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(71) Applicant: **GLAXOSMITHKLINE BIOLOGICALS**  
S.A. [BE/BE]; Rue de l'Institut 89, B-1330 Rixensart (BE).

(72) Inventor: **VANDE VELDE, Vincent**; GlaxoSmithKline  
Biologicals s.a., Rue de l'Institut 89, B-1330 Rixensart  
(BE).

(74) Agent: **ROBERTSON, James, Stuart**; GlaxoSmithKline,  
Global Patents CN925. 1, 980 Great West Road, Brentford,  
Middlesex TW8 9GS (GB).

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(54) Title: CALCIUM FLUORIDE COMPOSITIONS

(57) Abstract: Compositions comprising calcium fluoride composites comprising Ca, F, and an organic molecule are provided, as are methods for their use.



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TITLE  
CALCIUM FLUORIDE COMPOSITIONS

TECHNICAL FIELD

5           The present disclosure relates to composites for the stabilization of vaccine antigens and for enhancing the immune response against antigens used with the composites.

BACKGROUND

10           Subunit vaccines, for example recombinant protein/polypeptide antigens are only weakly immunogenic and thus there is a need for safe and effective adjuvants. Various adjuvants are known, including those comprising metallic salts such as alum, aluminum phosphate, and calcium phosphate. See, e.g., Lindblad (2004) *Vaccine* 22:3658-3668; Jiang et al (2004) *Vaccine* 23:693-698.

15           Thermo-stability of vaccines is desirable for practical and logistic reasons as thermostability of the vaccine reduces or avoids the requirement for cold-chain during worldwide distribution. Usually, lyophilisation techniques are applied to stabilize antigens. However, lyophilisation is not always possible or effective. Moreover, bypassing the costly and time consuming lyophilisation production step  
20           could increase the accessibility of the vaccine to a larger number of people in the world.

SUMMARY OF THE INVENTION

25           In one aspect, this disclosure provides calcium fluoride composites comprising Ca, F, and Z, wherein Z is an organic molecule. Methods for their production are provided. Methods for their use as adjuvants are also provided, as are methods for their use to stabilize antigens against temperature effects. Such methods include the use of some composites without lyophilization.

30           In a further aspect are provided calcium fluoride compositions comprising a calcium fluoride composite, said composite comprising Ca, F, and Z, wherein Z is an organic molecule.

          In a further aspect, are provided processes for making a calcium fluoride composite by sol gel precipitation comprising the steps of combining CaCl<sub>2</sub>, NaF,

and NaZ under precipitating conditions and collecting the water insoluble calcium fluoride composite. In a further aspect are provided products made by the process.

In a further aspect are provided adjuvant compositions comprising a calcium fluoride composition disclosed in the preceding aspects.

5 In a further aspect are provided processes for making the adjuvant compositions disclosed in the preceding aspects.

In a further aspect are provided immunogenic compositions comprising an antigen and an adjuvant composition as disclosed in the preceding aspects.

10 In a further aspect are provided processes for making immunogenic compositions as disclosed in the preceding aspects.

### BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Animal results obtained with HepB: antibody measurements (anti-HBs 14pII). Responses of the antigen are maintained when the antigen is adsorbed  
15 on the different carriers of the  $\text{CaF}_2$  family described herein.

Figure 2: Infrared spectra of batches 88331 07 compared to 88331 11. The infrared analysis shows the presence of  $\text{CaCO}_3$  of the Vaterite type.

Figure 3: Water solubility of  $\text{Ca}/\text{F}/\text{OH}$  composite, revealing that the composite is more soluble compared to the solubility of  $\text{CaF}_2$  reported in handbooks.

20 Figure 4: F4T formulation analyzed by SDS-PAGE analyses at  $t=0$ . Lane: 1, molecular weight standard; 2, sample buffer; 3,  $\text{CaF}_2/\text{CO}_3$  + liposome; 4, F4T +  $\text{CaF}_2$ ; 5, F4T +  $\text{CaF}_2$  + liposome; 6, F4T +  $\text{CaF}_2$ /cysteine; 7, F4T +  $\text{CaF}_2$ /cysteine + liposome; 8, F4T +  $\text{CaF}_2/\text{CO}_3$ ; 9, F4T +  $\text{CaF}_2/\text{CO}_3$  + liposome; 10, F4T. See Example 3B.

25 Figure 5: F4T formulation analyzed by SDS-PAGE after 1 month at  $4^\circ\text{C}$ . Lane: 1, molecular weight standard; 2, F4T bulk: bulk stored 1 month at  $-80^\circ\text{C}$  and thawed just before depot; 3, F4T bulk stored 1 month at  $4^\circ\text{C}$ ; 4, F4T formulated without inorganic and stored 1 month at  $4^\circ\text{C}$ ; 5, F4T +  $\text{CaF}_2$ ; 6, F4T +  $\text{CaF}_2$  + liposome; 7, F4T +  $\text{CaF}_2$ /cysteine; 8, F4T +  $\text{CaF}_2$ /cysteine + liposome; 9, F4T +  
30  $\text{CaF}_2/\text{CO}_3$ ; 10, F4T +  $\text{CaF}_2/\text{CO}_3$  + liposome. See Example 3B.

Figure 6: F4T formulations analyzed by SDS-PAGE after 1 month at  $30^\circ\text{C}$ . Lane: 1, molecular weight standard; 2, F4T bulk: bulk stored 1 month at  $-80^\circ\text{C}$  and thawed just before depot; 3, F4T bulk stored 1 month at  $30^\circ\text{C}$ ; 4, F4T formulated

without inorganic and stored 1 month at 30 C; 5, F4T + CaF<sub>2</sub>; 6, F4T + CaF<sub>2</sub> + liposome; 7, F4T + CaF<sub>2</sub>/cysteine; 8, F4T + CaF<sub>2</sub>/cysteine + liposome; 9, F4T + CaF<sub>2</sub>/CO<sub>3</sub>; 10, F4T + CaF<sub>2</sub>/CO<sub>3</sub> + liposome. Note the substantial degradation of lanes 3 and 4 (F4T without composite). See Example 3B.

5 Figure 7: Composite + **ClfA**<sub>Ni<sub>2</sub>3</sub> immunogenicity (antibodies). The immunogenicity of the antigen is maintained when the antigen is adsorbed on the different carriers. Mice were immunized with stabilized **ClfA**<sub>Ni<sub>2</sub>3</sub> composite (adsorbed on an inorganic carrier). The immunogenicity of these adsorbed composite in an emulsion formulation was carried out by ELISA- **ClfA**<sub>Ni<sub>2</sub>3</sub> composite (concentrations  
10 (µg/mL) on Post III. From left to right, non-treated, adsorbed on CaF<sub>2</sub>/CaCO<sub>3</sub>, adsorbed on CaF<sub>2</sub>/N-Ac-Cysteine, adsorbed on CaF<sub>2</sub>, and adsorbed on CaF<sub>2</sub>/Cysteine. See Example 4.

Figure 8: Infrared spectra of batches 8833152-7.

Figure 9: Immune Response of HepB adsorbed antigen. See Example 5.

15 Figure 10: Electron Microscopy photograph of calcium fluoride composites disclosed herein. Pictured are calcium fluoride composites disclosed in batch #10616125 (see Table 1 and the example entitled "Ca/F/N-Acetyl-cysteine batch # 10616125."

20 Figure 11: RSV neutralization titers in serum 14 days after the second immunization with rF antigen at two different doses adsorbed on different composites. See Example 6.

Figure 12: Anti-rF IgG concentrations in serum 14 days after the second immunization with rF antigen at two different doses adsorbed on different composites. See Example 6.

25 Figure 13: RSV titers in lungs 4 days after RSV challenge, according to various regimens composed of 2 µg rF and adjuvant. See Example 7.

Figures 14: Evaluation of composite-19F-DT formulations in the Balb/c mouse immunogenicity model. See Example 8.

30 Figure 15: Evaluation of composite-19F-DT formulations in the Balb/c mouse immunogenicity model (cont). See Example 8.

Figure 16: Evaluation of composite-19F-DT formulations in the Balb/c mouse immunogenicity model (cont). See Example 8.

Figure 17: Evaluation of composite-19F-DT formulations in the Balb/c mouse immunogenicity model (cont). See Example 8.

Figure 18: Animal Results of various composite-PRN. See Example 9.

5

## DETAILED DESCRIPTION

It is disclosed herein that adsorption of antigens to a water insoluble a calcium fluoride composite stabilizes the antigen against temperature dependent degradation. Moreover, it is disclosed that the calcium fluoride composites act as an adjuvant by increasing the immune response against an antigen adsorbed thereto.

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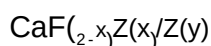
### Compositions

In some aspects are provided calcium fluoride compositions comprising a calcium fluoride composite, the composite comprising Ca, F, and Z. By "Z" is intended an organic (carbon-containing) molecule. By "composite" is intended a material that exists as a solid when dry, and that is insoluble, or poorly soluble, in pure water.

In some aspects, the composite comprises equal percentages w/w of Ca and F. In some aspects, the composite comprises a greater percentage Ca (w/w) than percentage F (w/w). By "percent X w/w" (where X is a molecule or element found in a composition) is intended the percentage of the total weight of the composition that is attributable to X. Thus, w/w in the present context means the dry weight. For compositions in which the relative ratios are known, the percent w/w may be determined mathematically. For instance, compositions of  $\text{CaF}_2$  comprise roughly 51% Ca and roughly 49% F (w.w): % w/w Ca =  $[(40\text{g/mol Ca} * 100)]/[40\text{g/mol Ca} + (2 * 19\text{g/mol F})] = 51$ ; % w/w F =  $[(2 * 19\text{g/mol F}) * 100]/[40\text{g/mol Ca} + (2 * 19\text{g/mol F})] = 49$ . Nonetheless, % w/w may also be determined by empirical methods. For instance, where the molecule in question is an acid or base, the percent w/w of that molecule may be determined by titration (where Z is carbonate, percent w/w/ carbonate can be determined by titration with HCl). Alternatively, where the molecule contains a fractional percent by weight of nitrogen, the percent w/w of that molecule may be determined by elementary analysis methods in which the amount of nitrogen is determined and then the total weight attributable to the nitrogen-containing molecule calculated using the molecular weight of the nitrogen-containing

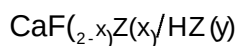
molecule. Instruments for this methodology are available commercially, for instance from Antek™, 300 Bammel Westfield Road, Houston, Texas 77090. Alternatively, percent w/w of an oxidizable organic molecule can be determined by oxydo-reduction titration methods, for example in the presence of potassium permanganate in the presence of sulfuric acid.

In some aspects, calcium fluoride composites as disclosed herein may be represented as follows:



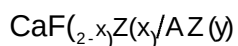
**Formula I**

where x is a non-negative number from 0 to 2, inclusive, and y is a non-negative number. In some aspects, y is a non-negative number from 0 to 2, inclusive. In some aspects, the sum of x and y together is a non-negative number of equal to or less than 2. In some aspects, x and y are not both zero. However, as will be understood given their formation, a calcium fluoride composite as described herein may not be uniform, but may rather comprise regions in which Z interacts with the rest of the constituents by primarily ionic or covalent interactions and regions in which Z interacts with the rest of the constituents through weak forces (represented by  $\text{Z}''$ ). In this context,  $\text{Z}_{(x)}$  represents the ionized form of Z and  $\text{Z}_{(y)}$  represents the unionized form of Z, such as HZ or AZ, or a mixture thereof, where A is a counterion. Such non-uniform composites may be represented as follows:



**Formula II**

or



**Formula III**

wherein neither x nor y are both not zero.

Calcium fluoride composites as disclosed herein will have the characteristics of forming a solid when dry, will be insoluble, or poorly soluble, in pure water, and exhibit an E.C.P. in the range of 5.0 to 11.0, inclusive.

In some aspects, Z comprises a functional group that forms an anion when ionized. Such functional groups include without limitation one or more functional groups selected from the group consisting of: hydroxyl, hydroxylate, hydroxo, oxo, N-hydroxylate, hydroxamate, N-oxide, bicarbonate, carbonate, carboxylate, fatty acid, thiolate, organic phosphate, dihydrogenophosphate, monohydrogenophosphate, monoesters of phosphoric acid, diesters of phosphoric acid, esters of phospholipid, phosphorothioate, sulphates, hydrogen sulphates, enolate, ascorbate, phosphoascorbate, phenolate, and imine-olates. In some aspects, the calcium fluoride composites herein comprise Z, where Z is an anionic organic molecule possessing an affinity for calcium and forming a water insoluble composite with calcium and fluoride.

In some aspects, the calcium fluoride composites herein comprise Z, where Z may be categorized as comprising a member of a chemical category selected from the group consisting of: hydroxyl, hydroxylates, hydroxo, oxo, N-hydroxylate, hydroxamate, N-oxide, bicarbonates, carbonates, carboxylates and dicarboxylate, salts of carboxylic-acids, salts of QS21, extract of bark of Quillaja saponaria, extract of immunological active saponine, salts of saturated or unsaturated fatty acid, salts of oleic acid, salts of amino-acids, thiolates, thiolactate, salt of thiol-compounds, salts of cysteine, salts of N-acetyl-cysteine, L-2-Oxo-4-thiazolidinecarboxylate, phosphates, dihydrogenophosphates, monohydrogenophosphate, salts of phosphoric-acids, monoesters of phosphoric acids and their salts, diesters of phosphoric acids and their salts, esters of 3-O-desacyl-4'-monophosphoryl lipid A, esters of 3D-MLA, MPL, esters of phospholipids, DOPC, dioleolyphosphatidic derivatives, phosphates from CPG motifs, phosphorothioates from CpG family, sulphates, hydrogen sulphates, salts of sulphuric acids, enolates, ascorbates, phosphoascorbate, phenolate, a-tocopherol, imine-olates, cytosine, methyl-cytosine, uracyl, thymine, barbituric acid, hypoxanthine, inosine, guanine, guanosine, 8-oxo-adenine, xanthine, uric acid, pteric acid, pteroylglutamic acid, folic acid, riboflavin, and lumiflavin.

In some aspects, the calcium fluoride composites herein comprise Z, where Z is selected from the group consisting of: N-acetyl cysteine; thiolactate; adipate; carbonate; folic acid; glutathione; and uric acid. In some aspects, the calcium fluoride

composites herein comprise Z, where Z is selected from the group consisting of: N-acetyl cysteine; adipate; carbonate; and folic acid.

In some aspects, the calcium fluoride composites herein comprise Z, where Z is N-acetyl cysteine, and the composite comprises between 51% Ca, 48% F, no more than 1% N-acetyl cysteine (w/w) and 37% Ca, 26% F, and 37% N-acetyl cysteine (w/w).

In some aspects, the calcium fluoride composites herein comprise Z, where Z is Z is thiolactate, and the composite comprises between 51% Ca, 48% F, no more than 1% thiolactate (w/w) and 42% Ca, 30% F, 28% thiolactate (w/w).

In some aspects, the calcium fluoride composites herein comprise Z, where Z is Z is adipate, and the composite comprises between 51% Ca, 48% F, no more than 1% adipate (w/w) and 38% Ca, 27% F, 35% adipate (w/w).

In some aspects, the calcium fluoride composites herein comprise Z, where Z is Z is carbonate, and the composite comprises between 51% Ca, 48% F, no more than 1% carbonate (w/w) and 48% Ca, 34% F, 18% carbonate (w/w).

In some aspects, the calcium fluoride composites herein comprise Z, where Z is Z is folic acid, and the composite comprises between 51% Ca, 48% F, no more than 1% folic acid (w/w) and 22% Ca, 16% F, 62% folic acid (w/w).

In some aspects, the calcium fluoride composites herein comprise Z, where Z is glutathione, and the composite comprises between 51% Ca, 48% F, no more than 1% glutathione (w/w) and 28% Ca, 20% F, 52% glutathione (w/w).

In some aspects, the calcium fluoride composites herein comprise Z, where Z is uric acid, and the composite comprises between 51% Ca, 48% F, and no more than 1% uric acid (w/w) and 36% Ca, 26% F, and 38% uric acid (w/w).

In some aspects, a calcium fluoride composite comprising Ca, F, and Z has the following composition (Chart 1):

**Chart 1:** Ca/F/Z %W/W calculation for various composites.

| Ca/F/Z %W/W calculation for various composites |  |                                                                                       |  |  |  |                     |  |  |  |
|------------------------------------------------|--|---------------------------------------------------------------------------------------|--|--|--|---------------------|--|--|--|
| based on                                       |  | Ca[(F <sub>2</sub> )100-% + (Z)%],<br>where % is 0, or 2 or 5 or 10 or 15 or 20 or 25 |  |  |  |                     |  |  |  |
|                                                |  | Z Formula Weight (FW)                                                                 |  |  |  | Mass/mole composite |  |  |  |

| Ca/F/Z %W/W calculation for various composites |      |                       |                                                                                       |                |      |                     |         |        |              |
|------------------------------------------------|------|-----------------------|---------------------------------------------------------------------------------------|----------------|------|---------------------|---------|--------|--------------|
| based on                                       |      |                       | Ca[(F <sub>2</sub> )100-% + (Σ)%],<br>where % is 0, or 2 or 5 or 10 or 15 or 20 or 25 |                |      |                     |         |        |              |
|                                                |      | Z Formula Weight (FW) |                                                                                       |                |      | Mass/mole composite |         |        |              |
| Ca/F/NAcefc/ICysteine                          |      |                       |                                                                                       | F <sub>2</sub> | Ca   | PM                  | Ca% w/w | F% w/w | NACys %      |
|                                                | %    | 161.0                 |                                                                                       | 38.0           | 40.0 |                     |         |        |              |
|                                                | 0.0  | 0.0                   | 100.0                                                                                 | 38.0           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0          |
|                                                | 2.0  | 3.2                   | 98.0                                                                                  | 37.2           | 40.0 | 80.5                | 49.7    | 46.3   | 4.0          |
|                                                | 5.0  | 8.1                   | 95.0                                                                                  | 36.1           | 40.0 | 84.2                | 47.5    | 42.9   | 9.6          |
|                                                | 10.0 | 16.1                  | 90.0                                                                                  | 34.2           | 40.0 | 90.3                | 44.3    | 37.9   | 17.8         |
|                                                | 15.0 | 24.2                  | 85.0                                                                                  | 32.3           | 40.0 | 96.5                | 41.5    | 33.5   | 25.0         |
|                                                | 20.0 | 32.2                  | 80.0                                                                                  | 30.4           | 40.0 | 102.6               | 39.0    | 29.6   | 31.4         |
|                                                | 25.0 | 40.3                  | 75.0                                                                                  | 28.5           | 40.0 | 108.8               | 36.8    | 26.2   | 37.0         |
|                                                |      |                       |                                                                                       |                |      |                     |         |        |              |
| Ca/F/Thiolactate                               |      |                       |                                                                                       | F <sub>2</sub> | Ca   | PM                  | Ca%     | F%     | Thiolactate% |
|                                                | %    | 104.0                 |                                                                                       | 38.0           | 40.0 |                     |         |        |              |
|                                                | 0.0  | 0.0                   | 100.0                                                                                 | 38.0           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0          |
|                                                | 2.0  | 2.1                   | 98.0                                                                                  | 37.2           | 40.0 | 79.3                | 50.4    | 46.9   | 2.6          |
|                                                | 5.0  | 5.2                   | 95.0                                                                                  | 36.1           | 40.0 | 81.3                | 49.2    | 44.4   | 6.4          |
|                                                | 10.0 | 10.4                  | 90.0                                                                                  | 34.2           | 40.0 | 84.6                | 47.3    | 40.4   | 12.3         |
|                                                | 15.0 | 15.6                  | 85.0                                                                                  | 32.3           | 40.0 | 87.9                | 45.5    | 36.7   | 17.7         |
|                                                | 20.0 | 20.8                  | 80.0                                                                                  | 30.4           | 40.0 | 91.2                | 43.9    | 33.3   | 22.8         |
|                                                | 25.0 | 26.0                  | 75.0                                                                                  | 28.5           | 40.0 | 94.5                | 42.3    | 30.2   | 27.5         |
|                                                |      |                       |                                                                                       |                |      |                     |         |        |              |
| Ca/F/Adipate                                   |      |                       |                                                                                       | F <sub>2</sub> | Ca   | PM                  | Ca%     | F%     | Adipate %    |
|                                                | %    | 144.0                 |                                                                                       | 38.0           | 40.0 |                     |         |        |              |
|                                                | 0.0  | 0.0                   | 100.0                                                                                 | 38.0           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0          |
|                                                | 2.0  | 2.9                   | 98.0                                                                                  | 37.2           | 40.0 | 80.1                | 49.9    | 46.5   | 3.6          |
|                                                | 5.0  | 7.2                   | 95.0                                                                                  | 36.1           | 40.0 | 83.3                | 48.0    | 43.3   | 8.6          |
|                                                | 10.0 | 14.4                  | 90.0                                                                                  | 34.2           | 40.0 | 88.6                | 45.1    | 38.6   | 16.3         |
|                                                | 15.0 | 21.6                  | 85.0                                                                                  | 32.3           | 40.0 | 93.9                | 42.6    | 34.4   | 23.0         |
|                                                | 20.0 | 28.8                  | 80.0                                                                                  | 30.4           | 40.0 | 99.2                | 40.3    | 30.6   | 29.0         |
|                                                | 25.0 | 36.0                  | 75.0                                                                                  | 28.5           | 40.0 | 104.5               | 38.3    | 27.3   | 34.4         |
|                                                |      |                       |                                                                                       |                |      |                     |         |        |              |

| Ca/F/Z %W/W calculation for various composites |      |                       |                                                                                       |                |      |                      |         |        |                    |
|------------------------------------------------|------|-----------------------|---------------------------------------------------------------------------------------|----------------|------|----------------------|---------|--------|--------------------|
| based on                                       |      |                       | Ca[(F <sub>2</sub> )100-% + (Σ)%],<br>where % is 0, or 2 or 5 or 10 or 15 or 20 or 25 |                |      |                      |         |        |                    |
|                                                |      | Z Formula Weight (FW) |                                                                                       |                |      | Mass/ mole composite |         |        |                    |
| Ca/F/Cysteine                                  |      |                       |                                                                                       | F <sub>2</sub> | Ca   | PM                   | Ca%     | F%     | Cysteine %         |
|                                                | %    | 119.0                 |                                                                                       | 38.0           | 40.0 |                      |         |        |                    |
|                                                | 0.0  | 0.0                   | 100.0                                                                                 | 38.0           | 40.0 | 78.0                 | 51.3    | 48.7   | 0.0                |
|                                                | 2.0  | 2.4                   | 98.0                                                                                  | 37.2           | 40.0 | 79.6                 | 50.2    | 46.8   | 3.0                |
|                                                | 5.0  | 6.0                   | 95.0                                                                                  | 36.1           | 40.0 | 82.1                 | 48.8    | 44.0   | 7.3                |
|                                                | 10.0 | 11.9                  | 90.0                                                                                  | 34.2           | 40.0 | 86.1                 | 46.5    | 39.7   | 13.8               |
|                                                | 15.0 | 17.9                  | 85.0                                                                                  | 32.3           | 40.0 | 90.2                 | 44.4    | 35.8   | 19.8               |
|                                                | 20.0 | 23.8                  | 80.0                                                                                  | 30.4           | 40.0 | 94.2                 | 42.5    | 32.3   | 25.3               |
|                                                | 25.0 | 29.8                  | 75.0                                                                                  | 28.5           | 40.0 | 98.3                 | 40.7    | 29.0   | 30.3               |
|                                                |      |                       |                                                                                       |                |      |                      |         |        |                    |
| Ca/F/Glutathione                               |      |                       |                                                                                       | F <sub>2</sub> | Ca   | PM                   | Ca% w/w | F% w/w | Glutathione%       |
|                                                | %    | 305.0                 |                                                                                       | 38.0           | 40.0 |                      |         |        |                    |
|                                                | 0.0  | 0.0                   | 100.0                                                                                 | 38.0           | 40.0 | 78.0                 | 51.3    | 48.7   | 0.0                |
|                                                | 2.0  | 6.1                   | 98.0                                                                                  | 37.2           | 40.0 | 83.3                 | 48.0    | 44.7   | 7.3                |
|                                                | 5.0  | 15.3                  | 95.0                                                                                  | 36.1           | 40.0 | 91.4                 | 43.8    | 39.5   | 16.7               |
|                                                | 10.0 | 30.5                  | 90.0                                                                                  | 34.2           | 40.0 | 104.7                | 38.2    | 32.7   | 29.1               |
|                                                | 15.0 | 45.8                  | 85.0                                                                                  | 32.3           | 40.0 | 118.1                | 33.9    | 27.4   | 38.8               |
|                                                | 20.0 | 61.0                  | 80.0                                                                                  | 30.4           | 40.0 | 131.4                | 30.4    | 23.1   | 46.4               |
|                                                | 25.0 | 76.3                  | 75.0                                                                                  | 28.5           | 40.0 | 144.8                | 27.6    | 19.7   | 52.7               |
|                                                |      |                       |                                                                                       |                |      |                      |         |        |                    |
| Ca/F/Glutathione oxide                         |      |                       |                                                                                       | F <sub>2</sub> | Ca   | PM                   | Ca% w/w | F% w/w | Glutathione oxide% |
|                                                | %    | 610.0                 |                                                                                       | 38.0           | 40.0 |                      |         |        |                    |
|                                                | 0.0  | 0.0                   | 100.0                                                                                 | 38.0           | 40.0 | 78.0                 | 51.3    | 48.7   | 0.0                |
|                                                | 2.0  | 12.2                  | 98.0                                                                                  | 37.2           | 40.0 | 89.4                 | 44.7    | 41.6   | 13.6               |
|                                                | 5.0  | 30.5                  | 95.0                                                                                  | 36.1           | 40.0 | 106.6                | 37.5    | 33.9   | 28.6               |
|                                                | 10.0 | 61.0                  | 90.0                                                                                  | 34.2           | 40.0 | 135.2                | 29.6    | 25.3   | 45.1               |
|                                                | 15.0 | 91.5                  | 85.0                                                                                  | 32.3           | 40.0 | 163.8                | 24.4    | 19.7   | 55.9               |
|                                                | 20.0 | 122.0                 | 80.0                                                                                  | 30.4           | 40.0 | 192.4                | 20.8    | 15.8   | 63.4               |
|                                                | 25.0 | 152.5                 | 75.0                                                                                  | 28.5           | 40.0 | 221.0                | 18.1    | 12.9   | 69.0               |

| Ca/F/Z %W/W calculation for various composites |      |                       |                                                                                      |                |      |                     |         |        |             |
|------------------------------------------------|------|-----------------------|--------------------------------------------------------------------------------------|----------------|------|---------------------|---------|--------|-------------|
| based on                                       |      |                       | Ca[(F <sub>2</sub> 100-% + (Σ)%],<br>where % is 0, or 2 or 5 or 10 or 15 or 20 or 25 |                |      |                     |         |        |             |
|                                                |      | Z Formula Weight (FW) |                                                                                      |                |      | Mass/mole composite |         |        |             |
| Ca/F/Uric acid                                 |      |                       |                                                                                      | F <sub>2</sub> | Ca   | PM                  | Ca% w/w | F% w/w | Uric acid%  |
|                                                | %    | 166.0                 |                                                                                      | 38.0           | 40.0 |                     |         |        |             |
|                                                | 0.0  | 0.0                   | 100.0                                                                                | 38.0           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0         |
|                                                | 2.0  | 3.3                   | 98.0                                                                                 | 37.2           | 40.0 | 80.6                | 49.7    | 46.2   | 4.1         |
|                                                | 5.0  | 8.3                   | 95.0                                                                                 | 36.1           | 40.0 | 84.4                | 47.4    | 42.8   | 9.8         |
|                                                | 10.0 | 16.6                  | 90.0                                                                                 | 34.2           | 40.0 | 90.8                | 44.1    | 37.7   | 18.3        |
|                                                | 15.0 | 24.9                  | 85.0                                                                                 | 32.3           | 40.0 | 97.2                | 41.2    | 33.2   | 25.6        |
|                                                | 20.0 | 33.2                  | 80.0                                                                                 | 30.4           | 40.0 | 103.6               | 38.6    | 29.3   | 32.0        |
|                                                | 25.0 | 41.5                  | 75.0                                                                                 | 28.5           | 40.0 | 110.0               | 36.4    | 25.9   | 37.7        |
|                                                |      |                       |                                                                                      |                |      |                     |         |        |             |
| Ca/F/Folic acid                                |      |                       |                                                                                      | F <sub>2</sub> | Ca   | PM                  | Ca% w/w | F% w/w | Folic acid% |
|                                                | %    | 439.0                 |                                                                                      | 38.0           | 40.0 |                     |         |        |             |
|                                                | 0.0  | 0.0                   | 100.0                                                                                | 38.0           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0         |
|                                                | 2.0  | 8.8                   | 98.0                                                                                 | 37.2           | 40.0 | 86.0                | 46.5    | 43.3   | 10.2        |
|                                                | 5.0  | 22.0                  | 95.0                                                                                 | 36.1           | 40.0 | 98.1                | 40.8    | 36.8   | 22.4        |
|                                                | 10.0 | 43.9                  | 90.0                                                                                 | 34.2           | 40.0 | 118.1               | 33.9    | 29.0   | 37.2        |
|                                                | 15.0 | 65.9                  | 85.0                                                                                 | 32.3           | 40.0 | 138.2               | 29.0    | 23.4   | 47.7        |
|                                                | 20.0 | 87.8                  | 80.0                                                                                 | 30.4           | 40.0 | 158.2               | 25.3    | 19.2   | 55.5        |
|                                                | 25.0 | 109.8                 | 75.0                                                                                 | 28.5           | 40.0 | 178.3               | 22.4    | 16.0   | 61.6        |
|                                                |      |                       |                                                                                      |                |      |                     |         |        |             |
| Ca/F/C03                                       |      |                       |                                                                                      | F <sub>2</sub> | Ca   | PM                  | Ca% w/w | F% w/w | Folic acid% |
|                                                | %    | 60.0                  |                                                                                      | 38.0           | 40.0 |                     |         |        |             |
|                                                | 0.0  | 0.0                   | 100.0                                                                                | 38.0           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0         |
|                                                | 2.0  | 1.2                   | 98.0                                                                                 | 37.2           | 40.0 | 78.4                | 51.0    | 47.5   | 1.5         |
|                                                | 5.0  | 3.0                   | 95.0                                                                                 | 36.1           | 40.0 | 79.1                | 50.6    | 45.6   | 3.8         |
|                                                | 10.0 | 6.0                   | 90.0                                                                                 | 34.2           | 40.0 | 80.2                | 49.9    | 42.6   | 7.5         |
|                                                | 15.0 | 9.0                   | 85.0                                                                                 | 32.3           | 40.0 | 81.3                | 49.2    | 39.7   | 11.1        |
|                                                | 20.0 | 12.0                  | 80.0                                                                                 | 30.4           | 40.0 | 82.4                | 48.5    | 36.9   | 14.6        |

| Ca/F/Z %W/W calculation for various composites |      |                       |                                                                                       |                |      |                     |         |        |                 |
|------------------------------------------------|------|-----------------------|---------------------------------------------------------------------------------------|----------------|------|---------------------|---------|--------|-----------------|
| based on                                       |      |                       | Ca[(F <sub>2</sub> )100-% + (Z)%],<br>where % is 0, or 2 or 5 or 10 or 15 or 20 or 25 |                |      |                     |         |        |                 |
|                                                |      | Z Formula Weight (FW) |                                                                                       |                |      | Mass/mole composite |         |        |                 |
|                                                | 25.0 | 15.0                  | 75.0                                                                                  | 28.5           | 40.0 | 83.5                | 47.9    | 34.1   | 18.0            |
|                                                |      |                       |                                                                                       |                |      |                     |         |        |                 |
| Ca/F/Hypoxanthine                              |      |                       |                                                                                       | F <sub>2</sub> | Ca   | PM                  | Ca% w/w | F% w/w | Hypo-xanthine % |
|                                                | %    | 135                   |                                                                                       | 38.0           | 40.0 |                     |         |        |                 |
|                                                | 0.0  | 0.0                   | 100.0                                                                                 | 38.0           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0             |
|                                                | 2.0  | 2.7                   | 98.0                                                                                  | 37.2           | 40.0 | 79.9                | 50.0    | 46.5   | 3.3             |
|                                                | 5.0  | 6.75                  | 95.0                                                                                  | 36.1           | 40.0 | 82.85               | 48.2    | 43.5   | 8.1             |
|                                                | 10.0 | 13.5                  | 90.0                                                                                  | 34.2           | 40.0 | 87.7                | 45.61   | 38.9   | 15.4            |
|                                                | 15.0 | 20.25                 | 85.0                                                                                  | 32.3           | 40.0 | 92.55               | 43.2    | 34.9   | 21.8            |
|                                                | 20.0 | 27.0                  | 80.0                                                                                  | 30.4           | 40.0 | 97.4                | 41.0    | 31.2   | 27.7            |
|                                                | 25.0 | 33.75                 | 75.0                                                                                  | 28.5           | 40.0 | 102.25              | 39.1    | 27.8   | 33.0            |
|                                                |      |                       |                                                                                       |                |      |                     |         |        |                 |
| Ca/F/Xanthine                                  |      |                       |                                                                                       | F <sub>2</sub> | Ca   | PM                  | Ca% w/w | F% w/w | Xanthine %      |
|                                                | %    | 151                   |                                                                                       | 38.0           | 40.0 |                     |         |        |                 |
|                                                | 0.0  | 0.0                   | 100.0                                                                                 | 38.0           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0             |
|                                                | 2.0  | 3.0                   | 98.0                                                                                  | 37.2           | 40.0 | 80.2                | 49.86   | 46.3   | 3.7             |
|                                                | 5.0  | 7.5                   | 95.0                                                                                  | 36.1           | 40.0 | 83.6                | 47.82   | 43.1   | 9.0             |
|                                                | 10.0 | 15.1                  | 90.0                                                                                  | 34.2           | 40.0 | 89.3                | 44.79   | 38.3   | 16.9            |
|                                                | 15.0 | 22.6                  | 85.0                                                                                  | 32.3           | 40.0 | 94.9                | 42.13   | 34.0   | 23.8            |
|                                                | 20.0 | 30.2                  | 80.0                                                                                  | 30.4           | 40.0 | 100.6               | 39.76   | 30.2   | 30.0            |
|                                                | 25.0 | 37.7                  | 75.0                                                                                  | 28.5           | 40.0 | 106.2               | 37.65   | 26.8   | 35.5            |
|                                                |      |                       |                                                                                       |                |      |                     |         |        |                 |
| Ca/F/Guanine                                   |      |                       |                                                                                       | F <sub>2</sub> | Ca   | PM                  | Ca% w/w | F% w/w | Guanine %       |
|                                                | %    | 150                   |                                                                                       | 38.0           | 40.0 |                     |         |        |                 |
|                                                | 0.0  | 0.0                   | 100.0                                                                                 | 38.0           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0             |

| Ca/F/Z %W/W calculation for various composites |      |                       |                                                                                       |                |      |                     |         |        |            |
|------------------------------------------------|------|-----------------------|---------------------------------------------------------------------------------------|----------------|------|---------------------|---------|--------|------------|
| based on                                       |      |                       | Ca[(F <sub>2</sub> )100-% + (Σ)%],<br>where % is 0, or 2 or 5 or 10 or 15 or 20 or 25 |                |      |                     |         |        |            |
|                                                |      | Z Formula Weight (FW) |                                                                                       |                |      | Mass/mole composite |         |        |            |
|                                                | 2.0  | 3.0                   | 98.0                                                                                  | 37.2           | 40.0 | 80.2                | 49.8    | 46.3   | 3.7        |
|                                                | 5.0  | 7.5                   | 95.0                                                                                  | 36.1           | 40.0 | 83.6                | 47.8    | 43.1   | 8.9        |
|                                                | 10.0 | 15.0                  | 90.0                                                                                  | 34.2           | 40.0 | 89.2                | 44.8    | 38.3   | 16.8       |
|                                                | 15.0 | 22.5                  | 85.0                                                                                  | 32.3           | 40.0 | 94.8                | 42.1    | 34.0   | 23.7       |
|                                                | 20.0 | 30.0                  | 80.0                                                                                  | 30.4           | 40.0 | 100.4               | 39.8    | 30.2   | 29.8       |
|                                                | 25.0 | 37.5                  | 75.0                                                                                  | 28.5           | 40.0 | 106                 | 37.7    | 26.8   | 35.3       |
|                                                |      |                       |                                                                                       |                |      |                     |         |        |            |
| Ca/F/Cytosine                                  |      |                       |                                                                                       | F <sub>2</sub> | Ca   | PM                  | Ca% w/w | F% w/w | Cytosine % |
|                                                | %    | 110                   |                                                                                       | 38.0           | 40.0 |                     |         |        |            |
|                                                | 0.0  | 0.0                   | 100.0                                                                                 | 38.0           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0        |
|                                                | 2.0  | 2.2                   | 98.0                                                                                  | 37.2           | 40.0 | 79.4                | 50.3    | 46.8   | 2.7        |
|                                                | 5.0  | 5.5                   | 95.0                                                                                  | 36.1           | 40.0 | 81.6                | 49.0    | 44.2   | 6.7        |
|                                                | 10.0 | 11.0                  | 90.0                                                                                  | 34.2           | 40.0 | 85.2                | 46.9    | 40.1   | 12.9       |
|                                                | 15.0 | 16.5                  | 85.0                                                                                  | 32.3           | 40.0 | 88.8                | 45.0    | 36.3   | 18.5       |
|                                                | 20.0 | 22.0                  | 80.0                                                                                  | 30.4           | 40.0 | 92.4                | 43.2    | 32.9   | 23.8       |
|                                                | 25.0 | 27.5                  | 75.0                                                                                  | 28.5           | 40.0 | 96.0                | 41.6    | 29.6   | 28.6       |
|                                                |      |                       |                                                                                       |                |      |                     |         |        |            |
| Ca/F/Thymine                                   |      |                       |                                                                                       | F <sub>2</sub> | Ca   | PM                  | Ca% w/w | F% w/w | Thymine %  |
|                                                | %    | 125                   |                                                                                       | 38.0           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0        |
|                                                | 0.0  | 0.0                   | 100.0                                                                                 | 38.0           | 40.0 | 79.7                | 50.1    | 46.6   | 3.1        |
|                                                | 2.0  | 2.5                   | 98.0                                                                                  | 37.2           | 40.0 | 82.3                | 48.5    | 43.8   | 7.5        |
|                                                | 5.0  | 6.25                  | 95.0                                                                                  | 36.1           | 40.0 | 86.7                | 46.1    | 39.4   | 14.4       |
|                                                | 10.0 | 12.5                  | 90.0                                                                                  | 34.2           | 40.0 | 91.0                | 43.9    | 35.4   | 20.6       |
|                                                | 15.0 | 18.7                  | 85.0                                                                                  | 32.3           | 40.0 | 95.4                | 41.9    | 31.8   | 26.2       |
|                                                | 20.0 | 25.0                  | 80.0                                                                                  | 30.4           | 40.0 | 99.7                | 40.1    | 28.5   | 31.3       |
|                                                | 25.0 | 31.2                  | 75.0                                                                                  | 28.5           | 40.0 | 78.0                | 51.3    | 48.7   | 0.0        |

In some aspects, the calcium fluoride compositions disclosed herein are pharmaceutically acceptable.

In some aspects, the calcium fluoride composites disclosed herein are in particulate form. In some aspects, the composite particles are in the nanoparticles or microparticles size range.

By "nanoparticles" is intended particles in the range of 1 nm - 999 nm, inclusive. Included within this definition are particles in the range of (A) between 50nm and 100nm, inclusive; between 45nm and 110nm, inclusive; between 40nm and 120nm, inclusive; between 35nm and 130nm, inclusive; between 30nm and 140nm, inclusive; between 25nm and 150nm, inclusive; between 20nm and 160nm, inclusive; between 15nm and 170nm, inclusive; between 10nm and 180nm, inclusive; (B) no less than 10nm, no less than 15nm, no less than 20nm, no less than 25nm; (C) no more than 150nm, no more than 200nm, no more than 250nm, no more than 300nm, no more than 350nm, no more than 400nm, no more than 450nm, no more than 500nm, no more than 550nm, no more than 600nm, no more than 650nm, no more than 700nm, no more than 750nm, no more than 800nm, no more than 850nm; or (D) roughly around 25 nm.

By "microparticles" is intended particles within the range of 1  $\mu\text{m}$  - 999  $\mu\text{m}$ , inclusive. Included within this definition are particles in the range of no more than 50 $\mu\text{m}$ , no more than 100 $\mu\text{m}$ , no more than 150 $\mu\text{m}$ , no more than 200 $\mu\text{m}$ , no more than 250 $\mu\text{m}$ , no more than 300 $\mu\text{m}$ , no more than 350 $\mu\text{m}$ , no more than 400 $\mu\text{m}$ , no more than 450 $\mu\text{m}$ , no more than 500 $\mu\text{m}$ , no more than 550 $\mu\text{m}$ , no more than 600 $\mu\text{m}$ , no more than 650 $\mu\text{m}$ , no more than 700 $\mu\text{m}$ , no more than 750 $\mu\text{m}$ , no more than 800 $\mu\text{m}$ , no more than 850 $\mu\text{m}$ , no more than 900 $\mu\text{m}$ , no more than 950 $\mu\text{m}$ .

In some aspects, the calcium fluoride compositions disclosed herein comprise more than one composite, where each composite comprises Ca, F, and Z as disclosed in the preceding paragraphs, and where each composite differs from the other by the percentage w/w of Ca, F, or Z, or by the chemical structure of Z.

In some aspects, the calcium fluoride compositions disclosed herein comprise an antigen, where the antigen is adsorbed to a calcium fluoride composite.

By "antigen" is intended a protein, polysaccharide, peptide, nucleic acid, protein-polysaccharide conjugates, molecule or hapten that is capable of raising an immune response in a human or animal. Antigens may be derived, homologous or

synthesized to mimic molecules from viruses, bacteria, parasites, protozoan or fungus. In an alternative embodiment of the invention the antigen derived, homologous or synthesized to mimic molecules from a tumor cell or neoplasia. In a further embodiment of the invention the antigen is derived, homologous or synthesized to mimic molecules from a substance implicated in allergy, Alzheimer's disease, atherosclerosis, obesity and nicotine-dependence.

Adsorption of albumin, chondroitin sulfate and glycoprotein onto calcium fluoride ( $\text{Ca F}_2$ ) was described Lindemann (1985) *Scandinavian Journal of Dental Research*, 93:381-83. Adhesion of microorganisms on  $\text{CaF}_2$  was reported Cheung et al. (2007) *Journal of applied Microbiology* 102:701-710. More recently, adsorption of ibuprofen on monodisperse  $\text{CaF}_2$  hollow nano-spheres was described Zhang et al. (2010) *Chem. Eur. J.* 16:5672-5680. Adsorptions of antigen on inorganic material are carried out by mixing antigen, in appropriate buffer, to a water suspension of the inorganic material in nano- or microparticle form. Optimization of time, temperature, pH, presence of salts and excipients are selected according to the conditions known or determined for the antigen. Depending of the nature and chemical composition of the antigen, at least one of the following adsorption mode of interaction may occur: adsorption by ligand exchange, by electrostatic forces or by hydrophobic forces. Antigen/inorganic ratio are optimized on a case per case basis. Available inorganic surface can be increased by using particles of smaller sizes.

In some aspects, antigens are adsorbed at room temperature over 2 hours under gentle agitation. In some aspects, the process of making a calcium fluoride composition comprises a step of adsorbing one or more antigens to the calcium fluoride composite during formation of the calcium fluoride composite. In some aspects, the process of making a calcium fluoride composition comprises a step of adsorbing one or more antigens to the calcium fluoride composite after formation of the calcium fluoride composite.

For various organic compounds (Z), the charge measured at the surface of the particle varies (see table 2A). This particle property may be utilized to optimize antigen adsorption by the electrostatic mode of interaction.

In some aspects, the calcium fluoride compositions disclosed herein are used in stabilizing an antigen. In some aspects of this use, the antigen is

thermostabilized. In some aspects of this use, the antigen is adsorbed to the calcium fluoride composite.

In some aspects, the calcium fluoride compositions disclosed herein are used in medicine. In some aspects, the calcium fluoride compositions disclosed herein are used in raising an immune response in a mammal. In some aspects, the calcium fluoride compositions disclosed herein are used in raising an immune response in a human. In some aspects, the calcium fluoride compositions disclosed herein are used in the prophylaxis and/or treatment of a mammal against disease caused by a virus, bacterium, or parasite. In some aspects, the calcium fluoride compositions disclosed herein are used in the prophylaxis and/or treatment of a human against disease caused by a virus, bacterium, or parasite. For such uses, the compositions disclosed herein may be delivered by administration to a subject in need thereof. Administration may be by a number of routes, including by delivery intramuscularly, subcutaneously, intradermally, sublingually, to the tonsils, or intranasally.

#### Processes for Making Compositions from Solid Particles

$\text{CaF}_2$  is available commercially. (Riedel de Haen™.) Pure  $\text{CaF}_2$  for use in the compositions disclosed herein may be prepared from solid  $\text{CaF}_2$  by the following scheme.

#### Scheme 1:

1.  $\text{CaF}_2$  solid particles are placed in a container.
2. Water is added.
3. The  $\text{CaF}_2$  + water is mixed.
4. The mixture is allowed to stand.
5. Up to or more than  $\frac{1}{2}$  of the supernatant is removed and replaced by water.
6. Steps 3 - 5 are repeated.
7.  $\text{CaF}_2$  solid is concentrated (by, e.g., centrifugation).

Composites for use in the compositions disclosed herein may be prepared by the following scheme. The starting constituents are available commercially.

Scheme 2:

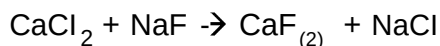
1. A solution comprising the selected compound Z is prepared.
2.  $\text{CaF}_2$  solid particles are added.
3. The  $\text{CaF}_2$  solid particles + solution comprising Z is mixed.
4. The mixture is allowed to stand.
5. Up to or more than  $\frac{1}{2}$  of the supernatant is removed and replaced by water.
8. Steps 3 - 5 are repeated.
6. The resulting solid is concentrated (by, e.g., centrifugation).

Processes for Making Compositions from Aqueous Solutions

Methods for synthesizing  $\text{CaF}_2$  from starting constituents in solution are known. For instance, preparation of nano-sized calcium fluoride by spray-drying following was reported by Sun et al. (2008) *Dental materials* 24:1 11-116, but this method has the disadvantage to use calcium hydroxide solution, which readily adsorbs  $\text{CO}_2$  from the air giving unwanted calcium carbonate contamination. See Kalinkin (2005) *Inorganic Materials* 41:1 1073-1 079. Nanoscale calcium fluoride may also be prepared according to Feldmann et al. (2006) *Small* 2:1 248-1 250, but this method has the disadvantage to use nitrate which even at trace level concentrations could be a problem for human injectable preparations.  $\text{CaF}_2$  may be synthesized by sol gel precipitation methodology.

Sol gel precipitation methodology is described in Nandiyanto et al. (2011) "Liquid-phase Synthesis of  $\text{CaF}_2$  Particles and Their Low Refractive Index Characterization" *KONA Powder and Particle Journal* 29:141-155. Nandiyanto indicates that certain parameters influence particle formation under the sol gel process. For instance, to influence the particle growing step, timing and temperature may be adjusted. Applicants modified the sol gel process by including washing steps as described generally in the following steps and in detail in the Examples. In some aspects, inclusion of washing steps is another way to decrease the particles growing step. It was observed that during washing by dilution, the concentration of starting materials was decreased. It is also expected that during washing, dilution of newly formed particles would occur.  $\text{CaF}_2$  for use in the compositions disclosed herein may be prepared by sol gel precipitation according to Reaction I as modified

by Scheme 3.



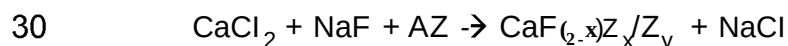
### Reaction I

#### 5 Scheme 3:

1. A solution comprising NaF is prepared (and sterilized by filtration). (NaF is available commercially.)
2. A solution comprising  $\text{CaC}^{\wedge}$  is prepared (and sterilized by filtration). ( $\text{CaCl}_2$  is available commercially.)
- 10 3. The solutions of step 1 and 2 are mixed.
4. The mixture is allowed to stand.
5. Up to or more than  $\frac{1}{2}$  of the supernatant is removed and replaced by water.
6. The retained liquid is mixed and the mixture is allowed to stand.
- 15 7. Steps 5 - 6 are repeated.
8. The resulting solid is concentrated (by, e.g., centrifugation).

Sol gel methodology was further modified for use in the present disclosure by the inclusion of Z in the reaction. In some aspects are provided processes for making a calcium fluoride composite by sol gel precipitation comprising the steps of combining  $\text{CaC}^{\wedge}$ , NaF, and NaZ under precipitating conditions and collecting the water insoluble calcium fluoride composite. In some aspects, the processes comprise a step of washing the calcium fluoride composite. In some aspects are provided processes for making a calcium fluoride composite by sol gel precipitation, comprising the steps of combining  $\text{CaCl}_2$ , NaF, and NaZ under precipitating conditions and collecting the water insoluble calcium fluoride composite.

Calcium fluoride composites for use in the compositions disclosed herein may be prepared according to Reaction II by following Scheme 4.



### Reaction II

where A is a metal, and x and y are as described in Formula I. In some aspects, A is Ca or Na.

Scheme 4:

1. A solution comprising the selected NaZ is prepared (and sterilized by filtration).
2. A solution comprising NaF is prepared (and sterilized by filtration).
- 5 3. A solution comprising  $\text{CaC}^{\wedge}$  is prepared (and sterilized by filtration).
4. The solutions of steps 1 and 2 are combined, then combined with the solution of step 3, then mixed.
5. The mixture is allowed to stand.
6. Up to or more than  $\frac{1}{2}$  of the supernatant is removed and replaced by
- 10 water.
7. The retained liquid is mixed and the mixture is allowed to stand.
8. Steps 6 - 7 are repeated.
9. The resulting solid is concentrated (by, e.g., centrifugation).

15 Alternatively, calcium fluoride composites for use in the compositions disclosed herein may be prepared according to Reaction II by following Scheme 5.

Scheme 5:

1. A solution comprising NaF is prepared (and sterilized by filtration).
2. A solution comprising  $\text{CaC}^{\wedge}$  and the selected organic Z is prepared
- 20 (and sterilized by filtration).
3. The solutions of steps 1 and 2 are mixed.
4. The mixture is allowed to stand.
5. Up to or more than  $\frac{1}{2}$  of the supernatant is removed and replaced by
- 25 water.
6. The retained liquid is mixed and the mixture is allowed to stand.
7. Steps 5 - 6 are repeated.
8. The resulting solid is concentrated (by, e.g., centrifugation).

30 Alternatively, calcium fluoride composites for use in the compositions disclosed herein may be prepared according to Reaction II by following Scheme 6.

Scheme 6:

1. A solution comprising NaF and the selected organic Z is prepared (and sterilized by filtration).

2. A solution comprising  $\text{CaC}^{\wedge}$  is prepared (and sterilized by filtration).
3. The solutions of steps 1 and 2 are mixed.
4. The mixture is allowed to stand.
5. Up to or more than  $\frac{1}{2}$  of the supernatant is removed and replaced by water.
6. The retained liquid is mixed and the mixture is allowed to stand.
7. Steps 5 - 6 are repeated.
8. The resulting solid is concentrated (by, e.g., centrifugation).

10 Alternatively, calcium fluoride composites for use in the compositions disclosed herein may be prepared using calcium ascorbate according to Scheme 7.

Scheme 7:

1. A solution comprising  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$  is prepared (and sterilized by filtration).
2. A solution comprising NaF is prepared (and sterilized by filtration).
3. The solutions of steps 1 and 2 are mixed.
4. The mixture is allowed to stand.
5. Up to or more than  $\frac{1}{2}$  of the supernatant is removed and replaced by water.
6. The retained liquid is mixed and the mixture is allowed to stand.
7. Steps 5 - 6 are repeated.
8. The resulting solid is concentrated (by, e.g., centrifugation).

25 In some aspects, the process of making a calcium fluoride composition comprises combining one or more antigens with  $\text{CaCl}_2$ , NaF, and NaZ under precipitating conditions. In some aspects, the process of making a calcium fluoride composition comprises a step of washing the calcium fluoride composite, wherein the washing step further comprises combining one or more antigens with the calcium fluoride composite. In some aspects, the process of making a calcium fluoride composition comprises a step of mixing the calcium fluoride composite with one or more antigens.

In some aspects, products made by the processes describe herein are

disclosed.

#### Adjuvant compositions

In some aspects are provided an adjuvant composition comprising a calcium fluoride composition as disclosed herein. By "adjuvant composition" is intended a calcium fluoride composition as disclosed herein that is capable of increasing an immune response against an antigen compared to administration of said antigen alone. In some aspects, adjuvant compositions as disclosed herein further comprise an immunostimulant.

In one aspect, this immunostimulant may be a saponin. A particularly suitable saponin for use in the present invention is Quil A and its derivatives. Quil A is a saponin preparation isolated from the South American tree *Quillaja Saponaria Molina* and was first described by Dalsgaard et al. in 1974 ("Saponin adjuvants", *Archiv. für die gesamte Virusforschung*, Vol. 44, Springer Verlag, Berlin, p243-254) to have adjuvant activity. Purified fragments of Quil A have been isolated by HPLC which retain adjuvant activity without the toxicity associated with Quil A (EP 0 362 278), for example QS7 and QS21 (also known as QA7 and QA21). QS-21 is a natural saponin derived from the bark of *Quillaja saponaria Molina*, which induces CD8+ cytotoxic T cells (CTLs), Th1 cells and a predominant IgG2a antibody response. QS21 is a preferred saponin in the context of the present invention.

In a suitable form of the present invention, the saponin adjuvant within the adjuvant composition is a derivative of *saponaria molina quil A*, preferably an immunologically active fraction of Quil A, such as QS-17 or QS-21, suitably QS-21.

In a specific aspect, QS21 is provided in its less reactogenic composition where it is quenched with an exogenous sterol, such as cholesterol for example. Several particular forms of less reactogenic compositions wherein QS21 is quenched with an exogenous cholesterol exist. In a specific aspect, the saponin /sterol is in the form of a liposome structure (WO 96/33739). In this aspect the liposomes suitably contain a neutral lipid, for example phosphatidylcholine, which is suitably non-crystalline at room temperature, for example egg yolk phosphatidylcholine, dioleoyl phosphatidylcholine (DOPC) or dilauryl phosphatidylcholine. The liposomes may also contain a charged lipid which increases the stability of the liposome-QS21 structure for liposomes composed of saturated lipids. In these cases the amount of

charged lipid is suitably 1-20% w/w, preferably 5-10%. The ratio of sterol to phospholipid is 1-50% (mol/mol), suitably 20-25%.

Suitable sterols include  $\beta$ -sitosterol, stigmasterol, ergosterol, ergocalciferol and cholesterol. In one particular aspect, the adjuvant composition comprises  
 5 cholesterol as sterol. These sterols are well known in the art, for example cholesterol is disclosed in the Merck Index, 11th Edn., page 341, as a naturally occurring sterol found in animal fat.

Where the active saponin fraction is QS21, the ratio of QS21 : sterol will typically be in the order of 1 : 100 to 1 : 1 (w/w), suitably between 1 : 10 to 1 : 1 (w/w),  
 10 and preferably 1 : 5 to 1 : 1 (w/w). Suitably excess sterol is present, the ratio of QS21 : sterol being at least 1 : 2 (w/w). In one aspect, the ratio of QS21 : sterol is 1 : 5 (w/w). The sterol is suitably cholesterol.

In another aspect, the adjuvant composition comprises an immunostimulant which is a Toll-like receptor 4 (TLR4) agonist. By "TLR agonist" it is meant a  
 15 component which is capable of causing a signaling response through a TLR signaling pathway, either as a direct ligand or indirectly through generation of endogenous or exogenous ligand (Sabroe et al, JI 2003 p1630-5). A TLR4 agonist is capable of causing a signally response through a TLR-4 signaling pathway. A suitable example of a TLR4 agonist is a lipopolysaccharide, suitably a non-toxic  
 20 derivative of lipid A, particularly monophosphoryl lipid A or more particularly 3-Deacylated monophosphoryl lipid A (3D - MPL).

3D-MPL is sold under the name MPL by GlaxoSmithKline Biologicals N.A. and is referred throughout the document as MPL or 3D-MPL. see, for example, US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094. 3D-MPL primarily  
 25 promotes CD4<sup>+</sup> T cell responses with an IFN- $\gamma$  (Th1) phenotype. 3D-MPL can be produced according to the methods disclosed in GB 2 220 211 A. Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 3, 4, 5 or 6 acylated chains. In the compositions of the present invention small particle 3D-MPL may be used to prepare the adjuvant composition. Small particle 3D-MPL has a particle size such  
 30 that it may be sterile-filtered through a 0.22  $\mu$ m filter. Such preparations are described in WO 94/21 292. Preferably, powdered 3D-MPL is used to prepare the adjuvant compositions of the present invention.

Other TLR4 agonists which can be used are aminoalkyl glucosaminide phosphates (AGPs) such as those disclosed in WO98/50399 or US patent No. 6,303, 347 (processes for preparation of AGPs are also disclosed), suitably RC527 or RC529 or pharmaceutically acceptable salts of AGPs as disclosed in US Patent  
5 No. 6,764,840. Some AGPs are TLR4 agonists, and some are TLR4 antagonists. Both are thought to be useful as immunostimulants.

Other suitable TLR-4 agonists are as described in WO2003/01 1223 and in WO 2003/099195, such as compound I, compound II and compound III disclosed on pages 4-5 of WO2003/01 1223 or on pages 3 - 4 of WO2003/0991 95 and in particular  
10 those compounds disclosed in WO2003/01 1223 as ER803022, ER803058, ER803732, ER804053, ER804057m ER804058, ER804059, ER804442, ER804680 and ER804764. For example, one suitable TLR-4 agonist is ER804057.

In a particular aspect, the adjuvant composition comprises both saponin and a TLR4 agonist. In a specific example, the adjuvant composition comprises QS21 and  
15 3D-MPL.

A TLR-4 agonist such as a lipopolysaccharide, such as 3D-MPL, can be used at amounts between 1 and 100µg per human dose of the adjuvant composition. 3D-MPL may be used at a level of about 50µg, for example between 40 to 60 µg, suitably between 45 to 55 µg or between 49 and 51 µg or 50µg. In a further aspect,  
20 the human dose of the adjuvant composition comprises 3D-MPL at a level of about 25µg, for example between 20 to 30µg, suitable between 21 to 29µg or between 22 to 28µg or between 28 and 27µg or between 24 and 26µg, or 25µg.

A saponin, such as QS21, can be used at amounts between 1 and 100µg per human dose of the adjuvant composition. QS21 may be used at a level of about  
25 50µg, for example between 40 - 60 µg, suitably between 45 to 55 µg or between 49 and 51 µg or 50µg. In a further aspect, the human dose of the adjuvant composition comprises QS21 at a level of about 25µg, for example between 20 to 30µg, suitable between 21 to 29µg or between 22 to 28µg or between 28 and 27µg or between 24 and 26µg, or 25µg.

Where both TLR4 agonist and saponin are present in the adjuvant composition, then the weight ratio of TLR4 agonist to saponin is suitably between 1 :5 to 5:1, suitably 1 :1. For example, where 3D-MPL is present at an amount of 50µg  
30

or 25µg, then suitably QS21 may also be present at an amount of 50µg or 25µg, respectively, per human dose of the adjuvant composition.

In one aspect, the immunostimulant is a TLR9 agonist, for example as set out in WO 2008/142133. In a specific example, said TLR9 agonist is an immunostimulatory oligonucleotide, in particular an oligonucleotide containing an unmethylated CpG motif. Such oligonucleotides are well known and are described, for example, in WO 96/02555, WO 99/33488 and US 5,865, 462. Suitable TLR9 agonists for use in the adjuvant compositions described herein are CpG containing oligonucleotides, optionally containing two or more dinucleotide CpG motifs separated by at least three, suitably at least six or more nucleotides. A CpG motif is a cytosine nucleotide followed by a Guanine nucleotide.

In one aspect the internucleotide bond in the oligonucleotide is phosphorodithioate, or possibly a phosphorothioate bond, although phosphodiester and other internucleotide bonds could also be used, including oligonucleotides with mixed internucleotide linkages. Methods for producing phosphorothioate oligonucleotides or phosphorodithioate are described in US5,666,153, US5,278,302 and WO95/26204. Oligonucleotide comprising different internucleotide linkages are contemplated, e.g. mixed phosphorothioate phosphodiester. Other internucleotide bonds which stabilize the oligonucleotide may be used.

Examples of CpG oligonucleotides suitable for inclusion in the adjuvant compositions described herein have the following sequences. In one aspect, these sequences contain phosphorothioate modified internucleotide linkages.

| CpG No.   | Sequence                                | SEQ ID NO |
|-----------|-----------------------------------------|-----------|
| 1826      | TCC ATG ACG TTC CTG ACG TT              | 1         |
| 1758      | TCT CCC AGC GTG CGC CAT                 | 2         |
| 1212      | ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG | 3         |
| 2006/7909 | TCG TCG TTT TGT CGT TTT GTC GTT         | 4         |
| 1668      | TCC ATG ACG TTC CTG ATG CT              | 5         |
| 5456      | TCG ACG TTT TCG GCG CGC GCC G           | 6         |

**Chart 2.** CpG oligos.

Alternative CpG oligonucleotides may comprise the sequences above in that they have inconsequential deletions or additions thereto.

In one aspect the immunostimulant is a tocol. Tocols are well known in the art and are described in EP0382271. In a particular aspect, the tocol is alpha-tocopherol

or a derivative thereof such as alpha-tocopherol succinate (also known as vitamin E succinate).

In one aspect, adjuvant compositions disclosed herein comprise an immunostimulant adsorbed to a calcium fluoride composite. In one aspect, adjuvant compositions comprise an immunostimulant adsorbed to a calcium fluoride composite, wherein said immunostimulant adsorbed to a calcium fluoride composite is MPL.

In one aspect is disclosed the adjuvant compositions disclosed herein for use in increasing the immune response against an antigen compared to an immune raised against said antigen when said antigen is administered with calcium fluoride composition or alone. In one aspect is disclosed the adjuvant compositions disclosed herein for use in increasing the immune response against an antigen compared to an immune raised against said antigen when said antigen is administered with calcium phosphate. The compositions disclosed herein may be delivered by administration to a subject in need thereof. Administration may be by a number of routes, including by delivery intramuscularly, subcutaneously, intradermally, sublingually, to the tonsils, or intranasally.

#### Processes for Making Adjuvant Compositions

In some aspects are disclosed processes for making an adjuvant composition as disclosed herein, comprising the steps of combining an immunostimulant with a calcium fluoride composite described herein. In some aspects are disclosed processes for making an adjuvant composition as disclosed herein, comprising the steps of adsorbing an antigen to a calcium fluoride composite as described herein.

#### Immunogenic Compositions

In some aspects are provided an immunogenic composition comprising an antigen and an adjuvant composition as described herein. In some aspects are provided an immunogenic composition as disclosed herein to be delivered intramuscularly, subcutaneously, intradermally, sublingually, to the tonsils, or intranasally.

In some aspects are provided an immunogenic composition as disclosed herein, where the composition wherein the pH of said composition is between about

pH5 and pH9. In some aspects are provided immunogenic compositions as disclosed herein that is suitable for human administration. In some aspects are provided an immunogenic composition as disclosed herein comprising one or more pharmaceutically acceptable excipients, in particular a buffer, a Tris buffer; or a histidine buffer. In some aspects are provided an immunogenic composition as disclosed herein, wherein the composition is prepared under aseptic conditions. In some aspects are provided an immunogenic composition as disclosed herein, wherein the composition is non-pyrogenic. In some aspects are provided an immunogenic composition as disclosed herein, where the composition is isotonic. In some aspects are provided an immunogenic composition as disclosed herein, where the composition comprises sugar or polyols.

In some aspects are provided an immunogenic composition as disclosed herein, where at least one antigen and at least one immunostimulant are adsorbed to a single type of composite as defined by percent w/w Ca, F, and Z and chemical structure of Z. In some aspects are provided an immunogenic composition as disclosed herein, where more than one antigen and more than one immunostimulant are adsorbed to a single type of composite as defined by percent w/w Ca, F, and Z and chemical structure of Z. In some aspects are provided an immunogenic composition as disclosed herein comprising at least a first and second type of composite as defined by percent w/w Ca, F, and Z and chemical structure of Z, wherein at least one antigen, at least one immunostimulant, or both, is adsorbed to said first type of composite, and wherein at least one antigen, at least one immunostimulant, or both, is adsorbed to said second type of composite. In some aspects are provided an immunogenic composition as disclosed herein comprising at least one composite as defined by percent w/w Ca, F, and Z and chemical structure of Z, wherein at least one antigen, at least one immunostimulant, or both, is adsorbed to said at least one composite, and wherein at least one antigen, at least one immunostimulant, or both, is adsorbed to a different metallic salt adjuvant. In some aspects, the second metallic salt adjuvant is calcium phosphate.

In some aspects there is provided immunogenic compositions disclosed herein for use in increasing the immune response against an antigen compared to an immune raised against said antigen when said antigen is administered with calcium fluoride composition or alone. In one aspect there is provided immunogenic

compositions disclosed herein for use in increasing the immune response against an antigen compared to an immune raised against said antigen when said antigen is administered with calcium phosphate. The compositions disclosed herein may be delivered by administration to a subject in need thereof. Administration may be by a number of routes, including by delivery intramuscularly, subcutaneously, intradermally, sublingually, to the tonsils, or intranasally.

#### Processes for Making Immunogenic Compositions

In some aspects are provided processes for making an immunogenic composition as disclosed herein, comprising the steps of combining a calcium fluoride composition described herein with an adjuvant composition disclosed herein.

#### Compositions and Methods for Reducing QS21 Reactogenicity

As described previously herein, QS21 is reactogenic and is typically provided in its less reactogenic composition (where it is quenched with an exogenous sterol, such as cholesterol for example). Several particular forms of less reactogenic compositions wherein QS21 is quenched with an exogenous cholesterol exist.

It was observed herein that QS21 combined with a calcium fluoride composition wherein Z is glutathione exhibits hemolytic activity levels (as measured by *in vitro* assay) comparable to the less compositions wherein QS21 is quenched with an exogenous cholesterol.

Accordingly, in some aspects are provided an adjuvant composition comprising a calcium fluoride composition as defined herein, wherein Z is glutathione, further comprising an immunologically active saponin fraction. In some aspects are provided an adjuvant composition, wherein the immunologically active saponin fraction is QS21. In some aspects are provided an adjuvant composition, wherein the QS21 is adsorbed to a calcium fluoride composite wherein Z is glutathione. In some aspects are provided an adjuvant composition wherein Z is glutathione for use in decreasing the hemolytic activity of QS21. In some aspects are provided a method for reducing the hemolytic activity of QS21 comprising the steps of combining QS21 with a calcium fluoride composite, wherein Z is glutathione. In some aspects are provided an immunogenic composition comprising an antigen and an adjuvant composition defined in the preceding paragraph. A suitable test to

determine haemolytic activity of immunologically active saponin fractions, including QS21, is described in Ronnberg et al. (1995) *Vaccine* 13:1 375-1 382.

#### Methods for Using Compositions

5           Methods are provided for the treatment or prevention of an infection or a disease caused by a virus, bacterium, or parasite in a mammal, said method comprising administering to said mammal a therapeutically effective amount of the calcium fluoride composition, the adjuvant composition, or the immunogenic composition described herein. Methods are provided for the treatment or prevention  
10 of an infection or a disease caused by a virus, bacterium, or parasite in a human, said method comprising administering to said human a therapeutically effective amount of the calcium fluoride composition, the adjuvant composition, or the immunogenic composition described herein.

          Methods are provided for inducing an immunogenic response in a mammal in  
15 need thereof, said method comprising administering to said mammal an effective amount of the calcium fluoride composition, the adjuvant composition, or the immunogenic composition described herein. Methods are provided for inducing an immunogenic response in a human in need thereof, said method comprising administering to said human an effective amount of the calcium fluoride composition,  
20 the adjuvant composition, or the immunogenic composition described herein.

          Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include  
25 plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. The term "plurality" refers to two or more. Additionally, numerical limitations given with respect to concentrations or levels of a substance, such as solution component concentrations or ratios thereof, and reaction conditions such as temperatures,  
30 pressures and cycle times are intended to be approximate. The term "about" used herein is intended to mean the amount  $\pm 10\%$ .

          The invention will be further described by reference to the following, non-limiting, figures and examples.

## EXAMPLES

Analytical Methods:*Equal Compensation Point*

5           Equal Compensation Point measurements: Measurements of Equal Compensation Point (E.C.P.) were carried out by potentiometric titration (J.R.Feldkamp et al., Journal of Pharmaceutical Sciences, 1981, Vol. 70, n°6 p 638-640). The results were presented in a global graph which is obtained by the juxtaposition of 4 different titration curves: two of them being measured in water and  
10   the two others measured in presence of various KCl (or KNO<sub>3</sub>) concentrations. For example in batch Ca/F/CO<sub>3</sub> # 88331 72A two Equal Compensation Point (E.C.P.) were obtained: 6.4 & 8.7 in the H<sub>2</sub>O/KCl system. In this case, below pH 6.4, the particle surface is charged negatively, between pH 6.4 and pH 8.7 the particle surface is charged positively, and above pH 8.7 the surface particle is charge  
15   negatively (Scheme 8). For comparison between the obtained E.C.P. H<sub>2</sub>O/KNO<sub>3</sub> values see Table 2A (herein below).

*Dry weight*

20           After homogenization of the suspension, an aliquot (10 ml) is evaporated to dry at 80°C during 5 days. The weight of the sample (in mg), represents the dry material quantity present in 10 ml of the suspension. This weight divided by ten represents the dry material quantity / ml of suspension.

*Infrared spectra*

25           The dry material (obtained as described herein) is hand ground and used as such for the infrared analysis. Few mg of sample were placed on the multi reflection holder of the Perkin Elmer FT-Infra Red instrument. Spectra were scanned in the % of transmittance mode from 4000cm<sup>-1</sup> to 600cm<sup>-1</sup>. It is interesting to note that organic material adsorbed on inorganic material give always very broad signals in  
30   infrared spectroscopy (compared to the pure organic material which gives very sharp signals).

          Examples of this were described for ibuprofen adsorbed on CaF<sub>2</sub> hollow sphere (C.Zhang et al., 2010, Chem. Eur. J. vol 16 p.5672-5680) or oleate

adsorbed fluorite (CaF<sub>2</sub>) (Handbook of Infrared Spectroscopy of Ultrathin Films. V.T. Tolstoy, I.V. Chernyshova and V.A. Skryshevsky. 2003 John Wiley & Sons, Inc.(page 552).

#### 5 *Nitrogen content by Antek*

Suspensions were injected without any other treatment in the Antek instrument. Therefore N concentrations were expressed in µgN/ml and represent the total N content found for both supernatant solution and adsorbed material. Analyses were also made on the last washing supernatant (W-10).

10

#### *Ca and F elementary analyses*

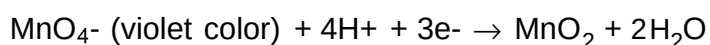
Suspensions containing 500mg dry material were filtered to recover the solid parts, which were calcinated. After mineralization, one part is used for Ca% determination (+/- 0.5 %) and the other part for F% determination (+/- 1%).

15

#### *Anti-Oxidant capacity*

Potassium permanganate (KMnO<sub>4</sub>), in presence of sulfuric acid solution, is one of the strongest oxidant. Violet permanganate anion is reduced to manganate oxide (MnO<sub>2</sub> brown color). This can further be reduced according to incolor Mn<sup>++</sup> cation, resulting in a 5 electrons exchange. In such conditions, most of organic matters were fully oxidized, while inorganic matters, such as CaF<sub>2</sub>, were insensitive.

20



25

Typically, five samples (0.1 ; 0.2; 0.3; 0.4 and 0.5ml of suspension) were placed in transparent polymeric container (avoid glass container when fluoride derivatives were placed in acid medium). To each of them 1ml of H<sub>2</sub>SO<sub>4</sub> 5M is added (add acid to water and never the reverse). Titration is carried out by drop by drop addition of KMnO<sub>4</sub> 1.0mM (violet color) until the discoloration persists during 3 minutes. Thus, anti-oxidant capacity, expressed in µI of KMnO<sub>4</sub> 1.0mM/ml of suspension, is obtained. Those values can be converted in µI of KMnO<sub>4</sub> 1.0mM/mg dry material. Similar titrations were carried out with known quantities of cysteine or N-acetyl-

30

cysteine solutions (1.0 mM). Thus, correlations between consumed quantities of  $\text{KMnO}_4$  and cysteine or N-acetyl-cysteine contents can be established. Furthermore, taking in account the weight of dry material present in the suspension, the quantities of organic materials (cysteine or N-acetyl-cysteine) per dry material can be  
 5 calculated and expressed in % w/w oxidizable organic material /dry weight.

### *Commercially available chemicals*

The following commercial products have been used:

- $\text{CaCO}_3$  solid: OMYA™ product OMYAPURE 35;
- 10 ▪  $\text{CaCO}_3$  solid particles: Sigma-Aldrich product 12010;
- Sodium fluoride: Merck™ product 1064490250;
- Calcium chloride: Merck™ product n° 1023780500;
- Cysteine: Aldrich™ product 168149; Cysteine: Merck product 1028380100;
- N-Acetyl-Cysteine: Sigma™ product A5099;
- 15 ▪ Thioglycerol: Sigma™ product 88640;
- Phosphoethanolamine: Sigma™ product P0503-100;
- Calcium fluoride: Riedel de Haen™ product 01123;
- Sodium bicarbonate: Merck™ product 1063295000;
- Sodium carbonate: Merck™ product 1063981000;
- 20 ▪ Calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ): Merck™ product 2382;
- Calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ): Sigma Aldrich™ product 12022;
- Disodium hydrogenophosphate dihydrate: Merck™ product 1065805000;
- Tri-sodium citrate: Merck™ product 1110371000;
- Sodium hydroxide: Merck™ product 1064981000;
- 25 ▪ Calcium ascorbate: Fluka™ product 11138;
- Glutathione: Merck™ product 104090.0050;
- Glutathione oxide: Sigma™ product G46265G;
- Thiolactic acid: Sigma Aldrich™ product T3,100-3;
- Adipic acid: Carlo ERBA™ product 401785;
- 30 ▪ Uric acid: Fluka™ product 51449;
- Calcium chloride hexahydrate: Merck™ product 102072.1000;
- Folic acid: Fluka™ product 01769;
- Hypoxanthine: Fluka product 56700

- Xanthine: Sigma™ product X7375-256
- Guanine: Aldrich™ product G 11950-1 00G
- Cytosine: Fluka™ product 30430
- Thymine: Sigma™ product T0376-5G.

5

#### Example 1. Characteristics of Sol-Gel Formations of Calcium Fluoride

Calcium Fluoride composites were formed and characterized by various methods. The results of this characterization are summarized in this example. The details of the formation of each batch mentioned in Example 1 may be found in

10 Example 2.

| <b>Table 1: Example of Calcium fluoride composites</b>                                                                                                                                                                                                                                                                 |                |            |                         |             |                       |                     |                  |                         |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|------------|-------------------------|-------------|-----------------------|---------------------|------------------|-------------------------|
| Quantities of NaF, CaCl <sub>2</sub> , and organic materials: column 3,4, and 5 respectively.                                                                                                                                                                                                                          |                |            |                         |             |                       |                     |                  |                         |
| Volume of the starting mix = Column 6. Final volume after concentration by centrifugation: column 7. Concentration of dry material: column 8. Total weight (column 9) is defined as the product of the concentration (mg dry material/ml column 8) by total volume at the final stage of preparation (ml in column 7). |                |            |                         |             |                       |                     |                  |                         |
| 1                                                                                                                                                                                                                                                                                                                      | 2              | 3          | 4                       | 5           | 6                     | 7                   | 8                | 9                       |
|                                                                                                                                                                                                                                                                                                                        | <b>Batch #</b> | <b>NaF</b> | <b>CaCl<sub>2</sub></b> | <b>Org.</b> | <b>Started volume</b> | <b>Final volume</b> | <b>Dry conc.</b> | <b>Total dry weight</b> |
|                                                                                                                                                                                                                                                                                                                        |                | <b>g</b>   |                         |             | <b>ml</b>             | <b>ml</b>           | <b>mg/ml</b>     | <b>g</b>                |
| <b>CaF<sub>2</sub></b>                                                                                                                                                                                                                                                                                                 |                |            |                         |             |                       |                     |                  |                         |
| CaF <sub>2</sub>                                                                                                                                                                                                                                                                                                       | 8833172D       | 1.68       | 2.22                    |             | 200                   | 35                  | 25.2             | 0.882                   |
| CaF <sub>2</sub>                                                                                                                                                                                                                                                                                                       | 8833190        | 8.42       | 11.11                   |             | 1000                  | 160                 | 33.21            | 5.313                   |
| CaF <sub>2</sub>                                                                                                                                                                                                                                                                                                       | 9440194        | 8.42       | 11.1                    |             | 1000                  | 160                 | 26.30            | 4.208                   |
| <b>Ca/F/OH</b>                                                                                                                                                                                                                                                                                                         |                |            |                         |             |                       |                     |                  |                         |
| CaF/OH                                                                                                                                                                                                                                                                                                                 | 11000123       | 4.2        | 11.11                   |             | 1000                  | 110                 | 14.25            | 1.567                   |
| <b>Ca/F /CO<sub>3</sub></b>                                                                                                                                                                                                                                                                                            |                |            |                         |             |                       |                     |                  |                         |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                  | 8833152        | 0.168      | 2.22                    | 1.908       | 200                   | 35                  | 45.2             | 1.582                   |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                  | 8833153        | 0.504      | 2.22                    | 1.484       | 200                   | 35                  | 41.7             | 1.459                   |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                  | 8833154        | 0.841      | 2.22                    | 1.060       | 200                   | 35                  | 40.5             | 1.417                   |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                  | 8833155        | 1.178      | 2.22                    | 0.636       | 200                   | 35                  | 42.7             | 1.494                   |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                  | 8833156        | 1.514      | 2.22                    | 0.212       | 200                   | 35                  | 39.2             | 1.372                   |

**Table 1: Example of Calcium fluoride composites**

| Quantities of NaF, CaC <sup>^</sup> , and organic materials: column 3,4, and 5 respectively.<br>Volume of the starting mix = Column 6. Final volume after concentration by centrifugation: column 7. Concentration of dry material: column 8. Total weight (column 9) is defined as the product of the concentration (mg dry material/ml column 8) by total volume at the final stage of preparation (ml in column 7). |           |       |                   |        |                |              |           |                  |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|-------|-------------------|--------|----------------|--------------|-----------|------------------|
| 1                                                                                                                                                                                                                                                                                                                                                                                                                      | 2         | 3     | 4                 | 5      | 6              | 7            | 8         | 9                |
|                                                                                                                                                                                                                                                                                                                                                                                                                        | Batch #   | NaF   | CaCl <sub>2</sub> | Org.   | Started volume | Final volume | Dry cone. | Total dry weight |
|                                                                                                                                                                                                                                                                                                                                                                                                                        |           | g     |                   |        | ml             | ml           | mg/ml     | g                |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                                                                                                                  | 88331 57  | 1.598 | 2.22              | 0.1 06 | 200            | 35           | 16.0      | 0.560            |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                                                                                                                  | 88331 72A | 1.51  | 2.22              | 0.1 68 | 200            | 35           | 8.28      | 0.289            |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                                                                                                                  | 88331 72B | 1.59  | 2.22              | 0.084  | 200            | 35           | 9.37      | 0.328            |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                                                                                                                  | 88331 72C | 1.66  | 2.22              | 0.01 6 | 200            | 35           | 18.5      | 0.647            |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                                                                                                                  | 94401 95  | 7.57  | 11.10             | 0.84   | 1000           | 160          | 14.6      | 2.336            |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                                                                                                                  | 99231 23  | 7.58  | 11.13             | 0.84   | 1000           | 160          | 16.27     | 2.603            |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                                                                                                                  | 99231 24  | 7.58  | 11.11             | 0.84   | 1000           | 160          | 19.67     | 3.147            |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                                                                                                                  | 11000080  | 0.84  | 11.1              | 7.70   | 1000           | 120          | 110.28    | 13.233           |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                                                                                                                  | 11000081  | 4.2   | 11.1              | 4.28   | 1000           | 170          | 40.79     | 6.934            |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                                                                                                                  | 11000082  | 5.88  | 11.1              | 2.57   | 1000           | 200          | 33.73     | 6.746            |
| Ca/F /CO <sub>3</sub>                                                                                                                                                                                                                                                                                                                                                                                                  | 11000083  | 7.56  | 11.1              | 0.86   | 1000           | 180          | 23.56     | 4.241            |
| <b>Ca/F /Ascorbate</b>                                                                                                                                                                                                                                                                                                                                                                                                 |           |       |                   |        |                |              |           |                  |
| Ca/F /Ascorbate                                                                                                                                                                                                                                                                                                                                                                                                        | 94401 98  | 4.20  | Ca ascor.<br>42.5 |        | 1000           | 250          | 13.5      | 3.375            |
| <b>Ca/F/ Cysteine</b>                                                                                                                                                                                                                                                                                                                                                                                                  |           |       |                   |        |                |              |           |                  |
| Ca/F/ Cysteine *                                                                                                                                                                                                                                                                                                                                                                                                       | 9440055   | 0.84  | 2.22              | 2.42   | 200            | 35           | 10.81     | 0.378            |
| Ca/F/ Cysteine *                                                                                                                                                                                                                                                                                                                                                                                                       | 9440056   | 0.84  | 2.22              | 2.42   | 200            | 35           | 13.20     | 0.462            |
| Ca/F/ Cysteine *                                                                                                                                                                                                                                                                                                                                                                                                       | 9440057   | 0.84  | 2.22              | 2.42   | 200            | 35           | 17.08     | 0.597            |
| Ca/F/ Cysteine *                                                                                                                                                                                                                                                                                                                                                                                                       | 9440058   | 0.84  | 2.22              | 2.42   | 200            | 35           | 8.72      | 0.305            |
| Ca/F/ Cysteine *                                                                                                                                                                                                                                                                                                                                                                                                       | 9440099   | 4.23  | 11.15             | 12.1 1 | 1000           | 160          | 20.84     | 3.334            |
| Ca/F/ Cysteine *                                                                                                                                                                                                                                                                                                                                                                                                       | 94401 97  | 4.21  | 11.10             | 12.1 0 | 1000           | 160          | 20.00     | 3.200            |

**Table 1: Example of Calcium fluoride composites**

| Quantities of NaF, CaC <sup>^</sup> , and organic materials: column 3,4, and 5 respectively.<br>Volume of the starting mix = Column 6. Final volume after concentration by centrifugation: column 7. Concentration of dry material: column 8. Total weight (column 9) is defined as the product of the concentration (mg dry material/ml column 8) by total volume at the final stage of preparation (ml in column 7). |           |       |                   |       |                |              |           |                  |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|-------|-------------------|-------|----------------|--------------|-----------|------------------|
| 1                                                                                                                                                                                                                                                                                                                                                                                                                      | 2         | 3     | 4                 | 5     | 6              | 7            | 8         | 9                |
|                                                                                                                                                                                                                                                                                                                                                                                                                        | Batch #   | NaF   | CaCl <sub>2</sub> | Org.  | Started volume | Final volume | Dry cone. | Total dry weight |
|                                                                                                                                                                                                                                                                                                                                                                                                                        |           | g     |                   |       | ml             | ml           | mg/ml     | g                |
| <b>Ca/F/N-Ac-Cyst.</b>                                                                                                                                                                                                                                                                                                                                                                                                 |           |       |                   |       |                |              |           |                  |
| Ca/F/N-Ac-Cyst.                                                                                                                                                                                                                                                                                                                                                                                                        | 94401 10  | 4.20  | 11.10             | 16.37 | 1000           | 160          | 10.36     | 1.657            |
| Ca/F/N-Ac-Cyst.                                                                                                                                                                                                                                                                                                                                                                                                        | 94401 96  | 4.20  | 11.1 1            | 16.30 | 1000           | 160          | 9.34      | 1.494            |
| Ca/F/N-Ac-Cyst.                                                                                                                                                                                                                                                                                                                                                                                                        | 106161 25 | 4.20  | 11.13             | 16.33 | 1000           | 160          | 9.30      | 1.488            |
| Ca/F/N-Ac-Cyst.                                                                                                                                                                                                                                                                                                                                                                                                        | 110001 01 | 4.21  | 11.1 7            | 16.34 | 1000           | 150          | 14.9      | 2.235            |
| <b>Ca/F/Glutathione</b>                                                                                                                                                                                                                                                                                                                                                                                                |           |       |                   |       |                |              |           |                  |
| CaF/Glutathione                                                                                                                                                                                                                                                                                                                                                                                                        | 106161 85 | 4.21  | 11.10             | 30.7  | 1000           | 250          | 18.43     | 4.607            |
| CaF/Glutathione                                                                                                                                                                                                                                                                                                                                                                                                        | 11000030  | 4.22  | 11.12             | 3.07  | 1000           | 160          | 27.2      | 4.352            |
| CaF/Glutathione                                                                                                                                                                                                                                                                                                                                                                                                        | 11000033  | 4.21  | 11.12             | 30.7  | 1000           | 175          | 23.5      | 4.1 12           |
| CaF/Glutathione                                                                                                                                                                                                                                                                                                                                                                                                        | 11000086  | 4.22  | 11.15             | 3.07  | 1000           | 170          | 24.76     | 4.209            |
| CaF/Glutathione                                                                                                                                                                                                                                                                                                                                                                                                        | 11000099  | 4.1 1 | 11.12             | 3.07  | 1000           | 180          | 23.1 9    | 4.1 74           |
| CaF/Glutathione                                                                                                                                                                                                                                                                                                                                                                                                        | 110001 94 | 4.20  | 11.13             | 3.09  | 1000           | 200          | 22.02     | 4.404            |
| <b>Ca/F/Glutathione oxide</b>                                                                                                                                                                                                                                                                                                                                                                                          |           |       |                   |       |                |              |           |                  |
| CaF/Glutathi. Oxi                                                                                                                                                                                                                                                                                                                                                                                                      | 106161 98 | 0.4   | 1.1 1             | 6.56  | 100            | 50           | 11.0      | 0.550            |
| CaF/Glutathi. Oxi                                                                                                                                                                                                                                                                                                                                                                                                      | 110001 39 | 4.20  | 11.15             | 3.22  | 1000           | 220          | 22.1 2    | 4.866            |
| CaF/Glutathi. Oxi                                                                                                                                                                                                                                                                                                                                                                                                      | 11000140  | 4.20  | 11.1 1            | 6.56  | 1000           | 220          | 22.6      | 4.972            |
| <b>Ca/F/thiolactate</b>                                                                                                                                                                                                                                                                                                                                                                                                |           |       |                   |       |                |              |           |                  |
| Ca/F/Thiolactate                                                                                                                                                                                                                                                                                                                                                                                                       | 11000031  | 4.21  | 11.12             | 10.6  | 1000           | 170          | 23.8      | 4.046            |
| Ca/F/Thiolactate                                                                                                                                                                                                                                                                                                                                                                                                       | 11000059  | 4.21  | 11.1 7            | 10.6  | 1000           | 160          | 10.6      | 1.696            |
| Ca/F/Thiolactate                                                                                                                                                                                                                                                                                                                                                                                                       | 11000060  | 4.1 2 | 11.1 7            | 10.6  | 1000           | 200          | 19.3      | 3.860            |
| <b>Ca/F/Adipate</b>                                                                                                                                                                                                                                                                                                                                                                                                    |           |       |                   |       |                |              |           |                  |

**Table 1: Example of Calcium fluoride composites**

| Quantities of NaF, CaCl <sub>2</sub> , and organic materials: column 3,4, and 5 respectively.<br>Volume of the starting mix = Column 6. Final volume after concentration by centrifugation: column 7. Concentration of dry material: column 8. Total weight (column 9) is defined as the product of the concentration (mg dry material/ml column 8) by total volume at the final stage of preparation (ml in column 7). |          |      |                             |       |                |              |           |                  |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|------|-----------------------------|-------|----------------|--------------|-----------|------------------|
| 1                                                                                                                                                                                                                                                                                                                                                                                                                       | 2        | 3    | 4                           | 5     | 6              | 7            | 8         | 9                |
|                                                                                                                                                                                                                                                                                                                                                                                                                         | Batch #  | NaF  | CaCl <sub>2</sub>           | Org.  | Started volume | Final volume | Dry conc. | Total dry weight |
|                                                                                                                                                                                                                                                                                                                                                                                                                         |          | g    |                             |       | ml             | ml           | mg/ml     | g                |
| Ca/F/Adipate                                                                                                                                                                                                                                                                                                                                                                                                            | 11000129 | 4.19 | 11.14                       | 7.3   | 1000           | 150          | 29.9      | 4.485            |
| Ca/F/Adipate                                                                                                                                                                                                                                                                                                                                                                                                            | 11481026 | 4.19 | 11.12                       | 7.31  | 1000           | 174          | 26.32     | 4.579            |
| Ca/F/Adipate                                                                                                                                                                                                                                                                                                                                                                                                            | 11481027 | 4.19 | 11.11                       | 7.31  | 1000           | 142          | 31.61     | 4.488            |
| <b>Ca/F/Urate</b>                                                                                                                                                                                                                                                                                                                                                                                                       |          |      |                             |       |                |              |           |                  |
| Ca/F/Urate                                                                                                                                                                                                                                                                                                                                                                                                              | 11000182 | 4.16 | 21.99<br>.6H <sub>2</sub> O | 0.132 | 1000           | 150          | 12.89     | 1.933            |
| <b>Ca/F/Folic acid</b>                                                                                                                                                                                                                                                                                                                                                                                                  |          |      |                             |       |                |              |           |                  |
| Ca/F/Folic acid                                                                                                                                                                                                                                                                                                                                                                                                         | 11481018 | 4.20 | 11.12                       | 0.448 | 1000           | 250          | 15.01     | 3.752            |
| <b>Ca/F/Hypoxanthine</b>                                                                                                                                                                                                                                                                                                                                                                                                |          |      |                             |       |                |              |           |                  |
| Ca/F/Hypoxanthine                                                                                                                                                                                                                                                                                                                                                                                                       | 11481198 | 4.21 | 14.73                       | 1.36  | 1000           | 200          | 13.09     | 2.618            |
| <b>Ca/F/Xanthine</b>                                                                                                                                                                                                                                                                                                                                                                                                    |          |      |                             |       |                |              |           |                  |
| Ca/F/Xanthine                                                                                                                                                                                                                                                                                                                                                                                                           | 11481199 | 4.22 | 14.71                       | 1.52  | 1000           | 200          | 20.3      | 4.060            |
| <b>Ca/F/Guanine</b>                                                                                                                                                                                                                                                                                                                                                                                                     |          |      |                             |       |                |              |           |                  |
| Ca/F/Guanine                                                                                                                                                                                                                                                                                                                                                                                                            | 11481195 | 4.21 | 14.71                       | 1.52  | 1000           | 180          | 21.58     | 3.884            |
| <b>Ca/F/Cytosine</b>                                                                                                                                                                                                                                                                                                                                                                                                    |          |      |                             |       |                |              |           |                  |
| Ca/F/Cytosine                                                                                                                                                                                                                                                                                                                                                                                                           | 11954009 | 4.22 | 14.73                       | 1.10  | 1000           | 100          | 13.74     | 1.374            |
| <b>Ca/F/Thymine</b>                                                                                                                                                                                                                                                                                                                                                                                                     |          |      |                             |       |                |              |           |                  |
| Ca/F/Thymine                                                                                                                                                                                                                                                                                                                                                                                                            | 11954064 | 4.22 | 14.75                       | 1.26  | 1000           | 180          | 9.8       | 1.767            |

Sol-gel formation allows one to influence the particle size by, for example, varying concentrations of starting solutions as disclosed in Nandiyanto. The use of various selected organic compounds in solutions allows one to obtain composite

particles possessing different surface charges (measured by their E.C.P. values, Table 2A).

**Table 2A: Starting solutions pH and surface charge (E.C.P.) of calcium fluoride composites.**

| Starting compounds     | Batch #  | Starting pH of each solutions and →pH of first wash | E.C.P.<br>H <sub>2</sub> O/KNO <sub>3</sub> |
|------------------------|----------|-----------------------------------------------------|---------------------------------------------|
| CaF <sub>2</sub>       | 8833190  | 7.15 + 7.11 → 9.44                                  | 6.9                                         |
|                        | 9440194  | 9.92 + 10.04 → 9.78                                 | 6.4                                         |
| Ca/F/N-Ac-Cysteine     | 9440110  | 8.35 + 9.26 → 8.47                                  | 7.3                                         |
|                        | 9440196  | 9.66 + 8.35 → 8.44                                  | 7.3                                         |
|                        | 10616125 | 9.01 + 8.30 → 8.44                                  | 7.3                                         |
| Ca/F/Ascorbate         | 9440198  | 9.08 + 9.49 → 6.65                                  | 7.3                                         |
| Ca/F/CO <sub>3</sub>   | 9440195  | 7.29 + 7.96 → 6.38                                  | 8.6                                         |
|                        | 9923123  | 7.25 + 7.72 → 6.43                                  | 8.3                                         |
|                        | 9923124  | 7.27 + 7.70 → 6.46                                  | 8.3                                         |
| Ca/F/Cysteine          | 9440099  | 8.22 + 9.22 → 8.27                                  | 8.4                                         |
|                        | 9440197  | 9.62 + 8.21 → 8.31                                  | 8.4                                         |
| Ca/F/Uric acid         | 11000182 | 8.60 + 6.24 → 5.39                                  | 8.4                                         |
| Ca/F/Glutathione oxide | 11000139 | 9.82 + 8.21 → 7.84                                  | 9.3                                         |
|                        | 11000140 | 9.82 + 8.12 → 8.07                                  | 9.8                                         |
| Ca/F/Glutathione       | 11000033 | 6.94 + 7.04 → 7.07                                  | 8.8                                         |
|                        | 10616185 | 8.90 + 8.57 → 8.74                                  | 9.1                                         |
|                        | 11000030 | 9.47 + 8.59 → 8.51                                  | 9.2                                         |
| Ca/F/Thiolactate       | 11000059 | 7.16 + 6.97 → 6.17                                  | 6.1                                         |
|                        | 11000031 | 9.56 + 9.54 → 9.73                                  | 6.7                                         |
|                        | 11000060 | 7.93 + 8.07 → 7.93                                  | 7.1                                         |
|                        | 11481063 | 9.15 + 8.50 → 8.87                                  | 6.9                                         |
|                        | 11481062 | 8.93 + 9.49 → 10.14                                 | 7.8                                         |
| Ca/Adipate             | 11000129 | 8.02 + 10.81 → 7.97                                 | 6.6                                         |
|                        | 11481044 | 6.99 + 9.51 → 6.93                                  | 7.2                                         |
|                        | 11481027 | 7.18 + 9.82 → 6.93                                  | 7.5                                         |
|                        | 11481059 | 7.26 + 5.76 → 7.05                                  | 7.9                                         |
|                        | 11481066 | 7.26 + 9.69 → 7.20                                  | 8.1                                         |

| Starting compounds  | Batch #   | Starting pH of each solutions and → pH of first wash | E.C.P.<br>H <sub>2</sub> O/KNO <sub>3</sub> |
|---------------------|-----------|------------------------------------------------------|---------------------------------------------|
| Ca/F/Thioglycollate | 11000032  | 9.57 + 11.49 → 11.04                                 | 10.4                                        |
| Ca/F/Hypoxanthine   | 11481 198 | 9.83 + 9.31 → 9.1 2                                  | 8.1                                         |
| Ca/F/Xanthine       | 11481 199 | 10.75 + 9.39^9.05                                    | 10.1                                        |
| Ca/F/Guanine        | 11481 195 | 12.52 + 8.48^1 1.66                                  | 6.8                                         |
| Ca/F/Cytosine       | 11954009  | 9.03 + 9.1 8 ^6.92                                   | 7.6                                         |
| Ca/F/Thymine        | 11954064  | 9.1 1 + 8.31 -> 8.66                                 | 8.5                                         |

When carbonate was simultaneously used during CaF<sub>2</sub> precipitation, Ca/F/CO<sub>3</sub> composite particles were obtained. By varying the relative quantities of bicarbonate or carbonate, composites exhibiting different surface properties were obtained (Table 2B).

**Table 2B: Comparison of E.C.P. values in the 8833172A-D series**

| Batches         | HCO <sub>3</sub> <sup>-</sup> / F <sup>-</sup> / Ca <sup>++</sup><br>mole ratio | % of CO <sub>3</sub><br>by titration | E.C.P.<br>H <sub>2</sub> O/KCl | E.C.P.<br>H <sub>2</sub> O/KNO <sub>3</sub> |
|-----------------|---------------------------------------------------------------------------------|--------------------------------------|--------------------------------|---------------------------------------------|
| <b>8833172A</b> | 2/36/20                                                                         | 3.5                                  | 6.4 and 8.7                    | 8.7                                         |
| <b>8833172B</b> | 1/38/20                                                                         | 1.1                                  | 6.3 and 8.4                    | 8.4                                         |
| <b>8833172C</b> | 0.2/39.6/20                                                                     | 0.09                                 | 5.1 and 9.1                    | 7.0                                         |
| <b>8833172D</b> | 0.0/40/20                                                                       | 0                                    | 7.8                            | 6.6                                         |

For low carbonate molar ratios the formed composite exhibits carbonate of the vaterite type. This was a surprise since other described vaterite formations indicate that high concentrations (CaCl<sub>2</sub> 1M + K<sub>2</sub>CO<sub>3</sub> 1M) were needed to obtain vaterite type of carbonate Mori et al. (2009) *Materials Science and Engineering* 29:1409-1414 and Parkin et al. (2009) *Optics Express* 17:21 944-21 955. The vaterite type of carbonate obtained by the method disclosed herein is of importance for adsorption of organic material possessing immunological properties (see experimental part: adsorption of MPL).

When cysteine was used during precipitation, Ca/F/cysteine composite particles were obtained. By varying the order of addition, composites exhibiting different surface properties were obtained (Table 3).

| <b>Table 3: Precipitations in presence Cysteine</b>        |                                                                       |         |                                                                       |         |                                                                       |         |                                                                       |         |
|------------------------------------------------------------|-----------------------------------------------------------------------|---------|-----------------------------------------------------------------------|---------|-----------------------------------------------------------------------|---------|-----------------------------------------------------------------------|---------|
| <b>(NaF + Cys) pH 8.18<br/>+ CaCl<sub>2</sub> pH 10.03</b> |                                                                       |         | <b>(CaCl<sub>2</sub> + Cys)<br/>pH 8.18 + NaF<br/>pH 8.78</b>         |         | <b>NaF pH 8.98 +<br/>(CaCl<sub>2</sub> + Cys)<br/>pH 8.19</b>         |         | <b>CaCl<sub>2</sub> pH 10.04<br/>+<br/>(NaF + Cys)<br/>pH 8.19</b>    |         |
| <b>Batch # 9440055</b>                                     |                                                                       |         | <b>Batch # 9440056</b>                                                |         | <b>Batch # 9440057</b>                                                |         | <b>Batch #<br/>9440058</b>                                            |         |
| Washing                                                    | pH                                                                    | mOsm/kg | pH                                                                    | mOsm/kg | pH                                                                    | mOsm/kg | pH                                                                    | mOsm/kg |
| W1                                                         | 8.03                                                                  | 409     | 8.25                                                                  | 411     | 8.26                                                                  | 405     | 8.05                                                                  | 410     |
| W2                                                         | 8.14                                                                  | 149     | 8.37                                                                  | 153     | 8.37                                                                  | 142     | 8.16                                                                  | 148     |
| W3                                                         | 8.19                                                                  | 69      | 8.42                                                                  | 71      | 8.44                                                                  | 65      | 8.27                                                                  | 71      |
| W4                                                         | 8.18                                                                  | 33      | 8.44                                                                  | 34      | 8.48                                                                  | 31      | 8.19                                                                  | 34      |
| W5                                                         | 8.36                                                                  | 16      | 8.84                                                                  | 25      | 8.85                                                                  | 15      | 8.36                                                                  | 17      |
| W6                                                         | 8.43                                                                  | 8       | 8.86                                                                  | 12      | 8.89                                                                  | 7       | 8.47                                                                  | 7       |
| W7                                                         | 8.47                                                                  | 4       | 8.91                                                                  | 3       | 8.91                                                                  | 3       | 8.51                                                                  | 3       |
| W8                                                         | 8.32                                                                  | 2       | 8.58                                                                  | 2       | 8.56                                                                  | 2       | 8.34                                                                  | 2       |
| W9                                                         | 8.15                                                                  | 1       | 8.33                                                                  | 1       | 8.48                                                                  | 1       | 8.36                                                                  | 1       |
| W10                                                        | 8.07                                                                  | 1       | 8.17                                                                  | 2       | 8.12                                                                  | 1       | 8.07                                                                  | 2       |
| Cone.                                                      | 10.81 mg/ml                                                           |         | 13.20 mg/ml                                                           |         | 17.08 mg/ml                                                           |         | 8.72 mg/ml                                                            |         |
| E.C.P.                                                     | H <sub>2</sub> O/KCl = 8.6<br>H <sub>2</sub> O/KNO <sub>3</sub> = 8.5 |         | H <sub>2</sub> O/KCl = 8.8<br>H <sub>2</sub> O/KNO <sub>3</sub> = 8.6 |         | H <sub>2</sub> O/KCl = 8.9<br>H <sub>2</sub> O/KNO <sub>3</sub> = 8.5 |         | H <sub>2</sub> O/KCl = 8.7<br>H <sub>2</sub> O/KNO <sub>3</sub> = 8.5 |         |
| pgN/ml                                                     | 193.5                                                                 |         | 292.8                                                                 |         | 412.9                                                                 |         | 187.1                                                                 |         |

Nanoparticles obtained herein exhibit higher solubility compared to handbook standard values (which were generally related to mono-crystals). For example, Figure 3 presents the water solubility of CaF<sub>2</sub>/OH nanoparticles batch 11000123.

- 5 This composite is more soluble compared to the solubility of CaF<sub>2</sub> reported in handbooks (0.14mM). Thus, these types of nano-composite particles are of great interest in the vaccine field using IM mode of administration.

Nitrogen content was analyzed by Antek as described in the Analytical Methods. From those results it is thought that a large majority of the nitrogen, 10 originated from the selected starting organic material used during the preparation, is located on the insoluble particles (See Table 4A).

**Table 4A: Nitrogen content by Antek analyses**

|                           | Batch #  | Theoretical<br>MgN/<br>ml<br>in<br>W10 | Antek                |                     | mg dry<br>material/<br>ml | $\mu$ g<br>N/mg<br>dry<br>material<br>(£) |
|---------------------------|----------|----------------------------------------|----------------------|---------------------|---------------------------|-------------------------------------------|
|                           |          |                                        | in W10<br>MgN/<br>ml | Total<br>MgN/<br>ml |                           |                                           |
| Ca/F/ Cysteine            | 9440055  | 15.6                                   | <b>35.1</b>          | <b>193.5</b>        | 10.8                      | 14.6                                      |
|                           | 9440056  | 15.6                                   | <b>53.8</b>          | <b>292.8</b>        | 13.2                      | 18.1                                      |
|                           | 9440057  | 15.6                                   | <b>42.3</b>          | <b>412.9</b>        | 17.08                     | 21.7                                      |
|                           | 9440058  | 15.6                                   | <b>43.5</b>          | <b>157.1</b>        | 8.72                      | 13.0                                      |
|                           | 9440099  | 17                                     | <b>8.5</b>           | <b>726</b>          | 20.84                     | 34.4                                      |
|                           | 9440197  | 17                                     | <b>34.6</b>          | <b>705.5</b>        | 20.00                     | 33.5                                      |
| Ca/F/N-Ac.Cysteine        | 9440110  | 17                                     | <b>26</b>            | <b>129</b>          | 10.36                     | 9.9                                       |
|                           | 9440196  | 17                                     | <b>22.6</b>          | <b>112.9</b>        | 9.34                      | 9.6                                       |
|                           | 10616125 | 17                                     | <b>37.2</b>          | <b>115.9</b>        | 9.30                      | 8.4                                       |
|                           | 11000101 | 18.2                                   | <b>11.7</b>          | <b>129.1</b>        | 14.9                      | 7.88                                      |
| Ca/F/Glutathione          | 10616185 | 32.8                                   | <b>44.1</b>          | <b>2062.4</b>       | 18.43                     | 109.5                                     |
|                           | 11000030 | 5.1                                    | <b>41</b>            | <b>2178</b>         | 27.20                     | 78.5                                      |
|                           | 11000033 | 4.4                                    | <b>55</b>            | <b>2526</b>         | 23.52                     | 105.0                                     |
|                           | 11000086 | 4.8                                    | <b>14.1</b>          | <b>2789.4</b>       | 24.76                     | 112.08                                    |
|                           | 11000099 | 4.5                                    | <b>139.5</b>         | <b>2425.2</b>       | 23.19                     | 98.56                                     |
|                           | 11000194 | 4.1                                    | <b>6.3</b>           | <b>2870</b>         | 22.02                     | 130                                       |
| Ca/F/Glutathione<br>oxide | 10616198 | 32.8                                   | <b>256</b>           | <b>2226</b>         | 11.0                      | 179.1                                     |
|                           | 11000139 | 3.6                                    | <b>70</b>            | <b>4130</b>         | 22.12                     | 183.5                                     |
|                           | 11000140 | 7.3                                    | <b>90</b>            | <b>4400</b>         | 22.6                      | 190.7                                     |
| Ca/F/Uric acid            | 11000182 | 0.04                                   | <b>7.1</b>           | <b>260.3</b>        | 12.89                     | 19.6                                      |
| Ca/F/Folic                | 11418018 | 0.77                                   | <b>0</b>             | <b>2034</b>         | 15.02                     | 135.4                                     |
| Ca/F/Hypoxanthine         | 11481198 |                                        | <b>95.9</b>          | <b>314.2</b>        | 13.1                      | 23.98                                     |
| Ca/F/Xanthine             | 11481199 |                                        | <b>183.6</b>         | <b>2185.2</b>       | 20.3                      | 107.6                                     |
| Ca/F/Guanine              | 11481195 |                                        | <b>196.4</b>         | <b>1253</b>         | 21.5                      | 49.14                                     |
| Ca/F/Cytosine             | 11954009 |                                        | <b>4.9</b>           | <b>0.3</b>          | 13.7                      | 0.02                                      |

**Table 4A: Nitrogen content by Antek analyses**

|              | Batch #  | Theor- | Antek    |             | mg dry | µg   |
|--------------|----------|--------|----------|-------------|--------|------|
| Ca/F/Thymine | 11954064 |        | <b>0</b> | <b>58.3</b> | 9.8    | 5.95 |

(E) = (Total MgN/ml - W10 µgN/ml) / mg dry material/ml  
W10 is the supernatant corresponding to the water washing step number 10.

Antioxidant capacity was used to determine % w/w oxidizable organic material /dry weight as described in the Analytical Methods. The results are shown in Table 4B.

**Table 4B: Anti-oxidant capacity of various CaF<sub>2</sub> based composites**

| Suspension                    | Batch # | Suspension<br>Concent. | Anti-<br>oxidant                  | capacity                            | Oxidizable<br>organic content |                                            |
|-------------------------------|---------|------------------------|-----------------------------------|-------------------------------------|-------------------------------|--------------------------------------------|
|                               |         | mg dry/ml              | µl KMnO <sub>4</sub><br>/ml susp. | µl<br>KMnO <sub>4</sub><br>/ mg dry | µmoles<br>Org./ml             | % <sub>w/w</sub><br>Org.<br>/dry<br>weight |
| <b>CaF<sub>2</sub></b>        | 8833190 | 33.2                   | < 50                              | < 1.5                               | NA                            | NA                                         |
| <b>Ca/F/Cysteine</b>          | 9440099 | 20.8                   | 7950                              | 382                                 | 6.81                          | 3.9                                        |
|                               | 9440197 | 20.0                   | 7926                              | 396                                 | 6.72                          | 4.0                                        |
| <b>Ca/F/N-Acetyl-Cysteine</b> | 9440110 | 10.36                  | 1843                              | 177                                 | 1.75                          | 2.4                                        |
|                               | 9440196 | 9.3                    | 3236                              | 239                                 | 3.03                          | 5.3                                        |

5

### Example 2. Formation of Composites

Various composites were formed according to the general schemes provided in the Detailed Description. Unless indicated to the contrary, all starting materials used herein were obtained commercially.

10

#### *1° Treatment of commercial solid particles*

**{CaCO<sub>3</sub>}solid water washing: batch # 88331 11**

Calcium carbonate from queries: OMYA® 264.8mg was treated with 250ml of water as described (Scheme 1). Supernatant pH is given (see Table 5).

15

| <b>Table 5: pH along the water washing steps and addition of various solutions on {CaCO<sub>3</sub>}solid particles</b> |                 |                |                 |                |                     |                |                 |                |
|-------------------------------------------------------------------------------------------------------------------------|-----------------|----------------|-----------------|----------------|---------------------|----------------|-----------------|----------------|
|                                                                                                                         | <b>88331 11</b> | <b>8833167</b> | <b>11000077</b> | <b>8833107</b> | <b>9923127</b>      | <b>8833164</b> | <b>88331 10</b> | <b>8833139</b> |
|                                                                                                                         |                 |                |                 | Starting pH    |                     |                |                 |                |
|                                                                                                                         |                 |                |                 | 7.43           | 7.48                | 7.48           | 8.1 8           | 8.1 1          |
| Washing                                                                                                                 | PH              | pH             | PH              | PH             | PH                  | PH             | 8.22            | 8.41           |
| W 1                                                                                                                     | 9.80            | 9.43           | 10.01           | 11.08          | 11.12               | 7.22           | 8.24            | 8.62           |
| W2                                                                                                                      | 9.66            | 9.33           | 9.92            | 11.01          | 11.14               | 7.04           | 8.29            | 8.8            |
| W3                                                                                                                      | 9.72            | 9.51           | 9.79            | 10.88          | 10.94               | 7.24           | 8.42            | 8.93           |
| W4                                                                                                                      | 9.67            | 9.43           | 9.72            | 10.71          | 10.83               | 8.25           | 8.52            | 9.08           |
| W5                                                                                                                      | 9.78            | 9.36           | 9.71            | 10.47          | 10.68               | 8.01           | 8.69            | 9.27           |
| W6                                                                                                                      | 9.71            | 9.47           | 9.70            | 10.21          | 10.44               | 8.92           | 8.8             | 9.44           |
| W7                                                                                                                      | 9.78            | 9.45           | 9.66            | 9.8            | 10.22               | 9.02           | 9.1 1           | 9.72           |
| W8                                                                                                                      | 9.82            | 9.57           | 9.57            | 9.57           | 9.83                | 9.74           | 9.32            | 9.84           |
| W9                                                                                                                      | 9.68            | 9.52           | 9.66            | 9.41           | 9.65                | 9.64           | 9.44            | 9.85           |
| W10                                                                                                                     | 9.45            | 9.45           | ND              | 9.1 5          | 9.1 8               | 9.66           | 64 %            | 62 %           |
| Yields                                                                                                                  | 82.4%           | 70.6%          | 83.8%           | 67 %           | 74.8%               | 84 %           | 9.4             | 9.9            |
| E.C.P.                                                                                                                  | 9.3             | 9.5            | 9.8             | 9.0            | 7.4 and 9.2         | 9.6            |                 |                |
| Elem. Analyses                                                                                                          |                 |                |                 | ND             | Ca:48%<br>F: 4 1.9% | ND             |                 |                |
| CO3 by titration                                                                                                        |                 |                |                 | ND             | 2.6%                | ND             |                 |                |

The final volume was 30ml and E.C.P. was measured on this suspension. Ten ml of this suspension was evaporated to dryness at 80°C and weighted. Yields were expressed in % compared to the weight of starting powder. Sample of dry material

5 was submitted to infrared analysis which shows the calcite type of CaCO<sub>3</sub>.

#### **{CaCO<sub>3</sub>}solid water washing: batch # 8833167**

Synthetic calcium carbonate, precipitated by bubbling CO<sub>2</sub> in a Ca(OH)<sub>2</sub> solution, from Mineral Technology® (Multiplex MM batch U203) 247.9mg was treated

10 as batch #88331 11 (Table 5).

**{CaCO<sub>3</sub>}solid water washing: batch # 11000077**

High Gravity Controlled Precipitation NPCC111 from NanoMaterials Technology® synthetic calcium carbonate, 0.828g was treated as batch #8833111 (Table 5).

5

**{CaCO<sub>3</sub>}solid treated with NaF solution: batch # 8833107**

Sodium fluoride (710.7mg) was dissolved in water and pH adjusted to pH 7.43, forming a total volume of 168 ml which was sterilized by filtration. To this solution, 265.1 mg of CaCO<sub>3</sub>Solid particles (OMYA®) were added. Water washing was carried out as described (Scheme 2). Supernatant pH is given (see Table 5), final volume being 30ml. Ten ml of this suspension was evaporated to dryness at 80°C and weighted. Yields were expressed in % compared to the weight of starting powder. Sample of dry material was submitted to infrared analysis which shows the presence of CaCO<sub>3</sub> of the Vaterite type (Figure 2).

15

**{CaCO<sub>3</sub>}solid treated with NaF solution: batch # 9923127**

2.6612g of CaCO<sub>3</sub>(OMYA®) was treated by 7.0843g NaF dissolved in 1Liter water, giving a starting pH of 9.52 and treated as for batch #8833107 (Table 5). The yields were 74.8%. Elementary Ca and F analyses were given (Table 5). Theoretical elementary composition of CaCO<sub>3</sub> is Ca: 40% and CO<sub>3</sub>: 60%; while CaF<sub>2</sub> gives Ca: 51.28% and F: 48.72%. Experimental data give Ca: 48% and F: 41.9% indicating that fluoride % is too low to be pure CaF<sub>2</sub> and Ca % is too low to be pure CaCO<sub>3</sub>. Sample of dry material was submitted to infrared analysis showing the presence of carbonate of vaterite type similar to the one of sample # 8833107 presented in fig.2. Titration by HCl indicates that this powder was only 2.6% carbonate. Thus, a composite of Ca/F/CO<sub>3</sub> was obtained in which the CaCO<sub>3</sub> part was of the vaterite type and thus differs from the starting CaCO<sub>3</sub> material.

25

**{CaCO<sub>3</sub>}solid treated with CaCl<sub>2</sub> solution: batch # 8833164**

Calcium chloride (2.0223 g) was dissolved in water and pH adjusted to pH 7.48, forming a total volume of 180 ml which was sterilized by filtration. To this solution, 276.8mg of CaCO<sub>3</sub>Solid particles (OMYA®) was added. Water washing and treatment was carried out as for batch # 8833107 (Table 5).

**{CaCO<sub>3</sub>}solid treated with Cysteine solution: batch # 8833110**

Cysteine (2.0111 g) was dissolved in water and pH adjusted to pH 8.18, forming a total volume of 168 ml which was sterilized by filtration. To this solution, 277.4mg of CaCO<sub>3</sub>Solid particles (OMYA®) was added. Water washing was carried out as described (Scheme 2). Supernatant pH is given (see Table 5).

**{CaCO<sub>3</sub>}solid treated with N-Acetyl-Cysteine solution: batch # 8833139**

N-Acetyl-cysteine (3.1058 g) was dissolved in water and pH adjusted to pH 8.11, forming a total volume of 180 ml which was sterilized by filtration. To this solution, 266.3mg of CaCO<sub>3</sub>Solid (Sigma-Aldrich) was added. Water washing was carried out as described (Scheme 2). Supernatant pH is given (see Table 5).

**{CaCO<sub>3</sub>}solid treated with thioglycerol batch # 8833114**

Thioglycerol (1.5ml) was dissolved in water and pH adjusted to pH 9.50, forming a total volume of 168 ml which was sterilized by filtration. To this solution, 263.5 mg (2.63mmoles) of CaCO<sub>3</sub>solid (OMYA®) was added. Water washing was carried out as described (Scheme 2). Supernatant pH is given in Table 6.

**Table 6: pH along the water washing steps after addition of various solutions on {CaCO<sub>3</sub>}solid particles**

|             | 883311 | 883310 | 883314 | 883314 | 883314 | 883314 | 883315 | 992313 | 992313 |
|-------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|             | 4      | 9      | 1      | 2      | 8      | 9      | 0      | 0      | 1      |
| Starting pH | 9.50   | 6.54   |        | 8.15   | 8.20   | 8.26   | 11.54  | 6.55   | 9.44   |
| Washing pH  | pH     | pH     | pH     | pH     | pH     | pH     | pH     | pH     | pH     |
| W1          | 9.45   | 6.66   | 6.62   | 8.15   | 8.24   | 8.5    | 11.53  | 6.58   | 9.49   |
| W2          | 9.51   | 6.74   | 6.27   | 8.19   | 8.36   | 8.64   | 11.38  | 6.61   | 9.53   |

**Table 6: pH along the water washing steps after addition of various solutions on {CaCO<sub>3</sub>}<sub>solid</sub> particles**

|                                     | 883311 | 883310 | 883314 | 883314 | 883314 | 883314 | 883315 | 992313              | 992313              |
|-------------------------------------|--------|--------|--------|--------|--------|--------|--------|---------------------|---------------------|
|                                     | 4      | 9      | 1      | 2      | 8      | 9      | 0      | 0                   | 1                   |
| W3                                  | 9.5    | 6.86   | 6.78   | 8.22   | 8.42   | 8.55   | 11.25  | 6.65                | 9.56                |
| W4                                  | 9.57   | 7.07   | 6.32   | 8.23   | 8.37   | 8.54   | 11.11  | 6.67                | 9.58                |
| W5                                  | 9.69   | 7.33   | 6.37   | 8.22   | 8.17   | 8.53   | 10.99  | 6.67                | 9.60                |
| W6                                  | 9.64   | 7.78   | 6.21   | 8.24   | 7.51   | 7.96   | 10.45  | 6.56                | 9.48                |
| W7                                  | 9.55   | 8.08   | 6.61   | 8.22   | 8.05   | 8.61   | 10.45  | 6.69                | 9.50                |
| W8                                  | 9.65   | 8.76   | 6.74   | 8.1    | 7.69   | 8.79   | 10.23  | 6.50                | 9.34                |
| W9                                  | 9.59   | 8.97   | 6.81   | 7.72   | 8.06   | 8.84   | 9.98   | 6.33                | 8.76                |
| W10                                 | 9.61   | 8.88   | 6.55   | 7.52   | 7.66   | 8.62   | 9.27   | 6.19                | 8.20                |
| Yields                              | 75 %   | 69 %   | 73 %   | 70 %   | 68 %   | 73 %   | 74 %   | 83 %                | 74 %                |
| E.C.P.<br>H <sub>2</sub> O/KCl      | 9.6    | 9.45   | 6 - 8  | < 4.5  | 7.4    | 5.6    | 9.2    | No<br>cross-<br>ing | No<br>cross-<br>ing |
| E.C.P.<br>H <sub>2</sub> O/KNO<br>3 |        |        | 7.2    | 7.2    | 7.4    | 6.1    | 8.6    | 7.4                 | 7.4                 |

**{CaCO<sub>3</sub>}<sub>solid</sub> treated with phosphoethanolamine batch # 8833109**

Phosphoethanolamine (2.3582 g) was dissolved in water and pH adjusted to pH 6.54, forming a total volume of 168 ml which was sterilized by filtration. To this solution, 270.6 mg of CaCO<sub>3</sub> solid particles (OMYA®) was added. Water washing was carried out as described (Scheme 2). Supernatant pH is given (see Table 6).

**{CaF<sub>2</sub>}<sub>solid</sub> water washing: batch # 8833141**

Calcium fluoride 316.0mg was treated with 250ml of water as described (Scheme 1). Supernatant pH is given (see Table 6).

**{CaF<sub>2</sub>}<sub>solid</sub> treated with Cysteine solution: batch # 8833142**

Cysteine (2.0735 g) (Merck) was dissolved in water and pH adjusted to pH 8.15, forming a total volume of 182 ml which was sterilized by filtration. To this

solution, 353.1 mg of calcium fluoride was added. Water washing was carried out as described (Scheme 2). Supernatant pH is given (see Table 6).

**{CaF<sub>2</sub>}solid treated with N-Acetyl-Cysteine solution: batch # 8833148**

5 N-Acetyl-Cysteine (3.01 924 g) was dissolved in water and pH adjusted to pH 8.20, forming a total volume of 180 ml which was sterilized by filtration. To this solution, 362.89 mg of calcium fluorite was added. Water washing was carried out as described (Scheme 2). Supernatant pH is given (see Table 6).

**10 {CaF<sub>2</sub>}solid treated with carbonate at pH 8.26 batch # 8833149**

Sodium bicarbonate (1.51 058 g) was dissolved in 180ml of water, a pH 8.28 was obtained. This solution was sterilized by filtration. To this solution, 377.99 mg of calcium fluoride was added. Water washing was carried out as described (Scheme 2). Supernatant pH is given (see Table 6).

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**{CaF<sub>2</sub>}solid treated with carbonate at pH 11.54 batch # 8833150**

Sodium carbonate (1.88348 g) was dissolved in 180ml of water, a pH 11.54 was obtained. This solution was sterilized by filtration. To this solution, 383.85 mg of calcium fluoride was added. Water washing was carried out as described (Scheme 2). Supernatant pH is given (see Table 6).

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**{CaF<sub>2</sub>}solid treated with phosphoethanolamine batch # 9923130**

Phosphoethanolamine (2.351 7 g) was dissolved in water and pH adjusted to pH 6.55, forming a total volume of 180 ml which was sterilized by filtration. To this solution, 351 .41 mg of calcium fluoride was added. Water washing was carried out as described (Scheme 2). Supernatant pH and osmotic pressure is given (see Table 6).

25

**{CaF<sub>2</sub>}solid treated with thioglycerol batch # 9923131**

30 Thioglycerol (1.5ml) was dissolved in water and pH adjusted to pH 9.44, forming a total volume of 180 ml which was sterilized by filtration. To this solution, 350.6 mg of calcium fluoride was added. Water washing was carried out as described (Scheme 2). Supernatant pH and osmotic pressure is given (see Table 6).

*2° Sol-gel precipitations***Na<sub>2</sub>HP0<sub>4</sub> solution added to CaCl<sub>2</sub> solution batch # 391080**

Calcium chloride dihydrate (1.8370g) was dissolved in 900ml of water giving a pH of 6.37. After sterilization by filtration, this solution was placed in 2 liters sterile Duran-Schott. Disodium hydrogenophosphate dihydrate (2.2250g) was dissolved in 900ml of water giving a pH of 9.32. After sterilization by filtration and under aseptic conditions, this solution is added to the CaCl<sub>2</sub> solution. After overnight decantation 1500ml of supernatant was discarded and replaced by 1500ml of sterile water. Those washing were repeated 12 times. Finally the suspension was concentrated by centrifugation to a total volume of 200ml. Those particles exhibit an E.C.P. H<sub>2</sub>O/KCL of 7.8 (see Table 7).

**CaCl<sub>2</sub> solution added to Na<sub>2</sub>HP0<sub>4</sub> solution batch 391082**

Disodium hydrogenophosphate dihydrate (2.22382g) was dissolved in 900ml of water. After sterilization by filtration, this solution placed in 2 liters sterile Duran-Schott. Calcium chloride dihydrate (1.83972g) was dissolved in 900ml. After sterilization by filtration and under aseptic conditions, this solution was added to the disodium hydrogenophosphate. The following treatments were similar to batch # 391 080 (Table 7).

**Na<sub>2</sub>HP0<sub>4</sub> and Citrate solution added to CaCl<sub>2</sub> solution batch # 391084**

Calcium chloride dihydrate (1.8413g) was dissolved in 900ml. After sterilization by filtration, this solution was placed in 2 liters sterile Duran-Schott. Disodium hydrogenophosphate dihydrate (2.2247g) was dissolved in 900ml of water and sterilized by filtration. Tri-sodium citrate (4.0259g) was dissolved in 180ml of water giving a pH of 8.49 and sterilized by filtration. Under aseptic condition the citrate solution was added to the disodium hydrogenophosphate solution and this mix was added to the CaCl<sub>2</sub> solution. The following treatments were similar to batch # 391 080 (Table 7).

**Na<sub>2</sub>HPO<sub>4</sub> and Lysine solution added to CaCl<sub>2</sub> solution batch # 391086**

Calcium chloride dihydrate (1.8350g) was dissolved in 900ml. After sterilization by filtration, this solution was placed in 2 liters sterile Duran-Schott. Disodium hydrogenophosphate dihydrate was dissolved in 900ml of water and sterilized by filtration. To 100ml of water was added to 15g of Lysine base. Hydrochloric acid (0.1 N) was added (40ml) to obtain a pH of 10.1. This solution was sterilized by filtration and added to the disodium hydrogenophosphate solution and this mix was added to the CaCl<sub>2</sub> solution. The following treatments were similar to batch # 391080 (Table 7).

**Table 7: Influence of soluble substance during the precipitation of calcium phosphate particles**

| Starting compounds                                                                                            | Batch # | E.C.P.<br>H <sub>2</sub> O/KCl |
|---------------------------------------------------------------------------------------------------------------|---------|--------------------------------|
| Na <sub>2</sub> HPO <sub>4</sub> solution added to CaCl <sub>2</sub> solution and washed with water           | 391080  | 7.8                            |
| CaCl <sub>2</sub> solution added to Na <sub>2</sub> HPO <sub>4</sub> solution and washed with water           | 391082  | 7.5                            |
| Na <sub>2</sub> HPO <sub>4</sub> + Citrate solution added to CaCl <sub>2</sub> solution and washed with water | 391084  | 9.2                            |
| Na <sub>2</sub> HPO <sub>4</sub> + Lysine solution added to CaCl <sub>2</sub> solution and washed with water  | 391086  | 8.4                            |

**CaF<sub>2</sub> batch # 88331 72D**

Sodium fluoride (8.4158g) was dissolved in 500ml of water and adjusted to pH 7.25. The solution was sterilized by filtration and 100ml of this solution was placed in a sterile 250ml Duran-Schott flask.

Calcium chloride (13.3555g) was dissolved in 600ml of water and adjusted to pH 7.07. The solution was sterilized by filtration and 100 ml of this solution added to the NaF solution. Water washing were carried out according to Scheme 3. Supernatant pH is given (see Table 8).

**CaF<sub>2</sub> batch # 8833190**

Sodium fluoride (8.4231 g) was dissolved in 500ml of water. The solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1093g) was dissolved in 500ml of water. The solution was sterilized by filtration and added to the NaF solution. Water washing were carried out according to Scheme 3. Supernatant pH is given in Table 8.

| <b>Table 8: Sol-gel precipitation of various Calcium fluoride composites.</b> |                                                                                   |                  |                    |                  |                                 |                |                                        |
|-------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------|--------------------|------------------|---------------------------------|----------------|----------------------------------------|
|                                                                               | <b>Precipitations in presence of decreasing bicarbonate quantities</b>            |                  |                    |                  | <b>Precipitation of Ca/F/OH</b> |                |                                        |
|                                                                               | <b>HCO<sub>3</sub><sup>-</sup> / F<sup>-</sup> / Ca<sup>++</sup> molar ratio:</b> |                  |                    |                  | <b>F/Ca molar ratio:</b>        |                |                                        |
|                                                                               | <b>2/36/20</b>                                                                    | <b>1/38/20</b>   | <b>0.2/39.6/20</b> | <b>0.0/40/20</b> | <b>2/1</b>                      | <b>2/1</b>     | <b>1/1</b>                             |
|                                                                               | <b>8833172 A</b>                                                                  | <b>8833172 B</b> | <b>8833172 C</b>   | <b>8833172 D</b> | <b>8833190</b>                  | <b>9440194</b> | <b>11000123</b>                        |
| Starting pH                                                                   | 8.53 + 8.07                                                                       | 8.44 + 8.07      | 8.21 + 8.07        | 7.37 + 8.07      |                                 |                |                                        |
| Washing                                                                       | pH                                                                                | pH               | pH                 | pH               | pH                              | pH             | pH                                     |
| W1                                                                            | 6.48                                                                              | 6.29             | 6.09               | 5.93             | 9.44                            | 9.78           | 5.93                                   |
| W2                                                                            | 6.8                                                                               | 6.71             | 6.63               | 6.1              | 7.15                            | 8.82           | 6.1                                    |
| W3                                                                            | 7.17                                                                              | 7.16             | 7.07               | 6.24             | 6.36                            | 7.13           | 6.24                                   |
| W4                                                                            | 7.23                                                                              | 7.27             | 7.16               | 6.13             | 6.47                            | 6.78           | 6.13                                   |
| W5                                                                            | 7.57                                                                              | 7.57             | 7.41               | 6.34             | 6.38                            | 6.52           | 6.34                                   |
| W6                                                                            | 7.54                                                                              | 7.48             | 7.18               | 6.14             | 6.27                            | 6.31           | 6.14                                   |
| W7                                                                            | 7.47                                                                              | 7.28             | 6.86               | 5.91             | 6.36                            | 6.25           | 5.91                                   |
| W8                                                                            | 7.38                                                                              | 7.17             | 6.84               | 6.09             | 6.25                            | 6.18           | 6.09                                   |
| W9                                                                            | 7.43                                                                              | 7.32             | 6.99               | 6.15             | 6.16                            | 6.44           | 6.15                                   |
| W10                                                                           | 6.98                                                                              | 6.9              | 6.6                | 5.83             | 6.02                            | 6.08           | 5.83                                   |
| Conc.                                                                         | 8.287mg/ml                                                                        | 9.377            | 18.52              | 25.274           | 33.21                           | 26.3           | 14.25                                  |
| CO <sub>3</sub>                                                               | 3.54%                                                                             | 1.13%            | 0.09%              | 0%               | NA                              | NA             | NA                                     |
| E.C.P.<br>H <sub>2</sub> O/KCl                                                | 8.7                                                                               | 8.4              | 9.1                | 7.8              | 0.8M =<br>7.3<br>3.2M=<br>8.3   | 0.8M =<br>7.6  | H <sub>2</sub> O/KCl<br>= 4.8 &<br>9.7 |
| E.C.P.                                                                        | 8.7                                                                               | 8.4              | 7.0                | 6.6              | 3.2M=                           | 3.2M=          | 7.5                                    |

| <b>Table 8: Sol-gel precipitation of various Calcium fluoride composites.</b> |                                                                        |                  |                    |                  |                                 |                              |                          |
|-------------------------------------------------------------------------------|------------------------------------------------------------------------|------------------|--------------------|------------------|---------------------------------|------------------------------|--------------------------|
|                                                                               | <b>Precipitations in presence of decreasing bicarbonate quantities</b> |                  |                    |                  | <b>Precipitation of Ca/F/OH</b> |                              |                          |
|                                                                               | <b>HCO<sub>3</sub><sup>-</sup> / F / Ca<sup>++</sup> molar ratio:</b>  |                  |                    |                  | <b>F/Ca molar ratio:</b>        |                              |                          |
|                                                                               | <b>2/36/20</b>                                                         | <b>1/38/20</b>   | <b>0.2/39.6/20</b> | <b>0.0/40/20</b> | <b>2/1</b>                      | <b>2/1</b>                   | <b>1/1</b>               |
|                                                                               | <b>8833172 A</b>                                                       | <b>8833172 B</b> | <b>8833172 C</b>   | <b>8833172 D</b> | <b>8833190</b>                  | <b>9440194</b>               | <b>11000123</b>          |
| H <sub>2</sub> O/KNO <sub>3</sub>                                             |                                                                        |                  |                    |                  | 6.9                             | 6.4;<br>0.8M=<br>6.6         |                          |
| Elementary analyses                                                           |                                                                        |                  |                    |                  | Ca:<br>52.8%<br>F:46.6%         | Ca:<br>55.2%<br>F: 44.7<br>% | Ca:49.7<br>%<br>F: 46.4% |

#### **CaF<sub>2</sub> batch # 9440194**

Sodium fluoride (8.4273g) was dissolved in 500ml of water. The solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1174g) was dissolved in 500ml of water. The solution was sterilized by filtration and added to the NaF solution. Water washing were carried out according to Scheme 3. Supernatant pH is given in Table 8.

#### **Ca/F/OH batch # 11000123**

Sodium fluoride (4.2055g) was dissolved in 504.2ml of water the pH was 9.40. The solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1107g) was dissolved in 508.2ml of water. The solution was sterilized by filtration and added to the NaF solution. Water washing were carried out according to Scheme 3. Supernatant pH is given in Table 8.

#### **Ca/F/CO<sub>3</sub> in the carbonate domain batches 8833152 to 8833157**

Na<sub>2</sub>CO<sub>3</sub>Solution: 10.6031g was dissolved in 500ml of water (obtained pH 11.61) and sterilized by 0.2μm filtration. NaF solution: 8.41413g was dissolved in 500ml water (obtained pH 9.66) and sterilized by 0.2μm filtration. CaCl<sub>2</sub> solution:

13.3250g was dissolved in 600ml water (obtained pH 10.07) and sterilized by 0.2 $\mu$ m filtration.

Starting with those solutions the Ca/F/ CO<sub>3</sub> batches 8833152 to 8833157 were carried out by following steps 1-3 according to table 9.

| <b>Table 9: Precipitation in presence of carbonate</b> |                                     |               |                 |                         |                       |          |           |
|--------------------------------------------------------|-------------------------------------|---------------|-----------------|-------------------------|-----------------------|----------|-----------|
|                                                        | <b>Step 1</b>                       | <b>Step 2</b> | <b>Obtained</b> | <b>Step 3</b>           | <b>mmole ratio</b>    |          |           |
| <b>Batch #</b>                                         | <b>Na<sub>2</sub>CO<sub>3</sub></b> | <b>NaF</b>    | <b>pH</b>       | <b>CaCl<sub>2</sub></b> | <b>CO<sub>3</sub></b> | <b>F</b> | <b>Ca</b> |
| <b>8833152</b>                                         | 90 ml                               | 10 ml         | 11.57           | 100 ml                  | 18                    | 4        | 20        |
| <b>8833153</b>                                         | 70 ml                               | 30 ml         | 11.52           | 100 ml                  | 14                    | 12       | 20        |
| <b>8833154</b>                                         | 50 ml                               | 50 ml         | 11.44           | 100 ml                  | 10                    | 20       | 20        |
| <b>8833155</b>                                         | 30 ml                               | 70 ml         | 11.34           | 100 ml                  | 6                     | 28       | 20        |
| <b>8833156</b>                                         | 10 ml                               | 90 ml         | 11.08           | 100 ml                  | 2                     | 36       | 20        |
| <b>8833157</b>                                         | 5 ml                                | 95 ml         | 10.96           | 100 ml                  | 1                     | 38       | 20        |

5

Water washing were carried out according to Scheme 6. Supernatant pH is given in Table 10.

| <b>Table 10: Precipitation of Ca/F/CO<sub>3</sub> in the carbonate domain</b> |                                                                              |                  |                  |                  |                  |                  |
|-------------------------------------------------------------------------------|------------------------------------------------------------------------------|------------------|------------------|------------------|------------------|------------------|
|                                                                               | <b>CO<sub>3</sub><sup>2-</sup>/F<sup>-</sup>/Ca<sup>++</sup> molar ratio</b> |                  |                  |                  |                  |                  |
| <b>Molar ratio:</b>                                                           | <b>18/04/20</b>                                                              | <b>14/12/20</b>  | <b>10/20/20</b>  | <b>6/28/20</b>   | <b>2/36/20</b>   | <b>1/38/20</b>   |
| <b>Batch #</b>                                                                | <b>8833152</b>                                                               | <b>8833153</b>   | <b>8833154</b>   | <b>8833155</b>   | <b>8833156</b>   | <b>8833157</b>   |
| Starting pH                                                                   | 11.57 +<br>10.07                                                             | 11.52 +<br>10.07 | 11.44 +<br>10.07 | 11.34 +<br>10.07 | 11.08 +<br>10.07 | 10.93 +<br>10.07 |
| Washing pH                                                                    | pH                                                                           | pH               | pH               | pH               | pH               | pH               |
| W1                                                                            | 9.66                                                                         | 9.89             | 10.19            | 10.24            | 9.8              | 10.19            |
| W2                                                                            | 9.55                                                                         | 9.98             | ND               | 10.29            | 9.87             | ND               |
| W3                                                                            | 9.55                                                                         | 9.96             | 10.15            | 10.18            | 9.81             | 10.15            |
| W4                                                                            | 9.55                                                                         | 9.82             | 10.07            | 10.05            | 9.74             | 10.07            |
| W5                                                                            | 8.81                                                                         | 9.09             | 9.52             | 9.53             | 9.22             | 9.52             |
| W6                                                                            | 9.45                                                                         | 9.49             | 9.68             | 9.76             | 9.58             | 9.68             |
| W7                                                                            | 9.48                                                                         | 9.52             | 9.61             | 9.74             | 9.54             | 9.61             |
| W8                                                                            | 9.43                                                                         | 9.46             | 9.57             | 9.67             | 9.5              | 9.57             |

| <b>Table 10: Precipitation of Ca/F/CO<sub>3</sub> in the carbonate domain</b> |                                                                              |                 |                 |                |                |                |
|-------------------------------------------------------------------------------|------------------------------------------------------------------------------|-----------------|-----------------|----------------|----------------|----------------|
|                                                                               | <b>CO<sub>3</sub><sup>2-</sup>/F<sup>-</sup>/Ca<sup>++</sup> molar ratio</b> |                 |                 |                |                |                |
| <b>Molar ratio:</b>                                                           | <b>18/04/20</b>                                                              | <b>14/12/20</b> | <b>10/20/20</b> | <b>6/28/20</b> | <b>2/36/20</b> | <b>1/38/20</b> |
| <b>Batch #</b>                                                                | <b>8833152</b>                                                               | <b>8833153</b>  | <b>8833154</b>  | <b>8833155</b> | <b>8833156</b> | <b>8833157</b> |
| W9                                                                            | 8.87                                                                         | 8.57            | 8.89            | 9.13           | 9.16           | 8.89           |
| W10                                                                           | 9.31                                                                         | 9.24            | 9.4             | 9.42           | 9.33           | 9.4            |
| Conc.                                                                         | 45.244                                                                       | 41.739          | 40.534          | 42.726         | 39.255         | 16.007         |
|                                                                               | mg/ml                                                                        | mg/ml           | mg/ml           | mg/ml          | mg/ml          | mg/ml          |
| % CO <sub>3</sub>                                                             | 86                                                                           | ND              | 75.8            | 49.1           | 11.4           | 4.5            |
| IR                                                                            | Fig.7                                                                        | Fig.7           | Fig.7           | Fig.7          | Fig.7          | Fig.7          |
| E.C.P.<br>/KCl                                                                | 9.2                                                                          | 9.3             | 9.3             | 9.4            | 9.3            | 8.9            |
| E.C.P.<br>/KNO <sub>3</sub>                                                   | 9.4                                                                          | 9.3             | 9.4             | 9.3            | 9.1            | 8.5            |

Thus, decreasing CO<sub>3</sub><sup>2-</sup> concentrations (or increasing the F<sup>-</sup> concentrations) gives less and less yields quantities, and lower W10 pH values.

5 Samples obtained at the highest F<sup>-</sup> concentration (#8833157) give the lowest E.C.P. value. This suggests that those obtained precipitates consist to CaCO<sub>3</sub> particles possibly containing increasing CaF<sub>2</sub> content as the F<sup>-</sup> concentrations were increased.

Thus, in the series Ca/F/CO<sub>3</sub> 8833152 → 8833157 we have:

- 10 - less and less CO<sub>3</sub><sup>2-</sup>, compensated by more and more F<sup>-</sup> (by starting mole ratio);
- similar concentrations in terms of dry weight mg/ml (except 8833157 which was much lower);
- decrease in CO<sub>3</sub><sup>2-</sup> contents (confirmed by titration and by IR (870cm<sup>-1</sup> see fig. 8);
- 15 - decrease of the calcite form (IR 1430 cm<sup>-1</sup> see fig. 8) in favor of the vaterite one (IR 1490 and 1420 cm<sup>-1</sup> see fig. 8); #8833155, #8833156 and #8833157 were rich in Vaterite type; #8833152 was poor in Vaterite and rich in Calcite. Vaterite gives a weak IR signal at 750cm<sup>-1</sup> and no signal at 713-

715cm<sup>-1</sup> ; while calcite exhibits IR signal at 713-715cm<sup>-1</sup> and no IR signal at 750cm<sup>-1</sup> (M. Sato and S. Matsuda; Zeitschrift für Kristallographie, 1969 vol. 129 p. 405-410);

- similar E.C.P. H<sub>2</sub>O/KCl values (except 8833157 which is lower);

5 - decrease in E.C.P. H<sub>2</sub>O/KNO<sub>3</sub> values at lower carbonate content.

### **Ca/F/CO<sub>3</sub> in the bicarbonate domain:**

Sodium bicarbonate solution: Sodium bicarbonate (8.4098g) was dissolved in 500ml of water (at this stage pH was 8.14) and sterilized by filtration. Sodium fluoride solution: Sodium fluoride (8.4158g) was dissolved in 500ml of water and the pH adjusted to 7.25. The solution was sterilized by filtration. Calcium chloride solution: Calcium chloride (13.3555g) was dissolved in 600ml of water and the pH adjusted to 8.07. The solution was sterilized by filtration

### **15 Ca/F/CO<sub>3</sub> in the bicarbonate domain batch # 8833172A**

Ten ml of sodium bicarbonate solution was placed in a sterile 250 ml Duran-Schott flask and 90 ml of the sodium fluoride solution was added, at this stage the pH was 8.53.

20 The calcium chloride solution (100ml) was added to the NaHCO<sub>3</sub> and NaF solutions. Water washing were carried out according to Scheme 4. Supernatant pH is given in Table 8.

### **Ca/F/CO<sub>3</sub> in the bicarbonate domain batch # 8833172B**

25 5 ml of sodium bicarbonate solution was placed in a sterile 250 ml Duran-Schott flask and 95 ml of the sodium fluoride solution was added, at this stage the pH was 8.44.

The calcium chloride solution (100ml) was added to the NaHCO<sub>3</sub> and NaF solutions. Water washing were carried out according to Scheme 4. Supernatant pH is given in Table 8.

30

**Ca/F/CO<sub>3</sub> in the bicarbonate domain batch # 88331 72C**

One ml of sodium bicarbonate solution was placed in a sterile 250 ml Duran-Schott flask and 99 ml of the sodium fluoride solution was added, at this stage the pH was 8.21 .

- 5 The calcium chloride solution (100ml) was added to the NaHCO<sub>3</sub> and NaF solutions. Water washing were carried out according to Scheme 4. Supernatant pH is given in Table 8.

HCl titrations carried out on batches 88331 72A, 88331 72B, 88331 72C and 88331 72D.

- 10 Presence of carbonate can be monitored by HCl titration. Comparisons were made by submitting similar quantities of nanoparticles, for example: 3.0ml of 88331 72A (at 8.28mg/ml), 2.7ml of 88331 72B (at 9.37mg/ml), 1.36ml of 88331 72C (at 18.52mg/ml) and 1ml of 88331 72D (at 25.27mg/ml), diluted when necessary in water to be at a total volume of 3ml each, and titrated by HCl 0.3N solution (table 8).

15

**Ca/F/CO<sub>3</sub> batch # 9440195**

Sodium bicarbonate (8.41 09g) was dissolved in 500ml of water (at this stage pH was 8.17) and sterilized by filtration. Sodium fluoride (8.4203g) was dissolved in 500ml of water and the pH adjusted to 7.29. The solution was sterilized by filtration.

- 20 Under aseptic conditions, 50ml of the bicarbonate solution and 450ml of the sodium fluoride solution was placed in a sterile 1L Duran-Schott. Calcium chloride (11.1089g) was dissolved in 500ml of water and the pH adjusted to 7.96. The solution was sterilized by filtration and added to the Duran-Schott container. Water washing were carried out according to Scheme 4. Supernatant pH and results of HCl
- 25 titrations are given (Table 11).

| <b>Table 11: : Precipitation of Ca/F/CO<sub>3</sub></b> |                                                                                  |                |                |                                                                                  |                     |                      |                    |
|---------------------------------------------------------|----------------------------------------------------------------------------------|----------------|----------------|----------------------------------------------------------------------------------|---------------------|----------------------|--------------------|
|                                                         | <b>HCO<sub>3</sub><sup>-</sup> / F<sup>-</sup> / Ca<sup>++</sup> molar ratio</b> |                |                | <b>CO<sub>3</sub><sup>2-</sup> / F<sup>-</sup> / Ca<sup>++</sup> molar ratio</b> |                     |                      |                    |
|                                                         | <b>2/36/20</b>                                                                   |                |                | <b>18.3 / 4 / 20</b>                                                             | <b>10.2/20 / 20</b> | <b>6.1 / 28 / 20</b> | <b>2 / 36 / 20</b> |
|                                                         | <b>9440195</b>                                                                   | <b>9923123</b> | <b>9923124</b> | <b>11000080</b>                                                                  | <b>11000081</b>     | <b>11000082</b>      | <b>11000083</b>    |
|                                                         | Starting pH                                                                      |                |                | Starting pH                                                                      |                     |                      |                    |
| <b>HCO<sub>3</sub><sup>-</sup></b>                      | 8.17                                                                             | 8.12           | 8.12           | 10.09                                                                            | 10.07               | 10.06                | 10.05              |

| Table 11: : Precipitation of Ca/F/CO <sub>3</sub> |                                                                               |              |              |                                                                               |              |               |             |
|---------------------------------------------------|-------------------------------------------------------------------------------|--------------|--------------|-------------------------------------------------------------------------------|--------------|---------------|-------------|
|                                                   | HCO <sub>3</sub> <sup>-</sup> / F <sup>-</sup> / Ca <sup>++</sup> molar ratio |              |              | CO <sub>3</sub> <sup>2-</sup> / F <sup>-</sup> / Ca <sup>++</sup> molar ratio |              |               |             |
|                                                   | 2/36/20                                                                       |              |              | 18.3 / 4 / 20                                                                 | 10.2/20 / 20 | 6.1 / 28 / 20 | 2 / 36 / 20 |
|                                                   | 9440195                                                                       | 9923123      | 9923124      | 11000080                                                                      | 11000081     | 11000082      | 11000083    |
| F <sup>-</sup>                                    | 7.29                                                                          | 7.25         | 7.27         |                                                                               |              |               |             |
| Ca <sup>++</sup>                                  | 7.96                                                                          | 7.72         | 7.70         | 10.1 7                                                                        | 10.1 7       | 10.1 7        | 10.1 7      |
| Washing                                           | pH                                                                            | pH           | PH           | PH                                                                            | PH           | PH            | PH          |
| W 1                                               | 6.38                                                                          | 6.43         | 6.46         | 7.55                                                                          | 7.41         | 7.92          | 7.68        |
| W2                                                | 6.78                                                                          | 6.86         | 6.98         | 6.93                                                                          | 7.39         | 8.1 8         | 8.03        |
| W3                                                | 7.1 3                                                                         | 7.1 2        | 7.34         | 7.08                                                                          | 7.73         | 8.38          | 7.84        |
| W4                                                | 7.43                                                                          | 7.54         | 7.65         | 7.1 7                                                                         | 8.04         | 8.78          | 7.49        |
| W5                                                | 7.46                                                                          | 7.63         | 7.80         | 7.69                                                                          | 8.45         | 8.87          | 7.45        |
| W6                                                | 7.48                                                                          | 7.85         | 7.81         | 8.1 5                                                                         | 8.88         | 9.07          | 8.46        |
| W7                                                | 7.54                                                                          | 7.87         | 7.81         | 8.52                                                                          | 9.27         | 9.33          | 8.1 9       |
| W8                                                | 7.56                                                                          | 7.74         | 7.70         | 8.74                                                                          | 9.39         | 9.34          | 8.28        |
| W9                                                | 7.53                                                                          | 7.87         | 7.85         | 9.1 0                                                                         | 9.54         | 9.40          | 8.23        |
| W10                                               | 7.64                                                                          | 7.98         | 7.91         | 9.32                                                                          | 9.60         | 9.57          | 8.55        |
| Cone.                                             | 14.6<br>mg/ml                                                                 | 16.27        | 19.67        | 110.28                                                                        | 40.79        | 33.73         | 23.56       |
| Ca % F%                                           | 53.2<br>46.                                                                   | 51.0<br>42.1 | 49.0<br>43.0 | ND                                                                            | 45.1 39.5    | 47.3 41.3     | 48.8 46.3   |
| E.C.P. /KCl                                       | 9.3                                                                           | 9.2          | 9.5          | ND                                                                            | 10.0         | 10.0          | 9.8         |
| E.C.P.KNO3                                        | 8.6                                                                           | 8.3          | 8.3          | ND                                                                            | 9.6          | = 9.5         | = 9.3       |
| % CO <sub>3</sub>                                 | 3.6%                                                                          | 2.9%         | 2.9%         | ND                                                                            | 44.25        | 23.40         | 6.1 5       |

### Ca/F/COs batch # 9923123

5 Sodium bicarbonate (8.41 22g) was dissolved in 500ml of water (at this stage pH was 8.1 2) and sterilized by filtration. Fifty ml of this solution was placed in a sterile 1 liter Duran-Schott flask. Sodium fluoride (8.42488g) was dissolved in 500ml of water and the pH adjusted to 7.25. The solution was sterilized by filtration and

450ml was added to the 1 liter Duran-Schott flask containing already the bicarbonate solution. Calcium chloride (11.1275g) was dissolved in 500ml of water and the pH adjusted to 7.72. The solution was sterilized by filtration and added to the NaHCO<sub>3</sub> and NaF solutions. Water washing were carried out according to Scheme 4.

5 Supernatant pH and results of HCl titrations: see Table 11.

### **Ca/F/CO<sub>3</sub> # 9923124**

50 ml of the sodium bicarbonate solution prepared for batch 9923123 was placed in a 1 liter sterile Duran-Schott flask. Sodium fluoride (8.42253g) was dissolved in 500ml of water and the pH adjusted to 7.27. The solution was sterilized by filtration and 450ml was added to the 1 liter Duran-Schott flask containing already the bicarbonate solution.

Calcium chloride (11.1111g) was dissolved in 500ml of water and the pH adjusted to 7.70. The solution was sterilized by filtration and added to the NaHCO<sub>3</sub> and NaF solutions. Water washing were carried out according to Scheme 4. Supernatant pH and results of HCl titrations were given (Table 11).

### **Ca/F/CO<sub>3</sub> in the carbonate domain 11000080-83**

Sodium carbonate solution: Sodium bicarbonate (17.11 g) was dissolved in 1000ml of water and NaOH was added to reach pH 10.09. This solution was sterilized by filtration. Sodium fluoride solution: Sodium fluoride (20.16g) was dissolved in 1200ml of water and the pH was 9.84. The solution was sterilized by filtration. CaC<sup>2+</sup> solution: 44.4g was dissolved in 2000ml water (obtained pH 10.17) and sterilized by 0.2μm filtration.

### **Ca/F/CO<sub>3</sub> 11000080:**

Using a 1L sterile Duran-Schott : 450ml of the carbonate solution was added to 50ml of sodium fluoride solution, resulting in a pH 10.09. Under laminar flow, 500ml of the calcium chloride solution was added. Water washing were carried out according to Scheme 4. Supernatant pH is given in Table 11.

**Ca/F/COs 11000081 :**

Using a 1L sterile Duran-Schott : 250ml of the carbonate solution was added to 250ml of sodium fluoride solution, resulting in a pH 10.07. Under laminar flow, 500ml of the calcium chloride solution was added. Water washing were carried out according to Scheme 4. Supernatant pH is given in Table 11.

**Ca/F/COs 11000082:**

Using a 1L sterile Duran-Schott : 150ml of the carbonate solution was added to 350ml of sodium fluoride solution, resulting in a pH 10.06. Under laminar flow, 500ml of the calcium chloride solution was added. Water washing were carried out according to Scheme 4. Supernatant pH is given in Table 11.

**Ca/F/COs 11000083:**

Using a 1L sterile Duran-Schott : 50ml of the carbonate solution was added to 450ml of sodium fluoride solution, resulting in a pH 10.05. Under laminar flow, 500ml of the calcium chloride solution was added. Water washing were carried out according to Scheme 8. Supernatant pH is given in Table 19.

**Ca/F/Ascorbic acid batch # 9440198**

Calcium ascorbate (42.6001g) was dissolved in 400ml of water (obtained pH = 7.35). Sodium hydroxide 17ml (0.5M) was added to reach pH8.99. Water was added (83ml) giving a pH 9.08 and this solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Sodium fluoride (4.2063g) was dissolved in 500ml water (obtained pH 9.49) and sterilized by filtration. This solution was added to the calcium ascorbate solution. The following day the mix was transferred in a 2L sterile Duran Schott flask and an additional 1 liter sterile water was added. An additional 15 days standing period was applied. Water washing were carried out according to Scheme 7. Supernatant pH is given in Table 12.

**Table 12:precipitation of Calcium fluoride Z composites**

| Batch #  | 9440198 | 9440099 | 9440197 | 9440110 | 9440196 | 10616125 | 11000101 |
|----------|---------|---------|---------|---------|---------|----------|----------|
| Starting | 9.08    | 9.22    | 9.00    | 8.96    | 9.26 +  | 9.66 +   | 9.01 +   |
| pH       | 9.49    | 8.22    |         |         | 8.35    | 8.35     | 8.30     |
| Washing  | pH      | pH      | pH      | pH      | pH      | pH       | pH       |

| <b>Table 12:precipitation of Calcium fluoride Z composites</b> |                    |                |                |                 |                |                 |                 |
|----------------------------------------------------------------|--------------------|----------------|----------------|-----------------|----------------|-----------------|-----------------|
| Batch #                                                        | <b>9440198</b>     | <b>9440099</b> | <b>9440197</b> | <b>94401 10</b> | <b>9440196</b> | <b>10616125</b> | <b>11000101</b> |
| W 1                                                            | 6.65               | 8.27           | 8.31           | 8.47            | 8.44           | 8.44            | 8.47            |
| W2                                                             | 6.67               | 8.45           | 8.41           | 8.62            | 8.58           | 8.54            | 8.62            |
| W3                                                             | 6.65               | 8.52           | 8.50           | 8.76            | 8.72           | 8.72            | 8.76            |
| W4                                                             | 6.67               | 8.60           | 8.65           | 8.92            | 8.88           | 8.87            | 8.89            |
| W5                                                             | 6.77               | 8.75           | 8.78           | 9.1 5           | 9.1 0          | 9.08            | 8.98            |
| W6                                                             | 6.64               | 8.97           | 9.03           | 9.43            | 9.38           | 9.34            | 9.02            |
| W7                                                             | 6.89               | 9.21           | 9.1 8          | 9.63            | 9.47           | 9.45            | 9.20            |
| W8                                                             | 6.86               | 9.22           | 9.1 8          | 9.47            | 9.1 5          | 8.93            | 8.64            |
| W9                                                             | 7.02               | 9.1 2          | 9.02           | 9.35            | 8.31           | 7.67            | 7.85            |
| W10                                                            | 7.1 2              | 8.96           | 8.64           | ND              | 7.05           | 7.26            | 7.37            |
| Cone.                                                          | 13.5<br>mg/ml      | 20.8<br>mg/ml  | 20.0mg/ml      | 10.3<br>mg/ml   | 9.34<br>mg/ml  | 9.30<br>mg/ml   | 14.9<br>mg/ml   |
| Ca% F%                                                         | 52.9<br>46.8       | 54.2<br>45.0   | 55.1<br>44.8   | 49.4<br>50.0    | 51.1<br>48.5   | 49.8 35.3       | 51.5 54.8       |
| E.C.P.<br>H <sub>2</sub> O/KCl                                 | 5.7 & 7.2<br>& 7.8 | 9.3            | 9.6            | 7.5             | 8.3            | 7.8             | 6.1 &<br>10.0   |
| E.C.P.<br>H <sub>2</sub> O/KNO <sub>3</sub>                    | 7.3                | 8.6            | 8.4            | 7.3             | 7.3            | 7.3             | 5.4 & 9.4       |
| pgN/ml                                                         | NA                 | 726            | 706            | 129             | 113            | 115.9           | 129.1           |

### Ca/F/Cysteine batch # 944055

5 Sodium fluoride 0.841 08g and cysteine 2.4221 6g (Merck) was dissolved in 84ml of water. Sodium hydroxide 0.5N (16ml) was added to reach pH 8.18. The solution was sterilized by filtration and placed in a 250ml sterile Duran-Schott flask. Calcium chloride 2.221 97g was dissolved in 100ml of water resulting in a pH 10.03 solution which was sterilized by filtration. Under sterile conditions this CaC<sup>+</sup> solution was added to the (NaF + Cysteine) solutions. Water washing and supernatant pH 10 are given in Table 3.

**Ca/F/Cysteine batch # 944056**

Calcium chloride 2 and cysteine 2.42838g (Merck) was dissolved in 80ml of water. Sodium hydroxide 0.5N (21 ml) was added to reach pH 8.18. The solution was sterilized by filtration and placed in a 250ml sterile Duran-Schott flask. Sodium fluoride 0.84168g was dissolved in 100ml of water resulting in a pH 8.78 solution which was sterilized by filtration. Under sterile conditions this NaF solution was added to the (CaF<sub>2</sub> + Cysteine) solution. Water washing, and supernatant pH are given (Table 3).

**10 Ca/F/Cysteine batch # 944057**

Sodium fluoride 0.84196g was dissolved in 100ml of water resulting in a pH 8.98 solution which was sterilized by filtration and placed in a 250ml sterile Duran-Schott flask. Calcium chloride 2.2265g and cysteine 2.42194g (Merck) was dissolved in 80ml of water. Sodium hydroxide 0.5N (21 ml) was added to reach pH 8.19. The solution was sterilized by filtration. Under sterile conditions this (CaF<sub>2</sub> + Cysteine) solution was added to the NaF solutions. Water washing and supernatant pH are given (Table 3).

**Ca/F/Cysteine batch # 944058**

Calcium chloride 2.2242g was dissolved in 100ml of water resulting in a pH 10.04 solution which was sterilized by filtration and placed in a 250ml sterile Duran-Schott flask. Sodium fluoride 0.84254g and cysteine 2.42793g (Merck) was dissolved in 83.5ml of water. Sodium hydroxide 0.5N (16.5ml) was added to reach pH 8.19. The solution was sterilized by filtration. Under sterile conditions this (NaF + Cysteine) solution was added to the CaF<sub>2</sub> solutions. Water washing and supernatant pH: see Table 3.

**Ca/F/Cysteine batch # 9440099**

Sodium fluoride (4.2287 g) was dissolved in 500ml of water, pH at this stage was 9.22, and sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1582g) and cysteine (12.1086 g) (Merck) was added and dissolved in 400ml of water (slightly violet color was obtained and pH = 5.58). Sodium hydroxide 0.5N was added (117ml) to reach pH 8.22 and this slightly violet solution was sterilized by filtration. The CaCl<sub>2</sub>-cysteine solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH and osmotic pressure is given (see Table 12).

**Ca/F/Cysteine batch # 9440197**

Sodium fluoride (4.215 g) was dissolved in 400ml of water (obtained pH = 9.62), sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1037g) was dissolved in 400ml of water (obtained pH = 10.06) and cysteine (12.1060 g) (Merck) was added (slightly violet color was obtained pH = 5.6). Sodium hydroxide 0.5N was added (107ml) to reach pH 8.21 and this slightly violet solution was sterilized by filtration. The CaCl<sub>2</sub>-cysteine solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 12).

**Ca/F/N-Acetyl-cysteine batch # 9440110**

Sodium fluoride (4.2058g g) was dissolved in 400ml of water (obtained pH = 9.26), and sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.10976g) was dissolved in 400ml of water and N-Acetyl-Cysteine (16.37876 g) was added (obtained pH = 1.67). Sodium hydroxide was added (0.550 g) and sodium hydroxide 0.5N was added (196 ml) to reach pH 8.35 and this slightly violet solution (coloration appears above pH 5) was sterilized by filtration. The CaCl<sub>2</sub>-N-acetyl-cysteine solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 12.

**Ca/F/N-Acetyl-cysteine batch # 9440196**

Sodium fluoride (4.2026g) was dissolved in 400ml of water (obtained pH = 9.66), and sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1139g) was dissolved in 400ml of water (obtained pH = 10.04) and N-Acetyl-Cysteine (16.3025 g) was added (obtained pH = 1.4). Sodium hydroxide (solid pellets Merck product 1064981 000 batch B0467298) was added (0.848 g) and sodium hydroxide 0.5N was added (172 ml) to reach pH 8.35 and this slightly violet solution (coloration appears above pH 5) was sterilized by filtration. The CaCl<sub>2</sub>-N-acetyl-cysteine solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 12).

**Ca/F/N-Acetyl-cysteine batch # 10616125**

Sodium fluoride (4.2026g) was dissolved in 500ml of water (obtained pH = 9.01), and sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1362g) was dissolved in 500ml of water (obtained pH = 10.04) and N-Acetyl-Cysteine (16.3313 g) was added (obtained pH = 1.63). Sodium hydroxide (solid pellets Merck product 1064981 000 batch B0467298008) was added until pH 8.02 and sodium hydroxide 0.5N was added (5 ml) to reach pH 8.30 and this slightly violet solution (coloration appears above pH 5) was sterilized by filtration. The CaCl<sub>2</sub>-N-acetyl-cysteine solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH: see Table 12.

**Ca/F/N-Acetyl-cysteine batch # 11000101**

Sodium fluoride (4.2101g) was dissolved in 500ml of water (obtained pH = 9.35), and sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1715g) was dissolved in 500ml of water (obtained pH = 9.97) and N-Acetyl-Cysteine (16.3469 g) was added (obtained pH = 1.79). Sodium hydroxide was added until pH 7.69 and sodium hydroxide 0.5N was added (5 ml) to reach pH 8.36 and this slightly violet solution (coloration appears above pH 5) was sterilized by filtration. The CaCl<sub>2</sub>-N-acetyl-cysteine solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 12).

**Ca/F/Glutathione batch # 10616185**

- Sodium fluoride (4.2098g) was dissolved in 500ml of water (obtained pH = 8.9), and sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1025g) was dissolved in 500ml of water (obtained pH = 10.00) and this solution filtered on 0.22 $\mu$ m and replaced in a clean 800ml Becker. Glutathione was added (obtained pH = 2.67). Sodium hydroxide was added to reach pH 8.57 and this solution was sterilized by filtration. The CaCl<sub>2</sub>-Glutathione solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 13.

**Table 13: Precipitation in presence of Glutathione**

| Batch #     | 11000033                          | 11000030                           | 11000086                     | 11000099                         | 10616185                    | 11000194                    |
|-------------|-----------------------------------|------------------------------------|------------------------------|----------------------------------|-----------------------------|-----------------------------|
| Starting pH | Ratio 1/1/1<br>pH6.9<br>7.04      | Ratio 1/1/0.1<br>pH 9.5<br>8.6     | Ratio 1/1/0.1<br>pH 9.3 8.6  | Ratio 1/1/0.1<br>pH 9.58<br>8.57 | Ratio 1/1/1<br>pH8.9 8.5    | Ratio 1/1/0.1<br>pH 9. 8.58 |
| Washing     | pH                                | pH                                 | pH                           | pH                               | pH                          | pH                          |
| W1          | 7.07                              | 8.51                               | 8.43                         | 8.41                             | 8.74                        | ND                          |
| W2          | 7.15                              | 8.66                               | 8.56                         | 8.54                             | 8.79                        | ND                          |
| W3          | 7.17                              | 8.74                               | 8.78                         | 8.65                             | 8.86                        | 8.51                        |
| W4          | 7.31                              | 8.88                               | 8.85                         | 8.78                             | 8.94                        | 8.71                        |
| W5          | 7.35                              | 9.03                               | 9.18                         | 9.02                             | 9.06                        | 8.81                        |
| W6          | 7.34                              | 9.14                               | 9.21                         | 9.21                             | 9.11                        | 8.84                        |
| W7          | 7.34                              | 9.28                               | 9.35                         | 9.34                             | 9.17                        | 9.07                        |
| W8          | 7.40                              | 9.27                               | 9.48                         | 9.24                             | 9.23                        | 9.20                        |
| W9          | 7.30                              | 9.22                               | 9.62                         | 9.39                             | 9.33                        | 9.38                        |
| W10         | 7.18                              | 9.17                               | 9.64                         | 9.44                             | 9.36                        | 9.54                        |
| Conc.       | 23.52mg/<br>ml                    | 27.20                              | 24.76                        | 23.19                            | 18.43                       | 22.02                       |
| Ca%<br>F%   | 45.8 43.3                         | 46.2 39.5                          | 45.6 44.0                    | 46.6 47.0                        | 39.7 37.5                   | 43.9 35.5                   |
| E.C.P.      | H <sub>2</sub> O/KCl<br>4.9 & 9.5 | H <sub>2</sub> O/KCl<br>4.8 & 10.1 | H <sub>2</sub> O/KCl<br>10.1 | H <sub>2</sub> O/KCl<br>10.1     | H <sub>2</sub> O/KCl<br>9.8 | H <sub>2</sub> O/KCl<br>9.8 |

**Table 13: Precipitation in presence of Glutathione**

| Batch # | 11000033                                 | 11000030 | 11000086 | 11000099 | 10616185 | 11000194 |
|---------|------------------------------------------|----------|----------|----------|----------|----------|
|         | H <sub>2</sub> O/KNO <sub>3</sub><br>8.8 | 9.2      | 10.1     | 10.4     | 9.1      | 9.1      |
| pgN/ml  | 2526                                     | 2178     | 2789     | 2425.2   | 2062.4   | 2870     |
| pgN/mg  | 105.0                                    | 78.5     | 112      | 98.56    | 109.51   | 130      |

**Ca/F/Glutathione batch # 11000030**

Sodium fluoride (4.2260g) was dissolved in 500ml of water (obtained pH = 9.47), and sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask.

- 5 Calcium chloride (11.120g) was dissolved in 500ml of water (obtained pH = 9.92) and this solution filtered on 0.22 $\mu$ m and replaced in a clean 800ml Becker. Glutathione (3.0726 g) was added (obtained pH = 2.72). Sodium hydroxide and sodium hydroxide 0.5M was added to reach pH 8.59 and this solution is sterilized by filtration. The CaCl<sub>2</sub>-Glutathione solution was added to the NaF solution. Water
- 10 washing were carried out according to Scheme 5. Supernatant pH was given in Table 13.

**Ca/F/Glutathione batch # 11000033**

- Sodium fluoride (4.2097g) was dissolved in 500ml of water (obtained pH = 9.35), HCl 0.3M was added to reach pH 6.94 and this solution sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask.

- Calcium chloride (11.117g) was dissolved in 500ml of water (obtained pH = 9.91) and this solution filtered on 0.22 $\mu$ m and replaced in a clean 800ml Becker. Glutathione (30.7 g) was added (obtained pH = 2.64). Sodium hydroxide and HCl
- 20 0.3M was added to reach pH 7.04 and this solution was sterilized by filtration. The CaCl<sub>2</sub>-Glutathione solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 13.

**Ca/F/Glutathione batch # 11000086**

Sodium fluoride (4.2235g) was dissolved in 500ml of water (obtained pH = 9.34), and this solution sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1431 g) was dissolved in 500ml of water (obtained pH = 9.92) and this solution filtered on 0.22 $\mu$ m and replaced in a clean 800ml Becker. Glutathione (3.0782 g) was added (obtained pH = 2.84). Sodium hydroxide and sodium hydroxide 0.5M was added to reach pH 8.59 and this solution was sterilized by filtration. The CaCl<sub>2</sub>-Glutathione solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 13.

**Ca/F/Glutathione batch # 11000099**

Sodium fluoride (4.1101g) was dissolved in 500ml of water (obtained pH = 9.58), and this solution sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.01228g) was dissolved in 500ml of water and this solution filtered on 0.22 $\mu$ m and replaced in a clean 800ml Becker (obtained pH = 10.05). Glutathione (3.0716 g) was added (obtained pH = 2.79). Sodium hydroxide and sodium hydroxide 0.5M was added to reach pH 8.57 and this solution was sterilized by filtration. The CaCl<sub>2</sub>-Glutathione solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 13.

**Ca/F/Glutathione batch # 11000194**

Sodium fluoride (4.2059g) was dissolved in 500ml of water (obtained pH = 9.24), and this solution sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1371 g) was dissolved in 500ml of water (obtained pH = 9.99) and this solution filtered on 0.22 $\mu$ m and replaced in a clean 800ml Becker. Glutathione (3.0912 g) was added (obtained pH = 3.07). Sodium hydroxide and sodium hydroxide 0.5M was added to reach pH 8.58 and this solution was sterilized by filtration. The CaCl<sub>2</sub>-Glutathione solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 13.

**Ca/F/Glutathione oxide batch # 10616198**

Sodium fluoride (0.4220g) was dissolved in 50ml of water (obtained pH = 8.64), and sterilized by filtration and placed in a sterile 100ml Duran-Schott flask. Calcium chloride (1.1112g) was dissolved in 30ml of water (obtained pH = 9.97) and

5 Glutathione oxide (6.5611 g) was added (obtained pH = 6.27). Sodium hydroxide 0.5N and 0.05M was added to reach pH 7.55 and this solution was sterilized by filtration. The CaCl<sub>2</sub>-Glutathione oxide solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 14.

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| <b>Table 14: Precipitation of Calcium fluoride composites and washing of CaPhosphate</b> |                        |                     |                     |                  |                   |                    |     |
|------------------------------------------------------------------------------------------|------------------------|---------------------|---------------------|------------------|-------------------|--------------------|-----|
| <b>Precipitation in presence of Glutathione oxide (GSSG) or uric or folic acid</b>       |                        |                     |                     |                  |                   | <b>CaPhosphate</b> |     |
|                                                                                          | <b>F/Ca/GSSG ratio</b> |                     |                     | <b>F/Ca/Uric</b> | <b>F/Ca/Folic</b> | <b>Brenntag</b>    |     |
|                                                                                          | <b>0.01/0.01/0.01</b>  | <b>0.1/0.1/0.05</b> | <b>0.1/0.1/0.01</b> |                  | <b>1/1/0.001</b>  | <b>2011-51</b>     |     |
|                                                                                          | <b>10616198</b>        | <b>11000139</b>     | <b>11000140</b>     | <b>11000182</b>  | <b>11481018</b>   | <b>11000160</b>    |     |
| Starting pH                                                                              | 8.64 7.55              | 9.82 8.21           | 9.82 8.12           | 8.60 6.24        | 6.98 9.73         | Scheme 1           |     |
| Washing                                                                                  | pH                     | pH                  | pH                  | pH               | pH                | pH                 | Osm |
| W1                                                                                       | 7.55                   | 7.84                | 8.07                | 5.33             | 5.65              | 6.21               | 292 |
| W2                                                                                       | 7.59                   | 7.66                | 8.10                | 5.49             | 5.75              | 6.42               | 145 |
| W3                                                                                       | 7.67                   | 7.84                | 8.14                | 5.58             | 5.72              | 6.60               | 73  |
| W4                                                                                       | 7.75                   | 7.95                | 8.07                | 5.65             | 5.73              | 6.77               | 36  |
| W5                                                                                       | 7.86                   | 7.75                | 8.25                | 5.76             | 5.73              | 6.81               | 19  |
| W6                                                                                       | 7.95                   | 7.71                | 8.57                | 5.85             | 5.81              | 7.02               | 8   |
| W7                                                                                       | 8.00                   | 7.98                | 8.41                | 5.93             | 5.92              | 7.14               | 3   |
| W8                                                                                       | 7.99                   | 8.02                | 8.52                | 6.05             | 6.16              | 6.76               | 1   |
| W9                                                                                       | 8.02                   | 7.89                | 8.50                | 6.01             | 6.08              | 6.66               | 0   |
| W10                                                                                      | 8.05                   | 7.93                | 8.45                | 5.99             | 6.09              | 6.77               | 0   |
| Conc. mg dry/ml                                                                          | 11.0                   | 22.1                | 22.6                | 12.82            | 15.02             | 21.9               |     |

| <b>Table 14: Precipitation of Calcium fluoride composites and washing of CaPhosphate</b> |                        |                     |                     |                  |                   |                    |
|------------------------------------------------------------------------------------------|------------------------|---------------------|---------------------|------------------|-------------------|--------------------|
| <b>Precipitation in presence of Glutathione oxide (GSSG) or uric or folic acid</b>       |                        |                     |                     |                  |                   | <b>CaPhosphate</b> |
|                                                                                          | <b>F/Ca/GSSG ratio</b> |                     |                     | <b>F/Ca/Uric</b> | <b>F/Ca/Folic</b> | <b>Brenntag</b>    |
|                                                                                          | <b>0.01/0.01/0.01</b>  | <b>0.1/0.1/0.05</b> | <b>0.1/0.1/0.01</b> |                  | <b>1/1/0.001</b>  | <b>201 1-51</b>    |
|                                                                                          | <b>10616198</b>        | <b>11000139</b>     | <b>11000140</b>     | <b>11000182</b>  | <b>11481018</b>   | <b>11000160</b>    |
| Ca% F%                                                                                   | Not Done               | 49.0 45.8           | 47.1 43.1           | 48.6 45.7        | 45.0 43.8         | Ca: 37.0%          |
| E.C.P. KCl                                                                               | 5.2 & 9.1              | 9.7                 | 9.9                 | No cross.        | 4.8               | 7.3                |
| E.C.P. KNOs                                                                              | 8.7                    | 9.3                 | 9.8                 | 8.4              | 8.1               | 7.6                |
| pgN/ml                                                                                   | 2226                   | 4 130               | 4400                | 260.3            | 2034              | NA                 |
| pgN/mg                                                                                   | 179.1                  | 183.5               | 190.7               | 19.69            | 135.4             | NA                 |

#### **Ca/F/Glutathione oxide batch # 11000139**

Sodium fluoride (4.20838g) was dissolved in 50ml of water (obtained pH = 9.82), and sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask.  
 Calcium chloride (1.1528g) was dissolved in 500ml of water (obtained pH = 9.97) and this solution filtered on 0.22 $\mu$ m and replaced in a clean 800ml Becker.  
 Glutathione oxide (3.2255 g) was added (obtained pH = 6.84). Sodium hydroxide and sodium hydroxide 0.5M was added to reach pH 8.21 and this solution was sterilized by filtration. The CaCl<sub>2</sub>-Glutathione oxide solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 14.

**Ca/F/Glutathione oxide batch # 11000140**

Sodium fluoride (4.20027g) was dissolved in 50ml of water (obtained pH = 9.82), and sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (1.1068g) was dissolved in 500ml of water (obtained pH = 9.94) and this solution filtered on 0.22 $\mu$ m and replaced in a clean 800ml Becker. Glutathione oxide (6.56150 g) was added (obtained pH = 6.55). Sodium hydroxide and sodium hydroxide 0.5M was added to reach pH 8.12 and this solution was sterilized by filtration. The CaCl<sub>2</sub>-Glutathione oxide solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 14.

**Ca/F/Thiolactate batch # 11000031**

Sodium fluoride (4.2078g) was dissolved in 500ml of water (obtained pH = 9.56), and sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1187g) was dissolved in 500ml of water (obtained pH = 9.89) and this solution filtered on 0.22 $\mu$ m and replaced in a clean 800ml Becker. Thiolactic acid (8.5ml) was added (obtained pH = 1.96). Sodium hydroxide was added to reach pH 9.54 and this solution was sterilized by filtration. The CaCl<sub>2</sub>-thiolactate solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 15.

| <b>Table 15: Precipitation of in presence of Adipate or Thiolactate</b> |                 |                 |                 |                 |                 |                 |
|-------------------------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| <b>Batch #</b>                                                          | <b>11000129</b> | <b>11481026</b> | <b>11481027</b> | <b>11000059</b> | <b>11000060</b> | <b>11000031</b> |
| Starting pH                                                             | 8.02<br>10.81   | 5.94<br>9.82    | 7.18<br>9.82    | 7.16 6.97       | 7.93<br>8.07    | 9.56<br>9.54    |
| Washing                                                                 | pH              | pH              | pH              | pH              | pH              | pH              |
| W1                                                                      | 7.97            | 5.56            | 6.93            | 6.17            | 7.93            | 9.73            |
| W2                                                                      | 7.75            | 5.66            | 6.95            | 6.28            | 8.13            | 9.85            |
| W3                                                                      | 7.58            | 5.72            | 6.94            | 6.07            | 8.09            | 10.00           |
| W4                                                                      | 7.50            | 5.72            | 6.84            | 6.04            | 8.51            | 10.12           |
| W5                                                                      | 7.58            | 5.99            | 6.92            | 5.87            | 8.73            | 10.23           |
| W6                                                                      | 7.08            | 6.06            | 6.79            | 5.86            | 8.96            | 10.36           |
| W7                                                                      | 6.65            | 6.20            | 6.78            | 5.91            | 9.23            | 10.43           |
| W8                                                                      | 6.52            | 6.28            | 6.72            | 5.96            | 9.49            | 10.42           |

| <b>Table 15: Precipitation of in presence of Adipate or Thiolactate</b> |                 |                 |                 |                 |                 |                 |
|-------------------------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| <b>Batch #</b>                                                          | <b>11000129</b> | <b>11481026</b> | <b>11481027</b> | <b>11000059</b> | <b>11000060</b> | <b>11000031</b> |
| W9                                                                      | 6.68            | 6.39            | 6.63            | 6.02            | 9.68            | 10.52           |
| W10                                                                     | 6.77            | 6.37            | 6.56            | 6.04            | 9.85            | 10.56           |
| Conc.                                                                   | 29.9mg/ml       | 26.32           | 31.61           | 10.6            | 19.36           | 23.88           |
| Ca% F%                                                                  | 50.0            | 46.3            | 45.1            | 50.1            | 49.5            | 47.2            |
|                                                                         | 44.4            | 42.2            | 44.7            | 43.2            | 46.1            | 42.7            |
| E.C.P.<br>H <sub>2</sub> O/KCl                                          | 6.6             | 7.2             | 6.7             | 9.5 ? &<br>10.2 | 5.5 & 8.4       | 5.4 & 9.5       |
| E.C.P.<br>KNO <sub>3</sub>                                              | 6.6             | 7.9             | 7.5             | 6.1             | 7.1             | 6.7 (9.5<br>?)  |
| % by<br>Titration                                                       | 4.4%            | 4.10%           | 5.27%           | 0.9%            | 1.8%            | 6%              |

#### **Ca/F/Thiolactate batch # 11000059**

Sodium fluoride (4.2146g) was dissolved in 500ml of water (obtained pH = 9.47), and HCl 0.03N as added to reach pH 7.16. This solution was sterilized by  
5 filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1780g) was dissolved in 500ml of water (obtained pH = 9.98) and this solution filtered on 0.22 $\mu$ m and replaced in a clean 800ml Becker. Thiolactic acid (8.5ml) was added (obtained pH = 2.05). Sodium hydroxide and NaOH 0.03M was added to reach pH 7.16 and this solution was sterilized by filtration. The CaCl<sub>2</sub>-thiolactate  
10 solution was added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH is given in Table 15.

#### **Ca/F/Thiolactate batch # 11000060**

Sodium fluoride (4.2161g) was dissolved in 500ml of water (obtained pH =  
15 9.45), and HCl 0.03N as added to reach pH 7.93. This solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1786g) was dissolved in 500ml of water (obtained pH = 9.96) and this solution filtered on 0.22 $\mu$ m and replaced in a clean 800ml Becker. Thiolactic acid (8.5ml) was added (obtained pH = 1.96). Sodium and NaOH 0.03M was added to reach pH 8.07  
20 and this solution was sterilized by filtration. The CaCl<sub>2</sub>-thiolactate solution was

added to the NaF solution. Water washing were carried out according to Scheme 5. Supernatant pH was given in Table 15.

**Ca/F/Adipic acid batch # 11000129**

5 Adipic acid (7.3070g) was dissolved in 500ml of water (obtained pH = 2.81). Sodium hydroxide was added to reach pH5.39. Sodium fluoride (4.1967g) was added; the pH at this stage was 5.59. More NaOH was added to reach pH 8.02, and this solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask.

10 Calcium chloride (11.1481g) was dissolved in 500ml of water (obtained pH =10.81). This solution was sterilized by filtration and poured into NaF/Adipate sterile solution. Water washing were carried out according to Scheme 6. Supernatant pH is given in Table 15).

**15 Ca/F/Adipic acid batch # 11481026**

Adipic acid (7.3174g) was dissolved in 500ml of water (obtained pH = 2.99). Sodium hydroxide was added to reach pH5.11. Sodium fluoride (4.1979g) was added; the pH at this stage was 5.48. More NaOH was added to reach pH 5.94, and this solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott  
20 flask.

Calcium chloride (11.1163g) was dissolved in 500ml of water (obtained pH =9.82). This solution was sterilized by filtration and poured into NaF/Adipate sterile solution. Water washing were carried out according to Scheme 6. Supernatant pH is given in Table 15.

25

**Ca/F/Adipic acid batch # 11481027**

Adipic acid (7.3151g) was dissolved in 500ml of water (obtained pH = 2.98). Sodium was added to reach pH5.36. Sodium fluoride (4.1995g) was added; the pH at this stage was 5.48. More NaOH was added to reach pH 7.18, and this solution  
30 was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask.

Calcium chloride (11.1209g) was dissolved in 500ml of water (obtained pH =9.82). This solution was sterilized by filtration and poured into NaF/Adipate sterile

solution. Water washing were carried out according to Scheme 6. Supernatant pH is given in Table 15.

**Ca/F/Uric acid batch # 11000182**

5 Sodium fluoride (4.1650g) was dissolved in 500ml of water (obtained pH = 9.52). Uric acid (0.13223g) was added by several small portions allowing time (overnight) for dissolution and compensate for pH drops by NaOH (0.5M) addition when needed; pH at this stage being 8.60. This solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride hexahydrate  
10 (21.9929g) was dissolved in 500ml of water (obtained pH =6.24). This solution was sterilized by filtration and poured into NaF/Urate sterile solution. Water washing were carried out according to Scheme 6. Supernatant pH: see Table 14.

**Ca/F/Folic acid (Vitamin M) batch # 11481018**

15 Sodium fluoride (4.2026g) was dissolved in 500ml of water (obtained pH = 9.33). Folic acid (0.4481 g) was added and pH drops to 5.74. NaOH 0.5M was added to reach pH 6.98 and this solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride (11.1183g) was dissolved in 500ml of water (obtained pH =9.73). This solution was sterilized by filtration and poured into  
20 NaF/Folate sterile solution. Water washing were carried out according to Scheme 6. Supernatant pH: see Table 14.

**Ca/F/Hypoxanthine batch 11481198**

Sodium fluoride (4.21 g) was dissolved in 500ml of water, 1.36g of  
25 hypoxanthine was added, NaOH 0.5M was added to reach pH 9.83 and this solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride dihydrate (14.73g) was dissolved in 500ml of water and 1ml of NaOH 0.05M was added to reach pH 9.31 and this solution was sterilized by filtration and poured into NaF/hypoxanthine sterile solution . Water washing were carried out according to  
30 Scheme 6. Supernatant pH see Table 15A.

**Ca/F/Xanthine batch 11481 199**

Sodium fluoride (4.22g) was dissolved in 500ml of water, 1.52g of Xanthine was added, NaOH 0.5M was added to reach pH 10.75 and this solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride dihydrate (14.71 g) was dissolved in 500ml of water and 1ml of NaOH 0.05M was added to reach pH 9.39 and this solution was sterilized by filtration and poured into NaF/Xanthine sterile solution . Water washing were carried out according to Scheme 6. Supernatant pH see Table 15A.

**Ca/F/Guanine batch 11481 195**

To 1.52g of Guanine in 50ml of water NaOH (solid pellets) (1.4g) were added. After dissolution 4.21 g of Sodium fluoride and 450ml of water were added. The pH of the solution was 12.52. This solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride dihydrate (14.71 g) was dissolved in 500ml of water and 0.5ml of NaOH 0.05M was added to reach pH 8.48 and this solution was sterilized by filtration and poured into NaF/guanine sterile solution . Water washing were carried out according to Scheme 6. Supernatant pH see Table 15A.

**Ca/F/Cytosine batch 11954009**

Sodium fluoride (4.22g) was dissolved in 500ml of water, 1.10g of cytosine was added (obtained pH 9.03) and this solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride dihydrate (14.73g) was dissolved in 500ml of water and 1ml of NaOH 0.05M was added to reach pH 9.18 and this solution was sterilized by filtration and poured into NaF/Cytosine sterile solution . Water washing were carried out according to Scheme 6. Supernatant pH see Table 15A.

**Ca/F/Thymine batch 11954064**

Sodium fluoride (4.22g) was dissolved in 500ml of water, 1.26g of hypoxanthine was added, NaOH 0.5M was added to reach pH 9.11 and this solution was sterilized by filtration and placed in a sterile 1 liter Duran-Schott flask. Calcium chloride dihydrate (14.75g) was dissolved in 500ml of water and 0.5ml of NaOH 0.05M was added to reach pH 8.31 and this solution was sterilized by filtration and

poured into NaF/Thymine sterile solution . Water washing were carried out according to Scheme 6. Supernatant pH see Table 15A.

| <b>Table 15A : Precipitation in presence of various organic materials</b> |                          |                               |                           |                           |                          |
|---------------------------------------------------------------------------|--------------------------|-------------------------------|---------------------------|---------------------------|--------------------------|
|                                                                           | <b>Ca/F/<br/>Guanine</b> | <b>Ca/F/<br/>Hypoxanthine</b> | <b>Ca/F/<br/>Xanthine</b> | <b>Ca/F/<br/>Cytosine</b> | <b>Ca/F/<br/>Thymine</b> |
| <b>Batch#</b>                                                             | <b>11481195</b>          | <b>11481198</b>               | <b>11481199</b>           | <b>11954009</b>           | <b>11954064</b>          |
| <b>Starting<br/>pH</b>                                                    | <b>12.52 8.48</b>        | <b>9.83 9.31</b>              | <b>10.75 9.39</b>         | <b>9.03 9.18</b>          | <b>9.11 8.31</b>         |
| <b>Washing<br/>pH</b>                                                     | <b>pH</b>                | <b>pH</b>                     | <b>pH</b>                 | <b>pH</b>                 | <b>pH</b>                |
| W1                                                                        | 11.66                    | 9.12                          | 9.05                      | 6.92                      | 8.66                     |
| W2                                                                        | 11.55                    | 9.21                          | 9.15                      | 6.68                      | 8.82                     |
| W3                                                                        | 11.54                    | 9.26                          | 9.28                      | 6.12                      | 8.86                     |
| W4                                                                        | 11.51                    | 9.46                          | 9.31                      | 6.15                      | 8.75                     |
| W5                                                                        | 11.50                    | 9.50                          | 8.48                      | 5.94                      | 8.23                     |
| W6                                                                        | 11.42                    | 9.62                          | 9.27                      | 5.99                      | 6.52                     |
| W7                                                                        | 11.32                    | 9.68                          | 9.49                      | 6.29                      | 6.37                     |
| W8                                                                        | 11.29                    | 9.67                          | 8.62                      | 5.90                      | 6.35                     |
| W9                                                                        | 11.29                    | 9.60                          | 9.52                      | 5.81                      | 6.63                     |
| W10                                                                       | 11.19                    | 9.56                          | 9.63                      | 5.82                      | 6.66                     |
| Conc.                                                                     | 21.5                     | 13.09                         | 20.3                      | 13.74                     | 9.8                      |
| Ca% F%                                                                    | 44.2 38.3                | 48.0 44.0                     | 48.7 44.3                 | 49.8 45.3                 | 43.7 45.8                |

#### **CaAdipate batch 11954096**

- 5            Calcium carbonate (5g ) was suspended in 500ml of water. Adipic acid (7.3g) was added. Additional water quantities were added until the volume reaches 750ml and the mix was heated at 60°C during 1 hour. This solution was sterilized by filtration (0.22 $\mu$ m filter). The resulting solution heated and concentrated by evaporation until about 280ml total volume. Crystals were separated from the supernatant and dried at 80°C during 5 days. This product is used as Calcium adipate reference during thermogravimetry measurements.
- 10

**Analysis of composite organic content by thermogravimetry.**

Dry material sample were submitted to thermogravimetry. Weight losses from RT to 600°C under N<sub>2</sub> and from 600°C to 800°C under O<sub>2</sub> were recorded and represent by difference the quantity of burned organic material.

| <b>Table 15B: Results of thermogravimetry analyses</b> |                                     |      |                                      |       |                                       |      |                                   |
|--------------------------------------------------------|-------------------------------------|------|--------------------------------------|-------|---------------------------------------|------|-----------------------------------|
| <b>Weight losses / samples</b>                         | <b>Between 0 and 200°C under N2</b> |      | <b>Between 200 et 600°C under N2</b> |       | <b>Between 600 et 800°C under Air</b> |      | <b>Residual material at 800°C</b> |
| <b>11000123<br/>CaF<sub>2</sub> (without organic)</b>  | 0.5%                                |      | 0.9%                                 |       | 0.1%                                  |      | 98.5%                             |
|                                                        |                                     | Δ    |                                      | Δ     |                                       | Δ    |                                   |
| <b>Thymine</b>                                         | 100%                                |      |                                      |       |                                       |      |                                   |
| <b>11954064<br/>Ca/F/Thymine</b>                       | 0.7%                                | 0.2% | 1.1%                                 | 0.2%  | 0.1%                                  | 0%   | 98.1%                             |
| <b>Adipic acid</b>                                     | 100%                                |      |                                      |       |                                       |      |                                   |
| <b>11954096<br/>Ca-Adipate</b>                         | 6.9%                                |      | 42.5%                                |       | 22.3%                                 |      | 28.3%                             |
| <b>11000129<br/>Ca/F/Adipate</b>                       | 1.5%                                | 1%   | 7.5%                                 | 6.6%  | 3.0%                                  | 2.9% | 88.0%                             |
| <b>11000060<br/>Ca/F/Thiolactate</b>                   | 1.1%                                | 0.6% | 2.4%                                 | 1.5%  | 0.3%                                  | 0.2% | 96.2%                             |
| <b>11000083<br/>Ca/F/CO<sub>3</sub></b>                | 2.3%                                | 1.8% | 2.0%                                 | 1.1%  | 2.1%                                  | 2.0% | 93.6%                             |
| <b>11000099<br/>Ca/F/Glutathion</b>                    | 2.3%                                | 1.8% | 5.7%                                 | 4.8%  | 2.1%                                  | 2.0% | 89.9%                             |
| <b>11000140<br/>Ca/F/Glutathione oxide</b>             | 3.2%                                | 2.7% | 13.3 %                               | 12.4% | 4.8%                                  | 4.7% | 78.7%                             |
| <b>11481018<br/>Ca/F/Folic</b>                         | 2.4%                                | 1.9% | 6.3%                                 | 5.4%  | 3.5%                                  | 3.4% | 87.8%                             |

|                                               |       |      |       |       |       |       |       |
|-----------------------------------------------|-------|------|-------|-------|-------|-------|-------|
| <b>11481064</b><br><b>Ca/F/Cysteine</b>       | 1.9%  | 1.4% | 3.1 % | 2.2%  | 0.2%  | 0.1 % | 94.8% |
| <b>11481 133</b><br><b>Ca/F/N-Ac-Cysteine</b> | 1.5%  | 1.0% | 2.4%  | 1.5%  | 0.3%  | 0.2%  | 95.8% |
| <b>Uric acid</b>                              | 0.1 % |      | 74.6% |       | 25.3% |       |       |
| <b>11481 186</b><br><b>Ca/F/Uric</b>          | 1.4%  | 0.9% | 1.9%  | 1.0%  | 0.4%  | 0.3%  | 96.3% |
| <b>11481 195</b><br><b>Ca/F/Guanine</b>       | 2.2%  | 1.7% | 5.0%  | 4.1 % | 2.5%  | 2.4%  | 90.3% |
| <b>Hypoxanthine</b>                           | 0.2%  |      | 50.6% |       | 49.2% |       |       |
| <b>11481 198</b><br><b>Ca/F/Hypoxanthine</b>  | 1.2%  | 0.7% | 2.0%  | 1.1 % | 0.5%  | 0.4%  | 96.3% |
| <b>Xanthine</b>                               | 0.3%  |      | 67.7% |       | 32.0% |       |       |
| <b>11481 199</b><br><b>Ca/F/Xanthine</b>      | 2.5%  | 2.0% | 3.8%  | 2.9%  | 1.4%  | 1.3%  | 92.3% |
| <b>11954009</b><br><b>Ca/F/Cytosine</b>       | 0.5%  | 0%   | 1.0%  | 0.1 % | 0.1 % | 0%    | 98.4% |

#### Example 3A. Adsorption of MPL

Adsorption of MPL (nanoparticles in water) on various inorganic particles were carried out under aseptic conditions and % of MPL adsorption measured by STEP® technology (space- and time-resolved extinction profile) using LumiSizer® instrument. Table 16 summarizes those adsorptions data and shows that presence of the vaterite type of carbonate in the calcium-fluoride-carbonate composite allows 100% adsorption of 100µg MPL on 500µg inorganic composite in 1ml water. Ca/F/N-Acetyl-cysteine (batch 1061 6125) gives similar results.

**Table 16: Estimated % of adsorption of 1169.6µg MPL on various carriers quantities based on the remaining supernatant % of transmitted light**

|                                                                                                                                                         | Carrier weights          |        |        |         |         | % CaCO <sub>3</sub> by titration |
|---------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|--------|--------|---------|---------|----------------------------------|
|                                                                                                                                                         | 1721pg                   | 3442pg | 6884pg | 11703Mg | 23407pg |                                  |
|                                                                                                                                                         | 100µg MPL/µg carrier/ ml |        |        |         |         |                                  |
| µg carrier/ ml →                                                                                                                                        | 125                      | 250    | 500    | 1000    | 2000    |                                  |
| CaF <sub>2</sub> (#9440194) t=0                                                                                                                         | ND                       | ND     | 20%    | 66%     | 100%    | 0                                |
| CaF <sub>2</sub> (#9440194) t=5days                                                                                                                     | ND                       | ND     | 66%    | ND      | ND      | NA                               |
| Ca/F/CO <sub>3</sub> (#9923124) t=0<br>Vaterite / Calcite ratio:<br>high                                                                                | ND                       | ND     | 66%    | 100%    | 100%    | 2.9                              |
| Ca/F/CO <sub>3</sub> (#9923124)<br>t=5days<br>Vaterite / Calcite ratio:<br>high                                                                         | ND                       | ND     | 100%   | ND      | ND      | ND                               |
| Ca/F/CO <sub>3</sub> (#8833157) t=0<br>CO <sub>3</sub> <sup>2-</sup> / F <sup>-</sup> / Ca <sup>2+</sup> : 1/38/20<br>Vaterite / Calcite ratio:<br>high | ND                       | ND     | 90%    | 80%     | ND      | 4.5                              |
| Ca/F/CO <sub>3</sub> (#8833156) t=0<br>CO <sub>3</sub> <sup>2-</sup> / F <sup>-</sup> / Ca <sup>2+</sup> : 2/36/20<br>Vaterite / Calcite ratio:<br>high | 66%                      | 70%    | 75%    | 80%     | ND      | 11.4                             |
| Ca/F/CO <sub>3</sub> (#8833152) t=0<br>CO <sub>3</sub> <sup>2-</sup> / F <sup>-</sup> / Ca <sup>2+</sup> : 18/04/20<br>Vaterite / Calcite ratio: low    | ND                       | ND     | 0%     | 0%      | ND      | 86                               |
| Ca/F/Cys. (#9440197) t=0                                                                                                                                | ND                       | ND     | 70%    | 100%    | ND      | NA                               |
| Ca/F/Cys. (#9440197) t=5<br>days                                                                                                                        | ND                       | ND     | 70%    | 100%    | ND      | NA                               |
| Ca/F/NACys. (#10616125)<br>t=0                                                                                                                          | ND                       | ND     | 70%    | 100%    | ND      | NA                               |
| Ca/F/NACys. (#10616125)<br>t=5d                                                                                                                         | ND                       | ND     | 100%   | 100%    | ND      | NA                               |

Example 3B. Stability and Thermostability of adsorbed antigen

Improvement of stability and thermostability of adsorbed antigen was demonstrated with F4T, a rather instable antigen. Table 17 summarizes the antigen adsorption conditions and final composition.

| Table 17: Formulation conditions of F4T proteins for stability studies |                                                     |   |                           |   |                                   |   |
|------------------------------------------------------------------------|-----------------------------------------------------|---|---------------------------|---|-----------------------------------|---|
| Inorganic<br>3mg dry material/ml                                       | CaF <sub>2</sub><br>#8833190                        |   | Ca/F/Cysteine<br>#9440099 |   | Ca/F/CO <sub>3</sub><br>#8833172C |   |
| Antigen                                                                | 225µg/ml                                            |   |                           |   |                                   |   |
| Buffer                                                                 | Tris 10mM, Phosphate 2.5mM, NaCl 5mM, Sorbitol 4.7% |   |                           |   |                                   |   |
| pH                                                                     | pH 7.5                                              |   | pH 8.0                    |   | pH 8.0                            |   |
| Immunostimulant*                                                       | -                                                   | + | -                         | + | -                                 | + |
| *Immunostimulant was of the GSK liposome family.                       |                                                     |   |                           |   |                                   |   |

Antigens adsorptions were determined by HPLC quantification carried out on the supernatant after centrifugation (to discard the antigen/inorganic part). No antigen in the supernatant was interpreted as equal to 100% of adsorbed antigen. Stability of adsorption was measured at t=0 and at t=1 month 4°C, while thermo-stability was determined after 1 month 30°C (Table 18).

| <b>Table 18: Adsorption of F4T measured by HPLC-RP</b> |     |               |                |
|--------------------------------------------------------|-----|---------------|----------------|
|                                                        | T=0 | T=1 month 4°C | T=1 month 30°C |
| F4T + CaF <sub>2</sub>                                 | 90  | 80            | 88             |
| F4T + CaF <sub>2</sub> + Liposome                      | 92  | 86            | 90             |
| F4T + Ca/F/Cysteine                                    | 100 | 94            | 93             |
| F4T + Ca/F/Cysteine + liposome                         | 100 | 95            | 93             |
| F4T + Ca/F/CO <sub>3</sub>                             | 93  | 85            | 91             |
| F4T + Ca/F/CO <sub>3</sub> + liposome                  | 94  | 89            | 91             |

Stability of F4T antigens profiles evaluations were carried out by SDS-PAGE analyses at t=0 (Figure 4) and compared at t=1 month 4°C (Figure 5), while thermo-stability was determined after 1 month at 30°C (Figure 6). Being adsorbed by

electrostatic forces, the antigen was released by the SDS-PAGE gel environment and applied experimental migration conditions. In all cases of this stability and thermo-stability measurements, there were impressive conservation of the antigens profile of the adsorbed material compared to none adsorbed control samples.

5

#### Example 4. Immune Response to ClfA<sub>N123</sub> and HepB antigens

##### *Immune Response of Composite-ClfA<sub>N123</sub> adsorbed antigen*

Adsorption of composite-ClfA<sub>N123</sub> was presented in Table 19 and formulations compositions were presented in Table 20.

10

| <b>Table 19: Adsorption of composite-ClfA<sub>N123</sub> measured by HPLC-SEC</b> |     |               |                |
|-----------------------------------------------------------------------------------|-----|---------------|----------------|
|                                                                                   | T=0 | T=1 month 4°C | T=1 month 37°C |
| CompositeClfA <sub>N123</sub> + CaF <sub>2</sub>                                  | 90  | 100           | 94             |
| CompositeClfA <sub>N123</sub> +<br>Ca/F/Cysteine                                  | 100 | 100           | 100            |
| CompositeClfA <sub>N123</sub> +<br>Ca/F/CO <sub>3</sub>                           | 91  | 100           | 100            |
|                                                                                   |     |               |                |

| <b>Table 20: Formulation of compositeClfA<sub>N123</sub> for animal studies</b> |            |                                          |                        |                              |                      |                         |
|---------------------------------------------------------------------------------|------------|------------------------------------------|------------------------|------------------------------|----------------------|-------------------------|
| <b>Groups</b>                                                                   |            | <b>Composite<br/>ClfA<sub>N123</sub></b> | <b>Buffer<br/>pH 6</b> | <b>Immuno-<br/>stimulant</b> | <b>Sorbito<br/>I</b> | <b>mOsmoles/<br/>kg</b> |
| None<br>adsorbed                                                                | -          | 60µg/ml                                  | Maleate<br>10mM        | Emulsion                     | 4.7%                 | 319                     |
| Ca/F/CO <sub>3</sub><br>#9440195                                                | 1mg/<br>ml | 60µg/ml                                  | Maleate<br>10mM        | Emulsion                     | 4.7%                 | 310                     |
| Ca/F/N-<br>Acetyl-<br>Cysteine<br>#9440196                                      | 1mg/<br>ml | 45µg/ml                                  | Maleate<br>7.5mM       | Emulsion                     | 3.5%                 | 235                     |
| CaF <sub>2</sub><br>#8833190                                                    | 1mg/<br>ml | 60µg/ml                                  | Maleate<br>10mM        | Emulsion                     | 4.7%                 | 314                     |
| Ca/F/                                                                           | 1mg/       | 60µg/ml                                  | Maleate                | Emulsion                     | 4.7%                 | 308                     |

| <b>Table 20: Formulation of compositeClfA<sub>N</sub>i23 for animal studies</b> |    |                                          |                        |                              |                      |                         |
|---------------------------------------------------------------------------------|----|------------------------------------------|------------------------|------------------------------|----------------------|-------------------------|
| <b>Groups</b>                                                                   |    | <b>Composite<br/>ClfA<sub>N</sub>i23</b> | <b>Buffer<br/>pH 6</b> | <b>Immuno-<br/>stimulant</b> | <b>Sorbito<br/>I</b> | <b>mOsmoles/<br/>kg</b> |
| Cysteine<br>#94401 97                                                           | ml |                                          | 10mM                   |                              |                      |                         |

As shown (Figure 7) the immunogenicity of the antigen was maintained when the antigen was adsorbed on the different carriers.

#### 5 Immune response of HepB adsorbed antigen

Formulations compositions are presented (Table 21).

| <b>Table 21: Animal antibodies obtained with Hepb (20µg) adsorbed on inorganic carrier (1240µg) and possible correlation with organic content of Calcium fluoride composites</b> |                |                        |                             |                           |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|------------------------|-----------------------------|---------------------------|
| <b>Formulations</b>                                                                                                                                                              | <b>Batch #</b> | <b>Antibody</b>        | <b>Performance</b>          | <b>Organic content</b>    |
|                                                                                                                                                                                  |                | <b>GMT+/-<br/>IC95</b> |                             | <b>µmoles/<br/>mg dry</b> |
| Engerix (AlOOH)                                                                                                                                                                  | A13AFO0024     | 6208                   | Reference                   | <b>NA</b>                 |
| Ca/F/Glutathione                                                                                                                                                                 | 11000099       | 6165                   | equivalent                  | <b>2.27</b>               |
| Ca/F/Glutathione oxide                                                                                                                                                           | 11000140       | 5850                   |                             | <b>2.34</b>               |
| Ca/F/CO <sub>3</sub>                                                                                                                                                             | 11000083       | 5742                   |                             | <b>1.02</b>               |
| Ca/F/adipate                                                                                                                                                                     | 11000129       | 4247                   | Statistically not different | <b>0.30</b>               |
| Ca/F/N-Acetyl-Cysteine                                                                                                                                                           | 11000101       | 4188                   |                             | <b>0.56</b>               |
| Ca/F/Thiolactate                                                                                                                                                                 | 11000060       | 3298                   | Lower                       | <b>0.17</b>               |
| Ca/F/OH                                                                                                                                                                          | 11000123       | 3078                   |                             | <b>NA</b>                 |
| Ca Phosphate                                                                                                                                                                     | 11000160       | 1475                   |                             | <b>NA</b>                 |

**Table 21** Animal experience LIMS 20130115, all formulated in 10mM TRIS pH 6.8 and sorbitol 4.7% except for Engerix which was in phosphate buffer and 150mM NaCl. CaPhosphate from Brenntag water washed according to Scheme 1 and Table

As shown, the antibody measurements (anti-HBs 14pII) (Figure 1) responses of the antigen were maintained when the antigen was adsorbed on the different carriers of the CaF<sub>2</sub> family described herein.

## 5 Example 5: Immune Response

### *Immune Response of HepB adsorbed antigen*

For this investigation of calcium fluoride composites in vivo, five calcium fluoride composites and AlOOH were selected from Example 4 for repetition using the same calcium fluoride composite batches as used in the Example 4; in addition  
 10 HepB adsorbed on ½ initial calcium fluoride composite quantity was selected for investigation (1240mg versus 600mg calcium fluoride composite). Further, previously untried calcium fluoride composites (containing a Z different from that of the previously tested batches) were selected for this investigation. The composites tested were as follows:

- 15     ▪ 1° AlOOH 1240µg
- 2° AlOOH 600Mg
- 3° CaF<sub>2</sub>/CO<sub>3</sub> 1240Mg
- 4° CaF<sub>2</sub>/CO<sub>3</sub> 600Mg
- 5° CaF<sub>2</sub>N-AcetylCysteine 1240pg
- 20     ▪ 6° CaF<sub>2</sub>N-AcetylCysteine 600Mg
- 7° CaF<sub>2</sub>/Glutathione 1240pg
- 8° CaF<sub>2</sub>/Glutathione oxide 1240pg
- 9° CaF<sub>2</sub>/Adipate 1240pg
- 10° CaF<sub>2</sub>/Uric acid 1240µg (new calcium fluoride composite)
- 25     - 11° CaF<sub>2</sub>/Uric acid 600µg (new calcium fluoride composite)
- 12° CaF<sub>2</sub>/folic acid 1240µg (new calcium fluoride composite)
- 13° CaF<sub>2</sub>/folic acid 600µg (new calcium fluoride composite)
- 14° HepB only (no carrier)

Preliminary results (without statistical treatment) are summarized in Figure 9 in the  
 30 same order from top (#1 = "Engerix-like" formula) to (# 14 hepB alone).

Example 6: Immunization of Balb/c mice with recombinant F protein adsorbed on different composites induces similar levels of RSV neutralizing and rF binding antibodies than Alum-hydroxide.

Adsorption measurements with composite were conducted with a recombinant RSV F protein (rF): five rF-composite formulations were selected for immunogenicity testing in Balb/C mice, in comparison with Alum hydroxide - or Calcium Phosphate-adsorbed rF (see Table 22).

| GROUP | ANTIGEN (RF) DOS<br>( $\mu$ G/ANIMAL) | ADJUVANT               | ADJUVANT DOSE<br>( $\mu$ G/ANIMAL) |
|-------|---------------------------------------|------------------------|------------------------------------|
| 1     | 2                                     | Alum hydroxide         | 50                                 |
| 2     | 0.1                                   | Alum hydroxide         | 50                                 |
| 3     | 2                                     | CaF2/Adipate           | 60                                 |
| 4     | 0.1                                   | CaF2/Adipate           | 60                                 |
| 5     | 2                                     | CaF2/Cysteine          | 60                                 |
| 6     | 0.1                                   | CaF2/Cysteine          | 60                                 |
| 7     | 2                                     | CaF2/N-acetyl-cysteine | 60                                 |
| 8     | 0.1                                   | CaF2/N-acetyl-cysteine | 60                                 |
| 9     | 2                                     | CaF2/Uric acid         | 60                                 |
| 10    | 0.1                                   | CaF2/Uric acid         | 60                                 |
| 11    | 2                                     | CaF2/Thiolactate       | 60                                 |
| 12    | 0.1                                   | CaF2/ Thiolactate      | 60                                 |
| 13    | 2                                     | CaPhosphate            | 60                                 |
| 14    | 0.1                                   | CaPhosphate            | 60                                 |

**Table 22:** experimental design of the mouse immunogenicity study

Groups of Balb/c mice (n=9/group) were immunized intra-muscularly twice at a 3-week interval with the formulations of Table 22. The rF antigen was used at two different doses with each of the selected adjuvants.

Sera from all mice were individually collected on Day 35 (14 days after the second immunization) and tested for the presence of RSV neutralizing antibodies using a plaque reduction assay and for the anti-rF IgG concentration by ELISA.

For the neutralization assay, serial dilutions of each serum were pre-incubated for 20 min with RSV A (Long strain) at 33°C. After incubation, the virus-serum mixture was transferred to plates previously seeded with Vero cells. On each plate, cells in one

column were incubated with virus only (100% infectivity) and 2 wells received no virus or serum (cell controls). Plates were incubated for 2 hours at 33°C, medium was removed and RSV medium containing 0.5% CMC (low viscosity carboxymethylcellulose) was added to all wells. The plates were incubated for 3 days at 33°C before immunofluorescence staining.

For ELISA, the statistical methods employed to compare different groups were Analysis of Variances (ANOVA 1 or ANOVA 2) on the log 10 values.

Results presented in FIG. 11 indicated that no significant difference could be observed between the neutralizing antibody response induced by any of the composites and alum hydroxide, at the two doses of antigen tested. In addition, at the 2 µg antigen dose, composite adipate induced significantly higher neutralizing antibody titers than the composite N-acetyl-cysteine and the composite uric acid. Calcium Phosphate was the less immunogenic adjuvant as it induced significantly lower neutralizing antibody titers than alum hydroxide (0.1 µg rF), composite adipate (2 µg rF), composite cysteine (2 µg rF) and composite uric acid (0.1 µg rF).

Very similar results were obtained when serum samples were tested for concentrations of anti-rF IgG (FIG. 12).

Example 7: Immunization of Balb/c mice with recombinant F protein adsorbed on different composites is able to significantly reduce RSV viral load in lungs following RSV challenge.

Based on the experiment described in Example 6, composite adipate, composite Cysteine and composite uric acid were selected for a mouse efficacy study, in comparison with Alum hydroxide. The experimental design is described in Table 23.

| GROUP | ANTIGEN (RF) DOSE<br>(µG/ANIMAL) | ADJUVANT       | ADJUVANT DOSE<br>(µG/ANIMAL) |
|-------|----------------------------------|----------------|------------------------------|
| 1     | 2                                | Alum hydroxide | 50                           |
| 2     | 2                                | CaF2/Adipate   | 50                           |
| 3     | 2                                | CaF2/Cysteine  | 60                           |
| 4     | 2                                | CaF2/Uric Acid | 60                           |
| 5     | 0 (PBS group)                    | None           | --                           |

**Table 23:** experimental design of the mouse efficacy study

Groups of Balb/c mice (n=8/group) were immunized intra-muscularly twice at a 3-week interval with the formulations of Table 23. Fourteen days after the second immunization, animals were challenged intra-nasally with  $1.54 \times 10^6$  pfu of RSV. To measure the efficacy of these exemplary vaccines, lungs were harvested 4 days post RSV challenge and individually weighed and homogenized. Serial dilutions (8 replicates each) of each lung homogenate were incubated with Vero cells and wells containing plaques were identified by immunofluorescence, 6 days after seeding. The viral titer was determined using the Spearman-Kärber method for TCID<sub>50</sub> calculation and was expressed per gram of lung. The statistical method employed to compare different groups was an ANOVA 1 on the log<sub>10</sub> values.

Results presented in FIG. 13 indicated that vaccination with 2 µg rF + composite Adipate was the only composite formulation able to completely abolish RSV replication in mouse lungs, as was vaccination with 2 µg rF + alum-OH. The two other composites tested (composite/Cysteine and composite/Uric Acid) did not completely prevent viral replication but significantly ( $P < 0.001$ ) reduced viral replication in the lungs. These results show that despite similar antibody responses, different composites can induce qualitatively different vaccine responses, as shown by the different degrees of inhibition of viral replication in RSV challenged Balb/c mice.

#### Example 8: Evaluation of Composite 19F-DT formulations in the Balb/c mouse immunogenicity model.

Ten different 19F-DT formulations were tested in the Balb/c mouse immunogenicity model through two experiments. Composite formulations were intramuscularly administered at the dose of 0.1 µg 19F-DT at days 0, 14 and 28. IgG levels and OPA titers were determined in individual sera collected at days 28 (ELISA and OPA except for the experience 20140179 made on pooled sera) and 42 (ELISA and OPA). All candidates except the Ca/F/Uric acid and  $\text{Ca}_3(\text{PO}_4)_2$  (used as a control) formulations reached the non-inferiority criteria versus  $\text{AlPO}_4$ . See Figures 14-17.

Example 9: Animal Results of Composite PRIM.

Briefly, groups of BALB/c mice (n=16/group) were immunized intramuscularly twice with a 2-week interval ; Sera from all mice were individually collected , fourteen days after the first immunization and seven days after the second immunization and tested for the presence of anti -PRN IgG antibodies according to the following protocol.

96-well plates were coated with PRN (6 pg/ml) in a carbonate-bicarbonate buffer (50mM) and incubated overnight at 4°C. After the saturation step with the PBS-BSA 1% buffer, mouse sera were diluted at 1/100 in PBS-BSA 0.2% Tween 0.05% and serially diluted in the wells from the plates (12 dilutions, step ½). An anti-mouse IgG coupled to the peroxidase was added (1/5000 dilution). Colorimetric reaction was observed after the addition of the peroxidase substrate (OPDA), and stopped with HCL 1M before reading by spectrophotometry (wavelengths: 490-620 nm). For each serum tested and standard added on each plate, a 4-parameter logistic curve was fit to the relationship between the OD and the dilution (Softmaxpro). This allowed the derivation of each sample titer expressed in STD titers.

| PRN (16µg/ml)                                   | GMT<br>+/- IC95 | GMT +/-<br>IC95 | upper-<br>GMT | GMT-<br>lower | upper-<br>GMT | GMT  |
|-------------------------------------------------|-----------------|-----------------|---------------|---------------|---------------|------|
|                                                 | 14PI            | 7PII            | 14PI          | 14PI          | 7PII          | 7PII |
| <b>PRN-AIOOH-like<br/>Composite( 160µg dry)</b> | 101             | 8878            | 65            | 40            | 7599          | 4094 |
| <b>PRN-AIOOH-like DTPa<br/>(248 µg dry)</b>     | 185             | 14945           | 56            | 43            | 6222          | 4393 |
| <b>PRN-CaF2-CO3</b>                             | 196             | 19110           | 45            | 36            | 9909          | 6525 |
| <b>PRN-CaF2-N-Acetyl-<br/>Cysteine</b>          | 164             | 16669           | 49            | 38            | 7230          | 5043 |
| <b>PRN-CaF2-Cysteine</b>                        | 190             | 13661           | 50            | 39            | 5433          | 3887 |
| <b>PRN-CaF2-Glutathione</b>                     | 186             | 16978           | 59            | 45            | 7261          | 5086 |
| <b>PRN-CaF2-Thiolactate</b>                     | 128             | 9073            | 43            | 32            | 5616          | 3469 |
| <b>PRN-CaF2-Folic acid</b>                      | 165             | 4743            | 113           | 67            | 4180          | 2222 |
| <b>PRN-CaF2-Adipate</b>                         | 368             | 11104           | 83            | 68            | 3979          | 2929 |

|                              |     |       |    |    |       |      |
|------------------------------|-----|-------|----|----|-------|------|
| <b>PRN-CaF2-Guanine</b>      | 129 | 16285 | 39 | 30 | 6535  | 4664 |
| <b>PRN-CaF2-Hypoxanthine</b> | 114 | 9608  | 48 | 34 | 10802 | 5085 |
| <b>PRN-CaF2-Xanthine</b>     | 153 | 19444 | 46 | 36 | 9107  | 6202 |
| <b>PRN-CaF2-Uric acid</b>    | 182 | 15306 | 40 | 33 | 5017  | 3779 |

**Table 24:** PRN antigen with various composites.

#### Example 10: Denge/Composite

Denge-4 formulated in 4.7% sorbitol in TRIS buffer at pH 8.0 was adsorbed on different composite to reach a final concentration of 4µg antigen per ml. After centrifugation, antigen was measured in the supernatant by ELISA. The 100 % ELISA value is given to similar Denge-4 formulation measured after centrifugation. Thus, low ELISA values indicate high adsorption of the antigen on composite. Table 25 indicates the composite quantities involved in each formulations.

| <b>Table 25: Adsorption of DENG-4 on various composite</b> |              |                                        |                                    |            |                                       |
|------------------------------------------------------------|--------------|----------------------------------------|------------------------------------|------------|---------------------------------------|
| Composite                                                  | Batch#       | Composite quantities (µg dry material) | Found Denge-4 ELISA concentrations | % recovery | % recovery relative ( / plain centri) |
| Denge-4                                                    | No Composite | 0                                      | 1.435                              | 35.9       | 100.0                                 |
| Ca/F/Cysteine                                              | 10616183     | 300                                    | <0.05                              | <1.3       | <3.5                                  |
| Ca/F/Adipate                                               | 11000129     | 150                                    | <0.05                              | <1.3       | <3.5                                  |
| Ca/F/CO <sub>3</sub>                                       | 11000083     | 300                                    | <0.05                              | <1.3       | <3.5                                  |
| Ca/F/N-Ac Cysteine                                         | 11481133     | 300                                    | <0.05                              | <1.3       | <3.5                                  |
| Ca/F/Xanthine                                              | 11481199     | 150                                    | <0.05                              | <1.3       | <3.5                                  |
| Ca/F/Uric                                                  | 11481186     | 300                                    | <0.05                              | <1.3       | <3.5                                  |
| Ca/F/Guanine                                               | 11481195     | 150                                    | <0.05                              | <1.3       | <3.5                                  |

Example 11: Adsorption of QS21

The immunostimulant QS21 is too hemolytic to be used in human vaccine by itself. Several formulations containing cholesterol avoid this problem. A hemolytic test are mandatory for new formulations involving quilliac extracts and derivatives (C.Kensil et al., 1991, J. Immunology, vol 146 (2) p. 431-437) (S. Soltysik et al. Vaccine 1995 vol 13 (15) p. 1403-1410) (B. Ronnberg et al., 1995, Vaccine, vol 13 (14) p.1375-1382) (BP. da Silva et al. 2005, Vaccine vol 23 p 1061-1070 and Oliveira-Freitas et al., 2006, Vaccine, vol 24 p 3909-3920 and C. Rodrigues et al., 2012, Chemistry and Biodiversity, vol 9 p. 58-67).

It was found that adsorption of QS21 on inorganic composites prevents hemolysis of chicken red blood cells without addition of cholesterol on the formulation. This was supported by the results obtained using Ca/F/Glutathione at 50, 25 and 12.5µg QS21 adsorbed on 6.7mg inorganic composite (batch 11000099) (Table 26).

| <b>Table 26 : Results of Hemolytic test for Ca/F/Glutathione</b> |                 |                   |             |             |               |             |
|------------------------------------------------------------------|-----------------|-------------------|-------------|-------------|---------------|-------------|
|                                                                  | <b>Carrier</b>  | <b>µg QS21/ml</b> |             |             |               |             |
|                                                                  | <b>11000099</b> | <b>100µg</b>      | <b>50µg</b> | <b>25µg</b> | <b>12.5µg</b> | <b>0µg</b>  |
| Delta DO                                                         | 6.7mg/ml        | 1.072             | 0.017       | -0.006      | -0.006        | 0           |
| Delta DO                                                         | No carrier      | 1.811             | 1.806       | 1.811       | 1.756         | 0           |
| %                                                                |                 | 59                | 0.9         | -0.3        | -0.3          | 0           |
| Status                                                           |                 | <b>Fail</b>       | <b>Pass</b> | <b>Pass</b> | <b>Pass</b>   | <b>Pass</b> |

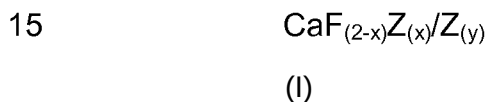
## WE CLAIM:

1. An adjuvant composition comprising a calcium fluoride composite, said composite comprising Ca, F, and Z, wherein Z is glutathione, further comprising an immunologically active saponin fraction.

2. An adjuvant composition of any preceding claim, wherein the composite comprises equal percentages w/w of Ca and F.

3. An adjuvant composition of any preceding claim, wherein the composite comprises a greater percentage Ca w/w than percentage F (w/w).

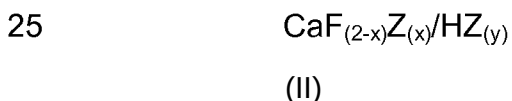
4. An adjuvant composition of any preceding claim, wherein the composite has formula (I):



and wherein where x is a non-negative number from 0 to 2, inclusive, and y is a non-negative number.

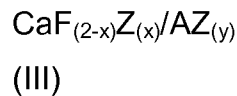
5. An adjuvant composition of claim 4, wherein the composite has a formula in which  $y = 0$ .

6. A calcium fluoride composition of any preceding claim, wherein the composite has formula (II):



and wherein the sum of x and y together is a non-negative number of equal to or less than 2, and neither x nor y is zero.

7. A calcium fluoride composition of any preceding claim, wherein the composite has formula (III):



5 and wherein the sum of x and y together is a non-negative number of equal to or less than 2, neither x nor y is zero, and A is a counterion.

8. An adjuvant composition of any preceding claim,  
wherein Z is glutathione,

10 and wherein the composite comprises between 51% Ca, 48% F, no more than 1% glutathione (w/w) and 28% Ca, 20% F, 52% glutathione (w/w).

9. An adjuvant composition of any preceding claim, wherein the composition is pharmaceutically acceptable.

15 10. An adjuvant composition of any preceding claim, wherein the calcium fluoride composite is in particulate form.

20 11. An adjuvant composition of claim 10, wherein the composite particles are in the nanoparticles or microparticles size domain.

12. An adjuvant composition of any preceding claim, further comprising a second composite wherein Z is not glutathione.

25 13. An adjuvant composition of any preceding claim comprising an antigen, wherein the antigen is adsorbed to a calcium fluoride composite.

14. An adjuvant composition of any preceding claim for use in medicine.

30 15. An adjuvant composition of any preceding claim for use in raising an immune response in a mammal.

16. An adjuvant composition of any preceding claim for use in raising an immune response in a human.

5 17. An adjuvant composition of any preceding claim for use in the prophylaxis and/or treatment of a mammal against disease caused by a virus, bacterium, or parasite.

10 18. An adjuvant composition of any preceding claim for use in the prophylaxis and/or treatment of a human against disease caused by a virus, bacterium, or parasite.

19. An adjuvant composition of any preceding claim for use in decreasing the hemolytic activity of QS21 .

15 20. A method for reducing the hemolytic activity of QS21 comprising combining the steps of combining QS21 with a calcium fluoride composite as claimed in any one of the preceding claims.

20 21. An immunogenic composition comprising an antigen and an adjuvant composition as defined in any one of the preceding claims.

25 22. A method for the treatment or prevention of an infection or a disease caused by a virus, bacterium, or parasite in a mammal, said method comprising administering to said mammal a therapeutically effective amount of an adjuvant composition of any preceding claim.

30 23. A method for the treatment or prevention of an infection or a disease caused by a virus, bacterium, or parasite in a human, said method comprising administering to said human a therapeutically effective amount of an adjuvant composition of any preceding claim.

24. A method for inducing an immunogenic response in a mammal in need thereof, said method comprising administering to said mammal an effective amount of an adjuvant composition of any preceding claim.

- 5           25. A method for inducing an immunogenic response in a human in need thereof, said method comprising administering to said human an effective amount of an adjuvant composition of any preceding claim.

**Fig. 1: Animal results obtained with HepB: antibody measurements (anti-HBs 14pII)**

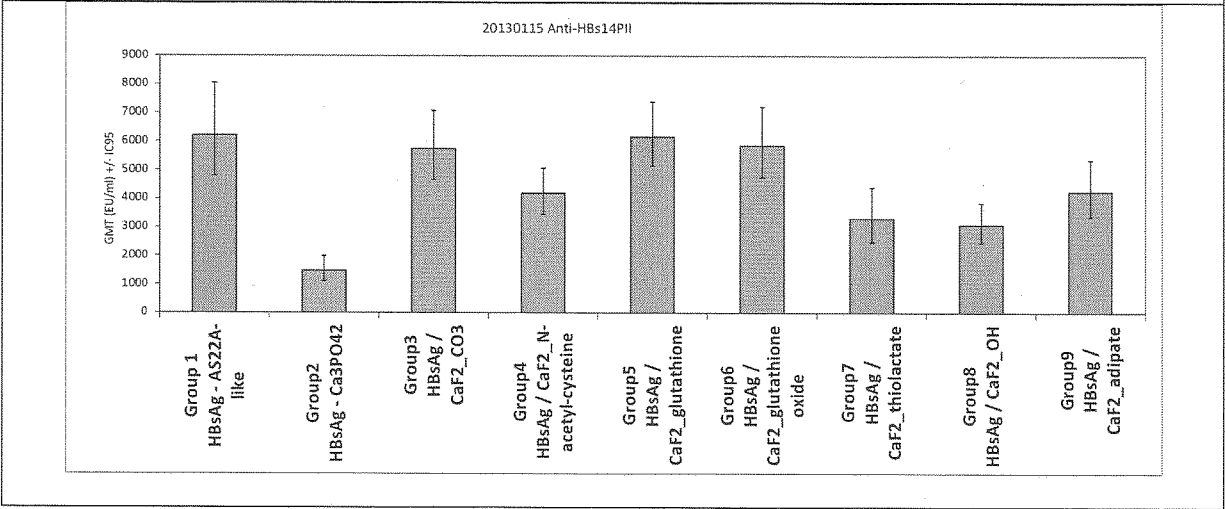


**FIG 1**

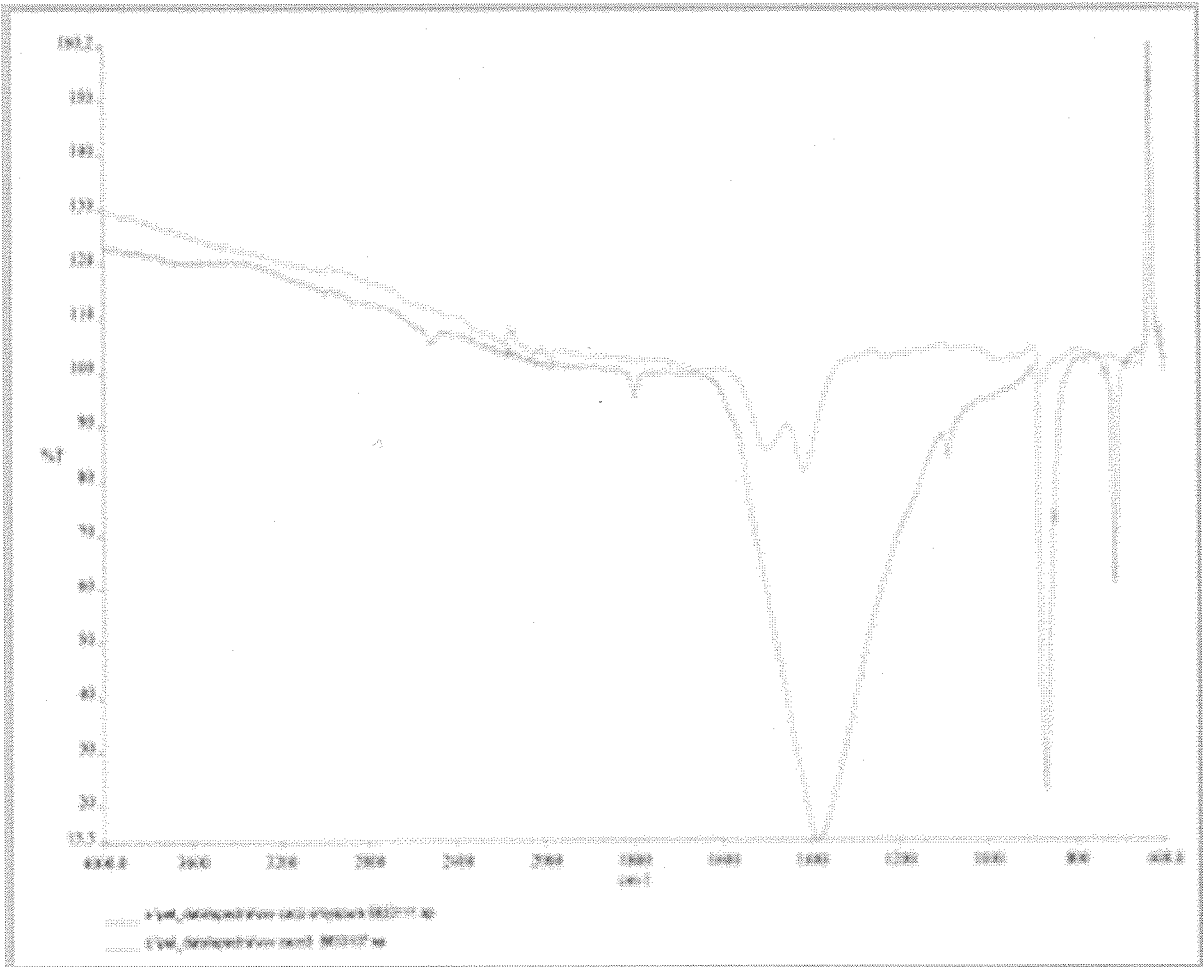
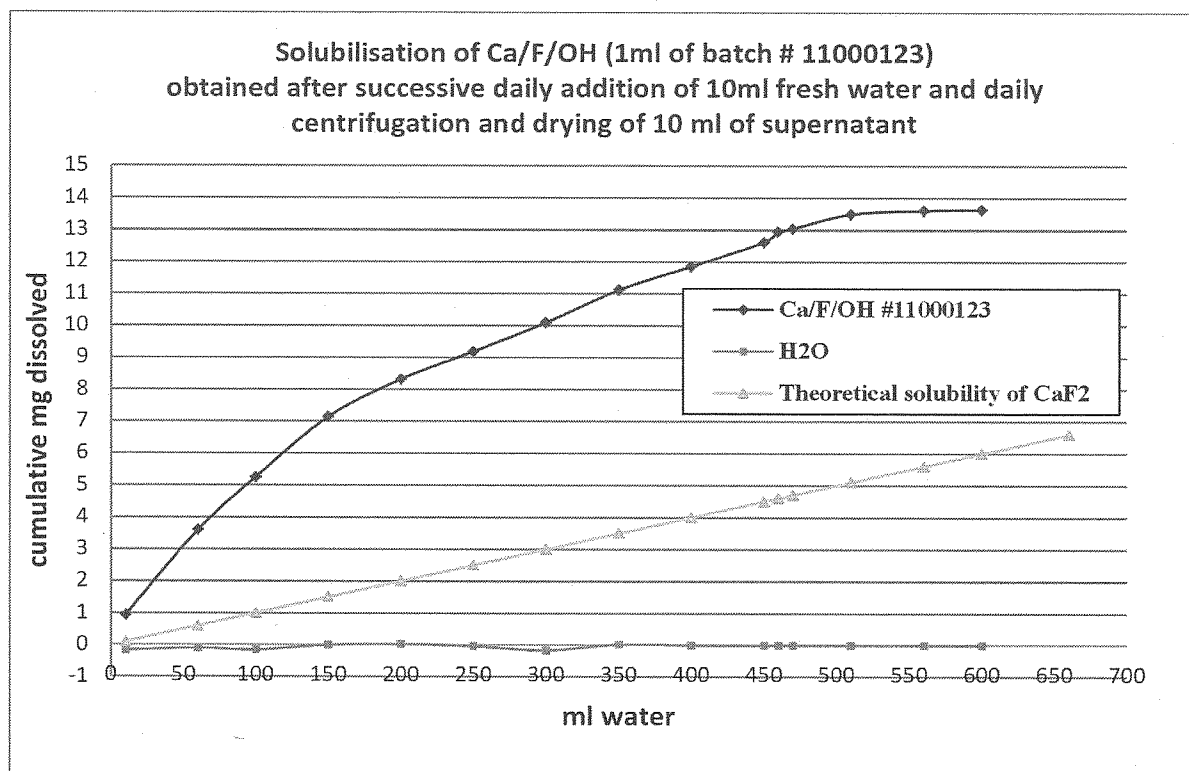
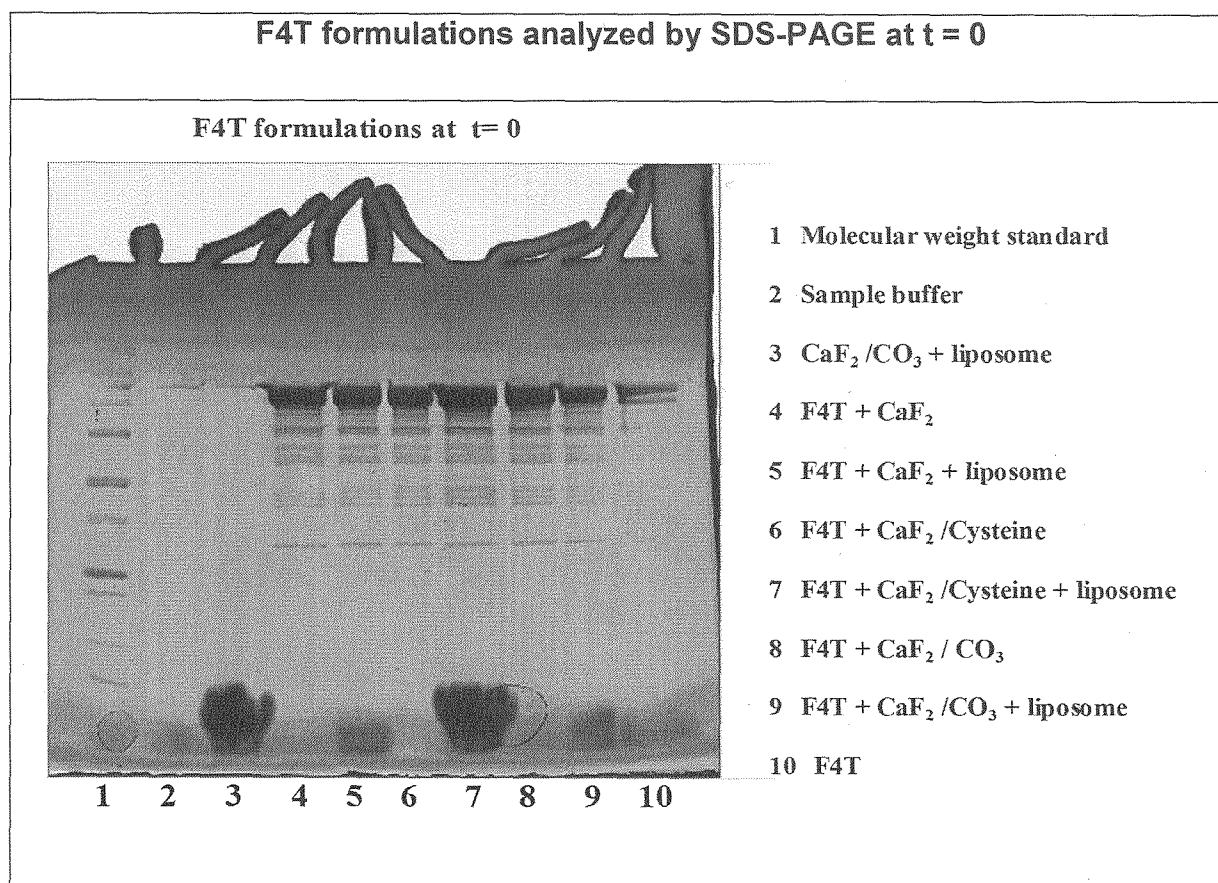


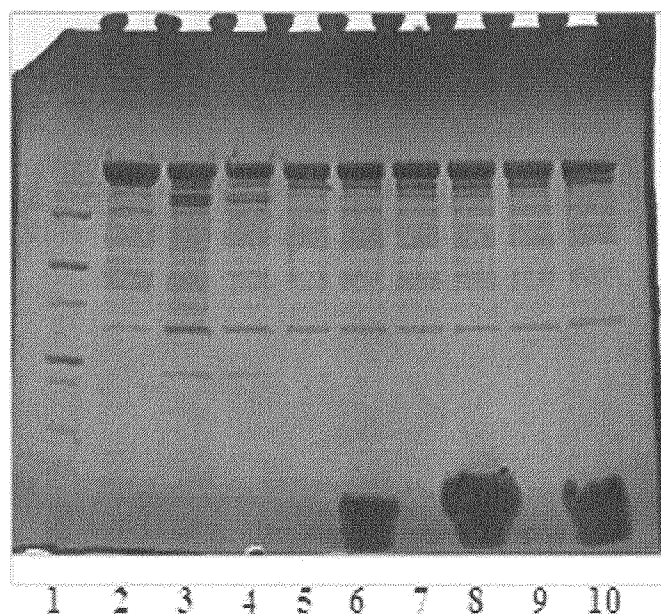
FIG 2

**FIG 3**

**FIG 4**

**F4T formulations analyzed by SDS-PAGE after 1 month at 4°C**

F4T formulations analyzed after 1 month at 4 °C

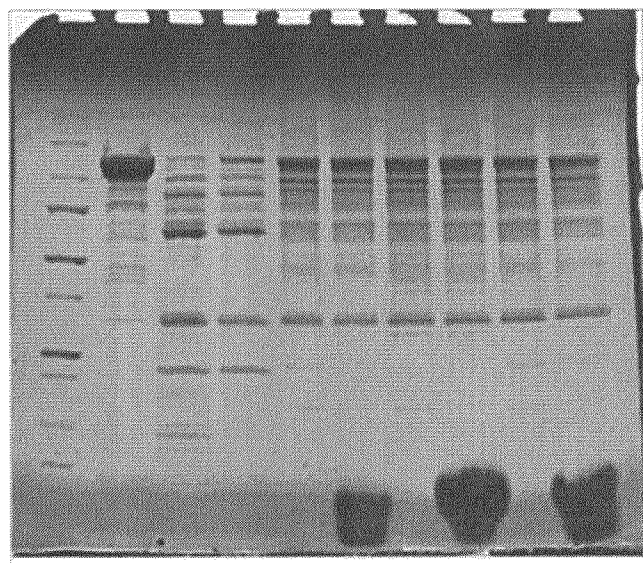


- 1 Molecular weight standard
- 2 F4T bulk: bulk stored 1 month at -80 °C and thawed just before depot
- 3 F4T bulk: stored 1 month at 4 °C
- 4 F4T formulated without inorganic and stored 1 month at 4 °C
- 5 F4T + CaF<sub>2</sub>
- 6 F4T + CaF<sub>2</sub> + liposome
- 7 F4T + CaF<sub>2</sub> Cysteine
- 8 F4T + CaF<sub>2</sub> Cysteine + liposome
- 9 F4T + CaF<sub>2</sub> / CO<sub>2</sub>
- 10 F4T + CaF<sub>2</sub> / CO<sub>2</sub> + liposome

**FIG 5**

**F4T formulations analyzed by SDS-PAGE after 1 month at 30°C**

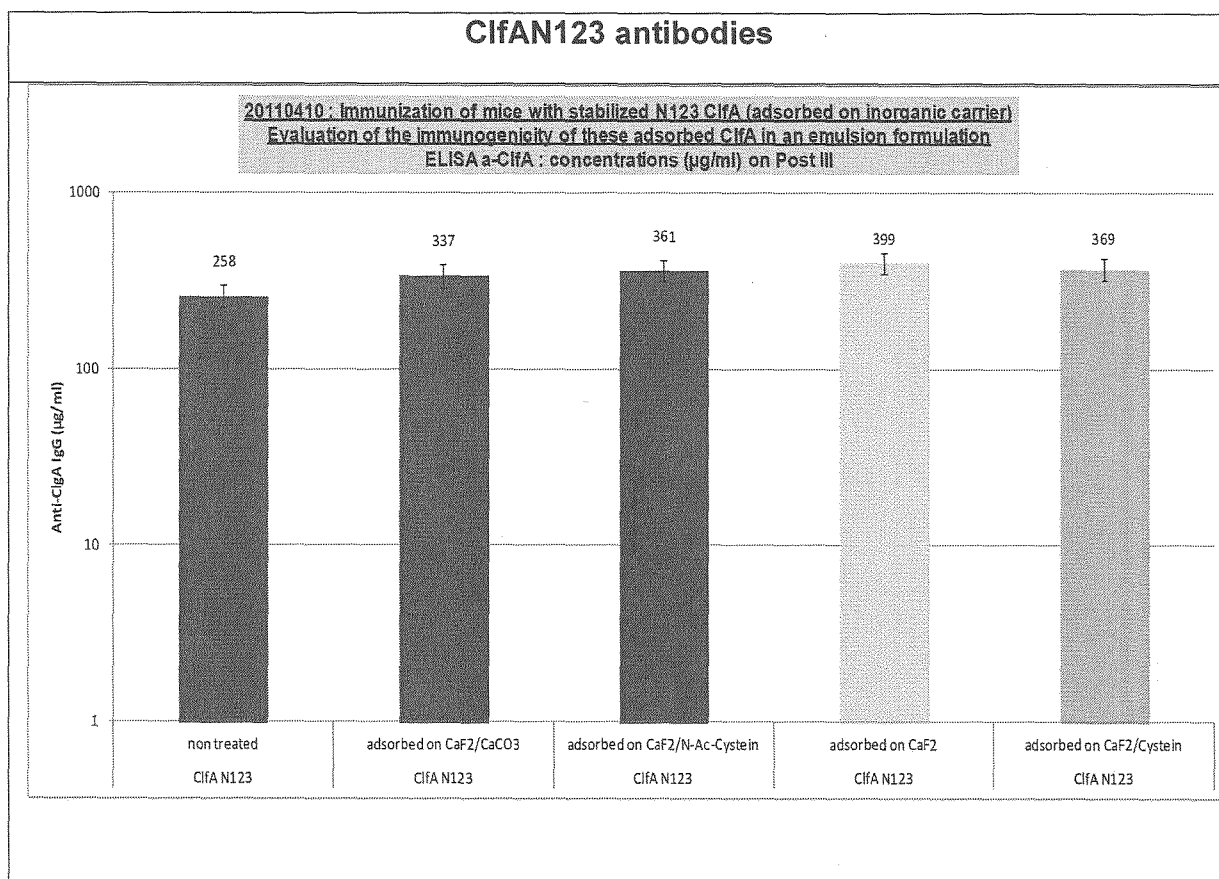
F4T formulations analyzed after 1 month at 30 C

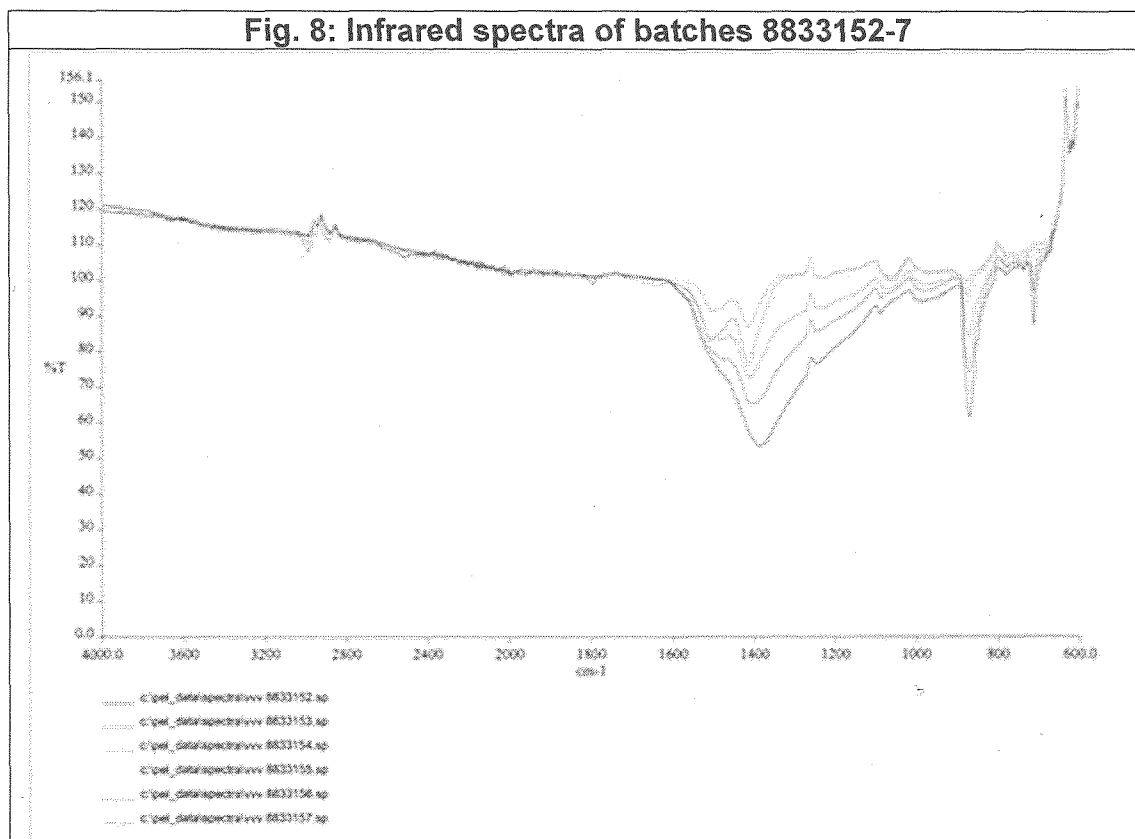


- 1 Molecular weight standard
- 2 F4T bulk stored 1 month at -80 C and thawed just before depot
- 3 F4T bulk stored 1 month at 30 C
- 4 F4T formulated without inorganic and stored 1 month at 30 C
- 5 F4T + CaF<sub>2</sub>
- 6 F4T + CaF<sub>2</sub> + liposome
- 7 F4T + CaF<sub>2</sub> / Cysteine
- 8 F4T + CaF<sub>2</sub> / Cysteine + liposome
- 9 F4T + CaF<sub>2</sub> / CO<sub>2</sub>
- 10 F4T + CaF<sub>2</sub> / CO<sub>2</sub> + liposome

1 2 3 4 5 6 7 8 9 10

**FIG 6**

**FIG 7**

**FIG 8**

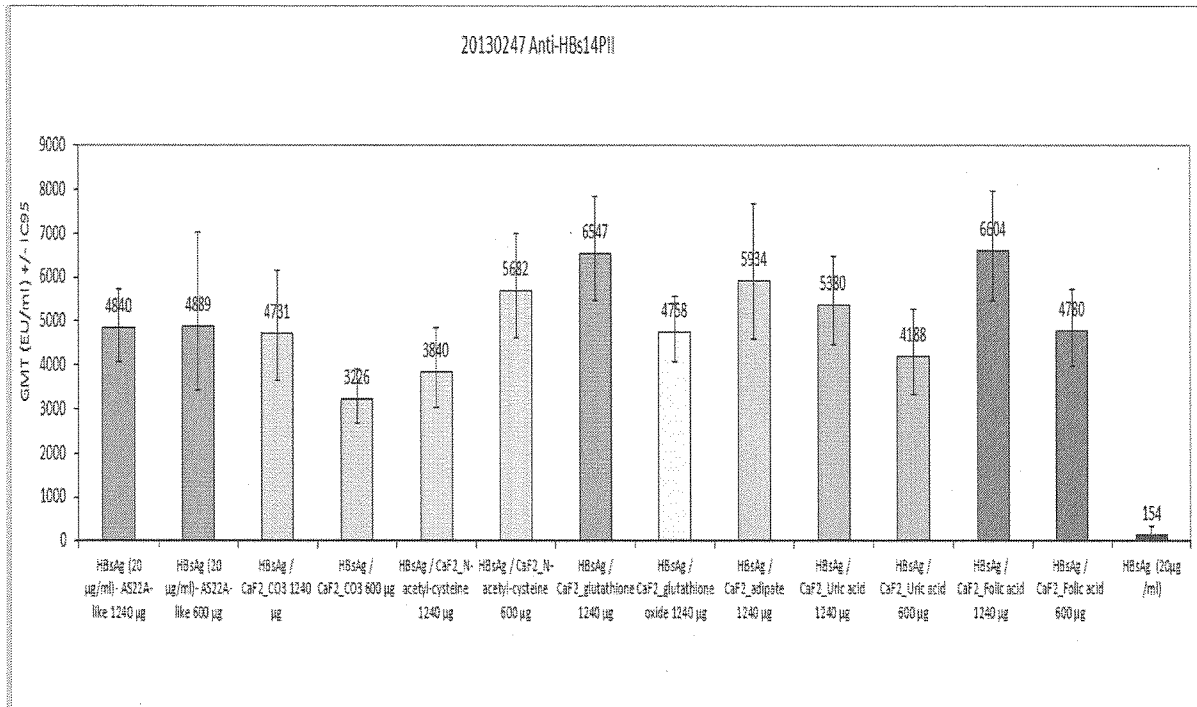
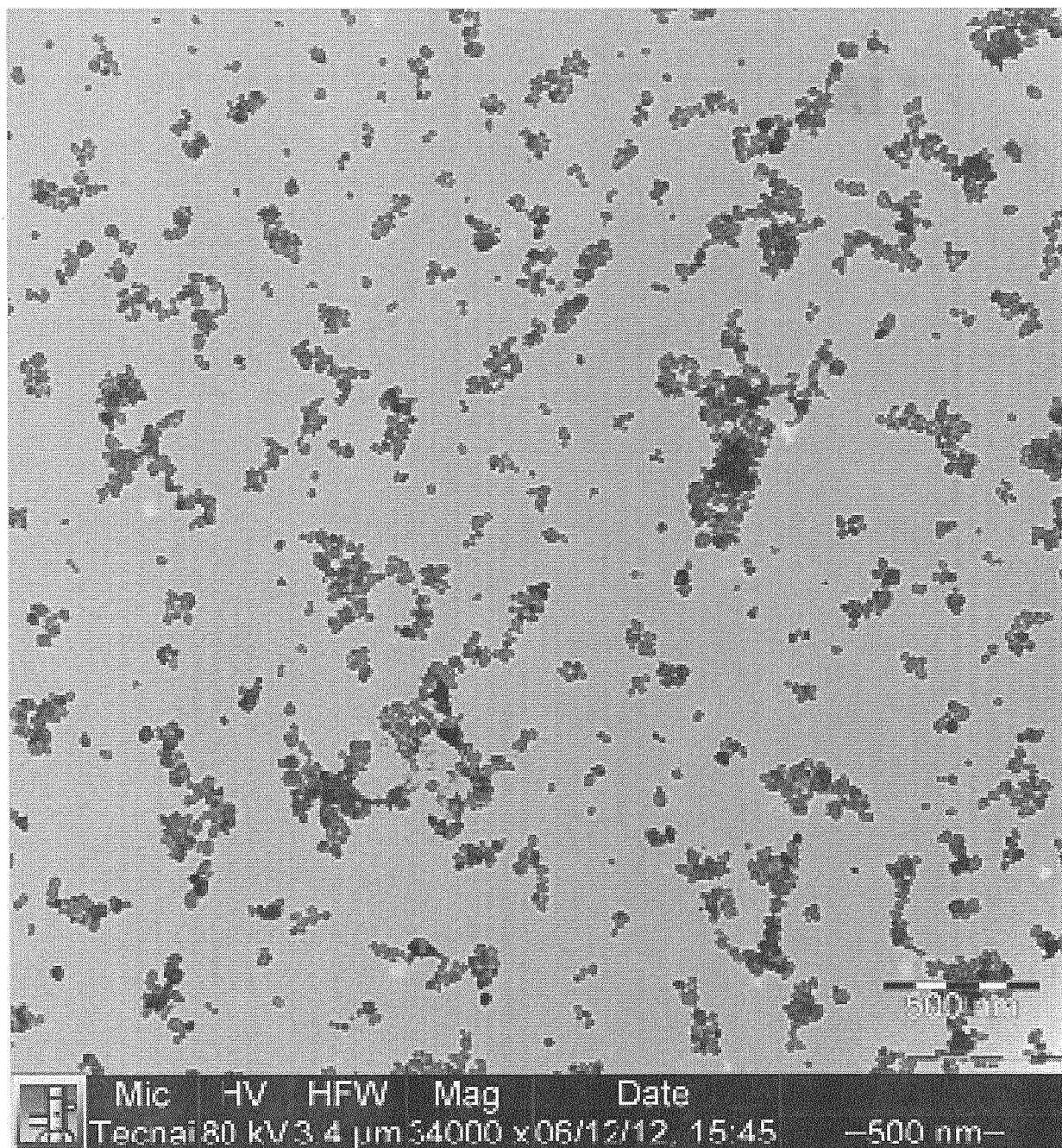


FIG 9

**FIG 10**

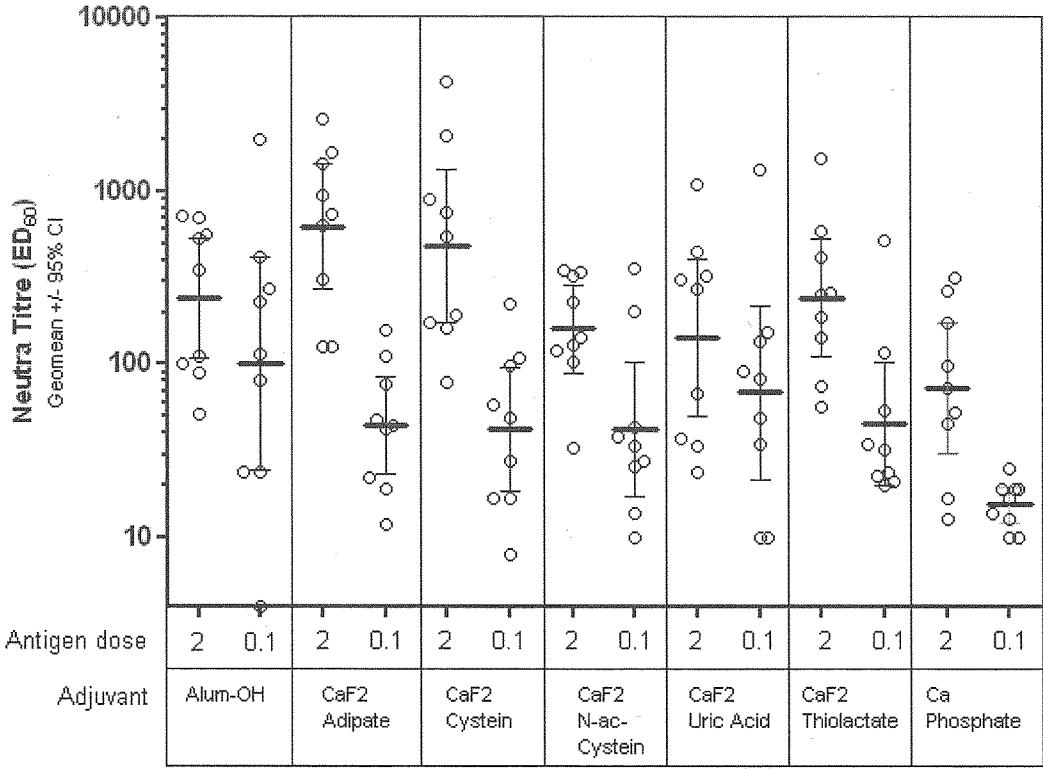


FIG 11

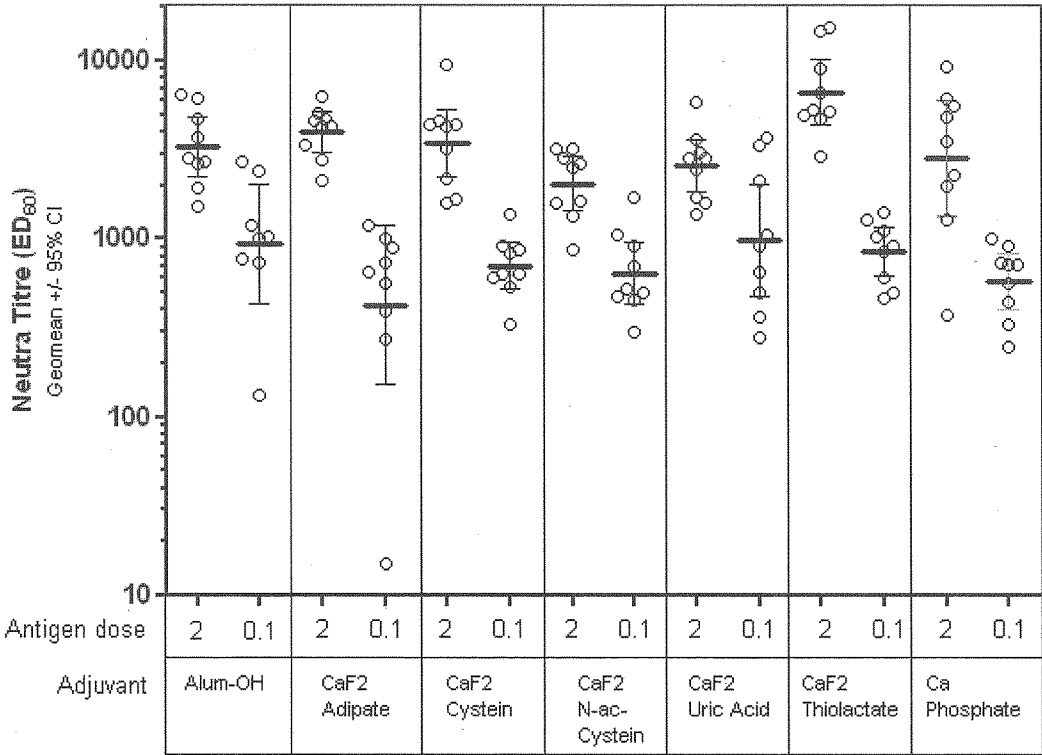


FIG 12

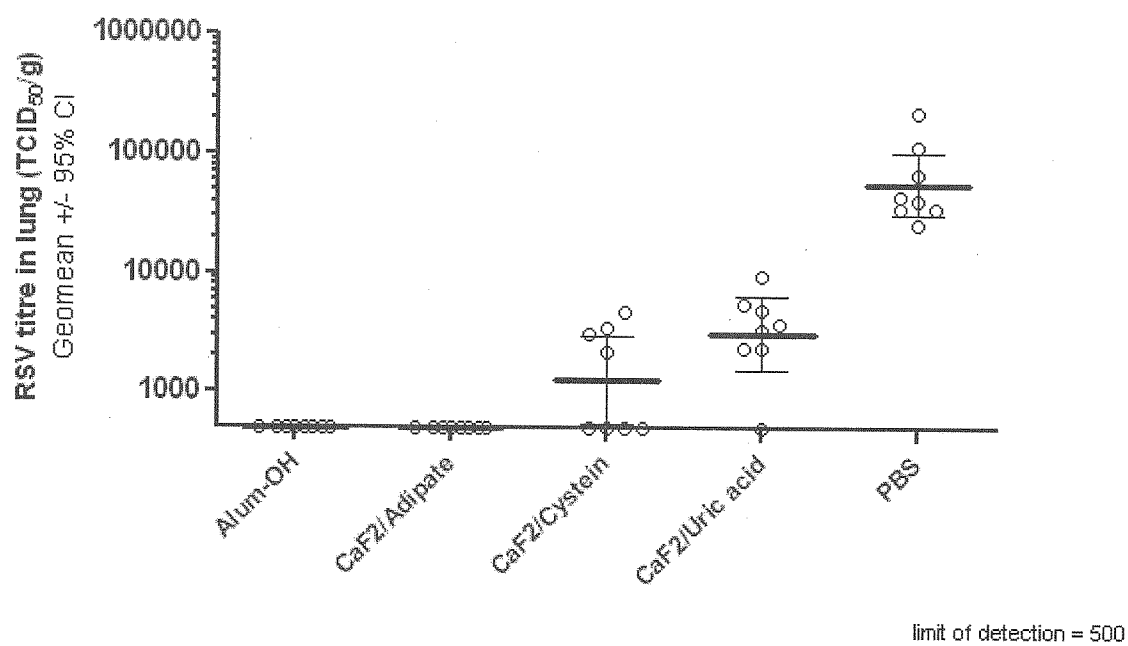


FIG 13

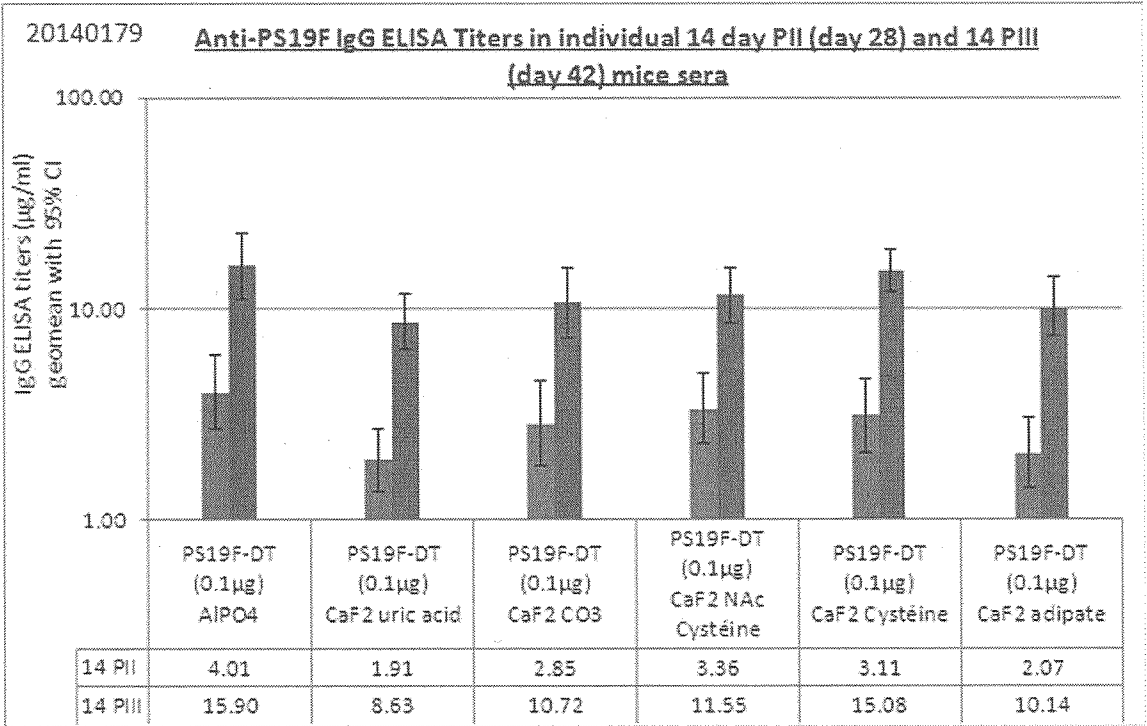
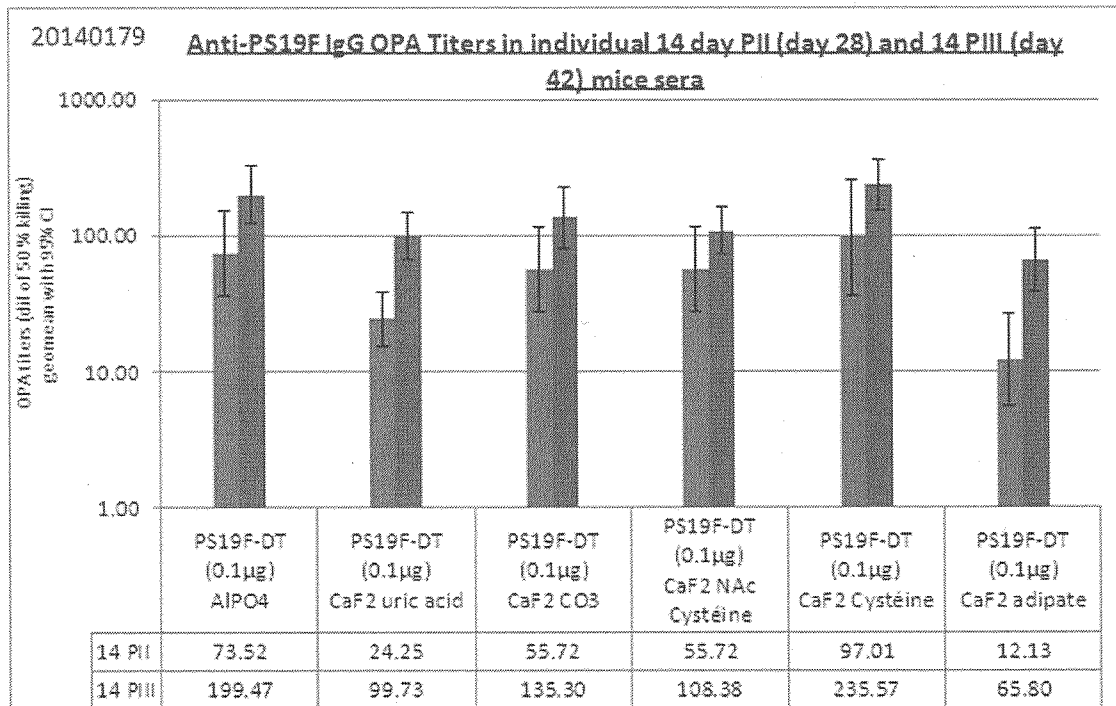


FIG 14

**FIG 15**

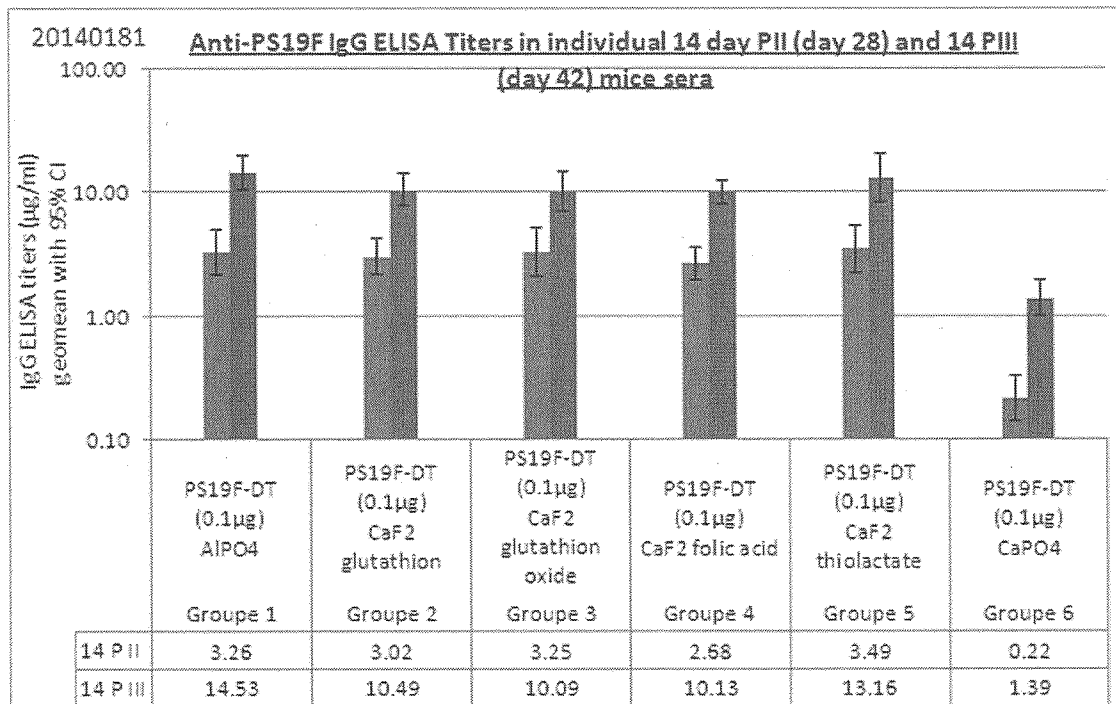


FIG 16

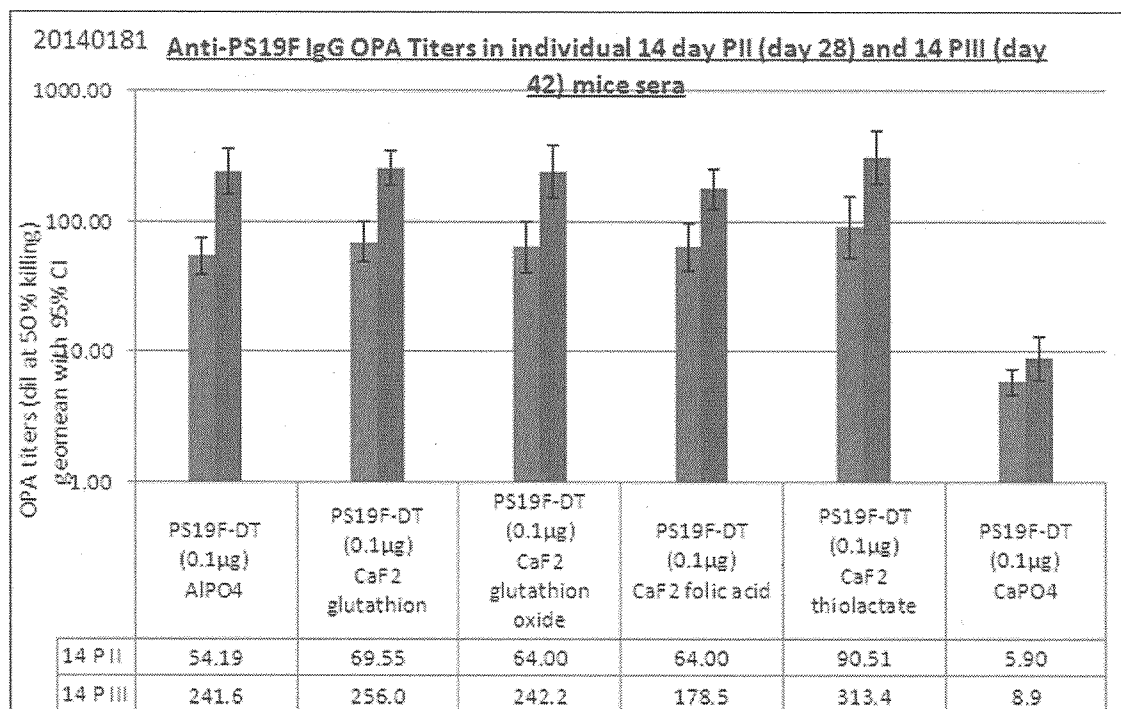


FIG 17

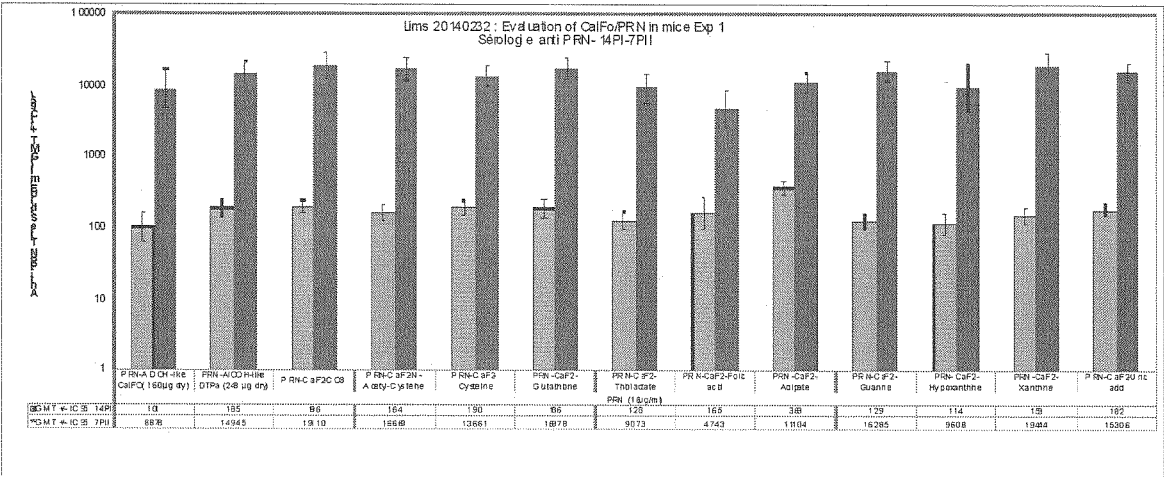


FIG 18

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/072740

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K39/39

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal , BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                 | Relevant to claim No. |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | wo 00/15194 A1 (ETEX CORP [US] )<br>23 March 2000 (2000-03-23)<br>abstract<br>examples 21-35<br>-----                                                              | 1-25                  |
| X         | wo 00/31120 A2 (ZAKRYTOE AKTSIONERNOE<br>OBSHEST [RU] ) 2 June 2000 (2000-06-02)<br>abstract<br>page 80, line 25 - line 27<br>claims 50, 52<br>example 18<br>----- | 1-25                  |
| A         | wo 2006/103112 A2 (LIDDS AB [SE] ;<br>LENNERNAES HANS [SE] ; LENNERNAES B0 [SE] ;<br>HUGOSSON JONA) 5 October 2006 (2006-10-05)<br>abstract<br>-----<br>-/--       | 1-25                  |



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

30 January 2015

Date of mailing of the international search report

17/02/2015

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040,  
Fax: (+31-70) 340-3016

Authorized officer

Mal amoussi , A

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/072740

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                    |                       |
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| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                 | Relevant to claim No. |
| A                                                    | <p>SUMIO HOSHI ET AL: "Adjuvant effects of fluoride on oral immunization of chickens",<br/>VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY,<br/>vol . 63, no. 3, 1 May 1998 (1998-05-01) ,<br/>pages 253-263 , XP055166105 ,<br/>ISSN: 0165-2427 , DOI :<br/>10.1016/0165-2427 (98)00103-2<br/>abstract</p> <p>-----</p> | 1-25                  |

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Information on patent family members

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