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ANTIOXIDANTS IN VIRGIN OLIVE OILS PRODUCED FROM TWO OLIVE CULTIVARS OF SLOVENE ISTRIA

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ABSTRACT

The content of biophenols and tocopherols in virgin olive oils is an important factor when evaluating their quality. In the present work, the differences in biophenols and tocopherols content of two major olive tree (Olea europaea *L.) cultivars in Slovene Istria – cv. 'Istrska belica' and 'Leccino' – based on 1997/98 and 1998/99 crops were examined. It was established that cv. 'Istrska belica' had higher total biophenols content than the tocopherols content compared to the cv. 'Leccino', which had higher tocopherols content. Furthermore, the differences in the extraction processes on the biophenols and tocopherols content in virgin olive oils crops 1999/2000 and 2000/2001 were examined, showing the dual phase decanter (DP) process gives better results. And – finally – the inadequate storage conditions (light and room temperature) effect was measured, confirming that the direct light speeds up the biophenols and tocopherols decomposition.*

Key words: virgin olive oil, biophenols, tocopherols, extraction process, HPLC, Slovene Istria

ANTIOSSIDANTI NEGLI OLII VERGINE D'OLIVA DELL'ISTRIA SLOVENA DI DUE CULTIVAR D'OLIVO

SINTESI

La quantità di biofenoli e tocoferoli contenuta nell'olio d'oliva è importante nella stima della sua qualità. L'articolo riporta il confronto tra le quantità di biofenoli e tocoferoli contenute in due cultivar dell'olivo (Olea europaea *L.), che crescono nell'Istria slovena – 'Bianchera Istriana' e 'Leccino' – negli anni 1997/98 e 1998/99. Gli autori hanno confermato che la quantità di biofenoli negli olii di Bianchera Istriana è più alta di quella contenuta negli olii di Leccino. Questi ultimi hanno però una quantità più alta di tocoferoli. Gli autori hanno inoltre confrontato l'influsso del processo di estrazione sul contenuto in biofenoli e tocoferoli e hanno dimostrato che il processo bifasico porta ad olii con una maggiore quantità di biofenoli e tocoferoli. Viene inoltre evidenziato l'effetto di un errato deposito (calore e luce) sulla quantità di biofenoli e tocoferoli negli olii e viene dimostrato che la luce diretta porta ad un aumento del decadimento di biofenoli e tocoferoli negli olii.*

Parole chiave: olio vergine d'oliva, biofenoli, tocoferoli, HPLC, produzione, Istria slovena

INTRODUCTION

Slovene Istra is the coastal part of Slovenia in the upper part of the Adriatic in the Mediterranean basin, world known for its diet based on virgin olive oils (VOO). Virgin olive oils contain many compounds of non triacylglycerolic origin – among them are dietary antioxidants biophenols (BP) and tocopherols (TOC) (Baldioli *et al.*, 1996; Tasioula-Margari & Okogeri, 2001). Virgin olive oil tocopherols are mostly (> 95%) composed of alpha-tocopherol isomer (Boskou, 1996). The content of biophenols (Uccella, 2001a, 2001b, 2001c) and tocopherols (Butinar *et al.*, 1999b) in virgin olive oils plays an important role when evaluating their overall quality. Biophenols are known to improve resistance to autooxidation, and they give the oil its characteristic fresh, fruity, piquant (sometimes fiercely fruity) flavour (Angerosa *et al.*, 2000). These compounds have a role in disease prevention (Owen *et al.*, 2000a) and prolong the shelf life of the virgin oil itself by preventing auto oxidative radical reactions (Blekas *et al.*, 1995; Dietary Reference Intakes, 2000; Caponio *et al.*, 2001).

The prevailing virgin olive oil biophenols are phenolic secoiridoide glucosides (SG). Both olive biophenols and tocopherols are secondary metabolites deriving from AcCoA (mevalonic acid) and phosphoenolpyruvate (shikimic acid) pathways (Ryan & Robards, 1998).

Olive biophenols from secoiridoide glucosides pathway are mainly oleuropein (structure 1 from Fig. 1), demethyloleuropein, ligstroside (structure 2 from Fig. 1), oleoside and their decomposition – hydrolytic/enzymatic products that during the processing of olives enter the lipophylic phase and enrich the virgin olive oils: among them are oleuropein aglycon (O-Agl) in two different tautomeric forms: hidroxy (structure 1b from Fig. 1) and aldehydic (structure 1a from Fig. 1), ligstroside aglycon (L-Agl), again in two different forms: hidroxy (structure 2b from Fig. 1) and aldehydic (structure 2a from Fig. 1), dialdehydic open form of decarboxymethyl oleuropein aglycon (DMO-dA) represented with the structure 1d from Fig. 1, dialdehydic open form of decarboxymethyl ligstroside aglycon (DML-dA) with the structure 2d from Fig. 1, tyrosol (Tyr) – structure 4 from Fig. 2 and hydroxytyrosol (Tyr-OH) – structure 3 from Fig. 2 (Cortesi *et al.*, 2002; Rovellini & Cortesi, 2002). The virgin olive oils' biophenolic segment is rich in lignans and two flavonoids: apigenin and luteolin (Cortesi *et al.*, 2002; Owen *et al.*, 2000b).

The content of biophenols and tocopherols in freshly pressed virgin olive oils depends on many factors – among them are the cultivar, cropping year, fruit ripeness and overall fruit condition (Gimeno *et al.*, 2002; Salvador *et al.*, 2003), climatic conditions (Patumi *et al.*, 2002; Manach *et al.*, 2004; Paz Aguilera *et al.*, 2005) and type and quality of the extraction process (Gutiérez

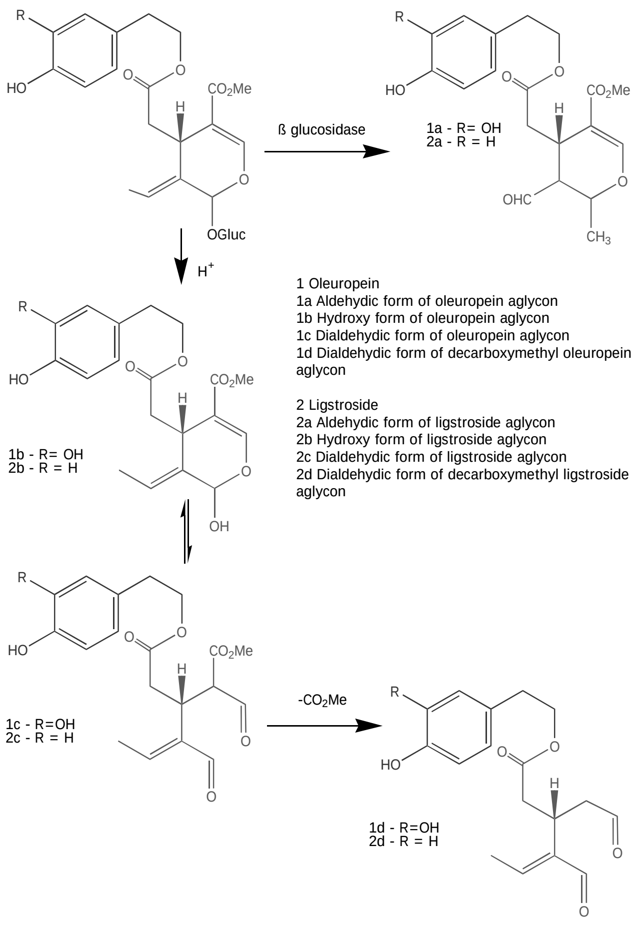
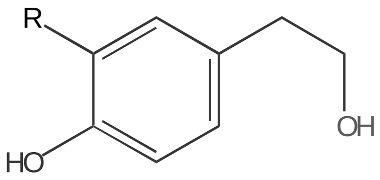


Fig. 1: Formation mechanisms of oleuropein and ligstroside biophenolic aglycons (Rovellini & Cortesi, 2002).
Sl. 1: Tvorbeni mehanizmi oleuropeinskih in ligstrozidnih biofenolnih aglikonov (Rovellini & Cortesi, 2002).

et al., 1999; Caponio & Catalano, 2001; Ranalli *et al.*, 2001). During storage, the content of simple biophenols Tyr and Tyr-OH depends on hydrolytic and enzymatic processes transforming complex secoiridoid glucosides in less complex ones (decomposition path as illustrated in Fig. 1) and on the oxidation of simple ortho biophenols – hydroxytyrosol. Tyrosol has negligible antioxidative activity, thus its content remains practically unchanged or even slightly increases due to ligstroside complex biophenols decomposing.

The aim of the present work was to examine the differences in biophenols and tocopherols content of the two major olive tree (*Olea europaea* L.) cultivars in Slovene Istra – cv. 'Istrska belica' (IB) and 'Leccino' (L) – based on 1997/98 and 1998/99 crops and to compare them to the values found in previous published work (Butinar *et al.*, 1999a, 1999b). Furthermore, we examined the effect of different extraction processes on biophenols and tocopherols content in virgin olive oils and the effect of inadequate storage conditions (light and room temperature) on total biophenols and tocopherols content and on content of HPLC determined secoiridoide originated biophenols as well.



3 - R=OH
4 - R=H

Fig. 2: Structures of tyrosol (4) and hydroxytyrosol (3).
Sl. 2: Strukturi tirosola (4) in hidroksitirosola (3).

MATERIALS AND METHODS

Materials

Cultivar influences. The research was performed on 21 virgin olive oil samples from the 1997/98 crop, analyzed in September 1998, and on 26 virgin olive oil samples from the 1998/99 crop, analyzed in October 1999. All virgin olive oil samples were stored in the dark, packed in dark-coloured air tight bottles at 15 ˚C. All virgin olive oil samples were pressed from the cv. 'Istrska belica' and 'Leccino' olives, or were mixtures of both cultivars that underwent the extraction process as a mixture.

Extraction process evaluation. The first part of evaluation was performed on oils from cv. 'Istrska belica' and 'Leccino' crop 1999/00, processed in two different olive-mills, first one using the percolation/centrifugation process (PC) and the second the centrifugal integral decanter (dual phase decanter, DP).

The second part of evaluation was performed on oils from cv. 'Istrska belica' and 'Leccino' crop 2000/01, processed in two commercially run olive-mills, both using the same extraction principle: centrifugal integral decanter, but being run under slightly different operation conditions due to each owner's strategies (DP-1 & DP-2).

Inadequate storage effects. The biophenols determinations were made on initially 21 virgin olive oil samples crop 1997/98, analyzed for the first time in September 1998. 11 out of those 21 samples were analyzed for the second time in May 1999, after being stored at 18 ˚C in dark-coloured bottles, and 6 out of these 11 samples were analyzed for the third time in May 2001, after being kept in transparent bottles at 18 ˚C.

The tocopherol determination on 4 virgin olive oil samples crop 1998/99 was performed for the first time in February 1999. The samples were stored in dark-coloured bottles in dark and cold place till June 1999 when the aliquot of the samples was put into transparent bottles and left on the laboratory shelf in full light until analyzed in May 2001.

Reference compounds. Tocopherol standards were obtained from Merck (Darmstadt, Germany). The calibration standard concentrations were spectrophotometrically checked according to A.O.C.S. method Ce 8–89 (AOCS, 1990) and (Balz *et al.*, 1996). Tyrosol was purchased from Fluka (Buchs, Switzerland). Hydroxytyrosol was prepared in our laboratory according to the publication of Baraldi (Baraldi *et al.*, 1983) and its purity checked with the aid of HPLC.

Methods

Extraction of biophenolic compounds and determination of total biophenols. The extraction and determination were performed according to Gutfinger's publication (Gutfinger, 1981).

HPLC analysis of biophenols. 100 μl of the biophenols extract (prepared as in 3.3.1) were put in the vial of the auto sampler of an Agilent 1050 quaternary pump HPLC system, equipped with an UV/VIS detector operating at 280E-9 m and Supelco 250 mm × 4.6 mm ODS column. 25 – 75 μl were injected into the system, the flow rate set to 1.0 ml/min, and the mobile phase used was a water/acetic acid and methanol gradient, which allowed the separation of the simple (Tyr and Tyr-OH) biophenols from the complex ones. The quantization of tyrosol and hydroxytyrosol was carried out by the external standard method. The response factor of tyrosol was used to quantitate the dialdehydic open form of decarboxymethyloleuropein aglycon and other biophenols.

Extraction of tocopherols. 500 mg of virgin olive oil sample were weighed in a 10 ml vial and 5.0 ml of methanol were added. The vial was capped and sonicated for 10 minutes. The methanol extract was refrigerated to allow the oil droplets to settle, filtered and transferred to the auto sampler vial (The New Expanded Supelco Reporter, 1993).

HPLC analysis of tocopherols. We used an Agilent system, equipped with a quaternary pump, auto injector and UV/VIS detector. Separation was achieved on a Supelco ODS 5μm, 4.6 x 250 mm column. The eluate absorbance was monitored at 290E-9 m. The mobile phase consisted of 2 vol. % MeOH in water. The flow during the analysis was 2 ml/min and the time necessary to separate all the peaks 10 minutes. The injection volume ranged from 50 – 75 μl of the methanol extract. The quantization of tocopherols (beta and gamma isomer co eluted) was carried out by the external standard method. For the alpha-tocopherol a 5 point calibration curve was constructed, which showed the R^2 = 0.9998 linearity in the range from 1 to 17 mg of the isomer/100 g of the oil.

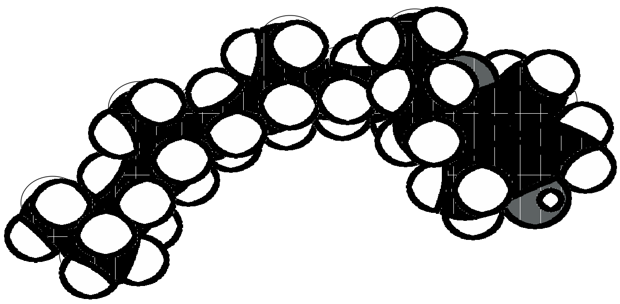


Fig. 3: Structure of alpha-tocopherol (white circles represent hydrogen atoms, black circles carbon and dotted circles oxygen atoms).
Sl. 3: Struktura alfa-tokoferola (beli krogci so vodikovi atomi, črni ogljikovi in šrafirani kisikovi).

RESULTS

Cultivar differences

Figures 4 and 5 show the average total biophenols content in virgin olive oils from the 1997/98 and 1998/99 crops and their comparison with tocopherols (Figs. 6 and 7). There are obvious differences between 'Leccino' and 'Istrska belica'. Virgin olive oils extracted from cultivar 'Leccino' have higher share in total tocopherols content compared to 'Istrska belica' oils, while in total biophenols content the ratio is turned around – 'Istrska belica' oils lead in total biophenols content.

Extraction process evaluation

Table 1 summarizes the cultivar, type of processing, total biophenols content, total tocopherols content, hydroxytyrosol, tyrosol, dialdehydic open form of decarboxymethyl oleuropein aglycon (DMO-dA) and total HPLC biophenols content for samples examined. The biophenols and tocopherols content are higher in both IB and L cultivars when using the dual phase decanter extraction process.

Tab. 1: Extraction processes data for virgin olive oil samples crop 1999/00: PC – percolation/centrifugation process; DP – centrifugal integral decanter (dual phase decanter).
Tab. 1: Podatki iz ekstrakcijskega procesa za vzorce deviškega oljčnega olja letnika 1999/00: PC – perkolacijsko/centrifugalni proces; DP – centrifugalno integralni dekanter (dvofazni dekanter).

Content	Cultivar			
	Istrska belica		Leccino	
	PC	DP	PC	DP
Total BP (mg/kg)	127	153	59	75
Total TOC (mg/100 g)	3.3	4.1	6.2	9.1
TyrOH (mg/kg)	5.9	3.7	0.4	1
Tyr (mg/kg)	12.9	7.0	4.9	6.1
DMO-dA (mg/kg)	4.9	14.9	0	0
Total HPLC BP (mg/kg)	164	213	80	90

Table 2 shows the cultivar, type of processing, total BP content, total TOC content, hydroxytyrosol, tyrosol, dialdehydic open form of decarboxymethyl oleuropein aglycon (DMO-dA) and total HPLC biophenols content for samples examined. Note the elevate total tocopherol amount in the extraction process DP-2 (probably due to added vegetation water during extraction process).

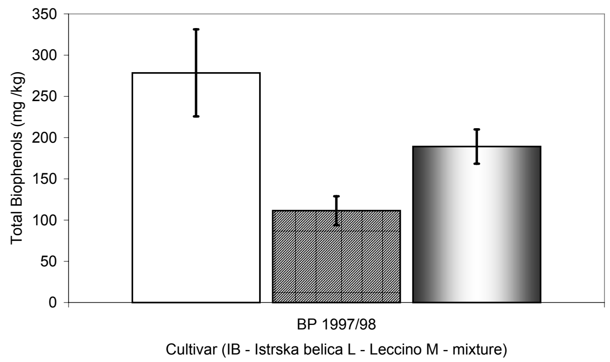


Fig. 4: Average total biophenol content for the oils from cultivars 'Istrska belica', 'Leccino' and mixtures of both produced from the 1997/98 crop olives (the error bars show the standard deviation).
Sl. 4: Povprečni skupni biofenoli v oljih sort 'Istrska belica', 'Leccino' in mešanic obeh iz oljk letnika 1997/98 (standardni odklon je označen z daljicami).

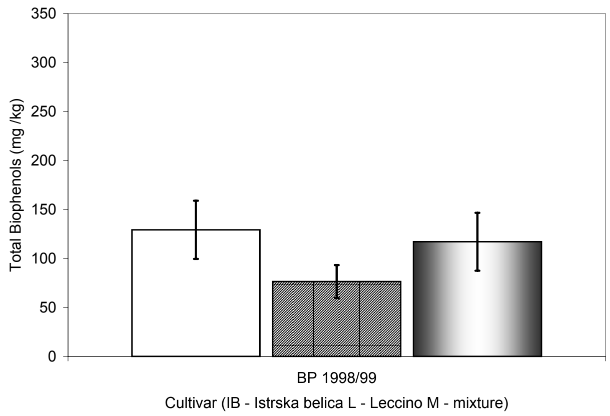


Fig. 5: Average total biophenol content for the oils from cultivars 'Istrska belica', 'Leccino' and mixtures of both produced from the 1998/99 crop olives (the error bars show the standard deviation).
Sl. 5: Povprečni skupni biofenoli v oljih sort 'Istrska belica', 'Leccino' in mešanic obeh sort iz oljk letnika 1998/99 (standardni odklon je označen z daljicami).

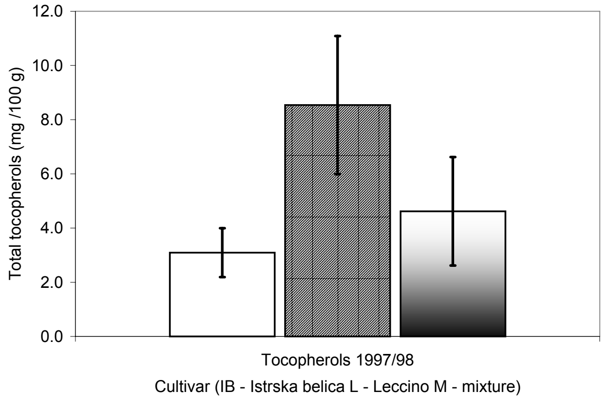


Fig. 6: Average total tocopherol content in the virgin olive oils from 'Istrska belica', Leccino' and mixtures of both cultivars from the 1997/98 crop (the error bars show the standard deviation).
Sl. 6: Povprečna skupna vsebnost tokoferolov v deviških oljčnih oljih sort 'Istrska belica', Leccino' in mešanic obeh sort letnika 1997/98 (standardni odklon je označen z daljicami).

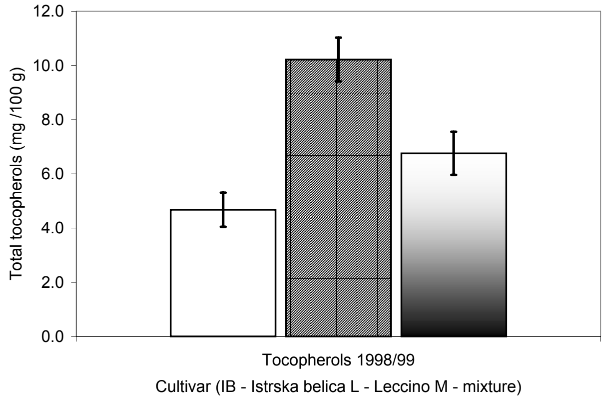


Fig. 7: Average total tocopherol content in the virgin olive oils from 'Istrska belica', Leccino' and mixtures of both cultivars from the 1998/99 crop (the error bars show the standard deviation).
Sl. 7: Povprečna skupna vsebnost tokoferolov v deviških oljčnih oljih sort 'Istrska belica', Leccino' in mešanic obeh sort letnika 1997/98 (kazalci so standardni odmik).

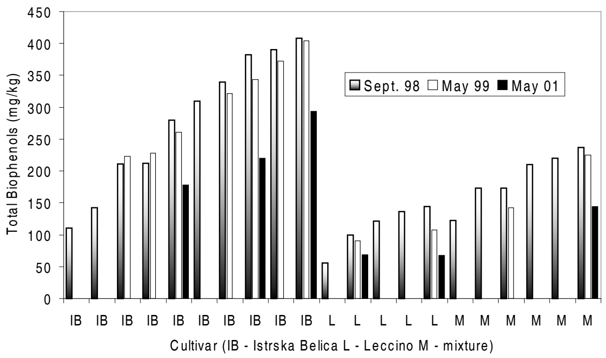


Fig. 8: Changes in the total biophenol content in selected virgin olive oils from the 1997/98 crop during 18 months of storage.
Sl. 8: Spremembe vsebnosti skupnih biofenolov v izbranih deviških oljčnih oljih letnika 1997/98 med 18-mesečnim hranjenjem.

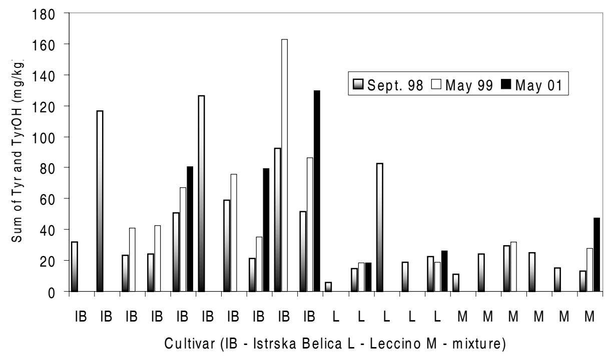


Fig. 9: Changes of the sum of Hydroxytyrosol and Tyrosol in selected virgin olive oils from the 1997/98 crop during 18 months of storage.
Sl. 9: Spremembe vsote hidroksitirosola in tirosola v izbranih deviških oljčnih oljih letnika 1997/98 med 18-mesečnim hranjenjem.

Tab. 2: Extraction processes data for virgin olive oil samples crop 2000/01: DP – centrifugal integral decanter (dual phase decanter).
Tab. 2: Podatki iz ekstrakcijskega procesa za vzorce deviškega oljčnega olja letnik 2000/01: DP – centrifugalno integralni dekanter (dvofazni dekanter).

Content	Cultivar			
	Istrska belica		Leccino	
	DP-1	DP-2	DP-1	DP-2
Total BP (mg/kg)	218	144	134	84
Total TOC (mg/100 g)	6.1	10	7.5	10,4
TyrOH (mg/kg)	5.4	11.3	2.9	3.4
Tyr (mg/kg)	4.3	8.6	9.2	6.5
DMO-dA (mg/kg)	87.6	41	37.9	13.8
Total HPLC BP (mg/kg)	279	193	233	125

Inadequate storage effects

Biophenols. We monitored changes in virgin olive oil samples crop 1997/98 in 3 time determinations. Figures 8, 9 and 10 show various biophenols relations in the ageing processes.

The 2 arrows in Figue 10 show the 'Leccino' samples running out of hydroxytyrosol, meaning that they lost their antiooxidative power from the biophenols species. The sum of tyrosol and hydroxytyrosol concentration in Figure 9 is rising, which demonstrates that the complex phenols are transformed into 'simple' ones and that Tyr is not being consumed, thus showing not being antioxidative potent.

Tocopherols. Figure 11 shows the influence of light on total tocopherols decomposition in virgin olive oil samples of 'Istrska belica' and 'Leccino' from crop 1998/99. The transparent bottle facilitates the passage of light into oil thus speeding the decomposition.

DISCUSSION AND CONCLUSIONS

Comparison of total biophenols content in the virgin olive oil samples from the olives harvested in the 1997/98 and 1998/99 crop years confirmed that several factors can influence the content of biophenols, such as: climatic conditions, harvesting and extraction process. Taking in regard the fact that olive orchards, cultivars, extraction facilities and harvesting time remained practically unchanged in the two crop seasons, we can speculate and conclude the climate has a major impact on the biophenols content, and even more so if we consider the fact biophenols are polar substances that can be rather easily leached out from the fruits or in the extraction process phase if their starting amount is relatively low. Secondly – the relative amount of total biophenols in the virgin oils processed from the cultivar 'Istrska belica' compared to the total biophenols content in the virgin oils processed from the cultivar 'Leccino' stays always higher, no matter what the actual absolute value might be (Figs. 4 and 5). When the total tocopherols content is considered, the virgin olive oils processed from cultivar 'Leccino' lead when compared to the oils processed from the cultivar 'Istrska belica', thus confirming our previous findings (Butinar *et al.*, 1999b).

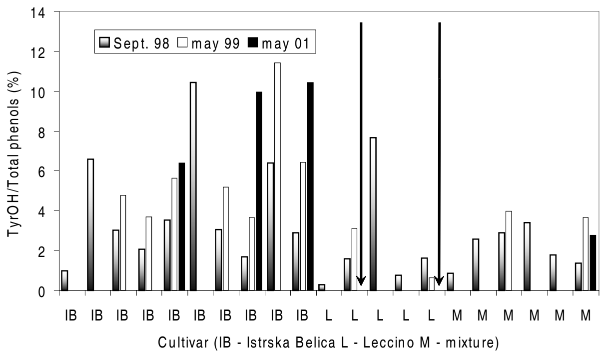


Fig. 10: Changes of the ratio Tyr-OH/total HPLC biophenols in virgin olive oils from the 1997/98 crop during 18 months of storage.
Sl. 10: Spremembe razmerja Hidroksitirosol/skupni HPLC biofenoli deviških oljčnih oljih letnika 1997/98 med 18-mesečnim hranjenjem.

It was shown how different centrifugation extraction processes can influence the total and HPLC biophenols content: percolation/centrifugation process vs. centrifugal/integral decanter process (Tab. 1) and how various ways of performing the same centrifugal/internal decanter (dual phase decanter) – DP-1 and DP-2 process can influence it as well (Tab. 2). It can be concluded that the process DP-2 was run with more water added compared to process DP-1 thus leaching the polar biophenols out of the oil. Consequently, the oil from process DP-1 is richer in total biophenols, total HPLC biophenols and complex biophenols (DMO-dA) meaning the amount of water added did not hydrolytically decompose the complex biophenols to simple ones (Tyr & TyrOH). The data for TyrOH in Table 2 demonstrate this: 5.4 *vs.* 11.3 mg/kg in IB oils and 2.9 *vs.* 3.4 mg/kg in L oils.

Figures 8, 9 and 10 clearly show how storage (inadequate storage conditions) influence the degradation process of complex biophenols towards the simple ones resulting in elevated degrees of hydroxytyrosol and tyrosol in the first stages then gradually changing to diminution of hydroxytyrosol degree (being antioxidatively active) not influencing the decay of tyrosol (not being antioxidatively active). The total biophenols content in some samples after 9 months of storage slightly increased (samples 3 and 4 for IB in Fig. 8). This can be explained either by considering the measurement uncertainty for the total BP determination (the differences being somewhat small) or by the fact the simple and complex biophenols have slightly different extinction coefficients when determining the absorbency in the total biophenols content determination using FC reagent. In the future work it would be more proper to report molar concentrations and not the mass ones.

The light and room temperature can considerably speed up the tocopherols decomposition; the light not absorbed in the dark-coloured bottles speeding the decomposition process even more (*e. g.* Fig. 11).

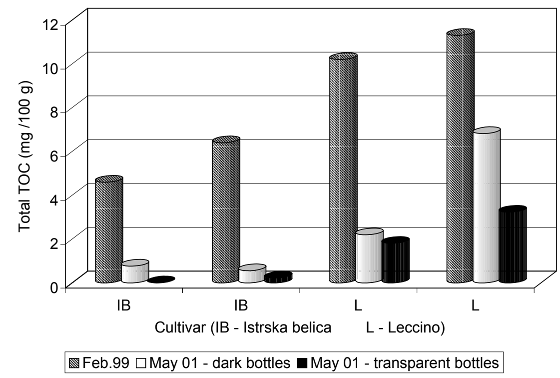


Fig. 11: Influence of light on the total tocopherol content in virgin olive oils from cv. 'Istrska belica' and cv. 'Leccino'.
Sl. 11: Vpliv svetlobe na vsebnost skupnih tokoferolov deviških oljčnih olj sort 'Istrska belica' in 'Leccino'.

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ANTIOKSIDANTI V DEVIŠKIH OLJČNIH OLJIH SLOVENSKE ISTRE IZ DVEH OLJČNIH SORT

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POVZETEK

*Slovenska Istra je del Mediterana, ki je svetovno znan po svoji specifični prehrani. Ena njenih najpomembnejših sestavin je deviško oljčno olje. Deviško oljčno olje vsebuje tudi sestavine netriacilglicerolnega izvora – med njimi za kakovost zelo pomembne antioksidante biofenole in tokoferole. Poznano je, da biofenoli povečujejo odpornost proti antioksidaciji in da olju dajejo značilen okus in vonj. Biofenoli in tokoferoli deviško oljčno olje ščitijo pred kvarjenjem, saj preprečujejo/dušijo reakcije avtooksidacije. Biofenoli deviških oljčnih olj so pretežno sekoiridoidno glukozidnega izvora. So oleuropein, ligstrozid, oleozid in predvsem v olju njihovi razpadni produkti: oleuropein aglikon, ligstrozid aglikon, odprta dialdehidna oblika dekarboksimetil oleuropein aglikona, odprta dialdehidna oblika dekarboksimetil ligstrozid aglikona, hidroksitirosol in tirosol. Na vsebnost biofenolov in tokoferolov v deviškem oljčnem olju vpliva veliko dejavnikov. Med hrambo se vsebnosti hidroksitirosola in tirosola spreminjata. Antioksidativna aktivnost tirosola je zanemarljiva, zato se njegova vsebnost v olju praktično ne spreminja ali pa se le rahlo povečuje. V pričujočem prispevku smo primerjali vsebnost biofenolov in tokoferolov v dveh kultivarjih oljke (*Olea europaea *L.), ki uspevata v Slovenski Istri – 'Istrska belica' in 'Leccino' – in sicer v dveh zaporednih letnikih 1997/98 in 1998/99 s podatki iz naših prejšnjih objav in jih potrdili. Potrdili smo, da je vsebnost biofenolov v oljih iz sorte 'Istrska belica' višja od vsebnosti biofenolov v oljih iz sorte 'Leccino', ki pa imajo višjo vsebnost tokoferolov v primerjavi z olji iz sorte 'Istrska belica'. Primerjali smo tudi vpliv ekstrakcijskega procesa na vsebnost biofenolov in tokoferolov in ugotovili, da daje 2-fazni ekstrakcijski proces (DP) olja, ki imajo višjo vsebnost tokoferolov in biofenolov ter da količina dodane vode pri procesu DP znatno vpliva na vsebnost omenjenih antioksidantov. Nenazadnje pa smo tudi preučili vpliv neustreznega skladiščenja (toplota in svetloba) na vsebnost biofenolov in tokoferolov v nekaterih vzorcih deviških oljčnih olj letnikov 1997/98 in 1998/99 ter pokazali, da (neposredni) vpliv svetlobe znatno pospeši razpad tokoferolov in biofenolov.*

Ključne besede: deviško oljčno olje, biofenoli, tokoferoli, HPLC, predelava, Slovenska Istra

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