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Quantification of synthetic phenolic antioxidants in dry foods by reversed-phase HPLC with photodiode array detection

Christian Perrin*, Liliane Meyer

Department of Quality and Safety Assurance, Nestlé Research Center, PO Box 44, Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland

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Abstract

Propyl gallate (PG), octyl gallate (OG), 2-and 3-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) are permitted in a limited number of food products according to local legislations, with individual maximum limits in each case. This study describes an in-house validated reversed-phase HPLC method for the quantitative determination of PG and BHA in gravies and dehydrated soups, BHA in bouillons, dehydrated meat and dry pet food, and OG in dehydrated meat. Two extraction methods were developed to optimise the recovery of the phenolic antioxidants. Methanol was more suitable for the extraction of BHA and OG from dehydrated meat, PG from gravies and BHA from hard and soft bouillons while hexane/2-propanol was more suitable for the extraction of BHA from gravies and dehydrated soups, and PG in dehydrated soups. On the other hand, PG could not be quantified accurately in soft bouillons using either of these extraction methods, due to a lack of selectivity. The relative standard deviation of repeatability was between 0.9 and 5.5% and recoveries from spiked samples in the ranges 85–106% for PG, 95–104% for BHA and 83–85% for OG. The procedure allows also the detection of 2 mg/kg of TBHQ and BHT, which are not permitted in the EU for use in dehydrated soups, bouillons, gravies and dehydrated meat.

Keywords: Phenolic antioxidants; Propyl gallate; Octyl gallate; BHA; BHT; TBHQ; Extraction methods; HPLC

1. Introduction

Synthetic phenolic antioxidants (SPAs) are widely used in food systems for prevention of lipid oxidation during processing and storage. This oxidation is responsible for the production of volatile compounds forming unpleasant flavours. The use of SPA in foodstuffs is strictly regulated and in the European Communities (EU) the directive 95/2/EC lays down the rules for their use. 2-and 3-*tert*-butyl-4-hydroxyanisole (BHA) is permitted in bouillons, gravies, dehydrated meat and dehydrated soups individually or combined with propyl gallate (PG) or octyl gallate (OG) or dodecyl gallate (DG) up to a maximum limit of 200 mg/kg, expressed on the fat content of the product. 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) is not permitted in these foods but it may be used in fats and oils. In the United States, *tert*-butylhydroquinone (TBHQ) is permitted and can be used alone or in combination with BHA and/or BHT up to 200 mg per kg of fat (Burdock, 1997). TBHQ is also permitted in Australia, Brazil, New Zealand and Philippines (Karovičová & Šimko, 2000b).

Many research experiments have been conducted to detect and quantify SPAs in foods. A particular emphasis has developed in the recovery procedures for antioxidants and in quantification procedures, including colorimetric methods, spectrophotometric methods in the UV range, paper and thin-layer chromatographic methods, gas and high-performance liquid chromatographic (HPLC) methods, and capillary electrophoresis. However, methods using the HPLC technique are the most widespread (Karovičová & Šimko, 2000a, 2000b).

PG, TBHQ, nordihydroguaiaretic acid (NDGA), OG, DG, BHA and BHT can be determined simultaneously in oils and fats after double extraction with 2 × 10 ml methanol followed by HPLC analysis according to the official methods of the AOAC, AOCS and IUPAC, i.e. on a C_{18} column using 5% acetic acid in water and 5% acetic acid in acetonitrile as eluents (AOAC Official Method 983.15, 1995; AOCS Official Method Ce 6-86, 1997; IUPAC Official Method 2.642, 1992). Recoveries

* Corresponding author. Fax +41-21-785-8553.
E-mail address: christian.perrin@rdls.nestle.com (C. Perrin).

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higher than 90% are obtained for all the antioxidants except for BHT (higher than 85%). The lowest concentration determined on 1-g test portions is 5 mg/kg. The official methods are suitable for the quantitative determination of antioxidants in fats and oils, but not for food products with a complex matrix, where higher sensitivity and selectivity are required.

Several HPLC procedures have been reported in the literature for the quantitative determination of SPAs in complex food products (Beaulieu & Hadziyev, 1982; Dieffenbacher, 1998; Gertz & Herrmann, 1983; Page & Charbonneau, 1989; Pinho, Ferreira, Oliveira & Ferreira, 2000; Rafecas, Guardiola, Illera, Codony & Boatella, 1998; Rustan, Damiano & Lesgards, 1993; Yamada, Miyata, Kato, Nakamura, Nishijima, Shibata & Ito, 1993) including pâtés, dehydrated onion and powder soups, dehydrated potatoes, frozen shrimps, paprika chips and infant formula powder. However, none of these methods has been applied to or validated for the analysis of phenolic antioxidants in bouillons, gravies, dehydrated meat and dry pet food. Quantitative determination of SPAs is an issue since incomplete extraction of antioxidants and co-extraction of potentially interfering substances may occur (Karovičová & Šimko, 2000a, 2000b). Due to the wide polarity range of SPA, several solvents have been used for the extraction of the antioxidants from food products with a complex matrix: hexane, petroleum ether and hexane mixed with acetonitrile and water were used to extract the fat and the antioxidants from the food matrix (Karovičová & Šimko, 2000b; Page & Charbonneau, 1989; Pinho et al., 2000; Rustan et al., 1993) and then the antioxidants were extracted with acetonitrile. However, the extraction into acetonitrile was not optimal since BHT recovery was low and high levels of interfering compounds were co-extracted (Karovičová & Šimko, 2000b). Recoveries higher than 91% were reported by extracting three times with acetonitrile (Page & Charbonneau, 1989). A direct extraction of the antioxidants from the food matrix with methanol or acetonitrile combined with 2-propanol and ethanol was also proposed by some authors (Dieffenbacher, 1998; Gertz & Herrmann, 1983; Rafecas et al., 1998; Yamada et al., 1993), with varied recoveries.

HPLC analysis of SPA is generally carried out using reversed-phase C_{18} columns and gradient elution techniques. UV detection at 280 nm is often used but electrochemical detection showed more sensitivity and selectivity (Karovičová & Šimko, 2000b; McCabe & Acworth, 1998; Rustan et al., 1993).

The aim of the present study was to develop a suitable method to quantify BHA (E 320) and PG (E 310) in bouillons, gravies, dehydrated soups, dehydrated meat and dry pet food, OG in dehydrated meat, and to detect TBHQ and BHT (E321), which are not permitted in the EU for use in dehydrated soups, bouillons, gravies, dehydrated meat.

2. Experimental

2.1. Equipment

High-performance liquid chromatograph HP 1050 from Agilent Technologies (Urdorf, Switzerland) equipped with a quaternary pump 79852 A, a UV-vis photodiode array detector G1306 A, an automatic sample injector 79855 A, a column oven 79856 AX, an on-line vacuum degasser G1303A and a ChemStation data software.

A HPLC column Supelcosil LC-18, 5 μm, 150 × 4.6 mm and a 2-cm Supelguard LC-18 cartridge, were purchased from Supelco (Buchs, Switzerland). A laboratory grinder, 20 000 rpm, was purchased from Framo-Geraetetechnik (Eisenbach, Germany). A centrifuge model Sigma 3K 15 with cooling system, max. 11,000 ***g***, was obtained from Fischer Scientific (Wohlen, Switzerland) with Nalgene 85-ml Teflon FEP tubes. An orbital shaker model IKA Werk KS500 (50–600/min) was purchased from Merck (Les Acacias-Geneva, Switzerland) and a rotary evaporator model RE-120 from Büchi (Flawil, Switzerland).

2.2. Chemicals

Propyl gallate (PG), octyl gallate (OG), 2-and 3-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) were purchased from Fluka Chemie (Basel, Switzerland). Methanol and 2-propanol (gradient grade for chromatography), *ortho*-phosphoric acid 85% and *n*-hexane (for liquid chromatography) were obtained from Merck (Les Acacias-Geneva, Switzerland). Acetonitrile (gradient grade for chromatography) was supplied by J.T. Baker (Deventer, Holland). Water was purified using a Milli-Q system from Millipore (Le Mont-sur-Lausanne, Switzerland).

2.3. Procedure

2.3.1. Preparation of standard solutions

A stock standard solution containing about 60 mg/100 ml of each of the following synthetic phenolic antioxidants: BHA, BHT, PG, OG was prepared in methanol and stored at 4 °C for a maximum of 1 week.

Another stock standard solution containing 60 mg/100 ml of TBHQ was prepared in methanol and stored at 4 °C for a maximum 2 of days.

The day of use, the standard working solution containing 12 μg/ml of each antioxidant was prepared by diluting 1.0 ml of stock solution of synthetic phenolic antioxidants and 1.0 ml of stock solution of TBHQ to 50 ml with methanol.

2.3.2. Extraction of synthetic phenolic antioxidants (SPAs) with methanol

Powdered samples were homogenised by mixing well with a spatula. Hard bouillon cubes and dry pet food were ground to a fine powder using a mortar and a laboratory grinder at 20 000 rpm for 2 min, respectively. Five grams of sample were weighed into a centrifuge tube. Twenty-five millilitres of methanol were added and the mixture was vigorously shaken for 10 min. using an orbital shaker and then centrifuged at 5000 ***g*** for 10 min. The supernatant was quantitatively transferred to a 100-ml round-bottomed flask and kept in the refrigerator at 4 °C. The procedure was repeated once and the combined methanolic extracts, kept in the round-bottomed flask, were then evaporated to dryness using a rotary evaporator under reduced pressure (< 100 mbar), at 40 °C. Ten millilitres of methanol were added to the residue and the mixture was well shaken by hand for 2 min. The solution was filtered through a 0.2-μm membrane filter before HPLC analysis.

2.3.3. Extraction of SPAs with hexane/2-propanol for fatty products (soft bouillons)

Soft bouillons were crushed and mixed well with a spatula to obtain an homogeneous paste. Five grams of sample were weighed into a centrifuge tube. Twenty-five millilitres of hexane/2-propanol 1:1 (v/v) were added and the mixture was vigorously shaken for 10 min using an orbital shaker and then centrifuged at 5000 ***g*** for 10 min. The supernatant was quantitatively transferred into a 100-ml round-bottomed flask and kept in the refrigerator at 4 °C. The procedure was repeated twice and the combined extracts, kept in the round-bottomed flask, were then evaporated to dryness using a rotary evaporator under reduced pressure (< 100 mbar), at 40 °C.

The residue was extracted with of 2×10 ml of methanol and the extracts were kept in a 50-ml round-bottomed flask before being evaporated to dryness under reduced pressure (< 100 mbar), at 40 °C. Ten millilitres of methanol were added to the residue and the mixture was well shaken by hand for 2 min. The solution was filtered through a 0.2 μm membrane filter before HPLC analysis.

2.3.4. Extraction of SPAs with hexane/2-propanol for powdered products (hard bouillons, gravies, dehydrated soups)

Powdered samples were homogenised by mixing well with a spatula. Five grams of sample were extracted with 2 × 25 ml of hexane/2-propanol 1:1 (v/v) as described in 2.3.3. Ten millilitres of methanol were then added to the residue and the mixture was well shaken by hand for 2 min. The solution was filtered through a 0.2-μm membrane filter before HPLC analysis.

2.3.5. HPLC analysis

SPAs were analysed in standard and sample solutions using gradient elution. Solvent A was water at pH 3.0 (acidified with phosphoric acid 1% v/v) and solvent B was methanol–acetonitrile 1:1 (v/v). Gradient conditions: 0–1 min, 28% B; 1–6 min, 28–40% B; 6–26 min, 40–90% B; 26–32 min, 90% B. To separate chromatographic interferences from PG it was sometimes necessary to start with 30% of solvent B. SPA were detected at 280 nm. The Supelcosil LC-18 column was at 30 °C, with a flow rate of 1.5 ml/min. Injection volume was 20 μl.

Each antioxidant was identified by comparing retention times and photodiode array spectra, in the range 220–320 nm, for standards and samples. Purity of each peak was checked so as to exclude any contribution from interfering peaks. Quantification was then done by comparing the areas of the corresponding peaks.

At the end of each working day, the whole chromatographic system was rinsed with water/methanol/acetonitrile 50:25:25 (v/v/v) for 30 min.

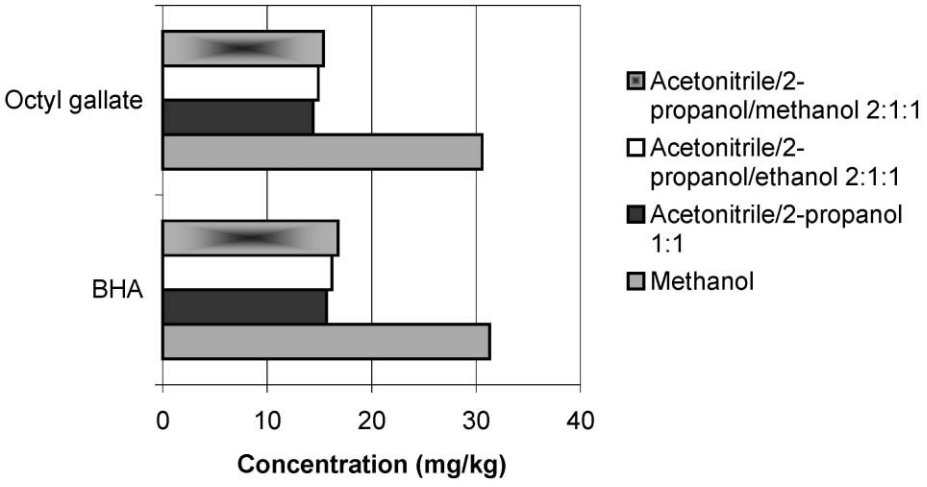

Fig. 1. Comparison of several solvents for extraction of BHA and OG from dehydrated chicken meat.

3. Results and discussion

3.1. Optimisation of the extraction procedures

Preliminary tests were carried out to determine the most appropriate extraction solvents to extract selectively the SPAs. Methanol was selected first since it was found to be suitable for the extraction of SPAs from fats and oils in our laboratory, although polar compounds may be co-extracted with the antioxidants. In order to cover a wide polarity range hexane/2-propanol 1:1 (v/v) was tested for the extraction of both the fat and the antioxidants more or less soluble in the fat. Three mixtures containing acetonitrile (acetonitrile/2-propanol 1:1; acetonitrile/2-propanol/ethanol 2:1:1 and acetonitrile/2-propanol/methanol 2:1:1) were also tested to extract SPAs from dehydrated meat.

All the tests described here were performed on dry foods that contained SPAs incorporated at the manufacturing stage.

3.1.1. BHA and octyl gallate (OG) in dehydrated meat

A comparison of results for extraction of BHA and octyl gallate from dried chicken meat using a single extraction with 10 ml of the various solvents is shown in Fig. 1. These results show that methanol extraction gives the highest recoveries of OG and BHA from dehydrated meat. In addition, the chromatogram and the photodiode array spectra showed no interference.

3.1.2. BHA in bouillons and gravies

BHA was extracted from gravy and bouillon samples with methanol (procedure 2.3.2) and with hexane/2-propanol 1:1 (procedure 2.3.3 or 2.3.4). The results

Table 1
Comparison of extraction solvents for analysis of synthetic phenolic antioxidants in bouillons and gravies

Sample	BHA (mg/kg)		Propyl gallate (mg/kg)	
	Methanol	Hexane/ 2-propanol 1:1	Methanol	Hexane/ 2-propanol 1:1
Beef bouillon 1	8.6	8.4	n.d.[b]	n.d.
Beef bouillon 2	n.d.	n.d.	7.7 [a]	4.2
Beef bouillon 3	n.d.	n.d.	16.4 [a]	5.1
Bouillon cubes	4.6	5.2	n.d.	n.d.
Chicken bouillon 1	n.d.	n.d.	5.8 [a]	2.6
Chicken bouillon 2	8.8	7.4	n.d.	n.d.
Gravy 1	n.d.	n.d.	12.3	6.0
Gravy 2	Strong interference	30.6	n.d.	n.d.

[a] The value includes the contribution of an interference.
[b] n.d., not detected.

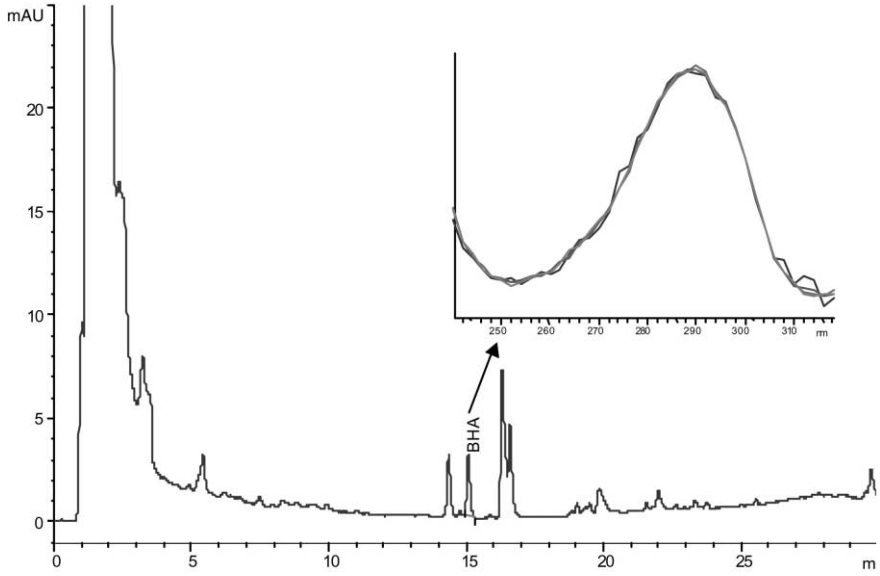

Fig. 2. Chromatogram of a beef bouillon containing BHA after extraction with methanol and comparison of normalised photodiode array spectra taken across the peak of BHA.

obtained were similar for bouillons as shown in Table 1. In addition, peak spectra for BHA showed no spectral impurity (Fig. 2). On the other hand, BHA could not be selectively extracted from the gravy product using methanol, since a strong chromatographic interference was observed.

3.1.3. Propyl gallate (PG) in bouillons and gravies

Products extracted with hexane/2-propanol 1:1 (v/v) showed less interferences at the beginning of the chromatogram than when they were extracted with methanol (Fig. 3a and b). Moreover, the recoveries of PG from spiked samples using hexane/2-propanol 1:1 were comparable with those obtained using methanol.

However, the results obtained for bouillons and gravies containing PG were higher using the extraction with methanol than using the double or triple extraction with hexane/2-propanol 1:1 (Table 1). Indeed, the extraction of PG using hexane/2-propanol 1:1 was incomplete, as demonstrated by re-extracting one gravy and one beef bouillon with 2×25 ml of methanol (Fig. 4).

The extraction of PG using methanol was selective for the gravy product but not for bouillons, as showed by the impurity obtained on the photodiode array spectra of the peak of PG (Fig. 5). The spectra obtained using hexane/2-propanol 1:1 also showed a little impurity. None of the proposed extraction procedures was found to be completely suitable for accurate quantification of

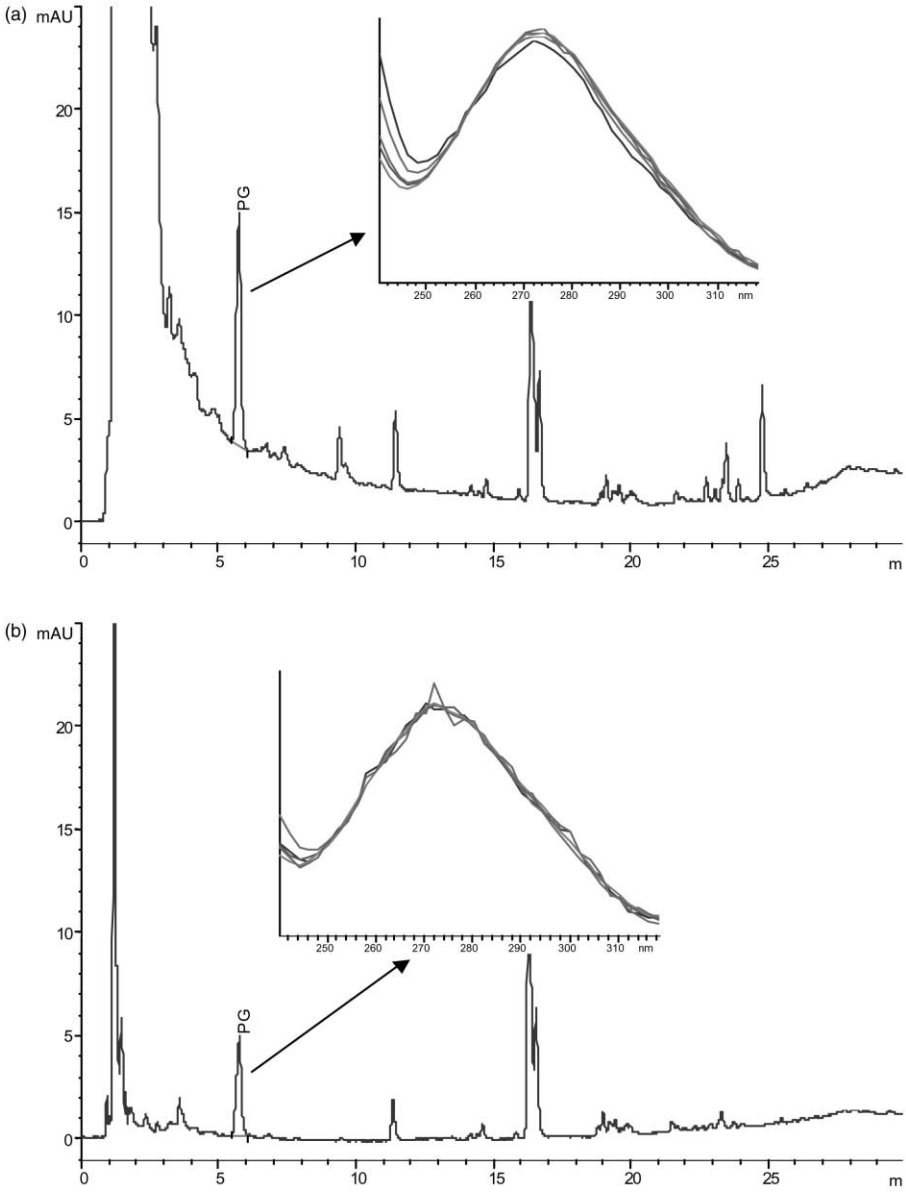

Fig. 3. (a) Chromatogram of a gravy containing PG after extraction with methanol and comparison of normalised photodiode array spectra taken across the peak of PG; (b) chromatogram of the same gravy after double extraction with hexane/2-propanol 1:1 (v/v) and comparison of normalised photodiode array spectra taken across the peak of PG.

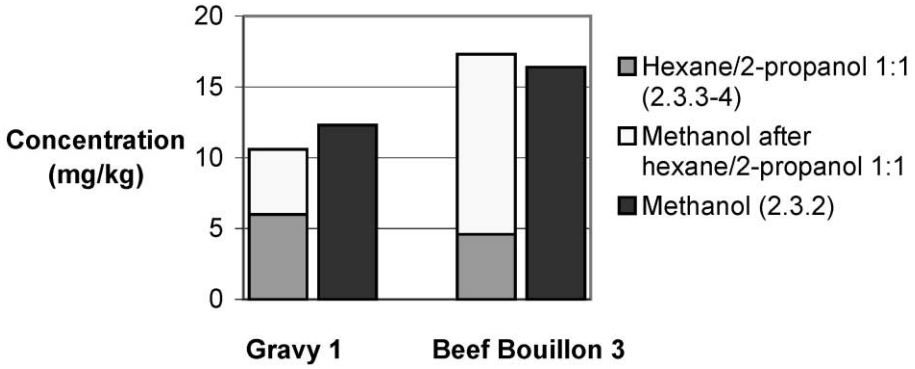

Fig. 4. Comparison of the influence of the extraction method on recovery of propyl gallate.

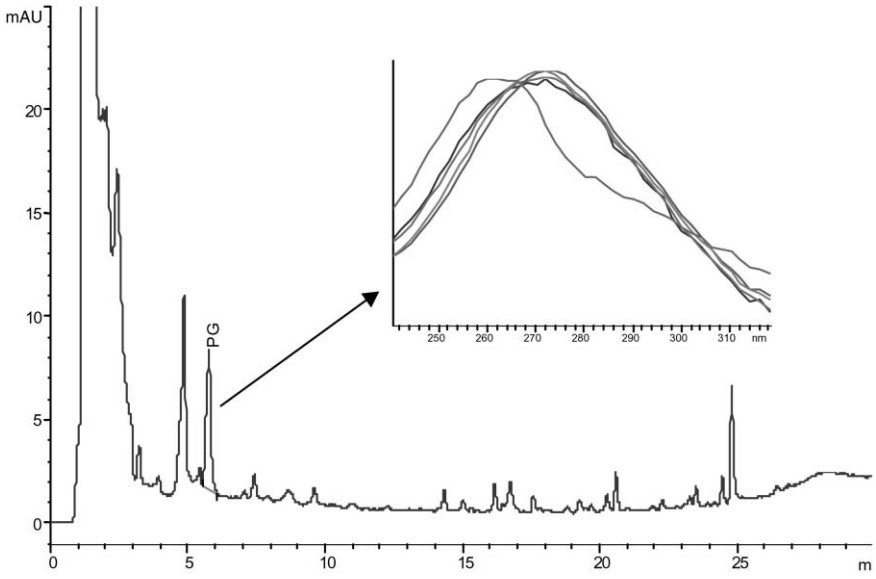

Fig. 5. Chromatogram a beef bouillon containing PG after extraction with methanol and comparison of normalised photodiode array spectra taken across the peak of PG.

PG in fatty (soft) bouillons and another more specific detection system, e.g. electrochemical or MS, is required for greater selectivity.

3.1.4. BHA and PG in dehydrated soups

The analysis of BHA and PG in dehydrated soups using the extraction with methanol and with hexane/2-propanol 1:1 yielded similar results except for BHA in one product (Table 2), where BHA extracted with methanol co-eluted with an interference. The chromatograms obtained using hexane/2-propanol 1:1 showed also less interfering peaks, in particular for retention times close to that of PG (about 6 min).

Table 2
Comparison of extraction solvents for analysis of synthetic phenolic antioxidants in dehydrated soups

Soup	BHA (mg/kg)		Propyl gallate (mg/kg)	
	Methanol	Hexane/ 2-propanol 1:1	Methanol	Hexane/ 2-propanol 1:1
1	10.2	9.8	n.d.[b]	n.d.
2	11.4	12.1	n.d.	n.d.
4	4.8[a]	1.6	2.1	1.9
5	7.4	6.9	n.d.	n.d.
6	n.d.	n.d.	0.6	0.8

[a] BHA co-eluted with an interference.
[b] n.d., not detected.

Table 3
Recovery of PG and BHA from spiked samples

Sample	Propyl gallate		BHA	
	Added (mg/kg)	Recovery (%)	Added (mg/kg)	Recovery (%)
Beef bouillon 1	20	106		
Beef bouillon 3	30	99	30	97
Bouillon cubes	9	94	8	95
Chicken bouillon 1	13	93	12	100
Chicken bouillon 4	13	85	13	100
Chicken bouillon 5			9	96
Chicken pâté	20	101	20	99
Dried chicken meat	120	96	130	100
Dried mutton meat	45	95		
Dry dog food	150	95	22	104
Gravy 3			9	98
Soup 5	20	96		

Table 4
Recovery of OG from spiked dehydrated meats

Sample	Octyl gallate	
	Added (mg/kg)	Recovery (%)
Dried mutton meat	19	83
Dried chicken meat	19	85

3.2. Method performance

3.2.1. Linearity

The linearity of the photodiode array detector response was tested for each compound by analysing standard solutions containing each SPA. Linear calibration curves were obtained, by plotting the peak area against the concentration of the respective compound, in the range 0.5–50 mg/l with a correlation coefficient of 0.9999 for each SPA.

3.2.2. Repeatability and intermediate reproducibility

Repeatability was checked by carrying out six replicate analyses on three bouillons, two dehydrated soups and one dry pet food containing BHA, one gravy containing PG and two dehydrated meats contaning BHA and OG. The relative standard deviation of repeatability (RSD) ranged from 0.9 to 5.5%.

Intermediate reproducibility was checked by carrying out duplicate analyses on the same products, on six different days. The RSD ranged from 1.2 to 11.7%.

3.2.3. Recovery experiments

Recovery was determined by spiking 12 products with different levels of PG and BHA and two dehydrated meats with one level of OG, using different extraction procedures (Tables 3 and 4). Recovery ranged from 85 to 106% for PG, from 95 to 104% for BHA and from 83 to 85% for OG in dehydrated meat. However, these results need to be viewed with caution since they are not representative of recoveries obtained from products containing SPAs.

3.3. Limit of detection for BHT and TBHQ

As the use of BHT and TBHQ is not permitted in culinary products in many countries, it was of interest to determine the detection limit for these antioxidants. One beef bouillon, one dried chicken meat and one dehydrated soup were spiked with 2 mg/kg each of BHT and TBHQ and analysed by HPLC after extraction with methanol. Both antioxidants were easily detected at this concentration.

4. Conclusions

The HPLC procedure proposed here is suitable for the quantitative determination of propyl gallate (PG) and 2-and 3-*tert*-butyl-4-hydroxyanisole (BHA) in gravies and dehydrated soups, BHA in bouillons, dehydrated meat and dry pet food, and octyl gallate (OG) in dehydrated meat. The method is precise (RSD of repeatability between 0.9 and 5.5%) and gives reasonable accuracy with recoveries of added antioxidants in the ranges 85–106% for PG, 95–104% for BHA and 83–85% for OG. It may be used to check the correct use of synthetic phenolic antioxidants in these food products.

PG cannot be quantitatively determined in fatty (soft) bouillons due to a lack of selectivity of the extraction methods against UV detection. Alternative detection methods like electrochemical or MS should be investigated. The procedure is also suitable for the detection of *tert*-butylhydroquinone (TBHQ) and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) at concentrations as low as 2 mg/kg.

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