Measuring the nutritional quality of local plant-based EUREGIO foods

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Abstract

In the recent years, the consumer choices have been focused on health-promoting plant-based food and their preferences are oriented towards regional foodstuff from local productions. Therefore, an important factor for vegetables grown Trentino-Alto Adige (Italy) is to point out the added value of alpine farming to evaluate the nutritional values of farming products. Omics technologies (e.g. genomics, transcriptomics, proteomics and metabolomics) are aimed at investigating the assessment of different pools of molecules and how they are translated into the structure, function, and dynamics of a biological system or systems in order to provide a comprehensive characterization of a specific organism. Research use the omics techniques to exhaustively understand the functionality of food components. Several sophisticated chromatographic methods, spectroscopic techniques and chemometric tools are applied to give an insight into a comprehensive overview of the intrinsic quality, typicality and regionality of specific plant-based foods in the present PhD thesis: apples and potatoes. The quality of these foods is evaluated by quantifying the secondary metabolites to investigate their nutraceutical values. The aim of this PhD project is to use several analytical techniques (LC-MS, UV-VIS) that are capable of comprehensively characterizing the food metabolome with particular emphasis on those components with high nutritional values. The data analysis and data handling of omics data requires advanced bioinformatic, statistical, and chemometric tools. Potatoes and apples are chosen as target matrices for these studies for their relevance in the local economy and for the peculiar chemical composition of particular interest for their health-promoting proprieties. The information is acquired using several sophisticated chromatographic and spectroscopic techniques, such as ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC– MS/MS) and UV/VIS. It is integrated to chemometric approaches (principal component analysis (PCA), partial least square regression (PLS), and data fusion) to achieve a comprehensive targeted chemical characterization. The sampling procedures gathers, in the case of the potatoes study, reference cultivars that may be found in the common retailers of Trentino/Alto-Adige and different production areas, the apples of 22 cultivars were harvest from the fields of the Laimburg Research Centre (Vadena, Italy) to guaranty comparability of the obtained data. Our results may be used as solid foundation for a reliable evaluation of apples and potatoes healthy "potential" value based on cutting-edge techniques, which are capable of providing comprehensive data regarding the alpine food quality parameters with high efficiency and reliability.

THE AIM OF THE THESIS

In the recent years, the interest in food quality assessment and consumer acceptance has been increased. Measuring food quality includes determining key product attributes that may be used to assess the conformance to those attributes in promoting the selection of health promoting and more palatable foods with high nutritional values. Therefore, the evaluation of the quality may be considered for selecting variables, which covers the entire production chain, from primary materials growth and harvest to the industrial processing and shelf-life definition. The investigation on the key variables, which are influenced by several factors (e.g. the cultivar, the pedo-climatic conditions, post-harvest treatments etc.) is a crucial point to assess the food quality. An additional information may be conducted on the consumers demand that influence the global food market and the presence of specific food in the retailers. For these reasons, the evaluation of the food quality is an important factor to guarantee the satisfaction of consumer and the beneficial proprieties. Another important aspect is the health condition of consumers that may depend on what they eat. It has been reported that multidisciplinary approaches may be useful to tackle the multifaceted challenges of obesity and healthy ageing.

This Ph.D. thesis aims to investigate informative metabolites, which are characterized by health-promoting proprieties, by using omics strategies based on various analytical techniques. The accumulation trend of the metabolites in plant-based food may be used to define reference patterns for quality assessment. The mission of this work is to lay scientific evidence to fight obesity and age-related chronic disease by valorizing locally produced food, thus providing new solutions and strategies for human diet.

The metaboloma of plant-based food is characterized by primary and secondary metabolites that are involved in the cell/plant metabolisms such as sugars, flavonoids, amino acids, vitamins, etc. Among these metabolites, the present work is focused on the characterization and quantification of metabolites that provide health benefits for consumers. The presence of high concentrations of beneficial compounds can then be considered a key property in defining the “overall quality” of specific foods, and can be used to direct agronomic practices and specific breeding and selection programs.

In this perspective, apples (Malus × domestica) and potatoes (Solanum tuberosum L.) are the main subjects of this thesis due to their relevance in the Trentino/Alto Adige region and for the important role that they have in the economy in this region. To date, there is a small number of studies that compare apple varieties cultivated in the

same environmental conditions with identical agricultural practices. Nevertheless, the metabolome of apples is extensively studied and reported. Moreover, recent studies hardly consider red-fleshed apples compared to non-pigmented fleshed cultivars, which are advisable to bring to the market new impacts due to the simultaneous presence of polyphenols and anthocyanins. From another point of view, the metaboloma of potatoes is hardly studied and the studies are generally, focus on specific chemical class. Our studies may play an important role in providing a wide range systematic characterization of the potato metabolome. Since the synthesis of metabolites is a complex biological process involving many signaling pathways, the multi omic approach is most suitable for examining these traits. Our results gave a picture of plant-based food metabolome perturbation.

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Author's declaration

I, Adriana Teresa Ceci, confirm that this is my own work and the use of material from other sources has been properly and fully acknowledged.

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CHAPTER 1

INTRODUCTION

Metabolomics is a part of "omics" science and it is an emerging field in food analysis, in general, in plant analysis, in which in-depth investigation of small molecules, including identification and quantification, is conducted. The study of metabolome involves a huge range of metabolites and their interaction with the biological system. The application fields are different, namely in agriculture food and safety for identification of markers in adulteration, food intake, food characterization and quality. The research in food Metabolomics is recently increasing due to the relationship between food consumption, human health and nutrition.

1.1 Environment, Food and Health

It has been extensively recognized that the intake of different types of functional food is inversely associated to the onset of chronic diseases, cancer, and cardiovascular disorders [1]. According to the World Health Organization (WHO) guideline, the essential nutrients are saturated fat, polyunsaturated fats, proteins, carbohydrates, fibre, mono- and

disaccarides and cholesterol. The most common accepted sources are fruit, vegetables, fish, olive oil, cereal and legumes [1]. Furthermore, in 2010, the United Nations Educational, Scientific and Cultural Organization (UNESCO) the Mediterranean diet has been defined as a 'cultural heritage of humanity' [1]. The Mediterranean diet involves the daily consumption of different nutrients, namely, monounsaturated fats, polyunsaturated fatty acids, a mixture of antioxidants (flavonoids, vitamins C and E) and fiber. Whereas, the intake of saturated fats and fatty acids should be controlled [2]. As reported by Menotti and Puddu [1], the incidence and mortality linked to cardiovascular diseases and coronary heart diseases are related to the eating habits and local characteristic of the population. This evidence is correlated to their agriculture and traditional eating patterns. Since the 1950, several studies have been published based on the relevance about healthy diet should include a high consumption of cereals, vegetables, legumes, fruits, nuts, raw olive oil, and fish [1]. Subsequently, meat, milk and its products, sugar and wine should be consumed in moderation. In detail, eggs should be consumed 2-3 times per week, white meat and fish few times a week and red meat a few times a month [3]. Since the beginning of the 20th century, the benefits of healthy eating have been already observed, which could be a role in prevention of diseases and in maintaining a healthy status. From the Second World War, the researches have started studying the health benefits of a healthful eating [1]. Therefore, the concept of Mediterranean diet was born in the mid-20th century and it is based on the eating behaviors of the Mediterranean population in the southern shores of Mediterranean sea [3].

The first study was conducted by Ancel Keys of the University of Minnesota about the inverse correlation between benefits of healthy eating and cardiovascular diseases [3]. This famous study, known as the "Seven Countries Study", which was begun in the 1950, it was conducted on eight nations of seven countries, namely USA, Netherlands, Italy, Croatia (former Yugoslavia), Serbia (former Yugoslavia), Greece and Japan [1].

The correlation between the different incidence and mortality rates in the onset of cardiovascular diseases and the dissimilarity in eating behaviours among individuals within populations has been highlighted and demonstrated for the first time [1]. In this study was pointed out the importance of the Mediterranean diet in preventing disease and chronic negative conditions. Indeed, high level of cholesterol, blood pressure, smoking and diabetes were recognized as risk factors for coronary heart disease. It has showed that healthy lifestyles, good eating behaviors and physical activity are linked to decrease the incidence of cardiovascular disease. The "Seven Countries Study" was considered as starting point for other studies, such the Zutphen Elderly Study. In this study, it was recognized that the flavonoids, which are naturally present in vegetables, fruits, and beverages, have an important role as antioxidants being them important scavengers of free radicals [4]. They measured the content of different class of flavonoids in several food and the information about the vital status and previous issues of all participants were recovered. They evaluated the correlation between the intake of flavonoids, obtained by black tea, onion and apples, and mortality after a coronary heart and myocardial infarction issues. Furthermore, the men of the town of Zutphen, in Netherlands, were subjected to a further study, in which the consumption of fish and coronary hearth disease were investigated [5]. The authors affirmed that the mortality caused by hearth diseases was lower after consuming 30 g of fish per day [5]. Another famous study is "French paradox" and this term was coined in 1980 [6]. It is refereed to the intriguing fact that French people had a low incidence of coronary artery anomalies, despite of eating a high content of saturated fat. The reason seemed to be linked to the consumption of wine that French people consumed it more than the others countries. In detail, red wine contained a high amount of flavonoids, which have beneficial effects on human health. Frankel et al. [6] provided useful information to explain the "French paradox", despite of the absorption and kinetic of flavonoids was hardly considered. Nevertheless, they affirmed

that the daily intake of phenolic components could reduce the oxidation of lipoproteins and then, reduce the onset of thrombotic and coronary artery disease [6].

During the conference on 'Diets of Mediterranean' held in 1993, a first series of guidelines about the beneficial effect of the Mediterranean diet on the human health are presented. These guidelines are shown graphically as a pyramid. The healthy eating pyramid was drawn for the first time in 1992 by US Department of Agriculture (USDA). Therefore, the Mediterranean diet pyramid is based on the scientific evidence, which have been proved since 1960 [7]. At the base of the Mediterranean diet pyramid, the most important daily food are graphically presented: whole grains, fruits, vegetables, beans, herbs, nuts and olive oil. Subsequently, fish, seafood, cheese and its products, and eggs are presented and they are occasionally consumed. Finally, red meat and sweets are eaten in limited amounts and they are reported on the top of pyramid [7]. As reported by Willett et al. [7], the maximum content of nutritional compounds for the human health could be achieved by a controlled processing, harvest season and freshness of food. Therefore, the Mediterranean diet is defined as a food patterns and dietary habits.

To date, the researchers are focused on evaluating the food nutritional quality by respecting the geography, food culture and season. The aim is to understand the correlation between the consumption of specific food and the biological functions in the human health. Therefore, the Food area of the project EUREGIO Environment Food and Health (EFH) aims to investigate the nutritional quality of local mountain foods in order to provide useful information to the population regarding the nutritional values of what they usually eat. The study collects several interdisciplinary approaches that includes nutrition, health, environment, economy, sustainability in environment, community, or society. The EUREGIO regions Trentino, South Tyrol, and Austria pay close attention to health situation in those Italian areas, with a special focus on important negative health conditions, such as obesity and metabolic diseases. The EFH project is focused on the

construction of a local map, based on the potential healthy proprieties of the local food, to design a nutritional workflow to fight obesity and metabolic human issues.

The genetic involvement in the onset of diseases has been already demonstrated [8]. The suitability in the selection of specific populations was highlighted to be potentially useful to investigate the correlation between environment and chronic issues due to the limited recombination events and shared factors such as, food and territory. Indeed, the EUREGIO area was chosen due to structure, historical, and political events, which influenced the "isolation" of these population from the rest of Italy [8]. In detail, the South Tyrol is an autonomous province located in northernmost of Italy and it is known as Trentino-Alto Adige/Südtirol. The most important city is Bolzano [8]. The location of Trentino-Alto Adige/Südtirol in Italy is shown in the Figure (1.1).

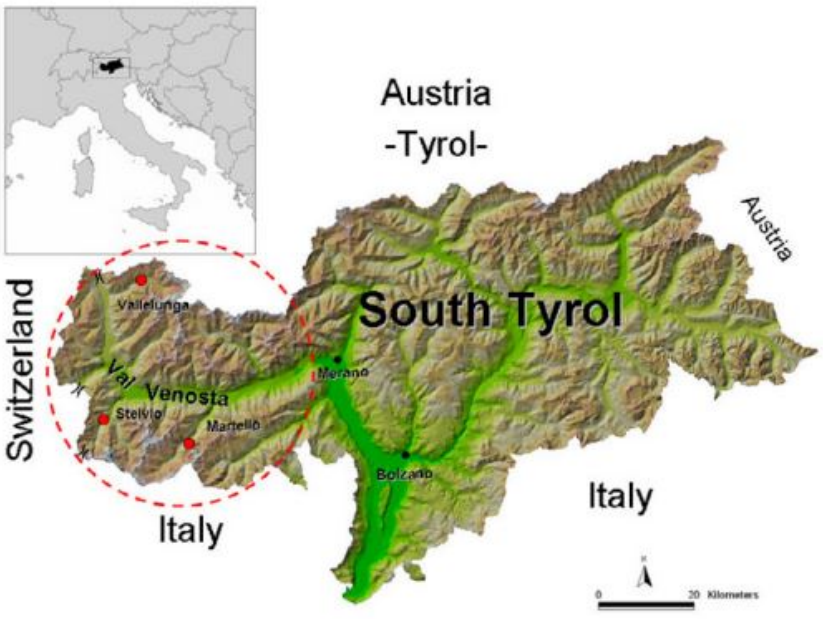

FIGURE 1.1. Location of South Tyrol in Italy, with a specific focus on Val Venosta. ©2007 Pattaro et al; licensee BioMed Central Ltd. (CC BY) license (http://creativecommons.org/licenses/by/2.0). [8].

Before the First World War (1914-18), the present italian region Trentino-Alto

Adige/Südtirol belonged to the Austro-Hungarian Empire, which included the Tyrol, Bolzano and Trentino areas [9]. After the war, Italy resulted a victor and all of these areas were occupied. The rise of fascism marked the start of a period of crucial importance to the history of Alto Adige [9]. The German population is forced to follow the laws of the new regime, this including mandatory study of the Italian language with the goal of erasing German customs. It was realized a severe italianization of Alto Adige/Südtirol by helping the Italian population to move to Alto Adige/Südtirol from other regions [9]. The aim of the Fascist regime was to italianise Alto Adige. Then, the German was secretly taught in the religious institutes to preserve the German culture. At the beginning of 1970s, the region Trentino-Alto Adige/Südtirol received Statute of Autonomy and the bilingualism was applied [9]. To date, Trento and Bolzano/Bozen are recognized as two provinces and one region. In this context, it was conducted a study called Cooperative Health Research In South Tyrol (CHRIS), which reported that cardiovascular and metabolic diseases are major public health issues amongst South Tyrolean population. It is likely that more than 40% of the adult population could suffer from at least one cardiovascular condition by 2030s. For 65 +-year-old people, cardiovascular diseases will become the main cause of death and considering the current ageing rate, this will result in a higher cost for the treatment of cardiovascular issues [10]. To date, the 20% of the population is obese and the 50% is in overweight. Within this context, the identification of the nutritional profile of EUREGIO foods is necessary to provide useful information to fight obesity and overweight in EUREGIO population. The aim is to find a new modern diet that may be used to oppose the overall increase in chronic diseases. In 2010, international scientific symposium was organized by Food and Agriculture Organization of the United Nations (FAO), in which the Mediterranean diet was defined as follow: *"Sustainable diets are those diets with low environmental impacts which contribute to food and nutrition security and to healthy life for present and future*

generations. Sustainable diets are protective and respectful of biodiversity and ecosystems, culturally acceptable, accessible, economically fair and affordable; nutritionally adequate, safe and healthy; while optimizing natural and human resource" [11]. Another interesting definition was given by UNESCO as follow: *"The Mediterranean Diet – derived from the Greek word díaita, way of life – is the set of skills, knowledge, rituals, symbols, and traditions, ranging from the landscape to the table, which in the Mediterranean basin concerns the crops, harvesting, picking, fishing, animal husbandry, conservation, processing, cooking, and particularly sharing and consuming of food"* [12]. Therefore, the foods play a fundamental role in preventing the onset of a chronic medical conditions [13]. Therefore, the aim of the Food area in the EFH project is to model an EUREGIO alpine diet using EUREGIO food based on the nutritional proprieties of Mediterranean diet.

1.2 Apple consumption

During the last decades, the interest towards the health benefits of fruits and vegetables has been continuously increased. Several studies already demonstrated that the bioactive compounds may modulate the onset of diseases, including chronic conditions, cardiovascular disorders, diabetes, cancer and degenerative status [14]. The capacity in preventing and reducing disease in humans may be linked to presence of phytochemicals, which contribute to the protective health benefits of these plant-based food. The most relevant compounds are the dietary polyphenols, which can be divided into flavonoids (60% of the total polyphenols) and phenolic acids (30% of the total polyphenols) [14]. It was reported that 1 g per day of polyphenols is eaten by the Western population [15]. After the consumption of monomeric or dimeric polyphenols, they are absorbed and subjected to the digestive enzymes of small intestine to undergo structural modifications. Then, the conjugation process occurs in the liver, which involves glucuronidation, methylation and

sulfation by phase II enzymes. The aim is to increase the solubility of the compounds. On the other hand, the polymeric polyphenols reach the colon and then they are metabolized by the colonic microbiota. The metabolites may have beneficial effects directly in the colon, or may be absorbed in the circulation [16]. Indeed, the glucuronide and sulfate conjugates are the most abundant metabolites found in the human biofluids after food intake [16].

Bonany et al. [17] showed how the consumers´ decisions are influenced by eating quality (flavour, taste and texture), appearance (fruit color), the quality of red skin color and the size of the apples. The breeding processes are focused on improving the consumers´ preferences by working on the availability of new apple varieties, which may satisfy the acceptability. It has been reported a strong correlation between firmness and texture, which are identified as determining features for acceptability on the customer´s part. Their data suggested that the preferences of the majority of the population are oriented towards firm and sweet apples, with a medium or low acidity [17].

To date, the cuisine of Trentino-Alto Adige is a mixture of Mediterranean diet, with a strong influence of mountain cooking and German habits. The cultural background and the history of Trentino-Alto Adige are likely to have appealed to the customers and influenced their expectations. However, the difference represented not only by the culture but also by the German language. Other important factors are the presence of small and medium-sized family farms, which contribute to the production of excellent quality of the local food. Furthermore, apples (Malus × Domestica) are 12.5% of all consumed fruit in the world, and it is widely accepted that they are beneficial both to consumers in good health, and consumers with specific pathologies such as hypercholesterolemia, cancer, cardiovascular disease [1]. The cultivation areas Trentino and South Tyrol are chosen because they are market leaders in producing apples in Italy. Furthermore, they supply one tenth of apples in EU member states, with a lot of different varieties [18]. In detail,

about 300 million kilos of apples per year are grown in Trentino-South Tyrol and the leader is the Val di Non in Trentino. German and Austrian traditions have influenced the recipes of making sweet and light pastry stuffed with apples, such as Apfelstrudel (apple Strudel), Apfelschmarren (apple fritters), Scheiterhaufen (bread and apples) and Apfelküchel (apple cake).

1.3 Potato consumption

The importance of potatoes was highlighted by FAO (The Food and Agriculture Organization) in 2008, when it has been promoted as “The International Year of the Potato". FAO declared that potatoes are the most common food produced, after maize, wheat, and rice. To date, FAO estimated that 160 countries cultivate potatoes, 4000 types of varieties are known in the literature and 368 million tonnes are generally produced [19, 20]. The industries are interested in measuring changing dietary patterns, and how they may be linked to the processing methods and cooking procedures. Indeed, potatoes are the most consumed tubers in the world, therefore, the determination, identification and quantification of secondary metabolites are determining factors in evaluating the nutritional value of potatoes [21]. The interest of the researchers is focused on the health proprieties of bioactive compounds in the potatoes [20, 21]. Indeed, potatoes are an important source of essential elements for human nutrition such as phenolic acids and flavonoids. The anti-oxidative, anti-inflammatory, and anti-carcinogenic activities of phenolic acids and flavonoids are well known [20, 21]. Regarding the chemical classes found in potatoes, which are reported by Akyol et al. [20], the major phenolic compounds are the phenolic acids, such as hydroxybenzoic acids and hydroxycinnamic acids. Additionally, flavonols, flavanols, and anthocyanins are found in the potato flesh [20]. The consumption of antioxidant-rich foods may remove the free radicals from the body, which may damage the cells and increase the onset of serious illness. Furthermore, emerging

evidence on the plant-based red and purple polyphenolic pigments, the anthocyanins, has reported regarding their significant results of their health benefits [22]. There is evidence that demonstrated the potential role in eating anthocyanin-rich foods. The anthocyanin-rich foods showed anti-inflammatory effects improved insulin resistance, and may be consumed by diabetic patients [22]. Jokioja et al. [22] suggested that the consumption of pigmented potatoes is a suitable strategy to increase the absorption of health-promoting anthocyanins. In the case of potatoes, the anthocyanins may differ in the number and position of hydroxy, methoxy, glycosyl, and acyl substituents [22]. The most relevant anthocyanins are the acylated anthocyanins and the acylation lends to the molecule several capacities (e.g. color stability, altered absorption, bioavailability, *in vivo* stability, and colonic degradation), which differ from nonacylated anthocyanins [22]. The application of omics techniques may be useful to gain insights in the modification of acylated and nonacylated anthocyanins. The *in vivo* experiments showed that the acylated anthocyanins were more stable in the upper gastrointestinal tract than the nonacylated anthocyanins, thus leading to reach the gut microbioma in their intact form. The impact on this site of the health-promoting anthocyanins may be promoted by the breakdown of the acylated anthocyanins and the production of phenolic metabolites. Nevertheless, the *in vitro* conditions hardly represented the postprandial human condition, indeed, there is a gap in the evidence regarding the metabolism of the acylated anthocyanins. Understanding that the potato are one of the most consumed food in the country, especially in Alto Adige (South Tyrol), they are grown in the harsher climate of the Val Pusteria valley. The harvest time of potatoes starts from the end of summer and almost 20 varieties are produced in South Tyrol every year [19].

1.4 Polyphenols and Antioxidants

The flavonoids are used by fruits and vegetables for their growth, protect against pathogens [23], color and flavor for attracting pollinating insects to help in seed and spore germination [14]. Furthermore, flavonoids defence the plants against the biotic and abiotic stresses and these metabolites have an important role as UV filters [23]. It has been reported that flavonoids are used by the plants as frost hardiness and drought resistance and they have the capacity to help plant controlling tissue internal temperature and coping with climatic variability and change (freezing and heating). Generally, the flavonoids have been divided into major classes: isoflavonoids, flavanones, flavanols, flavonols, flavones, and anthocyanidins [24].

Notably, the study conducted in apples by Vrhovsek et al. [15] reported that the flavanols represent 71-90%, hydroxycinnamates 4-18%, flavonols 1-11%, dihydrochalcones 2-6%, and anthocyanins 1-3% of the total apple flavonoids. Generally, the flavonoids are characterised by a skeleton with 15-carbon called "flavan" and a phenylpropanoid chain, which form two aromatic rings (A and B). The third ring, the C ring, is a heterocyclic pyran ring. Indeed, all of these chemical features make the chemical structure of flavonoids as a powerful scavenger of oxidative species. The chemical structure of each flavanoid may be modified by different chemical process and they are divided into the aforementioned groups, as shown in the Figure (1.2).

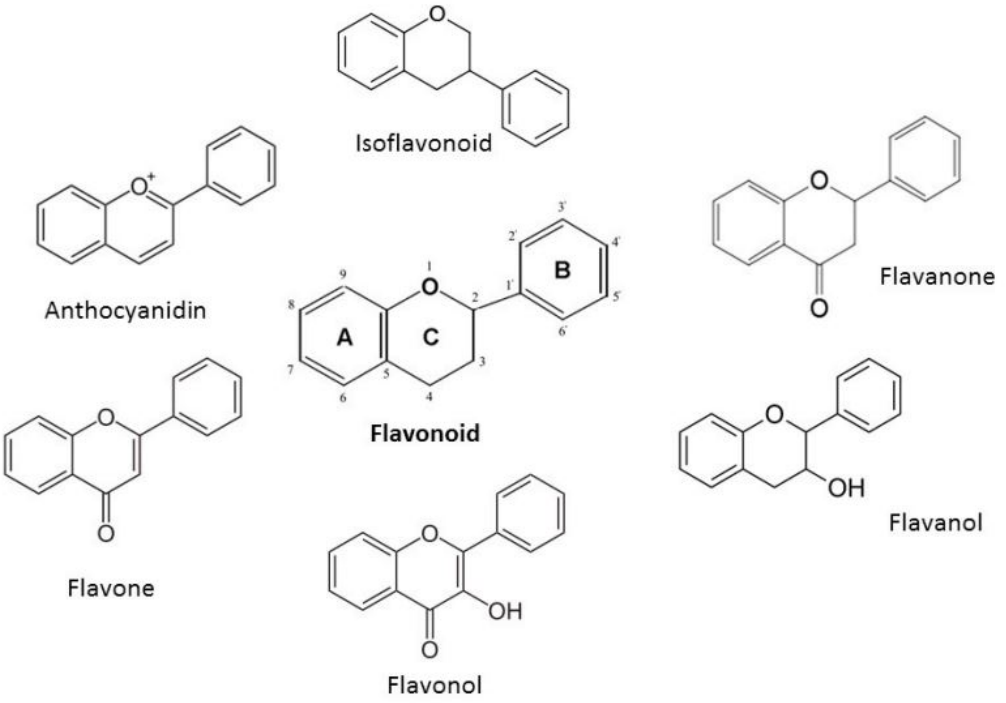

FIGURE 1.2. The main structures of flavonoids in apples. ©2020 by the authors. Licensee MDPI, Basel, Switzerland [24]. (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Additionally, all flavonoids are divided into several subclasses as reported in the Figure (1.3).

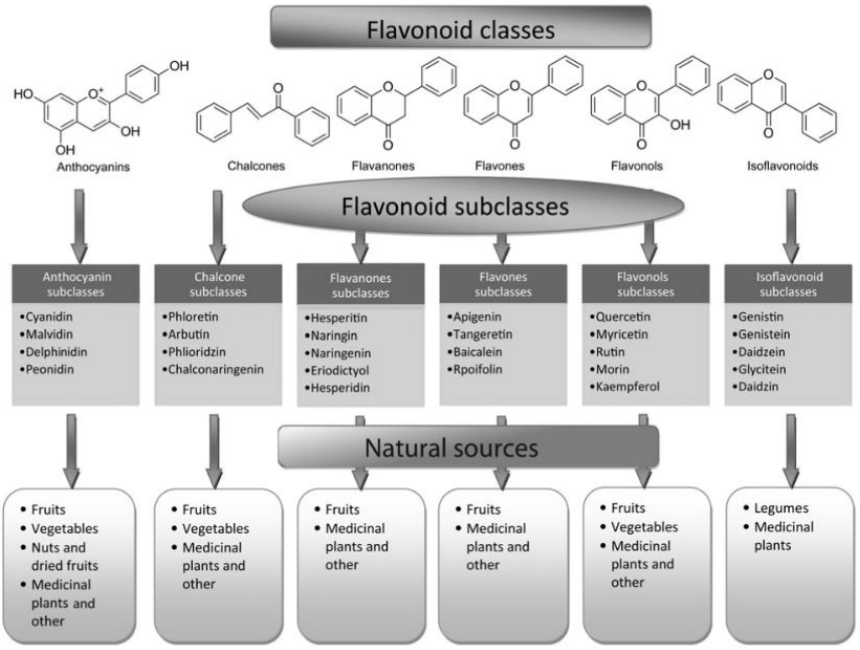

FIGURE 1.3. The main subclasses of flavonoids in different source types. © The Author(s) 2016 [23]. (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Flavanols: the flavanols (as known flavan-3-ols), the most abundant polyphenolic class in apples, are characterized by a saturated group, that is an unoxidized C ring and in the C3 position is bound a hydroxyl group. It is a common C6-C3-C6 structure [25]. The most representative polyphenolic compounds are the catechin and its stereoisomers, namely cis (-)-epicatechin) and trans (+)-catechin. The compounds are shown the Figure (1.4). The positions involved in determining the stereoisomerism are C2 and C3 positions in the molecule. The flavanol could be esterified with gallate groups [24]. Nevertheless, procyanidins are found to be representative in the apple chemical profile. The procyanidins are a mixture of the polymerized forms of (+)-catechin or (-)-epicatechin concatemers, namely procyanidin B1, B2 and C1 [26]. The procyanidin are oligomers and they are classified into A-type and B-type, based on the provenience. Generally, the monomers are bound by 4 $\rightarrow$ 8 carbon–carbon and a 2 $\rightarrow$ O7 ether bond in the type-A, whereas, 4

→ 8 carbon–carbon bond are characteristic in the B-type [25].

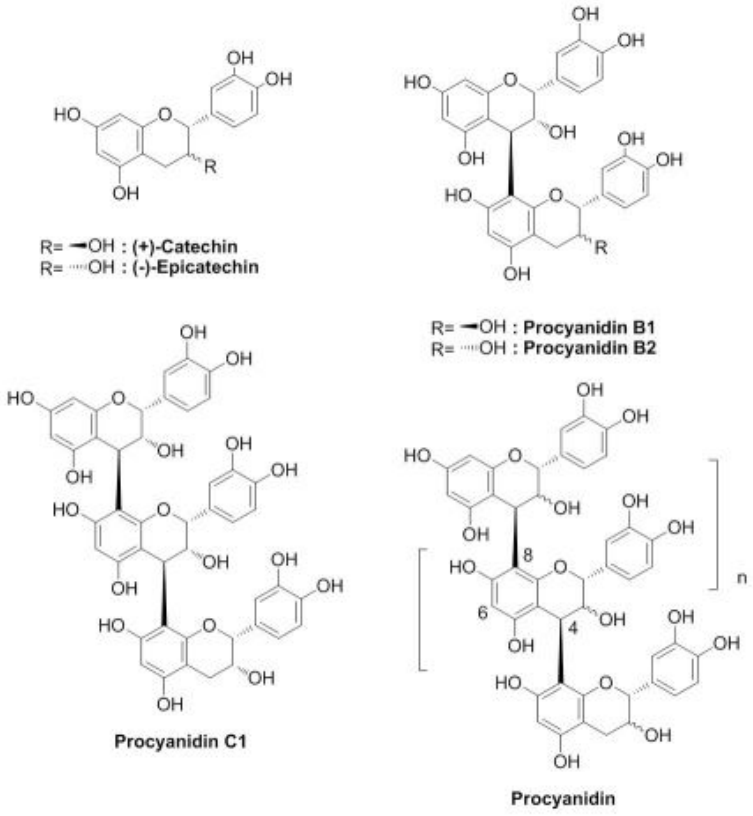

FIGURE 1.4. The main chemical structures of flavanols in apples. © The Author(s) 2016 [26]. (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

In the last decades, the role of catechin and its stereoisomers has been intensively investigated. The effects are reported: anti-inflammatory, anti-mutagenic, antihypertensive, antibacterial, and anti-carcinogenic [25]. They may protect from atherosclerosis [25]. Several studies showed that flavanols may inhibit the carcinogenesis, the tumor growth, cancer cell invasion, the angiogenesis of the tumor. The effectiveness of beneficial effects of catechins is proven in human experiments. It has been proven that the accumulation of reactive oxygen species (ROS) and radical nitrogen metabolites could contribute in the onset of the inflammation and chronic diseases in the human gut. The most common oxidant species are superoxide anion (O2-), hydroxyl radical (OH·),

and singlet oxygen ($^1O_2$). The ROS are produced during the normal metabolism of the cells, however, when the ROS exceed the usual content, they could contribute to disrupt the intestinal mucosa by destroying the integrity of the barrier of the gastrointestinal tract. The process of disruption involves the damage of cellular lipids, proteins, and other cellular components as well as the damage of DNA. Thus, it is leading to the activation of inflammatory cascade and the production of signaling pathways, namely nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) [25]. Additionally, the presence of procyanidins is fundamental. It has been reported by Masuda et al. [26] that procyanidins are able to protect articular cartilage against degeneration. Furthermore, Rauf et al. [27] reported that procyanidins reduced the oxidative stress caused by the ROS and thus providing lower levels in the amount of the inflammation compounds. Antioxidant, cardioprotective, neuroprotective, immunomodulatory, lipid lowering and anti-obesity, antidiabetic, anticancer, antimicrobial effects are reported [27].

In order to consider the beneficial effect of flavanols should be considered their availability after the intake. The catechins are subjected to three different reactions in the liver and intestine of the human: methylation, glucuronidation, and sulfation. The scope is to obtain the corresponding aglycones and then, they are absorbed into the human circulation. They undergo additional transformation driven by the gut microbiota and the beneficial effects are carried out by producing specific health metabolites [25]. The enterocyte-like differentiated Caco-2 cells are the most common cells, which are used to investigate the metabolism of metabolites by simulating the *in vivo* conditions [28]. Catechin and procyanidin C2 are absorbed at the level of the Caco-2 cell monolayer in the human intestinal epithelial, whereas, the degradation of procyanidin dimers B2 was evaluated *in vitro* carrying out a trial in an intestinal preparation [29]. Nevertheless, the absorption of procyanidin dimers is investigated in both rats and humans [29]. Shoji et al. [29] showed that the flavan-3-ols in the apples are absorbed faster than procyanidin

dimers and trimers due to the complexity in the chemical structures. Additionally, they may form complexes with mucosal proteins and it may influence the absorption of procyanidin. Furthermore, Ulaszewska et al. found five catechin metabolites after whole apple consumption [16].

Hydroxycinnamate: is the second class more abundant in the apples [15]. The most common hydroxycinnamates are chlorogenic acid (5-O-caffeoylquinic acid), which can be found with its 3-O- and 4-O-isomers, also known as cryptochlorogenic acid and neochlorogenic acid, respectively [30]. Other hydroxycinnamates are p-coumaric acid, caffeic acid, ferulic acid, and sinapic acid. Indeed, the chlorogenic acids are the esterified/etherified conjugates of these compounds. The chlorogenic acids are esters with caffeic acid and quinic acid. They are C6–C3 compounds. The main chemical structures of phenolic acids and hydroxycinnamates are shown in Figure (1.5) [31, 32].

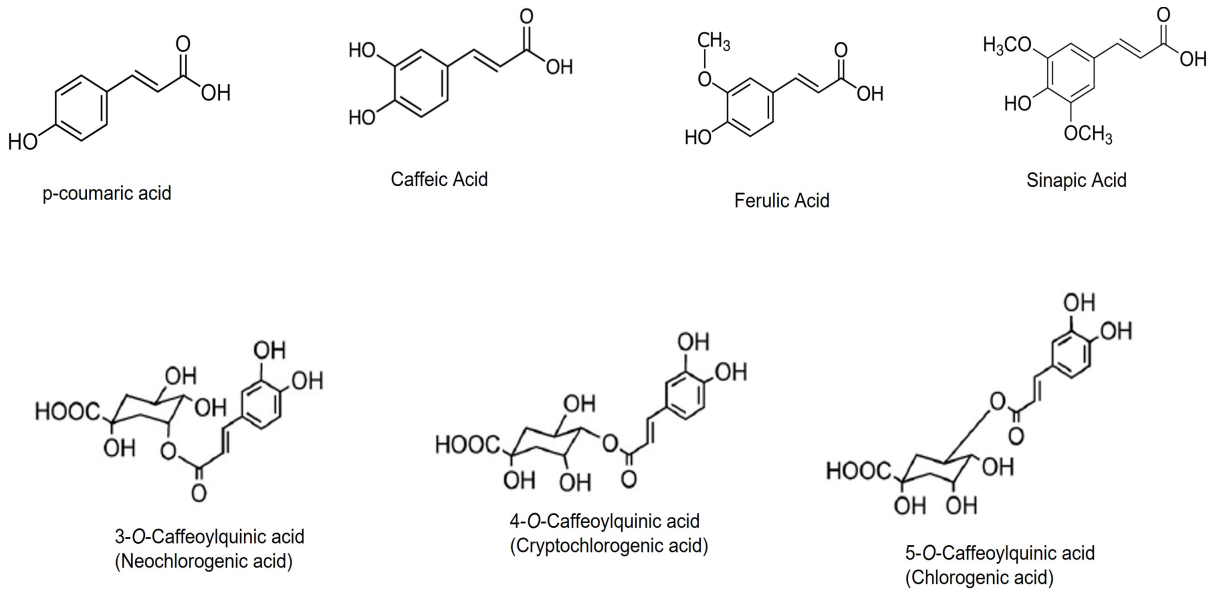

FIGURE 1.5. The main chemical structures of phenolic acids and hydroxycinnamates in apples adapted from [31]

The beneficial effects are reported, such as antibacterial and anti-inflammatory prop-

erties and they may protect from cardiovascular issues, diabetes and Alzheimer's disease [30]. Boyer and Liu [33] reported that chlorogenic acid had a very high scavenging activity. As mentioned before, the production of ROS enhanced the carcinogenesis and the intake of chlorogenic acids may protect from the cancer [33]. To date, the hydroxycinnamates could prevent the oxidative stress, which is involved in the development of chronic condition such as cancers, cardiovascular diseases, hypertension, neurodegenerative disorders [34]. Shahidi and Chandrasekara [34] reported that the hydroxycinnamates had a potential role as antioxidant *in vivo*. Indeed, the evaluation of their antioxidant activity *in vivo* is strongly dependent by their bioavailability [28]. The Caco-2 cells were used to estimate the behaviour of hydroxycinnamates in the human enterocytes including the expression of the enzymes involved in the metabolism of flavonoids. These cells were able to simulate the metabolism of hydroxycinnamates by the human small intestinal epithelium [28]. Kern et al. [28] defined that the most important pathways that occur are the glucuronidation and sulfation by producing hydroxycinnamate-glucuronides and methyl hydroxycinnamate-sulfates. Additionally, sulfate products were formed by ferulic acid, sinapic acid, and p-coumaric acid [28]. Therefore, the antioxidant proprieties may be attributed to the production of all these metabolites. Interesting results were reported by Spencer et al. [35], where the absorption of the hydroxycinnamates through the ileum and jejunum of rats is evaluated. They found that the absorption is lower in ileum than jejunum, but, it differed through the single hydroxycinnamates [35]. This discrepancy may be related to the susceptibility oh these compounds to the oxidation and the availability of hydroxyl groups to the glucuronidation process [35]. Furthermore, Ulaszewska et al. described that the chlorogenic acids and cinnamic acids are found in human biological fluids after consuming the whole apple [16].

Flavonols: another important class of polyphenols is flavonols. The flavonols are made up by a dihydroxy substitution of the B-ring called catechol, 2,3-unsaturation in

the C-ring and 4-carbonyl group [36]. The most important flavonols in plants are shown in the Figure (1.6).

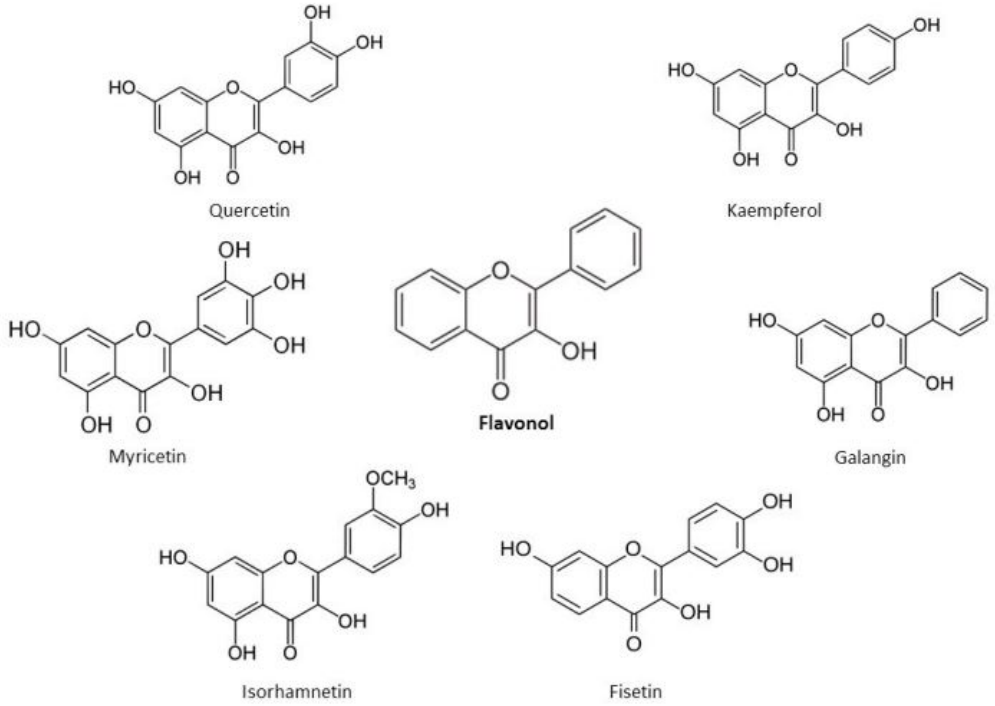

FIGURE 1.6. The main chemical structures of flavonols in apples. © 2020 by the authors. Licensee MDPI, Basel, Switzerland [24]. (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

In apples, quercetins are present as water-soluble glycosides [15]. Generally, the glycosylation occurs in the 3-position and the most common sugar residues are: D-glucose, D-galactose, L-rhamnose, L-arabinose, and D-xylose. The utilization of flavonols in supplementation of food products and the quercetin, the aglycone form, is applied in human clinical studies [36]. Nevertheless, it is studied that the amount of absorption of glycosides is more than aglycone. Boyer and Liu [33] reported that the quercetin intake may reduce the onset of lung cancer. Apples intake and quercetin intake may be linked to decrease the possibility in having thrombotic stroke, coronary heart disease or other cerebrovascular diseases. Ay et al. reported [37] several studies that demonstrated the

health benefits of quercetin. Quercetin is the major flavonols found in apples and it has been shown to exhibit cell death and cell cycle arrest in cancer cells by regulating the expression of oncogenes. It has been found that quercetins are able to induce the apoptosis in human prostate cancer cells. From another standpoint, and consistent to the objective of the health benefits of quercetin, this compound has a protective effect against the heart diseases and it has been demonstrated that quercetin could work as neuroprotective factor in the onset of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease [37]. In contrast, since the aglycone form of the quercetin has been used in cell culture studies, the researchers found difficulties in extrapolating information from *in vitro* experiments to compare to *in vivo* conditions. The biological activities of quercetin have been extensively reported such as anti-proliferation effects and the induction of apoptosis in cancer cells [38]. Nevertheless, the effect of quercetin on cells is hardly investigated and it seemed to be dependent on the concentration and on the cell type. For example, Spencer et al. [38] studied the possible metabolism of quercetin in human dermal fibroblasts and they demonstrated the abilities of quercetins to protect cells from the oxidative damage. Generally, the quercetins are subjected to glucuronidation and sulfation reactions after the intake. The glycosidic groups are cleaved by intestinal enzyme before subjecting them to glucuronidation and sulfation processes [35]. Spencer et al. [35] evaluated the adsorption of flavonoids in isolated preparation of jejunum and ileum from the rat small intestine. In the case of quercetins, the authors are able to find quercetin-3-glucoside, the hydrolysis product of the quercetin-3-glucoside, and rutin in jejunum. The rutin seemed to be absorbed in the original form [35]. In contrast, no quercetins are found in the ileum. It has been described that the glycosidic forms are cleaved by the microflora of the large intestine, and then they are absorbed. The glucuronidation of the flavonoids may be occurred both on the A-ring and B-ring in the flavonol structure. The glucuronidation occurred on the

B-ring seemed to be more influential than on A-ring, thus leading to decrease the antioxidant activity of the flavonol [35]. Boyer and Liu [33] reported that apples contained quercetin monoglycosides and rutin, which are generally less available to be absorbed. They evaluated the absorption of quercetin and quercetin-3-glucoside extracted by apple peels and the accumulation of these compounds is hardly observed in Caco-2 cells [33].

Dihydrochalcones: apples are an important source of dihydrochalcones and phloretin is the most abundant dihydrochalcones. The chemical structure is 2,6-dihydroxyacetophenone. Additionally, there is another interesting compound called phloridzin, which is a glucoside of phloretin where a beta-D-glucopyranosyl is attached at position 2' of phloretin. Vrhovsek et al. [15] found phloretin-xyloglucoside in the whole apple. The dihydrochalcones undergo chemical transformation by production phloretin glucuronide and phloretin sulfate—derived and these metabolites may be used as biomarkers of apple consumption [16]. The most important dihydrochalcones found in apples are shown in the Figure (1.7).

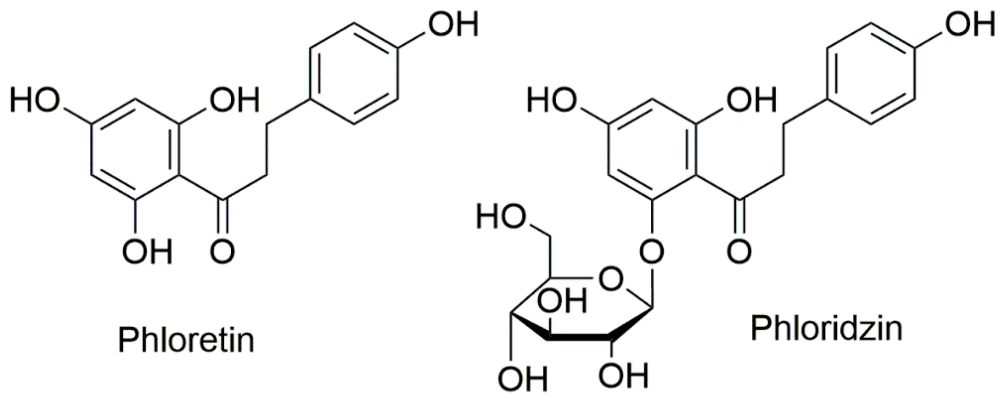

FIGURE 1.7. The main chemical structures of dihydrochalcones in apples. © 2020 by the authors. Licensee MDPI, Basel, Switzerland [39].

It has been reported that the phloridzin may be metabolized by intestinal hydrolases and the resulting aglycone is absorbed by the intestinal cells. The absorption occurred after the hydrolysis, which is conducted by lactase phloridzin hydrolase. As mentioned before, the glucuronidated and sulfated metabolites are the most common products that

may be found in the plasma [33]. Thus leading to an instability of the phloridzin because the O-glycosidic bond is hydrolyzed by intestinal glucosidases. In contrast, phloretin is well absorbed. Several studies have defined phloretin and phloridzin as inhibitor of the GLUT transporters and SGLT1 and SGLT2, the sodium glucose transporters (SGLTs) [40, 41]. GLUT transporters are able to move the glucose by following the concentration gradient, whereas SGLTs transporters collect glucose against the concentration gradient by pumping sodium in of the cells. SGLT1 is a high-affinity/low-capacity transporter and SGLT2 is a low-affinity/high-capacity transporter. In a renal tubular epithelial cell, the GLUT transporters are found in the basolateral side, whereas the SGLT2 are in the apical brush border membrane [41]. It has been widely studied that the SGLTs hardly compensated the high levels of the glucose in diabetics leading to the glucosuria. To date, the studies are focused on the inhibition of SGLTs to reduce the levels of glucose in patients with a hyperglycemic condition. The phloridzin is currently used as a prototype. Indeed, it has been shown that phloridzin was able to block the uptake of glucose into the brain, such as in the glucose-sensitive neurons in the hypothalamus [40].

1.5 Anthocyanins

The anthocyanins belong to the flavonoid group of polyphenols and they play an important role in attracting animals to promote the pollination process and in defending plants from ultraviolet (UV)-induced damage. The most common anthocyanins found in the plant-based food are cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin [22]. The main chemical structure of anthocyanin is C6-C3-C6 ring system of two hydroxysubstituted benzenes (A and B rings) linked to a C ring called a heterocyclic ring. The C ring is linked to A and B by a three-carbon bridge. The most important anthocyanins found in apples are shown in the Figure (1.8).

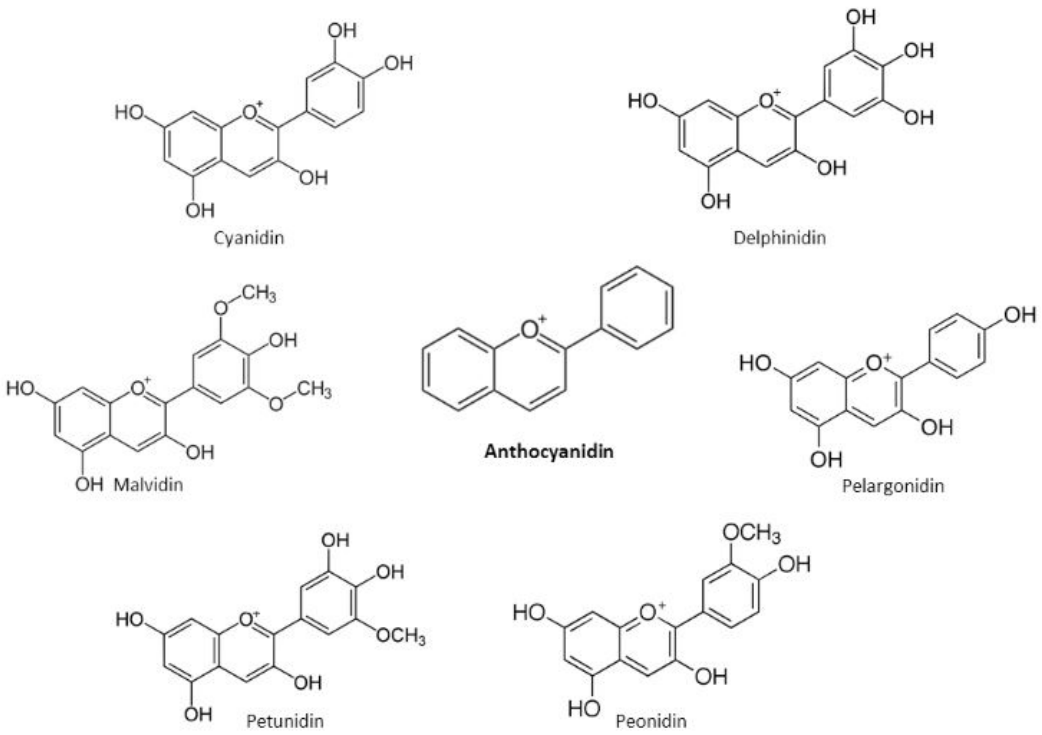

FIGURE 1.8. The main chemical structures of anthocyanidins in apples, the aglycones of anthocyanins. © 2020 by the authors. Licensee MDPI, Basel, Switzerland [24]. (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

The pH has a strong influence on the stability of the anthocyanin structure, indeed, the color of the solution changes in relation to the pH. At low pH, the solution is pink, purple at neutral conditions, greenish-yellow in basics, colorless in alkaline conditions. The common form of an anthocyanin consists of a sugar moiety linked to the hydroxy group of C3 to form 3-O-glycosides, such as glucose, rhamnose, galactose, xylose, arabinose, and rutinose. Other possible position may be in C-5 or C-7 [42]. The diglucoside derivatives may be 3,5-glycosylated and 3,7-glycosylated derivatives [42]. Additionally, the acyl group is added to the glycosyl moiety during the synthesis of these pigments in the citoplasm or in the vacuole of the plant-based food cells.

Anthocyanins are found and quantified in several vegetables, fruits, and beverages

such as: tomatoes, purple potatoes, eggplants, berries, apples, and wine [43]. Focusing on apples and potatoes, which are the major subject of this work, these plant-based food are characterized by differences in genetic traits that contribute in determining diversities in the phenotype [22]. The different concentrations in the amount and in the types of anthocyanins are determining factors in the levels of pigmentation to obtain partially or totally pigmented food [22]. Based on the anthocyanin backbone, the colours of the food tissues change. Oertel et al. [44] reported that pelargonidin has a orange-red shade, cyanidin has red shade, peonidin has pink shade, delphinidin has bluish-purple shade, petunidin has purple shade, and malvidin has reddish-purple shade [44]. The presence of specific anthocyanins contribute to give to the tissue a coloration patterns, which are high variable and the color of the food flesh may differ from the color of skin flesh [44].

After consuming anthocyanin-rich foods, the studies about the metabolism of anthocyanins are still controversial because there is a lack of evidence to support *in vivo* studies and these molecules are strongly susceptible to various factors of the human metabolism, such as temperature and pH. Jokioja et al. [22] reported in their comprehensive review the absorption, metabolism, distribution, and excretion of acylated and nonacylated anthocyanins.

In the recent years the interest in the anthocyanin-rich foods has increased and the anthocyanins are currently and commonly found in the human diet. Several studies reported that the intake of anthocyanins may improve the human health status by decreasing the risk of the onset of certain chronic diseases, such as cancer, cardiovascular, metabolic and degenerative diseases. The antioxidant proprieties of natural pigments are linked to their chemical structure, which is characterized by hydroxyl groups, the catechol moiety in the B ring, the oxonium ion in the C ring, hydroxylation and methylation patterns and acylation and glycosylation [42]. The production of ROS is a normal activity that occur in the human metabolism, however, when the ROS increase, the body functions

are altered and the cellular damage is promoted. Additionally, anthocyanins have a positive effects on ocular health status, glaucoma and ophthalmic. Furthermore, the most significant benefits are the potential antidiabetic activity, indeed, the intake of anthocyanin-rich foods contributes to normalize elevated glycemia and improve serum insulin level. This propriety is linked to the inhibition of the intestinal α-glucosidase by decreasing the liberation of glucose from the carbohydrates [43, 45], however, studies that simulate the *in vivo* conditions are still lacking [42]. Further health benefits are assigned to anthocyanins, such as cardiovascular protective effects, antimicrobial activity, positive effects against age-related cognitive decline, and anticancer proprieties [42].

1.6 The biosynthesis of flavonoids

The flavonoid pathway is extensively studied and reviewed and they are produced by the phenylpropanoid metabolic pathway. The initial step in the biosynthesis of flavanoids is the production of 4-coumaroyl-CoA using the amino acid phenylalanine by the phenylalanine ammonia-lyase (PAL) enzyme [31]. Then, three molecules of malonyl-CoA are condensed to 4-coumaroyl-CoA to generate backbone of flavonoids, called chalcone (2', 4', 6', 4-tetrahydroxychalcone) by the enzyme chalcone synthase (CHS) [31]. The isomerization of the chalcone produces flavanone by chalcone flavanone isomerase (CHI). From these metabolite, several subclasses of flavonoids are produced by different branches [31]. Based on the involved group of enzymes, such as isomerases, reductases, hydroxylases, which are present in the plants, the basic flavonoid skeleton is modified and the pathway diverges forming several subclasses [46]. The most studied branches of flavonoid pathway are the anthocyanin and proanthocyanidin pathways [31], which share the same precursors and some metabolic intermediates. The action of dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) determines the production of anthocyanin and proanthocyanidin (catechins and epicatechins) [46]. Therefore, the conversion of

flavan-3,4-diols to 3-OH-anthocyanidin metabolites by ANS is the crucial point in the synthesis of anthocyanin and proanthocyanidin [31]. The accumulation in the plant tissues of anthocyanin and/or proanthocyanidin may be encoded by several factors (e.g. environmental, genetic, or stress stimuli) [46]. The activity of enzymes determines the different accumulation of flavonoids. The production pathway of anthocyanins involves the synthesis of dihydrokaempferols, dihydroquercetins, and dihydromyricetins into anthocyanins with pelargonidin, cyanidin, and delphinidin backbones, respectively. The most common anthocyanins are cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin [22].

Therefore, the investigation in the flavonoid biosynthetic pathways is a crucial point to gain insights into the metaboloma of plant-based food.

The data presented in this thesis provide valuable information on the flavonoid metabolites in several plant-based food. We aim to explain quantitative differences of flavonoids using high precision analytical approaches and bioinformatic tools to elucidate the metaboloma of local plant-based EUREGIO foods.

1.7 Food Metabolomics: an overview

The food Metabolomics has recently risen as discipline for investigating food composition, safety, adulteration and quality. Since the metabolomics has been linked to reflect the physiological status of cells and tissues, the overall description in the composition of the food can be elucidated. It is well-known that the environment conditions and growing resources influence the accumulation and occurrence of metabolites. The analytical range in which the metabolites could be found is variable, indeed the size of molecules vary between 100-1500 Da and the concentration covers a huge range from the picomolar to millimolar [47]. Metabolomics is widely used to gain insight into the metabolic characteristics in food to assess the quality of what we eat. The metabolome of a food is

informative regarding the phenotype, the physiological status, the geographical origin, traceability and quality. As reported by Kim et al. [48], the food metabolomics is applied in food resources for identification of geographical origins, food intake to find important biomarkers in defining relationship between the consumption of a specific food and the human body and the processing protocol in the industry, as well as to investigate product quality, safety, organoleptic proprieties, and the effect of processing and storage technologies.

Therefore, the food Metabolomics is one of the most important field and its applications cover a huge number of areas. In detail, the object of the studies in the food Metabolomics are the metabolites, which are involved in the growth and the development of plants. The food Metabolome covers a huge range of classes of molecules, namely sugar, phenolic compounds, pigments, amino acids, steroids, amines, and carbohydrates. An interesting point of view is the determination of the relationship between environmental conditions, food consumption and possible variations in metabolic pathways in the human metabolism to define the nutritional and nutraceutical values of the food. The workflow of the food Metabolomics includes the selection of the sampling areas, the extraction procedures to extract the bioactive of compounds of interest, the choice of a suitable analytical platform to analyse the metabolites, the statistical analysis to handle the data, and the interpretation of the results in order to assign biological meanings. The variations in chemical profile and in the concentration of metabolites offer conceivable outputs in the metabolome, where the metabolic pathways are the inputs of the chemical reactions occurred.

1.7.1 The analytical platforms

The metabolome could be evaluated and measured by several instruments and tools to gain insight into more suitable information. It is suggested that the metabolites

could be identified and quantified by several procedures to obtain a summary of the physico-chemical situation. Generally, the instruments respect important criteria in food Metabolomics evaluation and investigation, namely reproducibility, high recovery, robustness, sensitivity, and sensibility. The most common platforms applied for the quantification and identification used in food Metabolomics are nuclear magnetic resonance (NMR) [49], liquid chromatography (LC) and gas chromatography (GC) coupled to a mass spectrometry (MS) [50, 51]. A better understating could be achieved by the implementation of a tandem MS (MS/MS) or MS^n obtain by a triple-quadrupole (QqQ) analyzer. Furthermore, the identification capabilities could be improved by high-resolution by time-of-flight (TOF), quadrupole time-of-flight (Q-TOF), and Orbitrap analyzers. The combination of these analytical tools allow us to perform and give insight into the metabolome and the metabolites are extensively profiled. To obtain a comprehensive profile of a metabolome, the first crucial step consists in the identification of the metabolites and this process could be a bottlenecks in food metabolomics, due to the instrumental response and the complexity of the food matrices. A good compromise is achieved by the application of ultrahigh pressure liquid chromatography (UHPLC), where an analytical column is used for the separation of multiple metabolites. These columns are made by a silica-particle-based stationary phase and the surface of the silica may be modified by various groups of non-polar or polar compounds to improve the interaction between analyte of interest and the stationary phase of the column. The choice of the column is connected to the properties of your target analytes. Another important factors is the mobile phase, which is flushed through the column. Then, the separation is achieved by the higher or lower affinity of the analyte towards with mobile or stationary phase. The analyte may have a different chemical attractions for the stationary phase and the separation occurs, which is based on the strength of chemical bounds that are formed by the metabolite and column. The retention time (RT) consists in the time taken by the

analyte to pass through a chromatography column and different metabolites normally have different RT. After the performed separation, the analyte is sent to the selected mass spectrometer, where the structure of a molecule is elucidated by its characteristic fragmentation pattern. The basic principle of mass spectrometry (MS) is to generate ions from the molecules, but also separating molecules by their mass-to-charge ration and to quantify them, which are coming from the LC. The analyte is separated by their mass-to-charge ratio (m/z) and the quantification and identification are carried out using respective m/z and abundance. A mass spectrometer consists of an ion source, a mass analyser, and a detector which are operated under high vacuum conditions. The mass spectrum is the result of a Mass Spectrometry analysis, which is graphically represented by signal intensity (ordinate) versus mass-to-charge ratios (m/z, abscissa). The peak is positioned based on its m/z, which is an ion that it is created from the analyte within the ion source. The intensity of the peak may be correlated to the abundance of that ion. Generally, the highest peak characterized by the highest m/z is called the molecular ion, M+•. The other peaks represent the fragment ion peaks. The most intense peak in a mass spectrum is known as base peak and all of mass spectral data are normalized to 100% relative intensity. The intensities of the peaks are presented as either peak heights or peak areas. The position of the signal, the m/z ratio, is determined from its centroid. As reported by Castro-Puyana and Herrero [52], the QqQ is defined by European Union (EU) Commission Decision 2002/657/EC as a powerful instrument to detect abolished or admitted substances in food. First of all, the analyte of interest may be compared to the its corresponding analytical standard by the RT. The QqQ analyzer is able to detect simultaneously a precursors ion and two subsequential ions (also called fragment ions. The fragments may be obtained by collision-induced dissociation (CID) and it is optimized of each analytical compound, which may be directly infused in the mass spectrometer. The triple quadrupole mass spectrometer (QqQ) is made of two mass filters (Q1 and Q3),

which contains four parallel, cylindrical metal rods. Q1 and Q3 are controlled by direct current (dc) and radio-frequency (rf) potentials. The collision cell, q2, is subjected to RF potential and it allows all ions to pass through the q2, knows as CID, as reported in the Figure (1.9).

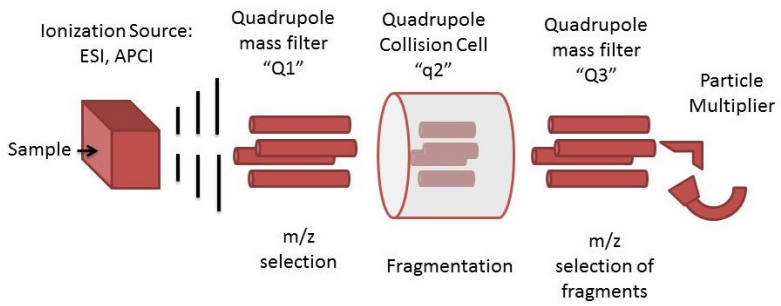

FIGURE 1.9. Schematic of a triple quadrupole mass spectrometer. © Wikipedia® [53]. (CC BY) license (https://creativecommons.org/compatiblelicenses).

The multiple reaction monitoring (MRM) is the most common quantification method where two fragments are generated by the parent ion. The most abundant one is used for quantification purpose, while the less abundant and their ratio are used for qualification purpose. The efficiency of UHPLC-MS is determined by the short time taken for carrying out an analysis in food Metabolomics. By using ultra-high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-QqQ-MS/MS) in MRM mode is possible to gain insight into a sensitive, precise and fast quantification of multiple molecule. To provide quantitative measures and characterization of locally grown food, our studies involves the utilisation of a powerful method: the UHPLC-QqQ-MS/MS.

1.7.2 Targeted and Untargeted in food Metabolomics

The coupling of different disciplines may be potentially used for providing more suitable information about the metabolome by offering a more comprehensive elucidation in the nutritional value of a food. Generally, the food metabolome is determined by the

evaluation of the global situation in the expression of metabolites. In accordance with the literature, the targeted analyses are focused on selected small molecules or a group of metabolites. The analytes of interest are identified by retention time and MRM mode. The publications are still increasing about the utilization of targeted analyse [54]. The targeted analyses allows to achieve a exhaustive characterization of specific compounds. The main advantage of targeted metabolomics is that the application of reference standards allows an accurate quantification and quantification of a selected subset of known molecules. Additionally, accuracy, selectivity and sensitivity are the most important features in these analyses, and since the user knows which molecules are being measured, the interpretation of data is easier to connect them in a biological meanings. Indeed, the optimization of chromatographic condition and MS values is carried out using a pure analytical standard. In contrast, to obtain a comprehensive study, the untargeted analysis is useful to reveal novel compounds and chemical unknowns. In untargated metabolomics the number of metabolites is larger than in targeted studies, therefore, the capability to obtain a more complete profile in a vegetable matrix is gained. A common strategy is to apply MRM mode in targeted metabolites where a molecular ion of a specific metabolite is selected, and then, it is fragmented.

Using analytical standard, it is possible to build a standard calibration curve of each analyte and then, the concentration of the specific metabolite in the vegetable matrix is calculated by interpolation. Quantitatively, the targeted metabolomics provides a in-depth insights into the compounds are likely present in a specific type of matrix. The quantification of metabolites helps in elucidating which compounds may be useful to distinguish samples within a group, or between groups of molecules. The major difference between targeted and untargeted is that the targeted measurements provide more accurate absolute quantification. It is reported that the targeted metabolomics is a hypothesis driven experiment [55].

1.7.3 Statistics

In targeted food Metabolomics, the user is focused on a particular metabolic pathway in which the involved metabolites are known. The appropriateness of a metabolite as biomarker has been already demonstrated in the literature to recognize characteristic features between groups [15]. Indeed, variation in the concentration of metabolites is widely reported as an important correlation between the levels of phytochemicals and the general status in a population. To date, it is possible to measure a huge number of metabolites simultaneously by using high-throughput instruments and tools. This is leading to an increment in the complexity of data matrices, which are obtained from different sources. The main challenge in the food Metabolomics field is to assess a biological meanings and interpretation of complex interaction from the analytes. Robust statistical methods are applied in food Metabolomics to answer to these questions. The analysis of high-throughput metabolomics data requires sophisticated statistical methods to visualize and summarize the results. This approach may be potentially used for highlighting differences in the levels of bioactive compounds in a group or between two groups to extract deductions and conclusions about the property of a data matrix [55]. The most common issue in statistical sampling is the definition of a "statistical sample", which is a subset of individuals from a within statistical population to obtain information about the features of the entire population. The most important factor of a statistical sample is the property of representativity to asses the suitability of the conclusion. When a statistical sampling procedure is drawn, the samples should be randomly chosen by a population distribution. Indeed, the assumption provides for sample distribution that adequately reflects the population distribution.

An important aspect in the statistical analysis is the distribution of the data around a value. The most relevant assumption is that the data are normally distributed. The normal distribution is driven by two parameters, which may be estimated: the mean

μ and the variance (the standard deviation) σ^2, or $\overline{\chi}$ and s^2 respectively. Nevertheless, since the analyses are subjected to measurement errors, which may influence the normal distribution of the data, a logarithmic transformation of the data is useful to respect the assumption.

The most common strategy in food Metabolomics is to apply univariate techniques based on the analysis of parameter-by-parameter in a group or between two or more groups (e.g. t-test and analysis of variance (ANOVA)). Nevertheless, in the common laboratory approach, the samples are extensively analysed to obtain more variable. Sometimes, the number of variable measured are more than the samples. An important feature may be to find the dependency between the variables [55]. In the analysis of metabolomics data the visualisation of a multitude of data is crucial, which are acquired by different analytical instruments. In the food Metabolomics the aims are to find differences or similarities between samples and to capture grouping features. The most popular of multivariate techniques are principal component analysis (PCA), partial least squares regression (PLS) and Data Fusion technique. All of these statistical tools may be applied to visualize large data sets, in particular when a lot of variables are acquired and quantified.

Principal component analysis (PCA): the PCA is a *unsupervised* method that is useful to compress the data obtained into a lower-dimensional data, without losing the variation in the data. In the PCA process, the variables are combined into principal components (PCs), in which the first PC captures the maximum variation possible. In most cases, more than one PC is needed, indeed the choice fells on the second component that explains the remaining variation. These two PCs are constructed by following the orthogonality between them and the amount of variance described. The PCs may be chosen by the user in order to capture the maximum variation possible. The data are

decomposed into a data matrix made up of rows corresponding to samples, and column corresponding to variables by obtaining three matrices. This decomposition is called singular value decomposition (SVD). Then, two matrices are extracted: the score matrix and loading matrix. The scores are representative of the position of the observations in the new coordinate system, which is characterized by the data transformed into scores. The loading reports the contribution of each variables into the PCs system. The plots used for reporting the score and loading matrices are called score and loading plots, in which the axes reports how much variation is described by the chosen PCs. The score plot is useful to show the position of the samples in the two first PCs and to capture grouping or metabolic pattern. The loading plot reports the contribution of each variable measured in the two first PCs. The variables are visualized as arrows that start from the origin. In detail, if the arrows point in the same direction, they are highly positive correlated. In contrast, a negative correlation is found when the direction of the arrows is opposite. Furthermore, the length of the arrow is determining how much the variation is described by a specific variable in the PCA. Score and loading plots may be combined to create a bi-plot, where the above-mentioned information is displayed.

The PCA reflects the structure of data and it is dependent on the treatment of the data. The scope of PCA is to show the dimensionality of data and to visualize the data. Being the PCA an exploratory tool, in food Metabolomics is used as a starting point to visualize the data. It is worthwhile applying mathematical operations to make the interpretation of data more suitable. When the data are obtained from mass spectrometers, in which the intensity of the peaks are your results, a common approach is to log-transform the data, in which each measurement called X is replaced by log(X). The purpose of this method is to achieve a better symmetric bell-shaped distribution in the data. Unfortunately, the outcomes, obtained by the analytical tools, hardly follow this assumption due to the complexity of the food matrices. This mathematical procedure

is left up to the user, based on the purpose of the subsequently statistical modelling. The log-transformation is useful to make data closer to the normal distribution, thus reducing or removing the skewness of the original data [56]. Another advantage in the application of log-transformation is to reduce the variability in the data due to the presence of outliers [56]. Subsequently, the mean-centering procedure is mandatory before submitting the data to the PCA. The mean-centering consists in the subtraction of the average value from each measurement and this process is called *column centering* due to the data matrix as a table. Furthermore, the autoscaling may be used, which is characterized by a mean of zero and a standard deviation of one. This process is adopted by the user when some variables may become dominant due to the greater quantity quantified. Thus, all variables have the same importance. Additionally, based on the aim, there is Pareto scaling as an alternative, in which variables with a high variance are more significant than variables with a low variance.

Partial least square regression (PLS): is a multivariate regression model and it is a *supervised* method. This approach has the capability to find a linear regression model by projecting the predicted variable and the observed variables. The PLS regression model finds the relationship between two data matrices X and Y to capture the maximum covariance between them. The maximum multidimensional variance direction in the Y space is explained by the multidimensional direction in the X space. Then, Y is a numerical variable, the field is the regression model. Otherwise, it is a classification model. The idea is to calculate the scores of X and Y [57]:

$$X = TB + E \tag{1.1}$$

$$Y = UQ + F \tag{1.2}$$

Equations (1.1) and (1.2) where X is a matrix of predictors $n \times m$ and Y is a matrix of responses $n \times p$. T and U are the projection of the *scores* of X and Y, respectively. B and Q are matrices of orthogonal *loading* matrices. E and F are the error terms. The algorithm of PLS is able to find the linear regression between X and Y [58].

Generally, the aim of PLS regression model is to find a particular metabolite profile that it is linked to the response. A typical application of PLS regression model as a prediction model is to predict a chemical compositions from spectral measurements (e.g. NIRS, IR, UV) [59]. Nevertheless, the PLS model tends to over-fitting, then, the selection of the optimal number of latent variables (nLVs) is a crucial step [60]. By definition, when the model describes the noise, instead of the connection between predictors and observations, is called over-fitting [60]. In contrast, another example of over-fitting is linked to the capacity of the model in memorizing the data and, thus leading to generalize the results. It is well-known that PLS is able to "learn" from the data due to the capacity of the model in remembering the data. A strategy to select nLVs is to use the cross-validation (CV), which tends to choose the model characterized by the lowest prediction error [60]. The CV is useful in selecting the smallest nLVs that provide the highest capacity in prediction. The CV procedure provides for leaving out a part of the data as test set, and the remaining is used for building the model, the training test. A good practise is to repeat building models by selecting different samples to compose the test set and training test, thus leading to calculate the cross-validation error. There are different CV procedures, namely, leave-one-out cross-validation (LOOCV), K-fold CV, Monte Carlo CV (MCCV), and repeated double cross-validation (rDCV) [60].

The performance in the prediction ability of the PLS model is obtained by the calculation of root-mean-square error of CV (RMSECV) or the coefficient of determination of CV, called Q^2. The goodness of the capacity in prediction of the optimized model is evaluated on the held-out test set. The range of the values is between 0 and 1. Ideally, the highest

value of Q^2 is indicating a goodness in selecting the optimal model.

The extraction of the regression coefficients in B (1.1) is an important aspect, which may contribute in understanding the importance of single features [57]. Indeed, the magnitude of the absolute values of the predictors is a crucial indication in evaluating which predictors are more suitable for the prediction [57].

Data Fusion: another powerful statistical tool that may be used in food Metabolomics is the *data fusion* procedure. In this case, the measurements are conducted on different analytical instruments, but using the same set of the samples. All of the databases are studied together to gain insight into a possible biological background. This method is useful when the user wants to conduct an in-depth investigation about the possible existing interconnection between different analytical measurements. Indeed, a common strategy in food Metabolomics is to analyze the matrices using various platforms e.g. LC-MS, GC-MS, NMR, and spectrophotometer. The combination of all these results may provide more information about similarities and differences between the samples. The data fusion model is a quantitative method that may be applied on a set of samples, which are measured by different analytical tools. The applications may be several [61]:

- *inter-platform*: the samples are analysed by several analytical instruments.
- *inter-sample*: different status/compartments of a sample is analysed by a metabolomics platform.
- *inter-omics*: the combination of the above-mentioned methods.

Since the number of variables acquired may be different between the analytical tools, a preprocessing of the data by scaling is required. Therefore, the variation obtained from one platform is not masked or dominated by the other analytical tool [61]. The data fusion may be used as exploratory tool to visualize possible clusters, outliers, or behaviour

patterns characteristic within or between groups. Additionally, it is possible that specific features may be determining in finding distinctive feature as biomarkers. Furthermore, an interesting application is to look for correlations within different datasets to study common and uncommon element between and/or within them. Multiple data fusion methods could be applied on different datasets, however, we decided to evaluate the efficiency of the Joint and Individual Variation Explained (JIVE) model to analyze distinctive and common parts within the datasets. The JIVE model works by estimating simultaneously which are the most important components shared between the datasets. The associations between different data types are explored and the relationship among them may be evaluated. Furthermore, each data types could be characterized by unique information useful to identify the distinctive components. The scaling mathematical procedure is mandatory, when the variables measured differ within the analytical tools used. The JIVE model is defined as an extension of PCA, in which the data types are decomposed into three terms: a low-rank approximation capturing joint variation across sources, low-rank approximations for structured variation individual to each source and residual noise [62]. A permutation test is used to estimate the ranks [62]. As in PCA, loadings and scores are calculated and they allow us to explain the common and distinctive parts without confusing them. An important aspect in JIVE is the orthogonality between the distinctive and common parts where the total variation is explained by the common and distinctive parts. The loadings and scores may be reported as plots just as happens in PCA plots. The common part captures the overall situation amongst the data sets, whereas the individual parts are evaluated as blocks separately. The individual part is orthogonal to the join part. The amount of the explained variation may be taken in the common part or in the individual part. The user has the possibility to evaluate the common part separately by the individual part, and each individual part may be consider as a separate block. Furthermore, the common and individual parts

could be combined to capture the explained variation between them. By analyzing the individual part, the useful information of each data type is taken into account. The combination of the common part, as a block, and one of the individual part is used to find an accurate estimation of what the selected individual part has in common (or not) with the common part [63].

CHAPTER 2

FIRST CONTRIBUTION

2.1 Preface

For the purposes of this research, I will present in this chapter 2 the results regarding a specific study conducted on the metabolic profile of 22 apple cultivars. I conceived the conceptualization and writing phases of this scientific contribution, and my interest is towards the data processing and the data analysis of the entire following study. My practical work is focused on the application of targeted analysis using the liquid chromatography coupled to the mass spectrometer to profile the metabolome of 22 apple varieties, thus giving in-depth information about the variation, in terms of concentration, in different apple cultivars. I applied the partial least square regression (PLS) approach to investigate a possible correlation between the metabolic profiles found in the apple pulp and apple skin for each variety under investigation in order to predict the antioxidant activity based on the polyphenolic profile.

Our investigation provides cultivar-specific metabolic profile of polyphenols in the pulp and antioxidant activities, polyphenolic content and total anthocyanin content

in the peel of 22 apple cultivars. The peel of each variety was characterized both on the sunny and the shady sides. All cultivars are true to type, accurately verified using molecular genetic tools. To reduce the effect of the environment on the metabolic profiles, all apple varieties were cultivated on M9 rootstocks in the same identical environment and agronomic conditions. The varieties were grouped into three groups: commercial, red-fleshed and, old, which were compared considering the metabolic pathway of polyphenols and anthocyanins. Moreover, a multivariate regression approach (PLS) was applied to investigate the cultivar-specific association between pulp and peel profiles.

Our results show that the variation in the concentration of phytochemicals is less pronounced in the old group, contrary to what is observed in the commercial apple varieties that demonstrate an extreme behavior. Surprisingly, the metabolome of red-fleshed cultivars is also extremely variable, even though this group of varieties is characterized by a strong phenotypic similarity.

The metabolic results of this work can be used to better define the overall quality of the local plant-based EUREGIO foods, with a special mention to 22 different apple cultivars that are grown in the same environment and agronomic conditions. These findings are significant for experiments studying the different chemical profiles of apple varieties in order to assess their benefits for the human health. A better understanding of chemical profiles of apples may lead to improved selection of specific apple varieties in order to drive the selection based on the bioactive compounds.

I believe that our findings could be used to stimulate the consumption and the diffusion of different apple varieties, encouraging at the same time the valorization and preservation of local cultivars. By reporting a new comparison data on the apple metabolome, we strongly believe that our findings would contribute proficiently to scientific community.

2.2 Metabolomic characterization of commercial, old, and red-fleshed apple varieties

metabolites

MDPI

Article

Metabolomic Characterization of Commercial, Old, and Red-Fleshed Apple Varieties

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Abstract: In this study, a metabolomic investigation was presented to correlate single polyphenolic compounds in apple pulp with quality characteristics such as antioxidant activity and content of phenolic compounds and anthocyanins in apple skin. Since the concentration of these compounds is influenced by environmental factors, the twenty-two apple cultivars originate from the same site. The polyphenolic compounds were analyzed by ultra-high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-QqQ-MS/MS). The antioxidant activity, phenolic content, and anthocyanins were evaluated on the sunny and the shady sides of apple skin by spectrometric assays. In old apple varieties, the measured parameters were higher than in the commercial and red-fleshed varieties. By contrast, the profile of flavan-3-ols and anthocyanins was variable amongst commercial and red-fleshed varieties. The partial least square (PLS) method was applied to investigate the association between the skin proprieties and the metabolic profile of the pulp. The highest coefficients of determination in prediction (Q2) were obtained for compounds quantified in old cultivars. These results provided information to define the old apple varieties as a reliable group based on the pathway of the antioxidant compounds and anthocyanins content. Our results show the possibility to find cultivars with promising health features based on their content of polyphenols suitable for commercialization or breeding.

Keywords: metabolomics; antioxidant activity; polyphenols; mass spectrometry

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1. Introduction

Chronic diseases, cardiovascular disorders, cancer, lack of physical activity, and obesity are well known threats for public health [1]. The WHO (World Health Organization) reports that the number of deaths in the world linked to these issues is dramatically increasing due to environmental degradation, malnutrition, and behavioral risk factors [2].

Regular consumption of vegetables and fruits has been continuously linked to a health-promoting lifestyle [3,4]. The WHO encourages a minimum of 400 g of fruit and vegetables per day [1]. Indeed, fruits and vegetables are essential sources of a large class of valuable compounds such as polyphenols, vitamins, and minerals [5–7].

Apples (*Malus* × *Domestica*) are 12.5% of all consumed fruit in the world [8], and it is widely accepted that they are beneficial both to consumers in good health, and consumers with specific pathologies such as hypercholesterolemia [8], cancer [4], cardiovascular disease [9], pulmonary disorders, and Alzheimer's disease [10]. Several studies highlighted the potential role of the antioxidants present in apples in preventing the formation of free radicals and reactive oxygen species (ROS) [11], which have been linked to chronic

disease and inflammation. The most important antioxidants in apples are polyphenolic compounds such as hydroxybenzoic acids, esters of caffeic acid and quinic acid, and quercetin, especially in their glycosylated forms. The major class of apple polyphenols is the flavan-3-ols, accounting for 71 to 90% of the total polyphenolic compounds in apples, namely epicatechin, catechin, procyanidin B2, and a large amount of procyanidin oligomers, and subsequently, hydroxycinnamates (4–18%), flavonols (1–11%), and dihydrochalcones (2–6%). The anthocyanins represent 1–3% of the total phenolic compounds and contain cyanidin glycosides (3-galactoside, 3-glucoside, 3-arabinoside, 3-xyloside) [12]. The presence of high concentrations of these beneficial compounds can then be considered a key property in defining the "overall quality" of specific apple cultivars [13], and can be used to direct agronomic practices and specific breeding and selection programs. Another important class of polyphenols is that of the anthocyanins. They are hypoglycemic and decrease the risk of type II diabetes [14].

In apples, antioxidant compounds are distributed in the fruit pericarp (which is often consumed) and in the fruit peel. In both sections, their concentration is dependent on several agronomic, environmental, and genetic factors [15]. For this reason, agronomical and environmental conditions should be kept as uniform as possible to compare different apple cultivars in order to highlight potential genetic patterns [16]. To date, however, as reported by Simmonds et al. [17], there are a few studies that compare apple varieties cultivated in the same environmental conditions with identical agricultural practices.

In this study, the different apple cultivars were categorized according to their phenotypic and commercial characteristics: old, commercial, and red-fleshed. Cultivars belonging to the old group originated between 1900 and 1950 according to Baric et al. [18], but they have lost popularity and consumer acceptance mainly due to deficits in productivity, homogeneity, storability, and/or shelf life [19]. The commercial group gathers the most consumed apple varieties currently available on the world apple market. Finally, the red-fleshed group includes a series of newly bred varieties characterized by a pigmented pericarp. This last group is considered particularly promising for future commercial exploitation for aesthetic and nutritional properties. The varieties present in this study are fingerprinted and validated based on the reference of Baric et al. [18] and they are commercially available [20].

Previous research by Farneti et al. [21] has suggested that the selection process of breeding and domestication did affect some, but not all, the phytochemicals contained in apples. However, also this extensive survey did not consider red-fleshed apples. While there is considerable interest in red-fleshed apples, due to the presence of the beneficial anthocyanins [22], it would be advisable to bring to the market new red-fleshed cultivar having both high concentration of polyphenols and anthocyanins.

In this study, metabolomics was applied to characterize the polyphenolic profile of 22 apple cultivars belonging to the varietal collection of Laimburg Research Centre (Auer (Ora), Italy). All cultivars were true to type, accurately verified using molecular genetic tools [18]. To reduce the effect of the environment on the metabolic profile of the cultivars, all apple varieties were cultivated on M9 rootstocks in the same identical environmental using agronomic conditions. The cultivation area Trentino and South Tyrol were chosen for this study because they are market leaders in producing apples in Italy. Furthermore, they supply one tenth of apples in EU member states, with a lot of different varieties [23]. The present investigation focuses on the profiling of the antioxidant properties and of the polyphenol composition of the pulp and peel of apples. Furthermore, a multivariate partial least squares regression approach was used to investigate the cultivar-specific association between pulp and peel profiles.

Our results showed that the variation in the level of phytochemicals is less pronounced in the old group, contrary to what was observed in the commercial apple varieties that demonstrate an extreme behavior. Furthermore, despite the fact that the red-fleshed apple cultivars belong to red-fleshed group, the concentration of metabolites is found to be extremely variable.

2. Results and Discussion

2.1. Principal Component Analysis (PCA) Used as an Exploratory Tool to Evaluate the Overall Apple Sample Distribution

To determine if the larger fraction of the variability in the concentration of phenolic compounds in apple pulp and the antioxidant activity in the peel could be associated to separation between the three apple groups (old, commercial, and red-fleshed), the full metabolomics dataset was analyzed using a principal component analysis (PCA). The results are reported in Figure 1. The PC1 vs. PC2 projection accounted for 49% of the total variance. In terms of the three apple classes (Figure 1a), the plot indicates that the three groups are separated enough. In particular, red-fleshed apples seem to be partially different from the others along PC1. The plot shows, however, the presence of one subcluster, and one red-fleshed is blended into commercial varieties. Indeed, the position of these three varieties in the PCA is highlighted in Figure 1b.

Looking at the old varieties (yellow color in Figure 1a), a general separation between cultivars was reported. Indeed, highly positive scores on the PC1 axis were observed, whereas the scores for other varieties were strongly negative on the PC2. In general, the loadings of these varieties showed a higher concentration of flavan-3-ols (catechin, epicatechin, procyanidin B1, procyanidin B2, and procyanidin C1) and antioxidant metabolites than the other samples.

The commercial varieties (blue color in Figure 1b) occupied the center of graphs, indicating that the variability within this group was not responsible for a large fraction of the variance of the overall dataset. Despite of this actual trend, Santana (SAN) showed a notable separation from the others commercial cultivars blending with one red-fleshed cultivar.

Regarding the old varieties, Wojdyło et al. [24] reported a good correlation between the antioxidant activity in vitro and the levels of procyanidins/flavan-3-ols. Thus, they supported our results, where a higher proportion of antioxidant and phenolic compounds was found in old cultivars compared to the other groups. Additionally, these results were consistent with other studies [12,25,26].

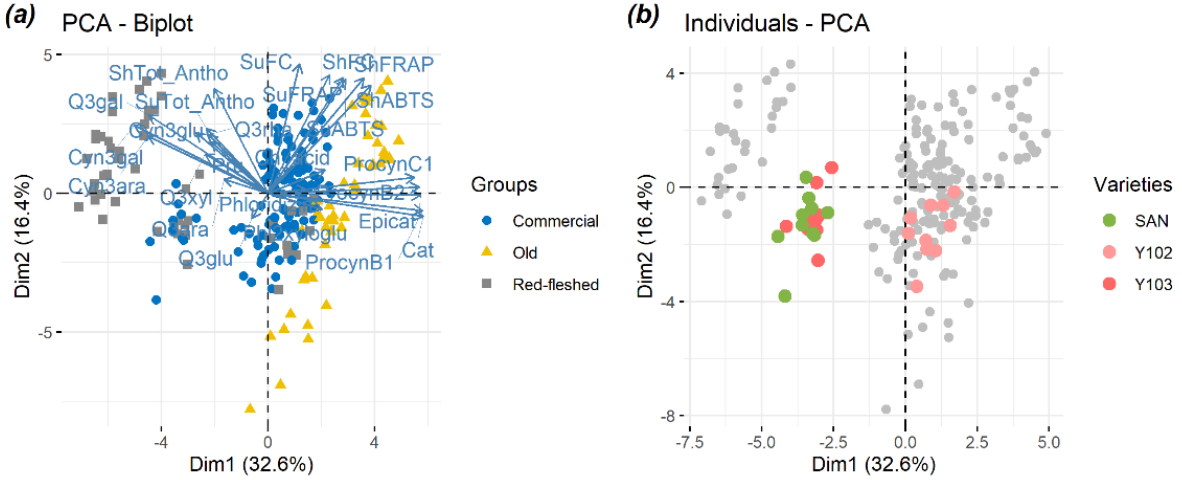

Figure 1. PCA of detected individual polyphenols in the pulp and of the antioxidant activities in the peel of 22 apple varieties. The colors (yellow, blue, and gray) indicate the apple classes (old, commercial, and red-fleshed). Loading is shown (**a**). PCA of the dataset where colors are used to highlight scores for three apple cultivars (one dot per apple shown): SAN, Y103, and Y102. The gray dots represent the other varieties. Scores are shown. (**b**). For apple variety names see Table 1.

Table 1. List of cultivars, code of cultivars, variety group, harvest days, storage days, and sampling days of apples analyzed in this study (d = day, CS = cold storage, SL = shelf life).

Apple Cultivars	Code	Groups	Harvest Date	+10 d CS	10 d CS + 3 d SL
Goldparmäne	GP	Old	20 August 2015	30 August 2015	2 September 2015
Kalterer Böhmer	KB	Old	15 September 2015	25 September 2015	28 September 2015
Kanada Renette	KR	Old	15 September 2015	25 September 2015	28 September 2015
Tiroler Spitzlederer	TS	Old	3 November 2015	13 November 2015	16 November 2015
Weisser Rosmarin	WR	Old	28 September 2015	8 October 2015	11 October 2015
Braeburn	B	Commercial	2 October 2015	12 October 2015	15 October 2015
Fuji (Brak)	F	Commercial	6 October 2015	16 October 2015	19 October 2015
Elstar	E	Commercial	20 August 2015	30 August 2015	2 September 2015
Gala (Simmons)	GA	Commercial	12 August 2015	22 August 2015	25 August 2015
Golden Delicious	GD	Commercial	14 September 2015	24 September 2015	27 September 2015
Granny Smith	GS	Commercial	24 September 2015	4 October 2015	7 October 2015
Nicoter	NIC	Commercial	16 September 2015	26 September 2015	29 September 2015
Lb 17906	LB	Commercial	27 October 2015	6 November 2015	9 November 2015
SQ159	SQ159	Commercial	17 September 2015	27 September 2015	30 September 2015
Rosy Glow	RG	Commercial	27 October 2015	6 November 2015	9 November 2015
Santana	SAN	Commercial	12 August 2015	22 August 2015	25 August 2015
Topaz	TOP	Commercial	21 September 2015	1 October 2015	4 October 2015
Bay 3484	BAY	Red-fleshed (type 1)	13 August 2015	23 August 2015	26 August 2015
Red-fleshed 2/Y103	Y103	Red-fleshed (type 2)	13 August 2015	23 August 2015	26 August 2015
Red-fleshed 3/Y102	Y102	Red-fleshed (type 2)	1 September 2015	11 September 2015	14 September 2015
Red-fleshed 4/R201	R210	Red-fleshed (type 1)	30 September 2015	10 October 2015	13 October 2015
RS-1	RS-1	Red-fleshed (type 1)	20 August 2015	30 August 2015	2 September 2015

As in the case of old varieties, red-fleshed cultivars showed a high degree of separation. As far as the red-fleshed group (gray color in Figure 1a) is concerned, the plot indicates negative scores on the PC1 and highly positive scores on the PC2. Surprisingly, Figure 1b shows that the scores are negative both on the two PCs for Y103 and Y102 and they show an interesting separation from the other red-fleshed cultivars. The presence of different subtypes of red-flesh varieties has been proposed on the basis of phenotypic characteristics (type 1 and type 2; see Section 2.2). In terms of loadings, red-fleshed varieties had a higher concentration of anthocyanins (cyanidin glucosides) and flavanols (quercetin glycosides) than commercial and old cultivars, with Y103 and Y102 being a notable exception (Figure 1b). Indeed, Y103 and Y102 were categorized as red-fleshed type 2, but the concentration of quercetin glycosides, cyanidin glycosides, and flavan-3-ols was extremely variable. In contrast with the studies of Bars-Cortina et al. [27], where they identified that the Rni allele, controlling the red color, co-segregated with the MdMYB10 gene, our findings suggest that the red-fleshed metabolome was extremely variable. This variability led to a different distribution of polyphenolic compounds in apples, even though they are categorized as red-fleshed cultivars. The PCA for metabolites in apple pulp and peel highlighted an interesting negative correlation between flavan-3-ols and anthocyanins. This antagonistic behavior is in agreement with the literature, and can be explained with the biosynthetic pathway of phenolic compounds [28]. Indeed, the synthesis of flavan-3-ols and anthocyanins is regulated by anthocyanidin reductase and anthocyanins synthase, which are responsible for the inverse production of these two major phenolic classes. Furthermore, it is demonstrated that the concentration of anthocyanins is especially dependent on the sunlight exposure [29].

The general trends in the accumulation of metabolites in the cultivars were well understood [12], a closer investigation of flavan-3-ols and anthocyanins was undertaken. Thus, two in-depth investigations were conducted in this study, on the occurrence and

distribution of flavan-3-ols and anthocyanins in apple pulps, and antioxidant activity in apple skin amongst these three variety groups [16].

2.2. Apple Pulp: The Polyphenolic Profile

The flesh being the most widely consumed part of an apple, a detailed characterization of compounds in the pulp was performed. Figure 2 shows the concentration of flavan-3-ols (catechin, epicatechin, procyanidin B1, B2, and C1) and anthocyanidins (cyanidin-3-arabinoside, cyanidin-3-galactoside, and cyanidin-3-glucoside) in the different varieties organized in the three macrogroups. The results of both the quantification and of the statistical analysis are reported in Tables S1 and S3, respectively.

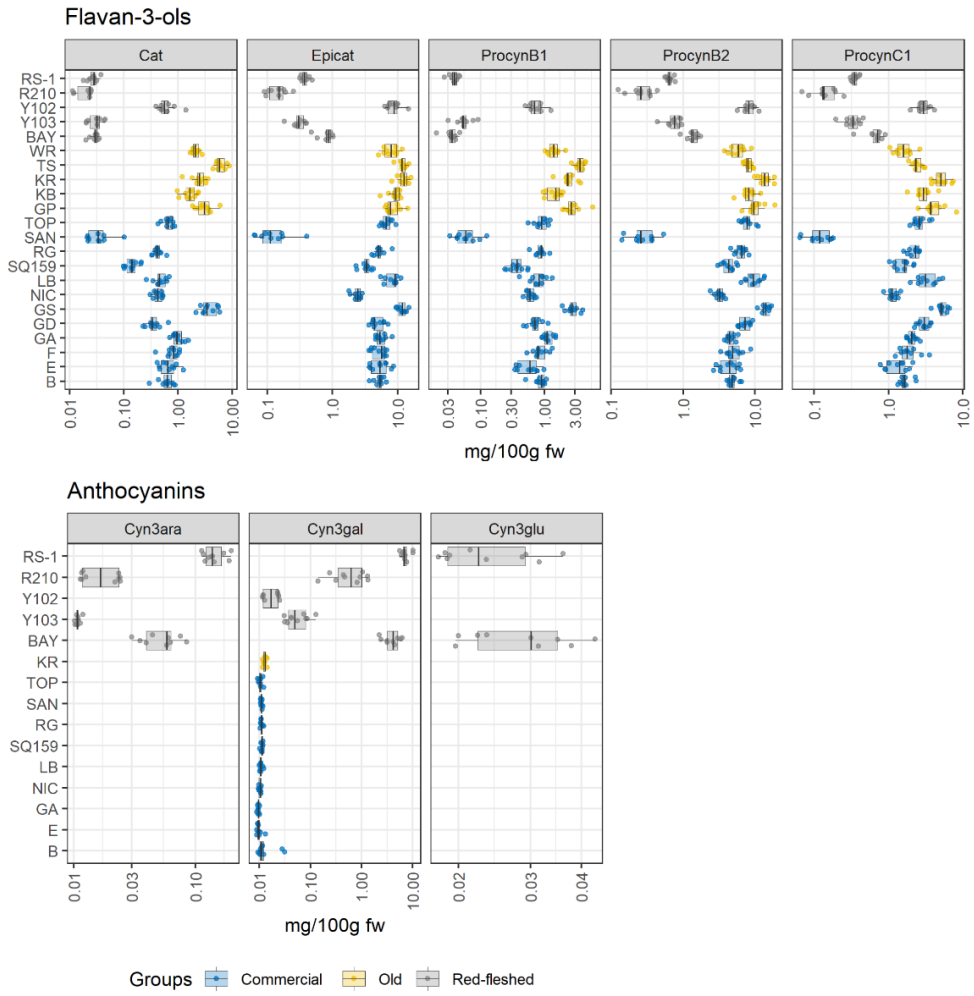

Figure 2. Concentration of flavan-3-ols and anthocyanins in the pulp of 22 apple varieties; 10 apples (biological replicates) per variety were analyzed. The concentrations of these metabolites were transformed in a log-10 scale. Varieties where anthocyanins were not detected have not been included in the graph. Cat = catechin, Epicat = epicatechin, ProcyanB1 = procyanidin B1, ProcyanB2 = procyanidin B2, ProcyanC1 = procyanidin C1, Cyn3ara = cyanidin-3-arabinoside, Cyn3gal = cyanidin-3-galactoside, Cyn3glu = cyanidin-3-glucoside, FW = fresh weight. For apple variety names see Table 1.

The boxplot of flavan-3-ols clearly showed that the concentration of these metabolites was less variable in old apple varieties than in the other groups. In contrast, a marked variability in the concentration of flavan-3-ols amongst the commercial and the red-fleshed groups was observed, where several cultivars stood out. In both classes, the major flavan-3-ols could differ by up to two orders of magnitude within a group. In the case of the commercial group, SAN and Granny Smith (GS) showed the lowest and highest contents, respectively.

The other varieties seemed to show an almost uniform level of these phenolic compounds. As far as the red-fleshed group is concerned, Y102 significantly stood out among the red-fleshed apple varieties due to the highest concentration of all flavan-3-ols. In contrast, Y103 followed the same behavior as the other red-fleshed cultivars.

The boxplot of anthocyanins demonstrated that the red-fleshed apple cultivars contained the highest number of pigments as expected and previously mentioned in the literature [27,30]. The cyanidin-3-galactoside (cyn3gal) was also detected in a few apple varieties belonging to the commercial group and in one old apple variety; however, the concentration was low. Remarkably, cyanidin-3-glucoside (cyn3glu) and cyanidin-3-arabinoside (cyn3ara) were not detected in the red-fleshed Y102 variety. Bay 3484 (BAY) and RS-1, instead, contained the highest amount of all cyanidins quantified, and cyn3glu was only detectable in these varieties. The reason could be linked to the limit of quantification (LOQ) of the analytical methods employed for the analysis of these compounds. Remarkably, the concentration of anthocyanins varied among the red-fleshed apple varieties, even though all these cultivars are grouped as red-fleshed [31].

In summary, the work showed that catechin, epicatechin, and procyanidin B2 were the most abundant flavan-3-ols detected in all groups, followed by procyanidin B1 and procyanidin C1. In contrast, Belviso et al. [19] found lower amounts of catechin and epicatechin in their varieties under investigation, but our results are in agreement with those reported by Simmonds et al. [17]. As reported by Belviso et al. [19], cyn3glu was not detected in old and commercial varieties. Giomaro et al. [32] observed a smaller amount of cyn3gal in all of their red-fleshed apples and found that this metabolite was the most abundant anthocyanin in apples [30,31]. Differences in the concentration of metabolites should be expected, as they do not only depend on the genotype, but also on agronomic factors, the harvest time, and on environmental factors, which lead to considerable biological variation, even when the varieties are grown in the same orchard [19].

In relation to anthocyanins, the pigmentation of apples was correlated to the dominant red-flesh *MYB10* genes [27,30]. Chagné et al. [30] did not report differences in the protein sequence between red-fleshed varieties and GS, apart from its differential expression [30]. In general, the higher concentration of anthocyanins in red-fleshed varieties compared to white-fleshed varieties has been already reported by Bars–Cortina et al. [27]. On the other hand, the varieties Y103 and Y102 showed low concentrations of anthocyanins (cyn3glu was not detected), despite being red-fleshed varieties. A possible explanation for why only two varieties (Y103 and Y102) out of five matched the data reported by Bars–Cortina et al. [27] could be linked to red fruit-flesh phenotypes [30]. The type-1 red-fleshed apples were characterized by a red coloration in stems, roots, leaves, and whole fruits; instead, the type-2 red-fleshed apples showed the reddening only in apple peels and new leaves [27]. Thus, Y103 and Y102 could refer to the type-2 red-fleshed, and RS-1, BAY, and R210 to the type-1 red-fleshed based on the concentration of anthocyanins.

Surprisingly, the red Y102 variety showed the lowest concentration of anthocyanins, but a high amount of flavan-3-ols. The occurrence and the distribution of flavan-3-ols and anthocyanins in the pulp of Y102 are similar like in the cortex of old apples varieties. This observation could be interpreted by considering the competitive pathway between flavan-3-ols and anthocyanins, which has been already reported by other studies [27,28]. These two phenolic subclasses utilized the same precursor: leucoanthocyanidins. Therefore, the competitive interaction of two different enzymes anthocyanidin reductase and anthocyanins synthase with the substrate could be responsible for differential biosynthesis of flavan-3-ols and anthocyanins, respectively [28].

It was noticed that the natural defense of apple trees against the apple scab infections (*Venturia inaequalis*) was linked to increment in the gene expression for the phenolic compounds [33]. Topaz (TOP) and Lb 17906 (LB), two scab-resistant apple cultivars, showed a considerable increment in the concentration of flavan-3-ols and these results were in agreement with the literature [33,34]. Noteworthy, Vanzo et al. [34] demonstrated that there is an upregulation in the polyphenolic pathway under organic production system both in

scab-resistant and scab-susceptible apple cultivars. Therefore, based on the investigated substances, our results support the reasonable possibility to use the increment in the levels of flavan-3-ols could be potentially associated with the resistance of cultivars to scab.

Another important aspect is the potential allergenicity of the apples due to the presence of high quantities of Mal d 3 allergen [35]. Indeed, SAN, a scab-resistant and hypoallergenic variety, has the lowest concentration of flavan-3-ols and these results agree with Vanzo et al. [34]. Kootstra et al. [36] reported that the SAN cultivar caused lower allergic effects than TOP, also known as a hypoallergenic apple. Remarkably, differences in the concentration of flavan-3-ols in SAN and TOP were found in our studies. Indeed, Simonato et al. [35] studied the hypoallergenic activity of five old cultivars, and they found a great diversity in the levels of phenolic compounds. Thus, it is providing the consideration that the phenolic profile could hardly be used to define the potential allergenicity of an apple cultivar in apple allergic individuals [36].

Our findings suggest that the occurrence and distribution of polyphenols seems to be more homogenous in the pulp of old apple varieties. In contrast, commercial and red-fleshed cultivars demonstrate a noticeable variation in the concentration of phenolic compounds.

2.3. Apple Skin: The Antioxidant Activity (AA), Total Polyphenolic Content (TPC), and Total Anthocyanins Content (Tot Antho)

The content of substances in apple skin is dependent on several factors, such as agronomic and environmental, and genetic ones [37]. This could imply that the response to sunlight exposure changes within cultivars. Figure 3 shows the results of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric-reducing antioxidant power (FRAP), which are used to evaluate the AA, and furthermore Tot Antho and TPC (measured with Folin–Ciocalteu (FC)) in the 22 apple varieties organized in three groups: old, commercial, and red-fleshed. The results of both the quantification and of the statistical analysis method are reported in Tables S2 and S3, respectively.

Old, commercial, and red-fleshed apple varieties showed a comparable trend in the AA and TPC, as evaluated by FRAP, ABTS, and FC, contrasting with the observation in the pulp.

Regarding the old group, Tiroler Spitzlederer (TS) and Kanada Renette (KR) had the highest antioxidant activity and phenolic content. In contrast, Weisser Rosmarin (WR) showed the lowest content of antioxidants and phenolic compounds. Goldenparmäne (GP) and Kalterer Böhmer (KB) presented a marked difference in the concentration of antioxidants and phenols between the sunny and the shady sides. In contrast, the plot of commercial apple varieties provided clear evidence that there was a pronounced difference on the antioxidant activity and phenolic content between the sunny and shady sides of apples. Indeed, this difference was more noticeable in Golden Delicious (GD), Nicoter (NIC), LB, SQ159 (SQ159), SAN, and TOP. In detail, these two sides of the cultivars SAN and NIC were in marked contrast. In addition, the influence of sun has a less noticeable effect on the distribution of antioxidant and phenolic compounds in the apple skin of the varieties Braeburn (B), Elstar (E), Fuji (F), Gala (GA), GS, and Rosy Glow (RG).

The plot of red-fleshed cultivars illustrates that only the skin of BAY was influenced by the sunlight, showing a higher pronounced effect on antioxidant activity and the content of polyphenols [37].

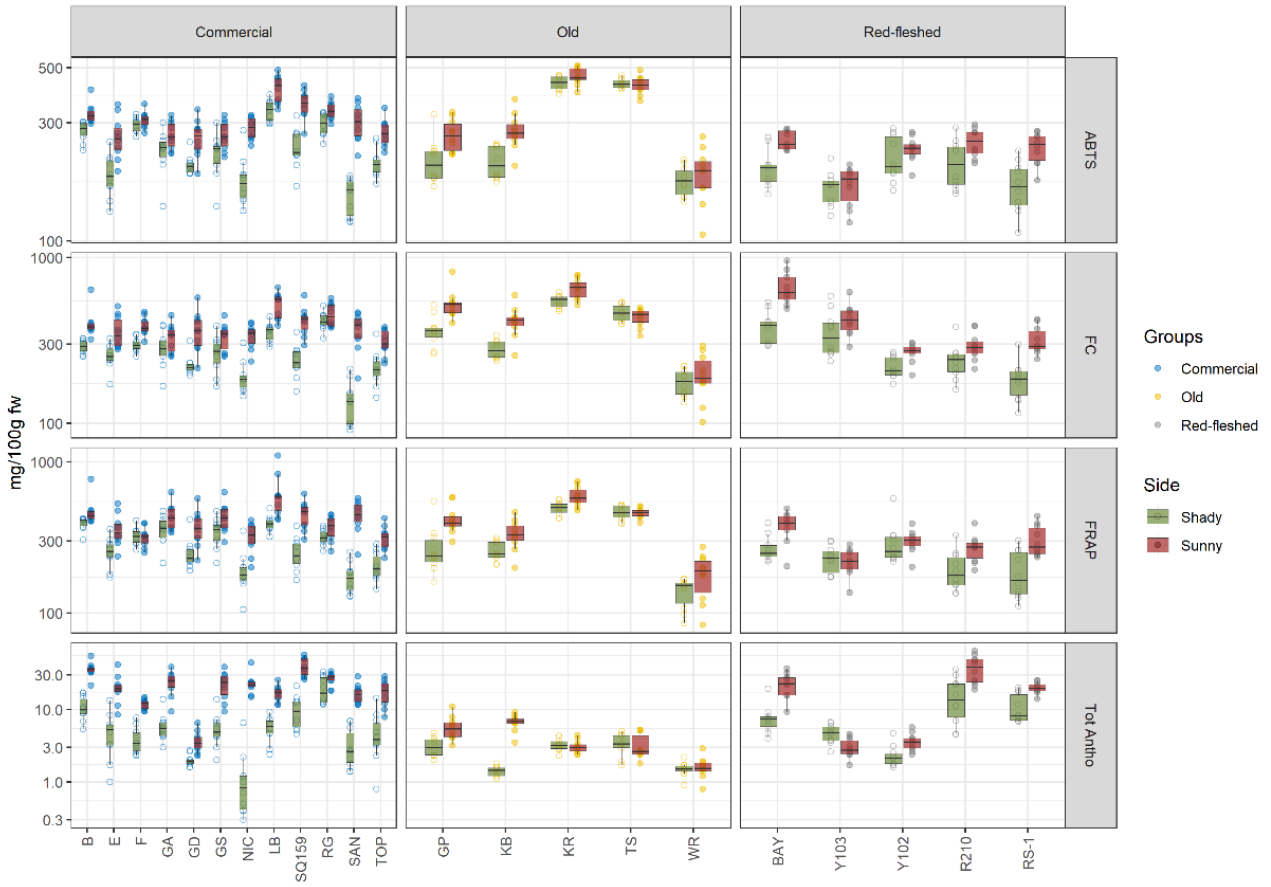

Figure 3. Results of FRAP, ABTS, FC, and Tot Antho in 22 apple varieties organized in three groups (old, commercial, and red-fleshed); 10 apples (biological replicates) per variety were sampled and the sunny and shadow exposed site of the apples were analyzed. The concentrations of these parameters were transformed in log-10 scale. FW = fresh weight. For apple variety names see Table 1.

The total anthocyanin content varied markedly between sunny and shady sides amongst all the three groups, where several cultivars stood out. Again, a more noticeable effect was observed in the commercial apple cultivars. The results of NIC stood out among the others, with a difference of almost two orders of magnitude in the total anthocyanin concentration. In contrast, the content of anthocyanins in the skins of old apple cultivars was less influenced by the sunlight, except for KB. Indeed, NIC and KB were bicolored varieties with a red overcolor on the sunny side [38,39]. Y103 and Y102 showed the lowest content of total anthocyanins and the exposure of fruits to the sun had a smaller effect on the skin pigmentation. In contrast, BAY, RS-1, and R210 have a higher content of anthocyanins, which varied considerably amongst these three cultivars. Since the peel is an important barrier protecting fruits from environment stress, these results suggest that exposure to sunlight leads to changes to the concentration of metabolites, even though the extent of the effect strongly depends on the variety. This observation is in agreement with previous studies [27,37] and can be rationalized with enzyme activities that are modulated by the exposure of fruits to the sun [27]. Furthermore, a higher level of anthocyanins in the peel than in the pulp is found, and this difference in the concentrations of phytochemicals in these different parts of apples is in accordance with other studies [25,40,41].

In general, the shady side of apples always showed a lower antioxidant, total polyphenolic, and total anthocyanin content. Indeed, the photosynthetic system and enzymatic activity is higher on the sun-exposed side than the shady side due to the upregulation of enzymes [37]. The overall upregulation can be interpreted considering the photoprotective role of flavonoids [37], which contribute to the antioxidant activity, total polyphenolic

content, and total anthocyanin content [26]. Since the concentration of phenylalanine and tyrosine, which are the most important precursors in flavonoid biosynthetic pathways in apples, has been found higher in sun-exposed peels [37], this higher concentration of the pathway precursors can be linked to the observed results. Our findings, however, suggest that some varieties are able to preserve biochemical characteristics despite the exposure to sunlight, especially those belonging to old and red-fleshed groups.

2.4. Prediction of the Antioxidant Activity in Apple Skin Based on the Polyphenolic Composition of Apple Pulp

Partial least square (PLS) regression was used to investigate the extent of the metabolic "association" between apple peel and apple pulp in three cultivar groups. The rationale behind this idea is to evaluate whether and to which extent the polyphenolic profile of the pulp can be used to predict the chemical characteristics of the skin. Separate models are then built and validated for the different parameters of the skin (ABTS, FC, FRAP, Tot Antho) in the three different variety groups for sunny and shady apples sides.

Since the antioxidant activity depends on the assay used, ABTS and FRAP are separately treated [28]. The FC assay is used to evaluate the conformity between the TPC in peel and polyphenolic profile in pulp. The results are shown in Figure 4 in terms validation R2 (Q2) of the model.

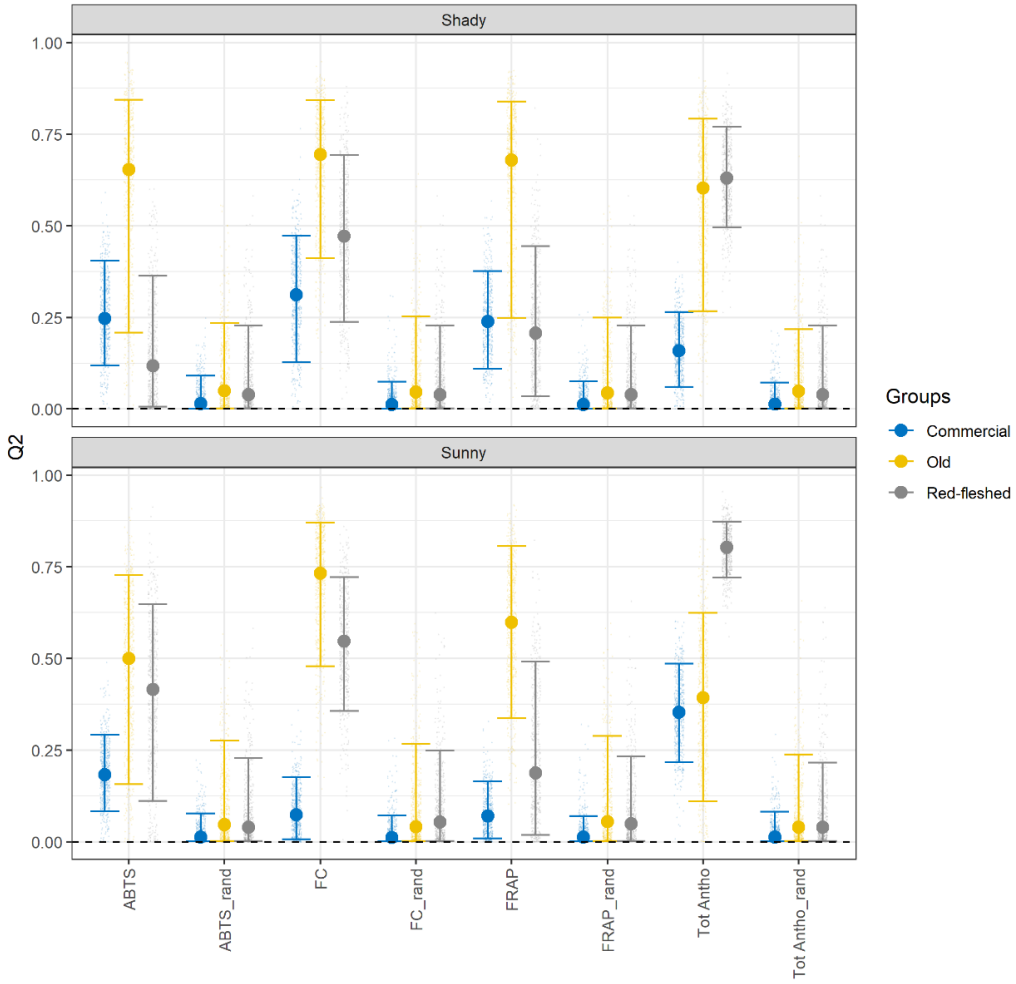

Figure 4. The quality of the PLS regression prediction model is measured by Q2 values using a 7-fold cross-validation (CV) repeated 10 times. The procedure of generating data, the splitting in train and test set, and fitting the models was set at 500 items. The points represent the median and the bars show the confidence intervals of 500 Q2 values. The confidence interval for the data is set at 99%.

In the plot, the Q2 of each model is compared with the predictive power of a model constructed on the same data in random order. When the confidence intervals of the data in the original dataset are overlapped to the confidence intervals of the data in the randomized dataset, the model was considered unable to perform a reliable prediction.

It is important to mention that the variability of Q2 is expected to be dependent on the number of apples per group, so fewer variable results were expected (and observed) for commercial varieties (12 cultivars, 10 biological replicates, 120 apples).

The plot of old varieties (yellow bars) clearly indicated that the PLS model showed a good predictive power for almost all assays considered, with median values of Q2 ranging from 0.5 and 0.7. A lower effectiveness was observed for ABTS and total anthocyanins content where the models did not perform better than random. For old varieties, a metabolic association between peel and pulp was supported. The situation was different for the commercial group (blue bars). In this case, PLS showed a lower effectiveness in predicting the properties of the peel. The confidence intervals for the predictions were narrower due to the higher number of samples, but Q2 never exceeded 0.5. In particular, the total anthocyanins content was only predicted reliably on the sunny side, whereas the results of other variables were predicted poorly. These results suggested a weak correlation between peel and pulp, which could be the sign of the presence of a more diverse metabolism within the commercial macro group.

In the class of red-fleshed cultivars, Q2 values varied from 0.1 to 0.7, indicating a large variability in the prediction ability (gray bars). These findings allowed us to emphasize once again that despite that the red-fleshed cultivars being grouped into one class, the concentration of phytochemicals was strongly variable. Figure 4 highlights the marked variability in the prediction of ABTS, FC, FRAP, and Tot Antho in the red-fleshed group. In contrast, the variation in the prediction ability is less pronounced in the old and the commercial groups, even when the median values of Q2 are low in the commercial varieties.

Looking in detail at the different parameters, the prediction of ABTS was achieved—even with different levels of accuracy—in the commercial and old groups, in contrast to what was observed in red-fleshed cultivars. PLS, instead, showed an efficient prediction ability for FC values in almost all groups, except for the sunny side of the commercial group. The prediction of FRAP was weak in the shady side of apples in commercial varieties and the sunny side of old varieties. Finally, the model was always able to predict the anthocyanin content, even when the median values of Q2 were low, such as in the commercial varieties. It is worth mentioning that the prediction of the total anthocyanin was more efficient in the sunny side than the shady side in the red-fleshed and the commercial groups, in contrast to what was observed in the old group.

Lim et al. [42] tried to predict the antioxidant activity in Australian fruits based on a metabolomic approach. PLS was used by Sahin et al. [43] to determine the antioxidant activity in fruit juices using high-performance liquid chromatography. The effect of storage in changes of antioxidant activity and total anthocyanin content in fruits was studied by Zheng et al. [44] using the PLS method. In both cases, the studies reported a positive correlation between the total polyphenolic content and antioxidant activity, whereas the individual polyphenolic compounds were hardly considered [13,45]. In this study, we confirmed a positive correlation, but we highlighted that the shady or sunny sides and the genotype of the variety contribute to determine the model performance.

Our results show that ABTS, FRAP, and FC assays followed different behaviors, even though they could be used to measure the AA and TPC in plant extract, respectively. Indeed, the ability of prediction was also highly variable between the sunny and the shady side of an apple. An exhaustive investigation was turned up by Csepregi et al. [46] regarding the main flavonoids that contribute to antioxidant activity in plant extract. They affirmed that the responses of a specific antioxidant assay depended on the functional groups bound on the main chemical structure of the polyphenols [46]. In addition, Tsao et al. [11] specified that the cis-configuration is more effective than the trans-configuration [27]. Furthermore, hydrogen bonds, which influenced the electron delocalization, could be

responsible for a diverse response of an assay [13]. Tsao et al. [47] used the Pearson correlation to establish that flavan-3-ols were the most reliable contributor to the antioxidant activity in apples and that procyanidins B2 and epicatechin were the most promising antioxidant polyphenolic compounds both in the peel and in the pulp [27]. Furthermore, Vanzani et al. [45] reported that apple oligomeric proanthocyanins had a higher antioxidant activity than their monomeric forms (catechin and epicatechin) [45]. Another important result was reported by Rossetto et al. [48], where they affirmed a synergic effect given by several antioxidants. These studies showed that a change in the polyphenolic composition and chemical characteristic of a phenolic compound could lead to discord outcomes of different assays.

Our results were in reasonable agreement with these literature data, confirming flavan-3-ols as the main contributor to the antioxidant activity of apples. Our findings showed that the apple varieties belonging to the old group were the most reliable cultivars and a metabolic "association" between peel and pulp of five different old varieties was found. Indeed, the best prediction ability was achieved in the old group due to the highest level of antioxidants present, namely flavan-3-ols.

Regarding the sunny/shady sides of an apple, Li et al. [37] reported that there is an upregulation of enzymes in the pathway of polyphenols due to their protective role against sunlight. In detail, the levels of anthocyanins, flavonols, and total phenolics were raised in the sun-exposed side of apples [37]. Another study showed that there was an increased concentration of carotenoids and quercetin glycosides in the sun-exposed peel [49]. Our results were not completely in agreement with these works; in fact, it is generally found that the Q2 values are higher in the sunny side than the shady side, but not in all groups. Indeed, our results suggest that the cultivar selection could influence the efficiency in the performance of the prediction model both in the sunny and the shady side. Given the abundance of anthocyanin pigments existing in red-fleshed apples, the prediction of Tot Antho was high in the sunny side in apples of the red-fleshed group [37]. Furthermore, the PLS regression model was able to predict Tot Antho on the sunny side of commercial apple varieties. In contrast, our method hardly predicted Tot Antho in old varieties, and it is reasonable to suppose that the pathway of flavan-3-ols was favored in these cultivars.

3. Materials and Methods

3.1. Chemical and Reagents

Formic acid (LC-MS grade) was obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile (LC-MS grade) and methanol (LC-MS grade) were purchased from VWR International Srl (Milan, Italy). Liquid nitrogen was purchased from Rivoira (Milan, Italy). Sodium fluoride, phosphoric acid, glacial acetic acid, TPTZ, ABTS, iron (III) chloride hexahydrate, Trolox, potassium chloride, (+)-catechin, sodium carbonate, F-C reagent, quercetin-3-xyloside, 3,4-dihydroxybenzoic acid, naringenin-7-glucoside, and phloridzin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Quercetin-3-rutinoside and potassium peroxydisulfate were purchased from Roth (Karlsruhe, Germany). Quercetin-3-glucoside, neochlorogenic acid, cryptochlorogenic acid, cyanidin-3-galactoside chloride, and malvidin-3-glucoside chloride were purchased from Extrasynthese (Genay, France).

(−)-Epicatechin, procyanidin B1, procyanidin B2, procyanidin C1, chlorogenic acid, cyanidin-3-glucoside chloride, cyanidin-3-arabinoside chloride, phloretin, quercetin-3-arabinoside, quercetin-3-galactoside, quercetin-4'-glucoside, and quercetin-3-rhamnoside were purchased from Phytolab (Vestenbergsgreuth, Germany). Sodium acetate anhydrous was purchased from CHEMSOLUTE® (Renningen, Germany). Hydrochloric acid from Fisher Chemical (Pittsburgh, UK). Deionized water was from MilliQ apparatus (Millipore Corp., Bedford, MA, USA).

3.2. Fruits Samples

In total, 219 apples of 22 cultivars were analyzed in this study and all cultivars were true to type, accurately identified using molecular tools [18]. The apple trees were grown

in the experimental orchard of Laimburg Research Centre in Auer (Ora, Italy) at 220 m a.s.l. on M9 rootstocks under identical climatic and agricultural conditions [23]. Ten fruits per cultivar were harvested from four different trees, considering their optimal harvest time between August and November 2015. Apples were collected from the central canopy of the trees, leaving the tops and the bottoms out. The fruits were stored at +2 °C for 10 days at normal atmosphere (95% RH) and at shelf-life conditions (at room temperature (RT) in the dark) for 3 days. The harvest days, cold storage, and shelf-life period are reported in Table 1.

The apples were peeled and cut into three equal equatorial discs choosing the central one. From the sunny and shady sides of each apple, a piece of 3 × 3 cm^2 were taken.

The central disc and both peel parts were immediately frozen separately in liquid nitrogen and freeze-dried using FreeZone Freeze Dry System (Labconco, MO, USA). All dried samples were milled to a fine powder using a commercial miller, packed, and stored in hermetic polyethylene bags at −80 °C up until the analysis.

3.3. Extraction of Samples

The extraction protocol used was adapted from Valls et al. [50]. Twenty-give mg of freeze-dried apple material were extracted with 1.83 mL of a mixture of water:methanol (80:20 *v*/*v*) containing sodium fluoride (100 mM) and acidified with H_3PO_4 (0.01 ng/µL). The mixture was shaken for 15 min and centrifugated at +5 °C at 14,000 rpm for 5 min. The supernatant was removed and stored at −80 °C until the analysis.

3.4. Analysis of Polyphenolic Profile of Apple Pulps

Each extract of apple pulp was analyzed one time by resulting in a complete dataset of 219 apple pulp analyses. A quality control sample (QC) was made by pooling of 100 µL of extract for each apple variety and the QC was injected every 10 analyses to control the absence of chromatographic drift. Stock solutions at a concentration of 2000 ng/µL for each phenolic standard were made in 50/50 Millipore water and methanol and then a mix solution at 50 ng/µL was prepared. This last one was serially diluted to working concentrations. The calibration curve of polyphenolic standards covered a range between 25 and 0.0025 ng/µL. The retention time, polarity, molecular weight, precursors, products, collision energy, RF lens, regression parameters, and linearity range of each compound was summarized in S4. As blank, 5 µL of Millipore water was injected every 15 analyses. The analyzed solution for each sample contained 5 µL of two internal standards, Malvidin-3-glucoside and Quercetin-4′-glucoside (0.1 ng/µL) to monitor the instrument performance, 45 µL of the extract (or working standard solution) and 50 µL of MilliQ water.

The UHPLC-QqQ-MS/MS analysis was performed using an UltiMate 3000 UHPLC system (Thermo Scientific, Waltham, MA, USA) coupled with a TSQ Quantiva (Thermo Scientific, Waltham, MA, USA) triple-stage quadrupole mass spectrometer. Separation of analytes was performed using a Hypersil GOLD™ HPLC (2.1 × 50 mm, 3 µm, Thermo Scientific, Waltham, MA, USA). The mobile phase was A (2.5% [*v*/*v*] formic acid in acetonitrile LC-MS analytical grade) and B (2.5% [*v*/*v*] formic acid in Millipore water). The gradient elution was 0–1 min (2.5% B), 1–10 min (16.5% B), 10–11.5 min (16.5% B), 11.5–12.5 min (23.5% B), 12.5–15 min (55% B), 15–15.5 min (95%), 15.5–17.5 min (95%), 17.5–18 min (2.5% B), and 18–21 min (2.5% B) with a consistent flow rate 0.4 mL/min. The column temperature and autosampler were set at +5 °C and +40 °C, respectively. The operations were controlled by the Chromeleon Chromatography Data System (CDS) (version 6.8) software and Thermo Xcalibur (version 3.0) software (both Thermo Scientific, Waltham, MA, USA). The source conditions were as follows: voltage 1500 V, vaporizer temperature 275 °C, capillary temperature 325 °C, sheath gas 40 arbitrary unit (AU), auxiliary gas 15 AU, sweep gas 2 AU, and collision gas (Argon) 1.5 mTorr. The operation was controlled by Thermo TSQ Quantiva (version 2.0) software (Thermo Scientific, Waltham, MA, USA). The acquisition of analytes was done in positive ionization mode with electrospray ionization (ESI) source. The operations were controlled, and the quantification was calculated using

Thermo TraceFinder (version 3.2) software (Thermo Scientific, Waltham, MA, USA). The polyphenols were identified by retention times and selected reaction monitoring (SRM) of reference compounds. The method was able to detect 22 polyphenols compounds and a total number of 17 polyphenols was found and quantified in the apple varieties under investigation. The individual polyphenolic compounds were grouped into six phenolic classes [11,25]: flavan-3-ols (procyanidin B1, B2, C1, (+)-catechin, (−)-epicatechin), anthocyanins (cyandin-3-galactoside, cyandin-3-glucoside, cyanindin-3-arabinoside), phenylpropanoids (chlorogenic acid), dihydrochalcones (phloridzin, phloretin-2′-xyloglucoside), flavanones (prunin), and flavonols (quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-xyloside, quercetin-3-rhamnoside, and quercetin-3-arabinoside). The quantification was carried out by external calibration curves of each compound. The results were normalized for the weight and expressed as the milligram of each compound per 100 g of sample on fresh weight.

3.5. Spectrophotometric Assays of Apple Peels

3.5.1. Total Polyphenolic Content (TPC)

The TPC was determined by the Folin–Ciocalteu method and adapted from Valls et al. [50]. Two hundred and fifty µL of deionized water and sixty µL of extracts was added to sixty µL of Folin–Ciocalteu reagent. The mixture was mixed at 12,000 rpm for 6 min at RT. Then, 630 µL of sodium carbonates (7.5% *w*/*v*) were added to the mixtures and they were mixed at RT for 90 min. The absorbance was recorded at 740 nm on a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, CA, USA) spectrophotometer, and referred to a standard curve of catechin (range 0–150 ng/µL). The results were normalized for the weight and expressed as milligram of catechin equivalents per 100 g of sample on fresh weight.

3.5.2. Total Anthocyanins Content (Tot Antho)

The Tot Antho was determined by pH differential method and adapted from Valls et al. [50]. Two dilutions of the same extract were made by adding 800 µL of potassium chloride (0.025 M, pH 1) to 200 µL of sample extracts and 800 µL of sodium acetate (0.4 M, pH 4.5) to 200 µL of sample extracts. The absorbance was recorded at 520 and 700 nm on a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, USA) spectrophotometer. Tot Antho was calculated using Lambert–Beer law (ε = 26,900 L/mol/cm, MW = 449.2 g/mol) from the measures at pH 1. The results were normalized for the weight and expressed as milligrams of cyanidin-3-glucoside equivalents per 100 g of sample on fresh weight.

3.5.3. Antioxidant Capacity Measurements (AA)

Antioxidant Activity, ABTS Assay

The AA was determined using Trolox equivalent antioxidant capacity (TEAC) assay and adapted from Valls et al. [50]. For the assay, 1970 µL of ABTS reagent was added to 30 µL of sample extract and the mixtures was incubated into dark for 10 min at RT. The decrease in absorbance was read at 734 nm using a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, CA, USA) spectrophotometer, and referred to a standard curve of Trolox (range 15.6–250 µM). The results were normalized for the weight and expressed as milligrams of Trolox equivalents per 100 g of sample on fresh weight.

Antioxidant Activity, FRAP Assay

The AA was determined by FRAP assay and adapted from Valls et al. [50]. For the assay, 960 µL of FRAP reagent was added to 60 µL of sample extract and 180 µL of MilliQ. The mixtures were incubated into dark at +37 °C for 90 min. The decrease in absorbance was read at 595 nm using a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, CA, USA) spectrophotometer, and referred to a standard curve of Trolox (range 15.6–250 µM). The results were normalized for the weight and expressed as milligrams of Trolox equivalents per 100 g of sample of fresh weight.

3.6. Statistical Analysis

3.6.1. The Quantification Analysis

An exploratory data analysis was made to check normal distribution of data and to assess the equality of variance (Shapiro–Wilkinson test and Levene's test). For all analyses carried out on polyphenolic metabolites in apple pulps and antioxidant activity in apple peels, the ANOVA assumptions were violated, even after mathematical log transformation of data due to the heterogeneous samples. The data were analyzed through multiple comparisons with Kruskal–Wallis nonparametric test to find differences between apple varieties. If the Kruskal–Wallis test was significant, a post-hoc analysis could be performed to determine which levels of the independent variable differ from each other level. A post-hoc test was using the criterium Fisher's least significant difference. The adjustment methods included the Bonferroni correction. The significant differences were accepted by <0.05 and represented by different letters (S3). For this purpose, the following R package was used: agricolae [51].

3.6.2. The Exploratory Tool: PCA

The dataset used for this purpose made of the data of chromatographic polyphenolic pattern of the apple pulp extracts—which presents multiple chemical compounds and their relative concentrations—and the data of AA, TPC, and Tot Antho of the apple peel extracts.

In the case of the LC/MS analysis, the dataset contained some missing values (S1), because certain compounds were not determined in apple pulp extracts caused by the concentration under the limits of quantification (LOQ). To prepare a complete dataset for statistical analysis, the missing values were handled by replacing them with random small values between zero and the limits of detection (LOD) [52].

In food chemistry studies, PCA is used to investigate where clusters within the samples exist in a dataset without assigning a prior membership [53]. To reduce the complexity of the dataset, the data were log10-transformed and scaled to unit variance to prevent some variables becoming dominant due to the greater quantity quantified. Then, the data were subjected to PCA [54].

In regard to PCA, based on the Kaiser criterion, the number of principal components (PC) is defined by eigenvalues higher than 1 which were considered "significant" in the data submission to the PCA analysis [53].

For this purpose, the following R packages were used: FactoMineR and factoextra [55,56].

3.6.3. The PLS Regression Analysis

The PLS approach covers a huge number of applications in different scientific fields and this regression model is commonly used to predict specific properties from the chemical composition in matrices [57–59]. The PLS models used the X matrix (the pulp dataset) and Y matrix (the skin dataset) simultaneously, finding the latent variables in X that best predict the latent variables in Y. The X-matrix consisted of 17×219 elements (number of polyphenolic compounds in columns $\times$ number of samples in rows). The Y-matrix was modified for each assay. The Y-matrix was made of 1×219 (number of one AA assay in columns (FRAP, ABTS, FC or Tot Antho) $\times$ number of samples in rows. To define the metabolic correlation between peel and pulp, the complete dataset was used, and the data were log-10 transformed, mean-centered, and auto-scaled prior to the multivariate modelling.

The method validation was carried out by using a repeated 7-fold cross-validation (CV). Thus, the data were shuffled and random sampled multiple times to make a robust model that covered the maximum number of samples in training and testing operations. The K parameter was an integer value, and it indicated that the dataset would be split into K folds randomly. Among the K folds, the K-1 was the train dataset, and the model was trained on it. The train dataset was made of 70% of the complete dataset. The remaining dataset (20% of the complete dataset) was used as a test set, and it was used to evaluate the performance of the model. The CV was repeated 10 times to optimize the model

parameters (such as the significant components) and the model was trained on the train set. The "goodness" of prediction of the optimized model was evaluated by predicting the Q2 value using the held-out test set. The statistical parameter named Q2 was used to demonstrate the ability of the PLS regression model in predicting the antioxidant activity, total polyphenolic content and the total anthocyanin content in apple peel based on the polyphenolic compounds quantified in apple pulp. In order to assess the variability of the results, the procedure of generating data, the splitting in train and the test and fitting models were repeated 500 times.

To validate the PLS models, one randomization sample was created by randomly assigning all measured values to each apple sample. The prediction was made as aforementioned and the Q2 generated from both dataset—the original dataset and the modified dataset—were compared.

For this purpose, the following R package was used: caret [60].

4. Conclusions

Fruits and vegetables are rich in bioactive compounds, which are able to prevent many chronic human diseases; the consumption of apples has been continuously linked to lower risk of the onset of cancer [11]. Our results support the evidence that consuming different apples belonging to several cultivars could provide several classes of polyphenols beneficial to human health. This study drew attention to 5 old, 12 commercial, and 5 red-fleshed apple varieties. We demonstrated the utility of the metabolomic approach to determine that the old cultivars were a reliable group based on the metabolic pathways of the polyphenolic compounds, the antioxidant properties, and total anthocyanin content. Furthermore, we noticed that the variability in the concentration of polyphenolic compounds was more pronounced in commercial and red-fleshed apple varieties. In particular, for the red-fleshed varieties, it was interesting to observe that there were some genotypes (Y102, BAY and RS-1) combining a significant concentration of anthocyanins and a high content of the other classes of polyphenols in the pulp. Indeed, Y102 showed a higher amount of flavan-3-ols (21.74 mg/100 g FW) compared to the most consumed GD (16.42 mg/100 g FW). Additionally, 2.94 mg/100 g FW and 1.45 mg/100 g FW of flavan-3-ols in the BAY and RS-1 varieties were reported, respectively. Furthermore, anthocyanins content of 0.02 mg/100 g FW, 4.14 mg/100 g FW and 7.50 mg/100 g FW were found in Y102, BAY, and RS-1, respectively, whereas in the GD they were absent. Moreover, a noticeably content of total anthocyanins in the peel of the sunny exposed side of the apples were found, 3.65 mg/100 g FW, 20.01 mg/100 g FW and 23.03 mg/100 g FW in Y102, RS-1, and BAY, respectively. The data suggested the consumption of the whole fruit and these red-fleshed cultivars could be potentially used as new functional apples. These findings were in agreement with the results obtained by the prediction model. Indeed, we were able to demonstrate the reliability of the partial least square regression as a powerful method to predict the antioxidant activity in the apple skins. The efficiency at maximum precision was achieved in the old group confirming that the variability in the concentration of metabolites was less pronounced in the old group considered for this survey.

During the last decades, consumers' perception of the quality of fruit has been influenced by specific features, namely crunchiness, shelf life, and juiciness. Currently, these criteria have been used to steer the breeding process towards an improvement in the consumer acceptability, at the expense of healthy chemical compounds, such as polyphenols. Thus, it is potentially leading to the commercialization of new varieties with lower nutritional values. The phenolic profile of the apples has not been taken into consideration when applying the breeding process. Our results showed that the evaluation of the metabolomic profile of the apples is a suitable approach to assess the quality of fruits in the breeding programs or to evaluate new functional apples to the human diet. In conclusion, our findings suggest that the old varieties could be considered as potential fruits for apple breeding programs, encouraging the valorization and preservation of local cultivars, and thus confirming the results found by other studies [12,25,35]. The aim was to contribute

new understandings towards selecting promising fruits with health features based on the metabolic pathways of the phenolic compounds and anthocyanins.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/metabo11060378/s1: Table S1: List of the apple cultivars (column A), polyphenolic classes (column D-I) and polyphenolic compounds (column J-Z) quantified in the pulp of the 22 cultivars included in the study (mean ± SD, n = 10). Results are expressed as mg/100 g FW, Table S2: List of the apple cultivars, the antioxidant activity (FRAP and ABTS), total polyphenolic content (FC) and total anthocyanin content annotated in the peel (sunny and shady sides of apples) of 22 cultivars included in the study (mean ± SD, n = 10). FRAP and ABTS are expressed as Trolox mg/100 g FW, FC are expressed as catechin mg/100 g FW, Tot Antho are expressed as cyanidin-3-glucoside mg/100 g FW, Table S3: Kruskal–Wallis nonparametric test, post-hoc analysis is using the criterium Fisher's least significant difference, the adjustment methods include the Bonferroni correction results on the antioxidant activity, total polyphenolic content, total anthocyanin content, the polyphenolic compounds, and polyphenolic classes in the apple cultivars included in the study, Table S4: Compounds analyzed with UHPLC-QqQ-MS/MS in this study, the corresponding retention time, ESI polarity, molecular weight, precursor ions, product ions, collision energy, RF lens, regression parameters, and linearity range are reported.

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References

1. World Health Organization. Available online: https://www.who.int/health-topics/nutrition (accessed on 3 March 2021).
2. Romagnolo, D.F.; Selmin, O.I. Mediterranean Diet and Prevention of Chronic Diseases. *Nutr. Today* **2017**, *52*, 208–222. [CrossRef]
3. Saura-Calixto, F.; Goñi, I. Definition of the Mediterranean diet based on bioactive compounds. *Crit. Rev. Food Sci. Nutr.* **2009**, *49*, 145–152. [CrossRef] [PubMed]
4. Shoji, T.; Miura, T. Apple Polyphenols in Cancer Prevention. In *Polyphenols in Human Health and Disease*; Acad Press: London, UK, 2014; Volume 2, pp. 1373–1383.
5. Liu, T.; Bassi, M.; Sadar, N.; Lubes, G.; Agnolet, S.; Stürz, B.; Guerra, W.; Robatscher, P.; Zanella, A.; Oberhuber, M. Non-destructive assessment of ascorbic acid in apples using near-infrared (NIR) spectroscopy together with partial least squares (PLS) regression. *Acta Hortic.* **2018**, 447–454. [CrossRef]
6. Denis, M.C.; Furtos, A.; Dudonné, S.; Montoudis, A.; Garofalo, C.; Desjardins, Y.; Delvin, E.; Levy, E. Apple peel polyphenols and their beneficial actions on oxidative stress and inflammation. *PLoS ONE* **2013**, *8*, e53725. [CrossRef]
7. Persic, M.; Mikulic-Petkovsek, M.; Slatnar, A.; Veberic, R. Chemical composition of apple fruit, juice and pomace and the correlation between phenolic content, enzymatic activity and browning. *LWT Food Sci. Technol.* **2017**, *82*, 23–31. [CrossRef]

8. Koutsos, A.; Riccadonna, S.; Ulaszewska, M.M.; Franceschi, P.; Trošt, K.; Galvin, A.; Braune, T.; Fava, F.; Perenzoni, D.; Mattivi, F.; et al. Two apples a day lower serum cholesterol and improve cardiometabolic biomarkers in mildly hypercholesterolemic adults: A randomized, controlled, crossover trial. *Am. J. Clin. Nutr.* **2020**, *111*, 307–318. [CrossRef] [PubMed]
9. Boyer, J.; Liu, R.H. Apple phytochemicals and their health benefits. *Nutr. J.* **2004**, *3*, 1–15. [CrossRef]
10. Hyson, D.A. A Comprehensive Review of Apples and Apple Components and Their Relationship to Human Health. *Adv. Nutr.* **2011**, *2*, 408–420. [CrossRef]
11. Tsao, R.; Yang, R.; Young, J.C.; Zhu, H. Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). *J. Agric. Food Chem.* **2003**, *51*, 6347–6353. [CrossRef]
12. Vrhovsek, U.; Rigo, A.; Tonon, D.; Mattivi, F. Quantitation of polyphenols in different apple varieties. *J. Agric. Food Chem.* **2004**, *52*, 6532–6538. [CrossRef]
13. Jacobo-Velázquez, D.A.; Cisneros-Zevallos, L. Correlations of antioxidant activity against phenolic content revisited: A new approach in data analysis for food and medicinal plants. *J. Food Sci.* **2009**, *74*, R107–R113. [CrossRef] [PubMed]
14. Jokioja, J.; Percival, J.; Philo, M.; Yang, B.; Kroon, P.A.; Linderborg, K.M. Phenolic Metabolites in the Urine and Plasma of Healthy Men After Acute Intake of Purple Potato Extract Rich in Methoxysubstituted Monoacylated Anthocyanins. *Mol. Nutr. Food Res.* **2021**, *65*, 2000898. [CrossRef]
15. Li, M.-J.; Ma, F.-W.; Zhang, M.; Pu, F. Distribution and metabolism of ascorbic acid in apple fruits (*Malus domestica* Borkh cv. Gala). *Plant Sci.* **2008**, *174*, 606–612. [CrossRef]
16. Cuthbertson, D.; Andrews, P.K.; Reganold, J.P.; Davies, N.M.; Lange, B.M. Utility of metabolomics toward assessing the metabolic basis of quality traits in apple fruit with an emphasis on antioxidants. *J. Agric. Food Chem.* **2012**, *60*, 8552–8560. [CrossRef] [PubMed]
17. Simmonds, M.S.; Howes, M.-J.R. Chapter 1-Profile of Compounds in Different Cultivars of Apple (Malus x domestica). In *Nutritional Composition of Fruit Cultivars*; Simmonds, M.S., Preedy, V.R., Eds.; Academic Press: San Diego, CA, USA, 2016; pp. 1–18. ISBN 978-0-12-408117-8.
18. Baric, S.; Storti, A.; Hofer, M.; Guerra, W.; Dalla Via, J. Molecular Genetic Identification of Apple Cultivars Based on Microsatellite DNA Analysis. I. The Database of 600 Validated Profiles. *Erwerbs Obstbau* **2020**, *62*, 117–154. [CrossRef]
19. Belviso, S.; Scursatone, B.; Re, G.; Zeppa, G. Novel Data on the Polyphenol Composition of Italian Ancient Apple Cultivars. *Int. J. Food Prop.* **2013**, *16*, 1507–1515. [CrossRef]
20. Kissabel. Available online: https://kissabel.com/ (accessed on 11 May 2021).
21. Farneti, B.; Masuero, D.; Costa, F.; Magnago, P.; Malnoy, M.; Costa, G.; Vrhovsek, U.; Mattivi, F. Is there room for improving the nutraceutical composition of apple? *J. Agric. Food Chem.* **2015**, *63*, 2750–2759. [CrossRef]
22. Pojer, E.; Mattivi, F.; Johnson, D.; Stockley, C.S. The Case for Anthocyanin Consumption to Promote Human Health: A Review. Compr. Rev. *Food Sci. Food Saf.* **2013**, *12*, 483–508. [CrossRef]
23. Bassi, M.; Lubes, G.; Bianchi, F.; Agnolet, S.; Ciesa, F.; Brunner, K.; Guerra, W.; Robatscher, P.; Oberhuber, M. Ascorbic acid content in apple pulp, peel, and monovarietal cloudy juices of 64 different cultivars. *Int. J. Food Prop.* **2017**, *20*, S2626–S2634. [CrossRef]
24. Wojdyło, A.; Oszmiański, J.; Laskowski, P. Polyphenolic compounds and antioxidant activity of new and old apple varieties. *J. Agric. Food Chem.* **2008**, *56*, 6520–6530. [CrossRef] [PubMed]
25. Jakobek, L.; Ištuk, J.; Buljeta, I.; Voća, S.; Žlabur, J.Š.; Babojelić, M.S. Traditional, Indigenous Apple Varieties, a Fruit with Potential for Beneficial Effects: Their Quality Traits and Bioactive Polyphenol Contents. *Foods* **2020**, *9*, 52. [CrossRef] [PubMed]
26. Kschonsek, J.; Wolfram, T.; Stöckl, A.; Böhm, V. Polyphenolic Compounds Analysis of Old and New Apple Cultivars and Contribution of Polyphenolic Profile to the In Vitro Antioxidant Capacity. *Antioxidants* **2018**, *7*, 20. [CrossRef] [PubMed]
27. Bars-Cortina, D.; Macià, A.; Iglesias, I.; Romero, M.P.; Motilva, M.J. Phytochemical Profiles of New Red-Fleshed Apple Varieties Compared with Traditional and New White-Fleshed Varieties. *J. Agric. Food Chem.* **2017**, *65*, 1684–1696. [CrossRef]
28. Henry-Kirk, R.A.; McGhie, T.K.; Andre, C.M.; Hellens, R.P.; Allan, A.C. Transcriptional analysis of apple fruit proanthocyanidin biosynthesis. *J. Exp. Bot.* **2012**, *63*, 5437–5450. [CrossRef]
29. Lancaster, J.E.; Dougall, D.K. Regulation of skin color in apples. *Crit. Rev. Plant Sci.* **1992**, *10*, 487–502. [CrossRef]
30. Chagné, D.; Lin-Wang, K.; Espley, R.V.; Volz, R.K.; How, N.M.; Rouse, S.; Brendolise, C.; Carlisle, C.M.; Kumar, S.; De Silva, N.; et al. An ancient duplication of apple MYB transcription factors is responsible for novel red fruit-flesh phenotypes. *Plant Physiol.* **2013**, *161*, 225–239. [CrossRef] [PubMed]
31. Espley, R.V.; Bovy, A.; Bava, C.; Jaeger, S.R.; Tomes, S.; Norling, C.; Crawford, J.; Rowan, D.; McGhie, T.K.; Brendolise, C.; et al. Analysis of genetically modified red-fleshed apples reveals effects on growth and consumer attributes. *Plant Biotechnol. J.* **2013**, *11*, 408–419. [CrossRef] [PubMed]
32. Giomaro, G.; Karioti, A.; Bilia, A.R.; Bucchini, A.; Giamperi, L.; Ricci, D.; Fraternale, D. Polyphenols profile and antioxidant activity of skin and pulp of a rare apple from Marche region (Italy). *Chem. Cent. J.* **2014**, *8*, 1–10. [CrossRef]
33. McClure, K.A.; Gong, Y.; Song, J.; Vinqvist-Tymchuk, M.; Palmer, L.C.; Fan, L.; Burgher-MacLellan, K.; Zhang, Z.; Celton, J.-M.; Forney, C.F.; et al. Genome-wide association studies in apple reveal loci of large effect controlling apple polyphenols. *Hortic. Res.* **2019**, *6*, 1–12. [CrossRef] [PubMed]
34. Vanzo, A.; Jenko, M.; Vrhovsek, U.; Stopar, M. Metabolomic profiling and sensorial quality of 'Golden Delicious', 'Liberty', 'Santana', and 'Topaz' apples grown using organic and integrated production systems. *J. Agric. Food Chem.* **2013**, *61*, 6580–6587. [CrossRef]

35. Simonato, B.; Marangon, M.; Vincenzi, S.; Vegro, M.; Pasini, G. Evaluation of the phenolic profile and immunoreactivity of Mal d 3 allergen in ancient apple cultivars from Italy. *J. Sci. Food Agric.* **2020**, *100*, 4978–4986. [CrossRef]
36. Kootstra, H.S.; Vlieg-Boerstra, B.J.; Dubois, A.E.J. Assessment of the reduced allergenic properties of the Santana apple. *Ann. Allergy Asthma Immunol.* **2007**, *99*, 522–525. [CrossRef]
37. Li, P.; Ma, F.; Cheng, L. Primary and secondary metabolism in the sun-exposed peel and the shaded peel of apple fruit. *Physiol. Plant.* **2013**, *148*, 9–24. [CrossRef] [PubMed]
38. Biosüedtirol. Available online: https://biosuedtirol.com/en/apple/nicoter-kanzi (accessed on 15 May 2021).
39. Pomiferous. Available online: https://pomiferous.com/applebyname/rosa-di-caldaro-id-5552 (accessed on 11 May 2021).
40. Zhang, X.; Xu, J.; Xu, Z.; Sun, X.; Zhu, J.; Zhang, Y. Analysis of Antioxidant Activity and Flavonoids Metabolites in Peel and Flesh of Red-Fleshed Apple Varieties. *Molecules* **2020**, *25*, 1968. [CrossRef] [PubMed]
41. Kalinowska, M.; Bielawska, A.; Lewandowska-Siwkiewicz, H.; Priebe, W.; Lewandowski, W. Apples: Content of phenolic compounds vs. variety, part of apple and cultivation model, extraction of phenolic compounds, biological properties. *Plant Physiol. Biochem.* **2014**, *84*, 169–188. [CrossRef]
42. Lim, V.; Gorji, S.G.; Daygon, V.D.; Fitzgerald, M. Untargeted and Targeted Metabolomic Profiling of Australian Indigenous Fruits. *Metabolites* **2020**, *10*, 114. [CrossRef]
43. Sahin, S.; Demir, C. Determination of Antioxidant Properties of Fruit Juice by Partial Least Squares and Principal Component Regression. *Int. J. Food Prop.* **2016**, *19*, 1455–1464. [CrossRef]
44. Zheng, H.; Jiang, L.; Lou, H.; Hu, Y.; Kong, X.; Lu, H. Application of artificial neural network (ANN) and partial least-squares regression (PLSR) to predict the changes of anthocyanins, ascorbic acid, Total phenols, flavonoids, and antioxidant activity during storage of red bayberry juice based on fractal analysis and red, green, and blue (RGB) intensity values. *J. Agric. Food Chem.* **2011**, *59*, 592–600. [CrossRef]
45. Vanzani, P.; Rossetto, M.; Rigo, A.; Vrhovsek, U.; Mattivi, F.; D'Amato, E.; Scarpa, M. Major phytochemicals in apple cultivars: Contribution to peroxyl radical trapping efficiency. *J. Agric. Food Chem.* **2005**, *53*, 3377–3382. [CrossRef]
46. Csepregi, K.; Neugart, S.; Schreiner, M.; Hideg, É. Comparative Evaluation of Total Antioxidant Capacities of Plant Polyphenols. *Molecules* **2016**, *21*, 208. [CrossRef]
47. Tsao, R.; Yang, R.; Xie, S.; Sockovie, E.; Khanizadeh, S. Which polyphenolic compounds contribute to the total antioxidant activities of apple? *J. Agric. Food Chem.* **2005**, *53*, 4989–4995. [CrossRef]
48. Rossetto, M.; Vanzani, P.; Mattivi, F.; Lunelli, M.; Scarpa, M.; Rigo, A. Synergistic antioxidant effect of catechin and malvidin 3-glucoside on free radical-initiated peroxidation of linoleic acid in micelles. *Arch. Biochem. Biophys.* **2002**, *408*, 239–245. [CrossRef]
49. Solovchenko, A.; Schmitz-Eiberger, M. Significance of skin flavonoids for UV-B-protection in apple fruits. *J. Exp. Bot.* **2003**, *54*, 1977–1984. [CrossRef] [PubMed]
50. Valls, J.; Agnolet, S.; Haas, F.; Struffi, I.; Ciesa, F.; Robatscher, P.; Oberhuber, M. Valorization of Lagrein grape pomace as a source of phenolic compounds: Analysis of the contents of anthocyanins, flavanols and antioxidant activity. *Eur. Food Res. Technol.* **2017**, *243*, 2211–2224. [CrossRef]
51. Mendiburu, F. Agricolae: Statistical Procedures for Agricultural Research. *R Package Version* **2010**, *1*, 1–8.
52. Wei, R.; Wang, J.; Su, M.; Jia, E.; Chen, S.; Chen, T.; Ni, Y. Missing Value Imputation Approach for Mass Spectrometry-based Metabolomics Data. *Sci. Rep.* **2018**, *8*, 1–10. [CrossRef]
53. Granato, D.; Santos, J.S.; Escher, G.B.; Ferreira, B.L.; Maggio, R.M. Use of principal component analysis (PCA) and hierarchical cluster analysis (HCA) for multivariate association between bioactive compounds and functional properties in foods: A critical perspective. *Trends Food Sci. Technol.* **2018**, *72*, 83–90. [CrossRef]
54. Feng, C.; Wang, H.; Lu, N.; Chen, T.; He, H.; Lu, Y.; Tu, X.M. Log-transformation and its implications for data analysis. *Shanghai Arch. Psychiatry* **2014**, *26*, 105–109. [CrossRef]
55. Lê, S.; Josse, J.; Husson, F. FactoMineR: An R Package for Multivariate Analysis. *J. Stat. Softw.* **2008**, *25*, 1–18. [CrossRef]
56. Extract and Visualize the Results of Multivariate Data Analyses factoextra (datanovia.com). Available online: https://rpkgs.datanovia.com/factoextra/index.html (accessed on 9 June 2021).
57. Aznar, M.; López, R.; Cacho, J.; Ferreira, V. Prediction of aged red wine aroma properties from aroma chemical composition. Partial least squares regression models. *J. Agric. Food Chem.* **2003**, *51*, 2700–2707. [CrossRef]
58. Cozzolino, D.; Kwiatkowski, M.J.; Dambergs, R.G.; Cynkar, W.U.; Janik, L.J.; Skouroumounis, G.; Gishen, M. Analysis of elements in wine using near infrared spectroscopy and partial least squares regression. *Talanta* **2008**, *74*, 711–716. [CrossRef] [PubMed]
59. Hauksson, J.; Bergqvist, G.; Bergsten, U.; Sjostrom, M.; Edlund, U. Prediction of basic wood properties for Norway spruce. Interpretation of Near Infrared Spectroscopy data using partial least squares regression. *Wood Sci. Technol.* **2001**, *35*, 475–485. [CrossRef]
60. Kuhn, M. Building Predictive Models in R Using the caret Package. *J. Stat. Softw.* **2008**, *28*, 1–26. [CrossRef]

2.2.1 Conclusion: First Contribution

The objective of this chapter 2 is to select specific apple cultivars, which are richer in bioactive compounds than the traditional ones, to support the evidence regarding that the consumption of different apple varieties is a valuable asset to human health due to the presence of several classes of phytochemicals. As far as I am concerned, the consumers' choices are guided towards the selection of apples based on specific features (e.g. visual aspects, crunchiness, shelf life, and sweetness) without considering their beneficial effects on the human health. It is worth remembering that the assumption of bioactive compounds is an important key in the prevention of many chronic human diseases. Additionally, it is remarkable that the evaluation of antioxidants, which are involved in several functions such us in food storage by increasing shelf life, is a crucial point in assigning strategies during the food production. Therefore, I hold the view that the development of prediction models, which are able to asses the antioxidant proprieties in the apples, is an important step in considering the the overall quality of this fruit.

CHAPTER 3

SECOND CONTRIBUTION

3.1 Preface

It was a great honor for me to present the first published article (reported in chapter 2) at the anniversary XXX International Conference on Polyphenols (ICP2020) organized by the University of Turku, Finland. The ICP2020 was as a fully virtual conference organized in 13-15 July 2021. This ICP2020TURKU contribution has been published as Polyphenols Communications in 2021.

3.2 Metabolomics investigation of Antioxidant Proprieties, Polyphenolic profile and, Anthocyanin content in Commercial, Ancient and Red- fleshed apple varieties

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Metabolomics investigation of Antioxidant Proprieties, Polyphenolic profile and, Anthocyanin content in Commercial, Ancient and Red-fleshed apple varieties

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MAIN CONCLUSION

By balancing the intake of different apples cultivars, several classes of polyphenols beneficial are provided to human diet. The maximum efficiency in predicting the antioxidant properties using the polyphenolic profile is achieved in old cultivars, where the variability in the concentration of phytochemicals is less marked, contrary to what is observed in the commercial and red-fleshed apples. The aim is to contribute a new understanding for selecting promising fruits with health features.

INTRODUCTION

The World Health Organization encourages a 400 g per day of fruits and vegetables due to the presence of healthy compounds such as polyphenols. Apples are 12.5% of all consumed fruits in the world [1]. The antioxidants have a potential role to prevent the formation of reactive oxygen species (ROS), which are implicated in the onset of chronic disease and inflammation. In apples, the concentration of the antioxidants compounds is differently distributed in the fruit pulp and in the fruit peel. Agronomic, environmental, and genetic factors influenced the levels of phenolic compounds in both sections. The environmental and the agronomic conditions in the present investigation are kept as uniform as possible [1]. To date there are a few numbers of studies where apple varieties, grown in the same site, are compared. In this study, twenty-two apple varieties are fingerprinted and validated, and they are categorized in three groups: old, commercial, and red-fleshed [2]. The old varieties originated between 1900 and 1950, however, they have lost the acceptability from the farmers and consumers due to the deficits in production, storability and/or shelf life. The commercial group gathers the most consumed apple fruits, and they are currently available on the world apple market. The red-fleshed apples are characterized by a pigmented pericarp, and they are considered as promising apple cultivars for their nutritional properties. Since, the breeding process had a important effect on the content of certain phytochemicals. The red-fleshed apple cultivars were not taken into consideration in these extensive surveys. Indeed, it would be considerable to promote new apple cultivars with a reliable ratio between polyphenols and anthocyanins in pulp and peel compartment. In this study a metabolomic investigation is carried out on the characterization of twenty-two apple cultivars to profile the antioxidant properties and the polyphenol composition of the pulp and peel of apples.

MATERIALS & METHODS

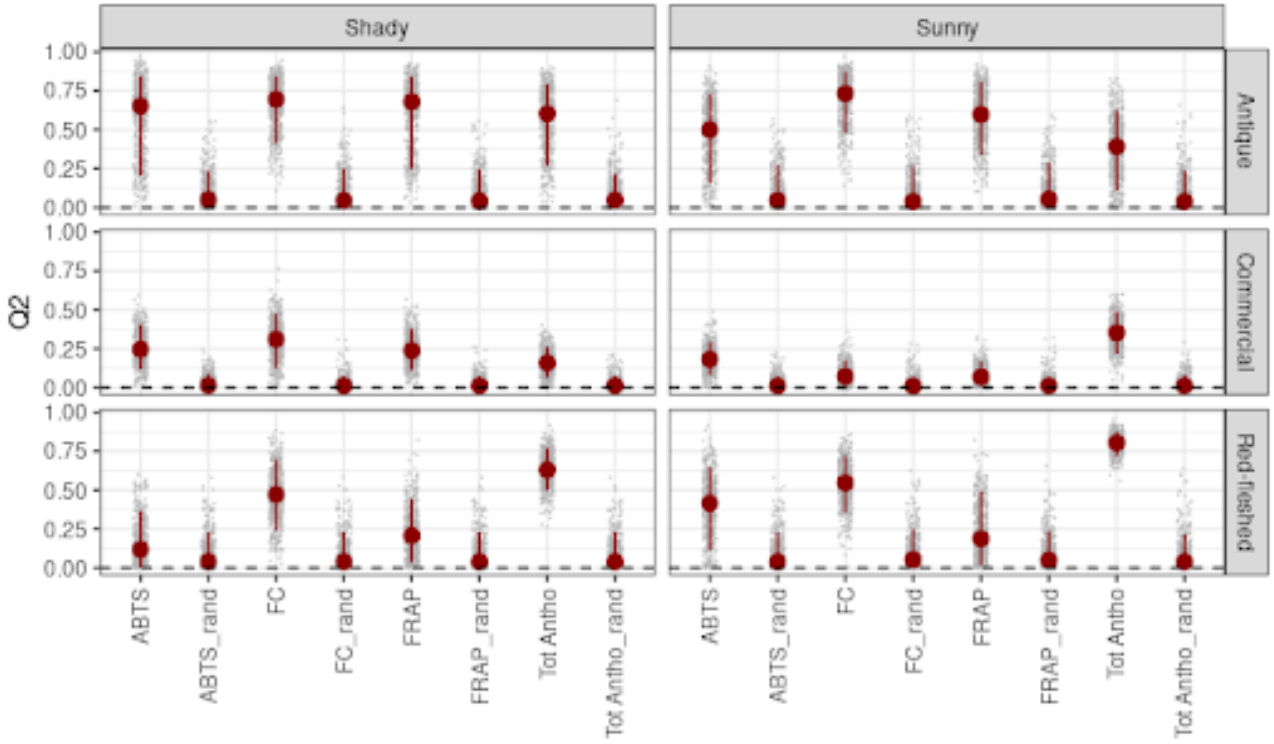
Twenty-two cultivars (10 biological replicates for each) were harvest in 2015 [4] Antioxidant activity, total polyphenolic content, total anthocyanin content assays were evaluated on the sunny and the shady sides of apple skin by FRAP, ABTS, Folin-Ciocalteu, and pH differential methods. Single polyphenols in pulp were quantified by UHPLC–MS/MS. Principal component analysis evaluated the separation between the three apple groups (old, commercial, and red-fleshed). The variability in concentration of flavan-3-ols and anthocyanins amongst groups is investigated. The partial least squares method predicted the antioxidant activity in the apple peel using the polyphenolic profile in apple pulp. The procedure of generating data, the splitting in train and the test and fitting models were

repeated 500 times. Q2 values of each model were compared to the same data in random order. The overlapping of the confidence intervals defined the reliability of the prediction power.

RESULTS & DISCUSSION

The PC1 and PC2 of the PCA (principal component analysis) applied on the whole dataset accounts for 49% of the total variance and the plot shows a good separation between the three groups. The PCA shows that the old varieties contain the highest amount of flavan-3-ols and antioxidant metabolites than the other samples [5]. The red-fleshed cultivars have a higher amount of anthocyanins and quercetin glycosides. In contrast, Y103 and Y102, two red-fleshed cultivars, are separated from the other red-fleshed varieties. The concentration of flavan-3-ols is less variable in old apple varieties than the other groups, where a marked variability in the concentration of flavan-3-ols is observed [5]. Y102 show the highest concentration of these metabolites and it could be linked to the red fruit-flesh phenotypes. The highest concentration of anthocyanins is found in the red-fleshed group, expect for Y102. The occurrence and the distribution of flavan-3-ols and anthocyanins in the apple pulps is attributed to the competitive pathway between these two polyphenolic classes. The presence of metabolites in the apple pulps seems to be more homogenous in old apple varieties [5].

The shady side of apples showed a lower antioxidant, total polyphenolic, and total anthocyanin content than the sunny side due to an up-regulation enzymatic activity in the sun-exposed side. A notably higher content of anthocyanins in the peel than in the pulp is found. Again, red-fleshed and old varieties are able to develop biochemical characteristics despite the exposure to sunlight. The best predictive power for almost all assays considered is achieved in the old group as show in the picture, which highlights a higher effectiveness in predicting the antioxidant properties of the peel using the polyphenolic profile [5]. In contrast, the evidence that there is a more diverse metabolism within the commercial and red-fleshed groups is confirmed by the large variability in the prediction ability [5].



REFERENCES

[1] Vrhovsek, U.; 2004. J. Agric. Food Chem 52: 6532–6538.
[2] Baric, S.; 2020. Erwerbs-Obstbau 62: 117–154
[3] Farneti, B.; 2015. J. Agric. Food Chem 63: 2750–2759.
[4] Valls, J.; 2017. European Food Research and Technology 243: 2211–2224.
[5] Ceci, A.T.; 2021. Metabolites 11: 378.

3.2.1 Conclusion: Second Contribution

Since I have no doubt that the sharing of a discovery and scientific findings amongst non scientists and scientists is an important way that such diffusion can take place, I decided to broaden my results during the ICP2020. The objective of this chapter 3 is to share health information that should be free to everyone. I believe that the main scope of a researcher is to raise awareness in eating healthfully.

CHAPTER 4

THIRD CONTRIBUTION

4.1 Preface

For the purposes of this research, I will present in this chapter 4 the results regarding a specific study conducted on 28 cooked potato samples, belonging to 18 different varieties, where a multiclass chemical characterization (total polyphenols, antioxidants, single phenolic compounds, and sugars) is conducted to gain insights regarding the characterization of the potato metabolome. My contributions for this chapter 4 covered all of steps for the preparation of the study, including the sampling procedure, the conceptualization of the study, the practical laboratory work, figures, and the application of a cutting-edge multivariate statistical method.

Our research was stimulated by a problem that emerged in the evaluation of the nutritional potential of potatoes (Solanum tuberosum L.): their high glycemic index (GI) is indeed causing an increment of the glycemic peak after consuming a meal of these tubers. This fact can cause significant health issues in those people who are characterized by specific pathological states like diabetes. It has been already demonstrated that the

simultaneous presence of anthocyanins and polyphenols in tubers decreases the glycemic peak, thus improving the glycemic control. Therefore, a careful selection of specific potato varieties could have a beneficial impact to promote healthy diets. Within this framework, our study presents the results of an extensive metabolic characterization of steam-cooked potato samples, belonging to 18 different varieties, which were categorized into four macro groups: white-, yellow-fleshed (both non-pigmented), red-skinned, and purple-fleshed (both pigmented) potatoes. The analyses produced three omic datasets (antioxidants including total polyphenolic content, polyphenols, and sugars) which were then jointly analyzed by using a data fusion approach based on Joint and Individual Variation Explained (JIVE). The application of this approach, which we believe to be innovative in this field of research, provides quantitative insights into shared and individual patterns present in the three datasets. In particular, our analysis showed that the partitioning between the non-pigmented and pigmented steam-cooked samples was common to all the three datasets. Conversely, red-skinned and purple-fleshed potatoes were separated by individual component of the polyphenolic dataset, thus suggesting that these two groups are characterized by specific patterns of polyphenolic constituents. The combination of individual components of polyphenol or sugar datasets with the joint component revealed that some potato varieties have a characteristic and diverging metabolome in contrast to their groups. Finally, the presence of specific metabolomic patterns is evaluated amongst the four groups, and the anthocyanin composition in red-skinned and purple-fleshed samples is investigated in-depth.

It is well-known that in potatoes the most evident phenotypic difference is the pigmentation of the flesh (or lack thereof), but this macroscopic difference is expected to affect the overall metabolome of the tubers. This is exactly what we demonstrate in our study, which is highlighting that the difference in pigmentation propagates to a large class of phytochemicals which should be considered together to select promising potato

varieties and increase the awareness of consumers.

In a wider perspective, our work offers objective data providing relevance and emphasis, and supporting consumers in the selection of health promoting potato varieties. Moreover, the application of the data fusion approach was useful to identify varieties rich in healthy bioactive compounds thus encouraging the valorization and preservation of locally produced potatoes.

4.2 Metabolomic characterization of pigmented and non-pigmented potato cultivars using a Joint and Individual Variation Explained (JIVE)

foods

MDPI

Article

Metabolomic Characterization of Pigmented and Non-Pigmented Potato Cultivars Using a Joint and Individual Variation Explained (JIVE)

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Abstract: Potatoes (*Solanum tuberosum* L.) are one of the most valuable agricultural crops, and the flesh of these tubers provides various classes of healthy compounds important for human nutrition. This work presents the results of a joint analysis of different chemical classes of compounds which provided insights on the metabolic characterization of pigmented and non-pigmented potato varieties collected from Italy. The identification of common or individual metabolic characteristics across the omic datasets (antioxidants, total polyphenolic content, polyphenols, and sugars) is conducted by Joint and Individual Variation Explained (JIVE), a data fusion multivariate approach. The common part of the multivariate model allowed the separation between non-pigmented and pigmented samples. Polyphenolic compounds were mainly responsible for the separation between purple-fleshed and red-skinned potatoes. An additional detailed analysis of the anthocyanin composition, including the acylated anthocyanins, allowed to pinpoint the diversities between the pigmented potato groups. Furthermore, the presence of an appreciable amount of hydroxycinnamic acids and anthocyanins in the purple-fleshed varieties, which are also characterized by a lower content of sugars, is found. Our results provide scientific evidence for the promotion of promising potato cultivars, which are characterized by a remarkable amount of various health benefit compounds.

Keywords: metabolomics; potatoes (*Solanum tuberosum* L.); metabolic characterization

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1. Introduction

According to FAO (Food and Agriculture Organization), potatoes are the most commonly food produced, after maize, wheat, and rice [1]. To date, FAO estimates that 160 countries cultivate potatoes, 4000 varieties are known, and 368 million tons are produced globally. The nutritional potential of potatoes is high because they are rich in various classes of valuable compounds (carbohydrates, proteins, fibers, vitamins, and microelements) and can also contain phenolic acids and flavonoids [2]. However, since their glycaemic index (GI) can be high (it covers a range from 23 to 111), their consumption should be controlled in the presence of specific pathological states like diabetes [3]. Despite this, the American Diabetes Association (ADA) advises to include them in a healthful meal plan [4], suggesting that diabetic people may consider implementing in their daily diet the intake of potatoes characterized by low-GI values. Amongst the different potato varieties, pigmented-fleshed potatoes have been the subject of increasing interest in the last few years showing considerable amounts of antioxidant compounds [5]. Their GI value is indeed reported to be lower compared with non-pigmented potatoes (yellow- and white-fleshed) [6], and the

contemporary presence of anthocyanins potentially increases their nutritional value [6,7]. It has been demonstrated that the presence of anthocyanins, combined with other phenolic compounds, may contribute to lowering the glycemic peak after consuming a meal of potatoes [6,7] by inhibiting the intestinal α-glucosidase. In particular, Jokioja et al. [7] have shown that a cooked yellow-fleshed potato portion enriched with purple-fleshed potato extract (rich in acylated anthocyanins and hydroxycinnamic acid derivatives), lowered postprandial glycemic response. This evidence is clearly suggesting that several metabolic features are concurring in defining the nutritional quality of the different potato varieties. To date, however, the literature exclusively reports studies which focus on specific chemical classes, and their results cannot be used for a wide range systematic characterization of the potato metabolome. In this paper, we present the results of an extensive metabolic characterization of 28 cooked potato samples, belonging to 18 different varieties, where a multiclass chemical characterization (total polyphenols, antioxidants, single phenolic compounds, and sugars) was used for a deeper and more comprehensive characterization of the potato metabolome. Following a classification proposed in the literature [2,5,8,9], the samples were organized in four macro groups: yellow-, white-fleshed (non-pigmented), red-skinned, and purple-fleshed (pigmented) potatoes. This broad characterization was complemented by a detailed characterization of the anthocyanins in the pigmented potato samples. Since potatoes are almost always consumed after cooking, we focused on steam cooked samples. Steaming was selected since it was able to retain the maximum amount of bioactive metabolites [10] while limiting in respect to deep-fried potato products the formation of metabolites causing concern, such as Maillard reaction products [11]. In order to investigate the association between the metabolic characteristics, the different datasets were jointly analyzed by a data fusion approach based on Joint and Individual Variation Explained (JIVE) [12]. This type of analysis complemented the characterization of the individual compounds and allowed us to assess the multivariate patterns in the whole dataset, highlighting characteristics that are shared or specifically associated to the different datasets. It is well-known that different degrees of pigmentation resulted in specific coloration patterns in the potato fleshes [2,5,8,9]. It might be the most evident phenotypic difference, however, there were larger genetic differences than the synthesis of pigments [13], thus translating in several types of potato metabolomes. The results of our analysis showed that differences between pigmented and non-pigmented potato varieties extended across the datasets, clearly indicating that the varietal differences in the capacity of synthesizing metabolites had a profound effect on the potato metabolism. At odds, the separation between red-skinned and purple-fleshed varieties was concentrated at the levels of the polyphenols. The in-depth characterization of the pigmented varieties highlighted the presence of specific patterns in anthocyanin composition which determines a clear separation between the different groups. We believe that the proposed approach could be useful to promote the selection of promising potato varieties to increase the awareness of the consumers in food selection.

2. Material and Methods

2.1. Reagents

Formic acid (LC-MS grade) was obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile (LC-MS grade) and methanol (LC-MS grade) were purchased from VWR International (Radnor, PA, USA). Sodium fluoride, phosphoric acid, glacial acetic acid, TPTZ, ABTS, iron (III) chloride hexahydrate, Trolox, potassium chloride, (+)-catechin, sodium carbonate, F-C reagent, quercetin-3-xyloside, 3,4-dihydroxybenzoic acid, caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, quercetin-3,4′-diglucoside, kaempferol-3-glucoside, catechin-2,3,4-$^{13}C_3$, ferulic acid-1,2,3-$^{13}C_3$, glucose, galactose, fructose, raffinose, sucrose, xylose were purchased from Sigma–Aldrich (St. Louis, MO, USA). Quercetin-3-rutinoside, gallic acid, 4-hydroxybenzoic acid, and potassium peroxydisulfate were purchased from Roth (Karlsruhe, Germany). Quercetin-3-glucoside, quercetin-7-glucoside, neochlorogenic acid, cryptochlorogenic acid, kaempferol-3-rutinoside, kaempferol-3-glucoside, kaempferol-

7-glucoside were purchased from Extrasynthese (Genay, France). (–)-Epicatechin, chlorogenic acid, rosmarinic acid, quercetin-3-sophoroside, quercetin-3-gentiobioside, quercetin-3-arabinoside, quercetin-3-galactoside, quercetin-4′-glucoside, and quercetin-3-rhamnoside were purchased from Phytolab (Vestenbergsgreuth, Germany). Sodium acetate anhydrous was purchased from CHEMSOLUTE® (Renningen, Germany). Hydrochloric acid from Fisher Chemical (Pittsburgh, UK). Deionized water was from MilliQ apparatus (Bedford, MA, USA). 4-Feruloylquinic acid, 5-feruloylquinic acid, kaempferol-3,4′-diglucoside, kaempferol-3-O-sophoroside were obtained from ChemFaces (Wuhan, China). Sodium hydroxide solution (50% in water) was obtained from Honeywell International Inc. (Charlotte, NC, USA).

2.2. Potato Materials

Fresh potato tubers were collected from 14 fields and four commercial retailers. In total, 28 potato samples belonging to 18 different varieties were obtained, and they were harvested in two harvest seasons (2019 and 2020). Two fields were repeated in two different years (2019 and 2020): Oris (BZ) and San Genesio (BZ), respectively. Detailed information is reported in Table 1.

From each sample, three biological replicates, each consisting of three to five tubers (approximately 150 g), were selected and treated separately. The potatoes were cleaned using tap water and dried with tissue paper. Each tuber was cut into small pieces (approximately 4×3 cm) without peeling the skin and then, they were steam-cooked.

Table 1. List of cultivars used in this study. The potato varieties are ordered by the group. The "sample ID" gathers the abbreviations used in the Figures 3–7.

Sample Number	Variety	Origin	Year	Sample ID	Group	Pigmentation	Skin Color	Pulp Color	Maturity	Shape
1	Belmondo	San Genesio (BZ)	2019	Belmondo (Sg19)	Yellow	Non-pigmented	Yellowish	Yellow	Early	Round-oval
2	Monalisa	San Genesio (BZ)	2020	Monalisa (Sg20)	Yellow	Non-pigmented	Golden-yellow	Golden-yellow	Medium early	Oval
3 4	Bettina	Oris (BZ)	2019 2020	Bettina (Sg19)	Yellow	Non-pigmented	Yellow	Light yellow	Medium early	Oval
5 6 7	Cicero	Oris (BZ) Oris (BZ) Funes (BZ)	2019 2020 2020	Cicero (Oris19) Cicero (Oris20) Cicero (Fun)	Yellow	Non-pigmented	Yellow	Pale Yellow	Medium early	Oval
8 9	Agria (Patata di Montagna)	Ret1 Ret2	2019	Agria (Ret1) Agria (Ret2)	Yellow	Non-pigmented	Yellow	Yellow	Early	Long oval
10	Selenella	Ret3	2019	Selenella	Yellow	Non-pigmented	Yellow	Yellow	Early	Oval
11	Colomba	Ret4	2019	Colomba	Yellow	Non-pigmented	Yellow	Yellow	Early	Round oval
12	Ditta	Merano	2020	Ditta	Yellow	Non-pigmented	Yellow	Light yellow	Early	Long oval
13	Vivaldi	Calceranica al lago (TN)	2020	Vivaldi	Yellow	Non-pigmented	Yellow	Light yellow	Medium early	Oval
14 15 16	Kennebec	Oris (BZ) Oris (BZ) Ravina (TN)	2019 2020 2020	Kennebec (Oris19) Kennebec (Oris20) Kennebec (Rav)	White	Non-pigmented	Yellow	White	Early	Oval
14	Red Scarlett	Calceranica al lago(TN)	2020	Red Scarlett	Red	Pigmented	Red	Light yellow	Medium early	Long oval
18	Desire	San Genesio (BZ)	2020	Desire (Sg20)	Red	Pigmented	Red	Light yellow	Medium late	Oval
19	Local red variety	Val di Ledro (TN)	2020	Local red variety	Red	Pigmented	Red	Light yellow	Medium late	Round oval
20 21 22	Margone	Val di Ledro (TN) Val di Gresta (TN) Baselga di Pinè (TN)	2020	Margone (Led) Margone (VdG) Margone (BdP)	Purple	Pigmented	Dark violet	Dark violet	Very late	Round oval
23 24 25	Violette	Sabaudia (LT) Piana del Fucino1 (AG) Piana del Fucino2 (AG)	2020	Violette (Saba) Violette (PdF1) Violette (Pdf2)	Purple	Pigmented	Dark violet	Dark violet	Very late	Long irregular
26	Vitellotte	Grotte di Castro (VT)	2020	Vitellotte	Purple	Pigmented	Dark violet	Dark violet	Very late	Long irregular
27	Delizia Blu	Grotte di Castro (VT)	2020	Delizia Blu	Purple	Pigmented	Dark violet	Dark violet	Very late	Round oval
28	Blaue St. Galler	Val Pusteria (BZ)	2020	Blaue St. Galler	Purple	Pigmented	Dark violet	Dark violet	Very late	Long irregular

2.3. Steam Cooking Method

Potato samples were cooked using a commercial steamer (Avance Collection Vaporiera HD9150/91, Philips). The cooking method was optimized based on the current literature [14] and the minimum cooking time was used to obtain an adequate palatability and taste in accordance with Italian eating habits [14]. The pieces of each biological replicate were arranged in a circle in the steamer baskets to reach uniform steaming and heating conditions. The food materials were cooked for 20 min. Then, the potato pieces were softly peeled off and stored at −80 °C. The samples were freeze-dried, ground to a fine powder in a grinder and stored into air-tight plastic zipper bags at −80 °C until the analyses.

2.4. Antioxidants, Total Polyphenolic Content, Polyphenols, and Sugars Extraction Method

The extraction protocol was adapted from Tierno et al. [15]. Powdered samples (100 mg) were extracted with 5 mL of methanol-water (70/30 v/v), acidified with HCl (to a final concentration of 0.12 mol/L). The mixture was vigorously shaken for 20 min using a multi-rotator, sonicated for 15 min and again vigorously shaken for 20 min. The supernatant was recovered, and the extraction operation was repeated. The methanolic extracts were combined (10 mL in total), then 1 mL was centrifuged at +5 °C at 14,000 rpm for 15 min. The supernatant was removed and stored at −80 °C until the analysis. For each biological replicate, three technical replicates were extracted.

2.5. Anthocyanin Extraction Method

The extraction protocol of Oertel et al. [9] was applied. Briefly, 100 mg of freeze-dried material was extracted with a mixture of 500 µL of methanol-water (70/30 v/v acidified with formic acid (0.2 mg/L). The sample was vortexed for 3 s at room temperature, shaken for 30 min on a thermomixer at +4 °C and centrifuged for 10 min at +4 °C with 12,000 rpm. The supernatant was recovered, and the extraction operation was repeated once again with the pellet. The combined extracts were stored at −80 °C until the analysis.

2.6. Spectrophotometric Assays

2.6.1. Total Polyphenolic Content (TPC)

The TPC was determined by the Folin–Ciocalteu method and adapted from Valls et al. [16]. 250 µL of deionized water and 60 µL of extracts was added to 60 µL of Folin–Ciocalteu reagent. The mixture was mixed at 12,000 rpm for 6 min at room temperature (RT). Then, 630 µL of sodium carbonate (7.5% w/v) were added to the mixtures, and they were mixed at RT for 90 min. The absorbance was recorded at 740 nm on a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, CA, USA) spectrophotometer, and referred to a standard curve of gallic acid (range 0–150 ng/µL). The results were normalized for the weight and expressed as mg of gallic acid equivalents per 100 g of fresh weight sample (Table S3).

2.6.2. Antioxidant Activity, ABTS Assay

It was determined using Trolox equivalent antioxidant capacity (TEAC) assay adapted from Valls et al. [16]. For the assay, 1970 µL of ABTS reagent was added to 30 µL of sample extract and the mixture was incubated into dark for 10 min at RT. The decrease in absorbance was read at 734 nm using a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, CA, USA) spectrophotometer, and referred to a standard curve of Trolox (range 15.6–250 ng/µL). The results were normalized for the weight and expressed as mg of Trolox equivalents per 100 g of fresh weight sample (Table S3).

2.6.3. Antioxidant Activity, FRAP Assay

It was determined by FRAP assay adapted from Valls et al. [16]. The purple-fleshed samples were prepared for the assay by diluting them 1:10 using the extraction solution (Section 2.4), whereas, the extracts of the yellow-, white-fleshed, and red-skinned potatoes were used without any further dilution. For the assay, 960 µL of FRAP reagent was added

to 60 µL of sample extract and 180 µL of MilliQ. The mixtures were incubated into dark at +37 °C for 90 min. The decrease in absorbance was read at 595 nm using a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, CA, USA) spectrophotometer, and referred to a standard curve of Trolox (range 15.6–250.0 ng/µL). The results were normalized for the weight and expressed as mg of Trolox equivalents per 100 g of fresh weight sample (Table S3).

2.6.4. Total Monomeric Content and Total Anthocyanins Content (Anto Mono and Anto Tot)

The Anto mono and Anto tot were determined by pH differential method and adapted from Valls et al. [16]. Two dilutions of the same extract were made by adding 800 µL of potassium chloride (0.025 M, pH 1) to 200 µL of sample extract and 800 µL of sodium acetate (0.4 M, pH 4.5) to 200 µL of sample extract, respectively. The absorbance was immediately recorded at 520 and 700 nm on a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, USA) spectrophotometer. The calculations were conducted using Lambert–Beer law (ε = 26,900 L/mol/cm, MW = 449.2 g/mol). The results were normalized for the weight and expressed as mg of cyanidin-3-glucoside equivalents per 100 g of fresh weight sample (Table S4).

2.7. Analysis of Polyphenolic Compounds

The analytical procedure was adapted from Ieri et al. [17]. An UltiMate 3000 UHPLC system (Thermo Scientific, Waltham, MA, USA) coupled with a TSQ Quantiva (Thermo Scientific, Waltham, MA, USA) triple-stage quadrupole mass spectrometer was used (UHPLC-QqQ-MS/MS). The quality control sample (QC) was made by pooling 500 µL of one yellow-fleshed variety extract, 500 µL of one white-fleshed variety extract, 500 µL of one red-skinned variety extract, and 500 µL of one purple-fleshed variety extract. Then, the resulting mixture was diluted 1:10 with extraction solution and used to control the absence of chromatographic drift and to check the stability of the instruments. A blank sample (solvent mixture) was used to verify absence of significant carry-over effect. The procedure for the calibration curve is adopted by Vrhovsek et al. [18]. The mother solutions of each analytical standard (2000 ng/µL) were prepared using methanol or a mixture of methanol/water (50/50 v/v. Four starting standard mixtures were prepared at 50 ng/µL representing the most known polyphenolic compounds (Table S1) following the current literature [2,18]. These starting standard mixtures were serially diluted to working concentrations. The calibration curve of polyphenolic standards covered a range between 10.0 and 0.0025 ng/µL. A mixture of two internal standards, which was prepared at 2 ng/µL using catechin-2,3,4-$^{13}C_3$ and ferulic acid-1,2,3-$^{13}C_3$, was used to monitor the instrument performance and an overall RSD% of 4.34% and RSD% 6.84% was respectively found. All potato samples were diluted 1:10 with extraction solution before analysis. Each injected solution was prepared by mixing 50 µL of potato sample (or QC, or standard mixture) with 50 µL (2 ng/µL) of internal standard solution, and they were analyzed in a randomized order, each 10 analyzes a QC was injected. Repeatability (intra-day and inter-day precision) was measured as RDS% after multiple injections (n = 10) of mixture of the standards at 1 ng/µL, 0.1 ng/µL, and 0.01 ng/µL. The RSD% for all the standards was calculated, which have been injected 10 times inter-day [17]. The mobile phase was B (0.1% [v/v] formic acid in acetonitrile LC-MS analytical grade) and A (0.1% [v/v] formic acid in Millipore water). The gradient elution was 0–1.5 min (5% B), 1.5–8 min (25% B), 8–10 min (55% B), 10–11 min (95% B), 11–12 min (95% B), 12–12.1 min (5%) with a flow rate 0.3 mL/min. The autosampler and column temperature were set at +5 °C and +40 °C, respectively. The separation of the compounds was performed using a Hypersil GOLD™ HPLC (2.1 × 100 mm, 1.9 µm, Thermo Scientific, Waltham, MA, USA) column with the corresponding pre-column. Injection volume was 1.5 µL. The operations were controlled by the Chromeleon Chromatography Data System (CDS) (version 6.8) software and Thermo Xcalibur (version 3.0) software (both Thermo Scientific, Waltham, MA, USA). The electrospray ion (ESI) source conditions were as follows: positive voltage 4000 V, negative voltage 3500 V, vaporizer temperature 300 °C,

capillary temperature 325 °C, sheath gas 40 arbitrary unit (AU), auxiliary gas 10 AU, sweep gas 0 AU. The collision gas (Argon) was set at 1.5 mTorr. Data processing was conducted using Tracefinder 3.2. The method was able to detect 27 polyphenolic compounds and a total of 15 polyphenols was quantified in the potato varieties under investigation. The retention time, ESI polarity, molecular weight, precursors, product ions, collision energy, RF lens, regression parameters, and linearity range of the calibration curve of each compound was summarized in Table S1. The quantification of each analyte was carried out by an external calibration curve using analyte/internal standard area ratios. The results were normalized for the sample weight and expressed as mg of each compound per 100 g of fresh weight sample (Table S3).

2.8. Analysis of Sugar Compounds

The analytical procedure was adapted from Eisenstecken et al. [19]. An ion chromatograph (IC) with pulsed amperometric detection (HPAE-PAD) was used to quantify individual sugars. The analysis was performed on ICS-5000 (Thermo Scientific Dionex, Sunnyvale, CA, USA). The separation of sugars was achieved by a Dionex CarboPac PA1 Analytical column (4 × 250 mm) and a Dionex CarboPac PA1 Guard column (4 × 50 mm). Yellow- and white-fleshed potato extracts were diluted using the extraction solution (Section 2.4) 1:10 before the injection, whereas, the extracts of the red-skinned and purple-fleshed potato samples were used without any further dilution. The calibration curve of sugar standards covered a range between 200.0 and 0.1 ng/µL. An isocratic condition with 50 mM sodium hydroxide (solvent A, 80%) and Millipore water (solvent B, 20%) for 17 min was used for the chromatographic separation of the sugar compounds, followed by a regeneration of the column with 200 mM NaOH (solvent C, 100%) for 10 min. Flow rate was set at 1.2 mL/min, injection volume was 20 µL and the column temperature +30 °C. An Au on PTFE disposable working electrode and a pH-Ag/AgCl reference electrode were used. All compounds were quantified with the calibration curve of each sugar. The operations were controlled by the Chromeleon Chromatography Data System (CDS) (version 6.8, Thermo Scientific, Waltham, MA, USA) software. The analytical features could be found in Table S5. The results were normalized for the sample weight and expressed as mg of each compound per 100 g of fresh weight sample (Table S3).

2.9. Analysis of Anthocyanins

The analytical procedure was adapted from Oertel et al. [9]. The analysis was performed on red-skinned and purple-fleshed potato samples. Separation was performed on a Waters Acquity UPLC system (Milford, MA, USA) using a RP Acquity UPLC BEH C18 column (130 A°, 2.1 × 150 mm, 1.7 µm, Waters, Milford, MA, USA), protected with an Acquity UPLC BEH C18 pre-column (130 A°, 2.1 × 5 mm, 1.7 µm, Waters, Milford, MA, USA). The following multistep linear gradient was used: 0–4 min (5–40% B), 4–9 min (40–55% B), 9–11 min (55–95% B), and an isocratic hold for 3 min. Solvent B was methanol/5% formic acid [v/v] and solvent A was water/5% formic acid [v/v], administered at a flow rate of 0.3 mL/min. The injection volume was 2 µL for both sample and standard solutions. Samples were kept at +6 °C during the analysis, the column at +40 °C. Mass spectrometry detection was performed on a Waters Xevo TQMS (Milford, MA, USA) instrument equipped with an electrospray (ESI) source. Capillary voltage was 3.5 kV in positive mode, the source was kept at 150 °C; desolvation temperature was 500 °C, cone gas flow, 50 L/h, and desolvation gas flow, 1000 L/h. Unit resolution was applied to each quadrupole. MRM (multiple-reaction monitoring) data analysis was conducted using MassLynx 4.1. All MRM parameters parameters and acronyms can be found in Table S2. All compounds were quantified with the calibration curve of cyanidin 3-glucoside. The results were normalized for the sample weight and expressed as mg cyanidin 3-glucoside per 100 g of fresh weight sample (Table S4).

2.10. Datasets

To conduct the data analysis in this study, the following datasets were generated: antioxidants (AA), polyphenols (poly), and sugars (sug) (Table 2).

Table 2. List of datasets used for the data fusion approach based on JIVE. Each dataset was identified by a dataset ID and gathered a specific number of analytes. The analytical methods were reported. More detailed information regarding the quantitative data, which composed the datasets, can be found in Table S3 (AA, Poly, Sug) and Table S4 (Anthocyanins).

Dataset	Dataset ID	N. of Analytes	Analytical Methods	JIVE Model
Antioxidants Total polyphenolic content	AA	3	Spectrophotometric assays (FRAP, ABTS, FC)	yes
Polyphenols	Poly	15	UHPLC-QqQ-MS/MS	yes
Sugar	Sug	7	IC-HPAE-PAD	yes
Anthocyanins	Anthocyanins	22	UHPLC-QqQ-MS/MS	no

2.11. JIVE, a Data Fusion Approach

Statistical analysis was performed using R software version 4.1.1. (R Foundation for Statistical Computing, Vienna, Austria) [20]. The shared and unshared components across the three datasets were investigated using the unsupervised multivariate approach Joint and Individual Variation Explained (JIVE) [12]. The missing values were imputed in all three datasets AA, poly, and sug (Table 2) by replacing them with a random value between zero value and the LOD (limit of detection) of the analytical method. The data were log-transformed, mean-centered, and scaled before JIVE analysis. The function used is called jive of the JIVE package [12]. The selection of the number of components was done by a permutation test to identify the number of components for common and distinct variation. The number of components were known as "ranks" [12], which were 1, 1, 3, 1 for joint, AA, poly, and sug datasets, respectively. Then, the JIVE results were submitted to the PCA (Principal Component Analysis) function of FactomineR package [21,22] to calculate loadings and score values. Based on the calculated rank, the loadings were extracted by the PCA results, and they were summarized in one dimension by using the facto_summarize function in R [22], which is able to subset and summarize the results of PCA. The resulting loadings from the facto_summarize function were used to calculate the variable importance of each polyphenol. The variable importance of each metabolite is calculated based on the results obtained by the variable's coordinates on the ranks and the dimension's eigenvalue's on the PCA results [22].

3. Results and Discussion

3.1. Interpretation of JIVE Model

JIVE is essentially a latent variable (LV) model, so its results can be graphically represented by score plots (similar to the one obtained by principal component analysis (PCA)) [12], in which the points represent the individual potato samples. The score plots show the projection of the data onto a set of "principal" components which represent joint and individual LVs. The joint component captures the variability common to the jointed datasets, while the individual components highlight the unique contributions of each dataset. In the score plots, the joint component is represented in the *y*-axis, whereas the *x*-axis is changed for each individual component of each dataset. These combined plots are used to investigate the similarity and/or dissimilarity amongst datasets. These score plots are shown in Figure 1 and this representation is known as a one-versus-the-rest approach.

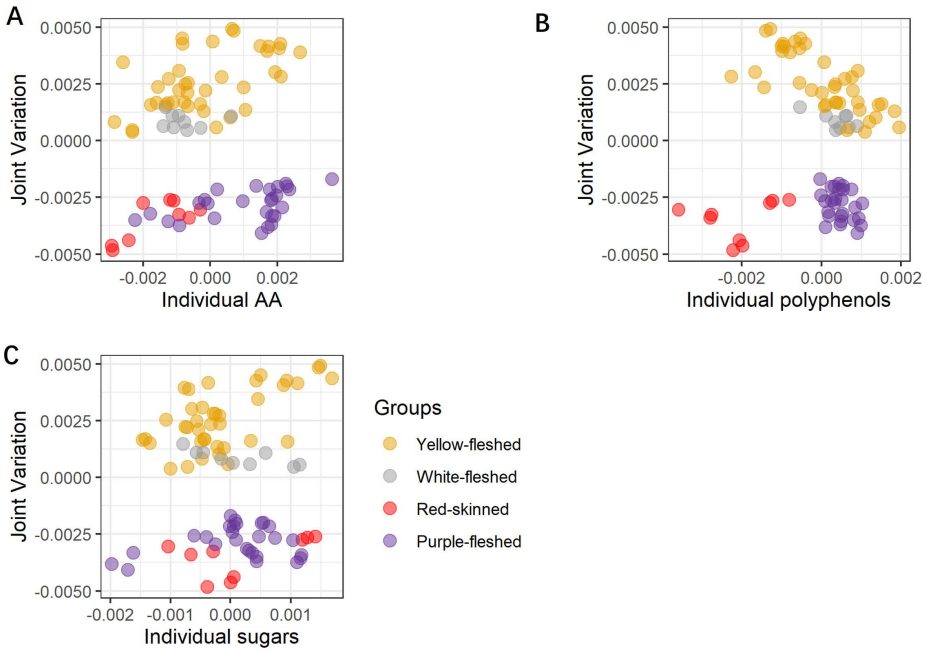

Figure 1. Score plots of the joint part of the three joined datasets, and the individual parts of the three separated datasets are reported as joint and poly (**A**), joint and sug (**B**), and joint and AA (**C**). The joint part gathers all three datasets (poly, sug, AA). The poly dataset includes the individual polyphenols, the sug dataset includes the individual sugars, and the AA dataset includes ABTS, FRAP, and FC assays. The 28 potato samples are organized into four groups (yellow-, white-fleshed, red-skinned, and purple-fleshed), which are highlighted using four different colors yellow, grey, red, and purple, respectively. For each steam-cooked potato sample, the three corresponding biological replicates are graphically presented as points and using the corresponding group color. AA = antioxidant activity, Poly = single polyphenols, Sug = sugars.

Looking at Figure 1A–C, a clear separation between the non-pigmented and pigmented steam-cooked potato samples is recognizable along the joint component, thus indicating that this type of partitioning is common to all three datasets. Further separation between the red-skinned and purple-fleshed samples is also visible (Figure 1B), but this separation is now concentrated along the individual components of the polyphenolic dataset, thus demonstrating that this class of compounds is exclusively able to differentiate among the two pigmented groups. Interestingly, the combination of polyphenols and common variation seem also to clearly separate the individual red-skinned varieties (local red variety, Desiree, and Red Scarlett), but a similar level of partitioning is not visible for the other three macro groups, thus providing evidence that each red-skinned potato may have a characteristic and distinctive metabolome. Looking at the individual component of the sugar dataset, a peculiar trend is observed for the red-skinned local red variety and the purple-fleshed Vitelotte, whose behavior prevents a complete separation of red-skinned and purple-fleshed varieties similar to the one observed for the polyphenols. The plot suggests that in terms of sugars, these two varieties are significantly divergent in contrast to the other members of the corresponding groups. The inspection of the variable importance in the different components of the JIVE model can be used to assess the specific contribution of the different metabolites and classes of metabolites to the individual and common components of the variability. In our case, the variable importance (VIP) of each compound is calculated using the reference [22] and shown in Figure 2.

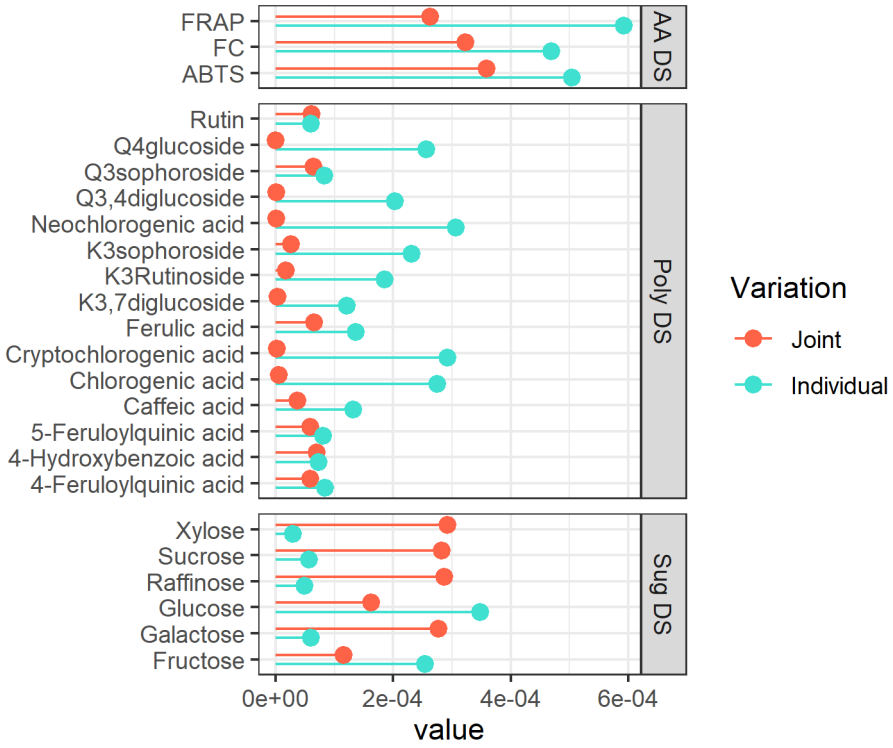

Figure 2. Variable importance (VIP) for each metabolite of the individual (turquoise lines) and Joint (tomato lines) parts. The VIP is calculated based on the rank extracted from the JIVE results. The ranks are 1, 3, 1 for the three separated datasets (AA, poly, and sug), respectively, whereas the joint part has a rank equal to 1 (see Section 2.11). AA = antioxidant activity, Poly = single polyphenols, Sug = sugars, DS = dataset, K = Kaempferol, Q = Quercetin.

The plot (Figure 2) suggests that the trends in the polyphenols are mainly captured by the individual component of the model (10 out of the 15 compounds showed a higher VIP for this component), while the variability of sugars mainly goes in the common part. In terms of interpretation, this means that the separation between pigmented and non-pigmented potatoes should be clearly visible in the sugar dataset, while the separation within the two pigmented groups should show up in the polyphenols dataset. Regarding the sugar dataset (sug DS), high values of VIPs for xylose, sucrose, and raffinose are observed demonstrating their responsibility in the separation between pigmented and non-pigmentated samples in the joint structure. On the contrary, considering the polyphenolic dataset (poly DS), high values of VIPs for the neochlorogenic, cryptochlorogenic, and chlorogenic acids are found, thus suggesting that they carry considerable value in the separation between pigmented and non-pigmentated samples too. In the case of the antioxidants, the picture is less clear-cut since this layer of data seemed to contribute almost in the same way to the individual and common components. A detailed discussion of the trends of the individual metabolites will be the subject of the following section.

3.2. Individual Metabolites

3.2.1. Antioxidant Activities and Total Polyphenolic Content

Figure 3 shows the antioxidant capacity (ABTS and FRAP assays) and the total polyphenols content (Folin–Ciocalteu (FC) assay) across the four groups of potatoes.

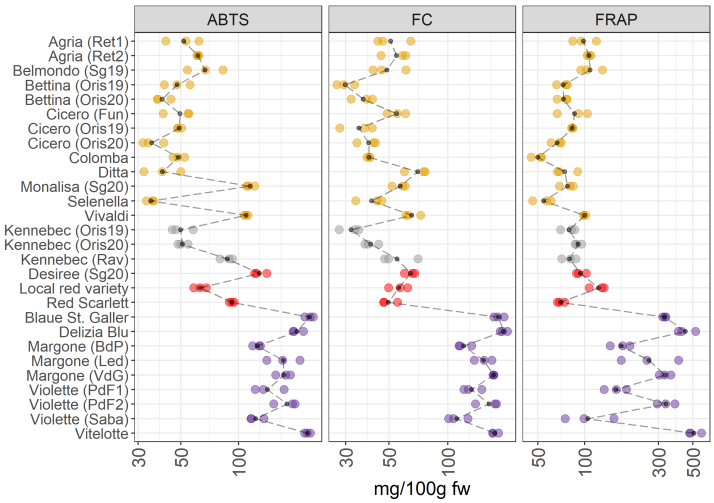

Figure 3. ABTS, FC, and FRAP in 28 potato varieties organized in four groups (yellow-, white-fleshed, red-skinned, and purple-fleshed); three biological replicates for each sample were analyzed. The black points represented the averages for each analyte calculated on three biological replicates. The black long dash lines showed the concentration trends amongst all the potato groups. The concentrations were transformed in a log-10-scale. fw = fresh weight. For more detailed information about potato varieties see Table 1.

The VIPs of the JIVE model (Figure 2) indicate that ABTS, FC and FRAP are mainly contributing to the individual part of the model, so a clear separation between between pigmented and non-pigmented varieties should not be the clearest structure in this dataset. This is confirmed by Figure 3, which shows a comparable amount of antioxidants and in the total polyphenolic content between yellow-, white-fleshed, and red-skinned groups. In contrast, the purple-fleshed potatoes have the highest antioxidant contents, in particular in the case of FC. Looking at the individual varieties, Vivaldi and Monalisa collected from San Genesio (Sg20, BZ, Italy) in 2020 (as yellow-fleshed potatoes), demonstrate higher values in the antioxidant capacity assay (ABTS), than the others yellow-fleshed samples. Additionally, the white-fleshed potatoes show a comparable content of antioxidants to yellow-fleshed samples, however, Kennebec from Rav (Ravina, TN, Italy) shows higher values of antioxidant activities (ABTS assay) than the other Kennebek cultivars collected in Oris (BZ, Italy). Additionally, there is a marked variability in the concentration of antioxidants in the red-skinned and purple-fleshed groups. Vitelotte and Blaue St. Galler cultivars, both belonging to the purple-fleshed group, show the highest levels of antioxidants and total polyphenolic content.

Years: Cicero, Bettina, and Kennebec potato varieties, which were cultivated in the same environment conditions (Oris, BZ, Italy), have been harvested in two consecutive years (2019 and 2020). These samples are indicated in the Figure 3 as Oris19 and Oris20 that means Oris-grown potatoes in 2019 and 2020. Differences in the total polyphenolic content and antioxidant properties were hardly observed both in yellow-fleshed (Cicero, Bettina) and in white-fleshed (Kennebec) potatoes.

Environmental conditions: In agreement with the published literature, our data show a noticeable variability in intra- and inter-groups (Figure 3). The intra-group variability is linked to environmental conditions which influences the accumulation of polyphenols in the crop [23]. At the same time, Lachman et al. [24] reported that there were significant differences in total polyphenolic content depending on the different places of growth.

The accumulation of phenolic compounds is regulated by the potato genotype, and several types of phenolics are synthesized. Additionally, the antioxidant potential of phenolic metabolites is influenced by the position of hydroxylation [23] and by the mean relative concentrations of the antioxidants found in the vegetable matrices. Several authors reported that the purple-fleshed varieties were characterized by a high content of

antioxidants, and that the content in the pigmented varieties may be 2-3 fold higher than in non-pigmented potatoes [5,25]. This was linked to the high content of polyphenols and anthocyanins, which demonstrated to have strong antioxidant properties [7,25,26]. Deuber et al. [27] reported that Vitelotte cultivar was characterized by the highest polyphenol and antioxidant content, thus confirming our results [23,27]. Furthermore, the same pattern is reported previously by Hamouz et al. [28], where they analyzed Vitelotte, Violette, and Blaue St. Galler varieties showing a very high content of antioxidants. Contrary to what is stated by Hamouz et al. [28], the Violette variety from Sabaudia (Saba) (LT, Italy) shows the lowest polyphenol content and antioxidants in the purple-fleshed group considered in this survey. The antioxidant activity was lower in the red-skinned potatoes due to the lower content in anthocyanins compared to purple-fleshed varieties [28]. The health effects of antioxidant compounds, mediated by their radical scavenging activity, are well-known [29] and other studies reported that the pigmented potato varieties had the highest antioxidant capacity [9,28,30]. Thus suggesting that these cultivars for selection for a healthy human diet.

3.2.2. Polyphenolic Compounds

An interesting variability of polyphenolic compounds is found amongst the potato groups under investigation (Figure 4).

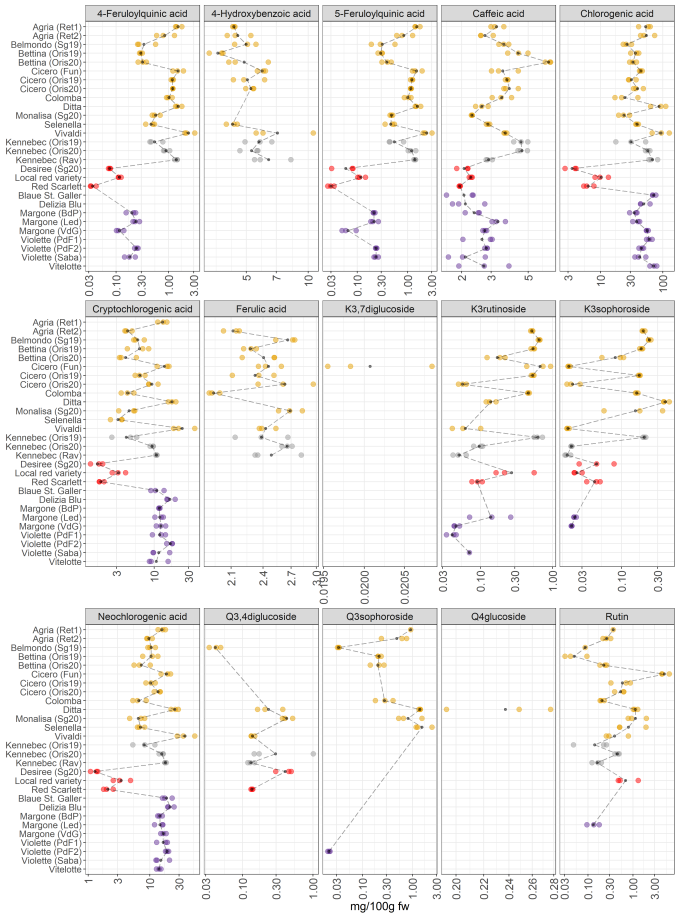

Figure 4. Polyphenolic compounds in 28 potato varieties organized in four groups (yellow-, white-, red-skinned, and purple-fleshed); three biological replicates for each sample were analyzed. The black points represented the averages for each analyte calculated on three biological replicates. The black long dash lines showed the concentration trends amongst all the potato groups. The concentrations were transformed in a log-10-scale. K = Kaempferol, Q = Quercetin, fw = fresh weight. For more detailed information about potato varieties see Table 1.

JIVE has indicated a strong role of this dataset in the differentiation of red-skinned and purple-fleshed potatoes (Figure 1B), and many polyphenols show a high importance in the individual component of the model. This differentiation should be then visible at the level of raw concentrations. Individual polyphenols (Figure 4) indeed show that red-skinned potatoes have the lowest concentration of many phenolic compounds. As already discussed, red-skinned varieties show very characteristic profiles.

As far as the individual metabolites are concerned, the plot shows an interesting variation in the concentration of 4-feruloylquinic and 5-feruloylquinic acids, which have been hardly considered in the literature [8] due to the scarce availability of pure certificated analytical standards. These metabolites are quantified and detected in a few pigmented samples: Violette variety from Piana del Fucino2 (PdF2) (AG, Italy) and Sabaudia (Saba) (LT, Italy) and in the variety of Margone collected from three cultivation areas in Trentino (Baselga di Pinè (BdP), Val di Gresta (VdG), and Val di Ledro (Led) (TN, Italy)), showing an appreciable concentration of 4-feruloylquinic and 5-feruloylquinic acids; in contrast, these two phenolic compounds are not detected in the cultivar Blaue St. Galler, collected in Val Pusteria (BZ, Italy). It is important to highlight that the individuals VIP of 4-feruloylquinic and 5-feruloylquinic acids (Figure 2) were slightly higher than the VIP of the joint component because these compounds were not detected in some cultivars (the missing values are imputed before the JIVE analysis). The reasons for the lack of quantification of some metabolites (4-hydroxybenzoic acid, 4-feruloylquinic acid, 5-feruloylquinic acid, ferulic acid, kaempferol and quercetin derivatives) in some potato cultivars was because these metabolites were present only in traces or were absent. Thus, meaning that the concentration of a specific compound was generally lower than its limit of quantification (LOQ). The LOQ of each analyte is reported in Table S1. Within the yellow-fleshed samples, considerable variability in the concentration of hydroxycinnamic acids (chlorogenic, cryptochlorogenic, and neochlorogenic acids) was found. The variety Vivaldi significantly stands out among the yellow-fleshed varieties due to the highest concentration of all isomers of chlorogenic acid. Additionally, this cultivar shows high antioxidant activity (Figure 3), and the positive correlation between the chlorogenic acids and antioxidant activities has been already reported [31]. Within the red-skinned group, the local red variety shows the highest amount of hydroxycinnamates acids. The amount of these hydroxycinnamic acids is comparable to the concentrations found in white-fleshed samples. It is recognized that there is a noteworthy amount of hydroxycinnamic acids in the purple-fleshed samples and the levels of these metabolites are comparable to the concentrations in yellow- and white-fleshed potatoes. Surprisingly, the concentration of hydroxycinnamic acids is strongly different between red-skinned and purple-fleshed samples. Therefore, our data confirm that the tissue-specific accumulation of some hydroxycinnamic acids may be cultivar-specific as well [8,9]. The VIP of hydroxycinnamic acids turned out to be extremely higher in the individual component than the joint ones, thus suggesting that they are the most influential factors in separating red-skinned and purple-fleshed groups (Figure 2). No missing values are reported (Figure 4). Some of the polyphenols strongly accumulate in yellow- and white-fleshed potatoes, especially kaempferol and quercetin derivatives. Indeed, our data show a pronounced variability in the concentration of kaempferol glycosides, and they are mostly present within yellow- and white-fleshed groups. Kaempferol-3-rutinoside and kaempferol-3-sophoroside are detected in all red-skinned varieties and a few purple-fleshed samples: Violette cultivar from Sabaudia (Saba) (LT, Italy) and Piana del Fucino1 (PdF1) (TN, Italy), and Margone cultivar (VdG and Led (both TN, Italy)). The VIP of kaempferol-3-rutinoside and kaempferol-3-sophoroside (Figure 2) are higher in the individual part, thus demonstrating that the presence of these two kaempferol glycosides may be used to distinguish the two pigmented groups considered for this survey. Regarding the quercetin glycosides, they are mostly detected in the yellow- and white-fleshed potato groups. Rutin, the most abundant quercetin found in potatoes [7,32], is also quantified in the local red variety (TN, Italy) and in the Violette from Piana del Fucino2 (PdF2) (TN, Italy). The quercetin-3,4-diglucoside is found in all red-skinned varieties and its amount is comparable

with the concentration found in yellow- and white-fleshed samples. The VIP of rutin in the individual and joint parts are comparable, indeed, a modest variability amongst the yellow-, white-fleshed and red-skinned groups is observed (Figure 2). In contrast, the individual VIP of quercetin-3,4-diglucoside is extremely higher than the joint VIP because the missing values are mostly imputed.

Years: Regarding the three varieties Cicero, Bettina, and Kennebec collected from Oris (BZ, Italy) in 2019 and 2020, the sampling year hardly influenced the concentration of polyphenols. Little variability in the concentration of hydroxycinnamic acid is observed. On the contrary, the amount of kaempferol-3-rutinoside and kaempferol-3-sophoroside is affected by the harvest year in the three cultivars under investigation. Indeed, the concentration of these compounds is higher in those cultivars collected in 2019 than in those harvested in 2020. In contrast, the rutin seems to be less variable between the two years. As confirmed by Reddivari et al. [33], the influence of the genotype on the polyphenolic profile was more significant than the effects given by specific climatic features of the given year. Nevertheless, it is worth reporting that the amount of polyphenols varied across their potato samples [33], thus supporting our results that show the effect of two years on the polyphenolic compounds concentrations in Cicero, Bettina, and Kennebec cultivars (Figure 4).

Environmental conditions: It is worth noting that our data show a remarkable variability in the concentration of phenolic compounds in potato samples belonging to the same varieties (Kennebec, Agria, Margone, and Violette), which are collected from different growing areas. Payyavula et al. [34] reported that the concentration of phenolics varied among the locations, and our data were in agreement with the authors [34,35]. Remarkably, the cultivars Kennebec, Cicero, and Bettina, which are harvested from Oris (BZ, Italy), show nearly the same concentration of polyphenols.

Chlorogenic acid is the major compound detected and quantified among the polyphenolic compound classes in all potato samples under investigation, and our findings are in accordance with the literature [14,32,34]. This bioactive compound had a strong antioxidant activity, indeed, the purple-fleshed potatoes have shown the highest amount in antioxidants (Figure 3). Deuber et al. [27] found the highest chlorogenic acid content in the Vitelotte cultivar in agreement with our study [27]. Additionally, Blaue St. Galler demonstrated a relevant concentration of chlorogenic acid (Figure 4). As reported by Ingallina et al. [8], chlorogenic acid was the strongest antioxidant compound, and the highest content of this metabolite was found in our study in Ditta, Vivaldi, and Vitelotte cultivars. Bassoli et al. [36] demonstrated that chlorogenic acid was able to inhibit glucose-6-phosphatase, which is involved in the pathway of gluconeogenesis and glycogenolysis and in the release of glucose in the blood controlling glycemia in type 2 diabetics. Therefore, the identification of those cultivars, in which the concentration of chlorogenic acid is high, could be potentially powerful for diabetic people. In the last decades, the attention towards phytochemicals has increased due to their well-known healthy properties [2]. Several authors reported the antioxidant properties of polyphenols, and their healthy properties have been deeper examined [2]. The antioxidant, anticancer, antiproliferative, and anti-inflammatory effects of potato extracts were studied in vivo and in vitro [7]. The two most abundant flavonols were rutin and kaempferol-3-rutinoside as reported by the review of Akyol et al. [2]. Surprisingly, Rommens et al. [13] tried to increase the production of kaempferol-3-rutinoside by modulating the flavonoid biosynthetic pathway. These metabolites undergo different metabolic transformations after the intake of flavonoid-rich foods [37]. A high amount of flavonoids is currently associated with different healthy properties. For example, the presence of quercetin derivatives may be useful for cardiovascular disease prevention and kaempferol derivatives are characterized by anti-inflammatory activities [37].

3.2.3. Sugars and Polyols

The main individual sugars quantified in potato samples were fructose, galactose, glucose, raffinose, sucrose, and xylose and are reported in Figure 5.

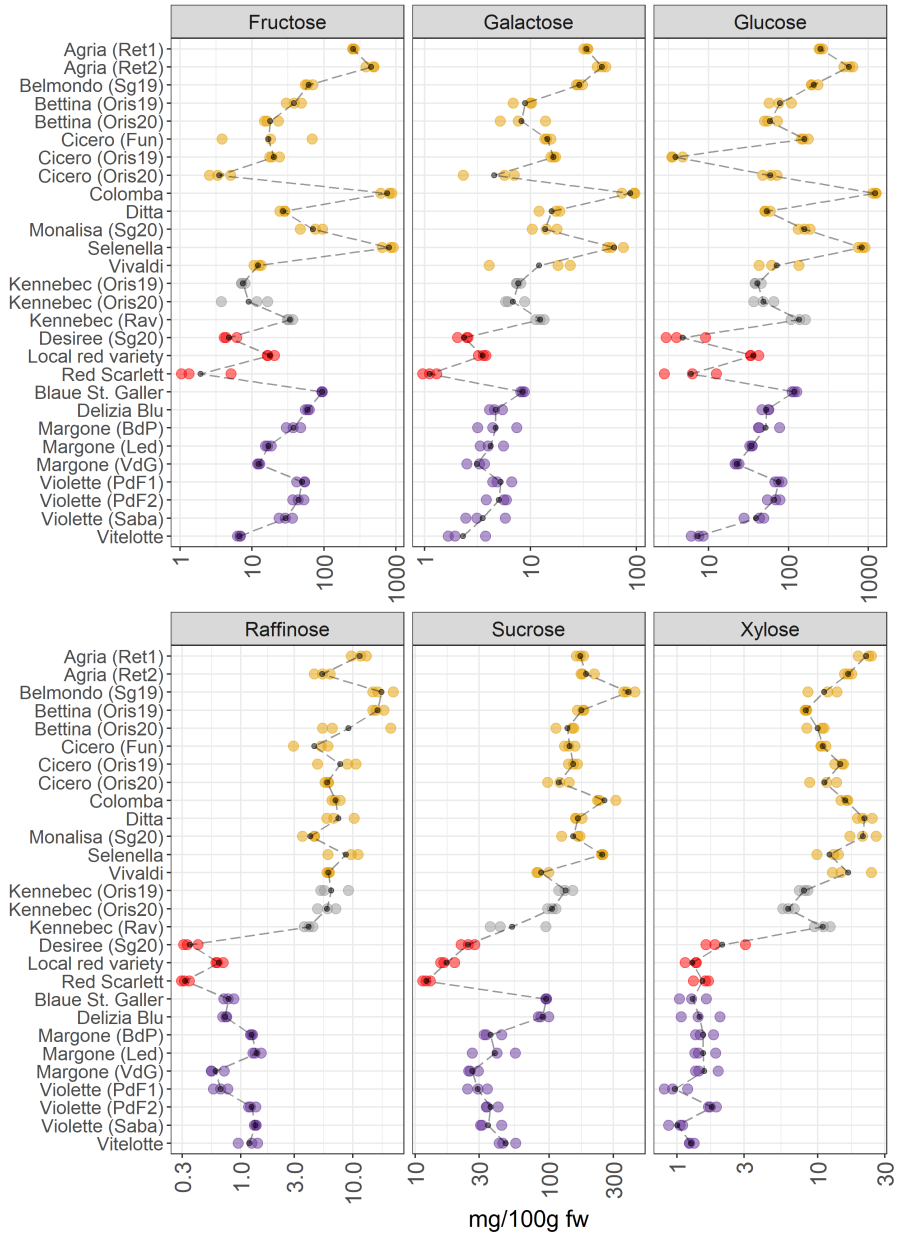

Figure 5. Content of individual sugars in 28 potato samples organized in four groups (yellow-, white-, red-skinned, and purple-fleshed); three biological replicates for each sample were analyzed. The black points represented the averages for each analyte calculated on three biological replicates. The black long dash lines showed the concentration trends amongst all the potato groups. The concentrations were transformed in a log-10-scale. fw = fresh weight. For more detailed information about potato varieties see Table 1.

The VIPs of JIVE (Figure 2) indicated that some of the members of this metabolic class (raffinose, xylose, sucrose, and galactose) were almost exclusively contributing to the joint part of the model. This result clearly shows up in the individual trend, where pigmented varieties are characterized by a strong reduction of the sugars raffinose, xylose, and sucrose (Figure 5). The profiles of glucose, galactose, and fructose showed a notable variability within the yellow-fleshed potato group. Conversely, the other three sugars show a lower variability within the same macro group. The white-fleshed potato samples show a comparable concentration of these carbohydrates with the yellow-fleshed group. As already pointed out during the discussion of the JIVE results, the trends observed for the red local variety and Vitelotte are somehow extreme in comparison to the other members

of the corresponding groups, thus providing evidence that the metabolome of these potato samples is peculiar and distinctive. In detail, the amount of sugars in the local red variety is comparable to the concentration of the carbohydrates found in the purple-fleshed varieties and this sample has the highest amount of glucose and fructose within the red-skinned group (Figure 5). Indeed, the scores show an interesting behavior of this potato sample in the individual component of the sugar dataset (Figure 1C), which is separated by the other red-skinned samples. Whereas the amount of sugars of Red Scarlet and Desiree varieties is the lowest detected amongst the groups. Another interesting observation may be done evaluating the trend observed for the Vitelotte cultivar, as a purple-fleshed variety, that demonstrates an interesting variation in the individual sugar profile. The amount of glucose and fructose is the lowest found within the purple-fleshed group and the Vitelotte sample has a sugar profile comparable to the red-skinned varieties. The scores of the Vitelotte cultivar are closer to the red-skinned group and it is well-differentiated by the other purple-fleshed samples (Figure 1C). The VIP of glucose and fructose are more influential in the individual component (Figure 2), thus suggesting that the concentration of these two carbohydrates may be useful to distinguish the red-skinned and purple-fleshed groups considered in this survey. Looking at the panels of the concentration of raffinose, sucrose, and xylose (Figure 5), the separation between non-pigmented and pigmented samples is clearly visible. These findings are confirmed by both what is observed in the score plot (Figure 1C), in which the two macro-groups are sharply noticeable, and the VIP that mainly has influential importance in the joint component (Figure 2).

Years: For Cicero, and Bettina, yellow-fleshed varieties obtained from Oris (BZ, Italy) in 2019 and 2020, a pronounced variation in the levels of sugars is observed. In contrast, the two Kennebec samples collected in Oris in 2019 and 2020 do not show differences in the levels of sugars. The reason could be linked to the different meteorological conditions and these cultivars react differently to the seasonal variation.

Environmental conditions: Looking at the same varieties of potatoes (Kennebec, Agria, Violette, and Margone), which are collected from different places, a slight variation is observed amongst these varieties within the groups. A more pronounced variation in the concentration levels of sugars is noticed in Kennebec and Margone samples. In detail, the Kennebec variety collected from Rav (TN, Italy) showed higher levels of sugars than those collected in Oris (BZ, Italy). It is worth noticing that the difference of altitude of the corresponding growing areas is about 600 m between Rav (around 200 m a. s. l.) and Oris (875 m a. s. l.). Similar results were observed in the study conducted by Andre et al. [35], where the influence of climatic cultivating conditions and altitude effects were investigated across different potato cultivars. Additionally, the most interesting differences in the glucose content between the two Agria samples obtained from commercial retailers are found.

It is well-known that fructose and glucose are produced in potatoes in equimolar amounts, even though the concentration of glucose is slightly higher than fructose (Figure 5). Glucose and fructose are reducing sugars and sucrose is a non-reducing sugar [38]. It is well known that the levels of these sugars depended on several factors (soil moisture, genotype, the levels of precipitation, irrigation, mineral nutrition, harvest time, and storage conditions) [38] and they are determining the quality of potatoes after storage and cooking and/or industrial processing [38]. Sowokinos et al. [38] reported that the sucrose content in different varieties was variable, and it depended on the reducing sugar (glucose and fructose) accumulation. Additionally, during storage, the carbohydrates were converted from starch, and the major sugars (glucose, fructose, and sucrose) are accumulated [39]. Therefore, the levels of sucrose provided important information in terms of harvest time, maturity, utilization practices, and the quality of potatoes for the selection of more suitable varieties for consumer preferences. Additionally, the potato cultivars containing less sugar should be preferred from consumers to reduce the impact on blood sugar. Therefore, the selection of potato varieties, which contain the lowest content of

carbohydrates, could play a potential role in preventing the onset of several dangerous health problems, including obesity and type 2 diabetes.

3.2.4. Anthocyanins

This last section focuses on a detailed investigation of the accumulation of anthocyanins in pigmented potatoes. Our results complement the studies already performed in recent years [5,7,9]. For this purpose, three red-skinned (Desiree, local red variety, and Red Scarlett) and five purple-fleshed varieties (Blaue St. Galler, Delizia Blue, Margone (from BdP, Led, and VdG), Violette (from PdF1, PdF2, and Saba), and Vitelotte) are collected, in total 12 samples of pigmented potatoes are thoroughly investigated in this study. Detailed information is reported in Table 1. The results are summarized in Figure 6.

Figure 6. Results of pH differential spectrophotometric method (Anto mono and Anto tot) and 22 single anthocyanins in 12 potato pigmented samples organized in two groups (red-skinned and purple-fleshed); three biological replicates for each sample were analyzed. The black points represented the averages for each analyte calculated on three biological replicates. The black long dash lines showed the concentration trends amongst all the potato groups. The concentrations were transformed in a log-10-scale. fw = fresh weight. Acronyms were reported in Table S2. For detailed information about potato varieties see Table 1.

We are able to detect and identify 22 anthocyanins in the potato extracts (Figure 6). The list of all acronyms can be found in Table S2. Our data show a pronounced difference between the accumulation of anthocyanins in red-skinned and purple-fleshed potatoes. Inter-varietal differences are observed, and a more pronounced variation in the content of anthocyanins is noted within the red-skinned potato varieties, showing that the local red variety have the highest levels of pigments in this group.

Total anthocyanin content and total monomeric anthocyanins: the total anthocyanin content (Anto tot) and total monomeric anthocyanins (Anto mono) in potatoes in the present study were analyzed by the pH differential spectrophotometric method. Generally, the highest content in monomeric anthocyanins and in total anthocyanins is found in purple-fleshed potato varieties. The Vitelotte, Blaue St. Galler and Delizia Blu cultivars turned out to be the varieties with the highest content of total anthocyanins and total monomeric anthocyanins. Additionally, within the red-skinned group, the local red variety has the highest amount of total and mono anthocyanins.

Cyanidin derivatives: Cyanidin 3-p-coumaroyl rutinoside-5-glucoside was tentatively annotated by Oertel et al. [9] for the first time. In our data, a variable accumulation pattern was observed within the red-skinned group, in which the local red variety has the highest amount of this compound. Whereas the concentration of the cyanidin derivative is higher in purple-fleshed varieties than the red-skinned group, a lower variability was observed within the purple group.

Malvidin derivatives: Several authors [10,23,26] reported that Violette is characterized by the highest levels of malvidin derivatives [23]. Our results (Figure 6) are in agreement with the literature [40], indeed, the Vitelotte cultivar contains the highest amount of malvidin 3-p-coumaroyl rutinoside-5-glucoside within the samples analyzed in this study.

Pelargonidin derivatives: our data show that the levels of acylated glycosides of pelargonidin are higher in red-skinned varieties than in purple-fleshed varieties, thus confirming that they are the most characteristic pigments in the red-skinned group [9]. The local red variety from Led has the highest levels of pelargonidin derivatives within the red-skinned group, in detail, the highest amount of pelargonidin feruloyl-xylosyl-glucosyl-galactoside is observed that was reported by Oertel et al. [9] for the first time. Lachman et al. [26] reported that pelargonidin 3-p-coumaric rutinoside-5-glucoside is the most abundant anthocyanin in potatoes and it was characteristic for red tuber tissue [9]. It is worth mentioning that the Blaue St. Galler variety shows the lowest content of pelargonidin derivatives.

Petunidin derivatives: petunidin derivatives are characteristic in the Blaue St. Galler variety and these results are confirmed by Lachman et al. [26]. In contrast the lowest amount of petunidin 3-p-coumaroylrutinoside-5-glucoside is observed in the Vitelotte cultivar, contrary to what it has been reported by Mulinacci et al. [10]. Within the red-skinned group, the highest amount of petunidin derivatives is found in the red local variety.

Peonidin derivatives: Blaue St. Galler contains the lowest concentration in peonidin derivatives within the purple-fleshed group, whereas the highest content is found in Violette (Saba) cultivar.

The literature reports that the most abundant anthocyanins in potates were: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin in acylated forms [7]. They are commonly acylated by caffeic, ferulic, and p-coumaric acids, for instance. Generally, the acylated anthocyanins were 98% of the total anthocyanin content in the potatoes [7] and the proportion of acylation differed between the red-skinned and purple-fleshed potato groups. Additionally, the acylated forms may be commonly linked to the rutinose, the most common glycosyl moiety, in position C3 [7], but also to other sugar moieties such as glucose, rhamnose, xylose, and arabinose. Jokioja et al. [41] described how the glycosyl moieties and the acylation influenced the chemical proprieties of the acylated anthocyanins, such as the stability of these compounds [41]. It has been reported that the different trend in the accumulation of anthocyanins in red-skinned and purple-fleshed varieties is a noteworthy factor in determining the tuber coloration patterns and the differentiation between the two pigmented groups [9]. Indeed, the proportions and the concentrations of acylated anthocyanins are variable across the pigmented potato varieties. However, all the anthocyanins under investigation has been detected in our samples, thus supporting existing results [41]. Numerous factors may influence the occurrence and accumulation of pigments in the flesh of potatoes [42]. Based on the type of acylated anthocyanin, the flesh of the pigmented tubers can be different. Indeed, Oertel et al. [9] reported that the presence of a specific anthocyanin backbone is responsible for the coloration patterns of

the tubers. For example, the authors [9] reported that the red-skinned varieties contained pelargonidin derivatives, whereas the purple-fleshed cultivars are characterized by petunidin derivatives, thus confirming our results. Additionally, a negative association between pelargonidin and petunidin derivatives is found. The biosynthesis of anthocyanins is influenced by internal factors, such as enzymes and sugar levels, and by external factors, such as climatic and environmental conditions [32]. The pigments are synthesized in the cytoplasm and endoplasmic reticulum membrane, and then they are accumulated in the vacuole [32]. Wang et al. [32] reported that purple-fleshed potatoes contained higher levels of enzyme PAL (phenylalanine ammonia lyase) than yellow-fleshed potatoes, which is involved in the anthocyanin accumulation. Being the potatoes consumed cooked, the cooking process is a crucial step in evaluating the amounts of acylated anthocyanins in pigmented tubers, indeed, the cooking treatments influenced the concentration of anthocyanins [10]. The anthocyanins were soluble in water and leached into the water during the boiling [43]. Mulinacci et al. [10] reported that the level of pigments between raw and cooked potatoes remained unaltered after microwaves cooking. Nevertheless, a decrease in the total anthocyanins content was observed in boiled potatoes [10]. This discrepancy could be due to the cooking methods and the cooking time, which influence the concentration of the phytochemicals in vegetables as reported in the literature [26,42]. Thus, leading to a increasing interest in selecting the most suitable cooking approach to preserve the maximum content of healthy compounds. It has been reported that the steaming method preserves the levels of pigments in potatoes compared to boiled or untreated material [42]. Additionally, the anthocyanins were strongly dependent on the pH and temperature of water leading to a certain loss of these pigments in comparison with the raw material during cooking [10]. Fossen et al. [44] reported that the color intensity of petanin and cyanidin-3-glucoside was affected by the pH variation. A degradation in the color intensity was observed until pH 5 for both pigments, whereas in alkaline conditions (pH 8.1) the coloration had a maximum [44]. Furthermore, the stability of anthocyanins during the storage was affected by the pH value [44]. Nevertheless, the aglycones of the anthocyanins were found to be bound to sugar moieties and the anthocyanins glycoside were more stable than the monoglucosides [45]. Fleschhut et al. [45] showed that anthocyanins were less stable at 23 °C than at 10 °C and decreased in the range between pH 4–9. The stability of the pigments in the foods was an important factor for the acceptability of the consumers [30]. The properties of anthocyanins have been already studied for their anticancer, anti-diabetes, and anti-inflammatory activity. Therefore, the actual research is focused on the bioavailability of anthocyanins in humans after the consumption of food rich in such compounds [41,45]. At odds, the biological activity and healthy potential of anthocyanins is mainly evaluated *in vitro* experiments. The bioavailability of anthocyanins is affected by several factors (e.g., pH, structural stability, acylation, absorption, and metabolism) [41]. Fleschhut et al. [45] highlighted the poor bioavailability of anthocyanins in humans due to the extensive biotransformation, which occurred at the colon level. Jokioja et al. [41] reported that the acylated anthocyanins are subjected to an extensive metabolism in humans [41]. The bioavailability of acylated anthocyanins depended on their chemical proprieties, the type of anthocyanin backbone, the acyl and sugar moieties, but also the presence of other compounds in the food matrices and the cooking method had an important role in the bioavailability of acylated anthocyanins. The scientific community agreed that the acylated anthocyanins, to which di- and trisaccharides are bound, are more stable compared to nonacylated anthocyanins or acylated anthocyanins with a monosaccharide conjugate [41]. Clinical studies were conducted on healthy, hypertensive, overweight, and obese volunteers [41] evidenced that the extracts of pigmented potatoes had an important role in avoiding the increase of both postprandial glucose and insulinemia [41]. Additionally, it has been proved that acylated anthocyanins had effects on postprandial inflammation, which caused by a high assumption of carbohydrates and fats, by controlling the postprandial glucose fluctuation. Therefore, the selection of pigmented tubers, which are characterized by a high amount of different types of acylated anthocyanins, is proposed

for human diet. Additionally, Moser et al. [46] showed that the purple-fleshed potatoes inhibited the glucose transportation in Caco-2 intestinal cells *in vitro*, thus highlighting the positive effects on glycemia and postprandial insulinemia [7,41].The anthocyanins are cleaved by enzymes of the gut bacteria into aglycon and phenolic acids supposing that the healthy effect of anthocyanins in humans is related to their metabolites [47]. Our results contribute to a better understanding of the metaboloma of eight pigmented potato varieties rich in health-promoting compounds to encourage the consumption of pigmented potatoes rich in different types of anthocyanins.

3.3. Identification of the Most Valuable Potato Varieties for Human Diets by Comparing the Different Metabolomes

A survey is conducted to identify the most favorable cultivars for human consumption by comparing the different metabolomes investigated. This aims to point consumers towards potato varieties characterized by a balanced content in single polyphenols, sugars, and single anthocyanins, including the acylated anthocyanins. For this claim, all single compounds, namely polyphenols (reported as total content of single polyphenols), sugars (reported as total sugar content), and single anthocyanins (reported as total content of single anthocyanins) were summarized for each potato sample. The summarized data are provided in the Tables S3 and S4 and they are graphically reported in Figure 7.

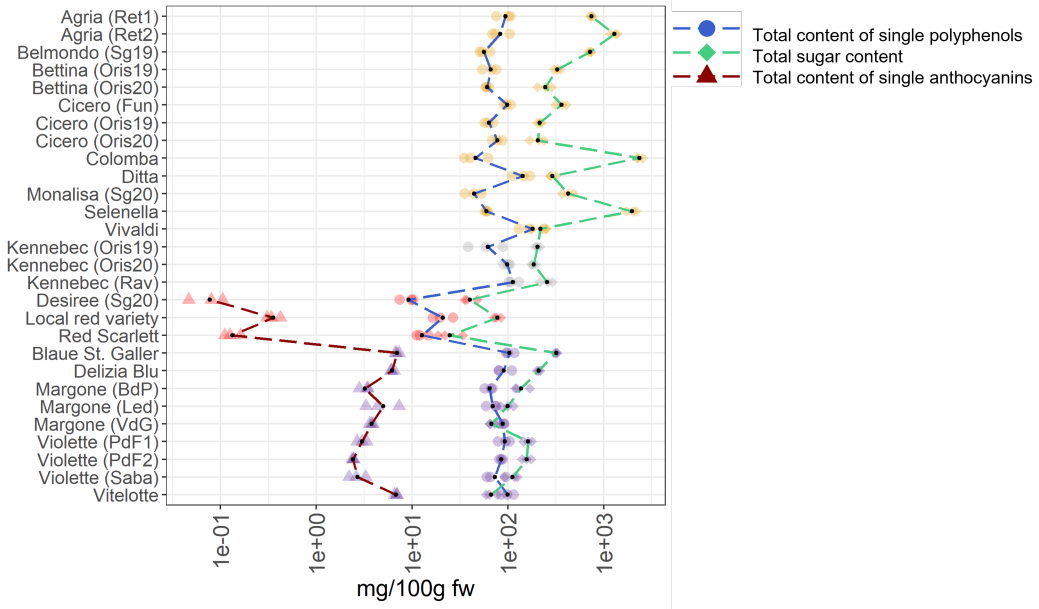

Figure 7. Total content of single polyphenols, total content of sugars, and total content of single anthocyanins in 28 potato varieties organized in four groups (yellow-, white-fleshed, red-skinned, and purple-fleshed); three biological replicates for each sample were analyzed. The black points represented the averages calculated on three biological replicates. The long dash lines showed the concentration trends amongst the potato groups. The concentrations were transformed in a log-10-scale. fw = fresh weight. For more detailed information about potato varieties see Table 1.

In the case of total content of single polyphenols, the most noticeable varieties are Vivaldi, Ditta, and Kennebec (Rav) showing concentrations of 180.17 mg/100 g fw, 142.42 mg/100 g fw, and 112.99 mg/100 g fw, respectively, that break the trend in the total content of polyphenols compared to other varieties. Notably, these varieties are belonging to the yellow- and white fleshed groups, highlighting that these groups are rich in polyphenols. Within the red-skinned potato samples, the highest concentrations of single polyphenols is found in the local red variety (20.87 mg/100 g fw). Indeed, a marked fluctuation in the total content of single polyphenols within this group is observed. Concerning the purple-fleshed varieties, the Blaue St. Galler cultivar shows the highest

total content of single polyphenols (103.84 mg/100 g fw), nearly compared to yellow- and white-fleshed potato samples. Interesting, the total content of single polyphenols in the purple-fleshed variety Margone from VdG, Led, and BdP, are showing notable concentrations and they are almost comparable between them, 88.14 mg/100 g fw, 69.28 mg/100 g fw, and 64.18 mg/100 g fw, respectively.

Regarding the total sugar content, the yellow-fleshed potato samples Colomba, Selenella, and Agria (Ret2), collected from different commercial retailers, show the highest values, namely 2,354.93 mg/100 g fw, 1,978.29 mg/100 g fw, and 1,287,86 mg/100 g fw, respectively. Notably, the total sugar content in these cultivars really stand out amongst all the samples studied and a high variability within the yellow-fleshed samples is observed, suggesting that potatoes from retailer have a higher sugar content than regionally produced ones. Indeed, the lowest total sugar content in this group is found in Ditta (290.02 mg/100 g fw) and Vivaldi (218.30 mg/100 g fw), both from local production. At odds, the total sugar content in the white-fleshed potato samples is comparable across them probably why only one variety (Kennebec) is collected in different locations and years, and a flat zone in the green dash line representing the total sugar content in Figure 7 is visible. On the contrary, in the pigmented samples, the total sugar content is significantly lower than in non-pigmented potatoes (Table S3). Within the red-skinned samples, which are all locally produced, the red local variety breaks the trend of the total sugar content due to the highest amount (77.26 mg/100 g fw), followed by the Desiree variety (40.10 mg/100 g fw) and the Red Scarlett variety (24.77 mg/100 g fw). Within the purple-fleshed potatoes, the highest sugar value in the Blaue St. Galle potato sample is found (319.00 mg/100 g fw), indeed, its content really stands out amongst all pigmented varieties (Figure 7). The most noticeable varieties in this group are Margone from Led and VdG, with a total sugar content of 99.53 mg/100 g fw and 67.27 mg/100 g fw, respectively, and Vitelotte with 66.77 mg/100 g fw.

Concerning the total content of single anthocyanins, the local red variety shows the highest value with 0.36 mg/100 g fw in the red-skinned potato group. As expected, a noteworthy increment in the trend of the total content of single anthocyanins in the purple-fleshed samples is observed (Figure 7). The variety of Margone, from Led, BdP, and VdG shows remarkable amounts of 4.99 mg/100 g fw, 3.22 mg/100 g fw, and 3.76 mg/100 g fw, respectively. The highest value in the total content of single anthocyanins is found in Blaue St. Galler (6.94 mg/100 g fw), which is locally produced, and is followed by the most famous and commercialized Vitelotte cultivar (6.78 mg/100 g fw). Indeed, the values of Blaue St. Galler and Vitelotte stand out within the purple-fleshed group (Figure 7). In summary, within non-pigmented potatoes, the most promising varieties are Ditta and Vivaldi due to the lowest total sugar content and remarkable values in the total content of single polyphenols. Concerning the red-skinned potato group, a noticeable total sugar content in the local red variety is well-counterbalanced by very high values in total content of single polyphenols and single anthocyanins. On the contrary, the total content of single polyphenols found in the purple-fleshed varieties Margone, collected in BdP, and Vitelotte is lower than the total sugar content in the same samples.

Our findings show that pigmented potatoes are characterized by a lower amount of sugars than non-pigmented potatoes, and by a higher content in single anthocyanins, and that locally produced varieties are the most promising ones for consumers.

4. Conclusions

In the habitual diet of Italian people, the presence of tubers with different skin and flesh colors is continuously increasing, thus stimulating the interest of researchers in evaluating their potential role in the human diet. In the present study, the metaboloma of four macro potato groups, that are yellow-, white-fleshed (non-pigmented), red-skinned, and purple-fleshed (pigmented) potatoes, was characterized by analyzing the antioxidant proprieties and the polyphenolic and sugar profiles. Furthermore, the anthocyanin profile in the pigmented potato samples was elucidated. The aims were to identify specific potato

cultivars rich in health-promoting compounds for the human diet and to promote the valorization of locally cultivated potato varieties. The results showed that the yellow- and white-fleshed potato samples, were characterized by low antioxidant proprieties, on the contrary, they had the highest content in single polyphenols and sugars. The red-skinned potato varieties showed similarities in the antioxidant properties compared to non-pigmented samples, however, the red-skinned samples were characterized by the lowest content of single polyphenols and sugars and showed a low anthocyanin content. The purple-fleshed varieties stand out because of the highest antioxidant activity and highest content of anthocyanins, moreover, they showed remarkable content of single polyphenols and a reasonable sugar content. Within the non-pigmented potatoes, the most promising varieties were the locally produced Ditta (BZ, Italy) and Vivaldi (TN, Italy) having the lowest amount of sugars and a remarkable content of polyphenols, followed by the varieties Bettina (BZ, Italy), Cicero (BZ, Italy), and Kennebec (BZ, Italy). Within the red-skinned group, the local red variety collected in Led (TN, Italy) showed the highest content in single polyphenols, sugars, and anthocyanins. The potatoes belonging to the purple-fleshed group are the most promising for human diet. A special interest was driven towards to the cultivar of Margone from local production. In particular, the best-balanced concentration in phytochemicals was found in the Margone sample from VdG (TN, Italy), characterized by high antioxidant propriety and polyphenolic content, a medium-low concentration of sugars, and an appreciable amount of anthocyanins, followed by the Margone samples from BdP (TN, Italy) and Led (TN, Italy). The Blaue St. Galler cultivar, collected from Val Pusteria (BZ, Italy), had the highest sugar content within the purple-fleshed group, however, this variety was well-counterbalanced by a moderate amount of polyphenols and a considerable amount of anthocyanins. Additionally, a good balance in the antioxidant propriety and in the concentration of polyphenols, sugars, and anthocyanins was found in the well-known, purple-fleshed variety cultivar Vitelotte (VT, Italy). In conclusion, the present study showed that locally produced potatoes, in particular the purple-fleshed varieties, contained the highest amounts of health promoting compounds, thus highlighting the importance of locally cultivated potatoes for human diet.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11121708/s1, Table S1: Polyphenol-Compounds analyzed with UHPLC-QqQ-MS/MS in this study, the corresponding compound class, compound number, compound name, retention time, ESI polarity, molecular weight, precursors, products, collision energy, RF lens, regression parameters and linearity range are reported. Table S2: Anthocyanins-Compounds analyzed with UHPLC-QqQ-MS/MS in this study, the corresponding compound name, compound name, acronym, retention time, transitions, declustering potential, entrance potential, collision energy, collision cell exit potential, the regression (r2) and slope of the cyanidin-3-glucoside are reported. Table S3: List of the potato samples (column A), varieties (column B), sugars (column D-I; dataset sug), antioxidant and total polyphenolic content (column J-L; dataset AA), polyphenolic compounds (column M-AA; dataset poly) quantified in the potato samples included in this study. Results are expressed as mg/100g fw. In column AB (total sugar content) the sum of all sugars are reported, in column AC (average of total sugar content) the average content of total sugars of the three biological replicates for each sample are reported, in column AD (total content of single polyphenols) the sum of all single polyphenols are reported, in column AE (average of total content of single polyphenols) the average content of the total single polyphenols of the three biological replicates for each sample are reported. Table S4: List of the potato samples (column A), varieties (column B), Total Monomeric Content and Total Anthocyanins Content (Anto mono and Anto tot) (column D–E) and single anthocyanins (column F-AA) quantified in the potato samples included in this study. Results are expressed as mg/100g fw. Acronym of anthocyanin-compounds are reported Table S2. In column AB (total content of single anthocyanins) the sum of single anthocyanins are reported, in column AC (average of total content of single anthocyanins) the average content of single anthocyanins of the three biological replicates for each sample are reported. Table S5: Compounds analyzed with ion chromatograph (IC) with pulsed amperometric detection (HPAE-PAD) in this

study, the corresponding compound number, compound name, retention time, molecular weight, regression parameters and linearity range are reported.

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Abbreviations

AA, antioxidants; ABTS, 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid; ADA, American Diabetes Association; Anto mono, Total Monomeric Content; Anto tot, Total Anthocyanins Content; a.s.l, above sea level; CDS, Chromatography Data System; ESI, Electrospray ionization; FAO, Food and Agriculture Organization; FC, Folin–Ciocalteu; FRAP, Ferric Reducing Antioxidant Power Assay; fw, fresh weight; GI, glycaemic index; HPAE-PAD, High-Performance Anion-Exchange chromatography with Pulsed Amperometric Detection; IC, ion chromatograph; JIVE, Joint and Individual Variation Explained; LC-MS, liquid chromatography-mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; LV, latent variable; MRM, Multiple-reaction monitoring; MW, molecular weight; PAL, phenylalanine ammonia lyase; PCA, principal component analysis; Polyphenols, poly; PTFE, Polytetrafluoroethylene; QC, quality control sample; RT, room temperature; RSD, relative standard deviation; SCA, simultaneous component analysis; Sugars, sug; SVD, Singular Value Decomposition; TEAC, Trolox equivalent antioxidant capacity; TPC, Total Polyphenolic Content; TPTZ, 2,4,6-Tris(2-pyridyl)- s-triazine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; UHPLC-QqQ-MS/MS, high-performance liquid chromatography coupled with triple quadrupole mass spectrometry; VIP, variable importance.

References

1. Thomas, G.; Sansonetti, G. (Eds.) *New Light on a Hidden Treasure: International Year of the Potato 2008, an End-of-Year Review*, 1st ed.; Food and Agriculture Organization of the United Nations: Rome, Italy, 2009.
2. Akyol, H.; Riciputi, Y.; Capanoglu, E.; Caboni, M.F.; Verardo, V. Phenolic Compounds in the Potato and Its Byproducts: An Overview. *Int. J. Mol. Sci.* **2016**, *17*, 835. [CrossRef] [PubMed]
3. Fernandes, G.; Velangi, A.; Wolever, T.M. Glycemic index of potatoes commonly consumed in North America. *J. Am. Diet. Assoc.* **2005**, *105*, 557–562. [CrossRef] [PubMed]
4. ADA. Myths about Diabetes. Available online: https://www.diabetes.org/diabetes-risk/prediabetes/myths-about-diabetes (accessed on 31 January 2022).

5. Bellumori, M.; Innocenti, M.; Michelozzi, M.; Cerretani, L.; Mulinacci, N. Coloured-fleshed potatoes after boiling: Promising sources of known antioxidant compounds. *J. Food Compos. Anal.* **2017**, *59*, 1–7. [CrossRef]
6. Ramdath, D.D.; Padhi, E.; Hawke, A.; Sivaramalingam, T.; Tsao, R. The glycemic index of pigmented potatoes is related to their polyphenol content. *Food Funct.* **2014**, *5*, 909–915. [CrossRef]
7. Jokioja, J.; Linderborg, K.M.; Kortesniemi, M.; Nuora, A.; Heinonen, J.; Sainio, T.; Viitanen, M.; Kallio, H.; Yang, B. Anthocyanin-rich extract from purple potatoes decreases postprandial glycemic response and affects inflammation markers in healthy men. *Food Chem.* **2020**, *310*, 125797. [CrossRef] [PubMed]
8. Ingallina, C.; Spano, M.; Sobolev, A.P.; Esposito, C.; Santarcangelo, C.; Baldi, A.; Daglia, M.; Mannina, L. Characterization of Local Products for Their Industrial Use: The Case of Italian Potato Cultivars Analyzed by Untargeted and Targeted Methodologies. *Foods* **2020**, *9*, 1216. [CrossRef]
9. Oertel, A.; Matros, A.; Hartmann, A.; Arapitsas, P.; Dehmer, K.J.; Martens, S.; Mock, H.P. Metabolite profiling of red and blue potatoes revealed cultivar and tissue specific patterns for anthocyanins and other polyphenols. *Planta* **2017**, *246*, 281–297. [CrossRef]
10. Mulinacci, N.; Ieri, F.; Giaccherini, C.; Innocenti, M.; Andrenelli, L.; Canova, G.; Saracchi, M.; Casiraghi, M.C. Effect of cooking on the anthocyanins, phenolic acids, glycoalkaloids, and resistant starch content in two pigmented cultivars of Solanum tuberosum L. *J. Agric. Food Chem.* **2008**, *56*, 11830–11837. [CrossRef]
11. Zhou, X.; Ulaszewska, M.M.; Cuparencu, C.; Gobba, C.D.; Vázquez-Manjarrez, N.; Gürdeniz, G.; Chen, J.; Mattivi, F.; Dragsted, L.O. Urine Metabolome Profiling Reveals Imprints of Food Heating Processes after Dietary Intervention with Differently Cooked Potatoes. *J. Agric. Food Chem.* **2020**, *68*, 6122–6131. [CrossRef]
12. Lock, E.F.; Hoadley, K.A.; Marron, J.S.; Nobel, A.B. Joint and individual variation explained (JIVE) for integrated analysis of multiple data types. *Ann. Appl. Stat.* **2013**, *7*, 523. [CrossRef]
13. Rommens, C.M.; Richael, C.M.; Yan, H.; Navarre, D.A.; Ye, J.; Krucker, M.; Swords, K. Engineered native pathways for high kaempferol and caffeoylquinate production in potato. *Plant Biotechnol. J.* **2008**, *6*, 870–886. [CrossRef] [PubMed]
14. Perla, V.; Holm, D.G.; Jayanty, S.S. Effects of cooking methods on polyphenols, pigments and antioxidant activity in potato tubers. *LWT-Food Sci. Technol.* **2012**, *45*, 161–171. [CrossRef]
15. Tierno, R.; Hornero-Méndez, D.; Gallardo-Guerrero, L.; López-Pardo, R.; de Galarreta, J.I.R. Effect of boiling on the total phenolic, anthocyanin and carotenoid concentrations of potato tubers from selected cultivars and introgressed breeding lines from native potato species. *J. Food Compos. Anal.* **2015**, *41*, 58–65. [CrossRef]
16. Valls, J.; Agnolet, S.; Haas, F.; Struffi, I.; Ciesa, F.; Robatscher, P.; Oberhuber, M. Valorization of Lagrein grape pomace as a source of phenolic compounds: Analysis of the contents of anthocyanins, flavanols and antioxidant activity. *Eur. Food Res. Technol.* **2017**, *243*, 2211–2224. [CrossRef]
17. Ieri, F.; Innocenti, M.; Andrenelli, L.; Vecchio, V.; Mulinacci, N. Rapid HPLC/DAD/MS method to determine phenolic acids, glycoalkaloids and anthocyanins in pigmented potatoes (Solanum tuberosum L.) and correlations with variety and geographical origin. *Food Chem.* **2011**, *125*, 750–759. [CrossRef]
18. Vrhovsek, U.; Masuero, D.; Gasperotti, M.; Franceschi, P.; Caputi, L.; Viola, R.; Mattivi, F. A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages. *J. Agric. Food Chem.* **2012**, *60*, 8831–8840. [CrossRef]
19. Eisenstecken, D.; Panarese, A.; Robatscher, P.; Huck, C.W.; Zanella, A.; Oberhuber, M. A Near Infrared Spectroscopy (NIRS) and Chemometric Approach to Improve Apple Fruit Quality Management: A Case Study on the Cultivars "Cripps Pink" and "Braeburn". *Molecules* **2015**, *20*, 13603–13619. [CrossRef]
20. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2017.
21. Lê, S.; Josse, J.; Husson, F. FactoMineR: An R package for multivariate analysis. *J. Stat. Softw.* **2008**, *25*, 1–18. [CrossRef]
22. Kassambara, A.; Mundt, F. Factoextra Extract and Visualize the Results of Multivariate Data Analyses. R Package Version 1.0.7. References—Scientific Research Publishing, 2020. Available online: https://scirp.org/reference/referencespapers.aspx?referenceid=3067217 (accessed on 31 January 2022).
23. Kita, A.; Bakowska-Barczak, A.; Hamouz, K.; Kułakowska, K.; Lisińska, G. The effect of frying on anthocyanin stability and antioxidant activity of crisps from red- and purple-fleshed potatoes (Solanum tuberosum L.). *J. Food Compos. Anal.* **2013**, *32*, 169–175. [CrossRef]
24. Lachman, J.; Hamouz, K.; Orsák, M.; Pivec, V.; Dvořák, P. The influence of flesh colour and growing locality on polyphenolic content and antioxidant activity in potatoes. *Sci. Hortic.* **2008**, *117*, 109–114. [CrossRef]
25. Silveira, A.C.; Orena, S.; Medel-Maraboli, M.; Escalona, V.H. Determination of some functional and sensory attributes and suitability of colored-and noncolored-flesh potatoes for different cooking methods. *Food Sci. Technol.* **2020**, *40*, 395–404. [CrossRef]
26. Lachman, J.; Hamouz, K.; Orsák, M.; Pivec, V.; Hejtmánková, K.; Pazderů, K.; Dvořák, P.; Čepl, J. Impact of selected factors—Cultivar, storage, cooking and baking on the content of anthocyanins in coloured-flesh potatoes. *Food Chem.* **2012**, *133*, 1107–1116. [CrossRef]
27. Deußer, H.; Guignard, C.; Hoffmann, L.; Evers, D. Polyphenol and glycoalkaloid contents in potato cultivars grown in Luxembourg. *Food Chem.* **2012**, *135*, 2814–2824. [CrossRef] [PubMed]

28. Hamouz, K.; Lachman, J.; Pazderů, K.; Tomášek, J.; Hejtmánková, K.; Pivec, V. Differences in anthocyanin content and antioxidant activity of potato tubers with different flesh colour. *Plant Soil Environ.* **2011**, *57*, 478–485. [CrossRef]
29. Hellmann, H.; Goyer, A.; Navarre, D.A. Antioxidants in Potatoes: A Functional View on One of the Major Food Crops Worldwide. *Molecules* **2021**, *26*, 2446. [CrossRef]
30. Li, A.; Xiao, R.; He, S.; An, X.; He, Y.; Wang, C.; Yin, S.; Wang, B.; Shi, X.; He, J. Research Advances of Purple Sweet Potato Anthocyanins: Extraction, Identification, Stability, Bioactivity, Application, and Biotransformation. *Molecules* **2019**, *24*, 3816. [CrossRef]
31. Xu, X.; Li, W.; Lu, Z.; Beta, T.; Hydamaka, A.W. Phenolic content, composition, antioxidant activity, and their changes during domestic cooking of potatoes. *J. Agric. Food. Chem.* **2009**, *57*, 10231–10236. [CrossRef]
32. Wang, S.; Pan, D.; Lv, X.; Song, X.; Qiu, Z.; Huang, C.; Huang, R.; Chen, W. Proteomic approach reveals that starch degradation contributes to anthocyanin accumulation in tuberous root of purple sweet potato. *J. Proteom.* **2016**, *143*, 298–305. [CrossRef]
33. Reddivari, L.; Hale, A.L.; Miller, J.C. Genotype, Location, and Year Influence Antioxidant Activity, Carotenoid Content, Phenolic Content, and Composition in Specialty Potatoes. *J. Agric. Food Chem.* **2007**, *55*, 8073–8079. [CrossRef]
34. Payyavula, R.S.; Navarre, D.A.; Kuhl, J.C.; Pantoja, A.; Pillai, S.S. Differential effects of environment on potato phenylpropanoid and carotenoid expression. *BMC Plant Biol.* **2012**, *12*, 1–17. [CrossRef]
35. André, C.M.; Oufir, M.; Hoffmann, L.; Hausman, J.F.; Rogez, H.; Larondelle, Y.; Evers, D. Influence of environment and genotype on polyphenol compounds and in vitro antioxidant capacity of native Andean potatoes (Solanum tuberosum L.). *J. Food Compos. Anal.* **2009**, *22*, 517–524. [CrossRef]
36. Bassoli, B.K.; Cassolla, P.; Borba-Murad, G.R.; Constantin, J.; Salgueiro-Pagadigorria, C.L.; Bazotte, R.B.; Silva, R.S.D.S.F.D.; Souza, H.M.D. Chlorogenic acid reduces the plasma glucose peak in the oral glucose tolerance test: Effects on hepatic glucose release and glycaemia. *Cell Biochem. Funct.* **2008**, *26*, 320–328. [CrossRef]
37. Dabeek, W.M.; Marra, M.V. Dietary Quercetin and Kaempferol: Bioavailability and Potential Cardiovascular-Related Bioactivity in Humans. *Nutrients* **2019**, *11*, 2288. [CrossRef] [PubMed]
38. Sowokinos, J.R. Relationship of harvest sucrose content to processing maturity and storage life of potatoes. *Am. Potato J.* **1978**, *55*, 333–344. [CrossRef]
39. Kumar, D.; Singh, B.P.; Kumar, P. An overview of the factors affecting sugar content of potatoes. *Ann. Appl. Biol.* **2004**, *145*, 247–256. [CrossRef]
40. Bontempo, P.; Carafa, V.; Grassi, R.; Basile, A.; Tenore, G.C.; Formisano, C.; Rigano, D.; Altucci, L. Antioxidant, antimicrobial and anti-proliferative activities of Solanum tuberosum L. var. Vitelotte. *Food Chem. Toxicol.* **2013**, *55*, 304–312. [CrossRef] [PubMed]
41. Jokioja, J.; Yang, B.; Linderborg, K.M. Acylated anthocyanins: A review on their bioavailability and effects on postprandial carbohydrate metabolism and inflammation. *Compr. Rev. Food Sci. Food Saf.* **2021**, *20*, 5570–5615. [CrossRef]
42. Lachman, J.; Hamouz, K.; Musilová, J.; Hejtmánková, K.; Kotíková, Z.; Pazderů, K.; Domkářová, J.; Pivec, V.; Cimr, J. Effect of peeling and three cooking methods on the content of selected phytochemicals in potato tubers with various colour of flesh. *Food Chem.* **2013**, *138*, 1189–1197. [CrossRef]
43. Tian, J.; Chen, J.; Ye, X.; Chen, S. Health benefits of the potato affected by domestic cooking: A review. *Food Chem.* **2016**, *202*, 165–175. [CrossRef]
44. Fossen, T.; Cabrita, L.; Andersen, O.M. Colour and stability of pure anthocyanins influenced by pH including the alkaline region. *Food Chem.* **1998**, *63*, 435–440. [CrossRef]
45. Fleschhut, J.; Kratzer, F.; Rechkemmer, G.; Kulling, S.E. Stability and biotransformation of various dietary anthocyanins in vitro. *Eur. J. Nutr.* **2006**, *45*, 7–18. [CrossRef] [PubMed]
46. Moser, S.; Aragon, I.; Furrer, A.; Klinken, J.W.V.; Kaczmarczyk, M.; Lee, B.H.; George, J.; Hamaker, B.R.; Mattes, R.; Ferruzzi, M.G. Potato phenolics impact starch digestion and glucose transport in model systems but translation to phenolic rich potato chips results in only modest modification of glycemic response in humans. *Nutr. Res.* **2018**, *52*, 57–70. [CrossRef] [PubMed]
47. Keppler, K.; Humpf, H.U. Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. *Bioorg. Med. Chem.* **2005**, *13*, 5195–5205. [CrossRef] [PubMed]

The Figure (4.1) represents the graphical abstract submitted to the open access journal "Foods".

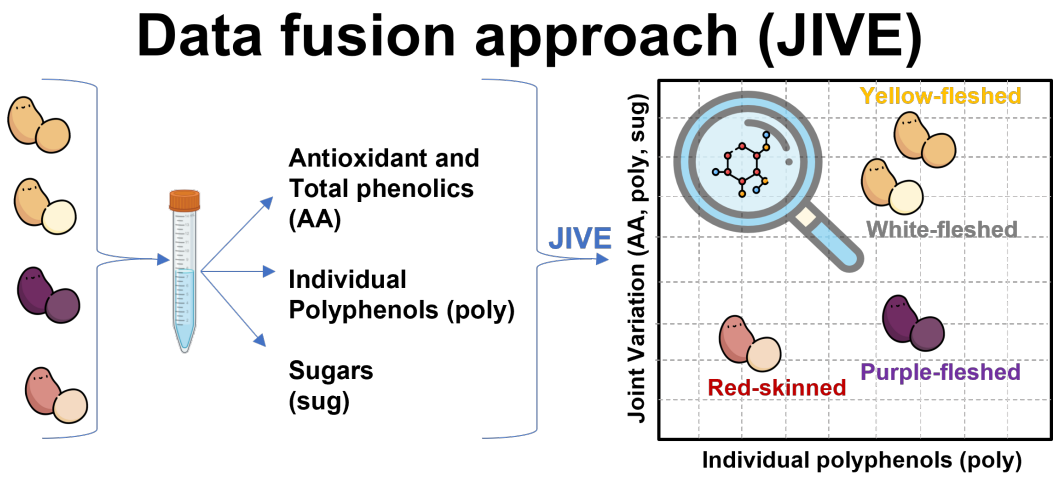

FIGURE 4.1. Graphical abstract submitted, a simplified representation of the study.

4.2.1 Conclusion: Third Contribution

The aim of this chapter 4 is to identify specific potato varieties that are rich in bioactive compounds by investigating different non-pigmented and pigmented potato metaboloma. Since potatoes are known for having high glycemic index (GI), which causes an increment of the glycemic peak, I deem it necessary to find a compromise between the high GI and the content of beneficial compounds. Therefore, the investigation of the chemical profile of different potato cultivars is a crucial point to identify their potential role in improving the awareness in the consumers' expectations. As far as I am concerned the most important beneficial compounds are antioxidants, polyphenols, sugars, and anthocyanins. Indeed, it is worth remembering that the presence of these phytochemicals (polyphenols and anthocyanins) are able to lower the glycemic peak after consuming a meal of potatoes. I consider that the promotion of the intake of potatoes, which are characterized by low-GI values and rich in beneficial metabolites, is useful to provide

scientific evidence for improving the the human diets.

CHAPTER 5

FOURTH CONTRIBUTION

5.1 Preface

For the purposes of this research, I will present in this chapter 5 the results regarding a specific study conducted on the metabolome of 9 red-fleshed apple varieties (both in peel and pulp sections) in order to provide information about their chemical profile. The investigation of these new emerging colored fruits has been stimulated by their high content of polyphenols and anthocyanins. The goal is to improve the knowledge about their health proprieties and to direct the breeding programs and agronomic practice towards red-fleshed cultivars rich in phytochemicals. My contributions for this chapter 5 covered all of the steps for the preparation of the study, including the sampling procedure, the conceptualization of the study, the practical laboratory work, figures, and the application of a cutting-edge multivariate statistical method. I was helped by Dr. Walther Guerra, the head of the *Instituts für Obst und Weinbau, Versuchszentrum Laimburg, (BZ, Italy)*, in selecting the most promising red-fleshed apple varieties for the human nutrition.

Appearance, colors, freshness and taste are the most important factors that are able to capture the interest of consumers. Therefore, the attention of stakeholders and producers is guided towards the choice of more attractive and delicious apple varieties. Additionally, the research gives insight into the selection of apple cultivars that are characterized by a high content of healthy compounds. Red-fleshed apples are recently capturing the approval of the population due to their novel appearance and appealing colors. To date, scientific evidences showed that these new apple varieties are characterized by a high content of healthy compounds such as phenolic compounds (flavonoids, flavanols and anthocyanins). The introduction of new fresh fruits in our eating habits is a crucial point to define a exact make-up of a diversified, balanced and healthy diet. The quality of the red-fleshed apple varieties is strongly influenced by several factors, such as temperature, sunlight, storage conditions etc., which have an impact on the texture, flavor, appearance of the fresh fruits. The domestication processes have played a part in determining a drop in the nutritional quality of the apple cultivars, however, the investigation of the chemical composition of these novel fruits is a fundamental step in defining the "overall quality" of specific apple cultivars before introducing them into the market. The goal of apple producers is to find a compromise between the astringent taste, which is given by a high content of flavonoids, and desirable traits (e.g. flavor, sweetness, and crispness).

5.2 Beta diversity analysis on the metabolome of the red-fleshed apple varieties

Authors: Adriana Teresa Ceci (A.T.C), Pietro Franceschi (P.F.), Walther Guerra (W.G.), Alexandra Lanz (A.L.), Michael Oberhuber (M.O.), Peter Robatscher (P.R.), and Fulvio Mattivi (P.F.) (manuscript in preparation).

5.2.1 Abstract

Red-fleshed apples have currently attracted the interest of producers and consumers because of their attractive color, appearance, freshness, as well as, their high nutritional value and healthy proprieties. Indeed, the red-fleshed cultivars are recognized as promising functional food due to the he simultaneous presence of high amount of anthocyanins (natural pigments) and polyphenols. In this section, we provide a comprehensive characterization of 9 red-fleshed apple varieties, manly focusing on their polyphenolic and anthocyanin profiles, total polyphenolic content, and the antioxidant activity determined by different *in vitro* methods. The chemical profile is evaluated in three apple sections, mainly, shady, sunny, and pulp sections. Additionally, the beta diversity measure is apply to evaluate the dissimilarity in terms of polyphenolic composition in different apple sections for clustering amongst 9 apple varieties. It is shown that the euclidean-based beta diversity measure captures compositional variability in the three apple sections efficiently, resulting in different clusters. The visualization of clusters is conducted using non-metric multidimensional scaling (NMDS) plots and the apples samples form groups according to the apple sections. Statistical tests are performed using ANOSIM and PERMANOVA to evaluate the significance. These findings contribute to select new approaches in the breeding development of red-fleshed apples with high nutritional potential.

Keywords: Red-fleshed apples, Phytochemical composition, Bioactive compounds Health-promoting properties, Beta-diversity measure.

5.2.2 Introduction

Apples are one of the most common crops cultivated in the world and they play an important socioeconomic role in the autonomous province of South Tyrol (Italy). This province has the biggest production area (19.000 ha) in Italy, and supplies up to 50% of

the national Italian apple market, 15% of the European and 2% of the global apple market [64]. The apple production is a dynamic and growing sector, and the competition in the European and global markets is constantly increasing. Consequently, the costumers are guided towards the choice of more nutritious and sustainable apple varieties. Indeed, selection of apple varieties is focused on apple varieties that are characterized by high nutritional values for the human nutrition. Therefore, stakeholders are interested in bringing in the markets new attractive apple varieties. The red-fleshed apple varieties appear to have a characteristic appearance with their broad spectrum of colors from pale pink to deep red colored flesh [65]. New promising apple varieties are the red-fleshed apples, which are characterized by the simultaneous presence of high amount of anthocyanins (natural pigments) and polyphenols [65]. The biosynthesis pathway route is divided into several branches and phenolic acids, polyphenols, and anthocyanins are produced in different amounts based on the involved enzymes [66]. In the habitual diet of Italian people, the presence of apples should be promoted and encouraged in order to stimulate the interest of researchers in evaluating their potential role in the human diet. Focusing on this, the red-fleshed variety studied in the present work demonstrated high nutritional values. On the other hand, the cultivation, production, harvesting time, and post-harvest conditions of red-fleshed fruits are more challenging and complex than conventional apple varieties [65]. Understanding the metabolome of the red-fleshed apples helps to improve breeding processes and to select promising apple varieties for the human nutrition. In the early stage of developing new varieties, the study of their metabolome plays an influential role in plant breeding. In this scenario, research gives the producers an insight into the quality attributes of apples and which apple types stimulate the interest of the consumers. The breeders are helped by the sensory trial and chemical analysis to target the best varieties and to steer the producers toward an appropriate selection, trademark and marketing campaign. The origin and evolution

of red-fleshed apples is still controversial, but several authors reported that these varieties have been dated back to 1890 [67]. The first trees were grown at the Zöschen Arboretum (Germany) by Georg Dieck and the seed were sent by the Russian lawyer and amateur botanist Vladislav E. Niedzwiecki, who was living in exile in Turkestan [67]. The seeds were known as *Malus niedzwetzkyana* that was a kind of apple native to certain parts of China, Afghanistan, Kazakhstan, Kyrgyzstan, and Uzbekistan and it was noted by Niedzwiecki for its peculiarities: red-fleshed, red-skinned fruit and red flowers [67]. Analyses on complete apple genome confirmed that all these red-fleshed apples originated from *M. sieversii f. niedzwetzkyana* and the cultivated species are *M. domestica var. niedzwetzkyana* [66]. The ancestor of *M. sieversii f. niedzwetzkyana*, the red-fleshed variant, is *M. sieversii* that is an ancient wild apple variety native to the mountains of Central Asia [66]. In 2012 has been identified and classified 3000 red-fleshed apple germplasm resources, including cultivars, wild species, and hybrids [67]. Generally, the commercialization in Europe of the red-fleshed apples was started in Northern Italy, Germany, and Spain. The biosynthesis pathway route is split into several branches and flavonoids and anthocyanins are produced in different amounts. It is worth observing that the metabolomic pathway in red-fleshed apples is expected to be towards the synthesis of anthocyanins, a higher amount of polyphenolic compounds is reported. Phenylalanine is a direct precursor and it is converted by phenylalanine ammonia lyase (PAL) into 4-coumaroyl-CoA and malonyl-CoA. Subsequently, the 4-coumaroyl- CoA is converted into dihydroflavonol and the anthocyanins, flavonols or other flavonoids are produced by dihydroflavonol. The production of flavanols and anthocyanins is competitive and it depends on the involved enzymes. A simplified representation of the flavonoid biosynthetic pathway leading to the three major classes is reported (5.1) [66].

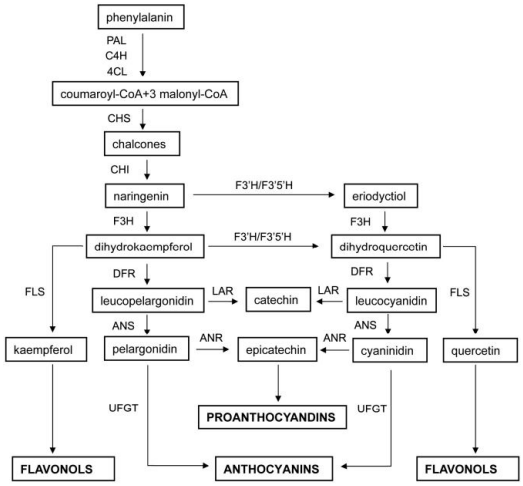

FIGURE 5.1. A simplified representation of the flavonoid biosynthetic pathway leading to the three major classes. © 2017 The Authors. The Plant Journal John Wiley Sons Ltd [66]. (CC BY) license (https://creativecommons.org/compatiblelicenses).

As it is possible to observe, the key enzyme of anthocyanin synthesis is dihydroflavonol 4-reductase (DFR) that competes with flavonol synthase (FLS) for the same substrates, dihydrokaempferol and dihydroquercetin. Additionally, leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) are the enzymes involved into the synthesis of catechin and epicatechin [66].

Transcription factors and the expression of structural genes are the determining factors in the anthocyanin biosynthesis, thus providing a different intensity of colors and pattern in the red-fleshed apples, which can be distinguished into red-fleshed and in red-skinned apples [67]. In apple, the most important regulatory genes are MYB transcription factors (TFs) associated with two-repeat (R2R3) class in the anthocyanin pathway. Additionally, it has been reported that basic helix-loop-helix (bHLH) TFs are able to interact closely with R2R3 MYB TFs [67, 68]. It has been reported that the R2R3 MYB, bHLH, and WD40 proteins, three TFs, collaborate in anthocyanidin production across all plant species reported to date, including apple [67].

In recent years, the genes involved in the apple coloration has been studied and three transcription factors, MdMYBA, MdMYB1, and MdMYB10 has been identified as the most common genes that responsible for the pigmentation. The three MdMYB genes are almost identical in nucleotide sequences, there are different alleles on linkage group 9 [69].

Regarding the control in the expression of anthocyanins in apple fruit skin, MdMYBA and MdMYB1 are isolated from two red skinned apple cultivar called 'Tsugaru' and 'Cripps Pink', respectively [68]. Additionally, the transcript of MdMYB1 increased when the skin of the apples is exposed to sunlight after keeping them under darkness [68]. MdMYBA is reported to be induced by UV-B irradiation and low-temperature treatment [68]. Other TFs are found to be responsible for skin coloration such as MdMYB3, MdTTG1, and MdJAZ18.

The regulation of the synthesis of anthocyanins in the flesh of the pigmented apples (called red-fleshed apples) is more complex than in the red-skinned apples [67]. The red-fleshed apples are characterized into two types: the Type 1 apples have a red coloration in the fruit flesh, skin, leaves, and other vegetative tissues, whereas, the Type 2 apples has the red pigmentation only in the flesh. The MdMYB10 gene is responsible for the red color in the Type 1 red-fleshed apples, in contrast, MdMYB10 gene is not present in Type 2 red-fleshed apples. Nevertheless, MdMYB110a gene is linked to the red pigmentation in Type 2 red-fleshed apples. Indeed, it is possible to observe a wider spectrum of colours in the Type 2 red-fleshed apples than in the Type 1 red-fleshed apples. Therefore, all of these TFs are determining factors in the regulation of the synthesis of anthocyanins in red-fleshed apples.

The most famous private breeder is Jean Luc Carrieres who created the most common apple red-fleshed varieties: RM1 e RS1 and they are taken in charge by Red Moon srl and commercialized under the umbrella brand: Red Moon®. RM1 e RS1 red-fleshed varieties

are scab-resistant, late flowering. RS-1 is harvested around early to mid-September, RM-1 around early to mid-October. Both are characterized by a high sugar and acid content. The Red Moon® brand has been promoted in 2018 during the the exhibition, held on the Berlin fairgrounds, called Fruit Logistica. Red Moon® is produced and marketed in Italy by Clementi Srl and Bio Meran and 100 hectares have been planted in the most suitable areas of Alto Adige and Veneto [70]. To date, two products, a red apple juice and dried apple slices, are present in the markets and the producers are trying to implement the fresh fruits, which are characterized by a bitter and astringency taste, in the fresh market. In this thesis, two RS-1 grown in Laimburg and Laces (planted 2017 and 2011, respectively) and two RM-1 grown at Laimburg (planted in 2011 and 2017, respectively) are examined. All of them are type 1 phenotype.

During the Fruit Attraction trade fair in Madrid (Spain), the Kissabel® umbrella brand has been promoted in 2017 with the Kissabel® Rouge, Kissabel® Orange, and Kissabel® Jaune lines. The selection program, which is called "Ifored", has been started from the early 1990s. in France and the main plant nurseries were Valois e Davodeau Ligonnière. The consortium in Italy, which has adhered to "Ifored", is Novamela that gathers Vog, VI.P, Melinda, La Trentina, and Rivoira agricultural cooperatives. To date, the most common apple red-fleshed varieties under Kissabel® umbrella brand are: R201 (a type 1 phenotype) e Y101 (a type 2 phenotype) and the plant patent in Europe has been applied on these two red-fleshed apple varieties [71]. Regarding the production of red-fleshed apples in South Tyrol (Italy), since 2010, 60 hybrids has been grown and tested by the Laimburg Research Centre and they are obtained from different origins BayOZ (Germany), Graminor (Norway), Fruture and Promofruit (Swiss), Howell Fruit Advantage (USA), Ifored and Jean Luc Carrieres (France) [71].

The commercialisation of red-fleshed apples is conducted under an umbrella brand e.g. Red Moon®, Kissabel®, Redlove®, etc [71]. In our studies some cultivars that belong

to Red Moon® and Kissabel® brands are analyzed. In this thesis, R201 grown at Ciardes and Laces (planted in 2016 and 2013, respectively) and Y102 (a type 2 phenotype) grown in Laces (planted in 2013) are examined.

It is well-known that apples are rich in phytonutrients which contribute to the human health. Due the fact that the metabolome of red fruits is still rather unknown, it is even more important to provide information about the chemical profile. Therefore, studying the chemical profile of new red-fleshed apple varieties is a crucial point to gain insights about their health benefits and nutritional value. Chemical analyses on the peels and on the pulps of 9 red-fleshed varieties are conducted, which are grown on different sea levels. A deeper characterization of the metabolome of red-fleshed apples is an important factor in improving the knowledge about their health proprieties.

The results reported in this contribution gain information regarding the metabolome of 9 varieties (both in peel and pulp sections) in order to help the introduction of these fresh fruits in the markets. The high content of healthy compounds should be considered as a key property in defining the “overall quality” of specific apple cultivars, and it is possible to direct the breeding programs and agronomic practices.

In this survey, a targeted metabolomics-based approach is used to comprehensively investigate those metabolites, better characterizing and discriminating the red-fleshed samples according to their phenotype and experimental location. In particular, we used a targeted screening based on different analytical methods combined with multivariate statistical analyses. This method is intended to be used for a deeper understanding about the profiling of red-fleshed apples. The chemical analyses are conducted on three sections of each apple: shady side, sunny side, and pulp and the chemical profile identified by UHPLC-QqQ-MS/MS and spectrophotometric assays.

The variability in the concentration of metabolites is evaluated in each apple section within single apple variety. The aim is to understand if the variability in the concentration

of polyphenols, antioxidants, and anthocyanins was bigger in the pulp, shady, and sunny sections, a beta-analysis is conducted on each section within the same variety. The analysis took into consideration not only the variability in the concentration of the compounds, but also the variability around the average.

The aim of this study is to increase the interest in new red-fleshed apple cultivar and to stimulate the awareness of the consumers regarding their potential role in preventing diseases. The analysis of whole profile of antioxidants, phenolic compounds, anthocyanins is carried out and different analytical methodologies are developed and optimized. There are a few studies that analysed the red-fleshed apple cultivar for commercial purposes [72].

5.2.3 Material and Method

5.2.3.1 Fruits Samples

In total, 90 apples of 9 cultivars were analyzed in this study. Apple trees were grown at the Laimburg Research Centre in Ora (242m a.s.l.), Laces (639m a.s.l.) and Ciardes (636m a.s.l.) in South Tyrol, Italy. The nine samples of apples are namely RS-1 Red Moon® (2017) Laimburg, RS-1 Red Moon® (2011) Laces, 108 (2017) Laimburg, CR28-1 (2015) Laimburg, RM-1 Red Moon® (2011) Laimburg, RM-1 Red Moon® (2017) Laimburg, Y102 Kissabel® (2013) Laces, R201 Kissabel® (2016) Ciardes, R201 Kissabel® (2013) The samples were harvested at their optimal harvest time between September and October 2020. Table 5.1 shows the list of red fleshed apple samples and acronyms that were analysed in this survey.

Table 5.1: List of red fleshed apple samples used in this study. The acronyms are displayed and they are used in the Figures 5.2 and 5.3

Sample number	Variety	Acronyms	Phenotype	Planting Year	Experimental location	Harvest time
1	RS-1 Red Moon®	RS1_319_27	Type 1	2017	Laimburg	01.09.2020
2	RS-1 Red Moon®	RS1_V135	Type 1	2011	Laces	10.09.2020
3	RM-1 Red Moon®	RM-1_11	Type 1	2011	Laimburg	12.10.2020
4	RM-1 Red Moon®	RM-1_17	Type 1	2017	Laimburg	29.09.2020
5	Y102 Kissabel®	Y102	Type 2	2013	Laces	30.09.2020
6	R201 Kissabel®	R201	Type 1	2016	Ciardes	07.10.2020
7	R201 Kissabel®	V220	Type 1	2013	Laces	07.10.2020
8	108	LB108	Type 1	2017	Laimburg	02.09.2020
9	CR28-1	CR28-1	Type 2	2015	Laimburg	22.09.2020

Apples of each sample were randomly collected from the central canopy avoiding the tops and bottoms of the trees from four different trees per sample. Apples were stored 10 days at 95% relative humidity and 1.3 °C (cold storage) and three days at 70% relative humidity and 25 °C (shelf life).

Sample preparation was done as in Ceci et al. [18] and reported in the (chapter 2) as first contribution. The apples were peeled and cut into three equal equatorial discs choosing the central one. From the sunny and shady sides of each apple, a piece of 3 × 3 cm^2 were taken. The central disc and both peel parts were immediately frozen separately in liquid nitrogen and freeze-dried using FreeZone Freeze Dry System (Labconco, MO, USA). All dried samples were milled to a fine powder using a commercial miller, packed, and stored in hermetic polyethylene bags at -80 °C up until the analysis.

5.2.3.2 Extraction of Samples

The extraction protocol was adapted from Ceci et al. [18] and reported in the (chapter 2) as first contribution. Twenty-five mg of freeze-dried apple material were extracted using 1.83 mL of a mixture of water:methanol (80:20 v/v) containing sodium fluoride (100 mM) and acidified with H3PO4 (0.01 ng/μL). The mixture was shaken for 15 min and centrifugated at +5 °C at 14,000 rpm for 5 min. The supernatant was removed and stored at -80 °C until the analysis.

5.2.3.3 Analyses on the apple pulps and peels

The following analytical methods were adapted from Ceci et al. [18] and reported in the (chapter 2) as first contribution.

Analysis of Polyphenolic Profile

An UltiMate 3000 UHPLC system (Thermo Scientific, Waltham, MA, USA) coupled with a TSQ Quantiva (Thermo Scientific, Waltham, MA, USA) triple-stage quadrupole mass spectrometer (UHPLC-QqQ-MS/MS). A Hypersil GOLD™ HPLC (2.1 × 50 mm, 3 um, Thermo Scientific, Waltham, MA, USA). The flow rate was kept 0.4 mL/min. The mobile phase was A (2.5% [v/v] formic acid in Millipore water) and B (2.5% [v/v] formic acid in acetonitrile LC-MS analytical grade) The gradient elution was 0–1 min (2.5% B), 1–10 min (16.5% B), 10–11.5 min (16.5% B), 11.5–12.5 min (23.5% B), 12.5–15 min (55% B), 15–15.5 min (95%), 15.5–17.5 min (95%), 17.5–18 min (2.5% B), and 18–21 min (2.5% B). The column temperature and autosampler were set at +5 °C and +40 °C, respectively. The source conditions were as follows: voltage 1500 V, vaporizer temperature 275 °C, capillary temperature 325 °C, sheath gas 40 arbitrary unit (AU), auxiliary gas 15 AU, sweep gas 2 AU, and collision gas (Argon) 1.5 mTorr. The polyphenols were identified by retention times and selected reaction monitoring (SRM) of reference compounds. The specific metabolite transitions and compound parameters are reported in the table 5.9. The analytical operations were handled by the Chromeleon Chromatography Data System (CDS) (version 6.8) software and Thermo Xcalibur (version 3.0) software (both Thermo Scientific, Waltham, MA, USA). the quantification was carried out by Thermo TraceFinder (version 3.2) software (Thermo Scientific, Waltham, MA, USA). The quantification was carried out by external calibration curves of each compound. The results were normalized for the weight and expressed as the milligram of each compound per 100 g of sample on fresh weight (see tables 5.6, 5.7, and 5.8).

Total Polyphenolic Content

The Total Polyphenolic Content was measured using the Folin–Ciocalteu reagent. The apple peel samples extracts were diluted 1:3 with extraction solution A. 250 μL of deionized water and 60 μL of extracts was added to 60 μL of Folin–Ciocalteu reagent. The mixture was mixed at 12,000 rpm for 6 min at RT. Then, 630 μL of sodium carbonates (7.5% w/v) were added to the mixtures and they were mixed at RT for 90 min. The absorbance was recorded at 740 nm on a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, CA, USA) spectrophotometer, and referred to a standard curve of catechin (range 0–150 ng/μL). The results were normalized for the weight and expressed as milligram of catechin equivalents per 100 g of sample on fresh weight (see tables 5.3, 5.4, and 5.5).

The Total Antioxidant Capacity

The total antioxidant capacity was measured using the FRAP (Ferric Reducing Antioxidant Power) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assays.

(a) FRAP assay: The extracts of apple peel samples were diluted 1:10 with extraction solution A. 960 μL of FRAP reagent was added to 60 μL of sample extract and 180 μL of MilliQ. The mixtures were incubated into dark at +37 °C for 90 min. The decrease in absorbance was read at 595 nm using a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, CA, USA) spectrophotometer, and referred to a standard curve of Trolox (range 15.6–250 μM). The results were normalized for the weight and expressed as milligrams of Trolox equivalents per 100 g of sample of fresh weight (see tables 5.3, 5.4, and 5.5).

(b) ABTS assay: 1970 μL of ABTS reagent was added to 30 μL of sample extract and the

mixtures was incubated into dark for 10 min at RT. The decrease in absorbance was read at 734 nm using a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, CA, USA) spectrophotometer, and referred to a standard curve of Trolox (range 15.6–250 μM). The results were normalized for the weight and expressed as milligrams of Trolox equivalents per 100 g of sample on fresh weight (see tables 5.3, 5.4, and 5.5).

pH Differential Method The monomeric and total anthocyanins were measured using the pH differential method. Two dilutions of the same extract were made by adding 800 μL of potassium chloride (0.025 M, pH 1) to 200 μL of sample extracts and 800 μL of sodium acetate (0.4 M, pH 4.5) to 200 μL of sample extracts. The absorbance was recorded at 520 and 700 nm on a Cary 60 UV–Vis (Agilent Technologies, Palo Alto, USA) spectrophotometer. Total Anthocyanins Content was calculated using Lambert–Beer law (ϵ = 26,900 L/mol/cm, MW = 449.2 g/mol) from the measures at pH 1. The results were normalized for the weight and expressed as milligrams of cyanidin-3-glucoside equivalents per 100 g of sample on fresh weight (see tables 5.3, 5.4, and 5.5).

5.2.3.4 The beta-diversity analysis

The beta diversity was defined by Legendre [73] as *"The variation in species composition among sites, or beta diversity, can be decomposed into replacement and richness difference."*. In our scenario, the communities are the polyphenolic compounds and the sites are three apple sections. Quantifying the differences among apple sections is an important step towards understanding how and why metabolites are distributed in the whole fruit. For our purpose, we used the beta-diversity analysis to evaluate the variability in the concentration of metabolites amongst three apple sections (sunny, shady, and pulp sides), and to investigate how much space is occupied by each singular section in terms of the changes of polyphenol concentrations.

Variation in polyphenolic composition, evaluated in terms of beta-diversity, from

the minimum to the maximum differentiation of each section within the same apple variety is visualized via non-metric multidimensional scaling (NMDS) ordination based on Euclidean distance using metaMDS() function [74]. NMDS is a exploratory tool to simplify multivariate data into a few important axes to facilitate recognition and interpretation of patterns and differences among groups [74].

The statistical significance of dissimilarities between the three apple sections for each apple variety is tested via analysis of similarity analysis (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) using 999 permutations. The functions were: anosim() and adonis2(), respectively. For this purpose, the VEGAN package is used [74].

ANOSIM is a statistical test that measures how much the dissimilarity between/among groups is significant based on a distance metric. The output are R value and the p-value. Mathematically, it is reported that when R value is higher than 1, the dissimilarities between/among groups is very high, in contrast, when the R value is lower than 0.1, the clouds are completely overlapped, reason for which the groups are similar. Additionally, the p-value is part of the ANOSIM output. To this end, the smaller the p-value, the more significant the dissimilarities in the compositions of the groups [75].

PERMANOVA is a non-parametric multivariate statistical permutation test. *"It compares groups of objects and test the null hypothesis that the centroids and dispersion of the groups as defined by measure space are equivalent for all groups. A rejection of the null hypothesis means that either the centroid and/or the spread of the objects is different between the groups. PERMANOVA draws tests for significance by comparing the actual F test result to that gained from random permutations of the objects between the groups. PERMANOVA tests for similarity based on a chosen distance measure"* [76] R^2 statistic is extracted, showing the percentage of the variance explained by the groups [75].

The three apple sections have been tested in pairs: shady versus sunny (Shady_VS_Sunny),

shady versus pulp (Shady_VS_Pulp), and sunny versus pulp (Sunny_VS_Pulp). In the Table 5.2 are shown the statistical results obtained by ANOSIM and PERMANOVA tests and they can be found in the (subsection 5.2.4).

In the case of the LC/MS analysis, the datasets contained some missing values 5.6, 5.7, and 5.8 because certain compounds are not determined in apple extracts caused by the concentration under the limits of quantification (LOQ). To prepare complete datasets for statistical analysis, the missing values are handled by replacing them with random small values between zero and the limits of detection (LOD) [77].

5.2.4 Results and Discussion

The beta-diversity is graphically reported as NMDS plot for each apple variety under investigation. The Fig. 5.2 shows that the chemical composition is a contributing factor in differentiating sunny, shady, and pulp sections of 9 apple varieties on NMDS plots.

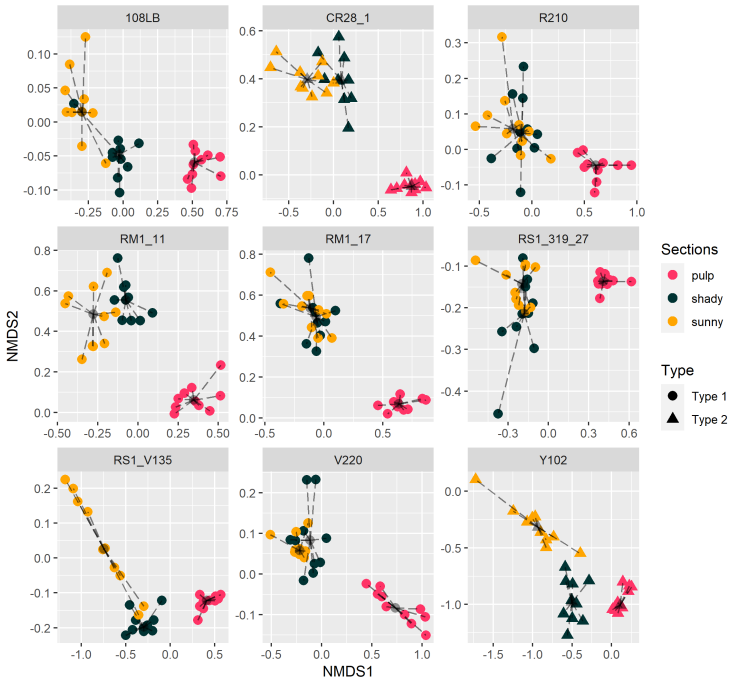

FIGURE 5.2. Schematic diagram of geometric partitioning for beta-analysis represented by NMDS method, showing for section = 3, of n = 10 sampling units (as biological replicates) per variety = 9 into a two-dimensional (bivariate, p = 2) Euclidean space (as NMDS1 and NMDS2 components). Each sampling unit is connected to its own group centroid (black dotted lines). The shapes (point and triangle) differentiate the red-fleshed types, and the colors the sections (red = pulp; yellow = sunny; and grey = shady) under investigation [78]. All information about 9 red-fleshed apple varieties are reported in the Table 5.1.

The polyphenolic metabolomic datasets is analyzed using beta-diversity analysis. According to beta-diversity analysis based on Euclidean distance of 90 apple samples (n = 10 for each variety = 9) deferentially enriched across three apple sectors. All apple pulp sections are significantly different (Table 5.2) from the apple skin (shady and sunny sections). Therefore, the strongest effect on the sample diversity is the variation in the concentration of the compounds between the pulp and skin and the separation exists along NMDS component 1 (NMDS1). Additionally, the beta-diversity analysis reveals that the NMDS2 provides for the separation between the two sectors sunny and shady sectors, thus indicating that the chemical composition between the two sections, reported

as shady and sunny sides, is influenced by the position of the apple on the tree and then the sun exposure. This assumption is confirmed by Ceci et al. [18] and reported in the (chapter 2) as first contribution. The sunny and shady sections are significantly different in the most of cultivars under investigation (Table 5.2).

This variation is notably higher in the 108LB, RM-1_11, and Y102 cultivars with apple samples partitioning into non-overlapping NMDS clusters based on apple sections. Thus, it is highlighting that the metaboloma in these three red-fleshed apple varieties is strongly variable across the three apple sections. While the shady metaboloma substantially overlaps with that of the sunny metaboloma, it appears to be restricted (lower dispersion) in R210, RM-1_17, RS1_319_27, and V220. Thus, it is suggesting that the metaboloma hardly differs the apple skin sections in these four red-fleshed apple varieties.

Noteworthy, the red dots (pulp section) occupy a narrower volume of space compared to the black (shady section) and yellow dots (sunny dots). Thus, it is meaning that polyphenolic community, as the polyphenolic profile, in the pulp section is less variable than the sunny and shady sections. Generally, the yellow dots are more spread from their centroids, thus confirming that the accumulation of polyphenolic compounds of the apples are greatly affected by environmental factors such as sun exposure [79].

Analysing the position of the centroids of each sector, they are mostly well-separated along NMDS1 and NMDS2. On the contrary, the sunny and shady sectors in the R210, RM1_17, RS1_319_27, and V220 apple varieties are characterized by overlapped centroids. Thus, it is suggesting that the polyphenolic community in the sunny and shady sectors is less variable in these varieties.

It is worth underling that NMDS plots are providing a graphical visualisation of the beta-diversity based on Euclidean distance of 90 apple samples. Indeed, clusters are found. Therefore, the statistical tests are needed to evaluate the statistically significant

differences between the three sections.

To examine the degree of similarity (ANOSIM test) and the level of variance (PERMANOVA test) between the three sections, the three apple sections have been tested in pairs: shady versus sunny (Shady_VS_Sunny), shady versus pulp (Shady_VS_Pulp), and sunny versus pulp (Sunny_VS_Pulp).

The ggplot package is used to graphically report the results obtained by ANOSIM and PERMANOVA tests in regards to their R values and R^2 values. The relative figures are shown in (5.3)

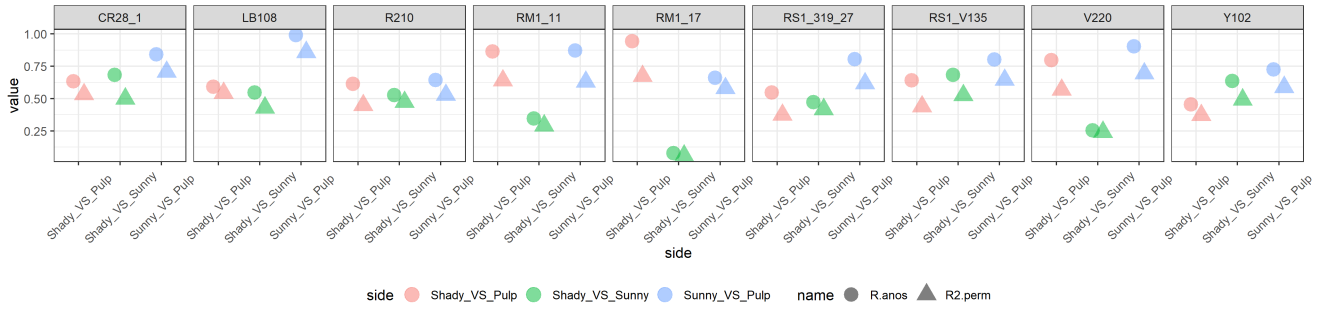

FIGURE 5.3. Results obtained by ANOSIM and PERMANOVA analyses in regards to their R value (point shape) and R^2 (triangle shape). The colors differentiate the the apple three sections under investigation [78]. All information about the 9 red-fleshed apple varieties are reported in the Table 5.1.

The statistical results obtained by ANOSIM and PERMANOVA tests are reported in the Table 5.2.

Table 5.2: The statistical results obtained by ANOSIM and PERMANOVA tests.

Apple Variety	**Pair**	**ANOSIM results**		**PERMANOVA results**		
		R	Sig	R2	F	Sig
RS1_319_27	Shady_VS_Pulp	0.546	0.001	0.3743	10.77	0.001
LB108	Shady_VS_Pulp	0.5909	0.001	0.5425	21.34	0.002
RS1_V135	Shady_VS_Pulp	0.6418	0.001	0.4393	14.1	0.001
CR28_1	Shady_VS_Pulp	0.6351	0.001	0.5323	20.48	0.001
RM1_17	Shady_VS_Pulp	0.9429	0.001	0.6739	37.21	0.001
Y102	Shady_VS_Pulp	0.4553	0.001	0.3721	10.67	0.001
R210	Shady_VS_Pulp	0.6133	0.001	0.4483	14.63	0.001
V220	Shady_VS_Pulp	0.7973	0.001	0.5677	23.64	0.001
RM1_11	Shady_VS_Pulp	0.864	0.001	0.639	31.86	0.001
RS1_319_27	Shady_VS_Sunny	0.4731	0.002	0.4177	12.91	0.001
LB108	Shady_VS_Sunny	0.5478	0.002	0.4302	13.59	0.001
RS1_V135	Shady_VS_Sunny	0.6824	0.001	0.5257	19.95	0.001
CR28_1	Shady_VS_Sunny	0.6833	0.001	0.4999	17.99	0.001
RM1_17	Shady_VS_Sunny	0.0782	0.12	0.0582	1.11	0.34
Y102	Shady_VS_Sunny	0.6364	0.001	0.4918	17.42	0.001
R210	Shady_VS_Sunny	0.5269	0.001	0.4734	16.18	0.001
V220	Shady_VS_Sunny	0.2551	0.002	0.2417	5.74	0.008
RM1_11	Shady_VS_Sunny	0.3467	0.001	0.2911	7.39	0.001
RS1_319_27	Sunny_VS_Pulp	0.8058	0.001	0.6174	29.05	0.001
LB108	Sunny_VS_Pulp	0.9904	0.001	0.8594	109.98	0.001
RS1_V135	Sunny_VS_Pulp	0.8038	0.001	0.6449	32.69	0.001
CR28_1	Sunny_VS_Pulp	0.8431	0.001	0.7058	43.19	0.001
RM1_17	Sunny_VS_Pulp	0.6622	0.001	0.5796	24.81	0.001
Y102	Sunny_VS_Pulp	0.7249	0.001	0.5887	25.76	0.001
R210	Sunny_VS_Pulp	0.6427	0.001	0.528	20.13	0.001
V220	Sunny_VS_Pulp	0.904	0.001	0.6942	40.86	0.001
RM1_11	Sunny_VS_Pulp	0.8713	0.001	0.6296	30.6	0.001

The beta-diversity values are significantly different based on the apple sections for the metabolomic data, though the level of variance explained by our metabolites is high.

The lowest R (ANOSIM result) and R^2 (PERMANOVA result) values are found in the Y102 variety (ANOSIM R = 0.4553, p-value = 0.001; PERMANOVA R^2 = 0.3721, F = 10.67, p-value = 0.001), however, the differences in Shady_VS_Pulp comparison are still statistically significant.

In contrast, lower R and R^2 for the pair Shady_VS_Sunny are found in regard to all cultivars, mostly in RM-1_11 (ANOSIM R = 0.3467, p-value = 0.001; PERMANOVA R^2 = 0.2911, F = 7.39, p-value = 0.001), RM-1_17 (ANOSIM R = 0.0782, p-value = 0.12; PERMANOVA R^2 = 0.0582, F = 1.11, p-value = 0.34), and V220 (ANOSIM R = 0.2551, p-value = 0.002; PERMANOVA R^2 = 0.2417, F = 5.74, p-value = 0.008). The pair Shady_VS_Sunny (point and triangle shapes) in the RM-1_17 cultivar shows a strong similarity and the variance explained is very low. The lowest R value indicates that the chemical profile between shady and sunny sections is similar (ANOSIM R = 0.0782, p-value = 0.12). Additionally, a lowest R^2 value is found in the RM-1_17 variety (PERMANOVA R^2 = 0.0582, F = 1.11, p-value = 0.34), thus meaning that the variability is not statistically significant between in the pair (Shady_VS_Sunny). Therefore, there is not a significant difference in beta-diversity based on the position of the apple on the three for the metabolomic data in this variety (RM-1_17), in contrast to what it is observed for the rest of the cultivars. Indeed, the centroids of the RM-1_17 variety in the figure (5.2) are completely overlapped. On the contrary, the centroids of sunny and shady sections in the V220 variety are almost overlapped, however, but the differences in Shady_VS_Sunny comparison are statistically significant. Thus, it is supporting the information that there is a significant difference between the sun-exposed side and the shaded side as a result of self-shading during its development. Regarding the pair Sunny_VS_Pulp, high R values are found in all cultivars under investigation. Therefore, there is a higher level of dissimilarity in the pair Sunny_VS_Pulp (ANOSIM results), thus meaning that the metabolome of the sun-exposed side of an apple is strongly influenced by external and internal factors. Therefore, it is distinguishable by the metabolome of the pulp. The PERMANOVA test supports these observations, indeed, a high level of variance is found in all cultivars. The major levels of dissimilarity (ANOSIM results) and the highest levels of variance explained (PERMANOVA results) in the LB108 variety

are found (ANOSIM R = 0.9904, p-value 0.001; PERMANOVA R^2 = 0.8593, F = 109.98 p-value = 0.001).

It is worth highlighting that the highest values in terms of variability, as polyphenolic community, in the pair Sunny_VS_Pulp are found compared to the variability calculated in others two pairs of sections within the same varieties (Shady_VS_Pulp and Shady_VS_Sunny). Thus, it is meaning that the pulp metabolome and the metabolome of sun-exposed side of apple fruit are strongly variable and divergent, supporting the evidence about the exposure of the skin to sun influence the accumulation of metabolites [80].

A detailed discussion of the trends of the individual metabolites will be the subject of the following pages. Figure 5.4 and Figure 5.5 show the box plots of the all metabolites quantified in 9 red-fleshed apple varieties.

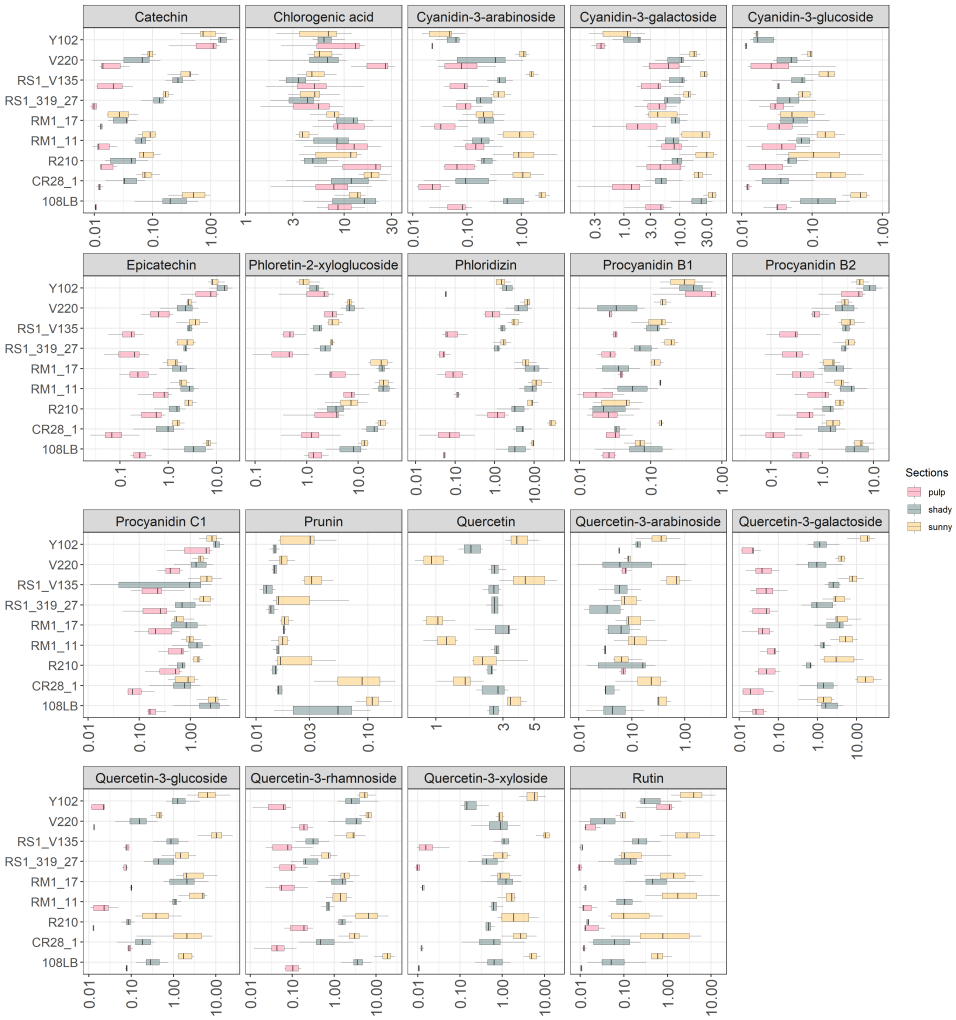

FIGURE 5.4. Concentration of flavonoids and anthocyanins in the pulp of 9 red-fleshed apple varieties. The sunny and shaded exposed site of the apples were analyzed. FW = fresh weight. The colors differentiate the sections (red = pulp; yellow = sunny; and grey = shady) under investigation [78]. All information about 9 red-fleshed apple varieties are reported in the Table 5.1.

The polyphenolic compounds are mostly detected and quantified in all 9 red-fleshed varieties, expect for prunin, quercetin, and quercetin-3-arabinoside that are not found in the pulp section. The main polyphenol classes found in all 9 red-fleshed varieties are anthocyanins, dihydrochalcones, and flavan-3-ols and these results are supported by Yuste et al. [65]. The overall tendency is that the polyphenolic compounds in the skin

(including the shady and sunny sections) are higher than those in the pulp [65, 72, 80], and the concentration of polyphenols is higher in sunny section than in shady section [72, 79].

A wide range of anthocyanins has been reported in the literature [65], and the most abundant is cyanidin-3-galactoside [81], which is around 95 % of the anthocyanin content [65].

As reported by Yuste et al. [65], catechin, epicatechin, and its polymerized forms (procyanindin B1, B2, and C1) are present in variable concentrations due to competitive pathway system between anthocyanins and flavan-3-ols.

In regards to anthocyanins and flavan-3-ols, the red-fleshed Y102 variety, a type-2 red-fleshed variety, shows the lowest concentration of anthocyanins, but a high amount of flavonoids. Y102 is characterized by a high amount of flavan-3-ols (catechin, epicatechin, procyanidin B1, procyanidin B2, and procyanidin C1). Additionally, it has been observed a considerable amount of quercetin glycosides in Y102 variety. On the contrary, the concentration of anthocyanins is the lowest compared to the other cultivars. The occurrence and the distribution of flavonoids and anthocyanins in Y102 are similar to the trends found in the same Y102 variety reported by Ceci et al. [18] and reported in the (chapter 2) as first contribution. Contrarily, the C28_1 variety is characterized by a higher content of anthocyanins than flavonoids, despite having been defined as type-2 red-fleshed variety. It is worth noticing that these two cultivars (Y102 and C28_1) are defined as red-fleshed type-2, however, their metaboloma is strongly divergent. As reported by Wang et al. [82] red-fleshed apples had a high amount flavan-3-ols and anthocyanins supporting our results.

Remarkably, the metaboloma of 108LB cultivar, a 1-type red-fleshed cultivar and cultivated by the Laimburg Research Centre, shows interesting results. The content of chlorogenic acid in the shady side is higher than the sunny side, on the contrary the

findings found in the other varieties. Additionally, the content of cyanidin-3-galactoside is extremely high and it is comparable with the RS1_V135 and RM1_11 varieties, which are commercialized under the umbrella brand: Red Moon®. Surprisingly, the content of cyanidin-3-galactoside in the sunny side found in the 108LB cultivar is higher than RS1_319_27 and RM1_17 varieties. 108LB cultivar has shown a noteworthy content of flavan-3-ols, thus making a special mention with regard to the amount of catechin, epicatechin, procyanidin B2, and procyanidin C1 in this variety that have demonstrated the highest found within the 1-type red-fleshed apples.

As reported by Bars-Cortina et al. [72], in the present survey the phloretin-2-xyloglucoside is the predominant dihydrochalcone found in the skin, and at a lower concentration level in the pulp [65]. As expected, the dihydrochalcone amount is higher in the apple peel than in the flesh for all of the apple varieties studied [72], and it has been found a higher concentration in the sunny section than the shady section [79]. Our findings show that the content of dihydrochalcones is extremely high in RM1_11, RM1_17, and CR28_1 varieties.

In regards to flavonols, the main flavonols quantified in all 9 apple varieties, in both skin and pulp, where the most abundant quercetin glycoside, which is quercetin-3-galactoside, followed by the quercetin-3-glucoside [72]. Our results match the literature where the concentrations of flavonols is lower in the pulp than those in the peel [65]. The general trend is that the sunny section in all varieties have a higher amount of quercetin glycosides than the shady section [79]. The quercetin is detected in the skin, but not in the pulp due to the limit of quantification.

Generally, our results show that the most abundant phenolic acid is the chlorogenic acid [65], and as reported by Yuste et al. [65] the main groups found in the red-fleshed apples are anthocyanins, dihydrochalcones, and flavan-3-ols that are confirming our findings.

It is worth observing that the metabolomic pathway in pigmented apples is expected to be towards the synthesis of anthocyanins especially for cyanidin-3-galactoside [81], higher amount of polyphenolic compounds especially for chlorogenic, phloretin-2-xyloglucoside, phoridizin, procyanidin B2, and quercetin glycosides are found [65, 81, 82]. These compounds are the most dominant phenolic compounds in all cultivars. It is noticeable that the concentration of the chlorogenic acid hardly differ amongst the three sections (pulp, shady, and sunny) within the cultivars. Surprisingly, the amount of chlorogenic acid in the pulp section is higher than the shady and sunny sections.

The literature reported that the flavones and flavanones are present at low concentration levels in both the flesh and skin [15].

It is known that there are two types of red-fleshed apples, namely type-1 and type-2. The type-1 has the MdMYB10 gene located on chromosome (ch.) 9 and the type-2 has the MdMYB110a gene located on ch. 17 [81]. The expression of MdMYB10 is increased in the type-1 and it is responsible for the reddish of apple pulp and foliage [81]. The regulation of these enzymes led to the biosynthesis of metabolites through the phenylpropanoid pathway [81].

It is already known that the type 1 red-fleshed apples, with a deep red pulp, are characterized by a high amount of phenolic compounds and anthocyanins, specially for chlorogenic acid and cyanidin-3-galactoside [81]. Additionally, the accumulation patterns of phenolic compounds in the type 2, with a pale-red flesh, has been considered suitable for the markets and consumption [72].

Differences in the concentration of metabolites are strongly influenced by agronomic factors, the harvest time, and on environmental factors, which lead to considerable biological variation [15], even when the varieties are defined as type-1 and type-2. Our results show that the metabolome of red-fleshed is extremely variable both between the two types and within the types [82]. Differences between Y102 and C28_1 cultivars are

representative examples; however, they share the genotype definition as "red-fleshed type-2", but not the metabolome, which lead to valuable metabolomic variation. Variability in the metaboloma of red-fleshed type-2 has been already reported by Ceci et al. [18] and reported in the (chapter 2) as first contribution.

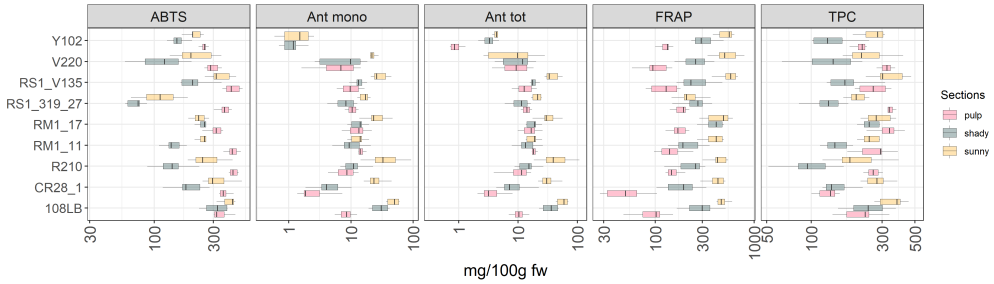

FIGURE 5.5. Results of ABTS, pH differential method (Anto mono, Ant tot), FRAP, TPC, in 9 red-fleshed apple varieties. The sunny and shadow exposed site of the apples were analyzed. FW = fresh weight; Anto mono = monomeric anthocyanin content, Anto tot = total anthocyanin content; TPC = total phenolic content. The colors differentiate the sections (red = pulp; yellow = sunny; and grey = shady) under investigation [78]. All information about 9 red-fleshed apple varieties are reported in the Table 5.1.

The overall tendency is that the accumulation of anthocyanins in sun-exposed side of the red-fleshed apples is more consistent than in the pulp and shaded sections. In detail, as expected, the red-fleshed type-1 apples have shown to be more concentrated in anthocyanins (including Ant mono and Ant tot) being characterized by a red pigmentation in whole fruit [80]. On the contrary, the Y102 and C28_1 varieties show lower anthocyanin content being two type-2 red-fleshed varieties being characterized by white flesh and

only fruit cortex accumulate red pigments [80]. Specifically, in the pulp section of Y102 variety the monomeric anthocyanin content (Anto mono) is not detected and the total anthocyanin content (Anto tot) is the lowest amongst all red-fleshed varieties. While, the C28_1 is characterized by a higher content of anthocyanins, despite having been defined as type-2 red-fleshed variety. It is worth underling that the anthocyanin content can vary from apple to apple [70].

The antioxidant activity (including ABTS and FRAP) shows diverging results. The results from FRAP assay follow the literature, where is already reported that the sun-exposed side is characterized by the highest amount of antioxidants compared to shaded and pulp sections [79]. Indeed, the photosynthetic system and enzymatic activity is higher on the sun-exposed side than the shady side due to the upregulation of enzymes and the potentiate production of antioxidant has been linked to the photoprotective role of flavonoids [79]. The ABTS assay reports more variable results, in which the pulp section is characterized by a higher content of antioxidant than sunny and pulp sections. Thus, it is meaning that the content of antioxidants in the red-fleshed apple varieties may depend on assay used to quantify the antioxidant activity [82]. Indeed, Wang et al. [82] reported that no universal assay is available that reflect the content of antioxidants in a matrix. It is important to use at least two methods to evaluate the antioxidant capacity *in vitro* studies. In this study, two antioxidant assays are used to obtain more accurate evaluations of antioxidant activities: ABTS and FRAP assays.

Once again, when the total polyphenolic content (TPC) in pulp and skin (including sunny and shady sections) is compared, the overall tendency is that TPC in the sun-exposed side is higher higher than pulp and shaded side. On the contrary, the pulp section of V220, RS1_319_27, and RM1_17 shows a higher TPC than the two other sections. The TPC has been related to the composition of polyphenols and anthocyanins. This assumption is also reported in the (chapter 4) as third contribution.

5.2.5 Conclusion

The benefits related to the consumption of pigmented fruits and vegetables is already reported [18, 81]. Our result contribute towards to select cultivars that have a high economic value as a functional food. Noteworthy, the red-fleshed apples were low allergy apple compared to white-fleshed apples [83]. Additionally, recent study reported that the red-fleshed apples are characterized by a higher antioxidant activity and anticancer cell proliferation ability than the white-fleshed varieties [84]. As reported by Yuste et al. [65], there is still scarce information regarding to the polyphenol metabolites and their effect on the human health, however, *in vitro* and *in vivo* studies demonstrated that the red-fleshed apples had several health outcomes in preventing human issues such as cancer, cardiovascular disorders, inflammation, and chronic diseases [65]. Therefore, our studies contribute to increase scientific knowledge about the metabolome of 9 red-fleshed apple varieties and their nutritional value and healthiness.

5.2.6 Conclusion: Fourth Contribution

The aim of this chapter 5 is to provide new knowledge on the composition of specific red-fleshed apple varieties, the composition of which may be beneficial and should be promoted. The investigation of chemical profiles of red-fleshed apple cultivars is a crucial point in estimating the “overall quality” of fruits. This could be useful to motivate the breeders to select healthier food, to bring on the markets more appreciable fruits, and to improve the awareness and the eating habits of the customers. Therefore, the role of my studies is to gain insights about the chemical profile of colored apples in order to find a good balance between the content of antioxidants, polyphenols, and anthocyanins.

Author Contributions: conceptualization, A.T.C., P.F., W.G., M.O., P.R. and F.M.; methodology, A.T.C., A.L., and P.R.; software, A.T.C., P.F., and A.L.; validation, A.T.C., P.F., and A.L.; formal analysis, A.T.C., P.F., A.L, M.O., P.R, and F.M.; investigation, A.T.C., P.R.

and F.M.; resources, M.O. and F.M.; data curation, A.T.C., P.F. and A.L.; writing—original draft preparation, A.T.C.; visualization, A.T.C., P.F., and A.L; supervision, P.F., P.R. and F.M.; project administration, W.G., M.O., P.R. and F.M.; funding acquisition, W.G., M.O., P.F. and F.M.

5.2.7 Supplementary Materials

Table 5.3: List of the apple variety, side, red-fleshed types, antioxidants (ABTS and FRAP assays), total polyphenolic content (TPC), and pH differential method (Anto mono and Anto tot) quantified in the pulp section of the 9 red-fleshed cultivars included in the study. Results are expressed as mg/100 g FW.

Variety	Side	Type	FRAP	ABTS	TPC	Ant mono	Ant tot
RS1_319_27	pulp	Type 1	200.21	372.13	329.5	12.95	16.69
RS1_319_27	pulp	Type 1	156.39	403.51	255.03	9.09	11.83
RS1_319_27	pulp	Type 1	200.22	390.39	365.76	11.8	15.48
RS1_319_27	pulp	Type 1	175.8	367.22	337.86	11.02	14.41
RS1_319_27	pulp	Type 1	140.03	340.06	288.73	9.94	13.17
RS1_319_27	pulp	Type 1	185.17	354.68	328.98	7.95	11.13
RS1_319_27	pulp	Type 1	201.13	352.13	335.85	12.01	15.68
RS1_319_27	pulp	Type 1	220.6	408.68	374.4	13.28	17.18
RS1_319_27	pulp	Type 1	208.75	423.59	357.52	8.2	11.46
RS1_319_27	pulp	Type 1	161.93	303.04	333.37	9.78	13.77
108LB	pulp	Type 1	73.06	305.38	161.24	5.52	6.98
108LB	pulp	Type 1	108.72	452.9	253	8.18	9.96
108LB	pulp	Type 1	114.62	396.53	239.2	6.87	9.13
108LB	pulp	Type 1	69.73	310.91	164.46	7.14	8.86
108LB	pulp	Type 1	79.3	321.55	200.15	8.82	10.61
108LB	pulp	Type 1	95.47	350.67	226.62	9.12	11.28
108LB	pulp	Type 1	47.75	304.33	139.19	6.47	7.76
108LB	pulp	Type 1	151.37	371.73	336.06	12.52	15.49
108LB	pulp	Type 1	111.49	303.44	246.46	12.06	14.48
108LB	pulp	Type 1	108.13	318.32	234.62	9.98	11.99
RS1_V135	pulp	Type 1	178.84	493.15	347.54	13.96	18.1
RS1_V135	pulp	Type 1	180.68	483.95	328.15	13.62	17.43
RS1_V135	pulp	Type 1	139.89	486.01	308.52	9.86	13.24
RS1_V135	pulp	Type 1	88.01	398.53	229.3	6.01	8.47
RS1_V135	pulp	Type 1	104.12	404.02	207.27	9.42	11.92
RS1_V135	pulp	Type 1	166.35	434.62	259.47	11.62	15.02
RS1_V135	pulp	Type 1	80.01	355.48	196.94	5.92	7.81
RS1_V135	pulp	Type 1	117.93	377.82	262.09	7.25	10.21
RS1_V135	pulp	Type 1	162.49	519.17	318.27	16.5	20.72
RS1_V135	pulp	Type 1	82.84	370.94	198.4	8.02	10.54
CR28_1	pulp	Type 2	28.98	420.85	155.94	1.5	4.5
CR28_1	pulp	Type 2	39.35	384	133.91	1.37	2.08
CR28_1	pulp	Type 2	64.82	335.14	145.12	3.54	4.1
CR28_1	pulp	Type 2	63.31	337.92	100.33	1.81	2.7
CR28_1	pulp	Type 2	30.72	338.83	140.35	1.82	2.41
CR28_1	pulp	Type 2	64.61	363.25	154.74	7.26	8.08
CR28_1	pulp	Type 2	27.56	368.73	76.15	1.9	2.99
CR28_1	pulp	Type 2	39.24	356.05	134.92	2.17	2.68
CR28_1	pulp	Type 2	104.01	349.14	133.01	5.28	6.47
CR28_1	pulp	Type 2	89.27	437.34	108.45	1.75	3.33
RM1_17	pulp	Type 1	152.59	275.31	291.57	7.63	10.38
RM1_17	pulp	Type 1	192.79	321.61	362	13.71	17.16
RM1_17	pulp	Type 1	127.23	245.08	235.76	9.46	12.28
RM1_17	pulp	Type 1	221.19	360.37	427.65	7.12	9.89
RM1_17	pulp	Type 1	205.04	351.4	374.17	14.78	18.11
RM1_17	pulp	Type 1	147.71	309.51	315.44	15.75	19.23
RM1_17	pulp	Type 1	172.83	329.94	354.11	15.74	19.39
RM1_17	pulp	Type 1	164.77	301.55	320.31	10.43	13.45
RM1_17	pulp	Type 1	162.62	296.68	296.23	12.96	16.06
RM1_17	pulp	Type 1	206.03	351.77	354.21	21.57	25.98
Y102	pulp	Type 2	152.09	251.47	231.8		1.06
Y102	pulp	Type 2	100	276.65	155.33		0.72
Y102	pulp	Type 2	142.36	263.82	236.61		0.79
Y102	pulp	Type 2	116.67	227.87	180.95		0.35
Y102	pulp	Type 2	129.83	257.97	218.72		0.90
Y102	pulp	Type 2	134.46	255.75	222.34		1.30
Y102	pulp	Type 2	122.9	240.82	218.01		0.71
Y102	pulp	Type 2	136.22	243.9	222.55		0.87
Y102	pulp	Type 2	117.29	254.13	202.99		0.82
Y102	pulp	Type 2	134.28	258.89	231.73		1.04
R210	pulp	Type 1	126.93	404.68	245.75	1.94	3.92
R210	pulp	Type 1	145.46	468.05	266.15	10.78	13.5
R210	pulp	Type 1	156.99	424.79	286.2	13.25	16.49
R210	pulp	Type 1	130.76	481.38	243.16	10.12	12.72
R210	pulp	Type 1	132.83	481.04	229.85	7.32	10.24
R210	pulp	Type 1	139.7	439.1	270.96	6.83	9.46
R210	pulp	Type 1	166.07	408.75	256.14	4.29	6.34
R210	pulp	Type 1	186.29	412.51	293.87	10.82	13.87
R210	pulp	Type 1	202.03	401.86	304.15	11.21	14.45
R210	pulp	Type 1	145.39	465.11	224.47	6.02	7.97
V220	pulp	Type 1	236.58	327.86	327.19	11.67	14.69
V220	pulp	Type 1	149.16	316.06	317.25	8.87	12.21
V220	pulp	Type 1	85.8	254.46	236.85	1.61	3.66
V220	pulp	Type 1	93.51	285	295.88	5.4	7.66
V220	pulp	Type 1	86.01	259.82	368.22	2.26	3.96
V220	pulp	Type 1	95.35	285.89	327.49	7.7	10.49
V220	pulp	Type 1	101.26	334.97	319.56	12.17	15.65
V220	pulp	Type 1	139.35	352.6	433.06	14.43	18.17
V220	pulp	Type 1	58.01	265.41	275.92	3.65	5.53
V220	pulp	Type 1	93.37	283.55	347.22	6.12	8.12
RM1_11	pulp	Type 1	98.34	404.53	296.91	9.72	13.51
RM1_11	pulp	Type 1	154.41	434.13	291.24	15.24	18.9
RM1_11	pulp	Type 1	113.53	463.64	293.65	13.86	17.48
RM1_11	pulp	Type 1	237.11	453.94	297	8.06	11.06
RM1_11	pulp	Type 1	172.07	362.47	211.83	13.74	17.11
RM1_11	pulp	Type 1	126.47	418.45	174.9	17.89	21.53
RM1_11	pulp	Type 1	128.07	404.13	250.65	15.4	19.83
RM1_11	pulp	Type 1	243.33	502.06	383.72	13.27	17.58
RM1_11	pulp	Type 1	96.95	471.24	328.37	14.28	18.41
RM1_11	pulp	Type 1	151.18	413.36	181.38	25.93	31.63

Table 5.4: List of the apple variety, side, red-fleshed types, antioxidants (ABTS and FRAP assays), total polyphenolic content (TPC), and pH differential method (Anto mono and Anto tot) quantified in the shady section of the 9 red-fleshed cultivars included in the study. Results are expressed as mg/100 g FW.

Variety	Side	Type	FRAP	ABTS	TPC	Ant mono	Ant tot
RS1_319_27	shady	Type 1	261.26	75.56	174.4	12.71	16.34
RS1_319_27	shady	Type 1	277.99	33.05	117.29	10.58	13.98
RS1_319_27	shady	Type 1	300.31	61.59	82.63	4.17	5.97
RS1_319_27	shady	Type 1	299.86	72.21	94.14	6.29	8.45
RS1_319_27	shady	Type 1	485.67	76.15	118.1	6.09	8.48
RS1_319_27	shady	Type 1	204.89	87.17	177.68	11.57	14.79
RS1_319_27	shady	Type 1	375.52	60.93	144.44	11.14	14.3
RS1_319_27	shady	Type 1	148.58	82.29	113.22	5.18	7.41
RS1_319_27	shady	Type 1	222.4	58.16	150.04	9.03	12.15
RS1_319_27	shady	Type 1	222.31	75.82	152.03	7.59	10.55
108LB	shady	Type 1	207.21	255.74	184.83	23.13	27.85
108LB	shady	Type 1	165.58	229.95	151.44	19.14	22.67
108LB	shady	Type 1	514.94	391.31	372.32	39.22	46.8
108LB	shady	Type 1	334.33	390.25	292.99	41.82	48.83
108LB	shady	Type 1	220.89	263.5	190.91	27.32	32.18
108LB	shady	Type 1	377.98	372.63	303.92	38.31	45.48
108LB	shady	Type 1	221.58	265.62	197.92	21.61	25.8
108LB	shady	Type 1	285.35	301.33	215.67	19.77	23.94
108LB	shady	Type 1	366.04	391.6	319.49	41.7	48.72
108LB	shady	Type 1	311.48	348.88	269.82	33.79	39.91
RS1_V135	shady	Type 1	343.06	283.14	231.61	18.09	23.3
RS1_V135	shady	Type 1	283.25	231.85	197.05	15.31	19.95
RS1_V135	shady	Type 1	164.54	162.75	128.9	11.9	15.72
RS1_V135	shady	Type 1	193.43	203.88	166.5	13.85	18.35
RS1_V135	shady	Type 1	191.17	169.28	138.64	13.47	17.71
RS1_V135	shady	Type 1	250.04	204.69	181.83	12.28	16.97
RS1_V135	shady	Type 1	210.21	164.45	134.58	9.43	12.72
RS1_V135	shady	Type 1	338.66	206.44	170.68	12.78	17.56
RS1_V135	shady	Type 1	476.85	349.8	314.55	22.89	29.44
RS1_V135	shady	Type 1	196.12	166.31	127.19	12.81	16.72
CR28_1	shady	Type 2	328.44	268.78	222.68	10.23	14.58
CR28_1	shady	Type 2	248.28	207.43	150.98	6.22	10.82
CR28_1	shady	Type 2	118.52	168.19	133.18	4.7	7.92
CR28_1	shady	Type 2	152.85	169.29	125.23	3.47	6.38
CR28_1	shady	Type 2	180.87	183.16	139.46	3.33	6.37
CR28_1	shady	Type 2	240.67	243.03	215.14	17.53	22.32
CR28_1	shady	Type 2	129.23	155.45	119.27	3.42	5.73
CR28_1	shady	Type 2	103.07	117.28	73.41	1.87	4.3
CR28_1	shady	Type 2	226.43	279.55	171.95	6.04	9.63
CR28_1	shady	Type 2	206.89	176.52	127.5	2.71	5.61
RM1_17	shady	Type 1	264.27	216.36	203.84	10.16	13.79
RM1_17	shady	Type 1	413.12	253.92	273.85	14.01	18.89
RM1_17	shady	Type 1	301.22	231.48	217.7	14.84	19.23
RM1_17	shady	Type 1	768.24	371.53	485.53	9.11	13.95
RM1_17	shady	Type 1	493.51	212.13	239.86	10.47	14.36
RM1_17	shady	Type 1	335.45	246.62	243.85	19.54	25.29
RM1_17	shady	Type 1	374.85	254.73	247.85	14.78	19.81
RM1_17	shady	Type 1	864.38	441.31	509.81	8.76	13.55
RM1_17	shady	Type 1	414.46	256.68	223.03	16.55	21.85
RM1_17	shady	Type 1	419.76	263.01	290.51	14.05	19.05
Y102	shady	Type 2	491.15	201.73	245.75	1.16	3.59
Y102	shady	Type 2	285.51	155.54	132.81	1.13	3.87
Y102	shady	Type 2	274.4	148.38	124.49	0.78	2.62
Y102	shady	Type 2	383.57	132.11	171.04	1.28	3.28
Y102	shady	Type 2	239.72	126.84	101.64	0.7	2.62
Y102	shady	Type 2	420.3	159.18	187.95	2.08	4.78
Y102	shady	Type 2	232.38	145.3	104.29	0.39	2.14
Y102	shady	Type 2	297.69	143.45	136.23	1.21	3.24
Y102	shady	Type 2	245.48	167.15	100.65	1.79	3.82
Y102	shady	Type 2	306.94	168.44	103.91	1.31	3.36
R210	shady	Type 1	260.52	144.73	124.88	6.84	9.64
R210	shady	Type 1	176.61	113.59	81.42	11.41	14.63
R210	shady	Type 1	258.1	159.02	103.99	22.61	26.74
R210	shady	Type 1	295.8	180.31	141.95	20.89	25.04
R210	shady	Type 1	178.01	133.3	85.05	12.89	15.84
R210	shady	Type 1	253.89	148.52	123.56	12.56	15.84
R210	shady	Type 1	287.75	87.83	61.48	6.77	8.7
R210	shady	Type 1	123.22	95.49	51.37	8.57	10.83
R210	shady	Type 1	322.11	231.14	166.1	10.39	17.13
R210	shady	Type 1	189.91	130.99	83.15	8.09	10.47
V220	shady	Type 1	373.93	193.45	221.12	14.65	10.72
V220	shady	Type 1	396.27	200.36	213.91	18.29	14.71
V220	shady	Type 1	287.4	135.13	165.05	9.55	13.51
V220	shady	Type 1	321.82	157.87	183.71	12.19	16.08
V220	shady	Type 1	217.31	83.3	102.94	3.04	5.48
V220	shady	Type 1	157.03	57.89	63.42	2.62	4.05
V220	shady	Type 1	199.43	107.36	119.49	10.02	13.32
V220	shady	Type 1	297.94	153.26	184.16	15.54	20.13
V220	shady	Type 1	229.19	86.93	100.57	3.55	5.93
V220	shady	Type 1	189.5	75.99	82.34	2.81	5.05
RM1_11	shady	Type 1	183.74	132.46	150.69	5.01	7.84
RM1_11	shady	Type 1	254.4	164.62	175.69	7.48	11.11
RM1_11	shady	Type 1	134.63	109.33	120	7.55	10.52
RM1_11	shady	Type 1	561.85	248.37	335.68	14.72	19.88
RM1_11	shady	Type 1	195.59	130.49	138.14	8.5	12.11
RM1_11	shady	Type 1	174.84	123.88	114.66	11.33	14.94
RM1_11	shady	Type 1	171.18	135.03	125.71	8.67	12.17
RM1_11	shady	Type 1	274.76	140.56	165.4	10.08	14.42
RM1_11	shady	Type 1	354.24	183.57	191.75	20.88	26.09
RM1_11	shady	Type 1	138.31	138.71	137.57	14.54	18.83

Table 5.5: List of the apple variety, side, red-fleshed types, antioxidants (ABTS and FRAP assays), total polyphenolic content (TPC), and pH differential method (Anto mono and Anto tot) quantified in the sunny section of the 9 red-fleshed cultivars included in the study. Results are expressed as mg/100 g FW.

Variety	Side	Type	FRAP	ABTS	TPC	Ant mono	Ant tot
RS1_319_27	sunny	Type 1	211.56	90.6	186.02	14.01	17.53
RS1_319_27	sunny	Type 1	145.8	81.82	169.67	12.69	16.52
RS1_319_27	sunny	Type 1	278.8	149.1	235.14	18.53	23.46
RS1_319_27	sunny	Type 1	200.74	115.9	194.34	18.22	22.88
RS1_319_27	sunny	Type 1	268	151.87	246.64	19.42	24.52
RS1_319_27	sunny	Type 1	169.04	186.48	331.26	18.14	24.61
RS1_319_27	sunny	Type 1	194.21	64.75	164.48	13.98	18.27
RS1_319_27	sunny	Type 1	214.61	84.96	208.04	15.92	19.99
RS1_319_27	sunny	Type 1	201.41	121.44	187.85	20.75	25.14
RS1_319_27	sunny	Type 1	362.13	106.8	207.33	12.72	17.03
108LB	sunny	Type 1	454.03	421.32	393.31	58.23	67.49
108LB	sunny	Type 1	430.36	428.3	380.26	44.06	52.33
108LB	sunny	Type 1	221.77	211.94	149.82	16.27	19.34
108LB	sunny	Type 1	429.7	427.74	398.12	60.29	70.19
108LB	sunny	Type 1	571.26	354.51	266.7	36.63	43.21
108LB	sunny	Type 1	418.78	432.26	344.01	56.55	66.36
108LB	sunny	Type 1	601.1	452.14	453.34	58.14	67.78
108LB	sunny	Type 1	500.52	444.52	427.35	40.18	48.49
108LB	sunny	Type 1	503.26	441.55	376.54	59.86	69.4
108LB	sunny	Type 1	476.31	319.65	275.39	38.22	45.01
RS1_V135	sunny	Type 1	640.63	459.85	472.36	41.17	52.52
RS1_V135	sunny	Type 1	687.28	302.57	280.77	25.51	32.56
RS1_V135	sunny	Type 1	564.16	312.48	296.1	20.96	28.05
RS1_V135	sunny	Type 1	655.17	424.4	445.15	44.78	56.5
RS1_V135	sunny	Type 1	498.5	256.69	233.32	23.61	30.06
RS1_V135	sunny	Type 1	565.33	290.7	309.39	26.38	33.85
RS1_V135	sunny	Type 1	664.19	450.05	469.08	39.22	50.69
RS1_V135	sunny	Type 1	374.86	304.35	321.14	22.44	29.69
RS1_V135	sunny	Type 1	422.51	342.95	289.27	24.55	31.64
RS1_V135	sunny	Type 1	596.79	319.61	296.73	27.47	35.91
CR28_1	sunny	Type 2	434.28	282.98	287.99	22.74	29.49
CR28_1	sunny	Type 2	332.95	271.23	285.93	30.38	38.23
CR28_1	sunny	Type 2	472.23	245.79	232.93	20.05	25.63
CR28_1	sunny	Type 2	362.6	250.49	261.03	16.17	21.7
CR28_1	sunny	Type 2	425.74	293.29	268.08	24	30.98
CR28_1	sunny	Type 2	735.52	511.43	486.45	45.09	55.93
CR28_1	sunny	Type 2	495.11	295.21	214.69	21.34	27.12
CR28_1	sunny	Type 2	512.77	425.13	380.19	29.28	36.98
CR28_1	sunny	Type 2	426.71	391.96	310.37	24.72	31.61
CR28_1	sunny	Type 2	286.47	296.86	228.23	17	22.48
RM1_17	sunny	Type 1	547.57	234.55	373.07	23.16	30.32
RM1_17	sunny	Type 1	359.98	221.77	272.34	23.03	29.23
RM1_17	sunny	Type 1	531.48	272.35	276.12	46.82	56.26
RM1_17	sunny	Type 1	610.77	278.05	353.98	15.32	20.61
RM1_17	sunny	Type 1	482.35	240.85	250.59	21.6	28.09
RM1_17	sunny	Type 1	332.32	188.87	214.76	31.45	38.2
RM1_17	sunny	Type 1	493.45	221.98	306.19	21.26	27.9
RM1_17	sunny	Type 1	279.52	136.13	142.83	13.65	17.52
RM1_17	sunny	Type 1	610.48	257.21	352.05	34.18	42.08
RM1_17	sunny	Type 1	341.17	211.32	238.83	32	38.65
Y102	sunny	Type 2	421.45	203.47	208.7	0.59	2.71
Y102	sunny	Type 2	383.81	167.06	190.91	1.66	4.46
Y102	sunny	Type 2	489.25	204.27	262.37	1.36	4
Y102	sunny	Type 2	436.81	204.1	225.76	2.18	4.57
Y102	sunny	Type 2	632.73	230.98	313.87	2.54	5.66
Y102	sunny	Type 2	558.65	235.14	298.06	0.63	3.67
Y102	sunny	Type 2	571.86	243.91	306.62	0.77	4.34
Y102	sunny	Type 2	562.36	203.47	256.85	1.76	4.34
Y102	sunny	Type 2	598.35	201.59	310.31	2.55	5.55
Y102	sunny	Type 2	628.03	252.07	298.22	1.18	4.04
R210	sunny	Type 1	421.45	197.13	141.3	14.3	18.35
R210	sunny	Type 1	300.97	209.5	158.38	23.25	28.36
R210	sunny	Type 1	450.13	293.4	228.23	46	54.09
R210	sunny	Type 1	545.07	331.34	262.78	64.21	75.21
R210	sunny	Type 1	841.9	430.16	388.65	92.46	107.92
R210	sunny	Type 1	546.91	342.6	272.76	54.03	63.96
R210	sunny	Type 1	426.75	238.34	171.65	22.54	27.98
R210	sunny	Type 1	406.95	247.67	169.8	31.91	38.25
R210	sunny	Type 1	409.16	242.95	193.12	32.92	39.64
R210	sunny	Type 1	265.02	186.81	119.03	28.72	34.23
V220	sunny	Type 1	393.37	161.11	185.96	22.89	24.65
V220	sunny	Type 1	800.56	349.26	416.72	21.02	28.29
V220	sunny	Type 1	593.45	165.91	168.64	5.49	9.55
V220	sunny	Type 1	567.67	220.85	254.05	27.77	12.19
V220	sunny	Type 1	337.88	133.37	163.42	13.04	3.04
V220	sunny	Type 1	443.48	172.99	198.86	23.54	2.62
V220	sunny	Type 1	443.9	224.74	228.5	20.26	10.02
V220	sunny	Type 1	673.09	336.57	322.09	25.51	15.54
V220	sunny	Type 1	684.04	313.62	300.25	21.18	3.55
V220	sunny	Type 1	449.05	176.05	207.3	22.17	2.81
RM1_11	sunny	Type 1	264.27	216.36	203.84	10.16	13.79
RM1_11	sunny	Type 1	413.12	253.92	273.85	14.01	18.89
RM1_11	sunny	Type 1	301.22	231.48	217.7	14.84	19.23
RM1_11	sunny	Type 1	768.24	371.53	485.53	9.11	13.95
RM1_11	sunny	Type 1	493.51	212.13	239.86	10.47	14.36
RM1_11	sunny	Type 1	335.45	246.62	243.85	19.54	25.29
RM1_11	sunny	Type 1	374.85	254.73	247.85	14.78	19.81
RM1_11	sunny	Type 1	864.38	441.31	509.81	8.76	13.55
RM1_11	sunny	Type 1	414.46	256.68	223.03	16.55	21.85
RM1_11	sunny	Type 1	419.76	263.01	290.51	14.05	19.05

Table 5.6: List of the apple variety, side, red-fleshed types, polyphenolic compounds quantified in the pulp section of the 9 red-fleshed cultivars included in the study. Results are expressed as mg/100 g FW.

Variety	Side	Type	Catechin	Chlorogenic acid	Cyanidin-3-arabinoside	Cyanidin-3-galactoside	Cyanidin-3-glucoside	Epicatechin	Phloretin-2-xyloglucoside	Phloridizin	Procyanidin B1	Procyanidin B2	Procyanidin C1	Prunin	Quercetin	Quercetin-3-rhamnoside	Quercetin-3-arabinoside	Quercetin-3-galactoside	Quercetin-3-glucoside	Quercetin-3-xyloside	Rutin
RS1_319_27	pulp	Type 1	0.01	7.05	0.14	7.65	0.06	0.25	0.47	0.06	0.03	0.48	0.38			0.1		0.06	0.08	0.01	0.01
RS1_319_27	pulp	Type 1	0.01	3.49	0.07	2.68	0.03	0.11	0.29	0.08	0.03	0.19	0.12			0.07		0.03	0.08	0.01	0.01
RS1_319_27	pulp	Type 1	0.01	9.73	0.19	8.75	0.04	0.4	1.11	0.16	0.03	0.56	0.51			0.24		0.1	0.09	0.02	0.01
RS1_319_27	pulp	Type 1	0.01	7	0.12	5.82	0.04	0.24	0.53	0.05	0.03	0.42	0.28			0.12		0.06	0.08	0.01	0.01
RS1_319_27	pulp	Type 1	0.01	4.48	0.09	4.42	0.04	0.18	0.45	0.01	0.03	0.28	0.24			0.1		0.05	0.08	0.01	0.01
RS1_319_27	pulp	Type 1	0.01	1.41	0.02	0.73	0.02	0.05	0.11	0.01	0.02	0.08	0.05			0.04		0.02	0.07	0.01	0.01
RS1_319_27	pulp	Type 1	0.01	1.78	0.04	1.55	0.02	0.06	0.09	0.05	0.02	0.09	0.08			0.04		0.02	0.06	0.01	0.01
RS1_319_27	pulp	Type 1	0.01	2.94	0.06	2.68	0.03	0.09	0.19	0.04	0.02	0.16	0.12			0.04		0.02	0.06	0.01	0.01
RS1_319_27	pulp	Type 1	0.02	9.08	0.09	4.16	0.03	0.25	0.54	0.05	0.03	0.39	0.35			0.13		0.07	0.08	0.02	0.01
RS1_319_27	pulp	Type 1	0.01	6.73	0.11	5.16	0.03	0.22	0.52	0.05	0.03	0.35	0.31			0.09		0.05	0.07	0.01	0.01
108LB	pulp	Type 1	0.01	16.16	0.08	4.78	0.02	0.41	2.3	0.05	0.03	0.59	0.34			0.16		0.05	0.08	0.01	0.01
108LB	pulp	Type 1	0.01	6.8	0.04	2.51	0.04	0.19	1.23	0.06	0.03	0.25	0.15			0.07		0.02	0.08	0.01	0.01
108LB	pulp	Type 1	0.01	9.21	0.04	2.45	0.03	0.21	1.48	0.26	0.02	0.26	0.16			0.1		0.02	0.08	0.01	0.01
108LB	pulp	Type 1	0.01	6.98	0.08	4.54	0.05	0.22	0.88	0.02	0.03	0.32	0.21			0.08		0.02	0.07	0.01	0.01
108LB	pulp	Type 1	0.01	11.97	0.1	5.87	0.04	0.46	2.68	0.05	0.02	0.61	0.34			0.14		0.04	0.08	0.01	0.01
108LB	pulp	Type 1	0.01	11.14	0.09	5.11	0.03	0.34	1.97	0.03	0.03	0.52	0.15			0.13		0.04	0.08	0.01	0.01
108LB	pulp	Type 1	0.01	8.06	0.09	4.69	0.03	0.31	1.22	0.05	0.02	0.46	0.16			0.11		0.03	0.07	0.01	0.01
108LB	pulp	Type 1	0.01	12.25	0.13	7.14	0.03	0.34	1.92	0.32	0.03	0.55	0.21			0.15		0.04	0.08	0.01	0.01
108LB	pulp	Type 1	0.01	1.82	0.02	1.02	0.01	0.06	0.23	0.05	0.02	0.08	0.05			0.02		0.01	0.07	0.01	0.01
108LB	pulp	Type 1	0.01	5.26	0.04	2.36	0.04	0.16	1.05	0.05	0.02	0.28	0.14			0.07		0.02	0.08	0.01	0.01
RS1_V135	pulp	Type 1	0.05	15.82	0.25	11.85	0.08	0.57	1.46	0.18	0.03	0.94	0.77			0.31		0.17	0.13	0.05	0.01
RS1_V135	pulp	Type 1	0.03	4.74	0.09	4.73	0.03	0.16	0.45	0.07	0.03	0.31	0.25			0.08		0.04	0.08	0.01	0.01
RS1_V135	pulp	Type 1	0.01	6.53	0.09	4.17	0.03	0.19	0.53	0.06	0.03	0.34	0.22			0.08		0.06	0.08	0.02	0.01
RS1_V135	pulp	Type 1	0.01	5.28	0.06	2.56	0.03	0.14	0.5	0.06	0.03	0.19	0.14			0.08		0.04	0.08	0.02	0.01
RS1_V135	pulp	Type 1	0.03	6.6	0.14	6.69	0.05	0.3	0.98	0.2	0.03	0.51	0.34			0.11		0.08	0.09	0.02	0.01
RS1_V135	pulp	Type 1	0.01	3.93	0.1	4.3	0.03	0.19	0.43	0.09	0.03	0.28	0.23			0.05		0.05	0.07	0.01	0.01
RS1_V135	pulp	Type 1	0.03	3.16	0.04	1.73	0.03	0.1	0.33	0.05	0.03	0.13	0.1			0.03		0.02	0.07	0.01	0.01
RS1_V135	pulp	Type 1	0.01	7.58	0.09	4.07	0.03	0.2	0.57	0.12	0.03	0.33	0.28			0.1		0.08	0.13	0.06	0.01
RS1_V135	pulp	Type 1	0.02	1.6	0.05	1.87	0.03	0.06	0.09	0.01	0.02	0.08	0.07			0.02		0.02	0.08	0.01	0.01
RS1_V135	pulp	Type 1	0.02	2.08	0.04	1.7	0.01	0.07	0.16	0.06	0.03	0.14	0.07			0.03		0.02	0.07	0.01	0.01
CR28_1	pulp	Type 2	0.01	19.07	0.04	2.93	0.01	0.25	4.62	0.17	0.06	0.41	0.2			0.13		0.04	0.1	0.01	0.01
CR28_1	pulp	Type 2	0.01	1.8	0.01	0.15	0.01	0.02	0.32	0.01	0.04	0.02	0.02			0.01		0.01	0.08	0.01	0.01
CR28_1	pulp	Type 2	0.01	11.37	0.02	1.75	0.01	0.11	1.85	0.14	0.03	0.16	0.11			0.07		0.08	0.09	0.01	0.01
CR28_1	pulp	Type 2	0.01	8.8	0.03	1.24	0.01	0.08	1.24	0.06	0.04	0.12	0.08			0.05		0.01	0.09	0.01	0.01
CR28_1	pulp	Type 2	0.01	6.98	0.01	0.52	0.01	0.06	1.25	0.08	0.02	0.08	0.06			0.04		0.01	0.08	0.01	0.01
CR28_1	pulp	Type 2	0.01	4.87	0.04	2.01	0.01	0.06	0.72	0.02	0.02	0.11	0.07			0.04		0.02	0.08	0.01	0.01
CR28_1	pulp	Type 2	0.01	3.35	0.01	0.6	0.01	0.04	0.53	0.06	0.02	0.04	0.02			0.02		0.01	0.08	0.01	0.01
CR28_1	pulp	Type 2	0.01	18.08	0.02	1.51	0.01	0.11	2.54	0.31	0.04	0.19	0.11			0.11		0.04	0.1	0.01	0.01
CR28_1	pulp	Type 2	0.01	9.44	0.05	3.07	0.01	0.12	1.41	0.1	0.02	0.19	0.12			0.06		0.07	0.1	0.01	0.01
CR28_1	pulp	Type 2	0.02	6.03	0.02	0.7	0.02	0.05	1.05	0.03	0.03	0.08	0.06			0.03		0.02	0.11	0.02	0.02
RM1_17	pulp	Type 1	0.01	22.61	0.08	4.33	0.09	0.58	6.93	0.2	0.04	1.03	0.54			0.15		0.08	0.1	0.01	0.01
RM1_17	pulp	Type 1	0.01	6.63	0.02	0.98	0.02	0.15	2.73	0.11	0.04	0.26	0.15			0.05		0.04	0.1	0.01	0.01
RM1_17	pulp	Type 1	0.01	8.05	0.04	1.77	0.01	0.2	2.97	0.05	0.03	0.27	0.17			0.04		0.04	0.1	0.01	0.01
RM1_17	pulp	Type 1	0.01	8.72	0.01	0.27	0.01	0.1	2.92	0.07	0.04	0.24	0.13			0.06		0.03	0.1	0.01	0.01
RM1_17	pulp	Type 1	0.01	4.77	0.02	0.81	0.02	0.1	1.44	0.03	0.03	0.13	0.08			0.02		0.01	0.08	0.01	0.01
RM1_17	pulp	Type 1	0.01	8.43	0.05	3.08	0.05	0.25	2.54	0.07	0.04	0.41	0.22			0.05		0.04	0.09	0.01	0.01
RM1_17	pulp	Type 1	0.01	17.41	0.06	4.25	0.05	0.39	5.81	0.16	0.04	0.7	0.47			0.13		0.06	0.1	0.01	0.01
RM1_17	pulp	Type 1	0.01	30.48	0.03	1.57	0.04	0.45	10.65	0.14	0.04	0.83	0.61			0.24		0.2	0.18	0.01	0.03
RM1_17	pulp	Type 1	0.01	12.51	0.1	5.68	0.06	0.36	5.12	0.16	0.04	0.62	0.35			0.08		0.05	0.1	0.04	0.01
RM1_17	pulp	Type 1	0.01	7.76	0.03	1.77	0.03	0.22	2.93	0.05	0.04	0.34	0.19			0.05		0.03	0.11	0.01	0.01
Y102	pulp	Type 2	1.25	13.95	0.02	0.44	0.01	11.31	3.48	0.06	0.84	7	2.69			0.09	0.06	0.01	0.01		1.25
Y102	pulp	Type 2	0.55	3.95	0.02	0.33	0.01	3.09	0.82	0.06	0.25	1.85	0.57			0.01	0.06	0.01	0.01		0.55
Y102	pulp	Type 2	1.44	16.23	0.02	0.45	0.01	11.09	1.88	0.39	0.93	6.31	2.55			0.07	0.06	0.01	0.02		1.44
Y102	pulp	Type 2	0.3	2.28	0.01	0.26	0.01	1.8	0.28	0.06	0.11	0.84	0.34			0.01	0.06	0.01	0.01		0.3
Y102	pulp	Type 2	1.08	14.26	0.02	0.48	0.01	7.42	2.24	0.06	0.72	5.23	2.39			0.07	0.07	0.02	0.02		1.08
Y102	pulp	Type 2	0.19	3.1	0.02	0.33	0.01	1.91	0.5	0.04	0.11	1.1	0.5			0.04	0.06	0.02	0.01		0.19
Y102	pulp	Type 2	0.59	12.25	0.01	0.28	0.01	6.33	2.28	0.05	0.58	4.89	1.86			0.05	0.05	0.02	0.02		0.59
Y102	pulp	Type 2	1.41	13.09	0.02	0.5	0.01	10.19	2.14	0.1	0.75	5.12	2.18			0.07	0.06	0.02	0.02		1.41
Y102	pulp	Type 2	1.12	15.37	0.02	0.42	0.01	7.69	2.71	0.06	0.7	5.2	1.73			0.02	0.07	0.04	0.02		1.12
Y102	pulp	Type 2	1.22	12.9	0.02	0.36	0.01	8.96	3.29	0.06	0.83	6.52	2.29			0.07	0.06	0.02	0.02		1.22
R210	pulp	Type 1	0.02	30.38	0.04	2.25	0.01	0.51	4.51	1.82	0.05	0.46	0.48			0.21	0.06	0.05	0.01		0.04
R210	pulp	Type 1	0.01	18.44	0.14	10.08	0.04	0.88	3.95	2.17	0.02	0.62	0.61			0.27	0.07	0.11	0.01		0.01
R210	pulp	Type 1	0.01	18.71	0.14	10.62	0.05	0.66	2.76	1.28	0.01	0.52	0.53			0.17	0.08	0.06	0.01		0.01
R210	pulp	Type 1	0.01	5.89	0.04	2.64	0.01	0.2	0.55	0.07	0.03	0.29	0.2			0.07	0.07	0.03	0.01		0.01
R210	pulp	Type 1	0.01	4.9	0.04	1.66	0.01	0.17	0.35	0.61	0.01	0.13	0.13			0.06	0.06	0.03	0.01		0.01
R210	pulp	Type 1	0.01	23.28	0.09	6.13	0.04	0.61	4.18	0.85	0.03	0.8	0.57			0.31	0.08	0.09	0.01		0.03
R210	pulp	Type 1	0.05	23.32	0.05	3.24	0.01	0.51	4.01	1.1	0.06	0.67	0.4			0.15	0.06	0.05	0.02		0.04
R210	pulp	Type 1	0.01	24.81	0.15	12.67	0.05	0.74	5.33	2.3	0.01	0.64	0.69			0.23	0.08	0.09	0.03		0.03
R210	pulp	Type 1	0.02	23.19	0.13	10.86	0.04	0.73	3.73	1.72	0.04	1.16	0.67			0.22	0.07	0.05	0.01		0.01
R210	pulp	Type 1	0.01	7.77	0.04	2.55	0.01	0.23	1.15	0.33	0.03	0.25	0.21			0.08	0.06	0.03	0.01		0.01
V220	pulp	Type 1	0.04	31.08	0.17	11.39	0.12	1.26	5.17	1.07	0.03	1.37	0.68			0.25	0.08	0.11	0.01		0.01
V220	pulp	Type 1	0.01	2.72	0.03	6.75	0.01	0.07	0.18	0.73	0.03	0.73	0.1			0.03	0.07	0.01	0.01		0.03
V220	pulp	Type 1	0.01	25.67	0.04	2.22	0.03	0.43	2.45	0.52	0.03	0.68	0.31			0.18	0.07	0.03	0.01		0.01
V220	pulp	Type 1	0.01	32.68	0.08	6.13	0.01	0.91	3.28	1.48	0.03	0.95	0.58			0.22	0.07	0.05	0.01		0.01
V220	pulp	Type 1	0.01	26.61	0.04	2.16	0.01	0.43	3.07	1.05	0.04	0.75	0.4			0.16	0.06	0.04	0.01		0.01
V220	pulp	Type 1	0.03	16.8	0.08	5.69	0.03	0.63	2.14	0.69	0.03	0.68	0.38			0.14	0.07	0.03	0.01		0.01
V220	pulp	Type 1	0.04	27.92	0.34	10.96	0.22	1.16	4.25	2	0.01	1.38	0.63			0.23	0.08	0.08	0.01		0.05
V220	pulp	Type 1	0.03	26.97	0.28	16	0.06	1.1	3.9	4.29	0.04	0.64	0.71			0.31	0.1	0.07	0.01		0.01
V220	pulp	Type 1	0.01	11.7	0.04	2.16	0.01	0.28	0.97	0.12	0.03	0.17	0.22			0.09	0.08	0.03	0.01		0.03
V220	pulp	Type 1	0.01	17.21	0.11	6.49	0.03	0.63	3.48	0.54	0.03	0.61	0.4			0.2	0.08	0.04	0.01		0.01
RM1_11	pulp	Type 1	0.02	21.56	0.13	7.24	0.05	0.92	9.33	0.1	0.03	1.14	0.67					0.08	0.03		0.02
RM1_11	pulp	Type 1	0.02	17.16	0.2	10.8	0.04	1.02	8.63	0.11	0.04	1.35	0.81					0.09	0.02		0.02
RM1_11	pulp	Type 1	0.01	7.62	0.11	5.18	0.02	0.43	5.14	0.09	0.01	0.29	0.31					0.05	0.02		0.01
RM1_11	pulp	Type 1	0.02	23.7	0.09	4.67	0.01	1.19	16.6	0.14	0.02	1.4	0.93					0.1	0.05		0.02
RM1_11	pulp	Type 1	0.01	17.08	0.16	8.68	0.06	0.74	5.59	0.12	0.01	1.3	0.72					0.08	0.02		0.01
RM1_11	pulp	Type 1	0.01	3.88	0.09	4.26	0.02	0.32	2.26	0.11	0.01	0.5	0.23					0.03	0.01		0.01
RM1_11	pulp	Type 1	0.01	10.6	0.2	10.82	0.06	0.83	7.34	0.12	0.02	1.14	0.67					0.07	0.02		0.01
RM1_11	pulp	Type 1	0.01	4.81	0.06	2.66	0.01	0.23	2	0.12	0.01	0.34	0.23					0.02	0.01		0.01
RM1_11	pulp	Type 1	0.01	11.63	0.23	10.36	0.03	0.82	7.87	0.21	0.01	0.6	0.59					0.08	0.01		0.01
RM1_11	pulp	Type 1	0.01	13.65	0.46	20.81	0.09	1.12	7.56	0.12	0.04	1.56	0.73					0.11	0.04		0.01

Table 5.7: List of the apple variety, side, red-fleshed types, polyphenolic compounds quantified in the shady section of the 9 red-fleshed cultivars included in the study. Results are expressed as mg/100 g FW.

Variety	Side	Type	Catechin	Chlorogenic acid	Cyanidin-3-arabinoside	Cyanidin-3-galactoside	Cyanidin-3-glucoside	Epicatechin	Phloretin-2-xyloglucoside	Phloridizin	Procyanidin B1	Procyanidin B2	Procyanidin C1	Prunin	Quercetin	Quercetin-3-rhamnoside	Quercetin-3-arabinoside	Quercetin-3-galactoside	Quercetin-3-glucoside	Quercetin-3-xyloside	Rutin
RS1_319_27	shady	Type 1	0.04	2.06	0.13	5.17	0.03	1.16	1.54	0.62	0.06	1.42	0.89	0.01	2.90	0.19	0.04	1.09	0.49	0.41	0.06
RS1_319_27	shady	Type 1	0.16	5.68	0.34	10.51	0.11	2.32	2.00	1.29	0.10	2.90	0.01	0.01	2.70	0.43	0.07	2.97	1.19	1.12	0.27
RS1_319_27	shady	Type 1	0.10	4.91	0.31	5.61	0.05	2.17	1.77	1.29	0.07	2.32	0.02	0.01	2.28	0.39	0.06	2.59	1.05	0.99	0.21
RS1_319_27	shady	Type 1	0.16	3.39	0.16	6.29	0.04	2.07	2.91	1.47	0.07	2.49	1.36	0.01	2.66	0.15	0.01	0.44	0.20	0.31	0.14
RS1_319_27	shady	Type 1	0.12	2.56	0.15	5.54	0.06	2.32	2.17	1.24	0.06	2.76	0.50	0.01	2.36	0.19	0.01	0.83	0.36	0.45	0.15
RS1_319_27	shady	Type 1	0.13	4.56	0.34	12.45	0.09	3.45	2.94	1.20	0.10	4.05	2.50	0.03	2.78	0.41	0.06	2.11	0.94	0.66	0.07
RS1_319_27	shady	Type 1	0.13	3.96	0.24	9.13	0.07	2.21	2.38	0.92	0.05	2.80	0.53	0.01	2.54	0.18	0.01	0.68	0.33	0.39	0.14
RS1_319_27	shady	Type 1	0.06	1.78	0.06	2.20	0.01	1.12	1.41	0.59	0.05	1.27	0.72	0.01	2.48	0.09	0.03	0.39	0.21	0.15	0.03
RS1_319_27	shady	Type 1	0.14	4.84	0.19	11.20	0.05	2.44	3.01	2.59	0.13	3.27	0.63	0.02	3.01	0.44	0.06	2.67	1.16	0.79	0.27
RS1_319_27	shady	Type 1	0.17	5.08	0.15	4.79	0.03	2.88	2.77	1.15	0.12	2.90	1.89	0.01	2.55	0.23	0.03	0.75	0.39	0.29	0.03
108LB	shady	Type 1	0.16	22.32	0.56	23.86	0.13	3.27	9.23	2.60	0.08	4.29	2.46	0.05	2.35	2.83	0.17	1.39	0.21	0.48	0.03
108LB	shady	Type 1	0.14	6.58	0.26	7.36	0.06	1.88	2.36	1.85	0.04	2.58	1.06	0.01	2.43	1.43	0.03	0.99	0.13	0.36	0.01
108LB	shady	Type 1	0.05	3.84	0.28	6.83	0.05	0.76	2.53	1.07	0.02	0.84	0.46	0.02	2.84	3.29	0.03	3.52	0.51	1.00	0.33
108LB	shady	Type 1	0.61	19.36	1.34	31.10	0.26	8.41	13.06	6.45	0.20	9.35	5.84	0.07	2.54	4.93	0.06	2.60	0.35	0.98	0.04
108LB	shady	Type 1	0.17	13.18	0.53	24.58	0.11	3.22	5.59	3.75	0.08	3.94	2.23	0.05	2.73	4.10	0.31	1.80	0.31	0.74	0.05
108LB	shady	Type 1	0.56	21.32	1.18	29.95	0.35	8.49	14.82	8.18	0.20	10.42	5.84	0.11	2.85	10.79	0.08	4.39	0.73	1.59	0.12
108LB	shady	Type 1	0.23	11.42	0.46	19.11	0.10	3.39	7.26	2.82	0.08	4.09	2.46	0.05	2.31	3.79	0.01	1.43	0.26	0.55	0.05
108LB	shady	Type 1	0.31	22.25	0.90	30.57	0.22	6.21	10.70	6.93	0.15	9.01	3.85	0.07	2.39	3.15	0.01	1.24	0.25	0.44	0.03
108LB	shady	Type 1	0.12	5.74	0.47	15.68	0.06	1.88	4.06	2.17	0.03	2.12	1.30	0.02	2.61	1.48	0.03	0.91	0.14	0.23	0.30
108LB	shady	Type 1	0.42	19.23	1.38	38.78	0.19	4.92	11.52	4.89	0.12	5.38	3.18	0.07	2.79	7.76	0.07	4.22	0.61	1.38	0.06
RS1_V135	shady	Type 1	0.17	3.12	0.50	13.34	0.07	3.11	1.74	1.43	0.13	3.53	2.58	0.01	2.80	0.32	0.07	2.73	0.99	1.20	0.28
RS1_V135	shady	Type 1	0.23	4.08	0.38	10.86	0.19	2.58	1.84	1.56	0.13	2.87	0.72	0.01	2.91	0.17	0.04	1.52	0.45	0.97	0.20
RS1_V135	shady	Type 1	0.09	2.19	0.30	6.25	0.05	1.05	0.73	0.48	0.02	1.15	1.18	0.01	2.35	0.12	0.02	3.41	0.33	0.63	0.15
RS1_V135	shady	Type 1	0.28	3.78	0.70	6.61	0.04	2.88	2.07	1.52	0.11	2.91	1.22	0.01	2.44	0.31	0.09	2.33	0.93	1.22	0.15
RS1_V135	shady	Type 1	0.34	4.03	0.58	13.59	0.08	2.65	1.17	1.23	0.08	2.75	0.01	0.01	2.36	0.21	0.05	1.93	0.66	0.99	0.23
RS1_V135	shady	Type 1	0.54	5.24	0.23	12.29	0.07	1.47	1.99	1.80	0.01	1.51	0.77	0.01	2.70	0.42	0.06	1.67	0.81	0.47	0.16
RS1_V135	shady	Type 1	0.26	2.52	0.41	4.71	0.08	3.28	1.60	1.83	0.13	3.63	0.01	0.01	2.46	0.51	0.06	3.91	1.88	1.50	0.72
RS1_V135	shady	Type 1	0.22	2.74	0.38	6.51	0.03	2.75	1.91	2.03	0.12	2.90	1.95	0.01	2.72	0.38	0.10	3.58	1.39	1.50	0.35
RS1_V135	shady	Type 1	0.29	5.66	0.36	11.27	0.11	4.91	4.17	2.88	0.18	5.55	0.02	0.02	3.90	0.77	0.15	9.14	2.25	4.07	0.44
RS1_V135	shady	Type 1	0.35	2.22	0.52	11.36	0.06	2.44	1.26	1.42	0.16	2.42	1.72	0.01	2.15	0.23	0.05	1.99	0.77	0.97	0.10
CR28_1	shady	Type 2	0.06	14.96	0.35	11.33	0.04	1.28	33.04	8.83	0.04	1.59	0.85	0.04	2.58	2.91	0.15	11.72	1.00	3.43	0.24
CR28_1	shady	Type 2	0.07	18.36	0.34	4.85	0.06	2.15	24.60	5.36	0.04	2.84	1.57	0.01	3.05	1.11	0.06	2.95	0.33	1.14	0.18
CR28_1	shady	Type 2	0.06	18.40	0.10	4.65	0.04	1.11	20.19	5.47	0.06	1.57	0.80	0.01	2.96	0.53	0.03	1.38	0.18	0.67	0.06
CR28_1	shady	Type 2	0.05	6.97	0.06	3.95	0.11	0.54	11.57	3.38	0.03	0.15	0.05	0.02	2.31	0.39	0.03	1.54	0.05	0.77	0.06
CR28_1	shady	Type 2	0.03	6.53	0.29	6.05	0.02	0.50	14.03	2.86	0.02	0.74	0.44	0.02	1.94	0.34	0.03	0.81	0.11	0.27	0.02
CR28_1	shady	Type 2	0.03	27.19	0.08	19.56	0.03	1.37	27.46	5.24	0.03	1.93	1.10	0.03	1.94	1.17	0.05	3.33	0.35	0.93	0.07
CR28_1	shady	Type 2	0.03	8.57	0.02	3.58	0.02	0.87	21.86	5.66	0.02	1.34	0.72	0.02	2.07	0.37	0.03	1.11	0.19	0.32	0.03
CR28_1	shady	Type 2	0.02	2.59	0.03	5.12	0.05	0.19	5.93	4.03	0.03	0.30	0.16	0.02	3.11	0.14	0.03	0.34	0.05	0.11	0.02
CR28_1	shady	Type 2	0.02	9.21	0.16	2.86	0.03	0.64	16.80	3.38	0.03	1.20	0.54	0.02	3.29	0.42	0.03	0.95	0.17	0.28	0.02
CR28_1	shady	Type 2	0.03	15.18	0.06	2.39	0.02	1.31	20.59	4.80	0.03	1.99	1.06	0.02	3.06	0.69	0.03	1.45	0.19	0.59	0.17
RM1_17	shady	Type 1	0.04	9.15	0.48	13.67	0.11	1.91	28.33	5.83	0.02	2.07	0.89	0.02	3.58	0.71	0.04	1.41	0.84	0.77	0.32
RM1_17	shady	Type 1	0.02	2.25	0.07	2.28	0.03	0.66	17.36	3.20	0.02	0.85	0.40	0.02	1.90	0.40	0.03	0.83	0.67	0.38	0.12
RM1_17	shady	Type 1	0.05	14.25	0.30	11.40	0.04	3.49	22.67	23.40	0.07	3.76	2.09	0.02	2.50	1.91	0.12	4.50	3.08	1.83	0.69
RM1_17	shady	Type 1	0.05	19.63	0.10	2.64	0.03	2.31	31.45	15.86	0.03	2.64	0.02	0.02	3.77	1.55	0.09	4.45	3.01	2.05	1.09
RM1_17	shady	Type 1	0.03	8.03	0.19	7.23	0.18	1.12	29.10	9.25	0.03	0.95	0.80	0.02	3.21	1.59	0.03	4.02	0.80	0.80	0.64
RM1_17	shady	Type 1	0.02	13.76	0.16	9.42	0.05	1.34	33.38	4.66	0.04	1.69	0.83	0.02	3.35	0.51	0.04	0.86	0.58	0.32	0.11
RM1_17	shady	Type 1	0.02	5.40	0.31	6.88	0.05	1.17	29.14	6.04	0.02	1.03	0.53	0.02	2.42	2.91	0.15	7.62	6.72	2.87	4.17
RM1_17	shady	Type 1	0.04	13.47	0.32	7.88	0.09	1.69	32.97	11.00	0.04	1.38	0.39	0.02	3.34	1.84	0.06	4.68	3.61	1.08	1.04
RM1_17	shady	Type 1	0.04	12.46	0.18	8.94	0.04	2.69	40.97	13.81	0.05	2.90	1.71	0.02	3.06	1.98	0.09	3.44	2.34	1.57	0.32
RM1_17	shady	Type 1	0.04	12.23	0.23	10.01	0.07	2.45	24.47	12.19	0.07	2.59	1.55	0.02	3.34	1.50	0.07	3.01	1.81	1.39	0.32
Y102	shady	Type 2	1.55	10.12	0.07	2.02	0.02	21.60	1.94	3.31	0.35	12.51	3.75	0.04	6.53	11.37	0.67	8.68	4.15	1.04	1.88
Y102	shady	Type 2	1.50	4.78	0.04	1.08	0.01	8.94	1.24	1.76	0.16	4.62	1.42	0.01	1.80	2.91	0.13	1.73	1.89	0.24	0.69
Y102	shady	Type 2	0.69	5.41	0.03	0.70	0.03	6.94	1.00	1.38	0.14	2.20	1.24	0.01	1.74	1.61	0.12	0.69	0.89	0.12	0.26
Y102	shady	Type 2	2.40	7.51	0.06	1.86	0.02	16.06	1.10	2.23	0.23	8.58	3.12	0.02	3.74	6.81	0.31	3.64	3.98	0.49	2.07
Y102	shady	Type 2	1.38	5.80	0.04	0.95	0.01	11.77	1.74	2.47	0.55	8.22	3.55	0.01	1.63	2.86	0.13	0.81	0.98	0.13	0.24
Y102	shady	Type 2	1.36	7.25	0.06	3.02	0.03	18.79	2.13	2.89	0.71	14.59	6.66	0.02	1.84	2.24	0.14	1.22	1.33	0.14	0.30
Y102	shady	Type 2	0.96	2.68	0.03	0.63	0.01	9.73	1.12	1.50	0.41	6.12	2.80	0.01	1.58	2.29	0.12	0.80	0.91	0.14	0.24
Y102	shady	Type 2	1.45	6.63	0.08	1.86	0.02	16.60	1.69	3.13	0.56	10.91	4.53	0.02	2.16	4.46	0.15	1.56	1.83	0.24	0.63
Y102	shady	Type 2	2.13	7.45	0.07	2.29	0.03	13.34	1.46	1.78	0.48	7.44	2.88	0.01	1.45	1.21	0.10	0.56	0.67	0.10	0.22
Y102	shady	Type 2	1.97	5.42	0.07	1.87	0.03	16.29	1.65	1.31	0.39	11.26	2.93	0.01	1.51	1.74	0.10	1.05	1.16	0.14	0.29
R210	shady	Type 1	0.05	7.06	0.15	3.54	0.04	1.76	7.20	7.57	0.07	2.30	1.36	0.01	2.27	2.28	0.28	0.66	0.12	0.67	0.01
R210	shady	Type 1	0.07	7.90	0.27	8.90	0.07	1.47	5.06	2.47	0.01	1.40	0.69	0.01	2.29	1.09	0.16	0.41	0.08	0.19	0.01
R210	shady	Type 1	0.02	4.72	0.35	17.63	0.05	1.33	1.85	2.79	0.02	1.30	0.65	0.02	2.70	1.32	0.16	0.57	0.08	0.40	0.02
R210	shady	Type 1	0.02	1.77	0.18	15.05	0.05	0.96	1.61	2.68	0.02	0.95	0.52	0.02	2.54	1.62	0.02	0.67	0.09	0.46	0.02
R210	shady	Type 1	0.04	3.63	0.22	7.16	0.04	1.55	2.62	3.09	0.03	1.47	0.71	0.01	2.48	1.26	0.07	0.49	0.06	0.45	0.01
R210	shady	Type 1	0.02	4.18	0.15	9.11	0.05	0.90	2.49	3.42	0.02	0.93	0.46	0.02	2.53	1.57	0.18	0.71	0.08	0.49	0.02
R210	shady	Type 1	0.04	3.83	0.18	5.02	0.06	1.57	3.98	5.29	0.04	1.58	0.79	0.01	2.32	1.89	0.21	0.68	0.10	0.54	0.01
R210	shady	Type 1	0.04	5.48	0.30	7.73	0.09	1.79	6.05	5.60	0.04	1.66	0.70	0.01	2.47	1.51	0.18	0.67	0.09	0.49	0.01
R210	shady	Type 1	0.03	4.85	0.19	11.39	0.06	0.51	3.47	1.15	0.02	0.67	0.37	0.02	2.61	0.44	0.02	0.30	0.03	0.16	0.02
R210	shady	Type 1	0.08	8.71	0.30	9.64	0.04	2.28	5.25	6.36	0.07	2.60	1.44	0.01	2.41	2.72	0.01	0.86	0.11	0.82	0.03
V220	shady	Type 1	0.05	8.81	0.56	19.11	0.10	2.18	6.42	4.06	0.03	2.12	1.34	0.03	2.73	3.87	0.05	1.71	0.22	1.13	0.06
V220	shady	Type 1	0.08	6.88	1.10	28.09	0.08	3.17	9.09	8.93	0.03	2.78	1.45	0.03	3.17	7.27	1.08	4.44	0.42	2.72	0.17
V220	shady	Type 1	0.14	6.56	0.55	10.61	0.06	4.09	11.23	7.10	0.08	5.00	2.78	0.02	2.78	4.32	0.26	1.31	0.23	1.34	0.05
V220	shady	Type 1	0.09	10.63	0.37	11.25	0.06	4.18	7.13	6.94	0.07	4.86	2.27	0.01	2.64	4.36	0.07	1.47	0.18	1.25	0.06
V220	shady	Type 1	0.03	5.98	0.04	8.16	0.03	0.67	4.55	1.89	0.01	0.66	0.48	0.01	2.59	0.73	0.03	0.28	0.04	0.18	0.03
V220	shady	Type 1	0.01	1.68	0.03	5.50	0.01	0.47	1.68	0.79	0.01	0.62	0.28	0.01	1.58	0.82	0.01	0.36	0.06	0.25	0.01
V220	shady	Type 1	0.07	8.53	0.30	11.84	0.04	2.32	7.03	3.20	0.04	3.16	1.21	0.01	2.47	2.03	0.03	0.61	0.12	0.53	0.01
V220	shady	Type 1	0.06	3.89	0.40	10.99	0.06	1.89	5.82	3.86	0.02	1.89	0.99	0.02	2.80	5.47	0.62	2.31	0.33	1.44	0.09
V220	shady	Type 1	0.11	8.80	0.10	3.64	0.04	3.01	13.29	6.54	0.08	4.56	2.25	0.01	2.37	2.93	0.01	0.70	0.14	0.73	0.03
V220	shady	Type 1	0.01	4.02	0.06	2.07	0.03	1.44	6.06	3.34	0.03	1.75	1.16	0.01	2.48	1.84	0.19	0.57	0.09	0.48	0.01
RM1_11	shady	Type 1	0.07	12.66	0.08	4.22	0.06	2.61	25.49	11.87	0.10	3.68	1.52	0.01	2.46	0.74	0.03	1.19	0.98	0.62	0.10
RM1_11	shady	Type 1	0.08	11.75	0.11	6.65	0.06	4.21	32.89	12.00	0.13	7.53	2.46	0.02	2.71	1.13	0.03	1.15	1.09	0.55	0.05
RM1_11	shady	Type 1	0.06	6.30	0.12	5.18	0.06	1.47	24.55	6.93	0.04	2.16	0.85	0.01	2.59	0.57	0.03	1.57	1.38	0.76	0.25
RM1_11	shady	Type 1	0.01	3.19	0.19	3.82	0.04	0.91	24.13	4.13	0.03	0.82	0.43	0.01	2.58	0.70	0.03	1.42	0.94	0.63	0.16
RM1_11	shady	Type 1	0.06	9.13	0.27	9.07	0.10	2.98	38.54	10.66	0.06	3.79	1.59	0.02	2.80	1.00	0.06	1.43	1.16	0.73	0.13
RM1_11	shady	Type 1	0.05	4.49	0.14	6.90	0.06	1.95	19.09	4.92	0.05	2.46	1.09	0.02	2.81	0.48	0.03	0.84	0.66	0.21	0.06
RM1_11	shady	Type 1	0.09	7.88	0.17	8.62	0.08	2.92	40.26	11.27	0.11	3.75	1.73	0.02	2.68	0.67	0.03	1.37	1.09	0.59	0.11
RM1_11	shady	Type 1	0.06	17.14	0.21	9.69	0.10	3.35	41.32	5.46	0.06	4.33	1.93	0.02	2.78	0.63	0.03	1.24	0.80	0.48	0.08
RM1_11	shady	Type 1	0.05	3.83	0.31	10.55	0.11	1.78	28.15	7.50	0.03	2.09	0.81	0.02	2.70	0.72	0.03	1.72	1.19	0.70	0.17
RM1_11	shady	Type 1	0.27	8.98	0.32	13.95	0.08	4.25	52.11	11.42	0.02	4.35	1.18	0.04	3.66	0.80	0.08	2.17	1.54	1.05	0.06

Table 5.8: List of the apple variety, side, red-fleshed types, polyphenolic compounds quantified in the sunny section of the 9 red-fleshed cultivars included in the study. Results are expressed as mg/100 g FW.

Variety	Side	Type	Catechin	Chlorogenic acid	Cyanidin-3-arabinoside	Cyanidin-3-galactoside	Cyanidin-3-glucoside	Epicatechin	Phloretin-2-xyloglucoside	Phloridizin	Procyanidin B1	Procyanidin B2	Procyanidin C1	Prunin	Quercetin	Quercetin-3-rhamnoside	Quercetin-3-arabinoside	Quercetin-3-galactoside	Quercetin-3-glucoside	Quercetin-3-xyloside	Rutin
RS1_319_27	sunny	Type 1	0.18	5.65	0.38	14.91	0.1	3.7	4.03	1.84	0.21	4.58	2.84	0.03		0.49	0.07	2.59	1.08	0.62	0.11
RS1_319_27	sunny	Type 1	0.16	5.64	0.28	14.11	0.07	1.41	2.82	1.41	0.14	2.82	1.41	0.01		0.71	0.07	2.82	1.41	0.99	0.08
RS1_319_27	sunny	Type 1	0.15	4.51	0.3	13.54	0.08	1.5	3.01	1.5	0.15	3.01	1.5	0.02		0.75	0.08	3.01	1.5	1.05	0.09
RS1_319_27	sunny	Type 1	0.08	1.55	0.27	7.9	0.09	1.41	1.54	0.92	0.16	1.63	1.13	0.02		0.27	0.05	1.35	0.63	0.34	0.09
RS1_319_27	sunny	Type 1	0.2	3.3	0.6	17.75	0.09	3.05	3.13	1.95	0.25	3.8	2.27	0.02		0.51	0.06	2.76	1.15	0.64	0.15
RS1_319_27	sunny	Type 1	0.19	6.79	0.65	19.62	0.07	3.36	3.53	2.64	0.22	4.39	2.51	0.07		2.7	0.38	5.72	9.5	4.27	1.23
RS1_319_27	sunny	Type 1	0.17	5.2	0.45	14.5	0.06	2.81	3	1.78	0.22	3.28	2	0.03		0.8	0.14	6.91	2.56	1.37	0.3
RS1_319_27	sunny	Type 1	0.16	4.83	0.32	16.09	0.1	1.61	3.22	1.61	0.16	3.22	1.61	0.02		0.8	0.08	3.22	1.61	1.13	0.08
RS1_319_27	sunny	Type 1	0.15	2.7	0.37	10.48	0.06	2.14	2.09	1.16	0.18	2.49	1.56	0.01		0.28	0.04	1.45	0.52	0.34	0.07
RS1_319_27	sunny	Type 1	0.23	9.02	0.51	11.32	0.03	3.38	3.23	2.07	0.22	4.65	2.53	0.03		1.19	0.15	7.18	3.41	1.61	0.46
108LB	sunny	Type 1	0.8	14.16	2.95	33.76	0.65	7.46	13.76	9.76	0.08	6.23	3.46	0.12	4.27	22.57	0.47	2.3	2.72	6.82	0.79
108LB	sunny	Type 1	0.34	13.35	1.79	31.88	0.34	7.52	11.81	9.55	0.03	3.97	2.05	0.1	3.25	13.86	0.3	0.98	1.23	4.66	0.5
108LB	sunny	Type 1	0.19	4.01	1.98	26.46	0.4	2.73	10.29	5.29	0.07	1.8	1.01	0.04	1.51	3.56	0.11	0.09	0.17	3.2	0.13
108LB	sunny	Type 1	0.98	13.96	2.31	45.63	0.61	8.97	15.05	11.98	0.18	6.97	4.07	0.16	4.17	28.22	0.43	2.06	2.72	7.07	0.77
108LB	sunny	Type 1	0.77	16.33	2.07	38.08	0.55	10.02	14.9	9.57	0.04	10.18	5.14	0.1	1.87	8.77	0.13	0.32	0.41	3.97	0.17
108LB	sunny	Type 1	0.32	7.96	2.68	31.43	0.27	6	12.44	8.63	0.08	2.15	1.32	0.1	3.57	16.16	0.34	1.14	1.56	5.1	0.42
108LB	sunny	Type 1	0.72	11.21	3.43	46.74	0.66	6.59	15.32	9.55	0.08	5.5	3.26	0.13	4.45	24.82	0.55	2.56	3.04	8.15	1.27
108LB	sunny	Type 1	0.28	15.74	2.26	43.21	0.34	5.15	11.62	9.75	0.06	5.72	2.65	0.13	3.34	20.49	0.52	2.7	3.05	5.64	2.41
108LB	sunny	Type 1	0.8	11.2	2.31	44.71	0.56	6.73	9.97	7.65	0.06	5.59	3.02	0.08	3.44	16.68	0.31	1.3	1.56	4.69	0.46
108LB	sunny	Type 1	0.36	14.76	2.83	38.48	0.43	6.18	16.23	9.01	0.1	6.02	3.1	0.12	3.22	20.52	0.31	1.56	1.91	4.42	0.71
RS1_V135	sunny	Type 1	0.51	1.18	1.19	7.76	0.08	1.48	3.28	2.85	0.15	0.94	0.9	0.02	6.45	2.86	0.4	6.31	9.7	10.47	2.58
RS1_V135	sunny	Type 1	0.62	7.15	1.33	31.06	0.12	6.51	4.25	4.58	0.09	6.47	2.63	0.03	2.82	1.01	0.38	3.35	3.71	5.9	0.82
RS1_V135	sunny	Type 1	0.18	5.81	1.58	22.96	0.15	3.09	2.97	2.23	0.07	3.29	2.04	0.05	4.4	3.14	0.67	9.92	10.68	10.43	3.56
RS1_V135	sunny	Type 1	0.31	8.38	3.92	51.86	0.21	4.64	4.12	4.05	0.1	4.34	2.93	0.07	7.3	5.63	1.33	7.58	25.1	20.17	12.2
RS1_V135	sunny	Type 1	0.45	6.4	1.69	27.89	0.09	3.6	2.73	2.72	0.14	3.48	2.1	0.03	3.39	2.04	0.73	6.89	7.5	9.43	1.45
RS1_V135	sunny	Type 1	0.43	4.37	1.45	27.57	0.21	3.72	2.51	2.77	0.14	3.52	2.06	0.04	4.05	2.81	0.66	10.29	11.19	11.94	3.03
RS1_V135	sunny	Type 1	0.31	3.34	2.09	11.8	0.18	2.62	4.52	5.27	0.16	2.04	1.41	0.03	6.08	3.04	0.8	12.43	14.81	13.23	6.29
RS1_V135	sunny	Type 1	0.12	4.87	1.58	23.71	0.21	2.06	2.63	2.13	0.05	2.08	1.26	0.04	5.56	3.89	0.98	15.47	16.34	13.5	8.1
RS1_V135	sunny	Type 1	0.44	4.54	1.47	32.3	0.2	6.68	5	3.62	0.2	6.79	4.13	0.03	3.2	0.92	0.35	2.64	3.04	6.28	0.67
RS1_V135	sunny	Type 1	0.44	4.11	1.42	29.58	0.16	3.71	2.56	3.36	0.18	3.77	2.53	0.03	4.23	1.94	0.71	7.9	8.46	10.03	1.64
CR28_1	sunny	Type 2	0.08	29.74	0.77	23.23	0.17	1.69	35.2	35.2	0.17	2.2	1.19	0.08	1.28	2.2	0.11	9.77	1.13	1.77	0.34
CR28_1	sunny	Type 2	0.05	19.51	2.61	37.81	0.54	1.52	32.07	32.07	0.12	1.39	0.99	0.14	1.62	3.63	0.28	21.62	3.29	3.09	3.63
CR28_1	sunny	Type 2	0.05	18.74	1.02	26.25	0.09	1.16	25.55	25.55	0.14	1.45	0.81	0.04	1.02	1.24	0.09	10.41	0.46	0.96	0.14
CR28_1	sunny	Type 2	0.09	23.6	0.67	19.88	0.11	1.72	38.11	38.11	0.14	2.36	1.2	0.09	1.33	3.44	0.19	13.11	1.28	2.41	0.34
CR28_1	sunny	Type 2	0.07	18.81	1.57	21.96	0.2	1.22	23.95	23.95	0.12	1.14	0.59	0.1	1.61	4.06	0.33	26.69	4.25	3.8	3.85
CR28_1	sunny	Type 2	0.14	26.58	1.3	37.82	0.33	2.2	38.91	38.91	0.16	2.27	1.44	0.16	2.88	6.41	0.41	40.87	4.72	6.57	1.98
CR28_1	sunny	Type 2	0.07	2.88	0.26	6.46	0.03	0.25	4.79	4.79	0.13	0.16	0.07	0.03	1.65	0.26	0.05	9.36	0.13	0.21	0.05
CR28_1	sunny	Type 2	0.1	13.98	1.52	21.11	0.31	1.21	21.58	21.58	0.14	0.97	0.47	0.18	2.18	4.97	0.47	44.51	8.44	5.85	6.02
CR28_1	sunny	Type 2	0.07	17.89	0.64	17.56	0.25	1.61	26.83	26.83	0.14	1.79	0.36	0.09	1.79	2.68	0.36	26.83	5.37	3.58	1.79
CR28_1	sunny	Type 2	0.1	15.23	1.1	17.29	0.14	1.79	27.2	27.2	0.14	2.13	1.18	0.05	1.33	2.54	0.19	10.72	0.98	1.98	0.21
RM1_17	sunny	Type 1	0.02	8.46	0.14	3.38	0.03	1.52	16.92	5.08	0.1	1.69	0.51	0.02	0.85	1.69	0.08	2.88	1.69	0.76	1.35
RM1_17	sunny	Type 1	0.03	8.53	0.2	4.83	0.03	1	24.2	5.08	0.1	1.27	0.53	0.02	0.82	1.4	0.08	3.04	2.68	0.84	1.22
RM1_17	sunny	Type 1	0.02	7.38	0.2	3.38	0.05	0.78	17.89	3.16	0.1	0.9	0.46	0.02	0.81	0.73	0.07	2.15	1.52	0.61	0.42
RM1_17	sunny	Type 1	0.04	4.49	0.14	2.59	0.04	0.92	35.74	9.65	0.14	0.96	0.47	0.02	1.37	3.1	0.27	10.75	8.44	2.39	5.43
RM1_17	sunny	Type 1	0.02	3.78	0.08	2.47	0.03	0.87	15.83	6.05	0.11	0.98	0.44	0.02	0.98	0.78	0.05	1.65	1.16	0.52	0.29
RM1_17	sunny	Type 1	0.02	7.01	0.18	2.63	0.05	1.58	17.53	5.26	0.11	1.75	0.53	0.02	0.88	1.75	0.09	2.98	1.75	0.79	1.4
RM1_17	sunny	Type 1	0.04	9.96	0.33	9.92	0.11	1.33	37.11	6.17	0.11	1.73	0.75	0.02	1.1	2.34	0.17	6.77	6.3	1.71	2.85
RM1_17	sunny	Type 1	0.02	6.5	0.22	6.5	0.11	1.95	32.5	6.5	0.13	2.17	0.65	0.02	1.08	2.17	0.11	3.68	2.17	0.97	1.73
RM1_17	sunny	Type 1	0.04	18.73	0.36	13.62	0.12	1.9	37.73	7.64	0.14	2.26	1.2	0.02	1.12	1.56	0.08	3.3	1.92	0.94	0.56
RM1_17	sunny	Type 1	0.06	8.94	0.49	14.05	0.15	1.55	46.72	11.71	0.15	1.53	0.76	0.02	1.72	3.97	0.19	13.15	10.98	2.89	6.31
Y102	sunny	Type 2	0.66	9.49	0.01	0.27	0.01	7.71	0.87	1.09	0.27	6.6	3.24	0.03	3.12	3.52	0.07	5.76	2.08	2.51	1.19
Y102	sunny	Type 2	1.42	8.33	0.05	1.53	0.02	14.81	1.23	1.8	0.33	5.46	2.73	0.02	4.59	7.19	0.46	21.01	7.51	6.84	4.45
Y102	sunny	Type 2	0.78	8.25	0.03	0.8	0.02	8.24	0.75	0.49	0.42	5.44	2.5	0.02	3.57	5.56	0.24	13.76	3.56	4.35	1.79
Y102	sunny	Type 2	1.82	5.78	0.09	2.41	0.03	14.57	0.83	1.52	0.75	8.19	4.18	0.03	3.74	4.73	0.41	18.38	4.83	5.72	2.51
Y102	sunny	Type 2	0.7	7.48	0.05	1.35	0.02	10.51	1.18	1.82	0.17	5.35	3.16	0.03	4.23	6.23	0.32	20.47	9.34	6.69	6.13
Y102	sunny	Type 2	1.32	11.87	0.05	1.19	0.02	10.81	1.81	2.6	0.49	7.72	3.57	0.05	5.42	9.66	4.58	21.33	10.18	10.79	6.19
Y102	sunny	Type 2	0.3	6.36	0.02	0.24	0.02	4.59	0.62	0.46	0.21	3.64	1.46	0.02	3.2	3.83	0.24	12.03	5.29	3.66	3.59
Y102	sunny	Type 2	0.9	2.3	0.05	1.17	0.02	7.72	0.71	1.46	0.4	5.2	2.04	0.03	3.76	5.11	0.43	19.59	9.67	5.8	6.31
Y102	sunny	Type 2	0.67	2.06	0.07	1.16	0.02	6.65	0.89	1.79	0.18	4.05	1.61	0.05	4.84	5.88	0.84	29.7	21.89	9.48	12.58
Y102	sunny	Type 2	0.34	2.99	0.02	0.34	0.02	8.13	0.71	1.12	0.14	4.6	2.11	0.02	3.36	3.79	0.12	8.41	3.19	3.23	1.63
R210	sunny	Type 1	0.08	3.48	0.51	15.46	0.05	2.94	15.25	16.02	0.08	2.64	1.71	0.02	1.98	4.36	0.05	1.67	0.25	1.34	0.06
R210	sunny	Type 1	0.06	11.13	0.8	33.93	0.09	2.34	9.62	6.72	0.04	1.95	1.4	0.01	1.82	2.99	0.04	1.18	0.16	0.87	0.04
R210	sunny	Type 1	0.11	6.44	1.93	43.36	0.3	3.86	3.38	9.14	0.05	2.82	1.66	0.03	2.74	18.9	0.16	11.5	0.98	5.6	0.52
R210	sunny	Type 1	0.06	12.65	1.29	36.09	0.12	2.16	4.67	6.95	0.05	1.75	1.07	0.03	4.19	6.08	0.11	3.61	0.45	1.86	0.11
R210	sunny	Type 1	0.14	12.17	4.6	47.37	0.99	3.75	9.75	13.01	0.03	2.61	1.56	0.05	4.51	19.68	0.09	14.73	1.57	7.33	0.78
R210	sunny	Type 1	0.14	13.88	1.86	41.91	0.31	3.19	9.35	8.73	0.02	2.3	1.38	0.03	2.56	11.15	0.09	10.9	0.9	5.01	0.56
R210	sunny	Type 1	0.06	4.75	0.75	21.62	0.04	2.48	9.63	9.17	0.06	2.34	1.42	0.01	1.93	7.23	0.06	2.43	0.34	1.85	0.09
R210	sunny	Type 1	0.09	15.02	1	27.17	0.14	2.77	5.62	8.85	0.05	2.13	1.34	0.02	2.32	9.02	0.07	4.11	0.43	2.61	0.14
R210	sunny	Type 1	0.02	13.53	0.3	9.99	0.03	0.7	2.28	2.34	0.02	0.67	0.34	0.02	1.84	2.68	0.05	1.4	0.16	0.83	0.05
R210	sunny	Type 1	0.02	4.5	0.69	18.75	0.05	0.84	4.53	3.17	0.02	0.6	0.35	0.02	1.8	1.58	0.05	1.2	0.13	0.77	0.05
V220	sunny	Type 1	0.08	5.83	0.8	18.37	0.1	2.33	6.41	4.36	0.11	2.39	1.42	0.02	1.02	5.13	0.09	2.83	0.29	0.81	0.1
V220	sunny	Type 1	0.11	7.67	1.34	19.16	0.1	3.83	7.67	7.67	0.19	3.83	1.92	0.02	0.96	7.67	0.1	4.79	0.57	0.96	0.1
V220	sunny	Type 1	0.08	4.25	0.98	14.49	0.07	2.83	5.67	5.67	0.14	2.83	1.42	0.01	0.71	5.67	0.07	3.54	0.42	0.71	0.07
V220	sunny	Type 1	0.1	7.59	1.09	20.34	0.1	3.59	8.49	7.81	0.16	3.69	2.23	0.02	1.17	7.8	0.1	3.39	0.39	1.04	0.11
V220	sunny	Type 1	0.06	5.38	0.91	10.23	0.06	1.77	6.98	5.15	0.1	1.91	1.09	0.01	0.71	4	0.04	1.49	0.21	0.49	0.06
V220	sunny	Type 1	0.1	9.55	0.77	23.88	0.09	2.54	6.87	7.81	0.14	2.34	1.26	0.02	1.23	8.02	0.09	3.92	0.46	1.11	0.17
V220	sunny	Type 1	0.09	4.47	1.41	37.13	0.16	3.03	6.19	8.28	0.14	2.95	1.41	0.04	1.98	16.25	0.16	10.2	0.94	2.46	0.44
V220	sunny	Type 1	0.08	4.93	1.12	14.78	0.1	2.46	7.39	6.57	0.15	2.46	1.64	0.02	0.82	5.75	0.08	4.93	0.49	0.82	0.08
V220	sunny	Type 1	0.11	7.19	1.24	17.97	0.09	2.7	6.29	7.19	0.18	2.7	1.8	0.02	0.9	6.29	0.09	5.39	0.54	0.9	0.09
V220	sunny	Type 1	0.08	4.95	1.16	16.52	0.15	2.48	6.61	5.78	0.17	3.3	1.65	0.02	0.83	6.61	0.08	4.13	0.5	0.83	0.08
RM1_11	sunny	Type 1	0.06	3.82	0.46	10.47	0.11	1.35	29.29	7.48	0.13	1.24	0.65	0.02	1.35	2	0.2	9.15	6.26	1.63	6.28
RM1_11	sunny	Type 1	0.06	3.21	0.45	10.33	0.06	1.95	37.49	15.5	0.14	2.24	1.1	0.02	0.91	1.22	0.05	2.41	1.91	0.77	0.31
RM1_11	sunny	Type 1	0.12	3.73	1.67	36.78	0.23	2.32	24.68	8.74	0.13	2.33	1.15	0.02	1.43	2.1	0.17	9.62	5.35	2.13	4.95
RM1_11	sunny	Type 1	0.09	3.5	1.75	34.99	0.16	1.75	26.24	15.75	0.14	2.62	0.87	0.02	1.05	0.87	0.09	5.25	5.25	1.75	1.75
RM1_11	sunny	Type 1	0.08	3.23	1.7	33.95	0.15	1.7	25.47	15.28	0.14	2.55	0.85	0.02	1.02	0.85	0.08	5.09	5.09	1.7	1.7
RM1_11	sunny	Type 1	0.09	4.81	1.18	27.23	0.24	2.81	32.31	11.07	0.14	2.84	1.46	0.02	1.03	1.6	0.09	5.14	3.82	1.38	0.88
RM1_11	sunny	Type 1	0.1	5.44	0.46	12.27	0.14	1.67	46.42	9.24	0.14	1.61	0.82	0.02	1.37	1.79	0.2	10.92	4.76	1.97	3.94
RM1_11	sunny	Type 1	0.03	3.92	0.37	10.06	0.07	1.1	21.18	6.93	0.1	1.15	0.5	0.01	0.81	0.64	0.05	2.13	1.3	0.6	0.37
RM1_11	sunny	Type 1	0.31	4.55	1.92	36.72	0.29	4.19	47.92	28.02	0.14	3.39	1.63	0.03	3.3	2.63	0.47	3.2	5.86	6.83	15.69
RM1_11	sunny	Type 1	0.12	3.53	0.73	23.47	0.15	2.41	38.59	11.28	0.14	2.39	1.06	0.02	1.37	1.1	0.14	5.36	1.99	1.2	0.71

Table 5.9: Compounds analyzed with UHPLC-QqQ-MS/MS in this study, the corresponding retention time (RT), ESI (Electron Spray Ionization) polarity, molecular weight (MW), precursors, products, collision energy, RF lens, regression parameters and linearity range are reported.

Compound Name	RT [min]	ESI Polarity	MW [Da]	Precursor [m/z]	Product [m/z]	Collision Energy [V]	RF Lens [V]	Regression (r2)	off set	slope	Instrumental linear range [µg/mL]	
											min	max
Neochlorogenic acid	1.01	+	354.31	355	163	10.3	46	0.9990	4.11E-04	5.11E-01	0.01	27.76
Procyanidin B1	1.37	+	578.52	579	291	10.3	89	0.9974	2.94E-02	5.23E-01	0.06	12.72
Catechin	1.58	+	290.27	291	123	15.1	60	0.9941	1.26E-02	5.04E+00	0.01	25
Chlorogenic acid	2.31	+	354.31	355	163	12.0	57	0.9969	5.53E-04	1.63E+00	0.003	26.83
Cryptochlorogenic acid	2.50	+	354.31	355	163	12.0	57	0.9945	7.09E-04	7.37E-01	0.01	24.5
Procyanidin B2	2.68	+	578.52	579	299	26.0	88	0.9950	2.66E-04	6.16E-02	0.02	26.22
Epicatechin	3.21	+	290.27	291	123	15.9	60	0.9955	1.88E-03	5.75E+00	0.01	25.625
Cyanidin-3-galactoside	3.71	+	484.84	449	287	22.0	75	0.9996	1.07E-01	5.36E+00	0.003	26.875
Procyanidin C1	4.17	+	866.77	867	407	27.8	102	0.9945	2.10E-03	3.78E-01	0.01	12.29
Cyanidin-3-glucoside	4.22	+	449.38	449	287	22.0	75	0.9989	1.75E-02	5.30E+00	0.005	12.65
Cyanidin-3-arabinoside	4.49	+	454.81	419	287	21.0	71	0.9975	1.44E-02	5.91E+00	0.006	27.75
Quercetin-3-galactoside	6.34	+	464.38	465	303	13.0	61	0.9958	1.85E-02	3.58E+00	0.01	26.34
Quercetin-3-glucoside	6.70	+	464.38	465	303	13.0	61	0.9965	1.75E-02	4.01E+00	0.01	26.61
Rutin	6.76	+	610.52	611	303	20.5	70	0.9984	1.65E-02	8.37E-01	0.03	23
Quercetin-3-xyloside	7.02	+	434.35	435	303	11.5	59	0.9979	4.04E-02	4.09E+00	0.02	25.36
Quercetin–3-arabinoside	7.32	+	434.35	435	303	11.5	59	0.9979	4.33E-02	8.17E+00	0.01	12.68
Prunin	7.63	+	434.39	435	273	13.7	61	0.0050	3.94E-03	1.81E+00	0.03	25.73
Quercetin-3-rhamnoside	7.97	+	448.38	449	303	10.3	53	0.9979	4.73E-03	1.39E+00	0.01	26.91
Phloretin-2-xyloglucoside*	8.02	+	568.52	569	107	30.0	60	0.9941	2.07E-03	6.48E-02	0.023	22.99
Phloridzin	8.63	+	436.41	437	275	10.3	60	0.9941	2.07E-03	4.07E-01	0.023	22.99
Quercetin	10.00	+	302.24	303	153	32.0	93	0.9783	1.57E-01	4.07E-01	0.41	29.39
Phloretin	12.59	+	274.26	275	107	18.5	55	0.9948	5.34E-02	1.08E+01	0.01	25.59

* Phloretin-2´-xyloglucoside: Amount is estimated by using the calibration curve of Phloridzin.

CHAPTER 6

CONCLUDING REMARKS

The high number of deaths related to chronic negative conditions, cardiovascular disorders, and non-communicable diseases is still increasing, thus stimulating the scientific community in looking for lifestyle changes and new dietary prevention strategies. One such plan is to increment the intake of plant-based foods rich in bioactive compounds, such as antioxidants, polyphenols, and anthocyanins. The consumers are interested in such quality and nutrition factors that include also physical characteristics (e.g. appearance, size, uniformity, maturity, firmness, skin, and flesh color) and sensory attributes (e.g. taste, aroma, texture, and crunchiness).

It has been shown that fruits and vegetables are rich in various healthy and functional compounds such as flavonoids, fibers, and vitamins. Among these phytochemicals, the phenolic profile, antioxidants proprieties, and the pattern of anthocyanins are the most frequently investigated. Fruits and vegetables have already demonstrated a wide range of functional proprieties including antioxidant, anticancer, and anti-diabetic and they are able to prevent cardiovascular diseases and age-related chronic issues. Cutting-edge methodologies have been developed in order to better characterize the metaboloma of

fruits and vegetables and the evaluation of their benefits on human health is continuously investigated. It has been reported that pulps and peels of fruits and leaves, and flowers of different plant species are the major source of phytochemicals, and the main compounds are flavonoids.

Owing to the effect of the selection of foods has been associated with the process of domestication in order to improve their attractiveness, there are significant differences in the concentration of phytochemicals that determine the commercialization of new varieties with lower nutritional values [15]. Therefore, the characterization of phytochemicals in fruits and vegetables is a crucial point in order to gain insights for future investigations. In this scenario, the scientific community is still focusing on different varieties of vegetables and fruits all over the world in order to improve their appearance, texture, and taste without losing the nutritional value through traditional breeding strategies.

The researchers are focused into the development of fruits and vegetables rich in anthocyanins and polyphenols using breeding processes in order to bring on the market new varieties with health-promoting proprieties. Indeed, the colored fruits are strongly attractive, and they represent a radical innovation in building healthy eating habit. The consumption of colored fruits is currently increasing, and the consumer are demonstrating high interest, despite of the people is hardly familiar with colored fruits and vegetables. Therefore, the studies are concentrated towards the phytochemical compositions and the health-promoting proprieties of the foods. As reported by Zeng et al. [85], it is important to investigate the chemical profile of the foods in order to prove their health-promising effects on the human health.

Nevertheless, the content of the phytochemicals is strongly affected by several factors (e.g. harvest time, environmental conditions, and geographical areas) and it changes across the varieties, thus indicating that the selection of new promising functional

foods is crucial for the success in building new healthy eating habit. Additionally, it is important to verify the effectiveness of the strategy *in vitro* and *in vivo* studies .

These studies are essential to assign the biological meaning that phytochemicals have when designing functional foods for the human health. This is because the metabolites are subjected to several transformations after the intake of foods, which are carried out by the microbiota of colon, thus stimulating the investigation of fruits and vegetables rich in bioactive compounds.

In this Ph.D. thesis, the variation in the content of several phytochemical compositions in selected fruit and vegetable species is explored. Different analytical methods prove to be useful to obtain information on metabolic profile of traditional Italian food products. More specifically, liquid chromatography–mass spectrometry, ion chromatography, and spectrophotometer analytical techniques are combined to gain insights on the chemical profile of specific foods. In detail, the chemical profiles of several apple and potatoes varieties are investigated that are mostly cultivated in South Tyrol and Trentino areas. Quantitative analyses of polyphenols, anthocyanins, sugars, and antioxidants are conducted. Additionally, the chemical profiles of new promising apple and potatoes varieties are reveled. The exploitation of several analytical techniques supports their utilization to make a comprehensive characterization of the metaboloma of several apple and potatoes varieties. Furthermore, different multivariate statistical approaches (PCA, PLS, and JIVE) are applied to ensure the reliability of the results.

The findings show that the content of bioactive compounds changes between cultivars and that flavonoids are the predominant secondary metabolites in apple and potato varieties. A wide range of compounds *in vitro* antioxidant proprieties and the chemical profiles (single polyphenols, sugars, and acylated anthocyanins)are detected and quantified in several apple and potatoes cultivars. In the chapter 2 the attention is drawn toward 5 old, 12 commercial, and 5 red-fleshed apple varieties. The results

demonstrate that the metabolomic profile of the apples is a suitable approach to assess the quality of fruits in the breeding programs. Regarding the chemical profile of apples, the most important flavonoids are polyphenols, anthocyanins, and dihydrochalcones. Furthermore, it has been confirmed that the concentration of metabolites in the flesh is lower than those in the peels. The white-fleshed apples are confirmed to be richer in flavan-3-ols, whereas the red-fleshed apples have a higher content of anthocyanins and dihydrochalcones. Based on the fact that all of apple varieties are locally produced, it has been found the most valuable for human consumption were Tiroler Spitzlederer (TS), Kanada Renette (KR), as old varieties, Granny Smith (GS), Lb 17906 (LB), as commercial cultivars, RS-1 (RS-1) red-fleshed (type 1), and red-fleshed 3/Y102 (Y102) as red-fleshed type 2, which have been identified within this study to be the most promising for human diet. The TS and KR were characterized by the highest amount of flavan-3-ols, moreover, small amount of cyanidin-3-galactoside (cyn3gal). GS and LB varieties demonstrated to have a considerable amount of flavan-3-ols, almost comparable with the old cultivars, and LB had a small content of cyn3gal. The RS-1 red-fleshed cultivar proved to have the highest anthocyanin content. Surprisingly, the content of flavan-3-ols Y102 cultivar was quantified to be the highest within the red-fleshed group, despite the lower content in anthocyanins.

The chapter 3 highlights what is already reported in the previous chapter as contribution in the XXX International Conference on Polyphenols (ICP2020). Overall, we showed to the scientific community during the ICP2020 that the old apple cultivars based on the metabolic pathways of the secondary metabolites are the most reliable apple group. The amount of phytochemicals in the old varieties is more stationary than the commercial and red-fleshed ones. On the contrary, a pronounced variability in the content of phytochemicals in the commercial and red-fleshed apple groups is observed. These results have stimulated the idea about the readability in the selection of apple cultivars

based on their phenolic pathway in order to select varieties with high nutritional values. Additionally, the old cultivars could be used as starting point to pinpoint new promising apple cultivars rich in bioactive compounds.

Regarding the metaboloma of potatoes, the results are reported in the chapter 4. The research is stimulated by a question that emerged in the evaluation of the nutritional potential of potatoes: their high glycemic index (GI). It is indeed causing an increment of the glycemic peak after consuming a meal of these tubers. Since potatoes are never consumed raw, in the studies the selected potato cultivars are cooked using a steamer. Indeed, the heat treatment favors the release of phytochemicals from the plant-based food, and it modulate the absorption of healthy compounds. The area of Trentino-Alto Adige has been considered as a suitable region to cultivate several potatoes cultivars; the study shed new light on the variety of Margone that demonstrated high nutritional values. The purple tubers have shown remarkable amounts of flavonoids such as polyphenols and anthocyanins. Within this framework, the study presents an extensive metabolic characterization of steam-cooked potato samples. Additionally, it was found that there are several types of potato metabolomes due to larger capacity in synthesizing phytochemicals. Indeed, it is demonstrated that differences between pigmented and non-pigmented varieties are determined by the accumulation of several metabolites, not only by the synthesis of pigments. Conversely, red-skinned and purple-fleshed potatoes are separated with the use of data analysis tools (JIVE) by the polyphenolic content, thus suggesting that these two groups are characterized by specific patterns of polyphenolic constituents. Additionally, a comprehensive characterization in the anthocyanin composition in red-skinned and purple-fleshed samples is reported. As far as the locally produced potatoes is concerned, Bettina, Cicero, and Kennebec varieties (non-pigmented potato samples), collected from Oris (BZ, Italy) and Ravina (TN, Italy), had a moderate content of sugars and polyphenols, moreover, the harvest seasons (2019

and 2020) of these varieties hardly affected the content of phytochemicals. Additionally, Ditta (BZ, Italy) and Vivaldi (TN, Italy) cultivars showed the lowest amount of sugars and the noticeable polyphenolic content within the yellow-fleshed group. Regarding the red-skinned potato samples, the local red variety is characterized by a higher amount of polyphenols, despite the modest content of sugars than the other two red-skinned cultivars.

The purple-fleshed varieties demonstrated to be most promising potato varieties. A special interest was focused on cultivar of Margone, that are BdP, Led, and VdG. In detail, a good balance in the concentration of phytochemicals is found in the purple-fleshed samples collected from VdG, indeed, this cultivar was characterized by higher contents in *in vitro* antioxidant proprieties and polyphenols, a lower amount of sugars, and an appreciable amount of acylated anthocyanins in comparison with the other purple-fleshed samples. Additionally, the Vitelotte cultivar, the most well-known purple-fleshed potato, showed the best balance in the concentration of these phytochemicals. It is worth emphasizing that cold temperatures stimulate the accumulation of anthocyanins [86], therefore the cultivation area of Trentino-Alto Adige may be considered as a suitable region to cultivated purple tubers with remarkable amounts of these compounds of interests

It is well-known that the most evident phenotypic difference in potatoes is the pigmentation of the flesh (or lack thereof), but this macroscopic difference is expected to affect the overall metabolome of the tubers. This is exactly what is demonstrated in the present study, which is highlighting that the difference in pigmentation propagates to a large class of phytochemicals which should be considered together to select promising potato varieties and increase the awareness of consumers.

In the chapter 5, a deeper characterization on the peels and on the pulps of red-fleshed cultivars is conducted and it has been proved that the amount of flavan-3-

ols, anthocyanins, and dihydrochalcones is extremely variable within the pigmented apples. We found that the amount of phytochemicals varies between and within the two red-fleshed apple types, despite of they are characterized into two genotypes (red-fleshed type 1 and type 2). The concentration of anthocyanins and dihydrochalcones in the red-fleshed apples has been confirmed to be higher than white-fleshed apples [65]. Contrarily, the amount of flavan-3-ols has been found variable within the red-fleshed apple cultivars, despite of the competitive synthesis between flavan-3-ols and anthocyanins. Some red-fleshed varieties have shown to be balanced in the content of flavan-3-ols and anthocyanins, associated to a higher amount in the concentration of dihydrochalcones. Once again, the Y102 variety, a red-fleshed type 2, demonstrated to be rich in flavan-3-ols (catechin, epicatechin, procyanidin B1, B2, and C1), on the contrary, a modest content in cyanidin glycosides. is registered. Another interesting variety is 108LB that shows a higher content in flavan-3-ols and cyanidin glycosides.

In this Ph.D. thesis, I studied 5 old, 12 commercial, and 5 red-fleshed apple varieties (chapter 2 and chapter 3), 28 cooked potato samples belonging to 18 different varieties (chapter 4), and 9 red-fleshed apple cultivars (chapter 5). The size of samples allowed to us to conduct an in-depth study regarding the metaboloma of non-colored and colored vegetables and fruits and, at the same time, to draw general conclusions about how to find a good balance between different classes of phytochemicals that should be taken. It should be remind, however, that metabolomics is an emerging tool to give a comprehensive measurement of all metabolites and a targeted analysis provides an accurate quantification of phytochemicals in plant-based foods. On-going and future studies of the metaboloma of local plant-based EUREGIO foods will include larger datasets, will be possible to apply better statistics, and allow us to make a more extensive comparison between the non-colored and colored vegetables and fruits. In particular, larger local samples are needed to build a local food map, based on the nutritional values of

local mountain foods in order to define the overall quality of food in Euregio regions (Trentino, South Tyrol, and Austria). To further investigate the metaboloma of different local plant-based EUREGIO, it would be interesting to apply other analytical methods in order to cover the maximum number of compounds from different chemical classes. For the nearest objects this can be achieved with untargeted approach that it is able to detect as many features as possible in a plant-based food matrix. In my opinion, the combination of targeted and untargeted approaches, provides crucial information on the production pathway of a huge range of metabolites. Future studies, therefore, should aim at improving our knowledge regarding the presence of phytochemicals in order to select the most promising fruits and vegetables, which can be used to fight obesity and age-related chronic diseases. I noticed a great potential of this resulting data in order to combine them with human nutrition.

By summarising, the aim of this PhD project was to define the overall quality of local plant-based EUREGIO foods by analyzing their metaboloma focusing on their chemical profile such as polyphenols, antioxidants, sugars, and anthocyanins. In detail, targeted metabolomic and multivariate statistical approaches are applied. The crucial point of this study is to assess the nutritional quality of the EUREGIO foods by evaluating a balance between the amount of phenolic compounds, antioxidants, sugars and anthocyanins. This research aims to identify new promising functional food based on quantitative analyses. Since there is a growing interest in the evaluation in the content of anthocyanins and polyphenols in pigmented and non-pigmented foods, thus stimulating a need to enhance the number of information on the chemical profile of these foods. The present study offers objective data providing relevance and emphasis and supporting consumers in the selection of health promoting plant-based food. Moreover, the application of different analytical tools is useful to identify varieties rich in healthy bioactive compounds, thus it is encouraging the valorization and preservation of locally produced plant-based food.

APPENDIX A

A.1 Student thesis

Co-supervision of the Bachelor Thesis of Alexandra Lanz at Bachelor Program: Biotechnology and Food Engineering Management Center Innnsbruck “Analysis of red fleshed apple varieties on their content of health-relevant metabolites". The resulting raw data obtained in the experiments are incorporated into this thesis and they are discussed into chapter 5.

A.2 Participation to Meetings/Conferences/Workshops

A.2.1 First year

EUREGIO-EFH WORKSHOP Socio-Economic Impact of Obesity and Healthy Ageing, UMIT – University for Health Sciences, Medical Informatics and Technology Hall in

Tirol, (Austria), 23.03.2019, Presentation of Poster "Measuring the nutritional quality of local plant based EUREGIO foods"

7th MS J-Day, “I giovani e la spettrometria di massa”, NOI Techpark Südtirol/Alto Adige – Bolzano, (Italy), 27.03.2019, Presentation of Poster "Identification of bioactive compounds with health-related potential by mass spectrometry"

6th MS Food, University of Camerino Auditorium Benedetto XIII Camerino (Italy), 25-27.09.2019, Presentation of Poster "Polyphenol composition of 12 apple cultivars grown in South Tyrol (Italy) determinate by ultra-high-performance liquid chromatography mass spectrometry (UHPLC-MS)".

EUREGIO Environment, Food & Health; Health First PhD-Workshop, NOI Techpark Südtirol/Alto Adige – Bolzano, (Italy), 03.10.2019, Oral Presentation of TITOLO TESI

A.2.2 Second year

8th MS J-Day, “I giovani e la spettrometria di massa”, 1st Online Edition – 09.07.2020, Presentation of Poster "Phenolic compounds quantification in raw and steamed Brassica vegetables from Trentino-Alto Adige (Italy) and Italian retailers".

A.2.3 Third year

ICP2020 Virtual Event XXX International Conference on Polyphenols, 13 – 15.07.2021, Oral Presentation of "Metabolomics investigation of antioxidant properties, polyphenolic profile and, anthocyanin content in commercial, ancient and red-fleshed apple varieties".

9th MS J-Day, “I giovani e la spettrometria di massa”, 2 Online Edition – 24.06.2021, Oral Presentation of "Metabolic association of health relevant metabolites between peel and pulp in apples of old, commercial, and red-fleshed cultivars".

Series of Lectures - 29.04.2022, Oral Presentation of "Metabolomic Characterization of Commercial, Old, and Red-Fleshed Apple Varieties".

BIBLIOGRAPHY

[1] A. Menotti and P. E. Puddu, “How the seven countries study contributed to the definition and development of the mediterranean diet concept: A 50-year journey,” *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 25, pp. 245–252, 2015.

[2] J. P. Mackenbach, “The mediterranean diet story illustrates that "why" questions are as important as "how" questions in disease explanation,” *Journal of Clinical Epidemiology*, vol. 60, pp. 105–109, 2007.

[3] R. Altomare, F. Cacciabaudo, G. Damiano, V. D. Palumbo, M. C. Gioviale, M. Bellavia, G. Tomasello, and A. I. L. Monte, “The mediterranean diet: A history of health,” *Iranian Journal of Public Health*, vol. 42, p. 449, 2013. [Online]. Available: /pmc/articles/PMC3684452//pmc/articles/PMC3684452/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3684452/

[4] M. G. Hertog, E. J. Feskens, D. Kromhout, M. G. Hertog, P. C. Hollman, M. G. Hertog, and M. B. Katan, “Dietary antioxidant flavonoids and risk of coronary heart disease: the zutphen elderly study,” *Lancet (London, England)*, vol. 342, pp. 1007–1011, 10 1993. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/8105262/

[5] D. Kromhout, E. B. Bosschieter, and C. de Lezenne Coulander, “The inverse relation between fish consumption and 20-year mortality from coronary heart disease,”

The New England journal of medicine, vol. 312, pp. 1205–1209, 5 1985. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/3990713/

[6] E. N. Frankel, J. B. German, J. E. Kinsella, E. Parks, and J. Kanner, "Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine," *Lancet (London, England)*, vol. 341, pp. 454–457, 2 1993. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/8094487/

[7] W. C. Willett, F. Sacks, A. Trichopoulou, G. Drescher, A. Ferro-Luzzi, E. Helsing, and D. Trichopoulos, "Mediterranean diet pyramid: a cultural model for healthy eating," *The American Journal of Clinical Nutrition*, vol. 61, pp. 1402S–1406S, 6 1995. [Online]. Available: https://academic.oup.com/ajcn/article/61/6/1402S/4651234

[8] C. Pattaro, F. Marroni, A. Riegler, D. Mascalzoni, I. Pichler, C. B. Volpato, U. D. Cero, A. D. Grandi, C. Egger, A. Eisendle, C. Fuchsberger, M. Gögele, S. Pedrotti, G. K. Pinggera, S. A. Stefanov, F. D. Vogl, C. J. Wiedermann, T. Meitinger, and P. P. Pramstaller, "The genetic study of three population microisolates in south tyrol (micros): Study design and epidemiological perspectives," *BMC Medical Genetics*, vol. 8, pp. 1–15, 6 2007. [Online]. Available: https://bmcmedgenet.biomedcentral.com/articles/10.1186/1471-2350-8-29

[9] J. de Meyer, "Food and agriculture organization of the united nations (fao) www.fao.org/nr/research-extension-systems/res-home/en." [Online]. Available: www.fao.org/

[10] C. Pattaro, M. Gögele, D. Mascalzoni, R. Melotti, C. Schwienbacher, A. Grandi, L. Foco, Y. D'Elia, B. Linder, C. Fuchsberger, C. Minelli, C. Egger, L. S. Kofink, S. Zanigni, T. Schäfer, M. F. Facheris, S. V. Smárason, A. Rossini, A. A. Hicks, H. Weiss, and P. P. Pramstaller, "The cooperative

health research in south tyrol (chris) study: rationale, objectives, and preliminary results," *Journal of Translational Medicine*, vol. 13, 11 2015. [Online]. Available: /pmc/articles/PMC4635524//pmc/articles/PMC4635524/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC4635524/

[11] B. Burlingame and S. Dernini, "Sustainable diets and biodiversity: directions and solutions for policy, research and action: proceedings of the intenational scientific symposium biodiversity and sustainable diets united against hunger, 3-5 november 2010, fao headquarters, rome," 2012.

[12] UNESCO, "Representative list of the intangible cultural heritage of humanity. available from: http://www.unesco.org/culture/ich/rl/00884," 2010.

[13] S. Dernini and E. M. Berry, "Mediterranean diet: From a healthy diet to a sustainable dietary pattern," *Frontiers in Nutrition*, vol. 2, 5 2015.

[14] D. A. Hyson, "A comprehensive review of apples and apple components and their relationship to human health," *Advances in Nutrition*, vol. 2, pp. 408–420, 9 2011.

[15] U. Vrhovsek, A. Rigo, D. Tonon, and F. Mattivi, "Quantitation of polyphenols in different apple varieties," *Journal of Agricultural and Food Chemistry*, vol. 52, pp. 6532–6538, 10 2004. [Online]. Available: https://pubs.acs.org/doi/abs/10.1021/jf049317z

[16] M. M. Ulaszewska, A. Koutsos, K. Trošt, J. Stanstrup, M. Garcia-Aloy, M. Scholz, F. Fava, F. Natella, C. Scaccini, U. Vrhovsek, K. Tuohy, J. Lovegrove, and F. Mattivi, "Two apples a day modulate human:microbiome co-metabolic processing of polyphenols, tyrosine and tryptophan," *European Journal of Nutrition*, vol. 59, pp. 3691–3714, 12 2020.

[17] J. Bonany, A. Buehler, J. Carbó, S. Codarin, F. Donati, G. Echeverria, S. Egger, W. Guerra, C. Hilaire, I. Höller, I. Iglesias, K. Jesionkowska, D. Konopacka, D. Kruczyńska, A. Martinelli, C. Pitiot, S. Sansavini, R. Stehr, and F. Schoorl, "Consumer eating quality acceptance of new apple varieties in different european countries," *Food Quality and Preference*, vol. 30, pp. 250–259, 12 2013.

[18] A. T. Ceci, M. Bassi, W. Guerra, M. Oberhuber, P. Robatscher, F. Mattivi, and P. Franceschi, "Metabolomic characterization of commercial, old, and red-fleshed apple varieties," *Metabolites*, vol. 11, 6 2021.

[19] The potato - International Year of the Potato 2008, "Triple quadrupole mass spectrometer - wikipedia," https://www.fao.org/potato-2008/en/potato/index.html, Last accessed 2021-12-06.

[20] H. Akyol, Y. Riciputi, E. Capanoglu, M. F. Caboni, and V. Verardo, "Phenolic compounds in the potato and its byproducts: An overview," *International Journal of Molecular Sciences*, vol. 17, 6 2016.

[21] F. Ieri, M. Innocenti, L. Andrenelli, V. Vecchio, and N. Mulinacci, "Rapid hplc/dad/ms method to determine phenolic acids, glycoalkaloids and anthocyanins in pigmented potatoes (solanum tuberosum l.) and correlations with variety and geographical origin," *Food Chemistry*, vol. 125, pp. 750–759, 3 2011.

[22] J. Jokioja, B. Yang, and K. M. Linderborg, "Acylated anthocyanins: A review on their bioavailability and effects on postprandial carbohydrate metabolism and inflammation," *Comprehensive Reviews in Food Science and Food Safety*, vol. 20, pp. 5570–5615, 11 2021.

[23] A. N. Panche, A. D. Diwan, and S. R. Chandra, "Flavonoids: an overview," *Journal of Nutritional Science*, vol. 5, pp. 1–15, 1 2016.

[Online]. Available: /pmc/articles/PMC5465813//pmc/articles/PMC5465813/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC5465813/

[24] D. M. Kopustinskiene, V. Jakstas, A. Savickas, and J. Bernatoniene, "Flavonoids as anticancer agents," *Nutrients*, vol. 12, 2 2020.

[25] F. Y. Fan, L. X. Sang, M. Jiang, and D. J. McPhee, "Catechins and their therapeutic benefits to inflammatory bowel disease," *Molecules : A Journal of Synthetic Chemistry and Natural Product Chemistry*, vol. 22, 3 2017. [Online]. Available: /pmc/articles/PMC6155401//pmc/articles/PMC6155401/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC6155401/

[26] I. Masuda, M. Koike, S. Nakashima, Y. Mizutani, Y. Ozawa, K. Watanabe, Y. Sawada, H. Sugiyama, A. Sugimoto, H. Nojiri, K. Sashihara, K. Yokote, and T. Shimizu, "Apple procyanidins promote mitochondrial biogenesis and proteoglycan biosynthesis in chondrocytes," *Scientific Reports 2018 8:1*, vol. 8, pp. 1–13, 5 2018. [Online]. Available: https://www.nature.com/articles/s41598-018-25348-1

[27] A. Rauf, M. Imran, T. Abu-Izneid, Iahtisham-Ul-Haq, S. Patel, X. Pan, S. Naz, A. S. Silva, F. Saeed, and H. A. R. Suleria, "Proanthocyanidins: A comprehensive review," *Biomedicine Pharmacotherapy*, vol. 116, p. 108999, 8 2019.

[28] S. M. Kern, R. N. Bennett, P. W. Needs, F. A. Mellon, P. A. Kroon, and M. T. Garcia-Conesa, "Characterization of metabolites of hydroxycinnamates in the in vitro model of human small intestinal epithelium caco-2 cells," *Journal of Agricultural and Food Chemistry*, vol. 51, pp. 7884–7891, 12 2003.

[29] T. Shoji, S. Masumoto, N. Moriichi, H. Akiyama, T. Kanda, Y. Ohtake, and Y. Goda, "Apple procyanidin oligomers absorption in rats after oral administration: Analysis of procyanidins in plasma using the porter method and high-performance

liquid chromatography/tandem mass spectrometry," *Journal of Agricultural and Food Chemistry*, vol. 54, pp. 884–892, 2 2006.

[30] M. H. Omar, W. Mullen, A. Stalmach, C. Auger, J. M. Rouanet, P. L. Teissedre, S. T. Caldwell, R. C. Hartley, and A. Crozier, "Absorption, disposition, metabolism, and excretion of [3- 14c]caffeic acid in rats," *Journal of Agricultural and Food Chemistry*, vol. 60, pp. 5205–5214, 5 2012.

[31] A. Crozier, I. B. Jaganath, and M. N. Clifford, "Phenols, polyphenols and tannins: An overview," *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet*, pp. 1–24, 11 2007.

[32] Z. Zhao and M. H. Moghadasian, "Bioavailability of hydroxycinnamates: A brief review of in vivo and in vitro studies," *Phytochemistry Reviews*, vol. 9, pp. 133–145, 3 2010.

[33] J. Boyer and R. H. Liu, "Apple phytochemicals and their health benefits," *Nutrition Journal*, vol. 3, p. 5, 12 2004. [Online]. Available: /pmc/articles/PMC442131//pmc/articles/PMC442131/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC442131/

[34] F. Shahidi and A. Chandrasekara, "Hydroxycinnamates and their in vitro and in vivo antioxidant activities," *Phytochemistry Reviews*, vol. 9, pp. 147–170, 3 2010.

[35] J. P. Spencer, G. Chowrimootoo, R. Choudhury, E. S. Debnam, S. K. Srai, and C. Rice-Evans, "The small intestine can both absorb and glucuronidate luminal flavonoids," *FEBS letters*, vol. 458, pp. 224–230, 9 1999. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/10481070/

[36] J. Lee and A. E. Mitchell, "Pharmacokinetics of quercetin absorption from apples and onions in healthy humans," *Journal of Agricultural and Food Chemistry*, vol. 60, pp. 3874–3881, 4 2012.

[37] M. Ay, A. Charli, H. Jin, V. Anantharam, A. Kanthasamy, and A. G. Kanthasamy, "Quercetin," *Nutraceuticals: Efficacy, Safety and Toxicity*, pp. 447–452, 1 2016.

[38] J. P. E. Spencer, G. G. C. Kuhnle, R. J. Williams, and C. Rice-Evans, "Intracellular metabolism and bioactivity of quercetin and its in vivo metabolites," *Biochem. J*, vol. 372, pp. 173–181, 2003.

[39] D. Zielinska, J. M. Laparra-Llopis, H. Zielinski, D. Szawara-Nowak, and J. A. Giménez-Bastida, "Role of apple phytochemicals, phloretin and phloridzin, in modulating processes related to intestinal inflammation," *Nutrients 2019, Vol. 11, Page 1173*, vol. 11, p. 1173, 5 2019. [Online]. Available: https://www.mdpi.com/2072-6643/11/5/1173/htmhttps://www.mdpi.com/2072-6643/11/5/1173

[40] G. Bebernitz, "Sodium–glucose cotransporters," *Comprehensive Medicinal Chemistry III*, pp. 491–511, 1 2017.

[41] J. J. Liu and T. W. Lee, "Sglt2 inhibitors for type 2 diabetes," *Annual Reports in Medicinal Chemistry*, vol. 46, pp. 103–115, 1 2011.

[42] A. Smeriglio, D. Barreca, E. Bellocco, and D. Trombetta, "Chemistry, pharmacology and health benefits of anthocyanins," *Phytotherapy Research*, vol. 30, pp. 1265–1286, 8 2016. [Online]. Available: https://onlinelibrary.wiley.com/doi/full/10.1002/ptr.5642https://onlinelibrary.wiley.com/doi/abs/10.1002/ptr.5642https://onlinelibrary.wiley.com/doi/10.1002/ptr.5642

[43] J. Jokioja, K. M. Linderborg, M. Kortesniemi, A. Nuora, J. Heinonen, T. Sainio, M. Viitanen, H. Kallio, and B. Yang, "Anthocyanin-rich extract from

purple potatoes decreases postprandial glycemic response and affects inflammation markers in healthy men," *Food Chemistry*, vol. 310, 4 2020. [Online]. Available: https://www.researchgate.net/publication/336833284_Anthocyanin-rich_extract_from_purple_potatoes_decreases_postprandial_glycemic_response_and_affects_inflammation_markers_in_healthy_men

[44] A. Oertel, A. Matros, A. Hartmann, P. Arapitsas, K. J. Dehmer, S. Martens, and H. P. Mock, "Metabolite profiling of red and blue potatoes revealed cultivar and tissue specific patterns for anthocyanins and other polyphenols," *Planta*, vol. 246, pp. 281–297, 8 2017.

[45] T. Fossen, L. Cabrita, and Øyvind M. Andersen, "Colour and stability of pure anthocyanins influenced by ph including the alkaline region," *Food Chemistry*, pp. 435–440, 1998. [Online]. Available: https://bibliotecadigital.ipb.pt/handle/10198/3206

[46] A. Mouradov and G. Spangenberg, "Flavonoids: A metabolic network mediating plants adaptation to their real estate," *Frontiers in Plant Science*, vol. 5, pp. 1–16, 11 2014.

[47] S. Lamichhane, P. Sen, A. M. Dickens, T. Hyötyläinen, and M. Orešič, "An overview of metabolomics data analysis: Current tools and future perspectives," *Comprehensive Analytical Chemistry*, vol. 82, pp. 387–413, 1 2018.

[48] S. Kim, J. Kim, E. J. Yun, and K. H. Kim, "Food metabolomics: From farm to human," *Current Opinion in Biotechnology*, vol. 37, pp. 16–23, 2 2016.

[49] S. Watermann, C. Schmitt, T. Schneider, and T. Hackl, "Comparison of regular, pure shift, and fast 2d nmr experiments for determination of the geographical origin of walnuts," *Metabolites*, vol. 11, 2021. [Online]. Available: https://www.mdpi.com/2218-1989/11/1/39

[50] U. Vrhovsek, D. Masuero, M. Gasperotti, P. Franceschi, L. Caputi, R. Viola, and F. Mattivi, "A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages," *Journal of Agricultural and Food Chemistry*, vol. 60, pp. 8831–8840, 2012, pMID: 22468648. [Online]. Available: https://doi.org/10.1021/jf2051569

[51] M. cialiè rosso, F. Stilo, C. Bicchi, M. Charron, G. Rosso, R. Menta, S. Reichenbach, C. Weinert, C. Mack, S. Kulling, and C. Cordero, "Combined untargeted and targeted fingerprinting by comprehensive two-dimensional gas chromatography to track compositional changes on hazelnut primary metabolome during roasting," *Applied Sciences*, vol. 11, p. 525, 11 2021.

[52] M. Castro-Puyana and M. Herrero, "Metabolomics approaches based on mass spectrometry for food safety, quality and traceability," *TrAC Trends in Analytical Chemistry*, vol. 52, pp. 74–87, 12 2013.

[53] Wikipedia, the free encyclopedia, "Triple quadrupole mass spectrometer - wikipedia," https://en.wikipedia.org/wiki/Triple_quadrupole_mass_spectrometer, Last accessed 2021-12-06.

[54] S. Piovesana, C. Cavaliere, A. Cerrato, C. M. Montone, A. Laganà, and A. L. Capriotti, "Developments and pitfalls in the characterization of phenolic compounds in food: From targeted analysis to metabolomics-based approaches," *TrAC Trends in Analytical Chemistry*, vol. 133, p. 116083, 12 2020.

[55] J. Bartel, J. Krumsiek, and F. J. Theis, "Statistical methods for the analysis of high-throughput metabolomics data," *Computational and Structural Biotechnology Journal*, vol. 4, p. e201301009, 1 2013.

[56] C. Feng, H. Wang, N. Lu, T. Chen, H. He, Y. Lu, and X. M. Tu, "Log-transformation and its implications for data analysis," *Shanghai archives of psychiatry*, vol. 26,

pp. 105–109, 4 2014. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/25092958https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4120293/

[57] G. Palermo, P. Piraino, and H. D. Zucht, "Performance of pls regression coefficients in selecting variables for each response of a multivariate pls for omics-type data," *Advances and applications in bioinformatics and chemistry : AABC*, vol. 2, p. 57, 2009. [Online]. Available: /pmc/articles/PMC3169946//pmc/articles/PMC3169946/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3169946/

[58] S. Wold, M. Sjöström, and L. Eriksson, "Pls-regression: a basic tool of chemometrics," *Chemometrics and Intelligent Laboratory Systems*, vol. 58, pp. 109–130, 10 2001.

[59] D. Eisenstecken, A. Panarese, P. Robatscher, C. W. Huck, A. Zanella, and M. Oberhuber, "A near infrared spectroscopy (nirs) and chemometric approach to improve apple fruit quality management: A case study on the cultivars "cripps pink" and "braeburn"," *Molecules (Basel, Switzerland)*, vol. 20, pp. 13 603–13 619, 8 2015. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/26213913/

[60] B. C. Deng, Y. H. Yun, Y. Z. Liang, D. S. Cao, Q. S. Xu, L. Z. Yi, and X. Huang, "A new strategy to prevent over-fitting in partial least squares models based on model population analysis," *Analytica Chimica Acta*, vol. 880, pp. 32–41, 6 2015.

[61] S. E. Richards, M. E. Dumas, J. M. Fonville, T. M. Ebbels, E. Holmes, and J. K. Nicholson, "Intra- and inter-omic fusion of metabolic profiling data in a systems biology framework," *Chemometrics and Intelligent Laboratory Systems*, vol. 104, pp. 121–131, 11 2010.

[62] M. J. O'Connell and E. F. Lock, "R.jive for exploration of multi-source molecular data," *Bioinformatics*, vol. 32, pp. 2877–2879, 9 2016.

[63] E. F. Lock, K. A. Hoadley, J. S. Marron, and A. B. Nobel, "Joint and individual variation explained (jive) for integrated analysis of multiple data types," *Annals of Applied Statistics*, vol. 7, pp. 523–542, 3 2013.

[64] "Apple-producing family farms in south tyrol: An agriculture innovation case study," https://authorservices.wiley.com/author-resources/Journal-Authors/licensing/licensing-info-faqs.html, Last accessed 2022-04-20.

[65] S. Yuste, I. A. Ludwig, M.-P. Romero, M.-J. Motilva, and L. Rubió, "New red-fleshed apple cultivars: a comprehensive review of processing effects, (poly)phenol bioavailability and biological effects," *Food function*, 2022. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/35419577/

[66] N. Wang, H. Xu, S. Jiang, Z. Zhang, N. Lu, H. Qiu, C. Qu, Y. Wang, S. Wu, and X. Chen, "Myb12 and myb22 play essential roles in proanthocyanidin and flavonol synthesis in red-fleshed apple (malus sieversii f. niedzwetzkyana)," *Plant Journal*, vol. 90, pp. 276–292, 4 2017.

[67] N. Wang, S. Jiang, Z. Zhang, H. Fang, H. Xu, Y. Wang, and X. Chen, "Horticulture research malus sieversii: the origin, flavonoid synthesis mechanism, and breeding of red-skinned and red-fleshed apples," *Horticulture Research*, vol. 5, p. 70, 2018. [Online]. Available: www.nature.com/hortres

[68] A. C. Allan, R. P. Hellens, and W. A. Laing, "Myb transcription factors that colour our fruit," *Trends in plant science*, vol. 13, pp. 99–102, 3 2008. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/18280199/

[69] S. Vimolmangkang, Y. Han, G. Wei, and S. S. Korban, "An apple myb transcription factor, mdmyb3, is involved in regulation of anthocyanin biosynthesis and flower development," *BMC Plant Biology*, vol. 13, p. 1, 2013. [Online]. Available: http://www.biomedcentral.com/1471-2229/13/176

[70] Simone Innerebner, "A surprise inside: New variety of red-fleshed apples – consorzio bia," https://www.consorziobia.eu/ENG/a-surprise-inside-new-variety-of-red-fleshed-apples/, Last accessed 2022-04-13.

[71] Walter Guerra, "Mele a polpa rossa, pronti al decollo - rivista di frutticoltura," https://rivistafrutticoltura.edagricole.it/featured/mele-a-polpa-rossa-pronti-al-decollo/, Last accessed 2022-04-13.

[72] D. Bars-Cortina, A. Macià, I. Iglesias, M. P. Romero, and M. J. Motilva, "Phytochemical profiles of new red-fleshed apple varieties compared with traditional and new white-fleshed varieties," *Journal of agricultural and food chemistry*, vol. 65, pp. 1684–1696, 3 2017. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/28191939/

[73] P. Legendre, "Interpreting the replacement and richness difference components of beta diversity," *Global Ecology and Biogeography*, vol. 23, pp. 1324–1334, 11 2014.

[74] J. Oksanen, "Package 'vegan' title community ecology package," 2022.

[75] M. J. Anderson, "Permutational multivariate analysis of variance (permanova)," *Wiley StatsRef: Statistics Reference Online*, pp. 1–15, 11 2017. [Online]. Available: https://onlinelibrary.wiley.com/doi/full/10.1002/9781118445112.stat07841https://onlinelibrary.wiley.com/doi/abs/10.1002/9781118445112.stat07841https://onlinelibrary.wiley.com/doi/10.1002/9781118445112.stat07841

[76] "Permutational analysis of variance - wikipedia," https://en.wikipedia.org/wiki/Permutational_analysis_of_variance, Last accessed 2022-05-11.

[77] R. Wei, J. Wang, M. Su, E. Jia, S. Chen, T. Chen, and Y. Ni, "Missing value imputation approach for mass spectrometry-based metabolomics data," *Scientific Reports 2018 8:1*, vol. 8, pp. 1–10, 1 2018. [Online]. Available: https://www.nature.com/articles/s41598-017-19120-0

[78] "Create elegant data visualisations using the grammar of graphics • ggplot2," lhttps://ggplot2.tidyverse.org/, Last accessed 2022-04-22.

[79] P. Li, F. Ma, and L. Cheng, "Primary and secondary metabolism in the sun-exposed peel and the shaded peel of apple fruit," *Physiologia plantarum*, vol. 148, pp. 9–24, 5 2013. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/22989296/

[80] X. Zhang, J. Xu, Z. Xu, X. Sun, J. Zhu, and Y. Zhang, "Analysis of antioxidant activity and flavonoids metabolites in peel and flesh of red-fleshed apple varieties," *Molecules (Basel, Switzerland)*, vol. 25, 4 2020. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/32340213/

[81] H. Sato, S. Otagaki, P. Saelai, S. Kondo, K. Shiratake, and S. Matsumoto, "Varietal differences in phenolic compounds metabolism of type 2 red-fleshed apples," *Scientia Horticulturae*, vol. 219, pp. 1–9, 5 2017.

[82] X. Wang, C. Li, D. Liang, Y. Zou, P. Li, and F. Ma, "Phenolic compounds and antioxidant activity in red-fleshed apples," *Journal of Functional Foods*, vol. 18, pp. 1086–1094, 10 2015.

[83] B. Nothegger, N. Reider, C. E. Covaciu, V. Cova, L. Ahammer, R. Eidelpes, J. Unterhauser, S. Platzgummer, M. Tollinger, T. Letschka, and K. Eisendle, "Allergen-specific immunotherapy with apples: selected cultivars could be a promising tool for birch pollen allergy," *Journal of the European Academy of Dermatology and Venereology*, vol. 34, p. 1286, 6 2020.

[Online]. Available: /pmc/articles/PMC7318684//pmc/articles/PMC7318684/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC7318684/

[84] C. X. Li, X. H. Zhao, W. F. Zuo, T. L. Zhang, Z. Y. Zhang, and X. S. Chen, "Phytochemical profiles, antioxidant, and antiproliferative activities of four red-fleshed apple varieties in china," *Journal of Food Science*, vol. 85, pp. 718–726, 3 2020.

[85] X. Zeng, H. Li, W. Jiang, Q. Li, Y. Xi, X. Wang, and J. Li, "Phytochemical compositions, health-promoting properties and food applications of crabapples: A review," *Food Chemistry*, vol. 386, p. 132789, 8 2022.

[86] R. S. Payyavula, D. A. Navarre, J. C. Kuhl, A. Pantoja, and S. S. Pillai, "Differential effects of environment on potato phenylpropanoid and carotenoid expression," *BMC Plant Biol.*, vol. 12(1), pp. 1–17, 3 2012. [Online]. Available: https://bmcplantbiol.biomedcentral.com/articles/10.1186/1471-2229-12-39