Metabolites of Apple

Apples represent a greater proportion of the worldwide fruit supply, due to their availability on the market and to the high number of existing cultivar varieties and apple-based products (fresh fruit, fruit juice, cider and crushed apples). Several studies on apple fruit metabolites are available, with most of them focusing on their healthy properties' evaluation. In general, the metabolic profile of apple fruits strongly correlates with most of their peculiar characteristics, such as taste, flavor and color. At the same time, many bioactive molecules could be identified as markers of a specific apple variety. Therefore, a complete description of the analytical protocols commonly used for apple metabolites' characterization and quantification could be useful for researchers involved in the identification of new phytochemical compounds from different apple varieties.

1. Introduction

Due to the beneficial properties of metabolites, the interest in the characterization and quantification of the metabolites—both primary and secondary—of plant-based foods is growing, and it often aims at the definition of food nutritional value, as well as of its quality and authenticity. In this context, foodomics technologies have emerged [1]. Foodomics has been defined as a new discipline that studies the food and nutrition domains through the application of advanced omics technologies for improving consumers' well-being, health and confidence [2]. Considering a large number of plant primary and secondary metabolites and their different properties, several analysis techniques have been developed for each class of compounds, which differ already starting from the extraction phase of metabolites [3]. The interest in nutritional parameters, such as sugars content, often identified as being responsible for obesity or diseases such as diabetes, determined the attention to develop analytical methods for the determination of simple sugars and polysaccharides [4]. Anyway, each class of metabolite, whether primary or secondary, needs a dedicated extraction and analysis method to offer the consumer as much information as possible regarding the functional values of the individual foods.

Malus domestica fruits, commonly known as apples, are one of the most consumed fruits worldwide, and they are generally recognized as an outstanding source of biologically active compounds, related to both functional and nutraceutical values [5] (**Figure 1**).
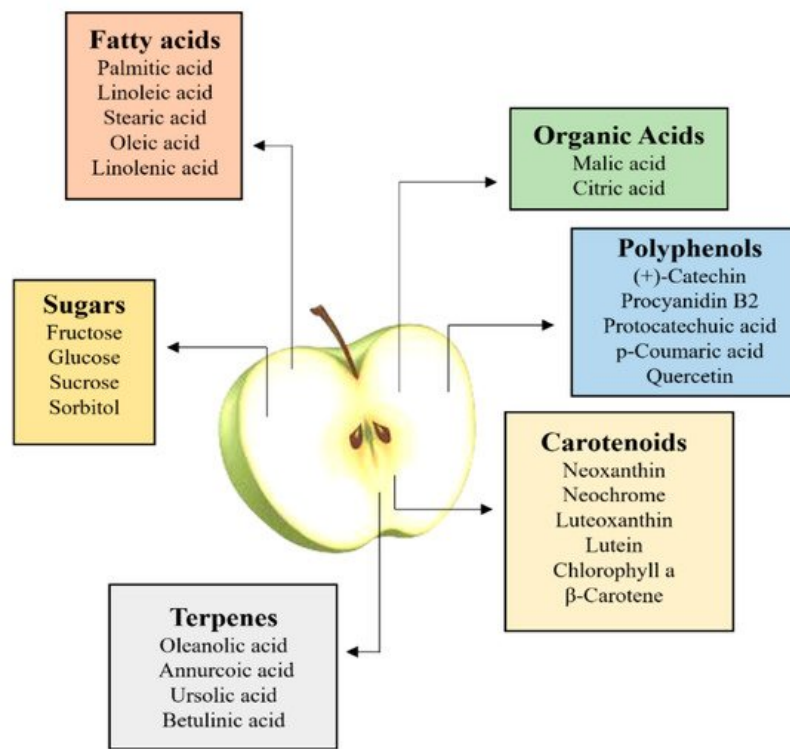
Figure 1. Main primary and secondary metabolites related to functional and nutraceutical values of apples.

Apples have a varied and well-balanced composition; more than 84% of their weight is represented by water, in which minerals such as K, Mg, Ca and Na and trace elements such as Zn, Mn, Cu, Fe, B, F, Se and Mo are dissolved [6]. Proteins and lipids represent a small energetic supply contribution to *Malus domestica* fruits, due to their relatively small concentrations [6]. On the contrary, the content of fibers, i.e., complex polysaccharides such as pectin, celluloses, hemicelluloses and lignin, is high in apples when compared with other fresh fruits [7]. Although a large variety of vitamins are present, the B complex vitamins are the most abundant. Moreover, apples are considered an important source of polyphenols, which are responsible for their well-known antioxidant properties [5].

Apples are commonly considered as healthy plant-based foods. They have a very low caloric impact. From a nutritional point of view, they contain a high number of sugars, balanced by the concentration of fibers [8]. In the last ten years, apples have been the subject of research and studies aiming to evaluate their effects on human health [9]. The daily intake of apples and apples-related products is often proposed in with weight control diets and has proved to have a statistically significant impact on weight reduction [10]. Recent epidemiological research shows how the risk of cardiovascular diseases and cancers could be reduced by the regular consumption of apples [11][12], due to their phenolic acids and flavonoids, i.e., molecules with antioxidant properties [12]. The nutritional value and health-related properties of apples depend on the bioavailability and daily intake of their nutrients and phytochemicals, as well as on their concentration [13]. Therefore, a complete evaluation of the apple's metabolites distribution is desirable.

2. Primary Metabolites: Extraction and Analysis

Primary metabolites are defined as chemical compounds which are necessary for plant growth, development and reproduction [14]. There are, basically, four main groups of primary metabolites in plant-based foods: sugars, amino-acids, fatty acids and nucleotides. Among them, sugars and fatty acids deserve special attention for apples, because they play a role in fruits' taste.

2.1. Sugars

For many fruits and vegetables, sweetness is an important parameter influencing their quality and is determined by the level of soluble sugars. Therefore, the determination of sugars and sweetness are of great importance in many fields of plant-food-science research [4]. In apples, sweetness is one of the most important quality parameters that determines the overall acceptability of the fruit, and it is affected mainly by fructose, glucose, sucrose and sorbitol concentrations [15]. Sorbitol and sucrose are biosynthetized in the leaves, and then they are translocated into the fruits, where they can be converted into fructose, glucose, malic acid and starch depending on the stage of fruit development. Sorbitol and sucrose represent almost the total translocated carbohydrates [15]. Although the individual sugar content may vary greatly between

and within apple cultivars, during fruit storage, the concentrations of sucrose tend to decrease due to the conversion to fructose and glucose [15].

Sugars quantification in apples is preceded by their extraction from the fruit tissues. The extraction of sugars is usually performed by using water as an extraction solvent, since it yields more reliable results. Indeed, Karkacier et al. [16] found that, with alcoholic extraction, some sugars may not adequately dissolve in proportion to their true concentration because of solvent vaporization, even at low temperatures. However, in general, a preliminary immersion in methanol/water solution of the fruit tissues before the extraction, is important to inhibit the invertase activity, thus avoiding the hydrolysis of sucrose into free sugars (glucose and fructose) [3].

As traditional colorimetric and iodometric methods are unable to quantitate sugars individually, non-structural carbohydrates in apples are analyzed by HPLC coupled with suitable columns and detectors, including refractive index detector (RI), evaporative light scattering detector (ELSD) and pulsed amperometric detector (PAD) [16][17][18]. HPLC coupled to diode-array detector is quite limited, as sugars do not absorb UV light at a wavelength longer than 200 nm [16]. Although less common, GC–MS-based methods for sugars' analysis have also been proposed. In these cases, preliminary derivatization stages with methoxyamine hydrochloride and N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) are needed [19].

One of the most used methods for the quantification of soluble carbohydrates, is HPLC–RI, being simple, rapid and economical [18]. In fact, an accurate HPLC–RI method for the simultaneous determination of glucose, fructose, sucrose and sorbitol in leaf and/or fruit peel from different apple cultivars was developed and validated by Filip et al. [18]. This method was found to be reproducible and sensitive. The HPLC–RI method was successfully applied to qualitative and quantitative establish the sugars of seven apple cultivars and two rootstocks originating from a germplasm collection [18]. Instead, Ma et al. [17] developed an HPLC–ELSD method to analyze sugars without derivatizations, with short analysis times (25 min) and good chromatographic separations. Recently, Yang et al. [20] also used the same method to quantify glucose, fructose, sucrose and sorbitol in "Orin" apples, founding that their content generally increased during the fruit-ripening periods and reached the maximum at full-ripening stage of 180 days after full-bloom stage [20]. ELSD does not suffer of limitations such as composition, flow rate of mobile phase and temperature. In this case, the detection is based on the ability of particles to cause photon scattering, thus with ELSD most compounds less volatile than the mobile phase could be detected, regardless the optical properties of the analytes.

The methods discussed above require considerable financial investment, advanced analytical skills and time. Moreover, they are destructive and require the use of hazardous chemicals and are labor intensive. As an alternative, more rapid, simple and non-destructive techniques, i.e., Fourier transform near-infrared (FT-NIR) spectroscopy with attenuated total reflection (ATR–FT-NIR), have been used to determine the sweetness as an internal quality attribute of fresh apples [21] [22]. By comparison of HPLC and FT-NIR for quantification of glucose, fructose and sucrose in intact apple fruit [21], it emerged that FT-NIR is more flexible and much faster than HPLC method.

2.2. Fatty Acids

The mesocarp, or pulp, of apples generally contains very low levels of lipid material (0.2–0.6%), and therefore it is not an important source of edible or industrial fats and oils [23]. Saturated and unsaturated fatty acids are, in fact, degraded to precursor molecules for straight-chain esters. However, despite their low levels of concentration, fatty acids (FAs) and lipids are important structural and metabolic constituents [24]. Indeed, alterations of the lipids constituting the cellular membranes, could cause serious problems to the cell's adaptability to stress conditions, thus resulting in fruit storage disorders. Moreover, FAs and lipids often play a crucial role as precursors of important volatile aroma and regulatory compounds cells [25]. Although there are differences among varieties and ripening stage, the content of saturated fatty acids is generally higher than that of the unsaturated ones in each apple variety. The optimal value of the ratio between the content of the latter and the former is around 30/70 or less [26]. Among the saturated and unsaturated FAs classes, palmitic acid (C16:0) and linoleic acid (C18:2) are the dominant compounds, respectively [26]. In addition to palmitic and linoleic acid, there are C18:0 (stearic acid), C18:1(oleic acid) and C18:3 (linolenic acid), whose concentration levels dynamically fluctuate during fruit growth and development. Other FAs, such as C20:0 (arachidonic acid), show only small variations, which may indicate that they are constituents of the fruit cuticle, rather than participants in actual fruit metabolism [25].

Gas chromatography (GC) coupled to flame ionization detector (FID) or mass spectrometric detector (MS) is the most commonly used technology for the detection of fatty acids in apples. The analysis of fatty acids by GC–FID and GC–MS-based analytical methods involve three main steps, i.e., their extraction from the sample matrix, their derivatization, and the GC–FID or GC–MS analysis [27]. Lipids extraction from apple tissues is performed with hexane, and it is often followed

by filtration of the supernatant through anhydrous sodium sulfate [28][29]. Soxhlet extraction with petroleum ether has also been used [29][30]. The derivatization is requested, because most fatty acids have high boiling points, thus being difficult to evaporate, and have a low FID response [31]. Fatty acids in apples are usually analyzed by GC–FID and GC–MS as methyl ester derivatives. The derivatization is typically made by treating apple extract with methanol and sulfuric acid under a stream of nitrogen, before the GC–MS analysis. Five fatty acids were identified as their methyl esters with this approach by Walia et al. [29] in apple fruits, i.e., oleic acid, linoleic acid, palmitic acid, stearic acid and arachidic acids. Since mass spectra of derivatized fatty acids rarely contain ions indicative of structural features (the positions of double bonds in the aliphatic chain, for example, cannot be determined), retention indices (RI) of the compounds relative to a mixture of n-alkanes are calculated and are typically used for their identification, alongside with a comparison of their mass spectral data with Wiley, NIST, NBS library and the literature data [29].

3. Secondary Metabolites

To date, the structures and the average content of primary metabolites occurring in apples, are well known, for their nutritional value [26]. Therefore, in recent years, research has been most focused on the extraction of secondary metabolites to be used in pharmacological studies for their beneficial properties [32]. Food and pharmaceutical studies have addressed their attention on fruits and vegetables' bioactive components considered healthy for the treatment and prevention of human diseases. Among the different classes of apples, secondary metabolites, polyphenols, carotenoids, organic acids and terpenes are the main phytochemicals [6].

Although several classes of bioactive compounds occurring in apples, their well-known antioxidant properties are mainly attributed to phenolic compounds. These compounds exhibit several of double bonds and hydroxyl groups in their structures, which are responsible of their antioxidant activity [33]. There are five major groups of polyphenolic compounds found in apples: hydroxycinnamic acids (primarily chlorogenic acid), flavan-3-ols, i.e., (+)-catechin, (−)-epicatechin and anthocyanidins, flavonols (mainly different quercetin glycosides), dihydrochalcones (such as phloridzin) and anthocyanins [34][35][36] (**Figure 2**). A high percentage (60%) of the total phenolic concentration in apple peel is represented by the monomeric and polymeric flavan-3-ols, while flavonols), hydroxycinnamic acids, dihydrochalcones and anthocyanins account, respectively, for the 18%, 9%, 8% and 5% of the total phenol content [37].
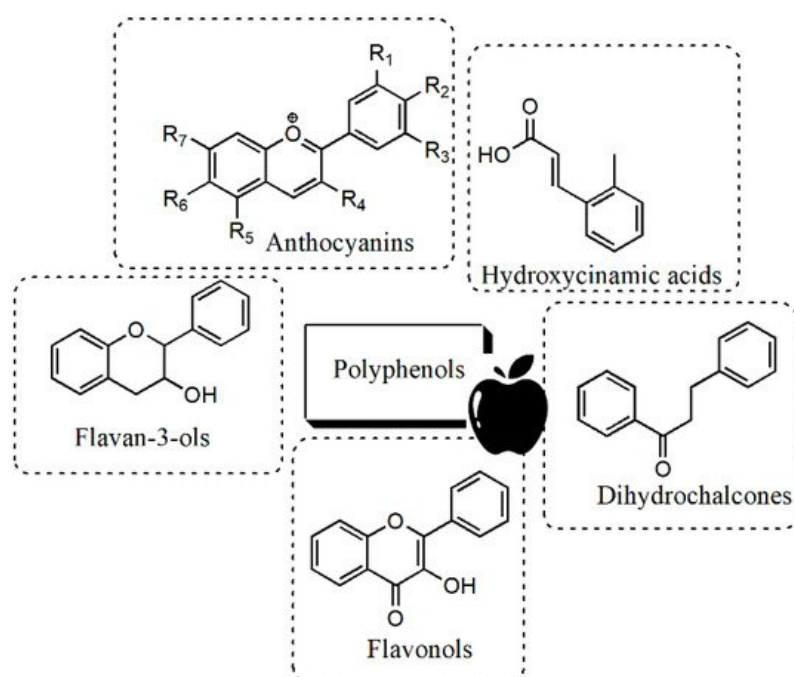

Figure 2. Main classes of polyphenols occurring in apples.

Carotenoid pigments in the skin of apples contribute to fruit coloration, and therefore to their attractiveness, but in the flesh, their concentrations are low. Indeed, fruits of commercial apple cultivars show relatively low concentrations of carotenoids (<2.5 µg/g of fresh weight), in comparison with non-commercial apples, such as the rootstock cultivar "Aotea", that show relatively high carotenoid concentrations [38].

Alongside with sugars, aromatic volatile compounds and organic acids are responsible for the taste and flavor of apples (**Figure 3**). In addition, organic acids are the main soluble constituents that influence the shelf life of fresh fruits and ripeness; consequently, they can be used as an index of consumer acceptability [39]. In cultivated apple, malic acid is the

predominantly detectable organic acid, while malic acid and citric acid are the predominant organic acids in wild apple species [40]. In regard to apple aroma profiles, many volatile organic compounds (VOCs) contribute to the overall sensory quality. These compounds include carboxylic esters, alcohols, aldehydes and ketones. Various terpenes have also been identified; however, they only contribute a relatively minor component of total VOCs produced [41]. Some of the terpene occurring in apples are α-farnesene, geranyl acetone and farnesol [42][43]. α-farnesene, which is an acyclic branched sesquiterpene, is highly occurring in ripe fruits; moreover, others monoterpenes, cyclic sesquiterpenes and terpene derivatives have also been identified in floral and vegetative tissues. Many of these compounds are constitutively produced in relatively low amounts also as response to insect infestation and they could directly affect apple pest behavior [41]. More polar and less volatile terpenes, i.e., triterpenoid compounds, have been also identified in apples, namely pomaceic, annurcoic, euscaphic, pomolic, corosolic, maslinic, oleanolic, betulinic and ursolic acid [44].
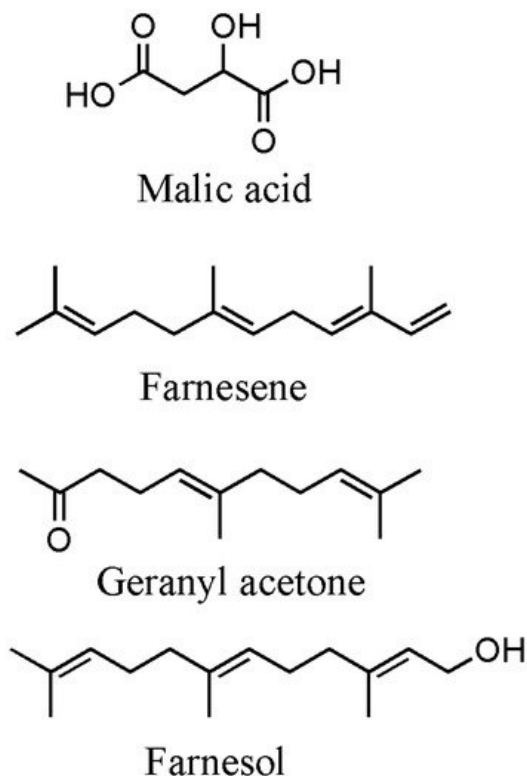

Figure 3. Main compounds responsible for the taste and flavor of apples.

3.1. Extraction

Phytochemical compounds from apples were extracted by using various extraction methods based on the application of different solvents and by heating and/or mixing. The most used techniques were Soxhlet extraction, maceration and hydro-distillation [45][46][47]. Conventional Soxhlet extraction still remains one of the most relevant approach to extract volatile compounds from apples [47]. The sample is placed in a thimble-holder, where it's slowly filled with condensed solvent from a distillation flask. Once the liquid has reached an overflow level, the whole contents of the thimble-holder is aspirated by a siphon, which unloads it back into the distillation flask, leaving the extracted analytes in the bulk liquid [29]. On the contrary, during maceration, the sample is immersed for a variable time in a solvent inside an airtight container in order to allow the analyte transferring [48]. Vacuum hydro-distillation, instead, uses water vapor to recover volatile and apolar components from fruit tissues, and, as Soxhlet, it has been mainly used for the extraction of apple aroma, because it gave the extracts closest to the fresh fruit [45]. The duration of the extraction process, and the large number of organic solvents used are the major drawbacks of these techniques. In fact, alternative approaches have emerged in an attempt to mitigate limitations of the conventional ones. The innovation is largely focused on finding technological solutions to diminish or even prevent the use of organic solvents in extraction processes, in order to obtain more highly purified products containing fewer additional toxins [49]. The new methods include solid–liquid extractions (namely microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE), accelerated solvent extraction (ASE), supercritical fluid extraction (SFE)) and solid-phase microextraction (SPME). Organic solvents, such as hexane, acetone, methanol, ethanol or water, have been generally used under atmospheric pressure. The choice of the solvent largely depended on the polarity of the analytes.

Extraction of polyphenols from apples has been carried out by using methods that differ for many variables, such as solvent, time, temperature and number of extractions; therefore, the data reported in the literature are not always easily

comparable. According to Bai et al. [50], the extraction of polyphenols from apples can easily be achieved via MAE, by using ethanol as extracting solvent, in a ratio to raw material of 22.9/1. Microwave-assisted extraction is based on the use of microwave energy to heat solvents in contact with a sample to allow the analytes partitioning from the sample matrix into the solvent [51]. Compared to Soxhlet and maceration, MAE results in a greater efficiency at a shorter time for the extraction of phenolic compounds from apple pomace. Moreover, accelerated operation can be regarded as a major advantage of MAE, which is useful especially at the industrial scale [46]. Bekele et al. [52] used a methanol extraction by adding pre-chilled MeOH (−20 °C) to apple pulp and extracting at 70 °C in a homogenizer. The extract was then centrifuged and dried under nitrogen flow. This extraction method was not selective for polyphenols; in fact, it allowed the extraction of many hydrophilic metabolites. Furthermore, this protocol of extraction was found to be not very efficient for complete extraction of all apple polyphenols, because less polar molecules could remain inside the matrix, which is discarded. In order to avoid this problem, a multi-step extraction can be used [53][54]. A first step involves the treatment of the dried apples cortex with hexane, to remove lipids, carotenoids and chlorophyll. Then, an extraction with methanol dissolves sugars, organic acids and phenolic compounds of low molecular weight. Lastly, the resulting residue is treated with aqueous acetone (4:6), in order to extract polymerized polyphenols. Sanoner et al. [53] applied this protocol by blending the apple powder with each solvent for 5 min, using an Ultra-Turrax blender, and the mixture was filtered through a G3 sintered glass filter. Moreover, UAE, ASE and SFE play an important role as real potential sustainable technique for industrial applications for polyphenols extraction. In recent works, ASE conditions have been optimized for the extraction of polyphenols from apple peel and pulp, achieving good recovery and repeatability. ASE allows to reduce the volume of solvent required for the extraction, to shorten the analysis time and the handling necessary to obtain precise results [55]. As regard to SFE, several works aimed to recover phenolic compounds applying CO_2 in supercritical conditions without and with ethanol (5%) as co-solvent. As CO_2 is non-polar, it is not a favorable solvent for polar polyphenols. However, the addition of organic co-solvents which could improve the solvating power and the yield of the extraction, such as ethanol, methanol and acetone, could be a suitable strategy [56]. Instead, Stefova et al. [57] used UAE with a methanol: water mixture for the extraction of polyphenols. Ultrasound can reduce the operating temperature of extraction for thermolabile compounds; moreover, the cavitation process that occurs during sonication causes the rupture of cell walls, thus enhancing solvent contact with available extractable cell material [58]. UAE has also been used for the extraction of organic acids; water or alcoholic mixtures were used as extraction solvents [26][40][59][60].

In regard to carotenoids and vitamins, their extraction from apple skin and pomace is usually carried out with acetone or a solution of acetone/petroleum ether as solvent [3][38]. Since carotenoids are strongly susceptible to oxidative degradation, due to the high number of conjugated double bonds, extraction must be performed in dark conditions or by adding antioxidant compounds, such as butylated hydroxytoluene (BHT), to the extraction solvent, in order to prevent photo-isomerization phenomena [61].

Unlike apple polar triterpenoids, such as betulinic and ursolic acid, whose extraction is carried out by using polar solvents (i.e., methanol and acetone) [62], a separate discussion is needed for the extraction of terpenes from apple tissues. As they are non-polar and highly volatile compounds, the better method for their extraction is the solid-phase microextraction (SPME), followed by their analysis by gas chromatographic (GC) methods. With SPME, the analytes are absorbed from the liquid or gaseous sample onto an absorbent coated fused silica fiber, which is part of the syringe needle, for a fixed time. The fiber is then inserted directly into a GC injection port for thermal desorption [63]. Other advantages of this technique are the absence of solvents and the possibility to separate and pre-concentrate the analytes in a single step [64]. Therefore, it is really helpful for the extraction of volatile compounds from apples, due to their low concentration and the complexity of the matrix [65]. As fiber coatings, DVB/CAR/PDMS (Divinylbenzene/Carboxen/Polydimethylsiloxane) and PDMS/DVB have been mainly used because they offer higher extraction efficiency and a clear pattern of volatile compounds [42][66]. Despite its several advantages, the limited amount of stationary phase on the fused silica fiber used for the SPME often does not ensure enough sensitivity and reproducibility. To overcome this problem, Madrera et al. [67] successfully applied stir bar sorption extraction (SBSE), a variation of SPME consisting on the use of magnetized bars covered with an absorbent polymer, for the extraction of apple pomace aroma. SBSE has the same advantages than SPME, but its sensibility increases around 100-fold because it uses a greater amount of stationary phase.

3.2. Chromatographic Methods

The determination of secondary metabolites in plant-based foods remains an analytical challenge, due to their low concentration and the complexity and diversity of their structures. The structural complexity of secondary metabolites often hinders most attempts to quantify these compounds by analytical methods not including preliminary separation steps [12][68]. In effect, to conduct a reliable detection and quantification of the main phytochemicals occurring in fruits, chromatographic methods (both LC and GC) either coupled with UV–Vis, fluorescence, or mass spectrometry (MS) detection, represent the gold standards methods [69][70][71][72][73]. The following paragraphs deepen on the

chromatographic methods that have been used for the determination of the main apple secondary metabolites, i.e., phenolics, organic acids, carotenoids and terpenes. The most common method for the separation and analysis of polyphenols and organic acids occurring in apple fruits is the high-performance liquid chromatography (HPLC) due to its high-resolution, efficiency, reproducibility and relatively short analysis time, without derivatization and no restriction on sample volatility [74][75][76][77][78][79]. Recent advances in apple phytochemicals analysis show a tendency for the application of environmentally friendly and faster techniques. This is evidenced, for example by the recently developed separative techniques, such as Ultrahigh-Performance Liquid Chromatography (UHPLC) and Ultra-Fast Liquid Chromatography that came from the evolution of packing materials used to improve resolution, and also contributed to such advances. Recently, UHPLC has been proposed for the analysis of polyphenols [80][81][82][83][84][85]. In general, peak efficiency and chromatographic resolution provided in UHPLC are higher than conventional HPLC. In addition, UHPLC methods can be considered more cost-effective because they typically need 80% less organic solvents than conventional HPLC methods [68]. Both HPLC and UHPLC can be easily coupled to a variety of detectors for polyphenols detection, including UV–Vis and MS.

To date, no single chromatographic methods capable of separating the different types of phenolic compounds, occurring in apples, are available. It is necessary to optimize the stationary phase, mobile phase, gradient elution, temperature and flow rate for each group of compounds [86]. Moreover, polyphenols stereochemistry, molecular weight, polarity and degree of polymerization could influence compounds retention. However, the reported methods for the separation of phenolics, as well as their glycosides, have been carried out mainly by reverse phase liquid chromatography (RPLC), on silica-based C18-bonded phase columns [57][83][87][88]. The average particle diameter of HPLC packings is typically 3–10 μm. With columns of smaller particle size, a larger number of plates per unit time is provided, with respect to columns with larger particle size [89]. As mobile phase, binary mixtures of aqueous formic acid or acetic acid and acetonitrile (ACN) or methanol (MeOH) as organic modifiers have been employed [57][83][87][88]. Typically, gradient elutions have been preferred, since multiple-step gradients are more suitable for complex mixtures, such as apple extracts. Although RPLC has been mostly chosen for apple polyphenols separation, Hollands et al. [90] used hydrophilic interaction chromatography (HILIC) to develop a robust and reliable analytical method for the extraction, separation and identification of monomeric and oligomeric procyanidins in apple extracts. HILIC separation mechanism is opposite to that of RP systems: polar stationary phase retains polar analytes, which are eluted by mixture of organic solvent (usually acetonitrile) and water [91]. Due to the complexity of procyanidins oligomers structures in the apple extract, normal phase silica columns were found to be not suitable for quantification purposes, particularly at a higher degree of polymerization. Instead, HILIC column ensured a better resolution of the chromatographic peaks [90].

As for phenolic compounds, reverse phase liquid chromatography is used also for the detection of organic acids by C18-bonded phase column. However, a method based on the use of an Aminex HPX cation-exchange column and an elution solvent consisting of sulfuric acid in bi-distilled water has been also reported for the quantification of malic acid [92]. The Aminex HPX-series of strong cation-exchange resins are prepared from a sulfonated polystyrene-divinylbenzene copolymer and are available in prepacked columns. One of the major problems experienced with the use of these columns are related to the co-elution of non-acid components and the poor resolution of the chromatographic peaks, thus limiting their use. Moreover, the ion exchange's separation mechanism implies that organic acids should be in their ionic form, so that a severe control of pH is required [93].

As regard detection, the most commonly used detector for HPLC is a variable-wavelength UV or UV–Vis detector, because both phenolics and organic acids absorb very well in the UV region [32][94][19][88][95][96][97][98][99]. The use of low UV detection wavelength, which ranged between 185 nm and 254 nm, allows to achieve high sensitivities in the determination of organic acids [100]. Moreover, for phenol compounds, HPLC–DAD provides extensive information; however, no single wavelength is ideal for monitoring all classes of phenolics, because they display absorbency maxima at different wavelengths (s belonging to flavanols, phenolics acids, dihydrochalcones and flavonols. Among them, quercetin glycosides were found to be the main polyphenols in the peel (203 ± 108 mg/100 g) and phenolic acids (10 ± 5 mg/100 g) in the flesh. Instead, by Liaudanskas et al. [95] flavonols were quantitated at 360 nm. The method implemented by these authors allowed the identification of 11 analytes: procyanidin B1, (+)-catechin, chlorogenic acid, procyanidin B2, (−)-epicatechin, rutin, hyperoside, isoquercitrin, avicularin, quercitrin and phloridzin. The elution order of quercetin-3-*O*-glycosides was as follows: quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (iso-quercitrin), quercetin-3-*O*-arabinoside (avicularin) and quercetin-3-*O*-rhamnoside (quercitrin), mainly according to other studies aimed at the evaluation of the quercetin derivatives profile in food extracts [69].

HPLC–UV for the quantification of polyphenols on *Malus* extracts allowed to reach limits of detection (LOD) and limits of quantification (LOQ) in the range 0.2–5.8 μg/mL and 0.1–7.1 μg/mL, respectively. Fluoresce detector (FL) in some cases offers higher selectivity and sensitivity compared to UV–Vis detection methods, so that it could be offered as a robust and

reliable alternative or to be complementary to UV–Vis detection systems. For example, Teleszko et al. [83] determined the polyphenolic profile in leaves and fruits of 2 cultivars of *Malus domestica* by UPLC–PDA–FL. In this case, the identification was also achieved, by using LC–MS/MS. Although LC–UV–Vis or LC–UV–FL are cheap and robust techniques for the quantification of polyphenols, their identification could be uniquely achieved through the comparison of retention times and UV–Vis spectra with those of authentic standards. Moreover, phenolic compounds are often linked to saccharidic moieties that are not UV-active, thus preventing the correct polyphenols identification.

Considering these difficulties, in many cases, it is necessary to use a more sensitive and selective detector such as a mass spectrometer to an LC system (LC–MS), as it allows unequivocal identification of the analytes thanks to the possibility to conduct MS/MS experiments [72][86][101][102][103][104]. Liquid chromatography coupled to mass spectrometry (LC–MS) or tandem mass spectrometry (LC–MS/MS) are among the most widely used techniques for the analysis of polyphenols occurring in apple fruits [105][106][107][108][109]. The employment of these methods is particularly helpful not only for their quantitative determination but also for their characterization and structural elucidation, especially when MS^n fragmentation can be achieved [68]. For the ionization of apple polyphenols in LC–MS, electrospray ionization (ESI) in negative mode has been, by far, the most generalized interface employed [106][108][110]. The negative ionization mode provides the highest sensitivity and results in limited fragmentation of flavonoids. Instead, for the identification of anthocyanins, positive ionization mode is mainly chosen, as it gives the best results [111]. As an extra-certainty to the molecular mass determination, the combination of both ionization modes (positive and negative) in MS^n scan could be implemented [13]. Other less common techniques used in the analysis of polyphenols areatmospheric pressure ionization techniques, such as atmospheric pressure chemical ionization (APCI). For instance, LC–APCI–MS in positive ionization mode was proposed for the characterization of apple polyphenols by Alonso-Salces et al. [112], who reported for the first time five isorhamnetin glycosides, two hydroxyphloretin glycosides and quercetin in apple peel.

As mass analyzers, multiple types are available and have been proposed for phenols detection, among them triple quadrupole (QqQ) [113], linear ion-trap [80], time-of-flight [80], Orbitrap [82] and QTrap [114], among others. QTrap mass analyzers are hybrid instruments combining a quadrupole and a liner ion-trap in a similar configuration to a QqQ instrument and they gaining popularity for the analysis of food products. LC–MS methods offer a better selectivity compared to LC–UV methods. In this regard, Verdu et al. [106] developed an UHPLC–UV and UHPLC–MS/MS for the quantification of phenolic compounds in apple juices. The developed methods were validated for 15 major compounds based on linearity, limits of detection and quantification, recovery and precision tests. A comparison of the quantifications showed that both UHPLC–UV and UHPLC–MS/MS had an excellent correlation for major compounds, quantified in 120 different samples. However, the slope value showed an overestimation of the UV detector for chlorogenic acid, explained by the co-elution of unknown UV-absorbing minor compounds, highlighting the advantage of using MS as detector and the selected reaction monitoring (SRM) mode to quantify highly concentrated samples. LC coupled to high-resolution mass spectrometry (HRMS), which provides accurate mass measurements, has recently obtained popularity due to its ability to give more comprehensive information concerning the exact molecular mass, elemental composition and detailed molecular structure of a given compound. LC–HRMS provides data of exceptional quality regarding apple metabolites. Indeed, it is currently used to aid in the identification of a broader range of phenolic compounds. High-resolution MS/MS has several advantages; indeed, they greatly improve the sensitivity and the accuracy of the mass measurements, thus allowing a simplified identification of the analytes and a differentiation between molecular formulas having the same nominal masses [108].

UV and MS are, often, both used for the identification and quantification of apple polyphenols [115][116]. Bizjak et al. [115] studied the changes of the concentrations of sugars, organic acids and a wide range of polyphenols as well as total phenolic compounds in the "Braeburn" apple peel during the advanced maturation of apples in two growing, by coupling HPLC to both detectors. A total of 21 phenolics, belonging to five groups, namely hydroxycinnamic acids, dihydrochalcones, flavonols, flavanols and anthocyanins, were identified and quantified. Identification was performed by comparing the retention times and their UV−Vis spectra from 200 to 600 nm and confirmed by MS and MS^2 data that were acquired in positive and negative ions mode by using full-scan-data-dependent MS scanning from *m/z* 115 to 2000. The results obtained could be useful to understand the evolution and highest concentration of primary and secondary metabolites in the last stages of apple ripening and their relation as well. Instead, Ramirez-Ambrosi et al. [13] used ultrahigh performance liquid chromatography with diode array detection coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry (UHPLC–DAD–ESI–Q-ToF-MS), in order to obtain polyphenolic profile of apples, apple pomace and apple juice from Asturian cider apples, in a single run of 22 min. This method allowed the automatic and simultaneous acquisition of accurate mass to charge values, overcoming chromatographic co-elution problems. With this technique, a large number of phenolic acids, organic acids and flavonoids were identified [13].

References

1. Bevilacqua, M.; Bro, R.; Marini, F.; Rinnan, Å.; Rasmussen, M.A.; Skov, T. Recent chemometrics advances for foodomics. TrAC Trends Anal. Chem. 2017, 96, 42–51.

2. Herrero, M.; Simò, C.; Garcia-Canasa, V.; Ibanez, E.; Cifuentes, A. Foodomics: MS-based strategies in modern food science and nutrition. Mass Spectrom. Rev. 2012, 31, 49–69.

3. Giordani, E.; Doumett, S.; Nin, S.; Del Bubba, M. Selected primary and secondary metabolites in fresh persimmon (Diospyros kaki Thunb.): A review of analytical methods and current knowledge of fruit composition and health benefits. Food Res. Int. 2011, 44, 1752–1767.

4. Magwaza, L.S.; Opara, U.L. Analytical methods for determination of sugars and sweetness of horticultural products-A review. Sci. Hortic. 2015, 184, 179–192.

5. Huber, G.M.; Rupasinghe, H.P.V. Phenolic profiles and antioxidant properties of apple skin extracts. J. Food Sci. 2009, 74, 693–700.

6. Feliciano, R.P.; Antunes, C.; Ramos, A.; Serra, A.T.; Figueira, M.E.; Duarte, C.M.M.; de Carvalho, A.; Bronze, M.R. Characterization of traditional and exotic apple varieties from Portugal. Part 1—Nutritional, phytochemical and sensory evaluation. J. Funct. Foods 2010, 2, 35–45.

7. Figuerola, F.; Hurtado, M.L.; Estévez, A.M.; Chiffelle, I.; Asenjo, F. Fibre concentrates from apple pomace and citrus peel as potential fibre sources for food enrichment. Food Chem. 2005, 91, 395–401.

8. Mohanta, S.; Singh, S.K.; Kumar, B.; Gulati, M.; Jyoti, J.; Som, S.; Panchal, S.; Melkani, I.; Banerjee, M.; Sinha, S.K.; et al. Solidification of liquid Modified Apple Polysaccharide by its adsorption on solid porous carriers through spray drying and evaluation of its potential as binding agent for tablets. Int. J. Biol. Macromol. 2018, 120, 1975–1998.

9. Ajanaku, C.; Echeme, J.; Mordi, R.; Bolade, O.; Okoye, S.; Jonathan, H.; Ejilude, O. In-vitro antibacterial, phytochemical, antimycobacterial activities and GC-MS analyses of Bidens pilosa leaf extract. J. Microbiol. Biotechnol. Food Sci. 2018, 8, 721–725.

10. Vieira, F.G.K.; Borges, G.D.S.C.; Copetti, C.; Amboni, R.D.D.M.C.; Denardi, F.; Fett, R. Physico-chemical and antioxidant properties of six apple cultivars (Malus domestica Borkh) grown in southern Brazil. Sci. Hortic. 2009, 122, 421–425.

11. Perry, A.; Rasmussen, H.; Johnson, E.J. Xanthophyll (lutein, zeaxanthin) content in fruits, vegetables and corn and egg products. J. Food Compos. Anal. 2009, 22, 9–15.

12. Ignat, I.; Volf, I.; Popa, V.I. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. Food Chem. 2011, 126, 1821–1835.

13. Ramirez-Ambrosi, M.; Abad-Garcia, B.; Viloria-Bernal, M.; Garmon-Lobato, S.; Berrueta, L.A.; Gallo, B. A new ultrahigh performance liquid chromatography with diode array detection coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry analytical strategy for fast analysis and improved characterization of phenolic compounds in ap. J. Chromatogr. A 2013, 1316, 78–91.

14. Canarini, A.; Kaiser, C.; Merchant, A.; Richter, A.; Wanek, W. Root exudation of primary metabolites: Mechanisms and their roles in plant responses to environmental stimuli. Front. Plant Sci. 2019, 10.

15. Guan, Y.; Peace, C.; Rudell, D.; Verma, S.; Evans, K. QTLs detected for individual sugars and soluble solids content in apple. Mol. Breed. 2015, 35, 1–13.

16. Karkacier, M.; Erbas, M.; Uslu, M.K.; Aksu, M. Comparison of different extraction and detection methods for sugars using amino-bonded phase HPLC. J. Chromatogr. Sci. 2003, 41, 331–333.

17. Ma, C.; Sun, Z.; Chen, C.; Zhang, L.; Zhu, S. Simultaneous separation and determination of fructose, sorbitol, glucose and sucrose in fruits by HPLC-ELSD. Food Chem. 2014, 145, 784–788.

18. Filip, M.; Vlassa, M.; Coman, V.; Halmagyi, A. Simultaneous determination of glucose, fructose, sucrose and sorbitol in the leaf and fruit peel of different apple cultivars by the HPLC-RI optimized method. Food Chem. 2016, 199, 653–659.

19. Zhang, Y.; Li, P.; Cheng, L. Developmental changes of carbohydrates, organic acids, amino acids, and phenolic compounds in "Honeycrisp" apple flesh. Food Chem. 2010, 123, 1013–1018.

20. Yang, S.; Meng, Z.; Li, Y.; Chen, R.; Yang, Y.; Zhao, Z. Evaluation of Physiological Characteristics, Soluble Sugars, Organic Acids and Volatile Compounds in "Orin" Apples (Malus domestica) at Different Ripening Stages. Molecules 2021, 26, 807.

21. Liu, Y.; Ying, Y.; Yu, H.; Fu, X. Comparison of the HPLC method and FT-NIR analysis for quantification of glucose, fructose, and sucrose in intact apple fruits. J. Agric. Food Chem. 2006, 54, 2810–2815.

22. Liu, Y.-d.; Ying, Y. Bin Measurement of sugar content in Fuji apples by FT-NIR spectroscopy. J. Zhejiang Univ. Sci. 2004, 5, 651–655.

23. Arain, S.; Sherazi, S.T.H.; Bhanger, M.I.; Memon, N.; Mahesar, S.A.; Rajput, M.T. Prospects of fatty acid profile and bioactive composition from lipid seeds for the discrimination of apple varieties with the application of chemometrics. Grasas y Aceites 2012, 63, 175–183.

24. Baeza-Jiménez, R.; López-Martínez, L.X.; García-Varela, R.; García, H.S. Lipids in fruits and vegetables: Chemistry and biological activities. Fruit Veg. Phytochem. Chem. Hum. Health Second Ed. 2017, 1, 423–449.

25. Song, J.; Bangerth, F. Fatty acids as precursors for aroma volatile biosynthesis in pre-climacteric and climacteric apple fruit. Postharvest Biol. Technol. 2003, 30, 113–121.

26. Wu, J.; Gao, H.; Zhao, L.; Liao, X.; Chen, F.; Wang, Z.; Hu, X. Chemical compositional characterization of some apple cultivars. Food Chem. 2007, 103, 88–93.

27. Chiu, H.H.; Kuo, C.H. Gas chromatography-mass spectrometry-based analytical strategies for fatty acid analysis in biological samples. J. Food Drug Anal. 2020, 28, 60–73.

28. Duroňová, K.; Márová, I.; Čertík, M.; Obruča, S. Changes in lipid composition of apple surface layer during long-term storage in controlled atmosphere. Chem. Pap. 2012, 66, 940–948.

29. Walia, M.; Rawat, K.; Bhushan, S.; Padwad, Y.S.; Singh, B. Fatty acid composition, physicochemical properties, antioxidant and cytotoxic activity of apple seed oil obtained from apple pomace. J. Sci. Food Agric. 2014, 94, 929–934.

30. Radenkovs, V.; Kviesis, J.; Juhnevica-Radenkova, K.; Valdovska, A.; Püssa, T.; Klavins, M.; Drudze, I. Valorization of wild apple (Malus spp.) by-products as a source of essential fatty acids, tocopherols and phytosterols with antimicrobial activity. Plants 2018, 7, 90.

31. Seppanen-Laakso, T.; Laakso, I.; Hiltunen, R. Analysis of fatty acids by gas chromatography, and its relevance to research on health and nutrition. Anal. Chim. Acta 2002, 465, 39–62.

32. 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.

33. 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.

34. 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.

35. Alonso-Salces, R.M.; Barranco, A.; Abad, B.; Berrueta, L.A.; Gallo, B.; Vicente, F. Polyphenolic Profiles of Basque Cider Apple Cultivars and Their Technological Properties. J. Agric. Food Chem. 2004, 52, 2938–2952.

36. Vanzani, P.; Rossetto, M.; Rigo, A.; Vrhovsek, U.; Mattivi, F.; D'Amato, E.; Scarpa, A.M. Major phytochemicals in apple cultivars: Contribution to peroxyl radical trapping efficiency. J. Agric. Food Chem. 2005, 53, 3377–3382.

37. Łata, B.; Trampczynska, A.; Paczesna, J. Cultivar variation in apple peel and whole fruit phenolic composition. Sci. Hortic. 2009, 121, 176–181.

38. Ampomah-Dwamena, C.; Deinoprat, S.; Lewis, D.; Sutherland, P.; Volz, R.; Allan, A.C. Metabolic and gene expression analysis of apple (Malus × domestica) carotenogenesis. J. Exp. Bot. 2012, 63, 4497–4511.

39. Mikulic-Petkovsek, M.; Schmitzer, V.; Slatnar, A.; Stampar, F.; Veberic, R. Composition of Sugars, Organic Acids, and Total Phenolics in 25 Wild or Cultivated Berry Species. J. Food Sci. 2012, 77, 1064–1070.

40. Ma, B.; Yuan, Y.; Gao, M.; Li, C.; Ogutu, C.; Li, M.; Ma, F. Determination of predominant organic acid components in malus species: Correlation with apple domestication. Metabolites 2018, 8, 74.

41. Nieuwenhuizen, N.J.; Green, S.A.; Chen, X.; Bailleul, E.J.D.; Matich, A.J.; Wang, M.Y.; Atkinson, R.G. Functional Genomics Reveals That a Compact Terpene Synthase Gene Family Can Account for Terpene Volatile Production in Apple. Plant Physiol. 2020, 161, 787–804.

42. Ferreira, L.; Perestrelo, R.; Caldeira, M.; Câmara, J.S. Characterization of volatile substances in apples from Rosaceae family by headspace solid-phase microextraction followed by GC-qMS. J. Sep. Sci. 2009, 32, 1875–1888.

43. Mehinagic, E.; Prost, C.; Demaimay, M. Optimization of extraction of apple aroma by dynamic headspace and influence of saliva on extraction of volatiles. J. Agric. Food Chem. 2004, 52, 5175–5182.

44. Sut, S.; Poloniato, G.; Malagoli, M.; Dall'acqua, S. Fragmentation of the main triterpene acids of apple by LC- APCI-MSn. J. Mass Spectrom. 2018, 53, 882–892.

45. Mehinagic, E.; Prost, C.; Demaimay, M. Representativeness of Apple Aroma Extract Obtained by Vacuum Hydrodistillation: Comparison of Two Concentration Techniques. J. Food Sci. 2003, 68, 2411–2415.

46. Rezaei, S.; Rezaei, K.; Haghighi, M.; Labbafi, M. Solvent and Solvent to Sample Ratio as Main Parameters in the Micro wave-assisted Extraction of Polyphenolic Compounds from Apple Pomace. Food Sci. Biotechnol. 2013, 22, 1269–1274.

47. Rowan, D.D. Volatile metabolites. Metabolites 2011, 1, 41–63.

48. Müller, E.; Berger, R.; Blass, E.; Sluyts, D.; Pfennig, A. Liquid-liquid Extraction. Encicl. Ind. Chem. 2000, 21, 250–303.

49. Naboulsi, I.; Aboulmouhajir, A. Plants extracts and secondary metabolites, their extraction methods and use in agriculture for controlling crop stresses and improving productivity: A review. Acad. J. Med. Plants 2018, 6, 223–240.

50. Bai, X.L.; Yue, T.L.; Yuan, Y.H.; Zhang, H.W. Optimization of microwave-assisted extraction of polyphenols from apple pomace using response surface methodology and HPLC analysis. J. Sep. Sci. 2010, 33, 3751–3758.

51. Eskilsson, C.S.; Bjorklund, E. Analytcal-scale microwave-assisted extrcation. J. Chromatogr. A 2000, 902, 227–250.

52. Bekele, E.A.; Annaratone, C.E.P.; Hertog, M.L.A.T.M.; Nicolai, B.M.; Geeraerd, A.H. Multi-response optimization of the extraction and derivatization protocol of selected polar metabolites from apple fruit tissue for GC-MS analysis. Anal. Chim. Acta 2014, 824, 42–56.

53. Sanoner, P.; Guyot, S.; Marnet, N.; Molle, D.; Drilleau, J.F. Polyphenol profiles of French cider apple varieties (Malus domestica sp.). J. Agric. Food Chem. 1999, 47, 4847–4853.

54. Guyot, S.; Marnet, N.; Laraba, D.; Sanoner, P.; Drilleau, J.F. Reversed-Phase HPLC following Thiolysis for Quantitative Estimation and Characterization of the Four Main Classes of Phenolic Compounds in Different Tissue Zones of a French Cider Apple Variety (Malus domestica Var. Kermerrien). J. Agric. Food Chem. 1998, 46, 1698–1705.

55. Alonso-Salces, R.M.; Korta, E.; Barranco, A.; Berrueta, L.A.; Gallo, B.; Vicente, F. Determination of polyphenolic profiles of Basque cider apple varieties using accelerated solvent extraction. J. Agric. Food Chem. 2001, 49, 3761–3767.

56. Ferrentino, G.; Morozova, K.; Mosibo, O.K.; Ramezani, M.; Scampicchio, M. Biorecovery of antioxidants from apple pomace by supercritical fluid extraction. J. Clean. Prod. 2018, 186, 253–261.

57. Stefova, M.; Petkovska, A.; Ugarkovic, S.; Stanoeva, J.P. Strategy for optimized use of LC-MSn for determination of the polyphenolic profiles of apple peel, flesh and leaves. Arab. J. Chem. 2019, 12, 5180–5186.

58. Pingret, D.; Fabiano-Tixier, A.S.; Le Bourvellec, C.; Renard, C.M.G.C.; Chemat, F. Lab and pilot-scale ultrasound-assisted water extraction of polyphenols from apple pomace. J. Food Eng. 2012, 111, 73–81.

59. Bizjak, J.; Weber, N.; Mikulic-Petkovsek, M.; Slatnar, A.; Stampar, F.; Alam, Z.; Stich, K.; Halbwirth, H.; Veberic, R. Influence of phostrade Ca on color development and anthocyanin content of "Braeburn" apple (Malus domestica Borkh.). HortScience 2013, 48, 193–199.

60. Feng, F.; Li, M.; Ma, F.; Cheng, L. Effects of location within the tree canopy on carbohydrates, organic acids, amino acids and phenolic compounds in the fruit peel and flesh from three apple (Malus 3 domestica) cultivars. Hortic. Res. 2014, 1, 1–7.

61. Rodríguez-Bernaldo de Quirós, A.; Costa, H.S. Analysis of carotenoids in vegetable and plasma samples: A review. J. Food Compos. Anal. 2006, 19, 97–111.

62. Fan, J.; Liao, D.; Zhang, X. Ultrasonic assisted extraction of ursolic acid from apple pomace: A novel and facile technique. Sep. Sci. Technol. 2016, 6395, 1344–1350.

63. Prosen, H.; Zupančič-Kralj, L. Solid-phase microextraction. TrAC Trends Anal. Chem. 1999, 18, 272–282.

64. Bianco, G.; Novario, G.; Zianni, R.; Cataldi, T.R.I. Comparison of two SPME fibers for the extraction of some off-flavor cork-taint compounds in bottled wines investigated by GC-HRMS. Anal. Bioanal. Chem. 2009, 393, 2019–2027.

65. Schmutzer, G.R.; Magdas, A.D.; David, L.I.; Moldovan, Z. Determination of the Volatile Components of Apple Juice Using Solid Phase Microextraction and Gas Chromatography-Mass Spectrometry. Anal. Lett. 2014, 47, 1683–1696.

66. Risticevic, S.; Souza-Silva, E.A.; Gionfriddo, E.; DeEll, J.R.; Cochran, J.; Hopkins, W.S.; Pawliszyn, J. Application of in vivo solid phase microextraction (SPME) in capturing metabolome of apple (Malus ×domestica Borkh.) fruit. Sci. Rep. 2020, 10, 1–11.

67. Sbse-gc-ms, S.; Rodr, R. Determination of Volatile Compounds in Apple Pomace by Stir Bar Sorptive Extraction and Gas Chromatography-Mass. J. Food Sci. 2011, 76, C1326–C1334.

68. Lucci, P.; Saurina, J.; Núñez, O. Trends in LC-MS and LC-HRMS analysis and characterization of polyphenols in food. TrAC Trends Anal. Chem. 2017, 88, 1–24.

69. Pascale, R.; Acquavia, M.A.; Cataldi, T.R.I.; Onzo, A.; Coviello, D.; Bufo, S.A.; Scrano, L.; Ciriello, R.; Guerrieri, A.; Bianco, G. Profiling of quercetin glycosides and acyl glycosides in sun-dried peperoni di Senise peppers (Capsicum annuu

m L.) by a combination of LC-ESI (—)-MS/MS and polarity prediction in reversed-phase separations. Anal. Bioanal. Chem. 2020, 412, 3005–3015.

70. Pascale, R.; Bianco, G.; Cataldi, T.R.I.; Buchicchio, A.; Losito, I.; Altieri, G.; Genovese, F.; Tauriello, A.; Di Renzo, G.C.; Lafiosca, M.C. Investigation of the Effects of Virgin Olive Oil Cleaning Systems on the Secoiridoid Aglycone Content Using High Performance Liquid Chromatography–Mass Spectrometry. J. Am. Oil Chem. Soc. 2018, 95, 665–671.

71. Pascale, R.; Bianco, G.; Cataldi, T.R.I.; Kopplin, P.S.; Bosco, F.; Vignola, L.; Uhl, J.; Lucio, M.; Milella, L. Mass spectrometry-based phytochemical screening for hypoglycemic activity of Fagioli di Sarconi beans (Phaseolus vulgaris L.). Food Chem. 2018, 242, 497–504.

72. Bianco, G.; Pascale, R.; Carbone, C.F.; Acquavia, M.A.; Cataldi, T.R.I.; Schmitt-Kopplin, P.; Buchicchio, A.; Russo, D.; Milella, L. Determination of soyasaponins in Fagioli di Sarconi beans (Phaseolus vulgaris L.) by LC-ESI-FTICR-MS and evaluation of their hypoglycemic activity. Anal. Bioanal. Chem. 2018, 410, 1561–1569.

73. Bianco, G.; Pascale, R.; Lelario, F.; Bufo, S.A.; Cataldi, T.R.I. Investigation of Glucosinolates by Mass Spectrometry. In Glucosinolates; Mérillon, J.-M., Ramawat, K.G., Eds.; Springer International Publishing: Cham, Switzerland, 2017; pp. 431–461. ISBN 978-3-319-25462-3.

74. Ventura, G.; Calvano, C.D.; Losito, I.; Bianco, G.; Pascale, R.; Palmisano, F.; Cataldi, T.R.I. Effect of pH and mobile phase additives on the chromatographic behaviour of an amide-embedded stationary phase: Cyanocobalamin and its diaminemonochloro-platinum(II) conjugate as a case study. J. Sep. Sci. 2019, 42, 1155–1162.

75. Pascale, R.; Bianco, G.; Coviello, D.; Cristina Lafiosca, M.; Masi, S.; Mancini, I.M.; Bufo, S.A.; Scrano, L.; Caniani, D. Validation of a liquid chromatography coupled with tandem mass spectrometry method for the determination of drugs in wastewater using a three-phase solvent system. J. Sep. Sci. 2020, 43, 886–895.

76. Natale, A.; Nardiello, D.; Palermo, C.; Muscarella, M.; Quinto, M.; Centonze, D. Development of an analytical method for the determination of polyphenolic compounds in vegetable origin samples by liquid chromatography and pulsed amperometric detection at a glassy carbon electrode. J. Chromatogr. A 2015, 1420, 66–73.

77. Dordevic, B.; Durovic, D.; Zec, G.; Radovic, A.; Vulic, T. Bio-chemical properties and susceptibility to fire blight (Erwinia amylovora Burrill) of scab-resistant apple cultivars (Malus domestica Borkh.). Folia Hortic. 2019, 31, 253–261.

78. Petkovska, A.; Gjamovski, V.; Stanoeva, J.P.; Stefova, M. Characterization of the polyphenolic profiles of peel, flesh and leaves of Malus domestica cultivars using UHPLC-DAD-HESI-MSn. Nat. Prod. Commun. 2017, 12, 35–42.

79. Marks, S.C.; Mullen, W.; Crozier, A. Flavonoid and chlorogenic acid profiles of English cider apples. J. Sci. Food Agric. 2007, 87, 719–728.

80. Jakobek, L.; García-Villalba, R.; Tomás-Barberán, F.A. Polyphenolic characterisation of old local apple varieties from Southeastern European region. J. Food Compos. Anal. 2013, 31, 199–211.

81. De Paepe, D.; Servaes, K.; Noten, B.; Diels, L.; De Loose, M.; Van Droogenbroeck, B.; Voorspoels, S. An improved mass spectrometric method for identification and quantification of phenolic compounds in apple fruits. Food Chem. 2013, 136, 368–375.

82. Navarro, M.; Moreira, I.; Arnaez, E.; Quesada, S.; Azofeifa, G.; Vargas, F.; Alvarado, D.; Chen, P. Polyphenolic characterization and antioxidant activity of malus domestica and prunus domestica cultivars from Costa Rica. Foods 2018, 7, 15.

83. Teleszko, M.; Wojdyło, A. Comparison of phenolic compounds and antioxidant potential between selected edible fruits and their leaves. J. Funct. Foods 2015, 14, 736–746.

84. Raudone, L.; Raudonis, R.; Liaudanskas, M.; Viskelis, J.; Pukalskas, A.; Janulis, V. Phenolic Profiles and Contribution of Individual Compounds to Antioxidant Activity of Apple Powders. J. Food Sci. 2016, 81, C1055–C1061.

85. Oszmiański, J.; Wojdyło, A.; Kolniak, J. Effect of pectinase treatment on extraction of antioxidant phenols from pomace, for the production of puree-enriched cloudy apple juices. Food Chem. 2011, 127, 623–631.

86. López-fernández, O.; Domínguez, R.; Pateiro, M.; Munekata, P.E.S.; Rocchetti, G.; Lorenzo, J.M. Determination of polyphenols using liquid chromatography–tandem mass spectrometry technique (LC–MS/MS): A review. Antioxidants 2020, 9, 479.

87. Serni, E.; Venir, E.; Romano, G.; Guerra, W.; Robatscher, P. Determination of Major Phenolics Content in Dried Apples from Three New Cultivars (Malus domestica Borkh.) Using HPLC-UV-FL with Pentafluorophenyl Stationary Phase. Food Anal. Methods 2020, 13, 863–871.

88. Wojdyło, A.; Oszmiański, J. Antioxidant activity modulated by polyphenol contents in apple and leaves during fruit development and ripening. Antioxidants 2020, 9, 567.

89. Wollgast, J.; Anklam, E. Review on polyphenols in Theobroma cacao: Changes in composition during the manufacture of chocolate and methodology for identification and quantification. Food Res. Int. 2000, 33, 423–447.

90. Hollands, W.J.; Voorspoels, S.; Jacobs, G.; Aaby, K.; Meisland, A.; Garcia-Villalba, R.; Tomas-Barberan, F.; Piskula, M. K.; Mawson, D.; Vovk, I.; et al. Development, validation and evaluation of an analytical method for the determination of monomeric and oligomeric procyanidins in apple extracts. J. Chromatogr. A 2017, 1495, 46–56.

91. Bernal, J.; Ares, A.M.; Pól, J.; Wiedmer, S.K. Hydrophilic interaction liquid chromatography in food analysis. J. Chromatogr. A 2011, 1218, 7438–7452.

92. Veberic, R.; Schmitzer, V.; Petkovsek, M.M.; Stampar, F. Impact of Shelf Life on Content of Primary and Secondary Metabolites in Apple (Malus domestica Borkh.). J. Food Sci. 2010, 75, 461–468.

93. Blake, J.D.; Clarke, M.L.; Richards, G.N. Determination of organic acids in sugar cane process juice by high-performance liquid chromatography: Improved resolution using dual aminex HPX-87H cation-exchange columns equilibrated to different temperatures. J. Chromatogr. A 1987, 398, 265–277.

94. Arnous, A.; Meyer, A.S. Comparison of methods for compositional characterization of grape (Vitis vinifera L.) and apple (Malus domestica) skins. Food Bioprod. Process. 2008, 86, 79–86.

95. Liaudanskas, M.; Viškelis, P.; Jakštas, V.; Raudonis, R.; Kviklys, D.; Milašius, A.; Janulis, V. Application of an optimized HPLC method for the detection of various phenolic compounds in apples from Lithuanian cultivars. J. Chem. 2014, 2014.

96. Sowa, A.; Zgórka, G.; Szykuba, A.; Franiczek, R.; Gbikowska, B.; Gamian, A.; Sroka, Z. Analysis of polyphenolic compounds in extracts from leaves of some malus domestica cultivars: Antiradical and antimicrobial analysis of these extracts. BioMed Res. Int. 2016, 2016.

97. Le Bourvellec, C.; Bouzerzour, K.; Ginies, C.; Regis, S.; Plé, Y.; Renard, C.M.G.C. Phenolic and polysaccharidic composition of applesauce is close to that of apple flesh. J. Food Compos. Anal. 2011, 24, 537–547.

98. Le Bourvellec, C.; Bureau, S.; Renard, C.M.G.C.; Plenet, D.; Gautier, H.; Touloumet, L.; Girard, T.; Simon, S. Cultivar and year rather than agricultural practices affect primary and secondary metabolites in apple fruit. PLoS ONE 2015, 10, e0141916.

99. Zhang, L.; Xu, Q.; You, Y.; Chen, W.; Xiao, Z.; Li, P.; Ma, F. Characterization of quercetin and its glycoside derivatives in Malus germplasm. Hortic. Environ. Biotechnol. 2018, 59, 909–917.

100. Mato, I.; Suárez-Luque, S.; Huidobro, J.F. A review of the analytical methods to determine organic acids in grape juices and wines. Food Res. Int. 2005, 38, 1175–1188.

101. Pascale, R.; Bianco, G.; Calace, S.; Masi, S.; Mancini, I.M.; Mazzone, G.; Caniani, D. Method development and optimization for the determination of benzene, toluene, ethylbenzene and xylenes in water at trace levels by static headspace extraction coupled to gas chromatography–barrier ionization discharge detection. J. Chromatogr. A 2018, 1548, 10–18.

102. Onzo, A.; Acquavia, M.A.; Cataldi, T.R.I.; Ligonzo, M.; Coviello, D.; Pascale, R.; Martelli, G.; Bondoni, M.; Scrano, L.; Bianco, G. Coceth sulfate characterization by electrospray ionization tandem mass spectrometry. Rapid Commun. Mass Spectrom. 2020, 34, 1–10.

103. Lelario, F.; De Maria, S.; Rivelli, A.R.; Russo, D.; Milella, L.; Bufo, S.A.; Scrano, L. LC-FTICR-MS and IRMPD in a Commercial Variety and and their Anticholinesterase and Antioxidant Activities. Toxins 2019, 11, 230.

104. Lelario, F.; Labella, C.; Napolitano, G.; Scrano, L.; Bufo, S.A. Fragmentation study of major spirosolane-type glycoalkaloids by collision-induced dissociation linear ion trap and infrared multiphoton dissociation Fourier transform ion cyclotron resonance mass spectrometry. Rapid Commun. Mass Spectrom. 2016, 30, 2395–2406.

105. Šavikin, K.; Živković, J.; Zdunić, G.; Godevac, D.; Dordević, B.; Dojčinović, B.; Dordević, N. Phenolic and mineral profiles of four Balkan indigenous apple cultivars monitored at two different maturity stages. J. Food Compos. Anal. 2014, 35, 101–111.

106. Verdu, C.F.; Gatto, J.; Freuze, I.; Richomme, P.; Laurens, F.; Guilet, D. Comparison of two methods, UHPLC-UV and UHPLC-MS/MS, for the quantification of polyphenols in cider apple juices. Molecules 2013, 18, 10213–10227.

107. Kolniak-Ostek, J.; Oszmiański, J.; Wojdyło, A. Effect of l-ascorbic acid addition on quality, polyphenolic compounds and antioxidant capacity of cloudy apple juices. Eur. Food Res. Technol. 2013, 236, 777–798.

108. Lee, J.; Chan, B.L.S.; Mitchell, A.E. Identification/quantification of free and bound phenolic acids in peel and pulp of apples (Malus domestica) using high resolution mass spectrometry (HRMS). Food Chem. 2017, 215, 301–310.

109. Reis, S.F.; Rai, D.K.; Abu-Ghannam, N. Water at room temperature as a solvent for the extraction of apple pomace phenolic compounds. Food Chem. 2012, 135, 1991–1998.

110. Malec, M.; Le Quéré, J.M.; Sotin, H.; Kolodziejczyk, K.; Bauduin, R.; Guyot, S. Polyphenol profiling of a red-fleshed apple cultivar and evaluation of the color extractability and stability in the juice. J. Agric. Food Chem. 2014, 62, 6944–6954.

111. 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.

112. Alonso-Salces, R.M.; Ndjoko, K.; Queiroz, E.F.; Ioset, J.R.; Hostettmann, K.; Berrueta, L.A.; Gallo, B.; Vicente, F. On-line characterisation of apple polyphenols by liquid chromatography coupled with mass spectrometry and ultraviolet absorbance detection. J. Chromatogr. A 2004, 1046, 89–100.

113. 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.

114. Todea, D.; Cadar, O.; Simedru, D.; Roman, C.; Tanaselia, C.; Suatean, I.; Naghiu, A. Determination of major-to-trace minerals and polyphenols in different apple cultivars. Not. Bot. Horti Agrobot. Cluj Napoca 2014, 42, 523–529.

115. Bizjak, J.; Mikulic-Petkovsek, M.; Stampar, F.; Veberic, R. Changes in primary metabolites and polyphenols in the peel of "braeburn" Apples (Malus domestica Borkh.) during advanced maturation. J. Agric. Food Chem. 2013, 61, 10283–10292.

116. De Bernonville, T.D.; Gaucher, M.; Guyot, S.; Durel, C.E.; Dat, J.F.; Brisset, M.N. The constitutive phenolic composition of two Malus×domestica genotypes is not responsible for their contrasted susceptibilities to fire blight. Environ. Exp. Bot. 2011, 74, 65–73.