PART A:

APPLICATION TO AMEND THE AUSTRALIA AND NEW ZEALAND FOOD STANDARD CODE FOR THE USE OF POTASSIUM POLYASPARTATE AS AN ADDITIVE FOR WINE

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Checklist for applications for substances added to food

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GENERAL REQUIREMENTS

A. SUMMARY

Potassium polyaspartate is the potassium salt of polyaspartic acid, produced from L-aspartic acid, which is a naturally occurring amino acid in wine, and potassium hydroxide (98% purity).

Studies have verified that potassium polyaspartate has stabilizing properties similar to those of the metatartaric acid (MTA) and carboxymethylcellulose (CMC), but is much more stable over time as well as filterable and unaffected by heat, which maintains the quality of the wine while increasing its cellaring or storage potential (shelf-life). Further, it does not have negative impacts on the sensory properties, such as colour, of the resultant wine. It is a completely biodegradable environmentally-friendly alternative to traditional polyanionic materials. Compared with physical stabilisation techniques such as cold stabilisation, electrodialysis and ion-exchange resins, use of potassium polyaspartate is labour, energy and water efficient, and hence cost competitive.

Potassium polyaspartate is thus proposed for use as a stabiliser against tartrate crystal precipitation in wine (red, rosé and white wine) at the typical use level of 100 to 200 mg/L and at a maximum use level of 300 mg/L, depending on the level of instability of the wine to be treated.

The European Food Safety Authority (EFSA) evaluated the safety of potassium polyaspartate as a food additive and in its opinion of 9 March 2016 (EFSA Journal, 2016)[1] concluded that there was no safety concern from the proposed use in wine at a maximum use level of 300 mg/L and typical levels in the range of 100-200 mg/L.

It has accordingly been approved and authorised for use as an additive for wine in the European Union (EU) in COMMISSION REGULATION (EU) 2017/1399 of 28 July 2017 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council and the Annex to Commission Regulation (EU) No 231/2012 as regards potassium polyaspartate and COMMISSION DELEGATED REGULATION (EU) 2017/1861 of 2 August 2017 amending Regulation (EC) No 606/2009 as regards certain oenological practices).[2] In addition, at the 50th session of the Codex Alimentarius Commission on Food Additives (JECFA), the EU and its Member States proposed that potassium polyaspartate used as a stabiliser in wine be added to the priority list of substances proposed for evaluation by JECFA.

To ensure consistency with international wine standards it is requested to amend the table to section S15—5 of Schedule 15 *Substances that may be used as food additives* in the food category 14.2.2 Wine, sparkling wine and fortified wine to include potassium polyaspartate as an additive, at a maximum of GMP.

It is also requested that Standard 4.5.1 Wine Production Requirements (Australia only) be amended to include polyaspartate in the Table to clause 3, at a maximum of GMP.

Standard 1.3.4 requires that substances added to food, including additives, comply with relevant specifications as detailed in the Code. Potassium polyaspartate meets the OIV specification (OIV-OENO 572-2017), which is one of the secondary references for specifications in Standard 1.3.4 (Identity and Purity). Therefore, no new specification is required for the Code.

[1] EFSA Journal 2016;14(3):4435

[2] http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R1399&from=EN

B. APPLICANT DETAILS

Applicant
ENARTIS PACIFIC PTY LTD

Contact person (Australia)

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Nature of applicant's business
Enartis Pacific Pty Ltd is part of the international Esseco Group S.r.l. which develops, produces, and sells inorganic chemical products and products for the winemaking industry worldwide. It offers sulphur derivatives, including sulphite crystals and solutions, liquid sulphur dioxide (compressed and liquefied gas), sodium hydrosulphite, sulphuric acid, and fuming sulphuric acid; and general chemical products, such as caustic potash, potassium carbonate and chlorinated derivatives, chloroparaffins, TMP, NPG, potassium and sodium formates, crushed sulphur, micronized and granulated sulphur, acetates, and ammonium carbonates. The company also offers processing aids and additives, including purifying agents, enzymes, yeasts, tannins, malolactic bacteria, stabilizers, and sulphiting agents, as well as research, wine testing, consultancy, and cellar services for the winemaking industry. It offers its products for various applications, including natural preservatives for foodstuffs, nutritional supplements and animal feeds, rubber vulcanizes, high-efficiency batteries production, pharmaceutical and personal care products, fixatives for photography and radiology, anti-fungal products and agricultural fertilizers, paper bleaching agents and furniture glues, airport runway de-icers, industrial detergents, and more. Esseco Group Srl was formerly known as Esseco Srl and changed its name to Esseco Group Srl in 2004. The company was founded in 1921 and is based in Trecate, Italy with operations worldwide. Other companies in the group include Enartis, Ever, Martin Vialatte, Oenofrance and Station Oenotechnique de Champagne.

Details of other individuals, companies or organisations associated with the application.
Companies or organisation associated with the application are ChemServices S.r.l. (based in Italy) and Enartis Pacific Pty Ltd (based in Australia); Tilly Bowden is the Managing Director of Enartis Pacific Pty Ltd.

C. PURPOSE OF THE APPLICATION

Purpose of application

The purpose of the Application is to allow the use of potassium polyaspartate as a food additive in wine under S15—5 of *Schedule 15 Substances that may be used as food additiv*es in the food category 14.2.2 Wine, sparkling wine and fortified wine *and Standard 4.5.1 Wine Production Requirements (Australia only),* in order to facilitate long-term stabilisation against tartrate crystal precipitation in wine without negative sensory changes, and in order to in order to ensure consistency with international wine standards thus facilitating the trade in wine, and to ensure that Australian wine is not at a competitive disadvantage compared with other winemaking countries and economies.

Standard(s) requiring amendment

The Applicant requests amendment to the:

- Table to Section S15—5 of Schedule 15 *Substances that may be used as food additives* in the food category 14.2.2 Wine, sparkling wine and fortified wine to include polyaspartate as an additive.

- Standard 4.5.1 *Wine Production Requirements (Australia only)* be amended to include polyaspartate in the Table to clause 3.

D. JUSTIFICATION FOR THE APPLICATION

a) Need for the proposed change

This application requesting an amendment to the Australia New Zealand Food Standards Code (FSC) to permit the addition of potassium polyaspartate to wine is for a stabilisation function.

The application provides an alternative to using less effective and efficient, and more expensive chemical and physical processes to stabilise wine against tartaric acid salt crystallization and precipitation. This is required in wines because tartrate crystals (potassium hydrogen tartrate and calcium tartrate) develop naturally in wine during storage and are the major cause of sediment in bottled wines. Although these tartrate crystals in wine do not pose a health risk, their presence affects the aesthetics and consumer acceptability of the wine. Accordingly, before the delivery for domestic or international trade, particularly white wine and sparkling wines have to be stabilised against tartrate salt precipitation.

b) Advantages of the proposed change over the status quo, taking into account any disadvantages

The simple and inexpensive method is the prevention of tartaric acid salt crystallization and precipitation in bottled wine instead of its removal pre-bottling by the addition of crystallization inhibitors.

The advantages of using potassium polyaspartate over the currently permitted chemicals to stabilise wine against tartaric acid salt crystallization and precipitation are that it can be used to stabilise wine for medium and long-term storage or cellaring, where the wine does not need to be consumed immediately on purchase due to increased potential for tartrate salt precipitation as is the case when metatartaric acid is alternatively used. It is also resistance to heat and filterable unlike other permitted chemicals.

In contrast to the use of carboxymethylcellulose which can selectively absorb proteins from wine, for example, sensory testing completed with expert winemaking panels indicates that there are no negative sensory (aroma and colour) impacts in using potassium polyaspartate.

The pre-bottling physical methods to remove tartaric acid salt crystals include prolonged storage of wine under low temperatures after which the wine is filtered and bottled. This method is relatively redundant given recent market requirements for fresh or young white wine, for example. A large-scale alternative is cold stabilisation of tartaric-unstable wines to precipitation temperatures of 2–4^{o}C, where tartrate salt is forced to precipitate after contact seeding with tartrate crystals which are subsequently removed by filtration. Cold stabilisation methods are both energy and water intensive. Electrodialysis and ion exchange are also physical methods, and again expensive and difficult for smaller wineries to adopt, also producing substantial greenhouse gas emissions, and a higher carbon footprint for consumers.

Accordingly, process efficiency will be improved without any negative quality impacts with the use of potassium polyaspartate for stabilisation. There are no perceived disadvantages to its addition to the FSC.

c) Evidence that the food industry generally or other specific companies have an interest in, or support, the proposed change

The identification of potassium polyaspartate as a suitable candidate to stabilise wine against tartaric acid salt crystallization and precipitation originated from the long-term European Stabiwine project

(FP7-SME-2012 SME-2012-2: Research for SME associations Grant Agreement n. 314903)[3]. The partners of the Stabiwine project included Esseco S.r.l, UMIL, Vinidea, CRA, ChemService and the AWRI.

The aim of the project was to test the effectiveness and suitability in winemaking of biopolymers, new or already used in other sectors, and to facilitate their authorisation in the EU in order to make these new practices available for EU SME winemakers. Economic, environmental and social benefits were expected outcomes for EU SME winemakers. A number of manuscripts in relation to the efficacy and safety of potassium polyaspartate in winemaking have been duly peer-review published.

Since 1 January 2018, the cumulative product volume sold in the EU is approximately 240 metric tons of liquid solutions. This volume refers to the Enartis sales exclusively within the EU, with the exception of France which is covered by another brand of Esseco Group. The total volume sold equates to some 1.7 to 1.8 million hectoliters of treated wine, both red, white and rosè. In addition, Enartis have some 350 established customers, that is, those who are repeatingly buying potassium polyaspartate, while approximately 800 customers are trialling it.

Subsequent to the approval of potassium polyaspartate for use in EU winemaking, several Australian wineries including Accolade Wines, Angove Family Winemakers and Casella Wines are currently evaluating potassium polyaspartate in small scale experiments. A letter of support from the Wine Industry Technical Advisory Committee (WITAC) of the Winemakers' Federation of Australia (WFA) is thus attached.

d) Details of the status of similar applications made in other countries by the applicant

The Applicant has also prepared and presented applications for registration of potassium polyaspartate in Chile, South Africa and the USA for assessment by the appropriate regulatory authorities. In addition, applications have been prepared for Canada and China to comply with their bilateral agreement obligations with the EU, especially where provisional authorisation may be granted. These countries are also significant export markets for Australian wine, as well as international competitors. Potassium polyaspartate can currently be used for winemaking in all countries of the European Union: Austria, Belgium, Bulgaria, Cyprus, Croatia, Denmark, Estonia, Finland, France, Germany, Greece, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, United Kingdom, Czech Republic, Romania, Slovakia, Slovenia, Spain, Sweden, Hungary. In addition, at the 50th session of the Codex Alimentarius Commission on Food Additives (JECFA), the EU and its Member States proposed that potassium polyaspartate used as a stabiliser in wine be added to the priority list of substances proposed for evaluation by JECFA.

D1. REGULATORY IMPACT INFORMATION

D.1.1 Costs and benefits

a) Costs and benefits to the consumers

There are no costs to consumers. Benefits to consumers include wine with longer cellaring/storage potential with improved sensory quality attributes consistent with consumers from, for example, the EU.

b) Costs and benefits to industry and business in general

There are no costs to industry and business. Permitting potassium polyaspartate for wine production provides an overall benefit, in terms of decreased production time and improved quality products at

[3] http://www.stabiwine.eu/

potentially lower costs of production. For example, use of potassium polyaspartate in wine production eliminates wine losses during the stabilization process and decreases substantially (up to 80%) both energy and water consumption, while decreasing labour and ancillary costs.

c) Costs and benefits to government

There will be no increased regulatory or enforcement costs for the government. Benefits include increased trade and innovation, consistency with the rules of major trading partners, and consistency with international obligations.

D.1.2 Impact on International Trade

Permission to use the additive potassium polyaspartate in winemaking, for Australian wine and for wine imported into Australia will:

- enable Australia to meet its obligations under the *Australian-European Union Agreement on Trade in Wine*;
- meet Australia's World Trade Organization obligations; and
- not competitively disadvantage Australia and Australian wine producers.

E. INFORMATION TO SUPPORT THE APPLICATION

a) Public health and safety issues

There are no negative public health and safety issues associated with consumption of potassium polyaspartate from wine. The Applicant believes that the approval of this application is consistent with FSANZ's obligation to protect public health and safety.

Authorisations and evaluations are also available for the sodium salt of polyaspartic acid, the polyaminoacid containing sodium instead of potassium. The sodium salt of polyaspartic acid is authorized for use in Australia[4] and the USA[5] as:

- a food contact substance as a dispersant for fillers and an anti-scale additive in sugar processing;
- a water treatment agent used as a scale inhibitor in cooling tower and boiler water applications, with properties of non-phosphor, non-nitrogen, non-pollution and complete biodegradation;
- a coadjuvant in the prevention of dental plaque; and
- an excipient in certain drugs.

b) Consumer choice issues

Inclusion of potassium polyaspartate will provide alternative cost and environmentally-friendly choices to current chemical and physical practices in the Australian wine industry. The Applicant believes that the approval of this application is consistent with FSANZ's obligations to ensure the provision of adequate information relating to food to enable consumers to make informed choices and prevent misleading or deceptive conduct.

c) Evidence of general food industry or specific company support

Subsequent to the approval of potassium polyaspartate for use in EU winemaking, several Australian wineries are evaluating assessing potassium polyaspartate in small scale experiments. A supporting letter from the Wine Industry Technical Advisory Committee (WITAC) of the Winemakers' Federation of Australia (WFA) is thus attached.

E.1. DATA REQUIREMENTS

The published literature has been reviewed to search for relevant references on polyaspartic acid. The methods used to identify relevant data, including the scope and criteria of literature searches, the database searched, the search strategy, language and time limitations, are described below together with search results. The full list of references together with results on another biopolymer is included in Annex number 5.3 by Restani 2012.

Literature search strategy:

The scope of literature search was to search for references relevant to safety or toxicity of polyaspartic acid/polyaspartate and its salts.

- Databases searched: EBSCO, EMBASE, MEDLINE (PubMed), MEDLINE (Ovid), Science Direct, Web of PubMed, TOXNET (which includes TOXLINE, DART, HSDB and ChemIDplus databases), SciFinder

[4]Australian Government. 2001 National Industrial Chemicals Notification and Assessment Scheme. File No. NA/932.

[5](US) Food and Drug Administration. 2007. Inventory of Effective Food Contact Substance (FCS) Notifications. FCN No.707.

Scholar. The Australian Wine Research Institute inhouse database was also utilised (www.awri.com.au).

- Keywords and Boolean operators: "Polyaspartic acid" OR "polyaspartate" AND "toxicity" OR "toxicology" OR "safety"
- Literature: both peer reviewed and grey literature
- Language: English
- Time limits: No
- Year of search: 2012 and updated 2017

E.1.1 Data related to safety studies

Summary of relevant data

Polyaspartic acid has been extensively investigated for its ability to protect against nephrotoxicity caused by gentamicin *in vivo* (rats and mice). Aminoglycoside antibiotics, such as gentamicin, accumulate in the kidney cortex and induce an acute tubular necrosis associated with a nonoliguric renal failure. It has also been studied for its ability to protect against nephrotoxicity caused by cadmium and as carrier of chemotherapeutic agents. It has been studied as drug carrier for colon-specific drug delivery, for the reduction of corticosteroids side effects during the treatment of inflammatory bowel diseases (IBD, ulcerative colitis and Crohn's disease), and against leukaemia.

Sodium polyaspartate has been toxicologically evaluated prior to applications to the Australian Government's *National Industrial Chemicals Notification and Assessment Scheme* (2001)[6] and US Food and Drug Administration (FDA) (2007)[7,8]. Data are confidential and only summary data are publicly available. Mutagenesis and genotoxicity tests were all negative. Acute toxicological studies did not raise concerns being LD50 above 2000 mg/kg b.w. Sub-acute oral toxicity studied performed in male and female Wistar rats, at daily dose of 0, 40, 400, 1000 mg/kg b.w. for two weeks showed the following results: NOAEL 40 mg/kg b.w. in male, NOAEL 1000 mg/kg b.w. in female. The toxic effects were localized in the urinary bladder, but were observed also in groups treated with L-aspartic acid sodium salt. Thus, the adverse effect in males could be due to the sodium salts but this is yet to be confirmed.

[6] Australian Government. 2001. National Industrial Chemicals Notification and Assessment Scheme. File No. NA/932. http://www.nicnas.gov.au/publications/car/new/na/nafullr/na0900fr/na932fr.pdf

[7] US FDA. 2007. Inventory of Effective Food Contact Substance (FCS) Notifications. FCN No. 707. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=fcsListing&id= 707

[8] http://www.fda.gov/downloads/Food/FoodIngredientsPackaging/FoodContactSubstancesFCS/UCM143781.pdf

Table Summary of toxicological data on polyaspartate sodium salt found in regulatory websites and scientific literature

Compound	Mutagenesis Genotoxicity	Acute toxicity Skin/eye irritation Skin sensitisation	Sub-acute Toxicity	Sub-chronic toxicity
Sodium polyaspartate	Negative in all below tests. Ames test Chromosomal aberration assay in Chinese hamster V79 cells; Forward mutation assay at the HPRT locus in Chinese hamster V79 cells;	Acute oral and dermal (rat) LD50>2000 mg/kg bw Not corrosive/irritant to skin/eye of rabbit Not a skin sensitiser in Guinea Pig	Rats, 2 weeks NOAEL= 40 mg/kg bw day (male)1 NOAEL=1000 mg/kg bw day (female)1	Not available

1 The adverse effect in urinary bladder was detected also in a control group treated with aspartic acid sodium salt. It has been hypothesized a role of sodium in this phenomenon.

Sodium polyaspartate gave low environmental toxicity on fish, Daphnia and algae and resulted to be significantly biodegradable in all tested conditions (inherent biodegradability; ready biodegradability; anaerobic biodegradability, modified Sturm test, biodegradation by two strains of *Pedobacter* spp. and *Sphingomonas* spp).

Relevant search results

Pharmacology

Polyaspartic acid has been extensively investigated for its ability to protect against nephrotoxicity caused by gentamicin *in vivo*. Aminoglycoside antibiotics, such as gentamicin, accumulate in the kidney cortex and induce an acute tubular necrosis associated with a nonoliguric renal failure.

Beauchamp et al. (1990) examined the influence of the co-administration of polyaspartic acid on these gentamicin-induced alterations, namely the lysosomal phospholipidosis and the increase in cell turnover. Investigators used an infused rat model in which animals received a total dose of 100 mg/kg bw of gentamicin over 12 h. Renal cortex was examined 2 h (day 0) and 48 h (day 2) after treatment. All animals received an injection of [3H]thymidine (200 microCi i.p.) Co-administration of polyaspartic acid (drug/polypeptide mass ratio 1:2.5) did not modify the drug serum levels. Yet, it was associated with an increased (approximately 35%) cortical content of gentamicin at day 0 and a significant protection against both biochemical changes (increase in lipid phosphorus levels and the decrease of the specific activity of acid sphyngomyelinase induced by gentamicin) and morphological signs (enlargement of lysosomes and deposition of myeloid bodies) due to lysosomal phospholipidosis in proximal tubular cells; and 3) an almost complete protection against increased cell turnover at day 2. This was assessed by dosing [3H]thymidine incorporation into DNA and the enumeration of S-phase cells after histoautoradiography.

Similar results were found by Kishore et al. (1990), who also compared the protective activity of poly-L-aspartate with two other polyanionic peptides, poly-L-glutamic and poly-D-glutamic acids. In this study, polyanionic peptides (250 mg/Kg bw in 0.5 mL of saline) were administered, alone or in association with gentamicin to female Sprague-Dawley rats for three consecutive days. Results suggested that only poly-L-aspartate and poly-D-glutamate were able to prevent the development of gentamicin-induced renal lysosomal phospholipidosis as assessed by key biochemical criteria (increase in lipid phosphorus and decrease of acid sphingomyelinase activity).

Kaloyanides and Ramsammy (1990) hypothesized that the protective effect of polyaspartate is directly associated with its ability to bind electrostatically gentamicin and other aminoglycoside antibiotics and thereby prevents these agents from binding to other anionic sites of cell membrane.

Swan et al. (1991) studied the long-term protective effect of polyaspartate in gentamicin induced nephrotoxicity in rats, that were divided in four groups (gentamicin-NaCl, polyaspartate-H2O, gentamicin-polyaspartate and NaCl-H2O). Within each group, animals were treated for seven or 14 days. The study confirmed the nephroprotective activity of polyaspartate, showing that there were no renal structural or functional consequences of polyaspartate given alone or with gentamicin. Creatinine clearance was similar in animals that received control, gentamicin alone and polyaspartate alone. Polyaspartate nephroprotection was confirmed also when increasing doses of gentamicin (80, 120 and 160 mg/Kg bw/day) were administered to rats for three, five, seven and 10 days, as shown by Swan et al. (1992). After seven days of treatment, renal injury was manifested by a significant elevation in serum creatinine levels in animals treated with 120 mg/Kg bw/day of gentamicin in comparison with those in all polyaspartate-exposed animals as well as in controls.

Todd and Hottendorf (1997) studied the effect of polyaspartate in reducing nephrotoxicity in cultured human proximal tubule (HPT) cell monolayers. Cells were treated basolaterally with medium containing gentamicin (2.2 mM) and either apically or basolaterally with medium containing polyaspartate (0.73 mM). Control HPT cells were treated with antibiotic-free growth medium or exposed apically and basolaterally to polyaspartate (0.73 mM) only. After 4 days of exposure, cell monolayers were placed into Ussing chambers to allow monitoring of transepithelial electrical properties. Gentamicin induced alterations in electrogenic transport (reflected by changes in short-circuit current) as well as alterations in paracellular properties (indicated by changes in transepithelial electrical resistance) were reduced in the presence of polyaspartate. Alterations resulting from selective basolateral exposure to gentamicin were unchanged in the case of apically applied polyaspartate and attenuated only when polyaspartic acid was added basolaterally.

Swan et al. (1991) also investigated polyaspartate nephroprotection against mercuric chloride and cis-platinum (cisP) in rats. Animals were divided into six groups: HgCl2-NaCl, HgCl2-polyaspartic acid (PAA), cisP-NaCl. cisP-PAA, PAA-H2O, and NaCl-H2O (control). HgCl2 was administered subcutaneously at 2 mg/Kg bw/day for 2 days, cisP was administered intraperitoneally at 6 mg/Kg bw/day for 3 days, and PAA was administered at 320 mg/Kg bw/day. Animals were sacrificed after 24 h. The clearance of creatinine was significantly lower in animals that received mercuric chloride (with or without polyaspartate) than in controls and rats that received polyaspartate alone. Likewise, animals that received cis-platinum were not protected by the presence of PAA, although renal function was slightly improved in polyaspartate-mercuric chloride group. These encouraging results led researchers to test PAA as carrier of others chemotherapeutic agents.

Zhang et al. (2008) tested nanoparticles of chitosan-polyaspartic acid-5-fluorouracil (CTSPasp-5Fu) in 32 nude BALB/c male mice inoculated subcutaneously near the nape with human SGC-7901 gastric carcinoma cell line (1x107 cell per mouse). When the tumours developed, the animals were randomly allocated into 4 groups with 8 mice in each groups: CTS-Pasp-5Fu (containing 5-Fu 1.25 g/L), 5-Fu (1.25

g/L), chitosan-polyaspartic acid, and control saline groups. Compared with control saline, the inhibition rates of tumour growth for the CTS-Pasp, 5-FU and CTS-Pasp-5Fu groups were 5.58%, 58.69% and 70.82%, respectively. Furthermore, there was a prominent degeneration and necrosis of tumour cells in the CTSPasp-5Fu group compared with the other three groups. The anti-tumour effects of CTS-Pasp-5Fu were better than those of 5-Fu at the same dose, and this preparation method enhanced the pharmacodynamics of the groups.

Polyaspartic acid was also evaluated as carrier for doxorubicin by Pratesi et al. (1985), to improve the therapeutic efficacy and to decrease the systemic drug toxicity. The effectiveness of doxorubicin alone, or linked to PAA, was tested against different tumours: the macrophage tumour J774 (in female BALB/c), Lewis lung carcinoma (in BDF1 mice), murine reticulum cell sarcoma M5076/73 (in female C57BL mice) and mammary adenocarcinoma (in a retired female c3H/He mice). The results indicated that the polymeric derivatives of doxorubicin had approximately 3-fold lower toxicity than the drug alone. In addition, the severity of specific toxic effects, including cardiotoxicity, were appreciably reduced following conjugation to PAA. The doxorubicin-PAA conjugate gave similar or rather greater therapeutic effects than free drug. This effect, more evident in the highly sensitive tumours, suggests an improvement of the therapeutic index of the polymer-linked drug.

Polyaspartic acid have shown to be a protective agent also against Cadmium (Cd), which is known to be a highly nephrotoxic heavy metal. Shibasaki et al. (1993) evaluated the effect of polyaspartate on Cd-induced nephrotoxicity in nineteen male Sprague-Dawley rats after short-term large-dose exposure to CdCl2. Fourteen rats were subcutaneously injected with $CdCl_2$ 3 mg/kg bw/day for 14 days, and seven of these rats were simultaneously injected with polyaspartate 500 mg/kg bw in the same manner. Five control rats were given daily subcutaneous injections of saline for 14 days. On day 10, CdCl2-exposed rats exhibited severe weight loss and higher urine volume than control rats; polyaspartate administration was associated with significant improvement in these parameters. Renal tubular function was evaluated by determining urinary N-acetyl-beta-Dglucosaminidase (NAG), alanine aminopeptidase (AAP) and fractional excretion of sodium (FENa), and percentage tubular reabsorption of phosphate (% TRP). When exposed to CdCl2, urinary NAG increased from 0.11±0.11 to 0.27±0.66 U/day, urinary AAP from 0.25±0.12 to 0.56±0.10 U/day, FENa from 0.75±0.02 to 1.2±0.88%, and %TRP from 0.85±0.01 to 0.78±0.02. All data were improved by treatment with polyaspartate. In particular, polyaspartate showed a marked efficacy in reducing the urinary lysosomal enzymes, such as NAG or AAP, and improving %TRP and FENa. There was no difference in Cd concentration in renal cortical tissue in untreated and PAA-treated rats, so that PAA mode of action in Cd-induced nephrotoxicity is unknown.

L-polyaspartic acid was investigated by Leopold and Friend (1995), as drug carrier for colon-specific drug delivery, for the reduction of corticosteroids side effects during the treatment of inflammatory bowel diseases (IBD, ulcerative colitis and Crohn's disease). The study was done with 12 male Sprague Dawley rats; two groups of five rats each were treated with dexamethasone (DX) and the ester pro-drug dexamethasone-poly(L-aspartate) (DXAsp), respectively; 2 rats were included as control animals. Drug and pro-drug solutions (corresponding to 1.18 mg DX/mL in dimethyl sulfoxide) were administered to two groups of rats (n=5) by intra-gastric infusion. The steady-state blood concentrations of DX were significantly lower after gastric administration of DXAsp, resulting in a less pronounced adreno-suppression compared to DX alone. Intra-gastric DXAsp administration resulted in a significantly higher DX concentration in the *cecum* and colon mucosa (blood ratios of 1.38 and 1.17, respectively) and the *cecum* muscle tissue compared to DX administration. This indicates that pro-drug cleavage takes place mainly in the large intestinal contents or tissues. Furthermore, DX concentrations after DXAsp administration were elevated in both mucosa and muscle tissues, making DXAsp interesting for the treatment not only of the inflamed mucosa in ulcerative colitis but also in Chron's disease.

Previously, Zunino et al. (1984) investigated the biological activity of daunorubicin bound to poly-L-aspartic acid (PAA) in comparison with free drug against leukaemia. C3H/He and BDF1 adult mice of both sexes were treated with daunorubicin and doxorubicin 0.1 mL/10 g body weight and administered i.v. or i.p. Polymeric derivatives were dissolved in distilled water or physiologic saline for i.p. or i.v. administration, respectively. Authors found a dose-dependent antitumor activity for both free and PAA-daunorubicin. However, at high doses (>4 mg/kg), the toxicity of free daunorubicin exceeded the therapeutic activity, resulting in a reduction of survival. This was not observed for PAA-daunorubicin. The conjugate showed also an increased effectiveness of daunorubicin.

Starting from studies performed on gentamicin, Sun et al. (2010) used the properties of polyaspartate (innocuity, hydrophilic biodegradability, solubility and degradability under physiological and biological conditions) to create a matrix to increase the ibuprofen dissolution and absorption. The matrix was created by introducing beta-ciclodextrin in Polyaspartate matrix (PASP-CD).

Polyaminoacids are also attractive candidates for the production of tissue-engineering matrices, including collagen-like and silk-like proteins. Cai et al. (2001) introduced polyaspartic acid as a bioactive moiety to modify poly(D,L-lactic acid) (PDLLA) film, widely used in tissue engineering. This binding improved the matrix hydrophilicity and biocompatibility and thus the cell affinity.

Polyaspartate was investigated by Guan et al. (2003), as a moderator of adhesion and the subsequent biofilm formation due to the activity of oral bacteria. The inhibition of bacterial adhesion by PA was assessed by a 30-min incubation with *Streptococcus sanguis* in a microtitre assay with the wells coated with hydroxyapatite (HAP) and an 18-h challenge with human salivary microflora in a HAP disc assay. The HAP disc assay showed that PA, both in aqueous solutions and in toothpaste, reduced the level of adhered microflora.

Sodium polyaspartate was studied as a stabilizing agent for clinical application. Maarek et al. (2001) investigated the use of polyaspartate as a stabilizing agent of indocyanine green (ICG), a compound widely used in cardiovascular, hepatic, and ophthalmologic studies. Application of this fluorescent dye has been handicapped by its poor stability in solution and by the complex dependence of its fluorescence intensity on concentration. Addition of sodium polyaspartate preserved the fluorescence of ICG and facilitated the use of this dye in vascular, ophthalmologic and hepatic studies.

Toxicology

The acute oral toxicity was performed in 3 Wistar rats, by using the OECD TG 423 test. Polyaspartic acid sodium salt was administered as a 42.8% aqueous solution (2000 mg/Kg pc), and the animals were observed for 14 days (Australian Government 2001). The LD50 resulted > 2000 mg/Kg. Neither mortality nor signs of toxicity were observed. The same results were found by Lanxess company In a more recent document, Lanxess reports a LD50 > 5000 mg/kg bw (LANXESS 2011).

In addition, a subacute study was carried out in which groups of male and female Wistar rats received the polymer by daily gavage for a period of four weeks, followed by a recovery period of two weeks. Dose levels were 0, 40, 200, and 1000 mg/Kg bw/day. Apart from microscopic effects on the urinary bladder that have been found to be species (rat) specific, there were no adverse effects in this study (Australian Government 2001).

Dermal toxicity was tested in 3 Wistar rats by using the OECD TG 402 test. Doses of 2400 mg/Kg bw of polyaspartic acid were administered under occlusive dressing for 24 hours, as 42.8% aqueous solution. Neither mortality nor signs of toxicity were observed. The LD50 was > 2400 mg/Kg (Australian Government 2001) or > 2000 mg/kg bw (LANXESS 2007) or >5000 mg/kg bw (LANXESS 2011).

Skin irritation was tested in 3 males of rabbit/Himalayan after administration of 0.5 mL of the polymer, in a 42.8% aqueous solution, under semi-occlusive dressing for 4 hours. Test was performed as indicated in the OECD TG 404 test method. No signs of erythema or oedema were seen in any of the animals at any of the observation times (Australian Government 2001).

Eye irritation was tested in 3 males of rabbit/Himalayan. Volumes of 0.1 mL of 42.8% aqueous solution placed into conjunctival sac of one eye of each animal was used. The test was performed as indicated in OECD TG 405 test method). Animals were observed for 60 min, 24, 48 and 72 hours. No signs of irritation of the cornea, iris or conjunctivae were observed in any animal at any of the observation times (Australian Government 2001).

Skin sensitisation was tested in guinea pigs (10 test and 5 control animals). The test was performed according to OECD TG 406. Polyaspartate polymer was not sensitising to the skin of guinea pigs

Genotoxicity

To test genotoxicity, Bayer used *Salmonella typhimurium* Reverse Mutation Assay. The test was performed in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, TA1537, according to the protocol described in OECD TG 471 test method. Polyaspartate concentration range used was 50-5000 microg/plate. The independent repeat test was performed using a 20 minute pre-incubation period; there were no signs of toxicity to any of the test strains at any of the concentrations employed. Solvent control plates gave counts in the expected range and positive controls produced significant increases in the revertant counts (Australian Government 2001).

Lanxess (2007) evaluated the polyaspartate *in vitro* genotoxicity, including a reverse mutation assay (Ames test) in *Salmonella typhymurium*; a chromosomal aberration assay in Chinese hamster V79 cells; and a forward mutation assay at the HPRT locus in Chinese hamster V79 cells. No evidence of genotoxic activity was observed in any of these assays.

Environmental effects

Tests on fish were performed using ten specimen fish per test concentration at a temperature of 21°C and using nominal concentrations of 3160 and 10000 mg/L. Observations were performed at 2, 24, 48, 72 and 96 hours. The results showed that no mortalities or sublethal effects were observed in the vessel containing 3160 mg/L of polyaspartate. After 96 h, 80% mortality was observed at a test concentration of 10000 mg/L (Australian Government 2001).

The immobilisation tests with Daphnia magna were performed under static conditions with observations performed at 24 and 48 hours. The test was performed using 10 daphnids per flask at a temperature of 20°C. The tests were conducted using nominal concentrations of 625, 1250, 2500, 5000 and 10000 mg/L. After 48 h, no immobilised daphnids were observed in the test vessels with less than 2500 mg/L of the notified polymer and 100 % mortality was observed after 48 h at test concentrations above 5000 mg/L. The 48-hour EC50 for polyaspartate to Daphnia magna is 3536 mg/L of the notified polymer.

Algae were exposed to the test substance at concentrations of 125, 395, 1248 and 3959 mg/L for 72 h at 24°C under constant illumination and shaking. After 72 h, the percentage inhibition of biomass for the test vessels containing 125, 395, 1248 and 3959 mg/L of the notified polymer was 2.3, 48.1, 84.3 and 92.2 %, respectively, and the percentage inhibition of growth rate was 0, 25.0, 66.7 and 66.7 %, respectively. The 72 h EbC50 (for biomass) and ErC50 (for growth rate) for the polymer to *Scenedesmus subspicatus* is 528 mg/L and 1070 mg/L, respectively (Australian Government 2001).

Biodegradability

Three studies were performed by Bayer to assess the biodegradability of polyaspartate. In the first one, aimed to assess "ready biodegradability" and performed in accordance with modified OECD Guideline 301 E, 74% of the test material degraded in 28 days (Australian Government 2001). In a second study aimed to assess "inherent biodegradability" and performed in accordance with OECD Guideline 302 E, 77% of the test material had degraded in 28 days and 81% had degraded in 56 days (LANXESS 2007).

In a third study, aimed to assess "anaerobic biodegradability" and performed in accordance with the IS0 1 1734 Test, 30% of polyaspartic acid sodium salt degraded under anaerobic conditions within 56 days (LANXESS 2007).

In addition, polyaspartate was evaluated for biodegradability in the modified Sturm test (OECD 301 E), in which degradation of the test compound was measured in terms of generated CO2 (LANXESS 2007). Degradation of polyaspartate was similar to control compound (glucose) while degradation of a standard polyacrylate was considerably lower. Under aerobic conditions, the probable degradation products would be sodium and nitrate ions, carbon dioxide and water. Anaerobic decomposition would ultimately yield sodium ions, methane, carbon dioxide, water, and ammonia.

In the papers by Tabata et al. (1999, 2000), the biodegradation of polyaspartic acid by two strains of *Pedobacter* spp. and *Sphingomonas* spp. was described. Bacteria from river water identified as a member of genus of *Sphingomonas* could degrade only polyaspartate having molecular weight below 5000 (low molecular-weight polymers), while the *Pedobacter* strain was able to degrade also high molecular weight polyaspartate. The biodegradability was measured by biochemical oxygen demand (BOD) assay and by gel permeation chromatography (GPC), after incubation of a freshwater sample taken from the river of Arakawa (Saitama, Japan) with 0.175 mg/mL of polyaspartate. The BOD-biodegradability of polyaspartate (low molecular weight) increased with time to reach about 78% within 15 days and was completely degraded within 12 days, as measured by GPC. As regarding polyaspartate with high molecular weight (5000-10000), a small portion of polymers and a small amount of low-molecular-weight-products were still present after 12 days.

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E.1.2 Data related to surveys on chemicals or other substances in food

Not available.

E.1.3 Data related to epidemiological/intervention studies in humans

Not applicable.

F. ASSESSMENT PROCEDURE

The Applicant suggests that the appropriate assessment procedure is General Procedure Level 1. There are no perceived health risks from its approval and the proposed use and use levels of potassium polyaspartate as a stabiliser in wine.

G. CONFIDENTIAL COMMERCIAL INFORMATION

There is no confidential commercial information incorporated in this application**.**

H. OTHER CONFIDENTIAL INFORMATION

There is no other confidential information incorporated in this application.

J. INTERNATIONAL AND OTHER STANDARDS

J.1 INTERNATIONAL STANDARDS

Potassium polyaspartate has been evaluated by the European Food Safety Authority (EFSA) and by the Organisation de la Vigne et du Vin (OIV) and the for winemaking. It was duly approved and authorised for use as an additive for wine in the European Union (EU) up to 10 g/hL (Commission Delegated Regulation (EU) 2017/1861 of 2 August 2017 amending Regulation (EC) No 606/2009 as regards certain oenological practices). It has also been included in the OIV International Code of Oenological Practices as follows (http://www.oiv.org/public/medias/5119/code-2017-en.pdf):

International Code of Oenological Practices
OIV Code Sheet – Issue 2017/01 II.3.3-17

3. WINES
3.3.15. TREATMENT WITH POTASSIUM POLYASPARTATE
(OENO 543/2016)

Definition:
Addition of potassium polyaspartate to wines

Objective:
Contribute to the tartaric stabilization of wines

Prescription:
a) The optimum dose of potassium polyaspartate used to stabilize wines, also those with a high degree of tartaric instability, must not exceed 10 g/hL. At higher doses, the potassium polyaspartate's (KPA) stabilizing performance is not improved and, in some cases, an increase of wine turbidity could be induced;
b) for red wines with high colloidal instability, prior treatment with bentonite is recommended;
c) the application of potassium polyaspartate must be in accordance with the prescriptions of the International Oenological Codex.
Recommendation:
Accepted.

Potassium polyaspartic acid is not listed by the US National Toxicology Program (NTP) or the International Agency for Research on Cancer (IARC), nor is it regulated as a carcinogen by the US Occupational Safety & Health Administration (OSHA). Authorisations and evaluations are available, however, for the sodium salt of polyaspartic acid (CAS 34345-47-6), the polyaminoacid containing sodium instead of potassium.

The sodium salt of polyaspartic acid is authorised for use in USA and Australia as a food contact substance, as a dispersant for fillers and an anti-scale additive in sugar processing; as a water treatment agent used as a scale inhibitor in cooling tower and boiler water applications, with properties of non-phosphor, non-nitrogen, non- pollution and complete biodegradation; as a coadjuvant in the prevention of dental plaque, and as an excipient in certain drugs.

J2. OTHER NATIONAL STANDARDS OR REGULATIONS

There are no other national standards or regulations for potassium polyaspartate.

K. STATUTORY DECLARATION

To be attached.

L. CHECKLIST

To be attached.

A. Technical information on the food additive

A.1 Nature and technological purpose of the additive

The proposed use of potassium polyaspartate is as a new food additive to be used as a stabilizer against tartrate crystal precipitation (anti-scaling additive) in wine (red, rosè and white wine) at the suggested use level of 100-200 mg/L (normal use level) and at the maximum use level of 300 mg/L.

A.1a Each of the technological purposes listed in Schedule 14 – Technological purposes performed by substances used as food additives that the additive fulfils.

The additive potassium polyaspartate is a stabiliser used in winemaking. From the Australia New Zealand Food Standards Code, Schedule 14, as follows:

Technological purposes

Purpose	*Sub-classes*	*Definition*
Stabiliser	binder, firming agent, water binding agent, foam stabiliser	maintains the homogeneous dispersion of two or more immiscible substances in a food

A.1b The reason why the food additive is needed to fulfil these purposes in each of the foods in which it is proposed to be used.

Wine, in its normal state, is naturally supersaturated with significant concentrations of potassium bitartrate and calcium tartrate. Potassium bitartrate can form crystals and precipitate from the wine during storage resulting in undesired sediment in the wine. Although potassium bitartrate crystals in wine do not pose a health risk, their presence affects the aesthetics and consumer acceptability of the wine. Accordingly, before the delivery for domestic or international trade, white wine and sparkling wines in particular, need to be stabilised against tartrate salt precipitation, which is from a natural grape source. The concentration of tartaric acid itself in the wine, alcohol, colloids, calcium, and potassium, the pH value of the wine, the duration and temperature of storage, and the surface (roughness) of the storage container all impact on tartrate salt precipitation in wine (Pilone and Berg 1965). Tartaric acid predominantly precipitates as the potassium salt followed by calcium salt.

Various physical and chemical techniques are currently applied during the winemaking process to prevent the formation of these deposits by reducing the concentration of potassium and tartaric acid in wines. Traditionally, part of the potassium bitartrate present in wines is removed by precipitation at low temperatures (cold stabilization) (Ribéreau-Gayon et al. 2006) but recently new techniques have been authorized such as electrodialysis (Moutounet and Escudier, 1991) and cation exchangers (Mourgue, 1993). Nonetheless, all these subtractive techniques have some disadvantages: cold stabilization is expensive and can sometimes be poorly selective with respect to phenols and polysaccharides (Gómez Benı´tez et al., 2003); electrodialysis requires a high initial investment for the machinery and involves high operating costs and, unlike cold stabilization, is sometimes excessively selective towards phenols and polysaccharides; finally, the use of cation exchangers is not recommended when the instability is due to an excessive tartaric acid content. Thus, these physical methods are expensive and difficult for smaller wineries to adopt.

Currently, the additives allowed by the European Union to stabilise wine to prevent the precipitation of tartrate into the relatively insoluble potassium bitartrate and calcium tartrate are metatartaric acid, cellulose gums and mannoproteins (Peynaud and Guimberteau 1961, Marchal and Jeandet 2009, Guise

et al. 2014). Metatartaric acid is very effective for all wine types but is unstable over time de-esterifying into tartaric acid within few months or after a few days under heating, and can be used only for wines with short shelf life; cellulose gums are effective and stable in white wines but can cause colour instability when used in red wines; mannoproteins have a variable effect and therefore need preliminary tests to evaluate their effectiveness and to determine the optimal dose, which is specific to the wine being treated (Claus et al., 2014; Crachereau et al. 2001; Gerbaud et al., 2010; Lubbers et al. 1993; Moine-Ledoux and Dubourdieu, 2002). Thus, among the crystallization inhibitors, only metatartaric acid is considered as a complete inhibitor of tartaric salt crystal nucleation and growth, until its hydrolysis occurs as the other additives merely slow crystal appearance and possibly their growth.

The presence of deposits of inorganic crystals in living organisms (biomineralization) is a ubiquitous phenomenon. Polyanionic proteins are the most important organic molecules that interact in living organisms with inorganic crystals, thus limiting their formation. The main anionic residues of the polyanionic proteins are aspartic acid and phosphorylated serine (approximately 80 mol.% of the total amino acid composition) (Joentgen et al., 2005). When dissolved in solution, these proteins can inhibit crystal nucleation and growth, thus modifying the morphology of the crystals that are being formed (Steinbüchel and Fahnestock, 2003). Moreover, the formation of scales has always been a common problem for many industrial processes and, in the past, the use of organic polymers with low or null biodegradability to limit the precipitation caused further environmental problems. With the aim of finding biodegradable polymers to control the precipitation of inorganic salts in liquid matrices, while still being eco-friendly, an attempt was made to mimic the structural and functional properties of polyanionic proteins (Sikes et al. 1991). These studies led to the development of polyaspartic acids: a new class of synthetic polyamides which are structural and functional analogues of subdomains of biomineralization- controlling proteins. Polyaspartic acids as homopolypeptides have not been found in nature and are not accessible via biotechnological procedures (Joentgen et al., 2005).

Polyaspartic acids are synthesized by the thermal polymerization of L-aspartic acid (ASP) or monoammonium malate resulting in polysuccinimide, which has a relatively low molecular weight, followed by hydrolysis. The presence of polycondensation accelerants, preferably phosphoric acid, results in polymers which are 100% biodegradable and have a higher molecular weight (up to 64 kDa) compared with other methods. The polymers synthesized without a catalyst have a lower molecular weight (10–15 kDa) and can only reach 70% biodegradability (Thombre and Sarwade, 2005).

Some of the first publications concerning the applications of polyaspartic acids involved the inhibition of calcium sulfate scale formation (Sarig and Shifrin, 1977). Sodium polyaspartate is now used to prevent fouling (by changing the crystal structure of calcium salts), as an additive in detergents, for the prevention of corrosion, as a coadjuvant in the prevention of dental plaque and as an excipient in certain drugs.

The aim of the European project STABIWINE (FP7, GA n. 314903, http://www.stabiwine.eu) was to identify new compounds for the tartaric and protein stabilization of wines. Comparative trials performed at laboratory level and then at industrial level with different kinds of polyaspartates, and specifically potassium polyaspartate, to verify their effectiveness as inhibitors of tartaric crystallization in wines, and against and some oenological additives already authorized, such as metatartaric acid. Potassium polyaspartate (KPA) added to wine even in very low dosage has been found to inhibit the formation of tartrate crystals; the natural wine composition is not altered by subtraction of salts, and no refrigeration or other physical process are required. The addition of the alternative additives metatartaric acid, carboxymethylcellulose and yeast mannoproteins, because of limited efficacy of the former two additives (see Figure below), or of cost of the third, these technologies are not universally permitted and applied.

Change in tartaric acid concentration (g/L) after cold test

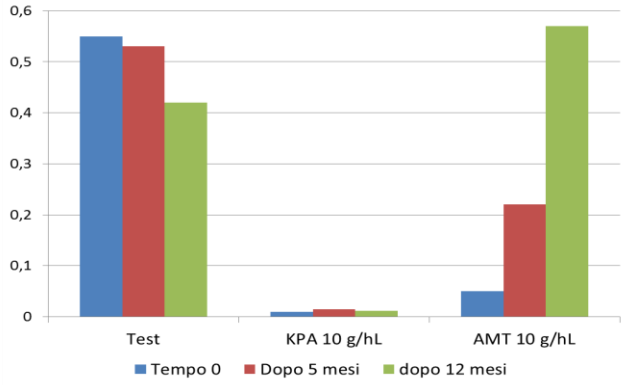


Figure: The difference in tartaric acid content in wines was determined by high performance liquid chromatography before and after storage at 4°C for 6 days (cold test). The difference in tartaric acid concentration before and afetr the cold test, due to precipitation of potassium tartrate, represents an index of stability/instability of the wine. Cabernet Sauvignon wine was treated with 100 mg/L potassium polyasparate (KPA) and metatartaric acid (AMT) and stored at -4°C for 6 days. The chane in tartaric acid concentration was determined after 0, 5 and 12 months storage compared to a control (test). Metatartaric acid's effectiveness was clearly lost within a few months (Bosso et al. 2015).

The additive potassium polyaspartate, therefore, presents significant qualitative, technological, environmental and economic advantages on practices currently applied by the international wine industry. Potassium polyaspartate is:

- is more efficient than all other additives in inhibiting the formation of tartrate crystals;
- it works in all wines of different types, origins and instability levels;
- has no effect on taste or aroma of wines, even at very high dosages;
- is stable over time, contrary to metatartaric acid;
- does not cause colour instability as for carboxymethylcellulose in some red wines;
- does not show fouling effect at membrane filtration;
- is produced from natural and renewable row materials; and
- is totally biodegradable and constitute no danger for the operators.

Addition of potassium polyaspartate has the lowest carbon and water footprint among all tartrate stability treatments, where cost of the treatment is very low, and it can be easily adopted even by the smallest wine producers.

The dose of potassium polyaspartate to be used depends on the level of instability of the wine to be treated. In most cases, an addition of 100 mg/L of potassium polyaspartate into wine, providing a 100 mg/L (100 ppm) concentration in the final product, is sufficient to obtain a complete inhibition of tartrate crystal formation during storage. In case of wines with very high levels of tartrate instability, the dose can increase up to 200 mg/L (200 ppm) and very occasionally to 300 mg/L (300 ppm). Therefore, a conservative approach has been used to calculate the consumer exposure by using 300 mg/L as maximum value (300 ppm).

Before addition to wine, the powder is first dissolved by agitation in a quantity of cold water or wine equivalent to approximately ten times the weight of polyaspartate. The low viscosity of this concentrated solution (20-30 centipoise) does not pose any handling problem. The concentrated solution is then incorporated into the wine through a pumping over operation or by dosage on-line, in order to uniformly disperse the additive in the whole mass of wine, avoiding aeration of the wine with

consequent oxidative reactions. After addition, the wine continues its production process toward final filtration, bottling and packaging.

A.1c Evidence that the amounts proposed to be added are consistent with achieving the technological purpose.

The effectiveness of polyaspartate in wine treatment was mainly assessed during the European research project StabiWine (FP7-SME-2012-2, n. 314903). A detailed summary of the experiments conducted during the project and their results are presented in Table 1.

The results of the efficacy studies showed that polyaspartic acids such as potassium polyaspartate have the capacity to stabilize red and white wines with a high level of tartrate instability. The effect was similar to the effect of metatartaric acid used at the same doses (100 mg/L).

In contrast to metatartaric acid, however, the polyaspartic acids maintained their stabilizing properties over time, for at least one year of aging. Differences between the tested polyaspartic acids were observed for the stability during storage and accordingly potassium polyaspartic acid (A-5KD) was chosen as it provided the best stability over time.

The effect of the presence of polyaspartic acids on colour stability in red wines was also studied. The colour stability was directly verified by monitoring the anthocyanin content and colour parameters of the control and treated wines during bottle aging. No differences were observed between the samples over time.

In some red wines an increase in turbidity was observed immediately after the addition of polyaspartic acids. Such turbidity did not increase during aging, and was prevented in samples treated with bentonite. It was thus considered advisable to plan a filtration step of the wine before bottling in case turbidity occurred. The efficiency of polyaspartic acids was unchanged after the filtration with 0.45 µm- filters, either at the addition, or after six months of bottle aging.

For white wine, the presence of polyaspartic acids did not cause turbidity and did not have any effect on colour parameters and colour evolution during bottle aging.

Table 1: Summary of the efficacy and stability experiments

The efficacy and stability of different polyaspartate salts (PAA) was studied during the StabiWine project. In the following table the experimental results are reported also for sodium polyaspartate (Na-PAA) even if not relevant to the current submission. The results from the first experiments were subsequently used to screen the different polyaspartic acids, where a decision made to focus on potassium polyaspartate (K-PAA).

Terms used: A-5DK = product code for potassium polyaspartate; KHT = potassium tartrate; CMC = carboxymethylcellulose; MTA = metatartaric acid; polyaspartate salts = PAAs

Short description of the experience	Typologies of wines	Results	Remarks
Experience 1 - ***Screening of 4 new PAAs*** (3 Na-PAAs and 1 K-PAA) to evaluate the stabilizing effect against	2 Cortese white wines, 2 Barbera red wines and 1 blend of	Good stabilizing effect similar to the control, in particular for the red wines. No differences among the PAAs in the stabilizing capacity	

Short description of the experience	Typologies of wines	Results	Remarks
KHT precipitations (by mini-contact and cold tests) in comparison with MTA. Dose of 100 mg/L (10 g/hL) for all the additives.	different red wines.		
Experience 2 - **Part A**: ***Screening of 4 new PAAs*** (3 Na-PAAs and 1 K-PAA: A-5DK) to evaluate the stabilizing effect against KHT precipitations (by mini-contact and cold tests) in comparison with MTA. Dose: 100 mg/L (10 g/hL) for all the additives. **Part B**: Evaluation of the effect of the 4 PAAs on colour stability and turbidity of red wines, in comparison with MTA and CMC.	**Part A:** 8 wines of different vintages (7 red wines and 1 white wine). **Part B**: 14 red wines from different vintages.	**Part A:** Good stabilizing effect similar to the control. No differences among the PAAs in the stabilizing capacity. Only in one case all the additives, included MTA, could only reduce the instability degree of the wine (highly unstable Barbera wine). **Part B**: No increase of wine turbidity was observed after the addition of the different products. For the PAAs trials an increase in instability was observed in 5 cases out 13.	
Experience 3 - ***Study of the stabilizing effect against tartaric precipitations*** (by mini-contact and cold tests) of 3 PAAs (2-Na and 1 KPA) in comparison with MTA, at the dose of 100 mg/L (10 g/hL), after the addition of the products and after 5 and 12 months of bottle aging. Study of the evolution over time of the aspartic acid content in wines. Evaluation of wine turbidity and colour stability test over time.	1 Cortese wine, 1 Barbera wine and 1 blend of different red wines.	The PAAs are more stable than MTA. After 1 year of bottle aging, all the white wines with added PAAs remained stable while the trials with added MTA became unstable. In the red wines the MTA lost its efficiency already after 5 months. The PAAs were less stable in the red wines than in the white one, and the behaviour varied with the type of wine: in any case, 1 trial out of 3 for both red wines resulted stable after 1 year of aging.	
Experience 4 -: *Study of the stabilizing effect against the tartaric precipitations* (by mini-contact and cold tests) of 4 new PAAs (3 Na-PAAs and 1 KPA: A-5DK)	1 Dolcetto red wine	The PAAs are much more stable than MTA. After 1 year of bottle aging, all the wines with added PAAs, including A-5DK, were already stable while the trials with added MTA were unstable after 3 months	

Short description of the experience	Typologies of wines	Results	Remarks
in comparison with MTA, at the dose of 100 mg/L (10 g/hL), after the addition of the products and after 3, 6 and 12 months of bottle aging. A comparison also concerned the trials with or without the addition of Arabic gum (AG). Determination of the aspartic acid content in wines after 12 months of bottle aging. Evaluation over time of wine turbidity and colour stability.		of bottle aging. After bottling and during aging, no effect of the addition of PAAs and MTA on the content of the total anthocyanins and flavonoids, colour stability and turbidity was observed. The colour/colloidal stability test, for which the PAAs trials were more unstable than the control and MTA trials, did not predict the behaviour of the wines during aging.	
Experience 5 - *Effect of the dose.* Study of the stabilizing effect against the tartaric precipitations (by mini-contact and cold tests) of 2 PAAs (1Na-PAA and 1 KPA: A-5DK) at 2 different doses (100 and 200 mg/L), measured 48 h after the addition and after 6 months of bottle aging. ***Effect on the oxidation process of a white wine.*** Study of the effect of 2 PAAs (1 Na-PAA and 1 KPA: A-5DK) at 2 different doses (100 and 200 mg/L) in comparison with the control on the colour stability of a white wine during aging.	1 white wine	The dose of 100 mg/L (10 g/hL) was enough to stabilize the wine. The behaviour at the cold test of the trials added of different doses of PAA was similar. On the contrary, significant differences between the two doses were observed by mini-contact test. The PAAs had no effect on the oxidation process of the white wine and on the reduction of the risk of browning.	
Experience 6 - ***Effect on the oxidation process of a white wine.*** Study of the effect of 1 KPA (A-5DK) at 2 different doses (100 and 200 mg/L) in comparison with the control on the	1 white wine (blend of Pecorino and Riesling)	The KPA had no effect on the oxidation process of the white wine and on the reduction of the risk of browning.	

Short description of the experience	Typologies of wines	Results	Remarks
colour stability of a white wine during aging.			
Experience 7 - *Study of the stabilizing effect* (by mini-contact and cold tests) against the tartaric precipitations of 1 KPA (A-5DK) at 2 different doses (100 and 200 mg/L) in white and red wines with different composition (pH=3.00 and pH=3.70; 11.5% and 15% of alcohol).	1 white wine and 1 Montepulciano d'Abruzzo red wine	The variation of the composition of the wine influenced the wine tartaric instability. The instability increased by increasing the pH and the alcoholic degree of the wines. The differences were more important in the white wine, more unstable, than in the red wine. In all cases the dose of 100 mg/L (10 g/hL) was enough to stabilize the wines. The behaviour at the cold test of the trials added of different doses of KPA was similar. On the contrary, significant differences between the two doses were observed by mini-contact test, but they were modest and without any practical interest.	
Experience 8 - *Study of the stabilizing effect* (by mini-contact and cold tests) against the tartaric precipitations of 1 KPA (A-5DK) at the dose of 100 mg/L on white wines with different composition (pH=3.00 and pH=3.70; addition of 2600 mg/L of polyphenols and no addition of polyphenols).	1 white wine	The variation of the wine composition influenced the wine tartaric instability. The instability increased by increasing the pH and by decreasing the polyphenols content of the wines. In all cases the dose of 100 mg/L (10 g/hL) of KPA was enough to stabilize the wines.	
Experience *9* - *Study of stabilizing effect* (by mini-contact and cold tests) against the tartaric precipitations of 1 KPA (A-5DK) at the dose of 100 mg/L on red wines originated from a same wine submitted to different fining treatment (no	1 Syrah red wine	The fining with gelatine and vegetal proteins did not modify the tartaric instability of the wines, while the bentonite treatment caused an increase in tartaric instability. The KPA stabilized all the wines against tartaric precipitations. The addition of KPA caused an increase of wine colloidal instability and	The results of the present experiment are very interesting from a practical point of view. A treatment with bentonite seems to solve the problem if the wine becomes turbid

Short description of the experience	Typologies of wines	Results	Remarks
treatment, bentonite, gelatine, gelatine + bentonite, vegetal proteins, vegetal proteins + bentonite). Study of the evolution over time of colour parameters, total anthocyanins and flavonoids content and turbidity (after 1, 3 and 6 months of bottle aging).		turbidity. When the wines were treated with bentonite before the addition of KPA this effect was not observed. During aging the turbid wines became limpid by sedimentation and no further haze appeared.	after KPA addition. The appearance of turbidity is fast, during the first days after the KPA addition and can be solved by filtering the wine. Some trials on the effect of filtration on the modification of the tartaric stability were also carried out.
Experience 10- *Study of the stabilizing effect* (by mini-contact and cold tests) against the tartaric precipitations of 1 KPA (A-5DK) at the dose of 100 mg/L on wines originated from the same white wine submitted to different fining treatment (no treatment, bentonite, casein, casein + bentonite, vegetal proteins, vegetal proteins+ bentonite). Study of the evolution over time of colour parameters and turbidity (after 1, 3 and 6 months of bottle aging).	1 white wine	The original wine resulted already stable and the fining with casein and vegetal proteins did not influence its tartaric stability; also, the treatment with bentonite had no effect. No relevant effect of KPA on turbidity and wine colour was observed after addition and during aging.	The addition of KPA did not cause the appearance of turbidity nor modification of colour in the white wines.
Experience 11 - Study of the tartaric stabilizing effect (by mini-contact and cold tests) of 1 KPA (A-5DK) in comparison with MTA, at the dose of 100 mg/L in wines originated from a same white wine submitted to different treatments (no treatment, addition of arabic gum, arabic gum+ filtration, addition of	1 white wine	The addition of KPA and MTA allowed to stabilize the wines against the KHT precipitation (cold test) in the wines originally instable. The wines added with arabic gum resulted less instable than the control. The filtration did not modify the stability degree of the wines. After 6 months of bottle aging the wines added with KPA were stable, while the MTA trials became as	The addition of KPA did not cause the appearance of turbidity nor modification of colour in the white wines. The filtration after the KPA addition did not modify the degree of wine tartaric stability.

Short description of the experience	Typologies of wines	Results	Remarks
tannins, tannins + filtration) after the addition of the products and after 6 months of bottle aging. Study of the evolution of the aspartic acid content, turbidity and colour parameters (A420 and CIELab parameters) over time.		unstable as the control. No effect of KPA on turbidity and wine colour was observed after addition and during aging.	

Thus, it can be concluded that polyaspartates are an effective stabilizing agent, able to inhibit the formation of tartrate crystals while preserving the original wine composition. Potassium polyaspartate is to be preferred to the sodium salt for use in wine, as in the first case provision of cations to wine with the treatment is negligible (less than 5% of the natural potassium content).

The addition of 100 mg/L (10 g/hL or 100 ppm) potassium polyaspartate has been shown to inhibit the formation of tartrate crystals in both white and red wines, even in case of high instability (up to 450 ΔμS/cm measured with mini-contact test). Its stabilizing effect has longer persistence than metatartaric acid which in an acid environment is de-esterified to tartaric acid and progressively loses its stabilizing capability in few months. Potassium polyaspartate is chemically stable in wine conditions and its effect remains unchanged for long periods of time.

In contrast to carboxymethyl cellulose (CMC), potassium polyaspartate is very easy to solubilize in water and in wine, and its addition to wine immediately before bottling does not affect its membrane filterability, even at 0.45 μm porosity.

Due to its high negative specific charge at wine pH, potassium polyaspartate has been shown to interact with wine colloids like proteins and polyphenols, increasing of turbidity of instable wines. Therefore, if required, a remedial procedure of wine fining with bentonite following the addition of potassium polyaspartate is recommended.

From a sensory perspective, no variation in taste and aroma of wines following stabilization with potassium polyaspartate has been reported, even following the addition of high dosages at 300 mg/L (30 g/hL or 300 ppm).

In some cases, a reduced sensitivity of potassium polyaspartate-added wine to oxidation was observed, probably to be linked with a chelating effect of the polymer toward bivalent cations (Fe^{2+}, Cu^{2+}) which are known to catalyze the oxidation reaction of some wine components.

A.1d If the food additive is a preservative, data to demonstrate its effectiveness in each of the food groups in which it is proposed to be used.

Not applicable.

A.1e Information is required on how the food additive is incorporated homogenously and stably into the different food matrices to which it is proposed to be added. Data should also be provided to address losses of the substance from the foods during normal shelf life conditions.

A.1.3.1 Summary of stability studies

The stability of the potassium polyaspartate (A-5D K/SD) in wine was assessed both directly and indirectly. Direct assessment was by measuring the concentration of aspartic acid unit (the potential degradation product of polyaspartate, that is also naturally present in wine) during the wine storage. Indirect assessment was by the evaluation of the effectiveness of the additive on the stability of various wine characteristics over time.

For this purpose, the potassium polyaspartate (A-5D K/SD) was added to different red and white wines, which were then bottled and analyzed during aging, after five and 12 months of storage. Only a small increase in the aspartic acid concentration was observed in all the wines after five and 12 months of bottle aging. After 5 months, a maximum increase of aspartic acid concentration of 1.3% for the white wine and 6.3% for the red wine was observed, while after 12 months the maximum increase of aspartic acid was 4.8% (white wine) and 7.5% (red wine) after addition of 100 mg/L potassium polyaspartate.

Similar results were obtained from two additional experiments on two wines (one white wine and one red wine), to which potassium polyaspartate (A-5D K/SD) was added, and monitored over time by assessing the tartaric stability and by quantifying the release of aspartic acid monomers.

After addition of 100 mg/L of potassium polyaspartate (A-5D K/SD) to a Dolcetto variety red wine, the concentration of aspartic acid after 12 months of storage showed that the difference between the treated and the control wine is limited to 1.75% of the potassium polyaspartate (A-5D K/SD) added to the wine. This value is close to the impurity of the commercial preparation potassium polyaspartate (A-5D K/SD) in aspartic acid (about 1%), residual of the row material used in the production process.

The tartaric stability was also monitored through analysis of tartaric acid content before and after cold storage, showing that the wine treated with potassium polyaspartate (A-5D K/SD) remained stable for 12 months of storage at room temperature, while the instability of control wine increased over time.

The same experiment was repeated on white wine. Results obtained after 6 and 12 months of storage at room temperature confirm that only the batch added of 100 mg/L of potassium polyaspartate (A-5D K/SD) remained stable. In fact, data obtained by analysis of free aspartic acid show a non-significant analytical difference between the control and the added wine, suggesting that also in white wine the degradation of potassium polyaspartate (A-5D K/SD) into its monomer is negligible.

Assuming that hydrolysis is the main reaction occurring in wine during storage, aspartic acid is the only degradation product formed. The quantification of the aspartic acid after storage shows a non-significant increase compared to the control, thus it can be concluded that the potassium polyaspartate (A-5D K/SD) is stable in wine for at least 12 months. This conclusion is also supported by the maintenance of the stabilization effect over time as observed in the experiment where the tartaric stability was measured.

A.1.3.2 Stability studies

The stability of the additive, as produced and in food during storage, have been evaluated as described below.

A.1.3.2.1 Stability of the additive as produced

Two samples of potassium polyaspartate (A5D K/SD, batch no. KHSH-070214-1) were stored for 8 weeks at 40°C and at 25°C, respectively. The determination of the dry matter content before and after storage did not show any difference as reported in the Table 2 below:

Table 2: % Dry matter in Samples A and B before and after storage

% Dry matter	T zero	After 8 weeks	Difference (%)
Sample A (40°C)	94.573	94.572	-0.001
Sample B (25°C)	94.566	94.569	0.003

As shown by the analysis of the four batches and reported in the specifications, as the dry matter content corresponds to almost 100% to potassium polyaspartate content, it can be stated that no degradation occurs during storage in accelerated conditions (40°C). Thus, a storage stability of at least four years is proposed for potassium polyaspartate (A5D K/SD, batch no. KHSH-070214-1)

Moreover, as presented in Table 3, analysis of two batches manufactured on 2012 shows that the content of potassium polyaspartate does not significantly differ (less than 5%) from the content detected in the two batches manufactured on 2014. Thus, it can be concluded that the active ingredient content is stable when potassium polyaspartate (A-5D K/SD) is stored also for long-term at room temperature. For more details please refer to the summary results listed in Tables 10 and 11 in this dossier.

Table 3: Content of potassium polyaspartate (KPA) in batches manufactured in 2012 and in 2014

Storage at ambient temperature	2012	2014	Difference (%)
Mean content of KPA	91.25	94.05	-3

A.1.3.2.2 Stability of the additive in water

Report:	Ref. 1.5/02, Marne S.K. (2014)
Title:	Stability Study of A-5D K SD in Water
Document No:	Study No. R/RA1385/SHA/14 (INTOX PVT. LTD.)
Guidelines:	None
GLP	Yes

Validated UV spectroscopic method was used for determination of concentration of test article in analytical grade water (please refer to point A.8.c of this dossier for a summary). The stability of the test article, potassium polyaspartate (A-5D K/SD, lot. No. KHKS-040412), in analytical grade water at room temperature for up to 24 hours was determined at two different dosages:

- 6.01 mg/mL (low dosage) formulation of the test article was prepared in analytical grade water. The concentration of test article in analytical grade water was determined before storage and was found to be 6.28 mg/mL. The formulation of the test article in analytical grade water was maintained at room temperature (27 ± 9°C) up to 24 hours. At the end of the 24-hour storage period, the concentration of the test article was determined and was found to be 6.19 mg/mL.

The % change in concentration of the test article in analytical grade water after 24 hours from its initial concentration (before storage) was found to be -1.43%; and

- 100.06 mg/mL (high dosage) formulation of the test article was prepared in analytical grade water. The concentration of the test article in analytical grade water was determined before storage and was found to be 98.42 mg/mL. The formulation of the test article in analytical grade water was maintained at room temperature (27 ± 9°C) up to 24 hours. At the end of the 24-hour storage period, the concentration of the test article was determined and was found to be 96.03 mg/mL. The % change in concentration of the test article in analytical grade water after 24 hours from its initial concentration (before storage) was found to be -2.42%.

The results show that the % change in the concentrations of the test article in analytical grade water after 24 hours at room temperature (27 ± 9°C) storage was found to be within the acceptance criteria of ± 20% from their initial concentrations (before storage).

Based on the results obtained in the study, it was concluded that potassium polyaspartate (A-5D K/SD) was stable in analytical grade water up to 24 hours at room temperature (27 ± 9°C).

A.1.3.2.3 Stability of the additive in wine

The physico-chemical stability of the additive during storage of the wine was mainly assessed during the European research project StabiWine (FP7-SME-2012-2, n. 314903).

A detailed summary of the experiments carried out during the project and their results are presented in Table 1. In particular, the stability of the additive was assessed both directly by measuring the concentration of aspartic acid during the wine storage and indirectly by the evaluation of the stability of the effectiveness of the products over time.

For this purpose, in a first study different polyaspartate salts (potassium polyaspartates manufactured by a different producer) were added to different red and white wines, which were then bottled and analyzed during aging. The longest storage period considered so far is one year of bottle aging. The concentration of aspartic acid in wines was quantified after five and 12 months of storage. Only a slight increase in the aspartic acid concentration was observed in all the wines after 12 months of bottle aging.

In Table 4, only the results of the experiments conducted with the potassium salt of potassium polyaspartate (PAA4) are reported.

After five months, a maximum increase of 1.3–6.3 mg/L compared to the control was observed for the white and the red wines, respectively. These values correspond to 1.3% (white wine) and 6.3% (red wine) by weight of the added amount of polyaspartate.

After 12 months, the PAA4 showed a maximum increase of 4.8–7.5 mg/L compared to the control, for the white and the red wine, respectively. These values correspond to 4.8% (white wine) and 7.5% (red wine) by weight of the added amount of polyaspartate (100 mg/L).

Table 4: Aspartic acid content in white and red wine after 5 and 12-months of storage

White wine [mg/L]				
Storage Time	**Sample**	**AA**	**Δ**	**% Δ**
After 5 months	control	6.1	-	-
	PAA4	7.4	1.3	**1.3%**
After 12 months	control	7.8	-	-
	PAA4	12.6	4.8	**4.8%**
Red wine CRA (red 2) [mg/L]				
Storage Time	**Sample**	**AA**	**Δ**	**% Δ**
After 5 month	control	7.9	-	-
	PAA4	14.2	6.3	**6.3%**
After 12 month	control	10.5	-	-
	PAA4	15.8	5.3	5.3%
Red wine CRA (red 3) [mg/L]				
Storage Time	**Sample**	**AA**	**Δ**	**% Δ**
After 5 month	control	4.5	-	-
	PAA4	8	3.5	**3.5%**
After 12 month	control	8.2	-	-
	PAA4	15.7	7.5	**7.5%**

Key words: AA = aspartic acid content expressed as mg/L; **Δ** = difference of aspartic acid content compared to control; % **Δ** = % of added potassium polyaspartate represented by **Δ**.

A similar experiment was repeated on two wines to which potassium polyaspartate (A-5D K/SD) was added which were monitored over time by assessing the tartaric stability and by quantifying the release of aspartic acid monomers.

Potassium polyaspartate (A-5D K/SD) (100 mg/L) was added to a Dolcetto red wine, and the tartaric stability was monitored through analysis of tartaric acid content before and after cold storage; the higher the difference between the two values, as reported below, the higher the tartaric instability of the wine. As shown in Table 5, the wine treated with potassium polyaspartate (A-5D K/SD) remained stable for 12 months of storage at room temperature, while the instability of control wine increased over time. In the same trial, metatartaric acid (MTA), the most common winemaking additive currently used for tartrate stabilization) was added to a batch of the same wine and its effectiveness was clearly lost within a few months. Data was processed with one-factor and two-factor ANOVA and Tukey's test to study the effect of the additives.

Table 5: Tartaric stability over time of a red wine to which potassium polyaspartate (A5D K/SD) was added, in comparison with a control and a red wine to which metatartaric acid (MTA) was added

Red wine (Dolcetto)

ΔH_2T (g/L)	48 hours	3 months	6 months	12 months
Control	0.158 b[1]	0.648 c	0.493 b	0.83 c
+100 mg/L A5D K/SD	0.035 a	0.025 c	0.008 a	0.00 a
+100 mg/L MTA	0.047 a	0.53 b	0.748 c	0.71 b

Key: [1]Different letters along the line discriminate treatments significantly different from one another ($p < 0.05$, Tukey's test).

The wine was also analyzed for its content of aspartic acid, the potential degradation product of polyaspartate, which is also one of the most represented amino acids naturally present in wine. As shown in Table 6, for the concentration of aspartic acid after 12 months of storage, the difference between the treated and the control wine is limited to 1.75 mg/L of aspartic acid (AA), which represents only 1.75% of the potassium polyaspartate (A5-D K/SD) added to the wine. This value is close to the impurity of the commercial preparation A5-D K/SD in aspartic acid (about 1%), residual of the row material used in the production process. Moreover, the data is coherent with the above observation on the maintenance of the stabilization effect over time documented above.

Table 6: Aspartic acid content (mg/L) in Dolcetto red wine after 12 months of storage

Storage time	Sample	AA (mg/L)	Change in AA (mg/L)	% Change in AA
12 months	control	1.55		
	A5D K/SD	3.30	1.75	1.5%

The same experiment was repeated with a white wine. The results as shown in Table 7 obtained after 6 months of storage at room temperature confirm that only the batch added of 100 mg/L of potassium polyaspartate (A5D K/SD) remained stable.

Table 7: Tartaric stability over time of a white wine to which potassium polyaspartate (A5D K/S) was in comparison with the control, and a white wine to which metatartaric acid (MTA) wad added

White wine

ΔH_2T (g/L)	48 hours	6 months
Control	0.32 a[1]	0.29 b
+100 mg/L A5D K/SD	0.08 b	0.02 a
+100 mg/L MTA	0.09 b	0.42 c

[1]Different letters along the line discriminate treatments significantly different from one another ($p < 0.05$, Tukey's test).

Data obtained by analysis of free aspartic acid show a non-significant analytical difference between the control and the added wine, suggesting that also in white wine the degradation of polyaspartate into its monomer is negligible. Assuming that hydrolysis is the main reaction occurring in wine during storage, aspartic acid is the only metabolite formed. The quantification of the aspartic acid after storage shows a non-significant increase compared to the control, thus it can be concluded that the A-5D K/SD is stable in wine for at least 12 months.

A.2 Information to enable identification of the additive

A.2.1 Identity of the substance

A.2.1.a Chemical name, when appropriate, according to IUPAC nomenclature rules

L-Aspartic Acid, Homopolymer, potassium salt

A.2.2.b CAS number, EINECS number (where appropriate), and other identification numbers

CAS no.: 64723-18-8

EC no.: E 456

A.2.2.c Synonyms, trade names, abbreviations

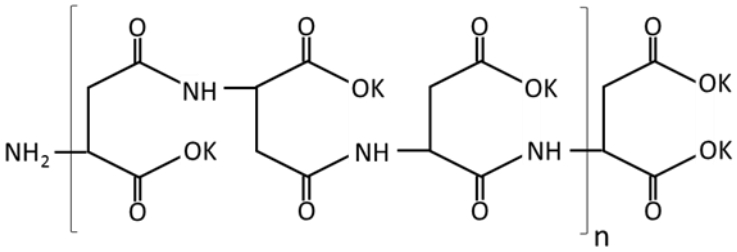
Trade name: Zenith Colour, Zenith UNO

Product code used in the dossier: A-5D K/SD

Other product codes and synonyms used in the dossier and in the study reports: A-5D K/SD; A-5D K SD; A-5DK/SD; A-5DK; KPA; potassium polyaspartate

A.2.2.d Chemical and structural formula and molecular weight or number average molecular weight and weight average molecular weight

Chemical formula: $[C_4H_5NO_3K]_n$

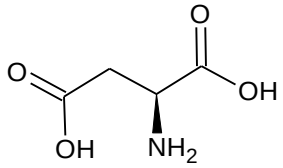


Structural formula:

Number average molecular weight: 1100 g/mol

Weight average molecular weight: 5300 g/mol

A.2.2.e Structural formulae of monomers and starting materials, other agents involved in the polymerisation

	Starting materials	
Name:	**L-Aspartic Acid monomer**	**Caustic Potash Liquid 45%**
IUPAC name:	L-Aspartic Acid	Potassium hydroxide
CAS no.:	56-84-8	1310-58-3
EC no.:	200-291-6	215-181-3
Chemical formula:	$C_4H_7NO_4$	KOH
Structural formula:		KOH
Molecular Weight:	133.10 g/mol	56.11 g/mol
Purity:	≥ 98.5%	45 – 47%

A.2.2.f Degree of substitution, percentages of substituted groups (where appropriate)

The proposed specification for the degree of substitution as potassium salt is set as 91.5% minimum. Please refer to Table 11 in this dossier for the results and to Section A.8.c for the description on the analytical method, which is based on IPC-OES technique.

A.3 Information on the chemical and physical properties of the additive

A summary of the physical and chemical properties of potassium polyaspartate (A-5D K/SD) is provided in the following table. The studies are provided in the annex to this application according to the assigned annex number.

Table 8: Summary of physical and chemical properties of potassium polyaspartate (A-5D K/SD)

Annex number	Study	Guideline	Test material	Findings	Reference
1.1.7/01	Appearance, colour and odour	EPA Guidelines OPPTS 830.6302, OPPTS 830.6303 and OPPTS 830.6304	Name: A-5DK SD Batch no.: KHKS 040412 Purity: 93%	The test material is a light brown solid powder without odour.	Brioschi M. (2014), report no. CH-501/2014
1.1.7/02	Solubility in water	OECD 105	Name: A-5DK SD Batch no.: KHKS 040412 Purity: 93%	Water: > 1000 g/L	Xiaohui Li (2014)
1.1.7/03	Solubility in other solvents	CIPAC MT 181	Name: A-5DK SD Batch no.: KHKS 040412 Purity: 93%	Xylene: < 5.0 g/L CH_2Cl_2: < 5.0 g/L Methanol: < 5.0 g/L Acetone: < 5.0 g/L Ethyl acetate: < 5.0 g/L n-Heptane: < 5.0 g/L	Brioschi M. (2014), report no. CH-501/2014
1.1.7/04	Particle size and distribution	CIPAC MT 59 and CIPAC MT 170	Name: A-5DK SD Batch no.: KHKS 040412 Purity: 93%	The test material has the following distribution: 57.36% greater than 0.125 mm; 24.27% from 0.125 to 0.075 mm; 17.66% from 0.075 to 0.045 mm; 0.48% lower than 0.045 mm.	Brioschi M. (2014), report no. CH-501/2014

Annex number	Study	Guideline	Test material	Findings	Reference
1.1.7/05	Spectra data	FT-IR	Name: A-5DK SD Batch no.: KHKS 040412 Purity: 93%	The FT-IR spectrum demonstrated characteristic peaks of A-5D K/SD.	Hailong Xia (2014a)
1.1.7/06	Spectra data	FT-IR	Name: L-aspartic acid Batch no.: 100206 Purity: >99.3%	The FT-IR spectrum demonstrated characteristic peaks of L-aspartic acid.	Hailong Xia (2014b)
1.1.7/07	Spectra data	H-NMR	Name: A-5DK SD Batch no.: KHKS 040412 Purity: 93%	The H-NMR spectrum demonstrated characteristic peaks of A-5D K/SD.	Kleps R. (2014a)
1.1.7/08	Spectra data	H-NMR	Name: L-aspartic acid Batch no.: 100206 Purity: >99.3%	The H-NMR spectrum demonstrated characteristic peaks of L-aspartic acid.	Kleps R. (2014b)
1.1.7/09	Spectra data	UV-Vis	Name: A-5DK SD Batch no.: KHKS 040412 Purity: 93%	There is no absorbance above 340 nm, that is in compliance with the L-aspartic acid	Hailong Xia (2014c)
1.1.7/10	Spectra data	UV-Vis	Name: L-aspartic acid Batch no.: 100206 Purity: >99.3%	There is no absorbance above 250 nm, that is in compliance with the L-aspartic acid	Hailong Xia (2014d)

A.4 Information on the impurity profile

A.4.a Specifications

The screening analysis of potassium polyaspartate, batch no. KHKS040412, was performed by the following steps:

A.4.a.1 Screening analysis of metals by ICP-OES of the dry matter sample

The ICP-OES analysis on the potassium polyaspartate dry matter resulted in a potassium content of 24% w/w, corresponding to a substitution degree of 94%. The content of each of the other metals is not greater than the trigger value for significant impurity of 0.1% w/w, and thus no inorganic fraction, except for the potassium salts, is present.

A.4.a.2 Determination of total nitrogen by Kjeldahl method in order to determine the purity of the polyaspartate

The purity of the organic fraction was verified through the determination of total nitrogen of the powder and the comparison of this value with the theoretical content in nitrogen of potassium polyaspartate according to its molecular formula. The batch of polyaspartate analyzed has a weight-average molecular weight of 5301, corresponding to the molecular weight of a polymer chain averagely composed by 34.2 monomers. The 34.2 molecules of nitrogen contained in such an average polymer have a molar weight of 479.6 i.e. 9.05% of the dry weight of polyaspartate. The experimental data of nitrogen content resulted 9.03%, corresponding to a 99.8% of the theoretical value.

Consequently, it can be stated that 99.8% of the dry weight of the powder is composed of a polymer of aspartic acid. The results of the above analysis confirm the following.

A.4.a.3 Determination of the free aspartic acid as a significant impurity in potassium polyaspartate as manufactured

The presence of free aspartic acid, residual from the polymerization process, has been determined by HPLC-FLD analysis of the polyaspartate solution. The concentration of free aspartic acid in the commercial preparation resulted to be very limited, below 1% dry weight.

Four typical production batches of potassium polyaspartate (A-5D K/SD) from the production plant were analysed for their content of active substance and impurities. The batches were selected to cover a manufacturing period ranging from 2012 to 2014. The information on the four batches tested is reported in Table 9 below:

Table 9: Information on the four batches tested

Batch no.	KHKS040412	KHKS-072512-1	KHKS-070214-1	KHKS-060414-1
Manufacturing date	04/04/2012	07/25/2012	07/02/2014	06/04/2014
Expiry date	04/03/2016	07/24/2016	07/01/2018	06/03/2018

Number-Average Molecular Weight (Mn)	1037.8 g/mol	1103.9 g/mol	1037.8 g/mol	901.3 g/mol
Weight-Average Molecular Weight (Mw)	5051.0 g/mol	5219 g/mol	5051.0 g/mol	5017.9 g/mol
Polydspersity Index (Mw/Mn) (P.I.)	4.867	4.728	4.867	5.567
Z-Average Molecular Weight (Mz)	11227.5 g/mol	11721.9 g/mol	11224.5 g/mol	11307.9 g/mol

Based on the results of the above analysis it can be stated that:

- the composition of the dry matter sample is almost potassium polyaspartate; and
- no significant impurities, except for the aspartic acid monomer, are contained in the potassium polyaspartate.

The results of the analysis of the four batches of potassium polyaspartate (A-5D K/SD or KPAA) as manufactured are reported in Table 10. The analytical methods are described in the Section A.8.a of this dossier.

Table 10: Four batch analysis results on potassium polyaspartate as manufactured

	Aspartic acid (% w/w)	**Loss of drying (% w/w)**	**KPA (% w/w)**
Analytical Method	HPLC-FLD	Gravimetric	By difference to 100%
Reference point	1.4.1/03	-	-
KHKS-040412	0.4	8.4	91.2
KHKS-072512-1	0.4	8.3	91.3
KHKS-070214-1	0.5	5.5	94.0
KHKS-060414-1	0.5	5.4	94.1
Mean	0.45	6.90	92.65
Max	0.5	8.4	94.1
Min	0.4	5.4	91.2
SD	0.06	1.68	1.62
Mean + 3sd	**0.63**	**11.94**	97.51
Mean -3sd	0.27	1.86	**87.79**
Proposed specifications	**Max 1.0**	**Max 11.0**	**Min 88.0**

Based on the values reported above the proposed specifications are as follow:

- Potassium polyaspartate: minimum 88.0 % w/w (based on mean minus three times the SD).
- Aspartic acid: maximum 1% w/w (based on mean plus three times the SD).

- Other significant impurities: No other impurities were found to be greater than 0.1% w/w.
- Loss of drying / Water: maximum 11.0% w/w (based on mean plus three times the SD).

The results of the analysis of the four batches of potassium polyaspartate (A-5D K/SD or KPAA) dry matter are reported in Table 11. The analytical methods used are described in the Section A.8.a of this dossier.

Table 11: Four batch analysis results on potassium polyaspartate dry matter

	Dry matter content analysis			
	Potassium content (% w/w)	**Degree of substitution (% w/w)**	**Nitrogen content (% w/w)**	**KPA (% w/w)**
Analytical Method	ICP-OES		Kjeldahl method	
Reference point	1.4.1/02		1.4.1/01	
KHKS-040412	24.0	94	7.90	99
KHKS-072512-1	24.5	96	7.95	101
KHKS-070214-1	24.1	94	7.95	100
KHKS-060414-1	24.6	96	7.91	100
Mean	24.3	95	7.92	100
Max	24.6	96	7.95	101
Min	24.0	94	7.90	99
SD	0.29	1.15	0.03	0.82
Mean + 3sd	25.17	98.45	8.02	102.46
Mean -3sd	23.43	**91.55**	7.84	**97.54**
Proposed specifications	-	**Min 91.5**	-	**Min 98.0**

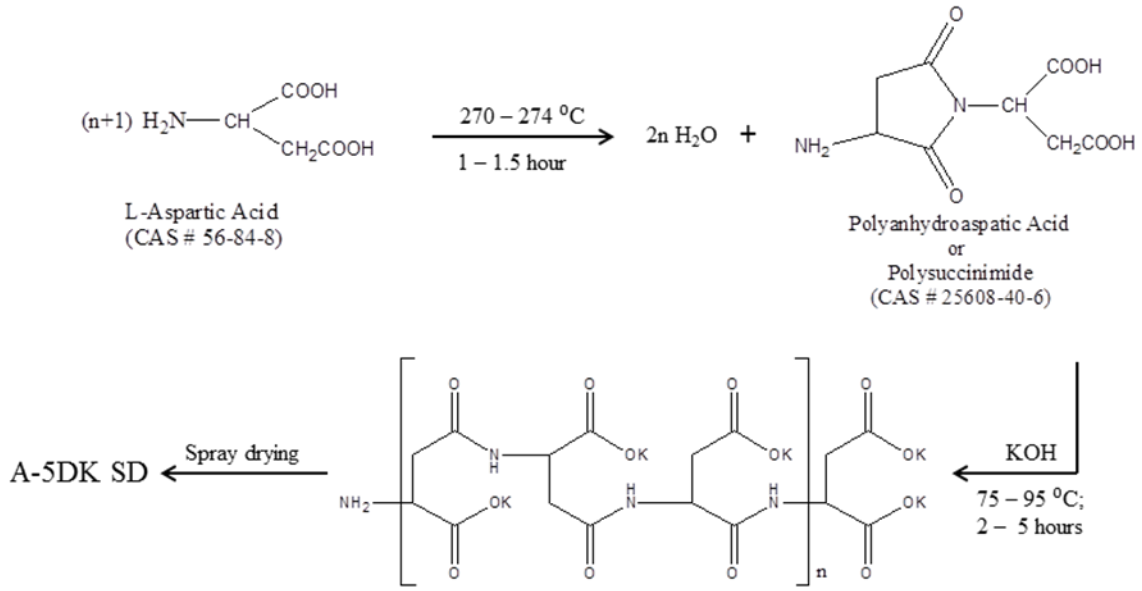
Based on the values reported above the proposed specifications on the dry matter material are as follows:

- Potassium polyaspartate: minimum 98.0 % w/w (based on mean minus three times the SD).
- Degree of substitutions: minimum 91.5% w/w (based on mean minus three times the SD).

The results of the analysis of the four batches of potassium polyaspartate (A-5D K/SD) support the proposed specification.

A.5 Manufacturing process

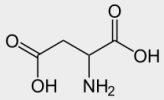
Potassium polyaspartate (A-5KD SD) is produced from L-aspartic acid according to the following reaction:



The thermic process transforms the aspartic acid into polysuccinimide that is insoluble. Polysuccinimide is then treated with potassium hydroxide under controlled conditions, thus allowing the opening of the ring and the polymerization of the units. Through this process a 40% solution of potassium polyaspartate at pH 8.3 is obtained. The last step of the production of the preparation potassium polyaspartate (A-5D K/SD) is the spray drying phase, whose result is a light tan powder at 92-95% dry matter.

Information on the two starting materials is as follows: L-aspartic acid monomer and caustic potash liquid 45%, used for the manufacturing of potassium polyaspartate (A-5D K/SD). The information provided is reported in Table 12.

Table 12: Information on the two starting materials used in the manufacture of potassium polyaspartate (A-5D K/SD)

	Starting materials	
Name:	L-Aspartic acid monomer	Caustic potash liquid 45%
IUPAC name:	L-Aspartic acid	Potassium hydroxide
CAS No.:	56-84-8	1310-58-3
EC No.:	200-291-6	215-181-3
Chemical formula:	$C_4H_7NO_4$	KOH
Structural formula:		
Molecular weight:	133.10 g/mol	56.11 g/mol
Purity	≥98.5%	45–47%

Analysis of residual potassium hydroxide (KOH) cannot be performed directly because it is not possible to differentiate between potassium (K) linked to potassium polyaspartate (A-5D K/SD) or potassium, hydroxide. Based on the proposed specification of 98% potassium polyaspartate (A-5D K/SD) in the dry material, residual potassium hydroxide cannot be greater than 2%. As the pH of wine ranges between 3 and 3.8, any residual KOH would be neutralised during the production and storage of wine.

A.6 Specification for identity and purity

Schedule 3	**Identity and purity**
S3—3	Substances with specifications in secondary sources
	(j) the International Oenological Codex (2017), Organisation Internationale de la Vigne et du Vin (OIV).

RESOLUTION OIV-OENO 572-2017

MONOGRAPH ON POTASSIUM POLYASPARTATE

THE GENERAL ASSEMBLY,

In view of Article 2, paragraph 2 iv of the Agreement of 3 April 2001 establishing the International Organisation of Vine and Wine,

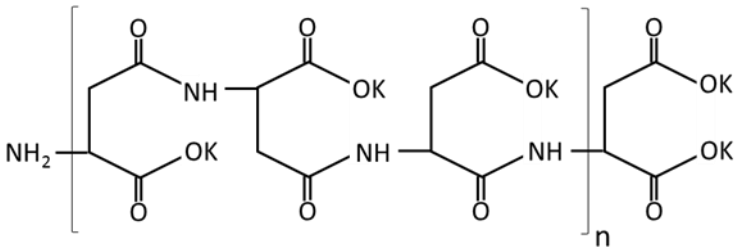
CONSIDERING the work of the "Specifications of Oenological Products" Expert Group,

CONSIDERING OIV Resolution OENO-TECHNO 14-543 'Treatment with potassium polyaspartate',

DECIDES to add the following monograph to the *International Oenological Codex*:

POTASSIUM POLYASPARTATE

Chemical name: Homopolymer of potassium L-aspartate or potassium polyaspartate
Chemical formula: $[C_4H_5NO_3K]_n$
Topological formula:



where n ≈ 30
CAS No.: 64723-18 -8

1. OBJECT, ORIGIN AND SCOPE OF APPLICATION

Oenological potassium polyaspartate is prepared exclusively from L-aspartic acid. The L-aspartic acid monomer used in the process is produced by fermentation. A thermal process converts the L-aspartic acid monomer into polysuccinimide, an insoluble compound. Polysuccinimide is then treated with potassium hydroxide under controlled conditions to obtain potassium polyaspartate. The potassium polyaspartate inhibits tartaric precipitation thanks to a 'colloid protector' effect. Potassium polyaspartate is effective for the tartaric stabilisation of wines.

2. SYNONYMS

Potassium polyaspartate, A-5D K/SD; A-5D K SD; A-5DK/SD; A-5DK; KPA.

3. LABELLING

The following indications should appear on the packaging labelling:

- the name and sales denomination,
- the statement 'Product for oenological use, limited use',
- any additives,
- instructions for use,
- the batch number and potassium polyaspartate content (purity) as well as the expiry date and storage conditions (temperature, humidity and aeration),
- the name or company name and address of the manufacturer, packager or supplier,
- the net quantity,
- the indication that the aspartic acid is sourced from genetically-modified organisms and the modified characteristic where relevant.

4. CHARACTERISATION

4.1 Description

Light-brown, odourless powder containing 90% dry matter. It is entirely soluble in water (> 1000 g/L) yet insoluble in organic solvents (< 5 g/L), with a shelf life of 4 years at room temperature.

4.2 Chemical formula

Potassium polyaspartate is a polymer composed of aspartic acid units, with the following general formula: $[C_4H_5NO_3K]_n$, where n corresponds to the average degree of polymerisation (n ≈ 30).

4.3 Degree of substitution

The degree of substitution of the potassium salt is at least 91.5% (in terms of anhydrous matter), in order to guarantee optimal solubility.

Assess the degree of substitution using the method described in Annex 1.

4.4 Molecular mass

Its average molecular mass, determined by gel permeation chromatography, is 5000 g/mol, which corresponds to the optimum efficiency of the product.

4.5 Composition

The purity of the product is verified by assaying the aspartic acid after total hydrolysis of the polymer and by comparing this value with the theoretical content of the monomer in the potassium polyaspartate according to its molecular formula. Refer to Annex 2 for the method description. The content of anhydrous potassium polyaspartate matter should be at least 98%.

5. TRIALS

5.1 Free aspartic acid content in potassium polyaspartate

The free aspartic acid content should be ≤ 2.0%.

Carry out the determination according to the method described in Annex 3.

5.2 Humidity – Loss due to dehydration

Determine the loss in mass of a gram of dry product kept in an oven for 12-24 hours at 105 ± 2 °C. The mass should be constant and the loss in mass should be less than 10%.

5.3 Metal content

Before determining the metals, mineralise the sample by means of acid digestion (HNO_3, H_2O_2 and HCl). Conduct the mineralisation in a microwave oven. The sample should not be crushed or dehydrated before mineralisation.

The reagents used for mineralisation are as follows: HNO_3 (65%) (Suprapur or similar), HCl (37%) (Suprapur or similar) and H_2O_2 (35%).

Introduce the polyaspartate sample (between 0.5 and 2 g) into a 100-mL calibrated flask before adding 25 mL HNO_3, 2 mL HCl and 3 mL H_2O_2. At this stage, subject the mixture to digestion in a microwave oven with a maximum power of 1200 W: 60% power for 1 min, 30% for 10 min, 15% for 3 min and 40% for 15 min. Subsequently, make the calibrated flask up to volume with double-distilled water. The determination of the metals is practised on the solution thus obtained.

5.3.1. Iron

Determine the iron according to the method described in Chapter II of the *International Oenological Codex*. The iron content should be below 10 mg/kg.

5.3.2. Arsenic

Determine the arsenic according to the method described in Chapter II of the *International Oenological Codex*. The arsenic content should be below 3 mg/kg.

5.3.3. Lead

Determine the lead according to the method described in Chapter II of the *International Oenological Codex*. The lead content should be below 2 mg/kg.

5.3.4. Mercury

Determine the mercury according to the method described in Chapter II of the *International Oenological Codex*. The mercury content should be below 1 mg/kg.

5.3.5. Cadmium

Determine the cadmium according to the method described in Chapter II of the *International Oenological Codex*. The cadmium content should be below 1 mg/kg.

ANNEX 1

1. Determination of the degree of substitution

1.1 Principle

The degree of substitution of commercial potassium polyaspartate is determined by the analysis of the potassium content using the ICP-OES method.

The determination of potassium is conducted using a calibration curve obtained by injecting five different concentrations of a reference standard solution.

To calculate the degree of substitution, the potassium concentration measured is compared to the theoretical content at 100% substitution.

1.2 Equipment

1.2.1 100-mL Volumetric flasks (class A)

1.2.2 Cyclonic atomisation chamber, standard quartz torch

1.2.3 Ultrasonic bath

1.2.4 Membrane filtration system with 0.45-µm porosity

1.3 Reagents

1.3.1 65% Nitric acid (HNO_3)

1.3.2 10 000 mg/L Potassium (K) standard solution (potassium ICP/DCP standard solution with 10 000 µg/mL 5% HNO_3)

1.3.3 Double-distilled water with superior resistivity of 10 MΩ.cm

1.3.4 Aqueous solution acidified with 0.5% HNO_3 (calibration blank), to be used as a diluent for the preparation of the calibration solutions

1.3.5 Calibration solutions prepared by dilution of the stock solution (point 1.3.2); the reference values are indicated below:

	STD 1	**STD 2**	**STD 3**	**STD 4**	**STD 5**
Potassium (mg/L)	200	400	600	1000	2000

1.4 Procedure

The preparation to be analysed (KPA) is dissolved in double-distilled water.

1.4.1 5000 mg/L KPA solution (a): weigh around 500 g (note the exact weight) directly into a 100-mL calibrated flask, make up to volume with double-distilled water (1.3.3) and stir in an ultrasonic bath (1.2.3) for at least 10 minutes. Filter using membranes with 0.45 µm porosity.

1.4.2 Prepare the five-point calibration curve with the standard solutions as indicated in point 1.3.5.

The results should be calculated from the average of three measurements.

If the concentration lies outside the calibration curve, the sample should be diluted so that its concentration falls within the calibration curve.

To calculate the degree of substitution, compare the potassium concentration measured to the theoretical content established at 100% substitution (see point 1.5).

1.5 Calculations

The potassium content is calculated by the processor of the acquisition software. The calculation to be conducted is as follows:

$$A = A' \times n \qquad \text{(a)}$$

where:

A: concentration of sample in mg/L

A': concentration of diluted sample in mg/L

n: dilution factor

The percentage of potassium in the KPA sample, expressed in dry weight, is calculated using formula (b):

$$\%K_{(dry\ weight)} = A\frac{100}{w}\frac{100}{(100-h\%)} \qquad \text{(b)}$$

where:

A: result of equation (a)

w: potassium polyaspartate in mg/L

h%: humidity of the sample, as a percentage

The degree of substitution (DS) is calculated using equation (c):

$$\%DS_K = \frac{\&K_{(dry\ weight)}}{\frac{MA_K}{MM_{KPAmonomer}}100} \qquad \text{(c)}$$

where:

MA_K: atomic mass of potassium

$MM_{KPAmonomer}$: calculated molecular mass of the polyaspartate monomer

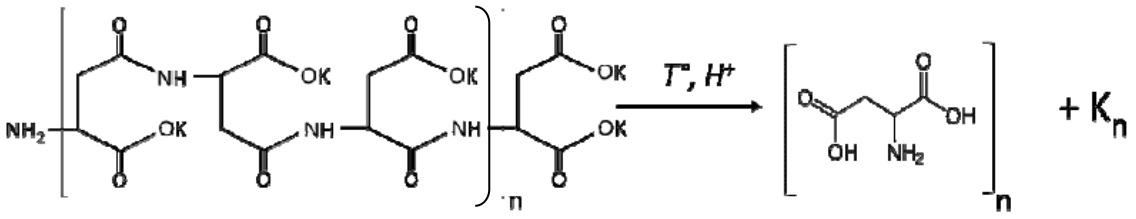
ANNEX 2

2. Determination of the purity of potassium polyaspartate

2.1 PRINCIPLE

Analysis by HPLC-FLD of the free aspartic acid content after acid hydrolysis.

The principle consists of determining the free aspartic acid by HPLC after acid hydrolysis of the KPA. This acid hydrolysis takes place under conditions allowing for the complete depolymerisation of the KPA:



Potassium polyaspartate — Aspartic acid

2.2 EQUIPMENT / APPARATUS

2.2.1 Hot plate for acid hydrolysis

2.2.2 4-mL Tinted-glass vials with screw cap

2.2.3 0.1 mg Precision weighing balance

2.2.4 Calibrated flasks

2.2.5 HPLC system including a quarternary pump, an automatic sampler, a thermostat and a fluorometric detector (FLD)

2.2.6 C18 column (e.g. Syncronis aQ C18, 4.6 x 250 mm; 5 µm [Thermo])

2.2.7 Filtration system with membranes of 0.2 µm porosity

2.3 REAGENTS AND SAMPLE PREPARATION

For acid hydrolysis

2.3.1 Potassium metabisulphite solution ($Na_2S_2O_2$) (CAS No. 16731-55-8) at a concentration of 10 g/L

2.3.2 6 M Hydrochloric acid (HCl)

2.3.3 5 M Sodium hydroxide (NaOH)

2.3.4 Double-distilled water with superior resistivity of 10 mΩ.cm

2.3.5 Potassium polyaspartate

For sample preparation

2.3.6 Aminocaproic acid ($C_6H_{13}NO_2$, CAS No.: 60-32-2)

2.4 PROCEDURE

The procedure comprises three steps:

- hot acid hydrolysis of the potassium polyaspartate sample,

- preparation of the samples for analysis by HPLC-FLD of the standard solutions that will determine the aspartic acid concentration,
- analysis of the free aspartic acid after hydrolysis by HPLC (see Annex 3).

2.4.1 Phase 1: acid hydrolysis

2.4.1.1 Transfer into a 4-mL vial (2.2.2):
0.2 mL 10 g/L sodium metabisulphite solution (2.3.1),
0.5 g potassium polyaspartate weighed to the nearest mg,
2 mL 6 N HCl (2.3.2).

2.4.1.2 Heat to 108 ± 2 °C for 72 hours (2.2.1).

2.4.1.3 Transfer to a 10-mL calibrated flask, add 2.4 mL 5 M NaOH (2.3.3) and make up to volume with double-distilled water (2.3.4).

2.4.2 Phase 2: preparation of the sample for HPLC analysis

2.4.2.1 Microfilter 5 mL of medium (2.4.1.3) at 0.20 µm (2.2.7) in a 20-mL calibrated flask.

2.4.2.2 Add 0.2 mL internal standard (aminocaproic acid) (2.3.6).

2.4.2.3 Make up to volume with double-distilled water.

2.4.3 Phase 3: Analysis of samples by HPLC (see Annex 3)

CALCULATIONS

The polyaspartate concentration (KPA) is calculated as follows:

KPA (mg/L) = (hydrolysed aspartic acid – free aspartic acid before hydrolysis) x f_{KPA}

where f_{KPA} = 1.15, which is the conversion factor of KPA into aspartic acid, calculated based on the ratio between the molecular mass of the KPA monomer (average MM of KPA A5DK SD monomers = 154) and the molecular mass of aspartic acid (133.1), as per the equation:

$$f_{KPA} = \frac{MM_{KPA\_monomer}}{MM_{aspartic\_acid}} = 1.15$$

where the free aspartic acid is determined according to Annex 3.

ANNEX 3

3. Determination of free aspartic acid

3.1 PRINCIPLE

The determination of aspartic acid in potassium polyaspartate as it was produced is carried out by HPLC coupled with fluorometric detection (FLD), after derivation of aspartic acid with ortho-phthalaldehyde (OPA). Potassium is determined using a calibration curve obtained by injecting the reference standard solutions.

3.2 EQUIPMENT / APPARATUS

3.2.1 Calibrated flasks

3.2.2 HPLC system including a quarternary pump, an automatic sampler, a thermostat and a fluorometric detector (FLD)

3.2.3 C18 column, e.g. Syncronis aQ C18, 4.6 x 250 mm; 5 µm

3.3 REAGENTS

3.3.1 Aspartic acid (D,L-aspartic acid, $C_4H_7NO_4$ ≥ 99 %, CAS No.: 617-45-8)

3.3.2 Solution 1: 8000 mg/L aspartic acid in double-distilled water

3.3.3 Solution 2: 200 mg/L aspartic acid in double-distilled water

3.3.4 Aminocaproic acid ($C_6H_{13}NO_2$, CAS No.: 60-32-2)

3.3.5 1000-mg/L aminocaproic acid stock solution in double-distilled water

3.3.6 Calibration solutions prepared by dilution of solution 1 (point 3.3.2) and solution 2 (3.3.3), whose reference values are indicated below:

	STD 1	**STD 2**	**STD 3**	**STD 4**	**STD 5**	**STD 6**
mL H_2O	18.8	19.0	15.0	19.750	19.375	18.750
mL Solution 1	-	-	-	0.250	0.625	1.250
mL Solution 2	0.2	1.0	5.0	-	-	-
Aspartic acid (mg/L)	**2**	**10**	**50**	**100**	**250**	**500**

3.3.7 Methanol for HPLC

3.3.8 Tetrahydrofuran for HPLC

3.3.9 Anhydrous sodium acetate (CAS No. 127-09-3)

3.3.10 Acetonitrile (CH_3CN) for HPLC

3.3.11 Sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$, CAS No. 1303-96-4)

3.3.12 O-phthalaldehyde (OPA): ($C_8H_6O_2$ ≥ 99%, CAS No.: 643-79-8)

3.3.13 Mercaptoethanol: (C_8H_6OS ≥ 99%, CAS No.: 60-24-2)

3.3.14 Double-distilled water with superior resistivity of 10 MΩ.cm

3.3.15 Derivation solution: in a 10-mL calibrated flask, introduce 100 mg OPA, 200 mL mercaptoethanol and 1 mL methanol, then make up to volume with a pH 10.5 buffer solution of 0.1 M sodium tetraborate decahydrate.

The solution should be prepared just before use since it degrades over the day following its preparation.

3.4 MOBILE PHASES

3.4.1 [Channel A]: ultra-pure water

3.4.2 [Channel B]: 0.05 M sodium acetate buffer/tetrahydrofuran (96:4; v/v)

3.4.3 [Channel C]: methanol

3.4.4 Channel D]: acetonitrile

3.5 PROCEDURE

The method consists of a reaction constituting the derivation of aspartic acid with the O-phtalaldehyde (OPA); the recovery rate for this process is 100%.

The instrumental parameters are as follows:
- temperature of the column: 40 °C,
- wavelength (λ): FLD Ex 340 nm, Em 450 nm,
- the separation is carried out in gradient mode (see point 3.4, Mobile phases):

Time (min)	% B	% C	% D	Flow (mL/min)
0.00	100.0	0.0	0.0	1.1
3.00	100.0	0.0	0.0	1.1
15.00	50.0	25.0	25.0	1.1
17.00	84.0	8.0	8.0	1.1
18.00	100.0	0.0	0.0	1.1
Run time: 21 min + 2 min downtime				

3.5.1 Prepare the calibration solutions by mixing 5.0 mL of the standard solution (3.3.6) and 0.2 mL of the internal standard solution (3.3.5) in a 20-mL calibrated flask, then make up to volume with double-distilled water and stir.

3.5.2 Dilute 5.0 µL of the sample (Annex 2, point 2.4.2) with 20 µL methanol, then derive with 0.5 µL OPA. Mix 10.0 µL of the thus-obtained solution 10 times in the injector, then inject after 0.5 min.

3.5.3 If the results exceed the upper limit of the calibration curve, dilute the sample and repeat the analytical procedure.

3.6 CALCULATIONS

The concentration of aspartic acid in the sample, expressed in mg/L, is obtained by applying the following formula:

$$Y = A \cdot f \cdot d$$

where: Y: concentration of aspartic acid in the sample, in mg/L

A: peak area of the chromatogram

f: response factor of the chromatogram peak

d: dilution factor

A.7 Information for food labelling

The class of food additive is stabliliser.

A.8 Analytical method for detection

Several analytical methods have been developed and validated in order to determine the active substance in different matrix. All methods are specific and fit-for-purpose.

A.8.a Description of the analytical methods for the determination of the active substance as manufactured

Determination of dry matter purity of potassium polyaspartate:

The following analytical method for the determination of the active substance as manufactured is provided in support of this assessment.

Report:	REF. 1.4.1/01, Vassanelli (2014)
Title:	Chemical Characterization of Potassium Polyaspartate
Document No:	01/2015 (Laboratory Enocentro srl)
Guidelines:	EEC guideline SANCO/3030/99 rev. 4 dated 11/07/00
GLP	No

Method:

The determination of the active ingredient (a.i.) is performed by Kjedahl method.

The determination of the total nitrogen contained in the potassium polyaspartate (KPA) is calculated by comparison between the percentage of nitrogen obtained with the analysis and the expected theoretical value, calculated on the basis of the molecular formula of the test item.

The lot of polyaspartate analyzed has a weight-average MW of 5301, corresponding to the molecular weight of a polymer chain composed by 34.2 monomers. The 34.2 molecules of N contained in such an average polymer have a molar weight of 479.6 i.e. 9.05% of the dry weight of polyaspartate.

Five repeatability tests at two levels were carried out to calculate the percentage of total nitrogen. The calculation formula is showed below:

$$\%Nitrogen_{(\text{dry weigh})\,experimental} = V_{H_2SO_4} \cdot f \cdot \frac{100}{w(g)} \cdot \frac{100}{(100 - h\%)}$$

where
V: mL of sulfuric acid 0.1 N used for titration
f: factor equal to 0.0014
h%: moisture percentage value in the sample

Test	Weight (g)	Sulfuric acid 0.1N (ml)	% N (dw) calculated	% N (dw) theoretical	% recovery
1	0.251	14.8	8.98	9.05	99.2
2	0.250	14.9	9.04	9.05	99.9
3	0.248	15.2	9.06	9.05	100.1

4	0.253	15.1	9.03	9.05	99.8
5	0.249	14.8	9.04	9.05	99.9
		Average	**9.03**		**99.8**

Test	Weight (g)	Sulfuric acid 0.1N (ml)	% N (dw) calculated	% N (dw) theoretical	% recovery
1	0.501	29.9	8.98	9.05	99.2
2	0.498	29.9	9.04	9.05	99.9
3	0.495	29.8	9.06	9.05	100.1
4	0.497	29.8	9.03	9.05	99.8
5	0.503	30.2	9.04	9.05	99.9
		Average	**9.03**		**99.8**

In the titration tests of the reaction blank, to verify the absence of interferences, the volume of sulfuric acid 0.1 N necessary for titration resulted lower than 0.1 mL (1 drop).

The experimental data of nitrogen content resulted 9.03%.

To calculate the purity of KPA in percentage the following formula is applied:

$$\%Purity_{KPA} = \frac{\%Nitrogen_{(\text{dry weigh})_{experimental}}}{\%Nitrogen_{(\text{dry weigh})_{theoretical}}} \cdot 100 \quad \text{(d)}$$

where:

$\%Nitrogen_{(\text{dry weigh})_{experimental}}$: calculated according to equation (e):

$$\%Nitrogen_{(\text{dry weigh})_{experimental}} = V_{H_2SO_4} \cdot f \cdot \frac{100}{w(g)} \cdot \frac{100}{(100 - h\%)} \quad \text{(e)}$$

where:
V: milliliters of sulfuric acid 0.1 N used for titration
w: weight (grams) of potassium polyaspartate
f: factor equal to 0.0014
h%: value in percentage of humidity of the sample

$$\%Nitrogen_{(\text{dry weigh})_{theoretical}} = \frac{AW_N}{MW_{KPAmonomer(SDexperimental)}} \cdot 100 \quad \text{(f)}$$

where:
AW_N: atomic weight of nitrogen
$MW_{KPAmonomer\ (SDexperimental)}$: molecular weight of potassium polyaspartate with substitution degree experimentally calculated through the formula (g):

$$MW_{KPAmonomer(SDexperimental)} = \frac{(MW_{KPAmonomer(SD100\%)} \cdot \%SD_K) + \left[MW_{KPAmonomer(SD0\%)} \cdot (100 - \%SD_K)\right]}{100} \quad \text{(g)}$$

The theoretical data obtained for nitrogen is 9.05% (dry weight), and the experimental data resulted 9.03%; this allows to state that the purity of the KPA analyzed is 99.8% according to equation (d).

Determination of degree of substitutions:

The following analytical method for the determination of the degree of substitution of potassium polyaspartate as manufactured is provided in support of this assessment.

Report:	REF. 1.4.1/02, Vassanelli (2014)
Title:	Chemical Characterization of Potassium Polyaspartate
Document No:	01/2015 (Laboratory Enocentro srl)
Guidelines:	EEC guideline SANCO/3030/99 rev. 4 dated 11/07/00
GLP	No

Method:
The determination of the degree of substitution of potassium polyaspartate as manufactured is performed by the analysis of the potassium content by ICP-OES method.

The quantification of potassium is achieved by a calibration curve obtained by injecting the reference standard solution at five concentrations.

To calculate the substitution degree, the measured potassium concentration is compared with the theoretical content with a substitution degree of 100%.

Validation:

Linearity: the linearity of the method was confirmed by analysis of five standard solutions in the range of 200 to 2000 mg/mL. Correlation coefficient r^2 of 0.9978.

Accuracy: recoveries of potassium in potassium polyaspartate were determined by spiking three aliquots of the potassium polyaspartate with the potassium reference material at three levels six times. The accuracy of the analytical method is reported as mean. Results are shown in Table 13.

Table 13: Accuracy data

	Average of 6 repetitions (mg/L) (a)	**Theoretical addition of potassium (mg/L) (b)**	**Experimental determination of potassium (mg/L)**	**% Recovery(**)**
Potassium solution 5000 mg/L KPA	1130	-	-	**-**
+ ≈250 mg/L Potassium	1385	250	255	102
+ ≈500 mg/L Potassium	1618	500	488	98
+ ≈750 mg/L Potassium	1855	750	725	97

(**) calculated by the formula (a-1130)/b·100

Precision: the repeatability (precision) of the analytical method was determined by calculating the mean amount and the standard deviation from analysis of the test item in eleven-fold. Observed repeatability's are summarised in Table 14.

Table 14: Precision data

Test item	a.i.	Number of Determinations	Mean amount (mg/L)	Standard deviation among repetition	Horwitz RSDr (%)
A-5D K/SD	potassium	11	1117.7	8.0	41.5

Limit of detection (LOD): is the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) within a stated confidence limit (generally 1%). The calculated LOD is 125 mg/L.

Limit of quantification (LOQ): is the lowest compound concentration that can be quantified in the analyzed matrix. The calculated LOQ is 375 mg/L.

Conclusions:
The analytical method was shown to be specific for the determination of potassium in the A-5D K/SD samples.

The range tested for potassium, from 200 to 2000 mg/mL, was found to be linear (correlation coefficient > 0.99).

The standard deviation among repetitions SDr was 8.0 for potassium and the Horwitz RSDr was 41.5% at a potassium concentration of 1117.7 mg/L. Since the relative standard deviation was lower than the Horwitz RSDr, the repeatability test for potassium was acceptable.

For the accuracy, the SANCO/3030/99 rev. 4 guideline requires mean recovery values in the range 90 to 110 % for content higher than 0.01 % w/w and lower than 0.1 % w/w.

Since all recovery values fulfilled these criteria, the accuracy of the analytical method can be considered acceptable.

As the method validation parameters, viz. specificity, linearity, accuracy, precision (repeatability), were found to be within the acceptance criteria it was concluded that the method was validated.

Based on the results obtained in the study it was concluded that the ICP-OES spectroscopic method is suitable to quantify the content of potassium in the technical material.

Determination of monomer as impurity:

The following analytical method for the determination of the monomer aspartic acid as significant impurity on potassium polyaspartate as manufactured is provided in support of this assessment.

Report:	REF. 1.4.1/03, Vassanelli (2014)
Title:	Chemical Characterization of Potassium Polyaspartate
Document No:	01/2015 (Laboratory Enocentro srl)
Guidelines:	EEC guideline SANCO/3030/99 rev. 4 dated 11/07/00
GLP	No

Method:
The determination of aspartic acid in the potassium polyaspartate (A-5D K/SD) as manufactured is performed by the HPLC chromatography with a fluorimetric detector (FLD), through a derivatization reaction of aspartic acid with o-Phthalaldehyde (OPA). The recovery obtained for such a process was evaluated and resulted 100%.

The quantification of potassium is achieved by a calibration curve obtained by injecting the reference standard solutions.

Linearity: Six standard solutions were prepared in the range 2 to 500 mg/L. Correlation coefficient, r^2 > 0.99.

A.8.b Description of the analytical methods for the determination of the active substance in food/wine

The following analytical method for the determination of the active substance in wine is provided in support of this assessment.

Report:	REF. 1.4.2/01, Vassanelli (2014)
Title:	Method for analysis of potassium polyaspartate in wine
Document No:	20150107 (Laboratory Enocentro srl)
Guidelines:	EEC guideline SANCO/3030/99 rev. 4 dated 11/07/00
GLP	No

Method:
The quantification of potassium polyaspartate in red/white wine is achieved by calculating the difference of the aspartic acid content before and after complete sample hydrolysis to aspartic acid monomer.

The determination of the aspartic acid is performed by HPLC using an external standard and FLD detector.

The quantification of aspartic acid is achieved by a calibration curve obtained by injecting the reference standard solutions.

The hydrolysis conditions are as follow: acid hydrolization at 180°C for 72 hours.

The chromatographic conditions are as follows:

- Oven temperature: 40°C
- Injection: 10 µl
- wavelenght (λ): FLD Ex 340 nm, Em 450 nm
- the separation is done under gradient:

Time	% sodium acetate buffer 0.05M/tetrahydrofurane (96:4)	% methanol	% acetonitrile	Flow
0.00	100.0	0.0	0.0	1.1
3.00	100.0	0.0	0.0	1.1
15.00	50.0	25.0	25.0	1.1
17.00	84.0	8.0	8.0	1.1
18.00	100.0	0.0	0.0	1.1
Stop time 21 min + 2 min post time				

Validation:

Specificity: interference was not observed from blank formulation solution.

Linearity: the linearity of the method was confirmed by analysis of six standard solutions in the range of 2 to 500 mg/mL. Correlation coefficient $r^2 \geq 0.99$.

Accuracy: recoveries of potassium polyaspartate in wine were determined by spiking three aliquots of two different kind of wine (red and white) with the potassium polyaspartate at three levels five times. The accuracy of the analytical method is reported as mean. Results are shown in Table 15.

Table 15: Accuracy data

	Average value of aspartic acid (mg/L)	Experimental KPA expressed as aspartic acid (mg/L) (added wine – no addition wine)	Theoretical added KPA(**) (mg/L)	% Recovery(*)
Red wine + No addition	81.5	-	-	**-**
Red wine + ≈50 mg/L	113.3	31.8	43.5	**73**
Red wine + ≈200 mg/L	228.2	147	174	**84**
Red wine + ≈500 mg/L	436.2	355	435	**82**
White wine + No addition	105.9	-	-	**-**
White wine + ≈50 mg/L	157.9	52	43.5	**119**
White wine + ≈200 mg/L	235.7	130	174	**75**
White wine + ≈500 mg/L	504.2	398	435	**92**

The recovery data are in accordance with the requirements of guideline SANCO 12495/2011, that indicates an acceptability range between 70% and 120%.

Precision: the repeatability (precision) of the analytical method was determined by calculating the mean amount and the relative standard deviation (% RSD) from analysis of the test item in five-fold. Observed repeatability's are summarised in Table 16.

Table 16: Precision data for aspartic acid

a.i.	Number of Determinations	Mean amount (mg/L)	Standard deviation (mg/L)	Horwitz RSDr (%)
Aspartic acid	5	16.29	0.36	1.14

Limit of detection (LOD): is the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) within a stated confidence limit (generally 1%). The calculated LOD is 0.7 mg/L

Limit of quantification (LOQ): is the lowest compound concentration that can be quantified in the analyzed matrix. The calculated LOQ is 2.1 mg/L

Accuracy of hydrolysis process: recoveries of aspartic acid in wine before and after hydrolysis were determined by spiking three aliquots of water with the aspartic acid standard material at three levels (25, 100 and 200 mg/L). The accuracy of the analytical method is reported as mean and are in the range of 99-100%, which are considered acceptable for the purpose of the method. Results are shown in Table 17.

Table 17: Accuracy data for aspartic acid

	Recovery	
	Added (mg/L)	(%)
pre-hydrolysis	25.4	99.2
post-hydrolysis	25.2	
pre-hydrolysis	109	99.1
post-hydrolysis	108	
pre-hydrolysis	213	100
post-hydrolysis	213	

Confirmatory: The identification of aspartic acid in wine is performed by LC-MS/MS chromatography according to the following instrumental conditions:

Method for analysis MS/MS:
- Scan type: MRM (scheduled MRM)
- Polarity: positive
- Run time: 8.0 min
- Ion spray voltage: 4500 V
- Temperature source: 300 °C
- Collision gas: 6 psi
- Curtain gas: 30 psi

Chromatography method:
- Oven Temperature: 25 °C
- Eluent A: 1% Acetic acid (liquid > 98%) in water (LCMS grade) + 5% Methanol (liquid for HPLC MS)
- Eluent B: Acetic Acid (liquid > 98%) in Methanol (liquid for HPLC MS)

- Eluent flux in analytical column: 0.5 mL/min
- Injection: 10 µl
- Analytical column: Hypercarb 100 x 2.1 mm, 5 µm particle size
- Pre-column: Hypercarb 10 x 3 mm, 5 µm particle size
- Gradient of the analytical column:

	%A	%B	Time (min)
1	100	0	0.0
2	100	0	0.2
3	0	100	10.2
4	0	100	12.2
5	100	0	12.3

Conclusions:
The analytical method was shown to be specific for the determination of aspartic acid in red and white wine samples.

The range tested for aspartic acid, from 5 to 500 mg/L, was found to be linear (correlation coefficient > 0.99).

The standard deviation among repetitions SDr was 0.36 for aspartic acid and the Horwitz RSDr was 1.14% at the aspartic acid concentration of 16.29 mg/L. Since the relative standard deviation was lower than the Horwitz RSDr, the repeatability test for potassium was acceptable.

For the accuracy, the SANCO 12495/2011 guideline requires mean recovery values in the range 70 to 120%. Since all recovery values fulfilled these criteria, the accuracy of the analytical method can be considered acceptable.

As the method validation parameters, viz. specificity, linearity, accuracy, precision (repeatability), were found to be within the acceptance criteria it was concluded that the method was validated.

Based on the results obtained in the study it was concluded that the HPLC-FLD method is suitable to quantify the content of KPA in wine.

A.8.c Description of the analytical methods for the determination of the active substance in water

The following analytical method for the determination of the active substance in water is provided in support of this assessment. The validation has been conducted to quantify the content of potassium polyaspartate (A-5D K/SD) in water, used as the vehicle in the subchronic toxicity study performed with potassium polyaspartate (A-5D K/SD) in rats (see Section B.2.b of this dossier for details).

Report:	REF. 1.4.3/01, Marne S.K. (2014)
Title:	Validation of Analytical Method for A-5D K SD
Document No:	Study No. R/RA1384/AMV/14 (INTOX PVT. LTD.)
Guidelines:	EEC guideline SANCO/3030/99 rev. 4 dated 11/07/00
GLP	Yes

Method:
The determination of the active substance potassium polyaspartate (A-5D K/SD) in water is performed by a UV analytical method.

The analytical method intended to be employed for potassium polyaspartate (A-5D K/SD) was supplied by the Applicant. The method of analysis is based on adding a cationic polymer to a buffered solution of anionic potassium polyaspartate. Turbidity developed, and the absorbance of the solution was measured at 420 nm.

Validation:
Specificity: interference was not observed from the blank solution.

Linearity: the linearity of the method was confirmed by analysis of five standard solutions in the range of 5 to 25 mg/L. Correlation coefficient r^2 of 0.9968.

Accuracy: accuracy of the method was determined by fortifying the test article at two different levels in duplicate. The accuracy of the analytical method is reported as mean. Results are shown in Table 18.

Table 18: Accuracy data for A-5D K/SD in A-5D K/SD

Spike code	Recovery		Mean Recovery
	Added (mg/L)	(%)	(%)
Spike Low A	5.03	92.19	92.00
Spike Low B	5.03	91.80	
Spike High A	10.06	94.44	94.48
Spike High B	10.06	94.51	
	Total mean recovery (%)		93.24

Precision: the repeatability (precision) of the analytical method was determined by calculating the mean amount and the relative standard deviation (% RSD) from analysis of the test item in five-fold. The results are summarised in Table 19.

Table 19: Precision data for A-5D K/SD in A-5D K/SD

Test item	a.i.	Number of Determinations	Mean amount (mg/L)	Standard deviation (mg/L)	Observed RSD (%)	Horwitz RSDr (%)
A-5D K/SD	A-5D K/SD	5	19.92	0.11	0.57	1.35

Conclusions:
The aim of this study was the validation of a UV analytical method to quantify the content of potassium polyaspartate (A-5D K/SD) in water. The analytical method was validated by determining its specificity, linearity, accuracy, precision (repeatability) and evaluating the same against the respective criteria of acceptance.

The method of analysis was found to be specific as a blank solution did not showed any interference. The linearity of the method was determined using five concentrations of the test article in the range 5.04 to 25.19 mg/L. Correlation coefficient (r) obtained from linear regression line was 0.9968, which was well within the acceptance criteria. Hence it was concluded that the method of analysis was linear in the tested range of concentrations.

The accuracy of the method was determined by fortifying test article at two different levels. The accuracy was reported on the basis of the % recovery of spiked concentration of the test article. The overall mean recovery was found to be 93.24%, which was within the acceptance criteria of 70-110%. Hence it was concluded that the method was accurate for the purpose of quantification.

The precision of the method was performed by injecting five replicate samples of the same concentration solution of the test article. The % RSD of % active ingredient in the five replicate samples was 0.57%, which was within the acceptance criteria of ± 1.35%. Hence it was concluded that method was precise for the purpose of quantification.

As the method validation parameters, viz. specificity, linearity, accuracy, precision (repeatability), were within the acceptance criteria it was concluded that the method was validated.

Based on the results obtained in the study it was concluded that the UV spectrophotometric method is suitable to quantify the content of potassium polyaspartate (A-5D K/SD) in water.

A.9 Potential additional purposes of the food additive when added to food

Not applicable.

B. Information related to the safety of the food additive

Overview and evaluation of toxicological data

The toxicological studies performed with potassium polyaspartate (A-5D K/SD) were carried out at Università degli Studi di Milano (Dipartimento di Scienze Farmacologiche e Biomolecolari) and at the GLP certified INTOX laboratory. The package of studies complies with European Commission and European Food Safety Authority's (EFSA) requirements for authorization of food additives, where this information was originally submitted in 2015 for evaluation. EFSA's scientific opinion was duly published on 17 March 2016, where the panel concluded that there was no safety concern from the proposed use and use levels of potassium polyaspartate (A-5D K/SD)[9] as a stabiliser in wine. An overview and evaluation of toxicological data is provided as follows.

Digestion and absorption

The Tier 1 *in vitro* studies carried out on different types of polyaspartate and on the test item potassium polyaspartate (A-5D K/ SD) show that:

1. the polyaspartate is negligibly digested both at gastric (by pepsin) and at intestinal level (by pancreatin). The proteolysis was always close to zero and no significant statistical difference was observed between data from the beginning to the end of enzymatic attack.

2. the absorption, and therefore the bioavailability, was evaluated using CaCo-2 cells, that represents the universally recognized model simulating the intestinal system. The absorption of polyaspartate result to be insignificant both before and after the simulated gastro-intestinal digestion.

The negligible digestion and the insignificant absorption allow to assume a minimal bioavailability of the polymer that, therefore, should be for the most part excreted with the faeces.

Cellular integrity

The potential effects of potassium polyaspartate (A-5D K/SD) on the integrity of the gut cells was investigated during the Tier 1 *in vitro* toxicokinetics study. The results showed as no significant modification of the cells treated with polyaspartate were observed al all tested concentrations.

Evaluation of the potential stimulation of immuno cello

The study was conducted on pro-myelocytic human cells THP-1, as surrogate of monocytes. Monitored parameters were the up-regulation of CD86 and release of IL8 that are considered markers of the activation. The results showed as potassium polyaspartate (A-5D K/SD) did not induce any activation of the immune system and, therefore, can be considered well tolerated.

Mutagenesis studies

Mutagenesis tests were carried out on *Salmonella typhimurium* according to OECD 471 and GLP principles. The study was conducted on bacteria strains TA1535, TA97a, TA98, TA100 e TA102. The doses were: 50, 150, 500, 1500 and 5000 micrograms/plate. The test was carried out with and without metabolic activation (with and without S9 from the test rat liver). The results showed as, under the

[9] https://www.efsa.europa.eu/en/efsajournal/pub/4435

study conditions, potassium polyaspartate (A-5D K/SD) did not show any mutagenic activity (DNA alteration).

Genotoxicity studies

In vitro test on micronucleus of human cultured lymphocytes was carried out according to OECD Guideline 487 and GLP principles. The study was performed to evaluate the potential clastogenesis and aneugenesis action of A-5D K / SD. In other words, to evaluate the capability of the substance to induce chromosomal breakages and to damage the genetic heritage. The study was conducted on cultures of human lymphocytes. The doses were: 500, 1500 and 5000 micrograms/mL. The study was carried out with and without metabolic activation (with and without S9 from rat liver). These results show, in the tested conditions, that potassium polyaspartate (A-5D K/SD) did not induce any chromosomal alteration.

Sub-chronic oral toxicity at 14 days

The test of sub-chronic oral toxicity with repeated dosage for 14 days was conducted according to OECD Guideline 407 and GLP principles. The test was conducted to define the doses of potassium polyaspartate (A-5D K/SD) of the 90-day study. Wistar rats (male and female) were daily administered with potassium polyaspartate (A-5D K/SD) in the diet by oral gavage at the doses of 60, 125, 250, 500, 1000 mg/Kg body weight/day. No significant differences between the control group (not fed with polyaspartate-added diet) and the treated groups were observed in any of the following parameters: mortality, body weight, food consumption, hematic parameters, liver enzymes. Moreover, no macro- or micro-alterations of the organs were observed. Therefore, the doses selected for the 90-day study were 250, 500 and 1000 mg/kg body weight.

Sub-chronic oral toxicity at 90 days

The test was carried out according to OECD Guideline 408 modified to introduce additional parameters from OECD 407, and GLP principles. The test was conducted to evaluate the oral toxicity after repeated administration potassium polyaspartate (A-5D K/SD) for 90 days. Wistar rats (male and female) were daily administered with potassium polyaspartate (A-5D K/SD) in the diet by oral gavage at the doses of 250, 500, 1000 mg/kg body weight/day. At all tested doses, no significant difference between the control group (no polyaspartate in the diet) and treated group was observed for the following parameters: mortality, body weight, food consumption, hematic parameters, blood concentration of liver enzymes (associated to hepatotoxicity) and thyroid hormones, urine analysis, oestrous cycle in females, histological examination of organs. As a conclusion, the 90-day no-effect level (90-day NOAEL) in Wistar rats is the maximum dosage used in the tests, i.e. 1000 mg/kg body weight/day.

B.1 Information on the toxicokinetics and metabolism of the food additive and, if necessary, its degradation products or major metabolites

B.1a Toxicokinetics and metabolism of potassium polyaspartate

Summary
Toxicokinetics is an application of pharmacokinetics to determine the relationship between the systemic exposure of a compound in experimental animals and its toxicity. Tier 1 absorption and *in vitro* gastrointestinal metabolism studies were performed with potassium polyaspartate (A-5D K/SD).

The study on digestibility of potassium polyaspartate (A-5D K/SD) was performed with an *in vitro* gastrointestinal model optimized using a sequential proteolytic attack with pepsin (porcine origin) and pancreatin (porcine origin) to simulate the human digestion at gastric (pepsine) and intestinal (pancreatin) level, respectively. The proteolysis of potassium polyaspartate (A-5D K/SD) was quantified by measuring the undigested proteins by microbiuret method (Itzhaki and Gill, 1964[10]) and the release of amino acids by ninhydrin method (Moore and Stein, 1954[11]; Moore, 1968[12]). The results showed that proteolysis was always close to zero and that there were no significant statistical differences between data from the beginning to the end of enzymatic attack.

The absorption of potassium polyaspartate (A-5D K/SD) was investigated in human colon adenocarcinoma CaCo-2 cells, which are universally recognized model to simulate intestinal barrier (Sambuy et al, 2005[13]). The absorption of digested and non-digested potassium polyaspartate (A-5D K/SD) through the intestinal barrier was measured by microbiuret assay and by quantification of aspartic acid in apical and basolateral compartments after 24-hour treatment at 37°C. No significant absorption was observed in all samples, before and after gastro-intestinal digestion.

The potential effect of potassium polyaspartate (A-5D K/SD) on the integrity of the gut cells was also investigated. No significant adverse effects on the integrity of the cells were observed at all concentrations of potassium polyaspartate (A-5D K/SD) tested in comparison with the control cells.

The Tier 1 absorption and *in vitro* gastrointestinal metabolism studies performed with potassium polyaspartate (A-5D K/SD) show that:

1. potassium polyaspartate is minimally digested both at the gastric level (by pepsin) and at the intestinal level (by pancreatin). The proteolysis was always close to zero and no significant statistical difference was observed between data from the beginning to the end of enzymatic attack;
2. the absorption and, therefore, the bioavailability of potassium polyaspartate was insignificant before and after the simulated gastro-intestinal digestion; and
3. there were no adverse effects observed on the gut cells following oral administration of potassium polyaspartate.

[10] Itzhaki RF and Gill DM. 1964. A micro-biuret method for estimating proteins. Analytical Biochemistry 9: 401-410.
[11] Moore S. and Stein WH. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. Journal of Biological Chemistry 211: 907-13
[12] Moore S. 1968. Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. Journal of Biological Chemistry 243: 6281-6283
[13] Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F. 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol. 21: 1-26

The minimal digestion and the insignificant absorption observed suggests that potassium polyaspartate is negligibly absorbed in the human gastrointestinal tract following oral administration and is excreted primarily unchanged in the faeces. Indeed, there was very limited breakdown to aspartic acid (less than 4%) in the simulated gastric digestion following incubation with porcine pepsin and pancreatin, where potassium polyaspartate does not show any adverse effects on the integrity of gut cells.

This conclusion is reinforced by physicochemical characteristics of potassium polyaspartate (i.e. its relatively high molecular weight and high hydrophilicity) and other data showing its low chemical reactivity and the lack of specific target organ toxicity (i.e. no genotoxicity in *in vitro* genotoxicity studies, and lack of adverse effects in the 90-day study).

Thus, it can be concluded with reasonable certainty that potassium polyaspartate (A-5D K/SD) is negligibly absorbed in the human gastrointestinal tract and that no Tier 2 or Tier 3 toxicokinetic testing is required. Moreover, polyaspartic is a polyaminoacid of aspartic acid, a non-essential amino acid naturally produced by the human body thus no adverse effects are expected following oral consumption of A-5D K/SD according to the proposed uses.

Any aspartic acid released, however, and subsequently absorbed would go into the amino acid pool. The majority of the material remained as polyaspartate following simulated gastric digestion *in vitro*. Based on 4% breakdown of aspartic acid, it can be estimated that the maximal amount of aspartic acid released would be 0.04 mg/kg bw per day at the typical use level and 0.07 mg/kg bw per day at the proposed maximum limit.

Tier 1 Absorption studies and in vitro gastrointestinal digestion studies

To assess gastro-intestinal digestibility and intestinal absorption of potassium polyaspartate (A-5D K/SD) the following *in vitro* tests were performed:

- *In vitro* assays using a sequential proteolytic attack with pepsin (porcine) and pancreatin (porcine) to simulate gastro-intestinal digestibility (proteolysis); and
- *in vitro* absorption in human colon adenocarcinoma CaCo-2 cells to simulate intestinal absorption.

In addition, the potential adverse effects of potassium polyaspartate on cellular integrity were analysed.

The full reports of these studies can be found in Annex numbers 4.1.2.1/01 and 4.5.3 by Restani 2015, and in Annex number 4.1.2.1/02 by Vassanelli 2015, and a summary of these studies is provided as follows.

Report:	P. Restani; February 9th 2015
Title:	Final report on the assessment of gastro-intestinal digestibility and intestinal absorption of potassium polyaspartate (A-5D K/SD) assayed by *in vitro* models
Guidelines:	No
GLP	No

Gastro-intestinal digestibility

An *in vitro* gastrointestinal model was optimized using a sequential proteolytic attack with pepsin and pancreatin to simulate the gastro-intestinal digestibility.

In vitro gastric (peptic) digestion

Aliquots of potassium polyaspartate (A-5D K/SD) (equivalent to 24 mg of polyaspartate) were suspended in 8 mL of 0.06 N HCl, pH 1-2, containing 0.05 mg/mL of pepsin from porcine gastric mucosa. The final potassium polyaspartate concentration was 3 mg/mL and the enzyme/protein ratio was 1:60 (w/w). Final pH value ranged between 1.27 and 2.80, where pepsin showed the highest activity. The samples were incubated for 5 min, 10 min or 2 h (120 min) in a Dubnoff water bath at 37 °C, shaken at 100 beats/min. To evaluate gastric digestion, an aliquot of samples was analysed by measuring the undigested proteins by microbiuret method (Itzhaki and Gill, 1964) and the release of amino acids by ninhydrin method (Moore and Stein, 1954; Moore, 1968). The remaining aliquot of samples was further digested with pancreatin.

In vitro pancreatic digestion

At the end of peptic digestion (2 h of peptic attack), a solution of borate buffer containing 0.5 mg/mL of pancreatin from porcine pancreas was added to each sample in the ratio 1:3.5 (v/v). The pH was adjusted to 6.8, the optimal pH for pancreatic enzymes, and the final pancreatin/potassium polyaspartate ratio was 1:21 (w:w). Pancreatic digestion lasted 4 or 24 hours. Samples at different times of hydrolysis (5 min, 10 min or 2 h of pepsin attack, or 2 h peptic + 4 or 24 h of pancreatic attack) were heated at 100°C for 10 min in a water bath to block the enzymatic activity. All digestions were performed in triplicate, and a 'time 0' sample was obtained by mixing all reagents, substrates and enzymes without incubation. In this case, the enzymatic activity was blocked at 100°C.

Proteolysis was quantified by measuring the undigested potassium polyaspartate by microbiuret method (Itzhaki and Gill, 1964); and the release of amino acids by ninhydrin method (Moore and Stein, 1954; Moore, 1968). Linear regression curves were obtained for both "gastric" and "pancreatic" conditions and for both methods. The linear regression curve to quantify undigested potassium polyaspartate by microbiuret method was obtained using potassium polyaspartate itself as a reference standard. It not possible to quantify proteolysis using bovine serum albumin BSA because of the specific composition of potassium polyaspartate. For the same reason, the linear regression curve to quantify the amino acids by ninhydrin method was obtained using standard solutions of L-aspartic acid. Considering the different reactivity of aspartic acid with ninhydrin at different pH values, two calibration curves were prepared: 1) standard solutions in 0.06 N HCl for peptic digestion; and 2) standard solutions in HCl/borate buffer for pancreatic attack.

Intestinal absorption

The absorption of potassium polyaspartate (A-5D K/SD) was investigated using differentiated human colon adenocarcinoma CaCo-2 cells as the universally recognized model to simulate intestinal barrier (Sambuy et al, 2005[14]). The potential effect of potassium polyaspartate on the integrity of the stomach and gut cells was investigated first. Non-cytotoxic concentrations were selected during these preliminary experiments where the barrier integrity was assessed by measuring the trans-epithelial electrical resistance (TEER). A solution of 20% ethanol was used as a positive control. Afterwards the absorption of digested and non-digested potassium polyaspartate through the intestinal barrier was measured by microbiuret assay and by quantification of aspartic acid through HPLC-FLD analysis after acid hydrolysis in apical and basolateral compartments after 24 hours treatment at 37°C.

[14] Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F. 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol. 21: 1-26

Cell differentiation

CaCo-2 cells were grown at 37°C in a 5% CO2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 1% (v/v) nonessential amino acids and 1% (v/v) L-glutamine, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin (culture medium). Cells were seeded in Transwell cell culture inserts and grown for 18–22 days. This time allows cells to differentiate in enterocytes, and the separation of the apical from the basolateral compartment, reproducing the *in vivo* organization of the intestinal mucosa. To evaluate the effect of different concentrations of potassium polyaspartate on monolayer integrity, differentiated CaCo-2 cells were exposed to the parent compound or its digestion products prepared as described below, and TEER was measured with a volt-ohm meter (Evohm; World Precision Instruments, Sarasota, FL, USA), 24 h after treatment. Before treatment, monolayers were washed once with culture medium without fetal calf serum, then 0.5 mL of culture medium without fetal calf serum containing A-5D K/SD was added in triplicate cultures in the apical compartment, while 1 mL of culture medium without fetal calf serum was added in basolateral compartment.

Cell viability

Prior to studying the effects of potassium polyaspartate (A-5D K/SD) on the integrity of the gastrointestinal barrier and its absorption, its cytotoxicity was assessed by the MTT test (Mosmann 1983). 100 µL of cells (1.5x105/mL) were seeded in a 96-well plate; when confluency was reached, cells were treated in quadruplicate with increasing concentrations of potassium polyaspartate in culture medium with and without fetal calf serum. After an incubation period of 24 h, 10 µL/well of MTT solution 7.5 mg/mL in culture medium was added. Cells were incubated for 3 h at 37°C, then medium was discarded and cells lysed in 100 µL/well of a mixture of HCl 1N:isopropanol (1:24, v/v). The absorbance of the resulting solutions was read at 595 nm in a microplate reader. The viability, expressed as percentage, is calculated as: Viability (%) = OD compound/OD control X 100.

Samples

Control of peptic digestion (B1): 8 mL of 0.06 N HCl (pH 1-2) containing 0.4 mg of pepsin from porcine gastric mucosa (EC 3.4.23.1, Merck, Damstadt, Germany); Control of peptic-pancreatic digestion (B2): 8 mL of 0.06 N HCl (pH 1-2) containing 0.4 mg of pepsin from porcine gastric mucosa (EC 3.4.23.1, Merck, Damstadt, Germany). This control sample was incubated for 2 h at 37°C and then added with pancreatin; Sample Time 0 (T0): Sample at T0; Sample Time 2+24 (T2h+24h): Sample obtained after 2 h of peptic digestion and 24 h of pancreatic digestion.

All digestions were performed in duplicate, and "control" samples (time 0, time 2+24h) were obtained by incubating all reagents and substrates, including enzymes but without A-5D K/SD. At the different times of hydrolysis, samples and controls were heated at 100°C for 10 min to stop the enzymatic activity. When necessary, the pH was adjusted approximately to 7 with 0.2 MnaOH to avoid cell toxicity. Each sample was freeze-dried and before use, the powder obtained was suspended in 2 mL of sterilised water and diluted as:

- Samples T2+24 and B2 (1%): 20 µL of solution is added with 1.980 mL of medium (Dulbecco's Modified Eagle Medium, DMEM).
- Samples T0 and B1 (20%): 400 µL of solution is added with 1.6 mL of medium (Dulbecco's Modified Eagle Medium, DMEM).

In parallel the following samples were prepared:

- Potassium polyaspartate (A-5D K/SD) solution (1 mg/mL): freeze-dried product was suspended in medium (Dulbecco's Modified Eagle Medium, DMEM) at the final concentration of 1 mg/mL.

- Potassium polyaspartate (A-5D K/SD) solution (0.5 mg/mL): freeze-dried product was suspended in medium at the final concentration of 0.5 mg/mL.

The absorption of 0.5 mg/mL through the CaCo-2 cell monolayer was measured in triplicate after incubation for 24 hours at 37°C.

Quantification of absorption through CaCo-2 monolayer by microbiuret test
Microbiuret assay was used to quantify proteins/polypeptides absorbed through the CaCo-2 monolayer. The absorbance measured at 310 nm is directly proportional to the concentration of polypeptides. Potassium polyaspartate (A-5D K/SD) was used to prepare the linear regression curves. After treatment, 50 and 100 µL of apical and basolateral portion deriving from the treatments with different samples were diluted to 1 mL with the corresponding digestion medium (0.06 N HCl, or a mixture of HCl and borate buffer as described previously) and added with 0.5 mL of 0.2% copper sulfate solution in 30% sodium hydroxide (in water) or sodium hydroxide (30% in water). After measuring absorbance at 310 nm, value B is subtracted from value A to eliminate unspecific interferences.

Assessment of absorption through CaCo-2 monolayer by quantification of aspartic acid
The absorption through CaCo-2 monolayer have been assessed by the quantification of aspartic acid in apical and basolateral compartments after 24 hours treatment at 37°C. The aspartic acid has been quantified with HPLC-FLD after acid hydrolysis at high temperature (108 ± 2°C for 72 hours).

Findings
Gastro-intestinal digestibility

Linear regression for quantification of undigested A-5D K/SD *by microbiuret method*
Both "gastric" and "pancreatic" conditions showed satisfactory linearity ranges (R^2 = 0.998 in gastric conditions; R^2 = 0.988 in pancreatic conditions).

Linear regression for quantification of amino acids released during digestion by ninhydrin method
Both "gastric" and "pancreatic" conditions showed satisfactory linearity ranges (R^2 = 0.999 in gastric conditions; R^2 = 0.992 in pancreatic conditions).

Sample analysis
The undigested potassium polyaspartate (A-5D K/SD) ranged between a mean value of 24.35 mg ± 0.88 SD (value of total protein before gastric digestion) up to a mean of 25.37 mg ± 2.7 SD (value of total protein after 2 hours gastric digestion and 24 hours pancreatic digestion). Thus the values before and after the gastric digestion showed no significant statistical differences. The free amino acid deriving from potassium polyaspartate proteolysis ranged between a mean value of 0.908 mg ± 0.099 SD (mg of total free aminoacids before gastric digestion) up to a mean of 0.956 mg ± 0.08 SD (mg of total free aminoacids after 2 hours gastric digestion and 24 hours pancreatic digestion). Thus, the values before and after the intestinal digestion showed no significant statistical differences.

Intestinal absorption

Cell viability
The MTT assay showed that potassium polyaspartate (A-5D K/SD) was well tolerated up to 2 mg/mL. Higher concentrations were not tested, since data were sufficient to optimize the test. The presence or absence of foetal calf serum (FCS) did not affect cell viability following potassium polyaspartate treatment.

Effect of potassium polyaspartate on intestinal membrane integrity
To evaluate the effect of potassium polyaspartate (A-5D K/SD) on monolayer integrity, the trans-epithelial electrical resistance (TEER) was measured at different times. Potassium polyaspartate (1 mg/mL) did not affect the intestinal membrane integrity. On the contrary, the positive control ethanol (20%) significantly affected membrane integrity.

Effect of digested and undigested potassium polyaspartate on CaCo-2 cell integrity
The effect of digested and undigested potassium polyaspartate (A-5D K/SD) was assessed in parallel. Ethanol 20% was used as positive control. Undigested potassium polyaspartate (0.5 and 1 mg/mL) and digested potassium polyaspartate (T0 and T2+24 h) did not affect TEER after 24 h of exposure. As expected, the positive control ethanol (20%) significantly affected membrane integrity.

Quantification of absorption through CaCo-2 monolayer by microbiuret test and quantification of aspartic acid
Microbiuret test: linear regression curves were obtained for gastric medium (T0) and pancreatic buffer (T2+24h) conditions. The calibration curves were built using Dulbecco's Modified Eagle's Medium to take into consideration possible interferences. The linearity was always satisfactory (R2 = 0.992 for gastric conditions; R2 = 0.985 for gastric-pancreatic conditions).

The results obtained by quantifying polypeptides (microbiuret method) and total aspartic acid after the absorption test through the CoCo-2 monolayer, are provided in Table 20.

All samples showed a similar behaviour before (T0) and after (T2+24h) gastro-intestinal digestion (no significant statistical difference), and no significant absorption was observed. Even though data show a certain variability (a high Standard Deviation), the results of absorption test can be considered reliable since both polyaspartate and aspartic acid were below the limit of detection of 0.7 mg/L in the basolateral portion of the model.

Table 20. Quantification of potassium polyaspartate (A-5D K/SD) absorption through the CaCo-2 cell monolayer

Sample	**A/B**	**Absorption measured by MicroBiuret method (peptides)**			**Absorption measured as aspartic acid (after acidic hydrolysis)**
		Expected concentration	**Determined concentration m±SD**	**Distribution (%) m±SD**	**Distribution (%) m±SD**
Potassium polyaspartate		1 mg/mL	1.237±0.29		
Time 0	A	2.4 mg/mL	2.03±0.63	100.0±31.0	100±13.7
Time 2+24h		2.4 mg/mL	2.06±1.23	101.5±59.7	118±54.8
Time 0	B	-	0.004	0	0
Time 2+24h		-	0	0	0

Legend: A= Apical; B= Basolateral.

Conclusions on Tier 1 absorption studies and *in vitro* gastrointestinal metabolism

Tier 1 absorption studies and *in vitro* gastrointestinal metabolism show that neither potassium polyaspartate (A-5D K/SD) or its breakdown products are digested in gastro-intestinal tract (proteolysis of A-5D K/SD close to zero) or absorbed from human colon adenocarcinoma CaCo-2 cells. It can be concluded with reasonable certainty that potassium polyaspartate is negligibly absorbed in the human gastrointestinal tract and no Tier 2 or Tier 3 toxicokinetic testing is required.

B.2 Information on the toxicity of the food additive and, if necessary, its degradation products and major metabolites

B.2.a Acute toxicity

Acute toxicity studies have been undertaken for the sodium salt of polyaspartate but not for potassium polyaspartate. These include studies by Bayer Australia Ltd in 2001 and Lanxess Deutschland GmbH in 2007, 2011 were undertaken as a requirement for notification of the polymer as an ingredient in liquid formulations for use within the water treatment industry, and for use as a dispersant for fillers and as an anti-scale additive during the processing/evaporation of raw beet and cane sugar juice, respectively.

A review of these studies is available in Annex number 5.3 by Restani 2012, and is also provided as follows.

3.1.1 Acute and sub-acute oral toxicity
The acute oral toxicity was performed in three Wistar rats, by using the OECD TG 423 test (http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECD_GL423.pdf). Polyaspartic acid sodium salt was administered as a 42.8% aqueous solution (2000 mg/Kg pc), and the animals were observed for 14 days (Australian Government 2001). The LD50 resulted > 2000 mg/Kg. Neither mortality nor signs of toxicity were observed. The same results were found by Lanxess. A more recent document reports a LD50 > 5000 mg/kg bw (Lanxess 2011).

In addition, a sub-acute study was carried out in which groups of male and female Wistar rats received the polymer by daily gavage for a period of four weeks, followed by a recovery period of two weeks. Dose levels were 0, 40, 200, and 1000 mg/Kg bw/day. Apart from microscopic effects on the urinary bladder that have been found to be species (rat) specific, there were no adverse effects in this study (Australian Government 2001).

3.1.2 Acute dermal toxicity
Dermal toxicity was tested in three Wistar rats by using the OECD TG 402 test (http://www.oecd.org/dataoecd/17/46/1948333.pdf). Doses of 2400 mg/Kg bw of polyaspartic acid were administered under occlusive dressing for 24 hours, as 42.8% aqueous solution. Neither mortality nor signs of toxicity were observed. The LD50 was > 2400 mg/Kg (Australian Government 2001) or > 2000 mg/kg bw (Lanxess 2007) or >5000 mg/kg bw (Lanxess 2011).

3.1.3 Skin irritation
Skin irritation was tested in three male Himalayan rabbits after administration of 0.5 mL of the polymer, in a 42.8% aqueous solution, under semi-occlusive dressing for 4 hours.
Test was performed as indicated in the OECD TG 404 test method (http://www.oecd.org/dataoecd/63/6/43302385.pdf). No signs of erythema or oedema were seen in any of the animals at any of the observation times (Australian Government 2001).

3.1.4 Eye irritation
Eye irritation was tested in three male Himalayan rabbits. Volumes of 0.1 mL of 42.8% aqueous solution placed into conjunctival sac of one eye of each animal was used. The test was performed as indicated in OECD TG 405 test method (http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECDtg405.pdf). Animals were observed for

60 min, 24, 48 and 72 hours. No signs of irritation of the cornea, iris or conjunctivae were observed in any animal at any of the observation times (Australian Government 2001).

3.1.5 Skin sensitisation

Skin sensitisation was tested in guinea pigs (ten test and five control animals). The test performed, as described in the OECD TG 406 method (http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/OECDtg406.pdf) according to the protocol listed in Table 21. The polyaspartate polymer was not sensitising to the skin of guinea pigs.

Table 21: Protocol for skin sensitisation test

Day	Treatment
0	• FCA diluted 1:1 with physiological saline • 5% polyaspartate polymer in physiological saline • 5% polyaspartate polymer in FCA
7	0.5 mL of 100% polyaspartate polymer was applied topically, under occlusive dressings for 48 hours, to same skin area
21 (challenge procedure)	0.5 mL of 25% polyaspartate polymer applied topically to dorsal area and flanks for 24 hours under occlusive dressing

FCA= Freunds Complete Adjuvant

3.2 Genotoxicity

To test genotoxicity, Bayer used the Reverse Mutation Assay, performed in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, TA1537, according to the protocol described in OECD TG 471 test method (http://www.oecd.org/dataoecd/18/31/1948418.pdf).

The polyaspartate concentration range used was 50-5000 µg/plate. The independent repeat test was performed using a 20-minute pre-incubation period; there were no signs of toxicity to any of the test strains at any of the concentrations employed. Solvent control plates gave counts in the expected range and positive controls produced significant increases in the revertant counts (Australian Government, 2001).

To test genotoxicity, Lanxess (2007) also used the Reverse Mutation Assay (Ames test) *in Salmonella typhymurium*, as well as a chromosomal aberration assay in Chinese hamster V79 cells and a forward mutation assay at the HPRT locus in Chinese hamster V79 cells. No evidence of genotoxic activity was observed in any of these assays.

Australian Government. 2001 National Industrial Chemicals Notification and Assessment Scheme. File No. NA/932. http://www.nicnas.gov.au/publications/car/new/na/nafullr/na0900fr/na932fr.pdf
LANXESS 2007. Food contact notification – Polyaspartic acid, sodium salt. http://www.fda.gov/downloads/Food/FoodIngredientsPackaging/FoodContactSubstan cesFCS/UCM143781.pdf LANXESS 2011. Safety Data Sheet Baypure DS 100/40%. http://bayferrox.com/en/products-applications-bfx/product-search/detail/baypureRds/? mode=fetch&lprm=aHR0cHM6Ly90ZWNoY2VudGVyLmxhbnhlc3MuY29tL2xhbnhlc3MvZW1lYS9kZS9tc2RzL21zZHNkb2NndWFyZC9CYXlwdXJlK0RTKzEwMC80M C5wZGY/ZG9jSWQ9ODk0NzQ5MQ==

B.2.b Short-term/Sub-chronic toxicity (Tier 1)

Summary

The ***Tier 1*** toxicity testing conducted with potassium polyaspartate (A-5D K/SD) in rats consisted of:

- A 14-day range-finding study performed to collect indication of target organs and to select appropriate doses for 90-day study
- A 90-day sub-chronic toxicity study (OECD TG 408), modified to include assessment of additional parameters described in the more recent guideline on repeated-dose 28-day oral toxicity study in rodents (OECD TG 407) to allow for the identification of chemicals with the potential to cause neurotoxic, immunological or reproductive organ effects or endocrine-mediated effects.

No toxicity was observed at the maximum dose level of 1000 mg/kg bw/day (no toxicological hazard identified) in the 90-day study performed with potassium polyaspartate in rats and a NOAEL of 1000 mg/kg bw/day was determined for potassium polyaspartate.

The full reports of these studies can be found in Annex numbers 4.3.2/01 and 4.3.2/02, and a summary of these studies is provided as follows.

Studies

Repeated Dose 14 Day Oral Toxicity Study of A-5D K SD in Wistar Rat (Dose Range Finding Study)

Summary
The Repeated Dose 14-Day Oral Toxicity Study of potassium polyaspartate (A-5D K/SD) in Wistar Rat (Dose Range Finding Study) was performed following the protocol (No. P/13969/SOR-14-DRF/14) prepared based on the recommendations made in 'The OECD Guidelines for Testing of Chemicals (OECD No. 407, Section 4: Health Effects) on conduct of 'Repeated Dose 28-day Oral Toxicity Study in Rodents' (Adopted: 03 October 2008)'.

Groups of five male and five female Wistar rats were administered potassium polyaspartate, by oral gavage daily at the doses of 60, 125, 250, 500 and 1000 mg/kg body weight for 14 days and were sacrificed on day 15 to evaluate its toxicity. A concurrent vehicle control group receiving analytical grade water at the dose of 10 mL/kg was also maintained.

The rats were examined daily for signs of toxicity, morbidity and mortality. Animals were subjected to detailed clinical examination before initiation of the study and weekly thereafter during the treatment period and at termination. Body weight and food consumption were recorded weekly. Laboratory investigations were performed on blood at termination of the study. All animals sacrificed terminally were subjected to a detailed necropsy and weights of kidneys, liver, adrenals, testes, spleen, brain and heart were recorded.

Potassium polyaspartate did not induce any mortality and treatment related clinical abnormalities in rats treated at and up to the dose of 1000 mg/kg body weight. No mortality or abnormal clinical signs were observed in vehicle control group animals.

Body weight gain and food consumption was not affected at and up to the dose of 1000 mg/kg body weight. The haematological parameters of haemoglobin, packed cell volume, total RBC count, total and differential WBC counts, RBC indices and platelet count of male and female rats, treated with the

test article at and up to the level of 1000 mg/kg were found to be comparable to those of the control animals at termination.

Potassium polyaspartate, at and up to the level of 1000 mg/kg, did not alter the plasma levels of total protein, albumin, globulin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, glucose, urea nitrogen, urea, creatinine and total cholesterol in male and female rats.

The values of absolute and relative organ weights of male and female rats treated with the test article, at and up to 1000 mg/kg were found to be comparable with those of the control rats at termination.

No gross pathological alterations were encountered in the rats sacrificed at termination of the study.

Based on these findings, the doses of potassium polyaspartate selected for the Repeated Dose 90-Day Oral Toxicity Study of potassium polyaspartate (A-5D K/SD) in Wistar Rat were 250, 500 and 1000 mg/kg body weight.

Report:	S. A. Gumaste; March, 06th 2014			
Title:	Repeated Dose 14-Day Oral Toxicity Study of A-5D K SD in Wistar Rat (Dose Range Finding Study)			
Document No.	R/13969/SOR-14-DRF/14			
Guidelines	OECD No. 407			
GLP	YES			
I. MATERIALS AND METHODS				
A. MATERIALS:				
1. Test Article Name	Potassium aspartate A-5D K SD			
Description :	Tan powder			
Lot/Batch #:	KHKS-040412			
CAS #:	64723-18-8			
Stability of test compound:	Not reported			
Date of manufacture:	04-04-2012			
Date of expiry:	04-03-2016			
2. Vehicle and/or positive control:	Analytical grade water			
3. Test animals -				
Species:	Rats (*Rattus norvegicus*)			
Strain:	Wistar			
Age:	Age at the start of treatment: 8 weeks.			
Weight at dosing:	Male Rats : 151 g to 190 g Female Rats : 130 g to 157 g			
	Male Rats	Mean	=	171.57 g (100%)
		Minimum	=	151 g (-11.99%)
		Maximum	=	190 g (+10.74%)
		n	=	30
	Female Rats	Mean	=	141.53 g (100%)
		Minimum	=	130 g (-8.15%)
		Maximum	=	157 g (+10.93%)
		n	=	30

Source:	INTOX PVT. LTD.
Acclimation period:	The animals were acclimatized for a period of eight days in the experimental room before start of the experiment.
Diet:	'Nutrilab' brand extruded pelleted rat feed manufactured by M/s Provimi Animal Nutrition India Ltd., Bangalore, was provided *ad libitum*. The feed is analyzed for nutritional components and environmental contaminants once in a year. Results of dietary analysis are provided by the manufacturer for each batch of diet.
Water:	Potable water, passed through an 'Aquaguard' water filter and subjected to ultra violet irradiation, was provided *ad libitum* in sterilized bottles with stainless steel sipper tubes. Water is analyzed half yearly for potability and once in a year for various environmental contaminants.
Housing:	Animals were housed in Room Number AR-12 in the experimental animal facility of INTOX PVT. LTD., maintained under appropriate barriers. Groups of five animals of similar sex being housed in one cage, in sterilized solid bottom polypropylene cages with stainless steel grill tops, facilities for food and water bottle, and with bedding of clean and sterilized paddy husk. Cages were suspended on movable stainless-steel racks.

B. STUDY DESIGN:

1. Animal assignment and treatment:

The selected male and female rats were assigned to control and different treatment groups as shown below:

G1	Vehicle control 10 mL/kg (Analytical grade water)	5 male and 5 female rats
G2	60 mg/kg	5 male and 5 female rats
G3	125 mg/kg	5 male and 5 female rats
G4	250 mg/kg	5 male and 5 female rats
G5	500 mg/kg	5 male and 5 female rats
G6	1000 mg/kg	5 male and 5 female rats

The test article was administered by oral gavage to each rat daily for 14 consecutive days at approximately the same time. Test article formulations were administered using an intubation needle fitted onto a disposable syringe of appropriate size (16G). The dosage volume administered to individual rats was adjusted according to its most recently recorded body weight. The dose volume was 10 mL/kg body weight. The vehicle control group rats received vehicle only i.e. analytical grade water at the dose volume of 10 mL/kg body weight.

3. Diet preparation and analysis:

Test article dosing formulations were prepared at appropriate concentrations to meet dosage requirements. The dosing formulations were prepared freshly each day before dosing. Analytical grade water was used as the vehicle control article. A-5D K/SD was dissolved in analytical grade water. Formulations prepared for different doses varied in concentrations to allow a constant dosage volume of 10 mL/kg body weight.

4. Statistics:

Using specific MS-Excel based computer programs, data was analyzed for statistical significance.
The body weight, organ weight, haematology and clinical chemistry data of different groups was compared by Bartlett's test for homogeneity. The data with homogeneous intra-group variances was subjected to one-way analysis of variance (ANOVA-Snedecor and Cochran, 1980). When `F' value was significant, Dunnett's pair wise comparison (Scheffe, 1953) of means of treated groups with control mean was performed individually. All analysis and comparisons were evaluated at 5% ($P<0.05$) level. Statistically significant differences ($P<0.05$) indicated, are designated by the superscripts in summary tables as stated below, where s+ / s- : Significantly higher (s+) / lower (s-) than the control group.

C. METHODS:

Observations:

1. Mortality:

Throughout the study, all cages were checked early on each working day and again in the afternoon to look for dead or moribund animals to allow necropsy examination to be carried out during the working hours of that day.

2. Clinical signs:

The rats were subjected to clinical examinations once daily during treatment period. All signs of ill health, together with any behavioural changes or reaction to treatment were recorded for individual animals. Dated and signed records of appearance change and disappearance of clinical signs were maintained on clinical history sheets.

3. Body weight:

The weight of each rat was recorded before initiation of treatment (day 0), weekly thereafter (day 7 and 14) and at necropsy (day 15).

4. Food consumption and compound intake:

The quantity of food consumed by rats in each cage was recorded weekly. Food intake per rat was calculated using the amount of food offered to and left in each cage in each group, and the number of rats in each cage.

5. Pathology

Clinical pathology

On completion of 14 days of treatment (i.e. on day 15), samples of blood were drawn, under light carbon dioxide anaesthesia, from the orbital plexus of all the male and female rats. The samples were collected in tubes containing K-EDTA for haematology and heparin for clinical chemistry as anticoagulants.

Animals were fasted overnight (approximately 18 hours) prior to blood collection but were having access to water *ad libitum.*

The estimations that were performed on blood samples have been listed below, together with an abbreviated title (used in Appendices and Tables).

Haematology

The following haematological parameters were determined using a 'Abbott, Cell-Dyn 3700 CS' Haematology Analyser (Abbott Park IL, 60064, USA): Haemoglobin (Hb), Packed cell volume (PCV), Total red cell count (Total RBC), Total white cell count (Total WBC), Platelets (Plat).

The following calculated RBC associated indices were recorded from the haematology analyzer: Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC).

Differential leukocyte count was made by using the 'Abbott, Cell-Dyn 3700 CS' Haematology Analyser (Abbott Park IL, 60064, USA)' Haematology Analyser.

Leucocytes were differentiated as, Neutrophils (N), Lymphocytes (L), Eosinophils (E), Monocytes (M) and Basophils (B).

Clinical chemistry:

Plasma samples were analyzed individually for determination of clinical chemistry parameters using Synchron CX-5 Pro Fully Automatic Random Access Analyser (Beckman Coulter, Inc., Miami, Florida, USA). These analyses were performed using commercially available diagnostic kits manufactured by Beckman Coulter, Inc., USA.

Plasma was analysed for the following parameters: Glucose (Glu), Urea Nitrogen (UN), Urea, Total Protein (Tot.Pro), Aspartate Amino transferase (AST), Alanine Amino transferase (ALT), Alkaline Phosphatase (Alp), Creatinine, Albumin, Globulin, Total Cholesterol.

6. Necropsy examination:

At termination of treatment period (i.e. on day 15), all rats were sacrificed by exsanguination under CO_2 anesthesia and complete necropsies were carried out.

Organ weights:

The following organs from all animals killed at the scheduled sacrifices were trimmed of any adherent tissue, as appropriate, fat and weighed wet as soon as possible to avoid drying: kidneys, liver, adrenals, testes, spleen, heart, brain.

Values of these organs as percent of necropsy body weights were estimated (relative organ weights).

Histopathology:

Histological examination was not carried out in the absence of any gross pathological change.

D RESULTS

Mortality

No mortality was observed in rats treated with test article at and up to 1000 mg/kg body weight. No mortality was observed in vehicle control group animals.

Clinical signs

In animals treated at and up to the dose of 1000 mg/kg, the daily general clinical examinations and weekly detailed clinical examinations did not reveal any treatment related incidence of clinical abnormalities.

Body weights

The body weight gain by male and female rats treated at and up to 1000 mg/kg body weight was found to be comparable to that by the control rats throughout the treatment period.

Food consumption

The values of average daily food consumption were not affected in both male and female rats treated at and up to 1000 mg/kg body weight.

The average daily food consumption per rat per day, computed over the period of two weeks, by male rats receiving test article at 60, 125, 250, 500 and 1000 mg/kg was 102%, 105%,107%, 106% and 105% respectively of that by control rats. Similarly, the average daily food consumption by female rats receiving the test article at 60, 125, 250, 500 and 1000 mg/kg was 112%, 104%, 113%, 118% and 105% respectively of that by control rats.

Haematology

At the end of treatment period and at the end of recovery period, the group mean values of haematological parameters such as hemoglobin, packed cell volume, total RBC count, RBC indices and platelet count of male and female rats, treated with test article at and up to the level of 1000 mg/kg, were found to be comparable with those of the control animals.

Although a statistically significant change was seen in eosinophilic count in male (G5) and in female (G3), it was not considered to be the treatment related change due to lack of dose related effect.

Clinical chemistry

The test article, up to the dose level of 1000 mg/kg, did not induce any changes in the plasma levels of total protein, albumin, globulin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, glucose, urea nitrogen, urea, creatinine and total cholesterol in male and female rats, at termination of the treatment.

Although a statistically significant change was seen in values of AST (G3 and G6 male), glucose (G4 male), blood urea nitrogen and urea (G5 males), whereas albumin (G3 and G6 female), ALP (G6 female) and total cholesterol in G5 females, it was not considered to be the treatment related change due to lack of dose related effect. Few individual deviations were noted both in treatment and control groups and hence none were considered of toxicological significance.

Organ weights

The values of absolute and relative weights of kidneys, liver, adrenals, testes, spleen, brain and heart of male / female rats treated with the test article at and up to 1000 mg/kg were found to be comparable to those of the control group rats at termination of the treatment period.

Although a statistically significant change was seen in absolute weights (male- liver and brain and female- kidneys and brain) and relative organ weights (male- kidneys and liver and female- kidney and brain), it was not considered to be the treatment related change due to lack of dose related effect.

Gross pathology

No gross pathological alterations were encountered in the rats sacrificed at termination of the study.

CONCLUSIONS

The present study involved daily oral administration of A-5D K/SD to groups of Wistar rats, five per sex per dose, at the doses of 60, 125, 250, 500 and 1000 mg/kg body weight for 14 days to evaluate its toxicity. The findings of this study were as follows:

- no mortality in rats treated with test article at and up to 1000 mg/kg body weight;
- no incidence of treatment related clinical abnormalities, at and up to the dose of 1000 mg/kg;
- no effect on the body weight gain by male and female rats treated at and up to 1000 mg/kg;
- no effect on average daily food consumption by the male and female rats treated at and up to the dose of 1000 mg/kg;

- no effect on the hematological and clinical chemistry parameters of male and female rats treated at and up to the dose of 1000 mg/kg;
- no significant alterations in the absolute and relative organ weights; and
- no treatment related gross pathological alterations in the tissues of male and female rats treated at and up to the level of 1000 mg/kg.

Based on these findings, the doses selected for the 'Repeated Dose 90-Day Oral Toxicity Study of potassium polyaspartate (A-5D K/SD) in Wistar Rat' were 250, 500 and 1000 mg/kg body weight.

Repeated Dose 90-Day Oral Toxicity Study of A-5D K SD in Wistar Rat

Executive Summary

Repeated Dose 90-Day Oral Toxicity Study of potassium polyaspartate (A-5D K/SD) in Wistar rats was performed in compliance with The OECD Guidelines for Testing of Chemicals (No. 408, Section 4: Health Effects) on conduct of 'Repeated Dose 90-Day Oral Toxicity Study in Rodents' (Adopted : 21 September, 1998) and in accordance with the mutually agreed protocol (No. P/13957/SOR-90/14) where additional parameters placing more emphasis on endocrine-related endpoints, as described in OECD Guideline for Testing of Chemicals (No. 407, Section 4: Health Effects) on conduct of "Repeated Dose 28-day Oral Toxicity Study in Rodents" (Adopted: 3 October 2008), were also included, as recommended in EFSA Guidance for submission for food additive evaluations (EFSA Journal 2012; 10(7):2760).

Groups of ten male and ten female Wistar rats were administered potassium aspartate (A-5D K/SD) by oral gavage daily at the doses of 250, 500 and 1000 mg/kg of body weight for 90 days and were sacrificed on day 91 to evaluate its toxicity. A concurrent control group of ten males and ten females receiving the vehicle, i.e. analytical grade water, at 5 ml/kg was also maintained for 90 days. Additionally, groups of five rats per sex which had received the vehicle at 5 ml/kg and the test article at the high dose level, i.e. 1000 mg/kg body weight, were further observed for a period of 28 days following the 90 days treatment, for assessment of reversibility, persistence or delayed occurrence of toxicity.

The rats were examined daily for signs of toxicity, morbidity and mortality. They were subjected to detailed clinical examination before initiation of the study and weekly thereafter during the treatment period, reversal period, and at termination. Ophthalmoscopic examination was conducted on control and high dose group animals before initiation of the study and at termination of treatment. In the thirteenth week of treatment animals were additionally examined for assessment of sensory reactivity, assessment of grip strength and motor activity. Body weight and food consumption were recorded weekly. Laboratory investigations were performed on blood and urine at termination of the treatment and at the end of recovery period. All animals sacrificed terminally were subjected to a detailed necropsy and weights of kidneys, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries and heart were recorded. Histopathological evaluation was performed on all tissues [(brain, spinal cord, eye, pituitary, thyroid, parathyroid, spleen, thymus, adrenals, pancreas, trachea, lungs, heart, aorta, oesophagus, stomach, duodenum, Jejunum, terminal ileum, colon, rectum, liver, kidneys, urinary bladder, prostate, seminal vesicle, epididymides, testes, ovaries, uterus, skin. Sciatic nerve, bone marrow (smear), mammary gland (females), mesenteric lymph node, axillary lymph node and salivary glands)] in all rats from the control and high dose groups.

There was no incidence of treatment-related mortality in rats treated with potassium aspartate at any of the dose levels. Potassium aspartate did not induce any remarkable or treatment related clinical abnormalities in rats treated at and up to the dose of 1000 mg/kg body weight. No mortality or

abnormal clinical signs were observed in the vehicle control animals. Ophthalmological examination did not reveal any treatment related ocular abnormalities. Also, the observations on sensory reactivity, grip strength and motor activity conducted in the thirteenth week of treatment did not reveal any neurotoxic potential of the test article. Body weight gain was not affected in male and female rats treated at and up to the dose of 1000 mg/kg and were found to be comparable to that by the control rats throughout the treatment period and also during the recovery period. Potassium aspartate did not have any adverse effect on the average daily food consumption by the male and female rats treated at any of the dose levels.

The haematological parameters of haemoglobin, packed cell volume, total RBC count, total and differential WBC counts, RBC indices, platelet count, activated partial thromboplastin time and prothrombin time of male and female rats treated with potassium aspartate were found to be comparable to those of the vehicle control animals at termination of the treatment, and also at the end of the recovery period.

Potassium aspartate did not alter the plasma levels of total protein, albumin, globulin, alanine aminotransferase, aminotransferase, alkaline phosphatase, glucose, creatinine, calcium, total cholesterol, phosphorous, total bilirubin, urea nitrogen, urea, sodium, potassium, thyroid hormones and triglycerides in male and female rats. The data on urinalysis indicated no adverse effect due to the treatment.

The values of absolute and relative weights of kidneys, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries and heart of male and female rats treated with test article, were found to be comparable with those of the control rats at the end of treatment period and also at the end of the recovery period.

Potassium aspartate did not have any adverse effect on the oestrus cycle of female rats treated at any of the dose levels.

No treatment related gross pathological changes were noted. Histopathological examination was performed on tissues of the control and high dose group animals, where the changes in the high dose group were incidental or comparable to the control group or unrelated to treatment.

Based on the findings of this study, the No-Observed-Adverse-Effect-Level (NOAEL) of potassium aspartate (A-5D K/SD) in Wistar rats, following oral administration for 90 days was found to be equal to or greater than 1000 mg/kg body weight.

Report:	S. A. Gumaste; April, 03rd 2014
Title:	Repeated Dose 90 Day Oral Toxicity Study of **A-5D K SD** in Wistar Rat
Document No.	R/13957/SOR-90/14
Guidelines	OECD No. 408
GLP	YES
I. MATERIALS AND METHODS	
A. MATERIALS:	
1. Test Article Name	Potassium aspartate A-5D K SD

Description :	Tan powder
Lot/Batch #:	KHKS-040412
CAS #:	64723-18-8
Stability of test compound:	Stable for 24 hours in analytical grade water
Date of manufacture:	04-04-2012
Date of expiry:	04-03-2016
2. Vehicle and/or positive control:	Analytical grade water
3. Test animals -	
Species:	Rats (*Rattus norvegicus*)
Strain:	Wistar
Age:	Age at the start of treatment: 8 weeks.
Weight at dosing:	Male Rats : 151 g to 196 g Female Rats : 130 g to 160 g
Source:	INTOX PVT. LTD.
Acclimation period:	The animals were acclimatized for a minimum period of seven days in the experimental room before start of the experiment.
Diet:	'Nutrilab' brand extruded pelleted rat feed manufactured by M/s Provimi Animal Nutrition India Ltd., Bangalore, was provided *ad libitum*. The feed is analyzed for nutritional components and environmental contaminants once in a year. Results of dietary analysis are provided by the manufacturer for each batch of diet.
Water:	Potable water, passed through 'Aquaguard' water filter and subjected to ultra violet irradiation, was provided *ad libitum* in sterilized bottles with stainless steel sipper tubes. Water is analyzed half yearly for potability and once in a year for various environmental contaminants.
Housing:	Animals were housed in Room Number AR-11 in the experimental animal facility of INTOX PVT. LTD., maintained under appropriate barriers. Groups of two / three animals of similar sex being housed in one cage, in sterilized solid bottom polypropylene cages with stainless steel grill tops, facilities for food and water bottle, and with bedding of clean and sterilized paddy husk. Cages were suspended on movable stainless-steel racks.

B. STUDY DESIGN:

1. Animal assignment and treatment:

The selected male and female rats were assigned to control and different treatment groups as shown below:

G1	Vehicle control 5 ml/kg (Analytical grade water)	10 male and 10 female rats

G2	Vehicle Control (Recovery) 5 ml/kg (Analytical grade water)	5 male and 5 female rats
G3	250 mg/kg (A-5D K SD)	10 male and 10 female rats
G4	500 mg/kg (A-5D K SD)	10 male and 10 female rats
G5	1000 mg/kg (A-5D K SD)	10 male and 10 female rats
G6	1000 mg/kg (A-5D K SD) (Recovery)	5 male and 5 female rats

The test article was administered by oral gavage to each rat daily for 90 consecutive days. Test article formulations were administered using an intubation needle (18/16 G) fitted onto a disposable syringe of appropriate size. The dosage volume administered to individual rat was adjusted according to its most recently recorded body weight. The dose volume was 5 ml/kg body weight. The vehicle control group rats received vehicle only i.e. analytical grade water at the dose volume of 5 ml/kg body weight.

3. Diet preparation and analysis:

Test article dosing formulations were prepared at appropriate concentrations to meet dosage level requirements. The dosing formulations were prepared freshly before daily dosing. A-5D K/SD was dissolved in analytical grade water. Formulations for different doses varied in concentrations to allow a constant dosage volume of 5 ml/kg body weight. Analytical grade water was used as vehicle control article.

4 Analysis of formulations for stability and concentration verification

Test article stability in analytical grade water by a validated analytical method has been established for 24 hours. A-5D K/SD was found stable for 24 hours in analytical grade water (Stability Study No. R/RA1385/14).

The test article formulations were subjected for verification of concentration thrice during the study period. Formulation analysis was performed at Analytical Chemistry Section of INTOX following the method provided by the Sponsor and validated at INTOX (Method Validation Study No. R/RA1384/14). The concentration of test article measured in the formulations prepared for dosing in the first week (on day 6), 7th week (on day 48) and 12th week (on day 79) of study, was close to the intended dose and was within acceptable limits. Results of formulation analysis (concentration verification) have been appended to the report (Appendix 14).

5. Statistics:

Using specific computer programs, data (body weight, organ weights, haematology, and clinical chemistry) were analyzed for statistical significance.

The body weight, haematology, clinical chemistry and organ weight data of different groups were compared by Bartlett's test for homogeneity. The data with homogeneous intra-group variances were subjected to one-way analysis of variance (ANOVA-Snedecor and Cochran, 1980). When the `F' value was significant, Dunnett's pair wise comparison (Scheffe, 1953) of means of treated groups with control mean was done individually.

All analysis and comparisons were evaluated at 5% ($P<0.05$) level. Statistically significant differences ($P<0.05$) indicated, are designated by the superscripts in summary tables as stated below
s+ / s- : Significantly higher (s+) / lower (s-) than the control group.

C. METHODS:

Observations:

1. Mortality

Throughout the study, all cages were checked early on each day and again in the afternoon to look for dead or moribund animals to allow necropsy examination to be carried out during the working hours of that day.

2. Clinical signs

The rats were subjected to clinical examinations once daily during the treatment and recovery periods. All signs of ill health, together with any behavioral changes or reaction to treatment were recorded for individual animals. Dated and signed records of appearance change and disappearance of clinical signs were maintained on clinical history sheets.

3. General clinical examinations

The rats were subjected to general cage side clinical examinations once daily during the treatment period and also during the recovery period.

4. Detailed clinical examinations

The rats were subjected to detailed clinical examinations before initiation of the treatment (to allow for within-subject comparisons) and weekly thereafter during the treatment and recovery periods. These observations were made outside the home cage in a standard arena and preferably at the same time. Signs noted were included, but not limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity such as lacrimation, piloerection, pupil size, and unusual respiratory pattern. Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards) were also recorded.

5. Ophthalmoscopy

At initiation of the experiment (prior to administration of the test article) and at termination of the treatment, the eyes of control and high dose group rats were examined by means of an ophthalmoscope (Heine-mini 3000).

After initial examination of eyes for 87 reflexes, the pupils were dilated using a Tropicamide ophthalmic solution to facilitate examination of fundus. These investigations were not extended to other groups and recovery groups, in the absence of any indication of treatment related abnormalities in the high dose group.

6. Functional observations

In the 13th week of treatment, all animals were examined for assessment of sensory reactivity, assessment of grip strength and motor activity. These include the functional observational battery suggested by Moser (Moser, 1989. Animal Behavioral Methods in Neurotoxicity Assessment).This neurological examination included :

1. Examinations in home-cage and open field
    a. Posture / Movement
    b. Respiration
    c. Palpebral closure
    d. Lacrimation
    e. Salivation
    f. Skin and hair coat
    g. Urination
    h. Defecation
    i. Locomotor activity
    j. Rearing

k. Gait

2. Manipulative examination / Responses to stimuli
    a. Tactile (touch) response
    b. Response to nociceptive stimuli (tail pinch)
    c. Pupil response to light
    d. Proprioception – Righting reflex
    e. Auditory response
    f. Head shaking
    g. Landing foot splay
    h. Grip strength

7. Body weight:

The weight of each rat was recorded before initiation of treatment (day 0), weekly thereafter and at necropsy. Weights of reversal group rats were recorded weekly during the post-treatment period and at necropsy.

8. Food consumption and compound intake:

The quantity of food consumed by rats in each cage was recorded weekly. Food intake per rat was calculated using the amount of food offered to and left in each cage in each group, and the number of rats in each cage. Food consumption of reversal group rats was recorded weekly during the post-treatment period. As the food offered to animals is in the form of firm steam extruded food pellets which are subjected to minimal spillage, the food consumption was not corrected for spillage.

9. Determination of oestrous cycle:

At termination of treatment (on day 90) and at termination of the reversal period (on day 118) the stage of oestrous cycle of all females was determined by taking vaginal smears.

10. Pathology:

Clinical pathology

After completion of 90 days of treatment (on day 91) and at termination of the reversal period (on day 119), samples of blood were drawn, under light carbon dioxide anesthesia, from the orbital plexus of all the male and female rats. The samples were collected in tubes containing K-EDTA, for haematology, and heparin, for clinical chemistry, as anticoagulants.

Animals were fasted overnight prior to blood collection, but had access to water ad libitum.

Also, urine samples from all the rats were collected at termination of the treatment period and at the end of the reversal period. Urine samples were collected using urine collection cages.

The estimations that were performed on blood and urine samples have been listed below.

Haematology:

The following estimations were performed using 'Abbott Cell Dyn 3700' Haematology Analyser (Abbott Park, IL 60064, USA): Haemoglobin (Hb), Packed cell volume (PCV), Total red cell count (Total RBC), Total white cell count (Total WBC), Platelets (Plat).

The following calculated RBC associated indices were recorded from the haematology analyzer: Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC).

Differential leukocyte count was made by using the 'Abbott, Cell-Dyn 3700' Haematology Analyser (Abbott Park IL, 60064, USA)' Haematology Analyser.

Leucocytes were differentiated as, Neutrophils (N), Lymphocytes (L), Eosinophils (E), Monocytes (M) and Basophils (B).

After completion of 90 days of treatment (on day 91) and at termination of the reversal period (on day 119), the following coagulation parameters were determined using 'Semi Automated Coagulation Analyser Start® 4, (Diagnostica Stago, France): Prothrombin Time (PT) – Seconds; Activated Partial Thromboplastin Time (APTT) – Seconds.

Clinical chemistry:

Plasma samples were analyzed individually for determination of clinical chemistry parameters using Synchron CX-5 Pro Fully Automatic Random Access Analyser (Beckman Coulter, Inc., Miami, Florida, USA). The analyses were performed using commercially available diagnostic kits manufactured by Beckman Coulter, Inc., USA.

The quantitative estimations of the thyroid hormones (T3, T4 and TSH) were performed by ELISA method using standard ELISA kits for rats. These estimations were done on ELISA Reader (Thermo Scientific GO., USA).

Plasma was analysed for the following parameters: Glucose (Glu), Urea Nitrogen (UN), Urea, Total Protein (Tot.Pro), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (Alp), Creatinine, Albumin, Globulin, Total Cholesterol; Total Bilirubin, Calcium, Phosphorous, Triglycerides, Triiodothyronine – T3, Thyroxine – T4, Thyroid Stimulating Hormone –TSH.

After completion of 90 days of treatment (on day 91) and at termination of the recovery period (on day 119) the following clinical chemistry parameters were determined using 'Easylyte Analyser': Sodium and Potassium.

11. Urinalysis

Urinalysis was performed on all animals, few days before the treatment period (day 86) and just before the end of the reversal period (day 116 and day 117).

Urine samples were collected using a battery of specially designed stainless-steel urine collection cages. Each rat was housed in this cage. Urine samples were collected over a period of about 2 to 4 hours. Food and water were not offered during this period.

Qualitative tests

Colour, Appearance, Specific gravity, pH, Protein, Glucose, Ketone, Bilirubin, Urobilinogen, Nitrite, Blood, Leucocytes, Volume.

Tests were performed using Multistix® 10 SG multiple reagent diagnostic strips manufactured by Siemens Healthcare Diagnostics Pty. Ltd., Bayswater Victoria 3153, Australia and were used as qualitative / semi-quantitative indicators of analyte concentration. The results were read using 'Clinitek Status' Urine Analyser' (Siemens Medical Solutions Diagnostics).

Microscopy

For microscopic examination, a drop of urine sample was spread on a microscope slide and examined for the presence of the following: Epithelial cells, Casts, Crystals, Other abnormal constituents, if any.

12. Necropsy examination

At termination of the treatment period (day 91) and at the end of the reversal period (day 119), all rats were sacrificed by exsanguinations under CO_2 anesthesia and complete necropsies were carried out. Necropsy was also performed on animals died during the study.

All the tissues listed in Appendix 13 of the report, from all animals, were preserved in 10% neutral buffered formalin. Testes and eyes were collected in modified Davidson's fixative. In addition, samples of any macroscopically abnormal tissues were preserved, along with samples of adjacent normal tissue where appropriate.

Organ weights:

The following organs from all animals killed at the scheduled sacrifices were trimmed of any adherent tissue, as appropriate, and weighed wet as soon as possible to avoid drying: kidneys, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries, and heart.

Values of these organs as percent of necropsy body weights were estimated (relative organ weights).

Histopathologycal examination:

Tissues collected for microscopic examination in this study were embedded in paraffin wax, sectioned at five micrometres and stained with haematoxylin and eosin.

Histopathological examination was conducted on the specified list of tissues including all macroscopically abnormal tissues of all animals from the control and high dose groups. The investigations were not extended to the lower dose groups and reversal groups in the absence of any treatment-related histological findings at the high dose level.

D RESULTS

Mortality

There was no incidence of any treatment related mortality amongst the rats treated with the test article at any of the dose levels. All treated animals survived throughout the treatment period of 90 days and also during the recovery period.

Clinical signs

The daily general clinical examinations and weekly detailed clinical examinations did not reveal any remarkable and treatment related incidence of clinical abnormalities.

Ophthalmoscopy

The terminal ophthalmologic examinations did not reveal any remarkable and treatment related incidence of ocular abnormalities.

Functional Observations (Neurological Examination)

The neurological examinations (functional observations) conducted in the thirteenth week of the study did not reveal any remarkable and treatment related incidence of neurological abnormalities. Also no findings, indicative of a neurotoxic potential of the test article, were encountered during these examinations.

Body weights

Body weight gain by male and female rats treated with the test article at and up to the dose of 1000 mg/kg of body weight, was found to be comparable to that by the control rats throughout the treatment period. Also during recovery period, the weight gain by male and female rats from the high dose group was found to be comparable to that by the control group rats.

Food consumption
The values of average daily food consumption by male and female rats treated with the test article at different dose levels, remained comparable to those of the control group rats.

The average daily food consumption per rat per day, computed over the period of 13 weeks, by male rats of group G3, G4 and G5 was 99%, 98%, and 97% of that by control rats. Similarly, the average daily food consumption by female rats of group G3, G4 and G5 was 98%, 99% and 103% respectively of that by control rats. After cessation of treatment the values of food intake during the recovery period were found to be comparable among the vehicle control and the high dose groups.

Haematology
At the end of the treatment period and also at the end of the recovery period, the group mean values of haematological parameters such as haemoglobin, packed cell volume, total and differential leucocyte counts, total RBC count, RBC indices, platelet count, activated partial thromboplastin time and prothrombin time of male and female rats, treated with the test article at and up to the level of 1000 mg/kg of body weight, were found to be comparable with those of the control animals.

Significant decrease in the value of neutrophils was observed in high dose group males as compared to control animals. The decrease in the value of neutrophils is marginal and not dose dependent and hence was not considered to be biologically significant.

Clinical chemistry
The test article, up to the dose level of 1000 mg/kg of body weight, did not induce any changes in the plasma levels of total protein, albumin, globulin, alanine aminotransferase, aminotransferase, alkaline phosphatase, glucose, urea nitrogen, urea, creatinine, total cholesterol, total bilirubin, sodium, potassium, calcium, phosphorous, thyroid hormones (T3, T4 and TSH) and triglycerides in male and female rats, at termination of the treatment and at the end of recovery period. The T4 values in three males and two females were below detectable range. This was observed in animals from treatment groups as well as from vehicle control group and not considered as treatment related change.

Although a statistically significant change (significantly lower) was seen in total cholesterol values in high dosed males, it was not considered to be a treatment related change due to lack of dose related effect. The decrease in the cholesterol values was marginal and incidental and of no toxicological significance.

Urinalysis
The data on urinalysis evaluated at termination of treatment and also at the end of the recovery period did not indicate any abnormality due to treatment with the test article. The data in treated animals and control animals was found to be comparable.

Oestrous cycle
Treatment with the test article did not result in any remarkable and treatment related incidence of oestrous cycle abnormalities in any of the female rats. Females from all control and treated groups exhibited the normal pattern of oestrus cycle.

Organ weights
The values of absolute and relative weights of kidneys, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries and heart of male / female rats treated with the test article were found to be comparable to those of the control group rats at termination of the treatment.

Although statistically significant changes were observed in a few organs (increased weights of testes, brain and thymus) in high dose recovery male rats at the end of recovery period, the changes observed were considered to be incidental and not treatment related as similar changes were not observed in these organs in high dose male rats at the end of treatment period.

Gross pathology

The test article, at and up to the dose level of 1000 mg/kg of body weight, did not induce any remarkable and treatment related gross pathological alterations in any of the tissues of treated rats, as evident at the detailed necropsy examination carried out at termination of the study and also at the end of recovery period. The only incidental gross pathological finding observed was adhesion of stomach with liver in a male rat treated at the dose of 250 mg/kg body weight. This being an isolated finding, considered as incidental and not related to treatment.

Histopathology

Histopathological examinations of the tissues of male and female rats from the control group and those treated at the high dose level did not reveal any significant and treatment related histopathological alterations.

Some incidental and spontaneous lesions observed in animals from the vehicle control and high dose group (1000 mg/kg) were – perivascular lymphocytic aggregation, peribronchial lymphoid tissue hyperplasia and foam cells in lungs; portal lymphocytic infiltration, chronic inflammatory foci and necrosis in liver; tubular dilatation and interstitial nephritis in kidneys; chronic trachitis in trachea; sub-mucosal lymphoid hyperplasia in stomach, ileum and colon; isolated incidence of lymphocytic infiltration in rectum and adhesions to serosa of stomach. Also, an isolated incidence of perivascular cuffing with lymphoid cells was observed in brain. All the microscopic changes noticed in this study appeared to be incidental as their frequency is very low and not dose dependent. In addition, the lesions were distributed with equal frequency in control and high dose animals and hence considered as incidental.

CONCLUSIONS

The present study involved daily oral administration of A-5D K/SD to groups of Wistar rats 10 per sex per dose, at the doses of 250, 500 and 1000 mg/kg of body weight for 90 days to evaluate its toxicity and reversibility of toxicity, if any. The findings of this study were as follows:

- no mortality at and up to the dose of 1000 mg/kg body weight;
- no incidence of treatment related clinical abnormalities, and no ocular toxicity and neurotoxicity, at and up to the dose of 1000 mg/kg body weight;
- no adverse effect on body weight gain by the male and female rats treated at and up to the dose of 1000 mg/kg body weight;
- no effect on average daily food consumption by the male and female rats treated at and up to the dose of 1000 mg/kg body weight;
- no effect on the hematological parameters of male and female rats treated at and up to the dose of 1000 mg/kg body weight;
- no effect on the clinical chemistry and urinalysis parameters of male and female rats treated at and up to the dose of 1000 mg/kg body weight;
- no significant alterations in the oestrous cycle of females at and up to 1000 mg/kg body weight;
- no significant alterations in the absolute and relative organ weights; and
- no treatment related gross and microscopic pathological alterations in the tissues of male and female rats treated at and up to the level of 1000 mg/kg body weight.

Based on the findings of this study, the No-Observed-Adverse-Effect-Level (NOAEL) of A-5D K/SD in Wistar rats, following oral administration for 90 days was found to be equal to or greater than 1000 mg/kg body weight.

Conclusions on Tier 1 sub-chronic toxicity testing
No toxicity was observed at the maximum dose level of 1000 mg/kg bw/day (no toxicological hazard identified) in the 90 days study performed with potassium aspartate (A-5D K/SD) in rats and a NOAEL of 1000 mg/kg bw/day has been identified for potassium aspartate (A-5D K/SD).

B.2.c Long-term toxicity and carcinogenicity

In view of the Tier 1 sub-chronic toxicity results, it was concluded that Tier 2 or Tier 3 chronic toxicity and carcinogenicity studies need not be performed with potassium polyaspartate (A-5D K/SD).

For example,

- The repeated dose 90-day oral toxicity study (OECD TG 408 modified with additional OECD TG 407 parameters) conducted with A-5D K/SD provided no evidence of mortality or toxicity effects; and

- Negligible absorption was found in *in vitro* absorption studies conducted with A-5D K/SD.

B.2.d Reproductive toxicity and B.2.e Developmental toxicity

The following relevant data from Tier 1 sub-chronic toxicity testing have been taken into account in order to decide about the need for reproductive and developmental testing in Tier 2.

- The repeated dose 90-day oral toxicity study (OECD TG 408 modified with additional OECD TG 407 parameters) conducted with potassium polyaspartate evidenced no effects on the reproductive organs and on the oestrous cycle.

- Negligible absorption has been found in *in vitro* absorption studies conducted with potassium polyaspartate.

Accordingly, it was concluded that it was unnecessary to conduct Tier 2 testing for reproductive and developmental toxicity studies with potassium polyaspartate.

In addition, the relevant data from Tier 1 *in vitro* absorption, *in vitro* genotoxicity and sub-chronic toxicity testing performed with potassium polyaspartate showed negligible absorption, no genotoxicity hazard, no systemic toxicity, no effects on the reproductive organs and on the oestrous cycle, no neurotoxicity, no immunotoxicity. Thus, it was concluded that additional studies on immunotoxicity, hypersensitivity and food intolerance, studies on neurotoxicity, endocrine activity and mechanisms and modes of action are not needed for A-5D K/SD.

Mechanisms and modes of action of potassium polyaspartate

No studies on the mode of action of potassium polyaspartate have been conducted being considered unnecessary because no adverse effects were observed in any of the toxicological studies performed with potassium polyaspartate. Moreover, aspartic acid is a non-essential amino acid, so the human body produces its own supply, and it can also be found in such food sources as dairy, beef, poultry,

sugar cane and molasses.

B.2.f Genotoxicity

Summary

Consistent with the recommendations of the European Food Safety Authority (EFSA, 2011[15]), the following two *in vitro* genotoxicity tests have been performed on potassium polyaspartate (A-5D K/SD), as the first step in genotoxicity testing:

- a bacterial reverse mutation assay (OECD TG 471); and
- an *in vitro* mammalian cell micronucleus test (OECD TG 487).

This combination of tests fulfils the basic requirements to cover the three genetic endpoints with the minimum number of tests; the bacterial reverse mutation assay covers gene mutations and the *in vitro* micronucleus test covers both structural and numerical chromosome aberrations. The addition of any further *in vitro* mammalian cell tests in a basic battery would significantly reduce specificity with no substantial gain in sensitivity (EFSA, 2011). The results of these tests are summarized as follows and the full reports are available from Annex numbers 4.2.2.1/01 and 4.2.2.2/01.

All *in vitro* genotoxicity tests undertaken which assessed potential for induction of gene mutation, structural (clastogenicity) and numerical (aneuploidy) chromosomal alternations, gave negative endpoints. Thus, it can be concluded with reasonable certainty that potassium polyaspartate (A-5D K/SD) is not a genotoxic hazard and that no further *in vitro* or *in vivo* genotoxicity testing is necessary. This conclusion is confirmed by other relevant data on potassium polyaspartate (A-5D K/SD), showing its low chemical reactivity (negligible absorption and digestibility in *in vitro* toxicokinetic studies) and the lack of specific target organ toxicity (90-day study).

Studies - Tier 1 Basic test battery

B.2.f.1 Bacterial reverse mutation assay (OECD TG 471)

Report:	J. P. Mane; May 12th 2014
Title:	*Salmonella typhimurium*, Reverse Mutation Assay of A-5D K/SD (Ames Test, OECD Guideline No. 471)
Document No.	Study No 13955
Guidelines:	OECD Guideline No. 471
GLP	YES

The Bacterial Reverse Mutation Assay (Ames Test) performed on potassium polyaspartate (A-5D K/SD) was carried out in compliance with the OECD Guidelines for Testing of Chemicals (No. 471, Section 4: Health Effects)[16] on the conduct of the "Bacterial Reverse Mutation Test", adopted on 21 July 1997, and in accordance with Organisation for Economic Co-operation and Development (OECD) Good Laboratory Practice (GLP) principles.

[15] EFSA, 2011. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. *EFSA Journal 2011;9(9):2379*

[16] http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en

Accordingly, potassium polyaspartate was evaluated in a pre-incubation assay to determine its ability to induce reverse mutation at selected histidine loci in five tester strains of *Salmonella typhimurium* viz. TA1535, TA97a, TA98, TA100 and TA102 in the presence and absence of a metabolic activation system (S9).
Based upon the preliminary tests conducted to assess the solubility/precipitation and cytotoxicity of potassium polyaspartate, the tester strains were exposed to the test article in triplicate cultures at the doses of 5000 µg, 1500 µg, 500 µg, 150 µg and 50 µg/plate both in the presence and absence of a metabolic activation system (S9). Liver S9, induced in Wistar rats by phenobarbitone with ß-naphthoflavone, was used for this purpose. Details are provided in Table 22.

Dimethyl sulfoxide was used as a vehicle. The exposed bacteria were plated onto minimal glucose agar medium supplemented with L-histidine. The plates were incubated at 37 ºC for 68 to 69 hours after which the histidine revertant colonies were counted and their frequency was compared with that in the vehicle control group. Concurrent positive control groups were also included in the experiment, as specified by the Test Guideline.

Findings:
The results indicated that the frequencies of histidine revertant colonies at all concentrations of potassium polyaspartate in strains TA1535, TA97a, TA98, TA100 and TA102, with and without the presence of a metabolic activation system, were comparable to those observed in the vehicle control group, as per the criteria employed for evaluation of mutagenic potential. Details are provided in Table 23.

Concurrent positive controls demonstrated sensitivity of the assay both in the presence and absence of metabolic activation. Plate counts for the spontaneous histidine revertant colonies in the vehicle control groups were found to be within the frequency ranges expected from the laboratory historical control data.

Table 22: Evaluation of preliminary cytotoxicity test

Concentration (µg/plate)	Without S9		Background Lawn		Mean	Multiples of Vehicle Control	With S9		Background Lawn		Mean	Multiples of Vehicle Control
	R1	R2	R1	R2			R1	R2	R1	R2		
5000	124	128	4+	4+	126.0	0.99	128	132	4+	4+	130.0	1.00
4000	132	136	4+	4+	134.0	1.06	134	128	4+	4+	131.0	1.01
3000	144	140	4+	4+	142.0	1.12	130	120	4+	4+	125.0	0.96
2000	144	132	4+	4+	138.0	1.09	124	134	4+	4+	129.0	0.99
1000	130	120	4+	4+	125.0	0.98	140	130	4+	4+	135.0	1.04
500	132	116	4+	4+	124.0	0.98	126	126	4+	4+	126.0	0.97
250	144	126	4+	4+	135.0	1.06	120	118	4+	4+	119.0	0.92
125	124	120	4+	4+	122.0	0.96	124	134	4+	4+	129.0	0.99
Vehicle Control	128	126	4+	4+	127.0	1.00	124	136	4+	4+	130.0	1.00

R1 and R2 refer to two replicates of culture plates of each test concentration.

Table 23: Summary data on Histidine Revertant Colonies

Treatment	Concentration		TA1535		TA97a		TA98		TA100		TA102	
	(µg/plate)	S9	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
A-5D K SD	5000	_	14.67	2.31	115.33	12.22	30.33	7.57	119.33	5.03	249.33	6.11
		+	13.33	2.52	132.00	2.00	24.67	3.51	120.00	12.00	242.67	8.33
	1500	_	14.00	4.00	122.67	3.06	26.33	4.73	140.00	17.09	224.00	8.00
		+	13.67	4.51	108.00	29.05	31.00	7.00	127.33	13.61	256.00	10.58
	500	_	21.00	3.61	125.33	5.03	21.00	4.00	130.00	19.08	237.33	4.62
		+	17.67	3.51	111.33	22.03	29.67	6.11	121.33	11.02	248.00	4.00
	150	_	13.33	3.06	94.67	18.58	19.33	1.53	129.33	20.13	261.33	12.86
		+	14.33	1.53	92.67	9.24	23.00	6.08	118.00	24.58	265.33	24.44
	50	_	15.33	4.51	124.67	4.62	23.00	2.00	134.67	7.02	250.67	6.11
		+	17.00	2.65	124.00	2.00	19.33	3.21	158.67	18.58	261.33	10.07
Vehicle Control												
Analytical grade water	100 µl	_	14.00	2.65	138.67	17.01	28.33	6.43	127.33	20.13	266.67	32.58
		+	21.00	7.00	115.33	8.33	23.33	4.93	123.33	14.19	252.00	8.00
Positive Controls												
Sodium azide	2	_	840.00	32.74	-	-	-	-	-	-	-	-
ICR 191	1	_	-	-	1008.00	141.53	-	-	-	-	-	-
4-Nitroquinoline-N-oxide	0,5	_	-	-	-	-	490.67	18.90	-	-	-	-
3-Methylmethane Sulphonate	1µl	_	-	-	-	-	-	-	1152.00	174.36	2232.00	119.47
2-Aminoanthracene	10	+	257.33	30.29	-	-	-	-	-	-	-	-
2-Aminofluorene	20	+	-	-	1210.67	76.87	617.33	58.01	1496.00	170.27	-	-
Danthron	30	+	-	-	-	-	-	-	-	-	2133.33	216.20

Conclusions of the Bacterial Reverse Mutation Assay:

It was concluded that, under the conditions of this Bacterial Reverse Mutation Assay (Ames Test), potassium polyaspartate (A-5D K/SD) is non-mutagenic in *Salmonella typhimurium* strains TA1535, TA97a, TA98, TA100 and TA102.

B.2.f.2 *In vitro* mammalian cell micronucleus test (OECD TG 487)

Report:	J. P. Mane; June 28th 2014
Title:	*In Vitro* Micronucleus Test of A-5D K SD in Cultured Human Lymphocytes
Document No.	R/13956/In vitro MNT/14
Guidelines	OECD No. 487 (2010)
GLP	YES

An *in vitro* Micronucleus test of potassium polyaspartate (A-5D K/SD) was carried out in compliance with the OECD Guidelines for Testing of Chemicals (No. 487, Section 4: Health Effects) and in accordance with OECD GLP principles.

The *in vitro* micronucleus assay is a genotoxicity test used for the detection of the chemicals that induce the formation of small membrane-bound DNA fragments such as micronuclei in the cytoplasm of the interphase cell. The *in vitro* mammalian micronucleus test of potassium polyaspartate in human peripheral blood lymphocytes was performed to evaluate the potential of the test article, or its metabolites, to cause clastogenic and aneugenic effects in cultured mammalian somatic cells, viz. human peripheral blood lymphocytes.

Cultures of human peripheral blood lymphocytes were exposed to potassium polyaspartate dissolved in analytical grade water at concentrations of 5000 µg/mL, 1500 µg/mL and 500 µg/mL (concentrations based upon the preliminary solubility/precipitation and cytotoxicity studies) with and without a metabolic activation system i.e. rat liver S9. Duplicate cultures were used at each concentration.

In experiments #1 and #2, cells were exposed to potassium polyaspartate for 3 hours and 20 hours, respectively, without the supplementary metabolic activation system. In experiment #3, conducted with the supplementary metabolic activation system, the cells were exposed for 3 hours to the test article 48 hours after the start of the culture. Cytochalasin B was added at 68 hours, while cell harvesting was performed at 96 hours after the start of the culture.

Positive, negative and vehicle controls, both with and without metabolic activation were tested concurrently with the test article. Analytical grade water was used as the vehicle. Mitomycin C and Vinblastine, known micronucleus forming agents, were employed as positive controls, at the concentration of 0.8 µg/mL and 0.08 µg/mL, respectively, for the experiments without the metabolic activation system, while Cyclophosphamide was employed at the concentration of 6.25 µg/mL for the experiment with the metabolic activation system. Each culture was harvested and slide preparations were made and stained with 5% Giemsa. Two thousand binucleated cells with well spread cytoplasm were evaluated microscopically for the presence of micronuclei, if any.

Findings:
A comparison of the % incidence of micronucleated binucleated cells (BNCs) for each of the three experiments conducted with potassium polyaspartate (A-5D K/SD) either with or without the metabolic activation system, did not reveal any biologically significant or dose-related increase. Also, there was no incidence of a biologically significant increase in the % incidence of micronucleated BNCs at any of the concentration levels in the cultures treated with potassium polyaspartate (A-5D K/SD).

Sensitivity of the test system and activity of S9 mix were demonstrated in the positive control group. The positive controls, viz. directly acting clastogen Mitomycin C, directly acting aneugen Vinblastine and indirectly acting clastogen Cyclophosphamide, induced significant increase in frequencies of micronucleated binucleated cells over the concurrent controls, which validated the test method.

Assessment of the cytokinesis-block proliferation index (CPBI) made during the preliminary cytotoxicity test and the main study indicated that potassium polyaspartate exerted no cytotoxic effects on the cultured human lymphocytes at the test concentration of 5000 µg/mL (3 hours exposure and 20 hours exposure). Details are provided in Tables 24, 25 and 26.

Table 24: Summary of incidence of micronucleated BNCs and cytotoxicity. Cultured Lymphocytes Treated for 3 hours without Metabolic Activation – Experiment #1

Group / Concentration (µg/ml)	Dose µg/ml	No. of Cells Analysed	No. of BNC with MN	% of BNC with MN	CBPI	Cytostasis %
Negative Control 0.9% Saline (w/v)	-	2012	2	0.10	1.95	-
Vehicle Control Analytical grade water	-	2033	4	0.20	1.94	-
Positive Control MMC	0.8	2249	31	1.38*	1.51	45.43
Positive Control VBL	0.08	2009	37	1.84*	1.56	40.55
Potassium polyaspartate A-5D K SD	5000	2016	6	0.30	1.88	6.17
	1500	2037	6	0.29	1.90	4.73
	500	2033	4	0.20	1.88	6.48

*p<0.05

Table 25: Summary of incidence of micronucleated BNCs and cytotoxicity. Cultured Lymphocytes Treated for 20 hours without Metabolic Activation – Experiment #2

Test/Control Article & Dose (µg/ml)	Dose µg/ml	No. of Cells Analysed	No. of BNC with MN	% of BNC with MN	CBPI	Cytostasis %
Negative Control 0.9% Saline (w/v)	-	2003	7	0.35	1.97	-
Vehicle Control Analytical grade water	-	2000	7	0.35	1.90	-
Positive Control MMC	0.8	2017	37	1.83*	1.56	37.45
Positive Control VBL	0.08	2000	34	1.70*	1.62	31.14
Potassium polyaspartate A-5D K SD	5000	2170	6	0.28	1.89	1.10
	1500	2079	5	0.24	1.90	- 0.33
	500	2000	6	0.30	1.87	2.53

*p<0.05

Table 26: Summary of incidence of micronucleated BNCs and cytotoxicity. Cultured Lymphocytes Treated for 3 hours with Metabolic Activation – Experiment #3

Test/Control Article & Dose (µg/ml)	Dose µg/ml	No. of Cells Analysed	No. of BNC with MN	% of BNC with MN	CBPI	Cytostasis %
Negative Control 0.9% Saline (w/v)	-	2009	6	0.30	1.92	-
Vehicle Control Analytical grade water	-	2065	4	0.19	1.86	-
Positive Control CPM	6.25	2013	40	2.00*	1.72	16.92
Potassium polyaspartate A-5D K SD	5000	2013	10	0.50	1.85	0.84
	1500	2019	5	0.25	1.84	1.85
	500	2008	5	0.25	1.85	0.99

*p<0.05

Conclusions of the *In vitro* mammalian cell micronucleus test:
Under the test conditions used, potassium polyaspartate (A-5D K/SD) did not induce chromosome breaks and/or gain or loss (i.e. it is not clastogenic and aneugenic) in cultured mammalian cells, viz. human peripheral blood lymphocytes.

Conclusions on genotoxicity hazard of potassium polyaspartate (A-5D K/SD) after the Tier 1 Basic test battery
All the *in vitro* genotoxicity endpoints are clearly negative and thus it can be concluded with reasonable certainty that potassium polyaspartate (A-5D K/SD) is not a genotoxic hazard and no further *in vitro* or *in vivo* genotoxicity testing is necessary.

This conclusion is confirmed by other relevant data on potassium polyaspartate (A-5D K/SD) showing its low chemical reactivity (negligible absorption in *in vitro* toxicokinetic studies), and its lack of specific target organ toxicity (90-day study). The lack of genotoxicity on the potassium salt is also confirmed from the genotoxicity data on the sodium salt of polyaspartic acid sourced from published literature and from existing registrations to the US FDA and FSANZ.

Tier 2 Follow-up of results from the basic test battery and Follow-up of results from Tier 2 by carcinogenicity studies and germ cell assays

Since the Tier 1 Basic test battery showed that potassium polyaspartate (A-5D K/SD) is not a genotoxic hazard, it was concluded that no follow-up *in vivo* testing was necessary.

B.2.g Special studies, such as neurotoxicity or immunotoxicity

No human studies with potassium polyaspartate (A-5D K/SD) have been performed. In addition, no human data are available given that is an application for registration of a *new* food additive. The likely

safety in humans at the proposed level of exposure has been demonstrated in the current application through a complete Tier 1 testing package (*in vitro* kinetics, 90-day study and *in vitro* genotoxicity) and a consumer safety assessment performed with EFSA FAIM model assuming worst use conditions. The Tier 1 testing package and the consumer safety assessment can be considered sufficient for safety assessment for single or short-term repeated administration of potassium polyaspartate (A-5D K/SD) to humans.

B.2.g.1 Neurotoxicity

The tiered approach to testing includes, at Tier 1, a 90-day study in rats (OECD TG 408) that involves investigation of the effect of the food additive on a number of parameters that may be indicative of a neurotoxic effect. These include: changes in clinical signs, functional observational battery, motor activity and brain weight relative to body weight in the absence of overt toxicity, and histopathological changes in this organ.

On the 13th week of treatment of the 90-day oral toxicity study performed with potassium polyaspartate (A-5D K/SD) in rats, as presented previously in pages 75-91 of this application, all rats were examined for assessment of sensory reactivity, assessment of grip strength and motor activity. These included the functional observational battery suggested by Moser 1989. The neurological examination included:

- Examinations in home-cage and open field: Posture/Movement, Respiration, Palpebral closure, Lacrimation, Salivation, Skin and hair coat, Urination, Defecation, Locomotor activity, Rearing and Gait

- Manipulative examination/Responses to stimuli: Tactile (touch) response, Response to nociceptive stimuli (tail pinch), Pupil response to light, Proprioception – Righting reflex, Auditory response, Head shaking, Landing foot splay

The neurological examinations (and functional observations) conducted in the thirteenth week of the 90-day study did not reveal any remarkable and treatment related incidence of neurological abnormalities. Also, no findings indicative of a neurotoxic potential of potassium polyaspartate (A-5D K/SD) were encountered during these examinations. Thus, it can be concluded that potassium polyaspartate (A-5D K/SD) is not neurotoxic and no Tier 2 or Tier 3 studies are necessary.

B.2.g.2 Immunotoxicity

The tiered approach to testing includes, at Tier 1, a 90-day study in rats (OECD TG 408). This study involved investigation of the effects of potassium polyaspartate (A-5D K/SD) on a number of parameters that may be indicative of an immunotoxic or immunomodulatory effect. These included changes in spleen and thymus weights relative to body weight in the absence of overt toxicity, histopathological changes in these and other organs of the immune system (e.g. bone marrow, lymph nodes and Peyer's patches), as well as changes in total serum protein, albumin:globulin ratio and in the haematological profile of the animals, notably in lymphocyte numbers and in the total and differential blood cell counts.

No effects from potassium polyaspartate were observed on any of the parameters that may be indicative of an immunotoxic or immunomodulatory effect.

The potential stimulation of immuno cello was assessed *in vitro* on pro-myelocytic human cells THP-

1, used as surrogate of monocytes. For details please refer to Annex number 4.5.3, Restani P., 2015. Part III – *In vitro* immunotoxicity. Prior to the study, the cytotoxicity of potassium polyaspartate was assessed through the MTT test. The THP-1 cells were diluted to 10^6 cells/mL in RPMI 1640, supplemented with 10% heated-inactivated fetal calf serum (media) and cultured in 37°C in 5% CO_2 incubator. Cytokine IL8 release (with ELISA) and CD86 expression (with flow cytometric analysis) were then assessed. For IL-8 release, 1.0 x 10^6 cells were seeded in 24-well plate. Cells were incubated for 24 h in the presence or absence of A-5D K/SD at 2 mg/mL. Lipopolysaccharide from *Escherichia coli* serotype 0127:B8 (LPS) 10 ng/mL was used as positive control.

The results showed that potassium polyaspartate did not induce any activation of the immune system (monitored parameters were up-regulation of CD86 and release of IL8). Thus, it can be concluded that potassium polyaspartate (A-5D K/SD) has no immunotoxicity and no Tier 2 or Tier 3 studies on immunotoxicity are necessary. In the positive control a statistical significant increase in both CD86 expression and IL-8 release was observed.

Allergy
At present there are no validated studies in laboratory animals which would allow assessment of the potential of a substance to cause allergic reactions in susceptible individuals following oral exposure. Studies on dermal or inhalation sensitisation may provide relevant information for possible hazards from occupational exposure to additives and could be helpful in assessing consumer safety even if their relevance to oral allergenicity remains unclear. Any available data on double-blind placebo-controlled oral food challenges, or prick testing in humans should be used. These data may be already available e.g. in the case where the food additive has already been studied for other studies such as in drugs.

Up to now no allergic reactions have been observed to the corresponding sodium salt of polyaspartate. Moreover, the evaluation of the potential stimulation of immuno cells conducted on pro-myelocytic human cells THP-1 showed that potassium polyaspartate (A5D K/SD) did not induce any activation of the immune system. Thus, there is low potential for potassium polyaspartate (A-5D K/SD) to cause allergic reactions.

Intolerance reactions
Intolerance reactions to food additives are not of immunological origin. They can be due to genetically defined metabolic specificities or to still other undefined causes (NIAID-Sponsored Expert Panel et al., 2010; Guandalini and Newland, 2011; Hayder and Bartholomaeus, 2011). Such reactions are difficult to predict and mostly rely on human studies reporting observations of adverse effects.

At present, no validated experimental *in vitro* and *in vivo* methods are available which would allow assessment of a substance's potential to cause intolerance reactions in susceptible individuals following oral exposure. Moreover, it is not feasible to undertake clinical studies of sufficient power prior to marketing. Any data from post-marketing surveillance may identify possible sensitive individuals.

Up to now no inborn errors of metabolism (IEM) have been reported for aspartic acid. Moreover, no intolerance reactions have been observed to the corresponding sodium salt of polyaspartate. Thus, potassium polyaspartate (A-5D K/SD) is not expected to cause intolerance reactions.

B.3 Safety assessment reports prepared by international agencies or other national government agencies, if available

B.3.1 European Food Safety Authority (EFSA)

On 24 February 2015, an application was submitted to the European Food Safety Authority (EFSA) for the authorisation of the use of potassium polyaspartate as a stabiliser in wine. The application was made available to the Member States pursuant to Article 4 of Regulation (EC) No 1331/2008. EFSA duly evaluated the safety of potassium polyaspartate as a food additive and in its opinion of 9 March 2016 concluded that there was no safety concern from the proposed use in wine at a maximum use level of 300 mg/L and typical levels in the range of 100-200 mg/L. The abstract of the safety assessment is as follows, and the complete published safety assessment is appended.

> Potassium polyaspartate (A-5D K/SD) is proposed for use as a stabiliser in wine, with a maximum use level of 300 mg/L and typical levels in the range of 100-200 mg/L. The data provided in support of the current application were in accordance with the Tier 1 requirement of the Guidance for submission for food additive evaluations issued by the ANS Panel in 2012. In the in vitro tests provided by the applicant, potassium polyaspartate (A-5D K/SD) showed minimal proteolytic digestion and no absorption of the intact compound. Potassium polyaspartate (A-5D K/SD) tested negative in a bacterial reverse mutation assay performed in accordance with OECD TG 471 and in an in vitro mammalian cell micronucleus test performed in accordance with OECD TG 487. From a 90-day oral toxicity study in rats performed in accordance with OECD TG 408, a no observed adverse effect level (NOAEL) was set at 1,000 mg/kg bw per day, the highest dose tested. The Panel considered these data as fulfilling the requirements for the evaluation of the new food additive and did not request additional testing for chronic toxicity and carcinogenicity, nor for reprotoxicity and developmental toxicity. Exposure estimates to potassium polyaspartate (A-5D K/SD) from its proposed use were calculated for both typical and maximum use levels. In the worst case scenario of high-level intakes of potassium polyaspartate (A-5D K/SD) when used at the maximum proposed use level of 300 mg/L, the maximum estimated intake would be 1.8 mg/kg bw per day in the elderly and 1.4 mg/kg bw per day in adults, resulting in a margin of safety of approximately 550. The Panel concluded that there was no safety concern from the proposed use and use levels of potassium polyaspartate (A-5D K/SD).

B.3.2 European Commission

On 28 July 2017, the European Commission amended the Annex II to Regulation (EC) No 1333/2008 and the Reg EU No 231/2012, by adding potassium polyaspartate to the list of food additives.

Conclusions on toxicological data and on safety criteria laid down in Article 6(1) of Regulation (EC) No 1333/2008.

The package of toxicological studies carried out on A-5D K/SD fulfills Regulation (EC) No 1333/2008 and EFSA requirements for a new food additive authorization. The results of toxicological studies show as A-5D K/ SD is negligible absorbed, it does not affect gut cells integrity and it does not induce any activation of the immune system. It is not mutagenic or genotoxic and it does not cause any toxic effect, even in case of repeated dosing (90 days NOAEL = 1000 mg/kg body weight /day, maximum tested dose). Thus it can be concluded that the proposed use of A-5D K/ SD as food additive for tartaric stabilization in white wine, rosè wine and red wine, does not represent a safety concern for any consumer group and fulfills safety criteria laid down in Article 6(1) of Regulation (EC) No 1333/2008.

Overall conclusions on potassium polyaspartate (*A-5D K/SD*) authorisation approval under Regulation (EC) No 1333/2008.

The proposed use of A-5D K/SD as food additive for tartaric stabilization in wine (functional class: stabilizer; food category: 14.2) does not represent a safety concern for consumers and for the environment and fulfills safety criteria laid down in Article 6(1) of Regulation (EC) No 1333/2008. A-5D K/SD enhances the keeping quality and stability of wine and aids in storage of wine, provided it is not used to disguise the effects of the use of faulty raw materials or of any undesirable practices or techniques, including unhygienic practices or techniques, during the course of any such activities. Thus, the proposed use of A-5D K/SD does not mislead the consumer and is of benefit to the consumer and also the requirements laid down in Article 6(2) of Regulation (EC) No 1333/2008 are fulfilled.

Thus, A-5D K/SD should be approved as a new food additive according to Regulation (EC) No 1333/2008.

B.4 Supplementary safety information

C. Information related to the dietary exposure to the food additive

C.1 A list of the food groups or foods proposed to contain the food additive, or changes to currently permitted foods

The only foods proposed to contain potassium polyaspartate are the alcoholic beverages, wine and wine products. The application for authorisation is for a new additive and there are no other sources of exposure.

C.2 The maximum proposed level or the concentration range of the food additive for each food group or food, or the proposed changes to the currently permitted levels.

The maximum use level of potassium polyaspartate is 300 mg/L.

C.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption.

From the Australian Bureau of Statistics for 2015-2016, the volume of wine available for consumption was 558.9 million litres, while per capita consumption of wine in Australia for those aged 15 years and over was 3.66 litres[17]. On a per day basis, this equates to 0.01 L. It is noted that the legal drinking age in Australia is 18 years. All states and territories prohibit people under this age to purchase alcohol or consumer alcohol in licensed premises or in public places.

From the Australian Institute of Health and Welfare for 2016, overall the most consumed alcoholic beverage was bottled wine. Regular strength beer was the main alcoholic beverage consumed by male drinkers while for female drinkers it was bottled wine[18]. The exception to this was for people aged 12–17 and females aged 18–24 where, for these groups, pre-mixed spirits was the main alcoholic beverage consumed, where only 18% of people aged 12-17 years had consumed any alcoholic beverages in 2016.

C.4 The percentage of the food group in which the food additive is proposed to be used or the percentage of the market likely to use the food additive

Based on average projected estimates across these markets that are now allowed to use potassium polyaspartate, namely France, Italy, Spain, Germany, South Africa, Chile and Eastern Europe, it is projected that 25% of all Australian wine will be produced with potassium polyaspartate after three years.

[17] http://www.abs.gov.au/ausstats/abs@.nsf/Latestproducts/4307.0.55.001Main%20Features32015-16?opendocument&tabname=Summary&prodno=4307.0.55.001&issue=2015-16&num=&view=

[18] https://www.aihw.gov.au/getmedia/15db8c15-7062-4cde-bfa4-3c2079f30af3/21028.pdf.aspx?inline=true

C.5 Information relating to the use of the food additive in other countries, if applicable

The EFSA guidance recommended use of the dedicated exposure assessment tool (FAIM), which is available on the EFSA website[19].

The input data required by the FAIM model are the food category, the use level and the ADI. In the case of this application for authorisation, the following were selected:

- Food category: 14.2 - Alcoholic beverages, including alcohol-free and low-alcohol counterparts
- Maximum use level: 300 mg/L
- Normal use level: 200 mg/L (worst case of normal use level)
- ADI: 10 mg/kg bw

The ADI proposed for potassium polyaspartate (A-5D K/SD) is based on the NOAEL of 1000 mg/kg bw/day (the highest tested dose) in the 90-day rat study and an uncertainty factor of 100 (inter species variation x intra-species variation = 10 x 10 = 100). This value is considered reasonable due to the limited absorption and limited metabolism showed by potassium polyaspartate in the cells of gastro-intestinal tract, the lack of adverse effects observed in the sub-chronic and genotoxicity studies, combined with its physico-chemical characteristics (relatively high molecular weight and high water solubility) confirming a low absorption and low chemical reactivity.

Moreover, polyaspartic acid is not listed by NTP, IARC or regulated as a carcinogen by OSHA, as detailed below.

Polyaspartic acid risk classification

- Environmental Protection Agency: No risk reported (EPA 2012)
- International Agency for Research on Cancer: Not listed (IARC 2012)
- National Institute for Occupational Safety and Health: Not listed (NIOSH 2012)
- US Occupational Safety and Health Administration: Not regulated (OSHA 2012)

Thus, it can be concluded as the use of 100 as uncertainty factor (10 for inter species variation, 10 for intra-species variation) in the ADI calculation for potassium polyaspartate (A-5D K/SD) is acceptable and no additional safety factor should be taken into account.

The outputs from the FAIM model obtained for A-5D K/SD are shown below in the following Tables 27 and 28.

[19] www.efsa.europa.eu/en/anstopics/docs/faimtemplate.xls

Table 27: Output of FAIM at maximum use level of 300 mg/L

A-5D K/SD (potassium polyaspartate)								
TOTAL ESTIMATED EXPOSURE: % OF THE ADI								
	MPL				Use levels (300 mg/L)			
	Range for mean across dietary surveys		Range for high level across dietary surveys		Range for mean across dietary surveys		Range for high level across dietary surveys	
	Min	Max	Min	Max	Min	Max	Min	Max
Toddlers	0.0	0.0	0.0	0.0	0.0	0.1	0.5	3.7
Children	0.0	0.0	0.0	0.0	0.0	0.6	0.0	14.9
Adolescents	0.0	0.0	0.0	0.0	0.0	3.2	0.4	77.6
Adults	0.0	0.0	0.0	0.0	2.6	16.4	18.1	95.2
The elderly	0.0	0.0	0.0	0.0	1.8	13.2	17.7	44.1

Table 28: Output of FAIM at normal use level of 200 mg/L

A-5D K/SD (potassium polyaspartate)								
TOTAL ESTIMATED EXPOSURE: % OF THE ADI								
	MPL				Use levels (200 mg/L)			
	Range for mean across dietary surveys		Range for high level across dietary surveys		Range for mean across dietary surveys		Range for high level across dietary surveys	
	Min	Max	Min	Max	Min	Max	Min	Max
Toddlers	0.0	0.0	0.0	0.0	0.0	0.0	0.4	2.5
Children	0.0	0.0	0.0	0.0	0.0	0.4	0.0	9.9
Adolescents	0.0	0.0	0.0	0.0	0.0	2.1	0.2	51.7
Adults	0.0	0.0	0.0	0.0	1.7	11.0	12.1	63.5
The elderly	0.0	0.0	0.0	0.0	1.2	8.8	11.8	29.4

It can be concluded that, even when adding the maximum use level of 300 mg/L, addition of potassium polyaspartate (A-5D K/SD) in winemaking does not represent a concern for consumers. It should be also considered that the estimated exposure of toddlers and children is not a realistic scenario because the current request for authorisation of potassium polyaspartate (A-5D K/SD) is for the use into wine, where the legal age for purchasing alcoholic beverages and being served in public place is 18 years.

C.6 For foods where consumption has changed in recent years, information on likely current food consumption

This includes any consumption information for foods where there has been a significant change in consumption since the most recent Australian and New Zealand NNSs which relate to the application. This can be based on market share data, or sales data, or on a similar market in another country.

N/A

C.7 Supplementary dietary exposure information

C.7.1 Excerpt of exposure data from Safety of potassium polyaspartate (A-5D K/SD) for use as a stabiliser in wine EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)

Food consumption data used for exposure assessment

EFSA Comprehensive European Food Consumption Database

Since 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) has been populated with national data on food consumption at a detailed level. Competent authorities in the European countries provide EFSA with data on the level of food consumption by the individual consumer from the most recent national dietary survey in their country (cf. Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment') (EFSA, 2011a). New consumption surveys recently[8] added to the Comprehensive database were also taken into account in this assessment[9].

The food consumption data gathered by EFSA were collected by different methodologies and thus direct country-to-country comparisons should be interpreted with caution. Depending on the food category and the level of detail used for exposure calculations, uncertainties could be introduced as a result of under-reporting and/or misreporting of the consumption amounts by subjects. Nevertheless, the EFSA Comprehensive Database represents the best available source of food consumption data across Europe at present.

Food consumption data used for the exposure assessment were from the population groups: infants, toddlers, children, adolescents, adults and the elderly. For the present assessment, food consumption data were available from 33 different dietary surveys carried out in 19 European countries (Table 29).

Table 29: Population groups considered for the exposure estimates of potassium polyaspartate (A-5D K/SD)

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From 4 months up to and including 11 months of age	Bulgaria, Denmark, Finland, Germany, Italy, UK
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Denmark, Finland, Germany, Italy, the Netherlands, Spain, UK
Children [(a)]	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Spain, Sweden, UK
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Finland, France, Germany, Italy, Latvia, Spain, Sweden, UK
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Romania, Spain, Sweden, UK
The elderly [(a)]	From 65 years of age and older	Austria, Belgium, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Romania, Sweden, UK

(a): The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merging of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011a).

Consumption records were codified according to the FoodEx classification system (EFSA, 2011b). Nomenclature from the FoodEx classification system has been linked to the Food Classification System (FCS) as presented in Annex II of Regulation (EC) No 1333/2008, part D, to perform exposure estimates. In practice, FoodEx food codes were matched to the FCS food categories.

Food categories selected for the exposure assessment of potassium polyaspartate (A-5D K/SD)
The food category in which the use of potassium polyaspartate (A-5D K/SD) is proposed for use was selected from the nomenclature of the EFSA Comprehensive Database (FoodEx classification system), at the most detailed level possible (up to FoodEx Level 3) (EFSA, 2011b).

Overall, all wine or fortified wine food categories available in the FoodEx nomenclature were included in the exposure assessment as shown in Table 30.

Table 30: Food categories available in the FoodEx nomenclature and considered for the exposure estimates of potassium polyaspartate (A-5D K/SD)

FoodEx Level 2	FoodEx Level 3	FoodEx Category No.
Wine	Wine (undefined)	A.14.02
	Wine, white	A.14.02.001
	Wine, white, sparkling	A.14.02.002
	Wine, red	A.14.02.003
	Wine, red, sparkling	A.14.02.004
Fortified and liqueur wines (e.g. vermouth, sherry, Madeira)	Fortified and liqueur wines (undefined)	A.14.03
	Vermouth	A.14.03.001
	Sherry	A.14.03.002

Exposure to potassium polyaspartate (A-5D K/SD) from its proposed use as a food additive

Estimate of exposure based on the Food Additives Intake Model (FAIM) template
The applicant has provided an estimate of exposure to potassium polyaspartate (A-5D K/SD) based on the output obtained using the FAIM model at the proposed ML of 300 mg/L and at the proposed typical use level of 200 mg/L in food category 14.2 Alcoholic beverages, including alcohol-free and low-alcohol counterparts (Documentation provided to EFSA n.1).

The Panel decided not to use the estimate of exposure generated from the FAIM tool and provided by the applicant because aggregation of the data resulted in an overestimate of the exposure.

The Panel therefore decided to perform a more refined assessment, limited to the consideration of the food categories described in Table 4.

Refined exposure assessment scenario
The exposure scenarios resulting from the refined assessment are presented in Table 31. Detailed results by population groups and survey are presented in Appendix following.

Table 31: Estimated exposure to potassium polyaspartate (A-5D K/SD) from its proposed use as a food additive: at the proposed use levels and at the proposed MLs

Estimated exposure (mg/kg bw per day)	Infants (4–11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (≥65 years)
Proposed typical use level: 200 mg/L						
Mean	<0.001	0–0.005	0–0.01	0–0.01	0.01–0.2	0.04–0.4
High level	<0.001	0–0.002	0–0.08	0–0.08	0–1.0	0.3–1.2
Proposed maximum Level: 300 mg/L						
Mean	<0.001	0–0.007	0–0.02	0–0.02	0.02–0.4	0.05–0.6
High level	<0.001	0–0.004	0–0.1	0–0.1	0–1.4	0.4–1.8

In consideration of the proposed use of potassium polyaspartate (A-5D K/SD) as a food additive, limited to wine, the Panel considered it appropriate to consider dietary exposure only in adults and in the elderly. The Panel acknowledged that data from the younger age groups (i.e. infants, toddlers, children and adolescents) showed some levels of intake from wine or other alcoholic consumption. Consumption of alcoholic beverages is not appropriate for these age groups, and these exposure estimates, which are very low, are most likely a result of the indirect consumption of alcoholic beverages (ranging from < 0.001 up to 0.1 mg/kg bw per day) as recipe ingredients of composite foods. Therefore, the Panel considered that these were not relevant for the current risk assessment.

The mean dietary exposure from the proposed typical use level of 200 mg/L ranged from 0.01 to 0.2 mg/kg bw per day in adults up to 0.04 to 0.4 mg/kg bw per day in the elderly. The high-level intake ranged from 0 to 1.0 in adults and from 0.3 to 1.2 mg/kg bw per day in the elderly.

At the proposed ML of 300 mg/L, the mean dietary exposure ranged from 0.02 to 0.4 mg/kg bw per day in adults up to 0.05 to 0.6 mg/kg bw per day in the elderly. The high-level intake ranged from 0 to 1.4 in the adults and from 0.4 to 1.8 mg/kg bw per day in the elderly.

Main food categories contributing to exposure to potassium polyaspartate (A-5D K/SD) using the proposed ML and typical level of use

Within the food categories selected to perform the current exposure assessment, the main categories contributing to exposure to potassium polyaspartate (A-5D K/SD) using both levels of 200 mg/L and 300 mg/L proposed by the applicant are presented in Table 32.

Table 32: Main food categories contributing to exposure to potassium polyaspartate (A-5D K/SD) using both level of 200 mg/L and 300 mg/L for the proposed uses (% min–max) and the number of surveys ≥5% contribution (*n*) in which each food category contributes

FoodEX Level 3	Infants	Toddler s	Children	Adolescents	Adults	The elderly
	Range of % contribution to the total exposure (number of surveys)[a]					
Wine (undefined)	100 (1)	90.2–100 (2)	7.6–78.8 (2)	12.4–100 (10)	6.1–85.5 (13)	5.8–97.9(8)
Wine, white	97.7–100 (3)	11.7–82.5 (4)	13.7–100 (14)	15.3–92.4 (13)	5.0–59.6 (17)	6.3–65.1 (13)
Wine, white, sparkling	–	–		7.0–100 (5)	8.3–22.9 (2)	–
Wine, red	–	9.8–100 (6)	5.8–72.2 (13)	5.4–94.5 (14)	6.8–77.8 (16)	21.5–81.7 (13)
Fortified and liqueur wines (undefined)	–	–	11.1 (1)	7.2–23.3 (2)	–	5.8 (1)
Vermouth	–	19.2 (1)	16.6–64.8 (2)	9.4 (1)	–	–
Sherry	–	–	10.8–17.5 (2)	17.1 (1)	–	5.7 (1)

–: no information available

(a): The total number of surveys may be greater than the total number of countries listed in Table 3 because some countries submitted more than one survey for a specific population.

Uncertainty analysis

Uncertainties in the exposure assessment of potassium polyaspartate (A-5D K/SD) for its proposed use as a food additive have been discussed above. In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2007), the following sources of uncertainties have been considered and are summarised in Table 33.

Overall, the Panel considered that the uncertainties identified would, in general, result in an overestimation of exposure to potassium polyaspartate (A-5D K/SD) as a food additive in European countries for the maximum level exposure scenario.

Table 33: Qualitative evaluation of influence of uncertainties on the dietary exposure estimate

Sources of uncertainties (a)	Direction
Consumption data: different methodologies/representativeness/under- reporting /misreporting/no portion size standard	+/-
Use of data from food consumption survey of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Correspondence of reported use levels and analytical data to the food items in the EFSA Comprehensive Food Consumption Database: uncertainties regarding which types of food the levels refer to	+/-
Maximum and typical proposed use level: levels considered applicable for all items within the entire food category	+/-
Maximum level exposure assessment scenario: all food categories at the maximum proposed use level	+
Uncertainty in possible national differences in use levels of food categories	+/–

(a): +, uncertainty with potential to cause over-estimation of exposure; –, uncertainty with potential to cause underestimation of exposure.

Appendix: Summary of total estimated exposure to potassium polyaspartate (A-5D K/SD) from its use as a stabiliser in wines for the maximum level scenario and the typical level scenario per population group and survey

	Number of subjects	Typical level scenario (200 mg/L)		Maximum level scenario (300 mg/L)	
		Mean	High level	Mean	High level
Infants					
Bulgaria (NUTRICHILD)	658	<0.001	0	<0.001	0
Germany (VELS)	159	<0.001	0	<0.001	0
Denmark (IAT 2006_07)	826	0	0	0	0
Finland (DIPP_2001_2009)	500	0	0	0	0
United Kingdom (DNSIYC_2011)	1366	0.001	0	0.001	0
Italy (INRAN_SCAI_2005_06)	12	<0.001		<0.001	
Toddlers					
Belgium (Regional_Flanders)	36	0.005		0.007	
Bulgaria (NUTRICHILD)	428	0.001	0	0.002	0
Germany (VELS)	348	0.001	0.002	0.001	0.004
Denmark (IAT 2006_07)	917	0	0	0	0
Spain (enKid)	17	0		0	
Finland (DIPP_2001_2009)	500	0	0	0	0
United Kingdom (NDNS-Rolling Programme Years 1–3)	185	0.001	0	0.002	0
United Kingdom (DNSIYC_2011)	1314	0.001	0	0.002	0
Italy (INRAN_SCAI_2005_06)	36	<0.001		<0.001	
Netherlands (VCP_kids)	322	0.001	0	0.001	0
Children					
Austria (ASNS_Children)	128	0.013	0.075	0.020	0.112
Belgium (Regional_Flanders)	625	<0.001	0	0.001	0
Bulgaria (NUTRICHILD)	433	0.001	0	0.001	0
Czech Republic (SISP04)	389	0.002	0	0.002	0
Germany (EsKiMo)	835	0.002	0.007	0.002	0.011
Germany (VELS)	293	0.001	0.004	0.001	0.006
Denmark (DANSDA 2005-08)	298	0.001	0	0.001	0
Spain (enKid)	156	0.001	0	0.001	0
Spain (NUT_INK05)	399	0	0	0	0
Finland (DIPP_2001_2009)	750	<0.001	0	<0.001	0
France (INCA2)	482	0.002	0.014	0.003	0.021
United Kingdom (NDNS-Rolling Programme Years 1–3)	651	0.001	0	0.001	0

Greece (Regional_Crete)	838	<0.001	0	0.001	0
Italy (INRAN_SCAI_2005_06)	193	0.003	0.001	0.005	0.002
Latvia (EFSA_TEST)	187	0	0	0	0
Netherlands (VCP_kids)	957	<0.001	0	<0.001	0
Netherlands (VCPBasis_AVL2007_2010)	447	0.001	0	0.001	0
Sweden (NFA)	1473	<0.001	0	<0.001	0
Adolescents					
Austria (ASNS_Children)	237	0.005	0.033	0.007	0.049
Belgium (Diet_National_2004)	576	0.014	0.075	0.021	0.113
Cyprus (Childhealth)	303	0.001	0	0.001	0
Czech Republic (SISP04)	298	0.005	0	0.008	0
Germany (National_Nutrition_Survey_II)	1011	0.008	0	0.012	0
Germany (EsKiMo)	393	<0.001	0.002	0.001	0.002
Denmark (DANSDA 2005-08)	377	0.008	0.058	0.012	0.087
Spain (AESAN_FIAB)	86	0.012	0.049	0.017	0.074
Spain (enKid)	209	<0.001	0	<0.001	0
Spain (NUT_INK05)	651	0.011	0	0.016	0
Finland (NWSSP07_08)	306	<0.001	0	0.001	0
France (INCA2)	973	0.007	0.024	0.011	0.036
United Kingdom (NDNS-Rolling Programme Years 1–3)	666	0.006	0.008	0.010	0.012
Italy (INRAN_SCAI_2005_06)	247	0.005	0.001	0.007	0.002
Latvia (EFSA_TEST)	453	<0.001	0	<0.001	0
Netherlands (VCPBasis_AVL2007_2010)	1142	0.009	0	0.013	0
Sweden (NFA)	1018	0	0	0	0
Adults					
Austria (ASNS_Adults)	308	0.079	0.439	0.118	0.658
Belgium (Diet_National_2004)	1292	0.197	0.964	0.296	1.446
Czech Republic (SISP04)	1666	0.109	0.749	0.163	1.124
Germany (National_Nutrition_Survey_II)	10419	0.132	0.772	0.198	1.157
Denmark (DANSDA 2005-08)	1739	0.246	0.911	0.370	1.367
Spain (AESAN)	410	0.064	0.403	0.095	0.604
Spain (AESAN_FIAB)	981	0.108	0.578	0.163	0.866
Finland (FINDIET2012)	1295	0.040	0.283	0.060	0.424
France (INCA2)	2276	0.208	0.962	0.312	1.443
United Kingdom (NDNS-Rolling Programme Years 1–3)	1266	0.165	0.798	0.247	1.197
Hungary (National_Repr_Surv)	1074	0.054	0.417	0.081	0.625
Ireland (NANS_2012)	1274	0.139	0.747	0.209	1.121
Italy (INRAN_SCAI_2005_06)	2313	0.196	0.821	0.293	1.231
Latvia (EFSA_TEST)	1271	0.012	0	0.019	0
Netherlands (VCPBasis_AVL2007_2010)	2057	0.122	0.749	0.183	1.124
Romania (Dieta_Pilot_Adults)	1254	0.050	0.306	0.075	0.459
Sweden (Riksmaten 2010)	1430	0.140	0.625	0.210	0.938

The elderly

Austria (ASNS_Adults)	92	0.150	0.625	0.225	0.938
Belgium (Diet_National_2004)	1215	0.190	0.913	0.285	1.370
Germany (National_Nutrition_Survey_II)	2496	0.142	0.781	0.212	1.172
Denmark (DANSDA 2005-08)	286	0.370	1.176	0.555	1.765
Finland (FINDIET2012)	413	0.036	0.342	0.054	0.514
France (INCA2)	348	0.299	1.049	0.448	1.573
United Kingdom (NDNS-Rolling Programme Years 1–3)	305	0.117	0.705	0.175	1.057
Hungary (National_Repr_Surv)	286	0.070	0.519	0.105	0.779
Ireland (NANS_2012)	226	0.093	0.539	0.140	0.809
Italy (INRAN_SCAI_2005_06)	518	0.272	1.009	0.408	1.514
Netherlands (VCPBasis_AVL2007_2010)	173	0.211	0.929	0.316	1.394
Netherlands (VCP-Elderly)	739	0.164	0.716	0.247	1.074
Romania (Dieta_Pilot_Adults)	128	0.048	0.263	0.072	0.395
Sweden (Riksmaten 2010)	367	0.189	0.700	0.284	1.050

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Annex of supporting studies and data

Annex number	Author	Year	Title Source (where different from company) Company, Report No. GLP or GEP status (where relevant) Published or Unpublished	Data protection claimed Y/N	Owner
1.1.7/01 & 1.1.7/03 & 1.1.7/04	Brioschi M.	2014	A-5D K SD: Determination of the Physico-chemical properties ChemService srl – Controlli e Ricerche, Study no. CH-501/2014 No GLP, unpublished	Y	Esseco srl
1.1.7/02	Xiaohui Li	2014	Water solubility of A-5D K / SD NanoChem Solutions Inc. No GLP, unpublished	Y	Nanochem*
1.1.7/05	Hailong Xia	2014	FT-IR Spectrospcopy of A-5D K / SD NanoChem Solutions Inc. No GLP, unpublished	Y	Nanochem*
1.1.7/06	Hailong Xia	2014	FT-IR Spectrospcopy of L-aspartic acid NanoChem Solutions Inc. No GLP, unpublished	Y	Nanochem*
1.1.7/07	Kleps R.	2014	1H NMR of A-5D K / SD NanoChem Solutions Inc. No GLP, unpublished	Y	Nanochem*
1.1.7/08	Kleps R.	2014	1H NMR of L-aspartic acid NanoChem Solutions Inc. No GLP, unpublished	Y	Nanochem*
1.1.7/09	Kleps R.	2014	UV-Vis of A-5D K / SD NanoChem Solutions Inc. No GLP, unpublished	Y	Nanochem*
1.1.7/10	Kleps R.	2014	UV-Vis of L-aspartic acid NanoChem Solutions Inc. No GLP, unpublished	Y	Nanochem*
1.2/01	Triulzi G.	2015	Potassium polyaspartate (KPA) stability statement. No GLP, unpublished	Y	Esseco srl
1.2/02	Marini S.	2014	Certificate of analysis of the 4 four batches tested. No GLP, unpublished	Y	Nanochem*
1.3/01	Fan G.	2014	Letter of Side Reaction Products No GLP, unpublished	Y	Nanochem*

Annex number	Author	Year	Title Source (where different from company) Company, Report No. GLP or GEP status (where relevant) Published or Unpublished	Data protection claimed Y/N	Owner
1.4.1/01 & 1.4.1/02 & 1.4.1/03	Vassanelli G.	2014	Chemical Characterization of Potassium Polyaspartate. Laboratory Enocentro srl, Report no. 01/2015 No GLP, unpublished	Y	Esseco srl
1.4.2/01	Vassanelli G.	2014	Method for analysis of potassium polyaspartate in wine. Laboratory Enocentro srl, Report no. 20150107 No GLP, unpublished	Y	Esseco srl
1.4.3/01	Marne S.K.	2014	Validation of Analytical Method for A-5D K SD INTOX PVT. LTD., Study no. R/RA1384/AMV/14 No GLP, unpublished	Y	Esseco srl
1.5/01	Unknown	2014	PAA Nanochem A5DK/SD KHKS-070214-1. Statement of stability. No GLP, unpublished	Y	Esseco srl
1.5/02	Marne S.K.	2014	Stability Study of A-5D K SD in Water INTOX PVT. LTD., Study no. R/RA1385/SHA/14 No GLP, unpublished	Y	Esseco srl
1.5/03	Bosso A.	2015	Work package 2 (WP2): Building enological know-how on Polyaminoacids Seventh Framework Programme (Theme SME-2012-2) Project: Stabiwine, Grant agreement No: 314903 No GLP, published	N	Stabiwine EU project
2	Various	Various	Existing authorisations and evaluations in USA, Australia and EU (Italy, Spain, France)	N	Various

Annex number	Author	Year	Title Source (where different from company) Company, Report No. GLP or GEP status (where relevant) Published or Unpublished	Data protection claimed Y/N	Owner
3.1.1/01	Bosso A.	2015	Work package 2 (WP2): Building enological know-how on Polyaminoacids. Seventh Framework Programme (Theme SME-2012-2) Project: Stabiwine, Grant agreement No: 314903 No GLP, published	N	Stabiwine EU project
4.1.2.1/01 & 4.5.3	Restani P.	2015	EU project Stabiwine Final Report by Università degli Studi di Milano (UMIL) DIPARTIMENTO DI SCIENZE FARMACOLOGICHE E BIOMOLECOLARI - DiSFeB., UNIVERSITY of MILANO No GLP, unpublished	N	Stabiwine EU project
4.1.2.1/02	Vassanelli G	2015	Potassium Polyaspartate Analysis of Samples from i*n vitro* Absorption test. Laboratory Enocentro Srl, No GLP, unpublished	Y	Esseco srl
4.2.2.1/01	Mane J. P.	2014	*Salmonella typhimurium*, Reverse Mutation Assay of A-5D K SD (Ames Test, OECD Guideline No. 471) INTOX PVT. LTD., Study no. 13955 GLP, unpublished	Y	Esseco srl
4.2.2.2/01	Mane J. P.	2014	*In Vitro* Micronucleus Test of A-5D K SD in Cultured Human Lymphocytes INTOX PVT. LTD., Study no. R/13956/In vitro MNT/14 GLP, unpublished	Y	Esseco srl
4.3.2/01	Gumaste S.A.	2014	Repeated Dose 14 Day Oral Toxicity Study of A-5D K SD in Wistar Rat (Dose Range Finding Study) INTOX PVT. LTD., Study no. R/13969/SOR-14-DRF/14 GLP, unpublished	Y	Esseco srl

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4.3.2/02	Gumaste S.A.	2014	Repeated Dose 90 Day Oral Toxicity Study of A-5D K SD in Wistar Rat INTOX PVT. LTD., Study no. R/13957/SOR-90/14 GLP, unpublished	Y	Esseco srl
5.3	Restani P.	2012	Deliverable D 3.1 Report on bibliography toxicology data search for polyaminoacids. Seventh Framework Programme (Theme SME-2012-2) Project: Stabiwine, Grant agreement No: 314903 No GLP, published	N	Stabiwine EU project

* Access to data granted by NanoChem Solutions Inc. Letter of Access enclosed to the current submission.