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(73) Patenthaver: **Stabilitech Biopharma Ltd, Unit 6, Sovereign Business Park , Albert Drive , Burgess Hill, West Sussex, RH15 9TY, Storbritannien**

(72) Opfinder: **Drew, Jeffrey, c/o Stabilitech Ltd., Unit 6, Sovereign Business Park, Albert Drive, Burgess Hill, Sussex RH15 9TY, Storbritannien**
**Woodward, David, c/o Stabilitech Ltd., Unit 6, Sovereign Business Park, Albert Drive, Burgess Hill, Sussex RH15 9TY, Storbritannien**
**Bainbridge, John, c/o Stabilitech Ltd., Unit 6, Sovereign Business Park, Albert Drive, Burgess Hill, Sussex RH15 9TY, Storbritannien**
**Corteyn, Amanda, c/o Stabilitech Ltd., Unit 6, Sovereign Business Park, Albert Drive, Burgess Hill, Burgess Hill, Sussex RH15 9TY, Storbritannien**

(74) Fuldmægtig i Danmark: **Zacco Denmark A/S, Arne Jacobsens Allé 15, 2300 København S, Danmark**

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# DESCRIPTION

## Field of the Invention

**[0001]** The invention relates to the stabilisation of viral particles.

## Background to the Invention

**[0002]** Some biological molecules are sufficiently stable that they can be isolated, purified and then stored in solution at room temperature. However, this is not possible for many materials and techniques involving storage at low temperature, addition of stabilizers or cryoprotectants, freeze-drying, vacuum-drying and air-drying have been tried to ensure shelf preservation.

**[0003]** Despite the availability of these techniques, some biological materials still show unsatisfactory levels of stability during storage and some techniques lead to added cost and inconvenience. For example, refrigerated transportation and storage is expensive, and any breaks in temperature control can result in reduced efficacy of the biological molecule. Further, refrigerated transport is often not available for the transport of medicines in countries in the developing world.

**[0004]** Also, the stresses of freeze-drying or lyophilisation can be very damaging to some biological materials. Freeze drying of biopharmaceuticals involves freezing solutions or suspensions of thermosensitive biomaterials, followed by primary and secondary drying. The technique is based on sublimation of water at subzero temperature under vacuum without the solution melting. Freeze-drying represents a key step for manufacturing solid protein and vaccine pharmaceuticals. The rate of water vapour diffusion from the frozen biomaterial is very low and therefore the process is time-consuming. Additionally, both the freezing and drying stages introduce stresses that are capable of unfolding or denaturing proteins.

**[0005]** WO 90/05182 describes a method of protecting proteins against denaturation on drying. The method comprises the steps of mixing an aqueous solution of the protein with a soluble cationic polyelectrolyte and a cyclic polyol and removing water from the solution. Diethylaminoethyldextran (DEAE-dextran) and chitosan are the preferred cationic polyelectrolytes, although polyethyleneimine is also mentioned as suitable.

**[0006]** WO-A-2006/0850082 reports a desiccated or preserved product comprising a sugar, a charged material such as a histone protein and a desiccation- or thermosensitive biological component. The sugar forms an amorphous solid matrix. However, the histone may have immunological consequences if the preserved biological component is administered to a human or animal.

**[0007]** WO 2008/114021 describes a method for preserving viral particles. The method comprises drying an aqueous solution of one or more sugars, a polyethyleneimine and the viral particles to form an amorphous solid matrix comprising the viral particles. The aqueous solution contains the polyethyleneimine at a concentration of 15µM or less based on the number-average molar mass ($M_n$) of the polyethyleneimine and the sugar concentration or, if more than one sugar is present, total sugar concentration is greater than 0.1M.

**[0008]** WO2008058035 teaches the lyophylization of VEE particles in the presence of a saccharide like sucrose, and of a plasticizer like DMSO.

## Summary of the Invention

**[0009]** The present inventors have found that viral preparations are preserved stably by (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, (b) methylsulfonylmethane and (c) sucrose, or sucrose and raffinose, or mannitol, during freeze-drying. Virus activity was preserved following subsequent heat challenge. Virus activity was also preserved during long-term stability tests. Virus activity may also be preserved in the aqueous solution prior to drying. The viruses were protected against damage caused by freeze-drying.

**[0010]** Accordingly, the present invention provides a method for preserving viral particles during freeze-drying comprising:

1. (a) providing an aqueous solution of:
    1. (i) viral particles from *Adenoviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Parvoviridae*, *Picornaviridae*, *Poxviridae, Herpesviridae, Togaviridae, Flaviviridae, Retroviridae, Filoviridae, Papillomaviridae, Caliciviridae, Coronaviridae, Reoviridae* or *Hepadnaviridae,*
    2. (ii) sucrose, or sucrose and raffinose, or mannitol, at a total sugar concentration of 0.1M to 3M,
    3. (iii) 0.1 to 1.5M of N,N-dimethylglycine or N,N,N-trimethylglycine, or a physiologically acceptable salt or ester thereof, and
    4. (iv) 0.1 to 1.5M of methylsulfonylmethane; and
2. (b) freeze-drying the solution to form a composition incorporating said viral particles.

**[0011]** The invention further provides a composition which is solid and freeze-dried, which comprises viral particles from *Herpesviridae, Togaviridae, Flaviviridae, Retroviridae, Filoviridae, Papillomaviridae, Caliciviridae, Coronaviridae, Reoviridae,Hepadnaviridae, Adenoviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Parvoviridae*, *Picornaviridae* or *Poxviridae,* and which is obtainable by a method of the invention.

## Brief Description of the Figures

**[0012]**

Figure 1 shows the results obtained in Reference Example 1. The ability of an excipient to help adenovirus withstand cycling between 37°C and -20°C was assessed. Dimethylsulfone (also called methylsulfonylmethane, MSM) was used as an excipient. p value summary: **=p<0.01, *=p<0.05. The error bars show the standard error of the mean (n = 4).

Figure 2A shows the temperature set for the shelf temperature of the VirTis Advantage freeze dryer used in various of the Examples.

Figure 2B shows the condenser temperature of the VirTis Advantage freeze dryer used in various of the Examples.

Figure 3 shows the results of the experiment of Reference Example 2 that investigated the effect of sugars and MSM on preservation of adenovirus during freeze drying. The error bars shown are the standard error of the mean (n = 3).

Figure 4 shows the results obtained in Reference Example 3 of adenovirus infectivity tested immediately after thawing as well as those of samples lyophilised after formulation with TMG (trimethylglycine) with or without sugars. Adenoviral activity stated as pfu/ml as assessed by counting cells positive for GFP expression. Error bars shown are the standard error of the mean (n = 3).

Figure 5 demonstrates the lyophilisation conditions used in Reference Example 4.

Figure 6 shows bar graphs demonstrating the virus titres of reconstituted samples used in Reference Example 4 following thermal challenge at 4°C or 37°C for 7 days. The starting titre of the input virus is also shown. The error bars represent standard error of the mean (n=3).

Figure 7 shows the results obtained in Reference Example 5 for adenovirus samples which were tested immediately after thawing ("*Pre-Lyophilisation*") as well as those of samples which were formulated in PBS (phosphate buffered saline) at DMG concentrations of 0.00M, 0.07M, 0.23M and 0.70M with and without sugars and which were subsequently lyophilised. Adenovirus activity stated as pfu/ml was assessed by counting cells positive for GFP (Green Fluorescent Protein) expression. The error bars shown are the standard error of the mean (n = 3).

Figure 8 shows the lyophilisation conditions used in Reference Examples 5 and 6.

Figure 9 shows the results obtained in Reference Example 6 for adenovirus samples tested immediately after defrosting as well as those of samples lyophilised after formulation with DMG with or without sugars and subsequently thermochallenged. (A) Adenovirus activity after lyophilisation and storage at +4°C for 7 days. (B) Adenovirus activity after lyophilisation and thermal challenge at +37°C for 7 days. Error bars denote standard of mean; n = 3 unless stated otherwise.

Figure 10 shows the shelf temperatures, condenser temperatures and vacuum conditions during freeze drying in the VirTis Advantage freeze-dryer in Reference Example 7.

Figure 11 shows the results obtained in Reference Example 7. Adenovirus activity stated as pfu/ml was assessed by counting cells positive for GFP. Error bars denote standard of the mean (n = 2). Significance was tested using a one way ANOVA followed by a bonferroni post test. The p value summaries are *=$p<0.05$ and **=$p<0.01$.

Figure 12 shows the appearance of the freeze-dried cakes obtained in Reference Example 8.

Figure 13 reports the results obtained in Reference Example 9. FD denotes freeze drying. Vials containing adenovirus and PBS only showed a much greater loss of virus titre compared to the vials containing adenovirus, mannitol and DMG. Error bars shown are the standard error of the mean (n = 2). Significance was tested using a one way ANOVA followed by a bonferroni post test. All values were compared to stock titre. The p value summaries are **=$p<0.01$ and ***=$p<0.001$.

Figure 14 shows the results obtained in Example 10 in which the ability of eleven formulations to stabilise adenovirus through freeze-drying and thermal challenge was assessed.

Figure 15 shows the results obtained in Example 11 in which the ability of eleven formulations to stabilise MVA through freeze-drying and thermal challenge was assessed.

Figure 16 shows a 3D representation of the design space in Reference Example 12. Balls represent formulations within the design space that were tested. This design is a three factor, full factorial screening design.

Figures 17 and 18 show the freeze-drying program used in Reference Example 12 and temperature readings from sensors during that program.

Figure 19 shows a residual normal probability plot for data from formulations containing DMG in Reference Example 12.

Figure 20 shows retained coefficients (effects) of the modelled data from formulations containing DMG in Reference Example 12. Error bars indicate significance if not crossing the origin.

Figure 21 shows retained coefficients (effects) of the modelled data from formulations containing SMM in Reference Example 12. Error bars indicate significance if not crossing the origin.

Figure 22 shows a residual normal probability plot for data from formulations containing SMM in Reference Example 12.

Figure 23 shows retained coefficients (effects) of the modelled data from formulations containing SMM in Reference Example 12 after inclusion of a nonspecific $2^{nd}$ order term. Error bars indicate significance if not crossing the origin.

Figure 24 shows a residual normal probability plot for data from formulations containing SMM in Reference Example 12.

Figure 25 shows retained coefficients (effects) of the modelled data from formulations containing TMG in Reference Example 12. Error bars indicate significance if not crossing the origin.

Figure 26 shows a residual normal probability plot for data from formulations containing TMG in Reference Example 12.

Figure 27 shows a 3D representation of the design space in Reference Example 13. Spheres represent formulations within the design space that were tested. This design is a Doehlert RSM design.

Figure 28 shows the freeze-drying program used in Reference Example 13.

Figure 29 summarises various statistics for the model derived from the data in Reference Example 13.

Figure 30 shows terms retained in the model in Reference Example 13 after fine tuning. Error bars not crossing the origin indicate a significant factor at the 95% C.I.

Figure 31 shows a surface response plot of predicted viral titre in formulations of DMG and sucrose using the model in Reference Example 13 at three different levels of raffinose - "Low" = raffinose at 0mM, "Mid" = raffinose at 150mM, "High" = raffinose at 300mM.

Figure 32 shows the settings and outputs from an optimum prediction based on the model of the data in Reference Example 13 generated using Monte-Carlo simulations. The predicted optima highlighted in this model are concentrations of sucrose = 0.5M, DMG= 1M, raffinose = 150mM.

Figures 33A and 33B show an optimum region plot from the Reference Example 13 data. The plots are at static raffinose levels = 0, 150, 272, 300mM. The variable plotted is recovered titre (pfu/ml). Figure 33A is a contour plot where a cross marks the predicted optimum. Figure 33B is an identical graph region highlighting region of the model where predicted recovered viral activity is greater than or equal to initial activity.

Figure 34 shows the freeze-drying program used in Reference Example 14.

Figure 35 shows recovered virus activity in Reference Example 14 as a percentage of starting titre at week 15 post lyophilisation. Error bars are standard error of the mean (n=2).

Figure 36 shows recovered virus activity over time at the accelerated stability temperature (+25°C) in Reference Example 14.

Figure 37 shows recovered virus activity over time at the stress testing temperature (+37°C) in Reference Example 14.

Figure 38 shows a 3D representation of the design space in Reference Example 15. Spheres represent formulations within the design space that are tested. This design is a Doehlert RSM design.

Figure 39 shows the lyophilisation conditions used in Reference Example 15.

Figure 40 summarises the statistics of the model in Reference Example 15 used to represent the data.

Figure 41 shows terms retained in the model in Reference Example 15 after fine tuning. Error bars not crossing the origin indicate a significant factor at the 95% C.I.

Figure 42 shows contours plot of recovered viral titre (TCID50/ml) with varying formulations in Reference Example 15.

Figure 43 shows a representation of the design space in Reference Example 16. Numbered circles represent formulations within the design space that are tested.

Figure 44 shows the lyophilisation conditions used in Reference Example 16.

Figure 45 summarises the statistics of the model in Reference Example 16 used to represent the data.

Figure 46 shows terms retained in the model in Reference Example 16 after fine tuning. Error bars not crossing the origin indicate a significant factor at the 95% C.I.

Figure 47 shows a surface response plot of the predicted recovered viral titre in formulations of DMG and mannitol using the model of Reference Example 16.

Figure 48 shows a screen capture of the settings and outputs from the optimum predictions based on the model of the data in Reference Example 16, generated using Monte-Carlo simulations. Iteration 48 highlighted in grey is the optimum formulation (1.0107M DMG).

## Detailed Description of the Invention

### Summary

**[0013]** The present invention relates to the preservation of viral particles by (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, (b) methylsulfonylmethane and (c) sucrose, or sucrose and raffinose, or mannitol. The viral particles are contacted with (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, (b) methylsulfonylmethane and (c) sucrose, or sucrose and raffinose, or mannitol, in an aqueous solution and the resulting solution in which the viral

particles are present is then freeze-dried to form a composition incorporating the viral particles.

**[0014]** The viral particles may therefore be admixed with an aqueous solution (*"preservation mixture"*) of (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, (b) methylsulfonylmethane and (c) sucrose, or sucrose and raffinose, or mannitol. The resulting solution is then freeze-dried to form a composition incorporating the viral particles. The freeze-dried composition may take the form of a cake or powder. The cake can be milled to a powder if required.

**[0015]** The invention enables virus structure and function to be preserved during the freeze-drying step. Virus activity following drying can thus be maintained. The presence of (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, (b) methylsulfonylmethane and (c) sucrose, or sucrose and raffinose, or mannitol, allows preservation of viral activity.

**[0016]** The preserved viral particles demonstrate improved thermal resistance allowing extension of shelf life, ease of storage and transport and obviating the need for a cold chain for distribution. The invention can thus provide protection as a cryoprotectant (protection against freeze damage), lyoprotectant (protection during freeze-drying) and/or a thermoprotectant (protection against temperatures higher or lower than 4°C).

**[0017]** In addition, the viral particles are preserved in the aqueous solution prior to the freeze-drying step. This allows the aqueous solution to be stored after preparation, until such time as the drying step can be carried out, without undue loss of viral activity.

**Viral particles**

**[0018]** The viral particles used in the present invention may be whole viruses such as live viruses, killed viruses, live attenuated viruses, inactivated viruses such as chemically inactivated viruses or virulent or non-virulent viruses. A live virus is capable of infecting and replicating within the host cell. A killed virus is inactivated and is unable to replicate within the host cell. The particles may be virus-like particles (VLPs) or nucleocapsids. The virus may be infectious to prokaryotic or eukaryotic cells. The virus may be a human or animal virus.

**[0019]** The viral particle is, or is derived from, a virus of the following families:

- *Adenoviridae* such as a human adenovirus or non-human adenovirus, for example human adenovirus A, B, C, D, E or F including human Ad5, Ad2, Ad4, Ad6, Ad7, Ad11, Ad14, Ad24, Ad26, Ad35 and Ad36 serotypes;
- *Caliciviridae* such as the norwalk virus;
- *Coronaviridae* such as human coronavirus 299E or OC43 and SARS-coronavirus;
- *Filoviridae* such as ebola virus;
- *Flaviviridae* such as yellow fever virus, west nile virus, dengue virus, hepatitis C virus;

- *Hepadnaviridae* such as hepatitis B virus;
- *Herpesviridae* such as herpes simplex virus e.g. HSV1 or HSV2, human herpesvirus 1, 3, 4, 5 or 6;
- *Orthomyxoviridae* such as influenzavirus A, B, C including but not limited to influenza A virus serotypes H1N1, H2N2, H3N2, H5N1, H7N7, H1N2, H9H2, H7N2, H7N3 and N10N7;
- *Papillomaviridae* such as human papilloma virus;
- *Paramyxoviridae* such as human parainfluenza virus 1, measles virus and mumps virus;
- *Parvoviridae* such as adeno-associated virus;
- *Picornaviridae* such as human poliovirus, foot and mouth disease virus (including serotypes O, A, C, SAT-1, SAT-2, SAT-3 and Asia-1);
- *Poxviridae* such as vaccinia virus, variola virus and avian poxvirus (fowlpox);
- *Reoviridae* such as bluetongue virus group;
- *Retroviridae* such as lentivirus including human immunodeficiency virus 1 and 2; and
- *Togaviridae* such as rubella virus.

**[0020]** In a preferred embodiment, the viral particle can be or can be derived from an *Adenoviridae*, *Orthomyxoviridae, Paramyxoviridae, Parvoviridae*, *Picornaviridae* or *Poxviridae* virus. In a particularly preferred embodiment, the viral particle can be or can be derived from an adenovirus, vaccinia virus, influenza virus, or measles virus. The virus can be Modified Vaccinia Virus Ankara (MVA) or a viral particle derived from MVA.

**[0021]** Virus-like particles (VLPs) include viral proteins derived from the structural proteins of a virus, but lack viral nucleic acid. When overexpressed, these viral structural proteins spontaneously self-assemble into particles. VLPs are replication incompetent. In some embodiments, the VLPs are viral proteins embedded within a lipid bilayer. Examples of VLPs includes phage-derived VLPs, human papillomavirus (HPV) L1 major capsid protein VLPs, Norwalk virus capsid protein VLPs and VLPs assembled from influenza virus structural proteins such as M1 protein, HA hemagglutinin protein and N1 neuraminidase protein.

**[0022]** Viral particles can be prepared using standard techniques well known to those skilled in the art. For example, a virus may be prepared by infecting cultured host cells with the virus strain that is to be used, allowing infection to progress such that the virus replicates in the cultured cells and can be released by standard methods known in the art for harvesting and purifying viruses.

**N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof**

**[0023]** The N,N-dimethylglycine or N,N,N-trimethylglycine may be present as a physiologically acceptable salt or ester thereof.

**[0024]** 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.

**[0025]** 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.

**[0026]** 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.

**[0027]** For the avoidance of doubt, the definitions include compounds in which the carboxylate anion is protonated to give -COOH and the ammonium 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.

**Sugars**

**[0028]** The sugars used in the present invention are sucrose, or sucrose and raffinose, or mannitol. When mannitol is used, cakes of improved appearance can be obtained on freeze-drying.

**[0029]** The presence of sugar may act to improve stability. The addition of sugar may also provide other benefits such as an altered lyophilisation cake and improved solubility for faster reconstitution. When one sugar is used, the sugar is preferably mannitol.

**[0030]** Preservation of viral activity is particularly effective when two or more sugars which are sucrose and raffinose are used in the preservation mixture. Sucrose is a disaccharide of glucose and fructose. Raffinose is a trisaccharide composed of galactose, fructose and glucose.

**Preservation procedure**

**[0031]** In the present invention, an aqueous solution comprising the viral particles, together with (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, (b) methylsulfonylmethane and (c) sucrose, or sucrose and raffinose, or mannitol, is freeze-dried. Any suitable aqueous solution may be used. The solution may be buffered. The solution may be a HEPES, phosphate-buffered, Tris-buffered or pure water

solution.

**[0032]** The solution may have a pH of from 2 to about 12 and may be buffered. The solution may be buffered with HEPES buffer, phosphate-buffer, Tris-buffer, sodium citrate buffer, bicine buffer (i.e. N,N-bis(2-hydroxyethyl) glycine buffer) or MOPS buffer (i.e. 3-(N-morpholino) propanesulfonic acid buffer). The solution may or may not contain NaCl. The solution may thus be a saline sodium citrate (SSC) buffered solution.

**[0033]** Generally a preparation of the viral particles is admixed with the preservation mixture, i.e. with an aqueous solution of a compound of (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, (b) methylsulfonylmethane and (c) sucrose, or sucrose and raffinose, or mannitol. The preservation mixture may itself be buffered. It may be a HEPES, phosphate-buffered, Tris-buffered or pure water solution.

**[0034]** Alternatively, the aqueous solution may typically consist, or consist essentially, of viral particles, (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, (b) methylsulfonylmethane and (c) sucrose, or sucrose and raffinose, or mannitol.

**[0035]** The concentrations of (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, (b) methylsulfonylmethane and (c) sucrose, or sucrose and raffinose, or mannitol, can be determined by routine experimentation. Optimised concentrations which result in the best stability can thus be selected. The N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, and (b) methylsulfonylmethane may act synergistically to improve stability.

**[0036]** The concentration of sugar (i.e. sucrose, or sucrose and raffinose, or mannitol) is at least 0.1M, at least 0.2M or at least 0.5M up to 3M, 2.5M or 2M. The sugar concentration is preferably from 0.2M to 2M. Alternatively, the sugar concentration or the total sugar concentration if more than one sugar is present may range from 0.15M to 2M or from 0.2M to 1M.

**[0037]** The concentration of the N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof in the aqueous solution for drying is in the range of 0.1M to 1.5M, preferably from 0.5M to 1.25M.

**[0038]** The concentration of the methylsulfonylmethane in the aqueous solution for drying is in the range of 0.1M to 1.5M, preferably from 0.5M to 1.25M.

**[0039]** Freeze-drying is used in the present invention. 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 optimized via routine experimentation.

**[0040]** On drying, a composition is formed which incorporates the viral particles. A matrix incorporating the viral particles is 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).

**[0041]** The sugar or sugars provide the amorphous matrix in the dried composition. The (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, and (b) methylsulfonylmethane are dispersed in the sugar matrix. The (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, and (b) methylsulfonylmethane are thus incorporated within the sugar matrix. The viral particles are incorporated within the sugar matrix too. The drying procedure can thus be effected by freeze-drying to form an amorphous cake within which the viral particles are incorporated.

**[0042]** The drying step is generally performed as soon as the aqueous solution has been prepared or shortly afterwards. Alternatively, the aqueous solution is typically stored prior to the drying step. The viral particle in the aqueous solution is preserved by the (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, (b) methylsulfonylmethane and (c) sucrose, or sucrose and raffinose, or mannitol, during storage.

**[0043]** The aqueous solution, or bulk intermediate solution, is generally stored for up to 5 years, for example up to 4 years, 3 years, 2 years or 1 year. Preferably the solution is stored for up to 6 months, more preferably up to 3 months or up to 2 months, for example 1 day to 1 month or 1 day to 1 week. Prior to drying, the solution is typically stored in a refrigerator or in a freezer. The temperature of a refrigerator is typically 2 to 8 °C, preferably 4 to 6°C, or for example about 4°C. The temperature of a freezer is typically -10 to -80°C, preferably -10 to -30°C, for example about -20°C.

**[0044]** The solution is typically stored in a sealed container, preferably a sealed inert plastic container, such as a bag or a bottle. The container is typically sterile. The volume of the bulk intermediate solution is typically 0.1 to 100 litres, preferably 0.5 to 100 litres, for example 0.5 to 50 litres, 1 to 20 litres or 5 to 10 litres. The container typically has a volume of 0.1 to 100 litres, preferably 0.5 to 100 litres, for example 0.5 to 50 litres, 1 to 20 litres or 5 to 10 litres.

**[0045]** The stored bulk intermediate solution is typically poured into a freeze-drying tray prior to the freeze-drying step.

**[0046]** Stable storage of the solution increases the flexibility of the manufacturing process. Thus, the solution can be easily stored, shipped and later dried.

***Freeze-drying***

**[0047]** Freeze-drying is a dehydration process typically used to preserve perishable material or make the material more convenient for transport. Freeze-drying represents a key step for manufacturing solid protein and vaccine pharmaceuticals. However, biological materials are subject to both freezing and drying stresses during the procedure, which are capable of unfolding or denaturing proteins. Furthermore, the rate of water vapour diffusion from the frozen biological material is very low and therefore the process is time-consuming. The preservation technique of the present invention enables biological materials to be protected against the desiccation and/or thermal stresses of the freeze-drying procedure.

**[0048]** There are three main stages to this technique namely 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, (Teu) in the case of simple crystalline products or glass transition temperature (Tg') in the case of amorphous products, i.e. below 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 primary drying stage.

**[0049]** 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.

**[0050]** 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.

***Dried composition***

**[0051]** A composition having a low residual moisture content can be obtained. A level of residual moisture content is achieved which offers long term preservation at greater than refrigeration temperatures e.g. within the range from 4°C to 56°C or more, or lower than refrigeration temperatures e.g. within the range from 0 to -70°C or below. The dried composition may thus have residual moisture content of 10% or less, 5% or less, 2% or less or 1% or less by weight. Preferably the residual moisture content is 0.5% or more 1% or more. Typically a dried composition has residual moisture content of from 0.5 to 10% by weight and preferably from 1 to 5% by weight.

**[0052]** The composition can be obtained in a dry powder form. A cake resulting from freeze-drying can be milled into 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.

**[0053]** The composition may typically consist, or consist essentially, of viral particles, (a) N,N-dimethylglycine or N,N,N-trimethylglycine or physiologically acceptable salts or esters thereof, (b) methylsulfonylmethane and (c) sucrose, or sucrose and raffinose, or mannitol.

**Measuring viral particle preservation**

**[0054]** Preservation in relation to viral particles refers to resistance of the viral particle to physical or chemical degradation and/or loss of biological activity such as nucleic acid or protein degradation, loss of transfection efficiency, loss of ability to stimulate a cellular or humoral immune response, loss of viral infectivity, loss of immunogenicity, loss of virus titre, loss of host cell response or loss of vaccine potency, under exposure to conditions of desiccation, freezing, temperatures below 0°C or below -25°C, freeze-drying, room temperature, temperatures above 0°C, above 25°C or above 30°C. Preferably, preservation according to the present invention comprises cryoprotection (protection against freeze damage), lyoprotection (protection during freeze-drying) and/or thermoprotection (protection against temperatures higher or lower than 4°C).

**[0055]** Methods of assaying for viral activity such as infectivity and/or immunogenicity are well known to those skilled in the art and include but are not limited to growth of a virus in a cell culture, detection of virus-specific antibody in blood, ability to elicit T and/or B cell responses, detection of viral antigens, detection of virus encoded DNA or RNA, or observation of virus particles using a microscope.

**[0056]** Further, the presence of a virus gives rise to morphological changes in the host cell, which can be measured to give an indication of viral activity. Detectable changes such as these in the host cell due to viral infection are known as cytopathic effect. Cytopathic effects may consist of cell rounding, disorientation, swelling or shrinking, death and detachment from the surface. Many viruses induce apoptosis (programmed cell death) in infected cells, measurable by techniques such as the TUNEL (Terminal uridine deoxynucleotidyl transferase dUTP nick end labelling) assay and other techniques well known to those skilled in the art.

**[0057]** Viruses may also affect the regulation of expression of the host cell genes and these genes can be analysed to give an indication of whether viral activity is present or not. Such techniques may involve the addition of reagents to the cell culture to complete an enzymatic or chemical reaction with a viral expression product. Furthermore, the viral genome may be modified in order to enhance detection of viral infectivity. For example, the viral genome may be genetically modified to express a marker that can be readily detected by phase contrast microscopy, fluorescence microscopy or by radioimaging. The marker may be an expressed

fluorescent protein such as GFP (Green Fluorescent Protein) or an expressed enzyme that may be involved in a colourimetric or radiolabelling reaction. The marker could also be a gene product that interrupts or inhibits a particular function of the cells being tested.

**[0058]** An assay for plaque-forming units can be used to measure viral infectivity and to indicate viral titre. In this assay, suitable host cells are grown on a flat surface until they form a monolayer of cells covering a plastic bottle or dish. The selection of a particular host cell will depend on the type of virus. Examples of suitable host cells include but are not limited to CHO, BHK, MDCK, 10T1/2, WEHI cells, COS, BSC 1, BSC 40, BMT 10, VERO, WI38, MRC5, A549, HT1080, 293, B-50, 3T3, NIH3T3, HepG2, Saos-2, Huh7, HEK293 and HeLa cells. The monolayer of host cells is then infected with the viral particles. The liquid medium is replaced with a semi-solid one so that any virus particles produced, as the result of an infection cannot move far from the site of their production. A plaque is produced when a virus particle infects a cell, replicates, and then kills that cell. A plaque refers to an area of cells in the monolayer which display a cytopathic effect, e.g. appearing round and darker than other cells under the microscope, or as white spots when visualized by eye; the plaque center may lack cells due to virus-induced lysis. The newly replicated virus infects surrounding cells and they too are killed. This process may be repeated several times. The cells are then stained with a dye such as methylene blue, which stains only living cells. The dead cells in the plaque do not stain and appear as unstained areas on a coloured background.

**[0059]** Each plaque is the result of infection of one cell by one virus followed by replication and spreading of that virus. However, viruses that do not kill cells may not produce plaques. A plaque refers to an area of cells in a monolayer which display a cytopathic effect, e.g. appearing round and darker than other cells under the microscope, or as white spots when visualized by eye; the plaque center may lack cells due to virus-induced lysis. An indication of viral titre is given by measuring "*plaque-forming units*" (PFU). Levels of viral infectivity can be measured in a sample of biological material preserved according to the present invention and compared to control samples such as freshly harvested virus or samples subjected to desiccation and/or thermal variation without addition of the preservation mixture as used in the present invention.

**[0060]** Some types of viral particles for use in the invention, such as viral proteins, VLPs, or some inactivated viruses do not have the ability to form plaques in the plaque assay. In this case, preservation can be measured by other methods such as methods for determining immunogenicity which are well known to those skilled in the art. For example, *in vivo* and *in vitro* assays for measuring antibody or cell-mediated host immune responses are known in the art and suitable for use in the present invention. For example, an antibody based immune response may be measured by comparing the amount, avidity and isotype distribution of serum antibodies in an animal model, before and after immunization using the preserved viral particle of the invention.

**Uses of the preserved viral particles of the invention**

***Vaccines***

**[0061]** The preserved viral particles of the present invention may find use as a vaccine. For example, preserved viral particles such as whole killed virus, live attenuated virus, chemically inactivated virus, VLPs or live viral vectors are suitable for use as a vaccine. As a vaccine the preserved viral particles of the invention may be used as antigens or to encode antigens such as viral proteins for the treatment or prevention of a number of conditions including but not limited to viral infection, sequelae of viral infection including but not limited to viral-induced toxicity, cancer and allergies. Such antigens contain one or more epitopes that will stimulate a host's immune system to generate a humoral and/or cellular antigen-specific response.

**[0062]** The preserved viral particles may be used to prevent or treat infection by viruses such as 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 and vaccinia virus. The vaccine may further be used to provide a suitable immune response against numerous veterinary diseases, such as foot and mouth disease (including serotypes O, A, C, SAT-1, SAT-2, SAT-3 and Asia-1), coronavirus, bluetongue, feline leukaemia virus, avian influenza, hendra and nipah virus, pestivirus, canine parvovirus and bovine viral diarrhoea virus. In one embodiment, the vaccine is a subunit, conjugate or multivalent vaccine. For example, the preserved vaccine of the invention may be used to treat infection by two or more different types of virus such as measles, mumps and rubella (e.g. MMR vaccine).

**[0063]** The preserved viral particle may be present in a composition which may further comprise appropriate buffers and additives such as antibiotics, adjuvants or other molecules that enhance presentation of vaccine antigens to specific cells of the immune system.

**[0064]** A variety of adjuvants well known in the art can be used in order to increase potency of the vaccine and/or modulate humoral and cellular immune responses. Suitable adjuvants include, but are not limited to, mineral salts (e.g., aluminium hydroxide ("alum"), aluminium phosphate, calcium phosphate), particulate adjuvants (e.g., virosomes, ISCOMS (structured complex of saponins and lipids)), microbial derivatives (e.g., MPL(monophosphoryl lipid A), CpG motifs, modified toxins including TLR adjuvants such as flagellin), plant derivatives (e.g., saponins (QS-21)) and endogenous immunostimulatory adjuvants (e.g., cytokines and any other substances that act as immunostimulating agents to enhance the effectiveness of the vaccine).

**[0065]** The vaccine composition is in a freeze-dried (lyophilised) form in order to provide for appropriate storage and maximize the shelf-life of the preparation. This will allow for stock piling of vaccine for prolonged periods of time and help maintain immunogenicity, potency and

efficacy. The preservation mixture for use in the present invention is particularly suited to preserve viral substances against desiccation and thermal stresses encountered during freeze-drying/lyophilisation protocols. Therefore, the preservation mixture is suitable for adding to the virus or viral particle soon after harvesting and before subjection of the sample to the freeze-drying procedure.

**[0066]** To measure the preservation of a vaccine, the potency of the vaccine can be measured using techniques well known to those skilled in the art. For example, the generation of a cellular or humoral immune response can be tested in an appropriate animal model by monitoring the generation of antibodies or immune cell responses to the vaccine. The ability of vaccine samples prepared in accordance with the method of the present invention to trigger an immune response may be compared with vaccines not subjected to the same preservation technique.

***Viral vectors***

**[0067]** A virus or viral vector preserved according to the method of the present invention can be used to transfer a heterologous gene or other nucleic acid sequence to target cells. Suitably, the heterologous sequence (i.e. transgene) encodes a protein or gene product which is capable of being expressed in the target cell. Suitable transgenes include desirable reporter genes, therapeutic genes and genes encoding immunogenic polypeptides (for use as vaccines). Gene therapy, an approach for treatment or prevention of diseases associated with defective gene expression, involves the insertion of a therapeutic gene into cells, followed by expression and production of the required proteins. This approach enables replacement of damaged genes or inhibition of expression of undesired genes. In particular, the preserved virus or viral vector may be used in gene therapy to transfer a therapeutic transgene or gene encoding immunogenic polypeptides to a patient.

**[0068]** In a preferred embodiment, the preserved viral particle is a live viral vector. By "live viral vector" is meant a live viral vector that is non-pathogenic or of low pathogenicity for the target species and in which has been inserted one or more genes encoding antigens that stimulate an immune response protective against other viruses or microorganisms, a reporter gene or a therapeutic protein. In particular, nucleic acid is introduced into the viral vector in such a way that it is still able to replicate thereby expressing a polypeptide encoded by the inserted nucleic acid sequence and in the case of a vaccine, eliciting an immune response in the infected host animal. In one embodiment, the live viral vector is an attenuated live viral vector i.e. is modified to be less virulent (disease-causing) than wildtype virus.

**[0069]** The basis of using recombinant viruses as potential vaccines involves the incorporation of specific genes from a pathogenic organism into the genome of a nonpathogenic or attenuated virus. The recombinant virus can then infect specific eukaryotic cells either in vivo or in vitro, and cause them to express the recombinant protein.

**[0070]** Live viral vector vaccines derived by the insertion of genes encoding sequences from disease organisms may be preferred over live attenuated vaccines, inactivated vaccines, subunit or DNA approaches. One of the most important safety features of live viral vectors is that the recipients may be immunized against specific antigens from pathogenic organisms without exposure to the disease agent itself. Safety is further regulated by the selection of a viral vector that is either attenuated for the host or unable to replicate in the host although still able to express the heterologous antigen of interest. A vaccine strain that has a history of safety in the target species offers an additional safety feature. Several systems have been developed in which the vector is deleted of essential genes and preparation of the vaccine is carried out in cell systems that provide the missing function.

**[0071]** A variety of vectors such as retroviral, lentiviral, herpes virus, poxvirus, adenoviral and adeno-associated viral vectors can be used for the delivery of heterologous genes to target cells. The heterologous gene of interest may be inserted into the viral vector. The preserved viral vectors of the invention may comprise for example a virus vector provided with an origin of replication, optionally a promoter for the expression of the heterologous gene and optionally a regulator of the promoter. For example, adenoviruses useful in the practice of the present invention can have deletions in the E1 and/or E3 and /or E4 region, or can otherwise be maximized for receiving heterologous DNA.

**[0072]** The viral vector may comprise a constitutive promoter such as a cytomegalovirus (CMV) promoter, SV40 large T antigen promoter, mouse mammary tumour virus LTR promoter, adenovirus major late promoter (MLP), the mouse mammary tumour virus LTR promoter, the SV40 early promoter, adenovirus promoters such as the adenovirus major late promoter (Ad MLP), HSV promoters (such as the HSV IE promoters), HPV promoters such as the HPV upstream regulatory region (URR) or rous sarcoma virus promoter together with other viral nucleic acid sequences operably linked to the heterologous gene of interest. Tissue-specific or inducible promoters can also be used to control expression of the heterologous gene of interest. Promoters may also be selected to be compatible with the host cell for which expression is designed.

**[0073]** The viral vector may also comprise other transcriptional modulator elements such as enhancers. Enhancers are broadly defined as a cis-acting agent, which when operably linked to a promoter/gene sequence, will increase transcription of that gene sequence. Enhancers can function from positions that are much further away from a sequence of interest than other expression control elements (e.g. promoters) and may operate when positioned in either orientation relative to the sequence of interest. Enhancers have been identified from a number of viral sources, including polyoma virus, BK virus, cytomegalovirus (CMV), adenovirus, simian virus 40 (SV40), Moloney sarcoma virus, bovine papilloma virus and Rous sarcoma virus. Examples of suitable enhancers include the SV40 early gene enhancer, the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus, and elements derived from human or murine CMV, for example, elements included in the CMV intron A sequence.

**[0074]** The viral vector containing a heterologous gene of interest may then be preserved according to the method of the invention before storage, subjecting to further preservation techniques such as lyophilisation, or administration to a patient or host cell.

**[0075]** Nucleic acids encoding for polypeptides known to display antiviral activity, immunomodulatory molecules such as cytokines (e.g. TNF-alpha, interleukins such as IL-6, and IL-2, interferons, colony stimulating factors such as GM-CSF), adjuvants and co-stimulatory and accessory molecules may be included in the preserved viral vector of the invention. Alternatively, such polypeptides may be provided separately, for example in the preservation mixture suitable for use in the invention or may be administrated simultaneously, sequentially or separately with preserved viral vectors of the invention.

**[0076]** Preferably, the preserved viral vector of the invention may be introduced into suitable host cells using a variety of viral techniques that are known in the art, such as for example infection with recombinant viral vectors such as retroviruses, herpes simplex virus and adenoviruses. Preferably, administration of the preserved viral vector of the invention containing a gene of interest is mediated by viral infection of a target cell.

**[0077]** A number of viral based systems have been developed for transfecting mammalian cells.

**[0078]** For example, a selected recombinant nucleic acid molecule can be inserted into a vector and packaged as retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo.* Retroviral vectors may be based upon the Moloney murine leukaemia virus (Mo-MLV). In a retroviral vector, one or more of the viral genes (gag, pol & env) are generally replaced with the gene of interest.

**[0079]** A number of adenovirus vectors are known. Adenovirus subgroup C serotypes 2 and 5 are commonly used as vectors. The wild type adenovirus genome is approximately 35kb of which up to 30kb can be replaced with foreign DNA.

**[0080]** There are four early transcriptional units (E1, E2, E3 & E4), which have regulatory functions, and a late transcript, which codes for structural proteins. Adenovirus vectors may have the E1 and/or E3 gene inactivated. The missing gene(s) may then be supplied in trans either by a helper virus, plasmid or integrated into a helper cell genome. Adenovirus vectors may use an E2a temperature sensitive mutant or an E4 deletion. Minimal adenovirus vectors may contain only the inverted terminal repeats (ITRs) & a packaging sequence around the transgene, all the necessary viral genes being provided in trans by a helper virus. Suitable adenoviral vectors thus include Ad5 vectors and simian adenovirus vectors.

**[0081]** Viral vectors may also be derived from the pox family of viruses, including vaccinia viruses and avian poxvirus such as fowlpox vaccines. For example, modified vaccinia virus Ankara (MVA) is a strain of vaccinia virus which does not replicate in most cell types, including

normal human tissues. A recombinant MVA vector may therefore be used to deliver a polypeptide. Additional types of virus such as adeno-associated virus (AAV) and herpes simplex virus (HSV) may also be used to develop suitable vector systems

***Administration***

**[0082]** Preserved vaccines or viral particles may be administered, in some instances after reconstitution of a freeze-dried product, to a subject *in vivo* using a variety of known routes and techniques. For example, the preserved vaccines can be provided as an injectable solution, suspension or emulsion and administered via parenteral, subcutaneous, oral, epidermal, intradermal, intramuscular, interarterial, intraperitoneal, intravenous injection using a conventional needle and syringe, or using a liquid jet injection system. Preserved vaccines may be administered topically to skin or mucosal tissue, such as nasally, intratrachealy, intestinal, sublingually, rectally or vaginally, or provided as a finely divided spray suitable for respiratory or pulmonary administration.

**[0083]** The methods described herein may further comprise the step of processing the mixture into a formulation suitable for administration as a liquid injection. Preferably, the method further comprises the step of processing the mixture into a formulation suitable for administration via ingestion or via the pulmonary route.

**[0084]** The preserved product is administered to a subject in an amount that is compatible with the dosage formulation and that will be prophylactically and/or therapeutically effective. The administration of the preserved product or vaccine of the invention may be for either *"prophylactic"* or *"therapeutic"* purpose. As used herein, the term *"therapeutic"* or *"treatment"* includes any of the following: the prevention of infection or reinfection; the reduction or elimination of symptoms; and the reduction or complete elimination of a pathogen. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

**[0085]** The following Examples illustrate the invention. The following materials, equipment and techniques were employed unless stated otherwise in the Examples:

**Materials**

**[0086]**

HEK-293 cells (ECACC 85120602)

Dimethylglycine DMG (Sigma D1156, Lot 077K1856)

Dimethylsulfone (MSM) (Sigma M81705, Lot 0001452516)

Sucrose (Sigma 16104, Lot 70040)

Raffinose (Sigma R0250, Lot 039K0016)

PBS (Sigma D8662, Lot 118K2339)

Water (Sigma W3500, Lots 8M0411 and RNBB1139)

Hydranal Methanol (Fluka 37817, Lot 8331D)

Hydranal Composite (Fluka 34805, Lot 8287A)

5ml glass vials (Adelphi Tubes VCD005)

14mm freeze drying stoppers (Adelphi Tubes FDIA14WG/B)

14mm caps (Adelphi Tubes CWPP14)

Adenovirus GFP (Vector Biolabs cat. 1060)

Measles virus strains 3A and 1A (a kind gift provided by P. Christian at NIBSC)

Dulbecco's Modified Eagles Medium (DMEM) (Sigma D5796, Lot RNBB1139)

Foetal Bovine Serum (FBS) (Sigma F7524, Lot 109K3395)

Penicillin Streptomycin (PS) (Sigma P4458, Lot 0409M00393)

Saline Sodium Citrate (SSC) (Sigma S6639, Lot 020M8404)

BHK-21 cell line (ECCAC CB2857)

HEK 293 (ECACC 85120602)

MVA (ATCC-VR-1508)

2ml glass vials (Adelphi Tubes VCDIN2R)

13mm freeze drying stoppers (Adelphi Tubes FDW13)

Crimps (Adelphi Tubes COTW13)

**Equipment**

**[0087]**

Advantage Freeze Dryer (VirTis)

HERA safe class II cabinet (Thermo Fisher)

VirTis Advantage freeze dryer (Biopharma Process Systems)

Binder $CO_2$ Incubator (Binder)

Binder APT line TM MK thermocycling test chamber (Binder)

Thermo Scientific MaxQ 4450 Incubator (Thermofiser)

KERN EW220-3NM balance (VWR)

Elcold -45°C freezer (VWR)

Forma 900 series -80°C freezer (Thermofisher)

Karl Fisher Volumetric Titrator (Mettler Toldeo)

DMIL LED Inverted Microscope (Leica, EQP#062)

ATL-84-1 Atlion Balance (Acculab, EQP#088)

IP250 37°C Incubator (LTE, EQP#016)

**Freeze drying protocol**

**[0088]** Samples were freeze dried by the VirTis Advantage freeze dryer, using the pre-programmed protocol lasting for approximately 3 days. Samples were frozen at - 40°C for 1 hour before a vacuum was applied, initially at 200 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 8 was extended until the samples were stoppered before releasing the vacuum. The drying cycle used is shown below:

| Step | Shelf temp (°C) | Time (mins) | Ramp/Hold | Vacuum (milliTorre) |
|---|---|---|---|---|
| 1 | -45 | 15 | H | - |
| 2 | -32 | 600 | R | 200 |
| 3 | -20 | 120 | R | 200 |
| 4 | -10 | 120 | R | 200 |
| 5 | 0 | 120 | R | 200 |
| 6 | 10 | 120 | R | 200 |
| 7 | 20 | 120 | R | 200 |
| 8 | 20 | 1250 | H | 400 |

**[0089]** In the primary drying phase the shelf temperature is raised to -32°C from - 45°C. The

secondary drying phase included a ramp to 20°C until the drying was completed. The condenser temperature was set to stay at a constant -80°C. Probes recorded shelf temperatures and condenser temperatures (see Figures 2A and 2B).

**Statistical Analysis**

**[0090]** A one way ANOVA test followed by a turkey pair wise comparison was performed to analyse significance between different excipients using PRISM Graphpad software version 4.00. The p value summaries are *=p< 0.10; **=p<0.05; ***=p < 0.005.

**[0091]** In some Examples the following values were calculated:

- $R^2$ = coefficient of determination. A measure of goodness of fit. $R^2$<0.5=low model significance.
- $Q^2$ = estimate of prediction precision. A measure of goodness of prediction. $Q^2$ should be >0.1 for a significant model. $Q^2$ should be >0.5 for a good model. $R^2$-$Q^2$ < 0.2 to 0.3
- Model validity (MV)= "a test of diverse model problems". Model validity < 0.25 = indicator of statistically significant model problems e.g. outliers, incorrect model / transformation.
- Reproducibility (Rep) = measure of variation between replicates compared to over all variability. Reproducibility > 0.5 implies significance.

**Reference Example 1**

**Freeze drying**

**[0092]** Each type of excipient (see Table 1 below) was made up as a stock and 250µl added to appropriately labelled 5ml glass vials. 50µl of adenovirus was then added to each vial. After vortexing, vials were loaded onto the VirTis Advantage freeze drier and freeze dried according to the protocol given in the general experimental techniques section above.

**Table 1 - Final Concentrations of excipient mixes**

| | | |
|---|---|---|
| PBS | Suc (1M) Raf (100mM) | Suc (1M) Raf (100mM), Dimethyl sulfone (1M) |

**Thermal challenge**

**[0093]** Following freeze drying, samples were incubated in a Binder APT line TM MK temperature test chamber. Samples were cycled through a temperature of 37°C for 12 hours, a one hour ramp to -20°C, 10 hours at -20°C followed by a one hour ramp to 37°C. Each cycle amounted to 24 hours and was repeated for a 2 week period before carrying out an adenovirus assay as described below.

**Adenovirus assay (GFP)**

**[0094]** 96 flat bottomed cell culture dishes (Jencons, UK) were seeded with HEK 293 cells (ECACC 85120602) at $10^5$ cells per ml (100µl per well) and maintained at 37°C with 5% $CO_2$. After achieving 90% confluence, vials containing the adenovirus plus excipient were reconstituted in 300µl PBS. A 1 in 10 dilution step was then taken by taking 20µl from the reconstituted vial and adding to 180µl of Dulbecco's Modified Eagle Medium (DMEM). A further 1 in 100 dilution (of the original sample) was performed by taking 20µl of the 1 in 10 dilution and adding it to 180µl of DMEM. 100µl of each of the resultant dilution (1 in 10 and 1 in 100) was then added to wells of the plate containing HEK 293 cells. Additionally, a further sample of adenovirus, from the same source and with the same titre (on storage at -80°C) used in the excipient treatments, was thawed and used to produce a 1 in 10 dilution series (in DMEM). Dilutions ranging from 1 in 10 to 1 in $10^6$ were also added to individual wells containing HEK 293s. At 48 hours post inoculation, the number of GFP (Green Fluorescent Protein) cells per well were counted using fluorescent microscopy, and this was subsequently converted to pfu/ml of the treated samples taking into account the volume applied and dilution of the inoculum.

**Results and Discussion**

**[0095]** This experiment was designed to assess the effect of cooling and heating on viral recovery in the presence of excipients during storage. The results demonstrate poor recovery in excipients containing sugars only or PBS (Figure 1). In excipients containing sugars plus dimethylsulfone, recovery was significantly higher.

**[0096]** Additionally, excipients containing dimethylsulfone showed little deterioration following heat and freeze challenge compared to control samples. The results indicate that thermoprotection during the FD process is essential as inadequate excipients such as sugars alone or PBS fail to produce any significant virus titre following FD. However when excipients containing dimethyl sulfone are used in conjunction with sugars, virus titre remains close to that of the original titre even during freeze thaw cycles.

**Reference Example 2**

**[0097]** Each type of excipient plus virus (see Table 2) was made up as a stock in PBS and 250µl added to appropriately labelled 5ml glass vials. All vials were prepared in triplicate. 50µl of adenovirus was added to each vial. After vortexing, rubber bungs were partially inserted and vials were loaded onto the VirTis advantage and freeze-dried (FD) according to the freeze drying protocol given in the general experimental techniques section above. Following freeze drying, samples were assessed for virus titre using the adenovirus assay described in Example 1.

**Table 2**

| Composition of excipients | Final Concentration of excipients |
|---|---|
| Suc / Raf + MSM | 1M Suc 100mM Raf 0.35M MSM |
| Suc / Raf + MSM | 1M Suc 100mM Raf 0.035M MSM |
| Suc / Raf + MSM | 1M Suc 100mM Raf 0.0035M MSM |
| Suc / Raf + MSM | 1M Suc 100mM Raf 0.35mM MSM |
| Suc / Raf + MSM | 1M Suc 100mM Raf 0.035mM MSM |
| MSM | 0.35M MSM |
| MSM | 0.035M MSM |
| MSM | 0.0035M MSM |
| MSM | 0.35mM MSM |
| Suc / Raf | 1M Suc 100mM Raf |
| PBS | PBS |

**[0098]** The results are shown in Figure 3. PBS, Sugars only and MSM only excipients gave poor recovery. Recovery of virus significantly increased when the excipient included MSM as well as sugars. The results showed a synergistic effect between MSM and sugars, whereas MSM used in isolation provided to be a poor stabilising excipient.

**Residual moisture protocol**

**[0099]** Some vials were taken for residual moisture measurement (see Table 3 below). Assessment of residual moisture was carried out using a volumetric Karl Fisher titrator. The titrator (Mettler Toledo) works on the principle that one mole of $I_2$ is consumed for each mole of $H_2O$. The titrator was validated using a 10mg/ml water standard (Sigma, UK).

**[0100]** Titration was carried out by weighing vials containing the dried excipient mixture using a balance (Kern, Germany). 1ml of liquid (hydranal methanol rapid and hydranol methanol composite, Fluka) from the chamber is transferred from the titration chamber to the glass vial using a 5ml syringe and needle. Once the excipient has dissolved the liquid is then taken back up into the syringe and the liquid injected into the titration chamber. The vial was reweighed

and the difference in weight (the weight of the excipient) was inputted into the titrator. The titrator then calculated the residual moisture.

**[0101]** Measurements indicate that the presence of MSM may assist in the drying of the cake during secondary drying.

**Table 3: Residual moisture as a percentage of freeze dried excipient mixture**

| | Suc (1M) Raf (100mM) | Suc (1M) Raf (100mM), MSM (0.35M) |
|---|---|---|
| Mean percentage moisture | 6.5 % | 4.6 % |

**Reference Example 3**

**[0102]** A mixture of excipient plus virus was prepared and processed as described in Example 2. The excipient contained TMG and optionally sugars. The final concentration of each component in the excipient before drying is shown in Table 4 below. All vials were prepared in triplicate.

**Table 4**

| | TMG (M) | Sucrose (M) | Raffinose (mM) | Virus |
|---|---|---|---|---|
| Test | 0.7 | 1 | 100 | Y |
| | 0.2 | 1 | 100 | Y |
| | 0.07 | 1 | 100 | Y |
| Excipient alone | 0.7 | 0 | 0 | Y |
| | 0.2 | 0 | 0 | Y |
| | 0.07 | 0 | 0 | Y |
| Assay controls | 0.7 | 1 | 100 | N |
| | 0.7 | 0 | 0 | N |

**[0103]** The results of using TMG (Trimethylglycine) in the excipient are shown in Figure 4. TMG appears to enhance recovery of adenoviral infectivity from lyophilised samples. However, the lowest concentration (0.07M) offers the greatest protection and increasing TMG concentration above this concentration reduces the protection offered. The 0.07M TMG treatment offered greater protection than sugars alone.

**Reference Example 4**

**[0104]** Example 4 describes experimentation to elucidate the interaction between S-methyl-L-

methionine (SMM), sucrose and raffinose as excipients in a freeze dried formulation of adenovrius.

**Preparation and lyophilisation of virus**

**[0105]** Recombinant adenovirus (Vector Biolabs) expressing enhanced GFP under a CMV promoter, and with a titre (pre-freeze) of $2x10^6$ pfu/ml, was removed from storage at -80°C and allowed to thaw. 50µl aliquots of the virus were diluted to 300µl in PBS containing a variable concentration of each of the excipients. A full list of excipient formulations tested can be seen in Table 5.

**Table 5 - Summary of excipient treatments, each treatment was made in triplicate**

| Sucrose (M) | Raffinose (mM) | SMM (M) | Thermal Challenge |
|---|---|---|---|
| 0.0 | 0 | 0.000 | 37 |
| 0.0 | 0 | 0.007 | 37 |
| 0.0 | 0 | 0.023 | 37 |
| 0.0 | 0 | 0.070 | 37 |
| 0.0 | 0 | 0.230 | 37 |
| 0.0 | 0 | 0.700 | 37 |
| 0.0 | 0 | 1.000 | 37 |
| 0.1 | 10 | 0.000 | 37 |
| 0.1 | 10 | 0.007 | 37 |
| 0.1 | 10 | 0.023 | 37 |
| 0.1 | 10 | 0.070 | 37 |
| 0.1 | 10 | 0.230 | 37 |
| 0.1 | 10 | 0.700 | 37 |
| 0.1 | 10 | 1.000 | 37 |
| 1.0 | 100 | 0.000 | 37 |
| 1.0 | 100 | 0.007 | 37 |
| 1.0 | 100 | 0.023 | 37 |
| 1.0 | 100 | 0.070 | 37 |
| 1.0 | 100 | 0.230 | 37 |
| 1.0 | 100 | 0.700 | 37 |
| 1.0 | 100 | 1.000 | 37 |
| 0.0 | 0 | 0.000 | 4 |
| 0.0 | 0 | 0.007 | 4 |
| 0.0 | 0 | 0.023 | 4 |

| Sucrose (M) | Raffinose (mM) | SMM (M) | Thermal Challenge |
|---|---|---|---|
| 0.0 | 0 | 0.070 | 4 |
| 0.0 | 0 | 0.230 | 4 |
| 0.0 | 0 | 0.700 | 4 |
| 0.0 | 0 | 1.000 | 4 |
| 0.1 | 10 | 0.000 | 4 |
| 0.1 | 10 | 0.007 | 4 |
| 0.1 | 10 | 0.023 | 4 |
| 0.1 | 10 | 0.070 | 4 |
| 0.1 | 10 | 0.230 | 4 |
| 0.1 | 10 | 0.700 | 4 |
| 0.1 | 10 | 1.000 | 4 |
| 1.0 | 100 | 0.000 | 4 |
| 1.0 | 100 | 0.007 | 4 |
| 1.0 | 100 | 0.023 | 4 |
| 1.0 | 100 | 0.070 | 4 |
| 1.0 | 100 | 0.230 | 4 |
| 1.0 | 100 | 0.700 | 4 |
| 1.0 | 100 | 1.000 | 4 |

**[0106]** Each treatment was made up in 6 replicate vials. These samples were prepared in 5ml glass vials, rubber bungs were partially inserted, and after vortexing were loaded onto the VirTis advantage and lyophilized under the conditions shown in Figure 5.

**Thermal challenge of lyophilised adenovirus**

**[0107]** After lyophilisation samples were immediately removed and 3 replicates of each treatment placed at 37°C for thermal challenge whilst the other 3 were stored at 4°C as post-lyophilisation controls. Thermal challenge was for 7 days, after which all the vials were returned to the control vials and all held at 4°C until it was practical to assay them.

**Assay of recovered infectious virus from rehydrated cakes**

**[0108]** 96 flat bottomed cell culture dishes (VWR, UK) were seeded with HEK 293 (*ECACC* 85120602) cells at $10^5$ cells per ml (100µl per well) and maintained at 37°C with 5% $CO_2$. After

achieving 90% confluence vials containing the adenovirus plus excipient were reconstituted in 300µl of PBS. The reconstituted samples were serially diluted 1:10 and 1:100 in DMEM plus 5% FBS. 100µl of each of the resulting diluted virus samples were then added to individual wells of the plate. After a further 48 hours, the number of GFP cells per well were counted using fluorescent microscopy.

**Protection of adenoviral infectivity during lyophilisation (Figure 6)**

**[0109]** The samples were assayed 33 days after lyophilisation, and in the case of the heat challenged samples after 7 days at 37°C followed by a further 26 days stored at 4°C. The results are shown in Figure 6A.

**[0110]** S-methyl-L-methionine alone shows a concentration dependent protection of adenovirus during lyophilisation. Increased S-methyl-L-methionine in the formulation gave an increase in the recovered viral infectivity from reconstituted samples in this concentration range. Co-formulation of S-methyl-L-methionine with the low concentration treatment of sugars (0.1M Sucrose, 10mM Raffinose) did not significantly alter this relationship. However, co-formulation of S-methyl-L-methionine with the high sugar treatment (1.0M Sucrose, 100mM Raffinose) did significantly enhance the recovery of viral infectivity at low S-methyl-L-methionine concentrations.

**[0111]** On this evidence the optimum formulation for protection of viral infectivity during lyophilisation would appear to be either a high concentration of S-methyl-L-methionine (>0.07M) with no sugars or a concentration of less than 0.23M S-methyl-L-methionine in co-formulations with high sugar concentrations (1.0M Sucrose, 100mM Raffinose).

**Protection of adenoviral infectivity during lyophilisation and thermal challenge at 37°C (Figure 6)**

**[0112]** In the absence of any sugars in the formulation S-methyl-L-methionine offers only very limited retention of viral infectivity during lyophilisation and subsequent thermo-challenge (Figure 6B). Even this limited protection is only seen at concentrations of 0.07M and above. Co-formulation with a low concentration of sugars (0.1M Sucrose, 10mM Raffinose) similarly offers little protection although efficacy may be enhanced at low S-methyl-L-methionine concentrations.

**[0113]** Co-formulation of S-methyl-L-methionine with a high concentration of sugars (1M Sucrose, 100mM Raffinose) demonstrates a clear enhancement of protection between 0.00M and 0.23M S-methyl-L-methionine, and this enhancement is well above an additive effect and could possibly therefore be considered true synergism at both 0.07M and 0.23M. The optimum concentrations appear to be S-methyl-L-methionine at between 0.05 and 0.1M formulated with

high sugar concentrations (1M Sucrose, 100mM Raffinose). However even in this range the recovery is around 2-3x$10^5$ pfu/ml which represents almost a log reduction over the assayed titre of the input virus.

**Reference Example 5**

**[0114]** Recombinant adenovirus (Vector Biolabs) expressing enhanced GFP (Green Fluorescent Protein) under a CMV promoter was formulated with excipient mixtures so that, after lyophilisation, levels of recovered infectious adenovirus could easily be assayed. Each type of excipient plus virus (see Table 6 below) was made up as a stock in PBS and 300µl added to appropriately labelled 5ml glass vials. After vortexing, rubber bungs were partially inserted and vials were loaded onto the VirTis Advantage freeze dryer and freeze-dried (FD) as according to the freeze-drying protocol given above. Following freeze drying, samples virus titre was assessed in an adenovirus assay as described below.

**Table 6 - Final concentrations of excipient mixes in Example 5**

| DMG (M) | Sucrose (M) | Raffinose (mM) | Virus |
|---|---|---|---|
| 0.7 | 1 | 100 | Y |
| 0.2 | 1 | 100 | Y |
| 0.07 | 1 | 100 | Y |
| 0.7 | 0 | 0 | Y |
| 0.2 | 0 | 0 | Y |
| 0.07 | 0 | 0 | Y |
| 0 | 1 | 100 | Y |
| 0 | 0 | 0 | Y |

**[0115]** Samples were freeze dried by the VirTis Advantage freeze dryer according to the protocol given in the general experimental techniques section above. Following freeze-drying, the samples were assayed in an adenovirus assay as described in Example 1.

**[0116]** The results as shown in Figure 7. Recovery of adenovirus lyophilised in PBS is typically low and this was reproduced in this experiment.

**[0117]** DMG alone provided protection of adenoviral infectivity during lyophilisation, and this compares favourably to sugars alone. The formulation of sugars and DMG demonstrated a dose-dependent protection. The highest concentration of DMG appears comparable to adenovirus pre-lyophilisation.

**Reference Example 6**

**[0118]** The experiment in this Example expands on the capacity of DMG to protect adenovirus during lyophilisation in conjunction with raffinose and sucrose, by exploring the capability of DMG to protect adenovirus during thermal challenge. Two concentrations of each of the sugars at a static ratio to each other were tested (High sugars = 1M Sucrose with 100mM Raffinose, Low sugars = 0.1M Sucrose with 10mM Raffinose), whilst five concentrations of DMG were investigated (0.007M, 0.023M, 0.070M, 0.230M, 0.700M).

**[0119]** A strain of adenovirus expressing GFP was formulated with the excipient mixtures so that, after lyophilisation and thermal treatment, levels of recovered infectious adenovirus could easily be assayed. Adenovirus was formulated with the excipients and lyophilized before storage at +4°C and +37°C for one week. Samples were subsequently inoculated to HEK293 cells and recovered virus assessed by counting the number of GFP-expressing cells at 48 hours post-inoculation.

**Materials and Methods**

***Preparation and lyophilisation of formulated virus***

**[0120]** Recombinant adenovirus (Vector Biolabs) expressing enhanced GFP under a CMV promoter, and with a titre (pre-freeze) of $2x10^6$ pfu/ml, was removed from storage at -80°C and allowed to thaw. 50µl aliquots of the virus were diluted to 300µl in PBS containing a variable concentration of each of the excipients. A full list of excipient formulations tested can be seen in Table 7 below. Each treatment was made up in 6 replicate 5ml vials. Rubber bungs were partially inserted, and after vortexing were loaded onto the VirTis Advantage freeze-dryer and lyophilised on program 10 (see Figure 8).

**Table 7 - Summary of excipient treatments, each treatment was made in triplicate**

| Sucrose (M) | Raffinose (mM) | DMG (M) | Thermal Challenge (°C) |
|---|---|---|---|
| 0.0 | 0 | 0.000 | 37 |
| 0.0 | 0 | 0.007 | 37 |
| 0.0 | 0 | 0.023 | 37 |
| 0.0 | 0 | 0.070 | 37 |
| 0.0 | 0 | 0.230 | 37 |
| 0.0 | 0 | 0.700 | 37 |
| 0.1 | 10 | 0.000 | 37 |
| 0.1 | 10 | 0.007 | 37 |
| 0.1 | 10 | 0.023 | 37 |
| 0.1 | 10 | 0.070 | 37 |

| Sucrose (M) | Raffinose (mM) | DMG (M) | Thermal Challenge (°C) |
|---|---|---|---|
| 0.1 | 10 | 0.230 | 37 |
| 0.1 | 10 | 0.700 | 37 |
| 1.0 | 100 | 0.000 | 37 |
| 1.0 | 100 | 0.007 | 37 |
| 1.0 | 100 | 0.023 | 37 |
| 1.0 | 100 | 0.070 | 37 |
| 1.0 | 100 | 0.230 | 37 |
| 1.0 | 100 | 0.700 | 37 |
| 0.0 | 0 | 0.000 | 4 |
| 0.0 | 0 | 0.007 | 4 |
| 0.0 | 0 | 0.023 | 4 |
| 0.0 | 0 | 0.070 | 4 |
| 0.0 | 0 | 0.230 | 4 |
| 0.0 | 0 | 0.700 | 4 |
| 0.1 | 10 | 0.000 | 4 |
| 0.1 | 10 | 0.007 | 4 |
| 0.1 | 10 | 0.023 | 4 |
| 0.1 | 10 | 0.070 | 4 |
| 0.1 | 10 | 0.230 | 4 |
| 0.1 | 10 | 0.700 | 4 |
| 1.0 | 100 | 0.000 | 4 |
| 1.0 | 100 | 0.007 | 4 |
| 1.0 | 100 | 0.023 | 4 |
| 1.0 | 100 | 0.070 | 4 |
| 1.0 | 100 | 0.230 | 4 |
| 1.0 | 100 | 0.700 | 4 |

***Thermal challenge of lyophilised adenovirus***

**[0121]** After lyophilisation, samples were immediately removed and 3 replicates of each treatment placed at +37°C for thermal challenge whilst the other 3 were stored at +4°C as post-lyophilisation controls. Thermal challenge was for 7 days, after which all the vials were returned to the control vials and held at +4°C until it was practical to assay them.

***Assay of recovered infectious virus from rehydrated cakes***

**[0122]** 96 flat bottomed cell culture dishes (VWR, UK) were seeded with HEK 293 (ECACC 85120602) cells at 105 cells per ml (100µl per well) and maintained at 37°C with 5% CO2. After achieving 90% confluence vials containing the adenovirus plus excipient were reconstituted in 300ml of PBS. The reconstituted samples were serially diluted 1:10 and 1:100 in DMEM plus 5% FBS. 100ml of each of the resulting diluted virus samples were then added to individual wells of the plate. After a further 48 hours, the number of GFP cells per well were counted using fluorescent microscopy. The results are shown in Figures 9A and 9B.

**Results and Discussion**

***Protection of adenoviral infectivity during lyophilisation (see Figure 9A)***

**[0123]** Samples stored at 4°C for the duration of the test period after lyophilisation were assayed as a proxy for post-lyophilisation controls and also as negative controls for the thermal-challenge. In the absence of any excipients the lyophilisation of adenovirus during this experiment reduced infectivity of the sample from $1.5x10^6$pfu/ml to less than $1.0x10^4$pfu/ml.

**[0124]** Use of DMG as an excipient with adenovirus enhanced recovery of viral infectivity after reconstitution of the freeze dried cakes. The optimal concentration of DMG for the protection of adenovirus during lyophilisation in this experiment appears to be 0.07M or greater. Using the excipients, recovered titres of between 7.5-$8.5x10^5$pfu/ml are readily achievable (compared to an input titre of $1.5x10^6$pfu/ml).

***Protection of adenoviral infectivity during lyophilisation and following thermal challenge at +37°C (see Figure 9B)***

**[0125]** No detectable recovery of viral infectivity was observed from vials containing no excipients (adenovirus in PBS) after thermal challenge at +37°C. This represents a very significant loss of viral infectivity over samples with an equivalent formulation held at +4°C.

**[0126]** It is possible to recover viral infectivity after thermal challenge from samples formulated with sugars alone (for example, 1.0M Sucrose, 100mM Raffinose). Unfortunately, the recovered viral titre is only $4.3x10^4$pfu/ml compared to $1.9x10^5$pfu/ml from samples held at 4°C for the duration of the test.

**[0127]** When DMG was used as the sole excipient, the optimum concentration of DMG appeared to be 0.07M or greater with recovery of around $7.5x10^5$pfu/ml. At DMG concentrations up to 0.07M (0.007-0.07M) there is a positive correlation between DMG concentration and recovered virus. Above 0.07M DMG its effect appears to be saturated.

**[0128]** Coformulation of adenovirus with the same lower concentration of sugars and DMG at 0.07M or above was at least as good as the equivalent DMG concentrations in the absence of any sugars and possibly gave a slight enhancement of the protective effect.

**[0129]** Coformulation of DMG at 0.023M or less, with the higher sugar concentration (1.0M Sucrose, 100mM Raffinose), enhanced recovery to levels comparable to those treatments in which the DMG effect was thought saturated. However, at DMG concentrations of 0.07M or above the addition of the high sugar concentration has no obvious benefit. These findings suggest that the addition of sugars to DMG formulations at the higher concentration (1.0M Sucrose, 100mM Raffinose) reduces the amount of DMG required to saturate its effect.

**Reference Example 7**

**[0130]** Recombinant adenovirus (Vector Biolabs) expressing enhanced GFP under a CMV promoter was formulated with excipient mixtures so that, after lyophilisation, levels of recovered infectious adenovirus could easily be assayed. Each type of excipient (see Table 8 below) was made up as a stock and 250µl added to appropriately labelled 2ml glass vials. 50µl of adenovirus was added to each vial from stocks. After vortexing, vials were loaded onto the VirTis Advantage freeze drier and freeze-dried.

**Table 8 - Final concentrations of excipient mixes for Example 7**

| | | |
|---|---|---|
| 0.7M DMG | 0.07M Mannitol | 0.7M DMG, 0.07M Mannitol |

***Freeze drying protocol***

**[0131]** Samples were freeze dried by the VirTis Advantage freeze dryer, using the pre-programmed protocol lasting for approximately 3 days. Samples were frozen at -40°C for 1 hour 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 9 was extended until the samples were stoppered before releasing the vacuum. The drying cycle used is shown in Table 9 below:

**Table 9 - Freeze drier conditions**

| Step | Shelf temp (°C) | Time (mins) | Ramp/Hold | Vacuum (milliTorre) |
|---|---|---|---|---|
| 1 | -45 | 15 | H | 300 |
| 2 | -34 | 30 | R | 300 |

| Step | Shelf temp (°C) | Time (mins) | Ramp/Hold | Vacuum (milliTorre) |
|---|---|---|---|---|
| 3 | -34 | 1200 | H | 300 |
| 4 | -20 | 120 | R | 300 |
| 5 | -10 | 120 | R | 300 |
| 6 | 0 | 120 | R | 300 |
| 7 | 10 | 120 | R | 80 |
| 8 | 20 | 1250 | H | 80 |
| 9 | 20 | 1250 | H | 80 |
| 10 | 20 | 1250 | H | 80 |

**[0132]** In the primary drying phase, the shelf temperature is held at -34°C. The secondary drying phase included a ramp to +20°C until the drying was completed. The condenser temperature was set to stay at a constant -80°C. Probes recorded shelf temperatures and condenser temperatures (see Figure 10).

***Adenovirus assay***

**[0133]** 96 flat bottomed cell culture dishes (Jencons, UK) were seeded with HEK 293 cells (ECACC 85120602) at $10^5$ cells per ml (100µl per well) and maintained at 37°C with 5% $CO_2$. After achieving 90% confluence, vials containing the adenovirus plus excipient were reconstituted in 1ml of Dulbecco's Minimum Essential Medium (DMEM) plus 5% Foetal Bovine Serum (FBS). A 1:10 dilution step was carried out by taking 100µl from the reconstituted vial and adding to 900µl of DMEM. 100µl of the resulting diluted virus was then added to the first row on the plate and a 1:2 dilution ran down the plate. The process was repeated with the next excipient. After a further 48 hours, the number of GFP cells per well were counted using fluorescent microscopy.

***Statistical Analysis***

**[0134]** A one way ANOVA test followed by a Bonferroni post test was performed to analyse significance between different excipients using PRISM Graphpad software version 4.00. The p value summaries are *=p< 0.05; **=p<0.01; ***=p < 0.001.

***Results and Discussion***

**[0135]** Figure 11 shows the benefit of a combination of mannitol and DMG on the preservation of adenovirus titre following freeze drying. Following freeze drying there was approximately half

a log drop in virus titre when DMG was used as an excipient on its own. When mannitol was the sole excipient the loss in titre was more significant than DMG with virus titre being reduced by 2 logs. When however both mannitol and DMG were used, there was no significant loss in titre and the appearance of freeze-dried cake improved.

**Reference Example 8**

**[0136]** The aim of the experiment in this Example was to assess cake formation. Example 8 was conducted in the same manner as Example 7 except that a broader panel of excipients mixes were employed. Each type of excipient (see Table 10 below) was made up as a stock and 300µl added to appropriately labelled 2ml glass vials. After vortexing, vials were loaded onto the VirTis Advantage freeze drier which was run according to the freeze drying protocol given in Table 9. Following freeze drying, samples were photographed and assessed for cake formation.

**Table 10 - Final concentrations of excipient mixes for Example 8**

| | | | | | |
|---|---|---|---|---|---|
| 0.7M DMG, 0.018M Mannitol | 0.7M DMG, 0.03M Mannitol | 0.7M DMG, 0.07M Mannitol | 0.7M DMG, 0.15M Mannitol | 0.7M DMG, 0.29M Mannitol | 0.7M DMG, 0.58M Mannitol |

**[0137]** The appearance of the lyophilised cakes following freeze drying was examined. The results are shown in Figure 12. Various concentrations of mannitol were used in the presence of 0.7M DMG. The highest mannitol concentration was 0.58M and the lowest was 0.018M. At the highest concentration of mannitol (0.58M), a white opaque cake was formed. As the concentration decreased, a less desirable transparent clear foam was formed.

**Reference Example 9**

**[0138]** Example 9 was conducted in the same manner as Example 7 except that only two types of excipients were prepared. The first excipient was the adenovirus in PBS made up to a final volume of 300ul. The second excipient was mannitol (0.58M) and DMG (0.7M) with the adenovirus in 2ml glass vials. After vortexing, vials were loaded onto the VirTis Advantage freeze drier and freeze-dried according to the freeze drying protocol given in Table 9. Following freeze drying, samples were either assayed for virus titre or heat treated for one week at +37°C and then assayed.

**[0139]** The results are shown in Figure 13. After freeze drying, there was a drop in virus titre of greater than half a log in the PBS controls. No significant loss in virus titre was seen in samples containing DMG and mannitol compared to the original virus stock. After heat treatment at +37°C, there was again a drop in virus titre of greater than half a log in the PBS controls. The virus titre in the samples containing DMG and mannitol declined by approximately 0.3 log

compared to the original stock titre.

**Example 10: Stablisation of adenovirus**

**Preparation and lyophilisation of virus**

**[0140]** Recombinant human adenovirus Ad5 (Vector Biolabs) expressing enhanced GFP (Green Fluorescent Protein) under a CMV promoter, and with a titre (pre-freeze) of $6.7x10^5$ pfu/ml in SSC, was removed from storage at -80°C and allowed to thaw. 50µl aliquots were added to 2 ml freeze-drying vials. To these 50µl virus samples was added 250µl of a formulation mixture composed of DMG, MSM and optionally sucrose. Each formulation mixture was made up in SSC. The concentration of DMG, MSM and sucrose in each formulation after addition to the virus sample is shown in Table 11:

**Table 11 - Tested formulations**

| Formulation | Sucrose | MSM | DMG |
|---|---|---|---|
| | (M) | (M) | (M) |
| 1 | 0.00 | 0.10 | 0.10 |
| 2 | 0.15 | 0.10 | 0.10 |
| 3 | 0.00 | 1.00 | 0.10 |
| 4 | 0.15 | 1.00 | 0.10 |
| 5 | 0.08 | 0.55 | 0.55 |
| 6 | 0.08 | 0.55 | 0.55 |
| 7 | 0.08 | 0.55 | 0.55 |
| 8 | 0.00 | 0.10 | 1.00 |
| 9 | 0.15 | 0.10 | 1.00 |
| 10 | 0.00 | 1.00 | 1.00 |
| 11 | 0.15 | 1.00 | 1.00 |

**[0141]** Rubber bungs were partially inserted. After vortexing, the vials were loaded onto a Virtis Advantage Plus EL85 freeze-dryer and lyophilised on program 4. Thus, samples were freeze dried using the drying cycles shown in Table 12 below. Samples were frozen at -45°C for 1 hour 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 -42°C. Step 11 was extended until the samples were stoppered before releasing the vacuum.

**Table 12 - Drying Cycles**

| Step | Shelf temp (°C) | Time (mins) | Ramp-Hold | Vacuum (milliTorre) |
|---|---|---|---|---|
| 1 | -45 | 30 | H | 300 |
| 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 |

**[0142]** In the thermal treatment, the shelf temperature was dropped to -40°C.

***Thermal challenge of lyophilised virus***

**[0143]** After lyophilisation, vials were immediately capped, removed, crimped and then placed at 37°C for thermal challenge. Thermal challenge was for 7 days, after which all the vials were returned to 4°C until it was practical to assay them.

***Assay of recovered infectious adenovirus from rehydrated cakes***

**[0144]** 96 flat bottomed cell culture dishes (VWR, UK) were seeded with HEK 293 (ECACC 85120602) cells at $10^5$ cells per ml (100µl per well) and maintained at 37°C with 5% $CO_2$. After achieving 90% confluence, cells were inoculated.

**[0145]** Vials containing adenovirus plus excipient were reconstituted in 300µl SSC. A 1 in 10 dilution step was then taken by taking 20 µl from the reconstituted vial and adding to 180µl of Dulbecco's Modified Eagle Medium (DMEM). A further 1 in 100 dilution (of the original sample) was performed by taking 20µl of the 1 in 10 dilution and adding it to 180µl of DMEM. 100µl of each of the resultant dilution (1 in 10 and 1 in 100) was then added to wells of the plate containing HEK 293 cells.

**[0146]** Additionally, a further sample of adenovirus, from the same source and with the same titre (on storage at -80°C) used in the excipient treatments, was thawed and used to produce a 1 in 10 dilution series (in DMEM + 10% FBS). Dilutions ranging from 1 in 10 to 1 in $10^6$ were

also added to individual wells containing HEK 293s. At 48 hours post inoculation, the number GFP (Green Fluorescent Protein) cells per well were counted using fluorescent microscopy, and this was subsequently converted to pfu/ml of the treated samples taking into account the volume applied and dilution of the inoculum.

***Results***

**[0147]** The results as shown in Figure 14. When the data was analysed by multiple linear regression (MLR) analysis using the MODDE 9.0 programme (Umetrics, Sweden), a synergistic effect was observed when MSM and DMG were used in combination and when DMG and sucrose were used in combination.

**Example 11: Stablisation of MVA**

***Preparation and lyophilisation of virus***

**[0148]** MVA was recovered from storage at -80°C and thawed. 50µl aliquots were added to 2 ml freeze-drying vials. To these virus samples was added 250µl of a formulation mixture listed in Table 11 above. Rubber bungs were partially inserted. After vortexing, the vials were loaded onto a Virtis Advantage Plus EL85 freeze-dryer and lyophilised on program 4 as described in Example 10.

***Thermal challenge of lyophilised virus***

**[0149]** After lyophilisation, vials were immediately capped, removed, crimped and then placed at 37°C for thermal challenge. Thermal challenge was for 7 days, after which all the vials were returned to 4°C until it was practical to assay them.

***Assay of infectious MVA recovered from rehydrated cakes***

**[0150]** MVA plus excipient were reconstituted in 300ml of SSC. The reconstituted samples were diluted and assayed.

**[0151]** Assay plates (96 wells) were seeded with BHK-21 cells (100µl per well, $10^5$ cells/ml). Cells were diluted in DMEM supplemented with 10% FBS, and 1% PS. The plates were placed at +37°C, + 5% $CO_2$ for 1 to 2 hours.

**[0152]** Meanwhile, a dilution series of the formulated MVA samples was prepared (in the same growth media) ranging from $10^{-1}$ to $10^{-4}$. Each dilution series was prepared 4 times. 35µl of each dilution was applied to individual wells containing BHK-21 cells and the wells were topped up with a further 65µl of media.

**[0153]** On day 6 after inoculation, the wells were scored for presence or absence of cytopathic effect (CPE) and $TCID_{50}$ calculated. These were then used to estimate the concentration of infectious MVA per ml in the thermo-challenged vials.

***Results***

**[0154]** The results are shown in Figure 15. The range of responses in this screening study was from 0.6-60.5% of starting titre (see Figure 15). This was assessed relative to a second aliquot of the virus held at -80°C until assay. Figure 15 shows the response to each formulation treatment as percentage of a positive control. The best performing formulation comprised 0.15M sucrose, 1M DMG, 1M MSM. Overall, the results strongly suggest that this combination of excipients has significant potential for the stabilisation of viruses in a freeze-dried setting.

**Reference Example 12**

**Materials**

**[0155]**

*Chemical*

| | Supplier | Product Code | Lot No. |
|---|---|---|---|
| 20x SSC | Sigma | S6639 | 020M8404 |
| Betaine | Sigma | B2629 | 069K1514 |
| Dimethyl glycine | Sigma | D1156 | 077K1856 |
| Dimethyl sulfone | Sigma | M81705 | 0001452516 |
| Dulbeccos Modified Eagles Medium | Sigma | D5796 | RNBB1139 |
| Foetal Bovine Serum | Sigma | F7524 | 109K3395 |
| Penicillin Streptomycin | Sigma | P4458 | 0409M0093 |
| Raffinose | Sigma | R0250 | 050M0053 |
| S-Methyl methionine | Sigma | 64382 | |
| Sucrose | Sigma | 16104 | SZB90120 |
| Water | Sigma | W3500 | 8M0411 |

| | Supplier | Product Code | Lot No. |
|---|---|---|---|
| X-Gen 500 P-Lin | Fermentas | R0521 | 00064973 |

*Biological*

| | Supplier | Product Code |
|---|---|---|
| Adenovirus | Vector Biolabs | Ad-CMV-GFP |
| BHK-21 cell line | ECACC | CB2857 |
| HEK 293 | ECACC | 85120602 |

*Other*

| | Manufacturer | Product Code |
|---|---|---|
| 5ml glass vials | Adelphi Tubes | VCD005 |
| 14mm freeze drying stoppers | Adelphi Tubes | FDIA14WG/B |
| 14mm caps | Adelphi Tubes | CWPP14 |
| 2ml glass vials | Adelphi Tubes | VCDIN2R |
| 13mm freeze drying stoppers | Adelphi Tubes | FDW13 |
| Crimps | Adelphi Tubes | COTW13 |

**Equipment**

**[0156]**

| | Manufacturer | Equipment No. |
|---|---|---|
| Virtis Advantage Plus EL85 Freeze Dryer | Virtis | EQP#084 |
| HERA safe class II cabinet | Thermo Fisher | EQP# 011 & 012 |
| DMIL LED Inverted Microscope | Leica | EQP#062 |
| Binder $CO_2$ Incubator | Binder | EQP#014 |
| Forma 900 series -80°C freezer | Thermofisher | EQP#015 |
| ATL-84-1 Atlion Balance | Acculab | EQP#088 |
| IP250 37°C Incubator | LTE | EQP#016 |

**Methods**

***Design of Experiment***

**[0157]** MODDE 9.0 was used to generate a three factor, two level full factorial screening

design (see Figure 16 showing coded values, and Table 13 showing actual concentrations applied). This design involves testing combinations of the excipients at the high and low levels of the tested range as well as replicated centre points. The replicated centre points give an indication of error in the experiment.

**[0158]** The design can model 1st order effects of each tested factor (excipient) and interactions between them, that is, determine the impact of the presence of the exipients to the formulation. It cannot model 2nd order of higher effects but can give an indication of whether they are present (curvature in the data). Second order effects result from covariance within the data, that is, two or more variables are dependent upon one another. Though 2nd order effects are expected, the intent is to use this simple screening study, with minimal treatments, in order to detect any effect of the excipient and then take forward any excipient that have an effect into a more sophisticated study that can model the effects more accurately.

***Stability of Adenovirus in a Lyophilised Setting***

***Preparation of and thermal challenge of formulated Adenovirus in a Freeze-Dried Setting***

**[0159]** Recombinant Adenovirus expressing enhanced GFP under a CMV promoter, with a titre (pre-freeze) of $6.7x10^5$ pfu/ml in saline sodium citrate (SSC), was removed from storage at -80°C and allowed to thaw at room temperature. Subsequently, 50µl aliquots of virus were added to 15 individual 2ml glass freeze-drying vials. To each vial 250µl of an excipient blend was admixed. The excipient blend formulations once mixed with virus are described in Table 13 and were made up in SSC.

**Table 13**

| Sample ID | Sucrose (M) | Raffinose (mM) | Excipient | Titre (pfu/ml) | | |
|---|---|---|---|---|---|---|
| | | | (M) | DMG | SMM | TMG |
| | | | | | | |
| 1 | 0.15 | 15.0 | 0.10 | 3.6E+3 | 6.0E+2* | 4.8E+3 |
| 2 | 1.50 | 15.0 | 0.10 | 6.0E+4 | 1.6E+5 | 1.0E+5 |
| 3 | 0.15 | 150.0 | 0.10 | 2.4E+3 | 6.0E+2* | 5.4E+3 |
| 4 | 1.50 | 150.0 | 0.10 | 9.0E+4 | 2.3E+5 | 9.0E+4 |
| 5 | 0.83 | 82.5 | 0.55 | 1.4E+5 | 1.7E+5 | 5.3E+4 |
| 6 | 0.83 | 82.5 | 0.55 | 1.6E+5 | 2.1E+5 | 1.2E+4 |
| 7 | 0.83 | 82.5 | 0.55 | 1.1E+5 | 2.7E5** | 7.8E+4 |

| Sample ID | Sucrose (M) | Raffinose (mM) | Excipient | Titre (pfu/ml) | | |
|---|---|---|---|---|---|---|
| | | | (M) | DMG | SMM | TMG |
| 8 | 0.15 | 15.0 | 1.00 | 1.9E+5 | 4.0E+4 | 2.9E+5 |
| 9 | 1.50 | 15.0 | 1.00 | 2.2E+5 | 9.0E+4 | 1.1E+5 |
| 10 | 0.15 | 150.0 | 1.00 | 1.9E+5 | 1.1E+5 | 3.1E+5 |
| 11 | 1.50 | 150.0 | 1.00 | 8.4E+4** | 1.7E+5 | 6.6E+3 |
| * =count below detectable levels \ assigned value of detection limit for ease of data transformation.<br>** = datapoint excluded during model fine tuning as an apparent outler | | | | | | |

**[0160]** Rubber bungs were partially inserted, and after vortexing were loaded onto a VirTis Advantage Freeze Dryer and lyophilised on program 4 (see Figure 17). After lyophilisation samples were immediately capped under vacuum, removed, crimped and placed at 37°C for thermal challenge. Thermal challenge was for 7 days, after which all the vials were held at 4°C until it was practical to assay them. Freeze-dried samples were reconstituted in 300µl SSC immediately prior to assay.

***Assay of adenovirus***

**[0161]** HEK 293 cells were prepared in 96 well flat bottomed cell culture dishes for inoculation by seeding at $10^5$ cells per ml (100µl per well) and maintained at 37°C with 5% $CO_2$. After 2 hours cells were inoculated as follows.

**[0162]** Thermo-challenged virus samples were diluted 1 in 10, and 1 in 100 in DMEM +10% FBS. 100µl of each of the resulting diluted virus samples were then added to individual wells of the assay plate. Additionally, a second aliquot of the original adenovirus in SSC was thawed from -80°C and a 10 fold dilution series (from 1 in 10 to 1 in 100,000) also prepared in DMEM +10% FBS. Two repeats of this positive control dilution series was inoculated to each 96 well plate used. After a further 48 hours, the number of GFP cells per well were counted using fluorescent microscopy.

**Results**

***General***

**[0163]** A good range of responses was observed in each experiment. Most yielded a range of

recovered viral activity of between just a few percent and 32-46% (see Table 14).

**Table 14 - model assessment parameters and range of responses for each excipient tested**

| Model Assessment Parameters | | | | | Data Spread (% Recovered Activity) | |
|---|---|---|---|---|---|---|
| $R^2$ | | $Q^2$ | MV | Rep. | Low | High |
| DMG | 0.95 | 0.88 | 0.94 | 0.89 | 0.36 | 32.84 |
| SMM | 0.5 | 0.4 | 0.71 | 0.88 | 0.09* | 40.30 |
| TMG | 0.75 | 0.54 | 0.88 | 0.60 | 0.72 | 46.27 |
| * = below detectable limit there for assigned threshold value to allow easier data transformation | | | | | | |

**[0164]** This spread of responses was sufficient to permit a suitable model to be applied. It is also indicative of a protective effect of the formulations.

**[0165]** In several models the lowest response was below the detection threshold of the assay. For ease of transforming datasets (log transformations) the response in these cases was assigned the level of the detection threshold, which in this case (taking to account the countable level and then allowing for dilution factors etc.) is $6x10^2$ pfu/ml.

**[0166]** Further modelling analyses are set out in Table 15 to 17.

**Table 15 - model assessment parameters from models where a non-specific 2nd order interaction is allowed**

| | $R^2$ | $Q^2$ | MV | Rep. |
|---|---|---|---|---|
| DMG | No curvature | | | |
| SMM | 0.95 | 0.66 | 0.89 | 0.88 |
| TMG | No improved model | | | |

**Table 16 - coefficients retained in the model after fine tuning**

| | Factors / Interactions | | | | | | |
|---|---|---|---|---|---|---|---|
| | NE | Suc | Raff | NExS | NExR | SxR | Curvature |
| DMG | ✓ | ✓ | | | | | No |
| SMM | * | ✓ | * | * | ▪ | | Yes |
| TMG | ✓ | * | ▪ | ✓ | ▪ | | Yes |

**[0167]** The following abbreviations/columns are present in Table 16

NE = novel excipient, Suc = sucrose, Raff = raffinose (all $1^{st}$ order effects).

NExS = interaction between NE and sucrose.

NExR = interaction between NE and raffinose.

SxR = interaction between sucrose and raffinose.

Curvature = indication of 2nd order effect.

* = non-significant term retained in model for model stability.

**Table 17 - coefficients retained in the model after fine tuning and inclusion of a hypothetical 2nd order effect**

| | Factors / Interactions | | | | | | |
|---|---|---|---|---|---|---|---|
| | **NE** | **Suc** | **Raff** | **NExS** | **NExR** | **SxR** | **2nd order term** |
| **DMG** | No curvature | | | | | | |
| **SMM** | ▪ | ✓ | ✓ | ✓ | ▪ | | ✓ |
| **TMG** | No improved model | | | | | | |

**[0168]** The following abbreviations/columns are present in Table 17

NE = novel excipient, Suc = sucrose, Raff = raffinose (all 1st order effects).

NExS = interaction between NE and sucrose.

NExR = interaction between NE and raffinose.

SxR = interaction between sucrose and raffinose.

Curvature = indication of 2nd order effect.

2nd order term = a 2nd order effect predicted by curvature in the data, that strengthens the models. The experimental design is unable to identify specific 2nd order effects.

***DMG***

**[0169]** A good spread of responses was found in this dataset (0.36-32.84% recovery) and notably the lowest response is above the detection limit (see Table 14). One data point, sample ID 11 (see Table 13) was eliminated from the analysis after being flagged as an obvious outlier during fine tuning of the model. The reason for this outlier is unknown but is presumed to be operator error. All four indicators of model strength are high (see Table 14), and no curvature was observed in the data (see Figure 19). Only two critical factors were identified by the model,

DMG and sucrose were each found to be significant positive 1st order effects (see Table 16 and Figure 20). No other effects or interactions were observed. Raffinose was not indicated as having an effect on the model and hence viral recovery in the range tested.

***SMM***

**[0170]** A good range of responses was observed in this dataset (0.09-40.30% recovery) (see Table 14), although, the lowest in this range was below the detection threshold. During model fine tuning one data point (sample ID 7) was eliminated from the analysis as an obvious outlier (see Table 13). The first model generated identified only sucrose as a critical factor (see Figure 21), however, it was necessary to retain non-significant factors (SMM, raffinose, and SMM*sucrose) within the model to achieve any sort of meaningful significance.

**[0171]** Even so, the model scores relatively poorly on $R^2$ and $Q^2$ (0.5 and 0.4 respectively). Figure 22 shows evidence of curvature in the model. Following this observation a new model was developed with the inclusion of a 2nd order effect. As in previous examples the specific 2nd order effect cannot be identified with this experimental design. The new model scored more highly on all four model assessment parameters. This model identified sucrose, and raffinose as 1st order effects as well as an interaction between SMM and sucrose and the putative 2nd order effect of one excipient. (see Figure 23). This new model showed no evidence of curvature within the model (see Figure 24).

***TMG***

**[0172]** A good spread of responses was observed in this dataset (0.72-46.27%) and all the data points were above the detectable threshold (see Table 13). Acceptable scores were generated for all four model assessment parameters (see Table 14). The model identifies a 1st order effect of TMG and an interaction between TMG and sucrose (see Table 16). Raffinose and sucrose are identified as non significant factors but sucrose is retained in the model to preserve the hierarchical model (see Figure 25). Figure 26 suggests curvature in the model; however, the model was not improved by the inclusion of a 2nd order interaction suggesting some other cause for the curvature.

**Reference Example 13**

**Materials**

**[0173]**

*Chemical*

| | Supplier | Product Code | Lot No. |
|---|---|---|---|
| 20x SSC | Sigma | S6639 | 020M8404 |
| Dimethyl glycine | Sigma | D1156 | 077K1856 |
| Dulbeccos Modified Eagles Medium | Sigma | D5796 | RNBB1139 |
| Foetal Bovine Serum | Sigma | F7524 | 109K3395 |
| Penicillin Streptomycin | Sigma | P4458 | 0409M0093 |
| Raffinose | Sigma | R0250 | 050M0053 |
| Sucrose | Sigma | 16104 | SZB90120 |
| Water | Sigma | W3500 | 8M0411 |

*Biological*

| | Supplier | Product Code |
|---|---|---|
| Adenovirus | Vector Biolabs | Ad-CMV-GFP |
| HEK 293 | ECACC | 85120602 |

Other

| | Manufacturer | Product Code |
|---|---|---|
| 2ml glass vials | Adelphi Tubes | VCDIN2R |
| 13mm freeze drying stoppers | Adelphi Tubes | FDW13 |
| Crimps | Adelphi Tubes | COTW13 |

**Equipment**

**[0174]**

| | Manufacturer | Equipment No. |
|---|---|---|
| Virtis Advantage Plus EL85 Freeze Dryer | Virtis | EQP#084 |
| HERA safe class II cabinet | Thermo Fisher | EQP# 011 & 012 |
| DMIL LED Inverted Microscope | Leica | EQP#062 |
| Binder $CO_2$ Incubator | Binder | EQP#014 |
| Forma 900 series -80°C freezer | Thermofisher | EQP#015 |
| ATL-84-1 Atlion Balance | Acculab | EQP#088 |
| IP250 37°C Incubator | LTE | EQP#016 |

**Methods**

***Design of Experiment***

**[0175]** MODDE 9.0 (Umetrics) was used to generate a Doehlert experimental design (see Figure 27). Doehlert designs are response surface modelling designs constructed from regular simplexes. They are easily extendable in different directions and new factors can be added to an existing design. Unlike regular formulation designs non-significant factors can be eliminated from the analysis and so do not become a confounding factor. Furthermore, different factors within the design are tested at a different number of levels, so it is possible to allocate more test levels to factors that are suspected of greater importance. Thus the excipients were tested at 7 levels, whilst sucrose was tested at 5 levels and raffinose at only 3 levels. This model retains the ability to model for second order effects and interactions. The design included 3 factors and 3 replicate centre-points resulting in 15 test samples.

**[0176]** Sucrose was tested between 0 and 1M. The upper level of sucrose was set at 1M because it has proved close to the limit for acceptable freeze-drying. It has also proved to be a highly successful level in prior studies, and in general higher sucrose concentrations are undesirable in parenterals. The lowest level of Sucrose was set at 0 M. Raffinose was tested over a range of 0 to 300mM although the nature of the Doehlert design meant that tested levels did not include 0mM, instead the following concentrations were tested; 27.5, 150.0, and 272.5mM.

**[0177]** DMG was tested over a linear range of 0 to 2M. It was possible to limit this range based on previous experiments in which the optimum concentration was frequently between 0.5 and 1.5M in a freeze-dried setting.

***Stability of Adenovirus in a Freeze-Dried Setting***

***Preparation of and thermal challenge of formulated Adenovirus in a Freeze-Dried Setting***

**[0178]** Recombinant Adenovirus expressing enhanced GFP under a CMV promoter, with a titre (pre-freeze) of $6.7x10^5$ pfu/ml in SSC, was removed from storage at -80°C and allowed to thaw. Subsequently, 50µl aliquots of virus were added to 15, 2ml, glass freeze-drying vials. To each vial 250µl of an excipient blend was admixed. The excipient blend formulations once mixed with virus are described in Table 18 and were made up in SSC.

**Table 18**

| Formulation No. | Sucrose | Raffinose | DMG | Titre (pfu/ml) |
|---|---|---|---|---|
| | (M) | (mM) | (M) | |
| 1 | 0.25 | 150.0 | 0.13 | 4.8E+04 |
| 2 | 0.75 | 150.0 | 0.13 | 1.3E+05 |
| 3 | 0.5 | 272.5 | 0.42 | 3.0E+05 |
| 4 | 0.25 | 27.5 | 0.71 | 2.2E+05 |
| 5 | 0.75 | 27.5 | 0.71 | 3.1E+05 |
| 6 | 0 | 150.0 | 1.00 | 3.1E+05 |
| 7 | 0.5 | 150.0 | 1.00 | 5.2E+05 |
| 8 | 0.5 | 150.0 | 1.00 | 3.7E+05 |
| 9 | 0.5 | 150.0 | 1.00 | 4.6E+05 |
| 10 | 1 | 150.0 | 1.00 | 4.7E+05 |
| 11 | 0.25 | 272.5 | 1.29 | 3.1E+05 |
| 12 | 0.75 | 272.5 | 1.29 | 3.3E+05 |
| 13 | 0.5 | 27.5 | 1.58 | 4.4E+05 |
| 14 | 0.25 | 150.0 | 1.87 | 1.4E+05 |
| 15 | 0.75 | 150.0 | 1.87 | 2.5E+05 |

**[0179]** Rubber bungs were partially inserted, and after vortexing were loaded onto a Virtis advantage freeze-dryer and lyophilised on program 4 (see Figure 28). After lyophilisation samples were immediately capped under vacuum, removed, crimped and placed at 37°C for thermal challenge. Thermal challenge was for 7 days, after which all the vials were held at 4°C until it was practical to assay them. Freeze-dried samples were reconstituted in 300µl SSC immediately prior to assay.

***Assay of Adenovirus***

**[0180]** HEK 293 cells were prepared in 96 well flat bottomed cell culture dishes for inoculation by seeding at $10^5$ cells per ml (100µl per well) and maintained at 37°C with 5% $CO_2$. After 2 hours cells were inoculated as follows.

**[0181]** Thermo-challenged virus samples were diluted 1 in 10, and 1 in 100 in DMEM +10% FBS. 100µl of each of the resulting diluted virus samples were then added to individual wells of the assay plate. Additionally, a second aliquot of the original Adenovirus in SSC was thawed from -80°C and a 10 fold dilution series (from 1 in 10 to 1 in 100,000) also prepared in DMEM +10% FBS. Two repeats of this positive control dilution series was inoculated to each 96 well plate used. After a further 48 hours, the number of GFP cells per well were counted using

fluorescent microscopy.

**Results**

**[0182]** A strong model was produced in which all four indicators suggested good significance (R2=0.93, Q2=0.79, Model Validity=0.98, Reproducability=0.68) (see Figure 29). Of these the figure for reproducibility is the only one that is slightly low, although it is well above 0.5. The reason for this value being slightly lower than has been customary could be the slightly higher variation between the replicated centrepoints or rather the level of variation between these is proportionally larger compared to the overall variation in the assay.

**[0183]** The model identified (see Figure 30) $1^{st}$ order effects of both sucrose and DMG as well as a 2nd order effect of DMG. No 1st or 2nd order effects of raffinose were observed. However, raffinose does have an interaction with DMG and thus the 1st order raffinose coefficient must be retained in the model to preserve the models hierarchical structure. Furthermore, the 2nd order raffinose effect was retained as it resulted in a stronger model (as assessed by the indicators shown in Figure 29 and discussed above). In any case the 2nd order raffinose effect was close to significance at the 90% C.I. and may be a genuine effect that simply cannot be conclusively detected over the range tested.

**[0184]** Figure 31 shows a series of 3D plots of recovered virus activity (Y-Axis) against varied sucrose (X-axis) and DMG (Z-axis) concentrations. "Low" denotes a raffinose concentration of 0mM, "Mid" denotes a raffinose concentration of 150mM and "High" denotes a raffinose concentration of 300mM.

**[0185]** Each plot shows the model at a different and static raffinose concentration. Improved preservation of adenovirus is achieved by increasing sucrose concentration. This trend continues beyond the tested range the experiment is unable to identify a true sucrose optimum. In contrast, the optimum DMG concentration is clearly within the tested range. Increasing Raffinose concentration appears to decrease the optimum DMG concentration.

**[0186]** Monte-Carlo simulations were used to predict an optimal formulation (see Figure 32). An optimum of 0.5M Sucrose, 1M DMG, 150mM Raffinose was predicted to yield a recovered virus titre of $4.2x10^5$ pfu/ml or 98% of that input before thermal challenge (based on a positive control which had a titre of $4.3x10^5$ pfu/ml).

**[0187]** The predicted optimum is shown on a contour plot (Figure 33a) which puts the optimum into context. The model predicts whole regions of the design space in which formulations would yield 100% or greater recovered virus activity. This region needs to be viewed as a plateau in the data within which close to zero loss of virus activity would be expected. Figure 33b highlights this region. The figure shows that as raffinose concentration is increased the region moves down the Y-axis (DMG concentration) and up the X-axis (sucrose concentration).

**Conclusions**

**[0188]** A formulation of DMG, sucrose and raffinose has been identified with significant potential for the preservation of adenovirus through lyophilisation and heat challenge. Models based on the data predict that recovery of 100% of viral activity is possible. This model, an optimum DMG concentration of between 0.5 and 1.5M was identified. The optimum sucrose concentration is beyond the tested range and also likely beyond other constraints of sucrose concentration. Raffinose does not appear to be a critical factor in this model.

**Reference Example 14**

**Materials**

**[0189]**

*Chemical*

| | Supplier | Product Code | Lot No. |
|---|---|---|---|
| 20x SSC | Sigma | S6639 | 020M8404 |
| Dimethyl glycine | Sigma | D1156 | 077K1856 |
| Dulbeccos Modified Eagles Medium | Sigma | D5796 | RNBB1139 |
| Foetal Bovine Serum | Sigma | F7524 | 109K3395 |
| Penicillin Streptomycin | Sigma | P4458 | 0409M0093 |
| Raffinose | Sigma | R0250 | 050M0053 |
| Sucrose | Sigma | 16104 | SZB90120 |
| Water | Sigma | W3500 | 8M0411 |

*Biological*

| | Supplier | Product Code |
|---|---|---|
| Adenovirus | Vector Biolabs | Ad-CMV-GFP |
| HEK 293 | ECACC | 85120602 |

*Other*

| | Manufacturer | Product Code |
|---|---|---|
| 2ml glass vials | Adelphi Tubes | VCDIN2R |
| 13mm freeze drying stoppers | Adelphi Tubes | FDW13 |
| Crimps | Adelphi Tubes | COTW13 |

**Equipment**

**[0190]**

| | Manufacturer | Equipment No. |
|---|---|---|
| Virtis Advantage Plus EL85 Freeze Dryer | Virtis | EQP#084 |
| HERA safe class II cabinet | Thermo Fisher | EQP# 011 & 012 |
| DMIL LED Inverted Microscope | Leica | EQP#062 |
| Binder $CO_2$ Incubator | Binder | EQP#014 |
| Forma 900 series -80°C freezer | Thermofisher | EQP#015 |
| ATL-84-1 Atlion Balance | Acculab | EQP#088 |
| IP250 +37°C Incubator | LTE | EQP#016 |
| +4°C long term sample fridge | LEC | EQP#090 |
| KB115 +25°C incubator | Binder | EQP#008 |

**Design of Experiment**

**[0191]** A long term stability study was planned to test putative optimal formulation of DMG, sucrose and raffinose for adenovirus in a lyophilised setting. Three formulations were tested:

| | |
|---|---|
| B | Adenovirus in SSC buffer alone; |
| B | Adenovirus in 0.5M sucrose and 150mM raffinose in SSC; and |
| B | Adenovirus in 0.5M sucrose, 150mM raffinose and 1M DMG also in SSC. |

**[0192]** A long-term stability testing temperature of +4°C±3 was selected. This is broadly consistent with standard industry guidelines for long-term testing of products intended for refrigerated storage (+5°C±3). An accelerated stability temperature of +25°C was adopted and a thermal challenge of +37°C was adopted to represent a stress testing temperature, or a further elevated accelerated thermal stability temperature.

**[0193]** The samples at 25°C and 37°C were tested 1, 2, 5 and 15 weeks post lyophilisation. The samples at +4°C were tested at 15 weeks post lyophilisation.

**Preparation of and thermal challenge of formulated adenovirus in a Freeze-Dried Setting**

**[0194]** Recombinant adenovirus expressing enhanced GFP under a CMV promoter, with a titre

(pre-freeze) of $6.7x10^5$ pfu/ml in SSC, was removed from storage at -80°C and allowed to thaw. Subsequently, 50µl aliquots of virus were added to 2ml glass freeze-drying vials. To each vial 250µl of an excipient blend was admixed. The excipient blend formulations used were as described above, namely (i) buffer alone (SSC), (ii) sugars (0.5M Sucrose, 150mM Raffinose in SSC), and (iii) a putative optimal formulation (0.5M Sucrose, 150mM Raffinose, 1M DMG, also in SSC).

**[0195]** Rubber bungs were partially inserted, and after vortexing were loaded onto a VirTis Advantage Freeze Dryer and lyophilised on program 1 (see Figure 34). After lyophilisation samples were immediately capped under vacuum, removed, crimped, and divided between the three thermal treatments. Subsequently, at each time point 2 vials of each formulation were recovered according to the schedule above and reconstituted in 300µl SSC immediately prior to assay.

**Assay of Adenovirus**

**[0196]** HEK 293 cells were prepared in 96 well flat bottomed cell culture dishes for inoculation by seeding at $10^5$ cells per ml (100µl per well) and maintained at 37°C with 5% $CO_2$. After 2 hours cells were inoculated as follows.

**[0197]** Thermo-challenged virus samples were recovered from thermo challenge as described above diluted 1 in 10, and 1 in 100 in DMEM +10% FBS. 100µl of each of the resulting diluted virus samples were then added to individual wells of the assay plate.

**[0198]** Additionally, a second aliquot of the original adenovirus in SSC was thawed from -80°C and a 10 fold dilution series (from 1 in 10 to 1 in 100,000) also prepared in DMEM +10% FBS. Two repeats of this positive control dilution series was inoculated to each 96 well plate used. After a further 48 hours, the number of GFP cells per well were counted using fluorescent microscopy.

**Results**

**[0199]** At 15 weeks, (see Figure 35) no virus activity was recovered from samples formulated in buffer alone (SSC). Formulation with sugars prevents some of this loss. However, only in those samples stored at +4°C are losses less than a full log drop i.e, recovered activities over 10% of starting titre. In this treatment at this time point losses are progressively worse with increasing temperature. Since elevated temperature is a standard mode of simulating longer thermal stability studies (accelerated stability) it is suggested that the losses in sugars have not reached endpoint at +4°C and that further losses over time can be expected.

**[0200]** Using the putative optimum formulation losses are further reduced. In fact at all three

storage temperatures losses are around a half log loss (33% recovered activity). The responses at all three temperatures are between 27.84 and 30.00% recovery which represent a loss of 0.52-0.54 Logs. There does not appear to be significant differences between the three temperatures (+4°C, 25°C and +37°C) with this formulation. This implies that either (a) the degradation has reached end-point and no further degradation over time can be expected, or (b) that the rate of decline has become so slow that the difference in the accelerated temperature studies cannot be detected.

**[0201]** Figures 36 and 37 further support these findings. At both, +25°C and +37°C, no virus activity is recovered from samples stored in buffer alone at any time-point. Those formulated in sugars alone retain some activity throughout. Their activity declines by a slightly greater degree and slightly more rapidly at the higher temperature (+37°C). In the putative optimal formulation there is a steeper decline in viral activity at +37°C but both temperatures decline to similar levels over time.

**[0202]** At both +25°C and +37°C with all the formulations tested the majority of the decline in virus activity occurs between t=0 and t=5 weeks. In fact, in the case of buffer alone and sugar formulations the vast majority of degradation occurs between t=0 and t=1 week. The value of the response used as t=0 was the titre of the virus prior to lyophilisation and thermal challenge whereas the time-points are plotted as weeks post thermal challenge. Consequently, the observed differences between t=0 and t=1 week are the sum of degradation during lyophilisation and during the first week of thermal challenge.

**Reference Example 15**

**Materials**

**[0203]**

*Chemical*

| | Supplier | Product Code | Lot No. |
|---|---|---|---|
| 20x SSC | Sigma | S6639 | 020M8404 |
| Dulbeccos Modified Eagles Medium | Sigma | D5796 | RNBB1139 |
| Foetal Bovine Serum | Sigma | F7524 | 109K3395 |
| Penicillin Streptomycin | Sigma | P4458 | 0409M0093 |
| Trimethyl glycine | Sigma | | |
| Water | Sigma | W3500 | 8M0411 |

*Biological*

| | Supplier | Product Code |
|---|---|---|
| BHK-21 cell line | ECACC | CB2857 |

| | Supplier | Product Code |
|---|---|---|
| MVA | ATCC | VR-1508 |

*Other*

| | Manufacturer | Product Code |
|---|---|---|
| 2ml glass vials | Adelphi Tubes | VCDIN2R |
| 13mm freeze drying stoppers | Adelphi Tubes | FDW13 |
| Crimps | Adelphi Tubes | COTW13 |

**Equipment**

**[0204]**

| | Manufacturer | Equipment No. |
|---|---|---|
| Virtis Advantage Plus EL85 Freeze Dryer | Virtis | EQP#096 |
| HERA safe class II cabinet | Thermo Fisher | EQP# 011 & 012 |
| DMIL LED Inverted Microscope | Leica | EQP#062 |
| Binder $CO_2$ Incubator | Binder | EQP#014 |
| Forma 900 series -80°C freezer | Thermofisher | EQP#015 |
| ATL-84-1 Atlion Balance | Acculab | EQP#088 |
| IP250 37°C Incubator | LTE | EQP#016 |

**Methods**

***Design of Experiment***

**[0205]** MODDE 9.0 (Umetrics) was used to generate a Doehlert experimental design (see Figure 38), as described in Example 13. Thus, TMG was tested at seven levels, whilst sucrose was tested at five and raffinose three. This model retains the ability to model for second order effects and interactions. The design included three factors and three replicate centre-points resulting in fifteen test samples.

**[0206]** Sucrose was tested between 0 and 1M. Raffinose was tested over a range of 0 to 300mM, although the nature of the Doehlert design meant that tested levels did not include 0mM. Instead the following ranges were tested: 27.5, 150.0, and 272.5mM. TMG was tested over a linear range of 0 to 2M.

***Preparation of and thermal challenge of formulated MVA in a Freeze-Dried Setting***

**[0207]** MVA was recovered from storage at -80°C and thawed. 50µl aliquots of the MVA were added to 2ml, glass freeze-drying vials, subsequently 250µl of an excipient blend was added to each vial. The excipient blend formulations once mixed with virus are described in Table 19 and were made up in SSC.

**Table 19**

| Formulation No. | Sucrose (M) | Raffinose (mM) | TMG (M) | Titre (pfu/ml) |
|---|---|---|---|---|
| 1 | 0.25 | 150.0 | 0.13 | 1.1E+05 |
| 2 | 0.75 | 150.0 | 0.13 | 7.6E+04 |
| 3 | 0.5 | 272.5 | 0.42 | 1.7E+05 |
| 4 | 0.25 | 27.5 | 0.71 | 4.8E+05 |
| 5 | 0.75 | 27.5 | 0.71 | 7.6E+05 |
| 6 | 0 | 150.0 | 1.00 | 4.8E+05 |
| 7 | 0.5 | 150.0 | 1.00 | 7.6E+05 |
| 8 | 0.5 | 150.0 | 1.00 | 7.6E+05 |
| 9 | 0.5 | 150.0 | 1.00 | 7.6E+05 |
| 10 | 1 | 150.0 | 1.00 | 1.2E+06 |
| 11 | 0.25 | 272.5 | 1.29 | 4.8E+05 |
| 12 | 0.75 | 272.5 | 1.29 | 7.6E+05 |
| 13 | 0.5 | 27.5 | 1.58 | 3.0E+05 |
| 14 | 0.25 | 150.0 | 1.87 | 7.6E+05* |
| 15 | 0.75 | 150.0 | 1.87 | 3.0E+05 |
| * indicates an outlier eliminated from the model | | | | |

**[0208]** Rubber bungs were partially inserted, and after vortexing were loaded onto a Virtis advantage freeze-dryer and lyophilised as described in Figure 39. After lyophilisation samples were immediately capped under vacuum, removed, crimped and placed at 37°C for thermal challenge. Thermal challenge was for 7 days, after which all the vials were returned to the control vials and held at 4°C until it was practical to assay them. Freeze-dried samples were reconstituted in 300µl SSC immediately prior to assay.

***Assay of MVA***

**[0209]** Assay plates (96 well) were seeded with BHK-21 cells (100µl per well, $10^5$ cells/ml). Cells were diluted in DMEM supplemented with 10% FBS, and 1% PS. The plates were placed

at +37°C, + 5% $CO_2$ for 1-2 hours.

**[0210]** Meanwhile, a 10 fold dilution series of the formulated MVA samples was prepared (in the same growth media) ranging from 1 in 10 to 1 in 10,000. Each dilution series was prepared 5 times. 100µl of each dilution was applied to individual wells containing BHK-21 cells (described above).

**[0211]** On 6 d p.i. the wells were scored for presence or absence of CPE and $TCID_{50}$ calculated. These were then used to estimate the concentration of infectious MVA per ml in the thermo-challenged vials.

**Results**

**[0212]** The data from this study is shown in Table 19. Responses varied from 6 to 92% of starting titre. During analysis, formulation number 14 was identified as an obvious outlier and excluded from this analysis. This enhanced model assessment parameters.

**[0213]** The model reported here (see Figures 40 and 41) demonstrates a $1^{st}$ order effect for sucrose. Raffinose was found to have no $1^{st}$ order effect but did demonstrate a $2^{nd}$ order interaction. TMG was not found to have a $1^{st}$ order effect in this study but a $2^{nd}$ order effect was identified. Finally, an interaction between sucrose and TMG was identified.

**[0214]** Figure 42 shows a contour plot of the model. The optimum TMG concentration is close to the centre of the model (around 1M), although this drifts slightly as you vary the other excipients. Raffinose also shows an optimum. Finally, as a general rule, the higher the sucrose concentration the better the preservation of MVA.

**[0215]** Monte-Carlo simulations identified an optimum of 1M sucrose, 1.14M TMG and 141.76mM raffinose and gave a predicted recovery of $1.14x10^6$ pfu/ml or 87.7% of starting titre.

**Reference Example 16**

**Materials**

**[0216]**
*Chemical*

| | Supplier | Product Code | Lot No. |
|---|---|---|---|
| 20x SSC | Sigma | S6639 | 020M8404 |
| Dimethyl glycine | Sigma | D1156 | 077K1856 |
| Dulbeccos Modified Eagles Medium | Sigma | D5796 | RNBB1139 |
| Foetal Bovine Serum | Sigma | F7524 | 109K3395 |
| Penicillin Streptomycin | Sigma | P4458 | 0409M0093 |
| Water | Sigma | W3500 | 8M0411 |

*Biological*

| | Supplier | Product Code |
|---|---|---|
| BHK-21 cell line | ECACC | CB2857 |
| MVA | ATCC | VR-1508 |

*Other*

| | Manufacturer | Product Code |
|---|---|---|
| 2ml glass vials | Adelphi Tubes | VCDIN2R |
| 13mm freeze drying stoppers | Adelphi Tubes | FDW13 |
| Crimps | Adelphi Tubes | COTW13 |

**Equipment**

**[0217]**

| | Manufacturer | Equipment No. |
|---|---|---|
| Virtis Advantage Plus EL85 Freeze Dryer | Virtis | EQP#096 |
| HERA safe class II cabinet | Thermo Fisher | EQP# 011 & 012 |
| DMIL LED Inverted Microscope | Leica | EQP#062 |
| Binder $CO_2$ Incubator | Binder | EQP#014 |
| Forma 900 series -80°C freezer | Thermofisher | EQP#015 |
| ATL-84-1 Atlion Balance | Acculab | EQP#088 |
| IP250 37°C Incubator | LTE | EQP#016 |

**Methods**

**[0218]** MODDE 9.0 was used to generate a Central Composite Face-Centred (CCF) design (see Figure 43). CCF designs are a form of Response Surface Modelling (RSM) design that tests only three levels of each factor but still supports a quadratic model. Unlike regular

formulation designs, non-significant factors can be eliminated from the analysis and so do not become a confounding factor.

***Preparation of and thermal challenge of formulated MVA in a freeze-dried setting***

**[0219]** MVA was recovered from storage at -80°C and thawed. 50µl aliquots of the MVA were added to 2ml glass freeze-drying vials. Subsequently 250µl of an excipient blend was added to each vial. The excipient blend formulations once mixed with virus are described in Table 20 and were made up in SSC.

**Table 20**

| Sample I.D. | DMG (M) | Mannitol (mM) | Titre (TCID50/ml) |
|---|---|---|---|
| 1 | 0 | 6 | 1.20E+5 |
| 2 | 2 | 6 | 3.00E+5 |
| 3 | 0 | 600 | 3.00E+5 |
| 4 | 2 | 600 | 1.90E+5 |
| 5 | 0 | 303 | 7.60E+5 |
| 6 | 2 | 303 | 1.90E+5 |
| 7 | 1 | 6 | 1.20E+6 |
| 8 | 1 | 600 | 1.20E+6 |
| 9 | 1 | 303 | 1.20E+6 |
| 10 | 1 | 303 | 1.20E+6 |
| 11 | 1 | 303 | 7.60E+5 |

**[0220]** Rubber bungs were partially inserted, and after vortexing were loaded onto a Virtis advantage freeze-dryer and lyophilised as described in Figure 44. After lyophilisation samples were immediately capped under vacuum, removed, crimped and placed at 37°C for thermal challenge. Thermal challenge was for 7 days, after which all the vials were returned to the control vials and held at 4°C until it was practical to assay them. Freeze-dried samples were reconstituted in 300µl SSC immediately prior to assay.

***Assay of MVA***

**[0221]** Assay plates (96 well) were seeded with BHK-21 cells (100µl per well, $10^5$ cells/ml). Cells were diluted in DMEM supplemented with 10% FBS, and 1% PS. The plates were placed at +37°C, + 5% $CO_2$ for 1-2 hours.

**[0222]** Meanwhile, a 10 fold dilution series of the formulated MVA samples was prepared in the

same growth media ranging from 1 in 10 to 1 in 10,000. Each dilution series was prepared 5 times. 100µl of each dilution was applied to individual wells containing BHK-21 cells (described above).

**[0223]** On 6 d p.i. the wells were scored for presence or absence of CPE and $TCID_{50}$ calculated. These were then used to estimate the concentration of infectious MVA per ml in the thermo-challenged vials.

**[0224]** Subsequently, a 2 fold dilution series of the formulated MVA samples was prepared ranging from 1 in 2,000 to 1 in 32,000. These dilutions were assayed separately but as before.

**Results**

**[0225]** The first pass of assaying these samples (LOG interval = 1) yielded only five levels of response and one of these was below the detection threshold. More importantly, six of the eleven treatments were above the maximum detection threshold. These samples were re-assayed (LOG interval = 0.3). The samples were held as liquid at +4°C between the two assays. Some samples gave a meaningful value (between maximum and minimum thresholds) in both assays. This allowed determination of loss between the two assays.

**[0226]** After the second pass assay no treatments yielded a titre below the detection threshold. For ease of transformation this treatment was assigned a response equal to the minimum detection threshold.

**[0227]** The model generated from this data is relatively strong. Three of four parameters of model validity score over 0.9 (R2=0.82, Q2=0.70, Model Validity=0.91, Reproducibility=0.70) (see Figure 45).

**[0228]** The model identified only one significant factor. DMG was found to have a second order (non-linear / quadratic) effect (see Figure 46).

**[0229]** Figure 47 shows the RSM model generated. It is effectively a simple DMG dose response curve that is not altered by mannitol within the tested concentration range. The dose response curve identifies a clear optimum DMG concentration, as do monte-carlo simulations (see Figure 48). The predicted optimum DMG concentration is 1.00M and predicted recovery of viral activity is 117% of starting titre.

# REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in

compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

**Patent documents cited in the description**


- WO9005182A **[0005]**
- WO20060850082A **[0006]**
- WO200811402 1A **[0007]**
- WO2008058035A **[0008]**

**Patentkrav**

**1.** Fremgangsmåde til bevaring af viruspartikler under frysetørring omfattende:

(a) tilvejebringelse af en vandig opløsning af:

(i) viruspartikler fra *Adenoviridae, Orthomyxoviridae, Paramyxoviridae, Parvoviridae, Picornaviridae, Poxviridae, Herpesviridae, Togaviridae, Flaviviridae, Retroviridae, Filoviridae, Papillomaviridae, Caliciviridae, Coronaviridae, Reoviridae* eller *Hepadnaviridae,*

(ii) saccharose, eller saccharose og raffinose, eller mannitol, ved en samlet sukkerkoncentration på 0,1 M til 3M,

(iii) 0,1 til 1,5 M N,N-dimethylglycin eller N,N,N-trimethylglycin, eller et fysiologisk acceptabelt salt eller en ester deraf, og

(iv) 0,1 til 1,5 M methylsulfonylmethan; og

(b) frysetørring af opløsningen for at danne en sammensætning, hvori viruspartiklerne er inkorporeret.

**2.** Fremgangsmåde ifølge krav 1, hvor den samlede sukkerkoncentration er fra 0,2 M til 2 M.

**3.** Fremgangsmåde ifølge krav 1 eller 2, hvor den vandige opløsning frysetørres i hætteglas eller ampuller, der derefter forsegles.

**4.** Fremgangsmåde ifølge krav 1 eller 2, hvor viruspartiklerne består af et levende live virus eller dræbt virus.

**5.** Fremgangsmåde ifølge krav 4, hvor det levende virus er fuldvirus eller levende svækket virus.

**6.** Sammensætning, der er fast og frysetørret, og som omfatter viruspartikler fra *Herpesviridae, Togaviridae, Flaviviridae, Retroviridae, Filoviridae, Papillomaviridae, Caliciviridae, Coronaviridae, Reoviridae, Hepadnaviridae, Adenoviridae, Orthomyxoviridae, Paramyxoviridae, Parvoviridae, Picornaviridae eller Poxviridae, og* som kan opnås ved hjælp af en fremgangsmåde som defineret i et hvilket som helst af krav 1 til 5.

# DRAWINGS

Fig. 1

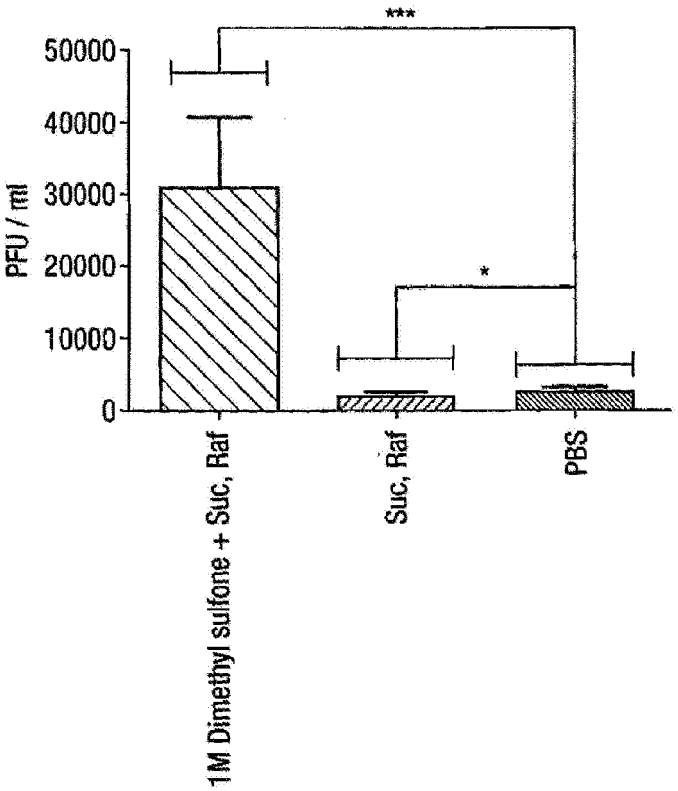


Fig. 2A

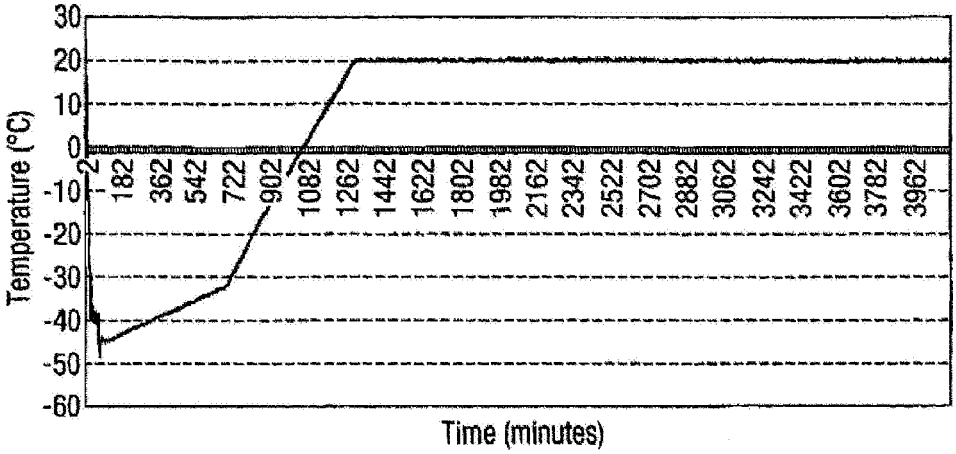

30
20
10
0
-10
-20
-30
-40
-50
-60
Temperature (°C)
2
182
362
542
722
902
1082
1262
1442
1622
1802
1982
2162
2342
2522
2702
2882
3062
3242
3422
3602
3782
3962
Time (minutes)

Fig. 2B

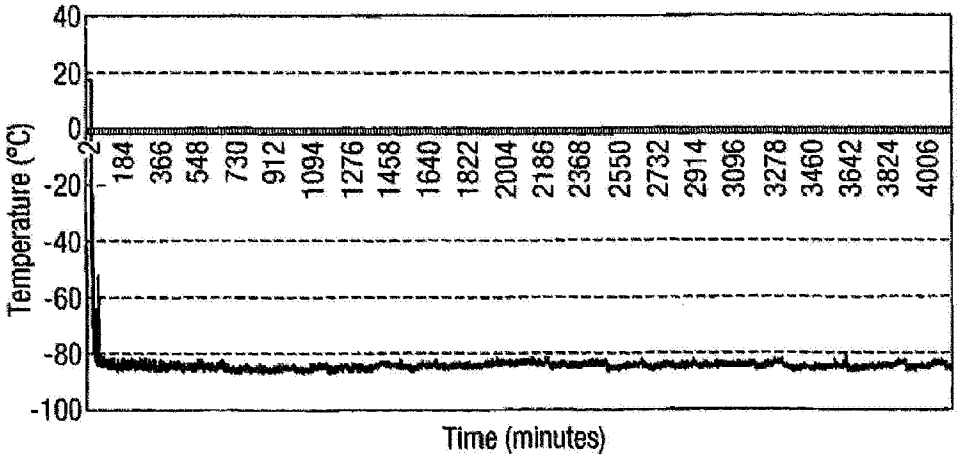

40
20
0
-20
-40
-60
-80
-100
Temperature (°C)
2
184
366
548
730
912
1094
1276
1458
1640
1822
2004
2186
2368
2550
2732
2914
3096
3278
3460
3642
3824
4006
Time (minutes)

Fig. 3

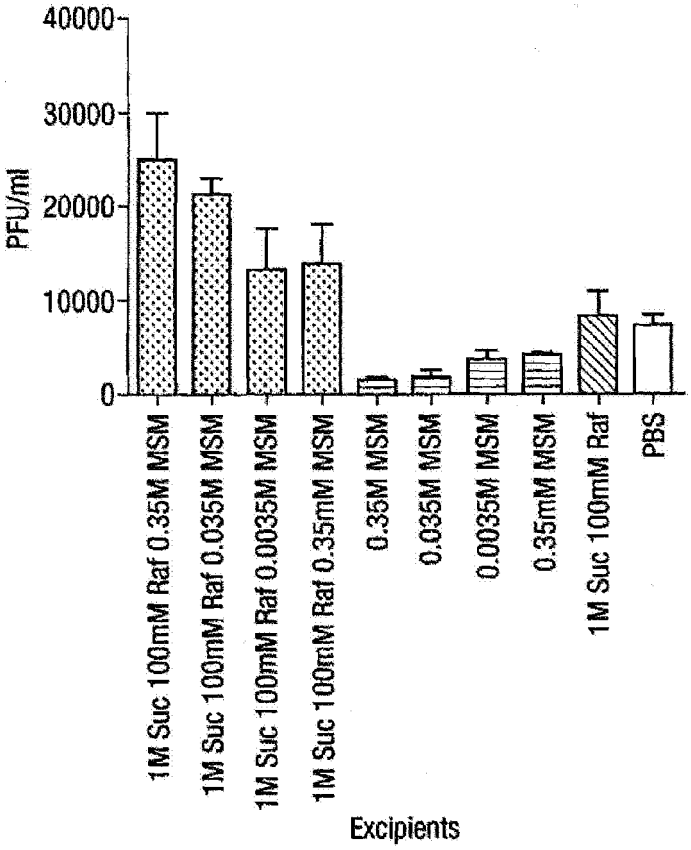

40000
30000
20000
10000
0
PFU/ml
1M Suc 100mM Raf 0.35M MSM
1M Suc 100mM Raf 0.035M MSM
1M Suc 100mM Raf 0.0035M MSM
1M Suc 100mM Raf 0.35mM MSM
0.35M MSM
0.035M MSM
0.0035M MSM
0.35mM MSM
1M Suc 100mM Raf
PBS
Excipients

Fig. 4

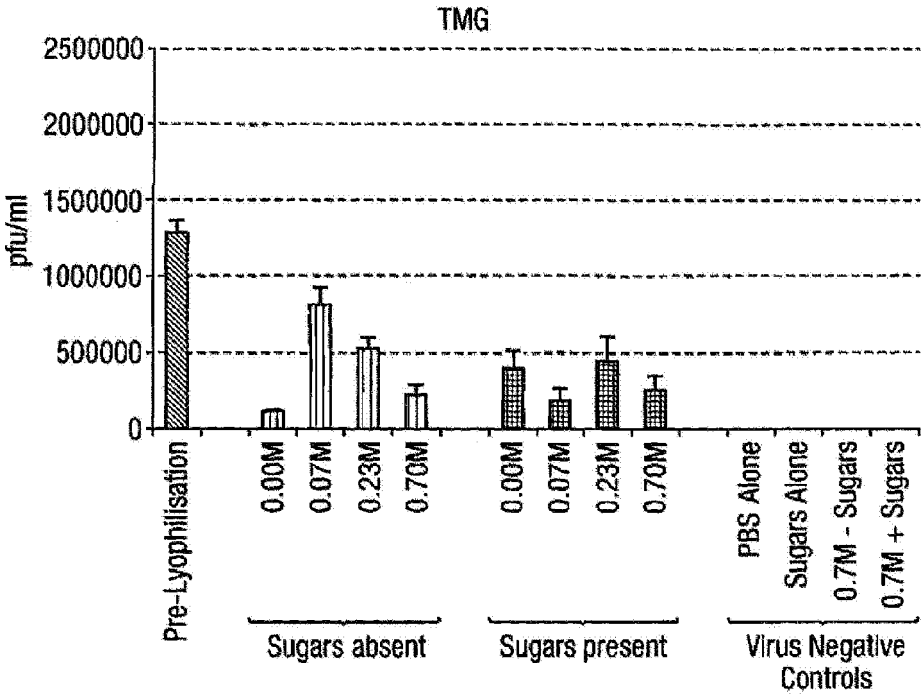

TMG
2500000
2000000
1500000
1000000
500000
0
pfu/ml
Pre-Lyophilisation
0.00M
0.07M
0.23M
0.70M
0.00M
0.07M
0.23M
0.70M
PBS Alone
Sugars Alone
0.7M - Sugars
0.7M + Sugars
Sugars absent
Sugars present
Virus Negative Controls

Fig. 5

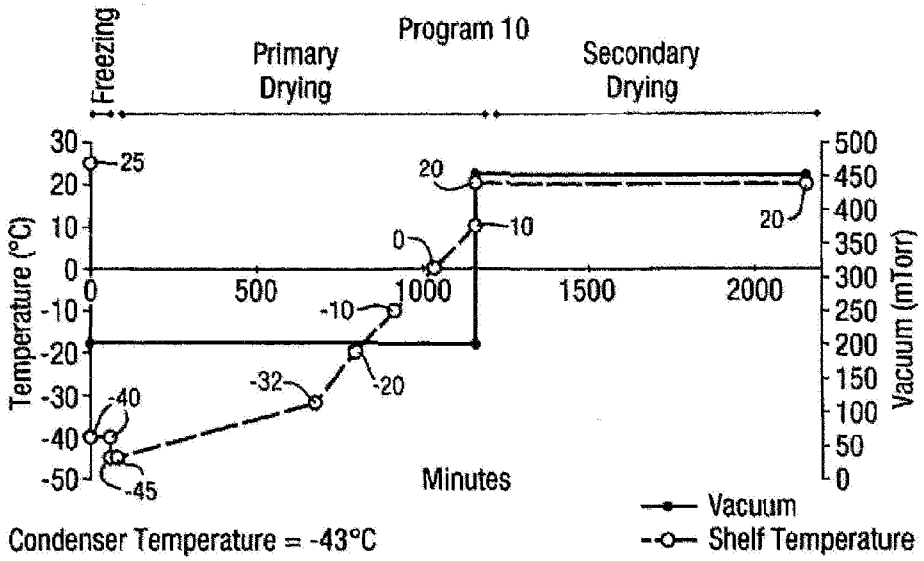

Program 10
Freezing
Primary Drying
Secondary Drying
Temperature (°C)
Vacuum (mTorr)
Minutes
Vacuum
Shelf Temperature
Condenser Temperature = -43°C

Fig. 6A

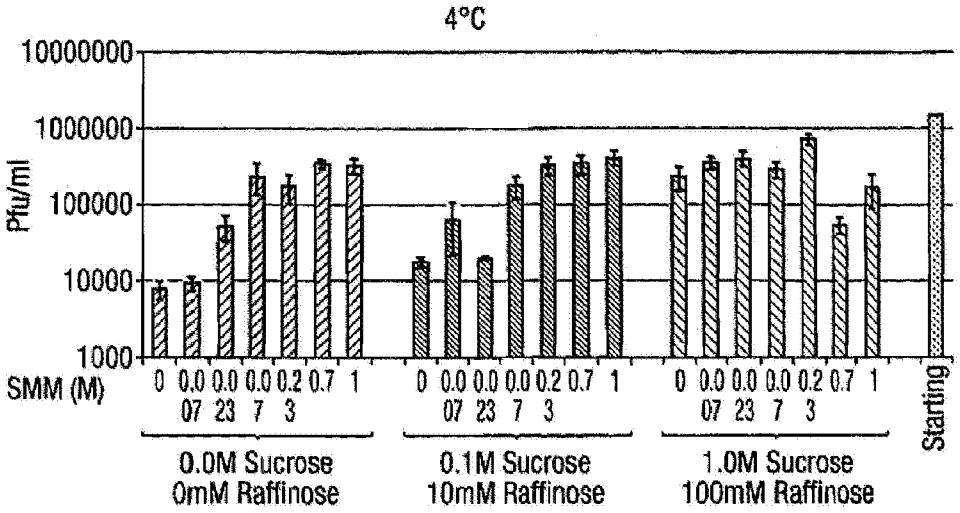

4°C
10000000
1000000
100000
10000
1000
Pfu/ml
SMM (M)
0 0.0 0.0 0.0 0.2 0.7 1
07 23 7 3
Starting
0.0M Sucrose
0mM Raffinose
0.1M Sucrose
10mM Raffinose
1.0M Sucrose
100mM Raffinose

Fig. 6B

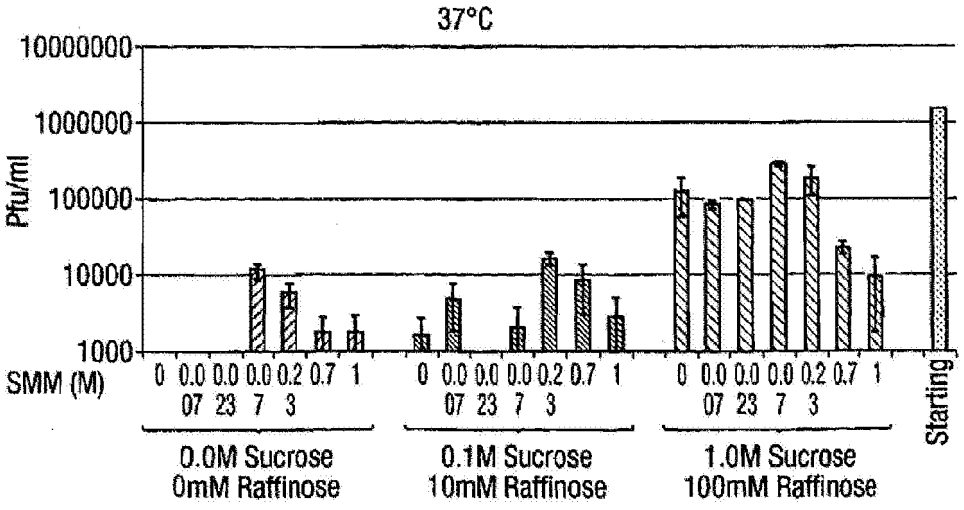

37°C
10000000
1000000
100000
10000
1000
Pfu/ml
SMM (M)
0 0.0 0.0 0.0 0.2 0.7 1
07 23 7 3
Starting
0.0M Sucrose
0mM Raffinose
0.1M Sucrose
10mM Raffinose
1.0M Sucrose
100mM Raffinose

Fig. 7

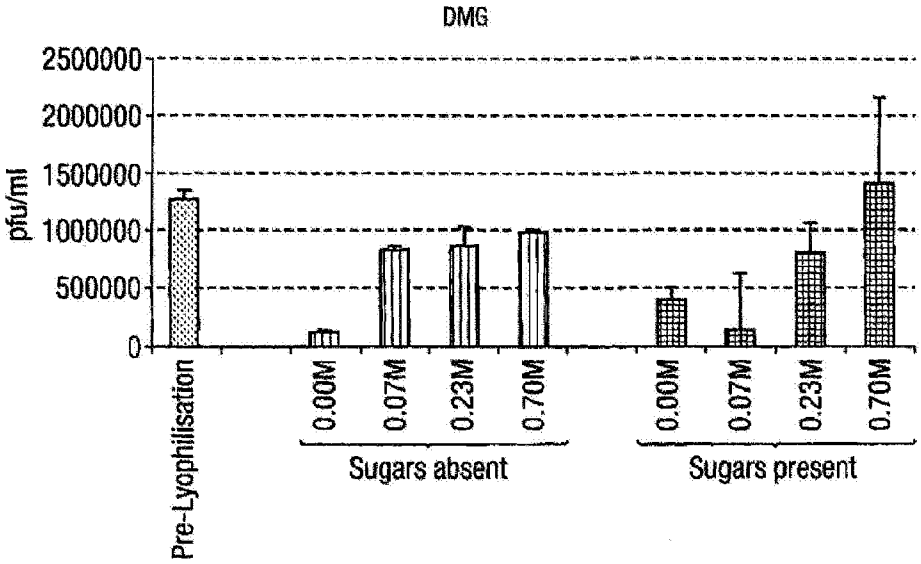

DMG
2500000
2000000
1500000
1000000
500000
0
pfu/ml
Pre-Lyophilisation
0.00M
0.07M
0.23M
0.70M
Sugars absent
0.00M
0.07M
0.23M
0.70M
Sugars present

Fig. 8

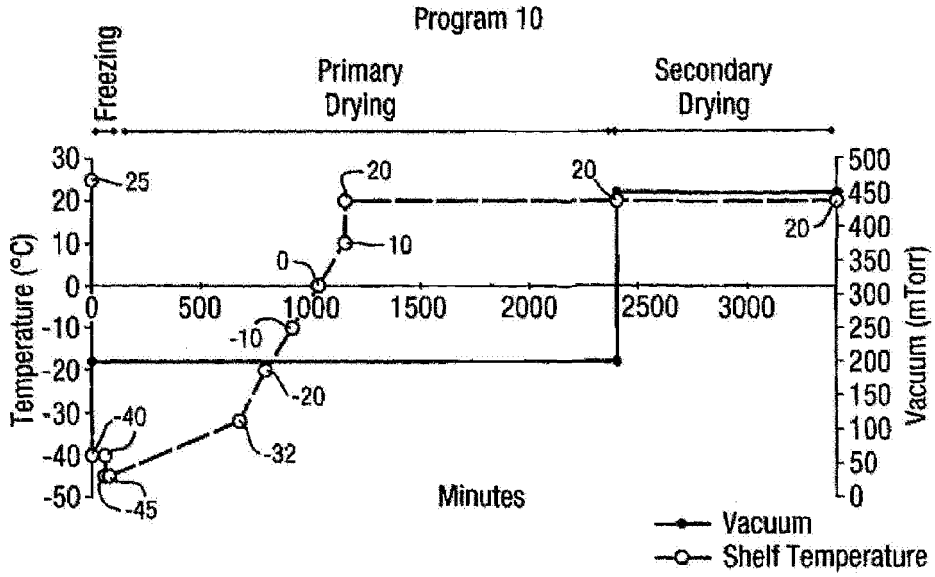

Program 10
Freezing
Primary Drying
Secondary Drying
Temperature (°C)
Vacuum (mTorr)
Minutes
Vacuum
Shelf Temperature

Fig. 9A

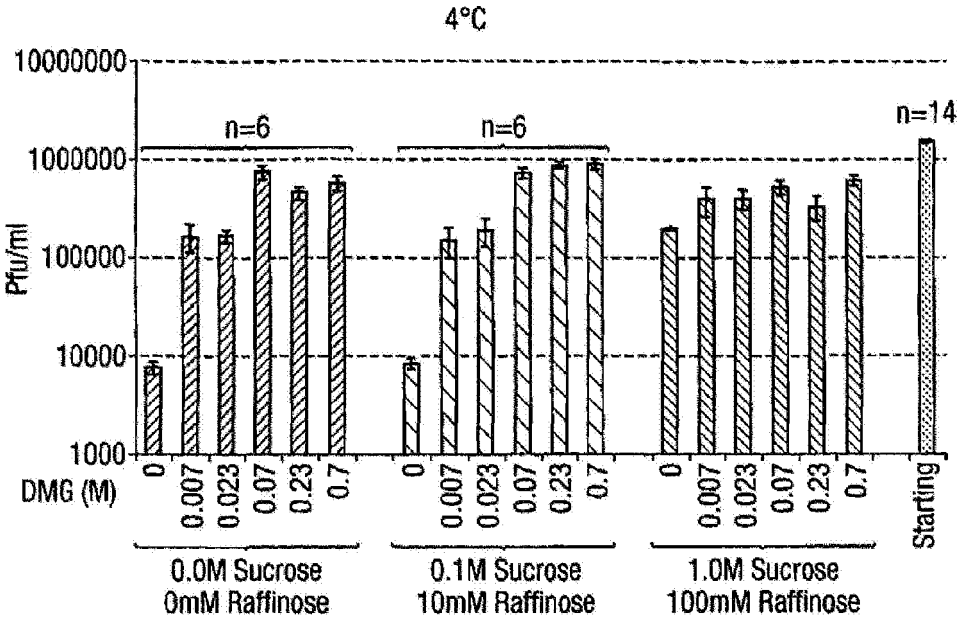

4°C
10000000
1000000
100000
10000
1000
Pfu/ml
n=6
n=6
n=14
DMG (M)
0
0.007
0.023
0.07
0.23
0.7
Starting
0.0M Sucrose
0mM Raffinose
0.1M Sucrose
10mM Raffinose
1.0M Sucrose
100mM Raffinose

Fig. 9B

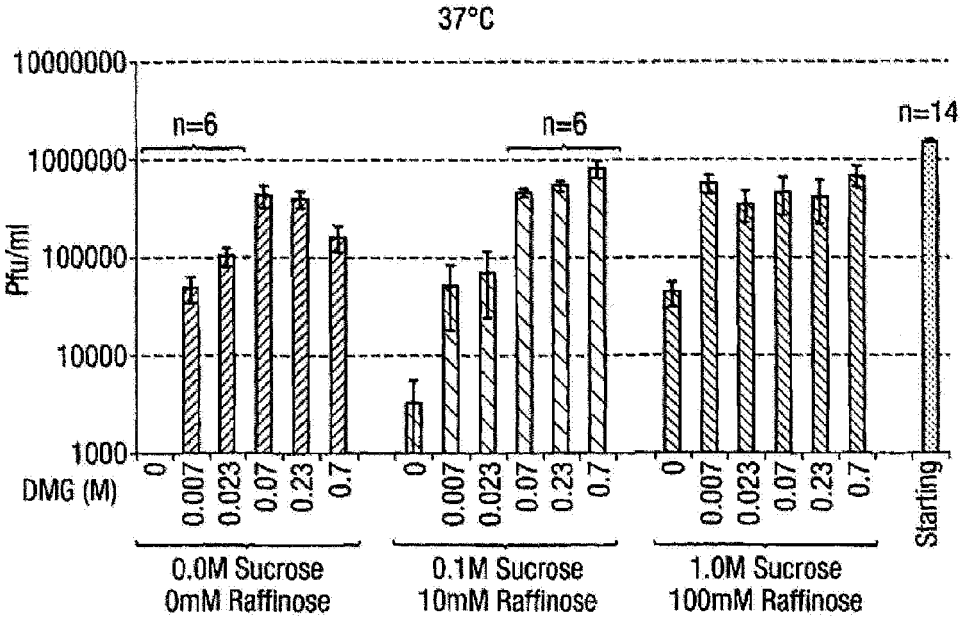

37°C
10000000
1000000
100000
10000
1000
Pfu/ml
n=6
n=6
n=14
DMG (M)
0
0.007
0.023
0.07
0.23
0.7
Starting
0.0M Sucrose
0mM Raffinose
0.1M Sucrose
10mM Raffinose
1.0M Sucrose
100mM Raffinose

Fig. 10

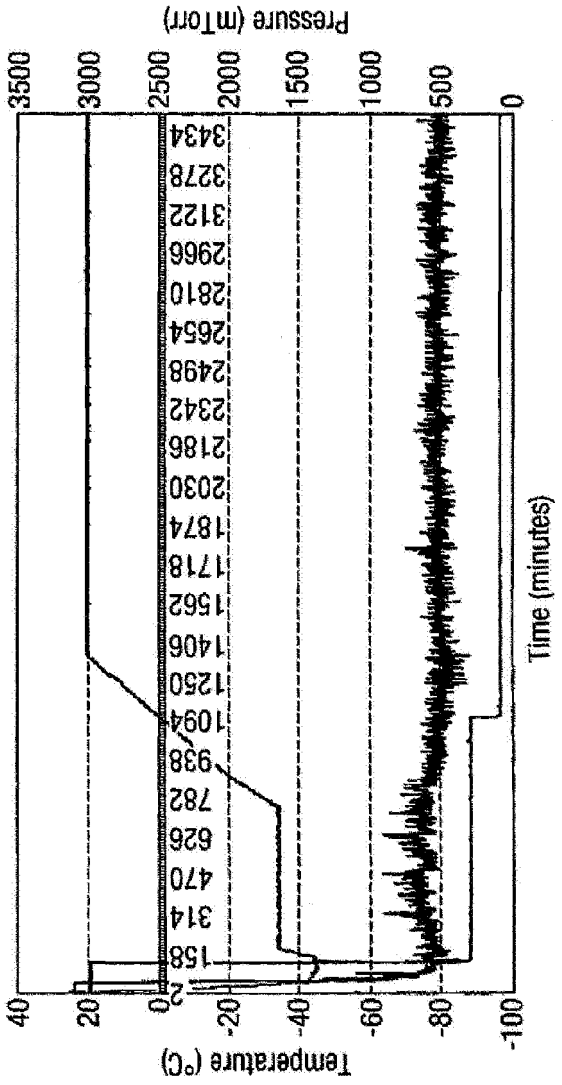

Pressure (mTorr)
3500
3000
2500
2000
1500
1000
500
0
3434
3278
3122
2966
2810
2654
2498
2342
2186
2030
1874
1718
1562
1406
1250
1094
938
782
626
470
314
158
2
Temperature (°C)
40
20
0
-20
-40
-60
-80
-100
Time (minutes)

Fig. 11

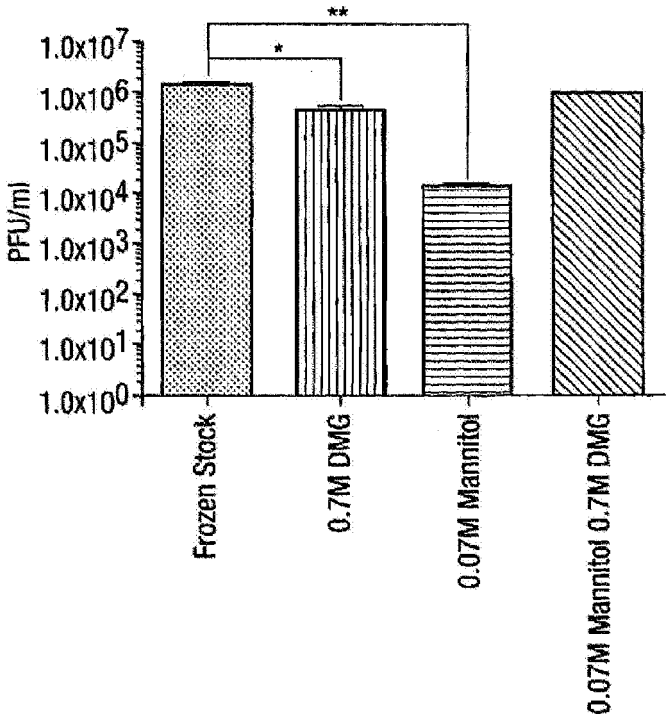

**
*
1.0x10^7
1.0x10^6
1.0x10^5
1.0x10^4
1.0x10^3
1.0x10^2
1.0x10^1
1.0x10^0
PFU/ml
Frozen Stock
0.7M DMG
0.07M Mannitol
0.07M Mannitol 0.7M DMG

Fig. 12

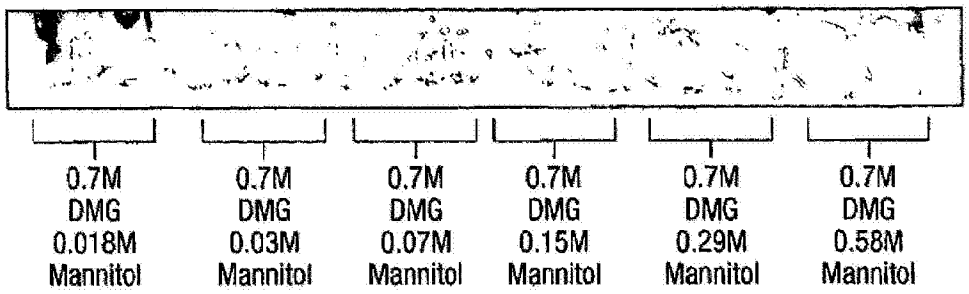

0.7M DMG 0.018M Mannitol
0.7M DMG 0.03M Mannitol
0.7M DMG 0.07M Mannitol
0.7M DMG 0.15M Mannitol
0.7M DMG 0.29M Mannitol
0.7M DMG 0.58M Mannitol

Fig. 13

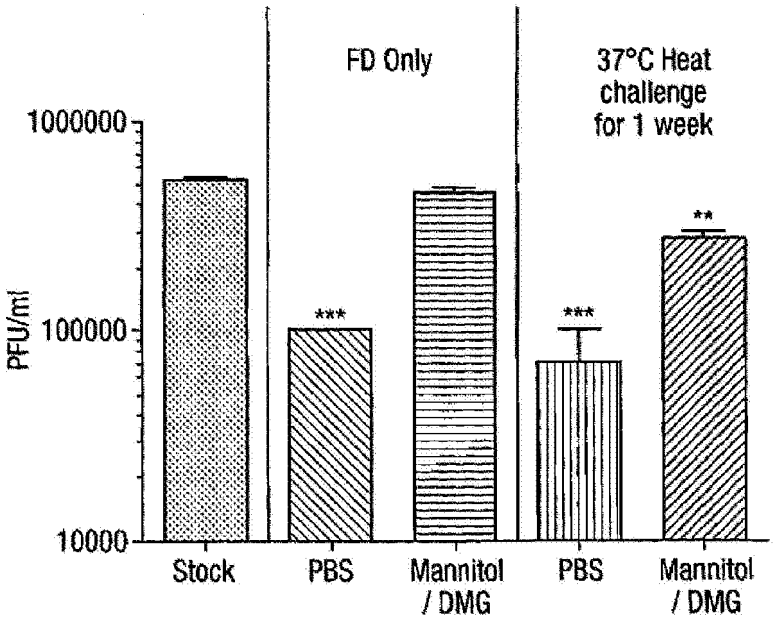

FD Only
37°C Heat challenge for 1 week
1000000
100000
10000
PFU/ml
***
***
**
Stock
PBS
Mannitol / DMG
PBS
Mannitol / DMG

Fig. 14

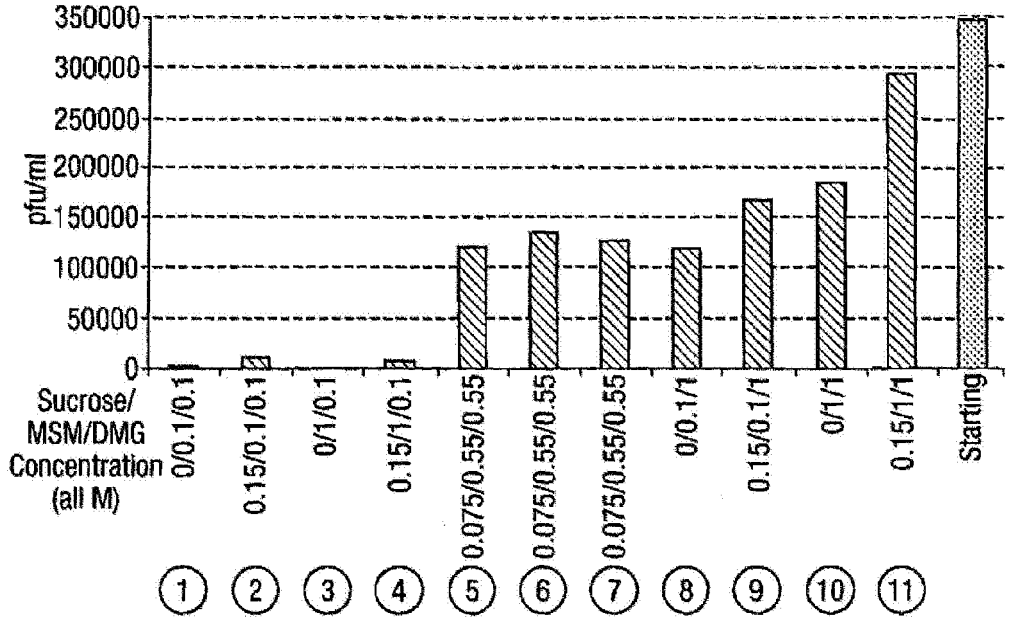

350000
300000
250000
200000
150000
100000
50000
0
pfu/ml
Sucrose/
MSM/DMG
Concentration
(all M)
0/0.1/0.1
0.15/0.1/0.1
0/1/0.1
0.15/1/0.1
0.075/0.55/0.55
0.075/0.55/0.55
0.075/0.55/0.55
0/0.1/1
0.15/0.1/1
0/1/1
0.15/1/1
Starting
1
2
3
4
5
6
7
8
9
10
11

Fig. 15

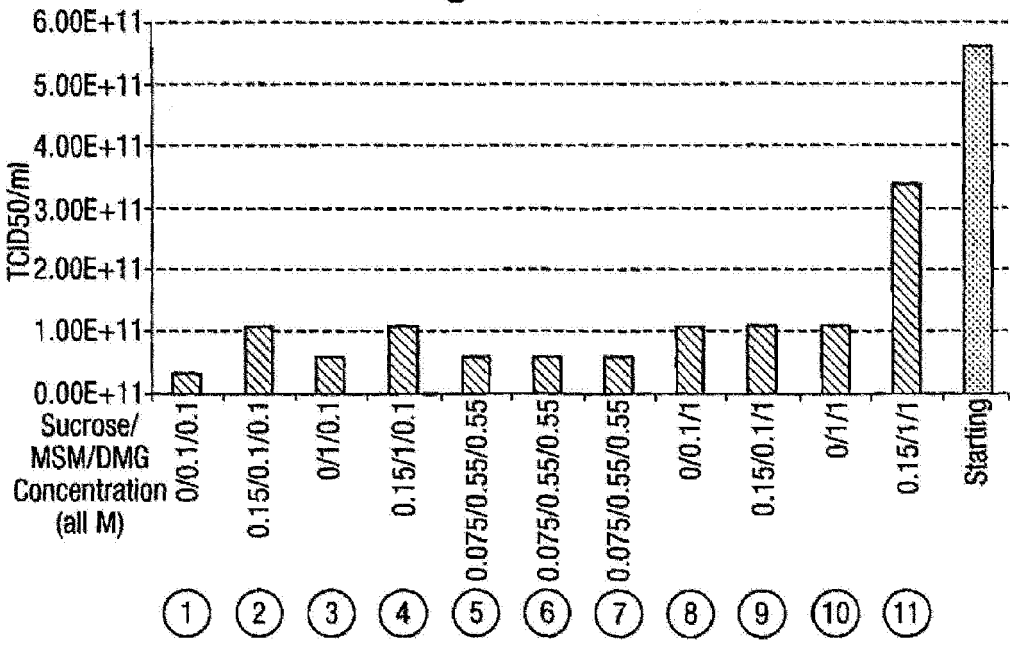

6.00E+11
5.00E+11
4.00E+11
3.00E+11
2.00E+11
1.00E+11
0.00E+11
TCID50/ml
Sucrose/
MSM/DMG
Concentration
(all M)
0/0.1/0.1
0.15/0.1/0.1
0/1/0.1
0.15/1/0.1
0.075/0.55/0.55
0.075/0.55/0.55
0.075/0.55/0.55
0/0.1/1
0.15/0.1/1
0/1/1
0.15/1/1
Starting
1
2
3
4
5
6
7
8
9
10
11

Fig. 16

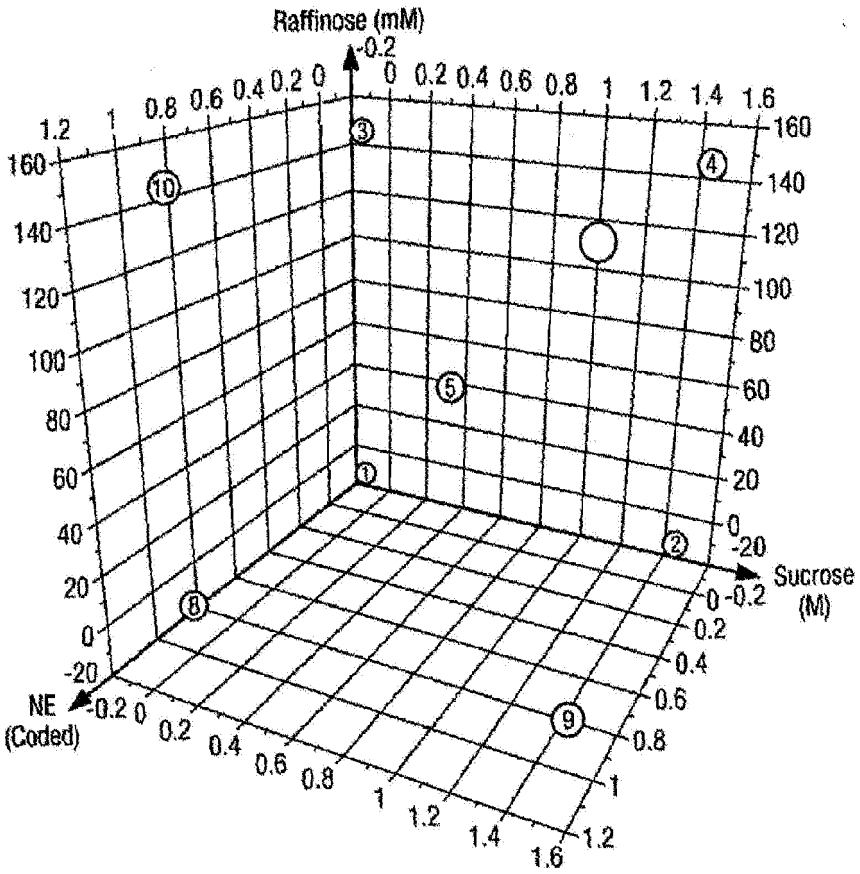

Raffinose (mM)
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
160
140
120
100
80
60
40
20
0
-20
Sucrose (M)
NE (Coded)
1
2
3
4
5
8
9
10

Fig. 17

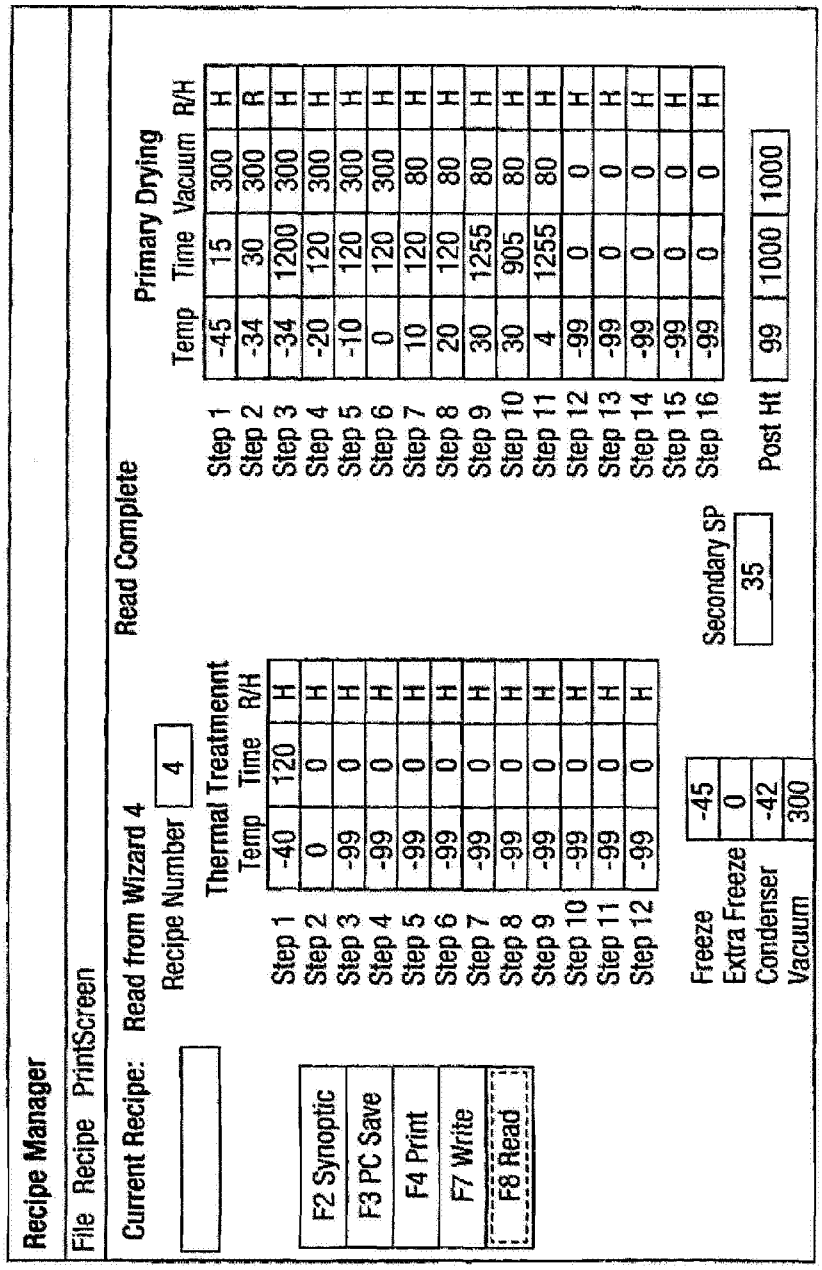

Recipe Manager
File Recipe PrintScreen
Current Recipe:
Read from Wizard 4
Read Complete
Recipe Number 4
Thermal Treatmennt
Temp Time R/H
Step 1 -40 120 H
Step 2 0 0 H
Step 3 -99 0 H
Step 4 -99 0 H
Step 5 -99 0 H
Step 6 -99 0 H
Step 7 -99 0 H
Step 8 -99 0 H
Step 9 -99 0 H
Step 10 -99 0 H
Step 11 -99 0 H
Step 12 -99 0 H
Primary Drying
Temp Time Vacuum R/H
Step 1 -45 15 300 H
Step 2 -34 30 300 R
Step 3 -34 1200 300 H
Step 4 -20 120 300 H
Step 5 -10 120 300 H
Step 6 0 120 300 H
Step 7 10 120 80 H
Step 8 20 120 80 H
Step 9 30 1255 80 H
Step 10 30 905 80 H
Step 11 4 1255 80 H
Step 12 -99 0 0 H
Step 13 -99 0 0 H
Step 14 -99 0 0 H
Step 15 -99 0 0 H
Step 16 -99 0 0 H
F2 Synoptic
F3 PC Save
F4 Print
F7 Write
F8 Read
Freeze -45
Extra Freeze 0
Condenser -42
Vacuum 300
Secondary SP 35
Post Ht 99 1000 1000

Fig. 18

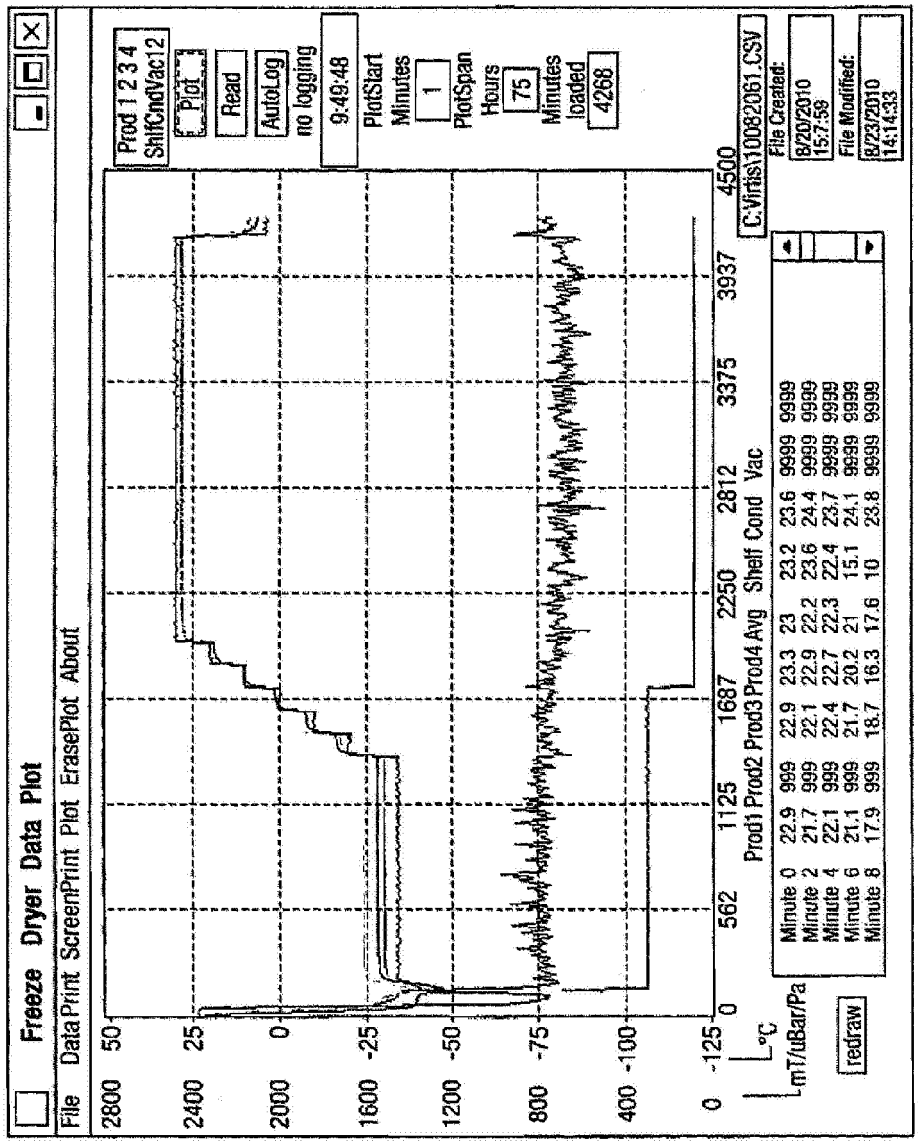
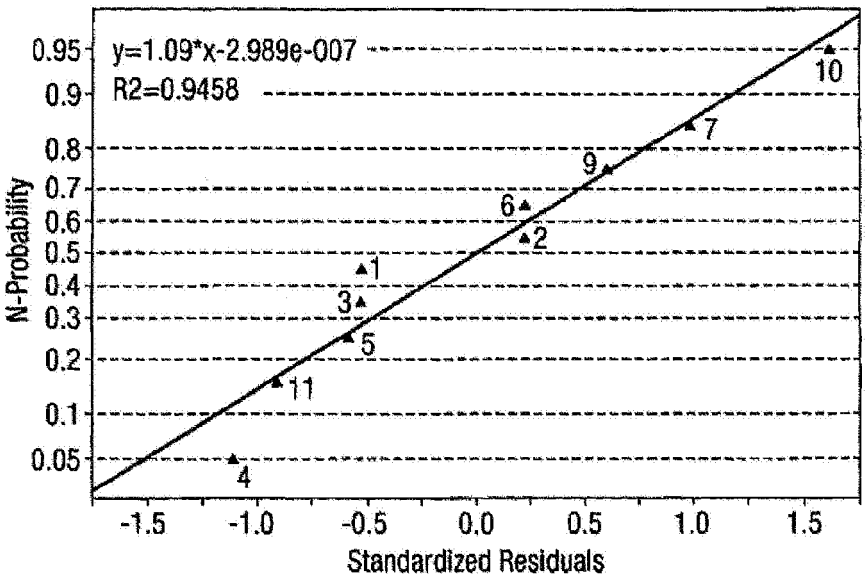
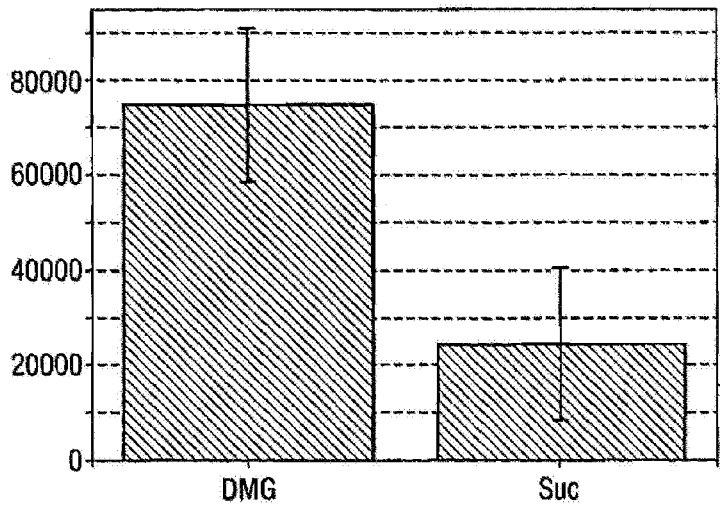

Freeze Dryer Data Plot
File DataPrint ScreenPrint Plot ErasePlot About
Prod 1 2 3 4
ShlfCndVac12
Plot
Read
AutoLog
no logging
9:49:48
PlotStart
Minutes
1
PlotSpan
Hours
75
Minutes
loaded
4268
redraw
°C
mT/uBar/Pa
Prod1 Prod2 Prod3 Prod4 Avg Shelf Cond Vac
C:\Virtis\10082061.CSV
File Created:
8/20/2010
15:7:58
File Modified:
8/23/2010
14:14:33

Fig. 19



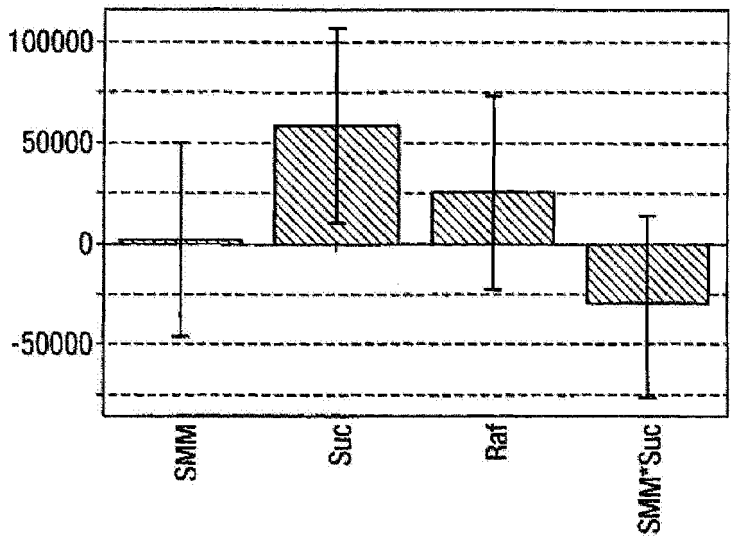

N=10 R2=0.949 RSD=1.982e+004
DF=7 Q2=0.886

Fig. 20



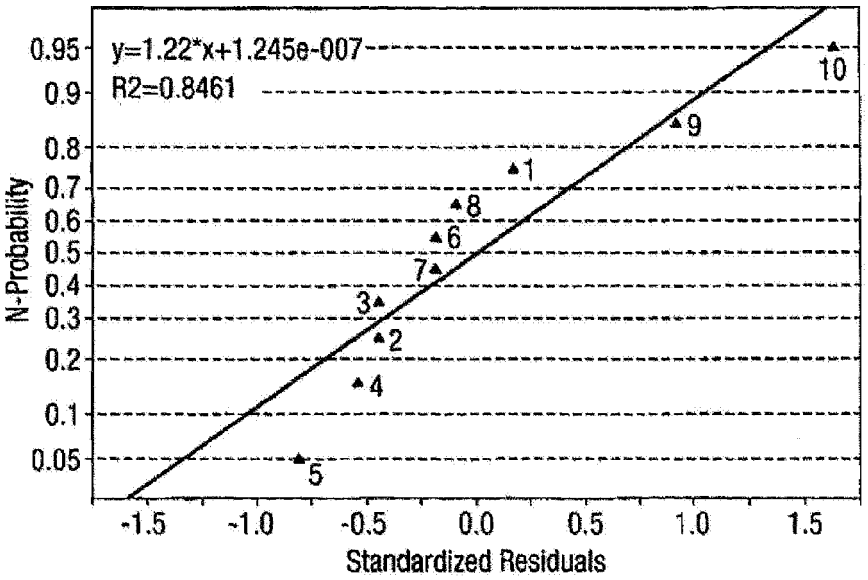

N=10 R2=0.949 RSD=1.982e+004
DF=7 Q2=0.886 Conf. lev.=0.95

Fig. 21



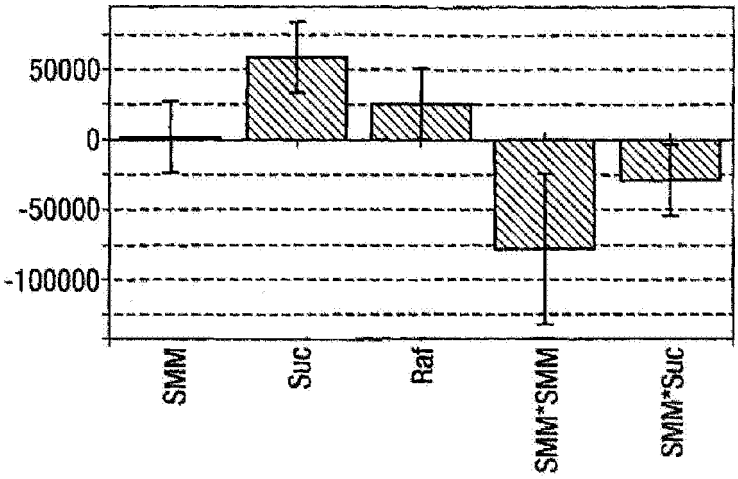

N=10 R2=0.748 RSD=5.615e+004
DF=5 Q2=0.508 Conf. lev.=0.95

Fig. 22



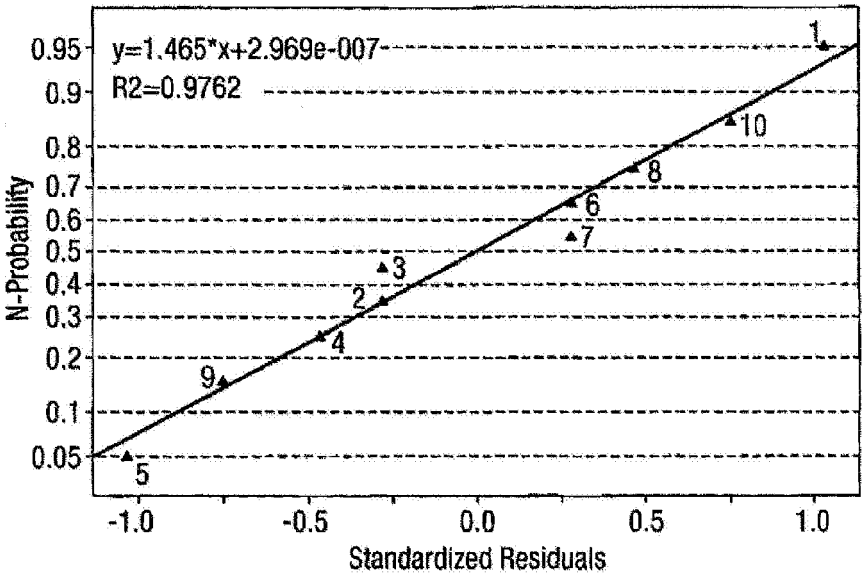

N=10 R2=0.748 RSD=5.615e+004
DF=5 Q2=0.508

Fig. 23



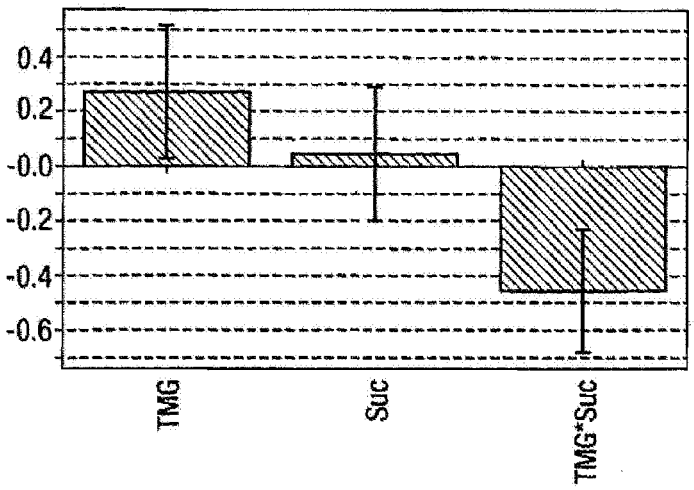

N=10 R2=0.955 RSD=2.669e+004
DF=4 Q2=0.666 Conf. lev.=0.95

Fig. 24



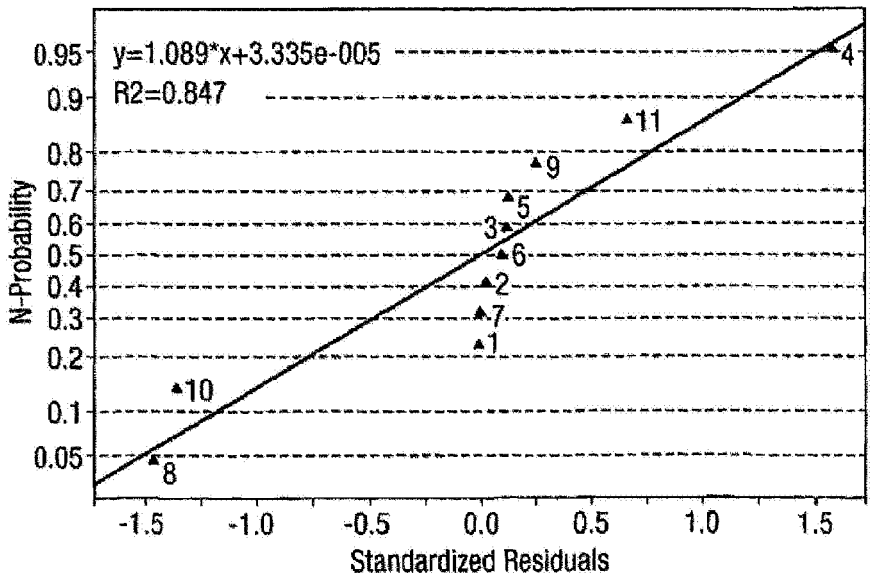

N=10 R2=0.955 RSD=2.669e+004
DF=4 Q2=0.666

Fig. 25




N=11 R2=0.753 RSD=0.4022
DF=7 Q2=0.542 Conf. lev.=0.90

Fig. 26




N=11 R2=0.753 RSD=0.4022
DF=7 Q2=0.542

Fig. 27

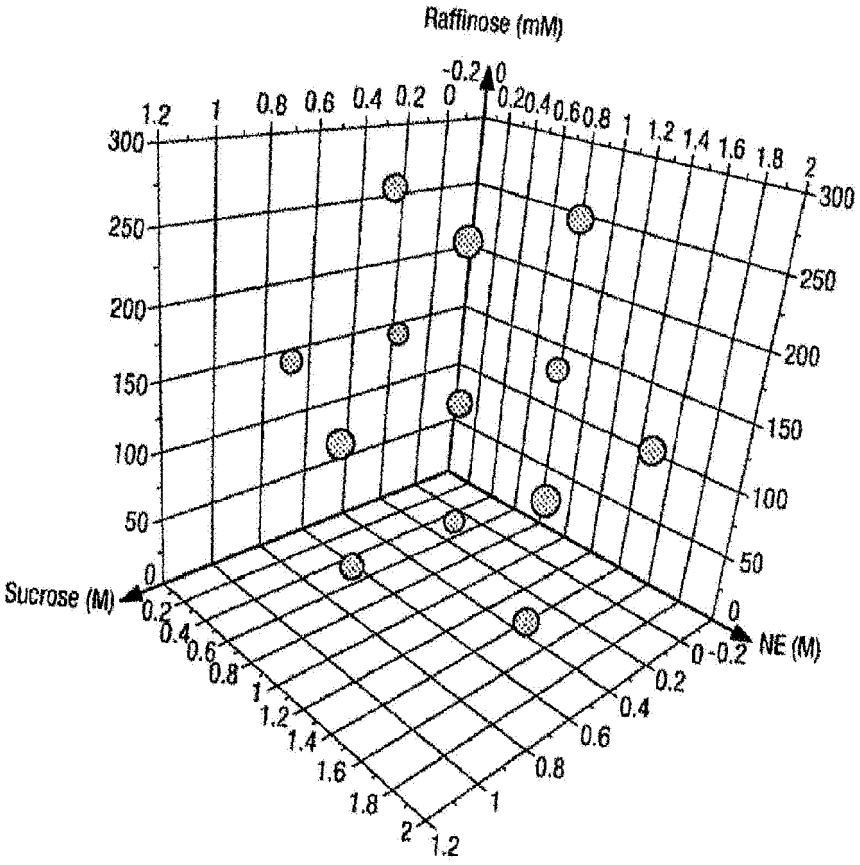

Raffinose (mM)
-0.2 0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8 2
1.2 1 0.8 0.6 0.4 0.2 0
300
250
200
150
100
50
0
Sucrose (M)
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8 2
NE (M)
0 -0.2 0.2 0.4 0.6 0.8 1 1.2

Fig. 28

**Recipe Manager**

File Recipe PrintScreen

Current Recipe: Read from Wizard 1 — Read Complete

Recipe Number 1

F2 Synoptic
F3 PC Save
F4 Print
F7 Write
F8 Read

**Thermal Treatmennt**

| | Temp | Time | R/H |
|---|---|---|---|
| Step 1 | -40 | 120 | H |
| Step 2 | 0 | 0 | R |
| Step 3 | 0 | 0 | R |
| Step 4 | 0 | 0 | R |
| Step 5 | 0 | 0 | R |
| Step 6 | 0 | 0 | R |
| Step 7 | 0 | 0 | R |
| Step 8 | 0 | 0 | R |
| Step 9 | 0 | 0 | R |
| Step 10 | 0 | 0 | R |
| Step 11 | 0 | 0 | R |
| Step 12 | 0 | 0 | R |

| | |
|---|---|
| Freeze | -40 |
| Extra Freeze | 0 |
| Condenser | -42 |
| Vacuum | 300 |

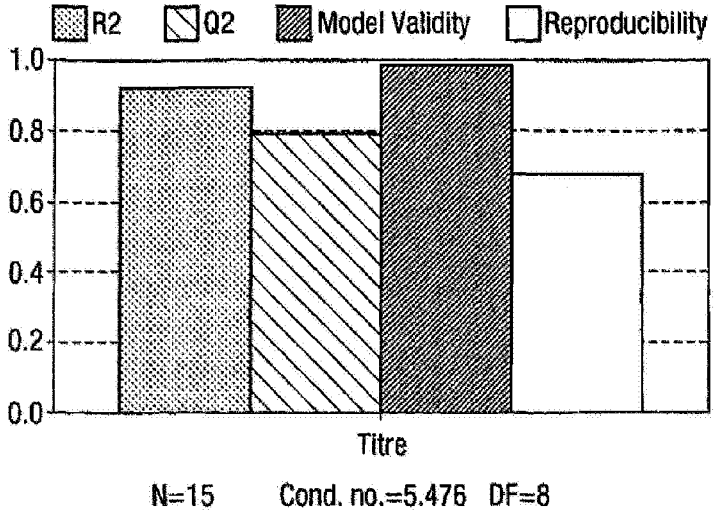
Secondary SP: 35

**Primary Drying**

| | Temp | Time | Vacuum | R/H |
|---|---|---|---|---|
| Step 1 | -45 | 15 | 100 | H |
| Step 2 | -36 | 30 | 100 | R |
| Step 3 | -36 | 600 | 100 | H |
| Step 4 | -36 | 1200 | 100 | H |
| Step 5 | -36 | 5 | 100 | H |
| Step 6 | -10 | 120 | 100 | H |
| Step 7 | 0 | 120 | 100 | H |
| Step 8 | 10 | 120 | 80 | H |
| Step 9 | 20 | 120 | 80 | H |
| Step 10 | 25 | 1255 | 80 | H |
| Step 11 | 4 | 1255 | 80 | H |
| Step 12 | 4 | 1255 | 80 | H |
| Step 13 | 4 | 1255 | 80 | H |
| Step 14 | 4 | 1255 | 80 | H |
| Step 15 | 0 | 0 | 0 | H |
| Step 16 | 0 | 0 | 0 | H |

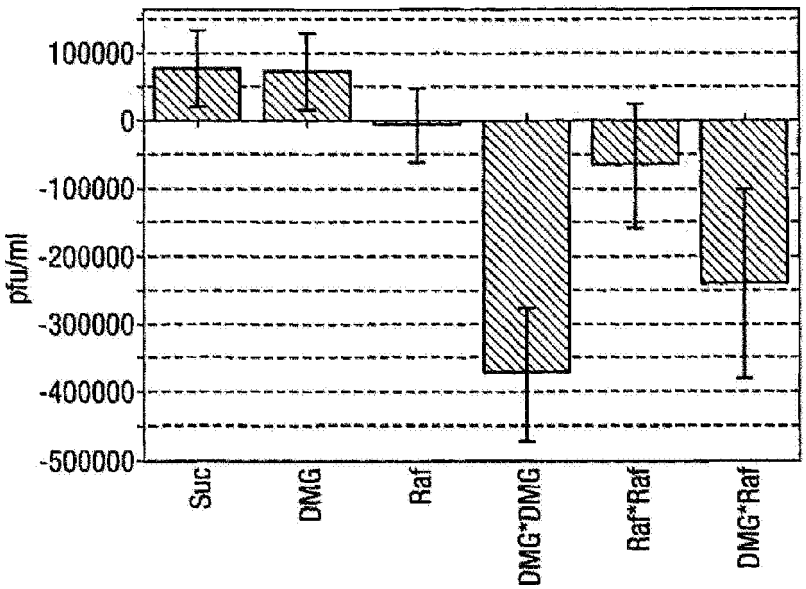
| | | | |
|---|---|---|---|
| Post Ht | 35 | 1000 | 450 |

Fig. 29




N=15 Cond. no.=5.476 DF=8

Fig. 30




N=15 R2=0.926 RSD=4.799e+004
DF=8 Q2=0.795 Conf. lev.=0.95

Fig. 31

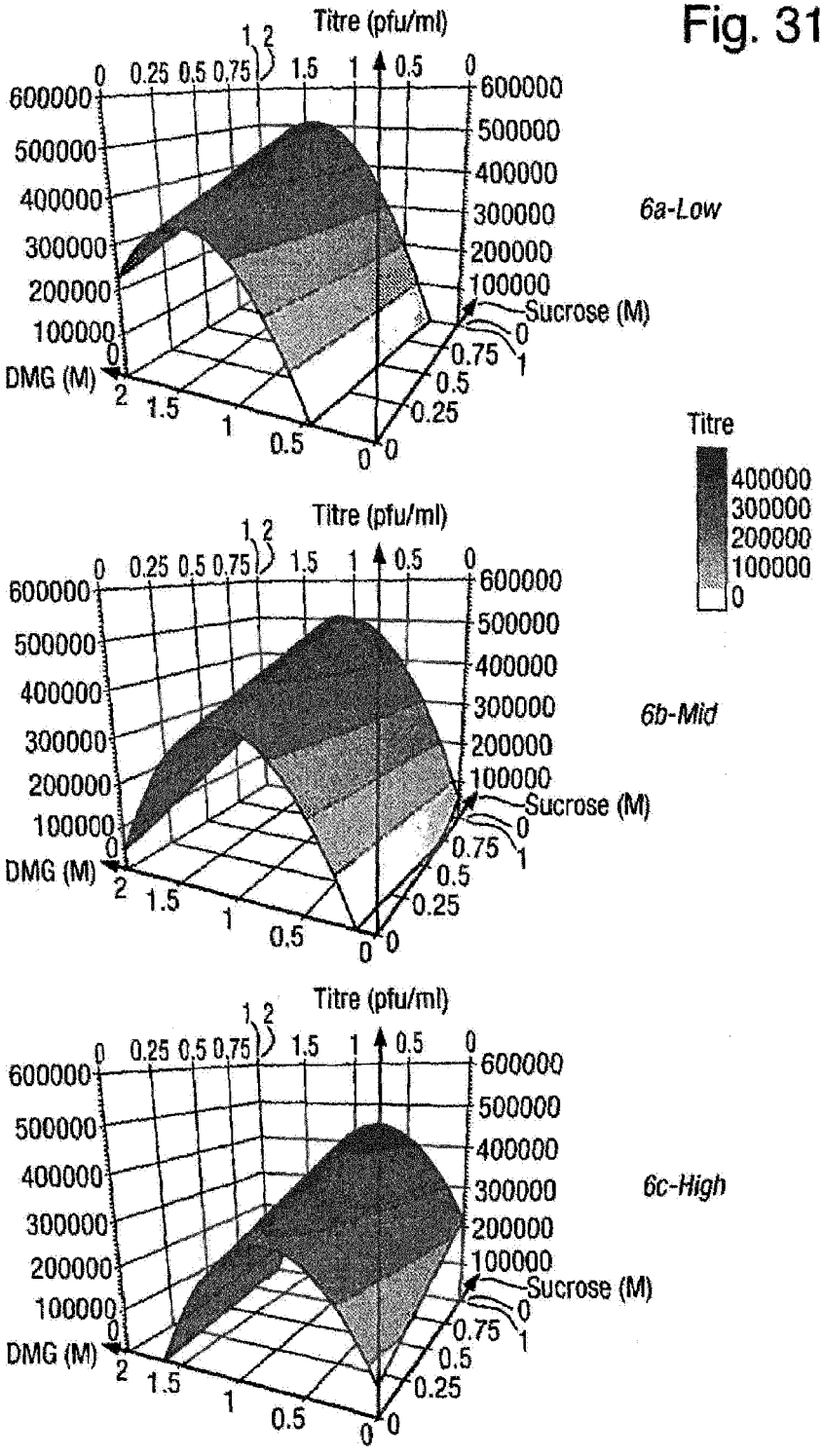
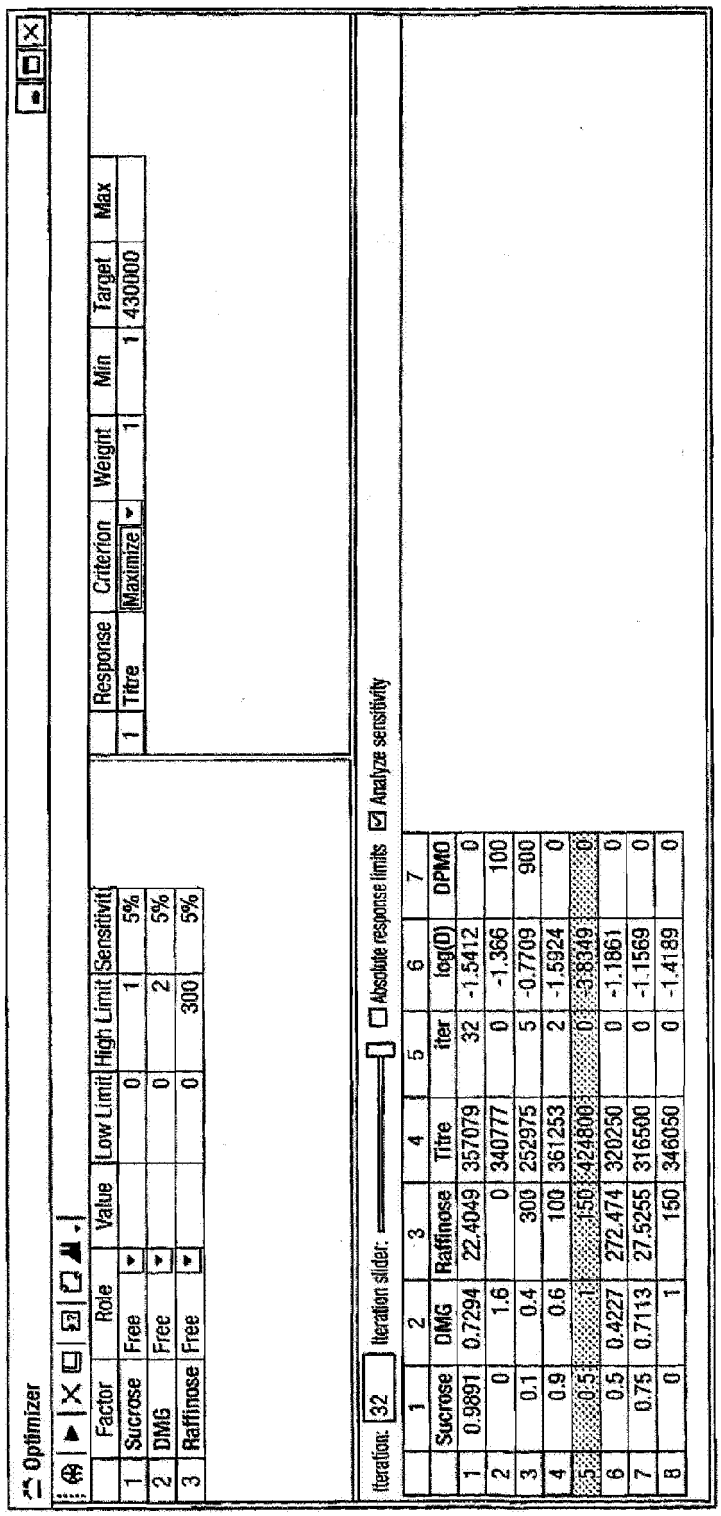

Titre (pfu/ml)
600000
500000
400000
300000
200000
100000
0
DMG (M)
2
1.5
1
0.5
0
Sucrose (M)
0
0.25
0.5
0.75
1
Titre
400000
300000
200000
100000
0
6a-Low
6b-Mid
6c-High

Fig. 32


Optimizer
Factor | Role | Value | Low Limit | High Limit | Sensitivity
1 Sucrose | Free | 0 | 1 | 5%
2 DMG | Free | 0 | 2 | 5%
3 Raffinose | Free | 0 | 300 | 5%
Response | Criterion | Weight | Min | Target | Max
1 Titre | Maximize | 1 | 1 | 430000
Iteration: 32
Iteration slider:
Absolute response limits
Analyze sensitivity
1 | 2 | 3 | 4 | 5 | 6 | 7
Sucrose | DMG | Raffinose | Titre | iter | log(D) | DPMO
1 0.9891 0.7294 22.4049 357079 32 -1.5412 0
2 0 1.6 0 340777 0 -1.366 100
3 0.1 0.4 300 252975 5 -0.7709 900
4 0.9 0.6 100 361253 2 -1.5924 0
5 0.5 1 150 424803 0 -38349 0
6 0.5 0.4227 272.474 320250 0 -1.1861 0
7 0.75 0.7113 27.5255 316500 0 -1.1569 0
8 0 1 150 346050 0 -1.4189 0

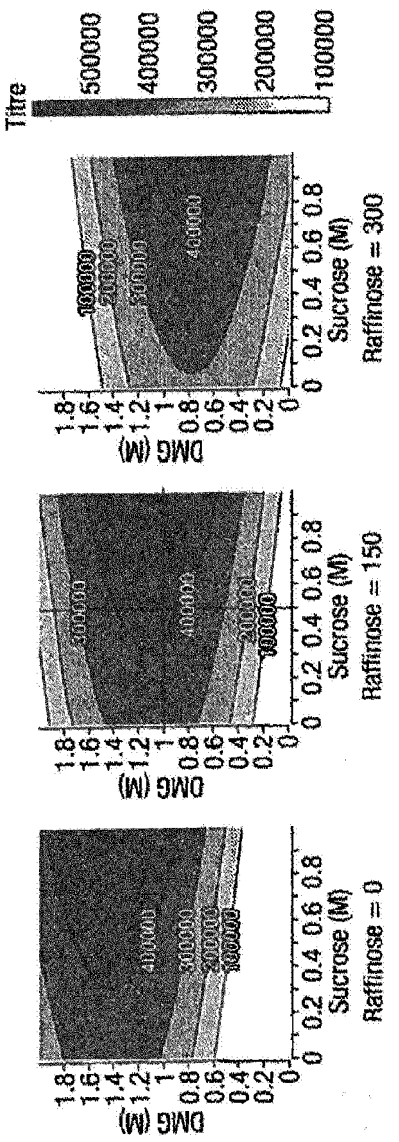



Fig. 33A

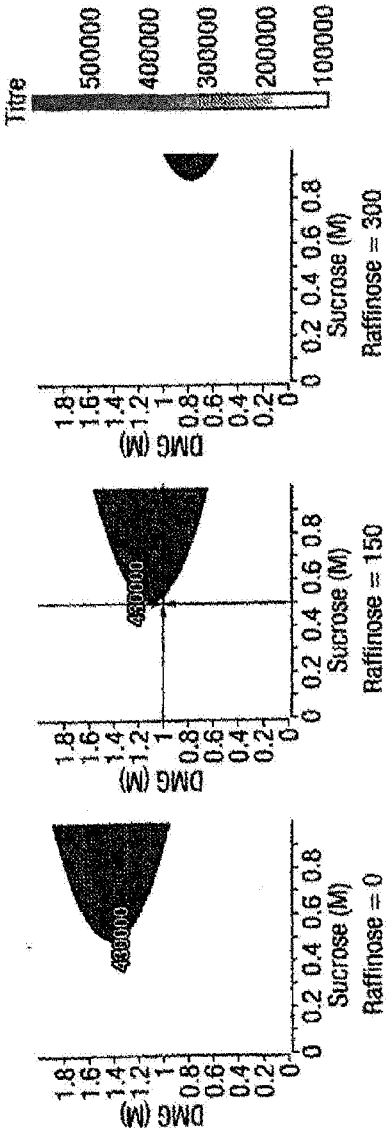



Fig. 33B

Fig. 34

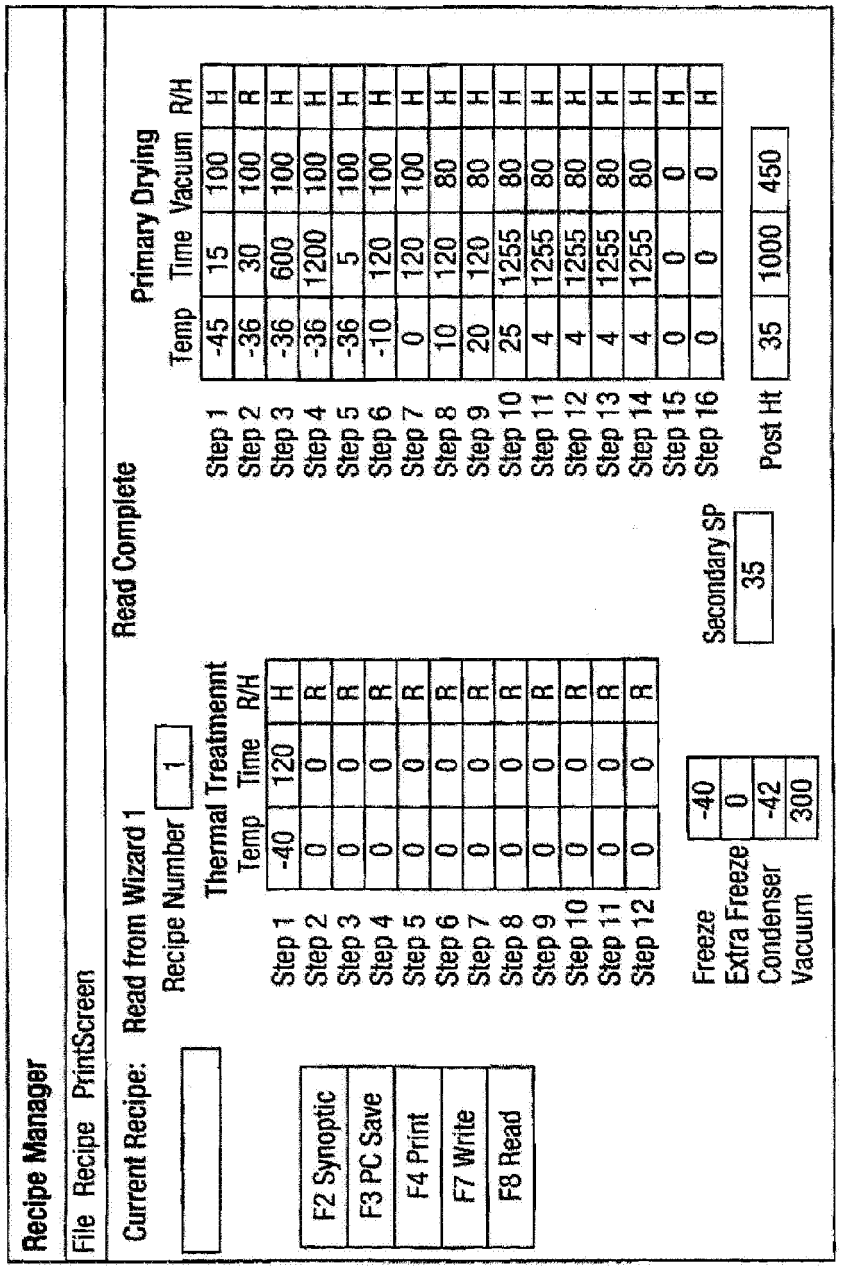

Recipe Manager
File Recipe PrintScreen
Current Recipe:
Read from Wizard 1
Recipe Number 1
Read Complete
Thermal Treatmennt
Temp Time R/H
Step 1 -40 120 H
Step 2 0 0 R
Step 3 0 0 R
Step 4 0 0 R
Step 5 0 0 R
Step 6 0 0 R
Step 7 0 0 R
Step 8 0 0 R
Step 9 0 0 R
Step 10 0 0 R
Step 11 0 0 R
Step 12 0 0 R
Primary Drying
Temp Time Vacuum R/H
Step 1 -45 15 100 H
Step 2 -36 30 100 R
Step 3 -36 600 100 H
Step 4 -36 1200 100 H
Step 5 -36 5 100 H
Step 6 -10 120 100 H
Step 7 0 120 100 H
Step 8 10 120 80 H
Step 9 20 120 80 H
Step 10 25 1255 80 H
Step 11 4 1255 80 H
Step 12 4 1255 80 H
Step 13 4 1255 80 H
Step 14 4 1255 80 H
Step 15 0 0 0 H
Step 16 0 0 0 H
F2 Synoptic
F3 PC Save
F4 Print
F7 Write
F8 Read
Freeze -40
Extra Freeze 0
Condenser -42
Vacuum 300
Secondary SP 35
Post Ht 35 1000 450

Fig. 35

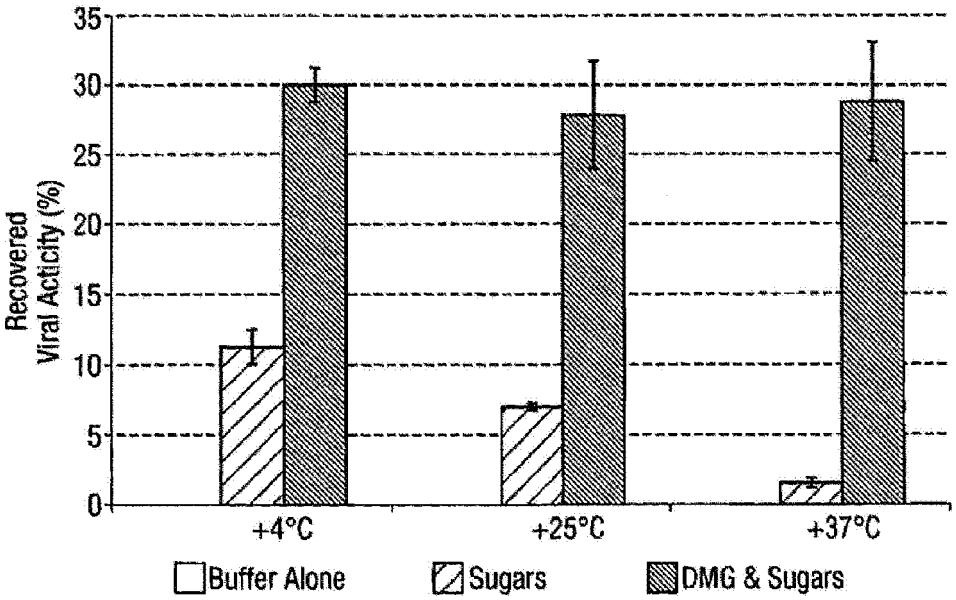

Recovered Viral Acticity (%)
35
30
25
20
15
10
5
0
+4°C
+25°C
+37°C
Buffer Alone
Sugars
DMG & Sugars

Fig. 36

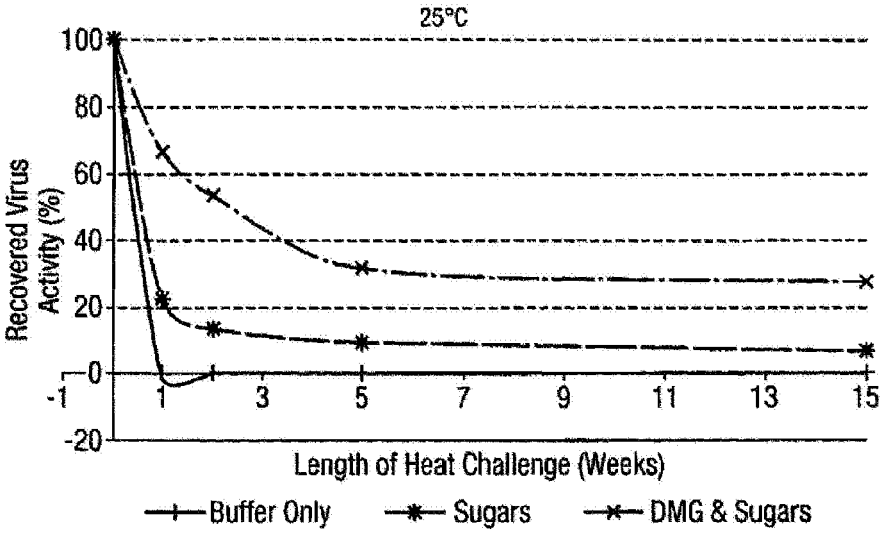

25°C
Recovered Virus Activity (%)
100
80
60
40
20
0
-1
-20
1
3
5
7
9
11
13
15
Length of Heat Challenge (Weeks)
Buffer Only
Sugars
DMG & Sugars

Fig. 37

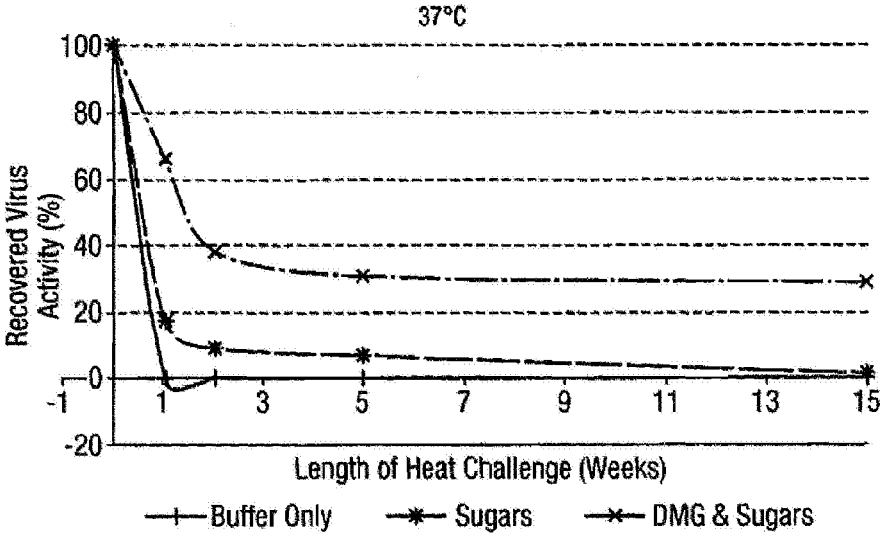

37°C
Recovered Virus Activity (%)
100
80
60
40
20
0
-1
-20
1
3
5
7
9
11
13
15
Length of Heat Challenge (Weeks)
Buffer Only
Sugars
DMG & Sugars

Fig. 38

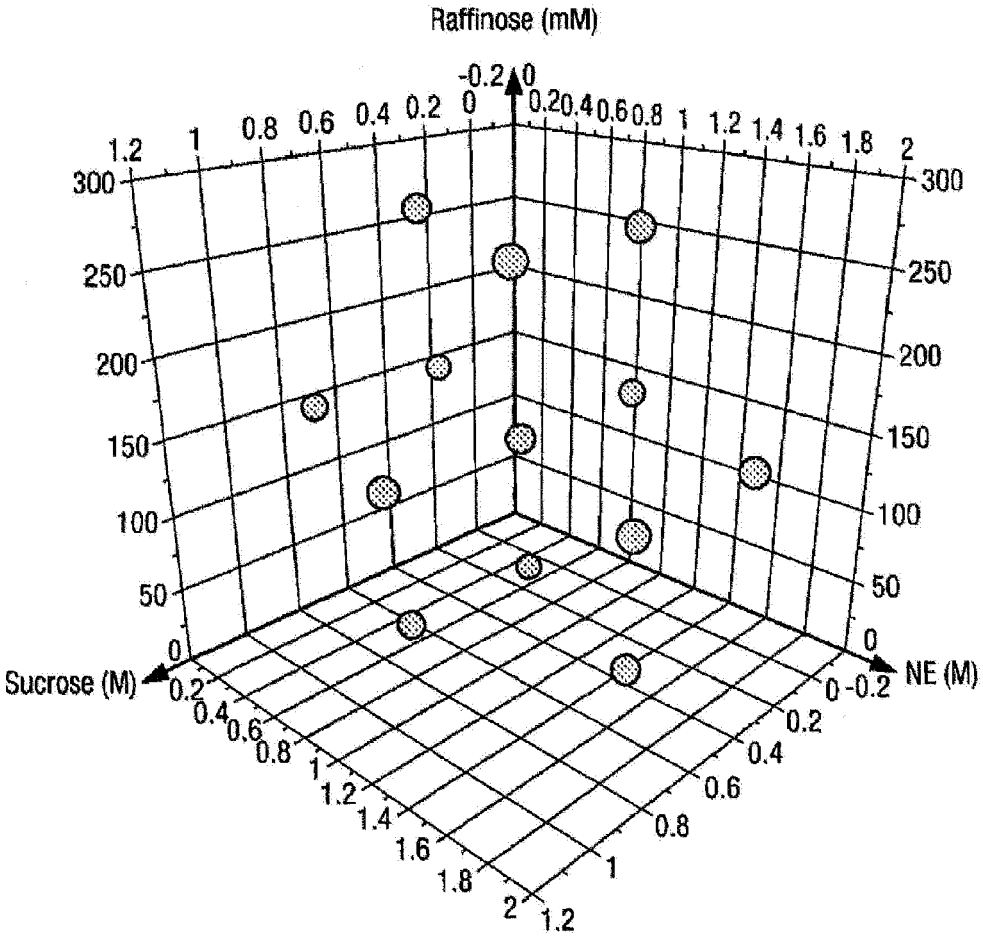

Raffinose (mM)
-0.2 0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8 2
300
250
200
150
100
50
0
Sucrose (M)
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8 2
1.2 1 0.8 0.6 0.4 0.2 0
NE (M)

Fig. 39

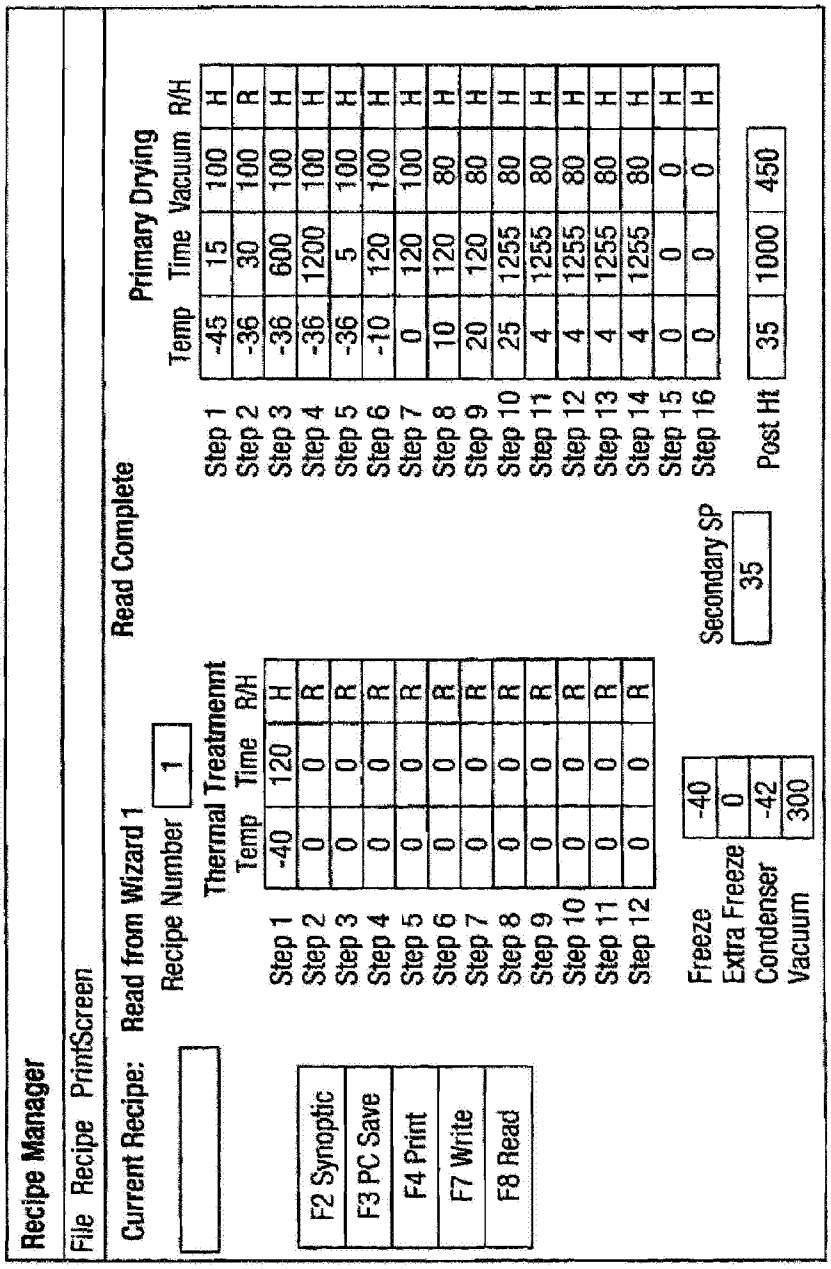

Recipe Manager
File Recipe PrintScreen
Current Recipe:
Read from Wizard 1
Read Complete
Recipe Number 1
Thermal Treatmennt
Temp Time R/H
Step 1 -40 120 H
Step 2 0 0 R
Step 3 0 0 R
Step 4 0 0 R
Step 5 0 0 R
Step 6 0 0 R
Step 7 0 0 R
Step 8 0 0 R
Step 9 0 0 R
Step 10 0 0 R
Step 11 0 0 R
Step 12 0 0 R
F2 Synoptic
F3 PC Save
F4 Print
F7 Write
F8 Read
Freeze -40
Extra Freeze 0
Condenser -42
Vacuum 300
Secondary SP 35
Primary Drying
Temp Time Vacuum R/H
Step 1 -45 15 100 H
Step 2 -36 30 100 R
Step 3 -36 600 100 H
Step 4 -36 1200 100 H
Step 5 -36 5 100 H
Step 6 -10 120 100 H
Step 7 0 120 100 H
Step 8 10 120 80 H
Step 9 20 120 80 H
Step 10 25 1255 80 H
Step 11 4 1255 80 H
Step 12 4 1255 80 H
Step 13 4 1255 80 H
Step 14 4 1255 80 H
Step 15 0 0 0 H
Step 16 0 0 0 H
Post Ht 35 1000 450

Fig. 40

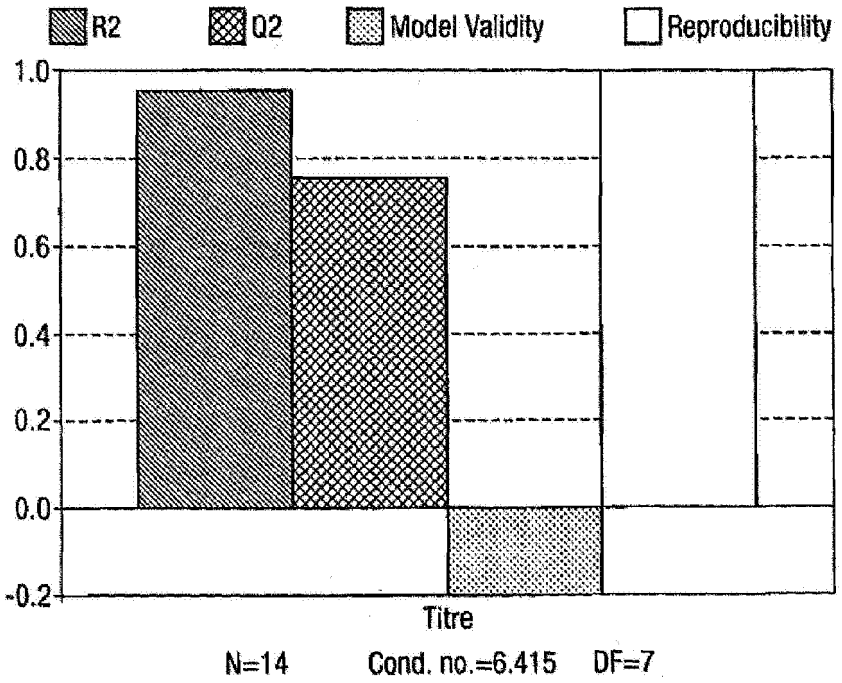

R2
Q2
Model Validity
Reproducibility
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Titre
N=14
Cond. no.=6.415
DF=7

Fig. 41

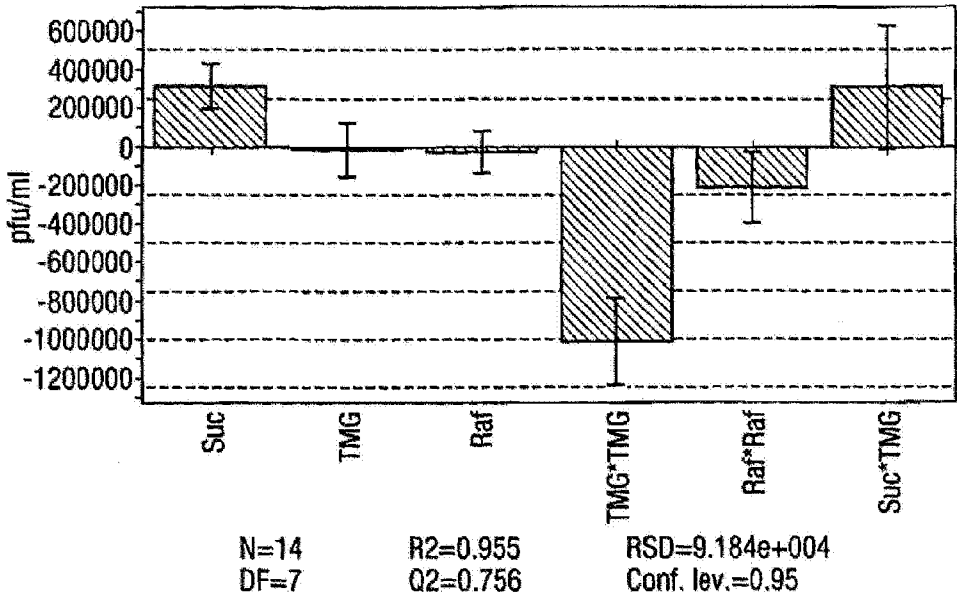

600000
400000
200000
0
-200000
-400000
-600000
-800000
-1000000
-1200000
pfu/ml
Suc
TMG
Raf
TMG*TMG
Raf*Raf
Suc*TMG
N=14
DF=7
R2=0.955
Q2=0.756
RSD=9.184e+004
Conf. lev.=0.95

Fig. 42

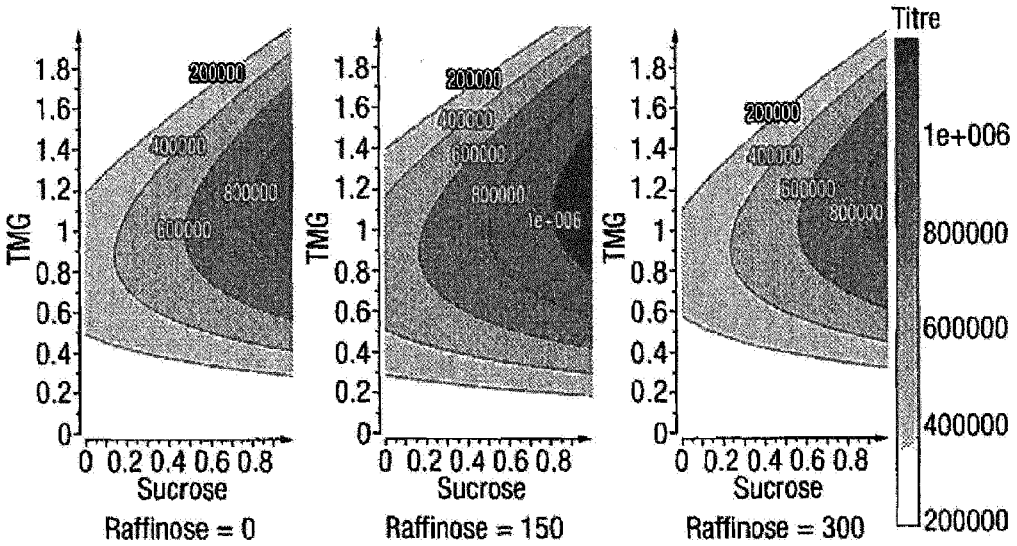

TMG
Sucrose
Raffinose = 0
Raffinose = 150
Raffinose = 300
Titre
1e+006
800000
600000
400000
200000

Fig. 43

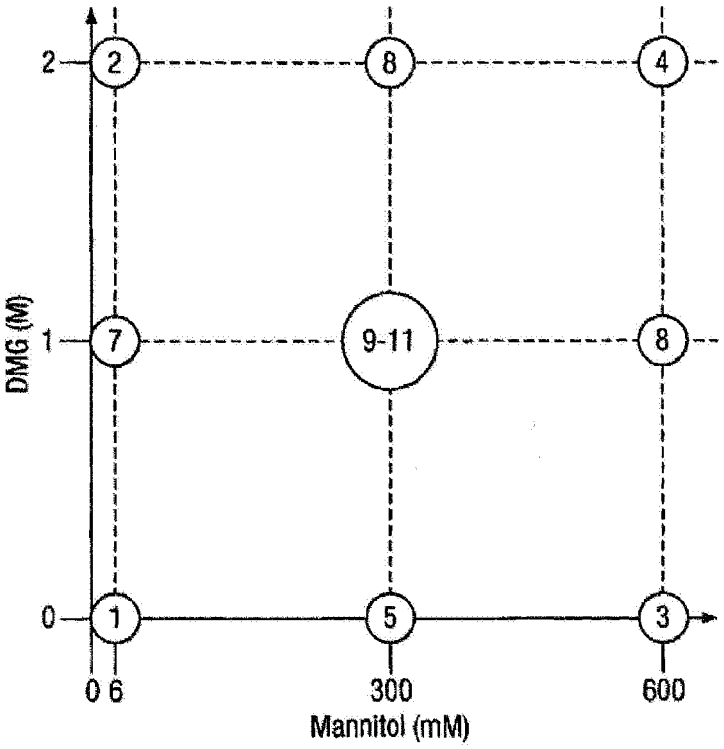


Fig. 44

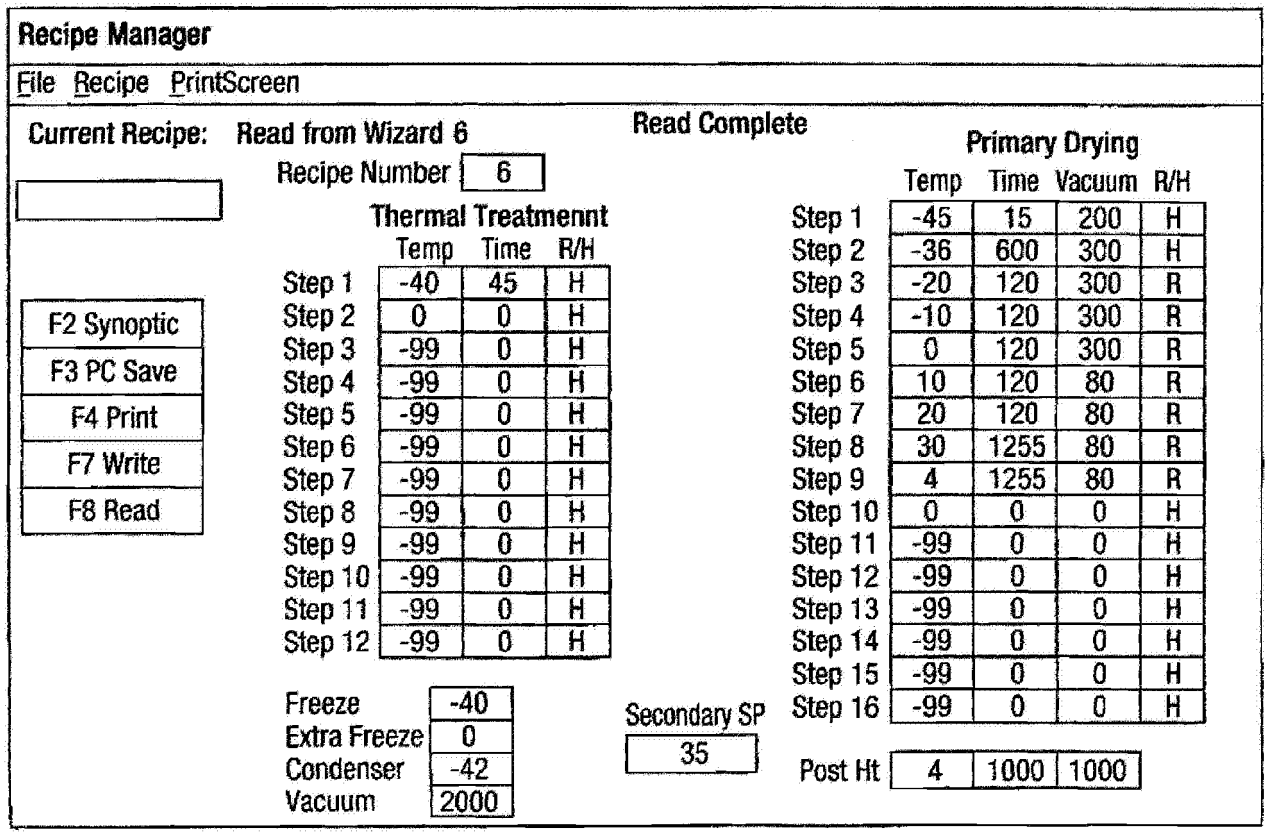



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Fig. 45

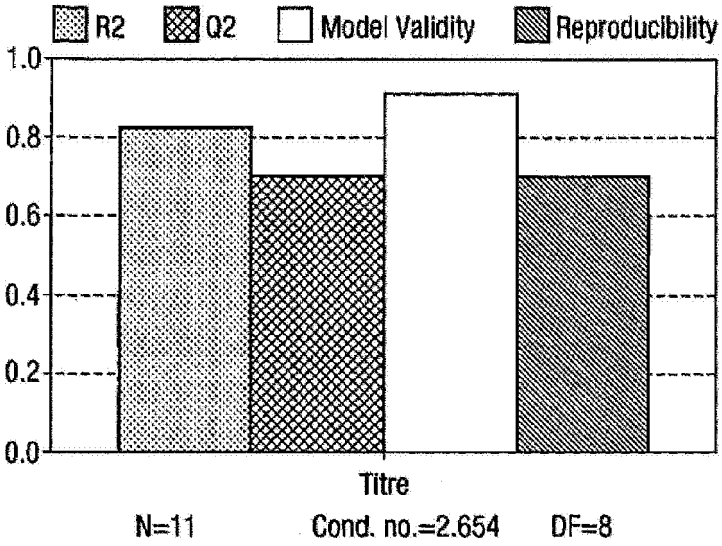

R2
Q2
Model Validity
Reproducibility
1.0
0.8
0.6
0.4
0.2
0.0
Titre
N=11
Cond. no.=2.654
DF=8

Fig. 46

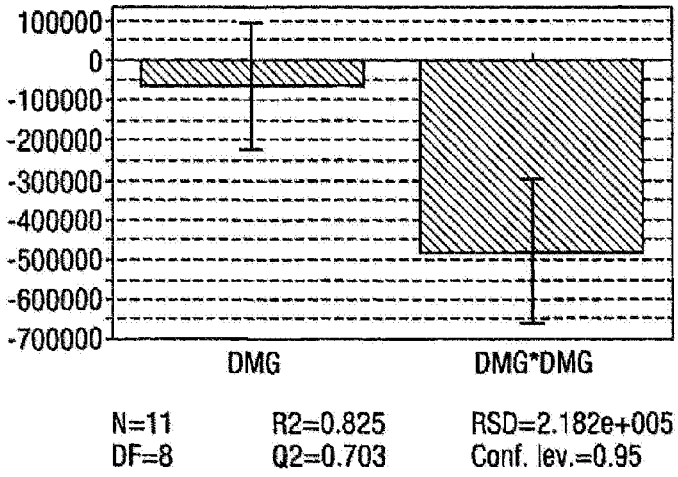

100000
0
-100000
-200000
-300000
-400000
-500000
-600000
-700000
DMG
DMG*DMG
N=11
DF=8
R2=0.825
Q2=0.703
RSD=2.182e+005
Conf. lev.=0.95

Fig. 47

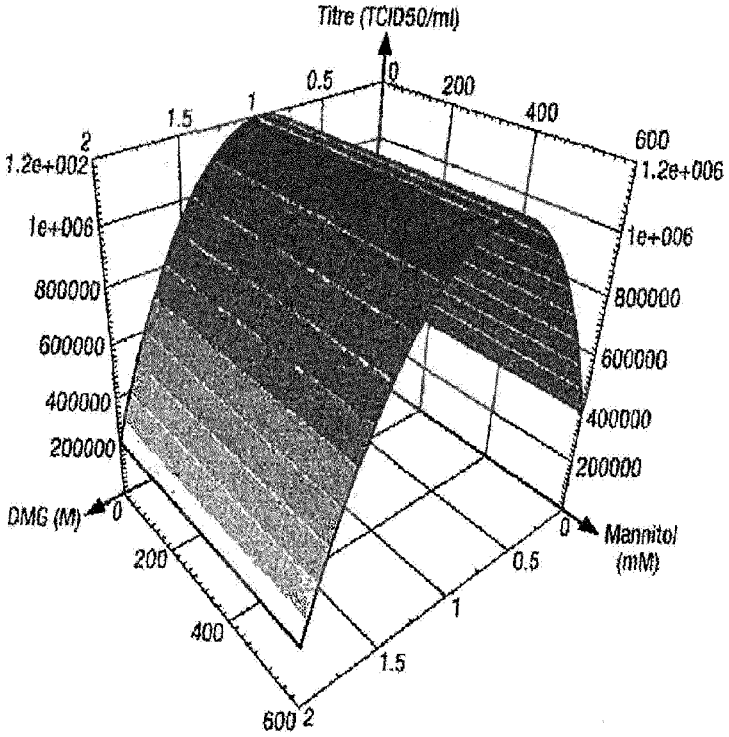
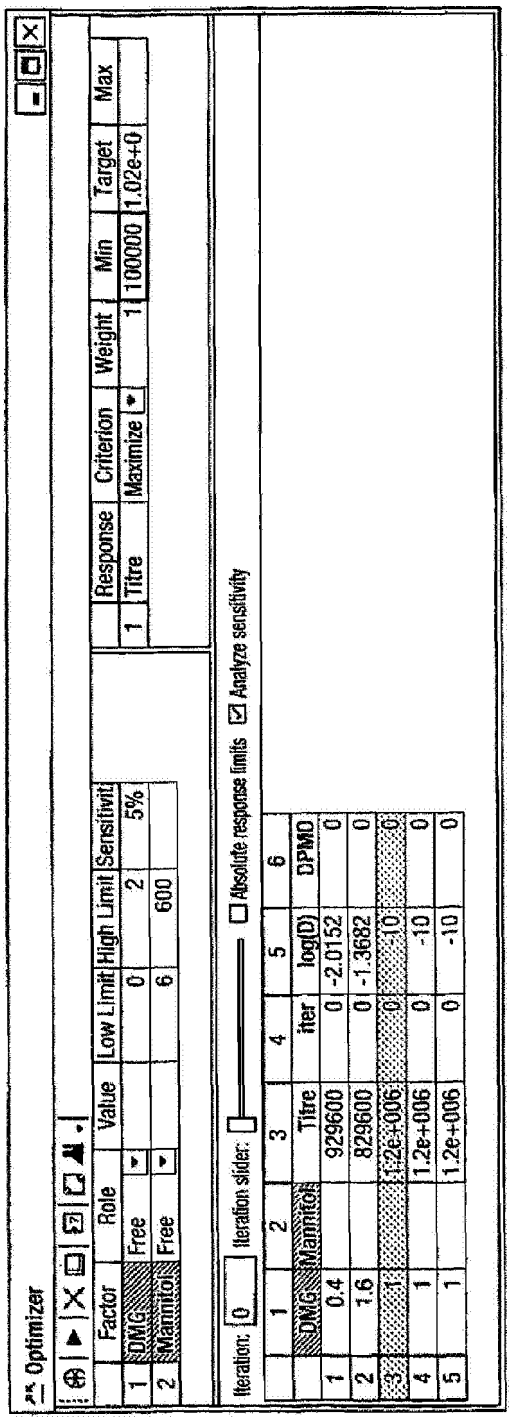

Titre (TCID50/ml)
1.2e+002
1e+006
800000
600000
400000
200000
0
0.5
1
1.5
2
0
200
400
600
1.2e+006
1e+006
800000
600000
400000
200000
DMG (M)
Mannitol (mM)

Fig. 48


Optimizer
Factor
Role
Value
Low Limit
High Limit
Sensitivity
DMG
Free
0
2
5%
Mannitol
Free
6
600
Response
Criterion
Weight
Min
Target
Max
Titre
Maximize
1
100000
1.02e+0
Iteration: 0
Iteration slider:
Absolute response limits
Analyze sensitivity
DMG
Mannitol
Titre
iter
log(D)
DPMD
0.4
1.6
929600
829600
1.2e+006
1.2e+006
1.2e+006
0
0
0
0
0
1
1
1
-2.0152
-1.3682
-10
-10
-10
0
0
0
0
0
0