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Ultrahigh-Pressure Liquid Chromatography Triple-Quadrupole Tandem Mass Spectrometry Quantitation of Polyphenols and Secoiridoids in California-Style Black Ripe Olives and Dry Salt-Cured Olives

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Supporting Information

ABSTRACT: The chemical composition of finished table olive products is influenced by the olive variety and the processing method used to debitter or cure table olives. Herein, a rapid ultrahigh-pressure liquid chromatography triple-quadrupole tandem mass spectrometry method, using dynamic multiple reaction monitoring, was developed for the quantitation of 12 predominant phenolic and secoiridoid compounds in olive fruit, including hydroxytyrosol, oleuropein, hydroxytyrosol-4-*O*-glucoside, luteolin-7-*O*-glucoside, rutin, verbascoside, oleoside-11-methyl ester, 2,6-dimethoxy-*p*-benzoquinone, phenolic acids (chlorogenic and *o*-coumaric acids), oleuropein aglycone, and ligstroside aglycone. Levels of these compounds were measured in fresh and California-style black ripe processed Manzanilla olives and two dry salt-cured olive varieties (Mission from California and Throuba Thassos from Greece). Results indicate that the variety and debittering processing method have strong impact on the profile of phenolic and secoiridoid compounds in table olives. The dry salt-cured olives contained higher amounts of most compounds studied, especially oleuropein (1459.5 ± 100.1 μg/g), whereas California-style black ripe olives had a significant reduction or loss of these bioactive compounds (e.g., oleuropein level at 36.7 ± 3.1 μg/g).

KEYWORDS: *table olives, polyphenols, secoiridoids, California-style black ripe, dry salt, UHPLC−QqQ MS/MS*

INTRODUCTION

Table olives from *Olea europaea* L. are a traditional product and an important component of the Mediterranean diet. World consumption of table olives is assessed at 2 521 500 tons in 2013.[1] There are three main categories of table olives based on the trade preparation methods, including green (or Spanish style), natural black (or Greek style), and California-style black ripe olives.[2] The California-style black ripe olives are the most widely consumed olive among American consumers. Olive fruit is a rich source of compounds with health-protecting activities and include hydroxytyrosol, oleuropein, and many other related biophenols and secoiridoid derivatives.[3] The complement of bioactives in the final olive products is influenced by the olive variety and the debittering method used in preparing the olives.[4] Debittering methods are of interest, because previous studies indicate that they can lead to olives with either increased or significantly reduced healthy properties.[5]

As a native to the Mediterranean, historically olives were first consumed as tree-ripened fruit. Olives lose bitterness through natural ripening processes (in Greece known as throuba or stafidolia).[6] Inhabitants of the Mediterranean area, who relied on olives as an essential part of their diet, developed various methods to preserve olives after harvesting. For many centuries, the basic ingredient used to preserve olives was salt. Additional ingredients used in olive preservation included honey, olive oil, vinegar, and vine juice.[6] The major bitter component of olives is oleuropein and its derivatives.[7] Oleuropein is removed during dry salt curing and can also be removed through other processes, including lye-assisted hydrolysis of oleuropein.[7−9] In Greece, olives are now produced primarily using brine (known as Greek style), dry salt, and lye (i.e., a 1−2% sodium hydroxide solution). In other countries, such as Spain, Italy, and the United States, most producers rely on lye to assist in debittering olives (Spanish-style and California-style black ripe olives).

Throuba Thassos olives (also called Stafidolies, twisted, or wrinkled olives) are a culturally important edible olive in Greece. Traditionally, this fruit is over-ripened on the tree, giving it a wrinkled appearance, and requires no additional processing to reduce bitterness. However, for economic reasons Throuba Thassos olives are now produced almost exclusively using dry salt processing to give a similar wrinkled appearance as tree-ripened olives. Despite the similar appearance, the two different types of processing lead to olives with different chemical profiles. In contrast to the naturally overripe olives,

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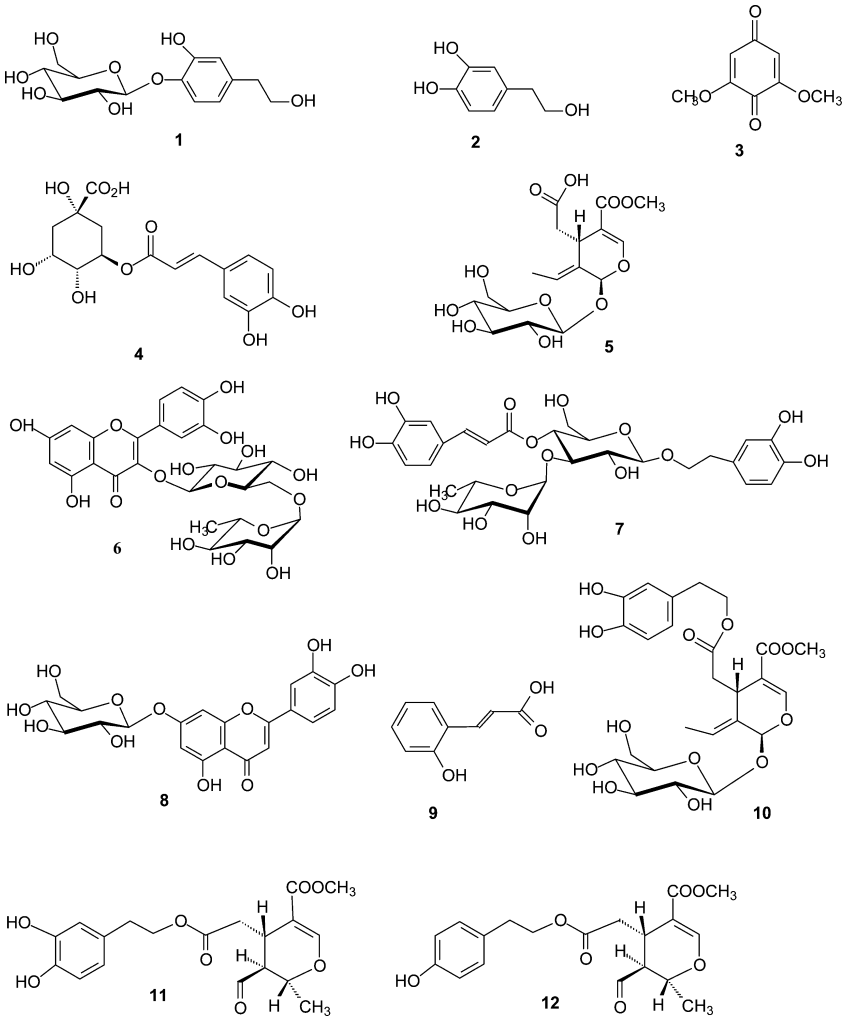

Figure 1. Structures of the studied compounds.

which contain very low amounts of oleuropein,[9] dry salt processing leads to a higher concentration of oleuropein,[5] highlighting the critical role of processing in the chemical composition of finished olives.[4] California-style black ripe olive processing methods involve harvesting olives before complete maturity (green). The raw olives are either directly treated with lye to remove bitterness or preserved in an acidic brine solution (0.2−0.4% calcium chloride, pH < 4.0) until they can be lye-treated and processed. A mild fermentation may occur during the initial brine storage. This style of processing results in olives with the lowest levels of oleuropein and bitterness.[10]

Herein, we describe a rapid ultrahigh-pressure liquid chromatography (UHPLC) triple-quadrupole tandem mass spectrometry (QqQ MS/MS) method using dynamic multiple reaction monitoring (dMRM) for the measurement of a range of key bitter and bioactive constituents in olives. The method was applied for the simultaneous quantitation of hydroxytyrosol-4-*O*-glucoside (**1**), hydroxytyrosol (**2**), 2,6-dimethoxy-*p*-benzoquinone (**3**), chlorogenic acid (**4**), oleoside-11-methyl ester (**5**), rutin (**6**), verbascoside (**7**), luteolin-7-*O*-glucoside (**8**), *o*-coumaric acid (**9**), oleuropein (**10**), oleuropein aglycone monoaldehydic form (**11**), and ligstroside aglycone monoaldehydic form (**12**) (Figure 1) in three different varieties of fresh and processed olives cured using either dry salt or California-style black ripe processing methods.

MATERIALS AND METHODS

Chemicals. High-performance liquid chromatography (HPLC)-grade formic acid, acetonitrile, hexane, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ).

Samples. Dry salt-cured Throuba Thassos olives were purchased from a local market in Davis, CA, and the producer was contacted for processing conditions. The Throuba Thassos olives were treated with 40% dry salt for 2 months. After curing, the olives were washed with water and dried at ambient temperature for 2−3 days before packaging. Dry salt Mission olives were purchased directly from the grower/processor at the local farmers' market in Davis, CA. According to the producer, the olives were treated with dry salt (1:1, w/w) for 3 months. After curing, the olives were washed with water and sun-dried. California-style black ripe olives were prepared using fresh Manzanilla olives collected in early October 2011 in Davis, CA. The fresh samples were analyzed on the day that they were collected. California-style black ripe olives were produced using typical commercial processing.[11] Briefly, the olives were prepared by immersion in a 1−2% sodium hydroxide solution over 3 consecutive days until the lye penetrated the cuticle layers, 1−2 mm of the pulp, and the pit. During the intervals between lye treatments, the fruit was suspended in water, in which air is bubbled for 24 h. Oxygen is required for the black color formation, an oxidation reaction that results from the oxidation and polymerization of *o*-diphenols, mainly hydroxytyrosol and caffeic acid. A ferrous gluconate solution (0.1%, w/v) was added for 24 h to fix the developed black color. Olives were then rinsed with water to neutralize the solution. Olives were packed in a 3% sodium chloride solution and sterilized at 121 °C for 15 min.

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Table 1. Mass Spectra Acquisition Properties of 12 Constituents Quantitated in Olive Fruit[a]

compound	compound name	mass	precursor ion	product ion	fragmentor voltage	collision energy	retention time
1	hydroxytyrosol 4-*O*-glucoside	316.12	315	153	115	12	1.55
2	hydroxytyrosol	154.06	153.1	123	100	8	2.15
3	2,6-dimethoxy-*p*-benzoquinone	168.04	169	141	110	12	3.4
4	chlorogenic acid	354.1	353.1	191	95	12	3.9
5	oleoside-11-methyl ester	404.13	403	223	90	10	4.25
6	rutin	610.15	609.1	300	220	40	4.55
7	verbascoside	624.21	623.2	161	210	36	4.92
8	luteolin-7-*O*-glucoside	448.1	447.1	285	135	16	5.55
9	*o*-coumaric acid	164.05	163	119	80	8	6.18
10	oleuropein	540.18	539.2	275.1	165	20	6.3
11	oleuropein aglycone monoaldehyde form	378.13	377.1	307	105	4	8.95
12	ligstroside aglycone monoaldehyde form	362.14	361.1	291.1	95	8	9.18

[a]All compounds were monitored in negative mode with the exception of compound 3, which was monitored in positive mode.

Reference Compounds. Hydroxytyrosol, oleuropein, rutin, chlorogenic acid, and *o*-coumaric acid were purchased from Extrasynthese (Genay, France), and their purity as stated by the supplier was >98%. Hydroxytyrosol-4-*O*-glucoside, luteolin-7-*O*-glucoside, verbascoside, oleoside-11-methyl ester, 2,6-dimethoxy-*p*-benzoquinone, oleuropein, and ligstroside aglycones were isolated after chromatographic purification from olives. The identity and purity of the isolated compounds was established using nuclear magnetic resonance (NMR) spectroscopy and, in all cases, was >95%.

Extraction and Sample Preparation. A random sampling of 20−30 olives was separated into three sub-samples. Each sub-sample was homogenized individually. A 10 g (wet weight) sample of olive pulp was removed and extracted immediately with 25 mL of MeOH/H_2O (4:1, v/v) in an ultrasonic bath for 45 min at 25 °C. A 25 mL aliquot of hexane was added for oil removal, and the mixture was shaken for 30 s. The supernatant was separated with centrifugation at 4000 rpm for 3 min. The hexane phase was removed, and a 1 mL aliquot of the MeOH/H_2O phase was filtered through a 0.45 μm filter (EMD Millipore, Billerica, MA), diluted with H_2O (1:50), and injected into the liquid chromatography−mass spectrometry (LC−MS).

UHPLC−MS/MS Analysis. Analysis of the 12 phenolic and secoiridoid compounds was performed on a 1290 Infinity UHPLC interfaced to a 6460 QqQ MS/MS with electrospray ionization (ESI) via Jet Stream technology (Agilent Technologies, Santa Clara, CA). The UHPLC was equipped with a binary pump with an integrated vacuum degasser (G4220A), an autosampler (G4226A) with a thermostat (G1330B), and a thermostated column compartment (G1316C). The column used was a 150 × 2.1 mm inner diameter, 2.7 μm, Poroshell 120 EC-C18 (Agilent Technologies, Santa Clara, CA). The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) with the following gradient program: 0−2.5 min, 10% B; 2.5−3.0 min, 10−25% B; 3.0−6.0 min, 25% B; 6.0−7.5 min, 25−40% B; 7.5−8.5 min, 40−95% B; and 8.5−9.5 min, 95% B. The flow rate was 0.4 mL/min, and the injection volume was 1.0 μL.

Negative and positive ESI modes were used. The drying gas temperatures and flow rate were 250 °C and 8 L/min, respectively. The sheath gas temperature and flow rate were 350 °C and 11 L/min, respectively, and the nebulizer gas pressure was 45 psi. The capillary voltage was 3.5 kV for positive and negative modes, and the nozzle voltage was 0 and 1.5 kV for positive and negative modes, respectively.

Dynamic MRM mode was used to analyze olive phenolic and secoiridoid compounds. A cycle time of 500 ms was used. Dwell times were automatically set using cycle time and peak overlap. Precursor ions and product ions were identified and optimized using MassHunter Optimizer (Agilent Technologies). A summary of the precursor ions and product ions used for quantitation is given in Table 1.

Calibration Curves. Standard stock solutions of 1.0 mg/mL were prepared in methanol. Stock solutions were diluted in MeOH/H_2O (4:1, v/v) to give a concentration ranging from 0.001 to 50 μg/mL. Stock solutions were used to identify the linear range for quantitation (LRQ), limit of detection (LOD; S/N > 3), and limit of quantitation (LOQ; S/N > 10) for each analyte. Olive samples were spiked to give concentrations of analytes at 0.002, 0.02, 0.2, 1, 2, and 5 mg/g of olive by diluting appropriate volumes of the stock standard solutions with MeOH/H_2O (4:1, v/v) and mixing with 10.0 g of olive flesh (cv. Manzanilla) after California-style black ripe processing. This olive matrix was selected because it presented the lowest concentrations of the analytes, making the construction of the calibration curves easier. The area of each analyte was measured in a blank matrix standard and then again after the addition of a known amount of standard analyte to the matrix. The difference in area measured for each analyte peak, before and after spiking, was directly correlated with the spiked amount to construct the calibration curve.

Method Validation. The method was checked for the linearity, precision [calculated as the relative standard deviation % (RSD %)], accuracy [evaluated as the relative percentage error % (Er %)], and sensitivity [defined by the limit of detection (LOD) and limit of quantitation (LOQ)].

RESULTS AND DISCUSSION

A UHPLC−QqQ MS/MS method using dynamic MRM was developed to quantitate a range of bitter and biologically active

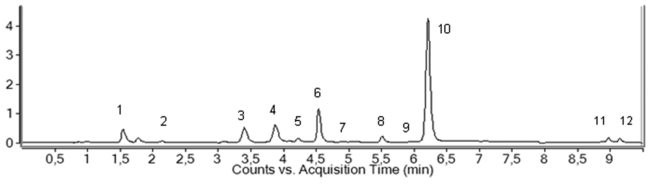

Figure 2. Total ion chromatogram of a mixture of the studied compounds. The numbers assigned to each peak correspond to each of the studied compounds.

phenolic and secoiridoid compounds in table olives. This method offers improved sensitivity, selectivity, and throughput (<10 min/run) in comparison to previously reported methods, as reviewed by Segura-Carretero et al.[12] The usefulness of the new method was demonstrated in the simultaneous quantitation of a broad range of phenolic and secoiridoid compounds in fresh and finished olive products generated by dry salt or California-style debittering methods. Rapid reliable methods for the quantitation of hydroxytyrosol, oleuropein, and their derivatives is of special interest because these compounds have been recently recognized by the European Union (EU) as agents protecting low-density lipoprotein (LDL) oxidation and, thus, offering cardiovascular protection.[13] Currently, this health claim is recognized only for olive oil. To date, several gas

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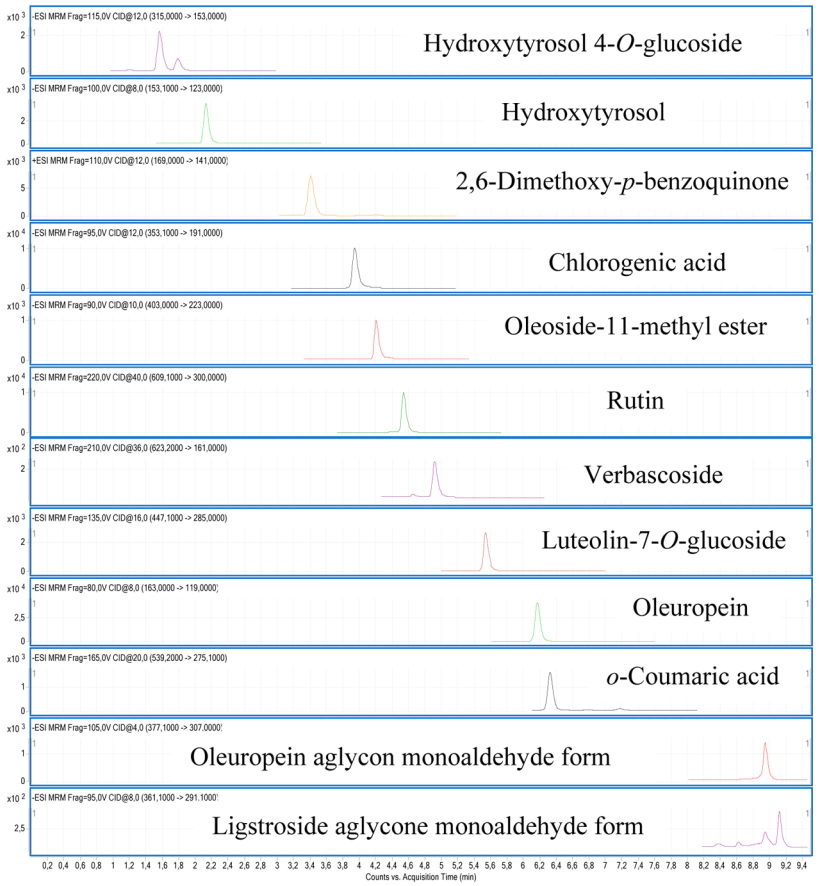

Figure 3. MRM-extracted chromatograms for each quantitated compound from a Mission olive sample.

Table 2. Concentration of Studied Compounds Expressed as Microgram Per Gram of Flesh (Wet Weight), Mean Values of Three Measurements

compound	compound name	Throuba Thassos dry salt	Mission dry salt	Manzanilla fresh	Manzanilla California style
1	hydroxytyrosol 4-*O*-glucoside	263.5 ± 19.1	1890.4 ± 150.5	163.5 ± 15.5	73.1 ± 5.5
2	hydroxytyrosol	195.1 ± 7.8	633.8 ± 55.1	894.5 ± 45.2	210.0 ± 18.8
3	2,6-dimethoxy-*p*-benzoquinone	7.0 ± 0.5	14.9 ± 1.2	916.9 ± 61.3	7.4 ± 0.6
4	chlorogenic acid	3.8 ± 0.2	20.8 ± 1.4	40.8 ± 3.7	ND[a]
5	oleoside-11-methyl ester	4238.1 ± 333.4	1393.8 ± 60.1	231.4 ± 19.9	7.9 ± 0.5
6	rutin	38.2 ± 2.5	194.8 ± 14.7	278.1 ± 20.5	ND
7	verbascoside	156.1 ± 10.1	14.9 ± 1.3	66.5 ± 6.1	ND
8	luteolin-7-*O*-glucoside	11.9 ± 1.0	276.3 ± 11.4	279 ± 18.3	4.5 ± 0.3
9	oleuropein	1459.5 ± 100.1	516.2 ± 44.3	9186.4 ± 530.2	36.7 ± 3.1
10	*o*-coumaric acid	ND	2.0 ± 0.5	1.8 ± 0.3	ND
11	oleuropein aglycone monoaldehyde form	50 ± 4.3	379.3 ± 30.2	2126 ± 190.1	ND
12	ligstroside aglycone monoaldehyde form	ND	74.6 ± 6.1	465.1 ± 34.8	ND

[a]ND = not detected.

chromatography/mass spectrometry (GC/MS), HPLC−ultraviolet (UV), or LC/MS methods are available for monitoring phenolic and secoiridoid compounds in olive products (e.g., olive oil, olive leaves, and olive fruits).[12] The GC/MS methods require derivatization and long analysis times (30−120 min).[14] The HPLC−UV methods have low sensitivity (e.g., LOQ = 1 μg/mL for hydroxytyrosol[5]) and are limited by long chromatographic separation times (20−100 min).[12] Additionally, in some cases, HPLC−UV methods are unable to resolve overlapped peaks,[15] making the simultaneous quantitation of numerous compounds difficult, especially in short separation times. Rapid LC/MS methods have been applied to unprocessed olives but are restricted to the quantitation of oleuropein and hydroxytyrosol.[16,17] A HPLC−Orbitrap MS/MS method for quantitation of nine compounds in fresh olives was recently reported.[18] The HPLC−Orbitrap and current UHPLC−QqQ methods present similar LODs and LOQs for oleuropein, hydroxytyrosol, oleuropein aglycone, and ligstroside aglycone, the only four common compounds studied using the two methods; however, the Orbitrap method presented a longer analysis time (31 min), and the quantitation range was restricted to higher concentrations (e.g., 1000−50 000 ng/mL compared to 100−12 500 ng/mL for oleuropein aglycone and 500−20 000 ng/mL versus 50−6250 ng/mL for hydroxytyr-

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osol). Moreover, the HPLC−Orbitrap method relies on calibration curves that were constructed by dilution of standard compounds in a different solvent (acetonitrile) than the solvent used for the dilution of the real samples (methanol and water). Extraction or dilution of oleocanthal and oleacein using methanol can have a negative impact on their levels as recently reported[19,20] and has the potential to impact other compounds (e.g., oleuropein aglycone and ligstroside aglycone) because these two compounds easily isomerize[20] and require well-controlled conditions for accurate measurements.

Recovery from the olive matrix varied. At high concentrations of added standards (1−5 ppm), recoveries were 85−95%, whereas at low concentrations (0.1−0.5 ppm), recoveries were 50−60%. For this reason, it was necessary to use calibration curves constructed with the addition of known amounts to olive matrix, instead of calibration curves constructed with pure compounds in solution. The method was checked for linearity, precision, accuracy, LOD, and LOQ, and the results were satisfactory. These results show that the method can be efficiently used for the measurement of target analytes. Intraday precision (repeatability) was in all cases <10%.

Special care was taken during the extraction to reduce the risk of enzymatic degradation of sensitive compounds, such as oleuropein and other glucosides, and for this purpose, the olive pulp was extracted immediately after crushing. However, specific experiments examining the role of the sample preparation procedure on the formation of aglycones were not performed.

A total ion chromatogram with all studied compounds (Figure 2) and a representative MRM-extracted chromatogram of Mission olives (Figure 3) demonstrate clearly resolved peaks. Although this study focused on quantitation of 12 targeted compounds, we were able to tentatively identify isomers, such as hydroxytyrosol-1-*O*-glucoside (1.8 min) and luteolin-4-*O*-glucoside (5.95 min), as well as tyrosol glucoside (salidroside), tyrosol, caffeic acid, and methoxytyrosol (data not shown). Tyrosol does not ionize well in ESI mode and, therefore, can only be quantitated at high concentrations. Because the levels of tyrosol were below the level of quantitation, it was not included in this study.

The levels of the 12 secoiridoid and phenolic compounds measured in fresh and California-style black ripe processed Manzanilla olives are given in Table 2. The levels of oleuropein (9186 ± 530 μg/g), oleuropein aglycone monoaldehyde (2126 ± 190 μg/g), and hydroxytyrosol (894.5 ± 45.2 μg/g) were measured in fresh olives (wet weight). After California-style black ripe processing, only hydroxytyrosol, hydroxytyrosol-4-*O*-glucoside, and oleuropein could be detected in levels of 210.0 ± 18.8, 73.1 ± 5.5, and 36.7 ± 3.1 μg/g wet weight, respectively. All of the other compounds presented concentrations lower than 10 μg/g wet weight. Chlorogenic acid, rutin, verbascoside, *o*-coumaric acid, oleuropein aglycone, and ligstroside aglycone could not be detected. These data indicate that California-style black ripe olive processing methods lead to significant reductions in the levels of the secoridoid and phenolic compounds evaluated in this study. This finding is in accordance with previous reports on phenolic compound change during California-style processing;[5] however, herein, a larger range of compounds was evaluated and with higher sensitivity.

In contrast to California-style olives, the dry salt Mission and Throuba Thassos olives presented relatively higher amounts of almost all studied compounds. For example, in Mission and Throuba Thassos olives, the levels were of oleuropein (516.2 ± 44.3 and 1459.5 ± 100.1 μg/g of wet weight), oleoside methyl ester (1393.8 ± 60.1 and 4238.1 ± 333.4 μg/g of wet weight), hydroxytyrosol (633.8 ± 55.1 and 195.1 ± 7.8 μg/g of wet weight), and hydroxytyrosol glucosides (1890.4 ± 150.5 and 263.5 ± 19.1 μg/g of wet weight) (Table 2). These findings support dry salt debittering methods as being advantageous for the retention of polyphenolic and secoiridoid constituents in table olives,[3] and they are not restricted to a specific variety; two commercial samples of two cultivars of different geographic origins presented similar retention of the targeted compounds.

The information presented herein demonstrates wide variation in the composition of a range of key bioactive phenolics in olives that is dependent upon both the variety and processing method used to create the olive product, and these factor need to be considered when developing possible health claims for table olives and their products.

■ ASSOCIATED CONTENT

Supporting Information

Linearity range, r^2, accuracy, LOD, and LOQ values for each compound in solution. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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