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Review

Determination of synthetic phenolic antioxidants in food by high-performance liquid chromatography

Jolana Karovičová[a,*], Peter Šimko[b]

[a]Department of Chemistry and Technology of Foods, Faculty of Chemical Technology, Slovak Technical University, Radlinského 9, 812 37 Bratislava, Slovak Republic

[b]Food Research Institute, Priemyselna 4, P.O. Box 25, 824 75 Bratislava, Slovak Republic

Abstract

This review deals with HPLC method to be used for the determination of synthetic phenolic antioxidants added to various foods. Sample preparation, isolation techniques, separation systems as well as detection methods used in applied food analysis procedures are discussed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Food analysis; Phenolic compounds; Antioxidants

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1. Introduction

Antioxidants are compounds that prolong the shelf life of foods by protecting them against deterioration caused by oxidation, such as fat rancidity and colour changes [1]. They are usually classified into two groups – natural antioxidants and synthetic antioxidants. Antioxidant technology plays an important role in utilisation of fats and oils as raw materials in food processing and in the marketing of foods containing fats under modern conditions. As such, the proper and effective use of antioxidants is dependent on a basic understanding of the chemistry

*Corresponding author.

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of fats, the mechanism of oxidation, and function of an antioxidant in counteracting this type of deterioration [2]. Antioxidants for fats and oils function by interfering with the formation of the free radicals that initiate and propagate oxidation. Knowledge of the mechanism of antioxidant performance reinforces several important aspects of antioxidant usage. Antioxidants have to form "stable" low-energy free radicals that will not further propagate the oxidation of fats and oils. From this point of view, the most convenient compounds are the phenolic compounds whose structure allows them to form low-energy radicals through stable resonance hybrids [1,62]. At present, synthetic phenolic antioxidants (SPAs) are being used as food additives, but because of their possible toxicity, most countries of the world have regulations for controlling the use of antioxidants in food applications. Such regulations identify specific approved antioxidants, establish permitted use levels, and include labelling requirements. However, differences among individual countries exists, i.e., antioxidant permitted in one country may be prohibited in another country. Internationally, the JECFA considers periodically food additives, including SPAs on the basis of all available scientific data to establish acceptable daily intake levels and specifications of identity and purity for the additives [3,4]. Some physical properties of SPAs are summarised in Ref. [1].

2. History

In the past, a lot of research experiments have been conducted to determine the presence and quantity of SPAs in foods. As shown in the literature reviews [1,5–11], a particular emphasis has been put on recovery procedures for antioxidants, and quantification procedures including colorimetric methods, spectrophotometric methods in the UV region, paper and thin-layer chromatographic methods, gas chromatography methods for free antioxidants and their derivatives, methods based on HPLC, electrochemical and miscellaneous techniques. As stated, the earlier spectroscopic techniques have been, at present, replaced by chromatographic methods.

3. HPLC

At present, HPLC determination of SPAs has became one of dominant analytical procedures when its advantages are based on simple sample treatment, possibility to use preseparation column in the HPLC system to remove impurities, possibility to change the polarity of mobile phase during analysis to separate and elute SPAs of different polarity, short analysis time and high reproducibility and sufficient detection limit.

3.1. Sample preparation

At the beginning of the HPLC use, with regard to the complexity and variety of matrices occurring in foods as well as their different properties and the evaluation level of techniques and equipment, it was practically impossible to use the only universal procedure for the determination of all SPAs. However, up to date modern procedures and techniques being used make it possible to determine all SPAs in one procedure. Because SPAs occur in foods at concentration levels usually up to 200 mg l^{-1}, there are usually used appropriate extraction and isolation techniques when individual procedures depend on various factors as follows: character of matrix (polar, semipolar, nonpolar), presence of interfering substances, number and physicochemical properties of SPAs to be determined. Determination may be also affected by the choice of suitable solvent to be used for extraction, because the polarities of individual SPAs are different [1]. Generally, effective extraction of SPAs from samples might not be easy because of their relatively low concentration in foods. Techniques of isolating SPAs from the food matrix are based on direct organic solvent extraction [12–15], steam distillation [16–18] or solid-phase extraction [19]. Problems of quantitative determination are usually associated with incomplete extraction of the SPAs, or with the coextraction of potentially interfering substances. For quantitative determination care has to be taken during solvent evaporation in vacuum to prevent the losses of BHT [13], or oxidation of TBHQ [20]. The use of pure solvents for extraction purposes is also crucial, as trace impurities may

cause losses of SPAs [7]. Also, the presence of peroxides in non freshly rectified solvents can cause loss of SPAs. A widely used isolation technique is direct extraction with solvents [12,21] especially in the case of low fat foods, with subsequent cleaning of the extracted matter. In this case, to eliminate interfering substances, liquid–liquid partition [13], fractionation using gel chromatography [21], column clean-up using a silica gel column [22], thin silica gel layers [23,24] or a Florisil column [25] have been used. The most suitable solvents for extracting SPAs from fats are acetonitrile [24,26–29] and water–alcohol mixtures. The fat is usually dissolved in hexane or light petroleum and SPAs are extracted into acetonitrile [20,24,26–28,30–32]. The disadvantages of acetonitrile extraction are that BHT recovery is low, and moderately high levels of interfering compounds are co-extracted. The advantage of aqueous methanolic extraction of SPAs from non polar solvent is that the fat is mostly excluded [14,33]. Hammond [14] described a methanolic extraction of a melted fat sample, heated to 40–50°C, followed by transfer of the mixture to a deep-freeze for a few hours to aid the solidification of any excess fat from methanol. The methanol layer was then decanted and filtered prior to addition of an internal standard and direct injection. A procedure for extracting BHA and BHT from vegetable oils has been reported by Phipps [13]. The oil was dissolved in *n*-heptane and extracted with DMSO. DMSO extracts were mixed with aqueous sodium chloride solution and the SPAs were back-extracted into light petroleum with following concentration and analysis. Mizutani et al. [34] derivatized BHT to BMMC using the quantitative Coppinger and Campbell reaction of BHT with bromine in methanol. Separation was carried out on a RadialPak μPorasil column using a hexane–2-propanol mixture as mobile phase. Detection of BMMC was monitored at 236 nm when BMMC gave a narrow symmetrical peak with linear dependence its height upon amount with minimum detectable amount 0.5 ng. Galensa [35] converted food additives, including BHA, BHT, PG, OG, DG, to their benzoylated derivatives using benzoyl chloride in pyridine. For separation of the derivatives, a concave gradient was used on a reversed-phase column. The method showed high selectivity and sensitivity, but it was time consuming, because the elution of derivatives took 1 h.

3.2. Stationary phases

At present, almost in all cases, reversed phases made of chemically bonded octadecyl silica of 5 μm particle size have been used for the separation of SPAs. In special works, i.e., for the effective separation of BHA isomers a γ-aminopropyl packing was used, modified with the *N*-3,5-dinitrobenzoylderivative of D-phenylglycine [36].

3.3. Detection systems

The most common detectors used for determination of SPAs are detectors which operate in the UV ranges of 233 or 280 nm [19,26,28,30,34–40]. Fluorimetric detectors are mainly used for determination of ethoxyquin [41–43] but also for BHA and BHT [44] and it is also possible to use electrochemical detectors [10,45,46]. A very progressive electrochemical detection system is based on measuring ‘‘array potential’’ of SPAs with previous oxidative removal of impurities [32].

3.4. Applications in food analysis

3.4.1. Determination of synthetic phenolic antioxidants in foods

A fast, sensitive and convenient method for determination of TBHQ in maize oils was described, when a sample was injected without any treatment [37]. The sample was injected directly onto a 25×0.4 cm I.D. column packed with LiChrosorb SI 60. A mobile phase of a mixture of dioxane and *n*-hexane and effluent was directed to the fluorescence detector which operated at 309 nm excitation wavelength and 340 emission wavelength. A recovery of 98.5% was obtained with a relative standard deviation (RSD) of 2.4%, when the detection limit of 6 ng was reached and average analysis time per sample was 5 min. A procedure for determining BHT, BHA and TBHQ in fats and oils onto either of two normal-phase columns was elaborated by Indyk and Woollard [39]. The procedure involves dilution of the sample in an isocratic ternary mobile phase consisting of hexane–

methylene chloride–acetonitrile, followed by HPLC on a silica or on a cyanopropyl column with UV detection at 280 nm. The method was successfully used for determining the three antioxidants in beef tallow and in palm, soy and maize oils. The limits of detection were 3 mg l^{-1} for BHT and BHA and 10 mg l^{-1} for TBHQ. On the other hand, attempts to elute NDGA, THBP and PG failed when peaks were broad, poorly defined and hence were unsuitable for quantification. Programmed solvent gradients also failed to achieve a satisfactory result. Anderson and van Niekerk [47] evaluated a simple HPLC procedure that requires neither extraction nor derivatization for the direct determination of six antioxidants, viz BHA, PG, OG, DG, TBHQ and NDGA in edible oils and fats. Separations were achieved using a 25 cm×0.45 cm column packed with 5 μm LiChrosorb DIOL using a mobile phase of hexane, 1,4-dioxane and acetonitrile with detection at 280 nm. The procedure may be applied to the analysis of groundnut, soybean, marula, sunflower, safflower and rapeseed oils, and beef fat. Interferences from coeluting materials restricts application of the procedure to determination of PG and NDGA in corn oil and only to NDGA in cottonseed oil. Under the HPLC conditions used, BHT is not resolved from the neutral lipids in the sample. The method is useful for the rapid determination of BHA, DG, TBHQ, OG, PG and NDGA at relatively low levels in numerous types of oils and fat. A qualitative and quantitative HPLC method for analysis of mixtures of 12 antioxidants was described by Grosset et al. [48]. For identification of the components present, gradient elution with a convex profile from water–methanol (35:65) to pure methanol used on a Waters 5 μm C_{18} column, with UV detection. PG was not separated by this system. For quantitative analysis, with UV and electrochemical detectors in series, the water–methanol mixture or pure methanol was used as the eluent, under isocratic conditions, with lithium perchlorate as supporting electrolyte. An applied potential ranging from +0.8 to +1.7 V allowed detection of all the antioxidants tested. Both modes of detection were very sensitive, with limits of detection as low as 61 pg. Andrikopoulos et al. [49] separated triglycerides, together with PG, OG, DG, BHA, TBHQ, BHT, Ionox 100, THBP and NDGA – the most commonly used SPAs to prevent oxidation of edible oils and fats, as well as the natural antioxidants tocopherols and α-tocopherol acetate by HPLC using a reversed-phase C_{18}-column and gradient elution with water–acetonitrile–methanol–isopropanol and UV detection. Except for dilution of the oil with isopropanol–hexane, no further sample preparation was required. Asap and Augustin [27] analysed TBHQ content in frying oil. After solubilisation in hexane, TBHQ was extracted into acetonitrile and analysed on an ODS column using acetonitrile–*n*-butanol–water as mobile phase. TBHQ was quantified at 292 nm. Ten laboratories collaboratively studied a HPLC method for the determination of PG, OG, DG, THBP, TBHQ, NDGA, BHA, Ionox 100 and BHT in butter oil [28]. A sample was mixed with hexane saturated with acetonitrile and SPAs were extracted with acetonitrile. After evaporation, the SPAs were analysed using a gradient liquid chromatographic system consisting of 5% acetic acid in water (A) and acetonitrile–methanol (1:1) (B). A linear gradient run from A–B (70:30) to 100% B over 10 min with hold until the last antioxidant (DG) was eluted at a flow-rate of 2 ml min^{-1} through a C_{18} bonded spherical silica column; detection at 280 nm. Although the greater efficiency of the columns packed with the spherical material compared with non-spherical material is evident, the results showed that separation using the latter material could also be acceptable. Therefore, the primary consideration in selecting the stationary phase should be that of acceptable separation, rather than packaging material itself. Overall mean recoveries as well as RSDs are summarised in Table 1. As found in this study, TBHQ was rapidly oxidised as had already been

Table 1
Overall mean recoveries and relative standard deviations to be calculated from values measured in collaborative test study

Analyte	Mean recovery (%)	RSD (%)
PG	100.9	8.55
THBP	97.8	17.4
TBHQ	103.4	25.6
NDGA	95.4	14.5
BHA	97.4	6.60
OG	93.6	9.64
Ionox	95.5	10.8
BHT	78.0	11.4
DG	96.2	7.35

stated before [20]. The oxidised product, with lower response, elutes between TBHQ and NDGA and increases as the parent TBHQ disappears. On the basis of the obtained results, the AOAC method 983.15 was modified [26]. A specific reversed-phase method for the determination of seven antioxidants (PG, THBP, TBHQ, BHA, BHT, OG, DG) in margarines by means of an internal standard was developed by Irache et al. [30]. The method is based on extraction with acetonitrile, rinsing the extract with acetonitrile–isopropanol and analysis by reversed-phase HPLC on a C_{18} Spherisorb ODS-2 column with an acetic acid–water–acetonitrile (5:70:25) mobile phase followed by a gradient to acetic acid–water–acetonitrile (5:5:90). The detector was operated at 280 nm and trials with seven antioxidants showed recoveries of 73.7–100.2% and detection limits of 0.5–1.1 μg g^{-1}. This procedure enables a good resolution between the peaks and avoids interferences due to the presence of some preservatives, i.e., sorbic and benzoic acids and methylparaben. This procedure was then applied to the determination of the stabilities of BHA, BHT and DG in a commercial margarine during storage at −18, 6, 20 and 50°C. A HPLC method using progressive electrochemical detection of SPAs was described by McCabe and Acworth [32]. Samples were mixed with hexane and SPAs extracted with acetonitrile. HPLC analysis of the extracts was performed without an evaporation step on a high-pressure Coul Array system in which analytes were detected on two coulometric array-cell modules, each containing four electrochemical sensors attached in series after the column. Analytes were separated on a Supelcosil LC-18, 5 μm, column using gradient elution and detected at potentials of −50, 0, 70, 250, 375, 500, 675 and 825 mV. To remove oxidative impurities to be coeluted with BHT, a guard cell with an applied potential of 900 mV was also placed in a system. The method was applied to the simultaneous detection of PG, THBP, TBHQ, NDGA, BHA, Ionox 100, OG, BHT and DG in butter, margarine, shortening, lard and hand cream (see Table 2). Advantages of the present method over the conventional AOAC method include more simple sample preparation, lower detection limits (10–200 pg for HPLC–electrochemical detection and 2000–10 000 pg for HPLC–UV), wider linear responses of electrochemical detection (three–four-orders of magnitude) to UV detection (two-orders of magnitude), higher sample throughput, lower solvent consumption and lower costs and time per analysis. Dieffenbacher [50] reported on a collaborative study organised by the Antioxidants Working Group of the AIIBP on determination of phenolic antioxidants (BHA and BHT) in foils, fats and dry foods (especially dried soup powders). Trials on the IUPAC HPLC method showed interference by co-extracted food constituents. A modification of this technique, using a methanol–methanolic, pH 3.5 KH_2PO_4 buffer (1:1, w/w) was tested. As found, this gave very rapid elution of co-extracted substances and hence reduced interference with antioxidant peaks. Method gave reproducible results for BHA and BHT in foods, and may also be used to detect these antioxidants in fats used in manufacture of soup mixes. Yankah et al. [44] described a HPLC method for determination of BHA, BHT and TBHQ contents in oils, foods and biological fluids. SPAs were separated and eluted on a reversed-phase column by a gradient of a mixture of water–acetonitrile–acetic acid (66.5:28.5:5) and a mixture of acetonitrile–acetic acid (95:5). Eluents were monitored at excitation and emission wavelengths of 280 and 310 nm, respectively. Calibration curves obtained using peak areas against concentration showed high coefficients of multiple determination (R^2 greater than 0.99) for all antioxidants. In oils, recoveries of known concentrations of added antioxidant standards were 98–99%. This method requires only simple sample extraction and purification prior to analysis and provides a relatively high percentage recovery. The method was applied successfully to the measurement of SPA concentrations in oils, soybean and total lipid extracted from horse mackerel, dried foods, smoke-flavoured and biological fluids.

TBHQ and BHA were determined together with preservatives and sweeteners after their separation by SPE [19]. Soy sauce was mixed with a HTA solution of pH 4.5 and hexadecyltrimethylammonium bromide as ion-pair reagent. After mixing thoroughly, and cleaning on a C_{18} cartridge, (additives were eluted with mixture of water and HTA, first fraction, and acetonitrile–water, second fraction) both fractions were combined, diluted in acetonitrile–water, filtered through a 0.2-μm membrane filter and ana-

Table 2
HPLC systems used for determination of synthetic phenolic antioxidants

Antioxidant	Sample/treatment	Stationary phase	Mobile phase	Detection	Ref.
PG, THBP, TBHQ, NDGA, BHA, BHT, Ionox 100, OG, DG	Oils, fats and butter oil. Extraction with acetonitrile from hexane solution	C_{18}-bonded spherical silica	(1) 5% Acetic acid in water (2) Acetonitrile–methanol (1:1); linear gradient from 30% (2) in (1) to 100% (2), flow-rate 2 ml min^{-1}	UV 280 nm	[26,28]
TBHQ, BHA	Dried roast beef, soy sauce, sugared fruit. Solid-phase extraction	5 μm C_{18} column, 25×4.6 mm I.D.	Acetonitrile–aqueous α-hydroxyisobutyric acid solution (pH 4.5) (2.2:3.4) or (2.4:3.6) (v/v) containing ion pairing reagent–hexadecyltrimethyl-ammonium bromide	UV 233 nm	[19]
BHA, DG, BHT, THBP, PG, TBHQ, OG,	Margarine. Extraction with acetonitrile from hexane solution	C_{18} Spherisorb ODS 2, 3 μm, 150×4 mm	Acetic acid–water–acetonitrile (5:70:25, v/v/v), for 4 min isocratically, then linear gradient to acetic acid–water–acetonitrile (5:5:90, v/v/v), flow-rate 1 ml min^{-1}	UV 280 nm	[30]
TBHQ	Maize oil	25×0.4 cm I.D. column packed with 5 μm LiChrosorb SI 60 (Merck)	Dioxane–*n*-hexane (24:76, v/v), flow-rate 3 ml min^{-1}	Fluorimetric detection, 309 nm excitation 340 nm emission	[37]
Isomers of BHA	Mixture of 2-BHA and 3-BHA	5 μm γ-aminopropyl packing, modified with *N*-3,5-dinitrobenzoyl derivative of D-phenylglycine	2-propanol–hexane (7:93, v/v) 1 ml min^{-1}	UV 288 nm	[36]
Isomers of BHA	Mixture of 2-BHA and 3-BHA	10×0.5 cm I.D. column packed with 3 μmHypersil ODS	Acetonitrile–water (2:3, v/v), flow-rate 2 ml min^{-1}	UV 228 nm	[38]
BHA, TBHQ, BHT	Oils, fats. Solving in mobile phase	Waters 10 μm μPorasil column (30 cm×3.9 mm I.D.), Rad Pak Cyano cartridge and Rad Pak silica cartridges (5 μm, 8 mm I.D.)	Hexane–methylene chloride–acetonitrile (85:9.5:5.5, v/v/v) Hexane–methylene chloride–acetonitrile (88.1:9.8:2.1, v/v/v) Flow-rate 0.8 ml min^{-1}	UV 280 nm	[39]
BHT	Poultry premix. Extraction into methanol, filtration and dilution with mobile phase	HIBAR LiChrosorb RP-18, 25 cm×4 mm I.D. column	Water–methanol (5:95), flow-rate 1 ml min^{-1}	UV 280 nm	[40]
BHT	Extruded potato snacks. Homogenisation in acetone–hexane (1:1, v/v)	Poly(styrene–divinylbenzene) PLgel, 100 Å, 5 μm, 300×7.7 mm and Spherisorb ODS 5 μm, 250×4.9 mm	Gradient: 0–15 min water, 15–17 min 0–20% acetonitrile, 17–30 min 20–80% acetonitrile, 30–35 min 80–100% acetonitrile, 35–40 min 100% acetonitrile	UV 254 nm	[51]
BHT, TBHQ, BHA, NDGA, PG	Carrot juices, powdered milk, appetizer cakes	LiChrocart RP 18	Acetonitrile–tetrahydrofuran–water	Electrochemical	[45]
PG, THBP, TBHQ, NDGA, BHA, BHT, Ionox 100, OG, DG	Oils. Dilution of the oil with isopropanol–hexane	Nucleosil C_{18}	Gradient elution with water–acetonitrile–methanol–isopropanol	UV diode array	[49]
TBHQ	Oils. Solution in hexane and extraction with acetonitrile	Hypersil ODS	Acetonitrile–*n*-butanol–water	UV 292 nm	[27]

Antioxidant	Sample / preparation	Column	Mobile phase	Detection	Ref.
Ethoxyquin	Paprika. Extraction into ethyl acetate	Spherisorb ODS-2 with particle size of 5 μm	Gradient: acetonitrile–water from 80:20 to 100% acetonitrile, acetonitrile–ethyl acetate (5:95), flow-rate 2 ml min^{-1}	UV 270 nm, fluorimetric detection 311 nm excitation, 444 nm emission	[41]
Ethoxyquin	Various meals and extruded pet foods. Extraction of grinded samples with acetonitrile	250×4.6 mm I.D. column packed with C_{18} octadecylsilane, 5 μm spherical, 100 Å pore size	Acetonitrile–0.01 *M* ammonium acetate (70:30, v/v), flow-rate 1.3 ml min^{-1}	Fluorimetric detection 360 nm excitation 432 nm emission	[42]
Ethoxyquin	Standard of ethoxyquin	250×4.6 mm I.D. column packed with ODS-silica	Methanol–water (90:10, v/v), flow-rate 1.0 ml min^{-1}	UV 254 and 380 nm, fluorimetric detection 360 nm excitation, 440 nm emission	[43]
TBHQ, BHA, BHT, gallates	Fats and oils. Extraction into methanol	BondaPak C_{18}	Gradient elution. Water+1% acetic acid–methanol+1% acetic acid from 50:50 to 10:90 (v/v)	280 nm	[58]
BHA, BHT, TBHQ	Model system	Partisil PXS ODS 2	0.05 *M* $LiClO_4$ in methanol–water 30:70, 65:35, 85:15 (v/v)	UV, fluorimetric and electrochemical in series	[46]
BHT, BHA, PG, OG, DG, ethyl gallate	Oils, fats. Solution in methanol	ODS Sil XII	Methanol–water (10:0, 9:1, 8:2, 7:3)	UV 280 nm	[59]
BHT, BHA, TBHQ	Fats, oils. Extraction from hexane solution with acetonitrile	LiChrosorb RP-18	Acetonitrile–water phosphoric acid Gradient elution	UV 280 nm	[29]
BHT, BHA	Butter, meat products, bakery. Extraction into methanol	ODS-Sil XI	Methanol–water (45:55, 65:25)	UV 280 nm	[60]
Ionox 330, Irganox 1076, BHT	Fats and oils. Dissolvation in chloroform	Nucleosil 10 C_{18}	Methanol–water	UV 280 nm	[61]
BHT	BHT. Derivatisation to cyclohexadienone	10×0.8 cm I.D. RadialPak μPorasil	Hexane–2-propanol (99:1, v/v), flow-rate 1 ml min^{-1}	UV 236 nm	[35]
BHA, BHT, NDGA, PG, OG, DG	BHA, BHT, NDGA, PG, OG, DG Derivatisation to benzoylated derivatives	Ultrasphere-ODS RP-18	(1) Acetonitrile–water (50:35, v/v) (2) Acetonitrile–water–*tert*.-butyl methyl ether (110:35:40, v/v/v), flow-rate 1 ml min^{-1}, gradient 2% of 2, that to 100% of 2 during 40 min, then to 2% of 2 during 10 min and 10 min isocratically	UV 230 nm	[50]
PG, THBP, TBHQ, NDGA, BHA, Ionox 100, OG, BHT, DG	Butter, lard, margarine, shortening, hand creams Extraction into acetonitrile after mixing with hexane	Supelcosil LC-18, 5 μm, 150×4.6 mm I.D.	(A) Water that contained 25 m*M* sodium acetate and 25 m*M* citric acid–methanol (95:5, v/v). (B) Water that contained 25 m*M* sodium acetate and 25 m*M* citric acid–methanol–acetonitrile (20:40:40). Gradient: initially 25% B with linear increase to 100% B over 12 min, hold for 8 min, return to 25% B and hold 10 min	Electrochemical UV 280 nm	[32]

lysed. Dried roast beef and sugared fruit were ground, and mixed with HTA solution of pH 4.5 and hexadecyltrimethylammonium bromide. After sonication followed filtration and the residue was washed with acetonitrile–water. Combined filtrates were precleaned by SPE and analysed. A mobile phase of acetonitrile–50 m*M* aqueous α-hydroxyisobutyric acid solution containing hexadecyltrimethylammonium bromide as ion pairing reagent and a C_{18} column with flow-rate 1.0 ml min^{-1} and detection at 233 nm, 15 food additives were separated. A combined system consisting of on-line non-aqueous SEC with reversed-phase chromatography was tested by Williams et al. [51] for the determination of BHT in extruded potato snacks. A sample was homogenised in acetone–hexane, then filtered and re-extracted with the same solvent. Combined filtrates were dried over sodium sulphate and the solvent was removed by rotary evaporation. Lipid residues were dissolved in toluene and injected into a poly(styrene–divinylbenzene) size-exclusion column using tetrahydrofuran as the mobile phase. Separated fractions of crude lipid extract were analysed on a reversed-phase Spherisorb ODS column using gradient elution. BHT was detected at 254 nm. As found, coupled non-aqueous SEC and reversed-phase chromatography may be used for the determination in crude lipid extracts of analytes having polarity equal or greater to that of phenol with detection limit of about 0.5 mg l^{-1}. Rustan et al. [45] used isocratic HPLC for the determination of α-, γ- and δ-tocopherol, BHT, BHA, PG, OG, DG, NDGA, TBHQ, ascorbyl palmitate and β-carotene in foods. A RP18 column was used in experiments and seven mobile phases based on various combinations of acetonitrile, methanol, water and tetrahydrofuran were tested. Trials with carrot juice, dried milk formula for infants and aperitif cakes showed that all 12 antioxidants could be determined by a single isocratic HPLC analysis. The optimum mobile phase was acetonitrile–tetrahydrofuran–water (65:20:2) for carrot juice and dried milk formula for babies, and acetonitrile–tetrahydrofuran–water (55:30:45) for aperitif cakes. Beker and Lovrec [40] developed a method for the determination of BHT in poultry premix. BHT was extracted from the sample with methanol, and the extract was filtered, and injected onto a HIBAR LiChrosorb RP-18 column. As a mobile phase, water–methanol (5:95) at a flow-rate of 1 ml min^{-1} was used and detection was at 280 nm. The recovery varied from 98.7 to 101.8% with RSDs from 1.58 to 3.05%.

3.4.2. Determination of etoxyquin

Ethoxyquin, a synthetic antioxidant, is not generally allowed for human consumption in foods, but it is being added to animal feed and to fruits as an antiscald agent [6,52]. Ethoxyquin is also used in the spice industry to effectively prevent carotenoid loss during postharvest handling. However, ethoxyquin treated paprika is unacceptable for some markets and consumers [53]. Perfetti et al. [54] described a method for the determination of ethoxyquin in paprika and chilli powder. Ethoxyquin was extracted from the spice with hexane and partitioned into 0.3 *M* HCl. After adjusting the solution to pH 13–14, ethoxyquin was extracted into hexane, and the hexane layer was evaporated to dryness. An acetonitrile solution of the residue was then analysed by reversed-phase HPLC with detection at 254 nm. The mobile phase was water–acetonitrile with ammonium acetate buffer. Recoveries from samples fortified at 50, 100 and 200 ppm averaged 92% with a RSD of 2.3%. The method was applied to a number of commercial samples of paprika and chilli powder. Ethoxyquin was found in paprika samples at levels up to 63 mg l^{-1} and in chilli powder samples at levels up to 20 mg l^{-1}. Perfetti et al. [55] described a method for the determination of ethoxyquin in milk. Milk solids were precipitated by adding acetonitrile, and the water–acetonitrile supernatant was washed with hexane to remove fat. Addition of NaCl caused the water–acetonitrile solution to separate into an aqueous phase and an acetonitrile phase, thus separating ethoxyquin from most water-soluble impurities. A large volume of water was then added to the acetonitrile layer and ethoxyquin was partitioned into hexane, and then removed at reduced pressure. The residue was dissolved in the mobile phase and analysed on a 250 mm×4.6 mm I.D. Ultrasphere ODS column using fluorescence detection (excitation at 230 nm, and emission at 418 nm) Water–acetonitrile with a diethylamineacetic acid buffer was used as the mobile phase. Recoveries from milk samples fortified at 1, 5 and 10 ng g^{-1} averaged 78% with an RSD of 5.0%. Low concentrations (less than 1 ng g^{-1}) of apparent ethoxyquin were detected in com-

mercial milk samples analysed by this method. A procedure for the analysis of fruit for residues of postharvest preservatives and ethoxyquin was described by Gieger [56]. Residues were extracted with dichloromethane with ultrasonic treatment. The extracts were analysed on a C_{18}, 5 μm column, with methanol–0.01 *M* ammonium acetate (61:39) as the mobile phase, adjusted to pH 7.5–7.7. The recovery of ethoxyquin was 65–78% and the detection limit was approximately 0.05 mg kg^{-1}. A method for the determination of ethoxyquin in paprika avoiding previous separation steps from other coloured substances was proposed by Vinas et al. [41]. Analysis is carried out by reversed-phase HPLC using gradient elution and UV detection at 270 nm. Using fluorimetric detection with excitation at 311 nm and emission at 444 nm, a detection limit of 0.2 μg ml^{-1} was reached. The method can be applied to determination of ethoxyquin in commercial samples in the presence of paprika (*Capsicum annuum*) carotenoids. A collaborative study for determination of ethoxyquin in various meals and extruded pet foods was done under Schreier and Greene [42]. Eleven laboratories took part in the study and determined ethoxyquin in acetonitrile extracts of grinded samples using a column packed with C_{18} octadecylsilane and a mixture of acetonitrile and ammonium acetate solution as a mobile phase. The method was tested for the analysis of 16 samples that contained ethoxyquin from 0.25 to 289 mg l^{-1}. Repeatability standard deviations ranged from 0.08 to 3.2 mg l^{-1}, and repeatability RSDs ranged from 4.5 to 32%. Reproducibility standard deviations ranged from 0.12 to 13 mg l^{-1} and reproducibility RSDs ranged from 4.5 to 55%. On the basis of the results it was recommended that the HPLC method for determination of ethoxyquin in feeds has been adopted as the first action method by AOAC International. Kato and Kanohta [43] dealt with the determination of degradation products of ethoxyquin using HPLC and GC–MS, and NMR spectrometry. For HPLC analysis an ODS column was used and methanol–water as the mobile phase. Ethoxyquin and its oxidised products were detected at 254 and 380 nm as well as fluorimetrically. As found, the main autooxidation product of ethoxyquin was its dimer, which converted into many other products upon exposure to daylight.

3.4.3. Other HPLC applications

HPLC is also a suitable method for the separation of BHA isomers. Commercially available BHA is a mixture of two positional isomers, an approx. 85:15 ratio of 3-BHA and 2-BHA. The former is approximately 2.4 times more effective as a food antioxidant than 2-BHA but 2-BHA is twice as effective as 3-BHA in inhibiting forestomach neoplasia in mice induced by benzo(*a*)pyrene. Ansari [36] used for the separation isocratic elution with 7% 2-propanol in hexane on a Pirkle Type I-A column, packed with 5 μm γ-aminopropyl packing, modified with the *N*-3,5-dinitrobenzoyl derivative of D-phenylglycine. The column effluent was monitored at 288 nm with a detection limit between 1 and 2 ng. Under these conditions, isomers were separated without derivatization, when the phenolic group of 3-BHA was sterically hindered by an *o-tert*.-butyl group and therefore could not interact with stationary phase that resulted in its rapid elution. A rapid, sensitive, reversed-phase HPLC method for the separation of BHA isomers was described by Berridge at al. [38]. Using a column packed with Hypersil ODS of 3 μm particle size and with a mobile phase consisting of acetonitrile–water it was possible to detect less than 0.5 ng of the isomers injected. The procedure is reliable and robust, and compared to another HPLC method, claimed to have a more stable, longer-lasting column requiring only occasional maintenance [38]. Hall et al. [57] compared FSCE and MECC techniques with HPLC analysis. Four major food grade antioxidants PG, BHA, BHT and TBHQ, were separated. Resolution of the four antioxidants was not successful with FSCE, but with MECC. Separation was completed with excellent resolution and efficiency within 6 min and pmol amounts of the antioxidants were detectable using UV absorption. In contrast, reversed-phase HPLC separation was not that efficient and required larger sample amounts and longer separation time.

4. Conclusions

The analytical determination of SPAs in various kinds of foods is a permanent task because of the interest of control and hygienic institutions. Variable content of individual components in foods can affect

determination by spectrophotometric methods. As shown, HPLC is a very useful, suitable method for the determination of SPAs in various kinds of foods, because it presents very reliable results within short analysis times. Moreover, with progress in the development of stationary phases it will be possible to also determine other food additives, i.e., preservatives, sweeteners, etc., simultaneously.

5. Nomenclature

AIIBP	Association Internationale de l'Industrie des Bouillons et Potages
AOAC	Association of Official Analytical Chemists
BHA	2(or 3)(*tert*.-Butyl)-4-hydroxyanisole
BHT	3,5-(*tert*.-Butyl)-4-hydroxytoluene
BMMC	2,6-Di-*tert*.-butyl-4-methyl-4-methoxy-2,5-cyclohexadienone
DG	Dodecyl gallate
DMSO	Dimethyl sulphoxide
FSCE	Free solution capillary electrophoresis
HPLC	High-performance liquid chromatography
HTA	α-Hydroxyisobutyric acid
Ionox-100	2,6-Di(*tert*.-butyl)-4-hydroxy-methylphenol
IUPAC	International Unit of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MECC	Micellar electrokinetic capillary chromatography
NDGA	Nordihydroguaiaretic acid
OG	Octyl gallate
PG	Propyl gallate
SEC	Size-exclusion chromatography
SPA	Synthetic phenolic antioxidant
SPE	Solid-phase extraction
TBHQ	*tert*.-Butylhydroquinone
THBP	2,4,5-Trihydroxybutyrophenone

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