![Food Standards Australia New Zealand logo]

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Supporting document 1

Risk and technical assessment – Application A1127

Processing Aids in Wine

Executive summary

The Winemakers' Federation of Australia (WFA) is seeking amendments to the Australia New Zealand Food Standards Code (the Code), to provide permissions for four new processing aids for use in the manufacture of wine.

The European Union (EU) has formally requested Australia authorise the use of these processing aids for use in wine and in response, the WFA have prepared this Application. Gaining permission for these processing aids will enable Australia to fulfil its treaty obligations under the Wine Agreement and also to satisfy World Trade Organisation obligations to ensure equal treatment with its trading partners.

Three of the four processing aids (chitin-glucan, PVI/PVP co-polymers, ammonium bisulphite (also known as ammonium hydrogen sulphite)) are permitted processing aids in the EU and the USA. The fourth, silver chloride, is permitted in the EU and the Republic of South Africa.

Three of the processing aids are removed by filtration or similar processes before bottling so negligible levels are expected in the final product. The fourth, ammonium bisulphite breaks down into nitrogen ions and is assimilated by yeast. FSANZ did note however, that sulphur dioxide (SO_2) is also produced during the breakdown of ammonium bisulphite, which may remain in the wine and have an incidental role as a preservative. The primary technological function for ammonium bisulphite is for a yeast nutrient and its use as such is technologically justified. SO_2 is a permitted food additive in wine under item 14.2.2 of the table to section 15—5.

The technical assessment concluded that the technological purposes of the four processing aids are justified at use levels consistent with good manufacturing practice (GMP), which limits the amount of the substances added to the lowest levels necessary to accomplish their intended function.

FSANZ has concluded that there are no public health and safety concerns from the proposed use of these substances as processing aids in the manufacture of wine.

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Part A: Chitin-glucan

1   Introduction

1.1   Description

Chitin-glucan is a fining agent of musts to reduce colloid content and cloudiness. It is also used to stabilise iron content in wines before bottling; preventing undesirable red colours in white wines. It also helps eliminate undesirable compounds such as metals and mycotoxins.

Characteristics of Chitin-glucan:

* extracted and purified from the mycelium of non-genetically modified *Aspergillus niger*.
* a natural, two polymer structure, covalently connected to form a three-dimensional network
* comprised of the polysaccharides chitin (repeat units N-acetyl-D-glucosamine) and 1,3-ß-glucan (repeat unit D-glucose)
* the chitin/glucan ratio ranges from 25:75 to 60:40.
* insoluble in wine
* the precipitates it forms with unwanted components in wine must are removed via filtration, racking, centrifugation or similar physical separation process.

1.2   Current permissions

1.2.1   International Organisation of Vine and Wine (OIV)

Resolutions:

* OIV/OENO 336B/2009 (Musts – fining using chitin-glucan)
* OIV/OENO 337B/2009 (Wines – fining using chitin-glucan)
* OIV/OENO 338B/2009 (Wines – treatment using chitin-glucan)
* OIV/OENO 339B/2009 (Wines – fining: complex modification of the existing sheet – chitin-glucan)

International Oenological Codex
* COEI-1-CHITCL:2009, OENO 367/2009 (Chitin-glucan)

1.2.2   European Union

Purpose

a.   reduction in the heavy metal content, particularly iron, lead, cadmium and copper
b.   prevention of ferric casse[1] and copper casse
c.   reduction of possible contaminants, especially ochratoxin A
d.   reduction in the populations of undesirable micro-organisms, in particular Brettanomyces; solely by means of treatment with chitosan.

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[1]Named after the element that causes the problem, such as an "ferric (iron) casse" or "copper casse", it is caused when these elements solidify and cloud the affected wine.

Requirements:

The concentrations to be used are determined after a qualification test. The maximum concentration (in grams per hectolitre – g/hL) related to each purpose used may not exceed:

- 100 g/hL for applications (a) and (b)
- 500 g/hL for application (c)
- 10 g/hL for application (d)
- sediments are removed using physical processes.

1.2.3   United States of America

Product	Technological use	Permitted amount
Wine	Clarification	100 g/100 L
	Fining	50 g/100 L
	Removal of trace metals	100 g/100 L
	Removal of potential mycotoxins	500 g/100 L
	Prevention of oxidation of colour	100 g/100 L
	Reduction of heat labile proteins	100 g/100 L
	Aid to filtration	50 g/100 L
Must	Clarification/removal of off-flavour	100 g/100 L
	Removal of potential mycotoxins	500 g/100 L
	Prevention of oxidation of colour	100 g/100 L

Note: in the USA, chitin-glucan is used as a processing aid during alcoholic beverage production and is subsequently removed by physical separation processes before bottling. Therefore, the above use concentrations do not constitute concentrations of chitin-glucan in the final product, from which chitin-glucan is absent.

1.3   Risk assessment questions

1.3.1   Technological function

Is the technological function clearly stated and does chitin-glucan achieve that function as a food processing aid in the quantity and form proposed?

1.3.2   Health and safety

What, if any are the potential public health and safety concerns arising from the use of chitin-glucan as a processing aid in the manufacture of wine in Australia and New Zealand?

2   Food technology assessment

2.1   Technological purpose

According to the OIV, chitin-glucan, when used as a processing aid in the manufacture of wine is a fining agent. The purpose of adding a fining agent preparation to wine is to soften or reduce its astringency and/or bitterness; remove proteins capable of haze formation; or reduce colour by the adsorption and precipitation of polymeric phenols and tannins.

It is also used for stabilising wines after alcoholic fermentation and stabilises the iron content in wine before bottling, preventing undesirable red colours in white wines. Information provided by the applicant shows that chitin-glucan also helps eliminate undesirable compounds such as metals and mycotoxins such as ochratoxin A (Table 1) Both the EU and USA permissions refer to the ability of chitin-glucan to remove metals and mycotoxins or micro-organisms from wine or wine must. The sediments that contain the chitin-glucan are removed from the must or wine at the end of the treatment by physical separation processes such as racking, centrifugation and/or filtration.

2.2 Technological justification

Test results for removing heavy metal and organic contaminants by chitin-glucan in wine were provided in the Application (Bornet and Teissedre 2008).

Red, white and sweet wines were spiked with either:

- iron (20 mg/L)
- lead (500 µg/L)
- cadmium (20 µg/L)
- organic contaminant- ochratoxin A (5 µg/L).

The wines were then treated with chitin-glucan at concentrations of 0.1 g/L, 0.5 g/L and 2 g/L. After 2 days, the levels of iron, lead and cadmium were measured. The average reduction of metals and ochratoxin A for each wine type is shown in Table 1.

Table 1: Average percentage reduction of metals and ochratoxin A in various wine types when treated with chitin-glucan.

Wine type	Average percentage reduction of contaminants in wines			
	Iron	Cadmium	Lead	Ochratoxin A
Red	81.5	43.0	53.5	70.0
White	61.5	12.0	57.5	58.9
Sweet	70.5	21.0	61.0	34.8

The reduction of these metals and organic contaminants through the use of chitin-glucan also improves the visual clarity of wines.

2.3 Chemical properties

Description

Chitin-glucan, the main component in the cell walls of the mycelium of *A. niger* is a purified ingredient in powder form, composed largely of two polysaccharides linked covalently to form a three-dimensional network:

- chitin, composed of repeating units of N-acetyl-D-glucosamine

- Beta (1,3) glucan, composed of repeating units of D-glucose

Chemical name:

Chitin-glucan

Common names:

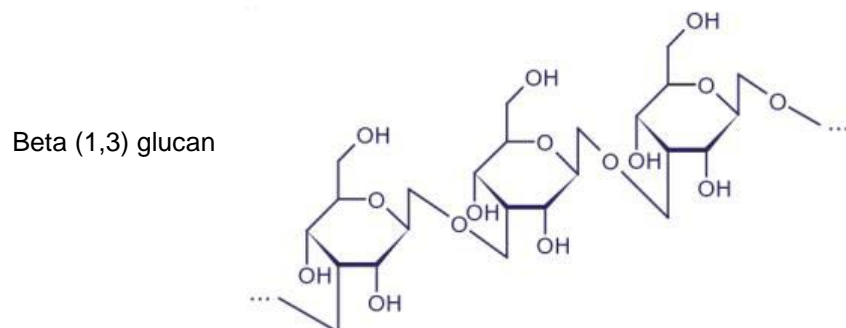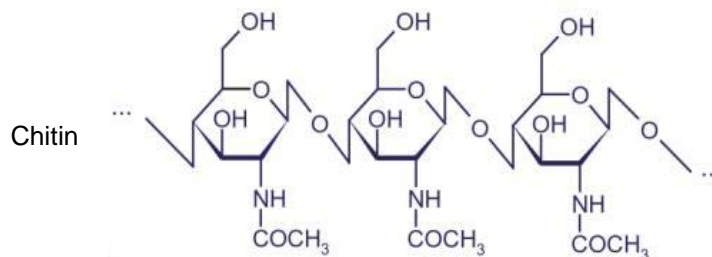Poly(N-acetyl-D-glucosamine)-poly(D-glucose) and 1,3-ß-glucan

CAS registry numbers:

Chitin (1398-61-4)

Beta (1,3) glucan (9041-22-9)

Chemical formula:

$[C_6H_{10}O_5]_m$ - $[C_8H_{13}NO_5]_n$

Structural formulas:

Chitin

Beta (1,3) glucan

2.4 Analytical method for detection

As chitin-glucan is insoluble in alcoholic beverages, the precipitates it forms with unwanted components in these beverages during processing require removal via filtration or similar processes. Tests to determine any residual chitin-glucan in wines are identified by the OIV in OIV INTERNATIONAL OENOLOGICAL CODEX Chitin-Glucan COEI-1-CHITGL: 2009.

2.5 Manufacturing process

Chitin-glucan is obtained from the mycelium of non-genetically-modified strains of *A. niger*, a microorganism employed in the food and pharmaceutical industries to produce citric acid. It is also used to produce a number of enzymes including α-amylase, cellulose, amyloglucosidase, catalase, glucose oxidase, lipase and pectinase.

Preparation of chitin–glucan from the biomass of *A. niger* is based on the alkali treatment of the biomass with NaOH solution (2.5%) at ambient temperature overnight. This is followed by an aggressive alkali treatment with concentrated NaOH (40–45%) at 130°C for 4–6 hours. The resultant white powder contains 32% polyaminosaccharide (chitin) and 15-20% glucan.

Other methods for chitin-glucan extraction from the biomass of *A. niger* involve alkaline extraction to remove the proteins and alkali-soluble polysaccharides. The purity of the product must be equal to or higher than 95 % as stipulated in the OIV specification.

2.6 Product specification

Any supplier of chitin-glucan would need to meet any specification set out in the Code, including the requirements set out in the OIV INTERNATIONAL OENOLOGICAL CODEX Chitin-Glucan COEI-1-CHITGL: 2009, which is one of the secondary references for specifications in S3—3(j) of the Code.

2.7 Food technology conclusion

FSANZ has concluded that the use of chitin-glucan is effective in reducing metals and a chosen organic contaminant in wine, as demonstrated by analysis of wines provided in the application. Chitin–glucan was shown to significantly reduce levels of iron, cadmium, lead and the organic contaminant, ochratoxin A, in various wine varieties. When added in a quantity and a form consistent with delivering the stated purpose, chitin-glucan is technologically justified for use as a processing aid for wine. It is removed before bottling and does not perform a technological purpose in the final food. Chitin–glucan will be permitted for use at good manufacturing practice (GMP) levels, which is the lowest possible level necessary to accomplish a desired effect. A specification for chitin-glucan which meets the requirements of the Code is provided by the OIV.

3 Hazard assessment

3.1 Toxicokinetics

It is not known whether chitin or beta-glucan is released from the chitin-glucan copolymer in the gastrointestinal tract following oral exposure (Jonker et al. 2010). The chitin-glucan copolymer itself, and chitin, are insoluble in water.

Only two chitinases have been identified in mammals, chitotriosidase and acidic mammalian chitinase (AMCase). Chitotriosidase is mainly secreted by macrophages and acts against chitin-containing parasites. AMCase has been identified in mice, rats, insectivorous bats, cynomolgus macaques (*Macaca fascicularis*) and humans.

It is highly expressed in stomach and lung, which is interpreted as indicating a dual digestive and immunological function. Chitinolytic activity in the gastrointestinal tract may also originate from enzymes produced by microorganisms. It is not known whether the level of AMCase activity in the human stomach is sufficient to effectively digest chitin associated with insects and crustaceans (Paoletti et al. 2007; Khousab and Yamabhai 2010; Strobel et al. 2013).

Chitosan, the deacetylated form of chitin, is soluble in water, particularly under acidic conditions, and would therefore be expected to be soluble in wine. Hydrolysis of chitosan could occur in the acid conditions of the stomach, and would produce chito-oligosaccharides (COS), of which smaller molecules would be absorbed from the gastrointestinal tract. Muanprasat and Chatsudthipong (2016) reviewed studies of the passage of COS through Caco-2 cell monolayers. COS of lower molecular weights, in the range 13 and 22 kDa, could pass through Caco-2 monolayers but those with a molecular weight of 230 kDa could not.

COS that are absorbed are principally distributed to the liver, spleen and kidneys, with some distribution to heart and lung. COS are degraded by lysozyme in plasma, liver, kidneys and urine, and are excreted in urine (Muanprasat and Chatsudthipong 2016).

The safety of chitin, chitosan, COS and beta-glucan are therefore relevant to this hazard assessment. Breakdown of beta-glucan would produce glucose, which is not of concern. FSANZ has approved chitosan from *A. niger* as a wine processing aid in 2013 (application A1077), and assigned an acceptable daily intake (ADI) of 'not specified'. Therefore, for this assessment, literature review regarding the safety of chitosan has been limited to studies published since 2012.

3.2   Genotoxicity studies

The genotoxicity of COS produced by depolymerisation of chitosan was assessed by Qin et al. (2006). COS differ from chitin and chitosan in that those of low molecular weight are well absorbed from the gastrointestinal tract. COS are therefore the logical compounds on which to perform genotoxicity assays. It may be reasonably concluded that if COS are not mutagenic or genotoxic, then the large, insoluble polymers from which they are derived would not be mutagenic or genotoxic either. On the basis of the reverse bacterial mutation assay, mouse micronucleus test and mouse sperm abnormality test, it was concluded that COS are not mutagenic or genotoxic.

3.2.1   *Reverse bacterial mutation assay (Ames test) of COS*

Salmonella typhimurium strains used for this assay (Qin et al. 2006) were TA97, TA98, TA100 and TA102. Bacteria were exposed to COS, dissolved in distilled water, at concentrations of 0.5, 5, 50, 500 and 5000 µg/plate, with and without S9 fraction metabolic activation system. Each assay was performed in triplicate. Negative (water and DMSO) and positive (1-aminofluorene, fenaminosulf, sodium azide and 1,8-dihydroxyanthraquinone) control assays were run simultaneously in duplicate. There was no significant increase in the number of revertant colonies in any of the four *S. typhimurium* strains, with or without S9 fraction, at any concentration of COS. The positive control articles caused an approximately threefold increase in the number of revertant colonies.

3.2.2   *Micronucleus test in mice*

Kunming strain mice, ranging in body weight from 25 to 30 g, were randomised to five groups, 5/sex/group (Qin et al. 2006). Mice were gavaged with COS, dissolved in distilled water, at 0, 1250, 2500 or 5000 mg/kg bw, in two divided doses 24 hours apart. The fifth group was the positive control group, and mice in this group were administered cyclophosphamide, 40 mg/kg in physiological saline, by IP injection. All mice were killed 6 h

after the final dose, and marrow cells from the sternum were fixed in methanol, stained with Giemsa stain and examined. The frequency of micronuclei was counted by examination of 1000 polychromatic erythrocytes/mouse.

The frequencies of micronuclei were not significantly different between the groups treated with COS and the negative control group, as determined by chi-square test. However, the frequencies of micronuclei in the positive control group were significantly increased.

3.2.3   *Sperm abnormality test in mice*

Male Kunming mice, weighing 27-35 g, were randomly assigned to five groups (5/group) (Qin et al. 2006). Mice were gavaged daily for five days with 0, 1250, 2500 or 5000 mg/kg bw/day COS in distilled water. The fifth group was the positive control group and was treated with 40 mg/kg bw cyclophosphamide in physiological saline by intraperitoneal injection. Mice were killed on the 35th day after the first dose, and epididymal smears were prepared, fixed with methanol and stained with 1% eosin. Sperm head morphology was assessed in 1000 sperm/mouse. The frequencies of sperm abnormalities in the three groups treated with COS were not significantly different to that of controls. In the positive control group, the frequency of sperm head abnormalities was significantly higher than in the negative control group or in the COS-treated groups.

3.2.4   **Other studies**

EFSA (2010) reviewed a GLP study report of a bacterial reverse mutation assay (Ames test) of a commercial chitin-glucan preparation with the commercial name KiOfine. KiOfine was non-mutagenic, with or without S9 fraction. EFSA (2010) also cited studies that showed a lack of genotoxicity of some compounds similar to chitin-glucan; 6-O-carboxymethylchitin-glucan and beta-glucan from barley.

3.3   **Animal studies**

3.3.1   *General toxicology studies on chitin-glucan*

13-week dietary study of chitin-glucan in rats

The test article for this study (Jonker et al. 2010) was chitin-glucan (94% purity; chitin: beta-glucan 30:70) derived from the mycelia of *A. niger*. Wistar rats, 20/sex/group, were individually housed under standard laboratory husbandry conditions. Rats were approximately six weeks old at the start of treatment. Chitin-glucan concentrations in the diet were 0, 1%, 5% and 10% w/w. Nutrient levels in the basal diet were in excess of requirements and allowed for 10% dilution. Food and water were provided *ad libitum*.

During the study rats were subject to daily clinical observations and weekly detailed examinations. In Week 13, 10 rats/sex/ group were subject to functional observational battery and motor activity assessment. Body weights and feed consumption were determined weekly. Water consumption was measured daily for 5-day periods during weeks 1, 6 and 12. All rats on study were subject to ophthalmological examination pre-study and during Week 13. Standard haematology and clinical chemistry parameters were determined for 10 rats/sex/group on Day 8, approximately mid-study and at scheduled termination. Urine was collected from the same rats during Week 13 for urinalysis and examination of sediment. At scheduled termination, adrenals, brain, full and empty caecum, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid with parathyroids, and uterus were weighed. A comprehensive list of organs was preserved for histopathology.

Analysis showed that actual chitin-glucan contents of the feed were close to target

concentrations and that the test article was homogeneously distributed in the feed. The overall mean daily intake of chitin-glucan in the 10% group was 6.6 g/kg bw in male rats and 7.0 g/kg bw in female rats. In the 5% group, the mean daily intakes were 3.2 g/kg bw in males and 3.4 g/kg bw in females. In the 1% group, mean daily intakes were 0.63 g/kg bw in males and 0.68 g/kg bw in females.

There were no treatment-related effects on clinical observations, motor activity, or performance during the functional observational battery, and no treatment-related ophthalmological findings. There were no significant differences in mean body weight. Rats in the 10% group consumed slightly more feed than rats in the control group. Water consumption was increased in males at all dose levels and in females in the 5% and 10% groups, although the difference from controls was not always statistically significant. No treatment-related changes were found in haematology, clinical chemistry, urinalysis or urine sediment.

Weights of full and empty caeca were significantly increased in males consuming ≥ 5% chitin-glucan and in females consuming 10% chitin-glucan, but there were no histopathological changes in the caecal walls. Other organ weights showed no effects of treatment. There were no gross or microscopic changes attributable to consumption of chitin-glucan in any organs.

Caecal enlargement is a common response in rats to a diet containing a large amount of carbohydrate that is poorly digested or absorbed. It is not considered to be a change of toxicological concern. Similarly, the higher food consumption was not regarded as toxicologically significant because it was a predictable response to a diet with a lower energy density than that of controls. The increased water consumption was not associated with any renal dysfunction or disease state associated with polydipsia, such as diabetes, and was therefore not considered to be an adverse effect.

It was concluded that the highest concentration in the diet, 10% w/w chitin-glucan, equivalent to doses of 6.6 and 7.0 g/kg bw/day in male and female rats respectively, was the no observed adverse effect level (NOAEL) in this study.

12-week dietary study of chitin-glucan in hamsters

This study (Berecochea-Lopez et al. 2009) was designed as an efficacy study rather than a toxicology study, although the parameters measured include some pertinent to toxicology.

Male Syrian golden hamsters, 12/group, were randomly assigned to three treatment groups. They were maintained under standard laboratory husbandry conditions and provided with food and water *ad libitum*. The diet was a hyperlipidaemic diet with a cholesterol content of 0.5% and a supplement of 15% lard at the expense of starch and sucrose. No selenium, vitamin C or vitamin E was added to the diet to moderate oxidative processes. The control group was gavaged with water daily, while the treatment groups were gavaged with suspensions of chitin-glucan in water, at either 21.4 mg/kg bw/day or 42.8 mg/kg bw/day. The doses of chitin-glucan were selected as being equivalent to 1.5 or 3.0 g/day for a 70 kg human being.

Food consumption was measured daily. At the end of the 12-week treatment period, hamsters were fasted overnight, bled by cardiac puncture, and killed. The liver was perfused with 0.15 mol/L KCl, *in situ*, removed, weighed, and sectioned. The liver was further processed for histopathology and for assays of glutathione peroxidase activity, superoxide dismutase activity, malondialdehyde, and cytosolic protein concentration. The left ventricle of the heart was collected for measurement of superoxide anion production. The thoracic aorta was removed, cleaned and weighed. Sections of aorta were analysed for cholesterol

concentration, while the aortic arch was processed for histopathology.

Treatment with chitin-glucan had no effect on food consumption. Feed conversion efficiency and weight gain were reduced 35% and 30%, respectively, in the 42.8 mg/kg bw/day group, but there were no effects on these parameters observed in the 21.4 mg/kg bw/day group. There were no adverse clinical observations in hamsters gavaged with chitin-glucan. Treatment with chitin-glucan had no effect on plasma total cholesterol or HDL-C, but plasma triglyceride was significantly decreased by treatment. Group mean plasma triglyceride of the 21.4 mg/kg bw/day group was decreased 20% relative to that of controls, while that of the 42.8 mg/kg bw/day group was decreased 39% relative to that of controls. Group mean superoxide anion production in the heart was significantly decreased (25% lower), relative to that of controls, in the 42.8 mg/kg bw/day group but not the 21.4 mg/kg bw/day group. Aortic cholesterol and aortic fatty streak accumulations were significantly decreased by feeding chitin-glucan. Both dose levels of chitin-glucan also significantly increased liver glutathione peroxidase activity and superoxide dismutase activity, relative to those of controls, and significantly decreased concentration of thiobarbituric acid reactive substance (assumed to be malondialdehyde). These effects were all considered to be beneficial rather than adverse.

While this study was primarily designed to show efficacy, it can be concluded that the NOAEL for chitin-glucan in the Syrian golden hamster is 42.8 mg/kg bw/day.

3.4   Other studies

EFSA (2010) reviewed an unpublished 28-day rat dietary study of chitin-glucan, conducted in compliance with GLP. No adverse effects were observed at the highest dose level, 10% of chitin-glucan in the feed, equivalent to 8 g/kg bw/day.

A 13-week subchronic toxicity study of chitin in F344 rats was published by Niho et al. (1999) but the text is in Japanese, with the exception of the abstract, figures and tables. The authors concluded that chitin was nontoxic at dietary concentrations up to 5%, the highest dose tested.

Rodent studies of COS were recently reviewed by Muanprasat and Chatsudthipong (2016). The acute oral LD_{50} for COS in mice is >10 g/kg bw. Oral administration of COS to mice for 50 days, at up to 500 mg/kg bw/d, had no adverse effects. A 90-day dietary study of COS in rats identified a NOAEL of 0.2% w/w, equivalent to 124 mg/kg bw/day in males and 142 mg/kg bw/day in females. The NOAEL was based on dermal inflammation of the forelimbs at 1% w/w COS in the diet. The mechanism of this effect was unknown (Muanprasat and Chatsudthipong 2016).

3.4.1   28-day gavage study of COS in rats

Sprague-Dawley rats, 9/sex/group, were individually housed under standard laboratory husbandry conditions (Kim et al. 2001). Rats were approximately 5 weeks old at the start of treatment. Rats were gavaged daily at a volume of 10 mL/kg with 0, 500, 1000 or 2000 mg/kg bw COS, of unspecified purity. Recorded parameters during the in-life phase were daily clinical observations, daily food consumption, and weekly body weight. Urine and blood were collected at the end of the study for urinalysis, haematology and serum chemistry, and rats were subject to gross necropsy. Liver, lung, kidney, spleen and gonad weights were recorded, and organs were preserved for histopathology. No treatment-related differences in survival or in-life parameters were found. There were no treatment-related effects on urinalysis or serum chemistry results. The male rats in the 2000 mg/kg bw/day group had an increase in total leucocyte count, relative to controls, that was statistically significant but which remained within normal biological range. No similar elevation was found in the female rats. Treatment had no effect on organ weights or histopathological findings. In

conclusion, the NOAEL of COS was the highest dose, 2000 mg/kg bw/day.

3.4.2 *Acute and 91-day oral gavage studies of a yeast-derived beta-glucan preparation in rats*

The test article in these two studies (Babíček et al. 2007) was a (1,3/1,6)-Beta-D-glucan extract from *Saccharomyces cerevisiae*, containing >75% w/w beta-glucan 1,3/1,6 and >80% w/w carbohydrate. The test article, identified as WGP® 3-6, was a powder and was administered to rats as a freshly-prepared suspension in sterile water.

For the acute study, Brl-Han: WIST@Jcl rats, 5 weeks old on receipt, were acclimated for a week under standard laboratory husbandry conditions before being assigned to control and treatment groups, 5 rats/sex/group. Rats were group-housed, 5/cage. Rats in the treatment group were dosed by oral gavage with WGP® 3-6 in suspension at 2000 mg/kg bw (20 mL/kg bw). Control rats were dosed with water at the same volume, 20 mL/kg bw. Rats were observed for 14 days. Bodyweights were recorded prior to dosing and on days 7 and 14. All rats survived to the end of the study and there were no differences between the control and treatment groups in clinical observations, bodyweight gain or findings on gross necropsy. It was concluded that the acute oral LD_{50} for WGP® 3-6 in rats was >2000 mg/kg bw.

Fischer-344 rats were used for the subchronic study. Rats were group-housed, 5/cage, under standard laboratory husbandry conditions and randomly assigned by weight to groups treated by oral gavage with WGP® 3-6 at 0, 2, 33.3 or 100 mg/kg bw/day for 91 days. The low-and mid-dose groups comprised 10 rats/sex/group. The control and high-dose groups comprised 20/sex/group, of which 10 rats/sex/group were assigned to recovery cohorts for a 14-day recovery phase.

Rats were 5-6 weeks old at study start. Clinical observations included twice-daily morbidity/mortality check, daily cage-side observations, and a weekly detailed clinical examination. Other in-life assessments were ophthalmic examination pre-study and prior to scheduled termination, weekly body weight measurement, weekly food consumption measurement, and functional and behavioural investigations which were conducted at an unspecified time near the end of the study. These included beam walking test, bar grip test, open field test and placing response assessment. Rats in the main study cohorts were killed following blood collection on Day 92 while rats in the recovery cohorts were killed following blood collection on Day 106. Blood was used for standard haematology and serum chemistry. All rats were subject to a full necropsy. Fresh weight was determined for liver, brain, heart, kidneys, spleen, adrenals, testes, epididymides, uterus, ovaries and thymus. Histopathology was conducted on a comprehensive list of tissues.

All rats survived to their scheduled termination, and treatment with WGP® 3-6 had no effects on clinical observations, ophthalmology findings, food consumption, bodyweight gain, neuromuscular function or behaviour. Statistically significant increases in group mean RBC, MCV and Hct, and decrease in group mean clotting time, in 100 mg/kg bw/day males on Day 92 remained within historical control ranges and were not observed in females. The authors noted that some unusually long clotting times occurred in males in the control group. Some clinical chemistry parameters also showed statistically significant but biologically minimal changes but there was no dose-response relationship evident. There were no treatment-related effects on findings at gross necropsy, organ weights, organ weight ratios or findings on histopathology. The authors concluded that the NOAEL for WGP® 3-6 in this study was 100 mg/kg bw/day, the highest dose tested.

3.4.3 28-day dietary study of barley beta-glucan in rats

The test article in this study (Jonker et al. 2010) was a high-purity (75.6%) Beta-glucan

preparation with the trade name Glucagel[TM]. Wistar rats, 5/sex/group, were group-housed under standard laboratory husbandry conditions and fed commercial rodent diet to which was added 0, 1, 5 or 10% Glucagel[TM]. The control diet itself contained approximately 0.8% Beta-glucan. Rats were observed daily for clinical signs, and were subject to detailed clinical assessment, including neurobehavioural assessment, weekly. A functional observational battery and motor activity assessment were conducted on days 22 through 23 of the in-life phase. Body weights were recorded weekly, and food consumption was estimated by weighing the feeders every 3 to 4 days. Water intake was also estimated, by weighing water bottles every 5 days. Rats were subject to ophthalmological assessment pre-study and this was repeated in week 4 for rats in the control and 10% Glucagel[TM] groups. Urine was collected overnight on Day 24. At the end of the study rats were anaesthetised for blood collection from the abdominal aorta, followed by termination by exsanguination. Rats were subject to gross necropsy and weights of adrenal, brain, full and empty caecum, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus and uterus, as applicable by sex, were recorded. The weighed organs were preserved for histopathology, in addition to aorta, colon, eyes, gut-associated lymphoid tissue, axillary and mesenteric lymph nodes, lungs, mammary gland (female), peripheral nerve, oesophagus, oviducts, pancreas, parathyroids, pituitary, prostate, rectum, salivary glands, seminal vesicles, small intestine, spinal cord, sternum, stomach, thyroid, trachea, urinary bladder and vagina, as sex-appropriate.

All rats survived to scheduled termination, and there were no treatment-related clinical effects noted on daily clinical assessment, weekly detailed clinical examination, functional observational battery or motor activity assessment. Treatment had no effect on ophthalmologic findings. There were no treatment-related effects on body weight, feed consumption or water intake. The group mean daily intake of the 10% Glucagel[TM] group was 7.7 g Glucagel[TM] /kg bw/day for male rats and 7.8 g Glucagel[TM] /kg bw/day for female rats, equivalent to 5.8 to 5.9 g beta-glucan/kg bw/day. No treatment-related changes were found in haematology. Compared to male controls, male rats in the 10% Glucagel[TM] group had significantly lower group mean values for plasma total cholesterol (88% of control value) and phospholipids (88% of control value), and significantly higher group mean urea (125% of control value). The group mean urea remained within normal range and there were no associated effects on other measures of renal function such as plasma creatinine, urinalysis, kidney weights or histopathology of the kidneys. The group mean weight of empty caecum was increased in male rats consuming ≥ 5% Glucagel[TM], and showed a dose-response relationship. The group mean weight of the empty caecum of the females in the 10% Glucagel[TM] group was approximately 40% higher than that of female controls, but this difference was not statistically significant. Consumption of Glucagel[TM] was not associated with gross or microscopic lesions in either sex.

Increase in caecal weight is a common response in rats to consumption of poorly digestible carbohydrate and is considered to be a physiological adaptation. Decreased concentration of serum cholesterol is a well-known effect of Beta-glucan in animals. The decreased plasma lipid levels in male rats were not accompanied by any adverse findings and were not considered to be toxicologically relevant. The NOAEL for dietary Glucagel[TM] in this study was therefore the highest dose level, 10% Glucagel[TM], equivalent to 5.8 to 5.9 g beta-glucan /kg bw/day.

3.5 Human Studies

3.5.1 6-week randomised controlled trial of chitin-glucan

This efficacy study (Bays et al. 2013) was a double-blind, placebo-controlled study of chitin-glucan derived from *A. niger* mycelia. The study was conducted at three different geographical sites. To be eligible to participate, subjects had to be generally healthy, aged

between 21 and 70, have a body mass index (BMI) between 18.5 and 34.9 kg/m^2, have fasting serum levels of LDC-C between 3.37 and 4.92 mmol/L, and be willing to take a supplement three times daily for 6 weeks. Grounds for exclusion included diabetes mellitus, cancer, gastrointestinal disease, cardiovascular disease, pregnancy, lactation, use of lipid altering drugs or supplements within 4 weeks of screening, > 5% change in bodyweight within 4 weeks of screening, or a known hypersensitivity or intolerance to fibre or fibre-containing products. Eligible subjects were randomised to receive one of four treatments: 4.5 g/day chitin-glucan, (n=34), 1.5 g/day chitin-glucan (n=33), 1.5 g/day chitin-glucan plus 135 mg/day olive extract (n=33) or rice flour as placebo (n=35). The treatments were administered in capsules and subjects were instructed to take three capsules three times a day, with water before each main meal, for 6 weeks. Pre-study blood testing included TSH, complete blood count and fasting serum triglycerides, LDL-C, total cholesterol and HDL-C. Subjects were asked to follow a maintenance diet regimen from two weeks prior to the start of treatment, through to the end of the study. No other supplements were used during the study.

Assessments at study start, week 4 and week 6 included body weights, blood pressure, fasting serum lipids, oxidised LDL, insulin, and urinary F2-isoprostane. Subjects were interviewed on adverse events, compliance with treatment and adherence to the diet.

Chitin-glucan for 6 weeks was generally well tolerated. Although some participants reported mild to moderate gastrointestinal complaints, there were no significant differences in the frequencies between any of the three treatment groups and the placebo control group. One subject taking 4.5 g chitin-glucan/day reported worsening of gastrointestinal reflux and one subject taking chitin-glucan and olive extract reported two events of increased frequency of bowel movements and excessive gas. There were no significant treatment-related changes in heart rate, body weight, haematology, clinical chemistry, or systolic or diastolic blood pressure. Administration of 4.5 g/day chitin-glucan significantly reduced oxidised LDL in serum, compared to placebo. The other two interventions had no significant effect on serum oxidised LDL. Supplementation with chitin-glucan did not significantly affect serum levels of total cholesterol, HDL-C, triglycerides, glucose, or insulin, and also had no effect on urinary F2-isoprostanes.

The mild to moderate gastrointestinal disturbances documented in this study are not considered to be adverse. Although this was not intended to be a study of safety or tolerance, it may be concluded from this study that 4.5 g/day chitin-glucan is not associated with any adverse effects in healthy human volunteers.

3.5.2   90-day single-blind, placebo-controlled randomised clinical study of chitosan

This study (Trivedi et al. 2016) was an efficacy study, investigating the efficacy of chitosan of fungal origin for weight reduction, but also assessed human tolerance of chitosan. 96 healthy adults, aged between 18 and 65 years and with BMI between 26 and 35 inclusive, were enrolled in the study. The chitosan used in the study was derived from *A. niger*. Chitosan was administered in capsules of 500 mg chitosan/capsule, and placebo was administered in identical capsules. Of the 96 subjects, 64 were randomised to the chitosan group and 32 to the placebo group. Participants were instructed to take one capsule in the morning, two 15 min prior to lunch, and two 15 min prior to dinner. Participants kept a written record of the number of capsules taken and time they were taken. Participants also kept a food diary, and were instructed to maintain their regular dietary habits. Participants visited their study centre prior to commencement of the study, on Day 1 of study, halfway through the study (Day 45 ±2) and at the end of the study (Day 90 ±3). Parameters measured at these visits including anthropometric determinations (upper abdominal circumference, hip circumference, waist circumference, waist to hip ratio), body composition measurements (BMI, body fat, visceral fat, muscle mass), vital signs (radial pulse, blood pressure, respiratory rate, body

temperature), glycated haemoglobin and selected serum lipid (triglyceride, HDL, LDL, VLDL) and biochemistry (urea, serum creatinine, ALT, AST) data.

Of the original enrolees, 86, 56 in the chitosan group and 30 in the placebo group, completed the study. Participants in the chitosan group experienced a statistically significant group mean weight loss of 3.24 kg relative to their weight at the start of the study, while those in the placebo group showed a minimal group mean weight loss of 130 g, which was not statistically significant. Only 4 participants in the chitosan group did not lose weight. Caloric intake did not change significantly in either group. Mean BMI decreased significantly in the chitosan group. Chitosan was associated with a significant decrease in glycated haemoglobin in participants who had a high glycaemic index at study start, while not affecting the glycaemic index of those who had normal glycaemic index at study start. There were no treatment-related effects on serum lipids or other serum biochemistry data. There were no treatment-related adverse events. Chitosan of fungal original was found to be safe and well-tolerated by all study participants.

3.5.3   Assessments by other regulatory agencies

The US FDA accepted a GRAS notice for chitin-glucan from *A. niger* on January 5, 2012, and designated it GRAS Notice No. GRN 000412. The GRAS notice was filed by KitoZyme S.A. and stated that chitin-glucan is GRAS for use in microbial stabilization, removal of contaminants, and/or clarification in alcoholic beverage production at levels between 10 and 500 g/hL (0.1 and 5 g/L).

A toxicological-hygienic certificate has been issued for chitin-glucan as a food additive by the State Committee for Health and Epidemiological Supervision of the Russian Federation (Kulev and Negrutsa 2015).

The EFSA Panel on Dietetic Products, Nutrition and Allergies published a scientific opinion on the safety of "KiOnutrime-CG™", a product containing more than 90% chitin-glucan, in 2010. The Panel concluded that the product is safe as a food ingredient at a proposed intake of 2 to 5 g/day (EFSA 2010).

3.6   Hazard assessment conclusions

Chitin-glucan is a permitted processing aid in the EU and is for sale in a number of countries with which the EU has wine agreements.

Chitin-glucan is a natural copolymer, composed of the polymers chitin and 1,3-beta-glucan. It is not known whether chitin-glucan can be broken down in the human gastrointestinal tract. If some breakdown is possible, the potential products could include chitin and B-glucan. Chitin could be deacetylated in the acidic environments of wine or the human stomach to chitosan, and chitosan could be hydrolysed in the gastrointestinal tract to COS. While systemic absorption of polymers and large molecules is negligible, COS of relatively low molecular weight could be absorbed. Breakdown of beta-glucan would produce glucose. Although the estimated intake of chitin-glucan through consumption of wine is negligible, evidence concerning the safety of the potential breakdown products, other than glucose, has been included in this assessment. Assessment of chitosan is limited to papers more recent than FSANZ's assessment of chitosan in 2013.

Results of genotoxicity assays of COS, including reverse bacterial mutation assay, mouse micronucleus test, mouse sperm abnormality test, are negative. Lack of genotoxicity has also been demonstrated for 6-O-carboxymethylchitin-glucan and beta-glucan.

No adverse effects of chitin-glucan, chitin or beta-glucan have been demonstrated in

subchronic studies in experimental animals at the highest doses tested. In a 28-day dietary study of chitin-glucan in rats, this was equivalent to 8 g/kg bw/d, while in a 13-week dietary study the highest doses were 6.6 g/kg bw/day in male rats and 7.0 g/kg bw/day in female rats. In Syrian golden hamsters, a 12-week dietary study identified the highest dose 42.8 mg/kg bw/d, as the NOAEL. Chitin was nontoxic in rats at dietary concentrations up to 5% for 13 weeks. The NOAEL for beta-glucan in a 28-day dietary study in rats was equivalent to 5.8 to 5.9 g/kg bw/d, while in a 91-day oral gavage study in rats it was 100 mg/kg bw/day, the highest dose tested.

Adverse effects of COS in a subchronic dietary study in rats were limited to dermal inflammation of the forelimbs at 1% w/w COS in the diet. The NOAEL was identified as the next lower concentration, 0.2% w/w, equivalent to 124 mg/kg bw/day in males and 142 mg/kg bw/day in females. The localised nature of the dermal lesions observed at the highest dose suggest that they are due to grooming of the coat, and therefore not relevant to human beings.

There were no adverse effects observed or reported in a 6-week randomised controlled trial of chitin-glucan at up to 4.5 g/day in healthy human volunteers. Similarly, in a 90-day trial of 2.5 g/day chitosan, no adverse effects were observed or reported in healthy human volunteers.

Based on the reviewed toxicological and human data, it is concluded that in the absence of any identifiable hazard, an ADI 'not specified' is appropriate for chitin-glucan.

4 Dietary exposure assessment

A dietary exposure assessment was not conducted because residual levels of chitin-glucan and its degradation products in wine are expected to be negligible.

5 Risk assessment conclusion

The use of chitin-glucan as a processing aid for the production of wine, as proposed in this Application, raises no public health and safety concerns.

Part B: PVI/PVP Co-polymers (Polyvinylimidazole-Polyvinylpyrrolidone)

1 Introduction

1.1 Description

Polyvinylimidazole-polyvinylpyrrolidone (PVI/PVP co-polymers) is an insoluble adsorbent resin used in winemaking with specificity for metals such as copper and iron.

PVI/PVP co-polymers function to:

- form precipitates in alcoholic beverages to reduce undesirably high concentrations of:

    - naturally occurring metal cation contamination in the must or contamination through must or wine treatment from winemaking equipment
    - copper enrichment following the treatment of wines by copper sulphate
    - iron, which forms complexes with tannins and phosphates, causing haze formation
    - aluminium.

- prevent the destruction of varietal thiols, thereby stabilising the flavours present in several white varietals such as Sauvignon Blanc and certain red wines
- prevent enzymatic and non-enzymatic browning reactions which are responsible for the undesirable brown discoloration of white and rosé wines
- prevent hazes and "pinking"[2] in wines

The technological purpose PVI/PVP co-polymers are as decolourants, clarifying, filtration and adsorbent agents. The precipitates formed by them in wine are removed via filtration or similar processes prior to bottling.

1.2 Current permissions

1.2.1 OIV resolutions and other OIV documents

Resolutions:

- OIV/OENO 1/2007 (Adsorbent copolymer treatment PVI/PVP (musts chapter)
- OIV/OENO 2/2007 (Adsorbent copolymer treatment PVI/PVP (wines chapter)
- OIV/OENO 262-2014 (PVI/PVP adsorbent copolymers – Codex)

International Oenological Codex:

- COEI-1-PVIPVP:2014, OENO 262/2014 (Adsorbent copolymers of polyvinylimidazole/polyvinylpyrrolidone (PVI/PVP))

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[2] Pinking may be a result of highly reductive conditions experienced during processing. When the wine is subsequently exposed to air the wine becomes pink.

1.2.2 European Union

Under EC Regulation 606/2009, PVI/PVP is permitted for reducing the copper, iron and metal content with the requirements being:

- Purpose:
    - to reduce excessively high concentrations of metals and to prevent defects caused by this excessively high content, such as ferric casse, through the addition of co-polymers that adsorb these metals.

- Requirements:
    - The added co-polymers must be eliminated by filtering within two days at most of their addition to the wine
    - In the case of musts, the co-polymers must be added no earlier than two days before filtering
    - The treatment is to be carried out under the responsibility of an oenologist or qualified technician
    - The adsorbent co-polymers used must comply with the requirements of the International Oenological Codex published by the International Organisation of Vine and Wine, especially as regards the maximum monomer content.

1.2.3 United States of America

PVI/PVP is permitted for beverages under Title 21 CFR 173.50 and FDA FCN No. 320. The amount used to treat the wine must not exceed 80 grams per 100 litres of wine.

1.3 Risk assessment questions

1.3.1 Technological function

Is the technological function clearly stated and do PVI/PVP co-polymers achieve that function as a food processing aid in the quantity and form proposed?

1.3.2 Health and safety

Are there any potential public health and safety concerns that may arise from the use of PVI/PVP co-polymers as a processing aid in the manufacture of wine in Australia and New Zealand?

2 Food technology assessment

2.1 Technological purpose

The Applicant has described the function of PVI/PVP co-polymers as insoluble adsorbent resins performing several functions in winemaking (Enartis), compared to PVP alone, namely:

- the specific adsorption of metals such as copper and iron, as well as for aluminium, allowing higher concentrations to be removed, thereby more efficiently preventing hazes in wines

- elimination of metals, such as copper, thus preventing the destruction of varietal thiols present in several white varietals such as Sauvignon Blanc, as well as in certain red wines

- the prevention of browning through the adsorption of phenolic compounds such as 3,4-dihydroxycinnamic acid derivatives, which otherwise enter into enzymatic and non-enzymatic reactions. The products of these reactions are responsible for the undesirable brown discoloration of white and rosé wines.

- prevention of "pinking" of white wines by eliminating catalysers of oxidation such as iron and copper and by reducing the polyphenolic content of wine.

The technological functions provided by the resins are as decolourants, clarifying, filtration and adsorbent agents.

2.2 Technological justification

The applicant provided a study on the influence of PVI/PVP co-polymers on the removal of metals in wines (Mira H, et al. 2007). The following were measured:

- copper (Cu)
- iron (Fe)
- lead (Pb)
- cadmium (Cd)
- aluminium (Al).

The removal of metals was shown to be more effective:

- when PVI/PVP co-polymers were applied to the wine than to the must
- in removal of Fe and Pb in white wines than in red
- in removal of Cu and Al in red wines than in white
- in general, the higher the PVI/PVP dose (figure 1).

In addition to metals removal, the PVI/PVP co-polymers had no significant effect on phenolic composition. Consequently, varying the use concentration did not significantly affect the wine's sensory characteristics regardless of whether PVI/PVP co-polymers were added to the must or to the wine (Mattivi et al 2000).
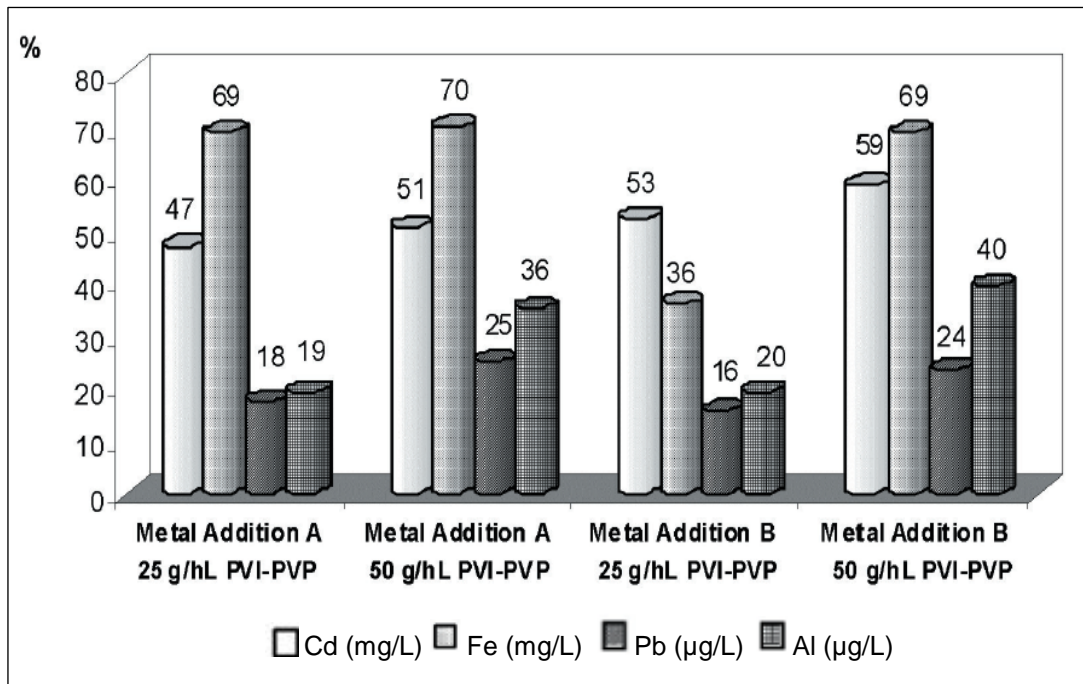
Figure 1. Percentage removal of metals (metal addition A: 5 mg/mL of iron and 0.5 mg/mL of copper; metal addition B: 15 mg/mL of Fe and 1 mg/mL of copper) by PVI/PVP co-polymers

PVI/PVP co-polymers meet the criteria of performing a technological purpose of a processing aid, such that they are used in the course of processing but do not perform a technological purpose in the final food. The precipitates formed by PVI/PVP co-polymers are removed from the wine by physical separation processes such as racking, centrifugation and/or filtration.

2.3   Chemical properties

Description   PVI/PVP consists of two separate monomers cross-linked to
                             form a three-dimensional network known as "adsorbent
                             PVI/PVP co-polymers" or "PVI/PVP co-polymers". They are
                             slightly-hygroscopic powders, insoluble in alcohol, water and
                             in most other liquids and act as fining agents in wines

Chemical name: Polyvinylimidazole-polyvinylpyrrolidone co-polymers
                             (PVI/PVP co-polymers)

Common names: Terpolymer of 1-vinylimidazole, 1-vinylpyrrolidone, and 1,3-
                             divinylimidazolidinone. Cross-linked copolymer of
                             vinylimidazole/vinylpyrrolidone

CAS registry number: N-vinylimidazole (1072-63-5)

(individual monomers)   N-vinyl-2-pyrrolidone (88-12-0)

Chemical formula: N-vinylimidazole ($C_5H_6N_2$)

(individual monomers)   N-vinyl-2-pyrrolidone (C_6H_9NO)

Molecular weight: N-vinylimidazole (94.115 Da)

(individual monomers)   N-vinyl-2-pyrrolidone (111.142 Da)

N-vinylimidazole
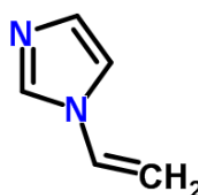
N-vinyl-2-pyrrolidone
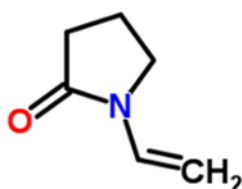
2.4 Analytical method for detection

OIV has developed analytical methods of detection for PVI/PVP co-polymers. In the European Union, treatment with PVI/PVP co-polymers must comply with the purity and identification specifications for authorised co-polymers, published in RESOLUTION OIV-OENO 262-2014 PVI/PVP ADSORBENT CO-POLYMERS – CODEX.

2.5 Manufacturing process

PVI/PVP co-polymers are manufactured by "popcorn" polymerisation; a free-radical mechanism that results in a polymer due to extensive crosslinking.

The monomers involved are:

- N-vinylimidazole   (CAS no. 1072-63-5)

and

- N-vinyl-2-pyrrolidone   (CAS no. 88-12-0)

with a ratio of 9:1 N-vinylimidazole:N-vinyl-2-pyrrolidone. A third monomer, N,N´-divinylimidazolidin-2-one (CAS no. 13811-50) is used as a crosslinking agent at a level of less than 2% by weight of the total amount of the monomers. The resultant product is an adsorbent resin which is insoluble in water, alcohol and in most other liquids. It has a number of functional imidazole groups which connect selectively to the various metals present in wine.

2.6 Product specification

Any supplier of PVI/PVP co-polymers would need to meet the requirements set out in the OIV INTERNATIONAL OENOLOGICAL CODEX PVI/PVP, COEI-1-PVIPVP 1: 2014, which is one of the secondary references for specifications in section S3—3(j).

2.7 Food technology conclusion

Based on a study that shows the efficiency in removing metals from wine and musts, FSANZ concludes PVI/PVP co-polymers to be effective in their role as a processing aid. Removing these metals also reduces the severity of wine spoilage through enzymatic and non-enzymatic reactions, and pinking of white wines. PVP/PVP also preserves the phenolic compounds in wine. At the usage levels permitted in the EU and the USA, PVI/PVP co-polymers are technologically justified for use as a processing aid. Furthermore, as the PVI/PVP co-polymers and the precipitates they form are removed through filtration, they do not perform a technological function in the final food. This means they meet the definition of "used as a processing aid" in the Code. PVI/PVP co-polymers will be permitted for use at GMP levels, which is the lowest possible level necessary to accomplish the intended effect. A specification for PVI/PVP co-polymers which meets the requirements of the Code is provided by the OIV.

3 Hazard Assessment

3.1 Toxicokinetics

Because PVI/PVP is an insoluble copolymer, absorption from the gastrointestinal tract would be negligible.

Analysis of a wine model solution by Schubert and Glomb (2010) after fining with PVI/PVP did not reveal detectable quantities of 2-pyrrolidone, but did reveal 18 µg/L imidazole, which

is a degradation product in wine. Other degradation products for which the wine was analysed were N-vinylimidazole and N-vinyl-2-pyrrolidone. The OIV specification for PVI/PVP sets limits for these and other degradation products; vinylpyrrolidone < than 5 mg/kg, vinylimidazole < 10 mg/kg, divinylimidazolidinone < 2 mg/kg, pyrrolidone < 50 mg/kg and imidazole < 50 mg/kg. Analysis of 140 wines revealed that 2-pyrrolidone is a natural constituent of wine in any case. The immediate precursors of this naturally occurring 2-pyrrolidone are 4-aminobutyramide and 4-aminobutyric acid (Schubert and Glomb 2010). In light of these findings, consideration of the safety of imidazole is included in this hazard assessment.

It is not clear whether the copolymer can be degraded into the polymers in the gastrointestinal lumen. No information was located on whether PVI can be absorbed, but absorption of PVP from the gastrointestinal tract is negligible (Gosselin et al 1976; Robinson et al 1990, as cited by BASF 1998).

3.2 Genotoxicity studies and animal studies

No studies of the genotoxicity of PVI/PVP, or toxicity studies in laboratory animals, were submitted or located.

The Enartis Safety Data Sheet for STABYL-PVI/PVP (copolymer of vinylimidazole and vinylpyrrolidone) states the acute oral LD_{50} in the rat is > 2000 mg/kg (Enartis 2015).

The BASF Safety Data Sheet for 1-vinylimidazole reports that the acute oral LD_{50} in the rat is 1040 mg/kg. Additional information in the Safety Data Sheet includes that 1-vinylimidazole is not mutagenic in bacteria or mammalian cell culture; that it is predicted to be an ocular irritant on the basis of the Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) assay but is not irritating or sensitising to the skin in animals; that repeated doses at high levels may be hepatotoxic; and that it is not a reproductive toxicant but may cause developmental effects at maternally toxic doses (BASF 2016).

Some imidazole derivatives have been found to be mutagenic and genotoxic, but these properties are confined to the nitroimidazoles (De Méo et al. 1992; Ebel et al. 2012).

The acute oral LD_{50} of imidazole in the rat is 970 mg/kg bw (Ebel et al. 2012). Clinical signs in acute poisoning are those of central nervous system stimulation, and include agitation, tremor, ataxia, convulsions and opisthotonus. Ebel et al. (2012) cited an unpublished 28-day repeat-dose oral gavage rat study by BASF that found a NOEL of imidazole of 62.5 mg/kg bw/day. Higher doses, up to 500 mg/kg bw/d, were associated with increased liver weight, which may have been an adaptive change.

3.3 Human Studies

No studies of the safety of PVI/PVP in human beings were submitted or located.

PVP is considered to be practically non-toxic in human beings. The oral lethal dose in humans is estimated to be above 15 g/kg bw.

No studies of the safety of PVI in human beings were submitted or located.

3.4 Assessments by other regulatory agencies

Polyvinylpyrrolidone-vinyl acetate (PVP/VA) copolymer has been assessed by the EFSA Panel on Food Additives and Nutrient Sources added to Food. The Panel concluded that the database is insufficient to establish an ADI. However, the Panel calculated a Margin of

Safety (MOS) from 43 to 120 for adults and from 63 to 175 for children. These MOS were considered to be sufficient, given the lack of absorption of PVP/VA, the fact that the NOAELs were the highest doses tested and that exposure estimates are based on worst case assumptions. From the maximum residual levels of vinyl acetate, Margins of Exposure (MOE) of > 106 were calculated, while the MOE for hydrazine were above 10000. The Panel concluded that the residual levels of hydrazine (up to a maximum of 1.0 mg/kg in the final product) are unlikely to be of safety concern, but considered it would be prudent to lower the level of hydrazine as far as reasonably achievable. The Panel concluded that the use of PVP/VA copolymer in solid food supplements as a binding/coating agent is unlikely to be of safety concern at the proposed uses (EFSA 2010).

PVP has been evaluated by JECFA in 1966, 1973, 1983, and most recently in 1986, and is currently assigned an ADI of 0–50 mg/kg bw (WHO, 1987).

3.5   Hazard assessment conclusion

Absorption of PVI/PVP from the gastrointestinal tract would be expected to be negligible, and information on acute toxicity in the rat indicates that toxicity is very low.

Potential breakdown products of PVI/PVP are PVI, PVP, 2-pyrrolidone and imidazole. There is a lack of information on the potential toxicity of PVI, but animal toxicity data for 1-vinylimidazole and imidazole indicate that they are of low toxicity. PVP is widely used in the pharmaceutical industry and absorption from the gastrointestinal tract is negligible. PVP is considered to be practically nontoxic in human beings. JECFA has set an ADI of 0–50 mg/kg bw for PVP. No information was located to suggest that PVI/PVP or its breakdown products pose a genotoxic hazard. PVI/PVP has been permitted for use in wine in the EU since 2009.

There is insufficient information to establish an ADI for PVI/PVP. However, it is concluded that PVI/PVP does not pose a hazard to consumers under the proposed conditions of use.

4   Dietary exposure assessment

A dietary exposure assessment was not conducted because residual levels of PVI/PVP and its degradation products in wine are expected to be negligible.

5   Risk assessment conclusion

The use of PVI/PVP as a processing aid for the production of wine, as proposed in this Application, raises no public health and safety concerns.

Part C: Ammonium Bisulphite (Ammonium hydrogen sulphite)

1 Introduction

1.1 Introduction

Ammonium bisulphite is a colourless crystal which is soluble in water and added to wine must via an aqueous solution. In its aqueous form, it is used exclusively for the fermentation of grape must. During fermentation ammonium bisulphite breaks down to provide ammonium (NH_4^+) ions which are directly assimilated by the yeast, promoting successful fermentation. These nitrogenous compounds, known as yeast assimilable nitrogen (YAN) are used exclusively for fermentation operations (JECFA 1974). Sulphur dioxide (SO_2) is also produced during the breakdown of the ammonium bisulphite. The SO_2 influences wine stability by inhibiting wild and spoilage yeasts and helps prevent oxidation and preserves the fruity flavour and freshness in wine.

1.2 Current permissions

1.2.1 OIV resolutions and other OIV documents

There are no OIV resolutions or OIV documents on ammonium bisulphite.

1.2.2 European Union

Under EC Regulation 1493/1999, ammonium bisulphite is permitted at levels of up to 0.2 g/L to encourage the growth in yeasts.

1.2.3 United States of America

There is no permission for use in the USA.

1.2.4 Republic of South Africa (RSA)

The RSA permits the use of ammonium bisulphite under the annex 1; list of oenological practices and processes authorised for originating in the Republic of South Africa. Addition of ammonium sulphate or ammonium bisulphite.

1.3 Risk assessment questions

1.3.1 Technological function

Is the technological function clearly stated and does ammonium bisulphite achieve that function as a food processing aid in the quantity and form proposed?

1.3.2 Health and safety

Are there any potential public health and safety concerns that may arise from the use of ammonium bisulphite as a processing aid in the manufacture of wine in Australia and New Zealand?

2 Food technology assessment

2.1 Technological purpose

Ammonium bisulphite has several functions in wine making. When broken down during fermentation it provides ammonium ions and sulphur dioxide (SO_2). The functions, as articulated in the application that each substance provide are:

- ammonium ions
    - a nitrogen source for nourishment of yeast, allowing efficient fermentation
    - influencing wine stability, particularly in white wine.

- sulphur dioxide
    - an antioxidant having a reductive effect. Captures oxygen and prevents oxidation
    - an antioxidase that destroys oxidases and prevents casse
    - an antimicrobial that inhibits the action of wild and spoilage yeasts, lactic and acetic bacteria.

2.2 Technological justification

Ammonium bisulphite is an efficient way to provide nutrition for the yeast during fermentation as it breaks down to nitrogenous ammonium ions, which are valuable nutrients for yeast. A deficiency of nitrogen can lead to slow or stuck fermentation necessitating supplementation to promote a clean and uninterrupted fermentation.

FSANZ noted that sulphur dioxide (SO_2) is produced during the breakdown of ammonium bisulphite. The primary technological function however for ammonium bisulphite is for a yeast nutrient and its use as such is technologically justified. SO_2 also inhibits wild and spoilage yeasts during fermentation. Generally, this SO_2 is supplemented by the further intentional addition of SO_2 as a food additive, preventing oxidation and preserving the fruity flavour and freshness of wine (British Columbia Amateur Winemakers Association).

Ammonium bisulphite is an alternative to a currently permitted processing aid; ammonium sulphite, which is a permitted microbial nutrient. Ammonium bisulphite in an aqueous form is considerably more effective and preferred when compared to alternatives that require mixing or individual addition at specific times of the fermentation process. In addition, ammonium bisulphite is safer to handle.

The ammonium ions produced via the breakdown of ammonium bisulphite meet the criteria of performing the primary technological purpose of a processing aid as they are consumed during the course of processing and have no function in the final product once fermentation is halted.

2.3 Chemical properties

Description: A white, crystalline solid with the formula $(NH_4)HSO_3$. It is the product of the half-neutralization of sulfuric acid by ammonia. It dissolves in water and is added to wine must via an aqueous solution.

Chemical name: Ammonium bisulphite

Common names: Ammonium hydrogen sulphite, Monoammoniumsulphite, Ammoniummonosulphite; Ammoniumhydrosulphite; Ammonium acid sulphite; Ammonium bisulphite solid; Ammonium sulphite hydrogen

CAS registry number: 10192-30-0

Chemical formula: $(NH_4)HSO_3$

Molecular weight: 99.11 g/mol

Structural formula:
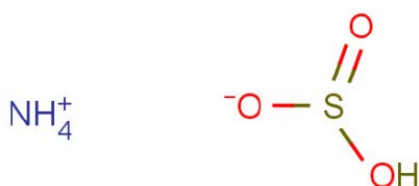
2.4 Analytical method for detection

Methods for identifying ammonium bisulphite are provided by the applicant by referencing the International Oenological Codex (IOC); Ammonium bisulphite COEI-1-AMMHYD: 2007.

Aqueous solutions of ammonium bisulphite produce reactions of ammonium (release of ammonia in the presence of sodium hydroxide when heated) and sulphur dioxide (filter paper soaked in potassium iodate and starch turns blue).

2.5 Manufacturing process

Concentrated sulphur dioxide is adsorbed in a solution of ammonia to make a concentrated solution of ammonium bisulphite. Pure ammonium bisulphite is a white crystalline solid. Commercially, however the aqueous solution is a transparent, slightly yellowy liquid with a slight odour of ammonium. It is available in various levels of concentration, with 60% concentration being common.

2.6   Product specification

Any supplier of ammonium bisulphite would need to meet the requirements set out in the OIV IOC Ammonium bisulphite COEI-1-AMMHYD: 2007, which is one of the secondary references for specifications in S3—3(j) of the Code.

2.7   Food technology conclusion

FSANZ concludes that the use of ammonium bisulphite is an efficient way to provide nutrients to the yeast as its primary function. FSANZ did note however, that sulphur dioxide (SO_2) is also produced during the breakdown of ammonium bisulphite. SO_2 produced during the breakdown of the ammonium bisulphite complements the endogenous SO_2 formed during fermentation and the intentionally added SO_2 providing a preservative function. The primary technological function for ammonium bisulphite is for a yeast nutrient and its use as such is technologically justified. Ammonium bisulphite can also be added at various stages of the wine making process and is preferred over alternatives for its effectiveness and from a safe handling perspective. The use of ammonium bisulphite as a processing aid is confirmed by the fact that the ammonium ions are metabolised by the yeast during fermentation, thereby effectively removing them from the final product. Ammonium bisulphite will be permitted for use at GMP levels, which is the lowest possible level necessary to accomplish the intended effect. A specification for ammonium bisulphite which meets the requirements of the Code is provided by the OIV.

3   Hazard assessment

3.1   Toxicity studies

The safety of sulphites has been the subject of extensive review by FSANZ under the recently completed Proposal 298. FSANZ concluded that the current ADI for sulphites, set by JECFA in 1973 and reiterated at subsequent JECFA reviews, is probably too low. The JECFA ADI is based on a NOAEL for gastric lesions in rats, but the findings are not consistently reproducible. There is no evidence that sulphites are developmental or reproductive toxicants, or that they are carcinogenic *in vivo*. Human toxicity is limited to hypersensitivity reactions in limited subpopulations. FSANZ concluded that a health based guidance value (HBGV) for sulphites cannot be determined until a robust long-term repeat dose study is completed.

There has been no new evidence on which to base a HBGV since Proposal 298 was completed. Therefore, there is no justification for undertaking further hazard assessment of sulphites at the current time.

3.2   Assessments by other regulatory agencies

The current JECFA ADI for dietary sulphite, 0–0.7 mg/kg bw, was set in 1973 and has been reiterated at subsequent JECFA reviews.

3.3   Hazard assessment conclusion

FSANZ has previously concluded that the current conservative JECFA ADI remains appropriate.

4   Dietary exposure assessment

A dietary exposure assessment was not conducted for this Application because any change

in dietary exposure to sulphites resulting from the use of ammonium bisulphite as a wine processing aid is expected to be negligible.

5   Risk assessment conclusion

The use of ammonium bisulphite as a processing aid for the production of wine, as proposed in this Application, raises no public health and safety concerns.

Part D: Silver chloride

1   Introduction

1.1   Introduction

Silver chloride (AgCl), a white crystalline solid, well known for its low solubility in water, occurs naturally as a mineral, chlorargyrite, or is synthesised by combining aqueous solutions of silver nitrate and sodium chloride.

It is used in wines to remove fermentation and storage-related abnormal odours caused by reduction reactions. It is added to wine must by way of an inert carrier such as diatomaceous earth, bentonite or kaolin.

The silver chloride reacts with sulphurous components, such as, hydrogen sulphide, disulphides, mercaptans or thioacetates[3] to form silver sulphide during the treatment. Silver chloride forms a precipitate with unwanted components and remains adsorbed by the inert carrier and together are separated by filtration. The likelihood of residues remaining in wine is very low, provided the wine is adequately filtered after treatment. Permission is being sought for addition of silver chloride as a processing aid to Standard 1.3.3 (d) Permitted decolourants, clarifying, filtration and adsorbent agents.

1.2   Current permissions

1.2.1   OIV resolutions and other OIV documents

Resolutions:

- OIV/OENO 145/2009 (Treatment with silver chloride)
- OIV-OENO 505-2014 (Monograph on silver chloride)

1.2.2   European Union

Amendment of EC Regulation 606/2009, permits addition to musts and wines at a maximum level of 1g/100L and under the conditions laid out in Annex 21 (OIV 2012).

1.3   Risk Assessment Questions

1.3.1   Technological function

Is the technological function clearly stated and does silver chloride achieve that function as a food processing aid in the quantity and form proposed?

1.3.2   Health and safety

Are there any potential public health and safety concerns that may arise from the use of silver chloride as a processing aid in the manufacture of wine in Australia and New Zealand?

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[3] This is a large group of malodorous sulphur compounds which are described by terms such as cabbage, struck flint or burnt rubber.

2 Food technology assessment

2.1 Technological purpose

Treating wines with silver chloride facilitates removal of any kind of sulphurous flavours or odours caused by components such as hydrogen sulphide, disulphides or thiols. Due to the high selectivity of silver chloride, it targets the sulphurous aromas, whilst leaving desirable aromas intact.

Silver chloride is applied to an inert carrier material[4] before addition to the wine. The precipitates of silver chloride that form with unwanted components in wines are removed via filtration or similar processes carried out a minimum of two days after application.

2.2 Technological justification

The functionality of silver chloride in removing unwanted odorous compounds has been shown to be justified at the recommended OIV use concentration of ≤ 1g/L. Tables 2 and 3 (Erbsloeh) show that the use of silver chloride is technologically justified when compared to traditional odorous compound removal by copper sulphate.

Table 2: Comparison of functionality of silver chloride and copper sulphate.

Processing aid	Solubility in Wine	Functionality	Removal from Wine
Silver Chloride	Hardly soluble, with good dispersion via an inert carrier.	Effective against difficult-to-treat sulphur off-flavours. Carrier material also supports binding of metal complexes	Complete removal of insoluble precipitates by filtration
Copper Sulphate	Highly soluble so needs to be added in surplus to guarantee functionality.	Only effective against H_2S (young wine stage) and easily accessible mercaptans. The excessive use of copper can cause bitter taste in wine.	Removal of excessive copper (limit value 1 mg/L) can only be achieved by blue fining

Table 3: Effectivity in odorous compound removal by silver chloride and copper sulphate in wine.

Odour name	Odour description	Effective in removing odour?	
		Copper sulphate	Silver Chloride
Sulphurous	Rotten egg	Yes	Yes
Thiole	Sulphur-like	Yes	Yes
Oligosulphide	Rubber/garlic	Yes	Yes
1,1 Ethandithiole	Rubber	Yes/No	Yes
Thioaceticester	Rubber/garlic	No	Yes

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[4] Refer to Standard 1.3.1 for permitted inert carriers.

2.3   Chemical properties

*Description*   A naturally occurring crystalline solid which upon heating
                                 converts to silver (and chlorine), which is signalled by greyish
                                 or purplish coloration to some samples.

Chemical name: Silver Chloride

Common names: Silver monochloride

CAS registry number: 7783-90-6

Chemical formula: AgCl

Molecular weight: 143.318 g/mol

Structural formula: Ag—Cl

2.4   Analytical method for detection

OIV (2014b) has developed analytical methods of detection for silver (Method OIV-MA-AS322-09 Type IV method, COMPENDIUM OF INTERNATIONAL METHODS OF ANALYSIS). Tests for silver chloride are provided in OIV (2014a).

2.5   Manufacturing process

Silver chloride is synthesised by combining aqueous solutions of silver nitrate and sodium chloride.

$AgNO_3(aq) + NaCl(aq) \rightarrow AgCl(s) + NaNO_3(aq)$
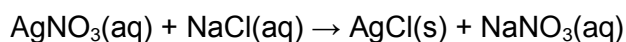
Silver chloride, in its pure state, is a white solid and should have a minimum purity of 99%. Determination of the silver content is conducted according to the atomic absorption spectrophotometry (AAS) method (7.8).

2.6   Product specification

Any supplier of silver chloride would need to meet the requirements set out in the meet the OIV INTERNATIONAL OENOLOGICAL CODEX Silver Chloride COEI-1-CHLARG: 2014 which is one of the secondary references for specifications in S3—3(j) of the Code.

2.7   Food technology conclusion

FSANZ considers silver chloride is effective in removing fermentation and abnormal storage-

related odours in wine. Evidence provided through wine industry research and development shows that silver chloride is successful in removing a wide range of fermentation and storage-related odours in wine. As silver chloride is able to be filtered from wine due to its attraction to an inert carrier, it meets the criteria of performing a technological purpose of a processing aid i.e. it is used in the course of processing but does not perform a technological function in the final food. Silver chloride will be permitted for use at GMP levels, which is the lowest possible level necessary to accomplish the intended effect. A specification for silver chloride which meets the requirements of the Code is provided by the OIV.

3 Hazard assessment

3.1 Toxicokinetics

There are few studies that present quantitative data on the absorption of silver from the gastrointestinal tract. Available data suggest that absorption of silver ranges from 0.4 to 10% (Hadrup and Lam, 2014).

Toxicokinetics of a single dose of silver ions, administered as silver acetate, were investigated by Boudreau et al. (2016) as part of a study comparing silver ions to silver nanoparticles. Male and female Sprague Dawley/CD-23 rats (2/sex/group) were administered a single dose of silver acetate or silver nanoparticles by oral gavage. The dose of silver acetate was 10 mg/kg bw. Blood, 100 µl/time point, was collected from the tail vein at 0, 5, 15 and 30 min, and 1, 2, 6, 8, 12, 24, 48 and 72 h, and analysed for silver content by ICP-MS. For rats of both sexes, the distribution $t_{1/2}$ for distribution was 4 to 5 h, the elimination $t_{1/2}$ was approximately 24 hours, and T_{max} was 12 hours. However, the AUC for female rats (12 µg.h/mL), was approximately double that of male rats (5 µg.h/mL).

The distribution and excretion of ionic silver, administered by oral gavage for 28 days as silver acetate, was investigated by Loeschner et al. (2011). Subjects were female Wistar Hannover Galas rats, 5 weeks old at the start of treatment and weighing approximately 107 g. Rats were pair-housed under standard laboratory husbandry conditions and provided ad libitum with feed and with water acidified with citric acid. Groups included a control group (n=10), a group dosed with silver nanoparticles (n=10), and the group dosed with silver acetate (n=9). The daily dose level of silver ions in the silver acetate was 9 mg/kg bw/day. Urine and faeces were collected over 24 from fasted rats in individual metabolism cages during week 3 of study. Rats were killed and exsanguinated on day 29, with necropsy and collection of blood, brain, stomach, liver, kidney, lungs and skeletal muscle. Slides for histopathology were prepared from liver, kidney and ileum. Silver concentrations were determined in tissues, blood plasma, urine and faeces by ICP-MS. Histological sections were processed for autometallography with toluidine blue as the counterstain. Tissues from one rat/group were processed for transmission and scanning electron microscopy. The highest silver concentrations, in decreasing order, in the silver acetate-treated rats were in kidney, lung, muscle, brain and plasma. Silver was also found to in liver, spleen, bone marrow, lymph nodes, skin, thyroid, heart, pancreas, and adrenal glands. The silver concentration was below the level of detection in the tissues of control rats. The concentration of silver in tissues varied considerably between individual rats in the silver acetate-treated group. Excretion of silver in urine was <0.1% while a high level of silver (49% ± 21%) was excreted in the faeces. The authors did not consider it possible to determine absorption of silver from this study, because of the likelihood of biliary excretion of silver, and of binding of silver to the intestinal epithelium. Autometallographic staining and transmission electron microscopy of the ileum showed that silver was deposited in the lamina propria, mainly in the tips of villi, and in the submucosal tissue. Energy dispersive x-ray spectroscopy showed that silver was associated with selenium and sulphur. In the liver, silver was located around central veins and portal tracts, and in Kupffer cells, while in the kidney, it was located in glomeruli and proximal tubules.

A study by van der Zande et al. (2012) provides additional information on absorption, distribution and elimination of silver ions administered to male Sprague-Dawley rats in the form of silver nitrate. Rats were maintained under standard laboratory husbandry conditions, not more than 3 per cage. They were approximately 8 weeks old and 245 g bw at the start of the study. Rats were randomly assigned to groups of 5 rats/group. There were five main study groups of which two were groups dosed with silver nanoparticles or a vehicle control. The results from those groups will not be discussed further here. The groups of relevance to this assessment were a group dosed with 9 mg/kg bw silver nitrate in water, and a control group dosed with water. There were two recovery cohorts of 5 rats/cohort for each of the silver nitrate treatment and the two silver nanoparticle treatments. Rats were dosed daily for 28 days by oral gavage, at a dose volume of 3.3 mL/kg bw. Rats were fasted for two hours prior to gavage. During the treatment phase, rats were weighed daily and bled (250 μL) from the tail vein weekly. Faeces samples were collected weekly for silver measurement. The main study groups were killed on day 29, with collection of liver, kidneys, lungs, heart, spleen, brain, bladder, testis, mesenteric lymph nodes, blood, stomach, small intestine, large intestine, small intestinal contents, and large intestinal contents. After day 29, recovery cohorts were weighed weekly. Weekly collection of blood and faeces continued. On days 36 and 84, groups of recovery cohort rats (5/group) were killed and subject to the same post-mortem procedures as the main study groups.

Excreted silver in the faeces was estimated to be >99% of the previous day's intake, implying that only a very small fraction of ingested silver is absorbed. Blood silver on day 29, one day after the last dose administration, was significantly lower than during the dosing period (group mean 59% of that on day 28), and one week after cessation of dosing, blood silver levels were not detectable, indicating a rapid clearance of silver from the circulation. Faecal silver levels were likewise not detectable 1 week after cessation of dosing. In tissues collected on day 29, silver levels were highest in the emptied parts of the gastrointestinal tract, followed by, in descending order, liver, spleen, testis, kidney, brain and lungs. Analysis of the tissues of rats killed on day 36 showed that one-week post-exposure, group mean silver concentrations in most tissues were less than 50% those measured on day 29, and silver was not detectable in most samples on day 84. Exceptions were brain, testis, kidney and spleen. In brain and testis, group mean concentrations of silver on day 36 were 70 to 100% those measured on day 29, while those in kidney and spleen were over 50%. The group mean silver levels in the brain were still above 90% of day 29 levels on day 84 (2 months' post-exposure), those in the testis were above 70% on day 84, and those in the kidney were more than 50%.

Boudreau et al. (2016) further investigated the distribution of silver ions in a 13-week repeat-dose oral gavage study in Sprague Dawley rats, from which information on distribution was obtained. Dose levels of silver acetate were 100, 200 and 400 mg/kg bw. The distribution of silver in tissues was determined by histopathology of a comprehensive range of tissues, as well as by transmission electron microscopy (TEM) and ICP-MS of selected tissues. The tissues subject to TEM were jejunum, proximal ileum, proximal colon, mesenteric lymph nodes, right kidney, median and left lateral lobes of the liver, and the spleen. These tissues, as well as blood, heart, uterine horn, and bone marrow were also subject to silver analysis by ICP-MS. For ICP-MS, the gastrointestinal tract tissues were emptied and flushed. With the exception of bone marrow, all tissues showed statistically significant, dose-related increased in silver concentrations relative to those in tissues from rats in the control group. Silver accumulations were significantly higher in the tissues of female rats compared to male rats, with the exception of the ileum. Blood levels of silver were not significantly higher after 12 weeks of dosing than after 1 week of dosing, indicating rapid clearance of silver. The highest levels of silver were found in kidney and spleen. In the jejunum, ileum and colon, silver accumulated as granules on the epithelial basement membrane, while in kidney they accumulated on the basement membranes of glomeruli. In the liver, the distribution of silver

granules was diffuse, while in the spleen they showed an affinity to extracellular membranes but were also found within macrophages. Energy-dispersive X-ray spectroscopy (EDS) was used to analyse the composition of the granules, and showed that selenium, sulphur and chloride were frequently found in association with silver.

In rats, ionic silver crosses the placenta and accumulates in foetal tissues (Charehsaz et al. 2016; see Section 1.8 below).

In addition to the tissues already mentioned above, ionic silver has been found to distribute to prostate, eyes, tongue, teeth, salivary glands, parathyroid glands (Hadrup and Lam 2014), tongue, teeth, salivary glands, choroid plexus of the brain, and choroid layer of the eye (ATSDR 1990) following oral exposure.

A woman was administered an oral dose of silver acetate radiolabelled with ^{110M}Ag. Whole-body counting showed that retention of silver was 21%, 20%, 19% and 18.7% of the administered dose after 1, 2, 8 and 30 weeks respectively (MacIntyre et al. 1978). Deposition of silver in the skin in human beings, causing a blue-grey discolouration called argyria, is considered to be irreversible (ATSDR 1990; Drake and Hazelwood 2005). Argyria may be considered to be a detoxification mechanism in that silver is sequestered in tissues in harmless form, as a complex with protein or as silver sulphide (Drake and Hazelwood 2005).

It is thought that absorbed silver forms complexes with proteins and nucleic acids, by binding to sulfhydryl, amino, carboxyl, phosphate and imidazole groups (ATSDR 1990; Drake and Hazelwood 2005)

Silver is eliminated primarily in the faeces (ATSDR 1990). Ligation of the bile duct significantly diminishes the faecal excretion of orally administered silver, which is consistent with biliary excretion being an important pathway (Hadrup and Lam 2014).

3.2   Genotoxicity studies

3.2.1   Reviews

The ATSDR reported in 1990 that they had found no studies on the genotoxic effects, in humans or animals, of oral exposure to silver or compounds of silver (ATSDR 1990). Hadrup and Lam (2014) cited an early (1975) study reporting negative results for silver chloride in a bacterial mutagenicity test using *Bacillus subtilis*, as well as a publication reporting lack of genotoxicity of silver iodide in assays including the bacterial reverse mutation (Ames) test, microsome test in human lymphocytes, and P388 lymphocyte leukaemia cells in mouse peritoneal cavity. Micronuclei were induced in TK6 lymphoblastoid cells in the presence of a high concentration of 5 nm silver nanoparticles. Hadrup and Lam (2014) concluded that silver has only limited genotoxic effects.

No studies concerning the carcinogenicity of oral exposure to silver or silver compounds were located.

Bacterial reverse mutation test

Bacterial strains used in this assay (Asakura et al. 2009) were *Salmonella typhimurium* strains TA100, TA1535, TA98 and TA1537, and *Escherichia coli* strain WP2*uvr*A/pKM101.Positive control substances for assays without S9 mix were 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide for TA98, TA100 and WP2*uvr*A/pKM191; sodium azide for TA1535; and 9-aminoacridine hydrochloride for TA1537. For assays with S9 mix, the positive control substance was 2-aminoanthracene. Silver was suspended in DMSO at 50 mg/mL and then serially diluted with the same solvent. Positive controls were dissolved in DMSO, with

the exception of sodium azide which was dissolved in water. A dose-range finding assay was conducted with silver concentrations of 5000, 1250, 313, 78.1, 19.5, 4.88, 1.22 and 0.305 µg/plate. The number of revertant colonies was less than twice that of the corresponding negative solvent control in all tester strains, with or without S9 mix, and there was no microbial toxicity. Doses of silver selected for the main test were therefore 5000, 2500, 1250, 625 and 313 µg/plate. For the main test, which was conducted by the pre-incubation method, three plates were used for each concentration as well as for positive and negative controls. Plates were incubated for 48 hours at 37ºC. The number of revertant colonies was less than twice that in the negative control plates at all doses of silver. No microbial toxicity was observed. Precipitation of silver occurred at ≥2500 µg silver, with and without S9 mix. The number of revertant colonies was increased, and more than double that in the negative controls, in the positive control plates. It was concluded that silver was not genotoxic in this assay.

Chromosomal aberration test in Chinese hamster lung cells in vitro

For the purpose of this test, (Asakura et al. 2009) silver was suspended in 1% sodium carboxymethylcellulose, which was also used as the negative control. Positive control substances were mitomycin C and benzo[a]pyrene. An initial cell growth inhibition test was carried out, with and without S9 mix, with 0.3 mL silver suspension at concentrations of 5000, 2500, 1250, 625 and 313 µg/mL. No cell growth inhibition was observed at any level. Therefore, the silver concentrations used for the pulse test were 5000, 2500 and 1250 µg/mL. The test was conducted in duplicate. Cells were incubated exposed to silver for six hours, washed, and incubated with fresh culture medium for a further 18 hours. Colcemid was added to arrest cells in metaphase, two hours before the end of incubation. Cell survival was measured, and two slides of fixed cells were prepared from each culture plate. One hundred cells in metaphase were examined for each slide. When results of the pulse test were negative, a further test was conducted using 24-hour continuous incubation with silver at concentrations of 5000, 2500, 1250, 625 and 313 µg/mL Mitomycin C was used as the positive control. The incidence of cells with structurally and numerically aberrant chromosomes was less than 5% for the silver-treated cells and the negative control cells in both the pulse and continuous tests, while the incidence of cells with structurally and numerically aberrant chromosomes was greater than 10% in both tests in the presence of positive control substances. It was concluded that silver was not genotoxic in this assay.

Rat micronucleus study

A micronucleus assay (Boudreau et al. 2016) was included in the 13-week repeat-dose oral gavage study. The micronucleus assay was conducted by flow cytometric analysis of peripheral blood collected from rats of both sexes during weeks 1, 4 and 12 of the study. Male and female rats treated with the highest dose of silver acetate, 400 mg/kg bw/d, had a small but significant increase in the frequency of micronucleated reticulocytes at Week 4 but not at Week 1. The authors noted that the rats in the 400 mg/kg bw/day exhibited severe gastrointestinal dysfunction and only one female rat, and no males, in the micronuclei sampling cohort in th400 mg/kg bw/day group survived to week 12. The authors did not draw any conclusions from the results of the micronucleus study.

3.3   Animal studies

3.3.1   General Toxicology Studies

Silver nitrate administered to rats in drinking water for 8.5 months, at a level corresponding to 81 mg silver/kg bw/d, retarded growth rate in rats (Matuk et al. 1981, as reviewed by Hadrup and Lam 2014). Silver nitrate administered in drinking water for 125 days, at a level equivalent to 14 mg/kg bw/d, affected activity levels in mice (Rungby and Danscher 1984, as

reviewed by Hadrup and Lam 2014).

28-day repeat-dose oral toxicity study in rats

This study (Hadrup et al. 2012) was conducted using Wistar Hannover Galas rats. Rats were four weeks old at study start. Female rats (44) were randomised into four groups and males (12) were randomised to two groups. Rats were pair-housed under standard laboratory husbandry conditions and provided with standard diet, and water acidified with citric acid, ad libitum. The vehicle control, administered to 10 females and six males, was 11.5 mg/mL polyvinylpyrrolidone (PVP) in water. Silver nanoparticles in aqueous PVP were administered at 2.25 mg Ag/kg bw/day to eight females, at 4.5 mg Ag/kg bw/day to eight females, and at 9.0 mg Ag/kg bw/day to 10 females and 6 males. A further group of eight females were dosed with 14 mg silver acetate/kg bw/d, equal to 9 mg silver/kg bw/day. All daily doses were divided into two administrations.

The results for rats treated with silver nanoparticles will not be reviewed here, because this assessment is of ionic silver.

In-life endpoints included survival, clinical observations, body weights, body weight gains, daily feed intake and daily water intake. On study day 18, rats were placed in individual metabolic cages for 24 hours and were provided with water but not feed while urine and faeces were collected. On day 28, rats were anesthetised with CO_2/O_2 and decapitated, with collection of blood for haematology and limited clinical chemistry (cholesterol, total protein, albumin, glucose, urea, creatinine, alanine aminotransferase and alkaline phosphatase. Urine was assayed for creatinine. Each rat was subject to complete necropsy with weighing of mesenteric lymph nodes, liver, spleen, kidneys, adrenals, testes, ovaries, heart and brain. From six rats/group, samples of liver, kidney, ileum and myocardium were fixed, processed to slides and stained with haematoxylin and eosin for histopathology. Sections from liver, kidney and ileum were also subject to TUNEL assay of apoptosis, and sections of liver and ileum were subject to immunohistochemical labelling for leucocyte marker/CD45. Caecum contents from all rats were sampled for PCR investigations of bacterial flora, including major bacterial phyla and expression of mRNA for silver resistance genes silCBA, silE, silP and silRS.

Treatment with silver acetate had no effect of survival or clinical observations. Group mean bodyweight gain was significantly lower than that of female controls in the intervals days 4-7 (47% of controls), days 11-14 (61% that of controls), and days 14-16 (19% that of controls) leading to a significantly lower group mean body weight gain over the study as a whole (79% that of controls) but group mean terminal body weight was not significantly lower than that of controls. Treatment with silver acetate did not significantly affect group mean feed intake, water intake, or haematology. Group mean ALP was significantly ($p<0.05$) elevated (140%) relative to that of female controls. A slight, but significantly ($p<0.05$) lower plasma urea (84%) was also found in silver acetate-treated rats. Urine creatinine and creatinine clearance were not affected by treatment. There were no gross lesions on necropsy, but group mean thymus weight, relative to body weight, was significantly ($p<0.05$) lower than that of control females (85%). No treatment-related microscopic lesions were found in liver, kidneys, ileum or myocardium, and there were no differences in the number of TUNEL-positive apoptotic cells in sections of liver, kidney or ileum. There was no treatment-related difference in leucocyte infiltration of liver or ileum, as determined by immunohistochemical labelling for leucocyte marker/CD45. Treatment with silver acetate did not affect the caecal populations of Firmicutes or Bacteroidetes, or the expression of silver resistance genes.

On the basis of decreased body weight gain, increased ALP and decreased relative thymus weight, the dose of 14 mg silver acetate/kg bw/d, equal to 9 mg silver/kg bw/day is considered to exceed the toxic threshold and to represent a LOAEL.

28-day repeat dose study in male rats

The design of this study (van der Zande et al. 2012) has been described in the toxicokinetics section, section 1.6 above. The dose of ionic silver used in the study was 9 mg silver/kg bw as silver nitrate, administered by daily oral gavage using water as the vehicle and control article. This dose level of silver had no effect on body weights, body weight gains, or clinical observations. There were no treatment-related effects on plasma AST activity, plasma ALT activity, levels of IgM or IgG in plasma, or proliferation of T or B cells isolated from the spleen or mesenteric lymph nodes. Similarly, treatment did not affect cytokine levels from lymphocytes isolated from the spleen or mesenteric lymph nodes and stimulated with lipopolysaccharides or concanavalin A. It may be concluded from these results that the NOAEL for silver in this study was 9 mg/kg bw/day.

13-week repeat dose oral gavage study in rats

Sprague Dawley rats (10/sex/group) were treated with 0, 100, 200 or 400 mg/kg bw/day silver acetate (Boudreau et al. 2016). Rats were approximately 7 weeks old at the start of treatment. Rats were gavaged twice daily, 12 hours apart, to a total daily volume of 20 mL/kg. The vehicle and control article was 0.1% methylcellulose in water. Rats were housed, 2/cage, under standard laboratory husbandry conditions. Food and water were provided *ad libitum*. Rats were subject to daily health checks and weekly detailed clinical observations. Food and water consumption were recorded weekly, and body weights were recorded daily. Blood was collected from tail veins at weeks 1, 4, 8 and 12 for determination of silver concentrations. Rats were fasted overnight prior to scheduled termination. At scheduled and unscheduled terminations, rats were weighed, anaesthetised with carbon dioxide, and exsanguinated by cardiac bleeding for haematology and clinical chemistry. A complete necropsy was performed on all animals. A comprehensive tissue list was collected for histopathology. Samples of ileum and ileal mucosa were collected for evaluations of intestinal microbiota and immune response evaluations. Samples for TEM, ICP-MS and micronucleus assay have been described in in Sections 14.2 and 14.3. Evaluation of reproductive parameters is described under Developmental and Reproductive Toxicology Studies, below.

High morbidity was observed in the 400 mg/kg bw/day group, with 7/10 female and 10/10 males terminated in moribund condition prior to the scheduled end of the in-life phase. Clinical signs in these rats were those of severe gastrointestinal signs, loss of body weight or failure to gain bodyweight, and unthrifty appearance. Treatment with silver acetate at ≥ 100 mg/kg bw/day had a statistically significant negative effect on body weight gain and terminal body weights in both sexes, although there were no consistent differences in food or water intake. MCV was significantly decreased in female rats treated with ≥ 200 mg/kg bw/day silver acetate and male rats treated with ≥ 100 mg/kg bw/day silver acetate. Serum alkaline phosphatase was significantly elevated in rats of both sexes treated with ≥ 200 mg/kg bw/day silver acetate, compared to sex-matched controls. Serum albumin, total protein and creatinine were significantly lower than those of controls in females treated with 400 mg/kg bw/day silver acetate, while serum alanine transferase was significantly higher. No comparison could be made with males for these parameters because no 400 mg/kg bw/day males survived to scheduled termination. The relative liver weights of males treated with ≥ 100 mg/kg bw/day silver acetate, and females treated with ≥ 200 mg/kg bw/day silver acetate were significantly higher than those of sex-matched controls. Treatment-related lesions found only in the 400 mg/kg bw/day group included mucosal hyperplasia of large and small intestines, and thymic atrophy or necrosis. However, a dose-related brown pigmentation of multiple organs, corresponding to microscopic, TEM and EDS findings of silver granule deposition, was found at all dose levels. The authors did not draw any conclusions regarding a NOAEL or LOAEL for silver acetate from this study, but from the results it may be concluded that a NOAEL was not identified and the LOAEL was the lowest dose

administered, 100 mg/kg bw/day silver acetate, equal to 64.6 mg silver/kg bw/day.

3.3.2 Developmental and Reproductive Toxicology Studies

Reviews

Oral administration of silver chloride, corresponding to 190 mg silver/kg bw/day, to female rats from GD1-GD20 resulted in increased post-implantation loss and death of all pups within 24 hours of birth (Shavlovski et al. (1995) as reviewed by Hadrup and Lam (2014)).

13-week repeat dose oral gavage study in rats

The 13-week repeat-dose oral gavage study (Boudreau et al. 2016), previously described under General Toxicology Studies, and included evaluation of some reproductive parameters. Oestrous cycling of the female rats on the study was assessed by examination of vaginal cells collected by vaginal lavage for each of 16 consecutive days prior to scheduled termination. The left testis and caudal epididymis of male rats were collected at termination to determine sperm counts, sperm motility and sperm morphology. There were no statistically significant treatment-related effects on oestrous cycles, or in sperm motility, testis sperm count, caudal epididymal sperm count, or sperm morphology between rats treated with up to 400 mg/kg bw/day silver acetate and controls.

Developmental toxicity study in rats

Female Sprague-Dawley rats, housed under controlled laboratory conditions, were assigned to 5 groups of 10 rats/group (Charehsaz et al. 2016). Rats were dosed by oral gavage from GD 7 to GD 20, at a daily volume of 4 mL/kg bw. The control group were dosed with water. Three groups were dosed with a silver nanoparticle suspension at doses of 0.2, 2 or 20 mg/kg bw/day. The fifth group was dosed with 20 mg Ag/kg bw/day as $AgNO_3$. Rats were pair-housed until GD18, when they were individually housed and monitored for parturition. Pups were counted and sexed on postnatal day (PND) 2, and dams and individual pups were then weighed and decapitated for collection of blood. Blood from pups in a litter was pooled. Brain, heart, lungs, liver, kidneys, spleen, ovaries and uterus were collected from dams, and brain, lungs, liver, kidneys, and stomachs with milk were collected from 6 to 7 pups per litter. Tissues processed for histopathology were brain, lung, spleen, heart, liver, kidney, uterus, ovaries from 5 dams/group, and brain, heart, liver, lung and kidney from one pup from each of those dams.

Silver in tissues and milk was determined by AAS. ALT, AST and IL-6 were measured in plasma. As markers of oxidative stress, superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and malondialdehyde were measured in lysed erythrocytes and in tissues, and plasma NO_2^- / NO_3^- ratios were also measured. Histopathology included immunohistochemistry for glial fibrillary acidic protein (GFAP) and neuron-specific nuclear protein (Neu-n).

The results for rats treated with silver nanoparticles will not be reviewed here, because this assessment is of ionic silver.

Dams gavaged with $AgNO_3$ showed no significant difference from controls in maternal body weight gain, organ weights, pregnancy length, number of implantations, number of foetal resorptions, litter size, sex distribution or pup weights. ALT, AST and IL-6 were not significantly elevated in dams or pups, relative to controls. However, dams treated with $AgNO_3$ showed a significant decrease in liver SOD and GPX, but a significant increase in brain SOD and GPx. The plasma NO_2^- / NO_3^- of AgNO3-treated dams was almost double that of controls. GPx was also significantly decreased in liver tissue, and significantly increased in

brain tissue, of pups of $AgNO_3$-treated dams. Catalase and malondialdehyde were not affected in dams or pups.

Silver levels in kidney, lung, liver, brain, plasma and lysed erythrocytes of pups from dams treated with $AgNO_3$ were consistently higher than those of pups of control dams, and the difference was statistically significant in lung.

Histopathological findings in dams treated with $AgNO_3$ included neuronal loss in the hippocampal pyramidal cells, accompanied by mild gliosis. Other histopathological findings associated with treatment were minimal hepatocellular vacuolation, minimal renal tubular necrosis with associated minimal inflammation, and minimal to mild inflammation in the lungs, with some histiocyte accumulation also observed in the lungs. There were no microscopic changes found in pups.

The dose of $AgNO_3$, 20 mg/kg bw/day, used in this study exceeded the threshold for maternal toxicity. It is apparent from this study that ionic silver crosses the placenta and accumulates in foetal tissues. While conventional markers of developmental toxicity in foetuses were unaffected, there was evidence of possible oxidative stress in foetuses.

3.3.3   Other studies

Three-month study of silver nitrate in the drinking water of male Wistar rats

Young male Wistar rats, of unspecified age but weighing 260-280 g prior to 2 weeks of acclimation, were maintained under standard laboratory husbandry conditions (Gueroui and Kechrid 2016). It is not specified whether the rats were group-housed or individually housed. Rats were randomly assigned to six groups of six rats/group. Group I, fed standard diet, was the control group. Group II were supplemented with vitamin E at 400 mg/kg diet, and selenium, 1 mg/mL, in drinking water. Group III rats were administered silver nitrate, 20 mg/L, in their drinking water. Group IV were administered silver nitrate, 20 mg/L, in their drinking water and dietary vitamin E at 400 mg/kg diet. Group V were given silver nitrate, 20 mg/L, and selenium, 1 mg/mL, in drinking water. Group VI were given silver nitrate, vitamin E and selenium. In-life parameters measured during three months of treatment included survival, clinical observations, body weight, and food intake. At the end of the in-life phase, rats were terminated with collection of blood and brain. Blood was used for clinical pathology while the brain was weighed and then transected longitudinally, with half fixed for histopathology and half processed for determination of parameters related to oxidation and antioxidants. The parameters measured included thiobarbituric acid-reactive substances (TBARS), brain vitamin E and glutathione levels, and brain activities of superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase, and catalase.

All rats survived to scheduled termination, and there were no adverse clinical observations related to silver nitrate. Silver nitrate had no apparent effect on body weight gain, food intake, water consumption or brain weight (expressed relative to body weight). The water intake of the $AgNO_3$+vit E+Se group (Group VI) was significantly lower than that of the $AgNO_3$ group (Group III) but in biological terms the difference was small (30.05 ±4.62 mL/rat/day versus 32.92 ±6.46 mL/rat/day). Compared to untreated controls (Group I), rats in the $AgNO_3$ group (Group III) had increases in serum cholesterol, LDH activity and lipase activity, and decreases in serum calcium, serum total protein and ALP activity. Supplementation with vitamin E and/or selenium ameliorated the effects of $AgNO_3$ on ALP, total protein, LDH and cholesterol. Silver nitrate induced an increase in brain TBARS level, relative to that of controls, that was ameliorated by vitamin E or selenium. Silver nitrate decreased brain vitamin E concentration but did not affect brain glutathione concentration or activities of glutathione peroxidase, glutathione-S-transferase, glutathione reductase or catalase. Silver

nitrate was, however, associated with a significant decrease in superoxide dismutase activity. Silver nitrate was associated with some cytoplasmic vacuolation of cerebral neurons, which was ameliorated by concurrent supplementation with vitamin E or selenium.

Although group mean water intake data are presented in this paper, group mean body weights are not available, and therefore the intake of silver nitrate in water cannot be expressed in mg/kg bw/day. For this reason, this study cannot be used to identify a NOAEL or LOAEL for silver in rats.

3.4 Human studies

The ATSDR (1990) found no reports of human fatalities, and no studies or case reports regarding respiratory, gastrointestinal, haematological, musculoskeletal, hepatic, renal, cardiovascular, immunological, developmental reproductive, genotoxic or carcinogenic effects in humans of oral exposure to silver or silver compounds.

There have been a number of case reports of generalised argyria in individuals who have ingested metallic silver or silver compounds over months or years. Silver-containing granules are visible in the dermis on microscopic examination of biopsies. The granules are distributed throughout the dermis but are most abundant in basement membrane and elastic fibres surrounding sweat glands. The pigmentation of the skin primarily affects areas exposed to sunlight, and is attributed to photoreduction of silver chloride and/or silver phosphate in the skin. X-ray dispersive analysis shows that the granules consist of silver complexed with sulphur and/or selenium. Argyria has been reported in association with chronic intake of silver acetate, silver nitrate, and metallic silver. Quantitative data on the level of intake leading to argyria are either unavailable or unreliable, and therefore a LOAEL for argyria has not been established.

The ATSDR (1990) located several reports of deposition of granules in the central nervous system, found on autopsy of argyric individuals. There was no evidence of neurotoxic effects of the granules, or of neurotoxic effects of oral exposure to silver.

3.4.1 Case report of neurotoxicity

In this case, a 75 year old retired electronic engineer was admitted to hospital with myoclonic seizures and diagnosed with corticobasal degeneration (Stepien et al. 2009).. The patient had a history of making 'colloidal silver' and had been consuming it multiple times a day for four years. A solution of silver found in his home contained 51.4 mg silver/L. The patient's serum concentration of silver was 67.7 µg/L, or 628.3 nmol/L (reference value 2.8 nmol/L). The authors of the case report acknowledged that development of neurodegenerative disease could be coincidental. They cited three other case reports of patients developing neurological disorders after chronic consumption of high levels of silver, however only two of those cases were seizure disorders. In all the cases, silver ingestion was high and/or of very long duration.

3.5 Assessments by other regulatory agencies

Silver was evaluated by JECFA in 1977 (WHO 1977) and by EFSA in 2016 (EFSA 2016), but no ADI was established by either committee, due to inadequate data. EFSA assigned to silver a specific migration limit of 0.05 mg/kg food (EFSA 2016).

3.6 Hazard assessment conclusion

FSANZ assessed the use of silver ions in 2006 and no toxicological concerns were found. This hazard assessment is therefore largely limited to information more recent than 2006.

Systemic absorption of ionic silver is low. Silver is rapidly cleared from the circulation but may be deposited in a wide range of tissues, where it is highly persistent but generally appears to be inert. Silver is primarily excreted in the faeces.

The available evidence indicates that silver is not genotoxic. No studies concerning cancer in animals or humans orally exposed to silver or silver compounds were found. A small number of subchronic repeat-dose studies in rats were found, in which a silver salt was administered orally to one group which was compared to a negative control group and groups administered silver nanoparticles. However, silver chloride, the silver salt that is the subject of this application was not the silver salt used in any of the studies. It is not possible to extrapolate from the silver compounds used in those studies to silver chloride. Although silver crosses the placenta, no evidence of reproductive or developmental toxicity of ionic silver was observed in rat studies.

Ionic silver appears to be of low toxicity in human beings. The most frequently reported effect of chronic ingestion of silver is argyria, a permanent blue-grey discolouration of the skin. A LOAEL for argyria has not been established, but it is a rare condition and considered unlikely to occur as a result of consumption of wine processed with silver.

No health-based guidance value for silver has been established by JECFA, on the basis of lack of data. The subchronic rat studies reviewed in this hazard assessment have been conducted since the last JECFA review of silver. The two rat studies that used the lowest dose level for silver produced different results at the only dose level used; 9 mg/kg bw/day. In neither study was silver chloride the silver salt administered, nor is it possible to extrapolate the results to silver chloride.

In conclusion, a health-based guidance value for silver chloride cannot be determined due to lack of appropriate studies. However, ionic silver appears to be of low toxicity.

4   Dietary exposure assessment

A dietary exposure assessment was not conducted because any residual levels of silver chloride in wine are expected to be negligible.

5   Risk assessment conclusion

The use of silver chloride as a processing aid for the production of wine, as proposed in this Application, raises no public health and safety concerns.

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