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SOLVENT EXTRACTION AND CHROMATOGRAPHIC DETERMINATION OF POLYPHENOLS IN OLIVE POMACE

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Summary

Olive oil mill waste obtained after two-phase olive oil extraction process was subjected to conventional liquid solvent extraction under different pH/temperature/duration conditions using different types of food-grade solvents. The independent variables were: solvent type (ethanol percentage), extraction temperature (20-90 °C), extraction time (30 min-24 h) and pH (2-10.3) of extraction solvent while the response variables were total phenolic content and antioxidant activity of obtained extracts. The optimum solvent extraction conditions for phenols were 120 min at 70 °C using 60% ethanol as extraction solvent, at solvent to sample ratio 5:1 (v/w). For quantification of major bioactive olive polyphenols hydroxytyrosol, tyrosol and oleuropein in obtained extracts, fast and simple RP-HPLC-DAD method was developed and validated. Oleuropein was presented in highest amounts with average value of 115.14±0.19 mg/kg of fresh olive pomace.

Keywords: olive pomace, extraction, HPLC-DAD, hydroxytyrosol, tyrosol, oleuropein

Introduction

The production of olive oil, the second most important agro-sector in Europe, has increased in recent years and, as the consequence, larger quantities of waste products associated with olive oil production are being disposed in the environment (olive leaves, olive pomace and olive mill waste water). They contribute significantly to excessive nutrient burdens in local ecosystems and represent ecological hazards (Aberg et al., 2004). The exploitation of olive waste from an environmental point of view may be approached in several ways: it can be used for energy generation; as fertilizer or soil conditioner; as herbicide or pesticide, as animal feed or in human consumption; for residual oil recovery; for production of various products (alcohols, biosurfactants, biopolymers, activated carbons) and for organic compounds recovery (pectin, phenolic antioxidants). Most studies dealing with biological activity of olive derived products have been focused on polyphenols as major active constituents. Olive pomace contains different phenolic compounds that can be divided in several classes: simple phenols (e.g., tyrosol (TS) and hydroxytyrosol (HTS)) cinnamic acid derivatives; flavonoids (e.g., apigenin, luteolin and rutin (quercetin-3-rutinoside)); and secoiridoids (e.g., oleuropein (OLE), oleuropein aglycone and de(carboxymethyl) oleuropein aglycone isomers) (Obied et al., 2007).

The potential of olive biophenols (OBPs) has already been recognized by competent authorities, such as EFSA (European Food Safety Authority). Extracts obtained from olives and olive mil wastes are generally regarded as safe (Soni et al., 2006; Obied et al., 2012) and reported pharmacological properties of particular OBPs include antioxidant, anti-inflammatory, cardiovascular, immunomodulatory, gastrointestinal, respiratory, autonomic, central nervous system, antimicrobial, anticancer and chemopreventive action (Obied et al., 2012). Pharmacologically active OBPs are abundant in different types of olive mill wastes, however the exact qualitative and quantitative composition of obtained extracts is significantly affected by the type of extraction (Kumar et al., 2006; Aliakbarian et al., 2011) and extraction conditions (time, temperature, pressure, solvent).

The increasing use of bioactive compounds in pharmaceutical, food and chemical industries sector such as points out the need of finding adequate extraction method for bioactive components from plant materials (Sasidharan et al., 2011). Bioactive compounds from plant materials can be extracted by various classical extraction techniques. Most of these techniques are based on the extracting power of different solvents in use and the application of heat and/or mixing (Azmir et al., 2013). The major challenges of conventional extraction techniques are longer extraction time, large solvent consumption, evaporation of the huge amount of solvent during the process, low extraction selectivity and thermal decomposition of thermo labile compounds (Luque de Castro and Garcia-Ayuso, 1998). Therefore, nonconventional extraction techniques such as ultrasound assisted extraction, enzyme-assisted

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extraction, microwave-assisted extraction, pulsed electric field assisted extraction, supercritical fluid extraction and pressurized liquid extraction have been introduced during the last two decades (Azmir et al., 2013). Those techniques have numerous advantages, especially in terms of sustainability and pollution prevention but can be conducted only in well equipped laboratories and in many cases, are demanding or not suitable for scale-up. Therefore, there is a constant need for further optimization of conventional extraction techniques in terms of efficiency, selectivity and sustainability. The aim of this work was to optimize the extraction of olive pomace polyphenols, using simple solvent extraction and only food-grade solvents with special emphasis set on HTS, TS and OLE as the major bioactive polyphenols in live. The additional goal was to develop and validate simple, fast and accurate procedure for their quantification in obtained extracts.

Materials and methods

Reagents and chemicals

Folin-Ciocalteu reagent, gallic acid, ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), tyrosol (4-hydroxyphenylethanol), hydroxytyrosol (3,4-dihydroxyphenylethanol) and oleuropein were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium carbonate and all solvents used throughout the experiments were obtained by Merck (Darmstadt, Germany). All other chemicals were from Kemika (Zagreb, Croatia).

Stock standard solutions

Stock standard solutions of TS, HTS and OLE were prepared in HPLC-grade water at 1 mg/mL level. Working standards were prepared prior to analysis by diluting appropriate volumes of stock solutions with HPLC grade water at 3, 9, 27, 81 and 243 μg/mL level.

Instrumentation

For chromatographic analysis, we used Agilent Life Sciences 1220 LC Gradient System equipped with a dual-channel gradient pump with degasser, autosampler, column oven and an additional Agilent 1260 Infinity Diode Array Detector (Agilent, Santa Clara, CA, USA). Chromatographic separation was performed on an Zorbax Eclipse Plus C18 reversed-phase column (250 x 4.6 mm, ID 5 μm) (Agilent, Santa Clara, CA, USA). Spectrophotometric analysis was conducted on UnicamUV4 UV-VIS spectrometer.

Chromatographic analysis of HTS, TS and OLE

For the chromatographic separation of HTS, TS and OLE a gradient elution program was used. A linear gradient was run with flow rate of 1 ml/min. The column was maintained at 40 °C throughout all experiments with the aid of an electronically controlled oven. All mobile phases were vacuum filtered through 0.45 μm membrane filter and degassed in an ultrasonic bath prior to HPLC analysis. For the validation of analytical method, the guidelines of the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use recommend the accomplishment of linearity, accuracy, precision, and sensitivity.

To evaluate the linearity of the method, standard solutions were prepared at five concentration levels containing 3, 9, 27, 81 and 243 ppm of TS, HTS and OLE. Five replicates at each concentration were analyzed. The linearity of the data was checked by performing linear least-squares regression analysis. Accuracy of the method was assessed by three quality control standards at three concentration levels, and was evaluated relative percentage error. The assay precision was evaluated by performing the assay at three levels (9, 27 and 243 ppm) in five replicates and calculating the RSD values. Intermediate precision was demonstrated by preparing standard solutions at three levels (9, 27 and 243 ppm) in three replicates on different days and calculation of RSD values. Recovery was evaluated as the ratio of the peak area for every substance in the spiked sample against that of the standard. Olive pomace samples were spiked at three different concentration levels. LOD was determined by preparing a solution that produced a response of about 3 and 10 times the baseline noise. The solution was injected three times, and the S/N (signal/noise) ratio recorded for each injection. Solution concentration is considered LOD if S/N ratio is between 3-10. LOQ was determined in the same manner but with an S/N ratio of 10-20.

Total phenolic content

Total phenolic content (TPC) was determined spectrophotometrically according to the method of Singleton and Rossi (1965) with some modifications. Briefly, adequatelly diluted olive pomace extracts (200 μL) were mixed with 1.35 mL of distilled water and 150 μL of Folin Ciocalteu reagent. After 5-minute incubation 1.5 mL of 6% Na_2CO_3 was added

to each reaction mixture and obtained solutions were incubated at 50 °C for 30 minutes. Absorbance readings were conducted at 725 nm and results were expressed as gallic acid equivalents (GAE).

TEAC assay

The TEAC (Trolox Equivalent Antioxidant Capacity) assay reflects the ability of hydrogen or electron-donating antioxidants to scavenge the ABTS•+ radical cation compared with that of Trolox. As described by Re et al. (1999). ABTS radical was prepared by mixing the equal volumes of 7 mM ABTS and 2.45 mM solution of $K_2S_2O_8$ and leaving the mixture overnight allowing the complete development of the chromophore radical. The reaction mixture was prepared by mixing 2.5 mL of adequately diluted $ABTS^{\bullet+}$ and 300 μL of adequately diluted sample and the absorbance was measured after 3 minutes. The quenching of initial absorbance was plotted against the Trolox concentration and obtained results were expressed as Trolox equivalents (TE).

Experimental design

Fresh olive pomace, obtained by the two-phase extraction process was delivered from the local olive-mill plant. Pomace samples (containing approximately 65% water, low amounts of pomace oil, olive pulp and pits) were homogenized, frozen immediately in the form of thin plates and used for the development and optimization of polyphenol extraction procedure. The independent variables were solvent type (ethanol percentage), extraction temperature, extraction time and pH of extraction solvent, while the response variables were total phenolic content (TPC) and antioxidant activity (TEAC) of obtained extracts (Table 1).

Table 1. Variables used in the process of the optimization of the extraction procedure

OPTIMIZATION VARIABLES	
INDEPENDENT VARIABLES	RESPONSE VARIABLES
SOLVENT TYPE *(water, 40% EtOH, 60% EtOH, 80% EtOH, 96% EtOH*)	TOTAL PHENOLIC CONTENT (*mg GAE/g*)
TIME OF EXTRACTION (*30 min, 60 min, 120 min, 300 min, 24 h*)	
TEMPERATURE (*room temperature, 20 °C, 50 °C, 70 °C, 90 °C*)	TEAC ANTIOXIDANT ACTIVITY (*mg TE/g*)
pH (*2, 6, 8.5, 10.3*)	

Extracts obtained under optimized conditions were analyzed for total phenolic content, tyrosol, hydroxytyrosol and oleuropein content, and TEAC. Chromatographic method for determination of hydroxytyrosol, tyrosol and oleuropein in obtained extracts was optimized and validated according to ICH guidelines (ICH, 1996). For HTS, TS and OLE determination, olive pomace extracts were freeze-dried immediately after extraction, dissolved in adequate volume of HPLC-grade water, filtered through 0.45 μm membrane filter and subjected to HPLC analysis.

Statistical analysis

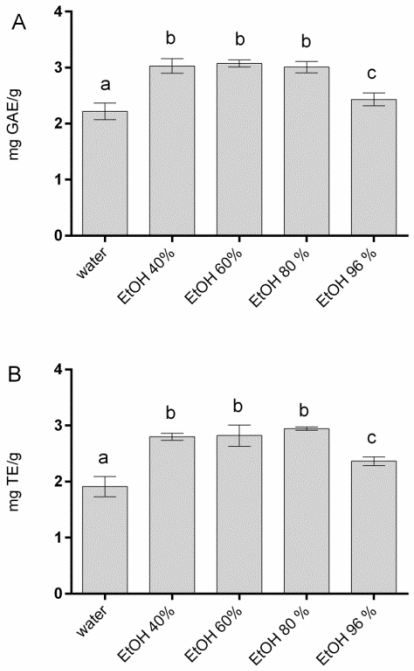
All analytical measurements were conducted at least in triplicates; the results were averaged and presented as means ± standard deviation. Analyses of variance ANOVA and post-hoc Tukey's test were used to compare significant differences in the values of response variables depending on the solvent composition and pH, extraction time and extraction temperature. Statistically significant influences were expressed using p values (Tukey's post hoc test, $p < 0.05$). Analyses were conducted using Prism GraphPad software (GraphPad Software, Inc., USA).

Results and discussion

Influence of different extraction parameters on TPC and TEAC

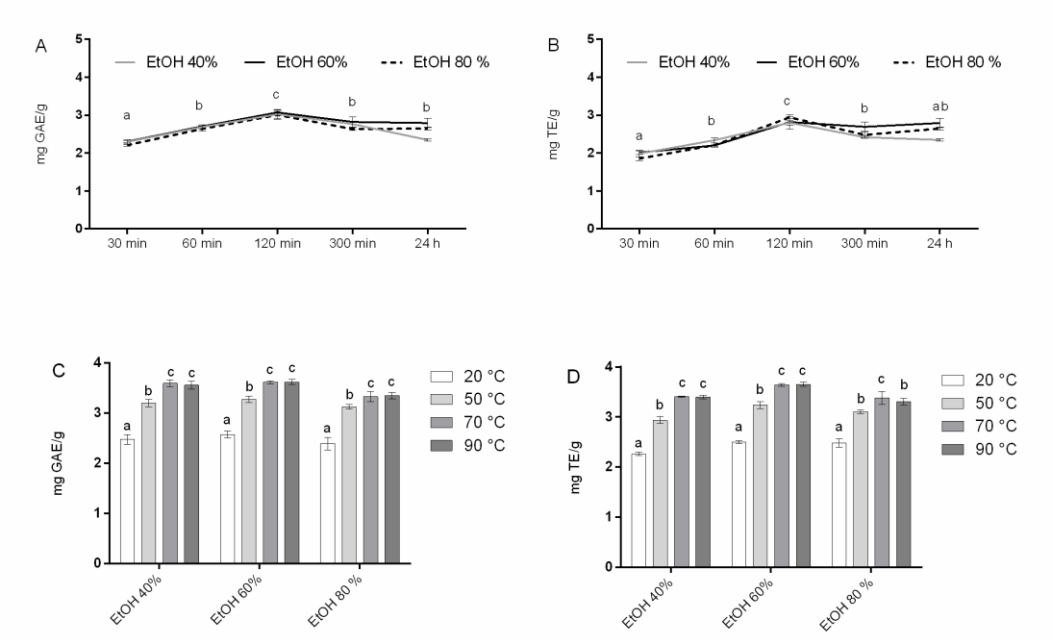
All olive pomace extracts contained significant amounts of polyphenolic compounds and showed antioxidant activity; however, obtained TPC and TEAC values differed significantly depending on the solvent polarity, pH and the duration and temperature of extraction. The influence of solvent on TPC and TEAC is presented in Fig. 1. Obtained results showed that significantly higher TPC and TEAC were obtained by using ethanol-water mixtures in comparison to pure water or 96% ethanol ($p<0.05$). Observed differences between TPC and TEAC content in extracts with 60% 40% or 80% ethanol were not statistically significant ($p>0.05$).

Extractions using different volume fractions of ethanol in water were used for the subsequent optimization of extraction procedure in terms of time of extraction and applied temperature. Obtained results are presented in Fig. 2.



[a,b,c]columns marked with the same letter belong to the same statistical group (p>0.05). Extractions were performed at pH=6, by shaking the mixtures at 70 °C (100 rpm) for 120 min. Sample to solvent ratio was 1:4.

Fig. 1. Impact of the solvent type on the TPC (A) and ABTS antiradical activity (B) of olive pomace extracts

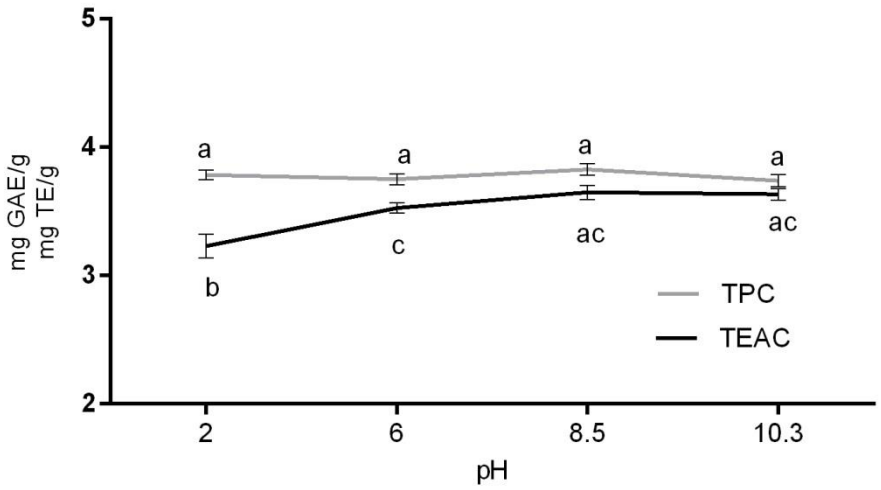


[a,b,c]data marked with the same letter belong to the same statistical group (p>0.05). Extractions were performed at pH 6, by shaking the mixtures at 70 °C (A, B), for 120 min (C, D). Sample to solvent ratio was 1:4.

Fig. 2. Impact of extraction time (A, B) and temperature (C, D) on the recovery of total phenols and ABTS antiradical activity of pomace extracts

By increasing the time of extraction from 30 to 120 min resulted with significant increase of polyphenolic content and antioxidant activity of obtained extracts for each investigated solvent. Further elongation of the extraction process to 300 min did not contribute to the efficiency of the extraction process, while after 24 h extraction significant decrease of TPC and TEAC was observed (Fig. 2A, Fig. 2B). Observed decrease in the extraction yield is probably due to degradation of polyphenolic compounds caused by hydrolysis, internal redox reactions and polymerization (Alonso-Salcez et al., 2001). Under the same extraction conditions, TPC and TEAC were comparable for all investigated extraction solvents (40%, 60% and 80% ethanol). Obtained results are consistent with the trends reported by Aliakbarian et al. (2011) who concluded that the prolongation of extraction time from 15 to 90 min significantly improves extraction of polyphenolic compounds from olive pomace in high-pressure-high temperature reactor, while longer extraction times promote degradation of polyphenols and negatively influences extraction yields. Similar trends were observed by Jerman et al. (2010) who optimized ultrasound-assisted solid liquid extraction of polyphenols from olive fruit.

Extraction temperature was also found as significant factor affecting the TPC and TEAC (Fig. 2C, Fig. 2D) and the higher extraction yields were obtained at the temperatures of 70 °C and higher. However, increasing the temperature above 70 °C did not produce any additional benefit; therefore 70 °C has been chosen as the optimal temperature for the extraction of olive pomace polyphenols. At 70 °C, significant differences were observed between the efficiency of tested extraction solvents; the efficiency of 80% ethanol was significantly lower in comparison to 40% and 60% ethanol. Obtained results indicate that the major polyphenolic compounds in olive pomace are thermostabile and/or that possible thermal degradation of polyphenols generates new polyphenolic compounds that retain antioxidant activity. Observed results are consistent with data of Herrero et al. (2011), who optimized the pressurized liquid extraction of olive pomace polyphenols using food-grade solvents and obtained the highest yields at high temperatures (150 °C and 200 °C for water and ethanol, respectively). Similarly, Aliakbarian et al. (2011) investigated extraction of olive pomace polyphenols by high-pressure-high temperature reactor and observed higher polyphenol yields at higher temperatures (optimal temperature was 180 °C). pH can also significantly influence the efficiency of extraction procedure, because polyphenolic compounds in plant material are often part of high molecular mass complexes that can be partially degraded under acidic or alkalic conditions enhancing in that way the extractability of phenolic compounds. On the other hand, extreme pH values can cause the degradation of phenolic compounds resulting in lower extraction yields. Therefore, the outcome depends on the nature of plant material and physicochemical characteristics of particular polyphenols. In case of olive pomace, the change of pH of the extraction solvent did not produce significant changes in TPC of obtained extracts; however significantly lower TEAC values were recorded in extracts obtained under acidic conditions (Fig. 3).



[a,b,c]data marked with the same letter belong to the same statistical group (p>0.05). Extractions were performed by shaking the mixtures at 70 °C (100 rpm for 120 min, using 60% ethanol as extraction solvent. Sample to solvent ratio was 1:4.

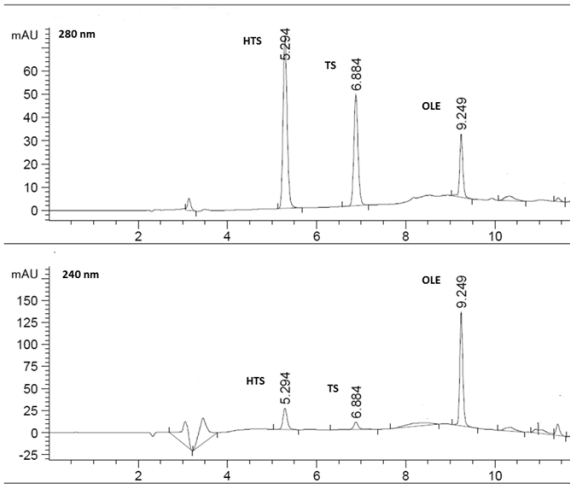
Fig. 3. Impact of pH on the recovery of total phenols and ABTS antiradical activity of pomace extracts

Development and validation of RP HPLC-DAD method for determination of HTS, TS and OLE in olive pomace extract

A

Time (min)	Solvent A[a] (%)	Solvent B[b] (%)	Flow-rate (ml/min)
0	90	10	0
4	80	20	4
8	40	60	8
11	30	70	11
15	90	10	15
20	90	10	20

B



C

Component	Regression equation	Correlation coefficient (r^2)
Hydroxytyrosol	y = 10.184x + 70.179	0.9859
Tyrosol	y = 11.709x - 4.3663	0.9998
Oleuropein	y = 21.527x - 75.091	0.9996

[a]*Solvent A: acetate buffer;* [b]*Solvent B: acetonitrile*

Fig. 4. RP HPLC-DAD method for determination of HTS, TS and OLE in olive pomace extracts: gradient elution program (A); chromatograms of reference standards (27 ppm) (B); regression equation and correlation coefficients (C)

For the chromatographic separation of HTS, TS and OLE a gradient elution program was used as shown in Fig. 4A. Solvent A was 0.05 M Na-acetate buffer (pH=5) and solvent B was acetonitrile.

UV spectra of all substances were recorded by diode array detection system and the maximum of absorbance were determined to be 240 nm for OLE and 280 nm for HTS and TS (Fig. 4B). Identification of the eluting peaks was performed by comparing their retention time values and the corresponding UV spectra with those of the standards. The linearity of the method was evaluated by linear regression analysis using five concentrations of tyrosol, hydroxytyrosol and oleuropein. Good linearity was achieved for all the analytes, as shown in Fig. 4C. For accuracy determination, three standard solutions at concentration levels 27, 81 and 243 ppm were analyzed. Obtained results were expressed as relative percentage error ranging from 1.33% - 4.04% for HTS; 1.99% - 3.42% for TS and 0.64% - 2.35% for OLE. Precision analysis was conducted by analyzing 5 replicates of standard solution at three concentration levels (9, 27 and 243 ppm). RSD values were ranged from 0.84 - 2.99 for HTS; 0.04 - 0.13 for TS and 0.86 - 3.45 for OLE. Intermediate precision was determined by analyzing three replicates of prepared standard solutions at three concentration levels on different days. Obtained RSD values were from 1.12 - 2.60 for HTS; 1.47 - 2.79 for TS and 0.67 - 2.38 for OLE. For recovery determination olive pomace samples were spiked with standard solutions of hydroxytyrosol, tyrosol and oleuropein at three concentration levels (27, 81 and 243 ppm). The obtained recovery was 100.9% - 102.4% for HTS; 99.3% - 101.3% for TS and 99.9% - 100.5% for OLE. The sensitivity of the method has been assessed by determining LOD values for HTS (1.5 ppm), TS (1.0 ppm) and OLE (2.0 ppm).

Applied method was found to be suitable for the analysis of olive pomace extracts due to simple sample preparation (it does not require any sample pre-treatment), short time of analysis and satisfying validation parameters. The only disadvantage of the method was its relatively low sensitivity especially inthe case of OLE; however, it was high enough for the analysis of obtained olive pomace extracts.

The content of HTS, TS and OLE in olive pomace extracts obtained under optimized conditions

Olive pomace extracts obtained under optimized extraction conditions (continuous shaking at 70 °C for 120 min) using 40% and 60% ethanol as extraction solvents were subjected to HPLC analysis in order to compare the efficiency the two extraction solvents. Namely, there were no significant differences between the two solvents regarding total phenol yields or antioxidant activity of obtained extracts. Namely, despite the wide variety of polyphenolic compounds present inolive oil and

pomace HTS, TS and OLE are considered to be the main polyphenolic bearers of the health-promoting properties of olive oil.

The only authorised health claim for olive oil, listed in the Regulation 432/2012 (EC, 2012), relates to the level of olive phenolic compounds and the impact on the protection of blood lipids from oxidative stress. The conditions of use of the claim are that it "may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g. OLE complex and TS) per 20 mg of olive oil. In order to bear the claim, information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 20 mg of olive oil (EFSA, 2011).

Obtained results are presented in Table 2, and clearly emphasize that using 60% ethanol (instead of 40% ethanol) results in small but statistically significant increase in HTS, TS and OLE content of obtained extracts.

Table 2. TPC, AA, HTS, TS and OLE content of ethanolic pomace extracts obtained under optimized conditions

Compound*	**40 % EtOH**	**60 % EtOH**
TPC *(mg GAE/g)*	3.59±0.07[a]	3.62±0.03[a]
AA (*mg TE/g)*	3.41±0.01[a]	3.64±0.03[a]
HTS *(mg/kg)*	75.46±0.33[a]	81.8±0.41[b]
TS *(mg/kg)*	82.08 ±0.13[a]	86.05±0.34[b]
OLE *(mg/kg)*	110.37±0.07[a]	115.14±0.19[b]

**values are expressed per g(kg) of fresh olive pomace. Differences between values in the same row marked with different letters are statistically significant ($p<0.05$).*

The amounts of HTS, TS and OLE are generally comparable to levels found in olive pomace by other authors. The content of bioactive polyphenols in olive pomace is variable and depends on numerous factors: olive cultivar, the olive oil extraction process and the type of pomace that remains as the by-product (traditional extraction, two-phase process or three-phase process, continuous combined percolation-centrifugation etc.) (Dermeche, 2013). However, our results show that OLE is the most abundant among analysed polyphenols in olive pomace which is consistent with observations of other authors (Cioffi et al., 2010; Rubio-Senet et al., 2013). They investigated more advanced extraction methods, using hydrothermally treated pomace, different types of organic solvents or applying higher temperatures or pressures.It is hard to compare the absolute yields of HTS, TS and OLE to ours since results obtained by different authors are often expressed in different ways (per g of dry extract, per g of dry pomace, per g of fresh pomace) without sufficient data that would allow re-calculation and comparison.

Conclusions

Extracts with different antioxidant (phenolics) concentrations and activities were obtained from fresh olive pomace by changing the conditions of conventional solvent extraction. For this purpose, RP HPLC-DAD method was validated and found to be suitable for the analysis of olive pomace extracts due to simple sample preparation (it does not require any sample pre-treatment), short time of analysis and satisfying validation parameters. Among different food-grade solvents, 60% ethanol was selected as the most appropriate solvent for the extraction of phenolic compounds from olive oil pomace under optimized conditions (120 min with shaking, 70 °C, 120 min). Satisfactory phenolic (antioxidant) yields prove that oil mill waste is a low-cost, renewable and abundant source of phenolic antioxidants and that simple solvent extraction which uses only food-grade solvents can be successfully applied to olive pomace.

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