UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Land, Environment Agriculture, and Forestry

Second Cycle Degree (MSc) in Food and Health

Analysis of Secondary Metabolites in Peel and Pulps of Local Apple Varieties of *Belluno* Region as Valuable Health Promoting Products

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Contents

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Abstract

Apples (Malus domestica Borkh) are cultivated worldwide and are the most widely grown species of Malus. This study aims to identify the secondary metabolites in the peels and pulps of fifteen local apple varieties of the *Belluno* region grown in Northeast Italy. The research will focus on the analysis of secondary metabolites in apple samples like chlorogenic acids, proanthocyanidins, flavonoids, chalcone, and triterpenes by high-performance liquid chromatography (HPLC) coupled with mass spectrometry (LC-DAD-MS). Results showed that peels have the highest content of Proanthocyanidins compared to pulps. Among cultivars, the pulps of *Renetta dei Carmelitani, Gelata Dall'Olo Tardiva, Abbondanza Rosso,* and peels of *Dal Sangue, Pom Prussian, and Dal Sangue* could be highlighted as sources of polyphenols than other varieties. Among these compounds, total triterpenes were in low amounts in all varieties.

Ancient *Belluno* region apples have valuable amounts of polyphenols among different apple cultivars that have been studied, due to their high content of bioactive compounds have made the food and nutrition market interested, thus replacing the existing chemical anti-oxidation ingredients in food to increase the nutritional value and health benefits.

Introduction

1.1 Background

‘An apple a day keeps the doctor away’.

The high participation of fruits and vegetables in the human diet is most important because they neutralize active oxygen species, which is hazardous to health. Plant tissue antioxidant capacity is associated with the activity of ‘‘free radical scavenging enzymes’’ (superoxide dismutase, catalase, peroxidase) and with the contents of antioxidant substances, mainly phenolic compounds, carotenoids, tocopherol, and ascorbic acid (Leja et al., 2003).

Reactive oxygen species are generated in all mammalian cells, partly because of normal intracellular metabolism in mitochondria and peroxisomes, and partly due to the activation of oxidant-producing enzymes in response to exogenous stimuli e.g., radiation, chemotherapeutics, cigarette smoke, and environmental toxins. Excessive accumulation of ROS can cause oxidative damage associated with many degenerative diseases such as aging, atherosclerosis, cancers, diabetes, and Alzheimer’s disease (Chen et al., 2014). The antioxidant defense can be enzymatic or non-enzymatic, as well as a repair system. Different plants contain important natural antioxidants because of their high bioactivity, and extremely low toxicity has been considerably used to prevent oxidative stress (Akbari et al., 2022).

Apple is one of the most popular fruits consumed worldwide, it has a good reservoir of bioactive polyphenols and is processed into various products. The fruit is reported to be harvested in excess (about a million tonnes) in 2020–2021. Data for 2019–2020 report consumption of 21.9 kg of apples per person annually (Gaharwar Let,2023).

Apples had the highest soluble free phenolics when compared to 10 other commonly consumed fruits. The antioxidant capacity of 20 fruits using the oxygen radical absorbance capacity assay (ORAC), ranked apples 8 out of 20 (Khanizadeh et al., 2008).

A pleasant combination of sweet and tart Flavors is a major factor in apples being consumed globally (with a world production of more than eighty million tons) as fresh fruit, juice, dried products, alcoholic beverages, or processed products such as candies and desserts (Kim et al., 2023). Therefore, apple fruits attracted attention in earlier in vitro and experimental studies, which can play a significant role in protecting general health.

1.2 Problem statement

There are up to three thousand apple varieties worldwide, but only a few of these are cultivated commercially, restricting the choice for consumers. Recently, many studies reported the phenolic composition of various apple cultivars grown in different countries or regions such as the Himalayas, Australia, Austria, Bosnia and Herzegovina, China, Italy, Japan, Norway, Poland, and Portugal. The phenolic composition and metabolic profiles of apples may differ from one to another depending on growing location, genetic, and environmental factors, and therefore differ between cultivars of the same species and even between different orchard environments. Additionally, the difference in natural antioxidant composition between cultivars, including phenolics, as well as physicochemical properties also affects the effectiveness of enzymatic browning inhibition in apple products (Arnold & Gramza-Michalowska, 2023). Metabolomic characterization could help map this metabolic diversity and facilitate the selection of healthy cultivars by breeders and consumers (Commisso et al., 2021).

Traditional ancient apple cultivars show good adaptability to the local environment and represent a valuable source for crop genetic variability (Kschonsek et al., 2018).

Ancient apple cultivars are still produced in Italy in local orchards, with one thousand varieties produced in the Italian territory, but about ten commercial varieties cover over 70% of the entire market (Preti R, Tarola A; 2020). Over 80% of the national crop comes from Northeast Italy: the region of Trentino Alto Adige represents the main producer (59.3%), followed by Veneto (13.8%), Emilia Romagna (9.6%) and Friuli Venezia Giulia (1.9%) (Giannetti et al., 2017).

Consumers are becoming increasingly aware of the importance of a diet rich in antioxidants and other beneficial elements, it is important to focus on consuming foods that are naturally grown and rich in bioactive compounds, while avoiding those that contain chemical substances. One way to meet this demand is by reviving ancient apple cultivars due to their unique composition, taste, resistance to pathogens, and adaptability to different climates and soil conditions.

Literature Review

2.1 Apple (Malus domestica Borkh.)

Currently, there are more than 7000 documented apple cultivars in the world. However, global production is dominated by relatively few well-adapted cultivars (‘Fuji’, ‘Gala’, ‘Golden’, ‘Granny Smith’ and ‘Delicious’), many of which are closely related to the detriment of the locally well-adapted apple (Mansoor et al., 2023).

Apple (Malus domestica Borkh.), a fruit from the Rosaceae family with many different varieties, is the most cultivated fruit across the world, ranking second for the total phenolic content after cranberry but having the highest free polyphenolic portion. The botanical characteristics of the apple have been described by depending on the rootstock, the apple tree has 1.5 to 7 m in height, with basal diameter ranges from 1 to 4.5 m. It is a hermaphrodite plant; some cultivars are partly self-fertile, but cross-pollination may also happen. It can also grow in a wide range of soil types. The expected yield varied from 20 to 70 tons/ha, in 3–10 years of full production period, depending on the cultivar or rootstock and site. Based on various estimations, there are over 7500 apple cultivars discovered from various regions in the world, but only around twenty cultivars are widely cultivated in the horticulture industry. Apple grows and is adaptable to various climates, but grows better in moderate humidity and cool temperatures, in the 35–50° latitude (Arnold M, et al; 2023 & Nkuimi Wandjou et al., 2020).

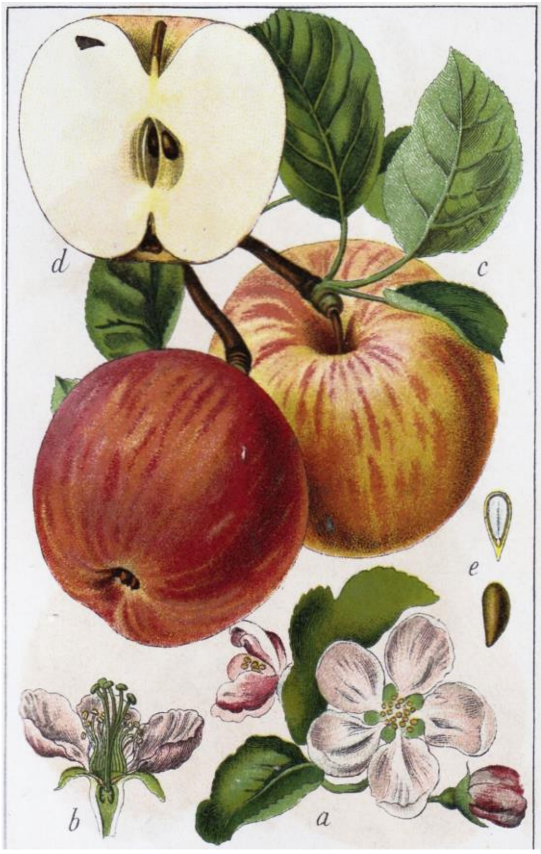

Fig 1: Botanical painting of Malus domestica, Jacob Sturm, 1796

2.1.1 Apple peel

In our diet, consumption of apple fruit or its products are a rich source of natural antioxidants and give up to 22% of total dietary phenolics. Apple peel has approximately 3- to 6-times higher flavonoids as compared to the flesh of an apple and apple peel also has unique flavonoids like quercetin glycosides, which is a richer amount compound in the apple flesh. The extract obtained from apple skin possesses strong antioxidant ability (Khalid et al., 2021). The compounds most commonly found in apple peels consist of procyanidins (mainly constituted by (−)-epicatechin, (+)-catechin, chlorogenic acid, phloridzin, and quercetin conjugates. Quercetin conjugates are found much more in the peel of apples (Karaman et al., 2013).

2.1.2 Apple pulp

In apple pulp, there is some (+)-catechin, procyanidins, (−)-epicatechin, and phloridzin, but these compounds are found in much lower concentrations than in the peels. Chlorogenic acid tends to be higher in the pulp than in the peel (Karaman et al., 2013).

2.2 Secondary metabolites

2.2.1 Polyphenol

Apples are an important source of polyphenols in the Western diet. Polyphenols are secondary plant metabolites that exhibit high antioxidant potential. Phenolic compounds in apples are of great importance for the tree, because they are involved in the natural defensive reactions of apples against various diseases and they act as stress-protective agents (Zupan et al., 2013). Polyphenols found in apples are of two classes: flavonoids (flavan-3-ols, dihydrochalcones, flavonols, anthocyanins) and phenolic acids (hydroxycinnamic acids) (Stefova et al., 2019). To this structurally diverse group of compounds different health benefits are attributed. The main apple phenolics belong to the subgroups hydroxycinnamic acid derivatives and flavonoids: dihydrochalcone-glycosides, flavanols, flavonol-glycosides, and anthocyanin-glycosides. Although phenolic structures, identified in different apple varieties are quite similar, marked differences in profiles and total contents have been reported (Kaeswurm et al., 2023).

It is known that phenolic compounds occur in plants in the form of glycosides or esterified with carboxylic acids, and apples have the highest number of free phenolics, among other edible fruits (Geană et al., 2021).

There are two classes of phenolic acids which include hydroxybenzoic acids (C6–C1), derived from benzoic acid, and hydroxycinnamic acids (C6-C3) which consist of a benzene ring coupled to a prop-2-enoic acid residue (-CH-CH-COOH). Both classes can be modified by hydroxylation (i.e. mono-, di-, or trihydric) and/or methoxylation of the aromatic ring. The functional activity of phenolic acids is dependent upon the degree and arrangement of hydroxylation and methylation on the aromatic ring. When caffeic acid is esterified to quinic acid (e.g., chlorogenic acid), in vitro antioxidant activity decreases whereas the dimer of caffeic acid demonstrates strong DPPH scavenging activity. Phenolic compounds exhibit excellent abilities to reduce and eliminate free radicals thereby providing antioxidant and anti-lipid peroxidation properties. One of the polyphenol mechanisms is the removal of free radicals by supplying hydrogen atoms or separate electrons from the phenol group and eliminating related enzymes, thereby preventing the production of free radicals and their intermediate products. Additionally, phenolic compounds can react with metal ions to inactivate the Fenton reaction. Understanding the range and distribution of phenolic acids in a food is critical to understanding the functionality of a food (Lee et al., 2017 & Li et al., 2021).

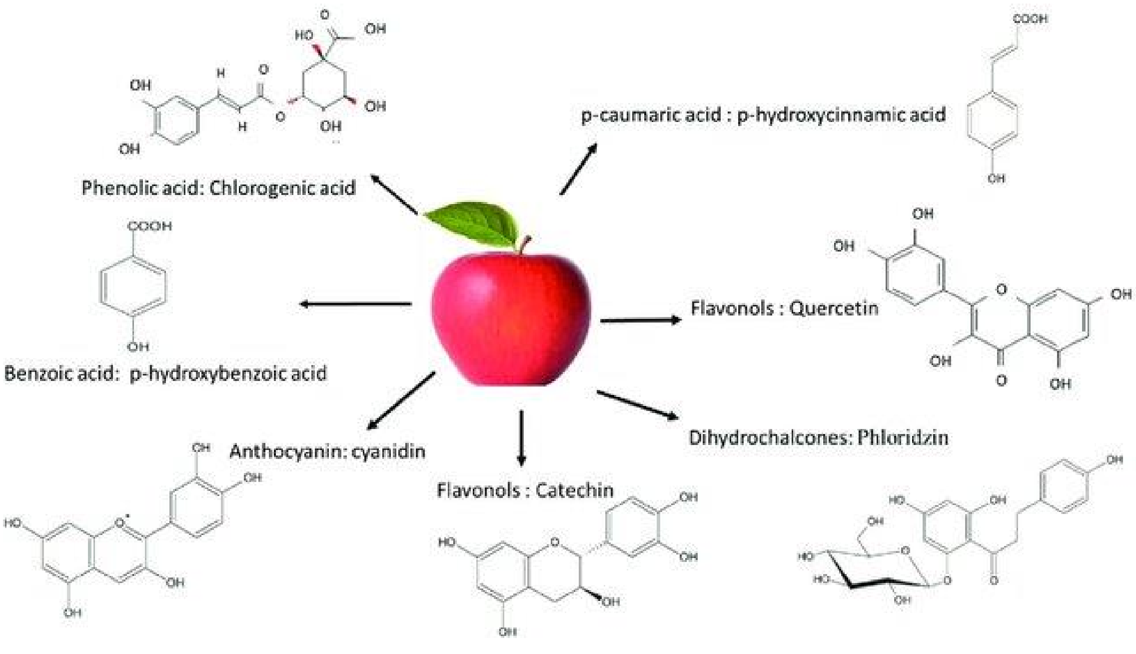

Fig 2: Chemical structures of Polyphenols

2.2.1.1 Flavonoids

Flavonoids are a very diverse group of polyphenolic compounds consisting of structurally related molecules having flavan, flavanone, flavone, isoflavone or chalcone skeleton recognized as an aglycone part of the molecule (Stefova et al., 2019).

Flavonoids can interact with other nutrients. Taking them together with oil increases the flavonoid bioavailability, and the oils enable the flavonoids to take place in the micelle composition. These micelles increase bile salt secretion and intestinal absorption of flavonoids. Flavonoids undergo metabolic transformations in the small intestine, liver, and kidney following intestinal absorption. Unabsorbed flavonoids remaining in the proximal intestine are further digested in the colon by microorganisms that can cleave their heterocyclic oxygen-containing rings, and the hydroxylated phenyl carboxylic acids formed can be absorbed. The highest plasma flavonoid concentration depends on the ingested flavonoid type but is usually reached 1-2 h after ingestion of food (Koseoğlu & Al-Taie, 2022).

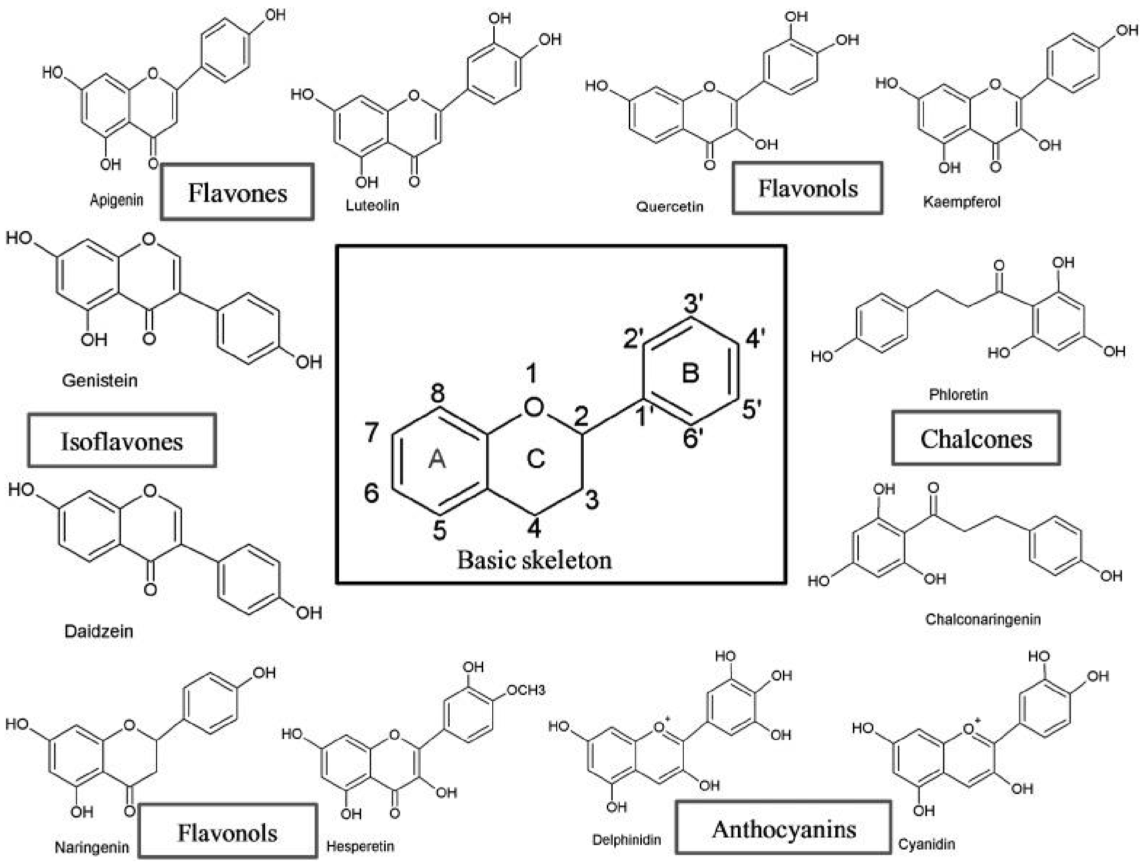

Fig 3: Structure of Flavonoids and their classes

2.2.1.2 Flavan 3 ols

Several literature reports documented the beneficial health effects of some apple phytochemicals. For instance, procyanidins from the flavan-3-ols class can suppress the production of reactive oxygen species (ROS) and the expression of pro-inflammatory agents by the inhibition of some pathways such as the NFκB one in vitro study (Nkuimi Wandjou et al., 2020). The functions of flavan-3-ols in plant physiology range from scavenging of free radicals to growth-regulating activity, from protein precipitation to DNA protection, and from hydration to impregnation of macromolecules of the cell wall (Inderjit et al., 1999). Flavan-3-ols (fig 4) constitute a flavonoid subclass naturally present in food as monomers catechin, epicatechin (C), and epicatechin (EC) oligomers, polymers (proanthocyanidins), and other derived compounds (such as theaflavins and thearubigins) most important antioxidants in both apple peel and flesh (Tsao et al., 2005 & Márquez Campos et al., 2020).

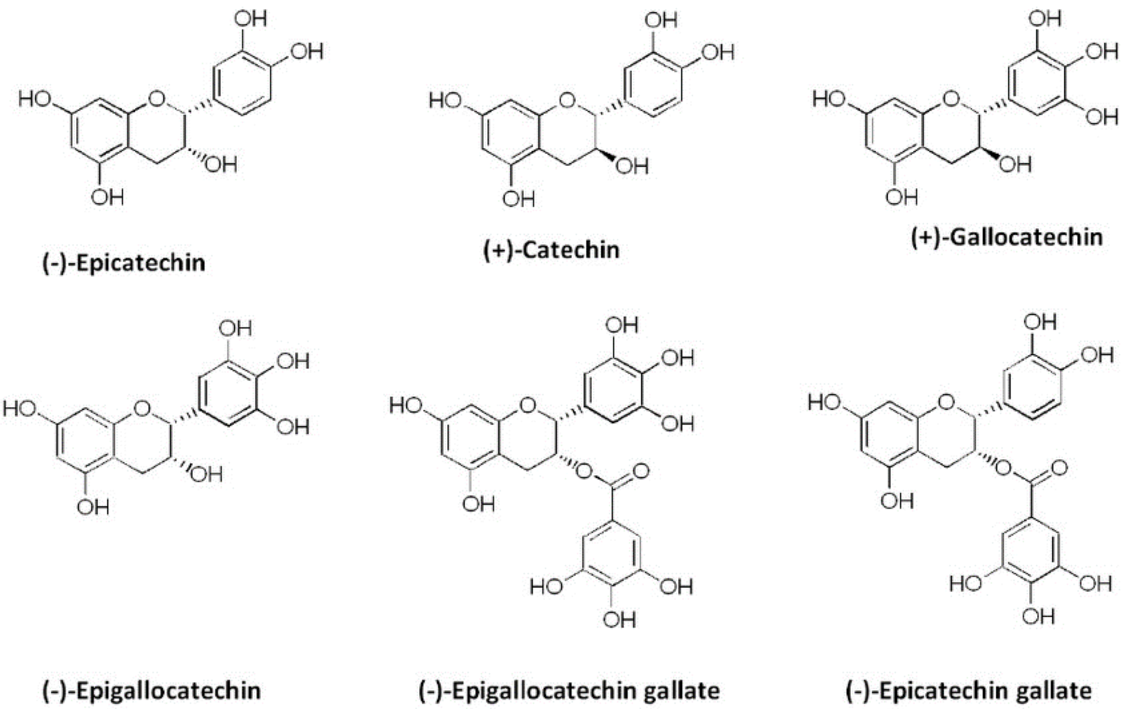

Fig 4: Chemical structures of Flavan-3-ols derivatives

2.2.1.3 Dihydrochalcones (DHCs)

Dihydrochalcones (mainly phloridzin, sieboldin, trilobate, and phloretin)(fig 5) represent the major flavonoid subgroup in apple green tissues. Although this class of phenolic compounds is found in huge amounts in some tissues (≈200 mg/g of leaf DW), their physiological significance remains unclear (Gaucher et al., 2013).

DHCs play an important role in plants against either biotic or abiotic stresses. Various studies have described the potential benefits of DHCs in human health, especially because of their antioxidant properties. Indeed, specific DHC could be effective in treating different human physiological disorders, notably diabetes, cancers, bone resorption, hypertension, and free-radial-involving disease, by inhibiting the formation of AGEs (advanced glycation end products)(Zhou et al., 2018).

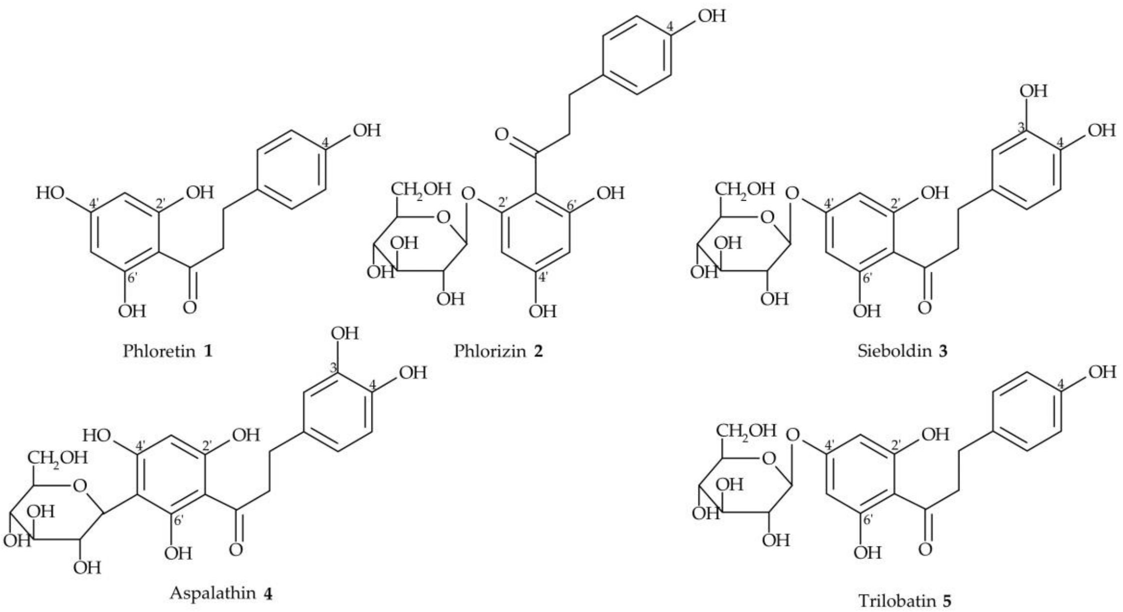

Fig 5: Chemical structures of Dihydrochalcones derivatives

2.2.1.4 Flavonols

Flavonols (fig 6) are one of the most widespread flavonoids in plants and the most prominent in foods are quercetin and kaempferol. Some of them have shown potential effects as inhibitors of some digestive enzymes (amylases, glucosidases, lipases) in the regulation of serum sugar levels. Furthermore, quercetin known as rutin appears to modulate some of the harmful effects associated with obesogenic diets and it has been associated with adipocyte browning (Millán-Laleona et al., 2023). Several lines of evidence suggest their protective role against damages induced by excessive UV and visible radiation (Merzlyak et al., 2005).

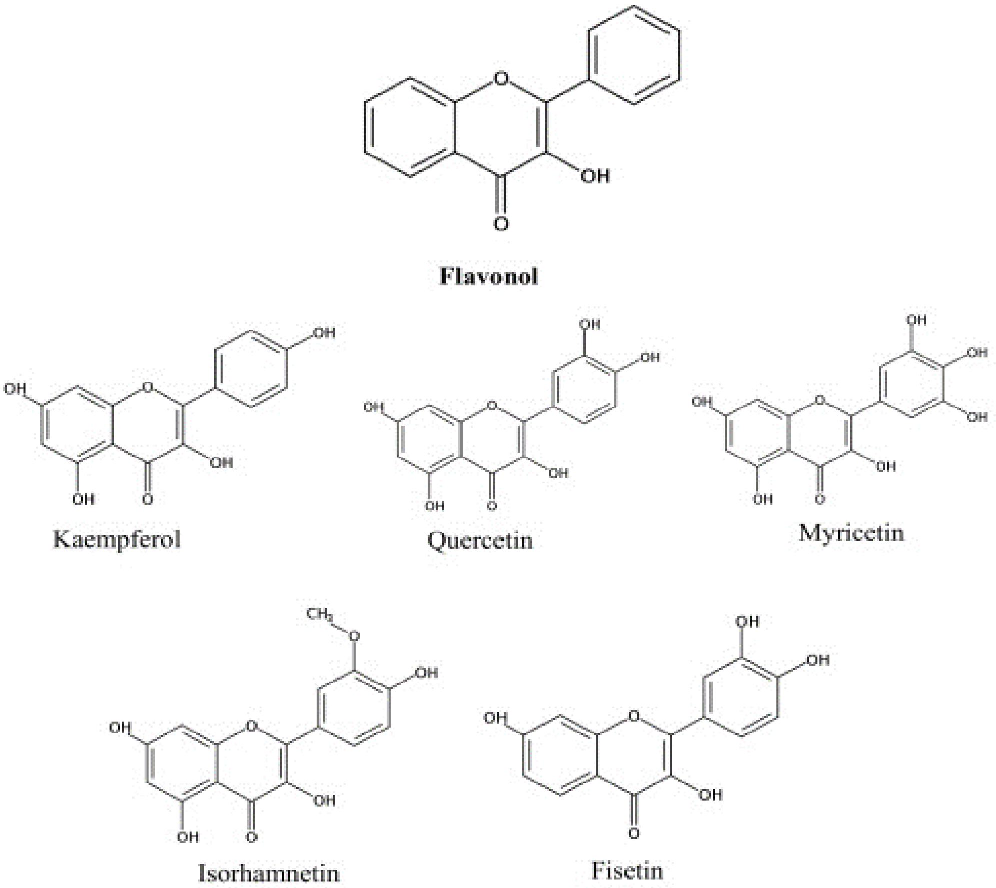

Fig 6: Chemical structures of flavonol derivatives

2.2.1.5 Anthocyanins

Anthocyanins (of the Greek anthos = flower and kianos = blue) (fig 7) are the most important pigments of vascular plants (Castañeda-Ovando et al., 2009). There is a huge variety of anthocyanins spread in nature based on an aromatic ring [A] bonded to a heterocyclic ring [C] that contains oxygen and also bonded by a carbon-carbon bond to a third aromatic ring [B] that exists alongside a variety of monosaccharides, including glucose, rhamnose, galactose, and xylose, and disaccharides consisting of rhamnose, gentian disaccharide, and sophora disaccharide to form glycosides (Liu et al., 2021 & Mena et al., 2014).

Anthocyanins protect plants from biotic attack and attract insect pollination; they also play roles in the response to biotic and abiotic stressors, scavenging oxygen-free radicals, and protecting plants from high light intensity. Anthocyanins have been recognized for their nutritional and health benefits in anti-cancer, anti-disease, and antioxidant activities (Gao et al., 2021).

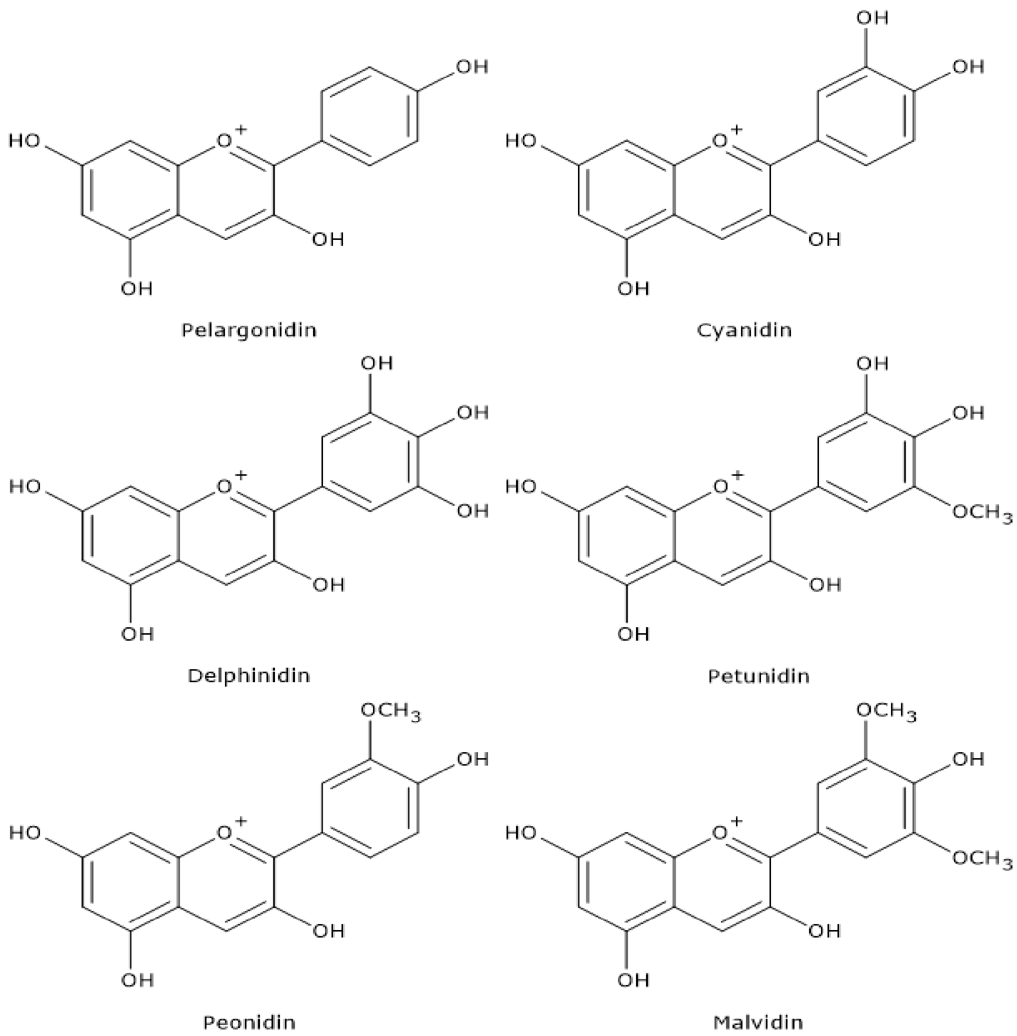

Fig 7: Chemical structures of anthocyanin derivatives

2.3 Bioactivity of apple polyphenols

2.3.1 Cardiovascular Disease (CVD)

Accumulating data indicate that certain dietary flavonoids, such as quercetin and epicatechin, positively impact cardiovascular health via effects on nitric oxide (NO) bioavailability, endothelial function, and blood pressure. Hypertension is the leading risk factor for CVD; endothelial dysfunction is an early event in the development of hypertension and CVD; and reduced NO bioavailability is closely connected to endothelial dysfunction and hypertension so lower risk of CVD with higher apple consumption could be mediated by the beneficial effect of apple skin on endothelial function, both acutely and chronically (Weichselbaum et al., 2010). The meta-analysis of epidemiological studies shows an inverse association between flavonol (together with flavone) intake and coronary heart disease and stroke (Perez-Vizcaino & Duarte, 2010).

2.3.2 Asthma and Pulmonary Function

The study surveyed nearly 600 individuals with asthma and 900 individuals without asthma in the United Kingdom about their diet and lifestyle. Total fruit and vegetable intake was weakly associated with asthma, and apple intake showed a stronger inverse relationship with asthma (Butland, 2000). Apple intake reduces pro-inflammatory cytokines in plasma thereby lowering airway inflammations and neutrophils in asthmatics. Gut microflora also plays a crucial role in the immune response to diet in asthma patients. Apple consumption leads to the production of metabolites with immunomodulatory effects such as short-chain fatty acids including ω-3 fatty acids which promote healthy gut functioning (Denis et al., 2013).

2.3.3 Anti-obesity and anti-diabetic effects

Obesity is a state of metabolic dysfunction that can lead to dyslipidemia and impaired glucose homeostasis. Apple polyphenols have been shown to ameliorate dyslipidemia/metabolic dysfunction in humans. The combination of obesity and obesity-triggered insulin resistance strongly contributes to the development of type 2 diabetes. In vitro, blood glucose-lowering action by apple (poly)phenols is well-documented, particularly in inhibiting carbohydrate-hydrolyzing enzymes, such as α-glucosidase and α-amylase enzymes (Yu et al., 2023). Supplementation of apple polyphenols (80% total polyphenols and 5% phloridizine) also demonstrated a reduced adipose mass in high-fat diet-fed mice. The gene expressions of leptin and adipose-specific genes were changed by apple polyphenols and correlated with their methylation patterns in adipose tissue (Lai et al., 2015).

2.3.4 Antiinflammation

Inflammation is involved in several stages of diseases such as asthma, diabetes, CVD, neurodegenerative diseases, or cancer. Genetic and environmental factors, such as diet, play an important role in inflammation. Flavonoids have anti-inflammatory properties through different mechanisms such as inhibition of regulatory enzymes and transcription factors that have an important role in the control of mediators involved in inflammation. The high antioxidative potential of phenolic compounds is a result of their structure; multiple double bonds as well as oxidation-prone hydroxyl and carbon groups allow them to accept several electrons per molecule. Consequently, flavonoids such as quercetin are probably the most investigated flavonoids that have a deep impact on several immune cells and immune mechanisms that are important in the inflammatory processes (Maleki et al., 2019, Woźniak et al., 2023 &Chagas et al., 2022).

2.3.5 Hepatoprotective

Liver diseases including viral hepatitis and autoimmune hepatitis represent major threats to human health worldwide. Apple polyphenol has a significant protective effect against acute hepatotoxicity (Wang et al., 2016). Liver fibrosis and cirrhosis is the main cause of the incidence of liver cirrhosis and hepatocellular carcinoma, Numerous halogenated chemicals like carbon tetrachloride (CCl4) have potential hepatotoxic effects due to cellular damage via oxidative stress and are used as substances to induce injuries in invivo model, pretreatment with phloretin, the main apple dihydrochalcone, significantly decreased CCl_4 -induced inhibition of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactic dehydrogenase (LDH) activities. Owing to its potent antioxidant and free radical scavenging activities, quercetin ameliorated (CCl_4)-induced oxidative stress and reduced levels of reactive oxygen species (ROS) and CYP2E1 expression in mice liver (Yousefi-Manesh et al., 2020).

Research Methodology

The separation of flavonoids from complex biological extracts often requires techniques such as high-performance liquid chromatography (HPLC) coupled with methodologies for detection and characterization (e.g., electrochemical, fluorescence, UV-vis, mass spectrometry).

This study was carried out to characterize extracts from dried pulp and peel of fifteen samples: Ruggine Rosso, Resertia Rosso, Abbondaza Rosso, Reneita Champagne, Dal Sangue, Roggine Dorata, Pom Prossian, Siror, Van. Renetta dei Cormehtam, San Baril Rosso, Bella Di Bosco Rossa, Abbondanza, Madonna D'agost, Ferrocesio, Gelata Pom Dall'oloTardivo of the apples (malus domestica) which are cultivated in Vecio Pomez were purchased from Belleuno region of the north in Italy. The samples were analyzed by High-Performance Liquid Chromatography coupled with a photodiode array detector and mass spectrometry (LC-DAD-MS).

3.1 Sample preparation

Apples of fifteen ancient varieties were collected in the Belluno region in Vecio Pomez. Samples were provided dried and each variety was obtained from pulps and peels (30 samples, Fig 8).

For preparing the stock of solvent, 500ml methanol and added 500 H_2O, 300 ml Formic acid 3%. 30-grained dried samples (1-15) related to pulps and peels, weighted,500 mg of samples transferred to Erlenmeyer. Added 25 ml solvents to every sample and ultrasound at room temperature for 15 min, Ultrasound can reduce the operating temperature of extraction for thermolabile compounds; moreover, the cavitation process that occurs during sonication causes the rupture of cell walls, thus enhancing solvent contact with available extractable cell material (Acquavia et al., 2021).

Take 1.5 ml from every sample in a microtube and centrifuge for 5 minutes with 3000 RPM. For the final step, take 1 ml from samples and then transfer them in vials for analysis by HPLC and Mass spectroscopy (Fig 9).

Fig 8: fifteen dried pulps and peels of Malus domestica

Fig 9: Step of sample preparation

Table 1: Sample Names

Sample name	Sample number	
	Pulps	**Peels**
Gelata Dall'Olo Tardiva	843	837
Ferrocesio	844	838
Madonna D'Agost	845	839
Abbondanza	846	840
Bella Di Bosco Rossa	847	841
San Baril Rosso	848	842
Ruggine Rosso	915	924
Reseretia	916	925
Abbondanza Rosso	917	926
Renetta Champagne	918	927
Dal Sangue	919	928
Ruggine Dorata	920	929
Pom Prussian	921	930
Siror	922	931
Renetta dei Carmelitani	923	932

3. 2 Instruments for measuring

An Agilent Eclipse SB C18 (50×4.6 mm) 1.8 µm was used as a stationary phase for analytical procedures. For LC-DAD-MS analysis an Agilent 1260 chromatograph equipped with a 1260 series diode array was used (Agilent, Santa Clara, CA, USA)(fig10). After the chromatography column, a “T” junction split the flow to DAD and Varian MS 500 mass spectrometer (Varian, Santa Clara, CA, USA). MS spectra were acquired using Electrospray (ESI) for polyphenols and triterpenes. For analyses, the spectrometer operated in negative ion mode acquiring spectra in the range *m/z* 50–1500.

Fig 10: HPLC & MASS Spectroscopy

3.3 Chemicals

Methanol, acetonitrile, formic acid, rutin, and phloridzin were obtained from Sigma-Aldrich (Milan, Italy). Procyanidin B2 and oleanolic acid were obtained from Extrasynthese (Genay Cedex, France).

3.4 Polyphenolic Analysis in Apple Couple HPLC

Separation of Polyphenols was achieved on an Agilent SBC18 (50×4.6 mm) 1.8µm using 1 %Formic acid in H2O (A), Acetonitrile (B), and Methanol (C) as mobile phase. A gradient program was used as follows: 0 ⟶ 0.5th min: A:B: C (95:5:0) ⟶ A:B: C (95:5:0), 0.5⟶5th min: A:B: C (95:5:0)⟶ A:B: C (85:15:0), 5⟶15th min: A:B: C (85:15:0) ⟶ A:B: C (60:30:10), 15⟶20th min: A:B: C (60:30:10) ⟶ A:B: C(20:70:10), 20⟶25th min: A:B: C (20:70:10) ⟶ A:B: C(0:90:10), 25⟶30th min: A:B: C (0:90:10) ⟶ A:B: C(0:90:10), 30⟶31st min: A:B: C (0:90:10) ⟶ A:B: C(95:5:0), 31 ⟶37th min: A:B: C (95:5:0)⟶ A:B: C(95:5:0). Flow rate was 0.750 ml/min. After the chromatography column, a "T" junction split the flow to DAD and Varian MS 500 mass spectrometer with ESI as an ion source.

3.5 DAD Detector and MASS Spectroscopy

For qualitative purposes, MSn spectra were used for identification of the compound, with the use of reference when available. For quantification, the DAD detector was set at 287 nm. Compounds were identified based on MS data and UV spectra and classified into four classes namely proanthocyanidins, chlorogenic acid derivatives, flavonoids, and chalcones. For quantification of these classes of compounds, the following reference compounds were used namely prontocyanidin B_2 (PAC B_2), Chlorogenic acid, Rutin, and Phloridzin. Standard solutions were prepared for the calibration curves in the concentration range of 9, 18, 45, 90 µg/mL.

Discussion & Results

4.1 Secondary Metabolite Fingerprinting of Apple

Phenolic compounds were identified in the extracts of the peel and pulp of apple fruits. Qualitative characterization was performed by comparison of UV spectra and mass spectra of each peak. For the analysis of apple compounds, a comprehensive characterization strategy was employed, utilizing retention times (m/z), along with a comparison to authentic standards based on the accurate mass of pseudomolecule $[M - H]^-$ ions for instance in Table 2 reported the identification of flavonoids and chalcone respectively according to Rutin, Quercetin galactoside, Quercetin hexoside, Quercetin pentoxide, Quercetin 3(2''acetyl hexoside), Quercetin hexoside, Phloretin-2-xyloglucoside, Quercetin rhamnoside, Rhamnetin glucoside, Phloretin-2-*O*-glucoside as standard compound. The rutin showed $[M - H]^-$ at m/z 609. For Quercetin galactoside the molecular ion $[M - H]^-$ was observed at m/z 463. Phloretin-2-xyloglucoside yielded MS^2 ion at m/z 567. Fragmentation data for each compound are presented and obtained by MS^2 from $[M - H]^-$ ions.

As depicted in Table 3 Flavonoids and chalcones in peels of all types of apples ranged from a retention time of 9.1 to 10.49 min significantly lower (0-0.05 mg/gr) in comparison with retention time at 10.49 and 10.7min (0.019-0.316 mg/gr). In addition, in retention time of 10.9 and 11 min, these amounts are 0 mg/gr in most types of apples. Following the results in pulp, the findings of these studies about Flavonoids and chalcones in pulps are more abundant than in peels. The total amounts of Flavonoids and chalcones in all types of apples in retention times of 9.3, 10.49, 10.7, and 11.6 min are respectively 11.187, 8.869 & 7.142, and 6.324 mg/gr which indicate that highest amounts. The peels of varieties Ferrocesio and Dal sangue have more content among fifteen apples.

The total phenolic content was compared with the literature. Some of the evaluated varieties have a similar total phenolic content as described previously for Himalayan crab apple *M. domestica* (25 – 43 mg GAE/g) (Dadwal et al., 2023), *e.g.*, Dal Sangue, Ferrocesio, and Siror. On the other hand, variety Madonna D'Agost showed the lowest total phenolic content. The peels have higher concentrations of these compounds as compared to pulp, similarly as it was reported previously (Sut et al., 2019). Other studies were focused on the evaluation of the total phenolic content of *M. domestica*, but the results were expressed as mg/100 g or mg/g of fresh weight (Preti & Tarola, 2021 & Bahukhandi et al., 2018) therefore, we are not able to compare the total phenolic content in this case.

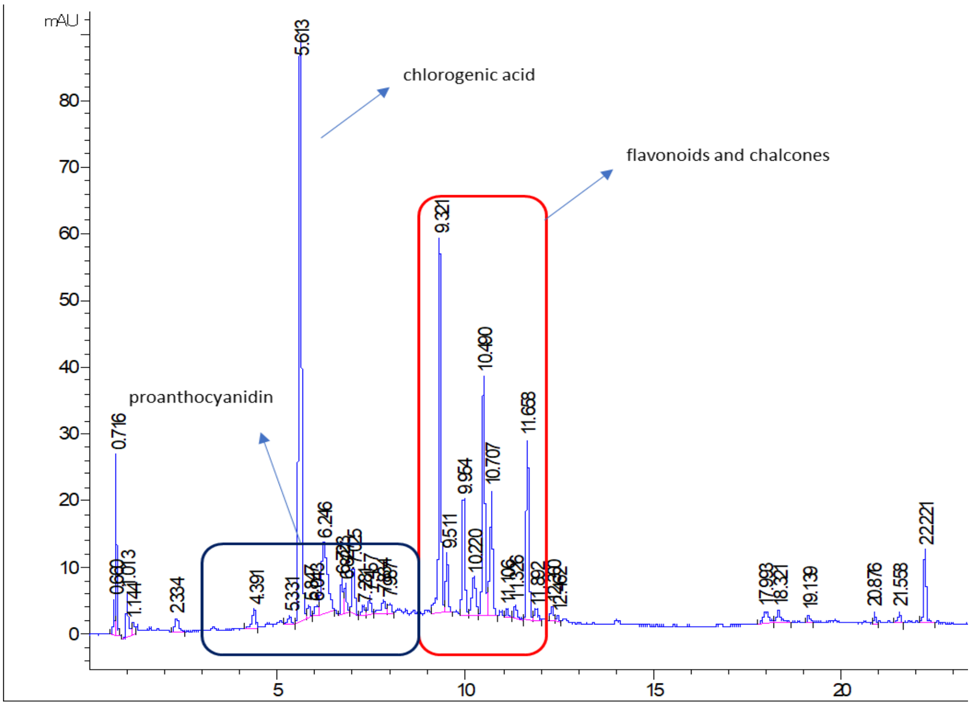

Fig 11: Exemplificative chromatogram at 287nm of Abbondanza peels, PAC, chlorogenic acid flavonoid, and chalcones are highlighted

Table 2: Identification of Phenolic Compounds in Apple according to their m/z value by using their spectral characteristics negative ions in HPLC-MS.

n.	RT (min)	$[M{-}H]^-$	Mass fragments	Compound
1	9.1	609	301	Rutin
2	9.3	463	301	Quercetin galactoside
3	9.5	463	301	Quercetin hexoside
4	9.9	433	301	Quercetin pentoxside
5	10.2	505	301	Quercetin 3(2''acetyl hexoside)
6.1	10.49	433	301	Quercetin hexoside
6.2	10.49	567		Phloretin-2-xyloglucoside
7	10.7	447	301	Quercetin rhamnoside
8	10.9	477	315	Rhamnetin glucoside
9	11	477	315	Rhamnetin glucoside
10	11.6	435		Phloretin-2-*O*-glucoside

Table 3: Flavonoids and chalcones in apple pulps and peels (mg/g)

RT (min)		9.1	9.3	9.5	9.9	10.2	10.49	10.49	10.7	10.9	11	11.6
Ion		**609**	**463**	**463**	**433**	**505**	**433**	**567**	**447**	**477**	**477**	**435**
Compound		**1**	**2**	**3**	**4**	**5**	**6.1**	**6.2**	**7**	**8**	**9**	**10**
Pulps	**Gelata Dall'Olo Tardiva**	0.007	0.022	0.016	0.010	0.001	0.015	0.139	0.038	0.000	0.000	0.029
	Ferrocesio	0.006	0.018	0.007	0.005	0.002	0.011	0.143	0.123	0.000	0.000	0.012
	Madonna D'Agost	0.000	0.002	0.005	0.004	0.003	0.002	0.019	0.016	0.000	0.000	0.002
	Abbondanza	0.001	0.020	0.041	0.023	0.001	0.029	0.051	0.057	0.000	0.000	0.022
	Bella Di Bosco Rossa	0.003	0.004	0.015	0.017	0.000	0.018	0.180	0.083	0.000	0.000	0.065
	San Baril Rosso	0.001	0.019	0.006	0.007	0.001	0.011	0.068	0.082	0.001	0.000	0.006
	Ruggine Rosso	0.002	0.001	0.015	0.022	0.002	0.046	0.124	0.077	0.007	0.000	0.044
	Reseretia	0.006	0.010	0.027	0.010	0.001	0.032	0.147	0.118	0.000	0.000	0.015
	Abbondanza Rosso	0.002	0.015	0.063	0.044	0.001	0.050	0.056	0.082	0.000	0.000	0.083
	Renetta Champagne	0.004	0.001	0.031	0.023	0.001	0.031	0.155	0.103	0.000	0.000	0.032
	Dal Sangue	0.014	0.007	0.020	0.008	0.002	0.019	0.097	0.100	0.000	0.000	0.009
	Ruggine Dorata	0.004	0.000	0.036	0.039	0.010	0.051	0.308	0.186	0.000	0.000	0.067
	Pom Prussian	0.005	0.012	0.019	0.013	0.000	0.028	0.110	0.141	0.000	0.000	0.018
	Siror	0.007	0.004	0.035	0.017	0.001	0.024	0.123	0.119	0.000	0.000	0.008
	Renetta dei Carmelitani	0.010	0.020	0.061	0.012	0.019	0.012	0.071	0.316	0.000	0.000	0.012
Peels	**Gelata Dall'Olo Tardiva**	0.135	0.206	0.150	0.107	0.001	0.178	0.622	0.125	0.013	0.033	0.368
	Ferrocesio	0.609	1.839	0.672	0.429	0.157	0.698	0.846	0.799	0.020	0.002	0.120
	Madonna D'Agost	0.017	0.216	0.045	0.097	0.005	0.197	0.212	0.231	0.002	0.001	0.047
	Abbondanza	0.058	1.027	0.211	0.350	0.060	0.678	0.187	0.394	0.088	0.030	0.307
	Bella Di Bosco Rossa	0.247	0.986	0.389	0.238	0.021	0.443	0.655	0.388	0.053	0.004	0.810
	San Baril Rosso	0.040	0.807	0.233	0.352	0.113	0.588	0.636	1.099	0.035	0.016	0.206
	Ruggine Rosso	0.016	0.159	0.070	0.085	0.001	0.198	0.687	0.182	0.010	0.002	0.616
	Reseretia	0.069	0.871	0.361	0.215	0.041	0.627	0.777	0.337	0.057	0.043	0.341
	Abbondanza Rosso	0.060	0.869	0.250	0.429	0.041	0.761	0.274	0.422	0.106	0.034	0.426
	Renetta Champagne	0.120	0.351	0.260	0.133	0.001	0.277	0.390	0.300	0.022	0.012	0.087
	Dal Sangue	0.280	1.377	0.623	0.360	0.008	0.730	0.576	0.522	0.055	0.138	0.095
	Ruggine Dorata	0.003	0.225	0.154	0.187	0.010	0.330	1.275	0.256	0.000	0.000	1.044
	Pom Prussian	0.126	1.370	0.298	0.387	0.009	0.630	0.642	0.680	0.006	0.001	0.072
	Siror	0.068	0.577	0.312	0.202	0.030	0.548	0.803	0.414	0.043	0.047	0.293
	Renetta dei Carmelitani	0.044	0.307	0.200	0.103	0.007	0.259	0.287	0.175	0.005	0.006	0.084

4.1.1 Pulp composition

Total polyphenols, triterpenes, and anthocyanidins in apple samples were measured by LC-DAD-MS, and quantitative data are reported in tables and figures below. According to Fig 12 and Table 4, Proanthocyanidins are the most abundant compounds compared to the other phenolic compounds. Chlorogenic acid derivatives (6.4mg/gr) and polyphenols (10.3 mg/gr) are most abundant in the pulps of the variety Renetta *dei Carmelitani.* Moreover, *Abbondanza Rosso* is the second variety richer in total phenolic compounds (9.2 m/gr).

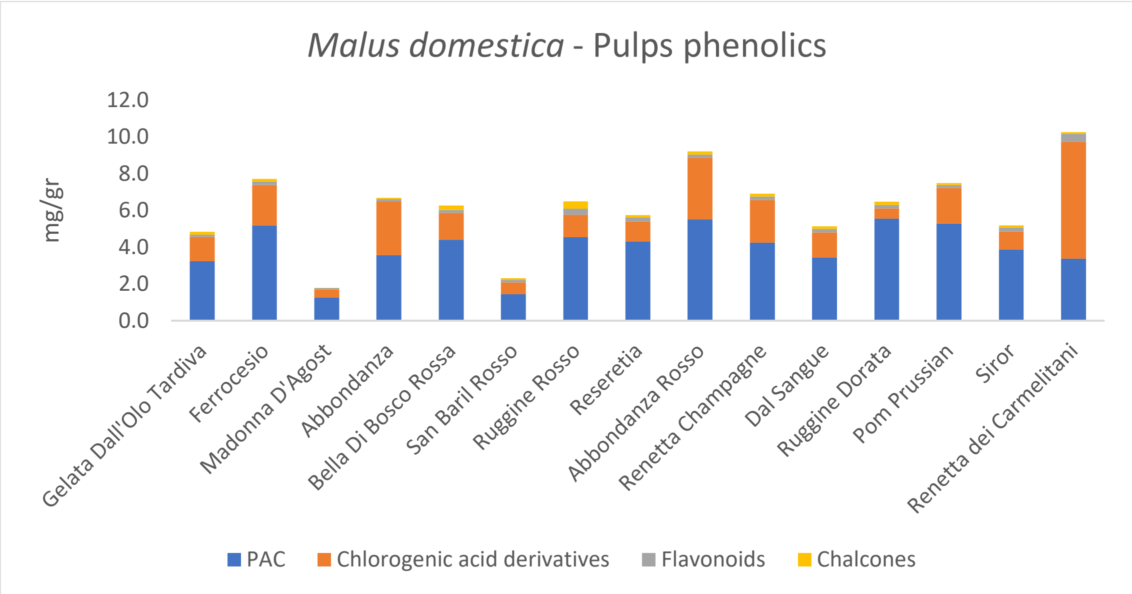

Fig 12: Total proanthocyanidin, chlorogenic acid derivatives, flavonoid, and chalcones in apple pulps

Table 4: Total proanthocyanidin, chlorogenic acid derivatives, flavonoid, and chalcones in apple pulps.

Apple Variety	**PAC mg/g**	**Chlorogenic acid derivatives mg/g**	**Flavonoids mg/g**	**Chalcones mg/g**	**Total polyphenols mg/g**
Gelata Dall'Olo Tardiva	3.2	1.3	0.1	0.2	4.8
Ferrocesio	5.2	2.2	0.2	0.2	7.7
Madonna D'Agost	1.3	0.5	0.1	0.0	1.8
Abbondanza	3.6	2.9	0.1	0.1	6.7
Bella Di Bosco Rossa	4.4	1.5	0.2	0.2	6.3
San Baril Rosso	1.4	0.6	0.1	0.1	2.3
Ruggine Rosso	4.5	1.2	0.3	0.4	6.5
Reseretia	4.3	1.1	0.2	0.1	5.7
Abbondanza Rosso	5.5	3.3	0.2	0.2	9.2
Renetta Champagne	4.2	2.3	0.2	0.2	6.9
Dal Sangue	3.4	1.4	0.2	0.1	5.1
Ruggine Dorata	5.6	0.5	0.2	0.2	6.5
Pom Prussian	5.3	1.9	0.2	0.1	7.5
Siror	3.9	1.0	0.2	0.1	5.2
Renetta dei Carmelitani	3.4	6.4	0.5	0.1	10.3

The amount of pulps triterpene exhibited in Table 5 is in the range of 0.4 to 1.1 mg/gr among 15 varieties. Higher amounts belong to *Gelata Dall'Olo Tardiva* (1.1 mg/gr) and *Renetta Champagne* (1mg/gr).

Table 5: Total triterpenes in apple pulps in mg/g

Apple Variety	mg/g total triterpene acids
Gelata Dall'Olo Tardiva	1.1
Ferrocesio	0.5
Madonna D'Agost	0.4
Abbondanza	0.6
Bella Di Bosco Rossa	0.7
San Baril Rosso	0.6
Ruggine Rosso	0.8
Reseretia	0.8
Abbondanza Rosso	0.9
Renetta Champagne	1.0
Dal Sangue	0.8
Ruggine Dorata	0.8
Pom Prussian	0.9
Siror	0.8
Renetta dei Carmelitani	0.9

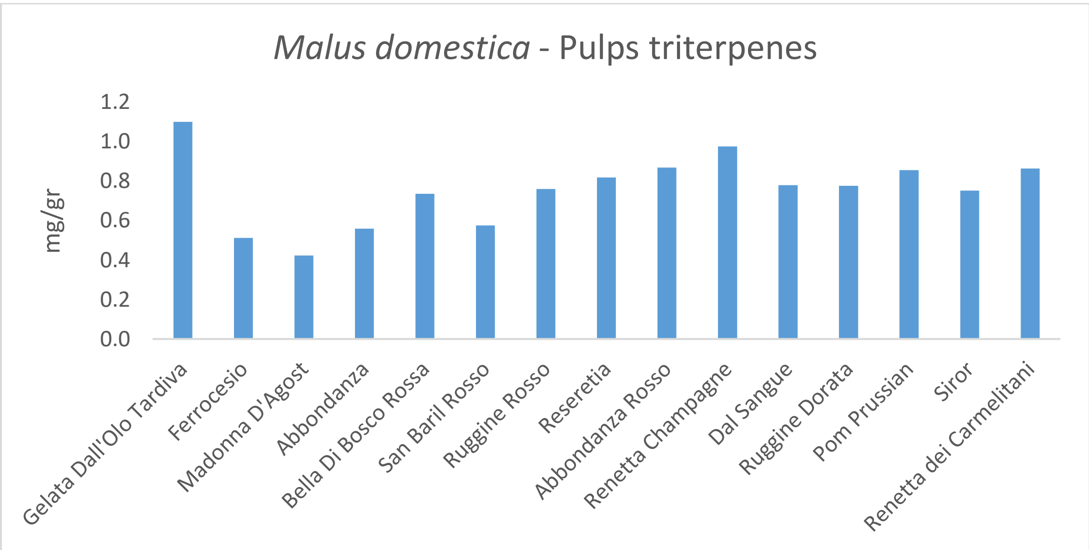

Fig 13: Total triterpenes in apple pulps

As shown in Table 6 and Fig 14, Antocyanidin in pulps of variety *Madonna D'Agost, Ruggine Dorata*, and *Siror* did not show, even in other apples that were in low amounts (10^3 mg/gr).

Table 6: Total anthocyanins in apple pulps in mg/g

Apple Variety	mg/g total anthocyanidins
Gelata Dall'Olo Tardiva	0.001
Ferrocesio	0.003
Madonna D'Agost	0.000
Abbondanza	0.005
Bella Di Bosco Rossa	0.004
San Baril Rosso	0.000
Ruggine Rosso	0.002
Reseretia	0.002
Abbondanza Rosso	0.036
Renetta Champagne	0.001
Dal Sangue	0.014
Ruggine Dorata	0.000
Pom Prussian	0.003
Siror	0.000
Renetta dei Carmelitani	0.005

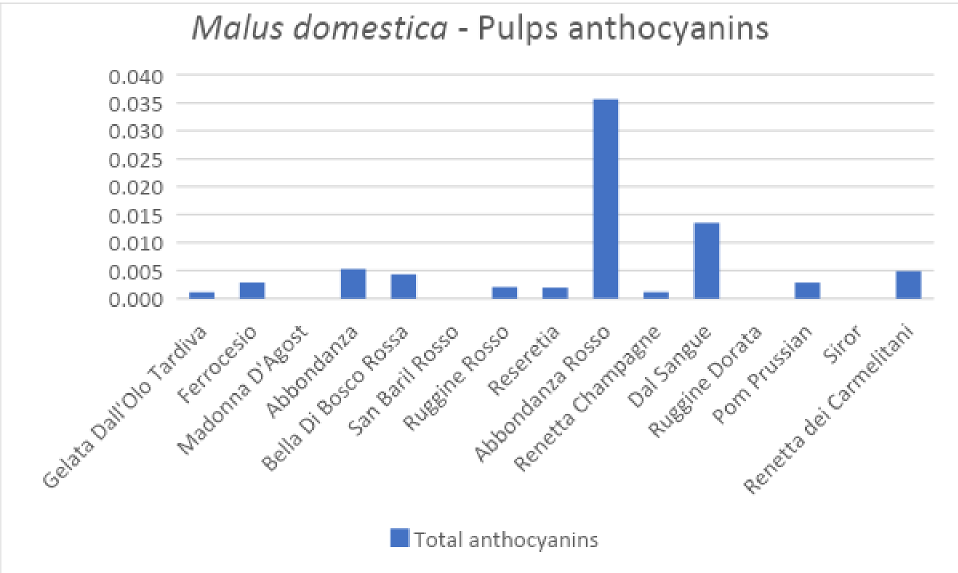

Fig 14: Total anthocyanins in apple pulps

4.1.2 Peel Composition

Table 7 and Fig 15 below show proanthocyanidins are the most abundant compounds compared to the other classes of phenolic compounds (chlorogenic acid derivatives, flavonoids, and chalcones). The peel of variety *Dal Sangue* is the richest in proanthocyanidins. We observed that the amount of flavonoid (5.1 mg/gr) and chalcone (7.3 mg/gr) derivatives are most abundant in the peel of the variety *Ferrocesio.* The high amount of Chlorogenic acid showed in *Abbondanza Rosso* (3.1mg/gr).

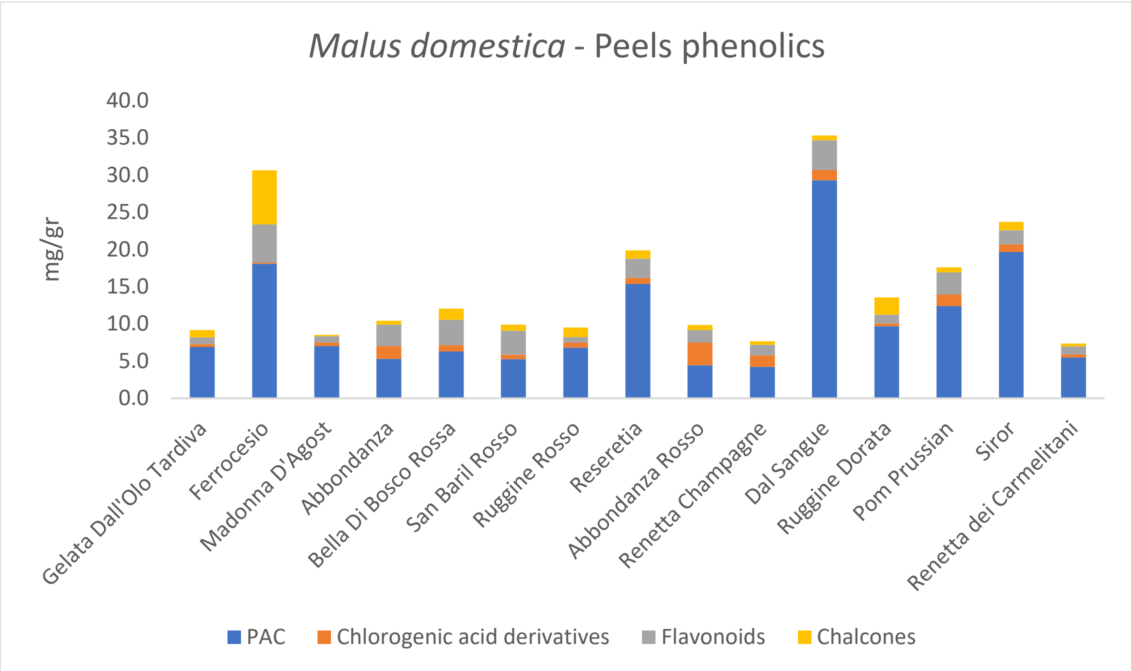

Fig 15: Total proanthocyanidin, chlorogenic acid derivatives, flavonoid, chalcones in apple peel

Table 7: Total proanthocyanidin, chlorogenic acid derivatives, flavonoid, and chalcones in apple peels.

Apple Variety	**PAC mg/g**	**Chlorogenic acid derivatives mg/g**	**Flavonoids mg/g**	**Chalcones mg/g**	**Total polyphenols mg/g**
Gelata Dall'Olo Tardiva	6.9	0.4	0.9	1.0	9.2
Ferrocesio	18.1	0.2	5.1	7.3	30.6
Madonna D'Agost	7.0	0.5	0.9	0.2	8.5
Abbondanza	5.3	1.7	2.9	0.5	10.4
Bella Di Bosco Rossa	6.3	0.9	3.4	1.5	12.0
San Baril Rosso	5.3	0.6	3.2	0.8	9.9
Ruggine Rosso	6.8	0.7	0.7	1.3	9.5
Reseretia	15.3	0.8	2.6	1.1	19.9
Abbondanza Rosso	4.4	3.1	1.7	0.7	9.9
Renetta Champagne	4.2	1.6	1.4	0.5	7.7
Dal Sangue	29.3	1.4	4.0	0.6	35.3
Ruggine Dorata	9.7	0.4	1.2	2.3	13.6
Pom Prussian	12.4	1.6	3.0	0.7	17.6
Siror	19.7	1.0	1.9	1.1	23.7
Renetta dei Carmelitani	5.5	0.4	1.1	0.4	7.4

As indicated in MS chromatograms (fig 16) of Madonna D'Agost, peels triterpenes. We observed five peaks in molecular ion $[M - H]^-$ between 19 and 24 min. Structures of main fragment ions of pomaceic, cuneataol 501 m/z, euscaphic 487 m/z, annurcoic 485 m/z, pomolic, madlinic, corosolic 471 m/z, betuilinic, oleanolic, ursolic acids 445 m/z are reported.

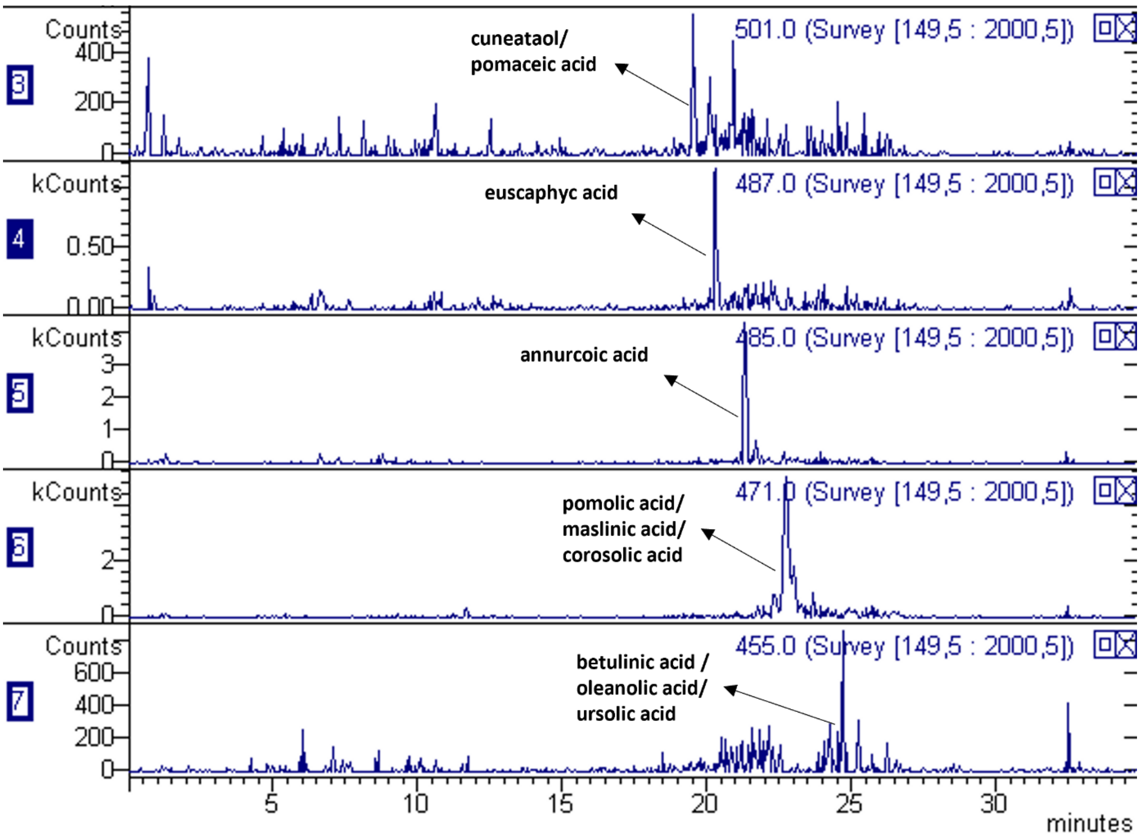

Fig 16: Exemplificative MS chromatograms of Madonna D'Agost peels triterpenes

Regarding to data in Table 8 and Fig 17, *Pom Prussian* and *Renetta Champagne* have higher total triterpene content 1.4 mg/gr and 1.3 mg/gr respectively in peels. In other varieties, total triterpene is observed in the range of 0.6 to 1mg/gr.

Table 8: Total triterpenes in apple peels in mg/g

Apple Variety	mg/g total triterpene acids
Gelata Dall'Olo Tardiva	0.9
Ferrocesio	0.6
Madonna D'Agost	0.9
Abbondanza	1.1
Bella Di Bosco Rossa	0.7
San Baril Rosso	0.9
Ruggine Rosso	1.1
Reseretia	1.0
Abbondanza Rosso	1.0
Renetta Champagne	1.3
Dal Sangue	1.0
Ruggine Dorata	1.1
Pom Prussian	1.4
Siror	1.2
Renetta dei Carmelitani	0.9

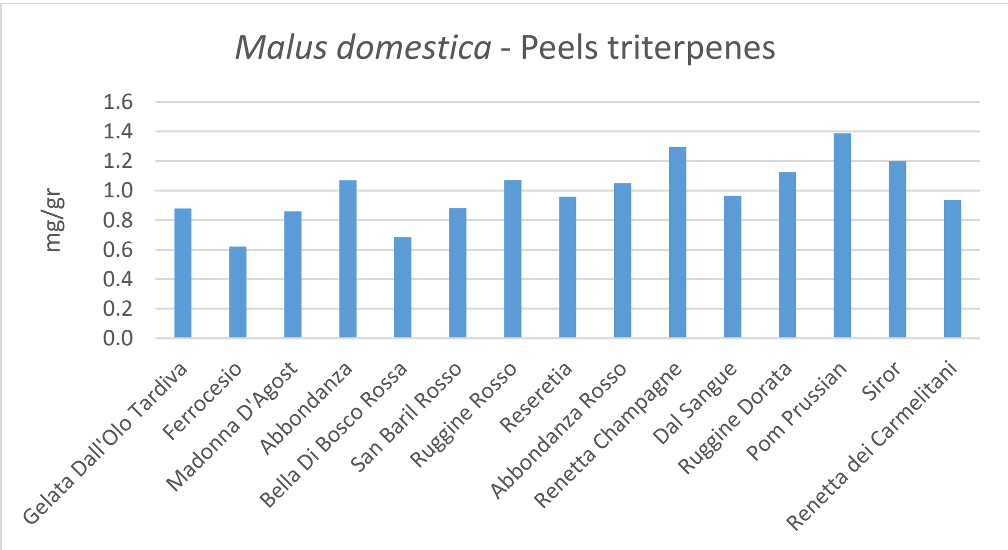

Fig 17: Total triterpenes in apple peels

According to Table 9 and Fig 18, all samples showed a lower content of total anthocyanidin in peels than other metabolites, which variety *Dal Sangue* and *Gelata Dall'Olo Tardiva* have higher (1.603 mg/gr) and lower (0.008mg/gr) amounts respectively.

Table 9: Total anthocyanins in apple peels in mg/g

Apple Variety	mg/g total cyanidins
Gelata Dall'Olo Tardiva	0.008
Ferrocesio	0.140
Madonna D'Agost	0.047
Abbondanza	0.371
Bella Di Bosco Rossa	0.073
San Baril Rosso	0.202
Ruggine Rosso	0.068
Reseretia	0.763
Abbondanza Rosso	0.899
Renetta Champagne	0.012
Dal Sangue	1.603
Ruggine Dorata	0.014
Pom Prussian	0.264
Siror	1.034
Renetta dei Carmelitani	0.063

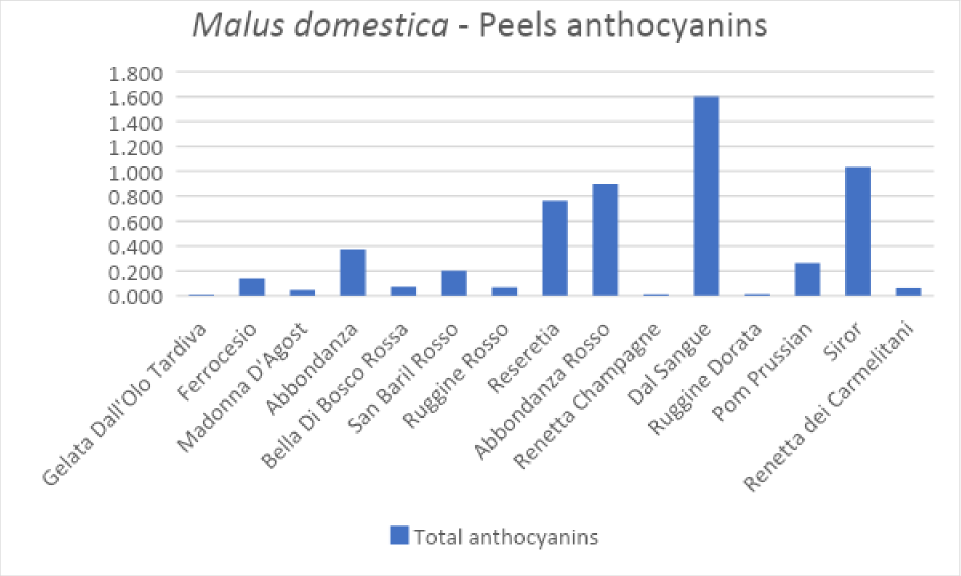

Fig 18: Total anthocyanins in apple peels

Sunlight is one of the environmental factors regulating gene expression and plant development, and ultraviolet-B (UV-B) is considered to be a major factor in increasing the synthesis of anthocyanins and flavonoids in plants (Liu et al., 2021). The differences in the polyphenol content among varieties could be the result of genetic variability. Namely, differences among genotypes accounted for 46 to 97% of the total variation in the concentration of total polyphenols and polyphenol groups in the flesh and the peel, except for the flavonols (quercetin derivatives). Flavonols in the peel are more susceptible to changes in the environment since they are sensitive to light and temperature changes. They protect the plant from UV radiation, and higher temperatures and exposure to light seem to promote their metabolism. In the studied apples, their high flavonol amount (quercetin derivatives) in the peel could be the result of environmental conditions and it could likely change from year to year. Environmental influence could be the reason for the lack of pattern for the categorization of apple varieties according to the peel polyphenols (Jakobek & Barron, 2016).

Conclusions

Recent advancements in pre-treatment procedures, separation techniques, and spectrometry methods have enabled us to analyze phenolic compounds qualitatively and quantitatively. The online coupling of liquid chromatography with mass spectrometry (LC-MS) has emerged as a useful tool for metabolic profiling of plant samples. The aim is to evaluate the phytochemical content in the peel and pulp of locally fifteen-grown apples from the *Belluno* region.

We have shown that apple peels had richer total polyphenol compounds (proanthocyanidin, chlorogenic acid derivatives, flavonoids, and chalcones), triterpenes, and total anthocyanins than pulps. On this basis, we conclude that a variety of *Ferrocesio, Dal Sangue, and Siror* have higher levels of polyphenols in both peel and pulps as can be seen in the chromatograms. On the other hand, the total anthocyanins in pulps of varieties *Siror, Madonna D'Agost, and San Baril Rosso* is zero while in peels, we found significantly more content.

As per dietary guidelines, fruit-rich diet is recommended. This fruit, with its high-added value compounds, has great potential for use as food additives and dietary supplements. The beneficiation of varietal patrimony through nutraceutical applications can offer new profitable opportunities for cultivation, strengthen the preservation of local biodiversity, and contribute to sustainable agroecological practices. This can be achieved by leveraging the antioxidant potential of plants, promoting biofortification, and developing high-added-value products from plant waste and by-products.

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