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(71) Applicant(s)
Stabilitech Ltd.

(72) Inventor(s)
Drew, Jeffrey;Woodward, David;Corteyn, Amanda

(74) Agent / Attorney
Spruson & Ferguson, L 35 St Martins Tower 31 Market St, Sydney, NSW, 2000

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(71) Applicant *(for all designated States except US)*: **STABILITECH LTD.** [GB/GB]; London Bioscience Innovation Centre, 2 Royal College Street, London NW1 ONH (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **DREW, Jeffrey** [GB/GB]; Stabilitech Ltd., Imperial Incubator, Bessemer Building Level 1, Imperial College London, South Kensington London SW7 2AZ (GB). **WOODWARD, David** [GB/GB]; Stabilitech Ltd., Imperial Incubator, Bessemer Building Level 1, Imperial College London, South Kensington London SW7 2AZ (GB). **CORTEYN, Amanda** [GB/GB]; Stabilitech Ltd., Imperial Incubator, Bessemer Building Level 1, Imperial College London, South Kensington London SW7 2AZ (GB).

(74) Agents: **WOODS, Geoffrey, Corlett** et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5JJ (GB).

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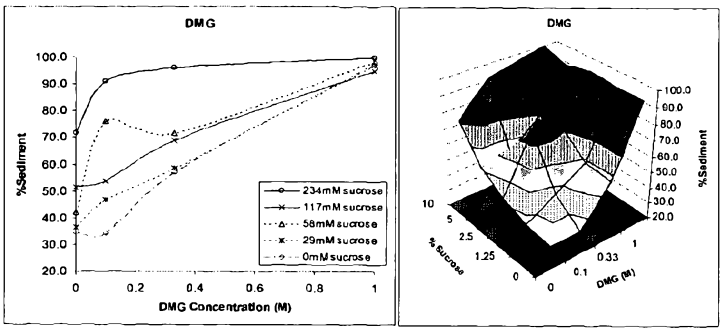
Figure 2



(57) Abstract: A method for preserving an aluminium-salt adjuvant during freezing or drying comprising freezing or drying an aqueous suspension or solution comprising: (a) an aluminium salt adjuvant; (b) a compound of formula (I) or a physiologically acceptable salt or ester thereof or a compound of formula (II) or a physiologically acceptable salt or ester thereof; and (c) optionally, one or more sugars.

WO 2011/121305 A3

METHOD FOR PRESERVING ALUM ADJUVANTS AND ALUM-ADJUVANTED VACCINES

Field of the Invention

The invention relates to a method for preserving an aluminium salt adjuvant during freezing or drying, typically during freezing or drying of a vaccine preparation comprising an aluminium salt adjuvant and one or more vaccine antigens.

Background to the Invention

Aluminium salt adjuvants are currently the most widely used adjuvants for human and veterinary vaccines. Aluminium adjuvant compounds include aluminium salts such as aluminium phosphate ($AlPO_4$) and aluminium hydroxide ($Al(OH)_3$) which are generically referred to in the field of vaccine adjuvants as *"alum"*. To provide adequate immunogenicity, it is thought that antigens must be adsorbed onto the surface of the adjuvant. It is believed that alum adjuvants act as an immune system stimulus as well as providing a depot of antigen at the site of administration (e.g. by injection) thereby providing a gradual and continuous release of antigen to stimulate antibody production. Aluminium adjuvants in their natural form are commonly known as gels, which are particulate suspensions in aqueous media.

The storage and transportation of alum-adjuvanted vaccines is problematic. Freeze-drying (lyophilisation) is a process frequently used to improve long-term stability of various protein preparations. Nevertheless, commercial vaccine compositions containing aluminium salt adjuvants cannot be freeze-dried without causing damage to the adjuvant structure. Freeze-drying causes the collapse of the gel structure of the adjuvant resulting in aggregation and precipitation of the adjuvant salt on resuspension in water. The effect is to significantly reduce the immunogenicity of the vaccine.

WO 01/93829 describes a method of preparing an adjuvanted vaccine comprising spray-drying or spray freeze-drying an aqueous solution comprising:

(a) from 0.1 to 0.95% by weight of an aluminium salt or calcium salt adjuvant having an antigen adsorbed therein;

(b) from 0.5 to 6% by weight of a saccharide;

(c) from 0.1 to 2% by weight of an amino acid or salt thereof; and

(d) from 0.02 to 1% by weight of a colloidal substance.

WO 2008/118691 describes a method of preparing an immunologically-active adjuvant-bound dried vaccine composition comprising (a) combining at least one aluminium-salt adjuvant, at least one buffer system, at least one glass-forming agent and at least one antigen to create a liquid vaccine formulation; (b) freezing the liquid vaccine formulation to create a frozen vaccine formulation; and (c) lyophilizing the frozen vaccine formulation to create a dried vaccine composition. The glass-forming agent is preferably trehalose.

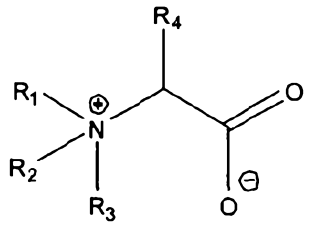
Summary of the Invention

Surprisingly, the present inventors found that structural damage to an aluminium salt adjuvant can be reduced by freezing or drying, in particular freeze-drying, the adjuvant in the presence of a compound of formula (I) or (II) or a physiologically acceptable salt or ester thereof. The additional presence of one or more sugars can lead to a further reduction in the structural damage to the adjuvant during freezing or drying.

Accordingly, the present invention provides a method for preserving an aluminium-salt adjuvant during freezing or drying comprising freezing or drying an aqueous suspension or solution comprising:

(a) an aluminium salt adjuvant;

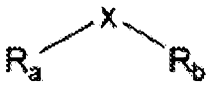
(b) a compound of formula (I) or a physiologically acceptable salt or ester thereof



wherein:

- R_1 represents hydrogen or C_{1-6} alkyl; and
- R_4 represents hydrogen; or
- R_1 and R_4 together with the atoms to which they are attached form a pyrrolidine ring;
- R_2 represents hydrogen, C_{1-6} alkyl or $-(CH_2)_{2-5}NHC(O)(CH_2)_{5-15}CH_3$; and
- R_3 represents C_{1-6} alkyl; or

a compound of formula (II) or a physiologically acceptable salt or ester thereof



(II)

wherein:

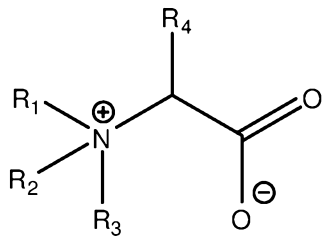
- X represents $-S(O)_2-$ or $-S^+(R_c)-$;
- R_a and R_b independently represent C_{1-6} alkyl; and
- R_c represents C_{1-6} alkyl substituted with a carboxylate anion and with an amine ($-NH_2$) moiety; and

(c) optionally, one or more sugars.

In one aspect there is provided a method for preserving an aluminium-salt adjuvant during freezing or freeze-drying comprising freezing or freeze-drying an aqueous suspension or solution comprising:

(a) an aluminium salt adjuvant;

(b) a compound of formula (I) or a physiologically acceptable salt or ester thereof



(I)

wherein:

R_1 represents hydrogen or C_{1-6} alkyl; and

R_4 represents hydrogen; or

R_1 and R_4 together with the atoms to which they are attached form a pyrrolidine ring;

R_2 represents hydrogen, C_{1-6} alkyl or $-(CH_2)_{2-5}NHC(O)(CH_2)_{5-15}CH_3$; and

R_3 represents C_{1-6} alkyl; and

(c) one or more sugars.

The present invention also provides:

- use of an excipient comprising (i) a compound of formula (I) or (II) of the invention or a physiologically acceptable salt or ester thereof and (ii) optionally, one or more sugars, for preserving an aluminium salt adjuvant during freezing or drying;
- a vaccine composition comprising: an aluminium-salt adjuvant; one or more antigens; a compound of formula (I) or (II) of the invention or a physiologically acceptable salt or ester thereof; and optionally, one or more sugars.
- a vaccine composition obtainable by the method of the invention; and

- use of an excipient comprising (i) a compound of formula (I) or (II) of the invention or a physiologically acceptable salt or ester thereof and (ii) optionally one or more sugars, as a resuspension agent for a vaccine composition.

The frozen or dried vaccine compositions facilitate appropriate storage and maximize the shelf-life of the compositions. The compositions can be stock piled for prolonged periods of time. The immunogenicity, potency and efficacy of the vaccines can thus be maintained. The compound of formula (I) or (II) or physiologically acceptable salt or ester thereof and the optional sugar(s) act as cryoprotectants and protect the aluminium salt adjuvants against the stresses encountered during freezing and also as a lyoprotectant during freeze-drying.

Brief Description of the Figures

Figure 1 shows the results of analysing adjuvants microscopically in the Reference Examples after freezing the aluminium hydroxide gel. Panel A shows an example of normal undamaged structure and panel B shows damaged agglomerated crystalline structure post-freezing of the aluminium hydroxide adjuvant.

Figure 2 shows the results of an adjuvant agglomeration assay after freezing an aluminium hydroxide gel in the presence of various concentrations of sucrose and dimethylglycine (DMG) in Example 1.

Figure 3 shows recovery of adjuvant ($Al(OH)_3$) after freeze-thaw in the formulations described in Example 2 containing sucrose and/or trimethylglycine (TMG) as assessed using an agglomeration assay.

Figure 4 shows recovery of adjuvant ($Al(OH)_3$) after freeze-thaw in the formulations described in Example 2 containing sucrose and/or S-methyl-L-methionine (SMM) or methylsulfonylmethane (MSM) as assessed by an agglomeration assay.

Figure 5 shows results of an adjuvant agglomeration assay after freeze-drying of an aluminium hydroxide gel in the presence of various concentrations of sucrose and dimethylglycine (DMG), trimethylglycine (TMG), S-methyl methionine (SMM) or sarcosine in Example 3.

Figure 6 shows the percentage of BSA bound to the adjuvant in Example 6 compared to the control.

Figure 7 shows the concentration of BSA bound to the adjuvant in Example 6.

Figure 8 shows the dot blot results from Example 7. Figure 8A shows the dot blot of the samples set out in Table 19 stored at 4°C. Figure 8B shows the dot blot of the samples set out in Table 19 stored at -80°C

Figure 9 shows more dot blot results from Example 7. Figure 9A shows the dot blot of the samples set out in Table 20 stored at 4°C. Figure 9B shows the dot blot of the samples set out in Table 20 stored at -80°C.

Detailed Description of the Invention

Summary

The present invention relates to the reduction and/or prevention of structural damage to aluminium salt vaccine adjuvants when frozen or dried, especially freeze-dried. Such structural damage is reduced or prevented by freezing or drying the adjuvant in the presence of a compound of formula (I) or (II) or physiologically acceptable salt or ester thereof and optionally (ii) one or more sugars.

The aluminium salt adjuvant, on which typically at least one antigen is adsorbed, is contacted with the compound of formula (I) or (II) or physiologically acceptable salt or ester thereof in aqueous solution. The resulting aqueous composition, in which one or more sugars may also be present, is then frozen or dried. When an antigen is present, the method is a method of preparing a vaccine composition comprising an aluminium salt adjuvant and at least one antigen. A vaccine preparation comprising the aluminium adjuvant can be thawed or reconstituted after freezing or drying respectively, prior to administration of the vaccine preparation to a patient.

The invention enables the structure and function of the aluminium adjuvant to be preserved during the freezing or drying step. The immunogenicity of aluminium adjuvanted vaccines following freezing or drying can consequently be maintained.

Aluminium salt adjuvant

Any type of aluminium salt suitable for use as an adjuvant may be used in the invention. The aluminium salt may be aluminium hydroxide ($Al(OH)_3$), aluminium phosphate ($AlPO_4$), aluminium hydrochloride, aluminium sulphate, ammonium alum, potassium alum or aluminium silicate. Preferably, the aluminium salt adjuvant used is aluminium hydroxide or aluminium phosphate. Most preferably, the aluminium salt adjuvant is aluminium hydroxide ($Al(OH)_3$).

Typically, the aluminium salt adjuvant takes the form of a hydrated gel made from an aluminium salt, the hydrated gel being a particulate suspension in aqueous media. The preparation of aluminium-salt adjuvants are well known to those skilled in the art. For example, aluminium hydroxide and aluminium phosphate adjuvants are generally prepared by exposing aqueous solutions of aluminium ions (typically as sulfates or chlorides) to alkaline conditions in a well-defined and controlled chemical environment, as known to those skilled in the art. Such methods can be used for example, to prepare an aluminium hydroxide or aluminium phosphate hydrated gel.

Antigen

An antigen suitable for use in the invention includes any immunogenic component of a vaccine. Thus, the antigen may be a protein, bacterial-specific protein, mucoprotein, glycoprotein, peptide, lipoprotein, polysaccharide, peptidoglycan, nucleoprotein or fusion protein.

The antigen may be derived from a microorganism (such as a bacterium, virus or fungus), a protozoan, a tumour, a malignant cell, a plant, an animal, a human, or an allergen. In one embodiment, the antigen is a protein but excludes a whole virus or virion.

The antigen may be synthetic, for example as derived using recombinant DNA techniques. The antigen may be a disease-related antigen such as a pathogen-related antigen, tumour-related antigen, allergy-related antigen, neural defect-related antigen, cardiovascular disease antigen, rheumatoid arthritis-related antigen. The antigen may be an inactivated or attenuated/detoxifed toxin (toxoid).

In particular, the pathogens from which the vaccine immunogen is derived may include human papilloma viruses (HPV), HIV, HSV2/HSV1, influenza virus (types A, B and C), para influenza virus, polio virus, RSV virus, rhinoviruses, rotaviruses, hepaptitis A virus, norwalk virus, enteroviruses, astroviruses, measles virus, mumps virus, varicella-zoster virus, cytomegalovirus, epstein-barr virus, adenoviruses, rubella virus, human T-cell lymphoma type I virus (HTLV-I), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus, poxvirus, vaccinia virus, Salmonella, Neisseria, Borrelia, Chlamydia, Clostridium such as C. difficile and C. tetani, Bordetella such as Bordetella pertussis, Corynebacterium such as C. diptheriae, Plasmodium, Coxoplasma, Pneumococcus, Meningococcus, Cryptococcus, Streptococcus, Vibriocholerae, Staphylococcus, Haemophilus, Bacillus such as Bacillus anthracis (anthrax), Escherichia, Candida, Aspergillus, Entamoeba, Giardia and Trypanasoma.

The vaccine may further be used to stimulate a suitable immune response against numerous veterinary diseases. The vaccine antigen may therefore be derived from a foot and mouth disease virus (including serotypes O, A, C, SAT-1, SAT-2, SAT-3 and Asia-1), coronavirus, bluetongue virus, feline leukaemia virus, avian influenza virus, hendra and nipah virus, pestivirus such as bovine viral diarrhoea virus and canine parvovirus.

Tumor-associated antigens include for example, melanoma-associated antigens, mammary cancer-associated antigens, colorectal cancer-associated antigens or prostate cancer-associated antigens

An allergen-related antigen includes any allergen antigen suitable for use in a vaccine to stimulate suppression of an allergic reaction in an individual to which the vaccine is administered (e.g. antigens derived from pollens, dust mites, insects, food allergens, dust, poisons, toxins, venoms and parasites).

Compound of formula (I) or (II) or physiologically acceptable salt or ester thereof

The compound of formula (I) and (II) may be present as a physiologically acceptable salt or ester thereof.

The salt is typically a salt with a physiologically acceptable acid and thus includes those formed with an inorganic acid such as hydrochloric or sulphuric acid or an organic acid such as citric, tartaric, malic, maleic, mandelic, fumaric or methanesulphonic acid. The hydrochloride salt is preferred.

The ester is typically a C_{1-6} alkyl ester, preferably a C_{1-4} alkyl ester. The ester may therefore be the methyl, ethyl, propyl, isopropyl, butyl, isobutyl or tert-butyl ester. The ethyl ester is preferred.

As used herein, a C_{1-6} alkyl group is preferably a C_{1-4} alkyl group. Preferred alkyl groups are selected from methyl, ethyl, propyl, isopropyl, butyl, isobutyl and tert-butyl. Methyl and ethyl are particularly preferred.

For the avoidance of doubt, the definitions of compounds of formula (I) and formula (II) also include compounds in which the carboxylate anion is protonated to give -COOH and the ammonium or sulfonium cation is associated with a pharmaceutically acceptable anion. Further, for the avoidance of doubt, the compounds defined above may be used in any tautomeric or enantiomeric form.

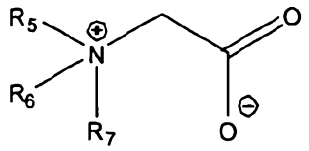
Compounds of formula (I)

Typically, R_1 represents hydrogen or C_{1-6} alkyl and R_4 represents hydrogen. Typically, R_2 represents hydrogen or C_{1-6} alkyl. Preferably, R_1 represents hydrogen or C_{1-6} alkyl, R_4 represents hydrogen and R_2 represents hydrogen or C_{1-6} alkyl.

Preferably, the compound of formula (I) is an N-C_{1-6} alkyl-, N,N-di(C_{1-6} alkyl)- or N,N,N-tri(C_{1-6} alkyl)-glycine or physiologically acceptable salt or ester thereof. The alkyl group is typically a C_{1-4} alkyl group. Preferred alkyl groups are selected from methyl, ethyl, propyl, isopropyl, butyl, isobutyl and tert-butyl. Methyl and ethyl are particularly preferred.

Preferred compound of formula (I) are N-methylglycine, N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof. N-Methyl-glycine is also called sarcosine. N,N-Dimethylglycine is also termed dimethylglycine (DMG) or 2-(dimethylamino)-acetic acid. N,N,N-trimethylglycine is termed trimethylglycine (TMG).

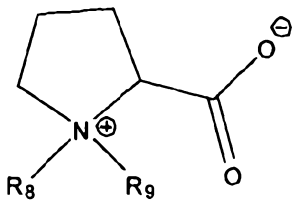
Alternatively, the compound of formula (I) is typically a glycine derivative of formula (IA) or a physiologically acceptable salt or ester thereof:



(IA)

wherein R_5 and R_6 independently represent C_{1-6} alkyl, for example C_{1-4} alkyl such as methyl or ethyl; and R_7 represents C_{1-6} alkyl, for example C_{1-4} alkyl such as methyl or ethyl, or $-(CH_2)_{2-5}NHC(O)(CH_2)_{5-15}CH_3$. Preferred compounds of formula (IA) are trimethylglycine (TMG) and cocamidopropyl betaine (CAPB) or physiologically acceptable salts or esters thereof.

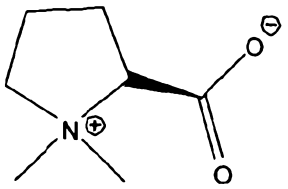
Alternatively, the compound of formula (I) is typically a proline derivative of formula (IB) or a physiologically acceptable salt or ester thereof:



(IB)

wherein R_8 and R_9 independently represent C_{1-6} alkyl, for example C_{1-4} alkyl such as methyl or ethyl. Preferably the compound of formula (IB) is an S-proline derivative. Preferably R_8 and R_9 both represent methyl; this compound is known as proline

betaine. S-proline betaine or physiologically acceptable salt or ester thereof is particularly preferred:



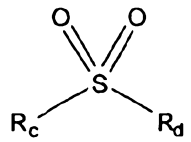
Compounds of formula (IA) or physiologically acceptable salts or esters thereof are preferred.

Most preferably, the compound of formula (I) is N, N-dimethylglycine or physiologically acceptable salt or ester thereof.

Compounds of formula (II)

Typically, the carboxylate and amine substituents of R_c are attached to the same carbon atom of the R_c alkyl moiety. Typically R_c is a C_{2-4} or C_{2-3} alkyl moiety.

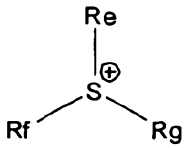
The compound of formula (II) is typically a sulfone compound of formula (IIA) or a physiologically acceptable salt or ester thereof:



(IIA)

wherein R_c and R_d independently represent C_{1-6} alkyl, for example C_{1-4} alkyl such as methyl or ethyl. A preferred sulfone compound is methylsulfonylmethane (MSM), which is also known as dimethylsulfone ($DMSO_2$).

The compound of formula (II) is typically a compound of formula (IIB) or a physiologically acceptable salt or ester thereof:



(IIB)

wherein R_e and R_f independently represent C_{1-6} alkyl, for example C_{1-4} alkyl such as methyl or ethyl, and R_g represents C_{1-6} alkyl, for example C_{1-4} alkyl such as methyl or ethyl, substituted with a carboxylate anion and with an amine ($-NH_2$) moiety. A preferred compound of formula (IIB) is S-methyl-L-methionine (SMM) or a physiologically acceptable salt or ester thereof.

Sugars

Sugars suitable for use in the present invention include reducing sugars such as glucose, fructose, glyceraldehydes, lactose, arabinose and maltose; and preferably non-reducing sugars such as sucrose and raffinose. The sugar may be a monosaccharide, disaccharide, trisaccharide, or other oligosaccharides. The term "*sugar*" includes sugar alcohols.

Monosaccharides such as galactose and mannose; dissaccharides such as sucrose, lactose and maltose; trisaccharides such as raffinose and tetrasaccharides such as stachyose are envisaged. Trehalose, umbelliferose, verbascose, isomaltose, cellobiose, maltulose, turanose, melezitose and melibiose are also suitable for use in the present invention. A suitable sugar alcohol is mannitol.

Two or more sugars may be present. Two, three or four sugars may be used. When one or more sugars are present in the aqueous suspension that is frozen or freeze-dried, preferably sucrose or sucrose and raffinose are present. Sucrose is a disaccharide of glucose and fructose. Raffinose is a trisaccharide composed of galactose, fructose and glucose.

Aqueous suspension to be frozen or dried

The aqueous suspension or solution to be frozen or dried can be prepared by admixing the aluminium salt adjuvant with an aqueous solution of the compound of formula (I) or (II) or physiologically acceptable salt or ester thereof. The compound of formula (I) or (II) or physiologically acceptable salt or ester thereof may in particular be selected from dimethylglycine, S-methyl-L-methionine, methylsulfonylmethane, sarcosine and trimethylglycine, and may for example be dimethylglycine, S-methyl-L-methionine, methylsulfonylmethane or trimethylglycine. Any suitable aqueous solution may be used. The solution may be buffered. The solution may be a HEPES, Tris-buffered, phosphate-buffered or pure water solution.

Optionally one or more sugars is dissolved in the aqueous solution prior to admixture with the adjuvant. Alternatively the sugar(s) can be admixed with the suspension of the adjuvant in the aqueous solution of the compound of formula (I) or (II) or physiologically acceptable salt or ester thereof.

Where present, the antigen(s) are generally adsorbed onto the adjuvant prior to admixture of the adjuvant with the aqueous solution of the compound of formula (I) or (II) or physiologically acceptable salt or ester thereof. The adjuvants can be prepared in the form of a hydrated gel and the antigen adsorbed into the hydrated gel. Antigen adsorption can be carried out using techniques well known to those skilled in the art. For example, for certain protein antigens, adsorption may best be carried out at a pH interval where the adjuvant and antigen will have opposite electrical charges, facilitating electrostatic attraction and adsorption. Protein adsorption for a particular antigen-adjuvant combination will depend on the nature of the antigen and the chemical environment (pH, ionic strength, presence of surfactants etc).

The concentrations of the compound of formula (I) or (II) or physiologically acceptable salt or ester thereof and of the or each sugar in the aqueous suspension or solution to be frozen or can be determined by routine experimentation. Optimised concentrations can thus be selected. The compound of formula (I) or (II) or physiologically acceptable salt or ester thereof can act synergistically with the sugar(s) to improve stability.

The concentration of the compound of formula (I) or (II) or physiologically acceptable salt or ester thereof in the aqueous suspension or solution is typically in the range of 0.001M or more, preferably in the range of 0.01M or more and more preferably 0.1M or more, for example from 0.1M to 5.0M. The particular concentration that is employed will depend on several factors including, where present, the nature of the antigen; the particular compound of formula (I) or (II) or physiologically acceptable salt or ester thereof being used; whether one or more sugar is being used and if so the identity of the sugar(s); and the particular freezing or drying procedure that is adopted. Thus:

- The concentration of a compound of formula (I) or a compound of formula (IA) or formula (IB), such as TMG, or a physiologically acceptable salt or ester thereof is preferably from 0.01M to 5M, from 0.1M to 5M, from 0.2M to 5.0M or from 0.1M to 1M.
- The concentration of a compound of formula (II) in which X represents -$S(O)_2$- or a compound of formula (IIA), such as MSM, or a physiologically acceptable salt or ester thereof is preferably from 0.01M to 4M, from 0.05M to 2M or from 0.07M to 1M or even to 0.53M.
- The concentration of a compound of formula (II) in which X represents -$S^+(R_c)$- or a compound of formula (IIB), such as S-methyl-L-methionine, or a physiologically acceptable salt or ester thereof is preferably from 0.01M to 5M, from 0.1M to 5M, from 0.2M to 3M or from 0.1M to 1M.
- The concentration of a compound of formula (I) which is a N,N-di(C_{1-6} alkyl)-, N,N,N-tri(C_{1-6} alkyl)-, or N-C_{1-6} alkyl-glycine, such as N,N-dimethylglycine, N,N,N-trimethylglycine, or N-methylglycine, or a physiologically acceptable salt or ester thereof is typically 0.01M or more and preferably 0.1M or more, for example from 0.1M to 5.0M, from 0.33M to 5.0M, from 0.5M to 4M or from 0.5M to 3M.
- The concentration of a compound of formula (I) which N,N-dimethylglycine (DMG) or a physiologically acceptable salt or ester thereof is typically 0.01M or more and preferably 0.1M or more, for example from 0.1M to 5.0M, from 0.33M to 5.0M,

from 0.5M to 4M or from 0.5M to 3M. Less DMG or DMG salt or ester can be employed when one or more sugars are present.

If one or more sugar(s) is used, the concentration of sugar or total concentration of sugar in the aqueous suspension or solution that is to be frozen or dried is typically 1M or less or 0.7M or less, for example 0.5M or less or 0.29M or less. A 10% w/v sucrose solution has a sucrose concentration of 0.29M. The sugar concentration or the total concentration may be down to 0.1mM, to 0.5mM, to 0.073M or to 0.146M.

When the compound of formula (I) or (II) or physiologically acceptable salt or ester thereof is DMG or a physiologically acceptable salt or ester thereof, the concentration of sugar, if present, in the aqueous suspension or solution for freezing or drying is typically 1M or less or 0.7M or less, for example 0.5M or less or 0.29M or less. A 10% w/v sucrose solution has a sucrose concentration of 0.29M. Preferably, the concentration of the sugar such as sucrose or raffinose or, if more than one sugar is present, the total concentration of sugar is 0.5M or less, 0.2M or less, 0.1M or less or 10mM or less. The minimum concentration of the sugar if present or, if more than one sugar is present, the minimum total concentration of sugar may be 0.01M, 0.1M or 0.2M. The sugar concentration, for example the concentration of sucrose or raffinose, or the total concentration if more than one sugar is present may thus be from 0.01M to 0.7M, from 0.029M to 0.5M, from 0.058M to 0.3M or from 0.1M to 0.3M. When the sugar is sucrose, the concentration of sucrose is preferably from 0.01 to 0.2M and the concentration of DMG or salt or ester thereof is preferably from 0.2 to 2M.

The particular concentration that is employed will depend on several factors including the nature of the antigen, the particular the compound of formula (I) or (II) or physiologically acceptable salt or ester thereof being used and the particular freezing or drying procedure that is adopted. The sugar concentration or the total concentration may be from 0.1mM to 0.7M, from 5mM to 0.7M, from 0.073M to 0.5M, or from 0.146M to 0.389M.

When the sugar is mannitol, the mannitol concentration is typically 0.2 to 1M or 0.2 to 0.8M, preferably 0.25 to 0.6M or 0.4 to 0.8M, for example 0.5 to 0.6M.

The most effective concentration of the compound of formula (I) or (II) or physiologically acceptable salt or ester thereof will depend on the particular type of compound used, whether it is used in combination with a sugar and the type of aluminium salt adjuvant that is used e.g. whether an aluminium hydroxide or aluminium phosphate adjuvant is used. Using a mixture of a compound of formula (I) or (II) or physiologically acceptable salt or ester thereof together with a sugar, the inventors have demonstrated that lower concentrations of each component can be used to achieve the same level of protection of the adjuvant as that obtained when each component is used separately.

Highly concentrated solutions of sugars have been known to give site-specific reactions when vaccine preparations containing such concentrated sugars are injected into patients. Therefore, the invention has the advantage that lower concentrations of sugars can be used when in combination with a compound of formula (I) or (II) or physiologically acceptable salt or ester thereof. As a result, when such vaccine preparations are reconstituted or thawed, the concentration of sugar is reduced and the likelihood of site-specific reaction is minimised.

Freezing/Drying

Freezing

Freezing is conducted by any suitable method. Freezing may thus be carried out by immersing in liquid nitrogen or liquid nitrogen vapour, placing in a freezer at a temperature of from –4°C to –80°C or using a dry ice and alcohol freezing bath. At atmospheric pressure, temperatures such as -4°C or below, -10°C or below, -15°C or below, -20°C or below, -25°C or below may be used.

Drying

Typically, drying is achieved by freeze-drying, vacuum drying, spray-drying, spray freeze-drying or fluid bed drying. Freeze-drying is preferred. By reducing the

water in the material and sealing the material in a vial, the material can be easily stored, shipped and later reconstituted to its original form. The drying conditions can be suitably optimised via routine experimentation.

On drying, a composition is formed which incorporates the viral particles. A matrix incorporating the viral particles is thus produced. The composition is typically an amorphous solid. A solid matrix, generally an amorphous solid matrix, is thus generally formed. By "*amorphous*" is meant non-structured and having no observable regular or repeated organization of molecules (i.e. non-crystalline). The drying procedure can be effected to form an amorphous cake e.g. by freeze-drying.

Freeze-drying

Freeze-drying can be carried out according to standard procedures. There are three main stages: freezing, primary drying and secondary drying. Freezing is typically performed using a freeze-drying machine. In this step, it is important to cool the biological material below its eutectic point, the lowest temperature at which the solid and liquid phase of the material can coexist. This ensures that sublimation rather than melting will occur in the following steps. Alternatively, amorphous materials do not have a eutectic point, but do have a critical point, below which the product must be maintained to prevent melt-back or collapse during primary and secondary drying.

During primary drying the pressure is controlled by the application of appropriate levels of vacuum whilst enough heat is supplied to enable the water to sublimate. At least 50%, typically 60 to 70%, of the water in the material is sublimated at this stage. Primary drying may be slow as too much heat could degrade or alter the structure of the biological material. A cold condenser chamber and/or condenser plates provide surfaces on which the water vapour is trapped by resolidification.

In the secondary drying process, water of hydration is removed by the further application of heat. Typically, the pressure is also lowered to encourage further drying. After completion of the freeze-drying process, the vacuum can either be

broken with an inert gas such as nitrogen prior to sealing or the material can be sealed under vacuum.

Vacuum drying

In certain embodiments, drying is carried out using vacuum desiccation at around 1300Pa. However vacuum desiccation is not essential to the invention and in other embodiments, the preservation mixture contacted with the viral particle is spun (i.e. rotary desiccation) or freeze-dried (as further described below). Advantageously, the method of the invention further comprises subjecting the preservation mixture containing the viral particle to a vacuum. Conveniently, the vacuum is applied at a pressure of 20,000Pa or less, preferably 10,000Pa or less. Advantageously, the vacuum is applied for a period of at least 10 hours, preferably 16 hours or more. As known to those skilled in the art, the period of vacuum application will depend on the size of the sample, the machinery used and other parameters.

Spray-drying and spray freeze-drying

In another embodiment, drying is achieved by spray-drying or spray freeze-drying the viral particles admixed with the preservation mixture of the invention. These techniques are well known to those skilled in the art and involve a method of drying a liquid feed through a gas e.g. air, oxygen-free gas or nitrogen or, in the case of spray freeze-drying, liquid nitrogen. The liquid feed is atomized into a spray of droplets. The droplets are then dried by contact with the gas in a drying chamber or with the liquid nitrogen.

Fluid bed drying

In a further embodiment, drying is achieved by fluid bed drying the viral particles admixed with the preservation mixture of the invention. This technique is well known to those skilled in the art and typically involves passing a gas (e.g. air) through a product layer under controlled velocity conditions to create a fluidized state.

The technique can involve the stages of drying, cooling, agglomeration, granulation and coating of particulate product materials.

Heat may be supplied by the fluidization gas and/or by other heating surfaces (e.g. panels or tubes) immersed in the fluidized layer. Cooling can be achieved using a cold gas and/or cooling surfaces immersed in the fluidized layer. The steps of agglomeration and granulation are well known to those skilled in the art and can be performed in various ways depending on the product properties to be achieved. Coating of particulate products such as powders, granules or tablets can be achieved by spraying a liquid on the fluidized particles under controlled conditions.

The composition that is produced by the freezing or drying is typically a solid matrix having a low residual moisture content. A level of residual moisture content is achieved which offers long term preservation of vaccine activity at temperatures greater than refrigeration temperatures, e.g. from 4°C to 56°C or more, or lower than refrigeration temperatures, e.g. from 0°C to −70°C or below. The composition that is produced according to the invention may thus have a residual moisture content of 5% or less, 2% or less or 1% or less by weight. Typically the composition has residual moisture content of from 0.1 to 5% or from 0.5 to 5%.

The composition can be obtained in dry powder form. A cake resulting from the drying, e.g. freeze-drying step can be milled to powder form. A solid composition according to the invention thus may take the form of free-flowing particles. The solid composition is typically provided as a powder in a sealed vial, ampoule or syringe. If for inhalation the powder can be provided in a dry powder inhaler. The solid matrix can alternatively be provided as a patch. A powder may be compressed into tablet form.

The composition may consist, or consist essentially, of: the aluminium-salt adjuvant; one or more antigens; the compound of formula (I) or (II) or a physiologically acceptable salt or ester thereof; and optionally one or more sugars.

Use of compositions of the invention

The frozen or dried vaccine compositions are converted into liquid form (aqueous solution) prior to administration to a patient. A frozen composition is thawed and diluted as necessary with e.g. phosphate-buffered saline or Water for Injections. A dried composition is reconstituted as an aqueous solution, for example by phosphate-buffered saline or Water for Injections. The resulting aqueous solution can then be administered, e.g. by injection, to a patient in need of vaccination.

The compound of formula (I) or (II) or a physiologically acceptable salt or ester thereof and, optionally, one or more sugars, typically acts as a resuspension agent for the vaccine composition, for example when it is converted into liquid form (aqueous solution) prior to administration to a patient.

Protection against adverse effects of freezing or drying

Aluminium salt adjuvants in their natural form are commonly in the form of gels that are particulate suspensions in aqueous media. Freezing or drying often causes structural alterations typified by an increased particle size with corresponding increased sedimentation rates and tighter packing of the sedimented solid compounds. Using the present invention, however, damage in the form of increased particle size, increased sedimentation rate and/or tighter packing of sedimented solids as a result of freezing or freeze-drying can be reduced.

Structural damage in the form of increased particle size with corresponding increased sedimentation rates and tighter packing of the sedimented solid compounds can been assessed using the adjuvant agglomeration assay described in Example 1. Other analytical methods for assessing the physiochemical characteristics of aluminium adjuvants before and after freezing or freeze-drying may also be used. For example, particle size distributions of the aluminium gel particles can be obtained using laser diffraction analysis, X-ray diffraction or infrared spectroscopy. Microscopy can also be used to visualise structural changes.

The following Examples illustrate the invention. A Reference Example is also provided.

Reference Example

Adjuvant

Aluminium hydroxide gel ($Al(OH)_3$) was obtained from Sigma (A8222) as a 13mg/ml solution (with a pH of 6.8).

Freezing the adjuvant

The adjuvant was frozen by being placed in a laboratory freezer where it was left overnight at -20°C. It was then allowed to thaw at and equilibrate to room temperature (approximately 20°C).

Microscopic analysis

Adjuvants were examined microscopically at a magnification of 100x. Examples of the normal, undamaged amorphous structure and damaged agglomerated crystalline structure post-freezing of aluminium hydroxide are shown in Figure 1. Photograph A shows the evenly distributed particulate suspension of undamaged adjuvant compared with photograph B which shows the formation of large agglomerated flat crystal structures typical of freeze-damaged adjuvant.

Example 1

Methods

The aluminium hydroxide adjuvant was obtained from Sigma (A8222) as a 13mg/ml solution at pH 6.8. Initially, 50µl volumes of the aluminium hydroxide were added to 100µl volumes of sucrose and/or a further excipient diluted in Dulbecco's phosphate buffered saline (PBS) in wells of 96 well flat bottomed microplates. The further excipient was DMG. A list of final concentrations of DMG and sucrose before freezing can be seen in Table 1 below.

The adjuvants were frozen at –20°C. After approximately 18 hours samples containing $Al(OH)_3$ were thawed and assessed for sediment levels as described using the adjuvant agglomeration assay described below.

Table 1

Excipient	Excipient Concentration (M)	Sucrose Concentration (mM)
DMG	1	234
DMG	0.33	234
DMG	0.1	234
DMG	0	234
DMG	1	117
DMG	0.33	117
DMG	0.1	117
DMG	0	117
DMG	1	58
DMG	0.33	58
DMG	0.1	58
DMG	0	58
DMG	1	29
DMG	0.33	29
DMG	0.1	29
DMG	0	29
DMG	1	0
DMG	0.33	0
DMG	0.1	0
DMG	0	0

Adjuvant agglomeration assays

The amount of agglomeration was assessed by taking up samples from each well into 100µl micropipettes, allowing resettling to occur for 1 hour at room temperature and then measuring the height of the sedimented gel as a percentage of the total height of the solution in the pipette. The height of the sedimented gel as a percentage of the total height of the solution in the pipette was expressed as % gel volume. The greater the % gel volume, the more structurally intact is the adjuvant.

Results and Discussion

Results from these studies are shown in two forms in Figure 2. Firstly simple XY scatter plots are shown and this is complimented by 3D sheet plots.

In the absence of any DMG or sucrose, only a 30% recovery of adjuvant was measured indicating a very significant loss in adjuvant structure had occurred during freeze thaw (~70% loss). Increasing the concentration of sucrose in the formulation increased the recovery of adjuvant to a maximum of ~70% at the highest concentration tested (234mM, approx 8% w/v).

Increasing the concentration of DMG alone increased the recovery of adjuvant. A good dose dependent response was observed and it was possible to achieve near 100% recovery with DMG alone.

Coformulation of the adjuvant with both DMG and sucrose significantly reduced the amount of DMG required to achieve near 100% recovery.

Example 2

Methods

The aluminium hydroxide adjuvant was obtained from Sigma (A8222) as a 13mg/ml solution at pH 6.8. Initially, 50µl volumes of the aluminium hydroxide adjuvant were added to 100µl volumes of sucrose and/or a further excipient diluted in Dulbecco's phosphate buffered saline (PBS) in wells of 96 well flat bottomed microplates. The further excipients were S-methyl-L-methionine, MSM and TMG. The adjuvants were frozen at –20°C. A list of final concentrations of sucrose and the further excipient before freezing can be seen in Table 2 below.

After approximately 18 hours samples containing $Al(OH)_3$ were thawed and assessed for sediment levels as described using the adjuvant agglomeration assay in Example 1.

Table 2

Further excipient	Concentration (M) of further excipient	Sucrose concentration (mM)
S-Methyl-L-methionine	1	234
S-Methyl-L-methionine	0.33	234
S-Methyl-L-methionine	0.1	234
S-Methyl-L-methionine	0	234
S-Methyl-L-methionine	1	117
S-Methyl-L-methionine	0.33	117
S-Methyl-L-methionine	0.1	117
S-Methyl-L-methionine	0	117
S-Methyl-L-methionine	1	58
S-Methyl-L-methionine	0.33	58
S-Methyl-L-methionine	0.1	58
S-Methyl-L-methionine	0	58
S-Methyl-L-methionine	1	29
S-Methyl-L-methionine	0.33	29
S-Methyl-L-methionine	0.1	29
S-Methyl-L-methionine	0	29
S-Methyl-L-methionine	1	0
S-Methyl-L-methionine	0.33	0
S-Methyl-L-methionine	0.1	0
S-Methyl-L-methionine	0	0
MSM	0.53	234
MSM	0.26	234
MSM	0.13	234
MSM	0.07	234
MSM	0	234
MSM	0.53	117
MSM	0.26	117
MSM	0.13	117
MSM	0.07	117
MSM	0	117
MSM	0.53	58
MSM	0.26	58
MSM	0.13	58
MSM	0.07	58
MSM	0	58
MSM	0.53	29
MSM	0.26	29
MSM	0.13	29

MSM	0.07	29
MSM	0	29
MSM	0.53	0
MSM	0.26	0
MSM	0.13	0
MSM	0.07	0
MSM	0	0
TMG	1	234
TMG	0.33	234
TMG	0.1	234
TMG	0	234
TMG	1	117
TMG	0.33	117
TMG	0.1	117
TMG	0	117
TMG	1	58
TMG	0.33	58
TMG	0.1	58
TMG	0	58
TMG	1	29
TMG	0.33	29
TMG	0.1	29
TMG	0	29
TMG	1	0
TMG	0.33	0
TMG	0.1	0
TMG	0	0

Results and Discussion

Results from these studies are shown in two forms in Figures 3 and 4. Firstly simple XY scatter plots are shown and this is complimented by 3D sheet plots.

In the absence of both sucrose and a further excipient only a 30% recovery of adjuvant was measured, indicating a very significant loss in adjuvant structure had occurred during freeze thaw (~70% loss). Increasing the concentration of sucrose in the formulation increased the recovery of adjuvant to a maximum of ~70% at the top concentration tested (234mM, approx 8% w/v).

Increasing the concentration of the further excipient alone (TMG and S-Methyl-L-methionine) increased the recovery of adjuvant. In each of these cases, a

good dose dependent response was observed and it was possible to achieve near 100% recovery with the further excipient alone.

Coformulation of the adjuvant with both sucrose and one of the further excipients (TMG or S-Methyl-L-methionine) significantly reduced the amount of the further excipient required to achieve near 100% recovery.

Example 3

Methods

The aluminium hydroxide adjuvant was obtained from Sigma (A8222) as a 13mg/ml solution at pH 6.8. A volume of adjuvant was centrifuged to form a pellet which was subsequently washed in 40mM HEPES + 25mM NaCl at pH 7.9 (twice) and re-suspended in half the original volume, resulting in an approximately 26 mg/ml solution. Into each vial was added 75 µl of 26 mg/ml adjuvant solution and 225 µl of relevant excipient (adjusted in concentration to account for added adjuvant volume) to equal the appropriate concentration, with each vial containing a final adjuvant concentration of 6.5 mg/ml. A list of final concentrations of excipients are set out in Table 4 below.

Samples were freeze dried by the VirTis Advantage freeze dryer, using the drying cycles shown in Table 3 below, lasting for approximately 3 days. Samples were frozen at -40°C for 2 hours before a vacuum was applied, initially at 300 milliTorre with a Thermo Savant VLP pump (Thermofisher, UK). Shelf temperature and vacuum were adjusted throughout the process and the condenser was maintained at -80°C. Step 11 was extended until the samples were stoppered before releasing the vacuum.

In the primary drying phase the shelf temperature was dropped to –45°C. The secondary drying phase included series of hold steps increasing in temperature up to 30°C until the drying was completed. Probes recorded shelf temperatures and condenser temperatures.

Table 3

Step	Shelf temp (°C)	Time (mins)	Ramp/Hold	Vacuum (milliTorre)
1	-45	15	H	-
2	-34	30	R	300
3	-34	1200	H	300
4	-20	120	H	300
5	-10	120	H	300
6	0	120	H	300
7	10	120	H	80
8	20	120	H	80
9	30	1255	H	80
10	30	905	H	80
11	4	1255	H	80

Adjuvant agglomeration assays

The vials containing freeze-dried adjuvant were reconstituted into 300 µl of purified water and vortexed. The amount of agglomeration was assessed by taking up samples from each well into 100µl micropipettes, allowing resettling to occur for 90 minutes at room temperature and then measuring the height of the sedimented gel as a percentage of the total height of the solution in the pipette. The height of the sedimented gel as a percentage of the total height of the solution in the pipette was expressed as % gel volume. The greater the % gel volume, the more structurally intact is the adjuvant.

Table 4

Further excipient	Conc. (mM) of further excipient	Conc. (mM) of sucrose	% gel volume
None	0	500	100
None	0	334	93.2
None	0	167	47.3
None	0	84.2	32.3
None	0	1	14.7
None	0	0	16.7
DMG	1	500	99.6
DMG	1	1	12.4

DMG	500	1	99.7
DMG	1	334	96.2
DMG	167	334	100
DMG	1	167	37.2
DMG	167	167	99.7
DMG	334	167	100
DMG	167	1	79.82
DMG	334	1	99.5
DMG	84	334	99.6
DMG	84	84	50.4
DMG	334	84	59.5
DMG	1	500	99.6
DMG	1	1	18.1
DMG	500	1	100
DMG	167	167	100
TMG	1	500	99.6
TMG	1	1	13.5
TMG	500	1	99.3
TMG	1	334	93.0
TMG	167	334	100
TMG	1	167	39.8
TMG	167	167	99.6
TMG	334	167	100
TMG	167	1	60.9
TMG	334	1	84.7
TMG	84	334	90.4
TMG	84	84	51.2
TMG	334	84	94.1
TMG	1	500	100
TMG	1	1	14.1
TMG	500	1	99.5
TMG	167	167	100
SMM	1	500	100
SMM	1	1	13.7
SMM	500	1	100
SMM	1	334	95.4
SMM	167	334	98.1
SMM	1	167	40.0
SMM	167	167	100
SMM	334	167	100
SMM	167	1	98.0
SMM	334	1	100
SMM	84	334	100

SMM	84	84	99.6
SMM	334	84	99.2
SMM	1	500	98.2
SMM	1	1	14.9
SMM	500	1	100
SMM	167	167	100
Sarcosine	1	500	98.1
Sarcosine	1	1	13.8
Sarcosine	500	1	100
Sarcosine	1	334	91.3
Sarcosine	167	334	98.2
Sarcosine	1	167	40.6
Sarcosine	167	167	98.5
Sarcosine	334	167	100
Sarcosine	167	1	50.7
Sarcosine	334	1	100
Sarcosine	84	334	97.2
Sarcosine	84	84	65.9
Sarcosine	334	84	100
Sarcosine	1	500	98.1
Sarcosine	1	1	14.1
Sarcosine	500	1	100
Sarcosine	167	167	98.4

Results and Discussion

The results are set out in Table 4 above and graphically in Figure 5. These confirm that adjuvant structure can be maintained upon freeze-drying adjuvant solutions in the presence of a range of concentrations of (i) DMG, TMG, S-methyl methionine or sarcosine, and (ii) sucrose. Generally, the cake quality is better for lower concentrations of the further excipient and higher concentrations of sucrose.

Example 4

Materials and equipment

Mannitol:	Sigma	Lot #: 077K0166
DMG:	Sigma	Lot # 077K0166

TMG	Sigma	Lot# 049K1529
SMM	Sigma	Lot # 001425374
Sarcosine	Sigma	Lot # 078K3727
Aluminium Hydroxide Gel	Sigma	Lot # 018K0761
Virtis Advantage Plus Freeze-Dryer:	Virtis	EQP # 084
Purified Water:	Sigma	Lot # RNBB2958
Micropipettes (capillary tubes):	Blaubrand,	Lot # 7091 44
Freeze Drying Vials:	Adelphi 2ml VCDIN2R	
Stoppers:	Adelphi FDW13 13mm	

Methods

Taking into account the 75 µl of adjuvant added per 300 µl freeze dry vial (1/4 volume, therefore 25% more concentrated) the following excipient mixes were created in HEPES buffer as 10ml master mixes:

Table 5

	Mannitol [M]				
	0.548	0.274	0.137	0.069	0 %
Excipient [mM]	500				
	250				
	125				
	62.5				
	31.25				
	15.63				
	0				

To each vial was added 75 µl of 26 mg/ml aluminium hydroxide adjuvant (which was prepared by centrifuging 13mg/ml aluminium hydroxide gel and resuspending the pellet in half the original volume) and 225 µl of appropriate excipient mix, as listed above. The vials were then stoppered before placing in the VirTis Advantage freeze dryer, using the drying cycles shown in Table 6 below.

Table 6

Step	Temperature (°C)	Time (minutes)	Vacuum (MTorr)
1	-40	45	500
2	-36	600	200
3	-20	120	300
4	-10	120	300
5	0	120	300
6	10	120	80
7	20	120	80
8	30	1255	80
9	4	1255	80

Table 6

After freeze drying the vials were stoppered under vacuum, capped and photographs were taken. Adjuvant agglomeration was assessed as set out in Example 3.

Results and Discussion

The results are set out in Table 7 to 10 below.

Table 7A

Mannitol [M]	0.548	0.548	0.548	0.548	0.548	0.548	0.548
DMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	100	100	98	97	98.5

Table 7B

Mannitol [M]	0.274	0.274	0.274	0.274	0.274	0.274	0.274
DMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	100	98	77.5	96	50

Table 7C

Mannitol [M]	0.137	0.137	0.137	0.137	0.137	0.137	0.137
DMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	98	100	96	73.3	43.75	46.2	32.5

Table 7D

Mannitol [M]	0.069	0.069	0.069	0.069	0.069	0.069	0.069
DMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	90	28.6	26.2	21.2	16.7

Table 7E

Mannitol [M]	0.041	0.041	0.041	0.041	0.041	0.041	0.041
DMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	99	99	90	42.5	27.0	18.8	12.5

Table 7F

Mannitol [M]	0.0206	0.0206	0.0206	0.0206	0.0206	0.0206	0.0206
DMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	99	98	95	41.5	25.0	14.0	12.5

Table 7G

Mannitol M	0	0	0	0	0	0	0
DMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	97	80	31.6	18.6	13.6	11.8

Table 8A

Mannitol M	0.548	0.548	0.548	0.548	0.548	0.548	0.548
TMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	100	100	97	94	92

Table 8B

Mannitol M	0.274	0.274	0.274	0.274	0.274	0.274	0.274
TMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	100	100	88.9	80	45

Table 8C

Mannitol M	0.137	0.137	0.137	0.137	0.137	0.137	0.137
TMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	98	87.5	50	50	38.6	35.7

Table 8D

Mannitol M	0.069	0.069	0.069	0.069	0.069	0.069	0.069
TMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	99	98	90	50	25	15.8	11.6

Table 8E

Mannitol M	0.041	0.041	0.041	0.041	0.041	0.041	0.041
TMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	98	50	25.0	15.8	11.6

Table 8F

Mannitol M	0.0206	0.0206	0.0206	0.0206	0.0206	0.0206	0.0206
TMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	98	96	80	44.0	20.0	16.6	13.5

Table 8G

Mannitol M	0	0	0	0	0	0	0
TMG [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	98	90	50	27.7	15.5	10.7	9.9

Table 9A

Mannitol M	0.548	0.548	0.548	0.548	0.548	0.548	0.548
SMM [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	100	98	95	100	95.0

Table 9B

Mannitol M	0.274	0.274	0.274	0.274	0.274	0.274	0.274
SMM [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	100	100	100	90	48

Table 9C

Mannitol M	0.137	0.137	0.137	0.137	0.137	0.137	0.137
SMM [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	98	100	98	98	62.5	50	31.9

Table 9D

Mannitol M	0.069	0.069	0.069	0.069	0.069	0.069	0.069
SMM [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	97	100	88.2	27.5	23.4	18.75

Table 9E

Mannitol M	0.041	0.041	0.041	0.041	0.041	0.041	0.041
SMM [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	97	97	78.6	24.7	15.7

Table 9F

Mannitol M	0.0206	0.0206	0.0206	0.0206	0.0206	0.0206	0.0206
SMM [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	98	98	90	98	45	23.8	12.5

Table 9G

Mannitol M	0	0	0	0	0	0	0
SMM [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	100	96	34.4	26.2	11.1

Table 10A

Mannitol M	0.548	0.548	0.548	0.548	0.548	0.548	0.548
Sarc. [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	97	88	97	88	89

Table 10B

Mannitol M	0.274	0.274	0.274	0.274	0.274	0.274	0.274
Sarc. [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	100	98	87.5	88.9	39.8

Table 10C

Mannitol M	0.137	0.137	0.137	0.137	0.137	0.137	0.137
Sarc. [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	98	100	61.2	40	35.7	33.7	30.8

Table 10D

Mannitol M	0.069	0.069	0.069	0.069	0.069	0.069	0.069
Sarc. [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	98	34.1	22.7	21.9	20.4

Table 10E

Mannitol M	0.041	0.041	0.041	0.041	0.041	0.041	0.041
Sarc. [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	98	28.6	20	14.8	13.1

Table 10F

Mannitol M	0.0206	0.0206	0.0206	0.0206	0.0206	0.0206	0.0206
Sarc. [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	97	97	80	38	20	13.5	12.6

Table 10G

Mannitol M	0	0	0	0	0	0	0
Sarc. [mM]	500	250	125	62.5	31.25	15.625	0
% Gel height	100	100	90	37.5	17.7	12.8	11.1

These results show the presence of DMG, TMG, SMM and sarcosine allows for reduction in the concentration of sugars without a reduction of adjuvant protection. This demonstrates the clear role of excipients in adjuvant stabilisation.

Example 5

Materials and equipment

Mannitol:	Sigma	Lot #: 077K0166
DMG:	Sigma	Lot # 077K0166
TMG	Sigma	Lot# 049K1529
SMM	Sigma	Lot # 001425374

Sarcosine	Sigma	Lot # 078K3727
Aluminium Hydroxide Gel	Sigma	Lot # 018K0761
Virtis Advantage Plus Freeze-Dryer:	Virtis	EQP # 084
Purified Water:	Sigma	Lot # RNBB2958
Micropipettes (capillary tubes):	Blaubrand,	Lot # 7091 44
Freeze Drying Vials:	Adelphi 2ml VCDIN2R	
Stoppers:	Adelphi FDW13 13mm	

Methods

Taking into account the 75 µl of adjuvant added per 300 µl freeze dry vial (1/4 volume, therefore 25% more concentrated) the following excipient mixes were created in HEPES buffer in 10ml master mixes:

Table 11

	Mannitol [M]					
	0.767	0.657	0.548	0.438	0.329	0
Excipient [M]	1.4					
	1.2					
	1.0					
	0.8					
	0.6					
	0					

To each vial was added 75 µl of 26 mg/ml aluminium hydroxide adjuvant (which was prepared by centrifuging 13mg/ml aluminium hydroxide gel and resuspending the pellet in half the original volume) and 225 µl of appropriate excipient mix, as listed above. The vials were then stoppered before placing in the freeze drier and run on the cycle set out in Table 12.

Table 12

Step	Temperature (°C)	Time (minutes)	Vacuum (MTorr)
1	-40	45	
2	-36	600	200
3	-20	120	300
4	-10	120	300
5	0	120	300
6	10	120	80
7	20	120	80
8	30	1255	80
9	4	1255	80

After freeze drying the vials were stoppered under vacuum, capped and photographs were taken. Adjuvant agglomeration was assessed as set out in Example 3.

Results and discussion

The results are set out in Table 13 and 14 below.

Table 13

Mannitol [M]	Excipient Type / Concentration [mM]	% Protection
0.767	DMG 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	100
	0.6 M	100
	0 M	86
0.657	DMG 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	100
	0.6 M	100
	0 M	75
0.548	DMG 1.4 M	100
	1.2 M	100
	1.0 M	100

	0.8 M	100
	0.6 M	100
	0 M	63
0.438	DMG 1.4 M	100
	1.2 M	100
	1.0 M	96
	0.8 M	100
	0.6 M	100
	0 M	50
0.329	DMG 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	96
	0.6 M	100
	0 M	23
0	DMG 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	96
	0.6 M	96
	0 M	11
0.767	TMG 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	100
	0.6 M	100
	0 M	90
0.657	TMG 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	98
	0.6 M	100
	0 M	80
0.548	TMG 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	100
	0.6 M	100
	0 M	70
0.438	TMG 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	100
	0.6 M	100
	0 M	50
0.329	TMG 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	96
	0.6 M	100
	0 M	50
0	TMG 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	100
	0.6 M	96
	0 M	14

Table 14

Mannitol [M]	Excipient Type / Concentration [mM]	% Protection
0.767	SMM 1.4 M	100
	1.2 M	100
	1.0 M	98
	0.8 M	98
	0.6 M	98
	0 M	82
0.657	SMM 1.4 M	100
	1.2 M	98
	1.0 M	98
	0.8 M	98
	0.6 M	100
	0 M	75
0.548	SMM 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	100
	0.6 M	100
	0 M	68
0.438	SMM 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	100
	0.6 M	98
	0 M	52
0.329	SMM 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	98
	0.6 M	100
	0 M	46
0	SMM 1.4 M	100
	1.2 M	100
	1.0 M	100
	0.8 M	100
	0.6 M	95
	0 M	12

These results show the presence of DMG, TMG and SMM allows for reduction in the concentration of sugars without a reduction of adjuvant protection. This demonstrates the clear role of excipients in adjuvant stabilisation.

Example 6

Introduction

Bovine serum albumin (BSA) is commonly used as a model in experiments where, for example, protein adsorption onto an adjuvant is to be measured.

Materials and equipment

BSA:	Sigma P5369	Lot 058K6061
Alhydrogel : 2%	Brenntag	Lot 4420
Mannitol:	Sigma	Lot #: 077K0166
DMG:	Sigma	Lot # 077K0166
TMG:	Sigma	Lot# 049K1529
SMM:	Sigma,	Lot # 001425374
DPBS:	Sigma	RNBB1286
Virtis Advantage Plus Freeze-Dryer:	Virtis	EQP # 084
Purified Water:	Sigma	Lot # RNBB2958
Freeze Drying Vials:	Adelphi 2ml VCDIN2R	
Stoppers:	Adelphi FDW13 13mm	
-20 °C Freezer:	Stabilitech EQP #	
Nunc 96-well ELISA plate		
Bradford Reagent:	Sigma B6916-500ML	Batch 080M4359
BioTek Plate Reader:	Stabilitech EQP: 027	

Protein adsorption

Alhydrogel (supplied at 2% stock (w/v)) was added to PBS containing BSA to equal a final 10 ml volume with concentration of 0.52% Alhydrogel and 200 μg/ml BSA. The protein adsorption step was incubated by gently rocking at room temperature before placing overnight at +4 °C.

The following excipient mixes were prepared in 5 ml volumes:

- 1.315 M (24%)Mannitol
- 1.315 M (24%) + 1.6 M DMG
- 1.315 M (24%) + 1.6 M TMG
- 1.315 M (24%) + 1.6 M SMM
- 1.096 M (20 %)Mannitol + 1.2 M DMG
- 1.096 M (20 %) + 1.2 M TMG
- 1.096 M (20 %) + 1.2 M SMM
- 0.877 M (16 %)Mannitol + 0.8 M DMG
- 0.877 M (16 %) + 0.8 M TMG
- 0.877 M (16 %) + 0.8 M SMM
- PBS

Adjuvant-Excipient Processing

The adjuvant was mixed with the excipient concentration in a 1:1 ratio (2ml + 2ml) to create half concentration of excipients above, 0.26 % Alhydrogel and 100 µg/ml BSA. This was incubated at +4 °C for 12 hours before being split off into 300 µl volumes which were either (a) frozen (-80°C), (b) lyophilised as set out in Table 15 below or (c) held at +4 °C as liquid.

Blanks of equivalent volumes were produced and processed as discussed where no protein was included as blanks for the protein assay.

Table 15

Step	Temperature (°C)	Time (minutes)	Vacuum (MTorr)
1	-40	45	
2	-36	600	200
3	-20	120	300
4	-10	120	300
5	0	120	300
6	10	120	80

7	20	120	80
8	30	1255	80
9	4	1255	80

After freeze drying the vials were stoppered under vacuum, capped and photographs were taken and cakes were scored on cake quality were scored on cake quality as described in Example 3.

The liquid, frozen and lyophilised vials were then placed at room temperature to equilibrate/thaw whilst the lyophilised vials were reconstituted in 300 µl of purified water and vortexed until complete reconstitution was observed.

Each of the 300 µl volumes was pulsed on the microfuge for 1 minute to pellet the adjuvant, the supernatant was discarded and the pellet was resuspended in equal volumes of PBS. This procedure was repeated 3 times to completely remove residual excipients from the adjuvant.

Protein assay (Bradford)

Each of the excipient combinations was run in duplicate with a duplicate counterpart blank (i.e. no protein). The liquid, lyophilised and frozen samples were run on separate plates. To standardise protein concentrations each plate was run with a standard curve starting with BSA at 200 µg/ml serially diluted down to 6.25 µg/ml. A volume of 50 µl of adjuvant sample was added to each well before adding 125 µl of Bradford solution (equilibrated to room temperature). The plates were transferred to the plate reader which was set on the plate shake mode (to keep adjuvant in suspension) for 5 minutes before each plate was read at an absorbance at 595nm.

Results

For each plate standard curves were produced (with blanks subtracted) and those standard curves (with y=mx+c equation) were used to ascertain the protein concentrations of the adjuvant samples with their respective blanks also subtracted.

Data was plotted as total protein concentration of the adjuvant sample/ml and also as a percentage of the PBS control.

The results are set out in Table 16 to 18 below and in Figures 6 and 7.

Table 16

Excip [M]	0.8M DMG + 0.66M Mann	0.8M TMG + 0.66M Mann	0.8M SMM + 0.66M Mann	0.66M Mann
% BSA (of PBS Ctrl.)	99.49	98.12	96.76	100.00

Table 17

Excip [M]	0.6M DMG + 0.548 M Mann	0.6M TMG + 0.548 M Mann	0.6M SMM + 0.548 M Mann
% BSA (of PBS Ctrl.)	97.27	101.877	100.68

Table 18

Excip [M]	0.4M DMG + 0.438 M Mann	0.4M TMG + 0.438 M Mann	0.4M SMM + 0.438 M Mann
% BSA (of PBS Ctrl.)	97.088	94.37	95.39

Discussion

. These mannitol and DMG, TMG and SMM concentrations resulted in close to complete structural preservation of the adjuvant structure.

The total protein results demonstrate that BSA levels adsorbed to the adjuvant were comparable across the mannitol-excipient ranges, and indeed to mannitol alone (0.66 M/12%) and PBS. This suggests that there was no elution of the protein caused by the excipients when introduced to pre-adsorbed Alhydrogel. This was also the case for PBS and mannitol. The liquid hold, lyophilisation and incidents of freeze-thaw did not exacerbate any elution.

This experiment shows that the protein is still adsorbed to the adjuvant under conditions where the structure of the adjuvant is preserved during lyophilisation.

Example 7

Introduction

This experiment compares a mannitol base with DMG, TMG, and SMM at levels that have previously been shown to protect adjuvant structure. It compares the antigenicity of the antibody bound to the alum both when the alum antibody has been kept at 4°C and when it has been freeze thawed, using a dot blot to probe the activity of the antibody in both storage methods.

Materials

Chemical

	Supplier	**Product code**	**Lot no.**
PBS x 10		-	-
Tween 20	Sigma	P1379	-
Skimmed milk powder	Marvel	-	-
Alhydrogel	Brenntag	-	4420
TMB Chromogen	Invitrogen	SB02	727643282A
Mouse mAb	Serotec	8437	5208x220610
Anti mouse HRP	Sigma	A0412	077K6008
Mannitol	Sigma	M1902	077K0166
DMG	Sigma	D1156	077K1856U
TMG	Sigma	B2629	049K1529
SMM	Sigma	12209121	0001423374

Other

	Supplier	**Product code**	**Lot no.**
Nitrocellulose membrane	Sigma	N8267	3110
Petri dish	Fisher	FB51504	264541

Equipment

	Manufacturer	Equipment No.
Rocker	Stuart Scientific	EQP#091
Balance	Sartorius	EQP#089
Forma 900 series -80°C freezer	Thermofisher	EQP#015
Scanner	Cannon	-

Methods

Mouse antibody adsorbed onto alum was freeze thawed and kept at 4°C in the presence of various excipients. This was assayed using a dot blot to see if the mouse antibody had retained its antigenicity.

2% alhydrogel solution was diluted to 0.52% with PBS and mouse antibody added to a concentration of 200μg/ml. This was allowed to mix for an hour at room temperature with agitation then put at 4°C over night. The alum-antibody solution was then diluted 1:1 with excipient solutions to give final excipient concentrations as listed below:

-0.657M mannitol

-0.657M mannitol+0.8M DMG

-0.657M mannitol+0.8M TMG

-0.657M mannitol+0.8M SMM

-PBS

These solutions were then split into two aliquots. One of each excipient was kept at 4°C, the other was stored at -80 °C until required.

Dot blot of retained mouse antibody activity

A nitrocellulose membrane was cut to the required size and 2μl of samples applied as dots. This was allowed to dry and then incubated in 10ml PBS +0.05% Tween 20+ 5% milk for 1hour at room temperature on a rocker. This solution was then removed and the membrane then incubated in 10ml of anti-mouse-HRP (horseradish peroxidase) diluted to 1:5000 in PBS +0.05% Tween 20+ 5% millk for

1hour at room temperature on a rocker. The membranes were then washed for 3 x 10 minutes with PBS +0.05% Tween 20. The membranes were blotted on tissue paper to remove excess buffer and then 10ml of TMB (tetramethylbenzidine) was put on to the membrane for 5 minutes. The TMB was then dabbed off and blot colour scanned.

Results

Figure 8 (Table 19 shows the layout of samples tested in Figure 8) shows that in both the liquid (Figure 8A) and freeze-thawed (Figure 8B) samples, all samples not containing antibody are negative as expected and the positive control of antibody only is strongly positive. In the liquid samples all the dots are similar at the same dilutions. The frozen samples are less consistent, especially between the samples in excipient and the PBS control sample. The PBS sample is weaker at the 1:500 dilution then the samples in the different excipients.

Figure 9 (Table 20 shows the layout of samples tested in Figure 9) shows results consistent with this. All the negative controls without antibody are negative, including the excipient only controls which show that the excipients are not interfering with the assay. The PBS samples are again weaker than the liquid samples when frozen, especially when compared to samples in excipient at 1:300 and 1:500.

Table 19 – Layout of samples tested in Figure 7

1. 0.52% alum only	**2.** Mouse mAb 50ug/ml 1:100	**3.** Mouse mAb 50ug/ml 1:500
4. Mouse mAb-12% mannitol-0.26% alum	**5.** Mouse mAb-12% mannitol-0.26% alum 1:100	**6.** Mouse mAb-12% mannitol-0.26% alum 1:500
7. Mouse mAb-12% mannitol-0.8M DMG-0.26% alum	**8.** Mouse mAb-12% mannitol-0.8M DMG-0.26% alum 1:100	**9.** Mouse mAb-12% mannitol-0.8M DMG-0.26% alum 1:500
10. Mouse mAb-12% mannitol-0.8M TMG-0.26% alum	**11.** Mouse mAb-12% mannitol-0.8M TMG-0.26% alum 1:100	**12.** Mouse mAb-12% mannitol-0.8M TMG-0.26% alum 1:500

13. Mouse mAb-12% mannitol-0.8M Vit U 0.26% alum	**14.** Mouse mAb-12% mannitol-0.8M Vit U 0.26% alum 1:100	**15.** Mouse mAb-12% mannitol-0.8M Vit U 0.26% alum 1:500
16. Mouse mAb-PBS-0.26% alum	**17.** Mouse mAb-PBS-0.26% alum 1:100	**18.** Mouse mAb-PBS-0.26% alum 1:500

Table 20 – layout of samples tested in Figure 8

1. 0.52% alum only	**2.** Mouse mAb 50ug/ml	**3.** 12% mannitol-0.8M Vit U 0.26% alum
4. Mouse mAb-12% mannitol-0.26% alum 1:100	**5.** Mouse mAb-12% mannitol-0.26% alum 1:300	**6.** Mouse mAb-12% mannitol-0.26% alum 1:500
7. Mouse mAb-12% mannitol-0.8M DMG-0.26% alum 1:100	**8.** Mouse mAb-12% mannitol-0.8M DMG-0.26% alum 1:300	**9.** Mouse mAb-12% mannitol-0.8M DMG-0.26% alum 1:500
10. Mouse mAb-12% mannitol-0.8M TMG-0.26% alum 1:100	**11.** Mouse mAb-12% mannitol-0.8M TMG-0.26% alum 1:300	**12.** Mouse mAb-12% mannitol-0.8M TMG-0.26% alum 1:500
13. Mouse mAb-12% mannitol-0.8M Vit U 0.26% alum 1:100	**14.** Mouse mAb-12% mannitol-0.8M Vit U 0.26% alum 1:100	**15.** Mouse mAb-12% mannitol-0.8M Vit U 0.26% alum 1:500
16. Mouse mAb-PBS-0.26% alum 1:100	**17.** Mouse mAb-PBS-0.26% alum 1:300	**18.** Mouse mAb-PBS-0.26% alum 1:500
19. 12% mannitol-0.26% alum	**20.** 12% mannitol-0.8M DMG-0.26% alum	**21.** 12% mannitol-0.8M TMG-0.26% alum

Conclusion

Both sets of results show weaker positive results for PBS only samples compared to samples containing excipients when frozen. This shows that the excipients are offering protection to the antibody with alum when compared to antibody with alum alone when the samples are freeze-thawed, as the antibody is retaining its antigenicity more efficiently.

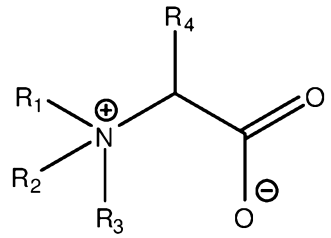
2011234268 04 Jun 2015

CLAIMS

1. A method for preserving an aluminium-salt adjuvant during freezing or freeze-drying comprising freezing or freeze-drying an aqueous suspension or solution comprising:

(a) an aluminium salt adjuvant;

(b) a compound of formula (I) or a physiologically acceptable salt or ester thereof



(I)

wherein:

R_1 represents hydrogen or C_{1-6} alkyl; and

R_4 represents hydrogen; or

R_1 and R_4 together with the atoms to which they are attached form a pyrrolidine ring;

R_2 represents hydrogen, C_{1-6} alkyl or $-(CH_2)_{2-5}NHC(O)(CH_2)_{5-15}CH_3$; and

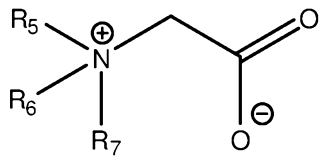
R_3 represents C_{1-6} alkyl; and

(c) one or more sugars.

2. The method according to claim 1 in which the aqueous suspension or solution further comprises at least one antigen.

3. The method according to claim 1 or claim 2 in which the compound of formula (I) is:

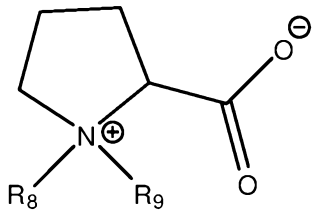
(a) a compound of formula (IA) or a physiologically acceptable salt or ester thereof



(IA)

wherein R_5 and R_6 independently represent C_{1-4} alkyl and R_7 represents C_{1-4} alkyl or $-(CH_2)_{2-5}NHC(O)(CH_2)_{5-15}CH_3$;

(b) a compound of formula (IB) or a physiologically acceptable salt or ester thereof:



(IB)

wherein R_8 and R_9 independently represent C_{1-4} alkyl;

(c) a N,N-di(C_{1-6} alkyl)-, N,N,N-tri(C_{1-6} alkyl)-, or N-C_{1-6} alkyl-glycine or a physiologically acceptable salt or ester thereof;

(d) N,N-dimethylglycine, N,N,N-trimethylglycine, or N-methylglycine or a physiologically acceptable salt or ester thereof;

(e) N,N-dimethylglycine or a physiologically acceptable salt or ester thereof; or

(f) trimethylglycine, cocamidopropyl betaine or proline betaine or a physiologically acceptable salt or ester thereof.

4. The method according to any one of the preceding claims wherein the aluminium salt adjuvant is aluminium phosphate or aluminium hydroxide.

5. The method according to any one of claims 2 to 4 wherein the or each antigen is provided absorbed on the adjuvant.

6. The method according to any one of the preceding claims wherein the concentration of the compound of formula (I) or physiologically acceptable salt or ester thereof is at least 0.1M.

7. The method according to any one of the preceding claims wherein

(i) one sugar is used, or

(ii) one sugar is used and (a) the sugar is sucrose, the concentration of sucrose is from 0.01 to 0.5M or from 0.01 to 0.2M and the concentration of the compound of formula (I) or physiologically acceptable salt or ester thereof is from 0.2 to 5M, or (b) the sugar is sucrose, the concentration of sucrose is from 0.01 to 0.5M or from 0.01 to 0.2M and the concentration of the compound of formula (I) or physiologically acceptable salt or ester thereof is from 0.2 to 2M, or (c) the sugar is mannitol, the concentration of mannitol is from 0.2 to 0.8M and the concentration of the compound of formula (I) or physiologically acceptable salt or ester thereof is from 0.5 to 1M, or

(d) the sugar is sucrose and the concentration of sucrose is from 0.01 to 0.7M or 0.01. to 0.6M or 0.01 to 0.5M.

8. The method according to any one of claims 1 to 6 wherein

(i) two or more sugars are used; or

(ii) two or more sugars are used and (a) sucrose is present with another sugar and the other sugar is raffinose, stachyose or a sugar alcohol, or (b) sucrose is present with another sugar and the other sugar is raffinose.

9. The method according to any one of the preceding claims wherein the suspension or solution is (a) freeze-dried, or (b) freeze-dried to form an amorphous solid matrix.

10. The method according to claim 9 wherein a dried amorphous solid matrix is formed and the solid matrix is provided in the form of a powder in a sealed vial, ampoule or syringe.

11. The method according to any one of any one of claims 1 to 8 wherein (a) the resulting cake is milled to form a powder and the powder is provided in a sealed vial, ampoule or syringe, or (b) the solid matrix forms part of tablet or capsule.

12. Use of an excipient comprising (i) a compound of formula (I) as defined in claim 1 or claim 3 or a physiologically acceptable salt or ester thereof and (ii) one or more sugars, for preserving an aluminium salt adjuvant during freezing or freeze-drying.

13. A vaccine composition comprising:

- an aluminium-salt adjuvant as defined in claim 1 or claim 4;
- one or more antigens;
- a compound of formula (I) as defined in claim 1 or claim 3 or a physiologically acceptable salt or ester thereof; and
- one or more sugars.

14. A vaccine composition obtainable by a method as defined in any one of claims 2 to 11.

15. Use of an excipient comprising (i) a compound of formula (I) as defined in claim 1 or claim 3 or a physiologically acceptable salt or ester thereof and (ii) one or more sugars, as a resuspension agent for a vaccine composition as defined in claim 13 or claim 14.

2011234268 04 Jun 2015

16. A method according to claim 1 and substantially as hereinbefore described with reference to any one of the examples.

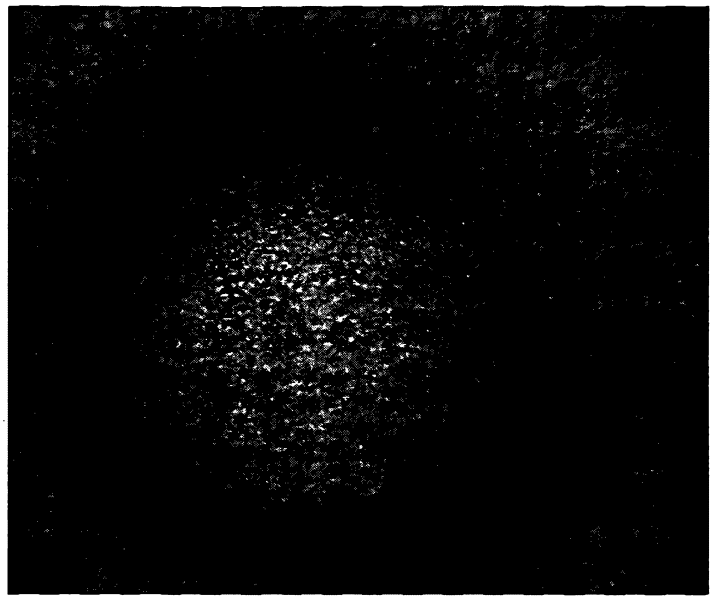
Stabilitech Ltd.

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON

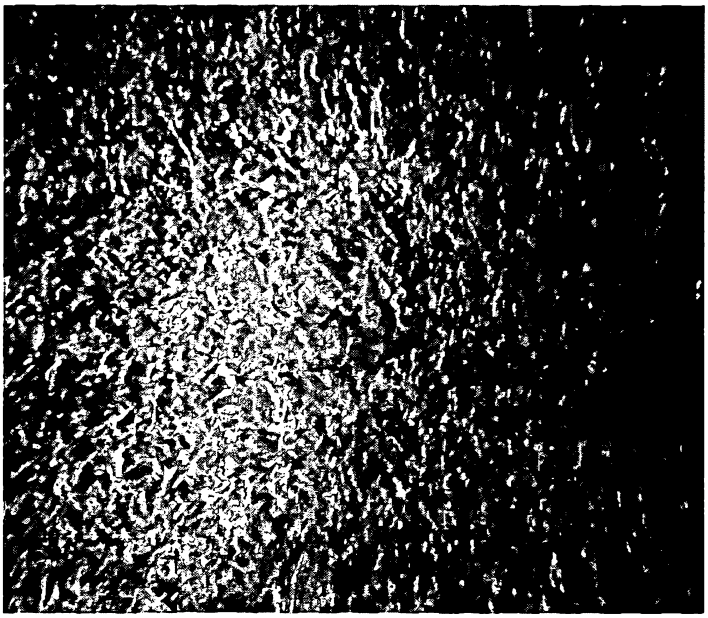
Figure 1

A



Normal adjuvant

B



Freeze damage

Figure 2

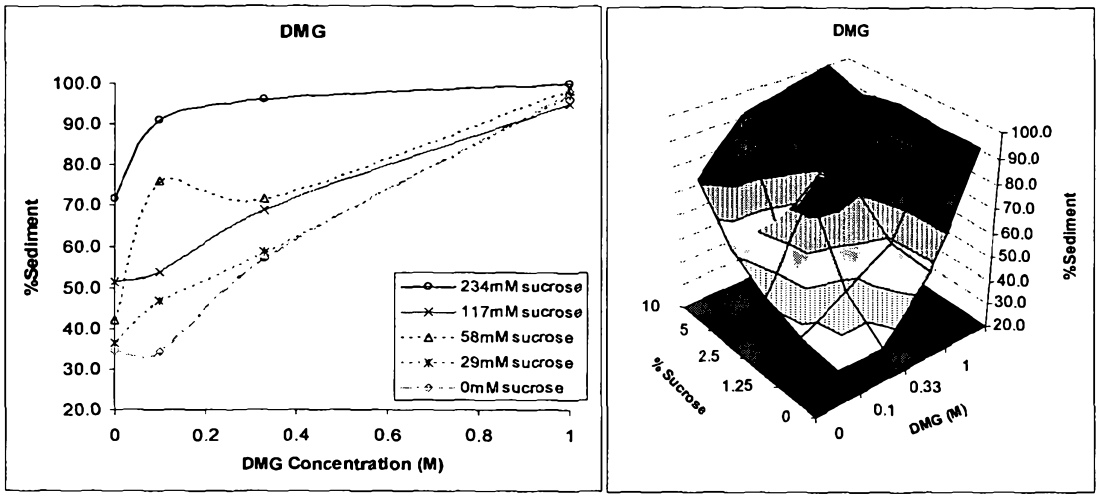


Figure 3

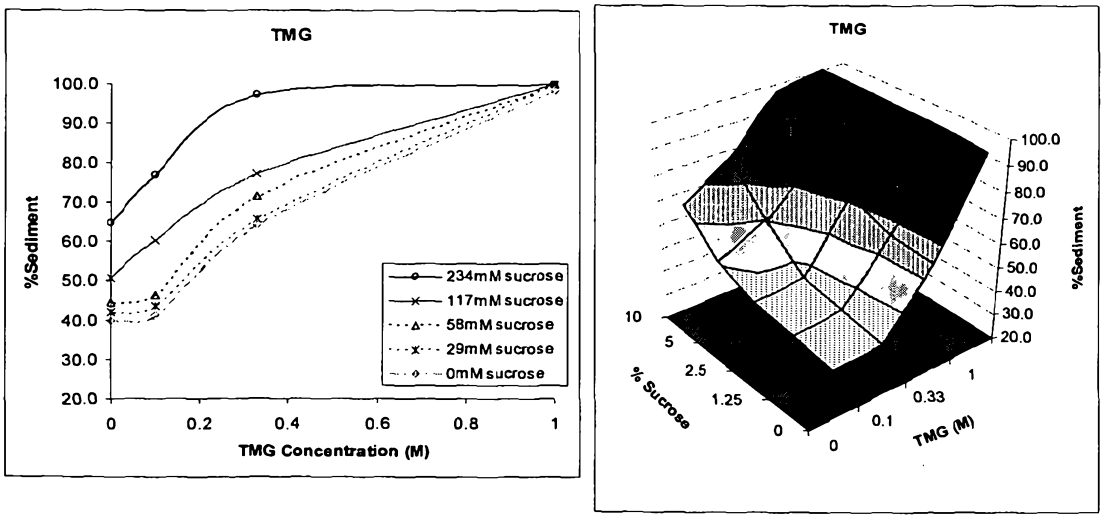


Figure 4

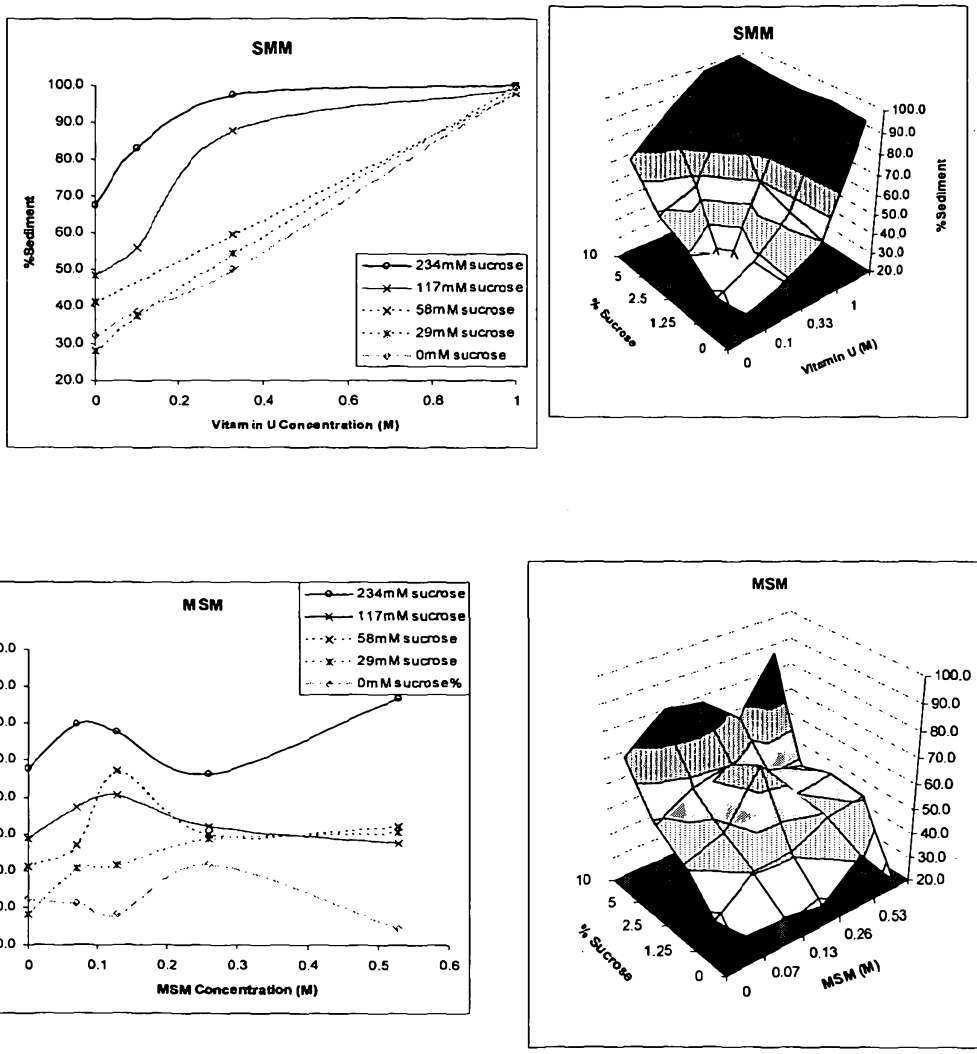

SMM
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
%Sediment
0
0.2
0.4
0.6
0.8
1
Vitamin U Concentration (M)
234mM sucrose
117mM sucrose
58mM sucrose
29mM sucrose
0mM sucrose
SMM
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
%Sediment
10
5
2.5
1.25
0
% Sucrose
0
0.1
0.33
1
Vitamin U (M)
MSM
234mM sucrose
117mM sucrose
58mM sucrose
29mM sucrose
0mM sucrose%
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
%Sediment
0
0.1
0.2
0.3
0.4
0.5
0.6
MSM Concentration (M)
MSM
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
%Sediment
10
5
2.5
1.25
0
% Sucrose
0
0.07
0.13
0.26
0.53
MSM (M)

Figure 5

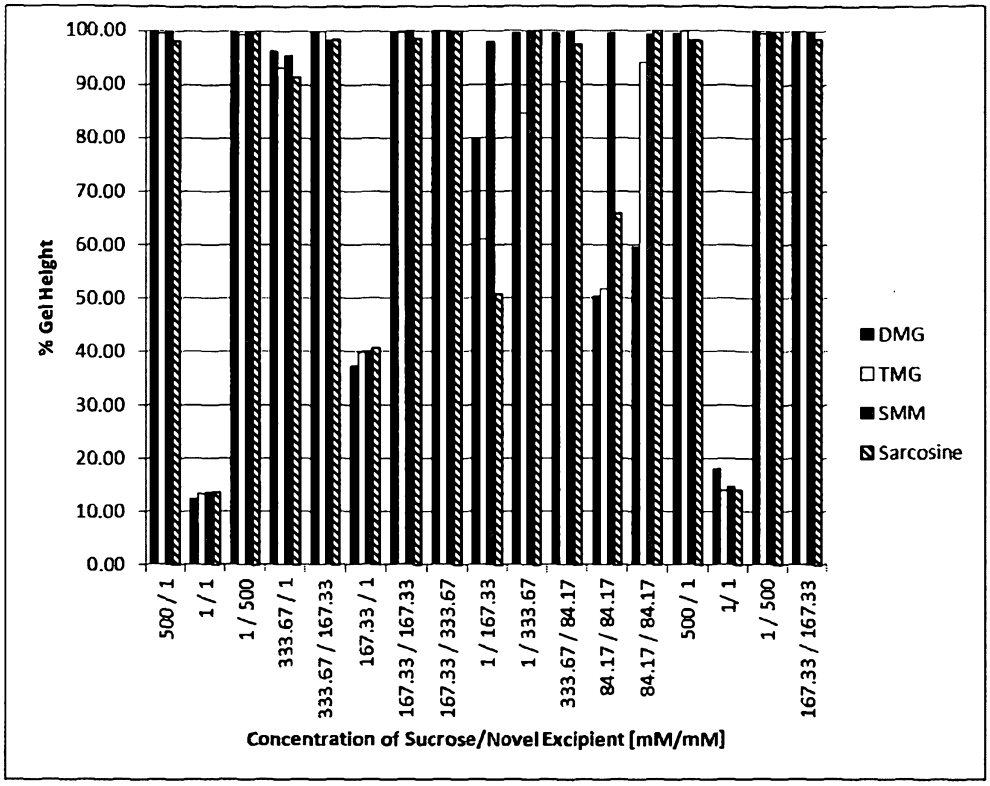

100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
% Gel Height
500 / 1
1 / 1
1 / 500
333.67 / 1
333.67 / 167.33
167.33 / 1
167.33 / 167.33
167.33 / 333.67
1 / 167.33
1 / 333.67
333.67 / 84.17
84.17 / 84.17
84.17 / 84.17
500 / 1
1/ 1
1 / 500
167.33 / 167.33
Concentration of Sucrose/Novel Excipient [mM/mM]
DMG
TMG
SMM
Sarcosine

Figure 6

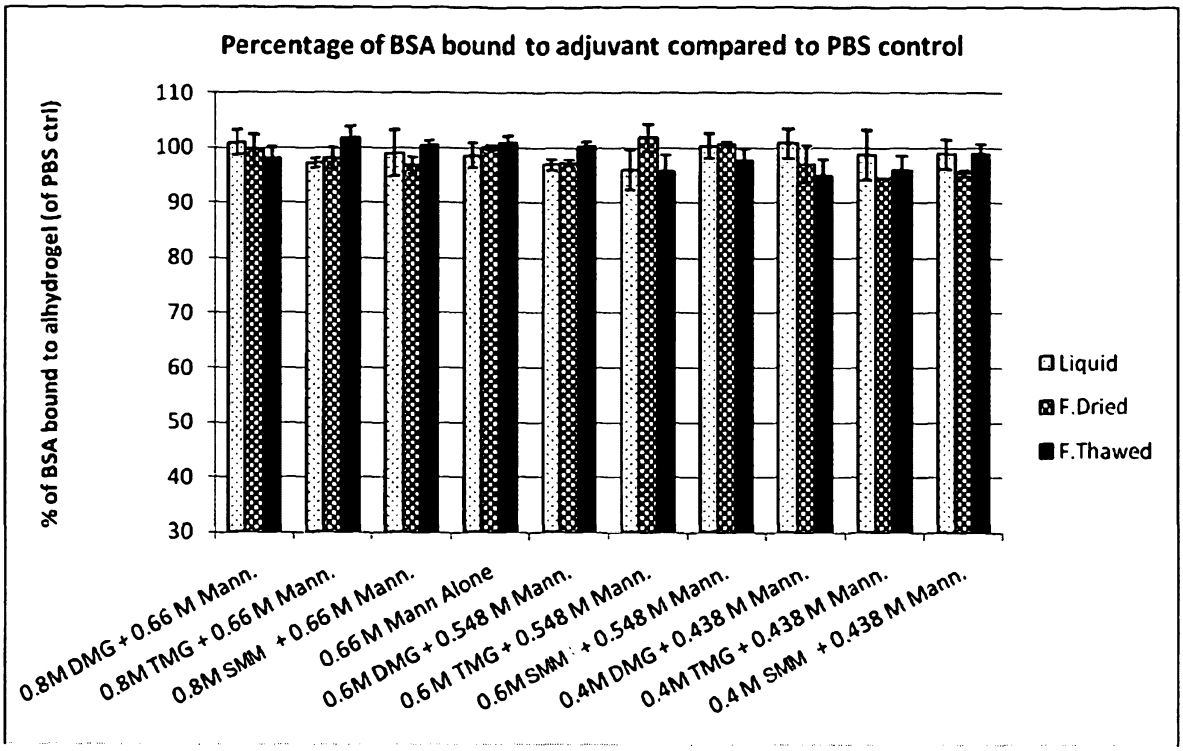

Percentage of BSA bound to adjuvant compared to PBS control
% of BSA bound to alhydrogel (of PBS ctrl)
110
100
90
80
70
60
50
40
30
Liquid
F.Dried
F.Thawed
0.8M DMG + 0.66 M Mann.
0.8M TMG + 0.66 M Mann.
0.8M SMM + 0.66 M Mann.
0.66 M Mann Alone
0.6M DMG + 0.548 M Mann.
0.6 M TMG + 0.548 M Mann.
0.6M SMM + 0.548 M Mann.
0.4M DMG + 0.438 M Mann.
0.4M TMG + 0.438 M Mann.
0.4 M SMM + 0.438 M Mann.

Figure 7

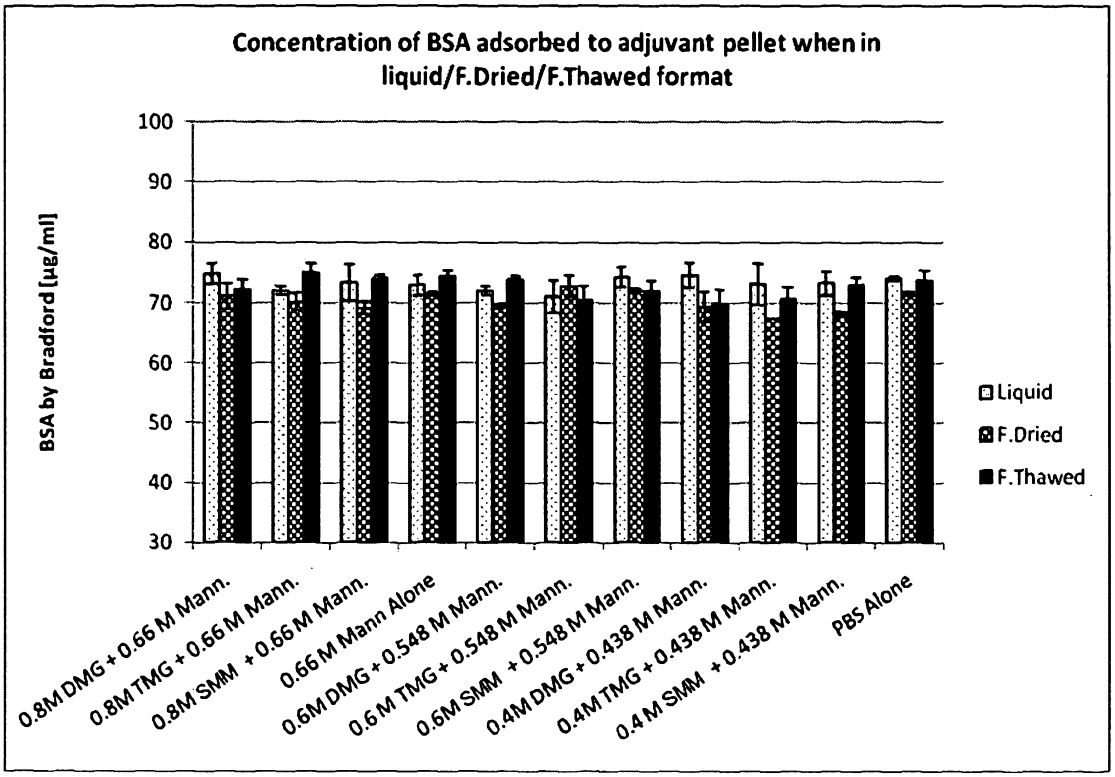

Concentration of BSA adsorbed to adjuvant pellet when in liquid/F.Dried/F.Thawed format
BSA by Bradford [µg/ml]
100
90
80
70
60
50
40
30
Liquid
F.Dried
F.Thawed
0.8M DMG + 0.66 M Mann.
0.8M TMG + 0.66 M Mann.
0.8M SMM + 0.66 M Mann.
0.66 M Mann Alone
0.6M DMG + 0.548 M Mann.
0.6 M TMG + 0.548 M Mann.
0.6M SMM + 0.548 M Mann.
0.4M DMG + 0.438 M Mann.
0.4M TMG + 0.438 M Mann.
0.4 M SMM + 0.438 M Mann.
PBS Alone

Figure 8

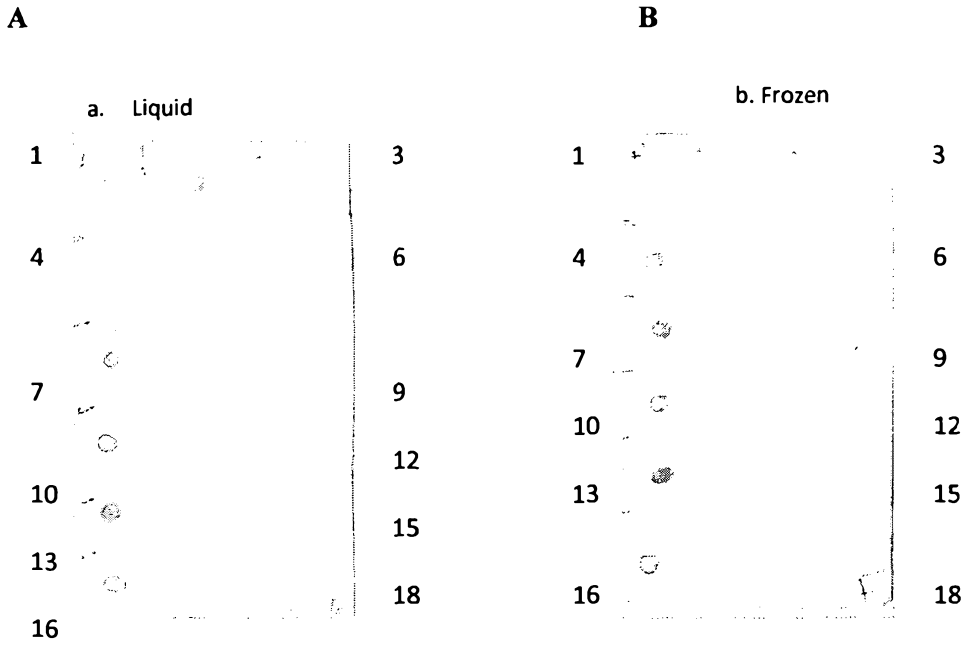


Figure 9