Bureau Nieuwe Voedingsmiddelen
Novel Foods Unit

Ice structuring protein

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Tweede beoordeling van de veiligheid voor de consument, volgens de Europese verordening 258/97 betreffende nieuwe voedingsmiddelen en nieuwe voedselingrediënten

Second opinion regarding consumer safety, in accordance with European Regulation 258/97 concerning novel foods and novel food ingredients

aan/to:

de Minister van Volksgezondheid, Welzijn en Sport
the Minister of Health, Welfare and Sport

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Bureau Nieuwe Voedingsmiddelen
CBG
Postbus 16229
2500 BE Den Haag
novelfoods@cbg-meb.nl
www.nieuwevoedingsmiddelen.nl

Novel Foods Unit
MEB
P.O. box 16229
2500 BE The Hague
The Netherlands
novelfoods@cbg-meb.nl
www.novel-foods.nl

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Beoordeling

Inleiding

Aan de orde is een tweede beoordeling volgens de Europese Verordening 258/97, over het gebruik als nieuw voedingsmiddel van het zogenoemde *Ice Structuring Protein* (verder aangeduid met de afkorting ISP). Deze aanduiding wordt gebruikt voor een aantal eiwitten die in de natuur in verschillende organismen voorkomen [1]. Deze eiwitten kunnen een zeer uiteenlopende structuur hebben, maar hebben gemeen dat ze weefsels beschermen tegen schade door koude, doordat de eiwitten het ontstaan van ijskristallen beïnvloeden. Deze aanvraag betreft het eiwit dat wordt aangeduid als ISP Type III HPLC 12. Dit ISP komt van nature voor in een zeevis, de Atlantische puitaal (*Macrozoarces americanus*). Voor commerciële productie wordt het eiwit echter gewonnen uit een genetisch gemodificeerde gist, die een synthetisch gen bevat dat codeert voor het ISP. De aanvraag is ingediend door de firma Unilever PLC, gevestigd in het Verenigd Koninkrijk. Deze firma wil het ISP toepassen bij de productie van consumptie-ijs. Tijdens het bevriezen zorgt het ISP voor het ontstaan van een gewenste structuur. Het eiwit blijft vervolgens aanwezig in het eindproduct.

In het kader van de desbetreffende Europese toelatingsprocedure is deze tweede beoordeling uitgevoerd door het Bureau Nieuwe Voedingsmiddelen van het College ter Beoordeling van Geneesmiddelen. Het bureau heeft hiervoor de Commissie Veiligheidsbeoordeling Nieuwe Voedingsmiddelen geraadpleegd, hierna genoemd ‘de commissie VNV’.

Eerste beoordeling

De eerste beoordeling van de aanvraag voor markttoelating is verricht in het Verenigd Koninkrijk door de *Advisory Committee on Novel Foods and Processes* (ACNFP) van de *Food Standards Agency*. De ACNFP concludeert dat ISP veilig kan worden gebruikt voor de bedoelde toepassing. Tevens is de ACNFP van mening dat producten zodanig moeten worden geëtiketteerd dat duidelijk is dat dit ingrediënt is geproduceerd door gebruik te maken van een genetisch gemodificeerde gist.

Bevindingen van de commissie VNV

De commissie VNV heeft geen bezwaar tegen de toelating van ISP voor de voorgestelde toepassing. Zij baseert haar oordeel op de informatie in het dossier (waarvan de samenvatting is opgenomen als bijlage A) en de eerste beoordeling door de ACNFP (bijlage B). Als achtergrondinformatie heeft het bureau ook gebruik gemaakt van de openbare verslagen van de beraadslagingen van de Britse ACNFP.

Keuze van het beoordelingsregime

Hoewel het ISP afkomstig is van een genetisch gemodificeerde gist, wordt het product niet beoordeeld volgens de Europese verordening 1829/2003 [2]. Dit is in overeenstemming met de benadering die wordt beschreven in een rapport van de Europese Commissie aan de Raad en het Europese Parlement over de tenuitvoerlegging van de genoemde verordening

[3]. Volgens dit document is de verordening niet van toepassing op voedingsmiddelen die worden geproduceerd door fermentatie, waarbij het genetisch gemodificeerde micro-organisme wordt gebruikt als een "technisch hulpmiddel" (*processing aid*) [a]. Dat wil onder meer zeggen dat het genetisch gemodificeerde micro-organisme in principe niet meer aanwezig is in het eindproduct. In het geval van deze aanvraag bestaat het nieuwe ingrediënt uit producten van een genetisch gemodificeerde gist, maar zijn de gistcellen zelf door microfiltratie verwijderd.

Gezien de technische functie van het gebruik van het ISP bij de bereiding van consumptie-ijs zou de vraag gesteld kunnen worden of het preparaat als een levensmiddelenadditief zou moeten worden gezien. Het is echter niet in te delen in één van de categorieën die worden genoemd in de Europese richtlijn over levensmiddelen additieven [4]. In december 2006 heeft het Permanent Comité voor de Voedselketen en de Diergezondheid besloten dat een veiligheidsbeoordeling van het ISP als nieuw voedingsmiddel geëigend is [5].

De commissie VNV heeft kennis genomen van de discussies over dit product. De commissie vindt het nogal gekunsteld om dit product van genetische modificatie te laten beoordelen als nieuw voedingsmiddel. De ACNFP heeft hier echter op een goede manier invulling aan gegeven door de oude hoofdstukindeling voor ggo-beoordelingen volgens de Europese Aanbeveling 97/618 te gebruiken, en daarbij de relatie aan te geven met de vereisten die worden genoemd in het handreikingsdocument van het EFSA GMO panel voor dit type producten [6,7].

Productieproces en productspecificaties

Voor de productie van het ISP wordt een genetisch gemodificeerde bakkersgist gebruikt. (afgeleid van *Saccharomyces cerevisiae*, stam CEN.PK) Deze gist bevat een synthetisch gen dat codeert voor een eiwit, dat qua aminozuursequentie identiek is aan het ISP uit de Atlantische puitaal. Dit ISP is 66 aminozuren lang en wordt uitgescheiden door de gistcellen. Door filtratiestappen worden de gistcellen verwijderd en wordt het ISP uit het cultuurmedium geconcentreerd. Het concentraat wordt gebufferd met citraat. Het uiteindelijke preparaat bevat naast het ISP ook andere eiwitten uit de gist, evenals suikers en zouten. Volgens de specificatie bevat het preparaat ten minste 5 g/l actief ISP, ten hoogste 2% as en 2mg/l zware metalen en is de pH 3.0 (+/- 0.5). Ook zijn er in de specificatie vereisten vastgelegd voor de microbiologische kwaliteit van het product. Het dossier bevat gedetailleerde analysegegevens van vijf commerciële productbatches. Daarnaast is een verder geconcentreerd preparaat geanalyseerd, dat voor het toxicologisch onderzoek in proefdieren is gebruikt. De ACNFP heeft vragen gesteld over de glycosylering van het ISP eiwit. Het blijkt dat circa 40% van het ISP in dit preparaat is geglycosyleerd, waardoor deze moleculen niet

[a] Een voetnoot bij Artikel 1 van Richtlijn 89/107/EEG luidt: "In deze richtlijn wordt onder „technisch hulpmiddel" verstaan: een stof die op zichzelf niet als voedselingrediënt wordt geconsumeerd, die bij de verwerking van grondstoffen, levensmiddelen of voedselingrediënten bewust wordt gebruikt om tijdens de bewerking of verwerking aan een bepaald technisch doel te beantwoorden en die kan leiden tot de onbedoelde maar technisch onvermijdelijke aanwezigheid van residuen van deze stof of derivaten ervan in het eindproduct, mits deze residuen geen gevaar voor de gezondheid opleveren en geen technologische gevolgen voor het eindproduct hebben."

actief zijn. Het geglycosyleerde eiwit is niet meegerekend in het gehalte aan ISP dat is weergegeven in tabel 2 van het dossier.

De commissie VNV meent dat voldoende informatie is gegeven over de productiemethode en de productspecificatie.

Geschiedenis van het bronorganisme

Het ISP wordt geproduceerd door een genetisch gemodificeerde bakkersgist (*Saccharomyces cerevisiae*), die is afgeleid van stam CEN.PK. De gemodificeerde gist is in Nederland in 2002 toegelaten als productieorganisme. De ACNFP concludeerde dat er voldoende bekend is over het gebruikte micro-organisme.

De commissie VNV deelt deze conclusie.

Analyse van de genetische modificatie en de effecten daarvan

Op basis van de bekende aminozuurvolgorde van het ISP uit de Atlantische puitaal werd een expressiecassette geconstrueerd voor de productie van dit eiwit in gist. Daarvoor werd een synthetisch gen gemaakt met een aangepast codongebruik, geflankeerd door een induceerbare promoter en een signaalsequentie. De gebruikte vector was zodanig ontworpen, dat het synthetische gen door homologe recombinatie kon integreren in het ribosomale DNA van de gist. Tevens bevat de expressiecassette een *leu2*-gen dat wordt gebruikt om een toename te induceren van het aantal kopieën van het ingebrachte DNA. Het resultaat hiervan is de aanwezigheid van circa 30-50 aaneengeschakelde kopieën van de expressiecassette op één plaats in het DNA van de gistcel. Het nieuwe DNA bevat geen antibioticumresistentie marker. Op verzoek van de ACNFP leverde de aanvrager experimentele bevestiging dat het nieuwe DNA slechts op één plaats in het genoom was geïntegreerd en dat daarbij geen nieuwe open leesramen waren ontstaan. Gegevens over genetische stabiliteit en over de expressie van het nieuwe DNA waren naar tevredenheid van de ACNFP verstrekt.

De commissie VNV heeft geen vragen over de analyse van de genetische modificatie en de effecten hiervan.

Inname

De aanvrager stelt dat ISP zal worden gebruikt in consumptie-ijs tot een gehalte van maximaal 0,01%. De ACNFP wijst erop dat dit globaal overeenkomt met gebruik van het ISP preparaat tot maximaal 0,2%, omdat het preparaat 5-8% ISP bevat. De aanvrager heeft voedselconsumptiegegevens uit het Verenigd Koninkrijk, Nederland en Frankrijk gebruikt om een schatting te maken van de mogelijke inname van ISP bij de voorgestelde toepassing. De gegevens uit het Verenigd Koninkrijk worden in de eerste beoordeling gedetailleerd besproken. In absolute zin werd de maximale inname geschat op 99 g ijs per dag (het 97,5^{e} percentiel van de jongens van 11 tot 14 jaar), wat overeen zou komen met 0,21 mg ISP per kg lichaamsgewicht. Terecht wijst de ACNFP erop dat bij jongere kinderen de inname van ISP per kg lichaamsgewicht hoger kan uitkomen. (0,38 mg/kg voor kinderen van 4 tot 6 jaar) Volgens consumptiegegevens uit Nederland zouden volwassenen het meeste ijs consumeren. (tot 100 g per dag voor het 95^{e} percentiel) Voor Frankrijk werd een gemiddelde ijsconsumptie van nog geen 10 gram per dag gerapporteerd.

Volgens de commissie VNV volstaat de gepresenteerde schatting van de inname van ISP bij de voorgestelde toepassing.

Eerdere blootstelling

De Atlantische puitaal heeft geen geschiedenis als voedingsmiddel in de EU, al wordt deze vis volgens het dossier geconsumeerd in het noordoosten van de Verenigde Staten. De ACNFP wijst erop dat gegevens over de inname van ISPs uit andere voedingsmiddelen niet bruikbaar zijn voor de veiligheidsbeoordeling. Het gaat hier namelijk om zeer uiteenlopende eiwitten, die alleen een vergelijkbare functie hebben in de natuur. Verder wijst men erop dat er wel een lange geschiedenis van consumptie bestaat voor bakkersgist. Dat is van belang omdat het ISP preparaat ook andere bestanddelen uit gistcellen bevat. De aanvrager heeft verklaard dat consumptie-ijs met het ISP sinds 2003 op de markt is in de Verenigde Staten. Ook in enkele andere landen zijn zulke producten al toegelaten.

De commissie VNV deelt de conclusie van de ACNFP dat de informatie over andere ISPs niet zonder meer bruikbaar is voor de veiligheidbeoordeling.

Voedingskundige informatie

Het directe effect van de voorgestelde toevoeging van het nieuwe ingrediënt aan consumptie-ijs is verwaarloosbaar. De aanvrager stelt in het dossier dat door de toevoeging van ISP consumptie-ijs met een iets andere samenstelling gemaakt kan worden. Zo zouden producten kunnen worden gemaakt met minder vet of toegevoegde suikers of met een hoger fruitgehalte.

Volgens de commissie VNV is er uit voedingskundig oogpunt geen sprake van nadelige effecten bij de voorgestelde toepassing.

Microbiologische informatie

De aanvrager heeft een specificatie opgesteld met maximale waarden voor de aanwezigheid van verschillende micro-organismen in het nieuwe ingrediënt. (volgens de aanvrager is dit in overeenstemming met de norm uit de Food Chemicals Codex voor voedingsenzymen) Daarnaast blijven de vereisten van kracht die in het algemeen van toepassing zijn voor consumptie-ijs. Het dossier beschrijft de microbiologische analyse van tien commerciële productbatches. De ACNFP concludeert dat de microbiologische veiligheid van het product is aangetoond.

De commissie VNV deelt deze conclusie.

Toxicologische informatie

De aanvrager heeft een geconcentreerde batch van het ISP preparaat gebruikt voor een 90-dagen oraal toxiciteitsonderzoek bij ratten. Bij dit onderzoek werd het ISP preparaat dagelijks toegediend via gavage in doses die 58, 290 en 580 mg ISP bevatten. De dosisgroepen bestonden uit 20 mannelijke en 20 vrouwelijke ratten. Bij afzonderlijke controlegroepen werd water of citroenzuur toegediend. Het onderzoek werd uitgevoerd volgens van toepassing zijnde internationale standaarden. Er werden geen afwijkingen gevonden, gerelateerd aan de toediening van ISP. De aanvrager concludeerde dat hieruit een NOAEL van 580 mg ISP per kg lichaamsgewicht per dag kan worden afgeleid. Bij de geschatte maximale inname van 0,38 mg ISP per kg lichaamsgewicht per dag voor kinderen van 4 tot 6 jaar betekent dit een veiligheidsfactor van 1500. Voor andere groepen zou deze veiligheidfactor nog hoger zijn.

Verder beschrijft het dossier vijf onderzoeken naar mogelijke genotoxiciteit. In geen van deze gevallen werden aanwijzingen voor genotoxiciteit gevonden.

In het dossier wordt uitgebreid ingegaan op mogelijke allergeniteit van het ISP. De eerste beoordeling bevat een overzicht van experimentele resultaten uit het dossier. Een vergelijking van de aminozuurvolgorde van het ISP met bekende allergenen leverde geen overeenkomsten op. Verder werd ISP *in vitro* goed afgebroken door pepsine. Onderzoek bij proefpersonen die allergisch zijn voor kabeljauw toonde aan dat men in de meeste gevallen wel reageerde op de Atlantische puitaal, maar niet op het ISP. De ACNFP vroeg de aanvrager naar mogelijke reacties tegen componenten uit de gist bij personen die allergisch zijn voor gist of schimmels. De aanvrager was van mening dat de bekende allergenen uit gist wel betrokken zijn bij inhalatie- of contactallergie, maar niet bij voedselallergie. De ACNFP was echter van mening dat een risico voor personen met gistallergie niet kon worden uitgesloten, en stelde dat op het etiket moet worden vermeld dat het product van gist afkomstig is. Ook het feit dat dit een genetisch gemodificeerde gist is, zou volgens de ACNFP op het etiket moeten worden vermeld.

Volgens de commissie VNV volstaat het uitgevoerde toxicologisch onderzoek voor dit product. De commissie betwijfelt of het uit veiligheidsoogpunt noodzakelijk is om op het etiket te vermelden dat ISP afkomstig is uit gist. De commissie spreekt zich niet uit over de vraag of het product zou moeten worden geëtiketteerd als afkomstig van genetische modificatie. Voor dergelijke etikettering bestaat aparte wetgeving, los van de veiligheidsbeoordeling in het kader van de verordening voor nieuwe voedingsmiddelen.

Conclusie

De commissie VNV deelt de mening van de ACNFP dat de veiligheid van het ISP bij de voorgestelde toepassing in consumptie-ijs voldoende is onderbouwd.

Referenties

1. Crevel RW, Fedyk JK, Spurgeon MJ. Antifreeze proteins: characteristics, occurrence and human exposure. Food Chem. Toxicol. 2002 Jul;40(7):899-903.

2. Verordening (EG) nr. 1829/2003 van het Europees Parlement en de Raad van 22 september 2003 inzake genetisch gemodificeerde levensmiddelen en diervoeders (Voor de EER relevante tekst). PB L 268 van 18.10.2003, blz. 1–23.

3. Verslag van de Commissie aan de Raad en het Europees Parlement over de uitvoering van Verordening (EG) nr. 1829/2003 van het Europees Parlement en de Raad inzake genetisch gemodificeerde levensmiddelen en diervoeders (http://eur-lex.europa.eu/LexUriServ/site/nl/com/2006/com2006_0626nl01.pdf).

4. Richtlijn 89/107/EEG van de Raad van 21 december 1988 betreffende de onderlinge aanpassing van de wetgevingen der Lid-Staten inzake levensmiddelenadditieven die in voor menselijke voeding bestemde waren mogen worden gebruikt. PB L 40 van 11.2.1989, blz. 27–33.

5. Standing Committee on the Food Chain and Animal Health, section toxicological safety of the food chain. Summary record of the meeting of 14 December 2006. (http://ec.europa.eu/food/committees/regulatory/scfcah/toxic/summary23_en.pdf)

6. 97/618/EG: Aanbeveling van de Commissie van 29 juli 1997 betreffende de wetenschappelijke aspecten en de presentatie van de informatie die nodig is om aanvragen voor het in de handel brengen van nieuwe voedingsmiddelen en nieuwe voedselingrediënten te ondersteunen alsmede het opstellen van de verslagen van de eerste beoordeling uit hoofde van Verordening (EG) nr. 258/97 van het Europees Parlement en de Raad (Voor de EER relevante tekst). PB L 253 van 16.09.1997, blz. 1-36
7. EFSA (2006): Guidance document of the scientific panel on genetically modified organisms for the risk assessment of genetically modified microorganisms and their derived products intended for food and feed use. The EFSA journal: 374, 1-115.

Assessment (English courtesy translation)

Introduction

The subject in question is a second assessment, in accordance with European Regulation 258/97, regarding the use of Ice Structuring Protein (referred to in this report by the abbreviation ISP) as a novel food. This designation is used for a number of proteins which occur naturally in various organisms [1]. The structure of these proteins can vary enormously, but they share the property of being able to protect tissues from damage in very cold conditions. They do this by influencing the development of ice crystals. This application relates to the protein designated as ISP Type III HPLC 12. This ISP occurs naturally in a marine fish native to the Atlantic, the ocean pout (*Macrozoarces americanus*). For the purpose of commercial production, however, the protein is harvested from a genetically modified yeast into which a synthetic gene coding for the ISP has been inserted. The application was submitted by Unilever PLC, which is based in the United Kingdom. This company wishes to use the ISP in the production of edible ices. The ISP ensures that the requisite ice structure develops during freezing. Afterwards, the protein is still present in the final product.

In the framework of the relevant European approval procedure, this second opinion was prepared by the Novel Foods Unit of the Medicines Evaluation Board. To this end, the Unit consulted the Committee on the Safety Assessment of Novel Foods (hereafter referred to as “the VNV Committee”).

Initial assessment

The initial assessment of the application for market authorisation was carried out by the Advisory Committee on Novel Foods and Processes (ACNFP) of the Food Standards Agency, in the United Kingdom. The ACNFP concluded that the ISP can be safely utilised for the use in question. In addition, the ACNFP takes the view that product labelling must clearly indicate that this ingredient is derived from a genetically modified yeast.

Findings of the VNV Committee

The VNV Committee has no objection to the authorisation of ISP for the proposed use. It bases its views on the information contained in the dossier (the summary of which is contained in Annex A), and on the initial assessment by the ACNFP (Annex B). The Unit has also used the public reports of the ACNFP’s deliberations as background information.

Choice of assessment regime

Although the ISP is derived from a genetically modified yeast, the product is not assessed in accordance with European Regulation 1829/2003 [2]. This is in agreement with the approach described in a European Commission report for the Council of Europe and the European Parliament, concerning the implementation of the regulation in question [3]. According to this document, the Regulation is not applicable to foods produced by fermentation, where the

genetically modified organism is used as a processing aid [a]. One aspect of this is that, in principle, the genetically modified microorganism is no longer present in the final product. In the case of this application, the novel ingredient consists of materials produced by a genetically modified yeast, however the yeast cells themselves have been removed by microfiltration.

In view of the technical function of the use of the ISP in the preparation of edible ices, it is reasonable to ask whether the preparation should be viewed as a food additive. However, this material does not fall into any of the categories described in the European Directive on food additives [4]. In December 2006, the Standing Committee on the Food Chain and Animal Health decided that a safety assessment of the ISP as a novel food would be appropriate [5].

The VNV Committee has examined the debate surrounding this product. The Committee feels that it is rather contrived to have this product of genetic modification assessed as a novel food. However, the ACNFP has managed to deal with this issue effectively by employing the old chapter structure for GMO assessments, in accordance with European Recommendation 97/618 [6]. In addition, it indicates the relationship to the requirements described in the guidance document of the European Food Safety Authority's (EFSA) Scientific Panel on Genetically Modified Organisms for products of this type [7].

Production process and product specifications

A genetically modified baker's yeast (derived from *Saccharomyces cerevisiae*, strain CEN.PK) is used for the production of the ISP. This yeast contains a synthetic gene coding for a protein which, in terms of its amino acid sequence, is identical to the ISP from the ocean pout. This ISP is 66 amino acids in length, and is excreted by the yeast cells. The yeast cells are removed by filtration, after which the ISP is concentrated from the culture medium by ultrafiltration. The concentrate is then buffered using citrate. In addition to the ISP, the final preparation also contains other yeast proteins, as well as sugars and salts. According to the specification, the preparation contains at least 5 g/l of active ISP. It also contains no more than 2% ash and 2 mg/l of heavy metals, and has a pH of 3.0 (+/- 0.5). The specification also sets out requirements with regard to the product's microbiological quality. The dossier contains detailed analytical data on five commercial batches. A more highly concentrated preparation used for toxicological testing in experimental animals was also analysed. The ACNFP posed various questions pertaining to the glycosylation of the ISP protein. Approximately 40% of the ISP in this preparation was found to be glycosylated, which means that these molecules are not active. The glycosylated protein was not included in the ISP concentration given in Table 2 of the dossier.

The VNV Committee takes the view that sufficient information has been provided concerning the production method and the product specification.

[a] A footnote to Article 1 of Directive 89/107/EC states that: "For the purpose of this Directive, 'processing aid' means any substance not consumed as a food ingredient by itself, intentionally used in the processing of raw materials, foods or their ingredients, to fulfil a certain technological purpose during treatment or processing and which may result in the unintentional but technically unavoidable presence of residues of the substance or its derivatives in the final product, provided that these residues do not present any health risk and do not have any technological effect on the finished product.."

History of the source organism

The ISP is produced by a genetically modified baker's yeast (*Saccharomyces cerevisiae*) derived from strain CEN.PK. The modified yeast was approved in 2002 for use as a production organism in the Netherlands. The ACNFP concluded that the nature of the microorganism being used in this case is sufficiently well understood.

The VNV Committee concurs with this conclusion.

Analysis of the genetic modification and of its effects

The known amino acid sequence of the ocean pout's ISP formed the basis for the construction of an expression cassette for the production of this protein in yeast. To this end, a synthetic gene with optimized codon use was prepared, flanked by an inducible promoter and a signal sequence. The vector used here was designed to enable the synthetic gene to integrate into the yeast's ribosomal DNA by homologous recombination. The expression cassette also contains a *leu2* gene, which is used to induce an increase in the number of copies of introduced DNA. This results in the presence of approximately 30-50 linked copies of the expression cassette at a single site in the yeast cell's DNA. The new DNA does not contain an antibiotic resistance marker. At the request of the ACNFP, the applicant provided experimental confirmation that the new DNA had only been integrated at a single site in the genome, and that this had not resulted in the creation of new open reading frames. The data provided to the ACNFP, regarding genetic stability and the expression of the new DNA, were found to be satisfactory.

The VNV Committee has no questions regarding the analysis of the genetic modification and its effects.

Intake

The applicant states that ISP will be used in edible ices, up to a maximum concentration of 0.01%. The ACNFP points out that this broadly corresponds to the use of the ISP preparation at concentrations of up to 0.2%, since the preparation contains 5-8% ISP. The applicant has used food consumption data from the United Kingdom, the Netherlands and France as the basis for an estimate of the possible intake of ISP in the proposed use. The data from the United Kingdom are discussed in detail in the initial assessment. In absolute terms, the maximum intake was estimated to be 99 g of edible ices per day (the 97.5th percentile of boys aged 11 to 14), which would correspond to 0.21 mg ISP per kg body weight. The ACNFP rightly points out that, in the case of younger children, the intake of ISP per kg body weight may be higher (0.38 mg/kg for children from four to six years of age). Consumption data from the Netherlands indicate that it is adults who consume the greatest quantity of edible ices (up to 100 g per day for the 95th percentile). Reports indicate that the average consumption of edible ices in France is less than 10 grams per day.

The VNV Committee feels that the presented estimate of ISP intake in the proposed use is adequate.

Previous exposure

While the dossier indicates that the ocean pout is eaten in the north-eastern United States, this fish has no history of food use in the EU. The ACNFP points out that data on the intake of ISPs from other foods are not usable for the purposes of the safety assessment. This is because the proteins involved are extremely varied, sharing only a comparable function in

the natural situation. Furthermore, it is pointed out that baker's yeast does have a long history of consumption. This is an important consideration, as the ISP preparation also contains other components of yeast cells. The applicant has stated that edible ices containing the ISP in question have been on the market in the United States since 2003. Such products have also been admitted in several other countries.

The VNV Committee concurs with the ACNFP's conclusion that information relating to other ISPs cannot automatically be used for the safety assessment.

Nutritional information

The direct effect of the proposed addition of the novel ingredient to edible ices is negligible. In the dossier, the applicant states that the addition of ISP will make it possible to produce edible ices with a slightly altered composition. This would enable products to be created that contain less fat or added sugars, or higher concentrations of fruit.

The VNV Committee takes the view that, from the nutritional point of view, the proposed use will not have any adverse effects.

Microbiological information

The applicant has drawn up a specification giving maximum values for the presence of various microorganisms in the novel ingredient (according to the applicant, this is in keeping with the standard set out in the Food Chemicals Codex for food enzymes). In addition, the requirements that are generally applicable to edible ices remain in effect. The dossier gives details of the microbiological analysis of ten commercial batches. The ACNFP concludes that the product's microbiological safety has been demonstrated.

The VNV Committee concurs with this conclusion.

Toxicological information

The applicant used a concentrated batch of the ISP preparation for a 90-day oral toxicity study in rats. In this study, the ISP preparation was administered daily, via gavage, at doses containing 58 mg, 290 mg and 580 mg of ISP. The dose groups consisted of 20 male and 20 female rats. Water or citric acid was administered to separate control groups. The study was carried out in accordance with the relevant international standards. No anomalies related to the administration of ISP were found. The applicant concludes that a NOAEL of 580 mg ISP per kg body weight per day can be derived from this work. The estimated maximum intake of 0.38 mg ISP per kg body weight per day for children of four to six years of age represents a safety factor of 1500. It is claimed that, for other groups, this safety factor would be even higher.

The dossier also describes five studies into potential genotoxicity. None of these studies found any evidence of genotoxicity.

The dossier explores the possible allergenicity of the ISP in depth. The initial assessment contains a summary of experimental results from the dossier. Comparisons of the ISP's amino acid sequence with known allergens revealed no similarities. In addition, the ISP was shown to be effectively broken down by pepsin *in vitro*. Studies in test subjects who are allergic to cod revealed that while most exhibited a reaction to the ocean pout, none reacted to the ISP itself. The ACNFP asked the applicant whether individuals who are allergic to yeasts or moulds might exhibit any reactions to the components of the yeast cells used. The applicant took the view that the known allergens of yeast are involved in inhalation

allergy or contact allergy, but not in food allergy. However, the ACNFP felt that the possibility of a risk to individuals with yeast allergy cannot be excluded. It therefore proposed that the label must indicate that the product was derived from yeast. According to the ACNFP, the fact that this yeast was genetically modified should also be stated on the label.

The VNV Committee feels that the toxicological studies carried out for this product were adequate. The Committee doubts whether, from the point of view of safety, it is necessary to indicate on the label that ISP is derived from yeast. The Committee does not express a view about whether the label should indicate that the product was derived from genetic modification. Labelling of this kind is governed by separate legislation, unrelated to the safety assessment in the context of the Regulation concerning novel foods.

Conclusion

The VNV Committee shares the ACNFP's view that the safety of the ISP for the proposed use in edible ices has been adequately demonstrated.

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De commissie / The Committee

- Prof. dr. G.J. Mulder, *voorzitter / chairman*
  emeritus hoogleraar toxicologie, Universiteit Leiden
  professor emeritus toxicology, Leiden University
- Prof. dr. C.A.F.M. Bruijnzeel-Koomen
  hoogleraar dermatologie/allergologie; UMC Utrecht
  professor of dermatology/allergology; University Medical Centre Utrecht
- Ir. E.J. Kok
  toxicoloog; RIKILT Instituut voor Voedselveiligheid, Wageningen
  toxicologist; RIKILT, Institute of Food Safety, Wageningen
- Dr. C.F. van Kreijl
  moleculair-bioloog (gepensioneerd); RIVM Bilthoven
  molecular biologist (retired); National Institute of Public Health and the Environment, Bilthoven
- Dr. F.M. Nagengast
  gastro-enteroloog; UMC St Radboud, Nijmegen
  gastro-enterologist; University Medical Centre St Radboud, Nijmegen
- Dr. ir. J.M.A. van Raaij
  voedingsfysioloog; Wageningen Universiteit en Researchcentrum; RIVM Bilthoven
  food physiologist; Wageningen University and Research Centre; National Institute of Public Health and the Environment, Bilthoven
- Prof. dr. ir. G. Schaafsma
  hoogleraar voeding; Wageningen Universiteit en Researchcentrum
  professor of nutrition; Wageningen University and Research Centre
- Dr. G.J.A. Speijers
  toxicoloog (gepensioneerd); RIVM Bilthoven
  toxicologist (retired); National Institute of Public Health and the Environment, Bilthoven
- Prof. dr. W.J. Stiekema
  hoogleraar bioinformatica; Wageningen Universiteit en Researchcentrum
  professor of bioinformatics; Wageningen University and Research Centre
- Dr. J.W. Tas, *adviseur / advisor*
  Ministerie van VWS, Den Haag
  Ministry of Health, Welfare and Sport, The Hague
- Prof. dr. W.M. de Vos
  hoogleraar microbiologie; Wageningen Universiteit en Researchcentrum
  professor of microbiology; Wageningen University and Research Centre
- Dr. R.A. Woutersen
  toxicoloog, toxicologisch patholoog; TNO Kwaliteit van Leven, Zeist
  toxicologist, toxicologic pathologist; TNO Quality of Life, Zeist

Bureau Nieuwe Voedingsmiddelen, CBG/ Novel Foods Unit, MEB

- Dr. C.M.A. van Rossum, *beoordelaar voedselveiligheid / scientific assessor food safety*
- Dr. M. Rutgers, *beoordelaar voedselveiligheid / scientific assessor food safety*
- Drs. E. van Galen, *hoofd BNV/ head NFU*
- Mr F. Zaidi, *management assistent/ management assistant*

Samenvatting van het dossier / Summary of the dossier

EXECUTIVE SUMMARY

Unilever is seeking approval in Europe under Regulation (EC) N^{o} 258/97 of the European Parliament and of the Council of 27th January 1997 concerning novel foods and novel food ingredients, for the use of a particular type of Ice structuring protein (ISP), Ice Structuring Protein Type III HPLC 12 preparation (ISP Type III HPLC 12 preparation) as a novel food ingredient in the production of edible ices. The term 'edible ices' encompasses ice cream, including dairy ice cream, milk ice, water ice, fruit ice, sorbets, frozen desserts and any similar products such as iced smoothies and products of which edible ice is a component. The ice structuring protein will be used in edible ice at a level not exceeding 0.01% by weight.

Regulatory approval for the use of ISP Type III HPLC 12 preparation has previously been obtained in Australia and New Zealand, Chile, Indonesia, Mexico and the Philippines based on local regulatory procedures. ISP Type III HPLC 12 preparation has been determined to be generally recognized as safe (GRAS) in the United States, and this determination was reviewed and accepted by the US Food and Drug Administration.

Using ISP during the manufacture of edible ices results in a variety of benefits to consumers including improved nutrition profiles (such as the amount of calories, fat, saturated fat, sugars and fruit), organoleptic properties (such as hardness, brittleness or creaminess and enhanced flavour delivery) and also greater temperature stability (an important factor in maintaining good quality products throughout the supply chain.) All of the product properties and benefits are a consequence of the increased connectivity of ice produced in the presence of ISP Type III HPLC 12 preparation.

Ice structuring proteins (ISPs) are naturally occurring proteins and peptides that bind to ice and are found widely in nature, for example, in cold water fish, vegetables, grains, lichens and bacteria. ISPs function to help organisms cope with very cold environments by both lowering the temperature at which ice crystals grow and by modifying the size and shape of the ice crystals that are formed so that the ice is less damaging to tissues.

ISP Type III was originally isolated from the ocean pout (*Macrozoarces americanus*), a cold-water fish found off the northeast coast of North America. This type of ISP consists of 12 isoforms that can be separated by high performance liquid chromatography (HPLC). Isoform HPLC 12 (ISP Type III HPLC 12), composed of 66 common amino acids, was selected for commercial development.

Sourcing ISPs from nature is currently not sustainable or economically feasible. Therefore Unilever has developed a contained-use production system which produces ISP Type III HPLC 12. The production system is based on fermentation using genetically modified baker's yeast which is an established approach common in the production of vitamins and enzymes e.g. chymosin used in making vegetarian cheese. The process includes a purification stage where the yeast cells are removed which yields an ISP Type III HPLC 12 preparation to a stringent specification and means that the ISP Type III HPLC 12 preparation does not contain any residual modified yeast cells.

As indicated, ISPs occur naturally in many foods consumed by man. Based on ISP concentrations in cold-water fish and the landings of such fish, the average consumption of fish ISP in the diet is estimated to be 1-10 mg/day in the USA and 50-500 mg/day in Iceland. Specifically, exposure resulting from consumption of the proposed products would be well within the estimated range of current population exposures to ISPs.

An extensive safety testing programme was undertaken for ISP Type III HPLC 12 preparation. Firstly, the scientific and medical literatures were reviewed to find whether any specific adverse effects had been attributed to ISPs in general, and to gauge population exposure to these proteins. Secondly, as allergenicity is a potential hazard of exposure to proteins, a detailed assessment of the allergenicity of ISP Type III was undertaken. The third element of the safety assessment was an evaluation of the general toxicity and genotoxicity of the ISP Type III HPLC 12 preparation, using standard toxicological methods which complied with OECD guidelines.

Amino acid sequence analysis and susceptibility to proteolytic breakdown were evaluated and neither indicated allergenic potential. The potential of ISP Type III to provoke a reaction in fish-allergic individuals was also tested and ISP Type III did not provoke skin prick test reactions, nor did it bind IgE from fish-allergic individuals. In addition, daily ingestion by volunteers of ISP Type III HPLC 12 preparation for eight weeks failed to generate a detectable immune response. These results demonstrate the safety of ISP Type III to persons allergic to fish, as well as to individuals potentially susceptible to producing IgE responses to proteins (atopic individuals). Based on data and observations outlined, it is concluded that ISP Type III HPLC 12 preparation presents no allergenic risk to fish-allergic individuals or the population at large.

The genotoxic potential of ISP Type III HPLC 12 preparation was assessed by a bacterial mutation assay, an *in vitro* chromosome aberration assay in human peripheral blood lymphocytes, a gene mutation assay in mouse lymphoma L5178Y cells, and an *in vivo* rat bone marrow micronucleus assay. There was no evidence of genotoxic activity in any of these tests.

A 13-week gavage study in rats was conducted to assess the potential for toxicity of ISP Type III HPLC 12 preparation, with the top dose of ISP Type III HPLC 12 being 580 mg/kg body weight/day. Lower doses were one-half and one-tenth the highest dose, by dilution. The results showed no differences between control and test groups in clinical signs, body weights, haematological parameters, clotting potential, in the biochemical composition of the blood, or in organ weights. There were also no macroscopic or microscopic findings due to the effects of the test material.

The highest dose tested in the 13-week rat study, 580 mg ISP Type III HPLC 12/kg body weight/day, was selected as the no observed adverse effect level (NOAEL) because of the lack of toxicity established by detailed observations. This was then used to derive a safe level of intake. A safety factor of 100 was used, based on the totality of analytical, animal, human, and *in vitro* data summarised in this document, general knowledge of proteins, and from the approaches to estimating a safety factor described in published articles. Therefore, a safe level of intake for the preparation was determined as the NOAEL divided by the safety factor, and calculated to be 5.8 mg ISP Type III HPLC 12/kg body weight /day.

The estimated daily intake (EDI) for the group that had the highest estimated edible ice intake in the UK (males aged 11-14 years old at the 97.5th percentile), is 0.21 mg of ISP Type III HPLC 12/kg body weight. This conservatively assumes that all the ice-cream eaten contains ISP at the highest proposed level of use i.e. 0.01% by weight and uses the average bodyweight recorded for this group (NDNS anthropometric data) of 47 kg. This EDI is 28-times less than a conservatively established safe level of intake.

Use of ISP preparation in products is not expected to significantly change population consumption of edible ices, but rather to influence product choice within that market.

In conclusion, available published data, experimental findings and calculations of projected consumption indicate that ISP Type III HPLC 12 preparation is safe for consumers under the intended conditions of use in edible ices.

Eerste beoordeling / First assessment

ADVISORY COMMITTEE ON NOVEL FOODS AND PROCESSES

OPINION ON AN APPLICATION UNDER THE NOVEL FOODS REGULATION FOR ICE STRUCTURING PROTEIN PREPARATION DERIVED FROM FERMENTED GENETICALLY MODIFIED BAKER'S YEAST *Saccharomyces cerevisiae* AS A FOOD INGREDIENT

Applicant:	**Unilever PLC**
Responsible Person:	**Dr George Gordon**
EC Classification:	**5.1**

Introduction

1. An application was submitted by Unilever PLC on 15 June 2006 for the authorisation of an ice structuring protein Type III HPLC 12 preparation derived from a fermented genetically modified baker's yeast as a novel food ingredient. A copy of the application dossier was placed on the FSA website for public consultation.
2. Ice structuring proteins (ISP) are naturally occurring proteins and peptides, which are found in a variety of living organisms such as fish. ISP protect them from damage to tissues in very cold conditions by lowering the temperature at which ice crystals grow and by modifying the size and shape of ice crystals. ISP found in ocean pout[1] are defined as Type I, II, III or IV. Twelve different ISP type III have been identified in the serum of ocean pout using high performance liquid chromatography (ISP Type III HPLC 1-12).
3. The applicant states that sourcing ISP Type III from ocean pout is not sustainable or economically feasible. The applicant has therefore developed a fermentation system using a genetically modified baker's yeast (*Saccharomyces cerevisiae*) carrying a synthetic gene encoding for the ISP Type III HPLC 12.
4. Unilever seeks approval to market its ISP Type III HPLC 12 preparation in edible ices at level not exceeding 0.2%. The presence of the ISP Type III HPLC 12 during the manufacture of frozen products, at the freezing stage, causes ice crystals to form in a particular way so that there are a large number of very small crystals. Normally, in these products, there are a small number of relatively large ice crystals. The continuing presence of the ISP is not necessary for the maintenance of the small crystal size once the product is frozen. Physical interactions between the very small ice crystals provide a

[1]Cold water fish found off the North East American coast (*Macrozoarces americanus)*

structure that differs from conventionally frozen iced products. This effect allows, for example, the production of ice cream with a low fat content.

5. The applicant's ISP Type III HPLC 12 preparation has already been authorised in Australia, New Zealand, Chile, Indonesia, Mexico, the United States and the Philippines under their local regulatory procedures[2]. In the EU, ingredients produced by fermentation using genetically modified micro-organisms, not present in the final product, do not fall under the scope of the regulation 1829/2003 on GM food and feed[3], and this therefore applies to Unilever's ISP preparation as the yeast cells are removed from the final product. In the EU, the proposed ISP Type III HPLC 12 preparation is considered to be a novel food ingredient as it has no significant history of consumption in the EU prior to 15 May 1997. It therefore falls under the scope of the novel food regulation (EC) 258/97 (Article 1(2)(d)). This was confirmed at the Standing Committee on Food Chain and Animal Health meeting of 14 December 2006[4], which concluded that the ISP preparation should be regarded as a novel ingredient and not as a food additive.

6. The application for authorisation of this preparation was prepared pursuant to Commission Recommendation 97/618/EC of 29 July 1997 concerning the scientific aspects and presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients. This preparation has been classified as a product of a GM microorganism, the host microorganism used for the genetic modification having a history of use as food or as a source of food in the Community under comparable conditions of preparation and intake (class 5.1).

7. EFSA have recently published their guidance document on the risk assessment of products derived from genetically modified microorganisms[5] (GMMs). The scope of this document includes food produced using GMMs irrespective of whether or not they fall under regulation 1829/2003. This guidance document describes three distinct groups of genetically modified microorganisms (GMMs). The ISP Type III HPLC 12 preparation would be classed as a group 2 GMM "*Complex products derived from GMMs but not containing viable GMMs nor unit length of any cloned (foreign) open reading frames (e.g. lysed cell*

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[2] See Food Standards Australia New Zealand initial assessment report (October 2004) http://www.foodstandards.gov.au/_srcfiles/A544_ISP_IAR.pdf and a response from the US FDA concerning the manufacturer's determination that the ISP preparation is Generally Regarded as Safe (April 2003) http://www.cfsan.fda.gov/~rdb/opa-g117.html

[3] "The status of food or feed produced by fermentation using genetically modified micro-organisms has to be clarified in the light of the recital no.16 of the Regulation. When the GM micro-organism is used as a processing aid, the food and the feed resulting from such production process are not to be considered as falling under the scope of the Regulation" [Extract of report from the Commission to the Council and the European Parliament on the implementation of Regulation (EC) no 1829/2003 of the European Parliament and of the Council on genetically modified food and feed (October 2006). See under item 10 at: http://eur-lex.europa.eu/LexUriServ/site/en/com/2006/com2006_0626en01.pdf]

[4] See under Item 7 at: http://ec.europa.eu/food/committees/regulatory/scfcah/toxic/summary23_en.pdf

[5] A summary of the information required of applications for the placing of food/feed products derived from GMMs on the market is provided in Section E Table 1 of the guidance document (pp52-58): http://www.efsa.europa.eu/etc/medialib/efsa/science/gmo/gmo_guidance/gmo_guidance_ej374.Par.0001.File.tmp/gmo_guidance_ej374_gmm.pdf

extracts, some feed enzymes, wine, some beers, etc.)" as, although it has been partially purified, the composition of the preparation has not been fully defined. A summary of the information required of applications for the placing of food/feed products derived from GMMs on the market is provided in Annex 1 below, with an indication of the corresponding sections of the application dossier.

I Specifications of the novel ingredient (NI)

Information on this aspect is provided on p. 10-12 of the application dossier

8. The novel food ingredient (NI) is a yeast-derived preparation containing a particular type of ISP known as ISP type III HPLC 12. Isoform HPLC 12 (ISP III-12) is the most functionally active form of type III ISP *in vitro* and is composed of 66 amino acids.
9. The NI is a light brown liquid and consists of ISP III-12 protein, glycosylated ISP III-12, and other components derived from the fermentation (proteins and peptides from yeast, sugars, acids and salt). The concentrate is stabilised with 10mM citric acid buffer.
10. The NI is produced according to Good Manufacturing Practice and the applicant has confirmed the following specification:
    - Assay – Not less than 5g/l active ISP type III HPLC 12
    - pH – 3.0 +/- 0.5
    - Ash – Not more than 2%
    - Heavy metals – Not more than 2mg/l
11. The applicant has provided compositional data on five commercial batches of the NI and data on one batch of concentrated NI in table 2 of the application dossier. Some key parameters from the commercial batches are summarised in the attached table A.
12. The applicant has stated that any variations observed between the batches are due to the concentration step employed during the production. The differences between the quantifiable and total solids are reflective of the cumulative variability inherent in the large number of analytical techniques used for characterisation. The analysis demonstrated a minimum mass balance of 97.9% (w/w) and the applicant has concluded that all batches of the NI were found to be homogenous.
13. The applicant has stated that the NI is stable at -20°C for extended periods without preservatives. The final commercial material will be shipped in frozen sealed containers and the recommended storage time will be 6 months.
14. The applicant was asked to provide further information on the variability between batches of the ISP preparation, including the extent and pattern of glycosylation. The applicant indicated that, compared with other yeast strains, the GM yeast strain used to produce the NI has a limited ability to glycosylate proteins, resulting in 52 to 65% of ISP III-12 proteins in the NI being unglycosylated. The amino acid sequence of the ISP III-12 has 8 theoretical sites for O-glycolysation. The applicant provided liquid chromatographic-mass spectroscopic analytical results on commercial batches of the NI showing that the pattern of glycosylation is constant between batches and has been shown to be unaffected by either process or media changes. The applicant has also

provided results from gel filtration chromatography on commercial batches of the NI indicating that 40% of the total ISP III-12 is glycosylated, of which 75% in glycoform I and 25% in glycoform II. The applicant highlighted that the glycosylated ISP III-12 is inactive and only the non-glycosylated ISP III-12 can bind to ice crystals. The applicant was of the view that the presence of glycosylated ISP III-12 in the NI will not affect its binding properties. In response to a request by the ACNFP, the applicant has also confirmed that the inactive glycosylated form of ISP protein has no function in the preparation. The application draws a parallel with the manufacture of food enzyme preparations, which are generally subjected to minimal processing in order to maintain high functional activity, resulting in varying degrees of purity. The applicant also points out that an extensive test regime has been carried out on the complete ISP preparation to ensure that it is safe for human consumption.

Discussion: *The Committee was satisfied with the applicant's explanation on the homogeneity of the NI (see paragraph 12 above). In response to the Committee's questions about the reason for glycosylated proteins being present in the NI, the applicant explained that these were inactive and that the partial purification process is designed to retain the maximum functional activity in the preparation. The Committee therefore accepted the applicant's proposed specification for the NI. The Committee also noted that the complete ISP preparation has been submitted to toxicological tests to verify that it is safe for human consumption (See section XIII).*

II. Effect of the production process applied to the novel food

Information on this aspect is provided on p. 13-14 of the application dossier

15. The production process involves fermentation with a genetically modified food grade yeast (*S. cerevisiae*) in sealed fermentation vessels (i.e. under contained use conditions). The applicant states that all steps in the production process are commonly used throughout the food industry. The 3 main steps are as follows:

    - Fermentation – the volume is scaled up in stages to the final production volume and protein production is then induced. ISP is secreted into the medium during a controlled phase of slow growth;
    - Cell removal – after fermentation the medium is filtered by microfiltration or filter press leaving a yeast cell free liquid. The yield and purity of the protein is increased by washing the remaining biomass with water;
    - Concentration – cell removal is followed by an ultrafiltration step which retains all material above 1kDa, including ISP which is 6kDa, but removes small molecules. The product is then packaged and stored in frozen sealed containers.

Discussion: *The Committee was satisfied with the applicant's proposed production process for the NI.*

III. History of the organism used as a source of the novel food

Information on this aspect is provided on p. 16 and Appendix 7 of the application dossier

16. The parent organism *Saccharomyces cerevisiae* has been widely used in the food industry for fermentation purposes for a very long period. The specific yeast strain used for production of ISP, a derivative of strain CEN.PK, has been classified in the Netherlands, under Council Directive 90/219/EC[6], as belonging to Group 1 AB. The applicant also noted that commercial production of ISP for markets outside Europe commenced in the second quarter of 2003.

Discussion: *The Committee was content with the information provided on the history of the GM Saccharomyces cerevisiae strain used by the applicant as a source of the NI.*

IV. Effect of the genetic modification on the properties of the host organism

Information on this aspect is provided in Appendix 1 of the application dossier

17. The expression vector contains a synthetic gene coding for ISP type III HPLC 12 originating from ocean pout. This ISP has the same amino acid sequence as the ocean pout ice protein, but the nucleotide sequence has been engineered to reflect optimal codon usage in yeast, thus maximising expression in this host.

18. The vector used to introduce the ISP expression cassette was designed to integrate the expression cassette into the ribosomal DNA (rDNA) of the yeast genome. The resulting yeast strain, CENPK338, contains a multicopy expression cassette inserted at the rDNA locus with no antibiotic resistance markers and no bacterial or fish DNA.

19. The applicant was asked to provide additional data on the molecular characterisation of the insert in the GM yeast. The additional molecular data provided included a more detailed description of the vector, the insertion event and its characterisation. Unlike most eukaryotes, insertion of transformed DNA into the yeast genome occurs through homologous recombination. In addition, the efficiency of targeting is increased in direct proportion to number of copies of the target gene. The integration system used by the applicant exploits these two features of homologous recombination in yeast by targeting the vector to the multicopy ribosomal DNA locus. After targeted integration into the yeast genome the copy number of the expression cassette was increased by selection under growth conditions that favoured yeast cells with multiple copies of the DNA insert.

20. Southern blotting (using a *LEU2 BstE2-EcoRV* DNA fragment as a probe) revealed two bands of the expected size, with a high intensity band of 6.2Kb representing the multicopy expression cassette and a faint band of 2.2Kb representing the chromosomal LEU2 gene. The copy number of the 6.2Kb fragment was estimated at 30-50 copies. The absence of any other bands was interpreted as indicating that the expression cassette was integrated exclusively as tandem repeats. PCR of the flanking sequences using appropriate 5' and 3' primer pairs for the rDNA locus and the insert revealed

[6] Council Directive 90/219/EC of 23 April 1990 on the contained use of genetically modified micro-organisms

bands of roughly the expected size for the 2 flanks; their size and identity was confirmed by cloning and sequencing. The absence of the ampicillin selectable marker was also confirmed by PCR.

21. Following the Committee's consideration of the above information, the applicant was asked to provide further data to demonstrate the absence of secondary integration sites in the genome of the host organism and on the sequence analysis of the flanking regions of the insertion site(s) to check whether this revealed the creation of any potential open reading frames in these regions. The applicant has not found any secondary integration sites in the genome of the *S. cerevisiae* used for producing the ISP preparation. The applicant provided a figure showing restriction maps of the DNA structure generated on integration of the cassette and the fragments detected. Results obtained using five different restriction enzyme digests did not demonstrate the presence of a secondary integration site and the applicant was of the view that it is unlikely that this site would be masked, following this digestion. The applicant concluded that the rDNA locus was the sole location for integration of the expression cassette. Finally, the applicant also highlighted that the mechanism of integration regenerates the existing NTS1 sequence in rDNA, as confirmed by sequencing of boundary fragments. Integration therefore did not lead to generation of any additional open reading frames.

Discussion: *The Committee agreed that the tests carried out by the applicant had confirmed that the inserted DNA had been integrated at the expected site. The Committee was reassured by the further information provided by the applicant which showed that there was only one integration site and that no additional open reading frames were generated.*

V. Genetic stability of the GMO

Information on this aspect is provided in Appendix 1 of the application dossier

22. Strain stability was measured after more than 70 generations of growth under non-selective conditions. The following parameters were compared:

    - Cell viability;
    - Presence of the ISP gene (as detected by PCR);
    - Structure of the integration site (as revealed by Southern blotting);
    - Protein expression levels (under inductive growth conditions).

23. The applicant states that no differences were found for any of the parameters measured after the period of growth used for comparison.

Discussion: *The Committee was content with the information provided by the applicant on the genetic stability of the genetically modified yeast used for the production of the NI.*

VI. Specificity of expression of novel genetic material

Information on this aspect is provided in Appendix 1 of the application dossier

24. Expression of the ISP is under the control of an inducible pGAL7 promoter that only permits high levels of expression of the protein in the presence of

galactose. Expression is repressed during growth in the presence of more than 0.5% glucose.

Discussion: *The Committee noted the above information and did not raise any concerns.*

VII. Transfer of genetic material from GM microorganisms

Information on this aspect is provided in Appendix 1 of the application dossier

25. The applicant has tested the ISP preparation for contamination with DNA derived from the inserted ice structuring protein gene using an ISP gene specific PCR assay. No DNA contamination was detectable using this approach. The detection limit was estimated at 2 x 10^{-10} g ISP plasmid DNA/g of lyophilised ISP protein preparation.

Discussion: *The Committee was satisfied that no DNA derived from the ISP gene inserted in the GM baker's yeast had been detected in the NI, at the limit of detection of the PCR method used.*

VIII. Ability of the GMM to survive in and colonise the human gut

Information on this aspect is provided in Appendix 1 of the application dossier

26. The production process is designed to remove all yeast cells from the ISP preparation and the final product should not contain any GM microorganism that could survive in or colonise the human gut.

Discussion: *The Committee was content that there will be a filtration step within the production process to remove the GM yeast cells. The GMM will therefore not be present in the NI. The Committee noted that yeast proteins will however be present (see section XIII).*

IX. Anticipated intake/extent of use of the novel ingredient

Information on this aspect is provided on p. 16-19 of the application dossier

27. The applicant intends to use the NI in edible ice products to improve their nutrition profiles, organoleptic properties (taste and mouthfeel) and stability. The term "edible ices" encompasses ice cream, including dairy ice cream, milk ice, water ice, fruit ice, sorbets, frozen desserts and similar products such as iced smoothies. The level of ISP will not exceed 0.01% by weight and will more commonly be less than 0.005% in the final product. As the ISP comprises 5-8% of the commercial product, the level of addition of the ISP preparation or the NI will be up to 0.2%.
28. The anticipated intake of ISP Type III HPLC 12 from its use in edible ices has been calculated using the latest UK NDNS data for children, young children and adults. Estimates are for consumers only, which means that only those who have consumed ice cream at some point during the survey period are included.
29. Results are given as daily edible intakes estimated as grams per day. Based on the information given the applicant estimates that boys aged 11-14 have the highest potential intake of edible ice per day with a high level (97.5th

percentile) intake of 99 g/day. Using the maximum proposed level of inclusion of the NI and the average recorded body weight for this group of 47 kg, the estimated daily intake is 0.21mg of ISP type III HPLC 12 /kg body weight. The applicant's estimates for each age group are presented in table B.

30. The NDNS surveys were carried out in 4 waves, covering January to March, April to June, July to September and October to December and the applicant has taken seasonal differences into consideration by providing estimates for each of the waves. This has found that, at the 97.5th percentile, for adults and children, there was only a small difference between the highest and the lowest consumption estimates, suggesting there is little change amongst those who consume edible ices in each season. However, there is a larger difference between the January to March wave and the June to September wave in the survey of young people (ages 4-16), where high level consumption (97.5th percentile) increases from 58 to 80 grams/day.

31. In order to complement the information provided in the original dossier, which gave seasonal data intake estimates for ISP from its use in edible ices only for the combined 4-18 age group, the UK Competent Authority asked the applicant to provide a breakdown of these estimates for the age bands 4-6 years, 7-10, 11-14 and 15-18, using the latest UK NDNS data (see below). The applicant provided the estimates of the daily consumption of edible ices by young people, broken down by both season and age (see Table C).

32. Although these estimates of high level consumption are less robust due to the relatively small number of consumers in each sub-group, the data show that the highest estimated intake, on a body weight basis, is in 4-6 year old children during the summer months (equivalent to 0.38. mg of ISP III-12 per kg bodyweight per day). Although this exceeds the highest estimates mentioned in the application dossier, the applicant points out that there is still a factor of 1500 between this and the NOEL of 5.8 g/kg bw/day observed in the animal feeding studies (expressed as ISP III-12).

33. The applicant has also estimated daily intakes of ice cream for the Netherlands using the 1997-98 Dutch National Food Consumption Survey and for France using CREDOC, Enquête individuelle et nationale sur les consommations alimentaires (INCA, 1999). In the Netherlands, the highest consumption of ice cream is found in adults, where high level consumption (95th centile) is 100g/day[7]. Ice cream consumption recorded in the French survey is lower, with an average value of less than 10 grams/day in all age groups.

Discussion: *The Committee considered that the consumption of the NI at the proposed levels of incorporation on edible ices did not raise any specific concerns.*

[7] Note: The Dutch values are averaged over only 2 days, compared with 7 days in the British surveys (or 4 days for pre-school children). On statistical grounds it is to be expected that the observed high level consumption of most foods will decrease as the survey period increases.

X. Information from previous human exposure to the novel ingredient or its source

Information on this aspect is provided on p.16 of the application dossier

34. ISP occur naturally in the in the blood of fish living in areas where the sea freezes, such as cod and herring, and so are normally consumed in the diet. They are also found in edible plants such as oats, rye, barley, wheat, carrot, potato, taproot and leaves of Brussels sprouts. However, despite their similar functionality, ISP have a range of different structures and it is not possible to draw any meaningful comparisons with the NI.

35. Although ocean pout, the fish from which the ISP that this application refers to was originally isolated, has no history of consumption in the European Community, it is consumed in North Eastern USA. The applicant suggests that eating a 200g portion of ocean pout would result in an intake between 120 and 420 mg of ISP type III. This is higher than the estimated daily intake from edible ices (see above).

36. In addition to the ISP and its glycosylated counterpart, the NI contains other components derived from the fermentation. *Saccharomyces cerevisiae* has a very long history of use in food production and there is therefore a long history of consumption of the yeast itself and its fermentation products.

37. The applicant states that edible ices containing the NI have been on the market in the USA since the second quarter of 2003 with no reported consumer issues. Similar products have also been on sale in other countries such as the Philippines since 2004.

Discussion: *The Committee was content with the information provided on previous human exposure to the NI and its yeast source.*

XI Nutritional information on the NF

Information on this aspect is provided on p.21 of the application dossier

38. As the NI will be used in edible ices at a level not exceeding 0.2% by weight (equivalent to 0.01% of the ISP component), the applicant has stated that no nutritional implications are expected. The NI's protein sequence is comprised of amino acids which are commonly found in the human diet and for this reason it would be digested as a protein according to normal metabolic processes and will not have any significant effect on total protein intake. The NI would not displace existing ingredients, although its use might facilitate the manufacture of ice cream products with as reduced fat content.

Discussion: *The Committee was satisfied with the nutritional information provided for the NI.*

XII. Microbiological information on the novel food

Information on this aspect is provided on p.21-22 of the application dossier

39. The microbiological specification for the NI is as follows:

Total microbial count	<3000/g
Coliforms	<10/g
Listeria spp.	Absent in 25g

Salmonella spp.	Absent in 25g
Yeast and mould count	<100/g (GM yeast absent by test)
Staphylococcus aureus	<10/g
Bacillus cereus	<100/g

40. Table 7 in the dossier summarises the microbiological analysis of 10 commercial batches of the NI.

41. The microbiological safety of the edible ices containing the NI will be ensured by using the accepted principles of good manufacturing practice and conditions for processing and distribution currently applied to edible ices. The applicant considers that no additional controls will be necessary.

Discussion: *The Committee was of the view that the microbiological safety of the NI had been demonstrated.*

XIII. Toxicological information on the novel food

Information on this aspect is provided on p.23-71 of the application dossier

42. The applicant has carried out an evaluation of the general toxicity and genotoxicity of the NI. The potential allergenicity of the NI was also assessed.

(a) Toxicological and genotoxicological assessments

Information on this aspect is provided on p.23-30, Appendices 6 and 10-15 of the application dossier

43. The applicant has provided details of a number of toxicological and genotoxicological studies carried out on the NI. The results of these studies are presented in the attached Table D. To increase their sensitivity these tests were conducted on a specially prepared batch of the NI, designated 201008, which was subjected to an additional concentration stage using ultrafiltration to remove excess water and low molecular weight components. The applicant was asked to provide additional information on batch 201008 of the NI regarding the way it was prepared and how its composition compares with the commercial product. The applicant explained that the final stage of the production process of the NI involves ultrafiltration with Synder spiral wound membrane modules (1 kDa). Batch 201008 is obtained by continuing the ultrafiltration for longer to obtain 30g/L of ISP III-12. Compositional comparison of batch 201008 with other batches of the NI is provided in table 2 of the dossier. The applicant has confirmed that the additional ultrafiltration does not modify the NI, as shown in study report AC000082 (Appendix 4 of the dossier).

44. The applicant concludes that the NI does not present any toxicological or genotoxicological potential.

(b) Allergenicity assessment

Information on this aspect is provided on p.30-66, Appendices 17-20 (study reports), Appendices 20 and 22 (publications)

45. A summary of the tests assessing the potential allergenicity of the NI is given in table 9 (Annex 1, p.31). The results of these tests are summarised in the attached Table E.

46. The applicant concludes that the NI is safe for both fish-allergic individuals and other consumers.

47. The applicant was asked to provide further details on the amino acid sequence analysis of ISP III-12. The applicant has therefore explained that the original amino acid sequence analysis of ISP III-12 against public protein databases was carried out in 2001/02. This analysis generated some false positives and was not repeated. In 2005, the amino acid sequence of ISP III-12 analysis was analysed again using a customised allergen database (FARRP AllergenOnline database) and a general protein database (NCBI non-redundant database). This analysis did not reveal any significant sequence alignment with known allergenic proteins. The applicant concluded that ISP III-12 is unlikely to be a food allergen.

48. The applicant was also asked whether any information exists on the potential for the GM *Saccharomyces cerevisiae* proteins present in the preparation to induce allergic reactions in individuals sensitised to *Candida* 'yeast' or other fungi. The applicant indicated that sensitisation to yeast proteins most occurs via the respiratory tract and via the skin, and there is no evidence to indicate that it arises from the consumption of foods and drinks containing *S. cerevisiae*. This conclusion is supported by the fact that the three allergens in *S. cerevisiae* namely enolase, manganese super-oxide dismutase and cyclophin have only been associated with inhalant and/or skin allergies. It is recognised that people with atopic dermatitis which is associated with allergic reaction to yeasts such as *Candida albicans*, *Pytirisporum ovale* and *Malassezia furfur* are likely to cross-react to S. cerevisiae proteins when challenged in skin prick tests or RASTs. The applicant however refered to conclusions from Kortekangas-Savolainen et al (1994) that "the IGE-mediated allergy to baker's yeast should not lead to the denial of bakery, brewery and wine products".

(c) Potential yeast (Saccharomyces cerevisiae) allergenicity assessment
Information on this aspect is provided on p.48-51 and table 16 of the application dossier

49. The applicant notes that three proteins identified in Saccharomyces cerevisiae are associated with inhalant and/or skin allergies and adds that "all the fish allergic subjects who were skin prick test positive to yeast in the above studies are able to consume foods containing yeast without adverse reaction" (see table 16, p.51). The applicant is therefore of the opinion that the yeast component of the NI does not pose a clinically significant allergic risk.

Discussion: *The Committee was satisfied with the toxicological assessment carried out by the applicant on the NI which showed that it is safe for human consumption at the proposed level of use. The Committee particularly discussed the following points:*

- ***Inflammatory potential of the NI*** *– during our public consultation on this application, a member of the public suggested a need to conduct studies to test long-term inflammation in both young and older animals. The Committee asked for expert advice on this point from specialists in animal pathology and immunology of the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment. They were of the view that the applicant had carried out all the appropriate studies needed to assess potential immunogenicity. The 90-day study did not show any indication of any effects on the immune system, whether inhibitory or stimulatory, and there were no clinical signs of inflammatory responses that*

might justify further investigations in this area. The Committee therefore concluded that the NI did not have any inflammatory potential.

- ***Animal models of sensitisation*** *- The Committee was of the opinion that animal models of sensitisation had improved in recent years and that the quote in the dossier from the 2001 WHO Rome conference to the effect that, "such models were at too early a stage of development to generate data for risk assessment", was no longer valid. The Committee discussed the value of having a study of sensitisation using the ISP preparation on an appropriate animal model and concluded that, in this case, this additional information was not necessary.*

- ***Amino acid sequence homology of ISP Type III-12 with A.niger superoxide dismutase -*** *The Committee noted that reference in the application to Baderschneider et al's (2002) findings that a match over a very short part of the amino acid sequences between a superoxide dismutase (allergen Asp f6) from Aspergillus fumigatus and ISP Type III was not significant and the Committee asked for an external expert view on that point. The expert's view was that the similarity between ISP type III and AspF6 was very low and it is very unlikely that Aspergillus allergic individuals will react to ISP. The Committee therefore concluded that the NI will not induce a reaction in Aspergillus allergic individuals*

- *.**Fish allergy –*** *The Committee queried whether the results from tests on cod-allergic people were sufficiently representative of fish allergy in general. The Food Standards Agency's allergy experts indicated that sera from cod allergic subjects are considered to be a relatively good candidate for assessment of whether the NI is likely to bind IgE of fish allergies. This is because of the high homology of parvalbumin (the major fish allergen) across fish species. Fish allergic individuals can be mono-sensitised to other non-parvalbumin allergens in fish, such as collagen, but this is relatively rare and it seems unlikely, although not impossible, that the non-parvalbumin allergens would cross-react with the ISP. Further, as cod allergic subjects, many of which also showed positive SPTs to ocean pout, eel pout and eel (indicating cross-reactivity among these species), did not react to the ISP preparation it seems unnecessary to extend the tests to subjects with other fish allergies besides cod. The Committee concluded that using the cod allergic individuals in Phase I of the assessment of the allergic potential of the ISP preparation is representative of the fish allergic population.*

- ***Yeast allergy*** *- The Committee did not agree with the applicant that the yeast proteins present in the ISP preparation did not present any potential allergenic risk and recommended that the labels of products containing the ISP preparation should indicate that it is derived from a yeast source.*

Labelling
Information on this aspect is provided on p.11 of the application dossier

50. The applicant proposes to describe the NI as “Ice Structuring Protein” in the list of ingredients of edible ices, consistent with other ISP-containing products on the market outside the EU.

Discussion: *The Committee considered whether the NI should be labelled as derived from a GM source. It was stated in a recent Commission report*[8] *that ingredients produced by fermentation using genetically modified micro-organisms not present in the final product do not fall under the scope of legislation on GM food and therefore do not need to be labelled as GM under this specific piece of legislation. This conclusion is shared by the Food Standards Agency and by the responsible authorities in other Member States and it can be applied to the NI, as the yeast cells are removed from the final product.*

The Committee was aware that other food ingredients derived from GM micro-organisms, such as enzymes used as processing aids and some highly-refined vitamins and amino acids, are not labelled to indicate their source. Nevertheless, the use of a synthetic gene sequence and the presence in the NI of a significant proportion of cellular by-products from the fermentation process such as yeast proteins (as noted in Section VIII above) made this a special case and the committee felt that the omission of this information through the absence of labelling could be potentially misleading to consumers.

The Committee therefore recommended that information should be provided to consumers indicating that the ingredient is manufactured using a GM yeast. This could be achieved either through information provided on food packaging or possibly via other easily-accessible routes, for example, via a reference to a website and the manufacturer's telephone careline.

The Committee noted that there was some misunderstanding among members of the public as to the source of the NI. Some think it to be a fish product that is therefore unsuitable for fish allergic individuals and vegetarians. The NI is not extracted from fish but from a non-animal source (yeast), which has been genetically modified by inserting a synthetic gene that provides a “blueprint” for a protein that has the same structure as one that is found in a type of fish. This confusion highlights the need for clear information about the NI to be made available to the public.

OVERALL DISCUSSION

51. The information supplied by the applicant offers sufficient reassurance that the consumption of the NI in edible ices does not give rise to any toxicological or allergenic concerns, other than those associated with the presence of yeast proteins.

[8] Section 10 of the Report from the Commission to the Council and the European Parliament on the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council on genetically modified food and feed, COM(2006) 626, October 2006. http://eurlex.europa.eu/LexUriServ/site/en/com/2006/com2006_0626en01.pdf

52. Regarding the labelling of the product, the applicant needs to comply with the Food Labelling Regulations 1996 (as amended). They should ensure that the labelling and presentation of the products adequately informs the consumer, particularly in relation to its consumption by yeast allergic individuals.

CONCLUSION

53. The Advisory Committee on Novel Foods and Processes is satisfied by the evidence provided by Unilever that the range of uses for its ice structuring protein preparation is acceptable, subject to the applicant's adherence to the proposed specification and the production parameters described above. The Committee recommends that products containing the ingredient should be labelled to indicate that it is derived from yeast. In order that consumers should be adequately informed and are not misled, the Committee also recommends that information should be provided to consumers in an easily-accessible format indicating that the ingredient is manufactured using a GM yeast.

July 2007

Annex 1

Comparison of requirements described in EFSA guidance on the risk assessment of genetically modified microorganisms (May 2006) against the data provided in the application dossier

A summary of the information required of applications for the placing of food/feed products derived from GMMs on the market is provided in Table 1 of the guidance document (pp52-58). The main categories are:

Category described in the EFSA guidance document	Corresponding section of the application dossier (see ACNFP/78/2)
Characteristics of the recipient or parental microorganism	Section III: History of the organism used as the source of the NF
Characteristics of the donor organism	Section X: Information from previous human exposure to the NF or its source (*Note: the ISP gene introduced into the production organism is synthetic, designed to code for the same protein that is found in fish)*
Description of the genetic modification process	Section IV: Effect of the genetic modification on the properties of the host organism
Information relating to the GMM and comparison of the GMM with its conventional counterpart	Section IV: Effect of the genetic modification on the properties of the host organism Section V: Genetic stability of the GMO Section VI: Specificity of expression of novel genetic material *(comparison between the GMM and its conventional counterpart is not applicable as there is no equivalent product from non-GM yeast)*
Information relating to the production process	Section II: Effect of the production process applied to the NF
Information relating to the production purification process	Section II: Effect of the production process applied to the NF
Description of the product	Section I: Specification of the NF Section XI: Microbiological information on the NF
Assessment of the presence of recombinant DNA and of the potential risk of gene transfer	Section VII: Transfer of genetic material from GM microorganisms Section VIII: Ability to survive in and colonise the human gut
Comparison of the GM product with its conventional counterpart	(not applicable as there is no conventional counterpart)
Considerations for human health and animal health of the GM product (including toxicity and allergenicity)	Section IX: Anticipated intake/extent of use of the NF Section XI: Nutritional information on the NF Section XIII: Toxicological information on the NF

Tables

Table A: Composition of batches of the commercial the ISP preparation

Batch	200030	200034	200046	201024	201083
Total protein (g/litre)	15.0	14.3	16.4	23.7	31.5
ISP III-12 (g/litre)	5.5	4.8	5.0	6.2	8.4
Protein breakdown (% of total)					
ISP III-12	*36%*	*34%*	*31%*	*27%*	*27%*
glycosylated ISP III-12	*22%*	*18%*	*20%*	*23%*	*25%*
yeast protein	*23%*	*24%*	*22%*	*29%*	*32%*
peptides	*20%*	*24%*	*28%*	*22%*	*17%*
Total solids (g/litre)	34.5	41.0	39.7	73.0	77.7
Unquantified solids* (g/litre)	3.0	10.0	6.4	20.6	8.4
Unquantified solids (% of total)	*9%*	*24%*	*16%*	*28%*	*11%*

* Quantified solids = Total Kjeldahl protein + mannose + citric acid + minerals (Na, K, Mg, Ca, PO_4)

Table B: Consumption of edible ices by British consumers

	Consumption of edible ices recorded in NDNS surveys (grams / day)					
Age	1.5-4.5	4-6	7-10	11-14	15-18	19-64
Proportion of consumers	42.9%	61.1%	59.3%	49.6%	35.7%	27.3%
Median (M / F)	16 / 15	17 / 14	18 / 17	22 / 18	16 / 13	17 / 17
High level (97.5th centile) M/F	62 / 64	59 / 73	63 / 64	99 / 76	83 / 71	78 / 73

Table C: Consumption of edible ices by British children, by season

			Centiles of consumption of edible ices recorded in 1997 NDNS survey (grams / day)					
Age groups	Wave	N	5^{th}	10^{th}	50^{th}	90^{th}	95^{th}	97.5^{th}
4-6 yrs	1	42	2.14	6.43	14.86	31.14	32.29	39.71
	2	57	4.29	5.00	15.00	40.43	57.00	57.14
	3	61	5.43	7.57	16.57	38.43	59.43	76.29
	4	57	5.16	7.14	16.86	36.43	45.43	45.57
7-10 yrs	1	45	5.00	5.43	17.14	43.00	50.43	51.71
	2	72	6.86	8.57	20.50	42.00	60.00	63.00
	3	102	7.57	8.57	21.07	51.86	63.00	77.71
	4	68	7.00	8.00	15.86	33.90	45.43	58.43
11-14 yrs	1	44	4.29	4.71	17.93	50.71	58.29	62.29
	2	70	4.86	6.93	17.71	51.71	61.71	84.57
	3	73	7.29	9.14	21.57	58.57	80.86	83.14
	4	45	4.86	5.43	16.71	43.14	49.29	60.00
15-18 yrs	1	26	5.00	6.86	12.43	33.00	35.14	60.57
	2	27	5.66	5.71	17.00	54.86	62.43	80.86
	3	55	5.71	7.14	15.43	45.86	70.86	83.00
	4	31	6.00	7.00	15.71	39.29	77.86	85.57

Wave 1: Jan – Mar, Wave 2: Apr – Jun , Wave 3: July – Sep and Wave 4: Oct – Dec

Table D: Toxicological and genotoxicological studies on the novel ingredient

Appendix to application dossier	Test material	Tests	Result
Appendix 9	ISP Type III HPLC 12 preparation Batch 201008[(1)]	90-day sub-chronic oral toxicity test in rats, at doses equivalent to 58, 290 and 580 mg ISP per kg bodyweight per day	NOAEL is 580mg ISP Type III HPLC 12/kg bw/day, equivalent to 6.9 – 12.1g of the NI/kg bw/day [(2)]
Appendix 11	ISP Type III HPLC 12 preparation Batch 201008 FD [(3)]	Bacterial reverse mutation assay using 4 strains of *Salmonella typhimurium*	Negative on 3 strains False-positive on 1 strain due to contamination with other microorgnisms
Appendix 12	ISP Type III HPLC 12 preparation Batch 2010034	Bacterial reverse mutation assay using 4 strains of *Salmonella typhimurium*	Negative on all strains
Appendix 13	ISP Type III HPLC 12 preparation Batch 201008 FD [(3)]	*In vitro* chromosome aberration assay in human peripheral blood lymphocytes	Negative
Appendix 14	ISP Type III HPLC 12 preparation Batch 201008 FD [(3)]	Gene mutation assay at the thymidine kinase locus of mouse lymphoma L5178Y cells	Negative
Appendix 15	ISP Type III HPLC 12 preparation Batch 201008 FD [(3)]	*In vivo* rat bone marrow micronucleus assay	Negative
Appendix 16	AFP III HPLC 12 preparation[(4)]	Randomised placebo controlled human trial to evaluate single ingestion	No toxicity detected

[(1)] Batch 201008 is a concentrated form (~5-fold) of the commercial preparation. Compositional data for batch 201008 and for 5 standard commercial batches of the NI can be found in the application dossier, Table 2, p.12.

[(2)] Calculation of the NOAEL expressed as NI containing between 4.8% and 8.4% of ISP Type III HPLC 12 (application dossier, Table 2, p12).

[(3)] Batch 201008 was too dilute for use in genotoxicity and was therefore freeze-dried to obtain ISP Type III HPLC 12 preparation Batch 201008 FD. No difference in composition, except for the water content, was observed between these two batches (application dossier, Appendix 5)

[(4)] The applicant has explained that "Anti- Freeze Protein (AFP) III HPLC 12" is used in this study report as an alternative name for ISP Type III HPLC 12 (application dossier, p.6)

Table E: Tests on the potential allergenicity of the novel ingredient

Dossier reference	Test material	Tests	Result
Appendix 21	-	Amino acid sequence analysis using BLAST and FASTA computer programmes	No primary sequence similarity with any known allergens, including fish allergens
Appendix 18 Figures 7-9 Table 22	ISP Type III HPLC 12 preparation	Pepsin hydrolysis resistance	Most of the peptides <2.3kD Low probability that ISP Type III HPLC 12 could elicit reaction

(a) Phase I Studies in 20 fish allergic individuals (cod)

-	Eel, eel pout, ocean pout	Skin prick testing	Positive
Table 13 Figure 4	Ocean pout extract (2 mg protein/mL) Freeze-dried ISP III HPLC 12 preparation (20 ng/mL to 200μg/mL)	IgE binding *in vitro* – RAST inhibition	18/20 subjects had IgE against ocean pout No binding of IgE to freeze-dried ISP preparation
Table 13	Nine different concentrations (3.5-fold dilutions) of ocean pout extract (max = 0.2mg/mL) and freeze-dried ISP III HPLC 12 preparation (max = 10 mg/mL)	IgE binding in vitro – Basophil histamine release	Positive for ocean pout extract Negative for freeze-dried ISP III HPLC 12 preparation

(b) Phase II Studies in 22 fish allergic individuals

Table 14	ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III Pure ISP Type III HPLC 12 preparation (no yeast proteins)	Skin prick testing	4 subjects reacted to both test materials These 4 subjects did not react to pure ISP Type III HPLC 12 preparation (no yeast proteins)
Tables 15 and 16 Figure 5	ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III Pure ISP Type III HPLC 12 preparation (no yeast proteins)	IgE binding *in vitro* – RAST inhibition	8 subjects were positive to ISP Type III HPLC 12 preparation including yeast protein. These 8 subjects included 3 of the 4 subjects who reacted positive to the skin prick testing. All 8 subjects did not react to pure ISP Type III HPLC 12 preparation (no yeast proteins) No binding of IgE to freeze-dried ISP preparation
-	ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III Pure ISP Type III HPLC 12 preparation (no yeast proteins)	IgE binding *in vitro* – *Basophil histamine release* to investigate positive skin prick test results	Positive for both materials Negative on pure ISP Type III HPLC 12 preparation (no yeast proteins)

(c) General allergy testing on 28 healthy adults

Table 17	ISP Type III HPLC 12 preparation	Antibody response to ingestion on 28 healthy adults without a history of previous consumption of ISP Type III with 8 controls	No observed clinical symptoms or biochemical changes associated with food allergy
Table 18	ISP Type III HPLC 12 preparation	Enzyme-linked immunosorbent assay (ELISA)	Negative
-	ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III Pure ISP Type III HPLC 12 preparation (no yeast proteins)	Skin prick testing	1 subject positive to both materials but negative on pure ISP Type III HPLC 12 preparation (no yeast proteins)
Table 19	ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III	IgE binding *in vitro* – RAST inhibition	Weak specific IgE response (peaking at week 4)
Table 20	ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III	IgE binding in vitro – Basophil histamine release	Negative
Figure 6	ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III	IgE binding *in vitro* – immunoblotting	Negative