Simultaneous Determination of Ascorbyl Palmitate and Nine Phenolic Antioxidants in Vegetable Oils and Edible Fats by HPLC

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ABSTRACT: This study describes an HPLC method for the simultaneous determination of ascorbyl palmitate (AP) and synthetic phenolic antioxidants (SPA) in vegetable oils and edible fats in a single run. To achieve this, citric acid was used in combination with isoascorbic acid for stabilization of AP in standard and sample solutions and for deactivation of oxidizing agents in the HPLC system. SPA and AP were directly extracted from samples with methanol containing 1 mg/mL each of citric acid and isoascorbic acid. HPLC analytical and guard columns were pretreated with 90% methanol/acetonitrile 1:1 (vol/vol), containing 4 mg/mL each of citric acid and isoascorbic acid, and 10% water at pH 3, for 30 min. Under these conditions, AP was stable for about 7 h at room temperature. The relative SD of repeatability for AP (0.5–3.6%) was comparable to that for SPA (0.3–2.8%). Average recovery from spiked samples was 100% for AP, 98–103% for SPA, and 85% for BHT (up to 90% using double extraction with methanol).

Paper no. J10279 in *JAOCS 80,* 115–118 (February 2003).

KEY WORDS: Ascorbyl palmitate, fats, HPLC, oils, phenolic antioxidants.

Ascorbyl palmitate (AP) is a lipophilic derivative of ascorbic acid that provides the antioxidant capacity of ascorbic acid to oils and fats (1). It functions mainly as an oxygen quencher and a reducing agent for heavy metal ions (2). Synthetic phenolic antioxidants (SPA) also have a major role in the prevention of the oxidation of oils and fats and function as free radical scavengers of the peroxy- and oxy-radicals formed during autoxidation (3).

In the United States, AP is listed in the Code of Federal Regulation as a chemical preservative with no specific limitations or restrictions. On the other hand, 2- and 3-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), propyl gallate (PG), octyl gallate (OG), and dodecyl gallate (DG) may be used individually or in combination up to a maximum limit of 200 mg/kg (4). In Europe, the use of antioxidants is regulated by Directive No. 95/2/EC (5). BHA is permitted in oils and fats individually or combined with PG, OG, or DG up to 200 mg/kg, whereas BHT is permitted up to only 100 mg/kg. AP may be used *quantum satis* whereas TBHQ is not allowed. Other SPA such as nordihydroguaiaretic acid (NDGA), 2′,4′,5′-trihydroxybutyrophenone (THBP), and 2,6-di-*tert*-butyl-4-hydroxymethylphenol (Ionox-100) are universally not permitted for use in foods.

PG, TBHQ, NDGA, OG, DG, BHA, and BHT are determined routinely in oils and fats after double extraction with methanol followed by HPLC analysis according to the official methods (6–8). Recoveries higher than 90% are obtained for all the antioxidants except for BHT (86–90%) (9). AP cannot be analyzed quantitatively with this method because AP is susceptible to degradation during the analytical procedure. However, AP has been quantitatively determined using HPLC (10,11) and amperometric flow injection analysis (12). Although AP is more stable in solution than ascorbic acid, palmitic acid in the 6-position does not prevent hydrolysis of the enediol system (13). Several extractants have been used in an attempt to prevent the hydrolysis of the lactone ring and to inhibit oxidation of ascorbic acid, including TCA, metaphosphoric acid, oxalic acid, and methanol (14). Other components such as EDTA, desferrioxamine, or diethylenetriaminepentaacetic acid were added to extracting solvents to chelate divalent metal cations (14). AP has been stabilized for up to 5 h at 25°C in 90% DMSO with 0.12% metaphosphoric acid and 0.05% ascorbic acid (15). Preliminary experiments carried out in our laboratory showed that methanol alone is not able to stabilize AP in solution. The addition of an oxygen quencher (isoascorbic acid) combined with an acid (trichloroacetic or citric) was necessary to prevent degradation of AP in solution. It was also observed that AP was strongly degraded during HPLC separation. This paper proposes an HPLC procedure for the simultaneous determination of AP and SPA with conditions preventing the degradation of AP.

EXPERIMENTAL PROCEDURES

Sources of chemicals. Acetonitrile (gradient grade) was purchased from J.T.Baker (Deventer, Holland). Methanol (gradient grade for chromatography), TCA, and *ortho*-phosphoric acid 85% were obtained from Merck (Les Acacias-Geneva, Switzerland). Ascorbyl palmitate, BHA, BHT, TBHQ, PG, OG, DG, NDGA, THBP, Ionox-100, D(−)-isoascorbic acid, and citric acid anhydrous were purchased from Fluka Chemie (Basel, Switzerland). Water was purified using a Milli-Q system from Millipore (Le Mont-sur-Lausanne, Switzerland).

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Preparation of standard solutions. A stock standard solution containing about 0.4 mg/mL of each of the following SPA—BHA, BHT, PG, DG, OG, NDGA, THBP, and Ionox-100—was prepared in methanol and stored at 4°C for 1 wk. Another stock standard solution containing 0.4 mg/mL of TBHQ was prepared in methanol and stored at 4°C for 24 h. A stock standard solution containing 0.4 mg/mL of AP was prepared in methanol containing 1 mg/mL citric acid and 1 mg/mL isoascorbic acid and stored at 4°C for 8 h.

The day of use, a standard working solution containing 16 µg/mL of each antioxidant was prepared by diluting 2.0 mL of stock solution of SPA, 2.0 mL of stock solution of TBHQ, and 2.0 mL of stock solution of AP to 50 mL with methanol containing 1 mg/mL citric acid and 1 mg/mL isoascorbic acid.

Extraction procedure. Solid fats were melted in advance in a water bath by warming to about 10°C above the melting temperature of the fat. Homogenized oil or fat (1 g) was weighed into a centrifuge tube. Ten milliliters of methanol containing 1 mg/mL citric acid and 1 mg/mL isoascorbic acid was added and the mixture was vigorously shaken for 5 min by hand and then centrifuged at 5,000 × *g* for 10 min. The supernatant was filtered through a 0.2 µm membrane filter before HPLC analysis.

HPLC analysis. HPLC separations were performed by an HP 1050 HPLC (Agilent Technologies, Urdorf, Switzerland) equipped with a quaternary pump (79852 A), a UV-vis photodiode array detector (G1306 A), a column oven (79856 AX), and ChemStation data software.

Before analysis, the HPLC system was pretreated with isoascorbic and citric acids, as described further in the Results and Discussion section. AP and SPA were analyzed in standard and sample solutions using gradient elution at a flow rate of 1.5 mL/min. Solvent A was water at pH 3.0 (acidified with phosphoric acid 1% vol/vol) and solvent B was methanol/acetonitrile 1:1 (vol/vol). Gradient conditions: 0–1 min, 35% B; 1–5 min, 35–45% B; 5–12 min, 45–90% B; 12–18 min, 90% B. The antioxidants (20 µL) were injected onto a SUPELCOSIL LC-18, 5 µm (150 × 4.6 mm i.d.) column, preceded by a 2-cm Supelguard LC-18 cartridge (Supelco, Buchs, Switzerland) at 30°C. 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. Detection of SPA was performed at 280 nm whereas AP was detected at 255 nm. Quantification was then done by comparing the peak area of each antioxidant in a sample with that of the corresponding peak of the standard. At the end of each working day, the whole chromatographic system was rinsed with water/methanol/acetonitrile 50:25:25 (by vol) for 30 min.

To shorten the time of analyses, experiments carried out on AP alone were achieved using isocratic elution with 90% mobile phase B. AP was thus eluted within 6 min.

Statistical analysis. Excel software (Microsoft, Redmond, WA) was used for linear regression analysis, to check the linearity of detector response, and for calculating mean and SD values.

RESULTS AND DISCUSSION

Stability of AP. The stability of AP in methanol with and without additives is shown in Figure 1. AP without additives degraded by 2% after 24 min, 8% after 1 h, and 20% after 2 h, whereas the degradation using additives was below 5% after 2 h. The best results were obtained using both isoascorbic acid (1 mg/mL), as oxygen quencher, and citric acid (1 mg/mL), as chelating agent. The degradation of AP after 3.6 h was only 1.6%.

Isoascorbic and citric acids were also very efficient for stabilizing AP in sample solutions. Indeed, no decrease in AP concentrations was noticed in extracts from corn oil, rapeseed oil, and palm olein (26–220 mg/kg AP), kept for 24 h at 4°C.

HPLC analysis. HPLC conditions (column and solvents) were the same as for the analysis of SPA in dry foods (9) but with a gradient of mobile phase shortened from 32 to 18 min. An example of an HPLC chromatogram for a standard solution containing AP and the nine SPA is shown in Figure 2. AP was eluted from the HPLC column within 16 min, which was faster than proposed by Dieffenbacher and Trisconi (10) for the separation of seven SPA and AP (25 min). The solvent mixture water/methanol/acetonitrile, without additives, was not able to prevent the degradation of AP by oxidation agents during HPLC analysis. AP injected into the HPLC was consumed immediately, leading to a drastic decrease in the concentration of AP followed by a slow increase over time. The nature of the oxidation agents was not determined but could be traces of divalent metal cations in the HPLC system. AP could be stabilized by passing 90% solvent B, containing 4 mg/mL isoascorbic acid and 4 mg/mL citric acid, through analytical and guard columns for 30 min. The columns were maintained at 30°C, and the flow rate was held at 1.5 mL/min. The columns were rinsed with 35% solvent B for 60 min before the analysis. This pretreatment allowed AP to be analyzed under stable conditions over about 7 h (Table 1).

Linearity. The linearity of the photodiode array detector was checked for each antioxidant by analyzing in triplicate nine

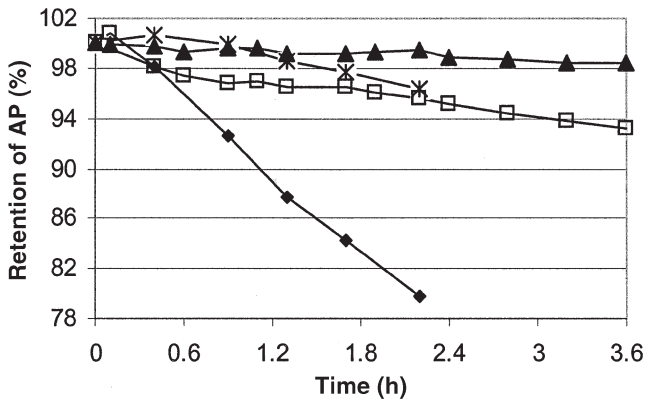

FIG. 1. Stability of ascorbyl palmitate (AP: ♦) in methanol (13 µg/mL), without and with additives. ✱: AP + isoascorbic acid (IAA, 1 mg/mL); ▲: AP + citric acid (1 mg/mL) + IAA (1 mg/mL); □: AP + TCA (2 mg/mL) + IAA (1 mg/mL).

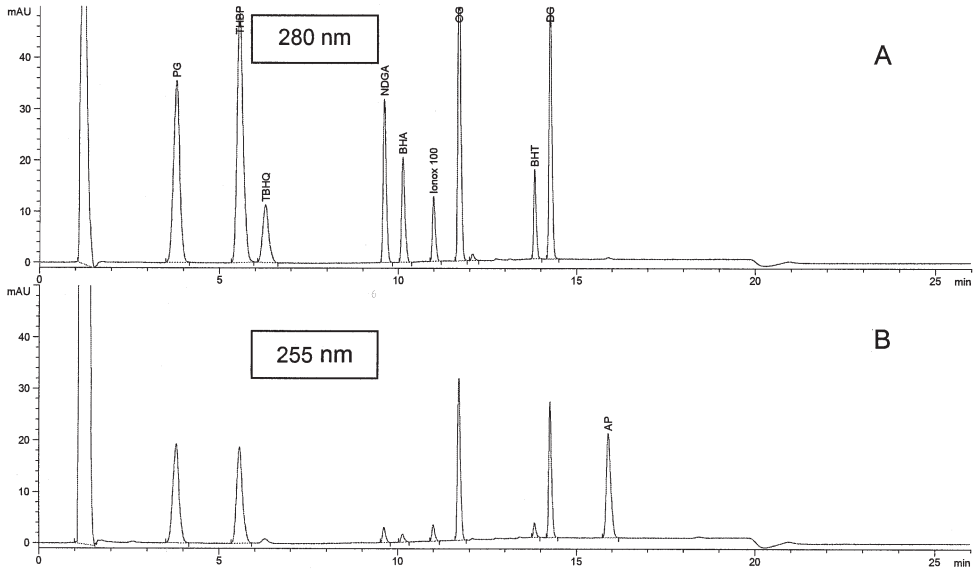

FIG. 2. Liquid chromatogram of a standard solution (20 µg/mL) of nine phenolic antioxidants (A) and AP (B), in methanol containing citric acid (1 mg/mL) and IAA (1 mg/mL). PG, propyl gallate; THBP, 2′,4′,5′-trihydroxybutyrophenone; TBHQ, *tert*-butyl-hydroquinone; NDGA, nordihydroguaiaretic acid; BHA, 2- and 3-*tert*-butyl-4-hydroxyanisole; Ionox-100, 2,6-di-*tert*-butyl-4-hydroxymethylphenol; OG, octyl gallate; BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; DG, dodecyl gallate; for other abbreviations see Figure 1.

standard solutions containing different levels of each antioxidant over the range 0.5–50 µg/mL. Linear regression analysis showed a linear relationship between peak area and concentration, with correlation coefficients between 0.9990 and 0.9999.

Repeatability. Repeatability of AP analysis was checked by carrying out six replicate analyses on one corn oil, one rapeseed oil, two palm oleins, and two fat mixes (75% palm olein, 18% palm kernel oil, 7% corn oil; and 44% palm olein, 31% palm kernel oil, 25% corn oil). The relative SD of repeatability (RSD) ranged from 0.5 to 3.6%. Repeatability of PG and BHA was checked by analyzing one chicken fat (PG: 36 mg/kg, BHA: 90 mg/kg), and the RSD were 0.5 and 0.3%, respectively. Repeatability for the other phenolic antioxidants, i.e., OG, DG, BHT, TBHQ, NDGA, THBP, and Ionox-100, was checked by analyzing the corn oil spiked with about 50 mg/kg of each of these antioxidants. The RSD was also satisfactory, ranging from 0.5 (OG) to 2.8% (BHT).

Recovery experiments. The rapeseed oil, the corn oil, and the fat mix containing 44% palm olein were spiked with different levels of antioxidants and analyzed using the procedure described above. Each sample was analyzed four times, and the results obtained are given in Table 2. Average recovery was 100% for AP, 98–103% for SPA, and 85% for BHT. A double extraction with methanol (10 mL) had been found

TABLE 1
Stability of the Analysis of AP and Phenolic Antioxidants in Methanol Containing Citric Acid (1 mg/mL) and Isoascorbic Acid (1 mg/mL)[a]

	Concentration of antioxidant (µg/mL)								
Antioxidant	0.5 h	1 h	2 h	4 h	6 h	7 h	Mean[b]	SD	RSD[c] (%)
PG	10.1	10.0	10.0	10.1	10.2	10.1	10.1	0.05	0.5
THBP	9.0	8.9	9.0	9.0	9.1	9.0	9.0	0.04	0.4
TBHQ	11.0	11.0	11.0	11.1	11.2	11.2	11.1	0.08	0.7
NDGA	8.9	8.9	8.9	9.0	9.0	8.9	8.9	0.05	0.5
BHA	13.1	13.0	13.1	13.2	13.3	13.2	13.2	0.07	0.5
Ionox-100	11.1	11.1	11.0	11.1	11.3	11.1	11.1	0.08	0.7
OG	10.8	10.8	10.8	10.9	10.9	10.8	10.9	0.05	0.5
BHT	9.9	9.9	10.0	10.1	10.0	9.9	10.0	0.06	0.6
DG	9.9	9.8	9.8	9.9	9.9	9.8	9.8	0.05	0.5
AP	9.7	9.6	9.5	9.7	9.8	9.8	9.6	0.12	1.2

[a]Over a period of about 7 h at room temperature.
[b]Mean of 14 determinations performed every 0.5 h.
[c]Relative SD. Abbreviations: PG, propyl gallate; THBP, 2′,4′,5′-trihydroxybutyrophenone; TBHQ, *tert*-butylhydroquinone; NDGA, nordihydroguaiaretic acid; BHA, 2- and 3-*tert*-butyl-4-hydroxyanisole; Ionox-100, 2,6-di-*tert*-butyl-4-hydroxymethylphenol; OG, octyl gallate; BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; DG, dodecyl gallate; AP, ascorbyl palmitate.

TABLE 2
Recovery of AP and Nine Synthetic Phenolic Antioxidants from Spiked Rapeseed Oil, Corn Oil, and Fat Mix (44% palm olein, 31% palm kernel oil, 25% corn oil)[a]

Antioxidant	Added (mg/kg)	Recovery range (%)	Average recovery (%)
PG	28–201	97–105	102
THBP	26–199	97–106	101
TBHQ	26–202	95–105	101
NDGA	26–200	98–110	103
BHA	26–200	94–101	98
Ionox-100	29–205	93–108	101
OG	24–198	97–103	100
BHT	37–209	83–89	85
DG	28–208	98–105	101
AP	30–97	97–103	100

[a]For abbreviations see Table 1.

necessary to obtain a more satisfactory recovery for BHT (86–90%) (9).

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[Received March 22, 2002; accepted October 19, 2002]