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Review

Analytical Methods for Extraction and Identification of Primary and Secondary Metabolites of Apple (*Malus domestica*) Fruits: A Review

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Abstract: 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. This review describes the analytical methods published in the last ten years, in order to analyze the most important primary and secondary metabolites of *Malus domestica* fruits. In detail, this review gives an account of the spectrophotometric, chromatographic and mass spectrometric methods. A discussion on the quantitative and qualitative analytical shortcomings for the identification of sugars, fatty acids, polyphenols, organic acids, carotenoids and terpenes found in apple fruits is reported.

Keywords: apple; metabolites; polyphenols; mass spectrometry–based analytical methods; GC–MS; LC–MS; LC–MS/MS

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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).

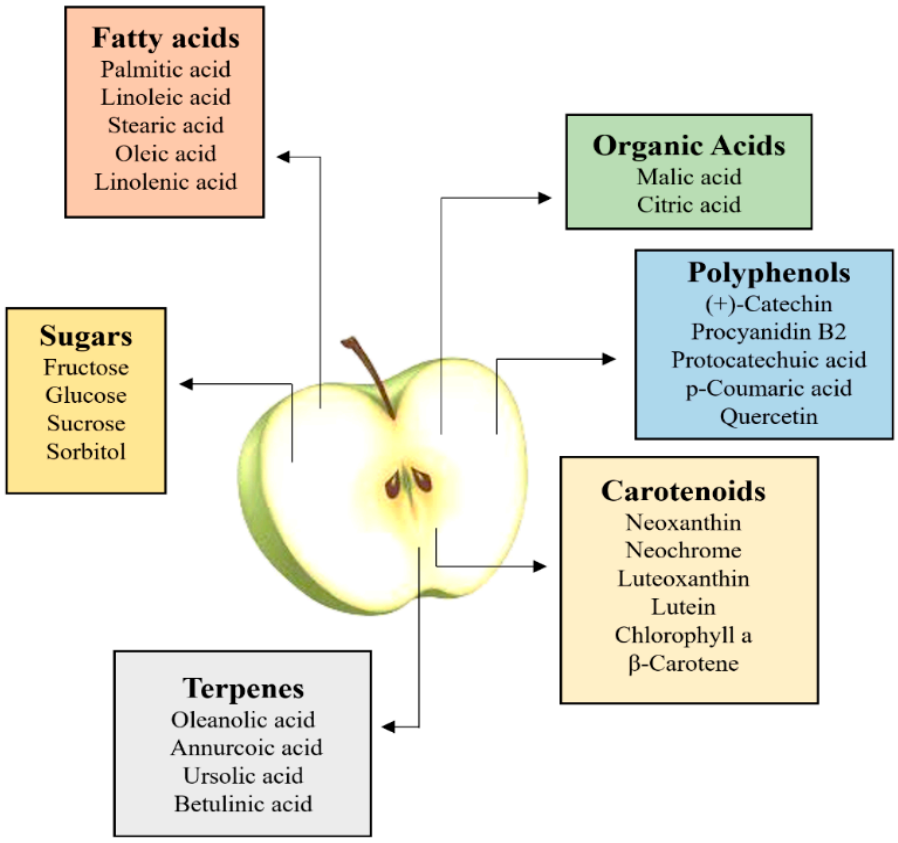

Figure 1. Main primary and secondary metabolites related to functional and nutraceutical values of apples.

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.

Over the years, many new *Malus domestica* species have been produced through new grafts and hybridizations, thus obtaining apple fruits with different chemical–physical characteristics and organoleptic properties [14]. The first evaluation regards the pulp, which usually has a yellow/white color and can be crunchy, pasty or floury, based on the percentage of pectin and cellulose [15]. The crunchiness or mellowness of the apple is affected by the water content, too, which is generally around the 85%. By varying this percentage, greater or less juiciness results [6]. The flavor that distinguishes the various apples species depends on the quantity of organic acids and sugars present in the pulp [16]. All the qualitative, physical and organoleptic parameters characterizing the existing apple varieties are listed in Table 1.

Table 1. Qualitative, physical and organoleptic parameters characterizing all the existing apple fruit varieties.

Apple Variety	Color	Dimension	Pulp	Flavor
Renetta	Yellow, tending to the green	Medium	Soft and pasty	Acid, medium sweetened
Golden Delicious	Gold yellow	Large	Crunchy and juicy	Very sweet
Stark Delicious	Bright red	Large	Crunchy and juicy	Sweet, little acidic
Granny Smith	Green	Medium	Crunchy	Acid
Fuji	Reddish with yellow-green streaks	Large	Crunchy and juicy	Sweet, little acidic
Pink Lady	Reddish/red	Medium	Crunchy	Sweet
Annurca	Bright red	Small	Crunchy	Acid
Royal Gala	Red with streaks	Small	Crunchy and juicy	Very sweet
Kanzi	Intense red or yellow	Medium	Hard and crunchy	Medium sweet and acid
Braeburn	Brown red	Medium–large	Crunchy	Medium sweet/acid
Morgenduft	Red with clear patches	Medium	Hard and juicy	Sweet and slightly acid

In addition to the apple fruit varieties listed in Table 1, some other types result from crosses between them [17]. Moreover, it should be considered that some small variations in each apple variety could occur, due to grafts and environmental conditions [16]. All the existing varieties of apple fruits show a characteristic profile of phytochemical components [18].

Like all fruits, apples have to meet commercial-quality parameters established by the European Commission Regulation (CE) N. 85/2004 15 January 2004. In detail, an apple fruit must be whole, healthy, clean, free of pathogens and free of flavors or odors other than usual, in order to be considered qualitatively relevant, from a commercial point of view.

In general, the phytochemical profile of apple fruits strongly correlates with most of their peculiar characteristics, such as taste, flavor and color. As previously said, it is difficult to estimate the number of the existing apple varieties among the world, due to the continuous changes and hybridizations performed [6].

However, it is possible to determine the phytochemical profile of apples to provide the real quantities of all natural compounds [19]. At the same time, new bioactive molecules could be identified as markers of a specific apple variety. Thus, an investigation of the complete metabolic profile of these fruits is mandatory. In this review, a discussion on the main analytical methods, differing in their extraction and detection protocols, developed in the last ten years for the investigation of primary and secondary metabolites occurring in apple fruits is provided. Particular attention is given to the more recent and cutting-edge techniques, thus making the readers could easily choose the methods that best suit their needs.

2. Apple Metabolites Analysis

Sugars, proteins, lipids, carotenoids, polyphenols and triterpenoids are present with different concentrations in apple fruits [20]. Since these phytochemical constituents have received greater attention in the last years, thanks to their beneficial properties, an overview of the main analytical methods applied for their analysis in apple fruits is useful [21]. Due to the pronounced chemo-variability observed in apple fruits, the availability of an update and comprehensive review dedicated to analytical methods, such as that reported in the present study, could be a useful tool in the standardization of apple fruit extracts, to be used in studies for various purposes. All the existing methods include a preliminary step, during which the extraction of the metabolites of interest from apple fruits is carried out. Successful metabolomic research, in fact, requires effective metabolite extraction. The sample pretreatment, which precedes metabolites analysis can widely vary depending on the type of matrix of interest (Table 2). If the samples are liquid, they can be analyzed directly after filtration and/or centrifugation; for solid samples, preliminary freeze-drying, grinding and extraction phases are required [22]. The extraction procedure is aimed at maximizing the amount and concentration of the compounds of interest and must be chosen according to the type of metabolites [23]. For this reason, extraction is probably the most critical step in the analysis of the plant and fruit metabolites. An ideal extraction should allow us to recover all metabolites of interest, without any chemical modifications. The extraction procedures differ according to the physicochemical properties of the compounds to be extracted, i.e., polarity and solubility [24]. Furthermore, after the extraction of metabolites, several methods could be employed in order to quantify them. Usually, spectrophotometric assays are used as a preliminary screening of the major classes of metabolites occurring in the fruits. They allow a general evaluation of the content of a specific metabolite class; however, they do not provide quantitative information about individual compounds [25]. On the other hand, higher sensitive and selective analytical techniques, such as mass spectrometry [26,27] or liquid chromatography coupled to mass spectrometry (LC–MS), are exploited for the separation and the detection of individual compounds, as well as for their structural characterization [28]. LC–MS is one of the most used techniques in foodomics, as well as in drugs analyses and in the environmental fields [29]. Starting from primary metabolites, with a special focus on sugars and fatty acids, a description of the extraction protocols of the different classes of metabolites occurring in apple fruits, as well as of the analytical techniques used for their analysis, is provided in the following sections. Particular attention will be addressed to secondary metabolites' extraction, detection and quantification, as they are the main compounds responsible for the well-known healthy properties of *Malus* fruits.

Table 2. Main extraction and detection methods used for apples primary and secondary metabolites, with their recommendations.

		Extraction and Detection Methods	Recommendations
Primary Metabolites	Sugars	Extraction LSE extraction with water as solvent Analysis HPLC–RI; HPLC–ELSD	• A preliminary immersion in methanol/water solution is important to avoid the hydrolysis of sucrose into free sugars; • HPLC–RI and HPLC–ELSD based methods are destructive, require the use of hazardous chemicals and are labor intensive. As an alternative, FT-NIR and ATR–FT-NIR are more rapid and simple to be used.
	Fatty acids	Extraction LSE extraction with hexane as solvent; Soxhlet extraction with petroleum ether Analysis GC–FID; GC–MS	• The derivatization is requested, because most fatty acids have high boiling points, thus being difficult to evaporate, and have a low FID response.

Table 2. *Cont.*

		Extraction and Detection Methods	Recommendations
Secondary Metabolites	Phenolics and organic acids	Extraction MAE or UAE extraction; Analysis HPLC–DAD; LC–MS/MS	• No single wavelength is ideal for monitoring all classes of phenolics, because they display absorbency maxima at different wavelengths; • As phenolic compounds are often linked to saccharidic moieties that are not UV-active, the correct polyphenols identification with LC–UV is not often feasible; • LC–MS/MS allow sensitive and selective analysis.
	Carotenoids	Extraction LSE extraction Analysis HPLC–DAD; LC–MS/MS	• Carotenoids are strongly susceptible to oxidative degradation, due to the high number of conjugated double bonds, thus extraction must be performed in dark conditions or by adding antioxidant compounds; • The λmax of individual carotenoids can vary depending on functional groups.
	Terpenes	Extraction SPME and SBSE extraction for volatile terpenes; UAE extraction for non-volatile triterpenoids; Analysis GC–FID; GC–MS for volatile terpenes; HPLC–DAD; LC–MS/MS for non-volatile triterpenoids	• It is highly recommended the combined use of retention index values (RI) and MS spectrum for the univocal peak assignment during analysis of volatile terpenes; • LC–DAD is often not useful, as these compounds absorb UV radiation weakly and only at wavelength of 200 nm.

3. Primary Metabolites: Extraction and Analysis

Primary metabolites are defined as chemical compounds which are necessary for plant growth, development and reproduction [30]. 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.

3.1. 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 [31]. 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 [31]. 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 [31].

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. [32] found that, with alcoholic extraction, some sugars may not adequately dissolve in proportion to their true concen-

tration 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) [32–34]. HPLC coupled to diode-array detector is quite limited, as sugars do not absorb UV light at a wavelength longer than 200 nm [32]. 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 [35].

One of the most used methods for the quantification of soluble carbohydrates, is HPLC–RI, being simple, rapid and economical [34]. 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. [34]. 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 [34]. Instead, Ma et al. [33] developed an HPLC–ELSD method to analyze sugars without derivatizations, with short analysis times (25 min) and good chromatographic separations. Recently, Yang et al. [36] 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 [36]. 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 [37,38]. By comparison of HPLC and FT-NIR for quantification of glucose, fructose and sucrose in intact apple fruit [37], it emerged that FT-NIR is more flexible and much faster than HPLC method.

3.2. 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 [39]. 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 [40]. 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 [41]. 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 [42]. Among the saturated and unsaturated FAs classes, palmitic acid (C16:0) and linoleic acid (C18:2) are the dominant compounds, respectively [42]. 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 fluc-

tuate 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 [41].

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 [43]. Lipids extraction from apple tissues is performed with hexane, and it is often followed by filtration of the supernatant through anhydrous sodium sulfate [44,45]. Soxhlet extraction with petroleum ether has also been used [45,46]. The derivatization is requested, because most fatty acids have high boiling points, thus being difficult to evaporate, and have a low FID response [47]. 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. [45] 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 [45].

4. Secondary Metabolites

To date, the structures and the average content of primary metabolites occurring in apples, are well known, for their nutritional value [42]. 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 [21]. 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 [48]. 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 [49–51] (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 [52].

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 [53].

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 [54]. 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 [55]. 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 [56]. Some of the terpene occurring in apples are α-farnesene, geranyl acetone and farnesol [57,58]. 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 [56]. 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 [59].

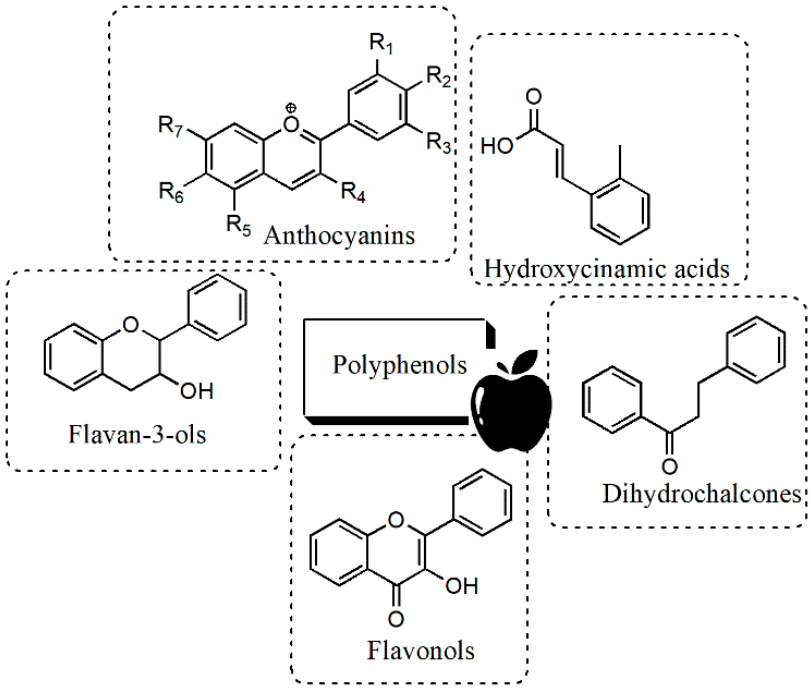

Figure 2. Main classes of polyphenols occurring in apples.

4.1. 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 [60–62]. Conventional Soxhlet extraction still remains one of the most relevant approach to extract volatile compounds from apples [62]. 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 [45]. 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 [63]. 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 [60]. 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 [64]. 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) (Table 3). 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.

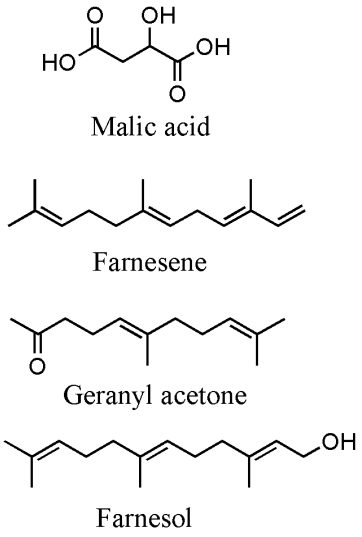

Figure 3. Main compounds responsible for the taste and flavor of apples.

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. [65], 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 [66]. 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 [61]. Bekele et al. [24] 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 [67,68]. 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. [67] 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 [69]. 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 [70]. Instead, Stefova et al. [71] 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 [72]. UAE has also been used for the extraction of organic acids; water or alcoholic mixtures were used as extraction solvents [42,55,73,74].

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 [3,53]. 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 [75].

Unlike apple polar triterpenoids, such as betulinic and ursolic acid, whose extraction is carried out by using polar solvents (i.e., methanol and acetone) [76], 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 [77]. Other advantages of this technique are the absence of solvents and the possibility to separate and pre-concentrate the analytes in a single step [78]. Therefore, it is really helpful for the extraction of volatile compounds from apples, due to their low concentration and the complexity of the matrix [79]. 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 [57,80]. 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. [81] 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.

4.2. Analytical Methods

The interest in the characterization and quantification of secondary metabolites of plant-based foods is growing and it often aims at the definition of food nutritional value as well as its quality and authenticity. Considering the large number of plant primary and secondary metabolites and their different properties and structures, different analytical techniques have been developed for each group or subgroup of phytochemicals [12]. Spectrophotometric assays have been largely used for the rough quantification of polyphenols and flavonoids. In the last decade, there has been an increasing request of more reliable and accurate analytical methods, with reduced operational time and costs, as well as with minimized use of hazardous chemicals [82–85], according to the green chemistry objectives [86]. The most common analytical methods used for the detection and/or quantification of the main classes of apples phytochemicals are the chromatographic ones.

4.2.1. Spectrophotometric Assays

The extraction of secondary metabolites is followed by their qualitative and/or quantitative analysis. Although the separation techniques, coupled with different detectors,

play a key role in the analysis of bioactive compounds, a number of spectrophotometric methods have been developed, in order to verify their presence or to quantify them in plant-based foods [87]. These assays are based on different principles and are mainly used to determine polyphenols and flavonoids.

For the rough quantification of polyphenols in apple fruits extract, colorimetric methods are widely used, due to their simplicity and high sensitivity. These include the Folin–Ciocalteu methods, used for the analysis of total polyphenols content (TPC), and the aluminum chloride ($AlCl_3$) assay for flavonoids determination [5,10,88,89]. The Folin–Ciocalteu assay is based on an electron-transfer reaction between the Folin–Ciocalteu reagent, which is a mixture of phosphomolybdic and phosphotungstic acid, with the polyphenol-based extract. In presence of phenols, under basic conditions, phosphomolybdic and phosphotungstic acid reduce themselves. Their reduced forms confer to the sample a blue color (λ_{max} = 495 nm). By evaluating the absorbance of the extract at 765 nm, it is possible to determine the total phenols concentration [10]. Viera et al. [10] used this assay in order to quantify the TPC in Fuji, Galaxy and other traditional Brazilian apples varieties. A great variation in terms of total phenolic content was observed by the authors among the apple cultivars (105.4–269.7 mg of gallic acid equivalents per 100 g of fresh matter). One of the main limits related to the quantification of total polyphenols through spectrophotometric assay, is due to interfering compounds, such as sugars, amino acids and ascorbic acid, which absorb in the same polyphenols region [90]. To avoid this problem, Vrhovsek et al. [88] made a cleanup procedure on a C-18 cartridge, prior TPC analysis. The average content of total polyphenols in the apple, evaluated by the Folin–Ciocalteu assay, was 110.2 mg/100 g of fresh fruit with significant differences depending on the apple variety.

Table 3. Overview of the methods used for the extraction of the main phytochemical classes occurring in apples.

Class	Extraction Technique	Solvents	References
Phenolics	Microwave-assisted extraction Solid–liquid extraction Ultrasound-assisted extraction Accelerated solvent extraction Supercritical fluid extraction	• Ethanol • Methanol • Acetone:water • Methanol:water	[61,65,67–72]
Organic acids	Ultrasound-assisted extraction	• Water • Ethanol:water • Methanol:water	[42,55,73,74]
Carotenoids	Solid–liquid extraction	• Acetone + BHT • Acetone/petroleum ether + BHT	[32,53]
Terpenes	Solid-phase microextraction Stir bar sorption extraction Ultrasound-assisted extraction	• / • / • Methanol • Acetone	[56,57,76,80]

Instead, the principle involved in $AlCl_3$ colorimetric method is that $AlCl_3$ forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonol, which show maximum of absorbance at 415 nm. The total flavonoid content of *Malus* fruits is generally, around 110–120 mg of catechin equivalents per 100 g of fresh matter [89].

Although these spectrophotometric assays are often used as preliminary test to evaluate the content of a specific metabolite class of apples, they are not selective and not useful for a sensitive quantification of individual compounds. Therefore, in the last years, more sensitive and selective methods, based on chromatographic techniques, have been continuously developed.

4.2.2. 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 [12,91]. 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 [92–96]. 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.

Phenolics and Organic Acids

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 [97–102]. 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 [103–108]. 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 [91]. 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 [109]. 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 [71,106,110,111]. 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 [112]. As mobile phase, binary mixtures of aqueous formic acid or acetic acid and acetonitrile (ACN) or methanol (MeOH) as organic modifiers have been employed [71,106,110,111]. 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. [113] 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 [114]. 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 [113].

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 [115]. 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 [116].

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 [21,28,35,111,117–121]. 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 [122]. 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 (Table 4).

Table 4. Absorption maximum (λ_{max}) of polyphenols occurring in apples and belonging to flavanols, phenolics acids, dihydrochalcones and flavonols classes. Reproduced under the terms of the Creative Common CC BY license which permits reuse in any medium, provided the original open access work is properly cited [21].

Group of Polyphenols	Phenolic Compound	λ_{max} (nm)
Flavanols	Procyanidin B1	281
	(+)-Catechin	281
	Procyanidin B2	281
	Procyanidin C1	275
	(−)-Epicatechin	280
	Procyanidin A2	280
Phenolic acids	Gallic acid	272
	Protocatechuic acid	261, 298
	5-O-Caffeoylquinic acid	326
	Caffeic acid	324
	p-Coumaric acid	310
	Ferulic acid	324
Dihydrochalcones	Phloretin-2-*O*-β-glucoside	287
Flavonols	Quercetin-3-*O*-galactoside	259, 348
	Quercetin-3-*O*-glucoside	259, 351
	Quercetin-3-*O*-rutinoside	259, 348
	Quercetin-3-*O*-xyloside	260, 348
	Quercetin-3-*O*-arabinoside	260, 347
	Quercetin-3-*O*-rhamnoside	260, 347
	Quercetin	256, 372

For comprehensive and simultaneous monitoring of different groups of polyphenols, Kschonsek et al. [21] set the detector at 254, 280 and 320 nm to identify and quantify, into fifteen different apple cultivars, twenty polyphenolic compounds 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. [117] 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 [92].

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. [106] 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 [95,109,123–126]. 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 [127–131]. 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 [91]. For the ionization of apple polyphenols in LC–MS, electrospray ionization (ESI) in negative mode has been, by far, the most generalized interface employed [128,130,132]. 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 [133]. 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. [134], 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) [135], linear ion-trap [103], time-of-flight [103], Orbitrap [105] and QTrap [136], 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. [128] 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 (see Supplementary Materials Table S1). 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 [130].

UV and MS are, often, both used for the identification and quantification of apple polyphenols [137,138]. Bizjak et al. [137] 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 (see Supplementary Materials Table S1) [13].

Carotenoids

The diversified structural characteristic of apple carotenoids requires accurate methods for their separation and identification. To date, many chromatographic techniques are available for the determination of carotenoids in plant-based foods; however, their characterization is more challenging due to similar molecular mass (structural isomers or geometrical isomers) and other derivatives [139]. Routinely, HPLC with UV–Vis, are used for the separation and quantification of carotenoids, occurring in apples [11,140,141], as these compounds show absorption in the visible region (400 and 500 nm), due to the long-conjugated double-bond system [142]. However, the λ_{max} of individual carotenoids can vary depending on functional groups (see Table 5). To confirm the occurrence of unknown carotenoids in a given sample, single run by HPLC (normal or reversed-phase) systems with isocratic or gradient elution are used. Typically, chromatographic separation of carotenoids is based on HPLC analysis using C18 and C30 columns. In general, C30 and C18 stationary phases are extremely employed for the separation of geometrical isomers. As regard the composition of the mobile phase, Perry et al. [11] quantified carotenoids in apples with a HPLC–UV–Vis (DAD) method, which involved the use of methanol/MTBE (2-methoxy-2methylpropane)/water (95:3:2, v/v, with 1.5% ammonium acetate in water) and methanol/MTBE/water (8:90:2, v/v, with 1.0% ammonium acetate in water). Instead, a binary-gradient elution with acetone and deionized water was used by Delgado-Pelayo et al. [140] for the characterization and the quantification of chlorophyll and carotenoid pigments in the peel and flesh of thirteen commercial apple cultivars, including red-skinned varieties (Ariane, Fuji (I) from Italy, Fuji (F) from France, Pink Lady, Royal Gala and Starking Red Chief), green-skinned varieties (Granny Smith, Green Doncella, Green Golden Delicious and Reina de Reineta), yellow-skinned and yellow-green and varieties (Golden Montaña, Golden Delicious and Golden Rosett). It should be noted that, for more reliable results, the identification of the pigment profile is often performed not only by comparing the UV–visible spectra and retention time, but also by comparing mass spectra with those of standard pigments and data in the literature [11,141]. Indeed, closely related carotenoids and their metabolites could co-elute in various analytical methods. Thus, although spectral confirmation of these carotenoids is based on characteristic UV–Vis spectra, obtained by PDA/DAD, additional characterization is required by MS analysis [139]. Remarkable milestones are provided by the development of analytical methods

for qualitative and quantitative analysis of carotenoids, through liquid chromatography coupled to mass spectrometry; first of all, the possibility to confirm their structures through tandem mass spectrometric experiments. MS/MS is used in order to find out the characteristic/typical fragmentation pattern of carotenoids, thus allowing their identification. The fragmentation patterns of the main carotenoids occurring in apples are reported in Table 5. Most of the MS analysis of carotenoids is performed, by using positive ion mode. APCI (atmospheric pressure chemical ionization) has become the most widely used ionization technique for various carotenoids in apples, because of high sensitivity [139]. Recently, also ultrahigh-performance liquid chromatography (UHPLC) technology has been used for the analysis of carotenoids in *Malus* fruits. As the UHPLC C30 stationary phase columns are not commercially available, C18 columns have been used [143].

Table 5. Wavelengths of maximum absorption λ_{max} and characteristic fragmentation pattern of the main carotenoids occurring in apples. Reproduced with the permission of Elsevier [140].

Pigment	λ_{max} (nm)	HPLC/MS (APCI+) Fragmentation Pattern *m/z*	
		$[M + H]^+$	Characteristic Fragments
all-*trans*-Neoxanthin	419, 444, 472	601	583 ($[M + H- 18]^+$), 565 ($[M + H-18-18]^+$), 547 ($[M + H-18-18-18]^+$)
9′-*cis*-Neoxanthin	415, 439, 468	601	583 ($[M + H-18]^+$), 565 ($[M + H-18-18]^+$), 547 ($[M + H-18-18-18]^+$)
Neochrome	401, 424, 452	601	583 ($[M + H-18]^+$, 565 ($[M + H-18-18]^+$), 547 ($[M + H-18-18-18]^+$)
all-*trans*-Violaxanthin	418, 443, 472	601	583 ($[M + H-18]^+$), 565 ($[M + H-18-18]^+$)
9-*cis*-Violaxanthin	411, 436, 468	601	583 ($[M + H-18]^+$), 565 ($[M + H-18-18]^+$)
13-*cis*-Violaxanthin	410, 435, 466	601	583 ($[M + H-18]^+$), 565 ($[M + H-18-18]^+$)
Luteoxanthin	400, 424, 451	601	583 ($[M + H-18]^+$), 565 ($[M + H-18-18]^+$)
all-*trans*-Antheraxanthin	424, 448, 476	585	567 ($[M + H-18]^+$), 549 ($[M + H-18-18]^+$), 505 ($[M + H-80]^+$)
all-*trans*-Zeaxanthin	428, 455, 481	569	551 ($[M + H-18]^+$),533 ($[M + H-18-18]^+$)
all-*trans*-Lutein	428, 448, 476	569	551 ($[M + H-18]^+$),533 ($[M + H-18-18]^+$)
9-*cis*-Lutein	330, 420, 444, 472	569	551 ($[M + H-18]^+$),533 ($[M + H-18-18]^+$)
13-*cis*-lutein	334, 418, 441, 470	569	551 ($[M + H-18]^+$),533 ($[M + H-18-18]^+$)
Chlorophyll b	457, 646	907	629 ($[M + H-278]^+$)
Chlorophyll b′	457, 646	907	629 ($[M + H-278]^+$)
Chlorophyll a	430, 662	893	615 ($[M + H-278]^+$)
Chlorophyll a′	430, 662	893	629 ($[M + H-278]^+$)
all-*trans*-β-Carotene	427, 454, 479	537	445 ($[M + H-92]^+$)

Terpenes

Terpenes are secondary metabolites obtained by the combinations of several isoprene units (C_5) and they can be grouped, based on the number of carbon atoms, in monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}) and triterpenes s(C_{30}). Terpenes with 15 carbons or less are typically volatile compounds, due to their small size and low polarity, and they represent in apples a minor component of total volatile organic compounds (VOCs), responsible for the fruits' aroma [56]. Some interesting triterpenoids, occurring in *Malus* fruits, are more polar and less volatile molecules, because they are linked to one or multiple polar groups: among them, oleanolic, annurcoic, ursolic and betulinic acid are the most important polar triterpenoids [144].

The detection and quantification of volatile terpenes in apples are widely performed through the use of the GC coupled to mass spectrometry (MS). Few works were based on GC coupled to flame ionization detector (FID) method [145,146]; however, GC–FID is one of the most used detectors in the flavor and fragrance field, due to its low cost and simplicity. More commonly, the nature of terpenes is investigated by using single quadrupole MS detectors [57,81,147]. The easy electron impact (EI) ionization ensures high reliability in compounds detection. However, problems in identification of monoterpenes and sesquiterpenes could be found, due to their structural similarities, which result in

similar MS spectra. To overcome these problems retention index values (RI) and MS spectrum interpretation should be used for a univocal peak assignment [92]. Compounds are identified using mass spectral libraries and linear retention indices, calculated from a series of n-alkanes from C_6 to C_{30}. Such an approach was used by Ferreira et al. [57] to identify several terpenes occurring in different apple varieties of *Malus domestica* Borkh from different geographic regions at Madeira Islands, including farnesol and α-farnesene. The authors found that for the whole fruit, terpenes accounted for 3.10% of the total GC peak area of the chromatograms. In order to increase the selectivity and sensitivity of the analytical method, Vrhovsek et al. [148] used tandem mass spectrometry (QqQ) for metabolite profiling of volatile compounds, including terpenes, in apples. The developed analytical method allowed the separation of co-eluted terpenes, with the same nominal masses, which could be not identified by conventional detectors such as FID and/or qMS. In this work, the triple quadrupole mass spectrometer operated in multiple selected reaction monitoring mode (MRM) and at the optimal collision energy for a given compound, the most intense fragmentation ion was chosen as a quantifier (Q) and the second most intense as a qualifier (q). The confirmation of molecules identity was achieved by comparing the q/Q ratio of samples and those of reference standards. The choose of two selected ions transition, one for quantification and one for confirmation, during MRM, ensured high selectivity and sensitivity, using the q/Q ratio as a confirmatory parameter.

As triterpenoids such as betulinic acid (BA) and ursolic acid (UA) are polar metabolites, they are not efficiently volatilized for analysis using GC. Thus, LC–MS is more suitable for their analysis [59,149–151]. LC coupled with ultraviolet (UV) or diode array detectors found a limited application in the study of apples triterpenes, as these compounds absorb UV radiation weakly and only at wavelength of 200 nm. Thus, only few studies reported the use of LC–DAD for triterpenoids analysis in apple fruits [152]. On the contrary, LC–MS/MS has been widely preferred, as it allows compounds unequivocal identification by examining their fragment ions, obtained through MS/MS experiments [153]. Sut and colleagues [59] investigated the fragmentation pathways of apple triterpenes, i.e., pomaceic, annurcoic, euscaphic, pomolic, corosolic, maslinic, betulinic, ursolic and oleanolic acid by LC–APCI–MS/MS, in order to allow their fast identification without the use of reference standards. Negative ion mode was the polarity chosen for the analytes. The main ions observed in ion trap and Q-TOF measurements for the considered triterpene acids are summarized in Table 6. The application of LC–MS/MS for triterpenoids analysis also ensures more sensitive quantification. Wildner et al. [149] developed a practical and reliable analytical method for the extraction, identification and quantification of UA and BA in apple peel extracts, by using LC coupled to MS with positive electrospray ionization mode (ESI+). An isocratic run, consisting of 80% acetonitrile and 20% ammonium acetate 10 mM pH 6.0, allowed the separation of the analytes. The developed method was validated in terms of sensibility, precision and accuracy. The LOD and LOQ values were 0.087 and 0.266 μg/mL for BA and 0.398 and 2.117 μg/mL for UA. For betulinic acid, LOD and LOQ values were lower compared to those obtained with the LC–DAD method developed by Butkevičiūtė et al. [152] (LOD: 0.15 μg/mL and LOQ: 0.45 μg/mL).

Table 6. Main fragments observed for the considered apple triterpene acids, low-resolution species were detected in ion trap (LR-IT), while high-resolution species were detected using Q-TOF (HR-Q-TOF). Reproduced with the permission of John Wiley and Sons [59].

Ion	Pomaceic Acid	Annurcoic Acid	Euscaphic Acid	Pomolic Acid	Corosolic Acid	Maslinic Acid	Betulinic Acid	Oleanolic Acid	Ursolic Acid
HR-MSQ-TOF	501.3222 (Δ + 1.2 ppm for $C_{30}H_{45}O_6$ 501.3216)	485.3281 (Δ + 2.9 ppm for $C_{30}H_{45}O_5$ 485.3267)	487.3415 (Δ − 1.6 ppm for $C_{30}H_{47}O_5$ 487.3423)	471.3474 (Δ + 0.4 ppm for $C_{30}H_{47}O_4$ 471.3473)	471.3475 (Δ + 0.4 ppm for $C_{30}H_{47}O_4$ 471.3473)	471.3475 (Δ + 0.6 ppm for $C_{30}H_{47}O_4$ 471.3473)	455.3529 (Δ + 0.9 ppm for $C_{30}H_{47}O_3$ 455.3525)	455.3522 (Δ − 0.7 ppm for $C_{30}H_{47}O_3$ 455.3525)	455.3531 (Δ + 1.3 ppm for $C_{30}H_{47}O_3$ 455.3525)
MS^2									
$[M-H-18]^-$	483.3110 (Δ − 3.9 ppm for $C_{30}H_{43}O_5$ 483.3111)	467.3170 (Δ + 0.9 ppm for $C_{30}H_{43}O_4$ 467.3161)	469.3304 (Δ − 3.0 ppm for $C_{30}H_{45}O_4$ 469.3318)	453.3359 (Δ − 2.2 ppm for $C_{30}H_{45}O_3$ 453.3369)					
$[M-H-40]^-$							415.3181 (Δ − 7.5 ppm for $C_{27}H_{43}O_3$ 415.3212)		
$[M-H-44]^-$	457.3303 (Δ − 3.3 ppm for $C_{29}H_{45}O_4$ 457.3318)	441.3355 (Δ − 3.2 ppm for $C_{29}H_{45}O_3$ 441.3369)							
$[M-H-46]^-$	455.3160 (Δ − 0.3 ppm for $C_{30}H_{43}O_4$ 455.3161)						409.3423 (Δ − 10.0 ppm for $C_{29}H_{45}O$ 409.3470)		
$[M-H-48]^-$					423.3250 (Δ − 3.1 ppm for $C_{29}H_{43}O_2$ 423.3263)	423.3253 (Δ + 2.4 ppm for $C_{29}H_{43}O_2$ 423.3263)	407.3314 (Δ − 3.2 ppm for $C_{29}H_{43}O$ 407.3314)	407.3314 (Δ − 3.2 ppm for $C_{29}H_{43}O$ 407.3314)	407.3304 (Δ − 2.5 ppm for $C_{29}H_{43}O$ 407.3314)
$[M-H-60]^-$	441.2984 (Δ − 4.8 ppm for $C_{28}H_{41}O_4$ 441.3005)			411.3250 (Δ − 3.2 ppm for $C_{28}H_{43}O_2$ 411.3263)					
$[M-H-62]^-$	439.2848 (Δ + 0.9 ppm for $C_{28}H_{39}O_4$ 439.2848)	423.3259 (Δ − 0.9 ppm for $C_{29}H_{43}O_2$ 423.3263)	425.3420 (Δ − 5.4 ppm for $C_{29}H_{45}O_2$ 425.3420)	409.3095 (Δ − 2.9 ppm for $C_{28}H_{41}O_2$ 409.3107)			393.3143 (Δ − 8.5 ppm for $C_{28}H_{41}O$ 393.3157)		

Table 6. *Cont.*

Ion	Pomaceic Acid	Annurcoic Acid	Euscaphic Acid	Pomolic Acid	Corosolic Acid	Maslinic Acid	Betulinic Acid	Oleanolic Acid	Ursolic Acid
$[M-H-64]^-$				407.3313 (Δ − 0.3 ppm for $C_{29}H_{43}O$ 407.3314)	407.2914 (Δ − 8.8 ppm for $C_{28}H_{39}O_2$ 407.2950)	407.6			
$[M-H-66]^-$					405.5	405.5			
$[M-H-78]^-$					393.3157 (Δ − 0.1 ppm for $C_{28}H_{41}O$ 393.3157)	393.6	377.5		
$[M-H-80]^-$		405.3149 (Δ − 2.0 ppm for $C_{29}H_{41}O$ 405.3157)	407.2934 (Δ − 3.9 ppm for $C_{28}H_{39}O_2$ 407.2934)						
$[M-H-93]^-$	409.3107 (Δ − 5.9 ppm for $C_{28}H_{41}O_2$ 409.3107)	393.3157 (Δ − 1.3 ppm for $C_{28}H_{41}O$ 393.3157)							
MS^3 *									
	409.6→379.5	423.6→405.7 423.6→393.6	425.6→407.6 425.6→405.6 425.6→393.5		423.5→407.5 423.5→405.5 423.5→393.5			407.6→391.6 407.6→377.6 407.6→365.4 407.6→363.4	407.6→391.5 407.6→378.5 407.6→377.5 407.6→206.6
MS^4 **									
	379.5→363.8 379.5→361.8 379.5→190.3 379.5→137.3	393.6→377.6 393.6→189.3	393.5→377.5 393.5→189.3		393.5→377.6 393.5→375.5 393.5→202.5				

* Fragment ions obtained by fragmentation of the precursor ion (left of the arrow), during MS^3 experiments. ** Fragment ions obtained by fragmentation of the precursor ion (left of the arrow), during MS^4 experiments.

5. Conclusions

In this review, a comprehensive description of extraction approaches and analytical methods, used for the determination of primary and secondary metabolites of apple fruits (*Malus domestica*), was provided. In detail, the extraction method mostly employed for the extraction of primary metabolites from apple tissues is the traditional extraction with solvent (LSE); for secondary metabolites, extraction with solvents at different polarities, assisted by microwave (MAE) or ultrasound (UAE), are commonly used. After extraction, spectrophotometric assays are often used to quantify apple phytochemicals, mainly polyphenols and flavonoids. However, spectrophotometric methods are not selective; therefore, chromatographic methods (LC and GC coupled to MS) are used, too. Overall, an analysis based on the LC–MS reaches a greater sensitivity, thus allowing us to identify the phytochemical components occurring in apples at very low concentrations.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/separations8070091/s1. Table S1: Overview of the analytical techniques used in the last 10 years (reported in chronological order) for the extraction and the analysis of secondary metabolites occurring in *Malus Domestica*.

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