Carrageenan
Handling/Processing

This technical report is limited in scope to focus only on Evaluation Question #10 and incorporates responses to specific questions that were requested by the National Organic Standards Board (NOSB) Handling Subcommittee. A full technical report on carrageenan was last published in 2011 (ICF 2011).

Evaluation Questions for Substances to be used in Organic Handling

Evaluation Question #10: Describe and summarize any reported effects upon human health from use of the petitioned substance (7 U.S.C. § 6517(c)(1)(A)(i), 7 U.S.C. §6517(c)(2)(A)(i)) and 7 U.S.C. § 6518(m)(4)).

Carrageenan (CAS # 9000-07-1) is an FDA-approved direct food additive with an average molecular weight of 200-800 kDa, and may be referred to as "undegraded" or "native" carrageenan in the literature. The actual molecular weight of food-grade carrageenan represents a spectrum of molecular weights that are naturally present in live seaweed. The kappa, iota or lambda formation of carrageenan is defined by the number and position of sulfate groups (Cian et al. 2015).

Differences between Carrageenan and Poligeenan

Poligeenan, also called "degraded carrageenan" or "C16" in the literature, is a distinctly different substance from carrageenan, although carrageenan is its raw material. Poligeenan (CAS# 53973-98-1) is an artificially formed polymer produced by subjecting carrageenan to extensive acid hydrolysis at low pH (0.9-1.3) and high temperatures (>80° C) for an extended period of time (McKim 2014). It is defined by the United States Adopted Names Council as having an average molecular weight of 10-20 kDa (Cohen and Ito 2006). It was developed in the 1960s to treat pain associated with ulcers, and its only application today is as a component of x-ray imaging diagnostic products (Watson 2008). Poligeenan is not an approved food additive and is not used in any food applications. The literature is in agreement that poligeenan causes ulcerations of the cecus and proximal colon in experimental animals, leading to its classification by the International Agency for Research on Cancer as a possible human carcinogen (Weiner 2014; Tobacman 2001).

It is possible that food-grade carrageenan may contain some low molecular weight fractions that are equivalent to poligeenan, although validated analytical methods to accurately measure the low molecular weight distributions of carrageenan are not fully developed or available to the industry (Cohen and Ito 2006). An analysis of the molecular weight distributions of 29 types of commercially available food-grade carrageenan demonstrated that none of the food-grade samples contained molecular weight fractions equivalent to poligeenan at a detection limit of about 5% (Uno, Omoto, et al. 2001a).

Degradation of Carrageenan in Digestive System

Several studies have investigated the potential of carrageenan degradation in the digestive tract. The research is not fully conclusive but seems to suggest that degradation is possible.

In an early *in vivo* study by Pittman, Golberg and Coulston (1975), carrageenan was given to guinea pigs, monkeys and rats via drinking water or in the diet. Fecal and liver samples were examined quantitatively by gel electrophoresis to determine changes in molecular weight of carrageenans after passing through the digestive tract. The study demonstrated that high molecular weight carrageenans are degraded to some extent as a result of their passage through the intestinal tract, although to what extent exactly is variable and not fully understood. Concentrations of carrageenan in the feces were 2-3 orders of magnitude greater than those in the liver, demonstrating that most of the administered dose was contained in the feces. Carrageenans present in the feces were reduced to approximately 100 kDa or less. The study made no conclusions regarding the influence that degradation might have on ulcerogenic potential. A critique of this study by Necas and Bartosikova (2013) suggested that the degradation of carrageenan in the digestive

system is of limited toxicological significance because ulceration was not detected in feeding studies, indicating that the carrageenan is not degraded to the same molecular weight as poligeenan.

In a more recent *in vivo* study, carrageenan with an average molecular weight of 832 kDa was given to rats via the diet at a level of 5% for one day, and no carrageenan for the second and third days (Uno, Omoto, et al. 2001b). Fecal samples were collected on each of the three days. The lowest average molecular weight detected over the three days was 718 kDa, indicating that some degradation did occur. In another study by Tache et al. (2000), the average molecular weight of carrageenan was not changed significantly during digestion by rats after being given 2.5% food-grade carrageenan via drinking water.

Polysaccharides such as carrageenan are depolymerized (degraded) in acid solution, and the rate of polymerization depends on pH and temperature (Capron, Yvon and Muller 1996). An early *in vitro* study by Ekstrom (1985) analyzed the rate of degradation through batch hydrolysis of 8 food-grade carrageenans in a simulated gastric fluid. The findings showed that after 2 hours in simulated gastric juice at pH 1.2, almost 90% of the carrageenan had a mass of <100 kDa and 25% had a mass of <20 kDa. At pH 1.9, the rate of degradation was much lower; after 2 hours, 65% of the carrageenan had a mass of <100 kDa and 10% had <20 kDa. Ekstrom's conclusion is that the acidity and rate of passage through the stomach will determine the degree of degradation of carrageenan. No conclusions were made regarding the possible toxicological implications of the degradation. At least two review articles have critiqued this study, noting that the conditions of the simulated gastric fluid are more extreme than would be expected to occur normally in the stomach during digestion (McKim 2014; Necas and Bartosikova 2013). Ekstrom's batch hydrolysis study was replicated more recently by Capron, Yvon, and Muller (1996) who found that after 6 hours at pH 1.2, the average molecular weight is greater than 200 kDa, which is much higher than Ekstrom's results. Capron, Yvon, and Muller (1996) also analyzed the rate of degradation in an artificial stomach which simulated more realistic conditions for human digestion, wherein the pH gradually decreases from 5 to about 2 or 1.5 over the course of 3 hours prior to gastric emptying (Capron, Yvon and Muller 1996). Findings from the artificial stomach experiment showed that under the most unfavorable conditions of gastric digestion (slow emptying rate and rapid acidification), about 10% of the carrageenan had a molecular weight <100 kDa.

The potential for carrageenan to be degraded in other parts of the digestive system has also been reviewed. The International Programme on Chemical Safety (IPCS) in cooperation with the Joint FAO/WHO Expert Committee on Food Additives (JECFA) acknowledged that carrageenan may be degraded in the gut, but suggested that that the effects of degradation might not be toxicologically significant (JECFA 1999). The report did not find evidence of degradation in the lower gut.

Enzymatic incompatibility in the intestines has been suggested to reduce the likelihood that carrageenan will degrade in significant amounts in the intestines. Carrageenan has a unique structure with alternating a-(1-3) and b-(1-4) glycosidic bonds. Intestinal enzymes such as lactase which are believed to be capable of depolymerizing carrageenan are only able to recognize and cleave the b-(1-4) bond; however, the actual existence and concentration of enteric enzymes capable of degrading carrageenan are not known (McKim 2014).

Inflammation and Ulceration

The effects of carrageenan on human health have been studied in depth over the past several decades, although there is not a lot of human clinical data on the topic. Studies have focused mainly on laboratory animals *in vivo*, as well as *in vitro* studies and on the material itself. Negative effects on animal subjects have been documented in some studies.

Several conclusions in the literature for animal feeding studies did not associate food-grade carrageenan fed in the diet with inflammation or ulceration, although some research does suggest an association. In a study by Weiner et al. (2007), rats were fed food-grade carrageenan for 90 days at rates up to 50 ppm in the diet. The carrageenan used in this study was specially formulated to comply with the European Commission's recommendation that no more than 5% of carrageenan fractions should have molecular

weight below 50 kDa (European Comission 2003). The findings showed no toxicologically significant differences between the high dose and the control, and no evidence of erosions, ulcerations, inflammation, regeneration, hyperplasia, or any other abnormalities of the gastrointestinal tract. Abraham et al. (1985) fed rats 5% food-grade carrageenan in the diet for 40 weeks and did not observe any significant histopathological effects. Tomarelli et al. (1974) fed 4% food-grade carrageenan in a milk powder to rats for 6 months and did not observe any abnormal cecum or colon tissue morphology or any evidence of ulceration. A study by Poulsen (1973) observed no ulcerations or erosions in the gastrointestinal tract of pigs that were fed dietary carrageenan, although some changes in intestinal flora were observed. One dietary study found a negative effect in guinea pigs. Grasso et al. (1973) identified multiple pin-point caecal and colonic ulcerations in guinea pigs after being fed 5% diet of carrageenan for 45 days. However, rats that were fed the same dietary concentration in the same study did not develop any signs of ulceration, leading the researchers to conclude that guinea pigs are a more sensitive species.

Feeding studies specific to infants have also occurred. In an early study by McGill et al. (1977), infant baboons were fed formula containing 1% (equivalent to highest concentration in commercially available human infant formula) or 5% native carrageenan. The findings showed that the carrageenan had no effect compared to the control on hematological or clinical variables or the microscopic appearance of the gastrointestinal tract. More recently, a 10-day study of neonatal mini pigs fed formula containing 0, 300 (0.03%) or 3000 (0.3%) mg/kg carrageenan (average molecular weight >663 kDa) showed no notable differences between the treatment groups in mucosal mast cell counts across the entire gastrointestinal tract (JECFA 2015). Another study of piglets fed formula containing 0, 300 (0.03%), 1000 (0.1%) or 2250 (0.225%) mg/kg carrageenan (average molecular weight >663 kDa) also showed no treatment-related effects on the gastrointestinal tract (JECFA 2015). In a study by Weiner et al. (2015), piglets were fed formula containing kappa and lambda carrageenan (average molecular weight >664 kDa) at concentrations of 0, 300 (equivalent concentration to commercial human infant formula), 1000 or 2250 ppm for 28 days. Histopathological findings did not show evidence of carrageenan-induced inflammation or ulceration of carrageenan-treated piglets (Weiner et al. 2015). Based on these infant feeding studies, the Joint FAO/WHO Expert Committee on Food Additives concluded that the use of carrageenan in infant formula at concentrations up to 1000mg/L is not of concern (JECFA 2015).

Results are mixed in animal studies that administered carrageenan through drinking water. One of the earliest studies of carrageenan-induced ulceration was performed by Watt and Marcus (1969), wherein guinea pigs were fed 1% undegraded carrageenan solution via drinking water. The findings showed evidence of ulcerative lesions, although conclusions were not made regarding the relevancy to humans. A later study by Benitz, Golberg and Coulston (1973) did not observe any intestinal abnormalities in rhesus monkeys given 1% carrageenan via drinking water or given 50-1250 mg/day carrageenan via a stomach tube.

Several *in vitro* studies have been performed to investigate carrageenan-induced effects on cell signaling pathways that contribute to inflammation, but without consensus among the reviewed research. A series of studies has shown that carrageenan can induce a complex inflammatory cascade in human intestinal epithelial cells[1] through an immune-mediated mechanism (Borthakur et al. 2012) and a reactive oxygen species (ROS)-mediated mechanism (Bhattacharyya, Dudeja and Tobacman 2008), which contribute to an inflammatory response. A feedback loop leads to extended inflammation (Bhattacharya et al. 2010a). The inflammatory cascade involves carrageenan-induced activation of toll-like receptor 4 (TLR4) and BCL10 (B-cell CLL/lymphoma 10) which leads to stimulation of nuclear factor kappa B (NF- kB) and induction of interleukin-8 (IL-8), both of which are proinflammatory (Borthakur et al. 2007; Bhattacharyya et al. 2010b; Bhattacharyya, Feferman, and Tobacman 2015). However, the ability for carrageenan to bind to TLR4 and trigger the inflammatory cascade has been challenged in the literature. A study by McKim, Wilga,

[1] Some studies used only normal intestinal cells (NCM460 cell line) derived from colonic mucosa from an individual adult human male (Bhattacharyya, Dudeja and Tobacman 2008). Other studies also included trials with normal intestinal epithelial cells derived from primary human colonic epithelial cells of patients undergoing colonic surgery (Borthakur et al. 2012).

Pregenzer, et al. (2015) of carrageenan activity towards TLR4 in human embryonic kidney cells[2] after 24 hours of exposure to carrageenan showed that carrageenan does not bind to TLR4, and therefore cannot be an agonist[3] for the human TLR4 signaling pathway.

A review article by Tobacman (2001) of animal studies on the effects of carrageenan and poligeenan on gastrointestinal health concluded that undegraded carrageenan is associated with intestinal ulcerations and neoplasms. The article attributed these issues to the contamination of undegraded carrageenan by components of low molecular weight, the spontaneous metabolism to lower molecular weight by acid hydrolysis under conditions of normal digestion, or the interactions with intestinal bacteria. The article is critiqued by several industry-funded researchers who note that Tobacman's conclusions for carrageenan are inappropriately extrapolated from studies performed with poligeenan (McKim 2014; Weiner 2014; Cohen and Ito 2006). Many of the studies referenced in the Tobacman review article that used food-grade carrageenan are included in this technical report to assess the potential for degradation and ulceration (Nicklin and Miller 1984; Rustia, Shubik and Patil 1980; Pittman, Golberg and Coulston 1975; Engster and Abraham 1976; Poulsen 1973; Benitz, Golberg and Coulston 1973; Grasso et al. 1973).

Definitive conclusions regarding the varying degrees of human susceptibility to inflammation effects of carrageenan cannot be made from the available literature. The Acceptable Daily Intake (ADI) for carrageenan is established as "not specified," meaning that the total dietary intake of carrageenan when used as a food additive does not represent appreciable risk to health (JECFA 2001). ADIs are intended to be universally applicable to all sectors of the population. However, since different animal species, different animals within the same species, and different human intestinal cell lines have produced different experimental results, it is reasonable to expect that humans may also experience varying degrees of sensitivity to carrageenan in the diet.

Absorption

The absorption capacity of carrageenan into the gastrointestinal tract has been shown to be affected by the molecular weight and form of carrageenan when administered through drinking water. An early study by Engster and Abraham (1976) demonstrated that artificially prepared low molecular weight (<107 kDa) iota-carrageenan fractions administered to guinea pigs via drinking water were absorbed in the cecal lamina propria and submucosal macrophages and subsequently caused ulceration. However, when fed in the diet, the iota fractions did not produce any inflammatory response in the cecum. Higher molecular weight fractions (>145 kDa) of iota-carrageenans administered via drinking water were not absorbed. Absorption of kappa or lambda carrageenan of all molecular weights (ranging from 5-516 kDa) did not occur when administered via drinking water. The researchers concluded that different forms of carrageenan of the same molecular weight can cause different effects in the guinea pig cecum. In a later study by Nicklin and Miller (1984), rats were given 0.5% high molecular weight food-grade carrageenan via drinking water for 90 days. The findings showed that small quantities of carrageenan were persorbed across the mucosal interface of the gut, but there were no observed abnormal histological features or pathological lesions attributable to the carrageenan treatment.

Carrageenan is mostly excreted in the feces. A feeding study of rats demonstrated that on average, 98% of carrageenan consumed is excreted in the feces (Tomarelli et al. 1974). An early study measured an excretion rate of 90-100% in the feces of rats fed carrageenan in the diet (Hawkins and Yaphe 1965). Another feeding study of rats estimated the recovery rate of about 90% (Uno, Omoto, et al. 2001b). Although these studies indicate that there may be a small percentage that is not excreted, there is no apparent evidence in the literature of animal feeding studies that carrageenan fed in the diet is absorbed in the gastrointestinal tract in toxicologically significant quantities.

[2] HEK293 cell line derived from human embryonic kidney cells originally sourced from an individual healthy aborted human fetus (www.hek293.com)

[3] An agonist is a molecule that combines with a receptor on a cell to trigger a physiological reaction

Tumor Promotion and Carcinogenicity

In vivo studies generally conclude that carrageenan does not initiate tumors, although conclusions regarding its role in the promotion of existing carcinogenic activity are mixed. Rustia, Shubik and Patil (1980) administered food-grade carrageenan to rats and hamsters at rates up to 5% in the diet over the lifetime, and found no statistically significant differences in the incidence of tumors. Hagiwara et al. (2001) studied the potential for carrageenan to promote tumors by injecting rats with a carcinogen (DMH) and then feeding 5% carrageenan in the diet for an additional 32 weeks. The histopathological analysis showed that carrageenan lacks tumor-promoting potential on DMH-induced colorectal carcinogenesis. Taché et al. (2000) studied the effect of carrageenan on the initiation and promotion of aberrant crypt foci (a precursor of tumors) and whether intestinal microflora is a contributor. Their animal model used conventional rats (containing their natural gut flora) and germ-free rats colonized with human fecal microflora (to simulate human colon) which were fed carrageenan in solid gel at rates up to 10%. The carrageenan-fed rats (both types) showed no indication of tumor initiation. To evaluate tumor promotion, rats (both types) were injected with a carcinogen (AOM) and then given carrageenan in drinking water or in gel at rates up to 2.5%. The findings showed that carrageenan did contribute to growth promotion of AOM-induced tumors in conventional rats at the highest dose, but did not promote growth in any of the human-fecal-affiliated rats. Calvert and Satchithanandam (1992) studied the effect of carrageenan on thymidine kinase activity (an indicator of cell proliferation) in the colonic mucosa. Rats were fed carrageenan at rates between 1% and 2.61% in the diet for 4 weeks. The findings showed significantly increased thymidine kinase activity only at the highest dose, which is equivalent to 100 times the maximum normal human intake. There were no histological abnormalities associated with the carrageenan treatments. From the above studies on the role of carrageenan in tumor promotion of existing carcinogenic activity, it is difficult to draw conclusions about how carrageenan may contribute hazardous risk to humans.

An *in vitro* study by Tobacman (1997) investigated the carcinogenic effects of carrageenan by exposing mammary myoepithelial cells to lambda-carrageenan at rates up to 0.0014%. The findings showed disruption of the internal cellular architecture of the carrageenan-treated cells, and suggested that there may be implications for mammary carcinogenesis. However, the article does not attempt to extrapolate the findings as evidence of risk for normal dietary consumption of carrageenan.

Carrageenan-induced cell signaling pathways that contribute to proliferation disorders have been studied in human colonic epithelial cells. A mechanism of carrageenan-induced Wnt signaling can lead to proliferative disorders and contribute to colon carcinogenesis as demonstrated in a study by Bhattacharyya, Feferman, Borthakur, et al. (2014).

Insulin Resistance and Diabetes

A series of studies beginning in 2012 have investigated carrageenan-induced effects on cell signaling pathways that inhibit insulin signaling leading to insulin resistance and glucose intolerance (Bhattacharyya et al. 2012). Insulin resistance is the principal feature of type 2 diabetes (Copps and White 2012). The mechanisms of the cell-signaling pathway are demonstrated in a recent study by Bhattacharyya, Feferman, and Tobacman (2015), wherein carrageenan-induced inflammatory and transcriptional cell-signaling cascades impair glucose tolerance resulting in insulin resistance.

In an *in vivo* experiment by Bhattacharyya, Feferman, Unterman, et al. (2015), mice were exposed to carrageenan (10 mg/L of lambda and kappa high molecular weight carrageenan delivered via drinking water), high fat diet (8% fat), or the combination of high fat diet and carrageenan, or untreated, for one year. The results showed that carrageenan exposure led to glucose intolerance after six days, and that carrageenan in combination with high fat diet produced earlier onset of fasting hyperglycemia, higher glucose levels, and exacerbated dyslipidemia, suggesting that carrageenan exposure may exacerbate harmful effects of a high fat diet and contribute to development of diabetes.

Relevancy of Non-Dietary Experimental Models

When carrageenan is used as a food additive, it is typically bound to a protein. As described in the 2011 Technical Report (ICF 2011), the ability of carrageenan to tightly bind to positively charged substances like salt ions and proteins is the reason that carrageenan is an effective stabilizer in food products. The kappa, iota or lambda formation of the carrageenan influences the interactions with proteins (Cian et al. 2015). Both kappa-carrageenan and iota-carrageenan are able to form helical structure in solution, allowing the formation of thermoreversible gels commonly used in foods and infant formulas, whereas lambda-carrageen cannot form helices and can therefore only produce highly viscous solutions (Uno, Omoto, et al. 2001a). These forms are blended in various proportions to satisfy particular food production requirements. In typical commercial food products, lambda-carrageen is a minor component in combination with kappa-carrageenan (JECFA 2015).

Because the presence of protein can impact the behavior of carrageenan, dietary studies are considered the most relevant. The U.S. FDA recommends 50 ppm (5%) of test material in the diet as the highest dose, since higher doses of non-nutritional substances can cause nutritional deficiencies (Weiner, Nuber, et al. 2007). The effects of higher dosages are likely due to nutritional deficiency rather than substance toxicity. Guinea pigs are the common subject in *in vivo* animal studies because this species is considered the most sensitive to intestinal effects. Neonatal pigs and mini pigs are appropriate models for human infants (JECFA 2015). Some concerns have been raised about experimental models that do not utilize a protein source, such as carrageenan administered via drinking water. The absence of a protein may increase the proportion of free carrageenan molecules available for hydrolysis and/or interaction with intestinal cells, which could result in findings that would otherwise not occur if carrageenan was consumed with food (McKim 2014).

Systemic injections of carrageenan are associated with acute inflammation, and are widely used in experimental pharmacology research (Weiner 2014). Approximately 400 research papers have cited the use of carrageenan-induced rat paw oedema to test the effectiveness of anti-inflammatory drugs. Typically in these studies, a solution of 1–3% lambda–carrageenan (non-gelling type) in saline is injected into the hind paw of the rat (Necas and Bartosikova 2013). The literature does not describe how these systemic injections of carrageenan are scientifically relevant to normal dietary intake of carrageenan. Non-gelling type carrageen is typically not used on its own in commercial food products. Injected carrageenan molecules are not subjected to the same action as they are through dietary intake and passage through the digestive tract, before they interact with cells.

There is disagreement in the literature regarding the applicability of some aspects of *in vitro* laboratory studies to the effects of carrageenan in humans as part of the diet. *In vitro* refers to an artificial environment outside of a living organism, such as in a petri dish or test tube, whereas *in vivo* studies are those that occur within a living organism, such as animal test subjects. *In vitro* models have useful applications in identifying cell signaling pathways, but are limited by their inability to completely duplicate the extensive interactions among cells and tissues occurring in an animal model (Hartung and Daston 2009). The relevancy of nearly all of the *in vitro* studies performed on the health effects of carrageenan is contested by McKim (2014), an *in vitro* toxicologist, in a review article prepared for and funded by FMC Corporation, a manufacturer of carrageenan. The concern appears to be that the *in vitro* models lack the functional mechanisms that are present in the intestinal tract *in vivo*, such as the absence of serum protein. The Joint FAO/WHO Expert Committee on Food Additives echoes the concerns of extrapolating *in vitro* findings to conclusions of risks *in vivo*. The cell linings of the gastrointestinal tract *in vivo* are protected by a mucous barrier that is not present in *in vitro* models (JECFA 2015).

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