From Olive Drupes to Olive Oil. An HPLC-Orbitrap-based Qualitative and Quantitative Exploration of Olive Key Metabolites

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Key words
- olive oil production
- olive drupes
- HPLC-Orbitrap-HRMS/MS
- oleocanthal
- oleacein

Abstract

The aim of the current study was the qualitative exploration and quantitative monitoring of key olive secondary metabolites in different production steps (drupes, paste, first and final oil) throughout a virgin olive oil production line. The Greek variety Koroneiki was selected as one of the most representative olives, which is rich in biological active compounds. For the first time, an HPLC-Orbitrap platform was employed for both qualitative and quantitative purposes. Fifty-two components belonging to phenyl alcohols, secoiridoids, flavonoids, triterpenes, and lactones were identified based on HRMS and HRMS/MS data. Nine biologically and chemically significant metabolites were quantitatively determined throughout the four production steps. Drupes and paste were found to be rich in several components, which are not present in the final oil. The current study discloses the chemical nature of different olive materials in a successive and integrated way and reveals new sources of high added value constituents of olives.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

received April 29, 2013
revised July 1, 2013
accepted August 11, 2013

Bibliography
DOI http://dx.doi.org/10.1055/s-0033-1350823
Published online September 26, 2013
Planta Med 2013; 79: 1576–1587 © Georg Thieme Verlag KG Stuttgart · New York · ISSN 0032-0943

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Introduction

In 2011, the European Food Safety Authority (EFSA) published a scientific opinion suggesting that virgin olive oil (VOO) could be considered as a beneficial health agent based on the numerous scientific publications that underline its biological impact. Most of those studies have shown associations between a VOO-rich diet and a decreased cardiovascular risk, stroke incidences, and type 2 diabetes. Specifically, VOO has been shown to possess antiatherosclerotic effects and its consumption has been shown to decrease plasma low-density lipoprotein (LDL) and cholesterol levels [1]. In other studies, VOO showed potent antihaemostatic and anti-inflammatory properties, as well as an improved effect on endothelial function and insulin sensitivity [2]. One of the most important groups of bioactive secondary metabolites in VOO is phenolic alcohols, e.g., tyrosol and hydroxytyrosol. These alcohols form esters with elenolic acid derivatives to give glycosylated secoiridoids, such as oleuropein and ligstroside, the corresponding aglycons and decarboxymethyl forms with different configurations of the elenolic acid moiety, e.g., oleacein and oleocanthal. These compounds show antimicrobilal, anti-inflammatory, and hypoglycaemic effects and are considered responsible for the antioxidant properties of VOO [3–5]. Apart from phenols and secoiridoids, triterpenes, such as maslinic and oleanolic acid, are also characteristic secondary metabolites, abundant in VOO, and contribute to several of its biological effects. **Fig. 1** and **Fig. 1S** of the supplementary data illustrate representative secondary metabolites found in VOO with a documented biological impact.

Despite the high significance of the olive polyphenols, their final concentration in the oil is really questionable, since the process during oil production can destroy, degrade, or simply remove to waste, large quantities of these valuable secondary metabolites. The production of oil is more dynamic than a simple extraction procedure, since important enzymatic functions are activated transforming initial bioactive molecules found in drupes to other derivatives, finally consumed in oil. Furthermore, the comprehension of the variability of crucial olive secondary metabolites throughout the olive oil production procedure is of great importance. The synthesis of several of these valuable components is often de-

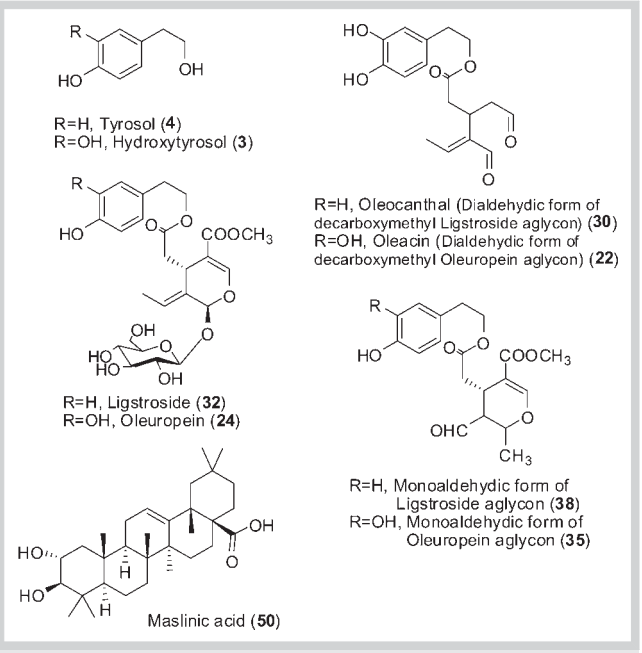

Fig. 1 Representative structures of olive secondary metabolites that have been quantified throughout the production steps of VOO (the numbering of compounds is in accordance with ◉ **Table 1**).

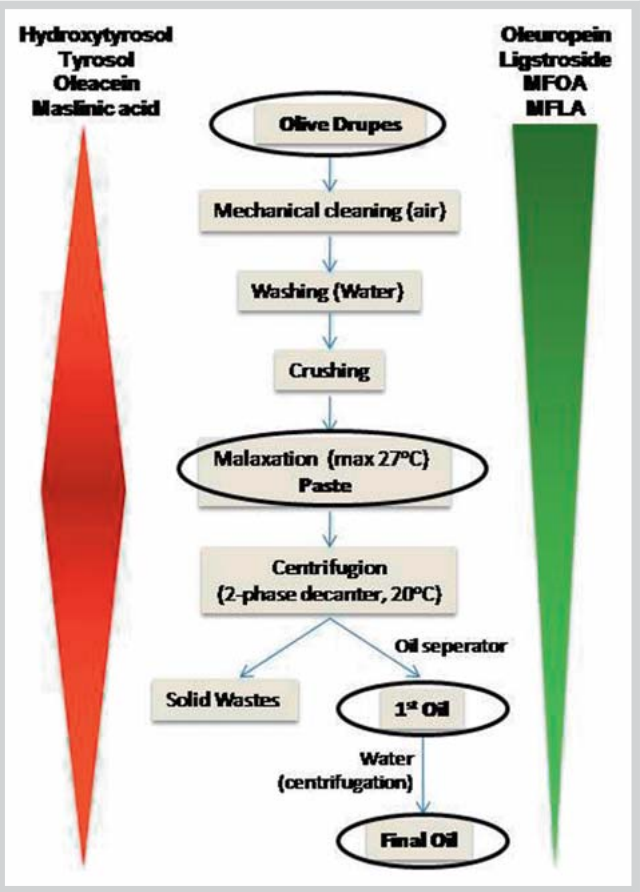

Fig. 2 Graphical illustration of the basic production steps of a two-phase olive oil mill. The different materials under investigation are highlighted as well as the quantitative alterations of some biologically important secondary metabolites. MFOA: monoaldehydic form of oleuropein aglycon; MFLA: monoaldehydic form of ligstroside aglycon.
(Color figure available online only.)

manding, time consuming, and expensive, e.g., oleocanthal [6, 7]. Additionally, in a modern consuming market, compounds of natural origin, rather than synthetic (organic, natural materials for food supplements and cosmetics), are requested. Thus, it is critical to explore the entire procedure of olive oil production qualitatively and quantitatively, and define the chemical nature of each intermediate material. Along these lines, the determination of new or optimum sources of key bioactives of olives is of great important for the isolation thereof or the creation of enriched extracts.

Up to now, studies are characterised by the lack of a holistic, complete, and successive approach that could investigate the entire metabolic content of different materials of the olive oil production process. Most of the studies concern determination of certain phenols in specific samples, such as single paste, drupes, or oil extracts. Consequently, this fragmented approach allows neither the comprehensive investigation of olive constituents nor their biotransformation, and therefore the quality of olive oil. Regarding the analytical methods used for the quantitative determinations of phenols and/or secoiridoids, most of the studies have been performed on the basis of the UV-Vis absorption [8–10]. There are a limited number of studies utilising LC-MS platforms for quantitative analysis of components in specific olive samples. Regarding the analysers, mostly triple quadrupoles (TQ) have been used [11, 12], while recently some studies incorporating TOF technology have been published [13].

HRMS analysers such as TOF and Orbitrap have become competitive tools for the characterisation of complicated components in complex matrices. The latest generations of HRMS instruments, such as Orbitrap platforms, show improved resolution and stability of accurate mass measurements, and at the same time great quantitative potential [14]. Given these advantages, Orbitrap-MS quantitative determination is an alluring platform for confident and rapid quantitative and, simultaneously, qualitative monitoring of small molecules in complex mixtures.

Thus, the aim of the present study was the qualitative and quantitative monitoring of important olive secondary metabolites, throughout the whole olive oil production procedure, incorporating an HPLC-LTQ-Orbitrap platform. The basic steps of the olive oil production procedure, from the drupes to final oil (drupes, paste, first oil, final oil, waste), at a commercial two-phase oil mill are defined and the most important Greek variety Koroneiki, at the late ripening stage, was investigated. A detailed HPLC-HRMS and HRMS/MS-based study for the complete characterisation of the chemical constituents of each production step was performed resulting in the identification of more than 50 secondary metabolites, with high confidence. Furthermore, given the fact that the published data are controversial, quantitative monitoring of nine main components of olives with high biological importance (hydroxytyrosol, tyrosol, oleacein, oleocanthal, oleuropein, ligstroside, monoaldehydic form of oleuropein aglycon – MFOA, monoaldehydic form of ligstroside aglycon – MFLA, and maslinic acid) was also carried out.

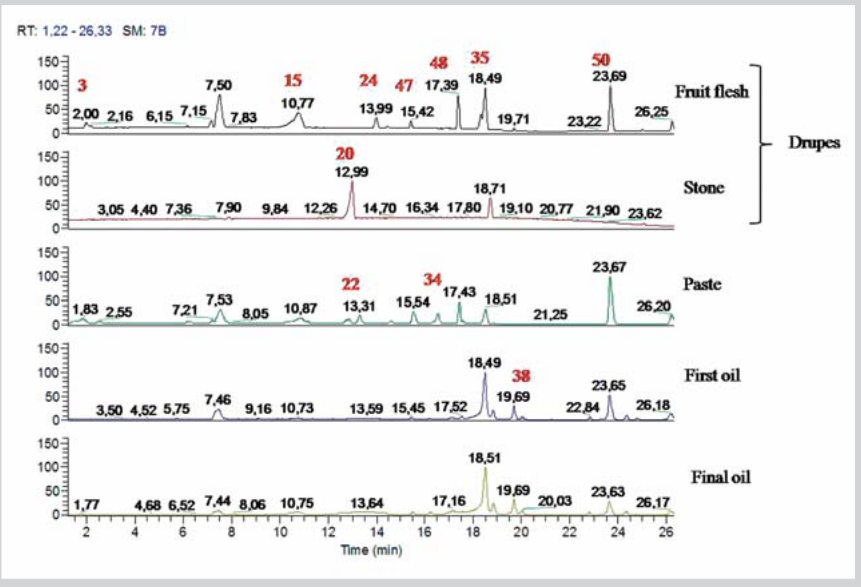

Fig. 3 Comparative base peak chromatograms of all the extracts from each step of the olive oil production procedure. Representative secondary metabolites are annotated.
(Color figure available online only.)

Results and Discussion

Even if olive oil is a widely investigated product due to its nutritional and therapeutic values, the studies regarding the constituents of olive drupes as well as the intermediate materials until the final olive oil production are limited. For instance, the number of studies concerning olive oil before refining and the paste during the malaxation procedure is relatively low. Furthermore, comparative data concerning the metabolic content between the flesh and the stone of olive drupes are also rather small [8, 15–18]. Thus, the first goal of the current study was the LC-HRMS and HRMS/MS profiling of the extracts originating from the different steps of the production of olive oil at a two-phase olive mill. **Fig. 2** illustrates the production procedure of a biphasic olive mill and the different materials under investigation.

The increased resolving power of 30 000, even higher in low mass regions (60 000–70 000), together with the highly accurate mass measurements (routinely $\Delta m < 2–3$ ppm) of the Orbitrap analyser, enabled the detection and identification of numerous compounds. Specifically, chromatographic (Rt) and spectrometric features such as UV absorbance and HRMS/MS data allowed for the detection and identification of 52 secondary metabolites and the performance of a comparative study for the presence of these constituents in the different production steps. It is important to mention that the suggested EC (Elemental Composition) not only for the pseudomolecular ions but also for HRMS/MS fragments as well as the respective indicative RDBeq values assisted drastically in the identification process [19]. **Table 1** summarises the results of the profiling study including some spectroscopic characteristics and major fragments of the detected compounds. The ESI ionisation method, in the negative mode, which has been effectively applied in the past for the detection of secondary metabolites in VOOs, was chosen [20, 21]. As expected, most of the detected compounds belong to phenyl alcohols, secoiridoids, phenolic acids, triterpenes, and lactones. **Fig. 3** illustrates the base peak chromatograms of all samples under investigation with the identification of some major peaks.

Furthermore, specific key olive secondary metabolites were quantitatively monitored throughout the two-phase VOO production. There are recent reports concerning the application of HPLC-MS/MS systems for the quantitative determination of several major olive phenolics, such as hydroxytyrosol, tyrosol, oleuropein, and others [12, 22]. However, the application of HPLC-LTQ-Orbitrap is something that has never been applied before. Specifically, nine secondary metabolites with an important biological impact, hydroxytyrosol, tyrosol, oleacein, oleocanthal, oleuropein, ligstroside, MFOA, MFLA, and maslinic acid, were quantified. The analysis was performed in the full scan mode using the ion extraction method for the quantification and detection of the analytes. The extracted ion chromatograms (XICs) of the compounds quantified are presented in **Fig. 4**. It is important to highlight that oleacein and oleocanthal are eluted as broad peaks. This chromatographic behaviour could be attributed to the dialdehydic system of both molecules that form hemiacetals when analysed in water or other protic solvents [12]. The results of the quantitative determination of the nine standard compounds are presented in **Fig. 5** and **Table 2**. All quantities are expressed as mg/kg of fresh weight and as mmol/kg of initial fresh weight. In the case of oil, it has been estimated that from 1 kg of olive drupes, 250 mL of olive oil is produced.

a) Phenyl alcohols and derivatives: In total, six simple phenyl alcohols (**1** to **6**) were detected in all olive samples (**Table 1**). This mainly concerns tyrosol, hydroxytyrosol, their derivatives and glycosylated forms and they are eluted at the first ten minutes of the run due to their strong hydrophilic nature. Only tyrosol (**4**), a precursor of ligstroside (**32**), and hydroxytyrosol (**3**), a precursor of oleuropein (**24**), were detected in all different materials of the production procedure while the corresponding glycosides (**5, 2i**, and **2ii**) were detected only in the drupes and paste, and are absent from both oils. This could be attributed to the activity of certain enzymes, e.g., β-glucosidase during the malaxation process being able to hydrolyse the glycosides to the respective aglycons. Interestingly, there is a large alteration of their quantities in the different samples (**Fig. 5** and **Table 2**).

Generally, it is accepted that hydroxytyrosol is a degradation product of oleuropein [23], a process which is enhanced by the ripening stage [24]. The drupes studied in the present study were at the latest maturity stage; however, the levels of hydroxytyrosol and tyrosol in this extract were relatively low (0.81 and 1.02 mmol/kg, respectively). In the same sample, the levels of

Table 1 Secondary metabolites detected in the different olive extracts. Rt = retention time; EC = elemental composition; RDBeq = ring double bond equivalent; $[M - H]^-$: *m/z* of the pseudomolecular ion. In "**bold**" are the compounds which were quantified.

	Compounds	Extracts	Rt (min)	EC	RDBeq	$[M - H]^-$ *m/z*	Main fragments (EC, RDBeq)
	Phenyl alcohols & derivatives						
1	3,4-Dihydroxy-phenyl glycol	1st oil, final oil	1.00	$C_8H_9O_4$	4.5	169.0510	–
2	Hydroxytyrosol hexoside (2 isomers)	Drupes, paste	i) 1.44 ii) 1.98	$C_{14}H_{19}O_8$	5.5	315.1088	153.0563 ($C_8H_9O_3$, 4.5)
3	**Hydroxytyrosol**	**All**	**1.79**	**$C_8H_9O_3$**	**4.5**	**153.0563**	**123.0456 ($C_7H_7O_2$, 4.5)**
4	**Tyrosol**	**All**	**3.29**	**$C_8H_9O_2$**	**4.5**	**137.0602**	**-**
5	Tyrosol hexoside	Drupes, paste	3.40	$C_{14}H_{19}O_7$	5.5	299.1138	–
6	Hydroxytyrosol acetate	1st oil, final oil	9.51	$C_{10}H_{11}O_4$	5.5	195.0666	–
	Secoiridoids and derivatives						
7	Hydroxylated products of the decarboxymethyl elenolic acid (two isomers)	Drupes, paste	i) 1.64, ii) 3.11	$C_9H_{11}O_5$	4.5	199.0616	181.0508 ($C_9H_9O_4$, 5.5) 155.0717 ($C_8H_{11}O_3$, 3.5) 111.0821 ($C_7H_{11}O$, 2.5)
8	Hydroxylated products of the decarboxyl elenolic acid (two isomers)	a. Drupes b. Paste	a. 3.77 b. i) 3.77, ii) 4.39	$C_{10}H_{13}O_5$	4.5	213. 0772	151.0768 ($C_9H_{11}O_2$, 4.5)
9	Hydroxylated form of elenolic acid	1st oil, final oil	4.83	$C_{11}H_{13}O_7$	5.5	257. 0669	–
10	Oleoside	Drupes, paste	5.56	$C_{16}H_{21}O_{11}$	6.5	389.1092	345.1195 ($C_{15}H_{21}O_9$, 5.5)
11	Secologanoside	Drupes, paste	5.77	$C_{16}H_{21}O_{11}$	6.5	389.1092	345.1195 ($C_{15}H_{21}O_9$, 5.5)
12	Glucosyl-methyloleoside	Paste	6.08	$C_{23}H_{33}O_{16}$	7	565.1778	–
13	Oleoside 11-methyl ester/oleoside 7-methyl ester/8-epikingiside (three isomers)	a. Drupes b. Paste	a. i) 6.61, ii) 8.47 b. i) 7.96, ii) 8.47	$C_{17}H_{23}O_{11}$	6.5	403.1249	223.0612 ($C_{11}H_{11}O_5$, 6.5)
14	Aldehydic form of decarboxyl elenolic acid	Drupes, paste	7.93	$C_{10}H_{15}O_5$	3.5	215. 0929	197.0823 ($C_{10}H_{13}O_4$, 4.5) 153.0926 ($C_9H_{13}O_2$, 3.5)
15	Elenolic acid.	All	10.77	$C_{11}H_{13}O_6$	5.5	241.0720	209.0457 ($C_{10}H_9O_5$, 6.5) 165.0561 ($C_9H_9O_3$, 5.5) 139.0404 ($C_7H_7O_3$, 4.5) 127.0405 ($C_6H_7O_3$, 3.5) 121.0300 ($C_7H_5O_2$, 5.5) 101.0250 ($C_4H_5O_3$, 2.5) 95.0509 (C_6H_7O, 3.5)
16	Dihydrooleuropein	Paste	11.62	$C_{25}H_{35}O_{13}$	8.5	543.2087	525.1980 ($C_{25}H_{33}O_2$, 9.5) 513.1982 ($C_{24}H_{33}O_{12}$, 8.5)
17	*Neo*-nuzhenide	Paste	12.28	$C_{31}H_{41}O_{18}$	11.5	701.2295	–
18	Hydroxytyrosol acyclodihydroelenolate	Drupes, paste	12.66	$C_{19}H_{25}O_8$	7.5	381.1557	363.1450 ($C_{19}H_{23}O_7$) 349.1289 ($C_{18}H_{21}O_7$) 331.1185 ($C_{18}H_{19}O_6$) 213.0761 ($C_{10}H_{13}O_5$, 4.5) 151.0768 ($C_9H_{11}O_2$, 4.5)
19	Caffeoyl-6-oleoside	Drupes, paste	12.72	$C_{25}H_{27}O_{14}$	12.5	551.1411	–
20	Nuzhenide	Drupes, paste	12.99	$C_{31}H_{41}O_{17}$	11.5	685.2332	523.1809 ($C_{25}H_{31}O_{12}$, 10.5) 453.1389 ($C_{21}H_{25}O_{11}$, 9.5) 421.1495 ($C_{21}H_{25}O_9$, 9.5) 299.1130 ($C_{14}H_{19}O_7$, 5.5)
21	Hydroxy-*O*-decarboxymethyl oleuropein aglycon/hydroxylated form of oleacein	Paste, 1st oil, Final oil	i) 8.31 (broad peak) ii) 13.00	$C_{17}H_{19}O_7$	8.5	335.1137	199.0614 ($C_9H_{11}O_5$, 4.5)
22	**Oleacein**	**All**	**13.22**	**$C_{17}H_{19}O_6$**	**8.5**	**319.1189**	**301.1082 ($C_{17}H_{17}O_5$, 9.5) 195.0663 ($C_{10}H_{11}O_4$, 5.5) 165.0560 ($C_9H_9O_3$, 5.5)**
23	Caffeoyl-6-secologanoside	Drupes, paste	13.63	$C_{25}H_{27}O_{14}$	12.5	551.1411	–
24	**Oleuropein**	**Drupes, paste**	**13.89**	**$C_{25}H_{31}O_{13}$**	**10.5**	**539.1773**	**377.1239 ($C_{19}H_{21}O_8$, 9.5) 307.0821 ($C_{15}H_{15}O_7$, 8.5) 275.0923 ($C_{15}H_{15}O_5$, 8.5)**
25	Oleuropein isomers	Drupes, paste	i) 13.25, ii) 14.87	$C_{25}H_{31}O_{13}$	10.5	539.1773	469.1352 ($C_{21}H_{25}O_{12}$, 9.5) 437.1458 ($C_{21}H_{25}O_{10}$, 9.5) 315.1084 ($C_{15}H_{19}O_8$, 5.5)
26	Hydroxy-oleuropein aglycon	1st oil, final oil	i) 14.06, ii) 16.88	$C_{19}H_{21}O_9$	9.5	393.1194	–
27	*p*-Coumaroyl-6-oleoside	Drupes, paste	14.22	$C_{25}H_{27}O_{13}$	12.5	535.1460	491.1560 ($C_{24}H_{27}O_{11}$, 11.5)
28	Oleuroside	Drupes, paste	14.43	$C_{25}H_{31}O_{13}$	10.5	539.1773	469.1352 ($C_{21}H_{25}O_{12}$, 9.5) 437.1458 ($C_{21}H_{25}O_{10}$, 9.5) 315.1084 ($C_{15}H_{19}O_8$, 5.5)

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Table 1 *Continued*

	Compounds	Extracts	Rt (min)	EC	RDBeq	$[M-H]^-$ *m/z*	Main fragments (EC, RDBeq)
29	Oleuropein aglycon derivative	Paste	14.91	$C_{19}H_{25}O_8$	7.5	365.1608	–
30	**Oleocanthal**	**All**	**16.64**	**$C_{17}H_{19}O_5$**	**8.5**	**303.1241**	**285.1132 ($C_{17}H_{17}O_4$, 9.5) 179.0715 ($C_{10}H_{11}O_3$, 5.5) 165.0559 ($C_9H_9O_3$, 5.5)**
31	Comselogoside (*p*-coumaroyl-6-secologanoside)	Drupes, paste	15.33	$C_{25}H_{27}O_{13}$	12.5	535.1460	491.1560 ($C_{24}H_{27}O_{11}$, 11.5) 265.0728 ($C_{13}H_{13}O_6$, 7.5)
32	**Ligstroside**	**Drupes, paste**	**15.64**	**$C_{25}H_{31}O_{12}$**	**10.5**	**523.1825**	**361.1291 ($C_{19}H_{21}O_7$, 9.5) 291.0876 ($C_{15}H_{15}O_6$, 8.5) 259.0971 ($C_{15}H_{15}O_4$, 8.5)**
33	Oleuropein aglycon	All	i) 15.91, ii) 16.86, iii) 18.65	$C_{19}H_{21}O_8$	9.5	377.1246	345.0979 ($C_{18}H_{17}O_7$, 10.5) 307.0821 ($C_{15}H_{15}O_7$, 8.5) 275.0923 ($C_{15}H_{15}O_5$, 8.5) 241.0718 ($C_{11}H_{13}O_6$, 5.5) only for Rt. 15.90)
34	Nuzhenide 11-methyl-oleoside	Drupes, paste	16.72	$C_{48}H_{63}O_{27}$	17.5	1071.3517	909.3005 ($C_{42}H_{53}O_{22}$, 16.5) 839.2578 ($C_{38}H_{47}O_{21}$, 15.5) 771.2332 ($C_{34}H_{43}O_{20}$, 13.5) 685.2332 ($C_{31}H_{41}O_{17}$, 11.5) 523.1809 ($C_{25}H_{31}O_{12}$, 10.5) 453.1389 ($C_{21}H_{25}O_{11}$, 9.5)
35	**Monoaldehydic form of oleuropein aglycon**	**All**	**18.51**	**$C_{19}H_{21}O_8$**	**9.5**	**377.1246**	**345.0979 ($C_{18}H_{17}O_7$, 10.5) 307.0821 ($C_{15}H_{15}O_7$, 8.5) 275.0923 ($C_{15}H_{15}O_5$, 8.5) 275.0561 ($C_{14}H_{11}O_6$, 9.5) 247.1000 ($C_{14}H_{15}O_4$, 7.5) 195.0666 ($C_{10}H_{11}O_4$, 5.5) 149.0248 ($C_8H_5O_4$, 6.5) 121.0307 ($C_7H_5O_3$, 5.5)**
36	6-O-[(2E)-2,6-Dimethyl-8-hydroxy-2-octenoyloxy] secologanoside	All	17.32	$C_{26}H_{37}O_{13}$	8.5	557.2242	513.2344 ($C_{25}H_{37}O_{11}$, 7.5) 345.1190 ($C_{15}H_{21}O_9$, 5.5)
37	Ligstroside aglycon	All	i) 17.24, ii) 18.37, iii) 19.85	$C_{19}H_{21}O_7$	9.5	361.1291	291.0876 ($C_{15}H_{15}O_6$, 8.5) 259.0971 ($C_{15}H_{15}O_4$, 8.5)
38	**Monoaldehydic form of ligstroside aglycon**	**All**	**19.63**	**$C_{19}H_{22}O_7$**	**9.5**	**361.1291**	**291.0876 ($C_{15}H_{15}O_6$, 8.5) 259.0971 ($C_{15}H_{15}O_4$, 8.5)**
	Phenolic acid derivatives						
39	*β*-Hydroxyverbascoside [Campneoside II]	Drupes, paste	8.68	$C_{29}H_{36}O_{16}$	12.5	639.1933	–
40	Verbascoside	Drupes, paste	10.91	$C_{29}H_{35}O_{15}$	12.5	623.1984	461.1670 ($C_{20}H_{29}O_{12}$, 6.5)
41	Isoacteoside	a. Drupes b. Paste	a.) 11.91 b. i) 10.94, ii) 11.91	$C_{29}H_{35}O_{15}$	12.5	623.1984	461.1669 ($C_{20}H_{29}O_{12}$, 6.5)
	Flavonoids						
42	Quercetin-3-*O*-rutinoside	Drupes, paste	9.83	$C_{27}H_{29}O_{16}$	13.5	609.1463	301.0349 ($C_{15}H_9O_7$, 11.5)
43	Luteolin hexosides	Drupes, Paste	i) 10.40, ii) 12.07, iii) 12.94	$C_{21}H_{19}O_{11}$	12.5	447.0937	285.0407 ($C_{15}H_9O_6$, 11.5)
44	Luteolin-7-*O*-rutinoside	Drupes, paste	10.78	$C_{27}H_{29}O_{15}$	13.5	593.1517	447.0934 ($C_{21}H_{19}O_{11}$, 12.5) 285.0408 ($C_{15}H_9O_6$, 11.5)
45	Apigenin-7-*O*-hexosides	Drupes	12.02	$C_{21}H_{19}O_{10}$	12.5	431.0988	269.0452 ($C_{15}H_9O_5$, 11.5)
46	Apigenin-7-*O*-hexosyl rhamnosides	Drupes, paste	i) 11.66 ii)12.15	$C_{27}H_{29}O_{14}$	13.5	577.1563	269.0453 ($C_{15}H_9O_5$, 11.5)
47	Luteolin	All	15.42	$C_{15}H_9O_6$	11.5	285.0407	217.0515 ($C_{12}H_9O_4$, 8.5) 199.0401 ($C_{12}H_7O_3$, 9.5) 175.0398 ($C_{10}H_7O_3$, 7.5)
48	Apigenin	All, minor in drupes – major in final oil	17.39	$C_{15}H_9O_5$	11.5	269.0458	–
	Triterpenes						
49	Oleanolic acid	All	26.06	$C_{30}H_{47}O_3$	7.5	455.3535	407.3321 ($C_{29}H_{43}O$, 8.5)
50	**Maslinic acid**	**All**	**23.50**	**$C_{30}H_{47}O_4$**	**7.5**	**471.3484**	**423.3268 ($C_{29}H_{43}O_2$, 8.5) 405.3158 ($C_{29}H_{41}O$, 9.5) 393.3161 ($C_{28}H_{41}O$, 8.5)**

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Table 1 *Continued*

	Compounds	Extracts	Rt (min)	EC	RDBeq	$[M - H]^-$ *m/z*	Main fragments (EC, RDBeq)
	Lactones						
51	(Z)-2-(5-Ethylidene-2-oxotetra-hydro-2H-pyran-4-yl)acetic acid	All, in drupes traces	6.24	$C_9H_{11}O_4$	4.5	183.0667	139.0768 ($C_8H_{11}O_2$, 3.5)
52	Lactone (ester with hydroxytyrosol)	Paste	12.57	$C_{17}H_{21}O_6$	7.5	321.1346	185.0821 ($C_9H_{13}O_4$, 3.5)

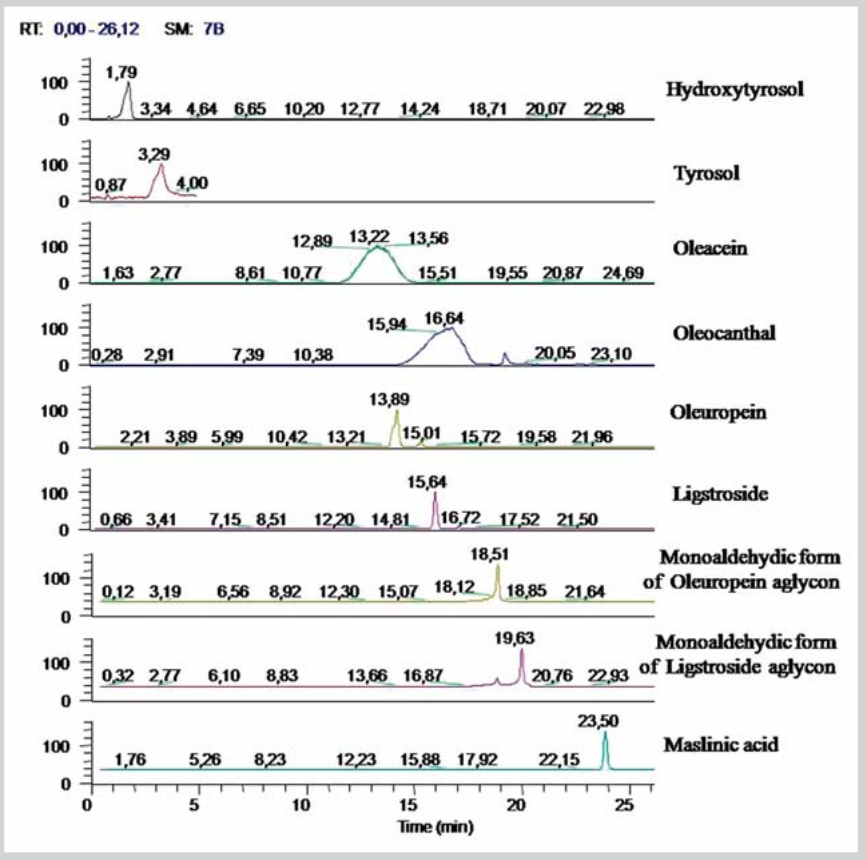

Fig. 4 Extracted ion chromatograms (XIC) of the pure compounds used for the quantitative determinations.
(Color figure available online only.)

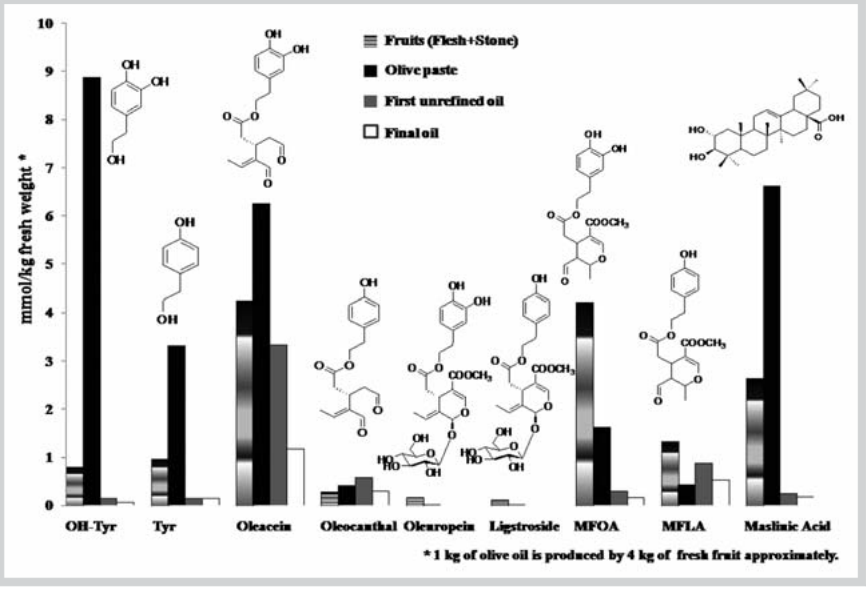

Fig. 5 Quantitative alterations (in mmol/kg fresh drupe weight) of the main selected secondary metabolites. (OH-Tyr: hydroxytyrosol, Tyr: tyrosol, MFOA: monoaldehydic form of oleuropein aglycon, MFLA: monoaldehydic form of ligstroside aglycon).

Table 2 Quantitative results (in mg/kg and mmol/kg of fresh drupe weight) of the nine selected main secondary metabolites in drupes, paste, first oil, and final oil.

	Drupes		Fruit paste		First oil		Final oil	
	mg/kg*	mmol/kg*	mg/kg	mmol/kg	mg/kg	mmol/kg	mg/kg	mmol/kg
Hydroxytyrosol	124.3	**0.81**	1366.4	**8.87**	5.8	**0.04**	2.4	**0.02**
Tyrosol	147.3	**1.02**	457.2	**3.31**	5.3	**0.04**	5	**0.04**
Oleacein	1361.3	**4.25**	2000.6	**6.25**	266.6	**0.83**	94.7	**0.30**
Oleocanthal	96.4	**0.32**	127.1	**0.42**	44.2	**0.15**	22.2	**0.07**
Oleuropein	96.4	**0.17**	13.5	**0.03**	0	**0**	0	**0**
Ligstroside	66.8	**0.13**	9.7	**0.01**	0	**0**	0	**0**
MFOA[1]	1599.7	**4.23**	616.7	**1.63**	110.6	**0.29**	66.3	**0.18**
MFLA[2]	483.3	**1.34**	159.3	**0.44**	79.3	**0.21**	48.5	**0.13**
Maslinic acid	1252.9	**2.65**	3123.2	**6.62**	29.5	**0.06**	20.9	**0.04**

* For the respective calculation of the results, it has to be mentioned that 1 kg of drupes gives approximately 250 mL of oil. Thus, the values for oil samples are equal to mg/250 mL of oil; [1] Monoaldehydic form of oleuropein aglycon; [2] Monoaldehydic form of ligstroside aglycon

oleuropein and ligstroside were also low (0.03 and 0.01 mmol/kg, respectively). This could imply that oleuropein and ligstroside degradation also leads to compounds other than hydroxytyrosol and tyrosol, as discussed below.

Concerning both components, there is an impressive increase during the malaxation of the paste (8.87 and 3.31 mmol/kg) that confirms that the enzymatic procedures promoted during kneading lead to degradation of several components that include tyrosol or hydroxytyrosol units in their basic structure and not only ligstroside and oleuropein. Finally, the levels of both compounds are drastically decreased in both oils indicating their loss in the solid wastes or their transformation to other constituents in VOO.

Two additional phenyl alcohols were also detected, but only in the oils and, specifically, the oxidation products hydroxytyrosol acetate (**6**) [20] and 3,4-dihydroxyphenyl glycol (**1**), with the latter being a hydroxylated derivative of hydroxytyrosol. It is considered to be a metabolite of hydroxytyrosol, but it has been found as a major constituent in naturally-debittered (in brine) edible olives [10]. In the present study, the fact that it is found only in oil samples implies a biotransformation during the oil production process. 3,4-Dihydroxyphenyl glycol is also mentioned as the phenyl moiety of a hydroxylated derivative of oleuropein found in drupes [25]. Nevertheless, this molecule was not traced in any sample in the present study.

b) Secoiridoids: Two of the most represenative secoiridoids found in olives are oleuropein (**24**) and ligstroside (**32**), which the hydrolysis thereof leads to the cleavage of the glycosidic bond and the formation of oleuropein (**33 i, ii**, and **iii**) and ligstroside aglycons (**37**), respectively. Due to the keto-enolic tautomeric equilibrium of the elenolic acid moiety, which involves the ring opening, several isomers of these aglycons can be formed. Fu et al. [26] detected 11 isomers of oleuropein aglycons in VOOs by ESI-TOF-MS. In the samples under investigation, four different isomers of oleuropein aglycon (**33 i, ii**, and **iii, 35**) (Rts 15.91, 16.86, 18.28, and 18.65 min) and respective derivatives for ligstroside aglycons (**37 i, ii**, and **iii, 38**) (17.24, 18.37, 19.50, and 19.85 min) have been detected. However, the isomers which were found in relatively large quantities in all our samples are MFOA and MFLA (**35** and **38**) at Rts 18.28 and 19.50 min, respectively (identified with comparison to the authentic sample), as shown in **Fig. 2S** (Supporting Information). Isomers at 15.91 and 17.24 min, respectively, are relatively more concentrated in the fruit paste extracts.

Critical for the identification of these components were the HRMS/MS data. **Fig. 3S** (Supporting Information) illustrates the HRMS/MS spectra of MFOA and its fragmentation mechanism is described in **Fig. 6**. Briefly, the loss of a methanol group from the carboxymethyl substitution results in the ions at *m/z* 345.0979 and *m/z* 327.0873 after a successive loss of H_2O. The fragment at *m/z* 307.0821 is formed by the loss of a C_4H_6O neutral fragment from the pseudomolecular ion [21], while a further $-OCH_3$ elimination gives the ion at *m/z* 275.0561 (RDB 9.5). This fragmentation pattern agrees with that suggested by Fu et al. [26] (ESI-TOF-MS) and Japon Lujan et al. [11] (ESI-Triple Quadripole MS/MS) for oleuropein aglycons. Fu et al. [26] refers to the fragment at 275.0900 as $C_{14}H_{11}O_6$. In the present analysis, the high resolving power of the Orbital analyser allowed the separation of two different ions in this mass area: a major fragment at *m/z* 275.0923 and a minor one at *m/z* 275.0561 (**Fig. 3S**, Supporting Information). The fragment that corresponds to $C_{14}H_{11}O_6$, prementioned in the literature, was the minor one at *m/z* 275.0561, RDB 9.5, while the major ion at *m/z* 275.0923 corresponds to the molecular formula $C_{15}H_{15}O_5$, RDB 8.5, and has not been mentioned before. It is suggested that, in this case, the fragment is formatted by the subsequent loss of the carboxymethyl, aldehydic, and methyl groups. Further elimination of the phenyl moiety and closing of the heteocyclic ring ends up with the ion at *m/z* 149.0248, which after the loss of a CO group, gives the ion at *m/z* 121.0307. Finally, the ion at *m/z* 195.0666 corresponds to the hydroxytyrosol acetyl ester that is formed through various cleavages of the iridoid part, as shown in **Fig. 6**.

All the other oleuropein aglycons (**33 i, ii**, and **iii**) present similar fragmentation motifs as presented in **Table 1**. However, the isomer at Rt 15.90 min gives a base peak at *m/z* 241.0718, which corresponds to the elenolic acid moiety ($C_{11}H_{13}O_6$). These findings are in accordance with Fu et al. [26] who detected this fragment in only one among eleven oleuropein aglycon isomers (ESI-TOF-MS). It can be suggested that this isomer corresponds to an elenolic moiety configuration that can be stably ionised at ESI^-, possibly compound **15** (**Fig. 1S**, Supporting Information). Regarding the fragmentation of oleuropein, it shares some common fragments with the aglycon, after the cleavage of the glucosidic bond (*m/z* 377.1246).

In the present study, drupes are characterised by low levels of hydroxytyrosol, oleuropein, tyrosol, and ligstroside, but they contain significant quantities of the respective aglycons (**33 i, ii,** and **iii, 37 i, ii,** and **iii**), especially the monoaldehydic forms (**35** and **38**) and the demethylcarboxylated dialdehydic derivatives oleacein (**22**) and oleocanthal (**30**). Concerning these two derivatives,

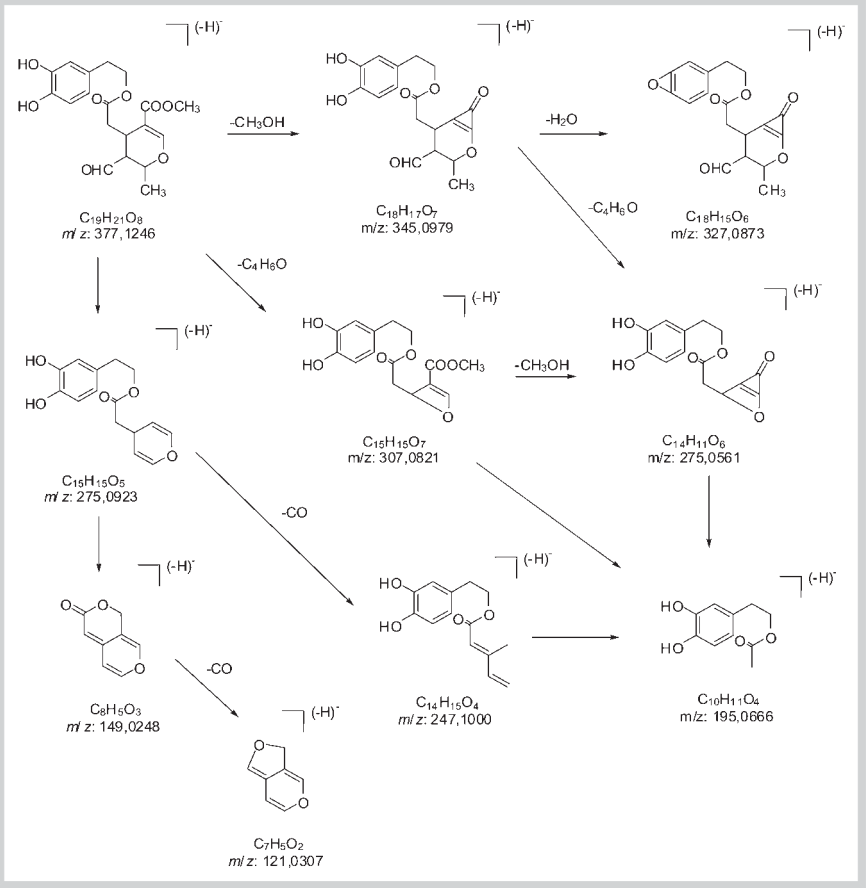

Fig. 6 Proposed fragmentation mechanism of the monoaldehydic form of the oleuropein aglycon (Rt. 18.28).

most studies, especially those dealing with olives at the early ripening stage, are not detected in drupes. Sivakumar et al. [24] has reported a complete absence of oleacein in some varieties; however, it was present in some others only in October and November. On the contrary, other authors have referred to their content increasing during ripening (together with that of oleoside and elenolic acid derivatives) due to mechanical or enzymatic degradation of oleuropein and ligstroside [27]. However, it has been shown that the oleacein content in olive drupes dramatically varies among different varieties (from 23–1.8 mg/g fresh weight) [27]. It is worth noting that mainly oleacein, and secondly oleocanthal, were found as major compounds in all production stages, including drupes.

Elenolic acid (**15**) and derivatives constitute the iridoid part of several important secondary olive metabolites, such as oleuropein and ligstroside, and are often mentioned as one of their hydrolysis products. The high resolving power of the Orbitrap analyser and the highly accurate measurements in the MS/MS level allowed for the identification of multiple fragments and possiblely the fragmentation mechanisms, which is described in **Fig. 4S** of the Supporting Information. Elenolic acid is present in all stages of the production procedure, while its hydroxylated form (**9**) was only found in the oil. On the contrary, its aldehydic derivative (**14**) was only found in the initial stages of the production procedure. Additionally, several other secoiridoids are found only in drupes and paste, a fact that implies degradation during malaxation, or partial removal to the solid wastes. More specifically, isomers of the hydroxylated products of decarboxymethyl and decarboxyl elenolic acid (**7** and **8**) were only detected in drupes and paste. The glucosylated elenolic acid, oleoside (**10**) (**Fig. 1**), and its isomer secologanoside (**11**) were also only detected in drupes and paste, together with their methyl esters (**13**) (**Fig. 1**), and their esters with *p*-coumaric (**27, 31**) and caffeic acid (**19, 23**). Esters with secologanoside have been mentioned before in olive drupes and their HRMS/MS fragmentation patterns have been described [28]. Glucosyl methyl oleoside (**12**) was detected in paste and was mentioned before as a possible biomarker in olive leaves [29].

Nuzhenide (**20**) (**Fig. 1S**, Supporting Information) is considered a major component of olive seeds. It together with its glucoside and MFOA were the major constituents of the extracted seeds. Nuzhenide was not detected in drupe flesh, but was found in paste in smaller quantities than in seeds. It could be suggested that its degradation during the malaxation process contributes to the accumulation of hydroxytyrosol in paste. Its hydroxylated analogue, *neo*-nuzhenide (**17**), previously detected as a micro-component in fruit flesh [25], was not detected in drupes, but in paste, further implying a possible hydroxylation of nuzhenide during the malaxation process. Nuzhenide 11-methyl-oleoside (**34**), a more complex derivative, was also found in drupes and paste. HRMS/MS fragments of nuzhenide have been mentioned before [30], (**Fig. 5S,** Supporting Information). It is worth mentioning that only in olive paste, at least five other peaks, possible isomers of oleuropein, were observed with identical HRMS data and a very similar Rt.

c) Other Phenols: Verbascoside (**40**), isoacteoside (**41**), and campneoside (**39**) are phenyl alcohols and caffeic acid sugar esters containing hydroxytyrosol or 3,4-dihydroxy-phenyl glycol moieties and are found in several olive extracts [30]. In the present study, they were only detected in drupes and paste, while they are ab-

sent from the first oil and the final product. This shows that either they were biodegradated or were simply lost in the waste. Previous literature data [31] mention their presence in solid wastes. Both verbascoside and isoacteoside give a characteristic fragment ion at *m/z* 461.1670 with an RDBeq value at 6.5 (HRMS/MS) that corresponds to the loss of the caffeoyl moiety.

Concerning flavonoids, their glycosides are accumulated in drupes and paste samples, and are hydrolysed to the respective aglycons during the production procedure. Three isomers of luteolin hexoside (**43 i, ii**, and **iii**) have been detected before in drupes and paste. Previously, 7- and 4-*O*-glucosides have been detected in olive samples, as well as the 6-*C*-glucoside homoorietin. In the same samples, luteolin rutinoside (**44**) was also detected, giving the characteristic fragments at *m/z* 447 and 285 of luteolin hexoside and luteolin aglycon (**47**), respectively. Luteolin has been detected in all samples, including oils, while the prementioned glucosides are completely absent from the oils implying degradation to the respective aglycon during the malaxation phase. The same happens with the apigenin aglycon (**48**), present in all samples, but as a minor component in drupes, while its glycosides (**45, 46 i** and **ii**) are present only in the initial stages of production. Quercetin rutinoside (**42**) was also detected in drupes and paste, but the respective aglycon was not detected further.

d) Triterpenes: The characteristic olive triterpenes, maslinic (**50**), and oleanolic (**49**) acids, can be detected throughout the whole production procedure. They are found in drupes, mainly the skin, but their presence in the olive stone has also been reported [15]. Throughout the oil production procedure these triterpenes are partially transferred in the oil and, in the present study, big quantitative alterations were observed throughout the production procedure. More specifically, maslinic acid is a major component in drupes and it significantly increases in paste (**Fig. 5** and **Table 2**). This probably has to be attributed to the kneading of the paste together with the stone. Allouche et al. [16] showed that during malaxation with stones, maslinic acid increases the final content in maslinic acid up to 30% in comparison to non-stoned malaxation. Concerning the two oil samples, it seems that in this case, there is also a large loss of maslinic acid in solid wastes and its content is seriously decreased in the final oil.

In order to proceed with the quantitative analysis, nine representative compounds previously isolated from our group were selected and calibration curves were constructed. The regressions obtained for each standard together with the ranges and the LODs and LOQs are presented in **Table 1S** of the Supporting Information. The standard errors for all levels of each calibration curve were calculated and back-calculated values in all counts did not exceed 15% which shows that the developed methodologies can be considered of adequate accuracy. Concerning the intra-assay precisions of our methods, relative standard deviations (RSD) of the analysis of five replicates of three different concentrations of each calibration curve was < 10%. Furthermore, concerning the intermediate precision, RSDs were < 15% in all cases. Accuracies of each calibration curve and interday precision results are presented in **Table 2S**, Supporting Information.

In all samples under investigation, the dihydroxylated derivatives (hydroxytyrosol part) were found to be more concentrated than the monohydroxylated derivatives (tyrosol part). For example, oleacein is 13 times more concentrated than oleocanthal in drupes, 18 times more in paste and four times more in the final oil. The relative concentration of the MFOA is three times higher than the respective ligstroside derivative in drupes and five times higher in paste. The only exception concerns the respective alcohols since hydroxytyrosol is abandoned more in lower levels in the drupe extracts than in tyrosol.

Furthermore, the compounds found in different extracts could be clearly divided into two groups; the components, which were mostly found in drupes and paste, on the one hand, and those present in the two oil samples on the other. The step that is crucial between these two "groups" of samples is the malaxation of the paste. It is known that interesting qualitative and quantitative alterations happen during the crushing of drupes and kneading. It has been shown that the malaxation, including the crushed seed, leads to a higher secoiridoid composition of the final oil, such as oleacein and oleocanthal, since the procedure induces the function of various endogenous enzymes that enhance biotransformations [17].

Contrary to most references in the literature, the major components in drupes were oleacein and MFOA (4.25 and 4.23 mmol/kg fresh weight, respectively). The levels of oleuropein on the other hand were relatively low, together with the levels of hydroxytyrosol (0.17 and 0.81 mmol/kg fresh weight, respectively), which is supposed to be an oleuropein degradation product. The same trend was observed for tyrosol and ligstroside, although the levels of tyrosol were generally higher. Herein oleuropein and hydroxytyrosol levels in drupes were significantly lower than the levels of oleacein and MFOA. The same is valid for the respective levels of ligstroside, tyrosol, oleocanthal, and MFLA in lower levels (0.13, 1.72, 0.32, 1.34 mmol/kg). Amiot et al. [32] refers to the reduction of oleuropein with maturation with a parallel increase of elenolic acid glycoside and demethyloleuropein, but not in accordance. However, demethyloleuropein was not detected in our drupe samples (and in no other sample category), implying a further degradation of this secoiridoid, probably to oleacein, the levels of which were increased in the drupes compared to the references. These findings could also alternatively imply that the degradation of oleuropein and ligstroside through natural maturation does not lead to the formation of hydroxytyrosol and tyrosol, but mainly to the respective monoaldehydic and dialdehydic aglycon forms. It could be suggested that during natural maturation the overexpressed enzyme β-glycosidase that hydrolyses oleuropein and ligstroside leads to the respective aglycons and other related derivatives [23]. This is well connected to the increased content in oleuropein and ligstroside aglycons, oleacein, and oleocanthal in the drupe extracts.

Regarding the paste, it seems that the malaxation process rapidly increases the levels of tyrosol, mainly hydroxytyrosol, whose levels are 8-fold higher compared to that in drupes (8.87 mmol/kg). It has been mentioned that hydroxytyrosol is a hydrolysis product of oleuropein. However, the low oleuropein content in drupes does not support this hypothesis. It seems more likely that hydroxytyrosol is a degradation product of the MFOA, whose levels are clearly decreased (1.63 mmol/kg). Of course, it also has to be mentioned that the large accumulation of hydroxytyrosol in paste could also be a result of other compounds that bear this moiety, e.g., nuzhenide, which is a compound found in large quantities in seeds, but only in traces in paste extracts, the mechanical or enzymatic degradation of which could lead to the increase of hydroxytyrosol. The trend is the same for tyrosol/MFLA (3.31 and 0.44 mmol/kg, respectively). The levels of oleuropein and ligstroside were further reduced and were both found at the limit of detection level (0.03 and 0.01 mmol/kg), which has already been well established [8].

The malaxation process also seems to greatly increase the levels of oleacein and oleocanthal, which has been mentioned before in the literature [9,27]. The great accumulation of the two dialdehydic components can be attributed to the increased enzymatic function during kneading that is further enhanced by the crushed seeds. Several researchers in the past combined the increase of phenols like oleacein with the rapid reduction of the oleuropein content at the initial stages of olive paste malaxation [8], however, this cannot be valid for our samples, since oleuropein and ligstroside levels were found to be really low in drupes. Thus, it could be suggested that those components originated from the respective monoaldehydic aglycons, as mentioned for hydroxytyrosol and tyrosol.

Luaces et al. [18] stated that the contribution of the olive stone plays a catalytic role in the production of the aldehydic forms of oleuropein and ligstroside aglycons. In our study, this cannot be generalised, as the accumulation of the dialdehydic derivatives and diminishing of the monoaldehydic analogues has been observed. Generally, it has to be highlighted that the increase of the phenolic content during malaxation is more due to the enzymatic activity of seed enzymes rather than as a result of phenolics found in seeds [22].

As far as the first (unrefined) oil is concerned, it seems that during the centrifugation procedure in the oil separator for the removal of solid wastes, high quantities of the all secondary metabolites are lost. Specifically, hydroxytyrosol, tyrosol, oleacein, and maslinic acid are the main components (among studied) that are mostly lost. This implies that the solid waste of the two-phase oil production procedure could be a very rich source of secoiridoids and phenols, mainly hydroxytyrosol and oleacein, and it could be worth it to explore this further for its content, in those compounds, in a preparative scale. However, it should be kept in mind that all these components are extremely sensitive and enzymatic procedures are probably still continuing after the separation of the oil. The final refinization process, by washing the dark first oil with water, further removes valuable constituents from the final product.

Concerning the final product, the oil, all phenolics calculated were found more concentrated in the final product (VOO) studied (var. Koroneiki) compared to other oils studied before [33]. For example, VOO obtained from Catalonia (Spain) and from Arbequina var. had almost ¼ the quantity of hydroxytyrosol, tyrosol, oleacein, and oleocanthal than that in Koroneiki VOO [12]. Some other literature data places the levels of oleacein and oleocanthal in the final oil a little bit higher than the ones found here: 1278–1633 mg oleacein/kg oil and 92–148 mg oleocanthal/kg oil [9]. This could be attributed to the harvesting period (23th of September until 18th of November). It seems that the findings here agree with the assumption that the contents of these two interesting molecules, considered to be formed with degradation and hydrolysis of other compounds, is increased when the initial drupe material is more mature. Oleacein and the MFOA were found to be the major phenols in the final product of this research. This observation is in agreement with previous data concerning the phenolic content in several Spanish oils [33].

Summarising, it seems that there is a clear diminishing trend of oleuropein/ligstroside and their monoaldehydic aglycon derivatives from drupes to olive oil and, in contrary, an accumulation of oleacin/oleocanthal and hydroxytyrosol/tyrosol. Generally, the levels of dihydroxylated derivatives are always higher compared to monohydroxylated derivatives, and oleacein is a major constituent in olive paste. On the other hand, it could be suggested that the late maturation of drupes favour its synthesis.

In the present work, an integrated study was carried out allowing the qualitative profiling as well as the quantitative monitoring of olive constituents in different steps of a single production line of a biphasic olive mill from the most important Greek variety Koroneiki. More than 50 secondary metabolites were identified with high confidence in all materials under investigation and important structural information was derived. An HPLC-LTQ-Orbitrap platform in MS and MS/MS levels was used for the first time for the simultaneous qualitative and quantitative analysis and proved to be an adequate system for the rapid characterisation of olive extracts. Furthermore, better insight regarding the total constituents of olive drupes (flesh and stone), paste, unrefined oil, and final oil was provided, while important correlations between the different constituents as well as between the different production phases were revealed. New data regarding biotransformation procedures were also derived contributing to the better understating of olive's chemical nature.

The LTQ-Orbitrap HRMS/MS proved to be an adequate system for the rapid and simultaneous qualitative and quantitative characterisation of olive extracts and allowed for the monitoring of secondary metabolite alterations throughout the procedure of VOO production.

Furthermore, this study led to the development of a procedure for the estimation of the final product value based on the quality of the initial fruits. It was shown that only a small portion of the beneficiary metabolites end up in the final product. There is a clear diminishing trend of oleuropein/ligstroside and their monoaldehydic aglycon derivatives and an accumulation of oleacin/oleocanthal and hydroxytyrosol/tyrosol from drupes to olive oil. The level of dihydroxylated derivatives are always higher compared to the monohydroxylated derivatives. Oleacin, hydroxytyrosol, as well as tyrosol and maslinic acid are major constituents in olive paste. Thus, olive paste could be an excellent starting material for their isolation. A great percentage of all compounds is lost in solid wastes of a two-phase olive mill and the final product contains only a small portion of secoiridoids and triterpenes, thus solid waste could also be considered a raw material for the isolation of valuable olive components.

Materials and Methods

▼

Plant material

Samples from each step of the oil production procedure were collected from a single production line in January 2012 from "Renieris" diphasic olive mill in Lakonia, Pelloponese, Greece. The olive variety was Koroneiki, one of the most popular in Greece. More specifically, samples were selected from the four major production steps: a) olive drupes, collected the first week of January 2012, b) olive paste from the malaxation process, c) unrefined oil that comes after the centrifuging of the malaxed paste and the discard of the solid waste, and d) final oil which is derived after the refinement procedure with centrifugation and washing of the first oil with pure water. Lyophilised olive drupes were deposited in the Laboratory herbarium, voucher specimen KO001.

Extraction procedure

A similar extraction procedure for solid (drupes and paste) and liquid (oils) samples was followed. Three batches of drupes and paste were first lyophilised in order to optimise the extraction

procedure [34]. Drupes lost 37% of their initial weight and paste 41%, on average. Initially, cyclohexane (cHex) was used for defatting purposes and the extraction of the phenolics was performed with methanol (MeOH). More specifically, three batches of 10 g of lyophilised drupes and paste were defatted with cHex (3 × 100 mL × 20 min) and the residue was exhaustively extracted with MeOH (3 × 100 mL × 20 min), using ultrasounds, at room temperature. Concerning the drupes, the flesh was separated from the stone and they were extracted separately in order to diminish enzymatic procedures and biochemical transformations [17, 18]. Finally, 1.2 g of methanolic extracts, on average, were obtained from both flesh and seeds. Also, three batches of olive oils (before and after washing), 100 mL each, were also defatted with cHex (3 × 100 mL) and then extracted with MeOH using liquid-liquid extraction, (3 × 100 mL). On average, 2.58 g and 8 g of methanolic extracts, respectively, were obtained by the two different oils. After evaporation to dryness, all the samples were kept at − 20 °C until the analyses.

Sample preparation

For cleanup and phenolic enrichment prior to analysis, all extracts were subjected to SPE, using diol cartridges (1 g; Supelco) according to a protocol previously applied for the manipulation of olive samples [22]. Briefly, after the activation of diol material with three column volumes of MeOH, the cartridges were conditioned with three column volumes of *n*-hexane (nHex). One hundred mg of each extract, diluted in nHex, were applied and eluted with five column volumes of the same solvent. After the removal of the lipophilic compounds, the phenolics were eluted with five column volumes of MeOH. Finally, cartridges were washed with three column volumes of MeOH/H_2O mixture (50/50). The recovery of all compounds quantified was estimated to be > 95%. The procedure was carried out on all extracts (5 extracts × 3 times) and the respective enriched samples were kept at − 20 °C prior to analyses.

Qualitative and quantitative analysis of the methanolic extracts

All analyses were performed on an Accela High Speed LC System equipped with a PDA detector and hyphenated to an LTQ-Orbitrap XL hybrid mass spectrometer, using an ESI ionisation probe, in the negative mode (Thermo Scientific). Separations were carried out on an Ascentis Express Fused-Core™ C18 column (100 × 2.1 mm i.d., 2.7 µm; Supelco). After optimisation, a 33-min LC gradient elution programme was developed enabling the efficient separation of the majority of the components. The flow rate was set to 400 µL/min and the solvent system was (A) water (H_2O), 0.1% acetic acid (AA, 0.1% v/v) and (B) acetonitrile (ACN). The elution programme was: 5% B for 2 min; 10% B in 2.5 min; 25% B in 11.5 min; 95% B in 13 min and hold for 2 min; back to 5% B in 0.5 min and conditioning for 2.5 min (total analysis time, 33 min). The injection volume was 5 µL. The HRMS and HRMS/MS data were acquired with a mass range of 100–1500 *m/z*. ESI conditions: capillary temperature 350 °C; capillary voltage − 3 V; tube lens − 43.46 V. Nitrogen was used as a sheath gas (30 arb) and an auxiliary gas (10 arb). For the HRMS/MS acquisitions, a data-dependent method including the detection (full scan) and fragmentation of the three most intense peaks per scan was used. The mass resolving power was 30 000 for both levels, and the normalised collision energy was set to 35.0% (q = 0.25) for the HRMS/MS experiments.

Qualitative analysis. A full scan as well as a data-dependent acquisition (full scan and MS/MS) was used for the profiling of all extracts under investigation. Based on spectrometric features such as suggested elemental composition (EC), ring double bond equivalents (RDBeq) values, as well as fragmentation patterns, the identification of olive constituents was performed.

Quantitative analysis. Nine standard compounds were used. Hydroxytyrosol, ligstroside, and MFOA were of synthetic origin (purity > 95%, HPLC-PDA). Tyrosol, oleacein, oleocanthal, oleuropein, MFLA, and maslinic acid were isolated previously in our laboratory from natural sources (purity > 92%, HPLC-PDA). Syringaldehyde was used as an IS and was obtained by Sigma-Aldrich. All pure compounds, IS, and crude extracts were analysed in a full scan mode (mass range 100–1500 *m/z*) and an ion extraction method using a mass window of 0.01 Da was used for the quantitation. For tyrosol, which is a difficult ionisable molecule under the above conditions, a smaller mass window was used (*m/z* 136.5–137.5).

Method validation. For the preparation of the calibration curves, at least seven different concentrations of each compound in ACN were prepared. Each point of the calibration curves was at least triplicated. The concentration of IS was stable in all samples. After the extraction of the pseudomolecular ions, peak areas were used for the calculations. The optimum models for the description of our data were the linear ($y = \alpha + \beta x$) or square ($y = \alpha x^2 + \beta x + \gamma$), depending on the analyte, where y is the (analytical signal/IS) peak area abundance ratio and x the analyte concentration.

The accuracy of the method was evaluated by the estimation of the relative standard errors for each level of the calibration curves. Intra-assay precision was evaluated by analysis of representative replicates at three different concentrations of each analyte (low, middle, and high) from the whole calibration range on the same day and is expressed as relative standard deviation (RSD). Intermediate precision was evaluated by analysing the same samples three times on three different days.

The sensitivity of the method was evaluated by the values of limit of detection (LOD) and limit of quantification (LOQ). LOD was calculated as the lowest concentrations tested yielding a signal-to-noise ratio of at least three and LOQ refers to a signal-to-noise ratio of ten. For this purpose, ten different acetonitrile samples were injected, and LOD and LOQ values were calculated using the signal-to-noise criterion of three and ten, respectively.

Supporting information

Chemical structures of representative secondary metabolites detected in olive samples, characteristic XICs chromatograms of oleuropein and ligstroside aglycons, the HRMS/MS spectrum of the monoaldehydic form of oleuropein aglycon, the HRMS/MS spectrum of the nuzhenide, and the proposed fragmentation mechanism of elenolic acid are available as Supporting Information. Also available are regressions, correlation coefficients, limits of detection, and limits of quantification of all standards, as well as interday (intermediate) precision and accuracy data for each standard regression.

Acknowledgements

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The authors would like to thank RENIERIS olive oil mills for providing the samples and information on the production procedure. This work has been carried out with the support of the

Commission of the European Community, Framework Program 7, OLITEC Project, No. 230 763.

Conflict of Interest

The authors declare no conflict of interest.

References

1 *Perez-Martinez P, Garcia-Rios A, Delgado-Lista J, Perez-Jimenez F, Lopez-Miranda J.* Mediterranean diet rich in olive oil and obesity, metabolic syndrome and diabetes mellitus. Curr Pharmacol Des 2011; 17: 769–777
2 *Lopez S, Bermudez B, Varelal LM, Ortega A, Jaramillo S, Abial R, Muriana FJG.* Olives and olive oil: diet and health impacts. CAB Rev 2012; 7: 1–10
3 *Cicerale S, Lucas LJ, Keast RSJ.* Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. Curr Opin Biotechnol 2012; 23: 129–135
4 *Hamden K, Allouche N, Damak M, Elfeki A.* Hypoglycemic and antioxidant effects of phenolic extracts and purified hydroxytyrosol from olive mill waste *in vitro* and in rats. Chembiol Interact 2009; 180: 421–432
5 *Visioli F, Bernardini E.* Extra Virgin Olive Oil's Polyphenols: Biological Activities. Curr Pharmacol Des 2011; 17: 786–804
6 *Smith III AB, Han Q, Breslin PAS, Beauchamp GK.* Synthesis and assignment of absolute configuration of (−)-oleocanthal: a potent, naturally occurring non-steroidal anti-inflammatory and anti-oxidant agent derived from extra virgin olive oils. Org Lett 2005; 7: 5075–5078
7 *Smith III AB, Sperry JB, Han Q.* Syntheses of (−)-oleocanthal, a natural NSAID found in extra virgin olive oil, the (−)-deacetoxy-oleuropein aglycone, and related analogues. J Org Chem 2007; 72: 6891–6900
8 *Gomez-Rico A, Inarejos-Garcia AM, Salvador MD, Fregapane G.* Effect of malaxation conditions on phenols and volatile profiles in olive paste and the corresponding virgin olive oils (*Olea europaea* L. Cv. Cornicabra). J Agric Food Chem 2009; 57: 3587–3595
9 *Morello JR, Romero MP, Motilva MJ.* Effect of the maturation process of the olive fruit on the phenolic fraction of drupes and oils from Arbequina, Farga, and Morrut cultivars. J Agric Food Chem 2004; 52: 6002–6009
10 *Rodriguez G, Lama A, Jaramillo S, Fuentes-Alventosa JM, Guillen R, Jimenez-Araujo A, Rodriguez-Arcos R, Fernandez-Bolanos J.* 3,4-Dihydroxyphenylglycol (DHPG): An important phenolic compound present in natural table olives. J Agric Food Chem 2009; 57: 6298–6304
11 *Japón Luján R, Priego Capote F, Marinas A, Luque De Castro MD.* Liquid chromatography/triple quadrupole tandem mass spectrometry with multiple reaction monitoring for optimal selection of transitions to evaluate nutraceuticals from olive-tree materials. Rapid Commun Mass Spectrom 2008; 22: 855–864
12 *Suárez M, Macia A, Romero MR, Motilva MJ.* Improved liquid chromatography tandem mass spectrometry method for the determination of phenolic compounds in virgin olive oil. J Chrom A 2008; 1214: 90–99
13 *Ouni Y, Taamali A, Gomez-Caravaca AM, Segura-Carretero A, Fernandez-Gutierrez A, Zarrouk M.* Characterization and quantification of phenolic compounds of extra-virgin olive oils according to their geographical origin by a rapid and resolutive LC-ESI-TOF MS method. Food Chem 2011; 127: 1263–1267
14 *Henry H, Sobhi HR, Scheibner O, Bromirski M, Nimkar SB, Rochat B.* Comparison between a high-resolution single-stage Orbitrap and a triple quadrupole mass spectrometer for quantitative analyses of drugs. Rapid Commun Mass Spectrom 2012; 26: 499–509
15 *Ranalli A, Pollastri L, Contento S, Di Loreto G, Iannucci E, Lucera L, Russi F.* Sterol and alcohol components of seed, pulp and whole olive fruit oils. Their use to characterize fruit variety by multivariates. J Sci Food Agric 2002; 82: 854–859
16 *Allouche Y, Uceda M, Jimenez A, Aguilera MP, Gaforio JJ, Beltran G.* Fruit quality and leaf and stone addition affect Picual virgin olive oil triterpenic content. J Agric Food Chem 2009; 57: 8998–9001
17 *Servili M, Taticchi A, Esposto S, Urbani S, Selvaggini R, Montedoro GF.* Effect of olive stoning on the volatile and phenolic composition of virgin olive oil. J Agric Food Chem 2007; 55: 7028–7035
18 *Luaces P, Romero C, Gutierez F, Snaz C, Perez AG.* Contribution of olive seed to the phenolic profile and related quality parameters of virgin olive oil. J Sci Food Agric 2007; 87: 2721–2727
19 *Tchoumtchoua J, Njamen D, Mbanya JC, Skaltsounis AL, Halabalaki M.* Structure-oriented UHPLC-LTQ Orbitrap-based approach as a dereplication strategy for the identification of isoflavonoids from *Amphimas pterocarpoides* crude extract. J Mass Spectrom 2013; 48: 561–575
20 *Guttierez-Rosales F, Rios JJ, Gomez-Rey ML.* Main polyphenols in the bitter taste of virgin olive oil. Structural confirmation by on-line high-performance liquid chromatography electronspray ionization mass spectrometry. J Agric Food Chem 2003; 51: 6021–6025
21 *Savarese M, De Marco E, Sacchi R.* Characteriztion of phenolic extracts from olives (*Olea europaea* cv. Pisciottana) by electrospray ionization mass spectrometry. Food Chem 2007; 105: 761–770
22 *Alarcón Flores MI, Romero-González R, Garrido Frenich A, Martínez Vidal JL.* Analysis of phenolic compounds in olive oil by solid-phase extraction and ultra high performance liquid chromatography–tandem mass spectrometry. Food Chem 2012; 134: 2465–2472
23 *Charoenprasert S, Mitchell A.* Factors influencing phenolic compounds in table olives (*Olea europaea*). J Agric Food Chem 2012; 60: 7081–7095
24 *Sivakumar G, Bati CB, Uccella N.* HPLC-MS screening of the antioxidant profile of Italian olive cultivars. Chem Nat Prod 2005; 41: 588–591
25 *Di Donna L, Mazzotti F, Napoli A, Salermo R, Sajjad A, Sindona G.* Secondary metabolism of olive secoiridoids. New microcomponents detected in drupes by electronspray ionization and high-resolution tandem mass spectrometry. Rapid Commun Mass Spectrom 2007; 21: 273–278
26 *Fu S, Arraez-Roman D, Menendez JA, Segura-Carretero A, Fernandez-Gutierrez A.* Characterization of isomers of oleuropein aglycon in olive oils by rapid-resolution liquid chromatography coupled to electrospray time-of-flight and ion trap tandem mass spectrometry. Rapid Commun Mass Spectrom 2009; 23: 51–59
27 *Gutierrez-Rosales F, Romero MP, Casanovas M, Molilva MJ, Minguez-Mosquera MI.* Metabolites involved in oleuropein accumulation and degradation in fruits of *Olea europaea* L.: Hojiblanca and Arbequina varieties. J Agric Food Chem 2010; 58: 12 924–12 933
28 *Jerman Klen T, Trebse P, Mozetic Vodopivec B.* Ultrasound-assisted solid liquid extraction (USLE) of olive fruit (*Olea europaea*) phenolic compounds. Food Chem 2012; 123: 175–182
29 *De Nino A, Mazzotti F, Morrone SP, Perri E, Raffeelli A, Sindona G.* Characterization of Cassanese olive cultivar through the identification of new trace components by ionspray mass spectrometry. J Mass Spectrom 1999; 34: 10–16
30 *Rigane G, Salem RB, Sayadi S, Bouaziz M.* Phenolic composition, isolation, and structure of a new deoxyloganic acid derivative from Dhokar and Gemri-Dhokar olive cultivars. J Food Sci 2011; 76: C965–C973
31 *Suárez M, Romero MP, Ramo T, Macià A, Motilva MJ.* Methods for preparing phenolic extracts from olive cake for potential application as food antioxidants. J Agric Food Chem 2009; 57: 1463–1472
32 *Amiot MJ, Fleuriet A, Macheix JJ.* Accumulation of oleuropein derivatives during olive maturation. Phytochemistry 1989; 28: 67–69
33 *Lozano-Sanchez J, Segura-Carretero A, Menendez JA, Oliveras-Ferraros C, Cerretani L, Fernandez-Gutierrez A.* Prediction of extra virgin olive oil varieties through their phenolic profile. Potential cytotoxic activity against human breast cancer cells. J Agric Food Chem 2010; 58: 9942–9955
34 *Rios JJ, Gutierrez-Rosales F.* Comparison of methods extracting phenolic compounds from lyophilised and fresh olive pulp. LWT-Food Sci Technol 2010; 43: 1285–1288