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TOX/2020/42

COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Follow-up discussion paper to "alternatives to plastic packaging for food & drinks packaging": allergenicity of chitin and chitosan based BBFCMs

Introduction

1. In May 2020, a paper entitled "scoping paper: alternatives to conventional plastics for food & drinks packaging (TOX/2020/24)" was taken to the COT. The Committee was asked to provide further guidance on the potential toxicological hazards associated with bio-based food contact materials (BBFCMs). Members noted that quantitative information was needed on contamination, degradation, and migration of chemicals and allergens during the manufacture of commercial BBFCMs, as well as environmental impacts after disposal, for example formation of micro/nano-plastics upon entering landfill or from energy-from-waste processes.

2. The Committee was also asked to advise on which BBFCMs require consideration in further detail. Due to the diversity of available BBFCMs, the Committee agreed that it would be helpful to focus on BBFCMs that are most or most likely to be used in the UK, either directly or through import, such as PLA plastic. The Secretariat agreed to identify the most widely used materials and other higher priority materials for further review. The Food Contact Materials (FCM) Policy team added that they have received enquiries on chitin-based BBFCMs and chitosan-based drinking straws regarding their allergenic content. Subsequently, this discussion paper focuses on the immunogenicity and allergenicity of chitin- and chitosan-based BBFCMs. Information on possible future priorities for review will be brought to a future meeting.

3. Chitin is the second most abundant polysaccharide on earth after cellulose and can be extracted from the cell walls of fungi, and from the exoskeletons of crustaceans and insects. Chitosan is commonly manufactured from chitin (chitosan exists naturally in only a few species of fungi such as zygomycetes). Chitosan is used in some food applications (see Table 2), whilst other chitin-based products are in development (see paragraphs 29-35).

4. FCM Policy have presently identified four businesses that made direct queries to the FSA about chitin/chitosan BBFCMs (as primary and secondary packaging), and a total of three businesses about chitosan-based drinking straws. Although no UK incidents have raised formally, there is one report of a potential reaction to the use of a chitosan-based straw in a pub which was reported to a local authority. It was concluded that the reaction was a result of the meal, though additional precautions were put in place concerning labelling.

Several pub chains have switched to using chitosan-based straws[1], and are required to include clear labelling.

Regulatory aspects

5. In March 2018, the EU approved a ban on a range of single-use plastics including drinking straws[2]. Thus, alternative materials for drinking straws such as chitosan have been developed by several companies such as CuanTec.

6. Chitosan is used as a food additive in Italy, Finland, Korea, and Japan because of its properties (Peter, 1997; Singla & Chawla, 2001).

7. Chitosan and chitin have not been officially classified as GRAS (generally recognised as safe) by the US Food and Drug Administration (US FDA). Rather, two biomedical companies have notified the US FDA of their view that the use of chitosan and chitin in specific food applications is GRAS. For example, the biomedical company KitoZyme views the use of chitosan (derived from *Aspergillus niger*) in alcoholic beverage production (with chitosan being removed from the beverages post-treatment, using physical separation processes) as GRAS. In their correspondence to KitoZyme, the US FDA (2011) concluded that: "based on the information provided by KitoZyme, as well as other information available to FDA, the agency has no questions at this time regarding KitoZyme's conclusion that chitosan from *A. niger* is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of chitosan". A similar statement was made by the US FDA to KitoZyme in respect of chitin being used in beverage production (US FDA, 2012). Presently, five notices appear on the FDA website for chitosan and chitin, which are as follows:

- *A.niger*-derived chitosan, used as a "secondary direct food ingredient in alcoholic beverage production at levels between 10 and 500 grams per hectoliter (100 liters)", by KitoZyme[3]
- Shrimp-derived chitosan, for "use in foods generally including meat and poultry, for multiple technical effects", by Primex[4]
- Shrimp-derived chitosan, for use as an "ingredient in food including meat and poultry products", by Primex[5]
- Shrimp-derived chitosan, for "use in foods in general for multiple technical effects in accordance with good manufacturing practice", by Primex[6]

[1] https://www.midsussextimes.co.uk/lifestyle/food-and-drink/warning-alternative-biodegradable-straws-may-be-unsuitable-vegetarians-and-vegans-953184

[2] https://www.bbc.co.uk/news/world-europe-45965605

[3]https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=397&sort=GRN_No&order=DESC&startrow=1&type=basic&search=chitosan

[4]https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=443&sort=GRN_No&order=DESC&startrow=1&type=basic&search=chitosan

[5]https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=170&sort=GRN_No&order=DESC&startrow=1&type=basic&search=chitosan

[6]https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=73&sort=GRN_No&order=DESC&startrow=1&type=basic&search=chitosan

- *A.niger*-derived chitin, for "use in microbial stabilization, removal of contaminants, and/or clarification in alcoholic beverage production at levels between 10 and 500 grams per hectolitre", by KitoZyme[7]

8. Recent research has addressed the development of composite films for food packaging with additional or enhanced properties such as antimicrobial and antioxidant activities. These "smart materials" have included the use of chitin or chitosan in their composition (see paragraphs 29-35).

9. In Europe, there are two regulations relevant to the use of "smart materials" used in food packaging: Commission Regulation (EC) Nos. 1935/2004 and 450/2009.

10. In European legislation, all materials and articles intended for contact with food must meet the requirements of the Framework Regulation (EC) No 1935/2004. The basic principle underlying this Regulation is detailed in Article 3 which states: "materials and articles, including active and intelligent materials and articles, shall be manufactured in compliance with good manufacturing practice so that, under normal or foreseeable conditions of use, they do not transfer their constituents to food in quantities which could: a) endanger human health; b) bring about an unacceptable change in the composition of the food; c) bring about a deterioration in the organoleptic characteristics thereof."

11. The use and authorisation of these smart materials and articles intended to come into contact with food is regulated under Commission Regulation (EC) No 450/2009, where overall migration limits (OMLs) and specific migration limits (SMLs) are considered. This regulation also establishes an EU-wide list of substances that can be used in the manufacture of these materials. Substances may only be added to the list once their safety has been evaluated by EFSA.

Chemistry & manufacturing process

Chitin

12. Chitin, the second most abundant polysaccharide on earth after cellulose, is found in the cell walls of fungi, and in the exoskeletons of crustaceans and insects (mammals lack chitin and the enzyme involved in its synthesis, chitin synthase). In situ, chitin is linked to other structural components, such as protein and glucan, to form a protein-chitin matrix (Romano *et al.*, 2007). The main components of crustacean shells are on a dry weight basis (depending on the species and season) are: 30-40% protein, 30-50% mineral salts, and 13-42% chitin (Kurita, 2006).

13. Chitin is commercially derived from the shells of crustaceans (principally shrimps and crabs) that are supplied in large quantities as a by-product from the

[7]https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=412&sort=GRN_No&order=DESC&startrow=1&type=basic&search=chitin

shellfish processing industries. With the emergence of the insect industry, Berezina & Hubert (2020) consider that commercial chitin will increasingly be derived from insects.

14. The extraction of chitin involves two steps: demineralisation and deproteinisation. Deproteinisation can be conducted using chemical methods, which are well established for the commercial preparation of chitin. NaOH is the preferential reagent for chemical methods, which is used to solubilise the proteins present; it is applied at concentration ranging from 0.125 to 5.0 M, at varying temperature (up to 160 °C) and treatment duration (from few minutes up to few days) (Younes & Rinaudo, 2015). In their review, Younes & Rinaudo (2015) noted that "the complete removal of protein is especially important for biomedical applications, as a percentage of the human population is allergic to shellfish, the primary culprit being the protein component". However, a strong alkali treatment can damage the chitin structure and/or result in deacetylation, giving only chitosan and no longer the chitin at the end of the process (Rinaudo et al., 2006; Vazquez et al., 2013).

15. Alternatively, enzymatic methods can be used for deproteinisation of chitin. Enzymatic methods more environmentally friendly than chemical methods, and also help to avoid unwanted changes to the chitin structure. Enzymatic methods utilise whole cell microorganisms (Xu *et al.*, 2008) or purified enzymes (De Holanda & Netto, 2006; Synowiecki & Al-Khateeb, 2000). However, deproteinisation levels achieved in such cases are generally lower than those obtained using alkaline treatments. As such, use of the enzymatic method is limited to laboratory scale studies (Gadgey & Bahekar, 2017).

16. Table 1 shows the percentage of deproteinisation achieved from some enzymatic and chemical chitin recovery methods. This table shows a large variation exists for the conditions of deproteinisation for chitin preparation, as well as the percentage of deproteinisation obtained.

Table 1: Methods for recovery of chitin from marine resources, and extent of deproteinisation (DP).

Method for deproteinisation	**Conditions of deproteinisation**	**DP (wt %)**	**References**
chemical	shrimp shells; "partial autolysis", then 0.62M NaOH (1:5 w/v) for 20 hours at ambient temperature; 5 samples	99.16 ± 0.12 – 99.45 ± 0.06	Toan (2009)
chemical	Shrimp shells; 1M NaOH for 24 h at 70°C	>99	Percot *et al.* (2003)
chemical	shrimp shells; 2M NaOH for 2-5 hours at 30-65°C; 4 samples, at varying shell:NaOH ratios	95.34 ± 0.38 – 96.83 ± 0.17	Bajaj *et al.* (2011)

chemical	shrimp waste; 1.25M NaOH at ratio of 1:20 (*w/v*) for 4 hours at 80°C	93.8 ± 1.38	Manni *et al.* (2010)
enzymatic	A21 protease enzyme/substrate 7, 75 U/mg (60 °C, 6 h)	88	Younes *et al.* (2012)
enzymatic	Alcalase (50 °C, 3 h)	80	Abdelmalek *et al.* (2017)
enzymatic	Sil-Al 4 × 4 TM inoculant, glucose, 30 °C, 7 days	91	Manni *et al.* (2010)
enzymatic	*S. marcescens, L. paracasei,* glucose, 30 °C, 7 days	68.9	Jung *et al.* (2007)
enzymatic	*L. acidophilus* SW01, glucose, 37 °C, 168 h	96.5	Duan *et al.* (2012)
enzymatic	Stabisil inoculant, lactose, 25 °C	40	Healy *et al.* (1994)
enzymatic	*L. lactis, T. turnirae,* glucose, 7 days	95.5	Aytekin & Elibol (2010)
enzymatic	*L. paracasei, S. marcescens*, glucose, 30 °C, 7 days	52.6	Jung *et al.* (2006)

17. Presently, a total of three chitin products are available from Sigma-Aldrich Chemical Company (UK), which are all derived from shrimp shells. Information on their compositional purity is as follows (percentage purity data appear to be lacking):

- Powder; practical grade; requires purification prior to use as a substrate for chitinase[8]
- Purified powder; suitable for analysis of chitinase; purified by a modification of the method of Hirano & Nagao (1988)[9] (N.B this study describes a method for the preparation of colloidal chitin from chitin powder that was provided by “Katakurachikkarin Ltd. Tokyo”, and does not appear to include a deproteinisation step).
- Coarse flakes; practical grade[10]

18. According to Berezina & Hubert (2020), “no completely effective method for the determination of this (chitin) purity exists. Usual techniques such as Fourier-transform infrared (FT-IR) spectroscopy or X-ray analysis are only qualitative, whereas some other techniques such as liquid nuclear magnetic resonance (NMR) or chromatography are impossible due to the high insolubility of the polymer. Therefore, the previously described “alkaline extraction” method is often applied (Hajji *et al.*, 2014; Rhazi & Desbrieres, 2000).” A modified

[8] https://www.sigmaaldrich.com/catalog/product/sigma/c7170?lang=en&region=GB
[9] https://www.sigmaaldrich.com/catalog/product/sigma/c9752?lang=en&region=GB
[10] https://www.sigmaaldrich.com/catalog/product/sigma/c9213?lang=en&region=GB

spectrophotometric method according to Lowry *et al.* (1951) is often used for quantification of protein in chitin samples (e.g. Bajaj *et al.,* 2011).

19. One of the main limitations of using chitin on a large commercial scale is its water insolubility. Therefore, derivates have been produced from chitin that are more water-soluble, of which chitosan is the most important commercially. The chemical structures of chitin and chitosan are shown in Figure 1.

Chitosan

20. Although chitosan is also insoluble in water, it is soluble in slightly acidic solutions (pH<6.5) in which the glucosamine units are converted into a soluble form, $R–NH_3^+$ (Qin *et al.*, 2006).

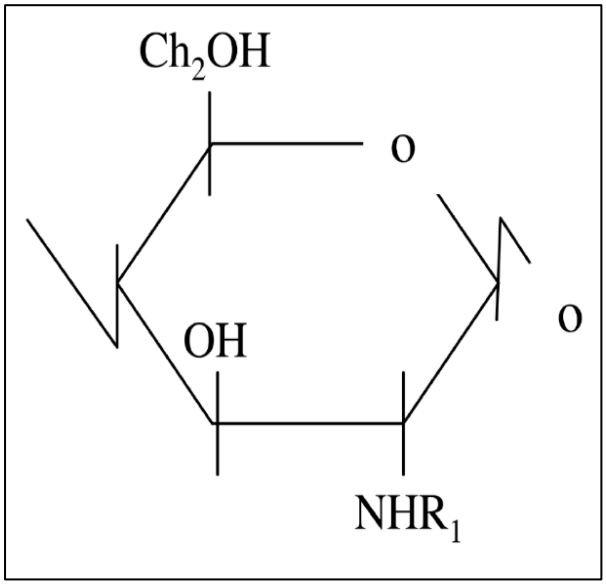

Figure 1: Chemical structures of chitin (R_1 = $COCH_3$) and chitosan (R_1 = H). Chitin is a high molecular weight β(1,4)-linked homopolymer of N-acetylglucosamine. It is found in three forms: α-chitin, β-chitin, and γ-chitin. α-chitin is the most abundant form found in nature, where the polymer folds back on itself to form antiparallel strands – this structure gives rise to intra-chain hydrogen bonding and consequently makes it the most stable form of chitin. Conversely, β-chitin is arranged in parallel strands, whilst γ-chitin (the least common form) is a mixed form, i.e., a combination of α and β forms. Chemical structure taken from Lee *et al.* (2008).

21. Chitosan exists naturally in only a few species of fungi such as zygomycetes (Muzarellu *et al.*, 1994). Therefore, chitosan is commonly manufactured from chitin, by removing acetyl groups (COCH3) from chitin though enzymatic or chemical methods. Chemical methods are used more extensively for commercial chitosan preparation than enzymatic methods because of their lower cost and suitability for mass production (No *et al.*, 1995). Usually, the chemical method involved sodium or potassium hydroxides at a concentration of 30-50% w/v, at high temperature (100ºC) (Aranaz *et al.* 2009).

22. Regardless of the method used however, the extent of N-deacetylation throughout the polymer is almost never complete as some acetamide groups usually remain (Abdulkarim *et al.*, 2013). This gives rise to different degrees of deacetylation (DD). The DD is generally defined as the glucosamine/N-acetyl glucosamine ratio. When the percentage of N-acetyl glucosamine > glucosamine, the polymer is called chitin. Conversely, when the percentage of glucosamine > N-acetyl glucosamine, the compound is called chitosan (Viarsagh *et al.*, 2010).

Chitosan has also been defined as chitin that is sufficiently deacetylated to form soluble amine salts (NTP, 2017).

23. Solubility of chitosan in aqueous, acidic media occurs when deacetylation of chitin reaches approximately 50% (Rinaudo, 2006), though in addition to the DD, chitosan solubility is also dependent on the molecular weight and the distribution of the remaining acetyl groups on the polymer (Kubota & Eguchi, 1997). Experiments conducted by Ottsy *et al.* (1996) show compositional heterogeneity in the chitosans, with chitin-like acid insoluble fractions with acetylated units between 88-95%, and fractions with acetylated units from 20-52%. The DD influences both chemical (e.g. solubility) and biological (e.g. bioavailability and biodegradability) properties of chitosan (Benhabiles *et al.* 2012; Park & Kim, 2010).

24. The high density of positive charges that are left on the amino groups after deacetylation make chitosan water-soluble and allows it to readily interact with negatively charged substances such as proteins, fatty acids, bile acids, and phospholipids. These interactions give rise to several properties of chitosan, including antimicrobial, antioxidant, and fat-binding properties, leading to several applications in the food industry (see Table 2).

Chitooligosaccharides

25. Chitooligosaccharides (COS), having a molecular weight of approximately 10kDa or less, are the depolymerised products of chitin or chitosan, and can be produced through chemical hydrolysis or enzymatic methods (Xia *et al.*, 2010). Enzymatic methods can use various enzymes including chitinase and chitosanase (Klinkesorn, 2013). Various non-specific enzymes can also break down chitosan including lysozymes, cellulases and lipases, which help with its biodegradation in nature (Raafat & Sahl, 2009). Chitosan with a molecular weight of ≤ 16KDa is considered a COS (Rajoka *et al.*, 2020). COS are water-soluble (Qin *et al.*, 2006), and have antioxidative, anti-inflammatory, and antibacterial effects (Huang *et al.*, 2016). However, COS have been observed to irritate intestinal epithelial mucosal tissues, stimulating them to hyperproduce mucin (Deters *et al.*, 2008).

ADME & toxicity

26. Results from Chae *et al.* (2005) indicate that absorption of chitosan from the gastrointestinal tract following oral exposure in rats is inversely related to its molecular weight: oral gavage administration of chitosan with molecular weights of 3.8, 7.5, 13, 22, or 230 kDa resulted in maximum plasma chitosan concentrations (Cmax) of 20.23, 9.30, 5.86, 4.32, or <0.5 µg/mL, respectively. Degradation of chitosan in vertebrates is thought to occur predominantly by lysozymes and bacterial enzymes in the colon (Kean & Thanou, 2010). The rate of biodegradation of chitosan in vivo is dependent on the DD (Yang *et al.*, 2007).

27. Studies designed to evaluate the effectiveness of chitosan as a weight-loss supplement suggest that it is well tolerated in humans. No adverse effects

were reported in male (4.5 g chitosan/ day) or female (2.5 g/ day) volunteers following oral chitosan administration for 12 days (Gades & Stern 2003, 2005). Additionally, no adverse effects were reported following oral administration of chitosan at up to 6.75 g per day for 8 weeks in male and female volunteers (Tapola *et al.*, 2008).

Beneficial properties & applications of chitin/chitosan

28. Chitosan has some useful properties, leading to its use as a preservative, a packaging additive, and a dietary supplement in the food industry (see Table 2).

Table 2: Some properties of chitosan and corresponding applications in the food industry.

Property	Description of property	Application in food
Antimicrobial	This antimicrobial activity has been linked to the positive charges of the C2 amino groups in the glucosamine monomers of chitosan. These positive charges may interact with the negatively charged microbial cell membrane, causing leakage of the intracellular constituents of the microorganisms and cell death (Dutta *et al.*, 2009). Another proposed mechanism is the ability of chitin and its derivatives to activate defence mechanisms of the host organisms, such as inducing chitinases and other pathogenesis-related proteins (El Ghaouth *et al.* 1992).	The antimicrobial and antioxidant properties of chitin and its derivatives has led to its application as a food preservative (Sethulekshmi 2014). Chitosan-based edible films can be consumed along with the product in the package (Yadav *et al.* 2019). These films appear in vacuum-packaged processed meat (Ouattara *et al.*, 2000), cheese (Fajardo *et al.* 2010), and other foods such as vegetables, fruits, grains, and fish (Sinha *et al.*, 2012). Chitosan can also be used as an inhibitor of browning in juices (Abdelmalek *et al.*, 2017), and an antioxidant in sausages (Arslan & Soyer, 2018).
Antioxidant	Chitosans may retard lipid oxidation by chelating ferrous ions present in meat (No *et al.*, 2007). NH2 groups may react with hydrogen ions to produce NH3 groups and may react with other free radicals (Xie *et al.*, 2001).	(see above)
Reduction of lipid absorption	It is claimed that chitosan, because of its cationic nature, binds to bile and fatty acids, which reduces their absorption and facilitates their excretion (Gallaher *et al.*, 2000). Another possible mechanism is that chitosan traps fat in the intestines by increasing the viscosity of the	Chitosan is sold as a dietary supplement, where manufacturer-recommended human consumption typically averages 14.3 mg chitosan/kg per day (based on a 70 kg

	intestinal contents and preventing the hydrolysis of triglycerides (Kanauchi *et al.*, 1995).	adult)[11,12]. On the basis of scientific data presented to EFSA in 2011, the Panel concluded that "a cause and effect relationship has been established between the consumption of chitosan and maintenance of normal blood LDL-cholesterol concentrations", and considered that in order to obtain this effect in adults, 3 g of chitosan should be consumed daily (EFSA, 2011).
Dietary fibre	Insoluble, non-digestible chitosan fibres have been used as a source of dietary fibre.	Industrial production of chitosan dietary fibres has occurred (Hughes, 2002).

Some chitin- and chitosan-based BBFCMs on the market or in research

29. Modifying chitosan by the addition of a metal enhances its antimicrobial activity compared to native chitosan (Du *et al.*, 2009). For example, the antimicrobial activity of chitosan- Zn^+ and chitosan-Ag^+ is higher than native chitosan (Zhang *et al.*, 2016; Wei *et al.*, 2009). Subsequently, some of the chitosan-based BBFCMs are nanoengineered to contain metal ions. For example, Yin et al. (2018) prepared carboxymethyl chitosan/poly(vinyl alcohol)/Cu blend film for packaging application. The tensile test and thermal gravimetric analysis revealed improved mechanical and thermal properties of chitosan after blending, while the copper ions loading improved the antibacterial activity.

30. Satam *et al.* (2018) developed a flexible packaging material comprised of alternating layers of chitin nanofibers and cellulose nanocrystals onto poly(lactic acid) (PLA) films. Satam *et al.* noted that it "can be applied to a variety of applications where oxygen permeability is a key problem, including packaging of foods".

31. ChitoClear®, a chitosan-based product for food packaging, is commercialised by Primex Company (Siglufjordur, Iceland). NorLife and Kitoflokk™ brands from Norwegian Chitosan (Kløfta, Norway) also manufactured for application in food and beverages (Ferreira *et al.* 2016).

32. The n-CHITOPACK project coordinated by Mavi, Italy was initiated with the objective of developing new chitin-based food packaging material by utilising chitin nanofibrils with other natural polymers (Morganti 2013).

[11] General Nutrition Centers Inc. GNC Total LeanTM chitosan with glucomannan. https://www.gnc.com/fiber/484711.html?productId=2459379

[12] Vitamin World Inc. Chitosan 500 mg. http://www.vitaminworld.com/fiber/chitosan-500mg-0070004945.html

33. Wu *et al.* (2019) developed a novel intelligent film by immobilizing 1%, 3% and 5% black rice bran anthocyanins (BACNs) into oxidized-chitin nanocrystals (O-ChNCs)/ chitosan (CS) matrix. The ultraviolet-visible spectrum of BACNs solutions showed colour variations from red to greyish green in a range of pH 2.0–12.0. The study authors concluded that the results showed that the CS/OChNCs/BACNs (COB) films containing 3% of BACNs (COB-3) were able to monitor the spoiling of fish and shrimp by visible colour changes. Therefore, the developed COB-3 films could be used as an intelligent food packaging for monitoring animal-based protein food spoilage.

34. Sahraee *et al.* (2017) developed gelatin-based bionanocomposite films (GNCFs) containing 0, 1, 3, and 5% zinc oxide nanoparticles (N-ZnO) and/or 0, 3, 5, and 10% chitin nanofibers. Simultaneous incorporation of chitin and ZnO nanoparticles in the GNCFs had the interactive effect on improving the physicochemical and antimicrobial properties of GNCFs. Sahraee *et al.* concluded that the GNCFs “showed better physical and antifungal properties than net gelatin films and can be applied for increasing storage life of packaged foods”.

35. Panariello *et al.* (2019) treated cellulose-based board packaging with chitosan and chitin nanofibrils (in varying ratios). Trials performed with packaged food demonstrated that chitin and chitosan were effective in reducing the microbial growth, thus allowing an increase of food shelf life. The study authors concluded that “the results confirmed that it will be reasonably possible to increase food safeness and to waste less food thanks to the use of a fully renewable and biodegradable packaging”.

Immunogenicity of chitin and chitosan

36. Chitin and chitosan are potential targets for recognition by mammalian immune system since mammalians lack such biopolymers naturally (Komi *et al.,* 2019). Thus, Patel & Goyal (2017) note in their review that “caution should be exercised while using it for food and therapeutic purposes”.

37. Upon exposure, chitin can be recognised by mammalian chitinases that bind and degrade chitin, and chitinase-like proteins which also bind chitin but are catalytically inactive (Funkhouser & Aronson, 2007). Furthermore, both chitin and chitosan particles are readily phagocytosed, supporting a role for recognition via specific receptor(s) mediating phagocytosis, though the receptor(s) remain to be determined (Bueter *et al.,* 2011).

38. Chitin and chitosan were first shown to be immunostimulating in the 1980’s. Chitin and chitosan were shown to activate macrophages and natural killer (NK) cells to express a number of pro-inflammatory cytokines such as IL-1, CSF, and IFN-γ; these effects led to enhanced cell-mediated cytotoxicity in mice, in addition to enhancement of antibody production and delayed-type hypersensitivity in guinea pigs (Nishimura *et al.,* 1984, 1985; Iida *et al*., 1987). In 1986, Suzuki *et al.*, through their analysis of splenic cell changes in cancerous mice, showed that the antitumor

mechanism of COS is to enhance acquired immunity by accelerating T-cell differentiation to increase cytotoxicity and maintain T-cell activity.

39. Patel & Goyal (2017) consider that desriptions of chitin having “exceptionally low” immunogenicity (e.g. Zhang *et al.*, 2011) are “misleading”. Indeed, there appears to be a more complex picture regarding the immunological properties of chitin. Lee *et al.* (2008) speculated that “when chitin containing pathogens enter a host, the innate anti-pathogen response contains oxidants and chitinases that induce chitin fragmentation. The resulting intermediate sized fragments, in turn, serve as an alarm signal to induce and amplify local inflammation by activating pattern recognition receptors and pathways like NF-κB. This would continue until the invader has been successfully dealt with and smaller chitin fragments are generated. These small fragments would induce molecules like IL-10 which feedback to control the local inflammatory response”.

40. Mammalian innate immune responses to chitin seem to depend on the size of the chitin fragments used to stimulate immune cells (Da Silva *et al.,* 2009). Very large (>100 μm) chitin fragments seem to be immunologically inert, while intermediate (40–70 μm) and small chitin (<40 μm) seem capable of activating macrophages and eliciting IL-17, TNF and IL-23 production via a range of pattern recognition receptors (PRRs) (Da Silva *et al.,* 2008). For example, intravenous administration of small chitin particles (1–10 μm) into the lung activated alveolar macrophages to express cytokines such as IL-12, tumour necrosis factor (TNF)-α, and IL-18 (Shibata *et al*., 1997).

41. Administration of chitin/chitosan beads (administered directly into the lungs, Reese *et al*., 2007) and microparticles (injected subcutaneously, Heseini *et al.*, 2016) have caused immune responses in mice.

42. Koller *et al.* (2011) showed that epidermal or epithelial cells can recognise chitins via PRRs, leading to cytokine/chemokine secretion. This may be important in the regulation of epidermal immunity, since chitin is expressed by microorganisms that are involved in some skin allergies.

43. The effect of chitosan as a novel adjuvant to an inactivated influenza vaccine was studied (Chang *et al.*, 2004). Here, BALB/c mice were abdominally inoculated with vaccine and chitosan together twice every three weeks. Blood serum was prepared and tested for levels of antibodies IgG, IgG1, and IgG2a as well as IgA antibody in nasal secretions. One week after the immunisation regimen, the mice were challenged with the deadly flu virus A/PR/8/34(H1N1) and the weights of the mice and levels of antibody protection were measured. The results indicated that using chitosan as an adjuvant increased the antibody content in serum remarkably and increased the antiviral defence in the mice, enhancing the immune reaction to the vaccine.

44. Huang *et al.* (2006) studied the anticancer activities of differently charged COS derivatives using three cancer-cell lines: HeLa, Hep3B, and SW480. Neutral red and MTT cell-viability studies revealed that highly charged COS derivatives could significantly reduce cancer-cell viability, regardless of their positive or negative charge. Furthermore, fluorescence microscopic observations and DNA fragmentation

studies confirmed that the anticancer effect of these highly charged COS derivatives were due to necrosis. However, the exact molecular mechanism for the anticancer activity of strongly charged COS compared to their poorly charged counterparts is not clear.

45. *Lactococcus lactis* and *Lactobacillus plantarum* have chitin-binding and/or chitinolytic proteins (Sánchez *et al.*, 2011). These bacteria are integral part of gut normal flora, fermented foods, and probiotic-fortified foods (Kim *et al.*, 2013; Todorov *et al.*, 2012). However, their inflammatory role in the gut has not been observed, indicating that if chitins accidently reach the gut, they are converted to some other, non-immunogenic form, and thus immune activation in gut does not occur (Patel & Goyal, 2017). Furthermore, Patel & Goyal (2017) stated that "excess chitin exposure is likely to be increasing chitinolytic bacteria in human microbiome".

46. The ability of chitin to activate a variety of innate (eosinophils, macrophages) and adaptive immune cells (IL-4/IL-13 expressing T helper type-2 lymphocytes) has recently been reviewed by Komi *et al.* (2019). Given these immunostimulating effects, Komi *et al.* concluded that:

- wide distribution of chitin makes its exposure inevitable; however, the avoidance of chitin exposure needs to be investigated;
- commercial shellfish chitin has been used in most chitin immunology studies, and our knowledge remains incomplete regarding other sources of chitin such as fungal chitin in similar studies; and,
- lacking novel methods for chitin purification may explain the conflicting data in the literature of immune responses to chitin.

Allergencity of chitin & chitosan

47. Incomplete deproteinisation of chitin may lead to the presence of allergenic proteins in the final material such as tropomyosin. Tropomyosin is the main allergenic protein in sea food, which can cause allergic reactions in sensitised individuals. Thus, some researchers do not recommend the use of chitosan in the diet of individuals who are allergic to crustaceans (Ylitalo *et al.*, 2002). The most widely accepted allergen reference doses for crustacean-derived protein are ED01 (where <1% of the allergic population may be expected to react) at 26.2 mg protein, and ED05 at 280 mg protein (Remington *et al.,* 2020). These reference values are derived from human food challenge data, and represent acute intake levels that elicit reactions in IgE-mediated food allergies.

48. Kato *et al.* (2005) reported a case of immediate-type allergy for chitosan-containing health food. The patient was a 47-year-old female who developed systemic urticaria and difficulty in breathing after oral ingestion of chitosan. Since skin tests (prick test and scratch patch test) were positive, the test was done using another commercial chitosan, and was positive. The patient was diagnosed as having chitosan-induced immediately-type allergy, and was instructed to avoid ingestion of chitosan. The patient developed no symptoms thereafter. The study authors concluded that chitosan may have functioned as a food allergen because of its molecular weight and general properties.

49. Bae *et al.* (2013) investigated the role of chitin and chitosan in inhibition of food allergic responses to peanuts. They treated C3H/HeJ mice with α-chitin, β-chitin, and β-chitosan for 6 weeks starting 1 week before peanut sensitisation. They evaluated the allergic symptoms 30-40 minutes after the oral ground whole peanut challenge, and reported the capability of chitin and chitosan to suppress the anaphylaxis symptoms from peanut-induced hypersensitivities. Moreover, peanut-specific IgE levels were reduced in mice treated with α-chitin and β-chitosan.

50. Chitosan has applications in various fields such as tissue engineering and biomedicine due to its low cost, biocompatibility, lack of toxicity, and biodegradability (Madhumathi *et al.*, 2009; Konovalova *et al.*, 2017). Wound dressings manufactured from chitosan are available for clinical use (Wedmore *et al.*, 2006). Chitosan is considered to be hemostatic due to its cationic nature (NTP, 2017), which supports its use in wound dressings. Waibel *et al.* (2011) investigated the safety of these "HemCon®" bandages, that were introduced in 2005 for US soldiers. Patients who reported shellfish allergy were recruited. Initial assessment included a detailed history, IgE skin prick testing (SPT), and serum testing to shellfish allergens. Participants who demonstrated specific shellfish IgE underwent a bandage challenge. Results: Nineteen participants were enrolled; 10 completed the study. Seven (70%) were male and the average age was 44.8 + 10 years. Nine (90%) reported a shrimp allergy history and five (50%) reported multiple shellfish allergies. All participants completing the study had positive SPT and serum IgE testing to at least one shellfish; eight (80%) had shrimp positive SPT and ten (100%) demonstrated shrimp-specific IgE. No participant had a positive SPT to chitosan powder or experienced an adverse reaction during bandage challenges. No protein bands were visualised during gel electrophoresis analysis of chitosan powder. The study authors concluded that all participants tolerated the HemCon bandage without reaction. This is the first study demonstrating the safety of this bandage in shellfish allergic subjects.

51. In 2010, EFSA assessed the safety of chitin-glucan as a novel food ingredient (EFSA, 2010). The product assessed was called "KiOnutrime-CG™", composed of >90 % chitin-glucan (the main component in the cell walls of Aspergillus niger, derived from a fermentation process), and ≤ 6 % protein, and intended to provide an intake of 2 to 5 g chitin-glucan/day. The Panel used a report showing no observed adverse effects at the highest dose administered (about 6.6 g/kg bw) in a 13-week rat study (TNO, 2009). Because this dose is approximately 80-fold higher than the maximum intended level of intake for humans on a g/kg bw basis, the Panel concluded that KiOnutrime-CG™ is safe as a food ingredient at the proposed conditions of use and the proposed intake levels. The Panel assessed the risk of allergenicity on the basis of some allergenic enzymes that are synthesised by *A. niger* such as beta-xylosidase. The Panel concluded that "an allergenic risk cannot be ruled out, but is expected not to be higher than the consumption of other *A. niger* derived products".

52. In 2019, EFSA evaluated the safety of the food enzyme chitinase from *Streptomyces violaceoruber*, intended to be used in baking processes (EFSA, 2019). The potential allergenicity of the chitinase (produced with the genetically modified S. violaceoruber strain pChi) was assessed by comparing its amino acid sequence with

those of known allergens according to the Scientific Panel on Genetically Modified Organisms (EFSA 2017). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, no match was found. No food allergic reactions to this chitinase have been reported in the literature. Although several cases of respiratory allergy following occupational inhalation of aerosols containing chitinase had been reported (Martel *et al.*, 2010; Patel and Goyal, 2017), several other studies had also shown that adults with occupational asthma to enzymes can ingest respiratory allergens without acquiring clinical symptoms of food allergy (Brisman, 2002; Poulsen, 2004; Armentia *et al.*, 2009). Therefore, the Panel considered that under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme could be excluded, that the likelihood of such reactions occurring was considered to be low. The Panel thus considered that there are no indications for food allergic reactions to this chitinase, and concluded that the food enzyme chitinase produced with the genetically modified S. violaceoruber strain pChi does not give rise to safety concerns arising from the toxicological studies and the production process under the intended conditions of use.

Questions on which the views of the Committee are sought:

I. Given the extent of deproteinisation of chitin during its manufacturing process using chemical methods, does the Committee have any comments on the risk of allergenicity from chitin- or chitosan-based BBFCMs on the basis of allergenic proteins that may be present?

II. Do the immunological properties of chitin or chitosan pose a health risk when used in BBFCMs?

III. Does the reported case of immediate-type allergy for a chitosan-containing health food (paragraph 48) represent a health risk to the general public?

IV. Is any further information sought from the Secretariat on chitin/chitosan-based BBFCMs?

Secretariat
September 2020

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