Study of surfactant-preservative and protein-preservative interactions in multi-dose injectable formulation

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Popular Science Summary

Interactions between components in multi-dose injectable formulations may lead to incompatibilities.

Preservatives are required in this type of formulations to ensure the sterility of the product during clinical use; however, it is known that their interaction with non-ionic surfactants and proteins can cause turbidity and protein aggregation.

But what are the consequences of these defects and how can they be avoided?

These incompatibilities may lead to a decrease in the efficacy of the medicinal product. Nevertheless, these effects occur for specific preservative concentrations. Hence, the need arises for a proper formulation away from concentrations in which the components may present incompatibilities.

This project aims to study in detail at what concentrations these defects become significant.

For this purpose, turbidity of samples containing these components is analysed. This characteristic is related to the concept known as cloud point in the case of surfactant, and aggregation in the case of protein.

A simple, accurate way to analyse the turbidity of a sample is by measuring the scattered light from a laser beam.

Thanks to ProbeDrum® equipment, it is possible to simultaneously perform concentration titrations and scattered light measurements with a detection angle of 90° (side scattering). In this way, any change in the turbidity of a sample containing surfactant and/or protein can be determined while small amounts of preservative are continuously added.

The components used for the study are phenol, polysorbate 80 and somatropin, as preservative, non-ionic surfactant and protein, respectively.

In addition, another aim of the project is to analyse the effect of other components also used in this type of formulations, such as salt and non-ionic tonicity adjusting agents, on these incompatibilities.

Results confirm that phenol causes a significant decrease in the cloud point of polysorbate 80 and an increased aggregation of somatropin. These effects appear at concentration ranges that can be used in multi-dose injectable formulations. In addition, the presence of salt and other tonicity agents reduces the amount of preservative required for the emergence of the cloud point of the surfactant.

Moreover, one of the most interesting results of the project shows that the increased aggregation due to preservative-protein interaction is strongly enhanced by the addition of salt from a very specific concentration. These results are supported by a structural analysis performed with CoSAXS beamline at Max IV.

Acknowledgement

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I would also like to thank my family and friends for their encouragement. I especially would like to express my most sincere gratitude to my parents, Mª de los Ángeles and Pedro, my brother Israel and Irene for their unconditional support at any time. Dedicated to my grandparents and Manuela.

Abstract

Multidose injectable formulations require a preservative such as phenol to ensure sterility and protection against microbial contamination during clinical use. However, it is known that the interaction between these components and non-ionic surfactants such as polysorbate 80, also found in this type of formulations, present incompatibilities, which can lead to decreases in the efficacy of the product. This incompatibility is linked to a decrease in the cloud point of the surfactant.

The presence of other common components in this type of formulations, such as salt, proteins or non-ionic tonicity adjusting agents are also known to influence this phenomenon.

Furthermore, it's known that preservatives can lead to increased aggregation in protein-based formulations as a consequence of preservative-protein interaction.

In order to study these incompatibilities, different concentration titrations were carried out in which the emergence of the cloud point was monitored by simultaneous measurement of light scattering. In general, these titrations consisted of the addition of phenol to samples containing polysorbate 80, salt, protein and tonicity adjusting agents in varying concentrations.

Additionally, preservative-protein interaction was studied by complementary concentration titrations and sample-specific analysis by small-angle X-ray scattering.

Results obtained in the study indicate that the presence of phenol, at concentrations within the range commonly used in multi-dose injectable formulations, leads to a reduction in the cloud point of polysorbate 80 down to 25ºC. In addition, the presence of other components such as salt, somatropin or mannitol is shown to decrease the phenol concentration required for the appearance of this phenomenon.

Moreover, results suggest that the interaction between somatropin and phenol leads to protein aggregation, which is significantly enhanced by the presence of salt.

Keywords: Phenol, polysorbate 80, cloud point, somatropin, aggregation, titration, light scattering, salt, surfactant, preservative, protein, tonicity adjusting agents, multi-dose injectable formulation.

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1. Aim

The aim of this project is to study how the presence of preservatives affects the cloud point of non-ionic surfactants, as well as the possible influence of other common components in multi-dose injectable formulations, such as salt, non-ionic tonicity adjusting agents and proteins, on the emergence of this phenomenon.

Furthermore, the possible protein aggregation resulting from the preservative-protein interaction and the influence of salt on this effect is studied.

2. Introduction

According to different studies, there are about 350 parenteral products on the world market, of which about one third are multi-dose formulations, such as fusion protein solutions, antivenoms and insulin products [1], [2]. They offer several advantages over single dose containers, such as patient convenience, less product waste and a considerable reduction in packaging by delivering multiple doses in a single vial [2].

On the one hand, microbial agents or preservatives are necessary in multi-dose parenteral formulations to ensure sterility and product protection from microbial contamination during patient use [3].

Some of the most commonly used microbial agents in parenteral products are organic preservatives such as phenol, 3-methyl phenol (m-cresol) or benzyl-alcohol [4]. For multi-dose formulations, the first two are currently the most widely used [1], [5].

Compared to phenol, m-cresol has a slightly improved antimicrobial activity. However, several studies report that the activity of the latter is reduced by the presence of non-ionic surfactants, such as polysorbates [1], [5]. Moreover, phenol is less prone to chemical degradation than m-cresol. Regarding the range of concentrations used for this type of formulations, in the case of phenol it varies from 0.15 to 0.50 wt% [4].

On the other hand, amphiphilic surfactants are commonly found in multi-dose injectable formulations. They are used to improve the stability of proteins in solution by associating with air-liquid interfaces and container walls. This helps to prevent protein surface adsorption and potential protein-protein interactions, which can lead to partial misfolding and aggregation. Additionally, they prolong pharmaceutical storage lifetime and improve drug safety profiles [1].

Polysorbate 80 (PS80) is a very common surfactant used in pharmaceutical formulations. It is a non-ionic amphiphilic compound consisting of a hydrophilic polyoxyethylene head group of approximately 20 monomeric units linked by an ester bond to a hydrophobic oleic acid tail [5].

PS80 is both an excellent surface adsorption stabiliser and biocompatible [6]. Its concentration range in this type of pharmaceutical formulations varies from 0.01 to 2 mg/mL [7].

At this point, it should be noted that multi-dose injectable formulations should be uniformly dispersed without turbidity or "cloudiness", which can be an indicator of an unstable solution that can lead to a decrease in the formulation efficacy [1].

However, clouding is a common phenomenon in non-ionic surfactants. As the temperature increases, the system becomes turbid and separates the phases. The temperature at which precipitation occurs is called cloud point. Here, a transition from a single-phase micellar solution to surfactant-rich and surfactant-poor phases takes place. The phase separation presumably occurs due to a decrease in hydration of the head group as it reaches a less polar conformation at higher temperature [8].

Water is believed to be a less effective solvent for hydrophilic groups (polyoxyethylene chain) at higher temperatures, which implies a dependence between cloud point and temperature [8]. In addition, it is known that the cloud point is a reversible phenomenon, so that a decrease in temperature can cause the turbidity in the system to disappear [9].

Another reported effect that makes it interesting to study is that non-ionic surfactants show optimal effectiveness when they are close to the cloud point [10].

Furthermore, the cloud point is very sensitive to the presence of additives in a system, even at very low concentrations [8], [10]. These additives modify the surfactant-solvent interactions, change the critical micellar concentration (cmc), the micelle size and the phase behaviour of surfactant solutions.

In order to achieve good solubilisation of the additives, remaining below the cloud point is crucial, which makes this parameter very important for the formulation [8].

Several studies have shown that the presence of additives has an important influence on the turbidity of surfactants. Many authors report a clear decrease in cloud point in the presence of salts. This reduction is greater the higher the salt concentration, and can lead to a decrease of more than 20°C [6], [8], [10].

In addition, other studies report that the presence of organic preservatives such phenol leads to a significant decrease of the cloud point of non-ionic surfactants [11], [12]. This decrease can reach values below 25ºC [12].

This effect may be a result of the preservative modifying the structure of the micelles by inserting itself between the hydrophilic head groups of the surfactant. This behaviour reduces the repulsion of the long hydrophobic tails of the surfactant, so that larger micelles can be formed [13]. This effect has been reported by other authors for small organic preservatives such as m-cresol [5], as shown in Figure 1.

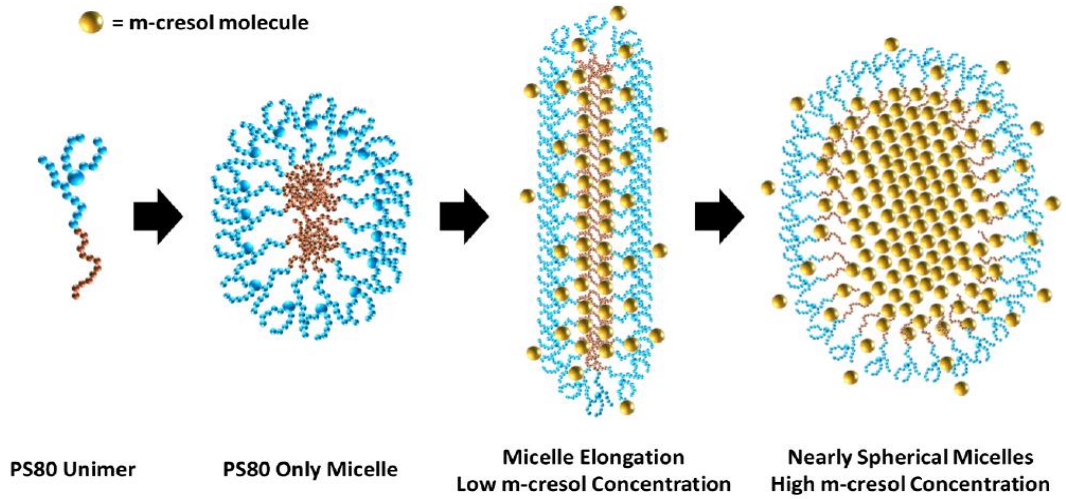

Figure 1. Approximate representations of a PS80 unimer (left), a PS80 micelle in deuterium oxide (left-centre), a PS80/m-cresol micelle in deuterium oxide with a low concentration of m-cresol (< 2 mg/mL) (centre right) and a PS80/m-cresol micelle in deuterium oxide with a high concentration of m-cresol (> 4.5 mg/mL) (right). The hydrophobic PS80 tails and m-cresol are mainly found in the micelle core, while the hydrophilic head group remains in the micelle shell. These illustrations are not to scale and are intended to show the changes in the overall morphology of the micelle with m-cresol concentration [5].

Additionally, the presence of an alcohol also affects the physical properties of water, altering its structure and increasing the lipophilic character of PS80 [9].

In the case of proteins, the presence of phenolic preservatives can lead to interactions that may result in increased aggregation, higher instability at elevated temperatures and chemical modifications [14]. It is also known that phenolic preservatives interact with protein and peptides by forming hydrogen bonds with the carboxyl group of these molecules [2]. However, aggregation is believed to be driven more by hydrophobic interactions than by other physical forces, such as hydrogen bonding, Van der Waals forces or electrostatic repulsion [15].

This behaviour has been reported for human growth hormone (hGH) [2], [15]. This protein is also called somatropin when it is produced by recombinantly modified cells and plays an essential role in growth, in other basal metabolic functions, in the regulation of glucose levels and in the regulation of muscle and fat mass [16].

Lastly, two other key components of multi-dose injectable formulations are buffering agents and tonicity adjusting agents.

Buffers are added to formulations to adjust and stabilise the pH, to avoid degradation during processing, storage and reconstitution and to optimise drug solubility and stability. For parenteral preparations, it is desirable that the pH of the product is close to physiological pH. Citrate buffers or phosphate buffered saline (PBS) in the range of 5-15 mM are typically used in formulations [4].

PBS is an isotonic buffer frequently used in biological applications because it closely mimics the pH, osmolarity and ion concentrations of the human body [17].

Regarding tonicity adjusting agents, parenteral formulations should be isotonic with human plasma to avoid pain, irritation and tissue damage at the site of administration. Some of the most commonly used tonicity adjusting agents are dextrose, glycerin and mannitol [4].

Therefore, after the analysis of the current situation, the need arises in the scientific community to investigate in detail how certain components used in multidose injectable formulations interact, and the regions in which they can be used together without the presence of turbidity that could lead to instability and a decrease in the efficacy of the formulations.

This project aims to contribute to the development of this research area by studying in detail the influence of common components in this type of formulations such as phenol, proteins, salt and other non-ionic tonicity adjusting agents on the decrease of the cloud point of PS80, as well as the increased protein aggregation resulting from preservative-protein interaction and the influence of salt on this effect.

3. Methodology

3.1. Instruments

3.1.1. Light scattering measurement with Probe Drum®

The way a sample diffracts and scatters light from a laser beam can reveal information about the sample such as the presence of turbidity [18].

Turbidity or "cloudiness" is an optical characteristic of a transparent liquid or solid sample that generally describes the clarity or haziness of the sample [19].

In liquids, it is caused by small suspended (undissolved) particles that have a different refractive index than the surrounding medium. This interference causes reflection, absorption and scattering, and thus a directional change of the radiated light. As the particle concentration increases, the intensity of the scattered light rises.

The two most important factors influencing the intensity and spatial distribution of scattered light are the particle size and the wavelength of the light.

The particle size in biological products usually ranges between 100-500 nm, as in the case for colloids [20].

Moreover, the wavelengths applied in turbidity measurements are usually in the near infrared range (NIR 700-1000 nm) to eliminate the influence of any coloured substances.

Since the angular intensity distribution of the scattered light around a particle is not symmetrical, the angle between the emission beam and the detector is crucial for the turbidity measurement. In this type of experiment, a detection angle of 90° (side scattering) is generally used as it is more sensitive to particles in the range described (100-500 nm) [20].

The equipment used for the measurement of light scattering is Probe Drum. This instrument enables automatic concentration titration in the range of microlitres, thanks to the action of a pump, while simultaneously measuring light scattering. In addition, it is equipped with an adjustable stirring system.

The instrument is fitted with a detector that is positioned at a 90° angle to the incoming light to measure right angle scattering (RALS). As previously mentioned, this method is the simplest and most suitable for monitoring changes in the composition of the type of samples analysed [18].

Light scattering is measured in Fluorescence mode by means of a laser beam with an excitation light wavelength of 637 nm.

This type of measurement can provide information about the turbidity of a sample with high precision as an additional component is added.

3.1.2. Structure analysis with Co-SAXS

The CoSAXS beamline of Max IV laboratory is a state-of-the-art, multi-purpose Small-Angle X-ray Scattering (SAXS) instrument that offers the possibility to use the inherent high coherent properties of the 3 GeV MAX IV ring through X-ray Photon Correlation Spectroscopy (XPCS) experiments [21].

It is designed to provide intense and optionally coherent illumination at the sample position, which allows coherent imaging and speckle contrast technique [22].

The X-ray beam hits the sample and most of it passes directly through the sample without interacting with it. Some X-rays are scattered and form a scattering pattern on the detector behind the sample. This detector is placed in an 18 m long vacuum tank to detect the weak signals without the X-rays being absorbed by the air. It is the scattering pattern which contains the information about the size and shape of the particles in the sample.

Small Angle X-ray Scattering (SAXS) is an analytical technique that measures the intensities of X-rays scattered by a sample as a function of the scattering angle. Measurements are made at very small angles, typically between 0.1 and 5 degrees. From Bragg's law, it is deduced that as the scattering angle decreases, increasingly larger structural features are probed [23].

Small Angle X-ray Scattering (SAXS) gives structural information from 1 nm to 1 μm length scales and it can provide complementary information about the shape, folding, aggregation, conformations and assembly state of macromolecules in solution [24].

This analysis is used in the study to obtain structural information, which cannot be revealed by light scattering, from samples containing protein in solution.

3.2. Experimental set up

The experiments were performed at the Department of Food Technology, Engineering and Nutrition, Faculty of Engineering, Lund University. All the equipment required to carry out the experimental work was provided by the department.

Chemicals used in the process, polysorbate 80 (CAS No: 9005-65-6), di-sodium hydrogen phosphate dihydrate (CAS No: 10028-24-7), potassium dihydrogen phosphate (CAS No: 7778-77-0), sodium chloride (CAS No: 7647-14-5), D(+)Glucose monohydrate (CAS No: 14431-43-7), D-Mannitol (CAS No: 69-65-8) and phenol (CAS No: 108-95-2), were obtained from Sigma-Aldrich (Germany). Somatropin was a gift from Ferring Pharmaceutical (Denmark). All other chemicals were PA grade.

3.2.1. Stock solutions

Phosphate buffered saline (PBS) 10 mM was used as buffer for all stock solutions. PBS was made from potassium dihydrogen phosphate and di-sodium hydrogen phosphate dihydrate and subsequently adjusted to pH 7 with HCl 0.1 M. The pH measurement was conducted with the Thermo Scientific Orion Star™ A215 pH/Conductivity Benchtop Multiparameter Meter.

Stock solutions were prepared and stirred for at least 1 hour. All solutions were stored at room temperature. Those containing PS80 were covered to avoid possible light damage.

In the case of somatropin, the agitation was done manually and very gently to avoid aggregation. The storage temperature was -18ºC and 4ºC for dry and diluted protein, respectively. The diluted protein concentration was measured with NanoDrop® ND-1000 Spectrophotometer. Results were analysed with Proteins A280 software where the parameters set were "other protein (E&MW)" for sample type, "17.67" for e (x1000) and "22.12" for molecular weight (kDa). It should be noted that somatropin had to be used within 3 consecutive days of dilution to avoid flawed results due to degradation.

3.2.2. Light scattering measurement during concentration titration

The performed experiments were concentration titrations in which spectra were recorded using two detectors. The first one recorded the range of 240-500 nm with 4 averages in the absorbance mode. The second one recorded the range 240-720 nm with 4 averages. Fluorescence mode was used for the latter with a 637 nm laser light and an interval time of 100 ms.

The equipment assembly was placed under the fume hood and the experiments were carried out at 25°C. When somatropin was involved, it was taken from the refrigerator 30 min before the experiments were performed to raise its temperature.

Generally, the initial content of the cuvettes was 800 μL and a total titrant volume of 150 μL was added in 5 μL steps. A baseline of 60 seconds, normal loading and mixing time of 30 seconds were set. In addition, intermittent stirring at a speed of 4 over 7 and an equilibration time of 60 seconds was established. Spectra were taken during equilibration at 10 second intervals.

Measurements were performed in a usual range of concentrations for the excipients tested. Nevertheless, in some cases, a wider span was considered in order to broaden the scope of the study. Most experiments were at least duplicates to ensure reproducibility of results.

Lastly, data analysis was conducted with the software Probe Drum Viewer and verified by visual inspection of the samples (Figure A25, attached in the appendix, shows the difference between samples with and without turbidity). The graphs obtained provide accurate information regarding the intensity of the light scattered at different wavelengths at different points of the titration.

3.2.3. Experimental series

The aim of the titrations was to accurately study the appearance of the cloud point and the behaviour prior to its appearance in the case of PS80, and protein aggregation in the case of somatropin. In this way, it is possible to analyse the effect caused by the interaction surfactant-preservative, and protein-preservative after the progressive addition of phenol, or sodium chloride, in cases where the preservative was already present in the initial sample. In addition, another purpose was to study the effect of other excipients on these behaviours.

For this purpose, the following experimental series was performed:

Experimental series P1. Phenol titration. Study of the effect of phenol on the cloud point of PS80 and analysis of the influence of different salt concentrations.

a. PS80
b. PS80 – NaCl 25 mM
c. PS80 – NaCl 0.15 M

Phenol 4 wt% was added as a titrant to a cuvette initially containing 800 μL of PS80 0.1 wt% and salt with different concentrations.

The experimental series P1.b was complemented by another concentration titration in which the phenol buffer also contained salt 25 mM.

Experimental series P2. Phenol titration. Study of the effect of different non-ionic tonicity adjusting agents on the cloud point of PS80.

a. PS80 – Glycerol 0.3 M
b. PS80 – Glucose 0.3 M
c. PS80 – Mannitol 0.3 M
d. PS80 – Mannitol 0.6 M
e. PS80 – Glucose 0.3 M – Mannitol 0.3 M

Phenol 4 wt% was added as a titrant to a cuvette initially containing 800 μL of PS80 0.1 wt% and a non-ionic tonicity adjusting agent with different concentrations.

Furthermore, the possible degradation over time when the tonicity adjusting agents were diluted was studied.

Experimental series P3. Phenol titration. Study of the effect of different PS80 concentrations on the cloud point.

a. PS80 0.10 wt%
b. PS80 0.25 wt%
c. PS80 0.50 wt%
d. PS80 0.75 wt%
e. PS80 1.00 wt%

Phenol 4 wt% was added as a titrant to a cuvette initially containing 800 μL of PS80 with different concentrations between 0.10 – 1.00 wt%.

Experimental series S1. Phenol titration. Analysis of the effect of the somatropin on the cloud point of PS80 and study of somatropin aggregation due to protein-preservative interaction.

a. PS80 – Somatropin
b. PS80 – Somatropin – Mannitol 0.3 M
c. Somatropin

Phenol 4 wt% was added as a titrant to a cuvette initially containing 800 μL of PS80 0.1 wt% and somatropin 10.32 ± 0.05 mg/mL. In addition, the combined effect of somatropin and a non-ionic tonicity adjusting agent was tested (b), as well as somatropin itself without the presence of a surfactant (c).

Experimental series S2. Phenol titration. Study of the influence of different salt concentrations on the cloud point of PS80 in samples containing somatropin and on somatropin aggregation due to protein-preservative interaction.

a. PS80 – Somatropin – NaCl 15 mM
b. PS80 – Somatropin – NaCl 20 mM
c. PS80 – Somatropin – NaCl 25 mM
d. PS80 – Somatropin – NaCl 50 mM
e. PS80 – Somatropin – NaCl 0.15 M
f. Somatropin – NaCl 20 mM
g. Somatropin – NaCl 25 mM
h. Somatropin – NaCl 50 mM

Phenol 4 wt% was added as a titrant to a cuvette initially containing 800 μL of PS80 0.1 wt%, somatropin 10.20 ± 0.17 mg/mL and salt with different concentrations (a-e). Moreover, a further series of titrations were carried out to examine the behaviour of somatropin and salt in the absence of surfactant (f-h).

For all these experimental series (P1, P2, P3, S1 and S2), a total titrant volume of 150 μL was added.

Experimental series N1. Sodium chloride titration. Study of the effect of different phenol/PS80 concentration ratios on the appearance of the cloud point of PS80.

a. PS80 – Phenol (77 μL - 0.35 wt%)
b. PS80 – Phenol (85 μL - 0.38 wt%)
c. PS80 – Phenol (90 μL - 0.40 wt%)
d. PS80 – Phenol (95 μL - 0.42 wt%)
e. PS80 – Phenol (100 μL - 0.44 wt%)
f. PS80 – Phenol (105 μL - 0.46 wt%)
g. PS80 – Phenol (110 μL - 0.48 wt%)
h. PS80 – Phenol (115 μL - 0.50 wt%)

Sodium chloride 5M (after filtration) was added as titrant to a cuvette initially containing 800 μL of 0.1 wt% PS80 plus an amount of phenol 4 wt% varying from 77 μL to 115 μL. The numbers in brackets represent the initial volume of phenol added to the cuvettes before titration and the related phenol concentration respectively.

In addition, the effect on the results of the prior addition of a limited amount of salt (30-100 μL) to the cuvette containing surfactant and preservative was studied.

For these experimental series (N1), a total titrant volume between 40 and 200 μL was added.

3.2.4. Small Angle X-ray Scattering analysis with CoSAXS beamline

The aim of this analysis was to obtain additional structural information on certain samples containing phenol, somatropin and salt, which presented interesting and unexpected results, for a deeper understanding.

The samples analysed are listed below, all dissolved in PBS 10 mM.

Sample CS1a. Somatropin (10.02 mg/mL)

Sample CS1b. Somatropin (10.02 mg/mL)-NaCl 25 mM

Sample CS1c. Somatropin (10.02 mg/mL)-NaCl 25mM-Phenol (0.36 wt%)

Sample CS1d. Somatropin (10.02 mg/mL)-Phenol (0.36 wt%)

Both salt and non-salt blanks were prepared.

This type of analysis was performed with CoSAXS beamline at Max IV and provided structural information from the 1nm to 1μm length scales. This is why the samples couldn't contain surfactant, as the micelles formed by the PS80 are larger.

4. Results

The data used for the analysis of the results are the graphics that provide information about the intensity of the light scattered by the laser at 637 nm as a function of the volume of titrant added. However, a range of 635-639 nm was used for the analysis of the results in order to avoid errors derived from possible deviations in the software.

Higher scattering values reflect increased turbidity in the system, which may be indicative of the emergence of the PS80 cloud point or an increase in protein aggregation.

The onset of sample clouding is, in most cases, well recognised in the graphs. Cloud point is considered to appear when a clear increase in the scattering intensity starts to be observed, where its value usually rises by more than 1000% in a range of less than 25 µL of titrant added. For a better understanding, this described behaviour can be observed in Figure 2.

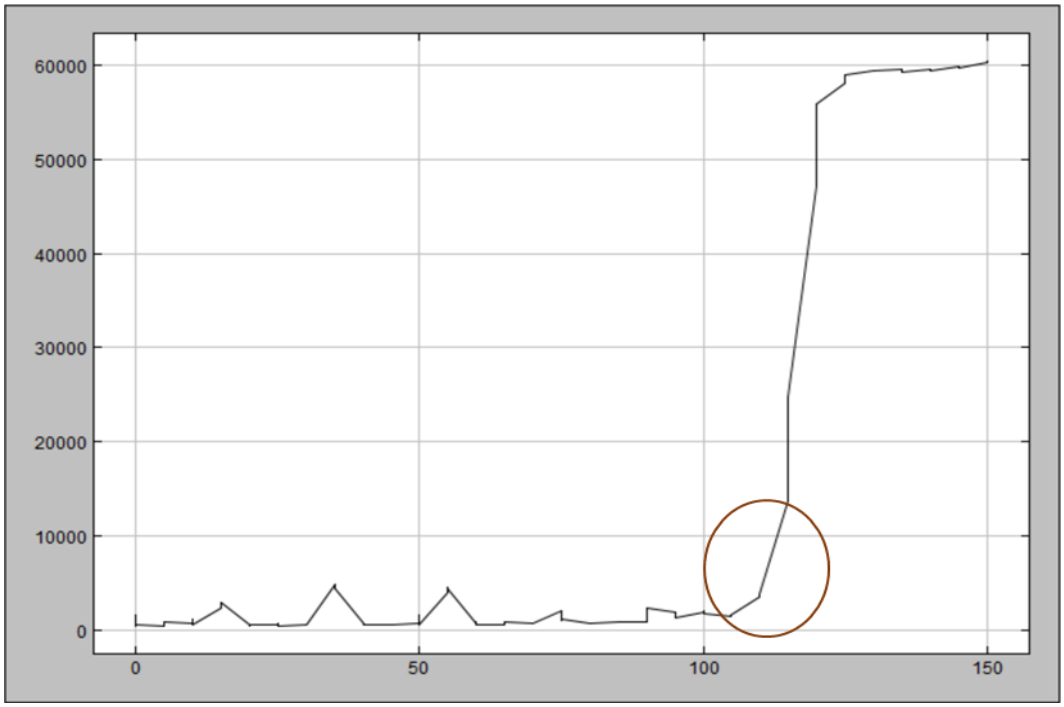

Figure 2. Standard graph representing a phenol titration, in this case P1a experiment (PS80 0.1wt%). The abrupt increase in scattering intensity values is indicative of the appearance of the cloud point. The X-axis represents the amount of titrant added and the Y-axis the light scattering intensity by the laser in the range 635-639 nm.

However, it is important to point out that, on many occasions, it is possible to find small rises caused by aggregates or precipitates that are subsequently dissolved after agitation, which do not correspond to the appearance of the cloud point. It is not possible to associate a specific intensity value from which the cloud point can be considered to have been reached, as the intensity values of the light scattering depend on the content of the samples analysed. It was therefore decided to analyse the data obtained graphically rather than numerically.

4.1. Phenol influence on the cloud point of PS80. Salt and non-ionic tonicity adjusting agent effect. Experimental series P1 and P2

Experimental series P1 and P2 were carried out to precisely determine the phenol influence on the cloud point of the PS80 at 25ºC. Moreover, the effect of salt and other tonicity adjusting agents is analysed.

Table 1 shows the amount of phenol required to reach the cloud point as well as the resulting phenol concentration for the entire experimental series P1 and P2.

Table 1. Cloud point. Influence of salt and non-ionic tonicity adjusting agents

Experiments	Experiments performed	Phenol added (μL)	Final phenol concentration (wt%)
P1a. PS80	10	110-115	0.49 ± 0.01
P1b. PS80-NaCl 25mM*	3	100-110	0.46 ± 0.02
P1c. PS80-NaCl 0.15M	2	95-100	0.43 ± 0.01
P2a. PS80-Glycerol 0.3M	1	105	0.46 ± 0.00
P2b. PS80-Glucose 0.3M	2	100-105	0.45 ± 0.01
P2c. PS80-Mannitol 0.3 M	2	95-100	0.43 ± 0.01
P2d. PS80-Mannitol 0.6M	2	75-80	0.35 ± 0.01
P2e. PS80-Glucose 0.3M--Mannitol 0.3M	4	60-70	0.30 ± 0.02

*Including experiments in which the phenol buffer also contained 25 mM salt as similar results were obtained for both tests.

The results show that, for an initial concentration of PS80 of 0.1wt% (final concentration 0.09 wt%), the cloud point appears at 25ºC after the addition of 110-115 μL of phenol, which is equivalent to a preservative concentration of 0.49±0.01 wt%.

Moreover, the presence of NaCl and other non-ionic tonicity adjusting agents considerably decreases the amount of phenol required to achieve this phenomenon, the more drastic the reduction, the higher the concentration of these components. Mannitol is the tonicity adjusting agent that contributes most to this decrease. In addition, the simultaneous presence of glucose and mannitol appears to have a significant effect.

Furthermore, anomalous results showed degradation in those samples containing tonicity adjusting, when repeating the experimental series P2a and P2c weeks after sample preparation.

Figure 3 shows graphically the results presented in Table 1.

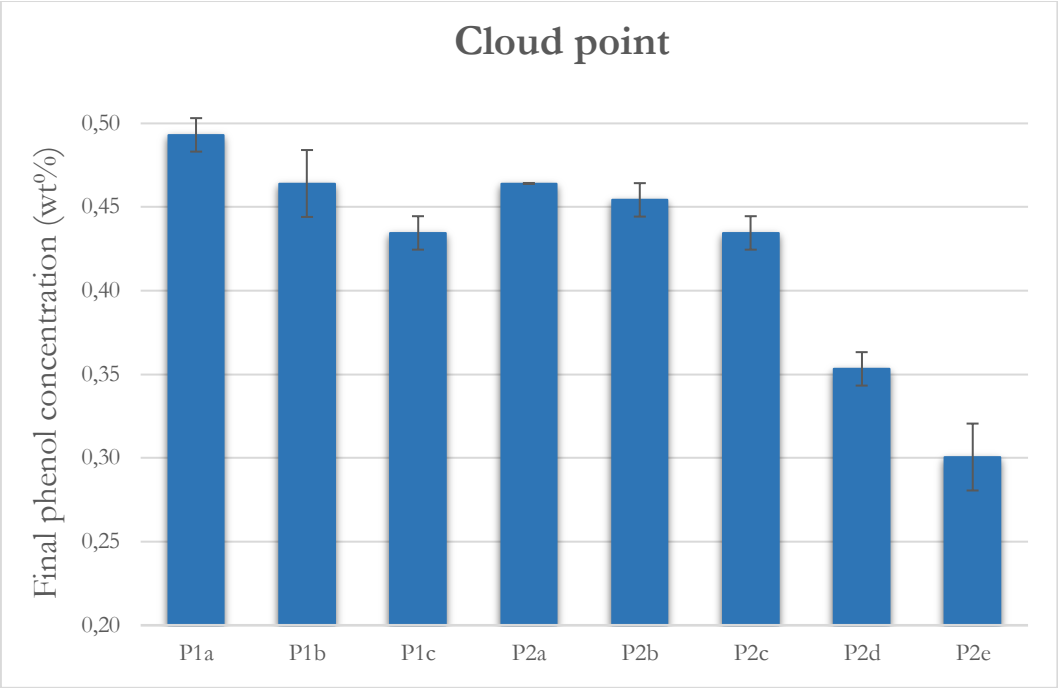

Figure 3. Phenol concentration required for the appearance of the cloud point of PS80 at 25ºC. Comparison of the results obtained in the experimental series P1 (samples with different salt concentration) and experimental series P2 (samples with different non-ionic tonicity agents).

In order to facilitate the understanding of the results, Figure A1 and Figure A2, which show the graphs obtained for experiments P1a and P1c, respectively, are attached in the appendix. In these graphs, the difference in phenol concentration required to reach the cloud point in samples containing different salt concentrations can be observed.

4.2. Influence of the surfactant concentration on the cloud point. Experimental series P3

Experimental series P3 was carried out to analyse the influence of PS80 concentration on the appearance of the cloud point. The results show irregular behaviour prior to the onset of this phenomenon.

It is possible to note the formation of several peaks corresponding to the formation and subsequent dissolution of precipitates. The effect is greater as the PS80 concentration increases. This is shown in Figures A3, A4 and A5 in the appendix, which represent experiments P3a, P3c and P3e, respectively.

Moreover, the appearance of the cloud point does not seem to be strongly influenced by the surfactant concentration. A small increase in the amount of preservative required is observed the higher the PS80 concentration. Nevertheless, quantitative analysis of the results in these cases is more challenging as the graphs are more complex and unreadable in the region where clouding starts.

4.3. Somatropin effect on the cloud point of PS80 and somatropin aggregation. Experimental series S1

Experimental series S1 was carried out to study the effect of somatropin on the cloud point of PS80 and somatropin aggregation due to its interaction with phenol.

Table 2 shows the amount of phenol needed to reach the cloud point as well as the resulting phenol concentration.

Table 2. Cloud point. Influence of somatropin.

Experiments	Experiments performed	Phenol added (µL)	Final phenol concentration (wt%)
S1a. PS80-Somatropin	3	105-115	0.47 ± 0.02
S1b. PS80-Somatropin--Mannitol 0.3M	2	85-95	0.40 ± 0.02

The results show that the presence of the protein slightly reduces the phenol concentration needed for the appearance of this phenomenon. Numerical values prove that 5 to 10 µL less phenol is required to reach the cloud point compared to similar experiments without somatropin (P1a, P2c). Moreover, experimental series S1b shows that the combination of somatropin and tonicity adjusting agent does not seem to have an additional effect on the results and on the emergence of the cloud point due to their interaction. Figure A6 shows the graph obtained in this experimental series.

In addition, the deviation in the results of the experimental series S1 is higher compared with similar experiments without somatropin (P1a, P2c).

Figure 2 shows the difference described above.

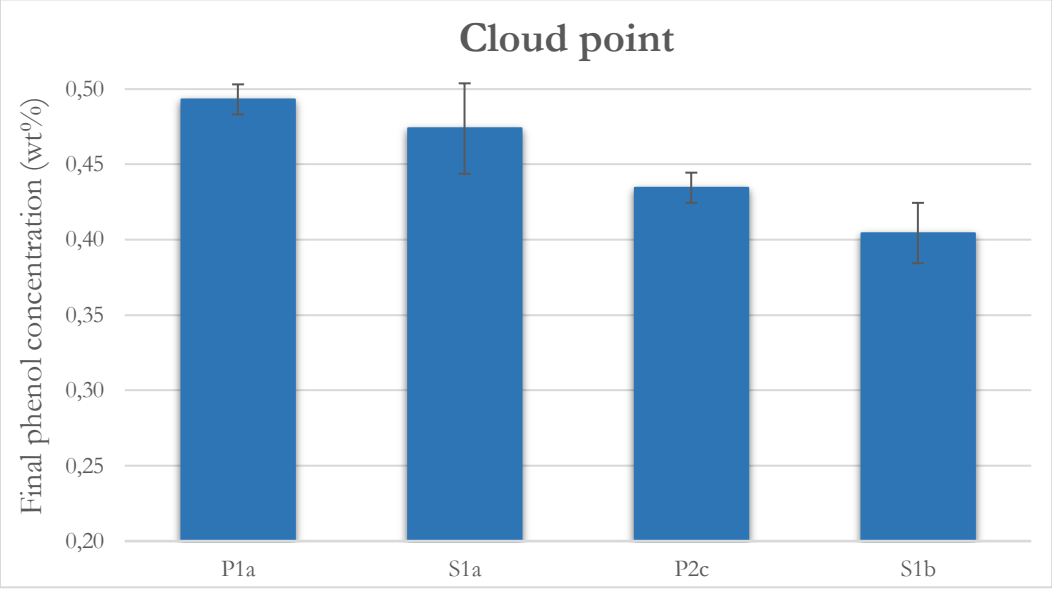

Figure 4. Phenol concentration required for the appearance of the cloud point in the experimental series P1a (PS80), S1a (PS80-Somatropin), P2c (PS80-Mannitol) and S1b (PS80-Somatropin-Mannitol). Comparison between samples with and without somatropin.

Furthermore, somatropin seems to have a small influence on the initial-middle phase of the titration prior to the appearance of the cloud point, by slightly increasing the scattering values. This behaviour seems to be corroborated by the experimental series S1c, which shows how this slight increase in scattering values is caused by the interaction between somatropin and phenol. This effect can be observed in Figures A7 and A8, which represent the plots obtained in experiments S1a and S1c respectively. It is also shown in Figure A6.

4.4. Influence of salt on the cloud point of PS80 in somatropin-containing samples and on somatropin aggregation. Experimental series S2

Experimental series S2 was performed to examine the influence of salt on the appearance of the cloud point in somatropin-containing samples and on the aggregation of somatropin. Salt does not seem to have a significant effect on the emergence of the cloud point. However, the study shows very interesting results in the initial stages of the titration, prior to the appearance of the cloud point. The intensity of the scattering in this region is higher than in the experimental series P1, P2 and S1.

Intensity values intensify as the salt concentration increases, reaching a point (for salt concentration 25 mM, experimental series S2c) where scattering intensity starts to achieve such high values that it results in the creation of a new medium-high scattering intensity region clearly discernible in the graphs. This area appears after the addition of 30-35 μL of phenol, which is equivalent to a preservative concentration of approximately 0.15 wt%. The mentioned area is shown in Figure 5.

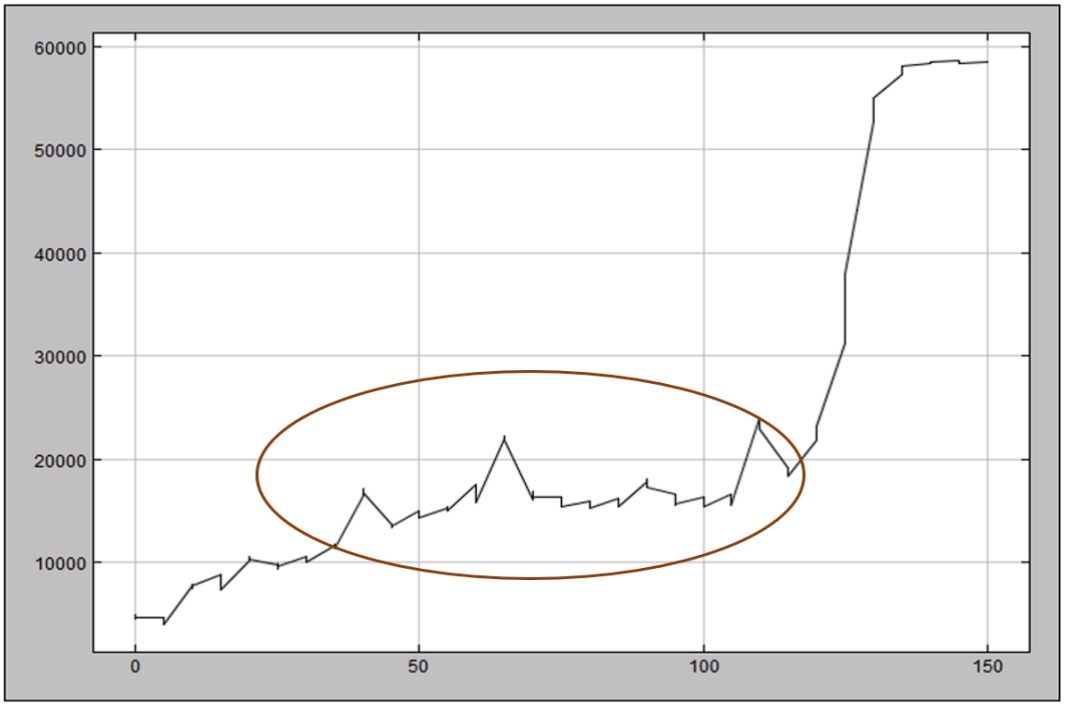

Figure 5. Phenol titration corresponding to experimental series S2c (PS80 0.1 wt%-Somatropin 10.37 mg/mL-NaCl 25 mM). Noteworthy, is the emergence of a new medium-high scattering intensity region.

Moreover, experimental series S2g (samples containing somatropin and NaCl 25 mM without the presence of PS80), shows rather high scattering values during the whole titration (without the existence of an abrupt increase due to the appearance of the cloud point, since there is no PS80). Figures A9 and A10 can be found in the appendices and show the results obtained in this experimental series.

This analysis is complemented with the experimental series P1b, containing PS80 and NaCl 25 mM without somatropin. In this case, no high scattering intensity values are observed prior to the appearance of the cloud point, which confirms that the increase in the values in the previous cases is related to phenol-somatropin-NaCl interaction. Figure A11 shows the graphs obtained in this series.

Additionally, plots obtained for the experiments containing NaCl 20 mM are included to show that the described effect does not appear until NaCl 25 mM concentration is reached. Figures A12 and A13 show the results obtained for experiments S2b and S2f.

As the salt concentration continues to increase, the results become more complex. For a concentration of 50 mM the behaviour gets unpredictable. This is observed, after obtaining completely random results in 3 different experimental series S2d. Similar behaviour is observed in the experimental series S2h, samples containing somatropin and NaCl 50 mM without the presence of PS80, after obtaining completely randomised plots for 2 experimental series S2h. Figures A14, A15, A16 and A17, A18 are included in the appendix and refer to the results obtained in the experimental runs S2d and S2h, respectively.

Finally, experimental series S2e (samples containing PS80, somatropin and NaCl 0.15M) shows turbidity instantaneously after the addition of phenol. Figure A19 in the appendix shows the result for this experiment.

4.5. Influence of salt concentration on samples with high preservative/surfactant ratio. Experimental series N1

Experimental series N1 was conducted to study how far salt concentration affects the appearance of the cloud point in samples that initially contained a high phenol/PS80 concentration ratio. Phenol concentrations used were very close to the spot at which the cloud point appears, (0.49±0.01 wt%), determined by P1a experimental series.

In order to study this effect, it is necessary to use another region that provides similar information for all cases, since the analysis region employed in the previous experimental series is practically immediate and unrecognisable.

Therefore, the region where scattering intensity values stabilise, after the drastic rise due to the appearance of the cloud point is selected for the analysis. This so-called "stable zone" region is reached equally in all series and is usually easily recognisable in the graphs. It appears, typically 10-20 μL after the cloud point. For a better understanding, the described region can be observed in Figure 6.

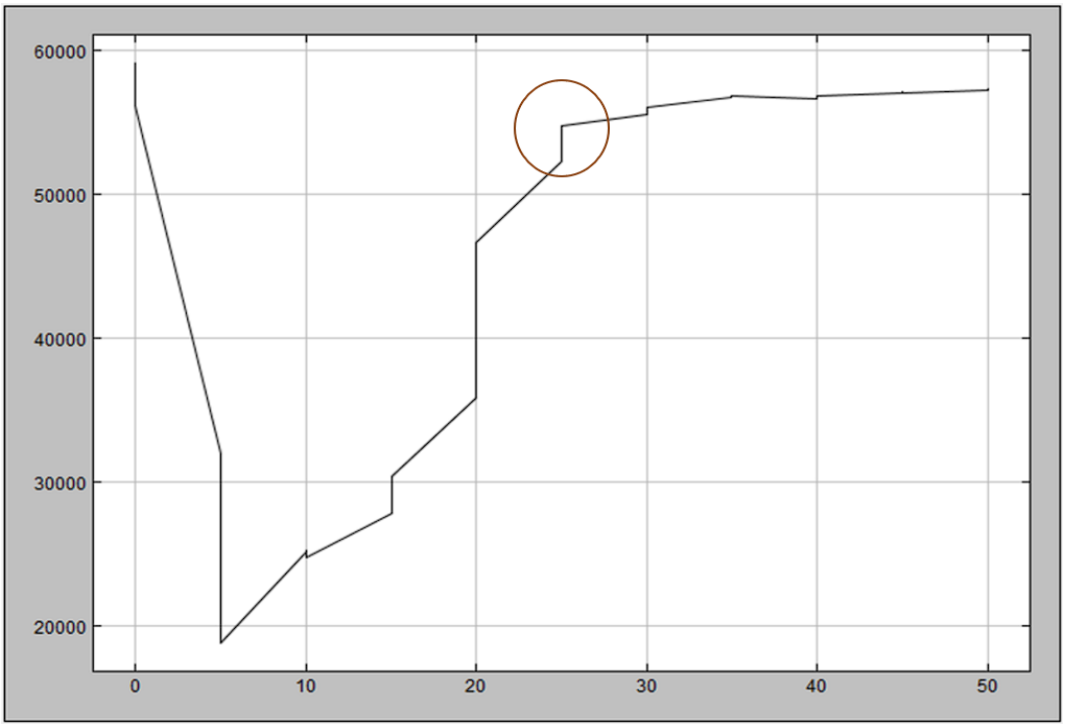

Figure 6. NaCl titration corresponding to experiment N1g (PS80 0.10 wt%-Phenol 0.48 wt%). The scattering intensity values reach stable values after the abrupt rise at the indicated point.

Table 3 shows the amount of salt required to be added to samples containing surfactant and preservative, to reach the stable zone mentioned above.

Table 3. Stable zone after cloud point. Influence of NaCl

Experiments	Experiments performed	NaCl added (µL)	Final NaCl concentration (M)
N1a. PS80-Phenol (77 µL - 0.35 wt%)	4	-	-
N1b. PS80-Phenol (85 µL - 0.38 wt%)	1	-	-
N1c. PS80-Phenol (90 µL - 0.40 wt%)	1	100	0.56
N1d. PS80-Phenol (95 µL - 0.42 wt%)	2	95	0.53
N1e. PS80-Phenol (100 µL - 0.44 wt%)	2	55	0.32
N1f. PS80-Phenol (105 µL - 0.46 wt%)	2	40	0.24
N1g. PS80-Phenol (110 µL - 0.48 wt%)	2	25	0.15
N1h. PS80-Phenol (115 µL - 0.50 wt%)	2	15	0.09

The results show that the higher the phenol concentration in the initial sample, the lower the amount of salt needed to reach the stable zone of high scattering intensity and, consequently, the cloud point.

However, the effect of NaCl starts to be relevant from a phenol concentration equal or higher than 0.40 wt%. For lower concentrations, the cloud point has not been achieved by adding NaCl.

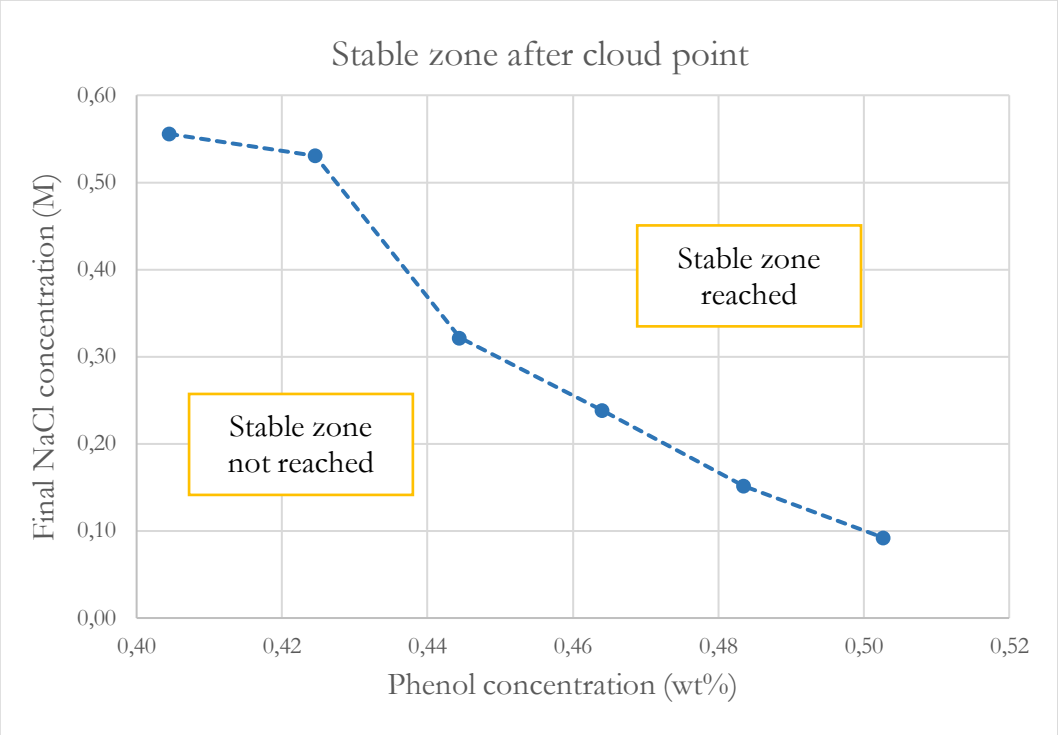

Figure 7. Salt required to reach the stable zone after cloud point, for a given phenol concentration. Below the curve, salt and phenol concentrations are not high enough to reach this region. Above the curve, any combination of concentrations is within the stable zone after cloud point. The points show the values given in table 3 for the experimental N1(c-h) series. The lines are only for connecting the markers.

Finally, the effect of the previous addition of a limited amount of salt to the cuvette before the beginning of the titration is studied. The results show a significant difference between this type of experiments and the experimental series N1 titrations. Continuous peak formation is observed, probably due to the formation and subsequent dissolution of precipitates. This behaviour can be noted in Figures A20, A21 and A22.

Additionally, in some cases, a clear delay in the appearance of both the cloud point and the subsequent stable zone is observed, compared to titrations containing the same concentrations of surfactant and preservative. This difference is shown in Figures A23 and A24.

4.6. Further study of the salt-protein-preservative interaction. CoSAXS analysis

This study was carried out in order to perform a more complete analysis of the unexpected region with medium-high scattering intensity values, shown in the experimental series S2c.

The results reveal a structural difference between the samples containing somatropin, NaCl and phenol (CS1c) and those containing somatropin and phenol (CS1d). This difference may be due to aggregation, being higher in the sample containing salt (CS1c).

This increased aggregation is observed due to a greater influence on the scattering data in the sample containing somatropin, salt and phenol. However, it is not possible to determine whether this is a consequence of a higher number of aggregates or larger aggregates.

The results obtained for the experimental series CS1c and CS1d are shown below.

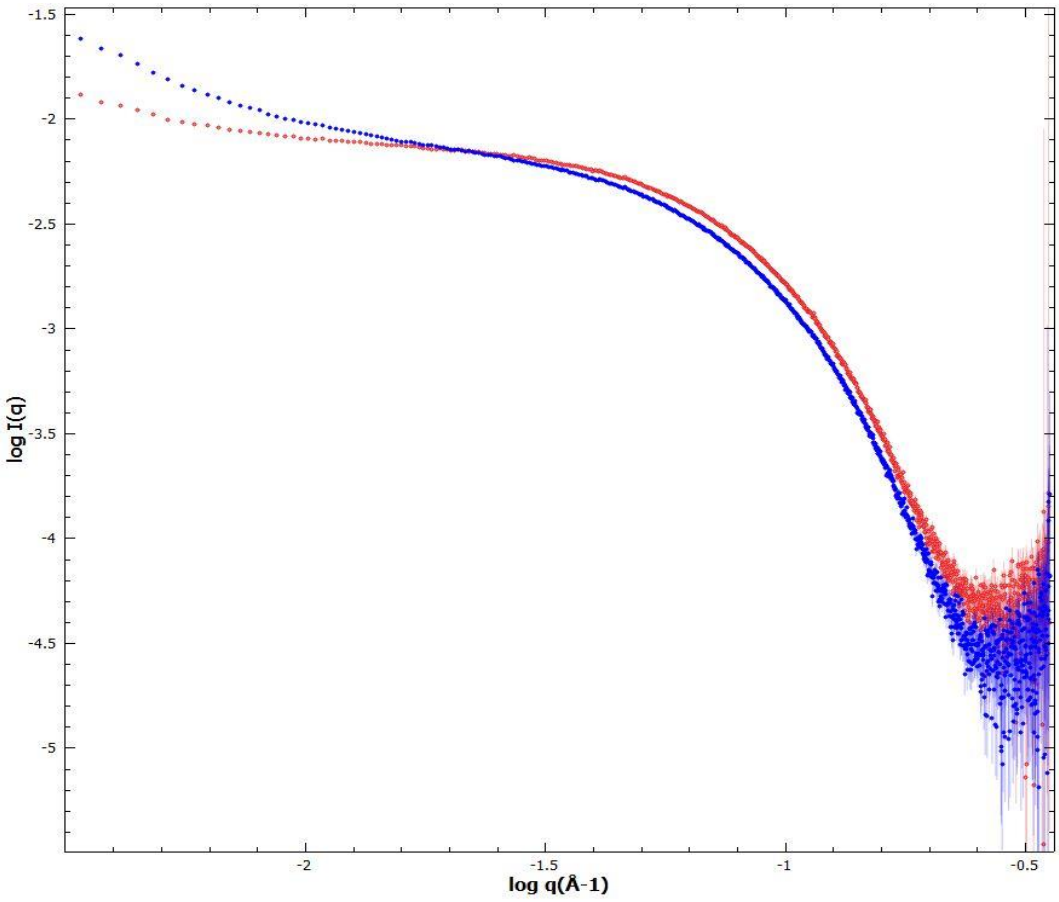

Figure 8. Structural information of samples CS1c (somatropin, NaCl and phenol) (blue) and CS1d (somatropin and phenol) (red), where I(q) represents the intensity for a given scattering angle, q. Comparison between samples with and without salt. Higher aggregation can be observed for the sample containing NaCl.

5. Discussion and conclusion

The main purpose of this project is to study the influence of common components in multi-dose injectable formulations such as phenol, salt, proteins and non-ionic tonic agents on the cloud point decrease of PS80.

Furthermore, the possible aggregation of somatropin due to its interaction with phenol is studied, as well as the influence of salt on this effect.

On the one hand, results show how phenol leads to a significant decrease of the cloud point of PS80 down to 25ºC. This phenomenon appears for a final PS80 concentration of 0.09 wt% from phenol concentrations of 0.49±0.01 wt%. These findings reaffirm the results already shown by other authors [11], [12].

The data also suggest that the presence of salt and other non-ionic tonicity adjusting agents (in common concentrations of use) can reduce phenol concentration required for the emergence of the cloud point at 25ºC to 0.43±0.01 wt%.

Furthermore, the results show that the higher the concentration of these components, the greater the decrease of the cloud point of PS80. This effect is also described by other authors who report the same effect for different salts [6], [10].

Regarding the influence of the PS80 concentration on the results, its variation does not seem to have a significant influence on this phenomenon. A small increase in the amount of preservative is required the higher the PS80 concentration.

On the other hand, this project also studies the influence of somatropin on the cloud point of PS80 and protein aggregation due to preservative-protein interaction.

The protein is shown to slightly reduce the phenol concentration required for the appearance of the cloud point of the PS80. Furthermore, it is observed that the combined presence of somatropin and mannitol has no additional influence on this effect or on increased protein aggregation.

However, higher scattering intensity values in the results seem to indicate somatropin aggregation caused by the preservative-protein interaction. This effect is also mentioned by other authors [2], [14], [15].

This aggregation appears to be significantly enhanced in the presence of NaCl, above a certain salt and preservative concentration. The most interesting case is the one observed in experimental series S2c, formulation that contains the limiting concentration of salt (25 mM) from which this effect starts to be significant. In this case, it only takes a phenol concentration of 0.15 wt% for this increased aggregation zone to appear.

For a better understanding, it was decided to further study the structural information of this region with CoSAXS beamline at Max IV. The results corroborate that samples containing protein, salt and preservative show a higher aggregation than those containing only protein and preservative.

Finally, the effect of the salt on the appearance of the cloud point in samples that initially contained a high phenol/PS80 concentration ratio was analysed. Despite a more complex analysis of the data, it is possible to observe that the addition of salt can cause the cloud point of PS80 to appear at 25°C, but from a certain phenol concentration.

Following the study, it is possible to confirm that the presence of preservatives such as phenol can reduce the cloud point of non-ionic surfactants as PS80 down to 25ºC in concentration ranges used in multi-dose injectable formulations.

Moreover, it is shown that the addition of other common components in this type of formulations, such as tonicity adjusting agents and proteins, reduces the concentration of preservative required for this cloud point decrease.

Additionally, results indicate that protein-preservative interaction leads to protein aggregation, which is significantly enhanced in the presence of salt.

For all these reasons, this study highlights the importance of proper design of formulations containing non-ionic surfactants and/or proteins in combination with phenolic preservatives to avoid incompatibilities and efficacy reduction due to cloudiness and aggregation.

5.1. Further development

This project suggests further research on the interaction between salt, phenol and somatropin, both with and without the presence of surfactants.

It would be interesting to study the reason and mechanism by which salt increases protein aggregation resulting from the preservative-protein interaction.

Moreover, and related to these experiments, it might be of interest to study other somatropin or NaCl concentrations. In the case of salt, it would be desirable for the concentration to be between the values at which interesting results have been obtained in this study (25-50 mM). It could also be interesting to further study this upper limit to try to understand the reason for the random results obtained for the same experiment. In this case, it would be necessary to analyse the influence of the methodology used to carry out the experiments, such as the order in which the components are added to the cuvette prior to titration.

Other interesting, related research could be the study of the effect other preservatives used in multi-dose injectable formulations such as m-cresol and benzyl alcohol, as well as the influence of other parameters such as agitation, temperature or pH on the cloud point of PS80 and protein aggregation.

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7. Appendices

The figures shown in the appendix are those obtained in Probe Drum viewer and are used to analyse the results. These graphs plot titrations. The X-axis represents the amount of titrant added and the Y-axis the light scattering intensity by the laser at the range 635-639 nm.

It should be noted that last titrations of the experiment phase were performed with a different polysorbate 80 and after readjustment of the Probe Drum configuration. This led to slightly deviated results. After several verifications, it was concluded that it is necessary to apply a correction factor. For the analysis of the results, 10 units less on the x-axis must be considered, i.e., 10 μL must be subtracted from the obtained results to obtain the appropriate values. The graphs marked with an asterisk (*) correspond to those experiments.

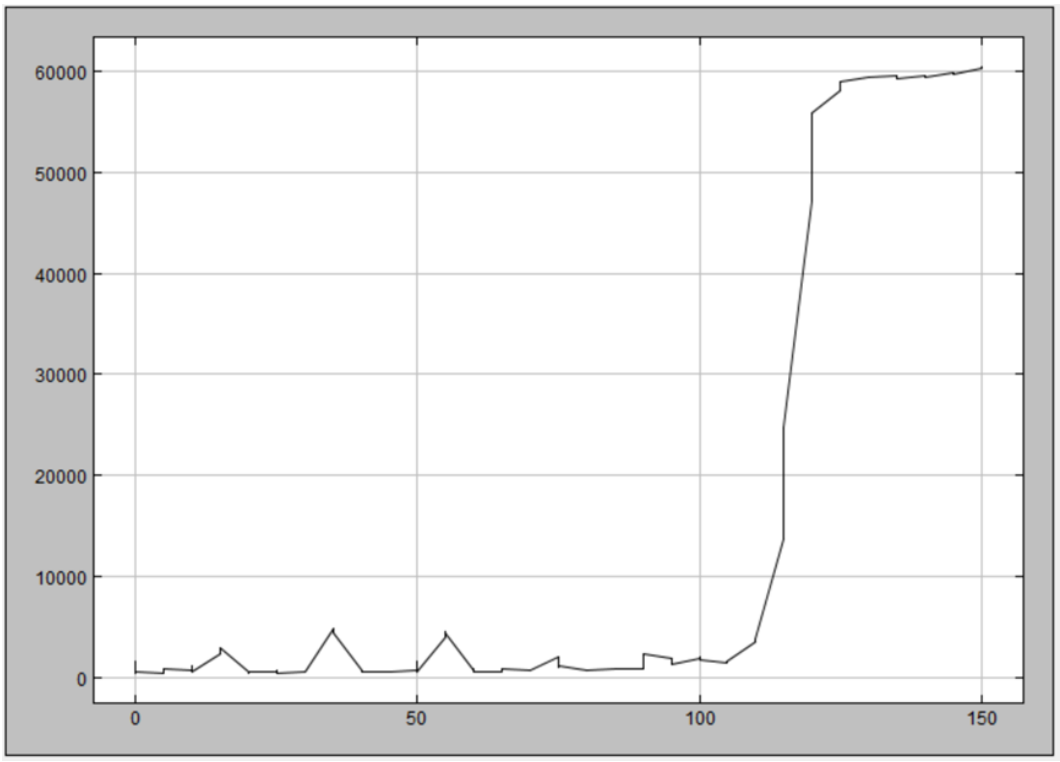

Figure A1. Phenol titration corresponding to experiment P1a (PS80 0.1wt% without salt). The amount of phenol required for the appearance of the cloud point of the PS80 at 25ºC is 110 μL.

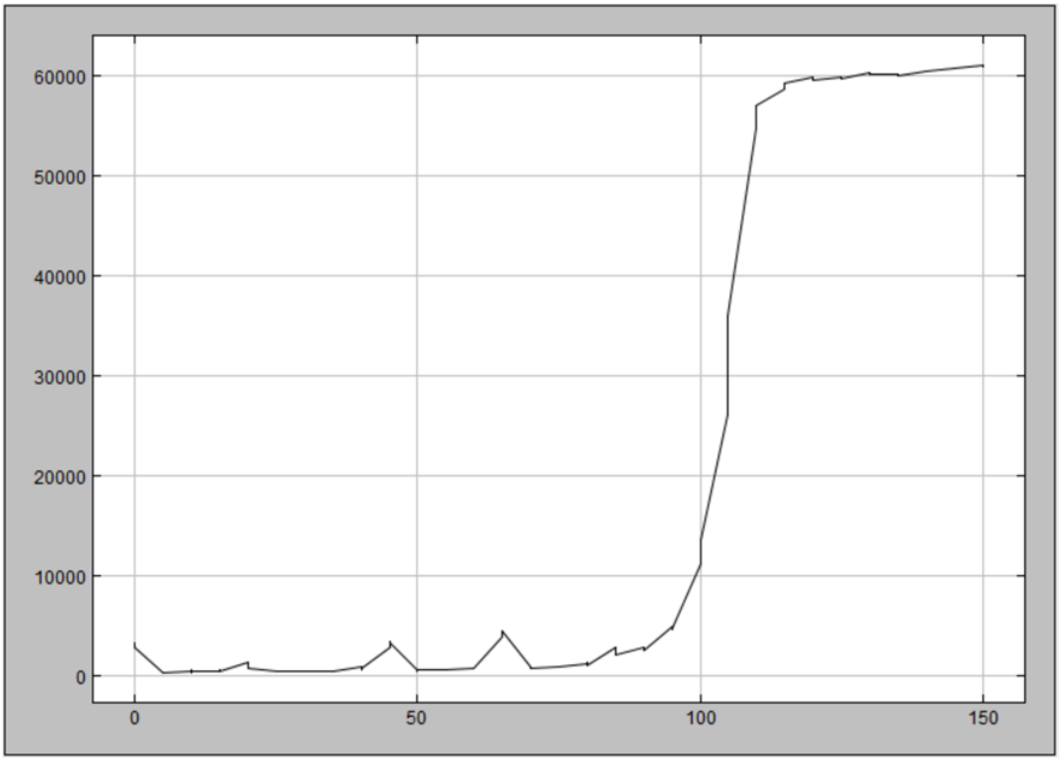

Figure A2. Phenol titration corresponding to experiment P1c (PS80 0.1wt%-NaCl 0,15M). The amount of phenol required for the appearance of the cloud point of the PS80 at 25ºC is 95 μL.

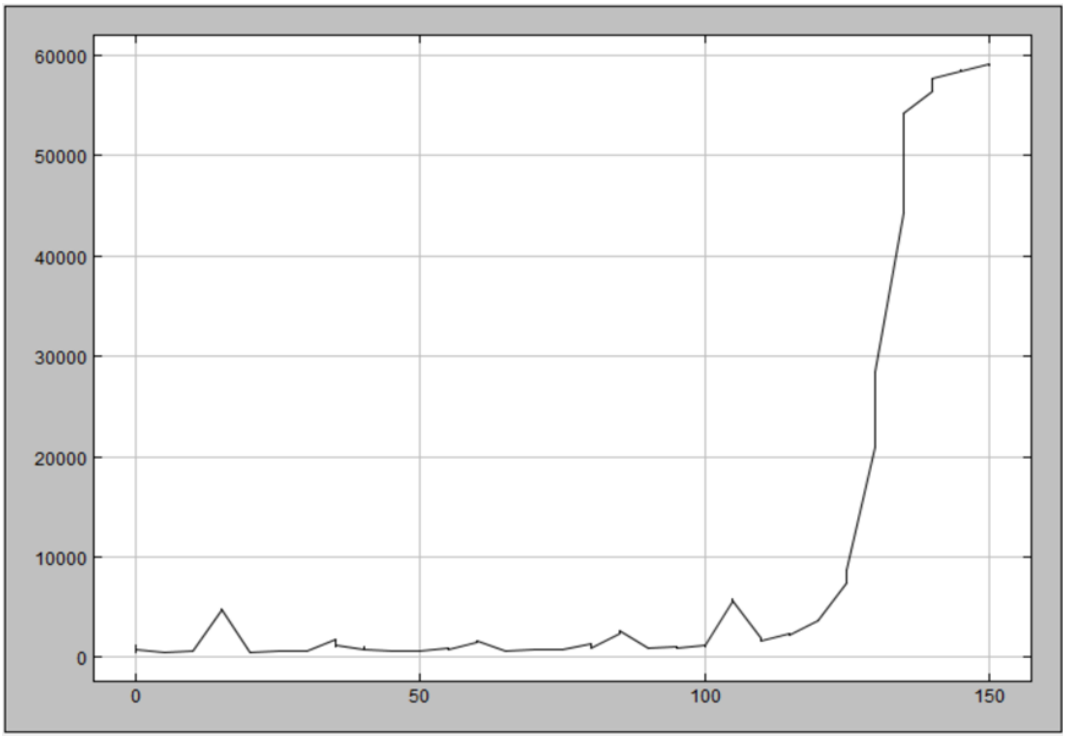

Figure A3. Phenol titration* corresponding to experiment P3a (PS80 0.1 wt%). No significant peaks are observed in the moments prior to the appearance of the cloud point.

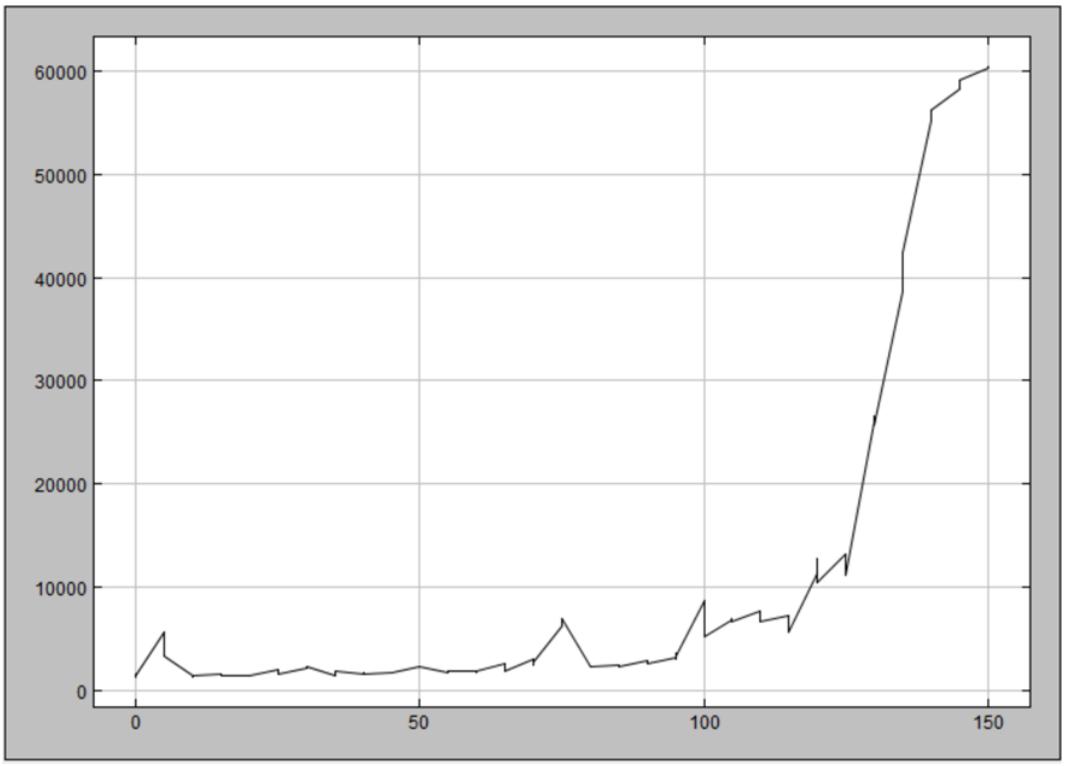

Figure A4. Phenol titration* corresponding to experiment P3c (PS80 0.5 wt%). Peaks are observed in the moments prior to the appearance of the cloud point.

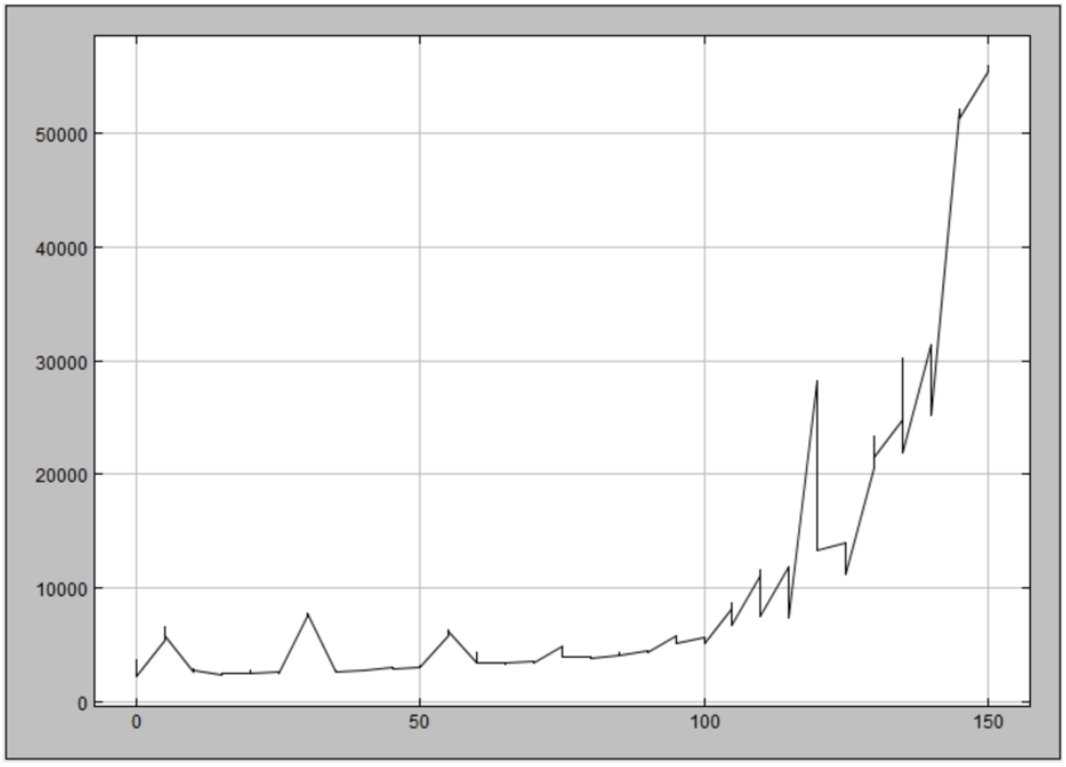

Figure A5. Phenol titration* corresponding to experiment P3e (PS80 1.0 wt%). More prominent peaks than in the P3c experiment are observed in the moments prior to the appearance of the cloud point.

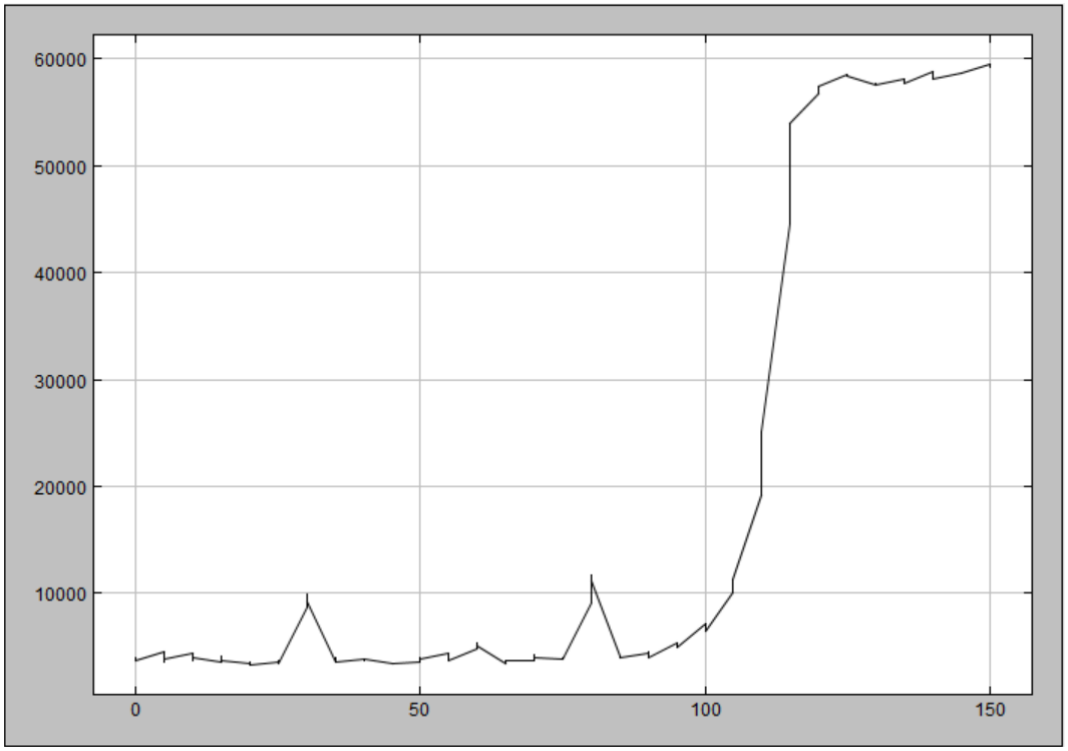

Figure A6. Phenol titration* corresponding to experiment S1b (PS80 0.1 wt%-Somatropin 10.27 mg/mL-Mannitol 0,3M). The presence of mannitol apparently has no effect on protein aggregation, in contrast to NaCl (see Figure 5).

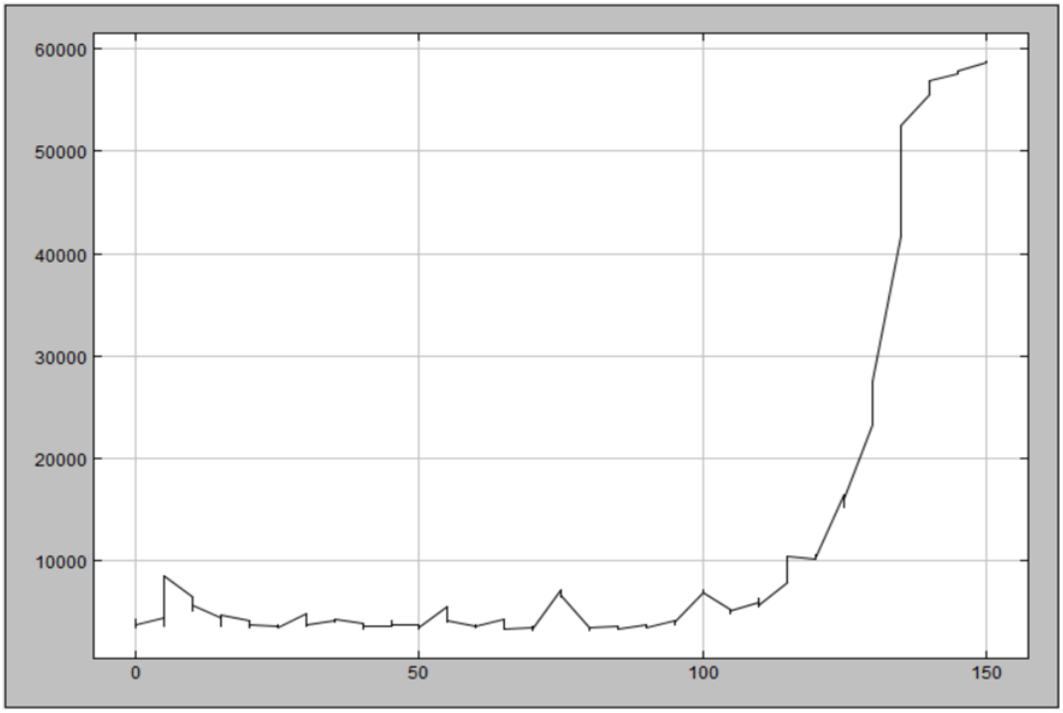

Figure A7. Phenol titration* corresponding to experiment S1a (PS80 0.1 wt%-Somatropin 10.37 mg/mL). Noteworthy, is the appearance of higher scattering values in the initial-middle phase of the titration than in the same case without somatropin, experiment P1 (PS80 0.1 wt%) (see Figure A3).

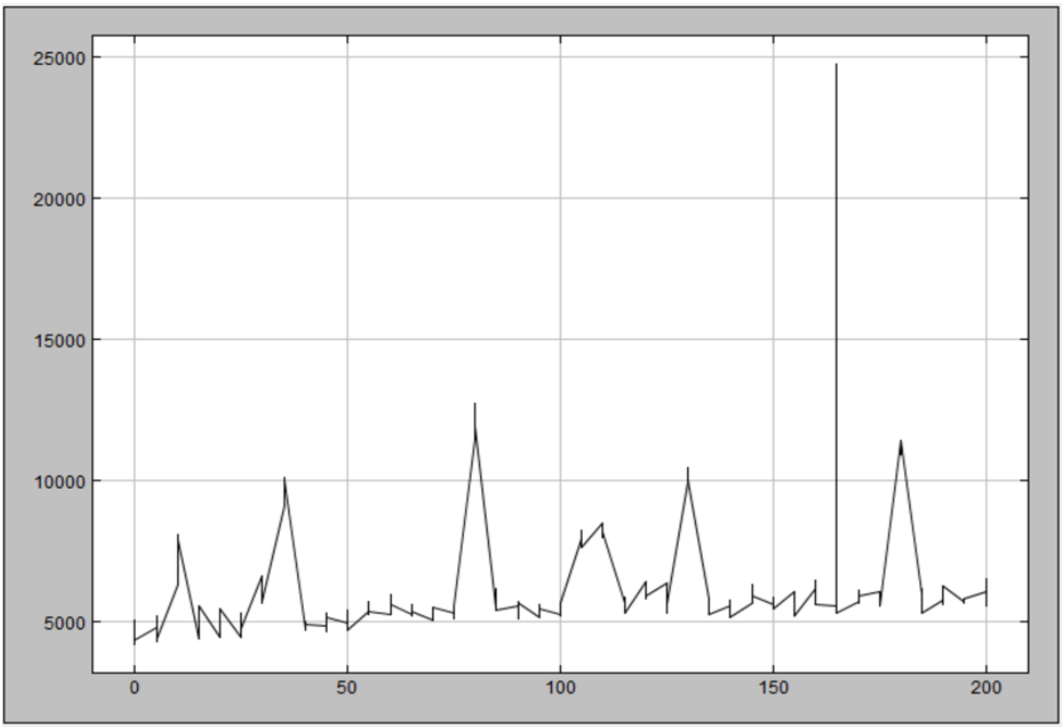

Figure A8. Phenol titration* corresponding to experiment S1c (Somatropin 10.35 mg/mL). There is no abrupt rise representing the appearance of the cloud point because there is no PS80 in the sample. However, scattering values are slightly high. This may be caused because of increased aggregation due to the protein-preservative interaction.

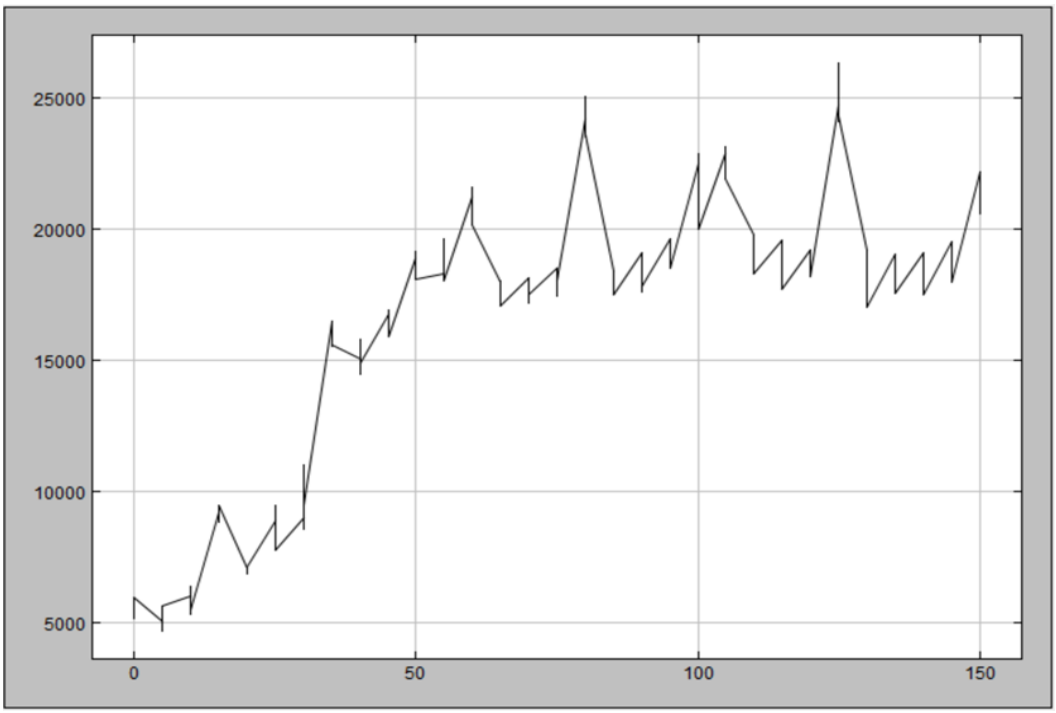

Figure A9. Phenol titration* corresponding to experiment S2g (1) (Somatropin 10.35 mg/mL-NaCl 25 mM). There is no abrupt rise representing the appearance of the cloud point because there is no PS80 in the sample. However, scattering values are rather high during the whole titration because salt enhanced protein aggregation.

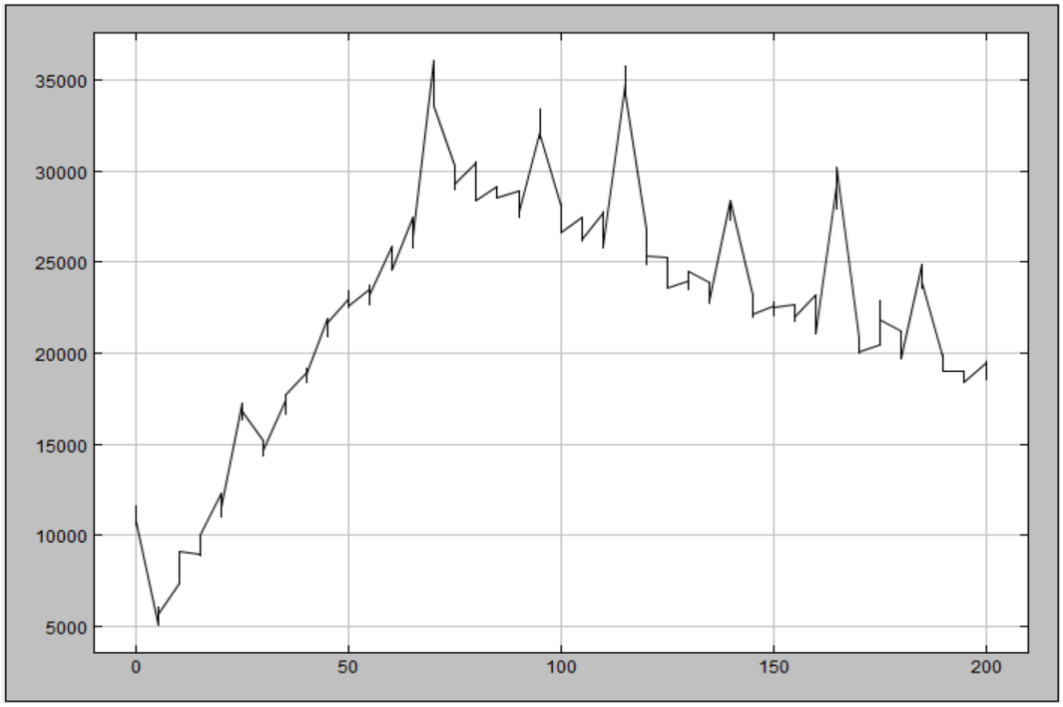

Figure A10. Phenol titration* corresponding to experiment S2g (2) (Somatropin 10.35 mg/mL-NaCl 25 mM). There is no abrupt rise representing the appearance of the cloud point because there is no PS80 in the sample. However, scattering values are rather high during the whole titration because salt enhanced protein aggregation. In this case there is a decrease in the intensity of the scattering values at the end of the titration.

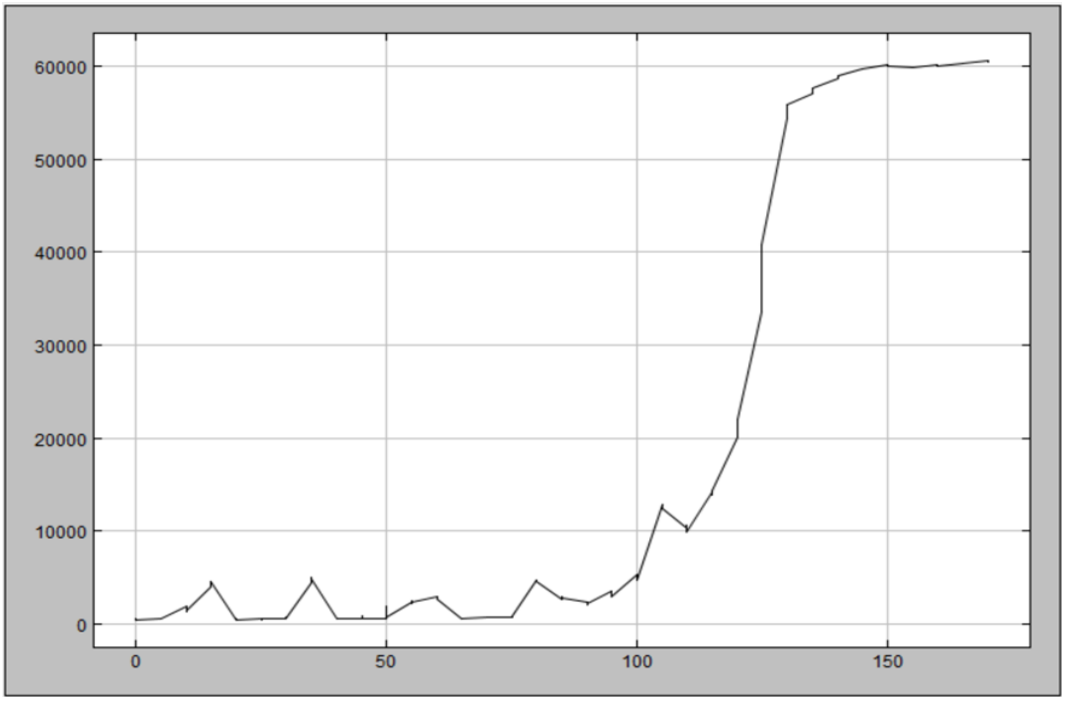

Figure A11. Phenol titration* corresponding to experiment P1b (PS80 0.1wt%-NaCl 0,25 mM). Compared to Figure 5, it can be noted that there is no previous high scattering intensity values region resulting from protein aggregation before the appearance of the cloud point.

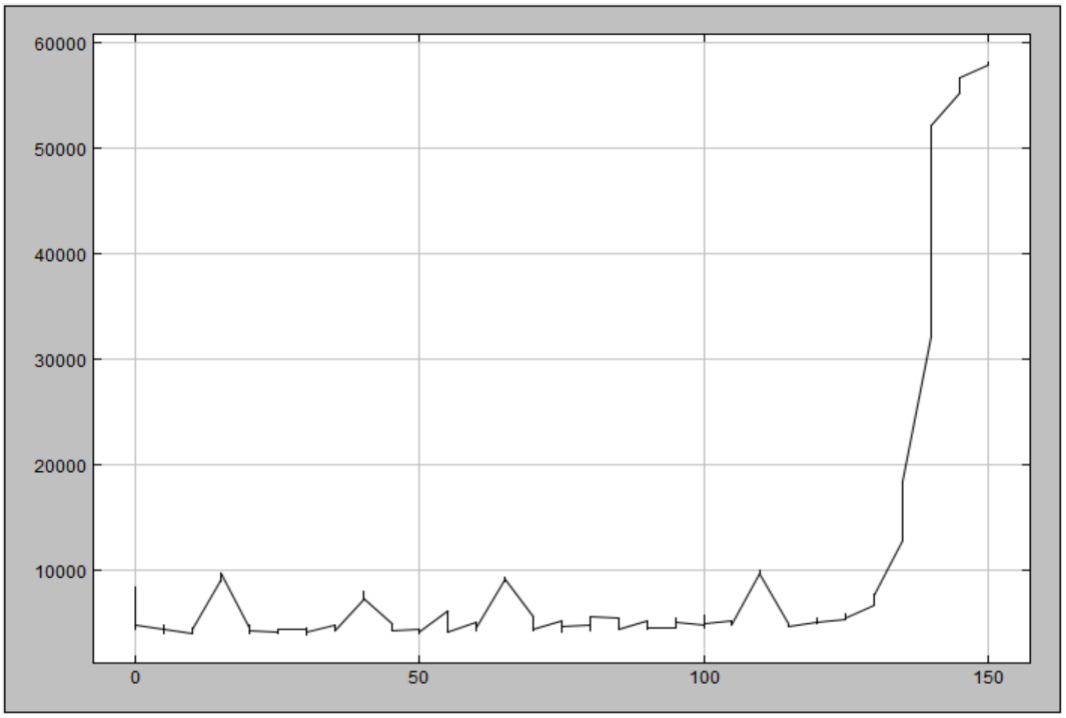

Figure A12. Phenol titration* corresponding to experiment S2b (PS80 0.1wt%-Somatropin 10.11 mg/mL-NaCl 20 mM). The initial-intermediate phase of the titration shows significantly lower scattering intensity values than in the case of the experimental series S2c (NaCl 25mM) (see Figure 5).

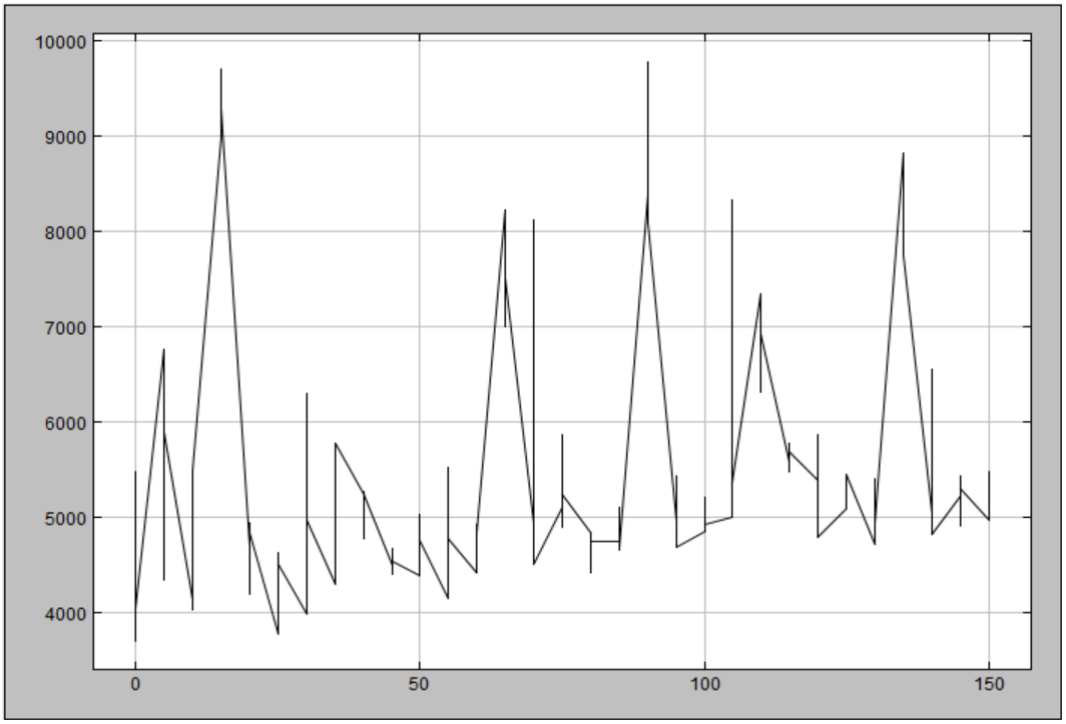

Figure A13. Phenol titration* corresponding to experiment S2f (Somatropin 10.11 mg/mL-NaCl 20 mM). Intermediate scattering values related to the results shown in Figure A12. However, significantly lower intensity values than in the case of the experimental S2g (NaCl 25 mM) series (see Figures A9 and A10).

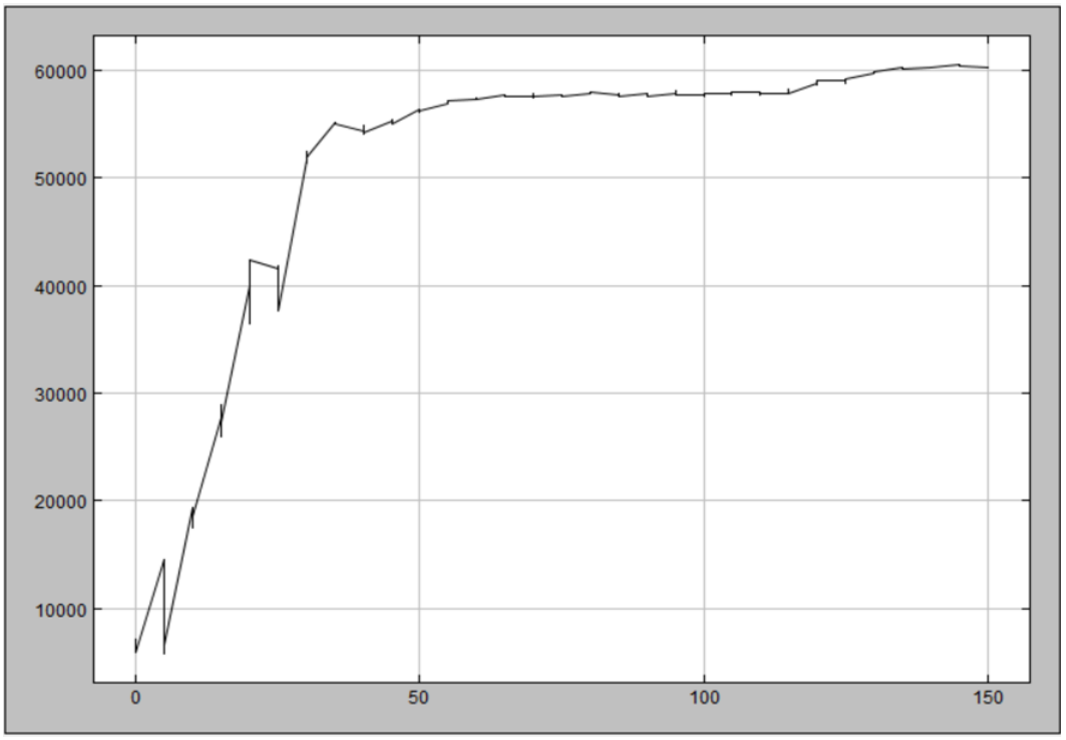

Figure A14. Phenol titration* corresponding to experiment S2d (1) (PS80 0.1 wt%-Somatropin 10.35 mg/mL-NaCl 50 mM). There is a sharp and immediate increase in scattering intensity values.

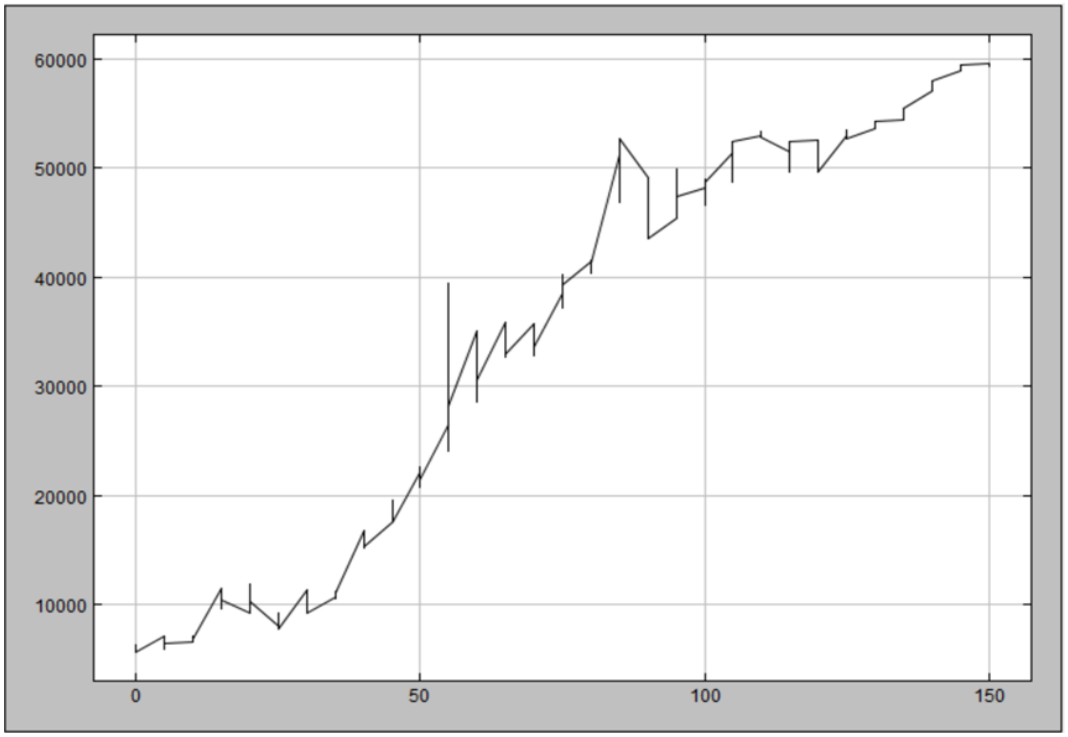

Figure A15. Phenol titration* corresponding to experiment S2d (2) (PS80 0.1 wt%-Somatropin 10.35 mg/mL-NaCl 50 mM). There is a progressive increase in the scattering intensity values. Significant difference with the graph shown in Figure A14.

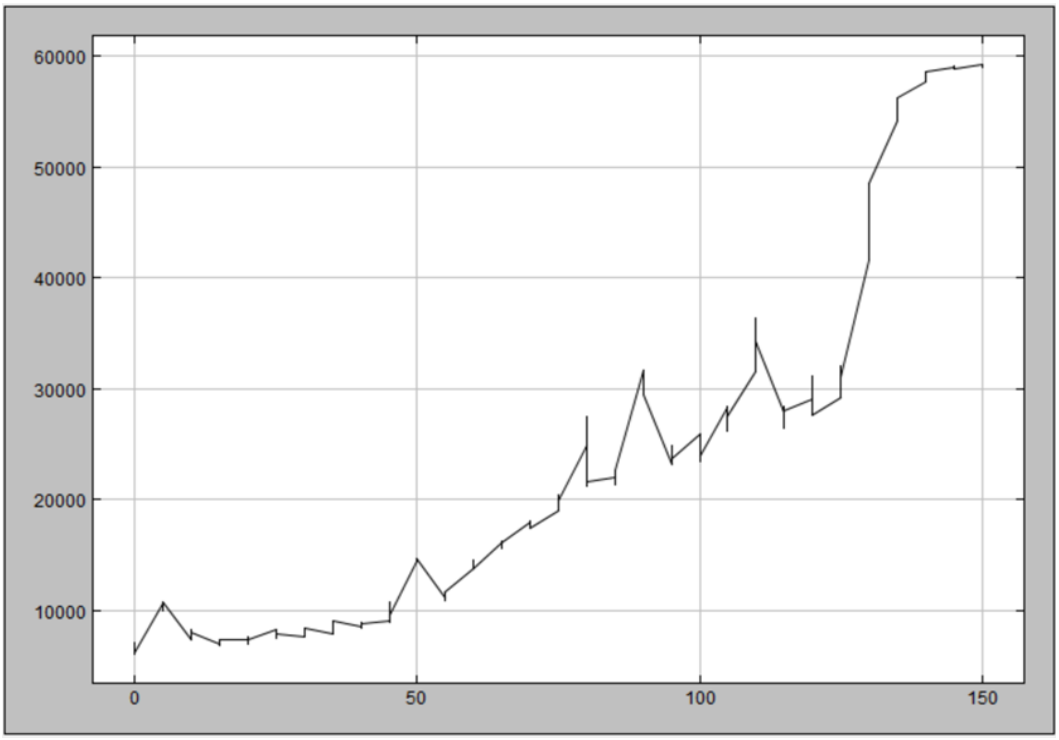

Figure A16. Phenol titration* corresponding to experiment S2d (3) (PS80 0.1 wt%-Somatropin 10.02 mg/mL-NaCl 50 mM). There is an initial region with low-intermediate scattering intensity values that later start to rise progressively. Finally, there is an abrupt increase. Significant difference with the graphs shown in Figures A14 and A15.

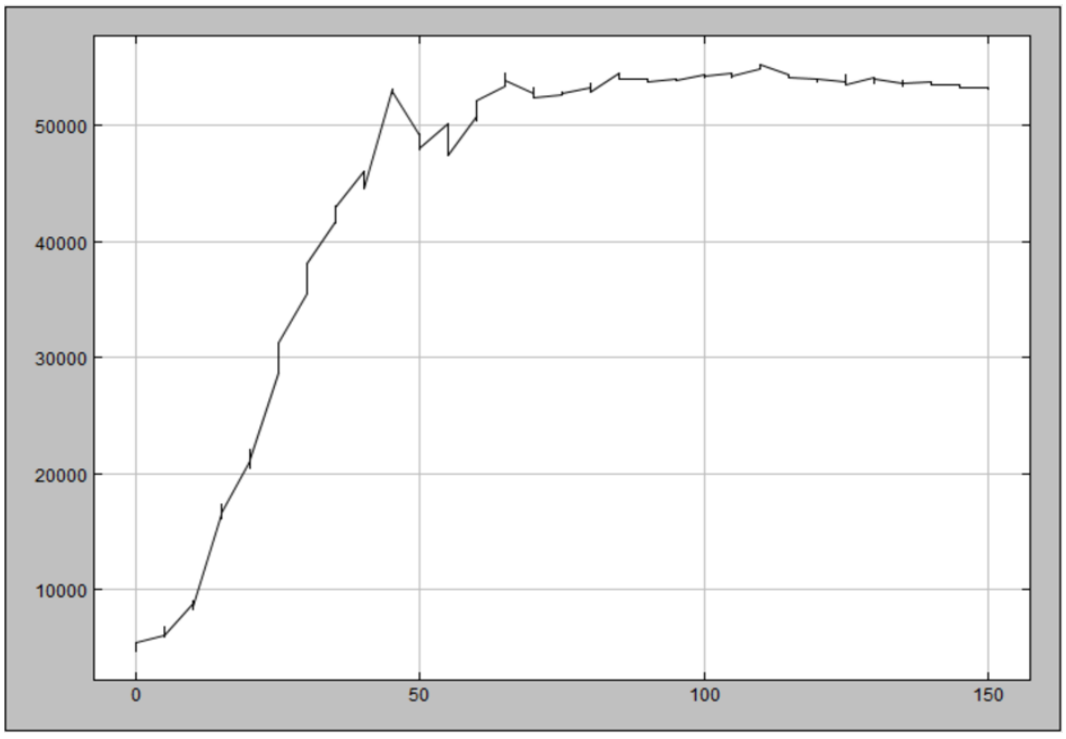

Figure A17. Phenol titration* corresponding to experiment S2h (1) (Somatropin 10.11 mg/mL-NaCl 50 mM). There is an abrupt increase in the scattering intensity values despite the fact that the sample does not contain PS80.

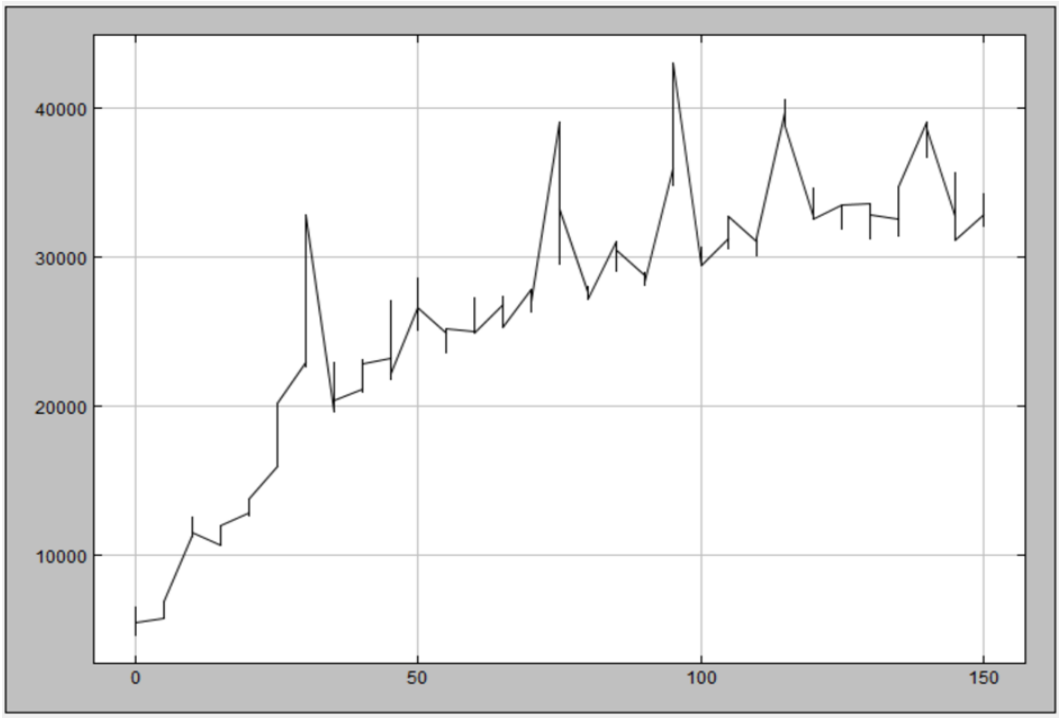

Figure A18. Phenol titration* corresponding to experiment S2h (2) (Somatropin 10.11 mg/mL-NaCl 50 mM). There is a progressive increase in the scattering intensity that reaches a high value. However, it is not as high as when the cloud point appears. Significant difference with the graph shown in Figure A17.

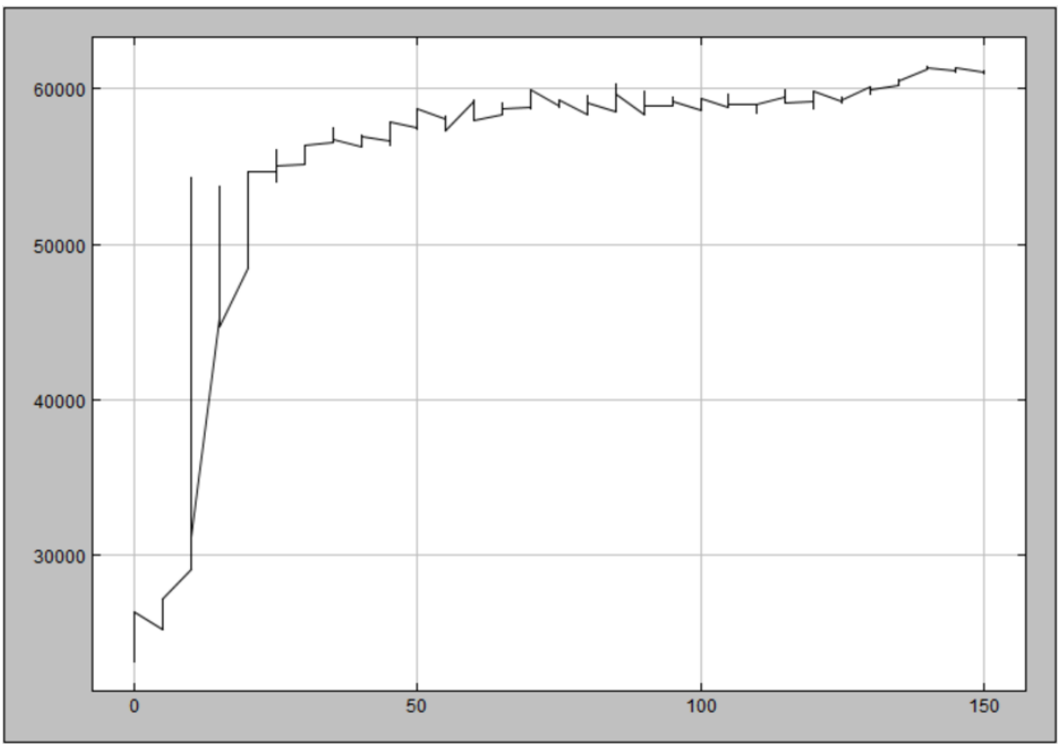

Figure A19. Phenol titration* corresponding to experiment S2e (2) (PS80 0.1 wt%-Somatropin 10.37 mg/mL-NaCl 0.15 M). Very high scattering intensity values from the beginning of the titration.

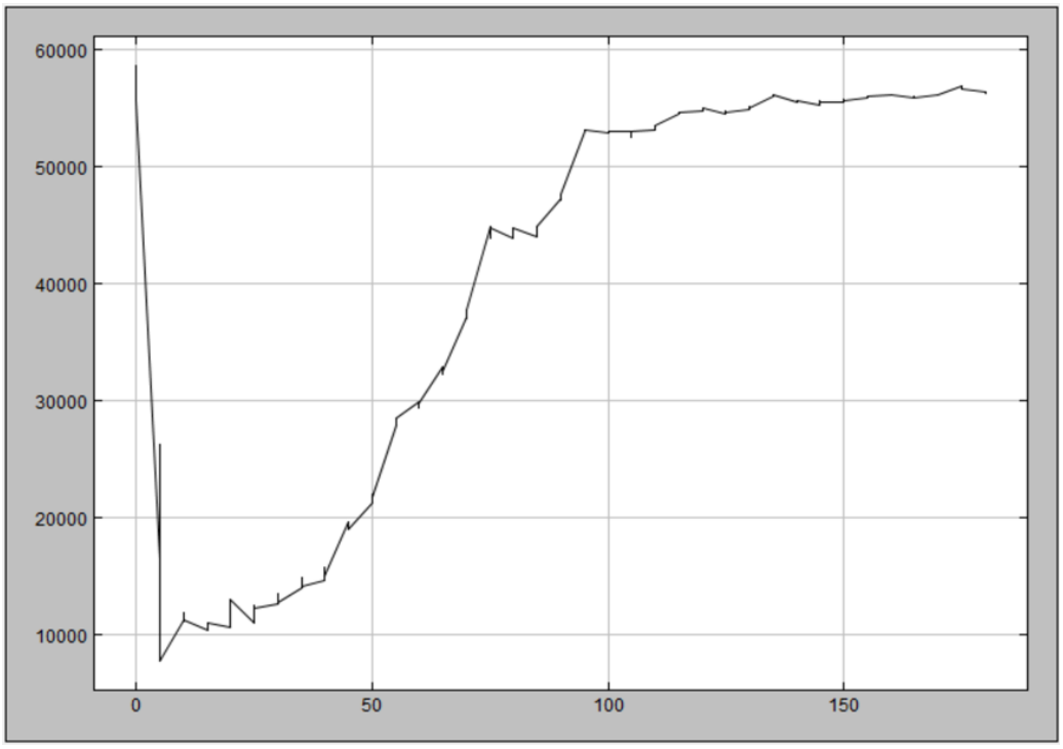

Figure A20. NaCl titration corresponding to experiment N1d (PS80 0.10 wt%-Phenol 0.42 wt%). There is no initial addition of salt to the cuvette. The stable zone is reached after the addition of 95 μL of NaCl 5 M.

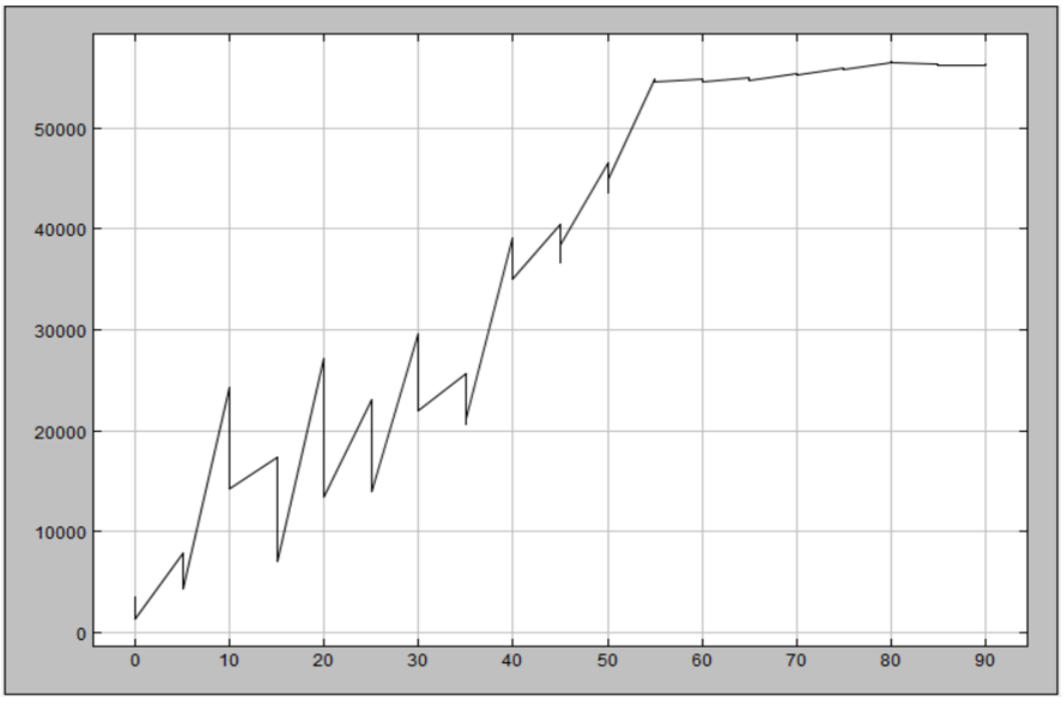

Figure A21. NaCl titration corresponding to experiment N1d(NaCl) (PS80 0.10 wt%-Phenol 0.42 wt% + 60 μL of NaCl 5 M). Before starting titration, 60 μl of NaCl 5M is added to the cuvette. Intermediate peaks appear which may represent the formation and subsequent dissolution of precipitates. The stable zone is reached after the addition of 55 μL of NaCl 5 M (total amount of salt equal to 105 μL).

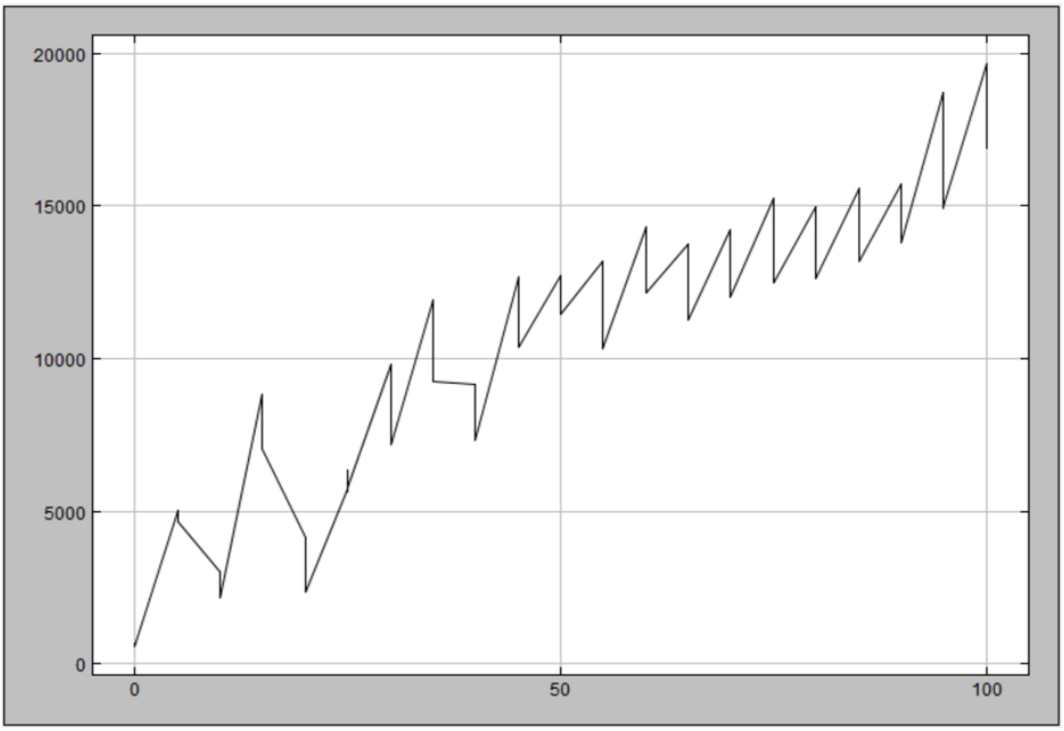

Figure A22. NaCl titration corresponding to experiment N1a(NaCl) (PS80 0.10 wt%-Phenol 0.35 wt% + 90 μL of NaCl 5 M). Before starting titration, 90 μl of NaCl 5M is added to the cuvette. Intermediate peaks appear which may represent the formation and subsequent dissolution of precipitates. Cloud point does not appear due to the low concentration of phenol present in the sample.

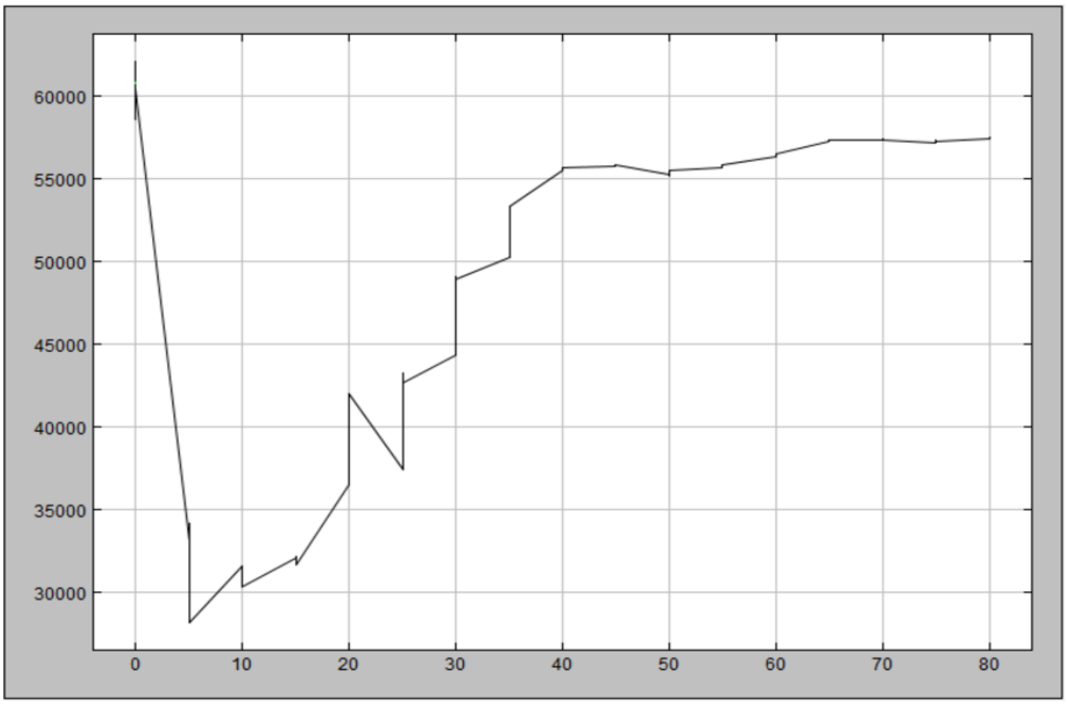

Figure A23. NaCl titration corresponding to experiment N1f (PS80 0.10 wt%-Phenol 0.46 wt%). There is no initial addition of salt to the cuvette. The stable zone is reached after the addition of 40 μL of NaCl 5 M.

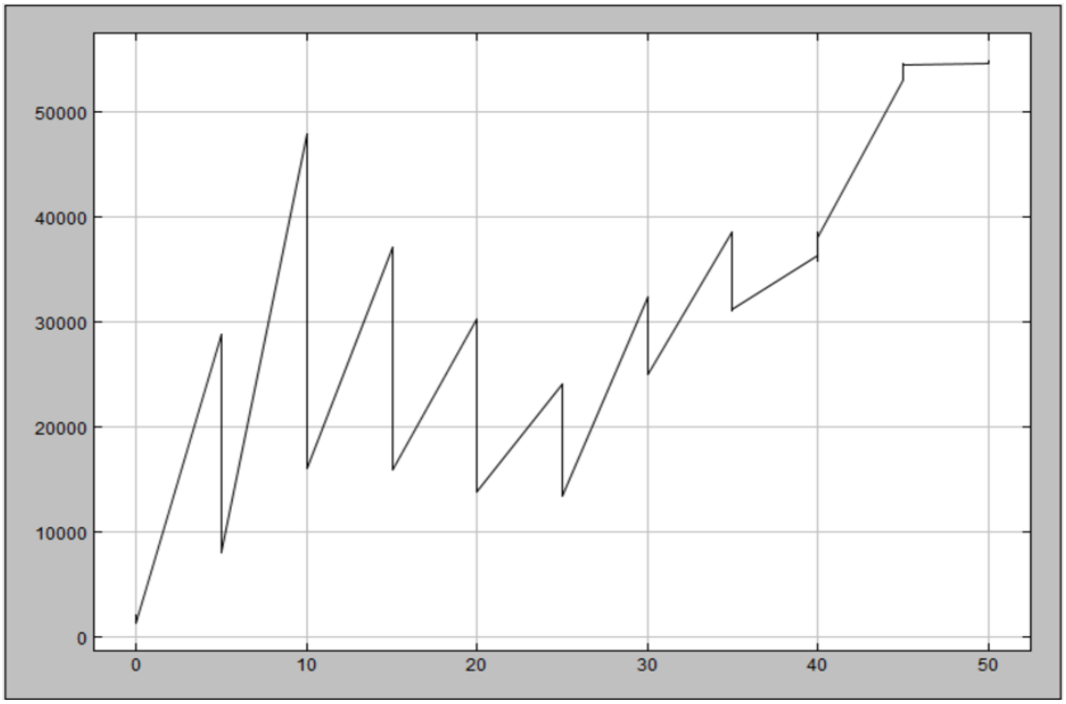

Figure A24. NaCl titration corresponding to experiment N1f(NaCl) (PS80 0.10 wt%-Phenol 0.46 wt% + 30 μL of NaCl 5 M). Before starting titration, 30 μl of NaCl 5M is added to the cuvette. Intermediate peaks appear which may represent the formation and subsequent dissolution of precipitates. The stable zone is reached after the addition of 45 μL of NaCl 5 M (total amount of salt equal to 75 μL). There is a clear delay in the appearance of the stable zone compared to Figure A23 (sample without NaCl in the cuvette).

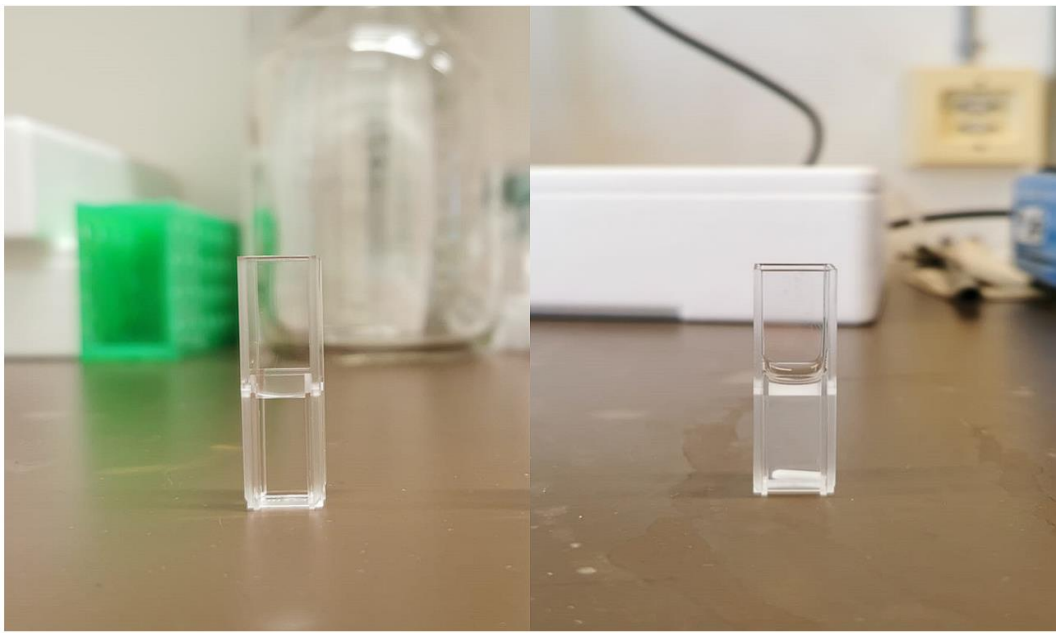

Figure A25. Visual inspection of two cuvettes. On the left, sample showing no turbidity (only contains PS80; no phenol has been added and therefore the cloud point has not been reached at 25ºC). On the right, sample showing turbidity (contains PS80 and the phenol necessary to reach the cloud point at 25ºC).