Olive Leaves Infuse and Decoct Production: Influence of Leaves Drying Conditions and Particle Size

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Taggiasca, a cultivar grown typically in western Liguria (Italy) and in the southern regions of France, is a tasty and sweet olive for table consumption and for the oil production. Olive oil production generates a huge amount a wastes. Besides the traditional olive mill wastewater and olive pomace, leaves of the trees are considered by-products and nowadays they are burned or used as animal feeds despite they have high content of bioactive compounds. Oleuropein, apigenin and vanillic acid are examples of polyphenols contained in olive leaves and their content changes depending on the cultivar, the region of growth and the harvesting time. The aim of this work is to valorise one of the by-products of the olive oil industry. The study aims to investigate the effect of the pre-treatment processes on the production of olive leaves infusion. Different drying techniques and particles size have been evaluated in order to produce an extract rich in phenolic compounds. Olive leaves (Taggiasca cultivar) were kindly provided by the Azienda Agricola Castellari company, Savona, Italy. Leaves were collected and immediately washed and dried at different temperatures (50, 60 and 70 °C) in a ventilated oven. Moreover, an additional drying at 70 °C was performed under nitrogen atmosphere. In order to evaluate the effect of the drying temperatures on total polyphenol (TP) and flavonoid (TF) yields and on antiradical power (ARP) of the extracts, samples were grinded (0.8-0.59 mm) using a laboratory mixer. Powders were then extracted using ethanol and analysed. Based on those results, the best drying condition was used to evaluate the influence of the matrix particle size on the extraction yield after ethanol extraction and teas production. Sieves (4, 9, 16, 20 and 30 Mesh) were used to obtain powder with particles distributed uniformly in the range $2.19 < A < 4.76$, $1.19 < B < 2.19$, $0.84 < C < 1.19$ and $0.59 < D < 0.84$ mm. Olive leaves teas were prepared as follow: 1) infusion, 200 ml of deionised boiling water were added to 2 g olive leaves powder and let them soak for 3 min without additional heating; 2) decoction, 200 ml cold water were added to 2 g of olive leaves powder and boiled for 15 min, waiting for 10 min after boiling before carrying on the analysis. After extracting with ethanol the dried matrix obtained at different temperatures, we found out that there was not statistical difference ($p<0.05$) in the TP (25.06 ± 1.00 mg_{GAE}/g_{DL}) and TF (22.23 ± 3.00 mg_{CE}/g_{DL}) content between samples obtained at 60, 70 °C and 70 °C under nitrogen flow. During the tea production, within each range of particles size (e.g. B), we found out that decoction is the process that allows to extract the highest quantity of total polyphenols (41.14 ± 2.56 mg_{GAE}/g_{DL}) and total flavonoids (45.27 ± 3.10 mg_{CE}/g_{DL}).

1. Introduction

Olive trees are largely widespread all over the world and their fruit are of major agricultural importance since they play a key role in the Mediterranean diet because of the nutritional values and chemical composition. Taggiasca is a tasty and sweet olive for table consumption even though it is mainly used for the oil production. Extra virgin olive oil contains a wide range of bioactive compounds, like unsaturated fatty acids and polyphenols, which are physiologically active and important for a healthy diet and longevity (Aliakbarian et al., 2011). Particularly, polyphenols compounds are secondary metabolites and possess high antioxidant, antimicrobial and anti-inflammatory properties. Several studies have shown their beneficial activity against common human diseases (cancer, cardiovascular diseases, etc.) (Buyukbalci and El, 2008; Palmieri et al.,

2012). Oil production generates a huge amount of wastes. Besides the traditional olive mill wastewater and olive pomace, leaves of the trees are considered by-products and nowadays they are burned or used as animal feeds despite they have high content of bioactive compounds. Oleuropein, apigenin and vanillic acid are examples of polyphenols contained in olive leaves and their content changes depending on the cultivar, the region of growth and the harvesting time (El and Karakaya, 2009). In order to exploit these compounds not only for medical application but also in the food industry (as dietary supplement or even simply as infusions) (Aliakbarian et al., 2015), recovery based on extraction processes is required. Several parameters influence the yield of extraction, but mainly the particle size and drying technique play a key role (Erbay and Icier, 2009). Drying allows to remove water, thus decreasing the moisture content and improving the shelf life of the product. Several techniques are able to remove moisture from the leaves and currently two of them are used for industrial applications: hot air-drying and freeze-drying. The first one is a relatively simple and fast process, but the high temperature could involve chemical modification of the bioactive compounds, leading to their degradation. On the other side, freeze-drying could represent a valid alternative where, thanks to the low temperature, moisture content is removed without any degradation process. Nevertheless, it is one of the most expensive techniques and the initial freezing process could affect the quality of the final product. For those reasons, a deep study of the drying techniques is required in order to fully exploit olive leaves potential (Fellows, 1988).

The aim of this work is to valorise one of the by-products of the olive oil industry. The study aims to investigate the effect of the pre-treatment processes on the production of olive leaves infusion. Different drying techniques and particles size have been evaluated in order to produce an extract rich in phenolic compounds. The yields of extraction have been determined by colorimetric analysis and HPLC-DAD.

2. Section headings

2.1 Materials

Olive leaves (Taggiasca cultivar) were kindly provided by the Azienda Agricola Castellari company, Savona, Italy. Leaves were collected and immediately washed and dried. Ethanol, methanol, acetonitrile, DPPH radical, Folin-Ciocalteu reagent and single phenolic standards were purchased from Sigma-Aldrich (Milan, Italy).

2.2 Sample preparation

Olive leaves were dried at different temperatures (50, 60 and 70 °C) in a ventilated oven (D-82152, MMM Medcenter, München, Germany) until reaching a proper value of humidity (less than 5 %). Moreover, an additional drying at 70 °C was performed under nitrogen atmosphere. In order to evaluate the effect of the drying temperatures on total polyphenols yield and on antiradical power of the extracts, samples were grinded using a laboratory mixer and separated by sieves (Mesh 20-30) to obtain a homogeneous powder (0.8-0.59 mm). Powders were then extracted and analysed. Based on these results, the best drying condition was used to evaluate the influence of the matrix particle size on the extraction yield. Sieves (4, 9, 16, 20 and 30 Mesh) were used to obtain powder with particles distributed uniformly in the range 2.19 < A < 4.76, 1.19 < B < 2.19, 0.84 < C < 1.19 and 0.59 < D < 0.84 mm.

2.3 Olive leaves teas preparation

Olive leave teas were prepared as follow:
* Infusion - 200 mL of deionised boiling water was added to 2 g olive leaves and let them soak for 3 min without additional heating;
* decoction - 200 mL cold deionised water was added to 2 g of olive leaves and boiled for 15 min, waiting for 10 min after boiling before carrying on the analysis.

Beside, phenolic fraction was extracted using a solution of ethanol/water (80:20 v/v) in order to compare the different yields. Briefly, 1.25 g of olive leaves powder were added to 10 ml of solvent and incubated for 24 h in dark conditions (Buyukbalci and El, 2008). At the end of each different extraction process, all of the samples were filtered through a paper filter (Whatman 40). The liquid fraction was centrifuged at 6000 ×g for 10 min (42426, ALC, Milan, Italy).

2.4 Total polyphenols and total flavonoids analyses

A modified version of the Folin-Ciocalteu assay (Casazza et al., 2011; Swain and Hillis, 1959) was used to quantify the total polyphenols (TP) content of the samples. Analysis were carried out at 725 nm using an UV-Vis spectrophotometer, model Lambda 25 (Perkin Elmer, Wellesley, MA) and the calibration curve was made using standard solutions of gallic acid in the range 0.01-1.00 mg/mL. Total Polyphenol yield (TPY) was

expressed as milligrams of gallic acid equivalents per gram of dried leaves (mg_{GAE}/g_{DL}) and it was calculated using the following equation:

$$TPY = \frac{ABS_{725}}{1.7} \times \frac{S}{DL} \quad\quad\quad (1)$$

where ABS_{725} is the absorbance at 725 nm, 1.7 is the slope of the calibration curve, S is the volume (mL) of the extractive solvent and DL are the grams of dried olive leaves powder.

Total flavonoid (TF) content of the olive leaves extracts was determined by the colorimetric method described by (Casazza et al., 2012). The absorbance was measured using the same spectrophotometer as above at 510 nm. Total flavonoid yield (TPY) was expressed as milligrams of catechin equivalents per gram of dried leaves (mg_{CE}/g_{DL}) and it was calculated using the following equation:

$$TFY = \frac{ABS_{510}}{2.3} \times \frac{S}{DL} \quad\quad\quad (2)$$

Where ABS_{510} is the absorbance at 510 nm, 2.3 is the slope of the calibration curve, S is the volume (mL) of the extractive solvent and DL are the grams of dried olive leaves powder.

2.5 Antiradical power determination

The antiradical power of extracts and teas was determined using the DPPH radical method. For each samples, 0.1 mL of nine different dilutions in methanol were prepared and mixed with 3.90 mL of DPPH· methanolic solution (9.15×10^{-5} mol/L). ARP was defined as $1/EC_{50}$ ($\mu g_{DPPH·}/\mu L_{extract}$).

2.6 HPLC-DAD analysis

Single phenolic compounds were detected and quantified by HPLC (Hewlett Packard, 1100 Series, Palo Alto, CA, USA) equipped with a C18 reverse-phase column (Model 201TP54, Vydac, Hesperia, CA, USA) coupled with a DAD detector, following the method described by Ferrari et al. (2014). Briefly, mobile phases were water/acetic acid (99:1, v/v) (solvent A) and methanol/acetonitrile (50:50, v/v) (solvent B), while the solvent gradient changed according to the following conditions: from 0 to 5 % B in 5 min, from 5 to 30 % B in 25 min, from 30 to 40 % B in 10 min, from 40 to 48 % B in 5 min, from 48 to 70 % B in 10 min, from 70 to 100 % B in 5 min and isocratic at 100 % B for 5 min. Runs were performed at 30 °C. Ferulic and Vanillic acids, Catechin, Epicatechin, Oleuropein and Apigenin were used as standards.

2.7 Statistical analysis

Statistical analysis was performed using ANOVA multiple comparison, followed by Tukey's post-hoc test, using a GraphPad Prism 6 software (San Diego, USA). Statistically significant values are presented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3. Results and Discussions

3.1 Effect of leaves drying methodology on polyphenol recovery

The results of the influence of drying condition on olive leaves' moisture content, total polyphenol and flavonoid yields and antiradical power of the extracts are reported in Table 1.

At all drying conditions, the moisture contents of samples were lower than 4%, which is an acceptable value to have an optimal storage of a dried product (Sinija et al., 2007; Vidović et al., 2014).

Table 1: olive leaves' moisture content, total polyphenol (TPY) and flavonoid (TFY) yields and antiradical power (ARP) of the extracts.

Temperature (°C)	Drying time (h)	Moisture content (%)	TPY (mg_{GAE}/g_{DL})	TFY (mg_{CAE}/g_{DL})	ARP ($mg_{DPPH}/mL_{extract}$)
50	14	3.46 ± 0.43^a	12.01 ± 0.60^a	6.79 ± 2.43^a	8.01 ± 0.32^a
60	10	3.65 ± 0.45^b	25.53 ± 1.04^b	22.25 ± 1.10^b	17.42 ± 1.56^b
70	7	$0.91 \pm 0.48^{a,b}$	23.75 ± 1.50^b	19.25 ± 0.64^b	$12.80 \pm 2.95^{a,b}$
70*	7	$0.30 \pm 0.03^{a,b}$	25.90 ± 1.95^b	25.18 ± 2.02^b	28.07 ± 4.90^c

*under N_2 flow

The TPYs ranging from 12.01±0.60 to 25.90±1.95 mg_{GAE}/g_{DL} were similar to those reported by Sahin and Samli (2013). The authors founded the maximum TP content of 25.06 mg_{GAE}/g_{DL} working with 50% of ethanol, 500 mg dried leaf to 10 mL solvent, and 60 min of extraction under ultrasounds.

After extracting the dried matrix obtained at different temperatures, we found out that there was not statistical difference ($p<0.05$) in the TP and TF content between samples obtained at 60, 70 °C and 70 °C under nitrogen flow. There was a slight difference in the antiradical power between the sample obtained at 70 °C under nitrogen flow and the other samples. Moreover, the extraction of dried matrix at 50 °C led to significantly lower values ($p<0.05$) of TP, TF and ARP. As can be observed, drying time had more influence on phenolic compound yields when compared to drying temperature. High temperature (60 °C) required shorter drying time than mild temperature (50 °C) to achieve the same final moisture content, while providing more bioactive compounds. In fact, working at 50 °C for 14 h instead of 60 °C for 10 h, TP and TF yields decreased of 53 and 69 %, respectively. Similar to our observation, Ahmad-Qasem et al. (2013) noticed that the best olive leaf drying conditions were at higher temperature (120 °C respect to 70 °C) for shorter times.

Table 2 shows the results of HPLC analysis. According to these data, extraction yields obtained at 50 °C are statistically lower than the one obtained at 60 °C: for example, oleuropein concentration in the sample dried at 60 °C for 10 h was more than 30 times higher than the one in the sample dried at 50 °C for 14 h. The same consideration was valid for catechin, vanillic acid and ferulic acid, while there was not statically difference for epicatechin and apigenin.

Table 2: main single phenolic compounds (mg/100g_{DL}) HPLC analysis of olive leaves extracts.

Drying temperature (°C)	Catechin	Vanillic acid	Epicatechin	Ferulic acid	Oleuropein	Apigenin
50	0.05 ± 0.01^a	0.08 ± 0.00^a	0.09 ± 0.01^a	0.01 ± 0.00^a	0.15 ± 0.01^a	0.05 ± 0.01^a
60	0.18 ± 0.03^b	0.14 ± 0.01^b	0.25 ± 0.02^a	0.14 ± 0.01^b	5.22 ± 0.76^b	0.05 ± 0.01^a
70	$0.12 \pm 0.00^{a,b}$	0.13 ± 0.00^b	0.27 ± 0.08^a	0.15 ± 0.01^b	2.98 ± 0.25^c	0.03 ± 0.00^a
70*	0.21 ± 0.05^b	0.17 ± 0.01^b	0.25 ± 0.02^a	0.15 ± 0.00^b	$3.68 \pm 0.03^{c,d}$	0.03 ± 0.00^a

*Drying under N_2 flow

3.2 Infuse and decoct phenolic concentrations

In order to evaluate the influence of particles size distribution on polyphenols' release during the decoction and infusion processes, dried olive leaves at 70 °C under N_2 flow were used. Moreover, extraction yields obtained by infusion and decoction processes have been compared to the one obtained by classic ethanol extraction.
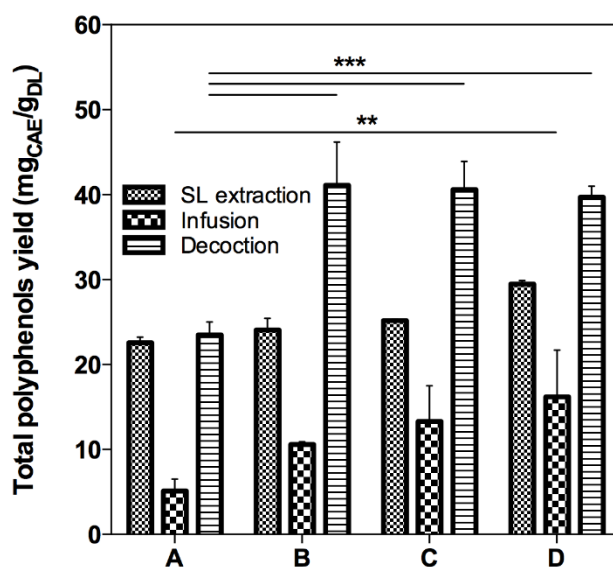

Figure 1: total polyphenol yield at different olive leaves particle size (2.19 < A < 4.76, 1.19 < B < 2.19, 0.84 < C < 1.19 and 0.59 < D < 0.84 mm).

In general, we obtained the same trend for both total polyphenol (Figure 1) and flavonoid (Figure 2) yields, with an increase of extraction yield as a function of the reduction of leaves particle size. Generally, the infusion process resulted in being the one with the lowest extraction yield.
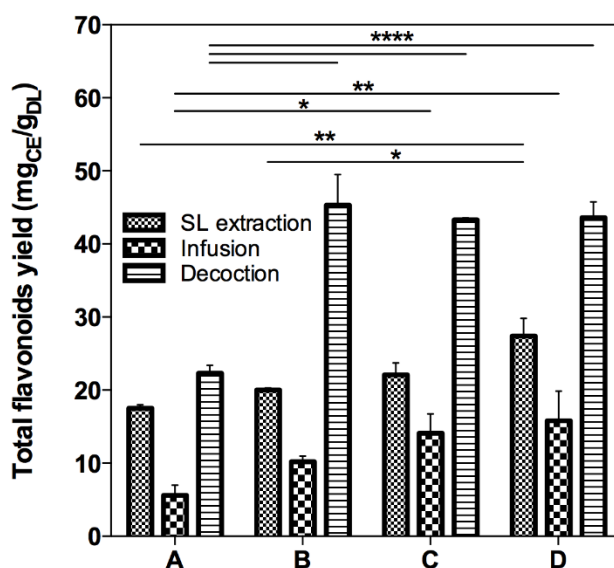

Figure 2: total flavonoid yield at different olive leaves particle size (2.19 < A < 4.76, 1.19 < B < 2.19, 0.84 < C < 1.19 and 0.59 < D < 0.84 mm).

Within each range of particles size, we found out that decoction is the process that allows to extract the highest quantity of total polyphenols and total flavonoids. The difference between this method and the other two is significant for high meshes (corresponding to low particles size), while it decreases going to lower value of mesh.

Table 3: main single phenolic compounds (mg/100g$_{DL}$) HPLC analysis of olive leaves teas obtained using leaves with different particle size (2.19 < A < 4.76, 1.19 < B < 2.19, 0.84 < C < 1.19 and 0.59 < D < 0.84 mm).

	Mesh	Catechin	Vanillic acid	Epicatechin	Ferulic acid	Oleuropein	Apigenin
SL extraction	A	0.11 ± 0.00[a]	0.12 ± 0.00[a,b,c]	0.16 ± 0.01[a]	0.12±0.01[a]	3.00 ± 0.25[a]	0.03 ± 0.00[a]
	B	0.12 ± 0.01[a]	0.14 ± 0.00[a,b,c]	0.18 ± 0.01[a]	0.14±0.01[a]	3.78 ± 0.21[a]	0.04 ± 0.00[a]
	C	0.18 ± 0.02[a,b]	0.13 ± 0.03[a,b,c]	0.19 ± 0.01[a]	0.15±0.01[a]	4.94 ± 0.16[a]	0.03 ± 0.00[a]
	D	0.18 ± 0.03[a,b]	0.14 ± 0.01[a,b,c]	0.25 ± 0.02[a]	0.14±0.01[a]	5.22 ± 0.16[a]	0.05 ± 0.01[a]
Infusion	A	0.28 ± 0.03[a,b,c]	0.07 ± 0.01[c]	0.41 ± 0.04[a,b]	0.14±0.02[a]	2.06 ± 0.19[a]	-
	B	0.31 ± 0.02[d]	0.09 ± 0.01[b,c]	0.46 ± 0.05[a,b]	0.17±0.04[a]	2.58 ± 0.49[a]	-
	C	0.43 ± 0.05[d,e]	0.16 ± 0.02[a,b,d]	0.53 ± 0.06[a,b]	0.24±0.03[a]	4.53 ± 0.52[a]	-
	D	0.50 ± 0.06[d]	0.16 ± 0.03[a,b,d]	0.75 ± 0.12[b,c]	0.27±0.03[a,b]	2.23 ± 0.31[a]	-
Decoction	A	0.25 ± 0.04[a,b,c]	0.15 ± 0.03[a,b,c]	0.40 ± 0.06[a,b]	0.13±0.01[a]	3.50 ± 0.52[a]	0.12 ± 0.02[a]
	B	0.78 ± 0.15[a,b,c]	0.23 ± 0.02[d]	1.28 ± 0.15[d]	0.47±0.08[c]	17.13 ± 2.55[b]	0.61 ± 0.10[b]
	C	0.62 ± 0.09[b,c,e]	0.20 ± 0.04[a,d]	1.00 ± 0.17[c,d]	0.42±0.07[b,c]	15.92 ± 2.81[b]	0.52 ± 0.11[b]
	D	0.78 ± 0.15[c,d,e]	0.19 ± 0.01[a,d]	0.97 ± 0.19[c,d]	0.40±0.06[b,c]	15.54 ± 2.06[b]	0.66 ± 0.10[b]

As concern the single phenolic compounds identified in olive leaves teas (Table 3), we can see that decoction leads to higher amount of oleuropein and apigenin (ten times higher than values obtained using the SL extraction). In general, the infusion process leads to phenolic recovery comparable to SL extraction, except for apigenin that has not been identified in any product.

4. Conclusions

In order to valorise one of the by-products of the olive oil industry, in this study, the effect of the pre-treatment processes on the production of olive leaves infusion and decoction were investigate. Different drying techniques and particles size of olive leaves have been evaluated in order to obtain a product rich in phenolic compounds. Predictably, the extraction yield of total polyphenols and flavonoids increased as a function of the

reduction of leaves particle size. The best drying condition was obtained at 70 °C under nitrogen atmosphere for a short period (7 h). Decoction process led to the higher phenolic compounds recovery regardless of the olive leaves particle size.

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