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Natural acidic deep eutectic solvent to obtain cellulose nanocrystals using the design of experience approach.

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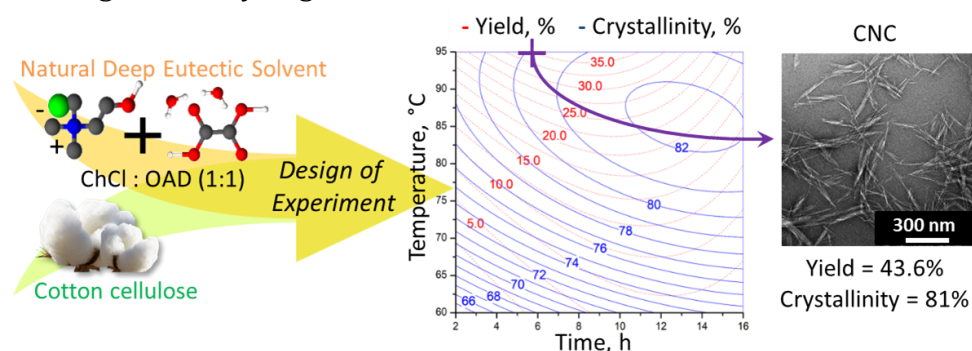
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Abstract

In this study, a new approach to optimize the cellulose nanocrystals (CNCs) extraction using acidic natural deep eutectic solvents (NADES) was introduced using, for the first time, design of experiment method. Choline chloride:oxalic acid dihydrate with a molar ratio of 1:1 was used to extract CNCs. Then, three most important parameters were varied to design the experiment: (i) cotton fibre concentrations, (ii) temperature and (iii) treatment time. Two outcomes were studied: the CNC yield and the crystallinity. The mathematical model for crystallinity perfectly described the experiments, while the model for CNC yield provided only a tendency. For a reaction time of 6 h at 95°C with a fibre concentration of 2%, the expected optimum CNC yield was approximately $35.5 \pm 2.7\%$ with a crystallinity index of $80 \pm 1\%$. The obtained experimental results confirmed the models with $43.6 \pm 1.9\%$ and $81 \pm 1\%$ for the CNC yield and the crystallinity index, respectively. This study shows that it is possible to predict the CNC yield and their crystallinity thanks to predictive mathematical models, which gives a great advantage to consider in the near future a scale up of the extraction of cellulose nanocrystals using this original family of green solvents.



Keywords: Cellulose nanocrystal – Natural Deep Eutectic Solvents – Design of Experiment – High crystallinity – Treatment optimisation

Highlights

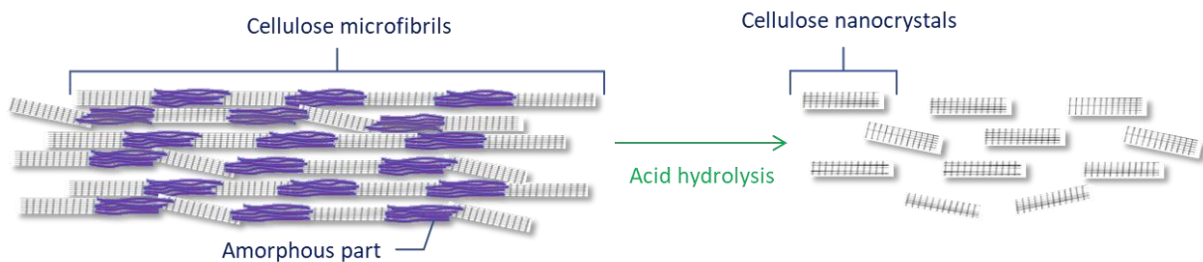
- Cellulose nanocrystals are successfully extracted using a natural deep eutectic solvent.

- The influence of the treatment time, temperature, and initial fiber concentration during the CNC extraction was studied and thoroughly described using mathematical models.
- 16 different parameter combinations are employed to build and to check the validity of these two mathematical models.
- Cellulose nanocrystals having optimal yield and crystallinity of 44.9% and 81%, respectively, were obtained.

1. Introduction

In 1947, Nickerson and Habrle were among the first to reveal the existence of a crystalline part on cellulosic fibres and isolate it using an acid hydrolysis process in 1947 (Nickerson and Habrle, 1947). Since the 1960s, cellulose nanocrystals (CNCs) have been extracted *via* sulfuric acid hydrolysis of the cellulose amorphous parts. CNCs have many characteristics that make them suitable for the development of innovative materials for various applications, e.g., the abundance of biosourced starting materials, their low density (1.6 g/cm³), and their high stiffness and specific surface area. Moreover, due to the presence of hydroxyl groups on their surface, CNCs are hydrophilic and reactive for post-modification to provide new functionalities. Biobased CNC nanoparticles are now commercially available (Reid, Villalobos, and Cranston, 2017), and they show promising results in various applications, such as biocomposites (Mariano, Kissi, and Dufresne, 2014), hydrogels (Du *et al.*, 2019), rheological modifiers (Gicquel *et al.*, 2019), tissue engineering and health care applications (Domingues, Gomes, and Reis 2014; Shankaran, 2018) and coatings (Mascheroni *et al.*, 2016). CNCs are rod-shaped particles with the following nanometric dimensions: their lengths are approximately 100 to 500 nm depending on the cellulose source (Bras *et al.* 2011) and their diameters are between 5 and 20 nm (Ramires and Dufresne, 2011; Isogai, 2020).

CNCs can be extracted by hydrolysing glycosidic bound of the amorphous part of cellulosic fibres. Sulfuric acid (H₂SO₄) is the most common acid used to obtain CNCs; indeed, this common procedure can be applied to any cellulose source with small adaptations, as sketched in scheme 1.



Scheme 1: Schematic representation of acid hydrolysis of cellulose fibre

Recently published reviews identify all the different conditions that can be used to extract cellulose nanocrystals with this “conventional hydrolysis”. (Dufresne 2017; Haldar and Purkait 2020). However, it was proved that the CNC yield, and their properties strongly depend of the cellulose sources and treatment conditions (time and temperature of treatment, and initial acid concentration). Thus, for example, Flauzino Neto *et al.* extracted cellulose nanocrystal from soy hull using H₂SO₄ 64% treatment, and

76 showed that prolonging the reaction by 10 min decrease the CNC length and reduce
77 their crystallinity (Flauzino Neto *et al.*, 2013).

78 However, other methods enable CNC extraction from biomass. For example, other types
79 of acids have been used, such as hydrochloric acid (Araki *et al.* 1999) or organic acid
80 (Filson and Dawsonandoh, 2009). Subcritical water (Novo *et al.* 2015) or ionic liquid
81 (Man *et al.* 2011) have also been used for cellulose nanocrystal extraction. More
82 recently, a new solution was proposed that consists of using acidic deep eutectic
83 solvents as reactive media (Zdanowicz, Wilpiszewska, and Szychaj 2018).

84 Deep eutectic solvents (DESs) are a new class of organic solvents that were introduced
85 for the first time in 2003 by Abbott *et al.*, who found that the eutectic mixture formed by
86 choline chloride and urea with a molar ratio of 1:2 exhibited a freezing point of 12°C,
87 which is considerably lower than that of either of the constituents (choline chloride =
88 302°C and urea = 133°C) (Abbott *et al.*, 2003). DESs are in a state of molten salt and ionic
89 liquid solvent, but compared to other solvents, they are easier to use and less toxic and
90 are composed of a Lewis or Brønsted acid and a base with a specific ratio. According to
91 the nature of these two constituents, DES can be classified into four types (Smith,
92 Abbott, and Ryder, 2014).

93 In the case of DES type III, the two constituents are a quaternary ammonium salt and a
94 hydrogen bond donor; the association of these compounds results in a eutectic mixture
95 *via* hydrogen bonding interactions between the hydrogen bond acceptor and the
96 hydrogen bond donor. The melting temperature of the mixture, far below that of its
97 individual constituents, can be explained by the fact that the strong hydrogen bonds
98 between the different compounds prevent the crystallization of each product (Francisco,
99 van den Bruinhorst, and Kroon, 2013). Moreover, if the DES is formulated with natural
100 compounds, the obtained mixture is referred to as natural deep eutectic solvent
101 (NADES), which can be considered a green solvent due to its nonvolatility, low toxicity,
102 and potential renewability, recyclability and biodegradability (Paiva *et al.* 2014; Vanda
103 *et al.*, 2018).

104 DES and NADES are easy to obtain and can be helpful in organic chemistry ; indeed, they
105 can replace some toxic organic solvents in a large number of application fields. In a
106 recent review, Zdanowicz *et al.* summarized the possible application range of DES and
107 NADES for polysaccharide processing (Zdanowicz, Wilpiszewska, and Szychaj, 2018).
108 One of these applications used of Type III NADES as an acidic hydrolytic solvent to
109 obtain CNCs. NADESs exhibit lower vapour pressures than aqueous solutions and thus
110 can be considered safer than aqueous solutions. Moreover, recent studies have claimed
111 the possibility of reusing DES five times without a decrease in their efficiency (Li *et al.*
112 2018; Liu *et al.*, 2017). These two advantages indicate that NADES should be more
113 deeply studied as a new method of cellulose nanocrystal production.

114 Many combinations of hydrogen bond donors and acceptors using different ratios can be
115 used to obtain acidic deep eutectic solvents that can hydrolyse the amorphous parts of
116 cellulose (Sirviö, Visanko, and Liimatainen, 2016; Ibrahim, Abdullah, and Sam, 2018;
117 Sirviö 2019). However, the most studied NADES was used for the first time by Sirviö *et*
118 *al.* in 2016 (Sirviö, Visanko, and Liimatainen, 2016). The researchers managed to extract
119 individual cellulose nanocrystals using a choline chloride:oxalic acid dihydrate
120 (ChCl:OAD 1:1) treatment from dissolving pulp. They used two different temperatures
121 (T=100 or 120°C) and a reaction time of 2 hours. After this pre-treatment, NADES was
122 removed using distilled water, and the suspension was disintegrated mechanically using
123 a microfluidizer. The CNCs produced at 120°C showed a high aspect ratio with a length
124 of approximately 353 ± 16 nm and a diameter of 9.9 ± 0.7 nm. Since this first study,

125 different studies using this NADES in different ratios, various experimental conditions,
126 and different cellulose sources have been conducted to extract CNCs. In 2017, Laitinen
127 *et al.* showed that only 30 minutes of treatment at 100°C is enough to extract CNC from
128 bleached birch Kraft pulp using ChCl:OAD 1:1 as the pre-treatment. The nanocrystals
129 obtained are non-charged and can be used as oil-water Pickering stabilizers (Laitinen *et*
130 *al.*, 2017). However, a faster way to produce CNCs using this solvent is to assist the pre-
131 treatment by microwave application. Using this technique, Liu *et al.* obtained a CNC
132 suspension in only 3 minutes of reaction (Liu *et al.*, 2017). More recently, Ling *et al.*
133 studied the effect of ChCl:OAD treatment on the cellulose nanocrystal structure. They
134 compared three ChCl:OAD molar ratios of 1:1, 1:2, and 1:3 at two temperatures of 80°C
135 and 100°C. In all cases, they managed to obtain cellulose nanocrystals, but lower
136 crystallinity and lamellar structures were observed for CNCs with a lower acid content
137 treatment. The CNCs obtained with a higher acid ratio (ChCl:OAD 1:3) were more
138 dispersed and exhibited a higher aspect ratio (Ling *et al.*, 2019). Inspired by the
139 conventional acid hydrolysis treatment with H₂SO₄, Yang *et al.* proposed the use of a
140 catalyst (FeCl₃ · 6H₂O) during DES treatment. They varied the temperature of the
141 treatment and the molar ratio of choline chloride, oxalic acid dihydrate, and catalyst and
142 found that the optimum conditions for the treatment were 80°C and 6 h of treatment in
143 ChCl:OAD:FeCl₃·6H₂O DES at a molar ratio of 1:4.43:0.1 (Yang *et al.*, 2019).

144 All these studies have been conducted recently and have not provided clear information
145 on the CNC yields. The possibilities to generate new acidic natural deep eutectic solvents
146 are almost infinite; indeed, simply changing the molar ratio between the constituents of
147 the solvent would result in a new solvent with new properties. The same is true if
148 another acid is used as the hydrogen bond donor. Therefore, the aim of the present
149 study is to examine the role of time and reaction temperature in the production of
150 cellulose nanocrystals. Our hypothesis is that there is an optimum of parameters for
151 obtaining higher yield of cellulose nanocrystals via DES treatment.

152 In this work, a design of experiment (DOE) approach is used to follow the yield of
153 cellulose nanocrystals and their crystallinity with the commonly used natural deep
154 eutectic solvent ChCl:OAD at a molar ratio of 1:1 by means of varying 3 parameters: (i)
155 the temperature (60-95°C), (ii) the reaction time (2-16 h) and (iii) the cotton fibre
156 cellulose concentration (1-2%). The DOE method allows process optimization by
157 performing a minimum number of experiments while varying the 3 parameters at the
158 same time. We first established a mathematical model to determine the optimal
159 conditions of acid hydrolysis to obtain the highest yield, and then, we tried to predict the
160 CNC crystallinity. Once the domain of the treatment conditions was perfectly described,
161 it was possible to choose the proper combination of parameters to obtain nanocrystals
162 with the desired yield and crystallinity.

163

164 **2. Materials and methods**

165 2.1. Materials

166 Dry sheets of commercially available of bleached and mechanically treated cotton
167 fibre obtained from the paper industry were used as cellulosic material (CELSUR, CS
168 21 DHS). Oxalic acid dihydrate (≥ 99%) and choline chloride (≥ 98%) were obtained
169 from Sigma-Aldrich, and sodium chloride (≥ 99%) was obtained from Roth.
170 Deionized water was used throughout the experiment.

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2.2. NADES treatment

173 The 1:1 molar ratio of acidic deep eutectic solvent was produced by stirring 63.0
174 grams of oxalic acid dihydrate and 69.8 grams of choline chloride continuously for 30
175 minutes at 95°C in a glass reactor. Small pieces of 3*3 mm cotton sheets (having a
176 weight about 1-2 mg) were added to the reactor. The cellulose concentration,
177 reaction time and temperature are the three variable parameters chosen to build the
178 design of the experiment with a total of 14 different sets of parameters (Ex: T=60°C,
179 t=16 h, c=2%). The treatment conditions to build the experimental domain in the
180 design of experiments were chosen based on preliminary tests, conditions used in
181 the literature and environmental aspects. For this reason, a lower range of
182 temperatures than those reported in the literature and longer reaction times were
183 tested.

184 At the end of the treatment, 200 ml of deionized water was added to the reactor to
185 quench the reaction and reduce the viscosity of the mixture. The treated cellulose
186 was washed and filtered through a membrane of 1 µm. The retained fibres were
187 dispersed in water and filtered again, and this action was repeated 3 times. The
188 filtrates were centrifuged at 10,000 rpm for 15 minutes with a small quantity of
189 sodium chloride, and the cellulose nanocrystals obtained were dialysed until the
190 conductivity of the sample was the same as that of deionized water.

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2.3. Design of experiment and data analysis

193 The software MINITAB (MINITAB ®, LLC) was used for the statistical design of
194 experiments and data analysis. To optimize the yield and crystallinity of the CNCs,
195 three important treatment variables are chosen: treatment time (X_1), reaction
196 temperature (X_2) and initial cellulose concentration (X_3). Their range and values are
197 shown in Table 1.

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Table 1: Reaction parameters and their values

Factor	Name	Range of actual and coded variables			
		Unit	-1	0	1
X_1	Reaction time	h	2	9	16
X_2	Temperature	°C	60	75.5	95
X_3	Concentration	%	1	1.5	2

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Central composite design (CDD) is commonly used for improving and optimizing processes to fit a model by the least-squares technique. The method contains three steps: (i) design and experiments; (ii) response surface modelling through regression; and (iii) optimization of parameters.

Using the CCD model, the nanocrystal yield and crystallinity can be modelled by two functions (Y_y and Y_c , respectively) of the three parameters; the equation can be written as follows (Equation 1):

Equation 1

$$Y_y \text{ or } Y_c = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1X_1 + b_{22}X_2X_2 + b_{33}X_3X_3$$

210

211 The predicted response (Y_c or Y_y) is correlated to the set of regression coefficients:
212 the intercept (b_0), the linear coefficients (b_1, b_2, b_3), the interaction coefficients ($b_{12},$
213 b_{13}, b_{23}) and the quadratic coefficients (b_{11}, b_{22}, b_{33}).
214 These different coefficients are calculated using the 14 treatment conditions
215 determined by the CDD method; 3 treatment conditions in the domain centre are
216 realized.

217

218 2.4. Nanocrystal and residual fibre analyses

219 **CNC and residual fibre (RF) yield**

220 As previously mentioned, residual fibres and cellulose nanocrystals were separated
221 after acid treatment, which enabled the determination of the real CNC yield before
222 the ultrasonic treatment.

223 The cellulose nanocrystal yield is calculated as the weight ratio of cellulose
224 nanocrystals (m_{CNC}) and the initial weight of cellulose fibres (m_0), as expressed in
225 Equation 2. At least two measurements were performed for each sample.

226 Equation 2

$$Y_{CNC} (\%) = ((m_{CNC} - m_0)) / m_0 \times 100$$

227

228 The residual fibre (RF) yield was also calculated using the same method (Equation
229 3).

230

Equation 3

$$Y_{RF} (\%) = ((m_{RF} - m_0)) / m_0 \times 100$$

231

232 **Crystallinity Index by x-ray diffraction (XRD)**

233 The crystallinity index (CI) of the cellulose nanocrystals was determined by the
234 amorphous subtraction method. The area under the curve of an amorphous standard
235 was subtracted from the sample area, and this difference was divided by the area of
236 the sample as expressed in Equation 4. The measurements were realized in the dry
237 CNC sample (overnight, 105°C) using an X'Pert Pro MDP instrument (Malvern
238 Panalytical) in reflection mode with the Bragg Brentano geometry. The anode was
239 composed of copper, and the wavelength was 1.5419 Angström. In this publication,
240 the amorphous sample was bleached birch cellulose pulp cryo-crushed for twenty
241 minutes using a Cryomill device (Retsch).

242

Equation 4

$$CI\%_{CNC} = (Area_{CNC} - Area_{amorphous}) / Area_{CNC} \times 100$$

243

244

245 **CNC sonicated suspension**

246 A determined quantity of CNC was diluted to a concentration of 10⁻² wt%. Using a
247 250-Watt sonication probe (Sonifer 250, Branson), the suspension was exposed to a
248 dispersive energy of 8.7 MJ per gram of dried CNC at 50% of maximum energy.

249

250 **Atomic force microscopy (AFM)**

251 One drop of the sonicated CNC suspension was deposited on a mica plate and dried
252 at room temperature overnight. Images were obtained using AFM (Dimension Icon)
253 in tapping mode, and the mean height of individual nanocrystals was obtained using
254 at least 50 measurements.

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Transmission electronic microscopy (TEM)

A few drops of the CNC sonicated suspensions were deposited onto a glow-discharged carbon-coated copper grid. After 2 min, the excess liquid was removed with filter paper, and a droplet of 2 wt% uranyl acetate was deposited onto the grid before completely drying the CNCs. The excess stain was removed, and the specimens were observed with a JEOL JEM 2100-Plus microscope operating at 200 kV. A minimum of 10 digital images were recorded at different locations with a Gatan Rio 16 camera, and the most representative image was used for the discussion.

Fourier transform infrared spectrometry (FTIR)

Infrared spectra were obtained from cotton cellulose fibres and residual fibres using a Perkin-Elmer Spectrum 65 instrument (PerkinElmer, USA). The fibres were dried at room temperature overnight, and a KBr pellet was pressed from the powder containing 2% fibre. Spectra were recorded in transmission mode between 4000 and 400 cm^{-1} with 16 scans.

Residual fibre morphology

Residual fibre morphology analysis (length, width, number of elements, etc.) was carried out using the MorFi device (MorFi compact, Techpap). Images of a suspension of residual fibres at 40 g/L between the measurement cells were acquired to perform the image analysis. Elements whose length exceeded 100 μm were counted as fibres and those with a length below 100 μm were counted as fines. This measurement was performed in triplicate.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy images were obtained with an ESEM instrument (Quanta 200, FEI, Japan) at an acceleration voltage of 10 kV. Wet samples of cotton fibres and residual fibres were air-dried and coated with carbon using a vacuum sputter coater. At least 20 images were taken, and the most representative images were used in the discussion.

3. Results and discussion

3.1. Model determination of the CNC yield.

The 17 conducted experiments are described in Table 2, and the obtained cellulose nanocrystal yields are reported in the penultimate column. The last column corresponds to the optimal normalization of the yield using the Cox-Box transformation method ($\lambda=0.33$) (Box and Cox, 1964).

Table 2: List of experimental parameters and obtained yields

Experiment Name	Concentration %	Time h	Temperature °C	CNC Yield %	Cox-Box transformation $\lambda = 1/3$
A	1.5	9	77.5	17.6	2.60
A'	1.5	9	77.5	22.8	2.83
A''	1.5	9	77.5	18.4	2.64
B	1	2	60	0.3	0.65

C	2	16	60	4.9	1.70
D	2	2	95	18.3	2.63
E	1	16	60	10.2	2.17
F	1	2	95	30.7	3.13
G	2	2	60	0.7	0.88
H	2	16	95	24.5	2.90
I	1	16	95	21.7	2.79
J	1.5	9	60	1.7	1.20
K	1.5	16	77.5	5.0	3.09
L	1.5	9	95	29.7	2.95
M	2	9	77.5	25.6	1.95
N	1.5	2	77.5	7.4	1.71
O	1	9	77.5	14.5	2.44

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Three replications were performed in the domain centre to assess the reproducibility of the treatment conditions (A, A' and A'').

The first response studied was the CNC yield. The coefficients of the models were determined according to the DOE results, and some insignificant interactions were detected and removed using the analysis of variance (ANOVA) method without degrading the model and maintaining a hierarchical structure. The final equation is expressed below (Equation 5), and the surface response of the CNC yield according to the temperature and the reaction time is plotted in Figure 1 **Erreur ! Source du renvoi introuvable.** A, whereas the coefficient p-values and regression coefficient are shown in Figure 1B.

Equation 5

$$Y_y(\%) = (-3.758 + 0.397 \times t(h) + 0.068 \times T(^{\circ}C) - 0.002 \times T(^{\circ}C) \times t(h) - 0.010 \times t^2(h))^3$$

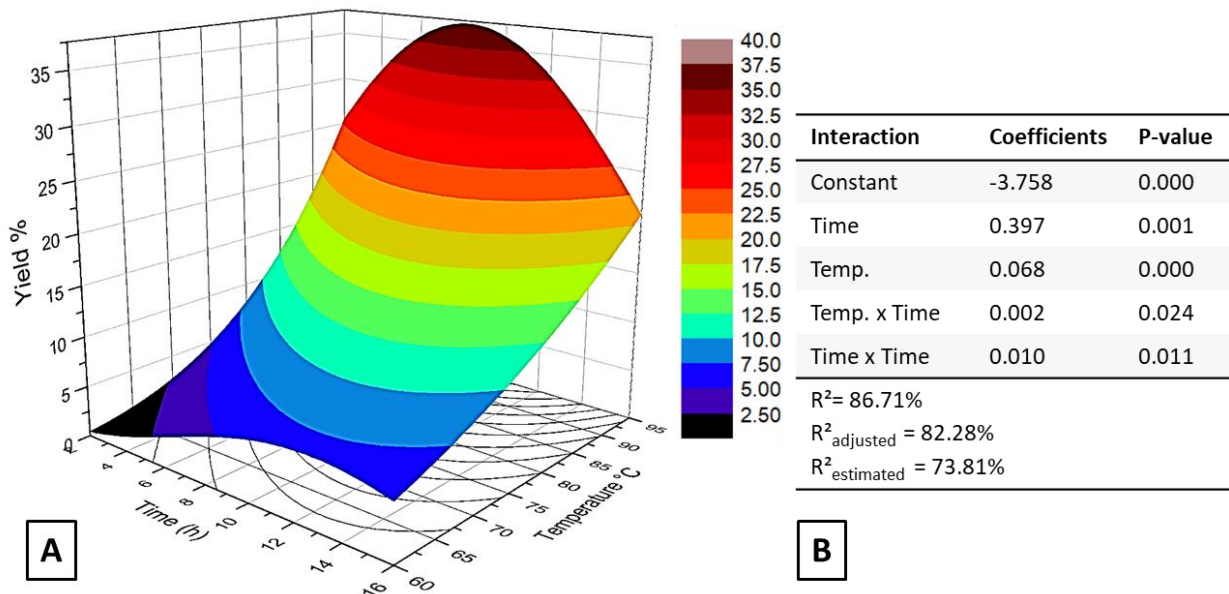


Figure 1: A. Surface response of the yield - B. Regression coefficients and p-values of the coefficients

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309 The fit of the model can be evaluated using the tree regression coefficients R-square
310 (R^2), adjusted R-square (R^2_{adjusted}), and estimated R-square ($R^2_{\text{estimated}}$); the closer
311 their values are to 100%, the better the model fits. In the current model, R^2 and
312 R^2_{adjusted} were higher than 80%, and the difference between R^2_{adjusted} and $R^2_{\text{estimated}}$
313 was lower than 10%; as a consequence, the model can be used with caution.

314
315 The first observation of this model is that the fibre concentration during the
316 treatment did not influence the final yield; indeed, all the mathematical terms of the
317 model, including the concentration, were negligible. The initial concentration range
318 of cellulosic cotton fibres (1-2%) was determined using preliminary experiments to
319 obtain a good dispersion of the fibres in the NADES while limiting the viscosity of the
320 reaction medium. Indeed, ChCl:OAD 1:1 is a viscous solvent, especially at low
321 temperature, and adding cellulose fibres further increases the global viscosity of the
322 mixture. The starting hypothesis consisted of the fact that increasing the fibre
323 concentration would reduce the efficiency of the treatment due to the limited
324 penetration of the chemicals into the cotton fibres. In practice, the small variation in
325 the concentration in the experimental domain did not allow us to observe the
326 influence of this parameter on the yield optimization because the viscosity of the
327 mixture was likely the same. This result also confirmed the large excess of acid,
328 which facilitated the hydrolysis of the amorphous part in the experimental domain.

329 As expected, the temperature played a crucial role in the extraction of the cellulose
330 nanocrystal. The higher the treatment temperature, the higher the final CNC yield.
331 This trend had already been observed by Sirviö in 2016 (Sirviö, Visanko, and
332 Liimatainen, 2016), but another study by Liu *et al.* showed that microwave
333 application reduced the CNC yield at high temperatures (Liu *et al.*, 2017). These
334 results are not truly in contradiction because the cellulosic fibre sources, as well as
335 the experimental conditions used, were different in the three studies (Table 3).
336 Moreover, in all cases, the yield in this approach increased with temperature and
337 started to decrease when the degradation took place.

338
339 The treatment time is also a very important parameter, and as observed in the
340 surface response (Figure 1), the CNC yield decreased after 8 or 9 hours of treatment.
341 Moreover, the residual fibres were brownish after this time, and after 16 h of
342 treatment, even the CNC nanoparticles became partially coloured, as already
343 observed by Liu *et al.*, who reported the same tendency. Both phenomena may be
344 due to the degradation of the cellulose material because of the strong condition of
345 treatment.

346
347 Since the theoretical mathematical model was defined, we proceeded with the next
348 step aiming at its validation by performing a new experiment within the domain. The
349 chosen parameters for this experiment targeted a theoretical maximum CNC yield of
350 $37.2 \pm 2.7\%$, which theoretically could be obtained after 8 hours and 13 minutes of
351 treatment at 95°C.

352
353 In this context, these optimal experimental conditions were tested in triplicate with a
354 chosen fibre concentration of 1%.

355 This set of parameters resulted in a CNC yield of approximately $44.9 \pm 2.5\%$. The
356 difference between the theoretical and experimental values was higher than
357 expected but still acceptable. In fact, even if a few points of higher yield were

358 observed, the standard deviation was a perfect fit with the theoretical value. Some
 359 hypotheses can be drawn to explain this slight discrepancy. The optimum point is
 360 located close to the maximum of the theoretical curve (maximum temperature
 361 T=95°C), whereas the mathematical model obtains a better fit in the centre. It is
 362 possible that some unknown parameters governing the studied reaction were
 363 neglected when building the model. Finally, the polynomial law is not perfectly
 364 suitable to properly describe the reaction.

365
 366 The cellulose nanocrystals (obtained using the treatment conditions ChCl:OAD, 95°C,
 367 8 h 13 min, c=1%) were characterized and can be compared to those of CNC obtained
 368 in the literature using deep eutectic solvents composed of choline chloride and oxalic
 369 acid dihydrate (Table 3).

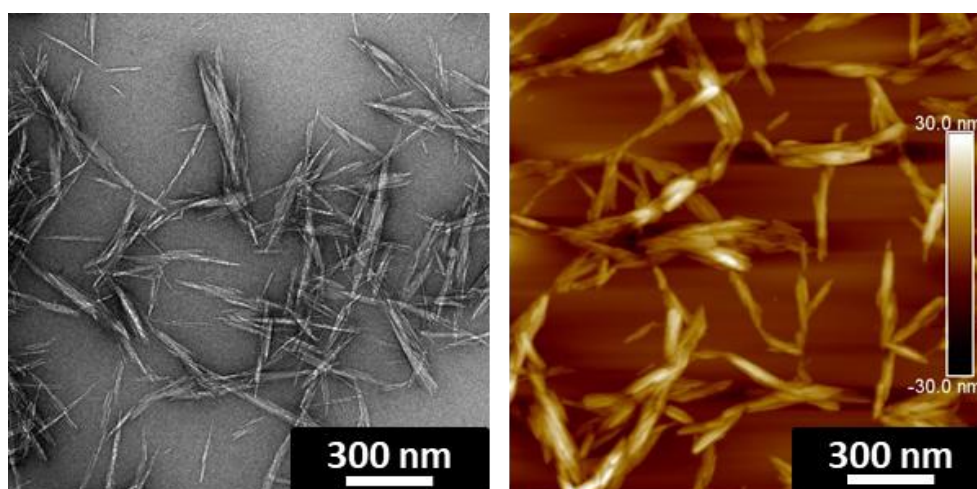
370
 371 Table 3: Overview of the different pre-treatment conditions using ChCl:OAD NADES
 372 for obtaining cellulose nanocrystals - adapted from Le Gars *et al.*, 2019

Cellulose source	Treatment (DES, ...)	Molar Ratio	Time	Temp. °C	Yield* %	Dimension nm	Ref
Dissolving pulp	ChCl : OAD	1:1	2 h	100	68	l = 390 ± 25 d = 13.6 ± 1.1	Sirviö, Visanko, and Liimatainen 2016
		1:1	2 h	120	73	l = 353 ± 16 d = 13.8 ± 0.7	
Dissolving pulp	ChCl : OAD	1:1	30 min	100	NA	l = 50 - 350 d = 3-8	Laitinen <i>et al.</i> 2017
Cotton fibre	ChCl : OAD	1:1	1 h	100	79.8	l = 194.1 d = 9.6 ± 2.9	Ling <i>et al.</i> 2019
	ChCl : OAD	1:2	1 h	100	80.0	l = 152.7 d = 6.1 ± 1.2	
	ChCl : OAD	1:3	1 h	100	81.6	l = 122.4 d = 4.7 ± 2.2	
Cotton fibre	ChCl : OAD Microwave assisted	1:1	3 min	80	74.2	l = 100-350 d = 3-25	Liu <i>et al.</i> 2017
		1:1	3 min	90	62.4	NA	
		1:1	3 min	100	57.8	l ≈ 150 d < 17	
Bleached eucalyptus kraft pulp	ChCl : OAD + catalyst: FeCl ₃ · 6H ₂ O (mmol/gDES)	1:4	7 h	80	86	l = 5152 ± 3328	Yang <i>et al.</i> 2019
		0	6 h	80	73	l = 270 ± 92	
		0.15	6 h	80	71	l = 258 ± 54	
		0.3	6 h	80	88	l = 5726 ± 3856	
Bleached cotton fibre: 14 exp.	ChCl : OAD	1:1	2-16 h	60-95	0.3-30.7	NA	This study
		1:1	8 h 13	95	CNC= 44.9% Fibre= 13.6%	l= 282 ± 46 d= 7.9 ± 1.9	

*Yield means yield of CNC and residual fibres except in our study
 NA: non-applicable

373

374 The CNC morphology was determined after sonication of the suspensions, and their
 375 height was deduced from AFM images, whereas their length was deduced from TEM
 376 images. Fifty measurements of individual CNC were conducted, and the mean height
 377 value was 7.9 ± 1.9 nm, while their length was approximately 282 ± 46 nm. These
 378 values are similar to those associated with the CNC dimensions obtained from cotton
 379 *via* classical acid hydrolysis (Dufresne, 2017) and those obtained by ChCl:OAD
 380 treatment (Liu *et al.*, 2017; Ling *et al.*, 2019). TEM and AFM images of our cellulose
 381 nanocrystals are displayed in Figure 2.
 382



383 Figure 2: Cellulose nanocrystals after treatment with ChCl:OAD (1:1), 95°C, 8 h 13 min: - Left.
 384 TEM image - Right. AFM image
 385

386 The obtained CNC yield was lower than the yield found in the literature. This
 387 difference can be attributed to the washing and separation steps after NADES
 388 treatment, which were different from those reported in the publications cited in
 389 Table 3. Indeed, in this work, residual fibres and CNC were separated before the
 390 ultrasonic treatment step and two distinguished suspension were obtained. The
 391 consequences are that some cellulose nanocrystals were removed along with the
 392 residual cellulose fibres and/or with the large CNC aggregates that were filtered out
 393 by the filtration membrane.

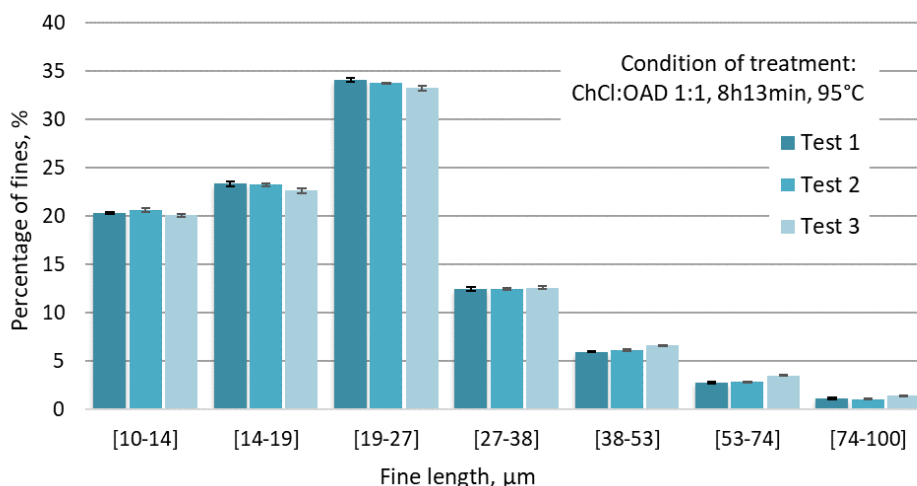
394 Using these “optimal” conditions, a small quantity of residual fibres was obtained
 395 (yield of fibres: 16.3%), and their morphology was compared with the initial
 396 dimension of cotton fibres (see Table 4). After the ChCl:OAD 1:1 treatment, the
 397 residual fibres exhibited a mean length divided by five and a number of fines
 398 multiplied by thirty-eight compared to the initial values, which confirmed the
 399 fragmentation capacity of NADES. Scanning electron microscopy images of fibres
 400 before and after treatment are shown in the supporting information (Figure S 1).
 401

402 Table 4: Morphology of residual fibres.

	Fibres		Fines	
	Mean length μm	Mean width μm	Fines content millions/g	Mean length μm
Reference Cotton Fibres	689 ± 10	25 ± 0	67 ± 3	49 ± 0
Residual Fibres	131 ± 1	21 ± 1	2531 ± 197	23 ± 1

403

404 The cotton fibre and the residual fibre morphology result from the optical analysis of
 405 the respective suspensions by the MorFi device. This device divides the counted
 406 elements into subcategories. Figure 3 shows an example of the measurement
 407 reproducibility obtained by comparing the mean length of fines contained in the
 408 residual fibre suspension for the three previous treatment conditions (8 h 13 min,
 409 95°C). According to the morphology analysis of the initial fibers, as expected, there is
 410 a big distribution of the fibres' size. As a consequence, big fibres or agglomerates
 411 would take much more time to be reduced to nanosize. This is the reason one, at the
 412 end of the reaction, some residual fibres are still present. Indeed, the quantity of this
 413 residue decrease with increasing reaction time.
 414



415 Figure 3: Fine mean length contained in the residual fibre suspension (measured in triplicate
 416 with ChCl:OAD 1:1, 8 h 13 min, 95°C treatment conditions)
 417

418 The infrared spectrum was obtained from the residual fibres and compared to that of
 419 the cotton fibres, and no new absorption peak was observed in the residual fibre
 420 spectrum. This result demonstrates that no chemical reaction took place on the
 421 cellulose (supporting information Figure S 2).
 422

423 3.2. Model determination for the crystallinity

424 To determine the theoretical equation of the cellulose nanocrystal crystallinity, the
 425 same methodology as that for the CNC yield was used. The crystallinity index (CI) of
 426 the cellulose nanocrystals previously obtained from the 17 experiments were
 427 evaluated by the amorphous subtraction method and are reported in Table 5.
 428

429 Table 5: List of experimental parameters and the obtained crystallinity

Experiment Name	Concentration %	Time H	Temperature °C	CNC CI %
Ref	-	-	-	79
A	1.5	9	77.5	79
A'	1.5	9	77.5	79
A''	1.5	9	77.5	77
B	1	2	60	61
C	2	16	60	73
D	2	2	95	78

E	1	16	60	71
F	1	2	95	78
G	2	2	60	65
H	2	16	95	80
I	1	16	95	80
J	1.5	9	60	66
K	1.5	16	77.5	80
L	1.5	9	95	81
M	2	9	77.5	80
N	1.5	2	77.5	72
O	1	9	77.5	78

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The amorphous subtraction method with cellulosic samples has been described in the literature (Isogai and Usuda, 1989), and over the last decade, it has been compared to other methods (Park *et al.*, 2010; Ahvenainen, Kontro, and Svedström, 2016). The main challenge of this method is choosing a good amorphous sample that fits the sample under investigation. In this publication, the chosen amorphous sample was bleached, cryo-crushed birch cellulose pulp.

As previously mentioned, three repetitions were performed in the domain centre to assess the reproducibility of the treatment conditions (A, A' and A''). This time, Box-Cox transformation was not required to analyse the data, and the obtained theoretical model is described by Equation 6.

Equation 6

$Y_{crystallinity}(\%)$

$$= -50.9 + 2.769 \times T(^{\circ}C) + 2.188 \times t(h) - 6.46 \times c(\%) - 0.01396 \times T^2(^{\circ}C) - 0.0362 \times t^2(h) + 4.90 \times c^2(\%) - 0.01429 \times T(^{\circ}C) \times t(h) - 0.0857 \times T(^{\circ}C) \times c(\%)$$

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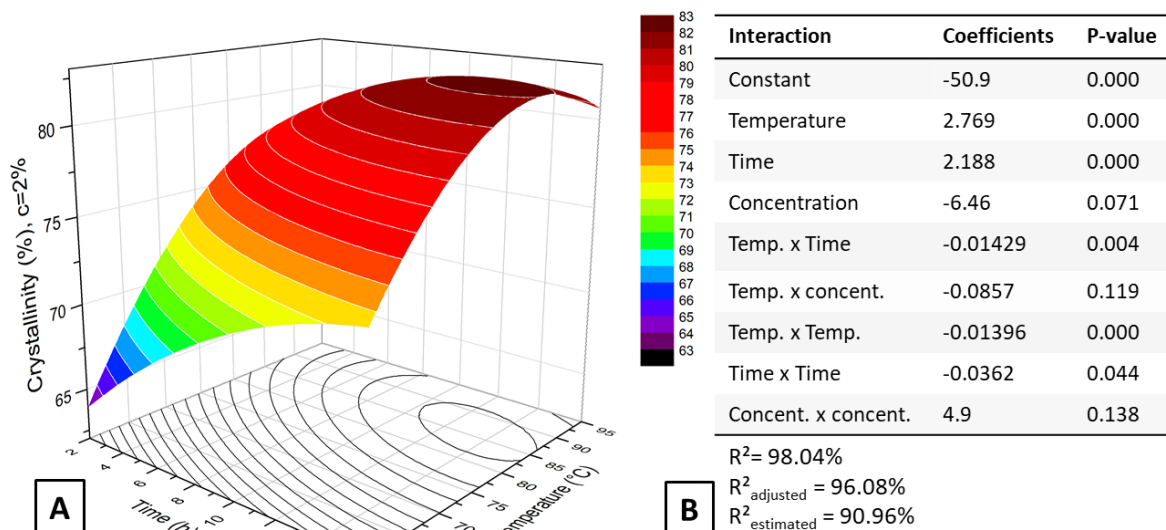
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In contrast to the CNC yield, the CNC crystallinity depended on the initial concentration of the cellulosic fibres. The surface response of the CNC crystallinity according to the temperature and the reaction time for a fixed concentration value of 2 wt% is plotted in Figure 4A, and the coefficient p-values and regression coefficient are shown in Figure 4B.



449
450 Figure 4: A. Surface response of the crystallinity (c= 2%) - B. Regression coefficients
451 and p-values of the coefficients.
452

453 The regression coefficients of this model were $R^2 = 98.0\%$ and $R^2_{\text{estimated}} = 91.0\%$, and
454 thus, the mathematical model can be used with the recommendation to test a new
455 point in the domain when designing the experimental strategy. The crystallinity of
456 the CNC obtained with the conditions for the yield optimization (T= 95°C, t= 8 h 13
457 min, c= 1%) was evaluated for the three repetitions. The theoretical value expected
458 for these parameters was CI%= 81 ± 1%, and the mean experimental value was 82 ±
459 1%. In conclusion, the model was able to predict the crystallinity of the obtained
460 CNCs in all experimental domains. The cellulose nanocrystal crystallinity seemed to
461 reach a maximum after approximately 13 h of treatment at 85°C using an initial
462 concentration of 2%. These crystallinity values were high but similar to those
463 reported by Ling *et al.* (Ling *et al.*, 2019). They obtained dispersed CNCs with a
464 crystallinity index of 75% using cotton fibres treated with ChCl:OAD 1:1 for 1 h at
465 80°C, and the theoretical model found in this study predicted a CI of 75% for the CNC
466 product treated at 80°C for 2 hours. It is worth mentioning the rather excellent
467 agreement between theoretical and experimental data in this section. Thus, it can be
468 concluded that: For low temperature experiments the crystallinity index decreased
469 most probably because of the formation of amorphous zones due to the swelling of
470 the fibres and before starting the hydrolysis step. Instead, for high temperature
471 experiment did not affect significantly the CI(%) because the swelling and the
472 hydrolysis took place simultaneously. In all cases, the changes on this parameter are
473 hard to ascertain because of the relatively high crystallinity of the starting materials
474 (CI(%) = 79%).
475

476 3.3. Response's combination

477 The strength of the design of the experimental method is the possibility of the
478 response's combination. Reporting the two models previously described for the CNC
479 yield (%) and the crystallinity index (%) in a two-dimensional graph simplifies the
480 choice of the parameters that can optimize the two responses simultaneously (Figure
481 5). The main objective of this graph is its capacity to predict CNC concentration and
482 crystallinity for a given set of parameters.

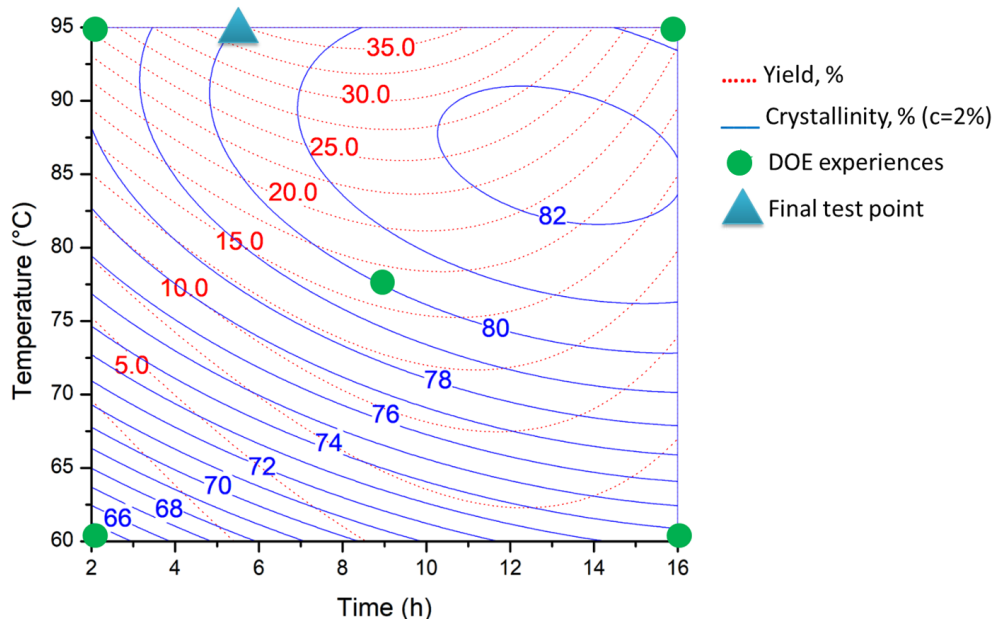


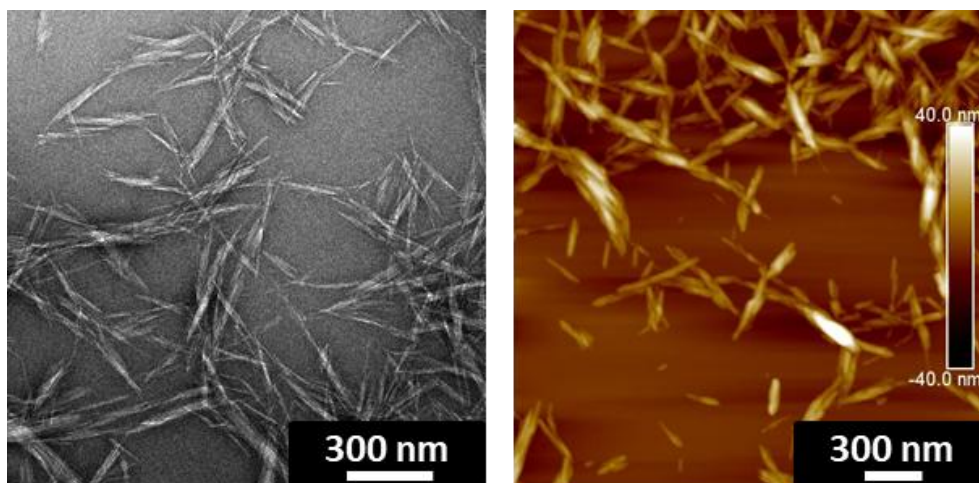
Figure 5: Overlay of the contour diagrams for the two studied responses: Yield% and Crystallinity% (c=2%).

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487 The actual aim was the production of cellulose nanocrystals in large quantities and, if
488 possible, at high concentrations. Therefore, the CNC yield was more relevant than the
489 crystallinity. Moreover, a short reaction time was recommended. Indeed, the
490 available CNC production processes using classical acid hydrolysis are carried out in
491 less than 1 hour. Furthermore, between 6 and 8 hours of treatment at 95°C, the gain
492 in CNC yield and crystallinity was very low. Considering this information, a new set
493 of parameters was chosen to carry out a final experiment: a reaction time of 6 hours
494 at a temperature of 95°C and an initial concentration of cotton cellulosic fibres of 2%
495 (bleu triangle in Figure 5).

496 For these parameters, the expected CNC yield was approximately $35.5 \pm 2.7\%$ with a
497 crystallinity index of $80 \pm 1\%$. Three experiments were carried out for
498 reproducibility, and the results were a CNC yield of $43.6 \pm 1.9\%$ and a crystallinity of
499 $81 \pm 1\%$. Similar to the previous experiments, the obtained CNC yield was higher
500 than expected and was relatively close to the yield obtained after 8 h 13 mins of
501 treatment ($44.9 \pm 2.5\%$). The mathematical model for CNC crystallinity was again
502 confirmed. The CNC morphology was determined after sonication of the suspensions,
503 and their height was obtained from AFM images, whereas their length was obtained
504 from TEM images. Fifty measurements of individual CNCs were conducted, and the
505 mean height value was found to be 11.3 ± 2.6 nm, while their length was
506 approximately 257 ± 54 nm. TEM and AFM images of these cellulose nanocrystals are
507 shown in Figure 6.

508



509

510 Figure 6: TEM (left) and AFM (right) images of CNCs obtained after 6 h of treatment at
511 95°C with a fibre concentration of 2%.

512 4. Conclusion

513

514 In this work, cellulose nanocrystals were successfully extracted using an acidic NADES
515 composed of choline chloride:oxalic acid dihydrate at a molar ratio of 1:1. To optimize
516 the treatment time and temperature in the acidic hydrolysis of the amorphous regions of
517 cellulose, the methodology of the design of the experiment was used. Two models were
518 established to characterize the evolution of the nanoparticle's crystallinity and the CNC
519 yield of the treatment. Our hypothesis has been confirmed and optima yield and CNC
520 crystallinity have been found thanks to the design of experiment approach. The first
521 model successfully described the crystallinity variation of CNCs according to the
522 different treatment parameters, while the second model was less accurate for describing
523 the experimental values and provided slightly higher values, although it followed the
524 same tendency. The obtained results are highly useful because, to the best of our
525 knowledge, this is the first time that this optimization work has been proposed. This
526 study shows that it is possible to predict the CNC yield CNC and their crystallinity thanks
527 to predictive mathematical models, which gives a great advantage to consider in the
528 near future a scale up of the extraction of cellulose nanocrystals using this original
529 family of green solvents.

530 The next step should be an evaluation of different cellulose sources and NADES
531 compositions by DOE to validate whether the methodology can be applied to any type of
532 CNC extraction by an acidic NADES.

533

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535

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540 Grenoble) for the cellulose amorphous reference used in the crystallinity study.

541

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543

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Supporting information

Use of a natural acidic deep eutectic solvent to obtain a high yield and crystallinity of cellulose nanocrystals using the design of experience approach.

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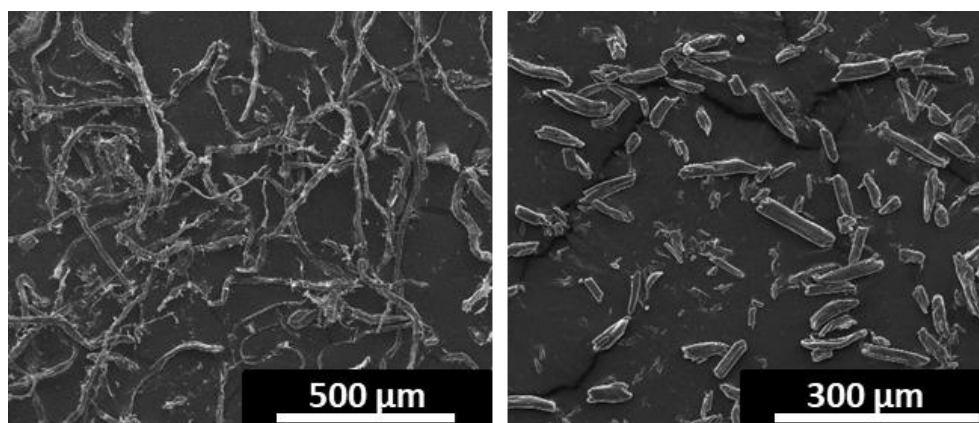


Figure S 1: SEM images of reference cotton fibres (left) and residual fibres after 8 h 13 min of treatment at 95°C (right)

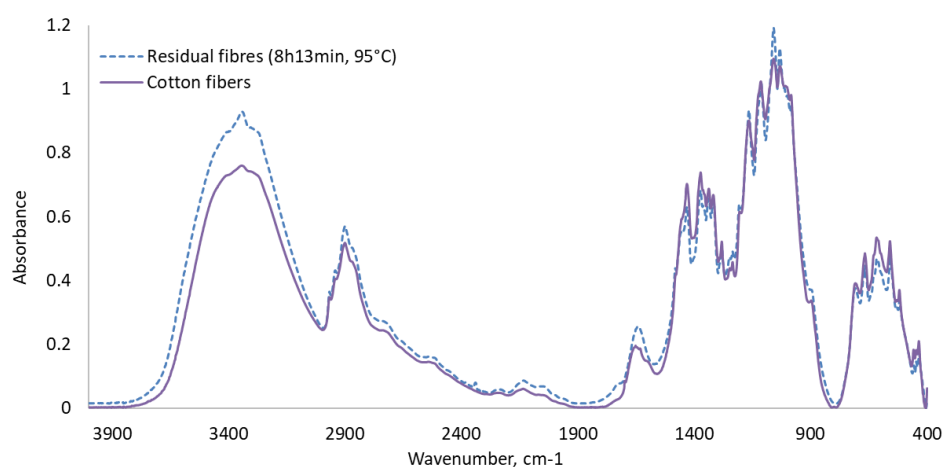


Figure S 2: Infrared spectra of cotton cellulosic fibres and residual fibres after 8 h 13 min of treatment at 95°C