Food Chemistry 123 (2010) 892–900

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Determination of lipid and protein hydroperoxides using the fluorescent probe diphenyl-1-pyrenylphosphine

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ARTICLE INFO

Article history:
Received 2 September 2009
Received in revised form 25 January 2010
Accepted 1 May 2010

Keywords:
Protein hydroperoxide
Lipid hydroperoxide
Diphenyl-1-pyrenylphosphine
DPPP
Method validation

ABSTRACT

By means of two alternative methods lipid and protein hydroperoxides (HP) were determined by fluorometry using the diphenyl-1-pyrenylphosphine (DPPP) probe. It has been shown that the formation of the fluorescence was influenced by the type of solvent and HP whereas the presence in the media of antioxidants such tocopherol and butylated hydroxytoluene had no effect. The combination of the chloroform:methanol (2:1, v/v) solvent mixture that is widely used for lipid extraction was combined with suitable solvents to develop a method with the maximum performance in determining HP in lipid extracts. Using a variety of lipids and lipid extracts, the final method proposed agreed well with the thiocyanate method for HP determination. In addition, the DPPP method was very sensitive, precise, accurate, free of interferences and specific for the determination of lipid soluble HP. DPPP can be also used to measure HP soluble in hydroalcoholic media. This alternative procedure showed a similar performance to its lipid soluble equivalent and was able to measure hydrogen peroxide promoted peroxidation of bovine serum albumin and water soluble HP in protein extracts. With the addition of triphenylphosphine the hydroalcoholic method is specific for the determination of protein HP.

1. Introduction

Organic hydroperoxides (HP) are oxidative mediators and products of peroxidation chain reactions produced by free radicals and enzymes such as lipoxygenase. They can be originated from the oxidation of unsaturated fatty acids and other lipid classes (Gutteridge, 1995) but also from proteins and amino acids (Gebicki, 1997; Gebicki & Gebicki, 1999).

Lipid HP are odourless but their formation and breakdown in foods lead to loss of nutrients with compounds with negative biological effects and off-flavours also being formed (Diplock et al., 1998; Esterbauer, Schaur, & Zollner, 1991; Nawar, 1996). Therefore, the food industry is interested in both inhibiting peroxidation and determining their concentration which gives an indication of the oxidative status, especially in a relatively non-oxidised product. In biological matrices, HP measurement, in connection with free radicals and other reactive oxygen species, has been used as indication of oxidative stress (Halliwell & Chirico, 1993; Mehrotra, Ling, Bekele, Gerbino, & Earle, 2001; Sivaram, Suresh, & Indira, 2003) which is associated with various diseases (Casetta, Govoni, & Granieri, 2005; Castro & Freeman, 2001; Esterbauer, Wag, & Puhl, 1993; Finkel, 1998) and ageing (Holbrook & Ikeyama, 2002; Stadtman & Berlett, 1997).

Until recently, proteins were not held to be biologically significant targets for reactive oxygen species but research has shown that protein form HP and can be responsible of DNA cross-linking (Gebicki & Gebicki, 1999). Mildly oxidised proteins are readily degraded and removed from the cell but when proteins are highly oxidised they aggregate and/or their solubility decreases which makes their elimination difficult (Cecarini et al., 2007; Grune, Merker, Sandig, & Davies, 2003). These highly oxidised proteins may contribute to several neurodegenerative diseases such as Alzheimer and may be involved in the ageing process (Grune et al., 2003; Stadtman, 2006; Widmer, Ziaja, & Grune, 2006). Therefore, highly oxidised proteins and protein HP can be useful markers of oxidative stress but there is a lack of simple and sensitive methods to determine these compounds.

Although various methods have been proposed to measure HP, the instability and diversity of HP in complex foods or biological matrices make it difficult to conduct accurate, sensitive and simple analysis. Chromatographic (GC and HPLC) and spectroscopic (NMR and ESR) methods showed high sensitivity, selectivity and reproducibility (Dobarganes & Velasco, 2002; Frankel, Neff, & Weisleder, 1990; Hughes, Smith, Horning, & Mitchell, 1983; Yamamoto, 1994; Yang et al., 1991) but not all laboratories have the necessary instrumentation so their application to routine analysis is compromised.

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doi:10.1016/j.foodchem.2010.05.003

More simple methods such as the widely accepted iodometric titration methods have inherent problems such as sensitivity, selectivity, and interference with contaminants and oxygen (Jessup, Dean, & Gebicki, 1994). The quantitative determination of HP by UV light absorption, normally at 232 nm, is based on the presence of HP on fatty acids with double bonds in a conjugated diene configuration. The major disadvantage of this method is that there can be fatty acid HP without a conjugated diene structure (e.g. HP on oleic acid) and conjugated dienes resulting from lipid oxidation that do not contain HP (e.g. 2,4-decadienal) (Dobarganes & Velasco, 2002).

Other methods based on the formation of iron complexes after reaction with the oxidising HP (thiocyanate and ferrous-oxidation-xylenol orange) were also proposed for their simplicity and improved sensitivity (Bou, Codony, Tres, Decker, & Guardiola, 2008; Dobarganes & Velasco, 2002; Nielsen, Timm-Heinrich, & Jacobsen, 2003; Shantha & Decker, 1994). However, these procedures are subjected to some possible interference caused by the presence of chelators, sources of iron or pigments which are common in foods and biological samples (Bou, Codony, et al., 2008). Finally, the measurement of total HP includes hydrogen peroxide, lipid and protein HP. Therefore, the correlations with food quality and biochemical pathways should take into account this fact or address this issue during analysis.

The fluorimetric determination of lipid hydroperoxides by means of the fluorescent probe diphenyl-1-pyrenylphosphine (DPPP) has been reported to be accurate and very sensitive in foodstuffs and biological materials using flow injection and HPLC post-column methods (Akasaka & Ohrui, 2000; Akasaka, Takamura, Ohrui, Meguro, & Hashimoto, 1996; Sohn, Taki, Ushio, & Ohshima, 2005). According to these latter authors, the phosphine moiety of the DPPP reacts with various HP producing a high intensity fluorescent DPPP oxide which is normally measured at 380 nm (excitation at 352 nm).

The aim of this work was to study and exploit DPPP's capabilities in terms of sensitivity, selectivity and lack of interference to determine the total content of lipid and protein HP. As for this, we developed two simple batch methods using that fluorescent probe for the specific determination of these two types of HP present in different food and biological samples.

2. Material and methods

2.1. Chemicals and standards

Diphenyl-1-pyrenylphosphine (DPPP) and 13(S)-hydroperoxide 9,11-octadecadienoic acid (HPODE) were from Cayman Chemical Co. (Ann Arbor, MI). Butylated hydroxytoluene (BHT), 30% hydrogen peroxide, *tert*-butyl hydroperoxide (TBHP), cumene hydroperoxide (CHP), *tert*-butylhydroquinone (TBHQ), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), triphenylphosphine (TPP) and bovine serum albumin (BSA) were from Sigma–Aldrich Co. (St. Louis, MO). Mixed tocopherols concentrate (Covi-ox® T-70) was from Cognis (Monheim, Germany). Solvents (HPLC grade) were from Fisher-Scientific (Pittsburgh, PA) and did not contain antioxidant or stabilising agents. The other chemicals used were of ACS grade and double-deionised water was used throughout. The purity of the HPODE was always checked before use by means of its molar extinction coefficient at 234 nm (λ_{234} = 25.6 M^{-1} cm^{-1}).

2.2. Influence of the reaction time for different HP and stability of the resulting DPPP oxide

To study the influence of the reaction time we added 200 μL of methanol containing different amounts of HP (ranging from 10 μM to 15 μM) and mixed with 100 μL of methanol containing 200 μM of DPPP. Samples and blanks were incubated at 60 °C under subdued light conditions in a water bath for different periods and cooled at 4 °C for 10 min, 20 min, 40 min, 60 min and 120 min in the darkness to stop the reaction. Under these conditions the fluorescence intensity of the formed DPPP oxide was maintained until 60–120 min of storage. For convenience, samples were hereafter stored for 20 min at 4 °C in the darkness to stop the reaction. Subsequently, methanol was added up to 9 mL, vortexed and an aliquot was then diluted five times and immediately read.

2.3. Influence of solvents

To study the influence of the solvents we added 200 μL of different solvents (methanol, butanol, hexane and chloroform) containing HP standards (CHP, TBHP, H_2O_2 and HPODE) at the same concentration and were mixed with 100 μL of the same solvent each containing 200 μM of DPPP. Because of its solubility, H_2O_2 was only dissolved in methanol and butanol. Samples and blanks were incubated for different periods at 60 °C under subdued light conditions in a water bath and then cooled at 4 °C for 20 min in the dark. Later on, the same solvent for each HP was added up to 9 mL, vortexed and an aliquot was then diluted five times and fluorescence was immediately determined.

In addition, 200 μL of 100% methanol, 1:2 (v/v) chloroform:methanol, 1:1 (v/v) chloroform:methanol, 2:1 (v/v) chloroform:methanol, or 100% chloroform containing 15 μM of CHP was mixed with 100 μL of either methanol or 1-butanol (hereafter referred simply as butanol) each containing 200 μM of DPPP. Samples and blanks were incubated for 3 h at 60 °C under subdued light conditions in a water bath and then cooled at 4 °C for 20 min in the dark. Afterwards, more methanol or butanol, depending on the previous solvent used to add DPPP, was added up to 9 mL, vortexed and an aliquot was then diluted five times and fluorescence was immediately determined.

2.4. Influence of antioxidants

A chloroform:methanol (2:1, v/v; 200 μL) solvent mixture solution containing 15 μM of CHP plus an antioxidant (BHT, TBHQ and tocopherol mix) either at 3 mM, 1.5 mM or 0.75 mM was mixed with 100 μL of butanol containing 130 μM of DPPP. Samples and blanks were incubated for 3 h at 60 °C under subdued light conditions in a water bath and then cooled for 20 min at 4 °C in the dark. Then, up to 9 mL of butanol was added, vortexed and a 2/3 dilution was made and fluorescence was immediately determined.

2.5. Final procedure for lipid HP

Varying lipid samples were weighed and dissolved in the chloroform:methanol (2:1, v/v) solvent mixture solution containing 2 mM BHT. Hereafter aliquots of these solutions are referred as sample volumes. Sample volumes of either 200 μL or 100 μL can be indistinctly used but a higher sensitivity was achieved when volumes of 100 μL were used. Results presented here were done by mixing this latter sample volume which was mixed with 100 μL of DPPP solution (130 μM DPPP dissolved in butanol containing 2 mM BHT). Those latter volumes were dispensed with positive-displacement pipettes. Then samples and blanks were incubated for 3 h at 60 °C under subdued light conditions. Subsequently, they were cooled by placing the reacted samples at 4 °C for 20 min in the darkness. Then, 9 mL of butanol was added, vortexed and a 2/3 dilution was made with butanol and fluorescence was immediately determined.