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Quantification of synthetic phenolic antioxidants in liver pâtés

O. Pinho, I.M.P.L.V.O. Ferreira*, M.B.P.P. Oliveira, M.A. Ferreira

CEQUP/Laboratório de Bromatologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha 164, 4050 Porto, Portugal

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Abstract

Pâtés are products with high fat levels and their consumption has increased in Portugal over the past few years. Owing to their composition, lipid oxidation is one of the primary mechanisms associated with deterioration of their quality. Therefore, antioxidants have become a useful group of food additives because they help to maintain the organoleptic quality of pâtés by avoiding rancidity. The aim of this work was the evaluation of seven synthetic antioxidants with phenolic structure, namely, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butyl hydroquinone (TBHQ), propyl (PG), octyl (OG) and dodecyl gallates (DG) and nordihydroguaiaretic acid (NDG), in liver pâtés available on the Portuguese retail market. The high performance liquid chromatography (HPLC) procedure described in AOAC for oils and fats was found to be inappropriate for pâtés, probably owing to their complex matrices. A modification of the Association of Official Analytical Chemists (AOAC) extraction procedure is thus presented. Extensive validation of this extraction procedure was carried out by recovery tests. Over 91% recoveries of added antioxidants were observed, except for PG and OG whose recoveries were 78.0 ± 2.2 and 82.1 ± 1.1 1%, respectively. The precision found was below 3.8%. No synthetic antioxidants were detected in six of the 12 assayed brands. One sample contained BHA and five samples contained NDG (concentrations ranged from trace levels to 26.3 ± 0.0 mg/kg of product). When pepper corn was added as an additive to pâtés, piperine (the main compound of pepper) appeared on the chromatogram but it did not interfere with the evaluation of the other antioxidants. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Liver pâtés; Synthetic antioxidants; Piperine; HPLC-UV

1. Introduction

Lipid oxidation is one of the primary mechanisms associated with quality deterioration in foods and specially in meat and fat products. The changes in quality are manifested by the formation of a number of volatile secondary products by adverse changes in flavour, colour, texture and nutritive value, and by the possible production of toxic compounds [Commission of the European Communities (CEC), 1989].

The pâté matrix is poor in natural antioxidants, because these are often lost during processing or storage, which justifies the need for the addition of exogenous antioxidants (Madhavi, Despande & Salunkhe, 1996).

Many synthetic compounds, which are characterised by a better antioxidant activity than natural antioxidants and are more easily available, have been used in a wide variety of food products. These synthetic antioxidants are mainly phenolic compounds and include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl, octyl and dodecyl gallates (PG, OG, DG), nordihydroguaiaretic acid (NDG) and *tert*-butyl hydroquinone (TBHQ). In spite of the aforementioned advantages the toxicological effects of these synthetic antioxidants have been the subject of controversy in recent years. Studies available in the literature (Clayson, Iverson, Nera, Lok, Rogers & Rodrigues, 1986; Madhavi et al., 1996) generally refer to the carcinogenic effect developed in rats owing to the presence of BHA and BHT at extremely high concentrations. Among the seven antioxidants mentioned above, only the first five listed are allowed in the EU and consequently in Portugal the use of the last two compounds in foodstuffs for antioxidant purposes was banned in the EU owing to toxicological reasons. Nevertheless, the considerable antioxidant efficacy of TBHQ and the wide use of NDG in some countries between 1950 and 1960 justified their inclusion in the control programme presented herein.

The aim of this paper was to quantify the seven synthetic antioxidants with phenolic structure in liver pâtés of

* Corresponding author. Fax: +351-2-200-3977.

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different origins existing on the Portuguese retail market in order to inform nutritionists and consumers about their eventual presence and concentration levels. No official method for the evaluation of levels of antioxidants in pâtés is reported in literature, hence we tried the Association of Official Analytical Chemists (AOAC) procedure 983.15 (Association of Official Analytical Chemists, 1990) described for oils and fats, and other methods used for the evaluation of antioxidants in margarines (Ferreira, Oliveira & Ferreira, 1993; Page, 1993; Pujol-Forn, 1980). However, certain difficulties were encountered during the extraction procedure, probably owing to the complexity of the matrices. Therefore, a modification of the AOAC extraction procedure was optimised. The chromatographic separation was carried out according to the AOAC procedure.

2. Materials and methods

2.1. Sampling

In order to have a working sample as representative as possible of the liver pâtés available on the Portuguese retail market, 12 brands of liver pâtés (two Portuguese brands and 10 imported from European Community Countries) which included seven brands of pork liver pâtés and five brands of fowl liver pâtés were randomly purchased. In order to evaluate the uniformity in composition of the antioxidants in each brand, three lots (each with a specific production date) were purchased over a period of one year. A total of 36 samples were analysed in triplicate.

2.2. Reagents and solutions

All reagents used in the various analyses were of pro-analyses (p.a.) grade purchased from Merck. The standards of the antioxidants were from Sigma Chemicals Company, Aldrich Chemicals Company and Poly-Science. The water for chromatography analysis had a resistance greater than 15 MΩ. It was filtered through a membrane of 0.45 μm porosity and subsequently degassed. The acetonitrile (Lichrosolv) used in the mobile phase was Merck "gradient grade".

Standard solutions were prepared in 2-propanol + acetonitrile (1 + 1). They were refrigerated and stored away from direct light.

The extraction solvents were *n*-hexane saturated with acetonitrile and acetonitrile saturated with *n*-hexane.

2.3. Apparatus and operating conditions

The chromatographic analyses were carried out in a Gilson high performance liquid chromatograph (HPLC) equipped with two pumps type 302 and 305 and a type 7125 Rheodyne Injector with a 20 μl loop. A Gilson variable wavelength UV–vis detector and an integrator Varian 4290 were also used. During validation studies, and to confirm the identity of the species in the real samples, a Jasco multiwavelength diode array detector MD-910 and Borwin PDA chromatography software were also used.

The chromatographic separation was achieved using a Spherisorb C_{18} ($S_{10}ODS_2$) chromatographic column, 5 μm, 250 mm × 4.6 mm i.d.

Typical operating conditions included the ultraviolet (UV) detector set at 280 nm with a sensitivity of 0.05 AUFS, room temperature and a flow rate of 2 ml/min.

The solvent system used for separation of phenolic antioxidants was equivalent to the AOAC method and included a gradient made with solution A 5% acetic acid in H_2O and solution B, acetonitrile with 5% of acetic acid. The linear gradient applied comprised a range which varied between 30% B in A to 100% B over a 10-min period, maintenance of upper limit conditions during 4 min and a return to the initial conditions within 2 min.

The analysed compounds were identified by chromatographic comparisons with authentic standards and confirmed by their associated spectra.

2.4. Extraction

In order to optimise the working conditions several attempts were performed in which different temperatures and small portions of solvent were used. The extraction procedure which gave the best results and which was consequently used in our sample analysis was as follows: a 1.5-g sample (weighed accurately to the nearest 0.001 g) was transferred quantitatively to a 50-ml beaker and washed five times with separate portions of *n*-hexane saturated with acetonitrile (ca. 5 ml each); each portion of extraction solvent was mixed with the pâté (at 60°C) for a fixed period of 5 min until complete dissolution of the fats, antioxidants and other apolar compounds in the liquid phase, all other constituents remained within the pâté matrix; each of the five extracts was then quantitatively transferred to the same 250-ml separatory funnel and the antioxidants were extracted with six 15-ml portions of acetonitrile saturated with *n*-hexane. Finally, the extracts of acetonitrile saturated with *n*-hexane were evaporated to dryness using a flash evaporator with a water bath set at 40°C, a vacuum source and water–ice condenser cooling.

The extract was redissolved with 2.5 ml of 2-propanol + acetonitrile (1 + 1).

2.5. Calibrations and calculations

Single standard solutions of antioxidants were prepared in order to establish elution times and spectra.

Quantification was based on the external standard method. Five mixed standards for seven antioxidants covering a broad concentration range were also prepared in order to establish calibration curves (concentrations: 3.1; 6.25; 18.8; 25 and 30 mg/l). Resulting peak heights were determined for duplicate 20-μl injections. Standard curves for each antioxidant were prepared by linear regression of peak height vs concentration.

2.6. Recovery study

Recovery studies were carried out on two different liver pâté samples that were separately spiked with 0.3 mg/ml of each antioxidant. Complete analyses were performed in triplicate by using the extraction procedure described above.

2.7. Statistical analysis

Data are presented as mean ± standard deviation. The results were statistically analysed by analysis of variance (ANOVA) followed by Fisher's PLSD test. Differences were considered significant for $p < 0.01$.

3. Results and discussion

3.1. Standard calibration curves

The seven phenolic antioxidants were well separated on the C_{18} column by using the assay conditions described herein as apparent from inspection of Fig. 1. A linear relationship was observed between the concentration of antioxidants and the UV absorbance at 280 nm. The regression equations for BHT, BHA, TBHQ, NDG, PG, OG and DG were $y = 0.0852\ x - 0.1624$; $y = 0.165\ x - 0.0911$; $y = 0.151\ x - 0.0627$; $y = 0.198\ x - 0.135$; $y = 0.0472\ x + 0.1867$; $y = 0.3641\ x - 0.618$ and $y = 0.0253\ x + 0.0015$, respectively. The correlation coefficient for each standard curve invariably exceeded 0.99 for all compounds.

3.2. Validity of the method

The reliability of the method, in terms of linearity, precision, recovery and sensitivity, was studied. The precision and accuracy of the extraction procedure was evaluated on two different pâté samples, one containing NDG and the other containing BHA. Six different extractions, as described above, were made for each sample and subsequently injected in duplicate. The relative standard deviation (RSD) was 3.7% and 3.8% for a concentration of 4.96 mg/kg of NDG and a concentration of 92.4 mg/kg of BHA, respectively.

The results obtained from the recovery studies are listed in Table 1. These results confirmed, on the one hand, the effectiveness of the extraction step, and on the other, the accuracy of the proposed methodology. The recoveries were generally over 91%, except for PG and DG. Some authors attribute this observation to differences in solubility of these two antioxidants when compared with that of the other five antioxidants; PG and DG have a lower solubility in fats and oils and a slightly higher solubility in water (Madhavi et al., 1996).

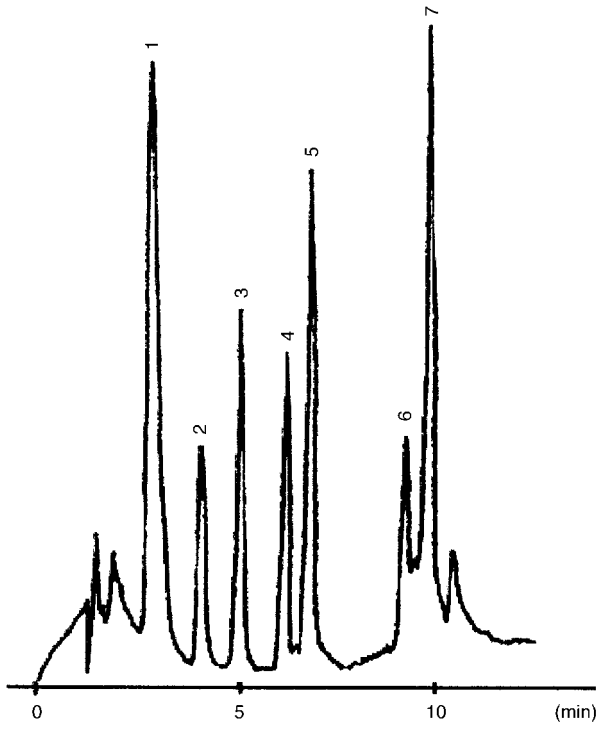

Fig. 1. Typical chromatogram of aqueous standard solutions (12.5 mg/l). (1) PG; (2) TBHQ; (3) NDG; (4) BHA; (5) OG; (6) DG; (7) BHT.

3.3. Chromatograms and results for liver pâtés

On the chromatograms of 10 of the 12 brands of the analysed liver pâtés an unidentified peak which was not OG but which was eluted with a retention time close to

Table 1
Statistical results for the recoveries obtained by the standard additions method

Antioxidant	Concentration[a] (mg/ml) ($n = 2$)	Recovery ± SD (%)
PG	0.30	78.0 ± 2.2
TBHQ	0.30	93.6 ± 1.1
NDG	0.30	91.0 ± 0.7
BHA	0.30	97.8 ± 0.4
OG	0.30	82.1 ± 1.1
BHT	0.30	99.9 ± 0.1
DG	0.30	94.3 ± 0.5

[a] Concentration of the antioxidant solution added to the pâté samples before analysis.

that of OG, was observed. Confirmation of the identity of this compound was achieved via several strategies. The composition of each sample according to the label contents was carefully studied and the same was made for the production technique of the liver pâtés (Frentz, 1982). A spice seemed to be the most probable ingredient responsible for the unknown peak.

Extractions were made as described above for pepper corn, paprika and malagueta pepper. Subsequently, the extracts were injected on the HPLC/diode array detection system under the described chromatographic conditions. Only the extract obtained from the pepper corn gave a peak with retention time and spectrum equivalent to those of the unknown peak found for the 10 liver pâté brands. Confirmation of piperine (the main component of pepper corn; Costa, 1981) in sample extracts was achieved via co-elution with the authentic standard and by varying the chromatographic conditions. Spectral equivalence was also observed throughout elution of the referred peak with a match factor against standard (calculated by the peak purity analysis program) that ranged between 997.78 and 999.53 for the 10 samples (a perfect match is defined instrumentally as 1000).

Fig. 2 shows a chromatogram of a liver pâté that contained 4.96 mg/kg NDG, and piperine. Standard OG was added to show that piperine did not interfere with the evaluation of the other antioxidants.

Results obtained from analysis of the seven synthetic antioxidants in 12 commercial brands of liver pâtés from the retail market (including pork liver pâtés and fowl liver pâtés) are presented in Table 2. The compounds found in the assayed samples were identified by comparing their UV–vis spectra in the 240–400 nm range with the library of spectra previously compiled by the authors.

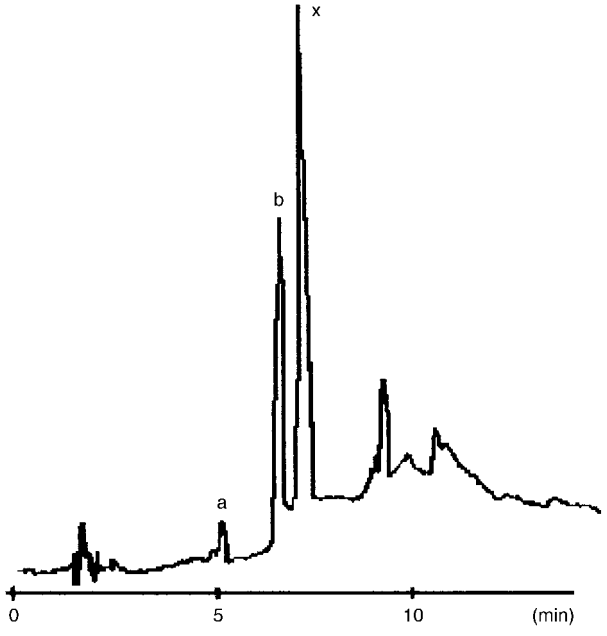

Fig. 2. Chromatograph of a liver pâté sample added with OG standard: (a) NDG, (b) OG (x) piperine.

Table 2
Results obtained for the analysis of synthetic antioxidants in liver pâtés

Brands[a]	Lots (mg/1000 g) A	B	C	Antioxidants found
1	nd[b]	nd	nd	–
2	$24.1 \pm 0.5^{c,d}$	26.3 ± 0.0^{d}	nd	NDG
3	92.4 ± 0.4^{d}	95.7 ± 0.0^{d}	nd	BHA
4	nd	nd	nd	–
5	Traces	Traces	Traces	NDG
6	$4.00 \pm 0.15^{c,d}$	$11.0 \pm 0.3^{c,d}$	$6.60 \pm 0.7^{c,d}$	NDG
7	$4.95 \pm 0.10^{c,d}$	$3.47 \pm 0.03^{c,d}$	$5.34 \pm 0.04^{c,d}$	NDG
8	nd	nd	nd	–
9	nd	nd	nd	–
10	nd	nd	nd	–
11	Traces	Traces	Traces	NDG
12	nd	nd	nd	–

[a] Brands 1–7 are different brands of pork liver pâtés; 7–12 are different brands of fowl liver pâtes, the letters A, B and C represent different lots: A, lot with oldest production date; B, lot with intermediate production date; C, lot with most recent prodution date.

[b] nd, not detected.

[c] Results are expressed as mean ± standard deviation ($n = 3$).

[d] Significant differences between the results were determined by ANOVA methodology followed by Fisher's PLSD test. Differences were considered significant for $p < 0.01$.

With regard to the composition of the liver pâtés in terms of antioxidants, no synthetic antioxidant was detectable in six of the 12 assayed brands; four of these brands corresponded to fowl liver pâtés. BHA was detected in one brand. NDG was found in five brands at completely different concentrations which varied from trace levels to 26.3 mg/kg of liver pâté. TBHQ was not detectable in any of the pâté in spite of its antioxidant potential.

All brands containing detectable antioxidants were characterised by the presence of a single antioxidant in all of the respective lots studied; however, highly significant differences ($p < 0.01$) were observed between different lots of the same brand, in terms of concentration.

4. Conclusions

Considering the complexity of the analysis, which included extraction, concentration, transfer and chromatographic determination, the results obtained for the validation of the method for the evaluation of seven antioxidants in pâtés must be considered satisfactory.

Furthermore, when pepper was included as an ingredient of the liver pâtés, piperine (the major component of the pepper corn; Costa, 1981) was extracted together with the antioxidant and appeared systematically on the chromatograms, but its presence did not interfere with the evaluation of the antioxidants.

The presence of NDG in five brands was a surprising observation since NDG is an antioxidant not suggested in the EC Directive 95/2/CE for food stuffs.

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