compounds was evaluated and compared. Tandem mass spectrometry (MS/MS) fragmentation was discussed for each family of compounds and the most characteristic and abundant product ions were selected to propose a selective and sensitive UHPLC–MS/MS method. The best results were obtained using APCI and APPI, while ESI provided the worst signal-to-noise ratio (S/N) for all compounds. For the analysis of olive oils, a simple solid phase extraction (SPE) with silica cartridges was applied before the determination by UHPLC–MS/MS (APCI and APPI) in multiple reaction monitoring (MRM) mode. Method quality parameters were stablished and the results demonstrate the good performance of the new methods, providing low limits of detection (0.004 – 0.9 mg L^{-1}), high extraction efficiencies (62 − 95%) and low matrix effects (<25%). The developed UHPLC–API–MS/MS (APCI and APPI) methods were applied to the analysis of olive oil samples and ß–carotene, pheophytin A, pheophytin B and lutein were detected and quantified in all of them at concentrations ranging from 0.1 to 9.5 mg L^{-1}.

Keywords: Natural pigments; olive oil; atmospheric pressure chemical ionization; atmospheric pressure photoionization; ultra-high performance liquid chromatography-tandem mass spectrometry

Introduction

In the olive oil production, olives are pressed in mills to get the juice by mechanical or other physical processes that do not change the taste, smell and color of the olive oil. These procedures give rise to products known as virgin olive oil (VOO) or extra virgin olive oil (EVOO). Oils that do not achieve certain organoleptic properties are considered defective – so-called lampante oil – not suitable for human consumption without a further refining. For commercialization, this oil is refined and improved by admixing 20–30% of VOO and the resulted product is referred as olive oil (OO) [1]. Because the color is an organoleptic parameter of olive oil and one of the most important characteristics for consumers to evaluate its quality, some producers have reduced the costly addition of 20-30% VOO and substituted it by the addition of a green dye to regreen the olive oil and to sell it as high quality product [2–3]. However, this practice is considered fraudulent in olive oils and their presence should be controlled.

Pigments such as chlorophylls and carotenoids are responsible of the olive oil color. Carotenoids present in olive oil are polyisoprenoid compounds constituted by a long alkyl chain and two cyclohexenyl rings in their structure, whereas chlorophylls are characterised by the presence of a chlorin structure (three pyrroles and one pyrroline coupled through four =CH– linkages) with a magnesium atom bonded to it (Fig. 1). Additionally, the chlorin ring can have different side chains, usually consisted in a phytol chain. These compounds are biosynthesised in nature, play an important role in the antioxidant metabolic pathways [4–6] and are unstable and sensitive to light, oxygen, acids and temperature. Due to these properties, chlorophylls are easy to degrade into pheophytins involving the release of the magnesium atom from the chlorin ring. This may occur owing to an inadequate storage or production processes of olive oil [7], resulting in color changes from green to yellow-brown [5,8]. The complexation of pheophytins

chlorin ring with Cu^{2+} yields the formation of green Cu-pheophytin complexes, which are much more stable and resistant to pH and temperature changes than chlorophylls due to the higher metal-chlorin ring bond energies [9]. These green copper chlorophyll complexes are commercialized as the food additive E-141i. However, although E-141i is allowed in food industry, its use in edible oils has been banned in the European Union being considered as a fraud [10].

Most of the published analytical methods for the determination of carotenoid and chlorophyll pigment families in olive oil matrixes are based on reversed-phase liquid chromatography (LC) [11,12]. LC columns with C_{30} stationary phases have been proposed for the analysis of carotenoids, but they provide a strong retention of the analytes especially for the most hydrophobic ones. In contrast, C_{18} columns are frequently preferred for the simultaneous analysis of chlorophylls and carotenoids, since they allow good pigment separation in shorter analysis time. In addition, UV–Vis is the detection system most commonly used for the LC analysis of chlorophylls and carotenoids, taking advantage of their chromophore groups [11,13,14]. However, the unequivocal identification and confirmation is one of the drawbacks of this methodology. Liquid chromatography coupled to mass spectrometry (LC–MS) has demonstrated to be an useful technique for the determination of these compounds in plants, grapes, wines and fruits [15–17]. Nevertheless, there are few studies for their analysis in olive oils and, most of them, are only focused on the characterization of chlorophyll [18] and Cu-chlorophyll derivative profiles [19,3] using either electrospray or atmospheric pressure chemical ionization (APCI) as ionization source. Atmospheric pressure photoionization (APPI) has also been applied in the determination of carotenoids by LC–MS but it has only been applied to standards [20]. Since, there is not any LC–MS method for the simultaneous determination of carotenoids, chlorophylls and chlorophyll derivatives in olive oil

samples so far, it would be interesting to evaluate the performance of different API sources for the ionization of these pigments and dyes and their applicability in the LC–MS analysis of olive oils.

The aim of this work was to study the ionization performance of carotenoids, chlorophylls and chlorophyll derivatives with three API sources (ESI, APCI and APPI) in order to identify which one provides the best performance. The API source selected was used to develop a new sensitive and selective ultra–high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method able to identify and quantify simultaneously natural pigments and E–141i that can be applicable to the detection of fraud in oil samples**.**

Materials and methods

Reagents and standards

Chlorophyll and carotenoid solid standards were purchased from Sigma-Aldrich (Steinheim, Germany) at purities higher than 90%. Standards of pheophytin A (PHE-A) and B (PHE-B) were obtained by acidification from their respective chlorophylls. Copper complexes of pheophytin A and B were obtained by adding an excess of copper (II) nitrate to the corresponding pheophytin. Cu-Pyropheophytin A was obtained from Cu-pheophytin A by refluxing-heating at 100 ˚ºC [21]. Chemical structures, acronyms and chemical formula of the studied compounds are shown in Fig. 1.

Individual stock standard solutions of chlorophylls (1,000 mg L^{-1}) were prepared in acetone, whereas acetonitrile was used to prepare carotenoid standard solutions (500 mg L^{-1}). An intermediate standard mixture containing all the target compounds (50 mg L^{-1}) were prepared monthly from stock standard solutions by appropriate dilution in acetonitrile:acetone (70:30, *v:v*). For quantification, calibration solutions of all the

pigments were prepared from the intermediate standard solution at concentrations ranging from 0.04 to 15 mg L_{-1} in acetonitrile:acetone (70:30, *v:v*). All these standard solutions were stored at -20ºC until their use.

Ethanol absolute for analysis was adquired from Panreac (Barcelona, Spain). Analytical reagent grade copper nitrate was purchased from VWR (Llinars del Vallès, Barcelona, Spain). Sodium sulfate anhydrous for analysis, ≥99.0%, toluene, chlorobenzene, tetrahydrofuran, anisole, potassium hydroxide (≥85%), ammonium acetate, acetic acid (≥ 99.5%), hexane, acetone for pesticide residue analysis (used as extraction solvent and for mobile phase), and methanol (MeOH), acetonitrile (ACN) and water of LC–MS grade were purchased from Sigma-Aldrich (Steinheim, Germany). Solvents used as components of the mobile phase were filtered through 0.22 µm pore size Nylon membrane filters (Whatman, Clifton, NJ, USA) before their use.

The nitrogen (99.95%) used for the atmospheric pressure ionization (API) sources (electrospray, APCI and APPI) was purchased from Linde (Barcelona, Spain) and the high-purity argon (Ar_1) (<99.999%) used as a collision-induced dissociation gas (CID gas) in the triple quadrupole instrument (QqQ) was purchased from Air Liquide (Madrid, Spain).

Instrumentation and UHPLC–MS/MS conditions

The chromatographic separation of natural pigments and copper derivatives was performed on an UHPLC system equipped with an Accela 1250 quaternary pump, an Accela autosampler and a column oven (Thermo Fisher Scientific, San Jose, CA, USA). An Accucore C_{18} column (100 mm × 2.1 mm id., 2.6 µm particle size) packed with superficially porous particles and purchased from Thermo Fisher Scientific was used as analytical column. The UHPLC system was coupled to a TSQ Quantum Ultra AM

(Thermo Fisher Scientific) mass spectrometer equipped with a triple quadrupole mass analyser. Three API sources could be swappable in the TSQ mass spectrometer, ESI, APCI and APPI (Thermo Fisher Scientific).

Two chromatographic separation methods were used. The first method (method 1) consisted in the separation of xanthophylls, chlorophylls and chlorophyll derivatives and the second one (method 2) for the analysis of ß–carotene. The mobile phase of method 1 consisted on water (solvent A), methanol (solvent B), acetonitrile (solvent C) and acetone (solvent D) working in a quaternary gradient elution mode. The gradient elution program started with 20% solvent A and 80% solvent C for 0.5 min followed by a linear gradient that raised up to 10% solvent B and 90% solvent C in 0.5 min. Then, this composition was kept in an isocratic step of 1.5 min. Afterwards, in a third stage, solvent D was introduced up to 50% and solvent C decreased to 50% during 1 min and these conditions were maintained in an isocratic step for 1 min. Finally, solvent D was raised up to 80% during 1 min and it was kept for 3 min more before returning to initial conditions. Method 2 was based on a binary gradient elution mode consisting in acetonitrile (solvent A) and acetone (solvent B). The initial conditions were 50:50 for 2 min followed by a linear gradient elution up to 20:80% in 3 minutes. This composition was kept in an isocratic stage for 1.5min before changing to initial conditions. In both cases, the injection volume was 10 µL, mobile phase flow-rate was 600 µL min^{-1} and the column oven temperature was held at 25°C during the chromatographic run.

Ionization sources working conditions were optimized by injecting 5 µL of a 5 µg mL^{-1} standard mixture in flow injection (FI) mode, which minimized the consumption of high cost pigment standards. The ionization source working parameters for ESI, APCI and APPI were as follow: nitrogen was used as sheath gas and auxiliary gas at flow rates of 70 and 50 a.u. (arbitrary units), respectively. The ion-transfer tube temperature was held

at 200°C while the vaporizer temperature was set at 300°C. In the case of ESI, the electrospray needle voltage was +3 kV, whereas in APCI the corona discharge current was set at +10 kV. For APPI a krypton lamp, which emits 10.6 eV photons, was used. The tube lens potential value was optimized for each compound obtaining the best responses from 85 to 145 V, depending on the compound. Regarding APPI source, direct photoionization and dopant-assisted ionization were compared using the mobile phase composition in order to simulate the optimum conditions. Several dopants (acetone, toluene, anisole, chlorobenzene and tetrahydrofuran) were post-column added into the mobile phase using a zero-dead volume T-piece. The optimal parameters were chosen based on the signal observed for CHL-A, LUT, β–CRIPT and VIO in FI mode. Chlorobenzene was selected as the most appropriate dopant and the concentration that provided the best response was 5% of the total amount of mobile phase flow rate.

The mass spectral data were acquired in full scan and product ion scan modes, while for quantification multiple reaction monitoring (MRM) mode was used, operating both quadrupoles (Q1 and Q3) at a resolution of 0.7 *m/z* full width half maximum (FWHM). In MRM, two transitions were monitored for each compound using 50 ms dwell time at 1.5 mTorr argon as collision gas pressure. MRM parameters such as the optimum collision energies (CE, eV), the MRM transitions (precursor-to-product ion) and the corresponding ion ratios are given in the supporting information (Table S1). The Xcalibur software *v*2.1 (Thermo Fisher Scientific) was used to control the UHPLC–API–MS/MS system and to acquire and process the MS data.

Samples

Several olive oil samples were analysed to study the applicability of the developed methods. The study was carried out with twelve different commercial olive oil samples,

(8 OO, 2 VOO and 2 EVOO) obtained from local supermarkets (Barcelona, Spain). Additionally, a commercial E–141i dye (a mixture of Cu-chlorophyll complexes), provided by SANCOLOR S.A. company (Barcelona, Spain) was also used in this study. E–141i is a liposoluble food additive composed of copper complexes of chlorophyll derivatives. This food additive product was characterized and among the chlorophyll derivatives the most abundant were those of cu-pyropheophytin A, cu-pheophytin A and cu-pheophytin B [2]. All samples were stored in dark at ambient temperature until their analysis.

Sample treatment

Olive oil samples were submitted to a sample treatment to obtain fat-free pigment extracts prior to their determination by UHPLC–MS/MS. For this purpose, solid phase extraction (SPE) using silica cartridges was used as first step. Briefly, pigments were extracted from olive oil samples using Supelclean™ LC–Si SPE cartridges (1.0 g, 6 mL) (Sigma-Aldrich, Steinheim, Germany) in a Visiprep System (Supelco, Bellefonte, PA, USA). SPE cartridges were first conditioned with 3 mL of hexane and afterwards 1.0 g of olive oil sample dissolved in 2 mL of hexane was loaded and passed through it. The cartridges were washed with hexane (15 mL) until total lipid removal and pigments were eluted adding acetone (5 mL). The hexane fraction, which contained lipids and the ß–carotene natural pigment, was kept for further analysis, while xanthophylls, chlorophylls and chlorophyll derivatives eluted in the acetone fraction. Subsequently, on to obtain an extract of ß–carotene free of lipids, the hexane fraction was saponified by adding 10 mL of a 10 % KOH in ethanol and 15 mL of water after 30 min. The hexane fraction containing the ß–carotene was cleaned up three times with water and three times more with a Na_2SO_4 aqueous solution. Both acetone (xanthophylls, chlorophylls and chlorophyll derivatives) and hexane (ß–carotene after saponification) fractions were

evaporated until dryness under a nitrogen stream at room temperature and re-constituted in 1.5 mL of acetonitrile:acetone (70:30, *v:v*). The re-constituted extracts were kept frozen at -18°C in the dark to avoid any degradation, photo-oxidation, but also to facilitate the precipitation of any possible remaining lipid. Finally, the supernatant was filtered through a 0.22 µm Nylon membrane filter and 10 µL were injected into the UHPLC–MS/MS system.

Results and discussion

Liquid Chromatography

In this study, a reversed-phase UHPLC column packed with superficially porous particles (Accucore C_{18}, see experimental section) was used to take advantage of the ultra-high performance provided by this column technology that should allow a high efficient chromatographic separation and short analysis time.

To optimize the chromatographic separation of xanthophylls, chlorophylls and chlorophyll derivatives, several mobile phase compositions and gradient elution programs were evaluated. In general, the high hydrophobicity (LogP: 8.70 – 16.53) and the low water solubility of these compounds made necessary to minimize the mobile phase water content. For this reason, the initial gradient elution program started at 20:80 water:methanol (*v/v*) (flow rate 300 µL min^{-1}) and it was linearly changed up to 10:90 methanol:acetonitrile (*v/v*). Under these conditions, xanthophylls eluted at 4 times the hold-up time (t_M) and before chlorophylls. Although some authors [11,12] used a small amount of an aqueous ammonium acetate solution at the initial conditions of the gradient elution, the substitution of this aqueous solution by just water made xanthophylls to elute earlier. This fact probably was due to the lower mobile phase ionic strength, which weakened the interaction of these analytes with the stationary phase. Additionally, the

coelution of neoxanthin (NEO) and violaxanthin (VIO) pigments had to be avoided, since they are isobaric compounds that also yield common product ions under tandem mass spectrometry conditions. The gradient elution program was optimized and an isocratic step of 0.5 min was necessary to achieve the separation of these compounds at base line, the final chromatographic resolution achieved was 1.9.

Under ternary gradient elution conditions (water:methanol:acetonitrile), chlorophylls and chlorophyll derivatives showed double chromatographic peaks. This can be attributed to the presence of epimer compounds [22] that gave the same response than the native compounds. Substituents in C−13_2 (methoxy group) and C−17_3 (phytyl group) (Fig. 1) in the epimer compounds are not in the same plane as in the native compounds. For this reason, the interaction between the stationary phase and the epimer compounds is favoured making the epimers to elute later than the native compounds. Furthermore, chlorophyll derivatives eluted in more than 25 minutes, thus it was necessary a quaternary elution program where acetone was added as the last step in order to increase the eluotropic strength at the end of the chromatogram and to shorten the analysis time. Finally, to further reduce the analysis time of chlorophylls and their derivatives, mobile phase flow rate was risen up to 600 µL min^{-1}. Fig. 2A shows the chromatogram of a natural pigment standards solution (xanthophylls, chlorophylls and chlorophyll derivatives, 2 µg g^{-1}) obtained under the optimum UHPLC conditions and as it can be seen, most of the compounds are separated at base line in less than 8 min, except CHL-B' and β–CRIPT that partially coeluted. Nevertheless, these two compounds did not show ion suppression/enhancement effect and they can be analysed individually by mass spectrometry thanks to the selection of non-interfering transitions (precursor-to-product ion). On the other hand, since ß–carotene is a non-polar compound, mobile phase components with high elutrophic strength such as acetonitrile and acetone were needed

280 to shorten the analysis time. Thereby, the obtained chromatogram for ß–carotene is shown
281 in Fig. 2B.

282 **Liquid Chromatography–Mass Spectrometry**

283 To study the atmospheric pressure ionization (API) behaviour of olive oil pigments, a
284 standard solution (10 mg L^{-1}) was injected in the UHPLC–MS/MS system (working
285 conditions section 2.2) using three API sources (ESI, APCI and APPI). Upon optimizing
286 the working parameters for each API source, it was found that vaporizer temperature and
287 tube lens offset voltage were the most critical ones. When the vaporizer temperature was
288 increased above 300°C, the signal of the protonated molecule ion decreased as a
289 consequence of its fragmentation by in-source collision induced dissociation (CID), thus
290 becoming the base peak of the mass spectrum the fragment ion originated from the loss
291 of water (Fig. S1 for VIO). Furthermore, Fig. 3 shows the effect of tube lens voltage in
292 the intensity of the base peak.This voltage value is compound dependent and an excessive
293 voltage would produce the in-source CID fragmentation [23]. The highest ion intensity
294 for chlorophylls was achieved at 140 V, while for carotenoids maximum responses were
295 obtained at 90 V. Moreover, these compounds experimented a significant in-source CID
296 fragmentation above this tube lens offset voltage that caused the decrease in the
297 protonated molecule ion intensity. Besides, the high polyene conjugation and the presence
298 of oxygen in these molecules, as well as the solvent system, have a significant influence
299 on the stability and formation of molecular ion and protonated molecule ion. Table 1
300 shows the ion assignment and the corresponding relative abundances observed using the
301 three API sources (ESI, APCI and APPI with chlorobenzene) in positive ion mode.

302 Under electrospray conditions, carotenoids (ß–CAR NEO, VIO, LUT and ß–CRIPT)
303 yielded the molecular ion $[M]^{+\bullet}$ (ß–CAR, *m/z* 536.5; NEO, *m/z* 600.4; VIO, *m/z* 600.4;

LUT, *m/z* 568.4; ß–CRIPT, *m/z* 552.4) as base peak as well as the ion $[M–H]^+$ (Rel. Ab. 35–60%), as can be seen in Fig. 4 for LUT. These ions may be generated *via* electrochemical oxidation in the electrospray needle [24,25]. Additionally, non in-source CID fragment ions were observed at significant intensities (relative abundance < 27%) for any of these analytes, but the oxidized ion $[M–H]^+$ showed a high tendency to generate adducts with the mobile phase components. Ions such as *m/z* 617.4 for NEO, *m/z* 617.4 for VIO and *m/z* 585.4 for LUT, shifted 18 units above the ion $[M–H]^+$ and they could be assigned to the water adduct ions $[M–H+H_2O]^+$. Furthermore, methanol adduct ions $[M–H+CH_3OH]^+$ such as *m/z* 599.4 for LUT and *m/z* 583.4 for ß–CRIPT were also observed.

Regarding chlorophylls the ions generated by ESI include both ions $[M+H]^+$ and $[M+Na]^+$, being the base peaks for CHL-A and CHL-B, respectively (Fig. 4). Additionally, chlorophylls and their epimers also showed the molecular ion $[M]^{+\bullet}$ as occurred with carotenoids. Under these conditions, the UHPLC–ESI–MS chromatogram showed a high background noise resulting in a low signal-to-noise ratio (S/N) for all compounds and as a consequence the limits of detection were relatively high ranging from 0.8 to 3 mg L^{-1}. Therefore, APCI and APPI were evaluated as alternatives to ESI in order to improve the signal intensity, since the gas-phase ionization mechanisms in these two API sources may be advantageous for the ionization of these highly conjugated compounds.

The ionization of chlorophylls and their derivatives (including epimer compounds) by APCI provided the ion $[M+H]^+$ (CHL-A, *m/z* 893.5; CHL-B, *m/z* 907.5; PHE-A, *m/z* 871.5; PHE-B, *m/z* 885.5; Cu-PHE-A, *m/z* 932.5; Cu-PHE-B, *m/z* 946.5; Cu-PyroPHEa, *m/z* 874.5) as base peak, as it was observed in ESI. Nevertheless, no adduct ions were generated and only a slight in-source CID fragmentation (relative abundance around 25%) was observed. However, unlike in ESI, xanthophylls showed a significant in-source

CID fragmentation in APCI, mainly due to the loss of a water molecule providing the ion $[M+H–H_2O]^+$ (NEO, *m/z* 583.4; VIO, *m/z* 583.4; LUT, *m/z* 551.4; ß-CRIPT, *m/z* 535.4). Nevertheless, in spite of the in-source fragmentation for xanthophylls, the ion $[M+H]^+$ continued being the base peak in the mass spectra of ß–CAR, VIO and ß–CRIPT. In the case of LUT and NEO, the in-source fragment ion $[M+H-H_2O]^+$ dominated both mass spectra as a consequence of the formation of a relatively stable allylic carbocation in the ε-ring. As an example, Fig. 4 shows the mass spectra obtained for CHL-A and LUT in APCI.

Regarding APPI, both chlorophyll and carotenoid families were ionized with no need of a dopant. The direct photoionization could be due to the high number of double bonds and electron-donor methyl groups in the chemical structure of these compounds, which results in low ionization potential values [26] that could facilitate the direct photoionization. Chlorophylls and their derivatives under direct photoionization provided the ion $[M+H]^+$ without significant in-source CID fragmentation, whereas most of carotenoids yielded the molecular ion $[M]^{+\bullet}$ (β–CRIPT and β–CAR always showed the ion $[M+H]^+$) and a low in-source CID fragmentation due to the loss of a water molecule.

Despite direct ionization occurred with these compound families, several dopants (acetone, toluene, chlorobenzene, anisole and tetrahydrofuran) were also tested in order to study their effect in the ionization efficiency to improve diagnostic ion signal. These studies were carried out using the mobile phase composition in order to simulate the optimum conditions for VIO, LUT, ß–CRIPT and CHL-A as model compounds and the ions observed are summarized in Table S2. Among the dopants evaluated, acetone, tetrahydrofuran and toluene allowed the ionization of the analytes in the same way, providing similar mass spectral patterns. VIO, ß–CRIPT and CHL-A yielded the ion $[M+H]^+$, while the ion $[M+H–H_2O]^+$ (in-source CID fragment) dominated the mass

spectrum of LUT. It must be noted that these dopants generally lack the capacity for charge exchange since fast self-protonation between a dopant radical ion $[D]^{+\bullet}$ and a neutral dopant molecule [D] could be the predominant ion-molecule reaction in the gas-phase, leading to the ion corresponding to the protonated dopant $[D+H]^{+}$. Afterwards, the analyte would be ionized *via* proton-transfer reactions to yield the ion $[M+H]^{+}$. However, anisole and chlorobenzene dopants could favour the charge exchange between the dopant molecular ion $[D]^{+\bullet}$ and the neutral analyte molecule [M] to yield the analyte molecular ion $[M]^{+\bullet}$. No in-source CID fragmentation occurred for carotenoids and only for LUT (Fig. 4) some fragment ions were observed at low relative abundance (<18%). Nevertheless, in the case of CHL-A (Fig. 4), in addition to the molecular ion $[M]^{+\bullet}$, proton-transfer reactions also occurred to yield the ion $[M+H]^{+}$. The analyte responses observed when working in both direct and dopant-assisted APPI modes were normalized to the highest signal for each compound in each case (Fig. S2). Generally, the highest relative signal intensity was obtained using chlorobenzene as dopant; although for LUT, the response was slightly higher using anisole. As a compromise, chlorobenzene was selected as the most suitable dopant for the APPI of the target compounds. Finally, Fig. 5 shows the comparison of the relative signal intensity of the base peak normalized to each compound in each ionization source (ESI, APCI and chlorobenzene assisted APPI). As can be observed, ESI shows the lowest response in all cases, so it was discarded for further studies. Although APPI provided the best results, APCI could also be considered as a good alternative for the analysis of these compounds since for most of them the response was only slightly lower than that obtained in APPI.

To improve the detectability, the sensitivity and to ensure the identification and quantitative determination of target compounds, tandem mass spectrometry was evaluated. The assignments of the main product ions are summarized in Table 2. Tandem

mass spectra of chlorophylls and carotenoids ions generated by APCI and APPI were studied. The corresponding product ions were characterized and the two most selective and abundant ones were selected for quantitative and confirmatory purposes when working in multiple reaction monitoring (MRM) mode. The product ion scan of chlorophylls and carotenoids were acquired at collision energies between 5 eV and 50 eV. The collision energies and the selected precursor and product ions as well as the ion ratios calculated for each compound are given in the supporting information (Table S1).

In the case of chlorophylls and their copper derivatives, the product ion scan for the precursor ion $[M+H]^+$ was obtained using an isolation window of 10 *m/z* in order to preservethe isotopic information of the product ions. Instead, for pheophytins, a standard isolation window of 1 *m/z* was used. Thereby, it was possible to confirm that all the product ions observed for chlorophylls and their copper derivatives kept the metal atom in their chemical structure (Fig. S3). Moreover, the fragmentation pattern of the chlorophyll family was characterized by the loss of the phytil chain 278 Da ($C_{20}H_{38}$) and the consecutive cleavage of the carboxymethyl ester (60 Da, $C_2H_4O_2$) at $C-13_2$ yielding the ion $[M+H- C_{22}H_{42}O_2]^+$, except for Cu-pyropheophytin A which lacked the β-keto ester. In addition, Cu-pyropheophytin A also showed product ions due to the carboxy-phytil loss (*m/z* 552, $[C_{32}H_{32}CuN_4O]^+$) and the cleavage at $C-17_3$ to lose CH_2CH_2COO-phytil (*m/z* 522, $[C_{30}H_{27}CuN_4O]^+$). Regarding chlorophyll epimers, the CID fragmentation behaviour is slightly different, even the similarity of the product ions observed, the relative abundances are quite different at the same collision energy. This fact could be explained because of the activation energy needed to fragment the epimer compound is lower than that required for the native compound, which could be related with the relative position of $C-13_2$ and $C-17_3$ (Fig. S4) that seems to stabilize the chemical structure in the case of the native compound.

Regarding carotenoids, the base peak ions observed in APCI and APPI were selected as precursor ions in tandem mass spectrometry. In all cases, the high polyene conjugation and the hydroxyl group in the chemical structure of xanthophylls were involved in the formation of the main characteristic and common product ions. A general fragmentation pattern consisting in consecutive losses shifted 14 Da (CH_2) and 28 Da (CH_2=CH_2) was observed as a result of the typical fragmentation of alkene chains. Additionally, product ions generated by dehydroxylation (loss of OH) or dehydration (loss of H_2O) from the respective precursor ion were also observed for all xanthophylls in both APCI and APPI.

For LUT the precursor ion was different in both APCI and APPI, but the two most abundant product ions observed, such as the ions at *m/z* 145 $[C_{11}H_{13}]^+$ and *m/z* 119 $[C_9H_{11}]^+$, were the same in both API sources. Moreover, the ion $[C_9H_{11}]^+$ (*m/z* 119) was the base peak in the product ion spectra of hydroxy carotenoids (ß–CRIPT and LUT) although it was also observed with lower intensity (40%) in the product ion spectra of ß–CAR,VIO and NEO with both API sources. Besides, the common product ion $[C_{11}H_{13}]^+$ (*m/z* 145) was also observed using APPI for all xanthophylls. Furthermore, both VIO and NEO have a cyclohexyl ring with an epoxide and an hydroxyl group which are involved in the formation of product ion at *m/z* 221 $[C_{14}H_{21}O_2]^+$ in APCI due to the loss of 380 Da.

Once the MS/MS conditions were established for each compound and the MRM transitions for quantification and confirmation (Table S1) purposes were selected, the performance of the UHPLC–MS/MS methods were evaluated using both APCI and APPI sources. Instrumental limit of detection (ILOD) and limit of quantitation (ILOQ) were calculated using standard solutions (Table 3). LODs based on a signal-to-noise ratio of 3:1 and LOQs based on a signal-to-noise ratio of 10:1 were determined by injecting 10 µL of standard solutions at low concentration levels. As can be seen, similar LODs values were obtained in both APCI and APPI except for LUT and VIO that showed slightly

429 better sensitivity in APCI. Nevertheless, LOD values were always lower than 0.2 mg L^{-1}
430 and down to 0.003 mg L^{-1} for the best cases. Besides, chlorophyll derivative values were
431 expected to be similar to the ones determined for their corresponding native compounds.
432 Considering that most of the natural pigments are usually expected at mg L^{-1} in olive oil,
433 these ILODs can be enough to detect and determine these compounds in the final olive
434 oil extracts.

435 **Sample analysis**

436 The application of the developed UHPLC–API–MS/MS methods for the determination
437 of pigments in olive oil samples required a previous sample treatment (extraction and
438 clean up) in order to achieve extracts clean enough before their analysis by UHPLC–
439 MS/MS. In this work, a SPE method using silica cartridges was applied as sample
440 treatment before the chromatographic analysis of natural pigments in olive oils. Acetone
441 extract contained the chlorophylls, chlorophyll derivatives and xantophylls, while the
442 saponified hexane extract contained the β–carotene.

443 Due to the lack of an olive oil sample free of target pigments, an olive oil sample (OO-
444 S8) spiked with target compounds at 4 mg L^{-1} (4 times higher than the endogenous
445 concentration determined in this sample) was used for the optimization of working
446 conditions and the estimation of quality parameters of the method. Olive oil sample and
447 a spiked olive oil sample were submitted to the sample treatment procedure and the
448 corresponding extracts were analysed by UHPLC–API–MS/MS in order to calculate the
449 SPE extraction efficiency (EE, %). Additionally, an aliquot of the olive oil sample extract
450 was also spiked with standards (4 mg L^{-1}) to evaluate the matrix effect (ME, %) in the
451 ionization by comparing it with the corresponding response of the standard at the same
452 concentration level. For most compounds, the SPE EE% ranged from 88% to 95% with
453 RSD% lower than 10%. Only CHL-A showed a lower EE% value (63%) owing to a

possible degradation of the added pigment into pheophytin due to the own acidity of the oil sample. ME (%) values ranged from 8% to 25% with RSD% values lower than 15%, which indicated that both APCI and APPI methods only showed a slightly matrix effect. This low ME% allowed us to use the external calibration method for the quantitative analysis of these compounds in olive oil samples. Moreover, method limits of quantification (MLOQs), defined as S/N of 10, ranged between 0.036 and 0.80 mg L^{-1} (Table 3). The linearity was satisfactory for all compounds within the concentration working range studied (0.04 – 15 mg L^{-1}), showing linear regression coefficients (R^2) higher than 0.998. In addition, run-to-run precision was estimated (concentration level ~ 4 mg L^{-1}) obtaining relative standard deviation values (n = 3, RSD%) lower than 7% in all cases. Trueness was also evaluated obtaining satisfactory results, with relative errors lower than 10%. These results show that the two UHPLC–API–MS/MS methods provide good performance and both could be proposed for the analysis of carotenoid and chlorophyll pigments, although APCI should be used if extra sensitivity is required, since APCI showed slightly better LODs than APPI. To our knowledge, there are no data published on UHPLC–MS/MS using APCI or APPI for the simultaneous determination of carotenoids, chlorophylls and the chlorophyll copper potential adulterant (E–141i) in olive oil samples.

In this work, in order to evaluate the feasibility of the developed SPE UHPLC–API–MS/MS methods a total of 12 olive oil samples were analysed in triplicate (n=3). Among the olive oil samples analysed, copper chlorophyll complexes were not detected, thus, indicating that all these selected samples were not adulterated with E–141i green dye. Moreover, neither ß–CRIPT nor NEO were detected above their MLOQ using both APCI and APPI sources. Besides, ß–CAR, PHE-A, PHE-B and LUT were quantified in all samples at concentration levels ranging from 0.1 to 9.5 mg L^{-1} (Fig. 6). Moreover, VIO,

was identified in all samples, although only in VOO and EVOO samples the concentration was above the MLOQ using both methods. However, VIO was not detected in OO samples with APPI method, but the better sensitivity of APCI allowed its detection at concentration levels close to MLOQ. As can be seen in Fig. 6, the results obtained with the proposed SPE UHPLC–MS/MS methods with APCI and APPI agreed.

For confirmatory purposes and for avoiding false positives, ion ratios between both quantitative and confirmatory transitions peak areas were compared with that obtained from the corresponding standards. For all the compounds detected in samples above the MLOQ, the ion ratio deviation ranged from 0.1 to 10% indicating the absence of false positive among the analysed samples.

Finally, since there was not any adulterated sample with E–141i, in order to test the feasibility of the method proposed for the analysis of Cu-chlorophyll complexes, two OO samples (OO-S2 and OO-S7) were spiked with E–141i to achieve low concentration levels on copper chlorophyll derivatives (0.09 – 0.16 mg L^{-1}) based in data found in the literature about its use in fraud practices [3]. These samples were submitted to the developed SPE UHPLC–API–MS/MS (APCI, APPI) methods. Both methods provided similar results (trueness 7-10%, RSD% 4-6%), demonstrating that both methods were able to detect and quantify these copper derivatives in olive oil samples. As an example, Fig. 7 shows the SPE UHPLC–APCI–MS/MS chromatograms obtained for (A) quantitation and (B) confirmation transitions in sample OO-S7 spiked with E–141i at 0.16 mg L^{-1}.

Conclusions

UHPLC–MS/MS using APCI and APPI sources has proved to be reliable and accurate method for the determination of carotenoids, chlorophylls and chlorophyll derivatives in

olive oils. The use of an UHPLC reversed-phase column (Accucore C18) and a quaternary gradient elution (water:methanol:acetonitrile:acetone) provided efficient chromatographic separation and resolution of all target compounds in a short analysis time (< 8 min). Furthermore, the results obtained in the ionization behaviour and MS/MS fragmentation studies showed that best ionization efficiencies were achieved using both APCI and APPI, being the predominant ions the protonated molecule $[M+H]^+$ for chlorophylls and their derivatives and the ions $[M+H]^+$, $[M+H-H_2O]^+$ and $[M]^{+\bullet}$ for carotenoids. Chlorophylls showed a common MS/MS fragmentation pattern based on the loss of the phytyl chain (278 Da; $C_{20}H_{38}$) and the consecutive cleavage of the carboxymethyl ester (60 Da, $C_{22}H_{42}O_2$) at C−13$_2$. While for carotenoids, the main product ions aroused from the fragmentation of the high polyene conjugated chain and the hydroxyl group. The combination of a simple SPE method and gas-phase ionization sources (APCI and APPI) allowed to keep the matrix effect under control, lower than 25%, and to use external calibration method for quantitative analysis. The good performances of the developed methods and the suitable results obtained in the analysis of olive oil samples have demonstrated their applicability and they can be proposed for the determination of the pigment profile as well as the detection of possible exogenous adulterants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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605

606 ***Figure Captions***

607 **Fig. 1** Chemical structures of studied natural pigments.

608 **Fig. 2** UHPLC–APCI–MS/MS chromatogram obtained from standard mixtures of target
609 compounds at a concentration of 2 mg L^{-1}. Chromatogram A: method 1;
610 chromatogram B: method 2. Compounds: (1) NEO; (2) VIO; (2') VIO'; (3) LUT;
611 (3') LUT'; (4) CHL-B; (4') CHL-B'; (5) β–CRIPT; (6) CHL-A; (6') CHL-A'; (7)
612 PHE-B; (8) Cu–PHE-B; (9) PHE-A; (9') PHE-A'; (10) Cu–PHE-A; (10') Cu–
613 PHE-A'; (11) Cu–PyroPHE-A; (12) β–CAR.

614 **Fig. 3** Effect of tube lens offset voltage on carotenoids and chlorophylls response in
615 APCI. Mass spectra of β–CRIPT (left) at a tube lens voltage of 90 V (up) and 140
616 V (bottom) and of Cu-PHE-A (right) at a voltage of 140 V (up) and 190 V
617 (bottom).

618 **Fig. 4** ESI, APCI and APPI (5% chlorobenzene as dopant) mass spectra of LUT and
619 CHL-A in positive-ion mode.

620 **Fig. 5** Comparison of target compounds responses (normalized chromatographic peak
621 height) using ESI, APCI and APPI (chlorobenzene as dopant) as ionization
622 sources.

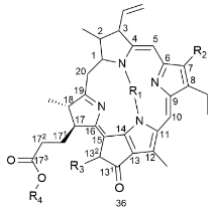
623 **Fig. 6** Individual concentrations of β-CAR, PHE-A, PHE-B and LUT for edible oils
624 obtained by UHPLC–APCI–MS/MS and UHPLC–APPI–MS/MS methods.

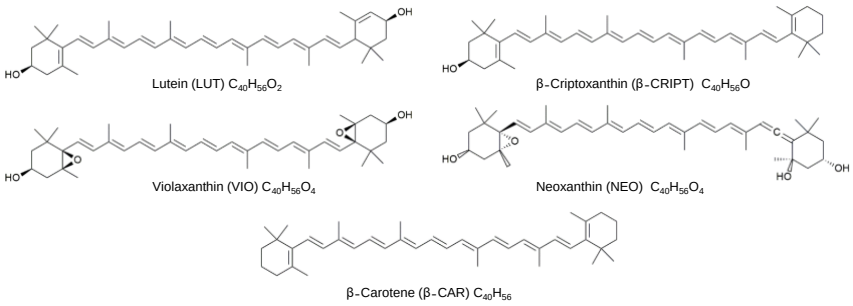
625 **Fig. 7** UHPLC–APCI–MS/MS chromatogram obtained for (A) quantitation and (B)
626 confirmation transitions from the analysis OO-S7 sample spiked with E–141i at
627 0.16 mg L^{-1}.

628

629 Figure1:

	R_1	R_2	R_3	R_4	Formula
CHL-A	Mg	CH_3	CH_3COOH	$C_{20}H_{39}$	$C_{55}H_{72}MgN_4O_5$
CHL-B	Mg	CHO	CH_3COOH	$C_{20}H_{39}$	$C_{55}H_{70}MgN_4O_6$
PHE-A	H_2	CH_3	CH_3COOH	$C_{20}H_{39}$	$C_{55}H_{74}N_4O_5$
PHE-B	H_2	CHO	CH_3COOH	$C_{20}H_{39}$	$C_{55}H_{72}N_4O_6$
Cu-PHE-A	Cu	CH_3	CH_3COOH	$C_{20}H_{39}$	$C_{55}H_{72}CuN_4O_5$
Cu-PHE-B	Cu	CHO	CH_3COOH	$C_{20}H_{39}$	$C_{55}H_{70}CuN_4O_6$
Cu-PyroPHE-A	Cu	CH_3	3H	$C_{20}H_{39}$	$C_{55}H_{71}CuN_4O_3$





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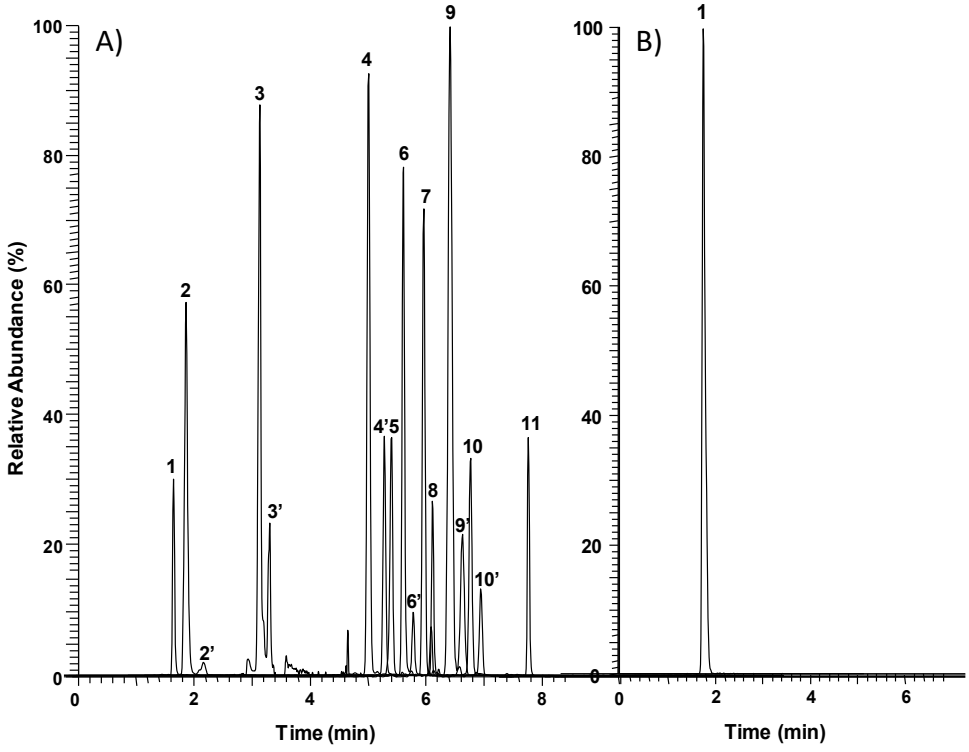
631 Figure 2:



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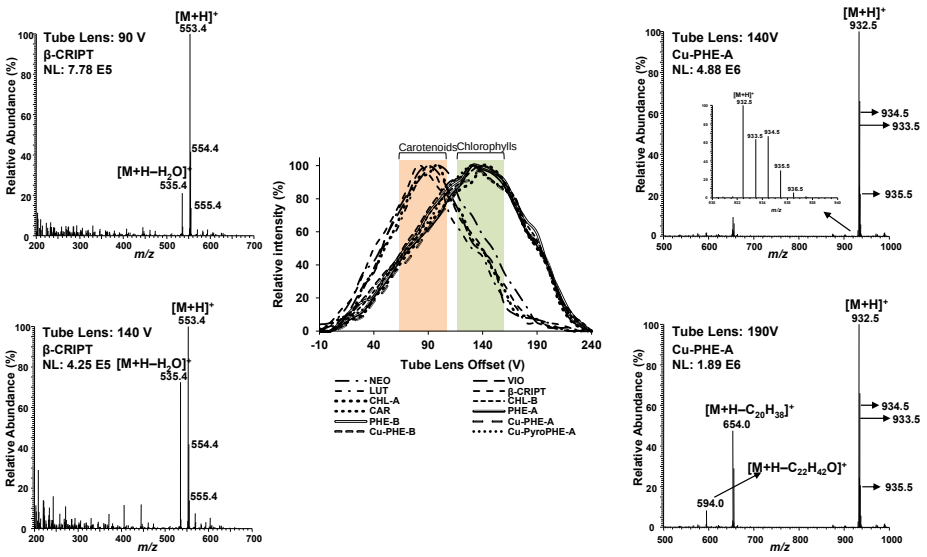

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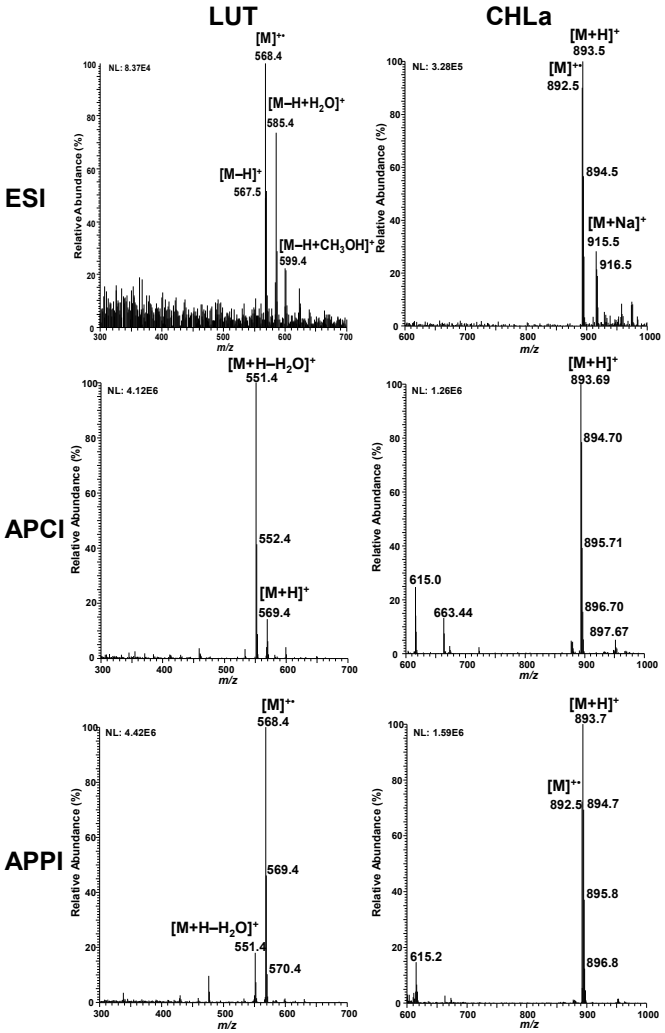

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638 Figure 5:

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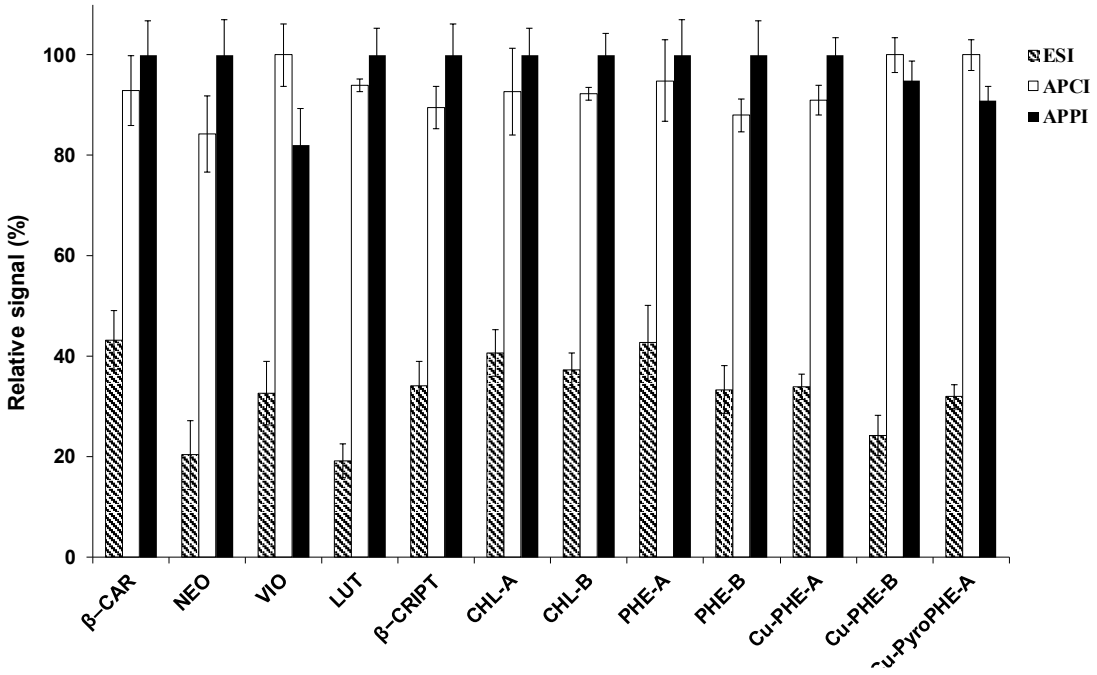

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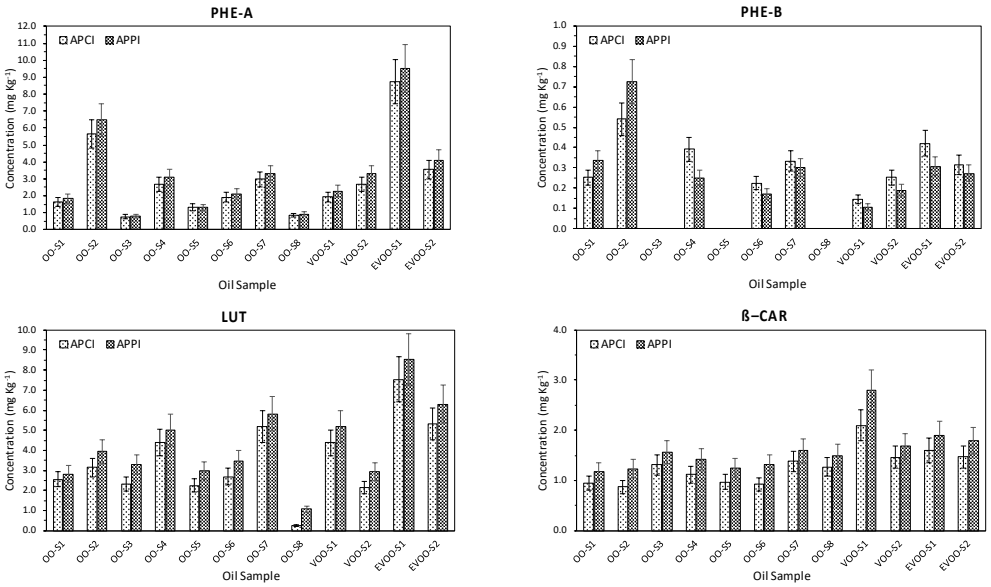

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645 Figure 7:

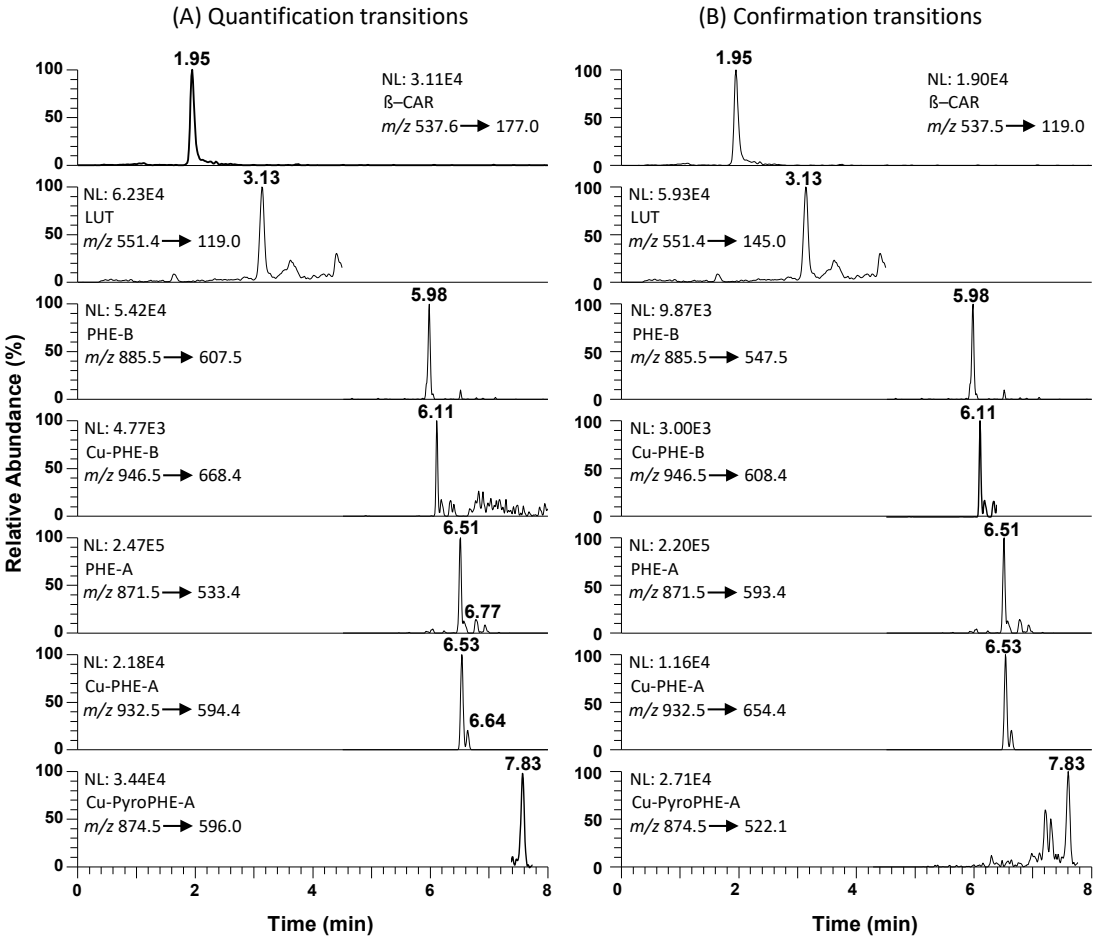

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Table 1: Assignment of ions generated in ESI, APCI and APPI (dopant:chlorobenzene) under optimal conditions

Compound	ESI		APCI		APPI	
	m/z (Rel. Ab. %)	Ion Assignment	*m/z* (Rel. Ab. %)	Ion Assignment	*m/z* (Rel. Ab. %)	Ion Assignment
β-CAR	536.5 (100)	$[M]^{+\cdot}$	537.6 (100)	$[M+H]^+$	537.6 (100)	$[M+H]^+$
NEO	617.4 (27)	$[M-H+H_2O]^+$	601.4 (67)	$[M+H]^+$	600.4 (100)	$[M]^{+\cdot}$
	600.4 (100)	$[M]^{+\cdot}$	583.4 (100)	$[M+H-H_2O]^+$	583.4 (24)	$[M+H-H_2O]^+$
	599.4 (32)	$[M-H]^+$	565.4 (56)	$[M+H-2H_2O]^+$		
VIO	617.4 (27)	$[M-H+H_2O]^+$	601.4 (100)	$[M+H]^+$	600.4 (100)	$[M]^{+\cdot}$
	600.4 (100)	$[M]^{+\cdot}$	583.4 (50)	$[M+H-H_2O]^+$	583.4 (13)	$[M+H-H_2O]^+$
	599.4 (36)	$[M-H]^+$				
LUT	599.4 (16)	$[M-H+CH_3OH]^+$	569.4 (18)	$[M+H]^+$	568.4 (100)	$[M]^{+\cdot}$
	585.4 (75)	$[M-H+H_2O]^+$	551.4 (100)	$[M+H-H_2O]^+$	551.4 (18)	$[M+H-H_2O]^+$
	568.4 (100)	$[M]^{+\cdot}$				
	567.4 (53)	$[M-H]^+$				
b-CRIPT	583.4 (22)	$[M-H+CH_3OH]^+$	553.4 (100)	$[M+H]^+$	553.4 (100)	$[M+H]^+$
	552.4 (100)	$[M]^{+\cdot}$	535.4 (21)	$[M+H-H_2O]^+$		
	551.4 (13)	$[M-H]^+$				
CHL-A	915.6 (27)	$[M+Na]^+$	893.5 (100)	$[M+H]^+$	893.5 (100)	$[M+H]^+$
	893.5 (100)	$[M+H]^+$	615.0 (24)	$[M+H-C_{20}H_{38}O_2]^+$	892.5 (70)	$[M]^{+\cdot}$
	892.5 (88)	$[M]^{+\cdot}$				
CHL-B	929.7 (100)	$[M+Na]^+$	907.5 (100)	$[M+H]^+$	907.5 (100)	$[M+H]^+$
	907.5 (26)	$[M+H]^+$	629.0 (19)	$[M+H-C_{20}H_{38}O_2]^+$	906.5 (50)	$[M]^{+\cdot}$
	906.5 (20)	$[M]^{+\cdot}$				
PHE-A	871.5 (100)	$[M+H]^+$	871.5 (100)	$[M+H]^+$	871.5 (100)	$[M+H]^+$
	870.5 (85)	$[M]^{+\cdot}$	593.0 (20)	$[M+H-C_{20}H_{38}O_2]^+$	870.5 (67)	$[M]^{+\cdot}$
PHE-B	885.5 (100)	$[M+H]^+$	885.5 (100)	$[M+H]^+$	885.5 (100)	$[M+H]^+$
	884.5 (75)	$[M]^{+\cdot}$	607.0 (18)	$[M+H-C_{20}H_{38}O_2]^+$	884.5 (62)	$[M]^{+\cdot}$
Cu-PHE-A	932.5 (100)	$[M+H]^+$	932.5 (100)	$[M+H]^+$	932.5 (100)	$[M+H]^+$
	931.5 (80)	$[M]^{+\cdot}$	654.0 (22)	$[M+H-C_{20}H_{38}O_2]^+$	931.5 (67)	$[M]^{+\cdot}$

Table 2: Ion assignment of product ions observed in MS/MS using APCI and APPI.

Compound	Ionization source	Precursor ion		Product Ion	
		m/z	Ion Assignment	*m/z*	Ion Assignment
β-CAR	APCI/APPI	537.6	$[M+H]^+$	177.0	$[M+H-C_{27}H_{36}]^+$
				119.0	$[M+H-C_{31}H_{46}]^+$
				105.0	$[M+H-C_{32}H_{48}]^+$
NEO	APCI	583.4	$[M+H-H_2O]^+$	221.0	$[M+H-C_{26}H_{36}O_2]^+$
				159.0	$[M+H-C_{28}H_{42}O_4]^+$
				119.0	$[M+H-C_{31}H_{46}O_4]^+$
	APPI	600.4	$[M]^+$	145.0	$[M-C_{29}H_{44}O_4]^+$
				171.6	$[M-C_{27}H_{41}O_4]^+$
				119.0	$[M-C_{31}H_{45}O_4]^+$
VIO	APCI	601.4	$[M+H]^+$	221.0	$[M+H-C_{26}H_{36}O_2]^+$
				165.0	$[M+H-C_{30}H_{44}O_2]^+$
				119.0	$[M+H-C_{31}H_{46}O_4]^+$
	APPI	600.4	$[M]^+$	145.0	$[M-C_{29}H_{44}O_4]^+$
				171.6	$[M-C_{27}H_{41}O_4]^+$
				119.0	$[M-C_{31}H_{45}O_4]^+$
LUT	APCI	551.4	$[M+H-H_2O]^+$	145.0	$[M+H-C_{29}H_{44}O_2]^+$
	APPI	168.4	$[M]^+$	119.0	$[M+H-C_{31}H_{46}O_2]^+$
				105.0	$[M+H-C_{32}H_{48}O_2]^+$
b-CRIPT	APCI/APPI	553.4	$[M+H]^+$	145.0	$[M+H-C_{31}H_{46}O]^+$
				119.0	$[M+H-C_{31}H_{46}O]^+$
				105.0	$[M+H-C_{30}H_{44}O]^+$
CHL-A	APCI/APPI	893.5	$[M+H]^+$	615.5	$[M+H-C_{20}H_{38}]^+$
				583.5	$[M+H-C_{21}H_{42}O]^+$
				555.5	$[M+H-C_{22}H_{42}O_2]^+$
CHL-B	APCI/APPI	907.5	$[M+H]^+$	629.5	$[M+H-C_{20}H_{38}]^+$
				597.5	$[M+H-C_{21}H_{42}O]^+$
				569.5	$[M+H-C_{22}H_{42}O_2]^+$
PHE-A	APCI/APPI	871.5	$[M+H]^+$	593.4	$[M+H-C_{20}H_{38}]^+$
				533.4	$[M+H-C_{22}H_{42}O_2]^+$
PHE-B	APCI/APPI	885.5	$[M+H]^+$	607.5	$[M+H-C_{20}H_{38}]^+$
				547.5	$[M+H-C_{22}H_{42}O_2]^+$
Cu-PHE-A	APCI/APPI	932.5	$[M+H]^+$	654.4	$[M+H-C_{20}H_{38}]^+$
				594.4	$[M+H-C_{22}H_{42}O_2]^+$
Cu-PHE-B	APCI/APPI	946.5	$[M+H]^+$	668.4	$[M+H-C_{20}H_{38}]^+$

Table 3: Quality parameters of UHPLC–MS/MS (APCI and APPI) methods.

Compound	ILOD (mg L^{-1})[a]		ILOQ (mg L^{-1})[a]		MLOQ (mg L^{-1})[a]		Concentration level (mg L^{-1})	Run-to-Run Precision (RSD %)		Trueness (Rel. Error %)	
	APCI	APPI	APCI	APPI	APCI	APPI		APCI	APPI	APCI	APPI
ß–CAR	0.03	0.06	0.1	0.2	0.3	0.3	4.5	3	5	4	4
NEO	0.2	0.15	0.8	0.5	0.9	0.6	4.9	2	3	2	3
VIO	0.02	0.03	0.08	0.1	0.1	0.2	4.8	1	1	-1	-0.2
LUT	0.003	0.06	0.01	0.2	0.01	0.2	4.2	1	1	1	3
ß–CRIPT	0.1	0.2	0.5	0.6	0.7	0.8	4.6	3	5	-5	-2
CHL-A	0.009	0.021	0.03	0.07	0.05	0.1	4.0	2	2	1	1
CHL-B	0.0009	0.001	0.003	0.004	0.004	0.005	5.0	3	2	3	3
PHE-A	0.01	0.015	0.05	0.05	0.06	0.07	4.3	3	4	-7	-3
PHE-B	0.003	0.006	0.01	0.02	0.03	0.06	4.5	4	2	2	5
Cu-PHE-A	0.009	0.006	0.03	0.02	0.05	0.04	0.2	5	2	10	12
Cu-PHE-B	0.006	0.009	0.02	0.03	0.03	0.05	0.1	5	3	8	6
Cu-PyroPHE-A	0.003	0.006	0.01	0.02	0.03	0.05	0.1	3	7	10	9

[a]Injection volume: 10 µL

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Supplementary Information

Simultaneous analysis of natural pigments and E-141i in olive oils by Liquid Chromatography–Tandem Mass Spectrometry

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682 **Supplementary Tables**

683 **Table S1:** MRM transitions, optimum collision energies (CE) and ion ratios used in UHPLC–MS/MS.

Compound	API source	Precursor ion (*m/z*)	Quantitation		Confirmation		Ion Ratio ± SD[a]
			Product ion (*m/z*)	CE (eV)	Product ion (*m/z*)	CE (eV)	
β-CAR	APCI/APPI	537.6	177.0	18	119.0	35	1.6 ± 0.03
NEO	APCI	583.4	221.0	30	159.0	40	2.2 ± 0.01
	APPI	600.4	145.0	40	171.6	40	2.3 ± 0.02
VIO	APCI	601.4	221.0	20	165.0	30	2.9 ± 0.01
	APPI	600.4	145.0	40	171.6	30	3.0 ± 0.01
LUT	APCI	551.4	119.0	40	145.0	40	1.1 ± 0.01
	APPI	568.4	145.0	35	119.0	45	1.1 ± 0.01
β-CRIPT	APCI/APPI	553.4	119.0	40	105.4	45	1.6/1.6 ± 0.02
CHL-A	APCI/APPI	893.5	555.5	40	615.5	20	1.7/1.8 ± 0.01
CHL-B	APCI/APPI	907.5	629.5	25	569.5	25	1.8/1.8 ± 0.02
PHE-A	APCI/APPI	871.5	533.4	50	593.4	45	1.1/1.1 ± 0.03
PHE-B	APCI/APPI	885.5	607.5	35	547.5	45	1.1/1.2 ± 0.02
Cu-PHE-A	APCI/APPI	932.5	594.4	40	654.4	25	1.9/1.9 ± 0.05
Cu-PHE-B	APCI/APPI	946.5	668.4	25	608.4	25	1.6/1.7 ± 0.07
Cy-PyroPHE-A	APCI/APPI	874.5	595.0	25	522.1	30	1.3/1.2 ± 0.06

684 [a] SD, standard deviation (n:5)

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686 **Table S2:** Assignment of ions observed for CHL-A, β-CRIPT, LUT and VIO in APPI using different dopants (post-column addition of 5%, *v/v*)

Pigment	*m/z*	Ion Assignment	No - dopant	Dopant		
				Acetone	THF	Toluene
			Rel. Ab. %	Rel. Ab. %	Rel. Ab. %	Rel. Ab. %
VIO	601.4	$[M+H]^+$	100	100	100	100
	583.4	$[M+H-H_2O]^+$	85	55	60	60
	565.4	$[M+H-2\cdot H_2O]^+$	18	10	10	10
LUT	551.4	$[M+H-H_2O]^+$	100	100	100	100
β-CRIPT	553.4	$[M+H]^+$	100	100	100	100
	535.4	$[M+H-H_2O]^+$	22	33	11	27
CHL-A	893.5	$[M+H]^+$	100	100	100	100

Pigment	*m/z*	Ion Assignment	Dopant	
			Anisole	Chlorobenzene Rel.
			Rel. Ab. %	Ab. %
VIO	600.4	$[M]^{+\bullet}$	100	100
	583.4	$[M+H-H_2O]^+$	5	12
LUT	568.4	$[M]^{+\bullet}$	100	100
	551.4	$[M+H-H_2O]^+$	14	18
β-CRIPT	553.4	$[M+H]^+$	100	100
CHL-A	893.5	$[M+H]^+$	100	100
	892.5	$[M]^{+\bullet}$	85	77

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Supplementary Figures

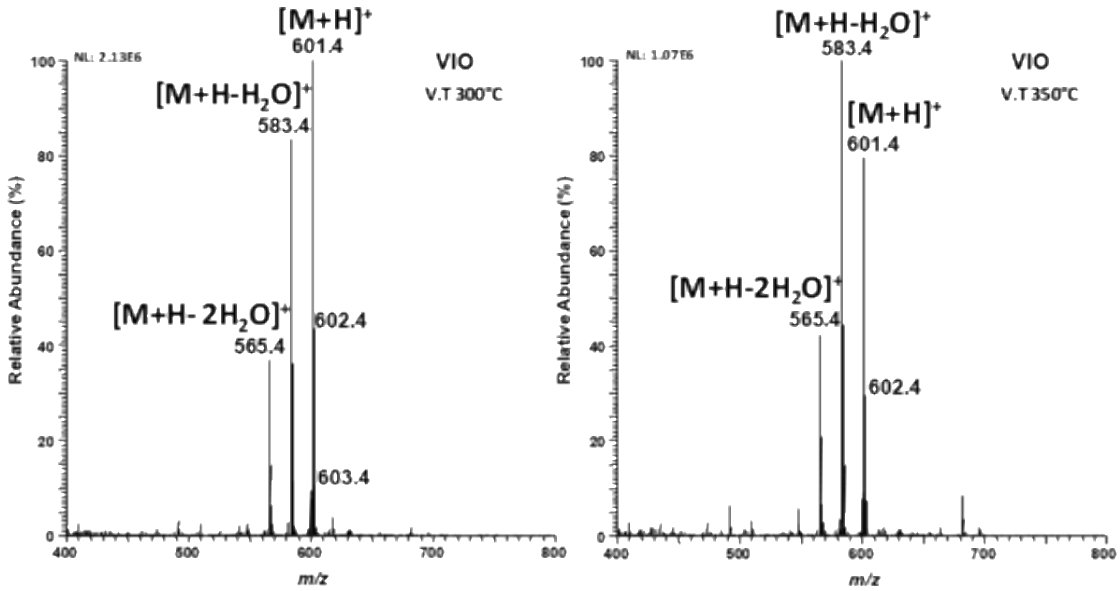

Figure S1: Effect of vaporizer temperature on the response of VIO in APCI source.

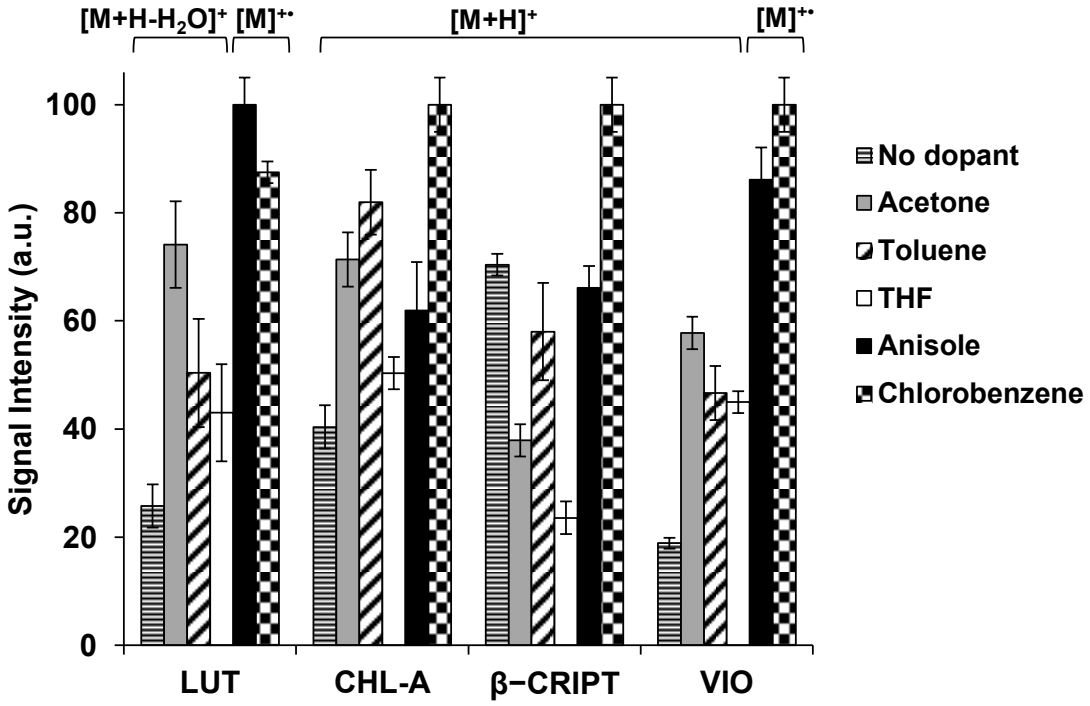

Figure S2: Effect of different dopants (post-column addition of 5% *v/v*) on the response of some selected photosynthetic pigments using APPI.

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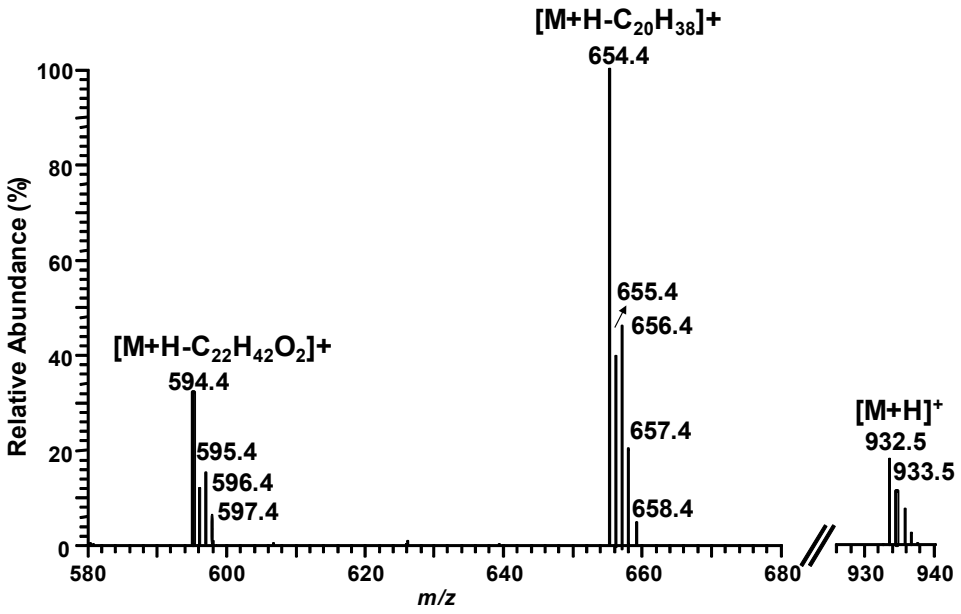

Figure S3: Tandem mass spectrum of Cu-PHE-A using [M+H]+ as precursor ion. Q1 isolation window: 10 Da FWHM.

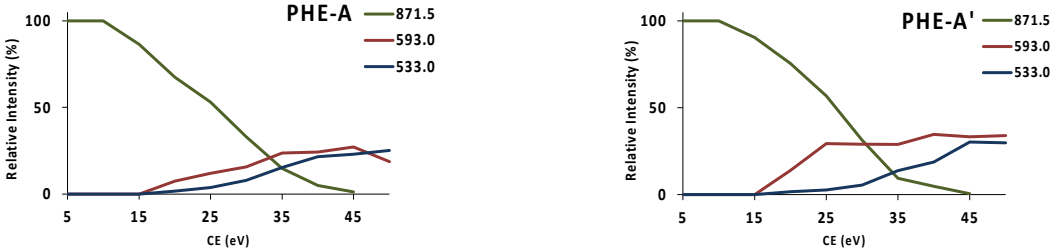

Figure S4: Effect of collision energy (CE) on the response of precursor and product ions for PHE-A and its epimer (PHE-A').