

# TREHALOSE MICRONIZATION USING GAS ANTISOLVENT PROCESS.

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The performances of gas antisolvent process for the precipitation of trehalose from aqueous solutions using isopropyl alcohol as modifier to enhance the solubility of water in supercritical carbon dioxide have been investigated using two different bench scale pilot plants.

The experimental tests have been performed using a new patented nozzle for the injection of the supercritical solvent and the solution into the particle precipitation vessel.

The objective of the work is to find the optimal performances of the GAS process, i.e. to maximize the trehalose yield.

All the experimental tests have been performed at 100 bar and 40 °C. Experimental conditions have been determined which allow to obtain trehalose yield of 98%. The influence of experimental conditions on the mean size of powders have been also determined.

## INTRODUCTION

The use of supercritical fluids in the pharmaceutical field for has been proposed for purification, micronization and encapsulation of therapeutic agents [1-5]. Another stimulating application for supercritical fluids is the stabilization of proteins in powder form [6-8].

Both aqueous and powder formulation of a protein therapeutic are affected by protein activity loss due to the irreversible destruction of primary, secondary or tertiary structures, referred as denaturation. Denaturation of proteins can occur both during the dehydration process and during the storage in aqueous solution or in powder form.

Protein therapeutic available in powder form are usually produced from aqueous solutions using lyophilisation. Several precautions must be taken in order to avoid damages that severe stressing steps such as freeze-thawing and drying can cause [9]. In fact, the first step in freeze-dried proteins formulation is the choice of conditions (pH, ionic strength, presence of stabilizers etc...) that guarantee the best protection against proteins unfolding and inactivation.

To reduce or avoid denaturation of proteins, some substances that are known to be very efficient in ameliorating dehydration-induced structural perturbations are used. Many of these substances belong to the group of carbohydrates. For example the disaccharide trehalose has been shown to be very effective [10, 11].

The stabilizing mechanism of these compounds as well as other stabilizers has not fully understood. The stability during drying and storage are explained by both the water

substitution and the vitrification hypotheses. The first states that stabilizers interact with the protein as water does by replacing the removed water and accounts for the thermodynamic control of drying process. The latter states that stabilizers are good glass formers and remain amorphous during and after drying so that they mechanically immobilizes proteins inside a glassy matrix.

A GAS process for the dehydration of proteins based on the use of supercritical fluids has the advantage of avoiding the freezing step, which is detrimental to protein stability, and to maintain the proteins in a favorable aqueous environment until the precipitation occurs. Another advantage relies on the mean size of powders produced, which is usually smaller than that obtained with classical processes like lyophilisation. The size of powders is important for the rapid and easy reconstitution of protein solution with an aqueous buffer prior to their use.

In addition, the stabilizer concentration in the aqueous solution can be determined only according the ratio protein/stabilizer needed for the protein stabilization, and not, as in the case of lyophilisation, to protect proteins during the production process.

The supercritical fluid normally used is carbon dioxide for its favorable properties, in combination with an important amount of a proper modifier to enhance the solubility of water in supercritical carbon dioxide.

To avoid the use of modifier some researchers have proposed to precipitate proteins from organic solutions [8], but the application of such method is limited to those proteins that are not damaged by such solvents.

As modifiers low molecular weight alcohols have been proposed. Ethanol is widely used for precipitation of proteins from aqueous solutions [12, 13]. The drawback of ethanol is that it can damage the protein structure causing their inactivation, even in the harmless supercritical carbon dioxide environment. It is known that isopropyl alcohol is less harmful of ethanol for protein inactivation, so its use is preferable as modifier for protein precipitation from aqueous solutions.

In this work the performances of isopropyl alcohol as modifier in the GAS process for the precipitation of trehalose from aqueous solutions are investigated using two different bench scale plants equipped with a new nozzle for the injection of aqueous solution and of supercritical carbon dioxide in the precipitation vessel [14].

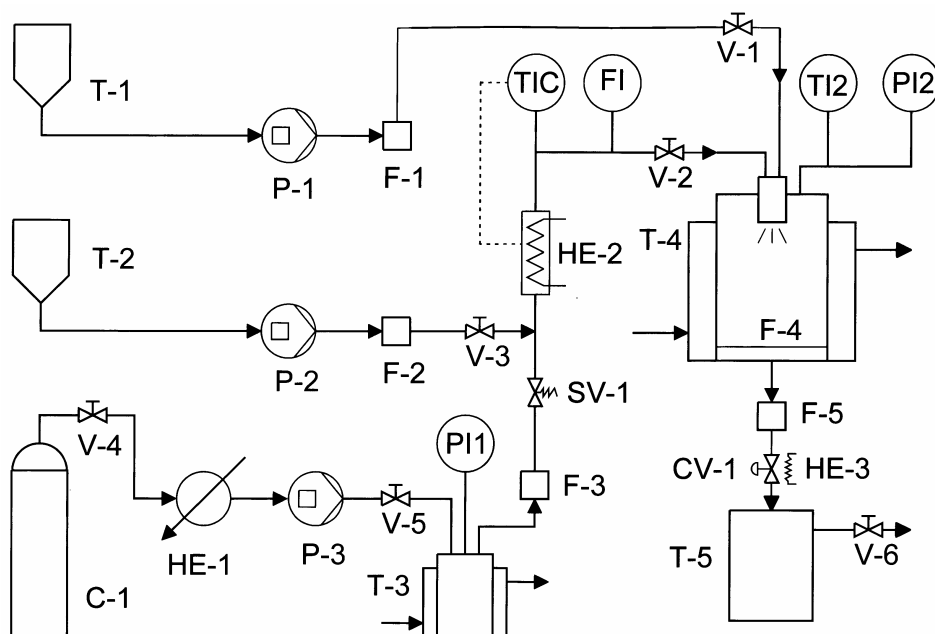
## **I - MATERIALS AND METHODS**

Trehalose was supplied by Sigma, carbon dioxide 99,9 was supplied by Rivoira and 99.8% isopropyl alcohol (IPA) by Fluka.

A PHILIPS SEM 505 electron scanning microscope was used to observe samples of the powders. Particle size distribution was determined by image analysis of SEM images.

Two different bench scale plant equipped with the new nozzle have been used, one is a self built apparatus and the other has been supplied by SEPAREX. The two plants are similar in principle, the latter being equipped with a full automated control and data acquisition system. The volume of the particle formation vessel is also different in the two plants: about 600 cm<sup>3</sup> for the self built plant and about 300 cm<sup>3</sup> for the SEPAREX plant.

A schematic of the experimental apparatus is shown in figure 1. Three high pressure metering pump are used to feed the liquid solution, the co-solvent and the carbon dioxide, the three streams are filtered through 5 µm filters. Carbon dioxide is converted to the gaseous state in the heated tank T-3, then it is mixed with the co-solvent and heated by means of the electric heater HE-2 to a temperature higher than that fixed in the precipitation vessel to take into account the temperature decrease for the pressure drop across the nozzle.



**Figure 1** : Schematic of experimental apparatus for GAS process. T-1: solution tank; T-2: Co-solvent tank; P-1: solution pump; P-2: co-solvent pump; P-3: supercritical fluid pump; F-1, F-2, F-3: line filters; HE-2: electrical heater; CV-1: micro metering valve.

The precipitation vessel T-4 is equipped with an internal basket with a 5  $\mu\text{m}$  sintered stainless steel filter at its bottom to recover the powders.

The duration of each experimental run was set to collect about 200 mg of powders. After stopping the feeding of solution and co-solvent pure carbon dioxide was fed to the precipitation vessel to dry the powders. An amount of carbon dioxide of about two precipitation vessel volume were enough to obtain dry powders. Particle size distribution was determined by image analysis of SEM images.

The nozzle used for the injection of aqueous solution and of supercritical carbon dioxide in the precipitation vessel is described in detail elsewhere [14, 15]. The basic characteristic of the nozzle is that it provides separate inlets for the supercritical fluid and for the solution, that come into contact in the precipitation vessel.

The experimental tests reported in this work have been performed using a nozzle with two outer orifices (diameter 60  $\mu\text{m}$ ) for carbon dioxide inlet and a central orifice (diameter 30  $\mu\text{m}$ ) for the liquid solution inlet.

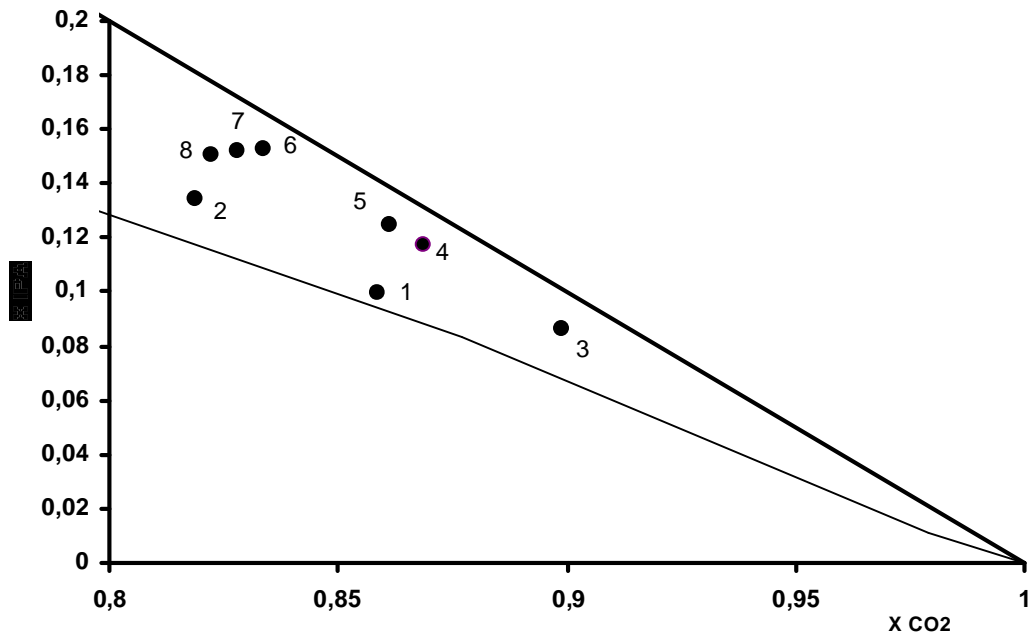
Isopropyl alcohol was used as modifier to enhance the solubility of water in supercritical carbon dioxide.

The phase behavior of the system carbon dioxide- water-isopropyl alcohol (IPA) is rather complex as it can give two phase and three phase equilibrium [16], but this is of limited concern in our case, as the experimental conditions in the GAS process are chosen in such a way to be outside the two phase region, in the carbon dioxide rich phase.

The experimental tests were aimed at finding the optimal performances of the GAS process maximizing the trehalose yield at constant carbon dioxide flow rate, changing the IPA flow rate.

	TEST No	1	2	3	4	5	6	7	8
CO <sub>2</sub> flow rate	gr/min	30	30	30	30	30	30	30	30
IPA flow rate	g/min	4.7	6.7	3.9	5.5	5.9	7.5	7.5	7.5
Solution flow rate	g/min	0.6	0.7	0.2	0.2	0.2	0.2	0.3	0.4
CO <sub>2</sub> mole fraction	-	0.859	0.819	0.898	0.869	0.861	0.833	0.828	0.822
IPA mole fraction	-	0.099	0.134	0.087	0.117	0.125	0.143	0.152	0.151
H <sub>2</sub> O mole fraction	-	0.042	0.047	0.015	0.014	0.014	0.014	0.020	0.027
CO <sub>2</sub> /water	-	50.0	42.9	150.0	150.0	150.0	150.0	100.0	75.0
CO <sub>2</sub> /IPA	-	6.3	4.5	7.6	5.4	5.0	4.0	4.0	4.0
IPA/ water	-	7.9	9.6	19.7	27.6	29.6	37.5	25	18.7
size	μm	-	-	-	2.3	-	2.3	2.4	2.8
Trehalose yield	%	0	0	17	47.7	61.3	70	98.0	88.7

**Table 1 :** Experimental conditions and results of precipitation of trehalose from aqueous solutions at 100 bar and 40 °C, trehalose concentration 2% by weight.



**Figure 2 :** Phase behavior of carbon dioxide-water-isopropyl alcohol (IPA).

□ equilibrium data [16]; ● experimental conditions of experimental tests.

All the experimental tests have been performed at 100 bar and 40 °C with a carbon dioxide flow rate of 30 g/min. The trehalose concentration of the aqueous solution is 2% by weight for all the experimental tests. The experimental conditions and the results are reported in table

1. The results reported for each experimental test are the mean of duplicated tests performed on both bench scale plant available (self built apparatus and SEPAREX plant). Trehalose yields obtained with the two plant are very close.

In figure 2 are reported the equilibrium data of the system carbon dioxide-water-isopropyl alcohol [16], together with the experimental conditions of each test.

For the experimental tests No. 1 and 2 the trehalose yield is 0, showing that there is lower limit for the IPA/water ratio that cannot be exceeded. Experimental tests No. 3, 4, 5 and 6 have been performed at a constant value CO<sub>2</sub>/water ratio of 150, with increasing values of IPA/water ratio, from 19.7 up to 37.5, correspondently the trehalose yield increases from 17 up to 70%. Tests 7 and 8 have the same CO<sub>2</sub>/IPA ratio as test No. 6, but the IPA/water ratio is decreased from 37.5 of the test No. 6, to 25 for test No. 7 and to 18.7 for the test No. 8, correspondingly, the thehalose yield reaches the value of 98.0% and then decreases to 88.7%. The results of the experimental tests show that there is a set of parameters that allows to maximize the trehalose yield.

For the powders obtained in the tests No. 4, 6, 7 and 8, the mean size has been determined by image analysis of SEM images. The mean size of the powders ranges fro 2.3 to 2.8 µm, showing that the different experimental conditions do not have a strong influence on the mean size of the powders.

## CONCLUSION

Precipitation of trehalose from aqueous solutions with the GAS process using isopropyl alcohol as modifier has been investigated in order to find the optimal performances of the process, i.e. maximizing trehalose yield.

The experimental tests were performed using two different bench scale plants equipped with a new nozzle for the injection of carbon dioxide and liquid solution in the precipitation vessel.

A maximum trehalose yield of 98% has been obtained for a CO<sub>2</sub>/IPA ratio of 4 and a IPA/water ratio of 25, at pressure of 100 bar and temperature of 40 °C.

The mean size of trehalose powder has been determined by image analysis of SEM images.

## Acknowledgements

We thank Mr. G. Spagnoli for setting up the experimental apparatus.

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