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Study of Polyphenol Profile and Antioxidant Capacity in Italian Ancient Apple Varieties by Liquid Chromatography

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***Abstract*—**Safeguarding, studying and enhancing biodiversity play an important and indispensable role in re-launching agriculture. The ancient local varieties are therefore a precious resource for genetic and health improvement. In order to protect biodiversity through the recovery and valorization of autochthonous varieties, in this study we analyzed 12 samples of four ancient apple cultivars representative of Friuli Venezia Giulia, selected by local farmers who work on a project for the recovery of ancient apple cultivars. The aim of this study is to evaluate the polyphenolic profile and the antioxidant capacity that characterize the organoleptic and functional qualities of this fruit species, besides having beneficial properties for health. In particular, for each variety, the following compounds were analyzed, both in the skins and in the pulp: gallic acid, catechin, chlorogenic acid, epicatechin, caffeic acid, coumaric acid, ferulic acid, rutin, phlorizin, phloretin and quercetin to highlight any differences in the edible parts of the apple. The analysis of individual phenolic compounds was performed by High Performance Liquid Chromatography (HPLC) coupled with a diode array UV detector (DAD), the antioxidant capacity was estimated using an *in vitro* essay based on a Free Radical Scavenging Method and the total phenolic compounds was determined using the Folin-Ciocalteau method. From the results, it is evident that the catechins are the most present polyphenols, reaching a value of 140-200 µg/g in the pulp and of 400-500 µg/g in the skin, with the prevalence of epicatechin. Catechins and phlorizin, a dihydrohalcone typical of apples, are always contained in larger quantities in the peel. Total phenolic compounds content was positively correlated with antioxidant activity in apple pulp (r^2 = 0,850) and peel (r^2 = 0,820). Comparing the results, differences between the varieties analyzed and between the edible parts (pulp and peel) of the apple were highlighted. In particular, apple peel is richer in polyphenolic compounds than pulp and flavonols are exclusively present in the peel. In conclusion, polyphenols, being antioxidant substances, have confirmed the benefits of fruit in the diet, especially as a prevention and treatment for degenerative diseases. They demonstrated to be also a good marker for the characterization of different apple cultivars. The importance of protecting biodiversity in agriculture was also highlighted through the exploitation of native products and ancient varieties of apples now forgotten.

Keywords Apple, biodiversity, polyphenols, antioxidant activity, HPLC-DAD, characterization.

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I. Introduction

In the second half of the twentieth century, the market has selected apple qualities to satisfy the requests of consumers, choosing the direction of the greatest profit with apples that are easy to keep and more eye-catching. Those that we commonly find in commerce, in fact, are the result of a very narrow selection that, over the years, has reduced to minimum the variety of apples grown for sale. In the world it is estimated there are at least 3000 varieties of apples, so we can realize how in reality the choice we have is really limited [1]. Italy presents a very high biodiversity potential for this fruit, but about ten commercial varieties cover over 70% of the entire market [2]. Although marketing strategies impose these conditions, in every region of Italy, agricultural biodiversity has not diminished and it can boast a generous amount of "forgotten" fruits. In fact, Italy produces over 1000 varieties of apples [1], and it is the third world producer and the seventh largest exporter of this fruit [2].

Biodiversity is invaluable because each fruit has its characteristics genetic and native varieties of a given territory, and therefore it is well adapted to geophysical and meteorological conditions as to be more resistant to parasites present in the area where they grow, thus minimizing pesticide treatments. Each variety has different maturation times; this gives the possibility of having a product without any particular forcing of production and without the need for long storages that reduce nutritional properties [3].

Apple is appreciated for its good taste and for several healthful nutrients it is well known to contain: carbohydrates, pectin, vitamins, microelements, dietary fiber and polyphenols. The main types of polyphenols in apples are flavan-3-ols, flavonols, dihydrochalcones, and phenolic acids [4]. Apple phenolic compounds are secondary plant metabolites that protect plant from pathogens' attacks. They have demonstrated to have many beneficial effects on human health, among others, reduced risk of some cancers, cardiovascular disease, asthma, and diabetes [5]. Genetic and agricultural practices can influence the profile and content of these compounds [6].

The preservation, study and enhancement of biodiversity play an important and indispensable role for relaunching Italian agriculture. The ancient local varieties constitute a valuable resource for genetic and health improvement. In order to protect biodiversity through the recovery and valorization of autochthonous varieties, in this study were analyzed four ancient apple varieties cultivated in Friuli

Venezia Giulia, a region in Northern Italy, with a great tradition in apple production.

II. Materials and Methods

Formic acid, hydrochloric acid, methanol, 1,1-diphenyl-2-picrilhydrazyl (DPPH) and Folin-Ciocalteau reagents, chlorogenic acid, [+]-catechin, [-]-epicatechin, phloretin, phloridzin, p-coumaric acid, gallic acid, caffeic acid, ferulic acid, quercetin and rutin were purchased from Sigma-Aldrich (Steinheim, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA) before use.

A. Sample Collection

The apple samples used in this study are native apple grafts with four different ancient varieties: Bella Donna, Calvilla bianca, Chei da la Fraula and Ruggine dorata, donated by a local farmer from Friuli Venezia Giulia. The samples were collected in late summer 2016, from three different trees for each variety, 2 kg per tree.

B. Sample Preparation

For each sample, approximately 5 g of pulp and peel were weighed separately, properly cut, and immediately homogenized in 10 mL of methanol containing 1% hydrochloric acid (HCl) in ultrasounds at temperature environment for 15 minutes. The organic solution was subsequently removed and inserted in a special container. A second extraction was therefore carried out on the apple sample left in the same way as described above. The volume extracts were finally combined and filtered on a 0.45 µm membrane (Millipore). The extracts were stored at -20 °C until the time of analysis.

C. HPLC Apparatus and Analysis Conditions

The HPLC analysis of polyphenols was performed on a Shimadzu, LC 6A liquid chromatographic system equipped with a degasser, a quaternary pump, a manual injector with a 20 µL loop, a column oven, and a Shimadzu, SPD-M6A diode array detector (DAD). The analytical column was an Altech C18 column (5 µm, 150 × 4.6 mm id, 5 um). The operating conditions were as follows: column temperature 25 °C, flow rate 0.8 mL/min injection volume 20 µL, monitoring wavelength range 190–600 nm. The mobile phase solvents were the following: (A) 1.7% formic acid in methanol and (B) 1.7% formic acid in water. Separation was achieved using the following gradients: 0-15 min 65% B, 20-28 min 20% B, 33 min 65% B. The compounds were monitored at 280 nm for gallic acid, catechin, epicatechin, rutin, phloridzin and phloretin, at 325 chlorogenic acid, caffeic acid, coumaric acid, ferulic acid and quercetin at 370 nm.

D. DPPH Radical Scavenging Activity

Aliquots (200 µL) of extracts were added to 3 mL of DPPH solution (6 × 10–5 mol/L) and the absorbance was determined at 515 nm after 30 minutes.

Results were calculated using the inhibition rate (*I*) of the radical cation, according to:

$$I\ \% = \frac{A0 - Af}{A0}.100$$

with: A0: radical cation initial absorbance, Af: final absorbance of the tested sample after incubation.

The DPPH radical scavenging activity of apple extracts was expressed as micrograms of gallic acid equivalents per gram of fresh peel or pulp (GAE= gallic acid equivalent antioxidant capacity).

E. Determination of Total Phenols Content

Total phenolics content was determined using the Folin–Ciocalteau method according to Singleton et al. [7]. Total phenolics content was expressed as milligrams GA equivalent (GAE) per gram FW.

III. Results and Discussion

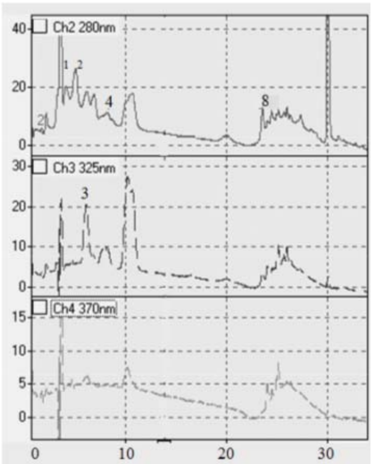
The analysis of the samples was carried out by means of a double extraction in acidified alcoholic solvent and ultrasound treatment in order to favor the extraction of the polyphenols in the organic solvent. The accuracy of the extraction was verified by recovery tests. The values obtained, by standard additions of the analytes to an apple sample, were depending on the compound, between 92% and 98%. In Fig. 1 are displayed chromatograms obtained from the analysis of the pulp and peel of the apple of the Bella Donna variety at the three different wavelengths chosen (280 nm, 325 nm, 370 nm). As can be seen in the peel, unlike the pulp, rutin and quercetin are also present.

From the data shown in Table I, it is evident that, among the 11 polyphenols determined in ancient apples, catechins are the most common, reaching a value of 140-200 µg/g in the pulp and 400-500 µg/g in the peel, with the prevalence of epicatechin. The total values (540-700 µg/g) are in agreement with those reported for 15 commercial apple varieties (100-700 µg/g) [6].

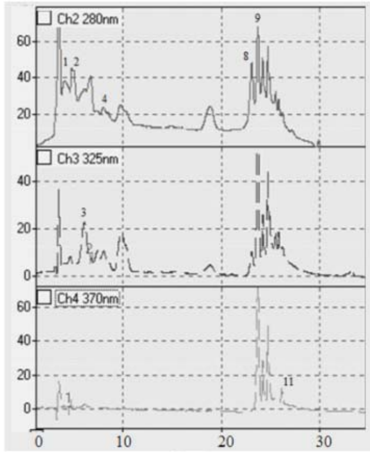
Catechins and floridzine, a typical hydrochalcone of apples, are always contained in greater quantities in the skin [8] and the obtained data confirm it, even if the concentration of florizine is influenced by the degree of maturation (it is greater in unripe apples) [9]. Flavanols, on the other hand, are exclusively present in the peel and the glycosylated, rutin form prevails over the free form, quercetin. The biosynthesis of these compounds is favored by solar UV radiation [10], moreover it depends on the color of the fruit, in fact the red apples have a greater biosynthesis capacity than the green and yellow ones. This is confirmed by the results of the analyses, given that the greater quantity of flavanoids is detected in the "Bella Donna" and "Chei de la Fraula" varieties, which, unlike the "Calvilla Bianca" and “Ruggine Dorata”, have a red brow [11].

Regarding non-flavonoid compounds, among cinnamic acids, chlorogenic acid is certainly the most present and, in the pulp, it is even the second most common compound after flavan-3-oli [12]. The other cinnamic acids, instead, have been found only in specific qualities of apples, for example caffeic acid in the variety "Calvilla" and ferulic acid in the "Chei da la

Fraula"; the coumaric acid was not detected in any of the four apples analyzed.



(a)



(b)

Fig. 1 Chromatogram of a sample of Bella Donna apple, pulp (a) and peel (b) recorded at the three operative wavelengths. Compounds: 1-gallic acid, 2-Catechin, 3-chlorogenic acid, 4-Epicatechin, 5-caffeic acid, 6-coumaric acid, 7-ferulic acid 8-phloridzin, 9-rutin, 10-phloretin, 11-quercetin

TABLE I
POLYPHENOL CONTENTS IN OF BELLA DONNA, CALVILLA BIANCA, CHEI DA LA FRAULA AND RUGGINE DORATA

Comp	Calvilla Bianca (μg/g)		Bella Donna (μg/g)		Chei da la Fraula (μg/g)		Ruggine dorata (μg/g)	
	Pulp	Peel	Pulp	Peel	Pulp	Peel	Pulp	Peel
Gallic	15,4±1,1	21,3± 1,6	17,2 ±1,2	25,5±0,4	12,7±1,6	24,7±0,6	13,6±0,8	21.5±2,6
Catechin	67,1± 0,1	143,2±8,2	107,8±2,9	156±2	51,8±5	125±2,1	90,6±4.3	170±12.8
Chlor	23,8±0,7	51,2±0,1	23,6± 0,8	47,8± 5,3	41,6±4,3	44,7±0,1	80.1±1.8	45.1±9,6
Epicat	138,4±13,4	258,0±9,8	58,6± 4,3	156,2±12,2	82,9±6	271,4±24	270±4.3	298,7±21,2
Caffeic	ND	14,4±5,2	ND	ND	ND	ND	ND	ND
Couma	ND	ND	ND	ND	ND	ND	ND	ND
Ferulic	ND	ND	ND	ND	ND	33,5±1	ND	ND
Phlorid	6,0± 0,5	85,5±1,2	26,2±0,3	131±14	6,5±2,	340,9±32,7	4,7±1.1	5.3±0.9
Rutin	ND	92,9±1,3	ND	176,9±12,2	ND	310±1,3	ND	155,2±18,4
Phlore	ND	ND	ND	ND	ND	ND	ND	ND
Querc	ND	7,9±0,4	ND	23,1±1,2	ND	12,1±1,5	2±0,6	33,7±7.8
∑c	250,7	674,4	233,4	716,5	195,5	1162,3	359,9	660,5

Data are expressed as means of three samples ± SD, ND= not detected.

TABLE II
TOTAL PHENOLICS (MGGAE)/G AND ANTIOXIDANT CAPACITY (UGGAE/G) IN THE FOUR APPLE VARIETIES STUDIED.

	Calvilla Bianca		Bella Donna		Chei da la Fraula		Ruggine dorata	
	Pulp	Peel	Pulp	Peel	Pulp	Peel	Pulp	Peel
Antiox capac (ugGAE/g)	33,5± 3,1	230,8± 8,8	28,8± 4,3	236,3± 9,8	29,5± 4,7	259,8± 12,5	28,9± 3,3	220,0±14,2
Total phenol (mgGAE/g)	1,5± 0,3	3,0± 0,6	1,8± 0,5	2,9± 0,6	1,3± 0,4	5,1± 0,9	1,6± 0,5	2,7± 0,6

Data are expressed as means of three samples ± SD.

Regardless of the variety, the peel has higher polyphenolic content than the pulp in accordance with what reported in literature [13], [14]. In detail, for the variety “Ruggine Dorata”, peel has about 50% more polyphenols than pulp, the varieties "Bella Donna" and "Calvilla Bianca" the content of the peel turns out to be about three times greater, while for the "Chei da la Fraula" it is even six times. This difference may be due to multiple factors, including the type of cultivar, the conditions of cultivation, growth, harvest and ripeness of the analyzed apples [15].

Data from the antioxidant activity test and from the total polyphenols content are in accordance with the results of the HPLC analysis (Table II). Apple peel shows higher antioxidant capacity in comparison to pulp in all the four apples analyzed. Antioxidant capacity for pulp ranged from 28.8 ugGAE/g in Bella Donna apples to 33.5 ugGAE/g in Cavilla Bianca. In peel the highest antioxidant capacity was found in Chei de la Fraula variety while the lowest is in Ruggine dorata.

The trend for the total polyphenols content follows exactly what described for the antioxidant capacity, with Chei de la Fraula peel having the highest content, almost ten times more than its pulp.

The result of the total polyphenol content and of antioxidant capacity was also examined in order to assess their correlation. They were positively correlated in apple pulp (r^2 = 0,850) and peel (r^2 = 0,820) and with the sum of the individual polyphenols determined (r^2 = 0,910). This evidence confirms the strong antioxidant properties of apple polyphenols [16].

IV. Conclusion

In this paper the quantitative analysis of 11 polyphenolic compounds (gallic acid, catechin, chlorogenic acid, epicatechin, caffeic acid, coumaric acid, caffeic acid, ferulic acid, florizine, rutin, floretin, quercetin) was carried out in samples of local apples from Friuli Venezia Giulia and Giulia grafted with four ancient varieties (Bella Donna, Calvilla Bianca, Chei da la Fraula and Ruggine Dorata). The analysis of individual polyphenols was performed by HPLC/DAD with satisfactory levels of performance of the method used in terms of repeatability, sensitivity and robustness. The apple extracts were also subject to test to quantify their antioxidant capacity and total polyphenols content.

Comparison of the results showed differences between the three varieties analyzed and between the edible parts (pulp and peel) of the apple. In particular, it is observed that: The distribution of phenolic compounds in the four varieties is similar but different between the pulp and the peel. The peel of the apples is richer in polyphenolic compounds than the pulp and it is therefore recommended to eat it even if it is often discarded. Flavonols are present exclusively in the peel. Antioxidant capacity values are in good accordance with the results of polyphenols levels, and show how the apple peel consumption can give protection against radicals even ten times more than pulp.

The high content of polyphenols in apples and therefore their beneficial effects on health have been confirmed. Furthermore, this study highlights the importance of protecting agricultural biodiversity through the enhancement of autochthonous products and ancient varieties of now forgotten apples, which represent a great genetic reservoir of beneficial compounds for human health. The interest in functional food, as ancient apples varieties demonstrated to be a promising starting point for re-launching agriculture in small local realities.

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