Evolution of Phenolic Compounds in Virgin Olive Oil During Storage

Luciano Cinquanta*, Marco Esti, and Ennio La Notte

Department of Agricultural, Food, Environmental and Microbiological Science and Technology, University of Molise, 86100 Campobasso, Italy

ABSTRACT: Phenolic compounds are of fundamental importance to the shelf life of virgin olive oils because of their antioxidative properties. In this paper, the evolution of simple and complex olive oil phenols during 18 mon of storage is studied by high-performance liquid chromatography (HPLC) analysis. The olive oils under examination were from various olive cultivars, harvested in two sectors in the same region at different stages of ripeness. The findings indicate that it is not the variety but rather the ripeness of the olives and the soil and climate that influence the phenol composition of virgin olive oil. In addition, a positive correlation was found between the age of the oils and the tyrosol to total phenols ratio. Lastly, gas chromatography–mass spectrometry analysis confirmed that the unidentified peaks detected by HPLC were of a phenolic nature.
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KEY WORDS: Antioxidants, caffeic acid, complex phenols, hydroxytyrosol, oleuropein, olive oil, olive ripeness, phenolic compounds, secoiridoid glucoside, tyrosol.

The amount of phenolic compounds in virgin olive oil is an important factor when evaluating the quality of virgin olive oil because natural phenols improve its resistance to oxidation (1–4) and its sharp bitter taste (5). These compounds have been correlated with the shelf life of oil and, in particular, its resistance to oxidation, which is mainly ensured by hydroxytyrosol and caffeic acid, which are ortho-diphenolic compounds (2,6–8). The other phenols (e.g., tyrosol and *p*-hydroxybenzoic, *o*-coumaric, and *p*-coumaric acids) have little or no antioxidant properties (9,10). However, many complex components of the polar fraction remain unidentified (11–13).

The content of phenolic derivatives in freshly made virgin olive oil is influenced by the variety, climatic conditions, fruit ripeness (14–19), and the oil extraction process used (15,20–23). During storage, the presence of these compounds depends on the hydrolytic processes that occur in the more complex forms (11) and on the oxidation of the ortho-diphenolic fraction. Thus, in-depth knowledge regarding the changes that phenolic substances undergo during storage could provide greater understanding of how the quality of olive oil is affected over time. The aim of this study was to examine the evolution of the simplest phenols, such as tyrosol and hydroxytyrosol, and the complex compounds in virgin olive oils during 18 mon of storage, taking into consideration the type of cultivar, harvest time, and area of origin.

*To whom correspondence should be addressed at Dipartimento di Scienze e Tecnologie Agro-Alimentari, Ambientali e Microbiologiche, Università degli Studi del Molise, via Tiberio 21/A, 86100 Campobasso, Italy.
E-mail: lanotte@hpsrv.unimol.it.

EXPERIMENTAL PROCEDURES

Materials and reference compounds. The 18 virgin olive oil samples were produced in the Molise region and were obtained from three varieties: Leccino, Gentile, and Rosciola. They were harvested at different stages of ripeness (25 October, 10 November, 25 November, and 10 December for Leccino only) in two production sectors in the region (A and B). All oil samples were obtained by industrial processing through pressure extraction and kept in 1-L bottles in a dark storeroom. The average temperatures during winter and summer were 6 and 12°C, respectively.

Tyrosol and α-tocopherol were produced by Fluka (Buchs, Switzerland); vanillic, caffeic and *p*-coumaric acids were produced by Sigma Chemical (St. Louis, MO), and bis (trimethylsilyl)-trifluoracetamide (BSTFA) was supplied by Merck (Darmstadt, Germany).

The following parameters were determined: free fatty acids, peroxide values, spectrophotometric properties, fatty acid methyl esters (24); total phenols (25); α-tocopherol (23); and minor polar components. The latter were also analyzed after 6, 12, and 18 mon of storage.

Extraction of minor polar components and high-performance liquid chromatography (HPLC) analysis. The polar fraction extract (12) was evaporated to dryness in a rotary evaporator at 40°C, and the residue was dissolved in methanol; 20 µL of this solution was injected into the HPLC system. The HPLC system consisted of a Waters 600E chromatograph (Milford, MA) with a Waters 150 mm × 4.6 mm C_{18} µ-Bondapak column with a same guard-column, coupled with a Waters 991 photodiode array detector.

The eluates were detected at 280 nm at 25°C, the flow rate was 1.3 mL/min, the mobile phase used was 2% acetic acid in water (W) and methanol (M) for a total running time of 50 min by using the following gradient: from 97%W–3%M to 80%W–20%M in 10 min, 60%W–40%M in 10 min,

45%W–55%M in 15 min, 0%W–100%M in 5 min until the end of the run. The quantitation of tyrosol, vanillic, caffeic, and *p*-coumaric acids was carried out by the external standard method. The response factor of tyrosol (Tyr) was used to quantitate hydroxytyrosol (OH-Tyr) and the total phenols. The identification of OH-Tyr was carried out on the basis of the mass spectrum recorded.

Gas–liquid chromatography–mass spectrometry (GC–MS). The eluates of the unidentified peaks were sampled separately during HPLC analysis and freeze-dried in an Edwards Modulyo (Crawley, Sussex, England). The samples were clarified, then derivatives were produced with 100 µL of BSTFA, according to Solinas (17), and GC–MS was performed with a Fisons MD 800 (Loughborough, Leicestershire, England), equipped with an on-column injection system, on a Supelco silica capillary column SE-54 (Bellefonte, PA) (30 m length; 0.25 mm i.d.; 0.25 µm film thickness). Carrier gas was helium, and the pressure on the head of the column was 40 KPa. The oven temperature was programmed to increase from 70 to 280°C at 2°C/min. The transfer line temperature was held at 250°C, and the ionizing voltage was 70 eV.

Statistical analysis. After checking that they were within the norm, the data were analyzed by StatView^{TM}SE (Abacus Concepts, Inc., Berkeley, CA) software. To calculate the confidence intervals and to perform the hypothesis test for a paired test, a single sample analysis with a paired samples procedure was adopted.

RESULTS AND DISCUSSION

Statistics regarding the parameters after 1 mon of oil storage [minimum, maximum, mean, and relative standard deviation (RSD)] are given in Table 1. Among the substances with antioxidant properties, the total phenols varied considerably (RSD = 31%), whereas the RSD value for α-tocopherol content was less than half of the amount for total phenols (RSD = 15%). However, the RSD value of oleic acid was low (4%), and the oleic acid to linoleic acid ratio was high (mean = 7.4).

Table 2 shows how the total phenols of the samples changed (determined by HPLC) during storage. In all virgin oil samples produced from the cultivars of both sectors, the riper the olives, the more the total phenol content decreased. This could be a result of increased esterase activity when the olives are at a more advanced stage of ripeness (26). This causes the degradation of oleuropein, the main secoiridoid glucoside in olives, and leads to the formation of compounds of both a phenolic and nonphenolic nature (16,27). Reduction in the total phenol content of the oils after 6, 12, and 18 mon of storage is a result of oxidation and hydrolytic activities, which increase during storage (11,12). In considering the influence of variety, soil and climate, the latter two appear to be the most important (21) in affecting the change in phenol composition of the oils during storage. In fact, greater variations were recorded in the total phenol levels of Leccino and Gentile, cultivated in different sectors, than between varieties cultivated in the same area.

Table 3 illustrates the evolution of OH-Tyr in the samples during storage. A typical rise and fall trend is observed in all samples, with the peaks varying both in height and in duration (6 or 12 mon). An element common to all samples after 1 mon of storage is, yet again, the low content in OH-Tyr and other simple phenols. The compounds involved were vanillic, caffeic, and *o*-coumaric acids; caffeic acid content was only a few ppm; and the others were even lower. After 1 mon, the mean OH-Tyr content was 3.5 mg/kg. This could result from hydrolysis of the phenolic substances during the oil extraction stage, which involves the formation of low molecular weight compounds with partition coefficients between vegetal water and oil that contribute to dispersion into water (28). There are more marked differences in OH-Tyr evolution after 6 and 12 mon of storage. It has been ascertained that this results from the hydrolysis of combined phenolic compounds, which, given the high antioxidant activity of OH-Tyr, ensures the stability of virgin olive oils (25). Therefore, the higher content of OH-Tyr after 6 mon of storage is a result of an increase in hydrolytic activities on complex phenols, probably

TABLE 1
Statistics on Olive Oil Composition

	Free fatty acids (%)	Peroxides (meq/kg)	α-Tocopherol (mg/100 g)	Total phenols (mg/kg)	Oleic/linoleic acid ratio			
Minimum	0.3	6	10	121	5.0			
Maximum	0.6	14	32	410	10.8			
Mean	0.4	10	20	258	7.4			
RSD[a]	24	26	15.3	31.2	20.1			
	Palmitic acid (%)	Palmitoleic acid (%)	Stearic acid (%)	Oleic acid (%)	Linoleic acid (%)	Linolenic acid (%)	Arachic acid (%)	Eicosenoic acid (%)
Minimum	11.4	0.7	2.3	64.6	6.9	0.6	0.3	0.1
Maximum	16.0	1.7	4.3	76.4	12.8	0.8	0.5	0.2
Mean	13.7	1.0	3.2	70.8	9.8	0.7	0.5	0.1
RSD	8.8	30.0	16.8	4.2	14.5	10.6	13.3	31.9

[a]Abbreviation: RSD, relative standard deviation.

TABLE 2
Changes in Total Phenols (mg/kg) in Oils[a]

Variety	Storage mon	Sector	Harvest time Oct. 25	Nov. 10	Nov. 25	Dec. 10
Leccino	1	A	318	270	230	121
	6	A	250	180	210	110
	12	A	120	81	91	40
	18	A	40	59	9	14
Leccino	1	B	272	188	188	138
	6	B	267	164	180	105
	12	B	183	125	30	90
	18	B	21	90	6	22
Gentile	1	A	411	382	226	—
	6	A	250	250	210	—
	12	A	150	98	190	—
	18	A	146	32	95	—
Gentile	1	B	403	190	182	—
	6	B	289	150	160	—
	12	B	66	109	60	—
	18	B	22	62	15	—
Rosciola	1	A	398	326	220	198
	6	A	310	150	112	72
	12	A	115	90	90	40
	18	A	79	30	42	12

[a]Stored for 1, 6, 12, and 18 mon, produced from olives harvested at different times in sectors A and B.

caused by the higher storage temperature in the summer. However, after 18 mon, the drop in the complex fraction results in a definite reduction in OH-Tyr and even its almost complete disappearance in 30% of the samples. Chromatograms of the phenolic fraction of one oil after 1, 6, and 18 mon of storage (Fig. 1) are given to support the described phenomenon. They illustrate the reduction of the complex fraction and, after 6 mon, the increase of the simple fraction. Spectral analysis of the GC–MS peaks of the complex phenolic fraction shows that peak number 5 [retention time (RT) = 32.6 min] has a mass spectrum that fits well with the OH-Tyr peak. Therefore, peak number 5 can be considered to be a complex phenol that contains OH-Tyr, and its hydrolysis results in the formation of OH-Tyr. In addition, the more mature the olives, the lower was the content of OH-Tyr in the oils. The total phenols content was affected in the same way,

TABLE 3
Changes in Hydroxytyrosol (mg/kg) in Oils[a]

Variety	Storage mon	Sector	Harvest time Oct. 25	Nov. 10	Nov. 25	Dec. 10
Leccino	1	A	4.5	1.1	2.8	1.3
	6	A	24.5	39.7	8.2	11.8
	12	A	11.6	18.3	5.9	16.0
	18	A	0.2	0.5	0.1	2.0
Leccino	1	B	8.8	1.9	15.9	2.1
	6	B	26.0	7.3	25.2	3.5
	12	B	31.0	33.1	2.5	9.7
	18	B	4.1	5.0	0.1	2.2
Gentile	1	A	2.6	2.8	1.8	—
	6	A	5.0	6.0	8.0	—
	12	A	53.5	5.7	2.3	—
	18	A	2.2	0.2	0.5	—
Gentile	1	B	2.8	3.5	3.9	—
	6	B	45.2	31.9	10.5	—
	12	B	20.0	18.2	2.2	—
	18	B	0.1	0.5	0.4	—
Rosciola	1	A	2.4	3.4	1.8	1.2
	6	A	5.1	28.8	3.6	6.7
	12	A	19.0	20.1	6.5	9.0
	18	A	4.2	3.0	0.1	2.0

[a]Stored for 1, 6, 12, and 18 mon, produced from olives harvested at different times in sectors A and B.

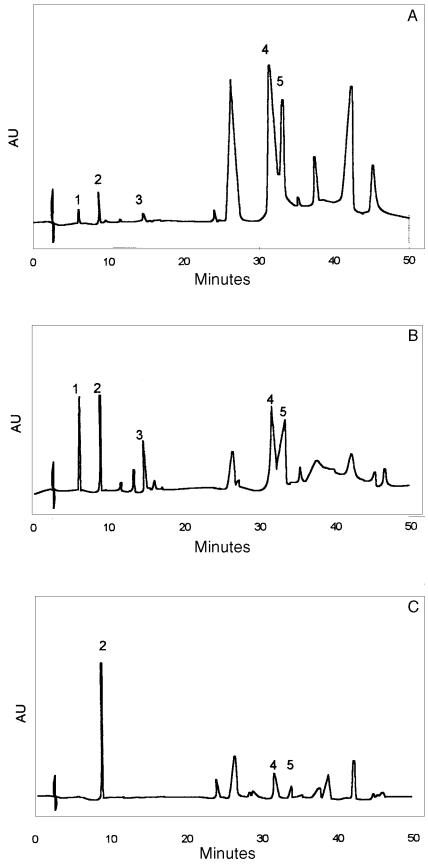

FIG. 1. Phenolic compound traces in olive oil after 1 (A), 6 (B), and 18 (C) mon of storage. Peak numbers are: (1) hydroxytyrosol; (2) tyrosol; (3) caffeic acid; (4) unidentified peak, retention time (RT) = 31.3 min; (5) unidentified peak, RT = 32.6 min.

although to a lesser extent. A similar trend as for OH-Tyr was observed for Tyr after 1, 6, and 12 mon of storage, although there were considerable differences between the samples (Table 4). However, after 18 mon, when Tyr is present in larger quantities, the differences, compared with OH-Tyr, become even more accentuated. Up to 12 mon, the kinetics of Tyr follow the same trend described for OH-Tyr. From GC–MS analyses, peak number 4 (RT = 31.3) can be considered to represent the complex phenol that contains Tyr. Tyr stability after 12 and 18 mon can be correlated with the fact that Tyr does not possess antioxidant activities and is therefore not easily degradable, whereas the complex phenolic fraction is drastically reduced. Moreover, the multiple box-and-whisker plots regarding the Tyr aggregates (Fig. 2) show that Tyr increases significantly after 6 mon (*t*-paired test = 5.4, $\alpha = 0.0001$) and becomes stable at a later stage.

After 1 mon, the low content and the slight differences in Tyr between the oils are a result, as for OH-Tyr, of the partition coefficient between vegetal water and oil during extraction. The median of Tyr content after 6 mon of storage is similar to its median after both 12 and 18 mon, thus confirming the stability of Tyr during storage. Moreover, changes in Tyr content appear greater after 6 mon than after either 12 or 18 mon, shown in Figure 2**,** where the central box is larger and the whiskers are longer. This is probably a result of the hydrolytic effects on the greater concentration and variety of the complex phenols in the oils after 6 mon. On the basis of these findings, we tried to formulate an age-index for olive oils based on the tyrosol/total phenols ratio (Fig. 3). To evaluate this index, the ratio values between the 10th and 90th percentile were considered for the different periods of storage. In our study, this enabled us to distinguish between freshly made oils and oils stored for 6 mon, when the ratio was under 4%; and for oils stored for 18 mon, when the tyrosol/total

TABLE 4
Changes in Tyrosol (mg/kg) in Oils[a]

Variety	Storage months	Sector	Harvest time			
			Oct. 25	Nov. 10	Nov. 25	Dec. 10
Leccino	1	A	2.7	1.0	2.5	1.1
	6	A	76.0	64.9	8.2	13.2
	12	A	8.6	20.3	7.2	22.0
	18	A	21.5	34.3	8.0	13.0
Leccino	1	B	3.0	1.4	5.3	3.2
	6	B	19.3	13.9	21.5	13.7
	12	B	22.6	15.0	3.8	17.5
	18	B	21.0	20.0	5.3	12.4
Gentile	1	A	3.0	2.1	1.8	—
	6	A	72.0	50.4	30.0	—
	12	A	60.6	8.5	53.9	—
	18	A	51.4	10.0	45.3	—
Gentile	1	B	4.3	5.4	3.2	—
	6	B	45.0	59.1	64.8	—
	12	B	51.0	46.7	60.2	—
	18	B	35.1	25.6	15.0	—
Rosciola	1	A	1.9	1.6	1.0	1.1
	6	A	8.9	28.6	5.5	7.6
	12	A	21.3	22.7	12.7	10.0
	18	A	26.6	26.0	12.2	6.1

[a]Stored for 1, 6, 12, and 18 mon, produced from olives harvested at different times in sectors A and B.

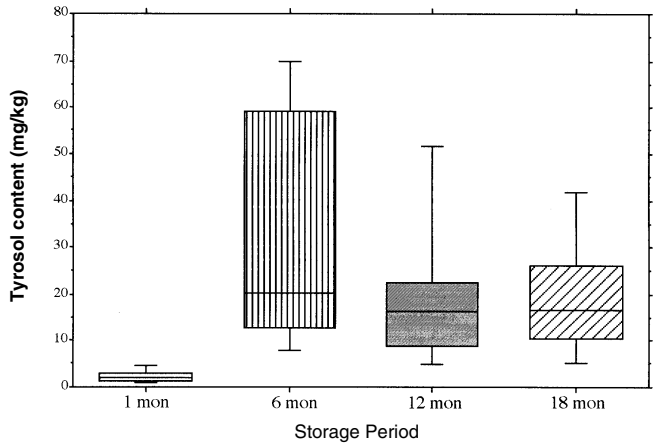

FIG. 2. Box-and-whisker plots of the tyrosol content in oil samples stored for 1, 6, 12, and 18 mon. The central box covers the middle 50% of the data values, between the lower and upper percentiles. The "whiskers" extend out from the 10th and 90th percentiles. The central line is at the median.

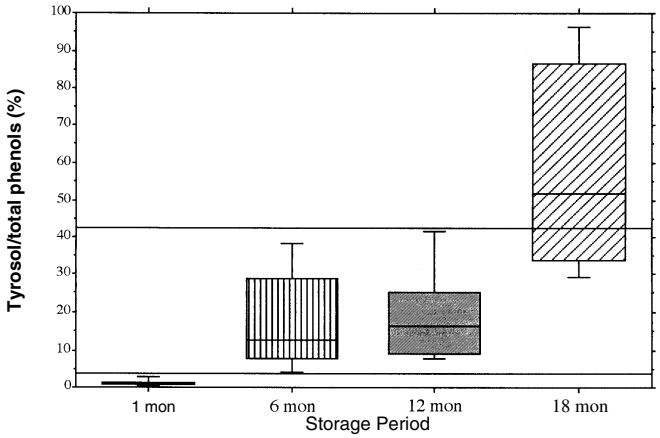

FIG. 3. Box-and-whisker plots of the tyrosol and total phenols ratio (%) in oil samples stored for 1, 6, 12, and 18 months. The central box covers the middle 50% of the data values, between the lower and upper percentiles. The "whiskers" extend out from the 10th and 90th percentiles. The central line is at the median.

phenols ratio was over 42%. However, the tyrosol/total phenols ratio did not permit a distinction to be made between oils stored between 6 and 18 mon when the ratio was between 4 and 42%.

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