10
Extraction of Natural Antioxidants

Vassiliki Oreopoulou
National Technical University of Athens
Athens, Greece

I. INTRODUCTION

Natural antioxidants are compounds from plant or animal sources that retard oxidative rancidity of oils, fats, and fat-soluble components, thus protecting them while delaying the development of unpleasant flavors and odors resulting from oxidation.

Antioxidants are present naturally in most raw food sources. Processing can remove or degrade some of these antioxidants. Therefore, a supplementation with suitable antioxidant compounds is needed to maintain acceptable quality of the products. Especially oils, fats, and products with a high fat content are susceptible to oxidation and require the addition of antioxidants. The most widely used antioxidants are synthetic ones, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ) and propyl gallate (PG). Doubts about the safety of synthetic antioxidants arose first in the 1960s and led to an increased interest and a broad research on natural antioxidants (1, 2). Natural antioxidants are primarily phenolic compounds that may occur in all parts of a plant. They are multifunctional and can act as free radical terminators, metal chelators, and singlet oxygen quenchers. The common plant phenolic antioxidants are tocopherols, flavonoids, and related compounds like coumarins, cinnamic acid derivatives and chalcones, phenolic diterpenes, and phenolic acids. Another widespread antioxidant in nature is ascorbic acid, but today its extraction from natural sources is not significant as all ascorbic acid used for food is chemically synthesized.

To be considered as an antioxidant for practical use, a compound extracted from a natural source must meet several criteria, including (a) absence of any

toxic or physiological effect, (b) no impartation of any strong odor, flavor, or color to the product, and (c) considerable antioxidant activity at small concentrations in the product. In fact, antioxidants present in or added to foods are functional at very small concentrations, usually up to 0.02%.

Usually, in literature surveys on antioxidants, compounds that have no antioxidant activity themselves but enhance the activity of antioxidants, called *synergists*, are included. This chapter covers the main sources and extraction processes of natural antioxidants and synergists.

II. SOURCES

The main sources for the extraction of natural antioxidants are of plant origin. Some animal sources, such as shrimp, have also been reported (3). The most common plant sources are (a) cereals, (b) citrus fruits, (c) cocoa and coffee beans, (d) herbs and spices, (e) oilseeds, (f) olives, (g) onion and garlic, (h) tea, as well as some miscellaneous products.

A. Cereals

Several cereals have been reported to possess antioxidant activity. Oat has been the most widely investigated because it has greater activity than the others and a bland flavor. Oat flour has been incorporated in dairy products, potato crisps, sausages, mayonnaise, etc., and improved their stability. Malt and barley flour or meal acted as antioxidants on fats too (2).

Extracts of groats and hulls from oat were more active than synthetic phenolic antioxidants in the protection of oils during frying and of emulsions (4, 5). The antioxidant activity of oat hulls and groats is attributed to several phenolic compounds, mainly ferulic, *p*-coumaric, caffeic, vanillic, *p*-hydroxybenzoic acids and vanillin (6). The extract of defatted oat kernels also showed antioxidant activity similar to BHT and PG that was attributed to caffeic and ferulic acid and their derivatives (7, 8). Oat hulls contain more phenolic acids than oat flour; therefore, oat hulls are more efficacious for antioxidant extraction (9).

Byproducts of other cereals could be used as raw materials for antioxidant extraction, too. Buckwheat hulls extracts showed a good antioxidant activity, which was attributed to the presence of dihydroxy phenolic components, flavonols, and flavone glucosides (10, 11).

B. Citrus Fruits

Citrus fruits and especially oranges contain antioxidant components (12, 13). These antioxidants are mainly concentrated in the outer part of the peel, called

flavedo. Extracts of orange and grapefruit flavedo had significant activity against *d*-limonene oxidation, but extracts of flavedo from lemon and lime had very little activity (13). On the contrary, extracts of lemon peel offer effective protection from peroxidative damage in living systems (14).

Antioxidant properties of extracts from orange flavedo were attributed to tocopherols (2, 13). Also citrus essential oils and terpenes showed antioxidant activity in several food products (2). However, flavonoids isolated from various citrus extracts seem to be the most important antioxidant components present in citrus fruits. More than 60 individual flavonoids have been identified in citrus fruits (15). Among them, flavanones are the most abundant, and especially hesperetin and naringenin are presented in the pulp of all citrus fruits (16). Highly methoxylated flavones occur in much lower concentration but exhibit the greatest antioxidant activity. Two isoflavones are referred as the main antioxidant components of osage orange peel (12), while a flavanone, eriocitrin and its aglycon, eriodictyol, are referred as the main antioxidant components of lemon peel (14). A review on citrus flavonoids and on the correlation of their structure to antioxidant activity has been presented by Benavente-Garcia et al. (17).

C. Cocoa and Coffee Bean

Cocoa bean husk powder and especially water and alcohol extracts were reported as potential antioxidants (18, 19). Fractions with antioxidant activity were isolated from the extracts, but the active substances were not identified. The brown pigment was suggested as the antioxidant factor (2), though it was not active to all substrates (20).

Roasted coffee powder showed antioxidant properties in oils. However, the antioxidant activity of the coffee powder was lower than that of its constituents: caffeic acid and quinic acid. Another potent antioxidant component in coffee is chlorogenic acid (2).

D. Herbs and Spices

Herbs and spices are the most broadly examined raw materials for the occurrence and extraction of antioxidants. Since the pioneer work of Chipault et al. (21), many researchers have reported results on the antioxidant efficiency of various herbs and spices and on the identification of active components. Though the results are sometimes contradictory, the extracts have in most cases higher activity than the relevant ground herbs or spices (2).

Rosemary has exhibited the greatest antioxidant activity of all spices and herbs. The extracts of rosemary were more effective than BHA and BHT in the protection of fat and of products with a high fat content (22–24). Bleached, odorless, and tasteless antioxidants have been produced from rosemary and com-

mercially exploited since the early 1980s (25, 26). The most active components of rosemary, identified in the earlier studies, were carnosol, rosemaridiphenol, and rosemariquinone (27–29). Nowadays several commercial rosemary extracts are available and their most active compounds are reported to be carnosol, carnosic acid, and rosemarinic acid (30–32).

Sage belongs to the same family as rosemary—the Labiatae family—and possesses almost equivalent antioxidant activity. Many investigations on rosemary antioxidant efficiency also include data on sage efficiency (2), and the same procedures for the extraction and purification of the antioxidant fraction from both spices have been suggested (25, 26). The main antioxidant constituents in sage were also found to be carnosol, carnosic acid, and rosemarinic acid, followed by other related phenolics (31, 33). In addition to the main sage species, i.e., *Salvia officinalis*, several other varieties have also shown remarkable antioxidant efficiency (34–36).

Other plants of the Labiatae family are potent sources of natural antioxidants (37). Oregano is one of the most promising. Extracts of oregano retarded lipid oxidation (37–40), while the essential oil of the spice also contained antioxidant components (41). Thyme seems to be another promising spice: extracts of the plant showed appreciable antioxidant activity (37, 42) and its essential oil was effective (43). Dittany and marjoram extracts exhibited antioxidant activity too (2, 37).

Several other herbs, spices, and medicinal plants are rich in antioxidant components (2, 42, 44–50).

E. Oilseeds

Oilseeds contain several antioxidant compounds that protect them against rancidity. Therefore, whole seeds or the byproducts obtained after oil extraction could be potential sources of antioxidants. Methanolic extracts of cottonseed exhibited antioxidant activity attributed mainly to flavonoids (51). Flavonoids are also claimed to be the major antioxidants of soybean methanolic extracts (52–54). Most of these flavonoids remain in the waste during soybean curd processing and could be recovered (54). According to Rhee et al. (55), cottonseed meal had a higher phenolic content and antioxidant activity than soybean meal. Canola meal is another potent source of antioxidants with a total phenolic content remarkably higher than both cottonseed and soybean meals (56). Other oilseeds studied with positive results include sunflower seed (57) and sesame seed (58).

F. Olives

Olives and olive oil are rich in antioxidant compounds, especially tocopherols. In addition to tocopherols, several other phenolic compounds, i.e., hydroxytyro-

sol, caffeic, protocatechuic, ferulic, syringic, and vanillic acids, were identified in the extracts of virgin olive oil and were found effective in prolonging the shelf life of the refined olive oil (59, 60). Rape, a major byproduct of olive oil production, was used for the extraction of antioxidants. The extracts contained various phenolic acids, catechol, and tyrosol, and inhibited oxidative deterioration of refined vegetable oils (61). Also, olive leaves contain phenolic antioxidant compounds, and some of them were identified (62–64).

G. Onion and Garlic

Onion contains appreciable amounts of the well-known flavonoid quercetin (16) and exhibits a remarkable antioxidant activity (65, 66). Onion skins are also rich in quercetin and quercetin derivatives, and their methanolic extracts were effective antioxidants (67, 68).

Garlic was found effective for some substrates, i.e., linoleic acid or minced pork (65), but ineffective in lard (44).

H. Tea

Tea leaves are a well-known source of natural antioxidants, especially catechins. Fresh or mildly processed commercial tea has been found to contain large quantities of these polyphenols, up to 30% of its dry mass (69). Comparable concentration has not been reported in any other foodstuff (70). Green tea extracts were 21–24% more effective at radical quenching than black tea extracts in both water and lipid-soluble media (71). Aqueous extracts of green tea retarded the oxidation of oils and fats more than commercial rosemary extract (72), and were also effective in emulsions (73) and fish meat (74). Commercial tea extracts are available with various catechin contents (74).

I. Miscellaneous Products

Various other fruits, vegetables, and grains contain considerable amounts of antioxidants (16, 48, 75). Among them those that could serve as potential sources for the extraction of antioxidants are the byproducts of agrofood industries or crops with a limited utilization due to damage or to low commercial profit.

Grape skins and seeds are a byproduct of the wine making industry, with a high phenolic content and a remarkable free radical scavenging capacity and antioxidant activity in oils (76–80). The antioxidant activity of citrus peel and pulp was mentioned above, and similar byproducts from other fruit processing industries can be used as well (81). Many other potential sources are referred, including bean hulls (82, 83), lupin derivatives (84), leaves of some agricultural products (67, 85), potato peel waste (86), and so forth.

III. EXTRACTION PROCESSES AND PARAMETERS

Although some of the raw materials mentioned above have been incorporated as dry powders to certain foods or food systems in order to protect them from oxidative deterioration, the use of extracts of the raw materials is suggested in most cases. The aim of extraction is to concentrate the antioxidant components of the raw material, apart from inert substances, so that the product of the extraction could be added to the food in smaller quantities.

The extraction process involves a more or less vigorous agitation of the ground raw material with the extraction solvent at ambient or elevated temperature and subsequent separation of the extract from the residue by filtration. Repeated extraction steps may be accomplished to increase the extract yield. Alternatively, a packed bed of the ground material can be used which is leached by the extraction solvent under refluxing conditions.

A. Solvent Selection

The solvent used for the extraction is of major importance for the recovery of the antioxidant components, the coextraction of undesirable substances, and the process yield. For the extraction of antioxidants from the majority of the sources mentioned above organic solvents have been used. Water has been used to a lesser extent, mainly for extracting antioxidants from tea and for the recovery of essential oils through steam distillation. In addition, water is used in mixtures with alcohols. Edible vegetable oils have been also reported and patented as extraction solvents.

Several studies focused on the efficiency of different organic solvents in extracting antioxidant components. The antioxidant components are of a phenolic nature; therefore, organic solvents of higher polarity are more effective in quantitative recovery of these substances than nonpolar solvents. For example, extracts obtained with methanol or ethanol from flour or hulls of cereals had higher antioxidant activity than extracts obtained with hexane, petroleum ether, ethyl ether or its mixtures with chlorophorm, acetone, and ethyl acetate (2, 4, 10). Also, the extraction of various aromatic herbs by hexane, ethyl acetate, or ethanol showed that the ethanol extracts were the most active in retarding the autoxidation process in lipid substrates (50). Similarly, in extraction of cocoa bean husks with various organic solvents, the alcoholic extracts were the most effective (18). In fact, methanol or ethanol are widely used for the extraction of antioxidants from rosemary, sage, and other herbs and spices, as well as from oilseed and other agroindustrial byproducts. Aqueous solutions of methanol are also used.

Although methanol and ethanol are suitable for quantitative extraction of total phenolics, many undesirable substances are coextracted, and purification is necessary to isolate the antioxidant fraction. Meanwhile, solvents of lower polarity may be used to recover and isolate special groups of phenolics. Thus ethyl ether yields a very low recovery of total phenols compared with 80% aqueous methanol, but it can extract the low molecular weight phenolic acids (87) which are good antioxidants. Both ethyl ether and hexane are suitable for the extraction of tocopherols and terpenoids with appreciable activity (40, 49). On the other hand, pure methanol or aqueous solutions of methanol may cause some decomposition to certain phenolic glucosides, which has not been detected with acetone, aqueous acetone, or ethyl acetate (87).

Results obtained by successive extractions of various raw materials with different solvents further indicated that the choice of the appropriate solvent depends on the raw material and the nature of the antioxidants it contains. Thus, in a sequential extraction of olive rape with hexane acetone and ethanol, the hexane extract, despite amounting to 9% of the dried rape, had very few polyphenols and lower activity than either the acetone or the ethanol extract, which had similar behavior and amounted to 4% and 7% of the dried rape, respectively (61). A similar result was obtained when cocoa byproducts were extracted with petroleum ether, or methanol, or successively extracted by petroleum ether followed by methanol. The methanol extract showed the highest activity which was further increased for the methanol extract obtained from the residue of petroleum ether extraction (88). Also, pretreatment of cocoa husks with petroleum ether and chloroform before alcoholic extraction improved the antioxidant properties of the alcohol extract (19).

In contrast in a sequential extraction of turmeric with hexane, benzene, and 80% aqueous methanol, all of the extracts had very good antioxidant properties but the benzene extract showed the highest activity. The yield of the hexane, benzene, and 80% aqueous methanol extracts were 1.98%, 4.15%, and 5.24% on a dry basis, respectively (89). When another spice, oregano, was successively extracted with hexane, ethyl ether, ethyl acetate, and ethanol, the hexane and ethyl ether extracts showed higher activity than the following extracts (40). The yield of hexane extraction amounted to 8.8% on a dry basis and the main antioxidant components were terpene derivatives. Yields of following extracts with ethyl ether and ethyl acetate were rather low (1.5% and 2.8% d.b., respectively), while the ethanol extract yield was 12.3% d.b. The antioxidant components of the ethanol extracts were mainly flavonoids, but a lot of other substances were coextracted; therefore, this extract had to be purified (90).

A comparative study of extraction yields and antioxidant activities of extracts obtained by different solvents and sequential extraction steps, from several aromatic herbs, was conducted by Dapkevicius et al. (46). Hydrodistillation was

used to isolate the essential oils and the yields were greatly dependent on the plant, (as high as 6.9%). Except for thyme essential oil, the rest showed no significant antioxidant activity. Extracts obtained by acetone after hydrodistillation or without previous hydrodistillation had good antioxidant activity. The yield was higher when the acetone extraction was conducted without a previous hydrodistillation step and amounted to 11.3% and 6.6% for the rosemary and sage extract, respectively. Methanol-water (1:1) extraction of the residues obtained from the acetone extraction resulted in extracts with yields higher than the acetone extraction (except for rosemary), but with lower antioxidant activity. In the same study, the water extract of the herbs obtained after hydrodistillation was tested and showed no antioxidant activity.

B. Effect of Extraction Parameters

The parameters that affect antioxidant recovery by aqueous ethanol extraction were investigated by Wettasinghe and Shahidi (92) during the extraction of an oilseed meal. The concentration of ethanol in the extraction medium affected markedly the recovery of antioxidants and best values lied in the range of 50–60%. The extraction temperature and time affected also the antioxidant activity of the extract. In particular, the activity increased with temperature and time up to 70°C and 60 min, respectively, and declined afterward. The optimal extraction conditions were determined through response surface methodology as 52% ethanol in water, 74°C, and 62 min. In the same study, aqueous methanol or aqueous acetone were tested as extraction media with slightly lower antioxidant activity than the ethanolic extracts.

Alcoholic extraction of other raw materials, like grape byproducts, is most effectively accomplished under acidic conditions. HCl acid is used and the pH is arranged around 3–3.5 (76). To avoid acid without decreasing phenolic recovery several sequential extraction steps with alcohols and alcohol-water mixtures were suggested (93). However, the hydrolysis of glucosidic bonds of polyphenols, induced by acid, is not necessarily undesirable as it might even increase the antioxidant activity of the relevant compounds.

Solvent extraction parameters for the isolation of phenolic antioxidants from wood hydrolysates were studied by Cruz et al. (94). They used ethyl acetate or ethyl ether as extraction solvents and found that ethyl acetate was more effective in quantitative removal of phenolics. A single-stage extraction at a solvent/hydrolysate volume ratio of 3:1 or a two-stage extraction at a solvent/hydrolysate volume ratio of 1:1, and a contact time of 30 min were adequate for the removal of 84% of the phenolics. Acidic pH conditions, i.e., pH 3, favored phenolic recovery, while temperature in the range of 10–40°C had no effect. The antioxidant activity coefficient of the extract obtained under the best conditions was equal to 64% of the relevant value of BHT.

Water extraction has been studied mainly for the extraction of various components from tea (95). The same kinetic model was fitted to the extraction of catechins, which are the main natural antioxidants present in tea, and of caffeine. The smaller catechin molecules (i.e., ungallated catechins) presented a higher rate of extraction, supporting the hypothesis that the rate-determining step is a diffusion one through the leaf matrix to the surface (69). Also the percent recovery after 20 min at 80°C of the gallated catechins decreased with increasing concentration of the tea, while the recovery of the ungallated catechins was not affected (96). Another parameter that affected extraction efficiency was the pH of the water. Increase of the pH from 6 to 7.6 resulted in decrease of catechin recovery due to epimerization (96). Temperature dependence of the rate of extraction of individual catechins was different (69). Therefore, extraction parameters may be selected to allow preferential extraction of some catechins instead of others. Caffeine is coextracted with catechins by water, and its rate of extraction is higher than the corresponding catechin value for green tea and lower for black tea. Also, the effect of temperature on the rate is greater for green than for black tea (69, 97).

Other parameters, which were proposed for the recovery of tea polyphenols, were extraction with water at 80°C, at a liquid-to-solid ratio of 14:1 (v/w), applying three successive extractions (98). For the recovery of flavonoids, ethanol-water mixtures (7:3), at a liquid-to-solid ratio of 5:1 (v/w), at ambient temperature were used with success (99).

C. Extraction Procedures

Several extraction procedures have been proposed and some of them have been patented. In most cases purification of the extracts is included.

An extraction and purification process for rosemary and sage was patented by Chang et al. (25). The solvent initially used was ethyl ether at a liquid-to-solid ratio of 2.4:1 and the extraction was conducted under reflux for 2 h. The residue obtained after filtration of the mixture was reextracted with ethyl ether under the same conditions, and the filtrates were combined and freed of solvent to yield up to 26% of crude antioxidant depending on the number of extractions. Methanol was also proposed as the extraction solvent in two reports (27, 91). To produce a bland, odorless, and tasteless antioxidant the crude extract was subjected to molecular distillation or vacuum steam distillation to yield approximately 10% of purified antioxidant. Kalsec Inc., which has the exclusive license to use this patent, modified the procedure to reduce color in its rosemary extract and manufactures two types of rosemary extracts with antioxidant inhibitors: an oil-soluble extract and a water-dispersible extract.

Sequential extraction of oregano with solvents of increasing polarity is a promising process. Hexane, ethyl acetate, and ethanol were used and the extrac-

tion was conducted under reflux through a fixed bed of the raw material. The extracts obtained by hexane and ethyl acetate were light colored and could be bleached with active carbon, if desired. The methanol extract had to be further purified in order to remove substances with no antioxidant activity. Final antioxidant solutions in oil were prepared by thorough mixing with refined vegetable oils and removal of the solvent under vacuum. Hexane extraction could be replaced by petroleum ether extraction with equal results. The process was also applied successfully to a sage species (*Salvia triloba* L.).

Except of organic solvents, solutions of potassium or sodium bicarbonate or disodium phosphate were also used in an extraction process conducted at 40–90°C under nitrogen. The extracts were subsequently decolorized, deodorized, and demineralized. The process was patented by Nestle SA (100) for the extraction of antioxidants from rosemary, sage, and parsley.

Oil extraction of ground spices with an edible animal or vegetable oil at 120–125°C was patented by Campbell Soup Company (101). The extract was separated from the spice solids by centrifugation and filtration, and deodorized by heating under vacuum while sparging with steam. Micronization of the ground spice in an edible oil was later proposed by Bracco et al. (26). The antioxidant components were separated from the lipid phase by molecular distillation either on falling film or on a centrifugal system.

Several other processes have been patented for the extraction of natural antioxidants (102, 103). These extracts are mainly characterized as flavorings and are usually approved as such by the legal organizations. However, they also have the ability to manage oxidation. Rosemary extracts are by far the most commercially exploited products and have been reported to represent about 40–50% of the antioxidant market in Europe (102).

D. Extraction with Supercritical CO_2

A relatively new process for the extraction of natural antioxidants involves supercritical CO_2 extraction. Extracts obtained by CO_2 extraction of various herbs at 30 MPa and 40°C had similar or higher antioxidant activity than the relevant acetone extracts (46). The yields obtained by supercritical CO_2 extraction were in most cases lower than the ones obtained by acetone extraction. Green tea was extracted by CO_2 at 31 MPa and 60°C, and the total yield as well as the yield of individual catechins was considerably lower than that obtained by ethanol or water extraction in a Soxhlet apparatus (104). The addition of aqueous ethanol solutions as cosolvents in the supercritical extraction increased the yield of catechins and the ratio of catechins to caffeic and galic acid. The increase was higher as the concentration of ethanol in the solutions increased up to 95%.

The effect of extraction conditions on total yield of solutes and on the antioxidant activity of the extracts was investigated. Esquivel et al. (105) used

summer savory and found that an increase of pressure above 12 MPa did not increase significantly the extraction yield that amounted approximately to 75% of that obtained by hexane. Extraction was conducted at 40°C and the other conditions determined for maximal solute recovery was the solvent-to-solid ratio, equal to 120 kg CO_2/h kg solid, and the duration of extraction, equal to 1 h. The extracts obtained from summer savory under the studied conditions did not show any antioxidant activity in oils at 120°C, as they consisted mainly of essential oils, while the residue of extraction was effective. Extraction of rosemary was accomplished on a pilot plant scale at 30–35 MPa and 40–60°C (106). The extracts were separated in two fractions with different antioxidant activity by arranging the conditions of pressure and temperature in two separator vessels. Methanol was also added as a modifier. The highest antioxidant activity was demonstrated by the extract obtained at 35 MPa and 50°C with no modifier added and separated in the first separator by a pressure reduction to 20 MPa, resulting in a density reduction from 0.9 to 0.78 g/mL.

Supercritical CO_2 extraction has also been proposed for the isolation of tocopherols from soy sludge (107). The most important raw material for the extraction of tocopherols is the deodorizer sludge obtained in the deodorization of vegetable oils and fats. The processing parameters (temperature, vacuum, quantity of injected steam) are critical for the yield of tocopherols. Besides the various tocopherols these distillates also contain sterols, hydrocarbons, flavor components, free fatty acids, and neutral triglycerides. The separation of tocopherols from other compounds is possible by esterification with a lower alcohol, followed by washing and vacuum distillation, by saponification, or by fractional liquid-liquid extraction. Additional purification steps may include molecular distillation, extraction, crystallization, or a combination of these processes (108). Due to the similar volatility of sterols, tocopherols, and fatty acids, it is quite difficult to separate them by fractional distillation and steam stripping at high vacuum. Recently, enzymatic processes have been used for hydrolysis of the neutral triglycerides to fatty acids (109) and esterification of free fatty acids to methyl esters (110) or butyl esters (109). The esterified products were then fractionated by distillation at high vacuum to isolate tocopherols and sterols together.

E. Purification

Purification of the crude extracts is essential, especially for the alcoholic extracts, in order to improve the antioxidant properties and to create a product with a light odor, taste, and color, suitable as a food additive. Vacuum steam distillation (25) and molecular distillation (26) are efficient methods, although they may affect the heat-sensitive natural products. Removal of undesirable components, such as lipids, carotenoids, and other fat-soluble materials, can be

done by washing the concentrated alcoholic extracts with an appropriate solvent, e.g., hexane (68). Isolation of an antioxidant fraction rich in flavonoids, from alcoholic extracts, was achieved through solvent evaporation under vacuum, dissolution of the solid residue in water, and liquid-liquid extraction with ethyl ether (90). Chloroform has been also suggested to remove caffeine or other pigments from aqueous extracts of flavonoids, such as extracts of tea. Subsequently an enriched flavonoid fraction was obtained by repeated extraction of the aqueous layer with equal volume of ethyl acetate (98, 99).

Fractionation with column liquid chromatography is suitable (27, 111) for the purification of solvent extracts, but a limitation for large-scale production arises from economic factors. A more convenient process proposed involves high-speed countercurrent chromatography with a multilayer coil separator-extractor. It permits the direct efficient separation of undesirable constituents, such as chlorophylls and carotenoids, from the crude spice extracts, in a single step, without the need of a clean-up step (112).

Another approach is membrane separation of the polyphenolic fraction, which was applied to green tea extracts in aqueous ethanol (113). The membrane separation focused on the removal of caffeine from the polyphenolic group of catechins, which are the main antioxidant components of green tea. Ethanol concentration affected the performance of the nanofiltration, and higher concentration (e.g., 80%) gave higher retention of catechins and better separation from caffeine.

Supercritical CO_2 fractionation of alcoholic extracts to isolate the antioxidant fraction has been also reported (114). The alcoholic extract was bleached through an active carbon column, mixed with thermally treated aluminosilicate, and resulting suspension evaporated to dryness under reduced pressure. The solid residue was extracted with CO_2 at 60°C at 10–40 MPa and at 100°C at 50 MPa, for 6 h, at CO_2 flow of 20 kg/h to give five fractions with higher antioxidant activity than the original extract. A total yield of 2.7% (calculated on the basis of air-dried plant material) was obtained.

REFERENCES

1. AL Bran. Toxicology and biochemistry of BHA and BHT. J Am Oil Chem Soc 52:372–375, 1975.
2. PA Dewdney, ML Meara. Natural fat-soluble antioxidants. Scientific and Technical Surveys No 96. The British Food Manufacturing Industries Research Association, 1977.
3. LJ De Rosenzweig Pasquel, JK Babbitt. Isolation and partial characterization of a natural antioxidant from shrimp (*Pandalus jordani*). J Food Sci 56:143–145, 1991.
4. KJ Duve, PJ White. Extraction and identification of antioxidants in oats. J Am Oil Chem Soc 68:365–370, 1991.

5. LL Tian, PJ White. Antioxidant activity of oat extract in soybean and cottonseed oils. J Am Oil Chem Soc 71:1079–1086, 1994.
6. Y Xing, PJ White. Identification and function of antioxidants from oat groats and hulls. J Am Oil Chem Soc 74:303–307, 1997.
7. DGH Daniels, HF Martin. Antioxidants in oats: monoesters of caffeic and ferulic acids. J Sci Food Agric 18:589–594, 1967.
8. DGH Daniels, HF Martin. Antioxidants in oats: glyceryl esters of caffeic and ferulic acids. J Sci Food Agric 19:710–714, 1968.
9. F Sosulski, K Krygier, L Hogge. Free, esterified and insoluble-bound phenolic acids. 3. Composition of phenolic acids in cereals and potato flours. J Agric Food Chem 30:337–340, 1982.
10. M Watanabe, Y Ohshita, T Tsushida. Antioxidant compounds from buckwheat (*Fagopyrum esculentum* Moench) hulls. J Agric Food Chem 45:1039–1044, 1997.
11. BD Oomah, G Mazza. Flavonoids and antioxidant activities in buckwheat. J Agric Food Chem 44:1746–1750, 1996.
12. JR Clopton. Antioxidant material from osage orange (Bois D'Arc) fruit. J Am Oil Chem Soc 30:156–159, 1953.
13. SV Ting, WF Newhall. The occurrence of a natural antioxidant in citrus fruit. J Food Sci 30:57–63, 1965.
14. Y Miyake, K Yamamoto, Y Morimitsu, T Osawa. Isolation of C-glucosyflavone from lemon peel and antioxidated avtivity of flavonoid compounds in lemon fruit. J Agric Food Chem 45:4619–4623, 1997.
15. R Horowitz, B Gentili. Flavonoid constituents in citrus. In: S. Nagy, PE Shaw, MK Vedhuis, eds. Citrus Science and Technology. Westport, CT: AVI Publishing, 1977, pp. 397–426.
16. U Justesen, P Knuthsen, T Leth. Quantitative analysis of flavonols, flavones, and flavonones in fruits, vegetables and beverages by high-performance liquid chromatography with photo-diode array and mass spectrometric detection. J Chromatogr 799:101–110, 1998.
17. O Benavente-Garcia, J Castillo, FR Marin, A Ortuno, JA Del Rio. Uses and properties of citrus flavonoids. J Agric Food Chem 45:4505–4515, 1997.
18. WS Mueller. Antioxidative properties of cacao and their effect on butter oil. J Dairy Sci 37:754–759, 1954.
19. AR Deschreider. Antioxidant properties of extracts of the shell of cacao beans, Inds Aliment Agric 75:759–766, 1958.
20. N Yamaguchi, S Naito, Y Yokoo. Studies on natural antioxidants. V. Antioxidative activity of pigment extracted from cacao bean husk. J Jap Soc Food Sci Techn 29:534–537, 1982.
21. J Chipault, G Mizumo, W Lundberg. The antioxidant properties of spices in foods. Food Technol 10:209–211, 1956.
22. B Ostric-Matijasevic. Application des composants antioxygenes extrais du romarin a l'augmentation de la stabilite du sandoux et de la viande. Rev Fr Corps Gras 8–9:443–452, 1963.
23. S Barbut, DB Josephson, AJ Maurer. Antioxidant properties of rosemary oleoresin in Turkey sausage. J Food Sci 50:1356–1359, 1985.

24. JI Gray, RL Crackel, R Cook, A Gastel, R Evans, DJ Buckley. Observations on the antioxidant properties and thermal stability of an oleoresin rosemary. 3rd International Exhibition and Conference on Ingredients and Additives, November 15–17, 1988.
25. SS Chang, B Ostric-Matijasevic, OAL Hsieh, C-L Huang. Natural antioxidants from rosemary and sage. J Food Sci 42:1102–1106, 1977.
26. U Bracco, J Loliger, J-L Viret. Production and use of natural antioxidants. J Am Oil Chem Soc 58:686–690, 1981.
27. JW Wu, M-H Lee, C-T Ho, SS Chang. Elucidation of the chemical structures of natural antioxidants isolated from rosemary. J Am Oil Chem Soc 59:339–345, 1982.
28. CM Houlihan, C-T Ho, SS Chang. Elucidation of the chemical structures of a novel antioxidant, rosemaridiphenol, isolated from rosemary. J Am Oil Chem Soc 61:1036–1039, 1984.
29. CM Houlihan, C-T Ho, SS Chang. The structure of rosemariquinine: a new antioxidant isolated from *Rosemarinus officinalis L.*. J Am Oil Chem Soc 62:96–98, 1985.
30. SL Richheimer, MW Bernart, GA King, MC Kent, DT Bailey. Antioxidant activity of lipid-soluble phenolic diterpenes from rosemary. J Am Oil Chem Soc 73: 507–514, 1996.
31. ME Cuvelier, H Richard, C Berset. Antioxidant activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary. J Am Oil Chem Soc 73:645–652, 1996.
32. PJ Hidalco, JL Ubera, MT Tena, M Valcarcel. Determination of the carnosic acid content in wild and cultivated *Rosemarinus officinalis*. J Agric Food Chem 46: 2624–2627, 1998.
33. ME Cuvelier, C Berset, H Richard. Antioxidant constituents in sage (*Salvia officinalis*). J Agric Food Chem 42:665–669, 1994.
34. J Protogeras, V Oreopoulou, C Tzia. Natural antioxidants from *Salvia triloba* for vegetable oils. Riv Ital Sostange Grasse 75:507–509, 1998.
35. J-P Yuan, H Chen, F Chen. Simultaneous determination of rosemarinic acid, lithospermic acid, and related phenolics in *Salvia miltiorrhiza* by HPLC. J Agric Food Chem 46:2651–2654, 1998.
36. XC Weng, W Wang. Antioxidant activity of compounds isolated from *Salvia plebeia*. Food Chem 71:489–493, 2000.
37. KD Economou, V Oreopoulou, CD Thomopoulos. Antioxidant activity of some plant extracts of the family Labiatae. J Am Oil Chem Soc 68:109–113, 1991.
38. N Nakatani, H Kikuzaki. A new antioxidative glucoside isolated from oregano (*Origanum vulgare* L.). Agric Biol Chem 51:2727–2732, 1987.
39. H Kikuzaki, N Nakatani. Structure of a new antioxidative phenolic acid from oregano (*Origanum vulgare* L.). Agric Biol Chem 53:519–524, 1989.
40. SA Vekiari, C Tzia, V Oreopoulou, CD Thomopoulos. Isolation of natural antioxidants from oregano. Riv Ital Sostanze Grasse 70:25–28, 1993.
41. M Milos, J Mastelic, I Jerkovic. Chemical composition and antioxidant effect of glycosidically bound volatile compounds from oregano (*Origanum vulgare* L. ssp. *Hitrum*). Food Chem 71:79–83, 2000.

42. K Schwarz, H Ernst. Evaluation of antioxidative constituents from thyme. J Sci Food Agric 70:217–223, 1996.
43. RS Farag, AZMA Badel, FM Hewedk, GSA El-Baroty. Antioxidant activity of some spice essential oils on linoleic acid oxidation in aqueous media. J Am Oil Chem Soc 66:792–799, 1989.
44. JK Huang, CS Wang, WH Chang. Studies on the antioxidant activities of spices grown in Taiwan I. J Chin Agric Chem Soc 19:200–207, 1981.
45. N Kalaimani, V Muraleedharan, K George Joseph. Antioxidant effect of bettel leaf extract on dry-cured fish. Fish Technol 21:37–40, 1984.
46. A Dapkevicius, R Veskutonis, TA van Beek, JPH Linssen. Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. J Sci Food Agric 77:140–146, 1998.
47. P Pietta, P Simonetti, P Mauri. Antioxidant activity of selected medicinal plants. J Agric Food Chem 46:4487–4490, 1998.
48. YS Velioglou, G Mazza, L Gao, BD Oomah. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. J Agric Food Chem 46: 4113–4117, 1998.
49. A Demo, C Petrakis, P Kefalas, D Boskou. Nutrient antioxidants in some herbs and Mediterranean plant leaves. Food Res Int 31:351–354,1998.
50. NV Vanishlieva, EM Marinova. Activity and mechanism of action of natural antioxidants in lipids. Recent Res Dev Oil Chem 2:1–9, 1998.
51. CC Whittern, EE Miller, DE Pratt. Cottonseed flavonoids as lipid antioxidants. J Am Oil Chem Soc 61:1075–1078, 1984.
52. Q-H Meng, P Lewis, K Wahala, H Aldercreutz, MJ Tikkanen. Incorporation of esterified soybean isoflavones with antioxidant activity into low density lipoprotein. Biochem Biophys Acta 1438:369–376, 1999.
53. A Hirota, S Taki, S Kawaii, M Yano, N Abe. DPPH radical–scavenging compounds from soybean miso and antiproliferative activity of isoflavones from soybean miso toward the cancer cell lines. Biosci Biotechnol Biochem 64:1038–1040, 2000.
54. E-A Bae, T-W Kwon, G-S Moon. Isoflavone content andantioxidative effects of soybeans, soybean curd and their by-products. J Korean Soc Food Sci Nutr 26: 371–375, 1997.
55. KS Rhee, YA Ziprin, KC Rhee. Antioxidant activity of methanolic extracts of various oilseed protein ingredients. J Food Sci 46:75–77, 1981.
56. F Shahidi. Food applications of natural antioxidants. In: SS Koseoglou, KC Rhee, RF Wilson, eds. Advances in Oils and Fats, Antioxidants, and Oilseed By-products, Vol. 2. Champaign, IL: AOCS Press, 1996, pp. 241–246.
57. A Lazani, M Cardillo, E Fedeli. The antioxidant activity of chlorogenic acid isolated from sunflower seeds. Riv Ital Sostange Grasse 56:273–275, 1979.
58. Y Fukuda, T Osawa, M Namiki, T Ozaki. Studies on antioxidative substances in sesame seed. Agric Biol Chem 49:301–306, 1985.
59. G Papadopoulos, D Boskou. Antioxidant effect of natural phenols on olive oil. J Am Oil Chem Soc 68:669–671, 1991.
60. MT Satue, S-W Huang, EN Frankel. Effect of natural antioxidants in virgin olive oil on oxidative stability of refined, bleached and deodorized olive oil. J Am Oil Chem Soc 72:1131–1137, 1995.

61. FZ Sheabar, I Neeman. Separation and concentration of natural antioxidants from the rape of olives. J Am Oil Chem Soc 65:990–993, 1988.
62. E Notte, N Romito. Autoxidation of olive oil: influence of polyphenols 1. Ind Agrarie 9:325–331, 1971.
63. FM Vela, AV Roncero. Antioxidantes naturales de las hojas de olivo II. Grasas Aceites 13:124–127, 1962.
64. O Benavente Garcia, J Castillo, J Lorente, A Ortuno, JA Del Rio. Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. Food Chem 68: 457–462, 2000.
65. S Naito, N Yamaguchi, Y Yokoo. Studies on natural antioxidants. II Antioxidative activities of vegetables of *Allium* species. J Jap Soc Food Sci Technol 28:291–296, 1981.
66. C Karastogiannidou. Effect of onion quercetin on oxidative stability of cook-chill chicken in vacuum-sealed containers. J Food Sci 64:978–981, 1999.
67. Y-H Chu, C-L Chang, H-F Hsu. Flavonoid content of several vegetables and their antioxidant activity. J Sci Food Agric 80:561–566, 2000.
68. HJ Suh, JM Lee, JS Cho, YS Kim, SH Chung. Radical scavenging compounds in onion skin. Food Res Int 32:659–664, 1999.
69. WE Price, JC Spitzer. The kinetics of extraction of individual flavonols and caffeine from a Japanese green tea (Sen Cha Uji Tsuyu) as a function of temperature. Food Chem 50:19–23, 1994.
70. WE Bronner, GR Beecher. Method for determining the content of catechins in tea infusions by high-performance liquid chromatography. J Chromatogr 805:137–142, 1998.
71. PT Gardner, DB McPhail, GG Duthie. Electron spin resonance spectroscopic assessment of the antioxidant potential of teas in aqueous and organic media. J Sci Food Agric 76:257–262, 1998.
72. Z-Y Chen, L-Y Wang, PT Chan, Z Zhang, HY Chung, C Liang. Antioxidative activity of green tea catechin extract compared with that of rosemary extract. J Am Oil Chem Soc 75:1141–1145, 1998
73. A Roedig-Penman, MH Gordon. Antioxidant properties of catechins and green tea extracts in model food emulsions. J Agric Food Chem 45:4267–4270, 1997.
74. Y He, F Shahidi. Antioxidant activity of green tea and its catechins in a fish meat model system. J Agric Food Chem 45:4262–4266, 1997.
75. CA Rice-Evans, NJ Miller, G Paganga. Antioxidant properties of phenolic compounds. Trends Plant Sci 2(4):152–159, 1997.
76. N Saint-Cricq de Gaulejac, Y Glories, N Vivas. Free radical scavenging effects of anthocyanins in red wine. Food Res Int 32:27–333, 1999.
77. N Saint-Cricq de Gaulejac, Y Glories, N Vivas.Comparative study of polyphenol scavenging activities assessed by different methods. J Agric Food Chem 47:425–431, 1999.
78. V Katalinic. Grape catechins-natural antioxidants. J Wine Res 10:15–23,1999.
79. N Gamez-Meza, JA Noriega-Rodriges, LA Medina-Juarez, J Ortega-Garcia, R Cazarez-Casanova, O Angulo-Guerrero. Antioxidant activity in soybean oil of extracts from Thomson grape bagasse. J Am Oil Chem Soc 76:1445–1447, 1999.

80. J Castillo, O Benavente-Garcia, J Lorente, M Alcaraz, A Redondo, A Ortuno, JA Del Rio. Antioxidant activity and radioprotective effects against chromosomal damage induced in vivo by X-rays of flavan-3-ols (procyanidins)from grape seeds (*Vitis vinifera*): comparative study versus other phenolic organic compounds. J Agric Food Chem 48:1738–1745, 2000.
81. Z Ju, WJ Bramlage. Phenolics and lipid-soluble antioxidants in fruit cuticle of apples and their antioxidant activities in model systems. Postharvest Biol Technol 16:107–118, 1999.
82. P-D Dur, WY Yen, P-C Du, G-C Yen. Antioxidant activity of Mung bean hulls. J Am Oil Chem Soc 74:1059–1063, 1997.
83. SN Onyenebo, NS Hettriarachchy. Effect of Navy bean hull extract on the oxidative stability of soy and sunflower oils. J Agric Food Chem 39:1701–1704, 1991.
84. E Tsaliki, V Lagouri, G Doxastakis. Evaluation of the antioxidant activity of lupin seed flour and derivatives (*Lupinus albus* ssp. Graecus). Food Chem 65:71–75, 1999.
85. BJF Hudson, SEO Mahgoub. Naturally-occuring antioxidants in leaf lipids. J Sci Food Agric 31:646–650, 1980.
86. D Rodrigues de Sotillo, M Hadley, ET Holm. Potato peel waste: stability and antioxidant activity of a freeze-dried extract. J Food Sci 59:1031–1033, 1994.
87. R Julkunen-Tiitto. Phenolic constituents in leaves of Nothern willows: Methods for the analysis of certain phenolics. J Agric Food Chem 33:213–217, 1985.
88. AH Azizah, NM Nik Ruslawati, T Swee Tee. Extraction and characterization of antioxidant from cocoa by-products. Food Chem 64:199–202, 1999.
89. S Chatterjee, SR Padwal Desai, P Thomas. Effect of γ-irradiation on the antioxidant activity of turmeric (*Curcuma longa* L.) extracts. Food Res Int 32:487–490, 1999.
90. SA Vekiari, V Oreopoulou, C Tzia, CD Thomopoulos. Oregano flavonoids as lipid antioxidants. J Am Oil Chem Soc 70:483–487, 1993.
91. SS Chang, Y Bao. U.S. Patent US 5,043,100 (1991).
92. M Wettasinghe, F Shahidi. Antioxidant and free radical-scavenging properties of ethanolic extracts of defatted borage (*Borago officinalis* L.) seeds. Food Chem 67: 399–414, 1999.
93. E. Revilla, J-M Ryan, G Martin-Ortega. Comparison of several procedures used for the extraction of anthocyanins from red grapes. J Agric Food Chem 46:4592–4597, 1998.
94. JM Cruz, JM Dominguez, H Dominguez, JC Parajo. Solvent extraction of hemicellulosic wood hydrolysates: a procedure useful for obtaining both detoxified fermentation media and polyphenols with antioxidant activity. Food Chem 67: 147–153, 1999.
95. M Spiro, D Jago. Kinetics and equilibria of tea infusion. Part III: Rotating disc experiment interprated by a steady-state model. J Chem Soc Faraday Trans I 78: 295–305, 1982.
96. Y Yoshida, M Kiso, T Goto. Efficiency of extraction of catechins from green tea. Food Chem 67:429–433, 1999.
97. M Spiro, D. Jaganyi, MC Broom. Kinetics and equilibria of tea infusion. Part IX:

The rates and temperature coefficients of caffeine extraction from green Chun Mee and Black Bukial teas. Food Chem 45:333–335, 1992.
98. A Zhang, PT Chan, YS Luk, WKK Ho, ZY Chen. Inhibitory effect of jasmine green tea epicatechin isomers on LDL-oxidation. J Nutr Biochem 8:334–340, 1997.
99. M Milovanovic, K Picuric-Jovanovic, B Vucelic-Radovic, Z Vrbaski. Antioxidants effects of flavonoids of *Anthriscus sylvestris* in lard. J Am Oil Chem Soc 73:773–776, 1996.
100. Nestle SA. Process for extracting antioxidants. British Patent 1,468,271 (1977).
101. Campbell Soup Company. Spice antioxidant principle and process for the extraction of the antioxidant principle from spice. British Patent 1,331,050, U.S. Patent 3,732,111 (1973).
102. BF Haumann. Antioxidants: Firms seeking products they can label as "natural" Inform 1(12):1002–1013, 1990.
103. J Giese. Antioxidants: Tools for preventing lipid oxidation. Food Technol 50(11): 73–81, 1996.
104. CJ Chang, K-L Chiu, Y-L Chen, C-Y Chang. Separation of catechins from green tea using carbon dioxide extraction. Food Chem 68:109–113, 2000.
105. MM Esquivel, MA Ribeiro, MG Bernardo-Gil. Supercritical extraction of savory oil: study of antioxidant activity and extract characterization. J Supercritical Fluids 14:129–138, 1999.
106. FJ Senorans, E Ibanez, S Cavero, J Tabera, G Reglero. Liquid chromatographic-mass spectrometric analysis of supercritical-fluid extracts of rosemary plants. J Chromatogr 870:491–499, 2000.
107. H Lee, BH Chung, YH Park. Concentration of tocopherols from soybean sludge by supercritical carbon dioxide. J Am Oil Chem Soc 68:571–573, 1991.
108. P Schuler. Natural antioxidants exploited commercially. In: BJF Hudson, ed. Food Antioxidants. London: Elsevier Applied Science, 1990, p 111.
109. S Ghosh, DK Bhattacharyya. Isolation of tocopherol and sterol concentrate from sunflower oil deodorizer distillate. J Am Oil Chem Soc 73:1271–1274, 1996.
110. S Ramamurthi, AR McCurdy. Enzymatic pretreatment of deodorizer distillate for concentration of sterols and tocopherols. J Am Oil Chem Soc 70:287–295, 1993.
111. S Nieto, A Garrido, J Sanhueza, LA Loyola, G Morales, F Leighton, A Valenzuela. Flavonoids as stabilizers in fish oil: an alternative to synthetic antioxidants. J Am Oil Chem Soc 70:773–778, 1993.
112. N Fischer, B Weinreich, S Nitz, F Drawert. Application of high-speed countercurrent chromatography for the separation and isolation of natural products. J Chromatogr 538:193–202, 1991.
113. V Nwuha. Novel studies on membrane extraction of bioactive components of green tea in organic solvents: Part I. J Food Eng 44:233–238, 2000.
114. Z Djarmati, RM Jankov, E Schwirtlich, B Djulinac, A Djordjevic. High antioxidant activity of extracts obtained from sage by supercritical CO_2 extraction. J Am Oil Chem Soc 68:731–734, 1991.