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Analysis of the crystallization process of a biopharmaceutical compound in the presence of impurities using process analytical technology (PAT) tools

Elena Simone,¹ Wei Zhang,^{2,3} Zoltan K. Nagy*^{1,3}

¹ *Department of Chemical Engineering, Loughborough University, Loughborough, LE11
3TU, U.K.*

² *New drug research and development center, North China Pharmaceutical group
Corporation, Shijiazhuang, China*

³ *School of Chemical Engineering, Purdue University, West Lafayette, 47907, Indiana,
United State*

Abstract

The crystallization of biopharmaceuticals can be problematic since, because the biosynthesis of these compounds is very difficult to control, they can present a significant amount of impurities that has to be eliminated. In fact, impurities can lead to changes in the properties of the drug that can significantly reduce its effectiveness or even put in danger the user.

The substance used in this work is vitamin B12 crude product extracted from fermentation.

The aim of this work is to exploit process analytical technology (PAT) tools to study the crystallization step of vitamin B12. Linear cooling crystallization experiments were performed using different conditions. The effects of solvent, cooling rate, seeding and purity of the initial material on the final size distribution and purity of the crystals were investigated through the use of UV/Vis spectroscopy, focused beam reflectance measurement (FBRM) and the CryPRINS software (Crystallization Process Informatics System).

It was found that impurities strongly inhibit the growth of vitamin B12 crystals, promoting nucleation and leading to a poor final crystal size distribution. Slow cooling can help in increasing the purity of the final product but also generates a broad crystal size distribution because of secondary nucleation. Preparing the solution with material already crystallized once and using purified seeds helped in obtaining a narrower crystal size distribution and also reduces breakage.

Keywords: PAT tools, biopharmaceutical, vitamin, crystallization, monitoring

1 **Introduction**

2 Over the last decade the investment in research and development of biopharmaceutical
3 companies has more than doubled: there is a great interest in formulating and optimizing the
4 manufacturing of bio-molecules and biopharmaceuticals such as proteins, lipids, vitamins,
5 hormones and DNA. Those compounds can be used in the treatment of serious diseases or
6 just as nutritional supplement.

7 Vitamin B12 (cobalamin) is one of the biggest and most complex vitamins and, in humans, it
8 is required to assist the actions of two enzymes: methionine synthase and (R)-methylmalonyl-
9 CoA mutase. It is commonly used to cure pernicious anemia and its deficiency determines an
10 increase in homocysteine levels that leads to a major risk for heart disease, stroke,
11 atherosclerosis and vascular diseases. Despite the lack of solid evidences on the importance
12 of vitamin B12 in maintaining normal myelination of nerve cells, its role in preventing many
13 neurological and psychiatric symptoms is becoming clearer and clearer. For these reasons,
14 every year, around 10 tons of the semisynthetic version of vitamin B12 (cyanocobalamin) are
15 produced by biofermentation from several bacterial species.¹

16 A schematic of vitamin B12 molecule is shown in Figure 1. The molecule is fairly large and
17 complex and can be divided in three parts: (1) a central corrin ring which contains four
18 ligands for the central cobalt ion, (2) a lower ligand donated by the 5,6-
19 dimethylbenzimidazole (DMBI) and (3) an upper ligand made from either an adenosyl group
20 or a methyl group.

21 Two pseudo-polymorphic forms were discovered by X-ray diffraction: a wet and a dry
22 crystalline structure.^{2,3} The main difference between the two is the presence of water
23 molecules in the crystal lattice that makes the unit cell of the wet form bigger than the dry.
24 Cyanocobalamin is mainly produced by biosynthetic fermentation processes, although a full
25 chemical synthesis (consisting of 70 steps) was developed in the early 70s.¹ High levels of

1 impurities are present in the biosynthesized vitamin B12 that can inhibit growth of the
2 crystals and strongly affect the final crystal size distribution together with the ease of
3 downstream processes (filtration in particular).

4 Process analytical technology (PAT) tools are widely used for the development of the
5 crystallization processes of many synthetic pharmaceuticals.⁴⁻⁶ The term PAT refers to “a
6 system for designing, analyzing, and controlling manufacturing through timely measurement
7 of critical quality and performance attributes of raw and in-process material and processes,
8 with the goal of ensuring final product quality”.⁷ PAT tools are the main element used in the
9 “Quality by Design” (QbD) approach that was introduced in the pharmaceutical industries in
10 order to minimize product waste due to mistakes in the manufacturing process. The QbD
11 concept consists in obtaining the desired quality of the product through the correct design of
12 the manufacturing process. In this approach some critical variables of the process, strictly
13 related to the quality of the product, are controlled during the manufacturing in order to
14 control the quality of the product itself.^{8,9} The QbD approach is well known and applied in
15 the pharmaceutical industry but only few examples are present for the manufacturing of
16 biopharmaceuticals.^{10,11} Despite the difficulty of controlling biological processes Near
17 infrared (NIR) and Raman spectroscopy, HPLC and image analysis were recently used for
18 some manufacturing steps of biomolecules.¹²⁻¹⁴

19 In this work the crystallization of vitamin B12 will be studied with PAT tools (ATR-UV/Vis,
20 FBRM and CryPRINS) and the effect of impurities on the growth and nucleation rate will be
21 analyzed. The produced crystals are analyzed with optical microscopy, HPLC and Raman.
22 Most of the impurities in cyanocobalamin have high levels of fluorescence and, therefore,
23 Raman can give an indication of the purity of the crystals since it is highly sensitive to this
24 phenomenon.^{15,16} However, for a precise quantification of the type and level of impurity in
25 vitamin B12 samples HPLC is necessary. The paper provides one of the first comprehensive

case studies that illustrate how an array of PAT tools can be used for the systematic understanding and design of a biopharmaceutical crystallization process.

Materials and methods

Equipment

Crude Vitamin B12 produced by fermentation was donated by Hebei Welcome Pharmaceutical Co., LTD (China). The raw material consisted in a dry powder containing around 7% of impurities. Those were mainly byproducts of the fermentation process (e.g. 50-carboxyl cyanocobalamin, 34-methyl cobalamin, 8-epi-cyanocobalamin and cyanocobalaminic lactate) and inert. Ultrapure water was obtained via a Millipore ultra-pure water system while ethanol was purchased by Fisher Scientific (purity >95% v/v). Pure water or solutions of water and ethanol were used as solvents for the experiments.

Experiments were conducted at both Loughborough University, UK and Purdue University, USA with similar, but not exactly identical instruments. Two 500 ml glass jacketed vessels and the same Raman system (an RXN1 Raman analyzer with immersion probe and 785 nm laser, Kaiser with iC Raman 4.1 software) were used. A simple schematic of the rig used for the experiments is shown in Figure 2. The experiments at Loughborough University were performed using an MSC621 Carl Zeiss ATR-UV/Vis spectrophotometer with Hellma ATR (type 661.822-UV) probe and a LabView based in-house software, a D600L Lasentec FBRM probe (Mettler Toledo with FBRM software V 6.7.0) and a Huber Ministat CC3 thermoregulator. The data from the FBRM, ATR-UV/Vis and the Huber is transmitted in real-time to the CryPRINS software (Crystallization Process Informatics System). This allows real-time monitoring and control of the FBRM counts, ATR-UV/Vis signal and the temperature, as well as setting a temperature profile and performing different control strategies. The instrumentation at Purdue University consisted in a Zeiss MCS621 UV/Vis

spectrophotometer with UV-VIS 190-720 nm Zeiss probe (Zeiss ProcessXplorer software version 1,3-Build 1.3.1.30), an FBRM G400 0.5-2000microns (Mettler Toledo with ic.FBRM software version 4.3) and a JULABO F25-ME thermoregulator. The samples of vitamin produced were analyzed using a Raman RXN1 microprobe (Kaiser) and an HPLC Agilent 1100 series (Hewlett Packard) with ChemStation software rev. A.09.03[1417].

Procedure for linear cooling experiments

Mixtures of water and ethanol and pure water were used as solvents for the linear cooling experiments. The concentrations used were about 0.118-0.13 g/g solvent in water and ethanol (saturation temperature of about 50 °C) and between 0.09 and 0.11 g/g solvent for water (saturation temperature of about 60 °C). The material was dissolved and the hot solution was filtered (using a filter paper) before starting the cooling profile because of the presence of insoluble particles in the raw material. The solubility of vitamin B12 in water at different temperatures was provided by the supplying company and is shown in Table 1, while data for ethanol/water mixtures at different temperatures was not available from the literature and the amount of material donated was too small to accurately measure it. However, two inferential curves of the solubility expressed as UV signal (absorbance value at 361 nm) were obtained in order to compare two solvents. Those curves can also be directly compared with the UV data in the Results and Discussion section (which is actually an absorbance and not a concentration value). The curves were acquired by slow heating (0.075 °C/min) of two slurries of vitamin B12 in water and ethanol/water. The amount of vitamin dissolved at any time and temperature is proportional to the absorbance value recorded. Figure 3 shows the inferential curves as well as the exact solubility in the two solvents at high temperatures (120 g/L ethanol/water solution at 53 °C, and 84 g/L water at 62 °C). The material was added to the solvent at ambient temperature and then dissolved by heating the solution and keeping it at high temperature for 15-20 min. In ethanol and water the higher temperature was around

75 °C while in pure water it was around 70 °C. Four cooling rates were used: -0.075, -0.1, -0.5 and -1 °C/min (slower, slow, fast and faster cooling), both seeded and unseeded experiments were performed. The cooling rates were chosen based on experience with similar types of crystals, equipment and scale.¹⁹⁻²¹ The final temperature after cooling was 5-6 °C. In the case of the seeded experiments the mass of seeds was of about 2.5 % of the total solid dissolved in the vessel. Seeds were obtained after three successive crystallizations of the raw material. The same seeds were used for all the seeded experiments: crystals were in the form of short needles with fairly homogenous width (around 40-60 µm) and variable length (up to around 200 µm). At the end of the profile, the temperature was kept constant for few hours (until both FBRM and ATR-UV/Vis signals stabilized); after that, crystals were filtered and washed with acetone. CryPRINS was used to select the desired heating/cooling rate and to monitor the UV and FBRM signals during the experiments. In addition to the linear cooling experiments, three consecutive crystallizations of the same material were conducted using ethanol and water or pure water as solvents. Recrystallizing the same material more than once allows obtaining very pure crystals but the overall yield of the process is penalized. These three experiments were conducted to check the purity that could be obtained by using multiple steps of crystallization compared to a single linear cooling profile. Table 2 shows the conditions of the experiments performed in this section.

HPLC analysis

The mobile phase for the HPLC analyses was prepared dissolving 7.4 g of $Na_2HPO_4 \cdot 2H_2O$ (HPLC purity grade, Fisher Scientific) in 740 ml of deionized water (from a Millipore Elix 5) and then adding 260 ml of methanol (Fisher Scientific). The solution pH was adjusted to 3.5 using H_3PO_4 (Fisons analytical reagents). The solid samples were dissolved in purified water (concentration of about 1 mg/ml). For the detection of the sample constituents the UV wavelength at 351 nm was used. The flow rate applied was 0.8 ml/min for a total running

time of 40 min for each sample. The temperature of the column was kept constant at 35 °C and the injection size was 20 µl. The type of column used was an Analytical Column Waters Spherisorb® 5µm C8 4.6×250 mm. The main peak at about 14 min corresponds to cyanocobalamin while the other peaks are byproducts (50-carboxyl cyanocobalamin, 34-methyl cobalamin, 8-Epi- cyanocobalamin, 7β,8β-cyanocobalamin lactate) and other unspecified impurities. All samples were analyzed at least twice and purity was calculated as the average relative area of the cyanocobalamin peak.

Principal component analysis on Raman spectroscopic data

Principal components analysis (PCA) is a dimension reduction technique for quantitative data. Given a set of data in more variables, with PCA it is possible to reduce the dimensions of the problem down to two or three, keeping at the same time, enough information to capture the inherent variability within the data. PCA is a method that models all the variations in the data set using orthogonal basis vectors (eigenvectors) which are called principal components in this analysis.¹⁷ If A is the $n \times m$ matrix of input data (n rows of spectra recorded at m wavelengths for example), PCA can decompose it in the following linear system:

$$A = CP^T + \varepsilon, \quad (1)$$

where C is the $n \times k$ matrix of k principal components' scores and P is the $m \times k$ matrix of the eigenvectors of A . The eigenvectors are called "loadings" in PCA. ε is a matrix containing the unexplained variance. Loadings and score for PCA can be used to interpret the data. The scores are usually represented in two-dimensional scatter plots. Often, a very high percentage of variability is gathered in the first few principal components so only a few of these plots are necessary. The scores plots are usually used to detect similarities, differences or other interesting relationships among samples. A PCA was performed over Raman spectroscopic data of vitamin B12 in order to determine a relation between the purity of the

analyzed crystals and their Raman spectra. Matlab 2013a was used for the calculation of the principal component scores (*pca* function).

Results and discussion

Linear cooling experiments

Simple linear cooling was applied to the raw and recrystallized vitamin B12 and the results were studied using FBRM and images from an optical microscope. The crystal size distribution (CSD) was found to be strongly affected by the purity of the material used and the cooling rate. Usually large crystals and a narrow crystal size distribution are preferred because they facilitate the downstream operations (such as filtration, washing and milling). For this reason a good quality CSD is characterized by large crystals of similar size. The solvent used did not have a significant effect on the CSD but it affects the final purity of the crystals. The effect of seeding was also analyzed and it was found that a slightly purer material can be obtained at the end of the crystallization using purified seed. Figure 4 shows the results of experiment 1 and experiment 2 described in Table 2, in which raw vitamin B12 was crystallized from an ethanol/water mixture at two different cooling rates (-0.5 and -0.1 °C/min, fast and slow cooling). From the microscopic images of crystals at the end of the two experiments (see Figure 4a and Figure 4b) it can be noticed that in both cases the size distributions look very broad with both small and large crystals; but a considerably larger size is clearly generated by the slow cooling shown in Figure 4b. This behaviour is similar to what normally happens for common pharmaceuticals such as paracetamol.¹⁸⁻²¹ In general, during a fast cooling experiment, nucleation happens at high supersaturation generating a large amount of small nuclei while in a slow cooling experiment, few nuclei are nucleated at low supersaturation. However, for paracetamol, a fast cooling generates a high number of total counts from the FBRM due to the large number of nuclei generated at high supersaturation. In the

case of Vitamin B12 the trend seems to be the opposite, as shown in Figure 5a and b. In experiment 1 (Figure 5a) when slow cooling was used, the number of counts was lower than in experiment 2 (Figure 5b) which was conducted at a fast cooling rate. Furthermore, the images from the microscope seem to contrast with the data from FBRM data since, clearly less and bigger particles were generated using slow cooling (Figure 4b) compared to fast cooling (Figure 4a). Additionally, the final square weighted mean chord length distribution is higher for the fast cooling rate (Figure 5a) than the slow one (Figure 5b). This discrepancy between images and FBRM data is related to the needle shape of the vitamin B12 crystals. As the particles grow in length, the shorter size of the crystals is counted by the FBRM more times than the longer giving the erroneous larger number of counts for small crystal size present in the vessel. A similar behavior for this probe was observed by Leyssens et al. for a small needle-like molecule prodrug antagonist called CDP323-2.²²

The longer the crystals are, the higher is the value of the total counts recorded by FBRM; and that is the reason why slow cooling of raw vitamin B12 generated a higher value of the total counts than fast cooling (70,600 #/meas. in the slow cooling against 44,000 #/meas. as shown in Figure 4a and 4b). However, considering the large amount of small crystals present at the end of both experiments (in particular see Figure 4a for experiment 1 and Figure 4c for experiment 3), it is clear that secondary nucleation prevails on growth for raw vitamin B12. This can be explained by the fact that the presence of impurities inhibits growth, and supersaturation is used mainly to nucleate new small crystals.

Some breakage is also present in the slow cooling after 400 min as shown in the CLD distribution of Figure 5d, where a reduction in the number of larger particles can be observed. Breakage of crystals also contributed to the higher number of counts in the case of slow cooling. Figure 6a and 6c show the FBRM and UV data for fast and slow cooling crystallization of a purified solution of vitamin B12 (experiment 3 and 4 of Table 2). The use

of recrystallized material with fewer impurities reduced considerably both primary and secondary nucleation during the cooling phase and promoted growth of the existing crystals. Figure 6b and 6d show the microscopic images of the resulting crystals; the crystal size distribution is narrower compared to experiment 1 and 2 and crystals are considerably larger with the maximum total counts that reaches only 5,200 #/meas. in the fast cooling experiment and 26,900#/meas. for the slow cooling. The effect of the shape of the crystals on the FBRM data is clearer in these two experiments because of the absence of breakage or secondary nucleation; crystals in Figure 6d are clearly longer than the ones shown in Figure 6b and the CLD (Figure 7a and 7b) present a bimodal shape with a growing peak at low sizes due to the increasing counts of the shorter size of the crystals.

It is clear that growth of vitamin B12 is strongly inhibited by impurities and very broad crystal size distributions are generated by secondary nucleation and breakage. Additionally, in some cases, multiple nucleation events can happen as shown in Figure 8b, c and d, which is also a clear indication of growth inhibited crystallization processes in the presence of impurities. Figure 8a shows the results for experiment 5 (Table 2) where vitamin B12 nucleated and grew throughout the entire cooling period. Figure 8b, 8c and 8d instead, show cases in which a second major nucleation event happened during the cooling phase. The UV signal dropped suddenly during the second nucleation and the counts increased at the same time. This phenomenon appeared with both raw and recrystallized material, for seeded and unseeded experiments and for both the solvents used. During these experiments growth was so inhibited that supersaturation accumulated during the cooling until it reached a level at which a second major nucleation event could happen. In these cases the final CLD is very broad and many small particles are present as a result of this double nucleation as shown in Figure 9 (for experiment 7).

Purity analysis using HPLC and Raman spectroscopy

A first qualitative screening of both the raw material and the crystals obtained by consecutive crystallizations (with increasing purity as shown in Table 3) was performed using Raman microscopy. The inert material filtered during the crystallizations was also analyzed. Figure 10a shows the Raman spectra of raw and filtered material; a strong fluorescence, due to impurities can be noticed (high intensity and no defined peaks). It is interesting to observe that just the filtration of the hot solution eliminates part of the fluorescent material. A loss of fluorescence due to less impurity can also be noticed in the samples of the consecutive crystallizations (Figure 10b). The number and position of the peaks is the same for all the samples but they tend to become broader and less intense as fluorescence (and, therefore impurity) increases. In order to have some quantitative information from Raman spectroscopy a principal component analysis was performed over the Raman spectra of the recrystallized material, the raw material and the filtered one. Figure 11a shows the score of principal component PC2 plotted versus the score of principal component PC1. The raw and filtered material points are isolated and very far from the crystallized samples because of their high impurity content. Figure 11b shows only the crystallized samples: a clear trend of the scores as a function of purity is present. This example provides an illustration for the non-conventional use of Raman spectroscopy for monitoring low concentration of impurities based on fluoresce. While fluorescence is in general undesirable in Raman spectroscopy, since it makes the interpretation of the significant peaks difficult, monitoring the amount of fluorescence in the Raman spectra can serve as a method of detecting the existence and changes in impurity levels in the case of fluorescent impurities.

The loss of impurity during the consecutive crystallizations also considerably improved the size of the crystals as shown in Figure 12. The raw material showed in Figure 12a is highly fluorescent and amorphous; particles are very small and hygroscopic and the colour is intense

red. Crystalline vitamin B12 is darker as shown in Figure 12b to 12d. The mean size of the crystals increases during the consecutive crystallization as a result of less inhibited growth. The results of HPLC analyses of the samples from selected experiments out of those performed are shown in Table 3. A higher purity can be reached if slow cooling rates are applied and seeding also helps in increasing the purification efficiency. The largest increase in purity was reached using raw material in an ethanol/water mixture with seeding and slow cooling (linear cooling experiment 14, increase in purity of 3.65%). The second crystallizations (using purified material) as expected present a lower increase in purity; however, even for this set of experiments, slow cooling and seeding helped reaching a higher purity. The effect of the solvent on the final crystal size distribution is negligible but choosing the suitable solvent seems to help increasing the purity of the product. It also seems not to affect purification efficiency since the final purity of similar experiments in different solvents is very close.

Conclusions

A set of PAT tools was used to study the crystallization in the presence of impurities of a small biomolecule produced by fermentation (vitamin B12). Materials produced by biosynthesis often contain a high amount of impurities that can significantly reduce the quality of the final crystals in term of size distribution. Multiple successive recrystallizations of the same material can help eliminating impurities and reaching a better crystal size distribution, but they also contribute to decrease the yield. Using PAT tools can help understanding the characteristics of the studied compound and developing a correct crystallization strategy to improve crystal size and purity of the final product. It was found that impurities strongly affect the growth rate, promoting nucleation and, in some cases, generating multiple nucleation events during the cooling profile. The final crystal

size distribution for these cases was found to be very broad, with very large crystals together with significant amounts of fines. Slow cooling of very impure material increases the probability of multiple nucleation events and, therefore, leads to a broad crystal size distribution. However, slow cooling was found to generate more pure crystals compared to fast cooling. The best quality of the crystals, in terms of size, was reached using already crystallized material to prepare the solution and slow cooling rates. Seeding was also found to help in increasing the purity of the final crystal while a change in the solvent composition did not largely affect purity or final CSD. The needle shape of the vitamin B12 crystals also had to be considered before interpreting the FBRM results. The growth of the long size of the needles generates an increase in the FBRM total counts that can be confused with nucleation. An analysis of the crystals with the optical microscope was necessary to interpret the counts and chord length distribution recorded by the FBRM. Monitoring the level of fluorescence due to impurities in the Raman spectrum, can serve as a method of detecting changes in the impurity concentrations in the crystalized products.

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1 **Table 1: Solubility of vitamin B12 in water at different temperature (data provided by Hebei Welcome**
2 **Pharmaceutical Co., LTD)**

Temperature (°C)	Solubility (g/l water)
10	9.25
20	10
25	11.5
30	13.5
45	24

3
4
5

1 **Table 2: Experiment conditions, slow and fast cooling of raw and crystallized material**

Experiment	Solvent	Vitamin concentration (g/g solvent)	Type of material	Cooling rate (°C/min)	Seeding
1	Ethanol/water	0.118	Raw	-0.5 (fast cooling)	No
2	Ethanol/water	0.118	Raw	-0.1 (slow cooling)	No
3	Water	0.097	Crystallized (more than once)	-0.5 (fast cooling)	No
4	Water	0.097	Crystallized (more than once)	-0.1 (slow cooling)	No
5	Water	0.089	Raw material	-0.1 (slow cooling)	No
6	Water	0.106	Crystallized	-0.075(slower cooling)	No
7	Water	0.089	Raw material	-0.1 (slow cooling)	Yes
8	Water	0.097	Crystallized	-0.1 (slow cooling)	Yes
9	Ethanol/water	0.119	Raw	-1 (faster cooling)	No
10	Ethanol/water	0.119	Raw	-1 (faster cooling)	Yes
11	Ethanol/water	0.119	Raw	-0.5 (fast cooling)	No
12	Ethanol/water	0.119	Raw	-0.5 (fast cooling)	Yes
13	Ethanol/water	0.119	Raw	-0.1 (slow cooling)	No
14	Ethanol/water	0.119	Raw	-0.1 (slow cooling)	Yes
15	Ethanol/water	0.13	Crystallized	-1 (faster cooling)	No
16	Ethanol/water	0.13	Crystallized	-0.5 (fast cooling)	No(150 rpm)
17	Ethanol/water	0.13	Crystallized	-0.5(fast cooling)	No(240rpm)
18	Ethanol/water	0.13	Crystallized	-0.5 (fast cooling)	Yes
19	Ethanol/water	0.13	Crystallized	-0.1 (slow cooling)	No
20	Ethanol/water	0.115	Raw	-0.5	No
21	Ethanol/water	0.137	From exp. 20	-0.5	No
22	Water	0.082	From exp. 21	-0.5	No

2

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Table 3: HPLC results for selected experiments performed

Sample	Purity (%)	Improvement in purity (%)	Solvent used	Material used	Cooling rate (°C/min)	Seeded
Raw material	93.11	0	n.a.	n.a.	n.a.	n.a.
First crystallization	96.14	3.03	n.a.	n.a.	n.a.	n.a.
Second crystallization	97.29	1.16	n.a.	n.a.	n.a.	n.a.
Third crystallization	98.03	0.74	n.a.	n.a.	n.a.	n.a.
LC experiment 8	97.54	1.24	Water	Crystallized	-0.1	Yes
LC experiment 9	96.35	3.24	Ethanol/water	Raw	-1	No
LC experiment 10	96.28	3.17	Ethanol/water	Raw	-1	Yes
LC experiment 11	96.28	3.17	Ethanol/water	Raw	-0.5	No
LC experiment 12	96.72	3.61	Ethanol/water	Raw	-0.5	Yes
LC experiment 13	96.51	3.40	Ethanol/water	Raw	-0.1	No
LC experiment 14	96.76	3.65	Ethanol/water	Raw	-0.1	Yes
LC experiment 15	97.66	0.94	Ethanol/water	Crystallized	-1	No
LC experiment 16	97.70	0.98	Ethanol/water	Crystallized	-0.5	No
LC experiment 17	97.72	1.00	Ethanol/water	Crystallized	-0.5	No
LC experiment 18	97.95	1.23	Ethanol/water	Crystallized	-0.5	Yes
LC experiment 19	97.77	1.05	Ethanol/water	Crystallized	-0.1	No
LC experiment 20	96.14	3.03	Ethanol/Water	Raw	-0.5	No
LC experiment 21	97.29	1.16	Ethanol/Water	Crystallized once	-0.5	No
LC experiment 22	98.03	0.74	Water	Crystallized twice	-0.5	No

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(a)

(b)

(c)

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