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(54) Title: PROCESS FOR PREPARING AN OIL-IN-WATER EMULSIFIED FOOD PRODUCT

(57) Abstract: The present invention relates to a process for preparing an oil-in-water emulsified food product having a pH of less than 7, comprising incubating egg yolk in the presence of an enzyme having phospholipase C activity to produce an egg yolk composition, mixing the egg yolk composition with oil and water, and preparing the oil-in-water emulsified food product. The invention further relates to the use of an egg yolk composition which has been treated with an enzyme having phospholipase C activity, to increase firmness of an oil-in-water emulsified food product



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PROCESS FOR PREPARING AN OIL-IN-WATER EMULSIFIED FOOD PRODUCT

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The present invention relates to a process for preparing an oil-in-water emulsified food product wherein an egg yolk composition is used and the use of an egg yolk composition to increase the firmness of an oil-in-water emulsified food product.

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Background

Eggs are widely used in food products. For instance in oil-in-water emulsified foods like mayonnaise, emulsified sauces and (salad) dressings, egg yolk or whole eggs are used because of its emulsifying and/or flavor enhancing properties. Mayonnaise typically contains about 65-70% of vegetable oil and at least 5 % egg yolk.

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In the last decades there has been an increasing need by consumers to reduce the calorie intake of foods and reduce the intake of cholesterol. Many studies have been performed to lower the content of eggs in oil-in-water emulsified food products, thereby lowering the cholesterol content of the food product. Reduction of the oil content will largely reduce the calorie content of the food product, as oil or fat is on weight base the highest contributor to the calorie content of a food product. However, the reduction of egg or oil can negatively influence the flavor, mouthfeel properties and/or functionality of in oil-in-water emulsified foods.

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A known method to increase the emulsifying properties of egg-yolk is by enzymatically treating the egg yolk with phospholipase A, such as phospholipase A1 or phospholipase A2, which converts phospholipids into lysophospholipids, for instance disclosed in US 4,034,124.

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In US6,660,312 B2 a method was developed to lower the cholesterol content of egg-yolk, while maintaining a high emulsion stability. Egg yolk was treated with phospholipase A2 and subsequently the egg yolk was treated with supercritical carbon dioxide. A disadvantage of this method is that it is a laborious and costly way of egg processing.

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EP2517578 discloses a method for the preparation of an egg composition wherein simultaneously a phospholipase A1 and a protease is used. The egg composition prepared this way exhibited improved functionality and viscosity, which permitted the use

of reduced amounts of the enzyme-treated egg yolk in the preparation of food products. A disadvantage of using a protease is that it is difficult to stop the proteolytic activity of the protease, which can result in small peptides with a bitter taste, and small peptides may negatively influence the final consistency of an emulsion.

5 Alternatively, CN102960770 discloses a method for preparing instant egg yolk powder wherein an egg yolk liquid is treated with phospholipase C. The egg-yolk liquid treated with phospholipase C exhibited an improved dispersibility in water, an increased thermal stability and good emulsion stability. CN102960770 does not relate to treating egg-yolk with phospholipase C to increase the firmness of an oil-in-water emulsified food
10 such as mayonnaise.

The aim of the present invention is an improved process for preparing an oil-in-water emulsified food product.

Summary

15 In one aspect the present invention relates to a process for preparing an oil-in-water emulsified food product having a pH value of less than 7, comprising incubating egg yolk in the presence of an enzyme having phospholipase C activity to produce an egg yolk composition, mixing the egg yolk composition with oil and water, and preparing the oil-in-water emulsified food product.

20 In another aspect the present invention relates to the use of an egg yolk composition treated with an enzyme having phospholipase C activity for increasing firmness of an oil-in-water emulsified food, and an oil-in-water emulsified food product obtainable by a process as disclosed herein.

In yet another aspect the present invention relates to a method for increasing
25 firmness of an egg yolk composition, comprising incubating egg yolk in the presence of an enzyme having phospholipase C activity to obtained increased firmness of the egg yolk composition. Surprisingly it was found that egg-yolk treated with phospholipase C was much firmer than egg-yolk treated with phospholipase A. It was found that an egg yolk composition as disclosed herein can be used oil-in-water emulsified food, such as
30 mayonnaise, emulsified sauces, dressings, and desserts with a lower oil-content.

Detailed description

In one aspect the present invention relates to a process for preparing an oil-in-water emulsified food product having a pH value of less than 7, comprising incubating

egg yolk in the presence of an enzyme having phospholipase C activity to produce an egg yolk composition, mixing the egg yolk composition with oil and water, and preparing the oil-in-water emulsified food product.

Incubating egg yolk in the presence of an enzyme having phospholipase C activity may be performed in any suitable way. Incubating is performed such that an enzyme having phospholipase C activity exhibits activity, as represented by the hydrolysis of phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) into diglyceride and phosphocholine and phosphoethanolamine respectively. Diglycerides are also known as diacylglycerides.

The wording "egg yolk" as disclosed herein refers to egg yolk or whole egg or any composition comprising egg yolk. Egg yolk as disclosed herein may be in a liquid form.

The egg yolk in a process or method as disclosed herein may be incubated with an enzyme having phospholipase C activity at any suitable temperature. The egg yolk may be incubated with an enzyme having phospholipase C activity at a temperature of between 5 and 65°C, such as between 10 and 60 °C, or between 20 and 55 °C.

Preferably, the firmness of the oil-in-water emulsified food product is increased in a process for preparing the oil-in-water emulsified food product. Accordingly, a process for preparing an oil-in-water emulsified food product may be a process comprising increasing firmness of an oil-in-water emulsified food product.

Firmness as used herein can be measured as compression in a texture analyser, for instance as disclosed in Dunnewind et al, (2004) J. of Texture Studies, 35, 603-620. Alternatively, firmness can be determined by viscometry, for instance by a Brookfield viscosimeter.

As defined herein the firmness of an oil-in-water emulsified food product prepared in a process as disclosed herein is increased as compared to an oil-in-water emulsified food product which is prepared with egg yolk that was not prepared with an enzyme having phospholipase C activity. Surprisingly it was found that an oil in water emulsified food product which was prepared with a phospholipase C treated egg yolk composition had an increased firmness as compared to a further identical oil-in-water emulsified food product that was prepared with egg yolk that had been treated with phospholipase A.

An oil-in-water emulsified food product has a pH of less than 7, such as a pH of between 2 and 6.5 or a pH of between 2.5 and 6, or a pH of between 3 and 5.5, or a pH of between 3.5 and 5, or a pH of between 4 and 4.5. An oil-in-water emulsified food

product having a pH of less than 7 has a better taste and microbial stability than an oil-in-water emulsified food product having a pH of 7 or higher.

A process according to the present invention may further comprise diluting the egg yolk or egg yolk composition with an aqueous solution. An aqueous solution can be water, a salt (NaCl) solution in water, a sugar solution in water or any other suitable aqueous solution, for instance water used in an oil-in-water emulsified food product. Diluting egg yolk of an egg yolk composition with the aqueous solution may be performed in any suitable way such as stirring with a spoon, a blender or a mixer.

Diluting the egg yolk or egg yolk composition with an aqueous solution can be performed at a ratio egg yolk (composition) to aqueous solution of 10:1 to 1:1, such as a ratio of 8:1 to 1:1, or a ratio of 5:1 to 1:1, or a ratio of 3:1 to 1:1 or a ratio of 2:1 to 1:1. Diluting egg yolk may be performed prior to incubating with phospholipase C or diluting the egg yolk composition may be performed after incubating with phospholipase C. Advantageously the processability of the egg yolk composition in a process or method as disclosed herein is improved after diluting the egg yolk or egg yolk composition with an aqueous solution.

The oil in the oil-in-water emulsified food product can be any type of food grade oil or fat from vegetable, algal or animal origin, for instance liquid oils such as sunflower oil, soy bean oil, rapeseed oil, canola oil or olive oil or any combination thereof.

Other ingredients for preparing an oil-in-water emulsified food product may be any suitable ingredient for preparing the oil-in-water emulsified food product, for instance sugar, salt, mustard, and / or vinegar. In addition gums, thickener hydrocolloids, native starches or modified starches, tomato-derived products, dairy derived products such as cream, dairy protein, buttermilk, or plant protein compositions as well as aromas, herbs, spices and antioxidants can be used to prepare an oil-in-water emulsified food product. Mixing an egg yolk composition with other ingredients in a process for preparing an oil-in-water food product as disclosed herein may be performed in any suitable way known to a skilled person in the art. For instance, such other ingredients may be added before, during or after mixing an egg yolk composition with oil and water.

An oil-in-water emulsified food may comprise any suitable amount of oil, usually between 5 and 80 wt/wt% of oil, such as between 10 and 75 wt/wt% oil, or between 20 and 70 wt/wt% of oil, or between 30 and 65 wt/wt% oil. Surprisingly, it was found that

also at lower oil contents the firmness of an oil-in-water emulsified food product produced in a process as disclosed herein was increased as compared to an oil-in-water emulsion that was prepared with untreated egg yolk.

An oil-in-water emulsified food product as disclosed herein may comprise an amount of 0.5 to 7 wt/wt% dry weight of egg yolk composition, such as an amount of 1 to 5 wt/wt% dry weight, or 1.5 to 4.5 wt/wt% dry weight, or 2 to 4 wt/wt% dry weight or 2.5 to 3.5 wt/wt% dry weight of egg yolk composition. The wt/wt% of egg yolk is defined as the weight of egg yolk relative to the total weight of the oil-water emulsified food product. Usually a standard liquid egg yolk contains approximately 50% dry matter. Accordingly, a process for preparing an oil-in-water emulsified food product may comprise a step of mixing the amounts of egg yolk composition indicated above with water and mixing in oil to prepare the oil-in-water emulsified food product. Optionally, other ingredients such as vinegar and/or mustard are added to the oil-in-water emulsified food.

The present invention also relates to an oil-in-water emulsified food product, obtainable by a process as disclosed herein.

An oil-in-water emulsified food product as disclosed herein may be a mayonnaise, an emulsified sauce (for cold and / or warm applications), a dressing or a dessert.

In another aspect the present invention relates to the use of an egg yolk composition treated with an enzyme having phospholipase C activity for increasing firmness of an oil-in-water emulsified food.

An enzyme which has phospholipase C activity in a process, use, or method disclosed herein, typically a phospholipase C, hydrolyses phospholipids just before the phosphate group releasing diacylglycerol (DAG) and a phosphate-containing head group. A phospholipase C as used herein may belong to enzyme classification EC 3.1.4.3, E.C. 3.1.4.11 (phosphoinositide phospholipase C), E.C. 3.1.4.12 (sphingomyelin phosphodiesterase), preferably a phospholipase C belongs to enzyme classification EC 3.1.4.3. Advantageously, an enzyme having phospholipase C activity in a method according to the present disclosure hydrolyses between 50 and 99%, such as between 60 and 98%, such as between 70 and 95%, such as between 80 and 90% of phospholipids present in egg yolk into diglyceride (1,2-diacyl-sn-glycerol) and phospho-choline (or choline phosphate) and phospho-ethanolamine (ethanolamine phosphate). It was surprisingly found that an egg yolk composition that has been incubated with an enzyme

having phospholipase C activity exhibited increased firmness and that an oil-in-water emulsified food prepared with the phospholipase C treated egg yolk composition was a stable emulsion and exhibited an increased firmness as compared to an oil-in-water emulsified food product prepared with egg-yolk that had not been treated with phospholipase C.

In one embodiment in a process, use or method as disclosed herein, egg yolk can be incubated with phospholipase C and another enzyme such as phospholipase A, for instance phospholipase A1 or A2 (E.C. 3.1.1.32 or E.C. 3.1.1.4, respectively). Accordingly, a process for preparing an oil-in-water emulsified food product having a pH of less than 7 as disclosed herein, may comprise incubating egg yolk in the presence of an enzyme having phospholipase C activity and an enzyme having phospholipase A activity to produce an egg yolk composition, mixing the egg yolk composition with oil and water, and preparing the oil-in-water emulsified food product. It was found that the processibility of an egg yolk composition that was treated with phospholipase C was improved when treated with phospholipase A, such as phospholipase A1 and / or phospholipase A2.

Any suitable enzyme having phospholipase C activity may be used in a process, use, or method as disclosed herein. An enzyme having phospholipase C activity, typically a phospholipase C, may be derived from any suitable organism for instance bacteria such a *Bacillus* sp. for instance *B. licheniformis*, *B. megaterium*, *B. subtilis*, *B. cereus*, *Pseudomonas* sp., or fungi, such a *Penicillium* sp. eg. *P. emersonii*, *Aspergillus*, eg. *A. niger* or *A. oryzae*, or from *Kinochaeta* sp.. A phospholipase C may for instance be an enzyme having an amino acid sequence according to SEQ ID NO: 1 disclosed in WO2003/089620, or SEQ ID NO: 175 having at least a mutation at position 63, 131 and/or 134, disclosed in WO2005086900, or SEQ ID NO: 176 having at least a mutation at amino acid position E41 disclosed in WO2008036863. A commercial phospholipase C product is for instance Purifine® PLC produced by DSM Food Specialties

The wording 'derived' in this context refers to the organism in which the enzyme is originally found, and does not refer to a host organism in which the enzyme may be produced. A phospholipase C may be produced in the original organism or synthetically produced, eg. via peptide synthesis, or the DNA encoding a phospholipase C may be synthesized and transformed and expressed in a host cell. An enzyme having phospholipase C activity in a method or process or use as disclosed

herein comprises an amino acid sequence that has at least 80% identity to the amino acid sequence according to SEQ ID NO: 1, such as at least 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or at least 99% identity to the amino acid sequence according to SEQ ID NO: 1. An enzyme having phospholipase C activity in a method or process or use as disclosed herein may comprise an amino acid sequence according to SEQ ID NO: 1.

Sequence identity, or sequence homology are used interchangeably herein. For the purpose of this invention, it is defined here that in order to determine the percentage of sequence homology or sequence identity of two amino acid sequences, the sequences are aligned for optimal comparison purposes. In order to optimize the alignment between the two sequences gaps may be introduced in any of the two sequences that are compared. Such alignment can be carried out over the full length of the sequences being compared. Alternatively, the alignment may be carried out over a shorter length, for example over about 20, about 50, about 100 or more amino acids. The sequence identity is the percentage of identical matches between the two sequences over the reported aligned region. The percent sequence identity between two amino acid sequences may be determined using the Needleman and Wunsch algorithm for the alignment of two sequences. (Needleman, S. B. and Wunsch, C. D. (1970) J. Mol. Biol. 48, 443-453). Amino acid sequences can be aligned by the algorithm. The Needleman-Wunsch algorithm has been implemented in the computer program NEEDLE. For the purpose of this invention the NEEDLE program from the EMBOSS package was used (version 2.8.0 or higher, EMBOSS: The European Molecular Biology Open Software Suite (2000) Rice, P. Longden, I. and Bleasby, A. Trends in Genetics 16, (6) pp276—277, <http://emboss.bioinformatics.nl/>). For protein sequences EBLOSUM62 is used for the substitution matrix. The optional parameters used are a gap-open penalty of 10 and a gap extension penalty of 0.5. The skilled person will appreciate that all these different parameters will yield slightly different results but that the overall percentage identity of two sequences is not significantly altered when using different algorithms.

After alignment by the program NEEDLE as described above the percentage of sequence identity between a query sequence and a sequence of the invention is calculated as follows: Number of corresponding positions in the alignment showing an identical amino acid in both sequences divided by the total length of the alignment after subtraction of the total number of gaps in the alignment. The identity as defined herein

can be obtained from NEEDLE by using the NOBRIEF option and is labeled in the output of the program as "longest-identity".

An enzyme having phospholipase C can be produced by any suitable technique known to a skilled person in the art, such as fermentation. During fermentation a
5 microorganism or host cell is cultivated in a suitable culture medium under conditions that allow expression of the enzyme having phospholipase C activity. Usually a phospholipase C is recovered from the culture medium.

An enzyme having phospholipase C activity may be used in a process or use as disclosed herein in substantially pure or pure or purified form. Substantially pure with
10 regard to the enzyme having phospholipase C activity refers to an enzyme preparation which contains at the most 50% of other protein material. A pure form of an enzyme or purified enzyme is an enzyme that is essentially free of other protein material, which means that less than 10%, preferably less than 9%, 8%, 7%, 6% or less than 5% of the proteins in an enzyme preparation comprising an enzyme having phospholipase C
15 activity is other protein material than the enzyme.

In another aspect the present invention relates to a method for increasing firmness of an egg yolk composition, comprising incubating egg yolk in the presence of an enzyme having phospholipase C activity to obtain increased firmness of the egg yolk composition. The firmness of an egg yolk composition obtained in a method as
20 disclosed herein is increased as compared to an egg yolk composition that was prepared with egg yolk that had been incubated without an enzyme having phospholipase C activity.

Firmness as used herein can be measured as compression in a texture analyser, for instance as disclosed in Dunnewind et al, (2004) J. of Texture Studies, 35, 603-620. Alternatively, firmness can be determined by viscometry, for instance by a
25 Brookfield viscosimeter.

Surprisingly, we found that using an egg yolk composition that has been incubated with phospholipase C can be used to increase firmness of an oil-in-water emulsified food product. This increased firmness can be used to prepare a very low fat
30 dressing (below 20% oil w/w), making use of extra structuring property of the PLC-incubated egg yolk

A method for increasing firmness of an egg yolk composition may comprise further incubating egg yolk with phospholipase A, such as phospholipase A1 or A2.

A method for increasing firmness of an egg yolk composition may comprise further steps such as pasteurizing or drying. The egg yolk composition may also be spray dried, to obtain a spray dried egg yolk composition. Spray drying and pasteurizing an egg yolk composition are known technologies to a skilled person in the art..

5 In another aspect, the present invention relates to the use of phospholipase C for improving functional properties of an egg white composition. An improved functional property may for instance be an improved foam quality. WO2014146660 discloses that the foam quality of an egg white composition can be improved by treating the egg white with a phospholipase A2, in particular, when egg
10 white is contaminated with egg yolk. We surprisingly found that when egg white is contaminated with egg yolk, treatment of the egg white that is contaminated with egg yolk with a phospholipase C, resulted in an improved foam quality of the resulting egg white composition.

15 The following examples illustrate the invention.

EXAMPLES

1. MATERIALS AND METHODS

MATERIALS

20 Enzymes: Purifine[®], phospholipase C (PLC), and Maxapal[®] A2, phospholipase A2 (PLA2) are both commercial products from DSM Food Specialties, Delft, the Netherlands.

Egg yolk and other egg products came from various suppliers, and so are ingredients
25 to make mayonnaises and emulsified sauces.

METHODS

Mayonnaise production

Method 1 – Production of a 75 wt% oil mayonnaise

30 **INGREDIENTS:** 30 g egg yolk (liquid) – or 15 g of egg yolk powder and 15 g of extra water, 91 g water, 2 g salt (NaCl), 10 g vinegar (5%) 375 g vegetable oil (soy or rapeseed). All ingredients at room temperature.

EQUIPMENT: Scale Timer Hand held kitchen high speed mixer/blender 2 x 1 litre plastic cans (1 for oil + 1 for mixing) Glass for vinegar

PROCEDURE: Weigh the ingredients in a 1 L beaker. Mix egg yolk and salt in water for 1 minute. Add oil gradually in a steady stream over 2 minutes while mixing with a hand held kitchen high speed mixer/blender. Mix for one additional minute after the oil addition. Add vinegar and mix for 30 seconds. Fill in suitable packaging (plastic bag or cups) and store 2-5°C for 24 hours. After at least 24 hours at 2-5°C the viscosity or product firmness is measured.

Method 2 – Production of different wt% oil mayonnaises

Mayonnaises (at 400 g scale) were prepared with native egg yolk, Purifine® C modified or Maxapal® A2 modified egg yolk by combining first all water phase ingredients (ingredients 2 to 7 in Table 1) and mixing (high shear for 5-6 minutes using a high shear device such as a Silverson mixer) the oil (70, 60 or 56 wt% slowly in the water phase while the beaker was cooled in ice water.

Table 1. Composition of 70, 60 and 56 wt% oil mayonnaise

	Ingredient	%
1	Sun flower oil	70, 60 or 56
2	Egg yolk	6.5
3	Sugar	2
4	Salt	1.5
5	Vinegar	2.5
6	Mustard	0.5
7	Water	17, 7 or 3
	Total	100

Characterization of phospholipid composition by ³¹P NMR

³¹P NMR was used to measure the conversion of phospholipids in egg yolk. In this method 50 mg of an egg yolk sample was dissolved in 1 ml of an aqueous solvent containing demineralized water with 10% deuteriumoxide (D₂O, Cambridge Isotope Laboratories, DLM-4), 25 mg/ml deoxycholic acid (Sigma D2510), 5,84 mg/ml EDTA di Na (Titriplex III, Merck 108418), and 5,45 mg/ml TRIS base, (Tris(hydroxymethyl)aminomethane, Merck 108387), of which the pH was adjusted to

pH 9 using 4N KOH and to which 2 mg/ml TIP internal standard (tri-isopropylphosphate, Aldrich 554669) (accurately weighed) was added.

The measurements were performed in a Bruker 400 MHz AvanceIII NMR spectrometer with a Prodigy BBO probe. The temperature of the probe head was set at 300K.

5 The measurement for quantification was performed with semi-quantitative parameters: 128 scans, 90° pulse, D1 = 5sec. As the relaxation times of the Internal Standard and the phospholipids are comparable, the calculation of the concentrations is accurate.

The following compounds were determined (Table 3 and 6): PC: phosphatidyl choline; LPC: lyso phosphatidyl choline; C-P; choline phosphate; PE: phosphatidyl
10 ethanolamine; LPE: phosphatidyl ethanolamine; E-P; ethanolamine phosphate; PI: phosphatidyl inositol; PO₄; free phosphate.

Product firmness and consistencies

The firmness of products was measured in compression using a TA-XT PLUS Texture
15 Analyzer (Stable Micro System), using the instrument settings as given in Table 2.

The compression measured with the mentioned texture analyzer was defined as 'product firmness'.

Table 2. Parameter setting of the TA-XT PLUS texture analyzer to measure
20 compression of the mayonnaises

Parameter ID	Parameter setting
Program	Compression
Force	Gram
Probe	½ inch cylinder Delrin P/ 0.5
Pre-test speed	1 mm/sec
Test speed	1 mm/ sec
Option Return to start	Yes
Distance	25 mm

Compression (3 measurements / jar) was measured of products after 2 days storage at 4°C; the temperature of the products during measurements was 4 - 10°C.

Consistencies were determined using a Brookfield viscometer as indicated in Example
25 1 and 2.

EXAMPLE 1. Effect of PLC and PLA2 treated egg yolk powder on firmness of mayonnaises

Pasteurized egg yolk (20 kg from Sanovo) was treated with 0.1% Purifine[®] PLC in a water bath set at 50°C for 4 hours. The viscous mass was diluted with an equal amount of water, and subsequently spray dried in a spray dryer with an inlet temperature of 180-200°C and outlet temperature of 75-80°C. The phospholipids composition was measured by ³¹P NMR (Table 3).

Table 3. Phospholipid composition of egg yolks in mol% measured by ³¹P NMR

	PLC treated 1	PLC treated 2
PC	12.2	3.1
LPC	5.1	7.5
C-P	60.0	62.2
PE	1.9	3.6
LPE	2.6	3.1
E-P	16.2	18.9
PI	1.9	1.7
free PO4	4.0	5.0
total	100	100

The results in Table 3 show that phospholipase C is capable of hydrolyzing 80-90% of the phospholipids PC and PE into C-P and E-P. A typical composition of standard egg yolk is shown in Table 6.

Mayonnaises were made according to method 1 described above with the PLC-treated egg yolk powder, or with Maxapal[®] A2 PLA2-treated egg yolk powder (produced by in a similar way as the PLC product), as well as mixtures of PLC and PLA2 treated powders.

Consistencies of the mayonnaises were determined by using Brookfield DV-E viscometer using Spindle D, helipath spindle, measured at 4-5°C after 1 day (Table 4).

Table 4. Brookfield viscosities in cP of mayonnaises made with PLC or PLA2 treated egg yolks or mixtures of both

Mayonnaises made with	Spindle speed		
	12 RPM	50 RPM	100 RPM
100% standard yolk powder (untreated)	35000	10000	5200
100% EMEY powder (PLA2 treated)	58000	16000	10500
100% PLC powder	118000	35000	15000
90% EMEY+10% PLC YP	80000	24500	12500
70% EMEY+30% PLC YP	85000	28000	13300
50% EMEY+ 50% PLC YP	97000	30000	16200

The results in Table 4 show that the mayonnaise made with PLC –treated egg yolk was most viscous and that mayonnaise made with PLA2 treated egg yolk – although much higher than non-enzyme treated egg yolk – was lower. Mixtures of PLC and PLA2 treated egg yolks gave intermediate consistencies of the mayonnaises.

To test the heat stability of the mayonnaises of Table 4, 50 g of the mayonnaises were heated in an oven at 110°C for 15 minutes. After this heat treatment no oil separation occurred in all the mayonnaises made with PLC or PLA2 treated egg yolk. In contrast oil separation did occur in the reference product made with untreated egg yolk.

Therefore, it was concluded that the mayonnaises made with PLC or PLA2 treated egg yolk were heat stable.

The mayonnaises were also heated in a microwave at full power for 15 seconds. The reference product fell apart, whereas the product made with PLC-treated egg yolk and with PLA2-treated egg yolk remained stable. The sample made with PLC-treated egg yolk was somewhat more grainy than the product made with PLA2 treated egg yolk.

EXAMPLE 2. Effect of water dilution on PLC-treated egg yolk firmness and viscosity

Liquid egg yolk (Beko, the Netherlands) as such, as well as egg yolk 1:1 diluted with tap water were treated with phospholipase C at 50°C for 4 hours. The viscosity and firmness of the yolk was measured using the Brookfield DV-II+Pro Extra using Helipath

spindle B and a texture analyzer TA-XT2 (Micro Stable Systems), respectively. The viscosity is given in mPa.s and the firmness is given in grams compression (Table 5). The results in Table 5 prove that the firmness of the egg yolk increases substantially after PLC treatment and that the increase is more contained after dilution with water.

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Table 5. Firmness and viscosity of egg yolk at 10°C after 2 hours incubation at 50°C with 0.1 % PLC / egg yolk w/w

10

	Texture analyzer TA-XT2	Brookfield Helipath spindle B viscosity
Beko egg yolk 24.11.2014	grams	mPa.s
Plain egg yolk	5.2	914
egg yolk treated with PLC	223	not measurable* too high
egg yolk diluted 1:1 with water	5,0	not measurable** too low
egg yolk diluted 1:1 with water treated with PLC	6.4	777

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Also whole egg (2 kg) was treated with PLC at 50°C for 3 hours. Enzyme treatment increased the consistency of the whole egg to a custard-like consistency.

EXAMPLE 3. Effect of oil level on firmness, heat stability and organoleptic properties of mayonnaises made with PLC and PLA2 treated egg yolk

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Pasteurized egg yolk (Beko, the Netherlands THT 24032014) was treated with 0.1% w/w Purifine® (30,000u/g; batch SF3011C) and 0.1% Maxapal® A2 (10,000 CPU/g; batch 613196801) at 50°C for 3 hours. For the PLC treatment the egg yolk was diluted 1:1 (w/w) with water. Directly after incubation of the egg yolk, samples were taken and frozen for ³¹P NMR analyses to check the phospholipid conversion. Just before ³¹P NMR analysis, 1000 mg NMR buffer pH 10.5 was added to 50-80 mg (accurately weighed) egg yolk (see method 2.2 above)

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The liquid enzyme-treated egg yolks were used to prepare 70, 60 and 56 % oil mayonnaises as described in method 2 above, using native (non-enzyme treated) egg yolk and PLC or PLA2 treated egg yolk. Other ingredients: Sunflower oil (PLUS private label); liquid egg yolk (BEKO, Netherlands); Sugar (CSM); Salt (Jozo tafel zout met

jodium; Extra fijn), Vinegar (Natuurazijn, Albert Heyn); Mustard (Kühne Franse mosterd mild); Water, potable tap water.

After preparation the mayonnaises were stored in plastic jars at 4°C, and used to assess firmness, heat stability and organoleptic properties.

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3.1. Phospholipid content and Firmness

The compositions of PLC and PLA2 treated egg yolk compositions were determined by ^{31}P NMR. The results in Table 6 show that PLC hydrolyzed almost all PC and PE into the phosphate esters C-P and E-P, and PLA2 hydrolyzed all PC and PE into the lyso form. Firmness of mayonnaises and emulsified sauces with various oil levels were measured by texture analysis according to method 2.3. The results are given in Table 7.

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Table 6. Phospholipid composition of egg yolks in mol% measured by ^{31}P NMR

	Ref egg yolk	PLC-treated egg yolk	PLA2-treated egg yolk
PC	76.57	5.74	1.75
LPC	3.16	2.09	77.49
C-P	0.00	71.95	0.00
PE	16.03	0.91	2.22
LPE	1.55	1.25	18.04
E-P	0.00	16.35	0.00
PI	2.69	1.70	0.51
free PO4	6.30	5.62	5.46

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Table 7. Firmness of final products by Texture Analysis compression measurement

Mayonnaise	Firmness (in gram)
70% oil reference	16.9
70% oil + PLC treated egg yolk	53.3
70% oil + PLA2 treated egg yolk	43.7
60% oil + PLC treated egg yolk	17.9
60% oil + PLA2 treated egg yolk	13.5
56% oil + PLC treated egg yolk	12.1

These results show that the 70% oil product made with PLC treated egg yolk is much firmer than the reference product made with untreated egg yolk, and also firmer than the product made with PLA2 treated egg yolk. As the products were all made with 6.5% wet egg yolk, the increased firmness is not due to the increased firmness of the egg yolk itself – the volume fraction of yolk is too low to be accountable for this increase. The firmness increase is therefore due to improved emulsifying properties of the PLC treated egg yolk.

3.2. Heat stability

The heat stability of the mayonnaises of Table 7 was assessed after a heat treatment in the microwave for 15 seconds at full power. Instable emulsions show oil separation, stable products show no oil leakage. Emulsified sauces prepared with PLC-treated egg yolk with 70 or 60% oil were heat stable after microwave treatment. Also with PLA2 modified egg yolk the resulting emulsified sauce is heat stable. In both cases no oil separation was observed. In contrast the reference emulsified sauce made with untreated egg yolk fully fell apart after heat treatment.

3.3. Organoleptic properties

Organoleptic properties of the mayonnaise-type sauces were evaluated in two groups of six or five internal, untrained persons. Products were compared to the reference in a blind test (i.e. coded by numbers not indicating the composition). First the reference product was tasted to get familiar with the product, followed by tasting of three products including the reference. The firmness perception as well as potential off smell or off taste were assessed. The following conclusions were drawn:

- No specific off-smell or off-taste was observed in any of all mayonnaises
- The taste profiles of 60 and 56 wt% oil mayonnaises did not show significant differences compared to the 70 wt% mayonnaises
- The textures of the 70wt% oil mayonnaises prepared with PLC treated egg yolk and PLA2 treated egg yolk were considered about equal, but these mayonnaises were much firmer than the 70% oil reference. The firmness of the 60 wt% mayonnaise made with PLC-treated egg yolk was considered comparable to that of the 70% oil reference.

EXAMPLE 4. Effect on firmness of low fat dressing (40 wt%) with or without PLC treatment in water phase incubated during preparation of the dressing

For a model low fat (40 wt%) dressing an adapted version of method 2 is used. The following ingredients were weighed and used in preparation of 1 liter of a starch paste: 37.2 g sugar (CSM), 22.3 g salt (Jozo), 9.3 g mustard (Kühne), 65.1 g Colflo 67 (Ingredion), 2.8 g guar gum (Hansacoll) and 2.8 g xanthan gum (Kelco). All ingredients were gradually added to the water, dissolved while heating and under constant mixing so that lumps were avoided. From this starch paste 161.4 gram was mixed with 11.1 gram egg yolk and 0.1% Phospholipase C on yolk base was added next to a reference without phospholipase C. The mixtures were incubated at 50°C for 3 hours. This resulted in a viscous liquid that remained pourable, pumpable and stirrable. By incubating the egg yolk in the water phase of the dressing, the viscosity was kept relatively low and processable. The mixtures were cooled down to room temperature, vinegar was added and mixed. Oil (120 gram) was gradually added in a steady stream while mixing (high shear for 5-6 minutes using a Silverson mixer as high shear device) in the water phase while the beaker was cooled in ice water. The liquid-enzyme treated egg yolk and a native non-enzyme treated egg yolk were used to prepare 40% oil low fat dressing. After preparation the dressings were stored in plastic jars, and used to assess firmness as shown in Table 8.

Table 8. Firmness of 40% dressings prepared with and without PLC

40% oil low fat dressing	
	Firmness in gram
Reference	21.1
With 0.1% PLC treated	29.6

The resulting dressing made with the PLC treated egg yolk starch paste mixture had a good appearance and a higher firmness than the reference product.

EXAMPLE 5. Effect phospholipase C treatment on foaming of egg white contaminated with egg yolk

In the following test egg white was mixed with small amounts of egg yolk to mimick contamination of egg white with egg yolk as can happen upon poor white/yolk separation.

Liquid egg white from Sanovo Foods. Egg yolk from fresh eggs was added in three levels, 0.2, 0.5 and 1.0% (w/w), and treated by 0.1% (w/w) Purifine® PLC enzyme at 50°C for 2 and 4 hours.

Enzyme is not added to the reference sample (only egg white) and it was also heated to 50°C for the same time as the other samples. Whipping test was done before addition of enzyme and after PLC incubation for the indicated time

Whipping test:

450 ml of egg white at 25°C was whipped for 90 sec in 2nd gear and then 90 sec in 3rd gear. A Hobart Mixer model C100 was used. After 180 seconds the foam was collected in a measuring cup 200 ml and then weighed on a balance to determine the density.

Table 9. Foam density of egg white treated with phospholipase C

Sample	Hydrolysis/ Heating time, Hours	Foam density after 180 seconds whipping [g/ml]
ref no enzyme	0	0.127
	2	0.21
	4	0.189
egg white + 0.2 % yolk	0	0.166
	2	0.118
	4	0.127
egg white + 0.5 % yolk	0	0.226
	2	0.138
	4	0.142
egg white + 1.0 % yolk	0	0.258
	2	0.166
	4	0.180

Table 9 shows the quality of the foam: the poorer the foam quality the higher the density. After incubation by phospholipase C all the egg white foam properties are improved, indicated by reduced density of the 2 and 4 hour samples compared to the sample at t=0.

CLAIMS

- 5 1. Process for preparing an oil-in-water emulsified food product having a pH of less than 7, comprising incubating egg yolk in the presence of an enzyme having phospholipase C activity to produce an egg yolk composition, mixing the egg yolk composition with oil and water, and preparing the oil-in-water emulsified food product,.
- 10 2. Process according to claim 1, wherein an amount of 0.5 to 7 wt/wt% dry weight of egg yolk composition is mixed with oil and water.
- 15 3. Process according to claim 1 or 2, further comprising diluting the egg yolk or egg yolk composition with an aqueous solution.
- 20 4. Process according to claim 3, wherein diluting the egg yolk or egg yolk composition with an aqueous solution is performed at a ratio egg yolk (composition) to aqueous solution of 5 : 1 to 1 : 1.
- 25 5. Process according to any one of the claims 1 to 4, wherein the process comprises increasing firmness of the oil-in-water emulsified food.
6. Process according to any one of claims 1 to 5, wherein the oil-in-water emulsified food product comprises between 5 and 80 wt/wt% oil.
- 30 7. Process according to any one of claims 1 to 6, wherein an oil-in-water emulsified food product is a mayonnaise, an emulsified sauce, a dressing or a dessert.
- 35 8. Process according to any one of the claims 1 to 7, further comprising incubating the egg yolk in the presence of an enzyme having phospholipase A activity.
9. Use of an egg yolk composition which has been treated with an enzyme having phospholipase C activity, to increase firmness of an oil-in-water emulsified food product.

10. Process according to any one of claims 1 to 8, or use according to claim 9 or method according to claims 12 or 13, wherein the phospholipase C has at least 90% identity to the amino acid sequence according to SEQ ID NO: 1.
- 5 11. Oil-in-water emulsified food product obtainable by a process according to any one of the claims 1 to 8, or 10.
12. Method for increasing firmness of an egg yolk composition, comprising incubating egg yolk in the presence of an enzyme having phospholipase C activity, wherein the firmness of the egg yolk composition is increased.
- 10 13. Method according to a claim 12, further comprising incubating the egg yolk with an enzyme having phospholipase A activity.

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/075134

A. CLASSIFICATION OF SUBJECT MATTER INV. A23L1/32 A23D7/005 A23L1/035 A23L1/24 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) A23L A23D		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
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Y	abstract page 667, column 1, paragraph 2 ----- -/-	3,4,13
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 11 January 2016		Date of mailing of the international search report 21/01/2016
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Schlegel, Birgit

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