Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) in relation to the use of hydrogen peroxide as a processing aid in the processing of blood products and cephalopods

Scientific Committee members

Rosaura Farré Rovira, Francisco Martín Bermudo, Ana María Cameán Fernández, Alberto Cepeda Sáez, Mariano Domingo Álvarez, Antonio Herrera Marteache, Félix Lorente Toledano, Mª Rosario Martín de Santos, Emilio Martínez de Victoria Muñoz, Mª Rosa Martínez Larrañaga, Antonio Martínez López, Cristina Nerín de la Puerta, Teresa Ortega Hernández-Agero, Perfecto Paseiro Losada, Catalina Picó Segura, Rosa María Pintó Solé, Antonio Pla Martínez, Daniel Ramón Vidal, Jordi Salas-Salvadó, Mª Carmen Vidal Carou

Secretary

Vicente Calderón Pascual

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Working Group

Alberto Cepeda Sáez (Coordinator)
Antonio Martínez López
Perfecto Paseiro Losada
Antonio Pla Martínez
Ricardo López Rodríguez (AESAN)

Abstract

The current study evaluates the possibility of increasing the use of hydrogen peroxide (oxygenated water-H_2O_2) as a processing aid in the processing of hemoderivatives and cephalopods.

Hydrogen peroxide is considered to be GRAS (Generally Recognized as Safe) by the FDA (Food and Drug Administration) and non-carcinogenic by the International Agency for Research on Cancer (IARC). In addition, the Scientific Committee on Consumer Products (SCCP) of the European Commission considers its use, up to 6%, in toothpaste to be safe.

In various countries, including some of the European Union, the use of this agent in foods has already been authorised, such as in the decontamination of water for human consumption and as a processing aid in intestines. In this report it has been proposed to increase its use in the bleaching of blood, and its fractions, and also on cephalopods due to bacteriostatic effect.

Regarding the use of hydrogen peroxide for the processing of cephalopods and hemoderivatives under the conditions proposed by the AFCA (Association of Manufacturers and Distributors of Food Additives and Supplements) and based on the data from the different studies that are brought together in this report, such as the absence of residues and the non-modification of lipid oxidation or proteins, the Scientific Committee concludes that hydrogen peroxide poses no risk to the consumer and can be used, always following the conditions included in this reported.

Key words

Hydrogen peroxide, cephalopods, hemoderivatives, processing aid.

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Background

The Association of Manufacturers and Distributors of Food Additives and Supplements (AFCA) and the Spanish Food and Drink Industry Federation (FIAB) have requested, accompanying their request with the corresponding report, authorisation for the use of hydrogen peroxide (H_2O_2) as a processing aid in the processing of the following food groups:

- Cephalopods.
- Blood products (whole blood, red blood cells and plasma).

To this end, the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) has assessed the safety of using hydrogen peroxide as a processing aid. In this respect, the Committee has established "Guidelines indicating the necessary documentation for the assessment of processing aids intended for use in human food" (AESAN, 2010). In accordance with the criteria established in the above-mentioned Guidelines, hydrogen peroxide is classified in situation 4: substance authorised in human food for which the acceptable daily intake has not been established and the use of which results in the technically unavoidable presence of residues.

In light of the above situation, aspects are assessed relating to the physical and chemical characteristics, technological function, residue studies and analytical methods, food safety studies and data (Level A) and studies on the consumption and assessment of the consumer level of exposure.

Considerations relating to hydrogen peroxide

1. Physico-chemical characteristics

Composition of the proposed product: hydrogen peroxide (CAS number: 7722-84-1) diluted in water at concentrations between 0.05 and 0.75% depending on the foods or groups of foods where it is to be used (referring to the concentration of the hydrogen peroxide solution that will be in direct contact with the food).

Reactivity

- Catalytic reactions: on silver, platinum, osmium and manganese.
- Addition reactions: may form compounds with ammonia or urea.
- Reduction reactions: reduces gold and silver oxides; converts ferricyanide to ferrocyanide; decomposes hypobromite to bromide.
- Oxidation reactions: the most common (arsenic, iron, lead...). They discolour sensitive materials including silk, ivory, feathers and others that do not withstand oxidation with chlorine, it has been used to remove chlorine residue thanks to its reducing action.

2. Technological function

Destination food or food group

The destination food or food group, and the maximum requested doses are as follows (Table 1):

Table 1. Destination food and maximum requested doses

Food or food group	Alleged technological use	Maximum dose
Cephalopods	Bacteriostat	0.05%/24 hours
Blood products: whole blood, red blood cells and plasma	Bleaching agent	0.75% (whole blood and red blood cells) and 0.1% in plasma/30 minutes

Alleged technological use

The alleged use is as bleaching agent or bacteriostat according to the sector in which it is used (Table 1).

Cephalopods

Cephalopods are highly perishable foods. In the food processing industries, while the product is conditioned, bacterial contamination may take place, which is also an indication that certain authorised additives (phosphates, citrates) do not prevent bacterial growth and the substitutes in the form of conservatives have technological disadvantages (undesirable colour and flavour). The main function is to control bacterial growth during the processing, in addition to preventing possible enzymatic degeneration. It acts as a bacteriostat and not as a bactericide.

Hydrogen peroxide is added after the skinning and gutting processes, as during the latter, released microorganisms would make the use of peroxide in the proposed concentrations inefficient. The dose is added directly at the processing plant by dilution.

No references have been found regarding the use of hydrogen peroxide combined with other types of permitted additive to obtain softening and whitening effects (polyphosphates, salt, citric acid, etc.). In the case of cephalopods, these mixtures are used to prevent changes in colour. The use of hydrogen peroxide limits the increase of pH caused by the use of additives alone.

Blood products

As indicated by the applicant, the globular fraction is equivalent to 2/3 parts of the total pork blood protein, and therefore huge quantities are produced every day which, if they cannot be used, may become a residue posing major environmental and health problems, unless disposed of correctly.

Hydrogen peroxide, in the proposed concentrations, oxidises the chromophore that gives colour to the haemoglobin (tetrapyrrolic group) and the red tone that prevents its use in human or animal food is lost. Therefore, a yellowish protein is obtained that maintains a large part of the nutritional value of the native protein (haemoglobin) and does not add any colour to the product to which it is added, and therefore can be better used by the meat processing industry.

3. List of authorised uses of hydrogen peroxide

Some of the authorised uses of hydrogen peroxide in the food sector are included in Table 2.

Table 2. List of authorised uses of hydrogen peroxide in food

	Authorised use	Reference
Europe	Regulation (EC) No 853/2004 establishes for finished gelatines (obtained from bones, hides and skins of farmed ruminant animals, pig skins and poultry skins) a hydrogen peroxide residue limit of 10 mg/kg (ppm).	(EU, 2004)
	Regulation (EC) No 123/2008 permits the use of hydrogen peroxide in the production of gelatine from products of animal origin.	(EU, 2008)
Spain	The use of hydrogen peroxide is authorised at a maximum dose of 5,000 ppm, in the bleaching of natural tripes.	Orden de 29 de octubre de 1986
	Its use is authorised to decontaminate water destined for human consumption.	Real Decreto 140/2003
France	Its use is authorised as a processing aid in tripes.	(Arrèté del Ministère de l'Economie, des Finances et de l'Industrie, 2006)
	Favourable toxicological assessment as a processing aid in the manufacture of infant formula whey.	(AFSSA, 2005, 2007)
	Favourable toxicological assessment, in solution with peracetic acid and acetic acid, for the microbiological decontamination of flours.	(AFSSA, 2006, 2010)
The United States	Recognised as GRAS (Generally Recognized As Safe) (21 CFR 184.1366) by the FDA (Food and Drug Administration), and its use is authorised in milk (0.05%), whey (0.04%), whey cheese coloured with annatto (0.05%), starch (0.15%), corn syrup (0.15%) and in emulsifiers (1.25%).	21 CFR 184.1366
	Its use is authorised (27CFR 240.1051) in wine (3 ppm) to facilitate secondary fermentations, with the condition that the final product does not contain residue, and to eliminate the colour of the red or black grape juice (500 ppm).	(FDA, 2011)
	Authorised for the treatment of poultry carcasses and organs (21 CFR 173.370).	21 CFR 173.370
	Included in the food additive data base (EAFUS) and in the list of "indirect" additives used in substances in contact with foods (CFSAN), both of the FDA.	–
Australia and New Zealand	Its use is authorised as a processing aid (bleaching agent) in foods, establishing a maximum residue of 5 mg/kg (ppm).	(ANZFSC, 2002)
Japan	Authorised as a bleaching agent for all types of food product, establishing as a limit to use that there are no residues in the final product.	(FFCR, 2010)
Taiwan	Authorised as a cleaning agent in all food products, except flours, provided there are no residues of this agent in food destined for human consumption.	(TFDA, 2009)

In addition, a report published by the Scientific Committee on Consumer Products (SCCP) of the Directorate General for Health and Consumer Protection of the European Commission (DG SANCO) states that the use of toothpastes, mouth rinses and tooth whiteners that contain up to 0.1% of hydrogen peroxide does not imply a risk to consumer health, and treatments for tooth whitening supervised by a dentist may contain up to 6% (SCCP, 2007).

Considerations on the studies and data relating to safety in the use of hydrogen peroxide

1. Toxicokinetics

Both the kinetics and the metabolism of hydrogen peroxide have been the subject of study by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC, 1992). Hydrogen peroxide is a normal product with an aerobic cellular metabolism as a result of a number of enzymatic reactions. In normal physiological conditions, the production of hydrogen peroxide in the liver is approximately 90 nmol/min/g of liver.

There are two main hydrogen peroxide metabolising enzymes, catalase and glutathione-peroxidase which control the peroxide concentration. Catalase is mainly found in the peroxisomes and is most active in the duodenum, liver, kidney, mucous membranes and highly vascularised tissues. In its presence, hydrogen peroxide is decomposed into water and oxygen, with a decomposition rate in human plasma of approximately 0.01-0.06 mmol/l/min. Catalase decomposes high concentrations of hydrogen peroxide whereas glutathione-oxidase is more efficient at low concentrations. Glutathione-peroxidase is found in the mitochondrions and reduces hydrogen peroxide to water, oxidising to gluthathione disulphide.

In the presence of transition metals, hydrogen peroxide can be reduced, in a *Haber-Weiss* reaction to highly reactive hydroxyl radicals and may cause a lipid peroxidation. The oxidative reactivity of hydrogen peroxide on biological molecules such as carbohydrates, proteins, fatty acids or nucleic acids is not significant in the absence of these transition metals.

An increased sensitivity of the erythrocytes to hydrogen peroxide is observed in the event of a deficiency of these enzymes (acatalasaemia and G6P-dehydrogenase).

Although in some studies, it has been observed that hydrogen peroxide increased enzymatic activity in rodent tissue, this has not been corroborated in other studies (ECETOC, 1992).

2. Effects on food

In this respect, the Scientific Committee estimated that one of the main aspects to consider is the toxic potential of the products formed as a consequence of using hydrogen peroxide, in particular metabolites resulting from the oxidation (potential formation of cholesterol or peroxides).

Cephalopods

Among the possible undesirable effects, the applicant has indicated that during the treatment with hydrogen peroxide, the components of the cephalopod may be modified. According to the Table of composition submitted, the fat and cholesterol contents given for squid, octopus and cuttlefish range

between 0.8-1.4 g/100 g and 112-233 mg/100 g, respectively. The average values given for fat and cholesterol in cephalopods are 1.3 g and 215 mg/100 g, respectively. Due to the use of hydrogen peroxide, there is an increased risk of oxidation of polyunsaturated fatty acids which would result in a musty flavour and some loss in nutritional value. However, cephalopods have a very low total fat content, which is almost all found in the entrails which are eliminated during preparation and prior to treatment with this agent. The use of hydrogen peroxide, on the other hand, may increase the content of cholesterol oxides in these products.

With respect to the proteins, only the sulphur-containing amino acids, methionine and cysteine are susceptible to modification (their SH-group would oxidise and form cystine, a reaction which takes place in the same way in the human metabolism).

The effects on carbohydrates are null. With respect to vitamins, it is stated that it destroys vitamin C although none of the foods considered are relevant sources of this vitamin.

Blood products

The data provided by the applicant indicate that, in the case of blood, the fat and cholesterol contents are 0.1 g and 190 mg/100 g, respectively. The analytical results conclude that the bleached product contains more ash as a result of the pH modification, it is more soluble and less red, no substantial changes are observed in the amino acids, the bleaching process contributes to reducing the total number of bacteria present in the end product, and moreover, the results for the peroxide index were negative.

3. Study of the effect of hydrogen peroxide on the cholesterol oxide content

Taking into consideration the data submitted by the applicant, the Scientific Committee considers that, although the fat content in these foods is low, the cholesterol content is not insignificant and therefore the effect of hydrogen peroxide on this component must be analysed.

The determination of cholesterol oxides is complex due to the low content, the difficulty of purification and separation, their instability and the lack of available patterns. The Scientific Committee considers that, in accordance with European directives, the correct identification of the same using mass spectrometry is to be recommended.

Gil et al. (2004) list the work carried out by different authors who specify the content of various cholesterol oxides found in meat and meat products and the huge variability existing depending on the species considered. Therefore, they conclude that the different sensitivity of the analytic techniques used for determining cholesterol oxides is one of the main causes of the variation and, therefore, it is necessary to continue the search for greater effectiveness in the techniques used.

To control the presence of cholesterol oxides, a separation of this fraction is generally recommended prior to the final chromatographic analysis. It is considered that the GC-MS and GC-MS/MS, following derivatization or directly, are the selected methods for determining cholesterol oxides, following solid phase separation of the triglycerides (Calvo et al., 2003).

In view of the bibliography consulted, (Valenzuela et al., 2002), it is possible to conclude that cholesterol oxides must be considered as undesirable products in our food, as saturated fatty acids,

the products of fatty acid oxidation, and the *trans* geometric and positional isomers are currently considered (Valenzuela et al., 1995). Among the biological effects attributed to cholesterol oxides the following should be mentioned: inhibition of cellular cholesterol synthesis; changes in membrane fluidity; induces apopthosis; inhibition of DNA synthesis; alteration of calcium homeostasis; thrombin-induced aggregation, possible mutagenicity and a greater atherogenicity than that of cholesterol.

The formation of cholesterol oxides does not depend exclusively on the action of hydrogen peroxide, but that the majority of the processing techniques, especially the cooking and irradiation treatments drastically increase the speed of formation (especially the temperature/presence of oxygen combination). In addition, the synthesis of cholesterol oxides can be reduced, using techniques such as packing in protective atmospheres and/or storage as frozen, and by the use of antioxidants.

A maximum intake of cholesterol oxides has not been specified, although in general, irrespective of that indicated by Valenzuela et al. (2002), the quantities found in the foods are below those which might be assumed to be harmful to health (Astiasarán et al., 2006). In this respect the same authors, Astiasarán et al. (2006), give figures for total cholesterol oxide contents in cooked meat samples ranging between 0.522 and 1.027 mg/100 g. In addition, they give figures for prawns subjected to different treatments: fresh (33.15 ppm), frozen (2.38 ppm), fresh grilled (55.43 ppm) and frozen grilled (13.06 ppm). In addition, it is indicated that there are studies available which establish a safe limit of <0.1 mg/day, although this reference is not backed by scientific evidence and, in turn, states that other authors give dangerous percentages of oxidation with respect to cholesterol of above 0.5%, and this figure is only exceeded in the case of microwave cooked salmon, with a value of 0.80% (Astiasarán et al., 2007). Other figures given in this work include: fish (salmon and prawns) and meat (hamburgers, chicken breasts, loin and frankfurter-type sausages) which raw have low cholesterol oxide content (0.003-0.552 mg/100 g food), significantly increasing after cooking (up to 0.7 mg/100 g food). Microwave cooking resulted in the greatest increase in cholesterol oxides in comparison with frying, grilling and roasting. The formation of cholesterol oxides was significantly reduced with vacuum packing. In addition, it was concluded that "the proven negative effects of cholesterol oxides, together with scientific evidence of their formation in foods and their absorption from foods, means that the intake of this type of compound in a normal diet must be determined and controlled".

In this respect, as an indicator of the loss of cholesterol and the consequent formation of oxides, the applicant has submitted information on the recovery percentage of a cholesterol pattern after treatment with hydrogen peroxide in different conditions. In concentrations higher (3.3%) than the maximum proposed by the applicant, the cholesterol losses and, therefore, the formation of cholesterol oxides, are minimal (1%), and are assumed to be much lower in the case of the treatment of complex matrices. Only when very high concentrations are used are cholesterol losses observed. These may be as much as 11% when hydrogen peroxide is applied (33%) directly on a dry cholesterol residue.

In the light of these reports, the Scientific Committee considers that, as the cholesterol oxide content in food depends on various factors such as the complexity of raw material, the culinary processes used, conservation processes, etc., it is not possible to establish a limit for their presence applicable to all the foods under evaluation in this report. Nevertheless, and considering that in no event do the regulations considered above establish a limit for these substances, their formation

should be minimised and must be the subject of a general study of the presence of cholesterol oxides in foods.

Study of the cholesterol oxide profile in cephalopods and blood products treated with hydrogen peroxide

For this purpose, the applicant submitted a study carried out to establish whether the cholesterol oxide profiles changed significantly when samples of cephalopods and blood products were subjected to treatment with hydrogen peroxide (H_2O_2).

This study used the analysis method validated by Menéndez et al. (2008). In accordance with the analytical recommendations mentioned above (section 3.3), a solid phase separation was made of the triglyceride fraction of interest, the extract was derivatized and subjected analysed by gas chromatography. The identification and confirmation of the analytes was carried out by mass spectrometry (GC-MS), in accordance with the guidelines of Commission Decision 2002/657/EC (EU, 2002). It is also important to note that to minimise any possible bias, the samples that underwent treatment were analysed in the same conditions as the control samples, in order to reject the possibility of artefacts generated during the analysis.

A total of 134 samples were analysed, including blood products (n=9) and three types of cephalopods: flying squid (n=66), squid (n=37) and cuttlefish (n=22). Samples which had not been subjected to any treatment were compared with samples sent to the laboratories by the companies and which had been treated with aqueous solutions of hydrogen peroxide at 0.05%, these were also compared with samples treated in the laboratory in accordance with the instructions of the companies at 0.05% (for 12 and 24 hours) and at 35% (12 hours) to simulate highly unfavourable conditions. With respect to the blood products, the samples sent to the laboratory by the companies had been treated with hydrogen peroxide at 0.75% for 15-30 minutes.

From this study, it is concluded that the samples of fresh cephalopods, the treated samples sent by the companies and the samples treated in the laboratory at the concentrations proposed, do not show any statistically significant differences ($p \leq 0.05$) in their cholesterol oxide profiles.

There are significant differences in the composition of some cholesterol oxides among the different types of matrices, but these differences are already observed in the untreated products.

The cholesterol oxides were mainly cholesterol epoxides, ß-Epoxy and α-Epoxy and 7-ketocholesterol, fully in line with those found in other foods. The 25-hydroxicholesterol, reputed to be one of the most toxic, was not observed in either untreated samples or those treated with hydrogen peroxide at 0.05%. Cholestanotriol is found in fresh samples always in very low concentrations, lower than those found in other foods according to the bibliography consulted. Moreover, these contents do not increase significantly in the treated samples.

With respect to the blood products, it should be highlighted that no significant statistical differences ($p \leq 0.05$) were observed between the untreated samples and those treated with hydrogen peroxide.

Levels of cholesterol oxides in the untreated blood product samples were higher than those usually found in foods ready for consumption or cooking. However, this is partly due to the fact that this is a product which is dried by atomisation at high temperatures (220-250 °C) which may explain these

values. On the other hand, it should be noted that this product is not consumed as such but is a prepared food in concentrated form. It should be noted that blood products are products used as ingredients in very small quantities in the preparation of meat products, in the order of 0.5-0.8 g/kg weight of meat. This is less than 1% of the weight of the meat which is subsequently processed. Therefore the cholesterol oxide content in the end product is much lower. In addition, a study carried out by the applicant on the effect of the treatment with hydrogen peroxide at 3% has shown that it does not affect either the nutritional value or the digestibility of the same (APC Europe, 2005).

In a recent review (Otaegui et al., 2010), has drawn up a list of the cholesterol oxides content in different fresh foods or foods subjected to industrial treatment. Quoting some examples, among the dairy products we have contents of 13.7-27.3 µg/g in butter (Pie et al., 1990) or 1.1 µg/g in powdered whole milk (Angulo et al., 1997); in egg-related products, quantities are given of 3.3-3.8 µg/g in pasteurised liquid egg (Guardiola, 1995) or 43.8-52.0 µg/g fat in dried egg pasta (Verardo et al., 2010). Among meat products the intervals range from 0.1 µg/g in beef (Boselli et al., 2009), 0.2 µg/g in poultry (Zubillaga and Marker, 1991), between 0.5-10.5 µg/g in fresh ham and 0.8 µg/g in cured ham (Sánchez et al., 2010) or between 0.6-18.7 µg/g in mortadella (Novelli et al., 1998). In fish and related products quantities are given of 0.7 µg/g fat in salmon (Echarte et al., 2001), 33.6 µg/g in anchovies (Shozen et al., 1997), 19.4 µg/g in Brazilian sardines (Saldanha et al., 2008), 119.9 µg/g fat in canned tuna (Zunin et al., 2001) and 18.1 mg/100 g in sun-dried shrimps (Soto et al., 2008).

In the study given here the total content of cholesterol oxides in the fresh samples were 0.6223 µg/g for flying squid, 2.2384 µg/g for squid and 0.2714 µg/g for cuttlefish. These are similar to those found in other fresh products, and could even be included among the foods with the lowest values and, of course, among the lowest values for the mentioned fishery products. In the case of squid, the values are slightly higher as in addition to the mantle, the tentacles, including the skin and underlying fat, were analysed.

In addition, the studies carried out of foods show a direct relation between the intensity of heat and the increased formation of cholesterol oxides (Osada et al., 1993) (Otaegui et al., 2010) and that, in addition, foods that are cooked and then stored frozen have higher contents than the raw samples that are stored frozen, indicating that even the cooking process may encourage oxidation during storage (Conchillo et al., 2005). In the work of Osada et al. (1993), in which cholesterol oxides were not observed in fresh squid, values of 0.146 µg/g were obtained for dry squid and 0.11 µg/g for cooked canned squid (n=3). With respect to the treatments to other fish and related products, Ohshima et al. (1993) indicate total values of cholesterol oxides for salted and dried anchovies of 127 µg/g dry weight compared to cooked and dried anchovies of 188 µg/g dry weight (an increase of 61 µg/g dry weight). Echarte et al. (2001) compared fresh salmon, salmon fried in olive oil, salmon fried in soya oil and roast salmon, and observed an increase of 2.24 µg/g fat in the salmon fried in olive oil, an increase of 2.61 µg/g fat in the salmon fried in soya oil and an increase of 6.64 µg/g fat in the roast salmon. Echarte et al. (2005) reported that the total values of cholesterol oxides for prawns sold fresh increased by 22.28 µg per g fat after grilling and that in prawns sold frozen the cholesterol oxide content only increased 10.68 µg/g fat after grilling. Saldanha and Bragagnolo (2007) observed that the total content of cholesterol oxides increased significantly in raw hake fillets from Patagonia, rising

from 8.00 µg/g dry weight on the first day of storage to 78.10 µg/g dry weight after 120 days of storage at -18 ºC (an increase of 70.1 µg/g dry weight) and in the case of grill roasted samples this rose from 18.50 to 122.30 µg/g dry weight after 120 days of storage at -18 ºC (an increase of 103.8 µg/g dry weight). This study was repeated with Brazilian sardines (Saldanha et al., 2008), confirming that the total content of cholesterol oxides increased significantly in raw Brazilian sardines, rising from 19.4 µg/g dry weight on the first day of storage to 115.2 µg/g dry weight after 120 days of storage at -18 ºC (an increase of 95.8 µg/g dry weight) and in the case of grilled sardines this rose from 41.6 to 177.9 µg/g dry weight after 120 days of storage at -18 ºC (an increase of 136.3 µg/g dry weight).

Smoking also seems to affect the cholesterol oxide contents and thus Pickova and Dutta (2003) give values of 6.23 µg/g of fat in fresh salmon roe compared to 93.06 µg/g of fat in smoked cod roe. Ohshima et al. (1993) give total cholesterol oxide contents for smoked salmon of 26.8 µg/g dry weight.

In the study submitted by the applicant, there are no statistically significant differences between the total cholesterol oxide content of treated and untreated cephalopod and blood product samples, therefore it is concluded that hydrogen peroxide does not appear to have a major influence on its formation in these foods. In fact, according to the results obtained, in some cases there is almost no increase in cholesterol oxides with respect to raw samples, and in those in which there is an increase (for example, those samples treated in the laboratory over a long period of time, 24 hours), this is very small. Very possibly, this is because the treatment with hydrogen peroxide recommended by manufacturers involves low quantities of hydrogen peroxide, and moreover this seems to decompose quickly. Nevertheless, it does seem important to establish a treatment level for hydrogen peroxide as, although the values of cholesterol oxides found in the least favourable conditions (direct treatment of cephalopods with hydrogen peroxide at 35%) are not high, they should nevertheless be controlled.

4. Effect of hydrogen peroxide on proteins

Although from a biomedical perspective, studies on the metabolism of protein oxidation have advanced significantly, there are very few studies which clarify and define the process of the oxidation of proteins present in foods and its consequences.

Protein oxidation was discovered at the start of the 20th century (Dakin, 1906), and was initially the subject of much research. However, this subject was soon abandoned due to the huge complexity revealed in the studies (lack of reliable identification of numerous centres of action, complexity in the explication of the mechanisms of action, lack of stability of the derivatives and in particular the lack of reliable analysis methods, etc.) (Davies, 2005).

The attack of oxidising agents on muscular proteins, among others, mainly causes losses in the -SH group and the generation of carbonyl compounds (aldehydes or ketones) (Xiong, 2000). Although hydrogen peroxide is a known oxidising agent, there are few studies that determine its effect on the proteins present in foods. Levine and Ciolino (1997) indicate that the treatment with hydrogen peroxide of biological cell cultures induces modifications in the DNA, but they stress that it is a poor indicator of the generation of carbonyl compounds by the proteins unless the cellular iron content is

increased by extracellular additions. In this respect, it should be noted that in the majority of cephalopods, iron is not present in large quantities given the structure of their circulatory system.

Recent studies of food models indicate that protein oxidation may lead to modifications to the jellification, emulsification, viscosity, solubility and hydration (Armenteros, 2010) or even to losses in aromas, colour, damage of the structure and loss of tenderness and juiciness in meat products (Lund et al., 2007). Rowe et al. (2004) have suggested that the oxidation of *post-mortem* proteins affects the texture of refrigerated beef. Carballo et al. (1991), Perlo et al. (1995), Jo et al. (2000) and Fernández et al. (2003) indicated in their works that the deterioration of the colour during the refrigerated storage of cooked meat could explain the degradation of certain nitropigments caused by the oxidation process, although none of the works indicate which mechanism is related to the oxidation of the proteins with this effect. In addition, the oxidation of the proteins may affect the nutritional value, probably either due to the destruction of amino acids or because when the proteins are modified, their digestibility may be altered (Estévez, 2005). In his doctoral thesis Estévez (2005) concludes that the oxidative deterioration of the proteins in cooked products affects certain quality parameters resulting in the discolouration of cooked sausages during refrigeration due to the degradation of hemic pigments and the deterioration of the texture of pâtés and sausages due to the possible generation of crossed links between proteins and the loss in their functionality.

Nevertheless, other authors specifically note that it is possible to use a controlled protein oxidation to improve the texture properties of certain foods or to create modified proteins which can be used as ingredients in the preparation of foods (Andersen, 2001).

The techniques which are used to detect the oxidation damage to proteins of animal origin are mainly adapted to those developed in biomedical research. Therefore, the quantification of carbonyl groups using the dinitrophenylhydrazine (DNPH) method has become the normal routine procedure in a large variety of meat products such as fresh meat, meat emulsions and cured meat products (Estévez and Cava, 2004) (Ventanas et al., 2006) (Lund et al., 2007). However, Estévez (2005) and Armenteros (2010) show that these methods significantly overestimate protein oxidation as they are not specific, and mass spectrometry methods are more reliable as they clearly identify the oxidation indicators (Armenteros et al., 2009) (Estévez et al., 2009). Therefore, this fact must be considered when assessing the data found in the scientific bibliography (possibly over-estimated) refereeing to protein oxidation in foods.

The French Agency for Food Safety (AFSSA) has studies which indicate that the treatment with hydrogen peroxide of whey destined for use in infant food, in concentrations of 200 mg of hydrogen peroxide/l whey, does not imply greater modifications of the protein oxidation or cholesterol oxidation, or the cysteine residue, in comparison with those obtained from heat treatments such as pasteurisation (AFSSA, 2005).

Lastly, it should be noted that scientific literature on this point often indicates that the protein oxidation is usually parallel to the lipid oxidation, thereby suggesting that both mechanisms may in some way be interconnected (Estévez, 2005) (Armenteros, 2010). Armenteros (2010) finds good correlation between both oxidations in meat products for example. It may therefore be inferred that when lipid oxidation is not very important as is the case in the studies given here on cephalopods and

blood products, then nor is the protein oxidation. This may be the reason that the majority of authors focus on the study of the presence of oxides of a lipid origin, given the difficulties in determining protein oxidation, as mentioned earlier.

5. Safety studies

With regards to the safety of hydrogen peroxide (H_2O_2), it is noted that it has been the subject of study, among others, of the Committee for Veterinary Medical Products, CVMP, of the European Medicines Agency (EMEA, 1996) and the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE, 2001). In the case of the CSTEE (2001) an intake through food products is estimated at between 0.033 and 0.13 mg H_2O_2/kg body weight/day, also establishing a No Observed Adverse Effect Level (NOAEL) of 30 mg H_2O_2/kg b.w., based on a trial on rats. No safe consumption threshold is indicated.

Subsequently, the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH, 2003) assessed the efficiency, and possible toxicity of a solution of peroxiacids, including hydrogen peroxide, used in the treatment of poultry carcasses. This assessment was based on the residues remaining after the treatment, and did not consider the possible formation of reaction compounds.

The SCVPH (2003) considers a NOAEL for hydrogen peroxide of 26 mg H_2O_2/kg b.w/day, although there is no associated Tolerable Daily Intake (TDI). The strategy used to assess the risk associated with the intake of this compound is that of the Margin of Safety, considering the estimated risk from the intake of hydrogen peroxide residues as negligible.

The opinion of the SCVPH was subsequently subject to review by the European Food Safety Authority (EFSA, 2005) who reached similar conclusions.

With respect to the toxicological assessment, EFSA considers the same NOAEL as the SCVPH for hydrogen peroxide, establishing:

- 26 mg/kg b.w./day for males and 37 mg/kg b.w./day for females, identified in a study over 90 days of catalase-deficient mice.
- 30 mg/kg b.w./day in a study of catheter-fed rats.

The potential exposure to peroxiacids and hydrogen peroxide considered by the EFSA, based on figures for the European adult meat intake, is 0.6 g/kg b.w./day for the average consumer, and 1.1 and 1.5 g/kg b.w./day for the 95 and 99 percentiles of excess consumption (average body weight of 60 kg). The EFSA also indicates, in line with the SCVPH, that the sub-products generated by the treatment of peroxiacid solutions (including hydrogen peroxide) do not entail unacceptable safety risks.

Together with the above-mentioned points, it is also of note, with respect to the assessment of its safety, that hydrogen peroxide is considered as non-carcinogenic by the International Agency for Research on Cancer (IARC, 1999).

Considerations on the studies and data relating to the presence of hydrogen peroxide residues

The applicant submitted an initial study on the possible presence of hydrogen peroxide residues in blood products and cephalopods.

Nevertheless the Scientific Committee, in view of the low number of samples analysed and the methods used, etc., viewed it as inadequate.

To determine the presence of hydrogen peroxide residues, the Committee considers it is more appropriate to use enzymatic spectrophotometric methods, as these are more reliable and easier to introduce when performing autocontrols in the food industries.

In this respect, the applicant then submitted another more complete study on the detection of hydrogen peroxide residue in which several methods were tried, but always under the premises that they were methods for which the applicable protocol would be of use in the processing industries.

This study concluded that recommended qualitative methods would include commercial test strips for the detection of H_2O_2 (*Quantofix*) (Detection limit: 1.5 mg H_2O_2/kg cephalopod) and the potassium iodide colorimetric method (Detection limit: 0.6 mg H_2O_2/kg cephalopod) and the recommended quantitative method would be a method based on the spectrophotometric detection of the coloured complex, xylenol-Fe^{3+}, a commercially available method (*PeroxiDetect® Kit*) (Detection limit: 1 mg H_2O_2/kg cephalopod). These methods are quick and easy to apply in any commercial laboratory. The validation of the quantitative method was presented. These methods were then applied for the detection of hydrogen peroxide residue in cephalopods. Specifically, on samples of flying squid, cuttlefish and squid (n=50) which had been treated experimentally by the companies and in the laboratory at concentrations of 0.05% hydrogen peroxide, corresponding to those indicated by the applicant for this type of food.

This work concluded that the presence of hydrogen peroxide was not observed at all in any of the cephalopod samples using the proposed methods. This is probably due to the fact that the hydrogen peroxide reacts well with the organic matter and quite quickly in the treatment conditions: the quantity of organic matter is very high with respect to that proposed for hydrogen peroxide and the pH of the extract is quite alkaline (pH 9.3), being a factor which accelerates the decomposition of this agent. These may both be factors which enhance the non-appearance of reside in these foods once they are no longer submerged in the treatment solution. Therefore, the hydrogen peroxide in the treatment concentrations proposed for the cephalopods does not leave residues and is considered to be acting as a processing aid.

These methods were not applied to blood product samples, as after treatment with hydrogen peroxide, this product was dried by atomisation at high temperatures (220-250 ºC), implying the total absence of hydrogen peroxide residue in the product. This has already been demonstrated in a study carried out of this product by APC Europe (2005).

Study of intake and assessment of the level of consumer exposure

1. Calculation of the estimated daily intake (EDI)

In order to assess exposure it is necessary to estimate the daily intake of hydrogen peroxide as a result of the possible presence of residues in the treated foods.

The figures available indicate that no hydrogen peroxide residues were observed in the foods that are the subject of this report. If hydrogen peroxide is considered as a processing aid, the residue should only be that which is technically unavoidable and with consideration for the detection limit of

the most sensitive analytic technique used (0.6 mg/kg), the hydrogen peroxide residues should not exceed this concentration. To calculate the estimated daily intake, the following two cases are considered: 0.6 mg/kg of hydrogen peroxide in the product ready for marketing and 1.5 mg/kg in the least favourable situation.

Given the average adult intake of foods or food groups in the study (AESAN, 2006) and a content in the foods of 1.5 mg/kg of hydrogen peroxide as residue, the exposure can be estimated as established below (Table 3). The intakes used are for "only consumers" given the low percentage of consumers of the foods considered here.

Table 3. Estimation of exposure to hydrogen peroxide in adults

Food	H_2O_2 content (mg/kg)	Average adult intake (g per person and day)	Adult intake in 97.5 percentile (g per person and day)	Assessment of average adult intake (mg H_2O_2 per person and day	Assessment of adult intake in 97.5 percentile (mg H_2O_2 per person and day
Squid, cuttlefish and similar	**1.5**	26.34	71.11	**0.0395**	**0.1067**
Canned squids and similar	**1.5**	29.07	77.43	**0.0436**	**0.1162**
Octopus	**1.5**	41.41	89.14	**0.0621**	**0.1337**
Blood	**1.5**	38.5	117.5	**0.0578**	**0.1763**
Total intake of H_2O_2 (mg per person and day)				**0.2030**	**0.5328**

In the above-mentioned conditions, assuming a maximum residue content of 1.5 mg/kg, the total consumption of hydrogen peroxide as residue in the foods under assessment would be approximately 0.2030 mg per person and day for the average consumer, and in the case of extreme consumers (97.5 percentile), 0.5328 mg per person and day. If we consider the average adult weight as 68.48 kg (AESAN, 2006) the intake in mg/kg b.w./day would be 0.0030 and 0.0078 for the average and extreme consumers, respectively.

If we consider a residue level of 0.6 mg/kg, the estimated daily intake for adults (average consumer) would be 0.0012 mg/kg b.w./day and for the extreme consumer 0.0031 mg/kg b.w./day.

Similarly, an estimation of exposure can be drawn up for children considering the average intake (AESAN, 2006) as established below (Table 4):

Table 4. Estimation of exposure to hydrogen peroxide in children

Food	H_2O_2 content (mg/kg)	Average child intake (g per person and day)	Child intake in 97.5 percentile (g per person and day)	Assessment of average child intake (mg H_2O_2 per person and day	Assessment of child intake in 97.5 percentile (mg H_2O_2 per person and day
Squid, cuttlefish and similar	**1.5**	26.27	74.23	**0.0394**	**0.1114**
Canned squids and similar	**1.5**	17.62	46.05	**0.0264**	**0.0691**
Octopus	**1.5**	49.58	74.1	**0.0744**	**0.1112**
Blood	**1.5**	0	0	**0**	**0**
Total intake of H_2O_2 (mg per person and day)				**0.1402**	**0.2916**

Assuming, as for the adults, a maximum residue content of 1.5 mg/kg, the total consumption in children of hydrogen peroxide as residue in the foods under assessment would be approximately 0.1402 mg per person and day for the average consumer, and in the case of extreme consumers (97.5 percentile), 0.2916 mg per person and day. Considering the average weight for children of 34.48 kg (AESAN, 2006), this intake would be equivalent to 0.0041 mg/kg b.w./day for the average consumer and 0.0085 mg/kg b.w./day for the extreme consumer.

If we consider a residue level of 0.6 mg/kg, the estimated daily intake for children (average consumer) would be 0.0016 mg/kg b.w./day and for the extreme consumer 0.0034 mg/kg b.w./day.

2. Calculation of the margin of exposure or margin of safety

Hydrogen peroxide is considered to be non-carcinogenic by the International Agency for Research on Cancer (IARC, 1999), therefore for the purposes of risk characterisation, it is only necessary to consider the non-carcinogenic effects, comparing the estimated daily intake with the tolerable daily intake (TDI). In this respect, the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH, 2003) established a NOAEL for hydrogen peroxide of 26 mg H_2O_2/kg b.w./day, although there is no TDI associated. This NOAEL has subsequently been used by EFSA in other assessments (EFSA, 2005).

The report from the SCVPH (2003) recognises that when there is no established TDI, the assessment must be made by applying the Margin of Safety (MOS). In this procedure, the NOAEL established in animals and expressed in mg/kg b.w./day is compared to the level to which the human is exposed. This MOS is the quotient between the NOAEL and the estimated daily intake, and in general an MOS of 100 is considered to be a safe level of exposure. However, the same report (SCVPH, 2003) also highlights that, in the case of peroxides, an MOS of 1,000 is to be recommended, considering the limited toxicological information available for these compounds. Therefore, in this case, an MOS of 1,000 should be considered as a safe level.

This procedure does not consider the differences in susceptibility between humans and animals or within animals or human beings; therefore, the MOS that indicates acceptable levels is relatively high.

Table 5 shows the calculation of the MOS for the exposure conditions estimated in the previous paragraph.

Table 5. Estimation of the margin of safety

	Adults		Children	
Parameter	**Average consumer**	**Extreme consumer**	**Average consumer**	**Extreme consumer**
Concentration of hydrogen peroxide (mg/kg)	1.5 (0.6 mg/kg)	1.5 (0.6 mg/kg)	1.5 (0.6 mg/kg)	1.5 (0.6 mg/kg)
H_2O_2 exposure (mg/kg b.w./day)	0.0030 (0.0012)	0.0078 (0.0031)	0.0041 (0.0016)	0.0085 (0.0034)
NOAEL (mg/kg/day)	26	26	26	26
Margin of safety (MOS)	**8,784 (21,849)**	**3,342 (8,360)**	**6,388 (15,951)**	**3,073 (7,692)**
Minimum required MOS	1,000	1,000	1,000	1,000

Therefore, it can be deduced that with the hydrogen residue peroxide level considered in the foods, there are no significant risks or adverse effects for health.

Conclusions of the Scientific Committee

1. With respect to the presence of hydrogen peroxide residues:
   a) The Scientific Committee considers that on the basis of the data provided in the different studies listed in this report relating to the use of hydrogen peroxide as a processing aid in the preparation of cephalopods and blood products, in the conditions proposed by the applicant, there is no risk to the consumer.
   b) For control in food industries involved in the use of hydrogen peroxide as a processing aid, the methods indicated in this report for the assessment of hydrogen peroxide residues should be adopted: as qualitative methods, commercial test strips should be used for the detection of H_2O_2, together with the potassium iodide colorimetric method, and as a quantitative method, a method based on the spectrophotometric detection of the coloured complex xylenol-Fe^{3+}, a commercially available method, should be adopted.
2. With respect to the increase and/or formation of cholesterol oxides in cephalopods and blood products treated with hydrogen peroxide in the proposed doses, this Committee considers that:
   a) These products already have quantifiable intrinsic contents of cholesterol oxides and the increase of this content after treatment is not significant ($p \leq 0.05$), as demonstrated in the studies provided for the comparison of samples treated with hydrogen peroxide and untreated samples.
   b) With respect to the formation of new cholesterol oxides, on the basis of the studies provided, it is concluded that the chromatographic profiles do not significantly ($p \leq 0.05$) contribute any new compound before and after treatment with hydrogen peroxide in the doses proposed for these products.

3. With respect to the effect of hydrogen peroxide on protein oxidation in treated samples, the bibliography consulted permits the conclusion that it is not important, as it takes place in parallel to lipid oxidation and both mechanisms are interconnected.
4. The use of hydrogen peroxide by the industry for the uses mentioned above and in the conditions given by the applicant should be subject to Good Manufacturing Practices.

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