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Review

Separation procedures for naturally occurring antioxidant phytochemicals

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Abstract

Phytochemicals in fruits, vegetables, spices and traditional herbal medicinal plants have been found to play protective roles against many human chronic diseases including cancer and cardiovascular diseases (CVD). These diseases are associated with oxidative stresses caused by excess free radicals and other reactive oxygen species. Antioxidant phytochemicals exert their effect by neutralizing these highly reactive radicals. Among the tens of thousands of phytochemicals found in our diets or traditional medicines, polyphenols and carotenoids stand out as the two most important groups of natural antioxidants. However, although collectively these phytochemicals are good antioxidants, the roles and effect of individual compounds are often not well known. Hundreds of carotenoids and thousands of polyphenols have been identified so far from various plants. A single plant could contain highly complex profiles of these compounds, which sometimes are labile to heat, air and light, and they may exist at very low concentrations in the plants. This makes the separation and detection of these antioxidant phytochemicals a challenging task. The present review focuses on the antioxidant activity, chemical types, sampling and sample processing procedures, and separation using various chromatographic and electrophoretic techniques. Detection and quantification using ultraviolet–visible–diode array and mass spectrometry will be discussed.

Keywords: Review; Antioxidant phytochemicals; Separation; Phytochemicals; Antioxidants; Carotenoids; Polyphenols; Flavonoids; HPLC; HSCCC; LC–MS; SFE; SFC

Contents

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1. Introduction

1.1. Antioxidant phytochemicals and human health

In recent years, many studies have shown that diets containing high content of phytochemicals can provide protection against various diseases. Approximately 90% of all cancer cases correlate with environmental factors, including one's dietary habits, and one-third of all cancer deaths in the United States are avoidable by changing dietary habits only [1,2]. These discoveries have rapidly amplified the consumer awareness of the potential benefits of naturally occurring compounds from plants in health promotion and maintenance, and researches in nutraceuticals and functional foods (NFF) and natural health products (NHP) have been hot topics in recent years [3–5]. The protective effects of fruits, vegetables and spices and herbs were found not only for cancer [5–9], but also other chronic diseases such as cardiovascular diseases (CVD) [10–18].

Among the causes of the major chronic health problems, harmful free radicals and reactive oxygen species (ROS) have been found to play an important role [19,20]. Radicals and ROS such as the superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and peroxy radical ($ROO^{\bullet}$) have been implicated as mediators of degenerative and chronic deteriorative, inflammatory, and autoimmune diseases [4,21], diabetes, vascular disease and hypertension [22–24], cancer and hyperplastic diseases [11,25], cataract formation [11,26], emphysema [27], arthritis, malaria, multiple sclerosis, myocardial ischemia-reperfusion injury [4], immune system decline, and brain dysfunction as well as the aging process [11].

Antioxidants such as Vitamins C and E are essential for the protection against ROS. However, the majority of the antioxidant activity of a fruit or vegetable may be from compounds such as phenolic acids and flavonoids, rather than from Vitamin C, E or β-carotene [28–32]. Intake of controlled diets rich in fruits and vegetables increased significantly the antioxidant capacity of plasma. This increase could not be explained by the increase in the plasma α-tocopherol or carotenoid concentration [33].

Antioxidant phytochemicals such as flavonoids are therefore the focus of many recent studies. The antioxidant activity of these compounds is predominantly determined by their structures, in particular the electron delocalization over an aromatic nucleus, in those based on a phenolic structure. When these compounds react with a free radical, it is the delocalization of the gained electron over the phenolic antioxidant, and the stabilization by the resonance effect of the aromatic nucleus, that prevents the continuation of the free radical chain reaction. This is often called radical scavenging, but polyphenolic compounds inhibit oxidation through a variety of mechanisms [34–37]. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) only tend to have one mode of action, i.e. via free radical scavenging, and are not able to sequester metal ions through the metal catalyzed route [4]. The anticancer activity of flavonoids has been attributed to a large variety of different mechanisms [38].

1.2. Chemical types and sources of antioxidant phytochemicals

Among the different groups of naturally occurring antioxidants from plants, carotenoids and polyphenolics are perhaps the two most important [39,40]. This review therefore will focus on the techniques used in the separation of these two major groups of antioxidants. Other antioxidant phytochemicals such as alkylamides in pepper and Echinacea will also be mentioned. Fig. 1 shows the chemical structures of typical polyphenolics, carotenoids and amides that are known to be antioxidants.

Carotenoids, including xanthophylls (oxygen-containing carotenoids) are naturally occurring coloured compounds that are abundant as pigments in plants. To date, about 500 and 600 specific carotenoids have been identified, mostly from plants and algae [41]. Carotenoids have the capacity to trap not only lipid peroxyl radicals, but also singlet oxygen species [42]. The essential role of carotenoids as a major dietary source of Vitamin A has been known for many years. Although all carotenoids contain extensive conjugated double bonds, individual carotenoids differ in their antioxidant potential in humans [43]. Some have no measurable antioxidant potential in vitro. The true antioxidant capacity of the most prevalent carotenoids in vivo is still in question. The antioxidant capacity of carotenoids may also be related to the structure. Larger conjugated system such as astaxanthin is known to have a higher antioxidant activity [44].

Polyphenolics is a highly inclusive term that covers many different subgroups of phenolic acids and flavonoids. More than 5000 polyphenolics, including over 2000 flavonoids have been identified, and the number is still growing [45]. Polyphenolics vary in structures: hydroxybenzoic acids and hydroxycinnamic acids have a single-ring structure, while

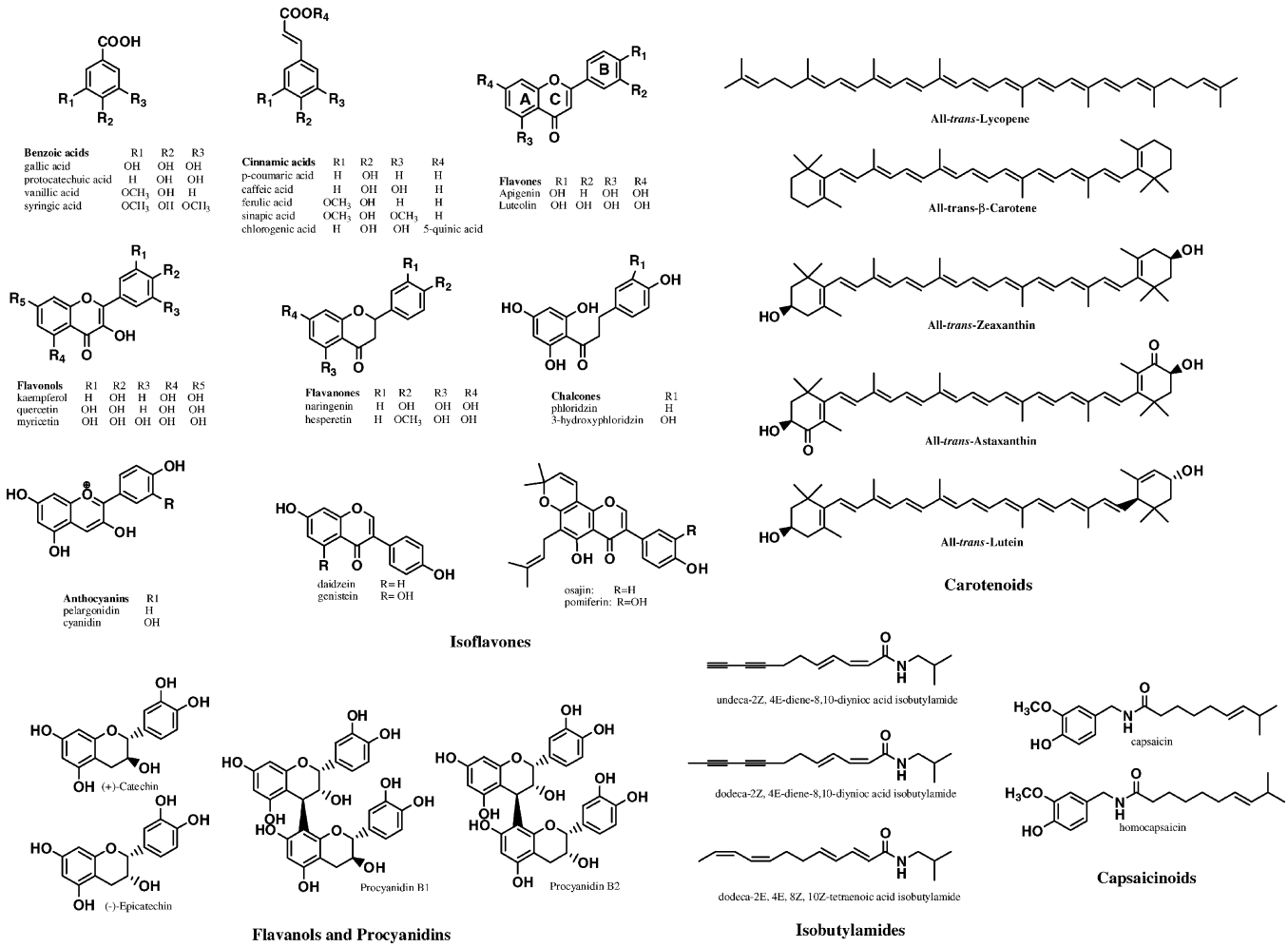

Fig. 1. Chemical structures of major antioxidant groups and representative individual antioxidant phytochemicals.

flavonoids can be further classified into anthocyanins, flavan-3-ols, flavones, flavanones and flavonols. Some of the flavonoids such as flavan-3-ols can be found in dimmers, trimers and polymers (Fig. 1). Many of the phenolics are often associated with sugar moieties that further complicate the phenolic profiles of plants [46]. Polyphenols are especially important antioxidants, because of their high redox potentials, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [47]. In addition, they have a metal chelating potential [48]. The antioxidant activity of the dietary polyphenolics is considered to be much greater than that of the essential vitamins, therefore contributing significantly to the health benefits of fruits [30].

Flavonoids and related polyphenols are ubiquitous in land plants, and have the general structure as shown in Fig. 1. Flavonoids generally consist of two benzene rings (rings A and B, Fig. 1) linked by an oxygen-containing heterocycle (ring C, Fig. 1). It should be noted that the chalcones are considered by many authorities to be members of the flavonoid family, despite lacking the heterocyclic ring C. The fused A and C rings are often collectively termed the flavonoid nucleus.

1.3. Evaluation of antioxidant activity

Many in vitro models and in vivo methods have been developed for the evaluation of antioxidant activity. However, the interpretation of results obtained from these model systems has to be dealt with caution due to the different methods being based on different mechanisms, resulting in considerably varied antioxidant activity. There is no perfect system available to help us know about the “true” antioxidant power or capacity of a single antioxidant or a complex medium of antioxidant phytochemicals [49,50].

The following are examples of the most frequently used simple in vitro models for the evaluation of total antioxidant activity.

Ferric reducing/antioxidant power (FRAP) assay: The FRAP assay was first introduced by Benzie and Strain [51] for measuring the total antioxidant activity. More recently this method has been modified for the 96-well microplate reader [52], giving better reproducibility and higher throughput of samples. The assay is based on the reducing power of a compound (antioxidant). A potential antioxidant will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); the latter forms

a blue complex (Fe^{2+}/2,4,6-tripyridyl-s-triazine (TPTZ), which increases the absorption at 593 nm. Stronger absorption at this wavelength therefore indicates higher reducing power of the phytochemical, thus, higher antioxidant activity.

β-Carotene–linoleic acid model system (β-CLAMS): The β-CLAMS method is based on the decolouration of β-carotene by the peroxides generated during the oxidation of linoleic acid at an elevated temperature [53,54]. This method has also been adapted for the 96-well microplate reader recently [52]. Readings are taken at 490 nm immediately after and typically at 15 min time intervals for 100–300 min. Flatter decaying curves indicate the presence of stronger antioxidants.

Oxygen radical absorption capacity (ORAC) method: The ORAC assay was developed by Cao et al. [55,56] and has been used to evaluate the antioxidant capacity of water-soluble phytochemicals. A fluorescent protein, R-phycoerythrin (R-PE) and a peroxyl radical generator, AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride were used in the assay. The excitation and emission wavelengths were set at 540 and 565 nm, respectively.

Thiobarbituric acid reactive substance (TBARS) method: During lipid peroxidation, lipid peroxides are formed, with a subsequent formation of peroxyl radicals, followed by a decomposition phase to yield aldehydes such as hexanal, malondialdehyde and 4-hydroxynonenal. This assay is based on the detection of a stable product, which is formed between aldehydes and thiobarbituric acid (TBA) in the aqueous phase. The production of TBARS was measured spectrophotometrically at 535 nm after an incubation period of 20 min at 80 °C [57].

Trolox equivalent antioxidant capacity (TEAC) method: This assay is based on the relative ability of antioxidants to scavenge the radical cation 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^+$). The radical is generated by the interaction of ABTS with the ferrylmyoglobin radical species, generated by the activation of metmyoglobin with H_2O_2. The extent of quenching of the ABTS radical is measured spectrophotometrically at 734 nm and compared with Trolox, a water-soluble Vitamin E analogue. Results are expressed as Trolox equivalents [58]. Other free radicals such as 2,2-diphenyl-1-picrylhydrazyl ($DPPH^•$) have also been used to measure antioxidant activities [59]. $DPPH^•$ shows an absorbance maximum at 515 nm which disappears upon reduction by an antioxidant phytochemical that has anti-radical property.

Photochemiluminescence (PCL) method [60]: PCL is based on an approximately 1000-fold acceleration of the oxidative reaction in vitro compared to normal conditions. This effect is achieved by optical excitation of a suitable photosensitizer, which exclusively results in the generation of the superoxide radical $O_2^{•-}$. The radicals are visualized with a chemiluminescent detection reagent. A synthetic fluorescent compound luminol is used in this assay. This compound plays a double role acting as both the photosensitizer and the radical reaction agent. A commercial instrument designed specifically for PCL is now available, and the author has found it quite a useful tool. Fig. 2 shows some typical curves of selected polyphenols and plant extracts.

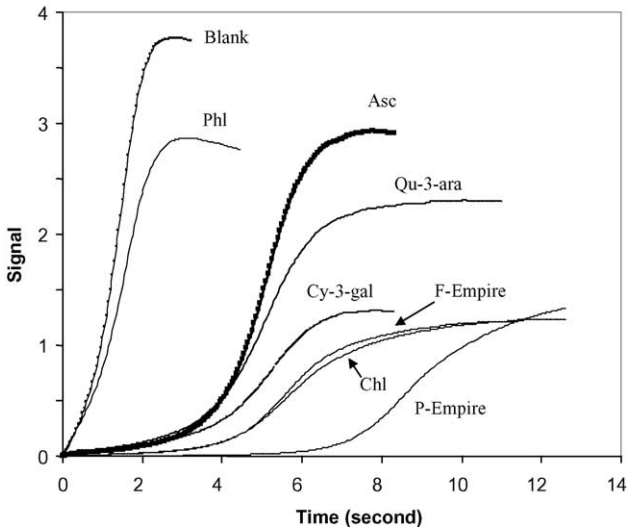

Fig. 2. Antioxidant activities of selected polyphenols and plant extracts measured by PCL method. The longer the lag phase, the stronger the antioxidant activity. The blank had the shortest lag phase. Phl: phloridzin; Asc: ascorbic acid; Qu-3-ara: quercetin-3-arabinoside; Cy-3-gal: cyanidin-3-galactoside; Chl: chlorogenic acid; F-Empire: extract of the Empire apple flesh; P-Empire: extract of the Empire apple peel. Apples were extracted with 70% aqueous methanol (1:1, w/v). Data for the extracts were obtained from a 50-fold dilution of the peel extract, and 10-fold dilution of the flesh extract. All other standards were in 10 μM concentration (R. Tsao et al., unpublished).

1.4. Sample collection, storage and extraction

Due to the vast reservoir of plants, the variation of different parts in a plant, and the diverse chemical structures and physicochemical properties of the antioxidant phytochemicals, it is nearly impossible to have any definitive procedure or protocol for the collection and storage of all plant materials. However, this is perhaps the most important step in the separation of antioxidant phytochemicals, because the aforementioned factors, and many others such as plant variety, growing location and season, may significantly affect the quantity and quality of the phytochemicals. Most traditional oriental medicines are harvested and dried for storage, while at other times, fresh or frozen plant materials have been used. The processing and storage conditions such as drying temperature and duration, thawing method, storage length and humidity therefore may also affect the outcome. In-depth discussion on this topic is beyond the scope of this review, however, it is the author's opinion that sample collection and storage conditions are essential and should be treated carefully.

Extraction method is also critical to the recovery of antioxidant phytochemicals. The nature of both plant materials and the bioactive components should be considered in order to achieve good extraction efficiency. Lipophilicity or hydrophilicity affects the solubility of a phytochemical in the ex-

tracting solvent, and conversely, polarity of a solvent also has an impact on the extraction efficiency. Some compounds such as lignans and procyanidins are often in bound or polymerized forms. Hydrolysis is therefore necessary before the extraction. Many different extraction methods exist for antioxidant phytochemicals, but most of them are based on solvent extraction using water, organic solvent or liquefied gas, or combinations of them under different temperature and pressure, although other methods such as physical press, filtration, steam distillation and solid adsorption (of liquid or head space) have also been used. Enzyme activity of the plants and the existence of oxygen and light during the extraction also impact the efficiency, therefore extreme care must be taken to avoid hydrolysis, oxidation [61,62] and/or isomerization [63]. Often, due to the analytical difficulties in later separation procedures, intentional hydrolysis for obtaining the aglycones of some flavonoids or derivatization of some fatty acids to esters may be incorporated into the extraction process.

The extraction procedure is determined by the types of antioxidants to be extracted and whether the objective is quantitative or qualitative. Polar antioxidants such as phenolic acids and glycosides of many flavonoids are generally extracted using water, alcohols or a mixture of water and alcohols. For antioxidants such as aglycones of some flavonoids and most carotenoids, non-aqueous solvents are used.

Methanol is more frequently used than ethanol due to its higher extraction efficiency. Aqueous methanol between 50 and 80% has been used for extracting hydroxycinnamic acids, and many subgroups of flavonoids. Higher water composition in the solvent can aid in the extraction of glycosides of these compounds, although due to the complexity of heterosidic combinations, certain groups of flavonoids, such as flavones and flavanols, are not generally characterized as intact compounds but in the form of their aglycones. For that reason, a hydrolysis procedure before or during extraction is required [64–67].

Solvent extraction offers good recovery of antioxidant phytochemicals from various samples, however, the use of large amount of organic solvents poses health and safety risks, and is environmentally unfriendly. There are many alternative methods that either eliminate or reduce significantly the use of organic solvents. Some of them offer identical, if not better, extraction efficiency and cost effectiveness. Methods such as solid-phase extraction (SPE) use solid absorbents to extract phytochemicals from liquid matrix such as juices. It is easy, rapid and economical compared to solvent extraction. However, SPE is perhaps more often used in sample cleanup, purification or pre-concentration than in extraction because of the selectivity and saturation of the absorbents. The following alternative extraction methods, microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE), due to their increasingly popular uses in the extraction of antioxidant phytochemicals, will be briefly discussed.

MAE is a relatively new extraction technique that combines microwave and the use of traditional solvent. In MAE, it is important that the extraction solvent has a good polarity, because solvents with high dielectric constants (polar) can absorb more microwave energy, therefore result in better extraction efficiency [68,69]. Water or other polar solvent is therefore often added as modifiers in order to achieve an optimal dielectric constant of the extraction solvent. However, disagreeing opinions also exist: when solvents of low dielectric constants are used, all the microwave energy may be directed to the sample material, the moisture inside the cellular structure absorbs the energy so quickly that it erupts and breaks the cell wall, releasing the phytochemicals to the surrounding solvent. Nonetheless, compared with the conventional solvent extraction, MAE offers many advantages: (1) shorter extraction time, often few minutes instead of hours; (2) less solvent; and (3) higher extraction efficiency.

Certain gases such as carbon dioxide (CO_2) can be liquefied to a state called supercritical fluid when the pressure and temperature are right. Characteristics of a supercritical fluid resemble both a gas and a liquid, and SFE takes advantages of such fluids. The gas-like characteristics help the fluids diffuse to the matrix and access to the phytochemicals, and the liquid-like characteristics provides good solvitation power. SFE has been used in recent years in many applications, and supercritical CO_2 is the most widely used solvent for many antioxidant phytochemicals. The most obvious merit of SFE is the cleanness. CO_2 is chemically inactive, has a low toxicity, and poses virtually no harm to the environment. However, SFE is a highly expensive technology. Extraction efficiency in CO_2 SFE can be optimized by changing the density of CO_2, modifier (e.g. organic solvent), modifier percentage, temperature, time and other parameters. Due to the apolar property of CO_2, it is most suitable for the extraction of antioxidants such as carotenoids and other relatively lipophilic antioxidants [70–72]. For most polyphenolic antioxidants, unfortunately, even though a good recovery rate can be achieved by changing the above-mentioned parameters (often by adding polar solvent), it often significantly offsets the many advantages that SFE offers. For example, in extracting polyphenol antioxidants from green tea, the best extraction yield was found in a system using 95% methanol and 5% CO_2 [73]. Others also found that significant amount of polar organic modifiers has to be added to obtain high extraction yield [74–79]. These studies showed that high concentrations of organic modifiers lead to reduced selectivity. Antioxidants in rosemary have been extracted using SFE [80,81], and it was found that supercritical CO_2 gave higher recovery than typical organic solvents [80].

PLE or accelerated solvent extraction (ASE) has been used for the extraction of bound residues of pesticides and other environmental contaminants. This technology has only recently been used for the extraction of antioxidant phytochemicals [82,83]. In PLE, fast and efficient extraction is achieved by applying high pressure and elevated temperature. It was found that using higher temperatures resulted in higher recovery rates [82,83]. At higher temperatures, although most phenolic antioxidants were stable, others such as catechin

and epicatechin were degraded (ca. 14% at 150 °C) [82]. In extracting catechin and epicatechin from tea and grape seed, it was found that among water, methanol, ethanol and ethyl acetate, methanol had the highest yield [83]. PLE was also applied to extract procyanidins [84].

2. Separation methods

2.1. Conventional chromatography

Paper, packed column and thin-layer chromatographic methods have been used for the separation and purification of many antioxidant phytochemicals. However, due to the lack of good separation efficiency and resolution, and the difficulties in detection, quantification and sensitivity, these conventional chromatographic techniques, particularly the paper chromatography (PC), are not being used as often as before. PC was used to separate flavonoids, cinnamic acids and coumarines from the different tissues and traditional medicinal preparations of dandelions (*Taraxacum officinale*) [85]. In this study, separation was achieved by using multiple 2DPC (two-dimensional) techniques on 3 mm Whatman paper and a mobile phase consisting of *n*-butanol–acetic acid–water (4:1:5) and 15% acetic acid. Thin-layer chromatography (TLC) and open column chromatography (CC) are still being used as separation tools for many antioxidant phytochemicals due to the convenience, low cost, simultaneous separation and detection of considerable amount of samples and the availability of new stationary phases [86]. A considerable number of TLC stationary phases were examined and compared for the separation of carotenoids in paprika. Among the alumina, silica, silica–diatomaceous earth (1:1, m/m), diatomaceous earth, cellulose, polyamide, cyano, diol and amino silica stationary phases, and different combinations of solvent systems, the best separations have been found in adsorption alumina TLC with hexane–chloroform mixtures as mobile phase [87]. The crude extracts of plant materials contain highly complex profiles of phytochemical antioxidants, and often, isocratic separation cannot achieve satisfactory separation. Multiple mobile phases, in regular or 2D TLC are therefore useful for good separation of antioxidant carotenoids from complicated plant materials or extracts [88–91]. Despite these applications, disadvantages such as large requirement in sample amount may restrict the use of TLC and CC because such amount is not always available. Recovery of the antioxidant phytochemicals from the TLC plates or CC could also be challenging [92,93]. The majority of the TLC and CC applications are in the fractionation and preliminary separation of antioxidant phytochemicals before they are separated, quantified and identified by HPLC or other high-performance separation techniques. TLC often has an additional role as a monitoring tool for CC fractionation. Using TLC and CC, nine antioxidants were separated and purified from the aerial parts of St. John's wort (*Hypericum hyssopifolium* L.) [94]. TLC and CC are also used, often in combination, in bioassay-guided fractionation of antioxidant phytochemicals. TLC was used to separate and identify phenolic acids and flavonoids in the water extracts of Lamiaceae family aromatic plants [95]. A strong antioxidant, rosmarinic acid was separated and purified from Summer savory (*Satureja hortensis* L.) using normal phase silica gel and reversed-phase C18 CC [96]. Similarly, several antioxidant phenolic acids and polyphenolics were isolated from the root sample of a traditional medicine *Polygonum multiflorum* Thunb using silica gel and Sephadex LH-20 CC [97]. TLC is also one of the main methods for class fractionation and speciation of lipids [98,99], and is used increasingly to determine the botanical origin, potency, and flavour potential of plant materials (e.g. herbs and spices) [100–102]. Many core and new TLC technologies have been identified and developed in recent years, including: (1) methods to provide a constant and optimum mobile phase velocity (forced flow and electroosmotically-driven flow), (2) video densitometry for recording multidimensional chromatograms, (3) in situ scanning mass spectrometry, and (4) bioactivity monitoring for selective detection [103]. These technologies, in combination with 2D, multiple development and coupled column–layer separation techniques could dramatically increase the use of TLC for the characterization of complex mixtures such as plant extracts containing phytochemical antioxidants [103].

2.2. Gas chromatography

Despite the high resolution and sensitivity of GC, due to the lack of volatility of the majority of plant derived antioxidants, its use in the separation has not been as popular as the high-performance liquid chromatography (HPLC). Application of GC is also limited because of the difficulty of large-scale separation and purification. Separation of antioxidant phytochemicals by GC has mostly been attempted for compounds in the essential oils of herbs. Depending on the physicochemical property of the antioxidants, columns of different polarity and lengths have been used in the separation. GC with a capillary column and a MS detector is the predominant system. A column with medium polarity (e.g. DB-5, with 5% biphenyl and 95% dimethylpolysiloxane) was found to give the best results in the separation of antioxidants from *Crataegus oxyacantha*, *Hamamelis virginiana* and *Hydrastis canadensis* [104]. The essential oil of *H. virginiana* showed the strongest antioxidant activity, and its major active component was identified to be 1,2,3-trihydroxybenzene, a phenolic compound by GC. Other studies also indicated that phenolic components in essential oils are the major contributor of the antioxidant activity [105,106]. Among the phenolic compounds of essential oils, carvacrol and thymol are probably the two most recognized antioxidants, typically found in thyme and oregano, respectively [107,108]. GC and GC–MS were used to separate alkylamides in Echinacea [109].

2.3. High-performance liquid chromatography

There is increasing need to know the photochemical profiles of antioxidants in different plants, and among different

varieties of the same plant, but conventional chromatographic techniques (PC, TLC and CC) in general lack the sensitivity and resolution that are often required for trace amount of antioxidant phytochemicals. GC meets these requirements, but its use is somewhat limited due to the non-volatility of many antioxidants. As most researchers would agree, HPLC is perhaps the most popular and reliable system among all chromatographic separation techniques for the separation of antioxidant phytochemicals. The versatility of HPLC is also aided by the different separation modes and types of detection methods, among which is the diode array detector (DAD) coupled with mass spectrometer (MS).

2.3.1. HPLC for carotenoid antioxidants

The lipophilic characteristics of carotenoids have made normal phase HPLC a more favourable choice for the separation of these phytochemical antioxidants. The majority of adsorption HPLC techniques used for the analysis of carotenoids employed silica stationary phase [86]. Separation of saponified carotenoids was carried out on a silica column (250 mm × 4.6 mm i.d., 5 μm) using gradient elution from 95% of light petroleum to 95% acetone [110]. With reversed-phase HPLC, C8 and C18 columns have been proven well suited for routine separations of carotenoids [111–113]. Piccaglia et al. [114] used a C18 column and achieved relatively good separation of free lutein, three lutein monoesters and five lutein diesters. However, for more complex samples, particularly those high in esters, a C30-column seems to have better separation and selectivity than the conventional C8 and C18 materials. RP C30 column is particularly a good choice for the separation of geometric isomers of carotenoids [115–121]. In a method by Sander et al. [122] it was found that with monomeric C18 column, non-polar carotenoid isomers were poorly resolved, and lutein and zeaxanthin were not separated. Better separation of the hydrocarbon carotenoids was possible with the polymeric C30 column. In monomeric C18 or C30 columns, the silica was treated so the surface has thorough endcapping, whereas in polymeric C18 or C30 columns, the material was synthesized from polyfunctional silanes which produce crosslinking of the hydrophobic phase on the silica surface. In the same paper Sander et al. also found that the retention behavior of lycopene varies dramatically with stationary phase properties. With monomeric C18 columns, lycopene usually elutes before α- and β-carotene, whereas with polymeric C18 and C30 columns, lycopene is strongly retained and elutes after these carotenoids [122]. Most recently, using C30 LC–MS, Breithaupt et al. were able to identify eight regioisomeric monoesters in addition to known lutein mono and diesters [123]. Geometric isomers of free carotenoids have been separated using mainly C30 columns, however, we recently developed a method using RP C18 column in combination with DAD and MS detection, and for the first time, separated several *cis* isomers of lutein diesters [124]. Several good review papers have been published in recent years on the separation of carotenoids, and readers are referred to those for more detailed discussions [111,125,126].

2.3.2. HPLC for polyphenolic antioxidants

For the separation of phenolic acids and flavonoids, the chromatographic conditions of the HPLC methods include the use of, almost exclusively, a reversed-phase C18 column; UV–vis diode array detector (DAD), and a binary solvent system containing acidified water (solvent A) and a polar organic solvent (solvent B). The separation normally requires 1 h at a flow rate of 1.0–1.5 mL/min. Solvent A usually includes aqueous acids or additives such as phosphate. Solvent B is normally pure or acidified methanol or acetonitrile. Vast amount of literature exists. Antioxidant flavonoids including rutin and chlorogenic acid in Solidago plants were separated using HPLC-UV [127]. Among the numerous separation systems, only a few procedures were developed to specifically measure polyphenolic concentrations in several commonly consumed foods [46]. Most of these methods have been developed to measure different groups of polyphenolics in a single plant, or a single or a few groups in multiple plant sources. Van Sumere et al. [128] indeed developed a good method that separated nearly 50 phenolic compounds from the rose flower pedals. However, some important antioxidant polyphenols such as procyanidins, chlorogenic acid and phloretin-glycosides were not included in their method. A method by Paganga et al. [129] and two other recent HPLC methods by Schieber et al. [130] and Shui and Leong [131] were developed for the separation and measurement of prominent flavonoids that are members of the subgroups of flavonoids; anthocyanins and procyanidins, however, were not included in their methods. Some methods such as those developed by Escarpa and Gonzalez [132,133], separated multiple groups of the most prominent phenolics with a relatively short analysis time, an obvious advantage for those who are interested in analyzing the major phenolic components. In shortening the analysis time, however, some minor or unknown compounds may have been missed due to co-elution. The co-elution may also affect the quantification of known compounds. Obtaining good resolution is considered to be the main difficulty for a method that is targeted for separation of multiple polyphenolic groups [131]. A method with improved separation was recently developed in the author's group using a binary mobile phase consisting of 6% acetic acid in 2 mM sodium acetate aqueous solution (v/v, final pH 2.55) (solvent A) and acetonitrile (solvent B) and a RP C18 column. The use of sodium acetate was key to the near baseline separation of 25 phenolics commonly found in fruits [134]. Such improved separation is particularly informative in terms of phytochemical profiling and quantification. Detection in HPLC is routinely achieved by UV absorption, often using DAD, however, DAD has mostly been used as a convenient multiple wavelength detector, and its versatility often appears to have been neglected [135]. The hydrophilicity of polyphenols is relative and it spans over a wide range. Oligomeric procyanidins for example are relatively less hydrophilic. Although RP-HPLC has been the primary separation means for the procyanidins, past studies illustrates the difficulty in determining the degree of polymerization of these antioxi-

dants. Hammerstone et al. therefore have developed a normal phase (NP) HPLC method that utilized a series of linear gradients of methanol into dichloromethane with constant amount of acetic acid and water [136]. For the detailed separation method for polyphenols, readers are referred to a recent book by C. Santos-Buelga and G. Williamson [137].

2.3.3. HPLC for other antioxidant phytochemicals

Although carotenoids and polyphenols are the two major antioxidant phytochemicals, many other naturally occurring antioxidants are found in plants [138]. Among them, alkylamides from the chrysanthemum plants and capsaicinoids from the chili peppers are strong antioxidants of particular interest. Isobutylamides in Echinacea were separated using a C8 or C18 HPLC with UV and MS detectors [139–142]. Using HPLC–DAD and HPLC–MS-SIR (selected ion recording), Luo et al. [139] were able to simultaneously separate and identify 12 isobutylamides and other phytochemicals in *Echinacea purpurea*. Capsaicin and related compounds also belong to amide group of phytochemicals and they are good antioxidants as well. Separation of capsaicinoids have been carried out using a MetaSil Basic C2–C8 RP-HPLC column and detected and identified by UV and MS or MS–MS [143].

2.3.4. HPLC with non-adsorption columns

The most frequently used NP and some RP-HPLC techniques for antioxidant phytochemicals are based on an adsorption/desorption mechanism. However, other modes have been used for the separation of some antioxidant phytochemicals. Procyanidins, for example, were separated by size-exclusion chromatography (SEC). Using a TSK gel α-2500 column, and a mobile phase consisting of acetone and 8 M urea (pH 2) (6:4), procyanidins with various degrees of polymerization were separated in native forms from apple and other plant extracts [144,145]. Some other separation modes such as ion exchange chromatography (IEC) have been used for the separation of antioxidant phytochemicals such as anthocyanins. However, these techniques are often used in combination with conventional RP-HPLC (e.g. C18). For instance, ion exchange resins such as Amberlite XAD-7 are often used to separate anthocyanins from other highly water soluble interference like sugars. Anthocyanins separated by IEC are often further purified on a Sephadex LH-20 column before finally being analyzed on a RP C18 column [146].

2.4. High-speed counter-current chromatography

Separation by counter-current chromatography (CCC) is based on the partition coefficient (*K*) of a phytochemical. High-speed CCC (HSCCC) is a relatively new technology and it is the most advanced CCC form in terms of partition efficiency and separation time. The separation in HSCCC is aided by pressure and centrifugal force; the latter is generated from both rotational and synchronous planetary motion of coiled columns. The force provides vigorous mixing between the two immiscible liquid phases, and retention of a very large fraction of the stationary phase [147]. Unlike other chromatographic techniques, HSCCC does not use solid support as the stationary phase, therefore has many advantages over conventional chromatography: (1) the elimination of sample loss caused by irrecoverable adsorption to the solid support matrix; (2) easy scale-up to larger fractionation system by simply changing the Teflon tubing coil (column) to larger sizes; (3) low-cost because it does not use expensive absorbents and columns; and (4) it reflects the real distribution profile of phytochemicals in a sample [148,149]. The most important step in developing a good HSCCC method is perhaps the determination of the *K*-value of an analyte in different two-phase liquid systems. This is normally done by dissolving a small amount of analyte in the same volume of each phase of the pre-equilibrated two-phase solvent system. The two solutions were mixed, shaken vigorously for 10 min, centrifuged at 4000 × *g* for 5 min to obtain a thorough equilibrium. An aliquot of each phase was then analyzed by HPLC or a spectrophotometer. The *K*-value was expressed as the concentration or absorbance of the phytochemical of interest in the upper phase divided by that in the lower phase. It is generally recognized that the *K*-value of the target antioxidant phytochemical must be in the range of 0.2–5 in a given two-phase system in order to obtain good separation [150]. Low *K*-values will result in a poor peak resolution, while high *K*-values tend to produce excessive sample band broadening [151]. In addition to the *K*-value, a suitable two-phase system should also have a satisfactory retention of the stationary phase and short settling time of the two solvents (<30 s) [151]. For more in-depth information on HSCCC theories readers are directed to a general review by Conway [152].

Although the first CCC separation of antioxidant phytochemicals was done nearly two decades ago by Putman and Butler for the separation of condensed tannins [153], a great number of HSCCC applications have been reported in the past several years, particularly in the separation and preparation of active ingredients from traditional herbal medicines including antioxidants [147–166]. Procyanidins in apple, were successfully separated by using type-J multilayer coil planet centrifugation with a two-phase solvent system composed of *tert*-butylmethylether–acetonitrile–water and/or a system containing methyl acetate–water [154,155]. Chlorogenic acid, an antioxidant found in apple and other fruits was separated with high purity and recovery rate from a traditional Chinese medicine *Flos Lonicerae* using HSCCC with a two-phase solvent system containing *n*-butanol–acetic acid–water (4:1:5) [147]. HSCCC has also been applied to separate more lipophilic phytochemicals such as carotenoids. A two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (5:5:6.5:3) was successfully used to separate a strong antioxidant astaxanthin [148].

2.5. Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is a new technology similar to HPLC, however, due to the use of super-

critical fluid such as carbon dioxide, it has several advantages over regular HPLC [167,168]: (1) the use of supercritical fluid such as carbon dioxide significantly reduces solvent waste, and makes it easier in removing the solvent when collecting fractions; (2) antioxidant phytochemicals have higher diffusivity in a supercritical fluid, because such liquid has low viscosity, therefore leads to more homogeneous diffusion of the antioxidant compounds into the packing materials, resulting in higher resolution and faster separation time; and (3) parameters such as temperature, pressure, and fluid composition of the mobile phase can be changed therefore give more venues for better separation [169].

Most of the SFC related literature is on the separation of relatively lipophilic antioxidants. This is not surprising because carbon dioxide, which is non-polar, is the most popular supercritical fluid. The simultaneous separation of *cis*- and *trans*-α- and β-carotenes was achieved using SFC [170,171]. Separation of geometric isomers of α- and β-carotene was also performed on a capillary column [172]. The results are very encouraging in terms of separation and gain in analysis time. Other antioxidants have also been separated by SFC. SFE extract of *Artemisia annua* L., was analyzed by supercritical fluid chromatography (SFC) using a capillary column, coupled with a flame ionization detector (FID). With optimized operating conditions, artemisinin and artemisinic acid were quantitatively extracted at a flow rate of 2 mL/min in less than 20 min. The supercritical fluid was composed of carbon dioxide and 3% methanol with temperature and pressure fixed at 50 °C and 15 MPa, respectively. Results were compared with two conventional liquid solvent extraction processes [173]. Natural α-tocopherol from γ- and δ-tocopherols were also separated by SFC, and the effects of pressure, temperature and the ethanol concentration in the mobile phase on the retention factor and resolution of tocopherols were studied comprehensively by Jiang et al. [174]. A method using normal-phase SFC with methanol as modifier has been developed for determination and quantification of the various indol-3-ylmethyl derivatives including ascorbigens formed from the glucobrassicin degradation product, indol-3-ylmethanol, under acidic conditions (pH 2–6) with and without the presence of ascorbic acid. The SFC method had detection limits in the 10–100 pmol range [175].

2.6. Capillary electrophoresis

Although HPLC stays as the most dominating separation technique for antioxidant phytochemicals, capillary electrophoresis (CE) is gaining popularity. Like HSCCC and SFC, CE is also a relatively new technique; however, it represents an alternative method for the analysis of different groups of antioxidant phytochemicals [176,177]. CE has several unique advantages compared to HPLC [178]: (1) it requires a very small sample size, (2) high efficiency due to non-parabolic fronting; (3) shorter analytical time; (4) low cost, particularly when use capillary zone electrophoresis (CZE) and fused-silica capillary; and (5) use no or only small amount of organic solvent therefore limits solvent waste [178–183]. Separation of antioxidant phytochemicals in capillary electrophoresis is based on the differences in mass to charge ratios of these compounds, and complex formation with tetraborate molecules when the phenolic compound has *ortho*-hydroxy groups. There are different modes in CE separations. CZE is the simplest mode and has been used to separate various types of antioxidant phytochemicals, particularly phenolic compounds [184–190]. Several CZE methods were developed for the separation of polyphenolic antioxidants such as epicatechin, catechin, quercetin, gentistic acid, caffeic acid, gallic acid and *trans*-resveratrol, myricetin and rutin in wine and grape samples [189,191]. Antioxidants in Ginkgo leaf infusates were also separated using a CZE system [192]. A recent CZE method was developed for the separation of anthocyanins in wine [193]. This method had comparable quantitative results with the HPLC method, but it significantly reduced the analysis time by nearly 75%. da Costa et al. separated anthocyanins from blackcurrant (*Ribes nigrum*) using CZE, and found that resolution and peak shapes of the anthocyanins were critically influenced by the pH of the running buffer and the presence of an organic solvent. Optimum qualitative separation was achieved on a fused-silica capillary with a phosphate running buffer containing 30% (v/v) acetonitrile at an apparent pH of 1.5 [194,195]. In CZE, modifiers such as organic solvents are often added to the running buffer to increase resolution of phytochemicals [182,196]. Addition of a modifier can reduce the viscosity, lower the zeta potential of the capillary wall, and increase selectivity and resolution. The major phenolic diterpenes responsible for the antioxidant properties of rosemary extracts, carnosol and carnosic acid, were separated by capillary zone electrophoresis (CZE) using a 56 cm long uncoated fused-silica capillary and a 50 mM disodium tetraborate buffer of pH 10.1. The CZE method had good reproducibility (relative standard deviation less than 5%) and the separation of carnosol and carnosic acid was accomplished in less than 11 min [197]. CZE was also used to separate puerarin, daidzein and rutin, antioxidants from the traditional Chinese medicinal plants, *Pueraria lobata* (Wild.) Ohwi and *Puerariae Radix* [198] and farrerol, quercetin, syringic acid, vanillic acid, 4-hydroxybenzoic acid, protocatechuic acid in *Rhododendron dauricum* L. [185].

Micellar electrokinetic chromatography (MEKC) uses surfactants such as sodium dodecyl sulfate (SDS) which form highly organized spherical micelles at concentrations above the critical micellar concentration (CMC) with the lipophilic tails toward the interior and the hydrophilic ends on the surface of the micelle. This creates an additional separation mechanism through partition. Compounds with different *K*-values can then partition differently between the micelles and the mobile phase (running buffer). This mode is particularly important for the separation of different neutral antioxidants. The CMC for SDS is typically 20 mM. The MEKC technique has been used for the separation of polyphenolic antioxidants [199–205]. Organic modifiers were also incorporated into the

MEKC system in some of these methods to increase the separation efficiency and resolution. Antioxidants from rosemary were determined using a new MEKC method, and found that MEKC had slightly lower reproducibility in peak area, but similar in retention time. However, the main advantage of MEKC is its much higher separation speed [206].

CE as a separation technology is still rapidly evolving and new modes of separation are being developed. Among them, a hybrid technique combining solid-phase matrix such as C18 and C30 polymers with high voltage electrophoresis has been found very useful. This new mode is called capillary electrochromatography (CEC). Sander et al. first reported the use of polymeric C30 stationary phases in CEC for the separation of carotenoid isomers [207,208]. This method was able to separate lycopene isomers, β-carotene isomers, α-carotene isomers, lutein isomers, zeaxanthin isomers and β-cryptoxanthin isomers in 35 min.

3. Quantification and online identification

The main purpose of this review is to give up-to-date information on the separation of antioxidants in plants. Methods for detection, quantification and online identification are inseparable from many of the above discussed separation techniques, however, they are ultimately a means for the confirmation of good separation. The above discussed separation modes (HPLC, CE, SFC, HSCCC) often share the same detection techniques, particularly UV–vis–DAD and MS detectors.

3.1. UV–vis and diode array detection

UV–vis spectrophotometry has been long used for quantitation of organic compounds that absorb light in the ultraviolet and visible region. Most antioxidant phytochemicals have highly conjugated double bond or aromatic systems that absorb light in this region. The application of UV–vis detector in separation technologies, particularly the combination of DAD and HPLC has gone beyond quantification by light absorbance. HPLC–DAD has played important roles in the identification of antioxidant phytochemicals, particularly polyphenolic compounds and carotenoids. In HPLC–DAD, the spectral information of known standards can be obtained online and saved as a library database. The UV–vis spectral data of all eluting peaks of a sample can be scanned, stored and later retrieved for comparison with the library data. A match of both UV–vis spectrum and retention time can lead to highly positive identification of an antioxidant phytochemical. As a detector, DAD is also capable of simultaneously detect and record chromatograms at different wavelengths. This feature significantly enhances the performance of the separation system, particularly when different groups of antioxidant phytochemicals are mixed in one sample. When proper wavelengths are chosen, e.g. at the maximum absorptions, all groups of antioxidant phytochemicals can be detected with the highest sensitivity [134]. An appropriate selection of the detection wavelength can also make possible the quantification of an unresolved or poorly resolved peak. DAD can also be used to examine the purity of a peak. In-depth discussion of HPLC–DAD and its use in identification of phytochemicals are beyond the scope of this paper, and there are several excellent reviews recently published if readers want to obtain further information [111,209].

Although UV–vis and DAD provide useful information for the identification of antioxidant phytochemicals, the use of conventional approaches based on spectra is often limited when samples contain very similar compounds. Unambiguous identification of structures cannot be done using UV–vis and DAD spectral data only [209]. For complete structural identification, other techniques such as MS and NMR are often necessary. Having said that, the combination of DAD and MS in HPLC has been a highly useful tool in the separation and determination of antioxidant phytochemicals. DAD is particularly useful in the selective detection of antioxidants with distinct UV–vis absorption patterns. Good examples of such compounds can be the carotenoids and anthocyanins. All carotenoids have the characteristic absorption pattern in the visible region between 410 and 470 nm. There are normally three absorption maxima at 410, 440 and 476 nm, although slight shifts may occur depending on the structural differences. A strong absorption at 330 nm indicates the *cis*-configuration [124]. In terms of polyphenolic antioxidants, certain subgroups can be separated by monitoring at different maximum UV–vis absorption (λ_{max}). The λ_{max} for the cinnamic acid and its derivatives is near 320 nm, and that for the benzoic acids, flavan-3-ols (including the dimers) and dihydrochalcones is about 280 nm. The λ_{max} for the flavonols is usually around 360 nm. Among the flavonoids, however, anthocyanins are the most unique subgroup because they absorb visible light near 520 nm when the molecules are in the flavylium cation status (when pH is low). This spectral characteristic gives anthocyanins advantages in being detected without the interference from other groups of phytochemicals. The maximum wavelength of absorption in the visible region for anthocyanins is found to be related to the substituent pattern in the B ring (Fig. 1).

3.2. Mass spectrometry and tandem mass spectrometry

Mass spectrometry (MS) can be carried out online coupled with chromatographic or electrophoretic techniques or offline as a stand-alone instrument. However, it is the former that provides unsurpassed opportunities in the identification and structure elucidation of antioxidant phytochemicals. There are two main types of ionization techniques for the antioxidant phytochemicals, the ion-spray techniques such as electrospray ionization (ESI), thermospray and atmospheric pressure chemical ionization (APCI), and the ion-desorption techniques which include fast atom bombardment (FAB), plasma desorption (PD), and matrix-assisted laser desorption ionization (MALDI). ESI and APCI are the two most widely used ionization methods for antioxidant phytochemi-

cals, and most commercial chromatography–mass spectrometry (LC–MS) instruments can accommodate both of these techniques. Although there is no clear line, ESI is more often used to ionize antioxidant molecules such as anthocyanins that are polar and exist as ions in aqueous solutions, and APCI is used for less polar and non-ionic antioxidants such as carotenoids [210]. APCI and ESI can be operated under both positive and negative ion modes (PI and NI). The most frequently used mass analyzers can also be separated into two main groups: analyzers based on ion beam transport such as magnetic field, time-of-flight (TOF), and quadruple mass filter; and those based on ion trapping technology. These analyzers vary in their capabilities with respect to resolution, accuracy and mass range. MS detector is critical for the identification of antioxidant phytochemicals because of the complex and diverse structures, and low concentrations in the plants. Sensitivity and selectivity of detection can be increased using tandem mass spectrometry, i.e. two (MS–MS) or more (MS^n) mass analyzers coupled in series. MS–MS and MS^n produce more fragmentation of the precursor and daughter ions, therefore, provide additional structural information for the identification of antioxidant phytochemicals. There are many excellent recent reviews on the application of LC–MS in quantitative and qualitative analyses of phytochemicals including antioxidants [121,135,195,211–215]. Flamini [216] has summarized the use of LC–MS in studies of polyphenols in grape extracts and wine. He specifically indicated that LC–MS techniques are the most effective tool in the study of the structure of anthocyanins, particularly the MS–MS approach which is a very powerful tool that permits anthocyanin aglycone and sugar moiety characterization. In the same review, other LC–MS techniques such as the matrix-assisted-laser-desorption-ionization–time-of-flight (MALDI–TOF) was also discussed by the author for the analysis of procyanidin oligomers.

Although PI-MS were used for the detection of various antioxidant phytochemicals, it was found that NI-MS methods, both APCI and ESI were excellent for flavonoid analysis, both in sensitivity and specific structural information [217]. Data reported in this paper showed that ESI was the method of choice for the analysis of low-molecular-mass phenols under NI mode, whereas flavan-3-ol compounds were well detected under both positive and negative ion. Negative LC–APCI–MS and low-energy collision induced dissociation (CID) MS–MS were used to provide molecular mass information and product-ion spectra of the flavonoid glycosides in some herbs [218]. Detection of phytochemicals including antioxidants has been subjected to many recent reviews [210,211].

4. Conclusion

The human health benefits of phytochemicals have been shown by many recent studies, and the roles of antioxidant phytochemicals as a whole have also been clearly demonstrated. However, despite the strong evidence that many groups of phytochemicals have good antioxidant activity both in vitro and in vivo, our knowledge about the biological function of individual antioxidant phytochemicals is lacking. Some of the biggest hurdles may include the low concentration, instability and difficulty in separation and detection of these bioactive compounds. In this review, the authors intend to condense some of the latest technologies that have been applied to the separation of antioxidant phytochemicals. The authors also provided some background information about the antioxidants chemistry and biochemistry, and their links to the health benefits. It is the authors' sincere wish that by emphasizing on the major antioxidant groups, i.e. carotenoids and polyphenolics, readers will be encouraged to carry out further studies on the development of new separation techniques and apply to these and other groups of antioxidant phytochemicals.

5. Nomenclature

2D	two-dimensional
AAPH	2,2′-azobis(2-amido-propane)dihydrochloride
ABTS	2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate
APCI	atmospheric pressure chemical ionization
ASE	accelerated solvent extraction
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CC	column chromatography
CCC	counter current chromatography
CE	capillary electrophoresis
CEC	capillary electrochromatography
CID	collisionally-induced dissociation
β-CLAMS	β-carotene–linoleic acid model system
CMC	critical micellar concentration
CVD	cardiovascular disease
CZE	capillary zone electrophoresis
DAD	diode array detector (or diode array detection)
ESI	electrospray ionization
FAB	fast atom bombardment
FRAP	ferric reducing/antioxidant power
GC	gas chromatography
GC–MS	gas chromatography–mass spectrometry
HPLC	high-performance liquid chromatography
HSCCC	high-speed counter current chromatography
IEC	ion exchange chromatography
K-value	partition coefficient
LC	liquid chromatography
LC–MS	liquid chromatography–mass spectrometry
MAE	microwave-assisted extraction
MALDI	matrix-assisted laser desorption ionization
MEKC	micellar electrokinetic chromatography
MS	mass spectrometer (or mass spectrometry)
MS–MS	tandem mass spectrometry
NHP	natural health products
NFF	nutraceuticals and functional foods

NI negative ion
NMR nuclear magnetic resonance
NP normal phase
$O_2^{\bullet -}$ superoxide anion
$^{\bullet}OH$ hydroxyl radical
ORAC oxygen radical absorption capacity
PC paper chromatography
PCL photochemiluminescence
PD plasma desorption
PI positive ion
PLE pressurized liquid extraction
P-PE R-phycoerythrin
$ROO^{\bullet}$ peroxyl radical
ROS reactive oxygen species
RP reversed-phase
SDS sodium dodecyl sulphate
SEC size-exclusion chromatography
SFC supercritical fluid chromatography
SFE supercritical fluid extraction
SIR selected ion recording
SPE solid-phase extraction
TBARS thiobarbituric acid reactive substance
TEAC trolox equivalent antioxidant capacity
TLC thin-layer chromatography
TOF time of flight
UV ultraviolet
vis visible

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